Science of the Total Environment 954 (2024) 176495

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

From small water bodies to lakes: Exploring the diversity of freshwater bacteria in an Alpine Biosphere Reserve

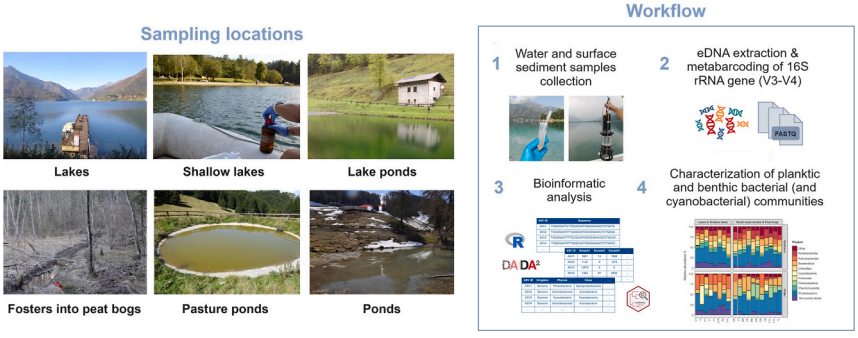
Sara Vettorazzo [a,b,*], Adriano Boscaini [a], Leonardo Cerasino [a], Nico Salmaso [a,b]

[a] Research and Innovation Centre, Fondazione Edmund Mach, via E. Mach 1, 38098 San Michele all'Adige, Italy
[b] NBFC, National Biodiversity Future Center, Palermo 90133, Italy

HIGHLIGHTS

- We investigated bacterial communities of water and surface sediments in Alpine waters.
- The majority (78 %) of amplicon sequence variants (ASVs) was unique to each sample.
- α-diversity was higher in sediments (median, 1469 ASVs) than in water (468).
- Benthic bacteria showed less variation between freshwater typologies.
- Planktic communities showed more phylogenetically and ecologically dissimilar ASVs.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Abasiofiok Mark Ibekwe

Keywords:
Small water bodies
Shallow lakes
Metabarcoding
Planktic bacteria
Benthic bacteria
Cyanobacteria

ABSTRACT

Small water bodies, although supporting high biodiversity, are often understudied in the Alpine region. In this work, we characterized the planktic and benthic bacterial communities, as well as the water chemistry, of a wide physiographic range of 19 freshwater bodies within an Alpine Biosphere Reserve, including ponds, pasture ponds, peat bogs, shallow lakes, and lakes. We collected both water and surface sediment samples, followed by metabarcoding analysis based on the V3-V4 regions of the 16S rRNA gene. We investigated the changes in biodiversity and the distribution of unique and shared amplicon sequence variants (ASVs) between water (11,829 ASVs) and surface sediment (19,145 ASVs) habitats, as well as across different freshwater typologies. The majority of ASVs (78 %) were unique to a single sample, highlighting the variability and uniqueness of bacterial communities in such freshwater bodies. Most freshwater environments showed higher α-diversity in sediment samples (median, 1469 ASVs) compared to water (468 ASVs). We found that water and sediment habitats harboured unique bacterial communities with significant differences in their taxonomic compositions. Benthic bacteria were associated with several biogeochemical and degradative processes occurring in the sediments, with no notable differences among freshwater typologies and with phylogenetically and ecologically similar species. Conversely, planktic communities showed greater heterogeneity: small water bodies and peat bogs were characterized by higher relative abundances of Patescibacteria (up to 33 %), while lakes and shallow lakes were dominated by Actinobacteriota (up to 36 %). Cyanobacteria (426 ASVs) were generally distributed at low abundances in both water and sediment habitats. Overall, our results provided essential insights into the

* Corresponding author at: Research and Innovation Centre, Fondazione Edmund Mach, via E. Mach 1, 38098 San Michele all'Adige, Italy.
E-mail address: sara.vettorazzo@fmach.it (S. Vettorazzo).

https://doi.org/10.1016/j.scitotenv.2024.176495
Received 20 April 2024; Received in revised form 19 September 2024; Accepted 22 September 2024
Available online 26 September 2024

bacterial ecology of understudied environments such as ponds and pasture ponds and highlighted the importance of further exploring their rich pelagic and benthic bacterial biodiversity.

1. Introduction

Small standing water bodies are abundant and widespread globally (Downing, 2010) and represent one of the most ecologically important freshwater habitats. They include all those small freshwaters that cannot be classified as lakes and that are characterized by limited surface area, scarce water depth, and small catchment areas, including a wide range of both natural and artificial bodies, both temporary and perennial, that are not easily categorized (Biggs et al., 2017; Stoch, 2005). Small standing water bodies vary in size, origin and water supply, and are characterized by a wide variation in physical and chemical characteristics, which in part reflects their small catchments and their strong influence by local conditions, land use, and hydrogeology. Due to their limited buffering capacity against environmental variation, their communities are more responsive to environmental changes than those of larger ecosystems (Biggs et al., 2017; David et al., 2021; Joniak et al., 2017; Kristensen and Globevnik, 2014). The shallowness of small water bodies allows the light penetration to the bottom sediments and the mixing of the water column, enabling a uniform distribution of nutrients and the potential growth of vegetation and algal mats, forming autotrophic benthic communities (Sigee, 2005; Stoch, 2005).

Small water bodies are widely recognized as true biodiversity hotspots with great value for nature conservation, often providing refuges for freshwater biodiversity and sustaining a higher proportion of rare, endemic, and endangered taxa than larger aquatic systems (Céréghino et al., 2014; Hill et al., 2021; Oertli et al., 2002; Scheffer et al., 2006). They contribute greatly to both local and regional biodiversity by encompassing a wide range of abiotic and biotic conditions that enhance the overall diversity and compositional dissimilarity of their ecological communities (Meerhoff and Beklioğlu, 2024).

Sediment and water are two distinct but closely connected environments in aquatic ecosystems, which interact closely through material deposition and resuspension (Huang et al., 2019; Ren et al., 2022). Depending on the physiography of the water body, sediment type, wind speed, and water turbulence, the mixing of the sediments can occur and affect the transparency of the water and photosynthesis (Scheffer, 2004; Stoch, 2005). In shallow water bodies, sediments are more easily and frequently resuspended than in deeper lakes and the cycle of sedimentation and resuspension can be rapid and continuous, usually supplying enough oxygen to maintain a superficial aerobic layer and allowing a more intensive benthic-pelagic coupling (Declerck et al., 2006; Scheffer, 2004).

Bacterial communities inhabiting water and sediment habitats differ in origin, diversity, and influencing factors (Gao et al., 2023). Bacteria generally increase by 3 to 5 orders of magnitude from the water to the surface sediments, where they show higher species richness and diversity. In sediments, bacteria play an important role in driving biogeochemical cycles and can influence water quality with their high metabolic activity, including the decomposition of organic matter as well as the storage and release of nutrients and dissolved substances (Scheffer, 2004; Schultz and Urban, 2008; Wetzel, 1983). In particular, the relative importance of sediments as a site for organic matter decomposition and bacterial production increases with decreasing water depth (Kalff, 2002), since the continuous supply of organic material and aerobic conditions of shallow waters provide optimal conditions for decomposing bacteria (Scheffer, 2004). Within the freshwater microbial communities, cyanobacteria are among the principal photosynthetic primary producers and can occur both in the pelagic zone and in the benthic mat, colonizing various types of sediments, macrophytes, and substrates. In waters characterized by high temperature, limited water exchange, and high nutrient availability, they can excessively proliferate and give rise to the formation of blooms that can potentially be toxic (Bauer et al., 2023; Ibelings et al., 2014; Kozak et al., 2019; Meriluoto et al., 2017). Extensive studies have been conducted on the compositions of the bacterial and cyanobacterial communities in lakes and shallow lakes (Chiriac et al., 2023; Farkas et al., 2020; Gao et al., 2023; Newton et al., 2011; Salmaso et al., 2024, Salmaso et al., 2018; Scheffer, 2004). However, information about small water bodies is much more limited (David et al., 2021; De Marco et al., 2014; Kozak et al., 2019; McMaster and Schindler, 2005). Indeed, despite their ecological relevance, small water bodies are largely excluded from water management planning and are among the least studied freshwater elements, leading to substantial gaps in knowledge about their biodiversity and functioning. Therefore, there is growing awareness of their important role in supporting freshwater biodiversity, highlighting the need to refocus attention on these ecosystems, as well as their sensitivity to anthropogenic disturbances and the importance of their protection and conservation (Biggs et al., 2017; Kelly-Quinn et al., 2017; Kristensen and Globevnik, 2014).

The objective of this work was to improve the knowledge of the biodiversity, and the ecological understanding of small water bodies' ecosystems located in an Alpine geographical area, with a focus on their bacterial and cyanobacterial communities. Specific objectives included: i) the quantitative assessment of changes in the bacterial community structure along a physiographic gradient of freshwater bodies, ranging from small ponds to lakes in the studied Alpine area; ii) the changes in biodiversity between the water and surface sediment habitats. The area examined in this study was the 'Ledro Alps and Judicaria' Biosphere Reserve, located in the north-eastern Alps. This Alpine region hosts a wide range of different freshwater bodies, from small ponds to mountain lakes, including wetlands, peat bogs, and artificial small basins used for agricultural and pasture purposes.

2. Materials and methods

2.1. The study site: the 'Ledro Alps and Judicaria' MAB UNESCO Biosphere Reserve

The 'Ledro Alps and Judicaria' Biosphere Reserve (hereafter, "Biosphere Reserve") is located in the Autonomous Province of Trento, in Northern Italy, and extends over 47,000 ha between Lake Garda (63 m a.s.l.) and the Brenta Dolomites (up to 3173 m a.s.l.). The area was recognized in 2015 as a UNESCO MAB Biosphere Reserve (www.mabalpiledrensijudicaria.tn.it). The Biosphere Reserve is made up of 89.6 % forests and grasslands, 8.6 % agricultural areas, and 1.8 % urbanized areas, and shows an anthropized alpine context with a strong agricultural and tourist vocation. The area is enriched by a high variety of freshwater bodies, from small ponds to mountain lakes, including wetlands, peat bogs as well as artificial small basins used for agricultural and pasture purposes. The 34 % of the surface area of the Biosphere Reserve consists of protected areas due to their rarity, fragility, and high level of biodiversity.

2.2. Study sites and sampling

Along an altitudinal and physiographical gradient, 19 sampling sites were selected across the Biosphere Reserve (Fig. 1, Supplementary Table 1) to represent a diverse range of freshwater bodies. Sampling sites included small ponds and pools, pasture ponds, fosters into peat bogs, lake ponds, shallow lakes, and lakes (Supplementary Fig. 1). The different freshwater typologies were categorized into two broad categories: "lakes and shallow lakes" (L-SLs) and "small water bodies and peat bogs" (SWB-PBs). The first group includes all the lakes (LL, LT),

shallow lakes (LA, LN, LV), and lake ponds (MST, PD, TF1) whereas the second group includes all the ponds and pools (MC, MN, MM, MB), pasture ponds (MT, BG, MS), and furrows into peat bogs (TF2, TF3, L1, TL). Sampling was carried out in 2019, between August and October (Supplementary Table 1). Both water and surface sediment samples were collected at each sampling location, obtaining 38 samples (19 for each habitat type). Water samples were collected differently according to the most suitable methods depending on the size and accessibility of the water body. For lakes, waters from the euphotic zones were collected from the centre of the basin in sterilized bottles using Hydrobios integrating water samplers (0–20 m; LL) and Niskin bottles (0–15 m; LT), while for shallow lakes, lake ponds, peat bogs and small water bodies, water samples were collected using sterilized buckets from the centre of the water body (0–20 cm; LA, LN) or telescopic samplers from the shores (0–20 cm; all the remaining water bodies). Surface sediment samples were collected in the littoral zone close to the shores with sterilized Pasteur pipettes and stored in Falcon tubes. All samples were stored in refrigerated containers and transferred to the laboratory for the successive analyses.

2.3. Chemistry and cyanotoxins

Chemical analyses were performed only on water samples, with standard methods following the protocols reported in APHA, et al. (2023). Conductivity (μS cm^{-1} at 20 °C) and pH were determined with electrodes specifically calibrated for low water mineralization and low organic content. Total alkalinity (mg L^{-1}) was measured by automatic titration with 0.01 N hydrochloric acid. The main ions (sodium, potassium, magnesium, calcium, chloride, and sulphate, mg L^{-1}) and nitrate (NO_3-N, μg L^{-1}) were determined by ion chromatography, while orthophosphate (SRP, μg L^{-1}), total phosphorus (TP, μg L^{-1}), total nitrogen (TN, μg L^{-1}), ammonium (NH_4-N, μg L^{-1}) and silica (SiO_2, mg L^{-1}) were measured by spectrophotometry. Bicarbonate (HCO_3^-, mg L^{-1}) and hardness (°F) were finally calculated from alkalinity and calcium + magnesium concentrations, respectively.

Cyanotoxins extraction and determination were performed following the methods described in Cerasino et al. (2017) and Cerasino and Salmaso (2012). The extracts were injected into an LC-MS system (UPLC Waters coupled to Sciex 4000 QTRAP) for the analysis and quantification (ng L^{-1}) of peptides (microcystins and nodularins) and toxic alkaloids (anatoxins, saxitoxins, cylindrospermopsins).

2.4. Environmental DNA analysis

Water samples were filtered on ISOPORE sterile filters with 0.22 μm pore size under a laminar flow cabinet within 24 h from sampling time, and filters were stored dry at −20 °C until DNA extraction. Environmental DNA (eDNA) from water and sediment samples was extracted using the DNeasy PowerWater® and the DNeasy PowerSoil® kits (MO BIO Laboratories, a Qiagen Company, USA), respectively. The quality and quantity of DNA extracted were detected by the NanoDrop (Thermo Fisher Scientific Inc., MA, USA). All the samples showed measurable concentrations of DNA above 2 ng μL^{-1} (mean ± SD, 28 ± 17 ng μL^{-1}).

Each DNA sample was subjected to PCR amplification (GeneAmp PCR System 9700, Thermo Fisher Scientific) by targeting and amplifying ~460 bp fragments of the V3-V4 hypervariable regions of the SSU 16S rRNA gene. The specific primer set used included 341F (5′ CCTACGGGNGGCWGCAG 3′) and 805Rmod (5′ GACTACNVGGGTWTCTAATCC 3′) (Apprill et al., 2015; Herlemann et al., 2011; Klindworth et al., 2013), with overhang Illumina adapters. These primers have been previously used in the assessment of bacterial biodiversity in aquatic environments, including the large southern perialpine lakes; a detailed description of PCR amplification and library construction can be found in Salmaso et al. (2018). The barcoded libraries were then pooled in equimolar concentrations by qPCR in a final library and checked on a Typestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA). The final amplicon library was sequenced on a lllumina® MiSeq (PE300) platform (MiSeq Control Software 2.5.0.5 and Real-Time Analysis software 1.18.54), to obtain paired-end reads of ~300 bp.

Raw sequences were demultiplexed using sample-specific barcodes and individually saved in forward and reverse FASTQ-formatted files. Sequences were deposited to the European Nucleotide Archive (ENA) with study accession number PRJEB74662.

2.5. Bioinformatic analysis

Primers were removed from raw sequences using Cutadapt 3.4 (Martin, 2011) implemented in the wrapper rmprim 0.11 in GitHub (github.com/hts-tools/metatools). Sequences were imported into R (R

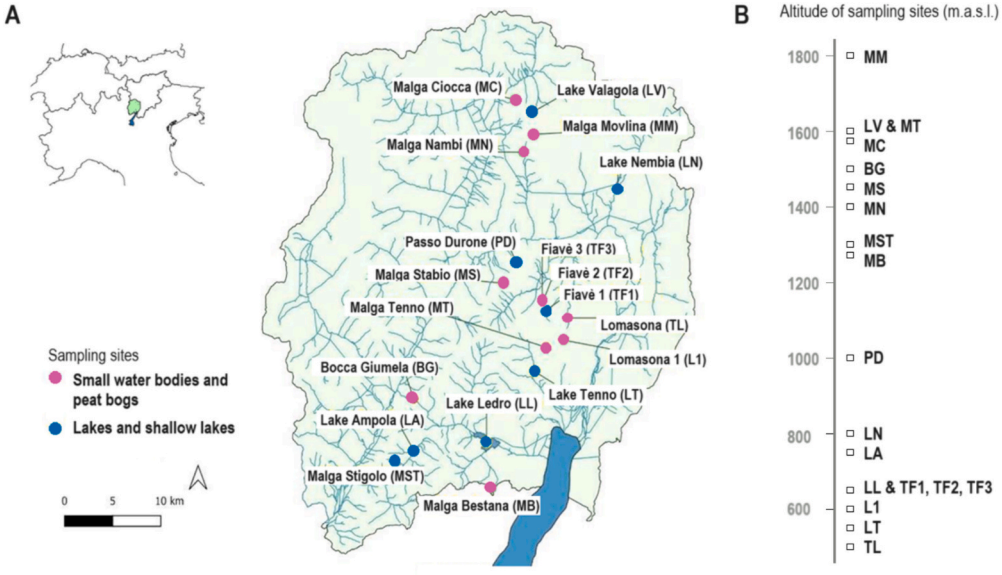


Fig. 1. (A) Map of the environmental sampling sites in the Alpi Ledrensi and Judicaria Biosphere Reserve, and its geographical location in Northern Italy. Samples are labeled with different colors according to the type of freshwater typologies. For each sampling location, the shortcode is reported in brackets. (B) Graphical representation of sampling sites' altitudes (m a.s.l.).

Core Team, 2022) and analysed according to the DADA2 pipeline (Callahan et al., 2016). The dada2 v1.20.0 R package was used, which implements the complete amplicon workflow to turn raw demultiplexed FASTQ files into quality filtered, merged, denoised, chimera-free, inferred sample sequences (ASVs, amplicon sequence variants), together with their abundances. Forward and reverse reads were truncated at 258 and 205 bp, respectively. The maximum number of expected errors allowed in a read was set to 1 and reads containing any Ns were discarded. Pairs of reads were merged requiring a minimum of 35 bp of overlap and merged reads not falling within the expected regions for this V3-V4 amplicon (400–430 bp) were removed. Taxonomic assignment up to the genus level was performed using the native implementation in DADA2 of the RDP naïve Bayesian classifier (Wang et al., 2007). As reference library sequences, the SILVA v138.1 NR 99 ribosomal reference database (Quast et al., 2012) was used, considering the training set already formatted for DADA2 (McLaren, 2021). To assign taxa only with a certain level of confidence, a 95 % minimum bootstrap confidence threshold was adopted for each taxonomic level. The complete and detailed bioinformatic pipeline used is deposited in the Zenodo repository (Salmaso et al., 2021).

2.6. Statistical data analysis

The ASVs abundance table (30,781 ASVs), taxonomy table, and environmental data were imported using the phyloseq v1.44.0 R package (McMurdie and Holmes, 2013). Taxonomic filtering was performed to remove sequences related to Archaea, chloroplast, and mitochondria, as well as unclassified bacterial sequences at the phylum level. Taxonomic filtration reduced the number of ASVs to 29,466. Raw abundances were then rarefied without replacement at the minimum sequencing depth (19,005), after checking that the rarefaction curves of each sample reached the plateau. After rarefaction, the number of ASVs considered in the further analysis was 28,629.

Downstream analysis and data visualization were performed using different R packages in addition to phyloseq, including: ANCOMBC v1.6.4 (Lin and Peddada, 2020), dendextend v1.17.1 (Galili, 2015), ggbiplot v0.55 (Vu, 2011), ggplot2 v3.5.1 (Wickham, 2016), iCAMP v1.5.12 (Ning et al., 2020), microbiome v1.21.1 (Lahti et al., 2017), phytools v2.3-0 (Revell, 2024), tidyverse v2.0.0 (Wickham et al., 2019), vegan v2.6-4 (Oksanen et al., 2022), VennDiagram v1.7.3 (Chen and Boutros, 2011).

The fraction of shared ASVs between sediment and water samples was visualized using Euler's diagrams. Alpha diversity was then quantified considering the observed number of ASVs (richness) and the Shannon diversity index (estimate_richness function, phyloseq). Differences in ASVs richness and Shannon diversity values between water and sediment samples were assessed using the non-parametric Kruskal and Wallis test (KW test). The structure of each community was then investigated through rank abundance dominance plots (Begon and Townsend, 2021; Whittaker, 1965), which display logarithmic species abundances against species rank order. After checking the different fitting models and related Akaike information criterion (AIC) (radfit function, vegan), log-normal distributions were fitted to each sample community rank abundance distribution (rad.lognormal function, vegan) and statistically evaluated with the Kolmogorov-Smirnov test (KS test).

Beta diversity was estimated using the Bray-Curtis (BC) dissimilarity computed on double square root-transformed abundances of ASVs. The BC dissimilarity matrix was then used to ordinate samples by non-metric multidimensional scaling (NMDS). NMDS analysis (metaMDS function, vegan) was carried out on all samples as well as on water and sediment samples separately. The 10 most abundant phyla were related to the NMDS ordination of all samples using vector fitting (envfit function, vegan), and the significance of vectors was estimated by 9999 permutations. Physical and chemical variables and altitude values (log-transformed) were used to ordinate water samples by principal components analysis (PCA; prcomp function) and, together with the 10 most abundant phyla, were related to the NMDS ordination of water samples using a second vector fitting analysis (9999 permutations). For water samples, a Mantel test (mantel function, vegan) was also performed to investigate correlations between the Euclidean distance matrix calculated using all log-transformed environmental and chemical variables and the BC dissimilarity matrix. Differences in bacterial composition between water and sediment samples were tested by permutational multivariate analysis of variance (PERMANOVA with 9999 permutations). Hierarchical cluster analysis was performed (hclust function, vegan) based on BC dissimilarities and considering Ward's linkage method (ward.D2).

To explore the differences in taxonomic composition between different habitats (water and sediment) as well as between different categories of freshwater typologies, the most abundant phyla were displayed using compositional bar plots. The Wilcoxon rank sum test (W test) was used to test differences in their relative abundance distribution. To explore changes in differential abundances of the ASVs identified at the genus level, the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC; Lin and Peddada, 2020) was applied on non-rarefied abundances and considering a minimum sample prevalence of 20 % (8 samples). The list of differently abundant genera was filtered considering a maximum adjusted p-value of 0.001. A qualitative analysis of the distribution of the Cyanobacteria phylum was then carried out, evaluating the prevalence of the most abundant genera. Differences in cyanobacterial ASVs richness distribution between water and sediment samples were tested with the non-parametric KW test.

To assess the community assembly processes and estimate the average phylogenetic distance between communities, beta-nearest taxon indexes (βNTI; Stegen et al., 2012) were computed using the functions pdist.big and bNTI.big (iCAMP). As inputs, phylogenetic trees were constructed by first aligning the ASVs sequences using MUSCLE v5 (−super5 algorithm; Edgar, 2022) and then by using FastTree, considering -gtr and -gamma options (Price et al., 2010). The unrooted trees were then rooted with the midpoint_root function (phytools). The βNTIs were estimated separately for each habitat group and for each habitat-freshwater typology group. With respect to the null model, βNTI values are considered to be statistically significant different if >2 or <-2 (indicating a major influence of determinism on assembly), while βNTI values between -2 and 2 indicate a major influence of stochasticity (Stegen et al., 2012).

3. Results

3.1. Chemical analysis of water samples

The 19 water samples from the Biosphere Reserve showed wide heterogeneity in physical and chemical variables (Fig. 2, Supplementary Table 2). pH values (Fig. 2A) ranged from 6.5 to 8.6 and were generally lower in the SWB-PBs group. In particular, pH values <7 were found in 4 small standing water bodies (MM, MS, MC, L1), indicating slightly more acidic conditions than in the other environments. Conductivity values ranged from 59 μS cm^{-1} in MM to 506 μS cm^{-1} in TF3 (where the highest concentrations of Cl and Ca content were detected). Conductivity of SWB-PBs water samples was significantly negatively correlated with altitude (Pearson's correlation, $r = -0.79$, $p = 0.004$). Silica concentrations (Fig. 2B) ranged between 0.6 mg L^{-1} (MS) and 6.6 mg L^{-1} (TL), and it was higher in a subset of SWB-PBs (mainly peat bogs). Nutrient concentrations were generally similar between most sampling locations, although some exceptions were highlighted in the SWB-PBs group (MC, MT, and TF3) (Fig. 2C-D). In particular, total nitrogen ranged between 289 μg L^{-1} (MS) and 3703 μg L^{-1} (TF3), while total phosphorous varied from 3 μg L^{-1} (LT, LN) to 367 μg L^{-1} (MT). The ordination of water samples by principal components analysis (PCA) (Fig. 2E), highlighted the decentralized positions of several samples (especially belonging to SWB-PBs group), such as the pond of Malga Ciocca (MC), which presented the highest content of ammonia, SRP, and potassium

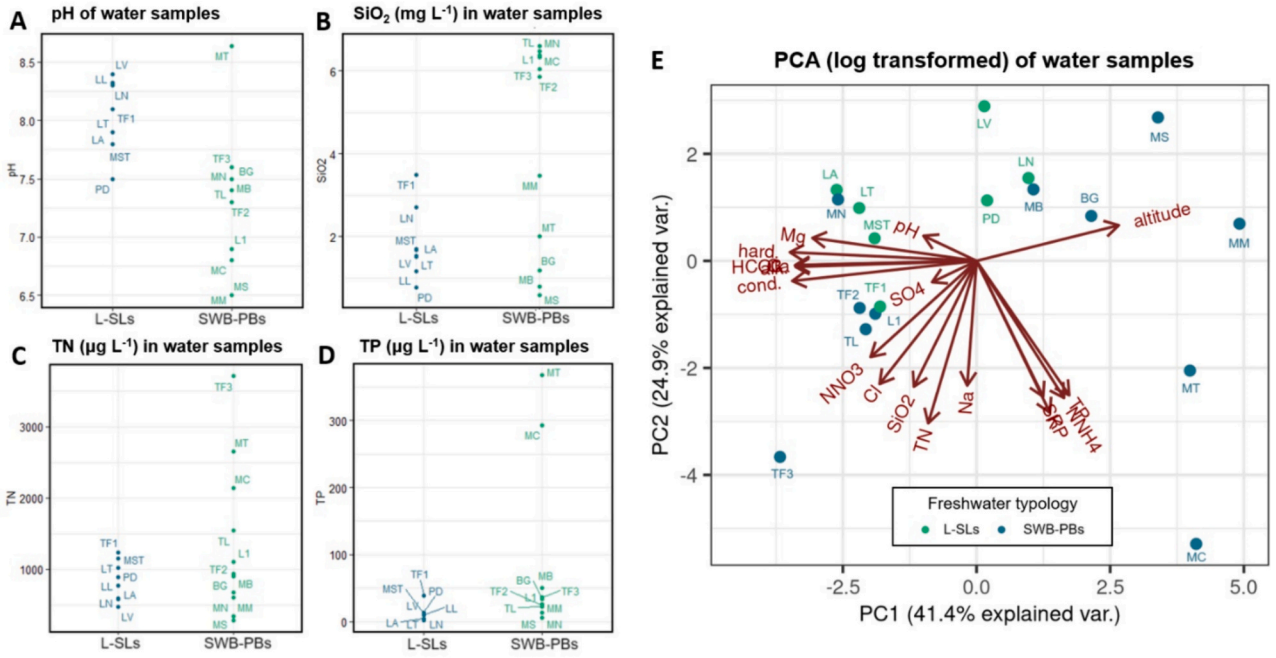


Fig. 2. (A) pH of water samples, separating the two categories of freshwater typologies. Concentrations of (B) SiO_2, (C) total nitrogen (TN), and (D) total phosphorus (TP) measured in water samples. (E) Principal components analysis (PCA) based on log-transformed chemical variables and altitude values of each sample; LL is not reported, because some chemical variables were missing. L-SLs: Lakes & Shallow lakes; SWB-PBs: Small water bodies & Peat bogs.

(Supplementary Table 2) as well as the second highest concentration of TP and the third highest one of TN.

3.2. ASVs distribution

After rarefaction at the minimum sequencing depth, 28,629 bacterial ASVs were analysed. Most ASVs (78 %) were unique to a single sample, highlighting the variability and uniqueness of bacterial communities in such freshwater bodies. Specifically, only 12 % and 4 % of ASVs were detected in two and three samples, respectively, whereas the 6 % was distributed in four or more samples.

Consistent with the generally low percentages of shared ASVs between all samples, only 8.2 % of ASVs were common to the two habitats (Fig. 3A). Indeed, 58.7 % and 33.1 % of ASVs were found to be

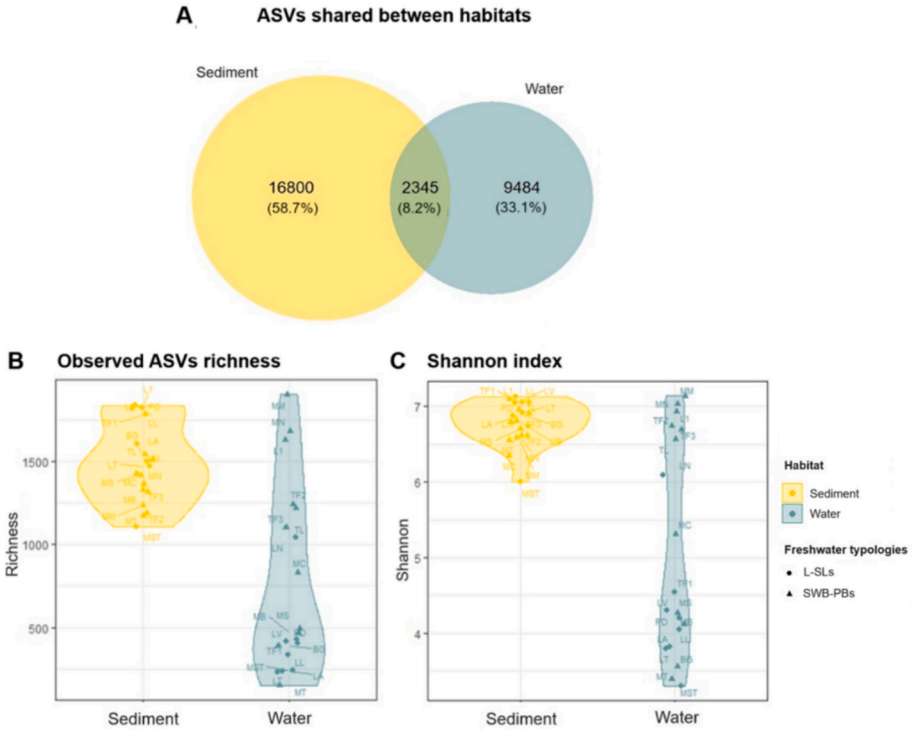


Fig. 3. Distribution of ASVs across the surface sediment and water samples. (A) Euler diagram showing the number of unique and shared ASVs between sediment and water samples. (B) Violin plots of the observed ASVs richness in water and sediment samples. (C) Violin plots of Shannon index values in water and sediment samples.

exclusively present in sediment and water samples, respectively. When considering each sampling location individually, the percentages of shared ASVs between water and sediment habitats ranged between 0.8 % and 11.5 % of the total number of ASVs of each location (Supplementary Table 3). Notably, also the total relative abundances of such shared ASVs resulted to be heterogeneous in the whole set of samples, ranging from 0.7 % to 87.8 %. However, no particular differences were highlighted among freshwater typologies. With some exceptions, the shared ASVs showed higher relative abundances in the water samples (Supplementary Table 3).

ASVs richness showed wider heterogeneity in water (range, 151–1903 ASVs) than in surface sediment samples (1105–1837 ASVs) (Fig. 3B). The difference in ASVs richness between water (median: 468) and sediment (median: 1469) samples was statistically significant (KW test, $p = 0.0002$). The same result was obtained for the Shannon index (Fig. 3C), which showed higher and less sparse values in sediment than in water samples. In particular, the dataset could be subdivided into two groups: one group showing lower ASVs richness (< 500) in water compared to sediment samples (> 1000), and one minor group displaying closer α-diversity values between water and sediment samples. This second group was represented by the shallow Lake Nembia (LN) and a subset of SWB-PBs consisting of 4 furrows into peat bogs and 2 small ponds (TL, TF2, TF3, L1, MM, MN). These latter sites will be referred to as 'peat bogs-like subgroup' in the rest of the manuscript.

In the Whittaker's rank abundance curves (Supplementary Fig. 2) species evenness is reflected in the shape of the fitting line. In general, the log-normal model was found to better fit the rank distribution of ASVs. However, 7 water samples (MB, MST, BG, MS, PD, MC, LT) showed a slightly better fit to the Mandelbrot model. The log-normal fitting was statistically significant in 30 out of 38 samples (KS test, $p < 0.03$). In general, planktic communities were characterized by steeper rank abundance curves than benthic ones. In Lake Nembia and in the peat bogs-like subgroup, the Whittaker's plots computed for the water and sediment samples showed similar shapes.

In the water compartment, 63 % of |βNTI| values were > 2. In particular, 20 % of βNTI were > 2, while 43 % < −2. In the sediment habitat, the fraction of |βNTI| > 2 was even higher, reaching the 78 % of the total pairwise comparisons (18 % of values >2 and 60 % < −2; Supplementary Fig. 3A). However, while the median βNTI in water was −1.1, the median βNTI in sediment was −3.4; the median βNTI reached the lowest value in the sediments of L-SLs (−6.4; Supplementary Fig. 3B). It is important to notice that the range of variability of the βNTI for all pairwise community comparisons was high, ranging from −14.7 to 11.2 for water habitats and from −13.5 to 21.1 for sediment habitats.

3.3. Bacterial ASVs distribution: taxonomic assignments

The 97 % of bacterial ASVs (with at least a phylum-level assignment) were classified at least up to the class level, followed by ASVs classified at the order (85 %), family (63 %), and genus level (32 %). Focusing on the phylum-level assignments (58 phyla) and considering the 10 most abundant phyla (Fig. 4A), a general distinct taxonomic composition between water and sediment samples was highlighted, supported also by the evaluation of the 5 most abundant ASVs of each sample (Supplementary Table 4). Overall, these 10 most dominant phyla contributed to a fraction of ASVs comprised between 91.3 % and 99.7 % for water and between 78.7 % and 96.2 % for sediments. In general, water samples were dominated by Proteobacteria (median: 35 %), Bacteroidota (17 %), Actinobacteria (7 %), Verrucomicrobiota (7 %), and Patescibacteria (4 %), while surface sediment samples were dominated by Proteobacteria (28 %), Bacteroidota (13 %), Chloroflexi (10 %), Verrucomicrobiota (10 %), Acidobacteriota (9 %), and Planctomycetota (7 %).

The relative abundances of 5 of these 10 most abundant phyla were significantly different in the two habitats (W test, $p < 0.04$; Fig. 4B). Sediment samples were statistically more enriched in Acidobacteriota, Chloroflexi, and Planctomycetota, while water samples were characterized by wide heterogeneity in the distribution of Actinobacteriota and Patescibacteria related to the different freshwater typologies. In particular, the water samples of L-SLs were enriched by Actinobacteriota (W test, $p = 0.01$), up to 36 % of total abundance (LA), while the water

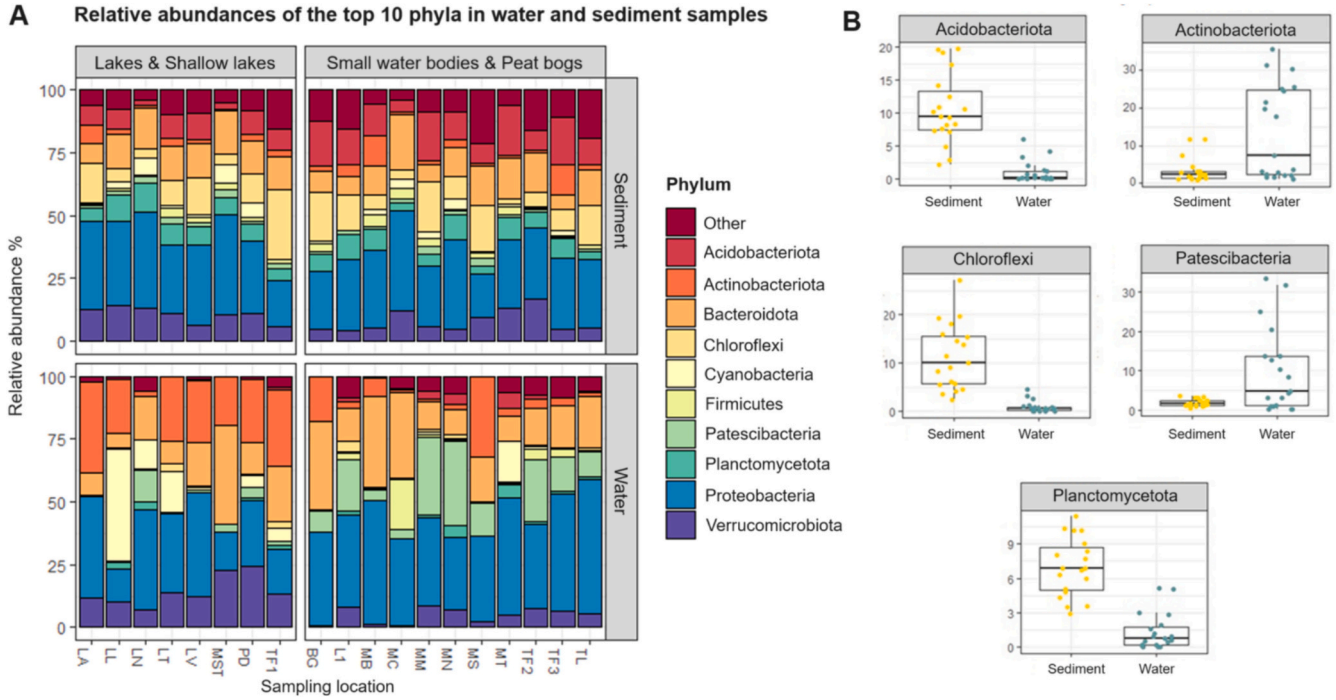


Fig. 4. (A) Bacterial composition of water and sediment samples; the relative abundances of the 10 most abundant phyla are shown. Samples are grouped according to the type of habitat and freshwater typology. (B) Boxplots of the distribution of the relative abundances of the phyla Acidobacteriota, Actinobacteriota, Chloroflexi, Patescibacteria and Planctomycetota in sediment and water samples; only the phyla in (A) with statistically significant differences in relative abundance in water and sediment samples were considered (Wilcoxon rank sum test, $p < 0.01$).

samples of SWB-PBs were characterized by higher abundances of Patescibacteria (W test, $p = 0.005$), up to 33 % of total abundance (MN).

In addition to the peculiarities of the two habitats, it is noteworthy that some locations showed high similarity in both their planktic and benthic bacterial community structures (cf. 3.2, Fig. 4), sharing also abundant ASVs. In particular, among the L-SLs samples, Lake Nembia (LN) exhibited the highest percentage of shared ASVs between the two habitats (11.5 %), which contributed to high fractions of the total relative abundances (47 % in water, 33 % in sediments; Supplementary Table 3). In water, the most abundant of such shared ASVs were two cyanobacteria (*Chamaesiphon* and *Cyanobium)* and one Proteobacteria (not further classified), while in sediments they were *Polymorphobacter* (Proteobacteria), *Luteolibacter* (Verrucomicrobiota), and *Cyanobium*. Considering the SWB-PBs category, the small pond of Malga Ciocca (MC) showed the highest percentage of shared ASVs (11.1 %), which were related to 63.5 % of the total abundance in water, and 27.5 % in sediments. In this case, the most abundant shared ASVs were *Pseudomonas* (Proteobacteria) and *Acinetobacter* (Proteobacteria) in both pelagic and benthic habitats.

3.4. Bacterial ASVs distribution: beta-diversity

Differences between sediment and water bacterial communities were summarized in the NMDS analysis (stress = 0.17, Fig. 5A). A statistically significant separation between water and sediment samples was observed (adonis test, $p = 0.001$), revealing planktic and benthic bacterial communities with distinct community structures. The vector fitting analysis computed on the NMDS ordination showed significant relationships ($p < 0.01$) between the distribution of these samples and the relative abundances of 7 out of the 10 most abundant phyla (Fig. 5A). Furthermore, water samples exhibited more dispersion and dissimilarity compared to sediment samples, which were more closely located. The BC dissimilarity values for water samples ranged between 0.56 and 0.99, whereas for sediment samples values were between 0.78 and 0.99.

The higher heterogeneity in water samples was investigated in a second NMDS analysis (Fig. 5B), which showed a clear separation between the peat bogs-like subgroup identified in section 3.2 (TL, TF2, TF3, L1, MM, MN) and the rest of the dataset, except for LN and MT, which were displayed far from the two groups. The vector fitting analysis computed on the NMDS ordination of the water samples (stress = 0.13, Fig. 5B), showed how such a different ordination of the peat bogs-like subgroup was associated ($p < 0.01$) with a major abundance of the Patescibacteria phylum. The configuration was also significantly related to other phyla, including Actinobacteriota in the direction of the L-SLs samples (congruent with the results of the section 3.3). Regarding water chemical and physical variables, only pH and SiO_2 showed significant relationships with this NMDS configuration ($p < 0.01$). A significant association of the peat bogs-like subgroup with a higher concentration of SiO_2 was detected (cf. Fig. 2B).

NMDS configurations were also supported by the hierarchical cluster analysis (HCA; Supplementary Fig. 4A), which showed a separation of the L-SLs water samples from their associated sediments, while several water samples of SWB-PBs clustered together with the respective sediments. Moreover, the HCA showed an evident separation between the waters of L-SLs and SWB-PBs, with the only exception of LN (Supplementary Fig. 4B).

Furthermore, statistically significant associations of bacterial community structure with environmental and chemical variables were found testing the correlation between the BC dissimilarity matrix of water samples and the Euclidean distance matrix computed based on all the log-transformed chemical variables measured (Mantel test, $p = 7e\text{-}04$).

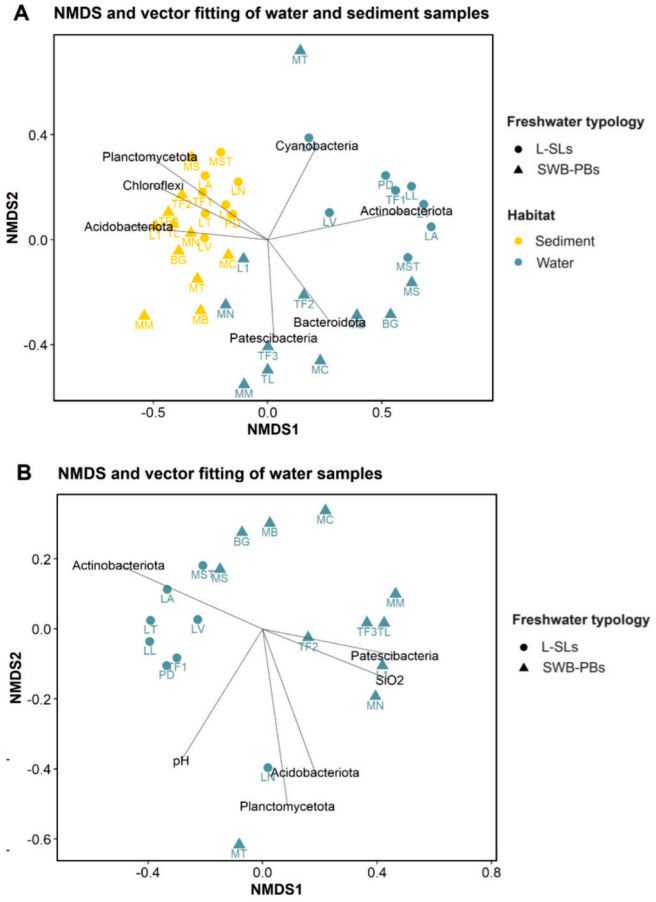


Fig. 5. (A) Non-metric multidimensional scaling (NMDS) ordination of water and sediment samples based on Bray-Curtis (BC) dissimilarity computed on double square root-transformed abundances of ASVs and vector fitting analysis based on the 10 most abundant phyla, reporting the statistically significant phyla ($p < 0.01$). Sample labels are colored differently according to habitat, and shaped according to the freshwater typology. (B) NMDS analysis of water samples (shaped differently according to the freshwater typology) and vector fitting analysis based on the 10 most abundant phyla and the water chemical variables ($p < 0.01$).

3.5. Bacterial ASVs distribution: ANCOM-BC analysis

The ANCOM-BC analysis was performed on 286 genera, which were present in at least 20 % of the dataset (8 samples), focusing only on the 32 % of ASVs assigned to a genus. ANCOM-BC results showed that 41 selected genera were significantly differentially distributed in water and sediment samples ($p < 0.001$; Fig. 6), supporting the different phyla distribution between the two habitats highlighted in the section 3.3. The total relative abundances of the genera varied between water bodies, especially considering water habitats (Supplementary Fig. 5).

Water and sediment samples were characterized by differently abundant genera belonging to Proteobacteria and Verrucomicrobiota, including *Limnohabitans* (Proteobacteria, 45 ASVs), which was detected in several water samples (0–21.5 %). The ASVs related to this genus constituted >17 % of the total relative abundance in 3 small water bodies, while resulting absent in almost all the sediments. Another example is *Chtoniobacter* (Verrucomicrobiota, 182 ASVs), which was detected in all sediment samples (0.15 %–2.7 %) and in several water samples with lower abundances (< 0.6 %).

Focusing on the different abundant phyla, the bacterial benthic community of the Biosphere Reserve was more enriched in genera belonging to Desulfobacteriota, Chloroflexi, Nitrospirota, Planctomycetota, and Acidobacteriota. Among them, ASVs assigned to *Geobacter* (Desulfobacteriota, 42 ASVs) were spread in almost all the sediments, even if covering low relative abundances (0–1.2 %). Then, ASVs related to *Anaerolinea* (Chloroflexi, 124 ASVs) and *Bryobacter* (Acidobacteriota,

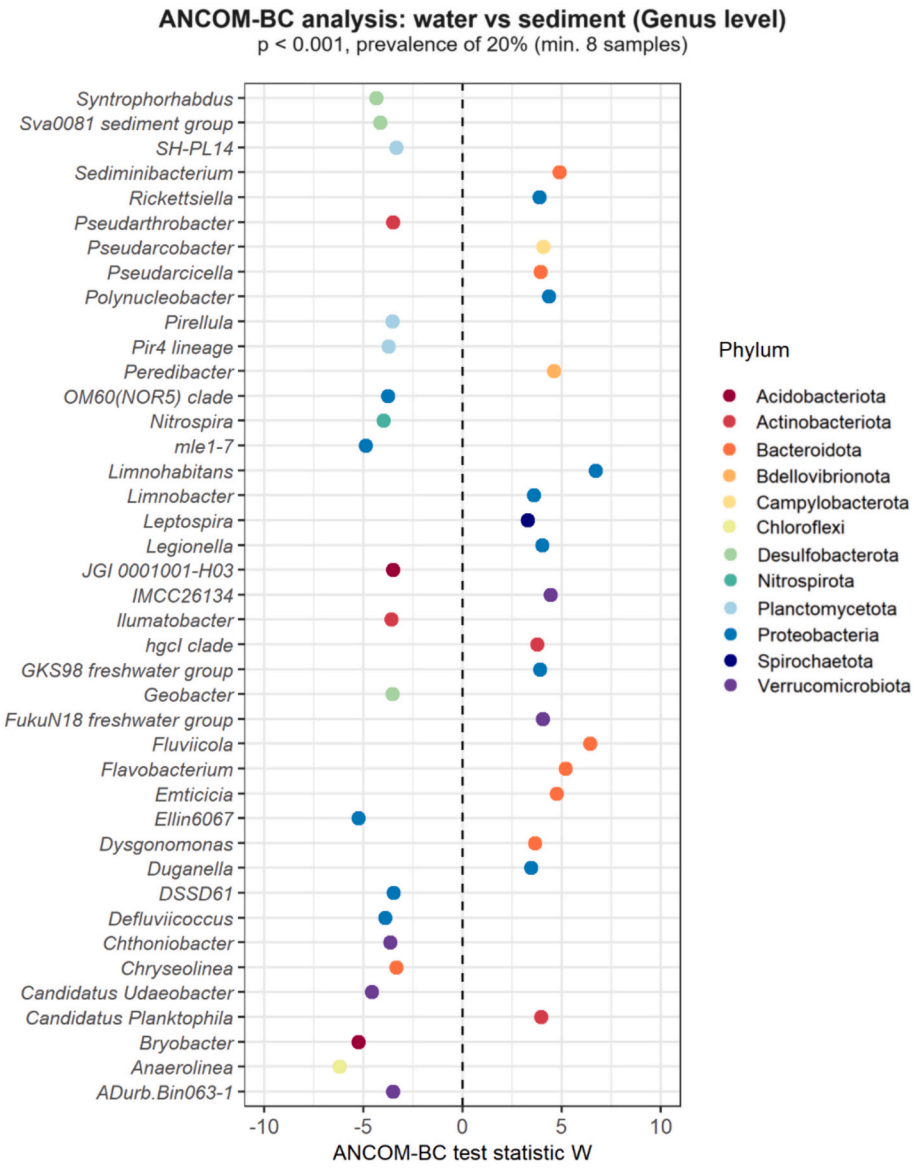


Fig. 6. ANCOM-BC analysis testing significant differences in the abundances of genera ($p < 0.001$) between waters and surface sediments. Only genera with a minimum prevalence of 20 % were considered (min 8 samples). Positive W statistic values are related to genera that are differentially enriched in water (right panel), while negative W statistic values indicate a higher enrichment in the sediment (left panel). The genera are colored differently according to their phylum.

92 ASVs) were also detected in almost all the sediments with variable abundances (0–2.7 % and 0.02–2.5 %, respectively). Other examples of abundant genera in benthic habitats were *Pirellula* (Planctomycetota, 259 ASVs, 0.06–4.0 %), and *Nitrospira* (Nitrospirota, 42 ASVs, 0–1.7 %). These latter five genera were almost absent in most of water samples or detected at low abundances in just a few cases. A notable exception is *Pirellula*, whose ASVs covered 3.8 % of the waters of a pasture pond (MT). Then, also a few genera from other phyla (Actinobacteriota and Bacteroidota) were found to be differently abundant in the sediments, but they were not among the most dominant ones (< 0.7 %).

On the other hand, the planktic bacterial community was more enriched in two genera from the Actinobacteriota phylum (Sporichthyaceae family): *hgcI clade* (17 ASVs, 0–13.6 %) and *Candidatus* Planktophila (8 ASVs, 0–9.6 %). Both were detected in almost all the L-SLs samples, with extremely variable abundances. In particular, the *hgcI clade* covered >12 % of the total abundance of three L-SLs locations (LA, LT, LV). However, they were generally absent in the SWB-PBs water samples, with just two exceptions (< 2.2 %), and they were also not detected (or just in traces) in the sediment samples. Then, six genera belonging to Bacteroidota were found to be more abundant in the open waters, including *Flavobacterium*. This genus was represented by numerous ASVs (414) in all the water samples (0.4–22.2 %) and was especially dominant (> 17 %) in 3 ponds (BG, MB, MC) and 1 lake pond (MST). *Flavobacterium* ASVs were also found in all the sediment samples but with lower abundances (0.1–4.7 %). Finally, 3 genera related to Campylobacterota, Spirochaetota, and Bdellovibrionota were significantly more abundant in the waters of the Biosphere Reserve.

It is worth noting that Patescibacteria representatives were missing in this analysis. This is because only 1.3 % of the total Patescibacteria ASVs were assigned up to the genus level (*TM7a* and *Candidatus* Saccharimonas), leaving the 99.7 % excluded from the ANCOM-BC analysis. In particular, the most abundant class was Parcubacteria, with approximately 50 % of its related ASVs being classified only up to the order level. Parcubacteria ASVs were particularly dominant in 8 SWB-PBs samples as well as in one shallow lake (> 6.4 %, max. of 30.2 %). In sediments, such ASVs always covered low relative abundances (0.2–2.3 %).

3.6. Bacterial ASVs distribution: focus on phylum Cyanobacteria

In the whole Biosphere Reserve, 426 ASVs were assigned to phylum Cyanobacteria. Differently from the analysis of the bacterial community, no significant differences in the cyanobacterial ASVs richness were found between water and sediment samples. In particular, cyanobacterial richness values ranged between 1 (TL) and 83 (LN) in sediment samples (median: 17 ASVs), and between 1 (MST) and 31 (LN) in water ones (median: 8 ASVs). Regarding taxonomy, 51 % of ASVs were classified up to the genus level (64 genera).

Water and sediment samples were mainly dominated by ASVs belonging to the Cyanobacteriales and Synechoccales orders (Fig. 7A). Focusing on the 10 most abundant genera (Fig. 7B), *Cyanobium* was the one with the highest mean relative abundance and the highest occurrence (Table 1), being detected in 23 samples and reaching a maximum relative abundance of 16 % (water of MT, a SWB-PB). *Candidatus* Obscuribacter was detected in 14 samples, although with a low relative abundance (maximum of 0.3 %), while *Pseudanabaena* was detected in 13 samples, with a maximum relative abundance of 1.3 %. All the other cyanobacterial genera identified were present in ≤11 samples (Table 1). Among the potentially toxigenic cyanobacteria of particular importance in the Alpine region, the genus *Planktothrix* was assigned to 15 ASVs (both in water and in sediment compartments) and detected in 11 samples, while *Tychonema* was assigned to 6 ASVs detected in 10 samples (only sediments).

In general, cyanobacterial ASVs were not among the most abundant and widespread taxa in the Biosphere Reserve. This was also in part supported by the detection of low concentrations of anatoxin-a (ATX) and microcystins (MCs) in only 5 out of 19 water samples investigated (Supplementary Table 5), which were mainly L-SLs. Even if not all these water samples were among the ones with the highest cyanobacterial abundances, they all showed ASVs assigned to potentially toxigenic cyanobacteria, such as *Microcystis* and *Planktothrix* (Fig. 7B, Supplementary Table 6).

Two L-SLs deserve special mention. First, Lake Nembia (LN) was the location with the highest richness in both water and sediment habitats, although with modest cyanobacterial relative abundances (4 % and 10 %, respectively). Waters of this shallow lake were dominated by *Chamaesiphon* and *Cyanobium*, and by a wide variety of cyanobacterial ASVs in the sediments, including members of the Microcystaceae family, and *Nodosilinea*, *Pseudanabaena* and *Cyanobium* (Supplementary Table 6). None of the cyanotoxins considered in the analysis were detected in this shallow lake. Then, Lake Ledro (LL) exhibited the highest relative abundance of total pelagic cyanobacterial ASVs (44 %), and it was also the site with the highest concentration of cyanotoxins in the water (Supplementary Table 5), showing the presence of both ATX and MCs (especially the congeners MCHTyR and MCRRdm). It's noteworthy that 38 % of the pelagic abundance in LL was assigned to the *Planktothrix* genus, represented by 2 different oligotypes corresponding to the two most abundant oligotypes previously identified in a large-scale metabarcoding survey in the Alpine region, defined as oligotypes "A" and "G" (Salmaso et al., 2024). Oligotype "A" contributed to 29 % of the total pelagic abundance in LL and was also found in traces in the water of MM and MN ponds.

Table 1
Occurrences of the 10 most abundant cyanobacterial genera identified, considering the total number of samples in which each genus was identified (Total column). Their occurrences considering each habitat separately are also reported (Water and Sediment columns). The last two columns report the maximum relative abundance of each cyanobacterial genus (Max rel. ab.), as well as the number of ASVs assigned (Num. ASVs).

Cyanobacterial genus assigned	Occurrence			Max rel. ab.	Num. ASVs
	Total	Water	Sediment		
Cyanobium PCC-6307	23	13	10	16.1 %	43
Candidatus Obscuribacter	14	7	7	0.3 %	5
Pseudanabaena PCC-9006	13	5	8	1.3 %	15
Planktothrix NIVA-CYA 15	11	5	6	37.9 %	15
Tychonema CCAP 1459-11B	10	0	10	1.3 %	6
Phormidium SAG 37.90	9	4	5	0.1 %	6
Microcystis PCC-7914	7	4	3	0.6 %	3
Nodosilinea PCC-7104	7	1	6	0.5 %	6
Leptolyngbya SAG 2411	7	1	6	0.1 %	2
Nostoc PCC-73102	6	1	5	0.4 %	6

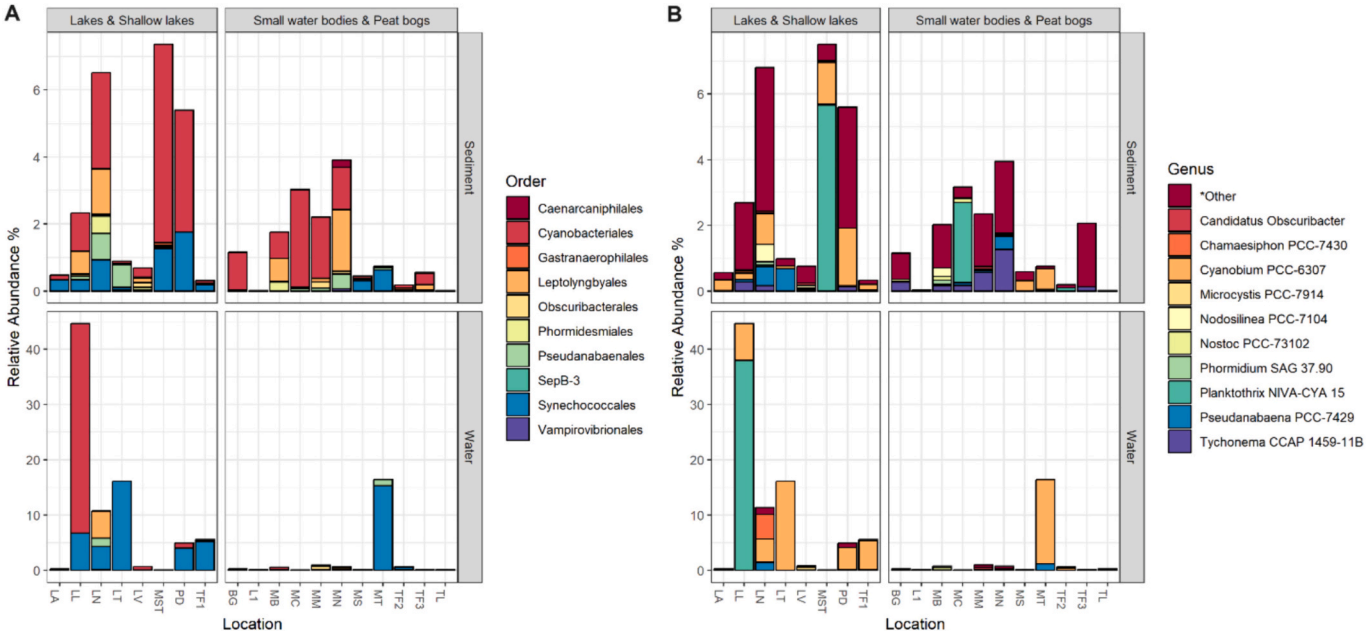


Fig. 7. Relative abundances of (A) cyanobacterial orders and (B) the 10 most abundant genera in the water and sediment samples. 'Other' includes both other classified and unclassified genera. Samples are grouped according to the habitat and freshwater typology. The y-axis scales are not the same for sediment and water habitats.

4. Discussion

This work investigated the differences between bacterial communities of water and surface sediment habitats across a wide physiographic gradient of freshwater bodies, ranging from small ponds and pools to deep lakes. The results provided an overall picture of the distribution of bacterial communities in a number of small Alpine water bodies that are not included in monitoring programmes, but which represent important sites of biodiversity in open waters and benthic habitats.

4.1. Distribution of bacterial ASVs in the Biosphere Reserve: community diversity of water and surface sediments

Water samples showed considerable heterogeneity in terms of α-diversity, highlighting how the structures and compositions of planktic bacterial communities are highly responsive to the different water body features. On the other hand, the bacterial composition of surface sediment samples showed greater consistency, with similar ASVs richness and Shannon diversity throughout the entire Biosphere Reserve. Both habitats, as well as each location, showed high proportions of rare and low abundance ASVs exclusively present in one (or few) samples. Such rare ASVs highly contributed to increasing the biodiversity and the richness of the samples, especially in the benthic habitats, but only partially contributed to the characterization and functioning of the community due to their stochastic nature (Salmaso et al., 2024; Zhang et al., 2016).

In general, sediment samples showed higher α-diversity than water ones. Several water samples were characterized by steeper Whittaker's rank abundance curves, indicating assemblages with high dominance of some abundant ASVs, while sediment samples showed flatter curves, indicating higher evenness and a major contribution of moderately abundant and rare ASVs. The high α-diversity in sediments could be attributed to the results of a long-term process of sediment erosion and accumulation from the watershed (Liu et al., 2018; Ren et al., 2019), and deposition of microbes and organic matter from the upper water layer, which provided a matrix of complex nutrients and solid surfaces for microbial growth (Wang et al., 2012). While the water compartment works as a carrier of biotic and abiotic elements, the sediments act as sinks for nutrient and carbon cycling (Battin et al., 2009; Li et al., 2023). The degradation of organic matter and aerobic and anaerobic respiration processes require specialized bacterial consortia that help to maintain the functionality of these habitats. Thus, uppermost sediments result in a global biogeochemical hotspot with high heterotrophic activity (Sauer et al., 2022; Wurzbacher et al., 2017), providing more niches for bacterial growth. In line with our results, bacterial communities in surface sediments showed richness and diversity exceeding that of water samples in several studies on different freshwater sources, such as lakes (Gao et al., 2023; Ren et al., 2019), shallow lakes (Zhong et al., 2023), thermokarst lakes (Ren et al., 2022), aquaculture ponds (Dai et al., 2021), rivers (Liu et al., 2018), and reservoirs (Qin et al., 2021).

However, this pattern of different richness between water and sediment samples was not consistent throughout the Biosphere Reserve. A specific group of water bodies, indicated as a 'peat bog-like subgroup', was particularly interesting as it showed high and similar richness in both water and sediment habitats, as well as similar evenness, according to their Whittaker's rank abundance curves. This group included 4 furrows into peat bogs and 2 small pools, representing the smallest and shallowest water bodies in the whole dataset and showing vegetation on their bottom. Moreover, these water bodies were also characterized by higher concentrations of SiO_2 in the water, which could be related to their small depths as well as to the degradation of organic matter and plant tissue (Harriss, 1967). In this type of shallow environment, the boundary between the water column and sediment is usually not well distinct (McMaster and Schindler, 2005), and even non-intense mixing events can lead to effective and constant resuspensions of sediment particles and cells, increasing the homogenization between the two compartments.

In addition to the 'peat bog-like subgroup', a similar pattern of richness was observed between the water and sediment habitats in a shallow lake (Lake Nembia, LN), which showed the highest percentage of shared ASVs. In part, this could be due to the results of wind-driven resuspensions and intense mixing events. Indeed, resuspended sediment bacteria could remain in the water compartment for days (Shao et al., 2013; Zhong et al., 2023). However, this question will remain open unless new studies will investigate this shallow lake from a temporal perspective.

The comparable richness pattern of water and sediment habitats in such a group of samples was not always linked to a higher percentage of shared ASVs. Indeed, even though some samples (like the furrows into peat bogs) showed similar richness between the two interfaces, they hosted different communities with a high fraction of exclusive ASVs. Nevertheless, a large proportion of the differences were due to ASVs or even genera that occurred at very low frequency in the entire sample set, suggesting, as highlighted in several other investigations, the high contribution of rarest taxa in shaping biodiversity. Nevertheless, rare ASVs occurring at very low frequencies and generally at low abundances cannot be of high discriminatory value (Lee et al., 2021; Salmaso et al., 2024; Zhang et al., 2016).

The community assembly analysis showed that the influences of deterministic and stochastic processes were different in the two habitats, even if a major role of deterministic processes was highlighted in both. βNTI values >2 (or <-2) indicated that phylogenetic turnover between a pair of communities was greater (or less) than expected by chance under random community assembly and governed primarily by niche-based processes and environmental filtering (Stegen et al., 2012). βNTI values <-2 were particularly observed in sediment habitats, especially if considering the sediments of L-SLs alone. This could suggest that the presence of similar environmental conditions in sediment across individual communities may have caused the occurrence, if not the same species, of phylogenetically and ecologically similar species. Nevertheless, the high dispersion of βNTI in the whole set of samples, highlighted the heterogeneity of the habitats considered in this analysis, particularly in the SWB-PBs water bodies.

4.2. Taxonomic composition of planktic and benthic bacterial communities

Sediment and water samples harboured different bacterial communities. Both habitats were characterized by high dominance of Proteobacteria, together with lower abundances of Verrucomicrobiota and Bacteroidota, but several other phyla resulted to be differentially abundant in the two compartments.

The benthic bacterial communities were mainly enriched with ASVs related to the Acidobacteriota, Chloroflexi, Planctomycetota, and Desulfobacterota phyla, which gather bacteria associated with several biogeochemical and degradative processes occurring in the sediments. The dominant presence of Acidobacteriota in freshwater sediments has been widely reported (Gao et al., 2022; Newton et al., 2011; Wang et al., 2012; Zhong et al., 2023). Acidobacteriota are an extremely diverse and ubiquitous phylum of mainly uncultured bacteria, especially abundant and dominant in soil habitats, but also found in aquatic systems and harsh environments, whose ecological knowledge is still fragmentary (Huber et al., 2022). In the Biosphere Reserve, an interesting acidobacterial genus differentially abundant in the sediments was *Bryobacter,* which embraces acidotolerant, aerobic, chemoorganotrophic bacteria, inhabiting acidic wetlands and soils. The only currently described species was isolated from a boreal peat bog (Dedysh, 2019). Besides Acidobacteriota, another peculiar phylum of the benthic community was Chloroflexi, which comprises a metabolic diverse group of microorganisms including aerobic and anaerobic photolithoautotrophic and chemoorganoheterotrophs bacteria, commonly detected in

sediments, soil, hot springs, and wastewater sludge (Hug et al., 2013; Speirs et al., 2019). Their greater abundance in freshwater sediments, compared to water, has been already reported in a shallow lake (Zhong et al., 2023). The sediments of the Biosphere Reserve resulted enriched also by representatives of Desulfobacterota phylum, which includes bacteria involved in sulphate reduction (Waite et al., 2020), contributing to anaerobic processes and sulphur cycling in sediment. An interesting example is the genus *Geobacter*, a known Fe(III)-reducing bacterium that was isolated from a diversity of sedimentary environments in which iron reduction is an important process (Coates et al., 1996).

Focusing on the bacterioplankton communities, water samples showed higher heterogeneity than sediments, mainly related to the different freshwater typologies. In particular, small water bodies and peat bogs were characterized by higher abundances of Patescibacteria, especially in the peat bog-like subgroup, while lakes and shallow lakes were in general more enriched by Actinobacteriota. The term Patescibacteria refers to a superphylum included in the recently described candidate phyla radiation (CPR; Castelle and Banfield, 2018), characterized by uncultured bacteria with reduced genome size, limited known biosynthetic and metabolic pathways, and small cell size to increase their limited metabolic potential. Patescibacteria have been observed in the pelagic communities and hypolimnia, as well as in anoxic and nutrient-limited groundwaters (Chiriac et al., 2023; Fujii et al., 2022; Tian et al., 2020). Within the Patescibacteria superphylum, the most abundant ASVs in the SWB-PBs group were related to Parcubacteria, which have been identified in a broad range of anoxic environments and indicated to be ectosymbionts or parasites of other organisms (Nelson and Stegen, 2015). David et al. (2021) showed the presence of Parcubacteria and other members of the CPR in some small and shallow freshwater ecosystems, where they resulted highly diverse and locally abundant, also in oxic conditions. Such an important prevalence of Parcubacteria (and Patescibacteria in general) in the bacterioplankton of small water bodies and peat bogs would be interesting to investigate further. On the other hand, the prevalence of Actinobacteriota in the bacterioplankton of L-SLs was expected, since several studies found Actinobacteriota to be among the most predominant groups in freshwater habitats (Gao et al., 2022; Ghai et al., 2014; Salmaso et al., 2018; Zhong et al., 2023). This phylum includes heterotrophic and generally aerobic bacteria with a wide and variable spectrum of morphologies, physiologies, and metabolic properties, including pathogens and plant and gastrointestinal commensals (Ventura et al., 2007). In particular, these bacteria are usually dominant in the surface water, and their abundance often decreases with decreasing oxygen concentrations (Newton et al., 2011). In the waters of the Biosphere Reserve, such a high abundance of Actinobacteriota in the L-SLs samples was mainly related to the dominance of the Sporichtyaceae family (order Frankiales), whose abundance covered almost 25–30 % in some deep and shallow lakes. Besides Actinobacteriota, the planktic communities of the L-SLs of the Biosphere Reserve, were also characterized by high abundances of *Limnohabitans* (β-Proteobacteria), a fast-growing bacterium with high rates of substrate uptake that is well known to have an important role in freshwater bacterioplankton communities (Kasalický et al., 2013; Newton et al., 2011). *Limnohabitans* was also one of the most abundant taxa detected in Lake Garda (Salmaso et al., 2018), a deep subalpine lake geographically close to the Biosphere Reserve.

Another important phylum to discuss is Cyanobacteria, which was detected in almost all water and sediment samples, with no significant differences in the distribution between the two habitats. However, cyanobacteria were not among the most widespread taxa in the Biosphere Reserve and their relative abundances were generally low, especially in the small water bodies. The most frequent cyanobacterial ASVs were assigned to the non-toxic and non-bloom forming genus *Cyanobium* (order Synechococcales), which belongs to the functional group of picocyanobacteria (< 2–3 μm) able to proliferate and adapt to various environmental conditions (Cabello-Yeves et al., 2018; Callieri et al., 2022; Salmaso et al., 2024). In addition to being the most frequent taxa in the Biosphere Reserve, they were often among the most abundant ones. They were dominant in the water sample from the only small water body site (an artificial pasture pond) where cyanobacteria covered a discrete fraction of the total pelagic community. However, among lakes and shallow lakes, two exceptions to this trend of low cyanobacterial abundance were highlighted. First, the shallow Lake Nembia once again demonstrated its unique characteristics, being the site with the highest cyanobacterial ASVs richness in both water and sediment habitats. Then, the second exception was Lake Ledro, where both microcystins and anatoxins were detected, and where ASVs assigned to *Planktothrix* made up more than a third of the pelagic bacterial community. Genus *Planktothrix*, which consists mainly of free-floating filamentous solitary species but also includes benthic strains, has been extensively studied due to its frequent red blooms in temperate freshwater ecosystems (Pancrace et al., 2017). Its presence in Lake Ledro has already been assessed and investigated (Boscaini et al., 2017; Cerasino and Salmaso, 2012). In particular, Lake Ledro has been characterized by the presence of *Planktothrix rubescens*, a bloom-forming species able to synthesize different microcystins with prevalence of the MC-RRdm variant (Cerasino et al., 2017), in line with our results. In addition, *Planktothrix* ASVs were found with modest abundance also in the sediments of a shallow lake pond (MST), as well as in a high-altitude pond (MC). Finally, another relevant toxigenic cyanobacterial genus detected was *Tychonema* (order Oscillatoriales), which includes filamentous planktic and benthic species potentially able to produce anatoxin-a (Shams et al., 2015). *Tychonema* ASVs were well distributed across the Biosphere Reserve, since they were detected in half of the sediment samples, in both freshwater typologies. It is important to highlight the potentiality of *Tychonema* (as well as other benthic Oscillatoriales) to constitute benthic mats producing the same range of anatoxins as planktic species, posing a less evident threat for animals and humans (Fastner et al., 2018; Salmaso et al., 2024). Nevertheless, no specific information about cyanotoxins in the sediments was collected in this study.

To conclude, the benthic communities resulted in general to be more conserved across the Biosphere Reserve and less influenced by the different freshwater typologies' features. They all showed high bacterial richness and some general concordant patterns of bacterial phyla compositions. On the other hand, the planktic communities better reflected, with their greater heterogeneity, the wide physiographic range of freshwater bodies investigated in this work. This opened also new questions about the different abundances of some bacterial taxa (e.g. Parcubacteria), as well as about the ecological role they could play in these small water bodies and peat bogs' bacterial communities.

4.3. Main constraints of the study and perspectives

Each water body was sampled only once, without any replication in time and space. This let us get only a single and unique frame of each water body, limiting the possibility of expanding the analysis in a temporal perspective and resulting in a limited number of samples that challenged the statistical analysis. Moreover, the lack of multiple samples in the timeline limited the ability to account for potential disturbance parameters, such as wind speed and wave force that might have influenced mixing processes, as well as possible localized pollution and contamination sources. This limitation is relevant also considering the wide variety of water bodies included in this investigation, which ranges from 'lakes and shallow lakes' (L-SLs; deep lakes, shallow lakes, and lake ponds) and 'small water bodies and peat bogs' (SWB-PBs; natural and pasture ponds, pools and furrows into peat bogs). Each broad category includes borderline habitats and a wide spectrum of variations in terms of depths, size, temperatures, elevation, water chemical variables, and water supply, which contribute to explaining the inter-site variability. In this respect, differences between water bodies and habitats should be interpreted with caution, as they only represent differences between sites in a limited time window. In the future, further research

approaches should be based on sampling that at least covers the main seasons and better accounts for potential spatial heterogeneity in water bodies.

To conclude, it should be also highlighted the limitations related to the use of short 16S rRNA reads in the classification of bacterial ASVs. Incorrect or questionable taxonomic assignments may occur with the RDP classifier, even considering a 95 % bootstrap threshold. The assignments reflect the highest level of taxonomic similarity found in the reference databases, which remain incomplete and lack a significant proportion of unclassified species or undetermined bacteria detected in environmental DNA analyses, especially if we consider poorly studied environments as the ones of this work. Moreover, the genetic information provided by amplicon sequencing analysis based on short reads is limited, and related bacterial genera may have 16S rRNA gene sequences with such a high degree of similarity that they are indistinguishable by considering only short fragments. For these reasons, ecological interpretation of the data was also hampered by the low proportion of ASVs classified up to the family and genus levels.

5. Conclusions

Our metabarcoding study revealed interesting information about the bacterial communities of both planktic and benthic habitats in a wide physiographic range of freshwater bodies, including small pools, ponds, pasture ponds, lake ponds, shallow lakes, and lakes. Each location showed its uniqueness in terms of water chemistry, and pelagic and benthic bacterial communities. However, similarities between the same habitats as well as among the same freshwater typologies were highlighted. We found statistically significant differences between water and surface sediment habitats in terms of bacterial richness and community taxonomic composition, also highlighting the presence of several differently abundant genera. Moreover, differences between freshwater typologies were also shown, especially in the planktic habitats, where bacterial communities exhibited differences in terms of richness and relative abundances of the main phyla according to the different freshwater typologies. On the other hand, benthic bacterial communities were found to be more similar and homogenous across the entire Biosphere Reserve and less influenced by the diverse range of physiographic features than the planktic counterpart.

Despite the limitations of the work and the fact that more data should be collected to draw more general conclusions, our study revealed important findings related to the bacterial ecology of these little-studied freshwater environments, highlighting their rich bacterial biodiversity and their high conservation value. This opens perspectives for further investigating their bacterial communities, also by applying other technologies such as full-shotgun metagenomics, to gain a better knowledge of their functional and ecological roles. A better understanding of the relationships between the differentially abundant bacteria and the diverse freshwater typologies, as well as a better characterization of the highly veiled diversity of bacterial taxa without clear similarity with the 16S rRNA gene reference databases, could provide interesting insights into the ecological roles of these peculiar freshwater and small water bodies.

CRediT authorship contribution statement

Sara Vettorazzo: Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Adriano Boscaini:** Writing – review & editing, Methodology, Conceptualization. **Leonardo Cerasino:** Writing – review & editing, Methodology. **Nico Salmaso:** Writing – review & editing, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw sequences have been deposited to the European Nucleotide Archive (ENA) with study accession number PRJEB74662.

Acknowledgements

The research was financially supported by Alpi Ledrensi e Judicaria, Riserva di Biosfera UNESCO, Programma sull'uomo e la biosfera (MAB). The analysis of the data was carried out in the framework of the National Biodiversity Future Centre (NBFC) funded by the Italian Ministry of University and Research and received funding from the European Union Next-GenerationEU (PNRR, Mission 4 Component 2, Investment 1.4, D. D. 1034 17/06/2022, Project CN00000033). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them. The authors want to thank the staff of MUSE - Museo delle Scienze (Trento) and of Agenzia Provinciale per la Protezione dell'Ambiente (APPA, Trento) for the support in field sampling and photo collection, and the staff of the Hydrochemistry and Sequencing Platforms at FEM, for the water chemistry and sequencing analysis. The authors want also to thank the referees for the insightful comments and suggestions that helped improve the quality of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.176495.

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