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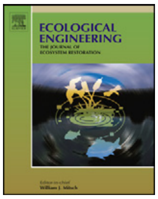

Effects of nitrate contamination and seasonal variation on the denitrification and greenhouse gas production in La Rocina Stream (Doñana National Park, SW Spain)

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ABSTRACT

Climatic influence (global warming and decreased rainfall) could lead to an increase in the ecological and toxicological effects of the pollution in aquatic ecosystems, especially contamination from agricultural nitrate (NO_3^-) fertilizers. Physicochemical properties of the surface waters and sediments of four selected sites varying in NO_3^- concentration along La Rocina Stream, which feeds Marisma del Rocio in Doñana National Park (South West, Spain), were studied. Electrical conductivity, pH, content in macro and microelements, total organic carbon and nitrogen, and dissolved carbon and nitrogen were affected by each sampling site and sampling time. Contaminant NO_3^- in surface water at the site with the highest NO_3^- concentration (ranged in 61.6–106.6 mg L^{-1}) was of inorganic origin, most probably from chemical fertilizers, as determined chemically (90% of the total dissolved nitrogen from NO_3^-) and by isotopic analysis of δ^{15}N-NO_3^-. Changes in seasonal weather conditions and hydrological effects at the sampling sites were also responsible for variations in some biological activities (dehydrogenase, β-glucosidase, arylsulphatase, acid phosphatase and urease) in sediments, as well as in the production of the greenhouse gases CO_2, CH_4 and N_2O. Both organic matter and NO_3^- contents influenced rates of gas production. Increased NO_3^- concentration also resulted in enhanced levels of potential denitrification measured as N_2O production. The denitrification process was affected by NO_3^- contamination and the rainfall regimen, increasing the greenhouse gases emissions (CO_2, CH_4 and especially N_2O) during the driest season in all sampling sites studied.

1. Introduction

Anthropogenic influence on the biogeochemical N cycle can produce important alterations of the cycle leading to concomitant environmental risks such as increased concentration of greenhouse gases, acidification of soils, streams and lakes, transfer of nitrogen through rivers to estuaries and coastal oceans, accelerated losses of biological diversity and human health and economy problems (Vitousek et al., 1997; Galloway et al., 2008; Mulholland et al., 2008). In aquatic ecosystems, water acidification, eutrophization, including occurrence of toxic algae, and toxicity of ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-) are the three major environmental problems due to inorganic nitrogen pollution (Camargo and Alonso, 2006). Furthermore, increasing global warming and decreased rainfall in some continental areas may increase ecological and toxicological effects of this type of environmental contamination (Camargo and Alonso, 2006). Abuse in utilization of nitrogenous chemical fertilizers has been shown to enhance emission of carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) greenhouse gases (Thornton and Valante, 1996; Merbach et al., 1996, 2001; Davidson and Verchot, 2000; Liu and Greaver, 2009). In addition to chemical fertilizers, release of greenhouse gases to the atmosphere can be induced by changes in precipitations, temperature, seasons, drought, regional deforestation, global warming, and El Niño events (Christensen et al., 1990; Smith et al., 2003; Davidson et al., 2004).

Wetlands are among the most important ecosystems on Earth because of their role in regulating global biogeochemical cycles. Climate change and anthropogenic effects may have significant impacts on coastal and inland wetlands (Mitsch and Gosselink, 2007; Olías et al., 2008). Accordingly, physicochemical and biolog-

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ical monitoring is needed for assessment of ecological risks due to freshwater pollution and to provide maximal information for adequate protection of aquatic ecosystems (Camargo, 1994). Several authors and reports have shown that NO_3^- contamination of soils and surface and groundwater is becoming more intense and frequent due to the great consume of inorganic nitrogen, mainly nitrate and ammonium salts from agrochemicals, of the intensive farming (Spalding and Exner, 1993; European Commission, 2002). Denitrification is the biological process by which NO_3^- can be transformed into molecular nitrogen (N_2) via formation of NO_2^- and nitric oxide (NO). Thus, it represents the major pathway by which NO_3^- can be removed from soils and waters to avoid NO_3^- accumulation and contamination. And yet, incomplete denitrification results in the production of the greenhouse gases NO and N_2O (Aulakh et al., 1992; Conrad, 1996; Groffman et al., 2006).

The European directive 91/676/CEE concerning NO_3^- contamination from agricultural sources defines the so-called "nitrate vulnerable zones" as reference areas of special environmental protection to prevent soil and water nitrate contaminations. An example is Doñana National Park (DNP), one of the most important wetlands in Europe covering an area around 60,000 ha in a marshy area of SW Spain, in the estuary of the Guadalquivir River. These water flows are susceptible of NO_3^- contamination from small urban areas in the surrounding of the park and agricultural practices allowed in the ecotone, where organic farming of strawberries and rice is common. This area is the most fertile and productive zone of Doñana as a result of its permanent humidity and of the fertilization it receives from the animals either living there or crossing it (Suso and Llamas, 1993).

Several authors have noted that surface and groundwater of DNP wetland are becoming polluted during the last 20 years. Suso and Llamas (1993) remarked that some wetlands and small streams could be depleted by groundwater extraction for agricultural reclamation, affecting negatively the quality of surface and groundwater. Olías et al. (2008) evaluated the water quality of the Almonte-Marismas aquifer (upon which DNP is located) and showed that it was affected by pollution of both agricultural and urban origins. They detected some shallow points located in the agricultural zones with high concentrations of NO_3^- and sulphates (SO_4^{2-}) from fertilizer pollution. Finally, Serrano et al. (2006) reviewed the aquatic systems of DNP and they focused on processes affecting water quality. They noted that there has been a considerable increase of NO_3^- concentration in the water flows of La Rocina and El Partido Streams during the past decade, probably due to the increase in cultivated land and fertilizer applications. They advise that the influence of this pollution on the eutrophication of the nearby marshes should not be overlooked.

Our research aim was to evaluate the anthropogenic (especially from agriculture) and seasonal influence in La Rocina Stream and how it could be affected by the physicochemical and biological characteristics of the surface water and its aquatic sediments, focusing in the NO_3^- contamination and its influence on the greenhouse gas production (CO_2, CH_4 and N_2O) and the denitrification process.

2. Materials and methods

2.1. Description and selection of sampling sites

In 1982, DNP was declared a Reserve of the Biosphere by UNESCO and a Wetland of International Interest per RAMSAR Convention. DNP wetland has three important surface water inputs (Arambarri et al., 1996; Serrano et al., 2006). Two natural streams called La Rocina and El Partido, in the north edge of the park, and the Guadiamar River, which represents the main water input of the wetlands and suffered the Aznalcollar mine spill in 1998 (Cabrera et al., 1999; Grimalt et al., 1999; Sierra et al., 2003; Olías et al., 2005).

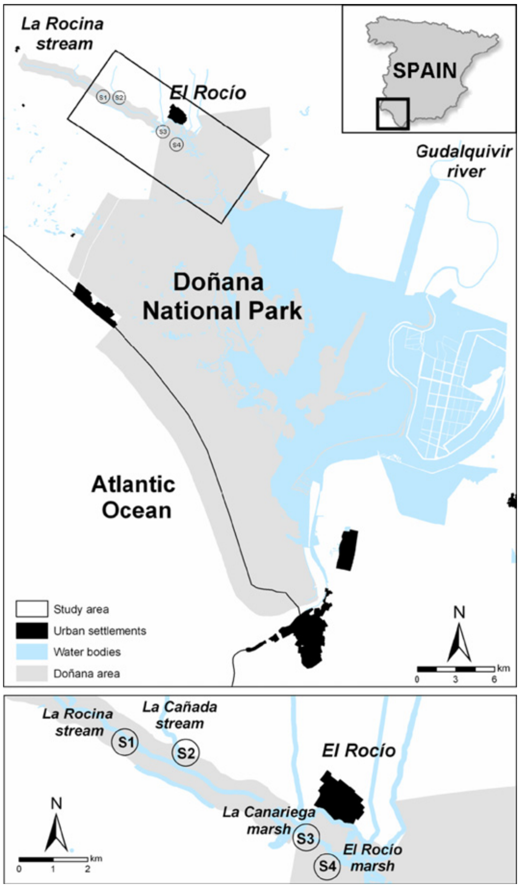

Fig. 1. Geographical situation of Doñana National Park (DNP) and La Rocina Stream. Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4).

The study was performed on La Rocina Stream, located in the north of the DNP wetland, which is one of the main natural streams feeding El Rocío marsh (Fig. 1). Selection of sampling sites was based on their NO_3^- content (in situ measurements using a Nitrate Test Kit, CHEMetrics Inc.) after screening of more than 25 points along the course of La Rocina basin (462 km^2) at the different sampling times. Four sampling sites along the course of La Rocina stream differing in their NO_3^- concentration were selected (Fig. 1). The Universal Transverse Mercator (UTM) coordinates for the sites were as follows: 29S 0718632, 4114294 for the lagoon of Palacio del Acebrón (S1); 29S 0717797, 4113881 for the small stream Arroyo de la Cañada (S2); 29S 0722653, 4111704 for the junction between the stream and the marsh called Vado de la Canariega (S3); and 29S 0723654, 4111088 for the El Rocío marsh (S4). S1 and S2 had the lowest and the highest NO_3^- concentration, respectively, whereas the values detected in S3 and S4 were between those detected in S1 and S2. Also, differences on hydrological morphology (S1: a small stream, S2: a lagoon, S3: the union between a stream and a marsh, S4: a marsh) and in riparian vegetation (not present in S3 and S4) were noted.

Samples were taken in October 2008 (T1) and January (T2), April (T3) and July (T4) 2009 in order to represent the pluvial regimen (dry and wet). Rainfall, relative humidity and air temperature were collected from the Manecorro RM1 meteorological station, which belongs to the Singular Scientific and Technological Installation (http://icts.ebd.csic.es/) of Doñana National Park located about 200 m away from S3, and from Estación Manual Palacio de Doñana (EM05, http://www-rbd.ebd.csic.es).

2.2. Physicochemical properties

Four replicates of the surface waters (approximately 1–2 m from the shore in streams and 3–4 m in lagoons for each replicate) and semi-disturbed sediments (0–10 cm from the upper layer using an EIJKELKAMP Peat sampler) were taken at each sampling site, placed in a portable fridge and processed in the laboratory within 24 h of sample collection. Subsets of samples from the sediments were lyophilized and kept frozen at −20 °C until use.

In water samples, pH and electrical conductivity (EC) were analyzed using a Basic 20 Crison pHmeter and a Basic 525 Crison conductimeter at the laboratory, respectively. After filtration through 0.45 μm filters, dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were determined using an automatic Shimadzu TOC-VCSN analyzer. NO_3^- and NO_2^- concentrations were estimated by ion chromatography (HPLC) using an IC-Pac anion HC (Waters) column at the facilities of Servicio de Instrumentación (EEZ-CSIC). NH_4^+ was determined by a colorimetric method based on Berthelot's reaction (Kempers and Zweers, 1986; Sommers et al., 1992), adding sodium citrate to complex divalent cations. Macro and microelements (P, K, Ca, Mg, Na, S, Fe, Cu, Mn, and Zn) were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) using an IRIS Intrepid II XDL (Thermo Fisher Scientific Inc.).

Texture of the sediments was determined in fresh samples according to the Spanish Official Methods for Soils and Waters (MAPA, 1974). NH_4^+, after 2 h extraction 1:20 (w/v) with 2 N KCl, and water extracted (1:20, w/v) NO_3^- and NO_2^- were also analyzed in fresh samples as indicated above. All other assays were performed in ground samples (0.2 mm) after lyophilization. pH and EC were measured after water extraction (1:5, w/v) for 2 h. Total organic carbon (TOC) and total nitrogen (TN) were determined using a LECO TruSpec CN Elemental Analyzer. DOC and TDN were obtained after 2 h water extraction 1:20 (w/v) and estimated as indicated for surface waters. Macro and microelements were analyzed by ICP-OES after microwave digestion with a mixture (1:1) HF:HCl. Organic nitrogen (N_{ORG}) was calculated as the difference between either TDN in surface waters or TN in sediments, and the content in inorganic nitrogen (N_{INORG}), considering N_{INORG} as $N\text{-}NO_3^- + N\text{-}NO_2^- + N\text{-}NH_4^+$.

2.3. Enzymatic analysis in sediments

A selection of some enzymatic activities related to the metabolic activity and the main biogeochemical cycles were measured in the freeze-dried sediments. Dehydrogenase was used as an estimation of overall microbial activity, β-glucosidase as the enzyme that catalyses the hydrolysis of disaccharides (C cycle), arylsulphatase as a measure of the enzymes catalyzing the hydrolysis of organic sulphate esters (S cycle), acid phosphatase as a measure of the enzymes responsible for the hydrolysis of phosphate esters (P cycle), and urease which catalyses the hydrolysis of urea to CO_2 and NH_3 (N cycle). Dehydrogenase was determined according to García et al. (1997), β-glucosidase, arylsulphatase and acid phosphatase, were determined as described by Tabatabai (1982) and urease activity was determined according to Kandeler and Gerber (1988). Briefly, these techniques were based on a controlled incubation of the sediments after adding the initial substrate (INT: 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-tetrazolium for dehydrogenase, *p*NG: 4-nitrophenyl-beta-D-glucopyranoside for β-glucosidase, *p*NPS: *p*-nitrophenyl sulphate for arylsulphatase, *p*NPP: 4-nitrophenyl phosphate for acid phosphatase and urea for urease activity, respectively) and measuring the ending product of each enzyme reaction colorimetrically (INTF: iodonitrotetrazolium formazan for dehydrogenase, *p*NP: *p*-nitrophenol for β-glucosidase, arylsulphatase, acid phosphatase and NH_4^+, measured as described above for water and sediment samples, for urease activity).

2.4. Gas emission (CO_2, CH_4 and N_2O) and denitrification potential of the sediments

The emission of CO_2, CH_4 and N_2O were measured after 24 h aerobic incubation (25 °C) of 20–30 g of the freshly collected sediments in 125 mL glass bottles. Gas concentrations were analyzed in the headspace by a Varian 4900 Gas Chromatograph with a PoraPlot Q column (10 m length, 0.15 mm internal diameter) and thermal conductivity detector (TCD). Denitrifying Enzyme Activity (DEA) and Denitrification Potential (DP) were also determined in the fresh sediments using an acetylene inhibition technique adapted from Simek et al. (2004). DEA is a measure of denitrifying enzymes in the sediment and reflects whether the environmental conditions of the sediments at the moment of sampling would induce the activity of the denitrifying bacteria, whereas DP represents a long-term denitrification potential, allowing the maximum regrowth of denitrifying bacteria (Tiedje, 1994). DEA was determined using an anerobic slurry prepared by mixing 25 g moist sediment and 25 ml of a solution containing 1 mM glucose, 1 mM KNO_3 and 1 g L^{-1} chloramphenicol (to prevent protein synthesis and growth) in a 125 mL glass bottle. The headspace was evacuated and flushed four times with He and 10 ml of acetylene were added. The samples were shaken at 25 °C and the concentration of N_2O was measured in the headspace after 30 and 60 min of incubation by gas chromatography, as previously described. DEA was calculated from the N_2O increase during a half an hour incubation (60–30 min) and using the Bunsen coefficient for the N_2O dissolved in water. DP was determined by mixing 6 g moist sediment with 5 mL of a solution containing 1 mM KNO_3 and 1 mM glucose in a 125 mL glass bottle. After evacuating and flushing the headspace four times with He, 10 ml of acetylene were added and the samples were incubated at 25 °C during 48 h. DP was calculated from the N_2O increase in the headspace after the second day of incubation and using the Bunsen coefficient for the N_2O dissolved in water.

2.5. Isotope measurements

$\delta^{15}N$ of NO_3^- was determined following the methodology described by Silva et al. (2000) with modifications. Water samples (10–30 l) were first filtered through Whatman filter paper and then passed through 0.45 μm filters (High Capacity GWV, Groundwater Sample Filter). Possible interferences from sulphate and phosphate in the samples were eliminated by adding an excess of $BaCl_2$, and dissolved CO_2 was removed by adding HCl and gentle heating. Water samples were then eluted through a cation exchange resin (AG 50W X8 100–200 mesh, Bio-Rad) to remove dissolved organic matter and the excess of Ba^{2+}, and passed through an anion exchange resin (AG1 X8 100–200 mesh, Bio-Rad) to retain NO_3^-. Finally, nitrate was eluted from the column by adding 1 N HCl, and the solution containing HNO_3 and HCl was neutralized with Ag_2O (Merck). The resulting AgCl precipitate was removed by filtration (0.45 mm membrane filter) leaving only Ag^+ and NO_3^- in solution. The solutions were freeze-dried yielding a pure, dry $AgNO_3$ precipitate.

Nitrogen isotope ratios and total nitrogen contents of $AgNO_3$ precipitates were determined by thermal de-composition in a

Table 1a
Physicochemical properties of the surface waters at the sampling sites.

Sampling site	Times	pH	EC[a]	DOC[b]	NO_3^{-b}	NO_2^{-b}	NH_4^{+b}	P[b]	K[b]	Ca[b]	Mg[b]	Na[b]	S[b]	Fe[b]	Cu[b]	Mn[b]	Zn[b]
S1	T1	6.62b	0.63a	17.7a	0.2c	nd	0.1	<0.6	9.0a	49.3a	20.9a	66.8a	61.2a	0.0d	nd	0.9	<0.01
	T2	6.57b	0.26c	16.7b	0.3b	0.6	nd	<0.6	4.8c	12.6b	6.0c	43.8b	7.0c	0.3a	0.1	<0.01	0.3
	T3	6.99b	0.12d	5.7d	0.6a	nd	nd	<0.6	2.0d	7.2c	3.3d	16.8c	2.4d	0.1c	0.1	<0.01	0.2
	T4	7.60a	0.33b	8.9c	nd	nd	nd	<0.6	5.2b	3.4d	8.6b	8.6d	46.6b	0.2b	<0.01	<0.01	0.2
S2	T1	7.75c	0.52c	28.3a	61.6d	0.1c	0.1	<0.6	10.9c	38.5b	6.8d	20.5c	16.2c	nd	nd	<0.01	<0.01
	T2	7.15d	0.57b	3.6c	106.6a	nd	nd	1.9a	16.0b	36.5c	11.2c	28.7b	18.5c	<0.01	0.1	0.1	<0.01
	T3	8.89a	0.62a	6.7b	101.6b	0.5b	0.1	1.2b	27.7a	56.9a	20.3b	51.6a	30.0b	<0.01	0.1	0.1	<0.01
	T4	7.96b	0.50d	3.1c	68.8c	1.2a	nd	0.6c	14.4b	15.1d	58.7a	16.6c	46.2a	0.1	<0.01	0.1	0.1
S3	T1	8.03b	1.22b	68.1b	1.9	0.5	0.3b	<0.6	24.3b	71.2a	25.3b	144.2a	84.1b	nd	<0.01	<0.01	nd
	T2	7.30c	0.44c	12.3c	3.0	0.4	nd	<0.6	5.5d	24.2d	7.9b	37.9c	14.9b	nd	0.1	<0.01	0.1
	T3	8.38a	0.34d	21.0c	nd	nd	0.2b	<0.6	9.7c	28.8c	12.2b	53.1b	9.2b	0.52	0.3	<0.01	0.5
	T4	8.03b	3.11a	135.2a	nd	nd	4.7a	2.3	41.3a	43.0b	30.1a	30.2d	581.3a	0.22	<0.01	<0.01	<0.01
S4	T1	7.95a	0.90a	77.7a	1.1c	0.1a	0.8b	<0.6	21.2a	70.3a	25.6b	103.4a	42.9b	<0.01	<0.01	0.2b	nd
	T2	7.54b	0.34d	6.8d	3.6b	0.2a	0.3d	<0.6	4.5d	23.0c	6.3d	25.4c	9.6d	<0.01	<0.01	0.2b	<0.01
	T3	8.32a	0.60c	12.9c	5.7a	nd	0.1c	<0.6	13.7c	51.4b	21.6c	79.4b	24.7c	<0.01	<0.01	<0.01	<0.01
	T4	7.51b	0.83b	16.3b	nd	nd	0.9a	<0.6	17.3b	14.1d	59.2a	26.0c	105.3a	<0.01	<0.01	1.2a	0.1
S		*	*	*	*	–	*	–	*	*	*	*	*	–	–	–	–
T		*	*	*	*	–	*	–	*	*	*	*	*	–	–	–	–
S × T		*	*	*	*	–	*	–	*	*	*	*	*	–	–	–	–

Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4). For each variable, at a given sampling site, values followed by the same letter are not statistically different according to Tukey's test at $p \leq 0.05$.

[a] Values of electrical conductivity (EC) are expressed in dS cm^{-1}.

[b] Values of dissolved organic carbon (DOC), NO_3^-, NO_2^- and NH_4^+ are expressed in mg L^{-1}. nd: no detected.

[*] $p \leq 0.05$.

Carlo Elba NC1500 elemental analyzer on line with a Delta Plus XL (ThermoQuest) mass spectrometer (EA-IRMS). The overall precision of analyses was ±0.1‰ for $\delta^{15}N$. The stable composition is reported as δ values per mil: $\delta = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$, where $R = {}^{15}N/{}^{14}N$ for $\delta^{15}N$. Nitrogen contents of the $AgNO_3$ samples were typically ~8%, indicating that no major contaminants were present in the precipitate. Commercial N_2 was used as the internal standard for the nitrogen isotopic analyses, contrasted with the international standard. $\delta^{15}N$ values for all samples were normalized against internationally accepted reference materials (IAEA N1, $\delta^{15}N$ = +0.4‰, IAEA N2, $\delta^{15}N$ = +20.3‰). The nitrogen isotope ratios of $AgNO_3$ generated from dissolved IAEA-NO-3 potassium nitrate were within +4.65‰ (n = 12), similar to the accepted value. Duplicate nitrogen isotope ratio determinations on $AgNO_3$ from laboratory nitrate samples were performed with a precision generally better than ±0.2‰. $\delta^{15}N$ values are reported with respect to air.

2.6. *Statistical analysis*

Differences between the different physicochemical parameters were checked out using the analysis of variance (ANOVA) and the Tukey post hoc test at $p < 0.05$. Pearson coefficients were calculated to obtained correlation between variables using the SSPS 17.0 program for Windows XP. A principal component analysis (PCA) was performed to analyze relationships among parameters concerning physicochemical characterization of the sediments, their enzymatic activities and gas production.

3. Results

3.1. *Meteorological data*

Total rainfall at DNP during the period of study was 299.9 mm, a value which is lower than that of 477.5 mm, which represents the mean rainfall for the previous 5 years. Main rainfall was registered in October 2008 (119.25 mm) and March–April 2009 (49.49 and 30.90 mm), respectively, coinciding with T1 and T3 sampling times. T2 (January 2009) presented 9.3 mm and T4 (July 2009) 0.11 mm, being the driest season. This pluvial regimen affected water dynamic in the sampling places studied especially at T4, transforming the stream and lagoon waters sampled in swamps (especially in S3 that presented a high eutrophization rate). Relative humidity was higher in autumn and winter seasons (T1: 73.79% and T2: 79.69%) than in spring and summer seasons (T3: 65.38% and T4: 48.68%), decreasing with air temperature (T1: 18.04 °C, T2: 9.73 °C, T3: 14.55 °C and T4: 25.31 °C).

3.2. *Surface waters: physicochemical characterization and isotopic analysis*

Surface water showed in general slightly basic pH values, especially in S2, S3 and S4 (average values of 7.94 and 7.83 in S2, S3 and S4, respectively, Table 1a). EC was related directly to total K, Ca, Mg, Na and S concentration in waters, and also to the sampling season. EC values typically varied within the range from 0.12 and 1.22 dS cm^{-1} depending on the sampling season. The highest EC values were generally recorded during rainy the season (T1), with the exception of the large EC value (3.11 dS cm^{-1}) registered in S3 during the driest season (T4), due to the eutrophization caused for the swamp water. In general, soluble organic matter was high at T1 (S1: 17.7, S2: 28.3 and S4: 77.7 mg L^{-1} of DOC) for all sampling sites studied with the exception of S3, that presented 135.2 mg L^{-1} of DOC at T4, due to the high water eutrophication (the P concentration at this location was the highest value for all sampling sites and seasons) which produced an elevated suspended algae content (green water colour by visual observation).

S2 presented higher TDN concentrations (in the range 21.6 and 9.4 mg L^{-1}) than the other three locations S1, S3 and S4 that presented an overall of 0.5, 6.2 and 2.3 mg L^{-1}, respectively (Table 1b). In S3 at the driest season (T4), TDN showed a value of 18.1 mg L^{-1} especially due to the high NH_4^+ content (Tables 1a and 1b). S2

Table 1b
Total dissolved nitrogen (TDN), dissolved organic carbon (DOC) and organic nitrogen (N_{ORG}) in the surface waters at the sampling sites.

Sampling site	Times	TDN (mg L^{-1})	DOC/DN	N_{ORG} (%)	N-NO_3^- (%)	N-NO_2^- (%)	N-NH_4^+ (%)
S1	T1	0.6b	29.5	79.5	7.5	–	13.0
	T2	0.7a	23.9	64.2	9.7	26.1	–
	T3	0.3d	19.0	54.8	45.2	–	–
	T4	0.4c	22.3	100.0	–	–	–
S2	T1	15.6b	1.8	10.1	89.2	0.2	0.5
	T2	15.3b	0.1	3.7	96.3	–	–
	T3	21.6a	0.3	4.2	94.7	0.7	0.4
	T4	9.4c	0.2	8.6	89.3	2.1	–
S3	T1	4.4b	15.5	81.5	9.8	3.5	5.3
	T2	1.1b	11.2	27.3	61.6	11.1	–
	T3	1.2b	17.5	87.0	–	–	13.0
	T4	18.1a	7.5	79.8	–	–	20.2
S4	T1	3.6a	21.6	75.0	6.9	0.8	17.3
	T2	1.1d	6.2	20.6	73.9	5.5	–
	T3	2.1c	6.1	35.0	61.3	–	3.7
	T4	2.3b	7.1	69.6	–	–	30.4

Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4). For TDN, at a given sampling site, values followed by the same letter are not statistically different according to Tukey's test at $p \leq 0.05$. $N_{ORG} = TDN - N_{INORG}$, where N_{INORG} (inorganic nitrogen) = N-NO_3^- + N-NO_2^- + N-NH_4^+. nd, no detected.

presented a large NO_3^- concentration (61.6, 106.6, 101.6 and 68.8 mg L^{-1} at T1, T2, T3 and T4, respectively) respect to the other sampling sites studied (less that 6 mg L^{-1}, Table 1a). These NO_3^- concentrations represented between 90 and 97% of the TDN of the surface waters (Table 1b). Also, this fact was noticed in DOC/TDN ratio values, being smaller in S2 (average of 0.6) than in S1, S3 and S4 (23.7, 12.9 and 10.2, respectively). These sampling sites presented an important organic nitrogen fraction (average of 74.6, 68.9 and 50.1%, respectively), not in S2 that was predominantly inorganic (average of 92.4% of NO_3^- respect to TDN content).

With the procedure used in this study, isotopic analysis of N-NO_3^- could be carried out only in S2, the site with the highest NO_3^- concentrations. Values of $\delta^{15}N$ ranged from −1.6 to +6‰ (AIR) with an average of −0.78‰ (AIR). This relatively low value, closed to that of the atmosphere air, indicates that contaminant NO_3^- was of inorganic origin because atmospheric air is used for their synthesis (Vitoria et al., 2004). Moreover, since mean average values of $\delta^{15}N$ for most inorganic Spanish fertilizers vary between −1 and +2‰ (AIR), being the total range between −4 and +6‰ (Otero et al., 2005), the stable isotopes of nitrogen indicate an origin related with fertilizers used in agricultural practices.

Table 2a
Physicochemical properties of the sediments at the sampling sites.

Sampling site	Times	pH (1:5)	EC (1:5)[a]	P[b]	K[b]	Ca[b]	Mg[b]	Na[b]	S[b]	Fe[b]	Cu[c]	Mn[c]	Zn[c]
S1	T1	5.44	860	0.12d	5.07c	7.28a	3.09b	0.71b	7.75b	16.39a	11c	252a	25b
	T2	5.51	390	0.45b	5.51b	5.89b	2.88b	0.71b	3.34d	15.40b	25a	163c	27b
	T3	3.98	565	0.83a	4.43d	5.28c	1.96c	0.55c	9.66a	13.47c	11c	98d	44a
	T4	5.90	178	0.32c	6.71a	6.89a	3.40a	0.82a	5.61c	13.42c	11c	189b	22b
S2	T1	5.05d	628a	0.12d	2.92b	5.04bc	1.72a	0.28a	3.80a	10.32b	8a	136a	16b
	T2	7.13c	82c	0.45a	3.21a	3.86c	1.79a	0.24b	0.65b	12.26a	4b	136a	17b
	T3	7.59b	153b	0.21c	1.92c	5.75b	0.92c	0.21bc	0.48c	5.06d	10a	114b	16b
	T4	8.26a	113bc	0.27b	2.02c	36.83a	1.33b	0.21c	0.52c	5.81c	9a	107b	19a
S3	T1	8.58a	90b	0.12b	0.68b	0.44c	0.44bc	0.10b	0.16a	2.57ab	5a	58a	7c
	T2	7.72c	84c	0.45a	0.91b	0.96a	0.62b	0.10b	0.15a	4.11a	4a	82a	11a
	T3	6.75d	39d	0.02c	0.57b	0.25c	0.28c	0.11b	0.14a	2.07b	2a	65a	17b
	T4	8.20b	101a	0.04c	1.29a	0.68b	0.76a	0.25a	0.12a	3.61ab	2a	69a	4d
S4	T1	7.36c	117c	<0.01	1.92d	1.32d	1.08c	0.23a	0.20a	5.66d	3d	140c	13d
	T2	7.55b	165b	<0.01	8.72b	5.58a	5.19a	0.48b	0.67a	26.60a	26a	313a	56a
	T3	6.81d	115c	0.17b	7.61c	2.78c	3.51b	0.49b	0.59a	13.67c	19c	150c	34c
	T4	7.68a	221a	0.33a	10.95a	4.37b	5.12a	0.80a	0.65a	19.37b	23b	256b	46b
S		*	*	*	*	*	*	*	*	*	*	*	*
T		*	*	*	*	*	*	*	*	*	*	*	*
S × T		*	*	*	*	*	*	*	*	*	*	*	*

Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4). For each variable, at a given sampling site, values followed by the same letter are not statistically different according to Tukey's test at $p \leq 0.05$.

[a] Values of EC (electrical conductivity) are expressed in μS cm^{-1}.

[b] For each variable, values are expressed in g kg^{-1} (sediment dry weight).

[c] For each variable, values are expressed in mg kg^{-1} (sediment dry weight).

* $p \leq 0.05$.

Table 2b
Total organic carbon (TOC), total nitrogen (TN), organic nitrogen (N_{ORG}), dissolved organic carbon (DOC) and dissolved nitrogen (DN) in sediments at the sampling sites.

Sampling site	Season	TOC[a]	TN[a]	TOC/TN	N_{ORG} (%)	NO_3^-[b]	NO_2^-[b]	NH_4^+[b]	DOC[b]	DN[b]
S1	T1	183.4a	5.7b	31.9	99.4	nd	nd	41b	1137c	70b
	T2	154.2b	9.0a	17.1	99.5	34	nd	44b	2580a	144a
	T3	99.8c	3.7c	27.3	99.3	7	nd	30c	332d	30c
	T4	206.0a	6.9b	29.8	99.1	nd	nd	79a	1934b	138a
S2	T1	78.4a	3.1a	25.5	99.6	32	nd	6c	280b	22bc
	T2	18.3b	1.0c	18.8	97.5	54	nd	16b	258b	23b
	T3	38.4b	1.6bc	23.7	97.8	3	nd	45a	174c	16c
	T4	22.7b	0.5c	42.9	96.8	nd	nd	20b	412a	42a
S3	T1	3.1a	0.3a	11.7	98.5	32a	nd	6c	163b	15b
	T2	3.4a	0.3a	12.2	97.8	4b	nd	7c	147b	17b
	T3	1.8b	0.2a	8.3	92.3	3b	nd	20b	25c	3c
	T4	1.7b	0.2a	11.0	89.9	nd	nd	26a	206a	33a
S4	T1	5.4d	0.4c	10.8	97.3	nd	nd	14c	310c	27c
	T2	16.6c	1.5b	10.9	98.6	4	nd	25b	382b	43b
	T3	25.2a	1.9a	12.6	97.7	nd	nd	55a	196d	19c
	T4	20.1b	1.9a	10.5	98.8	nd	nd	30b	669a	104a
S		*	*	*	*	–	–	*	*	*
T		*	*	*	*	–	–	*	*	*
S × T		*	*	*	*	–	–	*	*	*

Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4). For each variable, at a given sampling site, values followed by the same letter are not statistically different according to Tukey's test at $p \leq 0.05$.

[a] Values of TOC and TN are expressed in g kg^{-1} (sediment dry weight).

[b] Values of NO_3^-, NO_2^-, NH_4^+, DOC and DN are expressed in mg kg^{-1} (sediment dry weight). DOC and DN were obtained after 2 h water extraction (1:20, w/v) of the lyophilized sediments. N_{ORG} = TN – N_{INORG}, where N_{INORG} (inorganic nitrogen) = N-NO_3^- + N-NO_2^- + N-NH_4^+. nd, no detected.

[*] $p \leq 0.05$.

3.3. Sediments: physicochemical characterization, enzymatic activities and gas production

Sand constituted more than 85% of the components of the sediments. According to the corresponding contents in clay and silt, S1 and S2 were classified as loamy sand sediments, those from S3 as sand, and sandy loam for S4. Similar to surface waters, values of pH, EC and content in macro and microelements in sediments from the four sampling sites varied both among the sites and with the sampling time (Table 2a). Despite these differences, the values of TOC and TN were always higher in S1 than in the remaining sampling sites (Table 2b). Regardless of the sampling sites and sampling times, more than 90% of the nitrogen content in the sediments was of organic origin and, accordingly, the greatest values of DOC and TDN were also found in S1 (Table 2b). Similarly, β-glucosidase, dehydrogenase, urease acid phosphatase and arylsulphatase activities varied greatly with both the sampling sites and the sampling times (Fig. 2). Whereas S4 showed the highest values of dehy-

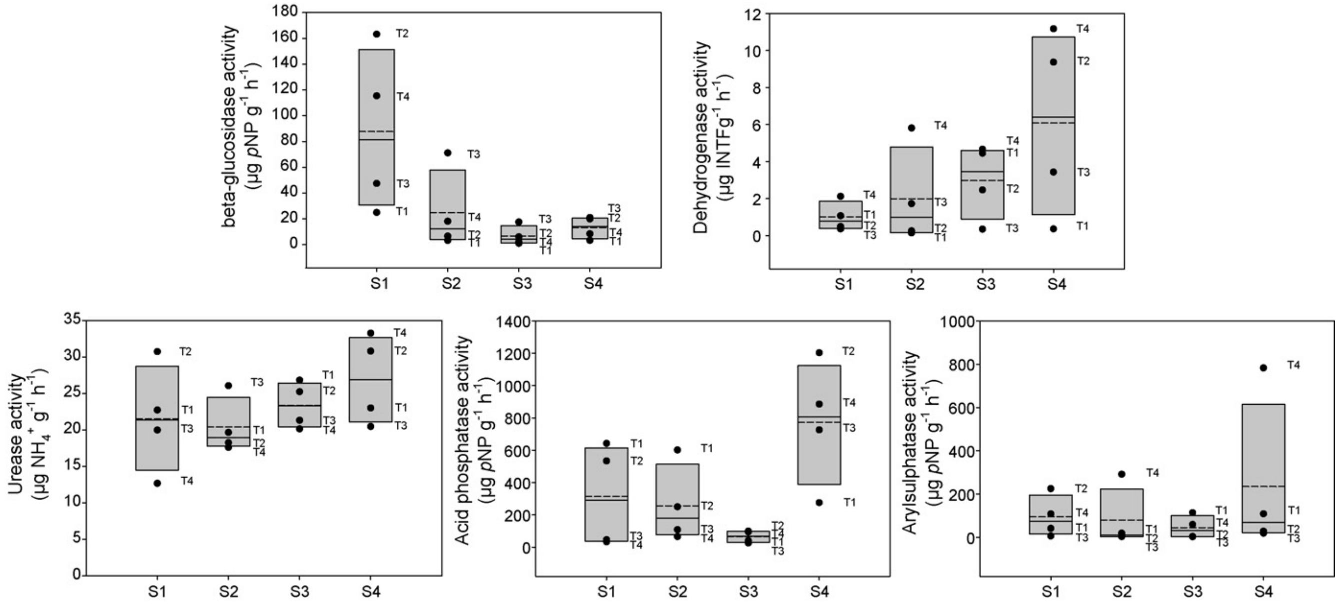

Fig. 2. β-Glucosidase, dehydrogenase, urease, acid phosphatase and arylsulphatase activities in sediments. Vertical boxes show the median (dash line), mean (solid line) and the 5th/95th percentiles. Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4).

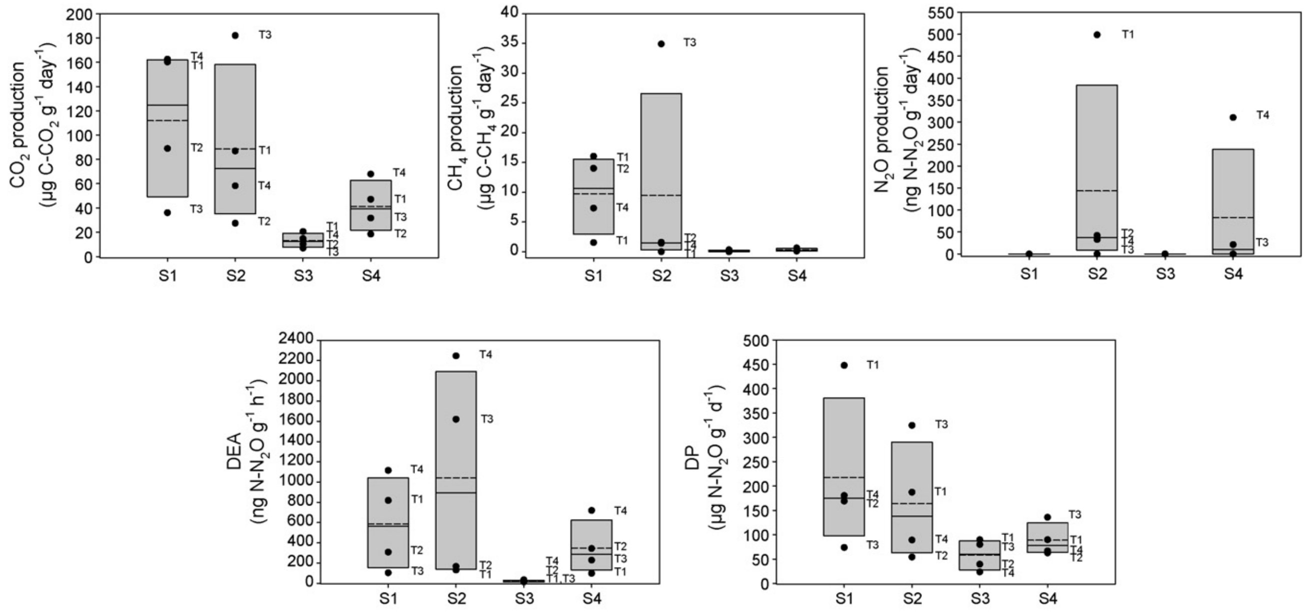

Fig. 3. CO$_2$, CH$_4$ and N$_2$O emissions, denitrifying enzymatic activity (DEA) and denitrification potential (DP) in sediments. The vertical boxes show the median (dash line), mean (solid line) and the 5th/95th percentiles. Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4).

drogenase (mean average value of 10.26 μg INTF g^{-1} h^{-1}), acid phophatase (mean average value of 23.5 μg *p*NP g^{-1} h^{-1}), urease (mean average value of 600 μg *p*NP g^{-1} h^{-1}) and arylsulphatase (mean average value of 400 μg *p*NP g^{-1} h^{-1}) activities, S1 was the site with the greatest values of β-glucosidase activity (mean average value of 90 μg *p*NP g^{-1} h^{-1}) (Fig. 2).

The mean average value of CO$_2$ produced at sampling sites S1, S2, S3 and S4 were 112, 89, 13, and 41 μg C-CO$_2$ g^{-1} day^{-1}, respectively (Fig. 3). Methane production from the sediments was also higher in S1 (mean average value of 9.7 μg C-CH$_4$ g^{-1} day^{-1}) than in S3 and S4 (mean average values of 0.2 and 0.3 μg C-CH$_4$ g^{-1} day^{-1}, respectively). Methane production occurred in S2 was in general low. Unexpectedly, values for S2 detected at T3 were the highest for all samples analyzed (34.9 μg C-CH$_4$ g^{-1} day^{-1}) (Fig. 3). S2, the site with the highest NO$_3{}^-$ contents in its surface water, and S4 showed maximal values of N$_2$O production with mean average values of 500 and 310 ng N-N$_2$O g^{-1} day^{-1}, respectively. Potential denitrification as assayed by DEA and DP showed that S1 and S2 have the highest potential for denitrification compared to S3 and S4. The highest values for DEA were obtained during the driest season (T4) with 1115, 2246 and 719 ng N-N$_2$O g^{-1} h^{-1} in S1, S2 and S4, respectively. Despite fluctuations at the sampling times, S1 and S2 also showed maximal values of DP with mean average values of 218, 164, 58 and 89 μg N-N$_2$O g^{-1} d^{-1} for S1, S2, S3 and S4, respectively.

3.4. Statistical analysis

Pearson correlation matrix revealed that TOC, TN, NH$_4{}^+$, DOC and TDN were positive and significantly ($p \leq 0.01$) correlated with CO$_2$ production and with β-glucosidase activity (Table 3). After PCA

Table 3
Pearson correlation matrix ($n = 16$) between the physicochemical properties, enzymatic activities and greenhouse gas emissions in sediments at four sampling sites along La Rocina Stream.

	pH	EC	TOC	TN	NH$_4{}^+$	DOC	DN	DH	GC	AS	AP	UR	CO$_2$	CH4	DEA	DP
pH																
EC	−0.770**															
TOC	−0.720**	0.676**														
TN	−0.698**	0.601*	0.932**													
NH$_4{}^+$	NS	NS	0.663**	0.644**												
DOC	NS	NS	0.815**	0.992**	0.612*											
DN	NS	NS	0.718**	0.815**	0.622*	0.932**										
DH	0.549*	NS	NS	NS	NS	NS	NS									
GC	NS	NS	0.707**	0.839**	0.689**	0.861**	0.736**	NS								
AS	NS	NS	NS	NS	NS	NS	0.521*	0.654**	NS							
AP	NS	NS	NS	NS	NS	NS	NS	0.509*	NS	NS						
UR	NS	NS	NS	NS	NS	NS	NS	0.499*	NS	NS	0.554*					
CO$_2$	NS	NS	0.714**	0.605**	0.625**	0.516*	NS	NS	0.542*	NS	NS	NS				
CH4	NS	NS	NS	NS	NS	NS	NS	NS	0.539*	NS	NS	NS	0.804**			
DEA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.606*	0.506*		
DP	NS	0.641**	0.632**	0.513**	NS	NS	NS	NS	NS	NS	NS	NS	0.854**	0.765**	NS	

NS, not significant; EC, electrical conductivity; TOC, total organic carbon; TN, total nitrogen; DOC, dissolved organic carbon; DN, dissolved nitrogen; DH, dehydrogenase activity; GC, β-glucosidase activity; AS, arylsulphatase activity; AP, acid phosphatase activity; UR, urease activity; DEA, denitrifying enzymatic activity; DP, denitrification potential.

[*] Significant at $p < 0.05$.

[**] Significant at $p < 0.01$.

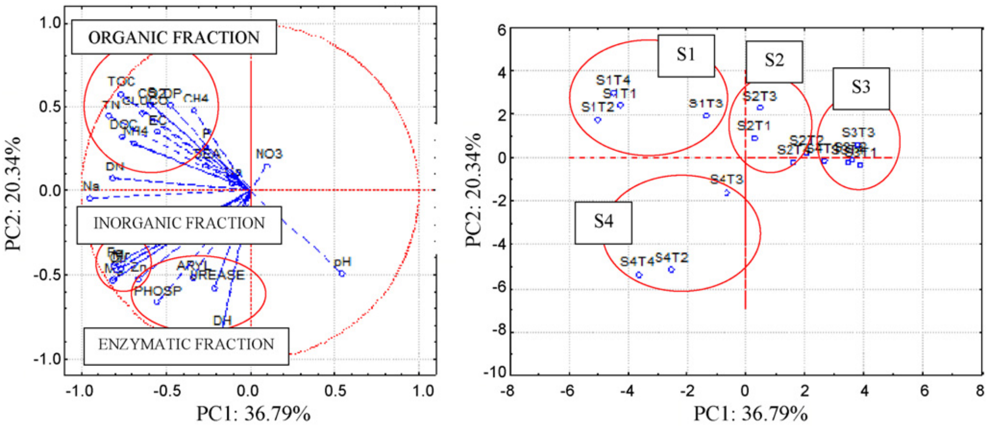

Fig. 4. (a) Principal component analysis performed on the whole set of measured sediments properties and (b), the spatial and seasonal distribution of the parameters. Sampling sites: Palacio del Acebrón (S1), Arroyo de la Cañada (S2), Vado de la Canariega (S3) and Marisma del Rocío (S4). Sampling times: October 2008 (T1), January 2009 (T2), April 2009 (T3) and July 2009 (T4).

analysis, except for NO_3^- and pH, the remaining parameters analyzed clustered in 3 main groups (Fig. 4a). The first cluster contained most of the parameters related with the organic fraction of the sediments (TOC, TN, DOC, TDN, NH_4^+, CO_2, CH_4, DP and β-glucosidase activity), the second cluster included the inorganic components (K, Ca, Mg, Fe, Cu, Mn and Zn), and the third cluster was composed of the enzymatic activities dehydrogenase, acid phophatase, urease and arylsulphatase. The first principal component (PC1) explained 36.79% of the total variance of the data, whereas the second principal component (PC2) was responsible for 20.34%. According to these two axes, the sampling sites were ordered as a function of the three clusters mentioned above (Fig. 4b). Consequently, S1 showed the highest values for the organic fraction and S4 for the enzymatic fraction. Nevertheless, sizes of the clusters indicated that seasonal variation affected much more to S1 and S4 than to S2 and S3.

4. Discussion

Although to a different extent depending on the sampling site, physicochemical properties of the surface waters sampled along La Rocina Stream were influenced by seasonal variation (especially rainfall regime). These results agree with those of Espinar and Serrano (2009) which indicate that development of temporary wetlands in DNP are influenced by climate and geology of the region. This is especially important in wetlands located in semiarid areas such as the South of Spain, where the climate is unpredictable and produces a wide range of hydrological conditions (Serrano et al., 2006). Thus, it is possible that rainfall, evaporation, groundwater discharge, biogeochemical interactions at the sediment–water interface affected chemical composition of the surface waters along the course of La Rocina Stream. Hydrological dynamic at each sampling site was affected by the sampling time. That was clearly visible in S3 at T4, the driest sampling time, where stream waters were transformed into swampy waters, and in S4 where desiccation almost emptied the El Rocio marsh.

Several authors have reported continuous increases in pollution (Suso and Llamas, 1993; Olías et al., 2008), and more precisely in NO_3^- content, in surface- and groundwater of DNP during the last two decades (González-Quesada et al., 1987; Serrano et al., 2006). Recently, contamination due to NO_3^- and SO_4^{2-} in the Doñana aquifer has been linked to utilization of agrochemicals during the agricultural practices that take place in the ecotone of the Park (Olías et al., 2008). Values of NO_3^- content in S2 were higher than the 50 mg L^{-1} defined by the European directive 91/676/CEE as the upper limit for NO_3^- contamination from agricultural sources (European Commission, 1991). At that site, concentration of inorganic N represented more than 90% of the TDN. In this study, based on isotopic analysis of the contaminant NO_3^- in surface waters of La Rocina Stream, we show that, at least in S2, they were of inorganic origin, more probably from chemical fertilizers.

The interpretation of the nutrients dynamic in aquatic ecosystems could be biased by the strong effects of hydrology on physicochemical (Espinar and Serrano, 2009). For that, microbiological processes involved in the principal biogeochemical cycles are needed (Faulwetter et al., 2009). Soil microorganisms mediate many processes that are of particular interest in freshwater wetland ecosystems where nutrient cycling is highly responsive to fluctuating hydrology and nutrients and soil gas releases may be sensitive to climate warming (Gutknecht et al., 2006). Determination of enzymatic activities in sediments of La Rocina Stream varied both among sampling sites and among sampling times. Although determinations of enzymatic activities in sediments are relatively scarce, previous analyses have shown they vary widely across the different wetland ecosystems examined (Gutknecht et al., 2006). In our study, dryness and temperature positively affected dehydrogenase, as values of activity were always greater at T4. Similarly, β-glucosidase activity correlated significantly with the content of the organic matter fraction, as the highest values of activity were detected in S1 and S2, the sites with the highest TOC concentrations. Similar results were reported by Williams and Jochem (2006) who showed that, despite the complex relationships between biological and environmental parameters, the kinetic of several ectoenzymes, among them β-glucosidase, were controlled by organic matter availability.

Wetlands play an important role in carbon cycle and in global climate change. The emission of greenhouse gases, especially CO_2, and CH_4, shows a large spatial and temporal variation due to the complex interactions between environmental variables and the microbiological processes leading to gas production. The carbon flux is related to many external factors, including soil environment, hydrological conditions, vegetation type and exogenous nitrogen (Ma and Lu, 2008). As revealed by Pearson correlation matrix (Table 3), CO_2 and CH_4 fluxes showed a strong seasonal influence, especially at S1 and S2, the sampling sites with the highest TOC concentrations. There is to note, however, that NO_3^- contamination increased production not only of N_2O, but also of CO_2 and CH_4. These results agree with those which show that alterations in the biogeochemical cycles in nature may lead to altered biogenic fluxes of CO_2, CH_4 and N_2O, the three main gases contributing to global warming (Liu and Greaver, 2009).

In addition to NO_3^-, denitrification correlated positively with the content of organic matter in the sediments. Accordingly, increased potential denitrification was observed at sites with the highest TOC values. Similar results were obtained during studies on denitrification and its relationship with organic carbon quality in three coastal wetland soils (Dodla et al., 2008). Also, Sirivedhin and Gray (2006) found that the sediment denitrification potential showed a positive relationship with the biodegradable organic carbon concentration produced by the periphytic algae in wetlands. Denitrification was also affected by the pluvial regime, as the highest values of DEA were registered during the driest season at each sampling time. Hernández and Mitsch (2007) founded that soil temperature, flood frequency and nitrate availability were important factors controlling denitrification in created wetlands. Davidson (1991) observed an increase N_2O production in dry season, and specially during drying and wetting cycles, caused by a temporal accumulation of mineral nitrogen into soil surface, which will become rapidly available to microbial biomass when dry soil is wetted.

5. Conclusions

The surface water of La Rocina Stream showed NO_3^- contamination, probably to agricultural sources. This contamination decreased along La Rocina basin and apparently, the superficial water body of DNP wetland was not affected. More research is needed to evaluate how the NO_3^- pollution could affect DNP groundwater. The environmental conditions such as precipitation rate, hydrological morphology and organic matter content greatly influenced the physicochemical characteristics of the surface waters of DNP wetland. The biological activity and greenhouse gas production in their aquatic sediments were also affected by these environmental parameters, specially the hydrology which had a major effect during the driest season. The denitrification process was affected by anthropogenic activity (nitrate contamination from agricultural practices) and the rainfall regimen, increasing the GHG emissions (CO_2, CH_4 and especially N_2O) during the driest season in all sampling sites studied.

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References

Arambarri, P., Cabrera, F., González-Quesada, R., 1996. Quality evaluation of the surface waters entering the Doñana National Park (SW Spain). Sci. Total Environ. 191, 185–196.

Aulakh, M.S., Doran, J.W., Mosier, A.R., 1992. Soil denitrification: significance, measurement, and effects of management. Adv. Soil Sci. 18, 1–52.

Cabrera, F., Clemente, L., Díaz Barrientos, E., López, R., Murillo, J.M., 1999. Heavy metal pollution of soils affected by the Guadiamar toxic flood. Sci. Total Environ. 242, 117–129.

Camargo, J.A., 1994. The importance of biological monitoring for the ecological risk assessment of freshwater pollution: a case study. Environ. Int. 20, 229–238.

Camargo, J.A., Alonso, A., 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. Environ. Int. 32, 831–849.

Christensen, P.B., Nielsen, L.P., Sorensen, J., Revsbech, N.P., 1990. Denitrification in nitrate-rich streams: diurnal and seasonal variation related to benthic oxygen metabolism. Limnol. Oceanogr. 35, 640–651.

Conrad, R., 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). Microbiol. Rev. 60, 609–640.

Davidson, E.A., 1991. Fluxes of nitrous oxide and nitric oxide from terrestrial ecosystems. In: Rogers, J.E., Whitman, W.B. (Eds.), Microbial Production and Consumption of Greenhouse Gases: Methane, Nitrogen Oxides and Halomethans. American Society of Microbiology, Washington, DC, pp. 219–235.

Davidson, E.A., Verchot, L.V., 2000. Testing the hole-in-the-pipe model of nitric and nitrous oxide emissions from soils using the TRAGNET database. Global Biogeochem. Cycles 14, 1035–1043.

Davidson, E.A., Ishida, F.Y., Nepstad, D.C., 2004. Effects of an experimental drought on soil emissions of carbon dioxide, methane, nitrous oxide, and nitric oxide in a moist tropical forest. Global Change Biol. 10, 718–730.

Dodla, S.K., Wang, J.J., DeLaune, R.D., Cook, R.L., 2008. Denitrification potential and its relation to organic carbon quality in three coastal wetland soils. Sci. Total Environ. 407, 471–480.

European Commission, 1991. Council Directive 91/676/EEC of 12 December 1991, concerning the protection of waters against pollution caused by nitrates from agricultural sources. Official J. Eur. Communities (L 375, 31/12/1991).

European Commission, 2002. Implementation of Council Directive 91/676/EEC concerning the protection of waters against pollution caused by nitrates from agricultural sources. Official J. Eur. Communities, http://ec.europa.eu/environment/water/water-nitrates/pdf/91_676_eec_en.pdf (accessed 01.06.10).

Espinar, J.L., Serrano, L., 2009. A quantitative hydrogeomorphic approach to the classification of temporary wetlands in the Doñana National Park (SW Spain). Aquat. Ecol. 43, 323–334.

Faulwetter, J.L., Gagnon, V., Sundberg, C., Chazarenc, F., Burr, M.D., Brisson, J., Camper, A.K., Stein, O.R., 2009. Microbial processes influencing performance of treatment wetlands: a review. Ecol. Eng. 35, 987–1004.

Galloway, J.N., Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. Science 320, 889–892.

García, C., Hernandez, T., Costa, F., 1997. Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun. Soil Sci. Plant Anal. 28, 123–134.

González-Quesada, R., Cabrera, F., Diaz, E., Arambarri, P., 1987. La calidad de las aguas del río Guadiamar y de los arroyos de la Rocina y el Partido en las proximidades de Doñana, SW de España. Limnética 3, 97–102.

Grimalt, J.O., Ferrer, M., MacPherson, E., 1999. The mine tailing accident in Aznalcollar. Sci. Total Environ. 242, 3–11.

Groffman, P.M., Altabet, M.A., Böhlke, J.K., Butterbach-Bahl, K., David, M.B., Firestone, M.K., Giblin, A.E., Kana, T.M., Nielsen, L.P., Voytek, M.A., 2006. Methods for measuring denitrification: diverse approaches to a difficult problem. Ecol. Appl. 16, 2091–2122.

Gutknecht, J.L.M., Goodman, R.M., Balser, T.C., 2006. Linking soil process and microbial ecology in freshwater wetland ecosystems. Plant Soil. 289, 17–34.

Hernández, M.E., Mitsch, W.J., 2007. Denitrification in created riverine wetlands: influence of hydrology and season. Ecol. Eng. 30, 70–88.

Kandeler, E., Gerber, H., 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol. Fertil. Soils. 6, 68–72.

Kempers, A.J., Zweers, A., 1986. Ammonium determination in soil extracts by the salicylate method. Commun. Soil Sci. Plant Anal. 17, 715–723.

Liu, L., Greaver, T.L., 2009. A review of nitrogen enrichment effects on three biogenic GHGs: the CO_2 sink may be largely offset by stimulated N_2O and CH_4 emission. Ecol. Lett. 12, 1103–1117.

Ma, A.N., Lu, J.J., 2008. The progress of research on carbon flux in wetland ecosystems. Wetland Sci. 6, 116–123.

MAPA, 1974. Métodos Oficiales de Análisis de Suelos y Aguas. Ministerio de Agricultura, Spain.

Merbach, W., Augustin, J., Kalettka, T., Jacob, H.J., 1996. Nitrous oxide and methane emissions from riparian areas of ponded depressions of Northeast Germany. J. Appl. Bot. Food Qual. 70, 134–136.

Merbach, W., Augustin, J., Gans, W., 2001. Nitrous oxide emission from fen mires in dependence of anthropogenic activities. J. Appl. Bot. Food Qual. 75, 118–123.

Mitsch, W.J., Gosselink, J.G., 2007. Wetlands, 4th ed. John Wiley & Son, Inc.

Mulholland, P.J., Helton, A.M., Poole, G.C., Hall Jr., R.O., Hamilton, S.K., Peterson, B.J., Tank, J.L., Ashkenas, L.R., Cooper, L.W., Dahm, C.N., Dodds, W.K., Findlay, S.E.G., Gregory, S.V., Grimm, N.B., Johnson, S.L., McDowell, W.H., Meyer, J.L., Valett, H.M., Webster, J.R., Arango, C.P., Beaulieu, J.J., Bernot, M.J., Burgin, A.J., Crenshaw, C.L., Johnson, L.T., Niederlehner, B.R., O'Brien, J.M., Potter, J.D., Sheibley, R.W., Sobota, D.J., Thomas, S.M., 2008. Stream denitrification across biomes and its response to anthropogenic nitrate loading. Nature 452, 202–205.

Olías, M., Cerón, J.C., Fernández, I., Moral, F., Rodríguez-Ramírez, A., 2005. State of contamination of the waters in the Guadiamar valley five years after the Aznalcóllar spill. Water, Air, Soil Pollut. 166, 103–119.

Olías, M., González, F., Cerón, J.C., Bolívar, J.P., González-Labajo, J., García-López, S., 2008. Water quality and distribution of trace elements in the Doñana aquifer (SW Spain). Environ. Geol. 55, 1555–1568.

Otero, N., Vitória, L., Soler, A., Canals, A., 2005. Fertiliser characterisation: major, trace and rare earth elements. Appl. Geochem. 20, 1473–1488.

Serrano, L., Reina, M., Martín, G., Reyes, I., Arechederra, A., León, D., Toja, J., 2006. The aquatic systems of Doñana (SW Spain): watersheds and frontiers. Limnética 25, 11–32.

Sierra, J., Montserrat, G., Martí, E., Garau, M.A., Cruañas, R., 2003. Contamination levels remaining in Aznalcóllar spill-affected soils (Spain) following pyritic sludge removal. Soil Sed. Cont. 12, 523–539.

Silva, S.R., Kendall, C., Wilkison, D.H., Ziegler, A.C., Chang, C.C.Y., Avanzino, R.J., 2000. A new method for collection of nitrate from fresh water and the analysis of nitrogen and oxygen isotope ratios. J. Hydrol. 228, 22–36.

Simek, M., Elhottová, D., Klimes, F., Hopkins, D.W., 2004. Emissions of N_2O and CO_2, denitrification measurements and soil properties in red clover and ryegrass stands. Soil Biol. Biochem. 36, 9–21.

Sirivedhin, T., Gray, K.A., 2006. Factors affecting denitrification rates in experimental wetlands: field and laboratory studies. Ecol. Eng. 26, 167–181.

Smith, R.S., Shiel, R.S., Bardgett, R.D., Millward, D., Corkhill, P., Rolph, G., Hobbs, P.J., Peacock, S., 2003. Soil microbial community, fertility, vegetation and diversity as targets in the restoration management of a meadow grassland. J. Appl. Ecol. 40, 51–64.

Sommers, S.G., Kjellerup, V., Kristjansen, O., 1992. Determination of total ammonium nitrogen in pig and cattle slurry: sample preparation and analysis. Acta Agric. Scand. B, Soil Plant Sci. 42, 146–151.

Spalding, R.F., Exner, M.E., 1993. Occurrence of nitrate in groundwater—a review. J. Environ. Qual. 22, 392–402.

Suso, J., Llamas, M.R., 1993. Influence of groundwater development on the Doñana National Park ecosystems (Spain). J. Hydrol. 141, 239–269.

Tabatabai, M.A., 1982. Soil enzymes. In: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2, Chemical and Microbial Properties 2. Soil Science Society of America, Madison, WI, pp. 903–947.

Thornton, F.C., Valante, R.J., 1996. Soil emissions of nitric oxide and nitrous oxide from no-till corn. Soil Sci. Soc. Am. J. 60, 1127–1133.

Tiedje, J.M., 1994. Denitrifiers. In: Bigham, J.M. (Ed.), Methods of Soil Analysis. Part 2. Microbiological and Biochemical Properties. Soil Science Society of America, Madison, WI, pp. 245–267.

Vitoria, L., Otero, N., Soler, A., Canals, A., 2004. Fertilizer characterization: isotopic data (N, S, O, C, and Sr). Environ. Sci. Technol. 38, 3254–3262.

Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H., Tilman, D.G., 1997. Human alteration of the global nitrogen cycle: sources and consequences. Ecol. Appl. 7, 737–750.

Williams, C.J., Jochem, F.J., 2006. Ectoenzyme kinetics in Florida Bay: implications for bacterial carbon source and nutrient status. Hydrobiologia 569, 113–127.