Abundance of denitrifying genes and microbial community structure in volcanic soils

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Abstract

Nitrous oxide (N_2O) is a potent greenhouse gas produced during denitrification, a process that includes several genes coding enzymes responsible for nitrogen (N) forms transformations. In volcanic ash soils of southern Chile, fertilization had increased over the last 10 years with implications for N_2O emissions. Despite this, little is still known about potential denitrification and the presence of denitrifying genes. In this study we report the abundance of denitrifying genes and a basal characterization of microbial communities in five volcanic ash soils with different levels of organic matter. The denitrifying genes determination showed that *nosZI* predominated over *nirK* and *nirS* in all soils suggesting a complete denitrification pathway, which would explain low N_2O losses in such soils. Estimation of total microorganisms studies using 16S and 18S rRNA showed that in these soils bacteria (10^9) was most abundant over archaea and fungi (10^7 ; $p < 0.05$). Sequences of DNA obtained by denaturant gradient gel electrophoresis (DGGE) indicated that Firmicutes, Acidobacteria and Proteobacteria are the main bacterial classes represented in Andisols (33, 28 and 23%, respectively) but with a lower diversity. More detailed studies about denitrifiers, gene-activity relationship and N_2O emissions are required.

Keywords : Volcanic soils, denitrification, nitrous oxide, denitrifying genes, microbial diversity

1. Introduction

Agricultural soils are the main source of nitrous oxide (N_2O) worldwide. Almost 300 times more influential than carbon dioxide (CO_2), N_2O contributes 10% to climate change each year and it is involved in the loss of the ozone layer (Thompson *et al.*, 2012). This gas is produced in the nitrogen (N) cycle, one of the major biogeochemical processes that is taking place in the biosphere. The cycle consists of two steps, nitrification and denitrification. The latter is an anaerobic respiration process involving a sequential and modular reduction of N species from nitrate (NO_3^-) or nitrite (NO_2^-) to nitric oxide (NO), nitrous oxide (N_2O) and nitrogen gas (N_2). Each one of these reductive steps is catalyzed by enzyme complexes: Nar, Nir, Nor and N_2or , respectively, which in turn are encoded by *narG* and *napA* genes for Nar, *nirK* and *nirS* for Nir, *norB* and *qnorB* for Nor, and *nosZ* for the complex N_2or (Philippot *et al.*, 2007). The description of this process has been mainly described in different bacterial communities, but its role in Archaea and Fungi domains it has only been recently studied (Maeda *et al.*, 2015).

In Chile, agriculture is mainly carried out in the central and southern region of the country on volcanic ash soils (Andisols, Alfisols and Ultisols) with a varied pattern of physicochemical properties including soil organic matter (SOM) content (Escudéy *et al.*, 2001). In Andisols, Alfaro and Salazar (2008) have reported higher N losses as NO_3^- whereas Vistoso *et al.* (2012) showed lower N_2O emissions after N application, suggesting that denitrification and N_2O emissions are limited and likely to be carried out completely, favoring N_2 formation. A preliminary study of the abundance of genes linked to the denitrification process showed that in two Andisols with high SOM, the presence of bacterial *nosZ* gene was abundant, supporting the idea that in these soils denitrification was carried out

comprehensively (Cardenas *et al.*, 2013). However, no information is available in relation to denitrifying genes in others soil types, neither the abundance and microbial composition related to denitrification in other domains.

The objectives of this study were i) to quantify the abundance of bacterial denitrifying genes (*nirK*, *nirS* and *nosZI*) in volcanic ash soils, and ii) to evaluate the bacterial diversity and its relative abundance to archaea and fungi domains in these soils.

2. Materials and Methods

2.1. Experimental sites and soil sampling

Five soil series were selected providing a range of SOM content: Collinco ($36^\circ 43'\text{S}$, $71^\circ 54'\text{W}$), Arrayán ($36^\circ 32'\text{S}$, $71^\circ 55'\text{W}$), Cudico ($40^\circ 39'\text{S}$, $73^\circ 21'\text{W}$), Osorno ($40^\circ 31'\text{S}$, $73^\circ 02'\text{W}$) and Nueva Braunau ($41^\circ 25'\text{S}$, $73^\circ 24'\text{W}$), with SOM contents of 4, 16, 15, 21 and 45%, respectively (Table 1). Soil samples (0 to 10 cm depth, $n=3$) were taken between august 2013 and march 2014 from permanent grassland sites with no recent history of N fertilizer application or grazing, and transported to INIA Remehue ($40^\circ 35'\text{S}$, $73^\circ 12'\text{W}$), where they were thoroughly mixed, sieved to 2 mm and stored at -80°C . The physicochemical analysis of soils (Table 1) was performed according to Sadzawka *et al.* (2006) and Lobos Ortega *et al.* (2016).

2.2. DNA extraction

Total DNA was extracted from 0.3 g of soil from each series using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., USA) and according to the manufacturer's instruction. The DNA was quantified by spectrophotometry using an Infinite M200 Nano-

Quant microplate reader (Tecan Trading AG; Switzerland) and its quality was evaluated by agarose gel electrophoresis in TAE 1x buffer stained with GelRed

(Biotium, USA) as mentioned in Armas-Ricard *et al* (2016). The DNA samples were stored in aliquots at -80 °C until amplification.

Table 1. Main physicochemical soil characteristics (0-10 cm depth; n=3).

Parameter	Collinco	Arrayán	Cudico	Osorno	Nueva Braunau
Sampling date	December'13	December'13	September'13	August'13	March'14
Soil type	Alfisol	Andisol	Ultisol	Andisol	Andisol
Classification ¹	Typic	Typic	Typic	Typic	Typic
	Rhodoxeralfs	Melanoxerands	Hapludults	Hapludands	Hapludands
Soil texture	Sandy clay	Sandy loam	Clay	Clay loam	Loam
SOM (%)	4.0 ± 0.06 ^d	14.9 ± 0.28 ^c	16.4 ± 0.48 ^c	21.4 ± 0.55 ^b	44.1 ± 1.20 ^a
C/N	10 ^c	13 ^b	13 ^b	14 ^b	17 ^a
N (mg kg ⁻¹)	4.5 ± 0.50 ^c	8.0 ± 0.01 ^{bc}	7.5 ± 0.29 ^c	67.8 ± 2.59 ^a	13.0 ± 0.71 ^b
P Olsen (mg kg ⁻¹)	2.5 ± 0.06 ^c	15.7 ± 0.20 ^d	23.3 ± 0.88 ^c	51.5 ± 0.67 ^a	36.0 ± 1.28 ^b
Al sat (%)	22.4 ± 0.18 ^a	0.28 ± 0.021 ^d	12.5 ± 0.28 ^b	0.72 ± 0.03 ^d	1.58 ± 0.565 ^c
Cu (mg kg ⁻¹)	1.98 ± 0.011 ^c	2.28 ± 0.020 ^c	5.74 ± 0.123 ^a	5.37 ± 0.128 ^a	4.33 ± 0.258 ^b
pH (H ₂ O)	5.37 ± 0.015 ^c	6.22 ± 0.006 ^a	5.52 ± 0.003 ^d	5.80 ± 0.014 ^b	5.70 ± 0.008 ^c

¹, Classification according to CIREN (2003); SOM: soil organic matter; C/N: carbon to nitrogen ratio; N, nitrogen; P, phosphorus; Al sat, aluminum saturation; Cu, Cu²⁺ concentration.

2.3. Quantitative PCR

Amplification of gene fragments was performed on a Rotor-Gene 6000 (Qiagen, Germany) using published primers (Table 2). Three replicates of each soil were used and run in triplicate. All amplifications were performed using the SensiFAST HRM Kit (Bioline Reagents Ltd.; UK), each reaction containing a final concentration of 1X SensiFAST HRM Mix, 400 nM Forward primer, 400 nM Reverse primer, 10 ng of DNA template and DNase-free water in

20 µL of reaction. Each amplification included a negative control without DNA (NTC).

For denitrifying genes, the copy number was normalized to account for soil moisture and to bacterial 16S rRNA to standardize by the total number of bacteria in the sample. The final genes copy numbers were reported as per gram of dry soil using the bulk density of the soil as measured at the collection site at the sampling time (Rowell, 1997). The potential transformation of N₂O to N₂ was estimated as *nosZI/nirK+nirS* ratio.

Table 2. Genes, primers and conditions used in the microbial determinations.

Gen	Primer	Sequence 5' – 3'	T annealing (°C)	Amplicon	Reference
16S rRNA	R907*	CCGTCAATTCMTTTGAGTTT	58	549 bp	Muyzer <i>et al.</i> ,
Bacteria	F358	CCTACGGGAGGCAGCAG			1993.
16S rRNA	UA1406R	ACG GGC GGT GWG TRC AA	55	655 bp	Baker, <i>et al.</i> ,
Archea	A751F	CCG ACG GTG AGR GRY GAA			2003.
18S rRNA	FR1	AIC CAT TCA ATC GGT AIT	53	390 bp	Vainio and
Fungi	FF390	CGA TAA CGA ACG AGA CCT			Hantula, 2000.
<i>nirK</i>	<i>nirK1F</i>	GGMATGGTKCCSTGGCA	63**/58	514 bp	Braker, <i>et al.</i> ,
	<i>nirK5R</i>	GCCTCGATCAGRTRTGTG			1998.
<i>nirS</i>	Cd3aF	G TSAACG TSAAGGARACSSGG	58	425 bp	Michotey <i>et al.</i> ,
	R3cd	GASTTCGGRTGSGTCTTGA			2000; 2004.
<i>nosZI</i>	<i>nosZ2F</i>	CGC RAC GGC AAS AAG GTS MSS GT	60	267 bp	Henry <i>et al.</i> ,
	<i>nosZ2R</i>	CAK RTG CAK SGC RTG GCA GAA			2006.

*The G-C Clamp sequence
CGCCCCGCCGCGCGCGCGGGCGGGGCGGGGGCACGGGGGGC
CGTCAATTCMTTTGAGTTT, was attached to the 5' end of the primer. ** Touchdown (1°C/cycle).

2.4. Calibration curves

To discard detrimental effects of PCR inhibitors, calibration curves with several DNA dilutions were constructed for each gene under study and tested as in Cardenas *et al.* (2013). The amplicons of each gene were cloned in *E. coli* JM109 using pGEM®-T Easy Cloning Vector (Promega, USA) according to the manufacturer's instructions. Plasmid DNA was purified using the PureYield™ Plasmid Miniprep System (Promega, USA) and its concentration was determined by spectrophotometry, as above. The presence of the inserts was verified by PCR with their specific primers and subsequent gel electrophoresis. The calibration curves since serial plasmid DNA showed a linear range between 106-1011 copy number, a R²>0.99 and the efficiency varied between 0.80-1.10.

2.5. Bacterial community analysis

Denaturant Gradient Gel Electrophoresis (DGGE) analysis for bacterial 16S rRNA was performed using a Cipher DGGEK-2001 system (C.B.S. Scientific, USA) and a GC clamp sequence attached to the 5' end of the forward primer. Briefly, 17 µL of PCR product was loaded onto an 8% (w/v) polyacrilamide gel with a 40–80% gradient. The electrophoresis was run for 17 h at 100 V and the gel was stained with GelRed (Biotium Inc., USA) for 30 min and photographed on a UV transilluminator (UVP, USA). Representative bands with a high reproducibility in DGGE gels were identified using the Doc-ItLS analysis software (UVP) and carefully excised with a scalpel to ensure the extraction of representative information about bacterial diversity.

The DNA was purified by the crush and soak protocol (Sambrook and Russell, 2001) and a second round of amplification was performed to ensure that excised bands did not contain multiple bands. Then, the amplicons were sequenced (Macrogen, Inc., Korea) and the chromatograms retrieved were compared to reported sequences at the National Center for Biotechnology Information (NCBI) and analyzed with BioEdit 7.2.5 and BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) programs. Taxonomic identification was performed with RDP Classifier (<http://rdp.cme.msu.edu/classifier/classifier.jsp>) and Ez-Taxon server (<http://www.ezbiocloud.net/eztaxon/identify>) databases. The total diversity was determined using the Shannon-Weaver index (Zar, 1999).

2.6. Statistical analysis

The gene copy number data were analyzed by a one-way ANOVA using the Statistica 7.0 package and comparisons were carried out with Fisher test. Differences were considered to be significant when the p value was ≤ 0.05 . The global relationship between gene abundances and the physicochemical soil parameters was performed using a multivariate generalized Procrustes analyses (Info-Gen) according to Gower (1975), and the relationship between SOM concentration and gene abundances was estimated using a lineal regression test. In both cases, JMP 10.0 was used as statistical package.

Table 3. Abundance of genes studied in volcanic ash soils ($n=3$). Data are showed as copy number g soil⁻¹ $\pm$ standard error of the mean.

	Collinco	Arrayán	Cudico	Osorno	Nueva Braunau
<i>nirK</i>	$8.6 \times 10^{-4} \pm 9.82 \times 10^{-5}^a$	$6.8 \times 10^{-4} \pm 1.93 \times 10^{-5}^a$	$7.6 \times 10^{-4} \pm 8.79 \times 10^{-5}^a$	$3.9 \times 10^{-4} \pm 2.58 \times 10^{-5}^b$	$4.6 \times 10^{-4} \pm 2.44 \times 10^{-5}^b$
<i>nirS</i>	$2.5 \times 10^{-4} \pm 2.85 \times 10^{-5}^{bc}$	$3.7 \times 10^{-4} \pm 5.31 \times 10^{-6}^a$	$3.1 \times 10^{-4} \pm 5.26 \times 10^{-5}^{ab}$	$1.8 \times 10^{-4} \pm 1.01 \times 10^{-5}^c$	$2.7 \times 10^{-4} \pm 1.29 \times 10^{-5}^b$
<i>nosZI</i>	$4.6 \times 10^0 \pm 9.13 \times 10^{-1}^c$	$1.2 \times 10^1 \pm 1.40 \times 10^0^a$	$5.5 \times 10^0 \pm 5.23 \times 10^{-1}^{bc}$	$8.3 \times 10^0 \pm 9.08 \times 10^{-1}^b$	$1.5 \times 10^1 \pm 1.00 \times 10^0^a$
<i>nosZI / nirK+nirS</i>	$4.1 \times 10^3 \pm 5.04 \times 10^2^d$	$1.1 \times 10^4 \pm 1.35 \times 10^3^c$	$5.5 \times 10^3 \pm 6.87 \times 10^2^d$	$1.4 \times 10^4 \pm 1.03 \times 10^3^b$	$2.0 \times 10^4 \pm 9.24 \times 10^2^a$
16S rRNA Bacteria	$3.5 \times 10^9 \pm 2.85 \times 10^8^a$	$3.8 \times 10^9 \pm 2.90 \times 10^8^a$	$4.6 \times 10^9 \pm 5.88 \times 10^8^a$	$3.6 \times 10^9 \pm 2.8 \times 10^8^a$	$4.1 \times 10^9 \pm 3.43 \times 10^8^a$
16S rRNA Archaea	$5.8 \times 10^7 \pm 3.21 \times 10^5^b$	$6.3 \times 10^7 \pm 2.31 \times 10^5^{ab}$	$6.6 \times 10^7 \pm 4.38 \times 10^5^a$	$3.0 \times 10^7 \pm 2.89 \times 10^5^c$	$2.8 \times 10^7 \pm 6.70 \times 10^6^c$
18S rRNA Fungi	$2.2 \times 10^7 \pm 8.63 \times 10^5^a$	$1.4 \times 10^7 \pm 2.29 \times 10^6^b$	$1.5 \times 10^7 \pm 8.84 \times 10^5^b$	$1.6 \times 10^7 \pm 1.80 \times 10^7^b$	$1.5 \times 10^7 \pm 1.76 \times 10^7^b$

Different letters indicate significant differences between soils ($p < 0.05$).

3. Results

3.1. Quantification of denitrifying genes

The quantification of denitrifying genes copy number showed that *nosZI* was the most abundant gene in all soils, followed by *nirK* and *nirS* ($p < 0.05$; Table 3). For each gene, significant differences were found between soils. The abundance of *nosZI* was higher in andisols (Nueva Braunau, Arrayán and Osorno) and lower in the ultisol. For *nirK* the abundance was relatively inverse to *nosZI* with lower levels in Osorno and Nueva Braunau, and higher values in Collinco, Cudico and Arrayán soils ($p < 0.05$; Table 3). The abundance of *nirS* gene was higher in Arrayán and Cudico, and lower in Osorno, Collinco and Nueva Braunau soils. In addition, the *nosZI/nirK+nirS* ratio was higher in andisols ($p < 0.05$; Table 3).

The Procraster analyses to relate gene abundances and the total physicochemical soil parameters show a high percentage of consensus (94.1%) whereas positive linear relationships were found between SOM content and *nosZI* gene copy number ($R^2 = 0.691$) and the *nosZI/nirK+nirS* ratio ($R^2 = 0.769$).

3.2. Abundance and diversity of microorganisms

The abundance of the bacterial 16S rRNA gene varied between 3.45×10^9 and 4.55×10^9 copy number g^{-1} , without significant differences among soils ($p > 0.05$; Table 3). For Archaea, 16S rRNA abundancy ranged

between 2.83×10^7 and 6.64×10^7 and was significantly lower than in bacteria ($p < 0.05$).

Differences among soils were observed between the Osorno and Nueva Braunau soil series containing lower abundancy whereas Cudico and Arrayán soils had higher contents (Table 3). Fungal 18S rRNA was lower than bacterial 16S rRNA and only showed a significant difference between Collinco and the others soils ($p < 0.05$).

The DGGE analysis for bacterial 16S rRNA showed high number of bands (> 100), with different migration and intensities between soils (data not showed). We obtained a total of 57 DNA sequences that were taxonomically classified to the Class level. Initially, nine sequences were inconsistent with those reported at NCBI but successfully assigned after RDP and/or EzTaxon analysis. The taxonomic diversity in all analyzed soil series corresponded to nine phyla and 21 classes (Table 4). The most predominant phyla in whole soils were Firmicutes (33%), Acidobacteria (28%) and Proteobacteria (23%). Firmicutes was predominant in Cudico (57%), Osorno (46%) and Nueva Braunau (50%) soils, respectively, whereas Acidobacteria predominated in Collinco (58%, Figure 1). Proteobacteria predominated in the Arrayán soil (62%). Other phyla less represented were Actinobacteria, Bacteroidetes, Chloroflexi, Deinococcus-Thermus, Gemmatimonadetes and Calsiderica (Figure 1). The Shannon-Wiener index showed that the total diversity was low ($H' < 1.0$) with similar values between soils (Table 5).

Table 4. Taxonomic identification of the DGGE bands.

Soil	Band	Gen Reference NCBI	Identity (%)	* Domain	Phylum	Class
Collinco	1	Uncultured bacterium clone MRD120_B12 16S ribosomal RNA gene, partial sequence	99	Bacteria	Bacteroidetes	Sphingobacteria
	2	Uncultured bacterium clone HL625 16S ribosomal RNA gene, partial sequence	93	Bacteria	Acidobacteria	Acidobacteria_Gp4
	3	Uncultured bacterium clone A1_a6 16S ribosomal RNA gene, partial sequence	91	Bacteria	Acidobacteria	Acidobacteria
	4	Uncultured bacterium partial 16S rRNA gene, DGGE band FRV03-7	83	Bacteria	Planctomycetes	Planctomycetacia
	5	Uncultured bacterium clone FCPO410 16S ribosomal RNA gene, complete sequence	88	Bacteria	Firmicutes	Clostridia
	6	Uncultured bacterium clone B184 16S ribosomal RNA gene, partial sequence	77	Bacteria	Proteobacteria	Gammaproteobacteria
	7	Uncultured Acidobacteria bacterium clone SCGR082 16S ribosomal RNA gene, partial sequence	89	Bacteria	Proteobacteria	Gammaproteobacteria
	8	Uncultured bacterium clone B2 16S ribosomal RNA gene, partial sequence	78	Bacteria	Deinococcus-Thermus	Deinococci
	9	Uncultured bacterium clone PASI_B10 16S ribosomal RNA gene, partial sequence	80	Bacteria**	Acidobacteria**	Solibacteres**
	10	16S rRNA amplicon fragment from a soil sample (ferralsol, Madagascar) resulting from a 16 days laboratory incubation experiment in the presence of 13C-enriched wheat-straw: Heavy-DNA fraction (DNA-SIP technique)	90	Bacteria	Actinobacteria	Actinobacteria
	11	16S rRNA amplicon fragment from a soil sample (ferralsol, Madagascar) resulting from a 16 days laboratory incubation experiment in the presence of 13C-enriched wheat-straw and a tropical perennate endogeic earthworm, <i>Poneroscolex corethrurus</i> : Light-DNA fraction (DNA-SIP technique)	88	Bacteria	Actinobacteria	Actinobacteria
	12	16S rRNA amplicon fragment from a soil sample (ferralsol, Madagascar) resulting from a 16 days laboratory incubation experiment in the presence of 13C-enriched wheat-straw: Light-DNA fraction (DNA-SIP technique)	92	Bacteria	Actinobacteria	Actinobacteria
Cudico	13	Uncultured soil bacterium isolate DGGE gel band 12 16S ribosomal RNA gene, partial sequence	77	Bacteria**	Firmicutes**	Bacilli**
	14	Uncultured bacterium partial 16S rRNA gene, isolate MOTU101, clone MEDIUM2_BAC565	92	Bacteria	Firmicutes	Bacilli
	15	Uncultured Firmicutes bacterium gene for 16S ribosomal RNA, partial sequence, isolate: DGGE band P11_B18	76	Bacteria	Firmicutes	Bacilli
	16	<i>Staphylococcus</i> sp. Drenje 1.6 16S ribosomal RNA gene, partial sequence	98	Bacteria	Firmicutes	Bacilli
	17	Uncultured <i>Terriblobus</i> sp. clone MA011 16S ribosomal RNA gene, partial sequence	80	Bacteria*	Acidobacteria*	Acidobacteria_Gp1*
	18	Uncultured soil bacterium isolate DGGE gel band 02 16S ribosomal RNA gene, partial sequence	81	Bacteria	Firmicutes	Negativicutes
	19	Uncultured bacterium partial 16S rRNA gene, clone CuiH21	81	Bacteria	Actinobacteria	Actinobacteria
	20	Uncultured Firmicutes bacterium partial 16S rRNA gene, clone EJIR08_21	86	Bacteria	Firmicutes	Bacilli
	21	Uncultured Acidobacteria bacterium clone SEG_08_135 16S ribosomal RNA gene, partial sequence	82	Bacteria**	Acidobacteria**	Acidobacteria_Gp4**
Arrayán	22	NCB unrated		Bacteria	Bacteroidetes	Flavobacteria
	23	Uncultured bacterium clone SII_2_47 16S ribosomal RNA gene, partial sequence	77	Bacteria	Firmicutes	Negativicutes
	24	Uncultured bacterium clone 0230 16S ribosomal RNA gene, partial sequence	92	Bacteria	Proteobacteria	Gammaproteobacteria
	25	Uncultured bacterium clone HLUC358 16S ribosomal RNA gene, partial sequence	90	Bacteria	Firmicutes	Clostridia
	26	Uncultured soil bacterium isolate DGGE gel band 17 16S ribosomal RNA gene, partial sequence	74	Bacteria**	Proteobacteria**	Gammaproteobacteria**
	27	Uncultured bacterium clone PS_B360 16S ribosomal RNA gene, partial sequence	96	Bacteria	Proteobacteria	Gammaproteobacteria
	28	Uncultured bacterium clone F3_E143 16S ribosomal RNA gene, partial sequence	86	Bacteria	Proteobacteria	Gammaproteobacteria
	29	Bacterium endosymbiont of <i>Ornithophagus Taurus</i> clone 3L2_NC1_019 16S ribosomal RNA gene, partial sequence	97	Bacteria	Proteobacteria	Gammaproteobacteria
	30	Uncultured organism clone ELU0149-T297-S-NCPRAMgAnA_000468 small subunit ribosomal RNA gene, partial sequence	73	Bacteria	Chloroflexi	Thermomicrobia
	31	Enterobacteriaceae bacterium M_Sw_ahS_07/11_1_1(1) 16S ribosomal RNA gene, partial sequence	97	Bacteria	Proteobacteria	Gammaproteobacteria
	32	<i>Oceanisphaera</i> sp. LAM-WHM-ZC 16S ribosomal RNA gene, partial sequence	76	Bacteria	Proteobacteria	Betaproteobacteria
	33	Uncultured alpha proteobacterium isolate DGGE gel band SPH-SW08-7 16S ribosomal RNA gene, partial sequence	97	Bacteria	Proteobacteria	Alphaproteobacteria
Osomo	34	Uncultured bacterium clone SiteB3Y_207 16S ribosomal RNA gene, partial sequence	90	Bacteria	Firmicutes	Bacilli
	35	Uncultured rumen bacterium clone MXAPIA-B1 16S ribosomal RNA gene, partial sequence	97	Bacteria	Firmicutes	Bacilli
	36	Uncultured bacterium clone 17d-22 16S ribosomal RNA gene, partial sequence	91	Bacteria	Firmicutes	Bacilli
	37	Uncultured Firmicutes bacterium partial 16S rRNA gene, clone DKBF_4	75	Bacteria	Deinococcus-Thermus	Deinococci
	38	<i>Bacillus lentus</i> 16S rRNA gene, isolate P26 5c1	96	Bacteria	Firmicutes	Bacilli
	39	Uncultured bacterium partial 16S rRNA gene, clone DKBF_5	76	Bacteria**	Proteobacteria**	Alphaproteobacteria**
	40	Uncultured bacterium clone BP_U4A_4g06 16S ribosomal RNA gene, partial sequence	91	Bacteria	Proteobacteria	Alphaproteobacteria
	41	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 953Se68u	82	Bacteria	Chlorobi	Ignavibacteria
	42	Uncultured bacterium clone D62 16S ribosomal RNA gene, partial sequence	82	Bacteria	Proteobacteria	Alphaproteobacteria
	43	Uncultured soil bacterium isolate DGGE gel band 11 16S ribosomal RNA gene, partial sequence	78	Bacteria	Firmicutes	Clostridia
	44	Uncultured bacterium RNA for 16S rRNA, partial sequence, clone: B1001R002_G23	92	Bacteria	Firmicutes	Clostridia
	45	Uncultured bacterium clone BP_U4B_2e10 16S ribosomal RNA gene, partial sequence	79	Bacteria	Planctomycetes	Planctomycetacia
S. Braunau	46	NCB unrated		Bacteria	Bacteroidetes	Sphingobacteria
	47	Uncultured bacterium clone AFEL3_aal4h05 16S ribosomal RNA gene, partial sequence	95	Bacteria	Firmicutes	Bacilli
	48	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 0502TCLN172	98	Bacteria	Firmicutes	Bacilli
	49	<i>Bacillus</i> sp. F3(2012) 16S ribosomal RNA gene, partial sequence	77	Bacteria**	Firmicutes**	Bacilli**
	50	Uncultured <i>Bacillus</i> sp. isolate DGGE gel band 5 16S ribosomal RNA gene, partial sequence	100	Bacteria	Firmicutes	Bacilli
	51	Uncultured soil bacterium isolate DGGE gel band 12 16S ribosomal RNA gene, partial sequence	76	Bacteria	Cyanobacteria / Chloroplast	Chloroplast
	52	Uncultured <i>Burkholderia</i> sp. isolate DGGE gel band 9TAT 16S ribosomal RNA gene, partial sequence	76	Bacteria	Proteobacteria	Deltaproteobacteria
	53	Uncultured soil bacterium isolate DGGE gel band 12 16S ribosomal RNA gene, partial sequence	74	Bacteria	Firmicutes	Clostridia
	54	Uncultured bacterium clone GB4XUSJ08JVLQW 16S ribosomal RNA gene, partial sequence	95	Bacteria	Acidobacteria	Acidobacteria_Gp1
	55	Uncultured bacterium RNA for 16S rRNA, partial sequence, clone: B1001R002_G23	96	Bacteria	Acidobacteria	Acidobacteria_Gp1
	56	Uncultured bacterium clone 16S-T6-3-9H 16S ribosomal RNA gene, partial sequence	98	Bacteria	Acidobacteria	Acidobacteria_Gp1
	57	Uncultured bacterium clone Upland_500_4065 16S ribosomal RNA gene, partial sequence	93	Bacteria	Gemmatimonadetes	Gemmatimonadales

*Sequences assigned using NCBI; **Sequences assigned using the EzTaxon database.

Figure 1. Phyla distribution of bacterial 16S rRNA in volcanic ash soils.

Table 5. Diversity of bacterial phyla of volcanic ash soils measured as Shannon-Wiener index. H' , diversity index; H' max, maximum diversity index; E, evenness.

Soil	H'	H' max	E
Collinco	0,6805	0,6989	0,9736
Arrayan	0,4480	0,6020	0,7466
Cudico	0,5010	0,6020	0,8322
Osorno	0,5695	0,6989	0,8148
Nueva Braunau	0,4894	0,6020	0,8129

4. Discussion

In this study we performed qPCR to determine the abundance of denitrifying genes in different soil series currently used in agricultural or livestock activities in southern Chile. Our findings showed that *nosZI* gene was more abundant than *nirK* and *nirS* in all soils with similar values to several reports from around the world (Henry *et al.*, 2006; Chronakova *et al.*, 2009; Cardenas *et al.*, 2013). In addition, andisols showed a higher *nosZI/nirK+nirS* ratio com-

pared to alfisol and ultisol. According to this, a high relationship was found between *nosZI* or the potential transformation of N_2O to N_2 (*nosZI/nirK+nirS* ratio) and SOM. These results suggest that during denitrification, most NO and N_2O are reduced to N_2 resulting in lower N_2O emissions. This hypothesis is supported by the fact that denitrification have been defined as a poor-represented process in Chilean andisols with lower N_2O emissions compared to others reports (Vistoso *et al.*, 2012).

However, it has been reported that levels of N_2O emissions could be uncoupled from genes abundance (Čuhel *et al.*, 2010), so our results must be analyzed on a wider context. Furthermore, the study of transcripts or the real enzyme activities remain as significant areas to be investigated.

Denitrifiers had been traditionally classified into two functional and mutually exclusive groups, those carrying *nirK* or *nirS* genes, which uses Cu^{2+} and cytochrome *cd1*, respectively, as co-factors (Philippot *et al.*, 2007). But recently the co-occurrence of both genes in microbial communities have been reported (Graf *et al.*, 2014). Regarding N_2O reductase, recent phylogeny studies have reported another gene coined *nosZII* which would have a significant contribution to N_2O losses, being associated to a different clade (Clade II; Jones *et al.*, 2012). This *nosZII* gene is very widespread and diverse, and more abundant than *nosZI* in bacteria and archaea. Preliminary results of our group show that bacterial *nosZII* is present in our soils and future studies are necessary to highlight and clarify the importance and function of this gene in volcanic ash soils. Recent reports indicate that both Archaea and Fungi have homologous and analogous enzymes to carry out the process of denitrification (Maeda *et al.*, 2015; Rusch, 2013), becoming a component to consider in future studies to cover the total contribution of microorganisms.

Chilean volcanic ash soils have been characterized in a limited way in terms of microbiota and its relation to denitrifiers. Our results using 16S and 18S rRNA indicates that Bacteria domain predominated in all evaluated soils, whereas Archaea is higher than Fungi. The 16S rRNA have been the universal marker used to obtain and estimate total abundance of bacteria and archaea in different samples (Prévost-Bouré *et al.*, 2014) including rhizosphere and bulk soil (Lagos *et al.* 2015). Despite being a multicopy gene in several species, its quantification is similar to *rpoB*, a new sin-

gle copy marker, validating their use in environmental samples (Deslippe *et al.*, 2014). In our study the copy number of bacterial 16S rRNA ranged between 3.45×10^8 to 4.55×10^8 being similar to another reports with soils showing a high SOM content (Kandeler *et al.* 2006; Cardenas *et al.* 2013). We found no significant differences between soils, despite the different physicochemical characteristics including SOM, pH or C/N ratio. These results suggest that in volcanic soils, the abundance of microorganisms could be linked to others factors like pH, water and oxygen content, organic C or the state of bioavailability of organic matter, regulating soil quality and plant nutrition (Čuhel *et al.*, 2010; Barea, 2015). A similar trend was found for fungal 18S rRNA but with Collinco soil showing a higher abundance. For archaeal 16S rRNA, the pattern was more heterogeneous with andisols showing the lower abundance. The gene copy number values reported here were similar to those reported by Cardenas *et al.* (2013) on similar soil types.

To assess the snapshot of the bacterial communities in our soils we performed DGGE assays, a molecular technique that is independent of culture and allows to study the diversity of communities (Muyzer *et al.* 1993). The pattern of bands obtained was different for each soil suggesting a differential composition. The sequence analysis only retrieved information to the Class level, and the total diversity measured as the Shannon-Weaver index was reduced probably because of the high taxonomic rank used in the allocation. This was presumably associated to the low number of DGGE bands isolated, which is recognized as the main limitation of the method. It is estimated that a single soil sample can generate a high number of bands (over 100 and more) corresponding to different communities (Nakatsu *et al.*, 2007). Nonetheless, the information available allowed for the analysis of differences in the most represented phyla. Thus, the phyla with greater representation in the analyzed soils

corresponded to Firmicutes, Acidobacteria and Proteobacteria, in agreement with Jorquera *et al.* (2010) in andisols. While these phyla are considered significant for the denitrification process, specific phylogenetic studies are required for denitrifying genes because they are uncoupled of the proposed 16S rRNA evolution (Goregues *et al.*, 2005). At the moment, we are performing DGGE using specific *nosZI* and *nosZII* primers.

Our results suggest that in volcanic soils of southern Chile, total denitrification can be expected under natural conditions which would explain the low N₂O emissions registered for agricultural and grassland soils (Muñoz *et al.*, 2011; Vistoso *et al.*, 2012; Hube *et al.*, 2016). Nevertheless, more studies including gene expression, enzyme activity and community structure of denitrifiers are required to link potential denitrification and the effect of soil macronutrients over N₂O emissions. This knowledge will be also relevant to develop future mitigation strategies appropriate for specific soil characteristics.

5. Conclusions

Our results show that in our volcanic ash soils bacterial *nosZI* predominates between denitrifying genes. The high ratio of *nosZI* over *nirK* and *nirS* in Andisols could suggest a higher denitrification potential. Compared to archaea and fungi, bacteria domain was most represented and includes Firmicutes, Acidobacteria and Proteobacteria classes, which have been implicated in soil denitrification. Further studies are necessary to understand soil N₂O emissions and its mitigation.

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