Chapter 11
Bacterioplankton

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11.1 Study Approaches

11.1.1 Microscopic Techniques (Direct Counts, FISH)

Long-term data on bacterioplankton or heterotrophic (HPP) and autotrophic (APP) picoplankton (microorganisms of <2 μm size) are not available from many lakes; exceptions include Lake Superior (Carrick et al. 2017), Kinneret (Hadas and Berman 1998), Biwa (Nagata 1984; Okuda et al. 2014), Tanganyika (Pirlot et al. 2005), and Kivu (Sarmento et al. 2008). The HPP studies in Lake Alchichica using DAPI direct counts' staining protocol (Porter and Feig 1980) were carried out from 1998 to March 2020 (Peštová et al. 2008; Macek et al. 2009; Hernández-Avilés et al. 2010; Bautista-Reyes 2011; Sánchez-Medina et al. 2016; Macek et al. 2020; Arrellano-Posadas et al. unpublished).

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During the sampling campaigns, it was found that the annual average of bacteria counts (derived from the whole water column sampling depth-weighted averages) was $2.04 \times 10^6 \pm 1.56 \times 10^6$ and 1.69×10^6 cells/mL as the average ± SD and median, respectively. However, excluding high HPP values (over 1×10^7 cells/mL from two samplings in a mixing period), the average changed to $1.93 \times 10^6 \pm 1.03 \times 10^6$ with a minimal change in median (1.67×10^6 cells/mL). By comparison, the sampling layer HPP counts varied between 4×10^5 and 3.26×10^7 cells/mL.

Besides the direct counts, bacterial biomass was analyzed using image analysis (ImageJ, USA with specific macros). As reported in Hernández-Avilés et al. (2012), the representative carbon content per bacterial cell during circulation and in the aerobic layers is 16.6 fg C/cell, while in the anaerobic hypolimnion is 19.4 fg C/cell. A detailed spatial and annual dynamics of HPP is discussed in Sect. 11.2. However, large purple sulfur bacteria were not included in this analysis and will be further discussed in Sect. 11.3.

After a few years, the unspecific direct counts technique was enhanced by implementing FISH (Hernández-Avilés et al. 2010, 2012), and consequently CARD-FISH techniques (Bautista-Reyes 2011; Bautista-Reyes and Macek 2012), which increased the hybridization efficiency (as percentage of bacteria hybridized with a triple EUB cocktail in direct DAPI counts). This implementation allowed complex studies of the prokaryotic composition in Lake Alchichica (period 2008–2009). The hybridization efficiency was approximately 40% in general, but it reached >60% in the well-established circulation. These results suggest that there is a high percentage of unknown microorganisms in Lake Alchichica, especially during the stratification period. Curiously, the highest EUB hybridization was accompanied by the lowest proportion of identified taxa (Bautista-Reyes 2011).

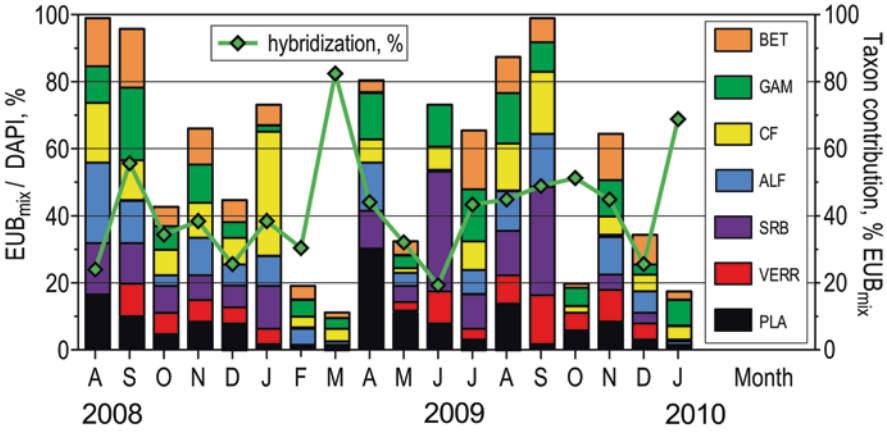

Fig. 11.1 Percentage of hybridized Bacteria (EUB_{mix}) in the FISH-DAPI assays and annual distribution of identified taxa in the Alchichica Lake water column, within EUB hybridized numbers: *BET* Betaproteobacteria, *GAM* Gammaproteobacteria, *CF* Cytophaga-Flavobacteria, *ALF* Alphaproteobacteria, *SRB* Sulfate reducing bacteria, *VERR* Verrucomicrobia and *PLA* Planctomycetes. (Modified from Bautista-Reyes 2011)

The results of CARD-FISH revealed the contribution of diverse aquatic bacterial taxonomic groups, such as Alphaproteobacteria (ALF), Gammaproteobacteria (GAM), Planctomycetes (PLA), and Cytophaga-Flavobacteria (CF) (Fig. 11.1). For example, ALF was the most abundant group in August 2008, but in September 2008 GAM abundance increased. CF dominated in January 2009, whereas PLA dominated the stable stratification. Sulfate-reducing bacteria (SRB), Betaproteobacteria (BET), and GAM were present mainly during stratification. These results also provide insight into the temporal variations of these taxa in Lake Alchichica associated with the hydrodynamic periods, i.e., the physical and chemical changes along the water column.

At the bottom of the metalimnion, which frequently coincided with the oxycline (only layers with DO >0.2 mg/L were integrated), the composition of bacteria showed quantitatively different results (Fig. 11.2). No evident dominance of any bacterial group was observed during the 2008 stratification period but was seen during October when BET dominated. In September 2009, an important contribution of SRB and CF was observed. Alphaproteobacteria (according to other studies, bacteria mainly associated with the genus *Paracoccus*; Hernández-Avilés et al. 2010) became proportionally important. These CARD-FISH results supported the idea that bacteria taxa contribution was continuously changing during the stratification period.

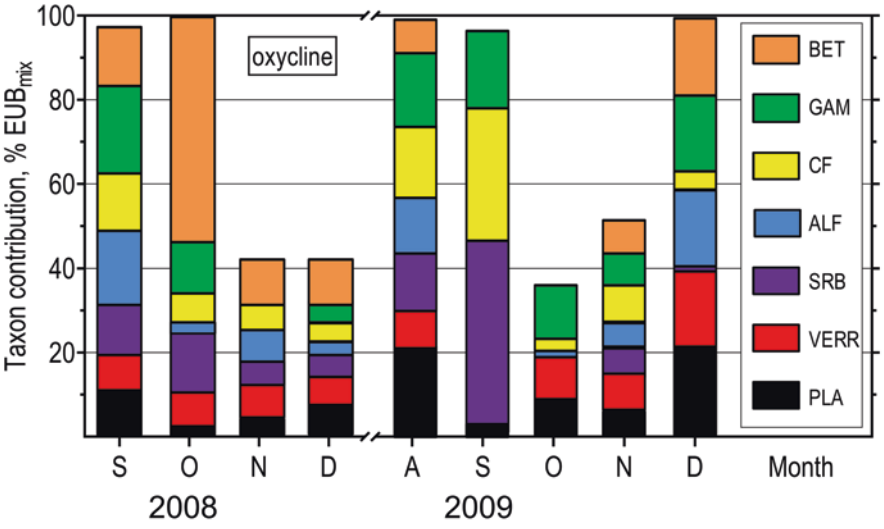

Fig. 11.2 Annual distribution of the identified taxa within EUB hybridized numbers in the oxycline (DO >0 mg/L). *BET* Betaproteobacteria, *GAM* Gammaproteobacteria, *CF* Cytophaga-Flavobacteria, *ALF* Alphaproteobacteria, *SRB* Sulfate reducing bacteria, *VERR* Verrucomicrobia and *PLA* Planctomycetes. (Modified from Bautista-Reyes 2011)

11.1.2 Culture-Independent Gene Marker Surveys

During the past decade (2010–to date), the diversity and metabolic potential of the bacterioplankton was also surveyed using new molecular tools that target gene sequences, particularly 16S rRNA sequences and functional gene markers related to biogeochemical processes. The implementation of new techniques allowed the study of the underestimated bacteria that were not detected by using hybridization approaches (Hernández-Avilés et al. 2010). The next-generation sequencing (NGS) surveys showed that the water column contains several bacteria, with an increasing number with depth (Fig. 11.3a). Further, the number of bacterial and archaeal taxa in the water column is larger during stratification than circulation, as suggested by the CARD-FISH assays (Fig. 11.2; Bautista-Reyes 2011)). For example, there are between 100 and 283 Amplicon Sequence Variants (ASVs) in circulation, whereas

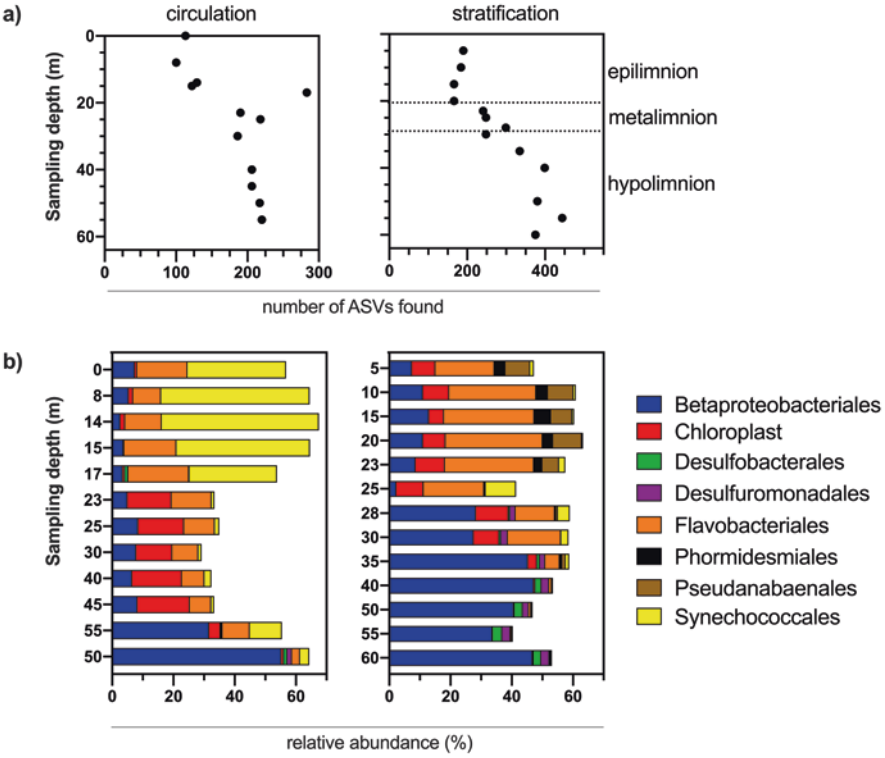

Fig. 11.3 Bacterial survey using the 16S rRNA gene and NGS in Lake Alchichica during the circulation and stratification. (**a**) Number of Amplicon Sequence Variants (ASVs) found in sampling depths. Phylotypes are defined as Amplicon Sequence Variants (ASVs), which are 16S rRNA sequences with 100% nucleotide identity. (**b**) Relative abundance of the main bacterial orders found in the 16S rRNA in the water column, including 16S rRNA sequences related to Chloroplast sequences

in stratification, this number increases to 166–444 ASVs. These variations in the water column are not just in the number of taxa, but also in their taxonomic affiliation (Fig. 11.3b). In circulation, unicellular cyanobacterial belonging to Synechococcales represent 30–60% of the relative abundance, and they are found with Flavobacteriales and diatoms (here mentioned as chloroplasts). However, at the well-established stratification, filamentous cyanobacteria are mostly found in the epilimnion (such as Phormidesmiales and Pseudoanabaenales); whereas some Synechococcales are in the metalimnion (these groups are further considered in Chap. 12, phytoplankton). Despite this high abundance of oxygenic phototrophs, it is also possible to observe diverse bacterial and archaeal taxa, such as Betaproteobacteriales in zones with DO <2 mg/L together with sulfate reducers such as Desulfobacterales and Desulfuromonadales.

Archaea has been also reported in the water column of Lake Alchichica. Hernández-Avilés et al. (2010) found archaeal microorganisms mainly during the stratification in the hypolimnion, yet these results showed low hybridization percentages with the ARCH915 probe. Using the same probe, Bautista-Reyes (2011) and Bautista-Reyes and Macek (2012) found that Archaea exhibited a very low abundance, generally below 5% of direct counts. In contrast, hybridized prokaryotes inside the ciliate cells included up to 10% of Archaea. The presence of archaea has now been confirmed by the 16S rRNA gene sequencing methods. Yet, their relative abundance is relatively low and represents up to 1.2% of the total 16S rRNA sequenced genes. The main microorganisms within this domain belong to the phylum Nanoarchaeaeota, which is also mainly found during the well-established stratification in the hypolimnion. However, Nanoarchaeota were described as interspecies symbionts with other Archaea from geothermal environments (St. John and Reysenbach 2019), yet, their possible host in Alchichica has not been identified.

At the genus level, *Flavobacterium* (order Flavobacteriales) is one of the most frequently found in the epilimnion, whereas *Planktosalinus* is the most abundant genus in the metalimnion. Regarding the order Betaproteobacteriales, the main group was MWH-UniP1, an aquatic group in the epilimnion which is associated with aerobic conditions.

Other gene markers have been used to survey the bacterioplankton in Lake Alchichica. These studies observed the diversity of bacteria related to the N and P cycles (Valdespino-Castillo et al. 2014, 2017; Pajares et al. 2017) and they have reinforced the biogeochemical functions of bacterioplankton in the lake, showing their relevance in elemental cycling. An interesting example is the diversity of microbial ectoenzymes related to phosphorus availability.

The prokaryotic genetic diversity related to the phosphorus cycling has been studied in the water column of Lake Alchichica during the circulation and stratification periods, together with their transcription patterns in a diel cycle (Valdespino-Castillo et al. 2014, 2017). In these studies, the diversity of genes encoding for alkaline phosphatases (*phoX* and *phoD*) and alkaline beta-propeller phytases (*bpp*) were surveyed using specific degenerated primers (reported in Sakurai et al. 2008; Huang et al. 2009; Sebastian and Ammerman 2009). Alkaline phosphatases and phytases are metalloenzymes whose expressions are known to be up-regulated

under phosphorus scarcity, although metal ions such as Ca^{+2}, Mg^{+2} and Zn^{+2} act as cofactors of the mentioned enzymes. Lake Alchichica water exhibits a particularly low Ca:Mg ratio and the diversity of these enzymes follows this environmental constraint (Valdespino-Castillo et al. 2014). These enzymes cleave ester and diester bonds to release phosphate from dissolved organic phosphorus (DOP), which is typically the largest fraction of phosphorus in aquatic systems (Dyhrman et al. 2007). Lake Alchichica's endorheic character was considered to study the natural seasonality of DOP utilization potential though microbial markers, finding seasonal changes in the identity of the DOP utilization microbes (Valdespino-Castillo et al. 2014, 2017) which also exhibited diurnal transcriptional patterns. The largest diversity of alkaline phosphatases *phoD* and *phoX* was related to Alpha-, Beta-, and Gammaproteobacteria, in addition to Bacteroidetes. Rhodobacteraceae (Alphaproteobacteria; e.g., *Rhodobacter* or *Paracoccus* genus), in addition to members of Betaproteobacteria: Rhizobiales (e.g., *Mesorhizobium* or *Agrobacterium*) and Burkholderiales (e.g., *Ramlibacter*, *Ralstonia*, and *Cupriavidus*) showed a close affiliation to the sequences found. Both phosphatases studied showed a close affiliation to phosphatases from Actinobacteria (e.g., *Geodermatophilus*, *Streptomyces*, and *Frankia*). Alkaline phosphatase *phoD* and *bpp* phytases were also related to Gammaproteobacteria (e.g., genera *Azotobacter* and *Pseudomonas*). Some *bpp* phytases were affiliated to Flavobacteriales (e.g., *Riemerella*). Flavobacteriales (*Bacteroidetes*) is an order that was later confirmed by 16S rRNA gene surveys as part of the epilimnion community. Finally, transcript sequences of *bpp* were also affiliated to *Scytonema*, a known diazotrophic cyanobacterium. The protein domain DUF839 detected in *phoX* has been recently described in the proteome of radiation resistant bacteria (Vishambra et al. 2017) as part of the proteins from their outer cell, including periplasmic and extracellular proteins. These recent findings converge with previous findings to point to the extracellular allocation (and function) of these enzymes.

11.2 Temporal and Spatial Dynamics of Heterotrophic Picoplankton

The HPP dynamics in Lake Alchichica follows a similar annual pattern, which is clearly linked to the warm-monomictic thermal regime of the lake (i.e., stratification and circulation periods) (Fig. 11.4a). HPP frequently culminates around the onset of- (between the end of December and January) and/or during the turnover (Fig. 11.4a). A second abundance peak is observed after the thermocline stabilization through June–July (maximum column mean 1.82×10^6 to 1.78×10^7 cells/mL, median of 4.11×10^6 cells/mL), which is occasionally higher than the first peak.

There is not a clear pattern in the distribution of HPP throughout the water column (Fig. 11.4b), as it varied notably. It was not proven that bacterioplankton should be concentrated around the bottom of the metalimnion, whereas local drop of

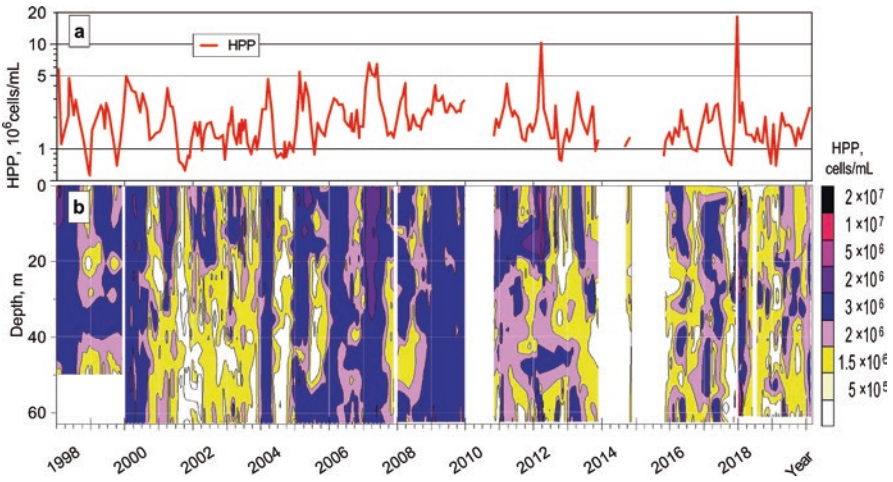

Fig. 11.4 (**a**) Depth weighted average counts of heterotrophic picoplankton, HPP (cells/mL); (**b**) HPP count isopleths (cells/mL)

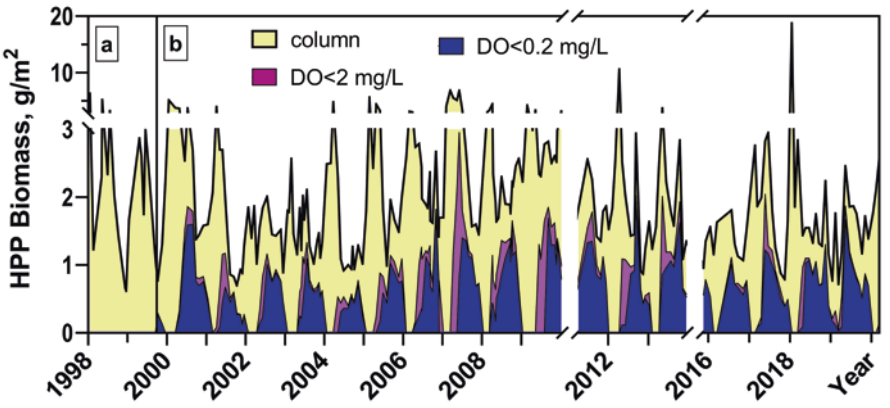

Fig. 11.5 Distribution of the heterotrophic picoplankton (HPP) carbon biomass (g/m^2) within the water column of Lake Alchichica. Total water column (yellow), microaerobic layer (DO <2 mg/L ->0.2 mg/L, violet) and anaerobic (DO <0.2 mg/L, blue) layer. Interval (**a**) represents 1998–2000 where only 5 depths were analyzed thus the oxygen dependent stratification was not evaluated, (**b**) represents a minimum of 10 sampled depths distributed according to the thermal and oxygen stratification

bacterioplankton biomass was observed in the fine scale-sampled upper hypolimnion, along with the peak of ciliate biomass. This behavior could be seen only in a detailed HPP stratification plots (e.g., Chap. 11.3, Fig. 11.8).

The HPP carbon biomass was calculated from the direct counts applying layer specific bacterial cell biomass (Hernández-Avilés et al. 2012). The biomass follows a slightly different pattern than that of direct counts due to the fact that the specific

cell biomass is higher in the anaerobic hypolimnion. However, it was sufficient to shift the maximum biomass peak to the early stratification from May to July, even though mixing period maxima were also observed (peaks from 35 to 295 μg/L, median 76 μg/L).

The HPP biomass was also integrated below the surface area (Fig. 11.5) to evaluate the importance of oxygen concentration in the HPP distribution. This exercise shows the relevance of the anaerobic hypolimnion HPP biomass (at DO <0.2 mg/L) within the total column. The anaerobic HPP biomass-contribution ranged from 60% to 70% between August and October, while in the whole stratification period it showed a mean of 42% (carbon).

Due to the observed relevance of the microorganisms and their metabolism in the oxycline, HPP biomass was analyzed in the layer with DO values between 0.2 and 2 mg/L (Fig. 11.5); in this case, the layer contributes in average only 12% to the total column biomass, with a median of 7%. The absolute maxima (up to 60%) were found to be related to the extended oxycline width (plane thermocline up to 8 m but oxycline with a depletion of 0.5 mg/(L m) through up to 10 m) during June–July stratification. The HPP biomass concentrated in the late stratification-oxycline (<2 m during November–December) was not apparently so important.

11.3 Temporal and Spatial Dynamics of Photosynthetic Anoxygenic Bacteria

Lake Alchichica displays a euphotic zone that includes the oxycline and upper anaerobic layers -the top of the hypolimnion- during the well-established stratification when the whole hypolimnion becomes anaerobic (see Chap. 7, physicochemical characteristics). These conditions explain the possible biological sulfate/sulfur reduction to hydrogen sulfide (see Chap. 17, the deep benthic zone). However, possible anoxygenic photosynthetic bacteria (APB) were not noted in the preparations inspected via epifluorescence microscopy using either DAPI staining or FISH techniques (Hernández-Avilés et al. 2010; Bautista-Reyes 2011; Bautista-Reyes and Macek 2012), possible anoxygenic photosynthetic bacteria (APB) were not observed in the preparations inspected via epifluorescence microscopy until 2012. Then, bacteria possessing bacteriochlorophylls were analyzed in the samples harvested on black 1 μm polycarbonate membranes through their infrared fluorescence but deep strata of the hypolimnion were not sampled in detail until 2017. The images were taken with an IR-sensitive camera excluding cyanobacteria and eukaryotic chlorophyll-possessing cells observed in visible color-images using the same filter sets (specific for chlorophyll *a* and phycobilins). The use of a camera was necessary because the IR autofluorescence was not bright enough for direct counting in the microscope (Fig. 11.6). In addition to the IR images, bacterial elemental sulfur deposits were counted in the Quantitative Protargol Stain (QPS) preparations (see Chap. 13) because a silver sulfide precipitate was observed in the cells (Fig. 11.6b).

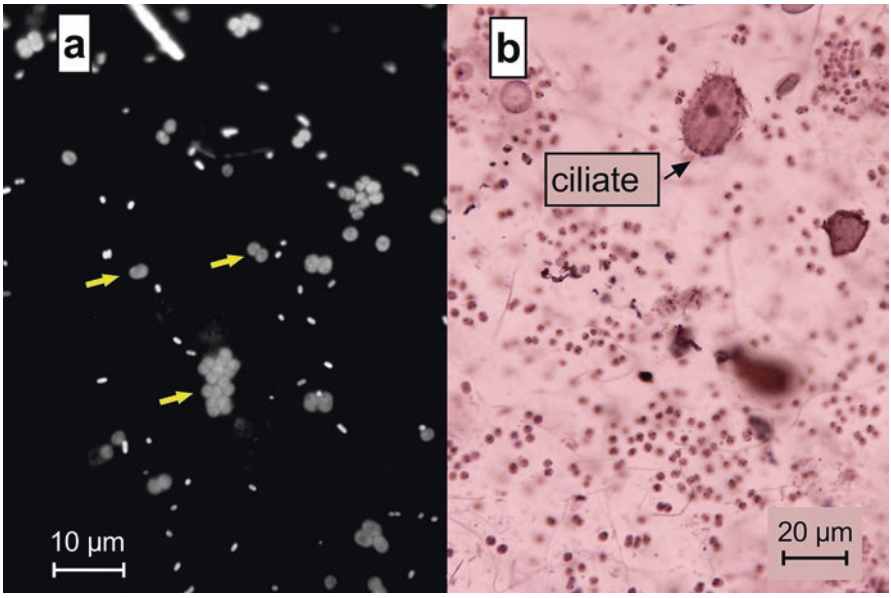

Fig. 11.6 Microscope analysis of anoxygenic photosynthetic bacteria in the water column of Lake Alchichica. (**a**) *Thiocapsa* sp. (marked with yellow arrows) and chlorophyll *a* containing microorganism including picocyanobacteria (bright spots). (**b**) Protargol-stained *Thiocapsa* sp. showing sulfur granules (black spots of silver sulfide) and a scuticociliate

The counts of purple sulfur bacteria (*Thiocapsa* sp.) showed that these microorganisms appear only during the well-established and late stratification (Fig. 11.7). Large *Thiocapsa* sp. was observed generally from July or August until December/January, reaching numbers up to nearly 10^5 cells/mL (Macek et al. 2020; Arellano-Posadas, unpublished). The maxima match with an exhaustion of oxygen and a photosynthetically active radiation (PAR) above 0.1%. The subsequent studies using a 16S rRNA amplicon sequencing strategy confirmed that sulfur oxidizing bacteria mainly belong to the orders Chromatiales and Ectothiorhodospirales, for which the genera *Thiocapsa* and *Thioalkalibrivio*, which are the most visibly abundant. These groups are characteristic of soda lakes (Sorokin et al. 2004; Kompantseva et al. 2007; Baatar et al. 2016).

Thiocapsa sp. direct counts were compared with molecular data using limnologic data as background (Figs. 11.7 and 11.8). In this case, the maximum of *Thiocapsa* sp. counts is found: (i) 2 m below the ciliate maximum biomass (mainly *Euplotes euryhalinus*), (ii) 1 m below the photosynthetic pigments' maximum (i.e., chlorophyll *a* and phycobilins) that matches with that of APP, and (iii) where the Eh reach negative values, and sulfide can be found at an analytically significant concentration. The relative abundance of 16S rRNA sequences related to *Thiocapsa* sp. (and here mentioned as 16S rDNA %) followed roughly the same pattern.

The presence of *Thiocapsa* sp. in a saline, warm monomictic lake such as Alchichica could be explained by its photo/biochemical versatility (van Gemerden

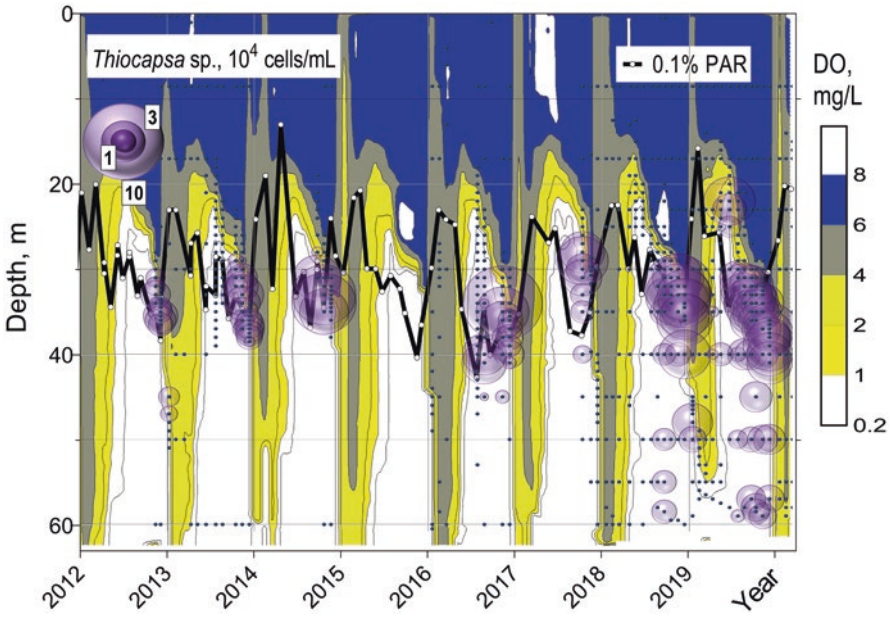

Fig. 11.7 Distribution of the anoxygenic photosynthetic bacteria (*Thiocapsa* sp., cells/mL, purple bubbles) in the water column of Lake Alchichica (2012–2020), quantified by microscopy. (The black dots identify the sampling depths). (Isopleths show the concentration of dissolved oxygen and the thick black line corresponds to 0.1% of photosynthetically active radiation (PAR) in the surface layer)

and Mas 1995; Hemp et al. 2016). This genus is very tolerant to aerobic conditions while it is able to switch between a phototrophic and chemolitotrophic metabolism. Furthermore, this genus was detected as the second most important photosynthetic sulfur bacterium competitive upon sulfide limitation and with higher PAR availability (Avetisyan et al. 2019).

11.4 Conclusions

Microbes, the unseen majority, are responsible for biogeochemical cycling and many other aspects of ecosystems' functioning. Nonetheless, their diversity and ecological properties remain poorly understood. This information is particularly scarce for tropical inland water bodies. The exploration of the microbial communities of Lake Alchichica dating back to the 1990s, constitutes a pioneer example in which spatial and temporal monitoring of the microbial communities is conducted in a deep, tropical lake. Particularly relevant and novel is the exploration of the hypolimnion as a non-homogeneous zone for microbial life. In this sense, the

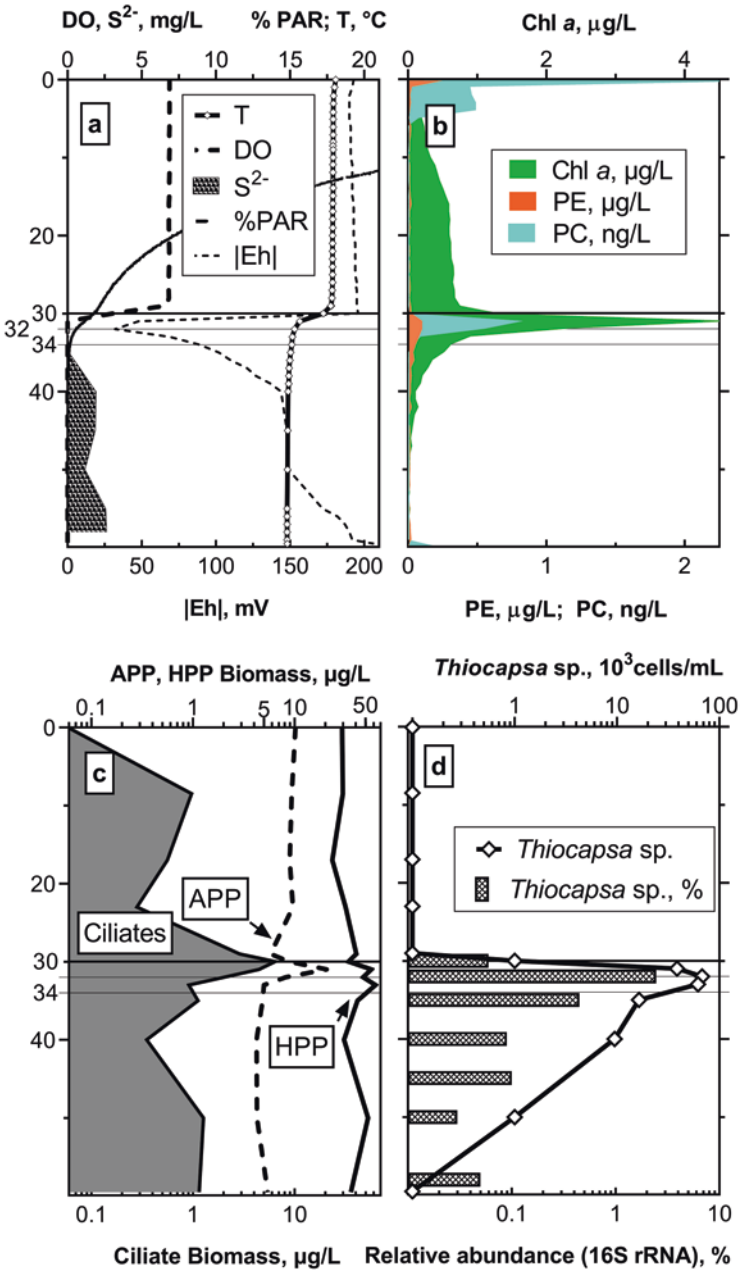

Fig. 11.8 Distribution of photosynthetic anoxygenic bacteria in the water column of Lake Alchichica. (**a**) Limnologic variables in Lake Alchichica (November 15, 2018): Dissolved oxygen (DO), sulfides (S^{2-}), %SPAR, temperature (T), and Redox potential (absolute value, |Ehl. (**b**) Chlorophyll *a*, Chl *a*, phycoerythrin, PE and phycocyanin, PC. (**c**) Biomass of heterotrophic (HPP) and autotrophic (APP) picoplankton, and ciliates; (**d**) Distribution of *Thiocapsa* sp. as direct counts and relative abundance of 16S rDNA sequences. Horizontal lines limit the thermocline and the redox potential drop to negative values

preliminary findings of microbial patterns below the oxygen minimum will contribute to a better understanding of the functioning of the aquatic ecosystem.

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