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Theoretical and Practical Aspects of the Quantification of Biodiversity among Microorganisms

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Theoretical and practical aspects of the quantification of biodiversity among microorganisms

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CONTENTS PAGE

SUMMARY

The quantification of biodiversity among microorganisms has to address both theoretical and practical aspects. Species concepts are often at variance with those applied in macroorganisms, and satisfactory concepts suitable for general use in bacteria and fungi have yet to be formulated. Molecular approaches have not yet provided a universal solution to this key issue. Quantification in habitats such as soil is difficult as isolation procedures yield only a small and skewed selection of the microorganisms present. Indices of taxonomic or phylogenetic diversity have potential in the quantification of microbial diversity at a range of ranks, but the non-equivalence of ranks and representatives of the taxa detected have to be addressed. Chemical and molecular methods have immense potential in the quantification of microbial diversity in environmental samples; 16*S* rRNA has shown particular promise with bacteria, but as yet the fungi lack a universal probe. A greater awareness of the limitations of existing approaches and methodologies used by microbiologists is needed, but significant progress can be anticipated as new technologies are developed and become more widely adopted.

1. INTRODUCTION

Microorganisms are the most ancient and numerous biotic entities on Earth, which through their metabolic diversity and genetic adaptability have successfully colonized every known ecological niche, including extreme environments inhospitable to other forms of life (Karl 1987). They play an integral and often unique role in the functioning of ecosystems and, through ecosystem interaction, in maintaining a sustainable biosphere. Indeed, the role of microorganisms in maintaining the dynamic equilibrium and integrity of the biosphere is so critical that the continued existence of Life is dependent on the sustained, microbially mediated transformation of matter in both terrestrial and aquatic environments. That almost all biological processes in the environment, either directly or indirectly, involve microorganisms is often overlooked, and the potential benefits of regulating, optimizing and exploiting microbial activity, largely unexplored (Zedan 1993).

Genetic diversity in microorganisms is so broad that it is not practical to consider cataloguing and preserving *ex situ* significant proportions of the diversity from anything but the most simple of natural habitats. For example, in the case of the fungal gene pool, the number of fungi currently maintained in service culture collections throughout the world represents about 17% of the 69 000 accepted fungal species which is but a small proportion of the estimated numbers of fungal species (Hawksworth 1991).

It is now accepted that the extent of microbial diversity has not been adequately characterized and

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there is an immense mismatch between knowledge of that diversity and its importance in both ecosystem processes and economic development (Hawksworth & Colwell 1992; Zedan 1993). Unlike macroecologists, microbial ecologists working on natural communities are faced with unique challenges posed by, for instance: (i) the very large number of individuals per sample, for example more than 10^9 organisms per gram of soil (Torsvik *et al.* 1990*a*,*b*); (ii) the problem of differentiating between different populations and the very high diversity at a relatively small scale, for example more than 10^4 species per gram of soil (Klug & Tiedje 1994); and (iii) the difficulty of defining a bacterial (Goodfellow & O'Donnell 1993) or fungal (Claridge & Boddy 1994) species, or some other unit that encompasses the appropriate level of diversity.

This paper draws attention to key theoretical and practical aspects of quantifying microbial diversity. Microbial diversity at the highest taxonomic ranks is discussed by Embley *et al.* elsewhere in this volume. Here, we consider the difficulties and suggest possible solutions to quantifying microbial diversity at the infra-familial taxonomic level, particularly genera and species.

2. THE BACTERIAL AND FUNGAL SPECIES CONCEPT

In keeping with most assessments of the extent of diversity in macroorganisms, most attempts to quantify microbial diversity to date have centred on species numbers. However, the species concept remains a difficult and controversial theme in both bacterial (Goodfellow & O'Donnell 1993) and fungal (Claridge & Boddy 1994) systematics.

In bacteria, estimates of species numbers have served primarily as a stimulus for a renewed debate on the need for a better, universal definition of the species. In the absence of such a definition, estimates of microbial diversity based on species richness (a simple count of the number of species present) and the comparability of such data with that of macroorganisms remain problematic.

Further, these concepts are not always directly comparable to species concepts in macroorganism groups, so that like is not always being compared with like.

(*a*) *The bacterial species*

The classical view is that bacterial species can be distinguished by a number of simple observable features, that is according to their phenotypic signatures. In practice, taxonomists working on different groups of organisms use different criteria for delineating taxa. Typical examples are the current treatments of the families *Bacillaceae* and *Enterobacteriaceae* with organisms in the first group markedly underspeciated (Rainey *et al.* 1994; White *et al.* 1994) whereas in the latter different generic designations are preserved for bacteria related at the species level (Palleroni 1994).

The subjective nature of the traditional species concept was recognized by Cowan (1978) who defined a species as 'a group of organisms defined more or less subjectively by criteria, chosen by the taxonomist to show to best advantage and as far as possible put into practice his individual concept of what a species is'. The fluid nature of this species concept lead to some microbial taxonomists being dubbed 'lumpers' and others as 'splitters'. Lumpers tend to emphasize the similarities between strains, thereby circumscribing relatively few taxa, whereas splitters highlight differences in the belief that the clarity inherent in small groups is paramount.

The limitations of the traditional phenotypic species concept is readily exemplified. Thus, the sugar tests used to distinguish *Bacillus circulans* from other facultative anaerobes are of no value in the circumscription of other bacilli such as *B. sphaericus* which have an oxidative metabolism and as such cannot metabolize carbohydrates (Priest 1993). It is now evident that this approach to the delineation of *Bacillus* species was flawed as sugar-utilizing strains generally formed homogeneous species whereas oxidative organisms were arranged into what proved to be genetically diverse groups largely because of their failure to metabolize carbohydrates.

The genetic concept of species has contributed little towards a more precise definition of bacterial species (Jones 1989). Most examples of horizontal gene transfer come from members of three genera which have a special capacity for the uptake and chromosomal incorporation of DNA (Maynard Smith *et al.* 1991). Such naturally transformable bacteria are found among both Gram-positive (e.g. *Bacillus*, *Streptococcus*) and Gram-negative (e.g. *Haemophilus*, *Neisseria*) genera (Stewart & Carlson 1986). Bacteria differing in DNA sequence by up to 20% can and do exchange chromosomal DNA (Maynard Smith *et al.* 1991). The exchange is usually local, often involving only a few hundred base pairs, and hence does not destroy the clonal population structure detected by methods such as protein electrophoresis. Interspecific transformation studies have revealed three distinct homology groups among strains of *Moraxella* and *Neisseria* (Bøvre 1980).

Although certain phages and plasmids have a broad host range, others will only mediate transfer between closely related strains. In the latter, host range is almost species specific (Jones 1989; Harwood 1993) due to factors such as the specificity of the host cell receptors. Although this might appear to provide a basis from which to develop a 'universal species' concept for bacteria, other plasmids are promiscuous in their conjugal transfer and appear to be able to mediate conjugation between members of most Gram-negative species. Although the basis of such extensive host ranges remains unknown, some of the plasmids concerned appear to have developed complex and highly flexible replicative systems. This has important ecological and evolutionary significance and serves to illustrate the potential fluidity of the bacterial genotype.

An alternative molecular approach to the definition

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

of a species (Wayne *et al.* 1987), which does not rely on the capability of organisms to exchange genetic information, is that based on percentage G + C composition and DNA:DNA hybridization. This molecular definition of species would recognize all strains with approximately 70% or more DNA–DNA relatedness *and* 5°C or less ΔT_m as belonging to the same species. This approach to the species concept is attractive as it can be applied to all prokaryotes irrespective of their growth requirements. Although this approach is generally considered reliable, it can, in practice, be difficult to generate a complete matrix of relatedness values between each and every strain.

The usual strategy in DNA homology experiments is to select a few organisms as reference strains and to compare all other strains against this limited set. This poses the problem of how well the taxonomic structure obtained using the reference set compares with that derived from a complete matrix in which all strain comparisons are made. This is not straightforward as it has been demonstrated (Hartford & Sneath 1988) that information on the underlying taxonomic structure is lost on selection of a small number of reference strains. Thus, considerable care and *a priori* knowledge is necessary in choosing reference strains that are representative of the taxonomic variance among isolates.

It will be clear from the above that an acceptable definition of the bacterial species concept has still to be obtained, and further that the way in which this is approached is distinct from the biological species stressed in macroorganism groups.

(*b*) *The fungal species*

The concept of the species is also unsatisfactory in mycology, although it has not yet attracted the level of debate seen in bacteriology. The wealth of morphological characters in many groups has led to an historical emphasis on discontinuities in these for species separations, ideally using discontinuities in more than a single character (Hawksworth 1974). More theoretical attention has focused on genetic-population aspects of refining the species concept (Burnett 1983; Brasier 1987; Perkins 1991; Claridge & Boddy 1994), yet in the 83% or so of known species not available in culture opportunities for experimental approaches both are limited and often impractical in view of scarcity of material. Further, as with bacteria, approaches have not been consistent across different groups of fungi, subdisciplines tending to evolve working practices acceptable to those within them. For example, carbohydrate assimilation tests and abilities to grow on particular media are used and accepted in yeasts, occurrences on particular hosts are of paramount importance in rust fungi (even in the absence of inoculation experiments), secondary metabolites are accorded major importance in some lichen-forming fungi (e.g. *Parmeliaceae*), and spore ornamentation at the scanning electron microscopy level has been emphasized in others (e.g. *Aspergillus*).

At the same time, a tremendous amount of variation is sometimes masked under a single fungus species name. It is becoming increasingly clear that a wide variety of fungi have a considerable number of 'vegetative incompatibility groups'; this phenomenon is known within meiotically (Brasier 1987) and mitotically (e.g. Ploetz 1990) reproducing groups where in effect there are biological species within morphospecies. In due course, an increase in the formal description of plant pathogens as species, but which cannot easily be distinguished morphologically, can be expected; one example is the recognition of *Ophiostoma novo-ulmi* as the causal agent of the European elm disease pandemic in the mid-1970s rather than continuing to include this within the morphologically almost identical *O. ulmi* (Brasier 1991).

(*c*) *The impact of molecular sequencing*

16*S* rRNA sequencing is being used increasingly to help describe new species (Li *et al.* 1994), to transfer previously described bacterial species to new genera (Ash *et al.* 1994), and to link mitotic fungi into sexually reproducing families and genera (LoBuglio & Taylor 1993). Although the application of such techniques has revolutionized understanding of the relationships amongst microorganisms above the genus level, it is not without problems in defining species. In yeasts, extensive data show that while well-defined species tend to have less than 1% nucleotide sequence difference in the 25*S*-63*S* rRNA subunit, related species tend to differ by 1–5% (Kurtzman & Robnett 1994). However, while all available 16*S* rRNA sequences of legionellae show marked sequence similarities, DNA–DNA hybridization studies of the same species may show less than 5% homology with the type strain of the genus, *Legionella pneumophila* (Brenner 1986; Fry *et al.* 1991). Similarly, Fox *et al.* (1992) have shown that although strains of *Bacillus globisporus* and *B. psychrophilus* share greater than 99.5% similarity in 16*S* rRNA sequence homology they show less than 50% reassociation in DNA pairing experiments. This suggests that in some cases strains regarded as belonging to different species may have near identical 16*S* rRNA sequences.

Ultimately, the question as to whether or not differences in rRNA sequences can be used to define more precisely a bacterial or fungal species will depend on whether the variability within a species is less than, and can be discriminated from, the variability at higher levels of taxonomic rank. This requires that more information is obtained on the variability in the 16*S* rRNA or other sequences among strains of the same species. Although such information is limited, A. G. O'Donnell, Y. Xia and T. M. Embley (unpublished data) have shown that within species of the genus *Azospirillum*, sequence homology over the V2 hypervariable region (Neefs *et al.* 1991) of the 16*S* rRNA was greater than 96% whereas that between species (over the V2 region) was in the range 85 to 95%.

The application of modern analytical techniques has led to dramatic improvements in bacterial and fungal systematics but the goal of a universally

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

accepted species concept remains as elusive as ever. Minimal standards have been published for the description of bacterial species within particular genera (Graham *et al.* 1991; Lévy-Frébault *et al.* 1992), but criteria for the recognition of species still vary considerably between bacterial genera. The minimal standards approach has not even been attempted in mycology.

The number of species in a genus to some extent reflects inherent natural diversity, but is also influenced by the criteria adopted to define the species and by how much taxonomic interest there is in a group. However, as pointed out above, this pragmatic approach to the definition and circumscription of species makes it very difficult to quantify microbial diversity in any meaningful way using the species as the basic unit of measurement.

3. QUANTIFYING MICROBIAL DIVERSITY IN AN ECOLOGICAL CONTEXT

Much of what is now known about the relationships between microorganisms that are not detectable by eye or with a hand lens is based on detailed experimental studies of those organisms after isolation from their natural habitat or host. Yet in assessing diversity for inventorying purposes, the challenge is how to quantify the microbial diversity of a given habitat or host. However, in the case of one of the major microbial habitats, soil, the complexity of relationships between the environments present and the microorganisms it contains results in major problems in defining habitat limits. Soil, is a matrix of solid, liquid and gaseous components interacting with the soil biota to provide a fluctuating and dynamic system (Burns 1983).

Microbial populations in soils vary spatially and temporally according to factors such as: the nature of the soil parent material; the availability of carbon sources; seasonal and diurnal variations in temperature, porosity and water holding capacity; and changes in electrolyte concentration, pH, redox and oxygen availability (Lee 1991). Given the complexity of the soil environment, the reliability of diversity estimates based on species concepts derived from taxonomic analyses of laboratory grown cultures is questionable. The immediate challenge facing microbial taxonomists and ecologists is how best to determine what needs to be known in order to quantify microbial diversity in natural environments.

The wide range of methods used to examine soils is a particular problem. Although there have been significant contributions drawing these together and explaining their value (Gams 1992; Wynn-Williams 1992), so few workers use absolutely identical protocols that comparisons between data sets cannot be made with confidence.

The situation is further complicated as, unlike the identification of the macroorganisms, microbial identification usually requires that the organism is extracted from its environment and then studied microscopically or in culture. By necessity, sampling methods differ between organisms and between habitats and as such are known to underestimate both the number and variety of species (Brock 1987; Schlegel & Jannasch 1991).

The spatial distribution of microorganisms in soil (Hattori 1988) and the need to overcome the wide range of microorganism–soil particle interactions (Ahmed & Oades 1984; Stotzky 1985) are the main limitations to quantitatively and representatively sampling soil microorganisms. Procedures used to dissociate microorganisms from particulate matter include shaking in diluent (Goodfellow *et al.* 1990), chelating agents (MacDonald 1986) and Tris buffer (Niepold *et al.* 1979), mild ultrasonication (Ramsey 1984) and repeated homogenization of soil in a variety of buffers followed by separation of extract from residue (Faegri *et al.* 1977). Hopkins *et al.* (1991) developed a multistage dispersion and differential centrifugation technique for representatively sampling microorganisms from soil. The efficiency of the method was assessed by determining the biomass recovered in extracts and residues compared to the soil at the onset.

In preliminary experiments the dispersion and differential centrifugation technique was found to be three to twelve times more effective in extracting actinomycete propagules from a range of soils than the conventional procedure of shaking soil in diluent (Atalan 1993). There was also evidence that different kinds of actinomycetes were isolated at different stages of the extraction procedure and that certain organisms were only recovered on isolation media seeded from inocula derived from the application of the new extraction procedure. These results suggest that actinomycete–soil interactions may limit quantitative and representative sampling of actinomycetes from soil and that the multistage dispersion and differential centrifugation technique is effective in breaking such interactions.

Another constraint on quantitative and representative sampling of bacteria from natural habitats is the dearth of suitable selective isolation procedures. The selectivity of isolation media is influenced by nutrient composition, pH, the presence of selective inhibitors, temperature and time of incubation. Innumerable media formulations have been recommended for the selective isolation of bacteria but the ingredients have usually been chosen empirically and the basis of their selectivity is not clear (Williams *et al.* 1984). It is now possible, using computer-assisted taxonomic procedures, to formulate and evaluate selective media in an objective way. Indeed, numerical taxonomic databases, which contain extensive information on the nutritional, physiological and antimicrobial sensitivity profiles of the constituent taxa, are ideal resources for formulating new selective media (Goodfellow & O'Donnell 1989). This taxonomic approach to selective isolation has been used successfully to isolate novel, rare and uncommon actinomycetes, notably streptomycetes, from diverse natural habitats (Vickers *et al.* 1984; Goodfellow & O'Donnell 1989; Atalan 1993).

In the case of fungi that can be detected by the unaided eye or a hand lens difficulties vary

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

considerably according to the biological or taxonomic group. For example, while perennial polypores and most lichen-forming fungi can be found at any time of the year, many others may be seasonal, have short-lived sporophores, or occur only on certain stages or parts of host plants or animals (e.g. decayed leaves, insect larvae). In the case of a forest, at least 30 discrete microhabitats can be recognized, almost all requiring separate techniques and often specialists for detection (Hawksworth *et al.* 1994). As an example, fungal inventory studies since 1969 on the 521 acre Slapton Ley National Nature Reserve in southwest England had generated a species list of 1919 fungi by the end of March 1994, of which 56 have been new to the U.K. and 21 described as new to science; that work has involved 58 mycologists and the actual total for this one site is now estimated at not less than 2500 species (D. L. Hawksworth, unpublished data).

These conditions make it difficult to base quantitative estimates of microbial diversity on a system of collection, isolation, classification and identification. Nonetheless, the issue urgently needs to be addressed if an 'All-Taxon Biodiversity Inventory' (ATBI) is ever to be accomplished at one site (Janzen 1993; Yoon 1993). Tentative first steps towards the development of protocols for the microbial component of such a project have been made (Hawksworth *et al.* 1994; Tiedje 1994), but concerted action is required. Not until at least one site is fully 'inventoried' will there be a known biota against which the efficacy of different sampling, isolation and assessment methods can be tested and extrapolitative methods calibrated for the assessment of species richness in other sites.

4. CAN INDICES USED TO QUANTIFY DIVERSITY IN MACROORGANISMS BE APPLIED TO MICROORGANISMS?

In assessing the biodiversity of a site, rather than relying solely on estimates of species richness, some account must be taken of the extent to which the species differ taxonomically. This is 'taxonomic diversity' and is considered to be more indicative of the biodiversity of a habitat than species richness. For example, at higher levels of taxonomic rank such as phlya, diversity in marine habitats is greater than that in terrestrial environments even though the number of species may be lower (Laserre 1992).

Measurements of taxonomic diversity require that microorganisms can be reliably identified to higher levels of the taxonomic hierarchy such as genus, family, order, and phylum. This is now feasible and can be done with some degree of reliability, particularly among the bacteria, and increasingly so with meiotically reproducing fungi. However, as the generic and suprageneric levels circumscribe genetic and phenotypic diversity subjectively this renders assessments of 'taxonomic diversity' based on formal ranks of limited value.

The lack of comparability of higher ranks is evident when taxa are studied by molecular phylogenetic methods. For example, in the fungi the *Oomycota* and *Myxomycota* are quite remote from each other and from the *Ascomycota*, *Basidiomycota* and *Zygomycota* cluster (Bruns *et al.* 1991) to the extent that these are now generally accepted as belonging to different kingdoms (Corliss 1993). This issue is explored by Embley *et al.* elsewhere in this volume.

Estimates of both species richness and higher levels of taxonomic diversity assume that all species or taxa are of equivalent value and as such should be given equal weight in the quantification of diversity for conservation purposes (Williams *et al.* 1991). However, among macroorganisms equivalence in the value of species has become less fashionable with species endemism and vulnerability used increasingly as additional criteria for site selection and conservation (Williams *et al.* 1991). Attempts to improve the usefulness of biodiversity indices have been made by Vane-Wright and colleagues applying fixed weights to terminal taxa, usually species, relative to their position in the classification ('root-weighting'; Vane-Wright *et al.* 1991). Biotas can then be compared by aggregating the taxonomic weights of all their species. Consequently, the total diversity score for any given system depends on both the number of species included and their taxonomic distinctness. This approach, and other variants of it, are critically reviewed by Faith (this volume).

The root-weighting approach has considerable promise as a means of quantifying microbial diversity, but to our knowledge, has yet to be applied. The major problem in its routine application in the foreseeable future is that it relies upon a sound taxonomic base, which for the reasons already outlined is seriously lacking for many of the microbial groups found in natural environments.

'Ecosystem diversity' aims to take account of all kinds of diversity within an ecosystem (Hawksworth & Ritchie 1993). Thus, in addition to estimates of species richness and abundance, the assessment accommodates factors such as community or habitat classification and measurements of structural diversity. Ecosystem diversity endeavours, therefore, to address some of the problems of scale and variability in natural habitats. According to Whittaker (1972) ecosystem diversity comprises: α-diversity, the diversity of species within a community or habitat (i.e. species richness); β-diversity, a measure of the rate and extent of change in species along a gradient between habitats; and γ-diversity, the richness in species over a range of habitats in a geographical region. Owing to difficulties in estimating the α-diversity of microbial populations, it is difficult to see how estimates of ecosystem diversity which take full account of the microbial diversity of a habitat can be derived unless 'species richness' is replaced with an alternative measure or index of microbial diversity which does not rely on the classification and identification of microorganisms.

Advances in molecular and chemical ecology which provide an estimate of microbial diversity without having to isolate the organisms show some promise (White 1983; Fredrickson *et al.* 1986; Giovannoni *et al.* 1990; Ward *et al.* 1990). However, as currently used, such approaches tend to be biased towards the more

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

dominant (abundant) organisms at the time of sampling. Because the relative abundance of microbial species in natural habitats is rarely equal, with a few species predominant among a larger group of common species, such estimates need to be interpreted cautiously.

5. CHEMICAL AND MOLECULAR APPROACHES TO QUANTIFYING MICROBIAL DIVERSITY

Attempts to quantify microbial diversity are all too readily thwarted by an inability to culture and identify a significant and representative proportion of the indigenous community. Non-culture methods of studying microbial populations in which specific macromolecules or biomarkers are extracted and compared have not been widely applied and are often treated suspiciously by many microbial ecologists because of possible changes in chemical composition during growth in natural environments (Tunlid & White 1992). Thus, the knowledge base against which their potential in quantifying diversity can be assessed is limited.

Although the efficiency with which biomolecules can be recovered from natural environments is affected by the physico-chemical and biological properties of the habitat, chemical markers have been used to study microbial populations in sediments, soils and waters. For example, White and his colleagues (White *et al.* 1979; White 1983) have pioneered the use of shifts in the phospholipid fatty acid composition of environmental samples to investigate change and diversity in bacterial communities. This type of approach, while lacking the resolution of taxonomic based methods, provides a simple measure or index of diversity which might be used to analyse both the α- and β-diversity of an ecosystem.

An alternative approach to the estimation of bacterial diversity is the analysis of 16*S* rRNA signatures. This technology is founded on the extensive 16*S* rRNA sequence databases used to determine the phylogenetic relationships of bacteria (Woese 1987) and makes possible the recognition of uncultured organisms using sequence signatures (Ward *et al.* 1990). None of the methodologies are without problems and each has its own strengths and weaknesses which may bias sequence recovery (Embley & Stackebrandt 1994). As with all biomolecules, whether the sample is representative of the indigenous population depends largely on the efficiency with which the nucleic acid (DNA or RNA) can be extracted from the environment. This extraction efficiency will depend on the prevailing physico-chemical environment at the time of sampling and on the biological properties, such as susceptibility to cell lysis, which may change with growth phase.

In the bulk DNA cloning approach (Pace *et al.* 1985; Schmidt *et al.* 1991) total DNA is isolated, partially digested with a restriction enzyme such as Sau 3A and cloned using a lambda vector. Genomic libraries generated in this way supposedly impose no selection bias on the recovery of rRNA genes from a particular group but it cannot be assumed that rDNAs from different organisms are comparable in their cloning efficiency. The major, practical disadvantage of this approach in analysing microbial populations is that most clones in the library will not contain rRNA genes (the predicted ratio is less than 0.5%; Schmidt *et al.* 1991) and finding them can be laborious and time consuming.

A second approach involves the isolation of total 16*S* rRNA from environmental samples which is then used as a template for reverse transcriptase to generate cDNA copies of the RNA. The copy rDNA is then cloned to make a library enriched in rRNA genes (Weller & Ward 1989; Ward *et al.* 1990; Weller *et al.* 1991). The disadvantages of this approach are that the reverse transcription of rRNA may not generate full length copies due to premature termination caused by secondary structure or base methylation of the rRNA. In terms of assessing the diversity of microbial communities, using the rRNA as template will tend to select for those organisms which contain large numbers of ribosomes at the time of sampling. Since ribosome content has been shown to relate to metabolic activity (Poulson *et al.* 1993), this type of analysis will select for the metabolically active components of the population. Thus, the diversity estimate may be biased towards particular groups of organisms.

The most widely applied system for analysing the variability in 16*S* rRNA signatures in environmental samples is based on the use of the polymerase chain reaction (PCR) together with specific primers to selectively amplify the genes coding for 16*S* rRNA (16*S* rDNA). The PCR products can then be cloned to produce a library which contains only 16*S* rDNA. As with the other molecular approaches, the success of this procedure in the analysis of microbial diversity depends upon the quality of the extracted DNA and on whether this is representative of the natural diversity of the sample. In addition, the PCR reaction selects for those sequences which bind most favourably with the primers (Reysenbach *et al.* 1992; Stackebrandt *et al.* 1993). For example, gene libraries produced from the same environmental DNA sample but generated using different primers and cloning systems, contained significantly divergent 16*S* rRNA sequence signatures (Embley & Stackebrandt 1994).

There is also some evidence indicating that the more abundant sequences account for most of the enzyme and are rapidly amplified during the PCR to large copy number (Embley & Stackebrandt 1994). Consequently, the amplified rDNA may not accurately reflect the true diversity of the bacteria present but that of the dominant populations. This problem may be overcome using specific restriction endonucleases which cut within the dominant rDNA sequences, eliminating them as PCR templates and enabling the amplification of other components of the bacterial community (Embley & Stackebrandt 1994).

In the fungi, the use of bulk PCR methods has not yet been attempted on environmental samples. Indeed, at this time there is no sure way of

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

ascertaining with confidence that proportion of RNA or DNA which is of fungal origin as no adequate universal probe for 'Fungi', or indeed the major fungal phyla, is yet available.

Despite these limitations, as a sufficient knowledge base develops, the use of the PCR to amplify 16*S* rRNA genes in particular will have the advantage that with amplification primers of different specificity, different levels in the taxonomic hierarchy, from kingdom to genus or species, can be targeted. This flexibility means that PCR and cloning techniques have the potential to be used in the quantification of not only 'species richness' but also 'taxonomic diversity'. As such they offer a particularly powerful and promising approach to the quantification of diversity in natural habitats.

6. CONCLUSIONS

Microbial diversity and its quantification can be interpreted in different ways depending on whether the perspective is ecological or taxonomic. To date much of the debate has focused on the taxonomic aspects of diversity, even though the questions asked are often ecological. This is perhaps due to the desire to apply the same principles to quantifying microbial diversity as are applied to macroorganisms. This is unlikely to be a particularly fruitful endeavour since the definition of species as applied in macroorganisms is not equivalent to that in many microorganisms.

Estimates of the microbial diversity in natural environments must accommodate the spatial and temporal variability in microbial populations. Scale effects, both temporal and spatial, are not only of fundamental importance in the quantification of biodiversity, but pose basic questions for microbial ecology the resolution of which could lead to the development of fundamental theories and hypotheses as to how microbial communities are structured in space and time; how they respond to environmental pressures and how diversity is connected to function (Klug & Tiedje 1994).

Spatial effects include an assessment of the relationship between community composition and scale. This is analogous to the area–species curve in macroecology but would require that appropriate measures of biodiversity are substituted for the classical eukaryotic species. The structure of such curves would be particularly important in predicting the location of undiscovered diversity and provide insight into how biodiversity changes relative to the environment. Such studies could be extended to include an evaluation of biodiversity in 'comparable' (e.g. the same soil type or similar vegetation cover) but geographically isolated habitats thereby providing information on microbial dispersal, evolution and selection (Klug & Tiedje 1994).

Temporal shifts in microbial diversity are brought about by changes in the environment of the microorganisms and may be induced by the organisms or imposed on the community from outside. A prerequisite to the quantification of diversity in natural samples is an understanding of the magnitude and level at which such changes operate. There is a need to know which taxonomic rank is most susceptible to change and what are the implications for estimates of microbial diversity at a given site.

Advances in the analysis and quantification of microbial diversity will undoubtedly require extensive collaborative, interdisciplinary studies which draw on the expertise of microbial taxonomists and ecologists. We are concerned that this pool of expertise is so low at this time, especially in countries rich in microbial diversity where even reference collections of cultures and specimens of described species are rarely available (Hawksworth & Ritchie 1993). Human resources rather than new methodologies are likely to be key limiting factors.

In addition to the development of new procedures, the efficacy and importance of existing techniques will need to be re-evaluated, and protocols developed to enable extrapolative approaches to be used in sites where limited resources preclude intensive studies. Biodiversity estimates will need to be based on stable, readily analysed properties of the microbial community. Estimates based on phenotypic, and to a lesser extent chemical techniques, are likely to reflect the physico-chemical environment and as such may be influenced by community composition and function at a particular site, at a given time. Sampling and collecting procedures need to be standardized also temporally, and in the case of larger fungi at least some lessons might be gained from experience of ornithologists confronted with somewhat parallel problems (Marchant 1983).

Analysis of microbial communities using rRNA sequences is likely to be less sensitive to variability in the physico-chemical environment providing the methodology can be improved and the bias in nucleic acid extraction and amplification procedures removed (Embley & Stackebrandt 1994).

Although significant steps have been taken over the last few years, the required technology remains in its infancy and is as yet unable to provide the tools necessary to quantify microbial diversity in anything but the simplest of natural habitats. Nevertheless, with an awareness of the limitations of existing methods and human resources, a re-assessment of the 'species' as the basic unit of microbial diversity, and the introduction of new methodologies, significant progress in the quantification of microbial diversity can be expected.

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