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**Distributions, abundances and activities of microbes associated
with the nitrogen cycle in riparian and stream sediments of a
river tributary**

Running Title: Riparian areas for microbial N cycle

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Abstract

River tributaries are ecologically important environments that function as sinks of inorganic nitrogen. To gain greater insight into the nitrogen cycle (N-cycle) in these environments, the distributions and activities of microbial populations involved in the N-cycle were studied in riparian and stream sediments of the Santa Fe River (SFR) tributaries located in northern Florida, USA. Riparian sediments were characterized by much higher organic matter content, and extracellular enzyme activities, including cellobiohydrolase, β -D-glucosidase, and phenol oxidase than stream sediments. Compared with stream sediments, riparian sediments exhibited significantly higher activities of nitrification, denitrification, dissimilatory nitrate reduction to ammonia (DNRA) and anaerobic ammonia oxidation; correspondingly, with higher copies of *amoA* (a biomarker for enumerating nitrifiers), *nirS* and *nirK* (for denitrifiers), and *nrfA* (for DNRA bacteria). Among N-cycle processes, denitrification showed the highest activities and the highest concentrations of the corresponding gene (*nirK* and *nirS*) copy numbers. In riparian sediments, substantial nitrification activities (6.3 mg-N·kg soil⁻¹·d⁻¹ average) and numbers of *amoA* copies (7.3×10⁷ copies·g soil⁻¹ average) were observed, and nitrification rates correlate with denitrification rates. The guild structures of denitrifiers and nitrifiers in riparian sediments differed significantly from those found in stream sediments, as revealed by analysis of *nirS* and archaeal *amoA* sequences. This study shows that riparian sediments serve as sinks for inorganic nitrogen loads from non-point sources of agricultural runoff, with nitrification and denitrification associated with elevated levels of carbon and nitrogen contents and extracellular enzyme activities.

Keywords: Nitrification, Denitrification, Riparian, N-cycle

49 **1. Introduction**

50 Streams and riparian wetlands serve as sinks of inorganic nitrogen (ION) loaded
51 through nitrogen-rich runoff from adjacent upland ecosystems (Holmes et al., 1996).
52 Removal of ION from those tributary systems typically relies on coupled nitrification and
53 denitrification reactions. Denitrification is the process of converting ION to the biologically
54 less reactive form N₂, which is removed from tributaries to the atmosphere (Jones and
55 Holmes, 1996); however, denitrifying microorganisms (denitrifiers) require nitrite or nitrate
56 as an electron acceptor, such that nitrification is a necessary prerequisite for denitrification.
57 Coupled denitrification and nitrification accelerates the ION removal rate in diverse
58 environments such as lake and river sediments (Seitzinger, 1994), natural marshes
59 (Thompson et al., 1995), and coastal sediments (Kaspar, 1983).

60 Even though nitrification and denitrification are functionally coupled, environmental
61 factors controlling the activities, diversities, and structures of nitrifying and denitrifying
62 prokaryotes differ in many terrestrial environments: nitrifiers are usually aerobic, autotrophic,
63 and sensitive to low pH (Ferguson et al., 2007); denitrifiers are facultative anaerobes and
64 heterotrophs, such that denitrification is controlled by low oxygen concentrations and
65 available carbon (Reddy and DeLaune, 2008). Fluctuating water tables in wetlands create an
66 alternating aerobic and anaerobic conditions (namely a redox-variable conditions), alternately
67 favoring nitrification and denitrification; thereby enhancing the nitrogen removal rate through
68 a tightly coupled denitrification and nitrification (Seitzinger, 1994; Pinay et al., 1995).
69 Analysis of microbial community structures is increasingly used as an information bank to
70 interpret the response of microbial populations of interest to environmental change; however,
71 little is known of the environmental controls on the structures and abundances of denitrifiers
72 and nitrifiers in variable redox systems such as shallow stream and riparian sediments.

In recent decades, extensive efforts have been made to investigate alternative nitrogen oxide-metabolizing pathways such as anaerobic ammonium oxidation (ANAMMOX) (Penton et al., 2006) and dissimilatory nitrate reduction to ammonium (DNRA) (An and Gardner, 2002). As these processes utilize common substrates (NO_2^- and/or NO_3^-) for denitrification, their activities could affect denitrification activities, dependent on environmental conditions. ANAMMOX was reported to contribute up to ~50% of N_2 production from marine ecosystems (Jetten et al., 2009), and up to ~30% in fresh water environments (Wenk et al., 2013). Many recent studies also showed that DNRA exceeded denitrification in a variety of environments, such as coastal ecosystems (Giblin et al., 2013) and a river estuary (Cao et al., 2016). Therefore, it is timely to re-examine the regulation of ION removal pathways from a comprehensive viewpoint.

This study aimed to evaluate the N-cycle with respect to the contributions of individual N-cycling populations to ION removal, their response to environmental factors, and interactions among populations in river tributaries. For this study, we determined the genetic structures and the potential activities of selected N-cycling populations, extracellular enzyme activities and geochemical parameters, and potential correlations between N-cycling populations and biogeochemical conditions from the riparian and aquatic stream in the Florida Santa Fe River (SFR) tributary selected for this study.

2. Materials and methods

2.1. Site description and sample process

The SFR of North Florida, USA, is approximately 121 km long and drains into the Suwannee River, which then empties into the Gulf of Mexico. The upper and mid regions of the SFR receive a number of tributaries draining land areas used for agricultural operations.

Two tributaries, designated T1 and T2 (Fig. S1), flow through or around the Boston Farm at the Santa Fe Ranch Beef Unit (BFSFEBU) of the University of Florida, which is home to approximately 300 heifers on 648 hectares, and the Holly Factory (HF), an ornamental plant nursery (Fig. S1). Nitrogen release from animal waste and fertilizer application is the primary input of nitrogen to these tributaries (Frisbee, 2007).

Triplicate cores (diameter 7.5 cm) were collected from the riparian and stream sediments at three sites, T1 (N 29°55'33", W 82°30'14"), T2U (at a T2-Upperstream: N 29°55'31", W 82°29'56") and T2D (at a T2-Downstream: N 29°55'17", W 82°30'01") along the tributaries T1 and T2, in different seasons (October 2007, and January, April, and July 2008). These sampling sites are surrounding the BFSFEBU and HF nursery; and as such represent the environments affected by the agricultural activities in different directions. Stream sediments were collected in mid points of streams, of which water depth was less than 50 cm, and riparian sediments were sampled near the stream (within 2 meters from the stream water body).

Surface sediments (0 to 3 cm) were characterized for physicochemical properties, incubations for determining extracellular enzyme activities (EEAs) and potential activities of N-cycling populations, and DNA extractions. Sediment samples were transferred from sampling sites on ice to the laboratory within one hour for processing. Triplicate soil cores (depth of 0 to 3 cm) collected from the riparian or the stream sediments within each site were composited to make enough sample volume for a series of experiments including, biogeochemical analysis and genetic studies. Approximately 10 g of sediments were stored at -80 °C until use for DNA isolation.

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2.2. *Soil physicochemical properties*

Inorganic N and extractable organic C (Ext-C) were extracted from sediment samples with 0.5 M K₂SO₄ (Bundy and Meisinger, 1994). The filtered extract was used for measuring NH₄⁺-N with a Seal AQ2 automated Discrete Analyzer (Mequon, WI, USA) according to the EPA Method 350.1 (EPA, 1993a). The NO₃⁻-N concentrations were analyzed with an Alpkem Rapid Flow Analyzer 300 Series (Clackamas, OR, USA) according to EPA Method 353.2 (EPA, 1993b). The K₂SO₄ extracts were digested using Kjeldahl block digestion for measuring extractable organic nitrogen (Ext-N), as described in EPA Method 351.2 (EPA, 1993c). Ext-C concentrations were measured with a Shimadzu TOC-5050A Total Organic C Analyzer equipped with an ASI-5000A auto sampler (Kyoto, Japan) according to the EPA Method 415.1 (EPA, 1974). Microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) in the sediments were determined by the differences between samples before and after chloroform fumigation-extraction (Brookes et al., 1985). Total nitrogen (TN) and total carbon (TC) concentrations were measured in sediments dried at 70°C for 3 days using a Thermo Electron Corp. Flash EA 1112 Series NC Soil Analyzer (Waltham, MA, USA; Nelson and Sommers, 1996).

2.3. *Potential microbial activities in N-cycle*

Potential nitrification rates (pNR) were determined as described by Berg and Rosswall (1985). Briefly, 10 g of wet sediments were amended with 1400 µg of NH₄⁺-N (as NH₄Cl) in 30 ml of deionized water, and incubated on a shaker at 24°C and 130 rpm for 7 days. After incubation, NO₃⁻-N concentration developed during the incubation was measured as described above.

Potential ANAMMOX rates (pNH₄oxR) were determined in soil incubation supplemented with ¹⁵N-NH₄⁺ and ¹⁴N-NO₂⁻ as described by Kuypers et al. (2003). Briefly, 10

g of wet sediments was incubated with 1500 μg of $^{15}\text{NH}_4\text{-N}$ in form of $^{15}\text{NH}_4\text{Cl}$ and 1400 μg of $^{14}\text{NO}_2\text{-N}$ in form of NaNO_2 under anaerobic conditions at 24°C . On 7th day incubation, $^{28}\text{N}_2$ and $^{29}\text{N}_2$ in the headspace were measured using a GC-IRMS (Thermo Finnigan MAT Delta Plus XL mass spectrometer, Waltham, MA, USA). The potential rates of ANAMMOX were calculated by the difference in the ratios of $^{28}\text{N}_2$ to $^{29}\text{N}_2$ between natural abundance and samples (Kuypers et al., 2003; Dalsgaard et al., 2005).

Potential denitrification rates (pDNR) in sediments were determined by the acetylene blocking method (Tiedje, 1982); 10 g of wet sediments was amended with 1400 μg of $\text{NO}_3\text{-N}$ (as KNO_3) and acetylene gas (12.5%). The N_2O produced in the headspace was measured at pre-determined intervals up to 4.5 h using a Shimadzu ECD GC 14-A (Kyoto, Japan).

Potential DNRA rates (pDNRAR) were measured as described by Stark and Hart (1996). Sediments (10 g) were incubated with 1500 μg of $^{15}\text{N-NO}_3\text{-N}$ (as K^{15}NO_3) under anaerobic condition at 24°C . On 7th day incubation, $^{15}\text{NH}_4\text{-N}$ in sediments was analyzed using a Thermo Finnigan MAT Delta Plus XL Mass Spectrophotometer equipped (Waltham, MA, USA) with a Costech Instrument Elemental Analyzer (Valencia, CA, USA) for flash combustion of solid material for N analysis.

2.4. Extracellular enzyme activities in organic matter decomposition

The activities of cellobiohydrolase (CBH; EC. 3.2.1.91) were spectrophotometrically determined as described by Linkins et al. (1990). Briefly, one gram (wet weight) of soil was incubated with 4-nitrophenyl- β -D-cellobioside (2 mM) as substrate and 9 ml distilled water for one day at 24°C . CBH activity was determined by measuring 4-nitrophenol at 410 nm during the incubation.

The β -D-glucosidase (Glu; EC. 3.2.1.21) activities were measured according to the protocol described by Hoppe (1993). Briefly, one gram of soil was incubated with 9 ml water

and 4-methylumbelliferyl (MUF)- β -D-glucoside (500 μ M) as substrate for 3 hours at 24 °C. The Glu activity was determined by measuring fluorescence from MUF in a Bio-Tek FL600 fluorometric plate reader (Winooski, USA).

Phenol oxidase (PhOx; EC. 1.10.3.21) activities were determined according to the method described by Pind et al. (1994). One gram of soil was incubated with L-dihydroxy phenylalanine (L-DOPA) as substrate and 9 ml water for 15 min at 24 °C. PhOx activity was determined by measuring 2-carboxy-2, 3-Dihydroindole-5,6-Quinone at 460 nm resulting from the enzymatic oxidation of L-DOPA.

2.5. Quantitative real-time PCR (qPCR)

DNA was isolated from 0.2 g (wet weight) of sediments using Power Soil DNA Isolation kit (MoBio, Carlsbad, CA, USA). All qPCRs were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR reaction mixtures contained 12.5 μ l of iQ SYBR green super-mix (Bio-Rad Laboratories, Hercules, CA, USA), 1 μ l of forward and 1 μ l of reverse primer (10 pmol· μ l⁻¹ each), and 2 μ l of DNA template in a final volume of 25 μ l.

Previously described primers and PCR conditions were used in this study for quantifying following genes using qPCR: bacteria 16S rRNA (Harms et al., 2003), *amoA* from ammonia oxidizing archaea (AOA) (Tournu et al., 2008) and from ammonia oxidizing bacteria (AOB) (Rotthauwe et al., 1997), *nirK* (Henry et al., 2004), *nirS* (Throbäck et al., 2004), *hzsA* (Harhangi et al., 2012), *nrfA* (Mohan et al., 2004), and *nifH* (Mehta et al., 2003). Primers used for this study are summarized in Table S1.

All qPCR runs included an image capture step (15 seconds at 80°C) after a final extension step of each cycle, and a melt curve analysis (increasing the temperature from 60 to 95°C in 0.5°C increments every 10 seconds) when the PCR amplification was completed.

All sample DNAs and standard DNAs were analyzed in triplicate. For every qPCR run, the standard plasmid DNA carrying the gene fragment of interest was included in the 96-well PCR plate. Standard DNA plasmids were prepared by cloning the target gene fragment amplified from sediment samples using the same primer used for qPCR. The insertion of the correct gene fragments in the standard plasmid DNA was confirmed by their sequences. A standard curve was constructed by plotting the relative fluorescent units at a threshold fluorescence value (C_T) versus the logarithm of copy number of the standard plasmid DNAs. The copy number in DNA solution was calculated by the following formula: molecules (gene copies) of DNA = (mass [in grams] $\times$ Avogadro's number)/(average molecular weight of base $\times$ template length) (Dorak, 2006), where the average weight of a base pair was assumed to be 650 Da. The PCR efficiency (E) was calculated from the slope of the standard curve by using the formula $E = 10^{-1/\text{slope}} - 1$ (Klein, 2002). All standard curves showed PCR efficiencies ranging from 85 to 98% (Table S1).

2.6. Sequence analysis for AOA *amoA* and *nirS*

AOA *amoA* was amplified from DNA using primers A19F/643R under PCR cycling conditions as described by Leininger et al. (2006). *nirS* was amplified using primers by *nirS* Cd3aF/*nirS* R3cd as described by Throbäck et al. (2004). The PCR products were ligated into pCRII-TOPO cloning vectors, and were transformed into chemically competent XL10-Gold® Ultracompetent Cells (Stratagene, CA, USA). Clones in randomly selected transformants using the selection LB plate supplemented with ampicillin (50 $\mu\text{g ml}^{-1}$) were submitted to the University of Florida Sequencing Core Laboratory (<http://www.biotech.ufl.edu/>) for sequencing the target insert fragments.

nirS and AOA *amoA* sequences obtained in this study were translated *in silico* into amino acid sequences for biomolecular analyses. For phylogenetic analysis, reference amino

acid sequences were retrieved from NCBI database (<http://www.ncbi.nlm.nih.gov/>). The amino acid sequences for *nirS* (thereafter refers as to NirS) and AOA *amoA* (AOA AmoA) were aligned with reference sequences and edited using ClustalX 2.0 (Thompson et al., 1997) and BioEdit v7.1.3 (Hall, 1999), respectively. Mothur v.1.32.1 (Schloss et al., 2009) was used to determine operational taxonomic units (OTUs) from the deduced amino acid sequences. OTUs were defined by 5% cutoff of dissimilarity in the amino acid sequences. The OTUs were used for calculation of alpha diversity measurements, Shannon indices, Chao1 richness estimators, and Good's coverage using Mothur. A maximum likelihood phylogenetic tree was constructed using amino acid sequences representing each OTU in MEGA version 5.2.1 (Tamura et al., 2011) with bootstrap analysis (1000 re-samplings).

OTU richness and diversity, and library coverage were calculated in Mothur. Protein distance matrices were calculated with PRODIST, using the Dayhoff PAM matrix algorithm in PHYLIP package version 3.69 (Felsenstein, 2005). Online analysis in Fast Unifrac package (Hamady et al., 2010) was used for Principal Coordinate Analysis (PCoA), P-test, and Unifrac significance test. The distance matrix from Phylip and Newick formatted phylogenetic trees were used as the input files for PCoA.

2.7. Statistical analyses

One way analysis of variance (ANOVA) tests were performed in JMP 10 (SAS Institute Inc., Cary, NC, USA) to assess differences between physicochemical parameters, activities of N-cycling population and EEAs, and the gene copy numbers measured in riparian and stream sediments seasonally sampled along study sites (T1, T2U and T2D). All post comparisons of means were accomplished using a Tukey-Kramer HSD test, adjusted for the overall error rates. P values <0.05 were considered significant. The relationships between qPCR data and physicochemical variables were analyzed using redundancy analysis (RDA) in Canoco

version 4.5 for Windows (ter Braak and Šmilauer, 2002). RDA was recommended when a Detrended Correspondence Analysis (DCA) revealed that the data exhibit a linear response to the environmental variables (Leps and Šmilauer, 2003). The statistical significances of axis and individual parameters were evaluated using a Monte Carlo permutation full-model with 999 unrestricted permutations. In addition, Spearman nonparametric correlation analysis was used to test for statistical correlations between two sets of above measured data.

2.8. Nucleotide sequence accession numbers

The GenBank accession numbers for sequences determined in this study are JN179403 to JN180086 for archaeal *amoA*, and JN179084 to 179402 for *nirS*.

3. Results

3.1. Soil physicochemical properties

An array of environmental parameters, including concentrations of N (TN, Ext-N, $\text{NH}_4^+\text{-N}$) and C species (TC, Ext-C) that are likely to serve as electron donors and acceptors, or microbial growth substrates were determined in the stream and riparian sediments of sites T1, T2U and T2D (Table S2). A fluctuation in those chemical concentrations was observed in seasonally collected samples from the three sites (Fig. 1); however, these fluctuation did not show a significant difference between seasons or sites within the riparian or stream sediments, except for Ext-C, which showed a significant difference in stream sediments between April and July ($P=0.016$ in Tukey-Kramer HSD; $P=0.04$, $F=4.5$, $DF=3.0$ in one-way ANOVA). In contrast, obvious differences in the measured chemical parameters were observed between riparian and stream sediments. Concentrations of TN (0.5 to 3.9 [1.96 average] g N·kg soil⁻¹) and $\text{NH}_4^+\text{-N}$ (7.4 to 32.7 [15.6 average] mg N·kg soil⁻¹) in riparian sediment soils were significantly higher than those in stream sediments (0.06 to 0.80 g [0.36

average] and 3.1 to 7.8 [5.4 average] mg N·kg soil⁻¹, respectively) (P<0.001, F=32.3 and 19.6, respectively). Likewise, riparian sediments contained significantly higher TC (10.0 to 74.4 [36.2 average] g C·kg soil⁻¹) and Ext-C concentrations (23.8 to 168.8 [103.9] mg C·kg soil⁻¹) than did stream sediments (0.8 to 8.6 [3.7 average] C g·kg soil⁻¹ and 7.2 to 138.0 [57.0 average] mg C·kg soil⁻¹, respectively) (P<0.01, F=36.9 and 9.1, respectively). Riparian concentrations of NO₃⁻-N and Ext-N were somewhat lower than in stream sediments; however, the differences were not significant. The pH was weakly acidic (4.0 to 6.6) in the tributary sediments, without a significant difference except for one case between T2U and T2D riparian sediments (P=0.018 in Tukey- Kramer HSD; P=0.023, F=5.9 in one-way ANOVA) (Table S2).

3.2. Potential activity of N-cycling populations

Potential microbial activities in N cycles were determined in laboratory incubations using the seasonally collected sediment samples in riparian and stream along sites T1, T2U and T2D. Amongst those measured activities, denitrifiers revealed the highest rate (pDNR), with a range from 1.0 to 64.7 mg N·kg soil⁻¹·d⁻¹, which were 4 to 29 times higher values in riparian sediments (22.5 mg N·kg soil⁻¹·d⁻¹ on average) than in stream sediments (2.7 mg N·kg soil⁻¹·d⁻¹ on average), with a significant P value of <0.01 (F=14.1) (Fig. 2). Nitrifiers also exhibited relatively high potential rates (pNR), ranging from 0.1 to 12.2 mg N·kg soil⁻¹·d⁻¹, in which riparian sediments were 2 to 6 times higher (6.3 mg N kg soil⁻¹·d⁻¹ average) than stream sediments (1.3 mg N·kg soil⁻¹·d⁻¹ average) (P<0.001, F=21.1). The potential DNRA rates (pDNRAR) in riparian and stream sediments were 0.28 mg N and 0.075 mg N·kg soil⁻¹·d⁻¹ average, respectively, with a significant P value of <0.05 (F=7.3), while pNH₄oxR averaged 0.11 and 0.08 mg N·kg soil⁻¹·d⁻¹, respectively.

In a few cases, the N-cycling rates were significantly different between sites or seasons. T1 and T2D showed significantly higher pNR compared with T2U ($P<0.05$ in Tukey-Kramer HSD; $P<0.05$, $F=3.9$ in one-way ANOVA). pDNRAR in January was significantly higher than in July ($P<0.05$ in Tukey-Kramer HSD; $P<0.05$, $F=3.9$ in one-way ANOVA) (Fig. 2).

3.3. Soil enzyme activities

CBH activities were 4 to 123 times higher in riparian sediments ($6.1 \text{ mmol}\cdot\text{g soil}^{-1}\cdot\text{h}^{-1}$ on average) than in stream sediments ($0.5 \text{ mmol}\cdot\text{g soil}^{-1}\cdot\text{h}^{-1}$ on average) ($P<0.001$, $F=30.8$) (Fig. 3). Glu activities in riparian and stream sediments averaged $0.07 \text{ mmol}\cdot\text{g soil}^{-1}\cdot\text{h}^{-1}$ and $0.001 \text{ mmol}\cdot\text{g soil}^{-1}\cdot\text{h}^{-1}$, respectively, with a significant P value of <0.001 ($F=21.7$). PhOx activities ranged from 0.002 to $0.13 \text{ nmol}\cdot\text{g soil}^{-1}\cdot\text{h}^{-1}$, with higher values in riparian than stream sediments (0.05 versus $0.02 \text{ nmol}\cdot\text{g soil}^{-1}\cdot\text{h}^{-1}$ average). EEAs were not significant different across sites or seasons.

3.4. Abundances of total soil bacteria and the microbes in N cycle

The abundance of total bacteria and selected genes involved in the N cycle of the SFR tributaries were estimated by copy numbers of corresponding genes using qPCR. Total bacterial numbers were enumerated using 16S rRNA gene copy numbers as proxy. The 16S rRNA gene copies ranged 1.3×10^9 to $1.1\times 10^{11}\cdot\text{g soil}^{-1}$, with a considerable seasonal fluctuation within each site. The copies measured in riparian sediments ($3.8\times 10^{10}\cdot\text{g sediment soil}^{-1}$ on average) were significantly higher compared with those in stream sediments ($2.7\times 10^9\cdot\text{g soil}^{-1}$ on average) ($P<0.001$, $F=17.7$) (Fig. 4).

Nitrifiers were enumerated using *amoA* in ammonia oxidizing bacteria (AOB) and archaea (AOA). AOA *amoA* copy (9.6×10^5 to $2.9\times 10^8\cdot\text{g soil}^{-1}$) outnumbered AOB *amoA*

copy (6.1×10^4 to $2.0 \times 10^7 \cdot \text{g soil}^{-1}$) in all sediment samples. Both genes exhibited higher copy numbers in riparian sediments than stream sediments: 6.9×10^7 vs $2.5 \times 10^6 \cdot \text{g soil}^{-1}$ average for AOA *amoA*, 3.9×10^6 vs $1.9 \times 10^5 \cdot \text{g soil}^{-1}$ average for AOB *amoA* ($P < 0.05$, $F = 5.6$ and 5.5 for AOA and AOB *amoA*, respectively).

nirK and *nirS* were employed to estimate the number of denitrifiers, which encode functionally equivalent but structurally different nitrite reductases, either a copper or a cytochrome cd1-containing enzyme, respectively (Zumft, 1997). In all sediment samples, *nirK* copies outnumbered *nirS* copies. The riparian sediments exhibited significantly higher copies for these genes than in the stream sediments: 1.4×10^8 vs $1.1 \times 10^7 \cdot \text{g soil}^{-1}$ for *nirS*, 8.6×10^8 vs $2.9 \times 10^7 \cdot \text{g soil}^{-1}$ for *nirK* average ($P < 0.01$, $F = 10.2$ and 18.5 respectively).

Gene *nrfA* was used for enumerating DNRA bacteria; *nrfA* encodes a periplasmic nitrite reductase, which catalyzes nitrite reduction using formate (Mohan et al., 2004). *nrfA* copies in riparian and stream sediments were $8.0 \times 10^6 \cdot \text{g soil}^{-1}$ and $6.1 \times 10^5 \cdot \text{g soil}^{-1}$ on average, respectively, with a significant P value of < 0.001 ($F = 20$).

N_2 -fixers were accounted by *nifH* (encoding dinitrogenase reductase; Raymond et al., 2004). The copy numbers of this gene were 4.5×10^8 and $2.6 \times 10^7 \cdot \text{g soil}^{-1}$ on average in riparian and stream sediments, respectively, showing a significant difference ($P < 0.001$, $F = 20$).

Although no significant difference was observed in most N-cycling gene copy numbers across sites and seasons, T2U exhibited significantly higher AOA *amoA* copies compared with T1 and T2D in stream sediments ($P < 0.01$ in Tukey-Kramer HSD; $P < 0.001$, $F = 16.7$ in one-way ANOVA).

An attempt was made to detect *hszA*, encoding a hydrazine synthase specifically present in ANAMMOX microbes; however, we were not successful in detecting this gene in any

sample with PCR using primers recently designed and widely applied for this gene (Harhangi et al., 2012).

3.5. Relationships between abundances and activities of microbes with environmental variables

RDA was performed to evaluate how the abundance and activities were related to the physicochemical parameters in the SFR tributary. The RDA results indicated that TC (39.6%: $P=0.001$, $F=63.95$), and TN (32.8%: $P=0.108$, $F=2.47$) explained the largest fraction of variation in microbial abundances and activities in the SFR tributary (87.1%: $P=0.002$, $F=10.52$). The angles of the arrows depicted in RDA biplot (Fig. 5) indicated that all the measured N-cycling gene copy numbers were positively correlated ($<90^\circ$ between angles) with NH_4^+ -N, Ext-C, TN, TC and Ext-N concentrations, while weakly or slightly negatively ($\geq 90^\circ$) correlated with NO_3^- -N concentrations and pH. Spearman's correlation analysis confirmed those RDA correlations (Table S3), showing that N-cycling genes were significantly positively correlated with NH_4^+ -N (correlation coefficient [ρ]=0.66-0.77, $P<0.001$), TN ($\rho=0.80$ -0.89, $P<0.001$), and TC ($\rho=0.80$ -0.88, $P<0.001$). Ext-C and Ext-N were also positively correlated with N-cycling gene copy numbers. pH and NO_3^- -N were negatively correlated with gene copy numbers.

All of the measured N-cycling rates were positively correlated with the abundance of copy numbers of the corresponding genes; i.e., pNR for AOA *amoA* ($\rho=0.35$, $P<0.05$) and AOB *amoA* copies ($\rho=0.66$, $P<0.001$), pDNR for *nirK* ($\rho=0.78$, $P<0.001$) and *nirS* copies ($\rho=0.81$, $P<0.001$), and pDNRAR for *nrfA* copies ($\rho=0.40$, $P=0.056$).

Activities of CBH, Glu and PhOx were also positively correlated with the N-cycling gene copy numbers and potential activities, with a significant P value in Spearman's

correlation analysis of most comparisons (Table S3).

N-cycling rates and EEAs were positively correlated with $\text{NH}_4^+\text{-N}$, Ext-C, TN, TC and Ext-N concentrations, and riparian sediments clustered independently of stream samples, showing a good agreement with the observation that most measured values were higher in riparian sediments than stream sediments.

3.6. Diversity and distribution of AOA amoA and nirS sequences

Diversity and distribution of nitrifiers and denitrifiers inhabiting the SFR tributaries were assessed from the AOA *amoA* and *nirS* sequences retrieved from riparian and stream sediments of sites T1, T2U and T2D. AOA *amoA* was selected as representative gene for nitrifiers due to its dominance over AOB in these samples. *nirS* was not selected by its dominance, but by the longer sequence length (approximately 425 bp) of its PCR amplicon rather than that of *nirK* (approximately 164 bp). The fact that *nirS* is more frequently observed in diverse bacterial communities than *nirK* in various sediments (Oakley et al., 2007; Dang et al., 2009) is another reason to select this gene for the diversity study.

3.6.1. AOA AmoA sequences

A total of 758 AOA *AmoA* sequences were obtained from the riparian and stream sediments of the three sites (T1, T2U and T2D), which were assigned to a total of 43 OTUs (Table S4). The Good's coverage estimate indicated that more than 94% of OTUs were picked up for this study from the sediments. Chao1 richness estimator predicted the presence of 10 to 26 OTUs in each location, with Shannon diversity indices ranging from 0.8 to 2.0. These diversity measurements indicated that the stream sediments support more diverse archaeal nitrifiers than riparian sediments (Table S4).

The 43 OTUs were distributed between 12 distinct phylogenetic clades (referred to as I

to XII) in ML tree (Fig. 6). These clades are divided into two previously defined groups: I.1a (including our clades I to V); and I.1b (clades VI to XII), represented by the genera *Nitrosopumilus* (a soil group) and *Nitrososphaera* (a marine group), respectively. Group I.1a dominated SFR tributaries, accounting for 63 to 98% of total AOA AmoA sequences from each location. Clade IV (group I.1a) dominated sites T1 and T2U (72 to 97% of total sequences) while T2D was dominated by clades II (27 to 41%) and VIII (28 to 37%) that belong to groups I.1a and I.1b, respectively.

The RDA biplot shows the correlation of each clade with physicochemical parameters (Fig. S2). Clade IV sequences dominate T1 and T2U and were positively correlated with TC, TN, Ext-C and Ext-N that were elevated in riparian sediments, while clades II and VIII dominate T2D and were negatively correlated with those parameters.

P test and Unifrac significance test indicated that AOA community structures from riparian sediments were significantly different from those from stream sediments ($P < 0.05$) (Table S5). PCoA analysis indicated that the AOA communities were clustered along sites rather than along riparian/stream (Fig. 6): the communities from T2U and T2D were clearly separated by axes P1 (explained 31% variables) and P2 (22% variables) from other sites, and T1 was also segregated from T2D.

3.6.2. *NirS* sequences

A total of 311 *NirS* sequences were retrieved from riparian and stream sites T1, T2U and T2D and were allotted to 147 OTUs. Coverage was estimated as 48 to 75% for each location (Table S4). The richness estimated for each location ranged from 26 to 118 OTUs, and Shannon's diversity index was 2.7 to 3.6, highest value for both estimations at the stream sediment community of T2D. There was a clear trend that stream sediments are more diverse than riparian sediments within each site (Table S4).

The 147 OTUs were distributed within 10 clades (referred to as I to X) in the ML phylogenetic tree (Fig. 7). The clades were mostly affiliated with NirS from *Proteobacteria*, without a substantial subclassification level. For instance, clade I includes *Pseudomonas* and *Achromobacter*, which belong to different classes (*Gamma*- and *Betaproteobacteria*, respectively). Clades III, V, and IX were dominant in riparian sediments, while clades VI and X dominated stream sediments (stacked column graph in Fig. 7).

The RDA biplot revealed that the clades that dominated riparian sediments (i.e., III, V, VII and IX) were positively correlated with TC, TN, Ext-C and Ext-N, while clades VI and X, which dominated stream sediments, were negatively correlated with those parameters (Fig. S2). Clades I, II and IV were positively correlated with pH.

P-test and Unifrac significance tests indicate that NirS assemblages from riparian sediments were significantly different from stream sediments ($P < 0.05$) (Table S5). The PCoA biplot shows that NirS assemblages from stream sediments were divided in the same direction by axis P1 (explained 28.62% variables), but not separated from all assemblages derived from riparian sediments; T2D riparian assemblage was oriented along the same direction (Fig. 7). The riparian assemblages were not separated by either axis.

4. Discussion

River tributaries are the first aquatic environmental compartments receiving non-point sources of agricultural runoff that may be highly enriched with IONs (Coffey, 1997; David et al., 2010). Tributaries may be broadly divided into aquatic and riparian zones. In the SFR tributaries, riparian sediments exhibited significantly higher levels of C (TC and Ext-C), N (TN, Ext-N, NH_4^+ -N) and EEAs (CBH, Glu and PhOx) compared with stream sediments. Riparian sediments also exhibited significantly higher activities (pNR, pDNR, pDNRAR, pNH₄oxR) and sizes (AOA/AOB *amoA*, *nirK/S*, *nrfA*, *nifH*) of N-cycling populations. The

elevated carbon and nitrogen contents in riparian sediments contribute to the increased activities and sizes of the N-cycling populations by providing the essential macro-nutrients. EEA is thought to provide labile carbon such as glucose and fatty acids derived from organic matter to N-cycling populations (Hume et al., 2002; Ding et al., 2013).

In wetland soils, the fluctuation of water tables strongly influences redox conditions. Riparian zone s are thought to have much broader redox ranges than stream sediments due to frequent fluctuations in water and air. The redox conditions strongly impact the rates of NH_4^+ diffusion/oxidation, and NO_3^- diffusion/reduction (Reddy et al., 1980). Hefting and colleagues (2004) reported that water table elevation primarily determined the N-cycle dynamics in riparian zones. The present study unveiled a new feature of the impact of redox potentials, that the alternating oxic and anoxic states enhance most N-cycling processes (abundance as well as activities). This coincident enhancement might be due to the fact that the redox variable conditions satisfies not only anaerobic processes (e.g., denitrification, DNRA, ANAMMOX, anaerobic N_2 -fixation), but also aerobic processes (e.g., ammonia oxidation, aerobic N_2 -fixation) by intermittently providing essential substrates from the atmosphere (e.g., O_2 and N_2).

The results obtained from the qPCR analysis and the potential activity for N-cycling populations illustrated the overall structure of N-cycle shaped by the SFR tributary. Denitrification was the key process, with the greatest activities and population sizes of the N-cycling processes tested. DNRA can compete with denitrification for nitrogen oxides in some environments, such as estuary sediments or ocean waters (An and Gardner, 2002; Gardner and McCarthy, 2009). One of the critical factors promoting DNRA is a high ratio of organic carbon (electron donors) to NO_3^- concentrations (Kelso et al., 1997; Silver et al., 2001). The SFR tributaries have a high Ext-C/ NO_3^- -N mole ratio (72 and 256 average in stream and riparian sediment, respectively), DNRA was expected to be favored; however, its activity and

gene copies remained at $\leq$ one magnitude of order that of denitrification (Figs. 2 and 4). This somewhat paradoxical observation might be due to the continuous supply of NO_3^- by nitrifiers, which showed much higher activities and gene copies than DNRA bacteria (Figs. 2 and 4). The oxic conditions in the SFR tributary might be another reason for the dominance of facultative anaerobic denitrifiers (most *Pseudomonas* in the SFR tributary [Fig. 7]) over DNRA bacteria comprising primarily obligatory fermentation or sulfide oxidizing anaerobes (Tiedje, 1988; Brettar and Rheinheimer, 1991).

ANAMMOX may also compete for NO_2^- in environments such as tidal marshes, estuarine sediments and the large lakes (Kuypers et al., 2003; Rich et al., 2008; Koop-Jakobsen and Gibli, 2009). However, its activity (pNH₄oxR) amounted to only 2.6% of pDNR in the SFR tributary. Moreover, *hszA* (characteristic of ANAMMOX bacteria) was not detected by PCR; therefore, ANAMMOX does not likely compete with denitrification in these sediments. NO_3^- concentration is a crucial factor determining the distribution of ANAMMOX (Algar and Vallino, 2014). In the low NO_3^- concentration (Fig. 1), ANAMMOX bacteria with an anaerobic chemolithoautotrophic trait seemed to be outcompeted by the aerobic heterotrophic *Pseudomonas* denitrifiers in the SFR tributaries.

The N-cycle structure determined in this study also indicated that the high activities of denitrifiers in SFR riparian sediments were dependent on the cooperative interaction with nitrifiers, which were 2 to 6 times higher activities in riparian than in stream sediments. Taking advantage of the high pDNR, which reached up to 64.7 mg N·g soil⁻¹·d⁻¹, denitrifiers quickly consumed the nitrogen oxides, leaving a residual pool as observed with the SFR tributary (<0.91 mg N·kg soil⁻¹). In such NO_3^- -N limiting situations, denitrification rates will be strongly controlled by nitrifying activity that provides nitrogen oxides from NH_4^+ -N, of which the concentrations observed here (3 to 33 mg N·kg soil⁻¹) are enough to satisfy the observed nitrification activity (0.1 to 12.2 mg N·kg sediment⁻¹·d⁻¹). Denitrification coupled

with nitrification was indicated by the positive correlation between pNR and pDNR, and between *amoA* copies and *nirS/K* copies in RDA biplot (Fig. 5). Unsurprisingly, denitrification coupled with nitrification has been observed in many other environments, including estuarine sediments (Jenkins and Kemp, 1984), rhizospheres in a flooded agroecosystem (Penton et al., 2013), in rice paddy rhizospheres (Arth et al., 1998), and in a river sediment (Sheibley et al., 2003).

In order to examine if the physicochemical parameters of riparian sediments influence assemblage structures differently from stream sediments, the assemblage compositions of nitrifiers and denitrifiers were determined by sequence analysis of AOA *amoA* and *nirS*. SFR tributary supported a diversity of AOA that were spread over 12 clades, and were affiliated with Groups I.1a (referred as to a soil group) and I.1b (referred as to a marine group) (Fig. 6). P value of <0.05 in P-test and Unifrac significance test indicated that the riparian AOA assemblages were significantly different from the stream assemblages (Table S5), suggesting that the different conditions of riparian and stream sediments apparently impacted the structure of AOA assemblages. RDA results indicated that AOA clades IV and V enriched in riparian sediments were positively correlated with TC, TN, Ext-C and Ext-N (Fig. S2), suggesting that those chemicals would stimulate them. It was also found that AOA assemblage structure was greatly impacted by local environmental conditions. For instance, clade IV dominated sites T1 and T2U ($\geq 72\%$ of total *amoA* sequences), but not site T2D ($\leq 15\%$) (Fig. 6). *Nitrosotalea* species belonging to clade IV was reported to be acidophilic (Lehtovirta-Morley et al., 2011; Zhou et al., 2015). Sites T1 and T2U are more acidic than T2D; such that, this clade might be selected at low pH.

The SFR tributary harbored a high diversity of denitrifiers, which were distributed between 10 distinct clades and were primarily affiliated with *Alpha*-, *Beta*- and *Gammaproteobacteria* (Fig. 7). Denitrifying assemblages in riparian sediments were

significantly different from the communities of stream sediments ($P < 0.05$ in P-test and Unifrac significance test) (Table S5), suggesting that the shaping of the assemblage structure was significantly influenced by the environmental conditions of riparian and stream sediments. RDA suggested that TC, TN, Ext-C and Ext-N act as environmental forces enriching the NirS-clades III, V, VII and IX, in riparian sediments (Fig. S2). Denitrifying communities were also statistically distinguished by sites ($P < 0.05$ in P-test and Unifrac significance test between sites) (Table S5), suggesting that the assemblage structures were not only affected by stream and riparian sediment conditions, but also by local site environmental conditions, and did not clustered according to either site or by the riparian/stream criteria in the PCoA biplot (Fig. 7), which suggests that the environmental parameters in the riparian and stream environments affect the assemblage structure as much as the site-specific environments. This suggests that the environmental gradients across the riparian and stream sediments act as filters differentiating the denitrifier genotypes between two habitats.

5. Conclusion

The results from multidirectional approaches employed in this study illustrate the genetic and functional structures of N-cycle established in a river tributary that has been recognized as hotspots mitigating ION input from non-point discharge of agricultural practices. The structures indicated denitrification is a central process in removing IONs from river tributaries under the support of nitrification activity that provides nitrogen oxides to denitrification via an ammonia oxidation pathway. It was also shown that riparian zones significantly elevate N-cycling processes with higher C and N concentrations, a higher EEA and a broader redox fluctuations than aquatic stream sediments. Moreover, AOA *amoA* and

nirS sequences indicated that the assemblage structures of nitrifiers and denitrifiers are shaped under the control of different environmental conditions present in riparian and stream sediments. Collectively, this comprehensive N-cycle structure extended our understanding on the relative contribution of each process to the ION removal in river tributary, the interaction between populations, and the assemblage structure responding to biogeochemical factors.

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Figure legends

Fig. 1. Physicochemistry profiles showing seasonal and spatial variation measured from riparian (circle symbols) and stream sediments (bar graphs) of Florida SFR tributaries. Seasons in seasonal plot: January, April, July and October from left; sites in spatial plot: T1, T2U, T2D from left. Error bar represents ± 1 SD ($n=3$ for each season, $n=4$ for each site). Box-and-Whisker plot was generated from the pooled data to present overall difference of each parameter between riparian and stream sediments. Boxes depict the medians (horizontal lines in the boxes) and the lower and upper quartiles (bottoms and tops of the boxes, respectively). The vertical bars (whiskers) show the highest and the lowest values, excluding outliers. Significant difference between samples was indicated with *** $P<0.001$, ** $P<0.01$, * $P<0.05$.

Fig. 2. Seasonal and spatial variation of potential rate of nitrification (pNR), denitrification (pDNR), DNRA (pDNRAR) and ANAMMOX (pNH₄oxR) in riparian (circle symbols) and stream sediments (bar graph) of the SFR tributaries. Error bar represents ± 1 SD (n=3 for each season, n=4 for each site). Box-and-Whisker plot representing overall pattern of each potential rate in riparian and stream sediments, with denotation of significant difference: *** P<0.001, ** P <0.01, * P<0.05.

Fig. 3. Seasonal and spatial variation of EEAs determined in the riparian (circle symbols) and stream sediments (bar graph) of the SFR tributaries: CBH (cellobiohydrolase), Glu (β -D-glucosidase), PhOx (phenol oxidase). Error bar represents ± 1 SD (n=3 for each season, n=4 for each site). Box-and-Whisker plot showing overall pattern of each activity between riparian and stream sediments, with a significant difference denoted with *** P<0.001, ** P<0.01, * P<0.05.

Fig. 4. Seasonal and temporal variation of gene copies for 16S rRNA and N-cycling populations measured from riparian (circle symbols) and stream sediments (bar graph) of SFR tributaries (bottom). Error bar represents ± 1 SE (n=3 for each season, n=4 for each site). Box-and-Whisker plot (top) representing the pooled data and significant difference between riparian and stream sediment soils (top). Significant difference was denoted with *** P<0.001, ** P<0.01, * P<0.05.

Fig. 5. Redundancy analysis (RDA) biplot representing relationship between N-cycling gene copies, potential activities of N-cycling populations, extracellular enzyme activities, and soil physicochemistry parameters determined in this study. White and gray circles indicate

samples collected from riparian and stream sediments, respectively. The first two axes explain 86.8% of the total canonical eigenvalues, with a significant Monte-Carlo test value ($P=0.002$).

Fig. 6. Maximum-Likelihood (ML) tree representing phylogenetic position of AOA AmoA sequences obtained from SFR tributary sediments. Values for nodes with over 50% bootstrap support are shown. The stacked-column graph (right upper) depicts the relative abundance of clades within riparian and stream sediment of each site. The clades that include our sequences with or without reference sequences are denoted by white color, while black-colored clades include only reference sequences. Unweighted PCoA biplot (right lower) shows phylogenetic differences of AOA community between samples. The circle size representing samples is correlated the abundance of AOA community estimated using qPCR.

Fig. 7. Phylogenetic distribution of NirS sequences detected in SFR tributary. Values for nodes with over 50% bootstrap support are shown. The clades including our sequences with or without reference sequences are denoted by white color, while black-colored clades contain only reference sequence. The stacked-column graph (right upper) shows the relative abundance of individual clades within riparian and stream sediments of each site. Unweighted PCoA biplot (right lower) shows the difference of the denitrifying communities between samples. The circle size indicating the community size measured within samples using by qPCR.

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