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Spatial scale and the diversity of benthic cyanobacteria and diatoms in a salina

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Abstract

We characterized the richness of benthic cyanobacteria and diatoms in a salina system using traditional and molecular biological methods. After determining the different morphotypes and 16S rRNA genes present in various localities within this hypersaline system, an analysis of the increase of organismal richness as a function of numbers of samples considered was carried out. We found that the spatial scales of sampling yielding significant increases in cumulative richness were those at which significant variations in environmental parameters (salinity, vertical microgradients) are known to exist, indicating that the presence of environmental gradients contributes to increased biodiversity. Additionally, we could use this type of cumulative analysis for the estimation, through asymptotic extrapolation, of the total richness of oxygenic phototrophs present in the entire salina system, and for the estimation of the average degree of dissemination of community members within the system. We found interesting differences between analyses based on morphotypes or 16S rRNA genes. The cumulative number of rRNA gene sequences exceeded that of morphotypes by more than two-fold. This indicates that many organisms possessing distinct 16S rRNA gene sequences could not be distinguished on the basis of morphology. Thus, some of the apparently widely distributed morphotypes may in fact conceal several ecologically independent genotypes.

Introduction

Reflecting on the notion that most microorganisms may have a cosmopolitan distribution, early microbiologists coined the expression that with respect to bacteria, 'everything is everywhere' (Baas-Becking, 1934). This hypothesis has pervaded microbiology until today. It is based on the assumption that microscopic organisms are efficiently dispersed over geographic distances, resulting in high local, but comparatively low global diversity (Fenchel, 1993). Indeed, small spatial areas may provide many ecological niches for microorganisms, and high local microbial richness has been described for many habitats (Tiedje, 1995; Pace, 1997). However, very limited data exist on the distribution of microorganisms in nature beyond the microscale. In fact, the impression of widespread occurrence of many microorganisms may to a considerable extent originate from poor taxonomic resolution.

We have investigated the richness and distribution of cyanobacteria and diatoms in a variety of microbial mats in a salina system in Baja California, Mexico. Detailed descriptions of this salina (Javor, 1989; Garcia-Pichel et al., 1994; Des Marais, 1995) and of the morphology of the mats studied (Garcia-Pichel et al., 1999; Nübel et al., 1999) have been published elsewhere. Cyanobacteria and diatoms dis-

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play considerable morphological variability, which is the basis for their current classification (Anagnostidis & Komárek, 1985; Castenholz & Waterbury, 1989; Round et al., 1990). Accordingly, 'floristic' studies have described the spatial distribution of morphology-defined taxa. For example, patterns of occurrence of morphotypes along environmental gradients in various hypersaline environments have been reported (Jørgensen et al., 1983; Ehrlich & Dor, 1985; Montoya T. & Golubic, 1991; Clavero et al., 1994). Moreover, for thermophilic cyanobacteria, the absence of particular morphotypes in some geothermal habitats suggests restrictions to dispersal at the global scale (Castenholz, 1996). However, limitations of morphology-based classification have also become apparent, particularly for morphologically more simple cyanobacteria (Castenholz, 1992; Komárek, 1996). Morphological groupings may (Garcia-Pichel et al., 1996) or may not (Ferris et al., 1996) represent phylogenetically coherent entities, and pleiomorphism may cause additional difficulties for microscopic identification (Garcia-Pichel et al., 1998). Thus, depending on the organisms and on the habitat studied, the analysis of cell and colony morphology may be misleading to an unknown extent, if the study of organismic diversity and distribution is intended. We investigated the richness of oxygenic phototrophs as reflected by their morphology and 16S rRNA genes. We compared richness differences among and within localities, and, using cumulative analyses, evaluated the use of such techniques to determine the spatial scales at which diversity increases. Additionally, we used comparisons of data based on either morphotypes or rRNA gene sequences to evaluate the power of morphological classification to delineate ecologically coherent units in this habitat across environmental gradients.

Materials and methods

Sampling

Microbial mats were sampled during the second to fourth weeks of April 1996 (mats P2, P4, P6, NC2, NC3) and 1997 (mats P3/4, P5, NC52). Sampling sites were located in evaporation ponds of the saltern in Guerrero Negro, Baja California Sur, Mexico, and in the salt marsh of the Ojo de Liebre Lagoon, as illustrated in Figure 1. Within the salina system, the samples analyzed covered the entire spectrum of salinity at which microbial mats occured, ranging from 6 to 16% (Figure 1). In addition, mats from upper tidal channels that had been exposed to natural diel variations of salinity were included (see Figure 1 for locations and ranges of salinity variation). Each of these sampling sites is referred to hereafter as a locality. Within each locality, triplicate cores 10–20 cm apart were subsampled. For light-microscopy, mat samples (core diameter, 4 mm) were fixed in 5% formaldehyde (wt/vol) and stored at 4 °C. For extractions of nucleic acids, mat samples (core diameter, 25 mm) were frozen on site, transported to the laboratory in liquid nitrogen, and stored at −80 °C until processed. Measurements of photosynthesis were performed in a field laboratory with microsensor techniques (Revsbech & Jørgensen, 1986). The maximum depth where gross photosynthesis was detectable varied from 1.5 to 4.8 mm (mean, 2.4 mm). These photic zones were sampled for the investigation of richness. Further results of these supplementary studies are reported elsewhere (Garcia-Pichel et al., 1999).

Analysis of morphotypes

The layers corresponding to photic zones were cut from formaldehyde-fixed mat samples with scalpel blades, and sectioned vertically into subcores of approximately 0.5×0.5 mm mat surface area. These pieces were placed on glass slides in one drop of water and chopped and stirred to achieve even distributions. Of each subcore, 25–40 randomly chosen phase-contrast microscopic fields were photographed at 400-fold magnification and cell counts were performed on projections of the resulting slides. Depending on the respective mat, 2000–3000 cells needed to be investigated to detect all rare and localized morphotypes in a single subcore, and thus to achieve stable richness estimates (Nübel et al., 1999).

Analysis of 16S rRNA genes

The layers corresponding to photic zones were aseptically cut from mat cores (100–400 mg, representing approximately 60 mm^2 of mat surface) and homogenized in Dounce tissue homogenizers (Novodirect, Kehl, Germany). Cell lysis and DNA extraction were performed as described previously (Nübel et al., 1997). Briefly, the suspensions were repeatedly frozen and thawed, and subsequently incubated in the presence of sodium dodecyl sulfate and proteinase K. Cell lysis was controlled microscopically. DNA was extracted by applying hexadecylmethylammonium bromide, phenol, chloroform, and

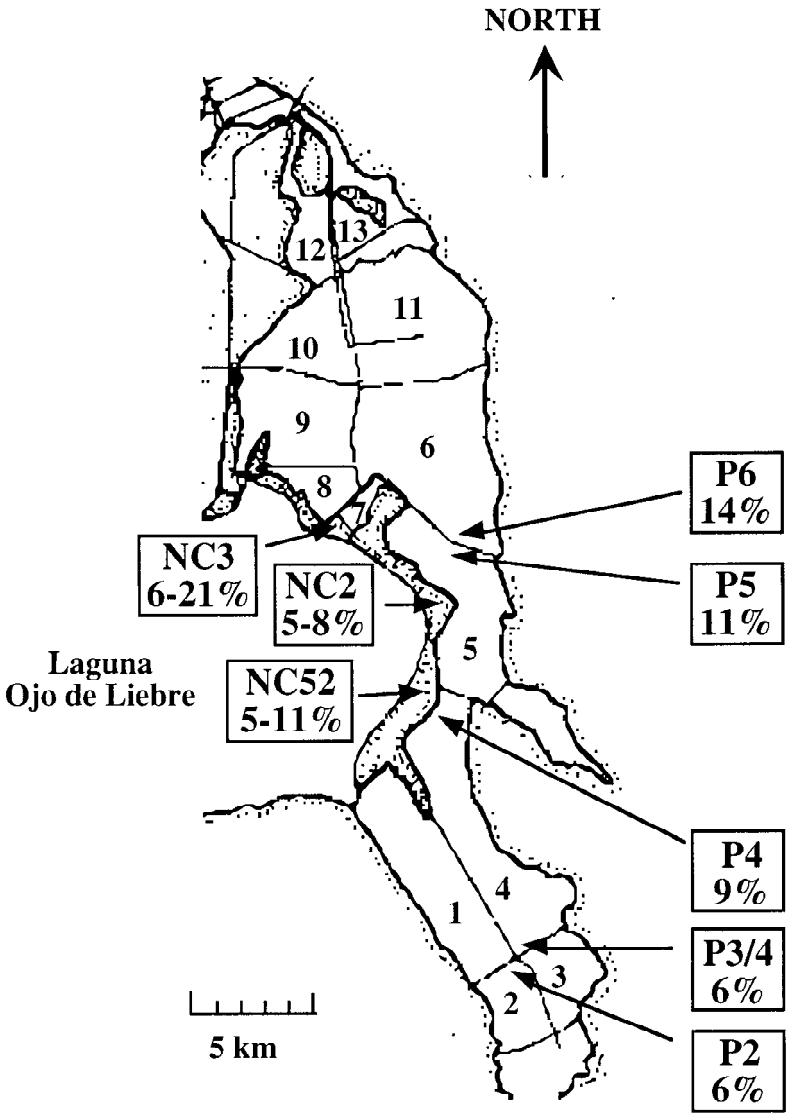

Figure 1. Map showing concentrator ponds of the saltern Exportadora de Sal, S. A. de C. V., Baja California Sur, Mexico. Sampling sites (localities) and salinities of brines, measured at time of collection, are indicated. In most localities salinitiy was virtually constant. In localities situated within tidal channels salinity varied with tidal cycles within the ranges indicated.

isoamylalcohol, and precipitated by addition of isopropyl alcohol. 10 ng of DNAs extracted from mat samples were added as templates to each 100 μl polymerase chain reaction (PCR) mixture. Oligonucleotide primers CYA359F and CYA781R were applied to selectively amplify 16S rRNA gene segments from cyanobacteria and plastids (Nübel, et al., 1997). Numbers in primer designations refer to 5′-ends of target signature sites in 16S rRNA genes (*Escherichia coli* nucleotide numbering (Brosius et al., 1981)). A 40-nucleotide GC-rich sequence was attached to the 5′-end of the primer CYA359F to improve the detection of sequence variation in amplified DNA fragments by subsequent denaturing gradient gel electrophoresis (DGGE (Nübel et al., 1997)). Amplification products generated by duplicate polymerase chain reactions with the same template DNAs were pooled and subsequently purified and concentrated by using the QIAquick PCR purification kit (Diagen, Düsseldorf, Germany). DNA concentrations in resulting solutions

were determined by comparisons to the Gibco low DNA mass standard (Gibco, Eggenstein, Germany) after agarose gel electrophoresis. 500 ng of DNA was applied onto denaturing gradient gels. DGGE separates DNA molecules with identical length but different sequences on the basis of their differential melting behaviour in a gradient of denaturants. It was performed as described previously (Nübel et al., 1997). Briefly, polyacrylamide gels with a denaturant gradient from 20% to 60% were used, electrophoreses were run for 3.5 h at 60 °C and 200 V, and subsequently DNA was stained by using ethidium bromide.

Estimation of richness

The estimation of the richness and diversity of morphotypes and 16S rRNA genes of oxygenic phototrophic microorganisms is described and discussed in detail elsewhere (Nübel et al., 1999). Briefly, cells and molecules were grouped into classes defined by unique cell and colony morphologies or nucleotide sequences, respectively. Ecological richness is considered to be the number of different classes. Cells and molecules from organisms other than oxygenic phototrophs were excluded from the analyses. Microscopically, diatoms and cyanobacteria could be distinguished from most other microorganisms due to their size, characteristic morphologies, and natural autofluorescence. 16S rRNA gene fragments were amplified by polymerase chain reaction (PCR) from cyanobacterial and plastid DNA after nucleic acid extraction from mat samples (Nübel et al., 1997). Phylum-specific amplification enabled the exclusion from the analyses of DNA from organisms other than oxygenic phototrophs (Nübel et al., 1997). Numbers of unique rRNA gene segments amplified were estimated after DGGE analysis of PCR products. With increasing numbers of bands detected in electrophoretic analyses the probability increases that classes cannot be discerned because they run at identical positions in the gel. However, based on the total distance of separation, average peak width, and the low numbers of peaks detected, this crowding effect was estimated to have only negligible effect on our DGGE results (Nübel et al., 1999).

Cumulative richness and its extrapolation

With increasing number of samples investigated, novel classes (morphotypes or 16S rRNA gene sequences) were detected that resulted in an increase of the cumulative richness. For example, the total number of different morphotypes detected in mats from localities P2 and P3/4 was 16 (five morphotypes occurred at both localities). Inclusion of locality P4 in the analysis added another five morphotypes that had not yet been observed in mats from P2 and P3/4, and so on. We pooled data obtained from individual localities and plotted the cumulative number of classes discovered as a function of the number of localities considered. The shape of the resulting accumulation curves depends on the sequence in which samples are added. In order to avoid this arbitrariness we computed and considered all possible accumulation sequences (Figure 2). The morphotype and gene sequence accumulation curves approach asymptotic maxima (S_{max}) which we estimated by extrapolation. For extrapolation two-parameter hyperbolas were fitted through means of cumulative richness calculated for an increasing number of localities as:

$$S(n) = \frac{S_{max} n}{K + n}$$

In this equation, S is the mean cumulative richness and n is the number of localities pooled. S_{max} and K are fitted constants, which were calculated by double inverse linear regression ($1/S(n)$ on $1/n$, Figure 2). $S(n) = S_{max}$ is the asymptote and S_{max} can be interpreted as the total richness to be expected in the system under study (Colwell & Coddington, 1994). We did not attempt to estimate confidence intervals for S_{max} and K because of questionable statistical validity of such a computation (Colwell and Coddington, 1994). The above equation is well known as the Michaelis-Menten equation of enzyme kinetics.

Table 1. Numbers of morphotypes

Locality	Triplicate subsamples			Cumulative total
P2	11	10	11	12
P3/4	7	7	8	9
P4	11	10	10	12
P5	7	6	8	8
P6	10	10	9	10
NC2	13	11	12	13
NC52	11	13	12	13
NC3	13	12	13	13

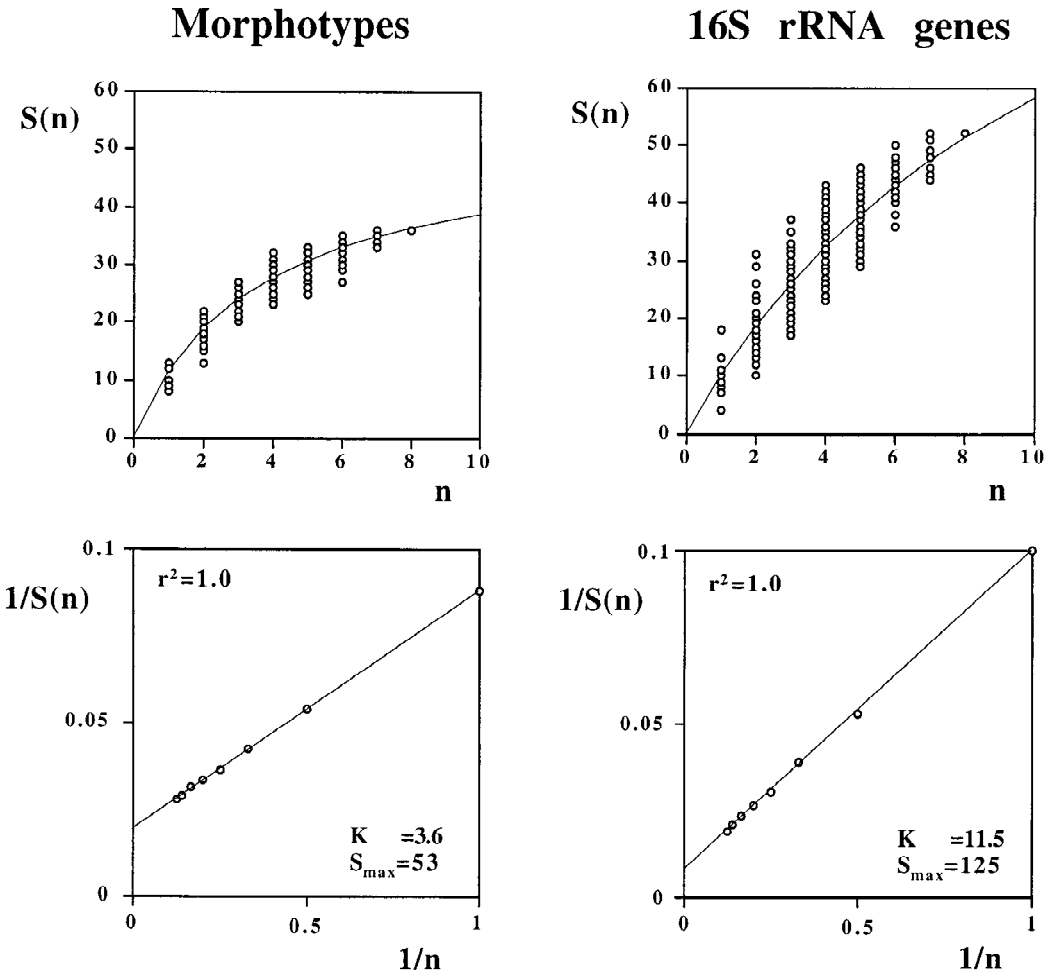

Figure 2. Cumulative number of classes, *S(n)*, plotted as functions of the number of localities, data from which were pooled, *n*. The left hand graphs show data from morphotype analyses, the right hand graphs show data from 16S rRNA gene analyses. In the upper graphs results from all possible combinations of localities were plotted to eliminate variation in curve shape due to accumulation order. Two-parameter hyperbolas were fitted through means of *S(n)* based on double-reciprocal plots as shown in the lower graphs. Pearson correlation coefficients, *r*, estimates of asymptotic total regional richness, S_{max}, and half saturation constants, *K*, are given.

Table 2. Numbers of 16S rRNA genes

Locality	Triplicate subsamples			Cumulative total
P2	10	12	12	12
P3/4	4	4	4	4
P4	10	10	10	10
P5	7	7	6	7
P6	10	10	10	10
NC2	12	12	12	13
NC52	13	12	12	13
NC3	14	17	17	18

Results and discussion

Spatial heterogeneity

The analyses performed permitted investigation of the heterogeneity of the distribution and diversity of cyanobacteria and diatoms at various spatial scales. Considerable patchiness in the organismal distribution was observed at the scale of cyanobacterial colonies and filaments (10–100 μm). Detailed microscopic analysis indicated that at least 2000–3000 cells per subsample needed to be investigated to achieve stable and repeatable estimates of morphotype richness (Nübel et al., 1999). In few cases, and to a lesser extent, this

type of heterogeneity was still apparent at the scale of millimeters. For example, tufts of diatoms were macroscopically visible on the surfaces of some of the mats. However, for all eight localities investigated, richness estimates based on either morphotypes or 16S rRNA genes were consistent among triplicate subsamples that had been taken several centimeters apart. The coefficients of variation for triplicates within localities were typically 5% and maximally 8%, indicating that heterogeneity was low within localities. As a result of this homogeneity, cumulative richness within localities was not much higher than that of the single subsamples (Tables 1 and 2). In contrast, we found marked differences among localities several kilometers apart. This is consistent with the assumption that the concentration of total salts in the brines overlying the microbial mats directly or indirectly determined the horizontal distribution of cyanobacteria and diatoms (Golubic, 1980; Ehrlich & Dor, 1985).

Estimation of regional richness

In the eight mat communities investigated, a total of 36 different morphotypes and 52 different 16S rRNA gene segments from oxygenic phototrophs were detected. Pooling of the data from an increasing number of localities resulted in increasing, but saturating, cumulative richness (Figure 2). Extrapolation of accumulation curves estimated the total numbers of different morphotypes and 16S rRNA genes from benthic oxygenic phototrophs probably present in the whole salina, S_{max}, to be 53 and 125, respectively. The mean richness detected at single localities, *meanS*, was 10.2 and 10.3 in the microscopic and molecular biological analyses, and the ratios $meanS/S_{max}$ accordingly were 0.19 and 0.08, respectively. These quotients are relative measures of the average dissemination of classes, theoretically ranging from zero (all classes are locally endemic) to one (all classes are ubiquitous). A dissemination quotient close to one may be reached in habitats such as soil, in which microbial community composition can be very homogeneous at a geographical scale of kilometers (Felske & Akkermans, 1998). The dissemination quotients determined in our study must be considered rather low, and are probably related to the marked physicochemical differences among sampling sites. However, it was observed that some morphotypes and rRNA genes were widespread among the various mat communities analysed, whereas others were found at few or single localities only (our unpublished results). While widespread occurrance through a wide range of salinity clearly indicates that an organism is euryhaline, restriction to certain communities may be caused by various other physicochemical factors or biological interactions. *K*, analogous to the half saturation constant K_m in enzyme kinetics, can be interpreted as the average number of samples needed to detect half of the richness in the system under study. These constants were found to be 3.6 and 11.5 for the analyses of morphotypes and 16S rRNA genes, respectively. In general, such estimates should have the potential for optimizing efforts in diversity surveys.

Morphotypes and 16S rRNA genes

We compared the richness of oxygenic phototrophs in microbial mats as reflected by their morphology and 16S rRNA gene sequences. Both approaches must be considered biased to an unknown extent, especially with respect to abundance determinations (discussed in detail in (Nübel et al., 1999)). However, when investigating samples within localities, both approaches repeatedly yielded similar results. Richness estimates based on 16S rRNA genes within localities correlated significantly with those obtained by morphotype analyses (Pearson correlation coefficient, r=0.940, p=0.001). Interestingly, the ratio of both richness estimates (means of triplicate analyses) equaled 1 in six of eight localities (Nübel et al., 1999). Thus, when analysing the diversity of cyanobacteria and diatoms at small spatial scale, morphotypes and 16S rRNA genes may seem equivalent. However, we found that morphology analysis detected significantly less richness than gene analysis when organism distribution was considered over larger spatial scales. The number of sequence-different 16S rRNA gene segments found in the eight microbial mats clearly exceeded the number of morphotypes identified. The lower degree of saturation (*K*, Figure 2) found for the latter analysis caused the estimates of S_{max} to differ more than two-fold.

The number of different 16S rRNA gene sequences found should be considered a minimum estimate of the underlying richness of 16S rRNA genes because oxygenic phototrophs may exist in the samples that do not contain the signature sequences for efficient PCR amplification (Nübel et al., 1997), and because amplified DNA molecules potentially may occur at the same position in the denaturing gradient gel despite differential sequences (Nübel et al., 1999). In turn, sequence analysis for the majority of bands indicated that most likely, none of the bands was derived

from any undesired amplification product (data not shown). Slightly different 16S rRNA gene sequences may exist in some organisms (Nübel et al., 1996), but this phenomenon seems to be rather rare among cultivated cyanobacteria and diatoms from hypersaline environments (our unpublished results).

With continued sampling sequence-different 16S rRNA genes accumulated at a faster rate and with a higher saturation value than morphotypes (Figure 2). Since the presence of strong limits to dispersal within this system of contiguous flow seem unlikely, the differences must indicate that many of the morphotypes concealed genotypes that are ecologically distinct and therefore occur at different sampling sites. Two reasons are plausible to explain the observation that these organisms have indistinguishable morphology, and probably both apply to some extent. First, morphology analysis may detect diversity at a phylogenetic level too high to correlate with ecophysiological diversity. In fact, once they are established, morphological features are rather conserved in phylogenetic lineages of bacteria in general (Siefert & Fox, 1998), and physiological differentiation may have occurred relatively later in the evolutionary history of the respective organisms. Alternatively, convergent evolution of morphology may have followed, or may have been part of, ecophysiological specializations. In any case, our results reflect the limitations of morphology-based classification, and indicate that the analysis of nucleic acid sequences may be more sensitive for the detection of the ecophysiological diversity of oxygenic phototrophic microorganisms. Clearly, there is a need for new cyanobacterial taxa, integrating information on the ecology and physiology of the respective organisms (Golubic, 1980; Anagnostidis & Komárek, 1985; Castenholz, 1992). For in-depth characterisations of ecophysiological capabilities of microorganisms, however, information retrievable from field observations will be insufficient in most cases. At best, differences in occurrence patterns may indicate some, but largely cryptic, divergence among distinguishable populations. In contrast, the bacteriological system of cyanobacterial classification (Castenholz & Waterbury, 1989) allows much more detailed experimental investigations on individual strains, but severely suffers from the difficulties inherent in the need for isolation and cultivation and currently is far from being complete (Castenholz, 1992). Molecular biology, however, offers powerful tools for the comparison of cultivated strains and their counterparts in the wild, as well as for guiding future isolation attempts towards the discovery of as yet unknown microorganisms.

Conclusions and outlook

It seems unlikely that limits to dispersal may isolate microbial populations only a few kilometers apart. The differential distribution of organisms must have been determined by their specific ecological demands. Accordingly, large increases in cumulative richness, detected by continued sampling, occurred at a spatial scale which included measurable variations in environmental parameters. We cannot assert at this point if this apparent relationship between microbial richness and environmental heterogeneity will hold in general for microbial communities, but it seems plausible in the light of niche specialization theory. If it does, then it should indicate that the spatial scales important for the accumulation of diversity may depend on the spatial structure of environmental gradients or boundaries. In habitats which show considerable physical heterogeneity at smaller scales (millimeter, centimeter), but not necessarily at larger scales (meter, kilometer), microbial richness may increase at different scales than those that were important in our system. It should prove interesting to extend this type of study to the global scale, as this may offer important information on extant global microbial diversity, for which data are simply non-existent. If cumulative analysis of similar habitats in widely separated geographical localities (for example, salina systems worldwide) should reveal small cumulative richness increases, this would speak for efficient dispersal at planetary scales. The dissemination quotients should give some information on the potential existence of limits to dispersal for a portion of the community members. Additionally, this type of analysis may facilitate the distinction of the presence of climatic borders for microbial communities or the determination of the space/time scales at which microbial dispersal occurs. It must be noted, however, that the primary structure of ribosomal RNA genes is highly conserved, and to identify ecologically coherent populations, it may even be necessary to analyse more variable nucleic acid sequences, such as those encoding proteins (Palys et al., 1997). Generally, ecologically and evolutionarily more meaningful species concepts for microorganisms may be developed in the future (Ward, 1998). Only then, when based on more equivalent units, the diversity and distribution in space

of plants, metazoa and unicellular organisms can be meaningfully compared.

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