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Environmental factors associated with heterotrophic nitrogen-fixing bacteria in water, sediment, and riparian soil of Suquía River

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ABSTRACT

In this study we investigated the environmental factors associated with biological nitrogen (N) fixation (BNF) in water, sediments, and riparian soil along a polluted river (Suquía River of Córdoba, Argentina). Here, we screened heterotrophic nitrogen-fixing bacteria and assessed the magnitude of BNF at different sites of Suquía River. To this aim, samples of the three habitats (riparian soil, water, and sediment) were collected from five polluted sites and one reference site during low and high flow water periods. In all samples the abundance of N-fixing bacteria was evaluated in solid nitrogen-free medium and the biological N fixation was measured by nitrogenase (Nase) enzyme activity using the acetylene reduction method. To identify the heterotrophic N-fixing taxa DNA of nine cultures isolated from sites with different Nase enzyme activity was extracted and the 16S rRNA gene was amplified and sequenced. In addition, the ammonia and organic carbon (C) content in all samples, the dissolved O_2 concentration in water, and the water content in riparian soil were measured. The N-fixing bacteria were detected in all study sites and habitats. The abundance of them correlated significantly with organic C content in sediment, and with water and organic C contents in riparian soil, whereas in water a negative correlation with dissolved O_2 was observed. In addition, the water and sediment Nase enzyme activity varied among sites during low flow period presenting significant correlation with ammonia and organic C contents in sediment. The identified taxonomic groups in the Suquía River are related to *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria* although the N-fixing capacity of them was not established. Altogether these findings demonstrate that BNF occurs in all habitats of Suquía River, being in sediments influenced mainly by the higher organic C present in the most polluted sites, while in riparian soil the organic C and water contents were the major abiotic factors that control the abundance of N-fixing bacteria. In Suquía River water the density of N-fixing bacteria were associated with low dissolved O_2 concentration. These data suggest that the BNF in Suquía River is a complex process that depends on numerous environmental factors that act together.

Introduction

Biological nitrogen fixation (BNF) plays a key role in the global cycle of nitrogen (N) since it is the main process by which atmospheric N is incorporated on the earth's surface (Kavadia et al., 2007; Farnelid and Riemann, 2008). BNF is carried out by prokaryotes called diazotrophs which contain nitrogenase (Nase) enzyme responsible for fixing atmospheric dinitrogen into NH_3, which is immediately incorporated to organic compounds (Paul, 2007; Farnelid and Riemann, 2008). BNF offers a natural means of providing N to the majority of ecosystems, but despite its importance in ecosystem function, ecological controls of N fixation are relatively poorly understood (Vitousek et al., 2002).

The N fixation activity is an alternative metabolism subject to a very wide variety of biotic (competitors for crucial resources and grazers that restrict distribution and abundance of N-fixing microorganisms) and abiotic controls (O_2, bioavailable organic compounds, ammonia) (Vitousek et al., 2002). Oxygen pressure inhibits BNF, due to the lower oxidation state of N respect to O_2.

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Therefore, O_2 concentration is one of the main factors limiting fixation activity and many of the physiological and morphological characteristics of diazotrophs are adaptations for protecting the Nase enzyme against O_2 (Wetzel, 2001; Paul, 2007; Farnelid and Riemann, 2008). Other limiting factor for N fixation activity is ammonia availability. Since BNF is a process that consumes great quantity of energy, microorganisms only fix N in absence of other sources of N that require lower energetic cost like ammonia (Paul, 2007). Furthermore, the availability of organic compounds of low molecular weight regulates N fixation in heterotrophs since organic compounds are the source of carbon (C) and energy for heterotrophic diazotrophs (Wetzel, 2001).

Aquatic ecosystems are very heterogeneous related to environmental conditions that regulate N fixation (Wetzel, 2001). For instance, it has been established that most of BNF by heterotrophs occurs in sediments (Wetzel, 2001). Moreover, Farnelid and Riemann (2008) affirm that soil and sediment aggregates are the main environments for heterotrophic N fixation due to their elevated organic matter content and low O_2 concentration produced by microbial respiration. Also, it has been reported that heterotrophic N fixation is closely associated with aquatic plant roots. Most importantly, plants are known to reduce O_2 concentration, by reduction of root respiration, and to provide a source of available carbon to soil and sediment diazotrophs (Černá et al., 2009).

At present, most of freshwater ecosystems are polluted and severely modified by channelization and dams, among others (Carpenter et al., 1998), which are additional factors that could affect BNF. While other processes of N cycle have been widely studied in aquatic ecosystems, little attention has been placed on BNF, particularly in lotic ecosystems with marked flow fluctuations. Moreover, it is unknown how river channelization and pollution influence BNF.

The Suquía River (Córdoba, Argentina), as most water courses running across big cities, receives complex pollutants from different sources. Furthermore, its watercourse has been modified by channelization, and it is particularly vulnerable to pollution due to its scarce and seasonal flow, short length and endorheic basin (Wunderlin et al., 2001; Merlo et al., 2011). For these reasons Suquía River is an excellent system to analyze the effect of multiple factors on the process of BNF in a lotic ecosystem.

In this study we investigated the environmental factors associated with BNF in water, sediment, and riparian soil along Suquía River. Here, we report heterotrophic N-fixing bacteria screening and the magnitude of BNF at sites of Suquía River with different degrees of pollution. We hypothesize that BNF is lower in polluted sites with elevated ammonia content, and higher in soil and sediment due to the larger availability of organic matter and lower O_2 concentrations.

Materials and methods

Study area

The Suquía River of Córdoba province (Argentina) begins at the San Roque Dam and flows mainly from west to east for about 200 km until Mar Chiquita Lake. The watershed is located in a semi-arid region, with a mean annual rainfall between 700 and 900 mm, concentrated from October to April. The mean temperatures are 10 °C in winter and 26 °C in summer. The San Roque Dam forms an artificial lake where recreational activities have promoted the urbanization of the lake shorelines and surroundings. Thirty km downstream from the dam, Suquía River enters to Córdoba city (1.29 million inhabitants). In the last 20 years, the city's population has almost doubled and growing industrialization has increased the risk of having toxic effluents discharged into the river. Near the eastern edge of the city, the Suquía River receives the sewage discharge from the Municipal Waste Water Treatment Plant (WWTP) (Merlo et al., 2011) (Fig. 1).

The flow regime of Suquía River is of exclusively pluvial origin, with a marked seasonality of the flow due to the irregular distribution of rainfall. The water flow estimation in high flow period (December to April), is greater than 15 $m^3\ s^{-1}$; whereas in low flow period (May–November) is 2.7 $m^3\ s^{-1}$.

Sampling design

Five known sites with different degree of pollution (Merlo et al., 2011 and Table 1) were selected along Suquía River: Córdoba city western border: Site 1 (S1), Córdoba city downtown: Site 2 (S2), Córdoba city eastern border: Site 3 (S3), Corazón de María village: Site 4 (S4), and Río Primero city: Site 5 (S5). In addition one reference site (RS): La Calera city was included (Table 1 and Fig. 1).

Study sites were sampled during low and high flow periods, August 2008 and March 2009, respectively. For each study site, samples were randomly taken along a 100 m linear transect on shoreline. Five samples of each habitat (water, sediment, and riparian soil: 0–20 cm) were collected in sterile containers. Water samples were stored at 4 °C until analysis. Approximately 10 g of soil and sediment samples were wet stored at 4 °C until the analysis of Nase enzyme activity was performed. Then, soil and sediment samples were air-dried for 24 h and sieved through a 2 mm mesh and stored at 4 °C for the chemical analysis and N-fixing bacteria screening according as described in Forster (1995). All the analysis was performed within 24–48 h of the sample collection.

Chemical analysis

Measured analytical parameters included: dissolved oxygen (WTW, Multiline F/Set 3), total organic carbon content (Nelson and Sommers, 1996), and ammonia content (Mulvaney, 1996). In addition the water content was measured in soil samples previous air-dried.

N-fixing bacteria screening

The screening of heterotrophic N-fixing bacteria was evaluated in each sample of water, sediment, and riparian soil by the ability to grow in agar plates containing solid nitrogen-free (NFB) medium (Döbereiner, 1995), using the pour plate technique. The cultures were incubated in dark at 28 °C for five days and the abundance of N-fixing bacteria were expressed as $\log_{10}$ of N-fixing bacteria per g of soil/sediment or mL of water ($\log g^{-1}$ or mL^{-1}).

Activity of N-fixing bacteria

BNF was measured by Nase enzyme activity using the acetylene reduction method (Alef, 1995). Briefly, four grams of freshly collected soil or sediment (five replicates per site) or 10 mL of water (five replicates per site) were sealed in 15 mL glass bottles. One mL of acetylene, freshly prepared from CaC_2, was added to each bottle. The bottles were vigorously shaken and then incubated in the dark at 28–30 °C. After 24 h an aliquot of 1 mL was taken to analyze the generated ethylene (C_2H_4) by gas chromatograph, using Shimadzu GC-8A with a flame detector and a Porapak column. The Nase enzyme activity was expressed as μg of C_2H_4 per kg of soil or sediment, or L of water (μg kg^{-1} or μg L^{-1}).

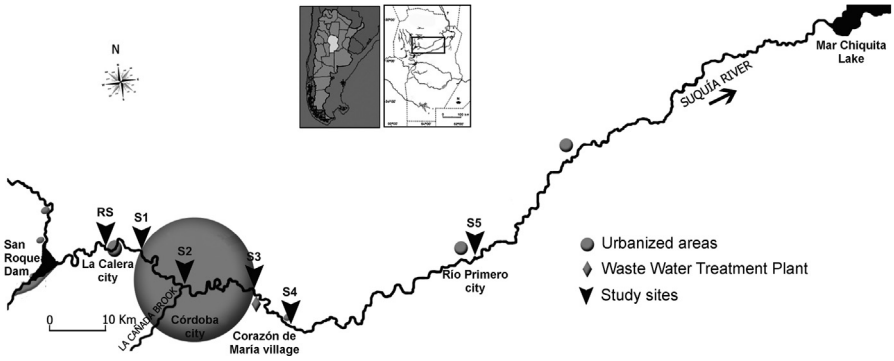

Fig. 1. Map of the Suquía River basin (Province of Córdoba, Argentina), showing the sampling sites.

PCR amplification of 16S rRNA gene and sequencing

To identify the heterotrophic N-fixing taxa and minimize non-diazotrophs growth, colonies of N-fixing bacteria differing in size, color, and shape were isolated into NFB medium repeatedly streaked on plates, and reisolated to obtain pure cultures. These bacteria were maintained into screw cap tubes containing 10 mL of solid NFB medium. Then, nine pure cultures from water sites with different Nase enzyme activity (RS, S1, and S2) were randomly selected and cultivated in liquid NFB media. They were represented with similar distribution and abundance in the five bacterial cultures performed from a same site. DNA of N-fixing bacteria in pure cultures was extracted according to Sambrook et al. (1989). DNA concentrations were determined using the Quant-iT^{TM} Broad-Range DNA Assay Kit (Invitrogen) according to manufacturer's protocol, and sample aliquots were standardized to contain 5–10 ng μL^{-1} in Tris–EDTA buffer.

Amplification of 16S rRNA gene from 10 ng of DNA was performed using 27f (GAG TTT GAT CCT GGC TCA) and 1492r (TAC GGY TAC CTT GTT ACG ACT T) primers (Lane, 1991). The PCR amplification was done in a total volume of 50 μL with 5 μL 10× PCR buffer (500 mM KCl, 20 mM $MgCl_2$, 200 mM Tris–HCl [pH 8.4]), 180 μM concentration of each deoxyribonucleoside triphosphate, 1 U of *Taq* polymerase (Invitrogen), and 0.4 μM of each primer. The following PCR conditions were used: a denaturation step of 5 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 3 min (Lu et al., 2006). The PCR products were visualized by electrophoresis in a 2% (w/v) agarose gel in 1.0× TAE buffer (40 mM Tris–acetate, 1 mM EDTA) stained with SYBR Safe (Life Technologies) and sequenced on both strands by Macrogen Inc. (Korea). DNA sequences were compared with sequences in the GenBank database. Nucleotide sequences were aligned using CLUSTALX 1.64b (Higgins et al., 1994). To construct phylogenic tree, based on nucleotide alignments, gene sequence distances were inferred by the neighbor-joining method. For each calculation, 1000 bootstrap resamplings were performed. Phylogenetic tree was constructed using MEGA 4.0 (Tamura et al., 2007).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained from each pure culture have been deposited in the GenBank nucleotide sequence database under the following accession numbers: KF939595–KF939603.

Statistical analysis

Differences of N-fixing bacteria abundance and activity among sites, water flow periods, and habitats (water, sediment, and riparian soil) were analyzed using three-factor ANOVA (site, habitat, and period). Means were compared using the least significant difference test (LSD) ($p \leq 0.05$). Besides, Pearson correlations were performed. Shapiro-Wilks test to evaluate deviation to normal distribution and Levene's test to corroborate homogeneity of variances were used. Non-parametric Spearman's correlation was used to analyze parameters which significantly differed from normal distribution. The data were then compared using non-parametric Kruskal–Wallis test. All analysis was performed using Infostat software (2001).

Table 1
Position, anthropogenic impact, and pollution information of Suquía River study sites.

River sites	Position	Anthropogenic impact	Pollution information
RS: Reference Site (La Calera city)	18.4 km downstream San Roque Dam, 488 m a.s.l., 31°21′45″ S and 64°20′99″ W	Eutrophic waters from the San Roque Dam and recreational use	*Escherichia coli* (log mL^{-1}) = 0 Fecal coliforms (log mL^{-1}) = 1.90 Conductivity (μS cm^{-1}) = 181.40
S1: Site 1 (Córdoba city west border)	17.1 km downstream RS, 417 m a.s.l., 31°23′07″ S and 64°14′15″ W	Sewage waters	*Escherichia coli* (log mL^{-1}) = 0 Fecal coliforms (log mL^{-1}) = 1.51 Conductivity (μS cm^{-1}) = 432.37
S2: Site 2 (Córdoba city down town)	12.1 km downstream S1, 393 m a.s.l., 31°23′82″ S and 64°14′62″ W	Industrial effluents, sewage waters and run-off from La Cañada brook	*Escherichia coli* (log mL^{-1}) = 0.23 Fecal coliforms (log mL^{-1}) = 2.69 Conductivity (μS cm^{-1}) = 972.30
S3: Site 3 (Córdoba city east border)	11 km downstream S2, 0.36 km upstream WWTP, 365 m a.s.l., 31°24′34″ S and 64°10′66″ W	Sewage waters, sand mining and trash in river banks	*Escherichia coli* (log mL^{-1}) = 0.30 Fecal coliforms (log mL^{-1}) = 2.49 Conductivity (μS cm^{-1}) = 929.00
S4: Site 4 (Corazón de María village)	12 km downstream WWTP, 341 m a.s.l., 31°26′81″ S and 63°59′45″ W	Sewage waters from waste water treatment plant (WWTP)	*Escherichia coli* (log mL^{-1}) = 0.62 Fecal coliforms (log mL^{-1}) = 2.88 Conductivity (μS cm^{-1}) = 952.30
S5: Site 5 (Río Primero city)	51.1 km downstream S4, 243 m a.s.l., 31°20′29″ S and 63°36′58″ W	Agricultural area and recreational use	*Escherichia coli* (log mL^{-1}) = 0.10 Fecal coliforms (log mL^{-1}) = 2.38 Conductivity (μS cm^{-1}) = 785.00

Table 2
Water chemical characteristics of Suquía River study sites during low and high flow periods. Values represent mean ± SD. *Significant differences between periods at each sampling site. Mean values with a common letters are not significantly different among sampling sites within the same period ($p > 0.05$). RS, reference site; S1, Site 1; S2, Site 2; S3, Site 3; S4, Site 4; S5, Site 5.

	RS	S1	S2	S3	S4	S5
Low flow						
Organic C ($mg\,L^{-1}$)	3.28 c* (±0.19)	5.58 bc (±0.99)	12.80 ab (±4.75)	10.97 ab (±5.96)	17.19 a (±4.67)	11.97 ab* (±5.88)
Ammonia ($mg\,L^{-1}$)	0.19 bc* (±0.18)	0.10 c (±0.04)	0.72 ab (±0.90)	0.23 bc (±0.07)	15.32 a* (±0.37)	1.86 ab (±3.77)
Dissolved O_2 ($mg\,L^{-1}$)	10.01 bc* (±0.08)	10.43 bc* (±0.39)	13.29 ab* (±0.54)	16.21 a* (±0.38)	3.18 d* (±0.36)	4.36 cd* (±0.89)
High flow						
Organic C ($mg\,L^{-1}$)	5.41 c* (±1.66)	12.70 ab (±15.22)	12.03 ab (±0.76)	10.05 bc (±1.06)	12.42 ab (±2.49)	27.49 a* (±8.92)
Ammonia ($mg\,L^{-1}$)	0.49 a* (±0.05)	0.12 c (±0.03)	0.40 ab (±0.10)	0.19 bc (±0.04)	1.18 a* (±0.63)	0.19 bc (±0.06)
Dissolved O_2 ($mg\,L^{-1}$)	6.87 cd* (±0.03)	7.26 bc* (±0.09)	8.28 a* (±0.47)	7.60 ab* (±0.15)	4.96 d* (±0.24)	7.11 bcd* (±1.08)

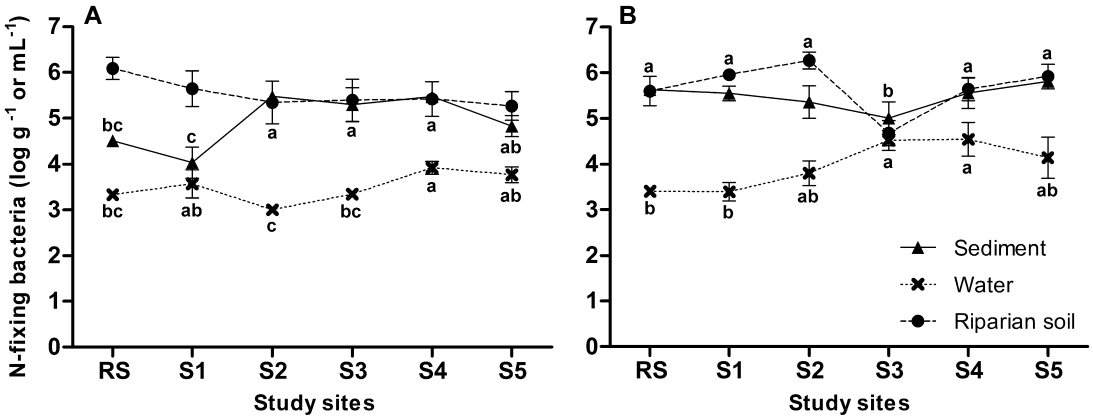

Fig. 2. Abundance of heterotrophic N-fixing bacteria ($log\,g^{-1}$ or mL^{-1}) in sediment, water, and riparian soil at Suquía River study sites in low (A) and high (B) flow periods. Bars indicate standard error. Sampling sites with common letters are not significantly different within the same period and habitat ($p > 0.05$).

Results

Chemical characteristics of water, sediment, and riparian soil

All analyzed parameters in Suquía River water varied significantly among study sites (Table 2). The lower value of organic C and ammonia was at RS and S1, respectively, compared to the other sites during high and low river flows. In contrast, the highest organic C levels were detected at S4 during low flow period and at S5 during high flow period. Besides, the lowest concentrations of dissolved O_2 were detected at S4 during both flow periods (Table 2). Chemical characteristics of sediment also showed variations among sites (Table 3). Organic C values were higher at S4 during low flow period, whereas in high flow period the higher values were determined at RS and S1 sites. On the other hand, ammonia content was higher at S4 during low and high flow periods (Table 3). Finally, riparian soil samples from RS, S1, and S2 sites presented the higher organic C levels in both low and high flows, while the lower ammonia content was observed at S3 during high flow period. Conversely, the water content of soil samples did not show significant differences among study sites at both low and high flow periods (Table 4).

Abundance and activity of N-fixing bacteria

The N-fixing bacteria were screened in solid nitrogen-free medium. In general, their abundance in soil and sediment was higher than the values found in the water during low and high flow periods (Fig. 2). Regarding the N-fixing bacteria quantified in water during low flow period, they were more abundant at S4 than RS, S2, and S3; also they had higher density at S1 and S5 than S2. In sediments its density increased downstream of S2 site, while no variations were detected in riparian soil among study sites (Fig. 2A). In high flow period the higher values of N-fixing bacteria in water were detected downstream of S2 site, while in riparian soil they presented lower abundance at S3 than in the other sites. The abundance of N-fixing bacteria did not show significant differences among sites in sediments (Fig. 2B). Moreover, comparing the abundance of N-fixing bacteria during high flow period respect to those observed during low flow period the most significant increases were detected in water at S2 and S3, and in sediments at RS, S1 and S5 (Fig. 3).

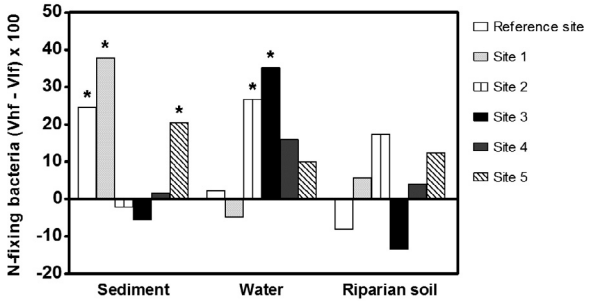

Fig. 3. Abundance of N-fixing bacteria in sediment, water, and riparian soil during high flow period (Vhf) minus the values found during low flow period (Vlf) at each Suquía River study sites expressed as percentage. (*) Significant increases during high flow period for each study site ($p < 0.05$).

Table 3
Sediment chemical characteristics of Suquía River study sites during low and high flow periods. Values represent mean ± SD. *Significant differences between periods at each sampling site. Mean values with a common letters are not significantly different among sampling sites within the same period ($p > 0.05$). RS, reference site; S1, Site 1; S2, Site 2; S3, Site 3; S4, Site 4; S5, Site 5.

	RS	S1	S2	S3	S4	S5
Low flow						
Ammonia (mg kg^{-1})	9.07 b (±4.87)	16.77 b (±16.93)	6.21 b (±3.42)	9.29 b (±6.87)	175.14 a (±187.19)	41.26 b (±70.88)
Organic C (g kg^{-1})	1.94 bc* (±1.71)	0.44 c* (±0.43)	19.08 ab (±23.65)	10.69 abc (±15.23)	25.67 a* (±22.75)	2.33 bc (±3.69)
High flow						
Ammonia (mg kg^{-1})	13.82 ab (±6.33)	11.18 abc (±7.39)	3.00 c (±0.56)	10.64 bc (±13.34)	30.25 a (±22.37)	15.99 ab (±12.91)
Organic C (g kg^{-1})	9.80 a* (±7.29)	10.65 a* (±9.41)	0.72 c (±0.20)	4.10 bc (±8.95)	2.01 bc* (±2.79)	8.71 ab (±8.03)

Table 4
Riparian soil chemical characteristics of Suquía River study sites during low and high flow periods. Values represent mean ± SD. *Significant differences between periods at each sampling site. Mean values with a common letters are not significantly different among sampling sites within the same period ($p > 0.05$). RS, reference site; S1, Site 1; S2, Site 2; S3, Site 3; S4, Site 4; S5, Site 5.

	RS	S1	S2	S3	S4	S5
Low flow						
Organic C (g kg^{-1})	15.35 ab (±6.52)	16.31 ab (±11.09)	24.31 a (±14.24)	0.98 c (±0.54)	2.14 c (±1.65)	10.64 bc (±9.78)
Ammonia (mg kg^{-1})	11.26 (±2.96)	7.70 (±3.36)	8.60* (±6.29)	7.89 (±3.05)	12.76 (±4.41)	7.49 (±3.72)
Water content (%)	33.98 (±11.24)	27.36 (±15.02)	36.16 (±20.01)	27.46 (±2.79)	11.42 (±11.52)	29.62 (±12.16)
High flow						
Organic C (g kg^{-1})	27.17 a (±9.44)	21.92 a (±5.84)	25.17 a (±2.22)	3.12 b (±4.49)	6.52 b (±5.83)	10.85 b (±5.73)
Ammonia (mg kg^{-1})	15.16 a (±8.50)	11.61 ab (±3.45)	17.39 a* (±4.46)	5.22 b (±1.53)	11.79 ab (±6.66)	10.75 ab (±3.66)
Water content (%)	33.95 (±5.32)	23.11 (±8.63)	34.40 (±1.75)	24.72 (±4.03)	22.67 (±10.80)	27.22 (±11.30)

BNF in water, sediment, and riparian soil samples was investigated by Nase enzyme activity measurement, which varied significantly among sites in water and sediment during low flow period, showing the highest activity at RS in water and at S4 in sediment (Table 5). During this flow period only S4 showed significant variation in enzyme activity among the studied habitats, with the highest value in sediment. No significant differences were detected for Nase enzyme activity among sites during high flow period; while in the same flow period, differences among habitats were observed at S5 with higher enzyme activity in the sediments (Table 5). Moreover, comparing the Nase enzyme activity during low flow period respect to those values during high flow period, higher activities were observed at RS and S1 in water samples and at S4 in sediment samples at low flow period (Table 5). No correlation was observed between N-fixing bacteria abundance and Nase enzyme activity in water, sediment, or riparian soil. However, the same analysis performed with the water values discriminated by site and period, revealed a significant correlation in RS samples obtained during low flow period ($r = 0.92$; $p = 0.001$).

Taxonomic identification of the isolated bacteria

To evaluate the phylogenetic diversity of the heterotrophic N-fixing bacteria isolates, the 16S rRNA was amplified by PCR. Approximately 1400 bp of the amplified 16S rRNA gene of nine heterotrophic N-fixing bacteria isolated from three different water

Table 5
Nitrogenase enzyme activity in water (μg C_2H_4 L^{-1}), sediment, and riparian soil (μg C_2H_4 kg^{-1}) from Suquía River study sites in low and high flow periods. Values represent mean ± SD. For each parameter, (*) indicates significant differences between periods at each sampling site and habitat. Mean values with a common lowercase or capital letters are not significantly different among sampling sites or habitat, respectively, within the same period ($p > 0.05$). RS, reference site; S1, Site 1; S2, Site 2; S3, Site 3; S4, Site 4; S5, Site 5.

	RS	S1	S2	S3	S4	S5
Low flow			μg C_2H_4 L^{-1} or kg^{-1}			
Water	8.18 a* (±5.30)	2.80 b* (±1.43)	2.04 b (±0.70)	2.92 b (±1.68)	3.27 b B (±2.09)	2.18 b (±0.20)
Sediment	13.16 b (±13.21)	35.83 b (±74.01)	12.51 b (±11.13)	2.96 b (±2.58)	87.12 a A* (±37.13)	17.66 b (±18.44)
Riparian soil	69.60 (±140.0)	5.43 (±3.28)	89.80 (±180.5)	6.48 (±6.13)	28.83 B (±32.38)	124.71 (±261.77)
High flow						
Water	0.93 (±0.50)	1.02 (±0.27)	4.80 (±5.64)	7.40 (±7.69)	1.92 (±1.02)	2.07 B (±0.77)
Sediment	6.12 (±6.65)	17.55 (±20.33)	5.44 (±6.54)	8.77 (±15.19)	8.41 (±6.85)	25.39 A (±21.30)
Riparian soil	7.37 (±9.53)	2.11 (±2.24)	15.86 (±14.21)	10.14 (±13.62)	13.76 (±15.27)	7.27 B (±5.61)

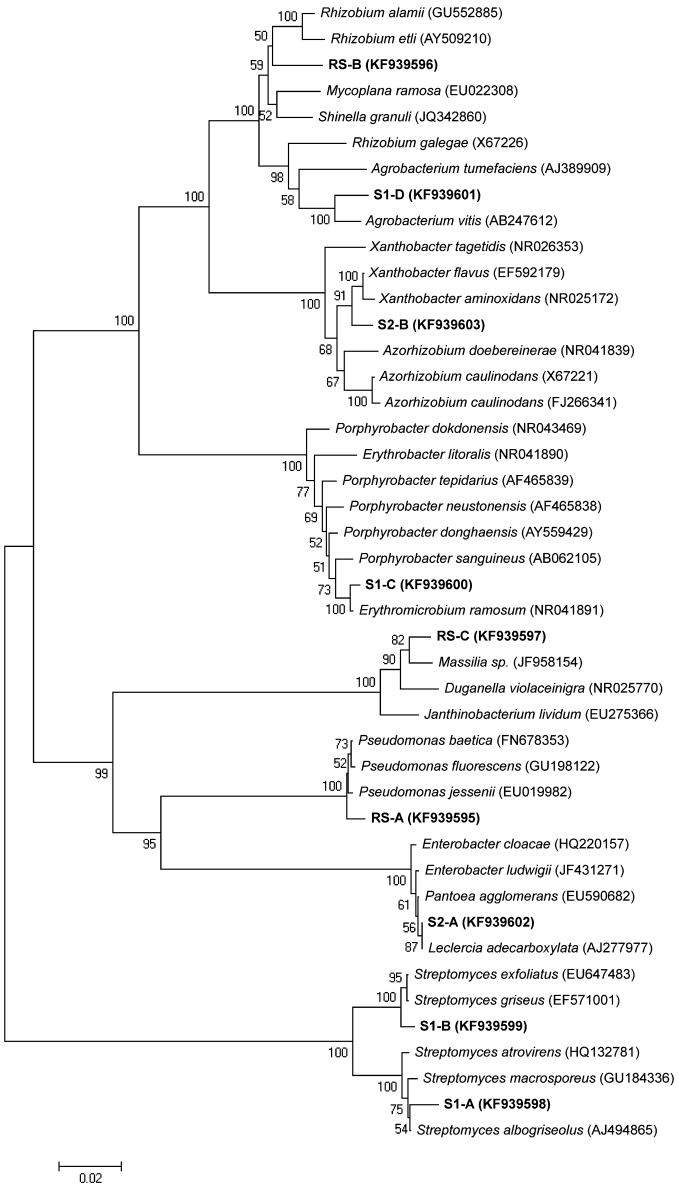

Fig. 4. Phylogenetic tree for 16S rRNA gene sequences obtained from strains isolated of Suquía River water. The 16S r RNA tree was based on DNA sequences of approximately 1400 bp. RS-A, -B, and -C: strains isolated from reference site; S1-A, -B, -C, and -D: strains isolated from site 1; S2-A and -B: strains isolated from site 2.

sites with different Nase enzyme activity (RS, S1, and S2) were sequenced. Three strains were isolated from RS (RS-A, -B, and -C), four from S1 (S1-A, -B, -C, and -D) and two from S2 (S2-A and -B). The selection was carried out on the basis that they had similar distribution and abundance in the five bacterial cultures performed from a same site. The data indicates that 16S rRNA gene sequences were represented by *Proteobacteria* and *Actinobacteria* phyla. Four sequences (RS-B, S1-C, S1-D and S2-B) were affiliated with *Alphaproteobacteria*. One sequence (RS-C) was associated with *Betaproteobacteria*. Two sequences (RS-A and S2-A) were aligned with *Gammaproteobacteria* class, and two sequences (S1-A and S1-B) were related to *Actinobacteria* (Fig. 4).

Environmental factors associated with BNF

The abundance of N-fixing bacteria showed significant correlations with organic C content in sediments ($r = 0.63$; $p < 0.0001$), and with organic C ($r = 0.45$; $p < 0.0001$) and water contents ($r = 0.32$; $p < 0.003$) in riparian soil. In the water samples, N-fixing bacteria abundance showed negative correlation with dissolved O_2 ($r = -0.43$; $p < 0.0006$). On the other hand, there were not significant correlations between Nase enzyme activity and the chemical characteristics of water and riparian soil. Conversely, in sediments, significant correlations between Nase enzyme activity and the ammonia ($r = 0.56$; $p < 0.0001$) and organic C ($r = 0.36$; $p < 0.01$) contents were detected.

Discussion

Abundance and activity of N-fixing bacteria

The presence of heterotrophic N-fixing bacteria in all Suquía River sites corroborates the importance given to heterotrophic fixation in aquatic ecosystems (Affourtit et al., 2001; Moisander et al., 2007; Hashimoto et al., 2012). Our findings indicate that, N-fixing bacteria abundance in water column was lower than in soil and sediment concordantly with the lowest organic compound levels. These observations were comparable to those data found in other aquatic ecosystems (Wetzel, 2001). Likewise, N-fixing bacteria abundance in riparian soil of Suquía River was similar to the density measured by Mantilla-Paredes et al. (2009) in flooded riparian soil of Amazonas River in Colombia (2.5×10^6 bacteria g^{-1} vs. 2.8×10^6 bacteria g^{-1}).

The N-fixing bacteria activity has been poorly studied in aquatic environments and most part of information belongs to marine and lacustrine environments (Burns et al., 2002; Steppe and Paerl, 2005; Farnelid and Riemann, 2008; Gamble et al., 2010). The values of Nase enzyme activity of Suquía River sediments were lower than those reported by Burns et al. (2002), who evaluated Nase enzyme activity in sediments of Chesapeake Bay of USA (20.07 $\mu g\, C_2H_4\, kg^{-1}$ vs. 520 $\mu g\, C_2H_4\, kg^{-1}$). These differences could be due to the lesser O_2 concentrations and the higher organic matter content detected in Chesapeake Bay sediments. Conversely, the values of Nase enzyme activity in Suquía River sediments were higher than those found in the rhizosphere sediments of a relatively pristine marsh of USA (10.36 ng $C_2H_4\, m^{-2}$) (Gamble et al., 2010). Comparing the Nase enzyme activity observed in riparian soil of Suquía River with the values detected by Mårtensson et al. (2009) in soil of rice crops, these were higher than our data (3640 $\mu g\, C_2H_4\, kg^{-1}$ vs. 31.78 $\mu g\, C_2H_4\, kg^{-1}$), whereas, Zhang et al. (2007) found that only a small fraction of diazotrophs present in rhizosphere soil of a river in Namibia actively fix N.

N-fixation in Suquía River water is probably carried out not only by phototrophs but also by heterotrophic bacteria that have different requirement to fix N. This is consistent with the presence of heterotrophic N-fixing bacteria in all the studied sites. Affourtit et al. (2001) determined that a diazotroph community of Neuse River of USA was mainly composed by heterotrophs, while in an estuarine environment, even though an increase in the cyanobacteria was observed, the heterotrophs proportion continue being significant. The lack of correlation between N-fixing bacteria abundance and Nase enzyme activity in Suquía River water could be due to the fact that N-fixation is an alternative metabolism that only takes place under certain conditions. In this regard, Steppe and Paerl (2005) did not find a relationship between N-fixing bacteria abundance and Nase enzyme activity in a marine intertidal microbial mat concluding that the higher Nase enzyme activity rates were produced during the night or in low photosynthetic periods. However, it has been informed that cyanobacteria with heterocystous are the more common phototrophs present in freshwater environments (Karl et al., 2002). Nevertheless, our results showed a strong correlation between heterotrophic N-fixing bacteria abundance and Nase enzyme activity in water at RS in low flow

period. It is well known that in lotic ecosystems of fast-flowing waters with canopy shade, like RS, heterotrophy predominates over autotrophy (Wetzel, 2001). Thus, it is probably that Nase enzyme activity in RS site was associated with heterotrophic and photoheterotrophic N-fixing bacteria (Sarma and Khattar, 1994; Feng et al., 2010; Ritchie and Johnson, 2012), which have adaptations to protect Nase enzyme from O_2 (Karl et al., 2002; Paul, 2007; Farnelid and Riemann, 2008). Moreover, the fact that Nase enzyme activity detected in Suquía River water during low flow period was higher than those values found in high flow conditions suggests that it could be associated with the lower temperatures registered in winter which favors the N_2 dissolution (Wetzel, 2001).

To our knowledge there is no previous data that estimate the global input of N by BNF in lotic ecosystems. If we assume a relation of 3:1 for C_2H_4:N_2 fixated (the production of 3 mol of C_2H_4 is equivalent to 1 mol of fixing N_2) (Werner, 1995), we can estimate that in Suquía River the N input by BNF per year is 61 ppm N (4 mg L^{-1} in water, 22 mg kg^{-1} in sediments, and 35 mg kg^{-1} in riparian soil).

The N-fixing community in lotic ecosystems has a key role in the cycle of N (Karl et al., 2002; Kavadia et al., 2007; Farnelid and Riemann, 2008). Although it is postulated that BNF is an unimportant process in eutrophic environments, Larsson et al. (2001) highlight the BNF contribution to the annual N input in eutrophic estuarine of Baltic Sea. Other studies also emphasize the importance of BNF in sea and terrestrial ecosystems (Karl et al., 2002; Vitousek et al., 2002; Abril et al., 2008). For example, Karl et al. (2002) informed that the global ocean BNF is about 100–200 Tg N $year^{-1}$, and Burns et al. (2002) estimated a BNF rate of 0.4 g N m^{-2} $year^{-1}$ in Chesapeake Bay sediments. Although it is difficult to compare these data with the value of global N input $year^{-1}$ mentioned above for Suquía River, due mainly to the different units used; we can conclude that Suquía River BNF is as important as those of the ocean or Chesapeake Bay.

Taxonomic identification of the isolated bacteria

Although the taxonomy of N-fixing bacteria have been studied in terrestrial ecosystems (Xie et al., 2003; Mårtensson et al., 2009; Xu et al., 2012), little information is currently available about freshwater aquatic environments, particularly in lotic ecosystems. Previous studies have considered that BNF in aquatic ecosystems is carried out mostly by cyanobacteria with a scarce participation of heterotrophic N-fixing bacteria. However, recent molecular studies showed that heterotrophic N-fixing bacteria are very diverse and widely distributed (Farnelid and Riemann, 2008), being in some aquatic ecosystems more diverse and abundant than cyanobacteria (Affourtit et al., 2001; Moisander et al., 2007; Hashimoto et al., 2012).

In the present study, all bacterial isolates contained 16S rRNA genes with more than 98% similarity to those 16S rRNA sequences registered in GenBank, with the exception of RS-B strain. Since RS-B strain sequence presented a similarity index of 97% with three different genera, deeper studies are needed to define if this strain belongs to one of these three genera or it is a novel genus (Peeters et al., 2011). Our results show that Suquía River has a great diversity of taxonomic groups, including *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* classes and *Actinobacteria* from *Actinomycetal* order. Although the N-fixing capacity of the bacteria identified in this study was not determined, Affourtit et al. (2001) found a great proportion of diazotrophs belonged to *Alphaproteobacteria* and *Gammaproteobacteria* in Neuse River water (USA). Besides, Zhang et al. (2008) identified a great proportion of diazotrophs belonging to *Alphaproteobacteria, Betaproteobacteria*, and *Gammaproteobacteria* from sediments of mangrove ecosystem sites in Sanya, Hainan Island, China. In contrast, *Alphaproteobacteria* have been poorly represented in the N-fixing sequences of bacteria associated with *Spartina alterniflora* of rhizosphere sediments in a marsh system (Gamble et al., 2010). However, since we did not measure the N-fixing ability in these isolates and the growth of non N-fixing bacteria in semi solid N free media has been previously reported (Beneduzi et al., 2013) it is not possible to rule out that some of them could represent non-diazotrophic bacteria.

Alphaproteobacteria was the major class represented in this study being three of them associated with *Rhizobiales* order and one with *Sphingomonadales* order. N fixation by symbiotic rhizobia occurs only when they are in symbiosis (Paul, 2007). However, some authors have described the N fixation capacity of some free living *Azorhizobium* strains (Dreyfus et al., 1983; Rashedul et al., 2009). The *Sphingomonadales* includes *Erythrobacter*, *Erythromicrobium* and *Porphyrobacter* genera and aggregated heterotrophic bacteria that synthesize bacteriochlorophyll a under aerobic conditions. Although the N fixation capacity of this group has not been demonstrated, Zehr et al. (2006) mentioned that probably some members of *Erythrobacter* are diazotrophs.

One of the isolated strains was associated with the *Betaproteobacteria* class including strains isolated from rhizosphere soil using N-fixing bacteria specific culture media (Hara et al., 2009; Xu et al., 2012). However, the N fixation capacity of these genera is scarcely studied.

Gammaproteobacteria class was represented by *Pseudomonadales* and *Enterobacteriales* orders. The *Pseudomonas* includes species capable to fix N (Jenni et al., 1989; Xie et al., 2003) present in aquatic environments like Sanya River sediments (Zhang et al., 2008), eutrophic lake water of Switzerland (Jenni et al., 1989), seawater of Japan (Hashimoto et al., 2012) and marsh rhizosphere sediments of United States (Gamble et al., 2010). Additionally, the N-fixation capacity of *Enterobacter* has been widely documented (Paul, 2007; Videira et al., 2012) including pathogenic and no pathogenic bacteria and many enterobacteria living in the intestine of warm-blooded animals (De Baere et al., 2001). Therefore, it is likely that S2-A strain could come from sewage source since it is well known the Suquía River sewage pollution (Merlo et al., 2011).

Finally, two isolated strains (S1-A and S1-B) were represented by *Actinomycetales* group. Even though little is known about the actinomycetes contribution to N fixation, Merrick (2004) reported diazotrophic bacteria from *Streptomyces* genus.

In summary, N-fixing bacteria are a diverse group, and since the N fixation genes are not distributed consistently among phylogenetic groups, genera including N-fixing bacteria and no fixing bacteria could be found in close taxonomic units (Affourtit et al., 2001).

Environmental factors associated with BNF

The N fixation capacity and the associated environmental factors have been widely investigated in microorganisms with agronomic importance. Nevertheless, little information is available about N-fixing community in aquatic ecosystems. The significant correlations between N-fixing bacteria abundance and chemical parameters found in this study are in agreement with the conditions needed to fix N, with the availability of organic compounds, and the low O_2 concentrations. While, the lack of correlation with ammonia content could be due to N-fixing bacteria use ammonia only when it is available due to the lower energetic cost of this process (Paul, 2007).

Even though the higher Nase enzyme activity in water was detected in the most oligotrophic site (RS) during low flow period (low ammonia and organic C content with high O_2 concentration), the lack of significant correlation between Nase enzyme activity with the chemical and physical variables indicates that the activity of N-fixing bacteria in water do not depend on the C source and

dissolved O_2 concentrations. It is widely accepted that O_2 inhibits the synthesis and Nase enzyme activity (Fay, 1992; Steppe and Paerl, 2005). In this sense, it has been documented that filamentous cyanobacteria have specialized cells (heterocystous) that protect Nase enzyme from O_2, while unicellular cyanobacteria temporarily separate the processes of fixation and photosynthesis (Fay, 1992; Wetzel, 2001). Therefore, these data indicates that the regulation of N fixation is a complex process that requires the convergence of several factors. For instance, the major abundance of N-fixing bacteria in water at S4 was linked to low O_2 concentration, which would favor Nase enzyme activity. Nevertheless, the high ammonia concentration produced by the pollution (Merlo et al., 2011) could be the cause of the low Nase enzyme activity. This is in agreement with some studies that detected a decrease in Nase enzyme activity due to the increase of N availability (ammonia and nitrate) in water (Forbes et al., 2008; Scott et al., 2008). Our results are in agreement with the data reported by Paerl et al. (1981) in lakes. These authors did not find a strong relationship between the ammonia content and N fixation activity. Moreover, nitrate should not act as a repressor of Nase enzyme, since the reduction of nitrate is energetically more expensive than BNF (Karl et al., 2002).

The fact that in sediments Nase enzyme activity was major in sites with higher organic C content and low dissolved O_2 concentration in water (for example S4 receives the sewage discharges from WWTP) is consistent with the established requirement (low O_2 concentration and available organic C compounds to produce the necessary ATP for Nase enzyme activity by heterotrophic N-fixing bacteria) (Wetzel, 2001; Kavadia et al., 2007; Farnelid and Riemann, 2008). Even though the ammonia content at S4 was high which does not favor N fixation activity, the high abundance of other microorganisms in the sediments of this site (Merlo et al., 2011) suggests that a great competition for this resource could occur (Farnelid and Riemann, 2008). In this sense, Kavadia et al. (2007) demonstrated that N-fixing populations could be affected by their competitors by two opposite ways: negatively due to the competition for common resources or positively due to the ammonia consumption by competitors. Hence, when there is high organic C concentration, the competition for it is low, while the high concentration of ammonia is consumed by heterotrophic microorganisms (Kavadia et al., 2007). This is in agreement with the high abundance of heterotrophic microorganisms detected in sediments at S4 by Merlo et al. (2011) and the higher Nase enzyme activity detected during low flow period (this study). In contrast, the low Nase enzyme activity detected at the same site during high flow period could be probably due to the increase of dissolved O_2 and the decrease of organic C by water drag (Merlo et al., 2011), which do not favor N fixation (Wetzel, 2001).

The lack of significant differences of Nase enzyme activity (among study sites and sampling periods) and significant correlations between Nase enzyme activity with the physical and chemical parameters of riparian soil could be due to hot spots of microbial activity occurring in soil. In hot spots, bacteria find optimal conditions for specific metabolic activity (McClain et al., 2003). Therefore, although the diazotrophic bacteria were detected in riparian soil, BNF could occur only when conditions are optimal, for example inside of aggregates with high quantity of organic matter and scarce O_2 concentration (Farnelid and Riemann, 2008) or in the rhizosphere that provides available organic C source and microaerophilic conditions (Černá et al., 2009). Furthermore, the availability of N to organisms could be greatly influenced by soil heterogeneity and may break the idealized dynamic of BNF.

Ecosystems are typically not homogeneous but rather complex systems, in which individuals are adapted to and interact with their local environments. The conditions that favor the growth and activity of diazotrophs greatly vary among them, since one environmental condition could select and favor the activity of a microorganism against another. For example, some heterotrophic diazotrophs are obligated anaerobes, while other diazotrophs could fix N in microaerophilic conditions or have adaptations to fix N in presence of O_2. Moreover, BNF is an alternative metabolism that occurs only when certain conditions converge (Wetzel, 2001; Vitousek et al., 2002; Paul, 2007).

In conclusion, this study demonstrates that BNF occurs in all the sites of the different analyzed habitats of Suquía River during high and low flow periods, being in sediments influenced mainly by the higher organic C present in the most polluted sites, particularly during low flow period when the pollutants are highly concentrated (Merlo et al., 2011). In addition, the low dissolved O_2 concentration and the competition with other microorganisms were additional environmental factors that modulate BNF in sediments. In riparian soil the organic C and water contents were the major abiotic factors that control the abundance of N fixing bacteria; whereas in Suquía River water the density of N fixing bacteria were associated with low dissolved O_2 concentration.

Finally, the phylogenetic diversity identified in the N-fixing bacteria isolates of Suquía River related to *Proteobacteria* and *Actinobacteria* phyla deserves future studies to establish the structure of this community.

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