SALINITY AND INORGANIC NITROGEN EFFECTS ON NITRIFICATION AND DENITRIFICATION RATES IN INTERTIDAL SEDIMENTS AND ROCKY BIOFILMS: DOURO RIVER ESTUARY, PORTUGAL

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ABSTRACT

The regulatory effect of salinity and inorganic nitrogen on nitrification and denitrification was investigated in intertidal sandy sediments and rocky biofilms of the Douro estuary. A seasonal sampling program and controlled experiments were conducted; nitrification and denitrification rates were measured in slurries using the difluoromethane and the acetylene inhibition techniques, respectively. Salinity did not show any regulatory control of denitrification for either environment, suggesting both denitrifier communities contained dominantly euryhaline bacteria. However, a clear stimulation of nitrification activity was observed when salinity increased from 0 to 15 psu (40% and 50%, for sediments and rocky biofilms, respectively). Nitrate availability clearly stimulated denitrification rates in sandy sediments, whereas in rocky biofilms no regulatory effect was evident. N_2O:N_2 ratio increased rapidly with NO_3^- enrichment (4% to 19% and 12% to 35%, for sediments and rocky biofilms, respectively). Ammonium enrichment experiments in sandy sediments showed a stimulation of nitrification activity by 35% for the 20 M of NH_4^+ treatment and a clear inhibitory effect when amended with 200 M of NH_4^+. Contrary, rocky biofilms nitrification was stimulated in 65% when 200 M of NH_4^+ was added. These results indicate that salinity and inorganic nitrogen increases in the Douro estuary could alter nitrification rates and also denitrification rates with a consequent N_2O accumulation.

Keywords: Nitrogen Effects, Nitrification, Denitrification, Rocky Biofilms, Salinity Effects, Sandy Sediments.

Introduction

The progressive increase of nitrogen loading and reduction of freshwater discharge into estuarine systems represent worldwide problems (Jickells, 1998; De Jonge *et al*., 2002; Montagna *et al*., 2002). In the Douro River estuary, the concentrations of NO_3^- inputs from freshwater increased dramatically during the 1990's (INAG, 2003) and additional nutrients will be added once several treatment plants whith minimal nutrient removal begin operation. On the other hand, due to water diversion for agriculture within the watershed and climate change, the estuary is facing a progressive decrease in freshwater input, which could consequently change salinity regimes.

Benthic microbial activity provides a key role for nitrogen transformations within the estuary. Under oxic conditions, nitrifying bacteria convert ammonium to NO_2^- and subsequently to NO_3^-. In the anoxic zones, denitrifying bacteria use NO_3^- or NO_2^- as electron acceptors, converting them into gaseous (N_2 or N_2O) forms. Both of these processes are considered important in reducing the land-derived N loading to coastal waters (Jensen *et al*., 1996, Seitzinger, 2000). Because benthic nitrification and denitrification influence the fate of inorganic nitrogen inputs into an estuary, understanding of the environmental factors that control those processes are of great ecological importance. Many relationships have been observed between salinity and inorganic nitrogen variations with sediment key nitrogen cycle processes (e.g. Ogilvie *et al*., 1997, Rysgaard *et al*., 1999), but often, it is difficult to relate the observed relationships to a single parameter, since many parameters together exhibit variation throughout an estuary. In the present study a seasonal sampling program and controlled experiments were performed, in order to understand the effect of the possible salinities changes and the inorganic nitrogen increase (NO_3^- and NH_4^+) on the nitrification and denitrification within intertidal sandy sediments and rocky biofilms of the Douro River estuary.

METHODS

Study area and sampling

The Douro River watershed drain 17% of the Iberian Peninsula (Fig. 1). This drowned granitic valley estuary is confined 21 km from the mouth by a hydroelectric power dam. The estuary averages 8 m in depth, has a semidiurnal tidal range of 2-3 m, temperatures between 8-25 °C, salinities from 0 to 35 psu and a residence time between 0.3 and 16.5 days (Vieira & Bordalo, 2000). Freshwater input into the estuary during the post-dam period (1985-2002) has been reduced to 505±56 $m^3 s^{-1}$ from 660±6 $m^3 s^{-1}$ between 1960-1984. The estuary water column on average contains 23 µM NH_4^+, 52 µM NO_3^- and 1 µM NO_2^-. Nitrate concentration in Crestuma dam has dramatically increased during the last ten years (Fig. 2). In addition, largely untreated sewage from approximately one million inhabitants is discharged within its lower reach.

This study was conducted in two different intertidal environments within the lower estuary (Fig. 1), intertidal rocky biofilms (*Enteromorpha sp*. colonizing zones), and intertidal sandy sediments. Both sites have been characterized, in previous studies, in terms of total organic matter and Chlorophyll *a* concentrations and in the case of sandy sediments also in terms of macrofauna, microphythobenthos and particle size distribution, (Magalhães *et al*., 2000, Magalhães *et al*., 2002, Magalhães *et al*., 2003).

At each site, monthly samples were taken between February 2002 to October 2002. Sediment cores (10 cores, 3 cm diameter and 10 cm depth) and rocky biofilms (about 100 ml, removed by scraping the rocks) and respective water column at the vicinity of each site were collected always at low tide. Enrichment experiments were performing in additional rocky biofilms and sediment collected during August 2002; estuarine brackish water and freshwater from the Douro River were also collected for nitrogen enrichments and for salinity variation experiments, respectively. All samples were kept cool and transported in the dark to the laboratory for processing no later than 1h after collection.

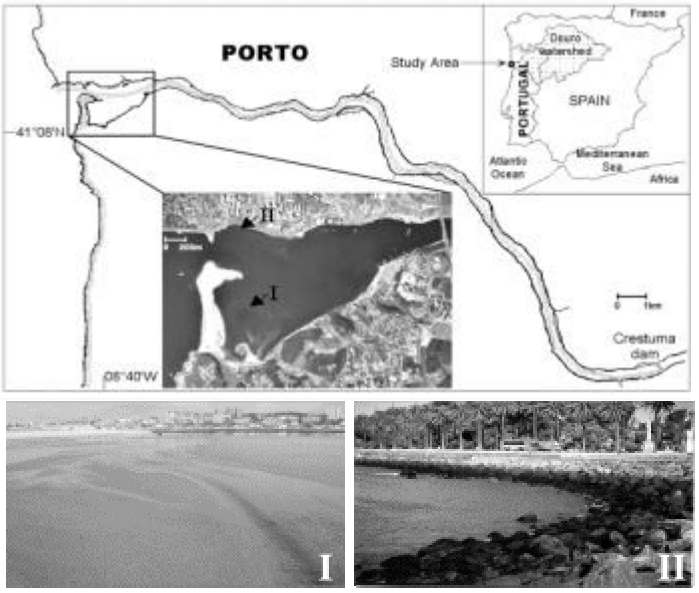

Figure 1-Douro River estuary and location of sampling sites.

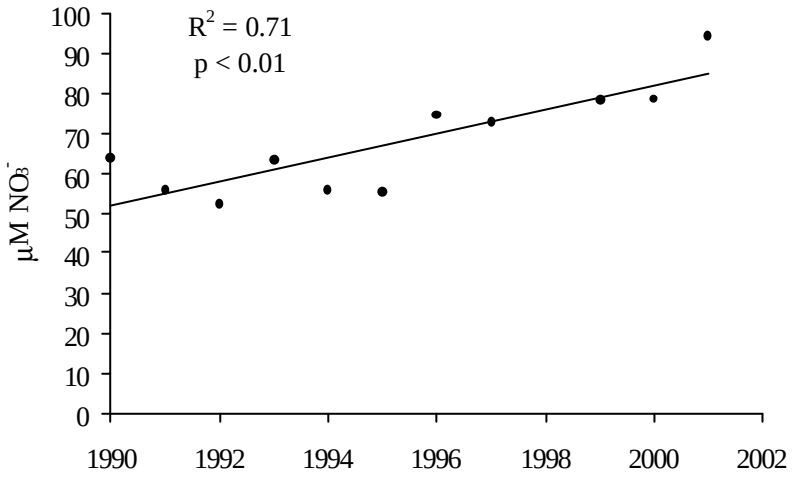

Figure 2 - Linear regression model between yearly averaged nitrate cencentration and time (1990-2001) in the Crestume-Lever reservoir. Data from the Portuguese Ministry of Environment (INAG, 2003).

Experimental Program

Denitrification rates were measured in triplicate using the acetylene inhibition technique of Sorensen (1978). The slurries were prepared by adding 10 ml of estuarine filtered water (0.2 µm) to a 50 ml serum bottle containing homogenized and weighted sandy sediment (around 3 g) or scraped rocky biofilm (around 1 g). Serum bottles were closed hermetically with butyl stoppers and aluminum crimp seals. The headspace was purged with helium and samples were run without and with acetylene (20% vol:vol). All samples were incubated in the dark for 4h at constant temperature (20 ºC) and stirring (70 rpm). At time zero, and time one, after headspace equilibration, 12 ml of gas sample were collected from each serum bottle and stored in a 12 ml evacuated serum vial for later analysis of N_2O. Time one gas sample was collected from each serum bottle by adding simultaneously 12 ml of a 3M NaCl solution (Joye et al., 1996).

Nitrification rates were measured in separated slurries by adding 25 ml of 0.2 µm filtered and oxygen saturated estuarine water to 50 ml serum bottles with homogenized and weighted sandy sediment (around 3 g) or rocky biofilm (around 1g), respectively. Samples were runed in triplicate without and with difluoromethane (DFM) (10% vol:vol), according to Miller

et al. (1998). All samples were incubated in the dark for 4h at constant temperature (20 ºC) and stirring (70 rpm). A time zero and time one 11 ml of overlying water samples were collected before and after the incubation period. These samples were centrifuged, 0.2 µm filtered and kept frozen (-20 ºC) for later NH_4^+ and NO_3^-+NO_2^- analysis.

In order to study salinity and nitrogen regulatory effects on nitrification and denitrification independently, additional experiments were performed during August 2002 at each site. Different concentrations of NO_3^- (0, 100 and 300 µM using a standard solution of KNO_3) and NH_4^+ (0, 20, 200 µM using a standard solution of $(NH_4)_2SO_4$) where added to the collected estuarine water (63 µM NO_3^-, 2 µM NH_4^- and 14 psu), and denitrification and nitrification measured in triplicate, as described above, for each different nitrate and ammonium treatment, respectively. The salinity effects were evaluated by amending the Douro River freshwater (60 µM NO_3^-, 5 µM NH_4^- and 0 psu) with salts using the artificial seawater formula (Cavanaugh, 1975), to achieve different salinities (0, 15 and 30 psu) and nitrification and denitrification measured as described above.

Analytic determinations

N_2 produced via denitrification was calculated as the difference between the N_2O produced with acetylene and the N_2O produced without acetylene. N_2O produced via denitrification was calculated by the N_2O accumulation in samples treated without acetylene. N_2O was quantified in triplicate using a Shimadzu gas chromatograph equipped with an electron-captured detector, according to Joye *et al*. (1996). Nitrification rates were calculated by the difference between NH_4^+ production measured in incubations without DFM and with DFM. Ammonium was quantified as described in Grasshoff *et al*. (1983) and nitrate plus nitrite assayed by an adaptation of the spongy cadmium reduction technique (Jones, 1984).

Results and Discussion

Values of monthly water salinity, time zero mean concentration of NO_3^- plus NO_2^- and NH_4^+ and relative release of N_2 and N_2O (N_2O:N_2 ratio) at each site for the seasonal sampling program are given in Table 1.

Table 1- Monthly incubation water salinity, time zero NO_3^- + NO_2^- and NH_4^+ concentration and N_2O:N_2 ratio (mean±standard error of the mean) for sandy sediment (site I) and rocky biofilms (site II); na: not available.

	Salinity (psu)		*NO_3^- + NO_2^- (mM)*		*NH_4^+ (mM)*		*N_2O:N_2 (%)*	
	Site I	Site II	*Site I*	*Site II*	*Site I*	*Site II*	*Site I*	*Site II*
Feb-02	22.7	14.9	34.5±1.1	51.7±0.1	21.1±0.7	37.0±1.1	6.5±0.5	0.0±0.0
Mar-02	15.1	4.5	31.9±0.9	99.8±0.1	21.9±0.5	25.6±1.2	7.0±0.1	41.0±1.6
Apr-02	16.0	16.0	39.59±0.3	49.6±0.5	38.6±1.9	67.0±5.2	1.3±0.1	6.5±0.3
May-02	24.0	20.1	21.5±0.1	38.3±1.8	36.9±0.8	51.65±4.3	0.4±0.0	9.2±0.7
Jun-02	26.8	16.5	5.2±0.3	41.4±1.1	100.0±3.7	97.6±1.1	0.6±0.0	0.0±0.0
Jul-02	25.0	24.8	12.8±0.2	31.5±1.0	22.0±1.9	158.0±5.6	0.1±0.0	16.6±2.1
Aug-02	13.9	13.9	65.0±0.6	61.1±1.3	28.4±1.1	14.9±2.2	1.4±0.1	5.0±0.8
Sep-02	13.2	16.2	36.0±0.5	37.4±0.7	na	101.5±4.2	0.8±0.0	0.8±0.1
Oct-02	22.7	14.9	34.5±1.1	51.7±0.1	21.1±0.7	37.0±1.1	0.8±0.0	0.0±0.0

Denitrification rates

Clear different magnitudes of denitrification rates were found during the monthly sampling period in sandy sediments (Fig. 3a). Higher rates of denitrification were observed in August (ANOVA, $p < 0.001$) and significantly lower rates were registered in June, July and October (ANOVA, $p < 0.05$). In sandy sediments, nitrate availability and salinity variations at time zero incubation water were clearly related to the measured denitrification rates (Fig. 3a). While a positive relationship was found between this process and nitrate concentration ($R^2 = 0.87$, $p < 0.001$, $n = 9$), denitrification rates were negatively related with salinity ($R^2 = 0.49$, $p = 0.04$, $n = 9$). The nitrate enrichment experiment corroborates with the seasonal program results since a progressive increase in denitrification (ANOVA $p < 0.001$) with the increasing of nitrate availability was found (Fig. 4a). However, in the salinity controlled experiment, no significant changes in denitrification rates between each treatment were observed (ANOVA, $p = 0.21$) (Fig. 4b). These results, together with the fact that during the surveys, nitrate concentration was strongly related to the salinity of estuarine water ($r = -0.81$, $p = 0.008$, $n = 9$) (Fig. 3a), suggests that salinity may not have a physiological effect on the denitrifying communities that inhabit sandy sediments. Thus, the significant relationship found between salinity and denitrification, occurred because nitrate concentration is a function of the salinity of the water rather than a process directly dependent of a given salinity.

In contrast to the results for intertidal sandy sediments, there was no seasonal signal for denitrification with regard to either salinity or nitrogen concentration in the intertidal rocky biofilms (Fig. 3b). These results are in concordance with the absence of a clear salinity regulatory effect in the salinity-controlled experiments, where no statistical differences between denitrification rates in the three salinities tested (ANOVA, $p = 0.61$) were found (Fig. 4d). The nitrate enrichment experiments showed a weak stimulation of denitrification activity between zero and 100 µM of NO_3^- additions (Fig. 4c). However, the lack of statistical differences between the different treatments performed, (ANOVA, $p = 0.40$), indicates that a nitrate concentration effect on denitrification rates was not evident. The apparent absence of a clear nitrate regulatory effect for rocky biofilms suggests that this denitrifier community was not nitrate limited. As in the case of the intertidal

sandy sediments, nitrate availability has also been identified as a primary factor in regulating benthic denitrification (e.g. Ogilvie *et al*., 1997). On the other hand, the absence of a clear physiologically salinity effect on the denitrification rates, contrary to what was observed by Rysgaard *et al*. (1999), indicates that euryhaline denitrifying bacteria inhabit both studied environments.

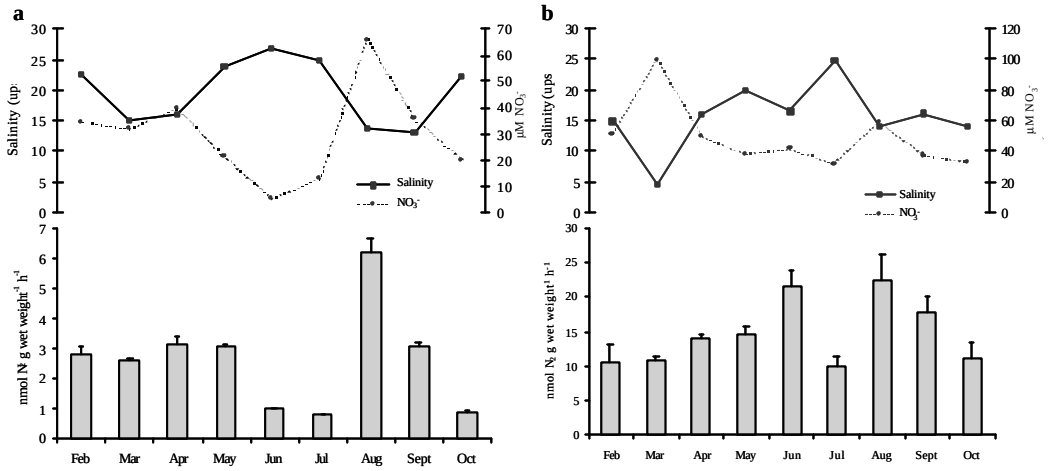

Figure 3 - Seasonal variation of denitrification rates and estuarine water salinity and time zero nitrate concentration (error bars = standard error of the mean of three replicates). a) sandy sediments; b) rocky biofilms.

In order to evaluate the magnitude of the N_2O accumulation during the denitrification process, the relative release of N_2 and N_2O (N_2O:N_2 ratio) was determined (Table 1). N_2O to N_2 ratios in sandy sediments were consistent with literature values (Seitzinger, 1988). However for the rocky biofilms, the obtained N_2O:N_2 ratios were much wider, with the higher value observed for the higher nitrate concentration (Table 1). For both sites, nitrate enrichment experiments showed relatively higher N_2O production with an increased of the amount of nitrate added, which agrees with other reports (Seitzinger *et al*., 1983). In the intertidal sandy sediments, N_2O:N_2 ratio rose from 3.5% at zero µM of NO_3^- addition to 18.8% at 100 µM of NO_3^- addition, maintaining this value for the 300 µM of NO_3^- addition. In the case of rocky biofilms, the N_2O:N_2 ratio increased was progressive with the increase of nitrate added (11.8% to 21.9% and 34.5%, respectively for 0 µM, 100 µM and 300 µM of NO_3^-). These results demonstrate a progressive decrease in denitrification efficiency with the increase in nitrate concentration, since a progressive accumulation of N_2O was observed. The increase of nitrous oxide production is of much concern since it is considered a potent greenhouse gas and a promoter of ozone depletion in the atmosphere (Dickinson & Ciceron, 1986).

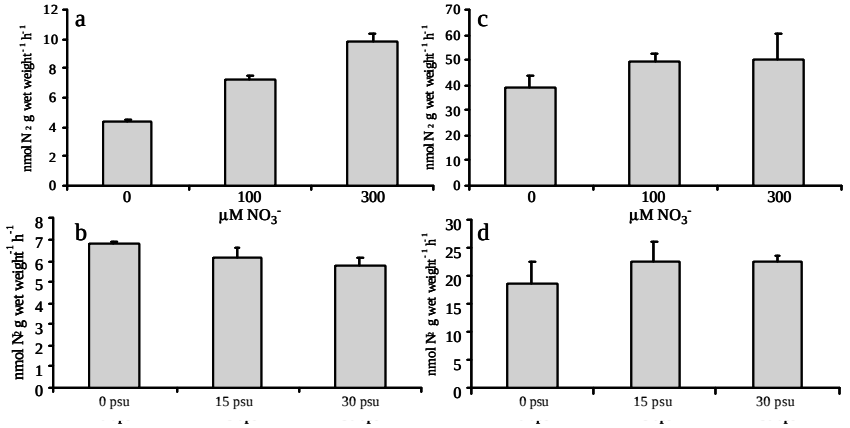

Figure 4 - Denitrification rates in sandy sediments (a, b) and rocky biofilms (c, d) at different salinities and nitrate treatments (error bars = standard error of the mean of three replicates). For nitrate treatments (a, c), estuarine brackish water was amended with 0 mM of NO_3^-, 100 mM of NO_3^- and 300 mM of NO_3^-. For the salinity treatments (b, d), Douro River freshwater was amended with different concentrations of salts to reach 0 psu, 15 psu and 30 psu.

NITRIFICATION RATES

Nitrification rates in sandy sediments showed a peak in June (ANOVA, $p < 0.001$) with lower values and slight variations for the other months (Fig. 5a). The seasonal pattern of ammonium concentration in time zero estuarine incubation water (Fig. 5a) was significantly and linearly related with the nitrification rates measured ($R^2 = 0.56$, $p = 0.02$, $n = 8$). Also a linear and positive relationship between salinity and nitrification rates was registered ($R^2 = 0.48$, $p < 0.04$, $n = 9$). Controlled experiments showed an independently regulatory effect of salinity and ammonium in the nitrification activity of sandy sediments (Fig 6a, b). A nitrification stimulation of 40% and 17% in the incubations performed at 15 psu and at 30 psu, respectively, compared to the incubation at 0 psu (Fig. 6b) was observed. In the ammonium enrichment experiment, a

stimulation of the nitrification activity of 35% was observed for the 20 µM of NH_4^+ treatment, but a clear inhibitory effect of the process was registered when 200 µM of NH_4^+ was added (Fig. 6a), suggesting that ammonium availability stimulates the nitrifying activity up to a certain point. According to the seasonal data, a significantly positive and linear relationship was found between nitrification rates and ammonium concentration within a range of 21.1 - 100.0 µM, suggesting that the sandy sediment nitrifying bacteria were stimulated, at least within such range.

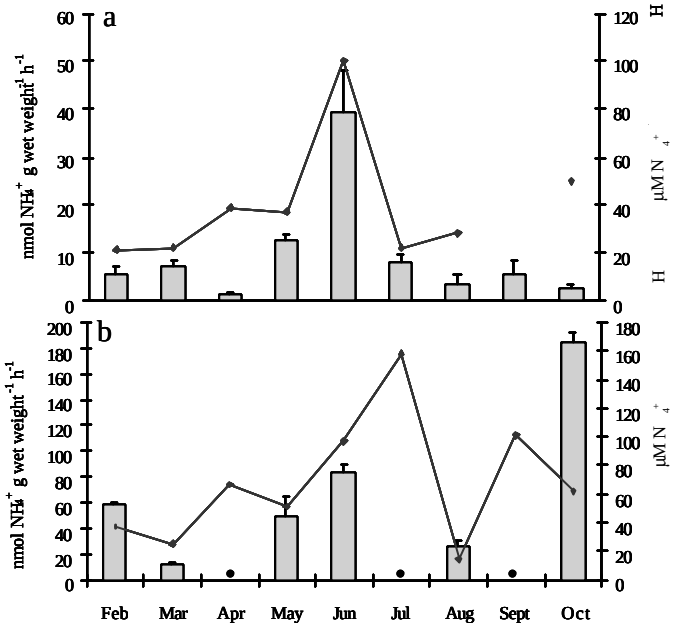

Figure 5 - Seasonal variation of nitrification and time zero NH_4^+ concentration (dark spots = null rates; error bars = standard error of the mean of three replicates). a) sandy sediments; b) rocky biofilms.

Correlation analysis did not show any relationship between seasonal nitrification rates in rocky biofilms and seasonal ammonium and salinity variations. Nevertheless, when in the control experiments ammonium concentration and salinity were isolated, a regulatory effect of these two parameters independently was demonstrated (Fig. 6c, d), with a clear stimulation of nitrification activity (65%) at the highest ammonium concentration tested (Fig 6c). With respect to the controlled salinity experiment, a stimulation of the nitrifiers activity by 50% was observed for the intermediated salinity, whereas an almost total inhibition of the process was registered for 30 psu (Fig. 6d). In contrast, recent studies have shown that a progressive salinity increase reduced the nitrification activity (Rysgaard *et al*., 1999, Campos *et al*., 2002). On the other hand, some culture experiments showed that estuarine isolates of ammonium oxidizers exhibited optimum nitrification rates at intermediate salinities (5-10 psu) (Jones & Hood, 1980) or even between 0-20 psu (MacFarlane & Herbert, 1984) with a subsequent reduction of the activity or inactivation for higher salinities. The lack of evidence for ammonium concentration and/or salinity as regulatory parameters for rocky biofilms during the seasonal surveys, suggests that other parameters could have an important role in regulating the nitrifier activity in these biofilms. Joye & Hollibaught (1995) suggested that sulfide could be an important regulatory factor for nitrification. Similar to the results of this study, several authors have demonstrated the strong ammonium regulatory effect of nitrification activity (e.g Berounsky & Nixon, 1993; Butturini *et al*., 2000). However, in this study is interesting to stress the differences between the responses of each studied environment to the ammonium treatments performed. While rocky biofilms nitrification was greatly stimulated at the higher concentration of ammonium, sandy sediments showed a clear inhibitory effect for such a high concentration of ammonium (Fig. 6a, c). All these results from the seasonal program and the controlled experiments suggests that salinity and ammonium concentration have a different but important regulatory effect on the nitrifier communities that inhabit sandy sediments and rocky biofilms of the intertidal areas of the Douro estuary. While rocky biofilms nitrifier communities showed much higher tolerance to high levels of ammonium concentrations compared with the sandy sediments, they do not tolerate as high salinities as the sandy sediments nitrifier community.

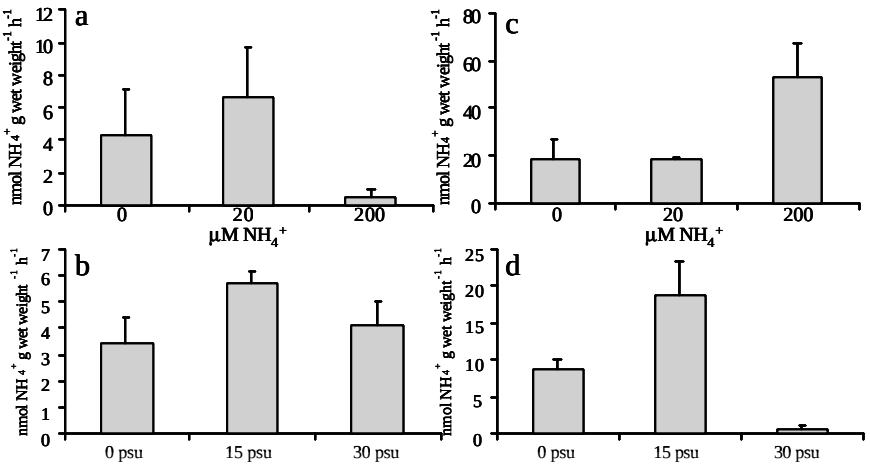

Figure 6- Nitrification rates in sandy sediments (a, b) and rocky biofilms (c, d) at different salinities and ammonium treatments (error bars = standard error of the mean of three replicates). For ammonium treatments (a, c), estuarine brackish water was amended with 0 mM of NH_4^+, 20 mM of NH_4^+ and 200 mM of NH_4^+. For the salinity treatments (b, d), Douro River freshwater was amended with different concentrations of salts to reach 0 psu, 15 psu and 30 psu.

CONCLUSIONS

- Denitrifying bacteria of intertidal sandy sediments and rocky biofilms were not physiologically influenced by the presence of sea salts, indicating that euryhaline denitrifying bacteria inhabit both environments.
- Increasing nitrate concentration clearly stimulated denitrification rates in sandy sediments; however in rocky biofilms this stimulation was not so evident, suggesting that these denitrifying communities are not nitrate limited.
- For both sites, it was demonstrated a decrease in denitrification efficiency with the increase in nitrate concentration, with a progressive accumulation of N_2O.
- While both communities showed optimal nitrification activity at intermediate salinities, sandy sediments nitrifiers tolerated better the increasing in salinity than the nitrifier communities from rocky biofilms.
- Ammonium concentration regulatory effect in the nitrification rates of both environments was demonstrated, and the different responses observed between sites, suggests that rocky biofilms nitrifier communities are more tolerant to higher ammonium concentrations.

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