Chapter 7
Towards a Microbial Conservation Perspective in High Mountain Lakes

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Abstract Microorganisms are fundamental components to maintain the ecological integrity of any ecosystem. Microscopic organisms have been, however, mostly excluded in conservation studies and microbiology has been developed as a scientific discipline lacking a natural history background. The detailed genetic studies carried out in the Aigüestortes i Estany de Sant Maurici National Park and recent works in the mostly scarce literature, show that the mostly oligotrophic and highly diluted waters in high mountain lakes hold a larger microbial phylogenetic uniqueness than expected and are reservoirs of large evolutionary potential, providing an overall natural history perspective for alpine archaea, bacteria, fungi and protists. Microbes arise as an important part of the biological richness of these environments that should be considered as a fundamental component of the natural heritage. Microbial ecologists are now closer than ever to deal with conservation biology concepts such as biological richness, extinction, biotic interactions, and ecosystems management. First insights emerge for establishing the microbial tolerance to different environmental conditions, for estimating which is the potentiality of survival and dispersal abilities in the different species, and for highlighting how the underappreciated microbiota will respond to stresses and disturbances brought by the global change. Warming and eutrophication may jeopardise the most idiosyncratic microbial populations that have found in these (ultra)oligotrophic and diluted systems the most appropriate conditions to thrive. Environmental managers and lawyers, citizen, and stakeholders, in general, have now access to scientifically informed advice for the unseen microbial life in the unexpectedly rich high mountain microbial ecosystems.

Keyword Bacteria · Archaea · Protists · Fungi · Biodiversity · Conservation biology · Lakes

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7.1   Introduction

Conservation biology is the scientific study of biodiversity oriented to protect species, habitats and ecosystems from unsustainable exploitation, uncontrolled extinctions and the increasing weakening of biological interactions. Conservation biology involves the interaction among apparently unrelated disciplines such as social and natural sciences, economics and computational and political sciences, among others, promoting integrative and transdisciplinary views on ecosystem health and aiming to formulate the scientific basis for the best practices in natural resource management. Unlike with plants and animals, microscopic organisms have been mostly excluded in conservation studies and microbiology has been developed as a scientific discipline lacking a natural history background (Margulis et al. 1986). Apparently, the study of microorganisms lacks naturalistic attractive and conservation-orientated perspectives because of microbial inconspicuousness, low probabilities of extinction and potential widespread distribution. Therefore, environmental managers and lawyers, citizens and stakeholders, in general, have obviated the fate of natural microbial communities among their daily worries and strategic planning beyond pathogens. Threats on microorganisms have been not considered as part of the natural resources management policies or in the estimation of the influence of human activities in nature. Ecosystem functioning carried out by microorganisms appears to rely on a high functional redundancy and its maintenance could be largely uncoupled to microbial biodiversity erosion (Wertz et al. 2006). Microorganisms, however, are the most abundant and widespread forms of life on Earth and encompass the highest taxonomic, metabolic, genetic, and functional diversity. They rule the whole biodiversity on Earth and their activities have a major ecosystem effect (Madigan et al. 2015). Conservation biology perspectives where the fundamental unit in the conservation of biodiversity is not the species but the habitat would probably apply very well for microorganisms.

In the early 90s of the past century, the International Programme of Biodiversity Science DIVERSITAS, a programme promoted among others by the International Council of Scientific Unions (ICSU) and now migrated to both Future Earth (http://www.futureearth.org/) and the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES), tried to reverse this lack of knowledge highlighting the crucial role that microbial biodiversity plays in the maintenance of many ecosystem services. The term "ecosystem services" describes ecosystem resources and processes that benefit human society, and its identification and value quantification can provide additional arguments for the protection of species and ecosystems that could easily reach public opinion and policy decisions (Daily et al. 2009). DIVERSITAS emphasised the immense genetic diversity of microorganisms and their crucial and unique roles as essential components of food chains and biogeochemical cycles and included "microbial biodiversity" within the nine fundamental cross-cutting research themes of critical importance for

biodiversity science. The Microbial Biodiversity programme called to develop innovative methods and techniques to accelerate the discovery and characterization of microbial diversity, and to establish reliable databases to collect and exchange information on the biological characteristics of microorganisms. It was particularly encouraged to improve the knowledge in freshwater microbial diversity to increase the available understanding of the effects of microbial diversity on aquatic ecosystem functioning, suppression of diseases organisms, provision of clean water through the respiration of organic carbon, denitrification and other metabolic processes, and to gain insights into the functioning and microbial regulation of biogeochemical cycles. The programme also urged to explore major issues concerning the conservation, origin and maintenance of microbial biodiversity.

In the past 20 years, and mainly over the past decade, microbial ecologists have developed, optimised and standardised powerful methods to capture microbial taxonomic and functional diversity. They have successfully combined multidisciplinary approaches from different scientific disciplines such as microbiology, ecology, molecular biology, bioinformatics and computational science and have efficiently linked available information on microbial diversity within a worldwide network. This sustained effort circumvented some of the methodological and conceptual concerns that had strongly limited the general perception of how important microbes are for Earth biodiversity and initiated the effective transplantation of concepts and basic knowledge of the general ecology grounded on plants and animals to microbial ecology. Efforts are now addressed to establish a predictive framework for the distribution and diversity of microorganisms, blurring the disciplinary boundaries that traditionally separated ecologists of tall and tiny. Currently, global initiatives such as the International Census of Marine Microbes (ICoMM), the Earth Microbiome (Gilbert et al. 2014) and the Human Microbiome projects (Methé et al. 2012), as well as international marine initiatives to profusely explore the microbial component in the surface (Tara Oceans Cruise, JCVI Global Ocean Survey, Ocean Sampling Day) and in the deep ocean (Malaspina Expedition) have been successfully promoted. The creation of an interdisciplinary Unified Microbiome Initiative to understand and harness the capabilities of the set of Earth's microbial ecosystems has been recently proposed (Alivisatos et al. 2015; Dubilier et al. 2015). Although, nothing equivalent is found for the freshwater realm despite the fact that the wide repertory of inland waters on Earth (Downing et al. 2006) contains a large, novel and unexplored microbiota (e.g. Hahn 2006; Esteban and Finlay 2010; Barberán and Casamayor 2011; Newton et al. 2011).

In the case of aquatic ecosystems from remote high-altitude mountain areas, the gap of knowledge is still big. Both the oligotrophic and highly diluted nature of the lake waters and the difficulties to collect samples in such painful to reach places have limited the interest among microbiologists to explore these isolated environments. Comparatively, such remote inland waters have remained less explored than lowland freshwaters. Although, aquatic microbes are abundant in the plankton

of these lakes (Felip et al. 1999), and the environmental heterogeneity at the local scale is larger than expected (Catalan et al. 1992, 2006). In addition, mountain lakes have been traditionally studied by limnologists with a background of ecology and phyto- and zooplankton biology (e.g. Catalan et al. 2006 and references therein, Tolotti et al. 2009) rather than by microbial ecologists with a background in microbiology and genetics. Consequently, the genetic diversity, taxonomic identity and ecological distribution of the unseen majority in genuinely high mountain systems have remained mostly unknown. A few investigations in the past years are however helping to fill this gap, primarily in the Alps (Pernthaler et al. 1998; Pérez and Sommaruga 2011), the Himalayas (Liu et al. 2006; Sommaruga and Casamayor 2009; Kammerlander et al. 2015), the high mountains of west USA (Nelson 2009; Hayden and Beman 2016) and, specially, in the Central Pyrenees within and around the Aiguestortes i Estany de Sant Maurici National Park (Catalan et al. 2006). Meteorologic variability, catchment inputs, and warming are key factors structuring microbial communities (Nelson 2009). These lakes are very sensitive to detect an excess of reactive N of human origin circulating through the atmosphere (Camarero and Catalan 2012). Some of these lakes are glacier-fed ecosystems and hold specific physical conditions, biodiversity and ecological functioning (Edwards et al. 2013; Peter and Sommaruga 2016). High mountain lakes formed by glaciers erosion are very comparable worldwide ecosystems, and the new lakes that are currently appearing after the glacial retreat in mountain areas offer great opportunities for ecological and limnological studies (Catalan and Rondon 2016). Microbial diversity in high-altitude aquatic ecosystems from highlands such as Tibetan lakes (Zhang et al. 2013) and Andean lakes (Ordoñez et al. 2009) are not considered here because of the consistent limnological, physicochemical and environmental differences mostly characteristic from the relatively flat terrain of plateau areas.

Probably, one of the most intensive and extensive limnological studies of alpine lakes to date have been carried out in the Central Pyrenees (Catalan et al. 2006). Extensive studies on microscopically conspicuous organisms have been already done (Catalan et al. 2015; Felip et al. 1999; Pla et al. 2003) and will be obviated here. Using environmental ribosomal RNA genes sequencing, a sample set representative of the lacustrine Pyrenean landscape heterogeneity has been studied in detail for bacteria, archaea and mostly inconspicuous microbial eukaryotes biodiversity, as will be presented further in this chapter. This alpine area contains the main freshwater lake district of south-west Europe, and constitutes a mosaic of highly diverse water bodies mimicking the geological diversity of the catchments (Catalan et al. 1992) that usually remain ice-covered for 4–7 months every year (typically from December to April). Very low concentrations of nutrients and salts (i.e. ultraoligotrophic and hypotonic waters), persistent extreme conditions (high UV exposure and water transparency, and low temperatures), and isolation might promote an adapted microbiota with a large number of specialist populations (Catalan et al. 2006). Although, lakes located at high altitudes efficiently integrate

information about changes in the catchment and land use and in the atmosphere and are very sensitive to environmental and global changes (Adrian et al. 2009), mainly remote depositions and global warming. These changes may induce rapid changes in the microbial community composition and potentially erode the most idiosyncratic populations leading to species decline by ecosystem degradation. Experimental evidence is however needed to test these hypotheses. New approaches to predict the impacts of global change at the microbial community level are also required for scientifically informed conservation and management of biodiversity and ecosystem services (Bellard et al. 2012).

7.2 A Biodiversity Unit for the Microbial World

Systematics and taxonomy are fundamental tools to track biological history understanding life forms origins and relationships and to help to organise biological complexity knowledge to support biological conservation, respectively (Cotterill 1995). Estimating the number of microbial species even at the order of magnitude is, however, a great challenge in biology and matter of intense debate in microbiology. Two of the main problems microbiologists have to successfully face this challenge are the own definition of the species concept and the little success bringing into a culture most of the wild microbes, respectively (Fig. 7.1). The species definition for bacteria requires individuals previously isolated and grown in culture and needs highly standardised laboratory protocols, comparative genomic information and a dataset of physiological and other phenotypical features. Thus, a pragmatic phylophenetic species concept for microbial taxonomists is only useful if pure cultures are available in the laboratory (Rosselló-Mora and Amann 2001). Currently, c. 13,000 bacterial and archaeal species are available in culture (Amann and Rosselló-Móra 2016). The last estimation using >20,000 microbial molecular surveys and mathematical modeling and scaling laws predicts the existence of between 10^{11}–10^{12} microbial species on Earth (Locey and Lennon 2016). If true, and according to the current number of catalogued microorganisms, that would mean that 99.999% of total microbial species are missing still. However, the lack of consensus ranges of several orders of magnitude. Estimations based on the empirical analysis of the bacterial and archaeal 16S rRNA gene sequences available in curated databases (c. 1.5 million full-length sequences), reduce the molecular census to a few millions Operational Taxonomic Units (OTU, 97% sequence identity "species-level" cutoff) (Schloss et al. 2016). In addition, it has also been predicted that environmental 16S rRNA gene sequences of the highest novelty are reaching a plateau and that most of the high microbial taxa will be discovered within a few years (Yarza et al. 2014). Still, to carry out the inventory and interpretation of the most abundant organisms on earth is a vast enterprise that will keep microbiologists busy for many decades. Telling apart microbial species is still highly controversial and difficult, and a consensus definition

of what should be the most operative and predictive unit to measure biological diversity in the microbial world is still under discussion (range 97–99% identity in 16S rRNA gene sequence, Kim et al. 2014). Luckily, diversity can be studied with any coherent and well-defined standardised unit as far as it is defined in a simple, clear, and unambiguous way. The ribosomal RNA genes are still the most successful proxy for a combined view of systematics and taxonomy in microorganisms circumventing the limitation that only a very small number of microbes can grow in culture media. Although, two intrinsic limitations should be considered. First, the use of a proxy too conservative for the "species level" that underestimates species diversity. Second, the fact that it fails to detect fast speciation processes in natural communities by horizontal gene flow. These genetic processes can maintain adaptability and provide ecological success to colonise particular environments (e.g. Llorens-Marés et al. 2017), leading to the emergence of a new "ecological species" nearly identical in the 16S rRNA gene sequence to a former population.

Altogether, microbial ecology is still in its infancy predicting species distributions across landscapes and identifying areas of high and low species richness, or highlighting whether or not vulnerable groups of microbial species or microbial processes exits, missing useful information for land management. There is considerable diversity to be explored yet, but the rate of new full-length sequences deposited in databases has consistently declined in the last years since next-generation sequencing (NGS) and high throughput has been expanded (Schloss et al. 2016). Although microbes can currently be reasonably well identified and classified in relation to each other allowing fast and proper universal
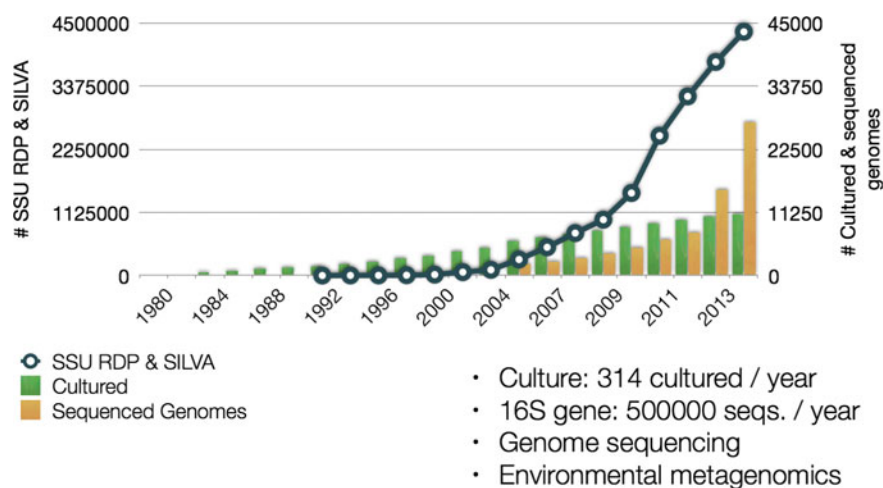

Fig. 7.1 Temporal trend and annual rates for the available number of pure cultures of bacteria and Archaea (*green label*), 16S rRNA gene sequences in curated databases (RDP and SILVA) and genomes (obtained from cultured strains and metagenomic surveys) (*orange label*). Data from Llorens-Marés 2015, Ph.D. thesis, University of Barcelona

communication among microbial ecologists, a high-quality census needs full-length 16S rRNA gene sequences and the short sequence lengths provided by current NGS methodologies need to be substantially improved (Amann and Rosselló-Mora 2016). Genomes and metagenomes currently available are growing exponentially (Fig. 7.1), and single cell genomics permits to reach the genetic potential of a microorganism without culturing. For the microbial world, the inadequacy of methods and conceptual separation of microbiology from natural sciences with strong ecological and evolutionary background such as zoology and botany should not be an unaffordable challenge anymore. In fact, microbial systems may push classical natural sciences disciplines and theoretical ecology forward to new unexplored frontiers. An integrative approach prevails today in the environmental sciences with an inclusive view on biological interactions networks (Faust and Raes 2012; Fuhrman et al. 2015), and on the integration of molecular biology at the community and ecosystem levels (Raes and Bork 2008). Merging community ecology and phylogenetics among co-occurring species can provide a new view for the study of microbial assemblages in situ (Barberán and Casamayor 2014). The use of phylogenetic approaches as a measure of biodiversity based on the phylogenetic difference between species (i.e. phylogenetic diversity, PD), offers new perspectives without previously fixing an operational taxonomic unit definition, and reduces to a single value the whole community complexity (see below). This approach may help to find patterns and to develop hypotheses based on the coexistence and adaptation of closely related species and to try to unveil the processes that shape community structure and composition.

7.3   A Natural History Perspective for Microorganisms in High Mountain Lakes

High-altitude mountain lakes hold a larger microbial biodiversity than could be initially expected in such very diluted waters. Typically, several hundred million prokaryotic cells and around a million of microscopic eukaryotes are present per litre of alpine lake water (Felip et al. 1999). In general, freshwater archaea in high mountain lakes show one to two orders of magnitude lower abundances than bacterial cells, and the richness within the Bacteria domain is substantially higher than within the Archaea. For instance, the analysis of the plankton in three connected shallow Pyrenean lakes within the Aiguestortes i Estany de Sant Maurici National Park estimated a bacterial richness of c. 2500 OTUs and an archaeal richness of c. 900 OTUs (Fig. 7.2, upper panel). These estimations required a sampling effort of c. 250,000 bacterial and c. 20,000 archaeal 16S rRNA gene sequences (Fig. 7.2, lower panel), only accessible through recent NGS technologies.
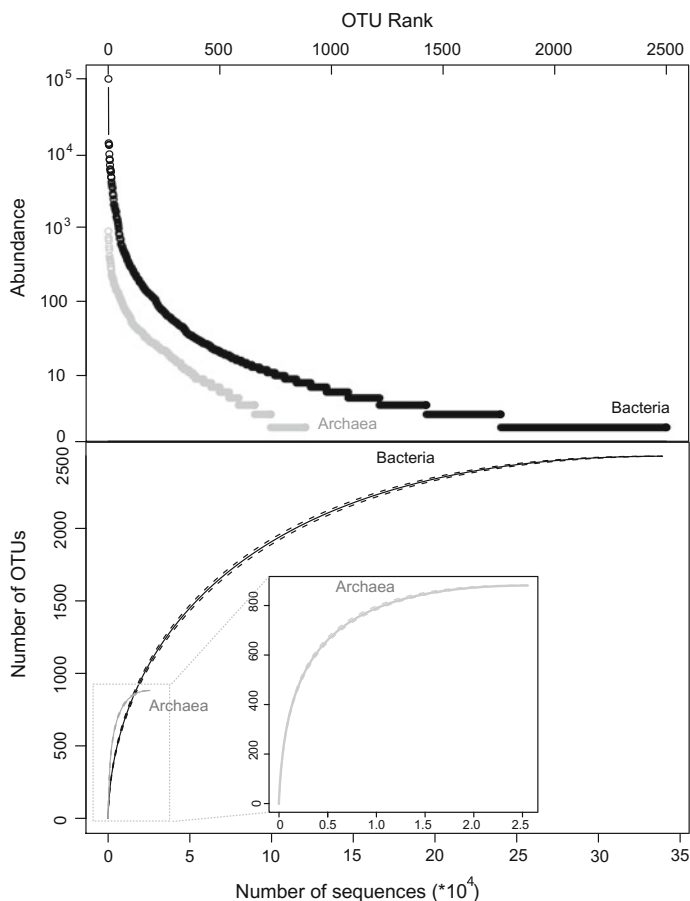
Fig. 7.2 Rank abundance (*top*) and rarefaction curves (*bottom*) for bacterial and archaeal OTUs inhabiting three connected shallow Pyrenean lakes communities within the Aigüestortes i Estany de Sant Maurici National Park. From Vila-Costa et al. (2013) with kind permission from Oxford University Press

7.3.1 Bacteria

The major bacterial taxa present in high mountain Pyrenean lakes are characteristic of the plankton present in worldwide freshwater environments and different from the oceans (Fig. 7.3, data from Barberán and Casamayor 2010). Bacteroidetes, Betaproteobacteria and Actinobacteria are the predominant groups in high-altitude lakes, with a significantly higher proportion of Betaproteobacteria and a lower proportion of Alphaproteobacteria than in lowland freshwaters (Barberán and Casamayor 2010). Also, the lakes' ecosystems can be synoptically sorted according to the evolutionary history contained in the whole bacterial assemblages without the
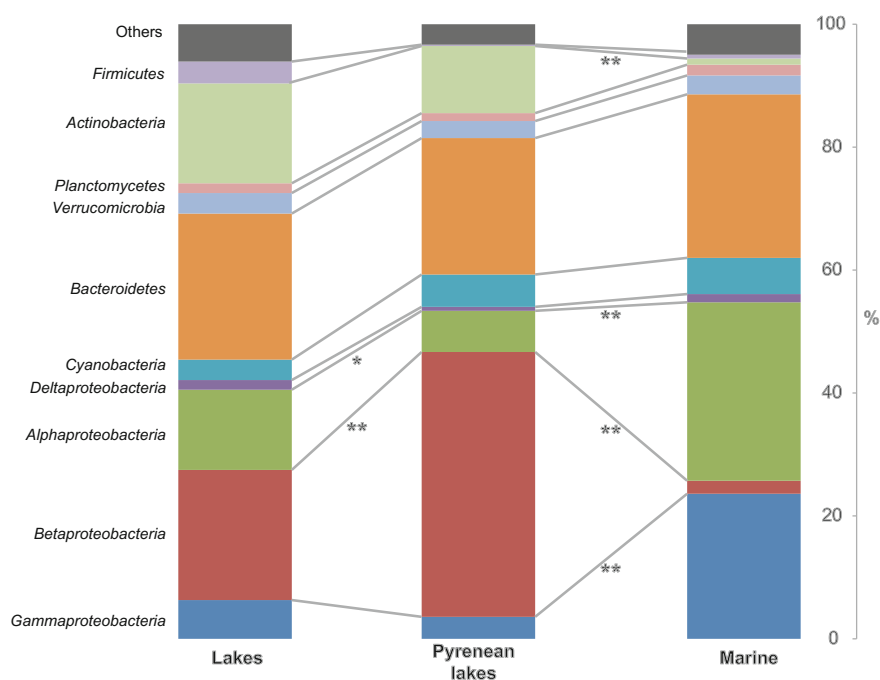
Fig. 7.3 Bacterial taxonomic composition of Pyrenean lakes, and comparison with a global meta-analysis of lakes and seas (Barberán and Casamayor 2010). Significant differences are shown (*$P < 0.05$, **$P < 0.01$, t-test). From Barberán et al. (2012), Ph.D. thesis, University of Barcelona

need of any particular taxonomic level using the phylogenetic dispersion of communities, through the phylogenetic diversity index (PD) (Barberán and Casamayor 2014). High or low levels of PD did not necessarily match those Pyrenean lakes with the highest or lowest OTUs richness, respectively, adding relevant information for stakeholders and managers and showing where most of the biological diversity accumulates (see Supporting information in Barberán and Casamayor 2014). Higher PD might result in higher functional diversity and versatility of the bacterial assemblages, but this assumption remains to be tested. This integrative tool also provides proper interbiome comparison. Interestingly, when the Pyrenean dataset is compared with a similar study of the plankton from surface waters in different seas and oceans (first 5 m sampled), and after correcting for unequal number of sequences between the studies, it arises that freshwater bacterial communities accumulated higher genetic dispersion than the very surface marine assemblages (Fig. 7.4). This result nicely contextualises any bacterial community circumventing culturing and species definitions limitations and agrees with the highest environmental heterogeneity present in the Pyrenean dataset analysed (see below). It also indicates that microbial diversity is a direct reflection of habitat diversity.

Fig. 7.4 The potential of phylogenetic diversity (PD) measures to direct intrabiome and interbiome comparisons. From Barberán and Casamayor (2014) with kind permission from John Wiley & Sons, Inc.
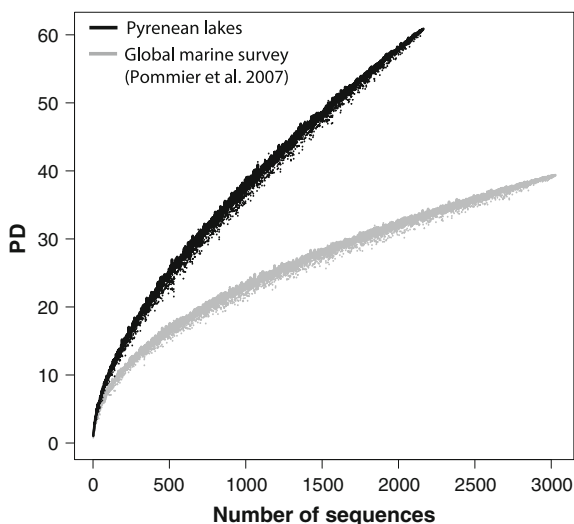

Fig. 7.5 Relationship between phylogenetic diversity (PD) and lake area. Significant linear regression line for the log-transformed relationship. From Barberán and Casamayor (2014) with kind permission from John Wiley & Sons, Inc.
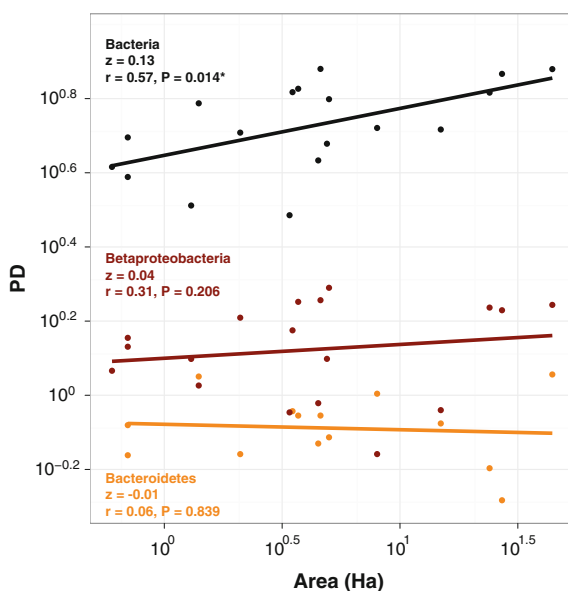
A significant relationship between Pyrenean lake area and PD of bacterial assemblages also exists (Fig. 7.5). Interestingly, the slope (z) of the log-transformed relationship (0.130) was similar to the value found in a study carried out in mountain lakes in Sierra Nevada (0.161) using OTUs obtained from a bacterial genetic fingerprinting profile (Fig. 7.6 panel A). The slope z is usually <1 meaning that, although the number of species increases with area, larger areas have in

proportion fewer species per unit area. In the case of bacteria, the z value (the slope of the species area relationship) is closer to the lower end, similar to the values reported for other planktonic organisms (Fig. 7.6 panel B). The species–area relationship (SAR) has been successfully used in macroecology and conservation biology to predict extinction according to the habitat reduction. In the case of microorganisms, how reliable are the extrapolation of the ends of the slope can be now better explored using massive sequencing technologies. Most microorganisms likely show long-distance dispersal abilities and large population sizes modulating the relative importance of niche, stochastic and historical processes that shape the structure of microbial communities (Barberán et al. 2014a), and first insights show that bacterial ubiquity may be a common pattern in high-altitude lakes worldwide (Sommaruga and Casamayor 2009). Cell dormancy, high persistence, and the fact that a single cell can generate a new population in a short time are microbial attributes that all together should be considered to accurately address the mechanisms that generate this pattern. Most probably, the presence of more available niches in larger lakes, a higher number of interactions, and more complex food webs may play a major role in determining bacterial richness and phylogenetic

Fig. 7.6 **a** Significant relationship (z value, the slope of the species area relationship) found between bacterial OTU richness and alpine lakes area in Sierra Nevada, SE Spain. **b** Comparison of z values among taxa. From Reche et al. (2005) with kind permission from John Wiley & Sons, Inc.
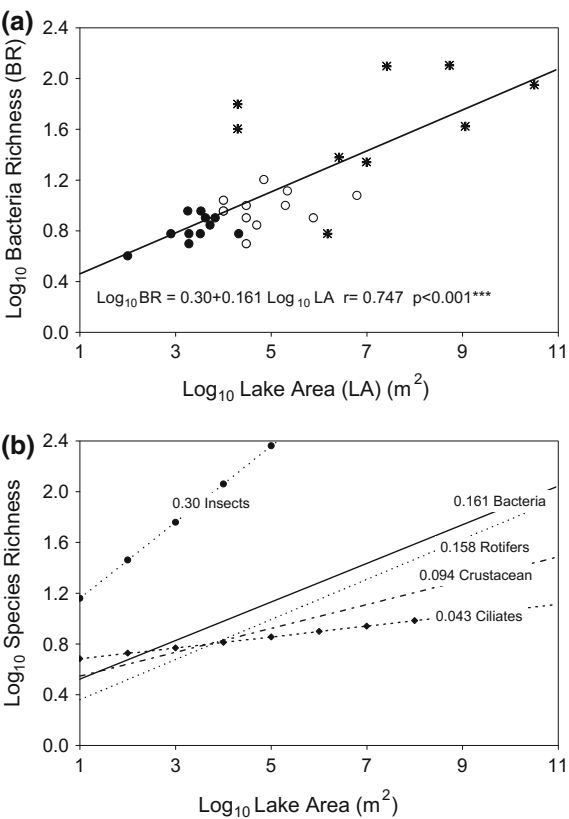
dispersion. Additional studies exploring the mechanisms that generate this pattern, the interbiome and empirical variability of the z values mentioned above, the variations with taxonomic resolution (Horner-Devine et al. 2004) and the limits and spatial scaling, are topics that microbial ecologists may be interested in developing further and high mountain lakes offer a very convenient natural scenario to explore it. If microorganisms are globally dispersed and cosmopolitan, the OTUs locally present in Pyrenean lakes will represent a large fraction of the cumulative species pool present in high mountain lakes around the world. Because of the high similarity of this type of environment across continents, this is a hypothesis that can be reasonably well tested.

The quantification of community similarity based on phylogenetic relatedness also provides new perspectives to bridge the gap between evolutionary and ecological analyses (Barberán et al. 2014a). Patterns capturing how phylogenetic community similarity is distributed along environmental gradients emerge without relying on any particular operational taxonomic unit definition (Barberán and Casamayor 2014). After testing for biogeographical patterns in Pyrenean lakes using distance matrix based on both environmental variables and geographical distance, environmental filtering and not spatial distance is most probably shaping the phylogenetic structure of the freshwater bacterial assemblages (Fig. 7.7). The high environmental richness within small distances in the Pyrenean lacustrine area, covered a gradient of environmental and trophic conditions of more than four units of pH, one order of magnitude of conductivity (from highly diluted to typical freshwater values), 10-fold phosphorous concentration, and 20-fold chlorophyll-a
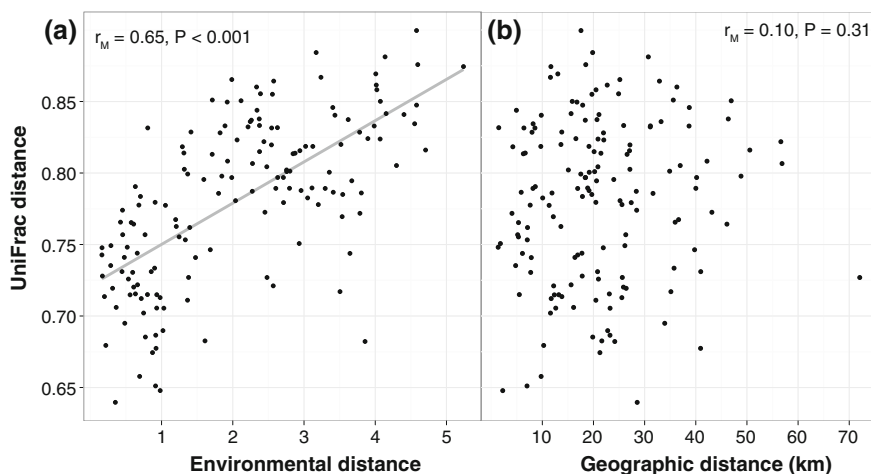
Fig. 7.7 Relationship between the UniFrac distance matrix and **a** the environmental Euclidean matrix (E) or **b** the spatial distance matrix (S). UniFrac is a β-diversity metric that quantifies community similarity based on phylogenetic relatedness. From Barberán and Casamayor (2014) with kind permission from John Wiley & Sons, Inc.
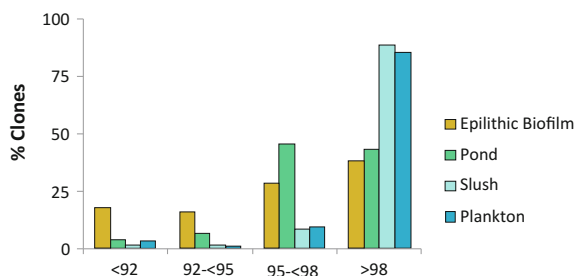
Fig. 7.8 Taxonomic novelty (percentage of identity with DNA sequences available in GenBank) for the bacterial 16S rRNA gene sequences found in the epilithon biofilm, plankton, slush (mixture of water and snow) and snow melting ponds in lakes of the Pyrenees. From Bartrons et al. (2012) with kind permission from Springer Publisher

content, being most of the lakes ultraoligotrophic or oligotrophic. Again, the higher than expected habitat richness provides a rich background for diverse planktonic bacterial communities.

Bacteria are also highly abundant and diverse in the epilithic biofilms of streams and lakes, although no consistent global elevational patterns in biodiversity for stream bacteria exist (Wang et al. 2017). These biofilms play, however, a relevant and specific biogeochemical role in mountain lakes (Vila-Costa et al. 2014). Bacteroidetes and Cyanobacteria are the most common groups found in the epilithon, whereas Actinobacteria were not detected and Betaproteobacteria were present in low abundances (Bartrons et al. 2012). Interestingly, most of the epilithic Bacteroidetes form distinct phylogenetic clusters and may represent particular poorly known ecotypes with a potentially major role in the organic matter cycling. In fact, the taxonomic novelty analysis for the bacterial 16S rRNA gene sequences showed that only 40% of the epilithon bacteria had been previously reported at the "species" level. Such value reached >80% in the slush (Llorens-Marès et al. 2012) or the plankton (Fig. 7.8). Interestingly, >25% of the epilithon species may potentially represent new bacterial families or even orders (identity in 16S rRNA gene < 95%). This idiosyncratic assemblage may be related to the large heterogeneity operating at the microscale, closer microbial interactions and coexistence of different physiologies and aerobic, anaerobic, phototrophic and chemotrophic metabolisms (Bartrons et al. 2012; Vila-Costa et al. 2014) a highly remarkable unexpected feature under such (ultra)oligotrophic prevailing conditions that deserves further studies. Potential for nitrogen fixation (i.e. presence of *nifH* genes) was also detected in the biofilms (Vila-Costa et al. 2014). Altogether, epilithic biofilms from mountain lakes and streams could hold a hotspot of microbial diversity, very rich in poorly known microbial species. The different taxa are substantially different from the bacterioplankton species and from previously reported gene sequences in databases, adding relevant spatial heterogeneity for the microorganisms in these environments.

7.3.2 Archaea

Archaea are commonly present in freshwater plankton, but most have remained unseen to aquatic ecologists and limnologists. For many years, archaea strains available in the laboratory were restricted to methanogens and microorganisms adapted to extreme temperature, pH and salinity. Apparently, the archaeal metabolic diversity and ecological distribution appeared more limited than their bacterial counterparts. In the most recent years, the environmental ribosomal RNA surveys unveiled Archaea as ubiquitous in freshwaters. Most of them but methanogens are distantly related to any laboratory strain, and there is ample room for new discoveries related to archaea and cold water habitats in lakes (Auguet et al. 2010). Curiously, freshwater archaeal richness and diversity appeared higher than in other biomes such as the oceans and soils after a meta-analysis of globally distributed clone libraries of the 16S rRNA gene (Auguet et al. 2010).

In the Pyrenean high mountain lakes, archaea are widespread and diverse. Archaea could be detected in 90% of surface waters in a large dataset of lakes examined (n = 313), with relative abundances generally up to 10% of the bacterioplankton sequences (Ortiz-Alvarez and Casamayor 2016). Alpine archaea belong to 13 different lineages (Fig. 7.9), with Pacearchaeota and Woesearchaeota as the most common groups, followed by Micrarchaeota–Diapherotrites (Euryarchaeota MEG cluster), Methanogens, Thermoplasmata and planktonic AOA (ammonia-oxidising Thaumarchaeota). Minor groups are related to the SM1K20 cluster, Aenigmarchaeota (Euryarchaeota DSEG cluster), MCG (Miscellaneous Crenarchaeotic Group, currently Bathyarchaeota) and soil AOA. In subsurface and bottom waters of deeper lakes, accumulations of AOA and Aenigmarchaeota are however detected (Auguet et al. 2012; Restrepo-Ortiz and Casamayor 2013). This extensive study in the Pyrenean lacustrine district unveiled the environmental preferences and habitat breadth for the different lineages. The species with wide niche breadth, i.e. generalists, were related to methanogens and Aenigmarchaeota, whereas the most specialists were Thermoplasmata, Micrarchaeota and AOA. Pacearchaeota and Woesearchaeota, the most abundant and widespread taxa, showed intermediate values (Ortiz-Alvarez and Casamayor 2016).

The metabolic potential of most lacustrine archaea and the impact in freshwater biogeochemical cycles are largely unknown. In some cases, the biogeochemical activities of Archaea can be environmentally traced by the study of functional genes coding for reactive enzymes such as the ammonia monooxygenase (Amo) present in AOA (Fernàndez-Guerra and Casamayor 2012). The Amo plays a fundamental role in the interconnection between N fixation and N losses, catalysing the oxidisation of NH_4^+ to NO_2^-. Nitrification helps to remove excessive ammonium nitrogen and prevent lakes from eutrophication. Thus, increasing evidence suggests that Archaea may play a significant role in ammonia oxidation in freshwaters in general, and specifically in alpine lakes with submerged vegetation (Vila-Costa et al. 2016). In fact, the interaction between microbial ecology and macrophytes ecology determines the ecosystem-level denitrification and the submerged vegetation landscape has a

Fig. 7.9 Archaeal lineages found in the Pyrenean lakes data set within the TACK, DPANN and Euryarchaeota superphyla. From Ortiz-Alvarez and Casamayor (2016) with kind permission from John Wiley & Sons, Inc.

major role in creating heterogeneity within the lakes that promotes microbial diversity (Vila-Costa et al. 2016). A symbiotic or parasitic lifestyle even with Bacteria has been suggested for Pacearchaeota and Woesearchaeota, probably related to small genomes sizes and limited metabolic capabilities (Spang et al. 2015; Ortiz-Alvarez and Casamayor 2016) but the gap of knowledge is certainly large.

The comparison of PDs for the whole archaeal assemblage and the AOA of Pyrenean lakes with globally distributed seas and soils unveiled again a phylogenetically rich freshwater assemblage in the Pyrenees, approximately twofold higher than in other biomes (Fig. 7.10). The regional diversity of AOA in the Pyrenees is of identical magnitude that the diversity globally observed in marine and soil habitats (amoA gene phylogenetic diversity by clone libraries). Apparently, Pyrenean lakes promote the growth of very diverse and distantly related archaeal communities probably because of the specific combination of persistently cold (ultra)oligotrophic waters, highly diluted and isolated water bodies, and

Fig. 7.10 Averaged PD for the archaeal 16S rRNA and amoA genes obtained in the Pyrenean study lakes compared with globally distributed marine and soil environmental studies. From Auguet and Casamayor (2013) with kind permission from Oxford University Press
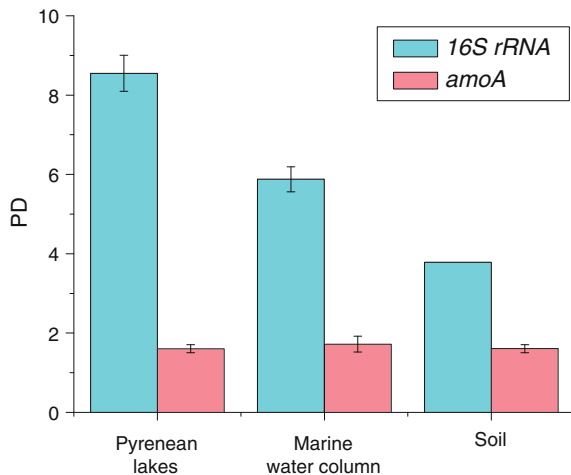
heterogeneous landscape. High mountain lakes are therefore natural laboratories of great interest to improve the current knowledge of archaeal biology and ecology, but perturbations in the trophic status and dissolved organic matter content may induce substantial changes in the archaeal community composition (Auguet and Casamayor 2013). Field experimentation should confirm whether or not warming and eutrophication are major threats for such idiosyncratic assemblages.

7.3.3 Protists and Fungi

Microeukaryotes are essential components of microbial food webs, and morphological criteria have been traditionally used to tell apart the different species and to successfully study the biology and ecology of the different taxa (e.g. diatoms, cryptophytes, chrysophytes, among others). Inconspicuous forms usually of the smallest sizes are however abundant and difficult to study because the cells lack morphological features for identification. Microscopic eukaryotes constitute much of the genetic diversity within the domain Eukarya and the development of genetic approaches mostly based on the 18S rRNA gene sequencing have revealed new previously undescribed clades and large diversity than expected present in nearly all the Eukarya lineages. As it occurs with their prokaryotic counterparts, most of them are uncultivable, but new molecular approaches combined with high-throughput sequencing technologies and bioinformatics analyses provide for the first time the possibility for detailed species inventories, although not free of limitations and biases (e.g. Stoeck et al. 2014; Triadó-Margarit and Casamayor 2012). More accurate preliminary estimations on how many different eukaryotic species exist, on their functions, and on the environments with high and low microbial eukaryotic

diversity can be obtained by comparison of both traditional and molecular technologies.

In a preliminary study of 11 selected high mountain Pyrenean lakes, the microeukaryotes major taxa found belonged to 9 high-rank taxonomic groups and 26 eukaryal classes. Predominant groups, both in abundance and in occurrence, were Chrysophyceae, Cryptophyta, uncultured Alveolata, pennate diatoms of the class Fragilariophyceae, Chlorophyceae, Dinophyceae and Fungi of the Chytridiomycota clade among others (Triadó-Margarit and Casamayor 2012) with most of the OTUs found exclusively affiliated to clusters formed by uncultured microorganisms. The genetic diversity within the Cryptophyta and Chlorophyceae was low and these groups also have the highest relatedness to cultured species. Cryptophyta contained the OTUs with the highest ubiquity in the dataset, but in general, most of the microeukaryote OTUs (>75% of them) were found at only one lake, highlighting the high potential of the whole Pyrenean lacustrine district to contain a high number of new microeukaryotes taxa. Overall, this study and a recent study in the Alps and Himalayan mountains (Kammerlander et al. 2015) unveiled the high mountain lakes habitat as an important biodiversity reservoir of genetically rich Stramenopiles (mostly Chrysophyceae), Alveolata (Ciliophora) and Opisthokonta (Fungi). A comparison with the community composition of marine and freshwater molecular samples (Fig. 7.11) shows the consistent dominance of Chrysophyceae in high-altitude lakes and Artic lakes (Charvet et al. 2012). Overall, Chrysophyceae were more widely distributed in lakes with high oligotrophic conditions. Trophic status modulates the changes in freshwater eukaryote community composition, and eutrophic lakes are less species-rich. Perturbation such as higher availability of reactive nitrogen introduced by atmospheric deposition may also change the community structure of the most sensible species (Kammerlander et al. 2015). Thus, preserving the cold and (ultra)oligotrophic characteristics of the high mountain lakes environment may be of great interest for the study of the ecology and evolution of such idiosyncratic protists but, again, field experimentation should be carried out to confirm these findings.

In fact, the genetic novelty level after a GenBank database search (Triadó-Margarit and Casamayor 2012) showed that many of the 18S rRNA gene sequences recovered in the Pyrenean survey were below the species-level cut-off most widely accepted for microeukaryotes (i.e. 98% identity, Caron et al. 2009). Mostly in the case of Rhizaria-Cercomonads, a set of small (about 10 μm) free-living heterotrophic flagellates difficult to identify under the microscope for species identity, for which c. 90% of the sequences would be new species, and Stramenopiles-Chrysophyceae with 30–40% of the sequences potentially as new species. Conversely, Pyrenean Cryptophyta and Chlorophyceae showed very low genetic novelty (<3% and <5% of new species, respectively). Other taxa with substantial novelty were found within the Opisthokonta (Fungi). Fungi have been invoked as a target group to develop a microbial perspective on conservation biology because both it is important by itself and the fact that fungi biodiversity and their ecosystems roles can benefit conservation in general (Griffith 2012; Heilmann-Clausen et al. 2015).
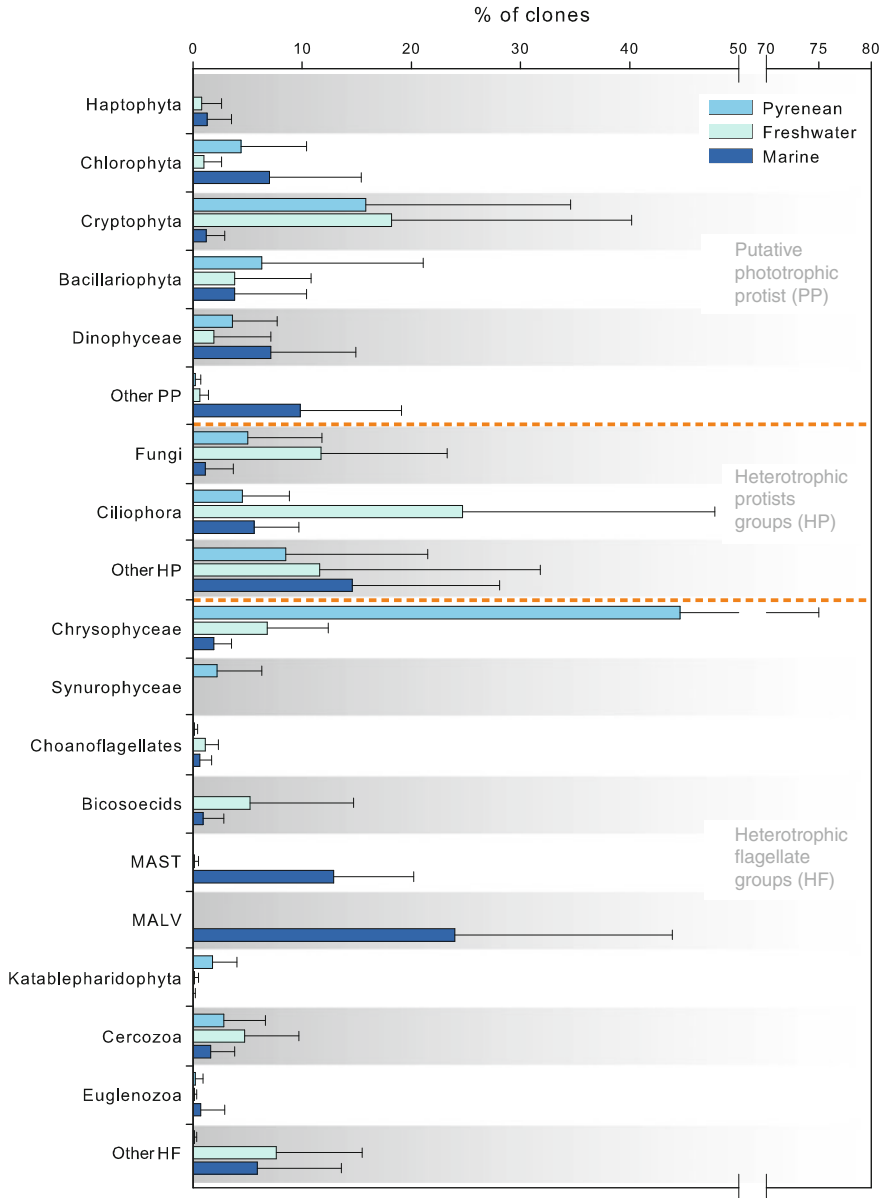
Fig. 7.11 Relative abundances of different microeukaryotes (i.e. <40 μm size) taxa identified by 18S rRNA gene sequencing in Pyrenean lakes and comparative analysis with marine and freshwater systems. *HF* (putative) heterotrophic flagellate groups; *HP* heterotrophic protists groups; *MAST* marine stramenopiles; *MALV* marine alveolates; *PP* (putative) phototrophic protists groups. From Triadó-Margarit and Casamayor (2012) with kind permission from John Wiley & Sons, Inc.

7.4 Towards a Microbial Conservation Perspective in High Mountain Lakes

Microorganisms are fundamental components to maintain the ecological integrity of any ecosystem but the exercise to define challenges and strategies (if needed) for microbial diversity conservation have only been seldom considered. Through the detailed studies carried out in the past years in the Aigüestortes i Estany de Sant Maurici National Park and recent few works available in the literature from other high mountain lakes districts such as the Alps, Himalayas and the western mountains of North America mentioned above, it arises that high mountain lakes hold a high microbial phylogenetic uniqueness and are reservoirs of large evolutionary potential. Microbes are therefore an important part of the biological richness of these environments that should be considered as a fundamental component of the natural heritage. However, we are in the very beginning for understanding microbial tolerance to different environmental conditions and how such underappreciated microbiota will respond to stresses and disturbances brought by the global change. How is the potentiality of survival and dispersal abilities for the different species, and whether or not the distribution of a single microbial population is restricted by the interactions established with other biological entities are also mostly unknown. Despite the advent of laboratory-friendly molecular methods and approaches, this incomplete knowledge and the lack in databases of enough microbial biogeographical studies of the high mountain realm to compare with, strongly limits the scientifically informed advice microbial ecologists can provide to managers dealing with the challenges for conservation of the whole high mountain landscape. Our basic knowledge of the biology and ecology of microorganisms will only improve after intensive inventories and worldwide-distributed field studies (Cotterill et al. 2007).

Whether or not there is any worry for microbes conservation in the high mountain ecosystem (and, in fact, in any other ecosystem) is yet a controversial and difficult to answer question, but global-scale warming and eutrophication may jeopardise the most idiosyncratic microbial populations that found in these areas the most appropriate conditions to grow. Conservation biology perspectives that rely mostly on habitat conservation apply very well for microorganisms. The microbial world may also nicely merge the compositionalist (i.e. entity-oriented through the lens of evolutionary ecology) and the functionalist (i.e. process-oriented through the lens of ecosystem ecology) views on conservation biology (Callicott et al. 1999). Conservation biology main concepts such as species, extinction, biotic interactions and management are however difficult to apply or poorly explored for microbes in situ. As mentioned above, microbial ecologists have managed somehow to sort out the problem of the species definition using either an operational and pragmatic biodiversity unit or phylogenetic approaches. Now they can sort out species rich and poor environments and explore how is the diversity of microbial life distributed around the planet and the relationships between microbial diversity, ecosystem health and biotic interactions (Faust and Raes 2012). The general concept that

biological diversity is threatened with extinction when an element is rare or when it is in decline do not fit very well for the microbial world for several reasons. For instance, the presence of resting or dormant life forms in the rare microbial biosphere with the potential to rapid response to environmental changes and to become an important member of the community. And also the low probability of local extinction because bacteria do not need to find a partner to reproduce and can remain viable in the rare biosphere for very long periods. Finally, the apparently high connectivity among ecosystems, in general, may provide continuous species transfers although there is a lack of experimental information on dispersal and extinction rates in the context of conservation. Airborne supply of species from adjacent or remote ecosystems to alpine areas (Hervàs and Casamayor 2009; Hervàs et al. 2009; Barberán et al. 2014b) may circumvent such extinction through global-scale dynamics, high dispersibility and high speciation in the local communities (Barberán et al. 2014a).

Fortunately, microbial ecologists and theoretical ecologists are now analysing in parallel large temporal datasets of microbial species using stochastic community assembly models and establishing distinctive extinction–colonisation signatures at the microbial taxa level. The concept of "colonisation" in this context is understood as the ability of a particular microbial taxon to appear within the most abundant members of the community, whereas "extinction" would be the process to fall into the rare biosphere and become undetectable in the best case. The method identifies for the first time those microbial taxa with an ephemeral dynamic (higher colonisation and higher extinction rates), and those that are more stable (lower colonisation, lower extinction) and it is a promising approach to applying a closer conservation view on both microbial populations and microbial habitats with a more dynamic perspective (Jiménez-Ontiveros et al. 2017). The inadequacy of knowledge on the microbial world needs the development of major educational programs (Barberán et al. 2016) with emphasis on the engagement of ecosystems managers and citizens to better understand the whole picture of the biosphere and the key role of microorganisms within it. Criteria such as microbial species richness, phylogenetic diversity, exclusive microbial species occurrences and the microbial conservation value of a habitat, are now available to support environmental managers and lawyer decisions, and to citizens and stakeholders in general. Examples of threatened areas of high microbial interest in which efforts should be more intense to preserve their biodiversity are now available (e.g. Casamayor et al. 2013). Disturbance and loss of habitats or massive habitat change by anthropogenic activities may indeed threaten microbes and led to local extinction as frequently as in macroorganisms. Ecosystem management in alpine areas should promote to maintain spots of habitat heterogeneity within the landscape matrix between lakes and within the lake to retain such phylogenetically rich and diverse natural heritage.

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