THE MICROBIOLOGICAL QUALITY OF WATER

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DAVID W. SUTCLIFFE

The impact of molecular biology on assessment of water quality: advantages and limitations of current techniques

J. R. SAUNDERS[1] AND V. A. SAUNDERS[2]

[1]School of Biological Sciences, University of Liverpool, PO Box 147, Liverpool L69 7ZB, UK

[2]School of Biomolecular Sciences, Liverpool John Moores University, Liverpool L3 3AF, UK

The advent of molecular biology has had a dramatic impact on all aspects of biology, not least applied microbial ecology. Microbiological testing of water has traditionally depended largely on culture techniques. Growing understanding that only a small proportion of microbial species are culturable, and that many microorganisms may attain a viable but non-culturable state, has promoted the development of novel approaches to monitoring pathogens in the environment. This has been paralleled by an increased awareness of the surprising genetic diversity of natural microbial populations. By targeting gene sequences that are specific for particular microorganisms, for example genes that encode diagnostic enzymes, or species-specific domains of conserved genes such as 16S ribosomal RNA coding sequences (*rrn* genes), the problems of culture can be avoided. Technical developments, notably in the area of *in vitro* amplification of DNA using the polymerase chain reaction (PCR), now permit routine detection and identification of specific microorganisms, even when present in very low numbers. Although the techniques of molecular biology have provided some very powerful tools for environmental microbiology, it should not be forgotten that these have their own drawbacks and biases in sampling. For example, molecular techniques are dependent on efficient lysis and recovery of nucleic acids from both vegetative forms and spores of microbial species that may differ radically when growing in the laboratory compared with the natural environment. Furthermore, PCR amplification can introduce its own bias depending on the nature of the oligonucleotide primers utilised. However, despite these potential caveats, it seems likely that a molecular biological approach, particularly with its potential for automation, will provide the mainstay of diagnostic technology for the foreseeable future.

Introduction

Traditional, culture-based methods for detection and identification of microorganisms were conceived largely for use in clinical situations, and their subsequent application to the open environment has proved to be inadequate. This is mainly because many natural environments are more dilute and nutrient-poor than clinical environments, and thus demand more sensitive and selective techniques for microbial detection. This is particularly so when considering water supplies, since not only is the detection of pathogens or indicator organisms paramount, but large-scale microbial activities, such as nitrification, may have significant impacts on water quality. The development of nucleic acid hybridisation and allied technologies has permitted the identification and quantification of specific genetic sequences in microbial communities and, in turn, provides a means of tracking microorganisms indirectly through properties of their genomes (Saunders & Saunders 1993; Saunders 1996). Importantly, DNA probes can be

employed to monitor the behaviour of microorganisms without the need for gene expression or host cultivation. Current technology now also opens the prospect of studies on microbial function within both native and polluted environments. This may be achieved by analysing the expression of key target genes that encode enzymatic functions crucial, for example, to a geochemical cycle.

A further advantage of using genetic manipulation in an environmental context is that genetic markers can be added or deleted from the genome of strains released into particular habitats, enabling complementary studies of identity and activity, and the monitoring and recapture of released strains. Tagging microorganisms with specific marker genes, or utilising genes unique to a particular species or group as markers, provides scope for determining the presence and activity of such organisms in the environment by phenotypic and/or genotypic identification (Morgan *et al.* 1989; Winstanley *et al.* 1989, 1991). The ability of microorganisms to adopt a viable but non-culturable state (Colwell *et al.* 1985) can drastically limit the utility of this mode of detection. To circumvent the culturability problem, methods can be employed that are based on detection of a specific marker gene sequence in total microbial DNA extracted directly from environmental samples. However, this methodology does not permit predictions to be made about the activity of the host organisms. Phenotypic analysis of the expression of marker genes can give indications of microbial responses to chemical and physical changes in the environment. The gene product might be detected by its biological activity or immunological reactivity and may allow direct detection *in situ* (see, for example, Morgan *et al.* 1989, 1991b; Winstanley *et al.* 1989).

The unreliability of techniques for culturing target organisms from environmental samples favours direct methods for detection (Table 1). However, some of these methods, such as DNA probing (Knight *et al.* 1990, 1991), generally require extractive procedures and hence the data samples obtained are used to reconstruct past events. Non-extractive techniques offer the potential for real time *in situ* detection, without perturbing the process under investigation. However, such direct detection may rely on expression of marker genes that can be variable in a changing environment. Clearly, the availability of a collection of methods that affords rapid and sensitive detection of the marker system in more than one guise is essential. It is, however, sensible to develop additional means of detecting organisms based on intrinsic features of the host, such as ribosomal RNA sequences, DNA fingerprints, or immunological markers. Thus host survival could continue to be monitored even if an introduced marker was lost.

The study of water microbiology is severely hindered by inherent and universal practical problems associated with investigation of interactions and functions of microscopic communities inhabiting complex micro-environments. For decades, the limitations of culture-based approaches, with their origins in medical and food microbiology, have held back understanding of the basic ecology of natural microbial populations. Cultivable organisms may form only 1% or less of the total bacteria present in soil and aquatic environments (Pickup & Saunders 1990; Morgan *et al.* 1991a; Pickup 1991). Furthermore, predictions of microbial activity cannot be made from laboratory-based studies conducted under artificial and optimised conditions. Laboratory enrichment cultures favour those species that exhibit the fastest growth rates, irrespective of their activity under natural environmental conditions. This is acceptable, even desirable, when attempting to detect the presence of human pathogens or indicators of faecal pollution, such as *Escherichia coli*. However, for the bacterial flora indigenous to the open environment, culture-based approaches may be entirely inappropriate: culture conditions have not been identified for many species, some may have prohibitively long generation times, and others may attain a viable, but non-culturable state. Viable but non-culturable cells (VBNC) of pathogens, for example *Vibrio* spp. (Colwell *et al.* 1985), in water supplies could

Table 1. *A list of molecular detection methods.*

Immuno-imaging

Immunocapture

Luminometry

DNA-DNA/RNA hydbridisation

Polymerase chain reaction (PCR)

Ligase chain reaction (LCR)

Flow cytometry and cell sorting

Table 2. *Practical detection limits of molecular methods.*

Technique	Approximate limits (cells per ml or g)
Viable non-selective culture	10^3
Viable selective culture	10^1
Fluorescent antibodies	2×10^1
Fluorescent oligonucleotides	10^5
Flow cytometry	5×10^2
DNA hybridisation	10^1 to 10^3
Polymerase chain reaction (PCR)	$10^0 \times 10^3$

Table 3. *Features of nucleic acid-based detection.*

Independent of culturability

Can be used to detect "VBNC" or dead cells

Potential to measure activity in viable cells

Can be automated

Extraction and amplification bias

Inability to distinguish live from dead?

Concordance of target gene with species?

Table 4. *Ribosomal RNA as a target for detecting bacteria.*

Ribosomal RNA (rRNA) is universal

Naturally amplified in ribosomes

rrn genes present in multiple copies in many microbial genomes

SSU, LSU and spacer regions of *rrn* operons contain high information content

Specific domains for identification and conserved regions for PCR amplification

present an unobserved potential threat to human health. It is not surprising therefore, that wherever culture-independent molecular biological techniques have been applied to natural habitats, populations have been revealed whose component bacteria are not represented in collections of cultured isolates (see, for example, Giovannoni *et al.* 1990a,b; Fuhrman *et al.* 1992; Liesack & Stackebrandt 1992; Ekendahl *et al.* 1994; Hales *et al.* 1996).

Molecular detection techniques in environmental microbiology

In addition to enabling the study of organisms that cannot be grown in culture, modern molecular techniques permit the direct detection of extremely small populations of viruses and microorganisms against a substantial background of other species. This is particularly appropriate to the problems of detecting pathogens within aquatic environments and water supplies. A variety of methodologies based on molecular biological techniques has been applied to the problems of microbial detection and identification in environmental microbiology (Table 1) (Pickup & Saunders 1990, 1996; Pickup 1991). The relative efficiencies of these techniques are given in Table 2. It is evident that molecular detection methods are currently no more efficient in absolute terms than traditional culture-based techniques. However, molecular genetic and immunological techniques such as immunocapture (Morgan *et al.* 1991b) can be used to detect and identify non-culturable or dead cells. This greatly extends their utility in water microbiology. Nucleic acid-based techniques have the widest applicability due to their many advantages (see Table 3), including the potential for automation which is essential if large numbers of samples are to be processed routinely. Direct recovery of DNA (and to a lesser extent RNA) from a diverse array of environments has now been described (see, for example, Fuhrman *et al.* 1988; Selenska & Klingmuller 1991; Tsai & Olsen 1992). Success in direct detection of microbial DNA from a wide range of natural and laboratory environments has most commonly been achieved by procedures that allow the *in vitro* amplification of nucleic acids using the polymerase chain reaction (PCR) Saiki *et al.* 1988), or less commonly the ligase chain reaction (LCR) (Backman 1992).

The enzymatic amplification of DNA by PCR has revolutionised many areas of biology, not least environmental microbiology. The ability to acquire meaningful diagnostic information from minute quantities of nucleic acids allows the presence of particular microorganisms to be inferred within hours of sampling. Molecular methods introduce their own biases, for example in the recovery of DNA or efficiency of enzymatic amplification *in vitro*. For example, difficulties may be encountered due to the low pH of many environmental samples which interferes with the extraction procedure, or the co-extraction of humic material which can inhibit anzyme activity of the thermostable DNA polymerases used in PCR (Fuhrman *et al.* 1988; Hales *et al.* 1996).

16S ribosomal RNA as a target for detection and identification of bacteria

Molecular biological techniques utilising 16S rRNA sequences as molecular targets have substantial advantages over traditional culture-based methods for characterisation of microbial populations (Table 4). The use of oligonucleotide probes complementary to specific regions of 16S rRNA may be discriminatory at the genus or species level, and are increasingly used to identify pathogens, examine the diversity of natural microbial populations, and characterise novel isolates, whilst avoiding a requirement to culture organisms (Stahl & Amman 1991; Stahl *et al.* 1988). Ribosomal RNA genes have now become established as targets of choice, since they contain conserved and variable sequences, within and between the *rrn* loci for 16S and 23S RNA, and a large database of sequence information now exists (Lane *et al.* 1985; Edwards *et al.* 1989; Embley *et al.* 1992; Larsen *et al.* 1993). This is certainly the case where

analysis of community structure and species composition is the objective, since few other genes can provide such a high information content. Comparison of sequence data from both laboratory cultures and natural biomass has supported the contention that considerable bacterial diversity exists within functional communities (Giovannoni *et al.* 1990a,b; Ward *et al.* 1990; Fuhrman *et al.* 1992). For example, Fuhrman *et al.* (1992) analysed 16S rRNA sequences of microorganisms isolated from sea water, and found only 70% sequence identity for the predominant sequence signature compared with those of previously described and culturable *Archaea*. Furthermore, many physiologically specialised microbial groups are phylogenetically coherent or restricted to a limited number of assemblages as detected by *rrn* sequence analysis (Woese *et al.* 1990).

Selection of appropriate oligonucleotide sequences to serve as probes and primers for PCR of 16S ribosomal DNA is generally straightforward, taking account of specificity and secondary structure properties that may alter access for the formation of a base-paired hybrid molecule. The application of PCR primers and probes has by necessity been optimised for genomic DNA sequences isolated from the available range of pure cultures for any genus or group (Stahl & Amann 1991). The individual oligonucleotides produced by exploiting such information may not be absolutely specific for the organisms actually present in an environment. However, their application to environmental samples is justified if distinct, but clearly phylogenetically-related sequences, are obtained directly, without culture, from environmental samples. Sequence information obtained by direct amplification may then be used to refine the amplification primer sequences in a reiterative process, as more and more sequence information becomes available.

Nitrifying bacteria – a model target for molecular analysis

The autrotrophic ammonia-oxidising (nitrifying) bacteria are crucial in the process of nitrification. They provide an ideal model target for the application of molecular techniques to natural environments, since the microorganisms concerned are slow-growing and difficult to culture, cardinal in an important environmental process. In targeting bacteria, the starting point is normally the phylogenetic information gained from sequence analysis of the 16S *rrn* genes of cultured ammonia-oxidising species (Head *et al.* 1993). In order to detect these bacteria, lakewater, sewage and sediment samples must be analysed using a nested PCR technique in which eubacterial rRNA genes are subjected to a secondary amplification (nested PCR) with *Nitrosomonas-* or *Nitrosospira*-specific primers (Hioms *et al.* 1995). The technique of nested PCR (Fig. 1) involves an initial amplification with primers universal to all eubacteria. This provides sufficient bacterial DNA to allow successful secondary amplification with primers specific for nitrifying bacteria. Despite the exquisite sensitivity and specificity of PCR reactions, direct amplification of DNA with primers specific for a particular group of bacteria often fails. Therefore nested PCR is a general technique applicable to a wide range of bacterial genera present at low numbers in environmental samples. The presence of *Nitrosospira* DNA, but not *Nitrosomonas* DNA, was demonstrated and confirmed by hybridisation of amplified DNA with an internal oligonucleotide probe (Hioms *et al.* 1995). Enrichments of lakewater and sediment samples, incubated for two weeks in the presence of ammonium, produced nitrite and were found to contain DNA from both *Nitrosospira* and *Nitrosomonas*. This demonstrates that *Nitrosospira* spp. are widespread in the environment, but are systematically under-represented in cultured isolates due to their slower growth rates in culture when compared with less numerous *Nitrosomonas* species. Repeated failure of *Nitrosomonas*-specific primers to amplify from DNA samples taken from environments known to be undergoing nitrification, even after nested PCR, is perhaps surprising (Hioms *et al.* 1995). DNA yields from PCR

Nested PCR

Universal
eubacterial
primer

Mixed species DNA including target

Universal
eubacterial primer   PCR I

Eubacterial
DNA
amplified

Specific
primer   PCR II   Specific
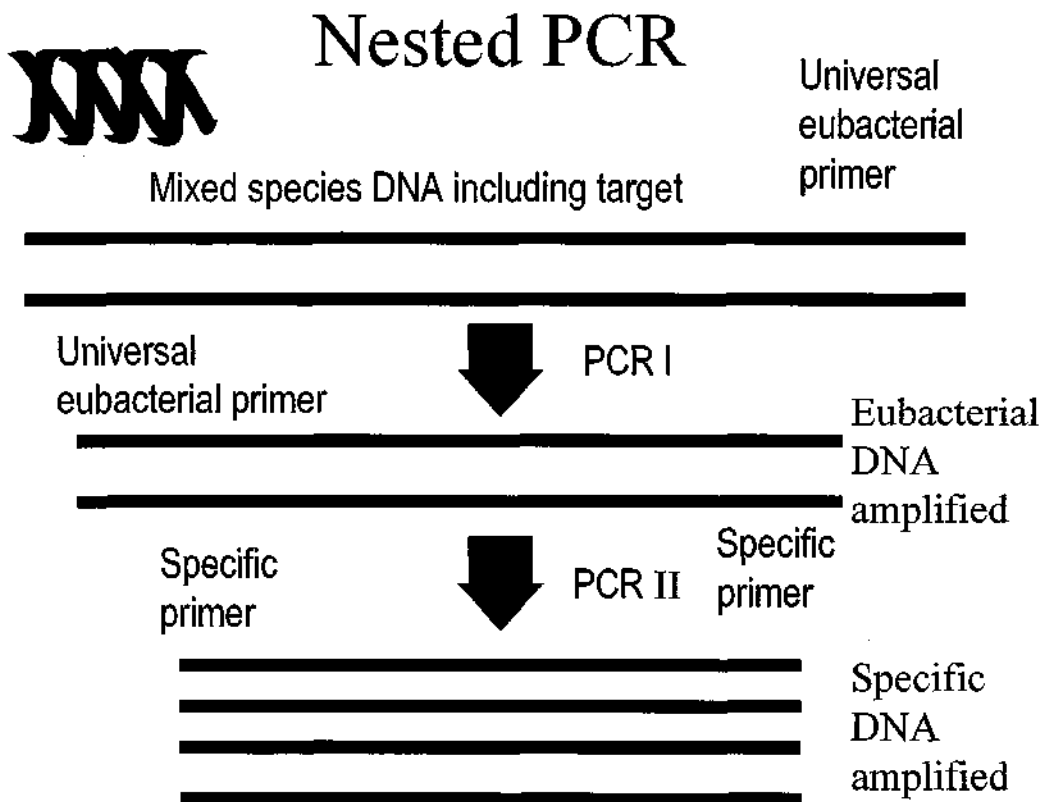primer

Specific
DNA
amplified

Figure 1. Nested PCR for the amplification of ribosomal DNA from specific bacterial groups. DNA is extracted from environmental samples and subjected to a primary amplification using "universal" oligonucleotide primers (in this case universal to Eubacteria), complementary to conserved regions at opposite ends of the 16S *rrn* gene. The product DNA from this first amplification is then used as a template for a second amplification using primers specific to regions conserved within the genus or group of interest, but internal (nested) to the original primers. This produces measurable amounts of group-specific DNA.

amplifications of pure cultures using *Nitrosomonas*-specific primers are consistently lower than those obtained with *Nitrosospira*-specific primers. Differential performance of primer combinations is an additional potential bias that can be introduced during the application of environmental PCR. However, the evidence suggests that *Nitrosomonas* rDNA is actually present at very low levels in both aquatic and terrestrial environments, despite nitrosomonads being the predominant species that are cultured. This is undoubtedly due to the ability of the *Nitrosomonas* spp. to outgrow their *Nitrospira* spp. competitors despite the fact that they are less numerous, and potentially less significant in terms of the process of environmental nitrification. This provides a general warning that traditional culture techniques may recover a sub-fraction of a microbial population that is subordinate in numbers and activity to more numerous, but poorly culturable or non-culturable, species.

Conclusions

Culture-based methods for monitoring bacterial populations are inevitably biased, under-representing both the numbers and diversity of bacteria present in aquatic and terrestrial

environments. Nevertheless, the newer approaches outlined here provide only partial solutions to this problem, since a further raft of biases may be introduced. Noteworthy amongst these are differential recovery of nucleic acids, and the specificity and efficiency of PCR amplification primers. It has become a common experience in microbial ecology, that the application of molecular techniques reveals a greater diversity of bacteria than is represented in cultured species. In addition to tracking the fate of pathogenic microorganisms, it is now possible using molecular technologies to address the comparative role of different community members, their relative contributions to nutrient cycling and bioremediation, and partitioning between active and dormant microbial populations.

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