Microbial Indicators of River Health—1997 Workshop

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Preface

Microorganisms may prove to be excellent indicators of river health since they orchestrate most ecosystem services, play a pivotal role in the transformation of nutrients, are numerous and can be simple to sample. With the exception of faecal indicators, microorganisms have not been used extensively as indicators of river health. The two key reasons for this are the lack of suitable methodologies and a lack of awareness of microbial ecology. Recently, several powerful methods have become available to detect microorganisms and/or their activities, in natural environments. These technologies range from gene probes to PCR to flow cytometry to chemical tracers. Armed with these technologies microbiologists are now gaining a clearer understanding of aquatic microbial ecology.

A workshop entitled 'Microbial Indicators of River Health' was held at Macquarie University, Sydney, on 31 July– 1 August 1997.

The purpose of the workshop was to investigate the use of microbial indicators for monitoring river health. The Day 1 program was comprised of short lectures and discussion on microbial indicators and techniques used for their assessment. On Day 2 there were laboratory demonstrations followed by round-table discussions.

The objectives of the workshop were to:

- increase awareness of the key role played by microorganisms in riverine ecosystem processes;
- define the needs of environmental managers;
- describe the types of processes that can be assessed by microbial monitoring;
- describe techniques used to assess microbial indicators;
- Consider the potential of microbial indicators for assessment of river health;
- consider key design features of sampling regimes and statistical analysis; and
- identify the most informative microbial indicators of river health

Seventy-eight delegates (plus 17 presenters) attended day one of the workshop, while day 2 of the workshop was restricted to 36 participants. Delegates at the workshop came from diverse organisations including Federal and State government agencies; local government; universities; water agencies; environmental consulting companies; and industry. We were also pleased to see that members of the public who were not representing particular bodies also attended. Participants came from all Australian States and Territories except the Northern Territory. There was one delegate from New Zealand.

The presenters of the workshop felt that they learnt a considerable amount from the participants and other presenters.

This book contains summaries of the papers presented at the workshop and some details of the techniques demonstrated during the laboratory sessions on day 2.

Duncan Veal
Macquarie University
February 1998

The National River Health Program and microbial indicators of river health

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National River Health Program Coordinator and Program Manager – Water Resources, LWRRDC

The National River Health Program was established in late 1993 following the Prime Minister's Statement on the Environment in 1992. Funded under that statement and by the Land and Water Resources Research and Development Corporation (LWRRDC), the program has had two components—the Monitoring River Health Initiative (MRHI) and the Environmental Flow R&D Initiative (EFMI). Phase 1 of the program (from 1993 to 1996) saw $12 million in R&D investment under those two initiatives, contributed and managed by the (then) Department of Environment, Sports and Territories, and LWRRDC. The program has received renewed funding for Phase 2, under the Rivercare Initiative of the Natural Heritage Trust (NHT).

The MRHI has had several major objectives:

- to develop and enhance techniques to monitor and assess the health of Australia's rivers;
- to establish a national approach to monitoring and assessing the health of Australia's rivers, in conjunction with the States and Territories; and
- to undertake the first national assessment of the instream health of Australia's rivers.

The National River Health Program's working definition of ecological health is as follows:

> *The ability of the aquatic ecosystem to support and maintain key ecological processes and a community of organisms with a species composition, diversity and functional organisation as comparable as possible to that of natural habitats within a region.*

During the R&D priorities review for Phase 1 of the MRHI conducted in 1993, the need to develop indicators of ecological health and to gain a fundamental understanding of the ecology of a number of biotic components of Australian river ecosystems was highlighted. As regards the latter, the review saw as imperative the development of a research capacity and training in microbial ecology and microbial indicators of river health.

To date, most MRHI investment has been focused on the development of a national river bioassessment system using macroinvertebrates, and to support research for the development of bioassessment systems with fish, algae and macrophytes. Recent research in microbial ecology has indicated the fundamental role that bacteria play in nutrient, energy and carbon cycling in river systems, and has highlighted the potential for developing bioassessment tools based on bacterial community composition, bacterial-mediated processes and population dynamics. However, understanding of bacterial roles in Australian riverine ecosystems is quite limited, and to this end a sub-program of the MRHI was developed following a review of R&D priorities by Duncan Veal and Barry Hart. Its primary objectives are:

- to develop microbial indicators of the ecological health of river systems;
- to develop skills in and awareness of microbial ecology and bioassessment in water resource management and environment agencies through workshops and training; and
- to build a capacity in microbial ecological research through postgraduate scholarships.

Some $350,000 was allocated to the sub-program under Phase 1 of the MRHI. The workshop on microbial indicators of river health is one the main activities of the sub-program in training and technology transfer in microbial techniques for research and assessment in aquatic bacterial ecology and bioassessment.

What do we know about the microbiology of aquatic systems in Australia?

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Introduction

The term 'microbiology' in this paper is limited to include the viruses, 'bacteria' (archaeabacteria, eubacteria including the cyanobacteria), fungi, and protozoans, but to exclude larger zooplankton, such as rotifers, crustaceans, etc. The systems examined are also limited, and exclude sewage-treatment facilities. Neither are issues concerned strongly with public-health microbiology (eg. faecal bacteria, potable water) examined in this paper. Given these delimitations, what is known about the microbiology of freshwater systems in Australia?

Historical perspective

The first question is: how much research work has been done in the past to study aquatic microbes? I made a first attempt at answering this question by examining the types of oral papers and poster presentations given at conferences of the two scientific societies most directly concerned with microbiological and limnological topics: the Australian Society for Limnology (ASL) and the Australian Society for Microbiology (ASM). Over the past five years, 4% of all presentations at ASL conferences dealt with microbes other than cyanobacteria; a further 8% were concerned with cyanobacteria. Less than 1% of presentations at ASM conferences (1991 and 1994 only) dealt with work on microbes in freshwaters. An examination of the contents of key limnological and microbiological texts (De Deckker and Williams 1986; Fenner 1990) reinforces the view that there has been only a limited research effort directed at the study of native microbes in the inland waters of Australia. What, then, is known about these microbes?

Planktonic environments

Studies in a number of paired river-billabong sites in the Murray–Darling Basin have shown there is a great difference in the abundance of bacteria (determined with epifluorescence microscopy) in rivers (about $1–5 \times 10^9$ cells/L) and wetlands (commonly $5–50 \times 10^9$ cells/L). However, it is difficult to apply traditional microscopy techniques to enumerate bacteria in rivers that are turbid, and these sorts of environments are common in inland Australia; other techniques such as flow cytometry or field-flow fractionation might be better suited to the task of enumerating planktonic bacteria. There is a good relationship between bacterial abundances and key biogeochemical indices (such as the activity of extracellular enzymes, including aminopeptidase and alkaline phosphatase) in billabongs, but not in rivers, where the best correlations for indices of bacterial activity are with loads of suspended solids. Close relationships between planktonic bacteria and phytoplankton have been demonstrated in one wetland, but whether this pattern holds more widely has yet to be shown. Bacterial productivity has been measured only rarely (with ^{3}H-leucine, and less reliably with the frequency of dividing cells), but extremely high productivities (exceeding 100 mg C/L/hour) have been found in some cases.

Variables such as those discussed above—total abundance, productivity, biogeochemical activity—are very much measures that typify environmental microbiology of the 1980s. Study of another key characteristic of planktonic bacterial communities—the description of the types of bacteria present—has had to wait on the development of suitable techniques, and these are largely molecular methods developed in the 1990s. The composition of planktonic bacterial assemblages has been studied in only one or two systems, using biomarkers (especially phospholipid fatty acid profiles) and 16S rRNA oligonucleotide probes, but these have been directed to answer very focused questions on selected groups of microbes. Methanotrophs, for instance, were estimated to account for up to one-third of all planktonic bacteria in the water column of Ryans 1 Billabong in NE Victoria. I could find no ecological-management work on the viral components of natural waters in Australia, and there has been (to my knowledge) only one study of the aquatic fungi (on the Hyphomycetes in streams around Canberra). Protozoa are also, from an aquatic perspective, *terra nullius*.

Benthic environments

There has been little research on the abundance, growth and community composition of bacteria in natural freshwater sediments in Australia. This might be because it is far harder to study benthic bacteria than it is to study planktonic bacteria. However, there has been a concerted effort to quantify carbon transformations in wetland sediments, especially the processes of methane production and consumption by bacteria. Methanogenic archaeabacteria account for 11–36% of all prokaryotes in wetland sediments, and much, perhaps most, of the carbon processed by bacteria in sediments seems to involve methane production and consumption. Fungi, estimated on the basis of the presence or absence of ergosterol, were not present in the sediments. Viruses and protozoans are, again, unstudied.

Biofilms

Some research has been conducted on freshwater biofilms, in both Victorian and South Australian systems. Bacterial abundance on biofilms on river red gum (*Eucalyptus camaldulensis*) is about 10–100 $\times$ 10^6 cells/cm^2. Biogeochemical significance, assayed via the measurement of extracellular enzyme activity, varies with the period over which the biofilms have been developing, as well as season and whether the biofilms are in the photic or aphotic zone. It is not known whether fungi are present in the biofilms, and studies of other microbiological components (eg. testate amoeba) are in their infancy.

Conclusions

In comparison with the lengthy and intricate amount of research conducted on the fauna (especially birds, fish and invertebrates) and, to a lesser extent, the flora of Australian inland waters, very little is known about the microbes in such environments.

Of all the microbial components (ie. viruses, archaeabacteria, eubacteria, fungi, and protozoans), the prokaryotes have been studied the best, but even then only for a very limited number of systems in south-eastern Australia and only over the past 10 years. Almost nothing is known about the bacterial components in other parts of Australia, and there is little likelihood that specifics gleaned from studies of freshwaters in the temperate south-east can be readily transferred to other regions, such as the monsoonal north or arid centre, or for other systems, such as inland saline waters. That aquatic bacteria can be abundant, metabolically active and highly productive suggests that they play a vital role not only from a biogeochemical perspective but also in aquatic food webs, but with the exception of one or two limited works on bacterivory, the importance of bacteria in either planktonic or benthic food webs has yet to be examined in Australia.

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Potential of microbial indicators of river health

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Environmental indicators

For the assessment of the ecological condition of the nation's rivers, both the National Water Quality Management Strategy and the National River Health Program are focusing on the use of biological indicators. Many different indicators have been suggested including macroinvertebrates, fish, algae, macrophytes and microorganisms. There has been considerable debate concerning the correlation of different indicators with ecosystem health. A conclusion that could be drawn from these debates is that the highest degree of correlation is generally to be found between the reported usefulness of a particular indicator and the discipline speciality of those advocating their usage. I am a microbiologist!

To keep the workshop presenters honest, it is worth while reviewing the criteria for the selection of indicators for State of the Environment (SoE) reporting and assessing various suggested microbial indicators against these criteria.

According to the State of Environment Reporting Unit, Department of Environment, Sports and Territories), an environmental indicator should:

- serve as a robust indicator of environmental change;
- reflect a fundamental or highly valued aspect of the environment;
- be either national in scope or applicable to regional environmental issues of national significance;
- provide an early warning of potential problems;
- be capable of being monitored to provide statistically verifiable and reproducible data that show trends over time and, preferably, apply to a broad range of environmental regions;
- be scientifically credible;
- be easy to understand;
- be monitored with relative ease
- be cost effective;
- be aggregative as possible (amenable to combination with other indicators to produce more general information about environmental conditions);
- have relevance to policy and management needs;
- contribute to monitoring of progress towards implementing commitments in nationally significant environmental policies;
- where possible and appropriate, facilitate community involvement;
- contribute to fulfilment of reporting obligations under international agreements;
- where possible, use existing commercial and managerial indicators;
- where possible, be consistent and comparable with other State and Territory indicators.

While no single indicator is expected to completely satisfy all these criteria, the best indicators are those that comply most fully with them.

Commonwealth state of the environment reporting uses a modified version of the pressure–state–response model originally developed by the OECD. Indicators are not only required for the state of the environment, but also for the pressures (human activities and natural events, such as floods, that affect the environment) and responses (actions taken in response to environmental problems.)

Allochthonous microbial indicators

In all aquatic environments, the microorganisms in the water body can be either autochthonous (produced within the waterbody) or allochthonous (derived from the surrounding terrestrial environment). In general, allochthonous microorganisms do not multiply or survive for extended periods in aquatic ecosystems. Both autochthonous and allochthonous microorganisms may be used as indicators of river health.

The presence of certain allochthonous microorganisms can be used to assess terrestrial inputs into aquatic systems. For example, the finding of bacteria of terrestrial origin in a river could be used to assess soil erosion, river flows and transport processes (Hart et al. 1996).

Allochthonous faecal coliforms, enterococci and coliphages (viruses that infect coliforms) have been used routinely for decades to monitor faecal contamination in water. In fact, these faecal indicator bacteria are probably the most commonly assessed biological indicators. Traditional methods for the measuring faecal indicators require a trained microbiologist, suitable facilities and typically about two days to get a result.

Recently, more rapid and simple methods have become available (Beebe et al. 1991). For coliforms and faecal coliforms these rapid methods depend on the detection of two enzymes, β-galactosidase and β-glucuronidase in a selective medium. β-galactosidase is detected using the chromogenic substrate O-nitrophenol-β,D-galactopyranosidase (ONPG) which is a colourless substrate that yields a yellow product (nitrophenol) when cleaved by β-galactosidase. β-glucuronidase activity is detected using a fluorogenic substrate, methyl-umbelliferyl-glucuronide (MUG) that yields a fluorescent blue product when cleaved by β-glucuronidase. Coliforms will turn the medium yellow, whereas faecal coliforms will turn the medium yellow and produce a fluorescent product. Commercial test-kits are available which consist of packaged, pre-weighed reagents that are simply added to water, incubated for 18 hours, then examined for yellow and fluorescent products. For quantification, the reagent-water sample mixture can be automatically dispensed into a 51-well disposable tray. Numbers of coliforms and faecal coliforms are determined after 18 hours incubation, using a most probable number technique. This assay can be conducted rapidly by individuals without training in microbiology and the results are easily interpreted. Given knowledge of what other microbial indicators of river health to test for, it should be possible to design similar, easy-to-use tests. Already a test for detecting enterococci in water has been developed (Fricker et al. 1995).

Autochthonous microbial indicators

The autochthonous microbial community structure can be used to assess river health in a similar way to the use of aquatic macroinvertebrates. For example, several phytoplankton species are particularly sensitive to heavy metals and can be used as indicators of heavy metal pollution (Kumari et al. 1991). Aniline is a ubiquitous chemical in the urban environment that is derived from the degradation of dyes, drugs and herbicides. The microbial degradation rate of aniline in urban rivers has been found to closely correlate with water quality (Osaki et al. 1991). The rate of aniline degradation or numbers of aniline degrading organisms could be used as biological indicator of chemical pollution.

The presence of microorganisms producing the enzyme catechol 2,3-dioxygenase (C23O) can be used to asses pollution by aromatic compounds (Joshi and Walia 1996). C23O is a key enzyme in the breakdown of aromatic compounds. Bacterial colonies expressing C23O are easily recognised as they produce the bright yellow degradation product, 2-hydroxymuconic semialdehyde, when sprayed with catechol. Detection using the polymerase chain reaction (PCR) of the genes encoding for the degradation of aromatic provides a more sensitive method of detection (Chandler and Brockman 1996).

Metabolic profiles

Phenotypic methods have traditionally been used as relatively simple and low-cost means of categorising diverse collections of microorganisms and for providing physiologically useful information. Traditionally, microbiologists have prepared a large number of different tests for characterising new isolates. A relatively new system called BIOLOG™ has been developed that is based on the ability to utilise 95 different carbon sources. Each carbon source is contained within a single well of a 96-well microtitre plate. Oxidation of a carbon source is detected by colour change caused by the reduction of a redox dye. The system is automated and a laser-based microtitre plate reader is used to obtain a metabolic profile that can be directly saved into a computer file. The profile is compared with a large data base to find the closest match.

Microbial communities (rather than individual isolates) can also be directly inoculated into the BIOLOG™ microplates and a metabolic fingerprint of the community obtained. Such metabolic fingerprints should reflect the metabolic capabilities of the microbial community under analysis, and by inference reflect the environmental conditions. Preliminary results from a collaborative research project between NSW Department of Land and Water Conservation and our laboratory, indicate that the metabolic profiles of sediment microbial communities differ between polluted and non-polluted rivers. Further, the results indicate that a nutrient poor, pristine river is home to greater metabolic diversity with higher variation between and within sites. The findings were consistent with a parallel survey of macroinvertebrate diversity. Using the BIOLOG™ system we have investigated the 95 carbon sources selected by the manufacturer for the BIOLOG™ GN plates. However, it is possible to design a BIOLOG™ plate for specific applications. Thus, carbon sources that are particularly discriminatory or relevant to riverine microbial communities could be used.

Flow cytometry

Flow cytometers have been described as automated microscopes with the advantage that cells can be analysed, quantitatively, at rates up to 40,000 per second. The principles of flow cytometry are explained briefly in the chapter by Holmes et al. in these proceedings. In river health assessment, major sub-populations of microorganisms can be identified using flow cytometry, without the need to culture cells. Fluorescent emission from endogenous pigments (chlorophyll-A and phycoerythrin) can be used to detect the phototrophs, and fluorescent DNA stains are used to determine total microbial numbers. When combined with light scatter measurements, ecologically diverse microbial groups can be discriminated.

One limitation of such studies is that they are depend on light scatter characteristics and autofluorescence alone to discriminate sub-populations. Improved phylogenetic discrimination of microorganisms can be achieved using hybridising fluorescent oligonucleotides of defined specificity to the ribosomes *in situ* within permeabilised whole cells (Delong et al. 1989).

Microbial biosensors

The use of microorganisms as biological indicators normally depends on the relative numbers of a particular autochthonous or allochthonous microorganism in the environment. However, it is also possible to use microorganisms as biosensors in toxicological tests. Three examples are discussed below.

Chemotaxis

Many bacteria respond to stimuli in the environment by movement, either towards an attractant or away from a repellent. Bacteria have chemosensors in their cell envelopes that detect chemicals in the environment and instruct the flagella how to respond. These chemosensors can be extremely sensitive in that they are able to detect concentration gradients over very small distances. Bacterial chemotaxis has been suggested as a simple and inexpensive tool to indicate the presence of pollutants, including hydrocarbons, heavy metals and organochlorines (Mitchell 1978, 1979). Owen and Winner (1991) have observed that copper concentrations of greater than 10 mg/L can cause the green alga *Chlamydomonas reinhardtii* to lose its flagella and that even higher concentrations cause encystation. Field data indicated that deflagellation was a more sensitive indicator of copper toxicity than population growth.

Stress responses

In response to harmful environmental conditions, cells produce specific proteins, often referred to as ‘stress proteins’. These responses are a universal biological response to both physical and chemical stress. The stress proteins include the heat shock response, starvation response and response to anaerobiosis. Pollutants such as cadmium, benzene, and several organochlorines, such as 4,4-dichoroaniline, hexachlorobenzene, pentachlorophenol and trichloroethylene, have been shown to induce stress proteins in *Escherichia coli* (Blom et al. 1992). These stress responses are rapid: it takes cells from 20 mins to 4 hours to adjust their cellular processes to the stressed environment. Such stress responses may be used as a rapid early warning indicator of ecosystem stress (Kjelleberg and Ashbolt, pers. comm.). Measurement of stress response may provide a more sensitive index of environmental stress than growth rate, since stress-protein synthesis is likely to be stimulated by very low pollutant concentrations at which little or no growth inhibition occurs (Blom et al. 1992).

Different suites of stress proteins have been found to be induced by different pollutants (Blom et al. 1992), suggesting that the method may have the potential to identify particular pollutants.

Stress-response proteins may be used as microbial bioindicators in two different ways. Either the indigenous microbial community can be examined directly for these proteins, or specific microorganisms with well-characterised stress proteins may be exposed to the environment (possibly immobilised on a ‘dipstick’) and examined for the stress-response (Kjelleberg and Ashbolt, pers. comm.). Currently, stress-response proteins are identified by 2-D gel electrophoresis. However, antibodies have been produced to specific stress response proteins enabling the rapid identification of stress responses. These antibodies could be incorporated into kits for rapid field evaluations. An alternative approach could be the use of reporter genes (eg. luciferase genes) inserted after the promoters of particular stress-response genes to indicate when these genes are active (Kjelleberg and Ashbolt, pers. comm.)

Microtoxicity assays

A toxicity bioassay based on monitoring the changes in light output from the marine bacterium *Photobacterium phosphoreum* has been developed (Kaiser and Palbrica 1991). In this assay the production of light by the bacterium is linked to cellular metabolism. Any substance that disrupts or inhibits any aspect of cellular metabolism can be detected. This assay is marketed commercially as

Microtox®. The assay is very sensitive and highly reproducible but does not provide information on what is causing the inhibition of cellular metabolism.

Conclusion

Microbial indicators could fulfil many of the SoE criteria for selection of indicators. The pivotal role of microorganisms in orchestrating ecosystem services, such as the cycling of nutrients, means that microbial communities (and thus potential microbial indicators) reflect fundamental environmental processes. Microbial indicators could also contribute to the management of processes that threaten these ecosystem services. Certainly, microbial indicators of faecal contamination play a key role in the management of wastewater systems. The rapid growth rate of many microorganisms means that microbial communities respond rapidly to a wide range of environmental perturbations. Thus, microbial indicators could provide early warning of potential problems. However, one disadvantage is that a microbial community may revert too quickly after a pollution incident (Hellawell 1986). The large numbers of microorganisms present in all environments means that they are capable of being monitored in a statistically verifiable manner. Microorganisms are ubiquitous, represent the largest component of the biomass, do not require trapping and samples are generally taken with relative ease. The requirement for a trained microbiologist has in the past limited the application of microbial indicators. However, test kits are becoming available for certain groups of microorganisms. These test kits enable non-specialists, eg. community-based groups, to conduct microbial analyses, and are facilitating microbial testing at remote locations.

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Use of extracellular enzyme activity to provide a biologically-based characterisation of dissolved organic matter in aquatic ecosystems

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Introduction

Dissolved organic carbon (DOC) in aquatic ecosystems is commonly the predominant form of organic carbon, with concentrations typically twice as high as particulate organic carbon (Thurman 1985). Despite this abundance, there has been a general inability to derive meaningful predictive measures of DOC quality or composition that would allow us to develop broad models of how DOC fuels microbial production. Clearly, an enhanced ability to trace DOC metabolism would improve our knowledge of how point and non-point inputs of dissolved materials may alter aquatic food webs or functioning.

Background

In the past few years, attempts to describe natural DOC have polarised, with bioassay approaches at one end of the spectrum and chemical characterisation at the other. Bioassays are powerful in that they rely on the microbial communities to yield information about which types of DOC support the highest growth, but they do not necessarily describe which factors are driving the observed variability in growth. Chemical characterisation has progressed greatly in the last five years, predominantly because of improvements in descriptions of the complex macromolecules (largely humic and fulvic acids; McKnight et al. (1994)) that comprise the bulk of the natural DOC pool. As yet, however, the description of bulk chemical composition has not yielded predictive measures of DOC quality.

In collaboration with Dr Robert L. Sinsabaugh, we have developed an approach using exoenzymatic activity to tighten the link between bacterial productivity and DOC composition. The premise underlying this approach is that bacteria will shift their elaboration of exoenzymes in response to shifts in DOC composition. A strength of this approach is that the changes in enzymes can be ascribed to shifts in abundance of particular classes of compounds such as plant polysaccharides versus proteinaceous compounds. Therefore, exoenzymes represent a higher-resolution, biologically-based tool for probing variability in DOC composition among systems. As one example, a set of large rivers in the north-eastern United States can be ordinated by enzyme activities. The patterns in enzymatic activity are correlated with the relative contribution of allochthonous carbon and direct measures of shifts in DOC composition such as the proportion of aromatic carbon compounds (Hopkinson, C. I. Buffam, J. Hobbie, J. Vallino, R. Hodson, M. A. Moran, J. Covert, E. Smith, J. Baross, B. Crump, B. Eversmeyer, F. Prahl, M. Perdue, S. Findlay and K. Foreman, unpublished data). Determining which enzymatic patterns are predictive of various bacterial processes (production, growth efficiency) is the logical next step in developing this approach to probing the DOC black box. We feel that this enzymatic bioassay encompasses strengths from both ends of the existing spectrum in approaches to DOC characterisation.

Case studies

The Hudson River: This approach has been applied in a study of organic matter loadings to the tidal freshwater Hudson River. There are several quantitatively large sources of allochthonous DOC in the Hudson River and their potential to contribute to heterotrophic bacterial growth was examined with bioassays (S. Findlay, R.L. Sinsabaugh, D.T. Fischer and P. Francini, unpublished data). Supply of DOC from the upper Hudson drainage basin and a large tributary in the mid-Hudson were sampled to represent terrestrially-derived sources. Two contrasting tidal wetlands have been shown to contribute DOC to the main stream and were used to represent emergent macrophyte sources. Release of DOC by diffusion from fine sediments supplies DOC originating from buried organic matter.

Extracellular enzyme activity was assayed by observing the time course of fluorescence of MUF-linked substrates ([enzyme:substrate]; Esterase:4-MUF-acetate; phosphatase:4-MUF-phosphate; leucine aminopeptidase: L-leucine 7-amido-4-methyl-coumarin; β-glucosidase:4-MUF-β-D-glucoside;

β-glucosidase:4-MUF-ß-D-glucoside; β-xylosidase:4-MUF-β-xyloside; β-N-acetylglucosaminidase:4-MUF-N-acetyl-β-glucosaminide) measured in 96-well microplates in a plate-reading spectrofluorometer (Perkin-Elmer LS50B). MUF-linked substrates (volume = 100 µL, conc. = 400 µM (MUF-acetate = 100 µM)) plus 150 µL of sample water are placed in a well and read repeatedly over 1–4 hours. These substrate concentrations are saturating as determined from kinetic assays, or at the upper limit of substrate solubility.

Bacterial growth varied among sources but differences were not large, indicating that all sources of DOC were capable of supporting bacterial growth at rates approximating summertime field values. Seasonal shifts in carbon availability were clear in several cases, with, for instance, greater growth on wetland-derived DOC at times of peak plant productivity. Seasonal differences in tributary DOC bioavailability were not large despite the well-known seasonality of tributary inputs. Activities of a suite of extracellular enzymes were used as a biologically-based characterisation of DOC from the various sources with shifts in allocation among enzymes apparent in many cases. The consistent induction of distinct suites of enzymes in response to DOC additions indicates that there are biologically relevant differences in composition among the sources. There was high temporal variability in enzyme activities in 'reference' treatments (no added DOC). Field samples collected over two years from stations spanning about 150 km of the river showed that temporal variability was much greater than large-scale spatial variability.

New Zealand streams: Streams draining catchments differing in land use are known to exhibit large changes in algal productivity, organic matter standing stocks and insect community composition. Microbial communities have not received as much attention and it is not clear how various microbial processes may respond to land-use influences on carbon supply. A suite of exoenzyme activities was assayed in three New Zealand streams draining pasture, native forest and a pine catchment (S. Findlay, C. Hickey, and J. Quinn, unpublished data). There were differences among catchments in activity of three of the five enzymes assayed (cellobiohydrolase, N-acetylglucosaminadase and dihydroxylphenylalanine oxidation). A principal-components analysis demonstrates that patterns of enzyme activity can be used to separate the three stream types.

To complement the field samples from different streams we also conducted a DOC addition experiment where sediment from a reach of Mangaotama Stream below the pasture and native forest streams (site M3, NZMS 260, S14 936789) was incubated with a variety of DOC sources known to occur within or above that stream reach. A bulk sediment sample from M3 was collected as for the individual locations in other streams. This sediment was incubated with five different water sources: (1) bulk water from PW2, (2) bulk water from NW5, (3) water from NW5 + algal DOC, (4) water from NW5 + seep DOC , and (5) water from NW5 + litter leachate.

The experimental addition of algal-leachate, leaf-litter leachate and high DOC water from a small seep resulted in marked shifts in epilithic enzyme activities one day after DOC additions. Oxidative enzymes showed a particularly strong response to additions of humic dissolved organic carbon. These changes did not persist, with activities seven days after DOC manipulation showing differences among treatments for only one of the four enzymes assayed. As for the field samples, a PCA showed large differences among treatments, indicating that exoenzyme patterns can be used to examine which DOC sources predominate in different streams. The qualitative patterns among enzymes are most useful in applying this approach as a 'fingerprint' of DOC sources. The separation of pasture from forested streams in the PCA indicates a common response of enzyme allocation in pasture versus native forested stream biofilms. We would predict that as streams revert to native forest cover either through land abandonment or restoration of riparian zones, enzyme patterns should shift toward those indicative of undisturbed native streams. The rapid although temporary response of enzyme patterns in the experiment shows that these patterns are quite dynamic with significant shifts in allocation after just 24 hours exposure to a different type of DOC. The disappearance of most statistically significant differences after one week suggests that the actual components of DOC causing these shifts are metabolised and depleted fairly rapidly. Measurements of the algal and litter exudates showed only slight DOC reduction after four days (16% and 26%, respectively), indicating that the components responsible for the enzyme induction are probably associated with only a small fraction of the DOC, which is rapidly lost from the batch exposure system. The pulsed nature of this experiment makes it likely that changes will be temporary, with more stable shifts only to be expected with a continuous addition of DOC from the various sources.

Future prospects

These studies show that patterns of enzyme activity can provide a sensitive indicator of differences among

sources of DOC. The challenges ahead include: (1) relating these compositional differences to differences in bioavailability, (2) separating shifts in enzyme induction from changes in microbial community composition, and (3) identifying the compounds or classes of compounds most responsible for generating variability in enzyme activities.

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Novel detection methods for specific microorganisms in the environment

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Introduction

The choice of microorganisms that are both representative and indicative of river health requires a detailed understanding their role in ecosystem processes. This, and the ability to detect and monitor these organisms, can be achieved only through a combination of traditional microbial ecology and the rapidly emerging molecular technologies.

The detection, identification and isolation of a wide range of microorganisms from the environment is an essential part of the study of microbial ecology and monitoring from both environmental and public health perspectives. In recent times, great emphasis was placed on the detection and enumeration of groups of bacteria indicative of pollution and contamination related to human health. However, their detection gives little insight into the microorganisms involved in ecosystem processes and, consequently, has little predictive value in environmental monitoring in the broadest sense. There are numerous strategies for the detection and isolation of bacteria from indigenous communities. These range from conventional bacterial methods to molecular techniques, which are constantly developing, complemented with electronic automated systems that can rapidly acquire and analyse large data sets. The novelty of new methodologies to assess the role of microorganisms in ecosystem processes lies in a combination of these approaches.

Limitations encountered

One of the most important considerations to take into account when applying any technique, whether it be molecular or classical, is the limitation imposed by either by the technique itself (eg. sensitivity) or by the environment (eg. contamination of sample). The limitations of any procedure often act as an indicator to future research priorities. For example, bacteria from most environmental samples are hard to culture: usually less than one percent can be isolated on artificial media. Therefore, very few microorganisms from any community have been described. This led to the hypothesis that many had adopted a specialised survival strategy ('non-culturable' but viable, NCBV; Roszak and Colwell (1987)) that precluded their re-culture. However, it is becoming increasingly likely that the NCBV state may be particular to a limited number of individuals. 'Non-culturability' in the environment *per se* may be solely due to our ignorance of the specialised conditions required to culture these organisms (Head et al. 1996). *Therefore, developing novel culture techniques should be a priority for the future*.

Molecular techniques also show limitations in sensitivity and through the introduction of bias. Analysis of microbial biodiversity by amplification of 16S ribosomal (r)RNA signal by polymerase chain reaction (PCR) may not be truly representative (like culture methods) of the indigenous community, because of biases in the PCR reaction or in the initial sampling strategy. PCR community analyses require extraction of total DNA, not all cells are susceptible to the lysing procedures and, consequently, their DNA will not contribute in the final sequence analysis. *Therefore, the development of realistic sampling strategies that are representative of the community under analysis is also a priority.*

Given an understanding of the limitations imposed on any community analysis, the arrival of novel molecular and classical techniques will continue to make a significant contribution to the detection of specific microorganisms and to an understanding of their role in ecosystem functions. The advantage of employing molecular detection strategies is that they do not rely on 'culturability' and the vast untapped proportion of the microbial community is therefore more readily accessible for analysis

Novel molecular techniques

There are many reviews of the application of molecular techniques to microbial ecology (eg. Pickup 1991; Prosser 1994). It would serve no purpose to repeat those descriptions here. Therefore, the aim of the section is to describe some of the novel

combinations of DNA-based and immunological techniques, combined with other technologies, that are applicable to the detection of specific microorganisms in microbial communities. There are two basic approaches common to whatever strategy is adopted : (i) to isolate specific cells for subsequent culture, enumeration and/or physiological analysis; and (ii) given that the majority of cells cannot be cultured, then cells can either be separated, purified and chemically fixed before enumeration and/or identification, or DNA is extracted and analysed directly for the presence of indicator genes or for phylogenetic analysis.

Immunological approaches

The use of either polyclonal or monoclonal antibodies offers a potentially sensitive and specific means of identifying environmentally important microorganisms. Antibodies can used to identify bacteria carrying specific marker gene products *(Pseudomonas putida* and enzyme linked immunosorbent assay ELISA; Morgan et al. (1989)) or specific strains such as *Nitrosomonas* spp., *Vibrio cholerae* and *Pseudomonas putida* (see Pickup 1991). When coupled to fluorescent molecules these probes permit visual observation by epifluorescence microscopy (Manz et al. 1995), highly sensitive detection by CCD cameras (Prosser et al. 1996) and rapid analysis by flow cytometry (Porter et al. 1996). One advantage of this approach is the maintenance of cell viability after labelling, allowing cell sorting and subsequent cell culture. Enrichment procedures, in general, often improved the effectiveness of isolation of culturable organisms from environmental samples, but introduced inefficiencies in time, and unwanted selectivity. These can be circumvented using fluorescence-activated cell sorting. Porter et al. (1993, 1995a,b, 1996) used this approach to selectively recover *E. coli* and *Ochrobacterum anthropi* naturally present in lake water subject to sewage discharge, to a purity of >70%. This approach is routinely used for enumeration of *Cryptosporidium parvum* with the cell sorter placing positive events on a microscope slide for confirmation (Vesey et al. 1994). Furthermore, dual labelling (Miller and Quarles 1990; two independent but specific probes with different fluorochromes) permits enhanced discrimination of specific cells from interfering particulates and indigenous bacteria, even between serotypes (eg. ammonia oxidising bacteria; Volsch et al. 1990).

An alternative immunological approach is to separate cells using immunomagnetic separation, an enrichment procedure that maintains the advantages of selectivity and speed while retaining culturability. The first environmental use of this method successfully isolated *Pseudomonas putida* from lake water using monoclonal antibodies specific to the host's flagella (Morgan et al. 1991). As with all immunomagnetic procedures, purification was achieved using magnetic retention of the bead-cell complex during a number of washing steps. This method has been extended to many species in a variety of environments including gram positive organisms (Mullins et al. 1995), sulfate reducers (Christensen et al. 1992) and *E. coli* O157 (Porter et al. 1997). Porter et al. (in press) replaced the antisera on the magnetic beads with lectins as an alternative approach. Despite some cross-reactivity, lectin-coated magnetic beads were capable of extracting cells from lake and river water and were capable of targeting specific groups of bacteria (eg. *E. coli*). The cells after extraction were suitable for direct PCR and coupled with fluoresceinated-lectins had the potential for discrimination by flow cytometry.

DNA-based methods

Efficient extraction of DNA representative of the microbial community from a specific environment/ habitat is the cornerstone of meaningful DNA-based analyses. Therefore, sample processing is a key issue, with the ultimate goal of presenting a contaminant-free cell or DNA suspension for molecular processing. Isolation of planktonic biomass from the aquatic environment is relatively straightforward using either filtration or tangential flow filtration (Pickup 1995). Soil and sediment processing requires either cells to be removed from the supporting matrix and chemically-fixed for whole cell analyses before lysis, or direct lysis on the solid matrix followed by DNA purification.

Fluorescent *in situ* hybridisation (FISH) uses fluorescent oligonucleotide probes specific to genera or species. The probes are often 30 bases long attached to a fluorochrome and target the 16S or 23S ribosomal RNA (rRNA; Porter 1996). These probes are applied to cell suspensions, thus the presence of specific microorganisms can be distinguished from non-target cells and quantified (eg. α-*proteobacteria*, sulfate reducers, *Pseudomonas fluorescens*; Amman *et al*. (1995)). In addition, quantification of the fluorescence signal gives an insight into the activity of the subset of the microbial community. Fluorescence in each case can be monitored either by epifluorescence microscopy, confocal microscopy (Manz et al. 1995), CCD camera technology or flow cytometry. Further developments of this approach have employed *in situ* PCR coupled with flow cytometry to detect specific gene sequences in intact cells (Porter et al. 1995a). This was achieved by including fluorescently-labelled dUTP. Using flow cytometry, cells with and without the target *xyl*E gene could then be discriminated (Porter et al. 1995b).

PCR has been widely applied to the detection of microorganisms in the environment. Organisms involved in key geochemical cycles detected by amplification directly from environmental samples or after enrichment include nitrifiers (Head et al. 1993; Hiorns et al. 1995), methanogens (Hales et al. 1996), methane oxidisers (MacDonald et al. 1996), sulfur oxidisers (Head et al. 1996) and sulfate reducers (see Amman et al. 1995), as examples. Clearly, PCR is a powerful tool but is subject to several limitations, notably the representative nature of the template DNA and its susceptibility to contamination. The overall sensitivity may be substantially reduced because of the large excess of non-target sequences and inhibitory substances in the sample. Miller et al. (1995) developed a PCR capture method to circumvent these problems. Target DNA was captured, immobilised and separated from the crude preparation using 5'-biotinylated probes coated on a spike attached to the reaction tube lid. The probes represent sequences upstream of the target region. The immobilised DNA was removed from the sample and separated from the probe using streptavidin-coated magnetic beads. The purified target DNA was then subjected to specific PCR. The signal obtained was devoid of false positives and represented a 10–100 fold increase in sensitivity. This has been applied to the detection of pathogens in faecal material (Miller et al. 1995) and freshwater rivers (Hermon-Taylor and Pickup, unpublished data) that are both non-culturable and occur in low numbers.

Conclusions

The availability of new techniques is advancing our understanding of microbial ecology and environmental microbiology. As a result of inherent limitations in each method it is unlikely that any one method is directly applicable to all environments. However, with developments in sampling strategy, existing and evolving methods will undoubtedly stimulate further investigations into this wide-ranging research topic.

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Phylogenetic approaches to the study of bacterial diversity in river ecosystems

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Introduction

Bacteria play key roles in energy flows and cycling of nutrients in aquatic ecosystems, and the balance and sustainability of river systems is dependent on the microbial community present. The nature of carbon and nutrient inputs, both autochthonous and allochthonous, is a major influence on the structure and diversity of aquatic bacterial communities, and is central to the concept and assessment of river health. Because of their metabolic diversity and relatively fast growth rates, bacterial communities are excellent candidates as rapid indicators of environmental change. However, the lack of knowledge of the basic structure and functioning of bacterial communities in Australian rivers is an obstacle to interpreting the response of bacterial communities to human (river regulation, pollution) and natural (flood, drought) perturbations.

Project aims

While most of what we know about the ecological functions of bacterial species has been derived from traditional culturing methods, it is now apparent that only a small proportion of bacterial species have so far been isolated (Hugenholtz and Pace 1996) and this gives a biased view of species diversity. As a result, decisions about the role of microorganisms in the environment are being made on the basis of scanty information. We aim in this project to overcome some of these deficiencies in knowledge by using the latest phylogenetic molecular methods to assess the structure and diversity of aquatic bacterial communities in tropical and subtropical rivers. We are focusing on lowland regulated rivers of major agricultural significance that are currently being studied from different perspectives in other National River Health Program projects. Based on the outcomes of our bacterial population assessment we will select bacterial indicator species which correlate with other biological and physicochemical parameters of river health. This project began in November 1996 and the preliminary results of a survey of the bacterial species diversity of surface sediments in the Condamine River will be used to illustrate the phylogenetic approach to the study of bacterial diversity in natural ecosystems. Two sites are currently under investigation. These are the relatively unimpacted Myall Creek, and Oakey Creek which receives sewage discharges.

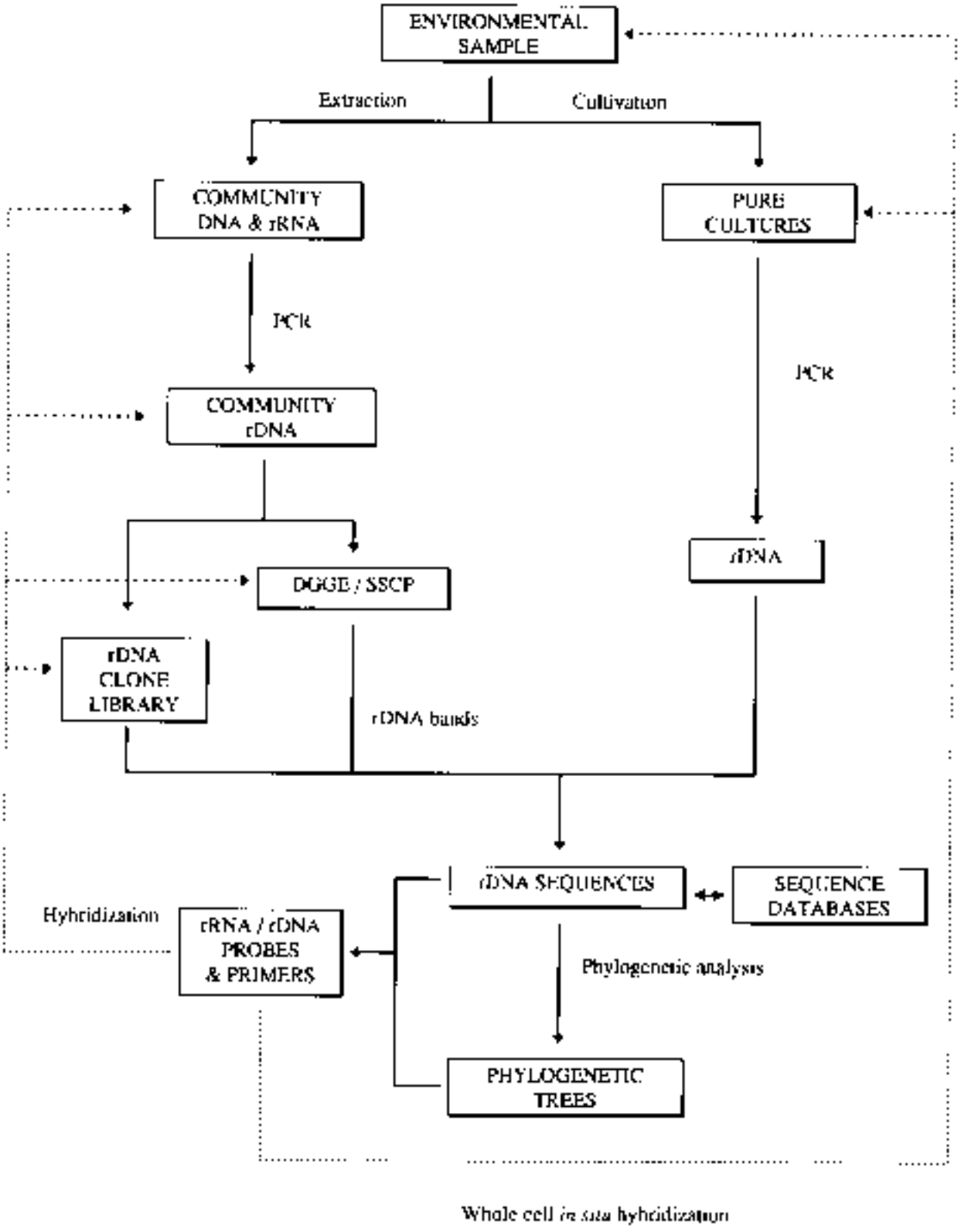
Molecular phylogenetic approach

The molecular phylogenetic approach entails the use of evolutionary significant molecules as a molecular signature that can be assigned to a particular species. In studies of prokaryotic microorganisms (eubacteria and archaeabacteria) the molecule of choice is 16S rRNA (ribosomal ribonucleic acid), selected because it occurs in all species, is structurally and functionally conserved, is of a size that allows the analysis of a statistically significant number of nucleotides which have evolved independently, and has conserved and variable regions (Woese 1987). Figure 1 illustrates diagramatically the molecular phylogenetic approach to the study of species diversity.

Due to rapid developments in nucleic acid sequencing techniques it is now practical to sequence the complete 16S rRNA molecule and probably the 16S rDNA gene. The sequences of most of the 3500 species of eubacteria and archaeabacteria are now available in international databases (Genbank, EMBL) and the Ribosomal Database Project (Maidak et al. 1994) for direct comparison via the Australian National Genomic Information Service (ANGIS) on the Internet.

Evolutionary relationships can be inferred by comparing the sequences of individual genes of a species (or phylotype). For genes with a high level of similarity with known species, the properties of the organism can be predicted, based on the properties of its known relatives, as all of the members of a particular phylogenetic group may be expected to have those characteristics which occur commonly in the group (Hugenholtz and Pace 1996). Whereas high similarity in sequences can be used for assignment to known species, distant relationships can be used to reveal new taxa for which further study is required to determine physiological function.

Figure 1. Schematic diagram of the molecular phylogenetic approach to the assessment of bacterial species diversity.



approach is that it depends solely on the ability to isolate the rRNA gene, thus allowing the study of bacterial diversity to be independent of the ability to culture the organism. This allows the inclusion of 'unculturable' or 'so far uncultured' organisms in the community analysis. Given that only a small proportion of bacterial species have so far been isolated in pure culture, the phylogenetic approach eliminates the culturing bias.

The culture-independent approach usually involves extraction of total genomic DNA (deoxyribonucleic acid) from the environmental sample, amplification of the rDNA by PCR (polymerase chain reaction) using universal primers (Lane, 1991), then cloning of the individual rRNA genes. Members of the clone library of recombinant cells containing rDNA inserts are randomly selected and the sequences determined. The derived sequences are aligned and compared with those of known species, other environmental clones, or cultures isolated from the same sample, to determine the species diversity of the clone library.

The clone library approach has been a great help in revealing the extent of bacterial and archaeal species diversity (eg. Liesack and Stackebrandt 1992). While it will continue to provide a means for providing baseline research information, the cloning methodology is too time-consuming, labour-intensive, and costly to be used for routine monitoring or in extensive ecological studies. Techniques such as denaturing gradient gel electrophoresis (DGGE) (Ferris et al. 1996; Murray et al. 1996; Muyzer et al. 1993) and single strand conformation polymorphism (SSCP) (Lee et al. 1996) provide alternative methods that overcome these deficiencies. In these latter methods the amplified rRNA gene fragments from each species present are separated by electrophoresis on polyacrylamide gels rather than by cloning. The electrophoresis patterns can then be used to follow changes in species diversity (Ferris and Ward 1997). If necessary, each rDNA band can be excised, PCR amplified, and sequenced to relate to the results from clone libraries or compared with database sequences (Ferris et al. 1996).

The advantage of the phylogenetic approach using 16S rDNA sequences is that no matter what method is used all the data can be related to the sequence representative of a species or phylotype whether that comes from a cultured organism or DNA from the environment. The sequence dataset can also be used to design rRNA-targeted fluorescently labelled oligonucleotide probes for direct visualisation of cells in the environment (Amann 1995; Amann et al. 1990, 1995). This technique, known as 'fluorescent in situ hybridisation' (FISH), exploits unique sequence regions of the 16S rRNA of each species, genus, or other taxon and allows the enumeration of species populations, and interactions with other members of the microbial community. Specific probes and PCR primers can also be designed from the sequences and used to probe community nucleic acids, rDNA clone libraries, DGGE bands, or isolates.

While the cloning approach has contributed to major advances in the accuracy of bacterial species identification and diversity assessment, it is not without its problems and care needs to be taken in the interpretation of results. Bias may be introduced: at the cell recovery and lysis step to release DNA; by adsorption of DNA to particulate matter; by PCR errors at the rDNA amplification and clone sequencing steps; in cloning; and in the selection of clones to represent the true diversity. The formation of chimeric inserts can also occur and needs to be checked. Overall though, using the molecular phylogenetic approach, considerable advances in the assessment of microbial diversity have been achieved which could not have been achieved by the culturing methods alone.

Methodology

DNA extraction

Samples of the first few centimetres of sediment were taken from the Myall and Oakey creeks in November of 1996. Genomic DNA was extracted according to the method of Zhou et al. (1996). Crude DNA extracts were further purified to remove humic material by cutting the high molecular weight DNA from low melting temperature agarose gels then using the Wizard PCR Preps DNA purification system (Promega) following the manufacturer's protocol.

Amplification of 16S rDNA

Primers conserved for the domain Bacteria 27f and 1492r (Lane 1991) were used to amplify the 16S rDNA in three separate reactions for each sample by PCR. PCR products were run on an agarose gel to check that the size corresponded to the 1500 bp size of the 16S rRNA gene, then were pooled and purified using Wizard PCR Preps DNA purification system (Promega) following the manufacturer's protocol.

16S rDNA clone library construction

Clone libraries of the 16S rDNA were constructed using the pGEM-T vector system (Promega) following the manufacturer's protocol. Clone colonies were then boiled and centrifuged and the resulting supernatant used as a template DNA in a PCR reaction using the plasmid specific primers T7 and SP6 (Promega) to amplify the 16S rDNA plasmid inserts. These PCR products were run on agarose gels to screen for any false positives clones.

DNA sequencing

PCR products were purified as before, quantified by electrophoresis on agarose gels with a low DNA mass ladder (Gibco BRL) and sequenced using ABI PRISM dye terminator cycle sequencing ready reaction kits (Perkin-Elmer) following the manufacturer's protocol. The reactions were run on an Applied Biosystems Model 373A DNA sequencer.

Phylogenetic analysis

Clone sequences were aligned manually with representative aligned bacterial 16S rDNA sequences obtained from the Ribosomal Database Project (RDP) (Maidak et al. 1994). Alignments and similarity matrices were performed using the 'ae2' editor (Larsen et al. 1993). A eubacterial consensus sequence was used as a guide to ensure correct alignment of homologous regions of the sequences (Lane 1991). To find possible nearest relatives, clone sequences were analysed using the program SIMILARITY RANK which is part of the RDP (Larsen et al. 1993) and the program BLAST (Altschul et al. 1990) using the non-redundant nucleotide database. Programs used to infer phylogenetic relationships are contained in the Phylogeny Inference Package (PHYLIP) version 3.5c (Felsenstein 1989). The DNADIST program was used to calculate evolutionary distances with the Jukes and Cantor model for nucleotide substitution (Jukes and Cantor 1969). Phylogenetic trees were constructed from evolutionary distance data by the neighbour-joining method (Nei and Saitou 1987), implemented through the program NEIGHBOR. A total of 100 bootstrap replicate resampling data sets for DNADIST were generated with SEQBOOT to test the statistical significance of branching topology, and the CONSENSE program was used to draw consensus trees.

Preliminary results

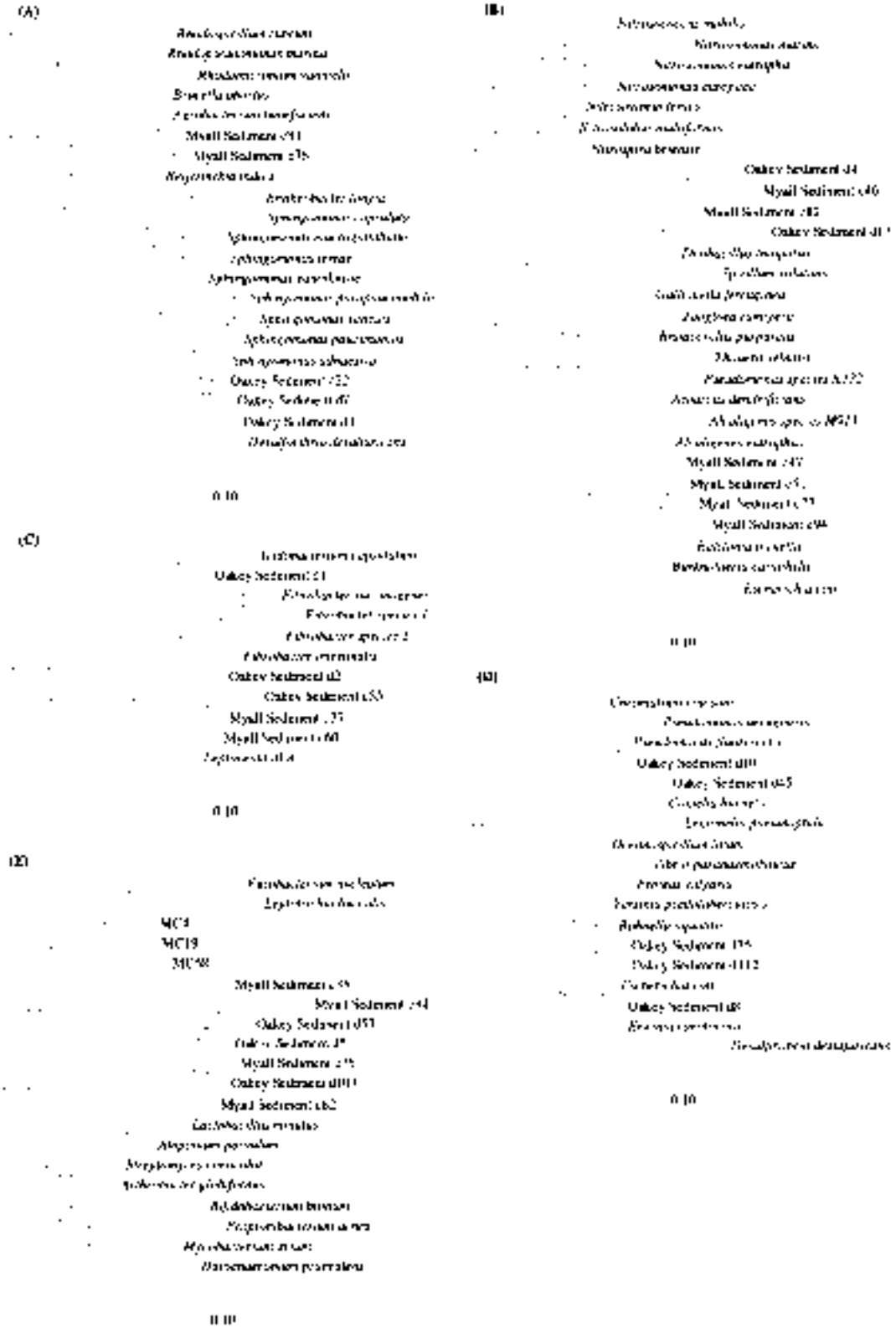
Table 1 summarises the results obtained to date on the analysis of rDNA clone libraries from the surface sediments of Myall Creek and Oakey Creek. The results show similarities and differences between the species diversities of the two sediments. The species diversity is lower than expected, probably because of the dominance of three groups of novel species for which no relative has been cultured before. Well known soil and aquatic species such as *Agrobacterium tumefaciens* (Fig. 2A), and *Alcaligenes eutrophus* (Fig. 2B) were found in Myall sediment, and *Pseudomonas fluorescens* and *Rahnella aquatilis* in Oakey sediment, and *Escherichia coli* of faecal origin in the Oakey sediment (Fig. 2D). By far the most significant result has been the detection of a novel phylotype in each of the β-Proteobacteria (Fig. 2B), the high mol% G+C Gram positive bacteria (Fig. 2E), and the *Fibrobacter* lineage (Fig. 2C). That these novel phylotypes occurred in both sediments suggests that they are important species in the surface sediments of rivers and not artefacts. The absence of sulfate-reducing bacteria is surprising but may indicate low populations or inappropriate redox conditions in the surface sediments or low populations.

Table 1. Distribution of sequences identified from the Myall Creek and Oakey Creek sediment 16S rDNA clone libraries.

Phylogenetic group	% Myall Creek (total 100 clones)	% Oakey Creek (total 100 clones)
α-Proteobacteria		
Total	3	3
Agrobacterium tumefaciens	3	–
Sphingomonas sp.	–	3
β-Proteobacteria		
Total	71	69
Alcaligenes eutrophus	8	–
Novel phylotype	63	69
γ-Proteobacteria		
Total	–	5
Rahnella aquatilis	–	2
Pseudomonas fluorescens	–	2
Escherichia coli	–	1
Fibrobacter		
Total	15	12
Acidobacterium capsulatum	–	1
Novel phylotype	15	11
High G+C Gram Positive		
Total	11	11
Novel phylotype	11	11

The β-Proteobacteria are highly represented in each clone library with 71% and 69% of clones from the Myall and Oakey creeks, respectively, found to belong to this group. Little diversity was found within the β-Proteobacteria (Fig. 2B) because 63% of such clones from the Myall Creek and all such clones from the Oakey Creek belong to a novel phylotype (94–98% sequence similarity). This phylotype has 87–89% sequence similarity to *Nitrospira briensis*, a representative of the *Nitrosomonas* group of nitrifying bacteria determined, from the phylogenetic tree of all sequences in the RDP, to be their closest relative (Larsen et al. 1993). However, the depth of the

Figure 2. 16S rDNA neighbour-joining trees of analysed clones. The bar reprsents 10 nucleotide substitutions per 100 nucleotides. (A) α-Proteobacteria: based on 368 bases with *Desulfovibro desulfuricans* used as the outgroup. (B) β-Protobacteria: based on 330 bases with *Escherichia coli* used as the outgroup. (C) *Fibrobacter* group: based on 348 bases with *Leptonema illini* used as the outgroup. (D) γ-Protobacteria: based on 368 bases with *D. desulfuricans* used as the outgroup. (E) High G + C Gram positive group: based on 329 bases with *Haloanaerobium praevalens* used as the outgroup.



phylogenetic relationship and the absence of a known cultured relative within the phylotype precludes the assignment of phenotypic characteristics to these bacteria at this stage. The Myall Creek sediment clones identified as being closely related to *Alcaligenes eutrophus* (Fig. 2B) show a 96.6–98% sequence similarity to that organism.

Eleven per cent of the clones in the libraries of both Myall and Oakey creeks belong to a novel phylotype in the high mol% G+C Gram positive bacteria phylum (Fig. 2E). These clones have pairwise sequence similarities in the range 92–98%, and are most closely but still distantly (74–81%) related to environmental clones MC4, MC19, and MC58 obtained from acidic eucalypt forested soil from Mount Coot-tha in Brisbane (Stackebrandt et al. 1993). The nearest cultured relatives are the *Atopobium* group but the novel unidentified clones show only a 74–79% sequence similarity to *Atopobium parvulum* the representative used in our analyses.

Another significant group of novel clones belongs to the *Fibrobacter* lineage (Fig. 2C). The clones which comprise 15% and 12%, respectively, of the clones in the Myall Creek and Oakey Creek clone libraries, are only distantly related to the genus *Fibrobacter*, an anaerobic cellulose-degrading bacterium. The novel phylotype belongs to a deep phylogenetic branch whose members share only 77–79% sequence similarity with *Fibrobacter intestinalis* and therefore probably represent a novel genus of unknown phenotype in the *Fibrobacter* group. Clone d1, however, has been identified more confidently with a 94.1% sequence similarity to *Acidobacterium capsulatum* (Fig. 2C).

Very few clones from either clone library were found to belong to the α-*Proteobacteria* (Table 1). Three clones from Myall Creek were identified as being highly related with sequence similarities of 99–99.5% to *Agrobacterium tumefaciens* (Fig. 2A), while three clones from Oakey Creek were identified as being highly related with sequence similarities of 98–99% with *Sphingomonas adhaesiva* (Fig. 2A).

Clones belonging to the γ-Proteobacteria came exclusively from the Oakey Creek sediment (Table 1, Fig. 2D). Clone d8 has a sequence similarity of 99.2% with *Escherichia coli*, while clones d35 and d112 are closely related to *Rahnella aquatilis* (98.2%), another member of the Enterobacteriaceae found in aquatic environments. Clones d10 and d45 are closely related (99.7% and 94.6%) to *Pseudomonas fluorescens*.

All unidentified clones require complete sequencing and further analysis to confirm their correct phylogenetic position, and to eliminate the possibility of chimeric origin.

Future directions

Work is in progress to complete the analysis of bacterial species diversity in samples from the water column and attached biofilm at the Myall and Oakey Creek sites. Phylogenetic probes are being designed to enumerate the population levels of the novel phylotypes in the sediments to confirm their significance in these ecosystems. The application of DGGE will be used to follow shifts in species diversity due to seasonal and physicochemical changes at different sites along the Condamine River.

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Microbial lipids in environmental research and monitoring

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Summary

The microbial community plays a key role in aquatic processes, including within Australian rivers. Innovative techniques are required to enumerate and characterise microbial communities as a prerequisite to determining river health. The use of microbial lipids enables biomass, community structure and nutritional status to be determined; in many cases the application of microbial lipid procedures will provide information not available using classical methods (eg. plate and direct microscopic counts). Components common to all cells provide a measure of total microbial biomass. Compounds restricted in distribution to subsets of the microbiota can be used as signatures for these groups, providing understanding of community structure, while nutritional status can be determined by measuring storage products or components that may change in concentration during environmental stress. During the past 20 years, microbial lipids (termed 'signature lipids') have been used increasingly by microbial ecologists in a wide range of environments. The application of microbial lipid assays provides powerful and sensitive tools to complement other procedures available for monitoring river health.

Background

Microbes play a significant role in aquatic environments, yet they are rarely considered in management protocols (Boon et al. 1996). This could be partly due to an overall lack of recognition or understanding of their role (Wetzel 1995; White 1995) and the fact that traditional techniques, including plating, are not applicable for environmental studies (White 1983). Molecular approaches offer enormous potential for studying bacterial consortia, but they are not yet sufficiently developed to be used routinely (Olsen et al. 1986; Ward et al. 1992). The combined use of molecular and signature lipid techniques will be advantageous for many field programs.

Signature lipid analyses offer many advantages for studying and monitoring microbes (White 1983). Of particular interest has been the cell membrane derived phospholipid fraction which is generally present in fixed proportions in viable eubacterial and archaeal cells. The measurement of lipid phosphate, phospholipid fatty acids (PLFA) and ether lipids (PLEL) provides sensitive, reproducible and quantitative measures of biomass and community structure of microbial assemblages. In this brief review, a variety of signature lipids commonly used will be described, an overview given of methods presently employed, and research applying these procedures to examine river health in Australia summarised.

Methods

Samples [bacteria, algae, water (usually suspended solids or particulate phase), sediments] are first extracted with organic solvents, such as the Bligh and Dyer (1959) mixture of chloroform–methanol–water. Total lipid is separated by chromatographic procedures with resultant fractions derivitised and analysed at nanogram, picogram or greater sensitivity by gas chromatography (GC), GC–mass spectrometry (GC–MS) and other instrumental facilities. Currently, instrumental procedures also are being assessed or

used in several laboratories for the initial sample extraction or fractionation; eg. supercritical fluid extraction and/or accelerated solvent extraction can be directly coupled to GC and GC–MS. Scope therefore exists for development of rapid signature lipid assays to be completed within 30 to 60 minutes or faster. When further developed, validated and used in environmental studies, application of rapid signature lipid procedures to river health monitoring programs will be possible.

Microbial signature lipids

Phospholipid fatty acids (PLFA)

Of the microbial signature lipids, the ester-linked PLFA have received the greatest interest in environmental studies with much of the pioneering led by White (1983) and associates. PLFA are found in the cell membranes of all viable eubacteria and degrade rapidly after cell death. Fatty acids have long been used in microbial taxonomy and close examination of PLFA profiles for environmental samples therefore provides insight into microbial community structure. Selected signature PLFA that are used routinely in environmental studies are listed in Table 1.

In combination with the measurement of microbial signature lipids described above, with appropriate experimental design, analysis of specific pollutants or other key environmental parameters also can be performed. Such studies provide an understanding of changes occurring to the microbial community that accompany or are caused by human or non-human activities. In laboratory studies the isomerisation of *cis* to *trans* monounsaturated fatty acids has been used as an indicator for the toxicity of organic compounds and measurement of the ratio of *cis* to *trans* monounsaturated fatty acids has wider application in natural habitats (Keweloh and Heipieper 1996; Guckert et al. 1986).

There have been studies to validate the use of PLFA and other signature lipids (Baulkwill *et al.* 1988), and PLFA measurements have been applied routinely to marine (including polar) environments, soil, indoor air, deep aquifers, the rhizosphere, artificial streams, bioremediation, biofouling, drinking water and many other fields.

Phospholipid ether lipids (PLEL)

The archaea are clearly differentiated from eubacteria based on membrane lipid composition. Unlike the PLFA present in all eubacteria, the phospholipids of archaea (methanogens, halophiles, thermoacidophiles) are unique and consist of isoprenoid ether-linked side-chains (Tornabene and Langworthy 1979); these lipids are termed phospholipid ether lipids (PLEL) (Table 1).

Table 1. Summary listing of selected microbial signature lipids and their sources.

Component	Source/comment
Phospholipid fatty acid	
total	total non-archaeal microbes
i&a17:1w7c	*Desulfovibrio* spp.
10Me16:0	*Desulfobacter* spp.
17:1 w6c	*Desulfobulbus* spp.
PUFA[a]	microeukaryotes
18:1w8c, 16:1w8c, 16:1w6c	methanotrophs
22:0–30:0	higher plants
10-OH18:0	*Cryptosporidium parvum*[b]
Phospholipid ether lipids (PLEL) (isoprenoid branched side-chains)	archaea (methanogens, thermoacidophiles, halophiles)
Lipopolysaccharide	gram negative bacteria
Sterols	(absent in bacteria)
coprostanol	human faecal contamination
dinosterol	dinoflagellates
ergosterol	fungi

[a] PUFA, polyunsaturated fatty acids.
[b] D.C. White, personal communication.

Sterols

Sterols are generally not found in bacteria and are common to eukaryotes. Like chlorophyll and carotenoid pigments they can be used to determine microeukaryote community structure. The 4-methyl sterol dinosterol (4,23,34-trimethyl-5α-cholestan-3β-ol) has been used as a marker for dinoflagellates for nearly 20 years and a number of other sterols also are used as specific signature lipids (Table 1). Coprostanol (5β-cholestanol) is produced in the digestive tract of higher animals by microbial degradation of cholesterol (Rosenfield and Gallagher 1964) and has proven a sensitive indicator of sewage pollution (eg. Hatcher and Mcgillivary 1979). The application of faecal sterol methodology in Australian coastal and inland waters was summarised in Nichols et al. (1996). Recent developments have seen the establishment of coprostanol concentrations equivalent to water quality guideline levels of bacterial indicators together with the ability to use faecal sterol profiles in combination with bacterial indicators to distinguish human, herbivore (eg. cows and sheep) and other sources of faecal pollution (Leeming 1996; Leeming et al. 1997; see also other papers in these proceedings).

Case studies of Australian rivers

Microbial signature lipids have been used by a number of Australian researchers, although to date not as routine monitoring tools. Studies of many of the compounds described above have been conducted in the Brunswick, Clarence, Derwent, Daintree, Hawkesbury-Nepean, Huon, Kangaroo, Mordialloc (Creek), Murray (WA), Serpentine, Williams and Yarra rivers and within Victorian wetlands including billabongs. Information from such investigations may eventually assist management decisions for natural ecosystems.

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Molecular markers and river health: bacteria, a cautionary tale

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A detailed description of the components of microbial communities involved in specific ecosystem functions will increase our ability assess river health through monitoring the presence and activity of microbial indicator species.

Background

The link between human health and water quality has traditionally been made through monitoring microbial indicators. Key indicator organisms, including *E. coli*, coliforms, faecal streptococci, *Cryptosporidium*, *Giardia*, campylobacters, *Pseudomonas aeruginosa* and viruses, have been used as a measure of water quality from a human health perspective particularly where consumption of potable water and water-associated leisure activities are concerned. Their importance in this respect should not be underestimated and the development of technologies for rapid water quality determination will continue to have a high priority. However, microbial indicators not directly associated with human health can be can be applied to examine anthropogenic effects on rivers. Hart et al. (1995) described their use as indicators of biodiversity leading to an understanding of their role in active ecosystem processes (Head et al. 1993; Hiorns et al. 1995; Ward 1996). These data may then be applied to ascertain whether key functional groups of organisms or species were present, excluded or enhanced when exposed, for example, to pollutant stress (Joye and Hollibaugh, 1995). Secondly, indicators can be used to assess functional efficiency of an ecosystem. However, this has traditionally been achieved through chemical process measurement (for example, nitrification (Hall 1984) and methanogenesis (Hall et al. 1995)). Where chemical measurements show a process to be failing, molecular tools can be applied to examine which component is affected and may lead to the implementation of a remediation strategy (Wagner et al. 1996).

The aim here is to describe key processes and our current ability to describe and detect the indicator species involved and to assess the limitations of this approach to river health. Two approaches are available for describing function microbial groups: (a) to target the organism or (b) to detect specific genes involved in the process.

Probes based on 16s rRNA sequences/immunological approaches

Nitrification and ammonia oxidising bacteria

Nitrification is a link between the reduced and oxidised sides of the nitrogen cycle and therefore is of fundamental importance to all ecosystems. In the aquatic environment, the importance of nitrification as an oxygen sink is well recognised and the presence of nitrifiers in a river environment is indicative of high biological oxygen demand (BOD; Hall 1984). Sewage discharge into a river containing a high ammonia concentration will enrich for nitrifiers and these in turn will remove oxygen from the river. The process is especially significant at night. Chemical analyses can determine the nitrification rate, but culture-based techniques for nitrifiers are very slow. Molecular techniques, particularly polymerase chain reaction (PCR) amplification of DNA or fluorescent *in situ* hybridisation (FISH) using specific fluorescent oligonucleotide probes, offer rapid systems for monitoring the presence of nitrifiers without the need to culture.

We attempted to describe the nitrifiers active within a lake in Cumbria, UK. Ribosomal (r)RNA genes amplified from lake water and sediments showed the presence of *Nitrosospira* spp. within the active nitrification region (Hiorns et al. 1995). This was achieved using a nested PCR whereby eubacterial 16S rRNA genes were amplified and these then formed the target for a second PCR reaction using nitrifier specific primers (Hiorns et al. 1995). *Nitrosomonas europaea*, the archetypal nitrifier, was not detected unless the samples were enriched with ammonium salts. In this example, culture, as in many cases, would not adequately describe the

ecologically-dominant ammonia oxidising species and the possible microbial indicator for this process in this system. Oligonucleotide probes and PCR primers are available to a number of nitrifiers (Head et al. 1993) which will significantly increase our ability to describe nitrifying communities in a number of environments (Mobarry et al. 1996). This approach has a direct application to sewage treatment and river management. Allied to this, immunological approaches permit the detection of specific serotypes in mixed populations and has some relevance in quantifying and monitoring the presence nitrifiers (Volsch et al. 1990).

Methanogenic and sulfate-reducing bacteria (SRB)

As with nitrification, molecular probes are available to analyse the bacteria involved in methanogenesis as more than 50 species have now been described (Reeve 1992). It is now possible to describe members of the methanogenic community without the need to culture (Raskin et al. 1994; Hales et al. 1996). Bacteria with methanogenic capabilities are strict anaerobes and are exclusive to the domain Archaea (Woese 1987). Their activity is oxygen-sensitive and thus sampling regimes for methanogenic bacteria require the exclusion of oxygen (Hall et al. 1996). In aquatic systems they are more likely to be associated with sediments where they exist on steep redox gradients. The presence of methanogens in the sediment is associated with stability, and their appearance in the planktonic state is indicative of sediment disruption or, more likely, an input source upstream. Similarly, functional sulfate-reducing bacteria are also mainly sediment-associated, relying on redox gradients to maximise activity. There are several probes specific to this group of organisms by which detection can be achieved either by PCR or using FISH (Stahl 1995; Devereux et al. 1996).

Direct detection of marker genes

Resistance genes

It is well recognised that contamination of the terrestrial and aquatic environments results in the selection of bacteria capable of either exhibiting active resistance to, or actively metabolising, the pollutants as a carbon source. These functions are often specified by a limit number of genes or operons which makes them indicative of the response of the microbial population to a particular pollutant. Specific genes can therefore be used as targets for a range of gene probes, PCR primers and antibodies to the gene product. There are numerous examples describing the detection of mercury-resistance genes (*mer*; Jeffrey et al. 1996), nickel-resistance genes (*nrf*; Pickup et al. 1997), antibiotic determinants (Smalla et al. 1993) and genes involved in hydrocarbon degradation (Halliersoulier et al. 1996; Joshi and Walia 1996).

Degradative genes

The enrichment of hydrocarbon degrading bacteria is often a consequence of low level but persistent infusion of pollutants or of catastrophic pollution events. Catechol 2,3 dioxygenase is one of the key enzymes in the degradation of aromatic hydrocarbons. It is encoded by a family of genes of which *xyl*E is the most studied. It was developed as a marker gene for monitoring the fate of genetically manipulated microorganisms (Pickup 1991). However, the presence of indigenous *xyl*E genes in the bacterial population leads to the correct inference that community has biodegradative potential. By default, this is probably due to selection of these organisms in the presence of aromatic hydrocarbons. Therefore, monitoring for the presence of this gene could indicate rising levels of aromatic pollutants and, conversely, its absence after a pollution event may be indicative of a recovery in the system. Chandler and Brockman (1996), for example, used a PCR-MPN method to quantify *xyl*E in fuel-contaminated soils, whereas Halliersoulier and co-workers (1996) used single PCR to quantify the presence of *xyl*E genes in soil. They found the detection limit to be 100 cells/g and that quantitative PCR was more sensitive that a comparative microbiological approach. A similar approach was used by Joshi and Walia (1996) to detect *xyl*E in polluted groundwater. They estimated the sensitivity of the PCR detection method to be 100 cells/mL. It is apparent that PCR detection of specific genes is an important advance whereby assessment can be quantified without culture of the host organisms.

Interpretation of indicator studies

It is important to note that the presence and significance of indicator organisms and marker genes requires careful interpretation. The presence of pathogens and/or indicators of faecal pollution above acceptable and defined levels provides unequivocal proof of the deterioration of water quality and its possible harmful effects on humans. The use of markers outside this context, namely in investigations of river health, is not as clearly defined. For example, the spread of antibiotic resistance is of major concern. An examination of antibiotic resistance profiles, and compilation of an antibiotic resistance index (Ab^r), for bacteria isolated from freshwater revealed that, taken in isolation, such data could be misinterpreted (Jones et al. 1986a,b). The Ab^r for bacteria from water bodies of higher nutrient status and receiving sewage discharges

was lower than those from water bodies not subjected to anthropogenic influences. The main factors affecting the resistance profiles obtained were the media composition and the trophic status of the water body. Jones et al. (1986a,b) found that the majority of the resistances observed were passive and variable if re-tested on different media (Jones et al. 1986a,b), and they proposed that many environmental assessments of Ab^r were misinterpreting the extent of resistance in the environment. Similarly, we a found novel transmissible nickel resistance plasmid in bacteria, and bacteria resistant to high levels of copper were isolated from an area not subjected to heavy metal pollution (Pickup 1989{not listed}; Pickup et al. 1997). A deterioration in environmental quality could not be inferred from any of these findings as chemical measurements did not detect pollutants in the system.

Despite some limitations, *xyl*E gene is a fairly robust marker for hydrocarbon pollution. It has been detected in both the terrestrial and aquatic environments (eg. Halliersoulier et al. 1996; Joshi and Walia 1996). Our experience with *xyl*E in freshwater systems arose from its use as a marker for genetically manipulated bacteria (Morgan et al. 1989). We determined the presence of *xyl*E in lakes of different trophic status and streams (R. Pickup, unpublished data). We were not able to detect *xyl*E^+ bacteria by direct plating but positive isolates were obtained by enrichment from sediments of all the lakes except the most oligotrophic. PCR detection, however, gave positive results only in lakes where hydrocarbons (usually entering through run-off) were detectable (Cranwell and Koul 1989). The detection limit of PCR (100 cells/mL), in this case distinguishes 'polluted' from 'non-polluted' water bodies. This was limited to the analysis of cores and did not apply to the water column where *xyl*E-containing organisms exist below the detection limit. However, where pollution is heavy, *xyl*E-containing organisms provide sufficient template to be readily amplified and quantified (Joshi and Walia 1996).

The presence of specific organisms associated with ecosystem functions whether detected by microbiological methods or by PCR techniques implies that conditions exist where they can function (eg. redox gradients permitting methanogenesis). If these organisms were present, and active due to selection following exposure to pollutants, then the implications for deterioration of river quality can be deduced. However, activity cannot be inferred from presence alone, which may be due to indirect causes such as run-off from peat areas and is probably transitory. For example, SRB can survive prolonged periods in the planktonic state in the presence of oxygen (Sass et al. 1997) and can be readily detected by culture from a number of rivers/streams in the UK with little or no sulfate loading (Hall, pers. comm.)

Therefore, at present, the use of indicator genes or organisms comes with the implicit requirement to assess the river conditions by more traditional means (eg. chemical analyses) and to relate activity to their presence. Without this approach misinterpretations in river quality may arise.

Advances with caution

When applied with care and with an appreciation of their limitations, molecular techniques will permit a greater insight into the organisms involved in ecosystem function, with the result that the robustness and confidence of predictions made through the presence of both indicator organisms and marker genes will increase. It must be noted that the most useful microbial indicators might not yet have been described.

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Distinguishing sources of faecal pollution in Australian inland and coastal waters

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Introduction

Faecal pollution of Australia's waterways continues to be a chronic problem, adding nutrients and degrading the water quality. Thermotolerant coliform bacteria (faecal coliforms) and faecal streptococci, are commonly used to indicate faecal contamination. The ratio between thermotolerant coliforms and faecal streptococci has previously been used to distinguishing human and herbivore-derived faecal matter by Geldreich (1976). However, this method is no longer recommended (Howell et al. 1995), so none of the currently used bacterial indicators on their own can distinguish different sources of faecal matter (Cabelli et al. 1983). The use of alternative indicators, in this case faecal sterols, in conjunction with existing bacterial indicators, offers a new way to distinguish sources of faecal contamination and monitor river health.

Faecal sterols

The most commonly known faecal sterol, coprostanol (5β-cholestan-3β-ol), is produced in the digestive tract of humans by the microbial hydrogenation of cholesterol (Rosenfield and Gallagher 1964). It has been proposed as a measure of human faecal pollution by a large number of researchers since the late 1960s (eg. Murtaugh and Bunch 1967; Dukta et al. 1974; Walker et al. 1982), but has not really been embraced as a sanitary indicator for sewage pollution because its presence is not considered as indicative of a health risk. Previous investigations of the overall sterol composition of animals and invertebrates were performed (see Walker et al. 1982), but largely to determine whether these animals produce coprostanol and if so, in what amounts relative to the weight of faeces, not relative to the overall composition of sterols.

In 1994, scientists from AWT-Ensight and CSIRO investigated differences in the neutral lipid composition and bacterial indicator profiles of faecal matter from a range of common animals (Leeming et al. 1994, 1996). It was identified that herbivores did have coprostanol in their faeces, but the dominant sterol was the C_{29} homologue of coprostanol, 24ethylcoprostanol (24ethyl5β(H)-cholestan-3β-ol). It was thought that by exploiting this difference, it would be possible to determine the contribution of faecal matter from these two sources relative to each other by calculating the ratio of coprostanol to 24ethylcoprostanol in human and herbivore faeces.

Animals such as dogs and birds are ubiquitous in urban areas, but either do not have coprostanol in their faeces or it is present in relatively trace amounts (Leeming et al. 1996). Therefore, the faecal matter from birds and dogs could be distinguished from humans and herbivores by comparing the *measured* abundances of bacterial indicators (eg. thermotolerant coliforms, faecal streptococci, enterococci) common to these animal groups with the abundance of bacterial indicators *expected* from human and herbivore sources based on faecal sterol concentrations. Further differences in the relative abundances of faecal coliforms and *Clostridium perfringens* spores were also observed (Leeming 1996) which could be exploited to distinguish contamination from birds and dogs.

Field trials

Subsequent field trials in Lake Tuggerah, NSW highlighted the potential to estimate the proportions of faecal pollution *in the water column* by calculating the ratios of bacterial indicator concentrations compared with faecal sterol data. It was recognised that the ratios of faecal sterols to bacterial indicators for faecal matter needed to be as accurate as possible and processes that might change the ratios during transport into receiving waters must be identified and understood. In response, the Water Services Association of Australia (WSAA) funded a project commencing in July 1996 to address these issues and to validate and refine the basis for distinguishing faecal pollution using faecal sterols and bacterial indicators.

In parallel to these investigations, more detailed field studies in Lake Macquarie (funded by Hunter Water), the Yarra River and Rippleside stormwater drains in Geelong (funded by the Victorian Environment Protection Authority) and other smaller studies for local councils were undertaken. In combination, these studies have built on a database about faecal pollution and its source(s) in a variety of environments and tested ongoing improvements to the broader experimental protocols.

The specific aims of the current investigations are to: (i) establish that differences in the presence and/or abundance of sterols and bacterial indicators for faeces from a wide range of animals are consistent and of diagnostic value; (ii) refine the precision and accuracy of the calculations used to fractionate the various sources of faecal matter; (iii) determine the relationships between, and relative persistence of, faecal sterols and bacterial indicators to account for possible differences; (iv) validate non-human sources by application of qualitative faeces-specific bacteriophage tests and/or polymerase chain reaction (PCR) assays; and (v) ground-truth a technique which can distinguish how much faecal matter is present in a polluted body receiving water from different animal groups (humans, herbivores, domestic pets [eg. dogs] and birds) to a degree of certainty suitable for management decisions. The primary aim of each of the parallel field studies, from the point of view of the funding agency, was to determine whether faecal pollution, measured by traditional bacterial indicators (ie. thermotolerant coliforms) was derived principally from human faecal matter. The supplementary aim was to identify and estimate contributions from non-human sources and provide feedback to the WSAA validation and refinement study.

Results

In summary, the results so far indicate that the differences in faecal sterols and bacterial indicators previously observed between the animal groups are consistent over a range of environments. The concentrations of faecal sterols and bacterial indicators measured can be variable, but the ratios are consistent within groups and are comparable to previously collected data (Leeming et al. 1994) and reference samples collected from diverse locations. Reference samples were collected from sewer mains or overflows, livestock saleyards, and bird resting sites etc. adjacent to field sites, and provided corroborating *in situ* ratios to estimate faecal contributions to specific environments. The independent use of four subgroups of bacterial indicators (*Escherichia coli*, faecal streptococci and enterococci, and thermotolerant coliforms) as a common denominator of all faecal pollution, also gave a higher degree of confidence to the estimates and enabled a likely range of contributions to be expressed. The majority of estimates from the independent calculations in the field studies, where the wider range of bacterial indicators was used, varied by less than 10%. Experiments to determine the relationships between, and relative persistence of, faecal sterols and bacterial indicators are still under way, as are efforts to confirm the identity of bacterial indicators from non-human and non-herbivore sources.

Results from field studies have shown the technique can distinguish between faecal contamination from humans and herbivores. Faecal contamination from dogs should also be evident because of the added distinguishing characteristic of *C. perfringens* spore abundance similar to the thermotolerant coliforms or faecal streptococci abundance. In the absence of a specific marker for bird faeces, the faecal profile of high thermotolerant coliforms and faecal streptococci and low faecal sterols cannot be unequivocally attributed to birds. In samples from Lake Tuggerah, the percentage of D^5 sterols (cholesterol and 24-ethylcholesterol) compared with the total sterols, including algal derived sterols, was higher (often more than 50%) at sites where coastal birdlife was prolific and the percentage of unexplained faecal contamination was calculated at greater than 90%. The faecal sterol signatures of seagulls (Leeming et al. 1996) and pelicans (Leeming et al., unpublished data), which are overwhelmingly dominated by D^5 sterols (> 80%), are thought to have noticeably elevated the proportion of cholesterol in these samples. However, a more definitive measure of faecal contamination from birds is needed to confirm this source.

Further issues

Additional factors which might affect or explain differences in the concentration of bacterial indicators compared with faecal biomarkers in the field are crucial to the application of this technique and include: (i) regrowth or re-suspension of bacterial indicators; (ii) inadvertent measurement of other species of bacteria; (iii) differences in bacterial enumeration between laboratories; and (iv) the effect of adhesion of faecal sterols and bacterial indicators to different particles and their potentially different settling rates. These issues are steadily being addressed. The crucial difference at this stage seems to be time and distance from the source. During wet weather, or when samples are collected close to discharge or entry points, faecal particulate matter has little time to degrade or settle. In such environments, the efficacy of the technique may be proportional to time and/or distance from entry or discharge.

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Linking river health monitoring with improving environmental quality

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Introduction

The development of new river health indicators promises to allow better assessment of river structure, function and environmental quality. For the greatest benefit, however, indicator technology and environmental management need to be linked. Such linkages should promote:

- interpretation of the environmental management significance of indicator information and generation of useable river health assessments; and
- responses which can be applied in a timely fashion, are environmentally and socially acceptable, and economically viable.

Such links are currently not well developed. For scientists the current major pre-occupations still appear to be methodology or academic argument (eg. this workshop; CRCFWE 1997). While scientific techniques and discussion can be of interest to environmental managers, they are not their major concern.

The aim of this paper is to alert microbiologists to some major preoccupations of environmental managers so that better linkages between river health indicators and environmental management may be developed.

Drivers of environmental management

To understand the needs of environmental managers, microbiologists should first be aware of major elements underpinning assessments of river health, especially:

1. *Total catchment management(TCM) principles*: These principles define the environmental management context for responses to a decline or improvement in river health.
2. *Environmental values (EVs) for water*: Examples of EVs are ‘protection of aquatic ecosystems’ and ‘protection of recreational water quality for primary contact’. In practice, these are sets of numerical and/or narrative criteria which define how the needs and wants of the community can be met for different water bodies (ANZECC 1992). Their achievement and maintenance on a sustainable basis are aims of environmental management activities. The definition of these numerical and narrative criteria may include desired values of selected microbial parameters.
3. *Broadly agreed and officially sanctioned environmental quality criteria*: Environmental managers require well-defined water quality criteria which reflect a scientific and environmental management consensus. Scientific argument about microbial indicator suitability, criteria, values etc. greatly limits their value, use and acceptance to environmental managers and the broader community.
4. *Practical environmental management systems(EMSs) and techniques*: For a definition of EMSs see below. To be of value for EMSs, an agreed indicator might:
   - add to or replace an indicator from those currently accepted;
   - aid in quantifying and qualifying EVs for a river system;
   - support suggested environmental responses to remediate any indicated problem;
   - act as a trigger for practical action to manage a river system or catchment.
5. *State of the Environment(SOE) or State of Catchment(SOC) reports*: These reports are an avenue for different spheres of government to report on the quality, quantity and conservation status of the environment. Together with indicators of river health they will increasingly define current and desired environmental values for river systems.

In addition to being linked with the above elements, there are various desirable attributes of river health indicators which will make their use feasible on a day-to-day basis:

- can be used to develop and/or refine criteria for quantifying river health;

- can be used in monitoring;
- aid diagnosis of common river health problems;
- work efficiently and reliably;
- provide information on river health adding to that from other technologies;
- products, processes, results and interpretations should be provided in a form suitable for communication to environmental managers, scientists from other disciplines and the broader community;
- should be linked, via triggers, to decision-related actions including management responses and community behaviour.

A future framework?

To strengthen the links between indicator technology and river management the following things need to happen:

- environmental managers clearly articulate to the scientific community what they want, why they want it and what they expect the indicator(s) to do;
- environmental managers become familiar with existing technologies, and microbiologists with environmental sampling regimes;
- microbiologists clearly explain how an agreed indicator acts as an indicator of river health;
- microbiologists work more closely with environmental managers to develop a consensus on 'best management practice' for selecting water quality criteria, measuring parameters, obtaining environmental values, interpreting data, public access to data (raw or interpreted), management responses etc.

Several possible approaches, available to promote such links, are listed below.

Incorporation into government conservation policy

Microorganisms are still poorly recognised as components of the biota, and microbiologists need to be involved in rectifying this. For example, the draft NSW Biodiversity Strategy acknowledges their importance but does not provide any further consideration for inclusion into the conservation activities of the State Government. Until the State is willing to address conservation of microorganisms it is unlikely local government will either.

Environmental values and national water quality criteria

Indicators should be linked to environmental values and national water quality criteria (eg. ANZECC 1992) through the addition of new criteria or the modification of existing ones. Current criteria focus on a limited range of microbes which may affect human health. In terms of the development of microbial indicators of river health, this practice can be viewed as restrictive and is probably not providing the best indicators for river health.

Environmental management systems

An environmental management system (EMS) is a set of arrangements that affects how an organisation goes about those of its activities that include environmental issues. EMSs are currently being standardised by the introduction of ISO 14000 (Standards Australia 1995a,b), a family of international standards and guidelines which provides a framework and management tools for achieving ecological and economic sustainability (Sutton 1996). The EMS process provides a framework within which river health indicators could be used. While the key place for indicator technology is in monitoring, microbiologists need to have input into other stages (policy, planning, implementation) of the development of the EMS. As EMSs are not static, involvement in any review would also be necessary. This would allow ongoing interaction between 'management' and 'science'.

State of the Environment reporting

State of the Environment reporting is an increasingly important tool for environmental improvement. Governments are required to compile these reports on an ongoing basis (eg. National five years, NSW two years, and local government annually). River health indicators have a clear role within this process to inform the broader community whether an riverine environment is being degraded or improved.

Expert system development

Lawrence (1997) has proposed the use of expert computer systems for managing water quality data collection, collation and interpretation. Such systems could be used for the following:

- simplifying the use of complex water quality guidelines by providing a standardised, interpretative assessment of river health;
- increasing access to such information where the provider clearly identifies target audiences; and
- estimating the probability of there being a detectable effect on the environment, eg. as with North American sediment quality guidelines (Long et al. 1995; Long and Morgan 1990).

Networking

Many indicator studies are currently undertaken in isolation, and duplication of effort is common. Therefore, creating and strengthening social and institutional communication between specialist ecologists and environmental managers is fundamental. The Trust, which has a major network facilitation role, is currently identifying and compiling a database of water quality monitoring site locations. This should assist contact between testing organisations and promote follow-up work at previously studied sites. Specialist researchers should also be aware that opportunities exist to work with community groups, who could assist with monitoring or provide the opportunity for 'before and after' river health studies through their catchment rehabilitation work.

Microbiology, management and the environment

While management is seldom recognised as part of the science of microbiology, it is, in fact, central to applied microbiology. The challenge for microbial ecologists is to recognise that their work must be applicable to stages, or to steps in, a larger management process.

The stages/steps which may be undertaken in managing river microbiology will depend on the functions, responsibilities and legislation applicable to the organisation undertaking them. Examples of process steps are shown in Table 1.

Trust experience during a Joint Recreational Water Quality Monitoring Program in 1996–97 illustrated a variety of issues to be addressed in implementing management processes which may impact on the introduction and application of novel indicator technology.

There is difficulty in defining 'river health' and as a consequence it is unclear which indicators are required and who should champion the introduction of new indicators (ie. researchers, analysts or managers). In addition, microbial indicators will compete with current and emerging plant and animal indicators.

While there is agreement on the need for monitoring there is disagreement on who should pay. The budgets for current monitoring tend to allow only for routine screening. Official sanctioning of the use of novel microbial indicators is no guarantee of their widespread use.

The increased use of sophisticated analytical techniques, statistics, expert systems, and quality control and assurance may produce more reliable data products, but they may also alienate testing organisations or make the use of microbial indicators in monitoring programs unaffordable. The latter is of particular concern for ongoing monitoring programs where some stakeholders come to believe that further testing will not show anything new and so be not worth financing.

Numerical criteria for assessing water quality can be criticised because of the uncertain significance of the results (Norris 1997). However, such criteria appear

Table 1. Possible process for managing recreational water quality

Stage of management process	*Environmental example*
1. Prerequisites: need objective and responsible agencies	Identify swimmer health as a responsibility of local government and others
2. Identify and obtain resources for work.	Council environment department funds
3. Undertake routine monitoring/screening	Water quality analyses for ANZECC recreation criteria
4. Identify problem	Coliform counts fail, Reports of ear infections to council health inspector
5. Undertake diagnostic testing at likely source	Undertake more intensive coliform monitoring
6. Identify cause/confirm source	high coliform discharge from leaky sewage pipe
7. Identify responses	repair pipe, replace sewerage system
8. Select response	repair pipe
9. Communicate response	council informs EPA/public of problem and proposed response
10. Apply response	repair pipe
11. Monitor effect of response	check river for elevated coliform counts
· If response ineffective go to 8.	search for alternate sources
· If response partially effective revise 7.	search for additional sources and reconsider 8.
· Response effective (desired outcome)	acceptable river coliform counts = **RIVER HEALTH MANAGED**

essential for environmental management as they can act as triggers for identifying response options and subsequent actions.

Access to data and data analyses from such programs is very contentious. There are concerns about data being out-of-date, data misinterpretation, the public information rights, responses by the media, legal liabilities in respect to follow-up and council/agency duty of care.

Conclusions

Environmental indicators have long played a crucial role in alerting society to environmental degradation. This is no longer sufficient for environmental managers. Ecologists are now also being asked how indicators can be used to identify environmental quality targets and what means should be used to achieve the latter as defined by their indicators.

This paper discusses this new emphasis and identifies linkages between indicator technology and their application to environmental management as a key issue to be addressed by scientists introducing new river health indicators. Scientists also need to be more aware of obstacles to the application of microbial indicators, and adapt their work accordingly.

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Statistical considerations for design and analysis of microbial indicators R&D

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Introduction

There are many different ways in which biota might be used to assess riverine health (Cranston et al. 1996). Microbial indicators for such investigations have rarely been discussed in the literature from a statistical viewpoint, although the same considerations apply as for all field-based environmental research. In this presentation I will seek to raise several issues to do with effective and efficient design of R& D for microbial indicators of river health. It is crucial to consider such issues at an early stage of this program rather than attempting at some later date to 'save' flawed data sets with less than optimal analytical approaches (Ward et al. 1986).

Importance of microbes = an opportunity

We are all aware of the important role that microbial processes play in ecosystems. Further, I contend that many of these form the bulk of ecosystemic functions within most rivers or billabongs. These include organic matter decomposition, mineralisation, denitrification, methanogenesis, etc. We may be able to design appropriate tests of these functional attributes that indirectly measure microbial activity. Thus, river health could be assessed by measuring these rates so that poor health would be defined as relatively lessened rates of selected functions. Most of these measurements would lead to reasonably straightforward univariate data analyses (with the caveats give below), with the added advantage that many microbial processes move much faster than their equivalents for bigger organisms. Speed of reaction is an advantage where rapid responses and early warning capabilities are critical but also imply that temporal scales of variability must be considered carefully.

Variability of microbial measures

The variability *per se* in space or time is not well understood for many (perhaps most) of the newer indirect measures of microbes. We need to document variation over a range of scales of time and space to determine the natural background noise against which any signal of riverine health must be judged. Naturally noisy systems generally require more measurements, to either increase the overall sample size, or partition variation across the difference scales (minutes to decades, microns to megametres) that otherwise could confound our interpretations. Understanding the form and degree of such variability is crucial to testing assumptions of many statistical analyses. This may even be exacerbated at smallest scales by the minuteness of microbes—because of their small size, potential to operate as 'passive particles' in their physical environments, and fast reaction times, it may be necessary to make more measurements at the smaller scales than say a ecologist concerned with macroinvertebrates, fish, riparian vegetation or even algae. I think that this is only a hypothesis at present but must remain so until we have some hierarchically sampled data to test it (see eg. Downes et al. (1993) for a macroinvertebrate example).

There is also a recent trend towards using measures of variability themselves to assess impacts. Originating from marine monitoring, the basis for this is that human activities often increase the variability of macroorganism species assemblages (Warwick and Clarke 1993).

Considerations of costs versus sample sizes

Some procedures may be too costly to afford enough sampling to capture the relevant variation. This may result in a low realised statistical power to determine environmental impacts (see Fairweather 1991). We need to apply clever approaches to minimise costs while ensuring interpretive flexibility such as double- or over-sampling in the field and archiving most material for later examination only if warranted. This would require means of storing microbial material without degrading its worth for later examination (eg. the macroorganism analogue is sample preservation). Separating variables into sets of surveillance

indicators (sampled often or intensively but cheaply, perhaps as surrogate measures) versus assessment indicators (examined infrequently due to greater expense) can be very useful where agreed triggers exist to increase the frequency of the assessment indicators (what Gray and Jensen (1993) called 'feedback monitoring').

Relative scales of variability of microbial and environmental measurements

The development of most methodologies for assessing riverine health use an overt comparison between organismic (dependent) variables and so-called 'environmental' (independent) variables (usually physico-chemical data related to pollution but sometimes also geomorphic or landscape data). Thus, the logical form of these comparisons is as some correlative test rather than a causal experiment. The statistical forms may be correlations or regressions (for continuous independent variables) versus analyses of variance or logistic regressions (for discrete independent variables), where the dependent data are continuous. Where the dependent variables are themselves discrete, then categorical data analyses like contingency tables are applicable. Basic statistics texts give guidance about most of these, and general statistics software such as SYSTAT, SAS, BMDP or SPSS cover them all. There are also more recent multivariate analogues for most of these tests (see below).

The key issue is that often the instrumental expense of chemical measurements may limit their sample sizes—the physico-chemistry may be measured rather more coarsely than the biological indicators. This makes the application of even a correlative test of underlying causation very difficult. To do this effectively, we must match the scales of data collection for both microbial and environmental data sets. Broader scale or more limited information for one set of variables severely limits the statistical models that can be applied in their analysis, even to the point where the most feasible analysis does not answer the initial question posed. A common form of this is spatial confounding (called 'pseudoreplication' by Hurlbert (1984)) where a lack of appropriate replication at a particular level of a design means that the error term (used as the denominator of many statistics) is inappropriate for the question posed. A way out of this that explicitly acknowledges the potential for meaningful variation at a number of levels is to explicitly estimate (via sampling) the variation both within and between experimental units. Hierarchical designs are appropriate for this (Downes et al. 1993; Underwood 1997).

Reference conditions and RIVPACS-type approaches

The macroinvertebrate component of the current NRHP uses a locally adapted variant (called AUSRIVAS) of the British RIVPACS methodology (Wright 1995) to assess the relative health of that riverine component. I will not go into much detail about that approach because I presume that Peter Davies will outline its philosophy (see also Norris 1995; Schofield and Davies 1996). Essentially this uses structured comparisons with reference conditions—sites that present a reasonable expectation of what biotic assemblages can be like under 'good' conditions. A model using several statistical components predicts what biota are expected in a test site given its location, altitude, stream order, biogeographic region, etc. Actual samples from the test site then are scored as a proportion of those expectations. A microbial analogue could be developed based on modelling the non-responsive aspects of the environment to predict microbial assemblages that are expected for that sort of site in good condition. At present we probably don't have the database to make such predictions but that is perhaps similar to the situation for RIVPACS in Australia in, say, 1993.

Univariate versus multivariate data

Many of the methods under discussion will yield a complex 'signature' (eg. of biomarkers like fatty acids) rather than a list of microbial species from a water or sediment sample. This suggests that often multi-signal variation must be analysed, in turn suggesting multivariate statistical methods. In the past these have been used in ecology for mainly descriptive purposes and the application to field situations of some older methods based on eigenvectors of data matrices (eg. PCA or DCA) are often limited by their own sets of assumptions. Newer methods like MDS were designed (often in the social sciences!) for handling a multiplicity of information types, data or signals, being based on the similarity amongst samples as determined by relative values of sets of variables. Until we understand some of the potential limitations (eg. multi-collinearity or other interrelationships amongst variables) for microbial data sets, then we would be well advised to use those multivariate techniques with the fewest assumptions.

That being said, there are some forms of microbial indicator data that do fit a univariate analysis model. These include the rates of a microbial process, the numbers of signatures (as a count like the number of morphospecies per sample for 'macroorganisms'), or some measure of the total microbial activity.

Descriptive analysis versus hypothesis testing

Again related mainly to multivariate methods, recent statistical and computing developments allow us to go beyond mere descriptions of assemblages and at least attempt to test statistical hypotheses about them. Computer-intensive methods are now readily available in desktop packages like PRIMER, PATN or CANOCO that allow simulations with your own multivariate data sets to implement permutation, Monte Carlo or distribution-free tests (Manly 1991; Good 1995; Clarke 1993) of whether one set of samples differs from another. Thus, multivariate hypothesis-testing is now more achievable as well as being a more realistic depiction of nature.

For example, taking the PRIMER package developed by the Plymouth Marine Laboratory in the UK, there are now randomisation analogues of ANOVA for detecting differences (called ANOSIM or analysis of similarities; see Clarke (1993)), tests for exploring which signatures differ most between samples (SIMPER or similarity percentages), estimates of multivariate variability (MVDISP; see Warwick and Clarke (1993)), and procedures for correlating biotic similarities with subsets of environmental variables (BIOENV; see Clarke (1993)). There are roughly analogous tests in the packages PATN & CANOCO.

Need for comparative trials

Having a valid basis for comparison is particularly important for evaluating a number of new(ish) or competing methodologies. One way to ensure this is to take established methods and compare how well they do in a variety of situations at retrieving (known) information about impacts. This has been done in deciding upon which marine monitoring methods are worthy of developing further (eg. see Bayne et al. (1986) for information on a trial of many methods in a fjord polluted with hydrocarbons). I think that LWRRDC needs to organise such direct comparisons for the NRHP R&D projects that do show promise. Only on that basis can we collectively decide which are the most applicable or useful methods, where we need to propagate their use, the skills needed to implement them, and build up experience with them in different situations. Where a technique is particularly expensive or specialised (eg. only a few instruments available in the country), then it can be difficult for quantitative specialists to get their hands (or computers!) onto such novel data to assess variability and other aspects of these data sets. I suggest then that proponents of the newer techniques be open to the examination of their data sets by statisticians and data analysts.

Advice and commentary

Throughout the workshop I also intend to provide relevant commentary on the sorts of data sets that the different techniques and approaches presented in the preceding papers might arrive at. This will be candid and rather off-the-cuff, but I see it as a good opportunity to raise further issues of particular interest to the proponents of particular methodologies.

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Notes for day 2 of the workshop

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Introduction

A key question for assessment of river health is what do we mean by 'river health'? The two extremes of opinion here are perhaps captured by the following definitions:

1. A healthy river is one which retains the biological attributes of river—it still has a full complement of riparian animals and plants.
2. A healthy river is one which performs the ecological functions we desire.

Both these 'definitions' acknowledge that a river is not just a geological feature (a moving body of water). What we recognise as a river is also a collection of plants and animals associated with defined biological and geological processes which have impacts beyond the immediate environment of the river.

Traditional methods of 'river health' assessment can be polarised at these two extremes by focusing on biodiversity surveys of aquatic invertebrates and vertebrates, or biochemical surveys of BOD, chemical pollutants etc.

Microbial indicators for river health may fall into either of these two broad categories through:

- Assessment of cells (essentially a biodiversity survey);
- Assessment of microbial activity (essentially a biochemical survey); or
- Assessment of genes (or gene products) as indicators of either cells or activity.

The following three papers outline current techniques for the assessment of cells, activity, and genes.

Cell detection methods for river health assessment

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Detection and counting of microbial cells as indicators of river health most closely parallels the use of aquatic invertebrates. It relies on the assumption that there is a population of microbial cells that is diagnostic of our definition of 'river'. Alteration of this population of cells changes the biological component of the river such that it no longer fits our definition—it is 'unhealthy' in the sense that it has become something else by virtue of an essential property being changed.

The advantages of this type of approach to river health assessment are that cells represent easily definable units and populations respond rapidly to disturbance. The disadvantage is that microorganisms are not readily visible: they are not part of the general image of a healthy river and in practice, politically at least, are surrogates for the more visible biological components of a river. In order to use microorganisms as surrogate indicators of river health we must first have some idea of the correlation between the microbiology and macrobiology of a 'healthy' river. Of particular importance is an understanding of the variation in space and time of microbial populations.

The major technological requirements for successful use of microbial cells as indicators of river health are:

(i) ability to process statistically significant samples (rapid counting of multiple samples);
(ii) ability to discriminate different types of cells; and
(iii) ability to determine the metabolic state (health) of each member of the population.

The small size and limited morphological variation of microorganisms has meant that each of these requirements has posed significant technological challenges.

A brief history of microbiological methods

The development of microbiology as a science has always been limited by the sensitivity of analytical techniques. Until recently the only technique capable of recording information from a single cell was micro-scopy. The simple morphology (at least in terms of size and shape) of most microbes severely limited the applications of microscopy. All analytical techniques of potential use in studies of microbial diversity were dependant on some method of signal amplification. For more than a century, the only method available for this was growth of the desired organism in pure culture. The limitations imposed by pure culture include: (i) bias towards dominant or fast-growing members of the community; (ii) most organisms cannot be cultured; and (iii) it is time-consuming. The challenge for microbiologists to overcome has been development of analytical techniques that allow measurement of microbial diversity without the need for growth of the organisms—that is, techniques that work on single cells.

Recent technological advances mean that just two techniques—flow cytometry and fluorescent *in situ* hybridisation—offer a wide range of possibilities for overcoming these problems and fulfilling the requirements for use of microbial cells as indicators of river health.

Flow cytometry and fluorescent *in situ* hybridisation

Why flow cytometry?

A flow cytometer has been described as an automated microscope, in that it is used to automatically determine the optical properties of a cell. There are two main reasons for use of flow cytometry: (1) it allows 'interrogation' of cells one at a time, ie. information is determined from individual cells and not a population; and (2) it is very rapid (typically 100–1000 and up to 30,000 cells/sec), allowing processing of statistically significant numbers of individuals.

Flow cytometry achieves this by coupling photodynamic measurements of cells with electronic data gathering. Individual cells are separated in a fast-

moving 'sheath' or stream of fluid and passed through a light beam. Any property of the cell which interacts with light can be determined (Figure 1). These include:

- size (*viz* shadow);
- surface texture (*viz* the shininess or graininess);
- refractivity (how much light is bent as it passes through the cell), and;
- fluorescence (many cells contain compounds which will fluoresce if irradiated by light

Flow cytometry can thus be used to rapidly analyse all free cells in a water sample. Much useful information with regard to discrimination of different types of cells in the sample is possible based on their optical properties. However, these few parameters are clearly insufficient to resolve all cell types and cannot always distinguish live from dead cells. This poses the greatest restriction to use of flow cytometry in river health assessment—insufficient resolution between cell types and metabolic states.

FISH to the rescue!

Fluorescent *in situ* hybridisation, or FISH, offers the solution to many of the restrictions of flow cytometry. Fluorescence is one of the parameters measurable in flow cytometry. The simplest way to increase the information content obtainable from cells in flow cytometry is by artificially labelling the cells with fluorescent tags which bind to specific subcellular targets. In principle, the only limitation to the versatility of fluorescence as a cell marker is the specificity of the delivery system for getting the fluor into the cell. Thus, cells can be discriminated on the basis of: (a) nucleic acid sequence—fluors attached to oligonucleotide probes; (b) antigens—fluors attached to antibodies; (c) viability—fluors which are only taken up by metabolically active cells; (d) cell cycle state—fluors which specifically bind to nucleic acids; and (e) biochemistry—substrates which release a fluor when acted on by a specific enzyme inside the cell.

By combining the sample-processing power of flow cytometry with fluorescent labelling techniques microorganisms can be readily analysed for viability and diversity, and useful information on their biochemistry can also be obtained. The advantages and disadvantages of various cell-labelling methods in potential river health applications are discussed below.

Determination of viability

Live cells have an electric potential across the cytoplasmic membrane which is absent from non-viable cells. This trans-membrane potential preferentially concentrates positively charged dyes, such as rhodamine 123 within the cell. In contrast, negatively charged dyes are excluded. Recently developed dyes, such as SYTOX, are likely to become widely used in flow cytometry as they are excitable at wavelengths commonly available on flow cytometers. All current 'viability' stains have limited use in environmental samples because they tend to bind non-specifically to particulate matter.

Cell diversity surveys

Oligonucleotide probes targeting ribosomal RNAs are now widely used in microbial ecology. This subject has been reviewed in detail recently. The chief advantages of using fluorescently labelled oligo

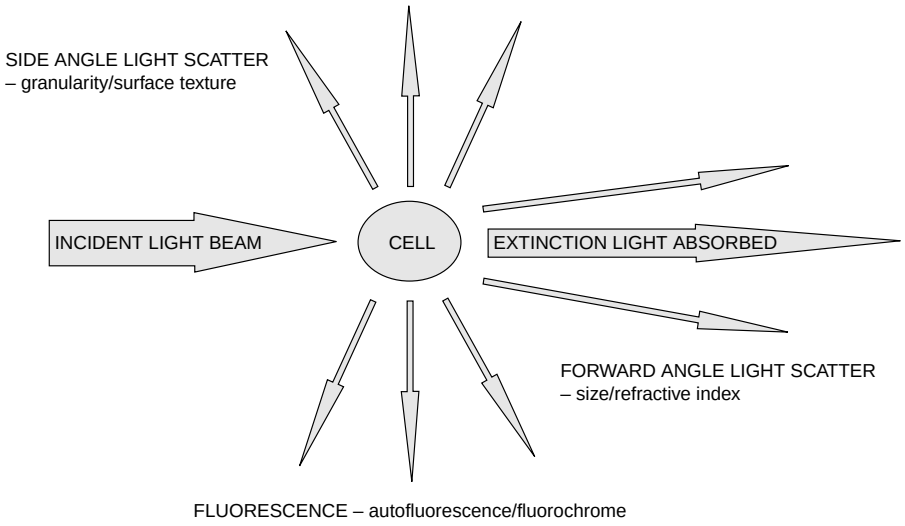

Figure 1. Interactions of light with a cell.

probes are: (i) they can target different 'levels' of phylogenetic relationship with high specificity, and (ii) they are easy to design and synthesise. Ribosomal-targeted oligo probes may also give information on cell viability, as there is a reasonably strong correlation between metabolic activity and ribosome number. The main disadvantage of such probes is that the sensitivity of the probes is not always adequate in natural samples. Improvements in the sensitivity of detection of fluorescence would make this the most generally applicable method for specific labelling of microbial cells.

Antibody probes can overcome the problem of sensitivity and achieve equivalent specificity. However, the flexibility in design of antibody probes is limited and initial manufacture of the antibodies is more time-consuming. In general antibody probes may only be designed against organisms which have previously been obtained in pure culture. However, the use of monoclonal antibodies is the present method of choice for monitoring a specific organism with flow cytometry.

Extending cell assessment to biochemistry/activity determinations

Many esterified fluorochromes remain non-fluorescent until cleaved by intracellular enzymes, whereupon a fluorescent product is released. Cells only become fluorescent after substrate cleavage by functional cytoplasmic enzymes and product retention by intact membranes. Thus, two cellular functions, esterase activity and membrane function, are tested.

The first studies using fluorogenic substrates involved the esterase substrate fluorescein di-acetate (FDA). Non-fluorescent FDA is cleaved by esterases in viable cells releasing fluorescein which stains the cells green. FDA is not an ideal substrate because fluorescein is only poorly retained by microorganisms. Derivatives are now available that have improved retention resulting from additional functional groups. For example, carboxy fluorescein di-acetate (CFDA) uses a carboxy group to reduce membrane permeability whilst chloromethyl fluorescein di-acetate (CMFD) includes a chloromethyl moiety that covalently links the esterase substrate to intracellular molecules.

The electron transport chain of respiring (viable) cells is capable of reducing the membrane permeant, nonfluorescent tetrazolium derivative 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) to a red fluorescent precipitate. If a cell is sufficiently active, enough precipitate will form for its discrimination from the background.

Many culture-based methods use specific biochemical assays to identify cell types possessing particular enzymes. For example, the enzyme β-galactosidase is found in faecal coliforms where it is involved in fermentation of the mammalian sugar lactose. Flow cytometric assays for β-galactosidase have been described that are applicable to bacterial and yeast cells with active β-galactosidases, either naturally present or 'transfected'. Currently, we are optimising the protocols to improve their discrimination as a possible means of detecting and confirming coliforms flow cytometrically.

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Assessment of microbial activity for river health

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Activity based measurements

Microbes can be described as small and relatively featureless organisms, thus, unlike other biologists, the microbiologist cannot rely on morphology as a defining characteristic. Furthermore, microbes in natural environments do not usually exist as free-living cells; rather they form complex assemblages or communities in close association with organic and inorganic particulate matter in the sediment and the water column. Thus, the detection and enumeration of microbes from these ecosystems can be problematic. To overcome these problems, microbial ecologists use a variety of biochemical and molecular techniques to characterise the structure and function of microbial communities. In this section, we will demonstrate some of the biochemical approaches for use of microbes as indicators of river health.

Fluorescein diacetate (FDA) hydrolysis

In assessments of river health, microbial biomass, microbial numbers and microbial activity may be important to the investigator. A number of methods have been developed to assess microbial activity including measuring CO_2-evolution, measuring ATP levels and measuring specific enzyme activities.

One of the simplest ways to assess microbial activity in environmental samples is to measure the rate of FDA-hydrolysis. FDA-hydrolysis is a simple assay for non-specific esterase activity. The assay depends on adding FDA, a colourless non-polar ester of acetate, to the sample. Non-specific microbial esterases then hydrolyse the FDA releasing fluorescein (Figure 1) which may be detected either colorimetrically or fluorometrically. Esterases have been found to be growth-linked in microorganisms, thus the amount of fluorescein released should be proportional to the amount of active microbial biomass. FDA-hydrolysis has been found to provide a crude, but often useful, indication of microbial activity in a variety of environments including, water, composts and soils.

Defined substrate technology (DST)

Defined enzyme substrates for detection of specific microorganisms

A number of products, based on ‘defined substrate technologies’ (DST), have recently become available for the detection of specific microorganisms. Unlike most microbial analyses, DST based methods are generally cheap, simple and can be carried out by non-specialists. These technologies could be used by community-based groups, such as Stream Watch.

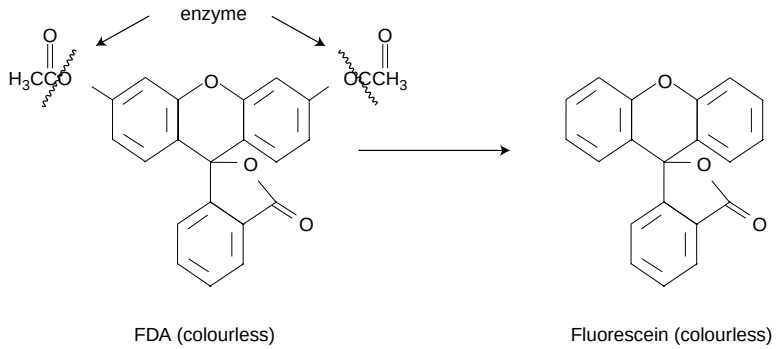

Figure 1. Hydrolysis of FDA (colourless) by non-specific microbial esterases to produce fluorescein (coloured)

With DST, detection, differentiation and identification of microorganisms is based on the presence or absence of enzymes. The defined substrates on which these enzymes act are both the indicator and the major carbon and energy sources within the medium. Chromogenic and fluorogenic substrates are available for a number of different enzyme classes enabling specific enzymes in these classes to be differentiated. These enzymes include aminopeptidases, esterases, glucosidases and deaminases.

For environmental applications, kits are available for detection of bacteria that are indicative of recent faecal contamination, eg. enterococci, coliforms and, specifically, *Escherichia coli.* However, it is feasible to develop similar kits for a wide range of other microorganisms of environmental significance.

In the session, 'Colilert' will be demonstrated. Similar products are produced by Millipore, Merck and Difco. Colilert is used for the simultaneous detection and confirmation of total coliforms and *E. coli*. It is based upon the use of indicator nutrients, salts, nitrogen and carbon sources that are specific to coliforms. Non-coliforms are suppressed and cannot metabolise the nutrients.

The nutrients are ONPG (ortho-nitrophenyl-β-D-galactopyranoside), which is metabolised by coliforms and produces a coloured end-product, and MUG (methylumberlliferyl-β-D-glucuronide) that is specifically metabolised by *E. coli* and is cleaved to give a fluorescent end-product. This test gives a result in l8–24 hours. Often a quantitative result rather than simple presence/absence is required and a simple disposable tray system, based on most probable number determinations, will be demonstrated.

Metabolic fingerprinting of microbial communities

Phenotypic methods have traditionally been used as a relatively simple and low cost method of categorising diverse collections of microorganisms.

Differentiation of individual microorganisms can be achieved by comparing the reactions of a large number of biochemical tests. This phenotypic approach can be time-consuming and tedious but the task has been simplified by the availability of commercial identification kits. Pure cultures of each strain are inoculated into the test panel and, after incubation, the reactions are compared with a database of known organisms for identification.

It would be impossible to identify each member of a microbial community in this way, as most microbes from natural environments cannot be isolated in pure culture. Rather than using these kits for identification of individual microbes, ecologists have used the BIOLOG™ system to study microbial communities. The tests are inoculated with environmental samples such as water and soil extracts rather than pure cultures to generate 'metabolic fingerprints' of the microbial community. The BIOLOG™ system consists of a 96-well microtitre plate with 95 different carbon sources and one control well (no carbon). Each well also contains mineral nutrients and the redox dye tetrazolium violet. The utilisation of the carbon source is indicated by the reduction of the dye, which turns from colourless to purple. The pattern of carbon source utilisation can be read in a spectrophotometric plate reader to generate the metabolic fingerprint.

The use of metabolic fingerprinting or substrate utilisation profiles has been used to study the impact of different ecological management practices and pollutants on both soil and aquatic microbial communities. The analysis of carbon source utilisation patterns in these studies is simplified by the use of multivariate statistics to condense the large data set which is generated from each sample. Principal component analysis (PCA) and canonical component analysis (CCA) are two statistical approaches that have been used to interpret this type of data.

The prospect exists to design BIOLOG™ plates specifically for environmental applications. Rather than using existing 95 carbon sources, plates can be designed using a reduced number of environmentally significant carbon sources that discriminate between different samples. Such plates may assess the ability of the microbial community to resist heavy metal concentrations or to degrade pollutants (such as organochlorines or aromatics).

Genes as a measure of microbial activity in rivers

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The development of new, microbially based, indicators of river health offers potential advantages to those monitoring or making decisions about management of river systems. These advantages arise because of the characteristics of microorganisms: they are central to many ecological processes, they occur in large numbers, and they have the ability to respond quickly to environmental perturbations. Hence, changes to riverine environments should cause direct and rapid effects on river microbiota.

Microbiologists agree that culture-based methods give a biased view of microbial ecology, and so in recent times there has been a move towards analysis of microbial DNA extracted directly from environmental samples. Can microbial ecologists agree on what genes might act as a indicators of river health? Can DNA studies be used to develop criteria to quantify river health? Are such studies practical in terms of efficiency, reliability and cost? What sort of sampling protocols need to be established? The experiments to answer these basic questions are only now being done in laboratories around the world.

Assessments of river health might be based on two different sorts of measures or questions:

1. Does the river still have its full complement of organisms?
2. Does the river still perform its full complement of ecological functions?

These assessments are not exclusive, but reflect two extremes of the different approaches to using DNA as a measure of river health.

In the first approach, phylogenetically-based DNA tests are applied to answer questions of how many organisms in particular taxa are present in an ecosystem. This approach is similar to counting numbers of macroinvertebrate morphospecies in rivers. The problems with such an approach lie in our lack of knowledge about the intrinsic variability of microbial species composition. There are few or no data available about the turnover of microbial species in space or in time. Such information will have to be obtained before meaningful measurements can be made on perturbations of microbial communities caused by environmental degradation.

The taxa of most use to monitoring river health also need to be identified. A number of taxa with direct effects on human health (coliforms, protozoans, viruses) have been monitored in the past, and will continue to be monitored in the future, but these do not allow assessment of river health in general. DNA-based tests can be designed to target particular groups whose presence is desirable or, more commonly, whose population dynamics might reflect deterioration of river quality (nitrifiers, cyanobacterial blooms). Ideally, organisms which are directly affected by identifiable pollutants or physical changes to the riverine environment are the best candidates for indicators.

In a second general type of approach to monitoring river health, specific ecosystem functions might be examined by analysis of genes involved in that function. For instance, the presence of a particular pollutant selects for microbial species able to resist or actively degrade that pollutant. There is now considerable information available on microbial genes involved in the degradation of xenobiotic compounds. One might think that DNA-based assays for these genes could reasonably be used as a direct indicator of environmental contamination. However, organisms capable of degrading man-made pollutants can also be present in pristine areas. A more relevant measure might be the number of such organisms, and the activity of the genes involved. To investigate gene activity, it may be necessary to examine microbial RNA rather than DNA, because the presence of RNA shows that a gene is being transcribed. For each candidate gene, considerable trials with known samples must be undertaken.

Despite the caution expressed in the previous paragraphs, it is clear that DNA-based methods will allow us to better understand the composition of

microbial communities, providing a framework for determining the contribution of individual community members to ecosystem function. With this knowledge, we should be in a better position to identify appropriate microbial indicators of river health. Once such indicators have been identified the development of DNA tests is a technical formality.

It is likely that the mainstay of DNA-based tests will be the polymerase chain reaction (PCR). This method has revolutionised biology and microbiology by providing a simple and effective means of DNA analysis. The PCR allows multiple rounds of DNA replication that result in the accumulation of a large amount of a specific DNA fragment, most often from a particular gene of interest. PCR can be used to monitor microbial populations because:

- generation of a PCR product is evidence for the presence of the targeted organism or gene;
- the amount of DNA produced can be related to the number of target genes in the original sample;
- PCR allows examination of microorganisms that cannot currently be cultured in the laboratory;
- PCR has the potential to be automated for analysis of large sample numbers.

In the laboratory session we will demonstrate the equipment and processes involved at each step of DNA based analyses of environmental samples. These steps involve:

- Extraction of DNA
- Amplification of specific genes using PCR
- Gel electrophoresis of the PCR products

Specific examples using PCR will be based on the assessment of spatial diversity of fungal species in pristine and impacted river systems, and on the differentiation of strains of *E. coli*.

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