

## **Anaerobic nitrification-denitrification mediated by Mn-oxides in meso-tidal sediments: implications for N<sub>2</sub> and N<sub>2</sub>O production**

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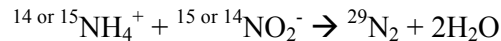
### **ABSTRACT**

Field measurements in the Arcachon Bay (south-west France) indicated anaerobic production of NO<sub>x</sub> via nitrification which was coupled to the reduction of Mn-oxides. To provide evidence for the occurrence of this process, laboratory microcosm experiments were set up. A <sup>15</sup>N tracer based approach was used to track if NO<sub>x</sub> produced through Mn-oxide mediated anaerobic nitrification would be reduced to N<sub>2</sub> via denitrification or anammox. We also hypothesized generation of the potent greenhouse gas - nitrous oxide (N<sub>2</sub>O) during nitrification-denitrification in the presence of Mn-oxides. The microcosms were prepared using sediment sectioned at varying depths (0-2.5, 2.5-4.5, 4.5-8.5, 8.5-12 and 12-17 cm) during two sampling campaigns in October (fall) and January (winter). Labelling with <sup>15</sup>NO<sub>3</sub><sup>-</sup> revealed low N<sub>2</sub> production originating from NO<sub>3</sub><sup>-</sup> in the water column (P<sub>w</sub>) which did not increase significantly on amendment with Mn-oxides during both sampling periods. However, for both seasons, a significant increase of N<sub>2</sub> produced via nitrification (P<sub>n</sub>) was observed upon addition of Mn-oxides reaching 76 fold enhancement at ≤ 2.5 cm. To support these results, sediment slurries of October were subjected to amendment of <sup>15</sup>NH<sub>4</sub><sup>+</sup>, <sup>14</sup>NO<sub>3</sub><sup>-</sup> with or without addition of Mn-oxides. A substantial production of P15 (N<sub>2</sub> production from <sup>15</sup>NH<sub>4</sub><sup>+</sup>) within 0-17 cm provided further evidence on nitrification-denitrification mediated by Mn-oxides probably with minimal intervention of anammox. In organically-rich sediments, anaerobic nitrification-denitrification mediated by Mn-oxides could play an important role in lowering re-mineralized NH<sub>4</sub><sup>+</sup> levels in the benthic system. As hypothesized, significant production of N<sub>2</sub>O through the pathway was observed revealing yet another mechanism for generation of the radiative gas.

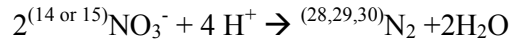
**Keywords:** Anaerobic; Anammox; Denitrification; Manganese oxides; Nitrification; Nitrous oxide; Sediments

## 1. Introduction

In estuarine systems, nitrogen (N) budget calculations show that only 10 to 25% of anthropogenically-derived N is exported to the sea (Schaefer and Alber, 2007) suggesting its local elimination. Two anaerobic processes are known to be responsible for nitrogen removal: (i) denitrification, which is the reduction of  $\text{NO}_3^-$  or  $\text{NO}_2^-$  to  $\text{N}_2$  and (ii) anammox (anaerobic ammonium oxidation) that couples oxidation of  $\text{NH}_4^+$  to  $\text{NO}_2^-$  reduction with  $\text{N}_2$  as the end product (Thamdrup and Dalsgaard 2002, Dalsgaard et al., 2003, Kuypers et al., 2003). Isotopic measurement (using  $^{15}\text{N}$  enrichment as tracer) of di-nitrogen species can discriminate between these two processes (Minjeaud et al., 2008; Risgaard-Petersen et al., 2003). The production of  $\text{N}_2$  via anammox has a 1/1 stoichiometry as follows:

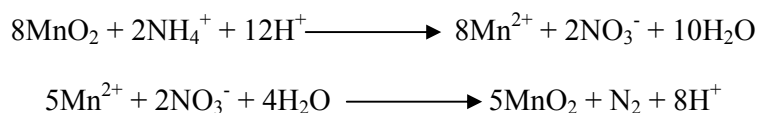


whereas, the production of  $\text{N}_2$  by denitrification follows a binomial distribution reflecting the frequency of  $^{15}\text{N}$  and  $^{14}\text{N}$  in the  $\text{NO}_2^-$  pool:



In marine ecosystems (anoxic water column, particles, sediments), the removal of nitrogen is mainly dominated by denitrification (Michotey and Bonin 1997; Kuypers et al., 2005; Minjeaud et al., 2009, Ward et al., 2009; Fernandes et al., 2012). The percentage of  $\text{N}_2$  production through anammox in sediments is variable (Dalsgaard et al., 2005). In coastal sediments, low anammox contribution has generally been recorded (Thamdrup and Dalsgaard 2002; Fernandes et al., 2012) while higher percentage of  $\text{N}_2$  production has been observed offshore e.g. continental slope sediments (Thamdrup and Dalsgaard 2002). In coastal sediments, high denitrification rates of up to  $0.22 \mu\text{mol N}_2 \text{ g}^{-1} \text{ h}^{-1}$  have been measured albeit pore water concentration of its substrates ( $\text{NO}_3^-$  and  $\text{NO}_2^-$ ) being in micro molar range (Fernandes et al., 2012). Isotopic  $\text{N}_2$  ratio measurements (Risgaard-Petersen et al., 2003) have solved this apparent contradiction. They showed that in sediments, denitrification from exogenous  $\text{NO}_3^-$  corresponds to less than 10% of the total rate (Hietanen and Kuparinen 2008; Minjeaud et al., 2009) while the main part of  $\text{NO}_3^-$  is formed *in situ* via nitrification. This suggests a close link between aerobic nitrification and denitrification in oxic-anoxic gradients. Among  $\text{NH}_4^+$  oxidation processes, the role of oxidized metal compounds in alternative anaerobic processes has often been suggested to explain the presence of unexpected peaks of  $\text{NO}_3^-$  in deep layers of sediment. After excluding the hypothesis of an enhanced supply of  $\text{NO}_3^-$  due to bioturbation, a local production of the nutrient due to reduction of metal-oxides has been suggested (Anschutz et al., 2000). Among them, manganese oxides (Mn-oxides) are commonly described as powerful oxidants capable of modifying or creating new pathways of oxidation in the N cycle (Luther et al., 1997; Hulth et al., 1999; Anschutz et al., 2000; Thamdrup & Dalsgaard, 2000; Deflandre et al., 2002; Mortimer et al., 2002, 2004; Hulth et al., 2005; Bartlett et al., 2007, 2008). Luther et al. (1997) have shown a strong link between Mn and N cycles and described several reactions causing short-cuts in the N cycle. Hulth et al. (1999) have described a thermodynamically favorable reaction of nitrification in anaerobiosis using Mn-oxides as the oxidant of  $\text{NH}_4^+$ . Anaerobic nitrification could use manganese as the electron acceptor to form  $\text{NO}_3^-$  (Mortimer et al., 2002; 2004; Luther and Popp 2002; Clément et al., 2005; Bartlett et al., 2008), which is then denitrified by

biologically-mediated denitrification or chemo-denitrification (Sørensen 1987; Aller 1990; Schulz et al., 1994; Murray et al., 1995; Luther et al., 1997) to form N<sub>2</sub> (Javanaud et al., 2011).



However, these results are mainly based on concentration of solutes and there is still few concrete evidence for the occurrence of anaerobic nitrification mediated by Mn-oxides.

Mn-oxides are widely distributed in anoxic marine sediments and are very reactive leading to several secondary reactions of diagenesis (Deflandre et al., 2002). However, due to complex recycling of redox species, these processes are difficult to discern and therefore poorly understood. Javanaud et al. (2011) used a denitrifying strain from Arcachon Bay sediments to demonstrate N<sub>2</sub> production from NH<sub>4</sub><sup>+</sup> through anaerobic nitrification-denitrification in the presence of Mn-oxides. Under nitrate-limited conditions, the authors suggested N<sub>2</sub>O accumulation through this pathway. In the present study, we used an isotopic approach (with <sup>15</sup>NO<sub>3</sub><sup>-</sup> or <sup>15</sup>NH<sub>4</sub><sup>+</sup>) to investigate the following: (1) down-core variation in N<sub>2</sub> production through anaerobic nitrification-denitrification by introducing Mn-oxides in sediment slurries from the Arcachon Bay (2) to verify the extent of N<sub>2</sub> production from NH<sub>4</sub><sup>+</sup> in the presence of Mn-oxides and (3) to test the hypothesis that Mn reduction coupled to anaerobic nitrification-denitrification could be important for N<sub>2</sub>O production in coastal sediments. Our results showed significant increase of N<sub>2</sub> production in slurry microcosms after addition of Mn-oxides which was mainly through anaerobic nitrification-denitrification rather than anammox. Elevated N<sub>2</sub>O production through the former pathway was also observed strengthening our hypothesis and re-affirming earlier observations made by Javanaud et al. (2011).

## 2. Methods

### 2.1. Study area

Arcachon Bay is a shallow meso-tidal lagoon (Deborde et al., 2008) 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 km<sup>2</sup>), and the intertidal area (117 km<sup>2</sup>) (Plus et al., 2010) made up of large tidal flats (Deborde et al., 2008). These tidal flats are mainly composed of muddy sand (grain size = 15 – 40 μm). Only 40 km<sup>2</sup> remains submerged at low tide (De Wit et al., 2005). The sampling station was located on a sloping mud flat (Fig. 1) in the southeast part of the Arcachon Bay. This area remained exposed to the atmosphere for ~ 6 h d<sup>-1</sup> and was composed of fine sediments of grain size ≈ 27 μm. This sampling site was chosen by all partners in the frame work of the PROTIDAL project. One of the aims of this project was to study the influence of successive emersion and immersion periods on benthic biogeochemical processes. The simultaneous determination of major redox biogenic and trace elements along with flux measurement at different interface (water-sediment, sediment-atmosphere, water-atmosphere, freshwater-seawater), macrofauna, seagrass and microbiological parameters in the transient conditions of the tidal environment were carried out.

## 2.2. Sampling and sample processing

Push cores of 8 cm diameter and 20 cm length were collected in October, 2007 and January, 2008 during low tide. Surface sediment temperature in October was around 10.0°C while in January it was 2.8°C. Eight sediment cores from an area of < 5 m<sup>2</sup> were collected, capped at both ends and transferred to the laboratory in an insulated box. For activity measurements, five replicate cores (core # 1 to 5) were sliced at 5 depth intervals i.e. 0-2.5, 2.5-4.5, 4.5-8.5, 8.5-12 and 12-17 cm within 3 h of retrieval. These depths were decided based on a preliminary profile of pore water Mn<sup>2+</sup> (data not shown) in the sediment column. Immediately after slicing, each slice from same depth of the different cores were pooled and conserved in sealed bag flushed under N<sub>2</sub>-atmosphere. The remaining three cores were processed for nutrient and metal analyses as follows:

Core # 6 – Analysis of pore water NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> (NO<sub>x</sub>)

Core # 7 – Analysis of pore water Mn<sup>2+</sup>

Core # 8 – Analysis of sediment extractable NH<sub>4</sub><sup>+</sup> and Mn-oxides (particulate fraction).

Core # 6 and 7 were processed within an hour of transportation to the onshore laboratory. Core # 8 was preserved at -20°C and processed for analysis of sediment extractable NH<sub>4</sub><sup>+</sup> and Mn-oxides within 48 h of collection.

## 2.3. Nutrient, dissolved and particulate Mn analyses

To gain a better resolution on the variation in nutrients with depth, sediment core was thawed and sectioned at 0.5, 1, or 2 cm intervals up 17 cm depth. Five millilitres of NO<sub>x</sub>-free artificial seawater (Baumann and Baumann, 1981) was added to 5 mL of sediment. The contents were homogenised to form slurry and centrifuged for 10 min at 5000 rpm (× 1803 g). The supernatant volumes were measured upon filtering through a 0.2-µm filter. NO<sub>3</sub><sup>-</sup> plus NO<sub>2</sub><sup>-</sup> analyses corresponding to NO<sub>x</sub> in the extracted pore water were analyzed with a continuous flow analyzer (AA3 autoanalyzer; SEAL Analytical; UK) according to Tréguer and Le Corre (1975). Ammonium concentrations were determined after KCl extraction (Laima 1993) using the fluorometric method (Kérouel and Aminot 1997). The dilution due to addition of synthetic seawater has been accounted for in the calculation of nutrients concentrations. Dissolved Mn was measured by flame atomic adsorption spectrometry (Model AA 300; Perkin Elmer; Waltham, MA) with ± 5% precision (Deborde et al., 2008). Sub-samples for analysis of Mn-oxides were dried in a hot air oven at 60 ± 2 °C for 48 h and disaggregated in an agate mortar before chemical treatment. An ascorbate reagent was used to extract the "amorphous readily microbially reducible phase" i.e. particulate Mn (Anschutz et al., 2005) from the sediment samples.

## 2.4. Synthesis of Mn-oxides

Since the crystallinity of MnO<sub>2</sub> provided by manufacturers does not correspond to MnO<sub>2</sub> freshly formed from oxidation of dissolved Mn (II) or particulate Mn (III) oxyhydroxides (Tebo et al., 2004), preparation of biologically active MnO<sub>2</sub> was performed in the laboratory using 0.1 M KMnO<sub>4</sub>, 0.1 M NaOH and 0.1 M MnCl<sub>2</sub> (with ratio volume of 2/4/3, respectively) (Laha and Luthy

1990). Mn-oxides were collected by centrifugation and washed several times with distilled water. This method of synthesis produces Mn-oxides structurally similar to naturally occurring mineral birnessite ((Na<sub>0.3</sub>Ca<sub>0.1</sub>K<sub>0.1</sub>) (Mn<sup>4+</sup>, Mn<sup>3+</sup>)<sub>2</sub> O<sub>4</sub>·1.5 H<sub>2</sub>O). Moreover, since the reactivity of Mn-oxide suspensions may change with long storage, they were used within 24 h after preparation to maintain bioavailability.

### 2.5. Estimation of N<sub>2</sub> production rates

Potential rates of anammox and denitrification were estimated by the method of Risgaard-Petersen et al. (2003), modified according to the work of Minjeaud et al. (2008). Briefly, 5 mL sediment samples from each section of the core were transferred to 22 mL headspace vials. Five mL of filter-sterilized seawater collected from the sampling site was added to the vials. The vials were capped with butyl rubber stoppers and crimped with aluminium seals. The headspace gas in the vials was replaced with helium gas by repeated vacuuming and purging. Vials containing oxygen free sediment suspension were pre-incubated for 3-5 h in order to eliminate any remaining traces of oxygen in the headspace. To re-affirm anaerobic conditions in the test vials, the oxygen level in gaseous phase was monitored by mass spectrometry until below the detectable limit of ~ 15 ppm. Positive pressure (0.15 MPa) was added to the headspace to prevent unintentional contamination with ambient air during the incubation and the gas sampling.

Two parallel incubations were also performed: (i) incubation with 100 μmol L<sup>-1</sup> <sup>15</sup>NO<sub>3</sub><sup>-</sup> (<sup>15</sup>N atom%, 98%) and (ii) incubation with 100 μmol L<sup>-1</sup> <sup>15</sup>NH<sub>4</sub><sup>+</sup> plus 100 μmol L<sup>-1</sup> <sup>14</sup>NO<sub>3</sub><sup>-</sup>. Each condition was duplicated with or without amendment of fresh Mn-oxides (final concentration adjusted to 100 μmol L<sup>-1</sup>). This resulted in sets of twelve vials, which were prepared in five replicates for each station. Argon at a final headspace concentration of 1% was added to all test vials as an internal standard for mass spectrometry. Microbial activities in each set were terminated after 0, 2, 4, 6 or 8 h at *in situ* temperature by injecting 0.25 mL 7 M ZnCl<sub>2</sub>.

The headspace gas samples were analysed by mass spectrometry (Quadrupole mass spectrometer *Anagaz 100*, MKS, Cheshire, UK) (Minjeaud et al., 2008) to quantify each N<sub>2</sub> isotope (<sup>28</sup>N<sub>2</sub>, <sup>29</sup>N<sub>2</sub> and <sup>30</sup>N<sub>2</sub>). Potential N<sub>2</sub> production, anammox and denitrification rates were calculated from the production rates of <sup>14</sup>N<sup>15</sup>N (<sup>29</sup>N<sub>2</sub>) and <sup>15</sup>N<sup>15</sup>N (<sup>30</sup>N<sub>2</sub>) in the test vials according to the method of Risgaard-Petersen et al. (2003) and Minjeaud et al. (2008). In the first set of incubations, N<sub>2</sub> production based on NO<sub>3</sub><sup>-</sup> originating from the overlying water (P<sub>w</sub>) or produced in the sediment via nitrification (P<sub>n</sub>) was quantified using the comprehensive method as outlined by Risgaard-Petersen et al. (2003). In the slurry systems, <sup>14</sup>N-NO<sub>3</sub><sup>-</sup> would be produced only from <sup>14</sup>N-NH<sub>4</sub><sup>+</sup> via nitrification and P<sub>n</sub> reflected the subsequent <sup>14</sup>N-NO<sub>3</sub><sup>-</sup> reduction to N<sub>2</sub>. Under strict anaerobic condition no P<sub>n</sub> could occur except if <sup>14</sup>N-NO<sub>3</sub><sup>-</sup> was produced by an alternative process. In the second set of incubations, we quantified N<sub>2</sub> production based on <sup>15</sup>NH<sub>4</sub><sup>+</sup> (P15) to confirm the stimulation of anaerobic oxidation of NH<sub>4</sub><sup>+</sup> to N<sub>2</sub> in presence of Mn-oxides. Production of P15 (N<sub>2</sub> production from <sup>15</sup>NH<sub>4</sub><sup>+</sup> labelling) permits to follow NH<sub>4</sub><sup>+</sup> oxidation to N<sub>2</sub> but does not discriminate anammox from P<sub>n</sub> (<sup>15</sup>NH<sub>4</sub><sup>+</sup> oxidation to NO<sub>x</sub> and its subsequent reduction to N<sub>2</sub>).

## 2.6. Nitrous oxide measurement

Nitrous oxide isotopes (molecular masses 45 and 46) produced in the incubation vials were quantified by mass spectrometry (Quadrupole mass spectrometer *Anagaz 100*, MKS, England). Total N<sub>2</sub>O produced was obtained by correction from isotopic fractionation (Lloyd & Scott, 1983) and the concentration has been expressed as nmol L<sup>-1</sup> h<sup>-1</sup>.

## 2.7. Statistical analysis

Student's *t*-test was performed on the dataset to evaluate the differences between activities of the N cycle using XLSTAT software 2008 (*Addinsoft*, Paris, France).

# 3. Results

## 3.1. Sediment characteristics

The down-core variations in the concentrations of major compounds involved in the N and Mn cycle during October and January have been depicted in Fig. 2. In October, the NH<sub>4</sub><sup>+</sup> concentration-depth profile was characterized by different concentration gradients through the sediment column. The profile showed two main convex-shaped zones of NH<sub>4</sub><sup>+</sup> generation i.e. from 0-6 and 10-17 cm. In contrast, the NH<sub>4</sub><sup>+</sup> concentration-depth profile in January was characterized by a constant concentration gradient. The highest measured values of NH<sub>4</sub><sup>+</sup> were obtained at the deeper depth during October and January reaching up to 277.0 μmol L<sup>-1</sup>. Profiles of oxidized forms of nitrogen (NO<sub>x</sub>) which corresponds to the sum of NO<sub>3</sub><sup>-</sup> plus NO<sub>2</sub><sup>-</sup> showed lower values compared to NH<sub>4</sub><sup>+</sup> (Fig. 2). The maximum concentrations of NO<sub>x</sub> were observed at the surface with values of up to 5.83 and 1.57 μmol L<sup>-1</sup> during October and January respectively. Though NO<sub>x</sub> concentration decreased to less than 1 μmol L<sup>-1</sup> below the surface, a peak was observed at around 7 cm depth during both sampling times.

Concentration of ascorbate-extractable Mn-oxides (hereafter referred as Mn-oxides) revealed that whatever the sampling time, the highest concentrations were observed within the first few millimetres. Thereafter, it decreased with depth to remain constant throughout the sediment column. During October, concentration of Mn-oxides in the sediment decreased from 1.11 μmol g<sup>-1</sup> at the surface to 0.63 μmol g<sup>-1</sup> at 17 cm. In January, Mn-oxides varied between 0.47 μmol g<sup>-1</sup> at the surface to 0.20 μmol g<sup>-1</sup> at 5.5 cm. In contrast to the particulate form of Mn, the concentration of its soluble form (Mn<sup>2+</sup>) was higher and its profiles presented varied patterns. In October, a major peak in pore water Mn<sup>2+</sup> concentration was seen below the surface (< 1 cm) corresponding to the zone of decrease in Mn-oxides. Below this depth, Mn<sup>2+</sup> concentration diminished except around 7 cm where concomitant peaks of Mn<sup>2+</sup> and NO<sub>x</sub> were noticed. However in January, concentrations of Mn<sup>2+</sup> were lower and never exceeded 2 μmol L<sup>-1</sup>. A peak in Mn<sup>2+</sup> concentration was seen below the surface (1-3 cm depth). Below 3 cm, a sharp decline in Mn<sup>2+</sup> concentration was observed.

## 3.2. Bacterial activities

Results for potential bacterial N<sub>2</sub>-producing activities (P<sub>tot</sub>) are tabulated in Table 1. Potential N<sub>2</sub> production rates varied by a factor of up to 3.2 (October) and 4.4 (January) along the sediment depth

(Table 1). This is largely due to high denitrification associated  $N_2$  production ( $29.09 \mu\text{mol L}^{-1} \text{h}^{-1}$ ) at the surface in January. Interestingly, the highest contribution of anammox to  $N_2$  production (21%) was also observed at the same depth during this period. Thus,  $^{15}\text{NO}_3^-$  labelling revealed denitrification to be the main process of  $N_2$  production with minimal contribution from anammox.

Estimation of  $N_2$  production based on  $\text{NO}_3^-$  from the overlying water ( $P_w$ ) or produced through the oxidation of  $\text{NH}_4^+$  in the sediment ( $P_n$ ) was further discriminated in microcosms with or without amendment with Mn-oxides according to Risgaard's calculations (Risgaard-Petersen et al., 2003). In October, potential  $N_2$  production through  $P_w$  was not significantly affected by addition of Mn-oxides (Fig. 3a). As expected under anaerobic conditions, very low natural  $P_n$  rates (without amendment of Mn-oxides) of  $\leq 1.23 \pm 0.80 \mu\text{mol L}^{-1} \text{h}^{-1}$  were observed except for the deeper layer where they were almost  $3 \times$  greater (Fig. 3b). In contrast, when Mn-oxides were added to the sediment slurries, a significant stimulation of  $P_n$  was observed between 0-4.5 cm with  $N_2$  production i.e.  $25.42 \pm 3.29 \mu\text{mol L}^{-1} \text{h}^{-1}$  at 0-2.5 cm ( $t$ -test,  $n = 6$ ,  $p < 0.04$ ) and  $9.04 \pm 0.59 \mu\text{mol L}^{-1} \text{h}^{-1}$  at 2.5-4.5 cm ( $t$ -test,  $n = 6$ ,  $p < 0.00001$ ). During January,  $P_w$  presented a conventional pattern with maximum activity in the upper layers followed by a decrease with depth (Fig. 3c). The benthic system was highly heterogeneous with elevated natural  $P_n$  rates under anaerobic conditions (Fig. 3d). On addition of Mn-oxides to the sediments, elevated  $N_2$  production was observed at all depths. Highest  $N_2$  production of  $16.02 \pm 9.94 \mu\text{mol L}^{-1} \text{h}^{-1}$  was recorded between 4.5-8.5 cm. It may be noted that larger standard deviation values for  $P_n$  rates observed in the presence of Mn-oxides are indicative of greater variability between test incubations during this sampling (Fig. 3d).

For samples collected during October, one set of test vials were spiked with  $^{15}\text{NH}_4^+$  and  $^{14}\text{NO}_3^-$  to examine  $N_2$  production based on  $^{15}\text{NH}_4^+$  (P15). The  $N_2$  produced could originate from anammox and/or nitrification coupled to denitrification since the labelling procedure did not permit to discriminate these two pathways. A significant increase in P15 ( $t$ -test,  $n = 6$ ,  $p < 0.05$ ) was observed at all depths in the presence of Mn-oxides (Fig. 4).

The production of  $N_2O$ , a powerful greenhouse gas, was also investigated at both sampling times with or without amendment of Mn-oxides (Fig. 5). Without Mn-oxides,  $N_2O$  production was almost undetectable at both sampling times and at all depths. With Mn-oxides, significant higher production of  $N_2O$  was observed whatever the sampling time ( $t$ -test,  $n = 10$ ,  $p < 0.0001$  for all samples), except during January at 8.5-12 cm. During October,  $N_2O$  production in the presence of Mn-oxides was maximal at 8.5-12 cm occurring at a rate of  $94.5 \pm 30.8 \text{ nmol } N_2O \text{ L}^{-1} \text{ h}^{-1}$ . In January, two maxima were observed i.e.  $37.9 \pm 5.2 \text{ nmol } N_2O \text{ L}^{-1} \text{ h}^{-1}$  in the upper 2.5 cm layer in which a peak of  $\text{Mn}^{2+}$  has also been recorded in the sediment column and between 12-17 cm ( $66.60 \pm 13.7 \text{ nmol } N_2O \text{ L}^{-1} \text{ h}^{-1}$ ) for which the concentration of nutrients at this depth are unfortunately not available.

#### 4. Discussion

Manganese oxides can be generated by a variety of processes mediated by presence of oxygen (Van Cappellen et al., 1998) and alternative oxidants such as iodate or nitrate (Anschutz et al., 2000; Deflandre et al., 2002). Field observation undertaken in the present study revealed that concentration of Mn-oxides in Arcachon sediments varied from 0.20 to  $1.11 \mu\text{mol g}^{-1}$  (Fig. 2). These values were 5 to  $67 \times$  lower than values reported from the Rhône estuary (Pastor et al., 2011) and continental shelf

sediment of Aquitaine merge (Hyacinthe et al., 2001; Anschutz et al., 2005). Maximum concentration of Mn-oxides was recorded in the first few millimetres of the sediment, followed by a decline with depth (Fig. 2). These Mn-oxides can be reduced to  $\text{Mn}^{2+}$  by indirect abiotic processes involving sulphur or intermediates of fermentation or by biotic processes such as bacterial respiration (Nealson and Saffarini 1994; Johnson et al., 1995). In the Arcachon sediments,  $\text{Mn}^{2+}$  concentration never exceeded  $5.5 \mu\text{mol L}^{-1}$  (Fig. 2). This value is lower to that reported for organically-rich marine sediments where the concentration of  $\text{Mn}^{2+}$  can vary from 18-216  $\mu\text{mol L}^{-1}$  (Mortimer et al., 2004). We also observed that between both sampling periods, relatively higher concentration of pore water  $\text{Mn}^{2+}$  was recorded in October (Fig. 2a) which could be attributed to reduction of Mn-oxides.

In Arcachon Bay, mineralization processes are intense because surficial intertidal flat sediments are rich in organic matter (Deborde et al., 2008). Products of microbial organic matter mineralisation, i.e.  $\text{NH}_4^+$  diffuse in sediments. During both the sampling periods, pore water  $\text{NH}_4^+$  concentrations showed different trends. In October, a strong vertical zonation was observed. Mineralization processes seemed to be more intense within 0-2 cm, whereas from 2-10 cm, production and consumption were more or less in equilibrium. In contrast, the  $\text{NH}_4^+$  concentration depth profile in January was characterized by a constant concentration gradient indicating that above 9 cm, concentration of the nutrient could be controlled more by diffusive transport.

It is generally accepted that in the presence of oxygen,  $\text{NH}_4^+$  can be oxidized to  $\text{NO}_2^-$  or  $\text{NO}_3^-$  through the nitrification pathway. These inorganic N compounds can diffuse from the oxic to anoxic layer and be subsequently reduced to  $\text{N}_2$  via denitrification or anammox leading to a significant loss of N.  $\text{NO}_x$  concentration in the Arcachon sediments was  $< 1 \mu\text{mol L}^{-1}$  (Fig. 2) except at the surface where a continuous replenishment of these compounds from the ambient seawater could be expected. The observed  $\text{NO}_x$  profiles could therefore be due to contribution of diffusion of the compounds from the overlying seawater, their indigenous production by nitrification and their consumption by dissimilative processes like denitrification or anammox that lead to  $\text{N}_2$  production. Our results have shown that denitrification was the main contributor to  $\text{N}_2$  production in the Arcachon sediments whereas anammox was almost undetectable except at 0-2.5 cm in January (Table 1).  $\text{NO}_x$  concentration and the relative size of the anammox population or their activity are important factors controlling transformation of  $\text{NH}_4^+$  through the anammox pathway (Trimmer et al., 2005). Therefore, favourable conditions for anammoxifiers mainly at  $< 2.5$  cm could have lead to high anammox activity. For other layers and samples,  $\text{N}_2$  production through denitrification superceeded that through anammox and ranged from  $5 \mu\text{mol L}^{-1} \text{h}^{-1}$  to  $29 \mu\text{mol L}^{-1} \text{h}^{-1}$  (Table 1). These values were of the same order of magnitude as those reported in coastal sediments from different geographic locations (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). In Mediterranean coastal sediments, Minjeaud et al. (2009) have also reported denitrification as the main process of  $\text{N}_2$  production with occasional occurrence of anammox at low rates. The relatively low rate of anammox recorded in the present study could not be an artifact of  $\text{NH}_4^+$  limitation in the slurries since concentration of the nutrient in the sediments was  $> 200 \mu\text{mol L}^{-1}$ . Thus, we can infer that  $\text{NO}_x$  reduction is routed through denitrification.



Earlier studies have reported coupling between nitrification and denitrification or anammox in an aerobic-anaerobic gradient. Under oxygen-depleted conditions, aerobic nitrification cannot sustain substrate for  $N_2$  production. Bartlett et al. (2007) have reported the occurrence of nitrification coupled to the reduction of Mn-oxides in anoxic marine sediments based on concomitant peaks of  $NO_x$  and  $Mn^{2+}$ . We occasionally recorded such peaks in the anoxic layer of the Arcachon sediments as reported earlier by Deborde et al. (2008). Based on studies by Mortimer et al. (2004), we interpreted this finding as  $NO_x$  production by anaerobic  $NH_4^+$  oxidation linked to reduction of Mn-oxides. Based on field observations, we assumed that  $NO_x$  produced due to anaerobic oxidation of  $NH_4^+$  would be further reduced to  $N_2$  via denitrification or anammox.

In this study, occurrence of anaerobic nitrification-denitrification mediated by Mn-oxides has been proved by development of an indirect approach using stable isotope tracer which permits to follow the origin of denitrified  $NO_3^-$  in the presence of Mn-oxides.  $^{15}N$  labelling revealed that  $N_2$  production based on  $NO_3^-$  originating from nitrification ( $P_n$ ) in October occurred in the deeper layer (12-17 cm) (Fig. 3b) whereas in January,  $P_n$  values of around  $5 \mu\text{mol L}^{-1} \text{h}^{-1}$  were observed through the sediment column (Fig. 3d). The detection of natural  $P_n$  in anoxic layers is therefore indicative of the occurrence of anaerobic oxidation of  $NH_4^+$  leading to  $NO_x$  production via the nitrification pathway.

Since sediment from Arcachon contained low concentration of Mn-oxides, impact of Mn-oxides amendment was analyzed. We observed that addition of Mn-oxides stimulated  $P_n$  production mainly between 0 and 4.5 cm during October (Fig. 3b) while in January,  $P_n$  increase was observed at all depths (Fig. 3d).  $P_n$  stimulation at  $< 4.5$  cm in October coincided with lowest values of natural  $P_n$  (without Mn-oxides addition) and the highest pore water  $Mn^{2+}$  concentration. This increase could be directly dependent on the lack of availability of substrate as outlined above along with a high abundance of reactive Mn in the sediments and  $NO_x$  reducing prokaryotes. The most prominent increase of  $P_n$  in the presence of Mn-oxides was observed for surface layers ( $< 4.5$  cm) during October. We confirmed this hypothesis by counter-labelling with  $^{15}NH_4^+$ ,  $^{14}NO_3^-$  and amendment with Mn-oxides. The increase in  $P_{15}$  ( $N_2$  production from  $^{15}NH_4^+$ ) (Fig. 4) indicated that  $^{15}NH_4^+$  was oxidized to  $NO_x$  and subsequently reduced to  $N_2$ . Thus, substantial stimulation of  $^{15}NH_4^+$  oxidation to  $N_2$  (based on increase in the rates of  $P_n$  and  $P_{15}$ ) provided indirect evidence on anaerobic nitrification-denitrification mediated by Mn-oxides. It could be argued that  $N_2$  production could be due to a stimulation of denitrification or anammox. Quite clearly, that was not the case since potential  $N_2$  production through  $NO_3^-$  from the water column ( $P_w$ ) was not significantly affected by addition of Mn-oxides. Furthermore, the increase of  $N_2$  production by Mn-oxides doesn't seem to be influenced by denitrification/anammox ratio. In consequence, we believe that Mn-oxides favour  $NO_x$  production and fuel  $N_2$  producing processes.

In Arcachon Bay, Rysgaard et al. (1996) have reported aerobic nitrification rate of  $3.3 \text{ mmol m}^{-2} \text{ d}^{-1}$  (Rysgaard et al., 1996) which is higher compared to those reported from other shallow marine sediments (Rysgaard et al., 1993, Gilbert et al., 1995). This high nitrification activity oxidized up to 22% of the  $NH_4^+$  produced from organic matter mineralisation. It was suggested that these high levels were probably caused by high rates of  $NH_4^+$  regeneration originating from decomposition of organic matter. Based on integration of anaerobic nitrification rates within 0-10 cm which were determined without amendment with Mn-oxides, we can estimate a rate of  $0.7 \text{ mmol m}^{-2} \text{ d}^{-1}$

corresponding to about 21% of aerobic nitrification rate previously reported (Rysgaard et al., 1996). The potential rates after amendment with Mn-oxides could reach up to  $14.4 \text{ mmol m}^{-2} \text{ d}^{-1}$  indicating that the process could be ecologically important in controlling  $\text{NH}_4^+$  accumulation in the benthic system.

For the first time, laboratory incubation experiments in the present study have shown a significant increase in  $\text{N}_2\text{O}$  production on addition of Mn-oxides during both samplings (Fig. 5). It should be noticed that maximum  $\text{N}_2\text{O}$  production in October corresponded to the zone where a secondary peak of  $\text{NO}_x$  and  $\text{Mn}^{2+}$  had been detected (Fig. 2a). Javanaud et al. (2011) have demonstrated bacterially-mediated  $\text{N}_2\text{O}$  production to occur in the absence of  $\text{NO}_3^-$  but with Mn-oxides and  $\text{NH}_4^+$  amendment giving an indirect proof of  $\text{NO}_x$  production. In the presence of Mn-oxides, denitrifiers could switch over to performing nitrification under nitrate-limited conditions and produce  $\text{NO}_3^-$ , which is further reduced to  $\text{N}_2\text{O}$  (Javanaud et al., 2011). The radiative gas  $\text{N}_2\text{O}$  is known to be produced as an intermediate of multiple pathways in natural environment. However, the mechanisms and contribution of ammonia oxidation pathways (nitrifier nitrification, nitrifier denitrification, and coupled nitrification-denitrification) and heterotrophic denitrification to  $\text{N}_2\text{O}$  production is poorly understood as well as their dependence on environmental factors like concentrations of oxygen and inorganic N compounds. Zhu et al. (2013) have shown  $\text{N}_2\text{O}$  production via nitrification to increase as  $\text{O}_2$  concentrations decreased from 21% to 0.5%. These authors suggest ammonia oxidation pathways and nitrifier denitrification as significant sources of  $\text{N}_2\text{O}$ .

## 5. Conclusion

Field observations from the Arcachon Bay provided indirect evidence on the occurrence of anaerobic nitrification coupled to the reduction of Mn-oxides. Further investigations using sediment slurry samples labelled with  $^{15}\text{NO}_3^-$  and amended with Mn-oxides showed significant increase in rate of  $\text{N}_2$  production mainly through anaerobic nitrification-denitrification. Elevated  $\text{N}_2$  production in incubations amended with  $^{15}\text{NH}_4^+$  and Mn-oxides further strengthened the origin of  $\text{N}_2$  through this pathway. The contribution of anammox in  $\text{N}_2$  production was found to be minimal. In oxygen-deficient zones, anaerobic  $\text{NO}_x$  production (through nitrification) mediated by Mn-oxides could be vital for the supply of substrate not only to denitrifiers but also to anammox bacteria that continuously need anaerobiosis and  $\text{NO}_2^-$  for maximal activity. As in the Arcachon Bay, where  $\text{NH}_4^+$  concentration in the sediments are  $100 \times$  higher than those of  $\text{NO}_x$ , the importance of anaerobic  $\text{NO}_x$  production mediated by Mn-oxides in eutrophication control could be more important than previously thought as it prevents  $\text{NH}_4^+$  accumulation. For the first time, our study revealed enhanced production of the greenhouse gas  $\text{N}_2\text{O}$  through anaerobic nitrification-denitrification in the presence of Mn-oxides. Thus, the process has the potential for stimulating  $\text{N}_2\text{O}$  productivity and its consequent emission from the benthic system during enhanced supply of Mn-oxides from the surficial layers via sediment re-working by bioturbators or physical mixing. Due to the complexity of the interactions between the different microbial communities involved in N and Mn cycles, further studies are necessary to identify major factors controlling the occurrence of anaerobic nitrification-denitrification mediated by Mn-oxides. This would provide a better understanding of the wider ecological significance of the process.

## Acknowledgements

The authors are thankful to CNRS (Centre National de la Recherche Scientifique) grants from the INSU -EC2CO –Microbien 2010 program and ANR Protidal and Izoflux. SOF is grateful to the Council of Scientific and Industrial Research (Govt. of India) for the award of *Research Associateship*, 2011. The authors are grateful to two anonymous reviewers who critically evaluated this manuscript and provided many useful suggestions that have helped us improve its quality. This is NIO contribution no. XXXX.

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## Legend to figures

**Fig. 1.** Location of sampling site (star) in the Arcachon bay. Grey areas indicate intertidal flats.

**Fig. 2.** Down-core profiles of principal compounds of N and Mn cycles ( $\text{NH}_4^+$ ,  $\text{NO}_x$ , pore water  $\text{Mn}^{2+}$ , Mn-oxides) in the sediment of Arcachon Bay during October 2007 (a) and January 2008 (b).  $\text{NO}_x$  corresponds to  $\text{NO}_2^-$  and  $\text{NO}_3^-$  content. Due to analytical problems, no data could be generated in January at  $>10$  cm.

**Fig. 3.** Down-core rates of potential  $\text{N}_2$  production through anammox + denitrification using  $\text{NO}_3^-$  from seawater ( $\text{P}_w$ ) (a) or  $\text{NO}_x$  produced in situ at the expense of ammonium ( $\text{P}_n$ ) (b) during October. In January,  $\text{N}_2$  production through  $\text{P}_w$  and  $\text{P}_n$  are shown in Fig. 3c and 3d respectively. Slurries have been subjected to  $^{15}\text{NO}_3^-$  amendment only (white bars) or with  $^{15}\text{NO}_3^-$  + Mn-oxides (hatched bars). Error bars represent SDs.

**Fig. 4.** Anaerobic  $\text{P}_{15}$  production rates in sediment slurry incubations during October. In the presence of  $^{15}\text{NH}_4^+$  and  $^{14}\text{NO}_3^-$  labelling,  $\text{P}_{15}$  corresponds to  $\text{N}_2$  production arising from anammox + denitrification and originating from the added  $^{15}\text{NH}_4^+$ . Slurries have been subjected to  $^{15}\text{NH}_4^+$  and  $^{14}\text{NO}_3^-$  without (white bars) or with Mn-oxides (grey bars). Error bars represent SDs.

**Fig. 5.** Down-core variations in production of nitrous oxide during October (a) and January (b). Slurries have been subjected to  $^{15}\text{NO}_3^-$  amendment only (white bars) or with  $^{15}\text{NO}_3^-$  + Mn-oxides (dark bars). Error bars represent SDs.

**Table 1**

Potential N<sub>2</sub> production rates expressed in  $\mu\text{mol L}^{-1} \text{h}^{-1}$ . Values in bracket are the percentage of anammox production.

Depth (cm)	P <sub>tot</sub> in October ( $\mu\text{mol L}^{-1} \text{h}^{-1}$ )	P <sub>tot</sub> in January ( $\mu\text{mol L}^{-1} \text{h}^{-1}$ )
0-2.5	8.49 (3.2%)	29.09 (21%)
2.5-4.5	6.27 (0%)	10.62 (3.8%)
4.5-8.5	6.67 (0%)	12.79 (0%)
8.5-12	5.26 (0%)	6.57 (1.5%)
12-17	16.76 (0%)	19.84 (3.8%)

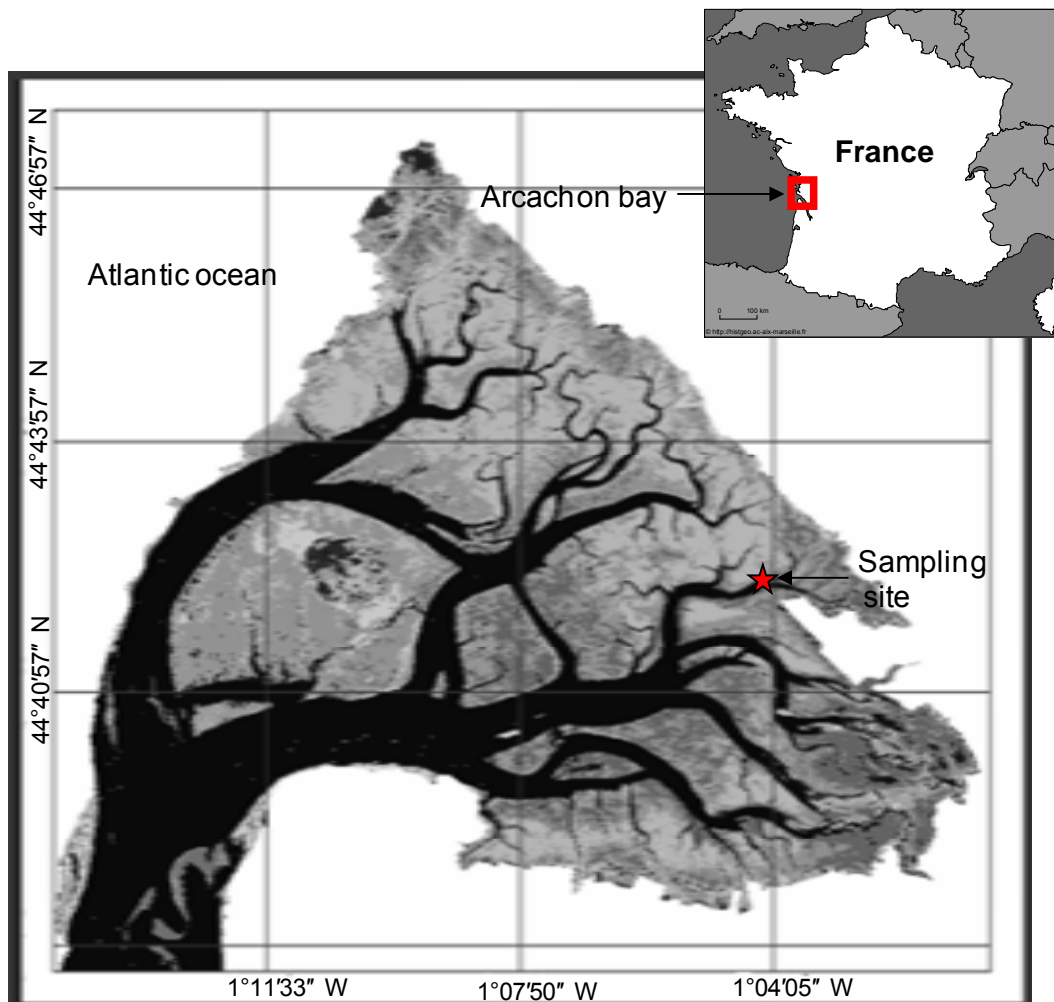


Fig. 1



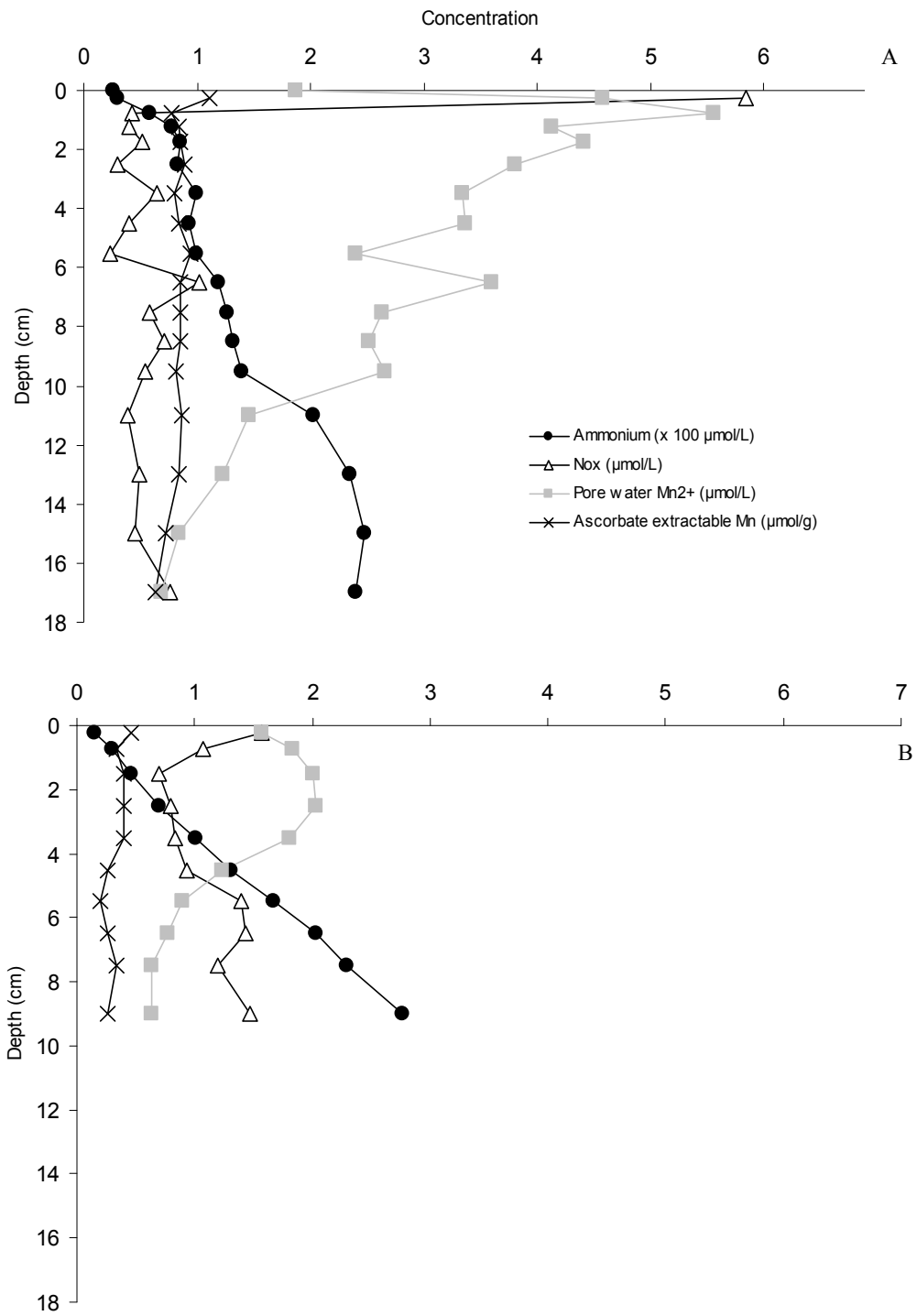


Fig. 2

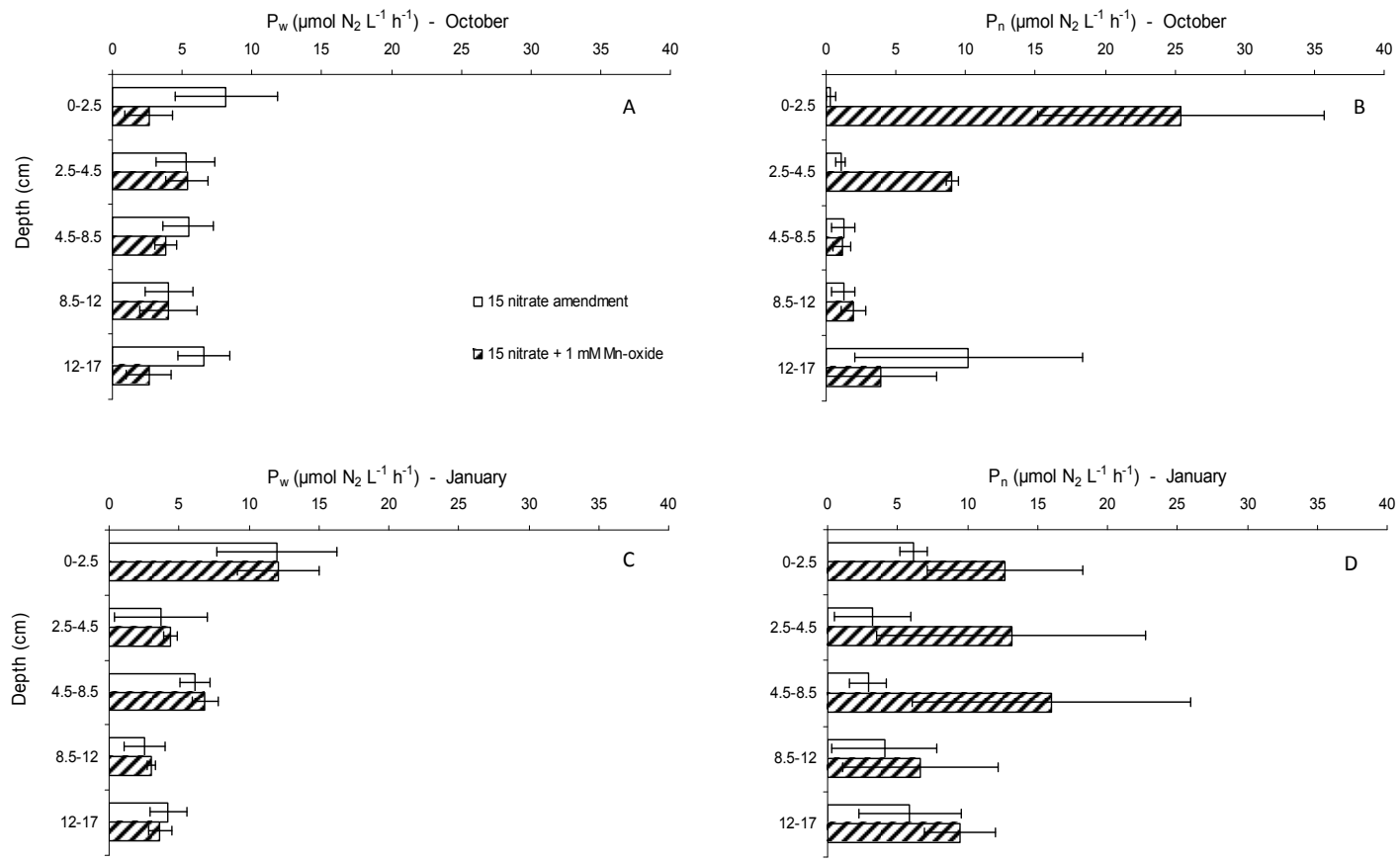


Fig. 3

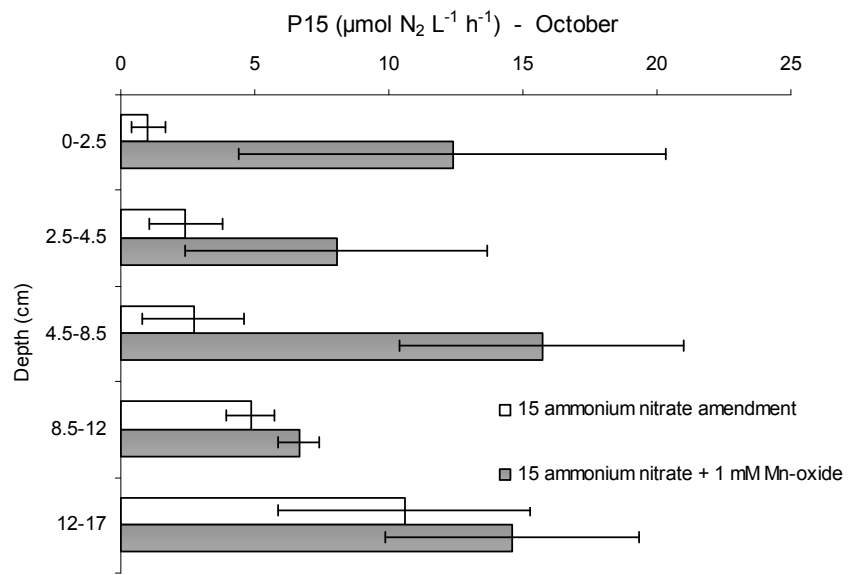


Fig. 4

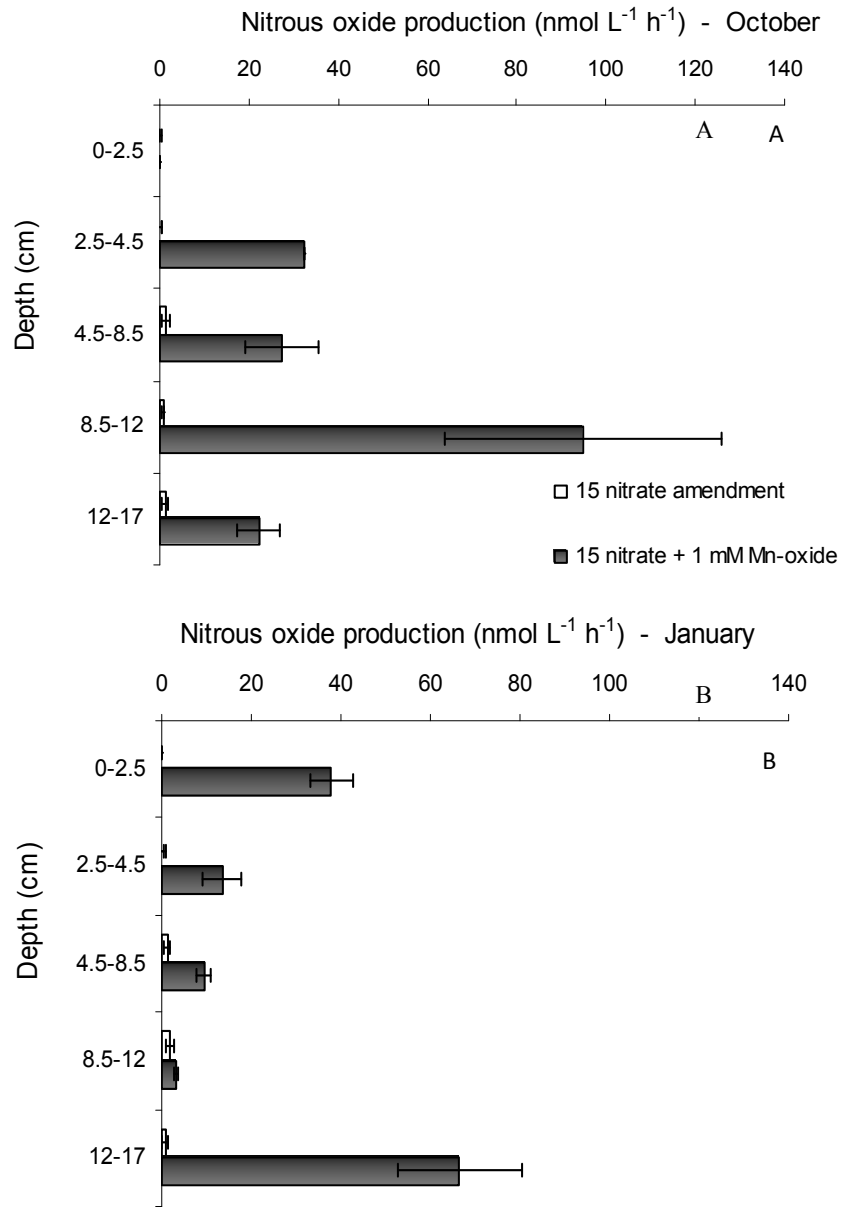


Fig. 5