Water Research 87 (2015) 193–201

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Investigation of denitrifying microbial communities within an agricultural drainage system fitted with low-grade weirs

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ARTICLE INFO

Article history:
Received 6 May 2015
Received in revised form
14 September 2015
Accepted 15 September 2015
Available online 16 September 2015

Keywords:
Nutrient reduction
Microbial ecology
Denitrification
Best management practice

ABSTRACT

Enhancing wetland characteristics in agricultural drainage ditches with the use of low-grade weirs, has been identified as a best management practice (BMP) to mitigate nutrient runoff from agriculture landscapes. A major objective of utilizing low-grade weirs as a BMP includes fostering environments suitable for the biogeochemical removal of nitrogen via denitrification. This study examined the spatial resolution of microbial communities involved in denitrification in agricultural drainage systems fitted with low-grade weirs. Appropriate sampling scales of microbial communities were investigated using 16S rRNA and denitrification functional genes *nosZ*, *nirS*, and *nirK* via quantitative polymerase chain reaction (qPCR) and terminal-restriction fragment length polymorphism (T-RFLP) analysis. Genes 16S rRNA, *nosZ*, and *nirS* were all successfully detected in soil samples, while *nirK* was below the detection limit throughout the study. Utilizing a combination of three sampling regimes (management, reach, catchment) was found to be effective in capturing microbial community patterns, as ANOVA results revealed *nosZ* gene abundance was significantly greater at the management rather than reach scale ($p = 0.045$; $F = 3.311$), although, no significant differences were observed in 16S rRNA or *nirS* between sampling scales ($p > 0.05$). A Pearson correlation matrix confirmed that 16S rRNA and *nosZ* gene abundances were positively correlated with soil carbon (C), nitrogen (N), and moisture, while *nirS* abundance was only positively correlated with soil C and soil moisture. This highlights the potential for wetland-like characteristics to be recovered in agricultural drainage systems, as weir proximity is observed to enhance soil moisture and conditions for N remediation. This study provides the basis for additional investigations of these unique environments in the Mississippi Alluvial Valley and a starting point for adaptive management to enhance agricultural drainage systems for microbial communities towards nutrient remediation goals.

1. Introduction

Natural and created wetlands have been identified as ecological systems for nitrogen (N) management, typically acting as sinks because of high levels of denitrification in their anaerobic soils (Mitsch et al., 2001). Scientists and land managers have aimed to identify innovative best management practices (BMPs) that marry biogeochemical principles of biological N removal with drainage needs of the agricultural community. Enhancing wetland characteristics in drainage ditches has been shown to decrease nutrient runoff from agriculture landscapes to adjacent aquatic ecosystems (Cooper et al., 2002; Kröger et al., 2008b; Moore et al., 2010). Adding controlled drainage practices in the form of low-grade weirs (hereafter "weirs") within drainage ditches also resulted in nutrient load reductions (Kröger et al., 2008a, 2011; Littlejohn et al., 2014). However, Mitsch et al. (2001) explains that wetlands formed using a controlled drainage technique are particularly vulnerable during flooding and their stability can be hard to predict. Semi-controlled and field experiments have reported that implementation of weirs has led to increased hydrologic residence time (HRT) (thus increased time for biogeochemical reactions) in drainage ditches (Kröger et al., 2008a; Littlejohn et al., 2014; Prince

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http://dx.doi.org/10.1016/j.watres.2015.09.028
0043-1354/

Czarnecki et al., 2014). Despite this observation, water quality results from Littlejohn et al. (2014) and Baker et al. (in review) reflect pronounced variability between storm-flow and low-flow sampling, by failing to show consistent reductions in N concentrations and loads. Variability in water quality suggests that perhaps weirs do not have enough of an impact on hydrology to achieve goals of mimicking wetlands by creating anoxic environments to enhance aquatic plant and microbial communities, driving biogeochemical N transformations and removal. Given the variability of the potential for weirs to reduce N in runoff in current literature and the dynamic nature of water quality, investigation of an endpoint that is directly linked to the biogeochemical N cycle and less variable within aquatic systems, is warranted to better understand the physiochemical and biological effects of weirs.

An important biogeochemical component of N cycling is denitrification, where nitrate (NO_3) is transformed to gaseous N and removed from aquatic environments, primarily as N_2O (nitrous oxide) and N_2 (di-nitrogen gas) (Ye et al., 1994; Philippot et al., 2002; Wallenstein et al., 2006). Functional genes (*nirK, nirS, cnorB, qnorB, nosZ*) have been identified in denitrifying microbes and are used to investigate soil health and allow for the characterization of microbial communities (Osborn et al., 2000). Investigation of these functional genes has also been instrumental in providing insight into denitrifying bacterial communities in a variety of environments (Henry et al., 2004; Dandie et al., 2007; Ahn and Peralta, 2009; Enwall et al., 2010; Bannert et al., 2011; Dandie et al., 2011).

Understanding these processes in relation to the surrounding environment is critical, as biogeochemical reactions involved in microbial denitrification are largely governed by environmental conditions. Oxygen, N, and carbon (C) availability, and their exchange between the water column and sediment, has been shown to affect the establishment of microbial biofilms and denitrification activity (Eriksson, 2001; Battin et al., 2003). Other environmental parameters affecting denitrification include pH, temperature, predation, and disturbances (Wallenstein et al., 2006). Microbial community responses to climate change, pollution, and anthropogenic influences have further emerged as important components to understanding ecosystem responses (Wallenstein et al., 2006). Recent literature has documented the influences of tillage management (Chèneby et al., 2009; Vargas Gil et al., 2009; Attard et al., 2010) and increasing agricultural intensity (Dell et al., 2008) on microbial community structure and function. Furthermore, Best and Jacobs (1997) reported that water management practices, such as BMPs, can also affect denitrification; implying that microbial processes would be affected.

Analysis of denitrifying microbial communities and chemical soil and water variables provides quantifiable data that offers a molecular perspective on BMP impacts to drainage systems on the agriculture landscape. Employing soil biogeochemical endpoints also offer a comprehensive understanding of how the ecosystem is responding to anthropogenic disturbances, and has the potential to identify conditions that enhance: 1) denitrifying functional gene abundance; 2) nitrate (NO_3) reductions; and 3) current nutrient management strategies. Prosser et al. (2007) deems developing an understanding of the ecology of microorganisms for the management of ecosystems and mitigation of climate change one of the greatest challenges of contemporary ecology. While long-term goals aim to develop a better understanding of the ecology of denitrifying organisms, it was deemed critical to first develop a foundational understanding of the distribution of denitrifying microbes in agricultural drainage ditches for the future investigation of the impacts of weirs on denitrifying microbial communities. This study is a preliminary investigation to determine appropriate sampling resolution of denitrifying microbial communities in agricultural drainage ditches. Justification for a multi-scale preliminary investigation of agricultural drainage systems with weirs is affirmed by Horner-Devine et al. (2004) stating that soil microorganisms are not randomly distributed; they exhibit different spatial patterns at different scales. It is anticipated that this work will provide a foundation for additional investigations of weir impacts in these systems that foster the development of mechanistic linkages between environmental and physiological processes, resource availability, and denitrifying microbial community dynamics.

Hypotheses of this investigation are 1) increasing sampling resolution (regime) will impact the abundance of microbial functional gene presence within an agricultural drainage ditch and 2) environmental parameters (soil C, N, and moisture) will also impact abundance of microbial functional gene presence within an agricultural drainage ditch. Investigation of these hypotheses were carried out through the following objectives: 1) determine an appropriate sampling regime for the further investigation of microbial communities in drainage systems and 2) identify relationships between soil C, N, and soil moisture and microbial communities. Functional genes involved in denitrification were quantified using quantitative polymerase chain reaction (qPCR) and 16S rRNA terminal-restriction fragment length polymorphisms profiles (TRFLP) were used to analyze microbial communities. Results of this study offer benefits for developing a better understanding of how weirs alter drainage environments in such a way that impacts microbial community structure and potential nutrient transformations. Understanding impacts of weirs on microbial communities and N cycling will also provide a greater understanding of how to improve management strategies to reduce N loading to the Gulf of Mexico.

2. Materials and methods

2.1. Study site

The study site is located on in Humphreys County, MS, USA (Fig. 1) within a sub-watershed chosen because of its priority status within the Mississippi River Basin Healthy Watersheds Initiative and listing on the Mississippi Department of Environmental Quality 303(d) list due to impaired waters (Upper Yazoo – Hydrologic unit code 08030206). The experimental ditch (Fig. 1) was fitted with four low-grade weirs, and chosen to investigate spatial distribution patterns of denitrifying microbial communities during fall 2012. A control ditch was not included in the experimental design as this preliminary investigation aimed to identify an appropriate sampling regime accounting for spatial heterogeneity within drainage ditches resulting from ephemeral hydrologic flow patterns and indiscriminate water pooling, while avoiding capturing noise associated with soil heterogeneity. It is general knowledge that soil exhibits extreme spatial heterogeneity at field scale. The drainage system is 1754 m long, approximately 10 m in width, and drains approximately 98 ha of agricultural land managed in a rotation of corn (*Zea mays* L.) and soybeans (*Glycine max* [L.] Merr.). Soil composition in the agricultural field surrounding the ditch is dominated by Alligator clay (43%), Forestdale silt loam (17%), and Forestdale silty clay loam (13%) (National Resources Conservation Service Web Soil Survey; retrieved May 4, 2012).

2.2. Soil and water sample collection

Soil microbial assessments were conducted at catchment, reach, and management scales. At the catchment scale, soil cores (3 cm diameter x 10 cm depth) were collected at 100-m transects along the length of the ditch, beginning directly in front of the first weir

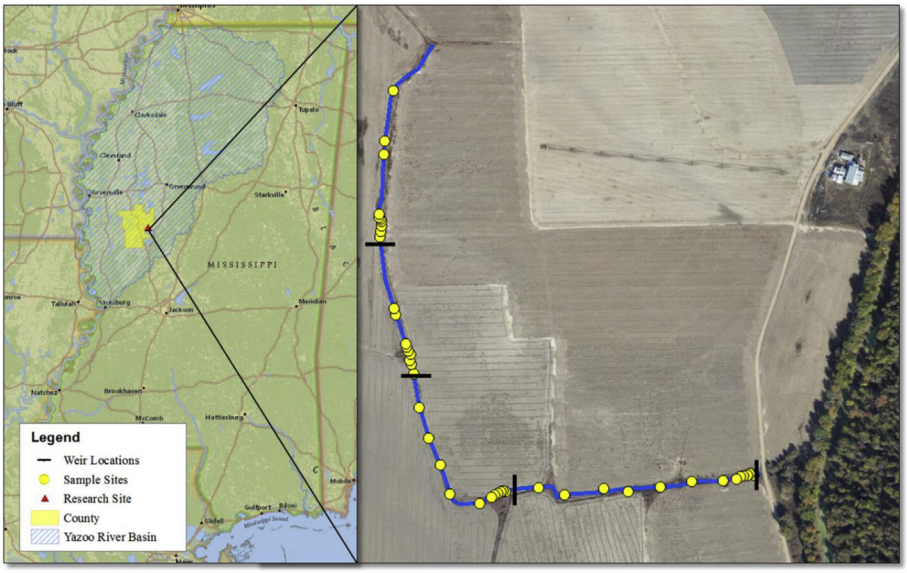

Fig. 1. The location of the experimental ditch within the Yazoo River Basin and Humphreys County, MS, USA are shown on the left. The research site with experimental drainage ditch, weir locations, and the respective sampling points where soil cores were collected for analysis are shown on the right.

(0 m). At the reach scale, soil cores were collected at 50-m intervals between weirs, beginning directly upstream of each weir (0 m) up to 250 m. At the management scale, soil cores were collected at 5-m intervals throughout the first 25 m directly upstream of each weir (0 m). Standardized sampling procedures were followed at each scale, where duplicate cores were collected from the center-most point of the thalweg at each transect. Samples collected along a single transect were combined in a sterile plastic bag to form a composite sample. Soil core samplers were sterilized with 90% ethanol prior to sampling at each transect. Soil samples were collected, stored on ice (~4 °C), and transported to the Mississippi State University Water Quality Lab within the same day. Duplicate soil cores were completely homogenized for analysis and made up a single unit; soil water content was determined (Hausenbuiller, 1975) subsequent to drying at 105 °C. Once soil moisture was determined samples were ground and analyzed for percent C and N using a Perkin–Elmer 2400 analyzer (PerkinElmer Inc., Waltham, MA, USA).

2.3. Microbial community analysis

Following homogenization of composite soil samples, aliquots (50 g) were stored at −80 °C until DNA extraction. DNA from each composite sample was extracted in triplicate sub-samples of 0.25 g (moist g) of soil using a QIamp® DNA Stool Mini Kit (Qiagen Inc.,Valencia, CA, USA) following the manufacturer's protocol; DNA from sub-samples was then pooled. Quality and quantity of DNA extracts were checked with a spectrophotometer (Nanodrop, Peqlab, Germany) prior to qPCR analysis. Extracted DNA was analyzed via qPCR to quantify the abundance of 16S rRNA and functional genes *nosZ, nirS,* and *nirK*. Positive control and standard curve DNA were generated from previously-amplified genes, extracted from ATCC isolates (Table 1) which were serially diluted to encompass 10^6 gene copies (gc) to 10^0 gc. All qPCR reactions were performed with an Applied Biosystems ABI Step One Plus real-time system using Applied Biosystems 96-well qPCR plates and qPCR-grade sealing film (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Polymerase chain reaction analysis followed protocols described by Bannert et al. (2011) which included an initial enzyme activation step; followed by cycling conditions specific to each gene assay (Table 1), and a melting curve. Primers specific to functional genes and PCR reaction mixtures are also described in Table 1. Inhibition controls were incorporated into each assay plate by using DNA extracts and serial spiked control DNA; sample template DNA was diluted to account for inhibition. A minimum detection limit of 5000 gc/dry g soil was set for all genes.

Community diversity analysis was performed via T-RFLP of the 16S rRNA gene. This method involved restriction endonuclease digestion using restriction enzymes of fluorescently-labeled qPCR products following Osborn et al. (2000). Restriction enzymes used were *Hha1* (catalog number R01375) and *Alu1* (catalog number R01395; New England Biolabs, Ipsich, MA, USA) and fluorescent primers used were Hex and Fam (Applied Biosystems, Foster City, CA, USA). Digestion products were mixed with fluorescently-labeled DNA size standard; fragments were then separated via gel electrophoresis and labeled fragments detected by laser using an ABI 3130 xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) (Osborn et al., 2000). Output fragments were called using Genemapper v4.1 with default settings.

2.4. Statistical analysis

Gene abundance, soil C, soil N, and soil moisture were analyzed using descriptive statistical methods, including Shapiro–Wilk and Levene tests to ensure normality and homogeneity of variance. All non-normal data was transformed using Box–Cox transformation (Box and Cox, 1964), prior to multivariate analysis. Analysis of variance (ANOVA) was used on transformed data to compare gene abundance between catchment, reach, and management scales. Multivariate analysis of transformed gene abundances, soil C, N, and moisture, distance from weirs, and distance from outflow was conducted using Principal Component Analysis (PCA) and a Pearson correlation matrix was calculated. All statistical analysis were conducted using Microsoft® Excel 2010/XLSTAT©-Pro 2012.1 (Addinsoft, Inc., Brooklyn, NY, USA).

Output from T-RFLP analysis included an electropherogram showing the microbial community profile as a series of colored peaks and a numerical table describing the size and height of each peak. T-RFLP profiles were processed and analyzed using T-REX software (Culman et al., 2009), which defines true peaks in the data,

Table 1
Methods used for qPCR quantification of functional genes extracted from soil samples. Methods include: primers used to amplify each functional gene, species used as a standard, master mix solution for amplification, amount of template rRNA used, respective amplicon lengths, thermal profiles, and the associated references from which thermal profiles were determined.

Target gene	Primer name	Standard species	Master mix	Template	Amplicon length	Thermal profile (qPCR)	Reference
16S rRNA	16SFOR 16SREV	*Pseudomonas aeruginosa (ATCC 27853)*	12.5 μl SYBR® Green Master Mix (Applied Biosystems), 0.50 μl each primer, 8.5 μl sterile water, 1 μl PVPP	2 μl	466	95 °C 10 min, 40 cycles (95 °C 15 s, 60 °C 1 min), 1 cycle (95 °C 15 s, 60 °C 1 min increasing 0.3 °C–95 °C 15 s)	(López-García et al., 2002 and López-García et al., 2001)
nosZ	nosZ1F nosZ1R	*Pseudomonas aeruginosa (ATCC 27853)*	12.5 μl SYBR® Green Master Mix (Applied Biosystems), 0.50 μl each primer, 8.5 μl sterile water, 1 μl PVPP	2 μl	259	95 °C 10 min, 40 cycles (95 °C 15 s, 60 °C 1 min), 1 cycle (95 °C 15 s, 60 °C 1 min increasing 0.3 °C–95 °C 15 s)	(Henry et al. 2006)
nirS	nirScd3aF nirSR3cd	*Pseudomonas aeruginosa (ATCC 27853)*	12.5 μl SYBR® Green Master Mix (Applied Biosystems), 0.50 μl each primer, 6.875 μl sterile water, 1 μl PVPP, 1 μl BSA (3%), 0.625 μl DMSO	2 μl	425	95 °C 10 min, 40 cycles (95 °C 45 s, 57 °C 45 s, 72 °C 45 s), 1 cycle (95 °C 15 s, 60 °C 1 min increasing 0.3 °C–95 °C 15 s)	(Kandeler et al., 2006)
nirK	nirK1040 nirK876	*Staphylococcus. epidermidis (ATCC 12228)*	12.5 μl SYBR® Green master mix (Applied Biosystems), 0.50 μl each primer, 6.875 μl sterile water, 1 μl PVPP, 1 μl BSA (3%), 0.625 μl DMSO	2 μl	165	95 °C 15 min, 6 cycles touchdown [95 °C 15 s, 63 °C 30 s(−1 °C per cycle), 72 °C 30 s, 80 °C 15 s], 40 cycles (95 °C 15 s, 58 °C 1 min), 1 cycle (95 °C 15 s, 60 °C 1 min increasing 0.3 °C –95 °C 15 s)	(Henry et al., 2004)

reconfigures peak alignment of terminal restriction fragment base-pair length, and incorporates experiment parameters to create environments which reflect microbial community profiles. Environmental parameters in the analysis included: spatial parameters which were binned into three groups comprised of distance from weir as 1 = 0–25 m; 2 = >25–100 m; 3 = >100 m and distance from outflow as 1 = 0–50 m; 2 = 51–100 m; 3 = >100–500 m; 4 = >500–1000 m; 5 = >1000 m). Output of this analysis included a two-way data matrix of interaction principal component axes (IPCA) that represent each "environment" or community profile about each sample. Principle component analysis (PCA) was utilized to determine how much influence each environmental parameter was having on each of the two most predominant axes. Identification of spatial patterns of denitrifying communities throughout a drainage system was used to identify areas of ditches that have likely been influenced by weirs and areas that have not, for the purpose of developing unbiased sampling regimes for further investigation of microbial communities in drainage ditches.

2.5. Geostatistical analysis

Empirical Bayesian Kriging (EBK) was used to visualize spatial patterns of denitrifying microbial communities in the experimental ditch. Data used was based on scaled sampling by utilizing transformed genes (gc/dry g soil) quantified by qPCR (16S, *nosZ,* and *nirS*). The EBK method offered several advantages in this study, most notably that it is more accurate than traditional kriging for smaller datasets (Pilz and Spöck, 2008). Additionally, EBK filters out a moderate trend in the data, accounts for the error introduced by estimating the underlying semivariogram, and has more accurate prediction of standard error than traditional methods. For a given distance (h), EBK uses a semivariogram model (γ) with the following form:

$$\gamma(\mathrm{h}) = \mathrm{Nugget} + \mathrm{b}|h|^{\alpha} \quad (1)$$

The nugget and b (slope) must be positive, and α (power) must be between 0.25 and 1.75. Under these restrictions, the parameters are estimated using restricted maximum likelihood. This semivariogram model does not have a range or sill parameter because the function has no upper bound. User-specified parameters for EBK analysis include subset size, the number of simulations, and the search neighborhood radius. These were set to one-half the sample size, 10,000, and two times the average nearest neighbor Manhattan distance, respectively. Kriging was performed using ArcGIS 10.1 Geostatistical Analyst (ESRI, Redlands, CA, USA).

3. Results

Quantification of eubacterial microbial communities by qPCR of 16S rRNA genes revealed average levels of communities to be 1.58×10^{10} gc/dry g of soil (Table 2). The average values for denitrification gene abundance revealed *nosZ* and *nirS* genes to be found at levels of 1.87×10^{7} and 5.47×10^{7} gc/dry g of soil, respectively. Denitrification gene, *nirK*, was below detection limits. When results of management, reach, and catchment sampling regimes were combined, gene abundances of 16S rRNA, *nosZ*, and *nirS* were found to vary throughout the length of the ditch channel (Fig. 2). Results of ANOVA on transformed values revealed no significant differences in 16S rRNA or *nirS* genes between the predetermined scales (management, reach, and catchment; data not shown); although, abundance of *nosZ* genes was found to be significantly greater at the management scale than at the reach scale ($p = 0.045$; $F = 3.311$).

To determine if weir proximity affects microbial community abundance, PCA was conducted in which transformed gene abundance data were considered active variables and distance from weir, soil moisture, soil C, and soil N were considered supplementary variables. Prior to PCA analysis, data was analyzed using a Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy and Bartlett's test of sphericity. Results of the KMO assessment were greater than 0.5 (0.647), indicating that sampling adequacy was satisfactory and factor analysis was appropriate. Bartlett's test of sphericity was significant, indicating that strength of variable relationships was strong, also supporting appropriateness of factor analysis. Output of PCA indicates that *nosZ*, *nirS*, and 16S rRNA were all positively correlated; this is supported by results of the Pearson correlation matrix (Table 3). Gene abundance vectors also appear to have positive relationships with C and N and soil moisture vectors; however, vectors close to the center of the correlation circle indicate that the strength of variable correlations are generally weaker. The Pearson correlation matrix confirms that 16S rRNA and *nosZ*

Table 2
Descriptive statistics of the quantification of functional genes 16S rRNA, *nosZ*, *nirS*, and *nirK* from all sampling sites within the drainage ditch in November 2012. Microbial DNA was extracted from soil samples and analyzed using qPCR.

Descriptive statistics	16S gc/dry g soil	*nosZ* gc/dry g soil	*nirS* gc/dry g soil	*nirK* gc/dry g soil
No. of observations	53	53	53	53
Minimum	7.53×10^8	1.42×10^6	1.88×10^5	BDL
Maximum	9.42×10^{10}	1.31×10^8	2.75×10^8	BDL
Median	1.14×10^{10}	7.36×10^6	3.60×10^7	BDL
Mean	1.58×10^{10}	1.87×10^7	5.47×10^7	BDL

*BDL refers to values that fell below detection limit of 5000 GU/g soil.

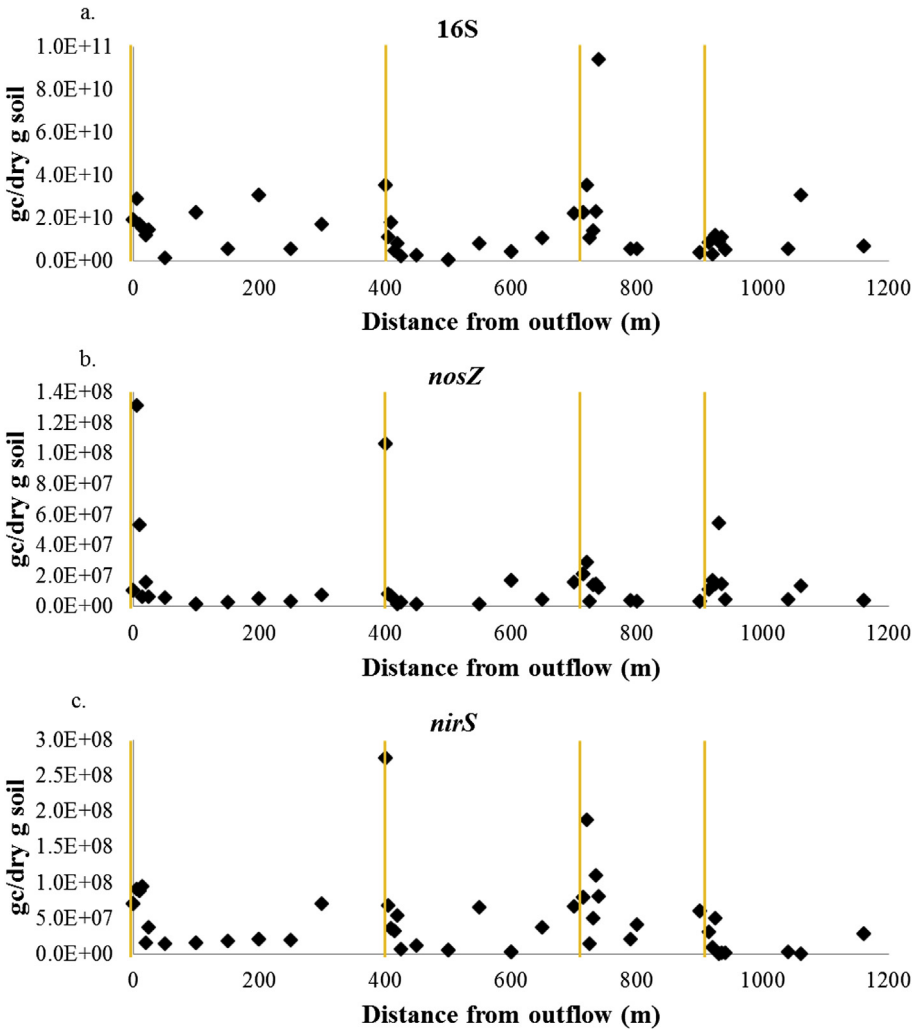

Fig. 2. Gene abundances (gc/dry g soil) of gene 16S rRNA (a), and denitrification functional genes *nosZ* (b), and *nirS* (c) found in soil microbes that were extracted from soil sampled throughout the length of the ditch, from the outflow to the inflow of respective channel. Locations of weirs in the channel are marked by yellow bar. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

gene abundances were positively correlated with soil C, N, and moisture, while *nirS* abundance was only positively correlated with soil C and soil moisture. Additionally, soil C and N were found to be positively correlated to each other, while soil N was also positively correlated with soil moisture. Distance from weirs was negatively correlated with *nosZ* abundance. While distance from weirs showed mostly negative relationships with genes and soil variables, distance from outflow showed more positive relationships with variables, as a significant positive correlation was found between distance from outflow and soil N. These results confirm relationships between soil microbial abundance and soil properties, as well as highlight the relationships between weirs and the denitrification gene *nosZ*. Spatial analysis of gene abundance was investigated using EBK for 16S rRNA, *nosZ*, and *nirS* gene abundances throughout the drainage ditch (Fig. 3). In general, model error rates were within acceptable limits, with the exception of *nirS*. Results of kriging indicated increased gene abundances in at least one of the four weirs in all genes analyzed.

Microbial community profiles were generated from T-RFLP data through T-REX as environments represented by IPCA axes. To identify significance in relationship patterns, a correlation analysis of IPCA1 and IPCA2 values, soil C, soil N, soil moisture, distance from

Table 3
Pearson (n) correlation matrix of gene abundances (active variables) and environmental parameters (supplementary variables) included in PCA; data was generated from analysis of parameter relationships using data from all of the samples collected from the drainage ditch in November 2012. All bold correlation values indicate significant relationships between measured parameters.

Variables	16S gc/dry g soil	*nosZ* gc/dry g soil	*nirS* gc/dry g soil	Distance from outflow	Distance from weir	Soil N (%)	Soil C (%)	Soil moisture (%)
16S gc/dry g soil	**1**	**0.477**	**0.552**	−0.194	−0.178	**0.447**	**0.441**	**0.323**
nosZ gc/dry g soil	**0.477**	**1**	**0.359**	0.026	**−0.336**	**0.592**	**0.519**	**0.380**
nirS gc/dry g soil	**0.552**	**0.359**	**1**	−0.256	−0.234	0.253	**0.277**	**0.378**
Distance from outflow	−0.194	0.026	−0.256	**1**	0.064	**0.313**	0.264	0.155
Distance from weir	−0.178	**−0.336**	−0.234	0.064	**1**	−0.099	−0.035	−0.146
Soil N (%)	**0.447**	**0.592**	0.253	**0.313**	−0.099	**1**	**0.831**	**0.582**
Soil C (%)	**0.441**	**0.519**	**0.277**	0.264	−0.035	**0.831**	**1**	0.230
Soil moisture (%)	**0.323**	**0.380**	**0.378**	0.155	−0.146	**0.582**	0.230	**1**

Values in bold are different from 0 with a significance level alpha = 0.05.

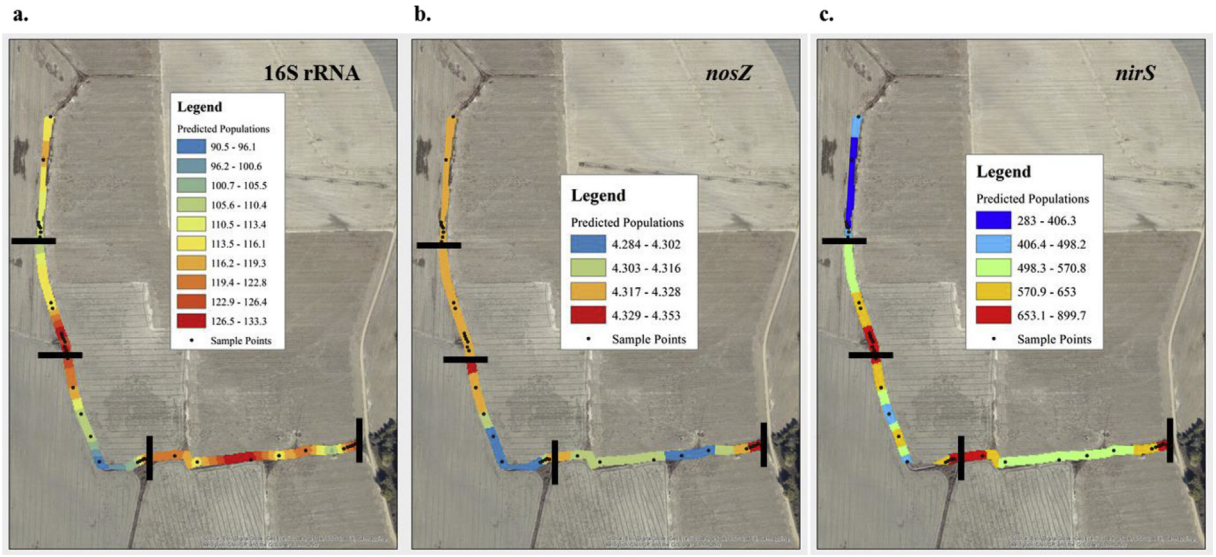

Fig. 3. Empirical Bayesian Kriging (EBK) of the spatial distribution of a) 16S rRNA, b) nosZ, and c) nirS transformed gene abundances (gc/dry g soil). Abundances were quantified using qPCR of samples extracted from soil cores collected throughout the length of the ditch in November 2012. Class breaks for each gene were determined by the model based on natural breaks in the data. Black bars indicate weir locations.

Table 4
Pearson (n) correlation matrix of interaction principal components axes (IPCA) about microbe community profiles (active variables) and environmental parameters (supplementary variables) included in PCA; community profiles were generated by T-REX software, which processed and analyzed T-RFLP results. Combining community profile data with environmental parameters for PCA analysis allowed for the identification of parameter relationships using data from all of the samples collected from the drainage ditch in November 2012. All bold correlation values indicate significant relationships between measured parameters.

Variables	IPCA1	IPCA2	Distance from outflow	From weir	Soil N (%)	Soil C (%)	Soil moisture (%)
IPCA1	**1**	**0.834**	**−0.485**	−0.056	0.039	0.070	0.009
IPCA2	**0.834**	**1**	−0.383	−0.252	0.072	0.208	0.136
Distance from outflow	**−0.485**	−0.383	**1**	−0.057	**0.433**	0.331	0.267
From Weir	−0.056	−0.252	−0.057	**1**	−0.209	−0.157	−0.277
Soil N (%)	0.039	0.072	**0.433**	−0.209	**1**	**0.816**	**0.538**
Soil C (%)	0.070	0.208	0.331	−0.157	**0.816**	**1**	0.191
Soil moisture (%)	0.009	0.136	0.267	−0.277	**0.538**	0.191	**1**

Values in bold are different from 0 with a significance level alpha = 0.05.

weir, and distance from outflow was conducted (Table 4). Analysis of all samples revealed that distance from outflow was the primary driver of IPCA1 while no primary driver was identified for IPCA2. Several secondary drivers of community structure were indicated by ordination figures and correlation analysis. Distance from outflow and weir were found to negatively influence IPCA1, while soil C and soil moisture had some positive influence on IPCA2. Many of the same trends in relationships between environmental parameters and microbial community structure paralleled those between environmental parameters and microbial abundance.

4. Discussion

4.1. Microbial abundance

This study evaluated the abundance of denitrification genes *nosZ, nirS, and nirK* and community composition throughout an agricultural drainage ditch to determine impacts to the ephemeral aquatic ecosystem following weir implementation. Different scales of sampling were investigated to determine the most appropriate regime to identify spatial patterns of microbial communities for

future investigation of multiple systems. Microbial abundance and composition and soil parameters were measured to determine if those parameters were influenced by weir proximity compared to more distal sampling locations. This data was also collected to identify relationships between soil parameters and the presence of denitrification functional gene abundance. Abundance of 16S rRNA genes ranged from 7.5×10^8 to 9.4×10^{10} (gc/dry g soil); values are comparable to results documented in previous investigations of soil microbes in a variety of different environments, including agricultural soils (Dandie et al., 2007), bioretention cells receiving urban runoff (Chen et al., 2013), and in constructed wetland soils receiving swine wastewater (Dong and Reddy, 2010). Similarly, results of *nosZ* and *nirS* concentrations, ranging between 1.9×10^5 and 2.8×10^8 (gc/dry g soil), were comparable to previously-documented abundances in agricultural soils (Dandie et al., 2007) and ephemeral wetland soils (Ma et al., 2008). Caution is warranted due to the nature of primer development, that primers may not amplify the full diversity of genes present in an environmental sample. Furthermore, the amplification of *nosZ* genes does not reflect whether or not microbes are capable of complete denitrification, i.e. if they are atypical *nosZ* genes (Sanford et al., 2012). Functional gene *nirK* was found below detection limits possibly due to 1) a lack of *nirK* genes in the soil, 2) inhibition of gene amplification as a consequence of soil properties, or 3) *nirK* gene primers not successfully amplifying gene fragments due to the high variability associated with *nirK* genes and their respective microbial populations. Successful amplification of the *nirK* functional gene in environmental samples has been reported (Henry et al., 2004; Dandie et al., 2011); however, the aforementioned studies reported soil properties with clay contents lower than those observed in the drainage ditch in this experiment. It has been well documented that the presence of humic substances, organic matter, and clay particles can bind nucleic acids and interfere with extraction procedures (Trevors, 1996; Hurt et al., 2001; England and Trevors, 2003). Dandie et al. (2011) also reported that amplification of *nirK*-bearing denitrifiers using targeted primers was unsuccessful even after exhaustive optimization of qPCR conditions and cycling parameters. Braker et al. (2000) similarly reported unsuccessful amplification of *nirK* genes in environmental samples from the Puget Sound, stating that amplification failures may have owed to a lack of relatedness between environmental genes and those used in primer design.

In previous studies, *nirK* primers were developed based on limited sequence information. It is likely that those gene fragments in the microbial populations in this study may be more distantly related from those used in the development of the *nirK* primers, rendering them undetectable. As a result of the cultivation-independent nature of most denitrification studies (Braker et al., 2000; Priemé et al., 2002), the development of *nirK* primers has been based on only a few sequences available at the time and have been tested on limited type strains. Most environmental studies produce partial sequences from unknown bacteria because they are not cultivated. More recently, a cultivation-dependent study of denitrifying bacteria reported that of 227 cultivated denitrifiers, 109 did not render an amplicon using five different primer sets (Heylen et al., 2006), further demonstrating the unsuitability of *nirK* primers as broad range amplification primers.

4.2. Sampling regimes

Statistical differences observed in *nosZ* genes and EBK results of increased gene abundance at one of four weirs for all genes indicate that a combination of all three sampling scales would be most effective in capturing community abundance and composition variability throughout the length of the ditch and within reaches, including influences of weirs. Results identified general increases in measured soil parameters as distance from the outflow increased, but within reaches of the system, general decreases in soil parameters were observed at close distances to weirs. Spatial kriging also highlighted increases in 16S rRNA gene abundances, as well as increases in *nosZ* and *nirS*, upstream of weirs, indicating that community distribution and composition may be impacted by the presence of weirs. Enwall et al. (2010) used varying distances for sample spacing in denitrifying community investigations, reporting ranges between 167 and 450 m, while Philippot et al. (2009) identified the presence of spatial autocorrelation within microbial communities at distances between 6 and 16 m. Points collected for this study were sampled at distances (5–100 m) which were encompassed by both studies; however, it should be noted that environments studied in both aforementioned articles were not conducted in directly comparable environments. Findings of the aforementioned studies and this investigation indicate that utilizing a sampling scale representative of the surrounding landscape heterogeneity will aid in identifying spatial patterns of soil microorganisms. By utilizing a combined sampling regime at multiple scales (catchment, reach, and management), within-ditch heterogeneity was successfully captured without being too sensitive (i.e. significant differences between all sampling points) or not sensitive enough (i.e. no observed spatial patterns). As patterns of denitrifying microbes were identified in relation to ecosystem management strategies and in a relatively unique environment, there is practical application for continued investigation of microbial communities within similar systems.

The abundance of denitrification functional genes relative to 16S rRNA, along with overall increases in microbial abundance upstream of weirs, provides evidence that weirs have the potential to effect proximal environments in such a way that is beneficial for denitrifying communities. As hydrology is considered the single most influential factor determining wetland characteristics (Mitsch and Gosselink, 2007), effects on water velocities and the enhancement of inundation following weir implementation are likely driving physiochemical changes in the immediate environment. When drainage ditches are not inundated, the presence of oxygen in aerobic soils inhibits the *nirS* enzymatic product, nitrite reductase, while the presence of NO_3 has been found to induce production of nitrite reductase (Maier et al., 1999). Nitrous oxide reductase, the product of *nosZ*, is even more oxygen-labile, while low pH has also been identified as an inhibitor to the enzyme production (Maier et al., 1999). Inundation of drainage ditches can neutralize pH and create anaerobic soils; oxygen-limited anaerobic soils would facilitate *nirS* and *nosZ* enzymatic production for the reduction of NO_3 and N_2O, respectively. While inundation has the potential to foster biogeochemical changes that provide more suitable acute habitat conditions for denitrifying microbes, sufficient amounts of C must also be available for denitrification to occur.

4.3. Environmental factors

General positive correlations between soil C and N with all genes also support the notion that environments where abundances and diversity are greatest, behind weirs, are representative of more wetland-like biogeochemical characteristics. Average amounts of C and N were 0.96% and 0.11%, respectively. These values are comparable to those found in mitigation wetlands in Piedmont, VA, USA, in which C and N both ranged between 1.1 and 2.2% (Ahn and Peralta, 2009). Values were slightly lower than those found in a constructed wetland near the study site in the Mississippi Delta, which reported that C and N both ranged between 1.5 and 2% (Weaver et al., 2012). However, C values were noticeably

lower than investigations of created wetlands in Spain, which found C to range between 3.3 and 13.5% (García-Lledó et al., 2011). Despite comparable soil N amounts, results from Baker et al.(in review) confirm that substantially more N is entering these systems from the agriculture landscape, and it's likely that high velocities of most runoff are insufficient for diffusion of N from the water column to the pore water to occur. This issue is emphasized by the fact that even if water were allowed time for diffusion, C could then become limiting. Bastviken et al. (2005) reported optimal C:N ratios in respect to cattails (*Typha* spp.) of 5:1 in wetlands, while an experiment targeting denitrifying bacteria reported optimal C:N ratios for initial NO_3 concentrations of 25, 50, 100, and 200 mg/l to be 5.5 ± 0.2, 4.5 ± 0.2, 4.0 ± 0.1, and 2.6 ± 0.1, respectively (Chiu and Chung, 2003). In the current study, C:N ratios were approximately 9:1, however, it's unknown whether the C is organic or bioavailable to microbial communities. Carbon limitation of denitrification could be prevented by adding a C source and further increasing hydraulic residence time. While high flows limit the applicability of bioreactors in this environment, it may be suitable to construct a controlled drainage structure made out of woody material; hence a C filter to foster denitrifying microbial activity. If acute physiochemical controls over denitrification induce stress on microbes over a long period of time, chronic stress will have a different impact on microbial communities.

Ephemeral drainage ditches experience annual hydrologic pulsing, as the Mississippi Delta region has a subtropical climate, receiving 128 cm of rainfall each year. Physical disturbances in the form of pulsing may result in chronic stress on organisms. Because of the molecular scale of microorganisms, primary effects are physiological, not physical (Schimel et al., 2007). While physiological changes likely regulate short-term responses to stress in soil communities and processes, over longer periods, shifts in microbial community composition are likely to regulate those (Schimel et al., 2007). Shifts would favor those that could adapt to variable environmental conditions and out-compete other microbes. Denitrification is considered a facultative anaerobic microbial process, in which an organism can gain energy through aerobic respiration and anaerobic respiration in the absence of oxygen, giving them an adaptive advantage over organisms, such as obligate aerobes or obligate anaerobes, which are only capable of one or the other of the aforementioned respiratory processes. This adaptive advantage lends credence as to why these denitrification functional genes are conserved throughout the general microbial population, exhibiting high phylogenetic diversity. More than 60 genera harbor denitrifying species; predominant denitrifiers include multiple *Psuedomonas (P.denitrificans, Pseudomonas aeruginosa, and P. stutzeri), Staphylococcus (S. meliloti), Alcaligenes (A. cycloclastes), Rhizobium sp*., and even archaea and fungi have been found to possess these genes (Zumft, 1997; Philippot et al., 2007). An investigation of hydrologic pulsing in wetlands reported that no distinct influences on the community structure of denitrifying bacteria was observed (Song et al., 2010), speculating that water-stress-tolerating denitrifying microbes could be selected by natural succession and competition in the system. Song et al. (2010) also observed that *nirS* gene copy number increased or remained constant during drying periods and dropped during re-flooding. Results from the aforementioned studies and the current study indicate that denitrifying microbes possess physiological adaptations that may allow them to withstand long-term stress from hydrologic pulsing disturbances. As periodic inundation occurs throughout the entire ditch thalweg during heavy rain events, this also offers support as to why *nosZ* and *nirS* functional genes were found throughout the ditch and why differences were not observed between sampling regimes. Positive correlations between denitrifying microbial abundance and diversity with proximity to weirs could, therefore, be indicative of extended inundation relieving chronic stress from pulsing events on microbial communities. Reducing chronic stress could decrease competition and foster more stable habitats for continued population growth and diversification.

5. Conclusion

This investigation allowed for the development of an appropriate sampling regime for the further investigation of microbial communities in drainage ditches. Utilizing a combination of all sampling regimes was found be to most effective in capturing microbial community patterns without capturing too much noise from environmental heterogeneity. The proximity of weirs was found to influence abundance and composition of microbial communities, and relationships between soil C, N, and microbial communities were identified. The potential for wetland-like characteristics to be recovered in agricultural drainage systems was also highlighted, suggesting that implementing weirs in these systems alters soil moisture and enhances conditions for N remediation. While weirs show the potential to foster microbial abundance and diversity for denitrification, further research utilizing N isotope tracers is warranted to determine if hydrologic variables, such as flow, are restricting N transport from agricultural runoff to drainage ditch sediments for microbial denitrification. Furthermore, the identification of microbes harboring denitrification functional genes in these systems which have been experiencing long-term pulsing disturbances offers a unique opportunity to examine microbial community structures and determine species that are predominant.

Acknowledgments

The authors would like to gratefully acknowledge the College of Forest Resources at Mississippi State University, Delta F.A.R.M., and study location land owners. Authors would also like to thank the Mississippi Agricultural Forestry and Experiment Station, and Forest and Wildlife Research Center for support.

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