Potential rates and environmental controls of anaerobic ammonium oxidation in estuarine sediments

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ABSTRACT: The importance of anaerobic ammonium oxidation (anammox)—a metabolic pathway that can generate dinitrogen—remains poorly described in temperate estuarine systems. We evaluated the relative importance of anammox and denitrification along the salinity gradient of the Cávado River estuary (NW Portugal) during a seasonal survey. Potential rates of anammox and denitrification were measured in anaerobic sediment slurries using 15N-labeled NO3− and NH4+ amendments. Production of 29N2 and 30N2 in the slurries was quantified using membrane inlet mass spectrometry (MIMS). Environmental parameters such as salinity, temperature and inorganic nitrogen were also monitored. Anammox and denitrification potentials in Cávado estuarine sediments varied from 0 to 3.3 and from 1.1 to 10.8 nmol N cm−3 wet sediment h−1, respectively. During 1 sampling occasion, anammox activity accounted for as much as 72% of the measured production of dinitrogen, while annual averages varied from 17 to 33% depending on location. Nitrate availability and temperature appeared to be the primary environmental controls of anammox. Higher concentrations of NO3− and intermediate temperatures in the estuarine water (14 to 16°C) supported higher anammox activities. Using 16S rRNA gene-specific primers, anammox-like bacterial sequences were recovered from Cávado sediments—corroborating the measured anammox activities. Our results support the environmental importance of anammox for removal of nitrogen in estuarine sediments, and suggest that this process is apparently regulated by few variables.

KEY WORDS: Anammox · Denitrification · Sediments · Estuary

INTRODUCTION

Microbial nitrogen cycling plays an important role in the maintenance of the biosphere (Gruber & Galloway 2008). In the last 100 yr, our perception of the nitrogen cycle has shifted from its role in promoting agricultural crop yields to an increasing awareness of the consequences of human interference (Galloway et al. 2008). Worldwide, the amount of soluble nitrogenous compounds entering estuaries and coastal waters from land, rivers and the atmosphere has increased dramatically in recent decades (Gruber & Galloway 2008). The anthropogenic influence on the global nitrogen cycle has contributed to a suite of environmental problems, such as the decrease in forest and grassland productivity and global climate change (Galloway et al. 2003). Also, the human disturbance of nitrogen fluxes may ag-
gravitate coastal eutrophication problems (Fulweiler et al. 2007).

Until recently, denitrification was thought to be the only major pathway that forms dinitrogen in the environment and removes inorganic nitrogen from aquatic systems (Herbert 1999). This process has been investigated thoroughly, revealing that a large fraction of the external nitrogen load delivered to coastal ecosystems is removed via sediment denitrification (e.g. Seitzinger et al. 2006), thereby reducing both anthropogenic eutrophication and nitrogen pollution of the open ocean (Seitzinger et al. 2006).

Indications that an ammonium oxidation pathway occurs in anoxic environments have appeared occasionally in the literature since 1941 (Hamm & Thompson 1941, Dalsgaard et al. 2005 and references therein). However, only in the 1990s was the process demonstrated to occur on wastewater sludge in a denitrifying reactor (Mulder et al. 1995). Later on, Thamdrup & Dalsgaard (2002) provided evidence of anammox activity in natural habitats, adding to our understanding of the biological nitrogen cycle. Anammox — anaerobic ammonium oxidation — uses nitrite as an electron acceptor, producing dinitrogen as the final product (van de Graaf et al. 1995). The process is mediated by obligatory anaerobic chemolithotrophic bacteria identified as members of the Planctomycetes (Strous et al. 1999). Anammox contributes significantly to dinitrogen production in the ocean, being recognized as a major sink for fixed inorganic nitrogen in anoxic water basins (Dalsgaard et al. 2003, Kuypers et al. 2005, Lam et al. 2009) and marine sediments, accounting for as much as 79% of dinitrogen production in continental shelf sediments (Thamdrup & Dalsgaard 2002, Engström et al. 2005). The fate of excess nitrogen in estuaries is determined by the activity of denitrifiers and anammox bacteria, although the relative importance of the 2 processes — anammox and denitrification — is still unclear, with anammox rates so far reported to account for between 0 and 30% of the measured production of dinitrogen (Trimmer et al. 2003, Rysgaard-Petersen et al. 2004, Rich et al. 2008, Dale et al. 2009, Dong et al. 2009, Nicholas & Trimmer 2009). Differences in the relative importance of denitrification and anammox may be controlled by the environmental variables that favor one process over the other.

The environmental factors that control rates of denitrification have been investigated in some detail (e.g. Cornwell et al. 1999, Magalhães et al. 2005a, Jensen et al. 2009). Nitrate (Christensen et al. 1990), organic carbon (Nixon 1981, van Luijn et al. 1999), and the activity of both benthic fauna and flora (Rysgaard et al. 1995, Cornwell et al. 1999) are among the most important controlling factors of denitrification so far identified.

However, information on the regulation of anammox in aquatic systems is scarce. Four main factors were previously reported to regulate anammox in aquatic sediments: temperature (Dalsgaard & Thamdrup 2002, Rysgaard et al. 2004), concentration of NO3− in the water (Trimmer et al. 2003, Risgaard-Petersen et al. 2004, Rysgaard et al. 2004, Rich et al. 2008, Nicholas & Trimmer 2009), concentration of NO2− in sediment (Meyer et al. 2005), and the content of organic carbon (Trimmer et al. 2003, Nicholas & Trimmer 2009).

In this study, we evaluated the spatial and temporal variation of anammox and denitrification along the Cávado River estuary and focused on identifying the environmental factors that regulate differences in the levels of anammox and the relative contribution of anammox to the production of dinitrogen.

MATERIALS AND METHODS

Study area and sampling

Sediment samples were collected from 3 intertidal sites at low tide along the salinity gradient in the Cávado River estuary (NW Portugal) (Fig. 1) in August and November 2007, and in January and April 2008. The Cávado River is 135 km in length with a watershed of 16 000 km2; it flows at an average rate of 66 m3 s−1 into the Atlantic Ocean at 41.54°N, 8.79°W. The Cávado estuary spreads over 2.56 km2 and experiences a semi-diurnal mesotidal regime (0.2 to 3.9 m). Freshwater nitrate inputs have ranged from 45 to 141 µM over the past 20 yr (annual mean values collected from the National Information System of Water Resources; INAG 2010). At each sampling site, a total of 10 sediment cores (3 cm diameter, 5 cm deep) were collected, combined, homogenized, and stored in sterile plastic bags. Pore-water samples were drawn using a pore-water ‘sipper’ with a perforated ending (Ø 1 to 2 mm holes) inserted to a depth of 5 cm and attached to a Teflon sampling tube; suction was provided by a hand-operated vacuum pump. Estuarine water was also collected at each site and stored in acid-cleaned polyethylene bottles. The water column temperature and salinity were measured in situ using a portable probe (YSI, model 30). Sediment oxidation−reduction potential (Eh) within the 5 cm depth was measured in situ using a platinum electrode (Hanna Instruments). The electrode
was regularly checked using a standard redox solution (HI7030, Hanna Instruments). Samples were immediately stored in a dark cooler and transported to the laboratory. In the laboratory, subsamples of sediment were separated and stored at −70°C for extraction of DNA. The estuarine water and pore-water from each site was immediately filtered (0.45 µm), and samples were processed according to the analytical and incubation procedures described below.

Analytical procedures

Concentrations of nitrite (NO$_2^-$) and ammonium (NH$_4^+$) in estuarine water and pore-water were determined colorimetrically using methods described in Grasshoff et al. (1983). Nitrate (NO$_3^-$) was assayed using an adaptation of the spongy cadmium reduction technique (Jones 1984). All the analyses were performed in triplicate, and standard curves were generated each day. The detection limit for NH$_4^+$, NO$_2^-$ and NO$_3^-$ was 0.1, 0.02 and 0.3 µM, respectively; the precision of all determinations was between 0.1 and 8%, depending on the particular nutrient concentration. Sediment organic matter (OM) was measured as a percentage of weight loss on ignition (500°C, 4 h). Grain size analysis was performed by dry sieving (Percival & Lindsay 1997). Determination of dissolved organic carbon (DOC) and total nitrogen (TN) was performed by high-temperature catalytic oxidation with a TOC-VCSN analyzer coupled to a total nitrogen-measuring unit (Shimadzu Instruments) according to Magalhães et al. (2008).

$^{15}$N sediment incubations and analysis

Potential anammox and denitrification activities were assessed in endpoint experiments, following the procedures of Risgaard-Petersen et al. (2004), with some modifications. For each homogenized sample and treatment, 3 replicates of 5 ml of sediment were transferred to Hungate glass tubes (Belco Glass) with a cut-bottom fitted with rubber stoppers; the tubes were flushed with argon. Ten milliliters of Ar-purged estuarine water were added, and the tubes were sealed, without headspace, using gas-tight caps with a septum. There were 3 treatments for each sample: (1) unamended control, (2) addition of 50 µM K$^{15}$NO$_3^-$ (>98% $^{15}$N atom, Isotec), and (3) addition of 5 µM $^{15}$NH$_4^+$ (>98% $^{15}$N atom, Isotec). 14NO$_3^-$ and 14NH$_4^+$ were supplied by the site water. One extra set of triplicate slurries (time zero) was ‘killed’ immediately by injection of 200 µl of a 7 M ZnCl$_2$ solution. Isotope solutions were added through the septum to the amended tubes. Slurries were incubated, in the dark, at in situ temperature (24, 16, 11 and 14°C for August, November, January and April campaigns, respectively). After 6 h, biological activity was stopped by the addition of ZnCl$_2$ (200 µl, 7 M). A 4 ml supernatant sub-sample was collected, immediately filtered (0.45 µm), and stored frozen for subsequent determination of NO$_3^-$, NO$_2^-$ and NH$_4^+$. The abundance of $^{29}$N$_2$ and $^{30}$N$_2$ was measured directly in the sediment slurry using a membrane inlet mass spectrometer (MIMS; Kana et al. 1994) consisting of a quadrupole mass spectrometer (QMS422, PfeifferBalzers) and fitted with a heated copper reduction column (Eyre et al. 2002). Relative levels of anammox and denitrification activities were calculated from the production of $^{29}$N$_2$ and $^{30}$N$_2$ in the samples amended with $^{15}$NO$_3^-$ using the equations of Thamdrup & Dalsgaard (2002). The isotopic dilution of the added $^{15}$N tracer was calculated from the ambient concentrations of NO$_3^-$ and NH$_4^+$ measured in the ‘killed’ controls and the amount of added $^{15}$N species. $^{15}$N
labeling varied from 32 to 65% of the nitrate pool, and from 4 to 16% of the ammonium pool. Linearity of the denitrification process during the incubation period was assessed in a previous time-series experiment using the acetylene blockage technique (data not shown).

**Molecular techniques**

Total community DNA was extracted from 1 g wet wt of sediment using a Power Soil DNA isolation kit (MoBio Laboratories), following the manufacturer’s instructions. A nested PCR approach was used to detect anammox bacterial 16S rRNA gene sequences in these sediments. In the first PCR we amplified Planctomycetales-specific 16S rRNA genes by using the Pla46 primer (Neef et al. 1998) and the 1392r universal bacterial primer (Muyzer et al. 1993). DNA amplification was performed using Ready-to-Go PCR beads (GE Healthcare) in reactions containing 1 µl of template DNA (5 to 15 ng) and 20 pmol of each primer in a final volume of 25 µl. All PCRs were performed with initial denaturation at 94°C for 4 min, followed by 40 cycles consisting of 94°C for 45 s, 59°C for 50 s, and 72°C for 3 min, with a final extension at 72°C for 5 min. The second PCR was performed with the anammox-specific 16S rRNA gene set of primers Amx 368F (Schmid et al. 2003) and Amx 820R (Schmid et al. 2000), with 1 µl of product from the first PCR reaction as template. Conditions for the second PCR were the same as those used for the first PCR, except that we used a 1 min extension at 72°C (Rich et al. 2008). PCR products were examined in 1.5% agarose gels by electrophoresis, and bands of the correct size were extracted and purified with the GFX PCR DNA and Gel Band purification kit (GE Healthcare). The amplicons were cloned using the TOPO TA cloning kit (Invitrogen). Plasmid-DNA was isolated using the GeneElute plasmid miniprep kit (Sigma). Clones were sequenced at STABVIDA Sequencing Facilities (Lisbon, Portugal). Sequences of the 16S rDNA inserts were determined by using M13 forward and reverse primers targeting vector sequences adjacent to the multiple cloning site. All sequences were compared to reference sequences from GenBank using the basic local alignment search tool (BLAST; Altschul et al. 1990). Sequences were aligned with Clustal W (Thompson et al. 1994) as implemented in Bioedit version 7.0.5 (Hall 1999). Neighbor-joining phylogenetic trees with bootstrapping were produced using MEGA software package version 5.0 (Tamura et al. 2011). Anammox bacteria-related sequences were published in GenBank under accession numbers JF797322 to JF797330.

**Data analysis**

Before statistical analysis was carried out, environmental data were tested for normality and homogeneity of variances. Temporal and spatial differences were examined using 1-way analysis of variance (1-way ANOVA) followed by a post hoc Tukey honestly significant difference (HSD) multi-comparison test. ANOVA and correlation analysis were performed using the software STATISTICA 6.0 (StatSoft). The significance level used for all tests was 0.05.

**RESULTS**

**Water and sediment characteristics**

Measured water, pore-water and sediment variables are summarized in Table 1. Nitrate, nitrite, DOC and TN concentrations correlated negatively with water salinity (r = −0.67, −0.74, −0.69 and −0.66, respectively; p < 0.05, n = 12), showing a longitudinal gradient of nutrients being supplied by the influx of freshwater. Cávado estuary sediments were composed mainly of gravel and coarse sand with a low content of organic matter (Table 1). The content of organic matter was higher in downstream sediments and correlated with salinity (r = 0.80, p < 0.01). Measurements of sediment redox potential showed that anoxic conditions were present occasionally.

**15N experiments**

Slurry 29N2 and 30N2 production was quantified as the end point concentration minus the background. In incubations with added 15NO3−, the production of 29N2 and 30N2 was detected in sediments from all locations (Table 2). The addition of 15NH4+ in the presence of ambient 14NO3− and 14NO2− produced 29N2 in sediments from sites CAV2 and CAV3. At the CAV1 site, during the first 3 surveys, the occurrence of anammox activity was not supported by the 15NH4+ incubations as no 29N2 production was detected. However, the production of 29N2 in our slurries might have been limited by the low 15N labeling of the NH4+ pool. No significant (p > 0.05) 30N2 production was noted in the presence of 15NH4+ (Table 2).
Table 2. Production of $^{29}$N$_2$ and $^{30}$N$_2$ (nmol cm$^{-3}$) in the slurries after incubation gen production. CAV1, CAV2 and CAV3 are sites in the Cávado River estuary, are averages ± SE (n = 3).

### Potential anammox and denitrification activities

Denitrification and anammox activities were measured in sediment slurries under anoxic conditions and during short (6 h) incubations to ensure $^{29/30}$N$_2$ concentration maxima during incubations.

The presence of measurable amounts of NO$_3^-$, NO$_2^-$ and NH$_4^+$ in the slurries at the end of the incubations confirmed that there had been no substrate limitation (data not shown). Denitrification potentials varied spatially, with higher magnitudes observed in the upper estuary ($\bar{X} = 7.8$ nmol cm$^{-3}$ wet sediment h$^{-1}$, CAV3) and lower values being found in the lower estuary ($\bar{X} = 4.6$ nmol cm$^{-3}$ wet sediment h$^{-1}$, CAV1; Fig. 2) (ANOVA, p < 0.05). However, denitrification at all sites and seasons was not linearly correlated with salinity, suggesting that salinity did not have a clear regulatory effect on the denitrification process in Cávado estuary. Moreover, no statistical relationship (p > 0.05) existed between denitrification potential and any of the measured environmental variables.

Potential anammox activities in the Cávado River estuarine sediments ranged from 0 to 3.34 nmol N cm$^{-3}$ h$^{-1}$ (Fig. 2). No significant spatial variations were found (p > 0.05); however, clear minima in anammox activity occurred in summer, when higher temperatures were measured. A scatter plot of incubation temperature and anammox data (Fig. 3a) indicates that anammox
increased up to an optimum temperature (14 to 16°C); activity tended to decrease after that. A progressive increase in potential anammox activity with the increase in availability of NO₃⁻ in the water column was revealed from the positive and linear correlation between these 2 variables (r = 0.75, p < 0.05, n = 12; Fig. 3b).

The relative contribution of anammox to dinitrogen production (ra) was as high as 72%, observed in April at CAV2 (Table 2), with annual averages of 17, 33 and 21% for sites CAV1, CAV2 and CAV3, respectively. TN and pore-water NO₃⁻ concentration were positively correlated with ra (r = 0.78 and r = 0.74, respectively; p < 0.05, n = 12).

Detection of anammox bacteria

A nested PCR approach to amplify the anammox-specific 16S rRNA gene was successful for CAV1 samples, in which anammox bacteria-related sequences were retrieved. The anammox sequences that we detected grouped within the genus Scalindua (Fig. 4). The clone C113b sequence was most similar (99%) to the 16S rRNA gene sequences retrieved from a hydrothermal vent located in the Guaymas basin, Mexico (Hirsch et al. 2011). Clone C12a sequence was most closely related (94% sequence identity) to a sequence from Lake Rassnitzer, Germany (Hamersley et al. 2009). Clone C15b sequence shared 97% sequence similarities with Scalindua wagneri (Schmid et al. 2003). The remaining clones from the CAV1 site positioned phylogenetically between known anammox bacteria and non-anammox members of the Planctomycetes. The 16S rRNA gene sequences retrieved from CAV2 sediments were related to uncultured bacteria outside the planctomycete clade (Fig. 4). No DNA amplification was achieved from CAV3 samples with the molecular approach used.

DISCUSSION

Denitrification potential measured in the Cávado River varied along the estuary, although activity values did not correlate with salinity. Indeed, the activity of denitrifying bacteria is not influenced by the presence of sea salts (Magalhães et al. 2005a), suggesting that halotolerant denitrifying bacteria might inhabit these regions. Nitrate availability is frequently identified as an important environmental control for natural denitrification activity because
maximum denitrification rates usually occur when concentrations of NO$_3^-$ in the water column are greatest (Ogilvie et al. 1997, Magalhães et al. 2005a,b). In the Cávado estuary, higher denitrification potentials were indeed measured at sites with higher concentrations of NO$_3^-$, although no significant relationship ($p > 0.05$) was found with any of the nitrogen species or environmental variables quantified. While spatial differences were observed in upper and lower estuarine locations (CAV1 and CAV3), no clear seasonal patterns of denitrification potential were detected (Fig. 2).

Potential anammox rates in the Cávado River estuary sediments ranged from 0 to 3.34 nmol N cm$^{-3}$ h$^{-1}$ (Fig. 2). Anammox activity was related positively to temperature up to intermediate temperatures of 14 to 16°C, after which activity tended to decrease (Fig. 3a). Similarly, Rysgaard et al. (2004) referred to the anammox response as being psychrophilic after they observed an optimum temperature of 12°C for the activity of anammox bacteria in Arctic sediments. Furthermore, Dalsgaard & Thamdrup (2002) reported a 15°C optimum in temperate shelf sediments. The anammox potentials reported here encompassed 12
different samples from an annual survey, but the temperature optimum was very similar to those reported in single-sample temperature incubation experiments (Dalsgaard & Thamdrup 2002, Rysgaard et al. 2004). Temperature was also described as a selective condition in an enrichment experiment of marine anammox bacteria (van de Vossenberg et al. 2008). The enriched cultures achieved rates similar to those of wastewater anammox bacteria, but at lower temperatures.

Our data also revealed a positive relationship between anammox and the concentration of NO$_3^-$ in the water column. The importance of NO$_3^-$ in regulating anammox has been additionally described in Rander Fjord (Risgaard-Petersen et al. 2004), where NO$_3^-$ was abundant in the water column, as well as in other estuarine systems (e.g. Trimmer et al. 2003, Meyer et al. 2005, Rich et al. 2008) and in arctic marine sediments (Rysgaard et al. 2004). Meyer et al. (2005) showed a strong correlation between NO$_2^-$ production in sediments and anammox activity using microscale pore-water profiles. In estuarine sediments, the supply of NO$_2^-$ via NO$_3^-$ reduction linked to sediment reactivity (e.g. organic matter content) was suggested to regulate the contribution of anammox to total dinitrogen production (Trimmer et al. 2003, Nicholls & Trimmer 2009). However, we observed no relationship between the content of organic matter and anammox activity in our study. The Cávado sediments were composed mainly of large-grain particles where oxic conditions are often present, suggesting that aerobic ammonium oxidation could also be a potential NO$_2^-$ supplier to suboxic sediment layers. Indeed, Nielsen et al (2009) registered high rates of NO$_2^-$ release predominantly associated with incomplete nitrification in sediments from the lower Weser estuary. Although these conditions might occur in situ, this hypothesis was not tested in our experiments—which were performed in anoxic slurry incubations that limit the aerobic oxidation of ammonium. Nonetheless, the relationship we found between the concentration of NO$_3^-$ in the water column and anammox activity suggests that anammox bacteria are fuelled by NO$_2^-$ produced through a NO$_3^-$-reduction process.

While NO$_3^-$ concentrations correlated well with salinity, we observed no variation in anammox activity along the salinity gradient, contrary to reports for other estuaries where anammox decreased seawards (Trimmer et al. 2003, Rich et al. 2008). Regarding the second anammox substrate, ammonium, a relationship between NH$_4^+$ and anammox is not usually detected because this nutrient is seldom limiting in sediments (Dalsgaard et al. 2005).

The percentage contribution of anammox to total production of dinitrogen (ra) was similar to the values documented previously in shelf sediments (Table 3), but was relatively high when compared to studies in shallow coastal (<10 m) and estuarine sediments (e.g. Thamdrup & Dalsgaard 2002, Nicholls & Trimmer 2009); these latter studies reported anammox as a minor process. If anything, the significance of the anammox reaction is probably underestimated, and the ra could be about 5 to 17% higher because our measurements were made in sediment slurries rather than from intact cores (Trimmer et al. 2006). On the other hand, anoxic conditions for anammox to occur might not always be present in Cávado estuarine sediments, altering the relative importance of the denitrification and anammox processes in the removal of nitrogen from this system.

Table 3. Potential anammox rates (nmol cm$^{-3}$ h$^{-1}$) and the contribution of anammox to the production of dinitrogen (ra %) measured in coastal sediments

<table>
<thead>
<tr>
<th>Location</th>
<th>Type of sediment</th>
<th>Anammox</th>
<th>ra %</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aarhus Bay</td>
<td>Subtidal (0–2 cm)</td>
<td>3.5</td>
<td>2</td>
<td>Thamdrup &amp; Dalsgaard (2002)</td>
</tr>
<tr>
<td>Skagerrak</td>
<td>Subtidal (0–2 cm)</td>
<td>1.3–4.1</td>
<td>24–67</td>
<td>Trimmer et al. (2003)</td>
</tr>
<tr>
<td>Thames estuary</td>
<td>Intertidal (0–2 cm)</td>
<td>0.2–9.8</td>
<td>&lt;1–8</td>
<td>Rysgaard et al. (2004)</td>
</tr>
<tr>
<td>Arctic marine sediments</td>
<td>Subtidal (0–4 cm)</td>
<td>0.2–15</td>
<td>1–35</td>
<td>Meyer et al. (2005)</td>
</tr>
<tr>
<td>Randers Fjord</td>
<td>Intertidal (0–0.5 cm)</td>
<td>3.8–11</td>
<td>5–26</td>
<td>Risgaard-Petersen et al. (2004)</td>
</tr>
<tr>
<td>Logan River, Australia</td>
<td>Mangrove sediment</td>
<td>0.5–8</td>
<td>0–9</td>
<td>Engström et al. (2005)</td>
</tr>
<tr>
<td>Skagerrak</td>
<td>Subtidal (0–2 cm)</td>
<td>0.55–3.5</td>
<td>15–79</td>
<td>Amano et al. (2007)</td>
</tr>
<tr>
<td>Long Island Sound</td>
<td>Subtidal (0–2 cm)</td>
<td>0.91–1.2</td>
<td>4–7</td>
<td>Rich et al. (2008)</td>
</tr>
<tr>
<td>Yodo estuary, Japan</td>
<td>Intertidal (0–2)</td>
<td>0.29–0.69</td>
<td>0–22</td>
<td>Dale et al. (2009)</td>
</tr>
<tr>
<td>Chesapeake Bay</td>
<td>Subtidal</td>
<td>0.51</td>
<td>&lt;1–11</td>
<td>Nicholls &amp; Trimmer (2009)</td>
</tr>
<tr>
<td>Cape Fear Estuary</td>
<td>Subtidal (0–3 cm)</td>
<td>3.8–16.5</td>
<td>0–3.34</td>
<td>Present study</td>
</tr>
</tbody>
</table>

*Incubation with $^{15}$NH$_4^+$
Taken together, our results suggest that the anamox process is probably modulated by only a few variables—which might explain the biogeographic stability in the magnitude of anamox activity in different regions (Table 3). Anamox potential rates in the Cávado River estuary sediments (0 to 3.34 nmol N cm⁻³ h⁻¹) were within the range reported for other sediments, such as temperate estuaries and coastal marine sediments (Table 3). Interestingly, as previously suggested (Meyer et al. 2005), such a parallel is usually not found for denitrification rates, whose magnitudes generally differ by several orders of magnitude between different natural ecosystems (e.g. Trimmer et al. 2003, Risgaard-Petersen et al. 2004, Dalsgaard et al. 2005). The variability in denitrification rates is consequently reflected in high variability in the relative importance of anamox to the total production of dinitrogen.

CONCLUSIONS

- The contribution of anamox to the overall production of dinitrogen found in this study was within the high range of values previously reported for other estuarine systems.
- Water column temperature and nitrate concentration were identified as important regulators of anamox activity in the Cávado estuary. These findings strengthen the role of these variables as prime environmental controls of the anamox process.
- These results reinforce the importance of the anamox reaction in removing fixed nitrogen from estuarine systems.

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