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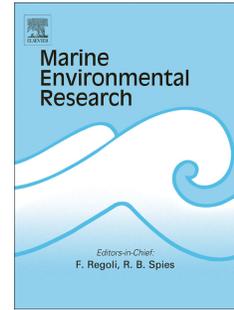
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1 Potential rates and environmental controls of denitrification and
2 nitrous oxide production in a temperate urbanized estuary

3
4 Catarina Teixeira^{*1,2}, Catarina Magalhães², Rui A.R. Boaventura³, Adriano A.
5 Bordalo^{1,2}

6
7 ¹Institute of Biomedical Sciences (ICBAS), University of Porto
8 Laboratory of Hydrobiology, Largo Prof. Abel Salazar 2, 4009-003 Porto, Portugal

9 ²Centre of Marine and Environmental Research (CIIMAR), University of Porto
10 Rua dos Bragas 289, 4050-123 Porto, Portugal.

11 ³Faculty of Engineering, University of Porto
12 Department of Chemistry, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

13
14
15 **ABSTRACT**

16
17 Denitrification may play a major role in inorganic nitrogen removal from estuarine
18 ecosystems, particularly in those subjected to increased nitrate and organic matter loads.
19 The Douro estuary (NW Portugal) suffers from both problems: freshwater input of
20 nitrate and organic load from untreated wastewater discharges. To assess how these
21 factors might control sediment denitrification, a 12-month survey was designed.
22 Denitrification potential and nitrous oxide (N₂O) production were measured at different
23 locations using the slurry acetylene blockage technique. Denitrification rate ranged from
24 0.4 to 38 nmol N g⁻¹ h⁻¹, increasing towards the river mouth following an urban
25 pollution gradient. N₂O production, a powerful greenhouse gas implicated on the
26 destruction of the ozone layer, was significantly related with sediment organic matter
27 and accounted for 0.5 - 47 % of the N gases produced. Additional enrichment
28 experiments were consistent with the results found in the environment, showing that
29 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

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

36

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

38

39

40 1. INTRODUCTION

41

42 Nitrogen is essential for all organisms, being a key player in many biogeochemical
43 cycles (Gruber and Galloway, 2008). Human-induced changes to the nitrogen cycle,
44 through the progressive increase of nitrogen inputs to coastal environments, created new
45 conditions that have already demonstrably altered biogeochemical fluxes of N
46 (Galloway et al., 2008; Schlesinger, 2009). These facts have led to a growing interest in
47 understanding the biogeochemical processes responsible for nitrogen removal from
48 natural ecosystems. Under anoxic conditions, denitrifying bacteria convert nitrate (NO_3^-)
49 or nitrite (NO_2^-) into gaseous forms, either dinitrogen (N_2) or nitrous oxide (N_2O).
50 This microbial-mediated process plays a particularly important role in nitrogen enriched
51 coastal systems, since it diminishes the amount of fixed nitrogen transported to the
52 ocean, and consequently reduces the nitrogen available for primary production
53 (Falfowski et al., 1998; Beman et al., 2005). In estuarine sediments, denitrification is
54 described to be capable of removing up to 44 % of oxidized nitrogen from the water
55 column (Ogilvie et al., 1997; Dong et al., 2000, Fear et al., 2005). Livingstone et al.
56 (2000) measured, in Belfast and Strangford Loughs, a denitrification potential capable
57 of removing 3 to 6 times the external nitrate load. Denitrification is also of current
58 interest because it contributes to the global biogenic N_2O emissions. Besides being a
59 precursor molecule for ozone-destroying NO radicals in the stratosphere (Dickinson and
60 Cicerone, 1986), N_2O is also a powerful greenhouse gas, with a global warming
61 potential 300 times higher than that of CO_2 (Dickinson and Cicerone, 1986; IPCC,
62 2001) and it is steadily increasing in the atmosphere (Khalil et al., 2002). Estuarine and
63 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

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

99

100

101 2. MATERIALS & METHODS

102

103 2.1 Study area and sampling sites

104 The Douro River estuary is located in NW Portugal at 41°08'N and 8°42'W. The estuary
105 has a semi-diurnal mesotidal regime and it drains an average of 505 m³s⁻¹ into the
106 Atlantic Ocean. As a function of seawater intrusion, it has been divided in 3 stretches
107 (Vieira and Bordalo, 2000), with average width and depth of respectively, 645 and 7.8
108 m for the upper estuary; 271 and 10 m for the middle estuary and 333 m and 7 m for the
109 lower estuary (Azevedo et al., 2008). Effluents of 8 wastewater treatment plants drain
110 into the estuary, without nutrient removal (Azevedo et al., 2008). Additionally,
111 freshwater inputs of oxidized nitrogen forms accounted for 10.4 x 10⁴ tonne in 2004
112 (INAG, 2004). In this study, four sites were chosen along an estuarine gradient (Fig. 1):
113 site I in the lower section, sites II and III in the middle section and site IV in the upper
114 section. Samples were taken monthly from October 2004 to September 2005. Subtidal
115 sediments (3-11 m depth) were collected with a Petite Ponar grab sampler (15 cm x 15
116 cm opening) operated from a boat. At each site, the contents of three grabs were pooled,
117 homogenized and stored in sterile plastic bags for sediment characterisation and
118 denitrification experiments. Simultaneously, near-bottom water samples were collected
119 with a 2 L Van Dorn bottle and stored in acid-cleaned polyethylene flasks for fecal
120 coliforms and inorganic nutrient analysis. Temperature, salinity, dissolved oxygen (DO)
121 and pH, were measured *in situ* using an YSI 6000 series CTD. Samples were
122 refrigerated in ice chests and transported in the dark to the laboratory for processing no
123 later than 1 h after collection.

124

125 2.2 Water and sediment analytical procedures

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

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

140

141 *2.3 Fecal coliform analysis*

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

149

150 *2.4 Denitrification measurements*

151 Denitrification potential and N_2O production rates were measured, for each sampling
152 site and monthly campaign, in triplicate sediment slurries using the acetylene inhibition
153 technique as described by Magalhães et al. (2005). Slurries consisted in 50 ml serum
154 bottles with 10 ml of incubation water (site water amended with 300 μM of nitrate and 2
155 mM of glucose) and 5 ml of homogenized sediment hermetically sealed with a butyl
156 stopper and aluminium crimp and purged 15 min with helium to remove oxygen (O_2).
157 The weight of wet sediment was registered for each serum bottle. Incubation sets with
158 and without acetylene addition (20 % vol:vol) were run in parallel. A separate set of
159 time zero samples was sacrificed immediately after acetylene addition, to quantify N_2O
160 levels at 0h. All samples were incubated in the dark for 4h at constant temperature (20
161 °C) and stirring (100 ppm). Linearity of the processes during the incubation period was
162 confirmed in previous experiments. At the end of incubation, 15 ml of headspace
163 sample was collected from each serum bottle (after shaking vigorously) by displacement
164 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).

182

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.

197

198 *2.6 Calculation of nitrogen loss*

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

206

207 *2.7 Data analysis*

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

226

227

228 3. RESULTS

229

230 *3.1 Water and sediment characteristics*

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

233 of values obtained during the surveyed period are presented. Higher water column NO_3^-
234 concentrations were measured in the upper stations, corresponding to the lowest
235 salinities. The inverse significant relation found between NO_3^- and salinity ($r=-0.80$,
236 $n=43$) confirms the continental origin of this nutrient. The urban stretch of the estuary
237 (lower and middle portions) showed an increased fecal contamination and ammonium
238 concentration. The decreasing ammonium: nitrate ratio along the estuary suggests that,
239 in Douro estuary, nitrogen inputs are primarily urban instead of agriculturally related.
240 Sediment characteristics varied widely along the estuary (Table 1). While the upper
241 estuary was characterised by sandy sediments with low organic content, higher organic
242 matter was present in the finer sediment areas from the lower and middle estuary.
243 Downstream sites were also characterised by permanent negative values of ORP
244 indicating low oxygen concentrations in sediments.

245

246 *3.2 Denitrification and N_2O production*

247 Significant monthly and spatial variability was observed for both denitrification and
248 N_2O production processes ($p<0.001$) (Fig. 2). Monthly variation detected had no
249 common temporal trend with the highest rates for each site being measured in different
250 months. Thus, no true seasonal variation was established. A clear spatial gradient was
251 found for the denitrification potential, the analysis of differences between sampling sites
252 (Fig. 3, Tukey HSD test, $p<0.05$) placed them in 3 groups, matching the 3 estuarine
253 sections, with higher values being found in the lower estuary and smaller values in the
254 upstream. As for the N_2O production, the measured values were significantly higher in
255 site III (Fig. 3, Tukey HSD test, $p<0.05$) comparing to the other sites. Denitrification
256 potential ranged from a minimum of $0.4 \text{ nmol N g ww}^{-1} \text{ h}^{-1}$ at site IV in March 2005,
257 and a maximum of $38 \text{ nmol N g ww}^{-1} \text{ h}^{-1}$ at site I in November 2004 (Fig. 2). Lower
258 N_2O production rates were observed at the upper section of the estuary (site IV), higher
259 N_2O production rates fluctuated between sites III, II and I according with the sampling
260 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
261 higher value being measured at site III where the $\text{N}_2\text{O}:\text{N}_2$ ratio reached 47 % (Fig. 4).

262

263 *3.3 Environmental controls*

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

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

275

276 *3.4 Carbon and nitrogen enrichment experiments*

277 Site water nitrate concentration used as incubation water in these experiments was 42.3
278 89.2 and 75.2 μM for site I, III and IV respectively. Nitrate addition stimulated
279 positively the denitrification rates in sediment from sites I ($r^2=0.98$, $n=12$) and III
280 ($r^2=0.90$, $n=12$) (Fig. 6). No significant effect on denitrification was detected with
281 increasing glucose concentrations at these sites. The addition of carbon only had a
282 positive response on organic-poor sandy sediments from site IV ($r^2=0.62$, $n=12$) (Fig.
283 6), although the difference between treatments was not significant (ANOVA, $p=0.16$).
284 Measured N_2O production rates were low, under $1.6 \text{ nmol N g ww}^{-1} \text{ h}^{-1}$ (Fig. 6), but
285 similar results to denitrification were obtained, with site I ($r^2=0.68$, $n=12$) and III
286 ($r^2=0.74$, $n=12$) being positively influenced by NO_3^- addition and site IV by glucose
287 addition ($r^2=0.75$, $n=12$). Addition of both nitrate and glucose produced a significant
288 positive effect on denitrification and N_2O production in sediment from site III when
289 compared when compared to single addition treatments (Tukey HSD test, $p<0.05$).

290

291 *3.5 Proportion of nitrogen loss*

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

296

297

298 4. DISCUSSION

299

300 The potential for denitrification and N₂O 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 (0.4-38 nmol N g ww⁻¹
303 h⁻¹) fall in the range of values reported for other ecosystems: like river sediments,
304 <0.01-260 nmol N g⁻¹ h⁻¹ (García-Ruiz et al., 1998) and 0-12 nmol N g⁻¹ h⁻¹ (Wall et al.,
305 2005); stream sediments, 0.71-340 nmol N g⁻¹ h⁻¹ (Arango et al., 2007) and coastal
306 aquifer sediments, up to 240 nmol N g⁻¹ 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 nmol N g⁻¹ h⁻¹ in
309 intertidal sandy sediments and 100 nmol N g⁻¹ 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 NO₃⁻ (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

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

372

373

374 5. CONCLUSION

375

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

382

383

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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).

