

Identification of potentially harmful bacterial genera of veterinary relevance in the Llanquihue urban wetlands

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ABSTRACT. Water bodies constitute natural, social, and ecological heritage under constant threat from the footprint of human action. They are the habitats of many species and play a key role in sustaining biodiversity in different ecosystems. However, anthropogenic activity can result in eutrophication of water bodies, favoring the excessive growth of microorganisms, which can be a risk factor for animal, human, and environmental health. The Agricultural and Livestock Service [Servicio Agrícola y Ganadero (SAG)] of Chile developed a list of Mandatory Reportable Diseases (MRD) to report suspected contagious diseases of veterinary relevance. In this sense, the detection of microorganisms in water bodies for the characterization of their sanitary status is important for the development of monitoring strategies and the prevention of possible diseases. This study aimed to determine the presence of potentially harmful bacteria in Llanquihue urban wetlands. Here, we used metagenomic sequencing of amplicons to characterize the composition of microorganisms in three urban wetlands in the city of Llanquihue, which could be a risk factor for animal health. Our main finding was the detection of genera that may be associated with MRD, in addition to other microbial genera that have pathogenic potential. On the other hand, we also report the presence of cyanobacteria genera that can be involved in toxin production in water bodies as another potential source of risk. This is the first study to use massive sequencing techniques to analyze the sanitary status of urban wetlands in the city of Llanquihue.

Keywords: Mandatory reportable diseases, Wetlands, DNA sequencing, bioinformatics.

INTRODUCTION

In the Los Lagos region of southern Chile, approximately 70% of the land corresponds to hydrographic basins. Rivers, lakes, wetlands, and groundwater have sustained the most important economic, social, and productive human activities, such as agriculture, livestock, forestry, and aquaculture, among others. However, poor management of water resources, excessive exploitation, and negligence in controlling anthropogenic organic and industrial waste harm ecosystems (Zedler & Kercher, 2005). These activities promote high levels of nutrients, such as nitrogen and phosphorous, in the aquatic network, a process called eutrophication (Akinawo, 2023). Eutrophication is one of the most worrying consequences of anthropogenic activity in ecosystems and water bodies (Bhagowati & Ahamad, 2019; Chislock et al., 2013; Khan & Mohammad, 2014; Pitois et al., 2001), favoring the excessive growth (blooms) of microorganisms that decrease the quality and availability of resources (Amorim & Moura, 2021; Paerl et al., 2014). In addition, microorganism blooms can be a risk factor for animal health because the abundance of certain pathogens in water bodies increases the risk of infectious or toxicological diseases in wildlife and domestic animals (Parlapani et al., 2023; Lapointe et al., 2015; Walters et al., 2011; Zinia & Kroeze, 2015).

The Agricultural and Livestock Service of Chile [Servicio Agrícola Ganadero (SAG)] has drawn up a list of Mandatory Reportable Diseases (MRD) of veterinary relevance

established for reporting suspected contagious diseases in animals (DECRETO 389 EXENTO, Ministerio de Agricultura, Chile, 2014). MRD can be caused by a wide variety of pathogens belonging to different bacterial genera. Some of these bacterial genera include *Mycobacterium*, *Brucella*, and *Mycoplasma*, which are involved in diseases such as bovine tuberculosis, paratuberculosis, brucellosis, and avian mycoplasmosis, among others (SAG, 2019). However, there are other diseases that, although not currently part of the MRD list, may constitute a risk to animal health, such as species members of *Flavobacterium*, *Microcystis*, and *Leptospira* genus. In the case of *Flavobacterium*, it has been reported that the species *Flavobacterium psychrophillum* can cause flavobacteriosis disease, being the second cause of mortality in fry and salmonids in culture center (Martínez, 2018). In addition, pathogenic species of the genus *Leptospira* are involved in a zoonosis with a worldwide distribution that occurs mainly in tropical, subtropical, and temperate zones (Céspedes, 2005). In this context, mammals such as horses, cows, dogs, cats, pigs, and rodents are highly relevant in the transmission of this pathogen because they are asymptomatic carriers (Faine, 1994). However, several reports suggest that animal health is not necessarily impaired only by pathogenic microorganisms, but also by microorganisms capable of producing toxic substances or products with noxious effects (Wood, 2016).

Cyanobacteria are widely distributed oxygenic, phototrophic microorganisms. Owing to eutrophication and the

progressive increase in temperature associated with climate change, an increase in cyanobacterial blooms has been reported in marine and freshwater ecosystems worldwide (Markensten *et al.*, 2010; Paerl & Huisman, 2008, 2009). One of the major concerns related to the presence of these microorganisms is the ability of some species to produce toxins (cyanotoxins) that can affect humans and animals as well as drastically decrease the health and quality of water bodies (Agrawal *et al.*, 2006; Briand *et al.*, 2003, 2005; Codd *et al.*, 1999; Codd *et al.*, 2005; Dadheech *et al.*, 2001; Oberhaus *et al.*, 2007). Cyanotoxins that damage the liver and/or nervous system are classified as hepatotoxins and neurotoxins (Dittmann *et al.*, 2013). It has been reported that hepatotoxic and neurotoxic cyanotoxins can cause acute lethal poisoning (Roset *et al.*, 2001). Microcystins are among the most common and toxic cyanotoxins in water bodies (Chorus & Welker, 2021), and toxins produced by members of the genus *Microcystis*. Microcystins can accumulate in environmentally exposed animal species, subsequently entering the food chain (Gkelis *et al.*, 2006; Papadimitriou *et al.*, 2010; Peng *et al.*, 2010; Xie *et al.*, 2005; Zhang *et al.*, 2007, 2009) and affecting a wide variety of species. Therefore, the presence of microorganisms with noxious effects in water bodies is an important public health concern.

Urban wetlands are ecosystems located within an urban radius where a wide variety of animal species can interact (Jisha & Puthur, 2021). However, in very few of these ecosystems, initiatives aimed at the identification, monitoring, and risk assessment against the presence of bacterial genera that are pathogenic or pose any potential risk to other animal species have been conducted. Currently, massive sequencing tools are widely used in the study of microbial communities in different environments and allow, among other things, the detection of pathogens (Bass *et al.*, 2023). In this study, we used amplicon sequencing to describe the composition of microorganisms in three urban wetlands in the city of Llanquihue, which may represent a risk factor for animal health.

MATERIAL AND METHODS

Samples

Water samples were collected from three wetlands in the city of Llanquihue (Figure 1), located in northern Patagonia, southern Chile. A volume of 2 liters of water samples was collected from the wetlands El Loto (41°15'16.4 "S 73°00'32.9 "W), Baquedano (41°15'01.2 "S 73°00'31.9 "W), and Las Ranas (41°15'43.4 "S 73°00'24.1 "W) in two different points of each wetland, using sterile glass bottles. The samples were recovered on May 5, 2021. The samples were transported using ice packs at 4°C for processing at the Institutional Laboratory of the Universidad San Sebastián, located in the city of Puerto Montt. The water was passed through MCE (Mixed Cellulose Ester) filters of 0.22 µm pore size, using a vacuum filtration system. The filters with biological material were stored in RNA Later (SIGMA, USA) at -20 °C until nucleic acid extraction.

Genomic DNA extraction

To recover gDNA, the filters were suspended in TRIS-EDTA-NaCl (STE) buffer at concentrations of 200 mM TRIS, 200 mM NaCl, and 20 mM EDTA prepared in DNase-free water. Enzymatic digestion of the cell wall was performed using 20 µL lysozyme (20 mg/mL) and 20 µL proteinase K (20 mg/mL), incubated for 1 h at 37 °C, and then the temperature was raised to 55 °C for an additional hour. During incubation, the solution was vortexed to allow the biological material of microorganisms to be released from the filters. The obtained solution was used for gDNA extraction using affinity columns provided in the AccuPrep® Genomic DNA Extraction kit (BIONEER, Korea), following the manufacturer's instructions.

DNA Amplicon Sequencing

A minimum of 200 ng of total DNA was sent to Novogene Sequencing Service (USA) for DNA amplicon sequencing of the 16S rRNA taxonomic marker. For this, the V3-V4 region (F: CCTAYGGGRBGCASCAG, R: GGACTACNNGG-GTATCTAAT) was amplified under previously established conditions (Behrendt *et al.*, 2011). The amplicons obtained were sequenced using Illumina Novaseq 6000 equipment. Sequencing was performed using paired ends with a length of 150 bp (150 bp paired-end) and an output of 3 gigabases per sample. Sequences were delivered in FASTQ format.

Bioinformatic Analysis

The obtained sequences were imported into the R language version 4.1.0 (R Core Team, 2013) to be processed following the DADA2 v1.16 pipeline (Callahan *et al.*, 2016) using the RStudio environment (Booth *et al.*, 2018). The preprocessing performed included inspecting quality profiles, filtering, and trimming of low-quality data. The obtained files were used to learn the error rates and generate an error model based on the data structure. Subsequently, the information obtained was combined and a sequence table was constructed to remove chimeric artifacts resulting from PCR amplification.

Taxonomic assignment was performed using the SILVA SSU V138 database (Quast *et al.*, 2013). The non-rarefied information was used to construct a phyloseq object (McMurdie & Holmes, 2013) to estimate taxonomic abundance and microbial diversity. The Microbiome package was used to estimate the core diversity (Lahti *et al.*, 2017). Finally, all data were plotted using the ggplot2 library (Ginestet, 2011) following the instructions of the phyloseq package (McMurdie & Holmes, 2013). Relative abundance was plotted using the average of the replicates from each site.

RESULTS AND DISCUSSION

Microbial diversity of urban wetlands from Llanquihue City

Using 16S rRNA amplicon sequencing of water samples from the El Loto, Las Ranas, and Baquedano wetlands, we performed taxonomic assignment of microbes that inhabit

these ecosystems. After describing the high diversity of microorganisms at the phylum level, sequencing data were separated into independent sets for each wetland studied (Figure 1A). The relative abundance at the phylum level was estimated for each wetland (Figure 1B). The results indicated a heterogeneous representation of the diversity of microorganisms present in the three water bodies considered in

this study, with Las Ranas being the wetland with the highest diversity (Figure 1C). It is worth noting the high abundance of cyanobacteria in the El Loto wetland, which was considerably higher than that in the Baquedano and Las Ranas wetlands. Additionally, other phyla including potentially pathogenic species such as *Actinobacteriota*, *Spirochaetota*, and *Bacteroidota* were observed.

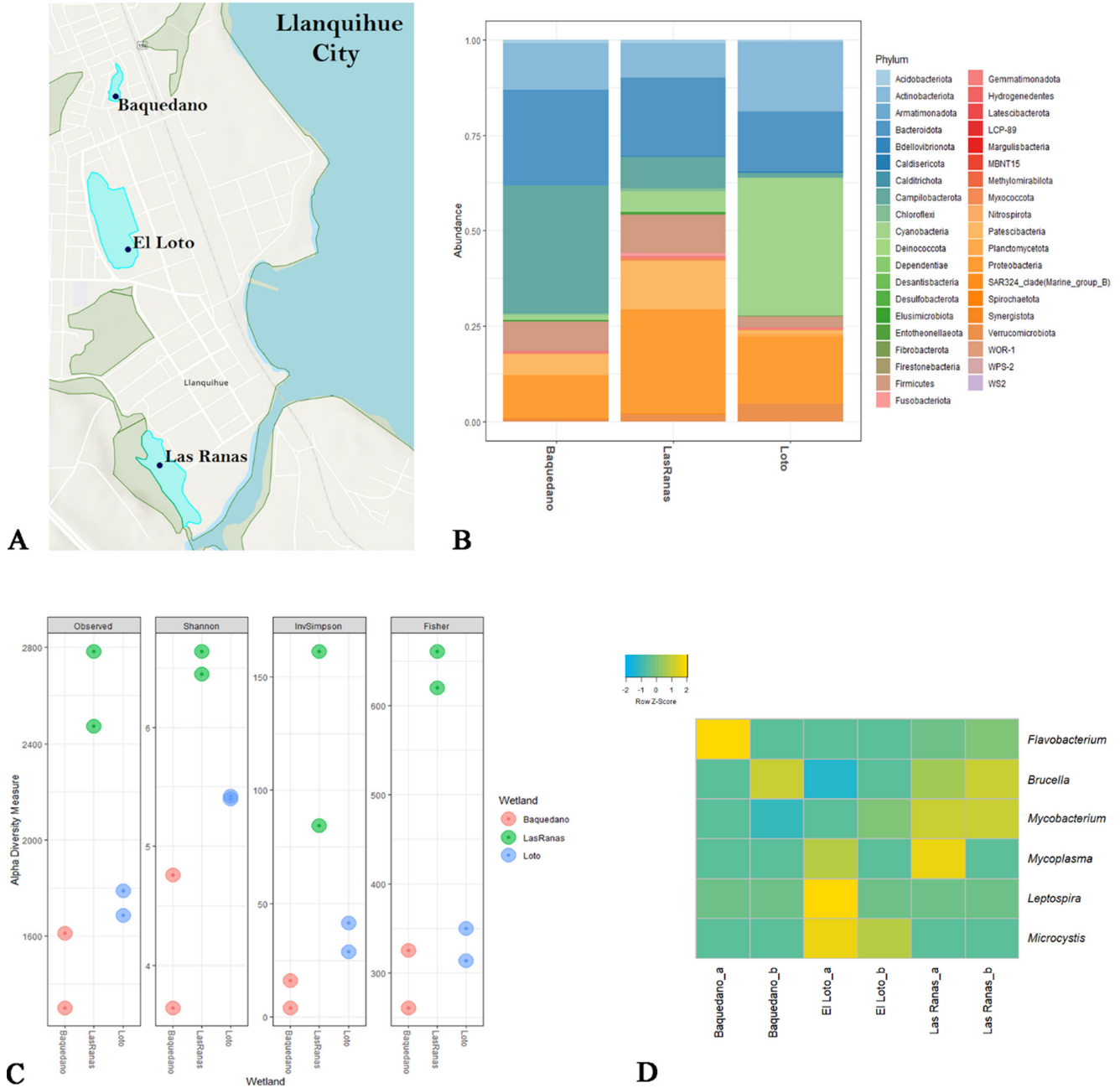


Figure 1. A) Three Urban wetlands located in Llanquihue city were selected for microbiological description. Baquedano wetland (1, 41°15'01.2"S 73°00'31.9"W), El Loto wetland (2, 41°15'16.4"S 73°00'32.9"W), Las Ranas wetland (3, 41°15'43.4"S 73°00'24.1"W). B) Relative abundance at phylum level obtained from urban wetlands. C) Measure of alpha diversity. D) Z-score of relative abundance of genera that may be associated with MDR or with harmful species.

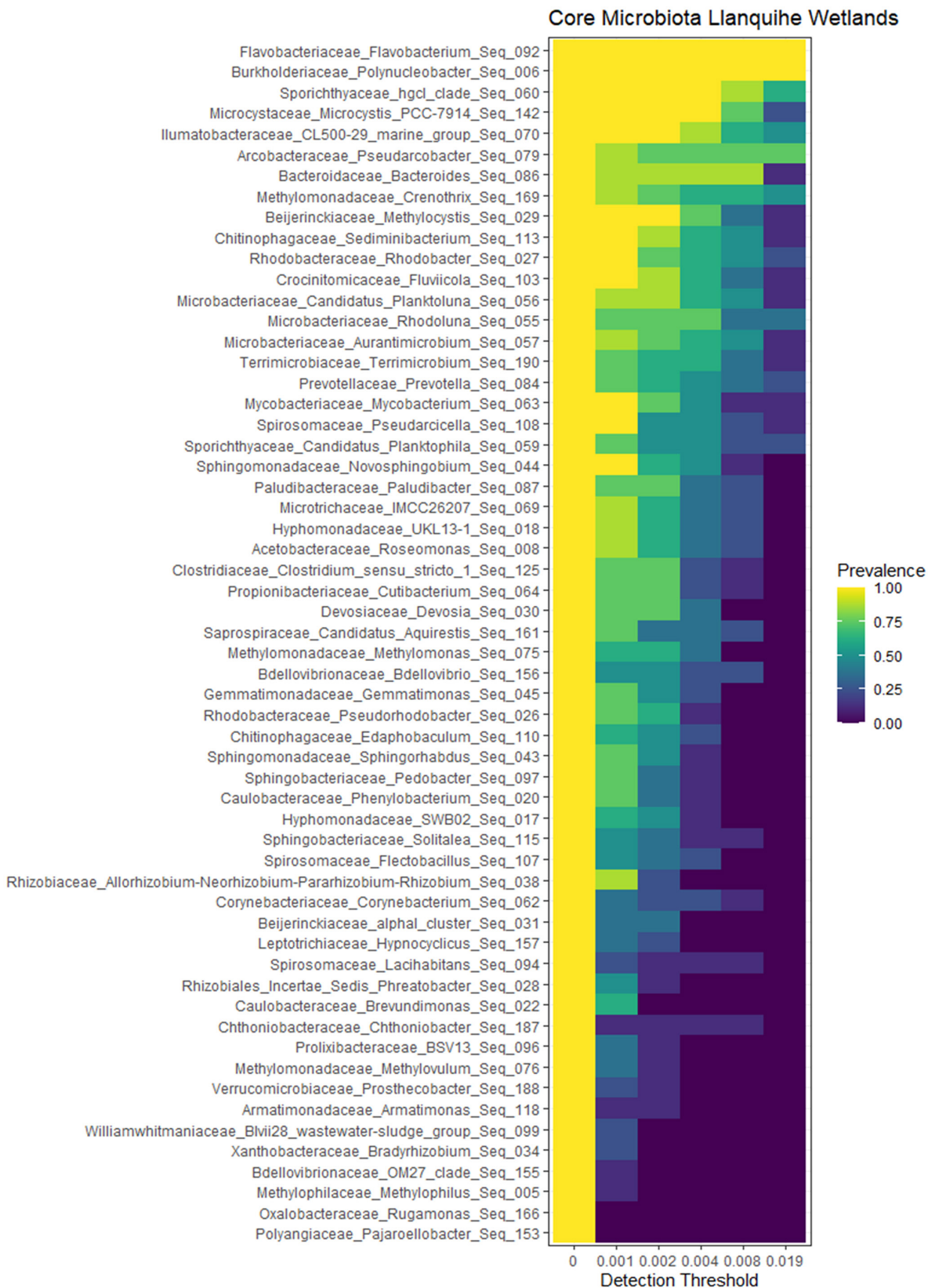


Figure 2. Core diversity analysis of the three urban wetlands. Taxonomic abundance data obtained from the wetlands were used to estimate the core of the most prevalent microorganisms inhabiting wetlands. The detected genera are ordered along the Y-axis

When examining the taxonomic classification at lower levels, we found the presence of genera that possess species with pathogenic potential, which are even present in the list of MRD. Figure 1D shows the presence of these genera in the three wetlands: *Mycobacterium*, *Brucella*, *Mycoplasma*, *Leptospira*, *Flavobacterium*, and *Microcystis*. It is well known that a variety of species belonging to these genera have serious implications on health (for reviews, please see Bierque *et al.*, 2020; Byndloss & Tsohis, 2016; Dow & Alvarez, 2022; Evangelista & Coburn, 2010; Garvey, 2020; Hilborn & Beasley, 2015; Irshath *et al.*, 2023; Loch & Faisal, 2015; Olea-Popelka *et al.*, 2017; Olsen & Palmer, 2014). Taken together, the presence of these bacterial genera corresponds to the first warning signal for the development of preventive measures.

Analysis of the Core diversity of the microbiota

Using the relative taxonomic abundance obtained, an analysis was carried out to identify the microorganisms that make up the microbial core, which consisted of the most prevalent shared microorganisms between niches (Shetty *et al.*, 2017) in the three Llanquihue wetlands analyzed (Figure 2). The results indicate that cyanobacteria belonging to the taxa *Flavobacterium*, *Polynucleobacter*, *Sporichthyaceae*, and *Microcystis* are the four top bacterial groups, which include species of health concern, such as *Flavobacterium* (Nematollahi *et al.*, 2003) and *Microcystis* (Carmichael, 1996). Interestingly, wetlands include genera that own species with pathogenic potential, such as *Roseomonas* and *Clostridium* (Mitchell *et al.*, 2022; Loch & Faisal, 2015; Nematollahi *et al.*, 2003). In addition, the genus *Microcystis* has toxicological potential (Carmichael, 1996).

The high abundance of genera with sanitary relevance, such as *Microcystis* and *Flavobacterium*, suggests that these water bodies can be reservoirs and dispersion media for MDRs and noxious species. In the case of *Microcystis* genera, there are few reports in Chile about the occurrence of a toxic bloom of *Microcystis aeruginosa* in lakes of the Biobío region, demonstrating that blooms occur periodically throughout the time, forming cumulative blooms during the summer; during the rest of the year, they can form a dispersive bloom (Almanza *et al.*, 2016). Additionally, Nimptsch *et al.* (2016) detected toxins associated with cyanobacterial blooms in lakes in northern Chilean Patagonia. However, no reports are available on the urban wetlands in the Los Lagos region. In addition, there is no database in this region with reports of animal toxicosis or human illnesses associated with ingestion of toxin-producing cyanobacteria. Nevertheless, it is necessary to develop preventive monitoring strategies for potentially toxic cyanobacterial species, as this phenomenon is on the rise worldwide and its impact on health, the environment, and the economy can be harmful, mainly because the management of *Microcystis* blooms is complex (Wilhelm *et al.*, 2020).

It is advisable to take preventive rather than reactionary action. In the microbial core, we only found the presence of *Bacteroides* and *Prevotella* (Figure 2). We detected the presence of genera whose species are described as belonging

to intestinal microbiota, such as *Prevotella* (Tett *et al.*, 2021), *Coprococcus* (Holdeman & Moore, 1974), *Bifidobacterium* (Bunesova *et al.*, 2014), and *Ruminococcus* (La Reau & Suen, 2018), which are in concordance with the fecal presence in the studied urban wetlands (data not shown).

Currently, the One Health perspective for research that unifies animal, human, and environmental health, requires multidisciplinary approaches to deepen the understanding of microbial communities that inhabit a given environment (Oliveira *et al.*, 2023; Banerjee & van der Heijden, 2023; Zinsstag *et al.*, 2018; Farschtschi *et al.*, 2022; Hilborn & Beasley, 2015). An increasing amount of evidence suggests that experimental strategies including global analyses of DNA, RNA, metabolites, and proteins (areas of research classified as “omics”) promising alternatives to conducting One Health studies (Gruszecka-Kosowska *et al.*, 2022; Tigistu-Sahle *et al.*, 2023) and the possibility to assess these studios in a variety of environments, ecosystems or conditions. Nevertheless, the presence of genera associated with MRD or noxious species raises the possibility that the same event may occur in other cities located around water bodies, which could affect the health of its inhabitants. Fortunately, the rise of molecular and DNA sequencing tools offers a new range of possibilities for the development of management strategies and monitoring of environmental microbial threats.

Further research is required to identify which species associated with MRD can be found in other water bodies in the region. It is necessary to implement techniques that allow characterization of the presence of microorganisms with sufficient resolution to determine the species inhabiting these ecosystems. Because water bodies are complex environments with a variety of microbial communities inhabiting them, Shotgun Metagenomics Sequencing may be an alternative to carry out deeper microbial and functional characterization of environmental communities. These methodologies are used for the massive detection of pathogen genomes in different environments and enable the study of interactions among human, animal, and environmental microbiomes (Trinh *et al.* 2018).

Authors' contributions

JDR wrote the manuscript and discussed results. DAM directed the study, conceptualized the experiments, performed bioinformatics analyses, and edited the manuscript. CO performed the experiments and conceptualized the figures. VC and CM contributed to reviewing the manuscript and discussed results. All authors agree with the final version of the manuscript.

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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