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State-of-the-art review

Thomas WEISSE

Institute for Limnology of the Austrian Academy of Sciences, Mondseestr. 9, A-5310 Mondsee, Austria,
e-mail: thomas.weisse@oeaw.ac.at

BIODIVERSITY OF FRESHWATER MICROORGANISMS – ACHIEVEMENTS, PROBLEMS, AND PERSPECTIVES

ABSTRACT**:** The extent and significance of the diversity of freshwater microbes is at present controversially debated. Until 1980 it was assumed that there are no freshwater-specific bacteria and that the total number of bacterial species is low. The advent of molecular tools over the last ten years revealed that there is a bacterial freshwater assemblage which is phylogenetically different from soil and marine bacteria; secondly, it became obvious that the total number of cultured bacterial species (~5900) underestimates bacterial diversity by several orders of magnitude. The current debate centres on 1) how to define a bacterial species and 2) if there is a microbial biogeography. The latter relates to the issue of ubiquity and cosmopolitanism, which is controversially discussed primarily in relation to eukaryotic microorganisms, namely ciliates. Although solid evidence is scarce, many microbial ecologists assume, in accordance with Baas Becking's famous 70-year old dictum – "*everything is everywhere, the environment selects*" – that freshwater microorganisms are easily dispersed and, therefore, potentially cosmopolitan. This review focuses on the often neglected second part of Baas Becking's metaphor. Evidence is accumulating rapidly that the environment does not simply act as a filter *sensu* Gleason's individualistic concept for widely dispersed microbes. Rather, prokaryotic and eukaryotic microorganisms have adapted to their specific habitat and perform better in this environment than newly invading congeners. There is an enormous ecophysiological diversity among closely related freshwater microbes which is neither obvious at the morphospecies level nor at the level of evolutionarily conserved genes, such as the small ribosomal RNA gene. Although this large diversity has been demonstrated for various groups of bacteria and protists, there is currently no measure available to compare microbial biodiversity across prokaryotic and eukaryotic domains. The current challenge is to link genetic divergence to ecophysiological diversity in the major taxa.

KEY WORDS: microbial diversity, freshwater, species problem, future perspectives

1. INTRODUCTION

There is no doubt that the main diversity of life is microbial; microorganisms, here defined as prokaryotes and eukaryotes invisible with the unaided eye (~ <1 mm), are found in each of the three domains of life (Fig. 1) and in nearly every major phylum. In particular, aquatic microbes are taxonomically diverse, highly abundant and they are present virtually everywhere. Yet, in spite of their significance for all biogeochemical processes, the biodiversity of microbes is little understood, relative to that of plants and animals. Microorganisms have been overlooked

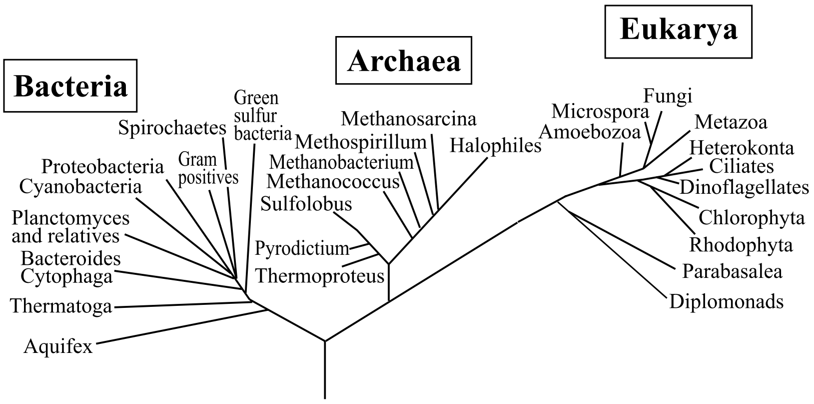

Fig. 1. The phylogenetic "tree of life" with the three domains Eubacteria, Archaea and Eukarya. For the sake of clarity not all organisms are shown from which sequence data are available.

in traditional ecological studies, owing to a lack of adequate methods to quantify and characterize free-living bacteria and minute eukaryotes. Their small size, the absence of diagnostic phenotypic characters, and the fact that many microorganisms are difficult to isolate and to rear in culture have limited the evaluation of their biodiversity (Dorigo *et al.* 2005). The advent of novel techniques such as direct counting by epifluorescense microscopy, image analysis, electron microscopy, flow cytometry, the development of protocols to measure growth, grazing loss and production rates and, most recently, molecular tools to identify as yet unculturable organisms, has revolutionized the field of microbial ecology during the past three decades (reviewed by Weisse 2003, Dorigo *et al.* 2005). The quantitative significance of aquatic microbes in pelagic food webs was detected in the 1970s and 1980s (Pomeroy 1974, Williams 1981, Azam *et al.* 1983, Porter *et al.* 1988). Since then, many case studies measured the magnitude and fate of bacterial and protist production. In recent years, the attention shifted from measuring bulk parameters such as, e. g. total bacterial abundance and production, towards addressing specific issues such as the response of a given bacterial or algal strain or community to a particular grazer (e.g. Šimek *et al.* 2002, Boenigk *et al.* 2004, Wu *et al.* 2004). Similarly, the impact of key environmental parameters, such as temperature or substrate supply (food), has been investigated at the species or strain level (Weisse and Montagnes 1998, Dini and Nyberg 1999, Weisse *et al.* 2001, 2002, Hahn and Pöckl 2005). Bacteria and small eukaryotes have been used as model organisms to address questions of general ecological relevance. Recent examples are the investigation of the speed of evolutionary adaptation in bacteria (Mongold *et al.* 1996, Cooper *et al.* 2001, Collins and Bell 2004), the adaptation of inducible defenses in ciliates (Kusch 1995), and the demonstration of chaotic patterns in a microbial food web with two bacterial prey and one ciliate predator species (Becks *et al.* 2005).

It is now clear that considering functional guilds, e.g. heterotrophic bacteria or algivorous protist, may be a valid approach for ecosystem models to quantify major metabolic pathways but is inadequate to portray the biodiversity of aquatic microbes. Further insight into the biodiversity of (aquatic) microbes is, at present, hampered by a lack of adequate conceptual models. Fundamental issues such as the identity and number of microbial species are still controversially debated (see next section). Current estimates of the number of bacterial species differ by several orders of magnitude (Table 1). It appears safe to conclude that <10%, perhaps <1%, of prokaryote diversity has been identified convincingly. Similarly, there is an ongoing debate as to whether the "true" number of ciliate species is close to 3000 or 30 000 species (Finlay *et al.* 1996, 1998, Finlay and

Table 1. Current estimates of total bacterial species numbers.

Total number of species	Based upon	Source and remarks
5900	Approved List of Bacterial Names, according to the International Code of Nomenclature of Bacteria and publication in the International Journal of Systematic Bacteriology (IJSB) / International Journal of Systematic and Evolutionary Microbiology (IJSEM)	http://www3.dsmz.de/bactnom/bactname.htm updated every 2 months
$10^6 - 10^7$	Assuming that between 0.1 to 1% of all bacteria have been cultured so far	This study
$10^5 - 10^7$	PCR community fingerprinting, DNA reassociation methods	Torsvik *et al.* (2002)
$10^9 - 10^{12}$	Estimated and extrapolated from bacterial DNA diversity in a soil sample of 30 g	Dykhuizen (1998); a highly theoretical approach, based upon crude assumptions

Fenchel 1999, Foissner 1999, Foissner *et al.* 2002). This debate is ultimately linked to the issue of microbial biogeography, discussed further below.

This paper summarizes the state of the art of aquatic microbial biodiversity, with emphasis on freshwater ecosystems. Since it is impossible to address comprehensively all levels of biodiversity, ranging from molecules to ecosystems, I will concentrate this overview on populations and species. This is not an exhaustive review; it cannot be when further case studies are being published every week. Rather, the goal is, by identifying current methodical and conceptual shortcomings, to give some perspectives for future research.

2. THE SPECIES PROBLEM

Evolutionary biologists are confronted with a colossal dilemma: almost 150 years after the publication of Darwin's classic on The Origin of Species (1859), it is not clear what a species is. There are over 20 competing species concepts available in the literature (see Mayden 1997 for a short review of contemporary species concepts), which can be grouped into 5 or 6 major categories (King 1993, Claridge *et al.* 1997, Rosselló-Mora and Amann 2001, Ogunseitan 2005). Even worse, biologists do not agree if a species is a taxonomic category only, i.e. an abstract concept, or an evolutionary unit ("real" species, see Claridge *et al.* 1997, Bachmann 1998, Hey 2001). This "most basic of biological problems" (King 1993) has been largely ignored in many biological disciplines, partly for practical purposes, and partly because many biologists dealt with sexually reproducing organisms with clear species boundaries according to the Biological Species Concept (BSC, Mayr 1942, 1963). For historic, ethnic and economic reasons, vertebrates have received considerably more attention than invertebrates in general, and microorganisms in particular, in major biological disciplines such as physiology, ecology and conservation biology. In retrospect, it appears fair to conclude that this anthropocentric point of view has hampered the search for a universal species concept.

The BSC became a widely accepted species concept because it has a solid theoretical foundation, it can be applied with ease, and, last but not least, its long-standing and authoritative promotion by its major advocate, Ernst Mayr (1904-2005). Even as a centenarian, he continued to write articles and books on major evolutionary issues (Mayr 2004). The BSC was attacked on many occasions and for various reasons, for instance, its difficulty in allowing for the extent of reproductive isolation in (hybrid) species (Simpson 1961) and its failure to explain convincingly the process of speciation. A curious corollary of the BSC is that no species could have existed for at least half the time that there has been life on Earth, because sexual reproduction evolved late in the evolution (Hull 1997). For botanists and microbiologists, the BSC is of little significance because it cannot be applied to asexually reproducing taxa, such as bacteria, many plants and many protists. There is, however, no alternative species concept available that defines bacterial and protist species across their respective realms convincingly. The morphospecies concept favoured by taxonomists has no solid theoretical foundation, whereas some highly theoretical evolutionary species concepts are not operational. The novel RNA and DNA sequence-based approaches to identifying microbial taxa do not solve the problem, since species circumscription uses empirically derived, arbitrary categories (Rosselló-Mora and Amann 2001, Stackebrandt *et al.* 2002). According to the International Committee on Systematic Bacteriology (Wayne *et al.* 1987, Stackebrandt *et al.* 2002), a bacterial species consists of a group of organisms with a DNA – DNA hybridization rate of ≥70% and a thermal denaturation midpoint (ΔT_m) of ≤5°C; the latter refers to a denaturation kinetic curve of a double-stranded DNA and denotes the temperature at which 50% of the DNA strands are denatured. The total DNA similarity of ≥70% and the (ΔT_m) of ≤5°C correspond to a sequence similarity of the 16S rRNA of ≥97% (Stackebrandt and Goebel 1994, Rosselló-Mora and Amann 2001), but this is a continuum with broad boundaries. Recent studies demonstrated that bacterial freshwater strains with identical 16S rRNA genes may have highly divergent genomes and ecophysiologies (Jaspers and Overmann 2004, Hahn and Pöckl 2005). Even if most homologous genes show extremely low sequence divergence, gene content can vary enormously, resulting from horizontal gene transfer (Gogarten and Townsend 2005). Accordingly, the boundaries between bacterial species may be fuzzy. This uncertainty in the species circumscription led Rosselló-Mora and Amann (2001), in their formulation of the phylophenetic species concept, postulate that a bacterial species is a "genomically coherent cluster of individual organisms that show a high degree of overall similarity in many independent characteristics".

It has been suggested for bacteria that biological diversity is not represented by nominal species but by ecotypes, the latter being defined as populations of organisms occupying the same ecological niche (Cohan 2001, 2002). It is, however, impossible to prove or disprove that two species share the same ecological niche, i.e. the niche is an useful ecological concept but not a (falsifiable) scientific hypothesis (Rigler and Peters 1995). Further, we are at present far from defining operationally complex microbial niches (Ogunseitan 2005). The term "ecotype" was first coined as a subspecific category for sexual plants (Turesson 1922) but, since then, it has been applied, *inter alia*, to many asexually reproducing plants and protists. Ecotypes are not only ecologically distinct populations but they are also genetically divergent clusters. Each ecotype has acquired predominantly neutral mutations, some of which may have been adaptive in a particular habitat. Evolutionary adaptation (microevolution), i.e. small-scale changes in gene frequencies in an isolated population over relatively few generations, becomes obvious by a changed phenotype (in terms of its morphology, physiology or behaviour). An experimental investigation of evolutionary adaptation is notoriously protracted and has thus been achieved only for some fast growing microorganisms (see Lenski 2004 and Rainey 2004 for short reviews, Collins and Bell 2004).

An analogous approach to the ecotype (Cohan 2001, 2002) or to the operational taxonomic unit (OTU, Green *et al.* 2004, Crump and Hobbie 2005) concept of microbiologists is taken for (sexually reproducing) macroorganisms in conservation biology, where biodiversity for many diverse taxa such as, e.g. Pacific salmon (Waples 1991) or Borneo's elephants (Fernando *et al.* 2003) is no longer measured in terms of (biological) species numbers but in terms of Evolutionary Significant Units (ESU; Ryder 1986). An ESU is defined pragmatically as a group of organisms that has undergone significant genetic divergence from other groups of the same species. Conservation biologists became aware that classical taxonomic species boundaries only describe a limited part of biodiversity, so conservation efforts that are restricted to this level may lead to a loss of evolutionary relevant variation present at the intraspecific level (Gosling 1999).

As with the bacteria, morphospecies designations of protists are equated with a 1 to 2% rDNA divergence (Jerome *et al.* 1996, Snoeyenbos-West *et al.* 2002, Katz *et al.* 2005). It is, however, well known following the pioneering work of Sonneborn on *Paramecium* (Sonneborn 1937, 1957) that morphologically indiscernible ciliates may harbour several cryptic species, with clearly differing physiologies and behaviours (consult Schlegel and Meisterfeld 2003 for a recent review on the species problem in protozoa). As already noted by Dini and Nyberg (Dini and Nyberg 1993), extensive intraspecific variation is a fact in ciliates and other protists (Weisse and Montagnes 1998, Kim *et al.* 2004, Lowe *et al.* 2005, Weisse and Lettner 2002). Figure 2 shows an example from a recent laboratory study and illustrates the ecological significance of seemingly minor differences in growth rates of freshwater ciliates (Weisse and Rammer 2006). It is, however, at present impossible to link the genotypic variation observed within nuclear ribosomal RNA or protein-coding genes to phenotypic divergence measured in complex ecophysiological processes, such as growth or feeding rates. The extent of genotypic and phenotypic clonal variation among common aquatic protists, and the widths and boundaries of their respective ecological niches, need to be better characterized. Protists that are indiscernible not just at the conservative 18S rRNA level, but which are identical in their non-coding ribosomal internally transcribed spacers (ITS), may nevertheless differ by up to 10% in their more variable genes, such as the mitochondrial cytochrome c oxidase I (Barth *et al.* 2006).

According to the foregoing considerations, the Cohesion Species Concept (Templeton 1989), the Phylogenetic Species Concept (Cracraft 1983) and the Ecological Species Concept (Van Valen 1976) appear more appropriate than the morphospecies concept to reveal "true" microbial diversity. The current challenge is to determine which level of a (sub)cluster, if any, corresponds to ecotypes (Palys *et al.* 1997, Cohan 2002) and, specifically for protists, to discover if

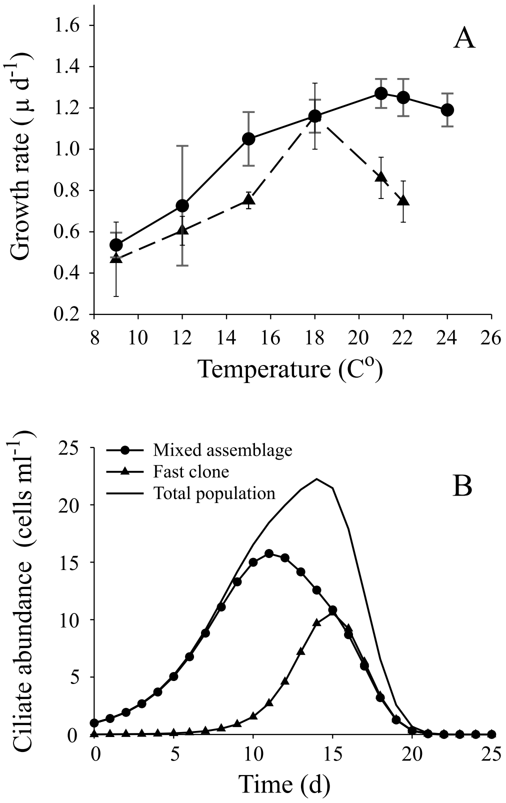

Fig. 2. Intraspecific differences in population growth rate of the planktonic freshwater ciliate *Rimostrombidium lacustris* (Foissner, Skogstad and Pratt) Petz and Foissner (Oligotrichida) and their potential ecological significance. Panel A shows temperature dependent growth rates (mean values and standard deviations) of two clones of *R. lacustris* measured in the laboratory; the clones were isolated in parallel from mesotrophic, subalpine Lake Mondsee (Austria) during early summer. Panel B shows the population dynamics of a hypothetical, non-clonal ciliate population (mixed assemblage, initial growth rate $\mu = 0.39\ d^{-1}$), a fast growing clone ($\mu = 0.43\ d^{-1}$), and the combined total ciliate population during model simulations over 25 d (modified after Weisse and Rammer 2006). Ciliate growth rate was related to temperature according to an empirically derived equation, and temperature was assumed to increase linearly from 6.8 to 13 °C over the study period. The population dynamics was modelled according to $N_t = N_0 * e^{(\mu - g)\,t}$, where N_0 and N_t are initial cell numbers and cell numbers at time t; the coefficient g denotes a loss term (grazing), which was initially low ($0.06\ d^{-1}$) and then increased at a rate of 25% per day. The model illustrates that a fast growing clone, dividing at a rate 10% higher than the average cells in the population, which initially represented only 1% of the total cell numbers, contributed 50% to the total population after 15 days (see Weisse and Rammer 2006 for further details).

there is any correspondence between DNA sequence clusters, ecotypes and (morphologically defined) biological species (Dini and Nyberg 1999, Foissner *et al.* 2001, Finlay 2004, Boenigk *et al.* 2005). In a more general context, the priority is to understand the interplay of molecular mechanisms with organismic and ecosystem biology (Jackson *et al.* 2002).

While there is now a close overall agreement between conventional and molecular taxonomy and phylogeny (Schlegel and Meisterfeld 2003, Lynn 2003), some notable discrepancies between morphological and molecular characterization have also been observed among several groups of free-living protozoa (e.g. Walochnik *et al.* 2002, Foissner *et al.* 2003, Katz *et al.* 2005). In some cases, the morphological and ultrastructural evidence cannot be reconciled with the phylogeny suggested by the evolution of the small ribosomal subunit (SSU rRNA) gene, and, at the present state of knowledge, the latter may also disagree with the phylogeny derived from the more variable protein nucleotide sequences, such as α-tubulin (Agatha 2004 and references therein). The disagreement between morphological and molecular evidence may, however, also reflect phenotypic plasticity, since an organism's phenotype is not fixed but is, within species-specific limits, dependent on the environment experienced during ontogeny (Huey and Berrigan 1996, Finlay 2004). Acclimation or acclimatisation to specific environmental conditions are more or less reversible, physiological examples of phenotypic plasticity (Huey and Berrigan 1996). The extent of phenotypic plasticity, i.e. the variability of a phenotype in response to environmental change, is also an irreversible genetic trait and may vary largely among different taxa. The true level of genetic divergence of protist ecotypes should become apparent when more protein-coding gene sequence data are available for various protist taxa.

The implications of the current uncertainty in defining species and ecotypes for estimating microbial biodiversity are obvious. A comprehensive understanding of the "true" diversity of extant microorganisms has not yet been achieved (Leander and Keeling 2003, Curtis and Sloan 2004) and quantification of the microbial diversity may even be futile (Wilson 1992). The relative significance of phenotypic plasticity and microevolution remain at present virtually unknown for bacteria and protists. All these issues are focal to the answers to three basic questions about aquatic microorganisms: "what is a species?", "how many species are there?" and "is microbial diversity fundamentally different from that of macroscopic organisms?" (Finlay and Fenchel 1999, Finlay *et al.* 2004). These questions have been raised for protists, as the outcome of an international meeting. In a more general context, we can ask with Curtis and Sloan (2004) "how can one measure microbial diversity and what does microbial diversity mean?".

3. SCALING − CATEGORIES OF BIODIVERSITY

The implications of the nature of the species concept are controversially discussed mainly among researchers working with eukaryote microorganisms. In contrast, prokaryote taxonomists agree that the current polyphasic species circumscription is acceptable and pragmatic, and covers the primary goal of taxonomy such as rapid and reliable identification of novel strains (Stackebrandt and Goebel 1994, Rosselló-Mora and Amann 2001, Stackebbrandt *et al.* 2002). Irrespective of the current uncertainty some authors conclude that the low species number of protists is a fact and that the protist diversity is fundamentally different from that of macroscopic organisms, resulting from their seeming global distribution and ubiquity (Finlay and Fenchel 1999, Finlay 2002, 2004). This claim builds upon the old, so-called Baas-Becking hypothesis, "everything is everywhere - the environment selects". The Dutch school of microbiologists, headed by Martinus Beijerinck and his successor Baas-Becking in Delft, believed that microorganisms would be globally dispersed and thrive, wherever a suitable habits exists (see Quispel 1998 for a short review of Baas-Becking's key publications that were published in the 1930s in Dutch, and Whitfield 2005 for a short summary of the "everything is everywhere" view).

Microbial diversity in any environment is not fixed but is a dynamic process of constant change. The species problem is therefore related to the mode of speciation (Mayr 1942). In the case of interbreeding populations, reproductive isolation is the sole criterion for the reality of species (Mayden 1997), leading to allopatric speciation. In contrast to sexually reproducing species, genetic exchange is not acting as a force of cohesion in asexual ecotypes (Cohan 2002). The force of cohesion that maintains the genetic identity of an ecotype is periodic natural selection, which can purge most or all genetic diversity from an asexual population (Atwood *et al.* 1951). Finlay and Fenchel (see references above) assume that, because the gene pool of each population is being swamped by new immigrants, local adaptation to specific habitats and allopatric speciation are of minor significance among free-living protists. Gene flow changes allele frequencies in a direction opposite to natural selection, that is, if the migration rate is large compared to selection, local adaptation is lost (Lenormand 2002, Padisák 2003). On the other hand, local diversity depends on the size of the reservoir community (metapopulation) from which new immigrants originate; if this is large, "chance alone will prevent physically identical communities from having the same, or sometimes even stable, communities" (Curtis and Sloan 2004). Note that immigration will be successful only if, among the flow of immigrants, there are some cells better adapted to the specific local environment than those already present. This is an important, implicit assumption of the "everything is everywhere" dictum.

There is evidence emerging that allopatric speciation should not be discouraged for microbes (Papke and Ward 2004). In particular, populations of "species" with cosmopolitan distributions may be sufficiently isolated to allow genetic divergence (Dini and Nyberg 1993, Foissner *et al.* 2001, Kurmayer *et al.* 2004), thus promoting allopatric speciation. There are also indications for sympatric speciation among aquatic protists despite a high dispersal potential (Darling *et al.* 2000 and references therein, Beszteri *et al.* 2005). Sympatric speciation leads to the origin of new species from a local population by colonizing different ecological niches within the same geographical zone; this is followed by prevention of gene flow due to intrinsic factors. Although this pattern of speciation is generally believed to play a minor role for species radiation (Mayr 1942, Ogunseitan 2005), sympatric speciation is a likely outcome of competition for resources (Dieckmann and Doebeli 1999). The intermediate case between allopatric and sympatric speciation, with limited migration/dispersal between divergent (sub-) populations, is known as parapatric speciation and may be the most general mode of speciation (Gavrilets 2003). It is clear that the mode of speciation relates to the range of migration, and this is obviously correlated to the size of the organisms. What appears to be sympatric for a fish in a lake, may be worlds apart for a bacterium.

Finlay (2002) identified a cutoff of ~1 mm in size for organisms with a ubiquitous distribution; in contrast to larger organisms, microorganisms <1 mm would not have any biogeography (Fig. 3). Note that in Finlay's hypothetical model, the abscissa represents organism length on a logarithmic scale but lacks dimensions except for the 1-mm cutoff shown by the arrow in Fig. 3. Further, I have added a scale for a characteristic area to Fig. 3 (top x-axis), because the size of organisms and their home range, where the organisms usually range in the course of a day or season, are linearly related to each other (Fig. 3, inset). The size of the home range is of utmost biological importance, because it sets the upper limits of biological interactions. According to Fig. 3, ubiquitous species (i.e., microorganisms) have typical home ranges varying between 10^{-6} and 10^{1} m², corresponding to linear dimensions ranging from 1 mm to several m. The typical linear home range dimensions of macroorganisms with biogeographies range from several m to several km. The significance of the home range may be illustrated using the following example: the home range of many mammals such as wolf, deer or elephant covers tens to hundreds of km². Similarly, salmon and other anadromous or catadromous fish species migrate over thousands of km in the course of their lifetime. Daily migrations of many fish species may reach several hundreds of

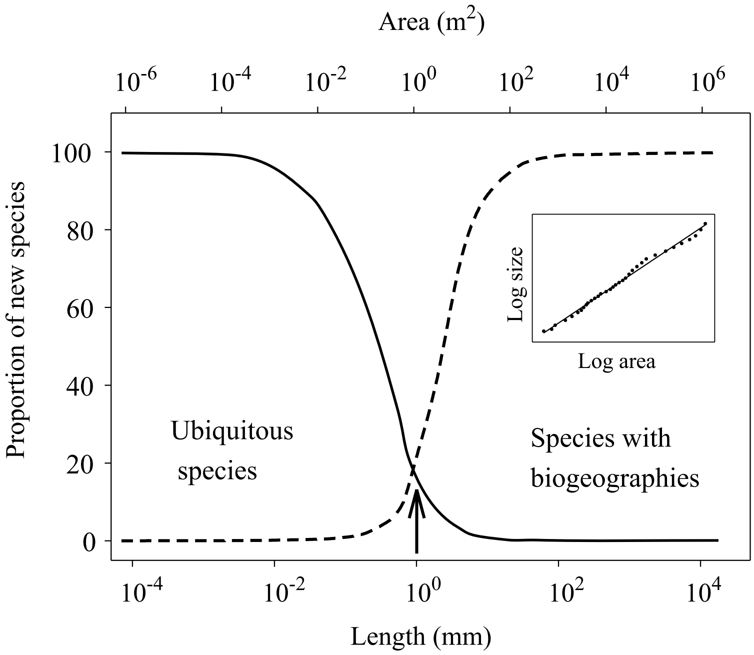

Fig. 3. A hypothetical model of the ubiquity-biogeography transition, which is assumed to be located in the vicinity of an organism size of ~1 mm (indicated by the arrow; modified after Finlay 2002). The solid curve denotes the proportion of new cosmopolitan species, which decreases with the size of the organisms, while the dashed curve denotes the proportion of new species with biogeographies, increasing with organism size. Since the size (length) of the organisms is correlated to the area of their home range (inset), the ubiquity/biogeography curves are also related to characteristic areas (top x-axis).

meters (Lucas and Baras 2001). Compare this home range to that of a freshwater microbe. Assuming a typical flagellate swimming speed of 0.2 mm s^{-1} (Fenchel 1987), a flagellate may migrate up to 17.3 m d^{-1} if it would constantly swim in one direction. Diel vertical migrations (DVM) over several meters are common among several freshwater phytoplankton species, and DVM exceeding 10 m have been reported (Sommer and Gliwicz 1986, Sommer 1988, Salonen and Rosenberg 2000). The range at which biological interactions such as competition and predation take place is, therefore, orders of magnitudes lower for aquatic microbes than it is for macroorganisms.

Finlay (2002) argues that the species to area curve is flat for microorganisms such as ciliates, i.e. increasing sampling area would only little increase the number of species found. This is in sharp contrast to insects, which show a linear increase in species richness with sampling area (in a double logarithmic plot; Fig 2 B in Finlay 2002). Although the microscale (α-) diversity of protists is high, the amount of change between two sites along a gradient (β-diversity) and the large scale (γ-) diversity appears to be low, i.e. the species composition will change little as one moves across a region or between regions. Such a pattern of diversity and low species ”turnover” (Whittaker 1970) is predicted by the model of high rates of dispersal and immigration within the neutral theory of community structure (Hubbel 2001, Bell 2001). However, according to the latter author, the neutral theory of abundance and diversity will fail at the taxonomic and geographical scales where specific adaptation has evolved. The neutral theory assumes that all members of a functional group are, on average, equally competitive, which stands in sharp contrast to the experience of all experimentally working physiologists and ecologists. In other words, Finlay’s hypothetical model and the neutral theory of abundance and diversity ignore the evolutionary significance of biotic interactions. Species to area relations must relate habitat size to the body size of the organisms to yield statistically sound results (Hillebrand and Blencker 2002).

In reality, an aquatic microbe may be dispersed over a much wider area than its home range, due to passive drifting by water currents, turbulence and mixing, and by transportation via wind, waterfowl and other vectors. However, a flagellate or ciliate has no chance to travel over a long distance as an active cell, simply because the lifespan of aquatic protists is in the order of hours to days. It must therefore be assumed that long distance dispersal is mediated via resting stages such as cysts known from, e.g. oligotrich freshwater ciliates (Müller and Wünsch 1999, Müller 2000, Müller *et al.* 2002) or chrysophyte flagellates (Cronberg and Sandgren 1986). Chrysophyte cysts are useful indicators for paleolimnological reconstruction of the past climate, because they are common in the sediment of lakes and are preserved for thousands of years (Cronberg 1986, Kamenik *et al.* 2001). Bacterial spores may survive even millions of years (summarized by Ogunseitan 2005). It is another, implicit assumption of the "everything is everywhere" dictum that all microorganisms are highly tolerant to stress, and may therefore survive long-distance travel (Hughes Martiny *et al.* 2006). Yet, similar to the issue of sexual reproduction, the significance and viability of resting stages remain at present unknown for >99 % of all extant freshwater microbes. Irrespective of this open question, it is clear that the scaling where specific adaptation may evolve in aquatic microbes is in the range of mm to m, certainly not km. Alpha-diversity of macroorganisms may be more comparable to β-, if not γ-diversity of microbes. The true microscale level (mm to m) of microbes has not yet been considered in published species to area curves; comparing the "species" turnover of elephants and bacteria in one plot with standard (logarithmic) scaling is therefore meaningless.

Assessing biodiversity in adequate terms does not relate only to spatial scaling; temporal scaling is of similar importance. The sampling frequency must relate to the generation time of the study organisms. One sample per year is usually enough to estimate the population size of common fish or macrozoobenthos species. The species composition of zooplankton and phytoplankton in a given water body may change within one to a few weeks. The significance of biotic interactions such as competition, predation and parasitism has been clearly documented for the occurrence and seasonal succession of eukaryotic freshwater plankton (Sommer *et al.* 1986, Sommer 1989). The investigation of temporal patterns of bacterioplankton community change is still in its infancy, but it is highly likely that similar processes lead to seasonal shifts in the bacterioplankton (Güde 1989, Pedrós-Alió 1989, Jürgens *et al.* 1999, Šimek *et al.* 1999, Van der Gucht *et al.* 2001, Becker *et al.* 2002, Zwisler *et al.* 2003, Crump and Hobbie 2005, Wu and Hahn 2006). A more thorough study of the temporal dimension of microbial biodiversity will certainly increase current estimates of the overall microbial diversity.

Finally, the taxonomic resolution is crucial for any record of biodiversity. As a consequence of the unresolved species problem (see above), this is a major issue in contemporary microbial ecology, which will be discussed in the following section.

4. NO BIOGEOGRAPHY OF AQUATIC MICROBES? – A MYTH UNRAVELED

The studies published until recently on the regional and local impact on species diversity were highly biased towards larger organisms and did not adequately investigate the degree of similarity between local and regional area for bacteria and protists (Hillebrand and Blencker 2002, Curtis and Sloan 2004). Biogeography has already been demonstrated for microorganisms living in isolated habitats, such as thermophilic archaebacteria (Whitaker *et al.* 2003) and cyanobacteria (Papke *et al.* 2003). Most freshwater phytoplankton species appear to be cosmopolitan, but there is also a considerable number of local or regional endemic algal species (reviewed by Padisák 2003). Recent evidence suggests that the similarity of bacterial and heterotrophic protist species composition decreases with increasing geographic distance (Hillebrand *et al.* 2001, Horner-Devine *et al.* 2004, Hughes

Martiny *et al.* 2006). The issue of microbial biogeography has been revitalized with new findings emerging from the advent of molecular techniques for the detection of as yet unculturable strains.

Twenty years after the detection of the quantitative significance of bacteria in pelagic food webs, Rheinheimer (1992) concluded in his textbook that there are no freshwater bacteria that would differ from bacteria found in soil and groundwater. With the advent of molecular tools for identifying aquatic microorganisms, it became obvious during the following decade that there is a set of freshwater bacteria clearly different from their marine counterparts (Glöckner *et al.* 2000, Zwart *et al.* 2002). Evidence is accumulating every week demonstrating that members of the α- and β-Proteobacteria, Bacteroidetes, and Actinobacteria form typical heterotrophic bacterial freshwater clusters (Hiorns *et al.* 1997, Methé *et al.* 1998, Lindström 2000, Hahn 2003, Hahn *et al.* 2003, Hahn and Pöckl 2005, Schauer and Hahn 2005, Crump and Hobbie 2005, Van der Gucht *et al.* 2005, Lindström *et al.* 2005, Langenheder *et al.* 2006). Similarly, the autotrophic single-celled Cyanobacteria form several clusters indigenous to freshwater (Ernst *et al.* 2003, Crosbie *et al.* 2003a, 2003b). Most recently, novel eukaryotic phylogenetic groups have been detected which seem to be typical for freshwater environments (Richards *et al.* 2005). It is common to most of these studies, that some taxa appear globally distributed while others show a more restricted distribution. The same conclusion holds true for protists (Coleman 2002, Mitchell and Meisterfeld 2005). Limited phylogenetic information in the SSU rRNA gene and taxon undersampling (Palys *et al.* 2000, Crosbie *et al.* 2003a) prevent, however, at present any firm conclusion on the extent of biogeography of most freshwater microbes.

The detection of microbial biogeography strongly depends on the taxonomic resolution of the analysis method (Mitchell and Meisterfeld 2005). In a salt marsh, Betaproteobacteria exhibited a significant taxa to area relationship for the 99% resolution of the 16S rDNA, but not for the 97% and 95% cutoff (Horner-Devine *et al.* 2004). Similarly, two neighbouring rivers in NE Massachusetts (USA) with remarkably synchronous bacterioplankton communities were similar at the 97% ribosomal DNA identity but differed in their diversity at the 99% identity (Crump and Hobbie 2005). Several recent studies suggest that the 16S rRNA gene is too conservative to identify OTU's, ecotypes or species of certain freshwater bacteria (Jaspers and Overmann 2004, Hahn and Pöckl 2005). The advent of molecular techniques with a higher taxonomic resolution such as PCR fingerprinting analyses revealed that each lake and pond has its own distinct bacterioplankton community (Yannarell and Triplett 2004, Hahn *et al.* 2005, Schauer and Hahn 2005, Langenheder *et al.* 2006). Figure 4 shows an example illustrating how the bacterioplankton composition may change along an environmental gradient.

Similar findings were reported for bacteria of the wide-spread *Polynucleobacter* group, which forms a monophyletic cluster of a minimum 16S rRNA sequence similarity of 95.8% within the Betaproteobacteria (Zwart *et al.* 2002, Hahn 2003, Wu and Hahn 2006). The *Polynucleobacter* cluster consists of at least four, species-like subclusters with >98% 16S rRNA gene similarity (Wu and Hahn 2006). A surprisingly low microdiversity was demonstrated for the narrow *Polynucleobacter* subcluster C (PnecC) in an alpine pond (Hahn *et al.* 2005), which further challenges the "everything is everywhere" theorem and contrasts recent investigations from coastal bacterioplankton populations, where an enormous genotypic diversity (>1600 coexisting phylotypes) was found at the 16S rRNA level (Acinas *et al.* 2004, Thompson *et al.* 2005). The question is, can these ribotypes be regarded as natural taxonomic units, i.e. do they represent species or ecotypes (Giovannoni 2004)? Since coexistence of more than 1000 ecological niches of sympatric bacteria appears unlikely, it is concluded that most of this genetic variation is neutral (Acinas *et al.* 2004, Giovannoni 2004).

Similar to bacteria, there is increasing evidence that previously recognized protist (morpho)species may actually have geographically patchy distributions in marine

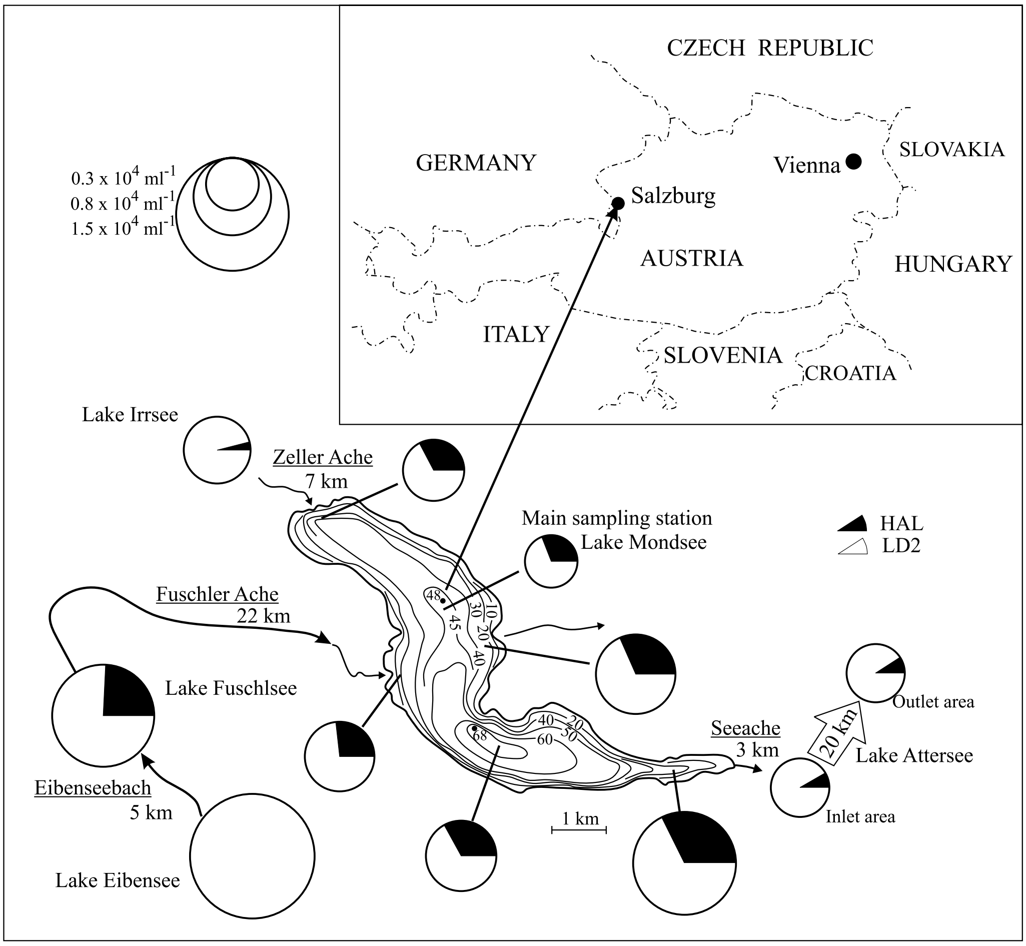

Fig. 4. Spatial distribution of the species-like subclusters HAL and LD2 within the bacterial SOL cluster (Fam. *Saprospiraceae*, modified from Schauer *et al.* 2006) in Lake Mondsee (Austria) and connecting lakes (Schauer *et al.* 2006). The study area is located 35 km east of the city of Salzburg (see map of Austria, on top). The size of the pie diagrams indicates the bacterial abundance. The relative contribution of the two bacterial subclusters was stable in each lake, but differed even in connected lakes (Irrsee and Mondsee, Mondsee and Attersee).

and fresh water (Kim *et al.* 2004, Katz *et al.* 2005). Again, detection of biogeography is related to the taxonomic level of resolution. Among the *Spumella*-like chrysophyte flagellates, a global distribution is suggested by 18S rRNA analysis, because identical ribotypes can be found on different continents; yet there are indications for ecological differences between these strains (Boenigk *et al.* 2005). Both for bacteria and for protists, the question is, what the sequence data actually show, and how a molecular view of microbial diversity can be compared with a species-based view of plant and animal ecology (Whitfield 2005, Hughes Martiny *et al.* 2006). Since the current the level of taxonomic resolution in microbial diversity studies is much coarser than in biogeography studies with macroorganisms, declaring that microbial "species" are cosmopoitan (Finlay 2002) might be equivalent to stating that a genus or family of birds is cosmopolitan (Huges Martiny *et al.* 2006). Summing up the evidence that has accumulated over the past five years, the conclusion is that there is no reason to further spread "the myth of ubiquity" (Lachance 2004). The reframed view (Katz *et al.* 2005, Hughes Martiny *et al.* 2006) is more exciting, since "we are beginning to see biogeographic patterns in microorganisms" (C. Horner-Devine, cited in Whitfield 2005).

5. THE SIGNIFICANCE OF LOCAL ADAPTATION

Local adaptation is the genetically fixed differentiation of individual populations among a given species in relation to particular environmental conditions. The adaptation becomes obvious in adaptive traits, increasing the Darwinian fitness of the respective genotypes, i.e. differentiation due to selectively neutral mutations does not imply adaptation. From the foregoing section, it is obvious that, in many cases, local adaptation cannot be studied in broad categories such as the 70% total DNA similarity (for prokaryotes), the morphospecies (for protists) or the SSU rRNA level (for bacteria and protists). As with the difficulty of identifying and comparing species across taxonomic domains, there is no "gold standard" to which local adaptation can be compared between prokaryotes and eukaryotes.

For prokaryotes, the term microdiversity has been introduced to describe the phenomenon of phylogenetically closely related but physiologically distinct populations (Moore *et al.* 1998, Jaspers and Overmann 2004). There is mounting evidence that freshwater bacteria with identical 16S rRNA gene sequences comprise strains with different physiological requirements, i.e. with different ecological niches. This has already been demonstrated experimentally for substrate use (Jaspers and Overmann 2004) and for temperature adaptation (Hahn and Pöckl 2005). In the latter study, the thermal niches of planktonic *Actinobacteria* corresponded to the habitat temperature of their origin.

Temperature adaptation within a nominal species has also been recorded for several planktonic ciliate species (Weisse and Montagnes 1998, Montagnes and Weisse 2000, Weisse *et al.* 2001). Protists with predominant or exclusively asexual reproduction have a (multi-)clonal population structure (Kusch 1998, Kusch *et al.* 2000, Kim *et al.* 2004, Lowe *et al.* 2005) with divergent genotypes and phenotypes. This intraspecific variability is obvious at the physiological but usually hidden at the morphological level. The concept that each (ciliate) morphospecies has a discrete phenotype and a unique ecological niche and that, therefore, biodiversity can be assessed in terms of (morpho)species numbers (Finlay *et al.* 1996, Finlay and Fenchel 2004), needs to be rejected (Weisse and Rammer 2006). The ecologically relevant realised or partial niche (Hutchinson 1965, Vandermere 1972) of a given, asexually reproducing species may vary considerably in space and time, primarily depending on the habitat and the clonal composition of the population. Bland Finlay, citing his own and Tom Fenchel's unpublished work, concedes that "clonal isolates of a single morphospecies from various locations worldwide retain physiological features that reflect the nature of the habitat from which they were taken" (Finlay 2004). Note, however, that a rigorous test of local adaptation among globally occurring aquatic protozoa is still lacking. If local adaptation is common, this will inherently affect ecosystem function, since, e.g. clone-specific production rates provide the amount of food available for metazoan predators. As Nanney pointed out (Nanney 1999, Nanney 2004, Nanney 2005), the functional diversity of ciliates (and, most likely, other protists) is considerably larger than is obvious at the morphospecies level, and adherence to the morphospecies concept will grossly underestimate the number of species, the number of niches, and the complexity of the ecosystem (Weisse and Rammer 2006).

6. IS MICROBIAL DIVERSITY FUNDAMENTALLY DIFFERENT? – DIRECTIONS FOR FUTURE RESEARCH

The advent of various molecular tools has collectively opened a new gate for the study of microbial diversity (Dorigo *et al.* 2005, Ogunseitan 2005). In prokaryotes, molecular techniques such as cloning and sequencing of ribosomal genes provide often the only means to assess the diversity of, as yet, unculturable organisms. The current challenge is to link genetic divergence to ecophysiological diversity in the major taxa. More data are needed to reveal if globally distributed microorganisms can be separated into individual metapopulations with limited genetic exchange. If this is the rule, the individual populations should show indications of local adaptation, both in the genotype and in the phenotype. The primary problem with the "everything is everywhere" view is that this is not a scientific hypothesis, because it is not falsifiable. Similarly, there is no point in arguing about the "true" number of ciliate and other protist species as long as vast areas such as Africa and the South American continent remain undersampled for major taxonomic groups (Foissner 1999, Foissner et al. 2002). The merit of this debate is that it stimulated further research.

From a conceptual point of view, the second proposition of Baas-Becking's axiom, "everything is everywhere, the environment selects", is more challenging than the better known first part. The conclusion that the environment selects is related to Gleason's individualistic concept (Gleason 1926) which interprets the relationship between coexisting species as the result of similarities in their requirements and tolerances. The environment acts as a filter, filtering out all those organisms whose properties do not fit to a given habitat. As Padisák (2003) pointed out, the absence of certain phytoplankton species from whole continents cannot be explained by the lack of suitable habitats. The significance of biotic interactions, in response to their habitat, needs to be explored in more detail for most microbial consortia. At present, aquatic microbial ecologists have, for the first time, the chance to identify patterns of microbial distribution and their interactions. The conceptual challenge is to compare the emerging patterns with those of plants and animals at equivalent taxonomic levels.

The consequences of the undoubtedly vast population sizes of aquatic microbes need to be analysed, both from an empirical and from a theoretical perspective. Is there a critical population size above which genetic drift plays no role, although the total number of mutations within a given taxon may be extremely high? What are the physical limits for sympatric and parapatric speciation among aquatic microbes, and how does the effect of environmental heterogeneity relate to divergence caused by (limited) dispersal? How important is lateral gene transfer for the establishment and maintenance of prokaryote biodiversity? Deciphering of an increasing number of microbial genomes will certainly provide at least partial answers to several of those questions. Yet, not everything is in the genes. Epigenetic inheritance systems (EISs, Jablonka *et al.* 1992, Jablonka and Lamb 1995) allow cells of different phenotype but identical genotype to transmit their phenotype to their offspring, even when the phenotype-inducing stimuli are absent.

The goal is to understand how (freshwater) microbes evolve and change, and to develop adequate methods and concepts (categories) to analyse those evolutionary shifts. Only once we have achieved a deeper understanding of the genotype-phenotype relationship of aquatic microbes can we conclude if their biodiversity is fundamentally different from that of macroorganisms. At present, it appears that it is not - what is fundamentally different, is our view of aquatic microbial diversity, which is still blurred. There is, however, reason to finish this review with an optimistic note. If microbial ecology keeps the momentum that is has gained during the past 20 years, some of the major open questions raised here will be answered within the next one or two decades.

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