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ARTICLE

Structure of denitrifying communities reducing N_2O at suboxic waters off northern Chile and Perú

Estructura de las comunidades desnitrificantes que reducen N_2O en aguas subóxicas del norte de Chile y Perú

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Resumen.- El gen *nosZ,* el cual codifica para la reducción de N_2O a N_2, fue usado para estudiar la estructura de las comunidades desnitrificantes en la zona de mínimo oxígeno frente a las costas de Chile y Perú, a través del polimorfismo de los fragmentos largos de restricción terminal (PFLRT) y clonamiento de los genes *nosZ*. El análisis del PFLRT mostró poca diversidad de genes *nosZ* en las profundidades subóxicas (núcleo de la zona de mínimo oxígeno-ZMO) comparado con las profundidades donde el O_2 varío ampliamente (límite superior de la zona de mínimo oxígeno o LSZMO). Las comunidades desnitrificantes-*nosZ* presentaron diferencias en su estructura entre localidades geográficas y tiempo de muestreo sugiriendo asociación con la variación en las condiciones ambientales. El análisis de correspondencia canónica indicó que los parámetros ambientales seleccionados como variables predictoras (N_2O, O_2, NH_4^+ y NO_2^-) explicaron bien las diferencias en la composición de la comunidad *nosZ* entre sitios de muestreo. El análisis filogenético mostró poca diversidad de secuencias *nosZ* y agrupó el 81% de los clones cerca al clúster de secuencias de sedimentos del Pacífico. Las secuencias no se relacionaron con ninguna secuencia *nosZ* reportada en agua de mar, o de bacterias desnitrificantes conocidas, demostrando la novedad de los filotipos encontrados en esta área.

Palabras clave: Pacífico sur este, gen *nosZ*, zona de mínimo oxígeno

Abstract.- The *nosZ* gene, which encodes for N_2O reduction to N_2, was used to study the structure of denitrifying communities in the oxygen minimum zone off Chilean and Peruvian coast throughout terminal restriction fragment length polymorphism (TRFLP) and cloning of *nosZ* genes. TRFLP analysis showed little diversity of *nosZ* genes at suboxic depths (Oxygen Minimum Zone´s core) compared with depths where O_2 largely varied (upper limit of OMZ or ULOMZ). The *nosZ*-denitrifying communities showed differences in its structure between geographical locations and time sampling suggesting an association with the shift in the environmental conditions. The canonical correspondence analysis showed that the environmental parameters selected as predictor variables (N_2O, O_2, NH_4^+ and NO_2^-) explained well the differences in *nosZ*-denitrifying community composition among sampling sites. The phylogenetic analysis showed little *nosZ* sequence diversity and grouped 81% of *nosZ*-clones near the cluster of sediments sequences from Pacific. Our sequences did not branch with any known denitrifying bacteria or seawater *nosZ*-sequences available, demonstrating the novelty of phylotypes founded in this area.

Key words: Eastern South Pacific, *nosZ* gene, Oxygen Minimum Zone

INTRODUCTION

The oxygen minimum zones (OMZ) of the eastern South Pacific Ocean (ESPO) along with other OMZ´s in the ocean have been identified as the major sites of denitrification (Codispoti & Packard 1980), where marked oxygen gradients in a reduced vertical scale (20-50 m) are found from oxygenated to suboxic/anoxic (in the OMZ´s core) conditions. Indeed, the OMZ off northern Chile and Perú is one of the shallowest and intense OMZ in the ocean with O_2 levels < 3 nM (Revsbech *et al*. 2009), which has been defined as functional anoxia *sensu* Thamdrup *et al*. (2012). Along that extreme O_2 gradient, NO_3^-, NO_2^- and N_2O gradients are observed as well, being N_2O, a potent greenhouse gas that shows high vertical variability (Farías *et al*. 2007). It is not clear yet which processes are responsible for huge N_2O accumulation at the upper limit of the oxygen minimum zone (ULOMZ) but it is clear that N_2O is depleted towards OMZ´s core, as O_2 declines and favors the N_2O reduction to N_2 by denitrifying process

(Farías *et al*. 2009). For example, N_2O concentrations as high as 172.7 nM off Peru (Codispoti *et al*. 1992) and 124 nM off northern Chile (Farías *et al*. 2007) were registered in subsurface water (below the mixed layer), constituting a large N_2O source estimated in ~12.78 Gg (Cornejo & Farías 2012). N_2O can be produced by nitrification and partial denitrification of bacteria and Archaea (Cabello *et al*. 2004, Castro-González & Farías 2004, Santoro *et al*. 2011, Loescher *et al*. 2012) as well as nitrifier denitrification (Shaw *et al*. 2006), processes which can co-occur at oxyclines and pycnoclines, while dissimilative N_2O to N_2 reduction at suboxic or anoxic condition takes place only by complete denitrification (Bange *et al*. 2010).

Since the partial denitrification is a significant N_2O source modifying oceanic N_2O reservoir, it is very important to know the communities responsible for reducing the N_2O to N_2 in the ESPO, taking into account that this step is developed only by denitrifiers possessing N_2O reductase. There is a wide variety of microorganisms, including over 40 genera of Bacteria, Archaea and fungi, which are capable of denitrifying and that are rarely strict anaerobes (Zumf 1997).

This work is one of the few studies that explore the diversity of communities related to the N_2O reduction in the suboxic water column off Chilean-Peruvian OMZ. There are only a few reports carried out in marine environments such as coastal sediments (Scala & Kerkof 1999, Mills *et al*. 2008), estuarine wetlands (Chon *et al*. 2011) and seawater (Stewart *et al*. 2012, Ganesh *et al*. 2013).

In this research, the functional marker *nosZ*-gene that encodes for N_2O reductase in the last step of denitrification was used. Although this gene is absent in some bacterial and archaeal species that harbor *nir* and *nor* genes, its high level of congruence with 16S rRNA phylogeny (Jones *et al*. 2008) indicates that it might be used to estimate the species-level novelty, as well as species-level diversity of denitrifiers in environmental samples. This marker can be useful to complement previous studies in the ESPO done with *nirS*-denitrifiers (Castro-González *et al*. 2005, Ward *et al*. 2009). We explore the molecular diversity and community structure of *nosZ*-type denitrifiers in the OMZ off northern Chile and Perú and interpret the relationship found between community structure and environmental parameters in the sampling areas.

Materials and methods

Study area and water sampling

The Figure 1 and Table 1 show locations and depth of sampled stations, they also indicate the different cruises and season in which seawater samples were taken. Depth range includes from 10 to 400 m depth and most of them were concentrated at the oxyclines. Seawater samples were collected in 12 L Niskin bottles attached to a CTDO rosette sampler. For DNA extractions, 5-8 L of seawater was filtered by vacuum consecutively through 20, 5 and 0.45 or 0.2 μm pore size membrane filters (Durapore, 47 mm diameter, Millipore, USA). The last filter was immediately immersed in 4.5 ml of sterile lysis buffer (20 mM Tris-HCl, pH 7.5-8.2, 50 mM EDTA, 20 mM NaCl) and stored in liquid nitrogen until DNA extraction.

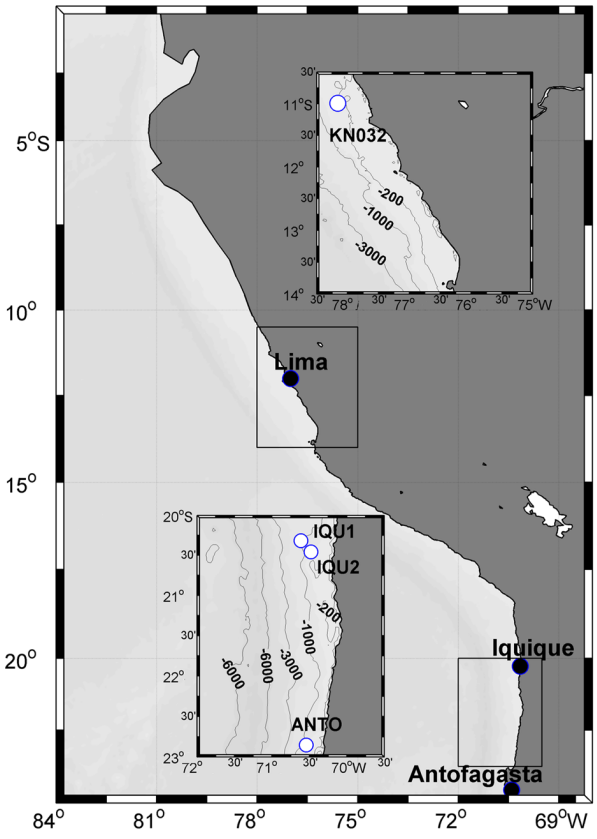

Figure 1. Study area showing the stations of sampling in the northern of Chile off Iquique (IQU1, IQU2) and off Antofagasta (ANTO); and off Lima, Perú (KN032). Elaborated by Juan Faúndez / Área de estudio mostrando las estaciones de muestreo en el norte de Chile frente a Iquique (IQU1, IQU2), frente a Antofagasta (ANTO) y frente a Lima, Perú (KN032). Elaborado por Juan Faúndez

Table 1. Locations (latitude and longitude) and biogeochemical data of stations studied in the OMZ off Chile and Perú.*mixed layer, **upper limit of OMZ / Localización (latitud y longitud) y datos biogeoquímicos de estaciones estudiadas en la ZMO frente a Chile y Perú.*capa de mezcla, **límite superior de la ZMO

Date of sampling	Summer December 2003	Autumn-March 2003				Winter-July 2004		Late spring October 2005
Station	ANTO-15	IQU1-10	IQU1-100	IQU1-300	IQU1-400	IQU2-40	IQU2-60	K032-40
Cruise	Dormido	CHUPS	CHUPS	CHUPS	CHUPS	Prodeploy	Prodeploy	KNOOR
Location	22º51'S	20º19'S	20º19'S	20º19'S	20º19'S	20º275'S	20º275'S	10º 59'S
	70º32'W	70º37'W	70º37'W	70º37'W	70º 37'W	70º287'W	70º287'W	78º 9'W
Km from coast	30	37	37	37	37	20.6	20.6	51
Depth (m)	15*	10*	100	300	400	40**	60**	40**
O_2 (µmol L^{-1})	104	167	8	14	20	24	12	2
N_2O (nmol L^{-1})	77.00	30.83	19.88	10.88	69.23	55.92	32.03	55.93
NO_3^- (µmol L^{-1})	4.75±0.14	4.28±0.24	9.75±0.14	19.00±0.59	20.57±0.92	12.70±1.61	12.00±0.60	13.81±0.50
NO_2^- (µmol L^{-1})	0.59±0.05	0.27±0.00	5.77±0.03	4.36±0.02	0.22±0.02	0.27±0.03	4.49±0.03	3.41±0.52
NH_4^+ (µmol L^{-1})	0.49±0.00	0.11±0.03	0.18±0.05	0.07±0.02	0.07±0.02	0.08±0.00	0.03±0.00	N.D
Simpson DI (1/D)	2.9	2.1	1.4	2.4	1.3	3.1	5.0	2.5

Geochemical analysis and experiments

Dissolved O_2 was analyzed by a modified Winkler method (Williams & Jenkinson 1982) based on a photometric end-point detector, a Dosimat 665 (Metrohom) and a chart recorder. Samples for N_2O were collected in 20 mL GC vials. A clean Tygon tube was used to fill each sample vial from the bottom, allowing at least 3 overflows to displace all bubbles in the vial, and then each sample was immediately poisoned with 50 µl of saturated Hg_2Cl and stored upside down, in the dark, at 4°C until analysis. N_2O was determined by Helium equilibration in the vial (McAullife 1971), followed by quantification with a Varian 3380 gas chromatograph using an electron capture detector maintained at 350°C. N_2O separation was achieved on a 3 m molecular column kept at 60°C, using an ultra-high purity 5% CH_4+Ar mixture as a carrier. The dissolved N_2O concentration in equilibrium with the Helium headspace was calculated in accordance with Weiss & Price (1980). Dissolved NO_2^- and NH_4^+ were measured immediately on board using standard colorimetric (Grasshoff *et al*. 1983) and flurometric techniques (Holmes *et al*. 1999), respectively; for NO_3^- analyses, the seawater samples were filtered by 0.7 µm Whatman GF-F glass filter on board and stored frozen until analysis using an automatic analyzer (Alpkem, Flow Solution IV) following the methodology described by Grasshoff *et al*. (1983).

DNA extraction and *nosZ* amplification

DNA extraction followed the procedure described by Castro-González *et al*. (2005). PCR amplifications of *nosZ* genes from total environmental DNA extracts (10 ng μl^{-1}) were performed with primers nos661F-nos1773R (Throbäck *et al*. 2004). The mix reaction (25 µl) contained 200 ng bovine serum albumin (Roche Applied Science, Indianapolis), 1 U RedAccuTaq Polymerase (Sigma), 200 µM each deoxyribonucleoside triphosphate, 4 pmol of each primer and 1 x PCR buffer provided with the enzyme. The primer nos661F was fluorescently labeled with FAM for T-RFLP analysis. Annealing temperatures during the first 10 touchdown cycles started with 59°C and were kept at 56°C during the following 30 cycles. Amplification products (1137-bp) were analyzed by electrophoresis using 2% (wt/vol) agarose gels followed by staining with ethidium bromide. Bands were visualized by UV excitation. Products of 3 replicate PCRs were combined. PCR-products were purified with the Quiaquick PCR purification kit (Qiagen) and quantified by spectrophotometry at 230, 260 and 280 nm.

NosZ T-RFLPs assay

Purified *nosZ* PCR products (100 ng) were digested with HinP1I (Thermo scientific) using 5U of 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 were separated with an automated DNA sequencer 310 (Applied Biosystems, Darmstadt, Germany). The lengths of fluorescently labeled terminal restriction fragments (T-RFs) were determined by comparison with the internal standard using GeneScan 3.71 software (Applied Biosystems).

Analysis of T-RFLPs, cloning and sequencing

Peaks >70-bp and >50 fluorescence units were included for the analysis. Patterns from different samples were normalized to identical total fluorescence units by an iterative standardization procedure (Dunbar *et al*. 2001). Relative abundance of T-RFs (%) was determined by calculating the ratio between height of a given peak and the normalized total peak height of each sample.

Taking into account the strong vertical chemical gradient permanently observed in the OMZ off Iquique, *nosZ*-communities present in upper limit (ULOMZ) and core of the OMZ were compared because the ULOMZ has a hypoxic condition and a N_2O maximum (IQU2 at 40 m), while OMZ core has a suboxic condition and a N_2O deficit (IQU1 to 100 m). *NosZ* PCR products (3 µl) obtained from these depths were cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Clones were screened for inserts of the proper size, and these inserts were sequenced as described previously (Avarahami *et al*. 2002). The *in silico* analysis of TRFLP from sequences was done with the T-DistinctiEnz[1].

Phylogenetic analysis

NosZ sequences were aligned with DNA sequences from the EMBL database with the Muscle 3.6 alignment software integrated in the BOSQUE software version 1.8 (Ramírez-Flandes & Ulloa 2008). A selection of environmental *nosZ* clones from marine sediment (Scala & Kerkof 1999, Mills *et al*. 2008), soils (Stres *et al*. 2004, Horn *et al*. 2006), wastewater (Sakano *et al*. 2002) and coastal seawater (unpublished, Accession #AB089825, AB089829 and AB089832) were chosen to cover the greatest possible sequence variation. The tree was reconstructed using sequences of partial gene fragments (1137-bp) with Neighbor Joining and Maximum Likelihood method integrated in the Bosque program. The tree topology was evaluated by bootstrap analysis using 100 resampling. One sequence representative of each OTU was submitted to the GenBank databases under accession number KF743125-KF743134.

Statistical methods

To visualize relationships between profiles, pairwise similarities were calculated using the Jaccard coefficient; these considered the presence (1) or absence (0) of T-RFs, the number of T-RFs common to 2 communities, and the total number of T-RFs observed. The diversity index (Simpson I/D) and similarity analysis were estimated and constructed with the Biodiversity Pro software version 2.0 (McAleece *et al*. 1997). Biogeochemical variables were linked to TRFLP patterns by Canonical Correspondence Analysis (CCA), which were performed using the PC-ORD program version 4.01 (MjM Software, Gleneden Beach, OR). CCA was used to (i) recognize patterns of *nosZ* gene composition with respect to the biogeochemical properties prevalent at each station, and (ii) to determine which environmental parameters contributed to the differences among stations.

Results

The TRFLP analysis (Fig. 2) showed a low richness of TRFs in the sampling area. The lowest number of TRFs (3) was founded at 100 m (IQU1-100) into the OMZ core and to 400 m (IQU1-400) depth in the lower oxycline off Iquique and the highest number (9) was founded at 60 m depth off Iquique (IQU2-60) within the ULOMZ. The results showed that the TRFs 77 and 710 were present in the Chilean OMZ off Antofagasta and Iquique, even in different years, but were not detected off Perú (KN032-40). The relative abundance of both TRFs was high, varying between 14-87% (TRF, 77-bp) and 18-68% (TRF, 710-bp) and they were distributed along the vertical profile done in the OMZ off Iquique (10-300 m depth for TRF-710 and 10-400 m depth for TRF-77), suggesting its adaptation to the chemical gradient observed in this area.

The TRFLP and similarity analysis of *nosZ*-communities (Fig. 3) indicated that off Iquique during 2003, the communities at depths of 10 m (IQU1-10) and 300 m (IQU1-300) were very similar (> 85%) because they shared the TRFs 77, 193, 200, 375 and 710-bp. The results also showed similarity (> 40%) between communities at 15 m off Antofagasta (ANTO-15) and those founded off Iquique to 10 (IQU1-10) and 300 m depth (IQU1-300), sharing the TRFs of 77-bp and 710-bp.

[1]<http:/www.bioinformatics.org/~docreza/rest_html/home/htm>

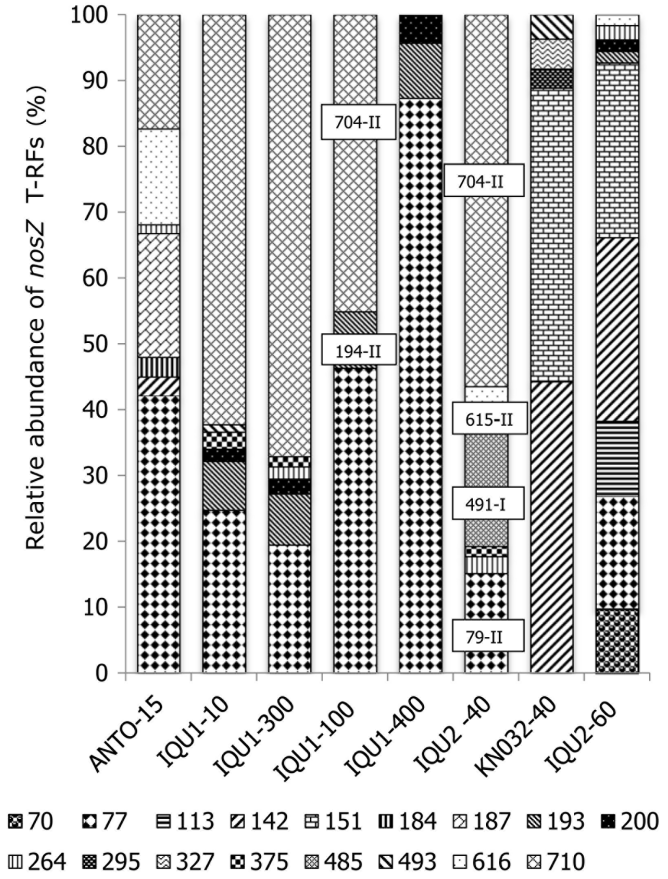

Figure 2. Terminal restriction fragment length polymorphism analysis of amplified *nosZ* genes hydrolyzed with HinP1I. Peak size in base pair is shown in the legend. X-axis, sampling station names (IQU1= Iquique at 2003, IQU2= Iquique at 2004, ANTO= Antofagasta at 2003, K032= Perú at 2005) and sampling depth (*e.g.*, IQU1-10, Iquique, sampling depth 10 m). Y-axis, relative abundance of T-RFs in percentage of total peak height. Sizes of T-RFs after *in silico* analysis of clones are shown on the respective experimental T-RF / Análisis del polimorfismo de los fragmentos largos de restricción terminal de genes *nosZ* amplificados e hidrolizados con HinP1I. El tamaño del pico en pares de bases se muestra en la leyenda. Eje X, nombre de las estaciones de muestreo (IQU1= Iquique en 2003, IQU2= Iquique en 2004, ANTO= Antofagasta en 2003, K032= Perú en 2005) y profundidad del muestreo (por ejemplo IQU1-10: Iquique, profundidad del muestreo 10 m). Eje Y, abundancia relativa de T-RFs expresada en relación a la abundancia total de T-RFs en cada muestra. El tamaño de T-RFs después del análisis de clones *in silico* se muestra sobre el respectivo T-RF experimental

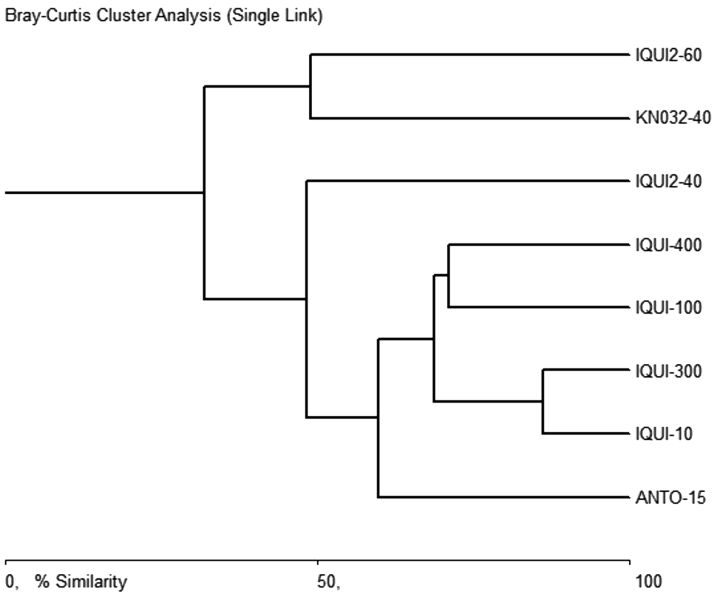

Figure 3. Dendrogram based on presence/absence of *nosZ* T-RFs show the similarity values (as percentage) between T-RFs obtained from amplicons digested with HinP1I. Sampling station names (IQU1= Iquique at 2003, IQU2= Iquique at 2004, ANTO= Antofagasta at 2003, K032= Perú at 2005) and sampling depth (*e.g.*, IQU1-10, Iquique, sampling depth 10 m) / Dendrograma basado en presencia/ausencia de TRFs *nosZ*, muestra los valores de similaridad (como porcentaje) entre los TRFs obtenidos desde amplicones digeridos con HinP1I. Nombre de las estaciones de muestreo (IQU1= Iquique en 2003, IQU2= Iquique en 2004, ANTO= Antofagasta en 2003, K032= Perú en 2005) y profundidad del muestreo (*e.g.*, IQU1-10, Iquique, profundidad del muestreo 10 m)

The TRFLP analysis (Fig. 2) also indicated that *nosZ*-communities from ULOMZ (Anto-15, IQU2-40 and IQU2-60) had a higher number of T-RFs than those observed in the OMZ core. Likewise, an increasing in the richness (Fig. 2) and diversity (Table 1) of TRFs was observed at the ULOMZ, associated also to the presence the exclusive TRFs to each depth: 187 and 184-bp (Anto-15), 70 and 113-bp (IQU2-60), 295 and 327-bp (K032-40), 485-bp (IQU2-40). Otherwise, the TRFs of 77, 264 and 616-bp were common to all samples and the relative abundance of the last TRF decreased with depth.

The *nosZ*-communities found at 60 m off Chile (IQU2-60) and at 40 m depth off Perú (KN032-40) grouped with a similarity of ~49% because they shared the T-RFs of 142 and 151-bp (Fig. 3). It is interesting to note that both TRFs increased its relative abundance from ~25% at the IQU2-60 station up to ~45% at the KN032-40 station in the OMZ Peruvian, where unique TRFs (295 and 327) were also observed (Fig. 2).

The diversity analysis (Table 1) showed high values (I/D= 3.1-5) between 40-60 m depths off Iquique and the lowest value (I/D= 1.3) at IQU1-400, where the *nosZ*-denitrifying community displayed low TRF's richness and little similarity with the communities at the ULOMZ (< 49%) and even with these ones recorded to 300 m depth (IQU1-300, ~45% similarity) (Fig. 3). Also to 400 m depth in the lower oxycline the TRF of 77-bp was the most abundant (~87%) and the second N_2O maxima was recorded (Table 1).

The CCA results (Fig. 4) showed that the environmental parameters selected as predictor variables (N_2O, O_2, NH_4^+ and NO_2^-) well explained the differences in *nosZ*-denitrifying community composition among sites. The composition of this community was explained in ~61% (Dimension 1, 43% and dimension 2, 18%, respectively) by such predictor variables. The sites separation along the first axis suggested that they differ in the *nosZ*-type community composition. The variation in *nosZ*-composition was positively correlated with O_2, N_2O and NH_4^+, and negatively with NO_2^-. The plot also showed that the distribution of *nosZ*-TRFs were scattered around the sampling point where they were more abundant. For example, the TRFs 710 was located near to IQU1-10 (10 m), IQU1-100 (100 m), IQU2-40 (40 m) and IQU1-300 (300 m); in these sites intermediate or low levels of N_2O, O_2, and NH_4^+ were detected. The TRF 616 was near to ANTO-15 where high O_2 and NH_4^+ levels were measured, and the T-RF 77 was near to IQUI-400 where high levels of N_2O were recorded.

The CCA analysis separated the Peruvian *nosZ*-communities (K03240) from the Chilean *nosZ*-communities (IQU2-60) and although they shared the TRF 151, 142 and 493 these ones were near to K03240 station for its major abundance in this area. In both localities high NO_2^- levels and similar NO_3^- levels were measured, however, the lowest O_2 level was detected in the K03240 station, which could explain the higher abundance of TRFs. Likewise, with this analysis the unique TRFs found in the ULOMZ samples were separated in the plot from the other TRFs. This was the case of TRFs of 184, 187-bp (Anto-15), 295, 327-bp (K032-40), 485-bp (IQU2-40) and 70,113-bp (IQU2-60).

Phylogenetic analysis

A total of 51 sequences (28 from 40 m depth and 23 from 100 m depth) were used for the phylogenetic analysis. The consensus of *nosZ*-tree (Fig. 5) revealed that the clones formed 2 clusters. The cluster I grouped (> 90% bootstrap) 2 unique clones with *Pseudomona stutzeri* (with 86% similarity) and with one clone isolated from wastewaters reactors; furthermore, this cluster grouped (> 99% bootstrap) 5 clones from 100 m depth with one clone isolated from soils (with 85% similarity). The cluster II was composed of 3 branches, the first one grouped (> 85% bootstrap) 37 sequences from 40 and 100 m depths with one sequence from Pacific's sediment (with 80% similarity), the other 2 branches had 3 and 4 sequences, respectively, which were unique in our analysis and also were grouped (80-97% bootstrap) with sequences from Pacific's sediments. Likewise, this cluster did not branch with any known denitrifying bacteria or with *nosZ*-sequences from Atlantic's sediments or seawater and the majority of DNA sequences presented a low similarity (60-83%) compared with other environmental sequences. This indicates that the OMZs off Chile and Perú contain uncharacterized *nosZ*-denitrifying organisms in the column water. In general, differences in the distribution of clones between 40 and 100 m depth in the phylogenetic analysis was not observed and the *nosZ*-sequences were not very diverse showing 3 OTUs and 8 ungrouped sequences both 99% and 97% of similarity, only (see Table S1). Comparable results were obtained with the similarity analysis (to 97% level) of amino acid *nosZ*-sequences where the same 3 OTUs along with 6 ungrouped sequences were observed (Table S2).

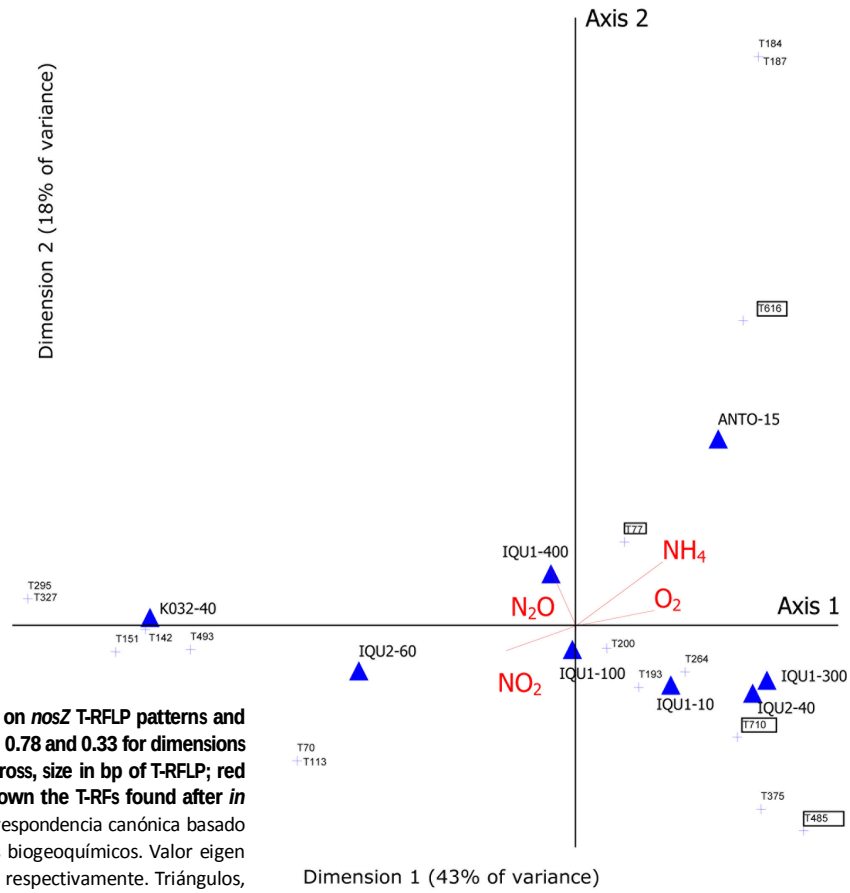

Figure 4. Canonical correspondence analysis based on *nosZ* T-RFLP patterns and biogeochemical parameters. Eigenvalues (500 runs), 0.78 and 0.33 for dimensions 1 and 2 respectively. Triangles, sampling stations; cross, size in bp of T-RFLP; red lines, biogeochemical parameters. The squares shown the T-RFs found after *in silico* analysis of clones sequences / Análisis de correspondencia canónica basado en patrones de T-RFLP de genes *nosZ* y parámetros biogeoquímicos. Valor eigen (500 corridas), 0,78 y 0,33 para dimensiones 1 y 2, respectivamente. Triángulos, estaciones de muestreo; cruces, tamaño en pb de T-RFLP; líneas rojas, parámetros biogeoquímicos. Los cuadrados muestran los T-RFs encontrados después del análisis de secuencias de clones *in silico*

Assignment of *nosZ* clones to T-RFs

Terminal restriction fragment length polymorphism analysis of clones combined with an *in silico* digestion of our *nosZ*-sequences showed that the majority of the sequences could be assigned to dominant T-RFs (Fig. 2). For the sample IQU2-40, clones corresponded to 4 TRFs (79, 491, 615 and 704) comprising ~90% of the total peak height and for the sample IQU1-100, clones were assigned to 2 TRFs (194 and 704) comprising ~53% of the total peak height. Terminal restriction fragments from *in silico* hydrolysis differed from the respective TRFs obtained experimentally by up to 6-bp (Fig. 2). For example, the 704-bp TRF of the *in silico* analysis using HinP1I corresponded to the experimental 710 TRF. Likewise, the 79, 194, 615 and 491-bp TRF calculated by *in silico* analysis corresponded to experimental TRF of 77, 193, 616 and 485-bp, respectively. Digestion with HinP1I yielded TRFs that were not generally unique to a single cluster in the tree. The cluster I grouped sequences with theoretical T-RF of 491 (I40-71), 104, 46, and 53-bp, however, the 2 last ones were not observed with the TRFLP analysis because only peaks of >70-bp were included to do it. The cluster II grouped in the first branch sequences of Iquique at 40 and 100 m depths with theoretical TRFs of 704-bp (92%) and 79-bp (8%). The second branch included TRF´s of 615-bp only, however, this TRF was not observed to 100 m depth off Iquique (IQU1-100) with the TRFLP analysis. The third branch in this cluster, included theoretical T-RFs of 79 (I40-89 clone), 194 (I100-56 and I100-55 clones) and 125-bp (I100-70 clone), but the last one was not detected in the TRFLP analysis.

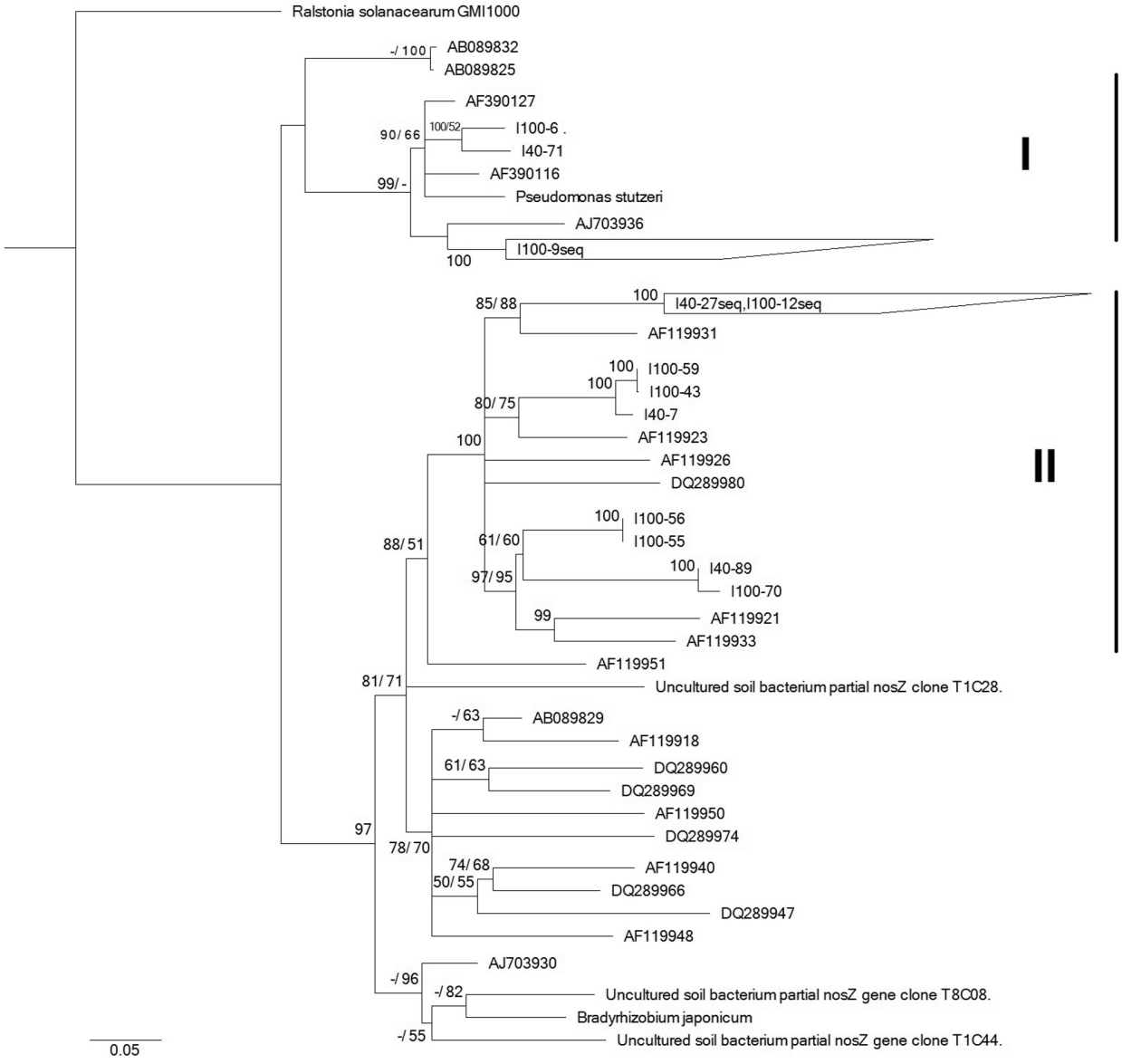

Figure 5. Phylogram for *nosZ* sequences based on partial gene fragments using distance matrix analysis (F84), Neighbor-Joining (NJ) and Maximum Likelihood (ML) method. The scale bar indicates 5% nucleotide substitutions. Bootstrap values greater than 50 from 100 replicate obtained by NJ at the numerator and ML at denominator are reported at the nodes. (-) indicated no bootstrap value reported. Clones obtained from coastal station off Iquique are designated as 'I' followed with numbers to indicate sampling depths and clone number. Roman numbers indicate sequence clusters obtained for the study area. The *nosZ* sequences of *Ralstonia solanacearum* GMI1000 served as out group / Árbol filogenético de secuencias *nosZ* basado en fragmentos parciales del gene usando métodos de matriz de distancia (F84), NJ y ML. La barra de la escala indica 5% de sustitución de nucleótidos. Se muestran en los nodos los valores bootstrap >50 obtenidos de 100 réplicas por NJ en el numerador y por ML en el denominador. El signo (-) indica que no hay un valor bootstrap reportado. Los clones obtenidos de la estación costera frente a Iquique son designados con 'I' seguido con números para indicar profundidad de muestreo y número del clon. Los números romanos indican los clúster de secuencias obtenidas en el área de estudio. La secuencia *nosZ* de *Ralstonia solanacearum* GMI1000 fue usada como grupo externo

Discussion

The structure of denitrifying communities with capacity to reduce N_2O to N_2 at suboxic waters off northern Chile and Perú was studied through the TRFLP analysis and cloning of *nosZ*-gene which encode for nitrous oxide reductase. Our data shown that *nosZ*-denitrifiers inhabit the ULOMZ and the core of the OMZ off Chile and Peru, below O_2 fluctuations between ~2-167 μM, however, they are few diverse both to taxonomic (DNA sequences) and functional level (amino acid sequences, Table S3). These results suggest that the *nosZ*-communities founded in this study have a lifestyle that is adaptable to the chemical (mainly O_2) and physical forcing of this area. It is in accordance with previous observation done in the OMZ off northern Chile which indicated that the surface oxic and well mixed waters (10-15 m depth), the hypoxic and suboxic waters in the ULOMZ (between 40-60 m) and the suboxic-anoxic waters into the core (100-300 m depth), mainly contained γ and δ-Proteobacteria, Bacteroidetes, Planctomycetes (Stevens & Ulloa 2008), and also anammox bacteria Candidatus '*Scalindua sorokinii*' (Galán *et al*. 2009) and even Cyanobacteria (Lavin *et al*. 2010, Fernández *et al*. 2011). Also, from a functional point of view, metatranscriptomic findings report the metabolic versatility of nitrifiers, archaea, anammox and denitrifiers type *nirS, nirK and nosZ* (Stewart *et al*. 2012) in the area; and other studies indicated the co-occurring and activity of ammonia-oxidizing β-proteobacteria, *Nitrosospira* spp. principally (Molina *et al*. 2007) and *nirS*-denitrifiers (Castro-González *et al*. 2005). These reports (with 16S RNA and functional genes) along with our results suggest that diverse microbial groups inhabit the OMZ in the ESPO.

The TRFLP´s results showed a low richness of *nosZ*-type denitrifiers in the sampling area, which was in accordance with cloning approach where only dominant TRFs were detected. However, changes in the composition (relative abundance and richness) of communities inhabiting the OMZ off Antofagasta, Iquique and Peru as well as along the column water off Iquique were evident. The lowest number of TRFs (3) was detected in the lower oxycline at 400 m depth (IQU1-400) and in the OMZ core off Iquique (IQU1-100) while the highest TRFs' richness (9 T-RFs) was observed in the ULOMZ at 60 m depth off Iquique (IQU2-60). Several TRFs were shared between all the stations and depths; in fact, the *nosZ*-community composition off Perú (at 40 m depth) was something similar (40%) although less diverse than those found off Iquique at 60 m depth (IQU2-60). Both results, suggest that some TRFs (corresponding to the T-RF 142 and 151-bp) of *nosZ*-type community are present along the OMZ in the ESPO, in permanent form, even in different season and geographic locations, probably by spatial similarity in some of the environmental conditions, given that, in both localities similar levels of N_2O, NO_3^- and NO_2^- were recorded during our study (Table 1).

The CCA analysis showed that the selected predictor environmental variables explained the differences in *nosZ*-denitrifying community composition among sites. The *nosZ*-communities were separated between some characteristics of the upper oxycline (TRFs: 616, 151, 142, 493, 70, 113, 295, 327, 184, 187) and the rest of TRFs into of core and/or the lower oxycline (TRF 77). Several studies have reported that the denitrifiers respond differently to environmental gradients of available nitrogen species and that the NH_4^+, NO_2^-, O_2 or NO_3^- could be determining the differences in its structure (Santoro *et al*. 2006, Hones & Hallin 2010). However, our results show that although the composition of *nosZ*-TRFs was positively correlated with O_2, N_2O and NH_4^+ and negatively with NO_2^- there is not a clear trend in this aspect, suggesting that probably other variables not measured in this study, could also impact the structure of *nosZ*-type denitrifiers. For example, organic carbon, metal availability and also C:N and nitrate/nitrite ratios have been reported to be important factors determining the community composition and structure of denitrifiers (Oakley *et al*. 2007, Ward *et al*. 2008, Mosier & Francis 2010, Lam *et al*. 2011).

The higher richness of T-RFs and diversity of communities in the ULOMZ than in the core suggest that there is a community shift based on the separation between them, which could be related to the strong physicochemical gradients observed among both layers, and also because the ULOMZ is a zone where the gases (O_2, N_2O) and nutrients (nitrite, ammonium) vary constantly by a strong microbial cycling of N and C (Molina *et al*. 2005, Cuevas & Morales 2006, Molina & Farías 2009) and where particulate material is trapped on a strong halocline (pycnocline) creating suboxic microsites within the oxygenated water column to sustain the denitrification process (Pantoja *et al*. 2004). In fact, this phenomenon has been supported recently in the ESPO by Ganesh *et al.* (2013) whose using a metagenomic approach, recorded *nosZ* genes between 70-1000 m depth off Chilean OMZ, with the peculiarity that their abundance was related directly with particle-associated niches. In the same sense, our data suggests that the variety of micro niches at oxyclines could be determining the observed differences in the structure of *nosZ*-communities inhabiting the ULOMZ off Chile and

Perú; because it was evident the presence and dominance of the T-RFs of 77-bp and 710-bp off Chile and not off Perú. Our results support the hypothesis of niche differentiation previously proposed for global data set of *nirS* and *nirK* communities (Jones & Hallin 2010), and for ammonia-oxidizing β-proteobacteria (Molina *et al*. 2007) and anammox bacteria (Galán *et al*. 2009) in the ESPO, which, could be a strategy of *nosZ*-community to be metabolically versatile in the ULOMZ to participate in several biologically mediated transformations of nitrogen and other elemental cycles as it has been argued by other researchers (Lam & Kuypers 2011).

On the other hand, the persistence of low O_2 levels into the core could be responsible of the low richness and diversity of *nosZ*-T-RFs recorded to 100 m depth. Our results suggest that although there are few organisms (detected as TRFs) that have the nitrous oxide reductase gene to this depth, there are some dominant TRFs (77 and 710) that could be in charge of the major part of reduction process of N_2O to N_2. Several studies have reported similar trends, *i.e.*, Jayakumar *et al.* (2009) reported that in the later stages of denitrification the dominance of a few phylotypes increased while the OTUs diversity decreased; Castro-González *et al*. (2005) observed lower diversity of *nirS*-phylotypes into the core than in the ULOMZ (Castro-González *et al*. 2005) and Steward *et al.* (2012) detected *nosZ* mRNAs only in the core (to 200 m depth) off Iquique, where high N_2O reduction rates (~3.2 μmol $L^{-1}d^{-1}$) were measured previously (Castro-González & Farías 2004).

Although *nosZ*-denitrifiers founded in this research have the nitrous oxide reductase gene to reduce N_2O to N_2 into the ULOMZ and into the core, it is necessary to develop specific studies to determine if all the organisms are active below these conditions and how their activities vary with O_2 and nutrient fluctuations. Until now, several studies in the area indicated that the denitrifying communities reduce more actively N_2O to N_2 in the core than in the ULOMZ (Castro-González & Farías 2004, Farías *et al*. 2007, 2009, Dalsgaard *et al*. 2012). This information is in accordance with a recent report that argued that although the *nosZ*-gene of Labrenzia-like denitrifying bacteria had a very broad biogeographical distribution inside and outside the OMZ of the Arabian Sea, they actively reduce N_2O only within the OMZ core with < 3.1 μM O_2 and > 3 μM NO_2^- (Wyman *et al.* 2013).

Our results also showed that in the OMZ off Iquique in the lower oxycline to 400 m depth, where levels of N_2O were high (69.23 μM), there is one dominant TRFs (TRF of 77-bp, ~88%) with the potential to reduce N_2O to N_2. However, our data suggests that the nitrous oxide reductase perhaps is being inhibited by high O_2 level (20 μM), contributing to the N_2O maxima usually found at this depth (Farías *et al*. 2007). In respect of the N_2O production by partial denitrification in marine environments it has been documented by several researchers in sediments (Gao *et al*. 2010) and suboxic areas, such as the Indian Shelf (Naqvi *et al*. 2000), the Arabian Sea (Naqvi *et al*. 2000), and in the upper oxycline (30-90 m depth) of the Chilean and Peruvian's OMZ (Farías *et al*. 2009). In the last case, it has been reported that the N_2O is produced below a wide range of O_2 (11.6-101 μM) at rates between 9.6-27.6 nmol $L^{-1}d^{-1}$ and that if denitrifiers are subject to increments of oxygen up to 48.5 μM the N_2O production can augment twofold (Castro-González & Farías 2004), demonstrating the strong inhibition of O_2 on N_2O reductase and the facultative capacity of denitrifiers in natural environments. However, we must keep in mind the possible uncoupling of denitrifying activity and community composition as it has been argued by Wallenstein *et al*. (2006) and Boyle *et al*. (2006). Several factors (environmental, biological, physical) not measured in this study could determine such behavior. For example, from the physical point of view, it has been reported that in one transient systems such as this upwelling area, significant amounts of N_2O can accumulate temporarily at the boundaries, when the system is shifting from oxic to sub-oxic conditions (Cornejo *et al*. 2007); and from the biological point of view, it has also been argued that changes in magnitude and timing of the carbon supply during the sampling times, the patchy distribution of heterotrophic denitrifying bacteria (Van Mooy *et al*. 2002, Vos & Montoya 2009, Bulow *et al*. 2010) and the dominance of anammox process in some periods and depths in respect to denitrification in the ESPO (Ward *et al*. 2009) it could be responsible of the decoupling observed between activity and composition of denitrifying communities.

The *nosZ*-communities inhabiting the ESPO although they are not very diverse, they are composed of new phylotypes and T-RFs which are present along the water column from oxic to sub-oxic conditions showing changes in their structure (OTUs richness, relative abundance and diversity) between geographical localities and between the ULOMZ and the core. Specific studies must be developed to determine with certainty if the *nosZ*-community is structured with base to niche differentiation, and if the community is metabolically active in the ULOMZ where major OTU's richness was found despite the strong variation of oxygen.

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Table S1. Operational Taxonomic Units (OTUs) obtained by similarity analysis (to 97%) of DNA sequences of *nosZ* gene found in the OMZ off Iquique to 100 m (I100) and 40 m depth (I40) / Unidades Taxonómicas Operacionales (OTU) obtenidas por análisis de similaridad (al 97%) de las secuencias de ADN del gen *nosZ* encontradas en la ZMO frente a Iquique a 100 m (I100) y 40 m de profundidad (I40)

OTU: 1	OTU: 2	OTU: 3	Ungrouped
I100-62	I40-8	I100-59	I100-70
I100-52	I40-72	I100-43	I100-6
I100-68	I100-5		I40-71
I100-66	I40-14		I40-7
	I40-43		I100-55
	I40-46		I40-89
	I40-100		I40-8
	I40-79		I40-72
	I100-2		
	I100-44		
	I40-47		
	I100-57		
	I40-44		
	I100-8		
	I40-41		
	I40-51		
	I40-75		
	I40-32		
	I100-61		
	I100-51		
	I100-4		
	I40-77		
	I40-10		
	I40-64		
	I40-90		
	I100-25		
	I40-83		
	I40-86		
	I40-92		
	I100-65		
	I100-7		
	I100-75		

Table S2. Operational Taxonomic Units (OTUs) obtained by nosZ amino acid similarity analysis (to 97%) found in the OMZ off Iquique to 100 m (I100) and 40 m depth (I40) / Unidades Taxonómicas Operacionales (OTU) obtenidas por análisis de similaridad de aminoácidos nosZ (al 97%) encontradas en la ZMO frente a Iquique a 100 m (I100) y 40 m de profundidad (I40)

OTU: 1	OTU: 2	OTU: 3	Ungrouped
I100-62	I40-7	I40-72	I100-70
I100-68	I100-59	I40-43	I100-6
I100-52	I100-43	I40-46	I40-71
I100-66		I40-47	I40-89
		I40-8	I100-55
		I100-2	I100-5
		I40-14	
		I100-65	
		I40-92	
		I40-100	
		I100-44	
		I40-79	
		I40-64	
		I40-44	
		I100-51	
		I100-57	
		I100-61	
		I40-10	
		I40-90	
		I40-86	
		I100-25	
		I40-77	
		I40-83	
		I40-32	
		I40-41	
		I40-51	
		I100-7	
		I100-8	
		I40-75	
		I100-4	
		I100-75	

Table S3. Similarity of amino acid sequences (to 97%) of nosZ gene found in the OMZ off Iquique to 100 m (I100) and 40 m depth (I40) / Similaridad de las secuencias de amino ácidos (al 97%) del gen nosZ encontradas en la ZMO frente a Iquique a 100 m (I100) y 40 m de profundidad (I40)

	I100-62	I100-68	I100-52	I100-63	I100-66	I40-71	I100-6	I40-89	I100-55	I100-56	I40-7	I100-43	I100-59	I100-70	I40-72	I40-43	I40-46	I40-47	I40-8	I100-65	I40-92	I40-96	I40-14	I40-100	100-44	I100-2
I100-62	100	98	98	99	99	86	87	60	59	59	60	60	60	55	57	59	59	59	59	59	59	59	59	59	59	59
I100-68	98	100	98	99	99	86	87	60	59	59	60	60	60	55	56	59	59	59	59	59	59	59	59	59	59	59
I100-52	98	98	100	99	99	87	87	60	60	60	60	60	61	55	57	60	60	60	59	59	60	60	60	60	59	60
I100-63	99	99	99	100	100	87	87	60	60	60	60	60	61	55	57	60	60	60	60	60	60	60	60	60	60	60
I100-66	99	99	99	100	100	87	87	60	60	60	60	60	61	55	57	60	60	60	59	59	60	60	60	60	59	60
I40-71	86	86	87	87	87	100	95	59	57	57	57	57	58	53	59	57	57	57	57	57	57	57	57	57	57	57
I100-6	87	87	87	87	87	95	100	58	56	56	57	57	57	51	56	56	56	56	56	56	56	56	56	56	56	56
I40-89	60	60	60	60	60	59	58	100	85	85	82	82	81	76	81	79	79	78	79	79	78	78	78	78	78	78
I100-55	59	59	60	60	60	57	56	85	100	100	82	83	83	82	86	83	83	82	82	82	82	82	82	82	82	82
I100-56	59	59	60	60	60	57	56	85	100	100	82	83	83	82	86	83	83	82	82	82	82	82	82	82	82	82
I40-7	60	60	60	60	60	57	57	82	82	82	100	99	99	82	88	83	83	82	82	82	82	82	82	82	82	82
I100-43	60	60	60	60	60	57	57	82	83	83	99	100	100	82	88	82	83	82	82	82	82	82	82	82	82	82
I100-59	60	60	61	61	61	58	57	81	83	83	99	100	100	82	88	83	83	82	82	82	82	82	82	82	82	82
I100-70	55	55	55	55	55	53	51	76	82	82	82	82	82	100	96	98	98	98	98	98	98	98	98	98	98	98
I40-72	57	56	57	57	57	59	56	81	86	86	88	88	88	96	100	97	97	99	98	99	98	98	99	98	98	98
I40-43	59	59	60	60	60	57	56	79	83	83	83	82	83	98	97	100	100	99	98	98	98	98	98	98	99	98
I40-46	59	59	60	60	60	57	56	79	83	83	83	83	83	98	97	100	100	99	98	99	99	99	98	99	99	99
I40-47	59	59	60	60	60	57	56	78	82	82	82	82	82	98	99	99	99	100	98	99	99	99	99	99	99	99
I40-8	59	59	59	60	59	57	56	79	82	82	82	82	82	98	98	98	98	98	100	99	99	99	98	99	99	99
I100-65	59	59	59	60	59	57	56	79	82	82	82	82	82	98	99	98	99	99	99	100	100	100	99	99	99	99
I40-92	59	59	60	60	60	57	56	78	82	82	82	82	82	98	98	98	99	99	99	100	100	100	99	99	99	99
I40-96	59	59	60	60	60	57	56	78	82	82	82	82	82	98	98	98	99	99	99	100	100	100	99	99	99	99
I40-14	59	59	60	60	60	57	56	78	82	82	82	82	82	98	99	98	98	99	98	99	99	99	100	99	99	99
I40-100	59	59	60	60	60	57	56	78	82	82	82	82	82	98	98	98	99	99	99	99	99	99	99	100	99	99
100-44	59	59	59	60	59	57	56	78	82	82	82	82	82	98	98	99	99	99	99	99	99	99	99	99	100	99
I100-2	59	59	60	60	60	57	56	78	82	82	82	82	82	98	98	98	99	99	99	99	99	99	99	99	99	100
I40-79	59	59	60	60	60	57	56	79	82	82	82	82	82	98	97	98	99	99	99	99	99	99	99	99	99	99
I40-64	60	59	60	60	60	57	57	78	82	82	83	82	82	98	98	98	99	99	99	99	99	99	99	99	99	99
I100-51	59	59	60	60	60	57	56	78	82	82	82	82	82	98	97	98	99	99	99	99	99	99	99	99	99	99
I100-57	59	59	60	60	60	57	56	79	82	82	82	82	82	98	98	98	99	99	99	99	99	99	99	99	99	99
I100-61	60	60	60	60	60	58	56	79	83	83	82	82	82	98	99	98	99	99	99	99	99	99	99	99	99	99
I40-44	59	59	60	60	60	57	56	78	82	82	82	82	82	98	97	98	99	99	99	99	99	99	99	99	99	99
I40-10	59	59	60	60	60	57	56	79	83	83	82	82	82	98	98	99	99	99	99	99	99	99	99	99	99	99
I100-8	59	59	60	60	60	57	56	79	82	82	82	82	82	98	98	99	99	99	99	99	99	99	100	99	99	99
I40-90	59	60	60	60	60	58	56	79	82	82	82	82	82	98	99	99	99	99	99	99	99	99	99	99	99	99
I40-86	59	59	60	60	60	57	56	79	82	82	82	82	82	98	99	99	99	99	99	99	99	99	99	99	99	99
I40-77	59	60	60	60	60	58	56	79	82	82	82	82	82	98	99	99	99	99	99	99	99	99	99	99	99	99
I40-83	59	60	60	60	60	58	56	79	82	82	82	82	82	98	98	99	99	99	99	99	99	99	99	99	99	99
I100-25	59	59	60	60	60	57	56	79	82	82	82	82	82	98	99	99	99	99	99	99	99	99	99	99	99	99
I100-7	59	59	60	60	60	57	56	79	83	83	82	82	82	98	99	99	99	99	99	99	99	99	99	99	99	99
I40-32	59	59	60	60	60	57	56	79	83	83	82	82	82	98	98	99	99	99	99	99	99	99	99	99	99	99
I40-41	60	60	60	60	60	58	56	79	83	83	82	82	82	98	99	99	99	99	99	99	99	99	99	99	99	99
I40-51	60	60	60	61	60	58	56	79	83	83	82	82	82	98	99	99	99	99	99	99	99	99	99	99	99	99
I100-4	60	60	60	60	60	58	56	79	83	83	83	82	83	98	99	99	99	99	99	100	100	100	99	100	100	100
I100-75	60	60	60	60	60	58	56	79	83	83	83	82	83	98	99	99	99	99	99	100	100	100	99	100	100	100
I40-75	60	60	60	60	60	58	56	79	83	83	83	82	83	98	99	99	99	99	99	100	100	100	99	100	100	100
I40-15	60	60	60	60	60	58	56	79	83	83	83	82	83	98	99	99	99	99	99	100	100	100	99	100	100	100
I40-74	59	60	60	60	60	58	56	79	83	83	82	82	82	98	99	99	99	99	99	100	100	100	99	100	100	100
I40-87	59	60	60	60	60	58	56	79	83	83	82	82	82	98	99	99	99	99	99	100	100	100	99	100	100	100
I40-88	59	60	60	60	60	58	56	79	83	83	82	82	82	98	99	99	99	99	99	100	100	100	99	100	100	100
I100-75	60	60	60	60	60	58	56	79	83	83	83	82	83	98	99	99	99	99	99	100	100	100	99	100	100	100

Table S3 continued / Continuación Tabla S3

	I40-79	I40-64	I100-51	I100-57	I100-61	I40-44	I40-10	I100-8	I40-90	I40-86	I40-77	I40-83	I100-25	I100-7	I40-32	I40-41	I40-51	I100-4	I100-75	I40-75	I40-15	I40-74	I40-87	I40-88	I100-75
I100-62	59	60	59	59	60	59	59	59	59	59	59	59	59	59	59	60	60	60	60	60	60	59	59	59	60
I100-68	59	59	59	59	60	59	59	59	60	59	60	60	59	59	59	60	60	60	60	60	60	60	60	60	60
I100-52	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60
I100-63	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	61	60	60	60	60	60	60	60	60
I100-66	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60
I40-71	57	57	57	57	58	57	57	57	58	57	58	58	57	57	57	58	58	58	58	58	58	58	58	58	58
I100-6	56	57	56	56	56	56	56	56	56	56	56	56	56	56	56	56	56	56	56	56	56	56	56	56	56
I40-89	79	78	78	79	79	78	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79	79
I100-55	82	82	82	82	83	82	83	82	82	82	82	82	82	83	83	83	83	83	83	83	83	83	83	83	83
I100-56	82	82	82	82	83	82	83	82	82	82	82	82	82	83	83	83	83	83	83	83	83	83	83	83	83
I40-7	82	83	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	83	83	83	83	82	82	82	83
I100-43	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82
I100-59	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	82	83	83	83	83	82	82	82	83
I100-70	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98
I40-72	97	98	97	98	99	97	98	98	99	99	99	98	99	99	98	99	99	99	99	99	99	99	99	99	99
I40-43	98	98	98	98	98	98	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
I40-46	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
I40-47	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
I40-8	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
I100-65	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100
I40-92	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100
I40-96	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100
I40-14	99	99	99	99	99	99	99	100	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
I40-100	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100
100-44	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100
I100-2	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100
I40-79	100	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100
I40-64	99	100	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100
I100-51	99	99	100	99	99	99	99	99	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100
I100-57	99	99	99	100	99	99	99	99	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100
I100-61	99	99	99	99	100	99	99	99	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100
I40-44	99	99	99	99	99	100	100	99	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100
I40-10	99	99	99	99	99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I100-8	99	99	99	99	99	99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I40-90	99	99	99	99	99	99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I40-86	99	99	99	99	99	99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I40-77	99	99	99	99	99	99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I40-83	99	99	99	99	99	99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I100-25	99	99	99	99	99	99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I100-7	99	99	99	99	99	99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I40-32	99	99	99	99	99	99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I40-41	99	99	99	99	99	99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I40-51	99	99	99	99	99	99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I100-4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I100-75	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I40-75	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I40-15	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I40-74	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I40-87	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I40-88	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
I100-75	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100