



# Structure and Functional Properties of Bacterial Communities in Surface Sediments of the Recently Declared Nutrient-Saturated Lake Villarrica in Southern Chile

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Received: 16 May 2022 / Accepted: 12 January 2023

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## Abstract

Lake Villarrica, one of Chile's main freshwater water bodies, was recently declared a nutrient-saturated lake due to increased phosphorus (P) and nitrogen (N) levels. Although a decontamination plan based on environmental parameters is being established, it does not consider microbial parameters. Here, we conducted high-throughput DNA sequencing and quantitative polymerase chain reaction (qPCR) analyses to reveal the structure and functional properties of bacterial communities in surface sediments collected from sites with contrasting anthropogenic pressures in Lake Villarrica. Alpha diversity revealed an elevated bacterial richness and diversity in the more anthropogenized sediments. The phylum Proteobacteria, Bacteroidetes, Acidobacteria, and Actinobacteria dominated the community. The principal coordinate analysis (PCoA) and redundancy analysis (RDA) showed significant differences in bacterial communities of sampling sites. Predicted functional analysis showed that N cycling functions (e.g., nitrification and denitrification) were significant. The microbial co-occurrence networks analysis suggested *Chitinophagaceae*, *Caldilineaceae*, *Planctomycetaceae*, and *Phycisphaerae* families as keystone taxa. Bacterial functional genes related to P (*phoC*, *phoD*, and *phoX*) and N (*nifH* and *nosZ*) cycling were detected in all samples by qPCR. In addition, an RDA related to N and P cycling revealed that physicochemical properties and functional genes were positively correlated with several nitrite-oxidizing, ammonia-oxidizing, and N-fixing bacterial genera. Finally, denitrifying gene (*nosZ*) was the most significant factor influencing the topological characteristics of co-occurrence networks and bacterial interactions. Our results represent one of a few approaches to elucidate the structure and role of bacterial communities in Chilean lake sediments, which might be helpful in conservation and decontamination plans.

**Keywords** Bacterial communities · Bacterial diversity · Functional genes · Lake sediments · Nutrients · Community structure

## Introduction

Freshwater bodies, such as rivers and lakes, are widely recognized as providers of Ecosystem Services (ES) supporting life on Earth [1]. Lake Villarrica (also known as Mallalaf-quén) is one of the most representative freshwater bodies

in southern Chile and a relevant ES provider to the local community. Its water mass is placed into the Toltén River basin at 230 m a.s.l., encompassing 176 km<sup>2</sup>, presenting an average depth of 120 m, a water renewal of 3–4 years, and typically monomictic and mesotrophic with a peaking thermal stratification during summer season [2]. In the Lake Villarrica hydrographic basin, two important cities (Villarrica and Pucón), historically harbor broad regional economic activities (such as tourism, sport fishing, agriculture, aquaculture, forestry, and animal husbandry), which have intensified over the last few decades. As a result of these activities, the city of Villarrica has experienced accelerated deterioration of water environment by increasing inputs of nutrients

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(mainly phosphorus [P] and nitrogen [N]) and pollutants into their waters [3]. Consequently, the Environmental Ministry of Chile declared this lake as a nutrient-saturated waterbody based on exceeding the maximum limits allowed for water transparency ( $\leq 4$  m), chlorophyll “a” ( $\geq 10 \mu\text{g L}^{-1}$ ), dissolved oxygen (DO;  $\leq 70\%$ ), dissolved P (DP;  $\geq 0.025 \text{ mg L}^{-1}$ ), total P (TP;  $\geq 0.025 \text{ mg L}^{-1}$ ), dissolved N (DN;  $\geq 0.03 \text{ mg L}^{-1}$ ), and total N (TN; DN;  $\geq 0.03 \text{ mg L}^{-1}$ ) during the last 2 years as consigned on the Decree no. 43 published in August 2018 (<https://www.bcn.cl/leychile/navegar?idNorma=1121466>). In addition, due to several events of strong Foehn winds during summer, locally called as “Puelche” wind, the mixing of its waters and bottom sediments has contributed to frequent cyanobacterial blooms [4]. In this context, the Chilean government has established a decontamination plan to monitor and decrease these environmental parameters. However, this plan does not consider actions based on biological parameters, such as the microbial communities in the water column and lacustrine sediments of the lake, which may have a relevant and practical significance for conservation and decontamination programs.

At a global level, it is well recognized that human activities result in increasing nutrient inputs (such as carbon [C], P, and N) and pollutant inputs (such as pesticides, antibiotics, and human pathogens) into aquatic systems [5]. For instance, P and N fertilizers are periodically applied to agricultural lands and subsequently deposited in the sediments of lakes of southern Chile by surface runoff waters, leachates, or as part of eroded soil particles, and positively influence the abundance and bloom of cyanobacteria in lakes [6]. Similarly, antibiotics in the  $\beta$ -lactam, quinolone, macrolide, tetracycline, and sulfonamide families are the most common agents found in Chilean impacted waters [7, 8], which can act as a selection pressure increasing antimicrobial resistance [9]. Recently, aquaculture centers surrounding Lake Villarrica have been implicated as major contributors of antibiotics in its waters [3].

Nutrient cycling in the Lake Villarrica sediments is likely a complex process involving a large number of bacterial taxa. To properly understand these cycles, the process must be analyzed in a holistic manner, where the relationship (including spatial and temporal connections) between nutrients, and bacterial communities as well as space and time, must be considered. Moreover, the relationship between nutrient cycling, eutrophication and bacterial communities must be estimated, and their contribution to the design and establishment of countermeasures need to be evaluated to prevent eutrophication's negative socio-economic impacts and maintaining the value of Lake Villarrica as an important ES provider in southern Chile. However, to our knowledge, there are no studies where bacterial communities are considered as a relevant driver of the fitness of ecosystems in Chilean lakes. In this context, the present study assessed to

investigate the structure and functional properties of bacterial communities in surface sediments with contrasting anthropogenic pressures from the nutrient-saturated Lake Villarrica. In this work, we employed high throughput DNA sequencing (HTS) and quantitative polymerase chain reaction (qPCR) approaches to evaluate the composition, predicted functions, co-occurrence networks, and functional genes involved in P and N cycling of bacterial communities in sediments from the five sampling points stated as relevant by the Chilean government to monitoring the Lake Villarrica.

## Materials and Methods

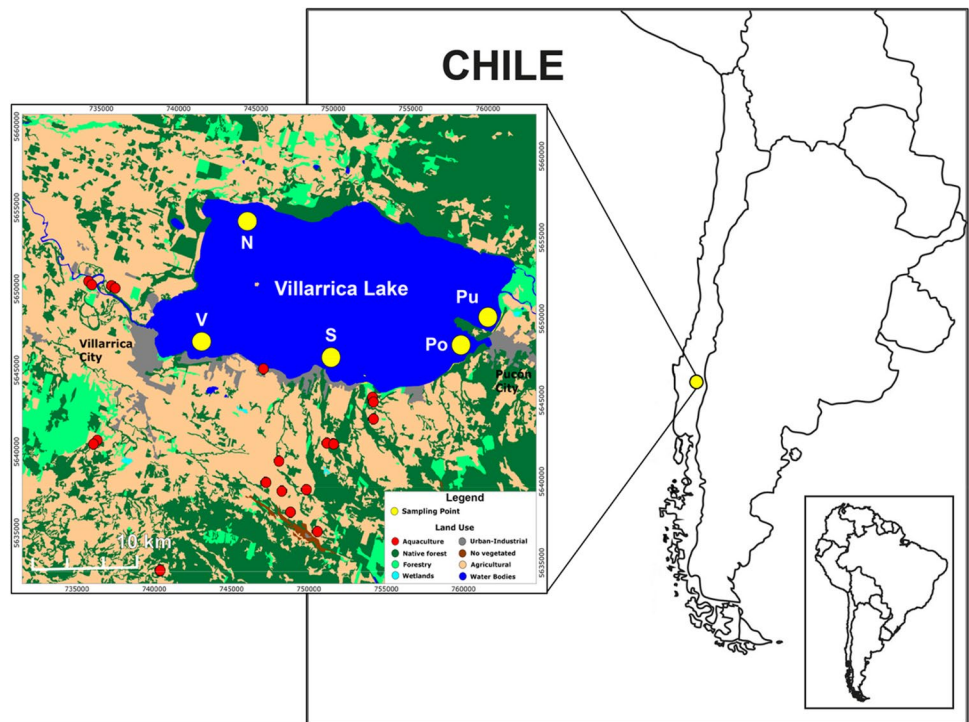
### Sampling Sites

The present study was spatially conducted considering five sampling sites which were defined and continuously monitored by the Regional Secretariat of Chile's Ministry of the Environment (Fig. 1). The sampling sites comprise Pucón city bay (Pu;  $39^{\circ}15'46.51''\text{S}$ ,  $71^{\circ}58'44.83''\text{W}$ ), Pucón city port (Po;  $39^{\circ}16'48.54''\text{S}$ ,  $71^{\circ}59'43.46''\text{W}$ ), South lake (S;  $39^{\circ}17'34.19''\text{S}$ ,  $72^{\circ}5'34.38''\text{W}$ ), Villarrica city bay (V;  $39^{\circ}17'6.26''\text{S}$ ,  $72^{\circ}11'13.27''\text{W}$ ), and North lake (N;  $39^{\circ}12'52.28''\text{S}$ ,  $72^{\circ}8'31.78''\text{W}$ ). In general terms, Pu, Po, S, and V are considered sampling sites with higher anthropic pressure, which are directly influenced by wastewater discharges (from  $0.6$  to  $7.84 \text{ Mg year}^{-1}$  of total phosphorus [TP] and from  $4.2$  to  $50.96 \text{ Mg year}^{-1}$  of total nitrogen [TN]), aquaculture ( $159.78 \text{ TP Mg year}^{-1}$  and  $835.23 \text{ TN Mg year}^{-1}$ ), and land use (mainly by agronomy and forestry;  $210.60 \text{ TP Mg year}^{-1}$  and  $793.21 \text{ TN Mg year}^{-1}$ ) from the whole shore between Villarrica and Pucón city and tributary effluents such as River Trancura. In contrast, because the main nutrients load almost only comes from grasslands ( $<0.001 \text{ TP}$  and  $\text{TN Mg year}^{-1}$ ) for the N sampling site, this was considered a zone under lower anthropic pressure than other studied sampling sites. Additionally, resulting from the particular hydrodynamic of Lake Villarrica, waters from the N site avoid mixing with the remaining lake zones for most of the year [3].

### Sampling Procedure

Quadruplicate composite samples of superficial sediments (10 cm depth) from Lake Villarrica were collected in March 2020 (summer) using a Petersen-like grab sampler. For the obtention of each composite sample, the Ohio Environmental Protection Agency recommendations for sediment sampling were followed [10]. Briefly, 3 sediment subsamples or grabs at equal volumes were randomly collected at each sampling site, thoroughly homogenized in a clean plastic container

**Fig. 1** Map showing the sampling sites for Lake Villarrica used in this study. The sampling sites were coded as Pucón city bay (Pu), Pucón city port (Po), south lake (S), Villarrica city bay (V), and north lake (N). The main soil uses of Villarrica Lake catchment are included



with an appropriate scoop, and an aliquot of that (500 mL) was placed into a sterile plastic flask and submitted as one of the composite replicates. This process was repeated four times in a 10-m radius until the whole of the replicates were completed. Immediately each composite sample was subject to a direct measurement of pH, temperature (Temp), dissolved oxygen (DO), electrical conductivity (EC), and oxidation–reduction potential (ORP) using an Edge® probe HI2020 (Hanna Instruments, Inc., Woonsocket, Rhode Island, USA). After sampling, sediment samples were cooled at 4°C and immediately transported to the Applied Microbial Ecology Laboratory (EMALAB) in the Universidad de La Frontera, Temuco (Chile) for processing. Each composite sample was separated into two different aliquot sets. Then, the first set was freeze-dried for chemical determination, and the second set was frozen at –80°C for DNA extraction.

### Chemical Properties of Sediment Samples

The contents of total carbon (TC) and TN in collected sediments were determined as follows. First, aliquots between 1.5 and 2.5 mg of sieved freeze-dried sediments (150 µm pore size) were determined on an automated elemental analyzer EA 3000 (Eurovector, Milano, IT) following the recommendations of Yang et al., [11]. Then, the elemental composition of TC and TN was calculated by interpolation into a calibration curve ( $r^2 = 0.98$ ) using EDTA as standard (99.4% purity; LECO®, USA) and expressed as mg of C or N per kg dw sediment ( $\text{mg kg}^{-1}$  dw).

Available phosphorus ( $P_{\text{Olsen}}$ ) was extracted using the 0.5 M Na-bicarbonate method and analyzed using the molybdate-blue method [12]. The organic matter (OM) contents were estimated by wet digestion [13]. Exchangeable cations ( $K^+$ ,  $Ca^{2+}$ ,  $Fe^{3+}$ ) were extracted with 1M  $CH_3 COONH_4$  at pH 7.0 and analyzed using flame atomic absorption spectrophotometry (FAAS) [14]. Exchangeable aluminum ( $Al^{3+}$ ) was extracted with 1M KCl and analyzed by FAAS [15]. The data obtained were analyzed by one-way ANOVA using Tukey's honestly significant difference (HSD;  $P \leq 0.05$ ) test.

### DNA Extraction from Sediment Samples

The extraction of genomic DNA from the sediments (~150 mg dry weight [dw]) was done using DNeasy® PowerBiofilm Kit (QIAGEN, Carlsbad, CA, USA) according to the manufacturer protocol. The DNA concentrations were determined with a broad range Quant-iT™ dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and measured using a Qubit4™ Fluorometer (Thermo Fisher Scientific). The DNA extracts were stored at –20°C until the microbial community and functional gene analyses.

### Composition and Predicted Functions of Bacterial Communities in Lake Sediments

The bacterial community compositions in DNA extracts from sediments were determined by HTS using the V4 hypervariable region of the 16S rRNA gene as described

by Zhang et al., [16]. The 16S rRNA gene libraries were built and sequenced using the dual indexing method [17] and barcoded primers on a MiSeq platform (Illumina, Inc, San Diego, California, USA). Raw sequence reads were trimmed and processed using SH17 to obtain high-quality data (QC > 35) [18, 19]. The trimmed sequences were aligned into operational taxonomic units (OTUs), and taxonomy was determined at a 3% dissimilarity cutoff based on the Greengene database using NINJA-OPS [20]. Non-related sequence reads (e.g., chloroplast and mitochondria) were removed via QIIME [21], and data were rarefied to 12,000 reads for biodiversity analyses. Raw sequencing data were deposited in the Sequence Read Archive (SRA) of NCBI under Accession Number PRJNA836640.

The richness (operational taxonomic units [OTUs] observed, abundance-based coverage estimate [ACE], and Chao1) and the diversity (coverage, Shannon index, and Simpson index) of the microbial community were analyzed using the mothur program ver. 1.42 and the R project (<https://www.r-project.org/>). The data obtained were contrasted by one-way ANOVA using Tukey's honestly significant difference (HSD;  $P \leq 0.05$ ) test. Differences in beta diversity among the community were evaluated by analysis of similarity (ANOSIM) using Bray-Curtis dissimilarity matrices [22, 23]. Molecular variance (AMOVA) was used to measure differences in sample clustering [24]. Principal coordinate analysis (PCoA) was performed to ordinate and visualize the samples. In addition, the FAPROTAX database and software were used to predict functions of the bacterial community members, which establish putative metabolic or other ecologically relevant functions as described by Louca et al., [25].

### Relationship Between Physicochemical Properties and Bacterial Community in Lake Sediments

The relationships among the environmental factors, microbial communities of sites, and bacterial abundance (species level) associated with N and P cycling were assessed with redundancy analysis (RDA). The significance of the model was tested by an ANOVA-like test using 999 permutations and the vegan package in R software version 4.1.2 [26]. The envfit function of the vegan library was assessed to explain the degree of variation by each factor.

### Co-occurrence Network of Bacterial Communities in Lake Sediments

The co-occurrence network was generated by using the WGCNA package based on the Spearman correlation matrix [27]. The operational taxonomic units (OTUs) were represented by the nodes, and the correlations between OTUs

were described as the edges in the topological graph, respectively. The appropriate similarity (0.807) was chosen during the network construction according to random matrix theory [28]. In addition, a false discovery rate (FDR) was used to adjust the  $P$  values and set up the threshold value of 0.05 [29]. The topological network properties were calculated via the *igraph* package [30]. The putative keystone taxa were determined based on the following thresholds: OTUs with degree >8, closeness centrality >0.15, betweenness centrality <0.025, and transitivity (clustering coefficient) >0.09 [31]. Gephi software was used to visualize the network image [32].

### Association of Network Topological Characteristics to Physicochemical Properties and Functional Genes in Bacterial Communities in Lake Sediments

The *igraph* package was also used to calculate the topological characteristics of each sample [27, 33]. The association between features of the co-occurrence network and physicochemical properties and functional genes was evaluated, as described previously [33], by using Spearman's rank correlation and  $P$  values that were adjusted using Benjamini and Hochberg FDR.

### Abundance of Bacterial Functional Genes in Lake Sediments

The abundance of total bacteria (16S rRNA genes) and the occurrence and abundance of bacterial functional genes involved in organic P mobilization (*phoC*, *phoD*, and *phoX* phosphatase genes and N fixation (*nifH*) and denitrification (*nosZ*), respectively, were determined by using qPCR and a StepOne Real-Time PCR System (ThermoFisher Scientific, Inc., Waltham, MA, USA) using PowerUp<sup>TM</sup> SYBR<sup>TM</sup> Green Master Mix (Applied Biosystems<sup>TM</sup>, Foster City, CA, USA) and ~25 ng  $\mu\text{L}^{-1}$  of DNA. The primer sets and conditions used for quantification of 16S rRNA [34], *phoC* [35], *phoD* [36], *phoX* [37], *nifH* [38], and *nosZ* [39] genes by qPCR are shown in Table 1. The copy numbers were calculated using a standard curve built for each targeted gene with dsDNA gBlock<sup>®</sup> Gene Fragments (Integrated DNA Technologies, Inc. Iowa, USA). The copy number was estimated with the equation '[concentration of the dsDNA gBlock<sup>®</sup> Gene Fragment in  $\text{ng } \mu\text{L}^{-1}$ ]  $\times$  [molecular weight in  $\text{fmol ng}^{-1}$ ]  $\times$  [Avogadro's number]' following the method described by Whelan et al., [40]. Absolute quantification (AQ) of bacterial genes was expressed as copy number per gram of dw (gene copy  $\text{g}^{-1}$  sediment dw) and used to determine the relative quantification (RQ) of each gene relative to the 16S rRNA gene as carried out by Campos et al., [41]. The data obtained from gene quantification assays were analyzed by using one-way ANOVA

**Table 1** Primer sets and PCR conditions used for the quantification of bacterial functional genes

| Gene        | Primer set              | Sequence (5'→3')                                | PCR conditions                                                                                                         | Amplicon size (bp) | Reference |
|-------------|-------------------------|-------------------------------------------------|------------------------------------------------------------------------------------------------------------------------|--------------------|-----------|
| 16S rRNA    | 799f<br>1115r           | AACMGGATTAGATACCCCKG<br>AGGGTTGCGCTCGTTG        | 10 min at 95 °C, and 40 cycles at 95 °C per 15 sec and 60 °C per 1 min, finalized by 15 sec at 95 °C                   | 316                | [30]      |
| <i>phoC</i> | phoc-A-F1<br>phoc-A-R1  | CGGCTCCTATCCGTCCGG<br>CAACATCGCTTTGCCAGTG       | 10 min at 95 °C, and 40 cycles at 95 °C per 15 sec and 60 °C per 1 min, finalized by 15 sec at 95 °C                   | 155                | [31]      |
| <i>phoD</i> | ALPS-F730<br>ALPS-R1101 | CAGTGGGACGACCACGAGGT<br>GAGGCCGATCGGCATGTCTG    | 20 sec at 95 °C, and 40 cycles at 95 °C per 3 se and 60 °C per 30 sec, finalized by 15 sec at 95 °C                    | 370                | [32]      |
| <i>phoX</i> | PhoX2F<br>PhoX2R        | GARGAGAACWTCACGGYTA<br>GATCTCGATGATRTGRCCRAAG   | 10 min at 95 °C, and 40 cycles at 95 °C per 15 sec, 52 °C per 15 sec and 72 °C per 1 min, finalized by 15 sec at 95 °C | 600                | [33]      |
| <i>nifH</i> | PolF<br>PolR            | TGCGAYCCSAARGCBGACTC<br>ATSGCCATCATYTCRCCGGA    | 10 min at 95 °C, and 40 cycles at 95 °C per 15 sec, 60 °C per 1 min, finalized by 15 sec at 95 °C                      | 342                | [34]      |
| <i>nosZ</i> | nosZ2F<br>nosZ2R        | CGCRACGGCAASAAGGTSMSST<br>CAKRTGCAKSGCRTGGCAGAA | 10 min at 95 °C, and 40 cycles at 95 °C per 15 sec, 60 °C per 1 min, finalized by 15 sec at 95 °C                      | 267                | [35]      |

and Tukey's honestly significant difference (HSD;  $P \leq 0.05$ ) test.

## Results

### Physical and Chemical properties of Sediment Samples

The physicochemical properties of sediment samples are summarized in Table 2. The pH and temperature in

sediments samples varied from 7.02 to 7.56 and 14.8 to 19.03°C, respectively. The DO ranged from 9.81 to 10.42 mg l<sup>-1</sup>, EC from 52.18 to 59 µS cm<sup>-1</sup>, and ORP from -229 to 222. Significantly ( $P \leq 0.05$ ) higher values of pH, EC and ORP were found in Pu samples, whereas the Po samples showed significantly ( $P \leq 0.05$ ) higher EC and temperature. Comparatively, significantly ( $P \leq 0.05$ ) higher values of TC (29.58 mg kg<sup>-1</sup>), TN (3.8 mg kg<sup>-1</sup>), P<sub>Olsen</sub> (25.98 mg kg<sup>-1</sup>) and OM (56.94 mg kg<sup>-1</sup>), K<sup>+</sup> (0.31 cmol<sub>(+)</sub> kg<sup>-1</sup>), Ca<sup>2+</sup> (8.13 cmol<sub>(+)</sub> kg<sup>-1</sup>), and Fe<sup>3+</sup> (138.44 cmol<sub>(+)</sub> kg<sup>-1</sup>) were observed in samples from the Villarrica city bay (V) site. Interestingly,

**Table 2** Physicochemical properties in sediment samples collected from the Villarrica Lake

| Samples                                    | Pucón city bay (Pu)                   | Pucón city port (Po) | South lake (S) | Villarrica city bay (V) | North lake (N)  |
|--------------------------------------------|---------------------------------------|----------------------|----------------|-------------------------|-----------------|
| pH                                         | 7.56±0.05 <sup>†</sup> a <sup>‡</sup> | 7.2±0.08 b           | 7.02±0.11 b    | 7.07±0.04 b             | 7.24±0.04 b     |
| DO (mg l <sup>-1</sup> )                   | 9.92±0.06 b                           | 9.81±0.07 b          | 10.42±0.03 a   | 10.41±0.07 a            | 10.35±0.08 a    |
| EC (µS cm <sup>-1</sup> )                  | 59.0±0.81 a                           | 58.98±0.42 a         | 56.13±1.08 b   | 52.18±0.13 c            | 56.25±0.34 ab   |
| ORP                                        | 222.13±10.08 a                        | 76.20±6.18 b         | 124.65±23.46 b | -229±9.36 c             | -187.48±26.31 c |
| Temperature (°C)                           | 14.8±0.39 c                           | 19.03±0.19 a         | 18.93±0.09 ab  | 18.15±0.03 b            | 18.75±0.09 ab   |
| TC (mg kg <sup>-1</sup> )                  | 3.6±0.18 b                            | 3.64±0.19 b          | 5.73±0.73 b    | 29.58±6.61 a            | 6.01±0.41 b     |
| TN (mg kg <sup>-1</sup> )                  | 0.91±0.01 b                           | 1.0±0.01 b           | 1.22±0.10 b    | 3.80±0.65 a             | 1.12±0.01 b     |
| P <sub>Olsen</sub> (mg kg <sup>-1</sup> )  | 9.15±0.60 b                           | 4.44±0.42 b          | 30.78±6.00 a   | 25.98±1.29 a            | 7.83±0.28 b     |
| OM (mg kg <sup>-1</sup> )                  | 8.47±3.31 b                           | 4.27±0.71 b          | 13.47±3.65 b   | 56.94±13.13 a           | 18.37±2.83 b    |
| K (cmol <sub>(+)</sub> kg <sup>-1</sup> )  | 0.07±0.01 bc                          | 0.04±0.01 c          | 0.12±0.01b c   | 0.31±0.07 a             | 0.18±0.01 ab    |
| Ca (cmol <sub>(+)</sub> kg <sup>-1</sup> ) | 1.41±0.17 c                           | 0.80±0.08 c          | 1.35±0.08 c    | 8.13±0.69 a             | 3.25±0.10 b     |
| Al (cmol <sub>(+)</sub> kg <sup>-1</sup> ) | 0.01±0.005 a                          | 0.01±0.001 a         | 0.01±0.001 a   | 0.01±0.001 a            | 0.01±0.002 a    |
| Fe (cmol <sub>(+)</sub> kg <sup>-1</sup> ) | 21.2±1.89 c                           | 39.7±1.77 c          | 25.78±1.68 c   | 138.44±12.32 a          | 110.04±4.06 b   |

DO dissolved oxygen; EC electro conductivity; ORP oxidation–reduction potential; T temperature; TC total carbon; TN total nitrogen; P<sub>Olsen</sub> available phosphorus; OM organic matter; K exchangeable potassium ion (K<sup>+</sup>); Ca exchangeable calcium ion (Ca<sup>2+</sup>); Al exchangeable aluminum ion (Al<sup>3+</sup>); Fe exchangeable iron ion (Fe<sup>3+</sup>)

<sup>†</sup>The values represent means ± standard error from  $n = 4$

<sup>‡</sup>Same lower letters in the same row represent significant differences (one-way ANOVA, Tukey HSD test,  $P \leq 0.05$ ) among samples.

the less anthropogenized N site showed higher values of DO ( $10.35 \text{ mg l}^{-1}$ ) and lower values for nutrients such as TC ( $6.01 \text{ mg kg}^{-1}$ ) and TN ( $1.12 \text{ mg kg}^{-1}$ ).  $P_{\text{Olsen}}$  ( $7.83 \text{ mg kg}^{-1}$ ) and OM ( $18.37 \text{ mg kg}^{-1}$ ) compared with most of the more anthropogenized sampling sites.

### Composition and Predicted Functions of Bacterial Communities in Lake Sediments

Sequencing resulted in 90.73 to 94.29% coverage of OTUs in the studied sediment samples (Table 3). Significantly ( $P \leq 0.05$ ) greater number of OTUs (define at the 97% similarity level) was observed in Pu (2,484) and S (2,323) samples compared to other samples (2,000, 1,839, and 1,724 in V, N and Po samples, respectively). Concerning the bacterial alpha diversity, Shannon index values ranged from 6.2 to 6.9, ACE from 2,790 to 4,603, Chao1 from 2,501 to 3,869, and 1/Simpson from 147.6 to 434.8. Interestingly, Shannon index analyses did not reveal significant ( $P \leq 0.05$ ) differences between sediment samples, but significantly ( $P \leq 0.05$ ) greater ACE, Chao1 and 1/Simpson index values were assigned to samples from the Pu site compared with the other more anthropogenized samples sites and the less anthropogenized N sampling site.

Assignment of taxonomic affiliation to members of bacterial communities indicated that Proteobacteria was the most abundant phylum in all sediment samples, with values ranging from 38.8% to 48.1% (Fig. 2A). This was followed by members of the phyla Bacteroidetes (10.8 to 20.1%), Acidobacteria (8.8 to 18.8%) and Actinobacteria (5.4 to 9.8%). A great diversity of minor taxa was also observed, highlighting members of the Verrucomicrobia (0.7 to 1.6%) and Aminicenantales (0.1 to 3.6%) phyla in all sediment samples, except Pu samples (Fig. 2B).

At the family level, greater relative abundances of taxa in the sediment samples were attributed to members of *Chitinophagaceae* (2.7 to 11.9%), *Xanthomonadales incertae solis* (0.4 to 7.2%) and *Nitrosomonadaceae* (0.8 to 3.9%). Despite this, our analyses revealed a higher accumulated abundance (28.3 to

44.3%) of “unclassified” taxa (Fig. 3A). Interestingly, Pu samples exhibited a very low relative abundance ( $< 0.4\%$ ) of *Desulfobacteriaceae* compared to other samples (1.2 to 5.7%). The minor taxa at family level were dominated by “less abundant” microbiota (15.4 to 29.3%), highlighted by BSV26 (0.6 to 2.2%) and *Cystobacteriaceae* (0.3 to 3.1%) families in the N, Po, and V samples, and *Planctomycetaceae* (1.6 to 1.8%) and *Hyphomicrobiaceae* (2.1 to 3%) families in Pu samples (Fig. 3B).

PCoA analyses showed a clear separation of bacterial communities between sampling sites (Fig. 4), where a significant separation was observed in Pu and Po samples in relation to the S, V, and N samples.

### Relationship Between Physical and Chemical Properties and Bacterial Community in Lake Sediments

The results from the RDA for the whole community demonstrated that all the physical and chemical properties of sediments explained 77.26% of the variability (Fig. 5). In this analysis, the RDA1 and RDA2 axis explained 65% of the bacterial community variability. In this context, the relationship between bacterial community and physical and chemical properties were explained in decrescent order by ORP (13.3%),  $\text{Ca}^{2+}$  (12.82%), EC (11.8%),  $\text{K}^+$  (9.5%),  $P_{\text{Olsen}}$  (9.11%), OM (7.29%), Temp (4.62%), pH (3.7%), DO (3.54%), and  $\text{Al}^{3+}$  (1.6%). The RDA revealed a clear spatial separation for bacterial communities of Pu and Po sites, whereas V, S, and N sites grouped them more closely. Then, Pu was positively related to Al and pH, whereas the V site and the less anthropogenized N site were positively related to  $P_{\text{Olsen}}$ , OM, DO,  $\text{K}^+$  and  $\text{Ca}^{2+}$ . Finally, the S site was positively related with  $\text{Al}^{3+}$ , pH,  $P_{\text{Olsen}}$ , OM, DO,  $\text{K}^+$ , and  $\text{Ca}^{2+}$ , whereas Po did not show any clear positive correlation.

### Predicted Functions of Sediment Microbiota

The predicted microbial functional groups in each sediment sample are shown in Fig. 6. In general terms, the

**Table 3** Richness and alpha diversity among bacterial communities in sediment samples collected from the Villarrica Lake

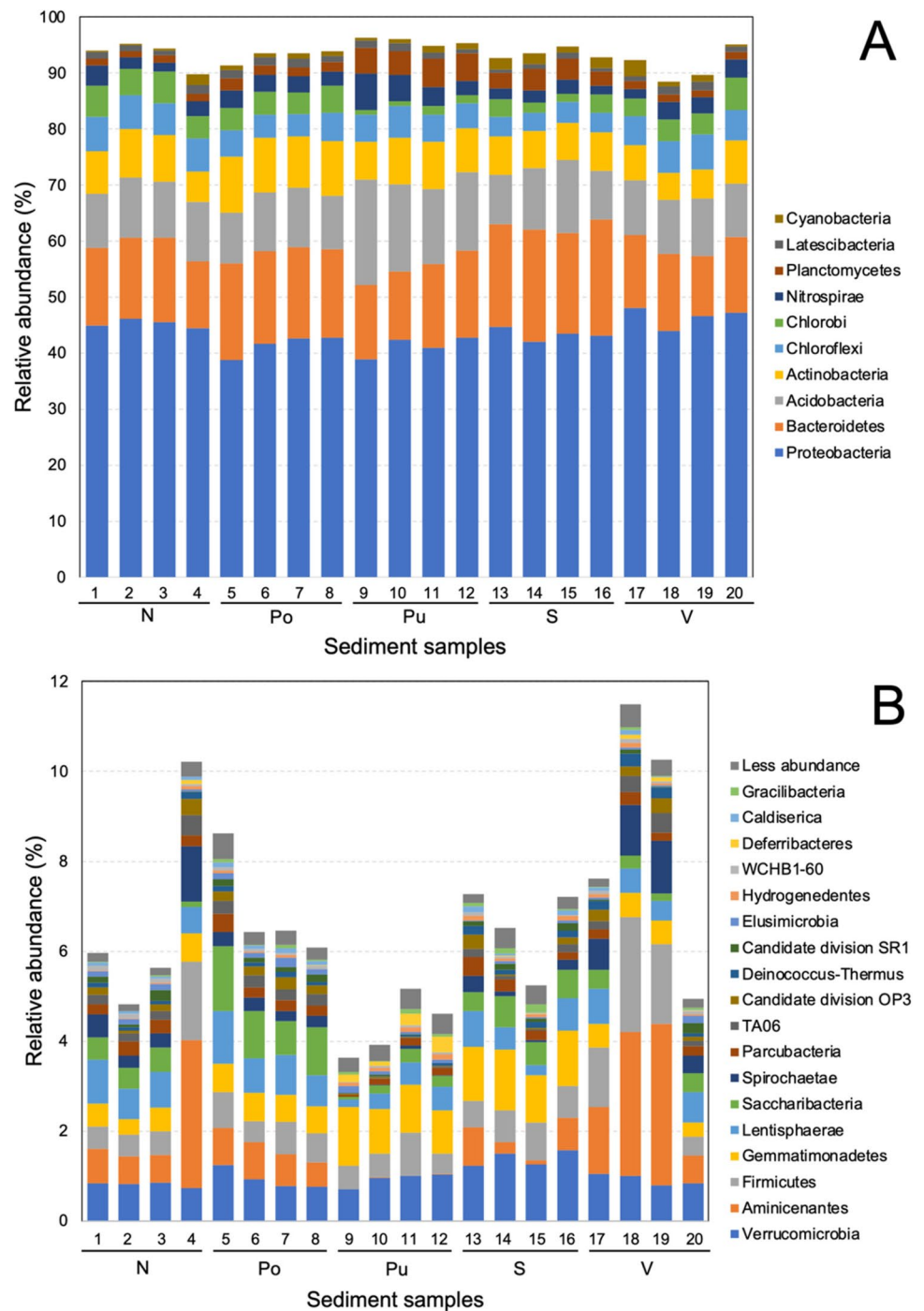
| Sample                  | Coverage (%)  | $S_{\text{obs}}^{\dagger}$ | Shannon      | ACE <sup>‡</sup> | Chao1        | 1/Simpson        |
|-------------------------|---------------|----------------------------|--------------|------------------|--------------|------------------|
| Pucón city bay (Pu)     | 90.73±0.44 c* | 2,484±134 a                | 6.90±0.1 a   | 4,603±188 a      | 3,869±151 a  | 434.78±60.8 a    |
| Pucón city port (Po)    | 94.29±0.52 a  | 1,724±129 c                | 6.24±0.015 a | 2,790±218 c      | 2,501±151 d  | 147.6±39.44 c    |
| South lake (S)          | 91.64±1.31 c  | 2,323±277 a                | 6.82±0.2 a   | 4,182±500 ab     | 3,567±151 ab | 388.35±116.75 a  |
| Villarrica city bay (V) | 92.78±0.65 b  | 2,000±160 b                | 6.55±0.23 a  | 3,725±328b       | 3,125±151 bc | 273.97±104.41 ab |
| North lake (N)          | 93.09±0.14 b  | 1,839±101 bc               | 6.33±0.21 a  | 3,774±119b       | 3,034±151 c  | 207.25±87.97 b   |

<sup>†</sup> $S_{\text{obs}}$ : number of OTUs observed at 97% similarity

<sup>‡</sup>ACE: abundance-based coverage estimate

\*The values represent mean ± standard deviation from  $n = 4$ . Sample groups sharing the same letter in each column did not vary significantly ( $P \leq 0.05$ ) by ANOVA followed by Tukey's post hoc test

**Fig. 2** Relative abundances of major (A) and minor (B) phylum-level taxa of bacterial communities in sediment samples of Lake Villarrica. The samples were coded as Pucón city bay (Pu), Pucón city port (Po), south lake (S), Villarrica city bay (V), and north lake (N)

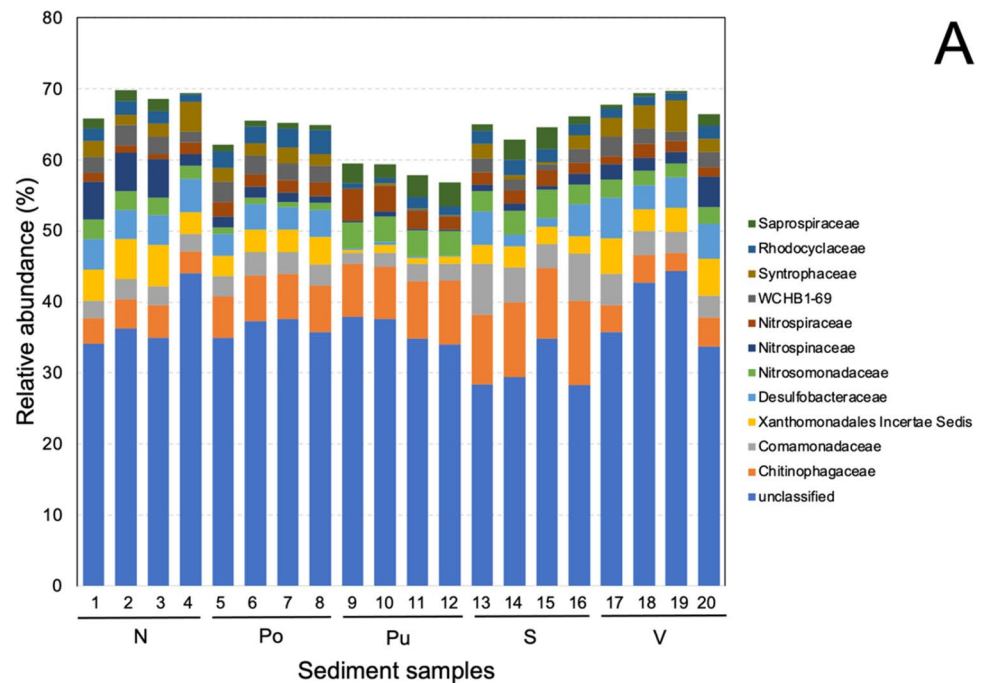


major functions were attributed to heterotrophy (15.2 to 23.4%), followed by aerobic heterotrophy (8.1 to 20.7%), nitrification (5.4 to 18.6%), and aerobic nitrite oxidation (2 to 11.8%) (Fig. 6A). Higher relative abundances were also assigned to respiration of sulfur compounds (2.5 to 9.7%) and sulfate respiration (2.4 to 9.7%) in all samples, except for Pu samples (<1.2%). When minor functional groups were analyzed (Fig. 6B), a greater abundance of functional assignments was attributed to hydrocarbon

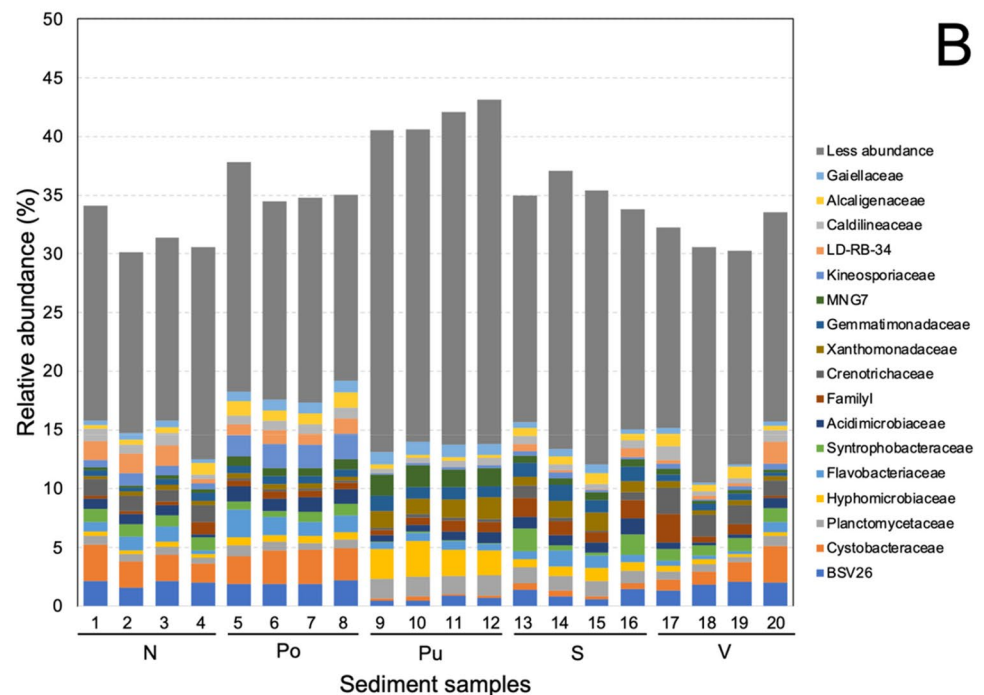
degradation (3.1 to 4.3%) and methanotrophy (2.9 to 4%) in the N and V samples, compared to the other samples (S, Po, and Pu), ranging from 0.4 to 1.8% and 0.5 to 1.7%, respectively. In contrast, samples from the S, Pu, and Po sites were dominated by microbes involved in photoautotrophy (1.1 to 2.7%), oxygenic photoautotrophy (1.6 to 2.3%), and nitrate reduction (0.9 to 2%). It is noteworthy that our analysis also predicted several functions involved in N cycling, such as N respiration, N fixation,

A

**Fig. 3** Relative abundances of major (A) and minor (B) family-level taxa of bacterial communities in sediment samples of Lake Villarrica. The samples were coded as Pucón city bay (Pu), Pucón city port (Po), south lake (S), Villarrica city bay (V), and north lake (N)



B



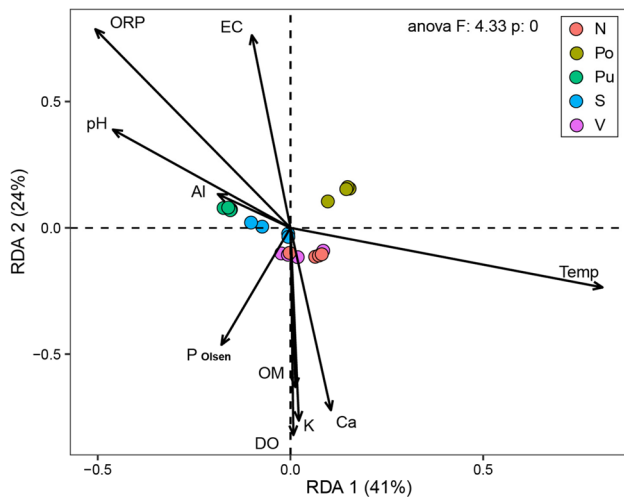
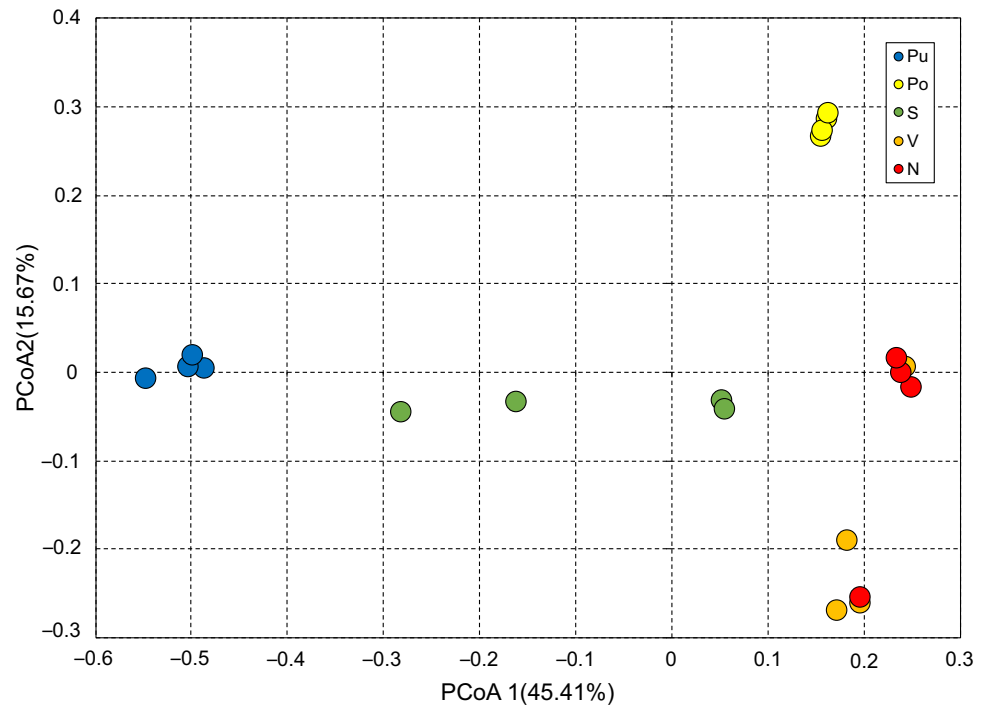
nitrite respiration, and denitrification of nitrate, nitrite, and nitrous oxide.

### Co-occurrence Network of Bacterial Communities in Lake Sediments

The co-occurrence network of the bacterial community in the lake sediments included 1,511 nodes (e.g., OTUs) and 2,544 edges. The average network diameter, modularity

index, and transitivity were 11.6, 0.6304, and 0.0640, respectively (Fig. 7). The analyses revealed the presence of four main putative keystone taxa represented by OTUs classified as members of the families *Chitinophagaceae* (*Feruginibacter* genus), *Caldilineaceae* (*Chloroflexi* genus), *Planctomycetaceae* (*Schlesneria* genus), and *Phycisphaerae*. Despite their implied importance, the relative abundance of these putative keystone taxa represented only 0.23% of the total bacterial community in sediment samples.

**Fig. 4** Principal coordinate analysis (PCoA) of bacterial communities in sediment samples of Lake Villarrica. The samples were coded as Pucón city bay (Pu), Pucón city port (Po), south lake (S), Villarrica city bay (V), and north lake (N)



**Fig. 5** Redundancy analysis (RDA) of physical and chemical properties (Temp, EC, ORP, pH,  $P_{Olsen}$ , OM, DO,  $Ca^{2+}$ ,  $K^+$ , and  $Al^{3+}$ ) and the whole bacterial community of each sediment sample collected from Villarrica Lake. The samples were coded as Pucón city bay (Pu), Pucón city port (Po), south lake (S), Villarrica city bay (V), and north lake (N)

### Abundance of Bacterial Functional Genes in Lake Sediments

The qPCR analyses revealed significantly ( $P \leq 0.05$ ) greater amounts of total bacteria in Pu and Po samples ( $34$  to  $3.9 \times 10^{11}$  copies of 16S rRNA gene  $g^{-1}$  sediment) compared to the S, V, and the less anthropogenized N samples

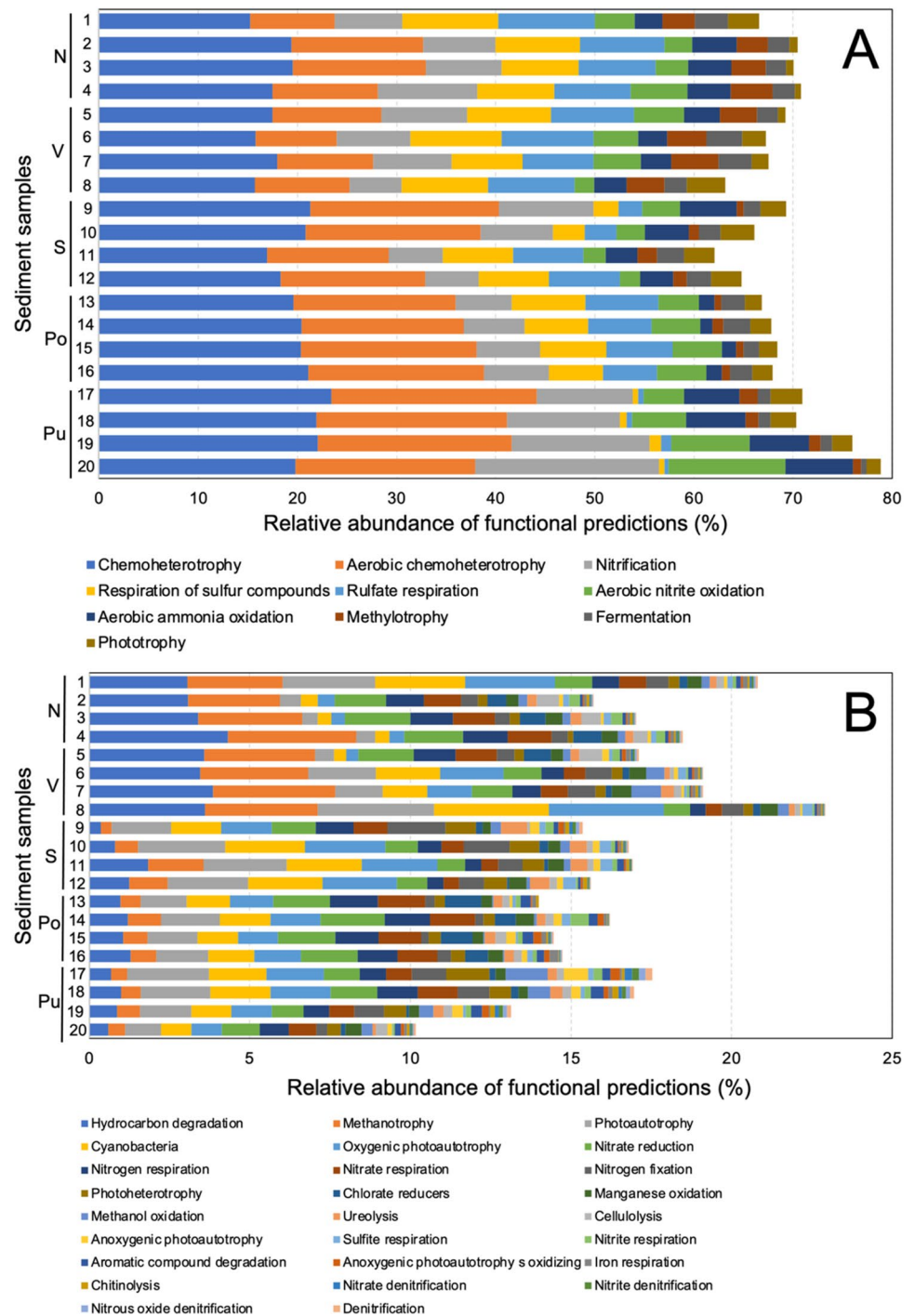
( $3.4$  to  $4.5 \times 10^{10}$  copies of 16S rRNA gene  $g^{-1}$  sediment) (Fig. 8A). In general, higher absolute abundances were observed in the *nifH* gene, with values from  $1$  to  $3.7 \times 10^8$  copies  $g^{-1}$  sediment, whereas the *phoD* gene showed the lower abundances with values ranging from  $3.5$  to  $9.9 \times 10^4$  copies  $g^{-1}$  sediment in most of the samples. The abundance of *phoC* genes ranged from  $4.2$  to  $8.3 \times 10^6$ , *phoX* genes from  $3.3$  to  $4.4 \times 10^5$ , and *nosZ* genes from  $0.4$  to  $1.7 \times 10^6$  copies  $g^{-1}$  sediment from the Po, S, and V samples. However, significantly lower ( $P \leq 0.05$ ) abundances of the *phoC* ( $4.8 \times 10^5$  copies  $g^{-1}$  sediment), *phoX* ( $2.4 \times 10^4$  copies  $g^{-1}$  sediment), and *nifH* ( $1 \times 10^7$  copies  $g^{-1}$  sediment) genes were coincidentally found in the Pu samples. It is noteworthy that N samples also presented peaking abundances for *phoC* ( $1.3 \times 10^7$  copies  $g^{-1}$  sediment), *phoX* ( $2.4 \times 10^5$  copies  $g^{-1}$  sediment), *nifH* ( $3.7 \times 10^8$  copies  $g^{-1}$  sediment), and *nosZ* ( $4.3 \times 10^6$  copies  $g^{-1}$  sediment) compared with some of the more anthropogenized samples.

Interestingly, there were higher relative abundances of each functional gene in relation to the total bacteria (functional gene/16S rRNA gene ratio) in samples from sites close to Villarrica city (V and N samples, ranging from  $10^{-5}$  to  $10^{-2}$ ) relative to those from sites near Pucón city (Pu and Po samples, ranging from  $10^{-8}$  to  $10^{-3}$ ) (Fig. 8B).

### Relationship Between Bacterial Abundance Associated with N and P Cycling Parameters

The results from RDA for physicochemical and gene abundances related to N (RDA-N) and P (RDA-P) cycling and

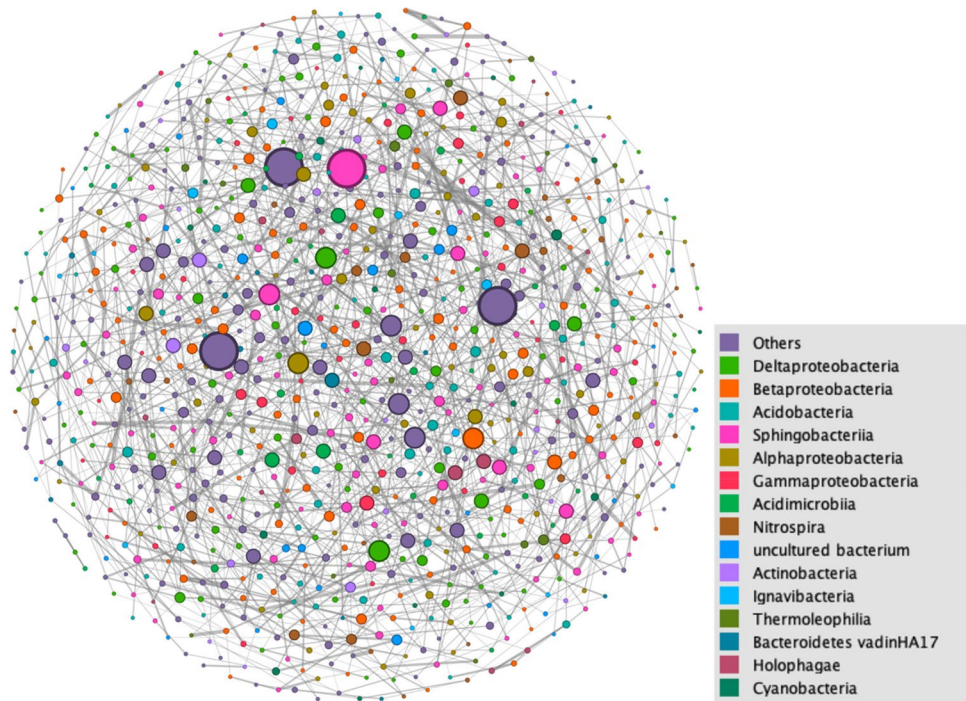
**Fig. 6** Relative abundances of major (A) and minor (B) predicted functional groups in sediment samples of Lake Villarrica. The samples were coded as Pucón city bay (Pu), Pucón city port (Po), south lake (S), Villarrica city bay (V), and north lake (N)



bacterial abundances and genus level, considering both explained 61 and 69.14 % of bacteria genus variability, respectively (Fig. 9a and b). In the case of RDA–N, the Temp, DO, TC, and OM factors explained significantly ( $P > 0.05$ ) between 33.6 and 65.2 % of the variation; meanwhile, *nosZ* and *nifH* gene abundance and TN explained between 32.8 and 61.8 %. This analysis found positive correlations among *Nitrobacter*, *Pseudomonas*, *Nitrospira*, and *Rhizobium* genera with DO, TN, OM, TC, *nifH* – and

*nosZ* gene abundances and most of the samples from Pu and Po sites. On the other hand, *Thauera*, *Defluviicoccus*, *Candidatus Competibacter*, and *Nitrosomonas* genera were positively related to Temp and most of the V and N samples. Finally, *Chryseolinea* and *Nitrosospira* genera were positively related to pH and most of the S samples (Fig. 9a). For the RDA–P, DO,  $P_{Olsen}$ , OM, TC, and TN factors explained significantly ( $P > 0.05$ ) between 29.8 and 81.4% of the variation; whilst *phoX* and *phoD* gene abundances were not

**Fig. 7** Co-occurrence network among bacterial communities in sediment samples of Lake Villarrica. The size of each node (OTU) is proportional to the number of connections. The size of edges connecting nodes represent both strong (Spearman's  $\rho > 0.807$ ) and significant ( $P < 0.05$ ). Node colors represent the taxa indicated in the legend



significantly and explained from 1.3 to 16.7 % of the variation. The clearest positive correlations were found among *Thiobacillus*, *Rhizobium*, and *Agromyces* genera with *phoX*,  $P_{Olsen}$ , OM, TC, and TN and some samples from Pu and N (Fig. 9b).

### Association of Network Topological Characteristics to Physicochemical Properties and Functional Genes in Bacterial Communities in Lake Sediments

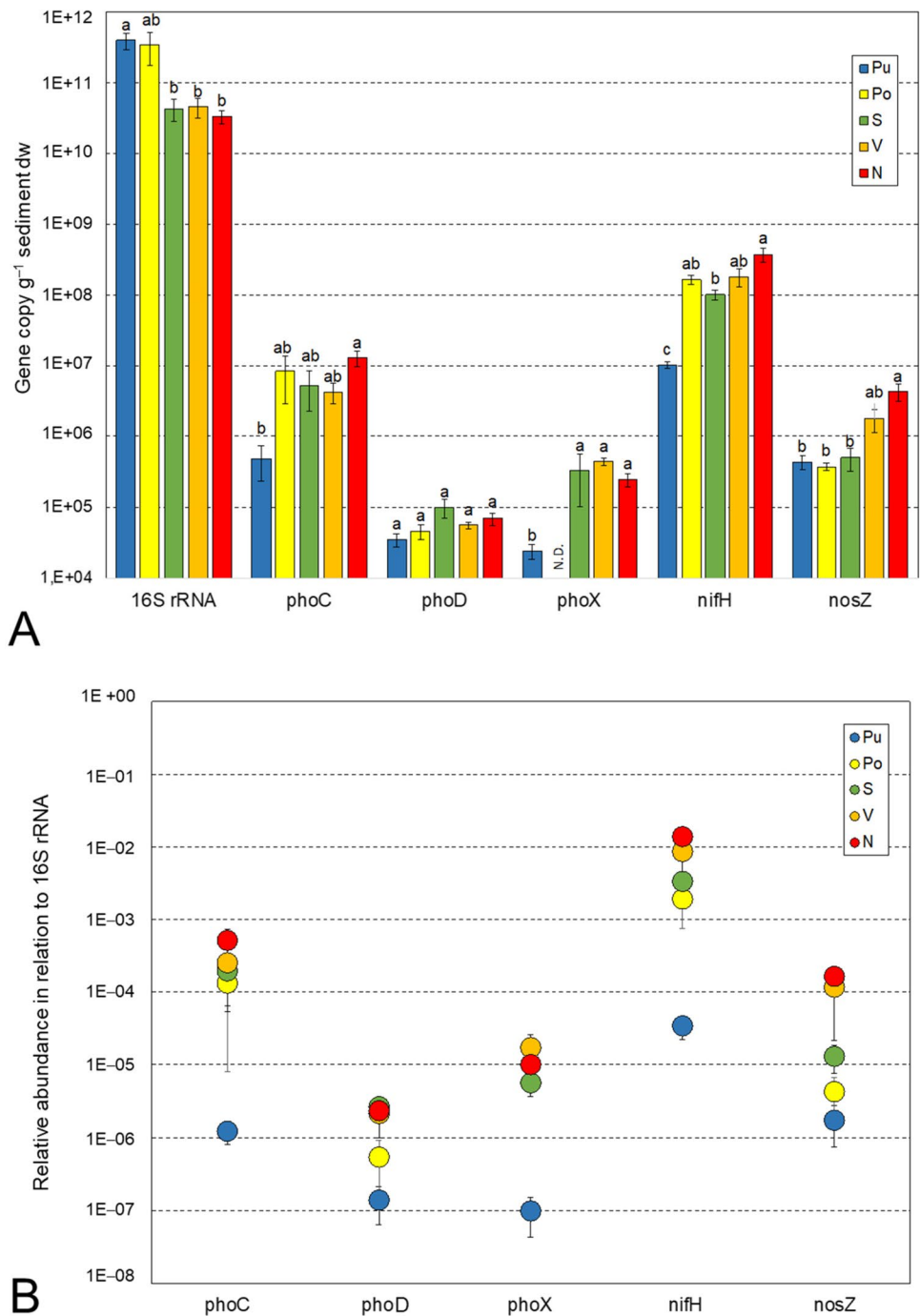
The association between the topological characteristics of the bacterial network and the physicochemical properties and bacterial functional genes present in the sediment samples from Lake Villarrica were also explored (Fig. 10). We observed that vertex number and edge number was positively correlated with pH ( $R$ -value  $> 0.6$ ,  $P$  value  $< 0.05$ ), but negatively correlated with TN ( $R$ -value  $< -0.7$ ,  $P$  value  $< 0.05$ ), Ca ( $R$ -value  $< -0.5$ ,  $P$  value  $< 0.05$ ), and Fe ( $R$ -value  $< -0.6$ ,  $P$  value  $< 0.05$ ). Vertex number was also positively correlated with EC ( $R$ -value  $> 0.6$ ,  $P$  value  $< 0.05$ ). The edge mean and the average nearest neighbor degree were negatively correlated with Ca ( $R$ -value  $< -0.4$ ,  $P$  value  $< 0.05$ ) and Fe ( $R$ -value  $< -0.4$ ,  $P$  value  $< 0.05$ ). While path length was negatively correlated with pH ( $R$ -value  $< -0.5$ ,  $P$  value  $< 0.05$ ) and ORP ( $R$ -value  $< -0.4$ ,  $P$  value  $< 0.05$ ), it was positively correlated with TC ( $R$ -value  $> 0.5$ ,  $P$  value  $< 0.05$ ), Ca ( $R$ -value  $> 0.3$ ,  $P$  value  $< 0.05$ ), and Fe ( $R$ -value  $> 0.5$ ,  $P$  value  $< 0.05$ ). Betweenness centralization, degree centralization, and transitivity were positively correlated with DO ( $R$ -value  $>$

0.2,  $P$  value  $< 0.05$ ). Modularity was negatively correlated with pH ( $R$ -value  $< -0.5$ ,  $P$  value  $< 0.05$ ) and positively correlated with  $P_{Olsen}$  ( $R$ -value  $> 0.2$ ,  $P$  value  $< 0.05$ ) and Ca ( $R$ -value  $> 0.3$ ,  $P$  value  $< 0.05$ ) and Fe ( $R$ -value  $> 0.5$ ,  $P$  value  $< 0.05$ ). The concentration of the *nosZ* gene in samples was positively correlated with average path length, betweenness centralization, degree centralization, modularity, and transitivity. In contrast, the *nifH* gene was negatively correlated with vertex number. The *phoC* gene was also positively correlated with betweenness centralization and density, but there were no significant associations between *phoD* and *phoX* genes to network topological characteristics ( $P > 0.05$ ).

### Discussion

In this study, we examined the relationships between bacterial communities, predicted functions, co-occurrence networks, and functional genes involved in P and N cycling in surface sediments with contrasting anthropogenic pressures from Lake Villarrica in Chile. In general, physicochemical analyses revealed that the sediment samples were neutral to weakly-alkaline pH (from 7 to 7.6) with temperatures ranging from 15 to 19°C (Table 2). Similar pH values have been observed in freshwater sediments from other eutrophic regions in the globe, such as Taihu, East Dongting, and Cao-hai lakes (from pH 7.1 to 7.8) in China, although some of these sediments had higher temperatures (from 29 to 33°C) [42–44]. Regarding DO, the N site showed a higher value

**Fig. 8** Absolute and relative abundance of bacterial genes involved in P (*phoC*, *phoD*, and *phoX*) and N (*nifH* and *nosZ*) cycling in sediment samples of Lake Villarrica. The samples were coded as Pucón city bay (Pu), Pucón city port (Po), south lake (S), Villarrica city bay (V), and north lake (N). Error bars represent standard error, and different lower letters denote statistical difference by one-way analysis of variance (ANOVA) and Tukey HSD test ( $P \leq 0.05$ ;  $n = 4$ ). N.D.: not detected

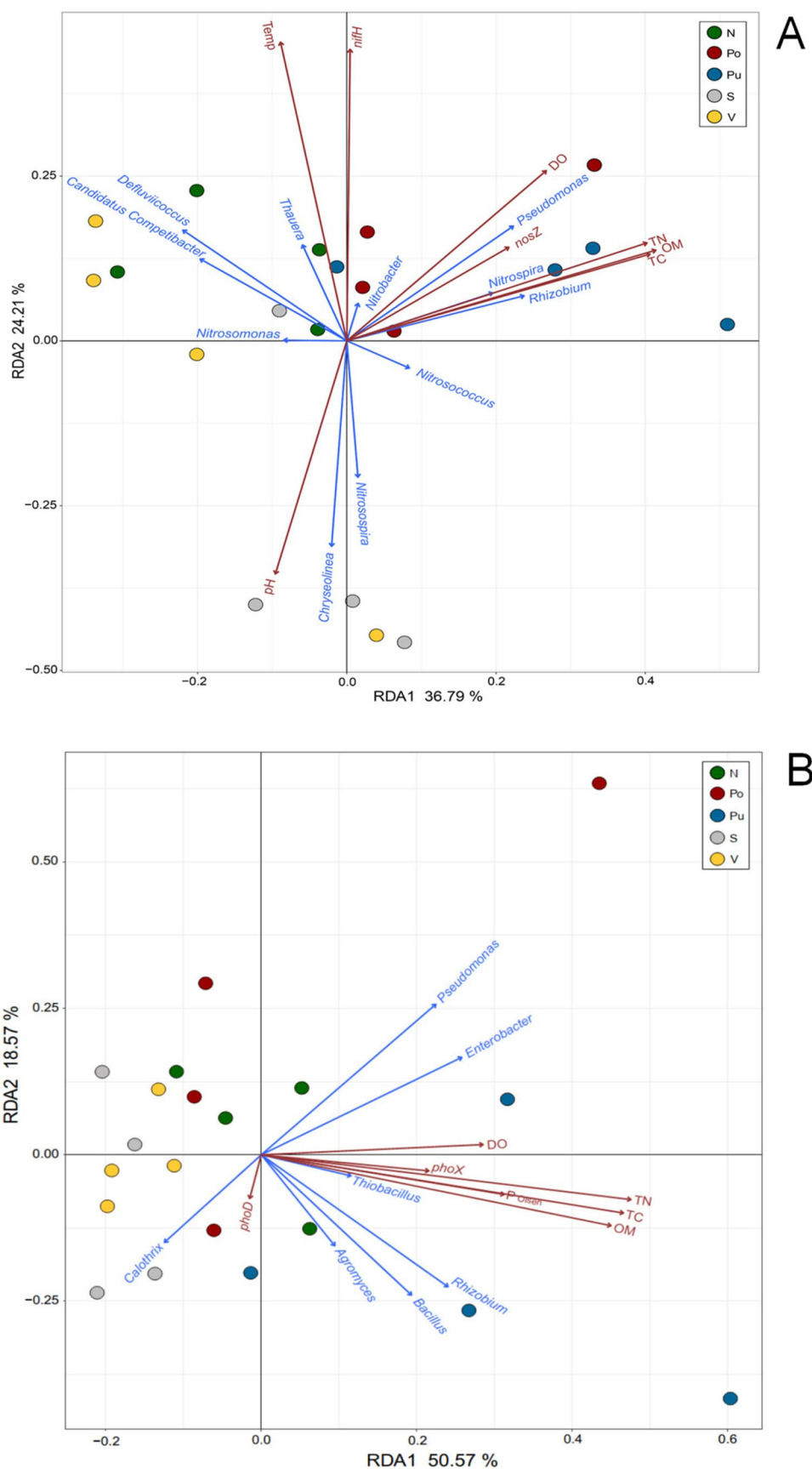


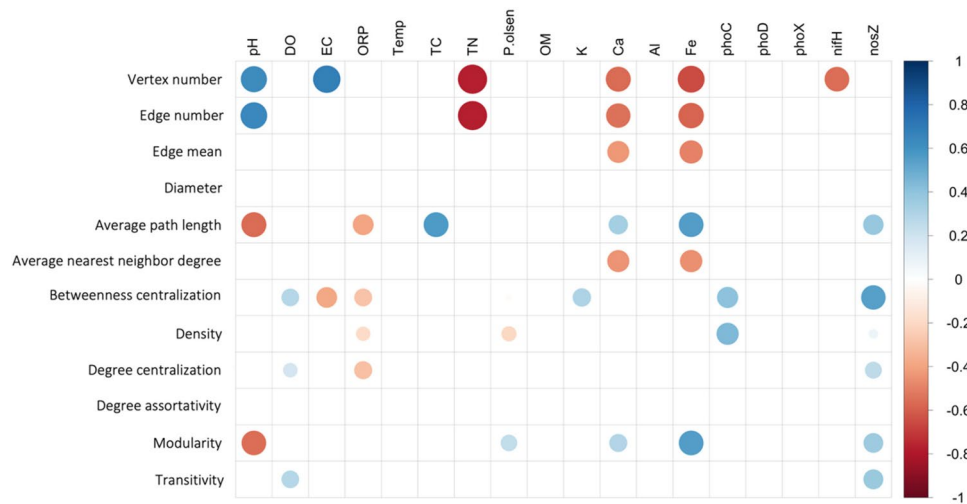
(10.35 mg l<sup>-1</sup>) compared to other sites such as Pu and Po (from 9.81 to 9.92 mg l<sup>-1</sup>), which support the idea about differences in the degree of anthropogenic pressure. Then, depletion of oxygen by sediment microbes has been as well reported in eutrophic freshwater ecosystems such as Chinese lakes and water reservoirs [45–48], Ganga river [49], and other anthropized Chilean rivers and lakes [6, 41].

In general terms, the values of TC, TN, P <sub>Olsen</sub>, and OM in our sediment samples (Table 2) were lower than those observed in sediments from the other eutrophic lakes,

including the Greek Lake Lysimachia (1,069 to 4,131 mg C kg<sup>-1</sup>, 790 to 3,060 mg N kg<sup>-1</sup>, and 310 to 760 mg P kg<sup>-1</sup>) [50] and Chinese lakes, including Lakes Caohai (507 to 521 mg P kg<sup>-1</sup>; [44]), Chaohu (3,520 mg C kg<sup>-1</sup>; [51]), Bosten (>1,500 to < 4,500 mg C kg<sup>-1</sup>, >1,500 to < 4,300 mg N kg<sup>-1</sup>, and >100 to <700 mg P kg<sup>-1</sup>; [48]), and Taihu (>600 to <1,000 mg P kg<sup>-1</sup>, >1,000 to <3,500 mg P kg<sup>-1</sup>, and >3 to <10% OM; [42, 43]). These differences might be justified by differences on the trophic degree of Lake Villarrica, which most part of the year present a mesotrophic status

**Fig. 9** Redundancy analysis (RDA) of physical and chemical properties (Temp, EC, ORP, pH,  $P_{Olsen}$ , OM, DO,  $Ca^{2+}$ ,  $K^+$ , and  $Al^{3+}$ ), gene abundances related to N (RDA-N; Fig. 9a) and P (RDA-P; Fig. 9b) cycling, and bacterial abundances at genus level from sediment samples collected from Villarrica Lake. The samples were coded as Pucón city bay (Pu), Pucón city port (Po), south lake (S), Villarrica city bay (V), and north lake (N)





**Fig. 10** Association of network topological characteristics to physicochemical properties and bacterial genes involved in P (*phoC*, *phoD*, and *phoX*) and N (*nifH* and *nosZ*) cycling in sediment samples of Lake Villarrica. DO: dissolved oxygen, EC: electrical conductivity, ORP: oxidation-reduction potential, Temp: temperature, TC: total carbon, TN: total nitrogen, P<sub>olsen</sub>: available phosphorus, OM: organic

matter, K: potassium, Ca: calcium, Al: aluminum, Fe: iron. The blue and red colors represent positive correlation and negative correlation, respectively. The association matrix was examined by Spearman's rank correlation test. *P* values were further adjusted through the Benjamini and Hochberg false discovery rate (FDR), and the matrix only keeps a correlation with *P* < 0.05

overpassing to eutrophic level only when the wind mix the bottom sediment and the peaking thermal stratification is present during summer [2, 4, 52]. In addition, our results also showed significantly (*P* ≤ 0.05) higher values of nutrients, OM, and exchangeable ions (K<sup>+</sup>, Ca<sup>2+</sup>, and Fe<sup>3+</sup>) in sampling sites close to Villarrica city (V samples) compared to other sampling sites (Po, Pu, S, and N samples). Spatial and temporal variations in physicochemical properties in sediments among sampling sites collected in the same lake have also been reported in studies performed in lakes located in China [49, 51], Greece [50], and India [53]. Most of these studies stated that differences are conditioned by differences in the degree of anthropogenic pressure of sites; however, the hydrodynamic and thermal stratification becomes an influential factor in mixing water and sediments from distant zones of a lake [54–56]. Then, even when the N site in Lake Villarrica is considered the less anthropogenized site, values in its physicochemical properties can approximate the ones presented in geographically closer sites (e.g., V and S) as an effect of hydrodynamic fluctuations during the summer season [2, 52].

In relation to the bacterial communities, DNA sequencing resulted in the identification of 1,724 to 2,484 observed OTUs in sediments. A similar number of OTUs (1,229 to 2,251) were observed in Lakes Taihu, East Dongting, and Huangda by Huang et al. [42], but lower compared to Lakes Caohai (3,034 to 3,199 OTUs) and Chaohu (12,236 to 12,527 OTUs) [44, 51]. Concerning the richness and alpha diversity, values of Shannon, ACE, and Chao1 indexes revealed a high bacterial diversity in our sediment

samples (Table 3). However, higher values and ranges have been reported in other lakes, such as Dianchi lake (6.1 to 9.9 of Shannon index; 1,431 to 3,992 of Chao1 index) and Chaohu lake (6.6 to 10.7 of Shannon index; 1,716 to 5,290 of ACE; 1,715 to 5,136 of Chao1 index) in China [51, 57]. In contrast, the Shannon, Chao1 and 1/Simpson (147.6 to 434.8) values we observed were higher compared to those reported in sediments from lakes located in colder regions, with values >2.6 to <3.8 of Simpson index and >6 to <14 of 1/Simpson index in Geneva Lake (Alps, Switzerland) [58], and values >1.7 to <3.2 of Simpson index and >30 to <150 of Chao1 index in Uchum lake (Siberia) [59]. Then, as nutrients control the primary productivity of lakes and temperature stimulates the growth of microorganisms [60], the more eutrophic lakes increases in richness and decrease in bacterial diversity [61] compared to slightly eutrophic lakes such as Lake Villarrica, which commonly drive higher values for both parameters [62].

DNA sequencing analysis also showed that the Proteobacteria (38.8% to 48.1%), Bacteroidetes (10.8 to 20.1%), Acidobacteria (8.8 to 18.8%), and Actinobacteria (5.4 to 9.8%) were the main dominant phyla in sediment samples from Lake Villarrica (Fig. 2). Similarly, the Proteobacteria (22.7 to 86.2%), Acidobacteria (12.9 to 34.7%), and Bacteroidetes (2.5 to 44.4%) were previously found as the most abundant phyla in three Chinese lakes (Taihu, Huangda, and East Dongting lakes) by Huang et al., [42]. Similar to previous reports [51, 63–65], we found that members of the phyla Verrucomicrobia and Aminicenantes were also present in lake sediments. So then, Proteobacteria, Bacteroidetes,

Acidobacteria, Actinobacteria, and Verrucomicrobia are typically the major phyla in eutrophic water reservoirs [46] and lakes [42] and rivers [51]. Furthermore, members of these phyla have been proposed as suitable environmental indicators of anthropogenic impact on lakes [66, 67].

At a fine taxonomic level, the *Chitinophagaceae* (2.7 to 11.9%), *Xanthomonadaceae* (0.4 to 7.2%), and *Nitroso-monadaceae* (0.8 to 3.9%) were found as the most abundant families in our sediment samples (Fig. 3). Similarly, *Chitinophagaceae* and *Xanthomonadaceae* families have been reported as dominant bacteria in eutrophic lake sediments in China [42, 45].

Differences of bacterial communities between sampling sites were clearly revealed by PCoA analysis (Fig. 4). Spatial and temporal differences in the structure of bacterial communities between sampling sites in the same lake or between different lakes are commonly revealed by beta diversity analysis, such as non-metric multidimensional scaling or PCoA [42, 57–59]. When whole bacterial communities of sampling sites and physicochemical parameters were scaled in the RDA (Fig. 5), the geographically closer sites Pu and Po and V, S, and N were grouped separately. This finding supports the idea about the influence of the thermal stratification and hydrodynamics of Lake Villarrica during summer on the nutrient and bacterial mobilization among closer sites. Then, Mahler et al., [68] demonstrated that the transportation of suspended nutrient-rich particles and attached bacteria drive noticeable shifts on the bacterial community composition across the karstic Lez aquifer in France. Similarly, Tan et al., [69] revealed that the heterogeneity of the bacterial communities from the Chinese Lake Taihu depended on sediment resuspension by wind, water temperature, and the influence of allochthonous bacteria.

Based FAPROTAX analyses estimated bacterial functions involved in heterotrophy (15.2 to 23.4%), aerobic heterotrophy (8.1 to 20.7%), nitrification (5.4 to 18.6%), aerobic nitrite oxidation (2 to 11.8%), and sulfur (2.5 to 9.7%) and sulfate (2.4 to 9.7%) respiration to be dominant in sediment samples (Fig. 6). At a more minor abundance level, diverse functions involved in N cycling (N respiration, N fixation, nitrite respiration, and denitrification of nitrate, nitrite, and nitrous oxide) were also highlighted. Sulfate-reducing bacteria, nitrifiers, methanogens, and methanotrophs were reported as key functional groups in sediments from the anthropogenically impacted Caohai lake in China [44]. In this context, microbial respiration reactions (such as aerobic, nitrate reduction, sulfate reduction, and methanogenesis) in sediments across five lakes (e.g., Greifen, Baldegg, Zug, Zurich, and Lucerne lakes) in Switzerland appear to be influenced by the trophic status of the lakes [70]. In addition, bacterial communities in sediments from Lake Poyang in China showed a higher relative abundance of genes associated with carbohydrate metabolism, carbon

fixation pathways, methane metabolism, anaerobic ammonium oxidation (also known as anammox), nitrogen fixation, and dissimilatory sulfate reduction than those bacterial communities present in the water column [71]. In Poyang lake, functional genes related to nitrate reduction and denitrification were also observed in sediments from emerged areas and inundated areas [64].

The results of co-occurrence network analysis suggested that the *Chitinophagaceae* (*Ferruginibacter* genus), *Caldilineaceae* (*Chloroflexi* genus), *Planctomycetaceae* (*Schlesneria* genus) and *Phycisphaerae* family members constituted the keystone taxa in sediments of Lake Villarrica (Fig. 7). Linear discriminant analysis (LDA), coupled with effect size (LEfSe), revealed that members of the *Solibacteraceae*, *Hyphomonadaceae*, *Ktedonobacteraceae*, and *Geobacteraceae* families were reported as biomarkers (defined as the taxa with significantly different distribution between the two sediments) of Lake Caohai, whereas members of the families *Bryobacteraceae* (*Bryobacter* genus), *Kofleriaceae* (*Haliangium* genus), *Micromonosporaceae* (*Luedemannella* genus), *Solibacteraceae* (*Candidatus Solibacter* genus), and *Gemmatimonadaceae* (*Gemmatimonas* genus) reported as pivotal taxa by network analysis [44]. Similarly, members of families *Desulfobacteraceae* and *Thermodesulfobacteriaceae* were indicated by LDA analysis as indicators of microbial groups in sediments from Taihu and Huangda lakes. In contrast, *Xanthomonadaceae*, *Chitinophagaceae*, *Flavobacteriaceae*, *Sphingomonadaceae*, and *Oxalobacteraceae* families were highlighted in the sediment samples from East Dongting lake [42]. In the case of *Caldilineaceae* (*Caldilineae* class), this family has been reported in sediments from Maslak, a small artificial lake located in a densely populated region of Turkey [72], and *Caldilineae* has recently been proposed as microbial bioindicator of environmental quality in sediments of lagoons contaminated by heavy metals from the Central Andes of Peru [73]. Interestingly, network analyses done in this current study predicted that members of the phylum Planctomycetes, particularly members of the classes *Planctomycetaceae* and *Phycisphaerae*, were keystone taxa in sediments from Lake Villarrica. Planctomycetes are aerobic or facultatively anaerobic, chemoheterotrophic bacteria widely distributed in aquatic environments (such as sea and lake sediments) where they play a pivotal role in global N cycling by anammox process that directly transform nitrite ( $\text{NO}_2^-$ ) and ammonium ( $\text{NH}_4^+$ ) ions into diatomic nitrogen ( $\text{N}_2$ ) [74, 75].

Counts of total bacteria revealed by qPCR ranged from  $10^{10}$  to  $10^{11}$  copies of 16S rRNA gene  $\text{g}^{-1}$  sediment with a clear spatial difference between the grouped more impacted Pu and Po sites concerning the less anthropogenized N and close related S and V sites (Fig. 8). Our counts of the 16S rRNA genes were close to those observed in sediments from Lake Geneva in Switzerland ( $>10^{10}$  to  $<10^{12}$  gene copies  $\text{g}^{-1}$

sediment) [76], but higher than those observed in sediments from Lake Caohai ( $>10^8$  to  $<10^{10}$  gene copies  $g^{-1}$  sediment) and Lake Waihai ( $>10^6$  to  $<10^{10}$  gene copies  $g^{-1}$  sediment) in China [57]. Compared to results from our study, 16S rRNA gene abundances were lower ( $10^8$  cells  $g^{-1}$  sediment) in sediments from Lakes Azul, Verde, Fogo, and Furnas in the Azores (Portugal) [77]. Therefore, since nutrient availability directly impacts bacterial abundance, significant differences are feasible between lakes or intra-sites of the same water body with different nutrient regimes [61, 62].

The abundances of functional genes revealed by qPCR analyses varied between sampling sites and targeted genes (Fig. 8), where lower abundances were registered for the *phoX* in Pu samples ( $2.4 \times 10^4$  copies  $g^{-1}$  sediment;  $10^{-8}$  ratio) and higher abundances were found for the *nifH* gene in N samples ( $3.7 \times 10^8$  gene copies  $g^{-1}$  sediment;  $10^{-2}$  ratio). Then, since activation of the *phoX* gene is negatively controlled by the abundance of P, sites more enriched with this element P usually harbor lower abundances of *phoX*-harboring bacterial communities [37]. Then, the expression of this starving gene might be modulated by the abundance of P, which has been observed in North American [78], Chinese [61, 79] freshwater ecosystems, and the coastal Budi lake in Chile [60]. On the other hand, higher abundances of *nifH*-harboring bacteria are commonly found in sites with lower N inputs, being nitrifying bacteria key in supplying the requirements of this nutrient for lake sediments [80]. Compared to the abundances of the *nosZ* we found in our studied sediment samples ( $10^5$  to  $10^6$  gene copies  $g^{-1}$  sediment), Bai et al., [57] reported higher abundances of the *nosZ* genes in sediments from Lake Caohai ( $>10^6$  to  $<10^7$  gene copies  $g^{-1}$  sediment), but lower abundances observed in sediments from Lake Waihai ( $>10^2$  to  $<10^6$  gene copies  $g^{-1}$  sediment). Moreover, these authors also reported higher *nosZ*/16S rRNA gene ratios ( $10^{-2}$  to  $10^{-5}$ ) in sediments from Lakes Caohai and Waihi compared to those observed in our study ( $10^{-4}$  to  $10^{-6}$ ) [57]. Similarly, *nosZ* and *nifH* normalized to the 16S rRNA gene abundance contributed to  $<1\%$  of the total microbial community in the majority of sampling sites in the Lake Ellesmere (New Zealand) [81]. Recently, Zhang et al., [48] reported the presence of  $10^5$  gene copies  $g^{-1}$  of bacterial *phoD* in suspended particles from Lake Taihu. In sediments from Lake Chaohu, the relative abundance of *nifH* in relation to 16S rRNA genes ranged from 0.22 to 7.2% of total bacterial whereas the relative abundance of *phoD* gene was higher than that of *phoX* gene in the most samples with up to 1.94% [82]. Lastly, Martins et al., [77] reported that anaerobic ammonia-oxidizers were the most abundant bacteria (4.5 to 16.6%) in sediments from Azorean eutrophic lakes (such as Verde, Azul, and Furnas) in Portugal, followed by nitrifying bacteria (0.8 to 13.0%), denitrifying bacteria (0.5 to 6.8%), and P-accumulating

bacteria ( $<0.3\%$ ), whereas denitrifying bacteria dominated (8.8%) sediments from Fogo, an oligo-mesotrophic lake.

When physicochemical parameters, gene abundances related to P and N cycling and relative abundances at the genus level were analyzed by the RDA-N and RDA-P (Fig. 9a and b), the variability was best explained by N-related parameters in the RDA-N than the P-related in the RDA-P. Similarly, RDA results showed that physicochemical properties, such as TN, OM, pH, DO, and TP, correlate well with the bacterial community distribution in sediments from Lakes Taihu and Poyang [43, 64]. Additionally, studies have shown differences in the correlations found in lakes with contrasting trophic states, where OM was positively associated with bacterial communities in Lake East Dongting, TN and ORP were positively associated with bacterial communities in Lake Huangda, and TN, TP, OM, and the temperature was associated with the variation in bacterial communities in Lake Taihu [42]. On the other hand, as the RDA-N suggests, several nitrifying and denitrifying bacterial genera were associated with some of the most anthropogenized sites. Therefore, the nitrite-oxidizing bacterial (NOB) genus *Nitrobacter* and *Nitrospira*, the ammonia-oxidizing (AOB) bacterial genus *Nitrosomonas*, the N-fixing genus *Rhizobium*, and *Nitrosospira*, and the denitrifying bacterial (DNB) genus *Pseudomonas*, and *Thauera* are abundant and well correlated with *nifH* and *nosZ* genes in eutrophic Turkish [72], Portuguese [77], and Chinese [43–45, 57, 61, 80] lakes, overall, in the most disturbed zones of these environments. Belonging the RDA-P, the associations among *phoX* and *phoD* genes and bacterial genera were less clear than observed in RDA-N. However, the occurrence of DNB-related genera such as *Thiobacillus* with the capability of reducing  $Fe^{3+}$  and potentially crosslinking this function and OM respiration with P release from sediments indicates relevant participation in P recycling [41, 83]. Hence, *Thiobacillus* has been reported as a significant genus in sediments of the eutrophic Chinese lakes [43, 82, 83], and North American rivers [84].

Similarly, with RDA, RDA-N, and RDA-P, when we explored the associations of network topological characteristics with physicochemical properties and bacterial functional genes in the sediment samples of Lake Villarrica, some physicochemical properties (e.g., pH, DO, Ca, and Fe) were significantly associated with topological characteristics of microbial co-occurrence networks suggesting their impact on bacterial interactions. For example, Zhang et al., [51] showed a positive effect of TN and TC on members of Ignavibacteria and Gemmatimonadetes classes but a negative effect on the dominant bacteria group (e.g., Deltaproteobacteria, Acidobacteria, and Gammaproteobacteria) in sediments from Lake Chaohu. In addition, *nosZ* was identified as the most frequent gene significantly associated with topological characteristics of microbial co-occurrence networks,

which may indicate that the denitrifying microbial populations can frequently influence bacterial interactions in the sediment of Lake Villarrica. Denitrifying bacteria, as well as anammox bacteria, in sediment samples were also highlighted by other analyses in our study (e.g., FAPROTAX, qPCR, and RDA–N). This suggests that bacteria involved in N cycling are highly relevant in sediments of Lake Villarrica, as they not only to release  $N_2$  to the atmosphere but also are keystone taxa for microbe-microbe interactions. Denitrifying bacteria and anammox bacteria have been described as responsible for losses of N from lakes in several studies [85–87], but their role in the microbial sedimentary community and interactions with other microorganisms in lakes is by far unknown.

## Conclusions

The present study revealed differences in physicochemical and microbial characteristics of bacterial communities of sediments samples with contrasting anthropogenic degrees from Lake Villarrica. Regarding physicochemical parameters, nutrient levels, such as total carbon (TC), organic matter (OM), total nitrogen (TN), and available phosphorus ( $P_{Olsen}$ ), were significantly higher in most of the more anthropogenized samples compared to the less anthropogenized one. Similarly, alpha diversity showed high bacterial richness and diversity in most of the more anthropogenized sediments, and several bacterial taxa (e.g., Proteobacteria, Bacteroidetes, and Acidobacteria phyla) related to eutrophication were abundant. On the other hand, despite differences in the anthropogenized influences evident in Lake Villarrica, the principal coordinate (PCoA) and redundancy (RDA) analyses showed similarities for bacterial community structure among sites with different anthropogenic degrees. These similarities might be attributed to the geographical closeness of some sites and the particular hydrodynamic characteristics of Lake Villarrica during summer. Later, nitrification and denitrification were significant functions detected in all sediment samples, a finding supported by the positive correlations among nutrients, functional genes related to N and P cycles, and several nitrite-oxidizing, ammonia-oxidizing, and N-fixing bacterial genera. The impact *nosZ* gene was the most significant parameter associated with changes in the topological characteristics of microbial co-occurrence networks, indicating the importance of N-cycling bacterial community and denitrifying microbial populations. Further studies on the reasons for spatial and temporal changes in the structure and interactions of bacterial communities, and some of their significant bacterial groups (such as denitrifying bacteria and anammox bacteria), might be relevant for the improvement or design of conservation and

decontamination plans for lakes and other freshwater ecosystems in southern Chile.

**Acknowledgements** The authors acknowledge to the Scientific and Technological Bioresource Nucleus (BIOREN) for the availability of Illumina MiSeq equipment (Fondequip, code EQM150126). The authors would also like to thank (1) “Dirección General del Territorio Marítimo y de Marina Mercante (DIRECTEMAR)” of Pucón city, (2) Biol. Bárbara Cisternas from ‘Gobernación Marítima’ of Valdivia city, and (3) Eng. Pablo Etcharren from Seremi del Medio Ambiente (Región de La Araucanía) of the Ministerio de Medio Ambiente de Chile provided support for selecting sampling sites and collecting sediment samples. Finally, the authors would like to acknowledge the Editor and two anonymous reviewers for their criticism reading and constructive commentaries, which improved the quality of our manuscript.

**Authors’ Contributions** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by M.A.C., Q.Z., J.J.A., J.I.R., T.R., E.C., C.R., and J.H. The first draft of the manuscript was written by M.A.C., Q.Z., and M.A.J. All authors commented, revised, and edited on previous versions of the manuscript. All authors read and approved the final manuscript.

**Funding Information** This study was funded by Chile-China Joint Projects on Water Resources Management from ANID and NSFC (NSFC190012 in Chile and Grant No. 51961125201 in China) (to M.A.C., J.J.A., E.C., C.R., J.B., L.Z., R.X. and M.A.J.), by International Cooperation Project Chile-USA from ANID (code REDES190079) (to, M.A.C., J.J.A., J.I.R., A.H., D.P.J., A.O. and M.A.J.), by FONDECYT project no. 1201386 and 1221228 (to J.J.A. and M.A.J.), by NSFC (42077386) (Q.Z.), and by Science and Technology Research Partnership for Sustainable Development (SATREPS, Japan) (code JPM-JSA1705) (to M.A.C., J.J.A., J.I.R., T.R. and M.A.J.).

## Declarations

**Conflict of Interest** The authors declare no competing interests.

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