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RESEARCH ARTICLE

Spatial Variation of *nosZ*-communities in the Low Oxygen Waters of Prado Hydroelectric (South-West of Colombia)

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Abstract: Denitrification is a process of reduction of nitrate to dinitrogen by facultative anaerobic microorganisms, which have functional genes encoding denitrification enzymes (reductases). The *nosZ* gene encoding the enzyme that reduces nitrous oxide to dinitrogen was utilized in this research, as molecular marker for denitrifying communities into low oxygen waters of Prado reservoir. Our objective was to analyze and compare the composition (richness and abundance) of *nosZ*-type denitrifiers in relationship with physicochemical variables (oxygen, pH, temperature, nitrate, nitrite and ammonium) in three areas of this dam: Isla del Sol, Lozanía and Tomogó which are distant and have different anthropogenic influences. For this, we performed DNA extraction, amplification, 454 pyrosequencing and phylogenetic analysis of *nosZ* gene. The Chao1 estimator and Shannon index were used for compare richness and diversity of *nosZ* gene; and the relationship between compositions of operational taxonomic units (OTU) with physicochemical variables was established by canonical correspondence analysis (CCA). In the reservoir 45 *nosZ*-OTUs to species level (80% similarity) were detected. Lozanía had the highest number of OTUs (25) and greatest diversity (S_{CHAO1}: 35; H: 2.0), compared to the other areas sampling. The phylogenetic analysis showed the presence of many OTUs (28 of 45) with low similarity to *Proteobacteria* group and high similarity with sequences of environmental clones reported previously. The CCA showed that the *nosZ*-community composition of Prado dam was related with low pH (6.2), oxygen (0.01mg / L) and nitrate (<0.25 mg / L) recorded in the water column.

Keywords: 454 sequencing, Dam, Denitrifiers, Diversity, Functional gene, Microbial community, *NosZ*gene.

INTRODUCTION

In the natural environments, the nitrate and nitrite is removing by a microbial process known as denitrification, in which dissimilatory oxidized nitrogen is used as an alternative electron acceptor for energy production when oxygen is limited (Zumft and Kroneck 2007). Denitrifying microorganisms are in environments where three conditions exist: (Alldredge and Cohen 1987) low oxygen levels, (American Public Health Association 1999) availability of nitrate (NO_3^-) and nitrite (NO_2^-), and (Baxter *et al.* 2013) availability of organic matter (OM) (Correa-Galeote *et al.* 2013). The denitrifiers are known to belong to more than 50 genera of bacteria, including members of the groups: *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroides*, and *Planctomyces*, however, some Archaea and Fungi can denitrify too (Zumft and Kroneck 2007).

Among the genes of the denitrification, the *nosZ*-gene encoding the nitrous oxide reductase is one of the molecular marker most widely implemented for phylogenetic and ecological analysis of denitrifying communities from natural environments (Braker *et al*. 2012, Jones and Hallin 2010). It has recently been established that 80% and 86% similarity of DNA and amino acid sequences respectively of *nosZ* gene, allows detection of the OTUs in different habitats (Palmer *et al*. 2009). Many studies attribute the similarity of *nosZ*-gene sequences mostly to bacteria of *Proteobacteria* group (Jones *et al*. 2013, Wyman *et al*. 2013). However, Jones *et al*. (2013) and Sanford *et al*. (2012) discuss the presence of

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nosZ gene into a wide range of groups of high abundance in different ecosystems with structural variations of gene. The *nosZ*-gene study has allowed to analyze denitrifying communities related with the reduction of nitrous oxide to dinitrogen in aquatic systems such as ocean (Zehr and Kudela 2011), oxygen minimum zones (Wyman *et al*. 2013, Castro-González *et al*. 2015), estuaries (Chon *et al*. 2011), streams (Baxter *et al*. 2013) and wastewater (Hou *et al.* 2012). However, it is important to study these organisms in eutrophic lakes and reservoirs where the denitrification plays an important role in the balance of N_2O production/reduction which can determine if such areas are source or sink of this greenhouse gas (Wang *et al.* 2013, Yu *et al.* 2014).

In Colombia, there are currently 26 dam in operation, and among them the Prado Reservoir is the fourth largest. This reservoir is a multi-purpose (electricity, irrigation, aquaculture, fisheries, recreation and tourism) tropical dam constructed for water storage of the Cunday and Negro Rivers and other minor tributaries in the south west of Colombia (Guevara *et al.* 2009). Likewise, this reservoir provides drinking water for the population despite its water quality being strongly influenced by agricultural activities and domestic discharge. This reservoir was constructed in 1973, it has an average depth of ~45 m and a surface area of ~3.900 hectares covered with water during all the year. Because of the high organic load and the flooded vegetation not removed during its inundation the Prado Reservoir has high H_2S and ammonia production in the hypolimnetic anoxic environment and it has been classified as hyper-eutrophic (Ducharme 1975, Márquez and Guillot 2001, Roldán 2003). In the surface waters Guevara *et al*. (2009) have reported alkalinity from 17.95 to 39.38mg/L, phosphates between 0.01–0.30mg/L, conductivity from 48.73 to 67.06 mS/cm and pH between 6.42 - 8.48. Recently, Castro-González and Torres *et al.* (2015) reported a strong oxycline (151 - 4 µM O_2) and variability of nutrients along the column water (between 0-10 m depth) with levels of nitrate and ammonium between 2.6 - 36 µM and of 2.8 to 50 µM respectively. Taking into account all these characteristics, the Prado dam is an ideal place to explore aspects about denitrifiers diversity, given its eutrophic condition, thermal stratification, and hypoxic/suboxic water column (Guevara *et al.* 2009, Perea and Villanueva 2010). Until today, in this reservoir there have been studies about fishes (Villa and Lozada 1999, 2004), zooplankton, phytoplankton (Guevara *et al.* 2009) and bacterioplankton (Canosa and Pinilla 2007), but not related to the diversity of denitrifying microorganisms, excepting a recent study developed by Castro-González (2014) about the TRFLP diversity of *nosZ*-denitrifying communities between hypoxic and suboxic conditions in the water column at Isla del Sol. For this, the goal of this study was to determine and compare the composition (richness/abundance) of *nosZ*-type denitrifiers in three places of this dam, through PCR and 454 pyrosequencing of this functional gene. Also, we analyzed the relationship between denitrifying community composition with the physicochemical variables (oxygen, pH, temperature, nitrate, nitrite and ammonium) prevalent in the water column of this hydroelectric.

MATERIALS AND METHODS

Study Sites, Sample Collection and Total DNA Extraction

The sampling was done in the Prado dam in the south west of Tolima, which is one of the biggest reservoirs in Colombia (1.254 Ha), characterized by its eutrophic conditions, physical-chemical stratification, low oxygen and high levels of organic matter and nutrient in the water column in contrast with others Colombian hydroelectric (Canosa and Pinilla 2007, Guevara *et al.* 2009, Márquez and Guillot 2001, Perea and Villanueva 2010, Roldán 2003).

The water samples were taken in September 2012 (dry season) from three places: Isla del sol (3°45'N - 74°51'W), Lozanía (3°52'N - 74°48'W) and Tomogó (3°43'N - 74°53'W), between 6-9 m depth where low oxygen levels were detected during sampling (Fig. **1**). These sampling areas were selected taking into account previous reports about spatial heterogeneity of limnological variables caused by differences in the influence of rivers, small tributaries and anthropogenic activities related with changes in nutrient concentration, water temperature, transparency, conductivity and pH (SODEIC 1993, Guevara 1993 *et al*. 2009).

Temperature, pH and dissolved oxygen were measured on board with a waterproof Oakton® multi-parameter. Eight liters of water were maintained under refrigeration until collecting the microorganisms on membranes by vacuum filtration. On board, 350 mL of freshwater samples were sampled, filtered (through GF/F membranes) and frozen for nutrient analyses. The concentration of ammonium (NH_4^+) was determined by Kjeldahl method, and the dissolved nitrate (NO_3^-) and nitrite (NO_2^-) were measured to be 220-275 nm y 510 nm respectively, with spectrophotometric techniques following the Standard Methods for the Examination of Water and Wastewater (American Public Health Association 1999).

Water samples (8 L) were filtered sequentially through Millipore membrane filters of 20 µm, 8 µm and 0.45 µm

pore size. The last one membrane was used for bacterial DNA extraction with the PowerWater® DNA isolation kit (MoBio Laboratories, Inc., CA, USA). The DNA extracts were visualized with etidium bromide on 0.9% agarose gels and were quantified by spectrophotometry 260/280 in a nanodrop.

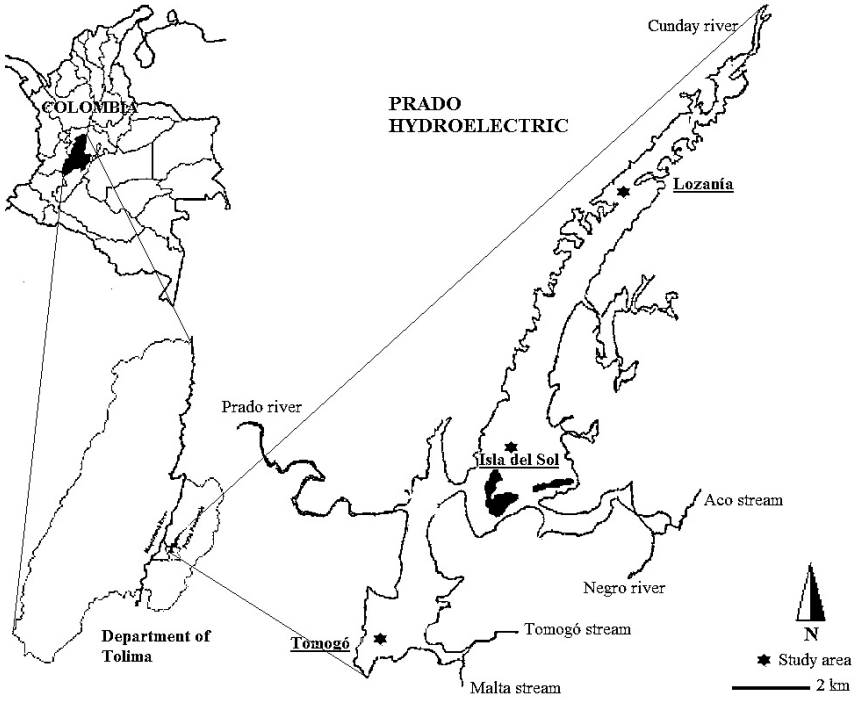

Fig. (1). Sampling areas in the Prado dam at south west of Tolima, Colombia.

Pyrosequencing and Sequence Filtering

Tag pyrosequencing of *nosZ* genes was used to study the microbial communities with the potential for N_2O reduction to N_2. DNA (10 µl, 20 ng/µl) from each sample was sent to the Research and Testing (R&T) Laboratory (Lubbock, TX). PCR amplification was performed using primers *nosZ*F and *nosZ*1622R (Kloos *et al.* 2001). Sequencing reactions utilized a Roche 454 GS-FLX+ instrument (Roche, Indianapolis, IN) with titanium reagents. Each study area had its specific barcode. The raw sequences processed and denoised by R & T Laboratory, where they were checked for elimination sequences with incorrect barcode and primers. After, a validation of sequences was performed according to Mao *et al*. (2013) and Palmer *et al*. (2012). As a result of this validation about 43% of sequences were eliminated and a total of 6697 sequences were used for the analysis (see Table **1**). In this process chimeras and duplicates were eliminated with USEARCH/UCHIME (Edgar *et al.* 2011) and DEREPLICATOR using the Fun Gene Pipeline (Fish *et al.* 2013). Ambiguous sequences or low quality regions (with Q-values less than 20) and sequences <350 bp were eliminated with Bio Edit v 7.1.11 (Hall 1999). Finally, no coding sequences for nitrous oxide reductase were eliminated with Blast2 go v2.6.6 (Conesa *et al.* 2005), using the non-redundant protein sequence database with an E-value cutoff of 0.001 (Mao *et al.* 2013).

Table 1. Steps followed for validation of denoised sequences obtained from Research and Testing laboratory.

Steps to validation of sequences	Sequences	
	#	%
Initial total number of sequences denoised following the R&T processing.	11.717	100
After checking for chimeric sequences	9.891	84,4
After checking for presence of ambiguous nucleotides	9.061	77,3
After checking sequences coding for *nosZ* gene	8.460	72,2
After checking for sequences <350 bp	6.740	57,5
After eliminate duplicate of sequences	6.697*	57,1

* A total of 6.697 sequences were validated for the analysis: 3.184 sequences for Lozania station (L6), 2.890 sequences for Tomogó station (T9) and 623 sequences for Isla del Sol station (IS7).

Composition and Diversity of *NosZ*-type Denitrifiers

The valid sequences were clustered as operational taxonomic units at species-level threshold distances of 20% (Palmer *et al.* 2009) based on DNA sequences. The OTUs were defined with DOTUR program [Distance-Based OTU and Richness (Schloss and Handelsman 2005)] using furthest neighbor assignment algorithm with 1.000 iterations. Later, *nosZ* sequences within OTUs that started with the barcode assigned to Tomogó, Isla del Sol and Lozanía were selected for recognizing the OTUs generated for each area. The coverage percent (%) of OTUs for the number of valid sequences, was calculated according to Good (1953) in Mao *et al*. (2013). The CHAO1 richness estimator, Shannon weaver diversity index and the Bray-Curtis similarity index were calculated with PAST v 3.0 (Hammer *et al.* 2001).

The phylogenetic tree was constructed with MEGA v 5.2.1 (Tamura *et al.* 2011). We selected 45 *in silico*-translated amino acid OTUs representative sequences, and multiple alignments were performed among OTU-sequences and *nosZ*-sequences from several environment (soil, sediment and water column) and bacterial cultures reported in the GenBank (http//:www.ncbi.nlm.nih.gov). The tree was reconstructed by the neighbor-joining algorithm using p-distances and bootstrap resampling based on 10.000 replicates, according to Palmer *et al*. (2012). The *Haloarcula marismortui* strain ATCC 43049 (AY596297) was used as the out-group sequence. All original 454 sequences were archived at NCBI Sequence Read Archive under accession SAMN03402215, SAMN03402334, SAMN03402335.

Statistical Analysis

The Shapiro-Wilk W test was used to estimate the normal distribution of OTUs abundances data in each study area, and the Kruskal-wallis test was used to estimate the statistical difference of *nosZ* genes abundances among study areas; using the PAST v 3.0 (Hammer *et al.* 2001). To evaluate the effect of physical-chemical variables in *nosZ*-OTU distribution, we used the canonical correspondence analysis (CCA) using the software Canoco for Windows 4.5 (Microcomputer Power, USA) (Ter Braak *et al.* 2002).

RESULTS

Physicochemical Variables

Among study areas the water average temperature was 26,4ºC, Isla del Sol registered the highest value (26,8°C) when compared with the others areas. The average pH in the sampling areas was 6,37 and Tomogó registered the highest value (6, 66) when compared with the others areas (with a pH of 6.2). The contents of O_2, NH_4^+, NO_3^- and NO_2^- showed great spatial differences among study areas. In the tree areas low levels of O_2 were recorded, but Tomogó had a high O_2 level (3,4mg/L) and Lozanía the lowest (0,01mg/L). Highest values of NH_4^+ (0,88mg/L) were reported in Tomogó, and the highest NO_3^- (0,78mg/L) and NO_2^- (0,079mg/L) levels were registered in Isla del Sol and Lozanía, respectively (Table **2**).

Distribution, Abundance and Diversity of *NosZ* OTUs

The sequences analysis showed that Lozanía had the highest number of sequences (3,184), followed by Tomogó (2,890) and Isla del Sol (623). From them 45 species-level OTUs were obtained by grouping to 80% of similarity. The greatest OTUs richness was observed in Tomogó (28) of which 18 were area-specific, followed by Lozanía (25) of which 10 were area-specific and finally Isla del Sol (12) of which two were area-specific. The data indicated that among study areas 5 OTUs were shared, except between Isla del Sol and Tomogó that did not share OTUs (Fig. **2A**). The average percentage of coverage, estimated with the number of OTUs (represented by one sequences) and the total number of sequences was 99,6%, indicating that the number of valid sequences used in this study was sufficient for the diversity, phylogeny and statistical analysis.

Approximately 65% of the *nosZ* sequences of Isla del Sol, Lozanía and Tomogó, were grouped into 2 or 3 OTUs (Fig. **2B**). The OTU01 was the most abundant (62%) in Isla Del Sol, the OTU27 was the most abundant (30%) in Lozanía and OTU21 was the most abundant (40%) in Tomogó.

Measurements of *nosZ*-OTUs diversity (CHAO1 estimator and Shannon index) from Lozanía and Tomogó were consistently higher than those of Isla del Sol; and the Bray Curtis similarity index was 0, 03 and 0,07 between Tomogó-Isla del Sol and Tomogó-Lozanía, respectively, suggesting differences among stations (Table **2**). The Shapiro-Wilk W test and the Kruskal-Wallis anova established significant differences between Isla del Sol and Lozanía (p-value: 0,003) and between Isla del Sol and Tomogó (p-value: 0, 0002).

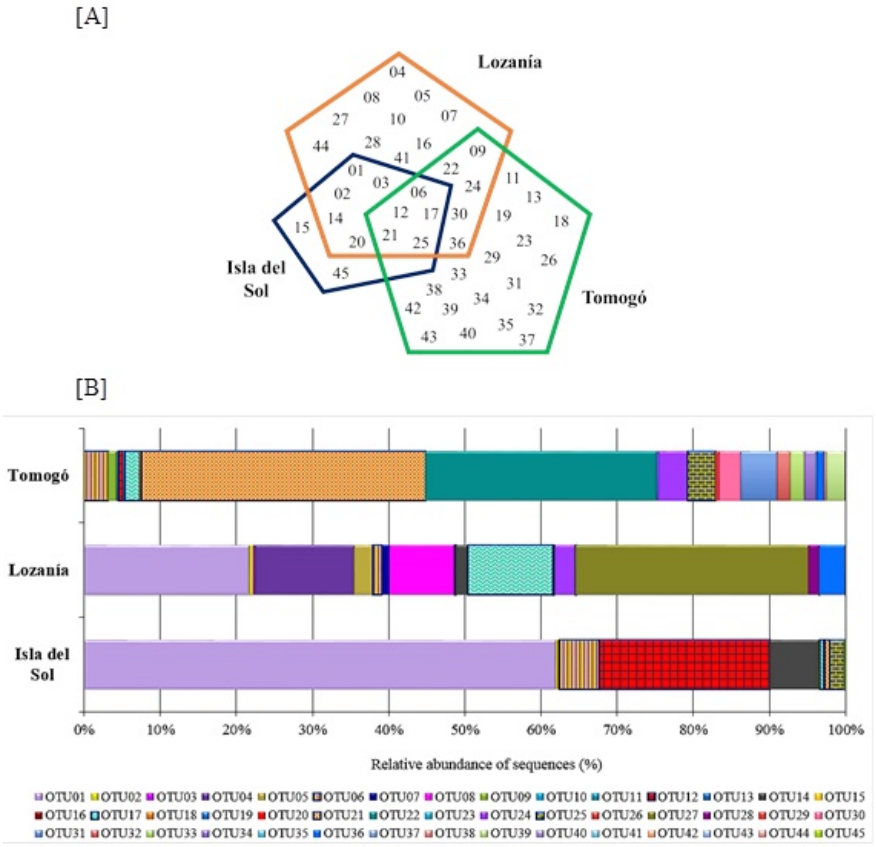

Fig. (2). Composition of *nosZ*-type OTUs from study areas of Prado reservoir (**A**): Number of *nosZ* OTUs founded in the study areas. (**B**): Relative abundance of *nosZ* OTUs between study areas.

Phylogenetic Analysis of *nosZ* OTUs

Phylogenetic analysis was done with 45 representative sequences of OTUs. In the phylogenetic tree two clusters were generated (Fig. **3**): the cluster one included 53% of the *nosZ*-OTUs sequences (OTU02, OTU6, OTU15, OTU33 and OTU34) which shared 60%-100% similarity with sequences of clones from soils, sediments, sewage and activated sludge. The 47% of remaining representative sequences had 80%-100% similarity with sequences of microorganisms isolated and cultured. Some of these OTUs were 80%-100% similar with *Proteobacteria nosZ* sequences, such as OTU21 with *Alcaligenes faecalis* A15 (100%); OTU04, OTU08 and OTU11 with *Bradyrhizobium* sp. TSA44 (80%, 90% and 87%); and OTU21, OTU22 and OTU25 with *Pseudomonas brassicacearum* PD5 (90% or 88%).

The cluster 2 grouped 9 OTUs the most of which had low similarity with *Proteobacteria* group (<50% similarity). Some OTUs (OTU03, OTU09, OTU10, OTU12 and OTU31) had a 40% and 60% similarity with *Azospirillum* sp TSO5. All OTUs showed <45% similarity with sequences of clones isolated from soil, seawater, freshwater and sediment.

Canonical Correspondence Analysis

In the analysis of the relationship among the composition of the *nosZ* community and the physicochemical variables in each study area, we observed that the studied environmental variables (oxygen, pH, nitrite and nitrate) explained the structure of the *nosZ*-community (Fig. **4**). Both axes explained 100% of total variation. The first axis explained 67, 3% of variation and was dominated by pH (weight correlation: 0, 98), O_2 (weight correlation: 0, 96) and NO_2^- (weight correlation: -0, 98). The second axis explained 32, 7% of variation and was dominated by NO_3^- (weight correlation: 0, 88) and temperature (weight correlation: 0,8).

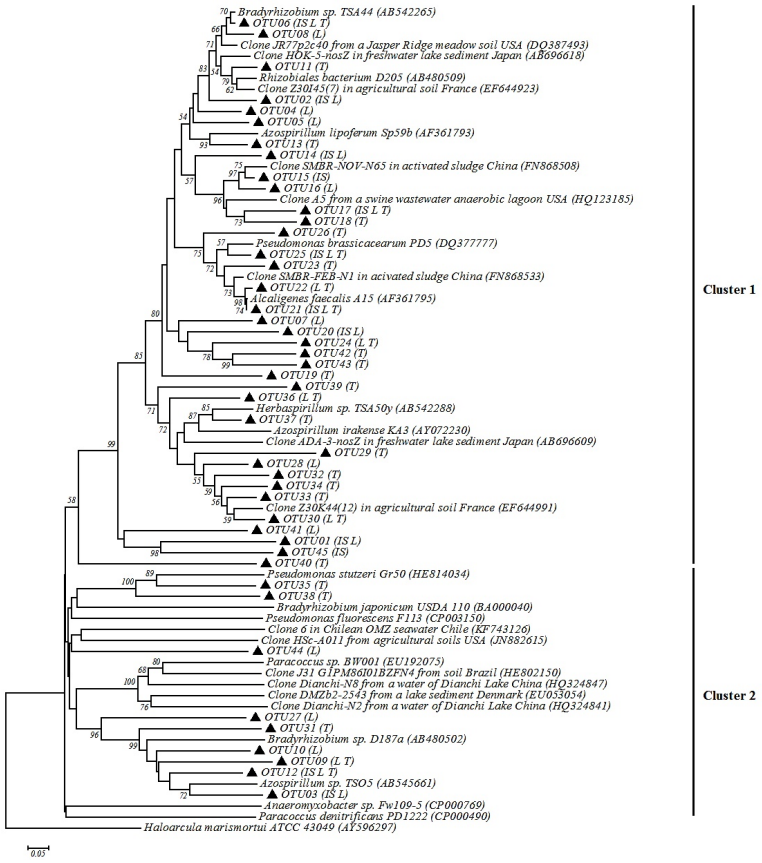

Fig. (3). Phylogenetic tree with the *nosZ* representative sequences (OTUs) from Prado reservoir water column Study areas are shown in parentheses (IS: Isla del Sol; L: Lozanía; T: Tomogó). The scale bar indicates the number of nucleotide substitutions per site.

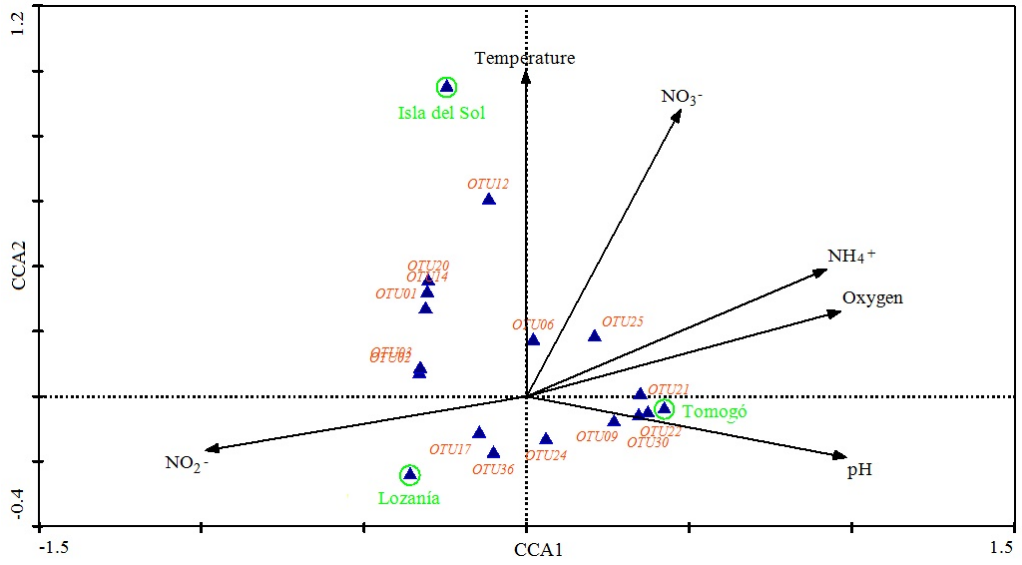

Fig. (4). Canonical correspondence analysis (CCA) of OTUs data and physicochemical variables in the three areas. Eigenvalues (500 runs), 0.703 and 0.342 for dimensions CCA1 and CCA2, respectively.

We observed that *nosZ*-communities composition from Isla del Sol were related with high NO_3^- and temperature levels, the communities composition in Tomogó were related with high pH and NH_4^+ levels, and intermediate oxygen level and the *nosZ*-community composition from Lozanía was associated with low O_2 and $NO_3^-$and higher NO_2^- level.

DISCUSSION

This is the first study in one Colombian's dam whose goal was explore the spatial variation of denitrifying communities using the *nosZ* functional gene that codes for nitrous oxide reductase. The values of temperature, pH and nitrate reported in the surface water at the study areas (Isla del Sol, Lozanía and Tomogó) are slightly lower than those reported previously (Canosa and Pinilla 2007, Castro-González 2014, Perea and Villanueva 2010) supporting the temporal variability recorded in the area (Guevara *et al.* 2009). Although profiles along water column of O_2 and nutrients are very limited for this dam, the oxygen, nitrate and ammonium levels quantified during this study support the hypoxic (even suboxic) and eutrophic condition of this reservoir which has been reported several decades ago (Ducharme 1975, SODEIC 1993, Roldán 2003)

The molecular analysis indicated that Lozanía contributed with a high number of sequences (3, 184), followed by Tomogó (2, 890) and Isla del Sol (623). Likewise, the richness and diversity analysis showed high values at Lozanía and Tomogó, and low values at Isla del Sol. Both results suggested spatial variation of *nosZ* denitrifying communities in the Prado dam. This data are related with the previous report of Canosa and Pinilla (2007) whose quantified low number of heterotrophs in the center of the dam in comparison with other stations, which was associated with lower chlorophyll level. Our results are interesting if we consider that previous studies had also reported spatial variability in dissolved organic carbon (DOC) availability which is a key substrate for the heterotrophic activity (Guevara *et al.* 2009). In this sense it is notorious that Guevara *et al*. (2009) recorded the highest DOC values at Tomogó (up to 11 mg/L), followed by Lozanía (9 mg/L) and by Isla del Sol (8mg/L) which are in agreement with our observations about a higher *nosZ* -diversity in the first two places where low O_2 levels plus enough organic substrate might be facilitating its metabolic activity.

Also, the turbidity could be another key factor in the study area because denitrifying microorganisms usually are associated to particles in suspension, where they found reduced niches required to sustain suboxic processes such as denitrification (Alldredge and Cohen 1987). In this sense, it is possible that the high values of turbidity reported for Lozanía and Tomogó by influence of tributaries (Guevara *et al.* 2009) could also be related with the higher values of diversity registered in both areas by particle-attached communities in comparison with Isla del Sol where the low levels of particles in suspension could be related with a free-living *nosZ*-denitrifying community less rich and abundant. In this scenario, the formation of microhabitats as well as the development of segregated niche in the water column of this reservoir could be taking place such as has been proposed for other aquatic environments (Jones *et al*. 2013, Wang *et al*. 2013).

For other hand, although our analysis suggested that the number of sequences used in this study were optimal for the number of OTUs detected in a 99,6% (Palmer and Horn 2012), one has to bear in mind the biases associated with the *nosZ*-gene amplification and sequencing because they may be influencing the differential amplification of some OTUs in each study area as has been reported by Magalhães *et al*. (2008) and Throbäck *et al*. (2004). Another possible explanation depends on how many *nosZ*-sequences are detected by our primers, in each study area; and whether the denitrifying genes are present or not with enough abundance. However, the low diversity detected at Isla del Sol in this study is consistent with previous results where a similar number of OTUs were detected to 9 m depth through *nosZ*-TRFLP analysis with other primers (Castro-González 2014).

This is one of the first studies which reports *nosZ*-OTUs generated with a 80% similarity between sequences obtained from water column of a dam and although this distance has been recommended for soil studies (Palmer *et al.* 2009) in this case it was successful to give us a good idea of *nosZ*-denitrifying diversity. Our results indicated that the OTUs richness founded in the Prado reservoir was similar to these reported from soils, where between 10 to 53 OTUs had been generated (Depkat-Jakob *et al.* 2012, Palmer *et al.* 2012); which suggest that in the column water of eutrophic environments can also inhabit a high diversity of organisms. In comparison with reports about *nosZ*-diversity in freshwater sediments (Huang *et al.* 2011, Jones and Hallin 2010, Magalhães *et al.* 2008, Palmer and Horn 2012) we detect a similar diversity, however, there are no studies about diversity of denitrifying communities in the water column of other dams or reservoirs for comparison. Likewise, our data suggest the coexistence of a variety of *nosZ*-communities to suboxic depths (6-9 m depth) in the Prado dam, which have the gene that code for reductase of nitrous oxide with the potential to develop N_2O reduction; however, it is necessary to develop more studies to determine the active transcription and functionality of this gene in the sampling areas.

Among the study areas, 5 OTUs (06, 12, 17, 21 and 25) were present in all areas and others OTUs were specific of each area (30 OTUs). This data suggest that in this dam we can observe organisms generalists and specialists such as

has been recently proposed for denitrifiers by Bowen *et al*. (2013) where the generalists can inhabit in a wide range of habitats unlike specialists. We observe the presence of many OTUs with low number of sequences (36 of 45 OTUs) which also has been reported in other studies as "rare biosphere" (Bowen *et al.* 2013, Huse *et al.* 2010, Kalscheur *et al.* 2012). This information suggest that several factor prevalent in the reservoir could favor the differentiation of ecological niches for different types of microorganisms, such as a wide range of substrates available, an intricate functional and ecological network between denitrifying bacteria, greater biological effectiveness (present only in specialists) and a great adaptive and evolutionary capacity, as has been proposed by others authors (Koeppel and Wu 2012, Logares *et al.* 2013, Matulich 2013).

The phylogenetic analysis showed that a few OTUs were 100% similar to previously reported sequences of *Alcaligenes faecalis* A15, *Bradyrhizobium* sp. TSA44 and *Pseudomonas brassicacearum* PD5. Our data are in accordance with preliminary reports about of the *Proteobacteria* that are ubiquitous and widely distributed in reservoirs, lakes, hot springs, activated sludge and soils (Hou *et al.* 2012, Wang *et al.* 2013, Yu *et al.* 2014). In this regard, in the Baiyangdian lake sediments and at Dianchi lake water column, affiliations of OTUs to *Rhizobiales* group (Wang *et al.* 2013, Wen *et al*. 2012) were recorded. Likewise, in the water column of Lake Dianchi (Wen *et al.* 2012) and wastewater (Hou *et al.* 2012) has been reported affiliation of OTUs with *Burkholderiales* and *Pseudomonadales* groups, respectively.

The presence of several OTUs (for example OTUs 45, 41, 15) with low similarity to *Proteobacteria* and high similarity to sequences of environmental clones reported previously, suggest that in the Prado dam a *nosZ*-community exists which is phylogenetically different and unique such as that been recorded in other natural environments (Bowen *et al*. 2013, Jones *et al.* 2013, Jones and Hallin 2010). This data indicate the coexistence of different bacteria in the reservoir with the potential to perform the reduction of N_2O to N_2, probably generating a functional redundancy that stabilizes the denitrification process under changing conditions (fluctuations of O_2 and nutrients), like that suggested for other habitats (Hou *et al.* 2012).

The environmental parameters selected as predictor variables (O_2, pH, temperature, NO_3^-, NH_4^+ and NO_2^-) explained the differences in *nosZ*-denitrifying community composition among sampling sites at the Prado dam. The *nosZ*-community composition was associated with hypoxic (~3.4mg/L O_2) and suboxic (0.01mg/L O_2) conditions recorded at Tomogó and Lozanía respectively, which support previous data about the presence of denitrifiers in the water column to O_2 levels between 1.1 - 4.1 mg/L (Castro-González 2014). In general, our results corroborate that the composition of *nosZ*-denitrifying microorganisms is related to low oxygen environments such as those founded in rivers (Baxter *et al.* 2013), estuaries (Magalhães *et al.* 2008), wastewater (Hou *et al.* 2012), lakes (Wen *et al.* 2012) and reservoirs (Yu *et al.* 2014).

Table 2. Physicochemical variables, richness, diversity and similarity analysis of *nosZ*-OTUs in the study areas of the Prado reservoir.

Study areas	mg/L				Richness of OTUs[1]	CHAO1[2]	H[3]	Bray-Curtis[4]		
	O_2	NH_4^+	NO_3^-	NO_2^-				Isla de Sol	Lozanía	Tomogó
Isla del Sol	1,2	0,59	0,78	<0,05	12	15	1,15	1		
Lozanía	0,01	<0,05	<0,25	0,079	25	35	2	0,24	1	
Tomogó	3,4	0,88	0,49	<0,005	28	28	1,96	0,03	0,07	1

Note: ºC, temperature; O_2, oxygen; NH_4^+, ammonium; NO_3^-, nitrate; NO_2^-, nitrite; OTU, operational taxonomic unit. [1]Number of OTUs detected; [2]CHAO1 richness estimator; [3]Shannon-Weaver diversity index; [4]Bray-Curtis index express the similarity among areas.

At Isla del Sol was founded a *nosZ*-community less diverse and rich whose composition was related with higher levels of NO_3^-, contrarily to observed at Lozanía were the *nosZ* community composition showing an inverse relationship with the nitrate levels. It is well known that the nitrate (NO_3^-) is required by denitrifying communities in estuaries, rivers, lakes and reservoirs (Zhao *et al*. 2015, Baxter *et al.* 2013, Magalhães *et al.* 2008, Wen *et al.* 2012) and for this case the results suggest that the denitrifying community at Lozanía could be using the nitrate actively because was evident that this ion decreased to 0.25 mg/L in comparison with the recorded at Isla del Sol (0.78 mg/L NO_3^-). Our data are similar to those reported by Magalhães *et al.* (2008) in the Douro estuary (Portugal) and corroborate arguments about different *nosZ*-groups could have different competitive advantages for denitrification when nitrate levels fluctuate in a system (Jones and Hallin 2010, Magalhães *et al.* 2008).

For the reservoir, nitrate levels are related to the planktonic and benthic activities (Márquez and Guillot 2001), and

the increase in nitrate level observed at Isla del Sol may be due to a higher nitrifying (to 1.2mg/L O_2) than denitrifying activity, to difference that observed at Lozanía where the lower O_2 level (0.01mg/L) favored the denitrification by a great variety of *nosZ*-OTUs. Our assessment is supported by studies showing as both ammonia and nitrite oxidation occurred at very low oxygen concentrations (even < 50 μM) in marine (Peng *et al.* 2015) and freshwater ecosystems (Wetzel 2001, Castro-González and Torres 2015).

For other hand, the *nosZ* genes composition -in Tomogó- was associated with a pH level of 6,7 which are in accordance with a previous report where *nosZ* community was active and diverse in a wide range of pH (6.1-8) (Palmer *et al.* 2012). The effect of pH on denitrification enzyme activity and diversity of denitrifying communities has also been reported for bacterial cultures (Saleh-lakha *et al.* 2009) and soils respectively (Enwall *et al*. 2005).

The bacterial diversity and composition between Isla del Sol and Tomogó differed significantly (β-diversity), although both localities are not so far between them. This suggest that differences in the studied physical-chemical variables and other factors not analyzed in this report such as availability of organic matter and dissolved organic carbon, particulate carbon/dissolved carbon ratio, oxygen and nutrients dial fluctuations and interaction with other micro-communities could be contributing to the differences founded in the structure of the microbial community between study areas.

CONCLUSION

This study is the first report that analyzes the microbial composition of a nitrogen functional community related with the reduction of the greenhouse gas, nitrous oxide to N_2. Our data demonstrate that such community is present, is diverse, rich and that its composition varies spatially in relationship with the environmental factors. This information is the base for future studies about of the activity and importance of this communities in the cycling of N_2O from this dam that will permit to determine if this reservoir is a source or sink of this greenhouse gas.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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