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Research Article

Denitrifying community structure variability in the Colombian Pacific

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ABSTRACT. Denitrifying communities were detected in the water column of the Colombian Pacific Basin, with oxygen levels between 6 and 56 µM. They were analyzed by Terminal Restriction Fragment Length Polymorphism (TRFLP) of functional genes nitrite reductase (*nirS*) and nitrous oxide reductase (*nosZ*), and by pyrosequencing the gene *nosZ*. These genes are responsible for the production and consumption of the greenhouse gas N_2O. The TRFLP analysis indicated that throughout the Colombia current, the composition of the *nirS*-type denitrifying community was more homogeneous than the *nosZ*-type community. The *nosZ*-type community presented the highest richness (up to 11 TRF) and diversity (Simpson Index 1/D = 4.85 and Shannon Index = 0.76) of terminal restriction fragments, as well as the higher richness of operational taxonomic units-OTUs (up to 71), obtained via pyrosequencing. Out of 84% of OTUs, 48% were assigned to the genus *Pseudomonas*, and 36% to environmental clones. These results indicate the presence of a *nirS*-community inhabiting the Colombian Pacific Basin, with low diversity and mostly restricted within 300 m depth. The *nosZ*-community showed similar richness and diversity of TRFs and OTUs to that reported for the South Pacific Ocean. These results demonstrate that although both denitrifying communities are present in the area, these communities differ in structure and dominance compared to previous reports for other denitrifying microorganisms.

Keywords: denitrifying communities, functional genes, Oxygen Minimum Zones, Colombian Pacific.

INTRODUCTION

Denitrification is an anaerobic respiratory process present in autotrophic and heterotrophic microorganisms over all three biological domains (Zumft, 1997). This process involves the progressive respiration of nitrate to nitrite, nitric and nitrous oxides, and eventually to N_2, and has been described in several environments, including oxygen minimum zones (OMZ) (Lipschultz *et al.*, 1990; Codispoti *et al.*, 2001; Nicholls *et al.*, 2007; Farías *et al.*, 2009; Jayakumar *et al.*, 2009; Ward *et al.*, 2009; Lam & Kuypers, 2011; Dalsgaard *et al.*, 2012). These zones are widespread throughout the water column, and occur when respiratory oxygen demand exceeds oxygen availability, due to the degradation of organic matter in poorly ventilated regions of the ocean.

These areas support microbial communities that mediate the cycling of nutrients and radiative trace gases such as methane (CH_4), nitrous oxide (N_2O) and carbon dioxide (CO_2) (Wright *et al.*, 2012). Depending on the dissolved oxygen levels within the water column, rapid denitrification can lead to the reduction, or the emission, of N_2O (Castro-González & Farías, 2004). However, evidence suggests that nitrification is the main source of N_2O in the ocean; this varies considerably between different ocean regions depending on dissolved oxygen levels, light, and ammonium availability for nitrifying activity (Nevison *et al.*, 2003).

N_2O has a radiative effect that is around 300 times that of CO_2 (Lashof & Ahuja, 1990), and it has been estimated that at least one-third of all natural N_2O emission is derived from the ocean, principally from OMZs (Naqvi *et al.*, 2010). In these areas, the primary challenge is to identify the microorganisms involved in nitrogen transformations and to understand the contribution and interaction of microorganisms with biogeochemical processes.

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One approach for studying denitrifying communities is through their functional genes because denitrifiers are found within a variety of phylogenetically unrelated groups, with over 50 genera (Zumft, 1997). Denitrification is distinguished from other forms of nitrate metabolism as it entails the following two steps: nitrite reduction and nitrous oxide reduction. The reductase required for these steps are coded by the *nir* and *nosZ* genes respectively, which have been used as functional markers to examine denitrifying communities over a variety of marine environments (Tiquia *et al.*, 2006; Magalhaes *et al.*, 2008, 2011; Mills *et al.*, 2008; Chon *et al.*, 2009; Jayakumar *et al.*, 2009a; Mosier & Francis, 2010). However, denitrifying organisms possess two types of functionally equivalent nitrite reductases, which are considered mutually exclusive to a single denitrifier, *i.e.*, both reductases will not occur within one denitrifier. One is a cytochrome cd_1 heme type reductase (*NirS*) encoded by the *nirS* gene and the other is the copper-oxide reductase (*nirK*) encoded by the *nirK* gene.

The *nirS* gene is the most commonly studied gene in marine environments, including estuarine and marine sediments (Nogales *et al.*, 2002), and seawater (Jayakumar *et al.*, 2004; Castro-González *et al.*, 2005). Its use has shown a link between functional diversity and ecosystem biogeochemistry (Jayakumar *et al.*, 2004), and low habitat selectivity, due to their tendency to overlap along environmental gradients (Jones & Hallin, 2010).

The *nosZ* gene, although absent in some bacterial and archaeal species that harbour *nir* and *nor* genes (Zumft & Kroneck, 2007), has been applied in several studies within marine environments, such as coastal sediments (Scala & Kerkhoff, 1999, 2000; Mills *et al.*, 2008), seawater (Wyman *et al.*, 2013), and estuarine wetlands (Chon *et al.*, 2009). This gene is a suitable functional marker as it has remained unchanged through evolutionary history, only one copy of the gene is present per organism, and it has a reliable assignment of the species-level Operational Taxonomic Unit (OTU) (Palmer *et al.*, 2009). Therefore, it can be used to estimate novelty at a species-level, as well as the species-level diversity of denitrifiers in environmental samples. This complements previous studies carried out in the OMZ with *nirS*-denitrifiers (Jayakumar *et al.*, 2004, 2009, 2009a; Castro-González *et al.*, 2005; Jones *et al.*, 2008).

Denitrifying bacteria have been detected in all the OMZs that occur within upwelling areas with high primary productivity, such as Chile and Peru in the Eastern Tropical Pacific (Castro-González *et al.*, 2005; Lam *et al.*, 2009; Ward *et al.*, 2009; Castro-González *et al.*, 2015). However, this is the first study investigating their presence in the Colombian Pacific Basin, which is an intermediate zone between two of the world's most prominent OMZs, the eastern north, and South Pacific.

The CPB has been characterized by oceanic upwelling (between 78-81°W and 2-5°N), coastal upwelling (between 8ºN and 2ºN), and oxygen-deficient conditions (<44.6 µM) in subsurface waters between 200-600 m depth (CCCP, 2002; NOAA National Oceanographic Data Center, 2008, 2009). A primary productivity of 1.51 g C m^{-2} d^{-1} (Gómez & Martinez, 2005), associated with a high flux of organic carbon to the seafloor, has been estimated for the Panamá Basin (Betancur & Martínez, 2003).

These conditions are characterized by strong temporal and spatial variations within the basin, due to fluctuations in coastal upwelling and seasonal river runoff (Rodríguez-Rubio & Stuardo, 2002; Bastidas-Salamanca *et al.*, 2006; Pennington *et al.*, 2006). Consequently, the Panama Basin is defined by three areas; northern eutrophic (between 8º-5.5ºN), mesotrophic (between ~5.5 y 2ºN) and southern eutrophic (to the south of 2ºN) (Betancur & Martínez, 2003).

Considering that the CPB presents both temporal and spatial variability, with biogeochemical conditions that may favor the development of denitrifier communities, as well as hosting anammox microorganisms that are also involved in the nitrogen cycle (Castro-González *et al.*, 2014), the main goal of this research is to explore the presence and composition of *nosZ* and *nirS* type-denitrifiers. This is achieved through contrasting sites along the basin using terminal restriction fragment length polymorphism (T-RFLP) analysis and *nosZ* gene pyrosequencing.

MATERIALS AND METHODS

Study area and water sampling

Seawater samples were collected along the Colombian Pacific Basin between 2-6ºN and 78-80ºW on September 2007 on board the PACIFICO XLV-ERFEN XLIII cruise, at five stations off Solano Bay (CPC01), Buenaventura (CPC14, CPC29, CPC45) and Tumaco (CPC33) (Fig. 1). The stations 1 and 33 were considered as coastal (<30 nautical miles from the coast), the station 14 and 29 were considered as of transition because they are in a mixing zone between coastal and oceanic waters (<90 nautical miles from the coast) and the station 45 was considered as oceanic.

Seawater samples were collected in 5 L Niskin bottles attached to a CTDO rosette sampler at 100, 300, and 500 m depth. The samples were manipulated on board as described in Castro-González *et al.* (2014), which included consecutive filtration steps through 20,

5 and 0.22 µm pore size membrane filters (Durapore, diameter 47 mm), and then preserved in lysis buffer at -80°C, until DNA extraction. Temperature, salinity and dissolved oxygen were measured with a CTD equipped with an O_2 sensor (Seabird SBE 43) for each station. The O_2 and nutrients profiles, were described previously in Castro-González *et al.* (2014).

DNA extraction and amplification of *nirS* and *nosZ* genes

DNA extraction, from bacteria collected on membrane filters (0.22 µm pore size), followed the procedure proposed by Castro-González *et al.* (2005). The DNA was quantified spectrophotometrically at 230, 260 and 280 nm. PCR amplification of *nirS* and *nosZ* genes of denitrifiers from environmental DNA extracts (10 ng μL^{-1}) were performed with primers Cd3aF-R3cd (Michotey *et al.,* 2000; Throbäck *et al.,* 2004) and nosZ1188F-nosZ1869R (Kloos *et al.,* 2001) respectively, to a final concentration of 10 pmol. RedAccuTaq Polymerase (0.5 µL) and, to optimize the amplification, 4 µg mL^{-1} Bovine Serum Albumin (BSA) were added to the reaction mix.

The *nirS* gene annealing temperatures during the first 10 touchdown cycles started with 58°C and were kept at 55°C during the following 30 cycles; denaturing and extension were done at 94°C for 30 s and 72°C for 40 s respectively. The *nosZ* gene annealing temperatures during the first 10 touchdown cycles started with 57°C and were kept at 56°C during the following 25 cycles; denaturing and extension were done at 94°C for 1 min and 72°C-1 min. Amplification products of *nirS* (400 pb) and *nosZ* (700 pb) were analyzed by electrophoresis using 2% (wt vol^{-1}) agarose gels followed by staining with ethidium bromide. Bands were visualized by UV excitation. Products of three replicate PCRs were combined. PCR-products were eluted and purified using the Wizard®SV Gel and PCR Clean-Up System (Promega, Mannheim, Germany).

***NosZ* and *nirS* terminal restriction fragment length polymorphism (TRFLP) analysis**

Purified *nosZ* and *nirS* PCR products (100 ng) were digested with *HhaI* and *MspI* respectively, using 5U of the enzyme in the manufacturer's recommended reaction buffers.

Digested products were cleaned with Autoseq G-50 columns (Amersham Biosciences) according to the manufacturer's recommendations. Aliquots (2 µL) of the digest were mixed with 12 µL of deionized formamide (Applera, Darmstadt, Germany) and 0.2 µL of an internal DNA length standard (X-Rhodamine mapMarkerR 50-1000 bp; BioVentures, Murfreesboro, TN). Terminal restriction fragments (TRF) were separated with an automated DNA sequencer 310 (Applied Biosystems). The lengths of fluorescently labeled T-RFs were determined by comparison with the internal standard using GeneScan 3.71 software (Applied Biosystems).

Analysis of TRFLPs

The peaks in the chromatography were discriminate by their height, then those presenting >50 fluorescence units and more than 36 bp in length were included in the analysis. Patterns from different samples were normalized to identical total fluorescence units by an iterative standardization procedure (Dunbar *et al.,* 2001). Relative abundance percentage of T-RFs was determined by calculating the ratio between the height of a given peak and the normalized total peak height of each sample.

To visualize similarities between T-RFLP profiles, pairwise similarities were calculated using the Bray Curtis coefficient; these considered the presence (1) or absence (0) of T-RFs, the number of T-RFs common to two communities, and the total number of T-RFs observed.

Pyrosequencing

454 GS-FLX Titanium Sequencing Technology (Roche, Switzerland) was used to study and compare the *nosZ*-microbial communities with the TRFLP analysis in the station CPC01500, CPC33300 and CPC45300 only. DNA (50 ng μL^{-1}) from each sample was sent to the Research and Testing Laboratory (Lubbock, TX, USA). PCR amplification was performed using primers *nosZF* and *nosZ*1622R (Kloos *et al.,* 2001).

Sequencing reactions utilized a Roche 454 GS-FLX+ instrument (Roche, Indianapolis, IN) following manufacturer's instructions. Following pyrosequencing, validation of raw sequences was performed according to Mao *et al.* (2011) and Palmer *et al.* (2012) where mismatches to the 5' primer were removed by annealed checking with ClustalW using MEGA v 5.2.1 software (Tamura *et al.,* 2011). Unspecific *nosZ* sequences were removed using Blast2go v 2.6.6 (Conesa *et al.,* 2005), chimeras along with exact duplicates of sequences were eliminated with the USEARCH/UCHIME (Edgar *et al.,* 2011) and DEREPLICATOR from FunGene pipeline (Fish *et al.,* 2013). Sequences containing any ambiguous bases or with size <350 bp were eliminated with BioEdit v 7.1.11 (Hall, 1999).

Analysis of *nosZ* sequences

The denoised *nosZ*-sequences were annealed with Clustal W v 2.1 software (Larkin *et al.,* 2007). The similitude between sequences was estimated with

DNADIST v 3.69 using the matrix of the distance calculated with the Jukes-Cantor model of PHYLIP software (Felsenstein, 2005). The sequences were clustered in operational taxonomic units (OTU) at species-level threshold distances of 20% (Palmer *et al.*, 2009) based on DNA sequences. The OTUs were defined using DOTUR program [Distance-Based OTU and Richness (Schloss & Handelsman, 2005)] using the furthest neighbor method with 1000 iterations. The OTUs taxonomic assignation was done by comparison with *nosZ* sequences from NCBI database using a similarity level >75%.

Statistical analysis

Diversity index (Shannon and Simpson) and similarity analysis for TRFLP analysis were estimated and constructed with the Biodiversity Pro software version 2.0. Richness and diversity index for the OTUs obtained by pyrosequencing were calculated with PAST v 3.0 software (Hammer *et al.*, 2001). The environmental variables such as salinity, temperature, and O_2, which were published previously by Castro-González *et al.* (2014) were linked to TRFLP patterns by Canonical Correspondence Analysis (CCA), which were performed using the PCord software version 4.01 (MjM Software, Gleneden Beach, OR). The CCA was also used for recognizing patterns between *nosZ* and *nirS* gene compositions among stations, based on relative abundance of T-RFs, and the environmental parameters.

RESULTS

Detection of *nosZ* and *nirS* communities in the study area

Unlike to the *nirS* gene, the *nosZ* gene was successfully amplified in the majority of sampling stations and depths. Both denitrifying communities were present at 300 m depth over all stations, with dissolved oxygen levels fluctuating between 6-13 µM.

Conversely, *nirS* was not detected at the CPC33 station, despite the lowest dissolved oxygen level (6 µM) was recorded in this area (Table 1). The data indicated that the TRF 378 is unique to Solano Bay and that this community had the highest abundance at 100 m depth. Data analysis suggests that although this TRF showed a greater richness at 300 m depth off Solano Bay, in accordance with the Shannon and Simpson diversity index, *nosZ*-type denitrifiers showed a greater diversity at 500 m depth (Table 2). This data demonstrates that microbial communities related to an N_2O reduction in the CPB are present along the water column over a wide range of oxygen levels.

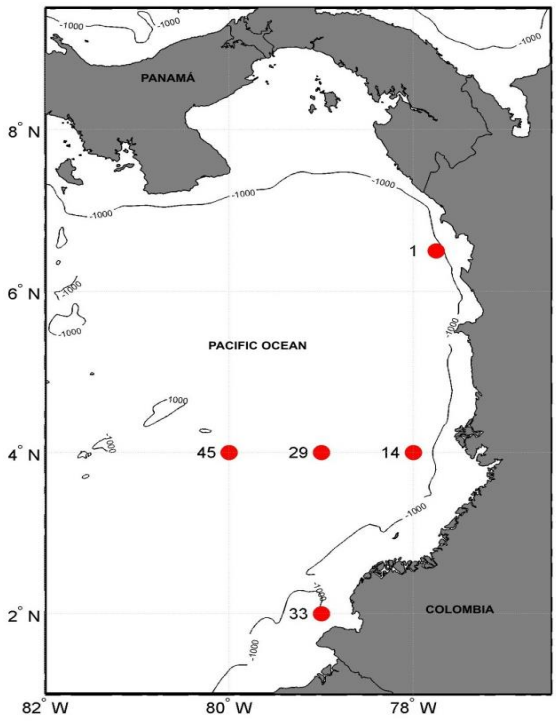

Figure 1. Location of sites (red circles) where seawater samples were taken during September 2007 at the DIMAR-CCCP oceanographic cruise.

TRFLP analysis

The TRFLP analysis (Fig. 2) showed that *nosZ*-type denitrifiers are present in all the stations along CPB at 300 m depth, with a total of 15 TRFs. In general, all TRFs were distributed throughout all the stations from north to south and including coastal and offshore areas. However, the TRFs 132, 140 and 378 were only present in the CPC01 station off Solano Bay, and the TRF 445 in the CPC14 station off Buenaventura.

The vertical composition of *nosZ*-type communities from coastal stations off Solano Bay and Buenaventura (Fig. 2) showed a lower number of TRFs off Buenaventura (8 TRFs) compared to Solano Bay (13 TRFs), suggesting a greater richness in the latter area (Table 2). The results showed that most communities were present between 100-500 m depth, with the TRFs 40, 43, 46, 126, 355, 428 at CPC29 station, and the TRFS 40, 43, 46, 126, 140, 323, 355 and 378 at CPC01 station. However, there were specific *nosZ*-communities at 300 and 500 m depth. At 300 m depth, the TRF 68 was observed in both stations, and the TRF 132, 370 and 428 were characteristic from CPC01 station. At 500 m depth, the TRFs 351 and 264 were typical of CPC29 and CPC01 stations, respectively.

NirS-type communities were studied along the CPB at 300 m depth and over a vertical profile off Solano Bay. At 300 m depth, *NirS* -TRFs (12, Fig. 3a) had

Table 1. Location and physical characteristics of seawater at the sampling stations where were detected the *nosZ* and *nirS* type communities for T-RFLP analysis. (+) = detected, (-) = not detected. *Samples where nosZ gene pyrosequencing was developed. Castro-González *et al*. (2014), previously published data of location, depth, oxygen, salinity, and temperature.

Station	Location	Depth (m)	O_2 µM	Salinity	TºC	*nosZ*/*nirS* gene detection
CPC01 off Solano Bay	06°30'1"N 77°45'W	100	33	34.87	14.5	+/+
		300	8	34.68	10.0	+/+
		500	12	34.62	7.9	+/+*
CPC14 off Buenaventura	04°00'N 78°00'W	100	47	34.90	14.3	-/-
		300	10	34.72	10.5	+/+
		500	15	34.64	8.3	-/-
CPC33 off Tumaco	02°00'N	100	56	34.92	14.2	-/-
	79°00'W	300	6	34.73	10.6	+/-*
		500	11	34.66	8.6	-/-
CPC29 off Buenaventura	04°00'N	100	45	34.89	14.8	+/-
	79°00'W	300	13	34.74	10.7	+/+
		500	22	34.61	7.7	+/-
CPC45 oceanic off	04°00'N	100	37	34.91	14.8	-/-
Buenaventura	80°00'W	300	7	34.74	10.9	+/+*
		500	16	34.62	7.8	-/-

Table 2. The richness of *nosZ*-type OTUs* and *nirS/nosZ* type-TRFs, and diversity index of TRFs of *nosZ* and *nirS* communities detected at the sampling stations along the Colombian Pacific.*Obtained by pyrosequencing. OTU: operational taxonomic unit, TRF: terminal restriction fragment.

Station	Depth (m)	OTUs richness *nosZ*	TRFs richness *nirS/nosZ*	Shannon Index *nos-Z*	Simpson Index (1/D) n*os-Z*	Shannon Index*nir-S*	Simpson Index (1/D) *nir-S*
CPC01 off Solano Bay	100	nd	6/6	0,62	3,43	0,59	3,35
	300		7/11	0,72	3,81	0,62	3,46
	500	71	5/7	0,76	4,85	0,59	3,31
CPC14 off Buenaventura	300		6/11	0,59	2,20	0,41	1,88
CPC33 off Tumaco	300	13	0/6	0,58	2,72	nd	nd
CPC29 off Buenaventura	100		0/5	0,63	3,94	nd	nd
	300		4/6	0,62	3,50	0,34	1,77
	500		0/6	0,68	4,13	nd	nd
CPC45 oceanic off Buenaventura	300	65	6/5	0,68	4,63	0,59	3,29

a lower richness compared to *nosZ* TRFs (15, Fig. 2a). The data in Figure 3 show that the TRFs 176, 220 and 259 were common throughout all the stations, from north to south and from the coast to offshore in the CPB. Additionally, although the TRF 112 was present in the coastal stations off Solano Bay (CPC01) and off Buenaventura (CPC14), it was more abundant in the latter station. Indeed, at 300 m depth, each station showed the following characteristic TRFs: CPC45: 166 and 272, CPC29: 59, CPC14: 37 and 89, CPC01: 107 and 262, suggesting that these communities are adapted to the prevalent environmental conditions at each station and depth. The vertical profile off Solano Bay presented a homogeneous distribution of *nirS*-communities for most of the TRFs between 100-500 m depth, with some variation in TRF abundance, suggesting that environmental conditions throughout the water column favor a wide distribution of TRFs. Likewise, the results showed a slight increase in TRF diversity (Table 2) and richness at 300 m due to the presence of the TRF 262 and 107, which are characte-ristic to this depth.

The similarity analysis for the *nosZ*-type community composition at 300 m depth is shown in the Figure 4. These communities showed very different results along (north to south) the Colombia current between Tumaco and Solano Bay, with similarity values between 13-28%. Higher similarities (62.7%) were observed between oceanic (CPC45) and transition station (CPC29) communities off Buenaventura (Fig. 4a).

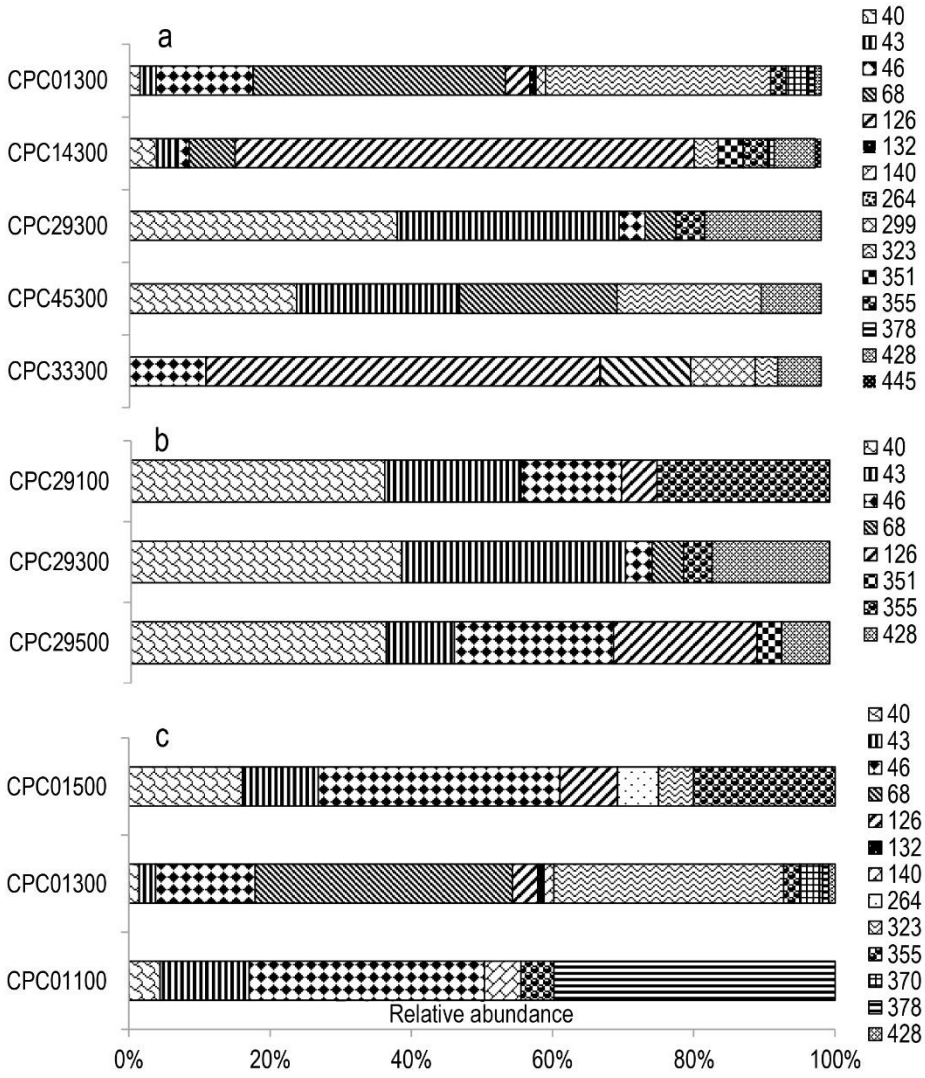

Figure 2. TRFLP analysis of *nosZ*-type denitrifiers along the a) CPB to 300 m depth, b) vertical profile off Buenaventura-CPC29 station, and c) off Solano Bay-CPC01 station. The legend indicated the size in base pairs of each fragment.

The communities present off Solano Bay, along with the vertical profile (Fig. 4b), showed a similarity ranging between 26-52.4%, indicating more similar communities at 100 and 500 m. The *nosZ* similarity analysis suggests that the TRFs present a unique composition at 300 m depth off Solano Bay. In contrast, along with the vertical profile at station CPC29, communities present a greater similarity (63.6-64.7) (Fig. 4c), indicating that the transition area off Buenaventura has a more homogeneous environment within the column water.

Along the CPB, at 300 m depth, results indicate similar communities, with a similarity analysis for *nirS*-type denitrifiers (Fig. 5) ranging between 21.5 to 78%.

The highest similarity was recorded between communities at CPC01 and CPC45 (78%), while the lowest similarity was observed for the communities inhabiting the CPC14 station (21.5%) (Fig. 5a). The TRFs along the vertical profile off Solano Bay had a high similarity (67-77%), especially for communities between 100 to 300 m depth.

The similarity analysis showed that along the CPB, at 300 m depth, *nirS* communities were more similar (up to 78%) than *nosZ* communities (up to 62.7%) (Fig. 5). Likewise, within the vertical profile off Solano Bay, the *nirS*-TRFs were more similar (up to 77%) compared to *nosZ*-TRFs (up to 52.4%).

These results show differences in the *nirS*-type and *nosZ*-type denitrifying community structure in the CPB, over both horizontal and vertical scales, suggesting that the environment influences the community composition. The results from the Canonical Correspondence Analysis (CAA) (Fig. 6) indicated that the environmental parameters selected as

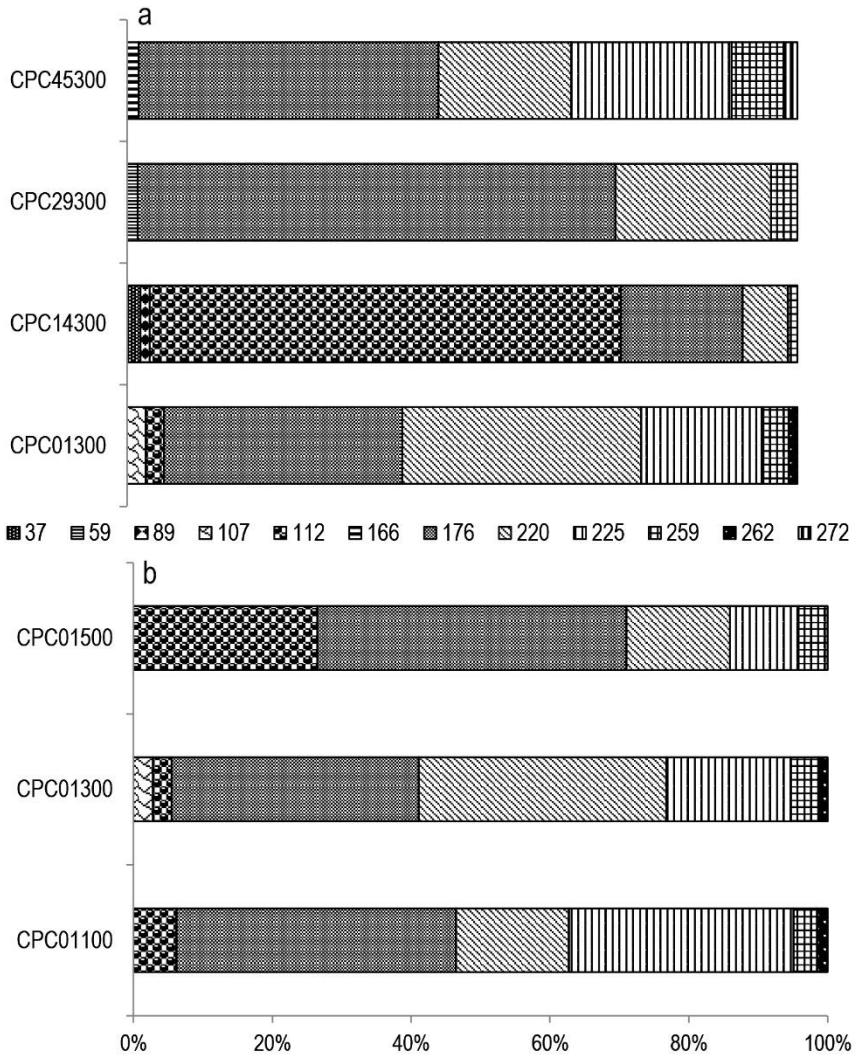

Figure 3. TRFLP analysis of *nirS*-type denitrifies at a) sampling stations to 300 m depth, and b) along to vertical profile at CPCO1 Station off Solano Bay. The legend indicates the size in base pairs of each fragment.

predictor variables (temperature, salinity and dissolved oxygen level) could account for 44.3% and 71.1% of *nosZ* and *nirS* community composition, respectively. The *nosZ*-type community composition was strongly correlated with dissolved oxygen, while the *nirS* community composition was related to salinity and temperature. In the CCA plot, *nirS*-TRFs showed a scattered and more abundant distribution around the sampling points, which agrees with the results from the TRFLP analysis showing a more homogeneous community in comparison with the *nosZ*-TRFs. Also, in the CCA, some unique *nirS*-TRFs (T59, T89, T37, T166, T107, and T272) and *nosZ*-TRFs (T186, T299, T132, T140, T264, T370, and T378) were identified.

Pyrosequencing analysis

A total of 26.628 sequences of the *nosZ*-type denitrifying community were obtained from the CPB. A total of 53% corresponded to the CPC01500 station, 35% to CPC45300 station, and 12% to CPC33300 station. A total of 94 OTUs were detected in the basin, with the highest number of OTUs (71) recorded from the coastal area off Solano Bay (CPC01500), followed by the offshore area (CPC45300) with 65 OTUs, and then by the coastal area of Tumaco (CPC33300) with 13 OTUs (Table 2, Fig. 7).

In general, 82% of sequences in the CPB were grouped into 20 *nosZ*-OTUs, the remaining 74 OTUs were less abundant. A total of nine *nosZ*-OTUs were shared between the stations 02, 03, 05, 06, 16, 44, 45, 46 and 48. Furthermore, a number of unique OTUs were detected at the following stations: CPC01500 with 26, CPC45300 with 21, and CPC33300 with one (Fig. 7a).

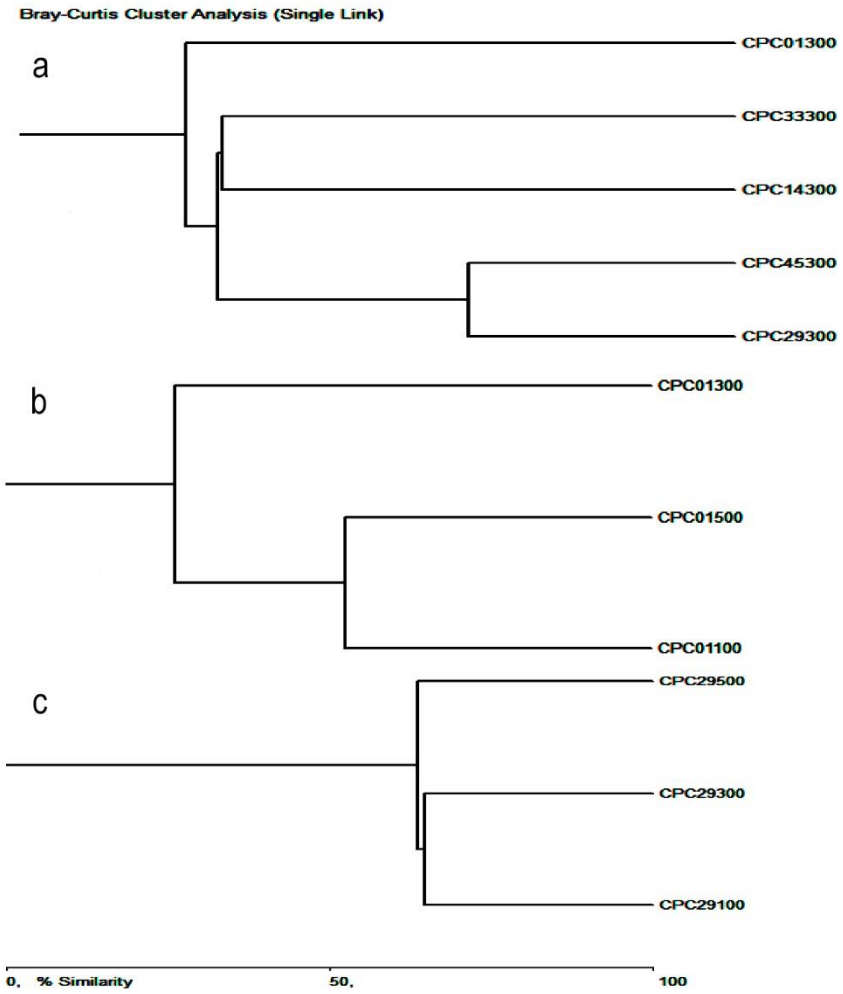

Figure 4. a) Similarity dendrogram between *nosZ*-type denitrifiers founded to 300 m depth in all the stations (from north to south) at Colombian Pacific Basin, b) in the vertical profile off Solano Bay, and c) in the vertical profile off Buenaventura.

A high proportion (85%) of the sequences correspond to the most abundant OTUs for each site, with 13 of 65 OTUs from the offshore station (CPC45300), 4 of 13 OTUs found off Tumaco (CPC33300 station) and 20 of 71 OTUs recorded off Solano Bay (CPC01500 station) (Fig. 7b).

The diversity analysis showed that the most diverse *nosZ*-communities were found off Solano Bay (CPC01500 station) (Simpson Index 1/D = 3.19), followed by the offshore area (CPC45300) (Simpson Index 1/D = 2.62), and finally by the coastal area off Tumaco (CPC33300) (Simpson Index 1/D = 1.66).

Taxonomic assignments carried out for 94 representative sequences of each OTU (Table 3) indicated there were a high number of microorganisms, such as *Pseudomonas* (48% of sequences), and environmental clones isolated from waters, sediments, and soils (36% of sequences), within the area. There was a minor abundance (12% of sequences) of organisms related to *Achromobacter*, *Bradyrhizobium*, *Marinobacter* and *Paracoccus*. The remaining 4% of sequences were not assigned, indicating the presence of novel denitrifying microorganisms in the CPB.

DISCUSSION

In general, the TRFLP analysis indicated that the *nirS* and *nosZ*- type denitrifiers are present along the Colombian Pacific Basin (CPB) from north to south, from coastal to offshore, and between 100-500 m depth. The highest richness and diversity of both communities was recorded off Solano Bay between 300 and 500 m depth, with levels of dissolved oxygen between 8-12 µM. The data also indicate that the denitrifying community, targeted with the *nirS* primers, had a low richness and diversity of OTUs (Table 2). The *nirS*-type community showed a more homogeneous structure along a vertical profile compared to the denitrifier community, targeted using the *nosZ* primers.

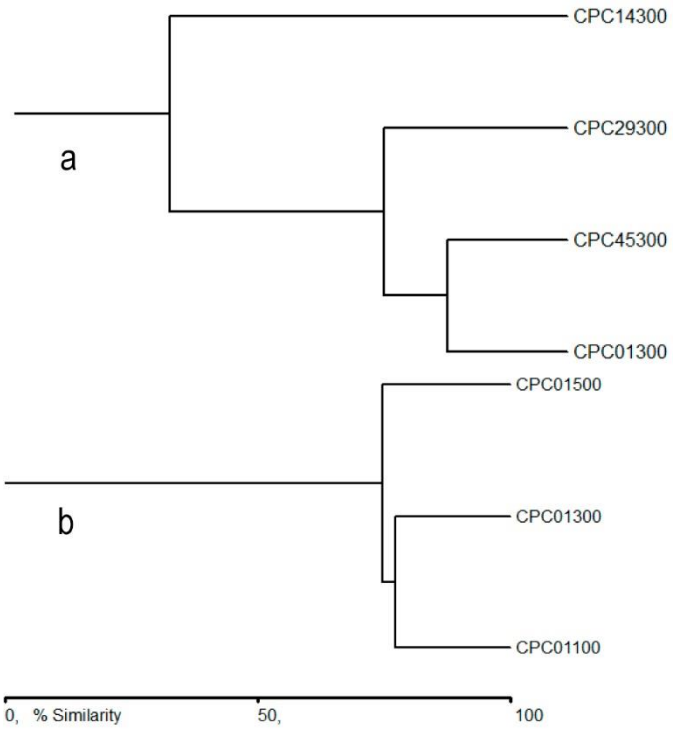

Figure 5. a) Similarity dendrogram for *nirS*-type denitrifiers founded along the CPB at the stations from north to south to 300 m depth, and b) along vertical profile at CPC01 station off Solano Bay.

These results agree with previous studies from oceanic areas that have indicated a low diversity in pelagic *nirS* communities (Castro-González *et al.*, 2004; Hannig *et al.*, 2006). The *nirS*-type denitrifiers from the Colombian Pacific were less diverse (Simpson Index: 1.77-3.46) compared to those detected in the Chilean OMZ (Simpson Index: 2.2-5.8). This study indicated a lower richness of OTUs (12) compared to those recorded off Chile (19), however, it is not possible to directly compare OTUs between both areas because the amplification was carried out with a different set of primers. Nonetheless, these differences may be attributed to a shallower OMZ off Chile compared to Colombia, thus leading to increased denitrifying activity in Chile. Likewise, the Chilean OMZ is characterized by high productivity, high nitrite (up to 5 µM) and low nitrate (0.3-14 µM) levels (Farías *et al.*, 2009). In comparison, the CPB has high nitrate (13-38 µM) and low nitrite (<0.07 µM) values, that indicate low denitrification activity in the suboxic waters.

Nevertheless, this is consistent with the general notion that, in terms of diversity, planktonic microbial communities are depleted compared to those in the sediments (Curtis *et al.*, 2002). Also, community diversity may be underestimated due to the type of primers used, since denitrifier communities related to *nirS* primers are less phylogenetically diverse compared with the communities related with the *nirK* or *nosZ* primers (Philippot *et al.*, 2009). Furthermore, *nirS* primers may not detect all the *nirS*-containing organisms (Throbäck *et al.*, 2004). Additionally, two further facts should be considered; first, that some denitrifying bacteria are able to metabolize up to 20-25 µM O_2 while requiring significantly lower oxygen levels to grow and maintain high abundances in the environment (Ulloa *et al.*, 2012). This is apparent for nitrite reducing bacteria, which may have a limited presence in the CPB due to higher levels of dissolved oxygen. Secondly, biases during PCR may favor the amplification of some OTUs over others (Oakley *et al.*, 2007).

However, there is also a plausible ecological explanation; as variations in vertical redox gradients (dissolved oxygen and nutrients) and particle size fraction between denitrifier community assemblies have been reported for *nirS*, *nosZ*, and *nirK* in the OMZ of the South Pacific (Ganesh *et al.*, 2014) and the Black Sea (Oakley *et al.*, 2007). In this sense, the community composition in the CPB is likely to be influenced by similar factors, considering that spatial variation has been reported for primary productivity (Rodríguez-Rubio & Stuardo, 2002; Bastidas-Salamanca *et al.*, 2006). Spatial variability influences the variation in the organic carbon flux in the basin (Betancur & Martínez, 2003), separating the eutrophic area to the north, between

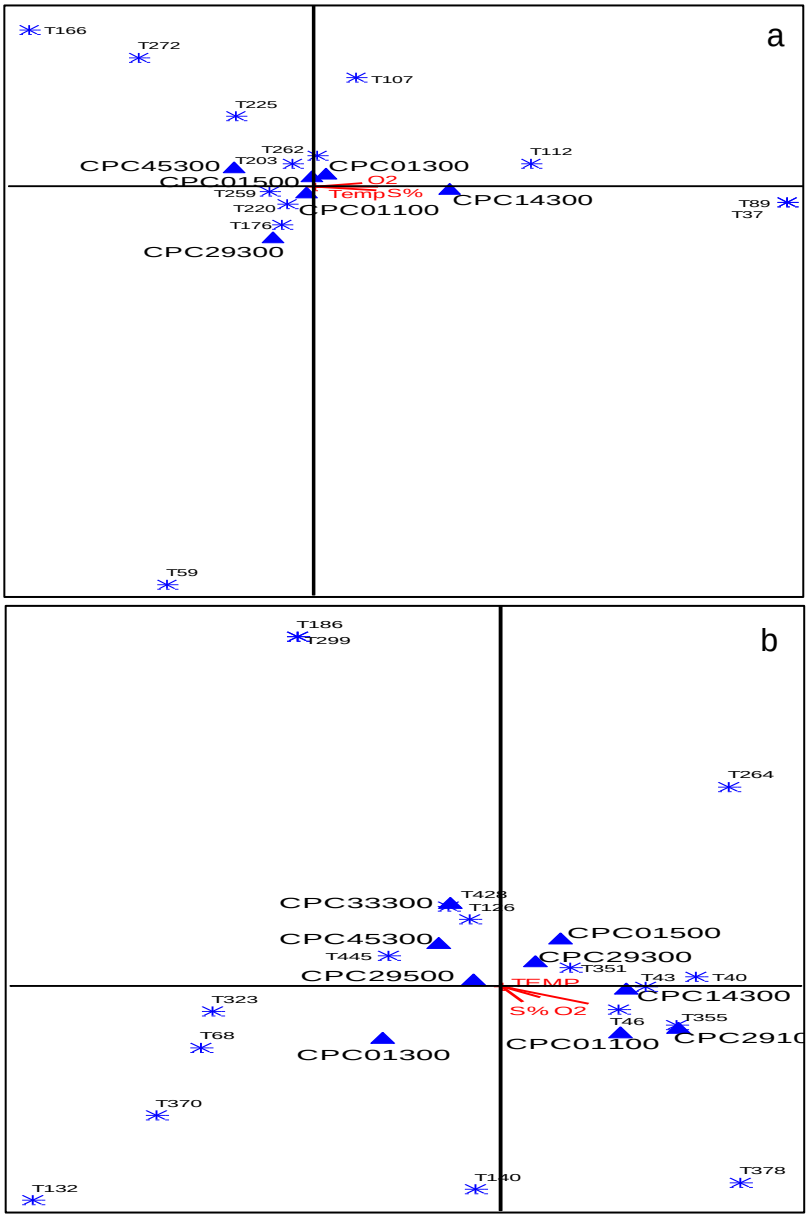

Figure 6. a) Canonical correspondence analysis of communities' *nirS*, b) *nosZ*-type communities in the Colombian Pacific Basin.

8-5.5ºN, from the mesotrophic region in the central basin (between 5.5 and 2ºN). However, more research is necessary to validate this hypothesis and determine the rate of reduction of nitrate to dinitrogen, the vertical N_2O distribution, as well as explore if the high nitrate level recorded along the water column are due to low reduction rates and the combined activity of the *nirS*-denitrifying and anammox communities (Castro-González *et al.*, 2014).

About the *nosZ* community, the molecular analysis suggested that at dissolved oxygen levels ranging between 7-12 µM, a rich and diverse community is present within the OMZ off Solano Bay (300-500 m depth) and the oceanic upwelling area (CPC45). A higher diversity of *nosZ*-OTUs was detected through pyrosequencing compared to the TRFLP analysis. Furthermore, a better community resolution was obtained through pyrosequencing using a reverse primer different to the primer used for TRFLP (the nosZ1622R primer are located into the amplified region by the nosZ1869R primer of *nosZ*-gene).

This study indicated a lower richness of *nirS*-TRF communities in the CPB compared with results from the Baltic Sea (Hannig *et al.*, 2006), the Arabian Sea (Jayakumar *et al.*, 2004, 2009) and the Eastern South Pacific (Castro-González *et al.*, 2005). On the other

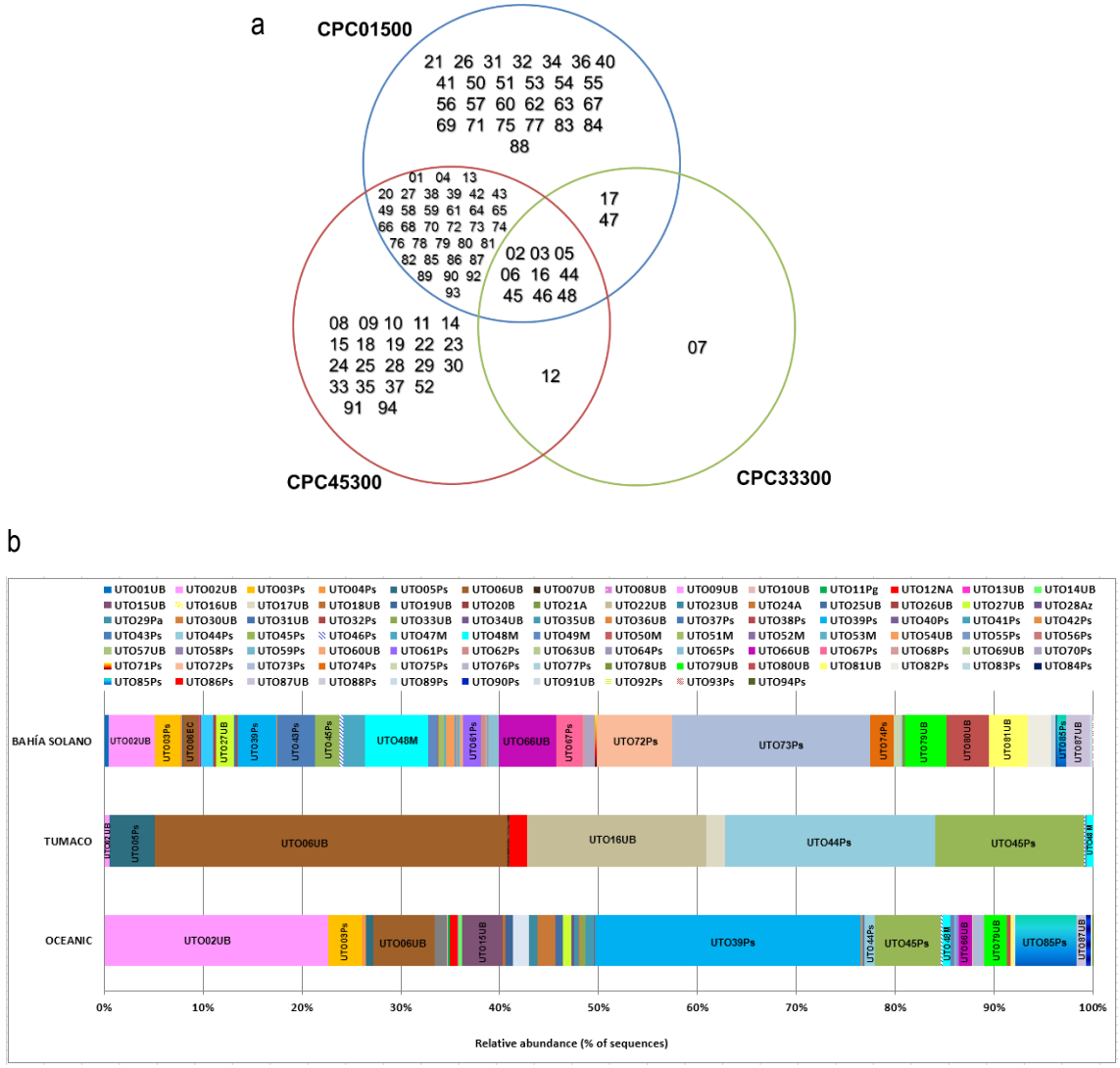

Figure 7. a) Distribution of *nosZ*-type OTUs between sampling areas and b) relative abundance of OTU at each station: Solano Bay (CPC01500),Tumaco (CPC33300), and Oceanic station (CPC45300). The letter next to number of OTU indicate some grade of similarity with *Achromobacter* (Ac), *Alcaligenes* (Al), *Azospirillum* sp. (Az), *Bradyrhizobium* sp.; *Marinobacter* sp. (M); *Paracoccus* sp. (Pa); *Pseudogulbenkiania* sp. (Pg); *Pseudomonas* sp. (Ps); Uncultured bacterium (UB) or No assigned (NA).

hand, the CPB has a similar richness of *nirS*-TRFs to those reported in the Black sea (Oakley *et al.*, 2007). However, it should be considered that these studies used different primer sets and restriction enzymes, and not all primers amplify with the different samples with the same efficiency, in fact, variation in primer performance has been reported between habitats (Bonilla-Rosso *et al.*, 2016).

This study observed the successful amplification of *nirS*-type denitrifiers in the CPB to depths of 300 m for all stations, suggesting that this gene occurs in a higher abundance to this specific depth, contrary to the OMZ off Chile. Within this zone, the *nirS*-community was present between 50-200 m depth, despite variations in nitrite, nitrate and dissolved oxygen. Additionally, at the OMZ core off Chile, the *nirS*-community structure was related to high nitrite levels and low dissolved oxygen, however in the CPB, water mass density could explain about 44% of the community composition, indicating that other factors, such as micronutrients or organic compounds, could be also contributing to the *nirS*-structure.

Few studies have been carried out on the *nosZ* gene in marine sediments and seawater (Scala & Kerkhoff, 1999, 2000; Nogales *et al.*, 2002; Mills *et al.*, 2008; Chon *et al.*, 2009; Fortunato *et al.*, 2009; Magalhaes *et*

Table 3. Percentage (%) of sequences, taxonomic affiliation, identity and e-value of 94 OTUs founded in the sampling areas off Solano Bay, Tumaco, and in the oceanic station.

UTO	% of sequences	Taxonomic affiliation	Identity (%)	e-value
UTO39Ps	11,6	*Pseudomonas* sp.	93	2,00E-179
UTO73Ps	11,0	*Pseudomonas stutzeri* DSM 10701	94	0,0
UTO02UB	10,6	Uncultured denitrifying bacterium: Laizhou Bay sediment	98	0,0
UTO06UB	7,0	Uncultured bacterium clone: DGGE band UZ4D nosZ	99	0,0
UTO45Ps	5,3	*Pseudomonas balearica* DSM 6083	95	2,00E-178
UTO72Ps	4,1	*Pseudomonas* sp.	93	0,0
UTO48M	4,1	*Marinobacter hydrocarbonoclasticus*	93	5,00E-153
UTO66UB	3,5	Uncultured bacterium clone: 2-15-1 putative nitrous oxide reductase (nosZ) gene	100	0,0
UTO79UB	3,0	Uncultured bacterium clone: DGGE band Zl-3	98	0,0
UTO44Ps	2,7	*Pseudomonas balearica DSM 6083*	97	0,0
UTO85Ps	2,7	*Pseudomonas stutzeri* strain SLG510A3-8	96	0,0
UTO03Ps	2,6	*Pseudomonas stutzeri* Gr50	99	0,0
UTO80UB	2,4	Uncultured bacterium clone: DGGE band Zl-3	99	0,0
UTO81UB	2,2	Uncultured bacterium clone: DGGE band Zl-3	99	0,0
UTO43Ps	2,0	*Pseudomonas stutzeri* DSM10701	99	0,0
UTO16UB	2,0	Uncultured bacterium clone: J7 G1PM8610AVM1E	91	3,00E-164
UTO87UB	1,5	Uncultured bacterium clone: TS4 NosZ	95	5,00E-173
UTO15UB	1,4	Uncultured bacterium clone: PYL-XJ-131	81	9,00E-100
UTO67Ps	1,4	*Pseudomonas stutzeri* DSM10701	93	1,00E-167
UTO82Ps	1,3	*Pseudomonas stutzeri* TR2	97	0,0
UTO74Ps	1,3	*Pseudomonas balearica DSM 6083*	93	2,00E-179
UTO27UB	1,2	Uncultured forest soil bacterium clone: DUNnos205	82	2,00E-77
UTO47M	1,2	*Marinobacter hydrocarbonoclasticus* NY-4	86	3,00E-137
UTO61Ps	1,0	*Pseudomonas stutzeri* DSM10701	94	0,0
UTO05Ps	0,8	*Pseudomonas stutzeri* SLG510A3-8	99	0,0
UTO21Al	0,7	*Alcaligenes faUBalis: P156*	88	3,00E-144
UTO24Ac	0,6	*Achromobacter xylosoxidans*: FDAARGOS_150	93	2,00E-179
UTO49M	0,6	*Marinobacter hydrocarbonoclasticus: ATCC 49840 chromosome*	96	0,0
UTO65Ps	0,6	*Pseudomonas stutzeri* DSM10701	99	0,0
UTO22UB	0,6	Uncultured bacterium clone: DGGE gel band HZ9D	91	8,00E-158
UTO70Ps	0,5	*Pseudomonas* sp.: Gr65	92	9,00E-170
UTO12NA	0,5	No assigned	0	0,0
UTO08UB	0,4	Uncultured bacterium clone 1-82	98	
UTO54UB	0,4	Uncultured denitrifying bacterium clone 7-nosZ-LZB putative	84	3,00E-118
UTO75Ps	0,4	*Pseudomonas stutzeri* strain: Gr46	94	0,0
UTO46Ps	0,3	*Pseudomonas balearica DSM 6083*	95	0,0
UTO51M	0,3	*Marinobacter* sp. CP1	87	1,00E-143
UTO23UB	0,3	Uncultured soil bacterium clone: E1-4	94	3,00E-175
UTO25UB	0,3	Uncultured bacterium clone: F1	80	1,00E-92
UTO35UB	0,3	Uncultured bacterium clone: Z88	99	0,0
UTO19UB	0,3	Uncultured bacterium clone: J7 G1PM86I01AVM1E	89	6,00E-153
UTO83Ps	0,3	*Pseudomonas stutzeri* DSM 10701	96	0,0
UTO01UB	0,3	Uncultured denitrifying bacterium clone: 34-nosZ-LZB	94	0,0
UTO62Ps	0,2	*Pseudomonas stutzeri* DSM10701	94	0,0
UTO04Ps	0,2	*Pseudomonas stutzeri* strain Gr50	98	0,0
UTO59Ps	0,2	*Pseudomonas stutzeri* strain Gr50	97	0,0
UTO17UB	0,2	Uncultured bacterium clone: J7 G1PM86I01AVM1E	91	9,00E-164
UTO90Ps	0,2	*Pseudomonas stutzeri* strain Gr50	98	0,0
UTO33UB	0,2	Uncultured denitrifying bacterium clone: 1-nosZ-LZB putative	95	0,0
UTO26UB	0,2	Uncultured alpha proteobacterium clone: pRSc_68	95	0,0
UTO78UB	0,2	Uncultured bacterium clone: 2-15-1 putative	98	0,0

Continuation

UTO	% of sequences	Taxonomic affiliation	Identity (%)	e-value
UTO29Pa	0,2	*Paracoccus* sp.	90	7,00E-152
UTO14UB	0,2	Uncultured bacterium clone: 1_100_nosZ putative	78	2,00E-63
UTO89Ps	0,2	*Pseudomonas stutzeri* strain Gr50	95	0,0
UTO31UB	0,1	Uncultured bacterium clone: 3_57_nosZ putative	86	1,00E-136
UTO28Az	0,1	*Azospirillum* sp.	81	7,00E-95
UTO60UB	0,1	Uncultured bacterium clone: 2-15-1 putative	99	0,0
UTO42Ps	0,1	*Pseudomonas balearica* DSM 6083	95	0,0
UTO68Ps	0,1	*Pseudomonas balearica* DSM 6083	90	9,00E-138
UTO53M	0,1	*Marinobacter vinifirmus* strain NBC33	94	4,00E-162
UTO55UB	0,1	Uncultured bacterium isolate DGGE gel band Z-5	88	3,00E-150
UTO64Ps	0,09	*Pseudomonas stutzeri* DSM10701	96	0,0
UTO77Ps	0,09	*Pseudomonas stutzeri* DSM10701	99	0,0
UTO18UB	0,08	Uncultured bacterium clone PYL-SMS-68	93	3,00E-162
UTO71Ps	0,08	*Pseudomonas* sp. Gr65	90	1,00E-162
UTO11Pg	0,07	*Pseudogulbenkiania* sp. YNH5A	84	2,00E-114
UTO57UB	0,07	Uncultured bacterium isolate: DGGE band Zl-3	92	3,00E-156
UTO84Ps	0,07	*Pseudomonas stutzeri* strain SLG510A3-8	95	0,0
UTO58Ps	0,07	*Pseudomonas stutzeri* strain Gr50	97	0,0
UTO63UB	0,07	Uncultured bacterium clone: 2-15-1 putative	97	0,0
UTO76UB	0,06	Uncultured denitrifying bacterium clone 34-nosZ-LZB putative	95	0,0
UTO38Ps	0,06	*Pseudomonas stutzeri* strain Gr50	93	0,0
UTO13UB	0,04	Uncultured bacterium clone 83-07	82	2,00E-83
UTO92Ps	0,04	*Pseudomonas stutzeri* strain Gr50	94	0,0
UTO86Ps	0,04	*Pseudomonas stutzeri* strain Gr46	95	0,0
UTO20B	0,04	*Bradyrhizobium* sp. BTAi1	99	0,0
UTO30UB	0,04	Uncultured bacterium clone: PYL-SMS-69	94	3,00E-175
UTO36UB	0,03	Uncultured denitrifying bacterium clone 40-nosZ-LZB putative	90	5,00E-154
UTO50M	0,03	*Marinobacter* sp. CP1	92	1,00E-174
UTO41Ps	0,03	*Pseudomonas stutzeri* strain DCP-Ps1	93	6,00E-179
UTO94Ps	0,03	*Pseudomonas stutzeri* strain SLG510A3-8	999	2,00E-95
UTO69UB	0,03	*Pseudomonas stutzeri* DSM10701	85	2,00E-128
UTO52M	0,01	*Marinobacter hydrocarbonoclasticus* strain NY-4	89	6,00E-147
UTO07UB	0,01	Uncultured bacterium clone Buji1-6	76	6,00E-71
UTO88Ps	0,01	*Pseudomonas stutzeri* strain Gr50	94	0,0
UTO93Ps	0,01	*Pseudomonas stutzeri* strain: TR2	93	6,00E-83
UTO37Ps	0,008	*Pseudomonas stutzeri* DSM 10701	91	2,00E-164
UTO09UB	0,004	Uncultured bacterium isolate DGGE gel band nosZ-band20	96	2,00E-172
UTO10UB	0,004	Uncultured bacterium clone: SMBR-FEB-N51	87	2,00E-120
UTO32Ps	0,004	*Pseudomonas sp.* strain Gr65	92	6,00E-114
UTO34UB	0,004	Uncultured denitrifying bacterium clone 39-nosZ-LZB putative	84	5,00E-123
UTO40Ps	0,004	*Pseudomonas stutzeri* DSM 10701	88	7,00E-146
UTO56Ps	0,004	*Pseudomonas balearica* DSM 6083	92	3,00E-175
UTO91UB	0,004	Uncultured denitrifying bacterium clone: 34-nosZ-LZB putative	96	1,00E-174

al., 2011; Bowen *et al.*, 2013; Ganesh *et al.*, 2014; Castro-González *et al.*, 2015; Raes *et al.*, 2016). These studies show an increased abundance and richness of *nosZ*-phylotypes in sediments compared to seawater. Within the water column, Castro-González *et al.* (2015) observed a similar richness of *nosZ* TRFs in the OMZ off Chile, as reported in this study.

Richness may be underestimated in the CPB, considering that recent reports indicate that nitrite and nitrous oxide reduction genes are more abundant in the particulate fraction (>1.6 µM) compared to the free-living fraction (0.22 µM) in the Chilean OMZ (Ganesh *et al.*, 2014) and in estuarine areas (Smith *et al.*, 2013). This study focused on the denitrifier community sampled using 0.22 µM filters, revealing a higher

richness of OTUs obtained through pyrosequencing of *nosZ*-gene compared to results from the OMZ off Chile, using shotgun metagenome sequencing and amplification of 16S rRNA by Ganesh *et al.* (2014).

In general, comparing the composition of marine microbial communities is a challenge, due to differences in methodology, size-fractionation, as well as variations in the water column conditions between samples. Likewise, several researchers (Stevens & Ulloa, 2008; Bryant *et al.*, 2012; Ganesh *et al.*, 2014) have suggested that taxonomic diversity and metabolic functions are influenced by multiple environmental parameters across diverse marine habitats.

This data demonstrates that microorganisms that inhabit the CPB, such as *Bradyrhizobium, Paracoccus, Marinobacter, Achromobacter,* as well as high abundances of *Pseudomonas,* display a low diversity of the denitrifying microbial genus. This data is similar to reports from the OMZ off Chile, where phylogenetic analysis has shown a reduced diversity of *nosZ*-type sequences and the similarity between some clones with *Pseudomona stutzeri*, or with clones isolated from marine sediments (Castro-González *et al.*, 2015).

Although all these genera have been reported in marine environments (Pinhassi *et al.*, 2004; Peressutti *et al.*, 2010; Zhu *et al.*, 2013), it is unusual to observe that one of the OTUs from 300 m depth is affiliated with *Bradyrhizobium* (99%), a strictly aerobic anoxygenic phototrophic bacterium, which has been reported in oxic oceanic surface water (Hu *et al.*, 2006). Nevertheless, it is important to consider that taxonomic diversity may be underestimated from the low number of *nosZ*-pyrosequences within the area in comparison with results obtained using the 16S rDNA, which varies from ~80.000-200.000 sequences (Peressutti *et al.*, 2010; Lee *et al.*, 2011).

To our knowledge, this is the first study that explores the use of Taq pyrosequencing technology with a denitrifying functional gene from seawater samples, with results indicating a wide variation in pyrosequences obtained between areas. These results agree with previous data, reporting elevated OTU richness in the particulate fraction (0.2-1.6 µm) at tropical non-OMZ sites (Brown *et al.*, 2009; Kembel *et al.*, 2011). Furthermore, a high number of TRFs and OTUs detected in the area indicate microdiversity, as proposed in previous studies (Acinas *et al.*, 2004; Peressutti *et al.*, 2010). Some pyrosequence related to environmental clones and *Pseudomonas* presented a similarity between 75-80%, which agrees with the recommended threshold for estimating *nosZ*-derived species-level OTUs (Palmer *et al.*, 2009).

The CCA results support numerous studies that report changes in the composition of functional microbial communities in response to environmental factors, however; in this case, physical factors such as temperature, salinity, and dissolved oxygen acquire a greater importance in structuring denitrifying communities. Different studies in marine environments have corroborated that denitrifying communities respond differently to environmental factors. In the case of the *nirS*-community, several studies (Chon *et al.*, 2009; Abell *et al.*, 2010, 2013; Mosier & Francis, 2010; Francis *et al.*, 2013) have found that the abundance, composition, and even *nirS* phylotype richness in estuarine sediments was correlated with a low C/N ratio, N total, chlorophyll-*a*, organic matter availability, and strongly related to salinity changes.

This data support the results of the CCA analysis, where salinity, temperature, and oxygen are the physical variables that determine spatial changes in the *nirS*-community structure along the water column in the CPB. Other studies have suggested that niche-based processes determine the distribution of *nirS* and *nirK* genotypes in sediments (Jones & Hallin, 2010) and in the OMZ (Ganesh *et al.*, 2014), indicating that *nar*, *nir*, *nor* and *nosZ*-denitrifying communities dominate the particulate fraction, where they are favored by oxygen and organic substrates in the surrounding water column.

Considering that denitrifying communities have particular requirements for metabolism, such as electrons acceptors (nitrate, nitrite, N_2O) and electron donors (organic matter), their availability in the water column could determine the occurrence of the following processes: nitrate reduction, nitrite reduction and N_2O reduction by different communities and at different rates. Furthermore, the interaction with other microorganisms, such as nitrifiers, nitrogen-fixing and anammox bacteria should be considered. Thus, considering the low values of predictor variables, as well as unique OTU distributions recorded within both communities in this study, it is likely that other unconsidered factors, such as the distribution of organic matter (dissolved and particulate), size-fractions of particles within communities, nutrients, and N_2O availability, could be important in determining the niche-based processes that take place in the CPB.

These results indicate that communities with the *nosZ*-gene could codify for the reduction of N_2O to N_2. The composition of *nosZ*-type communities is strongly related to dissolved oxygen levels, sustaining the notion that potentially important N_2O emissions occur from the Colombian Pacific. Also, *nosZ*-communities present at depth, with dissol-ved oxygen levels up to 56 µM into the CPB, are in accordance with other studies that reported the presence of *nosZ* transcripts in oxic and mesotrophic upper mixed layer waters (Wyman *et*

al., 2013; Raes *et al.*, 2016), as well as in shallower and oxic depths in the Chilean OMZ (Stewart *et al.*, 2012).

It was more challenging to determine the importance of other environmental parameters in the structure of *nirS* or *nosZ* denitrifier communities, due to limited information available on each site. However, both molecular methods do allow for the detection of differences in community structure between the two types of denitrifying communities.

The NH_4^+ (0.18-2 µM) and NO_3^- (10-40 µM) levels registered in the area Castro-González *et al.*, 2014 indicate an active microbial process involving organic matter remineralization and nitrification. Although there is no apparent NO_2^- or NO_3^- deficit along the water column, which is considered to be a signal of denitrification in the OMZs (Codispoti *et al.*, 2001; Farías *et al.*, 2007), the molecular results indicated that the denitrifying community is present and that its composition varies along the CPB.

From a biogeochemical point of view, these results suggest that if denitrification is occurring in the CPB, it is likely to take place at low rates in subsurface waters. First, because the organic carbon flux in this area is minor compared to the flux that occurs in OMZs associated with coastal upwelling, where high denitrification rates have been reported (Chang *et al.*, 2014). Second, because of dissimilative nitrate reduction rates to nitrite decrease under oxic conditions (Kalvelage *et al.*, 2011).

This research demonstrates the presence, richness and wide distribution of *nirS* and *nosZ*-denitrifying communities along the hypoxic water of the Colombia Current in the Tropical Pacific. This study established that Taq pyrosequencing technology of functional denitrification genes could be applied to compare seawater communities. In this case was able to identify a cosmopolitan marine genus as well as some novel sequences, in an environment where dissolved oxygen levels, temperature, and salinity are able to partially influence the structure and variation of the denitrifying community along the Colombian Pacific.

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