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Water and Sediment Bacterial Communities in a Small Mediterranean, Oxygen-Stratified, Saline Lake (Lake Alboraj, SE Spain)

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Abstract: Lake Alboraj, located in southeast Spain, was declared natural Microreserve and included into European Natura-2000 Network due to its contribution to environmental heritage. Unfortunately, the ecological status of the lake has changed dramatically, mainly due to the lowering of water table caused by groundwater abstractions for irrigation. It is a permanent small karstic lake whose surface has reduced in the last decades to nearly the third part of its historical water level. The water column shows a marked seasonal oxycline, that splits an aerobic upper layer (epilimnion) from an anaerobic layer below (hypolimnion). Sequencing 16S rRNA gene amplicons and applying chemical tools at epilimnion, hypolimnion and sediment, showed a clear gradient in the bacterial community structure, which support the co-existence of assimilatory and dissimilatory microbial mediated reactions. Results allows to infer that microbial stratification could provide various physical and chemical environments at different depths in the water column related to biogeochemical reactions providing N-S-C- recycling processes.

Keywords: eutrophic lake; oxycline; bacterial communities; 16S rRNA gene



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1. Introduction

In recent times, there is a growing interest in the study of the role of small water bodies in the ecology, biodiversity and provision of ecosystem services at a basin-scale [1]. These small sized lakes (less than 0.1 km² area) are attracting the attention of current worldwide research due to the active global role on environment dynamics that they play [2]. At the same time, they are highly vulnerable to different temporal and spatial-scale anthropic pressures, as well as to climate change. As a result, small lakes suffer eutrophication processes, water acidification and changes in the water balances, which alter their natural behaviour. Such modifications of the natural behaviour are related to changes in nutrient's dynamics, with the consequent influence in the amount of greenhouse gas-emissions and in the response of the system to water pollution.

Lake Alboraj is included in a Special Area of Conservation (SAC) of the European Natura 2000 Network (code ES4210011), due to ecologic reasons, since the area surrounding the lake maintains the presence of endemic halophytic flora growing on gypsum soils (the only known population of the endangered *Cistaceae* species *Helianthemum polygonoides*). The natural and cultural importance of these ecosystems makes their conservation a matter of environmental awareness by authorities, water users and environmental organisations. In this sense, a responsible use of natural resources is mandatory to guarantee sustainable conditions, which may be easily altered by anthropic pressures nowadays. This also generates a growing concern in the society because Alboraj “Microreserve” contributes to

an important biodiversity heritage at local and national level [3]. Nevertheless, from the 80's of the past century to the present, the ecological status of the lake has changed dramatically mainly due to changes in land uses produced in the hydrogeological boundary of the "Tobarra-Tedera-Pinilla" groundwater body, which formerly contributed to the formation and maintenance of the lake. The most noticeable modifications include the changes from dry-extensive to irrigated-intensive croplands facilitated by groundwater pumping, which has led to a lowering of the lake water table.

Even though some limnological approaches have been addressed on the Lake Alboraj [4–8], studies on stratification dynamics of the water column have recently started. Since 2017, a marked oxycline in the eutrophic lake has been observed [9,10], which can constitute a biogeochemical interface by providing aerobic and anaerobic conditions. The dissolved oxygen stratification patterns may originate different conditions in the environment, at different depths in the water column, that lead to different rates in nutrient recycling. Specifically, the turnover of nitrogen in the water column is highly relevant for assessing eutrophication processes in surface water bodies. In this line, the lake became eutrophic from an initial oligotrophic state due to the overgrowth of benthic cyanobacterial mats, which promoted a progressive burial of its submerged macrophyte meadows [10]. The multiple spatial variations in oxygen, N-species concentration, as well as land occupation, may act akin to dependent factors in the composition of bacterial communities and the processes they carry out [11]. Consequently, it affects the correct functioning of the aquatic ecosystem since it may be significantly altered by the activity of microorganisms. Despite bacteria can play an important role in the water column chemostratification, due to their close involvement in N-S-C speciation via redox interfaces, the study of bacterial populations through specific DNA applications in small lakes remain widely unaddressed [12]. Understanding environmental processes is the first step in forecasting lake evolution and, in the same way, a comprehension of the dynamics of stratification is required to prognosticate the past and the future of lakes [13].

The study of environmental DNA by culture-independent tools in Lake Alboraj represents a starting point for studying the microbial structure involved in the N-S-C cycles. In this sense, both the eutrophic character of the small lake and the persistence of an oxycline offer an opportunity for studying the bacterial structure changes across the water column in order to gain knowledge about the microorganisms involved in eutrophication processes as well as in greenhouse gas emissions (i.e., CO₂, N₂O, CH₄). Thus, in this study, novel techniques of next generation sequencing (NGS) are applied to obtain information about the differences existing in the bacterial communities present in the sediment and at different depths of the water column, with different chemical characteristics due to the stratification of the lake. The results obtained will serve as a first step in the study of the biogeological processes that may be happening in these saline environments.

2. Materials and Methods

2.1. Study Area

Lake Alboraj is located in SE Spain, a karstic-tectonic sinkhole formed about 700,000 years ago [14], which sustains a permanent waterbody. The region is influenced by a semiarid meso-Mediterranean climate, with an historic average annual precipitation below 380 mm, with a fresh autumn (average autumn temperature of 6.7 °C) and a hot summer (average summer temperature of 24.3 °C). As freshwater habitat, this lake has been classified as type 3190 "Lakes of gypsum karst" within Natural Habitat Types of Community Interest [4,15]. According to the lithology characteristics, the lake is hyposaline, calcium-sulphated, with a historic conductivity ranging from 2240–2496 mg/L (Regional Decree 182/2000; Declaration of the Micro-reserve Lake Alboraj and Natural Resources Management Plan of Lake Alboraj). The small endorheic lake basin (120 ha) is in an agricultural landscape, where eutrophication, salinisation and lowering of the water level are the main anthropic impacts. Groundwater and surface water withdrawals have caused the shrinkage of the lake from a flooded area of 16,000 m², in the early 80's of the past century, to 5500 m², in 2017 [16]. The

lake shrinking in turn has caused a salinity increase up to 5760–7680 mg/L [9]. Nowadays, the lake water table reaches 100 m in diameter, 5000 m² of surface and settle three deep dolines (5 m depth in its deepest doline) with shallow interdoline areas less than 2 m deep [10]. This geomorphological feature promotes the formation of a marked seasonal oxygen stratification pattern of the water column.

The water level fluctuates and very much depends on the precipitation regime, although groundwater inputs from a local shallow Pliocene aquifer formed in the surroundings prevent drying. From a bathymetry model, the maximum volume of stored water has been estimated in 0.018 hm³, corresponding to a laminar water area of 9500 m² and a maximum depth of 6.5 m (unpublished data). No water withdrawal or surface sewage discharges occur in the lake.

Typically, when the oxygen stratification occurs, the lake shows three different layers: the epilimnion, which comprises the upper aerobic layer; the metalimnion, the next layer in the depth profile that comprises from the depth at which oxygen starts to decrease to the depth at which anoxia is reached, i.e., the oxycline; and the hypolimnion, extends from the end of metalimnion to the lake bottom, i.e., the anaerobic layer. The oxygen depletion coupled with a strong gradient in physico-chemical parameters in the metalimnion.

2.2. Water and Sediment Samples for Chemistry and Bacterial Analysis

Samples for chemistry and bacterial analysis were collected on March 13, 2019, in the center of deepest doline from a boat (38°34'56.60148'' N and −1°39'12.76319'' W).

One liter of water for bacterial analysis and two liters of water for chemical parameters were collected with a Rüttner bottle at 1 m (W1; aerobic epilimnion) and 4 m (W4; anaerobic hypolimnion) deep. Glass bottles were sterilised in autoclave 120 °C for 15 min and were pre-rinsed several times with water sample before collection. Afterwards, bottles were sealed with a screw cap, avoiding headspace, kept in a cooler box in darkness and transported to the laboratory. At the laboratory, water samples were filtered immediately through nylon membranes with 0.45 µm pore diameter (Merck KGaA, Darmstadt, Germany) and stored in a cold room (4–6 °C) until further analysis in the next 48 h. Nylon membranes were frozen and kept at −20 °C to be used for further DNA analysis.

A sediment sample was collected at the bottom of deepest point of the lake (4.7 m deep at the sampling date). Sampling was performed by collecting the first 5 cm of the lake bottom sediments with a sediment corer. The sample, collected aseptically in a sterile 50 mL polypropylene tube, was sealed with a screw cap, kept in a cooler box in darkness and transported to the laboratory, for a time not exceeding 5 h.

2.3. Physico-Chemical and Pigment Profiling

Depth profiles of dissolved oxygen (DO), redox condition (Eh), pH, total dissolved solids (TDS) and temperature (T) were performed at 0.5-metre depth intervals from a boat on the same date as water and sediment sampling using a HQ40d portable Multi-Parameter Meter (Hach Company, Loveland, CO, USA). The accuracy of physico-chemical determinations was ±0.01 mg/L for DO, ±0.1 mV for Eh, ±0.002 for pH, ±0.5% for TDS and ±0.3 °C for T.

As a measure of phytoplankton biomass that could help in the interpretation of DNA analysis, pigment concentration profiling was also determined. The samples were taken with a Rüttner bottle at a 1-metre depth interval and transferred to 1 L opaque bottles and kept cold and dark in a cooler box. Phytoplankton chlorophyll *a* (algae and cyanobacteria) were determined by filtering 400 mL of lake water. Fiberglass filters (0.7 µm pore, 47 mm diameter) were frozen for 1 week to break algal cells. Pigment extraction was accomplished in a vial by adding 5 mL of 90% acetone and leaving it in the fridge at 4 °C at least 12 h up to a maximum incubation of 24 h. After performing spectrophotometry scanning, chlorophyll *a* was calculated by the trichromatic method [17].

2.4. Chemical Analysis

Alkalinity (HCO_3^- and CO_3^{2-}) was determined by titration in the laboratory with a Methrom 855 robotic titrosampler (Methrom 855, Methrom, Switzerland); 50 mL subsamples were treated with acid titration (0.05 M HCl) with a limit of detection of 1 mg/L and an accuracy of $\pm 0.2\%$. Dissolved Organic Carbon (DOC) and Total Dissolved Nitrogen (TN) concentration was determined by the high temperature combustion method using a Shimadzu TOC-V Analyzer and Total Nitrogen Measuring Unit. The aqueous concentration of Cl^- , SO_4^{2-} , F^- and Br^- was determined by ionic chromatography (IC) using a 930 Compact IC Flex Oven/SeS/PP/Deg system coupled 944 Professional UV/VIS detector Vario. For Cl^- , SO_4^{2-} , F^- and Br^- determinations, the limit of detection was 1 mg/L. The concentration of NO_3^- was determined by UV-vis spectrophotometry following the Griess reaction assay by cadmium reduction to nitrite (NO_2^-) [18]. The detection limit of NO_3^- measurements was 0.3 mg/L. NH_4^+ concentration was determined by indophenol blue method [19], with a detection limit of 0.02 mg/L. Accuracy for NO_3^- and NH_4^+ determinations was $\pm 1.0\%$. Sample treatments and chemical analyses were performed at the Biotechnology and Natural Resources laboratory (Institute for Regional Development, University of Castilla-La Mancha).

2.5. DNA Extraction and MiSeq Illumina Sequencing

Environmental DNA was extracted immediately after sampling to minimise significant DNA degradation. Prior to DNA extraction, water and sediment samples were aseptically homogenised by vortexing to prevent decantation or non-representative sampling. After homogenisation, water samples (W1 and W4) were filtered throughout nylon membranes with 0.45 μm pore diameter (Merck KGaA, Darmstadt, Germany), at a rate of 1 nylon membrane per 500 mL, approximately. For the DNA extraction from sediments, 0.5 g of sediment sample were used, while for the water samples, nylon membranes obtained after filtering were used. Environmental DNA extraction was performed using the NucleoSpin[®] Soil DNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions with slight adaptation for water samples. Nylon membranes were sterilely cut into small fragments by a scalpel, immersed into lysis buffer and vortexed. The resultant supernatant was taken to further proceed to DNA extraction following manufacturer's instructions. Yield, quality and purity of extracted DNA was quantified by 1% agarose gel and spectrophotometer, using a multimode lector Cytation 5 with the specific software Gen5 (Biotek, VT, USA). DNA yield and quantitation were measured by absorbance at wavelengths 260, 280 and 320 nm. Purity ratio $A_{260/280}$ was around 1.8 in water and sediment DNA. All extracted DNA samples were stored at $-20\text{ }^\circ\text{C}$ until further processing.

After DNA quality and quantity controls, amplicon sequencing (V3–V4 region) of 16S rRNA gene [20] was performed on DNA extracts on a MiSeq using v3 chemistry by Stabvida (Caparica, Portugal, Portugal). 16S amplicon PCR primers used in the library preparation was 5'-CCTACGGGNGGCWGCAG-3' with the Illumina adapter 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3' for forward sequencing and 5'-GACTACHVGGGTATCTAATCC-3' with the Illumina adapter 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3' for reverse sequencing. Final taxonomic assignment was obtained after amplicon data processing and sequence alignment using QIIME 2[™] v2018.6.0 [21] and the version 132 of SILVA database [22]. Generated sequences from epilimnion, hypolimnion and sediment were deposited in the GenBank database under the BioProject PRJNA734791 and BioSample accessions number SAMN19539535–SAMN19539537.

3. Results

3.1. Chemical and Ecological Changes in the Water Column

The lake showed a well-defined stratification pattern of the water column (Figure 1). DO profile defined three layers: epilimnion (0–2.5 m), oxycline (2.5–3.5 m) and hypolimnion (3.5–4.7 m). DO, T, TDS, Eh, pH and Chl *a* parameters were measured every 0.5 m up

to 4.7 m deep, or 4.5 m in case of Chl *a*, while all other parameters were measured at epilimnion and hypolimnion layers. In epilimnion, DO concentrations ranged from 13.6 to 15.2 mg/L and temperature decrease from 12.8 to 11.4 °C. Eh, pH and Chl *a* slightly varied, ranging from −80 to −83.1 mV, 8.31 to 8.38 and 57.4 to 67.4 mg/L, respectively, while TDS gradually increase with depth from 7776 to 7814 mg/L. The metalimnion was defined by a sharp change in DO (oxycline), reaching values of 0.06 mg/L at 3.5 m, in the top of the hypolimnion. T and pH both decrease from 10.2 to 8.7 °C and 8.21 to 7.72, respectively, while Eh started increasing up to −46.2 mV and Chl *a* reaches it maximum value of 127.3 mg/L. In the hypolimnion layer, DO remained close to 0 values, as well as all other parameters hardly changed their values. At the lake bottom was obtained 8.5 °C for T, 7.59 for pH, −39.9 mV for Eh and 7930 mg/L for TDS.

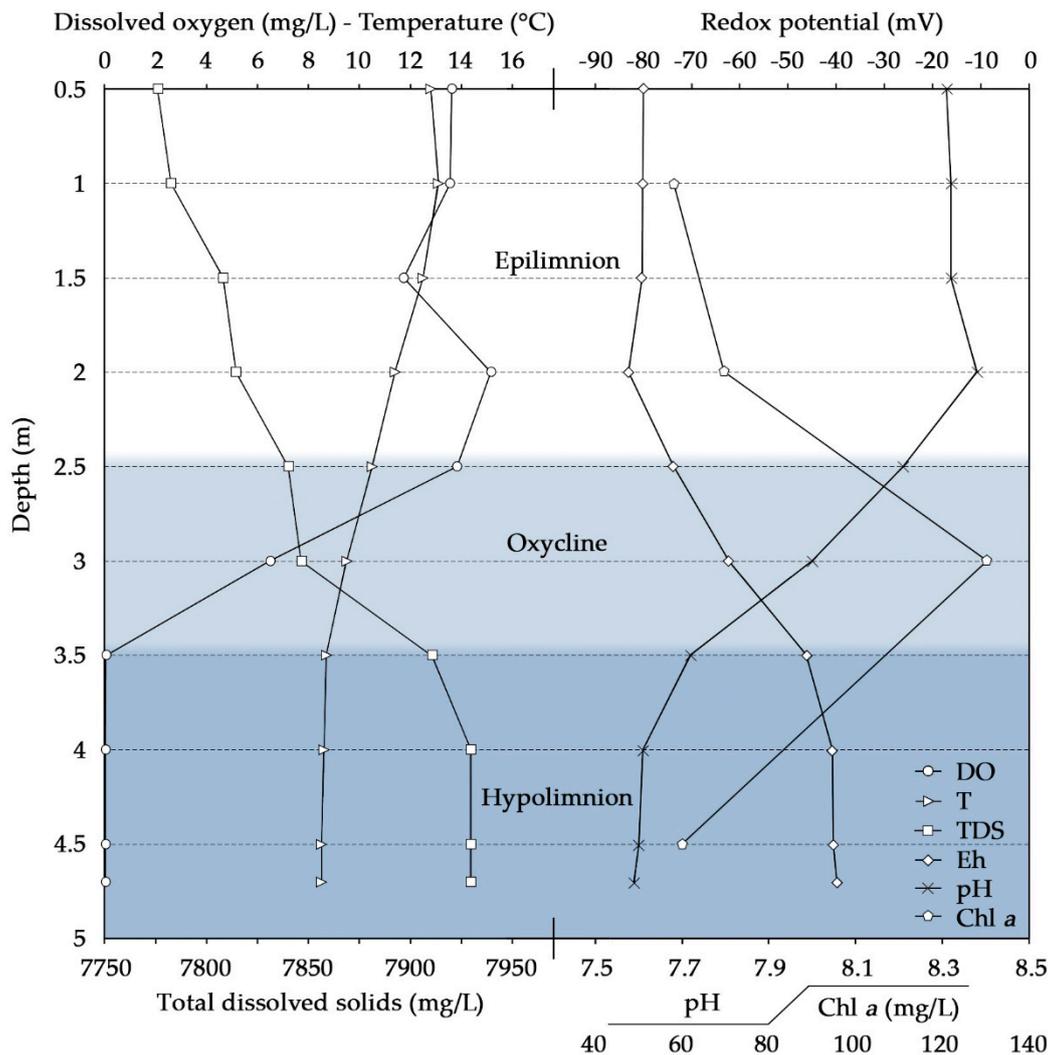


Figure 1. Physico-chemical properties of water column from Lake Alboraj were recorded during the spring oxycline state (13 March 2019).

The visual disappearance of the “Secchi disc” occurred at 0.9 m deep, denoting a turbid state of the lake. Regarding N-species, concentrations of NO_3^- , TIN and TON denoted a marked decrease from the epilimnion to the hypolimnion, while NH_4^+ ranged up in the deeper layer. SO_4^{2-} , Cl^- , F^- and Br^- also showed higher values at the epilimnion than hypolimnion (Table 1).

Table 1. Chemical and limnological values from water samples from Lake Alboraj in sampling campaign. Depth is given in meters and concentration of ions, in mg/L. -: no data available.

Depth	Epilimnion		Hypolimnion	
	1	4	4	4.5
NH ₄ ⁺	0.04	2.2		0.06
NO ₃ ⁻	10.3	0.6		4.7
TIN	2.4			1.1
TON	1.3			0.5
N _{total}	3.7	3.20		1.6
P _{total}	0.1			0.12
DOC	12.9	14.9		
HCO ₃ ⁻	379	398		-
SO ₄ ²⁻	6310	5426		-
Cl ⁻	1376	1187		-
F ⁻	8.7	7.5		-
Br ⁻	2.7	2.4		-

3.2. Comparative Analysis of the Microbial Structure

Water and sediment samples for microbial characterisation were taken in early spring coinciding with a period of a well-defined oxygen stratification pattern. The amplicon-based next generation sequencing analysis of the 16S rRNA V3-V4 region, carried out in water and sediment samples, provides a comprehensive screening of bacterial population. After denoising, from a total of 379,326 high-quality sequences representing the bacterial community, an average of $126,442 \pm 9509$ sequences was obtained per sample. Afterward, with a clustering threshold of 97% similarity level, a total of 682 non-singletons and unique features (OTUs) were identified with an average of 458 ± 132 OTUs per sample (Table 2). Regarding archaeal communities, the dataset reveals poorly enriched and characterised lineages. The sequences clustered in 8 OTU, at 97% similarity level, with very few reads per OTU. A minimum significant number of sequences must be clustered to be considered a valid OTU for classification purposes. For this reason, only the Bacteria domain was considered for community analysis.

Table 2. Number of sequences and OTUs generated and diversity estimators from MiSeq Illumina sequencing analysis.

Sample	Depth (m)	Habitat	Sequences Generated	OTUs Generated	Shannon	Phylogenetic Diversity
W1	1	Water, epilimnion	133,167	558	6.24	51.89
W4	4	Water, hypolimnion	115,563	507	5.75	51.00
S	4.7	Sediment	130,596	308	6.12	43.16

The diversity of prokaryotic population was compared between samples by rarefaction curve analysis of the estimated OTUs. Rarefaction curves showed a plateau stage in all samples, achieving a good coverage of the sequencing analysis of the entire community (Supplemental Figure S1). OTU-based alpha diversity measurement, as Shannon diversity index, decreased in depth at the water column, reaching the highest value in the epilimnion (W1) and the lowest value in the hypolimnion (W4). Prokaryotic diversity in sediments was similar to epilimnion diversity (Table 2).

Microbial communities of the studied zones varied greatly in taxonomic composition (Figures 2 and 3). The microbial structure associated showed different distribution based on the sample nature, even within depth-related water location. Proteobacteria phylum was widely present in all samples but differed in class composition either to water or sediment sample. Water samples were also represented by Cyanobacteria and Bacteroidetes phyla, whereas Actinobacteria and Firmicutes were characteristic from sediment phyla structure. The Proteobacterial *Alphaproteobacteria* and *Gammaproteobacteria* class, represented

by *Rhodobacteraceae* and *Burkholderiaceae*, respectively, contributed mostly to the spatial distribution of Lake Alboraj W1 sample (upper water sample, 1 m). OTUs clustering with members of *Hydrogenophilaceae* and *Saccharospirillaceae* families, within *Gammaproteobacteria* class, led the spatial distribution of W4 (deepest water sample, 4 m). *Oxyphotobacteria* members, within Cyanobacteria, also comprise a representative relative abundance in both water samples depth. Finally, sequences clustering with *Pseudomonadaceae*, *Burkholderiaceae* (both Gammaproteobacteria), *Propionibacteriaceae* (Actinobacteria) families and the *Bacilli* class contributed mostly to the spatial distribution of Lake Alboraj sediment (S) sample.

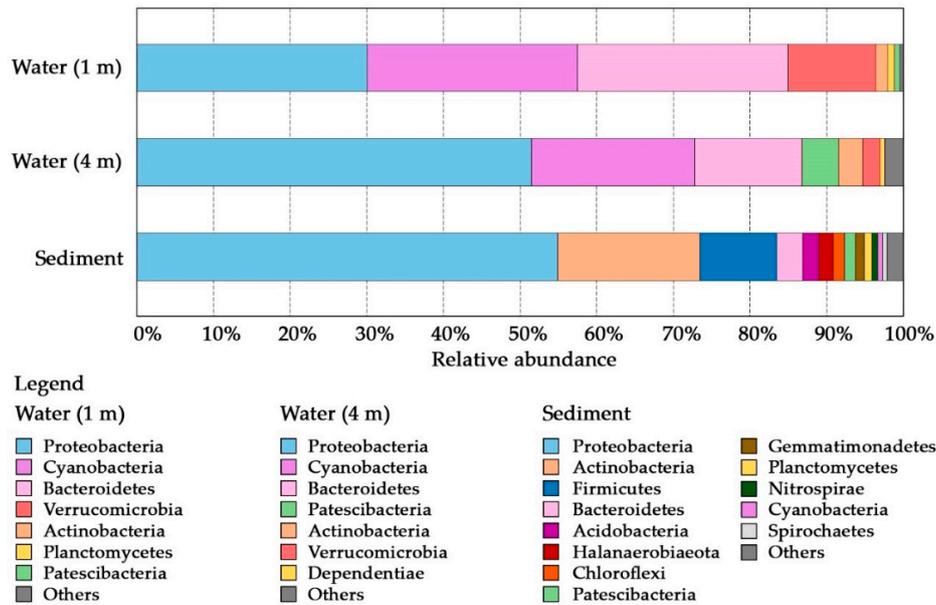


Figure 2. Relative abundance of major bacterial phyla from Lake Alboraj water and sediment. Only phyla with a relative abundance >0.5% of total bacterial population were included.

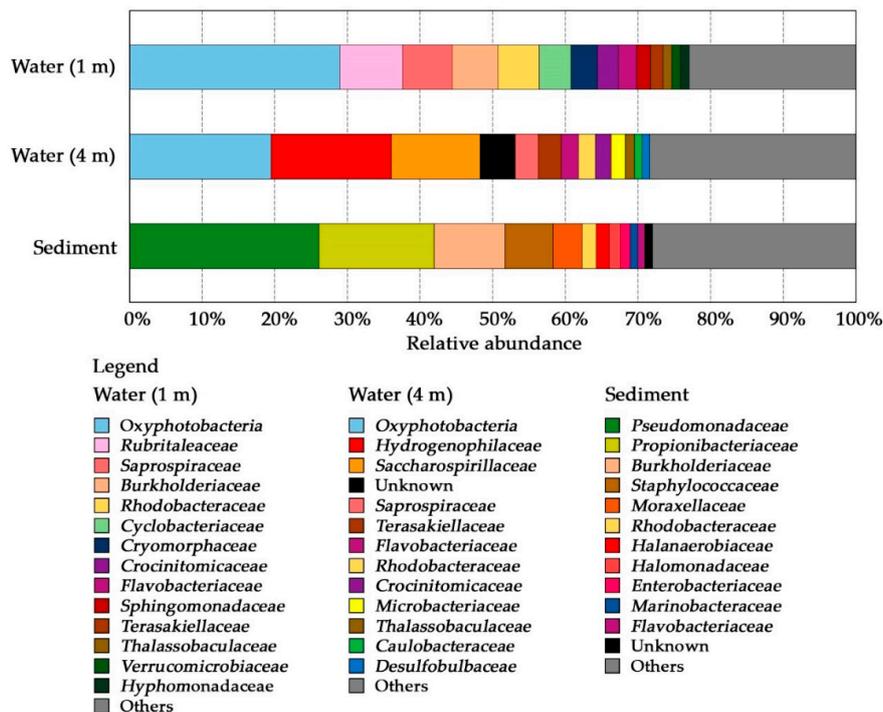


Figure 3. Relative abundance (%) of major families identified in water (1 m and 4 m) and sediment samples from Lake Alboraj. Only families with a relative abundance >1% of total bacterial population were included.

3.2.1. Water Associated Bacterial Communities

Bacterial community structure in the water column was more diverse than in sediments; results also showed that there were not large differences between epilimnion and hypolimnion bacterial richness (Figure 3). The sequencing of W1 clustered into 22 phyla, 36 classes, 158 families and 155 genera, while W4 clustered a total of 23 phyla, 39 classes, 171 families and 160 genera. In both cases, more than one third of the genera were unknown or uncultured. Water samples shared >85% of the main phyla, comprising Proteobacteria (W1, 30%; W4, 51%), Cyanobacteria (W1, 27%; W4, 21%) and Bacteroidetes (W1, 27%; W4, 14%). In addition, with the presence of the phylum Verrucomicrobia, the spatial distribution of the bacterial population covered up to 96% in W1. On the other hand, W4 reached a 97% of total spatial distribution with Patescibacteria, Actinobacteria and Verrucomicrobia. Despite of the similarity in phyla distribution, the water strata become divergent at the family level.

Oxyphotobacteria, the only family within Cyanobacteria, comprised 27% of relative abundance in W1, decreasing to 21% in W4. The second most abundant family was *Rubritaleaceae*, within Verrucomicrobia, contributing 8% in W1. An environment of low oxygen availability such as W4 habitat drastically limits family development because of its mandatory aerobic chemoheterotrophic metabolism [23]. The *Bacteroidia* relatives clustered mainly in *Saprospiraceae*, *Cyclobacteriaceae*, *Cryomorphaceae*, *Crocinitomicaceae* and *Flavobacteriaceae* families, accounting a total of 23% over the bacterial population in W1 sample. It is a clade strongly dependent on the availability of organic matter, with complex organic carbon sources transformation and decomposition (i.e., *Flavobacteriaceae*), saprophytism of microorganisms—algae or bacteria—(i.e., *Saprospiraceae*) [24]. Moreover, the clade involves other families without any known function due to the difficulties in isolating their members (i.e., *Cryomorphaceae*). Members of the family *Sphingomonadaceae* also were present, although only represented a 2% of the total spatial bacterial distribution in W1 and was described as xenobiotic compounds transformation [25,26].

As regards W4 sample, Cyanobacteria continue to be among the most abundant taxa, representing 20% of total identified sequences. Contrary to W1 sample, *Gammaproteobacteria* members as well as *Hydrogenophilaceae* and *Saccharospirillaceae* families increased their representation accounting for 30%, 19% and 14%, respectively. Little is known about these families and their metabolic functions. *Hydrogenophilaceae* had been poorly described as nitrogen fixers by Calvin-Benson cycle and reduced-sulphur compounds oxidisers [27]. Unknown family from the recent proposed lineage *Gracilibacteria* (currently, included in Patescibacteria phylum) was identified in a 5% of relative abundance in W4. *Alphaproteobacteria* clade was comprised mainly by *Terasakiellaceae*, *Rhodobacteraceae*, *Thalassobaculaceae* and *Caulobacteraceae*. Members of the family *Terasakiellaceae* and *Caulobacteraceae* were documented to be nitrogen fixers [28] and denitrifiers through nitrate reducing pathway [29], respectively, and may be important for nitrogen natural regulation. Furthermore, *Rhodobacteraceae* and *Desulfobulbaceae* families had been reported to play a key role in sulphur cycle. *Rhodobacteraceae* acts as sulphur, sulphide, thiosulphate or sulphate oxidiser to obtain carbon sources [30], while *Desulfobulbaceae* is a well-known sulphate-reducing bacteria family [31]. Finally, some members of the family *Microbacteriaceae* (Actinobacteria) appeared in W4. This family has been found to be associated to saprophytic processes by degradation of organic matter. They are also associated to phosphorus extraction under aerobic and anaerobic conditions or recalcitrant xenobiotic degradation [32,33].

3.2.2. Sediment Associated Microbial Communities

Lake Alboraj sediment bacterial communities presented the lowest diversity regarding bacterial taxa. The sequencing clustered a total of 28 phyla, 58 classes, 94 orders, 117 families and 109 genera. Most of the genera were unknown or uncultured. At the phylum level, Proteobacteria (55%), Actinobacteria (19%) and Firmicutes (10%) comprised the overall structure, accounting for 84% of relative abundance (Figure 2). Within Proteobacteria classes, *Gammaproteobacteria* (48%) was the dominant, which was related to sulphur cycle

processes. Moreover, *Alphaproteobacteria* and *Deltaproteobacteria* (4% and 3%, respectively), which are related to nitrate reduction and sulphur cycle, completed the main phylum. In addition to the Proteobacteria members, representative of the Actinobacteria phylum, were observed. Actinobacteria, the second phylum, was almost dominated by members of *Actinobacteria* class (17%). Firmicutes, the third most abundant phyla, was formed by *Bacilli* members (7%).

The most abundant family within the bacterial community (Figure 3) was *Pseudomonadaceae*, a *Gammaproteobacteria* with 26% of the total population. *Pseudomonadaceae* is closely reported to nitrogen cycling, including some species which have been reported to be able to perform aerobic or anaerobic denitrification processes commonly by nitrate-reduction or nitroaromatic compound degradation (i.e., fertilisers, pesticides) [34,35]. In addition, *Burkholderiaceae* family, with a 10% of relative abundance, has been reported to manage widespread adaptation to different ecological niches and can also perform very diverse metabolic functions (i.e., nitrogen fixation, aromatic compound catabolism) [36]. Other *Gammaproteobacteria* member with a 9% of relative abundance was *Moraxellaceae* family, which has saprophytic capacities. The members of this family have been described as active agents in decomposition and subsequent mineralisation of organic matter present in the environment [37]. In addition, although it is not a family related to nitrogen-cycle, some of its genera have been reported to be related to nitrate-reducing processes [38].

The Actinobacteria relatives present in Lake Alboraj sediment clustered mainly in the recent described *Propionibacteriaceae* family, accounting 16% of relative abundance of the total. Remarkably, its metabolic properties are monospecific, however, its metabolic functionality may change whether new species were included [39]. On the other hand, a well-known *Alphaproteobacteria* member was *Rhodobacteraceae*. This family is characterised by its metabolic multifunctionality on sulphur and carbon cycles, as well as the symbiotic association with other microorganisms [30], which makes it can be found widespread distributed throughout the lake. The noteworthy function of the family is the oxidation of sulphur, its simple compounds (sulphide, thiosulphate) or sulphate (in some cases) to obtain a carbon source [30]. Elemental sulphur is usually the end product of its metabolism. Another significant finding is the abundance of *Flavobacteriaceae* members, which is mainly particle-associated and strongly dependent on the availability of organic matter [24,40].

4. Discussion

To our knowledge, this study provides the first effort to investigate the bacterial community structure along depth at Lake Alboraj, an ecosystem which is subjected to self-regulation due to the forgotten human care and nature preserving. Results showed that the diversity of bacterial communities, according to the phylogenetic analysis, gives rise to different lineages along spatial location and habitat. Phylogenetic diversity indicated a longer evolutionary history in water samples, due to a phylogenetic distancing between the microorganisms that coexist in the environment [41–43]. Moreover, a non-metric multidimensional scaling (NMDS) analysis revealed clear differences among the bacterial community structure between water samples and sediment (Supplemental Figure S2). So, both phylogenetic diversity measurement and NMDS analysis suggest the coexistence of different bacteria community structures into the water column and water-sediment interface. Under an environment with conditions of low-oxygen and anoxia, microorganisms are expected to adapt their requirements towards metabolic strategies diversification to fill the expected oxygen-limiting niche. For that reason, the bacterial community changes in their distribution between the epilimnion and hypolimnion layers, and the sediment, coinciding with noticeable oxygen depletion in the water column. DO concentrations shift to lower concentrations in a few decimeters at 2.5 m water depth, describing a sharp oxycline (2.5–3.5 m) which was not linked to the thermocline (Figure 1). This result is not in line with the accepted consensus which states that in oxygen stratified lakes is a common process of the development of a thermocline [44]. The small dimension of the lake, a water column of about 4.7 m depth and an extension near to 5500 m², may not

promote the development of a temperature gradient during winter as well as an efficient oxygen exchange at the water-atmosphere interface. In fact, the existence of a complete anaerobic water column from late spring to late summer suggests that oxygen exchange with atmosphere can be limited to cold periods; during warm periods, a decrease in oxygen solubility matches with the augmentation of water temperature.

Regarding to processes involved in the N-cycle, a greater abundance of bacterial families was found in the sediment and hypolimnion layer (W4), compared to epilimnion (W1). Despite of the similarity in the uses of nitrogen compounds, the bacterial communities found in the sediment and W4 samples are dissimilar. Noticeably, the greater abundance of denitrifiers, such as members belonging to *Pseudomonadaceae*, *Burkholderiaceae* and *Halanaerobiaceae*, was detected in the sediment. In W4 sample, *Caulobacteraceae* and some members clustering in *Rhodobacteraceae* and *Hydrogenophillaceae*, were related to processes contributing to nitrogen turnover. Even though the presence of microorganisms identified as facultative aerobic denitrifiers were found in sample W1 (i.e., *Burkholderiaceae*, *Rhodobacteriaceae*), they have not such relative frequency such as in the deeper water layer and bottom lake sediment samples. This agrees with the fact that conventional biological nitrogen removal is achieved by two well-defined sequential stages, nitrification under aerobic conditions followed by denitrification under anoxic conditions. Therefore, in the oxic layer of the water column the denitrification is not such manageable by biological processes mediated by bacteria, which are commonly adapted to anaerobic conditions. Across the bacterial community clustered in W1, only *Burkholderiaceae* has been identified within the denitrification processes. Even though little is known about the metabolic capacities, this finding suggests that some bacteria are capable of change their metabolism in agreement with the environmental conditions and N-species availability [11]. The higher presence of denitrifier bacteria in the deeper zones of the water column, matches with a decrease in the concentration of nitrate (NO_3^-), from 10.33 mg/L, in the epilimnion, to 4.70–0.60 mg/L, in the hypolimnion. The higher number of denitrification related families in sediments could be explained by low oxygen habitat and the availability of NO_3^- and organic energy sources, since NH_4^+ is rarely limiting in sediments [45,46]. In W4, members belonging to *Saprospiraceae*, *Flavobacteriaceae*, *Crocinitomicaceae* and *Microbacteriaceae* families, which can perform saprophytic processes linked to decomposition of organic matter and microbial biomass [24], have also been identified.

The production and consumption of NH_4^+ can be driven under different microbially mediated processes linked to gradients of oxygen [47–49]. The development of an anaerobic hypolimnion is typical in eutrophic lakes and the processes can involve the increase of nutrients, such as NH_4^+ . At the water-sediment interface, processes such as ammonification can release NH_4^+ during organic matter decay. In this sense, it has been proposed that in the hypolimnion NH_4^+ was the major N species identified, and sediment release can contribute nearly 85% hypolimnetic NH_4^+ [47]. A decrease in NH_4^+ concentration is clearly detected from the hypolimnion (2.18 mg/L) to the epilimnion (0.09 mg/L). The nutrient availability found at hypolimnion may be stimulated by microbially-mediated processes at the water-sediment interface.

Dissimilatory nitrate reduction to ammonium (DNRA), may produce NH_4^+ under strictly anaerobic conditions [45]. A wide distribution group of sediment bacteria is capable to perform DNRA [50], which acts in Alboraj sediment as facultative anaerobes, *Enterobacteriaceae*, to aerobes, *Pseudomonadaceae*. Moreover, bacteria belonging to *Desulfuromonadaceae* family, which have been found to be associated to DNRA were also found in W4 sample. Bacterial structure results could support these findings where DNRA processes could take place in sediment by related members from *Gammaproteobacteria* and *Deltaproteobacteria*. Under anaerobic conditions, the oxidation of NH_4^+ can be driven under the presence of NO_2^- (anaerobic ammonium oxidation, anammox). According to present knowledge, anammox is carried out by a few anaerobic, autotrophic bacteria of a monophyletic clade in Planctomycetes [51]. Sediment gathered the conditions of anaerobic medium and substrate supplier necessary to keep families related to carried out anammox processes as *Pirellu-*

laceae, also poorly identified in W4 sample, within the phylum Planctomycetes. However, due to the limited information about factors controlling the processes in natural systems, knowledge about anammox in nature must continue as an ongoing effort to understand the distribution of the process that occur in the environment.

Noticeably, in the epilimnion, the nitrogen fixers are abundant. Oxygenic photoautotrophic cyanobacteria (*Oxyphotobacterias*) together with populations of *Terasakiellaceae* might be responsible for the primary productivity in the epilimnion. The proliferation of *Cyanobacteria* is strongly linked to biosphere's environmental perturbations, including nutrient enrichment [52] and climatic changes (i.e., hydrologic changes, drought and precipitation events hardening) [53]. Their role as atmospheric nitrogen fixers, as well as their capacity of symbiotic cooperation with other microorganisms are well known. Nevertheless, cyanobacterial bloom results in a massive decomposition of dead cells which is associated to cyanotoxins release to the environment [53]. Whenever toxicity occurs, it can cause harmful effects to microorganisms, leading to an unbalanced development of those communities. Moreover, in epilimnion layer, NH_4^+ can be assimilated by biomass as well as transformed into NO_3^- by nitrifiers under aerobic conditions (aerobic ammonium oxidation, nitrification). Therefore, nitrification may explain the appearance of NO_3^- in the epilimnion. Nonetheless, in our case study, the importance of external inputs (i.e., discharge of polluted groundwater, surface runoff) in nutrient availability cannot either be confirmed or discarded.

Biogeochemical S-cycle can be closely linked to microbial redox processes in aquatic systems [54,55]. Sulphate is an important end-product during microbially-mediated sulphide oxidation processes but can also be derived from the dissolution of sulphate-bearing minerals, as gypsum. Gypsum has been identified in the Triassic evaporitic rocks outcropping in the study area as well as in detrital grains in the Holocene sediments in which the lake is located [56]. The difference in SO_4^{2-} concentrations between the hypolimnion (6310 mg/L) and epilimnion (5426 mg/L) is important enough to dismiss the detection limit of the method. Nonetheless, a variety of sulphur related bacteria were identified in W1 and W4, which can perform coupled sulphate reduction–sulphide oxidation processes. At this interface, SO_4^{2-} can be used during sulphate-reducing bacteria processes to produce H_2S , which is re-oxidised in the epilimnion to SO_4^{2-} in the presence of oxygen. In this sense, S-cycle bacteria were better represented in anoxic hypolimnion layer, W4, than in the oxic epilimnion layer, W1. In W4 sample bacteria belonging to *Desulfobulbaceae*, *Rhodobacteraceae*, *Crocinitomicaceae* families, which include various sulphate-reducing bacteria [30,57] were found. High activity of sulphate-reducing bacteria is accompanied by production of H_2S , which acts as an electron donor for the chemolithotropic sulphur-oxidising bacteria. Microorganisms within the families *Rhodobacteraceae* and *Cyclobacteriaceae* found in the epilimnion are known sulphate-oxidising bacteria [58,59], which may participate in the activity of re-oxidising the bottom-produced H_2S to SO_4^{2-} in the upper oxic layer. Interestingly, both, the family *Methylomonadaceae*, identified in W4, and *Methylophagaceae*, identified in the sediment sample, are associated with H_2S oxidation processes and methane (CH_4) production. The availability of dissolved organic carbon is noticeable in the water column, but water samples do not show an important difference in DOC concentrations at W4 (14.9 mg/L) and W1 (12.9 mg/L). The consumption of organic matter by heterotrophs may lead to the production of CO_2 (g) which, in turn, can be reduced to CH_4 (g), under anaerobic conditions. The intensity of DO consuming processes by heterotrophic microorganisms may lead to the development of an oxygen deficient zone of different thickness in the lake. Depending on the rate of oxygen consumption processes and the renewal of oxygen at the water-atmosphere interface, a well-defined oxycline can be developed, with different thickness, in a poor ventilated water column, in absence of thermal stratification.

The bacterial distribution found matched with changes in the observed dissolved oxygen concentration decrease, from epilimnion to hypolimnion, chlorophyll concentrations and the distribution of some dissolved chemicals. These results support the idea that microbial structure plays an important role in the distribution and fate of chemical species.

The bacterial distribution in the bottom lake sediment and the stratified water column can promote the development of different interfaces in which redox conditions lead to the recycling of N, S and C-species.

Despite the importance of inherent interplay between native microorganisms and the environmental conditions in the development of a chemical stratification, there is still a paucity of evidence on what biochemical processes are being conducted by bacteria communities in Lake Alboraj. The interfaces, in turn, may offer the opportunity of learning about the distribution and fate of some pollutants, as well as deepening in the knowledge of the processes conducting to eutrophication of water bodies in which an oxycline is developed independently of the water volume stored and morphology of the lake.

5. Conclusions

The amplicon-based MiSeq Illumina sequencing allows obtaining a complete screening of the bacterial population inhabiting at different depths in the water column as well as in the sediment of Lake Alboraj. The composition of bacterial communities differs from the epilimnion to the hypolimnion, while the sediment holds a bacterial population different from those representing water samples. A greater abundance of bacteria families related to processes involved in the N-cycle was found in the sediment and hypolimnion layer (W4), although with different composition. A greater abundance of denitrifiers such as those belonging to families *Caulobacteraceae*, *Rhodobacteraceae* and *Hydrogenophilaceae* was detected in W4 samples, while in the sediment, members of the families *Pseudomonadaceae* and *Burkholderiaceae* and *Halanaerobiaceae*, related to processes that contribute to nitrogen turnover, were found. The higher presence of denitrifier bacteria in the deeper zones is consistent with the decrease in the concentration of NO_3^- detected. On the other hand, NH_4^+ concentration was higher in the hypolimnion. In this sense, a large group of bacteria capable to perform DNRA processes was found in the sediment, from facultative anaerobes such as *Enterobacteriaceae*, to aerobes such as *Pseudomonadaceae* or others akin to those from the family *Desulfuromonadaceae*.

As regards S-cycle, the results obtained showed the presence of a variety of sulphur related bacteria in the water samples, which can perform coupled sulphate reduction–sulphide oxidation processes. The anoxic hypolimnion layer showed a greater abundance of S-cycle related bacteria, including those belonging to the families *Desulfobulbaceae*, *Rhodobacteraceae*, *Crocinitomicaceae*, which can perform sulphate–reducing processes to produce H_2S . This H_2S may be re-oxidised in the presence of oxygen by known sulphate-oxidising bacteria such as *Rhodobacteraceae* and *Cyclobacteriaceae* found in the epilimnion.

This study has shed light on the bacterial community structure and its support to the co-existence of assimilatory and dissimilatory microbial mediated reactions. Differences in physico-chemical conditions in the water column are associated to a bacterial community structure adapted to the available nutrients. These dynamic biological processes might hold a key role in maintaining the ecological equilibrium of the aquatic ecosystem. Bacterial distribution points out that N-S-C biogeochemical cycles can be interconnected throughout interfaces: water-atmosphere, oxycline and water-sediment.

Future work is needed to understand the temporal changes in the structure of the bacterial communities through detailed monitoring that matches the changes in oxygen stratification patterns during the year. Detailed exploration of microbial community structure variation over a non-stratified situation and oxygen dynamic states, in addition to their function adjustment relationship, will be essential to deepen into the knowledge of the ecosystem development. Moreover, an exploration of the remaining microbial processes is still needed to understand the lake functioning and thus the resilience to its ongoing deteriorated ecological status.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11146309/s1>, Figure S1: Observed OTUs alpha rarefaction curve, Figure S2: Non-metric multidimensional scaling (NMDS) analysis of the weighted UniFrac distance of water and sediment samples from Lake Alboraj.

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