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Implications of denitrification in the ecological status of an urban river using enzymatic activities in sediments as an indicator

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ABSTRACT

A better understanding of the effects of a number of environmental factors on denitrification is vital for analyzing its role as nitrogen sink and providing deeper knowledge about the ecological status of a nitrate-rich ecosystem. Since few studies have addressed the occurrence and implications of denitrification in river sediments, and complexity of interactions among all these environmental factors makes comprehension of the process difficult, the potential of sediments from the Deba River to attenuate nitrate excess through denitrification was investigated. For this purpose, we adapted an *in vitro* method to measure activities of two enzymes contributing to the entire multiple-step nitrate reduction: Nitrate Reductase and Nitrite Reductase. The environmental features that influence both or single enzymatic activities were identified as oxygen availability, regulated directly by the moisture content or indirectly through the aerobic respiration, organic matter and nitrate content of sediments, and electrical conductivity and exchangeable sodium percentage of water. Additionally, our results showed that Nitrate Reductase catalyzes the principal limiting step of denitrification in sediments. Therefore, taking this enzymatic activity as an indicator, the southern part of the Deba River catchment presented low potential to denitrify but nitrate-limited sediments, whereas the middle and northern parts were characterized by high denitrification potential but nitrate-rich sediments. In general, this study on denitrifying enzymatic activities in sediments evaluates the suitability of the management of the effluents from wastewater treatment plants and municipal sewages to ensure a good ecological status of the Deba River.

Introduction

The declining quality of freshwater ecosystems has become an issue of global concern, especially in regions that have seen dramatic urban development and rapid industrialization. Indeed, the growing intensity and extent of untreated domestic and industrial wastewaters (Ali et al., 2011; Gyawali et al., 2012), or even effluents that are discharged from wastewater treatment systems (Akpor, 2011; Bhat and Pandit, 2014) are responsible for the degradation of the receiving water bodies.

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In this context, an effective water policy must take account of the vulnerability of aquatic ecosystems by the implementation of monitoring programmes for the catchment management. For a coherent and comprehensive classification of ecological status of rivers, the European Water Framework Directive (WFD, 2000/60/EC) establishes different biological, hydromorphological, chemical and physical/chemical parameters to be monitored by such programmes.

The biological indicators are exclusively relative to the aquatic flora, the benthic invertebrate fauna and the fishes living in water. However, bacteria have been recognized as an essential food for protists and invertebrates, constituting the base of benthic food webs (Alongi, 1994) and, consequently, contributing to higher trophic level production. More relevantly, microbial communities harbored in the sediment play a vital role in improving water quality due to their involvement in the biogeochemical cycles of nitrogen (N), phosphorous (P) and sulfur (S), as well as organic matter demineralization and biochemical degradation (Paerl and Pinckney, 1996; Holmer and Storkholm, 2001; Wu et al., 2012; Zhang et al., 2013). In particular, the microbial removal of nitrogen in aquatic ecosystems is of great interest for researches and managers since nitrate excess is linked to eutrophication, especially in coastal marine waters (Burgin and Hamilton, 2007). From the perspective of river water quality, complete denitrification still remains the most desirable nitrate removal pathway because (i) it represents a permanent nitrogen sink (Arce et al., 2015) and, (ii) it does not depend on the availability of additional end-products coupled to other N transformation processes or even S cycling (Burgin and Hamilton, 2007).

Alternatively, the proposed hydromorphological, chemical and physical/chemical parameters are considered to support the biological indicators — expressed in terms of composition and abundance. Nevertheless, factors affecting denitrification drive not only the abundance and diversity, but also the activity of the denitrifying community (Wallenstein et al., 2006; Veraart et al., 2017). Moreover, denitrification rates have not to be related to the abundance and richness of the denitrifiers (Hallin et al., 2009; Graham et al., 2010; Veraart et al., 2017), suggesting that the presence of a denitrifying community does not necessarily imply that it is operating in the ecosystem.

On the other hand, since biological indicators are known to be very sensitive to any ecosystem perturbation (Chaer et al., 2009; Guo et al., 2012), denitrifying activities could act as a potential assessor of the degrading effects of anthropogenic activities on the natural biochemical process of nitrogen. In recent decades, there has been a proliferation of researches into complete denitrification in river sediments (Steingruber et al., 2001; Wall et al., 2005; Tatariw et al., 2013; Liu et al., 2013; Arce et al., 2014) and there is now a deeper understanding of the abiotic factors disturbing the biological nitrate-reducing process. However, denitrification is a facultative anaerobic process consisting of the dissimilatory reduction of nitrate NO_3^- to N_2 by four steps: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$, and it is sequentially catalyzed by Nitrate Reductase (encoded by the gene *narG*), Nitrite Reductase (encoded by the genes *nirS*/*nirK*), Nitric Oxide Reductase (encoded by the gene *norB*) and Nitrous Oxide Reductase (encoded by the gene *nosZ*) enzymes (Zumft, 1997). The multiple interactions among all biotic and abiotic parameters make the process complex to understand, and investigation into single enzyme activity contributing to the entire nitrate-reduction reaction becomes crucial. For this purpose, numerous studies have examined all Reductases individually; however, these works have been primordially carried out in plants and soils.

Thus, the overall aim of this work was therefore to provide information on the activity of two Reductases (Nitrate Reductase and Nitrite Reductase) in surface sediments subject to different types of pollution (treated and untreated wastewaters discharge), in order to provide further knowledge of the effects of anthropogenic activities on denitrification, and consequently, on the ecological status of the Deba River urban catchment, using enzymatic activities as indicators. The specific objectives of this research were (i) to adapt an *in vitro* method for determining Nitrate Reductase and Nitrite Reductase activities in river surface sediments, by evaluating sample storage conditions, weight and moisture content, as well as liquid-to-solid ratio and duration of enzyme-extraction, incubation time and substrate availability, (ii) to analyze enzymatic activity rates during a rewetting period of samples, and (iii) to characterize physical/chemical properties of water and surface sediment samples and investigate their influence on enzymatic activities.

1. Materials and methods

1.1. Study area

The Deba River catchment (538 km^2) is located on the northeast coast of Spain (Gipuzkoa, Basque Country, Fig. 1). The Deba River (60 km) runs through the catchment towards the Bay of Biscay, receiving inflows from several tributaries (the Ego and the Oñati streams). A previous study (Martínez-Santos et al., 2015), offers an extensive description of the lithological characteristics, and main urban and industrial areas in the catchment.

Although the number of water treatment plants (WWTPs) has increased in the catchment in recent years, Borja et al. (2006) reportrted nitrogen loadings of over 9000 kg N/day/km^2 in the water body and classified the Deba River catchment as one of the most polluted rivers in the province of Gipuzkoa.

The Epele WWTP (Fig. 1) was put on continuous operation in May 2012; previously, organic-rich wastewaters from the towns of Arrasate and Oñati were discharged into the Deba River and Oñati stream *via* two sewers. However, the Basque Water Agency (URA, 2017), responsible for monitoring the chemical status of the rivers from the Basque Country, reported that the water body downstream of the Epele WWTP did not reach a "good status" for the concentration of nitrates in September, November and December 2016. On the other hand, despite being one of the largest towns in the area, the untreated sewage effluents of Ermua were previously discharged directly into the Ego stream. In June 2014, the sewer was connected to the Apraitz WWTP (Fig. 1) and the municipal wastewaters from Ermua are currently no longer discharged into the tributary. However, the Basque Water Agency still emphasizes the loss of natural characteristics of this stream due to infringements for ammonium, among other pollutants (URA, 2017).

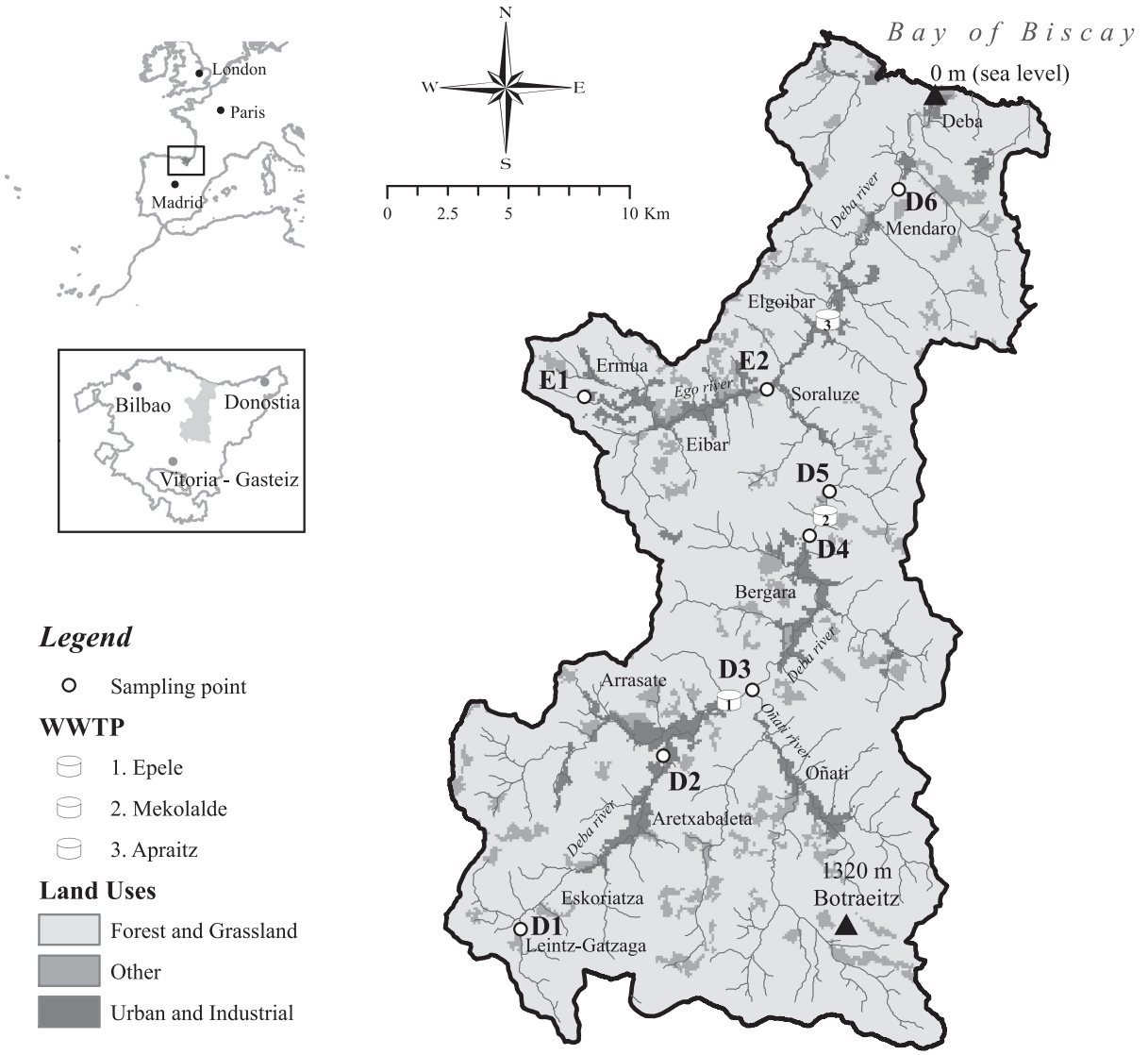

Fig. 1 – Location of the sampling sites, land uses, and wastewater treatment plants (WWTPs) in the Deba River catchment.

1.2. Field methodology

In October 2015, surface sediment samples were collected from six sampling sites (D1, D2, D3, D4, D5 and D6) along the main river bank and from two sampling sites (E1 and E2) in the Ego tributary. These sampling locations were chosen in order to differentiate the influence of natural processes from the effects of anthropogenic pollution sources on enzymatic activities from headwaters to the outlet. As per USEPA (2001), surface sediment subsamples (0–5 cm depth) from multiple points within each sampling site were collected using a sterilized plastic spoon, sieved through a sterile net with a mesh of 2 mm, composited in the field and sealed in sterile polyethylene bags. Water samples were also taken in sterile polyethylene bottles at all sampling sites. Electrical conductivity (EC), pH and redox potential (Eh) were measured *in situ* using a Crison EC-Meter Basic 30+, Crison Micro pH 2000 and Hach ORP/Redox sonde MTC101 with an Ag/AgCl electrode, respectively. All water and surface sediment samples were stored and refrigerated in the dark and transported to the Chemical and Environmental Engineering laboratory (University of the Basque Country) on the same day.

1.3. Laboratory methodology

Surface sediments were air-dried and ground with a pestle and mortar for homogenization. Before air-drying, the moisture content of the sediment samples was determined according to APHA-AWWA-WPCF, 1999. One replicate of each sample was not air-dried and was directly refrigerated at 4°C for the purposes of performing the experiment described below.

Total carbon (TC), nitrogen (TN) and sulfur (TS) were analyzed in the surface sediments using a TruSpec CHNS determinator (Leco Corporation). Volatile solids were determined using a muffle furnace as described in Method 2540 E of the Standard Methods (APHA-AWWA-WPCF, 1999). After incineration at 500°C, the percentage weight loss was considered to be representative of total organic carbon (TOC). The inorganic nitrogen in surface sediments was determined from a KCl 2 mol/L extraction

followed by colorimetric determination of NH_4^+ and NO_3^- content (Mulvaney, 1996) using a spectrophotometer Jasco V630.

Water samples were filtered through 0.45 μm filters. One replicate of each sample was acidified to pH < 2 with HNO_3 (65%) for cations (Ca^{2+}, Mg^{2+}, Na^+ and K^+) analysis using ICP-OES (Perkin Elmer Optima 2000). Anions (Cl^- and SO_4^{2-}) were measured in the non-acidified replicate using ion chromatography (DIONEX ICS 3000).

1.3.1. Measurement of enzymatic activities in surface sediment samples

The following is a description of the methodology applied for measuring enzymatic activity. It is based on studies by Antolín et al. (2010) and Kakutani et al. (1981), and adapted for a sediment matrix by appropriate tests conducted with the surface sediment sample from sampling site D6.

Air-dried surface sediment samples were humidified with Milli-Q until the moisture content was sufficient to ensure they were constantly moist (around 30% water). For 2 days, samples were acclimated at 13°C to simulate autumn river conditions (river water mean temperature in October 2015).

For crude enzyme extract, 1 g of humidified surface sediment was immersed in 12 mL of 50 mmol/L K-phosphate buffer (pH 7.5); 2 mmol/L EDTA; 2 mmol/L DTT; 1% (W/V) insoluble PVPP at 0°C and 1.5% soluble casein. The mixture was continuously homogenized in a rotatory shaker for 10 min. The crude extract was centrifuged at 4300 r/min for 5 min and the supernatant was stored on ice. All of the operations described above were performed in a temperature range of 0–4°C.

To perform *in vitro* Nitrate Reductase (NR) activity assay, 0.1 mL 50 mmol/L K-phosphate buffer (pH 7.5); 0.1 mL NADH (1 mg/mL); 0.2 mL KNO_3 0.1 mmol/L and 0.5 mL crude extract were made up to a final volume of 2 mL with Milli-Q. After 60 min incubation in the dark at 28°C, the reaction was stopped by submerging test tubes in an ice bath. Assay was run in triplicate and the NR activity was expressed as the amount (μmol) of nitrites generated per gram of dry sediment and per hour.

In vitro Nitrite Reductase (NiR) activity assay was performed using initial reaction mixture contained 2 μmol of KNO_2, 0.1 μmol of methyl viologen, 20 μmol of K-phosphate buffer (pH 7.0), and 0.1 mL of the crude extract in a total volume of 0.9 mL in a test tube. After the pre-incubation for 2 min at 30°C, the reaction was started by adding 0.1 mL of the freshly prepared dithionite solution containing 10 μmol of $NaHCO_3$ and 5 μmol of $Na_2S_2O_4$. After incubation for 90 min in the dark at 30°C, the reaction was stopped by vigorous shaking to oxidize reduced methyl viologen and $Na_2S_2O_4$, and chilled in an ice bath. An assay was run in triplicate and NiR activity was expressed as the amount (μmol) of nitrites consumed per gram of dry sediment and per hour.

For nitrite measurement, 0.8 and 0.6 mL aliquots of the incubation medium from NR and NiR, respectively, were diluted to 10 mL with Milli-Q water. The amount of nitrite in the reaction mixture was analyzed by adding 1 mL of 1% (W/V) sulphanilamide in HCl 1.5 mol/L and 1 mL of 0.02% (W/V) N-(1-naphthyl)-ethylenediamine dihydrochloride solution, and measuring the absorbance (540 nm) after 15 min by an ultraviolet spectrophotometer (Jasco V-630) (USEPA, 1979). A standard curve was prepared in the same way as the samples in the assays, but using aliquots of 0.8/0.6 mL of $NaNO_2$ standard solutions for NR and NiR, respectively.

1.3.2. Surface sediment sample weight, moisture conditions and storage

Sample preparation has a profound effect on the final outcome of enzyme extraction and its subsequent activity analysis. The weight-moisture tests were conducted by immersing 0.5, 1.0 and 1.5 g of air-dried (unhumidified) or wet surface sediment in enzyme-extracting dissolution. In addition, wet and humidified samples were analyzed to represent different sample storage conditions (wet or air-dried) affecting enzymatic activities with the optimal sample weight and moisture content. As both enzymatic activities were measured on aliquots from the same crude extract, NR activity was used as an indicator to select the best form of sample manipulation.

1.3.3. Liquid-to-solid ratio and duration of the enzymatic extraction

The step prior to the enzymatic activities assay involves the extraction of the enzymes synthesized by the denitrifying microorganism presented in the sediment. Based on previous studies (Alche et al., 2006; Martínez-Maqueda et al., 2013), a combination of mechanical homogenization with buffers was also used since it appears to be one of the best method even for strong cell disruption in plant tissues (Van Het Hof et al., 2000). Periplasmatic and membrane-bound NR, and periplasmatic NiR take part in the denitrification pathway in bacteria (Cabello et al., 2004). It is therefore crucial to ensure an optimum contact between sediment and enzyme-extracting dissolution to break the cytoplasmic membrane and extract both enzymes. Two liquid-to-solid ratios (5:1 and 12:1) found in the literature (Kaiser and Lewis, 1984; Antolín et al., 2010) were achieved by adding 5 and 12 mL of enzyme-extracting dissolution to 1 g of humidified surface sediment. Additionally, four operating scenarios were applied with different enzyme extracting times (5, 10, 30 and 60 min) and the liquid-to-solid ratio was set at 12:1. Since there is evidence suggesting that NR is more sensitive to inactivation depending on the composition of the extracting solution than NiR (Buczek, 1984), NR activity was also used as an indicator to establish the best enzymatic extraction procedure.

1.3.4. Initial nitrate concentration in the NR assay and enzymatic activities kinetics

Given that surface sediments contain nitrate, the influence on Nitrate Reductase activity of adding external substrate to the incubation medium was studied. Twenty four operating scenarios were applied combining different KNO_3 dissolution concentrations (0; control, 1, 10 and 100 mmol/L) and various incubation times (0, 15, 30, 45, 60, 75, 90, 105 and 120 min) when NR activity was measured. In addition, NiR activity assay was conducted at various incubation times (0, 15, 30, 45, 60, 75, 90, 105 and 120 min) when 0 (control) or 2 μmol of KNO_2 were added to a total volume of 0.9 mL in a test tube.

1.3.5. Effect of rewetting on enzymatic activity

A positive correlation has generally been found between microbial activity and water content (Iovieno and Baath, 2008). Indeed, rewetting of dry sediments has been shown to rapidly stimulate denitrification in Mediterranean temporary streams (Arce et al., 2014). However, there is little scholarship on the effect of prolonged rewetting on all Reductases that catalyze each step of the denitrification process in sediments. Therefore,

air-dried surface sediment samples were initially rewetted with Milli-Q (around 30% water) and kept moist for 5 days. The moisture content of the samples was monitored (adding Milli-Q water where necessary) and both enzymatic activities were measured daily.

1.4. Statistical analysis

A Shapiro–Wilk test was performed to check whether variables were normally distributed. Where necessary, data were log-transformed to ensure homogeneity of variance. The differences obtained by adjusting the parameters relative to adaptation of methodology, as well as the temporal and spatial variability of moisture content, enzymatic activities and nutrients during the rewetting period were analyzed using one-way ANOVA, taking $p < 0.05$ as significant, in accordance with Tukey's multiple range test. The physical/chemical effect of water and surface sediments on denitrifying microorganism activity was determined using Spearman correlation analysis (non-parametric test) and regression analysis between factors and NR or NiR activities. In addition, principal component analysis (PCA) was used to identify the main physical/chemical parameter controlling enzymatic activities in surface sediments from each sampling site. PCA with an eigenvalue greater than 1 was subjected to an orthogonal varimax rotation. This maximizes the variance to obtain a pattern of loadings for each factor that is as diverse as possible, thus lending itself to easier interpretation. Statistical processing of the data was performed using SPSS 22.0 software.

2. Results and discussion

2.1. Adaptation of *in vitro* assay of enzymatic activities for a sediment matrix

2.1.1. Sample preparation and enzymatic extraction

While no significant differences were found, wet surface sediment presented higher NiR activities than dry (not humidify) surface sediment, even where sample weights were different (the lowest increase was noted when 1.0 g of surface sediment sample was used in the assay). In addition, the replicate stored without previous air-drying showed a greater — though not significantly different — NR activity than the air-dried and humidified surface sediment sample (Table 1). It is well-known that moisture is one of the major environmental factors controlling microbial activity in soils, which is presumably lower in sediment with low soil water content (Hicks et al., 2003; Di et al., 2014). Given this circumstance, and in order to try to preserve the sample in the best conditions, surface sediments were air-dried before storage in the expectation that enzymes would become inactive. Rewetting dry surface sediments before enzymatic analysis will result in a microbial reactivation since organic matter becomes accessible for microbial degradation and promote microorganism growth (Iovieno and Baath, 2008).

An increase in nitrite generation rates with liquid-to-solid (L:S) ratio suggests that a higher volume of enzyme extraction dissolution improves contact between the solid and aqueous phases. Also, since buffer is required to maintain the stability of enzymes in both pH and ionic-strength terms, a high quantity would appear to prevent changes in the pH of the crude extract and modification of the proteins in the sediments (Laing and Christeller, 2004). Moreover, 10 min of continuous mixture shaking seems to be enough to promote the membrane break and facilitate efflux of the enzymes into the medium (Table 1). Indeed, Laing and Christeller (2004) recommend that the extraction procedure be performed rapidly to minimize exposure of enzymes to potentially damaging compounds upon cell breakage.

Table 1 – Nitrate Reductase (NR) activity in relation to varying parameters mentioned in the sample preparation and enzyme-extraction procedure.

	Parameter	Value		NR activity (μmol NO_2^--N generated/g/hr)
Sample preparation	Moisture and Weight	Dry (without humidify)	0.5 g	0.530 ± 0.012[a]
			1.0 g	0.232 ± 0.015[a]
			1.5 g	0.175 ± 0.021[a]
		Wet	0.5 g	0.844 ± 0.098[a]
			1.0 g	0.245 ± 0.008[a]
			1.5 g	0.196 ± 0.013[a]
	Storage	Dry (humidify)		0.686 ± 0.021[b]
		Wet		0.746 ± 0.033[b]
Enzymes extraction	L:S ratio	5:1		0.232 ± 0.015[c]
		12:1		0.581 ± 0.016[c]
	Duration	5 min		0.563 ± 0.047[d]
		10 min		0.746 ± 0.033[d]
		30 min		0.556 ± 0.008[d]
		60 min		0.615 ± 0.034[d]

Each value represents the mean of three replicate samples ± standard deviation. Different lowercase letters within the same parameter indicate that means are significantly different at $p = 0.05$.

Based on the results obtained from these different tests, and in order to reproduce faithfully the enzymatic activities occurring in the river environment, all crude enzyme extracts were obtained by immersing 1.0 g of surface sediment sample (previously humidified and acclimated for 2 days) in 12 mL of enzyme extraction dissolution and continuously shaking for 10 min.

2.1.2. Nitrate and nitrite reduction kinetics

The time course of nitrite accumulation during the initial 30 min of dark incubation of NR is characterized by lower NO_2^- generation when extra-nitrate is added (Fig. 2a). This suggests that excess substrate availability might decrease nitrite production until acclimatization and microbial demand increase. Indeed, other authors (Timpo and Neyra, 1983; Cazetta and Vasques Villela, 2004; Baloft et al., 2015) have found that, even if Nitrate Reductase activity in both leaf and stem tissues is enhanced by increases in nitrate supply, a high NO_3^- content exceeding plant demand promotes a decrease in NR activity. Nevertheless, increasing the concentration of added KNO_3 dissolution to 100 mmol/L results in a significant ($p < 0.05$) rise in the generation of nitrites over 60 min of incubation (Fig. 2a).

Zero-order kinetics could be used for modeling nitrite accumulation during the NR assay before maximum concentrations were achieved at 60 min, when KNO_3 is added to the incubation medium. In the case of the control replicate, surface

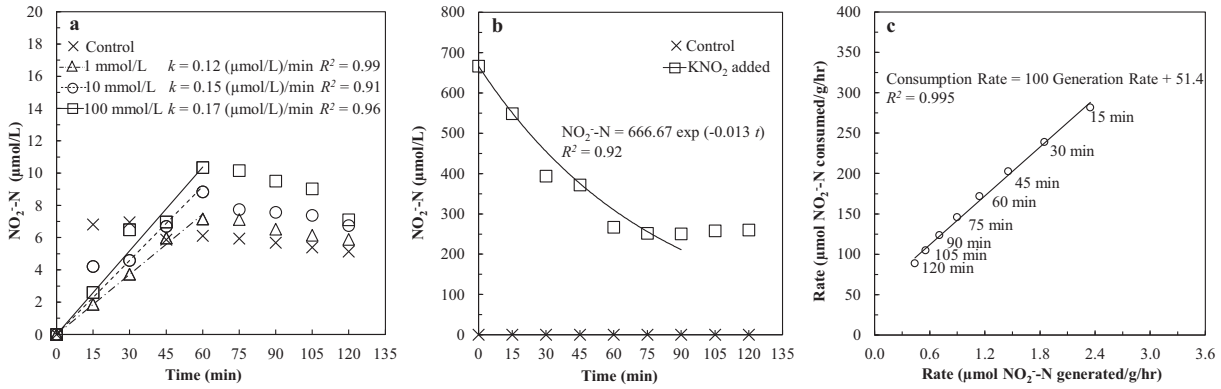

Fig. 2 – Time course of nitrite accumulation in the incubation medium (a) during the NR assay with varying external KNO_3 solution concentrations (zero-order models for nitrate reduction in the first hour of incubation were determined), and (b) during the NiR assay with and without KNO_2 addition (first-order model for nitrite reduction in the first 90 min of incubation was determined). The rate of nitrites consumption by NiR and the rate of nitrites generation by NR (with KNO_3 100 mmol/L dissolution) were calculated and compared (c). NR: Nitrate Reductase; NiR: Nitrite Reductase.

sediment NO_3^- content was sufficient for nitrites generation but significant variations ($p < 0.05$) along the incubation time were not observed. According to linear regression, nitrate reduction coefficient was greater for KNO_3 100 mmol/L dissolution (Fig. 2a). All these findings are in agreement with data from previous studies in both plants and soils (Jampeetong and Brix, 2009; Yu et al., 2012; Baloft et al., 2015), confirming that the addition of NO_3^- increases NR activity.

The NiR assay showed that the addition of external substrate (KNO_2) is crucial for enzyme activation since initial (t = 0 min) NO_2^- content in the incubation medium for the control sample was zero (Fig. 2b). First-order kinetics could be used for modeling nitrite accumulation during the NiR assay, just before achieving minimum concentration and adding KNO_2 (90 min). According to exponential regression, nitrite reduction coefficient was 0.013 min^{-1}. Consequently, a NR assay was conducted adding 0.2 mL of 100 mmol/L KNO_3 to the reaction medium and incubating for 60 min, while NiR assay was run by the addition of 2 μmol of KNO_2 and 90 min of incubation.

Finally, there is evidence suggesting that NR activity, considered as a key enzyme in nitrogen metabolism, is the main limiting step in nitrate assimilation in most macroalgae and higher plants (Lea, 1997; Cazetta and Vasques Villela, 2004; Chow, 2012; Klobus et al., 2013). Therefore, the activities for both enzymes under study were calculated from nitrites accumulation data throughout the incubation period, as represented in Fig. 2a and b (for NR activity, results obtained when KNO_3 100 mmol/L dissolution was added were taken into account) and compared in Fig. 2c. The slope of the linear adjustment (Fig. 2c) proves that 100 μmol of nitrites would be consumed by NiR for each μmol of nitrite generated by NR, verifying that NR activity is also the main limiting step in nitrate reduction in sediments. This finding could be the result of (a) the abundance of *nir* gene copies as opposed to *nar* gene copies in the surface sediment; however, since activity has not necessarily to be related to the abundance and richness of the denitrifying microorganisms, gene expression studies are required for a suitable identification of those genes contributing more to nitrite-reduction than to nitrate-reduction step, and/or (b) the low conversion rates achieved by NR, which consumed nearly all the NO_3^- in the surface sediment even though around 1000 times more nitrate was added to the incubation medium (Table 2).

2.2. Enzymatic activity rates throughout the rewetting period

After humidifying (t = 0 hr), the average moisture content increased to 25% ± 7% for all surface sediment samples. A slight decrease was observed to the end of the experiment (up to 39%), as shown by the fact that there were statistically significant temporal differences in moisture (Fig. 3a). Throughout surface sediments rewetting, NR activity rose to a peak value of 1.83 ± 0.21 μmol NO_2^--N generated/g/hr at 72 hr. After this time, a significant decrease was observed to the end of the experiment, evidenced by the relatively large variation (up to 55%) within the last 24 hr of the rewetting period (Fig. 3b). On the other hand, NiR activity exhibited a non-consistent temporal trend during the test with a moderate decrease (>27%) at 96 hr of the rewetting time (Fig. 3c). Values decreased at the first 24 hr of rewetting to a minimum of 123.1 ± 16.0 μmol NO_2^--N consumed g/hr, which increased within the next 48 hr, before diminishing again to 96.6 ± 3.1 μmol NO_2^--N generated/g/hr at 96 hr. NH_4^+ in the surface sediments tended to increase throughout experimental rewetting (Fig. 3d), and rose sharply to 2.52 ± 1.43 μmol NH_4^+-N/g at 96 hr, with concentrations of 2–7 times higher than for initial conditions (t = 0 hr). Finally, the NO_3^- content in surface sediments also underwent substantial changes during the test (Fig. 3e). All samples, except E2 and D6, exhibited a significant decrease (>42%) within the first 72 hr, just before a new rise to 75% of the initial condition (0.154 ± 0.096 μmol NO_3^--N/g) at 96 hr.

Median values of both enzymatic activities were not significantly different among sampling locations (Fig. 3b and c) due to the global effect of multiple parameters. Although it has been argued that O_2 might be an incomplete suppressor of denitrification (Wrage et al., 2004; Vega-Jarquin et al., 2008), the denitrifying enzymes are produced in near anaerobic conditions (van Spanning et al., 2007). Therefore, when the concentration of oxygen is high, the activity of the denitrifying enzymes is expected to be inhibited (van Spanning et al., 2007; Di et al.,

Table 2 – Water-surface sediment abiotic parameters and median values of NR and NiR activities measured in surface sediment for all sampling sites.

Parameter		Sampling site							
		D1	D2	D3	D4	D5	D6	E1	E2
Water	EC (μS/cm)	594	1313	844	578	558	497	457	470
	pH	7.8	8.2	8.0	7.9	7.9	7.7	7.9	7.6
	Eh (mV)	185	134	143	179	196	232	230	199
	ESP (%)	44.8	44.5	31.7	22.9	24.7	23.4	7.1	12.5
Surface sediment	TC (%)	3.3	3.3	5.5	3.3	3.9	2.8	2.4	5.7
	TOC (%)	2.8	1.8	2.6	1.6	1.9	1.3	1.9	1.7
	TN (%)	0.32	0.19	0.40	0.13	0.15	0.08	0.14	0.17
	TS (%)	0.08	0.35	0.22	0.02	0.15	0.02	0.01	0.05
	TC:TN (μmol TOC/μmol NO_3^--N)	12.1	20.1	16.2	29.8	30.0	40.3	19.9	39.0
	NH_4^+ (mg/kg)	9.0	4.3	20.1	7.3	6.4	3.6	3.3	36.1
	NO_3^- (mg/kg)	6.0	6.0	14.6	8.4	4.4	9.4	6.2	25.8
	TOC:TS (μmol TOC/μmol NO_3^--N)	34.4	5.3	12.0	79.3	12.4	64.0	188.6	33.0
	TOC:NO_3^- (μmol TOC/μmol NO_3^--N)	5335	3597	2106	2207	4906	1593	3578	747
	NR activity (μmol NO_2^--N generated/g/hr)	1.12	1.06	1.33	1.20	1.14	1.21	1.33	1.48
	NiR activity (μmol NO_2^--N consumed/g/hr)	125	111	130	113	116	110	127	114

EC: electrical conductivity; Eh: redox potential; ESP: exchangeable sodium percentage; TC: total carbon; TN: total nitrogen; TS: total sulfur; TOC: total organic carbon; NR: Nitrate Reductase; NiR: Nitrite Reductase.

2014). Aside from partial oxygen pressures in the gas phase, water-filled pore space is the most important regulatory factor of soil aeration as it represents a barrier to rapid O_2 diffusion, resulting in a strong link between oxygen availability and soil water content (Smith, 1990; Weier et al., 1993; Giles et al., 2012). In this context, Nitrite Reductase activity showed a strong positive correlation with surface sediment moisture content ($p = 0.541$ at 0.01 level) while Nitrate Reductase activity was not correlated to this environmental factor, indicating that interactions among diverse parameters lead to different water content influence.

Contrary to expectations, enzymatic activities were not correlated to substrate availability (NH_4^+ or NO_3^- in the surface sediments) during the rewetting period. Even in saturated sediments, particle size or geometry may affect capillary action, leaving air spaces in many pores and generating some aerobic microsites, where nitrate-reducing processes could be limited (Giles et al., 2012). Thus, while *in vitro* assays measured the anaerobic nitrate and nitrite reduction rates, during the rewetting period at 13°C both aerobic and anaerobic processes involved in nitrogen transformation might be occurring (e.g., ammonification, nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA), *Anammox*, *etc.*). NR activity, expressed as an average over two consecutive days, was compared to the variation in nitrate surface sediment content over the same period for all sampling sites. Both variables were positively correlated ($p = 0.451$ at 0.01 level), suggesting that anaerobic nitrate reduction catalyzed by NR enzyme took place when surface sediment samples were rewetted and acclimated at 13°C. However, if denitrification had been the only nitrate transformation process, the correlation would have been negative (the higher the NR activity, the greater the nitrate consumption). In addition, variation in nitrate and ammonium surface sediment contents (Δμmol NO_3^--N/g and Δμmol NH_4^+-N/g, respectively) over two consecutive days during the experiment were also positively correlated ($p = 0.624$ at 0.01 level). These results are in agreement with other authors, who reported that microbial ammonia oxidation is the first and rate-limiting step for subsequent nitrogen transformation and removal (Li et al., 2011; Zhi and Ji, 2014), since it produces substrate for Nitrate Reductase.

Finally, in the absence of a strong correlation between surface sediment moisture content and NR activity, aerobic respiration appears to play a crucial role in O_2 availability. Dissolved oxygen consumption by the oxidation of organic matter led to increasing anaerobic environmental conditions in the system, which were more favorable for denitrification (Tiedje, 1988; Giles et al., 2012; Zhi and Ji, 2014). According to previous studies (Hwang et al., 2005; Zhi and Ji, 2014), this relationship suggests an ecological and functional interaction between nitrifying and denitrifying microbial communities, inconsistent with any basic ecological premise that nitrification and denitrification are relatively independent and separate processes operating under different or contrasting conditions.

2.3. Environmental factors affecting enzymatic activities

Electrical conductivity, pH and Eh were measured in water samples of all sampling sites (Table 2). In addition, exchangeable sodium percentage (ESP) was calculated as $ESP = [Na^+] / [Na^+ + K^+ + Ca^{2+} + Mg^{2+}] \times 100\%$; where concentrations of cations are expressed in (mg/L) (Rietz and Haynes, 2003).

The pH in the water for this sampling campaign varies between 7.7 (D6) and 8.2 (D2), indicating a weakly alkaline environment but well-buffered surface waters. Redox potential shows positive values (134–232 mV) at all sampling sites. The highest values of electrical conductivity (1313 μS/cm) and most of the major ions (Na^+, K^+, Ca^{2+}, Mg^{2+}; Cl^-, SO_4^{2-}; data not included) are found in D2. Indeed, anhydrite and gypsum intercalations with limestone and sandstones (Wealden Facies evaporates) have been reported in the upper-part of the Deba catchment by Ábalos et al. (2008) and Iribar and Ábalos (2011). Consequently, the diffuse discharge of groundwater flows from evaporitic deposits, with high content of Na^+, Cl^- and SO_4^{2-}, might influence this ionic enrichment

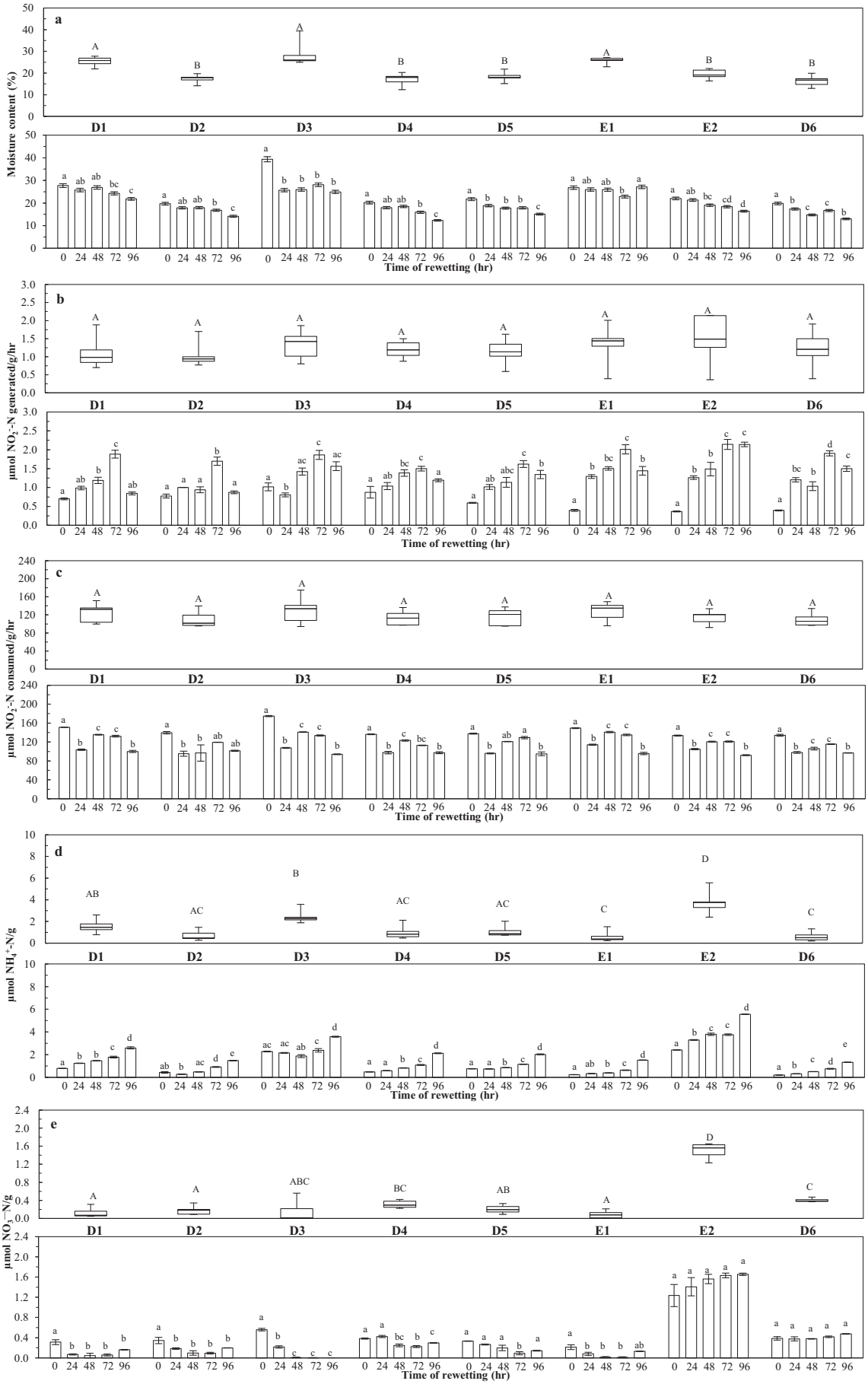
a
Moisture content (%)
D1 D2 D3 D4 D5 E1 E2 D6
Time of rewetting (hr)
b
μmol NO2−-N generated/g/hr
c
μmol NO2−-N consumed/g/hr
d
μmol NH4+-N/g
e
μmol NO3−-N/g
0 24 48 72 96

Table 3 – Regression analysis between median values of enzymatic activities and water- surface sediment abiotic characteristics.

Enzyme	Environmental factor	Regression
NR	Nitrate sediment content (mg NO_3^--N/g)	NR activity = 16.3 NO_3^- + 1.07 $R = 0.835$ $p = 0.010$
	TOC:NO_3^- (μmol TOC/μmol NO_3^--N)	NR activity = − 0.159 ln (TOC : NO_3^-) + 2.48 $R = 0.752$ $p = 0.031$
	Water electrical conductivity (μS/cm)	NR activity = − 0.002 EC + 2.23 $R = 0.810$ $p = 0.051$
	Exchangeable sodium percentage (%)	NR activity = − 0.007 ESP + 1.43 $R = 0.734$ $p = 0.038$
NiR	TOC (%)	NiR activity = 12.2 TOC + 94.6 $R = 0.793$ $p = 0.019$
	TC/TN (μmol total C/μmol total N)	NiR activity = − 13.2 ln (TC : TN) + 160 $R = 0.721$ $p = 0.044$

NiR: Nitrite Reductase.

(Martínez-Santos et al., 2015) and therefore the high values of the exchangeable sodium percentage (ESP) at D1 and D2 (44.8 and 44.5, respectively).

Total carbon (TC) and total organic carbon (TOC), total nitrogen (TN), total sulfur (TS), total ammonium (NH_4^+) and total nitrate (NO_3^-) were also analyzed in surface sediments of all sampling sites. Additionally, TC:TN, TOC:TS and TOC:NO_3^- ratios were calculated (Table 2).

Percentages of TN and total organic carbon (TOC) range from 0.08% to 0.40% and 1.3% to 2.8%, respectively. A significant correlation ($p = 0.762$ at 0.05 level) between these elements indicates that the primary source of nitrogen was the mineralization of organic matter. The spatial evolution of TOC reveals that its main sources were the natural processes and anthropogenic activities occurring throughout the catchment, such as the deposition of vegetation at D1 and E1, and WWTPs effluents at D5 and, especially at D3. On the other hand, the Ego tributary has the greatest spatial variability of nutrients; while at E1 ammonium and nitrate contents were 3.3 and 6.2 mg N/kg, respectively, E2 exhibited the highest values for these elements (36.1 N/kg of ammonium and 25.8 N/kg of nitrate). Surprisingly, the main river also shows high nutrients contents at D3 (Table 2). Frequently, the discharge of untreated wastewater or improperly treated effluents from WWTPs results in the deposition of large amounts of organic matter and nutrients (Naidoo and Olaniran, 2014). Even though D6 is located downstream of the Apraitz WWTP (Fig. 1), the decrease of TOC and the increase of nitrates compared with D5 suggest that this sampling site was more impacted by untreated urban wastewaters from the Ego tributary than by the effluents from the WWTP.

At the same time, the percentage of TS varies between 0.01% and 0.35%. Sulphate reduction below an oxygenated water column typically has TOC:TS ratios in the range of 1.5 to 5.0. A dramatic decrease in TOC:TS ratio was observed at D2, mainly caused by the high TS content (Table 2). This suggest that surface sediments might be deposited in periods of anoxia, although the redox potential measured in water indicates oxygenated conditions (Martínez-Santos et al., 2015).

Finally, although significant differences among sampling locations were not observed (Fig. 3b and c), the highest median value of Nitrate Reductase activities was computed in surface sediments at E2, followed by D3 > E1 > D6 > D4 > D5 > D1 > D2. In contrast, the greatest median value of NiR activities was observed at D3, followed by D1 > E1 > D5 > E2 > D4 > D2 > D6 (Table 2).

A number of environmental factors are known to control the rate of denitrification, including the O_2 and water content of soils, NO_3^-, carbon, pH, and temperature (Giles et al., 2012). After studying how enzymatic activities are directly or indirectly affected by O_2 and water content of sediments during the rewetting cycle, the effect of physical/chemical characteristics of the water and surface sediment on enzymatic activities was determined by regression analysis (Table 3).

Some authors have highlighted the importance of substrate availability as the major environmental factor controlling microbial activity in soils (Tiedje, 1988; Iovieno and Baath, 2008; Woodward et al., 2009; Yu et al., 2012; Giles et al., 2012). As expected, a positive relationship has been found between surface sediment nitrate concentrations and Nitrate Reductase activity (NRA, Table 3). NRA also showed a significantly positive correlation with N-NO_3^- concentration ($p = 0.857$ at 0.01 level), indicating a greater NR activity with a greater presence of the right form of nitrogen in surface sediments. On the other hand, while nitrate surface sediment content induces Nitrate Reductase activity, TOC is only positively correlated ($p = 0.857$ at 0.01 level) to NiR activity, displaying a linear pattern (Table 3). This may be explained by the same substrate affecting the variety of Reductases differently (Giles et al., 2012). Many studies have shown that C can affect the denitrifying capacity of soils (Dodla et al., 2008; Henry et al., 2008; Yu et al., 2012; Giles et al., 2012), since its degradation provides a source of electrons for denitrifying enzymes (Richardson, 2000). However, other authors have reported that nitrate is more rapidly reduced than nitrite when there is sufficient carbon supply (Lu et al., 2014). Conversely, Henry et al. (2008) found that the increasing percentage of the potential nitrate reduction rate was lower than that for potential denitrification rate in the soil microcosm amended with artificial root exudates. Observed responses

Fig. 3 – Moisture content (a), NR activity (b), NiR activity (c), ammonium content (d) and nitrate content (e) for all surface sediment samples are shown. Column plots represent values during the rewetting period (at 0, 24, 48, 72 and 96 hr), with bars indicating the standard error (SE). Boxplots represent the distribution of values for the complete rewetting period for each surface sediment sample. Different lowercase letters within a sample indicate that means are significantly different at $p = 0.05$ among rewetting times, while different uppercase letters indicate that medians are significantly different at $p = 0.05$ among sampling sites.

indicate that organic matter content in surface sediments was sufficient for nitrate reduction while it (organic matter) was vital for the occurrence of nitrite reduction.

Carbon to nitrogen ratio appears to affect both enzymatic activities negatively (Table 3), in the form of TOC:NO_3^- ratio to NR (p = −0.810 at 0.05 level) and as TC:TN ratio to NiR (p = −0,714 at 0.05 level). Zhi and Ji (2014) evaluated wastewater treatment performance in a wetland constructed by tidal flow under different C:N ratios (calculated as a quotient of chemical oxygen demand over ammonium-nitrogen in water), and concluded that a value greater than six was required to achieve complete denitrification. In our study, all surface sediment samples had high C:N ratios favored by nitrate-limited contents (Table 2). However, Burgin and Hamilton (2007) hypothesized different dissimilatory pathways of nitrate removal, and suggested that fermentative dissimilatory nitrate reduction to ammonium (DNRA) may be favored in nitrate-limited and labile carbon-rich environments over respiratory denitrification.

Finally, NR activity decreased with a rise of water EC (R = 0.824; p = 0.044 taking into account all sampling sites, except D2 and D3) and ESP (Table 3), illustrating the negative effect of these parameters on the denitrifying microorganisms (Wong and Dalal, 2008). With the increased EC, denitrifying bacteria growth and respiratory activity increased, lowering the efficiency of carbon use (Yu et al., 2012). In addition, under sodic conditions, toxicities of Na^+ and other accompanying ions (*e.g.*, Cl^- and HCO_3^-) along with water with a very high pH (around 8.0) also inhibit microbial growth (Zahran, 1997; Yu et al., 2012).

Since interactions among all environmental factors were considered responsible of the absence of significant spatial differences, PCA was used to identify the main factor controlling enzymatic activities at each sampling site. PCA produced two principal components, which together accounted for 68.2% of the total variance (35.8% for the Factor I and 32.4% for the Factor II). Fig. 4a summarizes the loadings of the factors; particularly noteworthy is the absolute value of the loadings more than 0.5 of the total variance. Factor I had strong loadings on NO_3^- and NH_4^+, while Factor II showed strong positive loadings on TOC, TOC:NO_3^-, and negative loadings on TC:TN. According to the influence of environmental parameters on enzymatic activities (Table 3), Factor I shows that substrate availability was a key factor for Nitrate Reductase activity. Additionally, Factor II shows that organic matter was efficiently utilized by Nitrite Reductase, whereas TC:TN or TOC:NO_3^- ratios had a strong repression effect on both enzymatic activities.

In a plot of the scores for the factors (Fig. 4b), surface sediment samples were clustered as expected. E2 and D3 were the sampling sites with the highest mean NR activities due to the high ammonium and nitrate loadings from the Epele WWTP and untreated wastewaters (UWW) discharge in the Ego stream, while the high TOC:NO_3^- ratio at D1 had a negative impact on this enzymatic activity favoring the fermentative dissimilatory nitrate reduction to ammonium (DNRA). In contrast, the sampling sites with the highest mean NiR activities (D3 and D1) can be seen to be distributed towards the positive side of Factor II induced by their high organic matter content.

It was noted that sampling locations inside the confidence ellipse (Fig. 4b) were influenced by more than one factor and, consequently, the effects of natural processes or anthropogenic activities on enzymatic activities could not be distinguished. For example, even though an increase in TOC was observed (Table 2), sampling sites up- and downstream of the Mekolalde WWTP (D4 and D5, respectively) are grouped together and they are not primarily influenced by organic matter loadings (Fig. 4b), suggesting that mineral parameters (ESP or EC) modified the effects of treated wastewaters discharge on NR and NiR activities. On the other hand, D2 had the lowest enzymatic activities even though it is not clearly associated with a main factor. Saline springs, which provide indirect evidence of the occurrence of evaporates, are common in this area and have a high Na^+, Cl^- and SO_4^{2-} concentration with an important presence of dissolved H_2S (Iribar and Ábalos, 2011). Moreover, the high sulfur content and the low TOC:TS ratio at D2 (Table 2) suggest that these surface sediments might have been deposited under periods of anoxia, favoring the biological sulphate reduction (Appelo and Postma, 2005) and the subsequent formation of metal sulphides (Fu and Wang, 2011; Martínez-Santos et al., 2015). In recent years, the existence of reduction of nitrate coupled with oxidation of reduced sulfur forms, including free sulphide (H_2S and S^{2-}) and elemental sulfur (S) has been documented in marine and freshwater ecosystems (Brettar and Rheinheimer, 1991; Brunet and Garcia-Gil, 1996; Burgin and Hamilton, 2007). It may therefore be concluded that the inhibition by sulphide and the high EC and ESP values observed for this site (Table 2) could explain the low enzymatic activities measured at D2.

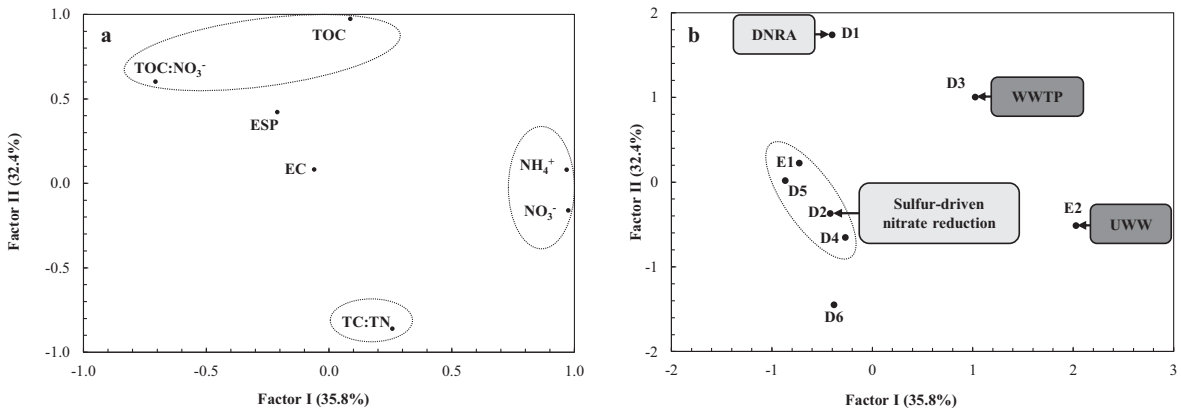

Fig. 4 – Loadings plot (a) and scores plot (b) of the factors to enzymatic activities in surface sediments for all sampling sites.

Finally, the response-time of the microbial communities harbored in the surface sediments for reducing nitrates, which has been previously considered the main limiting step of denitrification, were calculated from the nitrate content and the NR activity (Table 2). Considering an adequate optimization of the *in vitro* method for determining the enzymatic activities, the response-time represents a "theoretical minimum response-time" of the microbial communities. Despite the high NR activity, E2 and D3 presented the highest values (75 and 47 min, respectively) compared to the rest of sampling sites (17, 20, 23, 24, 30 and 33, for D5, E1, D1, D2, D4 and D6, respectively), indicating that the denitrifying activity might not be sufficient to support the high nutrient loadings from Ermua municipality and Epele WWTP, and to ensure the ecological health of the surrounding environment. In addition, although headwaters of the Ego tributary (E1) presented approximately 50% more NO_3^- than surface sediments from downstream of the Mekolalde WWTP (D5), they both showed the lowest response-time values. Therefore, the response-time highlights the importance of considering both the cumulative effects of the pollutants concerned (nitrates) and the purifying capacity of the recipient natural environment (denitrifying activity), as a more reliable reflect of the ecological status of an ecosystem.

3. Conclusions

The ecological effect of nitrate excess in rivers is a worldwide problem. In this context, denitrifying microbial communities, which are involved in the biological nitrate removal, determine the capacity of aquatic ecosystems to ensure a good ecological status. However, the European Water Framework Directive does not include the analysis of denitrifying activities in the monitoring programmes for the catchment management, as a more accurate indicator of the vulnerability of rivers against any ecosystem perturbation.

Several authors have evaluated the influence of numerous biotic and abiotic factors on denitrification in sediments. Nevertheless, since it comprises the reduction of NO_3^- to N_2 by four steps, the analysis of the effects of various physical/chemical parameters (moisture, organic matter and nitrate content of surface sediment, as well as electrical conductivity and exchangeable sodium percentage of water) on the activities of the enzymes — Nitrate Reductase and Nitrite Reductase — which catalyzed the first two stages of denitrification ($NO_3^- \rightarrow NO_2^-$ and $NO_2^- \rightarrow NO$, respectively) has been proposed. Thus, this work intends to contribute to a better comprehension of denitrification in sediments through deeper knowledge of each enzymatic activity contributing to the complete process. We also highlight the necessity of further analyzing and understanding of alternative microbially mediated nitrate removal pathways. In fact, natural processes like fermentative dissimilatory nitrate reduction to ammonium (DNRA) or sulfur-driven nitrate reduction seem to predominate over denitrification in the headwater of the main river.

Finally, this study suggests a reliable evaluation of the ecological status of a catchment based on considering both the accumulation of pollutants in sediments and the purifying capacity of the recipient natural environment. Nitrate Reductase activity, considered the main limiting step of denitrification, was almost always higher in surface sediments subject to pollution by treated or untreated wastewaters than in non-impacted ones. Specifically, the discharge of untreated urban wastewaters into the Ego stream until June 2014 and effluents from the Epele WWTP were largely responsible for inducing the greatest Nitrate Reductase activities. However, the highest nitrate contents in those same surface sediments indicated that denitrification might not be sufficient to support the high nutrient loadings and to ensure the ecological health of the surrounding environment.

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