

Article

Diversity of Bacteria in Lakes with Different Chlorophyll Content and Investigation of Their Respiratory Activity through a Long-Term Microcosm Experiment

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Abstract: Bacterial community structure and metabolism are critical factors for ecosystem functioning since they affect remineralization of nutrients and carbon flow. We used Illumina sequencing of 16SrRNA V3-V4 regions to investigate whether bacterial assemblage composition differs between four samples from two lakes in the geographic region of Epirus (Greece) characterized by distinct oligotrophic to eutrophic/hypereutrophic conditions as revealed by chlorophyll-a values. We found high similarity (>60%) for bacterial assemblages recovered from the two lakes when eutrophic/hypereutrophic conditions prevailed. Distinct bacterial communities appeared in oligotrophic and mesotrophic waters. Low temperature was occasionally an important factor in shaping the bacterial community. In parallel, microcosm experiments were performed to estimate respiration rates of bacterioplankton at in situ temperature and under a 2 °C temperature increase scenario. Differently assembled communities were found to display similar rates except under hypereutrophic conditions when respiration increased significantly, leading to hypoxic conditions. Temperature increase did not affect respiration rates. Overall this study indicated a clear differentiation of bacterial communities between sites of different trophic state. However, different communities responded similarly under a specific range of chlorophyll-a values and resisted small scale temperature perturbations. Different results were found for hypereutrophic conditions and this has implications for ecosystems functioning, given the increasing occurrence of eutrophication events.

Keywords: bacteria; diversity; respiration; lakes; temperature; trophic status; chlorophyll

1. Introduction

Bacteria play crucial roles in aquatic ecosystems as decomposers of organic matter, recyclers of nutrients, producers and consumers of greenhouse gases and intermediaries for the flow of energy and matter in food webs [1]. Quantification of bacterial cells is fundamental for aquatic microbiology [2], but it's not enough on its own for a thorough understanding of their contribution to ecosystem functioning, since bacterial abundance may have little, if any, correlation with bacterial activity [3–5]. Thus, in recent years, much effort has been devoted to explore bacterial diversity, so that scientists may answer unresolved questions and better understand previous findings.

Chlorophyll-a is a parameter widely used to describe ecosystems and their trophic state since it is linked to the availability of nutrients and organic substrates and to the onset and decline of phytoplankton blooms [6–8]. Several studies have shown positive correlations between bacterial abundance and chlorophyll-a [9]. Negative correlations have been interpreted as an indication of the dependence of bacteria on allochthonous organic matter [10]. Similarly, bacterial succession

in lakes may differ depending on whether the dominant available substrate is of phytoplankton or non-phytoplankton origin [11]. Changes in the composition of bacterial communities occur also depending on the phase of phytoplankton blooms and the dominant genera or species, affecting in turn dissolved organic carbon quantity and quality [12–14]. Although other parameters like environmental conditions [15] and top-down factors such as protistan grazing and viral lysis [11,16] may also have an impact on the structure of bacterial communities, distinct assemblages could be expected depending on the trophic status of the ecosystem as revealed by chlorophyll-a content. Indeed, a study in several lakes, rivers and reservoirs in Portugal indicated higher similarities for oligotrophic and mesotrophic water bodies compared to eutrophic/hypereutrophic [17]. However, a different pattern was revealed in reservoirs in Belgium where mesotrophic and eutrophic water bodies exhibited more similarities in respect to an oligotrophic reservoir [18]. A core bacterioplankton community was found across an oligotrophic to urban-eutrophic gradient in Lake Michigan [19]. Nevertheless, distinct bacterial assemblages were revealed in the two habitats and the authors suggested that oligotrophic conditions represented a strong selective force for specific, otherwise cosmopolitan, genera/lineages. This study also highlighted the role the watershed may have in shaping bacterial communities although recent findings from the Great Lakes region showed distinct assemblages for different aquatic habitats (creeks, a river, canals, stormwater outfalls and freshwater lakes) in the same watershed [20]. Trophic state affects not only the composition of bacterial communities but also their richness and evenness (i.e., diversity) since in eutrophic environments, habitat heterogeneity increases, favoring the co-occurrence of multiple taxa [21].

In this study, seasonal patterns of bacterial community composition were investigated in two lakes disturbed by human activities at different degrees. Ziros is a deep, dimictic, mesotrophic lake which is exposed to diffuse agricultural pollution [22] while Pamvotis is a shallow, polymictic, eutrophic/hypereutrophic lake impacted by point and non-point pollution related to the extended urbanization and intense agricultural activity in its catchment area [23]. Bacterioplankton diversity has been investigated in a limited number of freshwater ecosystems in the broader geographic area i.e., north-western Greece, e.g., [24–26], and to our knowledge, this is the first attempt to reveal the diversity of bacterioplankton communities in Greek lakes using high throughput sequencing. We hypothesize that bacterial community composition will converge in lakes/samplings with similar chlorophyll-a content and that taxa richness and diversity will increase in the more eutrophic waters. In parallel, we conducted a microcosm experiment [27] to estimate and compare the respiratory activity (i.e., oxygen consumption) of bacterial assemblages during long-term incubations. In this experiment we investigated also the effect of a temperature increase on bacterioplankton respiration, taking into account that global warming will increase surface water temperature of lakes. We tested a 2 °C increase since it has been estimated that in a scenario with a 5 °C air temperature increase there would be a ~3 °C rise in water temperature, for the most threatened, high latitude ecosystems [28]. This study thus contributes to the continuing effort to characterize freshwater lake bacterioplankton communities around the globe and tries to associate the structure of the communities with their metabolic performance.

2. Materials and Methods

2.1. Sampling—*In Situ* Abiotic and Biotic Parameters

Sampling was performed during the warm and the cold period of the year (summer 2016 and winter 2017) in the Ziros and Pamvotis lakes (Region of Epirus, NW Greece). Surface (0–20 cm) water was collected from one coastal site in each lake into 5 L well rinsed (10% HCl and milliQ water) polyethylene containers after filtration through a 180 µm plankton net. Samples were transferred to the laboratory at 4 °C in the dark for further processing. Physicochemical parameters (temperature, salinity, total dissolved solids and conductivity) were measured *in situ* with a Hach sension + EC5 conductivity meter and pH was determined using pH paper. Nitrates were determined by ion chromatography

in Shimadzu HPLC apparatus (detector Shimadzu CDD-10A VP, column shodex IC NI-424, column guard shodex IC NI-G, column oven Shimadzu CTO-10AC, pump Shimadzu LC-20AD). The samples were filtered through 2 μm filter paper and 20 μL of each sample were injected in HPLC equipped with a conductivity detector with flow rate 1.5 mL min^{-1} and shipping solvent 8 mM 4-Hydroxybenzoic acid + 2.8 mM Bis-Tris + 2 mM Phenylboronic acid + 0.005 mM CyDTA aq. Standard solutions of the above ions at concentrations ranging from 1 to 100 mg/L in seven levels were analyzed as the external calibration basis for quantification. The concentrations of total nitrogen (T-N), NH_4^+ , PO_4^{3-} and total phosphorus (T-P) of the samples were measured in a HACH DR/2010 Spectrophotometer. T-N and NH_4^+ in the water were determined spectrophotometrically by Total Kjeldahl (Nessler method) HACH method 8075 at the wavelength of 460 nm. T-P and PO_4^{3-} content were determined by the molybdenum blue method (HACH) after digestion with acid persulfate at 100 $^\circ\text{C}$ for 60 min when necessary at the wavelength of 880 nm.

Chlorophyll-a (chl_a) was determined as previously described in detail by Tsola et al. [8]. Water (0.5–2 L) was filtered through 0.7 μm (Sartorius, MGF, Goettingen, Germany) and chl_a was extracted at 4 $^\circ\text{C}$ in 90% acetone. After centrifugation at 4000 rpm, pigment concentration was determined spectrophotometrically using a U2800 Hitachi spectrophotometer. Dissolved oxygen (DO) concentration was measured as described below in ‘microcosm experiments’. In situ bacterial abundance was determined by epifluorescence microscopy after fixation with formalin 37% (2% *v/v* final concentration) and staining with DAPI [29]. At least 10 fields and 300 cells were counted on 0.2 μm filters (Isopore membrane filters 25 mm, Millipore, Burlington, MA, USA), using a Leica DM LS2 microscope under UV excitation at magnification X1000.

2.2. Bacterial Diversity

Bacterial diversity in each lake and sampling period was investigated through sequencing of the HV regions 3–4 of the 16S gene (~450bp). DNA extraction was performed with the MoBio PowerSoil Kit (MoBio Inc., Carlsbad, CA, USA) following its standard protocol. The 16S rRNA gene V3-V4 variable region PCR primers S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 [30] with barcode on the forward primer were used in a 28 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Gaithersburg, MD, USA). After amplification, pooled and purified PCR products were used to prepare illumina DNA library. Library preparation and sequencing was performed at MR DNA (Shallowater, TX, USA) on a MiSeq following the manufacturer’s guidelines. Sequences were processed using the MOTHUR software v1.30 [31], clustered in Operational Taxonomic Units (OTUs) at 97% sequence similarity and classified using the SILVA v132 SSU database [32]. Sequences from this study have been submitted to the NCBI Short Read Archive with BioProject ID PRJNA514081. All statistical analyses were done using PAST.3 software.

2.3. Microcosm Experiments

For the construction of microcosms, lake water was gently poured using a plastic tube into ~130 mL amber glass Winkler bottles. In total 6–8 duplicate microcosms were prepared for each lake and each temperature. Microcosms were incubated long-term (~14 days) [27], at in situ temperature, which ranged between 3.8 and 27.9 $^\circ\text{C}$ during samplings at the two ecosystems, and at temperature increased by 2 $^\circ\text{C}$. Every two days, duplicates from each incubation temperature were ‘sacrificed’ in order to determine the concentration of dissolved oxygen (DO) by the spectrophotometric Winkler method [33] as described in [8]. The method is suggested for suboxic to supersaturated with DO freshwaters. In brief, adequate volumes of the reagents manganese chloride (3M) and alkaline iodide (NaOH 8M, NaI 3M) were added to the samples. Samples were shaken, acidified with sulfuric acid (10 N) and the absorbance of the resulting from Winkler reactions solution ($\text{I}_2 + \text{I}_3^-$) was measured at 466 nm using a U2800 Hitachi spectrophotometer. Respiration rates were measured as the slope of changes in DO concentration (average values of duplicate samples) with time [34]. The Q_{10} value was calculated to estimate the effect of temperature change on respiration rates. The equation used

was $Q_{10} = (R_2/R_1)^{10/(T_2 - T_1)}$ where R_2 and R_1 are respiration rates at temperatures T_2 (in situ temperature +2 °C) and T_1 (in situ temperature) respectively [34]. We should mention here that experimental bottles contained the whole plankton heterotrophic community <180 µm. This was to avoid alterations of bacterial community composition caused by sequential filtrations [35] and in particular in high chlorophyll samples in which filter clogging interrupts flow rate at the initial steps of the filtration [8]. Nevertheless, we considered bacteria as the principal contributors to heterotrophic respiration [36,37] as well as that the encounter rate of bacteria and their predators in the 130 mL bottles would be low [3]. Finally, we assumed that microalgae abundance decreased due to enclosure e.g., [38,39] and that light limiting conditions did not allow them to recover.

3. Results

3.1. In Situ Abiotic and Biotic Parameters

During each sampling period, Ziros lake generally gave higher values of the various physicochemical parameters compared to Pamvotis. Temperature minima of 3.8 °C for Pamvotis and 10.4 °C for Ziros were found in winter. In summer, the respective temperatures were 21.7 and 27.9 °C. Conductivity in Pamvotis was $288 \pm 6 \mu\text{S cm}^{-1}$ and total dissolved solids (TDS) 190 mg/L (not determined in summer). In Ziros lake conductivity was $328 \mu\text{S cm}^{-1}$ in summer and $399 \mu\text{S cm}^{-1}$ in winter. The respective values for TDS were 210 and 253 mg/L. Total N (TN) and P (TP) concentration in Pamvotis ranged from 0.08 to 0.9 mg/L and from 0.7 to 1.2 mg/L respectively and minima were found in winter. In Ziros in winter TN was 0.3 mg/L and TP 0.5 mg/L. In summer the respective values were 0.2 and 0.6 mg/L. DO was always higher in Pamvotis and decreased from $10.40 \pm 0.02 \text{ mg/mL}$ in summer to $8.85 \pm 0.13 \text{ mg/mL}$ in winter (Figure 1). On the contrary in Ziros a low increase was found between summer ($5.95 \pm 0.26 \text{ mg/mL}$) and winter ($6.43 \pm 0.00 \text{ mg/mL}$). Chlorophyll-a concentration in Pamvotis was 26.26 µg/L in summer and dropped to 5.63 µg/L in winter. In Ziros lake maximum chl a was recorded in winter (8.69 µg/L, Figure 1). Bacterial abundance was similar in the two lakes in summer ($12.4 \pm 0.6 \text{ cells} \times 10^7 \text{ mL}^{-1}$) but followed different trends in winter (Figure 1). In particular in Pamvotis it increased reaching $34.4 \text{ cells} \times 10^7 \text{ mL}^{-1}$ and in Ziros it decreased to $7.73 \text{ cells} \times 10^7 \text{ mL}^{-1}$.

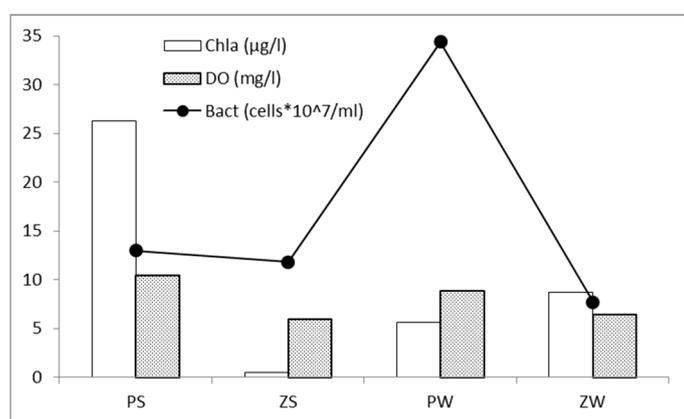


Figure 1. Chlorophyll-a (Chla), dissolved oxygen (DO) and bacterial abundance in Pamvotis (P) and Ziros (Z) Lakes in summer (S) and winter (W).

3.2. Bacterial Diversity

A total of 216,702 sequences and 1091 OTUs were obtained after the initial treatment of the data and removal of singletons, chloroplasts and eukaryotic sequences. The number of OTUs (richness) recorded in Pamvotis was 660 in summer, decreasing to 628 OTUs in winter. In Ziros Lake, richness did not vary between summer and winter (~600 OTUs). Richness and the Chao1 index indicated that the vast majority of OTUs have been revealed during the study (Figure 2). Shannon diversity showed the

lowest value in Ziros lake (2.88) and the highest in Pamvotis (4.53). Both the minimum and maximum occurred in summer. Evenness (Simpson 1-D) ranged between 0.87 and 0.97 in the different samples.

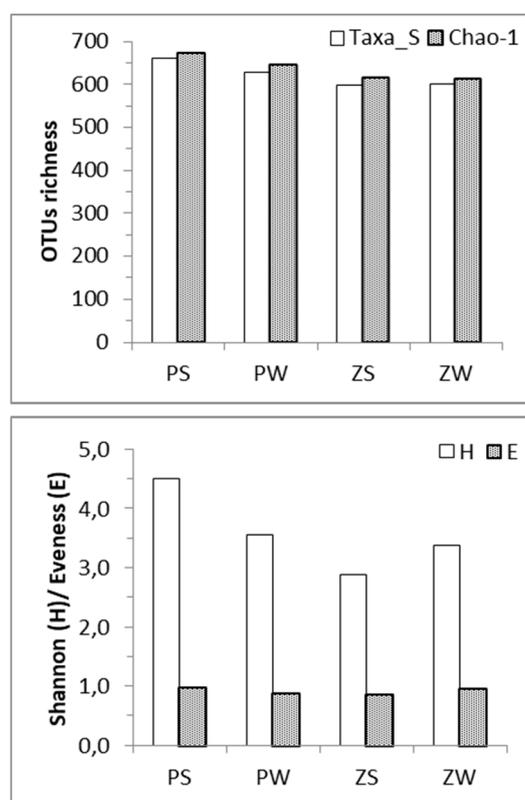


Figure 2. Top: Observed and estimated (Chao1) OTUs richness. Bottom: diversity (Shannon, H) and evenness (Simpson 1-D, E) indices in the two sampling sites (P,Z) and seasons (S,W).

The most dominant bacteria phyla were *Verrucomicrobia*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* followed by *Cyanobacteria* and *Planctomycetes* (cumulative 75%, Figure 3). *Actinobacteria* represented >30% in PS, ZS and ZW. In PW the most abundant phylum was *Verrucomicrobia* (>30%). *Proteobacteria* relative abundance was ~10% in Pamvotis but had higher contribution in Ziros lake both in summer (27.5%) and in winter (19.3%). *Bacteroidetes* represented 17–21% in different samples except in ZS where their contribution dropped significantly. At the family level *Sporichthyaceae* represented >22% in PS, ZS, ZW while *Rubritaleaceae* dominated in ZS (29.7%, Figure 3). Other abundant families representing more than 10% in different samples were: *Flavobacteriaceae* (16% in PW), *Methylophilaceae* (14.5% in ZW), *Methylacidiphilaceae* (13% in ZS) and SAR11 cladeIII (27.5% in ZS). At the lowest taxonomic level we found 6 to 47 OTUs representing 75% of total reads in different samples (Figure 3). OTUs which occurred in high abundances (>9%) in our samples included those affiliated to hgcI clade and unidentified *Sporichthyaceae*, CL500-29 group, *Flavobacterium*, unidentified *Methylophilaceae*, SAR11 Clade III and *Luteolibacter*. Cluster analysis using an unweighted pair-group average (UPGMA) algorithm and based on Morisita similarities between samples at the OTU level indicated similarities ~60% for ZW and PS. ZS and mainly PW samples were highly differentiated from the other lake communities (Figure 4). The environmental variables were subjected to PCA and components with loadings >0.5 were considered in a non-metric multidimensional scaling analysis (NMDS). NMDS (Figure 4) was applied using Bray-Curtis index. Four environmental variables resulted from the PCA analysis (chl_a, temperature, conductivity, TDS), were fitted a posteriori as arrows in order to estimate their relationship with bacterial community structure [40]. Coordinate 1 was related to temperature and to a lesser extent to chl_a while Coordinate 2 was related with TDS and conductivity and again, to a lesser extent to chl_a (Figure 4).

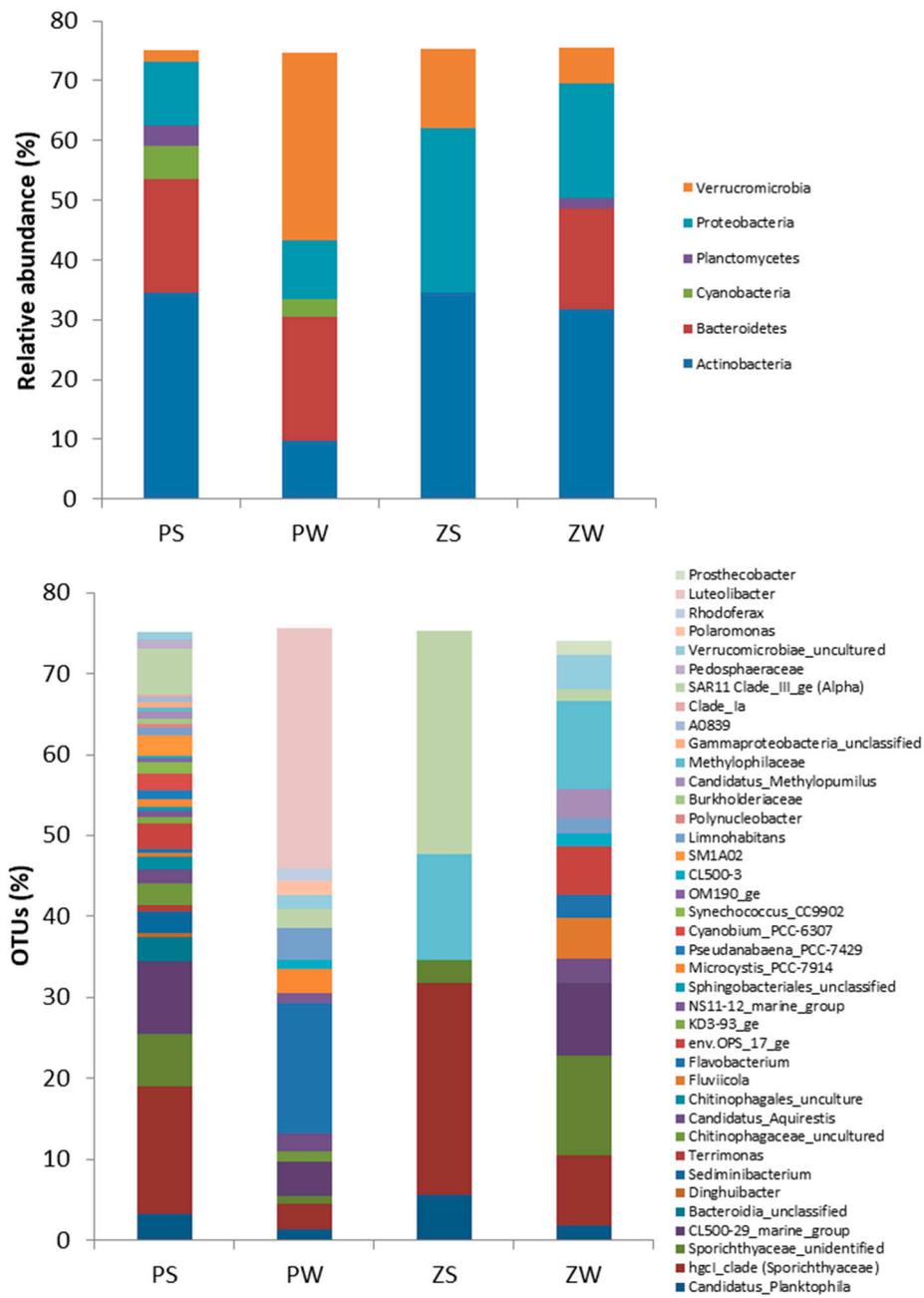


Figure 3. Relative abundance of dominant phyla and OTUs in Pamvotis (P) and Ziros (Z) Lakes in summer (S) and winter (W).

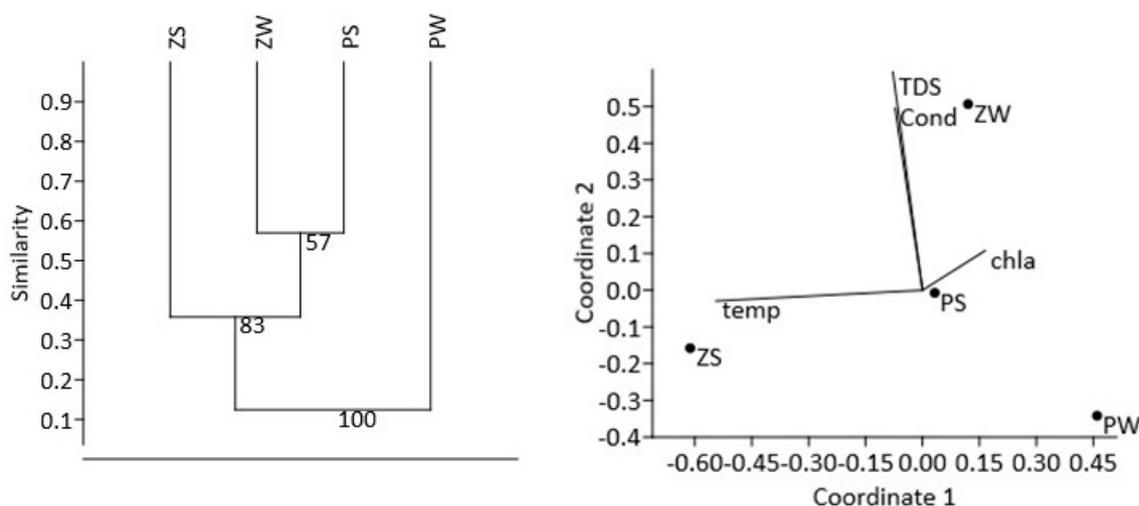


Figure 4. Cluster analysis based on Morisita similarities at the OTU level (**left**) and NMDS analysis based on Bray-Curtis index with fitted environmental variables (**right**) for Pamvotis (P) and Ziros (Z) samples in winter (W) and summer (S). Values at the nodes of the cluster correspond to bootstrap values. The correlation coefficients between each environmental variable and the NMDS scores are presented as vectors from the origin. The length of the vectors is arbitrarily scaled and only their directions and relative lengths should be considered. Temp: temperature, TDS: total dissolved solids, cond: conductivity, chla: chlorophyll-a.

3.3. Microcosm Experiments

Respiration rates (RR) were estimated during microcosm experiments (slope of linear regression analysis, $p < 0.01$, Figure 5). In Pamvotis, RR was $21.11 \mu\text{M}/\text{d}$ in summer, and at the end of the experiment, hypoxic conditions were observed in the experimental bottles ($19.33 \pm 5.40 \mu\text{M}$ or $0.62 \pm 0.17 \text{ mg}/\text{L}$). Lower RR were found in PW ($2.87 \mu\text{M}/\text{d}$) as well as in ZS ($2.50 \mu\text{M}/\text{d}$) and ZW ($2.76 \mu\text{M}/\text{d}$). The slopes of DO concentration over time increased with the rise of temperature by 2°C in PS, PW and ZS but decreased in ZW. We did not find any statistically significant differences between regression slopes for the two temperatures tested (in situ and in situ $+2^\circ\text{C}$, one-way ANCOVA $p > 0.05$). However, statistically significant differences were found for RR in PS microcosm when compared to the rest of the treatments (one-way ANCOVA $p < 0.001$). Respiration rates decreased at the beginning of the experiment and fluctuated thereafter (Figure 4). Respiration rates showed statistically significant correlation (Pearson's r) with chla ($r = 0.96$, $p < 0.05$), T-N ($r = 0.97$, $p < 0.05$), NH_4^+ ($r = 0.95$, $p < 0.001$), T-P ($r = 0.97$, $p < 0.05$) and PO_4^{3-} ($r = 0.97$, $p < 0.05$). Respiration rates at the beginning of the experiment were statistically correlated with T-P ($r = 0.99$, $p < 0.05$) and PO_4^{3-} ($r = 0.99$, $p < 0.05$) as well as with RR measured through the long-term incubation ($r = 0.99$, $p < 0.05$). The effect of temperature was estimated through Q_{10} which was found to increase in Pamvotis from summer (1.33) to winter (2.32). In Ziros lake Q_{10} peaked in summer (2.78) and decreased to < 1 in winter.

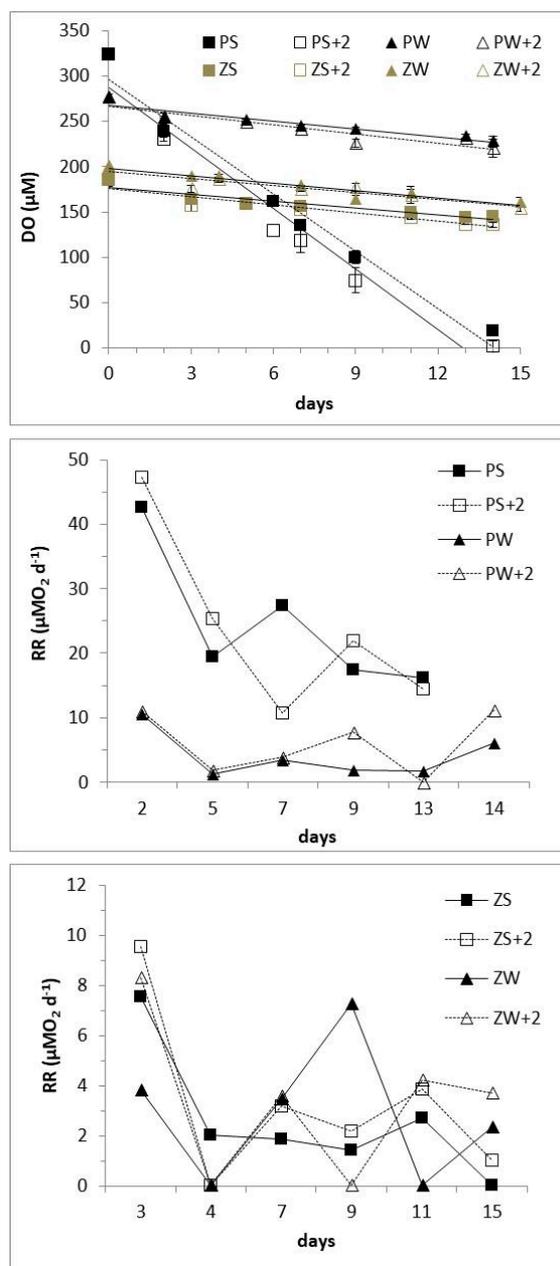


Figure 5. Top: changes in dissolved oxygen (DO) concentration with time. Slopes of regression lines (significance level $p < 0.05$) represent respiration rates (RR). **Middle and bottom:** changes in respiration rates (RR) with time. P: Pamvotis, Z: Ziros, S: summer, W: winter. Incubations at in situ temperature and at temperature increased by 2°C (+2).

4. Discussion

Bacterial diversity was investigated through Illumina sequencing of the hypervariable 3–4 regions of the 16S rRNA, in two lakes of different trophic state in the administrative region of Epirus in Greece. In particular, based on chlorophyll-a values and according to OECD [7] Pamvotis lake was characterized as hypereutrophic ($\sim 26 \mu\text{g/L}$) in summer. In winter, chl-a concentration decreased significantly ($\sim 6 \mu\text{g/L}$). Such low chl-a values have been previously found in Pamvotis only during the initial years following the start of operations of Ioannina's waste water treatment plant which received a part of the city's sewage [41]. They represent a threshold between mesotrophic and eutrophic conditions and in our study are probably associated with the extreme weather conditions i.e., the very low temperatures and the intense snowfall recorded in the area in previous days (www.meteo.gr).

In Ziros lake oligotrophic conditions prevailed in summer with chl_a values <1 µg/L. However, winter measurements support previous studies which characterized the lake as ‘mesotrophic with clear signals of eutrophication’ [22] since chl_a was found close to 9 µg/L, which represents eutrophic conditions. Classification according to TN compares well with that resulted from chl_a data except for ZS, which was found to be eutrophic in both sampling periods. TP values showed hypereutrophic conditions [7] in both lakes.

The bacterial communities were dominated by common freshwater phyla like *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, and *Verrucomicrobia* [42]. *Actinobacteria* with relative abundances >30% dominated all samples except PW (Pamvotis Winter), and consisted mainly of the cosmopolitan, eutrophic-waters associated, CL500-29 group and hgcl clade [43]. The ecological role of freshwater *Actinobacteria* was recently revealed through metagenomic analysis [44]. According to this analysis, *Actinobacteria* perform very well as degraders of recalcitrant organic matter received from the soil and the vascular plants of the watershed, but they are also excellent competitors for nutrients under oligotrophic conditions [44]. So, their predominance in the two lakes with rural and agricultural watersheds (along with urban and industrial activities in the case of Pamvotis) should probably be expected. On the other hand, Pamvotis is a lake with frequent and intense cyanobacterial blooms [45] and the low relative abundance of *Cyanobacteria* in our samples was surprising. A previous study [44] showed a negative correlation between *Actinobacteria* and *Cyanobacteria*, the former not being able to outcompete the latter under conditions that promote cyanobacterial blooms (i.e., high nutrient availability and temperature) and suggested that the predominance of *Actinobacteria* could be regarded as a sign of ‘impending ecological catastrophes’. A fine scale temporal study, during the warm period of the year that promotes the bloom in Pamvotis, would be needed to investigate if succession patterns exist for different autotrophic and heterotrophic bacterial taxa i.e., if *Cyanobacteria* replace *Actinobacteria* during the warm period of the year. However, we should also consider some methodological constraints. For example, the choice of 16S rRNA region may affect apparent community structure, although *Actinobacteria* and *Cyanobacteria* abundance profiles do not seem to differentiate for primers targeting different hypervariable regions [46]. Finally, it seems that the simultaneous use of molecular and microscopy/morphological techniques would help elucidate these controversies [47,48].

In Pamvotis lake in winter, *Actinobacteria* dropped to <10% and instead, *Verrucomicrobia* occurred in relative abundance >30%. These data support recent findings that *Verrucomicrobia* may constitute a high fraction of the bacterial communities [49]. The phylum has been associated with phytoplankton blooms and/or specific complex substrates [50,51] and single cell genomics have revealed genes for enzymes degrading a wide spectrum of polysaccharides by its representatives [52]. The vast majority of *Verrucomicrobia* (>29%) constituted of an OTU associated with the phycophilic/psychrotolerant genus *Luteolibacter* [53]. Thus low temperatures (3.8 °C), probably in combination with the presence of specific substances in the water column [51], triggered the growth and dominance of this particular taxon in Pamvotis lake in winter. Compared to *Actinobacteria* and *Verrucomicrobia*, *Bacteroidetes*’ ecological role in freshwater ecosystems is well known. They degrade autochthonous and allochthonous dissolved organic carbon and so they usually occur in high abundance under post-bloom conditions [54]. In this study, similarly to Schiaffino et al. [55], a higher contribution of *Bacteroidetes* to community structure was found under mesotrophic to hypereutrophic conditions than under oligotrophic conditions.

An indication of methylotrophic activity in Lake Ziros has been revealed through the detection of OTUs affiliated to *Methilophilaceae* (*Gammaproteobacteria*) considered as obligate or restricted facultative methylotrophs [56], in high relative abundance (<10%), both during the low and the high chl_a periods. In winter, ~4% of the sequences were affiliated to *Candidatus Methylopumilus*. Our data are few but they do not show a clear trend of methylotrophs abundance with chl_a, nutrient concentration or temperature as it has been suggested previously [57]. Ziros lake is surrounded by a dense forest of *Fraxinus angustifolia oxycarpa* and several other tree species. It is likely that methylotrophs are favored by the growth and decay of these plant species, which are sources of single-carbon (C1) substrates used for their metabolism [58].

Cluster analysis based on Morisita index showed a grouping (similarity >50%) of bacterial communities from eutrophic and hypereutrophic waters i.e., of those retrieved from Ziros in winter and Pamvotis in summer. Ziros summer (ZS, oligotrophic) and Pamvotis winter (PW, mesotrophic) samples occupied separate branches of the cluster with PW being the most dissimilar. These results differ from previous studies, which showed mesotrophic waters to exhibit similarities either to oligotrophic or to eutrophic waters [17,18]. The complete differentiation of the bacterial community in mesotrophic PW from the other samples supports the suggestion that extreme water physicochemical conditions, like the low temperatures recorded in this study, can shape communities and select taxa that cope well with harsh environment [59]. Actually, NMDS analysis indicated temperature as a factor affecting the community structure of PW. Thus a psychrophilic/psychrotolerant community developed in winter with high dissimilarities to those recorded in the other samplings. Shannon index of bacterial diversity increased with chl_a, in response to the higher spatial heterogeneity of the more productive waters [21]. This could also explain the relatively high similarity of bacterial communities in the two 'high' chl_a samples. It was interesting however that these communities did not show similarities in terms of their metabolic performance (respiratory rates) during the long-term microcosm experiment. Actually, in PS microcosms, the rate of decrease of DO (>20 mg/L/d) was higher compared to the rest of the microcosms (<0.2 mg/L/d) resulting in hypoxic conditions (<2 mg/L) at the end of the incubation period. On the other hand the highly differentiated communities of PW, ZW and ZS had similar respiration rates. Our results should be interpreted with caution and may not be extrapolated to natural conditions due to long-term incubations and associated bottle effects e.g., use of refractory carbon, growth of opportunistic species, accumulation of toxic byproducts, [60–62]. Furthermore, autotrophic respiration may have contributed to total respiration at least at the beginning of the experiment. However, based on the initial assumptions there is strong evidence that oxygen consumption in the bottles is mainly due to bacterial activity. Previous studies using long-term incubations estimated that heterotrophic bacteria contribute ~50% to community [36] and 87% to pico-planktonic size fraction [63] respiration. In this framework, the above findings have implications for ecosystems functioning since shifts in bacterial communities composition allow their assemblages to adapt to habitat 'peculiarities' not investigated here (e.g., substrate availability and quality), so that the outcome in terms of oxygen consumption is the same for the different assemblages i.e., they show similar respiration rates over a range of chl_a concentrations and temperatures found in the two lakes. For example, in Ziros lake in winter, 20 OTUs constituted 75% of total reads and were affiliated mainly to *Actinobacteria* (~32%), *Bacteroidetes* (~17%) and *Gammaproteobacteria* (~18%, Methylophilaceae but also *Limnohabitans*). In summer, fewer OTUs (6) constituted 75% of reads and besides *Actinobacteria* which had similar relative abundance as in winter, we found OTUs affiliated to the clade SAR11-III (*Alphaproteobacteria*) to thrive (~28%). SAR11-III has two lineages, the oligo/mesohaline SAR-IIIa and the freshwater SAR11-IIIb [64]. Since conductivity in Ziros was extremely low, we can speculate that the detected OTU in ZS was associated to the SAR11-IIIb ultramicrobacterium with the oligotrophic lifestyle [65]. As mentioned above, we expect bacterial community structure in the experimental bottles to change during the experiment and we believe that low temporal fluctuations of respiratory rates in the microcosms reflect these changes. The succession pattern is associated not only with the environmental conditions that prevail in the bottles [e.g., available organic substrates, 61] but also to the initial bacterial assemblage [66]. Qualitative changes in bacterial community composition have also been shown previously in freshwater plankton respiration experiments [67] and this is probably a reason for the lack of correlation between respiration and bacterial abundance, size or growth [4].

Respiration rates did not change significantly for a temperature rise of 2 °C; however, Q₁₀ index allowed for the investigation of possible temperature effects under the different conditions met in the two lakes. Our results indicated a high temperature effect at the cold temperatures recorded in Pamvotis in winter (3.8 °C) as has also been previously shown for marine waters [68]. On the contrary in Ziros lake the temperature effect was more pronounced in summer when high temperatures occurred (27.9 °C). It has been suggested that at high temperatures respiration is regulated by the availability of

substrates and/or demands for energy flow [5,69]. Thus, we could suggest that the high Q_{10} values are due to resource limitations associated with the oligotrophic conditions that prevailed.

The rates, at which ecosystem's nutrients recycle, are determined primarily by the actions of their constituent organisms [70]. While at a high taxonomic level we found similarities between samples, constituent OTUs differed reflecting heterogeneities in their habitats. It has been previously suggested that differently composed bacterial communities maintain similar large scale functions like respiration [71]. Our results, although based on few observations from two lakes characterized by distinct oligotrophic to eutrophic/hypereutrophic conditions, corroborate this hypothesis. Furthermore it seems that this functional redundancy might mitigate the impacts of warming in aquatic ecosystems. However, oxygen consumption increased under highly eutrophic conditions. These elevated levels of functioning (respiration) did not reflect a rise of bacterial abundance but are probably related to high competition between taxa when bacterial diversity increased [72]. These results indicate that distinct bacterial communities occur under different lake trophic states as revealed by chlorophyll-a and based on OECD classification. Further studies on a large network of freshwater lakes are needed to confirm such a pattern and to investigate whether water bodies of different trophic state have specific bacterial signatures. Thus, monitoring bacterial communities could become a useful tool for the assessment of the ecological quality of freshwater lakes.

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