Microbial Indicators of River Health

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Both the National Water Quality Management Strategy and the National River Health Program will focus on the use of *biological indicators* to assess the present ecological condition of the nation's rivers, and whether their management is leading to an improvement. A large number of biological indicators have been suggested over the years, including macroinvertebrates, fish, algae, macrophytes and microorganisms. Initially, the National River Health Program will be based largely on macroinvertebrates, although there is scope in the future to include fish and perhaps even algae.

The use of microorganisms as a new and potentially very sensitive biological indicator of river 'health' has also been advocated. Very large numbers of bacteria are present in every aquatic system, up to billions of individuals per litre and thousands of species per litre, and they respond rapidly to changed conditions, becoming active and starting to grow within periods of minutes to hours. They have a pivotal role in the transformation (spiralling, cycling) of carbon, all mineral nutrients and trace gases in all aquatic systems, including rivers and streams, and for this reason are extremely important in the 'bottom up' control of riverine ecosystems.

This review of the potential for microbial indicators to be used to assess river 'health' was commissioned by the Land and Water Resources Research and Development Corporation (LWRRDC). The objectives of the review are:

- to critically review current methodologies and approaches for utilising microbial indicators in the assessment of river and stream ecosystem 'health'
- to review studies and approaches that link microbial responses with other biotic responses used to assess river 'health'
- to recommend and rank in order of priority R&D requirements to improve the methodology of utilising microbial indicators in the assessment of river 'health'.

Role of microorganisms in rivers

Despite the fact that a wide range of microorganisms — bacteria, cyanobacteria, algae, protozoans and fungi — live in rivers, there is a major lack of knowledge about their role in these ecosystems. Because of this lack of detailed knowledge, the second chapter of the review provides some general information on microorganisms with a focus on bacteria. In particular, we review the types of bacteria living in rivers, the factors controlling their growth and activity, and their importance in food webs and in the cycling (spiralling) of carbon, nitrogen, phosphorus and sulphur. It has become increasingly clear over the past ten years or so that in addition to their traditional role as decomposers and re-mineralisers of organic matter, bacteria make up a significant component of all aquatic food webs. It has been estimated that bacterial production in planktonic systems may be around 20-30% of the primary production, with over 50% of the carbon fixed by the phytoplankton passing through the bacteria. In these systems, bacterial production makes up the major proportion of the total secondary production. There is less information on rivers, but one recent study of a slow-moving lowland river has shown that bacterial production was four times greater than the phytoplankton production, suggesting that other allochthonous sources of carbon were important in this river.

Current methods in aquatic microbiology

Aquatic microbial ecology is advancing extremely rapidly because of the development of a range of exciting new methods, many based on molecular biology techniques, and the application of new instrumental techniques, such as flow cytometry and field flow fractionation. The traditional microbial methods that have been used for many years to protect human populations against waterborne diseases are totally inadequate for studies of most natural microbial populations, since so few of the microorganisms involved in ecosystem processes have been (or are likely to be) identified. In the third chapter of the review we focus on recent developments in three areas: the identification and enumeration of microbes, the measurement of microbial biomass, and the measurement of microbial activity. We identify an extremely active field, with new techniques being developed regularly, and considerable scope for many of these to be applied to the study of microbial processes driving river ecosystems. It is clear that the most useful results will be forthcoming when several microbial indicators are used together.

However, despite the current activity, there is still much to be done. The review highlights four areas of concern: (i) considerably more work is needed to make the present microbial techniques more rapid and simpler to use; (ii) there needs to be much wider validation and testing of the methods in the field; (iii) there is an urgent need to increase the awareness of staff in environmental and water agencies to the new methodologies that are now available to assess microbial activities in river ecosystems; and (iv) there is a need to increase both the number and technical skill base of those working (in research institutions, environmental agencies and water agencies) in the aquatic microbial field over the next three to five years.

Use of microbial indicators

Microorganisms, have a number of potential advantages over other organisms as biological indicators of river 'health'. They are present in very large numbers, grow very rapidly and probably respond to ecosystem changes more rapidly than other biological components; certainly more rapidly than macroinvertebrates or fish. Chapter 4 reviews three possible uses of bacteria as biological indicators of pollution. First, we cover the use of microorganisms as indicators of pollution. Organisms such as *E. coli*, *Giardia* and *Cryptosporidium*, can be used as direct indicators of sewage or livestock pollution. Similarly, other groups of organisms (or enzymes or proteins associated with them) have been used to indicate pollution or stress by organic chemicals, heavy metals and heat.

The second use is as indicators of biodiversity. Here the principle is that a loss of biological diversity is an important indicator of failing ecosystem 'health'. It is somewhat surprising that microorganisms have not been used more for this purpose, given that they are the most diverse of the biological groups. Molecular techniques (eg. DNA-DNA hybridisation) are increasingly being used to measure changes in bacterial biodiversity caused by pollution.

The third use is as indicators of ecosystem function, where the aim is to measure the activity of key microbial processes that contribute to the functioning of that ecosystem. Methods in use include thymidine and leucine uptake for bacterial growth, respiration and enzyme activity for bacterial activity, and at the broader scale, measures of photosynthesis, heterotrophic activity, respiration, P/R ratio, enzyme activity, and specific microbial processes (eg. nitrogen fixation, nitrification, denitrification, sulphate reduction, methanogenesis and methane oxidation). The next few years will see a marked increase in the use of molecular biology techniques in studies of ecosystem processes.

However, before it is possible to use biodiversity or microbial processes as overall indicators of ecosystem 'health', there will need to be a considerably improved understanding of the variations in these key microbial processes in unimpacted ecosystems.

Links between microbial indicators and other biotic responses

There are very few published studies linking changes in the microbial community with other biotic responses. This is not surprising given the relatively recent ability to measure with any degree of ease and accuracy microbes and their activities in natural environments. Most microbial ecology research to date has concentrated on providing fundamental aquatic microbial ecological information. However, despite the lack of specific studies, it has been possible to predict that strong links will exist between microbial communities and macroinvertebrate species composition, algal biomass, and community metabolism. The opportunity exists in the near future to include measurement of microbial indicators in the new National Bioassessment of Rivers Program. There is still much to be done in (i) better defining the most appropriate microbial indicators to measure, (ii) working up more reliable protocols for the appropriate microbial methods, and (iii) training more scientists skilled in these microbial techniques.

Recommendations

Finally, Chapter 6 seeks to identify the major knowledge gaps that could limit the introduction of microbial indicators of river 'health'. The focus is specifically on knowledge gaps directly relevant to riverine microbial indicators, although many of these knowledge gaps are common to the application of microbial indicators to other aquatic and terrestrial ecosystems. Despite a small increase in activity in aquatic microbial ecology in Australia over recent years, there is still a major dearth of fundamental knowledge on the microbial assemblages and the processes they control in Australian aquatic ecosystems. This lack of fundamental information must be addressed urgently before it will be possible to use microorganisms, particularly bacteria, as indicators of river 'health'.

The recommendations are listed below:

Recommendation 1:

- that LWRRDC develop a strategy, in consultation with other research agencies (eg. Australian Research Council), aimed at increasing both the number and skill base of those working in the aquatic microbial field over the next three to five years. This strategy should (i) address the training needs at the postgraduate level through provision of post-graduate scholarships directed towards the development, assessment and application of new technologies for aquatic microbial ecology, and (ii) upgrade the knowledge base and technical skills within environmental agencies and water utilities by running workshops and short extension courses. Additional funding will be needed to address the fundamental aquatic microbial questions required to provide the research base needed to support (i) and (ii) above.

Recommendation 2:

- that LWRRDC fund a research program aimed at providing fundamental information about the microbial ecology of Australian aquatic ecosystems, with a focus on lowland river systems and on the eventual desire to produce microbial indicators for assessing the environmental 'health' of the aquatic ecosystems.

Recommendation 3:

- that LWRRDC increase its funding of research aimed at the development and field application of new microbial techniques (including those based on particular enzymes and stress-response proteins, rRNA probes and other molecular techniques) for the study of aquatic microbial ecology in Australia.

Recommendation 4:

- that LWRRDC establish a process to have conventional microbial methods become more accessible to water agencies in Australia. This will involve at least two components: first, the development of standard protocols; and second, the running of a series of training workshops and short courses throughout Australia.

Recommendation 5:

- that LWRRDC cooperate with instrument development groups with the aim of adapting existing and new instrumentation specifically for rapid river 'health' assessment.

Recommendation 6:

- that LWRRDC seek to establish a microbial component in the National River Health Monitoring Program. This will need careful planning which could be done by a small group, perhaps drawn from a range of institutions, to maximise expertise and diversity of opinion. The objective should be to have a microbial component introduced in approximately 18 months.

1. INTRODUCTION

Many Australian rivers are in poor condition, a fact given national focus by the massive algal bloom on the Darling River in 1991 which lasted three months and extended for over 1,000 km (DWR-NSW, 1992). However, algal blooms are only one of a large number of problems confronting Australia's rivers and streams that were highlighted in a recent Environment Protection Agency discussion document entitled '*Towards Healthy Rivers: The Ills Afflicting Our Rivers and How We Might Remedy Them*' (CEPA, 1992; CSIRO, 1992). The problems highlighted included river regulation; the clearing of catchment vegetation leading to increased erosion, greater turbidity and increased salinity; removal of essential riparian vegetation; 'river improvements' leading to the clearing of 'snag' habitats and the straightening of channels; introduction of alien biota; and pollution from organic matter, biocides, heavy metals and nutrients.

Over the years there have been many calls for a national approach to the management of water resources in Australia (eg. Pigram, 1986; Hart, 1993), but these have become more strident in recent times, spurred on by such major national events as the massive algal bloom on the Darling River in 1991 (DWR-NSW, 1992). Recently, two major initiatives have contributed towards the development of such a national approach to water quality management in Australia. The first was the establishment of a National Water Quality Management Strategy, a cooperative venture by the State and Federal Governments (ANZECC/AWRC, 1992), and the second was the Monitoring River Health Initiative announced as part of the Prime Minister's environment statement in December 1992. This latter has lead to the establishment of a joint National River Health Program[1], which has as its key activity the setting up of a national monitoring program to assess river 'health'.

Both the National Water Quality Management Strategy and the National River Health Program will focus on the use of *biological indicators* to assess the present ecological condition of the nation's rivers, and whether their management is leading to an improvement. A large number of biological indicators has been suggested over the years (Hellawell, 1986; Norris *et al.*, 1995), including macroinvertebrates, fish, algae, macrophytes and microorganisms. Initially, the National River Health Program will be based largely on macroinvertebrates, although there is scope in the future to include fish and perhaps even algae.

The use of microorganisms[2] as a new and potentially very sensitive biological indicator of river 'health' has also been advocated. Very large numbers of bacteria are present in every aquatic system, up to billions of individuals per litre and thousands of species per litre, and they respond rapidly to changed conditions, becoming active and starting to grow within periods of minutes to hours. They have a pivotal role in the transformation (spiralling, cycling) of carbon, mineral nutrients and trace gases in all aquatic systems, including rivers and streams, and for this reason are extremely important in the 'bottom up' control of riverine ecosystems.

However, despite the obvious importance of the microbial community in rivers and streams, there is still relatively little known about their ecology. In a recent review, Hobbie & Ford (1993) suggested that there is still a great need for information on such basic questions as: what types of microbes live in particular aquatic habitats, how many are there, what are they doing, how do they survive, what factors control their activity and numbers, and what role do they play in the cycling of carbon and nutrients?

The relative lack of a detailed understanding of aquatic microbial ecology has been primarily due to the great difficulties in measuring microbial numbers and their activity in nature. Many of the techniques that have been so successful in laboratory studies of microbial physiology or microbial-caused diseases are totally unsuitable for microbial ecology studies. Traditional plating and culture techniques measure considerably less than 1% of the natural populations in natural waters.

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1 The National River Processes and Management Program, is jointly run by the Land & Water Resources Research & Development Corporation (LWRRDC) and two components of DEST, the Commonwealth Environment Protection Agency (CEPA) and the Environmental Strategies Directorate. The program budget is $10 million over a period of five years, obtained by pooling the resources allocated under the Monitoring River Health Initiative with those of LWRRDC (Hart & Campbell, 1994).

2 Microorganisms consist of a large and diverse group of tiny organisms, including bacteria, cyanobacteria, algae, fungi, protozoa and viruses. However, this report focuses mostly upon bacteria.

Modern aquatic microbial ecology probably commenced in the mid-1970s with the introduction of epifluorescence microscopy to enumerate entire native bacterial assemblages (Hobbie *et al.*, 1977; Zimmermann & Meyer-Reil, 1974), which was followed soon after by the introduction of tritiated thymidine to measure bacterial division rates (Fuhrman & Azam, 1982). The past five years or so has seen the introduction of a further range of new microbial techniques (eg. molecular probes and relevant instrumentation such as flow cytometry), which has resulted in considerably more information on specific subsets of bacterial assemblages becoming available. However, despite modern aquatic microbial ecology being 20 years old, there has been very little work done on Australian freshwater systems until very recently (Boon, 1989a,b, 1992, 1993, 1994; Boon & Sorrell, 1991; Sorrell & Boon, 1992; Scholz & Boon, 1993a,b,c).

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- to recommend and rank in order of priority R&D requirements to improve the methodology of utilising microbial indicators in the assessment of river 'health'.

Because of the generally poor knowledge of the roles of microorganisms in rivers (in fact in any aquatic system), the second chapter of the review covers the types of microorganisms found in rivers, the main factors controlling their growth and activity, and the role of microorganisms in the spiralling (cycling) of carbon, phosphorus, nitrogen and sulphur in rivers. The third chapter covers current methods used in aquatic environmental microbiology, with considerable stress on newer methods based on immunological techniques and molecular probes, and on the application of instrumental techniques such as flow cytometry and field flow fractionation. A review of the uses of microbes as biological indicators is provided in chapter four under three sections — use as indicators of pollution, use as indicators of biodiversity and use as indicators of river ecosystem function. Chapter 5 contains a summary of the rather small amount of information linking microbial indicators with other biotic responses. Finally, Chapter 6 contains an assessment of the major knowledge gaps and a recommended research and development program to address these knowledge gaps.

2. ROLE OF MICROORGANISMS IN RIVERS

Despite the fact that a wide range of microorganisms — bacteria, cyanobacteria, algae, protazoans and fungi — live in rivers, there is a major lack of knowledge about their role in these ecosystems. Because of this lack of detailed knowledge, we provide in this chapter some general information on microorganisms, with a focus on bacteria. In particular, we review the types of bacteria living in rivers, the factors controlling their growth and activity, and their importance in food webs and in the cycling (spiralling) of carbon, nitrogen, phosphorus and sulphur. In addition to their traditional role as decomposers and remineralisers of organic matter, it has become increasingly clear over the past ten years or so that bacterial production is a significant component of all food webs. It has been estimated that bacterial production in planktonic systems may be around 20-30% of the primary production, with over 50% of the carbon fixed by the phytoplankton passing through the bacteria. Bacterial production often makes up the major proportion of the total secondary production. There is less information on rivers, but one recent study of a slow-moving lowland river has shown that bacterial production was four times greater than the phytoplankton production, suggesting that other allochthonous sources of carbon were important in this river.

2.1 GENERAL

Microorganisms represent an extremely large group of diverse organisms. The term microorganism is not of any phylogenetic significance but is simply a term of convenience, referring to those organisms too small to see without the aid of a microscope (typically <0.1 mm). Microorganisms include cyanobacteria (blue-green algae), bacteria, fungi, algae, protozoa and viruses. Three of these groups — the algae, fungi and protozoa — have the *eukaryotic* cell type (as do plants and animals), whereas the bacteria and cyanobacteria have the more simply organised *prokaryotic* cell type.

More recent evidence based on molecular-level genetic analysis and evolutionary considerations, has shown that the prokaryotes comprise two kingdoms, the *Archaebacteria* (Archaea) and the *Eubacteria* (Bacteria) (Woese, 1987; Woese *et al.*, 1990). The Archaea and Bacteria are no more related to each other than either is to the Eukaryotes. Thus, the traditional prokaryotic/eukaryotic classification of microorganisms is inappropriate when studying evolutionary/ecological interactions, and needs to be replaced with a three kingdom classification, Archaea, Bacteria and Eukarya (Eukaryotes); the first two of these kingdoms are exclusively microbial and the third (Eukarya) contains a number of microbial members (Woese *et al.*, 1990; Brock & Madigan, 1991).

The final group of microorganisms, the *viruses*, are acellular and are not capable of an independent existence. In fact, some consider viruses to be non-living. Viruses must infect other organisms in order to reproduce and virtually all life-forms are susceptible to viral infection. Viruses that infect bacteria and cyanobacteria are referred to as *bacteriophage* or simply phage.

Microorganisms are ubiquitous and every litre of river water will contain billions of individuals from thousands of species. Despite their small size, microorganisms are thought to constitute five to twenty-five times the total mass of animal life, both aquatic and terrestrial. Microorganisms have an amazing ability to increase in number. Given ideal conditions many bacteria will double in number every 20 to 60 minutes; some bacteria can even double their population every 10 minutes! This may not seem so staggering until it is considered that within 48 hours a single bacterium with a typical doubling time of 25 minutes will increase in biomass to an amount roughly equivalent to 4,000 times the mass of the earth. In natural environments, factors such as the availability of nutrients prevents such unlimited microbial growth. Even with such environmental resistance, typical doubling times for microorganisms in aquatic ecosystems are still relatively short, being measured in hours or days.

In all aquatic environments, the microorganisms including cyanobacteria, bacteria, algae, protozoa and fungi, make up a large proportion of the total biomass (Brock and Madigan, 1991). The microorganisms in water bodies can be both autochthonous (produced within the waterbody) and allochthonous (derived from the surrounding terrestrial environment). Allochthonous microorganisms may enter a water body from soil, animals, plant debris or sewage. However, such invading bacteria usually die off relatively quickly. The microbiota of river water normally shows a particularly close relationship with the surrounding terrestrial microbiota due to the constant injection of soil, water run-off and organic material, including sewage (Rheinheimer, 1991).

Bacteria may thus be useful biological indicators of river 'health' *per se*, and also of the interactions between the riverine environment and the surrounding terrestrial environment. For example, faecal coliform bacteria (bacteria found in the intestine) are useful indicators of sewage impact. Other terrestrial bacteria found in rivers could be used to assess soil erosion, river flows and transport processes. Generally, large lakes have received more scientific attention than rivers because they tend to be more self-contained and a larger proportion of the microbiota is autochthonous.

2.2 TYPES OF MICROORGANISMS

Ecologists normally consider an ecosystem in terms of the primary producers, the autotrophs, and the various trophic levels of consumer, the heterotrophs. *Autotrophs* gain energy from sunlight and carbon from CO_2-fixation. *Heterotrophs* gain both their carbon and energy from organic compounds that are ultimately derived from the autotrophs. Terms such as heterotroph and autotroph, that encompass considerations of both energy and carbon nutrition, are over-simplifications for microbiology. In the microbial world various permutations of sources of carbon and energy are possible. For example, microorganisms known as photoheterotrophs can gain energy from sunlight while simultaneously obtaining carbon from organic sources. Although we have divided microorganisms into heterotrophs and autotrophs here for convenience, it should be remembered that such terms can be misleading.

While much has been made of the role of bacteria as decomposers of organic matter (ie. heterotrophs), it is important to note that many bacteria (eg. nitrifiers, methanotrophs, methanogens, sulphur and iron bacteria, and cyanobacteria) are autotrophs which actually produce biological material and can be an extremely important component of the secondary production in aquatic food webs (see Section 2.4.1). The various types of heterotrophic and autotrophic bacteria are coved in the following section.

2.2.1 Heterotrophs

Bacteria

Bacteria all have the prokaryotic cell type. Heterotrophic bacteria are found within both kingdoms of the bacteria (Archaea and Eubacteria). The numbers and species composition of heterotrophic bacteria are largely controlled by the concentration of available organic material. In nutrient poor springs and groundwater, the numbers of bacteria are low, usually not more than a few thousand per mL. The predominant bacteria will be Gram-negative rods and prosthecate (stalked) bacteria (eg. *Hyphomicrobium*, *Caulobacteria*); *Gallionella* and *Pseudomonas* spp also often occur.

Along the length of the river, microbial biomass and diversity generally increase in response to the increase in available organic matter (Rheinheimer, 1991). Additionally, a shift in the microbial community occurs from oligotrophic bacteria in the headwater sections to more cosmopolitan bacteria from the families Pseudomonadaceae, Bacillaceae and Enterobacteriaceae. Further down the stream genera such as *Azotobacter*, *Vibrio*, *Spirillum*, *Thiobacillus*, *Micrococcus*, *Sarcina*, *Norcardia*, *Streptomyces* and *Cytophaga*, as well as spirochaetes and nitrifying bacteria, may appear (Rheinheimer, 1991). Generally, rivers have fluctuating conditions due to inflowing tributaries and sewage outflows and this is reflected in the microbial community. However, as will be covered later, these changes in the bacterial community with fluctuating environmental conditions have been poorly studied. Allochthonous bacteria, such as *Escherichia coli* and enterococci, have been used for many years as indicators of faecal contamination of water courses.

Fungi

All fungi are eukaryotic and heterotrophic. They are important decomposers in aquatic environments, being noted for the diverse range of extra-cellular enzymes they secrete enabling them to utilise a wide range of organic substrates for growth. Fungi are able to break down substances such as proteins, sugars, starch, fats, pectin, hemicellulose, cellulose, lignin and chitin. They probably play a more significant role than bacteria in the breakdown of the more recalcitrant materials such as lignin.

Representatives of all four classes of fungi are found in aquatic environments, either free living or more often growing on surfaces. The distribution and numbers of fungi are dependent upon the presence of plant and animal cells which they parasitise or consume. They are therefore rare in groundwater and springs because of low nutrient concentrations. As with bacteria, the factors governing the temporal and spatial distribution of fungi within riverine environments has been poorly studied, both overseas and in Australia (Thomas *et al.*, 1992).

Protozoa

Protozoa are eukaryotic microorganisms that feed on particulate or macromolecular materials by direct ingestion, phagocytosis (engulfing of particulate matter) or pinocytosis (taking up of fine droplets). Autochthonous protozoa are important bacterial predators in aquatic ecosystems, and probably play an important role in controlling bacterial and cyanobacterial populations in rivers. In soils, protozoa have been suggested as sensitive bioindicators of soil 'health'. Allochthonous protozoa, such as *Cryptosporidium*, *Giardia* and *Entomoeba histolytica*, introduced with faecal matter, are important waterborne human pathogens. These protozoa are able to produce specialised structures (cysts and oocysts) that enable them to survive in aquatic environments for long periods of time (months).

2.2.2 Autotrophs

Phototrophs

A range of photosynthetic microorganisms are found in riverine environments, including true algae, cyanobacteria (also commonly known as 'blue-green algae') and photosynthetic bacteria. The true algae are eukaryotes whereas the cyanobacteria and photosynthetic bacteria are prokaryotes. The cyanobacteria and true algae both carry out oxygenic photosynthesis (evolve O_2) and are often considered together, even though they are only distantly related phylogenetically. Other photosynthetic bacteria do not evolve O_2 and are referred to as anoxygenic phototrophs.

The extent of photosynthesis occurring in a particular waterbody is dependent on both the phototrophic population and the environmental conditions, such as nutrient availability, light and spectral distribution, and temperature. In shallow waterbodies much of the primary production may be carried out by spermatophytes and benthic algae, but in larger waterbodies algal phytoplankton are the main primary producers. In fresh waters, the most important phyla are the cyanobacteria, green algae (Chlorophyta) and the yellow brown algae (Crysophyta), including the diatoms.

The distribution of micro-algae is largely determined by environmental factors, such as light, temperature and nutrient supply. In fast-flowing lotic habitats, the micro-algae are usually epilithic (growing on rocks, stones or logs) or benthic (living in the bottom sediments). In calmer waters, the growth of planktonic algae may be very rapid if conditions are favourable, resulting in large accumulations of cells called an 'algal bloom'.

Many bacteria are able to gain energy from sunlight. Like algae and plants the cyanobacteria derive the ability to reduce CO_2 from the photolysis of H_2O; the evolution of O_2 is a product of this process. All other photosynthetic bacteria are unable to break down water, do not evolve O_2 and are described as anoxygenic. The anoxygenic photosynthetic bacteria obtain their reducing power for CO_2-fixation and biosynthesis from reduced compounds (eg. organic compounds such as lactate, reduced sulphur compounds or H_2). Anoxygenic photosynthetic bacteria are distinguished into the green and purple bacteria based on their photosynthetic pigments.

Algae and cyanobacteria fix CO_2 using the Calvin cycle, the same process that occurs in plants during the dark reaction (Brock & Madigan, 1991). Some of the photosynthetic bacteria also use the Calvin cycle, others use a reductive (reverse) TCA (tricarboxylic acid) cycle, and still others use a unique pathway of CO_2-fixation in which two molecules of CO_2 are converted into acetyl-CoA by the hydroxypropionate pathway. Although, aquatic micro-algae and many photosynthetic bacteria are basically autotrophic, many can exist heterotrophically in the dark through the assimilation of other organic compounds such as sugars and organic acids. Others are not able to grow in the dark, but their growth in light may be accelerated in the presence of organic substances, a phenomenon called photoassimilation or photoheterotrophy.

The green and purple bacteria are present in greatest numbers in stratified waters where light and H_2S are both present. Often the photosynthetic activity of these bacteria can be an important source of primary production and cyanobacterial blooms are known to occur in small lakes, ponds and geothermal springs rich in reduced sulphur compounds. The activities of these organisms are important in the cycling of sulphur in the biosphere.

The photosynthetic bacteria contain different photosynthetic pigments from the cyanobacteria and absorb light at different wavelengths. Pigment diversity is of considerable ecological significance. For example, by having different pigments cyanobacteria and photosynthetic bacteria are able to coexist in the same habitat, each using wavelengths of light not used by the other.

Chemolithotrophs

Chemolithotrophs oxidise inorganic substances to obtain energy. A number of reduced substances are used including iron, sulphur and nitrogen. These are often produced from anaerobic microbial activity, such as occurs in sediments. Chemolithotrophic bacteria are usually found at the boundary of reduced and oxygenated phases, typically the sediment surface, where both energy substrate and oxygen are readily available. Amongst the most important chemolithotrophs in aquatic systems are the sulphur and iron oxidising bacteria (eg. *Thiobacillus* spp.) and the nitrifying bacteria (*Nitrosomonas* and *Nitrobacter* spp.). Most chemolithotrophic bacteria fix CO_2, although some are able to utilise organic carbon sources to supplement CO_2-fixation.

In the following sections, the activities of some chemolithotrophic bacteria are listed and their significance to river health considered.

Nitrification

Nitrification involves the oxidation of ammonia (or ammonium ions) first to nitrite and then to nitrate.

$$NH_4^+ + \tfrac{3}{2}O_2 \rightarrow NO_2^- + 2H^+ + H_2O \qquad (\Delta G^\circ = -276 \text{ kJ/mole})$$

$$NO_2^- + \tfrac{1}{2}O_2 \rightarrow NO_3^- \qquad (\Delta G^\circ = -71 \text{ kJ/mole})$$

These two transformations are carried out by different microbial populations. Normally, the two processes are coupled and accumulation of nitrite is not observed. However, under conditions of environmental stress nitrite can accumulate. The nitrifying bacteria are important in the treatment of sewage by converting ammonia to nitrate. Vigorous and prolonged aeration is necessary for this process.

The occurrence of nitrite in drinking water is of concern, since nitrite can react chemically with amino compounds in food forming highly carcinogenic nitrosamines. Nitrate is not normally toxic, but in infants it may be reduced to nitrite in the gastrointestinal tract. Nitrite combines with haemoglobin in the blood causing respiratory distress know as the 'blue baby syndrome'. The accumulation of nitrate in groundwater can be a problem in agricultural areas which receive heavy loads of synthetic nitrogenous fertilisers.

Oxidative sulphur transformations

Reduced sulphur compounds can serve as an energy source for chemolithotrophic sulphide and sulphur oxidising bacteria in the presence of oxygen.

eg. $$H_2S + \tfrac{1}{2}O_2 \rightarrow S^\circ + H_2O \qquad (\Delta G^\circ = -210 \text{ kJ/mole})$$

$$S^\circ + \tfrac{3}{2}O_2 + H_2O \rightarrow H_2SO_4 \qquad (\Delta G^\circ = -627 \text{ kJ/mole})$$

The most important bacteria capable of oxidising reduced sulphur compounds are members of the genus *Thiobacillus*. These species accumulate globules of sulphur within their cells when H_2S is present, and then further oxidise the sulphur to sulphate when H_2S is absent.

The activities of sulphur oxidising bacteria can have a significant impact on river health. They can produce large amounts of acidic water in situations where pyrites (FeS_2) is exposed during the mining of coal and metal sulphide ores. This acidic water can then mobilise heavy metals from the mine workings which pollutes the river, killing fish, other aquatic animals and vegetation, and renders the water unsuitable for drinking or recreation. This is not a trivial problem; acid mine drainage from the Rum Jungle Mine in Northern Territory released 130 tonnes of copper; 100 tonnes of manganese, 40 tonnes of zinc and 13,000 tonnes of sulphate into the Finniss river system in one year (Moriarty & Veal, 1992). Similar acidic drainage can occur from areas where the soils contain pyrites (so called 'acid sulphate soils'), such as the coastal regions of northern New South Wales, resulting in significant reduction in the pH of rivers receiving this drainage.

2.2.3 Terminal electron acceptors

As discussed above, heterotrophs and chemolithotrophs generate energy via the oxidation of organic and inorganic energy rich compounds respectively. These processes result in the generation of electrons that must be disposed of using an electron acceptor (an oxidising agent). Oxygen is the most familiar electron acceptor and is used by all animals. However, microorganisms are able to use a variety of different electron acceptors, a factor that is particularly important in the biogeochemical cycling of elements in aquatic systems. This ability to use different electron acceptors has been used to classified microorganisms into different 'types'.

Aerobes and anaerobes

Aerobic microorganisms metabolise organic matter using O_2 as the terminal electron acceptor and produce CO_2 and H_2O (the reduced forms of O_2). This type of metabolism is known as *aerobic respiration.* Microorganisms are also able to use many other types of exogenous terminal electron acceptors, eg. sulphate and nitrate, reducing the electron acceptor (eg. SO_4^{2-} to H_2S) and excreting the end product from the cell. These alternative electron acceptors are only used in the absence of O_2. This type of metabolism is described as *anaerobic respiration*, and is discussed further below.

Many microorganisms, for example *Azotobacter* spp., have an absolute requirement for O_2 and are described as *obligate aerobes*. On the other hand, *facultative aerobes* (eg. *E. coli*) use O_2 if available, but will ferment if O_2 is limiting. Other microorganisms that can grow only in the absence of O_2 are known as *obligate anaerobes*. These include the methanogens, sulphate-reducing bacteria, and the clostridia. Another class of microorganisms, the *aerotolerant anaerobes*, are able to grow in the presence of O_2, but do not use it as an electron acceptor. These organisms are always fermentative and include the lactic acid bacteria (eg. *Lactobacillus* spp.).

Fermentation

Fermentation is an important process in aquatic systems, where organic matter is only partially metabolised. Fermenting bacteria use an intermediate of the fermentation pathway (eg. pyruvate) to accept electrons, rather than an exogenous terminal electron acceptor (eg. O_2, NO_3^-). The intermediate is reduced and this reduced product (eg. ethanol, lactic acid, propionic acid, formic acid, butyric acid, H_2, butanol and butanediol) is excreted from the cell.

In sediments, fermentation plays an important role in the decomposition of complex organic molecules, such as those found in plant residues, to smaller fermentation end-products. These end-products are then available as carbon and energy sources to other groups of anaerobic bacteria such as methanogens and sulphate reducers (see below).

Particular fermentation end-products or mixtures of end-products are characteristic of particular bacterial groups or even species of bacteria, and are commonly used to identify them. For example, the fermentation end-products of *E. coli* are used to confirm the identity of this organism when it is isolated from waters.

Anaerobic respiration

Anaerobic respiration involves the use of a terminal electron acceptor other than O_2, and only occurs in the absence of O_2. A variety of different terminal electron acceptors can be used by microorganisms including organic molecules (eg. fumarate) and inorganic ions (eg. SO_4^{2-}, Fe^{3+}, NO_3^- and HCO_3^-). Some of these terminal electron acceptors are discussed below. The energy yield from these different electron acceptors depends on the redox potential of the electron acceptor, and are generally in the order: $O_2 > NO_3^- > SO_4^{2-} > HCO_3^-$ > fermentation.

Sulphate-reducing bacteria

These use oxidised forms of sulphur as terminal electron acceptors in a manner similar to the use of O_2 by aerobes, but they produce H_2S rather than H_2O. In marine water, sulphate is present in quite high concentrations (ca. 28 mM) and this constitutes an enormous reservoir of oxidised sulphur. In fact, oceanic water contains 500 times more oxidation equivalents in the form of sulphate than in the form of oxygen. In marine sediments, around 25-50% of the carbon metabolised occurs via the sulphate reduction pathway. The three principal genera of the sulphate-reducing bacteria are *Desulfovibrio*, *Desulfomaculum* and *Desulfomonas*.

In freshwater environments, sulphate generally occurs at quite low concentrations and this often limits the activities of sulphate-reducing bacteria, so that methanogenesis is commonly more important than sulphate reduction in the metabolism of organic matter in freshwater sediments (Capone & Kiene, 1988). Sulphate reduction is also limited in aerobic environments because all sulphate-reducing bacteria are strict anaerobes, and because aerobic metabolism yields more energy. However, if a freshwater system is polluted with organic matter and/or sulphate, this can lead to the undesirable development of sulphate-reducing bacteria and the subsequent generation of undesirable H_2S. The H_2S produced can be quite toxic to other aquatic biota; the toxic effect of H_2S relates to its binding with iron in the cytochrome system of aerobic organisms. Sensitive organisms include nematodes, fish and plants. Some massive fish kills have been reported as a result of H_2S toxicity.

Nitrate-reducing bacteria

Nitrate can serve as a terminal electron acceptor for respiration in the absence of O_2 resulting in the production of the gaseous end products NO, N_2O and N_2. Two types of nitrate reducers are known.

(i) A variety of facultatively anaerobic bacteria, including Alcaligenes, Escherichia, Aeromonas, Enterobacter, Bacillus, Spirillum and Vibrio, are able to use nitrate as a terminal electron acceptor, converting it into nitrite. Approximately 40% of bacterial isolates from freshwater environments are able to reduce nitrate to nitrite. Generally, these organisms do not produce a gaseous end-product (ie. they do not denitrify). Nitrite produced by these species is excreted, or under suitable conditions will be reduced to ammonia (nitrate ammonification). Nitrate reduction plays an important role in sewage plants and in stagnant waters.

(ii) Denitrification is the production of gaseous end-products, NO, N_2O and N_2, from the reduction of nitrogen oxides. Denitrification is restricted to a few genera, including some species of Bacillus, Micrococcus, Aeromonas and Vibrio. The denitrification sequence is as follows:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Denitrification is an ecologically significant process because it leads to loss of fixed nitrogen, often a growth-limiting nutrient, from an ecosystem. Denitrification following nitrification (conversion of ammonia to nitrate) is often used to reduce the nitrogen content of wastewaters.

2.2.4 Methane oxidising and reducing bacteria

Methane has received considerable attention in recent years because of its contribution to global warming; the effect per molecule due to methane is 20 times greater than for CO_2. Freshwater sediments are the most important source of methane emissions from the earth's surface, accounting for 40-50% of the annual atmospheric methane flux (King, 1992). However, the amount of methane finally released to the atmosphere is only a tiny fraction of the total amount of methane produced, since oxidation of methane by aerobic microorganisms (methane oxidising bacteria) is thought to utilise over 90% of methane produced (King, 1992). This aerobic oxidation of methane is often confined to a thin layer, only a few millimeters thick, at the boundary of aerobic and anaerobic zones.

Methanogenesis (generation of methane) is restricted to a highly specialised group of strictly anaerobic bacteria. Most methanogens (Archaea) use the bicarbonate ion (HCO_3^-) as their terminal electron acceptor, and can utilise only a very restricted range of substrates (including H_2, methanol, formic acid and acetate). For example, with H_2:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G^o = -134 \text{ kJ/mole}$$

The methanogens are totally dependent on the activities of fermentative organisms to degrade complex organic matter (eg. cellulose, proteins and fats) to simple molecules (particularly acetate) that they can then use. It is interesting that the fermentative organisms are in turn dependent on the activities of the methanogens to remove acetate and H_2 that would otherwise accumulate and inhibit their activities.

The overall process of anaerobic decomposition of complex organic matter is shown in Figure 1. At least four different trophic groups are involved in the mineralisation of complex macromolecules, such as cellulose (Zeikus, 1977), in the anaerobic environment typical of most sediments. These are:

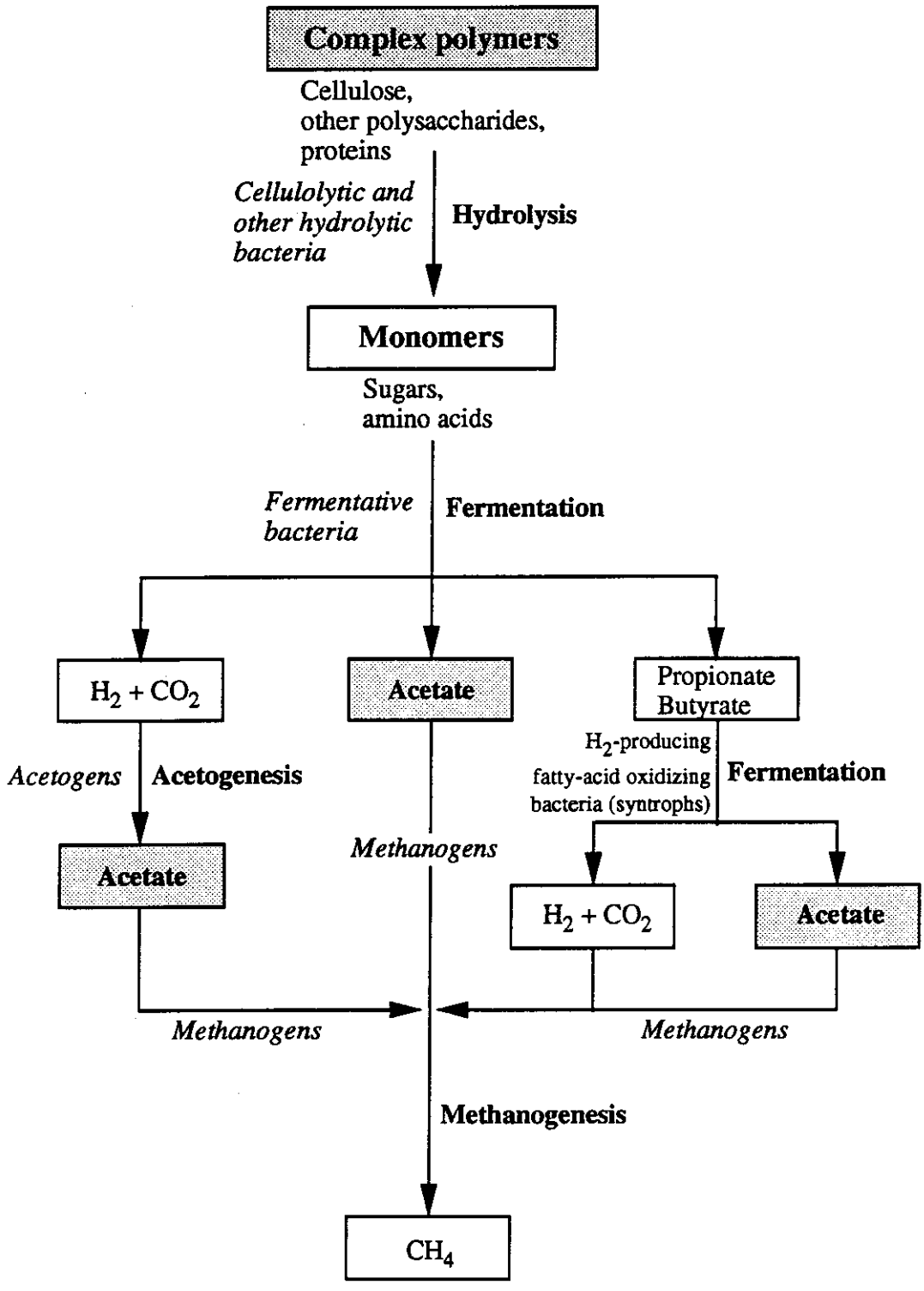
(i) *hydrolytic bacteria* that ferment complex polymeric compounds into a variety of reduced end-products such as organic acids and alcohols;

(ii) *hydrogen-producing acetogens* that ferment alcohols larger than methanol and organic acids larger than acetate into H_2 and acetate;

(iii) *homoacetogens* that ferment multi-carbon compounds, H_2 and one carbon compounds into organic acids via acetyl-CoA; and

(iv) *methanogens* that ferment H_2, CO_2, single C compounds and acetate into CH_4 and CO_2.

Figure 1: Anaerobic decomposition of organic matter (Modified from Brock & Madigan (1991))



Methanogens play a pivotal role in many anaerobic decomposing communities as they are responsible for removing fermentation end-products that, if allowed to accumulate, would limit further decomposition. Such metabolic interactions are not at all uncommon in natural microbial communities, having been been shown to be important in the decomposition of plant residues (Veal & Lynch, 1987), N_2-fixation in plant residues (Veal & Lynch, 1984) and the decomposition of recalcitrant pollutants such as PCBs (Davison *et al.*, 1994; Brown *et al.*, 1987a,b).

The importance of anaerobic processing of organic matter in low sulphate freshwater systems, where there is little competition from sulphate-reducers, has been shown by recent work on Ryan's Billabong (Sorrell & Boon, 1992; Bunn & Boon, 1993; Scholz & Boon, 1993a,b,c; Ross *et al.*, 1995). It was found that considerably more organic matter was metabolised via methanogenesis than via aerobic processes.

2.2.5 Viruses

The existence of viruses in aquatic ecosystems has been known since the 1940s (Zobell, 1946). Recent reports (Sieburth *et al.*, 1988; Bergh *et al.*, 1989), based on direct counting using epifluorescent microscopy, have revealed that there are 10^3 — 10^7 times more viruses in aquatic environments than previously thought (Paul, 1993). Most of the viruses found in aquatic ecosystems will probably be bacteriophage (viruses that have infected a bacterial cell). The numbers of viral particles found in water appear to mirror the bacterial population density (Paul, 1993). Phytoplankton blooms are correlated with increases in bacteriophage. Bergh *et al.* (1989) have estimated that one third of the bacteria in the water column might experience a bacteriophage attack each day, and for this reason bacteriophage probably play an important role in controlling bacterial and cyanobacterial population densities. It is possible that bacteriophage determination could be used as an indicator of ecosystem 'health' because their density often correlates well with that of their host.

2.3 FACTORS CONTROLLING GROWTH AND ACTIVITY

In this section we cover the influence of light, temperature and dissolved gases on the growth and activity of microorganisms. The other major factor controlling growth and activity — nutrients — is covered in Section 2.4.2.

2.3.1 Light

Light (together with nutrients) is a critical factor in determining the amount of inorganic carbon (CO_2) fixed into organic compounds through photosynthesis. The intensity and spectral composition of light penetrating the water surface has a profound effect on resident cyanobacterial and algal activity since it is available light that largely governs the potential for microbial activity.

Photosynthesis is dependent upon the spectral composition of light, which is altered by the selective absorption (and scattering) of different wavelengths as the incident light passes through the water column (Kirk, 1986). The absorption characteristics of natural waters is dependent upon the amount and type of dissolved and particulate matter present.

Phototrophs (eg. algae) use light of different wavelengths depending on their photosynthetic pigments. The main photosynthetic pigments are the chlorophylls which are able to capture light energy and use it directly for ATP production. Many alage have more than one chlorophyll, but most common are chlorophyll-a and chlorophyll-b. Accessory pigments and phycobilins are also able to absorb light energy that they then transfer to the chlorophylls. Carotenoid pigments may also act as accessory pigments, but their main function is probably to protect the organism from intense light or harmful wavelengths.

Cyanobacteria contain chlorophyll-a, but other bacteria contain different chlorophylls from those contained in algae. These bacteriochlorophylls differ from algal chlorophyll in chemical structure and the wavelengths of light they absorb. Generally, bacteriochlorophylls absorb light in the far-red and infra-red region (720-850 nm), a region which is only minimally absorbed by the water column and is poorly utilised by the various other phototrophs competing with the photosynthetic bacteria for light. This ability to use wavelengths of light that penetrates deep into the water column, and are not utilised by the algae, enables photosynthetic bacteria to live deeper in the water column, even below dense algal layers.

2.3.2 Temperature

Temperature can have a significant effect on the microbial community of riverine environments, particularly by affecting the rates of chemical reactions and the solubility of important gases (see Section 2.3.3). Microbial-mediated reactions and microbial growth reflect this temperature effect with growth rates of bacteria approximately doubling with every 10°C rise in temperature. Similarly, the rate of microbially-mediated processes like methanogenesis will increase with increases in temperature.

All microorganisms have a typical minimum, maximum and optimal temperature for growth (known as the cardinal temperatures). As different microorganisms have different cardinal temperatures, a change in water temperature can cause a significant shift in the microbial population. For example, Bianchi & Colwell (1985) found in nutrient-rich, oxygen-depleted water, resulting from a thermal effluent, that opportunistic bacteria including *Aeromonas* spp. became predominant, with fish kills occurring as a consequence. Human pathogens that require high temperature for growth, eg. *Naeglaria fowleri*, may be able to extend their range as a consequence of thermal pollution.

2.3.3 Dissolved gases

Oxygen and carbon dioxide are the two most important dissolved gases in aquatic environments, the first because of its critical importance in aerobic biological processes and involvement in redox (reduction-oxidation) reactions, and the second because of its role in the photosynthetic process and in controlling pH equilibria.

The dissolved oxygen concentration at equilibrium with atmospheric oxygen is a function of the water temperature, the partial pressure and the ionic strength of the water. The balance between microbial metabolism using oxygen and photosynthesis producing oxygen can alter the equilibrium concentration in the water column. Dissolved oxygen concentrations in excess of 100% saturation are found when there is a high level of photosynthetic activity, while values less than 100% saturation indicate that dissolved oxygen is being removed (by microbial respiration) at a rate faster than it can be replaced from the atmosphere. Dissolved O_2 depletion is often more serious in tropical waters because of the lower solubility of O_2 and increased microbial activity at higher temperatures. The dissolved O_2 concentration increases by 40% as the water temperature is lowered from 25°C to 4°C.

The concentration of carbon dioxide is also very important in natural waters since it helps to regulate the pH because of the equilibrium between dissolved carbon dioxide, bicarbonate ions, carbonate ions and hydrogen ions.

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + 2H^+$$

Additions of hydrogen ions or hydroxyl ions cause a shift in the equilibria to the left or right respectively, but the pH tends to remain stable if the buffering capacity of the system is not exceeded. Solubility of carbon dioxide in water is affected by many factors including temperature and salinity, but there is rarely a serious deficit in total carbon dioxide. This is largely due to the high solubility of carbon dioxide in water, which is approximately three times that of oxygen. Further, most photosynthetic cells appear to contain the enzyme carbonic anhydrase, which can convert bicarbonate ions to CO_2 (Fogg, 1972).

2.4 BACTERIAL PROCESSES IN RIVERS

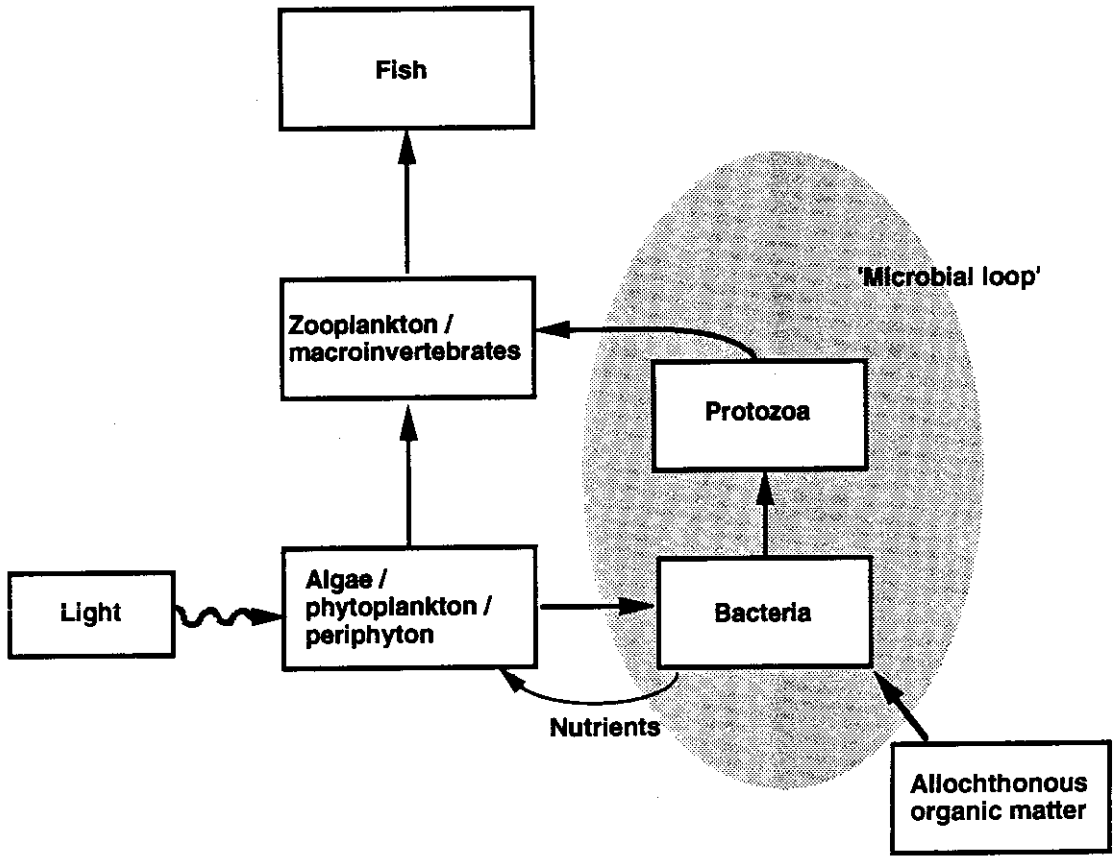
2.4.1 Microbial food web (microbial 'loop')

The understanding of the ecological significance of aquatic bacteria has undergone a dramatic change over the last two decades. The traditional role of bacteria as simply decomposers and remineralisers, converting organic matter into inorganic carbon and recycling nutrients to primary producers, has been significantly modified. In addition to the remineralisation function, bacteria also convert some of the organic matter into additional bacterial biomass, and this secondary bacterial production contributes an important food source in aquatic food webs. Figure 2 compares the traditional food chain (phytoplankton-zooplankton-fish) with a typical food web, showing particularly the 'microbial loop' where bacterial production is an important component of the food web (Azam *et al.*, 1983; Weisse, 1991). Porter *et al.* (1988) suggested that the term 'microbial loop' is potentially misleading in that it implies that the microbial component can be considered separate from the classic planktonic food web. They suggest it would be better if microbial processes were considered as an integral part of aquatic food webs.

Heterotrophic bacteria can utilise both dissolved and particulate organic matter, which may be either formed within the waterbody (eg. by phytoplankton) or transported from the catchment into the waterbody (eg. leaf detritus). In planktonic systems, bacterial production has been estimated to be around 20-30% of the primary production; production rates being 0.4 to 150 mg C m^{-3} d^{-1} or 120 to 2400 mg C m^{-2} d^{-1} on an areal basis (Cole *et al.*, 1988). It has been estimated that more than half the carbon fixed by phytoplankton moves through bacteria (Azam *et al.*, 1983, Cole *et al.*, 1988). The bacterial production represents a large component of the total secondary production and is roughly twice as large as the production of zooplankton for a given level of primary production.

There is less information on rivers. However, in a recent study of a large lowland river (Hudson River, New York), Findlay *et al.* (1991) showed that over a three year period bacterial production (250 mg C m^{-3} d^{-1} or ca. 1200 mg C m^{-2} d^{-1} integrated over a 5 m water column) was four times greater than the phytoplankton production. Obviously, in this river other sources of carbon are important in sustaining this high bacterial production.

Figure 2: Food web showing microbial loop



Bacterial numbers in most aquatic systems are relatively constant (10^8 -10^{10} cells L^{-1}), suggesting that production and losses are well balanced. Small flagellates and ciliates in the nanoplankton size range (2-20 μm) are presently considered to be the principal grazers of bacteria (and algal picoplankton) (Stockner & Porter, 1988; Berninger *et al.*, 1993), although viruses have also been flagged as being potentially important in controlling bacterial numbers in aquatic systems (Pace, 1988; Bergh *et al.*, 1989).

Heterotrophic bacteria are an important component of all aquatic ecosystems. In addition to their traditional role as decomposers and remineralisers of organic matter, it has become increasing clear that bacterial production is extremely important in all food webs.

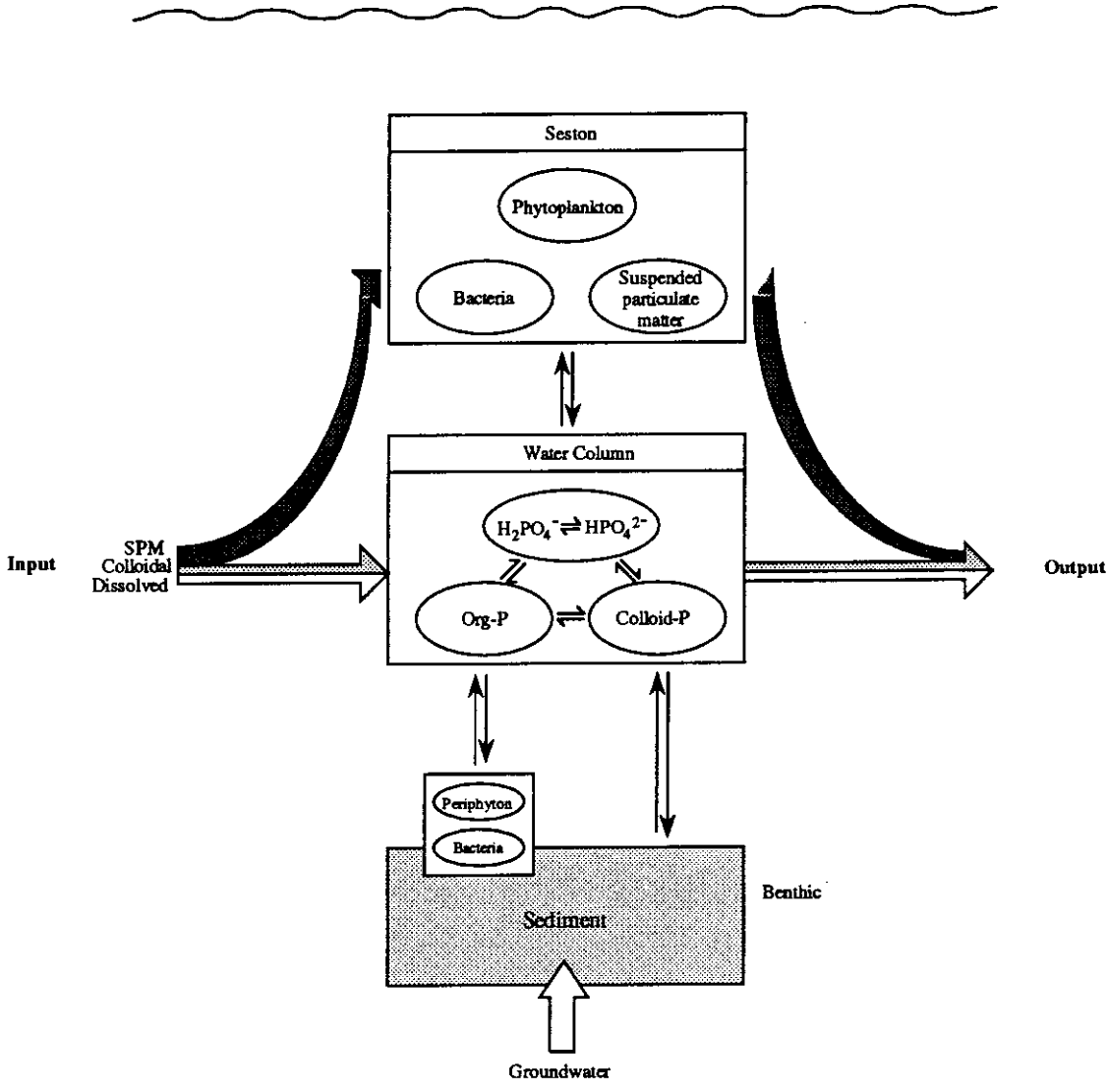
2.4.2 Biogeochemical cycles

The dynamics of materials, such as carbon, phosphorus, nitrogen and trace elements, is often more complicated in rivers and streams (running water or lotic systems) than in lakes and the oceans. In all aquatic systems, the behaviour of materials is coupled with the physical movement of the water. However, in rivers and streams these hydrologic and hydrodynamic factors have a much greater influence on behaviour than in other aquatic systems. Rivers and streams are essentially open systems, with allochthonous materials (dissolved and in suspension) transported through them, resulting in a net flux generally in the downstream direction.

For the purpose of understanding the dynamics of materials, a river or stream can be conceptualised as consisting of a number of *compartments*, with transfers of elements and materials between these compartments being possible via a number of *transfer pathways*. This model is known as a *biogeochemical cycle*. In rivers and streams, however, the concept of *material spiralling* has been introduced to indicate that in addition to the abiotic/biotic cycling, materials are transported through the system by the water flow (Newbold *et al.*, 1981; Mullholland *et al.*, 1985, 1990; Meyer *et al.*, 1988; Stream Solute Workshop, 1990; Hart *et al.*, 1992). Central to this concept is the index of uptake length (or spiral length — S_w), which is a measure of the rate of removal of a nutrient from the water relative to its downstream flux in the water column. S_w can also be viewed as a measure of the efficiency with which the ecosystem utilises available supplies of nutrients added to the system. Thus, large values of S_w indicate a low capacity for the system to utilise the particular element, while a small value of S_w indicates a high capacity for utilisation.

Figure 3 provides a model of a typical flowing water biogeochemical cycle (or spiral). In this rather simple model, three compartments are assumed to exist. The *water compartment* will consist of materials present in 'dissolved' and colloidal forms, with the possibility that there can be interactions between these two forms. The *seston compartment* will consist of two components, abiotic suspended particulate matter (SPM) transported by the river, and biological material such as bacteria and phytoplankton. Phytoplankton can make up a large proportion of the seston in large, slow moving rivers and regulated rivers (eg. in the River Murray, phytoplankton make up approximately 10-20% of the total seston concentration (typically 30-40 mg/L)), but will normally be a minor component in most upland streams and in highly turbid rivers. The *benthic compartment* consists of two components; the sediments, and the attached algae and microbial biofilms. This biological component is often a dominant part of the benthic compartment in small, rocky upland streams, but will be considerably less in larger, deep rivers. The actual composition of each of the three compartments, particularly the amount and type of biological material, will depend upon the type of river. For example, in small, fast flowing upland streams the biota is predominantly associated with the benthic compartment, while in larger rivers the biota will mostly be in the water column in the seston compartment, but depending upon the system there may also be a large biomass associated with the sediments.

Figure 3: Nutrient cycling



In this model, elements and materials are assumed to enter and leave each stream segment in three forms — soluble, colloidal and particulate. Of course, these are quite arbitary subdivisions, but they do permit us to emphasis some of the differences in reactivity and behaviour of trace nutrients (eg. P and N) associated with these three fractions. The downstream transport processes deliver the materials to reactive sites, where exchanges between sites can occur. These exchanges may include chemical transformations (eg. change in chemical species), sorption and desorption, and biological

processes, such as algal or bacterial uptake, bacterial oxidation and reduction, and invertebrate consumption of algae. In small rivers and streams, most of these transfers occur between the water compartment and the benthic compartment; however, in larger systems it is also possible that transfers between the water compartment and the seston compartment will occur. Transfers in the other direction, from the streambed (or phytoplankton) to the water column, result in release of materials to be subsequently transported further downstream. Retention is the net difference between uptake and release and will be flow-dependent (Stream Solute Workshop, 1990).

Bacteria are responsible for the biogeochemical cycling of all the elements in the biosphere. The bacterial processes occurring are basically the same in different aquatic environments although the species carrying out the processes may differ. Even major environmental changes (eg. increase in salinity) do not seem to affect these microbial processes, but merely regulate the microbial species present (Wood, 1965). However, although the broad details of the major biogeochemical processes are known, much less is known about the rates of these processes in the natural environment (Hobbie & Ford, 1993).

Discussions on the role of bacteria in the biogeochemical cycling of materials generally emphasise the importance of these organisms as decomposers of organic matter and thus as nutrient regenerators. However, there is now considerable evidence showing that bacteria can compete very effectively with algae for key nutrients (eg. phosphorus), and in this way may actually reduce algal growth rather than stimulate it via regeneration of nutrients (Currie & Kalff, 1984a,b,c; Cole *et al.*, 1988; Jansson, 1988, 1993). Certainly, the role of bacteria is considerably wider than just as decomposers and recyclers.

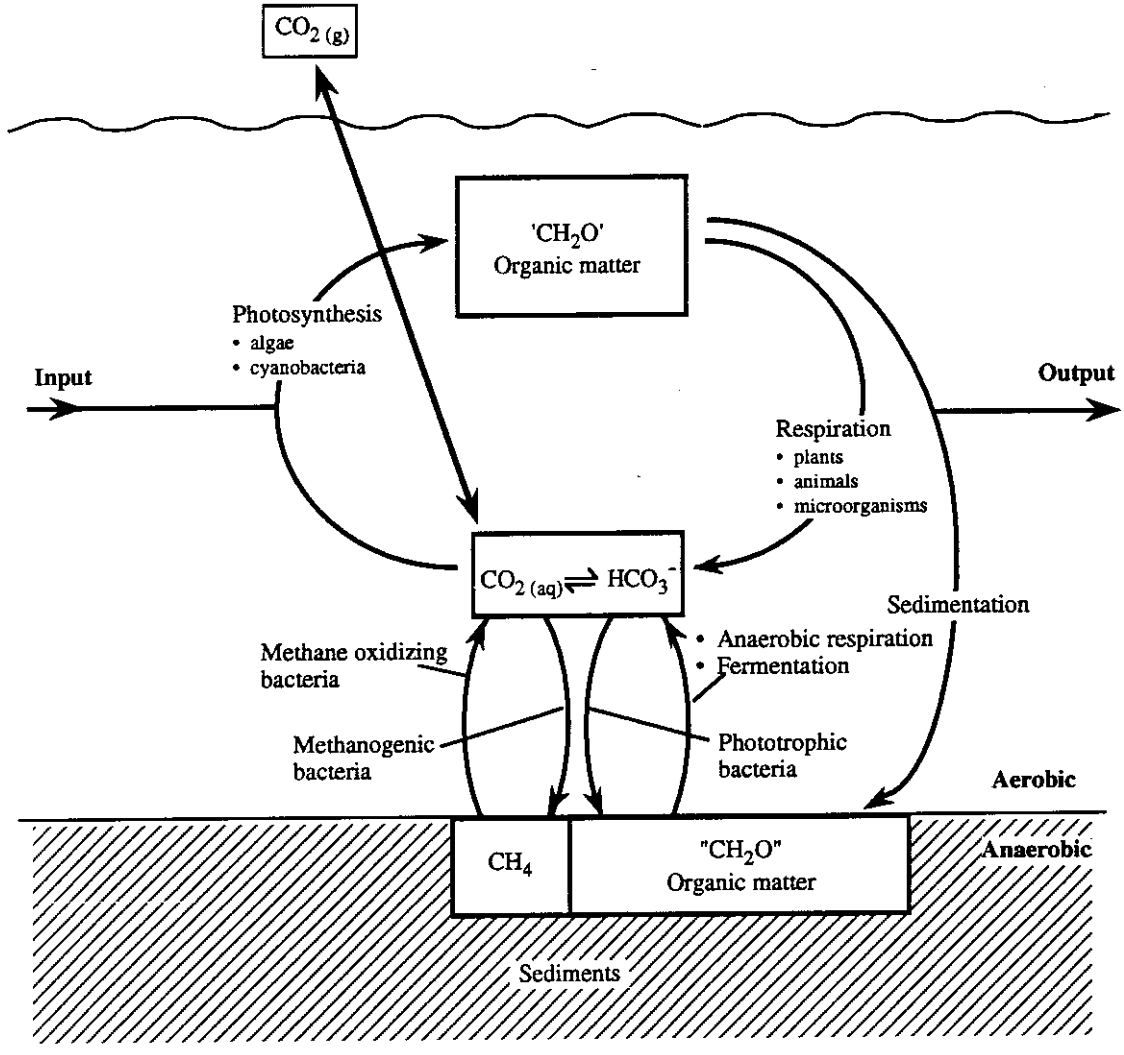
In this section we consider the role of bacteria in the biological transformations of carbon, nitrogen, phosphorus and sulphur. Although the cycling of these elements is considered separately here, it is important to recognise that these cycles are interrelated and cannot be separated from each other.

Carbon cycle

In rivers, the organic matter (substrates) for the growth of heterotrophs can be divided into two types, based largely on whether it is produced within or outside the system. *Autochthonous* substrates include organic matter (algae and bacteria) produced within the stream. In small streams, biofilms (consisting of a complex mixture of algae, microorganisms and polysaccharides) attached to rocks and logs are normally the most important (Lock, 1993), while in larger streams phytoplankton (and possibly bacteria) in the water column may become the dominant source of autochthonous matter. Exudation by phytoplankton and macroalgae may be an important source of organics. Algal products include small amounts of amino acids and larger amounts of short chain acids (such as glycolic acid) glycerol, carbohydrates and polysaccharides. *Allochthonous* substrates include organic matter (eg. leaves, bark) and nutrients derived from the catchment and transported to the stream via run-off and groundwater, or discharged directly to the stream (or tributary) from a wastewater treatment plant. Allochthonous material is particularly important in small streams where a large proportion of the organic matter and nutrients may originate from the surrounding terrestrial environment (Vannote *et al.*, 1980). A number of workers (eg. Hynes, 1975; Likens, 1984) have argued that stream ecology must link the stream with its catchment, and should encompass processes that occur below, beside and upslope of the stream channel.

Figure 4 shows the essential elements of a typical carbon cycle for a riverine system. Organic matter is either produced photosynthetically by algae and cyanobacteria under aerobic conditions, or by phototrophic bacteria under anaerobic conditions; additionally, allochthonous organic matter may be brought into the system. Removal of the organic matter can occur via a number of biological pathways, as well as by sedimentation and washout. Biological removal of organic matter (mainly algae and bacteria) occurs aerobically through grazing by animals (eg. macroinvertebrates, zooplankton) and decomposition by microorganisms, with the production of CO_2, or anaerobically via anaerobic respiration of fermentation. In some cases, particularly in systems containing anaerobic sediments, methane may also be produced within the sediments (by methanogenesis), but it is most likely that this methane will be oxidised at the sediment-water interface. Methanogenesis is commonly more important than sulphate reduction in the metabolism of organic matter in freshwater sediments, due to the generally low sulphate concentrations in freshwater systems; the reverse is generally true in marine and estuarine sediments where sulphate reduction is more common than methanogenesis (Capone & Kiene, 1988).

Figure 4: Typical carbon cycle in a river



Heterotrophic microorganisms are generally the first to use allochthonous or autochthonous matter in an aquatic ecosystem, assimilating part of it into energy-rich organic compounds, which can then be used by other heterotrophic organisms (eg. macroinvertebrates) as a source of food. During metabolism of this organic matter, part of the energy is converted into biomass and the remainder is dissipated as heat and is lost. Heterotrophic microorganisms are important in maintaining the energy flow in aquatic ecosystems by (i) assimilating dissolved and particulate organic molecules making them available to organisms higher up the food chain (eg. protozoa or filter feeding invertebrates), and (ii) mineralising organic compounds and releasing the inorganic minerals they contain (eg. P, N) which can be used by primary producers and other heterotrophs.

The proportion of the organic matter recycled by heterotrophic decomposition rather than being consumed by other trophic levels, varies considerably for system to system. Information now exists for the oceans and a number of limnetic systems (Pomeroy, 1974; Cole *et al.*, 1988), but there is still scant information for running waters. In planktonic systems, bacterial production has been estimated to be around 20-30% of the primary production, with more than half the carbon fixed by phytoplankton moving through bacteria (Cole *et al.*, 1988).

Bioremediation of organic pollutants

Microorganisms also play a major role in the natural amelioration of anthropogenic organic compounds in aquatic systems. Compounds such as aliphatic compounds (eg. petroleum products (Atlas & Bartha, 1992)), aromatic compounds (eg. biphenyl (Davison *et al.*, 1994), toluene (Wong & Dunn, 1974) and naphthalene (Dunn & Gansalus, 1973)), halogenated aliphatic and aromatic compounds (eg. chlorobiphenyl (Kamp & Chakrabart, 1974)), DDT (Bumpus & Aust, 1987), heptachlor and vinyl chloride can support microbial growth. Recently, even molecules that were considered recalcitrant to biodegradation (eg. polychlorinated biphenyls), have been shown to be

amenable to microbial degradation (Bedard *et al.*, 1986). Although there appear to be few anthropogenic organic molecules that are not subject to microbial degradation or modification, the physical and chemical conditions existing in a natural environments may not favour biodegradation.

Bioremediation is a new industry based on the activities of microorganisms to clean up contaminated sites. Bioremediation processes aim to create conditions that favour biodegradation of xenobiotic molecules (such as PCBs, DDT or oil spills). In the case of the Exxon Valdez, the oleophilic fertiliser 'Inipol' was found to be very effective in remediating the oil spill (Atlas & Bartha, 1992). Bioremediation technologies are likely to be used for the treatment of river sediments or groundwater contaminated with xenobiotic molecules. Although such technologies offer the prospect of remediation of aquatic ecosystems care needs to be exercised to ensure that xenobiotics are mineralised and not modified into more mobile forms.

Nitrogen cycle

Nitrogen plays a crucial role in primary productivity and is often the nutrient that limits primary productivity. The availability of nitrogen in aquatic ecosystems is dependent to a large extent on the breakdown and recycling of nitrogenous compounds through microbial decomposition. Nitrogen fixation, particularly by cyanobacteria, may also contribute significantly to nitrogen inputs to aquatic systems.

Figure 5 illustrates a typical nitrogen cycle for a riverine system. In aerobic waters, nitrate is the form of nitrogen commonly taken up by algae and bacteria to produce biomass. This nitrate is formed by a process called *nitrification*, where ammonia is microbially oxidised first to nitrite by *Nitrosomonas* species and then to nitrate by *Nitrobacter* species (Section 2.2.2). Total numbers of these bacteria are strongly correlated with the degree of eutrophication; nitrifying bacteria are rarely found in clean streams, but are usually found in eutrophic waters unless inhibited by oxygen depletion or toxic substances (Rheinheimer, 1991). Inland waters may have considerable allochthonous nitrogen inputs in the form of sewage, agricultural and urban run off. Much of this allochthonous input will be as organic nitrogen, for example the input from sewage. *Ammonification* is the breakdown of organic nitrogen producing ammonia. Ammonification is carried out by putrifying bacteria and fungi, this may be common in sewage laden waters.

Denitrification, the conversion of nitrate to nitrogen gas, is carried out by facultative anaerobic bacteria with nitrate acting as a terminal electron acceptor in anaerobic respiration (Section 2.2.3). Denitrification is regarded as the most important process for the removal of nitrogen from natural aquatic systems, although there is little experimental data to support this contention (Hobbie & Ford, 1993). The nitrate needed for the denitrification process mostly comes from *in situ* nitrification of planktonic or benthic organic matter, although considerable amounts may come from terrestrial inputs. In most systems, denitrification is tightly linked to nitrification and requires a delicate spatial arrangement between adjacent aerobic and anaerobic environments.

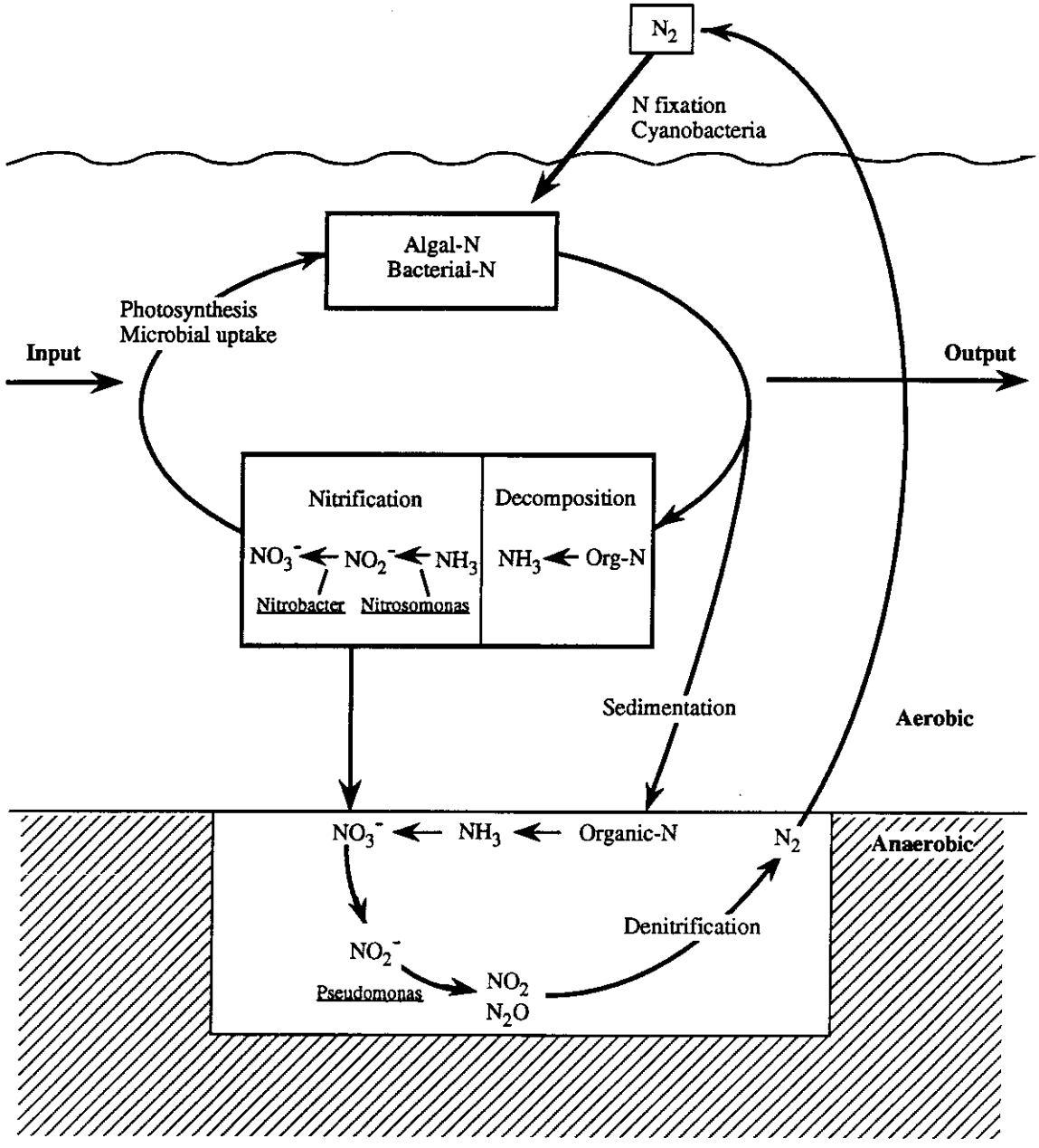
Losses of fixed nitrogen from a riverine ecosystem as a consequence of denitrification, sedimentation or export are offset either by allochthonous inputs or atmospheric N_2-fixation. Eukaryotic organisms, including plants, are unable to fix atmospheric N_2, this being a process exclusive to prokaryotic (bacterial) organisms. The fixation of N_2 is an energetically expensive process requiring *ca.* 20 ATP molecules per N_2-fixed molecule. The organisms also have a large requirement for phosphorus and to a lesser extent iron and molybdenum. Thus, the amount of N_2-fixation in a natural environment can be significantly limited by energy or elemental requirements.

Cyanobacteria normally make the most significant contribution to N_2-fixation in aquatic ecosystems. These N_2-fixing cyanobacteria may occur as symbionts of aquatic plants. For example, the N_2-fixing cyanobacterium *Anabaena azollae* grows symbiotically with the aquatic fern *Azolla* and can produce substantial levels of N_2-fixation. Together with the cyanobacteria, heterotrophic bacteria (eg. certain methanogens, sulphate reducing bacteria, clostridia, azospirilla and *Enterobacteriaceae*), may utilise plant residues as a source of carbon and energy for N_2-fixation (Veal & Lynch, 1984).

Phosphorus cycle

The cycling of phosphorus in aquatic systems is less influenced by microbial processes than either the nitrogen or sulphur cycles, but never-the-less microbial processes are still important. Figure 6 shows a simplified phosphorus cycle for a riverine system.

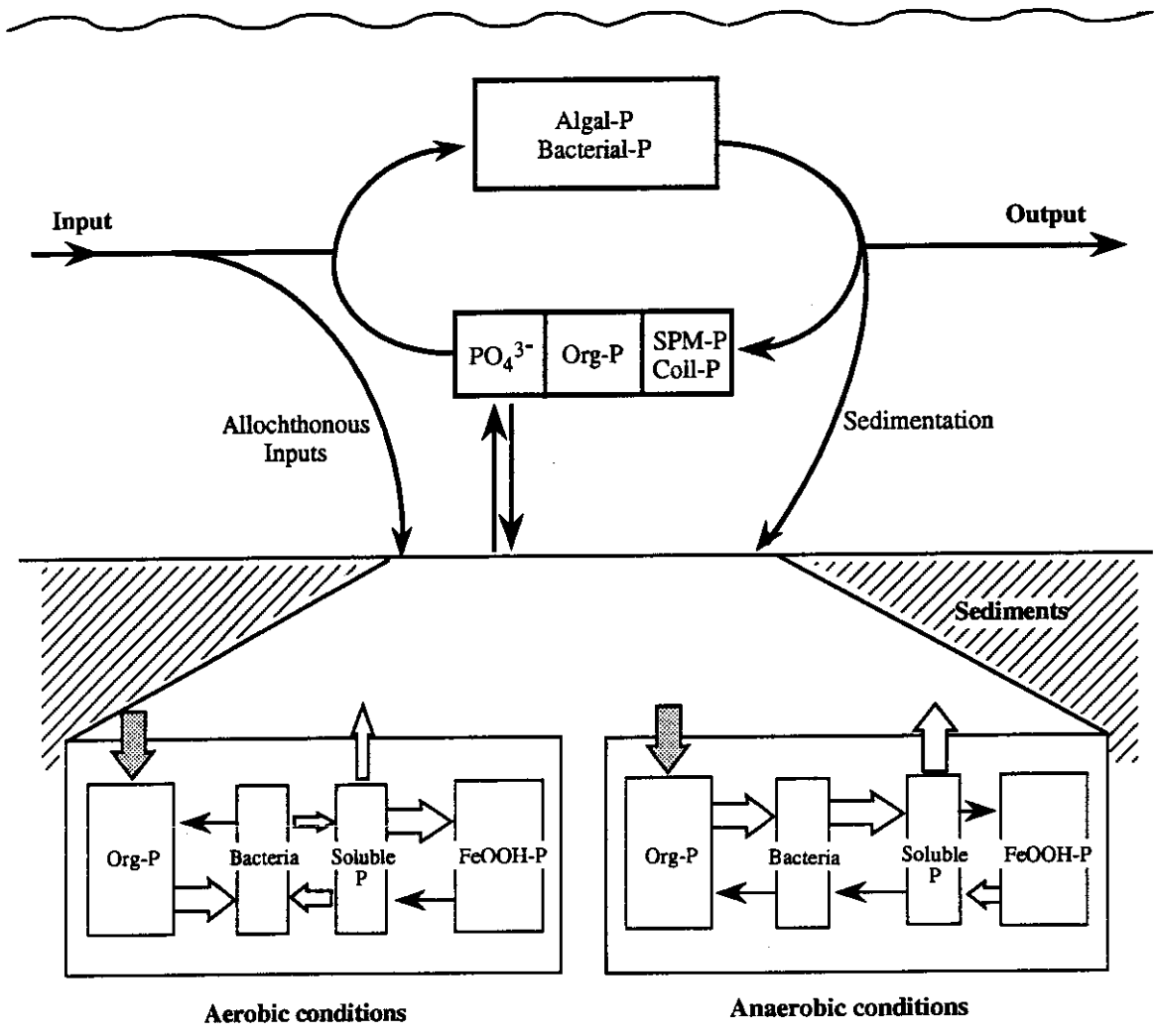
Figure 5: Typical nitrogen cycle



Inorganic forms of phosphorus are the most biologically available, although organic phosphorus compounds can also be utilised, but only after they have been hydrolysed to orthophosphate by phosphatase enzymes (Jansson *et al.*, 1988). It is now known that both algae and bacteria (cyanobacteria and other bacteria) compete quite effectively for available phosphorus in most aquatic systems (Currie & Kalff, 1984 a,b,c; Cole *et al.*, 1988; Jansson, 1988, 1993), so that the classical view that algae take up nutrients and bacteria regenerate nutrients by decomposition needs to be significantly modified. As noted above, bacteria can make up a significant proportion of the planktonic and benthic biomass, and often represents an important food source for higher heterotrophs. Most algae appear to be able to accumulate phosphorus in excess of their immediate needs for metabolism, and to store this as polyphosphates for use later when the external phosphorus concentration is reduced (Cembella *et al.*, 1984a,b). Both orthophosphate and organic phosphorus compounds are also released to the water column by growing algae and bacteria, and subsequently when they die and are decomposed by aerobic bacteria. Algae and bacteria may also be lost from the water column by sedimentation or washout of the system.

A question of some importance in lakes and large lowland rivers is the relative competition between bacteria and phytoplankton for the major nutrient phosphorus. Since bacteria are known to assimilate orthophosphate much more rapidly than phytoplankton (Currie & Kalff, 1984a,b,c; Jansson, 1988, 1993; Currie, 1990), it is of some interest to know how the phytoplankton obtain

Figure 6: Typical phosphorus cycle



sufficient phosphorus. Currie (1990) suggested three possible models: (i) that total P abundance determines algal abundance which in turn determines bacterial abundance, (ii) that phytoplankton and bacteria compete for the phosphorus, or (iii) that algal-bacterial competition for P does not directly determine their abundance, but that the phytoplankton and bacteria 'need' each other, the bacteria needing phytoplankton for vital organic carbon and the phytoplankton needing the bacteria to remineralise nutrients. There is now considerable support for the third model.

In small rivers and streams, where most of the primary production and bacterial secondary production occurs in microbial biofilms on the rocks and sediments, the cycling of nutrients such as phosphorus appears to be very tightly coupled within the biofilm (Lock, 1993). In fact, once a nutrient molecule enters a biofilm it is unlikely to be released (except when the film is sloughed off the substrate during a high flow event).

Phosphorus uptake and release from sediments is well known to be controlled by the redox condition in the sediments; under aerobic conditions phosphorus is retained by the sediments, while under anaerobic conditions, such as exist when a system becomes stratified, phosphorus is released from the sediments. The classical explanation for this observations has been that the sediment phosphorus is controlled by association with hydrous iron oxides, under aerobic conditions the hydrous iron oxides sorb orthophosphate and retain it in the sediments, while under anaerobic conditions the hydrous iron oxide is solublised due to the reduction of Fe(III) to Fe(II), and the orthophosphate is released to the interstitial waters from where it can diffuse to the overlying water column (Mortimer, 1941, 1942). Within this hypothesis, the only role of the bacteria was assumed to be to lower the redox potential by aerobic decomposition of sediment organic matter (ie. bacteria create the anaerobic conditions).

However, more recent studies suggest that while bacteria are certainly central in producing the anaerobic conditions, they also play a role in actively taking up (under aerobic conditions) and releasing (under anaerobic conditions) orthophosphate within the sediments (Gachter *et al.*, 1988; Gachter & Meyer, 1993; Davelaar, 1993; de Montigny & Prairie, 1993). Sinke *et al.* (1993) reported that 12-63% of the total P uptake by sediments was due to bacteria.

Sulphur cycle

Sulphur is an essential nutrient for microorganisms, but is usually plentiful and rarely limiting in rivers. Ecologically, sulphur is important because of the toxicity of certain compounds, such as H_2S and sulphuric acid, and because oxidation of sulphur compounds is important as an energy source for some aquatic bacteria.

Bacteria play major roles in both the oxidative and reductive parts of the sulphur cycle. Reduced inorganic sulphur (elemental S, H_2S, thiosulphate, sulphite) can be oxidised by a number of aquatic bacteria. In sediments, members of the genus *Thiobacillus* appear to be most important, while in the euphotic zone of the water column purple and green sulphur bacteria are the most important sulphur oxidisers. These oxidation processes generally produce sulphate and protons, and can result in a significant lowering of the water pH.

Perhaps more important in aquatic system is the sulphate-reduction, whereby sulphate is used as an electron acceptor in anaerobic respiration and H_2S is produced. Sulphate reduction occurs in anoxic sediments. If iron is present, ferrous sulphide will precipitate giving anoxic sediments their characteristic black colour. Sulphate reduction is often limited in freshwater systems because of a lack of sulphate.

3. METHODS IN AQUATIC MICROBIOLOGY

Aquatic microbial ecology is advancing extremely rapidly due mainly to the development of a range of exciting new methods, many based on molecular biology techniques, and the application of new instrumental techniques, such as flow cytometry and field flow fractionation. The traditional microbial methods, that have been used for many years to protect human populations against waterborne diseases, are totally inadequate for natural environments, since so few of the microorganism involved in ecosystem processes have been (or are likely to be) identified. This review concentrates on recent developments in three areas: the identification and enumeration of microbes, the measurement of microbial biomass, and the measurement of microbial activity. We identify an extremely active field, with new techniques being developed regularly, and considerable scope for many of these to be applied to the study of microbial processes driving river ecosystems. It is clear that the most useful results will be forthcoming when several microbial indicators are used together. Despite the current activity there is still much to be done. The review highlights four areas of concern: (i) considerably more work is needed to make the present microbial techniques more rapid and simpler to use; (ii) there needs to be much wider validation and testing of the methods in the field; (iii) there is an urgent need to increase the awareness of staff in environmental and water agencies to the new methodologies that are now available to assess microbial activities in river ecosystems; and (iv) there is a need to increase both the number and technical skill base of those working (in research institutions, environmental agencies and water agencies) in the aquatic microbial field over the next three to five years.

Studies of microorganisms in aquatic ecosystems are generally interested in identifying and enumerating species or groups of microorganisms present, and estimates of biomass, growth rate and activities. Techniques for obtaining basic information on microorganisms have been in standard use for years, but many of these traditional methods are limited in what they can tell us about aquatic microbial microorganisms. Traditional methods of microscope identification and microbial culture allow studies of culturable microorganisms. Unfortunately, the majority of aquatic microorganisms are not culturable. Furthermore, culture based methods rarely give information on the physiological or phylogeny of isolates. The last five to ten years has seen enormous increase in the number of new techniques that have become available, resulting in many new techniques for studying microorganisms. In this chapter, the traditional methods as well as new technologies are discussed with an emphasis on their usefulness and limitations in studying aquatic microorganisms. The traditional methods are only covered briefly but many handbooks and textbooks cover these methods in more detail (see Brock & Madigan, 1991; Atlas & Bartha, 1993; Kemp *et al.*, 1993).

3.1 TECHNIQUES FOR IDENTIFICATION AND ENUMERATION OF MICROORGANISMS

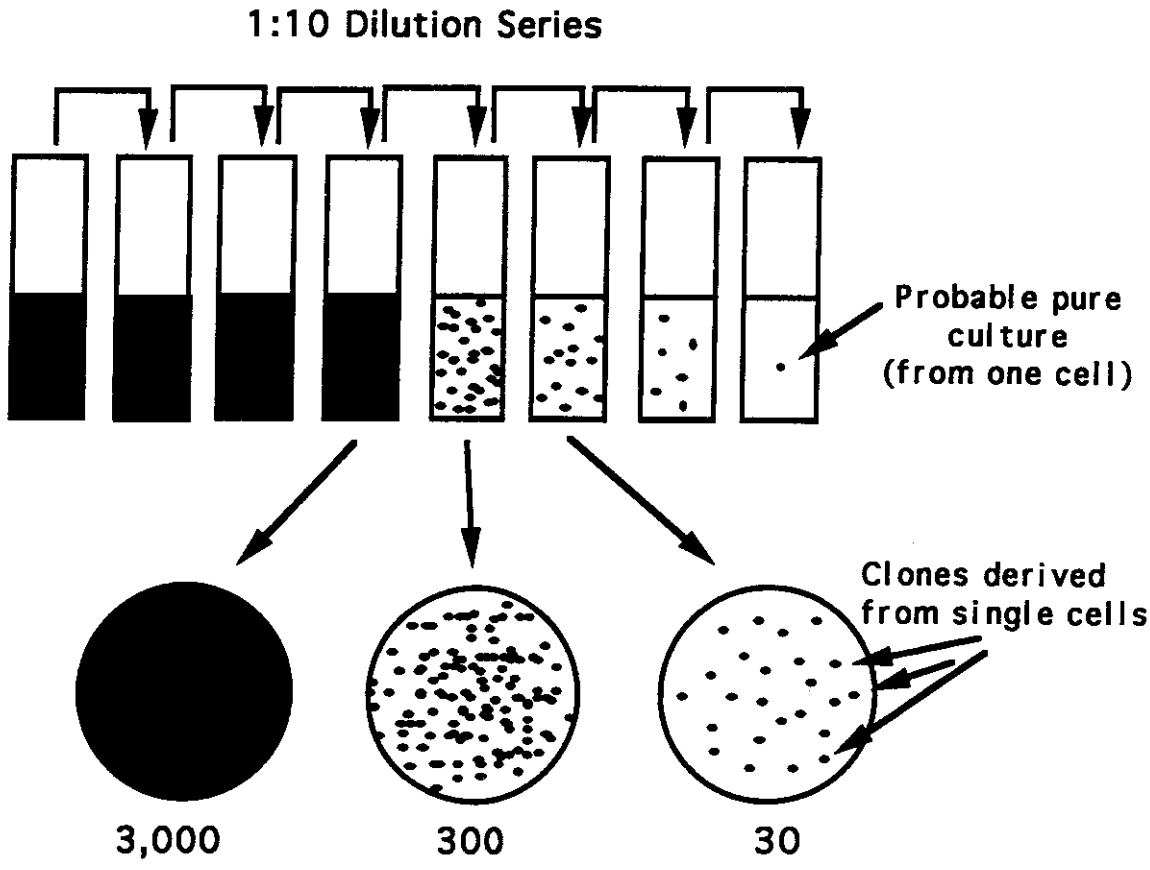
3.1.1 Viable cell counts (Plate methods)

The number of viable cells (those that are able to divide) is of interest in many cases. Traditionally, these are counted by determining the number of cells in the sample that are capable of forming colonies on a suitable agar plate medium (culture techniques).

Since native microorganisms vary widely in their nutritional and environmental requirements, some species, or groups of species, can be selected from a mixture of microorganisms by choosing the appropriate growth conditions. In fact, all culture media are to some extent selective as they all favour particular types of microorganisms. The procedure of adjusting culture conditions to select a particular organism is called *enrichment culture*. The sediment or water sample thought to contain the organism of interest is inoculated into a specific culture medium and then incubated under appropriate aeration, temperature and pH conditions. After an appropriate period of time the enrichment culture is examined for evidence of cell growth.

The traditional method for identifying a particular microorganism has been to isolate it as a pure culture then characterise it by various physiological, biochemical, and more recently serological means. The isolation procedure normally involves dilution of the microbial community to single cells followed by clonal growth of a pure culture on a suitable medium. After serial dilution of a sample, enumeration of microorganisms is achieved using techniques such as most probable number technique (dilution to extinction) or by spreading suitable dilutions on to agar to isolate clones (as colonies) which have arisen from a single cell (Figure 7).

Figure 7: Most Probable Number method of obtaining a pure culture of microorganisms



These techniques were developed at the end of last century and were largely responsible for the rapid advances seen in microbiology, during the early part of this century. Around 4,000 bacterial species have been described using such pure culture techniques (Holt, 1986-89; Starr, 1981). However, there is now good evidence to show that this number is well less than 1% of the total bacterial 'species' in nature (Torsvik *et al.*, 1990a,b).

There are major limitations with these traditional methods for analysing microbial communities. First, techniques that depend on dilution will only isolate the dominant microorganisms in the environment, since rare organisms will be diluted out. To overcome this problem microbiologists have developed a wide range of media that select for particular types, whilst selecting against the majority of the microbial community. However, the development of such selective media requires prior knowledge of the microorganism of interest. Second, a pre-requisite of these methods is that the organism must be culturable in laboratory medium, and as noted above most bacteria are non-culturable using currently available techniques so that viable counts obtained using culture techniques constitute between 0.0001% and 10% of counts obtained by direct microscopy. Finally, even when it is possible to culture microorganisms it can be difficult and very time consuming to identify novel organisms since morphological characters alone cannot be used to distinguish different types. Microbial ecologists have recently begun to develop a number of chemical, immunological and nucleic acid based techniques for detecting bacteria in the environment. These techniques are obviating the need to culture and are discussed in the following sections.

3.1.2 Microscope counts

The number of microorganisms in a population can be measured by direct counting under a microscope. Generally, cells are stained to make counting easier with the two most widely used stains being fluorochromes — 4',6-diamidino-2-phenylindole (DAPI) (Porter & Feig 1980) and acridine orange (AO) (Hobbie *et al.*, 1977); both these bind to the nucleic acids inside the cell.

Several differential (specific) stains are also available and these can be used to identify certain types of microbial cells and to classify them into broad categories, the most common being *Gram staining*. The Gram stain is one of the most useful staining procedures and is used to identify an unknown bacterium into one of two classes — Gram-positive (purple stain) or Gram-negative (red stain).

The colour of the stain is due to differences in the cell wall structure of these two bacterial types. Gram staining can only be effectively applied to dense cultures of isolated bacteria and for this reason cannot be used directly for environmental samples in the same way as DAPI and AO.

A number of microscope methods have been developed to determine whether native microorganisms are actively growing. These involve the use of stains such as the tetrazolium salt, iodonitrotetrazolium violet (INT), and the tetrazolium dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), as well as the antibiotic nalidixic acid. INT, a non-fluorescent stain, is perhaps the most used method for determining the proportion of metabolically active cells (Zimmerman *et al.*, 1978). Actively growing (respiring) cells oxidise INT with their electron transport system and deposit a red formazan product as crystals within the cell, which are then visible when cells are examined with a light microscope. It is often difficult to determine whether small cells, common in natural water samples, contain the red crystaline deposits of INT-formazan (Karl, 1986; Boon, 1991a). Thus, the INT method generally underestimates the actual proportion of metabolically-active bacteria, but still gives a better estimate than the viable count method. CTC staining is similar to INT except that the CTC-formazan product is viewed with a fluorescence microscope.

Numbers of actively growing bacteria can also be determined by incubation with nalidixic acid, an antibiotic that inhibits cell division (Kogure *et al.*, 1979). Nalidixic acid stops cell division by inhibiting DNA synthesis, which results in a lack of cross-wall formation so that growing cells elongate instead of dividing. This procedure, however, has problems in that recognising an elongated cell, especially when cells are small or slow growing, is subjective.

The direct counting of microorganisms in natural samples, using either the general or differential stains described above, has a number of disadvantages: (i) it is time consuming and laborious, (ii) it is difficult to identify most organisms on the basis of morphological characteristics only, (iii) it can often be difficult to differentiate between microorganisms and the vast number of other particles found in water samples, (iv) aggregates of microorganisms with other microorganisms and with other particles can also make counting very difficult, (v) dead cells are not distinguishable, and (vi) the method is not suitable for cell suspensions of low density. A bacterial concentration of approximately 10^6 bacteria per mL is required if one is to achieve a single bacterium per field of view on a microscope. Since many natural waters contain less than 10^6 bacteria per mL, some form of concentration is often required. Concentration methods include sedimentation, centrifugation or filtration. A most convenient method for direct counting of microorganisms is to pass a water sample through a membrane filter, stain the bacteria retained on the filter with a fluorochrome and examine the filter using epifluorescent microscopy (Hobbie *et al.*, 1977).

The capacity for bacterial counts in water samples using both DAPI and AO staining is now well developed in at least two laboratories in Australia — Water Studies Centre, Monash University, and Victoria University of Technology. Additionally, there is now some published information on bacterial numbers for several sites in south-eastern Australia (Boon, 1989a,b, 1990, 1991a,b, 1993, 1994), but most areas of Australia are unstudied.

3.1.3 Biomarkers

The use of biomarkers is a rapidly evolving area in which information from chemical analyses of whole organisms or cell fractions is used for classification and identification of microorganisms (Goodfellow & Minnikin, 1985). Increasingly sophisticated chemical procedures are now available to determine the lipid, amino-acid and carbohydrate and protein composition of microorganisms. Particular profiles of molecules, eg. lipids, may be characteristic of a certain group of microorganisms. Further, specific signature molecules may be characteristic of a particular microorganism. Information on these signature molecules or profiles is stored in databases to enable future identifications.

Lipid analysis has been used as a method to provide mainly qualitative information on the presence of certain groups of microorganisms and serves as an example of a biomarker. The method involves measurement of particular fatty acids (lipids) that are specific for particular microorganisms (eg. the rare fatty acid 18:1w8c (standard fatty acid nomenclature) is characteristic of type II methanotrophic bacteria) (Vestal & White, 1989; Manz *et al.*, 1993). The protocol involves extraction of the fatty acid with organic solvents, the fatty acid fraction is then dried and can be quantified by, for example, gas chromatography or flame ionisation detection. Work using the lipid biomarker technique has been performed in Australia (Boon & Sorrell, 1991; Scholz & Boon, 1993a,b,c).

There are several difficulties in the use of these chemical biomarkers. First, organisms have to be known and culturable in order to establish the data base, precluding the identification of truly novel organisms. Second, the production of lipids, amino acids and carbohydrates, is normally dependent on growth conditions which may limit the use of these biomarkers in environmental situations where growth rates can be quite variable. The methods are also often time consuming, tedious and difficult to undertake. Another difficulty is the accurate calculation of bacterial abundance from biomarker concentrations, since the conversion factors are quite variable. Despite these difficulties, biomarkers are still the best method available for determining assemblage composition and structure, although molecular probe techniques once better developed will most likely replace biomarkers for this purpose (P. Boon, Victoria University of Technology, personal communication, July 1994).

3.1.4 Immunological techniques

The use of specific antibodies offers a sensitive and specific means of identifying microorganisms without the need for culturing. Antibodies are produced in animals, such as rats, mice or rabbits, after injection with microbial cells or parts of cells and are able to bind with specific regions on a microbial cell (eg. antigens on surface) in a reaction that has been likened to a lock-and-key fit (Jain *et al.*, 1988). The covalent attachment of enzymes to antibody molecules creates a very functional immunological tool (Figure 8). Most antibody-based techniques employ some form of colorimetric or fluorometric detection system. The Enzyme-Linked Immunosorbent Assay (ELISA) method, for example, makes use of antibodies to which enzymes have been covalently bound. Typically linked enzymes include peroxidase, alkaline phosphatase, and beta-galactosidase, and these can all catalyse reactions whose products are coloured and can be measured in very low amounts. Once the antibodies have been made, ELISA assays require very little in the way of expensive equipment and are highly sensitive. They are widely used in clinical laboratories. ELISAs have been developed to detect serum antibodies to a variety of bacterial species, mostly pathogens.

Several genus- or species-directed, labelled antibodies are available to identify native microorganisms (Howgrave-Graham & Steyn, 1988; Manz *et al.*, 1993; Conway de Macario *et al.*, 1982). These antibodies have been raised against surface markers of defined pure cultures. This technique is restricted to detection of microorganisms that are sufficiently well characterised to be grown as pure culture, and other closely related microorganisms.

Antibodies have also been used to identify specific enzymes catalysing important ecological functions (eg. alkaline phosphatase which hydrolyses organic phosphorus compounds or the Rubisco enzyme responsible for CO_2-fixation (see Section 2.3.1). This technique can be used to detect such activities and enumerate microorganisms containing the target enzymes.

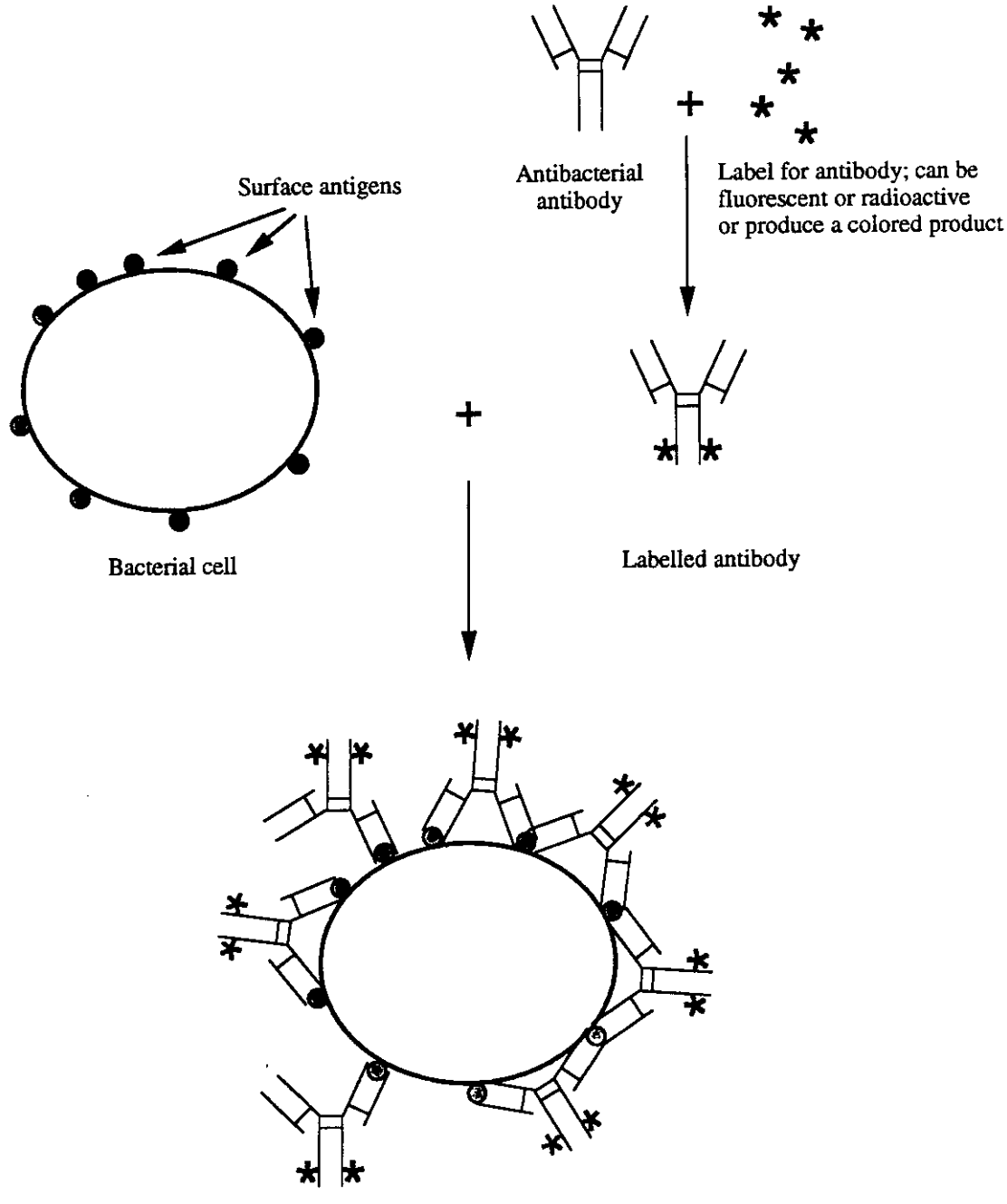
Antibody techniques have a number of difficulties, including: (i) the specificity of the antibodies in that they can cross-react with different cell types, (ii) they generally require pure cultures of microorganisms of interest, and (iii) they are difficult and time consuming to produce. In summary, immunological approaches are often too selective, and so are better used for intraspecies than interspecies analysis. This makes them good for pathological studies (eg. on serotypes), but less suitable for ecological studies.

3.1.5 Nucleic acid techniques

Nucleic acid techniques have greatly advanced in the past 5 to 10 years and have perhaps the best potential to help us to understand aquatic microorganisms and how they respond to environmental stresses. Nucleic acid techniques offer an extremely sensitive means of identification and enumeration of specific species or groups of aquatic microorganisms (Sayler & Layton, 1990) as well as giving us information on the activity of the microorganisms of interest. One of the main advantages of these techniques is that they do not require culturing of the microorganisms.

There have now been many applications of nucleic acid technology to environmental samples. Marine and freshwater systems, sediments, soils, aquifer materials, and waste water treatment processes have all been examined (Holben *et al.*, 1988; Stahl *et al.*, 1988; Steffan *et al.*, 1988; Devereux *et al.*, 1989; Schmidt *et al.*, 1991; Atlas *et al.*, 1992; Picard *et al.*, 1992; Rademaker *et al.*, 1992; Emond *et al.*, 1993; Kane *et al.*, 1993; Kramer & Singleton, 1993; Nakagwa & Yamasato, 1993; Poulsen *et al.*, 1993). While no standard protocol or probe has yet been utilised in a systematic manner for all environments, nucleic acid hybridisation technology has been successful in providing new information in virtually every application (Sayler & Layton, 1990). There is also much potential for developing kits using molecular probes that can be used routinely. Nucleic acid techniques are discussed below in some detail.

Figure 8: Direct staining method of using labelled antibodies to detect bacterial surface antigens (modified from Brock & Madigan, 1991)



Nucleic acid extraction

Many nucleic acid based techniques rely on extracting total nucleic acid, non-selectively, directly from the microbial community under study. When the extracted nucleic acid is to be used for probing, a 'clean' extraction is not always necessary. However purity is essential when nucleic acids are needed for PCR or cloning (see below). Two approaches to extract nucleic acids from environmental samples have been successfully used. The first involves separation of the bacteria from soil particles prior to lysis to release nucleic acids (Holben *et al.*, 1988), and the second involves direct extraction of nucleic acid from samples (Ogram *et al.*, 1987; Steffan *et al.*, 1988). Contaminants, such as humic materials and plant pigment degradation products, can interfere with molecular techniques and are generally removed with the addition of polyvinylpolypyrrolidone (PVPP), an insoluble polymer which binds humic and polyphenolic materials, preventing their copurification with DNA/RNA which occurs when using standard nucleic acid purification protocols (Holben *et al.*, 1988; Stephan and Atlas, 1988; Weller & Ward, 1989; Paul *et al.*, 1990).

Other techniques for purifying samples include the use of sephadex size exclusion columns and magnetic beads (Tsai & Olsen, 1992). Unfortunately, these latter procedures are labour intensive and give poor recovery of nucleic acids, and for these reasons have not been applied widely.

The extracted nucleic acids can be directly analysed in a number of ways (Figure 9). Three particularly useful techniques to provide information on *bacterial biodiversity* from the extracted DNA are:

(i) Rate of re-annealing — the DNA is dissociated (ie. the two DNA strands are separated) by raising its temperature (melting), and then allowed to reassociate (re-anneal) on cooling. The rate at which re-annealing occurs gives an indication of the number of species present, on the basis that the greater the diversity of DNA present the slower the rate of reassociation;

(ii) Cross hybridisation — DNA from one environment is radioactivity labelled, melted as described above and then allowed to reassociate with dissociated DNA from a different environment. The degree of cross-hybridisation (double strand formation) reveals the genetic relatedness of the two samples; and

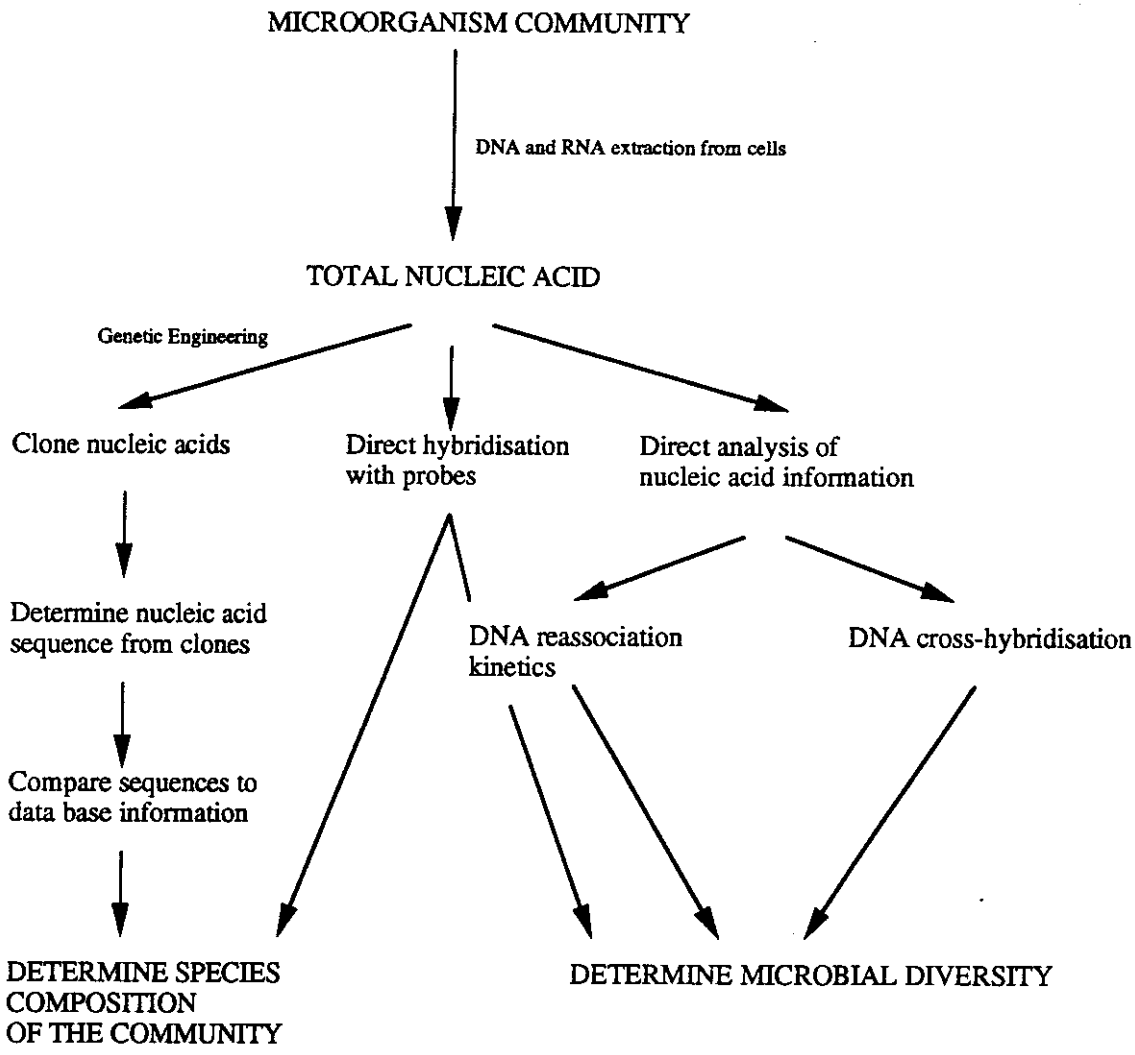
(iii) *Molecular probes* — extracted DNA or RNA can be probed with rRNA or gene probes directly using the hybridisation protocol described below.

These techniques can determine the temporal and spatial scales for changes in bacterial community composition, but cannot determine how these populations have changed, what components are different, and why the population has changed.

Hybridisation

Hybridisation is a useful method for detection of specific species or groups of microorganisms, or functional genes. This method involves using a chemically or radioactively labelled probe for the species, group or genes of interest. The probes are lengths of DNA or RNA and can vary from several nucleotides to several kilobases to entire organism genomes.

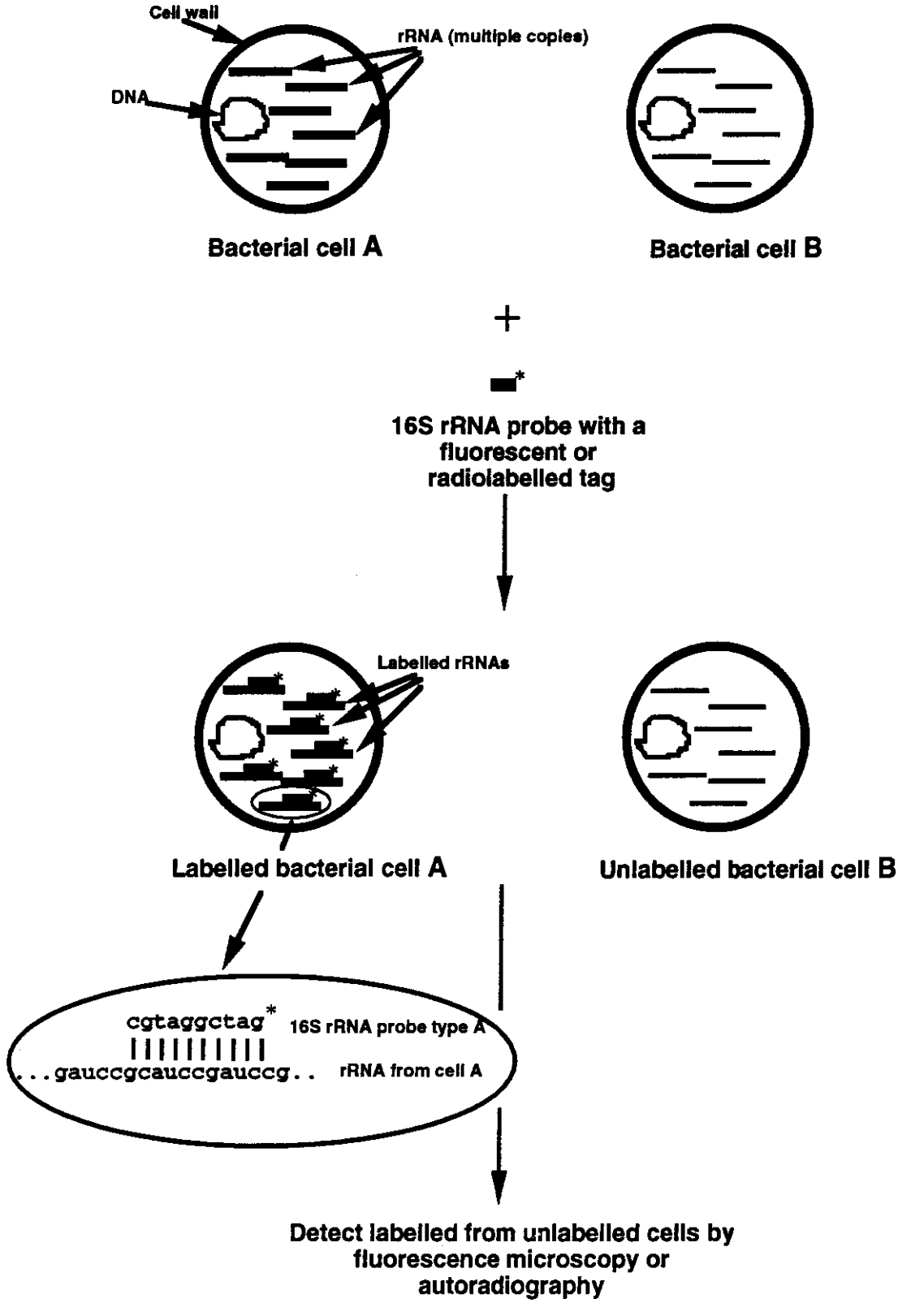
Figure 9: Nucleic acid analysis of microbial communities (modified from Pedros-Alio, 1993)



They bind to DNA or RNA within the cell of the targeted microorganism under specific conditions (Figure 10). Conditions that influence the degree of hybridisation include: temperature, contact time, salt concentration, the degree of mismatch between base pairs, and the length and concentration of the target and probe sequences. The organisms that hybridise to the probe can therefore be quantified, and a community profile of the relative composition of the population determined. As well the activity of the microorganisms may also be determined, depending on the procedure used.

There are two main types of probes currently being used for hybridisation; one is based on nucleic acid sequences of the ribosomal RNA (rRNA) genes, and the other is based on nucleic acid sequences for specific genes (eg. mercury resistance gene, toluene catabolism gene).

Figure 10: Generalised procedure for bacterial cell hybridisation 16S rRNA probe.



The rRNA probe is being extensively used at present and has proved to be applicable to all situations, whereas the specific gene probes are more limited in their use. Both are discussed below.

Ribosomal RNA

Ribosomal RNAs are very useful for identification of species or groups of species based on their phylogenetic classification (Woese, 1987). Several different types of rRNAs are present in prokaryotes and eukaryotes; most work has been done on the small ribosomal subunit (16S in prokaryotes and 18S in eukaryotes), with a smaller amount done on the large ribosomal subunit (23S in prokaryotes and 28S in eukaryotes) as well as the 5S rRNA subunit (in both prokaryotes and eukaryotes).

Here we answer the questions: what are rRNAs and why are they so useful? (see also Olsen *et al.*, 1986; Ward *et al.*, 1992). Ribosomal RNAs are essential components of the protein synthesis machinery in all cellular life forms and are functionally and evolutionally homologous in all organisms; therefore they are useful for studies of all microorganisms except viruses. Ribosomal RNAs are ancient molecules and are highly conserved in overall structure as well as in their nucleotide sequences. Thus homologous rRNAs are readily identified across kingdoms, with some sequence stretches being invariant (conserved) for all organisms, while others vary between organisms. The conserved sequences allows the alignment of variable sequences so that only homologous nucleotides are used in any phylogenetic analysis. Ribosomal RNAs constitute a significant component of cellular mass and are easily recovered from all types of organisms for analysis and accumulation of data base reference sequences. The rRNA genes seem to lack mechanisms for horizontal transfer between organisms, and for this reason, relationships between rRNAs reflect the evolutionary relationships between organisms. Because of these properties, researchers have been able to identify virtually any organism or group of related organisms on the basis of their rRNA. Species and group-specific rRNA probes can be designed to hybridise (see above for hybridisation protocol) to phylogenetically more or less conserved regions of the rRNA. Different levels of specificity can be achieved in the design of the probe. Many specific 16S rRNA probes are already available (see Ward *et al.*, 1992; Amann *et al.*, 1995; and Table 1). Particularly useful applications of rRNA probes are to define a group of microorganisms that has functional significance (eg. sulphate-reducing bacteria), or to detect a specific organism of interest (eg. the pathogen *Cryptosporidium* (Vesey *et al.* 1995, in press).

Table 1: Examples of phylogenetically related groups of microorganisms for which rRNA oligonucleotide probes are currently available

Target Organism	Source
all eubacteria	Giovannoni *et al.*, 1988
all eubacteria	Woese *et al.*, 1990
all archeabacteria	Giovannoni *et al.*, 1988
all archeabacteria	Amann *et al.*, 1990b
all archeabacteria	DeLong *et al.*, 1989
all eukaryotes	Giovannoni *et al.*, 1988
all eukaryotes	Amann *et al.*, 1990b
all organisms	Giovannoni *et al.*, 1988
all organisms	Amann *et al.*, 1990b
gram-positive bacteria with high G+C DNA content	Wagner *et al.*, 1994
Sulfate-reducing bacteria	Amann *et al.*, 1990b
alpha subclass of proteobacteria	Wagner *et al.*, 1993; Manz *et al.*, 1992
gamma subclass of proteobacteria	Wagner *et al.*, 1993; Manz *et al.*, 1992
beta subclass of proteobacteria	Wagner *et al.*, 1993; Manz *et al.*, 1992
Acinetobacter	Wagner *et al.*, 1994
Methylotrophic bacteria Type I	Tsien *et al.*, 1990
Methylotrophic bacteria Type II	Tsien *et al.*, 1990
Cytophaga-Flavobacterium cluster	Wagner *et al.*, 1994

To make a specific 16S rRNA probe, it is necessary to know the nucleic acid sequence of the target species or groups of species, and also the sequences of those species which are not to be targeted. This information can often be obtained from the 'The Ribosomal RNA Database Project' (Olsen *et al.*, 1991), which contains hundreds of 16S rRNA sequences from many different species and is accessible via computer E-Mail systems to all researchers.

Using molecular techniques enables bacteria and some other microorganisms to be studied in two ways, without the need for culturing:

- the rRNA can be extracted and the rRNA sequence determined by PCR and/or cloning methods. This can be done for pure culture or for mixed bacterial communities in water samples. The sequences obtained are then compared to database sequences to give information on the species or groups of species present in the sample.
- the bacterial community in a water sample can be probed with oligonucleotide sequences that will detect specific bacteria or groups of bacteria of interest.

Despite the advantages in using rRNA, it is important to note that there are some potential problems with the technique. These include:

(i) the accurate analysis of native bacteria by cloning and sequencing or hybridisation depends on the unbiased recovery of nucleic acids from the natural sample. There is evidence that sampling bias can result from cell recovery procedures that select against certain types of microorganisms (Goebel *et al.*, 1987; Hahn *et al.*, 1990a,b).

(ii) bias in the cloning procedure when using PCR can result in the naturally occurring rRNAs being amplified up in unequal proportions.

(iii) the reactivity of oligonucleotide rRNA probes cannot be predicted exactly. It is always possible that undiscovered sequence types may cross-react with a probe thought to be specific. Thus, it is essential that the specificity of any probe be tested empirically, a process that can be very time consuming.

(iv) rRNA probing of whole cells relies on the *in situ* penetration of the probe through the cell membrane, and with some bacteria permeability is a major problem.

(v) rRNA probes provide phylogenetic information that may or may not provide information on the functional role of that organism in the ecosystem under study.

Specific gene probes

An alternative to the 16S rRNA probe approach is to make probes for specific genes which define 'functional groups' of organisms. For example, mercury-resistance bacteria can be detected using the gene probe for the mercury resistant (*mer*) gene regardless of the phylogenetic groups of the bacteria containing this gene (Barkay *et al.*, 1985; Barkay & Olsen, 1986; Barkay *et al.*, 1989). It is possible that the identification of functional genes that are related to ecological processes could be used to predict the fate and indirectly the effects of pollutants in the environment. Unfortunately, there are relatively few of these types of probes because the gene of interest must have previously been characterised. The use of these probes has similar limitations to those described above for rRNA probes. In addition, these probes will not detect organisms that have a truly novel sequence for the particular activity of interest. Nevertheless they often provide phenotypic and genotypic selectivity which rRNA genes do not.

Whole cell hybridisations

Both of the above discussed probes can be used to evaluate the relative amounts of particular organism or groups of organisms present in a mixed population, by measuring the amount of a group-specific probe that is bound to whole cells in the mixed population. Two techniques have been developed:

(i) *Bulk dot blot* — fixed cells are vacuum blotted onto solid support membranes and hybridised under conditions specific for the chosen probe (Giovannoni *et al.*, 1988). Cells fixed with formaldehyde or glutaraldehyde (preferably prepared in the field) retain their ability to hybridise with probes even after months of storage at -70°C. This bulk dot blot method is best used to give an indication of the approximate numbers of different types of bacteria present.

If the technique is to be used for quantitative analysis of natural samples, the influence of variables, such as permeability of the fixed cells and accessibility of the probe target in the fixed preparation, must be investigated since these can influence the amount of the probe that is specifically bound to the cell. It is quite common for some form of pretreatment to be used to permeabilise the cell membrane (Fliss *et al.*, 1991; Zarda *et al.*, 1991; Amann *et al.*, 1992a; Hahn *et al.*, 1993), but despite this certain cell types (eg. some Gram positive species) may remain relatively impermeable to probes (Amann *et al.*, 1992a).

(ii) *Slide hybridisation* — a second approach to quantifying natural bacterial populations is to use hybridisation on microscope slides which allows individual cells to be distinguished. If the probe used contains a fluorescent label, the technique is called *Fluorescent In-Situ Hybridisation (FISH)*, but other labels, radioactivity and antibodies can also be used (Section 3.1.4). The technique involves first measuring the total number of cells (using DAPI or AO), and then determining the number of cells that hybridise with different probes. This approach provides a means of microscopically counting the constituents of a bacterial population. Since the number of labelled cells is determined and not the amount of probe bound, this method is less dependent upon variations in the efficiency of probe binding by different cell types. Cells that have been fixed, dried onto microscope slides and stored at -70°C will last for one year without significant loss in their ability to bind hybridisation probes, making this method readily applicable in field studies (Giovannoni *et al.*, 1988). With active cells, the degree of fluorescence depends on the life stage of the organism, while non-viable organisms will have little or no fluorescence.

Labelling probes

Molecular probes need to be labelled in some way to enable their measurement when bound to bacteria. Labels include: radioisotopes (Giovannoni *et al.*, 1988), fluorescence (DeLong *et al.*, 1989; Amann *et al.*, 1990a,b; Tsien *et al.*, 1990) and digoxigenin (Zarda *et al.*, 1991; Amann *et al.*, 1992b; Vilaro *et al.*, 1993). Each labelling technique has its advantages and disadvantages. For example, radioactively labelled probes are the smallest in size (Giovannoni *et al.*, 1988), and can generally penetrate the cell membrane more easily. Radioactive labels are better suited to bulk cell dot blots since they are very time consuming when used for slide hybridisation. Fluorescent labels (using fluorescein or rhodamine) are not useful for bulk cell dot blots because the results are difficult to quantify. They are, however, very effective when used for slide hybridisations. Background fluorescence in natural samples (eg. soil, sediment, plant or animal tissue) can make it difficult to identify cells to which fluorescent probes are attached. Digoxigenin-labelled probes provide the most sensitive means of selection for both bulk dot blots and slides hybridisations. However, this technique has a major problem in that the probe is made very large by the label and this results in major difficulties in the probe penetrating the cells.

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is now in common use for detecting native bacteria (reviews see Steffan & Atlas, 1988; Cox, 1993). PCR involves enzymatically amplifying a region of DNA or RNA defined by flanking primers, so that the DNA/RNA region becomes abundant enough to be detected or to be used in further manipulations. Therefore, PCR is a very useful technique for the detection of specific bacteria, especially when they are present in low numbers. It is also a very rapid technique, being capable of producing results on the same day when used to test aquatic systems, and has the potential to be used in kit form.

PCR involves three stages: (i) DNA is melted to convert double-stranded DNA to single-stranded DNA, (ii) primers are annealed to the target DNA, and (iii) the DNA is extended by nucleotide addition from the primers by action of DNA polymerase (Figure 11). The primers are designed to hybridise to regions of DNA flanking the desired target gene sequence. Melting the product DNA duplexes and repeating the process many times results in an exponential increase in the amount of target DNA.

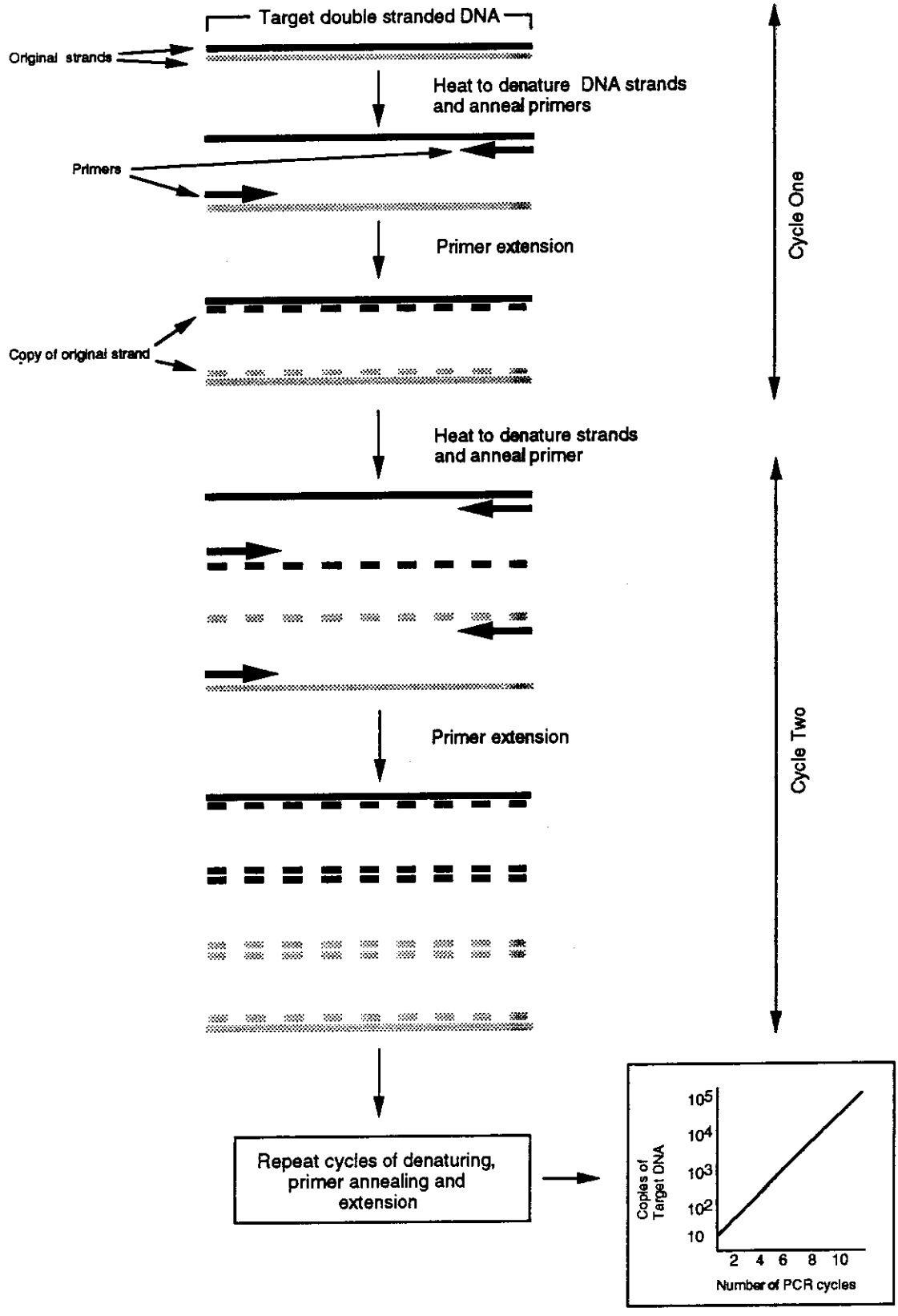
There are presently a number of applications of PCR in aquatic microbial ecology:

(i) as a tool to make cloning of genes or regions of DNA and RNA quicker and easier.

(ii) for the direct detection of bacteria in natural samples. This involves the detection of specific bacterial species (e.g pathogens) against a background of ambient bacteria. Several protocols are already available to detect toxigenic *E. coli* (Frankel *et al.*, 1989; Pollard *et al.*, 1990; Victor *et al.*, 1991) and others are being developed both in Australia and overseas.

(iii) quantitation of PCR amplified product. This is complicated by the fact that the amount of PCR products formed during the reaction increases exponentially, and therefore small differences in any of the parameters that effect the efficiency of amplification can dramatically affect the outcome of the reaction. Gilliland *et al.* (1990a, b) have developed a competitive PCR scheme in which target DNA is quantified by co-amplifying target DNA in the presence of known quantities of a competitive DNA. We predict that these quantification techniques will become increasingly sophisticated over the next few years.

Figure 11: The Polymerse Chain Reaction (PCR) for the amplification of target DNA sequences (modified from Brock and Madigan, 1991)

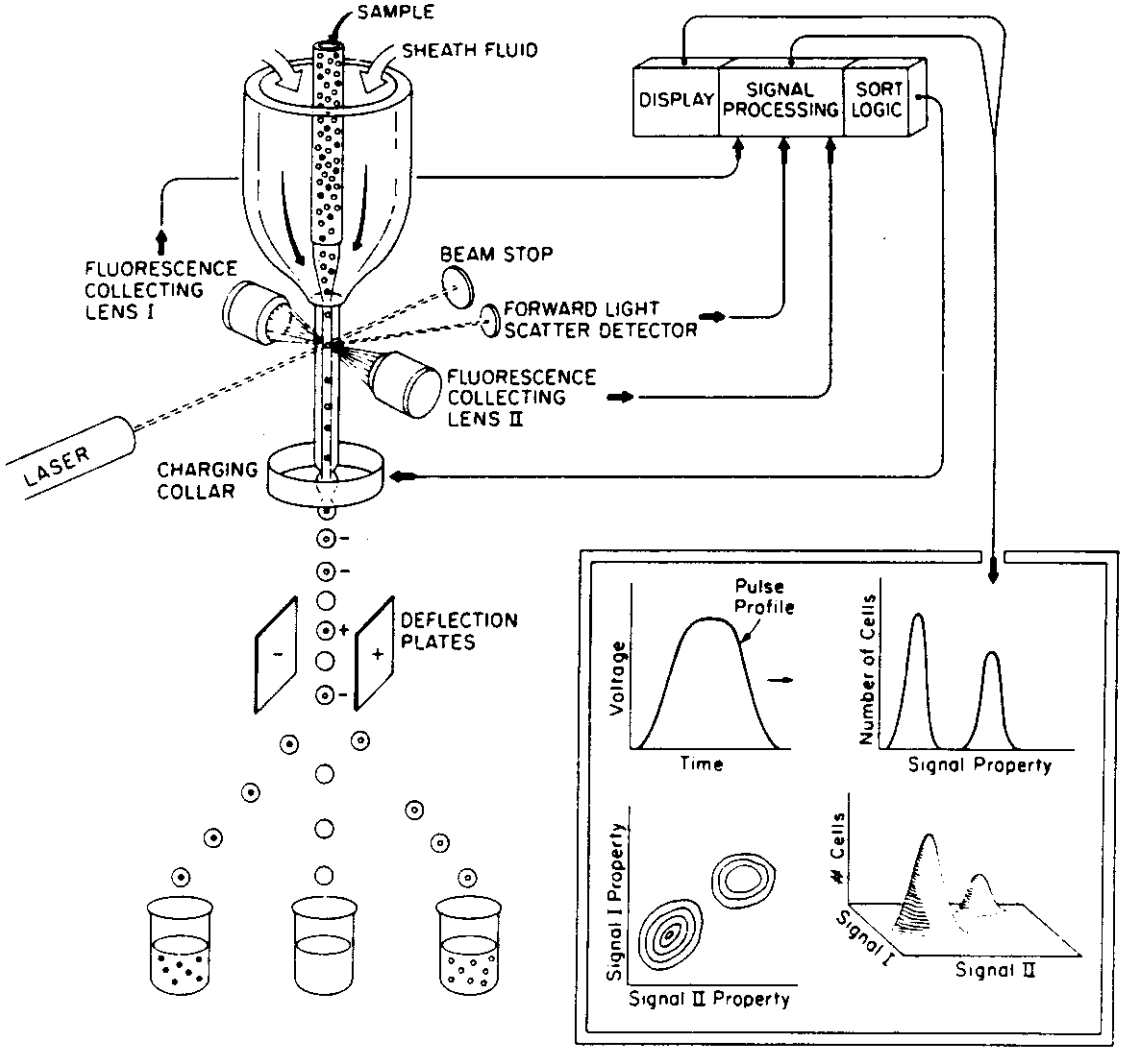


The application of PCR to natural samples is not without its limitations. For example, PCR applications require very pure samples of the target DNA or RNA, and also require knowledge of the DNA or protein sequence so that primers can be designed to amplify a target sequence (this latter requirement limits PCR applications to highly conserved genes, such as rRNA). There is also considerable potential for production of sequence artefacts during the PCR reaction (Liesack *et al.*, 1991) and contamination from outside sources that can be amplified, hence the need for strict controls.

3.1.6 Flow Cytometry

Flow cytometry is a very rapid method for quantitatively measuring the physical or chemical characteristics of cells. The cells are flowed in single file into a focused light beam at around 10 m s^{-1} and this allows approximately 2,000-10,000 particles s^{-1} to be analysed (Figure 12). The light source can either be a Hg-vapour lamp or one of an assortment of different lasers. Various parameters of individual cell, such as fluorescence (FL), forward angle light scatter (FALS) and side (90°) angle light scatter (SALS), can be measured simultaneously. Forward angle light scatter provides information on the size of a cell, while side angle light scatter correlates with how refractile the cell is to light. SALS is thought to provide information on the surface properties and internal structure of the cells. Fluorescence can be used to detect autofluorescence in the cell emanating from cellular components such as flavin nucleotides, pyridine, chlorophyll and other photosynthetic pigments. Fluorescence can also be used to identify fluorescent stains or specific tags attached to organisms of interest.

Figure 12: Flow cytometer/cell sorter (modified from Kemp *et al.*, 1993)



In natural samples, FALS, SALS and autofluorescence alone can provide sufficient information to enable clustering of cells into particular 'types' (Yentsch, 1990). Differences based on autofluorescence of phycoerythrin (orange) and chlorophyll (red) pigments have been used to rapidly discriminate and enumerate cyanobacterial phototrophs in samples containing other phytoplankton (Yentsch *et al.*, 1983; Olson *et al.*, 1985; Li & Wood, 1988). This ability to discriminate and enumerate microorganisms allows the rapid assessment of biodiversity to be achieved. Flow cytometry can also be used to count microorganisms in various 'groups' allowing enumeration of indicator groups (eg. cyanobacteria) which are recognised as important in assessing river health.

A spectacularly successful application of flow cytometry was the discovery of *Prochloron*-type phototrophs in the oceans (Chisholm *et al.*, 1988). Prochlorophytes are non-culturable prokaryotes that are phylogenetically closely related to the progenitors of the chloroplasts found in algae and plants. The prochlorophytes however contain different fluorescent pigment from the cyanobacteria, and it was this difference that enabled their detection in the ocean using flow cytometry (Chisholm *et al.*, 1988).

It is unlikely that specific bacteria can be identified in natural samples using only FALS, SALS and autofluorescence (Vesey *et al.*, 1994a). Usually, it is necessary to label the cells of interest with fluorescent molecules such as fluorescein isothiocyanate (FITC), R-phycoerythrin (RPE) or alophycocyanine (APC). A wide range of fluorescent labels is available. They can be distinguished from each other because they absorb and emit light at different wavelengths. Cells can be tagged using specific antibodies, lectins or nucleic acid probes conjugated to particular fluorochromes. These labels may be used individually or in combination to permit simultaneous detection of different organisms in the same sample or improved discrimination of a particular organism (Vesey *et al.*, 1994a).

Flow cytometry has been use for the rapid detection of specific non-culturable microorganisms in water samples. For example, Vesey and co-workers (Vesey *et al.*, 1993a,b, 1994a) have described the use of a sorting flow cytometer to detect the enteropathogens *Cryptosporidium* oocysts and *Giardia* cysts in concentrated water samples. Sensitivities as high as 1 cyst or oocyst in 10 litres of water have been achieved. These methods are now routinely used to detect these organisms in drinking waters (Vesey *et al.*, 1993b; 1994b).

Thus, flow cytometry has considerable potential as a rapid technique for the measurement of bacterial indicators. This technique can analyse 10,000 -1,000,000 cells per minute, and is readily automated. A number of parameters may be measured simultaneously enabling a variety of microbial indicators to be analysed simultaneously. The technology does not require the microbial bioindicator to be cultured, thus allowing almost instantaneous results to be obtained and enabling the analysis of non-culturable or slow growing organisms. When combined with nucleic acid or antibody methods, flow cytometry has the potential to enumerate microorganisms at the strain, species, genus or kingdom level. Alternatively, particular functional microbial groups may be enumerated using either nucleic acid or antibody technology. For example, Orellana *et al.* (1988) made antibodies to ribulose-1,5-bisphosphate carboxylase oxygenease (Rubisco), the enzyme involved in the fixation of CO_2 in most organisms capable of CO_2-fixation, and used these antibodies in the indirect immunofluorescence analysis of phytoplankton using flow cytometry.

We believe that flow cytometry will revolutionise detection methods used in environmental microbiology and microbial ecology. At present, the high cost of the instruments (>$A150,000), combined with the relatively small number of standard protocols for water samples, is limiting the applications of these instruments. Developments in progress, particularly in Australia, directed at the analysis of environmental samples, will increase the range of organisms that can be detected and enumerated using this technology, and decrease the cost and complexity of the instrumentation.

3.1.7 Confocal scanning laser microscopy

Digital image analysis with confocal scanning laser microscopy, is another relatively new technique that has great potential for providing better information about the spatial distribution, gene exchange, and to some extent, growth in complex natural microbial communities. Its advantage over normal epifluorescence microscopy is that it has much better resolving power (it can scan through a bacteria cell) and background fluorescence interference is eliminated. At present, the equipment is expensive and the technique rather complicated. However, the advantages offered by the technique in improving knowledge of the role of microorganisms in natural environments, means that its use will increase in the future.

3.2 METHODS FOR ESTIMATING MICROBIAL BIOMASS

Measurements of bacterial biomass are critical to understanding the ecological role of bacteria in aquatic systems, since biomass measurements allow evaluation of the bacterial population's potential activity in the environment, and its potential as a food source for higher trophic levels. However, most of the methods currently used to estimate bacterial biomass in natural samples produce rather crude estimates (Sharma *et al.*, 1993). In this section we review two methods based on the total microbial population — epifluorescence biovolume-biomass measurement and measurement of concentrations of certain biochemical compounds present in the microbial cells (eg. DNA, proteins, phospholipids, chlorophyll), and a new technique called Sedimentation field flow fractionation that shows great promise in being able to provide more accurate measurement of bacterial biomass in natural samples.

3.2.1 Biomass from biovolume estimates

Several microscopy techniques are available to estimate biomass, the most widely used being the direct epifluorescence method (Daley, 1979; Newell *et al.*, 1986; Fry, 1988; Bratbak, 1993). This involves using a number of fluorochromes to stain microorganisms, the most commonly used being DAPI and Acridine Orange. The cell biovolume is then estimated, and a suitable biovolume:carbon conversion factor applied to this figure to estimate the biomass. The disadvantages of this approach are: (i) the very significant errors in accurately estimating numbers and biovolume of bacterial cells under the microscope, and (ii) the possible inappropriateness of the current biovolume:carbon conversion factors that have all been determined using planktonic bacteria. Two newer methods that will lead to improvements in accuracy will be image analysis with CCD to quantify fluorescent emission, and confocal scanning laser microscopy (Section 3.1.7).

3.2.2 Biomass from specific indicator biochemicals

DNA

Concentrations of DNA are maintained in relatively constant proportions within microorganisms, and can therefore be used for biomass estimation. The reaction of DNA with a fluorescent dye (eg. ethidium bromide or Hoechst 33258) and subsequent spectrofluorometric detection, is generally use for environmental samples. In these assays, the DNA needs to be carefully purified to prevent interference. Additionally, the presence of eukaryotic DNA also needs to be carefully controlled.

Proteins

Although the concentrations of proteins are quite easily measured (eg. Lowry *et al.*, 1951), the use of protein measurement for estimating the biomass of microorganisms is limited to situations where the background protein levels from non-microbial sources are negligible, and by the fact that different microorganisms contain different amounts of protein. Because of these constraints, total protein measurements have only limited applicability for environmental samples.

ATP

Although ATP is present in all microorganisms, the actual concentration in a particular cell can vary widely because of changes in nutritional or physiological conditions, and the ATP:C ratio accordingly is known to vary from less than 50 to greater than 400. For this reason, and the failure of ATP to distinguish between bacteria, protozoa and algae, ATP measures are now rarely used to estimate bacterial biomass.

Phospholipids

Total phospholipid analysis is used to estimate biomass since phospholipids are a major and relatively constant component of the membranes of microorganisms (White *et al.*, 1979). More detailed information on different groups of microorganisms present can be obtained if the phospholipids are separated using techniques such as gas chromatography — mass spectrometry (White, 1983a; White, 1983b; Parkes, 1987). Boon and co-workers have analysed phospholipid profiles for microorganisms in Australian billabongs and found significant amounts of signature phospholipids for groups such as methanotrophic bacteria (Boon & Sorrell, 1991; Scholz & Boon, 1993a,b,c).

Muramic acid

Muramic acid, a constituent of bacterial cell walls but not algal cells, has also been used to estimate biomass, since there are quite well known relationships between muramic acid content and bacterial biomass (Moriarty, 1975, 1977, 1978). The conversion of muramic acid concentrations to biomass assumes that all Gram-positive bacteria have a specific and different amount of muramic acid in their cell walls from Gram-negative bacteria. In reality, there is a gradient of concentrations of muramic acid in Gram-positive and Gram-negative bacteria, and erroneous estimates of the proportions will yield inaccurate estimates of biomass in systems such as soils, where Gram-negative bacteria may not be present in high proportions. This is less of a problem for native aquatic bacteria since they are mostly Gram-negative. A bigger problem is caused by the fact that these Gram-negative bacteria contain less than 5% of the muramic acid content of Gram-positive bacteria, leading to a reduced sensitivity of the method for aquatic bacteria.

Photosynthetic pigments

In the absence of plants, it is possible to estimate numbers of photosynthetic microorganisms by measuring chlorophyll or other photosynthetic pigments. Chlorophyll-a, the dominant photosynthetic pigment in cyanobacteria and algae, is a useful measure of the biomass of these photosynthetic microorganisms even though there may not be a constant relationship between biomass and chlorophyll content (Banse, 1977).

3.2.3 Sedimentation field flow fractionation

Sedimentation field flow fractionation (SdFFF), can be used to enumerate bacteria and determine their density, size and mass. This technique is being developed at the Water Studies Centre, Monash University; early work has shown great promise with estimates of bacterial biomass having approximately 6-fold lower error than for conventional methods (Sharma *et al.*, 1993).

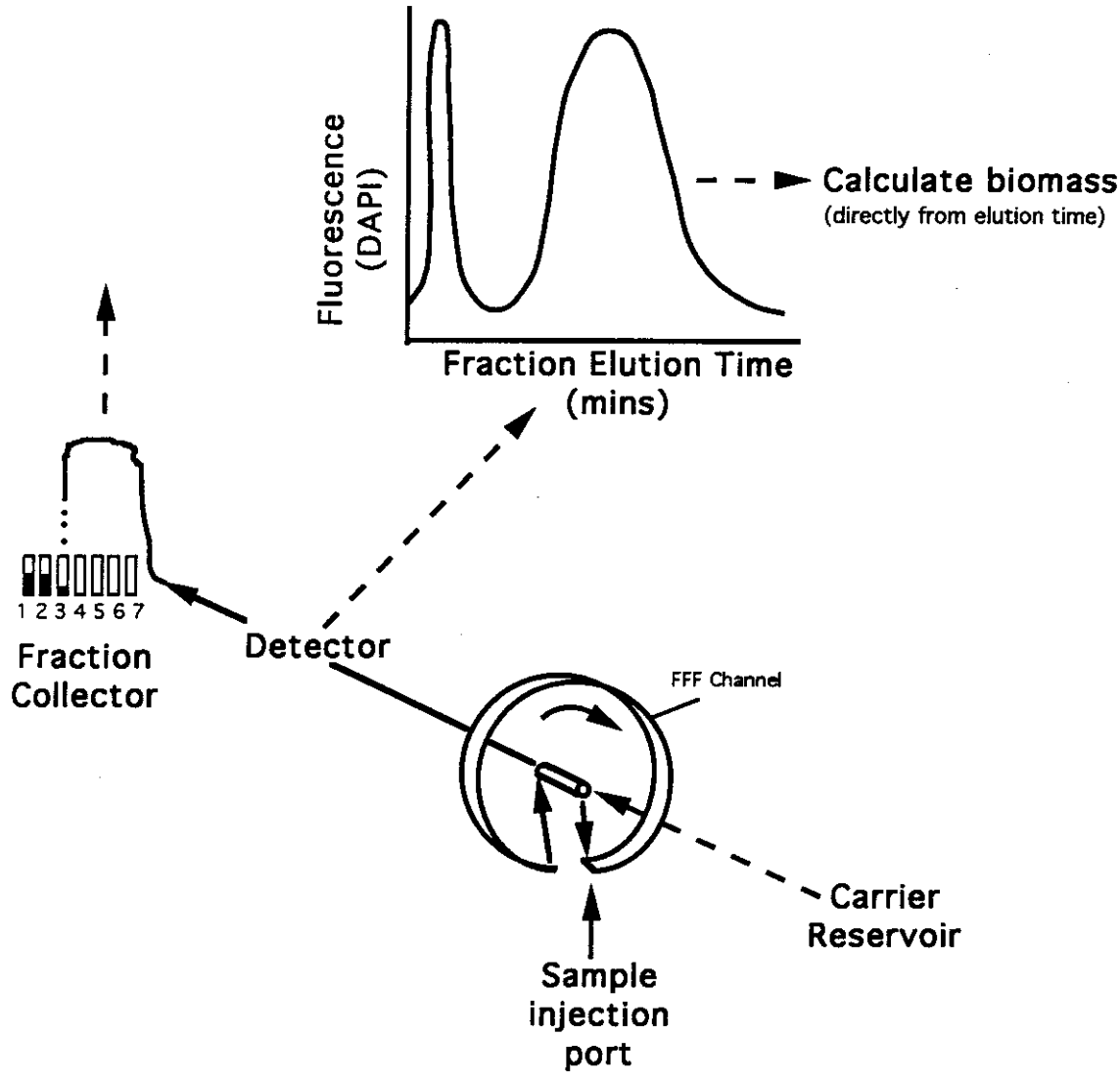
SdFFF is a separation technique in which separation of the cells is achieved in a narrow channel, after which the components are eluted into a sensitive detector and, if required, a fraction collector (Figure 13). The sample is injected at one end of the channel which spins such that a centrifugal field is applied perpendicular to the flat face of the channel, causing particles of different effective mass to be forced to one of the channel walls. After a 'relaxation period', when particles adjust their position relative to the wall due to Brownian motion, the channel flow is turned on. Because the SdFFF separation channel is empty, the carrier fluid develops a parabolic velocity profile with highest flow rates in the centre of the channel and velocities approaching zero near each channel wall, and will sweep bacteria of different buoyant masses downstream at different velocities. Sample components are subsequently flushed into a detector. The biomass of the cells in each fraction can then be directly calculated based on the characteristic elution time from the SdFFF channel.

At present, work is being undertaken at the Water Studies Centre to combine the SdFFF technique with molecular probe technology, so that the biomass of subsets of the bacterial population that hybridise to specific probes can be determine in addition to the total biomass of a given population.

3.3 METHODS FOR MEASURING RATES OF BACTERIAL GROWTH AND ACTIVITY

Biomass cannot be directly related to growth rates of bacteria because many bacteria are thought to be in a starved or dormant condition in the aquatic environments. Therefore, measurement of bacterial activity is important in understanding ecosystem function. Many methods are available for measuring the rates of bacterial activity. However, one of the main difficulties with these techniques is that most require manipulation of the system in ways that can alter the micro-environment that controls the rate of bacterial activity. For example, samples taken from a river for measurement of bacterial activity by addition of radioisotopes, will not experience the same water flow and nutrient exchange as the *in situ* sample. Techniques using short incubation times have been developed to partially overcome these problems, since these short assay periods guard against the tendency for samples removed from their surroundings to change in non-specific ways with time and ensure that the measurements obtained are representative of the sample as it existed in the natural environment. Furthermore, if bacteria are metabolically active in an environment, but are present in relatively small numbers, the activity assay may not be sensitive enough to measure whether a reaction has occurred.

Figure 13: Sedimentation field flow fractionation



3.3.1 Microbial growth

The growth rate of the total bacterial population can be determined by measuring processes that occur in all microorganisms; examples include measuring DNA synthesis (Karl, 1986; Moriarty, 1986), protein production (Kirshman *et al.*, 1985; Simon & Azam, 1989) or lipid production (Moriarty *et al.*, 1985). Isotopic methods are probably the most widely used means of evaluating growth of native bacteria because they are extremely sensitive and highly specific. The growth of major groups of microorganisms can be determined separately, for example using $^{14}CO_2$ uptake for algae and cyanobacteria, and tritiated thymidine (^{3}HTdr) uptake for heterotrophic aerobes and fermenters (Riemann & Bell, 1992).

Thymidine uptake

The thymidine method measures the incorporation of [^{3}H]-labelled thymidine into DNA in heterotrophic bacteria, the incorporation rate being related to the rate of bacterial production per unit volume or area per unit time (Riemann & Bell, 1992). Standard conversion factors are available to estimate growth rate from the thymidine incorporation rate. These factors are based on the assumption that DNA is synthesised in growing cells at a rate proportional to biomass, and that the rate of DNA synthesis reflects the growth rate of the bacteria. An advantage of the technique is that cyanobacteria cannot incorporate ^{3}H-thymidine into DNA (Bern, 1985), or do so too slowly for accurate measurement; the latter is also true for nitrifiers.

Leucine uptake

The rate of protein synthesis has also been used as a measure of growth, since proteins constitute a major proportion of the mass in a microbial cell. ^{3}H-leucine is used for this method as it readily incorporates into proteins (Kirshman *et al.*, 1985; Riemann & Bell, 1992). The advantage of this method over ^{3}H-thymidine is that it is orders of magnitude more sensitive, provided all bacteria are growing rapidly (Simon & Azam, 1989). The ^{3}H-thymidine and ^{3}H-leucine methods are complementary in that they measure different aspects of microbial growth (Boulton & Boon, 1991). The ^{3}H-thymidine method measures DNA synthesis, and therefore cell division, whereas the ^{3}H-leucine method measures metabolism. If unbalanced growth occurs, as for example in a natural environment where growth is severly limited by organic carbon, the leucine method will greatly overestimate the actual production of new cells. Thus, use of both methods simultaneously will generate the most reliable estimates of bacterial production (Daneri *et al.*, 1994).

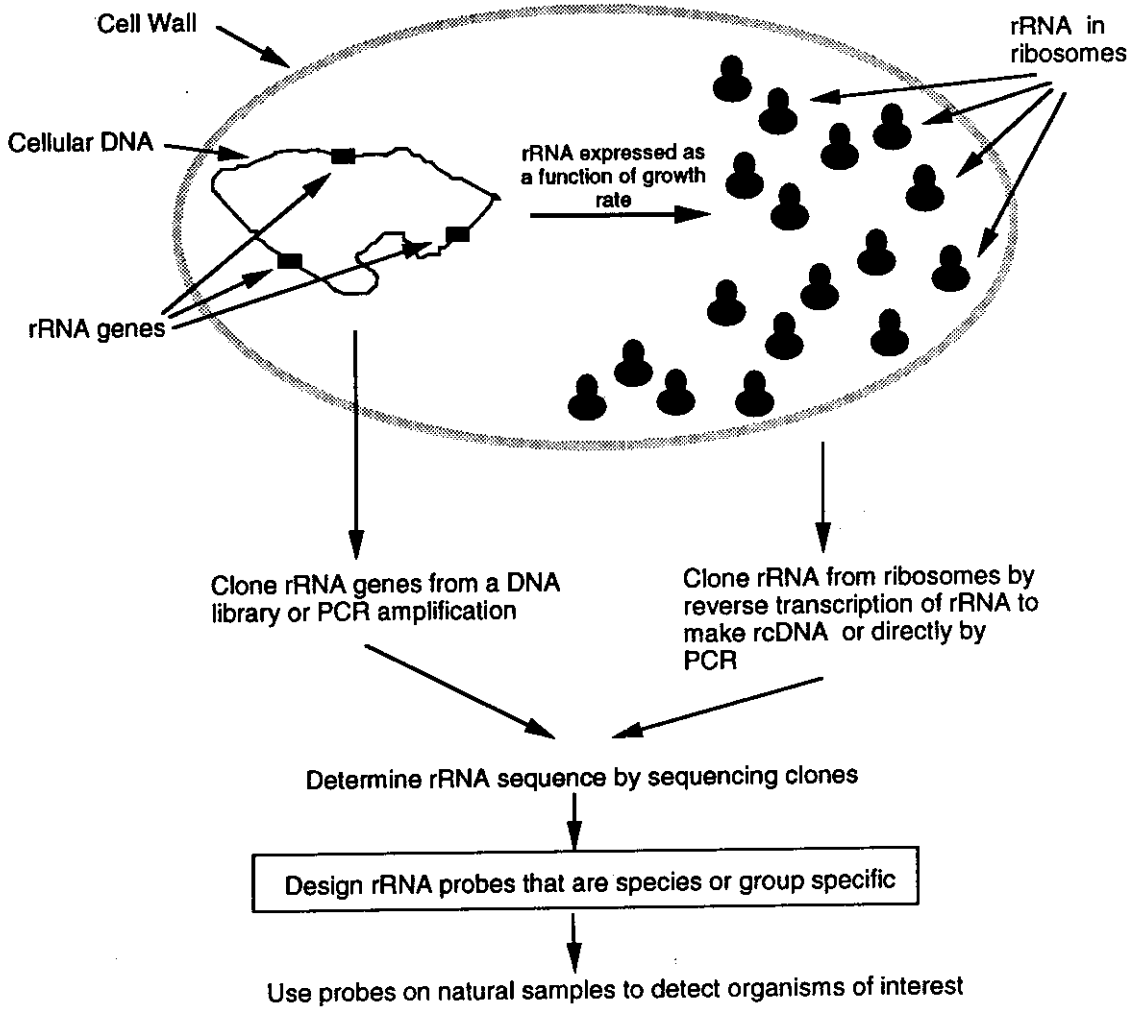
Lipids

Another method used to measure microbial growth rates is incorporation of radiolabelled orthophosphate (^{32}P-PO_4^{3-}) into phospholipids (Moriarty *et al.*, 1985; Moriarty *et al.*, 1990).

Nucleic acid techniques

Use of rRNA probes to measure metabolic activity has also been reported (Ingraham *et al.*, 1983). This method is based on the observation that the rRNA content of cells is proportional to growth rate over a wide range of growth rates (Figure 14). Thus, measurement of the amount of a specific probe hybridised per unit of biomass will provide an estimate of the metabolic activity (ratio of actively growing to dormant cells) in a particular microbial population. This technique is still in the early stages of development and more studies are needed before it can be confidently applied to environmental samples.

Figure 14: Ribosomal RNA expression and overview of strategy of rRNA probe design (modified from Ward *et al.*, 1992)



3.3.2 Microbial activity

Microbial activity is often a more sensitive indicator of microbial responses to changing environmental conditions than are other parameters (Boulton & Boon, 1991). In this section we review three methods used to measure microbial activity.

Respiration

The rate of consumption of dissolved organic carbon (DOC) compounds by heterotrophic bacteria can be measured by adding tracer amounts of radiolabelled ^{14}C-labelled DOC to the system and following the fate of the ^{14}C (ie. incorporation into bacterial biomass or releases as respired CO_2) over time (Wright & Hobbie, 1966; Hobbie & Crawford, 1969). It is important to have controls for these experiments to show that the transformations being measured are due to bacterial processes and not, for example, strictly chemical transformations.

P/R ratios

The ratio of gross primary production (P) to community respiration (R) is used to indicate whether a river or stream ecosystem is a net producer (P/R >1) or consumer (P/R <1) of organic matter. The terms 'autotrophic' and 'heterotrophic' are often used to classify stream ecosystems having P/R ratios >1 and <1 respectively.

A large number of factors can change the P/R ratio in a particular stream ecosystem, including: changes in primary production, changes in the amount and type of allochthonous organic matter input to the stream, changes in the relative rates of heterotrophic respiration of autochthonous and allochthonous organic matter, and the proportions of the macroinvertebrate biomass feeding upon autochthonous and allochthonous organic matter. Such ecosystem changes can be caused by such activities as clearing the riparian vegetation (that could alter the P/R ratio by allowing more light to enter the stream which would stimulate more primary production, and by reducing the input of allochthonous organic matter), the addition of nutrients (stimulate both primary production and growth of microbes), or the addition of organic-rich effluents (would stimulate heterotrophic respiration).

There have been only four studies of metabolism in Australian rivers and streams. Unpublished work cited by Pidgeon and Cairns (1981) suggested that a small stream near Armidale, NSW, partially shaded by natural eucalyptus vegetation, was autotrophic with a P/R ratio of 1.2. Lake *et al.* (1987) concluded that, assuming this type of stream was widespread in temperate Australia, a significant proportion of Australian streams may be autotrophic rather than heterotrophic. Chessman (1985) measured metabolism in the upper Latrobe River in Victoria and reported very low P/R ratios (0.05-0.5) as well as very low rates of net daily metabolism (-1.88 to -4.02 g O_2 m^{-2} day^{-1}). More recently, Davies (1993) measured metabolism in three relatively undisturbed upland streams of the northern jarrah forest in Western Australia. Using hemispherical chambers to measure net metabolism in three habitat types — cobbles, riffles and organic depositional areas, he found that on an annual basis (24 h) respiration exceeded gross primary production, although both were very low (mean annual GPP (0.03 to 0.65 g C m^{-2} day^{-1}; community respiration 0.04 to 0.72 g C m^{-2} day^{-1}; net daily metabolism -0.02 to -0.07 g C m^{-2} day^{-1}). The low metabolic rates in these streams was attributed to the very low nutrient concentrations and to the low 'quality' of the allochthonous matter (mainly *Eucalyptus marginata*). Treadwell (Monash University, personal communication, June 1994) has also measured metabolism at four sites within the Acheron River system in Victoria using *in situ* perspex chambers placed over plastic trays that were buried at each site and allowed to colonise for 4 weeks. He found negative net daily metabolism (indicating heterotrophic conditions) at all sites (exception was one site which had positive values in spring). There was considerable variation between the sites and within the one site with season. Gross primary production ranged from 0.009 to 10.6 g O_2 m^{-2} day^{-1}, 24 h respiration from 0.14 to 15.80 g O_2 m^{-2} day^{-1}, and net daily metabolism from +4.50 to -12.20 g O_2 m^{-2} day^{-1}. Correlation analysis indicated that metabolism was influenced by site, benthic organic matter, stream water temperature, chlorophyll-a concentration and PAR at the stream bed.

Specific measures of rates of microbial activity

A large variety of enzyme assays can also be used to measure the activity of native microorganisms (Morgan & Pickup, 1993). Enzyme activities give an indication of the transformation of organic matter in the aquatic environment. Some, such as measurement of dehydrogenase, esterase and phosphatase activities, measure the general activities of a relatively large portion of the microbial community, while others, such as measurement of cellulase, chitinase, and nitrogenase activities, measure the metabolic functions of small but important segments of the microbial community.

In Australia, enzyme activities have been reported for a number of billabongs (Boon 1989a,b, 1990, Boon *et al.*, 1990). Alkaline phosphatase and aminopeptidase were studied intensively because they were the most active enzymes in the billabongs and were known to be important in the regeneration of phosphorus and nitrogen (Boon *et al.*, 1990). These studies allowed comparisons of activity between different aquatic systems.

A number of isotopic methods are now available for the measurement of specific microbial processes, and these are widely used for determining microbial activity (Blackburn & Blackburn, 1993). Examples include:

- the measurement of photosynthesis by the light-dependent uptake of $^{14}CO_2$ into microbial cells;
- heterotrophic activity from the ^{14}C glucose turnover rate;
- intermediate carbon metabolites production rate by the turnover of acetate (Christensen & Blackburn, 1982);
- sulfate reduction by the rate of conversion of $^{35}SO_4^{2-}$ to $H_2{}^{35}S$ (Blackburn & Blackburn, 1993);
- rate of organic nitrogen mineralisation by the dilution of added $^{15}NH_4^+$ (Blackburn & Henriksen, 1983) or the turnover of labelled amino acids or urea (Lund & Blackburn, 1989);
- nitrification rate by the use of inhibitors (eg. acetylene) that lead to an accumulation of NH_4^+ (Sloth *et al.*, 1992);
- rates of denitrification by the inhibition of N_2O reduction by acetylene, a method with some problems (Seitzinger, 1988). A better method involves addition of $^{15}NO_3^-$ to water overlying sediments and measuring the rate of production of $^{14}N^{15}N$ and $^{15}N^{15}N$ (Nielsen, 1992). Exclusive production of $^{14}N^{15}N$ indicates denitrification from sediment-produced NO_3^-, whereas $^{15}N^{15}N$ production is dependent on sources of NO_3^- external to the sediment; and
- methanogenesis by measuring the conversion of $^{14}CO_2$ to $^{14}CH_4$ in the presence of a suitable reductant like H_2.

4. MICROBIAL INDICATORS AND THEIR APPLICATION

Microorganisms, and particularly bacteria, have a number of potential advantages over other organisms as biological indicators of river 'health'. They are present in very large numbers, grow very rapidly, are responsible for many ecosystem functions, and probably respond to ecosystem changes more rapidly than other biological components, certainly more rapidly than macroinvertebrates or fish. This chapter reviews three possible uses of bacteria as biological indicators of pollution. First, we cover the use of microorganisms as indicators of pollution. Organisms such as E. coli, Giardia and Cryptosporidium, can be used as direct indicators of sewage or livestock pollution. Similarly, other groups of organisms (or enzymes or proteins associated with them) have been used to indicate pollution or stress by organic chemicals, heavy metals and heat.

The second use is as indicators of biodiversity. Here the principal is that a loss of biological diversity is an important indicator of failing ecosystem 'health'. It is somewhat surprising that microorganisms have not been used more for this purpose, given that they are the most diverse of the biological groups. Molecular techniques (eg. DNA-DNA hybridisation) are increasingly being used to measure changes in bacterial biodiversity caused by pollution.

The third use is as indicators of ecosystem function, where the aim is to measure the activity of key microbial processes that contribute to the functioning of an ecosystem. Methods in use include thymidine and leucine uptake for bacterial growth, respiration and enzyme activity for bacterial activity, and at the broader scale, measures of photosynthesis, heterotrophic activity, respiration, P/R ratio, enzyme activity, and specific microbial processes (eg. nitrogen fixation, nitrification, denitrification, sulphate reduction, methanogenesis and methane oxidation) have all been used. Additionally, the next few years will see a marked increase in the use of molecular biology techniques in studies of ecosystem processes.

However, before it is possible to use biodiversity or microbial processes as overall indicators of ecosystem 'health', there will need to be a considerably improved understanding of the variations in these key microbial processes in unimpacted ecosystems.

Microorganisms, and particularly bacteria, have a number of potential advantages over other organisms as biological indicators of river 'health'. Their small size means that they are rapidly disseminated by currents and this, when combined with their very large numbers, makes the design of sampling regimes easier and results more statistically reliable. They also grow very rapidly and probably respond to ecosystem changes more rapidly than other biological components, certainly more rapidly than macroinvertebrates and fish. With the further development of microbial techniques, it can be confidently predicted that microbial analyses will be increasingly automated and the results more rapidly available. This contrasts with other biological indicators where often specialist or semi-specialist taxonomic skills are required to identify particular individual species within a sample.

As noted above, the short generation time of bacteria results in very rapid changes in community structure compared with other biological indicator organisms. This has advantages in that microorganisms can act as an early warning system of change, but also has the disadvantage in that intermittent pollution may be difficult to detect because the microbial community reverts too rapidly after a pollution incident (Hellawell, 1986).

In addition to changes at the level of species composition of microbial communities, the genetic flexibility available to prokaryotes in the form of high mutation rates, haploidy and genes borne on mobile genetic elements (eg. plasmids), allows populations to respond rapidly to changing environmental conditions (Terzaghi & O'Hara, 1990; Veal *et al.*, 1992). Plasmids are circular pieces of DNA that are largely independent of the chromosome. They generally encode genes that are not essential for central metabolism, but which may encode for various resistance traits (eg. antibiotic resistance, heavy metal resistance, ultraviolet light) or novel catabolic functions (eg. toluene, naphthalene, camphor or chlorobiphenyl) or pathogenicity determinants (see Veal *et al.*, 1992). Plasmids can be transferred from one cell to another without the need for cell division. Thus, plasmid-borne genes may be much more rapidly disseminated within bacterial communities than would be predicted from normal inheritance. Such transfer of plasmids is of concern for public health officials especially with the introduction of large numbers of bacteria carrying antibiotic resistance or pathogenicity determinant plasmids in wastewaters from hospitals or intensive

livestock or agricultural operations. Transfer of recombinant genes on plasmids is also central to the debate concerning the environmental release of genetically engineered microorganisms. The concern here is that such bacteria may transfer their recombinant genes to other organisms potentially creating hazardous hybrids. Plasmids encoding for phenotypes for resistances (eg. Hg^r) or catabolic functions (eg. aromatic degradation) may also serve a useful function as bioindicators of pollution.

4.1 BACTERIAL INDICATORS OF POLLUTION

Riverine pollution is caused by the introduction of organic or inorganic materials, or through physical changes to the river (eg. changes in temperature, flow or habitat). Here we discuss the use of microrganisms as direct indicators of pollution. Their use as ecosystem indicators that can pick-up the latter physical changes is covered in Section 4.3.

4.1.1 Direct Indicators

Sewage pollution

Microbiological water quality is normally assessed by the presence or absence of certain 'indicator' bacteria, such as coliforms (*E. coli*), enterococci and coliphage (viruses that infect coliforms). These 'indicator' bacteria are not normally a problem in their own right but are used to indicate recent faecal contamination, and hence the possibility that pathogenic organisms may also be present. They were selected largely because they could be quickly and safely cultured in the laboratory and were relatively easy to identify. Additionally, they grow slowly or not at all in the environment, are present in faeces in far greater numbers than any pathogens, and should die at a slower rate than the pathogens.

A considerable amount has been written on the use of faecal coliforms and faecal enterococci as indicators of sewage contamination. It is not appropriate to provide an exhaustive list of work done in this field, but we will summarise some points salient to the use of these organisms as bioindicators of river 'health'.

A total dependence on these indicator bacteria can lead to some false conclusions. For example, it is now established that the faecal coliform, *E. coli,* can grow in drinking water in the absence of faecal contamination (MacKay & Ridley, 1983; Camper *et al.*, 1991), leading to false positive results. Of rather more concern is the fact that both human viruses and pathogenic protozoans of faecal origin have been reported in waters when indicator bacteria were absent (Melnick & Gerba, 1980; Badenoch, 1990). Even pathogenic bacteria like *Salmonella* spp., which are taxonomically closely related to *E. coli*, can be recovered after the apparent disappearance of indicator organisms (Bianchi & Colwell, 1985). Further complications include the formation of non-culturable but viable bacteria (Byrd *et al.*, 1992; Turpin *et al.*, 1993), which has resulted in a 100-fold underestimation of *E. coli* numbers (Lewis *et al.*, 1991).

Traditional methods for the determination of faecal indicator organisms require a trained microbiologist and microbiology facilities. This has limited the wider application of these bioindicators. Recently, more rapid and simple methods for the detection of coliform bacteria and *E.coli* specifically have become available (Beebe *et al.*, 1991; Edberg *et al.*, 1988, 1989; Lewis & Mak, 1989). These rapid methods detect coliforms through the detection of the enzyme β-galactosidase, a reaction that gives a yellow product in the medium. *E. coli* is detected specifically through the detection of β-glucuronidase, which acts on methyl umbelliferyl glucuronide (MUG) to produce a fluorescent product that can be measured using UV-light (Edberg *et al.*, 1990). One commercially available product (COLILERT), a simple colorimetric assay, gives results within 24 hours and is suitable for use by a non-microbiologist with access to only limited facilities. COLILERT has gained approval from the US EPA for drinking water and is now widely used thoughout the the water industry in that country. An Australian product, which is based on the same principle as COLILERT and designed specifically for environmental applications (Peterson, 1993; Apte & Batley, 1992), will indicate, within one hour, if a waterbody contains greater than the safe bathing water limit of 300 coliforms/100 mL of water.

Faecal bacteria provide a quick, relatively cheap and generally reliable method to assess faecal contamination of riverine environments. Because of the importance of microbiologically safe drinking and recreational waters, extensive literature and experience on the use of faecal bacteria as bioindicators of water quality exists. The advent of new tests for faecal coliforms that negate the requirement for trained microbiologists and extensive microbiology facilities will greatly extend the availability of such tests. We envisage that these new tests will be suitable for remote or community testing of river 'health'.

A number of other indicator organisms for sewage contamination have been suggested, including bacteriophages (viruses that infect bacteria) (Havelaar, 1993), endospores of *Clostridium perfringens*, reovirus, and cysts of *Giardia intestinalis*. Many of these organisms are environmentally more robust than current indicators, and may provide longer term indicators of sewage contamination. However, it must be understood that no single group of indicator microorganisms will always be suitable (Elliot & Colwell, 1985). Furthermore, different groups of pathogens may need to be monitored in different water types. For example, in eutrophic freshwaters, cyanobacteria, *Aeromonas hydrophila* and *Giardia intestinalis* are of concern, while in estuarine waters *Vibrio vulnificus,* hepatitis A, Norwalk virus and *Cryptosporidium* oocysts are of concern (Ashbolt & Veal, 1994).

Livestock pollution

Cryptosporidium is now recognised worldwide as being amongst the most common causes of diarrhoeal disease in man (Current & Garcia, 1991). No chemotherapy is available for *Cryptosporidium* and in most individuals, cryptosporidiosis is a self limiting disease lasting 5-10 days. In immunosuppressed individuals, cryptosporidiosis is life threatening and is known to be a significant cause of death in AIDS patients. *Cryptosporidium* is transmitted via oocysts that are environmentally robust and highly infectious structures. *Cryptosporidium* is of considerable concern to water utilities because (i) waterborne transmission is well documented (Badenoch, 1990), (ii) these oocysts are known to remain viable for long periods of time in the water, considerably longer than indicator organisms used in water testing (waterborne outbreaks of cryptosporidiosis have occurred which have been traced to water treatment plants that have met all current microbiological and chemical standards (Current & Garcia, 1991; Veal *et al.*, 1995)), (iii) the oocysts are resistant to standard chlorination treatment used to disinfect drinking water, and (iv) *Cryptosporidium* has a very low infectious dose, as few as 10 oocysts can cause infection.

Cryptosporidium oocysts may prove to be a useful marker of livestock impact on riverine environments in Australia because *Cryptosporidium* appears to be a common, but often unrecognised, source of 'scour' in calves. An individual calf can produce up to 10^7 oocyst per gram of faeces per day. Many of the outbreaks of cryptosporidiosis appear to be related to contamination of water with cattle faecal matter (Badenoch, 1990). However, further work is required to establish the environmental link between cattle and oocysts in the riverine environments. This deficiency may be rectified by an Australia wide survey currently in progress on *Cryptosporidium* oocysts in the aquatic environment, and the relationship between oocysts and land use. This survey, funded by the Urban Water Research Association of Australia, is being conducted by Macquarie University in association with Australian Water Technologies. With further information, the numbers of *Cryptosporidium* oocysts in rivers may be useful as a measure of healthy drinking waters. The technology to detect these organisms rapidly, sensitively and reliably is available and was developed in Australia (Vesey *et al.*, 1994a,b).

Algal pollution

Certain species of algae (eg. cyanobacteria), in addition to being useful biological indicators of pollution, may themselves constitute a pollutant particularly when present in large numbers (Verhoeven, 1992). Thus, algae have been regarded as an incident-specific indicator of algal blooms rather than a core indicator of ecosystem 'health'. Flow cytometry has the potential to be a rapid, automated system for enumerating cyanobacteria in water (Vesey *et al.*, 1994a,b).

4.1.2 Response indicators

Organic matter

The input of large quantities of organic matter, such as paper pulp or starch wastes from the food industry, into a waterbody often results in undesirable biotic changes. Decomposition of this organic matter by heterotrophic microorganisms results in an enormous biological oxygen demand (BOD), which can deplete the dissolved oxygen content of the water. In fact, if the BOD is sufficiently high, the dissolved oxygen will become exhausted and undesirable anaerobic processes will take over. These anaerobic processes will result in the production of ammonia and hydrogen sulphide, which in themselves may be harmful to the biological community. Such an outcome is a very obvious biological indicator of organic pollution.

Aromatic compounds

Pickup (Freshwater Biological Association, UK, personal communication 1994) has used the abundance of the enzyme catechol 2,3 dioxygenase (C23O) to assess the potential of a site for aerobic degradation of aromatic pollutants in combination with ^{14}C-labelled hexadecane to assess potential degradation of aliphatic hydrocarbons. The enzyme C23O catalyses the ring cleavage of catechol to the bright yellow product, 2-hydroxymuconic semialdehyde (Winstanley *et al.*, 1989). Bacterial colonies expressing the C23O enzyme produce a yellow pigment when sprayed with catechol and are easily recognised. In addition, Pickup (unpublished data) has used the presence of the C23O enzyme to assess whether sites have been subjected to mild pollution (eg. hydrocarbon pollution from boats). In Lake Windermere, higher levels of C23O were found in bacteria from water and sediments taken from polluted areas. Only in pristine oligotrophic lakes were C23O-free bacteria detected. Pickup and colleages are moving to determine the class of C23O enzymes in polluted and non-polluted systems.

This approach appears to offer considerable potential for assessing the 'health' of Australian rivers as it provides a sensitive indicator of aromatic pollution and its bioavailability. The assay should even provide an indication of recent pollution events as it will take time for bacteria possessing C23O genes to be diluted from the population. These C23O genes are often plasmid-encoded (Pickup *et al.*, 1990; Winstanley *et al.*, 1991), and are likely to be rapidly disseminated through the microbial population. Therefore, the response of a bioindicator such as the C23O enzyme is likely to be very rapid.

This approach may also be applied to other xenobiotic molecules. For example, other biodegradative capacities such as dehalogenation (the removal of a halogen from an organic molecule) could be used in a similar manner to assess organochloride pollution and the potential of the indigenous microbial community to remediate such pollution. This technique could have considerable application in areas thought to be polluted by organochloride pesticides.

Osaki *et al.* (1991) found that the rate of microbial degradation of aniline in an urban river was closely correlated with water quality. Aniline, an ubiquitous chemical in the urban environment that is derived from the degradation of dyes, drugs and herbicides, is also a common metabolite of the microbial transformation of nitroaromatic compounds. These authors suggested that the microbial degradability of aniline in river water could provide an indicator of water pollution.

Heavy metals

Microorganisms can interact with heavy metals in one of three ways:

(i) the metal may adversely affect a particular microbial activity (eg. methanogenesis) which may in turn result in changes in microbial processes (for example, a decreased rate of organic decomposition and a change in the end-products of decomposition);

(ii) the microbial community may biologically transform the heavy metal in an attempt to detoxify it. Alternatively, microbial activity may increase bioavailability or mobility of the heavy metal, resulting in increased toxicity or bioaccumulation in the food chain (Inverson & Brinckman, 1978). For example, mercury salts, although toxic, are normally rapidly excreted from the body. However, in anaerobic sediments, some microorganisms are capable of methylating mercury; methylmercury is lipophilic and readily concentrated by a number of organisms, particularly shellfish and long-lived marine fish. Unlike inorganic mercury and phenylmercury compounds, methylmercury is only slowly excreted and tends to magnify in aquatic food chains; and

(iii) the numbers of microorganisms tolerant of a particular heavy metal may increase in the population, even when the metal concentration is increased to a relatively low concentration. Such an increase in the numbers of heavy metal-resistant bacteria will result from selection and then multiplication of metal-resistant bacteria. The rate of increase of heavy metal resistance in bacterial populations may be accentuated by transfer of heavy metal-resistant plasmids (Veal *et al.*, 1992). Increased resistance to heavy metals has been demonstrated in sediments from the Mediterranean (Gauthier *et al.*, 1981) and Chesapeake Bay, USA (Colwell & Nelson, 1975). The numbers of heavy metal-resistant microorganisms may be useful as sensitive indicators of heavy metal pollution.

As well as resistance, the relative sensitivity of microorganisms can be used as an indicator of heavy metal pollution. Kumari *et al.* (1991) found that several phytoplankton species could be used as indicators of heavy metal pollution in the River Moosi, India, and Whitton (1979) has associated the abundant growth of *Stigeoclonium tenue*, combined with the complete absence of *Cladophora glomerata*, with combined high nutrient and heavy metal levels. A particularly novel indicator of copper toxicity has been described by Owen & Winner (1991). They observed that copper concentrations of <10 mg L^{-1} cause cells of the green alga *Chlamydomonas reinhardtii* to lose their flagella and that higher concentrations cause them to encyst. Field data indicated that deflagellation was a more sensitive indicator of copper toxicity than population growth.

In marine environments, benthic seaweeds have been identified as good indicators of heavy metal pollution (Shimshock *et al.*, 1992). They are sessile and can be used to characterise a location, are good accumulators of metals, demonstrate a linear relationship between amount of uptake and exposure concentration, are easily collected, and are not subject to short term and erratic changes but act as indicators of the average concentration of metals. A significant body of research exists on heavy metal uptake and content in various marine algae. We found no such body of literature for riverine macro-algae and suggest by analogy to the marine environment that freshwater algae may prove to be useful bioindicators of heavy metal pollution.

Thermal pollution

The electricity industry is the major contributor to thermal pollution in aquatic environments. Large amounts of cooling water are returned to source causing the temperature to increase by 5-10°C. In common with other forms of pollution, this stress has the effect of decreasing bacterial diversity with concomitant increase in numbers within particular genera. This is demonstrated by increases in the numbers of the Gram-negative rods *Pseudomonas* and *Flavobacterium* found in power station effluent discharge ponds. Fish diseases, such as those caused by the bacteria Myxobacteria and *Aeromonas* spp. and aquatic phycomycete fungi, also show increases in thermally polluted waters, particularly if the waters are also eutrophic. Coliforms, and presumably related human pathogens, may also survive longer in warmer waters. The protozoan human pathogen *Naeglaria fowleri* is restricted to waters with temperatures around 40°C and the range of this organism could be extended as a consequence of thermal pollution.

Thermal pollution may also result in unwanted increases in certain autotrophic communities, particularly Chlorophyceae and cyanobacteria. Additionally, thermal pollution will lower the solubility of O_2 in water and may result in undesirable O_2-exhaustion and anaerobic processes becoming more prevalent.

4.1.3 Physiological responses as bioindicators

Use of microorganisms as biological indicators normally depends on the relative growth of a particular microorganism or group of organisms. There are, however, toxicological tests which measure the response of microorganisms as a bioindicator of various stressors. Some of the most common of these are reviewed in this section.

Chemotaxis

Many microorganisms will respond to stimuli in the environment by movement. In bacteria, the flagellum provides a mechanism for swimming towards an attractant or away from a repellent. Bacteria have chemosensors in the cell envelope that detect chemicals in the environment and instruct the flagella to respond. These chemosensors are 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 PCB's (Mitchell, 1978, 1979).

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. Stress proteins include the heat shock response (Neidhardt *et al.*, 1984), the SOS response (Walker, 1984), starvation response (Ostling *et al.*, 1993) and anaerobiosis (Spector *et al.*, 1986). Pollutants such as cadmium, benzene, chlorpyrivos, 2,4-dichloroaniline, dioctyphtalate, hexchlorobenzene, pentachlorophenol, trichloroethylene, tetrapropylbenzosulfonate; 6-amino-7-chloro-5,8-dioxoquinoline and ethanol have all been shown to induce stress proteins in *Escherichia coli* (Blom *et al.*, 1992).

The stress responses are rapid, taking from 20 min to 4 hours for cells to adjust their cellular processes to the stressed environment. Such stress responses may be used as a rapid early warning indicator of general ecosystem stress (S. Kjelleberg & N. Ashbolt, University of NSW, personal communication, June 1994). Measurement of stress response proteins as a bioindicator has the advantage that they are a more sensitive index of stress than growth rate, since stress-protein synthesis is likely to be stimulated at very low pollutant concentrations at which little or no growth inhibition occurs (Blom *et al.*, 1992).

The nature of the stress-response proteins produced may provide a quite specific method to identify particular pollutants. With nine different pollutants, no single set of proteins was found to be a universal, non-specific response in *Escherichia coli* (Blom *et al.*, 1992). Although some of the proteins were commonly induced by more than one type of stress agent, at least 50% of the proteins were unique to a specific chemical. These results indicate that it is conceivable to identify individual pollutants in the environment by the specific stress response that they elicit (S. Kjelleberg & N. Ashbolt, University of NSW, personal communication, June 1994).

Stress-response proteins may be used in two ways as bioindicators; either the microbial community can be examined directly for these proteins, or specific microorganisms with well-characterised stress responses (eg. *E. coli, Vibrio* spp. or *Salmonella typhimurium*) may be exposed to the environment (possibly immobilised on a 'dipstick') and examined for the stress responses (S. Kjelleberg & N. Ashbolt, University of NSW, personal communication, June 1994). Currently, stress-response proteins are identified by two dimensional polyacrylamide gel electrophoresis. However, a range of monoclonal antibodies is available for specific stress-response proteins, enabling the rapid identification of stress responses and the production of 'kits' which could be used for rapid field evaluations. An alternative approach could be to use reporter genes (eg. luciferase genes) inserted after the promoters of particular stress-response genes to indicate when these genes are active (S. Kjelleberg & N. Ashbolt, University of NSW, personal communication, June 1994). Information on the molecular biology of stress responses is becoming available that would enable the development of such reporter systems.

Use of stress-responses is a particularly novel approach to the development of bioindicators of river 'health'. This approach has many potential advantages. It is rapid, can assess general or specific stressors, measures bioavailability of pollutants, is an ideal technology for the development of kits that should be economical and, since similar stress-responses are found in all organisms, it should provide a good indicator of ecosystem 'health.' This technology still requires further development and verification under field conditions before it would be suitable for assessing river 'health'.

Ames test

The Ames test utilises a series of bacterial strains of *Salmonella typhimurium* to test the mutagenicity of drugs, agrochemicals, industrial compounds, food additives, drinks, cosmetics, and air and water pollutants. The test is based on measurement of back mutation rates in the histidine cistron in *Salmonella typhimurium*. Mutants that are auxotrophic (require) for histidine are treated with the chemical of interest and the numbers of bacteria able to grow on histidine-free media (back mutants) after treatment is determined. This method is rapid and the rate of back mutation provides a quantitative measure of mutagenicity. The Ames test could have applications in river 'health' assessment using concentrated water samples to determine the potential mutagenicity of compounds in the water. We are unaware of any work on river 'health' using this technique.

4.2 INDICATORS OF BACTERIAL BIODIVERSITY

4.2.1 General

For other organisms (eg. vertebrates, plants), it is generally accepted that biological diversity (ecosystem, species, and genetic diversity) is of intrinsic value and thus should be conserved. Even for invertebrates this concept is beginning to gain acceptance. It is also generally accepted that loss of biological diversity (ie. numbers of species) is an important indicator of failing ecosystem 'health'. The desirability of preserving biological diversity is enshrined in many international agreements. The recent Federal Government State of the Environment initiative recognises the significance of biological diversity and its assessment.

Microorganisms are undoubtedly the most diverse of all the 'biological groups' (Veal, 1995). However, microorganisms are generally ignored in assessments of biodiversity. Further, the concept of microbial biodiversity does not seem to have gained the same degree of acceptance as plant or animal biological diversity, either in the general or scientific community. This is of some concern considering the pivitol role microorganisms play in maintaining ecosystem functions (Chapter 2).

The major problems with assessing microbial biodiversity are methodological. Using traditional techniques, assessments of microbial biological diversity are out of the question (Veal, 1993). This is because the methods to isolate and identify all but a tiny fraction of the microorganisms present are not available. Even if all microorganisms could be isolated, the time and resources required to isolate and identify the thousands of microbial species from a single sample (eg. one gram of river sediment) would make such assessments prohibitively expensive.

A further problem with the assessment of microbial biodiversity stems from the basic unit of biodiversity — 'the species'. The species concept depends on barriers to genetic exchange between different species and to a large degree of phenotypic stability. It is important to recognise that the biological species concept was developed for sexually reproducing populations of eukaryotes, and has been described as a barely useful concept when applied to prokaryotes (Brock, 1987). Bacterial reproduction is asexual and bacteria exist as a series of more or less closely related clonal lines. Genetic exchange can occur between these clonal lines via a variety of different mechanisms (Mazodier & Davis, 1991; Veal *et al.*, 1992). This genetic exchange can occur between phylogenetically distantly related lines. Consequently, in prokaryotes, genetic exchange is not restricted by 'species' barriers. The bacterial phenotype is also potentially very flexible because of high mutation rates and expression of those mutations due to the haploid nature of bacteria. When such mutations occur in areas of the genome responsible for taxonomic features this can result in classifying a particular strain into a different species or even genus. For example, the inability of *E.coli* to utilise citrate as a sole carbon source is taxonomically important in distinguishing *Escherichia* from other members of the enterobacteriaceace such as *Klebsiella*. Two chromosomal mutations have been shown to convert citrate negative strains to citrate positive (Hall, 1982). Such mutations would result in the reclassification of *E.coli* into a different genus!

Despite the genetic plasticity of bacteria it is possible to cluster them into related types. For convenience a bacterial species has been arbitrarily defined as a series of strains with approximately 70% or greater DNA homology (Wayne *et al.*, 1987), although the exact level below which organisms are considered to belong to different species varies considerably. In general, the more studied a genus of bacteria the more species it will contain.

4.2.2 Measurement of microbial biodiversity

The increasing use of molecular methods in microbial ecology (see Chapter 3) is now making the assessment of bacterial biodiversity feasible. Although there have now been several studies of bacterial biodiversity using molecular techniques, we were only able to find one riverine example (Palmer *et al.*, 1994). Below we describe three examples, including the river case, to demonstrate the power of these techniques. Detailed reviews of the application of these molecular methods have been completed by Ward *et al.* (1992), Pedros-Alio (1993) and Bull *et al.* (1992).

Octopus Springs

Ward and his colleagues (Ward *et al.*, 1990a,b) have examined the 16S rRNA photosynthetic microbial mat at Octopus Springs, Yellowstone National Park. The spring is hot and stable with respect to many environmental features that vary in other habitats. This selectively hot and peculiarly stable environment, arguably, should support a less diverse microbial community than less stable and less selective environments. This mat has been studied by numerous microbiologists for over 30 years, using conventional means, and is considered to be one of the best characterised microbial communities. Using a 16S rRNA analysis, fifteen unique 16S rRNA sequences were identified, none of which matched with the sequences of organisms isolated from similar geothermal habitats. More significantly, in only one case did the sequence bear any resemblance to a recognised bacterial phylum. Thus, it can be concluded that this environment is dominated by non-culturable organisms.

Norwegian soil and sediment

A Norwegian study of microbial diversity in a beech-forest soil, and marine sediments, using the rate of re-annealing analysis method, described in Section 3.1.5, has been reported by Torsvik *et al.* (1990a,b). They found between 4,000 and 5,000 different bacterial species in each gram of soil taken from each environment. Furthermore, no overlap in species between the two environments was found. In this study the genetic codes had to differ by at least an arbitrary 30% to be considered as separate species.

River water

Palmer *et al.* (1994) reported a study in which the diversity of microbial communities upstream and downstream of an industrial waste discharge was measured in an attempt to assess the biological impact of the discharge. DNA was isolated from the microbial communities colonising artificial substrates located upstream and downstream of the discharge, and the similarity of these communities compared by DNA-DNA hybridisation. This method involves mixing the DNA from two sites (eg. upstream and downstream), heating to make the DNAs single stranded, then re-annealing, and finally measuring the concentration of DNA after re-annealing and comparing this with the amount of DNA which rehybridised with itself in a control (the upstream sample). The method is based on the fact that the more similar the two microbial populations, the greater the amount of DNA that would be formed on re-annealing (Lee & Fuhrman, 1990). Palmer *et al.* (1994) reported that the DNA hybridisation results indicated that the upstream microbial communities were more similar to each other than with the downstream sites, but that these differences were not statistically significant. Problems with this study included small sample sizes and highly variable background on the hybridisation membranes resulting from the rather insensitive detection methods used (DNA was labelled with biotinlyated-7-dATP, radioactive DNA labels would have been more sensitive).

4.3 MICROBIAL INDICATORS OF ECOSYSTEM FUNCTION

Microorganisms mediate a wide variety of ecosystem processes (eg. cycling of nutrients, breakdown of organic matter) that maintain the biosphere (Section 2.5), and any loss in the microbial biodiversity will undoubtedly have undesirable consequences on the system. In the previous section we noted the emergence of molecular biological techniques to measure loss in microbial biodiversity in aquatic systems subjected to pollution. With time we believe such measures of loss of microbial biodiversity may provide a useful comparative index of ecosystem 'health'.

Another, perhaps more direct way to assess the 'health' of an aquatic ecosystem is to measure the activity of key microbial processes that contribute to the functioning of that ecosystem. In recent years, a number of very useful methods have been developed to measure bacterial or microorganism activity either at the cell level or at the larger-scale. In the former case, the methods of choice include thymidine and leucine uptake for bacterial growth, and respiration and enzyme activity for bacterial activity; these methods are reviewed in Section 3.3. At the broader scale, measures of photosynthesis, heterotrophic activity, respiration, P/R ratio, enzyme activity, and specific microbial processes (eg. nitrogen fixation, nitrification, denitrification, sulphate reduction, methanogenesis and methane oxidation) have all been used. Unfortunately, there has been so little study of these ecosystem functions that it is not yet possible to relate measured changes in the heterotrophic or denitrification activity, or the P/R ratio, with the overall functioning (and hence 'health') of the ecosystem. In fact, in most cases it is still not possible to state with any certainty what the variations with time in these activities are in a 'healthy' ecosystem.

A recent study on methanogenesis in Ryans Billabong using molecular probes has shown the potential power of these new tools to assist in understanding detailed ecosystem processes (Ross *et al.*, 1995). We predict that the introduction of molecular biology techniques into studies of ecosystem processes will lead to a significant increase in understanding, and eventually may be used as overall indicators of ecosystem 'health'.

5. LINKS BETWEEN MICROBIAL INDICATORS AND OTHER BIOTIC RESPONSES

There are very few published studies linking changes in the microbial community with other biotic responses. This is not surprising given the relatively recent ability to measure with any degree of ease and accuracy microbes and their activities in natural environments. Most microbial ecology research to date has concentrated on providing fundamental aquatic microbial ecological information. However, despite the lack of specific studies, it has been possible to predict that strong links will exist between microbial communities and macroinvertebrate species composition, algal biomass, and community metabolism. The opportunity exists in the near future to include measurement of microbial indicators in the new National Bioassessment of Rivers Program. There is still much to be done in (i) better defining the most appropriate microbial indicators to measure, (ii) working up more reliable protocols for the appropriate microbial methods, and (iii) training more scientists skilled in these microbial techniques.

River managers commonly strive to determine the links (quantitative and qualitative) that exist between indicators of ecological 'health' and various disturbances or stressors. For example, if the conductivity of a waterway is increased, perhaps because of a discharge of saline water, the manager wants to know the level of change at which an adverse effect on the biota will occur. In situations where gross effects occur, such as fish kills caused by excess concentrations of heavy metals or pesticides, it is relatively easy to link the adverse effect back to the particular toxicant or toxicant mixture.

However, waterways managers are increasingly being faced with the need to manage for far more subtle effects, and in these cases the links are not always so obvious. An example is the massive cyanobacterial bloom that occurred in the Darling River in 1991, where a number of factors were postulated as the cause. These included: high phosphorus concentrations, increased light penetration because of reduced turbidity, and very low river flows providing the right conditions for blue-green algal growth. Clearly, all three factors played a part in the accelerated algal growth, and it is very difficult (and probably foolish) to try to single out one particular factor.

An emerging trend in aquatic ecosystem management, is the use of 'sets' of indicators rather than single indicators to assess ecosystem 'health' (ANZECC, 1992; Hart *et al.*, 1993). In the case of rivers and streams, an appropriate indicator set might include physico-chemical water quality indicators, biological indicators, sediment indicators, habitat indicators and riparian zone indicators.

5.1 AVAILABLE INFORMATION

There have been a number of attempts to link physico-chemical water quality indicators with biological indicators, the most successful being related to predictions of macroinvertebrate communities (eg. Campbell *et al.*, 1982; Hellawell, 1986; Smith, 1987; Wright *et al.*, 1988; Metcalfe-Smith, 1992; Maasdam & Smith, 1994). The links are quite well established for toxicants (eg. heavy metals, pesticides), where there is often a considerable amount of laboratory-based ecotoxicological information available (ANZECC, 1992; Chapman, 1995).

There have, however, been far fewer attempts to link various biotic indictators with each other. This lack of information is perhaps understandable given the complexity of interactions that occur between the various biological communities, and the fact that a particular stressor may influence one biological community and have little influence on others. In a recent review of the factors reponsible for algal blooms in Australian aquatic systems, Harris (1994) suggested that a whole system view was needed in which both 'top down' and 'bottom up' factors (and the interactions between them) will need to be considered.

This present review has identified a number of potential advantages in using microbial indicators to assess the ecological 'health' of rivers. In particular, since microorganisms respond rapidly to most stresses and are a pivotal component of all aquatic ecosystems, is seems likely that they will act as rapid 'early warning' indicators of adverse change. Importantly, as was discussed in Chapter 3, there are now available a number of methods (mostly routine) for potentially useful microbial indicators, including total bacterial numbers, numbers of active bacteria, bacterial production rates and in some cases numbers of organisms belonging to particular functional groups (eg. methanogens). There is also considerable activity worldwide in the development of increasingly rapid, specific and automated methods for the measurement of various aspects of aquatic microbial populations.

Unfortunately, there are very few published studies linking changes in the microbial community with changes in other biological communities. This is hardly surprising given the relatively recent ability to measure with any degree of ease and accuracy microbes and their activities in natural environments. Because of this, and the general lack of expertise, throughout the world but particularly in Australia, most researchers have concentrated to date on providing fundamental aquatic microbial ecological information, such as that required to answer the following questions: what types are where, how many there are, what are they doing, how do they survive, what controls their activity and numbers, and what roles do they play in the large cycles of carbon, trace gases and nutrients (Hobbie & Ford, 1993).

In one of the few studies of its type, Chappell & Goulder (1992) found the extracellular enzyme activity (glycosidase, phosphatase and sulphatase) in rock epilithon in headwater streams in northen England was correlated with water quality (eg. pH, temperature, conductivity, absorbance at 320 nm) and epilithic microbial variables (eg. bacterial activity, total bacterial numbers and chlorophyll-a), but not with stream variables such as water velocity and depth, or stone size. In studies of enzyme activity in Australian freshwater systems, Boon (1989a, 1990) found a good relationship with suspended solids concentration but not with nutrient concentrations. A subsequent study in Ryans Billabong produced good relationships between enzyme activity on suspended matter and the incidence of cyanobacterial blooms, bacterial abundances, extracellular aminopeptidase activity and amino acid concentration (Boon, 1993).

5.2 POSSIBLE BIOTIC LINKS

Despite this dearth of specific studies linking changes in microbial indicators with other biotic indicators, it is possible to note here a number of biotic responses that might be linked to changes in the microbial community.

Although a large number of biological indicators have been suggested as being relevant for assessing the general 'health' of rivers (Hellawell, 1986), only a small number are in common use. These include: bacteria, protozoa, algae (particularly for lakes and reservoirs), macroinvertebrates, macrophytes and fish. The best indicators that might be used to assess the 'health' of Australian rivers was the focus of an international conference on 'The Use of Biota to Assess Water Quality' held at the University of Canberra in September 1993 (Norris *et al.*, 1995). The concensus from this conference was that *aquatic macroinvertebrates* (to family level of taxonomic discrimination) and *fish* should be adopted as the most immediately useful indicators of river 'health', and that these should used in conjunction with *habitat assessment* and the measurement of a small set of *core physico-chemical indicators* (pH, conductivity, turbidity, total phosphorus, total nitrogen and total organic carbon). Furthermore, it was recommended that algae and ecosystem function methods (eg. P/R ratios) showed considerable promise as ecosystem 'health' indicators, but that more research was needed before these indicators could be used with any confidence. The conference also strongly recommended that methods for *data analysis and reporting* need to be considered at the outset of the program and adequate resources set aside for this purpose, and that any national project must have *strong management* if it is to be successful.

Here we comment upon the possible links between these commonly used biological indicators and microbial indicators.

5.2.1 Macroinvertebrate species composition

For rivers and streams, macroinvertebrates have emerged as the clearly preferred indicator for most agencies (eg. Wright *et al.*, 1985, 1988; Moss *et al.*, 1987; Metcalfe-Smith, 1992; Norris *et al.*, 1995). The macroinvertebrate community comprises a wide range of organisms which offer the possibility of varied responses to different environmental stresses (Norris *et al.*, 1995). Additionally, a number of relatively standard protocols for macroinvertebrate sampling and analysis are now available (Klemm *et al.*, 1990; Plafkin *et al.*, 1989; Wright *et al.*, 1988).

Macroinvertebrate community data are the basis for the new National Biossessment of Rivers Program as part of the National River Health Program which commenced in 1994 in all states and territories in Australia. The program aims to improve the knowledge of: (i) the 'health' of Australia's rivers, (ii) the impacts of pollution on river ecosystems, and (iii) the effectiveness of river management actions. The program is based on the UK RIVPACS approach (Wright *et al.*, 1985; Moss *et al.*, 1987). A rapid, robust and standardised sampling protocol has been developed and is being used by all agencies. The protocol also lays out an approach to sampling and data analysis.

Given the potential advantages in using microbial indicators to assess river 'health', particularly their much more rapid response to changes compared with macroinvertebrates, we believe it would be sensible to investigate the possibility of including some measurement of microbial indicators in the national bioassessment program in the near future. As a first stage, this could involve the collection of baseline bacterial information leading to a better understanding of the links between the microbial indicators measured and macroinvertebrate community structure. Then, as the knowledge of aquatic bacteria improved, it would be possible to include one or more bacterial indicators generally in the national program.

5.2.2 Algal biomass

Changes in both biomass and species composition of algae have been used to indicate eutrophication of aquatic systems (OECD, 1982; AWRC, 1983; White, 1983b; Harris, 1994 Carpenter *et al.*, 1995). These increases in algal biomass, expected as aquatic systems become more eutrophic, should also be reflected in increased bacterial numbers and possibly changes in the species composition. Bacterial numbers have been found to correlate strongly with algal numbers in many aquatic systems (Cole *et al.*, 1988; Rheinheimer, 1991). This is not surprising given that algae provide a very important 'food' source for bacteria, although whether a net increase in bacterial biomass occurs as a result of increased algal biomass is also dependent upon protozoan grazing rates. For this reason it may be that bacterial production or specific growth rates may be better indicators of heterotrophic processes than bacterial biomass.

Despite this general link between bacterial numbers and algal biomass, there is no information presently available from which to judge what we should regard as 'typical' bacterial numbers (or groups) in 'normal' and 'eutrophic' rivers and streams. Clearly, considerably more work needs to be done before bacterial numbers (or some other more appropriate indicator, eg. bacterial production) can be used to indicate changes in the eutrophic status of rivers.

5.2.3 Metabolism

The ratio of gross primary production (P) to community respiration (R) has been used to indicate whether a river of stream ecosystem is a net producer (P/R >1) or consumer (P/R <1) of organic matter (Vannote *et al.*, 1980; Minshall *et al.*, 1983; Chessman, 1985; Meyer & Edwards, 1990). The terms 'autotrophic' and 'heterotrophic' are often used to classify stream ecosystems having P/R ratios greater than 1 and less than 1 respectively.

A large number of factors can change the P/R ratio in a particular stream ecosystem. These include: changes in primary production, changes in the amount and type of allochthonous organic matter input to the stream, changes in the relative rates of heterotrophic respiration of autochthonous and allochthonous organic matter, and the relative proportions of the macroinvertebrate community feeding upon autochthonous and allochthonous organic matter. Such ecosystem changes can be caused by activities such as clearing the riparian vegetation (that could alter the P/R ratio by allowing more light to enter the stream which would stimulate more primary production, and by reducing the input of allochthonous organic matter), the addition of nutrients (which would stimulate both primary production and growth of microbes), and the addition of organic-rich effluents (which would stimulate heterotrophic respiration).

Guidelines based on ecosystem function, as reflected by P/R ratios, as an indicator of ecosystem 'health', generally require that such ratios do not vary significantly from those of similar local unimpacted systems, or that a particular system is not changed such that the relative balance between allochthonous and autochthonous energy pathways is maintained (ANZECC, 1992; Hart *et al.*, 1993). However, at present we have so little basic information on the levels of metabolism in Australian rivers and streams (see Section 3.3.2), the factors influencing changes in these levels, and the natural variations expected, that it will be some years before adequate guidelines can be developed.

Clearly, because of the vital role of bacteria in the metabolism of organic matter in rivers and streams, changes in stream metabolism as reflected in the P/R ratio (or net daily metabolism (NDM)) should also be reflected in a number of indicators of the microbial community. However, again the lack of basic information about the microbial communities in these systems makes it impossible to say more than the obvious, that changes in ecosystem metabolism will be linked to various microbial indicators.

5.3 LINKS WITH THE NATIONAL MONITORING PROGRAM

The establishment of a national biological monitoring program, which is presently underway, represents an ideal opportunity to collect basic information on microbial indicators in Australian rivers and streams. The national program will focus on macroinvertebrate communities, but will also seek to link changes in macroinvertebrate communities with other environmental variables such as physico-chemical water quality and stream characteristics (eg. altitude, substrate, habitat condition). It is proposed that in time this national program will also include other biological indicators, particularly fish and perhaps also algae.

The opportunity exists to include microbial indicators in this program. However, we suggest that this is two to three years distant since there is still much to be done in (i) better defining the most appropriate microbial indicators to measure, (ii) working up more reliable protocols for the appropriate microbial methods, and (iii) training more scientists skilled in these microbial techniques. Work should start immediately on planning for the introduction of the measurement of microbial indicators in this program.

6. KNOWLEDGE GAPS AND R&D NEEDED

This chapter seeks to identify the major knowledge gaps that could limit the introduction of microbial indicators of river 'health'. The focus is specifically on knowledge gaps directly relevant to riverine microbial indicators, although many of these knowledge gaps are common to the application of microbial indicators to other aquatic and terrestrial ecosystems. Despite a small increase in the activity in aquatic microbial ecology in Australia over recent years, there is still a major dearth of fundamental knowledge on the microbial assemblages and the processes they control in Australian aquatic ecosystems. This lack of fundamental information must be addressed urgently before it will be possible to use microorganisms, particularly bacteria, as indicators of river 'health'.

Bacteria play a pivotal role in the maintenance of ecosystem health, because they are so important in carrying out processes, such as decomposition of organic matter and nutrient recycling, that are essential for an ecosystem to function properly. Considering the critical role bacteria play, it is perhaps surprising that they have not been used more extensively as indicators of ecosystem 'health'. Limitations in the methods available to measure microorganisms and their functions in natural environments has been the main reason for microbial indicators not being adopted earlier. However, recent technological advances, particularly in the area of molecular biology, are now providing the tools to study microorganisms and microbial processes in natural environments. Many of these technological advances could be adapted to provide the tools to routinely measure microbial numbers and their functions in aquatic ecosystems.

LWRRDC could play a key role in encouraging the development and application of such routine methods. Development, application and interpretation of results from such methods is likely to be hampered in the near future by a critical shortage of microbiologists and molecular biologists with environmental skills. LWRRDC, in association with other research agencies, should encourage the training in these new techniques both through the provision of post-graduate scholarships and extension courses to upgrade skills within environmental agencies.

6.1 SKILL BASE IN AQUATIC MICROBIAL ECOLOGY

We comment briefly upon the general lack of aquatic microbial ecologists in Australia before addressing specific knowledge gaps. The paucity of Australian expertise in microbial ecology generally has been identified by Moriarty & Veal (1992). At the undergraduate level, microbiology has traditionally serviced the needs of medicine, veterinary science, dentistry and agriculture resulting in a separation of microbiology from developments in the main stream of biology and ecology. Students studying ecology or environmental science would be expected to gain a firm understanding of plant and animal science, yet most will not have any exposure to microbiology. In 1991, only 14 courses covering microbial ecology or environmental microbiology were available in Australia (Moriarty & Veal, 1992). Although some new courses in microbial ecology have been introduced since this survey, the number of students specialising in microbial ecology or related disciplines is still relatively small. This lack of exposure to microbial ecology at the undergraduate level leads to a correspondingly small number of postgraduate students in this field. Unless there is a significant increase in the number of students undertaking research in microbial ecology there will continue to be major problems in undertaking the fundamental research required to apply microbial indicators in Australia.

There is also an urgent need to increase the awareness of staff in resource management, environmental and water agencies, first to the roles of microorganisms in riverine environments, and second to the new methodologies that are now available to assess microbial activities in aquatic ecosystems. Clearly, LWRRDC can play an important role in addressing this problem.

Recommendation 1:

That LWRRDC develop a strategy, in consultation with other research agencies (eg. Australian Research Council), aimed at increasing both the number and skill base of those working in the aquatic microbial field over the next three to five years. This strategy should (i) address the training needs at the postgraduate level through provision of post-graduate scholarships directed towards the development, assessment and application of new technologies for aquatic microbial ecology, and (ii) upgrade the knowledge base and technical skills within environmental agencies and water utilities by running workshops and short extension courses. Additional funding will be needed to address the fundamental aquatic microbial questions required to provide the research base needed to support (i) and (ii) above.

6.2 FUNDAMENTAL KNOWLEDGE OF MICROBIAL ECOLOGY

Despite major increases in knowledge over the past 20 years (Ford, 1993), there is still much to be learned about the roles of microorganisms in the functioning of aquatic ecosystems. This lack of information is particularly noticeable for running waters, which have not been studied as intensively as other systems because they are so dynamic, especially in Australia. For example, there have been only five studies that have addressed any aspect of the microbial ecology of Australian rivers (Boon, 1989a, 1990, 1991a, 1993; Thomas *et al.*, 1992). The majority of the small amount of microbial ecology work that has been done on Australian freshwater systems has focused on billabongs (Boon, 1989b, 1991a,b, 1994; Boon *et al.*, 1990; Boon & Sorrell, 1991; Sorrell & Boon, 1992; Sholz & Boon, 1993a,b,c; Ross *et al.*, 1995).

For Australian freshwaters, there is an urgent need for more fundamental information about the important ecosystem processes (from a microbial viewpoint); processes such as carbon metabolism, nitrification and denitrification, and phosphorus cycling. This will require measurement of the total numbers of specific microbial functional groups and their activity, the main environmental factors that influence them, and 'typical' changes in response to these environmental factors. This basic research is needed to underpin the usefulness of any microbial indicators adopted in the near future.

Recommendation 2:

That LWRRDC fund a research program aimed at providing fundamental information about the microbial ecology of Australian aquatic ecosystems, with a focus on lowland river systems and on the eventual desire to produce microbial indicators for assessing the environmental 'health' of the aquatic ecosystems.

6.3 WHICH MICROBIAL INDICATORS TO USE?

There is no consensus in the literature regarding the most appropriate microbial indicators to use to assess river 'health'. Again this is understandable given the lack of attention to the possible use of microorganisms for anything but sewage pollution. Below we list some of the most obvious knowledge gaps that need to be addressed in order that microbial indicators can be defined and introduced for the purpose of assessing river 'health' in Australia.

6.3.1 Pollution

Bacteria, such as coliforms and *E. coli*, have been used for many years as indicators of sewage pollution (Chapter 4). There seems little doubt that this use will continue into the future, despite the many problems associated with them (Ashbolt & Veal, 1994), since they are now well accepted by managers and the public. Their continued use will be assisted by the availability of new simple, rapid techniques for their measurement.

Additionally, we can expect that specific antibody and molecular probes will be developed over the next few years that will allow the rapid detection of a range of waterborne pathogens, such as *Aeromonas* sp., *Salmonella* sp., *Giardia intestinalis* and *Cryptosporidium*. In fact, molecular probes for detecting a range of protozoan and bacterial pathogens are being developed by Australian Water Technologies in association with the University of New South Wales and Macquarie University Centre for Analytical Biotechnology (Cox, 1993).

The response of certain microorganisms to particular pollutants, eg. heavy metals, has also been used as a bioindicator (Section 4.1.3). Perhaps the best known example is the Ames test, which uses the bacterium *Salmonella typhimurium* to test for mutagenicity in wastewater and other samples; a major problem with the use of the Ames test for natural waters is the need to concentrate the samples.

The production of particular proteins (stress-response proteins) by all bacterial cells in response to harmful environmental conditions is another physiological response that has considerable potential to be exploited as a bioindicator. The responses are rapid, more sensitive than growth rate, and can provide general or specific information on pollutants. In addition, work is underway at the University of NSW to produce a simple 'kit', based on the use of monoclonal antibody technology, that will enable the rapid identification of stress responses in the field (Prof S. Kjelleberg, University of NSW, personal communication, July 1994). The method still needs further development and verification under field conditions before it would be suitable for assessing river 'health'.

6.3.2 Biodiversity

Loss of biological diversity (ie. numbers of species) is generally accepted as an important indicator of failing ecosystem 'health'. The concept of biological diversity is well established in virtually all areas of biology, but is rarely considered with regards to bacteria. This is perhaps due to the methodological and conceptual difficulties associated with assessing bacterial biodiversity. However, molecular methods are now becoming available that allow at least comparative assessments of baterial biodiversity. Preliminary data indicate that biological diversity is lost when an environment becomes stressed, for example by pollution. Measures of bacterial biodiversity will not provide information about ecosystem functions, but may provide an indication of the changes occurring within the ecosystem. The trick then will be to assess the importance of the change.

Although it is now possible to measure reasonably routinely total numbers of bacterial species and shifts in bacterial populations (eg. DNA annealing — Section 3.2.2), only one study has been done in an aquatic ecosystem. There would be considerable value in establishing a small pilot research project to obtain some 'typical' data for a number of river types, and then to use these data to further assess the usefulness of the method as a river 'health' indicator.

6.4 METHODS DEVELOPMENT

As noted above, the use of bacteria in assessing Australian river 'health' is presently hampered by the lack of information about the most appropriate species, groups of species or microbial processes to use as indicators. Additionally, there is also a lack of well developed protocols for measuring these indicators and for interpreting the results. However, we can confidently predict that given the rapidly developing knowledge base, the required information and protocols will soon be available to allow the development of microbial methodologies for the assessment of river 'health'.

6.4.1 Technology transfer

Many of the new microbial methods have been developed to satisfy academic research needs and are not currently suitable for routine applications. Close liaison between academic institutions and potential end users, such as environmental agencies and water utilities, is required, to ensure the effective transfer, evaluation and application of these technologies once suitable bacterial species or groups of species are recognised as indicators. Development of rapid, reliable and simple to interpret tests suitable for field use should be a priority.

6.4.2 Sampling

There has been considerable discussion recently regarding the generally poor statistical design adopted in water quality and biological monitoring programs (Underwood, 1991, 1992, 1993). The development of robust and statistically valid microbial sampling methods will be a crucial component of any protocols established for the assessment of river 'health'. Factors that must be considered in the proper design of microbial sampling programs for river 'health' include: what part(s) of the aquatic system (eg. water column, sediments, particular habitat) should be sampled, how often, how many replicates are needed, what is a representative sample size, and how should samples be preserved and stored. Present sampling protocols for assessing the microbial quality of recreational and potable waters for faecal contamination will be inadequate for assessing ecosystem 'health'. Work currently underway at the Water Studies Centre, Monash University (funded by LWRRDC) will address some of the above sampling questions.

6.4.3 Methods

Methods for assessing native bacteria can be classified into three groups:

(i) *Methods to identify and enumerate bacterial species* — include culture techniques for particular organisms (eg. pathogens) and new molecular probes which can determine functional groups (eg. nitrifer population), phylogenetic groups (eg. Enterobacteriaceae) or individual microbial species (eg. *Cryptosporidium* sp., *Salmonella sp.*).

(ii) *Methods to measure bacterial numbers and bacterial biomass* — include direct counts using DNA stains such as DAPI and acridine orange, perhaps coupled with new separation techniques such as field flow fractionation.

(iii) *Methods to measure microbial growth and activity* — include vital stains (eg. INT and CTC) for measuring whether native microorganisms are actively growing, thymidine or leucine incorporation into DNA or protein to measure growth rate, measures of photosynthesis and respiration (and hence P/R ratios), and measures of the activity of microbially-mediated processes (eg. N_2 fixation using the acetylene reduction assay, denitrification measured using the acetylene blockage assay, and biodegradative abilities, such as the ability to degrade aromatic compound).

Australian researchers are currently quite active in developing and using new techniques in all three areas. For example, a collaborative project involving Macquarie University and Australian Water Technologies is well advanced to couple flow cytometry with new immunological and molecular methods for the determination of *Cryptosporidium* and *Giardia* in concentrated natural water samples. Additionally, research is underway at the Water Studies Centre, Monash University using field flow fractionation to provide an improved method for measuring microbial biomass, and in applying a range of molecular methods to the study of microbial groups and processes in Australian rivers.

Analysis kits

Simple and reliable 'kits' are now available for the measurement of specific 'indicator' bacteria (for example, COLILERT to detect *E. coli* in potable water (Beebe *et al.*, 1991)) and kits to detect coliforms in recreational waters (Apte & Batley, 1992). It seems very likely that the development of simple, easy to use protocols will be expanded over the next five years to include many waterborne bacterial and protozoan pathogens (see Cox *et al.*, 1993). For example, a PCR kit for the detection of the bacterial pathogen *Aeromonas sp.* is being developed jointly by Australian Water Technologies (AWT) in association with the University of NSW (Cox, 1993). Work is also progressing well on a joint AWT/Macquarie University project to develop flow cytometric kits for rapid enumeration of microorganisms in water. The first of these kits for the protozoan parasites *Cryptosporidium* and *Giardia* in should be available in the second half of 1997.

New protocols to detect indicators of anthropogenic inputs into riverine environments (eg. the detection of enzymes important in aromatic degradation (Section 4.1.2), are also likely to become available in the near future. The measurement of stress-response proteins has been identified in this review as a novel, potentially useful indicator of ecosystem 'health' (Section 4.1.3). These could be used as bioindicators in two ways; either examine the microbial community directly for these proteins or specific microorganisms with well-characterised stress responses (eg. *E. coli)* may be exposed to the environment (possibly immobilised on a 'dipstick') and examined for the stress responses. A range of monoclonal antibodies are available for specific stress-response proteins, enabling the rapid identification of stress responses and the production of 'kits' that could be used for rapid field evaluations.

Molecular probes

The review of methods currently used in aquatic microbiology (Chapter 3) concluded that new methods based on nucleic acid technology, particularly those based on ribosomal RNA molecular probes, show great promise for determining the presence and numbers of specific microbial species or groups of species (eg. denitrifiers, methanogens), and also to measure the metabolic activity of the species or group. This technique relies on the availability of appropriate rRNA probes. Many rRNA probes are now available, with new probes becoming available all the time. Australian workers can now access this new information very rapidly (ie. generally before it is published in the scientific literature) via the 'Ribosomal RNA Database Project' located in US and accessible via E-Mail.

The design of new rRNA probes is relatively specialised, requiring the extraction of DNA from the particular microorganism, sequencing of the DNA, comparison of the DNA sequence to the ribosomal RNA database, and selection of an appropriate sequence to be used as the probe. Once the sequence has been determined the probe can be synthesised, a tag (fluorescent or radioactive) is attached, and the probe tested against a battery of control species to ensure specificity. At present in Australia there is a lack of researchers with the expertise to design new molecular probes for aquatic microorganisms. For this reason, it makes more sense that the major effort be put into gaining experience and knowledge by applying molecular techniques already available.

It is possible that considerable advances could be made in Australia, both in the fundamental understanding of the microbial ecology of rivers and in the application of microbial indicators of river 'health', if a specialist molecular biology group (or commercial company) were contracted to develop rRNA probes for particular functional genes (eg. sulphate reducers, denitrifiers) of relevance to aquatic microbial ecologists.

Recommendation 3:

That LWRRDC increase its funding of research aimed at the development and field application of new microbial techniques (including those based on particular enzymes and stress-response proteins, rRNA probes and other molecular techniques) for the study of aquatic microbial ecology in Australia.

'Conventional' methods

The most useful of the available 'conventional' methods are: direct counts using DAPI or acridine orange to determine total bacterial numbers; use of INT or CTC staining to determine the number of actively growing cells; and incorporation of ^{3}H-thymidine and ^{3}H-leucine uptake to measure bacterial growth rate. These are all readily available, but are not used on any routine basis by resource management agencies. The following will need to occur if these methods are to become more accessible: (i) standard protocols for their use must be developed, and (ii) a series of training workshops and short courses should be run throughout Australia.

Recommendation 4:

That LWRRDC establish a process to have conventional microbial methods become more accessible to water agencies in Australia. This will involve at least two components: first the development of standard protocols, and second the running of a series of training workshops and short courses throughout Australia.

Technological advances

Two particular technologies were identified in Chapter 3 as having the potential to significantly advance the identification and enumeration, and measurement of biomass of natural populations of aquatic microorganisms, mainly when coupled with some of the new molecular probes discussed above. Flow cytometry, especially when combined with antibody and molecular probe technology, has considerable potential as an extremely rapid method for enumerating particular microorganism species or functional groups of organisms (see Section 3.1.6 for details). In fact, routine methods based on flow cytometry, and developed in Australia (Vesey *et al.*, 1993a,b,c, 1994a,b), are already available to detect the enteropathogens *Cryptosporidium* and *Giardia* in concentrated water samples. Application of this technique is presently limited by the high cost of flow cytometers and the relatively small number of standard protocols for water samples. However, Australian-led developments in progress, directed particularly at environmental analysis, will increase the range of organisms that can be detected and enumerated using this technology, and should also decrease the cost and complexity of the instrumentation.

Sedimentation field flow fractionation (SdFFF) is another, relatively new technique, that has considerable potential, having recently been used with considerable success to measure both numbers and biomass of bacteria cells (Sharma *et al.*, 1993). Microbial biomass measurements using this technique are approximately six times more accurate than with conventional methods. When combined with molecular probe technology, field flow fractionation should allow the measurement of biomass of both a given population and of subsets of the population that hybridise to specific probes. Protocols to combine molecular probe and field flow fractionation technologies are currently being developed at the Water Studies Centre, Monash University.

The above methods will lead to the automated analysis of samples, enabling more objective, extensive and statistically valid sampling regimes. Such automated systems will also allow for more effective use of the limited number of suitably trained personnel.

Recommendation 5:

That LWRRDC cooperate with instrument development groups with the aim of adapting existing and new instrumentation specifically for rapid river 'health' assessment.

6.5 LINKS TO OTHER BIOTIC INDICES

In Chapter 5 we noted that there are very few published studies linking changes in the microbial community with other biotic responses. We attributed this to the relatively recent ability to measure microbes and their activities in natural environments with any degree of ease and accuracy, and to the focus of the small number of Australian microbial ecologists on providing fundamental information on aquatic microbial processes.

Despite this lack of specific studies, it was possible to predict that strong links should exist between microbial communities and macroinvertebrate species composition, algal biomass, and community metabolism in riverine ecosystems. However, the details of these links are not yet known. An important question that needs to be addressed is: will it help managers if the links between microbial indicators and other biotic indices are better quantified? We believe the answer ultimately is yes, but given the present state of knowledge about microbial indicators, the search for such links should be afforded a low priority. Much more important will be the fundamental science needed to better understand the microbial processes operating in riverine ecosystems.

The opportunity exists to include some measurement of microbial indicators in the new National Bioassessment of Rivers Program. Through such a program, it would then be possible to collect information that would contribute to a generally improved understanding of microbial processes in Australian rivers, and in particular would lead to a better understanding of the links between the microbial indicators measured and macroinvertebrate community structure (the latter is the basis of the present national program). For this to occur, however, there is much to be done in (i) better defining the most appropriate microbial indicators to measure, (ii) working up more reliable protocols for the appropriate microbial methods, and (iii) training more scientists skilled in these microbial techniques.

Recommendation 6:

That LWRRDC seek to establish a microbial component in the National River Health Monitoring Program. This will need careful planning which could be done by a small group, perhaps drawn from a range of institutions to maximise expertise and diversity of opinion. The objective should be to have a microbial component introduced in around 18 months.

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8. REFERENCES

Amann, R. L., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. & Stahl, D. A. (1990a). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology*, 56(6), 1919-1925.

Amann, R. L., Krumholz, L. & Stahl, D. A. (1990b). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of Bacteriology*, 172(2), 762-770.

Amann, R. L., Ludwig, W. & Scheifer, K. (1992a). Identification and *in situ* detection of individual bacterial cells. *FEMS Microbiology Letters*, 100, 45-50.

Amann, R., Ludwig, W. & Schleifer, K.-H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbial Reviews*, 59(1), 143-169.

Amann, R. L., Stromley, J., Devereux, R., Key, R. & Stahl, D. A. (1992b). Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Applied and Environmental Microbiology*, 58(2), 614-623.

ANZECC (1992). *Australian Water Quality Guidelines for Fresh and Marine Waters.* Canberra: Australian & New Zealand Environment & Conservation Council.

ANZECC/AWRC (1992). *National Water Quality Management Strategy: Water Quality Towards a National Policy —Discussion Paper.* Canberra: Australian & New Zealand Environment & Conservation Council, and Australian Water Resources Council.

Apte, S. C. & Batley, G. E. (1992). Rapid detection of sewage contamination in natural waters. *Chemistry in Australia,* 59, 397-399.

Ashbolt, N. J. & Veal, D. A. (1994). Testing the waters for a redundant indicator. *Today's Life Science*, 6(6), 28-29.

Atlas, R. M. & Bartha, R. (1992). Hydrocarbon biodegradation and oil spill bioremediation. In K. C. Marshall (Ed.), *Advances in Microbial Ecology* (pp. 287-338). New York: Plenum Press.

Atlas, R. M. & Bartha, R. (1993). *Microbial Ecology: Fundamentals and Applications* (third ed.). New York: The Benjamin/Cummings Publishing Company, Inc.

Atlas, R. M., Sayler, G., Burlage, R. S. & Bej, A. K. (1992). Molecular approaches for environmental monitoring of microorgamisms. *BioTechniques*, 12(5), 706-714.

AWRC (1983). *Proceedings of Eutrophication Workshop.* Canberra, Australia: AWRC Conference Series No. 7, Aust. Govt. Publishing Service.

Azam, F., Fenchel, T., Field, J. G., Gray, J. S., Meyer-Reil, L. A. & Thingstad, F. (1983). The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser.,* 10, 257-263.

Badenoch, J. (1990). *Cryptosporidium in Water Supplies.* London: HMSO.

Banse, K. (1977). Determining the carbon to chlorophyll ratio of natural phytoplankton. *Marine Biology*, 41, 199-212.

Barkay, T., Fouts, D. L. & Olsen, B. H. (1985). Preparation of DNA gene probe for detection of mercury resistance genes in gram-negative bacterial communities. *Applied and Environmental Microbiology*, 49(3), 686-692.

Barkay, T., Liebert, C. & Gillman, M. (1989). Hybridisation of DNA probes with whole-community genome for detection of genes that encode microbial responses to pollutants: *mer* and Hg^{2+} resistance. *Applied and Environmental Microbiology*, 55(6), 1574-1577.

Barkay, T. & Olson, B. H. (1986). Phenotypic and genotypic adaptation of Aerobic heterotrophic sediment bacteria communities to mercury stress. *Applied and Environmental Microbiology,* 52(2), 403-406.

Bedard, D. L., Unterman, R., Bopp, L. H., Harber, M. L. & Johnson, C. (1986). Rapid assay for screening and characterising microorgansisms for the ability to degrade polychlorinated biphenyls. *Applied and Environmental Microbiology*, 51, 761-768.

Beebe, J. L., Kelley, J. K., Crooks, R. L. & Cada, R. L. (1991). Performance of the COLILERT presence/abscence test compared to membrane filtration method for coliform analysis of potatable water samples. In J. R. Hall & G. D. Glysson (Eds.), *Monitoring Water in the 1990's. Meeting New Challenges* (pp. 455-462). Philadelphia PA: American Society for Testing and Materials.

Bergh, O., Borsheim, K. Y., Bratbak, G. & Heldal, M. (1989). High abundance of viruses found in aquatic environments. *Nature*, 340, 467-468.

Bern, L. (1985). Autoradiographic studies of [*methyl*-^{3}H]thymidine incorporation into a cyanobacterium (*Microcystis wesenbergii*) — bacterium association and in selected algae and bacterium. *Applied and Environmental Microbiology*, 49, 232.

Berninger, U.-G., Wickham, S. A. & Finlay, B. J. (1993). Trophic coupling within the microbial food web: a study with fine temporal resolution in a eutrophic freshwater ecosystem. *Freshwater Biology*, 30, 419-432.

Betts, W.B., Casemore, D., Fricker, C., Smith, H. & Watkins, J. (1945). Protozoan Parasites in Water. Royal Society for Chemistry (Cambridge, U.K.) 131-138.

Bianchi, M. & Colwell, R. R. (1985). Microbial indicators of environmental water quality: the role of microorganisms in the assessment and prediction of changes in the marine environment induced by human activities. In J. Salanki (Ed.), *Biological Monitoring of the State of the Environment: Bioindicators* (pp. 5-15). Oxford: IRL Press.

Blackburn, T. H. & Blackburn, N. D. (1993). Rates of microbial processes in sediments. *Phil. Trans. R. Soc. Lond. A*, 344, 49-58.

Blackburn, T. H. & Henriksen, K. (1983). Nitrogen cycling in different types of sediments from Danish waters. *Limnology & Oceanography*, 28, 477-493.

Blom, A., Harder, W. & Matin, A. (1992). Unique and overlapping pollutant stress proteins of *Escherichia coli*. *Applied and Environmental Microbiology*, 58(1), 331-334.

Boon, P. I. (1989a). Organic matter degradation and nutrient regeneration in Australian freshwaters: I. Methods for exoenzyme assays in turbid aquatic environments. *Arch. Hydrobiol.*, 115(3), 339-359.

Boon, P. I. (1989b). Relationships between actinomycete populations and organic matter degradation in Lake Mulwala, south eastern Australia. *Regulated Rivers*, 4(4), 409-418.

Boon, P. I. (1990). Organic matter degradation and nutrient regeneration in Australian freshwaters: II. Spatial and temporal variation, and relation with environmental conditions. *Arch. Hydrobiol.*, 117(4), 405-436.

Boon, P. I. (1991a). Bacterial assemblages in rivers and billabongs of southeastern Australia. *Microbial Ecology*, 22, 27-52.

Boon, P. I. (1991b). Enzyme activities in billabongs of southeastern Australia. In R. J. Chrost (Ed.), *Microbial Enzymes in Aquatic Environments* (pp. 286-297). Berlin: Springer-Verlag.

Boon, P. I. (1992). Antibiotic resistance of aquatic bacteria and its implications for limnological research. *Australian Journal of Marine and Freshwater Research*, 43, 847-859.

Boon, P. I. (1993). Organic matter degradation and nutrient regeneration in Australian freshwaters: III. Size fractionation of phosphatase activity. *Arch. Hydrobiol.*, 126, 339-360.

Boon, P. I. (1994). Discrimination of algal and bacterial alkaline phosphatases with a differential-inhibition technique. *Australian Journal of Marine and Freshwater Research*, 45, 83-107.

Boon, P. I., Frankenberg, J., Hillman, T. J., Oliver, R. L. & Shiel, R. J. (1990). Billabongs. In N. MacKay & D. Eastburn (Eds.), *Understanding the River Murray* (pp. 184-198). Canberra: Murray Darling Basin Commission.

Boon, P. I. & Sorrell, B. K. (1991). Biogeochemistry of billabong sediments. I. The effect of macrophytes. *Freshwater Biology*, 26, 209-226.

Boulton, A. J. & Boon, P. I. (1991). A review of methodology used to measure leaf litter decomposition in lotic environments: time to turn over an old leaf? *Australian Journal of Marine and Freshwater Research*, 42, 1-43.

Bratbak, G. (1993). Microscope methods for measuring bacterial biovolume: epifluorescence microscopy, scanning electron microscopy and transmission electron microscopy. In P. F. Kemp, B. F. Sherr, E. B. Sherr & J. J. Cole (Eds.), *Handbook of Methods in Aquatic Microbial Ecology* (pp. 309-317). Florida: CRC Press, Inc.

Brock, T. D. (1987). The study of microorganisms *in situ*: progress and probles. In M. Fletcher, T. R. G. Gray & J. G. Jones (Eds.), *Ecology of Microbial Communities* (pp. 1-20). Cambridge: Cambridge University Press.

Brock, T. D. & Madigan, M. T. (1991). *Biology of Microorganisms* (sixth ed.). New Jersey: Prentice Hall.

Brown, J. F., Jr., Bedard, D. L., Brennan, M. J., Carnahan, J. C., Feng, H. & Wagner, R. E. (1987a). Polychlorinated biphenyl dechlorination in aquatic sediments. *Science*, 236, 709-712.

Brown, J. F., Jr., Wagner, R. E., Feng, H., Bedard, D. L., Brennan, M. J., Carnahan, J. C. & May, R. J. (1987b). Environmental dechlorination of PCBs. *Environmental Toxicology and Chemistry*, 6, 579-593.

Bull, A. T., Goodfellow, M. & Slater, J. H. (1992). Biodiversity as a source of innovation in biotechnology. *Ann. Rev. Microbiol.*, 46, 219-252.

Bumpus, J. A. & Aust, S. D. (1987). Biodegradation of DDT (1,1,1-Trichloro-2,2-Bis(4-Chlorophenyl-Ethane) by the white rot fungus Phanerochaete chrysosporium. *Applied and Environmental Microbiology*, 53(9), 2001-2008.

Bunn, S.E. & Boon, P. I. (1993). What sources of organic carbon drive food webs in billabongs?: a study based on stable isotope analysis. *Oecologia*, 96, 85-94.

Byrd, J. J., Leahy, J.G. & Colwell, R. R. (1992). Determination of plasmid DNA concentration maintained by non-culturable *Escherichia coli* in marine microcosms. *Applied and Environmental Microbiology*, 58, 2266-2270.

Campbell, I. C., Macmillan, L. A., Smith, A. J. & McKaige, M. E. (1982). *The Benthic Invertebrates of the Yarra River and its Tributaries*. Melbourne, Australia: Environmental Studies Series No 362, Ministry of Conservation.

Camper, A. K., McFeters, G. A., Characklis, W. G. & Jones, W. L. (1991). Growth kinetics of coliform bacteria under conditions relevant to drinking water distribution systems. *Applied and Environmental Microbiology*, 57, 2233-2239.

Capone, D. G. & Kiene, R. P. (1988). Comparison of microbial dynamics in marine and freshwater sediments: contrasts in anaerobic carbon catabolism. *Limnology and Oceanography*, 33, 725-749.

Carpenter, S. R., Christensen, D. L., Cole, J. J., Cottingham, K. L., Xi, H., Hodgson, J. R., Kitchell, J. F., Knight, S. E., Pace, M. L., Post, D. M., Schindler, D. E. & Voichick, N. (1995). Biological control of eutrophication in lakes. *Environmental Science and Technology*, 29, 784-786.

Cembella, A. D., Antia, N. J. & Harrison, P. J. (1984a). The utilization of inorganic and organic phosphorus compounds as nutrients by eukaryotic microalgae: a multidisciplinary perspective, Part 1. *CRC Crit. Rev. Microbiol.*, 10(4), 317-391.

Cembella, A. D., Antia, N. J. & Harrison, P. J. (1984b). The utilization of inorganic and organic phosphorus compounds as nutrients by eukaryotic microalgae: a multidisciplinary perspective, Part 2. *CRC Crit. Rev. Microbiol.*, 11, 13-117.

CEPA (1992). *Towards Healthy Rivers: The Ills Afflicting Our Rivers and How We Might Remedy Them* Discussion Paper, Comonwealth Environment Protection Agency, Canberra.

Chapman, J. C. (1995). The role of ecotoxicity testing in assessing water quality. *Australian Journal of Ecology*, 20(1), 20-27.

Chapman, P. M. (1992). Ecosystem health synthesis: can we get there from here? *Journal of Aquatic Ecosystem Health*, 1, 69-79.

Chappell, K. R. & Goulder, R. (1992). Epilithic extracellular enzyme activity in acid and calcareous headstreams. *Archiv Fuer Hydrobiologie*, 125(2), 129-148.

Chessman, B. C. (1985). Estimates of ecosystem metabolism in the La Trobe River, Victoria. *Australian Journal of Marine and Freshwater Research*, 36, 873-880.

Chisholm, S. W., Olson, R. J., Zettler, R. E., Goericke, R., Waterbury, J. & Welschmeyer, N. (1988). A novel free-living prochlorophyte occurs at high cell concentrations in the oceanic euphotic zone. *Nature*, 334, 340-343.

Choudhary, S. K. & Bilgrami, K. S. (1992). Studies on phytoplankton productivity of River Ganga at Sultanganj and Bhagalpur, India. *Polskie Archiwum Hydrobiologii*, 38(3-4), 375-379.

Christensen, D. & Blackburn, T. H. (1982). Turnover of ^{14}C labelled acetate in marine sediments. *Marine Biology*, 71, 113-119.

Cole, J. J., Findlay, S. & Pace, M. L. (1988). Bacterial production in fresh and saltwater ecosystems: a cross-system overview. *Mar. Ecol. Prog. Ser.*, 43, 1-10.

Colwell, R. R. & Nelson, J. D. (1975). *Metabolism of Mercury Compounds in Microorganisms.* Rhode Island.

Conway de Macario, E. C., Wolin, M. J. & Macario, A. J. L. (1982). Antibody analysis of relationships among methanogenic bacteria. *Journal of Bacteriology*, 149, 316-319.

Cox, P. (1993). PCR for detection of pathogens in water. In D. A. Veal (Ed.), *Water Microbiology for the 21st Century* Sydney: Australian Environmental Flow Cytometry Group, Macquarie University.

CSIRO (1992). *Towards Healthy Rivers*, Consultancy Report No 92/44, Division of Water Resources, CSIRO, Canberra.

Current, W. L. & Garcia, L. S. (1991). Cryptosporidiosis. *Clin. Microbiol. Rev.*, 4(3), 325-358.

Currie, D. J. (1990). Large scale variability and interactions amongst phytoplankton, bacterioplankton and phosphorus. *Limnology & Oceanography*, 35, 1437-1455.

Currie, D. J. & Kalff, J. (1984a). A comparison of the abilities of freshwater algae and bacteria to acquire and retain phosphorus. *Limnology & Oceanography*, 29, 298-310.

Currie, D. J. & Kalff, J. (1984b). The relative importance of bacterioplankton and phytoplankton in phosphorus uptake in freshwaters. *Limnology & Oceanography*, 29, 311-321.

Currie, D. J. & Kalff, J. (1984c). Can bacteria outcompete phytoplankton for phosphorus? A chemostat test. *Microbial Ecology*, 10, 205-216.

Daley, R. J. (1979). Direct epifluorescent enumeration of native aquatic bacteria: uses, limitations, and comparative accuracy. In J. W. Costerton & R. R. Colwell (Eds.), *Native Aquatic Bacteria: Enumeration, Activity, and Ecology* (pp. 29-45). Philadelphia: American Society for Testing and Materials.

Daneri, G., Riemann, B. & Williams, P. J. (1994). In situ bacterial production and growth yield measured by tymidine and leucine and fractionated dark oxygen uptake. *Journal of Plankton Research*, 16, 105-113.

Davelaar, D. (1993). Ecological significance of bacterial polyphosphate metabolism in sediments. *Hydrobiologia*, 253, 179-192.

Davies, P. M. (1993). *Ecosystem Ecology of Upland Streams of the Northern Jarrah Forest, Western Australia.* PhD, Univ. Western Australia.

Davison, A. D., Csellner, A., Karuso, P. H. & Veal, D. A. (1994). Community interactions and characterisation of a mixed microbial consortium capable of growth on biphenyl. *FEMS Microbiology Ecology*, 14, 133-146.

de Montigny, C. & Prairie, Y. T. (1993). The relative importance of biological and chemical processes in the release of phosphorus from a highly organic sediment. *Hydrobiologia*, 253, 141-150.

DeLong, E. F., Wickham, G. S. & Pace, N. R. (1989). Phylogenetic strains: ribosomal RNA-based probes for the identification of single cells. *Science*, 234, 1360-1363.

Devereux, R., Delaney, M., Widdel, F. & Stahl, D. A. (1989). Natural relationships among sulfate-reducing eubacteria. *Journal of Bacteriology*, 171(12), 6689-6695.

Dunn, N. W. & Gansalus, I. C. (1973). Transmissible plasmid coding early enzymes of naphtalene oxidation in *Pseudomonas putita*. *Journal of Bacteriology*, 114, 974-979.

DWR-NSW (1992). *Blue-Green Algae: Final Report.* NSW Blue-Green Algal Task Force, Department of Water Resources, Parramatta, NSW.

Edberg, S. C., Allen, M. J. & Smith, D. B. (1988). National field evaluation of a defined substrate method for simultaneous enumeration of total coliforms and *Escherichia coli* from drinking water: Comparison with the standard multiple tube fermentation method. *Applied and Environmental Microbiology*, 54(6), 1595-1601.

Edberg, S. C., Allen, M. J. & Smith, D. B. (1989). National field evaluation of a defined substrate method for the simultaneous detection of total coliforms and *Escherichia coli* from drinking water: Comparison with presence-abscence techniques. *Applied and Environmental Microbiology*, 55(4), 1003-1008.

Edberg, S. C., Allen, M. J., Smith, D. B. & Kriz, N. J. (1990). Enumeration of total coliforms and *Escherichia coli* from water by defined substrate technology. *Applied and Environmental Microbiology*, 56(2), 366-369.

Elliot, E. L. & Colwell, R. R. (1985). Indicator organisms for estuarine and marine waters. *FEMS Microbiol. Rev.*, 32, 61-79.

Emond, E., Fliss, I. & Pandia, S. (1993). A ribosomal DNA fragment of Listeria monocytogenes and its use as a genus-specific probe in an aqueous-phase hybridization assay. *Applied and Environmental Microbiology*, 59(8), 2690-2697.

Findlay, S., Pace, M. L., Lints, D., Cole, J. J., Caraco, N. F. & Peierls, B. L. (1991). Weak coupling of bacterial and algal production in a heterotrophic ecosystem: the Hudson River estuary. *Limnology & Oceanography*, 36, 268-278.

Fliss, I., Emond, E., Simard, R. E. & Pandian, S. (1991). A rapid and efficient method of lysis of *Listeria* and other gram-positive bacteria using mutanolysin. *BioTechniques*, 11(4), 453-457.

Fogg, G. E. (1972). *Photosynthesis.* London: English University Press.

Ford, T. E. (Ed.). (1993). *Aquatic Microbiology: An Ecological Approach.* Melbourne: Blackwell Scientific Publications.

Frankel, G., Giron, J. A., Valmassol, J. & Schoolnik, G. K. (1989). Multi-gene amplification: simultaneous detection of three virulence genes in diarrhoeal stool. *Molecular Microbiology* 3, 1729-1734.

Fry, J. C. (1988). Determination of biomass. In B. Austin (Ed.), *Methods in Aquatic Bacteriology* (pp. 27-72). Chichester: Wiley.

Fuhrman, J. A. & Azam, F. A. (1982). Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. *Marine Biology*, 66, 109-120.

Gachter, R. & Meyer, J. S. (1993). The role of microorganisms in mobilization and fixation of phosphorus in sediments. *Hydrobiologia*, 253, 103-121.

Gachter, R., Meyer, J. S. & Mares, A. (1988). Contribution of bacteria to release and fixation of phosphorus in lake sediments. *Limnology & Oceanography*, 33, 1542-1558.

Gauthier, M. J., Flatau, G. & Bernard, P. (1981). Tolerance au plomb, au cadmium et resistance aux antibiotiques chez les bacteries heterotrophes de sediments maris portuaires ou littoraux. *Rev. Int. Oceanogr. Med.*, 63-64, 65-83.

Gilliland, G., Perrin, S., Blanchard, K. & Bunn, H. F. (1990a). Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction. *Proceedings of the National Academy of Sciences of the United States of America*, 87, 2725-2729.

Gilliland, G., Perrin, S. & Bunn, H. F. (1990b). Competitive PCR for quantitation of mRNA. In M. Innis, D. Gelfand, D. Sninsky & T. White (Eds.), *PCR Protocols: A Guide to Methods and Applications* (pp. 60-69). New York: Academic Press.

Giovannoni, S. J., DeLong, E. F., Olsen, G. J. & Pace, N. R. (1988). Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. *Journal of Bacteriology*, 179(2), 720-726.

Goebel, U. B., Geiser, A. & Stanbridge, E. J. (1987). Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between *Mycoplasma* species. *Journal of General Microbiology*, 133, 1969-1974.

Goodfellow, M. & Minnikin, D. E. (Eds.). (1985). *Chemical Methods in Bacterial Systematics.* London: Academic.

Hahn, D., Amann, R. I. & Zeyer, J. (1993). Detection of mRNA in *Streptomyces* cells by whole-cell hybridization with digoxigenin-labeled probes. *Applied and Environmental Microbiology*, 59, 2753-2757.

Hahn, D., Kester, R., Starrenburg, M. J. C. & Akkermans, A. D. L. (1990a). Extraction of ribosomal RNA from soil for detection of *Frankia* with oligonucleotide probes. *Arch. Microbiol.*, 154, 329-335.

Hahn, D., Starrenburg, M. J. C. & Akkermans, D. J. C. (1990b). Oligonucleotide probes that hybridise with rRNA as a tool to study *Frankia* strains in root nodules. *Applied and Environmental Microbiology*, 56, 1342-1346.

Hall, B. G. (1982). Chromosomal mutation for citrate utilization by *Escherichia coli* K-12. *Journal of Bacteriology*, 151, 269-273.

Harris, G. P. (1994). *Nutrient Loadings and Algal Blooms in Australian Waters —A Discussion Paper.* Canberra: LWRRDC Technical Paper.

Hart, B. T. (1993). A national approach to river management in Australia. *Search*, 24, 125-130.

Hart, B. T., Angehrn-Bettinazzi, C., Campbell, I. C. & Jones, M. J. (1993). Australian water quality guidelines: role in protecting ecosystem health. *Journal of Aquatic Ecosystem Health*, 2, 151-163.

Hart, B. T. & Campbell, I. C. (1994). Assessment of river 'health' in Australia. In M. C. Uys (Ed.), *Classification of Rivers and Environmental Health Indicators* (pp. 117-189). Pretoria, South Africa: Proc. South African — Australian Workshop, 7-14 Feb., Cape Town, Water Research Commission Report No. TT 63/94.

Hart, B. T., Freeman, P. & McKelvie, I. D. (1992). Whole-stream phosphorus release studies: variations in uptake length with initial phosphorus concentration. *Hydrobiologia*, 235/236, 573-584.

Havelaar, A. (1993). Bacteriophages as models of human enteric viruses in the environment. *ASM News*, 59, 614-618.

Hellawell, J. M. (1986). *Biological Indicators of Freshwater Pollution and Environmental Management.* London: Elsevier Applied Science Publ.

Hobbie, J. E. & Crawford, C. C. (1969). Respiration for bacterial uptake of dissolved organic compounds in natural waters. *Limnology & Oceanography*, 14, 528-.

Hobbie, J. E., Daley, R. J. & Jasper, S. (1977). Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology*, 33, 1225-1228.

Hobbie, J. E. & Ford, T. E. (1993). A perspective on the ecology of aquatic microbes. In T. E. Ford (Ed.), *Aquatic Microbiology: An Ecological Approach* (pp. 1-14). Melbourne: Blackwell Scientific Publications.

Holben, W. E., Jansson, J. K., Chelm, B. K. & Tiedje, J. M. (1988). DNA probe method for the detection of specific microorganisms in the soil bacterial community. *Applied and Environmental Microbiology*, 54(3), 703-711.

Holt, J. G. (Ed.). (1986-1989). *Bergey's Manual of Sytematic Bacteriology.* Baltimore: Williams & Wilkins.

Howgrave-Graham, A. R. & Steyn, P. L. (1988). Application of the fluorescent-antibody technique for the detection of *Sphaerotilus natans* in activated sludge. *Applied and Environmental Microbiology*, 54, 799-802.

Hynes, H. B. N. (1975). The stream and its valley. *Verh. Internat. Verin. Limnol.*, 19, 1-15.

Ingraham, L. J., Maaloe, O. & Neidhardt, F. C. (1983). *Growth of the Bacterial Cell.* Sutherland, Mass.: Sinauer Associates.

Inverson, W. P. & Brinckman, F. E. (1978). Microbial metabolism of heavy metals. In R. Mitchell (Ed.), *Water Pollution Microbiology*, New York: Wiley Inter-Sciences.

Jain, R. K., Burlage, R. S. & Sayler, G. S. (1988). Methods for detecting recombinant DNA in the environment. *CRC Critical Reviews in Biotechnology*, 8(1), 33-84.

Jansson, M. (1988). Phosphate uptake and utilization by bacteria and algae. *Hydrobiologia*, 170, 177-189.

Jansson, M. (1993). Uptake, exchange and excretion of orthophosphate in phosphate-starved *Scenedesmus quadricauda* and *Pseudomonas* K7. *Limnology & Oceanography*, 38, 1162-1178.

Jansson, M., Olsson, H. & Pettersson, K. (1988). Phosphatases: origin, characteristics and function in lakes. *Hydrobiologia*, 170, 157-175.

Kamp, P. F. & Chakrabart, A. M. (1974). Plasmids specifying p-chlorobiphenyl degredation in enteric bacteria. In K. N. Timmis & A. Puhler (Eds.), *Plasmids of Medical, Environmental and Commercial Importance* (pp. 275-285). Amsterdam: Elsevier/North Holland Biomed. Press.

Kane, M. D., Poulsen, L. K. & Stahl, D. A. (1993). Monitoring the enrichment and isolation of sulfate-reducing bacteria by oligonucleotide hybridisation probes designed form environmentally derived 16S rRNA sequences. *Applied and Environmental Microbiology*, 59(3), 682-686.

Karl, D. M. (1986). Determination of *in situ* microbial biomass, viability, metabolism, and growth. In J. S. Poindexter & E. R. Leadbetter (Eds.), *Bacteria in Nature. Vol 2. Methods and Special Applications in Bacterial Ecology* (pp. 85-176). New York: Plenum.

Kemp, P. F., Sherr, B. F., Sherr, E. B. & Cole, J. J. (Eds.). (1993). *Handbook of Methods in Aquatic Microbial Ecology.* Ann Arbor: Lewis Publishers.

King, G. M. (1992). Ecological aspects of methane oxidation, a key determinant of global methane dynamics. In K. C. Marshall (Ed.), *Advances in Microbial Ecology.* New York: Plenum Press.

Kirk, J. T. O. (1986). *Light and Photosynthesis in Aquatic Ecosystems.* Melbourne, Australia: Cambridge University Press.

Kirshman, D., K'nees, E. & Hodson, R. (1985). Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic ecosystems. *Applied and Environmental Microbiology*, 49, 599-607.

Klemm, D. J., Lewis, P. A., Fulk, F. & Lazorchak, J. M. (1990). *Macroinvertebrate Field and Laboratory Methods for Evaluating the Biological Integrity of Surface Waters.* Washington: EPA/600/4-90/030, US Environmental Protection Agency.

Kogure, K., Simidu, U. & Taga, N. (1979). A tentative direct microscopic method for counting living marine bacteria. *Canadian Journal of Microbiology*, 25, 415-420.

Kramer, J. G. & Singleton, F. L. (1993). Measure of rRNA variations in natural communities of microorganisms on the southeastern U.S. continental shelf. *Applied and Environmental Microbiology*, 59(8), 2430-2436.

Kumari, J. N., Venkateswarlu, V. & Rajkumar, B. (1991). Heavy metal pollution and phytoplankton in the River Moosi (Hyderbad), India. *International Journal of Environmental Studies*, 38, 157-164.

Lake, P. S., Barmuta, L. A., Boulton, A. J., Campbell, I. C. & St Clair, R. M. (1987). Australian streams and northern hemisphere stream ecology: comparisons and problems. *Proceedings of the Ecological Society of Australia,* 14, 61-82.

Lee, S. & Fuhrman, J. A. (1990). DNA hybridisation to compare species compositions of natural bacterioplankton assemblages. *Applied and Environmental Microbiology*, 56, 739-746.

Lewis, C. M. & Mak, J. L. (1989). Comparison of membrane filtration and autoanalysis Colilert presence-absence techniques for analysis of total coliforms and *Eschericia coli* in drinking water samples. *Applied and Environmental Microbiology*, 55, 3091-3094.

Lewis, G.D., Grey-Young, G.M. & Loutit, M.W. (1991). In R.G Bell, T. M. Hume, T. R. Healy (Eds.), *Costal Engineering — Climate for Change.* Proceedings of 10th Australasian Conference on Costal and Ocean Engineering, Auckland: New Zealand.

Li, W. K. W. & Wood, A. M. (1988). Vertical distribution of North Atlantic ultraplankton: analysis by flow cytometry and epifluorescence microscopy. *Deep-sea Research*, 35, 1615-1638.

Liesack, W., Weyland, H. & Stackebrandt, E. (1991). Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. *Microbial Ecology*, 21, 191-198.

Likens, G. E. (1984). Beyond the shoreline: a watershed ecosystem approach. *Verh. Internat. Verein. Limnol.*, 22, 1-22.

Lock, M. A. (1993). Attached microbial communities in rivers. In T. E. Ford (Ed.), *Aquatic Microbiology: An Ecological Approach* (pp. 113-138). Melbourne: Blackwell Scientific Publications.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin Phenol reagent. *Journal of Biological Chemistry*, 193, 265-275.

Lund, B. A. & Blackburn, T. H. (1989). Urea turnover in a coastal marine sediment measured by a ^{14}C-urea short term incubation. *Journal of Microbiol. Methods*, 9, 297-308.

Maasdam, R. & Smith, D. G. (1994). New Zealand's national river quality network, 2. Relationships between physico-chemical data and environmental factors. *New Zealand Journal of Marine and Freshwater Research*, 28, 37-54.

MacKay, S. & Ridley, P. (1983). In T.M. Smyth (Ed.), *Proceedings of Tenth Federal Convention.* Sydney: Australian Water and Waste Water Association.

Manz, W., Amann, R., Ludwig, W., Wagner, M. & Schleifer, K.H. (1992). Phylogenetic oligonucleotide probes for the major subclasses of Proteobacteria: problems and solutions. *System. Appl. Microbiology*, 15, 593-600.

Manz, W., Szewzyk, U., Ericsson, P., Amann, R., Schleifer, K. & Stenstrom, T. A. (1993). *In situ* identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23 S rRNA-directed fluorescent oligonucleotide probes. *Applied and Environmental Microbiology*, 59(7), 2293-2298.

Mazodier, P. & Davies, J. (1991). Gene transfer between distantly related bacteria. *Ann. Rev. Genet.*, 25, 147-171.

Melnick, J. L. & Gerba, C. P. (1980). *CRC Crit. Rev. Environ. Contr.*, 10, 65.

Metcalfe-Smith, J. L. (1992). Biological water quality assessment of rivers based on macroinvertebrate communities. In P. Calow & G. E. Petts (Eds.), *Rivers Handbook*. Oxford: Blackwell Scientific Publications.

Meyer, J. L. & Edwards, R. T. (1990). Ecosystem metabolism and turnover of organic carbon along a blackwater river continuum. *Ecology*, 71, 668-677.

Meyer, J. L., McDowell, W. H., Bott, T. L., Elwood, J. W., Ishizaki, C., Melack, J. M., Peckarsky, B. L., Peterson, B. J. & Rublee, P. A. (1988). Elemental dynamics in streams. *Journal of the North American Benthological Society*, 7(4), 410-432.

Minshall, G. W., Petersen, R. C., Cummins, K. W., Bott, T. L., Sedell, R., Cushing, C. E. & Vanote, R. L. (1983). Interbiome comparison of stream ecosystem dynamics. *Ecological Monographs*, 53(1), 1-25.

Mitchell (Ed.). (1978). *Water Pollution Microbiology*. New York: Wiley Inter-Sciences.

Mitchell, R. (1979). Environmental effects on microbial processes. In R. R. Colwell & J. Foster (Eds.), *Aquatic Microbial Ecology* (pp. 25-34). Maryland, USA: University of Maryland.

Morgan, J. A. W. & Pickup, R. W. (1993a). Acitivity of microbial peptidases, oxidases and esterases in lake waters of varying trophic status. *Canadian Journal of Microbiology*, 39, 795-803.

Moriarty, D. J. W. (1975). A method for estimating the biomass of bacteria in aquatic sediments and its application in trophic studies. *Oecologia*, 20, 219-229.

Moriarty, D. J. W. (1977). Improved method using muramic acid to estimate biomass of bacteria in sediments. *Oecologia*, 26, 317-323.

Moriarty, D. J. W. (1978). Estimation of bacterial biomass in water and sediments using muramic acid. In M. W. Loutit & J. A. R. Miles (Eds.), *Microbial Ecology* (pp. 31-33). Berlin: Springer-Verlag.

Moriarty, D. J. W. (1986). Measurement of bacterial growth rates in aqueous systems form rates of nucleic acid synthesis. In K. C. Marshall (Ed.), *Advances in Microbial Ecology* (pp. 245-292). New York: Plenum.

Moriarty, D. J. W., Roberts, D. G. & Pollard, P. C. (1990). Primary and bacterial productivity of tropical seagrass communities in the Gulf of Carpentaria, Australia. *Mar. Ecol. Prog. Ser.*, 61, 145-157.

Moriarty, D. J. W. & Veal, D. A. (1992). Increasing awareness of microbial ecology. *Search*, 23(3), 100-103.

Moriarty, D. J. W., White, D. C. & Wassenburg, T. J. (1985). A convenient method for measuring rates of phospholipid synthesis in seawater and sediments: its relevance to the determination of bacterial productivity and the disturbance artifacts introduced by measurements. *Journal of Microbiological Methods*, 3, 321-330.

Mortimer, C. H. (1941). The exchange of dissolved substances between mud and water in lakes, 1 and 2. *Journal of Ecology*, 29, 280-329.

Mortimer, C. H. (1942). The exchange of dissolved substances between mud and water in lakes, 3 and 4. *Journal of Ecology*, 30, 147-201.

Moss, D., Furse, M. T., Wright, J. F. & Armitage, P. D. (1987). The prediction of the macroinvertebrate fauna of unpolluted running-water sites in Great Britain using environmental data. *Freshwater Biology*, 17, 41-52.

Mulholland, P. J., Newbold, J. D., Elwood, J. W. & Ferren, L. A. (1985). Phosphorus spiralling in a woodland stream: seasonal variations. *Ecology*, 66(3), 1012-1023.

Mulholland, P. J., Steinman, A. D. & Elwood, J. W. (1990). Measurement of phosphorus uptake length in streams: Comparisons of radiotracer and stable PO_4 releases. *Can. J. Fish. Aquatic Sci.*, 47(12), 2351-2357.

Nakagawa, Y. & Yamasato, K. (1993). Phylogenetic diversity of the genus *Cytophaga* revealed by 16S rRNA sequencing and menaquinone analysis. *Journal of General Microbiology*, 139, 1155-1161.

Neidhardt, F., VanBogeien, R. & Vaughn, V. (1984). The genetics and regulation of heat-shock proteins. *Ann. Rev. Genet.*, 18, 295-329.

Newbold, J. D., Elwood, J. W., O'Neill, R. V. & Van Winkle, W. (1981). Measuring nutrient spiralling in streams. *Can. J. Fish. Aquatic Sci.*, 38, 860-863.

Newell, S. Y., Fallon, R. D. & Tabor, P. S. (1986). Direct microscopy of natural assemblages. In J. S. Poindexter & E. R. Leadbetter (Eds.), *Bacteria in Nature. Vol 2. Methods and Special Applications in Bacterial Ecology* (pp. 1-48). New York: Plenum.

Nielsen, J. (1992). Denitrification in sediments determined from nitrogen isotope pairing. *FEMS Microbiol. Ecol.*, 86, 357-362.

Norris, R., Hart, B. T., Finlayson, M. & Norris, K. R. (Eds.). (1995). *Use of Biota to Assess Water Quality.* Melbourne: Special Issue Australian Journal of Ecology, Blackwell Science.

OECD (1982). *Eutrophication of Waters: Monitoring, Assessment and Control.* Paris: Organisation for Economic Cooperation and Development.

Ogram, A., Sayler, G. S. & Barkay, T. (1987). The extraction and purification of microbial DNA from sediments. *Microbiological Methods*, 7, 57-66.

Olsen, G. J., Lane, D. J., Giovannoni, S. J. & Pace, N. R. (1986). Microbial ecology and evolution: a ribosomal RNA approach. *Ann. Rev. Microbiol.*, 40, 337-365.

Olsen, G. J., Larsen, N. & Woese, C. R. (1991). The ribosomal RNA database project. *Nucleic Acids Research*, 19, 2017-2021.

Olsen, R. J., Vaulot, D. & Chisholm, S. W. (1985). Marine phytoplankton distributions measured using shipboard flow cytometry. *Deep-sea Research*, 32, 129-144.

Orellana, M. V., Perry, M. J. & Watson, B. A. (1988). Probes for assessing single cell primary production: antibodies against ribulose 1,5 bisphosphate carboxylase (RuBPCASE) and peridinin/chlorophyll a (PCP). In C. M. Yentsch, F. C. Maque & P. K. Horan (Eds.), *Immunochemical Approaches to Costal Estuarine and Oceanographic Questions. Lecture Notes on Costal and Estuarine Studies No. 25* (pp. 243-262). New York: Springer Verlag.

Osaki, Y., Matsueda, T., Nagase, M., Ogo, A. & Takahashi, K. (1991). The microbial degradability of aniline in river water and an attempt to use the level of the biodegradability as an indicator of water pollution. *Eisei Kagaku*, 37(5), 411-417.

Ostling, J., Homquist, L., Flardh, K., Svenblad, B. Jouper-Jaan, A. & Kjelleberg, S. (1993). Starvation and Recovery of *Vibrio*. In S. Kjelleberg (Ed.), *Starvation in Bacteria* (pp. 103-127). New York: Academic Press.

Owen, H. A. & Winner, R. W. (1991). Deflagellation of *Chlamydomonas reinhardtii* (Chlorophyceae) as an indicator of copper toxicity. *Journal of Phycology*, 27(3 Suppl), 55.

Pace, M. L. (1988). Bacterial mortality and the fate of bacterial production. *Hydrobiologia*, 159, 41-49.

Palmer, S. E., Niederlehner, B. R. & Cairns, J. (1994). Assessment of pollution impact on aquatic microbial communities based on DNA hybridization and protozoan identification: preliminary method development and comparison. *Journal of Aquatic Ecosystem Health*, 3, 35-44.

Parkes, R. J. (1987). Analysis of microbial communities within sediments using biomarkers. In M. Fletcher, T. R. G. Gray, & J. G. Jones (Eds.), *Ecology of Microbial Communities* (pp. 147-177). Cambridge: Cambridge University Press.

Paul, J. H. (1993). The advances and limitations of methodology. In T. E. Ford (Ed.), *Aquatic Microbiology* (pp. 15-46). Boston: Blackwell Scientific Publications.

Paul, J. H., Cazares, L. & Thurmond, J. (1990). Amplification of the *rbcL* gene from dissolved and particulate DNA from aquatic environments. *Applied and Environmental Microbiology*, 56, 1963-1966.

Pedros-Alio, C. (1993). Diversity of bacterioplankton. *Trends in Ecology and Evolution*, 8(3), 86-90.

Peterson, S. (1993). Rapid faecal coliform test. In D. Veal (Ed.), *Water Microbiology for the 21st Century*. Sydney: Macquarie University.

Picard, C., Ponsonnet, C., Paget, E., Nesme, X. & Simonet, P. (1992). Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Applied and Environmental Microbiology*, 58(9), 2717-2722.

Pickup, R. W., Simon, B. M., Jones, J. G., Saunders, J. R., Carter, J. P., Morgan, J. A. W., Winstanley, C. & Raitt, F. C. (1990). Survival of laboratory and freshwater bacteria carrying an extrachromosomal xylE gene in freshwater microcosms. In J. C. Fry & M. J. Day (Eds.), *Bacterial Genetics in Natural Environments* (pp. 89-99). London: Chapman and Hall.

Pidgeon, R. W. J. & Cairns, S. C. (1981). Decomposition and colonisation by invertebrates of native and exotic leaf material in a small stream in New England (Australia). *Hydrobiologia,* 77, 113-127.

Pigram, J. J. (1986). *Issues in the Management of Australia's Water Resources.* Melbourne, Australia: Longman Cheshire Pty Ltd.

Plafkin, J. L., Barbour, M. T., Porter, K. D., Gross, S. K. & Hughes, R. M. (1989). *Rapid Bioassessment Protocols for use in Streams and Rivers. Benthic Macroinvertebrates and Fish.* Washington, DC: EPA/444/4-89/001, Office of Water Regulations and Standards, US Environmental Protection Agency.

Pollard, D. R., Johnson, W. M., Lior, H., Tyler, S. D. & Rozee, K. R. (1990). Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. *Journal of Clinical Microbiology,* 28, 540-545.

Pomeroy, L. R. (Ed.). (1974). *Cycles of Essential Elements.* Stroudsburg, Penn: Dowden, Hutchinson and Ross.

Porter, K. G. & Feig, Y. S. (1980). The use of DAPI for identifying and counting aquatic microflora. *Limnology & Oceanography*, 25, 943-948.

Porter, K. G., Paerl, H., Hodson, R., Pace, M., Priscu, J., Riemann, B., Scavia, D. & Stockner, J. (1988). Microbial interactions in lake food webs. In S. R. Carpenter (Ed.), *Complex Interactions in Lake Communities* (pp. 209-227). Heidelberg: Springer Verlag.

Poulsen, L. K., Ballard, G. & Stahl, D. A. (1993). Use of rRNA flourescence *in situ* hybridization for measuring the activity of single cells in young established biofilms. *Applied and Environmental Microbiology*, 59(5), 1354-1360.

Rademaker, C. M. A., Wolfhagen, M. J. H. M., Jansze, M., Oteman, M., Fluit, A. C., Glerum, J. H. & Verhoef, J. (1992). Digoxigenin labelled DNA probes for rapid detection of enterotoxigenic, enteropathogenic and Vero cytotoxin producing *Escherichia coli* in faecal samples. *Journal of Microbiological Methods*, 15, 121-127.

Rheinheimer, G. (1991). *Aquatic Microbiology,* 4th Edition. Brisbane: John Wiley & Sons.

Riemann, B. & Bell, R. T. (1992). Advances in estimating bacterial biomass and growth in aquatic systems. *Arch. Hydrobiol.*, 118, 385-402.

Ross, J. L., Boon, P. I. & Hart, B. T. (1995). Detection and quantification with 16S rRNA probes of planktonic methylotrophic bacteria in a freshwater floodplain lake. *Limnology & Oceanography*, (submitted).

Sayler, G. S. & Layton, A. C. (1990). Environmental application of nucleic acid hybridization. *Ann. Rev. Microbiology*, 44, 625-648.

Schmidt, T. M., DeLong, E. F. & Pace, N. R. (1991). Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *Journal of Bacteriology*, 173(14), 4371-4378.

Scholz, O. & Boon, P. I. (1993a). Alkaline phosphatase, aminopeptidase and -D glucosidase activities associated with billabong periphyton. *Arch. Hydrobiol.*, 126, 429-443.

Scholz, O. & Boon, P. I. (1993b). Biofilms on submerged river red gum (*Eucalyptus camaldulensis* Dehnh. Myrtaceae) wood in billabongs: an analysis of bacterial assemblages using phospholipid profiles. *Hydrobiologia*, 259, 169-178.

Scholz, O. & Boon, P. I. (1993c). Biofilm development and extracellular enzyme activities on wood in billabongs of south-eastern Australia. *Freshwater Biology*, 30, 359-368.

Seitzinger, S. P. (1988). Denitrification in freshwater and coastal marine ecosystems: Ecological and geochemical significance. *Limnology & Oceanography*, 33, 702-724.

Shahwani, S. M. A. & Horan, N. J. (1991). The use of protozoa to indicate changes in the performance of activated sludge plants. *Water Research,* 25, 633-638.

Sharma, R. V., Edwards, R. T. & Beckett, R. (1993). Physical characterization and quantification of bacteria by sedimentation field-flow fractionation. *Applied and Environmental Microbiology*, 56(6), 1864-1875.

Shimshock, N., Sennefelder, G., Dueker, M., Thurberg, F. & Yarish, C. (1992). Patterns of metal accumulation in *Laminaria longicruris* from Long Island Sound (Connecticut). *Archives of Environmental Contamination and Toxicology*, 22(3), 305-312.

Sieburth, J. M., Johnson, P. W. & Hargraves, P. (1988). Ultrastructure and ecology of Aurecoccus anophageferens gen. et sp. Nov. (Chrysophyceae): the dominant picoplankter during a bloom in Narragansett Bay, Rhode Island, Summer 1985. *Journal of Phycology*, 24, 416-425.

Simon, M. & Azam, F. (1989). Protein content and protein synthesis rates of planktonic marine bacteris. *Marine Ecology, Progress Series*, 51, 201-213.

Sinke, A. J. C., Cottaar, F. H. M. & Keizer, P. (1993). A method to determine the contribution of bacteria to phosphate uptake by aerobic freshwater sediments. *Limnology & Oceanography*, 38(5), 1081-1087.

Sloth, N. P., Nielsen, L. P. & Blackburn, T. H. (1992). Measurement of nitrification in sediment cores using acetylene inhibition. *Limnology & Oceanography*, 37, 1108-1112.

Smith, D. G. (1987). *Water Quality Indexes for use in New Zealand's Rivers and Streams*. No. 12. Water Quality Centre, Hamilton.

Smith, H. V. & Rose, J. B. (1990). Waterborne cryptosporidiosis. *Parasitology Today*, 6, 8-12.

Sorrell, B. K. & Boon, P. I. (1992). Biogeochemistry of billabong sediments. II. Seasonal variations in methane production. *Freshwater Biology*, 27, 435-445.

Spector, M., Alibada, Z., Gonzales, T. & Foster, J. (1986). Global control in *Salmonella typhimurium*: two-dimensional electrophoretic analysis of starvation-, anaerobiosis-, and heat shock-inducible proteins. *Journal of Bacteriology*, 168, 420-424.

Stahl, D. A., Flesher, B., Mansfield, H. R. & Montgomery, L. (1988). Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Applied and Environmental Microbiology*, 54(5), 1079-1084.

Starr, M. P. (Ed.). (1981). *The Prokaryotes: A Handbook on Habitats, Isolation and Identification.* New York: Springer Verlag.

Steffan, R. J., Goksoyr, J., Bej, A. K. & Atlas, R. M. (1988). Recovery of DNA from soils and sediments. *Applied and Environmental Microbiology*, 54(12), 2908-2915.

Stephan, R. T. & Atlas, R. M. (1988). DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. *Applied and Environmental Microbiology*, 54(9), 2185-2192.

Stockner, J. G. & Porter, K. G. (1988). Microbial food webs in freshwater planktonic ecosystems. In S. R. Carpenter (Ed.), *Complex Interactions in Lake Communities* (pp. 69-83). Heidelberg: Springer Verlag.

Stream Solute Workshop (1990). Concepts and methods for assessing solute dynamics in stream ecosystems. *Journal of the North American Benthological Society*, 9(2), 95-119.

Terzaghi, E. & O'Hara, M. (1990). Microbial Plasticity: The Relevance to Microbial Ecology. In K. C. Marshall (Ed.), *Advances in Microbial Ecology* New York: Plenum Press.

Thomas, K., Chilvers, G. A. & Norris, R. H. (1992). Aquatic hyphomycetes from different substates: Substrate preference and seasonal occurrence. *Australian Journal of Marine and Freshwater Research*, 43(2), 491-509.

Torsvik, V., Goksoyr, J., Daae, F., Sorheim, R., Michalsen, J. & Salte, K. (1993). Diversity of microbial communities determined by DNA reassociation technique. *Trends in Microbial Ecology*, 375-378.

Torsvik, V., Goksoyr, J. & Daae, F. L. (1990a). High diversity in DNA of soil bacteria. *Applied and Environmental Microbiology*, 56(3), 782-787.

Torsvik, V., Salte, K., Sorheim, R. & Goksoyr, J. (1990b). Comparison of phenotypic diverstiy and DNA heterogeneity in a population of soil bacteria. *Applied and Environmental Microbiology*, 56(3), 776-781.

Tsai, Y. & Olsen, B. H. (1992). Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Applied and Environmental Microbiology,* 58(7), 2292-2295.

Tsien, H. C., Bratina, B. J., Tsuji, K. & Hanson, R. S. (1990). Use of oligodeoxynucleotide signature probes for identification of physiological groups of methylotrophic bacteria. *Applied and Environmental Microbiology,* 56(9), 2858-2865.

Turpin, P. E., Maycroft, K. A., Rowlands, C. L. & Wellington, E. M. H. (1993). Viable but non-culturable salmonellas in soil. *Journal of Applied Bacteriology,* 74, 421-427.

Underwood, A. J. (1991). Beyond BACI: Experimental designs for detecting human environmental impacts on temporal variations in natural populations. *Australian Journal of Marine and Freshwater Research,* 42, 569-87.

Underwood, A. J. (1992). Beyond BACI: the detection of environmental impacts on populations in the real, but variable, world. *J. Exp. Mar. Biol. Ecol.*

Underwood, A. J. (1993). The mechanism of spatially replicated sampling programmes to detect environmental impacts in a variable world. *Australian Journal of Ecology,* 18, 99-116.

Vannote, R. L., Minshall, G. W., Cummins, K. W., Sedell, J. R. & Cushing, C. E. (1980). The river continuum concept. *Can. J. Fish. Aquatic Sci.,* 37, 130-137.

Veal, D. A. (1993). Assessment of Microbial Biodiversity. In A. J. Beattie (Ed.), *Rapid Biodiversity Assessment*; Proceedings of the Biodiversity Assessment Workshop, Macquarie University, Sydney, 1993 Sydney: Research Unit for Biodiversity and Bioresources, Macquarie University.

Veal, D. A. (1995). The microscopic life forms. In A. J. Beattie (Ed.), *Biodiversity: Australia's Living Wealth* (pp. 56-67). Sydney: Reed Books.

Veal, D. A., Faulker, B., Vesey, G. & Grohmann, G. (1995). *Cryptosporidium*: the cunning contaminant. *Australian Microbiology,* 16(3), 12-14.

Veal, D. A. & Lynch, J. M. (1984). Associative cellulolysis and dinitrogen fixation by cocultures of *Trichoderma harziarnum* and *Clostridium butyricum. Nature,* 310, 695-697.

Veal, D. A. & Lynch, J. M. (1987). Effects of $(NH_4)_2SO_4$ on cellulolysis and N_2-fixation by cocultures of *Trichoderma harziarnum* and *Clostridium butyricum. Journal of Applied Bacteriology,* 63, 245-253.

Veal, D. A., Stokes, H. W. & Daggard, G. (1992). Genetic Exchange in Natural Microbial Communities. In K. C. Marshall (Ed.), *Advances in Microbial Ecology* (pp. 383-430). New York: Plenum Press.

Verhoeven, T. J. (1992). *Blue-green Algae: Interim Report of the NSW Blue-Green Algae Task Force.* NSW Govt., Sydney.

Vesey, G., Ashbolt, N., Walner, G., Dorsch, M., Williams, K. & Veal, D. A. (1995). Fluorescent *in situ* hybridisation using ribosomal RNA probes and flow cytometry for detecting and determining the viability of *Cryptosporidium* and *Giardia* in water.

Vesey, G., Fricker, C. R., Veal, D. A. & Ashbolt, N. (in press-b). New methods for the concentration and detection of *Cryptosporidium* and *Giardia* in water. *Water Science & Technology.*

Vesey, G., Hutton, P. E., Champion, A. C., Ashbolt, N. J., Williams, K. L., Warton, A. & Veal, D. A. (1994a). Application of flow cytometric methods for the routine detection of *Cryptosporidium* and *Giardia* in water. *Journal of Cytometry.*

Vesey, G., Narai, J., Ashbolt, N., Williams, K. & Veal, D. A. (1994b). Detection of specific micororganisms in environmental samples using flow cytometry. In Z. Darzynkiewicz & P. Robinson (Eds.), *Methods in Cell Biology* (pp. 488-521). New York: Academic Press.

Vesey, G., Slade, J., Byrne, M., Shepherd, K. & Fricker, C. R. (1993a). A new method for the concentration of Cryptosporidium oocysts from water. *Journal of Applied Bacteriology,* 75, 82-86.

Vesey, G., Slade, J. S., Bryne, M., Shepherd, K., Dennis, P. J. & Fricker, C. R. (1993b). The application of flow cytometry to the detection of pathogenic protozoa in water. In *Society of Applied Bacteriology Technical Series.* Oxford: Blackwell Scientific Publications.

Vesey, G., Slade, J. S., Bryne, M., Shepherd, K., Dennis, P. J. & Fricker, C. R. (1993c). Routine monitoring of *Cryptosporidium* oocysts in water using flow cytometry. *Journal of Applied Bacteriology,* 75, 87-90.

Vestal, J. R. & White, D. C. (1989). Lipid analysis in microbial ecology. *BioScience,* 39, 535-541.

Victor, T., DuToit, R., van Zyl, J., Bester, A. J. and van Helden, P. D. (1991). Improved method for the routine identification of toxigenic *Escherichia coli* by DNA amplification of a conserved region of the heat-labile toxin A subunit. *Journal of Clinical Microbiology*, 29, 158-161.

Vilaro, M., Jaulhac, B., Rifai, S., Nicolini, P., Piemont, V. & Monteil, H. (1993). Digoxigenin-labeled probes for detection of genes coding for enterotoxins and toxic shock syndrome toxin-1 from staphylococcal strains. *Journal of Microbiological Methods*, 18, 83-90.

Wagner, M., Amann, R., Lemmer, H. & Schleifer, K.-H. (1993). Probing activated sludge with oligonucleotides specific for proteobacteria: Inadequacy of culture-dependent methods for describing microbial community structure. *Applied and Environmental Microbiology*, 59, 1520-1525.

Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D. & Schleifer, K., -H. (1994). Development of an rRNA-targeted oligonucleotide probe specific for the genus Acinetobacter and its application for in situ monitoring in activated sludge. *Applied and Environmental Microbiology*, 60, 792-800.

Walker, G. (1984). Mutagenesis and incucible responses to deoxyribonucleic acid damage in *Escherichia coli. Microbiology*, 48, 60-93.

Ward, D. M., Bateson, M. M., Weller, R. & Ruff-Roberts, A. L. (1992). Ribosomal RNA analysis of microorganisms as they occur in nature. *Advances in Microbial Ecology*, 12, 219-286.

Ward, D. M., Weller, R. & Bateson, M. M. (1990a). 16S rRNA sequences reveal numerous uncultured inhabitants of a well-studied thermal community. *FEMS Microbiol. Rev.*, 75, 105-116.

Ward, D. M., Weller, R. & Bateson, M. M. (1990b). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature*, 345, 63-65.

Wayne, L. G., Brenner, D. L., Colwell, R. R., Grimont, P. A. D., Kandler, O. & al., e. (1987). Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.*, 37, 463-464.

Weisse, T. (1991). The microbial food web and its sensitivity to eutrophication and contaminant enrichment: a cross-system overview. *Int. Revue ges. Hydrobiol.*, 76, 327-337.

Weller, R. & Ward, D. M. (1989). Selective recovery of 16S rRNA sequences from natural microbial communities in the form of cDNA. *Applied and Environmental Microbiology*, 55(7), 1818-1822.

White, D. C. (1983a). Analysis of microorganisms in term of quantity and activity in natural environments. In J. H. Slater, R. Whittenbury, & J. W. T. Wimpenny (Eds.), *Microbes in their Natural Environments* (pp. 37-66). Cambridge: Cambridge University Press.

White, D. C., Davis, W. M., Nickels, J. S., King, J. D. & Bobbie, R. J. (1979). Determination of the sedimentary microbial biomass by extractable lipid phosphate. *Oecologia (Berlin)*, 40, 51-62.

White, E. (1983b). Lake eutrophication in New Zealand — a comparison with other countries of the Organisation for Economic Co-operation and Development. *New Zealand Journal of Marine and Freshwater Research*, 17, 437-444.

Whitton, B. A. (1979). Plants as indicators of river quality. In A. James & L. Evison (Eds.), *Biological Indicators of Water Quality*, New York: John Wiley & Sons.

Winstanley, C., Morgan, J. A. W., Pickup, R. W., Jones, J. G. & Saunders, J. R. (1989). Differential regulation of lambda and pR promoters by a cI reprssor in a broad-host range thermoregulated plasmid marker system. *Applied and Environmental Microbiology*, 55, 771-777.

Winstanley, C., Morgan, J. A. W., Pickup, R. W. & Saunders, J. R. (1991). Use of a xylE marker gene to monitor survival of recombinant *Pseudomonas putida* populations in lake water by culture on nonselective media. *Applied and Environmental Microbiology,* 57(7), 1905-1913.

Woese, C. R. (1987). Bacterial Evolution. *Microbial Reviews*, 51(2), 221-271.

Woese, C. R., Kander, O. & Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains archaea, bacteria and eucarya. *Proc. Nat. Acad. Sci.,* 87, 4576-4579.

Wong, C. L. & Dunn, N. W. (1974). Transmissible plamid coding for the degredation of benzoate and m-toluate in *Pseudomonas aravilla* mt-2. *Genetical Research*, 23, 227-230.

Wood, E. J. F. (1965). *Marine Microbial Ecology*, London: Chapman Hall.

Wright, J. F., Armitage, P. D., Furse, M. T. & Moss, D. (1985). The classification and prediction of macroinvertebrate communities in British rivers. *Ann. Report Freshwater Biological Assoc.,* 53, 80-93.

Wright, J. F., Armitage, P. D., Furse, M. T. & Moss, D. (1988). A new approach to the biological surveillance of river quality using macroinvertebrates. *Verh. Internat. Verein. Limnol.,* 23, 1548-1552.

Wright, R. T. & Hobbie, J. E. (1966). Use of glucose and acetate by bacteria and algae in aquatic ecosystems. *Ecology,* 47, 447-.

Yentsch, C. M. (1990). Environmental Health: Flow cytometric methods to assess our water world. In Z. DarzynKiewicz & H. A. Crissman (Eds.), *Methods in Cell Biology* (pp. 575-612). San Diego: Academic Press.

Yentsch, C. M., Horan, P. K., Muirhead, K., Dortch, Q., Haugen, E., Legendre, L., Murphy, L. S., Perry, M. J., Phinney, D. A., Pomponi, S. A., Spinrod, R. W., Wood, M., Yentsch, C. S. & Zahuranec, B. J. (1983). Flow cytometry and cell sorting: a technique for analysis and sorting aquatic particles. *Limnology & Oceanography*, 28, 1275-1280.

Zarda, B., Amann, R., Wallner, G. & Schleifer, K. (1991). Identification of single bacterial cells using digoxigenin-labelled rRNA-targeted oligonucleotides. *Journal of General Microbiology,* 137, 2823-2830.

Zeikus, J. G. (1977). The biology of methanogenic bacteria. *Biological Reviews*, 41, 514-541.

Zimmermann, R., Iturriaga, R. & Becker-Birch, J. (1978). Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. *Applied and Environmental Microbiology*, 36, 926-935.

Zimmermann, R. & Meyer-Reil, L.-A. (1974). A new method for fluorescence staining of bacterial populations on membrane filters. *Kiel. Meeresforsch.*, 30, 24-27.

Zobell, C. (1946). *Marine Microbiology,* Waltham: Chronica Botanical.

SUPPLEMENT TO LWRRDC CONSULTANCY UMO25: MICROBIAL INDICATORS OF RIVER HEALTH

RECOMMENDATIONS FOR RESEARCH

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This document contains more specific recommendations on research priorities contained in our review of Microbial Indicators of River Health, as requested by the National River Health Program Committee.

As indicated in the Review, research is needed in the following areas:

1. Structure and diversity of microbial communities;
2. Microbial community function;
3. Development of new techniques; and
4. Links between microbial and traditional physico-chemical and biological indices.

Research in the above areas 1 and 2 should be given highest priority at this time. Appropriate techniques are already available to obtain good microbial ecological information. What is desperately needed in Australia, however, is that these techniques be applied to real systems. Linking the microbial and other indicators should come from sensibly designed field studies designed to answer key questions nested within areas 1 and 2 above.

1. Structure and diversity of selected or key components of the microbial community

Only after we understand the structure and variability of microbial communities in natural systems can we expect to understand how these communities change when subject to stresses. The biological diversity (genetic potential) of the microbial population is also important to understand. There is now considerable evidence that a lack of biological diversity in a population indicates an unhealthy aquatic system. Microbes have the potential to be especially useful indicators of diversity, since microbial studies involve organisms from two entire kingdoms, the eubacteria and archaebacteria (Figure 15), whereas for other biological indicators only a small branch of the phylogenetic tree is being considered (eg. the diversity of fish involves consideration of differences within one branch of the eukaryotic kingdom — Figure 15). Rapid methods for the assessment of microbial diversity based on extracted nucleic acids are becoming available. Such rapid methods contrast markedly with laborious, traditional taxonomy-based approaches that are currently used to assess biodiversity in all other biological groups.

Research should be undertaken to determine:

- microbial diversity within a site (bacterial diversity and genetic potential);
- similarity of communities between sites; and
- effects of environmental perturbations on microbial diversity and community structure.

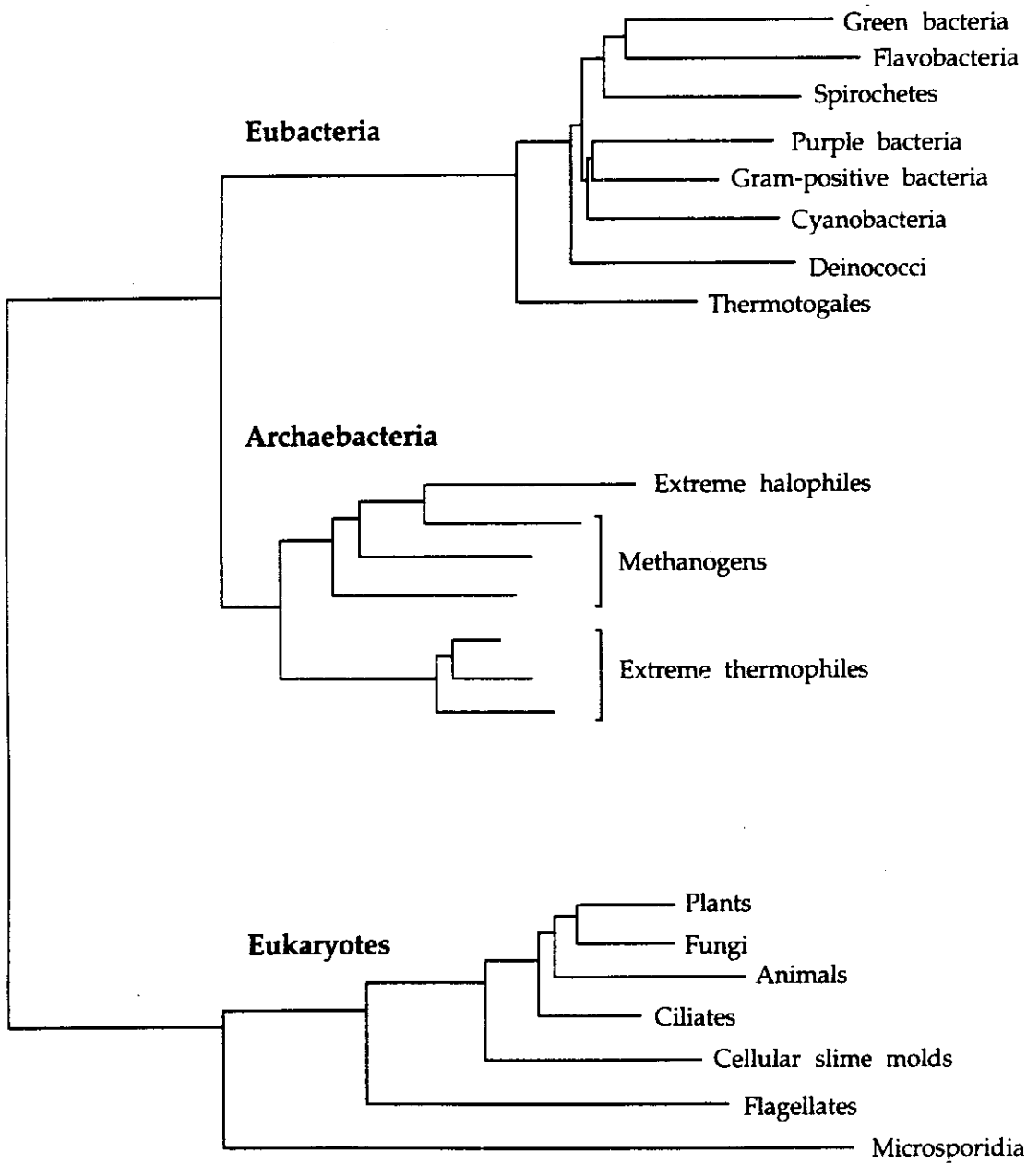
2. Microbial community functioning

Measurements of microbial community functions are important in understanding the processes occurring in a given environment, how these processes are affected by environmental stresses, and how well a system can recover from a stress. It is also important to understand the relative importance of microbes and other components of the community (eg. plants or macroinvertebrates) in processing of nutrients.

Research should be undertaken to determine:

- 'activity' measure of the community (eg. enzymes, thymidine and leucine incorporation);
- response by specific microorganisms to environmental perturbations, as assessed for example by stress proteins; and
- response by microbial communities to environmental perturbations, as assessed by molecular probes (eg. functional genes, rRNA).

Figure 15: Universal phylogenetic tree.



This tree is based on comparative sequencing of 16S or 18S ribosomal RNA. Note the three major domains of living organisms: the eubacteria (Bacteria), the archaebacteria (Archaea), and the eucaryotes (Eucarya). The evolutionary distance between two groups of organisms is proportional to the cumulative horizontal distance between the end of a branch and the node that joins the two groups.

3. Development of new techniques

Many new and exciting protocols are being developed. It is only recently with the advent of molecular biology that many environmental microbes could be studied. The development and application of molecular and other methods needs to be ongoing. More rapid and accurate ways of monitoring aquatic systems need to be developed. Ultimately, we believe a range of kits, based on techniques identified below, will be developed for routine use by water and natural resource management agencies.

Research is needed to develop new protocols using:

- oligonucleotide probes/gene probes/polymerase chain reaction techniques;
- field flow fractionation/flow cytometry;
- stress proteins/antibodies; and
- enzyme activities.

4. Links between microbial and traditional indices

Very few links exist between microbes and other indices of aquatic health. It is important to do comparisons. Different indicator groups (ie. microbes, macroinvertebrates, etc) could be useful in different environments or provide different information about the environmental stresses.

Research is needed to link microbial measures with:

- biological indicators (eg. macroinvertebrates/fish/plants); and
- physico-chemical water quality indicators.

General

It should be possible for research projects to be developed which combine a number of methodological approaches within the one project. For example, taxonomic diversity could be studied using either flow cytometry or molecular biology, or both. Similarly, measures of microbial functions could be investigated using thymidine and leucine uptake methods or measurements of activity of functional genes.

Differences between the microbial community in different parts of the aquatic system also need to be studied. For example, examining the microorganisms in the water column, sediments and biofilms may provide different information on environmental changes. Equally, comparison between systems using the same technique (eg. diversity measures, flow cytometry) would be interesting since it is possible that certain techniques work best on particular systems and not so well on others.

Investigators should also be encouraged to establish research links with existing projects assessing river health. A cross disciplinary approach will help to identify links between microbial and other biological and physico-chemical indicators, enable comparisons to be made between different measures of river health, and help to identify the most effective suite of indicators.