

Nitrate reduction activity determined with a continuous flow-through system in sediments from a subantarctic coastal environment

Actividad nitrato reductora determinada con un sistema de flujo continuo en sedimentos de un ambiente costero subantártico

Américo I. Torres¹, Mónica N. Gil¹ and José Luis Esteves¹

¹Laboratorio de Oceanografía Química y Contaminación de Aguas, Centro Nacional Patagónico, (CONICET), Boulevard Brown 2915, (9120) Puerto Madryn, Chubut, Argentina. americo@cenpat.edu.ar

Resumen.- La sedimentación y acumulación de detritos orgánicos autóctonos y alóctonos en el sedimento marino, favorece la actividad microbiana, consume el oxígeno disuelto y disminuye el potencial de óxido-reducción. En condiciones anaeróbicas otros aceptores de electrones son utilizados en los procesos de oxidación de la materia orgánica. La reducción de nitrato es un proceso importante que está relacionado con el ciclo del nitrógeno y la oxidación. El objetivo de este estudio fue estimar la actividad nitrato reductora en sedimentos de la Bahía Encerrada, la cual se encuentra sometida a una fuerte influencia antropogénica desde la ciudad de Ushuaia. Las muestras de sedimento fueron tomadas en septiembre de 2007 con draga Van-Veen y almacenadas en oscuridad. La actividad nitrato reductora fue estimada *in vitro* mediante un sistema de flujo continuo. La tasa de incorporación de nitrato se aproximó a una cinética del tipo Michaelis-Menten donde los valores de K_m y V_{max} fueron 1028 μM y 2,7 $\mu\text{mol g}^{-1} \text{h}^{-1}$, respectivamente. Se concluye que el nitrato es utilizado preponderantemente como aceptor final de electrones durante la oxidación de compuestos orgánicos en el sedimento de Bahía Encerrada.

Palabras clave: Nitrato, amonio, reactor, desnitrificación, Patagonia argentina

Abstract.- Sedimentation and accumulation of autochthonous and allochthonous organic detritus in marine sediments stimulate microbial activity, consume dissolved oxygen and decrease redox potential. In anaerobic conditions, other electron acceptors are utilized in organic matter oxidation process. Nitrate reduction is an important pathway which is related with nitrogen cycling and oxidation. The purpose of this study was to assess the nitrate reduction activity in sediments of Encerrada Bay, which is under a strong anthropogenic influence from Ushuaia city. Sediment samples were taken in September 2007 with a Van-Veen dredge and stored in darkness. Nitrate reduction activity was estimated *in vitro* using continuous flow-through system. Nitrate uptake rate followed a Michaelis-Menten kinetic where K_m and V_{max} values were equal to 1028 μM and 2.7 $\mu\text{mol g}^{-1} \text{h}^{-1}$, respectively. We concluded that nitrate is used principally as terminal electron acceptor during the oxidation of organic compounds in sediment of Encerrada Bay.

Key words: Nitrate, ammonium, reactor, denitrification, Argentinean Patagonia

INTRODUCTION

Nitrogen is the most abundant element in the terrestrial atmosphere and constitutes an essential nutrient to organisms since it is fundamental to the structures of biomolecules and biochemical processes that define life (Camargo & Alonso 2006, Francis *et al.* 2007). The nitrogen in the water column of a coastal system subjected to anthropogenic impact can come principally from two different inputs: external discharges (such as domestic and industrial sewages or agriculture activities) and benthic recycling. Independently of its origin, nitrogen

transformations in sediments significantly contribute in the trophic state as well as in the biogeochemical cycling of coastal environments (Clavero *et al.* 2000, Niencheski & Jahnke 2002). The role of marine sediments as a source or sink for nitrogen is governed by the relative rates of several anaerobic and aerobic reactions. Concentration of different chemical species of nitrogen in sediment is mainly controlled by the rate of the following processes: ammonium production from organic matter (ammonification), nitrate production from ammonium

oxidation (nitrification), anaerobic ammonium oxidation (anammox) to gaseous nitrogen, reduction of nitrate to ammonium (assimilative or dissimilative reduction) or to molecular nitrogen gas (denitrification) (Bange *et al.* 2005, Risgaard-Petersen *et al.* 2005, Francis *et al.* 2007). The ecological consequence of the latter is that nitrogen is unavailable to most of the producers such as phytoplankton and bacteria unless it is transformed into organic nitrogen during its fixation (An & Gardner 2002, Bange *et al.* 2005). On the other hand, the coupled nitrification-denitrification activity is one of the few natural processes capable of removing nitrogen from coastal waters and of counteracting eutrophication (Macreadie *et al.* 2006).

Microbiological reduction of nitrate to ammonium occurs under anaerobic conditions and it is carried out predominantly by chemoheterotrophic bacteria (Sánchez & Sanabria 2009). In this case, nitrate is used as terminal electron acceptor during the oxidation of organic compounds (Maier *et al.* 2000). This process can represent between 20-70% of the total reduction of nitrate, particularly in sediments with high organic matter content and a low oxidation-reduction potential (Esteves *et al.* 1986, Christensen *et al.* 2000, Sánchez & Sanabria 2009). Inhibition of denitrification process reinforces the enrichment of nutrients in the water column allowing an increase in the ammonium recycled rate from sediment and favoring the development of primary production and retention of nitrogen in the environment (Christensen *et al.* 2000, Cabrita & Brotas 2000).

Denitrification activity in sediment can be affected by temperature, pH, dissolved oxygen, redox potential, nitrate and ammonium concentrations and organic matter content (Christensen *et al.* 2000, An & Gardner 2002, Tomaszek & Czerwieniec 2003). Increases in the temperature and pH (between 6 and 8) stimulate the nitrate reduction activity, while high ammonium concentration inhibits it. The measurement of nitrate reduction activity in sediments can be carried out using batch system methods which have the advantage of simplicity. However, in these methods the accumulation of metabolic compounds (*e.g.*, ammonium) may inhibit denitrifying activity. On the other hand, the continuous flow-through sediment-water system allows that the metabolites formed during the process are continuously removed from the reactor, and thus do not disturb bacterial metabolic activity. The reactor represents a microcosm with some

controlled variables that allow us to determine the kinetic constants to be used for estimate the natural functioning in Encerrada Bay.

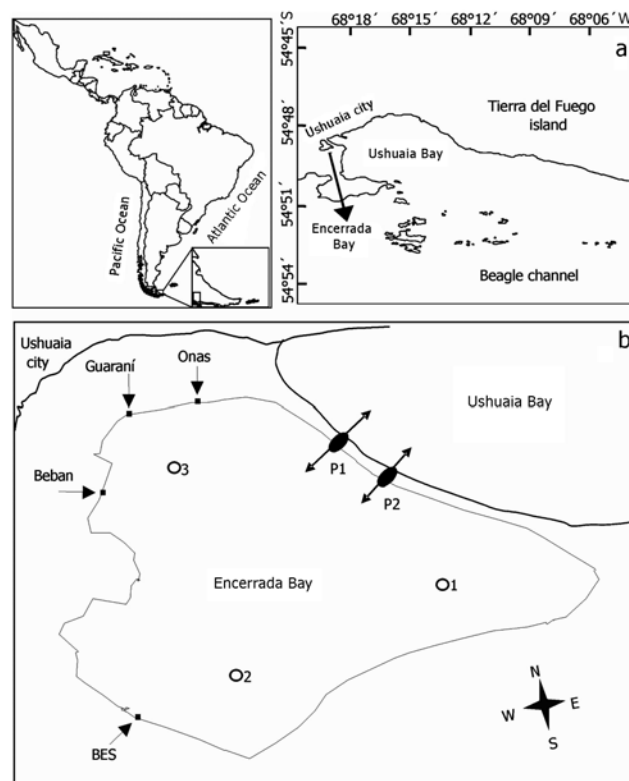
The aim of the present study was to assess the *in vitro* nitrate reduction activity using a continuous flow-through system in sediments from Encerrada Bay. Taking into account anoxic condition and the lack of nitrate and nitrite in pore water we expect to find a significant nitrate reduction activity in the superficial layer of sediment in this ecosystem.

MATERIALS AND METHODS

STUDY AREA

Encerrada Bay (54°48.9'S; 68°19.0'W), referred to hereafter as EB, is a semi-closed coastal system and it is located close to Ushuaia city in Tierra del Fuego province (Argentina) (Fig. 1). This bay has an area of 0.27 km² and a mean depth of 0.8 m. It is separated from Ushuaia Bay by artificial pathways, which communicate both Bays through two vents (P1 and P2 of 4.5 m long each), allowing water exchange in each semi-diurnal tidal cycle. EB has a high level of eutrophication due to the input of domestic and industrial wastewater through several discharges (Onas, Guaraní and Beban sewages and Buena Esperanza stream) from Ushuaia city. Although dissolved oxygen level in the water column remained close to saturation, Torres *et al.* (2009) observed anoxic sediment conditions, with predominantly black coloration and a brown thin layer (<5 mm) on the top of sediment in some sites of the bay, which is evidence that dissolved oxygen is incorporated only in this section. This suggests that coupled nitrification-denitrification could be restricted to that thin layer and only in some places of EB. Torres *et al.* 2009 also found average sediment porosity of 63.1 ± 4.7%, grain size below 63 µm, organic matter content between 8 and 19%, ranges of pore water and total ammonium concentrations of 0.14-0.59 µmol cm⁻³ and 0.41-0.92 µmol cm⁻³ wet sediment, respectively and undetectable pore water nitrate concentrations (in accordance with negative values of redox potential). Benthic fluxes in the sediment-water interface showed consumption of dissolved oxygen and nitrate by sediment and release of ammonium and phosphate into the water column (Torres *et al.* 2009). In such cases, when the available dissolved oxygen is not enough to oxidize the organic matter, nitrate is expected to be used as terminal electron acceptor.

Figure 1. a) Map of the study area. b) Position of Buena Esperanza Stream (BES), Onas, Guaraní and Beban runoffs and vents (P1 and P2) that communicate to Encerrada and Ushuaia Bay. The three circles indicate the sampled sites in Encerrada Bays / a) Mapa del área de estudio. b) Posición de las descargas: Arroyo Buena Esperanza (BES), Onas, Guaraní y Beban y de las aberturas (P1 y P2) que comunican las Bahías Encerrada y Ushuaia. Los tres círculos indican los sitios muestreados en Bahía Encerrada



SEDIMENT SAMPLING

Sediment was collected at three stations in EB with a Van-Veen dredge from a boat, in September 2007 (Fig. 1). The superficial layer (<1 cm) was separated on board using spatulas and then placed in hermetic plastic containers. A layer of 1 cm of seawater was added *in situ* and then stored at 4°C in dark conditions until the beginning of experiences, within 10 days from sampling day. Immediately before the beginning of each experience, the three sediment samples were homogenized in an oxygen-free glove box, filled with an inert gas (N₂).

NITRATE REDUCTION ACTIVITY DETERMINATION

Nitrate reduction activity was measured *in vitro* using the continuous flow-through system method developed by Northby (1976) and adapted by Esteves *et al.* (1986) to microbiological processes (Fig. 2). The Northby's equation for a continuous flow-through system at time 't' can be written as:

$$[J_0 X_i(t) + R_c(t)] - [J_0 X_0(t)] = \frac{d}{dt} [W \cdot X_0(t)] \quad (1)$$

where J_0 : flow of initial seawater solution enriched with nitrate (in this study KNO₃ was used) (l h⁻¹); X_i and X_0 : input and output nitrate concentrations (μmol l⁻¹), respectively; R_c : nitrate consumption (μmol h⁻¹); and W : dry weight of sediment in the reactor (g). In the steady-state the second member of the equation 1 is equal to zero and the nitrate reduction rate ($R_c NO_3^-$), defined as function of the dry weight of sediment introduced into the reactor (μmol h⁻¹ g⁻¹) is obtained as follows:

$$R_c NO_3^- = R_c / W = J_0 (X_0 - X_i) / W \quad (2)$$

Negative values of ($R_c NO_3^-$) correspond to nitrate uptake by microbial community.

EXPERIMENTAL DESIGN

Experiences were carried out by triplicate and different nitrate concentrations (25, 100, 200, 400 μM) were used. The seawater was filtered, enriched with nitrate and sterilized in autoclave during 20 min under 1.5 atmospheres to obtain low dissolved oxygen levels. Inside the reactor, the sediment (8.8 ± 2.6 g dry weight) was retained on a

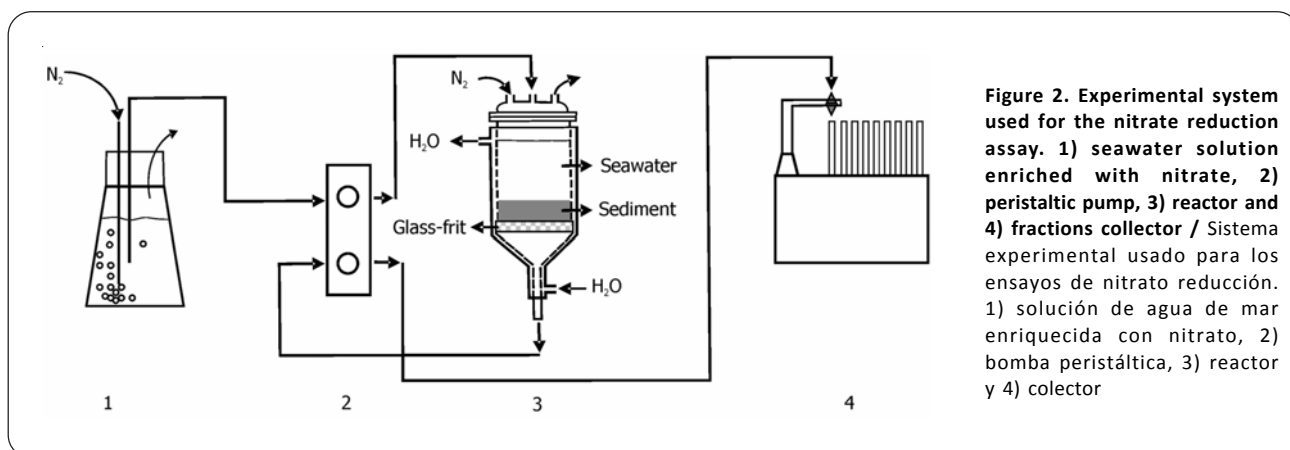


Figure 2. Experimental system used for the nitrate reduction assay. 1) seawater solution enriched with nitrate, 2) peristaltic pump, 3) reactor and 4) fractions collector / Sistema experimental usado para los ensayos de nitrato reducción. 1) solución de agua de mar enriquecida con nitrato, 2) bomba peristáltica, 3) reactor y 4) colector

glass-frit (10 μm porosity). Low oxygen atmosphere was obtained by purging the system with nitrogen gas. A temperature of $17 \pm 1^\circ\text{C}$ was maintained by water circulation around each reactor using a thermostatic bath (Mgw Lauda Kzr). We expected an increased nitrate reduction activity at that temperature in EB, which is the typical summer value in seawater (Torres *et al.* 2009). The experiences were run in dark conditions in order to avoid the activity of photosynthetic microorganisms. Each reactor was supplied with enriched seawater at a constant flow (36 ml h^{-1}) through a Technicon peristaltic pump. The flow value was defined according to Esteves *et al.* (1986) and Montes (1994). The experiences lasted 9 h. After the first 4 h (enough time to achieve stability of the system) and every 30 min, a fraction of 15 ml was taken from the reactor outflow. Immediately, ammonium concentration was measured in a subsample of 5 ml, according to Strickland & Parsons (1972). The remaining volume was stored at -20°C for determination of nitrate (NO_3^-) and nitrite (NO_2^-) concentrations following the Strickland & Parsons (1972) methods modified by automatic process of Technicon¹ (1977).

KINETIC CONSTANTS DETERMINATION

The ($R_c \text{NO}_3^-$) values were plotted as function of initial nitrate concentration in the reactor and double reciprocal relationship between them was applied. The Michaelis-Menten function was fitted to the data by least-square regression:

$$V = V_{\max} \cdot X / K_m + X \quad (3)$$

where V : nitrate uptake rate ($R_c \text{NO}_3^-$) ($\mu\text{mol g}^{-1} \text{h}^{-1}$), V_{\max} : maximum uptake rate ($\mu\text{mol g}^{-1} \text{h}^{-1}$), X : the nitrate concentration ($\mu\text{mol l}^{-1}$), and K_m : half-saturation constant (μM).

RESULTS

Nitrate concentration within each reactor increased until reaching the steady state nearly 300 min after the beginning of each experience (Fig. 3). Final value was always lower than that in the initial solution (between 9% and 96% in the experiences N° 10 and 6, respectively), indicating a consumption of this ion. Similarly, nitrite concentration increased in each experience during approximately 330 min until stationary state (not shown), but reaching a final value that was higher than that in the inlet (from 0.5 to $92 \mu\text{mol l}^{-1}$ according to the treatment). This pattern suggests production of this ion as an intermediate compound during the nitrate reduction process (Table 1). On the other hand, ammonium in the steady state showed higher concentrations than those in the initial solution for each treatment (Table 1). The differences between initial and final concentrations ranged from 0.2 to $150 \mu\text{mol l}^{-1}$, suggesting a production of this ion.

The calculated nitrate reduction activities ($R_c \text{NO}_3^-$) ranged from -1.7 to $-0.012 \mu\text{mol g}^{-1} \text{h}^{-1}$ (Table 2). The relationship between $R_c \text{NO}_3^-$ and initial nitrate concentrations is presented in Fig. 4a, and double reciprocal relationship between those parameters in Fig. 4b. Nitrate uptake rates exhibited saturation kinetics, such

¹Technicon. 1977. AutoAnalyzer II. Technicon Industrial Systems. Tarrytown, New York. 10591. Technicon Instruments Corporation.

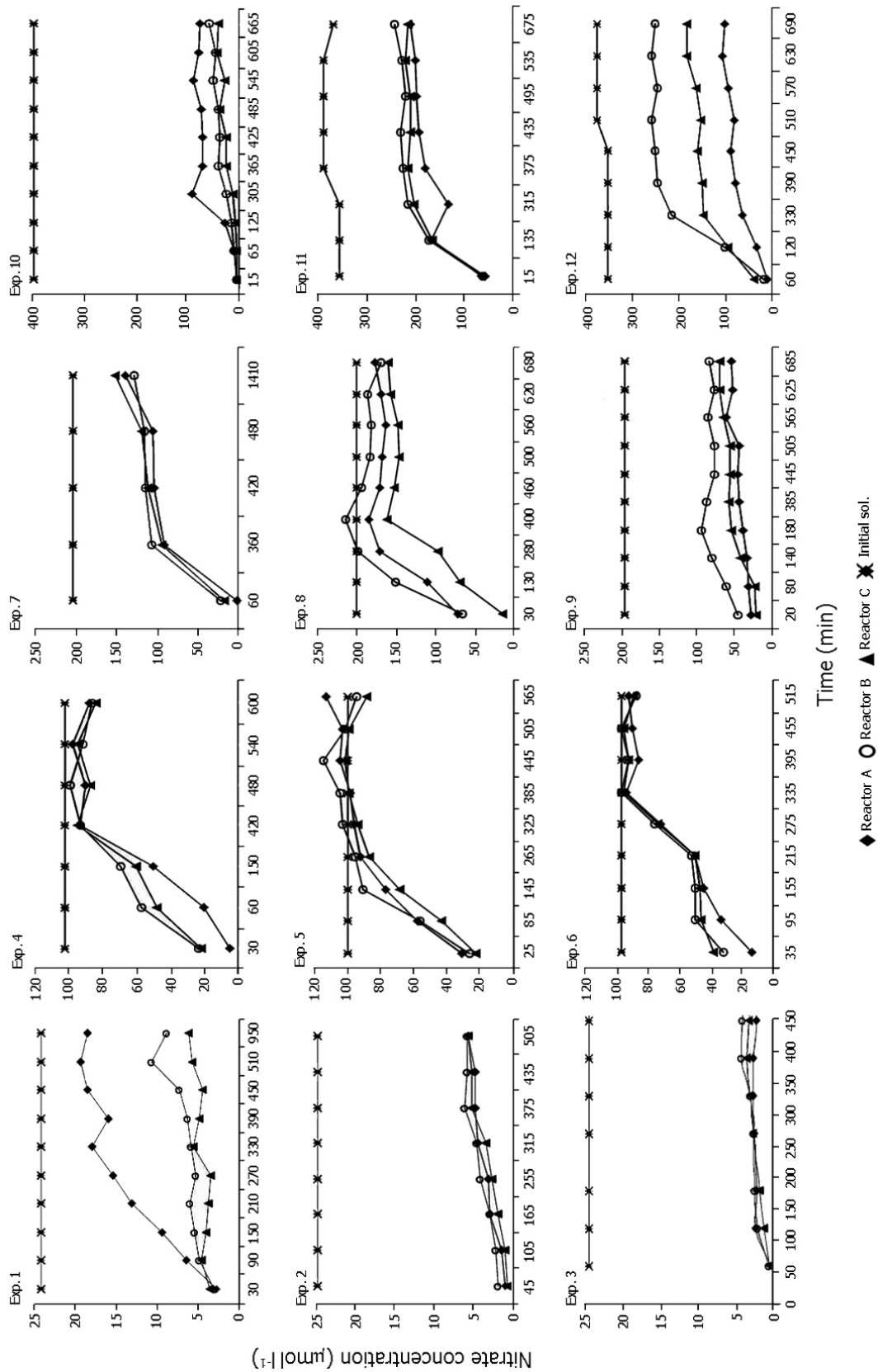


Figure 3. Nitrate concentration evolution in the liquid percolated through each reactor (A, B, C) in function of the time. In experiences 1-3, 4-6, 7-9 and 10-12 the initial nitrate concentrations were 25, 100, 200 and 400 µmol l⁻¹, respectively / Evolución de la concentración de nitrato en el líquido percolado de cada reactor (A, B, C) en función del tiempo. En las experiencias 1-3, 4-6, 7-9 y 10-12 las concentraciones iniciales de nitrato fueron 25, 100, 200 y 400 µmol l⁻¹, respectivamente

Table 1. Nitrate, nitrite and ammonium concentrations in the initial solution and the liquid percolated through of each reactor (A, B, C). The flow was constant in all experiences (0.036 l h⁻¹). Weights of wet sediment utilized in each reactor are shown / Concentraciones de nitrato, nitrito y amonio en la solución inicial y en el líquido percolado de cada reactor (A, B, C). El flujo fue constante en todas las experiencias (0,036 l h⁻¹). Se indican los pesos de sedimento seco utilizado en cada reactor

N° experience	Weight of wet sediment (g)			Concentration in the initial solution (μmol l ⁻¹)			Average nitrate concentration in the liquid percolated at steady state (μmol l ⁻¹)			Average nitrite concentration in the liquid percolated at steady state (μmol l ⁻¹)			Average ammonium concentration in the liquid percolated at steady state (μmol l ⁻¹)		
	A	B	C	nitrate	nitrite	ammonium	A	B	C	A	B	C	A	B	C
1	11.67	8.67	9.29	24.3	0.07	0.97	5.36	7.80	18.13	1.19	1.21	1.24	5.53	5.99	8.00
2	13.70	11.49	11.19	24.6	0.13	0.97	4.96	5.64	4.98	1.44	1.17	1.56	1.70	1.21	1.21
3	8.50	9.12	14.72	24.6	0.38	0.97	3.76	4.24	2.76	1.27	1.72	0.90	49.70	12.90	19.62
4	13.95	10.71	10.96	102.4	0.38	9.89	89.99	92.60	92.25	10.23	9.01	14.96	116.63	65.59	120.34
5	11.32	9.01	8.88	100.1	5.01	9.89	97.07	103.88	104.81	12.65	12.88	12.25	113.41	110.23	119.35
6	6.16	7.88	6.09	97.6	4.22	9.89	93.92	94.30	91.20	8.28	8.45	8.23	87.61	87.94	83.23
7	5.07	5.86	6.69	204.2	5.32	14.97	120.36	127.59	116.79	7.51	9.47	8.13	83.33	31.82	165.00
8	6.83	4.68	7.98	200.1	5.32	14.97	155.23	179.19	170.23	11.71	12.81	12.43	33.89	51.93	35.87
9	7.22	7.72	6.80	197.0	5.32	14.97	67.64	81.36	54.80	12.01	20.58	15.97	52.37	41.59	44.12
10	6.75	9.54	11.03	400.0	5.20	6.63	37.57	52.07	81.05	8.65	15.70	25.11	77.20	46.75	37.27
11	10.64	8.91	8.08	375.4	9.57	14.46	215.95	231.95	203.88	35.59	37.37	38.43	17.64	13.30	26.17
12	9.66	4.44	7.25	353.5	5.20	7.03	176.80	252.67	101.33	67.00	97.40	49.10	74.00	33.17	120.40

Table 2. Nitrate reduction activities ($R_c NO_3^-$) in each reactor (A, B, C) estimated from *in vitro* experiences / Actividad nitrato reductora ($R_c NO_3^-$) en cada reactor (A, B, C) estimada a partir de las experiencias *in vitro*

Exp./reactor	$R_c NO_3^-$ ($\mu\text{mol g}^{-1} \text{h}^{-1}$)		
	A	B	C
1	-0.073	-0.068	-0.019
2	-0.063	-0.060	-0.052
3	-0.051	-0.081	-0.093
4	-0.041	-0.033	-0.026
5	-0.012	-0.015	-0.015
6	-0.022	-0.015	-0.037
7	-0.451	-0.471	-0.620
8	-0.202	-0.161	-0.158
9	-0.685	-0.539	-0.709
10	-1.183	-1.314	-1.701
11	-0.711	-0.579	-0.581
12	-0.877	-0.818	-0.939

as that predicted by the Michaelis-Menten model. Kinetic parameters obtained were $V_{max} = 2.7 \mu\text{mol g}^{-1} \text{h}^{-1}$ and $K_m = 1028 \mu\text{M}$.

DISCUSSION

Nitrate reduction activity in anaerobic sediments represents an important alternative pathway as a source of electron acceptors for oxidation of organic matter (Sánchez & Sanabria 2009). In environments with high eutrophication, as in EB, dissolved oxygen concentration is rapidly depleted in superficial layer of sediment. In deeper layers only restricted or facultative bacterial metabolisms can be involved in the degradation of organic matter. In spite of negative values of redox potential in sediment and the non detectable concentrations of nitrate and nitrite in pore water, high concentrations of both nitrogen compounds in water column of EB were measured (Torres *et al.* 2009). This fact suggests an intense bacterial activity that uses nitrate as terminal electron acceptor in the upper layer of sediment and no inhibition in nitrate reduction activity has been observed. Eventhough ammonium increase was observed, significance of dissimilative reduction of nitrate relative to denitrification, can not be assessed with this methodology.

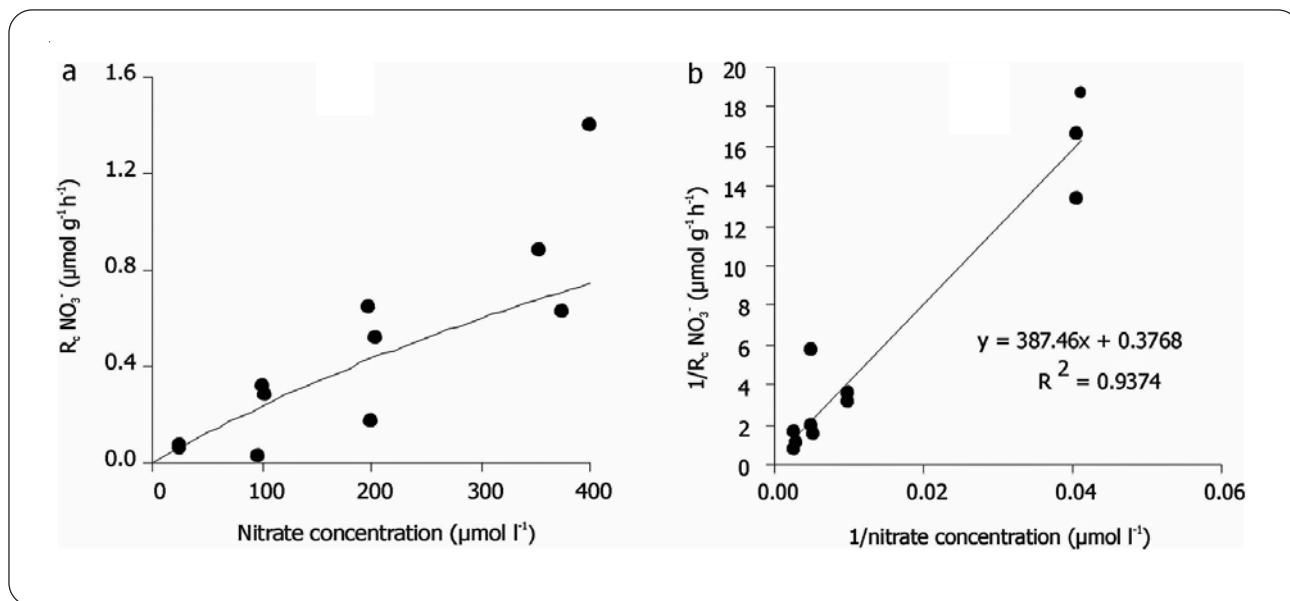


Figure 4. a) Nitrate reduction activity ($R_c NO_3^-$) as function of initial nitrate concentration (black points); and curve was fitted by use of the Michaelis-Menten equation (line); b) Double reciprocal plot of the nitrate concentration vs nitrate reduction activity / a) Actividad nitrato reductora ($R_c NO_3^-$) como función de la concentración inicial de nitrato (puntos negros); y la curva fue ajustada mediante la ecuación de Michaelis-Menten (línea); b) Doble recíproca de la concentración de nitrato vs la actividad nitrato reductora

Kinetic parameters of nitrate reduction activity adjusted to Michaelis-Menten model were one or two orders of magnitude greater than those determined by Montes (1994) in coastal systems of low latitude in Argentinean Patagonia. This author determined in petroleum impacted sediments and with low organic matter content (< 2%) a maximum uptake rate $V_{max} = 25.6 \cdot 10^{-3} \mu\text{mol g}^{-1} \text{h}^{-1}$ and half-saturation constant $K_m = 48 \mu\text{M}$ at a temperature of 17°C. Furthermore, Montes (1994) reported inhibition of this activity using a nitrate concentration of 500 $\mu\text{mol l}^{-1}$. On the other hand, Esteves *et al.* (1986) found a $V_{max} = 168.0 \cdot 10^{-3} \mu\text{mol g}^{-1} \text{h}^{-1}$ and a half-saturation constant $K_m = 78 \mu\text{M}$ in sediments with organic matter content <1% from Mediterranean Sea (France). The kinetic constants found in this study were in the same order of magnitude than those informed by An & Gardner (2002) in sediments from Baffin Bay (Southern USA). However, the obtained parameters were lower than those determined by Jorgensen & Sorensen (1988) in sediments of Norminde's fjord (Denmark) with organic matter content between 5-15% and greater than those measured by Kaspar (1983) in intertidal sediments of New Zealand.

Keeping in mind the equation 3, and considering kinetic parameters obtained in this study, average density of sediment ($1.26 \pm 0.07 \text{ g cm}^{-3}$), and the range of variation of nitrate concentration in water column (4-40 μM) measured by Torres *et al.* (2009), it is possible to estimate *in situ* nitrate flux in the sediment-water interface by surface unit ($\sim 1 \text{ m}^2$). Assuming that total nitrate reduction in sediment of EB is restricted to superficial layer of 0.005 m we should expect nitrate fluxes between 66 and 637 $\mu\text{mol m}^{-2} \text{h}^{-1}$. These values are in the same order of magnitude than fluxes obtained *in situ* through opaque benthic chambers (20 and 416 $\mu\text{mol m}^{-2} \text{h}^{-1}$) in the same coastal environment (Torres *et al.* 2009). According to Kaspar (1983) and Enoksson & Samuelsson (1987) denitrification process in several sediment types, including mud, marsh, sand and eelgrass bed, can represent between 70 and 95% of total nitrate consumption. In that case, for a nitrate flux of 637 $\mu\text{mol m}^{-2} \text{h}^{-1}$, the amount of released molecular nitrogen in EB would be between 446 and 605 $\mu\text{mol m}^{-2} \text{h}^{-1}$. This would produce a loss of this nutrient by the system of about 2728-3293 Kg N per month.

The employed methodology in this study, allowed us to estimate nitrate uptake rate and provides information about the use of nitrate as terminal electron acceptor in an eutrophic system; ammonium or molecular nitrogen production can not be adequately explained. This

methodology can be applied easily in those environments in which the systems *in situ* are affected by strong marine currents, deep sea sediment or sewage discharges (Miller-Way & Twilley 1996). Other advantage of this method, in addition to good reproducibility and quickness, is the small amount of sediment required, which permits the treatment of a large number of samples and also can be applied on board (Esteves *et al.* 1986, Miller-Way & Twilley 1996).

We conclude that in an eutrophic bay as EB, the system acts as a sink of nitrate. The nitrate flux from the water column to the sediment is significant and is used principally as terminal electron acceptor during the oxidation of organic matter. Further researches are required to quantify whether the reduced nitrate is used for ammonium production (dissimilative reduction) or molecular nitrogen production (denitrification), in order to get a better understanding of nitrogen cycling in this environment.

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