Accepted Manuscript

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Authors: Catarina Teixeira, Catarina Magalhães, Rui A.R. Boaventura, Adriano A. Bordalo

PII: S0141-1136(10)00104-2

DOI: [10.1016/j.marenvres.2010.07.001](https://doi.org/10.1016/j.marenvres.2010.07.001)

Reference: MERE 3463

To appear in: *Marine Environmental Research*

Received Date: 29 December 2009

Revised Date: 13 July 2010

Accepted Date: 14 July 2010

Please cite this article as: Teixeira, C., Magalhães, C., Boaventura, R.A.R., Bordalo, A.A. Potential rates and environmental controls of denitrification and nitrous oxide production in a temperate urbanized estuary, *Marine Environmental Research* (2010), doi: 10.1016/j.marenvres.2010.07.001

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Potential rates and environmental controls of denitrification and nitrous oxide production in a temperate urbanized estuary

Catarina Teixeira^{*1,2}, Catarina Magalhães², Rui A.R. Boaventura³, Adriano A. Bordalo^{1,2}

¹Institute of Biomedical Sciences (ICBAS), University of Porto

Laboratory of Hydrobiology, Largo Prof. Abel Salazar 2, 4009-003 Porto, Portugal

²Centre of Marine and Environmental Research (CIIMAR), University of Porto

Rua dos Bragas 289, 4050-123 Porto, Portugal.

³Faculty of Engineering, University of Porto

Department of Chemistry, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

ABSTRACT

Denitrification may play a major role in inorganic nitrogen removal from estuarine ecosystems, particularly in those subjected to increased nitrate and organic matter loads. The Douro estuary (NW Portugal) suffers from both problems: freshwater input of nitrate and organic load from untreated wastewater discharges. To assess how these factors might control sediment denitrification, a 12-month survey was designed. Denitrification potential and nitrous oxide (N₂O) production were measured at different locations using the slurry acetylene blockage technique. Denitrification rate ranged from 0.4 to 38 nmol N g⁻¹ h⁻¹, increasing towards the river mouth following an urban pollution gradient. N₂O production, a powerful greenhouse gas implicated on the destruction of the ozone layer, was significantly related with sediment organic matter and accounted for 0.5 - 47 % of the N gases produced. Additional enrichment experiments were consistent with the results found in the environment, showing that sediments from the upper less urban stretch of the estuary, mostly sandy, respond

*Corresponding author: Phone: +351222062285. Fax: +351222062284. E-mail: catarina@icbas.up.pt

positively to carbon and, inversely, in organic rich sediments from the lower estuary, the denitrification potential was limited by nitrate availability. The obtained results confirmed denitrification as an important process for the removal of nitrate in estuaries. The presence of wastewater discharges appears to stimulate nitrogen removal but also the production of N_2O , a powerful greenhouse gas, exacerbating the N_2O : N_2 ratio and thus should be controlled.

Keywords: Denitrification; nitrous oxide; nitrate; urban pollution; estuaries; sediments

1. INTRODUCTION

Nitrogen is essential for all organisms, being a key player in many biogeochemical cycles (Gruber and Galloway, 2008). Human-induced changes to the nitrogen cycle, through the progressive increase of nitrogen inputs to coastal environments, created new conditions that have already demonstrably altered biogeochemical fluxes of N (Galloway et al., 2008; Schlesinger, 2009). These facts have led to a growing interest in understanding the biogeochemical processes responsible for nitrogen removal from natural ecosystems. Under anoxic conditions, denitrifying bacteria convert nitrate (NO_3^-) or nitrite (NO_2^-) into gaseous forms, either dinitrogen (N_2) or nitrous oxide (N_2O). This microbial-mediated process plays a particularly important role in nitrogen enriched coastal systems, since it diminishes the amount of fixed nitrogen transported to the ocean, and consequently reduces the nitrogen available for primary production (Falfowski et al., 1998; Beman et al., 2005). In estuarine sediments, denitrification is described to be capable of removing up to 44 % of oxidized nitrogen from the water column (Ogilvie et al., 1997; Dong et al., 2000, Fear et al., 2005). Livingstone et al. (2000) measured, in Belfast and Strangford Loughs, a denitrification potential capable of removing 3 to 6 times the external nitrate load. Denitrification is also of current interest because it contributes to the global biogenic N_2O emissions. Besides being a precursor molecule for ozone-destroying NO radicals in the stratosphere (Dickinson and Cicerone, 1986), N_2O is also a powerful greenhouse gas, with a global warming potential 300 times higher than that of CO_2 (Dickinson and Cicerone, 1986; IPCC, 2001) and it is steadily increasing in the atmosphere (Khalil et al., 2002). Estuarine and coastal areas have been referred as active sites for N_2O production (Seitzinger and

64 Kroeze, 1998; Bange, 2006), accounting for as much as 60% of the total marine N₂O
 65 emissions (Bange et al., 1996). Because benthic denitrification and N₂O emission
 66 influence the inorganic nitrogen budget in estuaries and climate change, respectively, it
 67 is essential to understand how environmental factors control this biogeochemical
 68 process. Numerous investigators have correlated higher denitrification rates with the
 69 progressive increasing of NO₃⁻ concentrations, showing the importance of NO₃⁻ as a
 70 regulatory factor of denitrification (Christensen et al., 1990; Rysgaard et al., 1995;
 71 Ogilvie et al., 1997; Trimmer et al., 1998; Dong et al., 2000; Magalhães et al., 2005).
 72 Nitrate has also been found to stimulate N₂O emissions (Magalhães et al., 2005; Stow et
 73 al., 2005,). The availability of organic carbon (Nixon, 1981; van Luijn et al., 1999) and
 74 changes in oxygen regimes (Cornwell et al., 1999; de Bie et al., 2002) have also been
 75 recognized as major environmental controls of denitrification and N₂O production. In
 76 addition, the presence of infauna affects the rates of denitrification, since infaunal
 77 activity typically results in complex patterns of oxic and anoxic microenvironments that
 78 significantly affect the mixing of O₂ and NO₃⁻ from the sediment surface to deeper
 79 layers (Rysgaard et al. 1995). The presence of green algae, seagrass and
 80 microphytobenthos in shallow and intertidal soft bottom embayments and estuaries
 81 could compete with denitrification for the nitrate available in the pore water and also
 82 diminish denitrification activity through the high rates of O₂ production during
 83 photosynthesis (Sundbäck and Miles, 2000). Due to the highly dynamic interactions
 84 between environmental variables, denitrification rates and N₂O emissions can vary
 85 widely between seemingly similar systems (Nixon et al., 1996; Herbert, 1999). The
 86 Douro River is one of the longest rivers in the Iberian Peninsula (930 km), sharing its
 87 watershed between Portugal and Spain. The estuary is physically confined to a 21 km
 88 stretch limited upstream by a hydroelectric power dam. The Douro estuary area is
 89 heavily urbanized with over one million inhabitants concentrated mainly in the lower
 90 and middle stretches of the catchment. This highly modified water body faces two
 91 major problems, the freshwater input of nitrate and the bacterial contamination from
 92 untreated wastewater discharges (Azevedo et al., 2008). Although the denitrification-
 93 nitrate relationship is well established, the effects of sewage inputs on ambient
 94 denitrification are less often addressed. In this study, we quantified denitrification and
 95 N₂O production monthly in subtidal sediments collected from four sites along the
 96 estuary; simultaneously we measured water nutrient concentrations, fecal bacteria (as an

indicator of wastewater inputs) and sediment characteristics. Additionally, enrichment experiments were performed to assess carbon and/or carbon limitation.

2. MATERIALS & METHODS

2.1 Study area and sampling sites

The Douro River estuary is located in NW Portugal at 41°08'N and 8°42'W. The estuary has a semi-diurnal mesotidal regime and it drains an average of 505 m³ s⁻¹ into the Atlantic Ocean. As a function of seawater intrusion, it has been divided in 3 stretches (Vieira and Bordalo, 2000), with average width and depth of respectively, 645 and 7.8 m for the upper estuary; 271 and 10 m for the middle estuary and 333 m and 7 m for the lower estuary (Azevedo et al., 2008). Effluents of 8 wastewater treatment plants drain into the estuary, without nutrient removal (Azevedo et al., 2008). Additionally, freshwater inputs of oxidized nitrogen forms accounted for 10.4 x 10⁴ tonne in 2004 (INAG, 2004). In this study, four sites were chosen along an estuarine gradient (Fig. 1): site I in the lower section, sites II and III in the middle section and site IV in the upper section. Samples were taken monthly from October 2004 to September 2005. Subtidal sediments (3-11 m depth) were collected with a Petite Ponar grab sampler (15 cm x 15 cm opening) operated from a boat. At each site, the contents of three grabs were pooled, homogenized and stored in sterile plastic bags for sediment characterisation and denitrification experiments. Simultaneously, near-bottom water samples were collected with a 2 L Van Dorn bottle and stored in acid-cleaned polyethylene flasks for fecal coliforms and inorganic nutrient analysis. Temperature, salinity, dissolved oxygen (DO) and pH, were measured *in situ* using an YSI 6000 series CTD. Samples were refrigerated in ice chests and transported in the dark to the laboratory for processing no later than 1 h after collection.

2.2 Water and sediment analytical procedures

Inorganic nutrient analyses were performed in the estuarine overlying water of the subtidal sediments of each site. The water concentration of nitrite (NO₂⁻), ammonium (NH₄⁺) and orthophosphate (PO₄³⁻) ions were determined colorimetrically using methods described in Grasshoff et al. (1983). The nitrate (NO₃⁻) anion was assayed using an adaptation of the spongy cadmium reduction technique (Jones, 1984). All the

analyses were performed in triplicate and quantified using daily standard curves. The detection limit was 0.03, 0.1, 0.02 and 0.3 μM respectively for PO_4^{3-} , NH_4^+ , NO_2^- , NO_3^- and the precision of all determinations was in the range 0.1 - 8%, depending on the particular nutrient concentration. Sediment organic matter (OM) was measured as percentage of weight loss by ignition (500°C, 4 h) from the 60°C dried sediment. Grain size analysis was performed by dry sieving in samples previously treated with hydrogen peroxide (Percival and Lindsay, 1997). The oxidation-reduction potential (ORP) of fresh homogenised sediment was measured using a platinum electrode (Hanna Instruments).

2.3 Fecal coliform analysis

The quantitative determination of fecal coliforms (FC) in estuarine water samples was assayed according to the ISO 9308/1 standard (ISO, 1990). Bacteria were concentrated onto 0.45- μm sterile membrane filters, which were placed in Petri dishes containing mFC culture media (Merck) and incubated at 44.5°C. Fecal coliform bacteria colonies were counted after 24 h of incubation period. Data from triplicate analyses was averaged and expressed as colony-forming units (CFU) per 100 ml of estuarine water sample.

2.4 Denitrification measurements

Denitrification potential and N_2O production rates were measured, for each sampling site and monthly campaign, in triplicate sediment slurries using the acetylene inhibition technique as described by Magalhães et al. (2005). Slurries consisted in 50 ml serum bottles with 10 ml of incubation water (site water amended with 300 μM of nitrate and 2 mM of glucose) and 5 ml of homogenized sediment hermetically sealed with a butyl stopper and aluminium crimp and purged 15 min with helium to remove oxygen (O_2). The weight of wet sediment was registered for each serum bottle. Incubation sets with and without acetylene addition (20 % vol:vol) were run in parallel. A separate set of time zero samples was sacrificed immediately after acetylene addition, to quantify N_2O levels at 0h. All samples were incubated in the dark for 4h at constant temperature (20 °C) and stirring (100 ppm). Linearity of the processes during the incubation period was confirmed in previous experiments. At the end of incubation, 15 ml of headspace sample was collected from each serum bottle (after shaking vigorously) by displacement with 3 M NaCl solution (Joye and Pearl, 1993). The N_2O analysis was determined with

165 a gas chromatograph (Varian 3800) fitted with a ^{63}Ni electron captor detector (ECD)
 166 held at 250 °C and an automatic back flush valve to prevent C_2H_2 contamination of the
 167 detector. Separation was achieved on two HayeSep D columns (0.9 and 1.8 m), kept at
 168 80 °C, using a mixture of 5 % methane in argon as a carrier gas. Concentration was
 169 calculated using a standard curve generated using purified N_2O (N_2O in He, Scott
 170 Specialty Gas), and the instrument detection limit was approximately 10 nM N_2O . No
 171 production of nitrous oxide was detected at time zero samples. Nitrous oxide production
 172 rates were given by the N_2O accumulation in treatments without C_2H_2 and the potential
 173 denitrification rates (N_2 plus N_2O production) by the N_2O produced in acetylene
 174 incubations. This technique is known to have some important limitations; incomplete
 175 inhibition of N_2O reductase and/or decoupling of nitrification-denitrification may lead
 176 to rate underestimation. On the other hand, the mixing of the substrata may stimulate
 177 denitrification (Bernot, 2003). When using C_2H_2 inhibition together with NO_3^- and
 178 glucose additions we are estimating the denitrifier enzymatic potential of the sediments,
 179 instead of actual rates. However, the method is simple and cost-effective, being a useful
 180 tool for relative comparisons of denitrification across aquatic areas and seasons
 181 (Groffman et al., 2006).

183 *2.5 Carbon and nitrogen enrichment experiments*

184 After analyzing the monthly results we noticed that denitrification was not related with
 185 water column nitrate. Enrichment experiments were designed to determine if carbon (C)
 186 and (or) nitrogen (N) availability could be limiting sediment denitrification. As similar
 187 results were obtained for site II and III, we revisited only one of those sites as a
 188 representative of the middle estuary. Enrichment experiments were conducted in
 189 additional sediment samples collected in May 2006. Sediment and water samples were
 190 collected from lower, middle and upper sections of the estuary (at sites I, III and IV). In
 191 N treatments, slurry incubation water was amended with 100 μM , 200 μM or 300 μM of
 192 nitrate, using a standard solution of KNO_3 . For C treatments, incubation water was
 193 amended with progressive additions of glucose: 0.5 mM, 1 mM and 2 mM. Unamended
 194 controls and combined treatments with 300 μM nitrate and 2 mM glucose were also
 195 performed. Denitrification and N_2O production rates were measured in triplicate slurries
 196 for each treatment using the procedure described above.

198 *2.6 Calculation of nitrogen loss*

Estimates of the proportions of NO_3^- lost due to denitrification were calculated relative to the amount of NO_3^- flowing over 1 m of estuary channel (see Kemp and Dodds, 2002 for details). Briefly, the nitrate load over 1 m of channel length was calculated from water column nitrate concentration, discharge and channel width. Denitrification rates per square meter were roughly calculated using the sampling grab opening area and volume. Denitrification values were then compared to nitrate load. Annual mean values for each site were used.

2.7 Data analysis

Before statistical analysis was carried out, all data were log-transformed, i.e., $\log(x+1)$, to attain homogeneity of the variances and normality options. Spatial variation and monthly differences within each site were examined using analysis of variance (one-way ANOVA) followed by a post hoc Tukey honestly significant difference (HSD) multi-comparison test. Linear regression was used to analyse data from the N and C enrichment experiments. The relationships between denitrification and N_2O production rates and the environmental variables were explored using redundancy analysis (RDA). RDA is the direct extension of multiple regression to the modelling of multivariate response data (Legendre & Legendre, 1998). Highly correlated variables were examined using variance inflation factors and removed when proved to contribute little information to the ordination (ter Braak and Šmilauer, 2002). Intra-set correlations were used to examine the relative contribution of the environmental variables to the separate ordination axis. The significance of the generated ordination axes was determined using unrestricted Monte Carlo permutation tests (499 permutations). The significance level used for all tests was 0.05. Statistical analyses of data were performed using the software STATISTICA 6.0 (StatSoft, Tulsa, USA), with the exception of the RDA that was performed using the software CANOCO for Windows 4.5 (Biometris, Wageningen, The Netherlands).

3. RESULTS

3.1 Water and sediment characteristics

Sampling sites water column and sediment characterization in terms of some physical, chemical and biological parameters are summarised in Table 1. The average and range

of values obtained during the surveyed period are presented. Higher water column NO_3^- concentrations were measured in the upper stations, corresponding to the lowest salinities. The inverse significant relation found between NO_3^- and salinity ($r=-0.80$, $n=43$) confirms the continental origin of this nutrient. The urban stretch of the estuary (lower and middle portions) showed an increased fecal contamination and ammonium concentration. The decreasing ammonium: nitrate ratio along the estuary suggests that, in Douro estuary, nitrogen inputs are primarily urban instead of agriculturally related. Sediment characteristics varied widely along the estuary (Table 1). While the upper estuary was characterised by sandy sediments with low organic content, higher organic matter was present in the finer sediment areas from the lower and middle estuary. Downstream sites were also characterised by permanent negative values of ORP indicating low oxygen concentrations in sediments.

3.2 Denitrification and N_2O production

Significant monthly and spatial variability was observed for both denitrification and N_2O production processes ($p<0.001$) (Fig. 2). Monthly variation detected had no common temporal trend with the highest rates for each site being measured in different months. Thus, no true seasonal variation was established. A clear spatial gradient was found for the denitrification potential, the analysis of differences between sampling sites (Fig. 3, Tukey HSD test, $p<0.05$) placed them in 3 groups, matching the 3 estuarine sections, with higher values being found in the lower estuary and smaller values in the upstream. As for the N_2O production, the measured values were significantly higher in site III (Fig. 3, Tukey HSD test, $p<0.05$) comparing to the other sites. Denitrification potential ranged from a minimum of $0.4 \text{ nmol N g ww}^{-1} \text{ h}^{-1}$ at site IV in March 2005, and a maximum of $38 \text{ nmol N g ww}^{-1} \text{ h}^{-1}$ at site I in November 2004 (Fig. 2). Lower N_2O production rates were observed at the upper section of the estuary (site IV), higher N_2O production rates fluctuated between sites III, II and I according with the sampling month (Fig. 2). N_2O production ranged from 0.03 to $3.40 \text{ nmol N g ww}^{-1} \text{ h}^{-1}$ (Fig. 2), the higher value being measured at site III where the $\text{N}_2\text{O}:\text{N}_2$ ratio reached 47 % (Fig. 4).

3.3 Environmental controls

Relationships between denitrification potential and N_2O production with the environmental variables measured were examined using redundancy analysis. The results of the RDA analysis are presented in the triplot ordination diagram of Fig. 5. The

first two axes of the RDA analysis accounted for 62.1% of the overall variability of the data. The first RDA axis (horizontal) accounted for 55.6% of the total variability with sediment ORP, ammonium concentration and fecal coliforms being the best explanatory variables. Thus, the denitrification potential seems to be influenced by an urban pollution gradient. On the other hand, the production of N_2O seems to be related to sediment characteristics, being influenced by grain size and organic matter content. Unrestricted Monte Carlo permutation tests indicated that measured rates were significantly related to both axes.

3.4 Carbon and nitrogen enrichment experiments

Site water nitrate concentration used as incubation water in these experiments was 42.3 89.2 and 75.2 μM for site I, III and IV respectively. Nitrate addition stimulated positively the denitrification rates in sediment from sites I ($r^2=0.98$, $n=12$) and III ($r^2=0.90$, $n=12$) (Fig. 6). No significant effect on denitrification was detected with increasing glucose concentrations at these sites. The addition of carbon only had a positive response on organic-poor sandy sediments from site IV ($r^2=0.62$, $n=12$) (Fig. 6), although the difference between treatments was not significant (ANOVA, $p=0.16$). Measured N_2O production rates were low, under 1.6 $nmol\ N\ g\ ww^{-1}\ h^{-1}$ (Fig. 6), but similar results to denitrification were obtained, with site I ($r^2=0.68$, $n=12$) and III ($r^2=0.74$, $n=12$) being positively influenced by NO_3^- addition and site IV by glucose addition ($r^2=0.75$, $n=12$). Addition of both nitrate and glucose produced a significant positive effect on denitrification and N_2O production in sediment from site III when compared when compared to single addition treatments (Tukey HSD test, $p<0.05$).

3.5 Proportion of nitrogen loss

Proportions of N lost through denitrification were calculated for each site (table 2). Variation of denitrification rates along the estuary was contrary to the nitrate load. The proportion of NO_3^- denitrified relative to the NO_3^- load per metre increased from site IV to site I, where denitrification potential exceeded 3 times the nitrate load.

4. DISCUSSION

300 The potential for denitrification and N_2O production was observed in subtidal sediments
 301 throughout the Douro River estuary although the monthly survey did not revealed
 302 process seasonality. Denitrification rates measured in this study ($0.4\text{--}38 \text{ nmol N g ww}^{-1}$
 303 h^{-1}) fall in the range of values reported for other ecosystems: like river sediments,
 304 $<0.01\text{--}260 \text{ nmol N g}^{-1} \text{ h}^{-1}$ (García-Ruiz et al., 1998) and $0\text{--}12 \text{ nmol N g}^{-1} \text{ h}^{-1}$ (Wall et al.,
 305 2005); stream sediments, $0.71\text{--}340 \text{ nmol N g}^{-1} \text{ h}^{-1}$ (Arango et al., 2007) and coastal
 306 aquifer sediments, up to $240 \text{ nmol N g}^{-1} \text{ h}^{-1}$ (Aelion et al., 1997). In a previous study,
 307 Magalhães et al. (2005) registered high amplitudes of denitrification rates according to
 308 the different sites and substrates studied; with an average of $20 \text{ nmol N g}^{-1} \text{ h}^{-1}$ in
 309 intertidal sandy sediments and $100 \text{ nmol N g}^{-1} \text{ h}^{-1}$ in rocky biofilms from the Douro
 310 estuary. Water column nitrate concentrations decreased from upstream to the mouth of
 311 the estuary, a common feature found in other estuaries (Barnes and Owens, 1998; Dong
 312 et al., 2000). However, unlike these studies the sediment denitrification potential
 313 behaved inversely, increasing from upstream to downstream. Interestingly, our results
 314 suggested that denitrification potentials were influenced by the pollution gradient in the
 315 estuary, since positive relationships with ammonia concentration and fecal coliform
 316 contamination were recorded. Increases in water column ammonia concentration could
 317 stimulate sediment nitrification (Magalhães et al., 2005) and consequently
 318 denitrification by the coupling of both processes. However, the study area was mainly
 319 characterized by anaerobic sediments where nitrification was expected to be
 320 insignificant and denitrification to be fuelled by water column NO_3^- (Rysgaard et al.,
 321 1994). Higher denitrification rates were measured in the lower estuary followed by the
 322 middle estuary corresponding to the urban pressure gradient. Other studies also refer to
 323 higher denitrification rates related to sewage outfalls (Ogilvie, 1997; Bartkow and Udy,
 324 2004). Sewage disposal and detritus deposition may be responsible for the oxygen
 325 depletion found in the sediments, favouring nitrate respiration (Sørensen et al., 1979).
 326 Also it may be a source of labile organic matter, although this hypothesis was not tested.
 327 Denitrification is known to be related with organic carbon contents in both sediments
 328 and soils (Knowles, 1982). The supply of heterotrophic bacteria by the effluents is also
 329 a possibility (Servais et al., 1999). The lack of a relationship between measured
 330 denitrification rates and nitrate values led us to perform additional enrichment
 331 experiments. These experiments revealed that nitrate addition did stimulate
 332 denitrification in sediments from site I and III meaning that actual denitrification rates
 333 in these sites may be lower than measured. The same stimulation was not observed in

334 sediments from the upper estuary where lower denitrification capacity was found, these
 335 sediments although receiving high nitrate loads were characterised by larger particles
 336 with low organic content. Amendment experiments showed a linear response of
 337 denitrification to glucose additions at this site. Water column NO_3^- as a non-limiting
 338 substrate was previously described in high NO_3^- waters (Wall et al. 2005).
 339 To evaluate the significance of denitrification in Douro estuary the proportions of NO_3^-
 340 lost due to denitrification were calculated relative to the amount of NO_3^- flowing over 1
 341 m of estuary channel (Kemp and Dodds, 2002). Results showed a wide range of values
 342 along the estuary, from 0.1 upstream to a potential removal capability of 3 times the
 343 nitrate load at site I downstream. Higher ratios of denitrification potential to N input,
 344 ratios of 3 and 6, were described in Belfast and Strangford Loughs, Ireland by
 345 Livingstone et al., 1999.
 346 Increased N_2O production rates have been reported to be associated with more eutrophic
 347 environments coupled to high NO_3^- availability and consequently to higher
 348 denitrification rates (Seitzinger and Nixon, 1985; Middelburg et al., 1995; Kenny et al.,
 349 2004). In this study, however, N_2O production was related to sediment organic matter
 350 content, and interestingly the highest N_2O production did not coincide with the highest
 351 denitrification rates. Higher production rates of N_2O are known to be related to low
 352 oxygen concentrations (de Bie et al., 2002) and/or the H_2S inhibition of N_2O reduction
 353 to N_2 during denitrification (Sørensen et al., 1980; Senga et al., 2006). We did not
 354 measure H_2S to test that effect, however our results suggest that denitrification may not
 355 be the only source of N_2O . Dissimilatory reduction to ammonia (DNRA) might also
 356 produce N_2O (Smith and Zimmerman, 1981) and could be occurring simultaneously to
 357 denitrification in our slurries. DNRA was previously found to be favoured by reduced
 358 and C-rich environments (Buresh and Patrick Jr, 1981), conditions present in the
 359 sediments at site III. To evaluate the magnitude of the N_2O accumulation during
 360 denitrification, the relative production of N_2 and N_2O ($\text{N}_2\text{O}:\text{N}_2$ ratio) was calculated
 361 (Fig. 4). High $\text{N}_2\text{O}:\text{N}_2$ ratios were found, especially at site III, where values were
 362 persistently higher than 5 %. Seitzinger et al. (2000) reported that very small values (< 6
 363 %) were usually found in the aquatic environments although significantly higher values
 364 were also found in nitrogen-enriched rivers (García-Ruiz et al., 1998; McMahon and
 365 Dennehy, 1999). The potential effect of anthropogenic activities on nitrous oxide
 366 production from aquatic sediments was previously demonstrated, showing that N_2O
 367 emissions increased markedly as the rate of N loading to the system increased

(Seitzinger et al., 2000). Concerning the powerful greenhouse effect of N_2O and its recognized role as the most important ozone depleting gas emitted to the atmosphere (Ravishankara et al., 2009), the higher $N_2O:N_2$ ratio is undesirable, and thus control of organic input to estuaries could be an effective way of reducing the ratio.

5. CONCLUSION

Higher rates of denitrification and N_2O production were found in the more urbanized stretches of the estuary. Interestingly, the variables that better explained the observed variability were sediment ORP, ammonium and fecal coliforms for denitrification, and organic matter for N_2O production. The presence of wastewater discharges appears to stimulate nitrogen removal but also N_2O production, exacerbating the $N_2O:N_2$ ratio, which should be controlled.

Acknowledgements

We thank to Rita Teixeira and Ana Machado for their assistance in field and laboratory work. We also thank 3 anonymous reviewers for comments and suggestions on a previous version of the manuscript. This work was funded by the Portuguese Science and Technology Foundation (FCT) through a Ph.D. fellowship to C. Teixeira (POCTI/CTA/39034/2001), and a Postdoc fellowship to C. Magalhães (SFRH/BPD/14929/2004).

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Table 1. Water and sediment characteristics of the benthic sites. (Data represent means of monthly measurements $\pm$ SE, n=12).

	I	II	III	IV
km from mouth	1.5	3.5	5	12
Temp. (°C)	13.8 $\pm$ 0.68	14.0 $\pm$ 0.98	13.8 $\pm$ 0.86	14.6 $\pm$ 1.40
Salinity	25.7 $\pm$ 2.32	18.5 $\pm$ 2.58	22.7 $\pm$ 2.34	10.6 $\pm$ 2.53
DO (%)	107 $\pm$ 5.5	106 $\pm$ 5.4	101 $\pm$ 4.7	104 $\pm$ 5.3
pH	7.8 $\pm$ 0.1	7.8 $\pm$ 0.1	7.8 $\pm$ 0.1	7.7 $\pm$ 0.1
NO ₃ ⁻ (μM)	29.4 $\pm$ 5.9	43.2 $\pm$ 5.7	43.0 $\pm$ 5.9	73.8 $\pm$ 9.9
NO ₂ ⁻ (μM)	0.51 $\pm$ 0.06	0.64 $\pm$ 0.06	0.61 $\pm$ 0.05	0.85 $\pm$ 0.09
NH ₄ ⁺ (μM)	7.79 $\pm$ 0.96	8.02 $\pm$ 0.51	6.90 $\pm$ 0.51	5.34 $\pm$ 0.48
PO ₄ ³⁻ (μM)	0.91 $\pm$ 0.12	1.06 $\pm$ 0.07	1.00 $\pm$ 0.08	1.27 $\pm$ 0.07
NH ₄ ⁺ / NO ₃ ⁻	0.21 $\pm$ 0.03	0.18 $\pm$ 0.03	0.14 $\pm$ 0.03	0.07 $\pm$ 0.01
FC (CFU/100mL)	6,358 $\pm$ 1278	10,167 $\pm$ 5073	3,517 $\pm$ 632	1,219 $\pm$ 223
Sediment OM (% dry weight)	1.2 $\pm$ 0.2	3.1 $\pm$ 0.8	10.8 $\pm$ 0.8	0.36 $\pm$ 0.02
Sediment ORP (mV)	-304 $\pm$ 36.5	-251 $\pm$ 40.8	-329 $\pm$ 19.6	99 $\pm$ 63.2
% silt and clay (< 63 μm)	2.2 $\pm$ 0.4	8.5 $\pm$ 2.8	30.6 $\pm$ 4.0	0.2 $\pm$ 0.1
% gravel (> 2 mm)	67.2 $\pm$ 4.8	39.9 $\pm$ 7.6	2.6 $\pm$ 0.7	50.1 $\pm$ 2.2

Table 2. Proportions of N lost through denitrification.

		Denitrification ^(a)	Nitrate load ^(b)	Prop. N loss ^(c)
Downstream	Site I	28.6	10.3	3.0
	Site II	20.9	43.2	0.5
	Site III	16.5	60.4	0.3
Upstream	Site IV	8.9	83.0	0.1

^(a) Denitrification measured in $\text{kg N}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$.

^(b) Nitrate load measured in $\text{kg N}\cdot\text{m}^{-1}\cdot\text{d}^{-1}$.

^(c) Proportion of nitrate load denitrified per metre of stream length.

Figure captions

Figure 1. Location of sampling sites, wastewater treatment plants and tributaries discharging into de estuary. Limits of estuarine stretches (upper, middle and lower) according to Vieira and Bordalo (2000).

Figure 2. Monthly variation of denitrification potential (DNF) and N_2O production rates in the Douro river sediments (error bars = standard error of the mean of three replicates).

Figure 3. Mean annual denitrification and N_2O production rates (± 1 SE) of sampling stations. Different characters indicate significant differences between means (one-way ANOVA followed Tukey HSD test results, $p < 0.05$).

Figure 4. Monthly N_2O : N_2 ratio values calculated for each site.

Figure 5. RDA ordination plot showing the relationship between denitrification potential (DNF) and N_2O production (N_2O) rates and measured environmental variables.

Figure 6. Denitrification rates N_2O production rates measured in different NO_3^- (N) and glucose (C) treatments (error bars = standard error of the mean of three replicates).