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Environmental Monitoring of Bacteria

Edited by

Clive Edwards

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Preface

Environmental microbiology has emerged as an extremely active, exciting, and important area of research during the last few years and has challenged some long-held principles of traditional microbiology. For example, the numbers of colony-forming units obtained on solid agar media have always been assumed to reflect the numbers of viable bacteria present in any given sample. However, it is now known that many bacteria cultivable in the laboratory adopt unusual dormant states when subjected to the nutrient-limited conditions common in many ecosystems, which, in turn, makes these bacteria difficult to culture. This partially explains our current inability to culture the vast majority of bacteria known to reside in natural environments. Knowledge of the presence of some species in a natural environment can be of crucial importance, particularly with respect to detection of pathogenic species or to monitoring the fate and survival of genetically manipulated organisms within ecosystems. Therefore, there has been much effort generated to devising new and novel methods for detection, identification, and recovery of microorganisms from natural habitats.

Central to many of these methods has been the development and application of the techniques of molecular biology to environmental microbiology. These have resulted in direct investigations of microbial populations based on DNA analysis without the need for any cell culture. There is no doubt that this would not have been possible without the revolution in computer technology that has led to the development of highly accessible databases of vast amounts of information. Molecular biology methods have been harnessed and exploited also in the development of biophysical methods that enable rapid and automated analysis of microorganisms from natural environments.

Environmental Monitoring of Bacteria presents these new developments as a series of chapters that describe the principles of different techniques, how these techniques have been applied, and, for most of these, easy to follow protocols providing immediate access to a given method. Most of the presentations are directed at the bacterial world, which has been most extensively studied, but eukaryotic microorganisms are also referred to where

possible. *Environmental Monitoring of Bacteria* is aimed at any environmental microbiologist, from the undergraduate level upwards, who needs information that facilitates immediate access to techniques that enable the study of bacterial ecology as communities or single cells or at the molecular level.

I am grateful to the many colleagues and collaborators who have made their contributions to this book. Much of the research described was made possible by funding from the Natural Environment Research Council, who recognized the importance of molecular ecology at an early stage enabling it to flourish in the UK.

Clive Edwards

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1

Some Problems Posed by Natural Environments for Monitoring Microorganisms

Clive Edwards

1. Introduction

1.1. Traditional Microbiology

The history and emergence of microbiology as a scientific discipline are intimately linked with developments of methods for isolation, enrichment, growth, and maintenance of microorganisms in the laboratory as pure cultures to enable subsequent biochemical and genetic analyses. This approach has been extremely successful for the manipulation and exploitation of microorganisms in terms of disease control and eradication, development of biotechnological processes, and the evolution of sophisticated molecular genetic techniques. It has also resulted in some bacterial species being studied more than others, and some have become model species, the properties of which are then inferred for all microorganisms. Examples include the widespread use of *Escherichia coli* (model Gram-negative), *Bacillus subtilis* (Gram-positive), *Streptomyces coelicolor* (industrially important mycelial prokaryotes), *Saccharomyces cerevisiae* (model for yeast), and *Aspergillus* spp. (widely studied fungus). Unfortunately, it is often the case that experimental protocols developed for model species are not transposable to other bacteria. This gulf is often most apparent when deductions made from organisms grown as pure cultures in the laboratory are assumed to apply to those occurring in natural environments.

Until recently, the properties of microorganisms in their normal habitats were not considered important, and no doubt there was an assumption that they could not be greatly different from those encountered in the laboratory. However, it is now generally accepted that natural environments are severely nutrient limited ***(1)*** and that in soils and aquatic and sediment habitats, heterotrophic

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bacteria, in particular, have evolved strategies for coping with conditions of extreme nutrient limitation. Some species, such as *Bacillus*, that have a more limited metabolic repertoire adopt a primitive response involving sporulation ***(2)***. Other species have evolved other mechanisms that are only now beginning to be understood by microbiologists. There is no doubt that the prospect of releasing, either deliberately or accidentally, genetically manipulated microorganisms (GMMOs) into open environments has provided a huge impetus for the study of microorganisms in natural environments. As a result of more rational and concerted studies, a number of problematical areas have been identified. These suggest that it is difficult, if not impossible, to audit the total microbial diversity of a natural environment. These problem areas not only impact on our ability to monitor the microbial diversity of habitats but also challenge the many long-held and cherished dogmas of traditional laboratory-based microbiology.

2. Culturability

An important feature of natural environments is that only a small proportion of the bacteria present can be cultured by traditional methods, and this ranges from approx 0.01 to 10% ***(3)*** depending on nutritional status and type of environment. This means that it is difficult to gage the total bacterial diversity present within a given sample. The reasons for this are complicated and probably multifaceted, but include a lack of knowledge concerning the metabolic requirements of many species that prohibits suitable isolation media to be devised; the presence of species, normally culturable, that have adopted a state of metabolic shutdown, which means that they cannot be easily reisolated; and dormant species that are resistant to traditional methods of culture. Many terms have been proposed for such species including dormant, dwarf cells or, more often, viable but nonculturable (VBNC) cells. No single definition has proved satisfactory and the occurrence of VBNC states has proved controversial. There is no doubt that such a state can be demonstrated for starving cultures of many bacteria in laboratory experiments, but it is unlikely that it is a stable physiologic phenotype in natural environments; rather it is a transient property of bacteria as they transit through prolonged nutrient limitation to cell death. However, it is an important phenomenon as witnessed by the fact that some bacterial pathogens are able to cause disease yet fail to be detected or enumerated by the classical cultural techniques of microbiology ***(4)***. Such observations are important for understanding the dissemination and occurrence of pathogens in the environment as well as for predicting the fate and consequences of releasing GMMOs into open environments.

A consequence of nonculturability observed in microbial communities in natural environments is that it is difficult to assess whether the inability to culture is because a large proportion of observable intact cells are dead. The

problem of assessing true viability of bacterial populations and relating this to culturability has been addressed by many investigators. Recently, a number of viability dyes have been developed and tested to enumerate the proportion of live/dead cells in many ecosystems and laboratory model systems. These are mainly fluorescent dyes and are listed in **Table 1**. Their modes of action include membrane potential-dependent uptake (dead cells fail to generate a membrane potential); intracellular cleavage of colorless dye conjugates resulting in the release of a fluorochrome that is only retained (and therefore stains) intracellularly by live cells; metabolic activity-dependent dyes; exclusion mechanisms seen only in live cells. They have been applied to many experimental systems (*see* **ref. *5*** for review) and, on the whole, have demonstrated the probability that a large proportion of bacteria that are nonculturable remain live, as judged by testing with the numerous fluorescent dyes listed in **Table 1**. A firm conclusion that can be drawn from all the studies of viability and culturability of bacteria in natural environments is that they appear to be metabolically, and often morphologically, different from cultures grown in rich laboratory media. This is true for many bacterial species that are normally easily cultured by traditional methods in the laboratory but for some reason become recalcitrant to such methods when they are exposed to the rigors of natural ecosystems.

Recently Bloomfield et al. ***(6)*** have advanced a possible explanation for the occurrence or development of VBNC states. They propose that the failure to recover cells subjected to inimical processes such as starvation is the result of an oxidative-mediated suicide of the cell. It is well known that bacteria exposed to inimical processes undergo both biochemical and morphological adaptations to enable them to survive an environmentally imposed stress such as heat shock or nutrient limitation ***(7–9)***. An important consequence is a massive reduction of growth rate to near zero and the induction of high-affinity substrate uptake pathways. Transfer of such organisms to rich culture media leads to rapid switching on and flooding of metabolic pathways that cannot be coupled immediately to growth. Oxidation of substrates leads to overproduction of superoxide and free radicals, resulting in many of the cells being killed. There is much evidence now available to support this proposal, arising from increased understanding of the alternative pathways of gene expression that exist in microorganisms.

3. Sensor-Regulated Pathways of Gene Expression

In bacteria, many stimulus-response networks have been identified, and these are often associated with adaptations to changes in the external physical and chemical conditions. These networks have also been proposed to form part of a larger, global cellular regulatory network that responds to a variety of environmental stresses ***(10)***. It has been found that there may be overlaps

Table 1
Some Fluorescent Dyes That Have Been Used to Assess Microbial Viability[a]

Dye	Mode of Action	Applications
Dihexyl oxacarbocyanine	Membrane potential	Flow cytometric detection of bacteria
Rhodamine 123	Membrane potential	Microscopic enumeration of viable bacteria Flow cytometric assessment of viability in: 1. A range of Gram-positive and negative bacteria; 2. *Micrococcus luteus* 3. *Staphylococcus aureus*
bis-(1,3-dibutylbarbituric acid) pentamethine oxonol (Oxonol)	Membrane potential	Enumeration of dead cells
Fluorescein diacetate (FDA)	Intracellular esterase cleavage to release fluorescein which is retained within cells possessing an intact membrane	Microscopic detection of viable Gram-positive bacteria; viable mycobacteria; viable soil bacteria; viable aquatic bacteria Flow cytometric enumeration of metabolically active marine microalgae; viable *Bacillus subtilis*
Carboxyfluorescein diacetate (CFDA)	As for FDA	Flow cytometric enumeration and identification of viable compost bacteria; Flow cytometric assessment of viability in *Saccharomyces cerevisiae*
2',7'-Bis-(2-carboxyethyl) 5(6)-carboxyfluorescein acetoxymethylester	As for FDA	Flow cytometric assessment of viability of a range of bacterial species
Calcein acetoxymethylester	As for FDA	Microscopic detection of viable protozoa Flow cytometric assessment of viability of a range of bacterial species
Fluorescein di-β-D-galactopyranoside (FGP)	Intracellular enzymic cleavage, dye retention only by cells with intact membrane	Flow cytometric-activated cell sorting of viable yeasts and bacteria
Chemchrome Y	As for FGP	Flow cytometric detection of *Candida albicans*

Table 1 *(continued)*

Chemchrome B	As for FGP	Flow cytometric enumeration of viability of a range of bacterial species Analysis of viability of genetically modified *B. subtilis* in compost Resuscitation of VBNC *Vibrio vulnificus*
5-cyano-2,3-ditolyl tetrazolium chloride (CTC)	Respiratory activity	Microscopic detection of active aquatic bacteria Flow cytometric detection of respiring *M. luteus* Analysis of dormancy in *M. luteus*
Mithramycin	Staining elongated cells (viable) after prolonged incubation in the presence of nalidixic acid	FCM monitoring of viable *Yersinia ruckeri*
Propidium iodide	Dye exclusion by live cells	Microscopic detection of viable protozoa and yeasts
4',6-diamidino-2-phenylindole	Dye exclusion	Microscopic detection of *Cryptospiridium parvum* oocysts

[a]Data are taken from ref. 5.

between component networks in that proteins induced by one stress response may also be induced by other stresses. A generic model for stimulus-response networks is shown in **Fig. 1**. External fluctuations in such factors as essential nutrients, temperature, ultraviolet (UV) radiation, or chemicals and mutagens are detected by molecules within the cell that transmit the information, sometimes via an interrelated series of transmitter molecules, to the genome, resulting in the expression of specific genes that encode for proteins that enable the cell to adapt to or withstand the external stress. Well-studied examples include the heat-shock response regulon in *E. coli* and the SOS response to UV light damage of DNA. These types of sensor systems are reversible in that when conditions revert to the original levels, the new pathways of gene expression are switched off. These types of responses can alter the properties of cells quite drastically when the environmental stress is in operation.

4. Altered Physiological and Morphological States Arising From Nutrient Limitation or Starvation

Our understanding of the ways in which bacteria behave in natural environments and how they may exhibit grossly different physiological states has

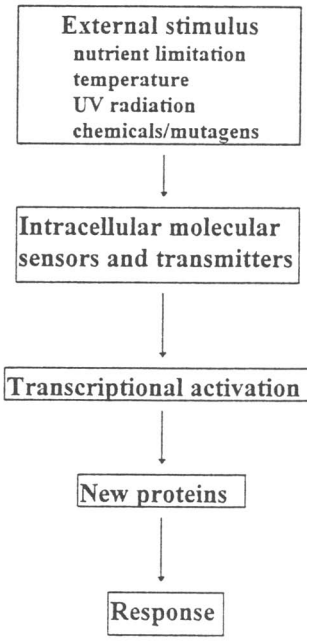

Fig. 1. Sensor regulator systems.

received a great deal of impetus from studies of the responses of enteric bacteria to prolonged culture in stationary phase. Early work identified that bacteria such as *E. coli* are capable of prolonged survival during extended periods of starvation (*see* **ref. *11***). Other work on the properties of stationary phase *E. coli* ***(12,13)*** and *Salmonella typhimurium* ***(14,15)*** cells identified a sigma factor named σ^S, that altered the specificity of RNA polymerase in order to induce an alternative pattern of gene expression. More recently, the role and regulatory properties of this sigma factor have been more clearly defined, and some of the main groups of genes whose expression is dependent on σ^S-directed RNA polymerase transcription and the functions they encode are given in **Table 2**. By analogy with these laboratory-based studies on stationary phase survival, it is likely that in nutrient-limited natural environments, bacteria will have undergone a program of altered gene expression that results in a cell that has distinct and different properties compared with those encountered in cells grown in nutrient-rich laboratory media. The proteins that are synthesized during starvation as a result of σ^S-controlled gene expression often are collectively referred to as starvation-inducible proteins (sti proteins), and their expression and synthesis have been extensively studied in *Vibrio* spp. ***(9,16)***.

Table 2
Some σ^S Regulated Genes[a]

Genes	Function
*kat*E and *kat*G (catalases HPI and HPII)	Prevention of DNA damage by H_2O_2.
*xth*A (exonuclease III)	Repair of H_2O_2 and UV-radiation damage.
*bol*A, *fic*	Overexpression results in stable spherical cells. *fic*$^-$ mutants are short rods.
spv genes (*Salmonella* plasmid virulence genes)	Transcription from *spv*A (of *spv*ABCD operon) promoter is σ^S-dependent. *rpo*S$^-$ mutants are 1000-fold less virulent.
ots BA operon (responsible for synthesis of the compatible solute trehalose)	Osmoprotection (*rpo*S$^-$ cells more sensitive to osmotic changes)
Unknown genes distinct from σ^{32} mediated gene expression (heat shock sigma factor)	Thermotolerance, may also be partially mediated by *ots*BA.
*glg*S	Glycogen synthesis.
Expression of a family of genes strongly induced by anaerobiosis is also moderately induced by σ^S; include a cytochrome oxidase, hydrogenase 1, and acid phosphatase	Anaerobically induced genes.
*osm*B and *osm*Y	Membrane and cell envelope functions.
Microcin C7, a peptide antibiotic that inhibits protein synthesis	Synthesis and excretion of microcins.

[a]Data are taken from **refs. *14*** and ***15***.

Starvation biology is now an active research area, and the physiology and ecology of slow growth or dormant cells has been reviewed in a thought-provoking review by Koch ***(17)***. A further complication that emerged from studies of nongrowing stationary phase bacteria was the proposal that under such conditions, mutants arose during starvation (after 12-d incubation) that had superior survival properties exhibited as the ability to outgrow 1-d-old cells from young cultures ***(13)***. This mutant phenotype was called GASP and was proposed to arise owing to mutations in *rpo*S. This study and other work raised the controversial possibility that mutations in the starving state could be directed ***(18)*** which in terms of evolutionary theory had Lamarckian implications. Recently these controversies were resolved by a demonstration that stationary phase mutations were not directed at selected genes, but occurred throughout the genome within a subpopulation of stressed cells via a recombination-

dependent process ***(19)***. This resolution depends on the emergence of a hypermutable subpopulation within a population of cells exposed to an inimical process such as starvation. This itself has profound consequences for our understanding of bacterial behavior in environments that regularly impose some type of stress on the resident bacterial population. It implies that cells can radically shuffle their genetic makeup in response to external factors, that mutations can be used in an attempt to ensure survival of a subpopulation of stressed individuals, and that such a phenomenon may help to explain such factors as the emergence of GASP mutants in stationary-phase cultures, resistance to bactericidal agents and antibiotics, as well as other adaptations such as survival of pathogens after exposure to a host's immune defenses.

5. Measurements of *In Situ* Activities

Because of the metabolically shut down states that heterotrophic and possibly other bacterial species exhibit in natural environments, measurements of *in situ* activity are difficult, especially for an individual species. It is probably more realistic to measure whole processes that may be mediated by many different species (e.g., the nitrogen cycle). However, this approach also poses problems for the investigator because bacterial populations that mediate important biogeochemical cycles often comprise producers and consumers of the end products alongside each other. One approach for measuring *in situ* activities is to concentrate on processes that have gaseous products. **Table 3** lists the major gaseous products and substrates for a variety of environmentally important processes, and it is immediately apparent that producers and consumers can coexist, e.g., nitrifiers and denitrifiers. Even the strictly anaerobic methanogens and aerobic methanotrophs can occupy the same layers within soil ***(20)***. A generic summary of the effects of nutrient limitation on bacterial properties in natural environments is given in **Table 4**. This underpins the observations made on the effects of *rpo*S expression in stationary-phase cultures and reinforces the argument that bacteria in natural environments can exhibit markedly different properties. With respect to detection and isolation, many of the changes pose challenges for modern methods. For example, the reduction of cellular rRNA as a result of a downshift of growth rate makes direct detection by fluorescent whole-cell hybridization with fluorescent oligonucleotide probes more difficult. The low metabolic activities also make *in situ* assessment of activity and viability extremely difficult. These problems are particularly important for detection of GMMOs in natural environments or for exploiting a GMMO for environmental processes such as *in situ* bioremediation. However, as continually stressed in the preceding sections, altered patterns of gene expression mean that laboratory activities may not be reproduced or possible. This is at last beginning to be recognized, and there has

Table 3
Microbial Production and Consumption of Gases

Gas	Producers	Consumers
Hydrogen	Nitrogen fixers; fermentation	Heterotrophs; methanogens; sulfur reducers
Carbon dioxide	Aerobic respiration; fermentation	Autotrophic bacteria
Carbon monoxide	Uncharacterized anaerobes	Ammonia oxidizers; carboxydotrophs
Nitrous oxide	Nitrifiers; denitrifiers	Denitrifiers
Nitric oxide	Nitrifiers; denitrifiers	Denitrifiers; heterotrophs and methanotrophs
Nitrogen	Denitrifiers	Nitrogen fixers
Methane	Methanogens	Methanotrophs

Table 4
Some of the Responses of Bacteria to Starvation

Response	Examples
Reductive division—ultramicrobacteria	Soil bacteria; marine bacteria; *E. coli*
Protein turnover—synthesis of sti proteins	*S. typhimurium E. coli Vibrio* sp. S14
Reduction in total cellular RNA	Marine *Vibrio*; numerous examples
Long-lived mRNA molecules	*Vibrio* S14
DNA levels remain constant or increase	Numerous examples
Reduced metabolic activity	*Vibrio* sp. ANT 300
Morphologic changes	*Vibrio* sp. S14
Altered physiology—development of new resistance properties	*Vibrio* sp. S14 *E. coli*; various examples
Changed antigenicity—new surface structures synthesized	*Vibrio; Escherichia, Salmonella*
Cells may become viable but noncultivable or dormant	Numerous examples, particularly Gram-negative pathogens
Mutations in *rpo*S^{-}, cells more competitive for resuscitation and survival	*E. coli*

[a]Data are taken from ref. 5.

been at least one demonstration of improved *in situ* biotransformation by placing the genes for phenol transformation under the control of promoters, such *rpo*S, that are active only under starvation conditions ***(21)***. Future work in this area will require a fuller understanding of the programs of gene expression under nutrient limitation conditions as well as a better appreciation of the molecular biology involved. Recently, attempts have been made to correlate

activity of cells *in situ* using microscopic methods to relate a cell's rRNA content (a measure of growth rate) to its degradative rates for a variety of substrates. The approach proved useful for model pure cultures, but would be extremely labor intensive to analyze unknown bacterial populations in soils and water ***(22)***.

6. Quorum Sensing and Resuscitation—Signal Molecules

Many Gram-negative bacterial species are now known to regulate gene expression in response to population size. This results in group behavior of bacterial populations that requires intercellular communication, generally by means of diffusible autoinducing molecules, now identified as *N*-3-(oxohexanoyl) homoserine lactone (HSL) or its derivatives. This process has been termed *"quorum sensing,"* which is characterized by bacteria synthesizing acyl-HSLs as signal molecules in a cell density-dependent manner. Originally, the process was discovered in the luminescent bacterium *Vibrio fischeri*, which only luminesces when present at high densities, cells at lower densities do not emit light. Our understanding of quorum sensing has been greatly enhanced by further studies of this system for which two genes are important: *lux*R, which encodes an autoinducer-responsive transcriptional activator, and *lux*I, which encodes a protein required for autoinducer synthesis. Not surprisingly other factors also impinge on luminescence gene expression. *lux*R requires activation by cyclic AMP (cAMP) and the cAMP receptor protein, iron, can influence luminescence expression and FNR exerts an effect on *lux*R. These studies on *V. fischeri* have resulted in the discovery of other quorum sensing–dependent activities for which homologues of *lux*R and *lux*I have been identified or proposed ***(23)***.

This means that integration of quorum sensing effector molecules with other global regulator systems can result in complex and sophisticated interactions. More important, it would seem that these may not be restricted to the signal producing species alone. Shaw et al. ***(24)*** developed a thin-layer chromatographic method for detecting and characterizing *N*-acyl homoserine lactone signal molecules. They then tested HSL signal molecules such as *N*-butanoyl-L-HSL, 3-oxo-, 3-hydroxy, and 3-unsubstituted derivatives purified from a variety of Gram-negative species in an assay. This tested the ability of HSL and its derivatives to induce gene expression of a gene in *Agrobacterium tumefaciens* that was regulated by autoinduction and that was fused to *lac*Z in order to assay gene expression and autoinduction. The investigators showed that signal molecules from different bacteria could be assayed in this way, which means that in the heterogeneous populations found in natural environments, the synthesis of HSL molecules by a single species can affect the activities of other species present within the same environment. A summary of the

Table 5
Processes reported to Be Dependent on Quorum-Sensing Autoinduction[a]

Bacterial species	HSL-induced activity
Pseudomonas aeruginosa	*las*B, which encodes elastase, a metalloprotease important for pathogenicity
V. fischeri	*lux*R, the product of which activates luminescence genes
Rhizobium leguminosarum	Regulation of catalase activity
E. coli	Cell division via expression of *fts*QA genes
Erwinia caratovora	Production of extracellular enzymes that results in tissue maceration in soft rotting of fruits and vegetables; also carbepenem antibiotic synthesis
Serratia liquefaciens	Initiation of differentiation
A. tumefaciens	Ti plasmid conjugal transfer
M. luteus	Resuscitation and regrowth of dormant cells by an HSL-like signaling molecule

[a]Data are taken from ref. 23.

cell density–dependent activity of HSL and its derivatives is given in **Table 5**. Of particular interest in the detection and monitoring of bacteria in natural environments is the possibility of cross induction of different activities between species, the relationship between autoinducers such as HSL with other global regulatory signals such as *rpo*S, and the possibility that resuscitation of bacteria in nonculturable states may be population density dependent and/or reliant on the production of an autoinducing molecular signal.

7. Summary

Analysis of the microbial diversity of the biosphere by traditional cultural methods under represents the true environmental diversity. This realization has been central in driving microbial ecologists to embrace and develop new methods for analysis, which is important for many key areas of biology. Microorganisms drive the chemistry of natural environments, and without them life would not be possible on this planet ***(25)***. The ability to monitor individual species or complex communities is therefore important because such factors as increased levels of pollution and global warming effects may upset the balance of communities and possibly their activities. Such changes may be extremely important if they affect pivotal species such as the nitrifiers. Interestingly, the search for better methods of analysis for environmental microbiology, particularly for nonculturable species, has also changed our preconceptions regarding the properties of microorganisms inferred from laboratory cultures. As this chapter has highlighted, bacteria inhabiting their natural environments, to which they have adapted and evolved over millions of years, may exhibit

totally different properties and interactions to those seen in laboratory cultures. There is no doubt that application of new methods for analysis that are presented in this book herald a rich future for recognizing new species of microorganisms, understanding how they interact in complex communities, and how their activities can be understood and possibly manipulated for environmental biotechnological purposes.

References

1. Gottschal, J. C. (1990) Phenotypic responses to environmental changes. *FEMS Microbiol. Ecol.* **74,** 93–102.
2. Errington, J. (1993) *Bacillus subtilis:* regulation of gene expression and control of morphogenesis. *Microbiol. Rev.* **57,** 1–33.
3. Pickup, R. (1991) Development of methods for the detection of specific bacteria in the environment. *J. Gen. Microbiol.* **137,** 1009–1019.
4. Colwell, R. R., Brayton, P., Herrington, D., Tall, B., Huq, A., and Levine, M. M. (1996) Viable but non culturable *Vibrio cholerae* 01 revert to a cultivable state in the human intestine. *World J. Microbiol. Biotechnol.* **12,** 28–31.
5. Edwards, C. (1996) Assessment of viability of bacteria by flow cytometry, in *Flow Cytometry Applications in Cell Culture* (Al-Rubeai, M. and Emery, A. N., eds.), Marcel Dekker, New York, pp. 291–310.
6. Bloomfield, S. F., Stewart, G. S. A., Dodd, C. E. R., Booth, I. R., and Power, E. G. M. (1998) The viable but non-culturable phenomenon explained? *Microbiology* **144,** 1–3.
7. Munro, P. M., Flatau, G. N., Clement, R. L., and Gautier, M. J. (1995) Influence of the RpoS (KatF) sigma factor on maintenance of viability and culturability of *Escherichia coli* and *Salmonella typhimurium* in seawater. *Appl. Environ. Microbiol.* **61,** 1853–1858.
8. Kaprelyants, A. S. and Kell, D. B. (1993) Dormancy in stationary phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. *Appl. Environ. Microbiol.* **59,** 3187–3196.
9. Oliver, J. D. (1995) The viable but non-culturable state in the human pathogen *Vibrio vulnificus*. *FEMS Microbiol. Lett.* **133,** 203–208.
10. Doull, J. L. and Vining, L. C. (1995) Global physiological controls, in *Genetics and Biochemistry of antibiotic production*, (Vining, L. C. and Stuttard, C., eds.), Butterworth-Heinemann, Boston, pp. 9–63.
11. Porter, J., Edwards, C., and Pickup, R. (1995) Rapid assessment of physiological status of *Escherichia coli* using fluorescent probes. *J. Appl. Bacteriol.* **79,** 399–408.
12. Kolter, R., Siegele, D. A., and Tormo, A. (1993) The stationary phase of the bacterial life cycle. *Ann. Rev. Microbiol.* **47,** 855–874.
13. Zambrano, M. M. and Kolter, R. (1996) GASPing for life in stationary phase. *Cell* **86,** 181–184.
14. Loewen, P. C. and Hengge-Aronis R. (1994) The role of the sigma factor σ^s (KatF) in bacterial global regulation. *Ann. Rev. Microbiol.* **48,** 53–80.

15. Foster, J. W. and Spector, M. P. (1995) How *Salmonella* survive against all the odds. *Ann. Rev. Microbiol.* **49,** 145–174.
16. Nystrom, T., Albertson, N. H., Flardh, K., and Kjelleberg, S. (1990) Physiological and molecular adaptation to starvation and recovery from starvation by the marine *Vibrio* sp. S14. *FEMS Microbiol. Ecol.* **74,** 129–140.
17. Koch, A. L. (1997) Microbial physiology and ecology of slow growth. *Microbiol. Mol. Biol. Rev.* **61,** 305–318.
18. Cairns, J. and Foster, P. L. (1991) Adaptive reversion of a frameshift mutation in *Escherichia coli*. *Genetics* **128,** 695–701.
19. Rosenberg, S. M. (1997) Mutation for survival. *Curr. Opin. Genetics Dev.* **7,** 829–834.
20. Hales, B. A., Edwards, C., Ritchie, D. A., Hall, G., Pickup, R. W., and Saunders, J. R. (1996) Isolation and identification of methanogen-specific DNA from blanket bog peat using PCR amplification and sequence analysis. *Appl. Environ. Microbiol.* **61,** 668–675.
21. Matin, A., Little, C. D., Fraley, C. D., and Keyhan, M. (1995) Use of starvation promoters to limit growth and select for trichloroethylene and phenol transformation activity in recombinant *Escherichia coli*. *Appl. Environ. Microbiol.* **61,** 3323–3328.
22. Whiteley, A. S., O'Donnell, A. G., MacNaughton, S. J., and Barer, M. R. (1996) Cytochemical co-localisation and quantitation of phenotypic and genotypic characteristics of individual bacterial cells. *Appl. Environ. Microbiol.* **62,** 1873–1879.
23. Fuqua, C., Winans, S. C., and Greenberg, P. R. (1997) Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum sensing transcriptional regulators. *Ann. Rev. Microbiol.* **50,** 727–751.
24. Shaw, P. D., Ping, G., Daly, S. L., Cha, C., Cronan, J. E., Reinhart, K. L., and Farrand, S. K. (1997) Detecting and characterising N-acyl-homoserine lactone signal molecules by thin layer chromatography. *Proc. Natl. Acad. Sci. USA* **94,** 6036–6041.
25. Pace, N. R. (1996) New perspectives on the natural microbial world: molecular microbial ecology. *ASM News* **62,** 463–470.

2

Sampling Sediment and Soil

Use of Coring Devices

Roger Pickup, Glenn Rhodes, and Grahame Hall

1. Introduction

1.1. Sampling Natural Environments

Individually, molecular and classical microbial ecology techniques are powerful tools in microbial ecology, but both are limited with respect to relating the presence and/or diversity of microorganisms to their function/activity in that environment ***(1,2)***. By combining the two approaches this obstacle can be overcome. Paramount in this respect are *in situ* sampling procedures that produce samples in a form representative of that environment or habitat ***(3)***.

To determine the roles played by microorganisms in a particular habitat, some form of procedure has to be undertaken to obtain representative samples upon which representative measurements can be made ***(3,4)***. There are usually three options available. First, a sample can be removed from an environment and returned to the laboratory for analysis. This approach is often synonymous with "destructive sampling," which renders the sample nonrepresentative of the environment from which it is removed (e.g., grab sample from benthic environment; *see* **ref. *4***). The nonrepresentative nature of the sample is owing to complete or partial loss of functional integrity. This is particularly apparent when studying geochemical processes that rely on redox gradients or those directly affected by the ingress of oxygen ***(5)***. Consequently, any process measurements made are no longer representative of that environment. Second, a sample can be removed from the environment while attempting to maintain *"in situ"* conditions during transportation and subsequent laboratory analysis. During the laboratory analysis, the sample can be maintained as close as pos-

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sible to *"in situ"* conditions, with one or two parameters being varied for experimental purposes. Further development of this principle led to systems that are often termed "microcosms." These can vary from the simple two-phase systems (lake water/air or soil/air) to the complex (three-phase flow-through sediment/lakewater systems; *see* **ref. *6***). Microcosms have provided model systems with which to study survival, movement, transport, gene transfer, and microbial interactions (*see* **ref. *7***). A third option is to perform the experiments in the field, with a minimum of disturbance to the habitat. This is the least flexible of the options, and only a limited range of parameters can be measured, e.g., methane flux from upland soils ***(8)***. These limitations arise owing to logistical constraints particularly when the transportation of delicate equipment to remote locations is required.

1.2. Sampling Aquatic Environments

This section focuses on sediments; however, sampling of the water column has been reviewed by Herbert ***(4)*** (*see* also Chapter 3). Grabs and corers represent the main types of sediment sampler.

1.2.1. Grab Samplers

There are a considerable number of grab samplers available. All have their advantages and disadvantages and none suit all environments ***(4)***. For *in situ* sampling, all grabs are not appropriate because the sediment obtained is mixed. In general, grab samplers penetrate the sediment by approx 10 cm and cover an area of 0.1–0.2 m^2 ***(4)***. Grab samplers include the Petersen grab, van Veen grab, Shipek grab, Ekman grab, Okean grab, and the Smith-McIntyre grab, all of which can be operated from a boat; however, they differ in size and complexity of operation, with the latter suitable for sampling continental shelf sediments ***(4)***. Larger amounts of sediments can be obtained using the Reineck box sampler in which the increase in sample size is compromised by its relatively large size and cumbersome nature ***(4)***.

1.2.2. Core Samplers

The most crucial aspect of corer design is the retention of the core on removal from the sediment environment. This is particularly important for corers penetrating the sediments to depths >10–20 cm and those in which access to the device occurs at the surface after retrieval ***(4)***. Other important features that are characteristic of all corers are the compression of the sediment and disturbance of the fine upper layers. Both are inevitable consequences of the coring operation. The simplest corer consists of a perspex tube (e.g., 5 cm diameter, 30 cm length; *see* **ref. *4***), that can be driven into sediments by hand in shallow waters (or intertidal zones) or manipulated by divers. After removal

from sediment, the ends of the tube can be sealed with bungs, preventing loss of the core material in transit. Several larger devices that are remotely operated are available for sampling at depth. The gravity corers ***(9)***, akin to small missiles attached to a rope, penetrate the sediment and the core is collected in the central Perspex core tube. The most notable disadvantage of this type of device is that it causes both disturbance and compression of the core material. The Emery and Dietz corer, the gravity corer with external retaining devices, and the Sholkovitz corer produce cores of increasing length in the marine environment (up to 3 m; *see* **ref. *10***). The Makereth corer retrieves long intact freshwater sediments cores ***(11)***. This corer permits the removal of undisturbed sediment of up to 6 m in length from lake-water environments. Operation of the apparatus is pneumatic (**Fig. 1**), whereby hydrostatic pressure acts on a cylindrical anchor chamber that embeds into the sediment on the lake bed. The anchor chamber holds the apparatus firmly in place while the corer tube is driven downward into the sediment by means of compressed air. Once coring is completed, the anchor chamber is automatically filled with air, the coring tube is removed from the sediment, and the whole apparatus is recovered to the surface by buoyancy lift (**Fig. 1**) and returned to the laboratory by boat. The core is extruded back on dry land and carried to the laboratory as four covered sections.

Frame-mounted corers minimize both disturbance and compression. The best example of this type is the "Jenkin surface-mud sampler" (**Fig. 2** ***[12]***), which retrieves 30–40-cm cores with overlying water sealed at both ends. The number of manipulations that can be performed on Jenkin core tubes samples demonstrates their versatility. Extrusion of the core, sectioning followed by processing the sample is the most common manipulation performed. However, the intact core and overlying water can be set up as a microcosm, and processes measured after incubating under varying conditions or after addition of substrates, e.g., acetate ***(13)***. Some tubes have been adapted with a spiral of sampling ports that allow substrate additions or sample removal at a variety of depths ***(13)***. This versatility is shared by others in this group, which includes the Craib sampler and simultaneous multicore samplers, both of which work on the same principle ***(4)***.

1.3. Sampling Soil Environments

Despite the heterogeneity of the soil environment, there are few methods for obtaining soil cores. The basic principle is to use either a large rubber mallet to hammer the core tube into the soil or a cutting device applied with a downward pressure to ensure penetration ***(5)***. Core tubes can comprise a variety of lengths and diameters. Obstructions in the soil such as stones, rocks, and branches and fibrous material, in general, can prevent successful penetration and affect the

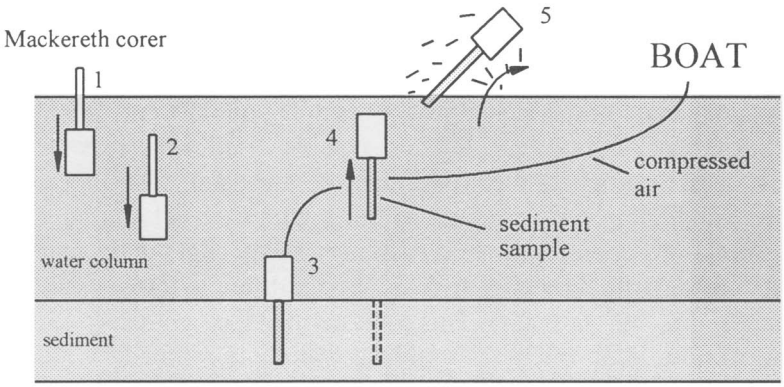

Fig. 1. The Mackereth corer: retrieval of 6-m sediment cores. The Mackereth corer descends the water column (2) from the surface (1) and embeds itself firmly in the sediment (3), and the internal core tube is extruded into the sediment. The entire apparatus returns to the surface by flotation using compressed air (4) and is collected by the surface craft and returned to the laboratory for processing (5).

integrity of the core after removal. Once removed, the core can be transported to the laboratory. Maintenance of anaerobic conditions is possible using the appropriate apparatus and will be discussed in the next section, which focuses on sampling peat soils and the maintenance of anaerobic conditions. However, the apparatus and procedure are directly applicable to sampling of soil types.

1.3.1. Peat Sampling

The fibrous nature of undisturbed surface peat prevents intact cores from being sampled without distortion of the vertical profile. The transition between aerobic conditions in the surface layers and the water-logged anaerobic horizons is characterized by steep oxygen concentration gradients ***(14)***. Such gradients play an important role in the vertical distribution of microbial populations ***(15)***. To accurately determine the vertical distribution of microbial activities within the peat, any compaction of the profile or disturbance of the redox conditions should be avoided. Moreover, the exposure of some obligate anaerobic bacteria to air, e.g., the methanogens, even for short periods, could affect the rate of methane production ***(16,17)*** and, therefore, reduce the activity of the population relative to the undisturbed condition.

Two strategies have been used to avoid compaction and oxygen contamination when sampling peat cores. One approach is to cut the peat, using a long knife ***(18)*** or other cutting device ***(19)***, to the shape and depth of the sample tube, which is then inserted into the preformed space. The initial cutting of the peat could introduce oxygen to the deep anaerobic layers, and, thus, the

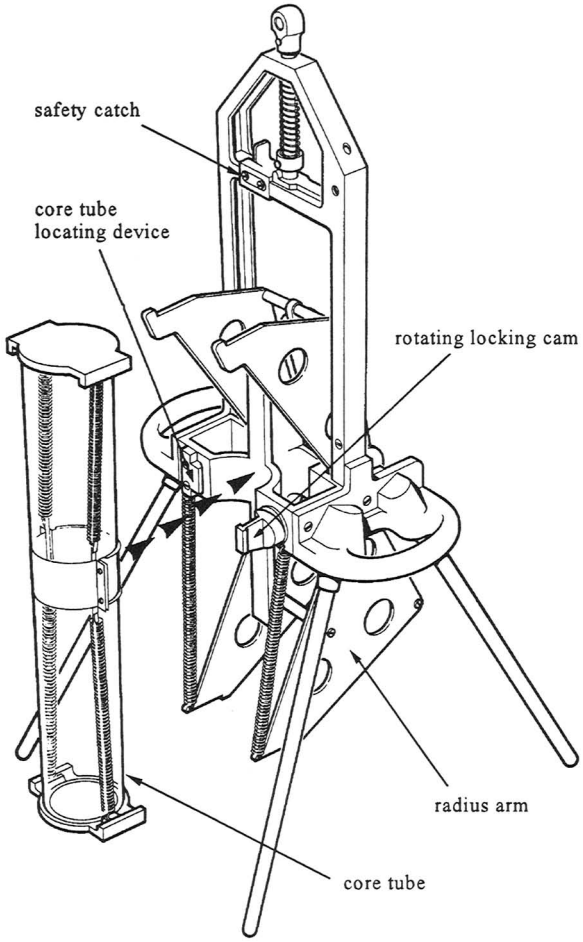

Fig. 2. The Jenkin corer. This device uses detachable core tubes to retrieve 30-cm sediment cores and overlying water.

shape of the cut should be accurate to avoid compaction of the peat on insertion of the sample tube. In the second approach, a cutting device is attached to the base of the sample tube. The tube (and cutter) can be rotated on the surface of the peat, cutting the fibrous peat deposits while enclosing the peat core in the sample tube ***(20)***, thereby reducing the potential for oxygen contamination. The core sample tubes must be excavated from the peat deposits ***(18)*** because attempts to remove these directly would result in the core sample remaining in place or, at best, breaking along its length.

Methods used to section the core, and obtain subsamples from depth, should also avoid distortion of the profile and exposure of anaerobic layers to oxygen.

Many reports describe the gas flushing of incubation chambers to establish anaerobic conditions prior to the determination of methane production *(21,22)*. This would imply that the sample material had been exposed to oxygen at some stage during the sectioning procedure. Moreover, many samples are slurried, which removes the spatial relationship among different populations of organisms. These associations are known to be important for the activity of different physiological groups of bacteria *(23)*, and, if possible, the structure of the sample should be maintained *(24,25)*. Methods that sample and section cores of peat and avoid exposure of the anaerobic layers to oxidizing conditions must benefit the interpretation of activity measurements.

2. Materials

2.1. Sampling Freshwater Sediments with the Jenkin Surface-Mud Core Sampler

1. Jenkin surface-mud sampler complete with rope.
2. Core tubes.
3. Sediment extruder.
4. Boat and safety equipment.

2.2. Sampling and Analysis of Peat Cores

1. Acrylic core tubes.
2. Cutter.
3. Appropriate seals.
4. Peripherals (*see* **Notes 11–27**; **Fig. 3**; **ref. *5***).

3. Methods

3.1. Sampling Freshwater Sediments with the Jenkin Surface-Mud Core Sampler

1. Position boat over sampling site.
2. Load core tube into Jenkin sample (*see* **Note 1**).
3. Lift suspension rod attached to rope, cock sample (*see* **Note 2**), and push safety catch to the "on" position (*see* **Note 3**).
4. Take the weight of sampler on support rope and lower into the water (*see* **Note 4**).
5. Release safety catch.
6. Gently and smoothly lower the sampler into sediments (*see* **Note 5**).
7. Wait for the spring mechanism to operate and for the sampler to settle (*see* **Note 6**).
8. Haul the sampler, keeping it vertical, back to the surface.
9. Remove sample tube and place in a suitable holder prior to extrusion.

3.2. Extrusion of the Core (see Note 7)

1. The mechanical extrusion unit is attached to the work surface.
2. Fix core tube firmly to the extrusion unit.

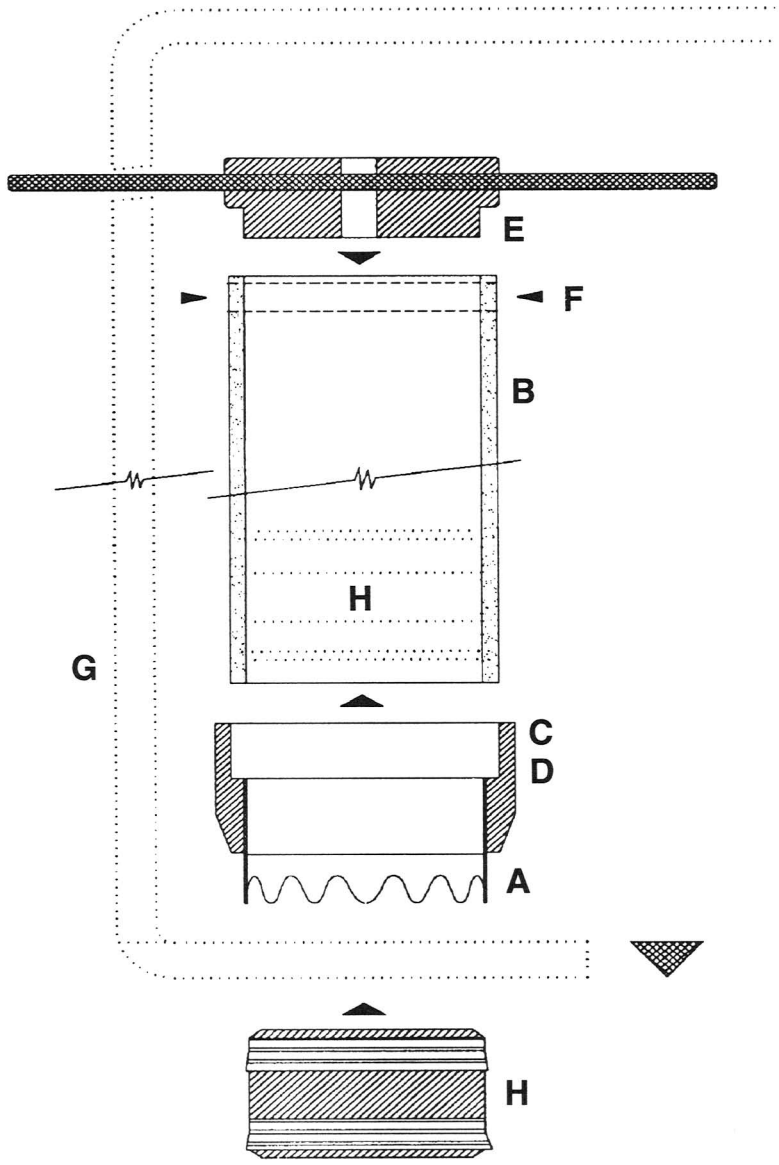

Fig. 3. Schematic diagram of the anaerobic peat sampling device including location of gas tight piston applied after retrieval of the core prior to transport to laboratory.

3. Locate extrusion rod in bottom extrusion plate in the core tube and lock in place.
4. Release the securing gate on the core tube bottom lid.
5. Remove top lid and siphon overlying water to just above the core surface (*see* **Note 8**).

6. Fit the sample collecting spout to the top of the tube.
7. Use the handwheel on the extrusion rod to extrude the core sediment.
8. Extrude the desired amount of sediment (*see* **Note 9**).
9. Remove the section of core into an appropriate vessel (*see* **Note 10**).
10. Repeat **steps 8–10** as appropriate.

3.3. Sampling Peat Cores

1. Identify sampling site for core extraction and place the specially designed sampler into position (*see* **Notes 11** and **12**).
2. Insert the tightly fitting PVC insert/"tommy" bar (**Fig. 3E**) into the top of the core tube and secure with a large Jubilee clip (**Fig. 3F**) positioned on the outside (*see* **Note 13**).
3. Carefully cut away the surface vegetation of the peat bog and place the assembled core tube upright on the peat surface.
4. Cut through the peat, holding the tommy bar and move with a rotating motion (both clockwise and counterclockwise), with slight downward pressure and isolate the core in the sample tube (*see* **Note 14**).
5. Remove the peat core and cutter from the peat bog using a metal rod with a right-angled bend at each end (**Fig. 3G**) (*see* **Note 15**).
6. Trim the bottom of the peat core extending from the cutter with a sharp knife.
7. Fit the gastight piston (**Fig. 3H**) by inserting through the cutter and up into the core tube (*see* **Note 16**).
8. Remove the cutter (*see* **Notes 17** and **18**).
9. Store the core(s) upright during transportation to the laboratory.

3.3.1. Sectioning the Peat Core Under Anaerobic Technique (see ***Note 19***).

1. Anaerobic sectioning is performed in a flexible gas hood flushed with nitrogen gas (**Note 20**).
2. The peat core is mechanically extruded into short lengths (12.5 cm) of acrylic core tube, which also serve as incubation chambers using a device (*see* **Note 21** and **Fig. 4A**).
3. A new incubation chamber is then inserted into the upper assembly and passed into the gas hood for the operations to be repeated as the core is sectioned further.

3.3.2. Incubation and Methane Analysis (see ***Note 22***)

1. Allow the incubation vessels to stand for 48 h (*see* Note 23).
2. Flush the headspace with nitrogen for 5 min after 24 and 48 h (*see* **Note 24**).
3. Remove subsamples of the headspace (usually 0.5 mL) at regular intervals (at least three) for analysis of methane concentration (*see* **Note 25** and **26**).
4. After incubation, dry all the sections of peat to a constant weight at 60°C for dry wt determination (*see* **Note 27**).

3.4. Future Developments

When peat cores were exposed to air, the methanogenic activities were on average 43% lower (range 12–74%; ***[5]***). Even brief exposure resulted in a

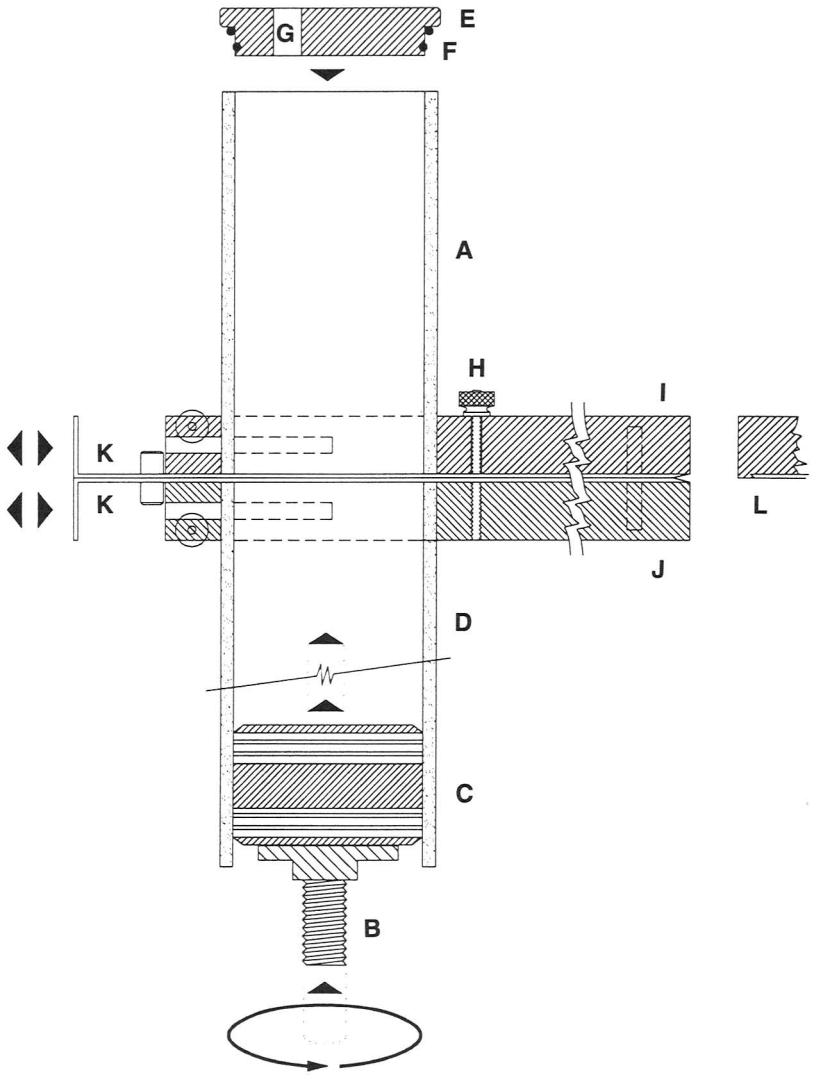

Fig. 4. Schematic diagram showing the peat sectioning device attaching the sample tube to the incubation section.

decline of 39% (9–66%), thus highlighting the benefit of *in situ* sampling procedures. Future developments lie not in the design of new procedures (many are already available), but in the recognition of their relevance and importance, when combined with the ever expanding suite of molecular techniques, to many facets of microbial ecology in which activity and function are related to biodiversity and population/community analyses.

4. Notes

1. Refer to the manual for instructions on operation and maintenance ***(12)***. Tubes are located in the apparatus with the tube harness flange facing inward.
2. Once the tube is securely attached to the sampler, the machine is cocked by a downward pressure on the radius arm cross bar until the radius arm is locked in the primed position.
3. The safety catch is pushed to the "on" position.
4. The sampler is lowered so that the legs are in the water before the safety catch is released.
5. Any uneven movement during lowering will activate the spring mechanism of the sampler.
6. Once the sampler comes into contact with the sediments (the lowering rope becomes slack), the spring mechanism will activate. The sampler should be left for a short period to settle prior to lifting.
7. The text assumes that the operator has purchased the appropriate equipment and has access to the operating manual, highlights the main operation of the unit, and provides useful hints on its efficient use.
8. Careful siphoning avoids disturbing the top sediment.
9. One revolution of the handwheel has a pitch of 2 mm.
10. This procedure can be carried out anaerobically using the gas hood detailed in **Note 20.**
11. The core tube dimensions are 0.5 m in length and 7.0 cm internal diameter.
12. The sampler comprises a sinuous, toothed cylindrical cutter (**Fig. 3A**) attached to the bottom of a length of acrylic core tubing (**Fig. 3B**). The cutter, whose internal diameter is exactly that of the core tube, is housed in a PVC sleeve (**Fig. 3C**) that fits tightly over the core tube. The sleeve has an internally machined rebate (**Fig. 3D**) that locates the core tube immediately above the cutter. The sleeve is secured to the outer wall of the core tube using adhesive tape.
13. The insert has a removable horizontal "tommy" bar passing through it and also a hole to allow air to escape during the coring process.
14. Any compaction of the peat profile (e.g., sinking of the peat core) can be readily observed through the transparent acrylic tubing and the rejected core.
15. The lower horizontal part is triangular in cross section and sharpened on each edge. The sharpened leading edge cuts through the peat as the rod is inserted adjacent to the core tube, to a depth just below the cutter (previously marked on the vertical section of the rod). The rod is rotated 360°, and the sharpened side edges cut through the peat immediately below the cutter at the base of the sample tube. This operation is facilitated by the upper horizontal part of the rod, which is quite long. The lower horizontal section is then located on the cutter, and the whole core tube is gently eased from the peat.
16. The piston is made of PVC with two external distributor seals located in recessed grooves.
17. The cutter can now be attached to another core tube.
18. It is not possible to exclude oxygen while the piston is inserted, and, therefore, the standard protocol of always sampling the peat at least 10 cm deeper

than the depth required is necessary. This provides a "buffer" zone that may be exposed to, and consume, oxygen while the depth of peat to be subsampled is protected.

19. For anaerobic procedures, replicate peat cores are sectioned at 1.0-cm intervals from 3.0 to 9.0 cm. All gassing procedures use oxygen-free nitrogen that has been passed over a heated copper catalyst (BASF, R3-11) to remove contaminating traces of oxygen. The flow rate of gas is approx 2 $L \cdot min^{-1}$.
20. The anaerobic hood is made from heavy-gage polyethylene bags, which allows manipulation of the equipment from the outside and access to the sectioning equipment from the bottom. The hood is flushed with nitrogen after the peat core has been prepared for the surface section to be removed.
21. The device is operated by a screw thread (**Fig. 4B**) with a pitch of 2 mm which locates on the piston (**Fig. 3G**; **Fig. 4C**) used to seal the bottom of the peat core (**Fig. 3B**; **Fig. 4D**).

    a. Prior to extrusion, the incubation chambers are sealed at the top with a PVC cap (**Fig. 4E**) that has an internal "O" ring to hold it in position and a larger "O" ring (**Fig. 4F**) that forms a seal between the machined flat edge of the tube and the shoulder of the cap. The top cap also contains a butyl rubber septum (**Fig. 4G**) that allows venting, gassing, or sampling of the headspace.
    b. The sample tube and the incubation chamber are connected by the sectioning device shown in **Fig. 4**. This consists of two assemblies that are joined together (**Fig. 4H**). The bottom of the upper assembly (**Fig. 4I**) and the top of the lower assembly (**Fig. 4J**) are sealed by removable metal plates (**Fig. 4K**) that are sharpened on their leading edge. These are located in finely machined dovetailed rebates (**Fig. 4L**), and when the assemblies are joined, the plates lie adjacent to each other.
    c. The lower assembly is placed over the sample tube and fixed in position so that the top edge of the tube touches the metal plate.
    d. The plate is removed and the peat core extruded until the surface is level with the top of the tube.
    e. The incubation chamber is placed in the upper assembly so that the bottom of the tube is against the metal plate.
    f. The assemblies are joined together and fixed in position on top of the core tube using the screw threads and bolts (**Fig. 4H**).
    g. The incubation chamber is flushed with nitrogen for 5 min before the plates are removed, and the core is extruded by the required amount (typically 1.0 cm).
    h. The peat is cut by replacing the plates. This isolates the cut section within the incubation chamber while closing the cut surface of the main peat core.
    i. The upper assembly, which includes the incubation chamber, is removed, leaving the lower assembly in place and sealing the remainder of the core sample.
    j. The plate from the upper assembly is removed while simultaneously inserting another PVC sealing cap. This is identical to the top seal but without the septum vent.

k. The extruded peat plug is therefore isolated in the incubation chamber. Gas-tight seals of the incubation chamber are ensured throughout the entire incubation period by securing the top and bottom caps with a 150-mm "G" cramp.

22. All incubations are performed at 20°C.
23. This allows the gases present in the peat sections (either dissolved or in gas bubbles) to equilibrate with the headspace.
24. The headspace volume is replaced approx 25 times.
25. All syringes and needles used for the removal of subsamples from the incubation vessels are preflushed with nitrogen.
26. Methane is analyzed using a Perkin Elmer 3500 gas chromatograph equipped with a Poropak N column and flame ionization detector. Injection of the gas sample is by a 0.1-mL gas loop attached to a gas sample valve. The gas loop is flushed with at least four times its volume prior to injecting the sample to the column.
27. Methane accumulation is linear throughout incubation, and rates are calculated from the slope and corrected for dry wt of the peat sections.

References

1. Hall, G. H., Jones, J. G., Pickup, R. W., and Simon, B. M. (1990) Methods to study the bacterial ecology of freshwater environments. *Methods Microbiol.* **23,** 181–210.
2. Pickup, R. W. (1991) Molecular methods for the detection of specific bacteria in the environment. *J. Gen. Microbiol.* **137,** 1009–1119.
3. Pickup, R. W. (1995) *Sampling and Detecting Bacterial Populations in Natural Environments*, Society for General Microbiology Symposium, series 52, Cambridge University Press, Cambridge, UK, pp. 298–315.
4. Herbert, R. A. (1990) Methods for enumerating microorganisms and determining biomass in natural environments. *Methods Microbiol.* **19,** 1–40.
5. Hall G. H., Simon, B. M., and Pickup, R. W. (1996) Methane production in blanket bog peat: a procedure for sampling, sectioning and incubating samples whilst maintaining anaerobic conditions. *Soil Biol. Biochem.* **28,** 9–15.
6. Morgan, J. A. W., Rhodes, G., Pickup, R. W., Winstanley, C., and Saunders, J. R. (1992) The effect of microcosm design on the survival of recombinant microorganisms in lake water. *Microbial Releases* **1,** 155–160.
7. Trevors, J. T. (1988) Use of microcosms to study genetic interactions between microorganisms. *Microbiol. Sci.* **5,** 132–136.
8. Baker, J. M., Norman, J. M., and Bland, W. L. (1992) Field scale application of flux measurement by conditional sampling. *Agricul. Forestry Meterol.* **62,** 31–52.
9. Fenchel, T. (1967) The ecology of the microbenthos. I. Quantitative importance of ciliates as compred with metazoans in various types of sediments. *Ophelia* **4,** 121–137.
10. Hood, M. A. (1992) Experimental methods for the study of fate and transport of microorgansims in aquatic systems, in *Microbial Ecology: Principles, Methods and Applications* (Levin, M. A., Seidler, R. M., and Rogul, M., eds.), McGraw Hill, New York, pp. 511–524.

11. Mackereth, F. J. H. (1958) A portable core sampler for lake deposits. *Limnol. Oceanogr.* **3,** 181–191.
12. Ohnstad, F. R. and Jones, J. G. (1982) The Jenkin surface mud sampler: user manual, Occasional Publication No. 15. Freshwater Biological Association, Titus Wilson Kendal, Cumbria, UK, pp. 45.
13. Jones, J. G. and Simon, B. M. (1984) Measure of microbial turnover of carbon in anoxic freshwater sediments: cautionary comments *J. Microbiol. Methods* **3,** 47–55.
14. Benstead, J. and Lloyd, D. (1994) Direct mass spectrometric measurement of gases in peat cores. *FEMS Microbiol. Ecol.* **13,** 233–240
15. Williams, R. T. and Crawford, R. L. (1984) Methane production in Minnesota peatlands. *Appl. Environ. Microbiol.* **47,** 1266–1271.
16. Boone, D. R. (1991) Ecology of methanogenesis, in *Microbial Production and Consumption of Greenhouse Gases: Methane, Nitrogen Oxides, and Halomethanes* (Rogers, J. E. and Whitman. W. B., eds.)**,** American Society for Microbiology, Washington, DC, pp. 57–70.
17. Fetzer, S., Friedhelm, B. and Conrad, R. (1993) Sensitivity of methanogenic bacteria from paddy soil to oxygen and desiccation. *FEMS Microbiol. Ecol.* **12,** 107–115
18. Freeman, C., Lock M. A. and Reynolds, B. (1993) Fluxes of CO**2,** CH_4 and N_2O from a Welsh peatland following simulation of a water table draw-down: potential feedback to climatic-change. *Biogeochemistry* **19,** 51–60.
19. Clymo, R. S. (1988) A high resolution sampler of surface peat. *Functional Ecol.* 2 425–431.
20. Williams, B. L. and Wheatley, R. E. (1992) Mineral nitrogen dynamics in poorly drained blanket peat. *Biol. Fertility Soils* **13,** 96–101.
21. Moore, T. R. and Knowles, R. (1990) Methane emissions from fen, bog and swamp peatlands in Quebec. *Biogeochemistry* **11,** 45–62.
22. Prieme, A. (1994) Production and emission of methane in a brackish and a freshwater wetland. *Soil Biol. Biochem.* **26,** 7–18
23. Jones, J. G., and Simon, B. M. (1985) Interaction of acetogens and methanogens in anaerobic freshwater sediments. *Appl. Environ. Microbiol.* **49,** 944–948
24. Yavitt, J. B., Downey, D. M., Lancaster, E., and Lang, G. E. (1990) Methane consumption in decomposing Sphagnum-derived peat. *Soil Biol. Biochem.* **22,** 441–447.
25. Yavitt, J. B. and Lang, G. E. (1990) Methane production and sulphate reduction in two Appalachian peatlands. *Biogeochemistry* **10,** 81–104.

3

Sampling Water Bodies

Tangential Flow Filtration

Roger Pickup, Helen Mallinson, and Glenn Rhodes

1. Introduction

The study of microbial community structure via analysis of total community DNA, or by the application of fluorescent oligonucleotide probes by fluorescent *in situ* hybridization, has become a valuable tool for understanding microbial diversity and abundance in a range of environments. However, one of the major limitations to research into microbial communities, and consequently the detection of microorganisms in the environment, is an inability to isolate and culture the vast majority of microorganisms. Because of the nonrepresentative nature of culturable techniques, sampling the "total" community is often the preferred option. Soil is a difficult medium to process and offers many impediments, particularly with respect to removal of cells from the soil matrix, whereas water is probably the most amenable medium to sample and process. The study of microbial community structure requires a representative sample of that community for processing in a form that is free from contaminants that will interfere with the analysis. This section details the contribution that tangential flow filtration (TFF) can make to this goal.

Traditionally, concentration of particles (biological and nonbiological) was carried by either centrifugation or "dead end" membrane filtration (standard filtration techniques; ***(1)*** under vacuum through either 0.2 or 0.4-µm pore size filters. Obvious disadvantages of this technique are the limits placed on the total volume of sample that can be processed, rapidity, and subsequent damage to cells on the membrane. TFF ***(2)*** offers an alternative strategy that bypasses these problems because it permits large volumes of water to be processed in the field ***(3)***. Particles >0.2 µm in diameter are concentrated not by retention directly

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against the filter, but within the void volume of the TFF unit, which increases in particulate concentration as more water is processed. The final concentrated solution (termed "retentate") is retained within the unit by setting up a back pressure. Once released, the concentrate is flushed through into a suitable container. The particulate matter from 100–200 L of lake water can be reduced to a volume of approx 500 mL. If required, this concentrate can be resuspended in as little as 10 mL after centrifugation. This represents a concentration factor of approx 10,000-fold. The cells are now amenable to direct DNA extraction followed by polymerase chain reaction (PCR) amplification. In addition, enrichment cultures can be set up using the concentrate as an initial inoculum.

This method of concentration has been used for a variety of microorganisms including hematopoietic necrosis viruses ***(4)***, pico- and nanoplankton in the marine environment ***(5,6,7)***, ammonia-oxidizing bacteria ***(8)***, *Giardia* cysts ***(9)***, algae from freshwater ***(10)***, protists from estuarine and marine environments ***(11)***, and for the assessment of trophic status of lakes ***(12)*** and PCR detection of the insertion element, IS*1* ***(13)***.

The apparatus used was the Millipore Pellicon cassette system (Millipure Ltd., Watford, UK) comprising a filtration cell (TFF unit), pump and retentate, and filtrate reservoirs (**Fig. 1**). The sample is pumped through the filtration cell whereby a flow, directed in parallel to the surface of the filtration membrane, is created ***(5)***. This flow keeps the particles suspended in the retentate. As the sample passes the filter surface, only a certain fraction of the medium (permeate; filtered water) passes through the membrane and is either collected in the filtrate reservoir or discarded. The retentate (concentrated sample) is recirculated through the retentate reservoir until the sample and then the retentate reservoirs are empty. The retentate is then collected from the filtration cell. The following section describes, as an example, the application of TFF as detailed by Hiorns et al. ***(8)*** that involved sampling a defined depth of lake water for ammonia-oxidizing bacteria.

2. Materials

1. Millipore Pellicon tangential flow systems equipped with a 3X Durapore microporous membrane cassette GVLP00005 with a rated pore size of 0.2 µm and a filtration area of 4.6 m^2 per membrane. The unit was set up as shown in **Fig. 1**. The sample was circulated by means of a Millipore variable-speed peristaltic tubing pump (XX80 3G2 30) fitted with 1.2-cm silicone tubing (internal diameter).
2. Oxygen/temperature profile meter.
3. Sample tubing with "T"-shaped inlet and weighted end (*see* **Note 1**).
4. 1-L sterile Schott bottle.
5. Two 500-mL sterile Schott bottles.

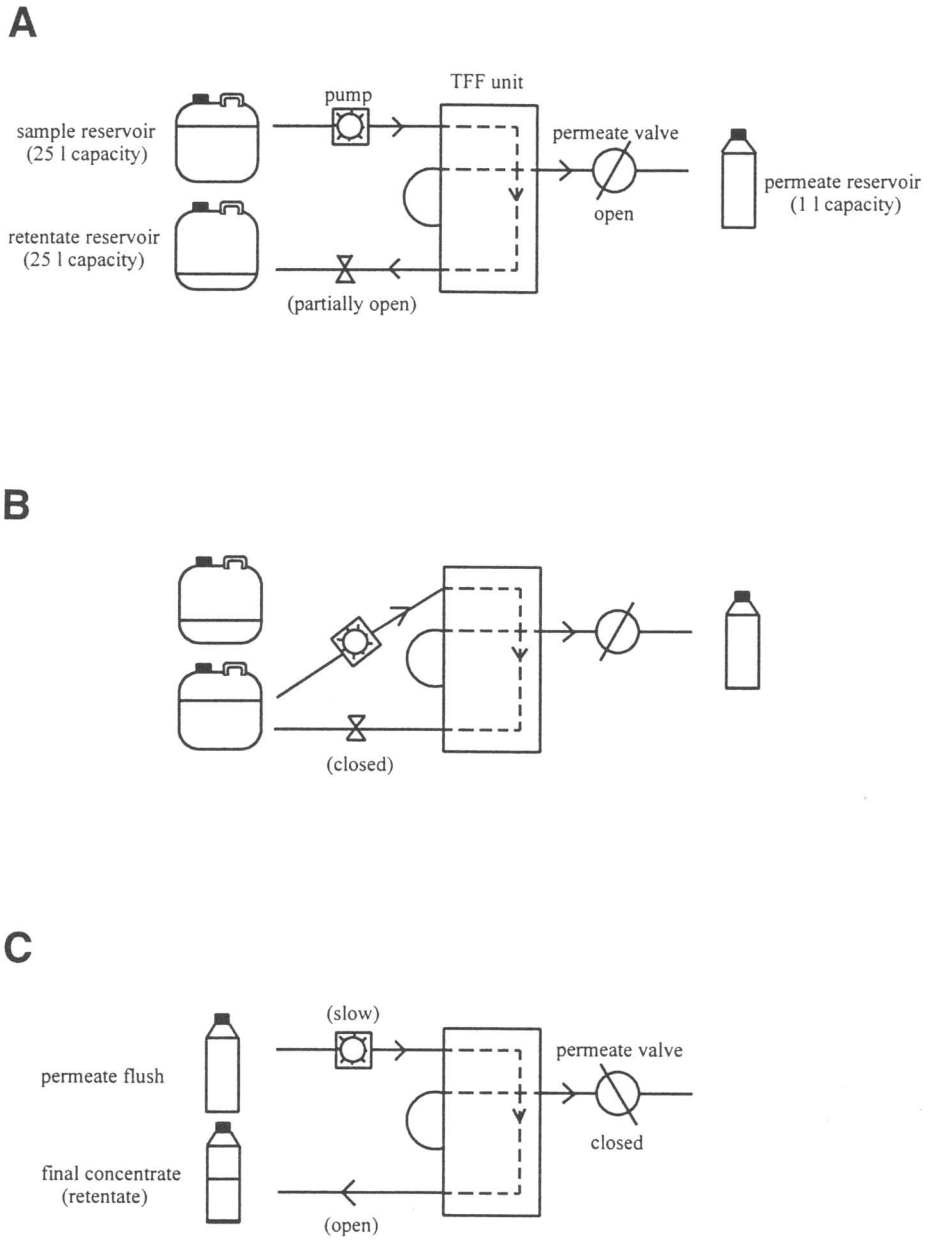

Fig. 1. Configuration of the tangential flow filtration unit during various stages of operation. **(A)** Initial set up with sample input and retentate output into appropriate reservoirs, permeate is collected; transfer to next sample reservoir as required. **(B)** When all sample reservoirs have been processed, retentate reservoir is reduced. **(C)** Permeate is used to flush out the final concentrate.

6. A 240-V petrol generator.
7. Five sample bottles (25 L).
8. Sampling boat with anchor and life jackets.

3. Methods

3.1. Sample Collection

1. Secure sample boat at sample site by mooring or by anchor.
2. Carry out oxygen/temperature depth profile at sampling site and identify oxy/thermocline.
3. Connect sample tube/weight via peristaltic pump to the TFF unit.
4. Connect pump to 240-V power generator and switch on.
5. Place sampling tube in water.
6. Fill sample tube with surface water and switch off pump (*see* **Note 2**).
7. Lower sample tube to required depth and stabilize (*see* **Note 3**).
8. Pump water and discard the void volume.
9. Collect required volume in prewashed 25-L containers (*see* **Note 4**).
10. Stow away sample tube (*see* **Note 5**).
11. Return the samples for TFF.

3.2. Tangential Flow Filtration

1. Set up TFF system with three filters in the filtration unit (*see* **Note 6**).
2. Connect sampling and retentate tubing to unit and check that they are firmly secured (*see* **Note 7**).
3. Insert sample tube into the sample reservoir and retentate tube into retentate reservoir (**Fig. 1A**; see **Note 8**).
4. Switch on pump, open permeate valve, and start pumping sample into the TFF unit.
5. Create a back pressure to between 1 and 2 bar using a valve or clip on the retentate tube to regulate retentate output flow (**Fig. 1A**; *see* **Note 9**).
6. Collect 1 L of permeate in sterile bottle and retain for later use, the remaining permeate is discarded (**Fig. 1A**; *see* **Note 10**).
7. Monitor TFF unit and transfer sample tubes to sample reservoirs as required (*see* **Note 11**).
8. After last sample reservoir has been processed, transfer the sample tube to the retentate reservoir and close retentate valve and continue pumping (**Fig. 1B**).
9. Once the retentate reservoir is empty, stop the pump, transfer the sample tube to the permeate bottle collected earlier, and close the permeate output valve (**Fig. 1C**).
10. Place retentate tube in 500-mL sample bottle and open retentate valve (**Fig. 1C**).
11. Slowly pump permeate through the TFF unit and collect the first 500 mL of retentate output (**Fig. 1C**).
12. Collect a second 500-mL sample.
13. Secure screw tops on the bottles and store on ice for future processing.

14. Clean TFF filter cassette unit at earliest opportunity (*see* **Note 12**).
15. Process samples as required (*see* **Note 13**).

4. Notes

1. The length of the tube is determined by the required depth of sampling.
2. Filling the tube with surface water reduces buoyancy.
3. Stabilizing refers to allowing the sample tube to unfold and reach the required depth. It is important to secure the boat so that the tube is vertical and not dragged with movement of the boat. For river sampling, it is possible to use a telescopic pole attached to the weighted sample tube to obtain water from midstream or beyond. In addition, if samples need to be taken at depth, then sufficient pipe needs to extend beyond the maximum pole length.
4. Containers were rinsed with lake water to remove any traces of detergent from laboratory washings.
5. By reversing the pump, the void volume will be expelled, making the tubing easier to gather in.
6. Each filter is separated from the unit and each other with silicone gaskets (supplied by manufacturer). The unit is tightened to required torque using a TFF tool.
7. The sample inlet tube connects the sample bottle to the unit, the retentate pipe connects the unit to the retentate reservoir, and the outlet pipe allows the permeate to be collected or discarded.
8. When retentate output is low (*see* **Note 9**), the retentate and sample tubes may be placed into the first sample reservoir. As the sample reservoir empties, it becomes the retentate reservoir, and the sample tube is transferred to the next sample reservoir.
9. The TFF pressure (input and output) depends on three interrelated features: the concentration of particulates, pump speed, and the back pressure created by the valve/clip on the retentate pipe, hence retentate flow rate. To maintain the required pressure from waters with a high concentration of particulates (e.g., eutrophic water or algal bloom) will require higher retentate flow and slower pump speed. In this case, the retentate output tube should be located in a separate reservoir. For oligotrophic waters (low particulate concentration), low retentate flow (virtually zero) and high pump speed will maintain the pressure, and both sample and retentate tubes can be located in the same reservoir at least until the first sample reservoir is empty.
10. The permeate is required to flush through the final retentate solution at the end of the procedure.
11. The pressure gages should be continuously monitored and the working pressures maintained. Any elevation or depression can be corrected by adjusting the pump speed or retentate valve. However, it is also important to monitor tube distortion, particularly on the sample input at the sanitary clip to the unit. Any distortion should be alleviated by reducing the pump speed. TFF will still continue if the pressure drops below 1 bar.
12. Filters should be cleaned in accordance with manufacturer's instructions.

13. Conditions used for sampling ammonia oxidizers from Esthwaite water (Cumbria, UK; ***[8]***) are as follows: sample depth, 8 m (oxycline); sample volume, 80 L; retentate volume, 1 L; DNA extraction ***(7)***; PCR ***(8)***. The above protocol was sufficient to demonstrate that *Nitrosospira* spp. were widespread in the environment ***(8)***.

References

1. Hall, G. H., Jones, J. G., Pickup, R. W., and Simon, B. M. (1990) Methods to study the bacterial ecology of freshwater environments. *Methods Miccrobiol.* **22,** 181–210.
2. Ludwig, K. and Oshaughnessey, K. (1989) Tangential-flow filtration: a technical review. *Am. Biotechnol.* **7,** 41–44.
3. Pickup, R. W. (1995) Sampling and detecting bacterial populations in natural environments. Society for General *Microbiology* Symposium series 52, Cambridge University Press, Cambridge, UK, pp. 298–315.
4. Batts, W. N. and Winton, J. R. (1989) Concentration of infectious hematopoietic necrosis virus from water samples by tangential flow filtration and polyethylene glycol precipitation. *Can. J. Fisheries Aquatic Sci.* **46,** 964–968.
5. Barthel, K. G., Schneider, G., Gradinger, R., and Lenz, J. (1989) Concentration of live picoplankton and nanoplankton by means of tangential flow filtration. *J. Plankton Res.* **11,** 1213–1221.
6. Giovannoni, S. J., Delong, E. F., Schmidt, T. M., and Pace, N. R. (1990) Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. *Appl. Environ. Microbiol.* **56,** 2572–2575.
7. Schmidt, T. M., Delong, E. F., and Pace, N. R. (1991) Analysis of a marine picoplankton community by 16S ribosomal RNA gene cloning and sequencing. *J. Bacteriol.* **173,** 4371–4378.
8. Hiorns, W. D., Hastings R. C., Head, I. M., McCarthy, A. J., Saunders, J. R., Pickup, R. W., and Hall, G. H. (1995) Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidising bacteria from lakewater and sediments. *Microbiology* **141,** 2793–2800.
9. Hastie, J. C., Kelly, P. J., and Brown, T. J. (1992) Concentrating *Giardia* cysts in water by tangential flow filtration compared with centrifugation. *NZ J. Marine Freshwater Res.* **26,** 275–278.
10. Petrusevski, B., Bolier, G., Van Breemen, A. N., and Alaerts, G. J. (1995) Tangential flow filtration: a method to concentrate fresh water algae. *Water Res.* **29,** 1419–1424.
11. Snyder, R. A. and Hoch, M. P. (1996) Consequences of protist stimulated bacterial production for estimating protist growth efficiencies. *Hydrobiologia* **341,** 113–123.
12. Morgan, J. A. W. and Pickup, R. W. (1993) Activity of microbial peptidases, oxidases, and esterases in lake waters of varying trophic status. *Can. J. Microbiol.* **39,** 796–803.
13. Pickup, R. W., Rhodes, G., and Saunders, J. R. (1995) Extraction of microbial DNA from aquatic ecosystems: Freshwater, in *Molecular Microbial Ecology* (Akkermans, A. D. L. and Van Elsas, J. D., eds.) Kluwer, The Netherlands, Section 1.1.2, pp. 1–11.

4

Dielectrophoresis

D. W. Pimbley, P. D. Patel, and C. J. Robertson

1. Introduction

1.1. Rapid Microbiologic Analyses

The increasing trend toward quality assurance programs and hazard analysis, consumer demand for a wide variety of wholesome foods, and legislative pressures (e.g., the U.K. Food Safety Act) have increased the need for more rapid microbiological analyses. Although hazard analysis and critical control point (HAACP) programs have reduced the emphasis on end product testing, microbiological analyses have a particular role in environmental monitoring, validation, verification, and to ensure compliance to legislative specifications (e.g., EC (European Community) microbiological criteria ***(1)***. Although extremely sensitive and not particularly capital intensive (except where automation is required), the classical, cultural-based microbiological techniques are time consuming, labor-intensive, and give results that are only of retrospective value. For example, a typical pathogen test (e.g., *Salmonella*) can include pre-enrichment, selective enrichment, selective plating, and confirmation can take up to 7 d to complete.

Significant progress has been made in "rapid" techniques for microbiological enumeration and detection of pathogens that reduces the analysis time quite significantly. For the estimation of total viable flora, there are techniques such as the direct epifluorescent filter technique (DEFT) ***(2)***, adenosine triphosphate (ATP) bioluminescence ***(3)***, impedance ***(4)*** and, more recently, fluorocytometry ***(5)*** and biosensors ***(6)***. For the detection of food-poisoning microorganisms, techniques based on enzyme-linked immunosorbent assay (ELISA), DNA probes and polymerase chain reaction (PCR), latex agglutination, electrical techniques (e.g., Bactometer, Malthus, and RABIT), and metabolic "marker"-based techniques are available ***(7)***. These alternatives to the

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classical cultural methods for the detection of microorganisms in foods are more rapid, but are generally lacking in sensitivity, and most techniques for pathogens still require a period of cultural enrichment of up to 48 h. They are also prone to interference from the components of the sample and sample turbidity or, in the case of pathogen analysis, prone to crossreactions from nontarget microorganisms, particularly closely phylogenetically related species. Problems such as quenching in ATP bioluminescence and nonspecific staining in the DEFT can result in over- or underestimation of microbial numbers; nonspecific reactions in ELISAs and inhibition of PCR may result in false-positive or -negative results. The speed, reliability, and robustness of these modern techniques can be dramatically increased if simple and efficient methods are developed that allow rapid isolation of the target microorganism from complex food matrices. There is a need, therefore, for real-time techniques that can reduce or eliminate the cultural stages.

A range of separation techniques has been reported that exploits to varying degrees, the cell surface characteristics (e.g., charge, antigenicity, and hydrophobicity) of microorganisms, including techniques based on immunomagnetic particles ***(8)***, ion exchange resins ***(9)***, differential centrifugation ***(10)***, aqueous biphasic systems ***(11)***, and dielectrophoresis ***(12)***.

Numerous potential applications of dielectrophoresis have been described, including the separation and manipulation of nonbiological and biological particles. Biological applications include the separation of viable and nonviable yeast cells ***(13)***, cancer cells ***(14)***, and erythrocytes ***(15)***. This chapter focuses on the application of dielectrophoresis to the isolation and concentration of microorganisms from food, beverage, and environmental samples.

1.2. Principles of Dielectrophoresis

Dielectrophoresis has been defined as the motion of a neutral or charged particle (e.g., microbial cell) that has undergone polarization as a result of being placed in a nonuniform electrical field ***(15)***. The nonuniformity of the electric field results in a nonuniform force distribution on the now polarized particle (known as a dipole), causing the particle to move toward the region of highest field intensity (**Fig. 1**).

The theory has been well developed by Pohl ***(12)***, who has shown that the dielectrophoretic force F_d can be given as:

$$F_d = (\text{particle volume}) \cdot (\text{polarizability}) \cdot (\text{local field}) \cdot (\text{field gradient}) \quad (1)$$

or

$$F_d = \alpha V \cdot \Delta\varepsilon \cdot |E_{\text{local}}| \cdot |E_{\text{local}} \quad (2)$$

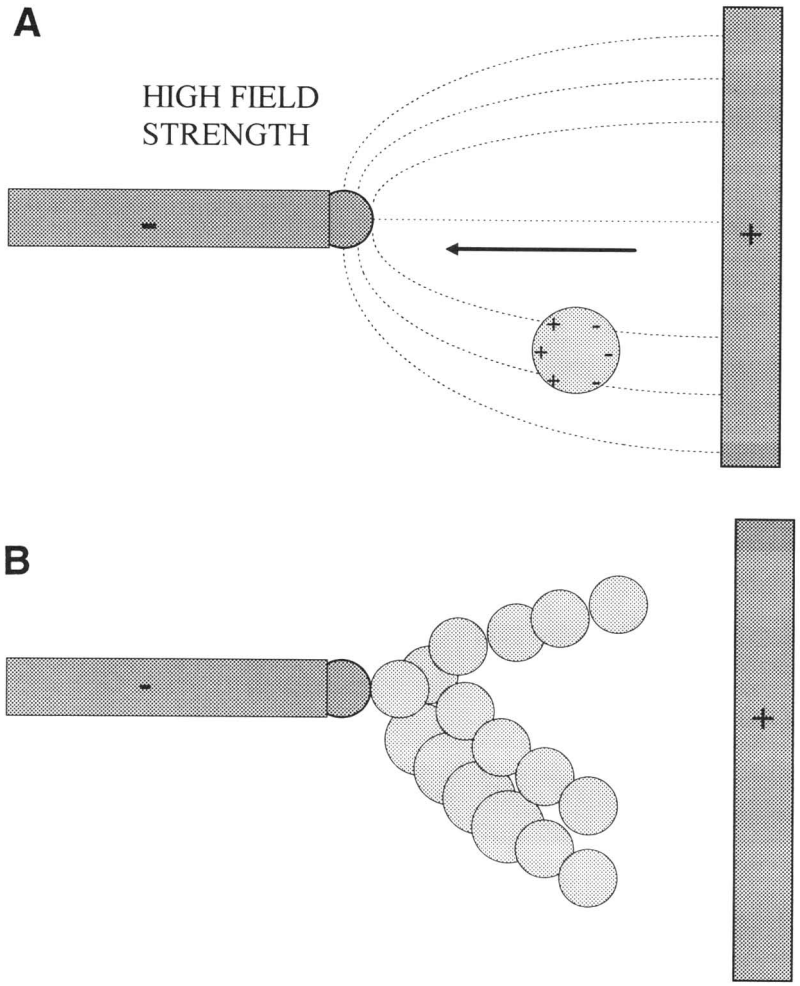

Fig. 1. Principles of dielectrophoresis (adapted from Pohl, 1978). The application of a nonuniform electric field to a suspension of microbial cells induces polarization of the cells, which then move toward the region of highest field strength. This movement is determined by the dielectric properties (conductivity and permittivity) of the cells and the suspending medium, and not simply their charge.

For a spherical electrode geometry:

$$F_d = \varepsilon \cdot V)/r^5 \tag{3}$$

and for a cylindrical geometry:

$$F_d = \infty(\varepsilon \cdot V)/r^3 \tag{4}$$

where ε is the difference in permittivity between the particle and medium, *V* is the volume of the particle, and *r* is the distance of the particle from the high-field (sharp) electrode.

It can be inferred from these equations that the dielectrophoretic force: increases with particle size, is stronger with cylindrical rather than spherical particles, and decreases rapidly with distance.

Unlike electrophoresis, in which movement is largely determined by the overall charge on the particle, dielectrophoretic movement is a function of the dielectric properties (conductivity and permittivity) of the particle and the suspending medium. The dielectric properties of a material are characterized by the specific electrical conductivity (or electrical resistivity) and the permittivity. The conductivity is determined by the density and efficacy of charge transport mechanisms within the material and can be determined by measuring the current that flows through a sample of the material at a given voltage. The permittivity of a material gives a measure of the ability of the positive and negative charges within a material to separate (i.e., polarize) under the effect of an applied electric field. The permittivity may be found by measuring the electrical capacitance of an electrical chamber that is filled with a sample of the material. The dielectric properties of fluids are strongly temperature dependent, so conductivity and permittivity of test materials must be measured as a function of temperature.

If the permittivity of the particle is greater than that of the surrounding fluid, then the body will move toward the regions of highest electric field intensity (known as positive dielectrophoresis). Conversely, if the permittivity of the particle is less than that of the surrounding fluid, then the particle will be forced toward the regions of lowest electric field intensity (known as negative dielectrophoresis). Thus, both positive and negative dielectrophoresis can be used to separate microbial cells from food suspensions and other matrices, by manipulating the conductivity of the suspending medium and the frequency of the applied electric field.

Although the movement of a particle in a nonuniform electric field is largely determined by the frequency of the electric field and conductivity of the suspending medium, the dielectric characteristics of the particle also influence its dielectrophoretic behavior. For a particle to migrate to the region of high-field intensity, it must exhibit a higher specific polarizability than the suspending medium. In microbial cells, various cell surface components (e.g., proteins, polysaccharides, and teichoic acids) and intracellular components (e.g., proteins, sugar, RNA, and DNA) contribute to the overall polarization. Since the contribution of these components will vary with cell physiology, age, and species, specific cell types give a characteristic collection spectrum over a range of frequencies ***(15)***.

1.3. Design of Dielectrophoresis Chambers

2.1.1. Two-Dimensional (2D) and Three-Dimensional (3D) Dielectrophoretic Chambers

The simplest form of dielectrophoretic chamber consists of a pair of electrodes suspended in the test suspension. Early chambers were constructed with wire or pin electrodes ***(15)***, but advances in microfabrication and photolithography have made possible the construction of multielement, paired electrode arrays, usually gold deposited onto a glass or ceramic substrate. The electrodes are connected to a high-frequency power supply to generate the nonuniform electric field. **Plate 1** shows the dielectrophoretic collection of *Micrococcus luteus* from a small volume (100 μL) of an aqueous suspension using an interdigitated electrode array. The microbial cells typically collect at the electrodes in long chains known as "pearl chains" (**Plate 2**).

To isolate microorganisms from larger volumes of food homogenates and to maximize the dielectrophoretic collection it is necessary to construct more complex flow-through chambers. The flow-through dielectrophoresis system used in our laboratory is shown in **Fig. 2**. It comprises a 3D dielectrophoretic chamber connected to a peristaltic pump (which circulates test sample through the chamber) and a high-frequency power source. In a flow-through dielectrophoretic chamber, there are three factors that affect the dielectrophoretic collection of microbial cells: the dielectrophoretic force pulling the cells toward the high-field region of the chamber, the fluid flow rate acting at right angles to the dielectrophoretic force, and the ionic composition of the medium. Thus, the dielectrophoretic field must be sufficiently strong to attract and hold microbial cells within the chamber. In theory, the dielectrophoretic field decreases as the inverse fifth power of the distance from the electrode; more effective separation of microorganisms is achieved only with closely spaced electrode arrays. In our laboratory, we have used chambers containing electrodes of a few microns in width and spacing to achieve the high-field strengths. With a nonoptimized version of a flow-through dielectrophoretic chamber, up to 90% of *Escherichia coli* in low-conductivity (<20 μS/cm) aqueous suspension can be collected in the chamber and eluted into a small volume of buffer (Pimbley, D. W. and Patel, P. D., unpublished data). Other factors that need to be considered in the design of dielectrophoretic chambers include the avoidance of heating, minimization of dead flow and turbulence, and elimination of electrolytic effects. Finite element analysis can be used to model the field and field divergence within preprototype dielectrophoretic chambers and to identify the optimum electrode configuration.

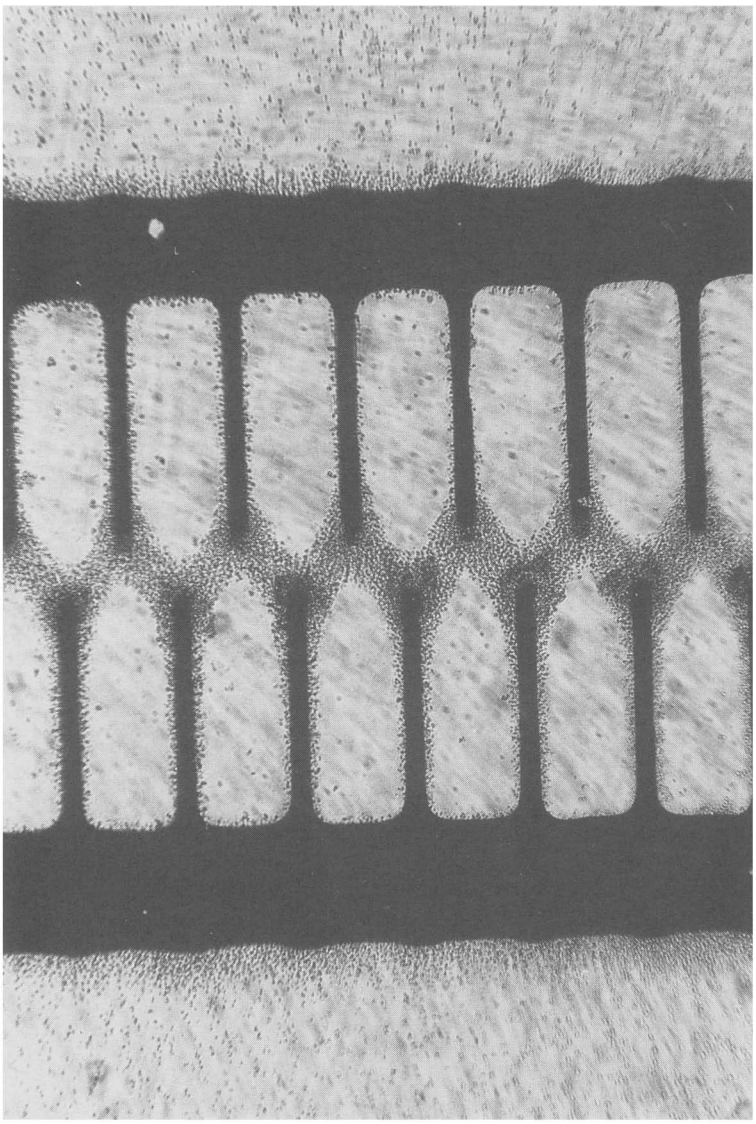

Plate 1. Dielectrophoretic collection of *Micrococcus luteus* using an interdigitated electrode array at 100 KHz, 9 V (×500 magnification).

Higher applied voltages allow a faster flow rate to be used and, hence, enhance dielectrophoretic isolation of microorganisms from aqueous suspension. More efficient separation can also be achieved by reducing "dead space" between electrodes. The "penalty" of higher voltages is that they can lead to

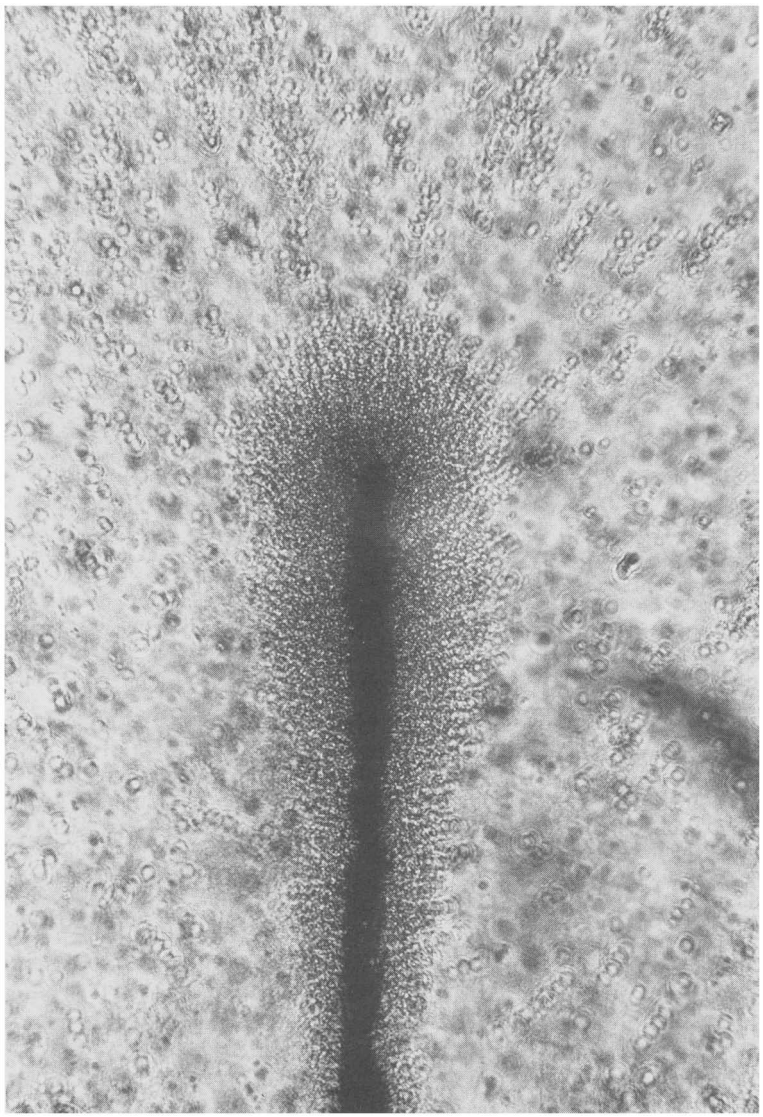

Plate 2. Dielectrophoretic collection of *Micrococcus luteus* using an interdigitated electrode array at 100 KHz, 9 V showing "pearl chain" formations (×1000 magnification).

excessive heat production in the chamber. Other factors, such as the chamber dimensions, construction materials, and the high conductivity of the test suspension, can also influence heat production.

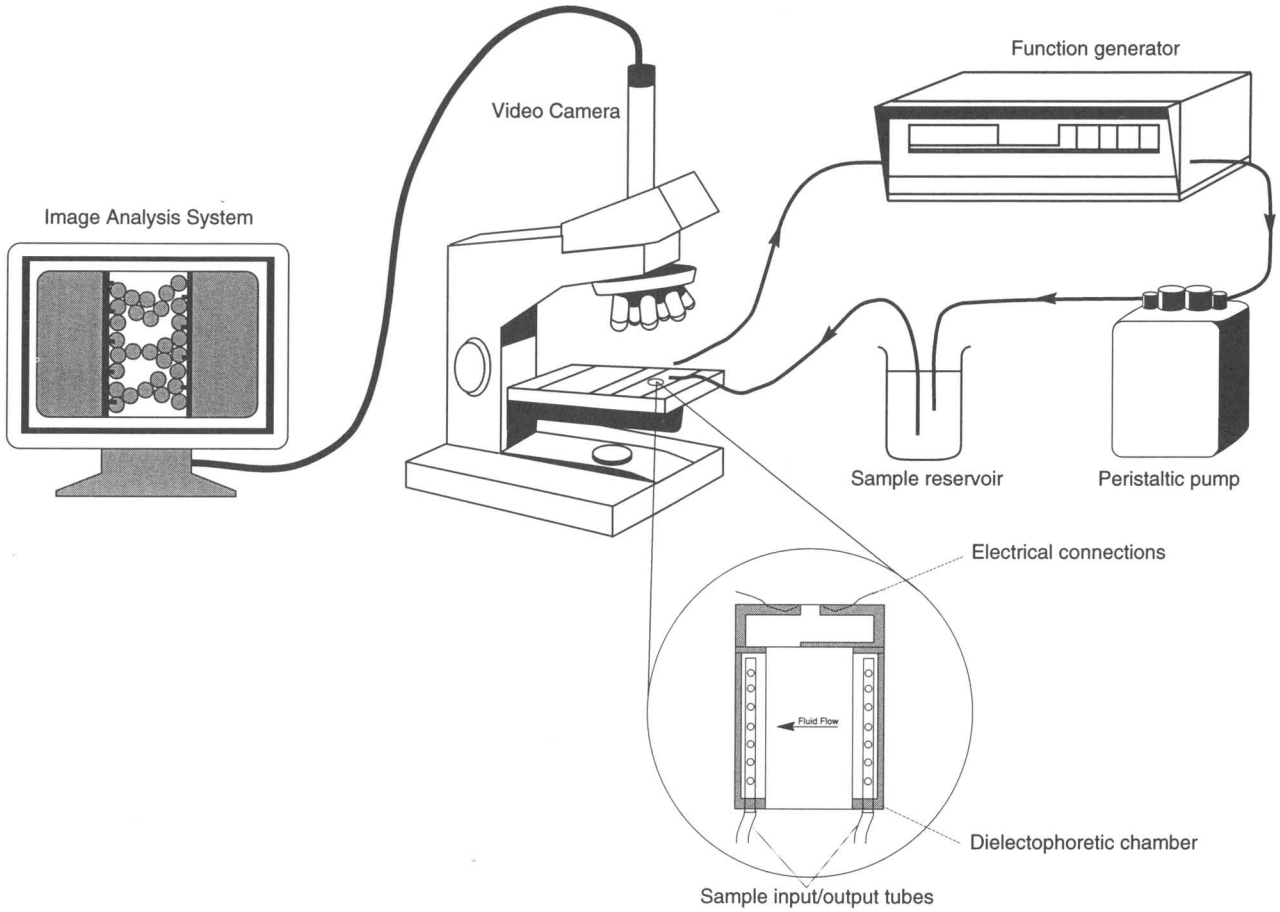

Fig. 2. System for the dielectrophoretic collection of microorganisms.

Dielectrophoretic collection of microorganisms can be measured in various ways. One of the simplest is to measure the length of the "pearl chains" of bacteria using a microscope and micrometer ***(15)***. Another approach is to use a spectrophotometer to measure optical density, either within the chamber or in the outlet tube ***(16)***, but this requires high concentrations of microbial cells (>10^8 *cfu*/mL). More sophisticated image analysis systems have been used to measure much lower concentrations of cells ***(17)***.

1.3.2. Traveling-Wave Dielectrophoretic Electrode Arrays

In a conventional dielectrophoretic chamber, the sample suspension containing microorganisms is passed over the electrode array, and the microorganisms are collected and then eluted. In a traveling-wave dielectrophoretic device, microorganisms are propelled through a stationary, supporting fluid by applying different phases of a high-frequency electrical signal in sequence to alternate pairs of electrodes. The velocity of the particles depends on the dielectric force exerted by the electrodes, which in turn depends on the field distribution and the dielectric properties of the particles and the fluid. Dedicated interface electronics and software have been developed to drive these electrode arrays. Traveling-wave dielectrophoretic devices have been used to separate microparticles ***(18)***, and it has been suggested that they could form the basis of microconveyor belt systems ***(19)***. However, the potential of these devices for food analysis has not yet been evaluated.

1.4. Factors Affecting Dielectrophoresis of Microorganisms

The efficiency of dielectrophoretic collection of microorganisms is influenced by various factors, including signal frequency, voltage, sample flow rate, sample conductivity (permittivity), temperature, and the dielectric characteristics of the microbial cell. For example, differences in the cell wall structures of Gram-negative (protein and lipopolysaccharide) and Gram-positive (teichoic acid and peptidoglycan) have been exploited to separate these groups of bacteria ***(20)*** and viable and nonviable cells of *Saccharomyces cerevisiae* have been separated using combined dielectrophoresis and electrorotation ***(21)***.

The frequency over which microorganisms can be collected by dielectrophoresis ranges from approx 1 kHz to 20 MHz. Over this range microorganisms exhibit typical frequency-dependent collection profiles (**Fig. 3**), which can be used, to some extent, to resolve mixtures of pure cultures of different cells types, species, or groups. In practice, for most bacteria the profiles generally overlap, so complete resolution of two populations is rarely achieved. The maximum collection rate normally occurs at a frequency around 200 kHz, but this can vary with the type of organism and cultural state. Dielectrophoretic collection increases with increasing voltage, but at voltages above 20 V, heat-

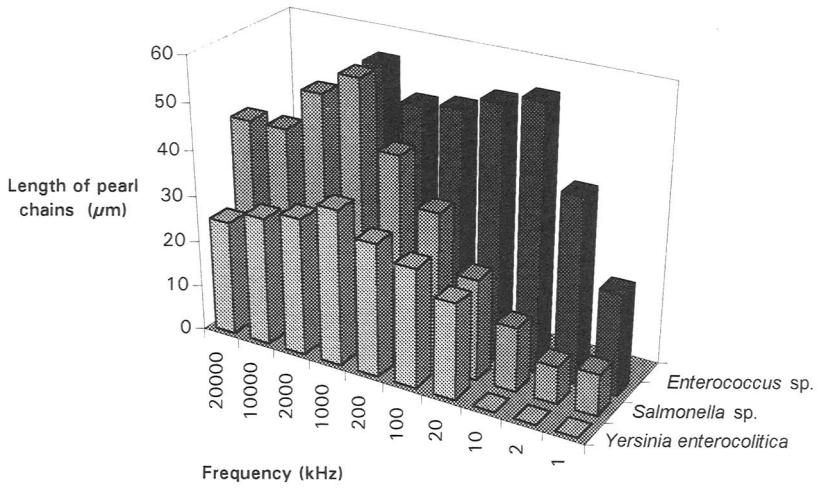

Fig. 3. Frequency-dependent dielectrophoretic collection profiles for various bacteria.

ing effects within the chamber can reduce the collection efficiency by causing thermal currents, and the heating also reduces the viability of collected microorganisms, unless the chamber is maintained at low ambient temperature using a cooling bath or Peltier device.

1.5. Techniques for Reducing the Permittivity of Microbial Suspensions

As previously indicated, the dielectrophoretic force is strongly influenced by the ionic strength (conductivity) of the medium. Highly polar substances (indicated by a high permittivity), such as salts, are attracted to the regions of high-field intensity and, if they are present at high concentrations, prevent the bacteria from collecting at the electrode. Since microbiological analysis involves the suspension and growth of target microorganism in high-conductivity broth media, some form of pretreatment (e.g., dilution in deionized water) is required to reduce the dielectrophoretic interference from dissolved salts. In tests with a typical nonselective broth culture medium and two selective media, it was found that a 1:1000 dilution of the broths was required (equivalent to a conductivity of <100 μS and a relative permittivity of <100) before any significant dielectrophoretic collection of the bacteria could be observed (**Fig. 4**).

For positive dielectrophoretic isolation and concentration of microorganisms to occur, the relative permittivity of the sample suspension must be lower than that of the microbial cells (100–1000). This can be achieved in various ways, including dilution in deionized water, desalting, deionization, and, possibly,

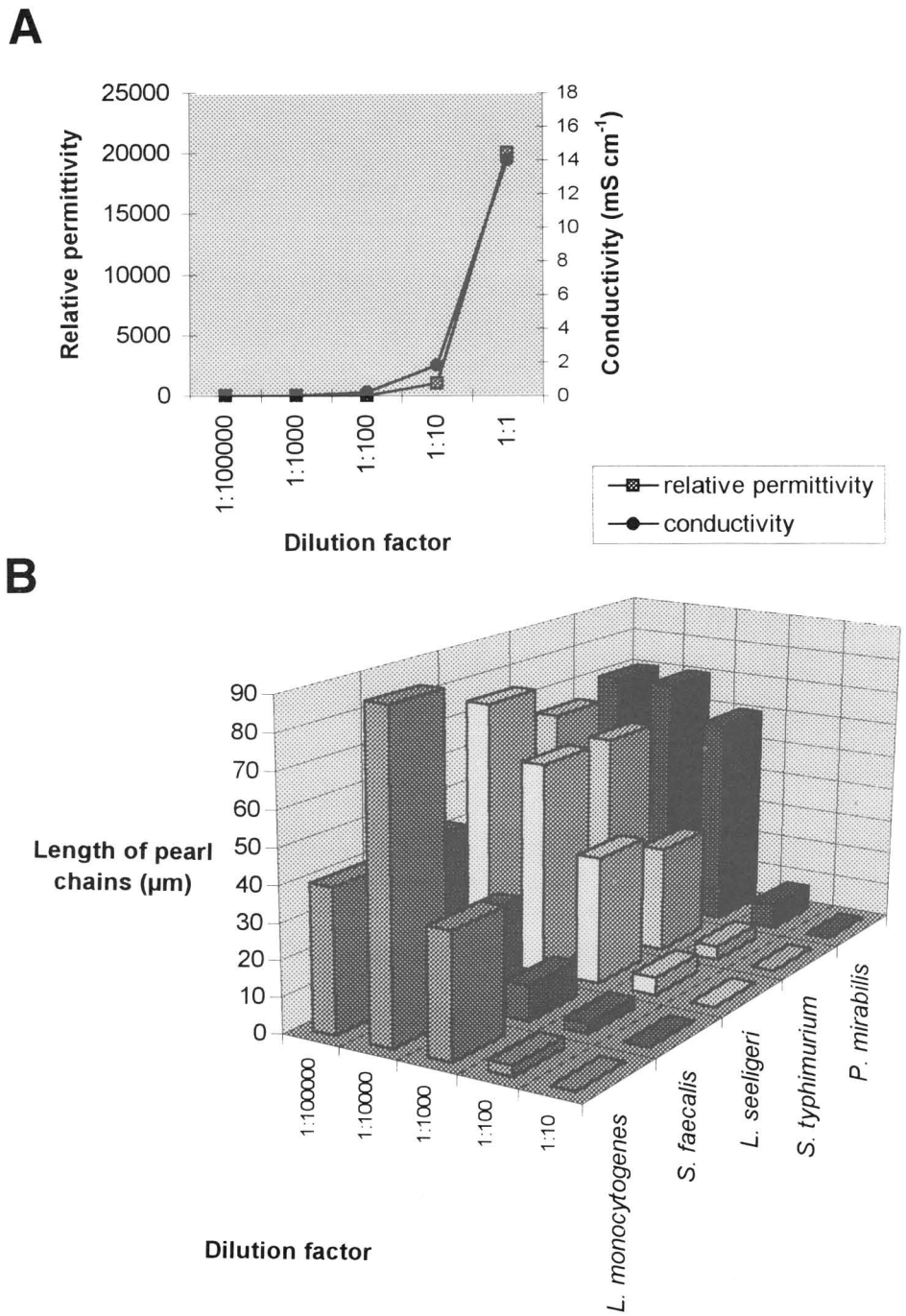

Fig. 4. Effect of dilution of buffered peptone water (BPW) in deionized water on **(A)** dielectric properties, and **(B)** dielectrophoretic collection of various bacteria at 100 kHz, 10 V.

the use of frequencies above 1 MHz. However, since dilution also reduces the level of the target microorganisms and would therefore reduce the sensitivity of a dielectrophoresis-based detection system, an alternative approach is preferred.

We have investigated a range of desalting techniques, including dialysis cassettes (Slide-A-Lyzer, Pierce & Warriner), dialysis chambers (Spin Biodialyser, Sialomed Inc.) and minicolumns of desalting gels (PD10, Pharmacia). Dialysis cassettes and chambers are simple to use but relatively slow, desalting 5–15 mL volumes of food homogenates in 3–5 h with good recovery of the total microbial flora (>95%). Columns of desalting gels are much faster (5–10 min), but the proportion of total microbial flora eluted in fractions with low conductivity is relatively low (<20%). To address these problems, a novel, rapid desalting technique based on dialysis chambers was developed, capable of reducing the conductivity of up to 5-mL volumes of a food suspension starting from >2000 μS/cm to <200 μS/cm in 15 min, without significant loss of microorganisms (Pimbley, D. W. and Patel, P. D., unpublished data). This process also clarifies the food suspension. The resulting microbial suspension has been shown to exhibit high recoveries when subjected to dielectrophoretic processes, as illustrated in the following section.

1.6. Application of Dielectrophoresis

Microorganisms can be broadly categorized as useful (e.g., starter culture bacteria), pathogenic (e.g., *Salmonella*, *Listeria*), spoilage, and indicator (e.g., coliforms). Dielectrophoresis has potential applications for the separation of all four groups from environmental and food matrices. The advantages of dielectrophoresis over other separation techniques are that it is rapid (<15 min), and that it can be used to separate microorganisms from relative complex matrices to give a clear suspension suitable for analysis by a modern detection system. As a generic system, dielectrophoresis can be used to separate the total microbial flora (including pathogens and spoilage microorganisms) from the sample prior to analysis using one of the modern detection systems (e.g., ATP bioluminescence). Alternatively, there appears to be potential for the specific dielectrophoretic separation of target microorganisms such as pathogens, using antibody-coupled molecules to modify the dielectrophoretic behavior of the microorganism (Pimbley, D. W. and Patel, P. D., unpublished data).

Differences in the cell surface characteristics of subpopulations of microorganisms caused by injury (e.g., by heating, freezing, or chemical damage) can be exploited in the separation of microorganisms using dielectrophoresis. There is also evidence that microorganisms can be characterized according to their dielectrophoretic collection profiles at different frequencies and conductivities ***(22)***.

2. Materials

2.1. Separation of Total Microbial Flora from Foods

1. Dielectrophoretic electrode chamber (*see* **Note 1**).
2. Function generator with a frequency range of 0-20 MHz and a voltage range of 1–20 V (e.g., Thander TG2001, Thander Electronics Ltd., Huntingdon, UK) (*see* **Note 2**).
3. Image analysis system, comprising a light microscope (e.g., Olympus BH-2 Olympus GmbH, Germany), a video camera (e.g., Hitachi KP-C500, Hitachi-Denshi, Tokyo, Japan), video text overlay (e.g., Linkham VTO 232, Linkam, Tadworth, UK), and a visual display unit (e.g., Hitachi VM-920K).
4. Conductivity meter (e.g., Horiba C172, Horiba instruments, Kyoto, Japan).
5. Peristaltic pump capable of handling up to 1 mL · min^{-1} (e.g., Gilson Minipuls, Gilson Inc., Middleton, USA).
6. Magnetic stirrer.
7. Sample homogenizer (e.g., Stomacher 400, Seward Ltd., London, UK).
8. Sterile diluent (e.g., Maximum recovery diluent, Oxoid Ltd., Basingstoke, UK).
9. System for estimating numbers of microorganisms (*see* **Note 3**).
10. Coarse, glass-fiber prefilter (e.g., Glassfibe prefilter, Whatman International Ltd., Maidstone, UK).
11. Dialysis cassettes (Slide-A-Lyzer 10K, Pierce and Warriner Ltd., Chester, UK) or dialysis chamber (Spin Biodialyzer, Sialomed Inc., Columbia, SC) with 0.6-µm polycarbonate membranes.

2.2. Dielectrophoresis in Environmental Monitoring

The protozoan parasite *Cryptosporidium parvum* is recognized as a significant water-borne human pathogen, that has a low infective dose and is resistant to commonly used disinfection methods such as chlorination. Current detection methods are time-consuming and unreliable because the organism is often present at low levels of contamination, necessitating the filtration of large volumes of water. A rapid, automated dielectrophoresis system for the differentiation of ozone-treated, autoclaved, and untreated oocysts of *C. parvum* from water has been described ***(23)***.

1. Dielectrophoretic chamber (*see* **Note 1**).
2. Microcomputer to set pulse voltage and frequency applied to the electrodes and to control the pump and timings (e.g., Epson PC AX2, Seiko-Epson Corp., Suwasi, Japan).
3. Pulse/function generator (e.g., Hewlett Packard 8116A, Hewlett Packard, Englewood, CO).
4. Peristaltic pump (e.g., Gilson Minipuls 3).
5. Microscope with facility for transmitted and reflected light (e.g., Nikon Labphot-2, Nikon Corp., Tokyo, Japan).
6. Solid-state color camera (e.g., Hitachi KP-C500).
7. S-VHS video cassette recorder (e.g., NEC DS 6000K, Yokyo, Japan).

8. 0.5-m*M* sodium dodecyl sulphate (SDS) (Sigma-Aldrich Co. Ltd., Poole, UK).
9. Stock suspension containing approx 10^8 *C. parvum* oocysts mL^{-1}.

3. Methods

3.1. Separation of Total Microbial Flora from Foods

A generic protocol has been developed for the separation of total microbial flora from food as outlined in **Fig. 5** and detailed next.

1. Homogenize sample 1:4 in sterile diluent using a stomacher or similar homogenizer.
2. Using a sterile syringe, pass 10 mL of homogenate through a sterile, coarse (50-µm) glass-fiber filter (e.g., Whatman GF) to remove particulate matter.
3. Transfer aliquot of the filtered sample to a desalting device (e.g., 5-mL Biodialyser dialysis chamber or 5-mL dialysis cassette).
4. Dialyze against 1 L of deionized water with stirring (both devices have magnets for use with magnetic stirrers) at 20°C until conductivity falls below 100 µS/cm (2 to 3 h).
5. Transfer a 5-mL aliquot of desalted homogenate to the dielectrophoresis system reservoir and recirculate through the chamber for 15 min at a flow rate of 0.5 mL/min with a signal of 200 kHz, 20 V applied to the electrodes from a function generator (*see* **Note 2**).
6. Elute the collected bacteria by turning off the electrical signal and flushing the chamber with 0.5 mL of sterile diluent.
7. Analyze the resulting clarified suspension of microorganisms (*see* **Note 3**).

Using a microfabricated 3D flow-through dielectrophoretic chamber and the previous protocol, we have demonstrated the rapid separation of total microbial flora from suspensions of various foods. A patented, rapid desalting technique ***(24)*** was used to rapidly reduce the conductivity of the samples from >2000 µS/cm to between 41 and 59 µS/cm within 15 min. **Table 1** shows that between 88 and 94% of the total microbial flora of desalted homogenates of chicken, minced beef, and skimmed milk powder (SMP) was collected in the chamber. The desalting and dielectrophoresis also removed particulate matter, leaving a clear suspension of microorganisms.

The dielectrophoretic separation of spoilage microorganisms *Kluyveromyces lactis* and *Pseudomonas aeruginosa* from untreated, high-conductivity lager beer (750 µS/cm) and mineral water (560 µS/cm) has also been demonstrated in static 2D dielectrophoretic chambers (Pimbley, D. W. and Patel, P. D., unpublished data) using higher signal frequencies (2 MHz). Similarly, spores of *Geotrichum candidum, Mucor plumbeus,* and *Penicillium* spp. have been separated from pasteurized whole milk, diluted 1 in 10 in distilled water, at a frequency of 20 MHz, with no dielectrophoretic effect on the milk casein micelles.

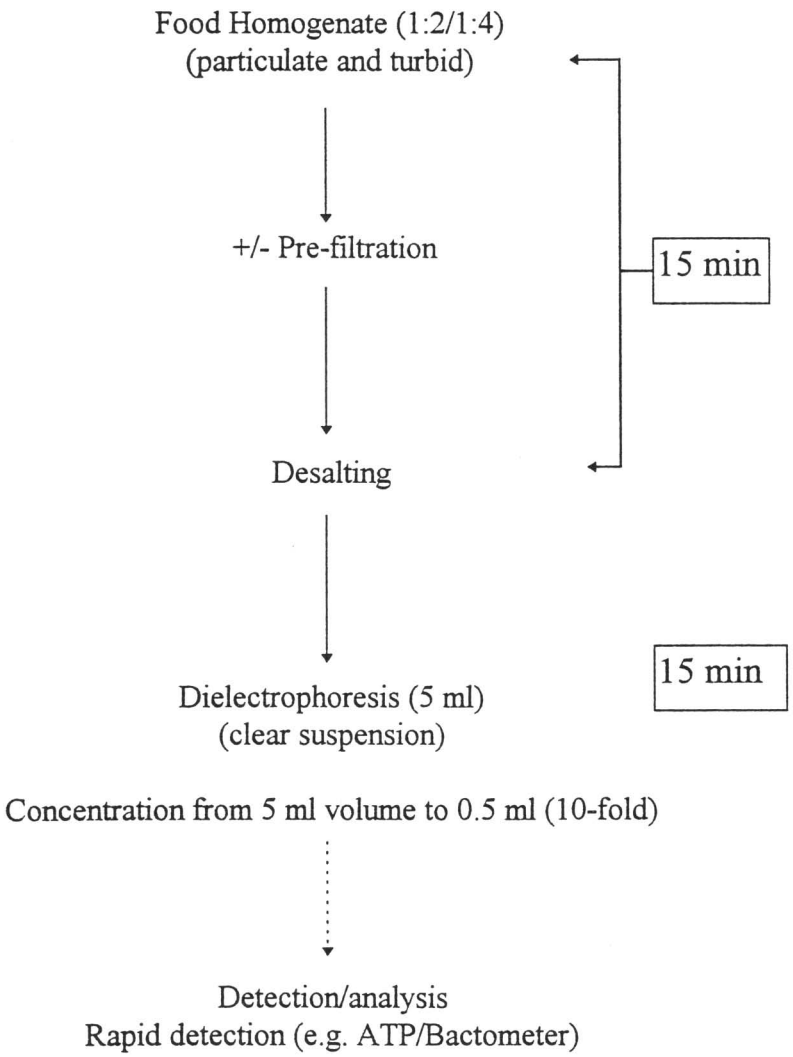

Fig. 5. Outline protocol for the dielectrophoretic separation of total viable microbial flora from food homogenates at 200 kHz and 20 V.

3.2. Preparation of Untreated C. parvum *Oocysts*

1. Suspend 1 mL of a stock suspension of oocysts in 20 mL of 0.5 m*M* SDS solution.
2. Centrifuge for 10 min at 12,000*g*.
3. Draw off supernatant and resuspend pellet in 4 mL of 0.5 m*M* SDS solution.

Table 1
Dielectrophoretic Separation of Total Microbial Flora from Chicken, Minced Beef and Skimmed Milk Powder (SMP) Homogenates After Rapid Desalting

Parameters	Chicken	Minced beef	SMP
Starting conductivity (μS/cm)	>2,000	>2,000	>2,000
Final conductivity (μS/cm)	41	54	59
Total viable count (cfu/mL)[a]	1.3×10^4	1.6×10^6	4.8×10^7
Before dielectrophoresis			
Total viable count (cfu/mL)	1.5×10^3	1.9×10^5	3.1×10^6
After dielectrophoresis			
% depletion	88	88	94

[a]cfu = colony forming units.

3.2.1. Preparation of Autoclaved C. parvum Oocysts

1. Autoclave a small volume of stock suspension of oocysts (10^8/mL) at 121°C for 10 min.
2. Proceed as for untreated oocysts (**Subheading 3.2.**).

3.2.2. Preparation of Ozone-Treated C. parvum Oocysts

1. Treat a 10-mL sample of stock oocyst suspension with 3.3 mg/L of ozone.
2. Centrifuge for 10 min at 10,000*g*.
3. Draw off supernatant and resuspend pellet in 4 mL of 0.02 m*M* SDS solution.

3.3. Dielectrophoretic Differentiation of Untreated and Treated C. parvum Oocysts (see Note 4)

1. Transfer test suspension of oocysts to the dielectrophoresis system sample reservoir.
2. Pump sample through dielectrophoretic chamber for 10 s at a flow rate of 1.5 mL/min.
3. Reduce flow rate to 0.05 mL/min and apply signal at frequency of 1 kHz.
4. Remove signal and continue pumping for 5 s.
5. Increase flow rate to 1.5 mL/min for 5 s.
6. Increment frequency.
7. Repeat steps **1–6** at signal frequency between 1 kHz and 50 MHz to generate collection spectra (*see* **Note 5**).
8. On completion of experiments, replay video recording and using digital freeze-frame facility to count oocysts collected on and between electrodes.

3.4. Future Prospects

In 1978 Pohl ***(15)*** predicted a bright future for biological dielectrophoresis. Certainly great strides have been made in the technology; in particular, the advent of microfabrication, microelectronics, and optical imaging systems have led to significant improvements in the efficiency of dielectrophoretic chambers and detection of the separated particles. Despite these advances, the full potential of dielectrophoresis as a technique for separating microorganisms from food and environmental samples has yet to be realized commercially for a variety of reasons, mostly dealing with sample preparation. As indicated earlier, the main parameters controlling the dielectrophoretic collection of microorganisms are frequency, conductivity and permittivity. Most microbiological techniques involve suspension of the test sample in a high-conductivity solution (e.g., diluent or microbial culture medium), which can significantly reduce positive dielectrophoretic collection at frequencies below the 1–10 MHz range. In addition, the highly particulate nature of some samples can interfere with collection by blocking the dielectrophoretic chamber. A novel and simple desalting procedure has been developed to overcome these problems ***(24)***. Another possible solution to the problem of high conductivity is the use of negative dielectrophoresis, a phenomenon that has been observed in high-conductivity samples, but has not yet been fully exploited.

Despite the apparent lack of commercial interest in the application of dielectrophoresis in environmental microbiology, rapid progress is being made in biomedical and biotechnological applications, such as the dielectrophoretic manipulation of submicron particles (e.g., viruses). It is likely that advances in dielectrophoretic chamber design, coupled with improved techniques for modifying the dielectrophoretic response of microorganisms, will lead to the development of efficient dielectrophoresis-based systems for the real-time separation and detection of microorganisms from food and environmental samples.

4. Notes

1. Dielectrophoresis chambers are not available commercially. The chambers used at Leatherhead Food RA were designed and fabricated by ERA Technology Ltd. The University of York (Dr. W. Betts) and University of Wales, Bangor (Dr. R. Pethig) also have expertise in the construction of dielectrophoretic systems.
2. The output of the Thandar TG2001 function generator is adequate for small volume (<0.5 mL) dielectrophoretic chambers. Larger dielectrophoretic chambers with more extensive electrode arrays may require a function generator with a higher power output.
3. The majority of bacterial cells are released from the electrodes as soon as the electrical signal is removed. However, the addition of 0.1% w/v Tween-80 to the diluent may improve the recovery of microorganisms.

4. For examples on how microorganisms can be estimated using plate counting, ATP bioluminescence, and electrical impedance, *see* **refs. *3***, ***4***, and ***25***, respectively.
5. **Steps 2–6** are controlled automatically using the microcomputer.
6. The dielectrophoretic response of *C. parvum* oocysts treated with ozone has been shown to be dose dependent, and consistent with a decrease in internal conductivity predicted by a mathematical model for two-shell spherical particles ***(26)***.

Acknowledgments

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References

1. Anonymous (1989) Council directive on hygiene and health problems affecting the production and the placing of egg products. OJ No. 32 (L212), 7/22, European Commission, Luxemborg, pp. 87–100.
2. Pettifer, G. L., Kroll, R. G. Farr, L. J., and Betts, R. P. (1989) DEFT: recent developments for foods and beverages, in *Rapid Microbiological Methods for Foods, Beverages and Pharmaceuticals* (Stannard, C. J., Pettit, S. B., and Skinner, F. A., eds), Society for Applied Bacteriology Technical Series No. 25. Blackwell Scientific, Oxford, UK, pp. 33–46.
3. Kyriakides, A. L. and Patel, P. D. (1994) Luminescence techniques for microbiological analysis of foods, in *Rapid Analysis Techniques in Food Microbiology* (Patel, P. D., ed.), Chapman and Hall, London, UK, pp. 196–231.
4. Arnott, M. L. (1993) Impedance microbiology in food quality control, in *Instrumentation and Sensors for the Food Industry* (Kress-Rogers, E., ed.), Butterworth-Heinemann, Oxford, pp. 499–520.
5. Brailsford, M. A. and Gatley, S. (1993) Industrial applications of flow cytometry for the rapid detection of microorganisms, in *New Techniques in Food and Beverage Microbiology*, Society for Applied Bacteriology Technical Series No. 31 (Kroll, R. G., Gilmour, A., and Sussman, M., eds.), Blackwell Scientific, pp. 87–100.
6. Wagner, G. and Guilault, G. G., eds. (1994) *Food Biosensor Analysis*. Marcel Dekker, New York.
7. Patel, P. D., ed. (1994) *Rapid Analysis Techniques in Microbiology*. Chapman and Hall, London, UK.
8. Patel, P. D. and Blackburn, C. de W. (1991) Detection of food poisoning agents using immunomagnetic particles, in *Magnetic Separation Techniques Applied to Cellular and Molecular Biology* (Kemshead, J. T., ed.), Cromwell, Broughton, Gilford, UK, pp. 93–105.

9. Patel, P. D., Wood, J. M., and Gibbs, P. A. (1983) Physico-chemical interaction of microorganisms with the cation exchange resin Bio-Rex 70: a preliminary application of the technique to foods. *Leatherhead Food Res. Asso. Res. Rep.* No. 437, Letherhead, UK.
10. Rodrigues-Szule, U. M., Ventoura, G. Mackey, B. M., and Payne, M. J. (1996) Rapid physicochemical detachment, separation and concentration of bacteria from beef surfaces. *J. Appl. Bacteriol*. **80(6),** 673–681.
11. Betts, R. P. (1994) The separation and rapid detection of microorganisms, in *Rapid Methods and Automation in Microbiology and Immunology* (Spencer, R. C., Wright, E. P., and Newsom, S. W. B., eds.), Athenaeum, pp. 107–120.
12. Pohl, H. A. and Crane, J. S. (1971) Dielectrophoresis of cells. *Biophys. J*. **11,** 711–727.
13. Markx, G. H., Talary, M. S., and Pethig, R. (1994) Separation of viable and non-viable yeast using dielectrophoresis. *J. Biotechnol.* **32(1),** 29–37.
14. Becker, F. F., Wang, X.-B., Huang, Y., Vykoukal, J., Gascoyne, P. R. C., and Pethig, R. (1994) The removal of human leukaemia cells from blood using interdigitated microelectrodes. *J. Phys. D: Appl. Phys*. **27,** 2659–2662.
15. Pohl, H. A. (1978) *Dielectrophoresis.* Cambridge University Press, Cambridge, UK.
16. Price, J. A. R., Burt, J. P. H., and Pethig, R. (1987) An optical technique for measurement of cell dielectrophoresis. Electrostatics 1987. Proc. 7th Int. Conference on Electrostatic Phenomena (Sproston, J. L., ed.), IOP Publishing, Oxford, UK, pp. 75–79.
17. Gascoyne, P. R. C., Huang, Y., Pethig, R., Vykoukal, J., and Becker, F. F. (1992) Dielectrophoretic separation of mammalian cells studied by computerized image analysis. *Measurement Sci. Tech*. **3(5),** 439–445.
18. Hagedorn, R., Fuhr, G., Muller, T., and Gimsa, J. (1992) Travelling-wave dielectrophoresis of microparticles. *Electrophoresis* **13(1–2)**, 49–54.
19. Talery, M. S., Burt, J. P. H, Tame, J. A., and Pethig, R. (1996) Electromanipulation and separation of cells using travelling electric fields. *J. Phys. D: Appl. Phys*. **29,** 2198–2203.
20. Markx, G. H., Huang, Y., Zhou, X.-F, and Pethig, R. (1994) Dielectrophoretic characterisation and separation of microorganisms. *Microbiology* **140,** 585–591.
21. Huang, Y., Holzel, R., Pethig, R., and Wang, X. B. (1992) Differences in the ac electrodynamics of viable and non-viable yeast cells determined through combined dielectrophoresis and electrorotation studies. *Phys. Med. Biol*. **37(7),** 1499–1517.
22. Markx, G. H., Dyda, P. A., and Pethig, R. (1996) Dielectrophoretic separation of bacteria using a conductivity gradient. *J. Biotechnol.* **51(2),** 175–180.
23. Archer, G. P., Betts, W. B., and Haigh, T. (1993) Rapid differentiation of untreated, autoclaved and ozone-treated *Cryptosporidium parvum* oocysts using dielectrophoresis. *Microbios.* **73,** 165–172.
24. Patel, P. D. and Pimbley, D. W. (1995) Dielectrophoresis. PCT Patent Application No. 950346. 9. Filed by UK MAFF, London, UK.
25. *Bacteriological Analytical Manual.* (1995) Food and Drug Administration, 8th ed., AOAC International.
26. Quinn, C. M., Archer, G. P., Betts, W. B., and O'Neill, J. G. (1996) Dose-dependent dielectrophoretic response of *Cryptosporidium* oocysts treated with ozone. *Lett. Appl. Microbiol*. **22(3),** 224–228.

5

Flow Cytometry and Cell Sorting

Rapid Analysis and Separation of Individual Bacterial Cells from Natural Environments

Jonathan Porter

1. Introduction

Effective monitoring of bacteria in the environment is a technical and methodological challenge. Detection and analysis of whole cells or marker molecules from entities as small as individual bacteria is a difficult task. Often it is necessary to increase the number of cells present in a sample using a culture step, before attempting the analysis step. In ecological studies, such indirect methods are easily criticized because they select for bacteria suited to the culture conditions at the expense of the majority of bacteria present (although such methods are used routinely for bacteriological quality control). However, many developments in methodologies have direct monitoring as their goal, i.e., analysis of bacteria from a given sample without a culture step. A further problem of the study of naturally occurring bacteria is the numbers of cells involved. Bacterial populations consist of immense numbers, and finding specific cells against a background that may consist of billions of nontarget cells is time-consuming and difficult.

One technique that offers a solution to these problems is that of flow cytometry (FCM) and cell sorting. FCM cannot solve all the methodological problems of environmental bacteriology, and its application is limited in certain situations. However, the claims of the manufacturers and of the few microbiological devotees are absolutely true. FCM can analyze thousands of bacterial cells, one at a time, every second. It can generate data on millions of individual cells, and even standard instruments are sensitive enough to have little difficulty in detecting bacterial spores. The information gained from each

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cell is invaluable, especially when the huge and ever-expanding range of fluorescent marker dyes is brought into play. Finally, the option of cell sorting allows physical separation of specific cells of interest, onto solid culture media, or into defined buffers, enriched and purified to allow successful application of most culture or molecular biological techniques. FCM is not a stand-alone method, but should be thought of as an analysis technique that leads to biologically relevant data and sample processing.

1.1. Principles of FCM and Cell Sorting

FCM instrument specifications may vary tremendously but all follow the same basic principle. A flow cytometer in its simplest form is an automated microscope. The cell sorting option, available on some machines, takes the process further by allowing physical separation of cells of interest. Traditional microscopy involves examination of a static, slide-mounted specimen. If a fluid stream were set to flow over the surface of the slide along a defined path, and individual cells were fed into this fluid stream, sequential examination of many different cells would be possible. Provided that each cell arrived singly and separately, and remained in the field of view for a sufficient length of time, one would be able to obtain all the desired information about it. The next step is to measure light entering the cells, and light scattered by the cells, by appropriate light detectors, and to feed their signals into a computer with appropriate software. Additional use of fluorescent labels for the cells expands the range of measurements. Because fluorescence emission is generally of a longer wavelength than the excitation (source) light, both can be measured simultaneously. This is the basic principle of flow cytometric analysis.

The entire setup is achieved through the use of several distinct systems within the instrument. These systems are described briefly here, and more detailed discussion can be found in Melamed et al. ***(1)*** and in Shapiro ***(2)***.

For reproducible results, every sample particle must be exposed to the same amount of light; thus all cells must follow the same path as closely as possible. Hydrodynamic focusing is utilized to achieve this. Pressurized water passing through a conical nozzle flows in a laminar fashion (sheath fluid). The sample, containing suspended cells, is introduced into the center of the sheath fluid (**Fig. 1**). Sample fluid velocity increases rapidly as it contacts the sheath fluid. This acceleration has the effect of increasing the distance between cells in the sample. Because the flow is laminar and the velocity of the two fluid streams become equal, the sample flow is constrained to the center of the fluid stream; i.e., the sample is hydrodynamically focused.

The width of the exciting light path must be greater than the width of this fluid stream to ensure uniform illumination. Sensitive cytometry requires intense excitation light, and thus light sources (usually mercury arc lamp or

laser) are focused into a small area. Dual-laser instruments increase the range of parameters that can be analyzed. Elimination of background scattered light by an appropriate optical design is also critical to obtain the best signal-to-noise ratio (SNR). Background noise is inherent, but as cells pass through the sensing region, light that is scattered and/or emitted is collected by an optical system.

A commonly implemented system is the jet-in-air delivery system (**Fig. 1**), which refers to the fact that the sheath and sample fluid pass through the nozzle as discussed previously, through the exciting light beam, and into a waste collection system as a jet of water without contact with a surface. Instruments using this type of configuration are produced by several manufacturers (*see* **Subheading 2.**). In such a system, light is collected by objective lenses, positioned to gather as much light as possible from the area where the cells meet the light beam, and as little light as possible from other places. For jet-in-air systems, these lenses generally have a low numerical aperture (e.g., about 0.6), which collects scattered light and fluorescence from a reasonably large area. Collected light is then delivered to the photocathode of a detector. Use of a low numerical aperture lens sacrifices some precision for the benefit of ensuring that cells are always in focus. However, other machines may be configured differently. The jet-in-air system requires high flow rates for laminar flow stability, and also is analyzed by a laser beam hitting a stream of water, which can cause greater inherent background light scatter than alternative, nonsorting options. Indeed, so much light is scattered by this arrangement that obscuration, or blocking bars, are required to prevent background light detection.

A common alternative is the use of a dark-field microscopy setup, in which the fluid stream is directed onto a microscope cover slip. Instruments of this type often use a mercury arc-lamp light source, such as the Skatron Argus (now marketed as the Bryte system by Bio-Rad), Partec, or Bruker (*see* **Subheading 2.**). Hydrodynamic focusing is still used to keep the sample stream maintained centrally, although the cells are confined to a more narrow core. Light is collected through a high numerical aperture (e.g., 1.3) microscope oil-immersion objective lens on the opposite side of the cover slip. Lower sheath fluid velocities can be used to maintain a stable flow. This design allows a minimum of reflecting surfaces (all of which are perpendicular to the light path), a low sheath fluid flow rate, low background light scatter, and use of efficient collecting lenses. However, light has to be collected from a smaller area in order for the sample cells to remain in focus. A very high SNR is obtained, and precise analysis of small particles (such as bacteria) is easily achieved. A flow chamber of this type is not as amenable to sorting as the jet-in-air system, although a flow-switching apparatus is available from Partec (see **Subheading 2.**). Jet-in-air systems may also be modified to uti-

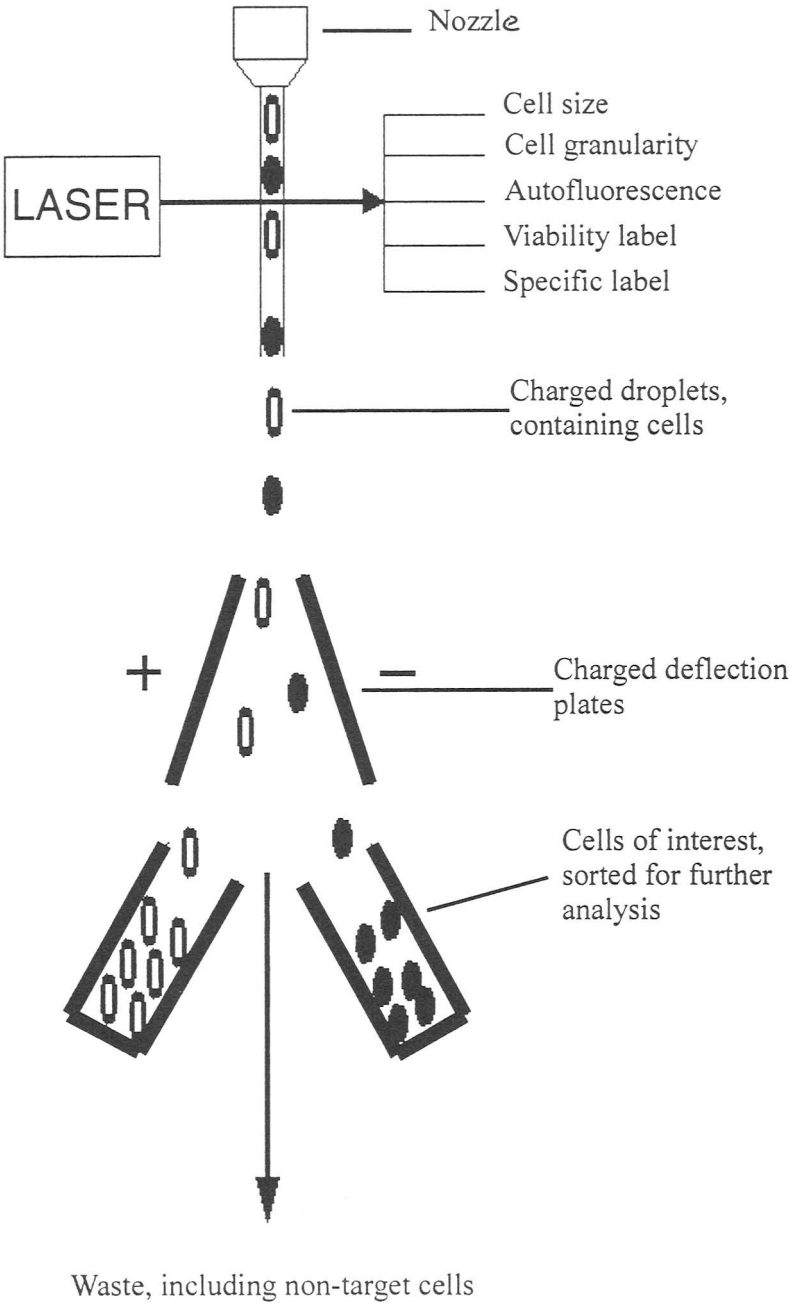

Fig. 1. Diagrammatic representation of the principles of FCM and cell sorting. A suspension of cells is fed slowly into the center of a rapid stream of sheath fluid pass-

lize oil-immersion light collection. More details on such systems are given in Steen ***(3)***.

Collected light is passed to a detector such as a photomultiplier tube (PMT) or a photodiode. Such detectors convert the light signal into an electrical pulse. A PMT is useful for low light level applications because, as the name suggests, the tube is able to multiply the received signal to produce a valuable electrical signal. Essentially cells are detected as pulses of current that greatly exceed background noise. These pulses are short-lived; thus, digital/analog converters and small capacitors are used to provide a "memory" for the computer. Linear and/or logarithmic amplification circuitry is also used to aid signal recognition from the detectors. Incoming data are used by the computer software to plot various histograms of light intensity against number (**Fig. 2**). Such plots can also be used to construct multiparameter histograms (**Fig. 2**), allowing sophisticated data analysis. Recent developments have trained neural networks to recognize subpopulations of cells of interest, further automating the data analysis process. Automatic sample loading, machine cleaning, and data storage allow "hands-off" use of the instrument.

Cell sorting can complete the process by physically separating subpopulations within the sample on the basis of measured cellular parameters. Vibration of the nozzle through which the sample passes, using a piezoelectric crystal device at a precise frequency, causes droplet formation of the fluid stream. Droplets break away from the stream at a defined distance from the light/fluid intersect (**Fig. 1**). Measurement of flow rate, droplet size, and droplet break-off point allows the machine to track any particle once it has passed through the light beam. When a particle passes through the light beam, scattered light and fluorescence are measured, and the system will decide whether to sort that particle based on operator-directed criteria derived from light scatter or fluorescence characteristics. Maximum and minimum values of these form the basis of a sort window. The machine will then decide whether that particle (i.e., target cell, nontarget cell, or dirt particle) is of interest (i.e., to be sorted). The only contact left with the particles at this point is through the sheath fluid, which can be positively or negatively electrically charged (**Fig. 1**). As a particle of interest passes beyond the laser beam, and toward the droplet break-off point, a charge is applied the instant after the preceding droplet has formed and

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ing through a conical nozzle, to achieve hydrodynamic focusing. The stream of cells passes through a beam of light, and scattered light and emitted fluorescence are detected. Vibration of the nozzle causes droplets to form, ideally containing one cell per droplet. Rapid charging of the sheath fluid enables the droplets to be charged at the point of break-off, if required. Charged droplets (containing cells) are then deflected by charged plates into collection tubes.

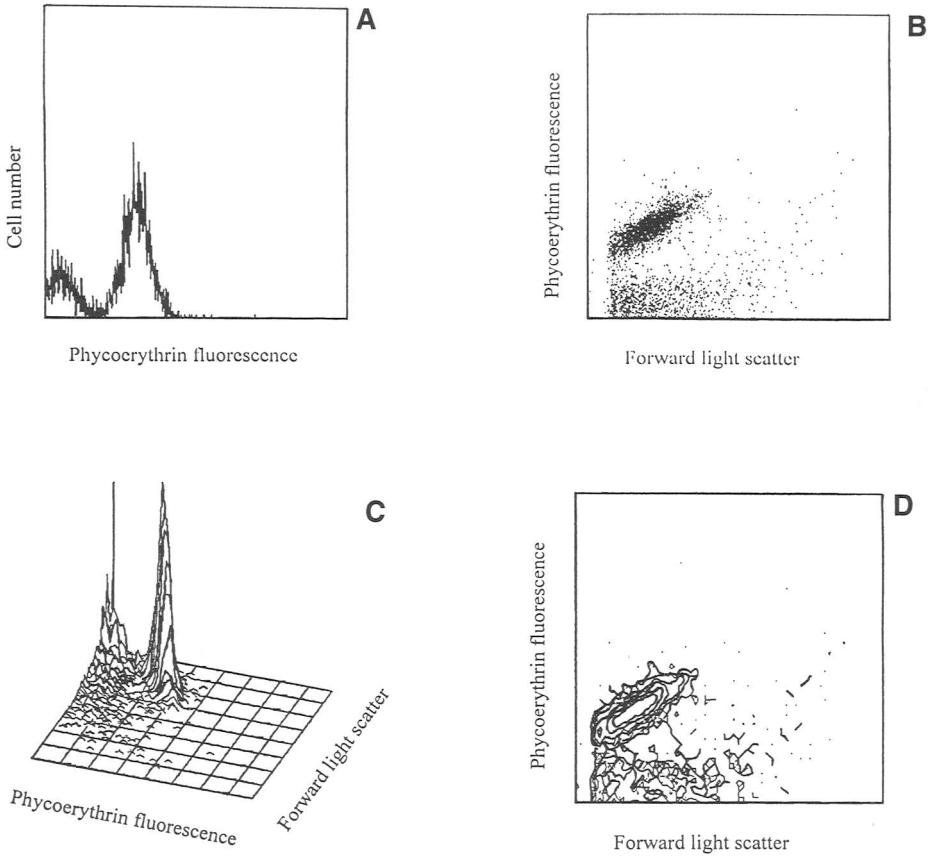

Fig. 2. Examples of data output from a flow cytometer. Bacterial cells were labeled immunofluorescently in sewage effluent. In all the examples, two populations can be seen. The population with least fluorescence represents background material and nonspecific labeling with the antisera. The second, more fluorescent population represents target cells. Discrimination of background and target cells was more than sufficient to allow highly successful cell sorting of the target cells. **(A)** frequency histogram of relative fluorescence (logarithmic scale) against number of cells; **(B)** dot-plot of immunofluorescence against forward light scatter, in which each dot represents one event; **(C)** three-dimensional histogram of fluorescence intensity (*x*-axis), light scatter (*y*-axis), and cell number (*z*-axis); **(D)** contour plot of fluorescence intensity against light scatter, in which concentric contours represent cell density.

broken off. The charge is applied until the particle-containing droplet has broken away. Thus, the droplet remains charged and falls through the space between two charged deflection plates (**Fig. 1**). Then, the droplet is deflected into a sterile collection tube (**Fig. 1**). Thus a flow cytometer/cell sorter has the

ability to label particles after analysis in one of three ways (positively, negatively, or neutrally charged) and has the potential to produce a highly enriched sample of targeted cells. Recent developments involve "half-charging" droplets. Reducing the total charge on droplets reduces the distance that droplets are deflected, thus allowing four sort labels. Other collection devices include microtiter tray wells or microscope slides. Efficiency will depend on the sort mode used, effective machine setup, and the ability to produce a sample in which cells are separate, discrete, and labeled. Sorting lowers analysis rates slightly from normal operation, but it is still possible to handle approx 2000 events/s using standard commercial machines, whereas upgrades may allow sorting of 20,000 events/s.

As previously outlined, the principle of FCM is the gathering of suspended particles singly and separately into a sensing region, where they are passed through a light beam of uniform wavelength and intensity. Each particle receives a uniform illumination for a short period of time (typically <10 μs) and emits a burst of scattered light and fluorescence over all angles. Light detectors (PMTs) produce an electrical signal that is proportional to the optical signal, to be stored and analyzed. From this signal, a measure of light scatter or fluorescence intensity can be calculated, and histograms or dot-plots of each parameter can be produced. FCM can thus measure several parameters simultaneously for several thousand cells each second.

2. Materials

2.1. Major FCM Manufacturers and Suppliers

The following list contains most major cytometer manufacturers and some major reagent suppliers. A more comprehensive list, enabling users to get individual cytometer components is given in Shapiro ***(2)***. Many of the instrument manufacturers also sell cytometry reagents. Sources of antibodies are not included, because there are few sources for antibodies against most bacteria, and many researchers raise their own.

1. Aber Instruments, Science Park, Aberystwyth, UK, SY23 3AH. Tel. +44 (0) 1970 615284; fax. +44 (0) 1970 615455. (Microcyte diode laser cytometer, compact and truly portable instrument, designed for the detection of bacterial sized-particles and allowing accurate counts.)
2. Becton Dickinson Immunocytometry Systems, 2350 Qume Drive, San Jose, CA 95131-1807, USA. Tel. (800) 223 8226; fax. (800) 223 8226. (Major cytometer manufacturer, with a wide range of sorting and nonsorting machines and reagents.)
3. Bio-Rad SPD S.r.l., Flow Cytometry Unit, Via Modigliani, 5/7 20090 Segrate, Milano, Italy. Tel. 39 2 21609 460; fax. 39 2 21609 499. (Bryte HS mercury-arc lamp cytometer [originally marketed as the Skatron Argus], volumetric sample injection and high precision when analyzing bacteria.)

4. Bruker Spectrospin S.A., 34 Rue de l'Industrie, F-67160 Wissembourg, France. Tel. (88) 73 68 00; fax (88) 73 68 79. (Mercury-arc lamp cytometer, high precision for small particles.)
5. Chemunex SA, 41, Rue du 11 Nov. 1918, 94700 Maisons-Alfort, France. Tel. (1) 43 969 200; fax. (1) 43 960 115. (ChemFlow II argon laser cytometer and associated reagents, marketed as a robust, easy-to-use instrument designed for industrial microbiology.)
6. Coulter Corporation, P.O. Box 169015, Miami, FL 33116-9015, USA. Tel. 1 305/380 3800; fax. 1 305/380 8313 (Major instrument manufacturer, wide range of instruments and reagents.)
7. Cytomation, 400 E. Horsetooth Road, Fort Collins, CO 80525, USA. Tel. (970) 226 2200; fax. 970 226 0107. (Specialists in high-speed analysis and sorting options, providing upgrades to existing instruments, and manufacturer of high-speed sorters.)
8. Molecular Probes, Inc., P.O. Box 22010, Eugene, OR 97402-0469, USA. Tel. (541) 465 8300; fax. (541) 344 6504. (Main supplier of fluorescent probes, dyes and calibration beads for all applications, technical assistance available).
9. Partec GmbH, Otto-Hahn-Strasse 32, D-48161 Munster, Germany. Tel. (49) 2534 80080; fax. (49) 2534 80090. (Laser and mercury arc lamp based instruments, commercially produced piezoelectric fluidic switching system for contained, aerosol-free cell sorting.).
10. Polysciences Inc., 400 Valley Road, Warrington, PA 18976-9990, USA. Tel. (215) 343 6484; fax. (215) 343 0214. (General reagents and calibration beads.)
11. Sigma Aldrich, Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178-9916, USA. Tel. (314) 771 5750; fax. (314) 771 5757. (General reagents.)

3. Methods

3.1. Applying FCM and Cell Sorting to Bacterial Populations

FCM is a powerful and versatile technique and, as such, can be used to answer many biological questions. However, as indicated in **Subheading 1.1.**, sample preparation and instrument setup determine the success of any analysis. Thus, the first step in the procedure is to establish a cell extraction method that leaves representative cells in a suspension free from particles large enough to block the nozzle of the instrument. Laboratory cultures are generally suitable without a cleanup step, although some workers have filtered culture media before use. All buffers and laboratory reagents must be filtered at least once before use, and it must be borne in mind that a sterile solution does not equate to a particle-free solution. It is often preferable to rinse glassware, and so forth, with particle free solutions prior to use. Sample preparation may range from none at all (e.g., analysis of lake water bacteria) to extensive blending/centrifugation/enrichment steps (e.g., processing soil samples). A large amount of sample preparation may introduce bias into the composition of the final cell suspension. Generally, aquatic samples require only a settling step, dilution, or

crude filtering through nylon mesh before analysis, resulting in minimal change. FCM analysis of bacteria from more challenging environments has been performed, but the degree of success has depended on the ease and effectiveness of cell extraction.

Having obtained a cell suspension that will not block or clog the FCM fluid tubing, it will be necessary to label cells to distinguish them from noncellular particles. Invariably this will require a fluorescent label, although some morphologically distinct cells (e.g., *Achromatium oxaliferum*; Pickup, R. W. and Head, I. M., personal communication) can be differentiated from background using inherent light scatter characteristics. However, these exceptions are rare and a fluorescence discriminator label is required for the majority of bacteria. Some bacterial populations can be distinguished on the basis of autofluorescence of specific pigments, but many other procedures require an added label. Choice of fluorescent label is dependent on the experimental aims. Samples may be probed for a total bacterial cell count, a viable or active cell count, a specific cell count, or an indication of cell macromolecular content (e.g., DNA, RNA, or total protein). Some of the dyes typically used are listed in **Tables 1** and **2**.

Sample labeling protocols are obviously dictated by the fluorescent dye being used. In some cases, dye binding is strongly influenced by salt concentration, which has caused problems in the study of marine bacteria. Protocols often require washing and resuspension steps to remove unbound dye. If enumeration of cells is important in the experimental aims, it may often be better to amend samples with concentrated buffer, and/or choose dyes and protocols that do not require washing steps, to avoid cell damage and loss.

An immense (and ever-increasing) range of fluorescent probes now exists for biological research. Many of these probes have been developed for mammalian cell biology, but bacterial applications are rapidly increasing. A summary of the major dyes can be found in **Table 1** (general fluorescent labels) and **Table 2** (fluorescent viability probes), together with examples of their use in flow cytometric studies of bacteria; details of the method used in each case can be found in the table references. The majority of these applications have used dyes for enumeration and viability assessment. Specific detection is achieved through the use of standard immunofluorescence labels such as fluorescein or phycoerythrin (**Table 1**) or through fluorescent *in situ* hybridization (FISH) using ribosomal RNA-directed oligonucleotides. These oligonucleotides are often labeled with standard dyes, although modern, brighter alternatives have been investigated ***(4)***.

Instrument setup is of great importance. The machine needs to be cleaned regularly and sterilized to prevent dirt and/or biofilm accumulation, and regular maintenance is important. Calibration is usually achieved through the use

Table 1
General Fluorochromes Used to Label Bacteria for FCM

Fluorochrome	Target molecules	References
Fluorescein and tetramethylrhodamine	General fluorescent labels, via isothiocyanate group; e.g,. total cell protein, antisera, oligonucleotides	*4,11,12,14–25, 28,39,40, 41,70*
Phycoerythrin	Conjugated to protein, usually for immunofluorescence	*18,19,25*
Ethidium bromide	Double-stranded nucleic acid, often with mithramycin	*32, 42, 43,45,46*
Propidium Iodide	Labeling double-stranded nucleic acid	*11–13,17,25, 40,41,44,70*
Mithramycin	G/C rich DNA, often used with ethidium bromide	*28,42,43,45,46*
Chromomycin A_3	G/C rich DNA	*25,47,48*
Hoechst 33342 or 33258	A/T rich DNA	*14,15,34,41,47-50*
4',6-Diamidino-2-phenylindole	Nucleic acid dye	*49,51–56*
SYBR Green I	Nucleic acid	*57*
YOYO-I, YO-PRO-I	Cyanine dye-based nucleic acid	*58*
PicoGreen	Double-stranded nucleic acid	*58*

Table 2
Fluorescent Probes Used to Assess Bacterial Viability by FCM

Fluorochrome	Cell function measured	References
Rhodamine 123	Membrane potential	*13,26,28-31,41,59–62,70*
Dihexylocarbocyanine dyes	Membrane potential (several forms exist)	*31,59,63*
Fluorescein diacetate	Enzyme activity, membrane integrity	*59*
Carboxyfluorescein diacetate	Enzyme activity, membrane integrity	*13,26,35,36,60*
Bis-carboxyethyl-carboxyfluorescein acetoxymethyl ester	Enzyme activity, membrane integrity	*13,25,26,35*
Calcein acetoxymethyl ester	Enzyme activity, membrane integrity	*26*
Chemchrome B	Enzyme activity, membrane integrity	*13,25–27,35,36,60*
Cyanoditolyl tetrazolium chloride	Respiratory activity	*28,34,64,65*
Ethidium bromide	Double-stranded nucleic acid	*32*
Propidium iodide	Double-stranded nucleic acid	*62,67*
BacLight	Commercial kit, nucleic acid	*30,31–35*
Oxonol dyes	Accumulate in dead cell membranes	*27,31,62,65–68*
Calcofluor White	Nucleic acid	*31*
PoPro	Nucleic acid	*29*
SYTOX Green	Nucleic acid	*30,69*

of monodisperse fluorescent beads, which are available in a variety of sizes. For bacterial studies, 0.5 to 2.0-µm beads are probably the most relevant sizes to use for instrument alignment. Subsequent data handling and analysis will depend on the instrument specifications. However, most machines now save files in a flow cytometry standard format. Several software packages (including freeware examples) exist that help in FCM data analysis.

3.2. Environmental Monitoring of Bacteria Using FCM and Cell Sorting

FCM and cell sorting have found many applications in microbiology. **Tables 1** and **2** give an idea of how the technology has been used to monitor bacteria. Several reviews also exist as an introduction to the area of FCM in environmental bacteriology ***(5–8)***. The use of FCM is increasing and has already helped considerably in certain situations, e.g., discovery of the significant marine genus *Prochlorococcus* ***(9)***. Observations using photosynthetic pigments and DNA analysis have shown the limitations of traditional methods for analyzing photosynthetic marine bacterial plankton biomass ***(10)***.

3.2.1. Specific Detection of Bacteria Using FCM

Immunological methods are well established in bacteriology and have much to offer in conjunction with FCM as specific labels for detection, enumeration, and purification procedures. Problems with production, specificity, epitope expression in stressed cells, and labeling of background material are well known; however, extremely sensitive and specific detection is possible, including the labeling of intracellular molecules. Significant improvements in detection, and SNRs have been made by combining labeling with monoclonal antibodies and propidium iodide for the detection of *Legionella* in cooling waters ***(11)*** and the detection of *Flavobacterium* in soil ***(12)***. Despite these improvements, FCM has proved to be of only limited value when working in soil ***(12,13),*** although other highly particulate environments such as feces and activated sludge have been successfully analyzed ***(14–16)***. However, the most successful immunofluorescent FCM applications to environmental bacteriology have used sewage or water samples ***(11,17–19)***. Porter et al. ***(18)*** used polyclonal antibodies to detect *Escherichia coli* in lake water and sewage. Positive events were sorted and plated onto selective media for confirmation. Single fluorochrome, indirect labeling protocols of this type, however, are susceptible to nonspecific binding of antibodies ***(19)***.

FISH methods label ribosomal RNA (rRNA) sequences inside intact cells. Many of these studies are performed and analyzed on microscope slides. The review of Amann et al. ***(4)*** gives a comprehensive background to FISH and discusses sample preparation, probe design, and hybridization conditions. FCM

analyses of mixed populations of cultured cells have been performed ***(20)***. Wallner et al. ***(21)*** optimized methods for bacterial analysis by FISH and FCM and subsequently used these to probe the microflora of activated sludge directly ***(16)***. Data such as these, and those from microscopic observations, have demonstrated that the fluorescence signal obtained is proportional to the ribosome content of the cell, and, hence, in nutrient-poor environments, low ribosome content may render cells difficult to detect.

One method for overcoming the detection limits of FISH may be to amplify target nucleic acid sequences inside whole cells. This approach has been developed in histopathology, in which increased sensitivity is needed to detect viral infections. The approach may be used as a method for labeling cells containing particular genes. It has been possible to perform the procedure on cultured bacterial cells, to detect a plasmid encoded gene ***(22)*** or the 16S rRNA gene ***(23)***. The latter study also demonstrated reverse transcription and amplification from mRNA inside whole cells.

3.2.2. Viability Assessment of Bacteria Using FCM

Fluorescent probes exist for a range of metabolic functions, that aim to reflect cell viability without the need for culture. Bacteriologists have not yet developed an assay that unambiguously demonstrates viability ***(6***,2***4)***. Whether fluorescent viability probes used either separately or in combinations actually reflect viability is open to question. However, the use of FCM has contributed to the increasing confidence in the data. Because many of the fluorescent viability probes share common excitation/emission wavelengths, they are difficult to use for simultaneous sample labeling. The speed of FCM has enabled researchers to process multiple subsamples of cells within an acceptable time period ***(25–27)***. In these studies, cell viability was assessed by the ability to be cultured on solid agar media, response to nutrient addition (nalidixic acid assay or direct viable count), maintenance of a membrane potential (using rhodamine 123 and oxonol dyes) and maintenance of membrane integrity, and intracellular membrane activity (using fluorogenic ester dyes). Total counts were also made, using microscopy and immunofluorescence. The studies show that greater variation is obtained from FCM counting than from microscopy. This variation arises from operator error, and nonuniform, day-to-day instrument operation, and emphasizes the need for quality control. However, the flow cytometric/fluorescent probe viability estimates were more similar to the direct viable (nalidixic acid) count procedure than to the culturable count. Use of these fluorescent viability probes is applicable to studying active cells, or cells whose stress response includes maintenance of metabolic activity.

Membrane permeability to nucleic acid stains has also been used as a viability indicator. The assay works on the basis that live cells maintain membrane

integrity and function and exclude the dye, which dead or damaged cells are unable to do. Thus, the dye enters the cell and labels nucleic acid. Work using the Gram-positive bacterium *Micrococcus luteus* has shown that the stress response appears to be one of metabolic shutdown, i.e., dormancy. Dormant *Micrococcus* cells did not form colonies on solid media, accumulated a nucleic acid dye, and failed to accumulate the membrane potential probe rhodamine 123 ***(28,29)***. However, appropriate treatment (e.g., with intercell signalling molecules) showed that dormant cells could be resuscitated ***(28,29)***. When breaking dormancy, these cells initially showed a decreased permeability to the nucleic acid dye, followed by accumulation of rhodamine 123 and ultimately became culturable. Only the use of FCM enabled such intricate measurements of this complex phenomenon. Other investigators have successfully used membrane permeability to a nucleic acid label as an indicator of viability ***(30–35)***. Fluorescent viability probe measurements may suffer from further complexity if efflux pumps lead to a false recording of viability. If these, or other, direct methods gain acceptance, they will be both more rapid and more accurate than culture techniques for monitoring viability of bacteria from a variety of sources ***(36)***.

4. Discussion and Future Prospects

FCM has already proven useful for (environmental) microbiology, and new advances in instrument design and data processing are constantly improving the technique. Although of limited application in particulate environments, FCM is ideally suited for analysis of aquatic bacteria. The instrumentation can be partly automated for routine use, and data analysis is more sophisticated than ever. Neural networks can be trained to recognize subpopulations of microorganisms from raw data and are being developed for examination of naturally fluorescent marine and freshwater planktonic species ***(37)***. Gauci et al. ***(38)*** demonstrated the use of spectral fingerprinting, to determine the spectral properties of particles at hundreds of wavelengths simultaneously. Particles are assigned to previously characterized optical fingerprints. Pulsed laser sources may also have several applications in bacteriology, allowing time-resolved fluorescence analysis to discriminate particles according to differences in their fluorescent lifetimes. Other developments include the production of cytometers specifically designed to detect microorganisms, including a battery-operated, portable FCM, with a diode laser and fixed optical alignment (eliminating machine setup and focusing) allowing field use ***(6)***. Advances such as these should help to establish FCM as a necessary, routine instrument in environmental microbiological studies.

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References

1. Melamed, M. R., Lindmo, T., and Mendehlsohn, M. L., eds. (1990) *Flow Cytometry and Sorting*. Wiley-Liss, New York.
2. Shapiro, H. M. (1988) Practical Flow *Cytometry*. Alan R. Liss, New York.
3. Steen, H. B. (1990) Characteristics of flow cytometers, in *Flow Cytometry and Sorting* (Melamed, M. R., Lindmo, T., and Mendehlsohn, M. L., eds.), Wiley-Liss, NY, pp. 11–25.
4. Amann, R. I., Ludwig, W., and Schleifer, K.-H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59,** 143–169.
5. Lloyd, D., ed. (1993) *Flow Cytometry in Microbiology*. Springer-Verlag, London.
6. Davey, H. M. and Kell, D. B. (1996) Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiol. Rev.* **60,** 641–696.
7. Porter, J., Deere, D., Pickup, R., and Edwards, C. (1996) Fluorescent probes and flow cytometry: new insights into environmental bacteriology. *Cytometry* **23,** 91–96.
8. Porter, J., Deere, D., Hardman, M., Edwards, C., and Pickup, R. (1997) Go with the flow-use of flow cytometry in environmental microbiology. *FEMS Microbiol. Ecol.*, **24,** 93–101.
9. Chisholm, S. W., Olson, R. J., Zettler, E. R., Goeriche, R., Waterbury, J. B., and Welschmeyer, N. A. (1988) A novel free-living prochlorophyte abundant in the oceanic euphotic zone. *Nature* **334,** 340–343.
10. Campbell, L., Nolla, H. A., and Vaulot, D. (1994) The importance of *Prochlorococcus* to community structure in the central North Pacific Ocean. *Limnol. Oceanogr.* **39,** 954–961.
11. Tyndall, R. L., Hand, R. E., Jr., Mann, R. C., Evans, C., and Jernigan, R. (1985) Application of flow cytometry to detection and characterization of *Legionella* spp. *Appl. Environ. Microbiol.* **49,** 852–857.
12. Page, S. and Burns, R. G. (1991) Flow cytometry as a means of enumerating bacteria introduced into soil. *Soil. Biol. Biochem.* **23,** 1025–1028.
13. Porter, J., Pickup, R. W., and Edwards, C. (1997) Evaluation of flow cytometric methods for the detection and viability assessment of bacteria in soil. *Soil Biol. Biochem.* **29,** 91–100.
14. Van der Waaij, L. A., Mesander, G., Limburg, P. C., and van der Waaij, D. (1994) Direct flow cytometry of anaerobic bacteria in human feces. *Cytometry* **16,** 270–279.
15. Van der Waaij, L. A., Limberg, P. C., Mesander, G., and van der Waaij, D. (1996) *In vivo* IgA coating of anaerobic bacteria in human faeces. *Gut* **38,** 348–354.
16. Wallner, G., Erhart, R., and Amann, R. (1995) Flow cytometric analysis of activated sludge with rRNA-targeted probes. *Appl. Environ. Microbiol.* **61,** 1859–1866.
17. Volsch, A., Nader, W. F., Geiss, H. K., Nebe, G., and Birr, C. (1990) Detection and analysis of two serotypes of ammonia-oxidizing bacteria in sewage plants by flow cytometry. *Appl. Environ. Microbiol.* **56,** 2430–2435.

18. Porter, J., Edwards, C., Morgan, J. A. W., and Pickup, R. W. (1993) Rapid, automated separation of specific bacteria from lakewater and sewage using flow cytometry and cell sorting. *Appl. Environ. Microbiol.* **59,** 3327–3333.
19. Porter, J., Pickup, R. W., Robinson, J., and Edwards, C. (1995) Recovery of a bacterial sub-population from sewage using immunofluorescent flow cytometry and cell sorting. *FEMS Microbiol. Lett.* **133,** 195–199.
20. Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R., and Stahl, D. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations. *Appl. Environ. Microbiol.* **56,** 1919–1925.
21. Wallner, G., Amann, R., and Beisker, W. (1993) Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14,** 136–143.
22. Porter, J., Pickup, R., and Edwards, C. (1995) Flow cytometric detection of specific genes in genetically modified bacteria using *in situ* polymerase chain reaction. *FEMS Microbiol. Lett.* **134,** 51–56.
23. Hodson, R. E., Dustman, W. A., Garg, R. P., and Moran, M. A. (1995) *In situ* PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. *Appl. Environ. Microbiol.* **61,** 4074–4082.
24. Nebe-von Caron, G., and Badley, R. A. (1995) Viability assessment of bacteria in mixed populations using flow cytometry. *J. Microsc.* **179,** 55–66.
25. Porter, J., Edwards, C., and Pickup, R. W. (1995) Rapid assessment of physiological status in *Escherichia coli* using fluorescent probes. *J. Appl. Bacteriol.* **79,** 399–408.
26. Diaper, J. P. and. Edwards, C. (1994) The use of fluorogenic esters to detect viable bacteria by flow cytometry. *J. Appl. Bacteriol.* **77,** 221–228.
27. Deere, D., Porter, J., Pickup, R., and Edwards, C. (1996) Direct analysis of starved *Aeromonas salmonicida*. *J. Fish Dis.* **19,** 459–467.
28. Kaprelyants, A. S. and D. B. Kell. (1993) Dormancy in stationary-phase cultures of Micrococcus luteus: flow cytometric analysis of starvation and resuscitation. *Appl. Environ. Microbiol.* **59,** 3187–3196.
29. Votyakova, T. V., Kaprelyants, K. S., and Kell, D. B. (1994) Influence of viable cells on the resuscitation of dormant cells in *Micrococcus luteus* cultures held in an extended stationary phase: the population effect. *Appl. Environ. Microbiol.* **60,** 3284–3291.
30. Langsrud, S., and Sundheim, G. (1996) Flow cytometry for rapid assessment of viability after exposure to a quaternary ammonium compound. *J. Appl. Bacteriol.* **81,** 411–418.
31. Mason, D. J., Lopez-Amoros, R., Allman, R., Stark, J. M., and Lloyd, D. (1995) The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. *J. Appl. Bacteriol.* **78,** 309–315.
32. Jernaes, M. W. and Steen, H. B. (1994) Staining of *Escherichia coli* for flow cytometry: influx and efflux of ethidium bromide. *Cytometry* **17,** 302–309.

33. Terzieva, S., Donnelly, J., Ulevicius, V., Grinshpun, S. A., Willeke, K., Stelma, G. N., and Brenner, K. P. (1996) Comparison of methods for detection and enumeration of airborne microorganisms collected by liquid impingement. *Appl. Environ. Microbiol.* **62,** 2264–2272.
34. Joux, F., LeBaron, P., and Troussellier, M. (1997) Succession of cellular states in a *Salmonella typhimurium* population during starvation in artificial seawater microcosms. *FEMS Microbiol. Ecol.* **22,** 65–76.
35. Jacobsen, C. N., Rasmussen, J., and Jakobsen, M. (1997) Viability staining and flow cytometric detection of *Listeria monocytogenes*. *J. Microbiol. Methods* **28,** 35–43.
36. Porter, J., Diaper, J., Edwards, C., and Pickup, R. (1995) Direct measurements of natural planktonic bacterial community viability by flow cytometry. *Appl. Environ. Microbiol.* **61,** 2783–2786.
37. Wilkins, M. F., Boddy, L., Morris, C. W., and Jonker, R. (1996) A comparison of some neural and non-neural methods for identification of phytoplankton from flow cytometry data. *Comp. Appl. Biosci.* **12,** 9–18.
38. Gauci, M. R., Vesey, G., Narai, J., Veal, D., Williams, K. L., and Piper, J. A. (1996) Observation of single cell fluorescence in laser flow cytometry. *Cytometry* **25,** 388–393.
39. Allman, R., Hahn, A. C., Phillips, A. P., Martin, K. L., and Lloyd, D. (1990) Growth of *Azotobacter vinelandii* with correlation of Coulter cell size, flow cytometric parameters, and ultrastructure. *Cytometry* **11,** 822–831.
40. Miller, J. S. and Quarles, J. M. (1990) Flow cytometric identification of microorganisms by dual staining with FITC and PI. *Cytometry* **11,** 667–675.
41. Diaper, J. P. and Edwards, C. (1994) Survival of *Staphylococcus aureus* in lakewater monitored by flow cytometry. *Microbiology* **140,** 35–42.
42. Skarstad, K., Steen, H. B., and Boye, E. (1983) Cell cycle parameters of slowly growing *Escherichia coli* B/r studied by flow cytometry. *J. Bacteriol.* **154,** 656–662.
43. Skarstad, K., Steen, H. B., and Boye, E. (1985) *Escherichia coli* DNA distributions measured by flow cytometry and compared with theoretical computer simulations. *J. Bacteriol.* **163,** 661–668.
44. DeLeo, P. C. and Baveye, P. (1996) Enumeration and biomass estimation of bacteria in aquifer microcosm studies by flow cytometry. *Appl. Environ. Microbiol.* **62,** 4580–4586.
45. Allman, R., Hann, A. C., Manchee, R., and Lloyd, D. (1992) Characterization of bacteria by multiparameter flow cytometry. *J. Appl. Bacteriol.* **73,** 438–444.
46. Thorsen, B. K., Enger, O., Norland, S., and Hoff, K. A. (1992) Long term survival of *Yersinia ruckeri* at different salinities studied by microscopical and flow cytometric methods. *Appl. Environ. Microbiol.* **58,** 1624–1628.
47. van Dilla, M. A., Langlois, R. G., Pinkel, D., Yajko, D., and Hadley, W. K. (1982) Bacterial characterization by flow cytometry. *Science* **220,** 620–622.
48. Sanders, C. A., Yajko, D. M., Nassos, P. S. Hyun, W. C., Fulwyler, M. J., and Hadley, W. K. (1991) Detection and analysis by dual laser flow cytometry of bacteriophage T4 DNA inside *Escherichia coli*. *Cytometry* **12,** 167–171.

49. Monger, B. C. and Landry, M. R. (1993) Flow cytometric analysis of marine bacteria with Hoechst 33342. *Appl. Environ. Microbiol.* **59,** 905–911.
50. Montford, P. and Baleux, B. (1994) Effects of environmental factors present in the St. Lawrence estuary (Quebec, Canada) on experimental survival of *Salmonella salamae* as determined by flow cytometry. *Can. J. Microbiol.* **40,** 712–719.
51. Troussellier, M., Courties, C., and Zettelmaier, S. (1995) Flow cytometric analysis of coastal lagoon bacterioplankton and picophytoplankton: fixation and storage effects. *Estuarine, Coastal Shelf Sci.* **40,** 621–633.
52. Robertson, B. R. and Button, D. K. (1989) Characterizing aquatic bacteria according to population, cell size and apparent DNA content by flow cytometry. *Cytometry* **10,** 70–76.
53. Button, D. K. and Robertson, B. R. (1989) Kinetics of bacterial processes in natural aquatic systems based on biomass as determined by high resolution flow cytometry. *Cytometry* **10,** 558–563.
54. Button, D. K., Schut, F., Quang, P., Martin, R., and Robertson, B. R. (1993) Viability and isolation of marine bacteria by dilution culture: theory, procedures and initial results. *Appl. Environ. Microbiol.* **59,** 881–891.
55. Schut, F., de Vries, E. J., Gottschal, J. C., Robertson, B. R., Harper, W., Prins, R. A., and Button, D. K. (1993) Isolation of typical marine bacteria by dilution culture: growth, maintenance and characteristics of isolates under laboratory conditions. *Appl. Environ. Microbiol.* **59,** 2150–2160.
56. Montford, P. and Baleux, B. (1992) Comparison of flow cytometry and epifluorescence microscopy for counting bacteria in aquatic ecosystems. *Cytometry* **13,** 188–192.
57. Marie, D., Partensky, F., Jacquet, S., and Vaulot, D. (1997) Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Appl. Environ. Microbiol.* **63,** 186–193.
58. Marie, D., Vaulot, D., and Partensky, . F (1996) Application of the novel nucleic acid dyes YoYo-1, Yo-Pro-1, and Picogreen for flow cytometric analysis of marine prokaryotes. *Appl. Environ. Microbiol.* **62,** 1649–1655
59. Diaper, J. P., Tither, K., and Edwards, C. (1992) Rapid assessment of bacterial viability by flow cytometry. *Appl. Microbiol. Biotechnol.* **38,** 268–272.
60. Diaper, J. P. and Edwards, C. (1994) Flow cytometric detection of viable bacteria from compost. *FEMS Microbiol. Ecol.* **14,** 213–220.
61. Kaprelyants, A. S., and Kell, D. B. (1992) Rapid assessment of bacterial viability and vitality by Rhodamine 123 and flow cytometry. *J. Appl. Bacteriol.* **72,** 410–422.
62. Lopez-Amoros, R., Comas, J., and Vives-Rego, J. (1995) Flow cytometric assessment of *Escherichia coli* and *Salmonella typhimurium* starvation-survival in seawater using rhodamine **123,** propidium iodide and oxonol. *Appl. Environ. Microbiol.* **61,** 2521–2526.
63. Montford, P. and Baleux, B. (1996) Cell cycle characteristics and changes in membrane potential during growth of *Escherichia coli* as determined by a cyanine fluorescent dye and flow cytometry. J. Microbiol. Meth. **25,** 79–86.

64. Kaprelyants, A. S. and Kell, D. B. (1993) The use of 5-cyano–**2,**3-ditolyl tetrazolium chloride and flow cytometry for the visualization of respiratory activity in individual cells of Micrococcus luteus. *J. Microbiol. Methods* **17,** 115–122.
65. Lopez-Amoros, R., Mason, D. J., and Lloyd, D. (1995) Use of two oxonols and a fluorescent tetrazolium dye to monitor starvation of *Escherichia coli* in seawater by flow cytometry. *J. Microbiol. Meth.* **22,** 165–176.
66. Deere, D., Porter, J., Edwards, C., and Pickup, R. (1995) Evaluation of the suitability of bis-(1,3-dibutylbarbituric acid) trimethine oxonol for flow cytometric assessment of bacterial viability. *FEMS Microbiol. Lett.* **130,** 165–170.
67. Jepras, R. I., Carter, J., Pearson, S. C., Paul, F. E., and Wilkinson, M. J. (1995) Development of a robust flow cytometric assay for determining numbers of viable bacteria. *Appl. Environ. Microbiol.* **61,** 2696–2701.
68. Mason, D. J., Allman, R. Stark, J. M., and Lloyd, D. (1994) Rapid estimation of bacterial antibiotic susceptibility with flow cytometry. *J. Microsc.* **176,** 8–16.
69. Roth, B. L., Poot, M., Yue, S. T., and Millard, P. J. (1997) Bacterial viability and antibiotic susceptibility testing with SYTOX green nucleic acid stain. *Appl. Environ. Microbiol.* **63,** 2421–2431.
70. Morgan, J. A. W., Rhodes, G., and Pickup, R. W. (1993) Survival of nonculturable Aeromonas salmonicida in lake water. *Appl. Environ. Microbiol.* **59,** 874–880.

6

Magnetic Particle–Based Separation Techniques for Monitoring Bacteria from Natural Environments

Jonathan Porter and Roger Pickup

1. Introduction

Physical separation of either intact target cells or specific molecules from many environments can result in a suspension free of contaminating particles, nontarget cells and biological inhibitors and highly enriched in the target cells or molecules of interest. The processed sample will be ready for the next part of the overall experimental protocol; e.g., a culture step, or a molecular biological procedure, and greater confidence in a successful outcome will be achieved. A range of methods exist for whole-cell extractions from a variety of environments. Such methods may aim to obtain a clean suspension that is representative of the bulk cell population, or may aim to target specific cells. On occasion, it is necessary to obtain a clean bulk cell suspension prior to specific cell extraction. This chapter deals with the separation of specific cells, either intact or targeting a marker molecule of interest. Methods for intact cell separations include flow cytometric cell sorting ***(1)***, optical trapping ***(2)***, micromanipulation ***(3)***, dielectrophoresis ***(4)***, ultrasound sedimentation ***(5)***, sedimentation field-flow fractionation ***(6)*** and elutriation ***(7,8)***. The general applicability and the degree of selectivity that can be achieved for the cell selection varies with each method. Of special importance in the context of this book is the environment from which the samples are taken, and the presence of nontarget particulate material, which can easily foul sensitive instruments, clog filters or hamper microscopic observations. One approach that has proven feasible from environments as challenging as feces, plant tissue, or soil is the use of magnetic particle separation technology.

The advantage offered by magnetic bead cell sorting is the ability to separate specific cells from bulk populations rapidly and simply, offering increased

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sensitivity and specificity. Physical separations based on cell densities (e.g., ultrasound sedimentation, elutriation, sedimentation field-flow fractionation) do not offer specificity and have other disadvantages (e.g., requirement of high cell numbers, large final sample dilution, and the necessity for specialized equipment). Micromanipulation and optical trapping have much to offer bacterial ecologists, and are effective at obtaining pure isolates. However, the procedures are time-consuming and lack initial specificity. Flow cytometric cell sorting is an effective and powerful tool, wherein each individual cell is examined and a decision is made whether or not to sort. The ability to examine single cells has much to offer microbiology, but flow cytometric cell sorting requires expensive, specialized instrumentation, and is less robust and less rapid than magnetic separations. Magnetic separations are durable, require a minimum of specialist equipment, and are easily performed on the bench.

1.1. Principles of Magnetic Particle–Based Separations

Magnetic particles are available in a variety of types and sizes, but the principle of the technique is the same for all types, and is outlined in **Fig. 1**. The magnetic particle is coated with a hapten that recognizes a receptor on the target cell wall or molecule. The haptens used to date are generally antibodies, although alternatives such as lectins have been investigated. A cell suspension is mixed with the labeled magnetic particles, and the hapten given time to react with the cells (**Fig. 1A**). The time required for this is selected so as to allow reaction with all target cells within the suspension without allowing nonspecific cell attachment (e.g., early stages of biofilm establishment). The suspension containing the cells and beads is then placed within a magnetic field, and the magnetic particles (with attached cells) are allowed to concentrate (**Fig. 1B**). The particles will remain held in place for the period that the magnetic force is present (**Fig. 1C**); thus, a suitably designed apparatus allows for the removal of the remainder of the suspension (containing nontarget cells and other particles), and the addition of fresh, defined buffer (**Fig. 1D**). Repeating the concentration step allows cell washing to be performed for as many times as necessary; generally only one or two washes yield suspensions that are adequately cleaned. Cells can then be removed from the particles for further study, although some applications allow use of the cells while still attached to the particles.

The magnetic separation step has been demonstrated to be applicable in highly particulate environments, including soil. The limiting step is thus generally the hapten used, and its reactivity after attachment to the beads. Magnetic particles can be purchased in a variety of forms, from ready-to-use kits to uncoated particles ready for user labeling. Available formats include the following:

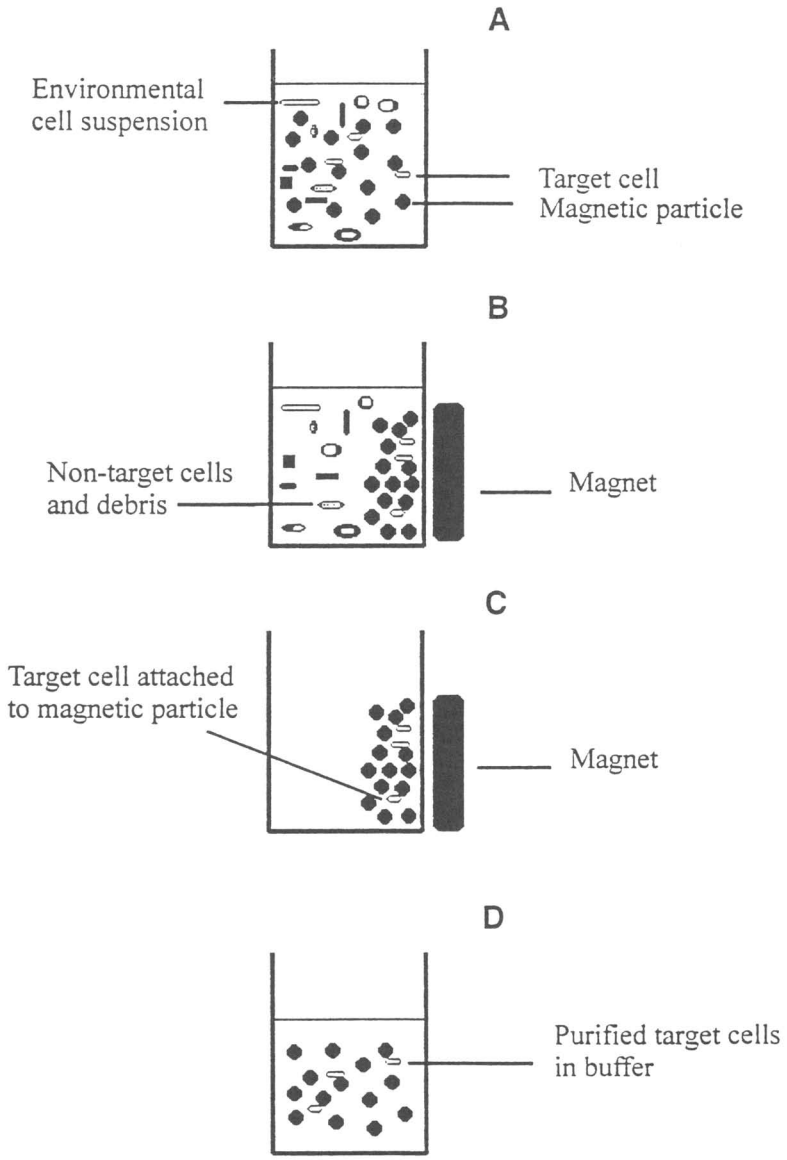

Fig. 1. Purification of target cells using a magnetic particle-based separation technique. **(A)** Cell suspension mixed with labeled magnetic particles. **(B)** Mixture placed in magnetic field. **(C)** Supernatant (containing nontarget cells) is removed. **(D)** Purified cells are resuspended in buffer solution.

1. Magnetic particles coated with a polymer that allows covalent linkage of monoclonal antibodies (MAbs) that recognize the target cells (primary labeling).
2. Magnetic particles coated with MAbs from all common laboratory animals. Particles labeled in this way allow all combinations of reactions with individual laboratory antibodies raised in other animals (secondary or affinity labeling). As an example, a user has raised an MAb in a mouse cell line that shows the desired specificity with the target cells. Purchase of magnetic particles coated with antimouse antibodies raised in rat, rabbit, or sheep will allow secondary labeling of beads with the mouse MAb. The correct presentation of the original antibodies cannot be guaranteed using this method, and the subsequent binding capacity of the antibody may be reduced. The separation procedure can be performed in one of two ways: either the coated beads are labeled with the antibody and then allowed to react with the cells, or the antibody is allowed to react with the cells and then the coated beads are added to react with the antibody. A further alternative to this approach is the use of protein A or protein G coatings to provide the link between the antibody and the magnetic particle.
3. Streptavidin-coated particles are available, thus allowing use of biotinylated antibodies in either of the alternative uses previously outlined.
4. Uncoated beads or activated beads are available, allowing the user to coat the particles directly, by a method of choice.

The range of magnetic particles commercially available also offers the user flexibility. Virtually all commonly used particles are superparamagnetic; i.e., they show magnetic properties when in a magnetic field but contain no residual activity when removed from the magnetic field. Commonly used are polystyrene-based, uniform spheres (beads), which contain many small magnetizable particles homogeneously distributed within the matrix. The entire bead is coated with a polymer to final diameters of a few micrometers, providing reactive groups for labeling, and also lowering the total surface area of the bead suspension. Colloidal particles (e.g., 40–100 nm) are also available with very large surface areas, but require far stronger magnetic forces for concentration, such as neodymium magnets ***(9)*** or high-gradient magnetic technology ***(10)***. Porous glass may also be used as a carrier for the iron oxide, and the pores dramatically increase reactive surface area. Larger magnetic beads require only normal magnets, and theoretically allow several cells to bind to each bead. Multiple colloidal particles bind to each cell for separation. Both approaches concentrate cells effectively in an extremely low stress manner. Some applications of such systems using mammalian cells have suggested that use of larger beads may adversely affect mammalian cell viability, and that multiple attachment points of the larger beads to the mammalian cells can cause problems if detachment is required at a later stage. Most reports using bacterial cell systems have found that cell viability is not affected by the beads. However, use of larger beads for bacterial separations could result in nonspecific entrapment

of cells within pore spaces between beads during concentration. Entrapment would be minimal if colloidal magnetic particles were used. Additionally, colloidal material has less effect on the optical properties of cells, does not aggregate or settle (thus avoiding the necessity of mixing when isolating very fragile cells), and shows more rapid cell binding than larger beads. However, use of larger beads requires far less time for separation to occur under normal conditions.

As well as positive selection of target cells, it is also possible to enrich for specific cell types by depletion of other cells, an approach that may be advantageous in some situations. Additionally, it is often possible to detach bound cells from the magnetic particles, although procedures such as the polymerase chain reaction (PCR), or DNA sequencing, are not affected *per se* by low levels of the particles.

1.2. Application of Magnetic Particle–Based Separations

Magnetic cell separations are rapid, robust, and straightforward procedures, and ideal for processing large cell numbers and searching for rare cells. In addition to separating whole cells, the approach is also amenable to the detection of specific molecules. Recognition and attachment of cells to the magnetic particles is of critical importance, and is probably the limiting step in the procedure. If this is effective, the procedure will selectively remove cells of interest for further study. Without the ability to provide a selective link between target cells and the magnetic particle, the procedure cannot work. For whole-cell extractions, the target receptor must therefore be expressed on the bacterial cell wall under appropriate environmental conditions. Whole-cell extractions of bacteria from environmental samples have targeted structures such as flagella, or cell-wall markers such as serotypes of pathogenic bacteria, or sugar residues on cell-wall proteins. When extraction of specific molecules is required, the choice of target can be extended to include intracellular markers, including nucleic acids and proteins. Whatever the target molecule, specificity and avidity of the hapten must be confirmed prior to attempting magnetic separation.

Having obtained a suitable hapten, it is necessary to attach it to the magnetic particles. As outlined previously, this can be achieved by direct coating of the particle or through an indirect procedure. Direct labeling requires the particles to be coated with a polymer that will accept the hapten. This is achieved through the use of well-established systems for covalently linking proteins, such as tosyl groups ***(86)***. Direct labeling of beads is relatively straightforward, and a procedure should be provided by the manufacturer. Secondary labeling generally involves use of commercially available magnetic particles that are coated with an affinity molecule, such as an immunoglobulin or protein A. Use of this

kind of system enables user-raised antisera to be adapted to magnetic separations, but the reactivity of the antisera cannot be guaranteed to remain the same when attached to the bead surface. In such a case, it may still be possible to use the antibody by following an indirect magnetic capture protocol, in which the cells are incubated with the antibody, before adding beads that have a coating with affinity for the antibody. A similar option for affinity labeling involves the use of a recognition system for a label that is coupled to the user's antisera, e.g., streptavidin/biotin. Use of such a system depends on the commercial availability of biotinylated antisera, or the willingness of the user to biotinylate antisera in-house. Again, this is a relatively straightforward procedure ***(86)***.

Physical separation using magnetic beads requires effective mixing to enable the label, the beads, and the cells to come into contact. Aquatic environmental samples need only resuspension in (or the addition of concentrated) buffer before magnetic separation. Samples containing solid material will need dispersal prior to the procedure. Standard procedures for food samples rely on the use of a stomacher to bring cells into suspension. It is possible to include filters in the stomacher bag to remove large particles and produce a clean suspension in a single step. Other environments, such as soils and sediments, usually rely on blending/centrifugation steps in particular buffers for optimal dispersal. Soil dispersal requires optimization for each soil textural type, and probably for individual soils. Use of ion-exchange resins has been shown to improve clay mineral dispersal in some soils. Again, a crude filtration or settlement step (often achieved by centrifugation) will help produce a cleaner suspension from which to extract target cells or molecules.

The hapten that is being used is then required to react with the target molecules in the suspension. If the hapten is attached to the beads, this simply means adding labeled beads to the cell suspension. However, the hapten may be allowed to react with the target molecules and the beads added to capture the hapten. In either case, the use of a blocking reagent to prevent nonspecific reactions may be necessary. Blocking reagents are often proteins such as bovine serum albumin (BSA), milk-derived protein, or gelatin. Samples are incubated to allow maximum cell binding with the hapten. Generally, the reaction time is approx 30 min. Increasing this time may not increase recoveries. However, density of the beads has been demonstrated to be of significance, and generally requires bead numbers greatly in excess of the target cell numbers.

After incubation, the beads can be washed by repeated concentration and resuspension in fresh buffer. Many reports have used a nonionic detergent such as 0.05–0.1% Tween-20, as well as maintaining the blocking reagent, in the wash buffer. The cells can be attached firmly to the beads, and may remain

attached during vortexing. However, this may vary with the hapten used, and more gentle washing (e.g., aspiration through a pipet tip) may be preferred. Washing steps will remove weakly, nonspecifically bound cells and also cells entrapped in the pore spaces if large magnetic beads are used. Purified cells are ready for analysis at this stage, whether by culture or otherwise. If a culture step is used, the magnetic bead separation may be considered a more rapid alternative to a preenrichment culture step. Alternatively, for highly sensitive detection, magnetic separation can be performed on enrichment broth samples. Target molecules such as nucleic acids should also be sufficiently clean as to allow molecular biological procedures. Many reactions are able to tolerate magnetic particles up to certain levels. Magnetic supports such as porous glass show greater thermal stability (e.g., during PCR cycling) than some polymer-based beads. Alternatively, cells or molecules can be removed from the beads, e.g., by boiling in detergent, or by adding a competing agent for the hapten binding site.

1.3. Environmental Monitoring of Bacteria Using Magnetic Particle–Based Separations

Magnetic particle separation techniques have been demonstrated to improve detection limits for monitoring bacteria in many situations. One of the largest subject areas has been its use in food microbiology, predominantly using commercially available kits. After magnetic separation, detection and confirmation of target cell recovery has been achieved by culture-based methods (**Table 1**) or direct detection (**Table 2**). In both cases, sensitivity has generally been improved by lowering the numbers of nontarget bacteria and other contaminants. Achieving a clean sample, enriched in target bacteria, has enabled savings in overall detection time. The IMS (immuno-magnetic separation) procedure has now been successfully applied to a wide range of sample types (**Tables 1** and **2**). The majority of reports use the straightforward approach of mixing an environmental cell suspension with beads ready-coated in the appropriate hapten. Removing the supernatant generally leaves concentrated beads that can be used for normal spread plates or direct detection. This approach has been successful in water ***(11)*** and soil ***(12,13)***, targeting specific bacteria via antisera ***(11–13,87)*** or targeting indigenous populations using lectins ***(19,88)***. However, variations on this theme do exist. Jones and Van Vuurde ***(18)*** captured magnetic beads directly onto a flat magnetic surface, subsequently rubbing the magnet over the surface of an agar plate to allow culture (magnetic fishing). Indirect capture, in which target cells are allowed to react with a labeled antibody, before being captured by use of beads which react with the primary antibody label, has been successfully applied ***(12,14,40)***.

Table 1
Examples of Culture-Based Detection of Bacteria after Magnetic-Particle Separation

Target organism	Hapten	Environment	References
Streptomyces lividans	MAb	Soil	***12***
Streptosporangium fragile	MAb	Soil	***13***
Thermodesulfotomaculum mobile	MAb/PAb	Seawater	***15***
Rhizobium	Unfractionated serum	Culture	***14***
Pseudomonas putida	MAb	Culture, lake water	***11***
Pseudomonas stutzeri	PAb	Seawater	***40***
Staphylococcus aureus	Lectin, PAb	Milk	***78,79,97***
Listeria monocytogenes	Lectin, MAb	Milk, cheese, ham	***78,79,96***
Indigenous bacteria	Lectin (concanavalin A)	Soil, river water	***19,88***
Xanthomonas campestris pv. *pelargonii*	PAb	Leaf surface	***18***
Erwinia carotovora subsp. *atroseptica*	PAb	Potato peel	***29***
Aeromonas salmonicida	MAb	Water, salmon lice	***33***
Vibrio parahaemolyticus	Commercial PAb	Mixed cultures	***37***
Salmonella spp.	Commercial kit	Wide variety of foods	***62-66,69–71***
Salmonella serogroup C1	MAb	Blood, stools	***38***
Escherichia coli O157	Commercial kit	Human and bovine faeces, beef, milk, rectal swabs, ice cream, body fluids, soil, pond water, slurry, grass	***24,42,46–48,50,52–61,87***

Use of magnetic-bead cell sorting has been proposed as an acceptable alternative to enrichment culture in some applications ***(64)***. This may be of great benefit to the food industry, reducing the 24-h enrichment period to under 1 h, but still yielding an isolate for confirmatory purposes. Magnetic separations compare well with traditional culture methods for both true- and false-positive and negative isolations ***(64–66)***. However, detection of very low numbers of target cells may still require a selective enrichment step ***(24)***.

Magnetic-bead-based detection technology has been developed further with DNA being the target rather than a specific host cell. Millar et al. ***(91)*** developed a magnetic system whereby sequences of DNA from the flanking regions of specific insertion elements or genes were attached to the beads. When mixed with DNA extract, the beads were able to capture specific target DNA of quality suitable for PCR. In addition, this system removed false positives and other PCR artifacts ***(91)***. Jacobsen ***(92)*** also developed a magnetic capture hybridization-PCR system (MCH-PCR) to detect the *lux* gene from an engineered strain of *Pseudomonas fluorescens* released onto barley root. After hybridization of the DNA extract with beads carrying a single stranded capture probe, the beads were separated and this step removed the hybrid DNA from its surrounding contaminants (e.g., humic acids) with a detection limit of 40 cells $\cdot$ cm^{-1} barley root. Using the same methodology but combined with reverse transcriptase-PCR (RT-PCR), a rapid and reliable procedure for detecting poliovirus in groundwater was developed that is readily adaptable for other viral pathogens ***(93)***. In this method, a biotinylated oligonucleotide capture was hybridized to poliovirus-RNA in solution. Streptivadin-coated magnetic beads removed the RNA-oligonucleotide hybrid from the sample prior to RT-PCR ***(93)***. In addition to detection of single species, MCH-PCR has been used to differentiate between strains of *Bacillus cereus* and *B. thuringiensis* using a specific gene as a marker ***(94)***.

1.4. Discussion and Future Prospects

The rapidity and simplicity of magnetic separations allows the processing of large numbers of both cells and samples. Commonly, researchers have targeted specific bacteria via cell antigens. Other investigators have used marker DNA as a promoter to detect surface expression of an epitope introduced into target cells and have used this epitope as a means to track released cells using immunofluorescence ***(77)***. An approach such as this would be suited to IMS, and would allow monitoring of released bacteria, possibly for biotechnological purposes.

Magnetic separations have almost always included use of a paramagnetic particle, relying on the hapten to provide the bridge between the cell and the

Table 2
Examples of Direct Detection of Bacteria after Magnetic-Particle Separation

Target organism	Detection method[a]	Hapten	Environment	References
Bacterial toxins (botulinus A, cholera staphylococcal enterotoxin)	ECL	MAb	—	***89***
Hepatitis A	RT-PCR	MAb	River and seawater, oysters	***73,75***
Rotavirus (group A)	RT-PCR	MAb	Fresh and sea water	***74***
Oral spirocetes	PCR	Cross-reactive MAb	Subgingival plaque	***20***
Bacillus thuringienesis	PCR	—	Soil	***43***
Bacillus anthracis	ECL	PAb	Soil	***76***
Bacillus stearothermophilus	ELISA	PAb	Milk, foodstuffs, soil	45
Helicobacter pylori	PCR	PAb	Water. feces	***28***
Alteromonas sp.	PCR	—	—	***44***
Mycobacterium avium	PCR	MAb	Feces	***21***
E. carotvora subsp. *carotovora*	PCR	PAb	Potato peel	***22,29***
Erwinia chrysanthemi	PCR	PAb	Potato peel	***29***
Salmonella	ELISA	Commercial kit	Foodstuffs	***39,68***
Salmonella enteritidis	ELISA, ECL	Commercial kit	Eggs, freshwater, serum	***17,25,31,32,36***
Salmonella typhimurium (including common surface antigen 1)	PCR, ECL	—	wide variety of foodstuffs, freshwater, human serum	***67,68,70,71,95***
Salmonella serogroups O-6,7	ELISA	MAb	Food	***38, 51***
Salmonellae serogroup D	PCR	—	Human feces	***25,39***
Salmonella virchow	Conductance	Commercial kit	Skim milk powder	***36***
L. monocytogenes	PCR	MAb	Cheese	***78***

Bordetella pertussis	PCR	PAb	Nasal pharyngeal aspirates	***23***
Xanthomonas axonopodis pv. *citri*	PCR/DIANA	PAb	Leaf extracts	***26***
Porphyromonas gingivalis	PCR	MAb	Mixed cultures	***27***
Yersinia enterococcolitica O:3	PCR/DIANA	MAb, PAb	Foodstuffs, feces, tonsil swabs	***30,72***
Shigella dysenteriae	ELISA	MAb	Feces	***34,35***
Shigella flexneri	PCR	MAb	—	***34,35***
E. coli O157	PCR, ECL	Commercial kit	Fresh water, foodstuffs	***25,41,49***

[a]ECL, Electrochemiluminescent; RT-PCR, reverse transcriptase-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; DIANA, detection of immobilized amplified nucleic acid.

particle. This allows specificity by use of different haptens, but makes separation of indigenous bacterial populations difficult. Such separations may have a role to play in environmental bacteriology, as a means of obtaining clean cell suspensions that are representative of the natural population. Attempts to achieve this using lectins as haptens have met with only limited success ***(88)***. It may be possible to reverse the bridge between the cells and the magnetic particle by coating the particles with a sugar, or by utilizing bacterial lectins ***(90)***, but with either approach it is highly unlikely that selectivity will be avoided. However, Zborowski et al. ***(85)*** demonstrated more general labeling and separation of bacterial cells by coating them in the lanthanide metal erbium, as Er^{3+}. This trivalent cation has an exceptionally high magnetic dipole moment, and a high affinity for many cell surfaces. Incubation of cell suspensions with $ErCl_3$ was sufficient to impart sufficient magnetic moment to concentrate cells when passed in solution over neodymium-iron-boron magnets. Approaches such as this, or use of magnetic colloids, with the extremely powerful neodymium-iron-boron magnets may allow for development of automated separations, because they avoid problems with settlement inherent with larger particles.

The selection of commercially available kits for use with magnetic separations continues to increase, and it is likely that any latex agglutination kits (e.g., *Pseudomonas pseudomallei* ***[84]***; *Cryptococcus neoformans* ***[83])*** could be easily adapted for IMS. Another area that may be of interest for some purposes could utilize the enterobacterial common antigen ***(16,80–82)***. The speed of the magnetic separation process may enable the use of more general antigens for capture purposes, allowing the introduction of greater selectivity subsequently, increasing the reproducibility and success rate of assays by using a highly enriched and purified cell sample.

2. Materials

2.1. General Apparatus and Consumables

1. Magnetic particle concentrator.
2. End-over-end shaker.
3. Stomacher or blender.
4. Quantitative protein assay (e.g., Bio-Rad Protein assay kits).

2.2. Direct Labeling of Magnetic Beads with a User-Produced Antibody

1. Selected antibody in an appropriate form for attachment to beads.
2. Magnetic particles suitably coated for labeling (*see* **Note 1**).

3. Labeling buffer: phosphate buffered saline (PBS), pH 7.4 (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 dissolved in approx 800 mL dH_2O; adjust pH and make up to 1 L).
4. Wash buffer: PBS, pH 7.4, containing 0.1% BSA.

2.3. Direct Labeling of Magnetic Beads with Lectins

1. Selected lectin in an appropriate form for attachment to beads.
2. Labeling buffer: sodium borate buffer, pH 9.5 (*I*05.1 g citric acid, 30.9 g boric acid, 69.0 g NaH_2PO_4; adjust pH with conc. NaOH).
3. Wash buffer: PBS, pH 7.3, amended with 0.1% BSA.

2.4. Preenrichment for Target Bacteria

General enrichment medium (e.g., buffered peptone water) or selective enrichment medium (e.g., enterobacteriaceae enrichment broth or an antibiotic-supplemented enrichment medium).

3. Methods

3.1. Direct Labeling of Magnetic Beads with a User-Produced Antibody

1. Quantify the protein in the purified hapten suspension (antibody).
2. Suspend the antibody in labeling buffer to a final concentration of 400 µg/mL of protein.
3. Suspend the magnetic particles thoroughly and aliquot a sufficient amount into a sterile microtube (*see* **Note 2**).
4. Wash the aliquoted stock beads three times by concentrating them in the magnetic field, removing the supernatant while holding the beads in the magnetic field, and resuspend in fresh PBS labeling buffer. Finally, resuspend to a concentration approximately double that of the stock in PBS.
5. Mix equal volumes of the washed beads and the antibody (*see* **Note 3**).
6. Incubate at 4°C for 18-24 h with end-over-end shaking.
7. Concentrate the particles and wash three times in PBS/BSA wash buffer.
8. Resuspend in PBS/BSA and store at 4°C.

3.2. Direct Labeling of Magnetic Beads with Lectins

1. Suspend the lectin in labeling buffer to a final concentration of 400 µg of protein/mL in borate buffer.
2. Suspend the magnetic particles thoroughly and aliquot a sufficient amount into a sterile microtube (*see* **Note 2**).
3. Wash the aliquoted stock beads three times by concentrating them in the magnetic field, removing the supernatant while holding the beads in the magnetic field, and resuspend in fresh borate buffer. Finally, resuspend to a concentration approximately double that of the stock in borate buffer.
4. Mix equal volumes of the washed beads and the lectin (*see* **Note 3**).

5. Incubate at 4°C for 18–24 h with end-over-end shaking.
6. Concentrate the particles and wash three times in PBS/BSA.
7. Resuspend in PBS/BSA and store at 4°C.

3.3. Preparing a Cell Suspension

There are several methods available to prepare the initial cell suspension depending on the source of the target cells (*see* **Note 4**).

1. From water the cell suspension can be obtained directly from the source with no preconcentration, by centrifugation of a range of water sample volumes (10 mL to 10 L), by tangential flow filtration from larger volume up to 100 L (*see* Chapter 3) or by other methods ***(5,6)***.
2. From soil several methods are available for dissociation of bacterial cells from the soil matrix ***(12,13,19)*** thereby generating a final cell suspension (*see* **Note 5**).
3. General isolation of bacteria from foodstuffs, such as meat and cheese, involves removal of a defined weight of sample followed by homogenization (using a stomacher), and often preculture is carried out in, e.g., buffered peptone water ***(45–47,49***; *see* **Note 6**).

3.4. Separation of Target Bacteria (Note 7)

3.4.1. Using Antisera

1. For lake water ***(11)***; take a 1-mL lake-water subsample (cell suspension) from the main sample and place in a 5-mL glass test tube.
2. Add 100 µL of 10X PBS (*see* **Note 8**) and gently mix.
3. Add 30 µL of bead suspension (antimouse immunoglobulin G [IgG] sheep antibodies coupled to a *P. putida*–specific monoclonal antibody at 10^8 beads · mL^{-1}***)*** to the previous mixture and incubate at 20°C for 15 min.
4. Attract the beads and bead-cell complex to the side of the test tube by placing it in the magnetic particle concentrator (*see* **Note 9**).
5. Pipet off the supernatant and wash the beads by adding 1 mL of 1X PBS.
6. Repeat **step 5** two more times.
7. Resuspend beads and bead-cell complexes in desired final volume of 1X PBS prior to further manipulation (*see* **Note 10**).

3.4.2. Lectins

1. Lectin-bound beads ***(78)***: add 50 µL of lectin-activated beads (tosyl-activated liganded to a specific lectin; approx 10^8 beads mL^{-1}***)*** to the sample and incubate at 4°C for 3 h with end-over-end mixing (*see* **Note 11**)
2. The beads are concentrated as in **steps 4–6**.
3. Release cells from beads using competing sugars specific to the lectin in use prior to further manipulation (*see* **Note 12**).

4. Notes

1. Particles may be purchased activated for labeling (e.g., tosyl activated) or coated with protein A, or with antibodies against immunoglobulins from most animals.

2. The stability of the labeled beads may vary (depending on the hapten used), and also the preservative added by the manufacturer will be removed prior to labeling. Thus, it is preferable to label small amounts of beads and discard unused beads after 2 wk. The amount of beads labeled will therefore depend on how much work is anticipated over a 2-wk period. Some investigators have noted the presence of large particulates in some colloid preparations, which should be removed by a brief incubation in the magnetic field prior to labeling.
3. Check colloid surface area and labeling requirements.
4. Optimization experiments will require samples to be spiked with target cells; these can be added to the concentrations required.
5. The cell concentration can be adjusted by dilution or centrifugation and resuspension in a defined volume of diluent.
6. Preculture serves as an enrichment step and can be carried out on any sample type using media that specifically enriches the target bacteria. The disadvantage of this approach is that any direct quantification of the target cells will be compromised.
7. There is no universal method for immunomagnetic separation. Only by optimization can the most appropriate experimental conditions be determined.
8. 10X PBS consists of 80 g of NaCl, 2 g of KCl, 1.4 g of Na_2HPO_4, 2.4 g of KH_2PO_4 in 800 mL distilled water (pH adjusted to 7.4 with HCl, sterilized by autoclaving).
9. The magnetic particle concentrator was supplied by Dynal (Bromborough, UK); alternatively, a standard bar magnet could be used.
10. Further manipulation could involve culture (cell removal achieved by sonication or vortexing; ***[11]***) or direct counting using acridine orange ***(11)***.
11. Payne et al. ***(78)*** recommend that magnetic-bead liganded lectins are the most efficient for separating bacteria from culture and foods.
12. Recovery of cells was found to be specific, with the majority of bacterial cells released from beads by incubation with competing sugar ***(78)***. The numbers of beads:numbers of cells was found to be important for successful separation ***(88)***, and was optimized at 450 µL cell suspension with 25 µL of labeled beads (approx 0.25 mg, or $1.5–1.75 \times 10^7$ beads) for cell suspensions containing $5.0 \times 10^3–2.0 \times 10^5$ cfu mL^{-1} ***(88)***. Greater cell densities were not tested in detail, and recovery was poor using cell densities lower than 5.0×10^3 *cfu* mL^{-1}. Addition of fewer magnetic beads reduced recovery, whereas addition of greater numbers of magnetic beads did not improve recovery. Additionally, it was found that washing steps played a significant role in specific cell recovery. Washing beads after magnetic separation by vortexing in fresh buffer gave poor recoveries, with the majority of the cells being found in the supernatant after the first wash. However, washing the beads by addition of fresh buffer and either inversion or gentle aspiration through a 1-mL disposable pipet tip improved recovery, yet retained specificity as cells were removed by aspiration and incubation in the presence of competing sugar.

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References

1. Porter, J., Edwards, C., Morgan, J. A. W., and Pickup, R. W. (1993) Rapid, automated separation of specific bacteria from lake water and sewage by flow cytometry and cell sorting. *Appl. Environ. Microbiol.* **59,** 3327–3333.
2. Mitchell, J. G., Weller, R., Beconi, M., Sell, J., and Holland, J. (1993) A practical optical trap for manipulating and isolating bacteria from complex microbial communities. *Microb. Ecol.* **25,** 113–119.
3. Bradford, D., Hugenholtz, P., Seviour, E. M., Cunningham, M. A., Stratton, H., Seviour, R. J., and Blackall, L. L. (1996) 16S ribosomal RNA analysis of isolates obtained from Gram-negative, filamentous bacteria micromanipulated from activated-sludge. *Syst. Appl. Microbiol.* **19,** 334–343.
4. Markx, G. H., Huang, Y., Zhou, X.-F., and Pethig, R. (1994) Dielectrophoretic charcaterization and separation of micro-organisms. *Microbiology* **140,** 585–591.
5. Limaye, M. S., Hawkes, J. J., and Coakley, W. T. (1996) Ultrasonic standing wave removal of microorganisms from suspension in small batch systems. *J. Microbiol. Methods* **27,** 211–220.
6. Sharma, R. V., Edwards, R. T., and Beckett, R. (1993) Physical characterization and quantification of bacteria by sedimentation field-flow fractionation. *Appl. Environ. Microbiol.* **59,** 1864–1875.
7. Hopkins, D. W., O'Donnell, A. G., and MacNaughton, S. J. (1991) Evaluation of a dispersion and elutriation technique for sampling microorganisms from soil. *Soil Biol. Biochem.* **23,** 227–232.
8. Pomponi, S. A. and Cucci, T. L. (1989) Separation and concentration of phytoplankton populations using centrifugal elutriation. *Cytometry* **10,** 580–586.
9. Bifulco, J. M. and Schaefer, F. W. (1993) Antibody-magnetite method for selective concentration of Giardia lamblia cysts from water samples. *Appl. Environ. Microbiol.* **59,** 772–776.
10. Miltenyi, S., Muller, W., Weichel, W., and Radbruch, A. (1990) High gradient magnetic cell separation with MACS. *Cytometry* **11,** 231–238.
11. Morgan, J. A. W., Winstanley, C., Pickup, R. W**., and** Saunders, J. R. (1991) The rapid immunocapture of *Pseudomonas putida* cells from lakewater using bacterial flagella. *Appl. Environ. Microbiol.* **57**, 503–509.
12. Wipat, A., Wellington, E. M. H., and Saunders, V. A. (1994) Monoclonal-antibodies for *Streptomyces lividans* and their use for immunomagnetic capture of spores from soil. *Microbiology* **140,** 2067–2076.
13. Mullins, P. H., Gurtler, H., and Wellington, E. M. H. (1995) Selective recovery of *Streptosporangium fragile* from soil by indirect immunomagnetic capture. *Microbiology* **141,** 2149–2156.
14. Dye, M. (1994) The enrichment of *Rhizobium* from a model system using immunomagnetic separation. *J. Microbiol. Methods* **19,** 235–245.
15. Christensen, B., Torsvik, T., and Lien, T. (1992) Immunomagnetically captured thermophilic sulfate-reducing bacteria from North-sea-oil field waters. *Appl. Environ. Microbiol.* **58,** 1244–1248.

16. Levasseur, S., Husson, M. O., Leitz, R., Merlin, F., Laurent, F., Peladan, F., Drocourt, J. L., Leclerc, H., van Hoegaerden, M. (1992) Rapid detection of members of the family Enterobacteriaceae by a monoclonal antibody. *Appl. Environ. Microbiol.* **58,** 1524–1529.
17. Gehring, A. G., Crawford, C. G., Mazenko, R. S., van Houten, L. J., and Brewster, J. D. (1996) Enzyme-linked immunomagnetic electrochemical detection of *Salmonella typhimurium*. *J. Immunol. Methods* **195,** 15–25.
18. Jones, J. B. and van Vuurde, J. W. L. (1996) Immunomagnetic isolation of *Xanthomonas campestris* pv. *pelargonii*. *J. Appl. Bacteriol.* **81,** 78–82.
19. Porter, J., Pickup, R. W., and Edwards, C. (1997) Evaluation of flow cytometric methods for the detection and viability assessment of bacteria in soil. *Soil Biol. Biochem.* **29**, 91–100.
20. Choi, B. K., Wyss, C., and Gobel, U. B. (1996) Phylogenetic analysis of pathogen related oral spirochetes. *J. Clin. Microbiol.* **34,** 1922–1925.
21. Li, Z. M., Bai, G. H., von Reyn, C. F., Marino, P., Brennan, M. J., Gine, N., and Morris, S. L. (1996) Rapid detection of *Mycobacterium avium* in stool samples from AIDS patients by immunomagnetic PCR. *J. Clin. Microbiol.* **34,** 1903–1907.
22. van der Wolf, J. M., Hyman, L. J., Jones, D. A. C., Grevesse, C., van Beckhoven, J. R. C. M., van Vuurde, J. W. L., and Perombelon, M. C. M. (1996) Immunomagnetic separation of *Erwinia carotovora* subsp. *atroseptica* from potato peel extracts to improve detection on a crystal violet pectate medium or by PCR. *J. Appl. Bacteriol.* **80,** 487–495.
23. Stark, M., Reizenstein, E., Uhlen, M., and Lundeberg, J. (1996) Immunomagnetic separation and solid-phase detection of *Bordetella pertussis*. *J. Clin. Microbiol.* **34,** 778–784.
24. Bennett, A. R., MacPhee, S., and Betts, R. P. (1996) The isolation and detection of *Escherichia coli* O157 by use of immunomagnetic separation and immunoassay procedures. *Lett. Appl. Microbiol.* **22,** 237–243.
25. Yu, H. and Bruno, J. G. (1996). Immunomagnetic-electrochemiluminescent detection of *Escherichia coli* O157 and *Salmonella typhimurium* in foods and environmental water samples. *Appl. Environ. Microbiol.* **62,** 587–592.
26. Hartung, J. S., Pruvost, O. P., Villemot, I., and Alvarez, A. (1996) Rapid and sensitive colorimetric detection of *Xanthomonas axonopodis* pv. *citri* by immunocapture and a nested polymerase chain reaction assay. *Phytopathology* **86,** 95–101.
27. Benkirane, R. M., Guillot, E., and Mouton, C. (1995) Immunomagnetic PCR and DNA probe for detection and identification of Porphyromonas gingivalis. *J. Clin. Microbiol.* **33,** 2908–2912.
28. Enroth, H. and Engstrand, L. (1995) Immunomagnetic separation and PCR for detection of *Helicobacter pylori* in water and stool specimens. *J. Clin. Microbiol.* **33,** 2162–2165.
29. van der Wolf, J. M., van Beckhoven, J. R. C. M., de Vries, P. M., and van Vuurde, J. W. L. (1994) Verification of ELISA results by immunomagnetic isolation of antigens from extracts and analysis with SDS-PAGE and western blotting, demonstrated for Erwinia spp. in potatoes. *J. Appl. Bacteriol.* **77,** 160–168.

30. Kapperud, G., Vardund, T., Skjerve, E., Hornes, E., and Michaelsen, T. E. (1993) Detection of pathogenic *Yersinia enterocolitica* in foods and water by immunomagnetic separation, nested polymerase chain reaction and colorimetric detection of amplified DNA. *Appl. Environ. Microbiol.* **59,** 2938–2944.
31. Widjojoatmodjo, M. N., Fluit, A. C., Torensma, R., and Verhoef, J. (1993) Comparison of immunomagnetic beads coated with protein A, protein G, or goat anti-mouse immunoglobulins—applications in enzyme immunoassays and immunomagnetic separations. *J. Microbiol. Methods* **165,** 11–19.
32. Widjojoatmodjo, M. N., Fluit, A. C., Torensma, R., Keller, B. H. I., and Verhoef, J. (1991) Evaluation of the magnetic immuno PCR assay for rapid detection of *Salmonella*. *Eur. J. Clin. Microbiol. Infect. Dis.* **10,** 935–938.
33. Nese, L. and Enger, O. (1993) Isolation of *Aeromonas salmonicida* from salmon lice (*Lepeophtheirus salmonis*) and marine plankton. *Diseases of Aquatic Organisms* **16,** 79–81.
34. Islam, D., Tzipori, S., Islam, M., and Lindberg, A. A. (1993) Rapid detection of Shigella dysenteriae and Shigella flexneri in feces by an immunomagnetic assay with monoclonal antibodies. *Eur. J. Clin. Microbiol. Infect. Dis.* **12,** 25–32.
35. Islam, D., and Lindbergh, A. A. (1992) Detection of *Shigella dysenteriae* Type 1 and *Shigella flexneri* in feces by immunomagnetic isolation and polymerase chain reaction. *J. Clin. Microbiol.* **30,** 2801–2806.
36. Parmar, N., Easter, M. C., and Forsythe, S. J. (1992) The detection of Salmonella enteriditis and *Salmonella typhimurium* using immunomagnetic separation and conductance microbiology. *Lett. Appl. Microbiol.* **15,** 175–178.
37. Tomoyasu, T. (1992) Development of the immunomagnetic enrichment method selective for *Vibrio parahaemolyticus* serotype K and its application to food poisoning study. *Appl. Environ. Microbiol.* **58,** 2679–2682.
38. Luk, J. M. C. and Lindeberg, A. A. (1991) Rapid and sensitive detection of *Salmonella* (O–6, 7) by immunomagnetic monoclonal antibody-based assays. *J. Immunol. Methods* **137,** 1–8.
39. Luk, J. M. C., Kongmuang, U., Tsang, R. S. W., and Lindeberg, A. A. (1997) An enzyme-linked immunosorbent assay to detect PCR products of the rfbS gene from serogroup D salmonellae: a rapid screening prototype. *J. Clin. Microbiol.* **35,** 714–718.
40. Bard, D. G. and Ward, B. B. (1997) A species-specific bacterial productivity method using immunomagnetic separation and radiotracer experiments. *J. Microbiol. Methods* **28,** 207–219.
41. Bruno, J. G., Yu, H., Kilian, J. P., and Moore, A. A. (1996) Development of an immunomagnetic assay system for rapid detection of bacteria and leukocytes in body fluids. *J. Mol. Recognition* **9,** 474–479.
42. Besser, T. E., Hancock, D. D., Pritchett, L. C., McRae, E. M., Rice, D. H., and Tarr, P. I. (1997) Duration of detection of fecal excretion of *Escherichia coli* O157:H7 in cattle. *J. Infect. Dis.* **175,** 726–729.
43. Damgaard, P. H., Jacobsen, C. S., and Sorenson, J. (1996) Development and application of a primer set for specific detection of *Bacillus thuringiensis* and *Bacil-*

lus cereus in soil using magnetic capture hybridization and PCR amplification. *Syst. Appl. Microbiol.* **19,** 436–441.
44. Yu, H., Bruno, J. G., Cheng, T. C., Calomiris, J. J., and Goode, M. T. (1995) A comparative study of PCR detection and quantitation by electrochemiluminescence and fluorescence. *Journal of Bioluminescence and Chemiluminescence* **10,** 239–245.
45. Blake, M. R. and Weiner, B. C. (1997) Immunomagnetic detection of *Bacillus stearothermophilus* spores in food and environmnetal samples. *Appl. Environ. Microbiol.* **63,** 1643–1646.
46. Restaino, L., Frampton, E. W., Irbe, R. M., and Allison, D. R. K. (1997) A 5 h screening and 24 h confirmation procedure for detecting *Escherichia coli* O157:H7 in beef using direct epifluorescent microscopy and immunomagnetic separation. *Lett. Appl. Microbiol.* **24,** 401–404.
47. Walsh, L., Dooge, D., and Hill, C. (1997) Screening for *Escherichia coli* O157:H7 in Irish ground beef using two commercial detection systems. *Irish Vet. J.* **50,** 111.
48. Besser, T. E., Hancock, D. D., Pritchett, L. C., McRae, E. M., Rice, D. H., and Tarr, P. I. (1997) Duration of detection of fecal excretion of *Escherichia coli* O157:H7 in cattle. *J. Infect. Dis.* **175,** 726–729.
49. Gooding, C. M. and Choudary, P. V. (1997) Rapid and sensitive immunomagnetic separation-polymerase chain reaction method for the detection of *Escherichia coli* O157:H7 in raw milk and ice cream. *J. Dairy Res.* **64,** 87–93.
50. Bolton, E. J., Crozier, L., and Wilkinson, J. K. (1996) Isolation of *Escherichia coli* O157 from raw meat products. *Lett. Appl. Microbiol.* **23,** 317–321.
51. Gehring, A. G., Crawford, C. G., Mazenko, R. S., van Houten, L. J., and Brewster, J. D. (1996) Enzyme-linked immunomagnetic electrochemical detection of *Salmonella typhimurium. J. Immunol. Methods* **195,** 15–25.
52. Chapman, P. A. and Siddons, C. A. (1996) Evaluation of a commercial enzyme-immunoassay (EHEC-TEK) for detecting *Escherichia coli* O157 in beef and beef products. Food Microbiol. **13,** 175–182.
53. Chapman, P. A. and Siddons, C. A. (1996) A comparison of immunomagnetic separation and direct culture for the isolation of verocytotoxin-producing *Escherichia coli* O157 from cases of bloody, non-bloody diarrhea and asymptomatic contacts. *J. Med. Microbiol.* **44,** 267–271.
54. Karch, H., Janetzkimittmann, C., Aleksic, S., and Datz, M. (1996) Isolation of enterohaemorrhagic *Escherichia coli* O157 strains from patients with hemolytic-uremic syndrome using immuno-magnetic separation, DNA-based methods and direct culture. *J. Clin. Microbiol.* **34,** 516–519.
55. Cubbon, M. D., Coia, J. E., Hanson, M. F., and Thomson-Carter, F. M. (1996) A comparison of immunomagnetic separation, direct culture and polymerase chain reaction for the detection of verocytotoxin producing *Escherichia coli* O157 in human feces. *J. Med. Microbiol.* **44,** 219–222.
56. Sanderson, M. W., Gay, J. M., Hancock, D. D., Gay, C. C., Fox, L., and Besser, T. E. (1995) Sensitivity of bacteriological culture for detection of *Escherichia coli* O157:H7 in bovine feces. *J. Clin. Microbiol.* **33,** 2616–2619.

57. Jinneman, K. C., Trost, P. A., Hill, W. E., Weagent, S. D., Bryant, J. L., Kaysner, C. A., and Wekell, M. M. (1995) Comparison of template preparation methods from foods for amplification of *Escherichia coli* O157 Shiga-like toxins type I and type II DNA by multiplex polymerase chain reaction. *J. Food Protect.* **58,** 722–726.
58. Bennett, A. R., MacPhee, S., and Betts, R. P. (1995) Evaluation of methods for the isolation and detection of *Escherichia coli* O157 in minced beef. *Lett. Appl. Microbiol.* **20,** 375–379.
59. Weagant, S. D., Bryant, J. L., and Jinneman, K. C. (1995) An improved rapid technique for isolation of *Escherichia coli* O157:H7 from foods. *J. Food Protect.* **58,** 7–12.
60. Wright, D. J., Chapman, P. A., and Siddons, C. A. (1994) Immunomagnetic separation as a sensitive method for isolating *Escherichia coli* O157 from food samples. *Epidemiol. Infect.* **113,** 31–39.
61. Chapman, P. A., Wright, D. J., and Siddons, C. A. (1994) A comparison of immunomagnetic separation and direct culture for the isolation of verocytotoxin-producing *Escherichia coli* O157 from bovine feces. *J. Med. Microbiol.* **40,** 424–427.
62. Hanai, K., Satake, M., Nakanishi, H., and Venkateswaran, K. (1997) Comparison of commercially available kits with standard methods for detection of *Salmonella* strains in foods. *Appl. Environ. Microbiol.* **63,** 775–778.
63. Poppe, C., Elliott, L. A., and Duncan, C. L. (1996) Evaluation of immunomagnetic separation in combination with modified semi-solid Rappaport-Vassiliadis medium and Rambach agar for the isolation of *Salmonella*. *J. Microbiol. Methods* **25,** 237–244.
64. Mansfield, L. and Forsythe, S. (1996) Collaborative ring-trial of Dynabeads anti-Salmonella for immunomagnetic separation of stressed *Salmonella* cells from herbs and spices. *Int. J. Food Microbiol.* **29,** 41–47.
65. Coleman, D. J., Nye, K. J., Chick, K. E., and Gagg, C. M. (1995) A comparison of immunomagnetic separation plus enrichment with conventional *Salmonella* culture in the examination of raw sausages. *Lett. Appl. Microbiol.* **21,** 249–251.
66. Coleman, D. J., Chick, K. E., and Nye, K. J. (1995) An evaluation of immunomagnetic separation for the detection of salmonellas in raw chicken carcasses. *Lett. Appl. Microbiol.* **21,** 152–154.
67. Holt, P. S., Gast, R. K., and Greene, C. R. (1995) Rapid detection of Salmonella enteriditis in pooled liquid egg samples using a magnetic bead-ELISA system. *J. Food Protect.* **58,** 967–972.
68. Cudjoe, K. S., Hagtvedt, T., and Dainty, R. (1995) Immunomagnetic separation of *Salmonella* from foods and their detection using immunomagnetic particle (IMP) ELISA. *Int. J. Food Microbiol.* **27,** 11–25.
69. Dziadkowiec, D., Mansfield, L., and Forsythe, S. (1995) The detection of *Salmonella* in skimmed milk powder enrichments using conventional methods and immunomagnetic separations. *Lett. Appl. Microbiol.* **20,** 361–364.

70. Cudjoe, K. S., Krona, R., and Olsen, T. E. (1994) IMS—a new selective enrichment technique for detection of *Salmonella* in foods. *Int. J. Food Microbiol.* **23,** 159–165.
71. Cudjoe, K. S., Krona, R., Gron, B., and Olsen, T. E. (1994) Use of ferrous sulphate and immunomagnetic separation to recover *Salmonella enteritidis* from raw eggs. *Int. J. Food Microbiol.* **23,** 149–158.
72. Rasmussen, H. N., Rasmussen, O. F., Christensen, H., and Olsen, J. E. (1995) Detection of *Yersinia enterocolitica* O-3 in fecal samples and tonsil swabs from pigs using IMS and PCR. *J. Appl. Bacteriol.* **78,** 563–568.
73. Monceyron, C. and Grinde, B. (1994) Detection of hepatitis A virus in clinical and environmental samples by immunomagnetic separation and PCR. *J. Virol. Methods* **46,** 157–166.
74. Grinde, B., Jonassen, T. O., and Ushijima, H. (1995) Sensitive detection of group-A rotaviruses by immunomagnetic separation and reverse transcription polymerase chain reaction. *J. Virol. Methods* **55,** 327–338.
75. Lopez-Sabater, E. I., Deng, M. Y., and Cliver, D. O. (1997) Magnetic immunoseparation PCR assay (MIPA) for detection of hepatitis A virus (HAV) in American oyster (*Crassostrea virginica*). *Lett. Appl. Microbiol.* **24,** 101–124.
76. Bruno, J. G. and Yu, H. (1996) Immunomagnetic-electrochemiluminescent detection of *Bacillus anthracis* spores in soil matrices. *Appl. Environ. Microbiol.* **62,** 3474–3476.
77. Cebolla, A., Guzman, C., and de Lorenzo, V. (1996) Nondisruptive detection of activity of catabolic promoters of *Pseudomonas putida* with an antigenic surface reporter system. *Appl. Environ. Microbiol.* **62,** 214–220.
78. Payne, M. J., Campbell, S., Patchett, R. A., and Kroll, R. G. (1992) The use of immobilized lectins in the separation of *Staphylococcus aureus*, *Escherichia coli*, *Listeria* and *Salmonella* spp. from pure cultures and foods. *J. Appl. Bacteriol.* **73**, 41–52.
79. Payne, M. J., Campbell, S., and Kroll, R. G. (1993) Separation of bacteria using agglutinins isolated from invertebrates. *J. Appl. Bacteriol.* **74,** 276–283
80. Kuhn, H.-M., Meier-Dieter, U., and Mayer, H. (1988) ECA, the common enterobacterial antigen. *FEMS Microbiol. Rev.* **54,** 195–222.
81. Sompolinksy, D., Hertz, J. B., Hoiby, N., Jensen, K., Mansa, B., and Samra, Z. (1980) An antigen common to a wide range of bacteria. 1. The isolation of a 'common antigen' from *Pseudomonas aeruginosa*. *APMIS* **88,** 143–149.
82. Sompolinksy, D., Hertz, J. B., Hoiby, N., Jensen, K., Mansa, B., Pedersen, V. B., and Samra, Z. (1980). An antigen common to a wide range of bacteria. 2. A biochemical study of a 'common antigen' from *Pseudomonas aeruginosa*. *APMIS* **88,** 253–260.
83. Currie, B. P., Freundich, L. F., Soto, M. A., and Casadevall, A. (1993) False-negative cerebrospinal fluid cryptococcal latex agglutination tests for patients with culture-positive cryptococcal meningitis. *J. Clin. Microbiol.* **31**, 2519–2522.
84. Smith, M. D., Wuthiekanun, V., Walsh, A. L., and Pitt, T. L. (1993) Latex agglutination test for identification of *Pseudomonas pseudomallei. J. Clin. Pathol.* **46**, 374–375.

85. Zborowski, M., Malchesky, P. S., Jan, T.-F., and Hall, G. S. (1992) Quantitative separation of bacteria in saline solution using lanthanide Er(III) and a magnetic field. *J. Gen. Microbiol.* **138**, 63–68.
86. Harlow, E. and Lane, D. (1988) *Antibodies, a Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
87. Porter, J., Mobbs, K., Hart, C. A., Saunders, J. R., Pickup, R. W., and Edwards, C. (1997) Detection, distribution and probable fate of *Escherichia coli* O157 from asymptomatic cattle on a dairy farm. *J. Appl. Microbiol.* **83**, 297–306.
88. Porter, J., Robinson, J., Pickup, R., and Edwards, C. (1998) An evaluation of lectin-mediated magnetic bead cell sorting for the targeted separation of enteric bacteria. *J. Appl. Microbiol.*, **84,** 722–732.
89. Gattomenking, D. L., Yu, H., Bruno, J. G., Goode, M. T., Miller, M., and Zulich, A. W. (1995) Sensitive detection of biotoxoids and bacterial spores using an immunomagnetic electrochemiluminescence sensor. *Biosensors Bioelectronics* **10**, 501–507.
90. Lelwalaguruge, J., Ascencio, F., Ljungh, A., and Wadstrom, T. (1993) Rapid detection and characterization of sialic acid specific lectins of *Helicobacter pylori*. *APMIS* **101**, 695–702.
91. Millar, D. S., Withey, S. J., Tizard, M. L. V., Ford, J. G., and Hermon-Taylor, J. (1995) Solid phase hybridization capture of low abundance target DNA sequences-application to the polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium aviam* subsp *silviticum. Anal. Biochem.* **226**, 325–330.
92. Jacobsen, C. S. (1995) Microscale detection of specific bacterial-DNA in soil with a magnetic capture-hybridization and PCR amplification assay. *Appl. Environ. Microbiol.* 61, 3347–3352.
93. Regan, P. M. and Margolin, A. B. (1997) Development of a nucleic acid capture probe with reverse transcriptase-polymerase chain reaction to detect poliovirus in groundwater. *J. Virol. Methods* **64**, 65–72.
94. Damgaard, P. H. Jacobsen, C. S., and Sorensen, J. (1996) Development and application of a primer set for specific detection of *Bacillus thuringiensis* and *Bacillus cereus* in soil using magnetic capture hybridisation and PCR amplification. *Sys. Appl. Microbiol.* **19**, 436–441.
95. Benkirane, R., Guinet, R., and Delaunay, T. (1992) Purification and immunological studies of the cross reaction between the 65 kilodalton Gonococcal parietal lectin and an antigen common to a wide range of bacteria. *Infect. Immun.* **60,** 3468–3471.
96. Skjerve, E., Rorvik, L. M., and Olsvik, O. (1990) Detection of *Listeria monocytogenes* in foods by immunomagnetic separation. *Appl. Environ. Microbiol.* **56,** 3478–3481.
97. Johne, B., Jarp, J., and Haaheim, L. R. (1989) *Staphylococcus aureus* exoplolysaccharide in vivo demonstrated by immunomagnetic separation and electron microscopy. *J. Clin. Microbiol.* **27,** 1631–1635.

7

DNA Extraction from Natural Environments

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1. Introduction

Until recently, studies on microbial communities present in natural environments relied on conventional optical microscopic observation and cultivation-based approaches. Although these traditional approaches remain valuable, they have a number of limitations. The most commonly cited limitation stems from the finding that the majority of microbial cells in natural environments cannot be cultured in the laboratory—the phenomenon of nonculturability. The fraction of bacteria in soil, e.g., which can be cultured forms only approx 0.3% of the total number of cells that are observed microscopically ***(1)***. By contrast, newer methods based on the use of molecular biology methods to analyze total extracted DNA from natural specimens, potentially sample the entire population and, thus, provide a better representative picture of the total microbial community.

Various molecular approaches, none of which require prior cultivation, have been developed. Some examine cellular components such as phospholipid fatty acids ***(2)*** whereas others are directed at analyzing nucleic acids either *in situ* within cells or following prior nucleic acid extraction ***(1)***. These techniques permit a more comprehensive understanding of particular environmental issues. This chapter focuses on the extraction of DNA from natural soil and sediment samples, but the questions, techniques, and form of analysis also apply to other complex matrices such as food, organic material, animal tissues, and so on.

Any specific natural environment will usually contain a variety of prokaryotes and eukaryotes. This biotic component frequently represents a significant proportion of mass present in environmental samples and is, in addition, diverse in composition ***(3)***. Although the general importance of their activities in natu-

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ral ecosystem processes is recognized, an understanding of the complex composition, diversity, and functioning of these biotic components is necessary ***(4)***. With this understanding, the likelihood of predicting the consequences of environmental change is enhanced.

There is an underlying assumption that the DNA extraction methods used produce molecules that are representative of the target community. The choice of method should be appropriate for the biological questions being addressed and the molecular analyzes that are planned ***(5)***. Of the DNA isolation methods that have been described, there is a division between those that do and those that do not separate cells of interest from the environmental sample prior to cell lysis. In the former (*indirect*) case, this separation can be carried out by methods such as sucrose gradient centrifugation ***(6)***. More commonly, lysis of cells while still within the environmental matrix is used (*direct*). This latter approach provides an extract containing DNA from either live or dead lysed prokaryotic and eukaryotic cells in addition to any extractable extracellular DNA ***(7)*** persisting in the sample.

In practical terms, the isolated DNA must be of sufficient quality for use in the most demanding of the anticipated biological procedures. For example polymerase chain reaction (PCR) amplification requires DNA of a higher purity than that that can be used for restriction endonuclease analyzes. To optimize the quality of subsequent analyzes, certain other considerations are important. These include maximizing the lysis of target cells and recovering the maximum quantity of DNA from the extraction milieu when possible. Moreover, the isolated DNA ideally should be of high mol wt, which requires extraction procedures that minimize shearing. Other desirable features of a technique include rapidity, high sample throughput, and the use of as few steps and reagents as possible to reduce the chance of introducing contaminating material. Many DNA extraction protocols have been published. This lack of procedural uniformity stems in part from the variable nature of the samples to be analyzed, with soil and sediment samples proving particularly recalcitrant owing to enzymatic inhibitors that coextract with DNA. In this chapter, we describe a method that has reliably provided DNA suitable for PCR amplification for each of the various samples (soil, sediment, feces, plant leaf, and water) processed, and make reference where possible to other protocols that may prove more appropriate for the examination of particular samples.

2. Materials

Before embarking on DNA extraction, several factors should be considered (*see* **Note 1**). Reference is made to other methods of DNA extraction (*see* **Note 2**), and some information is provided for the extraction of RNA as well (*see* **Note 3**).

2.1. Sampling

1. Gamma-irradiated plasticware appropriate to the size and composition of the sample (*see* **Note 4**).
2. Sampling equipment relevant to the study environment (*see* **Note 5**).

2.2. DNA Extraction

1. Environmental sample (*see* **Note 6**).
2. Balance.
3. Gamma-irradiated plastic centrifuge tubes (15 and 50 mL).
4. Benchtop centrifuge suitable for 15- and 50-mL tubes.
5. Water bath (capable of operating to 70°C).
6. Bead-beating machine with glass beads and beating vessels (*see* **Note 7**).
7. Ultracentrifuge and ultracentrifuge tubes.
8. 1-mL gamma-irradiated syringes and 1.5-in. needles.
9. Ultraviolet (UV) transilluminator.
10. Ice machine.
11. Refrigerator.
12. 1.5-mL microcentrifuge tubes.
13. Microcentrifuge.

2.3. DNA Visualization

Agarose; electrophoresis buffer of choice—either Tris-borate-EDTA or Tris-acetate-EDTA, DNA stain, e.g., ethidium bromide; loading dye.

3. Methods

3.2. DNA Extraction

1. Weigh an amount of sample (1 g is suitable for soil/sediment samples) into the bead-beating vessel already containing 0.5 grams of autoclaved glass beads (*see* **Note 8**).
2. Add 5 mL of extraction buffer (*1%* sodium dodecyl sulfate in 0.12 *M* Na_2HPO_4, pH 8.0).
3. Bead beat (for this machine, 30 s on setting 1) and allow to settle for 15 min.
4. Pour contents into a 15-mL centrifuge tube and heat at 70°C for 1 h.
5. Cool rapidly on ice and centrifuge at 2800*g* for 10 min at 4°C.
6. Transfer the supernate to a 50-mL centrifuge tube and hold on ice.
7. Add 5 mL of fresh extraction buffer to the pellet.
8. Vortex to resuspend the pellet and heat at 70°C for 1 h.
9. Cool rapidly on ice and centrifuge at 2800*g* for 10 min at 4°C.
10. Transfer the supernate to the 50-mL centrifuge tube and hold on ice.
11. Repeat **steps 7–10**.
12. Centrifuge the pooled supernates at 8000*g* for 30 min at 4°C.
13. Transfer the clear supernate to a fresh 50-mL tube and add polyethylene glycol 6000 (to a final concentration of 15%) and NaCl (to 10% of the volume of the supernate).

14. Precipitate the DNA overnight at 4°C and pellet by centrifugation at 5000*g* for 10 min at 4°C.
15. Discard the supernate and resuspend the pellet in 8 mL of TE buffer (10 m*M* Tris-1 m*M* EDTA, pH 8.0).
16. Add 100 µL of 10 mg/mL ethidium bromide and 8 g of cesium chloride.
17. Transfer the contents to an ultracentrifuge tube.
18. After sealing and balancing the tubes, centrifuge for 18 h at 18°C, using 50,000 rpm in a Beckman Ti75 rotor or equivalent (Beckman, High Wycombe, UK).
19. Extract the single DNA band, visualized on the UV transilluminator, from the gradient using a sterile syringe and needle, piercing the side of the tube.
20. Shake the DNA with an equal volume of cesium chloride-saturated isopropanol.
21. Remove the (pink) layer containing ethidium bromide.
22. Repeat **steps 20** and **21** twice more.
23. Dialyze the sample overnight in TE buffer.
24. Precipitate DNA with 0.1 vol of sodium acetate ***(3 M***, pH 4.8) and 2.5 volume of ethanol (100%) overnight at 4°C.
25. Pellet the DNA by centrifugation at 13,000*g* for 30 min at 4°C.
26. Remove the supernate and wash the DNA pellet twice with 70% ethanol.
27. Resuspend in an appropriate volume of water (e.g., 100 µL) (*see* **Note 9**).

3.3. DNA Visualization

1. Prepare an agarose gel (0.7% agarose in TBE or TAE buffer) containing 2 µL of 10 mg/mL ethidium bromide.
2. Load a portion (e.g., 10 µL of sample) and appropriate mol wt markers (e.g., kilobase ladder) into the agarose gel.
3. Following electrophoresis at 100 V for 1 h, inspect the gel, using a UV transilluminator, for the size and integrity of DNA extracted (*see* **Note 10**).
4. Estimation can then be made of the quantity of material extracted (*see* **Note 11**).

4. Notes

1. The design and execution of the sampling regime is of fundamental importance; consider factors such as the spatial arrangement and need for replicate samples to ensure statistical consistency and reduce variability also, remember that natural environments are in flux and that it may not be possible to repeat a sampling regime.
2. Monitoring is important in a wide number of environments, with different environments varying greatly in the number of microbes present and the nature of the environmental matrix.
   a. Sequences of microbial origin (viral, archaeal, bacterial, and/or fungal) have been amplified from DNA isolated from *terrestrial environments* such as soils ***(8–14)***, peat bog material ***(15,16)***, landfill ***(17)***, and subsurfaces ***(18)***; from *aquatic environments* such as seawater ***(19,20)***, freshwater sediments ***(21,22)***, hot spring sediments ***(23)***, marine sediments ***(9,24)***, biofilm microsections ***(25)***, marine microbial mats ***(26)***, hydrothermal vents ***(27)***, and hypersaline

lakes ***(28)***; from *clinical samples* ***(29)*** including: dental plaque ***(30)***, blood and blood products ***(31,32)***, bronchoalveolar lavage fluids ***(33)***; from *food* ***(34)*** including: milk ***(35)***, oysters ***(36)***, and cheese and sausage ***(37)***; and *from plants* ***(38,39)*** including: cankered wood ***(40)***, seagrass leaves ***(41)***, the fungal component of lichens ***(42)***, and lichens and their symbionts ***(43)***.

b. Methods have been developed for specific applications, e.g., extracting microbial DNA free from "contaminating" plant DNA from compost ***(44)*** or the use of differences in the specific gravity of fungal spores to separate mixtures of different fungal genera prior to DNA extraction ***(45)***. Yet, other methods have been developed to extract DNA from many plant, bacterial, and fungal species ***(46,47)***. Some methods, such as that of Kehrmeyer et al. ***(48)***, couple the extraction of DNA with other cellular components such as lipids, from the same soil or sediment sample.

3. Studies investigating biological or biochemical function within environments are becoming increasingly common as the technology advances and the need for environmental monitoring increases. This approach also adds an important new dimension to studies of biological flux in microbial ecology. Moran et al. ***(49)*** showed that rRNA could be recovered from bacteria in various environmental samples including sediment and soil. A hydroxyapatite spin-column method has been shown to be effective in extracting DNA and RNA rapidly from natural sediments ***(50)***. It has also been shown that nucleic acid extracts can be used to compare the active and total bacterial community using temperature-gradient gel electrophoresis profiles of ribosomal sequences ***(51)***.
4. Gamma-irradiated, plastic, Universal screw-capped containers and centrifuge tubes, such as Falcon tubes (e.g., Alpha Labs, Eastleigh, UK), are used routinely for sampling soil and surface sediments of up to 15 g, with grass and other surface plant growth first removed to expose bare soil.
5. The sampling equipment used varies according to the environment, the sample size, and the depth of sample to be taken. These can range from flamed spatulas, coring devices (surface soil and sediment sampling), boring equipment (deep subsurface, aquifer, and so on), to tangential flow filtration units (water samples). In the latter example, cell concentration, e.g., using micropore membranes or by centrifugation, is often required to overcome problems of the low microbial biomass in natural waters.
6. Samples should be processed as soon as possible to avoid changes during storage. When storage is essential, samples are frozen at –20°C or below, if possible. Prolonged storage of samples at 4°C can result in changes in detection level (*see* **ref. *52***). Samples for RNA extraction should be processed quickly and not stored.
7. A B. Braun cell homogenizer is used here with 0.17–0.18-mm diameter glass beads selected for bacterial cell lysis.
8. The method described here is modified from previous studies ***(9,53,54)***. To ensure that the method provides a representative sample of DNA from the environmental sample—the original reason for taking a mo lecular approach—it is important to lyse the cells as efficiently as possible. This method combines physi-

cal (bead beating and heat shocking) as well as chemical (SDS) methods to maximize lysis.

Other techniques have been described to increase the proportion of cells lysed including the use of small ***(1***00 mg) soil samples ***(55)***, and increasing bead-beating time to disrupt indigenous bacteria ***(56)***. Proteinase K treatment (*see* **ref. *57***) and freeze-thawing cycles (*see* **ref. *6***) have also been incorporated into different protocols to increase cell lysis. Although estimates vary considerably, up to 96% of cells present in samples of marine sediments have been lysed in one study ***(24)***.

9. This removes many substances inhibitory to enzymes used in molecular biological procedures. Humic acids, found frequently in samples from natural environments, are known inhibitors of enzymes such as *Taq* DNA polymerase ***(58)***. Compounds such as polyvinyl polypyrrolidone, which reduces the effect of inhibitory substances by absorbing humic compounds ***(38,59,60)***, or hexadecyltrimethylammonium bromide which overcomes the effects of inhibitory chemicals that coextract with DNA ***(6,61,62)***, have been recommended in other DNA extraction procedures. Other purification steps which have been used include; agarose gel electrophoresis (*see* **refs. *46,63,64)***, electroelution ***(22,65)***, passage through Elutip d columns (Schleicher and Schuell, Kingston-upon-Thames, UK) ***(55)***, ion exchange chromatography ***(66)***, and magnetic capture-hybridization ***(67)***.
10. The requirement is for the extracted DNA to be of high *quality*—both high purity and high mol wt. DNA that has been sheared to a significant extent may generate more chimeric sequences during PCR amplification as a result of the coamplification of homologous genes ***(68)***. In practical terms, this can lead to the description of nonexistent species or the misinterpretation of the level of bacterial diversity.
11. The *yield* of total DNA varies widely according to the extraction process and the environmental sample. Methods are available that can be used to quantify the extracted nucleic acids and that will also provide an estimate of the purity of the sample. As examples, van Elsas et al. ***(56)*** found that yields of total DNA, extracted from five different soils, varied from 2 to 35 µg/g of soil. Similarly, a range of between 2.5 and 26.9 µg/g of soil was found for eight soils of different organic carbon, clay content, and pH ***(57)***, and in a separate study, yields from different soils ranged between 6.1 and 54.0 µg/g of soil ***(62)***. For sediments, 47 µg/g of sediment has been recovered ***(24)***.

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References

1. Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microb. Rev.* **59,** 143–169.
2. Bardgett, R. D., Leemans, D. K., Cook, R., and Hobbs, P. J. (1997) Seasonality of the soil biota of grazed and ungrazed hill grasslands. *Soil Biol. Biochem.* **29,** 1285–1294.

3. Killham, K. (1994) *Soil Ecology.* Cambridge Unversity Press, Cambridge, UK.
4. O'Donnell, A. G., Goodfellow, M., and Hawksworth, D. L. (1994) Theoretical and practical aspects of the quantification of biodiversity among microorganisms. *Phil. Trans. Roy. Soc.* B. **345,** 65–73.
5. Leff, L. G., Dana, J. R., McArthur, J. V., and Shimkets, L. J. (1995) Comparison of methods of DNA extraction from stream sediments. *Appl. Environ. Microbiol.* **61,** 1141–1143.
6. Tsushima, S., Hasebe, A., Komoto, Y., Carter, J. P., Miyashita, K., Yokoyama, K., and Pickup, R. W. (1995) Detection of genetically engineered microorganisms in paddy soil using a simple and rapid nested Polymerase Chain-Reaction method. *Soil Biol. Biochem.* **27,** 219–227.
7. Trevors, J. T. (1996) DNA in soil—adsorption, genetic-transformation, molecular evolution and genetic microchip. *Antonie van Leeuwenhoek Int J. Gen. Mol. Microbiol.* **70,** 1–10.
8. Liesack, W., and Stackebrandt, E. (1992) Occurrence of novel groups of the domain bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *J. Bacteriol.* **174,** 5072–5078.
9. Bruce, K. D., Hiorns, W. D., Hobman, J. L., Osborn, A. M., Strike, P., and Ritchie, D. A. (1992) Amplification of DNA from native populations of soil bacteria by using the Polymerase Chain Reaction. *Appl. Environ. Microbiol.* **58,** 3413–3416.
10. Smalla, K., Cresswell, N., Mendoncahagler, L. C., Wolters, A., and vanElsas, J. D. (1993) Rapid DNA extraction protocol from soil for Polymerase Chain Reaction mediated amplification. *J. Appl. Bacteriol.* **74,** 78–85.
11. Silva, M. C., More, M. I., and Batt, C. A. (1995) Development of a molecular detection method for naphthalene degrading pseudomonads. *FEMS Microbiol. Ecol.* **18,** 225–235.
12. Ueda, T., Suga, Y., and Matsuguchi, T. (1995) Molecular phylogenetic analysis of a soil microbial community in a soybean field. *Eur. J. Soil Sci.* **46,** 415–421.
13. Borneman, J., Skroch, P. W., O'Sullivan, K. M., Palus, J. A., Rumjanek, N. G., Jansen, J. L., Nienhuis, J., and Triplett, E. W. (1996) Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl. Environ. Microbiol.* **62,** 1935–1943.
14. Rosado, A. S., Seldin, L., Wolters, A. C., and vanElsas, J. D. (1996) Quantitative 16S rDNA targeted polymerase chain-reaction and oligonucleotide hybridization for the detection of *Paenibacillus azotofixans* in soil and the wheat rhizosphere. *FEMS Microbiol. Ecol.* **19,** 153–164.
15. Hales, B. A., Edwards, C., Ritchie, D. A., Hall, G., Pickup, R. W., and Saunders, J. R. (1996) Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Appl. Environ. Microbiol.* **62,** 668–675.
16. Rheims, H., Sproer, C., Rainey, F. A., and Stackebrandt, E. (1996) Molecular biological evidence for the occurrence of uncultured members of the *Actinomycete* line of descent in different environments and geographical locations. *Microbiology* **142,** 2863–2870.

17. Wyndham, R. C., Nakatsu, C., Peel, M., Cashore, A., Ng, J., and Szilagyi, F. (1994) Distribution of the catabolic transposon Tn*5271* in a groundwater bioremediation system. *Appl. Environ. Microbiol.* **60,** 86–93.
18. Boivinjahns, V., Ruimy, R., Bianchi, A., Daumas, S., and Christen, R. (1996) Bacterial diversity in a deep-subsurface clay environment. *Appl. Environ. Microbiol.* **62,** 3405–3412.
19. Tamanaishacoori, Z., Jolivetgougeon, A., and Cormier, M. (1996) Comparison of direct PCR and PCR amplification after DNA extraction for the detection of vaible enterotoxigenic *Escherichia coli* in laboratory microcosms. *J. Microbiol. Methods* **26,** 21–26.
20. Teske, A., Wawer, C., Muyzer, G., and Ramsing, N. B. (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR amplified ribosomal DNA fragments. *Appl. Environ. Microbiol.* **62,** 1405–1415.
21. Rochelle, P. A., Fry, J. C., Parkes, R. J., and Weightman, A. J. (1992) DNA extraction for 16S ribosomal RNA gene analysis to determine genetic diversity in deep sediment communities. *FEMS Microbiol. Lett.* **100,** 59–65.
22. Chandler, D. P., Fredrickson, J. K., and Brockman, F. J. (1997) Effect of PCR template concentration on the composition and distribution of total community 16S rDNA clone libraries. *Mol. Ecol.* **6,** 475–482.
23. Barns, S. M., Fundyga, R. E., Jeffries, M. W., and Pace, N. R. (1994) Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proc. Natl. Acad. Sci. USA* **91,** 1609–1613.
24. Gray, J. P. and Herwig, R. P. (1996) Phylogenetic analysis of the bacterial communities in marine sediments. *Appl. Environ. Microbiol.* **62,** 4049–4059.
25. Taylor, D. G., Breen, A., and Bishop, P. L. (1997) Determination of phenol degrader distribution in biofilms using gene probes. *Water Res.* **31,** 119–129.
26. Muyzer, G., Dewaal, E. C., and Uitterlinden, A. G. (1993) Profiling of complex microbial-populations by denaturing gradient gel electrophoresis analysis of Polymerase Chain Reaction amplified genes coding for 16S ribosomal RNA. *Appl. Environ. Microbiol.* **59,** 695–700.
27. Muyzer, G., Teske, A., Wirsen, C. O., and Jannasch, H. W. (1995) Phylogenetic-relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel-electrophoresis of 16S rDNA fragments. *Arch. Microbiol*. **164,** 165–172.
28. Martinez-Murcia, A. J., Acinas, S. G. and Rodriguez-Valera, F., (1995) Evaluation of prokaryotic diversity by restriction digestion of 16S rDNA directly amplified from hypersaline environments. *FEMS Microb. Ecol.* **17,** 247–255.
29. Delamballerie, X., Zandotti, C., Vignoli, C., Bollet, C. and Demicco, P. (1992) A one step microbial DNA extraction method using Chelex–100 suitable for gene amplification. *Res. Microbiol.* **143,** 785–790.
30. Parrish, K. D. and Greenberg, E. P. (1995). A rapid method for extraction and purification of DNA from dental plaque. *Appl. Environ. Microbiol.* **61,** 4120–4123.

31. Golbang, N., Burnie, J. P., Klapper, P. E., Bostock, A. and Williamson, P. (1996) Sensitive and universal method for microbial DNA extraction from blood products. *J. Clin. Pathol.* **49,** 861–863.
32. Tagliaferro, L., Corbelli, M., Maietta, G., Pellegrino, V., and Pignatelli, P. (1995) Use of a rapid and simple method to extract proviral DNA in the identification of HIV–1 by PCR. *Microbiologica* **18,** 303–306.
33. Jaulhac, B., Nowicki, M., Bornstein, N., Meunier, O., Prevost, G., Piemont, Y., Fleurette, J., and Monteil, H. (1992) Detection of *Legionella* spp. in bronchoalveolar lavage fluids by DNA amplification. *J. Clin. Microbiol.* **30,** 920–924.
34. Candrian, U. (1995) Polymerase Chain Reaction in food microbiology. *J. Microbiol. Methods* **23,** 89–103.
35. Tola, S., Angioi, A., Rocchigiani, A. M., Idini, G., Manunta, D., Galleri, G., and Leori, G. (1997) Detection of *Mycoplasma agalactiae* in sheep milk samples by Polymerase Chain Reaction. *Vet. Microbiol.* **54,** 17–22.
36. Brauns, L. A., Hudson, M. C., and Oliver, J. D. (1991) Use of the Polymerase Chain-Reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. *Appl. Environ. Microbiol.* **57,** 2651–2655.
37. Ehrmann, M., Ludwig, W., and Schleifer, K. H. (1994) Reverse dot blot hybridization—a useful method for the direct identification of Lactic Acid bacteria in fermented food. *FEMS Microbiol. Lett.* **117,** 143–150.
38. Audy, P., Braat, C. E., Saindon, G., Huang, H. C., and Laroche, A. (1996) A rapid and sensitive PCR based assay for concurrent detection of bacteria causing common and halo blights in bean seed. *Phytopathology* **86,** 361–366.
39. Volossiouk, T., Robb, E. J., and Nazar, R. N. (1995) Direct DNA extraction for PCR mediated assays of soil organisms. *Appl. Environ. Microbiol.* **61,** 3972–3976.
40. Brown, A. E., Muthumeenakshi, S., Sreenivasaprasad, S., Mills, P. R., and Swinburne, T. R. (1993) A PCR primer specific to *Cylindrocarpon heteronema* for detection of the pathogen in apple wood. *FEMS Microbiol. Lett.* **108,** 117–120.
41. Weidner, S., Arnold, W., and Puhler, A. (1996) Diversity of uncultured microorganisms associated with the seagrass *Halophila stipulacea* estimated by Restriction Fragment Length Polymorphism analysis of PCR amplified 16S ribosomal RNA genes. *Appl. Environ. Microbiol.* **62,** 766–771.
42. Grube, M., Depriest, P. T., Gargas, A., and Hafellner, J. (1995) DNA isolation from lichen ascomata. *Mycol. Res.* **99,** 1321–1324.
43. Armaleo, D. and Clerc, P. (1995) A rapid and inexpensive method for the purification of DNA from lichens and their symbionts. *Lichenologist* **27,** 207–213.
44. Malik, M., Kain, J., Pettigrew, C., and Ogram, A. (1994) Purification and molecular analysis of microbial DNA from compost. *J. Microbiol. Methods* **20,** 183–196
45. Okuda, T., Yanagisawa, A., Fujimori, F., Nishizuka, Y., Takehana, Y., and Sugiyama, M. (1995) New isolation methods and Polymerase Chain Reaction strain discrimination techniques for natural products screening programs. *Can. J. Bot.* **73,** S 946-S 954.
46. Porteous, L. A., Armstrong, J. L., Seidler, R. J., and Watrud, L. S. (1994) An effective method to extract DNA from environmental samples for polymerase

chain reaction amplification and DNA fingerprint analysis. *Curr. Microbiol*. **29,** 301–307.

47. Rudi, K., Kroken, M., Dahlberg, O. J., Deggerdal, A., Jakobsen, K. S., and Larsen, F. (1997) Rapid, universal method to isolate PCR ready DNA using magnetic beads. *Biotechniques* **22,** 506–511.
48. Kehrmeyer, S. R., Applegate, B. M., Pinkart, H. C., Hedrick, D. B, White, D. C., and Sayler, G. S. (1996) Combined Lipid/DNA extraction method for environmental samples. *J. Microbiol. Methods* **25,** 153–163.
49. Moran, M. A., Torsvik, V. L., Torsvik, T., and Hodson, R. E. (1993) Direct extraction and purification of ribosomal RNA for ecological-studies. *Appl. Environ. Microbiol.* **59,** 915–918.
50. Purdy, K. J., Embley, T. M., Takii, S., and Nedwell, D. B. (1996) Rapid extraction of DNA and ribosomal RNA from sediments by a novel hydroxyapatite spin-column method. *Appl. Environ. Microbiol.* **62,** 3905–3907.
51. Felske, A., Engelen, B., Nubel, U., and Backhaus, H. (1996) Direct ribosome isolation from soil to extract bacterial ribosomal RNA for community analysis. *Appl. Environ. Microbiol.* **62,** 4162–4167.
52. Josephson, K. L., Gerba, C. P., and Pepper, I. L. (1993) Polymerase chain-reaction detection of nonviable bacterial pathogens. *Appl. Environ. Microbiol.* **59,** 3513–3515.
53. Selenska, S., and Klingmuller, W. (1991) DNA recovery and direct detection of Tn*5* sequences from soil. *Lett. Appl. Microbiol.* **13,** 21–24.
54. Bruce, K. D., Osborn, A. M., Pearson, A. J., Strike, P., and Ritchie, D. A. (1995) Genetic diversity within *mer* genes directly amplified from communities of noncultivated soil and sediment bacteria. *Mol. Ecol.* **4,** 605–612.
55. Picard, C., Ponsonnet, C., Paget, E., Nesme, X., and Simonet, P. (1992) Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain-reaction. *Appl. Environ. Microbiol.* **58,** 2717–2722.
56. vanElsas, J. D., Mantynen, V., and Wolters, A. C. (1997) Soil DNA extraction and assessment of the fate of *Mycobacterium chlorophenolicum* strain PCP-1 in different soils by 16S ribosomal RNA gene sequence based most-probable-number PCR and immunofluorescence. *Biol. Fertil. Soils* **24,** 188–195.
57. Zhou, J. Z., Bruns, M. A., and Tiedje, J. M. (1996) DNA recovery from soils of diverse composition. *Appl. Environ. Microbiol.* **62,** 316–322.
58. Tebbe, C. C. and Vahjen, W. (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Appl. Environ. Microbiol.* **59,** 2657–2665.
59. Porteous, L. A. and Armstrong, J. L. (1993) A simple mini-method to extract DNA directly from soil for use with Polymerase Chain Reaction amplification. *Curr. Microbiol*. **27,** 115–118.
60. Berthelet, M., Whyte, L. G., and Greer, C. W. (1996) Rapid, direct extraction of DNA from soils for PCR analysis using polyvinylpolypyrrolidone spin columns. *FEMS Microbiol. Lett.* **138,** 17–22.

61. Cho, J. C., Lee, D. H., Cho, Y. C., Cho, J. C., and Kim, S. J. (1996) Direct extraction of DNA from soil for amplification of 16S ribosomal RNA gene sequences by Polymerase Chain-Reaction. *J. Microbiol.* **34,** 229–235.
62. Wikstrom, P., Wiklund, A., Andersson, A. C., and Forsman, M. (1996) DNA recovery and PCR quantification of catechol **2,**3-dioxygenase genes from different soil types. *J. Biotechnol.* **52,** 107–120.
63. Erb, R. W., and Wagnerdobler, I. (1993) Detection of polychloriated biphenyl degradation genes in polluted sediments by direct DNA extraction and polymerase chain-reaction. *Appl. Environ. Microbiol.* **59,** 4065–4073.
64. Herrick, J. B., Madsen, E. L., Batt, C. A., and Ghiorse, W. C. (1993) Polymerase Chain Reaction amplification of naphthalene catabolic and 16S ribosomal RNA gene sequences from indigenous sediment bacteria. *Appl. Environ. Microbiol.* **59,** 687–694.
65. Vescio, P. A. and Nierzwickibauer, S. A. (1995) Extraction and purification of PCR amplifiable DNA from lacustrine subsurface sediments. *J. Microbiol. Methods* ***21,*** 225–233.
66. Lovell, C. R. and Piceno, Y. (1994) Purification of DNA from estuarine sediments. *J. Microbiol. Methods* **20,** 161–174.
67. Jacobsen, C. S. (1995) Microscale detection of specific bacterial DNA in soil with a magnetic capture-hybridization and PCR amplification assay. *Appl. Environ. Microbiol.* ***61,*** 3347–3352.
68. Wang, G. C. Y. and Wang, Y. (1996) The frequency of chimeric molecules as a consequence of PCR coamplification of 16S ribosomal RNA genes from different bacterial species. *Microbiology* **142,** 1107–1114.

8

Automated Sequencing of DNA Retrieved from Environmental Samples

Mathew Upton

1. Introduction

The advent of molecular techniques has revolutionized our understanding of microbial ecology, and their use in environmental microbiology is widespread. Many of the recent studies investigating the microbial flora of diverse ecosystems have adopted a common approach of targeted amplification of gene sequences from total extracted DNA using the polymerase chain reaction (PCR) followed by cloning and sequence analysis of amplimers. The study of 16S rRNA gene sequences has dominated these studies ***(1,2)***. Such studies have developed so rapidly because of the availability of automated sequencing techniques, which greatly reduce the processing time of retrieved DNA molecules and allow the analysis of a sufficient number of clones to make findings more representative of the diversity present within a system. This chapter covers the principles and use of automated DNA sequencing systems.

1.1. Principles of Automated Sequencing of DNA

Although several automated DNA sequence analysis systems are presently available (e.g., LiCor; Pharmacia LKB, Uppsala, Sweden), the information presented in this chapter is based on use of the Applied Biosystems ([ABI] Perkin-Elmer, Warrington, UK) 373A DNA Sequencing System because it is the most commonly used machine.

Automated DNA sequencing strategies are based on the chain termination method conceived by Sanger et al. ***(3)*** and follow the general protocol shown in **Fig. 1**. DNA polymerase is used to copy a single-stranded DNA (ssDNA) template molecule by adding deoxynucleotide triphosphates (dNTP) to the 3' end of a primer whose target is located 5' of the region of DNA to be sequenced. In addition to template DNA, DNA polymerase, buffer, primer, and dNTPs, the

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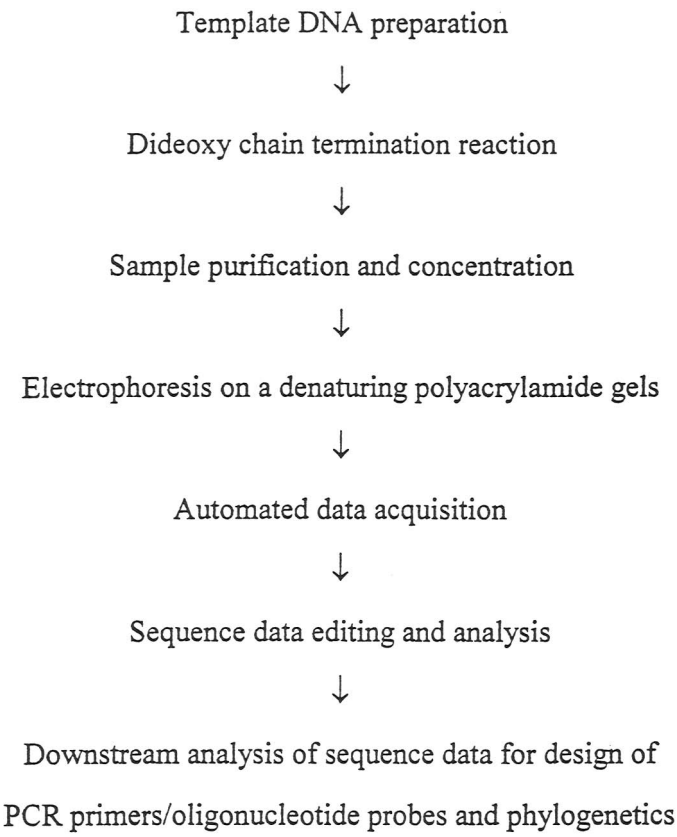

Fig. 1. A schematic representation of the processes involved in the analysis of DNA samples from environmental samples.

reaction mixture contains dideoxynucleotide triphosphates (ddNTP), which will be randomly incorporated into the synthesized DNA strand. Since DNA polymerases require a free 3' hydroxyl group for the enzymatic formation of a phosphodiester bond with incoming dNTPs, incorporation of a ddNTP results in chain termination, and reaction tubes will contain a mixture of synthesized DNA strands of different lengths.

Classically, ddNTP molecules were labeled with radioisotopes, and separate reactions carried out for each of the four ddNTPs (ddATP, ddCTP, ddGTP, and ddTTP) were subject to electrophoresis on adjacent lanes of a polyacrylamide gel. The development of fluorescent dye labeling of dNTPs for DNA sequencing ***(4,5)*** allows single reaction vessels and electrophoresis in the same lane, because each ddNTP is conjugated to a reporter molecule that emits light

at a unique wavelength when excited by laser light. The use of *Taq* polymerase allows double-stranded DNA (dsDNA) to be thermally denatured and results in linear amplification of synthesized DNA. This cycle sequencing approach gives greater yield and more accurate results than the standard reactions with heat-labile DNA polymerase.

A variation of the dye terminator method detailed previously uses fluorescently labeled primers and unlabeled ddNTPs to produce terminated DNA strands. Dye primer sequencing requires four reaction vessels, one for each ddNTP, although the products are combined and subject to electrophoresis in a single lane of the gel. Cycle sequencing can be performed using dye primers and *Taq* polymerase to increase signal strength. Dye primer sequencing is a favorable approach when cloning with vectors containing targets for standard dye primers such as -21M13, M13 Reverse, and so on, because labeled primers are commercially available (ABI).

The selection of a particular sequencing chemistry is dictated by the requirements and experience of the operator and the type of template to be analyzed. *Taq* polymerase cycle sequencing has the obvious advantage of increasing product yield, and the elevated reaction temperature reduces nonspecific primer annealing and the effects of secondary structure in template molecules. Although dye primer sequencing requires a reaction vessel for each ddNTP, increasing cost and the risk of operator error, and secondary structures can cause false stops, the approach gives accurate (98%) readings of DNA sequence up to 500 bp.

Dye terminator cycle sequencing with *Taq* polymerase is perhaps the most commonly used technique and is best suited for analysis of PCR products. Dye terminator sequencing has several advantages over primer sequencing: the reaction can be performed in one tube; false stops will not be detected by analysis software; and accurate (98%) sequence data can be obtained from most templates. Owing to the popularity of the dye terminator approach, this chapter focuses on this technique.

Following the chain termination reaction, irrespective of the chemistry used, samples are loaded on single lanes in a polyacrylamide denaturing gel and subjected to vertical electrophoresis for up to 12 h, separating the labeled DNA fragments according to size. Standard gels contain either 4.75 or 6.0% acrylamide. Gels cast with 4.75% acrylamide are useful for determining the sequence of bases close to the primer, and 6.0% gels will give good resolution of bases distal to the primer. During electrophoresis, a laser beam scans across the gel 2400 times per hour. On each scan, the laser passes through one of four filters exciting the reporter molecules, and fluorescence is detected by a photomultiplier tube. An electronic signal corresponding to the type and amount of fluorescence detected is sent to the data analysis software and stored for future

processing. When data collection is complete, automated analysis is initiated, and the results are stored as edited data files and printed as a chromatogram of the fluorescence peaks detected. Edited data files can be further analyzed using Macintosh software or exported in a PC format for use on compatible machines. Sequence data can then be imported into one of several programs available commercially, or via the Internet, for the design of PCR primers and oligonucleotides.

2. Materials

2.1. Preparation of Template DNA from PCR

1. Sterile, ultrapure distilled water (dH_2O) and 0.5-mL microfuge tubes.
2. Reagents for PCR: *Taq* polymerase and buffer, dNTPs, primers specific to target gene, and mineral oil.
3. Agarose (e.g., Sigma, Poole, UK) and running buffer of choice or reagents for oligonucleotide probing (e.g., Boehringer Mannheim DIG Labeling and Detection Kit).
4. Materials for purification of DNA template either by electrophoresis through low melting point agarose (e.g., SeaPlaque or SeaPrep, FMC BioProducts, Rockland, ME) or by centrifugation (e.g., Centricon-100 Micro-Concentrator columns; MicroSpin S-400 HR Columns, Pharmacia Biotech; QIAquick PCR Purification Columns, QIAGEN).

2.2. Alkaline Lysis Isolation of Vector

1. Solution I: 50 m*M* glucose, 25 m*M* Tris-HCl, pH 8.0, 10 m*M* ethylenediaminetetraacetic acid (EDTA), autoclave at 10 lb/in^2 for 15 min.
2. Solution II: 0.2 *N* NaOH, from 10 *N* stock, 1% sodium dodecyl sulfate, from 20% (v/v) stock.
3. 5 *M* potassium acetate (pH 4.8): Add 11.5 mL of glacial acetic acid and 28.5 mL of H_2O to 60 mL of 5 *M* potassium acetate and mix well.
4. Solutions of 5 *M* NaCl and 13% PEG_{8000} sterilized by autoclaving.
5. 95 and 70% ethanol.
6. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0.
7. Tris-saturated phenol.
8. Chloroform.
9. 3 *M* sodium acetate solution, pH 5.2.

2.3. Cycle Sequencing Reactions with Dye Terminators

1. Dye Terminator Ready Reaction Cycle Sequencing Kit (ABI).
2. Reagents for PCR: 0.5-mL microfuge tubes, *Taq* polymerase and buffer, dNTPs, primers (either specific to the target gene or the vector), and mineral oil.

2.4. Electrophoresis of Samples Using Denaturing Polyacrylamide Gels

1. dH_2O, warm dH_2O, Alconox (Alconox, New York, NY) and lint-free paper.
2. Permacel tape (Permacel, Preferred Tape Inc., Tulsa, OK) and clamps for gel plates.

3. 40% acrylamide solution: 19:1 acrylamide:bis-acrylamide.
4. Mixed-bed ion exchange resin (Sigma, Poole, UK).
5. 10% (w/v in dH_2O) freshly prepared ammonium persulfate solution.
6. *N,N,N′,N′*-Tetramethylethylenediamine (TMED, Sigma).
7. 10X TBE buffer: 108 g Tris, 55 g boric acid, 8.3 g Na_2EDTA, 1 L dH_2O. Check that the pH is 8.3 and prepare again if the pH is different.
8. Blue dextran loading buffer: 1 µL loading buffer (50 m*M* EDTA, pH 8.0, 30 mg/mL blue Dextran) and 5 µL deionized formamide. Formamide prepared by mixing 10 mL of formamide with 1 g of mixed bed resin for 15–20 min, filtering through paper and storing in aliquots at –20°C.

3. Methods

The following section details the techniques required for preparation of DNA template produced by PCR and the subsequent automated analysis on an ABI 373A Sequencing System following dye terminator cycle sequencing reactions. The protocols have been adapted from the user's manual, which can be referred to for more detailed information and for descriptions of preparation and analysis of DNA template from other sources.

3.1. Preparation of Template DNA from PCR

Poor quality template is the most common cause of error in DNA sequencing, and preparation should be performed with meticulous attention to detail of the procedures used. The quantity of template will also affect the outcome of sequencing reactions and has to be optimized for production of consistent data (*see* **Note 1**).

3.1.1. Reamplification of Template DNA from Bacterial Colonies

1. Suspend single colonies arising from cloning experiments in 100 µL sterile double-distilled H_2O (ddH_2O).
2. Heat to 98°C for 10 min.
3. Use 1–5 µL as template in PCR using the primers and conditions of the original amplification reaction.
4. Check for presence of inserts by gel electrophoresis or oligonucleotide probing.
5. Remove unused enzyme, buffer, primers, and dNTPs from template DNA by gel electrophoresis or centrifugation through one of the many commercially available columns.
6. Quality and quantity of the DNA can be reassessed by gel electrophoresis with a mol wt standard.

*3.1.2. Isolation of Vector (Alkaline Lysis Procedure) (*see ***Note 2****)*

1. Harvest the cells from a 500-mL culture by centrifugation at 6000*g*, and resuspend the pellet in 10 mL of Solution I containing 5 mg/mL of lysozyme.
2. Stand at room temperature for 5 min in a Beckman (Palo Alto, CA) SW27 polyallomer tube (or equivalent).

3. Add 20 mL of freshly prepared Solution II; mix the contents by inverting the tube gently and stand on ice for 10 min.
4. Add 15 mL of ice-cold 5 *M* potassium acetate (pH 4.8) and stand on ice for 10 min.
5. Centrifuge the tube at 23,000g for 20 min at 4°C.
6. Transfer equal quantities of the supernatant to each of two 30-mL Corex tubes and add 0.16 vol of 5 *M* NaCl and an equal volume of PEG_{8000} (Sigma). Mix well and stand on ice for 20 min.
7. Pellet the DNA by centrifugation at 12,000*g* for 30 min at room temperature (*see* **Note 3**).
8. Wash the pellet in 70% ethanol at room temperature and dry under vacuum.
9. Resuspend the DNA in 400 µL of TE buffer.
10. Add an equal volume of phenol; mix and centrifuge for 1 min.
11. Remove the aqueous phase, add 400 µL of chloroform, mix the solution, and centrifuge for 30 s. Repeat this step once (*see* **Note 4**).
12. Add 40 µL of sodium acetate and 1 mL of 95% ethanol and cool to –70°C for 20 min.
13. Remove all traces of ethanol and dry under vacuum. Resuspend the DNA pellet in 20 µL of TE buffer.

3.2. Cycle Sequencing Reactions with Dye Terminators

1. Add 4 µL of cocktail mix from the Dye Terminator Ready Reaction Cycle Sequencing Kit (ABI) to sterile 0.5-mL microfuge tubes.
2. Add 1 µL of 20 pmol primer to each tube (*see* **Note 5**).
3. Add template DNA to individual tubes at the following concentrations: PCR product, 20–200 ng; ssDNA, 0.25–0.50 µg; dsDNA, 1.0 µg.
4. Place tubes in a PCR thermal cycling machine and perform the cycle sequencing reactions under the following conditions: 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min; soak at 4°C.

3.3. Electrophoresis of Samples on Denaturing Polyacrylamide Gels

Secondary to the quality and quantity of template DNA used in sequencing reactions, the quality of the polyacrylamide gel and its careful preparation is important for the consistent production of accurate data. Glass plates should not be used if scratched or chipped, and all reagents should be ultrapure and filtered to remove particulate matter that may interfere with detection of fluorescent signals.

Data recorded during electrophoresis is stored in analyzed and raw formats. Sequence data can be output as a printout of the chromatogram produced by the laser scanning of the gel and as Macintosh- or PC-compatible sequence files. Further details of sequence analysis can be found in Chapter 9.

3.3.1. Preparation and Casting of Polyacrylamide Gels

Because the procedure for setting up and running gels on automated sequencing systems will vary greatly for different makes of machine, reference should be made to the relevant user's manuals.

1. Wash glass plates, comb, and spacers with Alconox and warm water, rinse with warm water followed by dH_2O, and dry with lint-free paper.
2. Align plates with spacers. Clamp one edge and seal the opposite side and corners with Permacel tape, excluding any air bubbles. Repeat to seal the other side.
3. To 25 mL dH_2O add 40 g of urea, 9.5 mL (for 4.75% gel; add 12 mL for 6% gel) of 40% stock acrylamide solution, and 1 g of mixed-bed resin. Heat and gently stir the mixture until the urea crystals begin to dissolve. Remove from the heat and continue to mix until the crystals are completely dissolved.
4. Degas the solution for 5 min using a 0.2-µm vacuum filter unit (*see* **Note 6**).
5. Add the degassed solution to a 100-mL measuring cylinder containing 8 mL of 10X TBE buffer, and make the volume up to 80 mL with dH_2O.
6. Pour the solution into a 150-mL beaker and mix in 400 µL of 10% ammonium persulphate solution and 45 µL of TMED, avoiding air bubbles.
7. Carefully fill the plates to 3–5 cm from the top, allowing all air bubbles to escape.
8. Lay the plates flat, wet the casting comb in 1X TBE buffer, insert between the plates, and clamp in position. Allow the gel to polymerize for at least 2 h at room temperature, but do not use a gel after more than 18–24 h.
9. Remove all tape and clamps from the gel, wash all traces of acrylamide from the plates with dH_2O, and allow them to air dry.

3.3.2. Gel Loading

1. Remove unincorporated dye terminators by centrifugation of reaction products though spin columns (e.g., Centri-Sep, Princeton Separations; MicroSpin S-400 HR Columns, Pharmacia Biotech, Uppsala, Sweden).
2. When using a 24 well comb, add 4 to 5 µL blue dextran loading buffer to each sample; use only 3 µL of loading buffer for a 36-well comb (*see* **Note 7**).
3. Briefly vortex and centrifuge samples before heating to 90°C for 2 min and store on ice for no more than 1 h.
4. Carefully flush all wells with 1X TBE buffer. Load samples in odd-numbered lanes and loading buffer in lanes 0 and 25 or 37 (*see* **Note 8**).
5. Run all samples into the gel by electrophoresis for approx 5 min. Flush all wells with 1X TBE buffer and load even numbered lanes. Complete the sequencing run.

3.4. Concluding Comments

Although the analysis of DNA sequences is rarely used directly in environmental monitoring, it is an important precursor to many of the methods involved. Automated DNA sequencing techniques are now widely available and their use decreases the time and effort required to develop new detection methods. In contrast to the radionucleotides used for labeling of DNA fragments in classical DNA sequencing approaches, the fluorescently labeled primers and ddNTPs used by automated systems are nonhazardous and have long shelf

lives. The major drawback of this technology is the initial capital investment. However, with rapidly developing techniques, machines are becoming available that reportedly are able to read lengths of over 1200 bp and perform bidirectional sequencing in one run. Such advances will reduce running costs and can only serve to facilitate a greater understanding of microbial diversity.

4. Notes

1. Template DNA for sequence analysis in environmental microbiology is most commonly obtained following PCR and cloning, although shotgun cloning approaches using unamplified extracted DNA have been used. Mixed DNA species amplified by the PCR are separated using standard cloning techniques. Vectors commonly used for cloning include pUC *(6)*, pGEM (Promega Madison, WI), and Bluescript (Stratagene, Cambridge, UK) series. These vectors contain a variety of priming sites to facilitate cloning and sequence analysis. Templates for sequencing reactions can be obtained by amplification of the target gene from bacterial colonies using specific primers, or by isolation of the vector followed by sequencing from priming sites in the vector or the target gene.
2. The alkaline lysis procedure is based on that of Birnboim and Doly *(7)*. Commercially available preparation methods include QUIAGEN columns and Promega Magic Minipreps (Promega).
3. Centrifugation of samples at 4°C may result in pelleting of salts.
4. Removal of all traces of phenol is essential as it can affect dye performance.
5. Targets for sequencing primers can be in the vector arms, and can be used to sequence cloned DNA without prior knowledge of sequence of the insert, or specific hybridization sites are chosen within the cloned DNA.
6. Degassing the solution for the same length of time for every gel ensures reproducible results.
7. Formamide is added to loading buffer so that DNA fragments migrate as discreet bands and blue dextran assists in sample visualization.
8. The ABI 373A Sequencing System can run either 24 or 36 samples. If using all lanes, care must be taken to avoid overflow of samples into adjacent wells. In addition, for the recognition of lanes the system's automatic lane tracker requires discreet spaces between samples. To achieve this, and to reduce the risk of sample overflow, wells are loaded alternately, odd-numbered first and then even numbered ones with brief periods of electrophoresis between loading.

References

1. Barns, S. M., Delwiche, C. F., Palmer, J. D., and Pace, N. R. (1996) Perspectives on archaeal diversity, thermophily and monophyly from environmental sequences. *Proc. Natl. Acad. Sci. USA* **93,** 9188–9193.
2. Borneman, J., Skroch, P. W., O'Sullivan, K. M., Palus, J. A., Rumjanek, N. G., Janses, J. L., Nienhuis, J., and Triplett, E. W. (1996) Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl. Environ. Microbiol.* **62,** 1935–1943.

3. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
4. Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Connell, C. R., Heiner, C., Kent, S. B. H., and Hood, L. E. (1986) Fluorescence detection in automated DNA sequence analysis. *Nature* **321,** 674–679.
5. Connell, C. R., Fung, S., Heiner, C., Bridgham, J., Chakerian, V., Heron, E., Jones, B., Menchen, S., Mordan, W., Raff, M., Recknor, M., Smith, L. M., Springer, J., Woo, S., and Hunkapiller, M. W. (1987) Automated DNA sequence analysis. *BioTechneques* **5,** 342–348.
6. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33,** 103–119.
7. Birnboim, H. C., and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7,** 1513.

9

Analysis of DNA Sequences

Mathew Upton

1. Introduction

Molecular biological methods are now commonly used to detect bacteria in diverse environments ranging from soils, sediments, and sludges ***(1,2)*** to plant ***(3)*** and mammalian ***(4,5)*** tissue and food or water samples ***(6,7)***. The techniques most widely used in detection methods are the polymerase chain reaction (PCR) and oligonucleotide probing. PCR exploits primers targeting a region of ribosomal RNA (rRNA) known to be specific to the organism of interest ***(8)***, or functional genes for metabolic pathways ***(9)*** and toxins ***(10)*** exclusive to certain bacteria, and facilitates qualitative or quantitative detection of target organisms in natural environments ***(11)***. Oligonucleotides can be labeled with radionucleotides or with chemiluminescent or fluorescent reporter molecules and used to probe nucleic acids extracted from samples ***(4)***, again giving qualitative or quantitative information regarding the occurrence of target organisms. In addition, fluorescently labeled olignucleotides are increasingly being used in combination with flow cytometry for cell counting or sorting ***(12,13)***, and with confocal laser scanning microscopy ***(2,14)*** to generate *in situ* data revealing close spatial associations of organisms in environmental samples.

Although analysis of DNA sequences is rarely used directly in environmental monitoring, many approaches rely on the analysis of DNA sequence data for design of primers and probes. This chapter follows the analysis of methanogen DNA sequences recovered from peat bog samples as an example of the types of sequence analyses that are performed in environmental microbiology. The information presented is intended to be a guide and the details for operating the programs used or listed are readily available at the addresses given.

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2. Materials

The following analyses are easily performed using PowerMac and PC workstations. An Internet connection is necessary for access to some software packages and transfer of files between the hard drive and remote sites using the file transfer program (FTP). A laser printer is useful for printing good quality phylogenetic trees and sequence alignments.

3. Methods

3.1. Sequence Data Editing

Initially, sequenced fragments of target DNA can be aligned and edited using one of the packages listed in **Table 1**. This list is not exhaustive and others can be found by following links at Internet sites such as the Ribosomal Database Project (RDP). Many of the packages are available free and can be downloaded over the Internet.

The following protocol was used in the analysis of four sequence fragments of the α-subunit of the methyl coenzyme M reductase (MCR) gene of methanogens that had been amplified from cloned environmental DNA by PCR with primers ME1 and ME2 (**Fig. 1**) ***(15)***. Automated sequencing was performed using an Applied Biosystems Inc. (ABI; Perkin-Elmer, Warrington, UK) 373A Sequencing System using dye terminator chemistry with primers ME1, 2, 4, and 5 (*see* **Note 1**).

1. Data from the ABI 373A Sequencing System are output from automated analysis software as a Macintosh sequence file and as a printout of the chromatogram produced by the laser scanning of the gel during electrophoresis (**Fig. 2**) (*see* **Note 2**).
2. Visually inspect chromatograms, which are printed in color for easy interpretation, for mistakes made by the automated base-calling software (*see* **Note 3**).
3. Import sequence fragments ME1, 2, 4, and 5 into the Sequence Navigator package (ABI). Using the chromatogram for reference, delete poor-quality data from both ends of the fragments (usually 10–50 bases at the 5' end and approx 100–200 bases from the 3' terminus). Overlap align fragments 1 with 4 and 2 with 5 to form a pair of full-length sequences and reverse complement the latter strand. Perform a comparative overlap of the two strands. Using the "Create Shadows" feature of the package, compare the forward and reverse strands and correct any mismatches by reference to the relevant chromatograms. Compute a consensus from the edited sequences and export it to a new folder as a "GCG" format file. The sequence can also be translated to amino acids in any of the possible reading frames.
4. Copy the exported file to a PC-compatible floppy disk using the Apple file convert program (unless using a PowerMac, which can write directly to a PC-for-

Table 1
Some Sources of Software for Analysis of DNA Sequence Data

Facility[a]	Function	Source/access information/reference
Sequence navigator	Sequence editing	ABI, Perkin-Elmer
Factura	Sequence editing	ABI, Perkin-Elmer
Oligo	Primer design/analysis software	National Biosciences
MacDNASIS Pro	Primer design/analysis software	Hitachi Software Engineering America
OPD	Database of oligonucleotide probe sequences	http://www.cme.msu.edu/OPD
GenBank	Searchable database of DNA sequences	http://www.ncbi.nlm.nih.gov/
EMBL	Searchable database of DNA sequences	http://www.ebi.ac.uk/ebi_home.html
DDBJ	Searchable database of DNA sequences	http://www.ddbj.nig.ac.jp/
GSDB	Searchable database of DNA sequences	http://www.ncgr.org/gsbd/
Lasergene	Sequence editing, database reference, primer and probe design, restriction site analysis	http://www.dnastar.com
Wisconsin Package (GCG)	Sequence editing, database reference, primer and probe design, restriction site analysis, phylogenetic analysis	University of Wisconsin, Genetics Computer Group
SEQNET	Access to major databases and the GCG editing and phylogenetics programs	http://www.dl.ac.uk/SEQNET/home.html
RDP	Searchable database of rRNA sequences, phylogenetics programs	http://rdp.life.uiuc.edu/
PAUP	Phylogenetic analysis	***15***
PHYLIP	Phylogenetic analysis	http://evolution.genetics.washington.edu/phylip.html
ClustalW	Tree alignment	http://www.no.embnet.org/phylogeny.html#ClustalW
TreeView	Viewing files from tree drawing packages	http://taxomony.zoology.gla.ac.uk/rod/treeview.html

[a]OPD, Oligonucleotide Probe Database; GenBank, run by the National Institute of Health (NIH), USA; EMBL, European Molecular Biology Laboratory, database run by European Bioinformatics Institute, UK; DDBJ, DNA Data Bank of Japan; GSDB, Genome Sequence Database; RDP, Ribosomal Database Project; PAUP, Phylogenetic Analysis Using Parsimony; SEQNET, Sequence Network; PHYLIP, Phylogeny Inference Package.

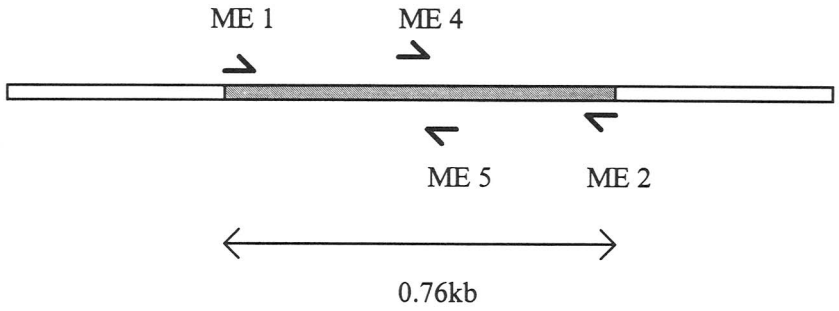

Fig. 1. Sequence fragments of the α subunit of the methyl coenzyme M reductase gene.

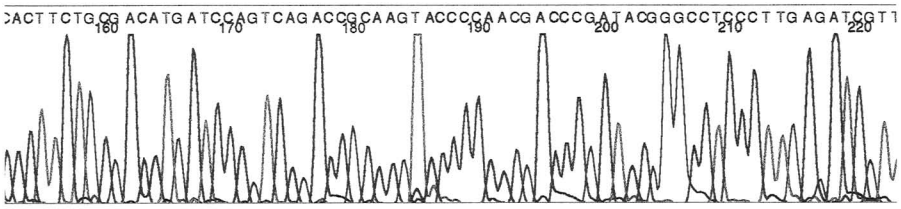

Fig. 2. Section of a chromatogram produced by the ABI 373A Sequencing System. The peaks, and the bases called from them, can be printed in color (A, green; C, blue; G, black; T, red) to assist in interpretation.

matted disk). Using FTP, transfer the file from the floppy disk into UNIX file space with access to the GCG (Wisconsin Package, University of Wisconsin, Genetics Computer Group, Madison, WI) suite of programs.

5. Perform a FASTA search against the GenBank and EMBL DNA sequence databases to check the similarity of the MCR sequence to those previously submitted to the databases (*see* **Note 4**).

3.2. Downstream Analysis of DNA Sequence Data

3.2.1. Sequencing Primer Design

The following procedure was used to create onward sequencing primer ME4 and was repeated using sequence ME2 for design of primer ME5.

1. In the GCG facility, align edited sequences from primer ME1 using the PILEUP program (*see* **Note 5**).
2. Visually inspect the 3' end of aligned sequences from the pileup. Identify a region of the alignment in which all sequences share homology and the following criteria are satisfied:

a. Primers should be 17–30 nucleotides in length with a G+C content of 40–60% and a T_m of at least 45°C.
b. There should be no regions of self-complementarity that result in hairpin loop formation. This is especially important at the 3' end of a primer.
c. Ideally, primer sequences should contain no mismatches with target regions. If it is not possible to target a suitable site, degenerate bases should be located at the 5' end of the primer sequence because synthesis is affected only by unstable binding at the 3' end.
d. Primers that target regions of secondary structure in template DNA will not perform optimally even when synthesized to the above specifications (**Subheading 3.2.1.2.a,b,c**).

3.2.2. PCR Primer Design

1. Import the sequence to be screened for PCR primer pairs into the GCG package at the SEQNET facility (*see* **Table 1**).
2. Run the PRIME program using the imported sequence as a target. Set the desired parameters such as primer and product length. The program automatically scans the possible primer pairs and rejects any that do not satisfy a range of requirements, including those that form primer dimers and those with different T_m values (*see* **Note 6**).
3. Primer specificity should be assessed by performing BLAST/FASTA searches against relevant databases (e.g., GenBank, EMBL) or using the Check Probe facility in the RDP (*see* **Note 7**).

3.2.3. Oligonucleotide Probe Design

1. Import the sequence that the probe is to target into the GCG package running in UNIX workspace.
2. Using the PILEUP program, align the sequence with corresponding sequences from both closely related and distant taxa (*see* **Note 8**).
3. Visually inspect the MSF file produced by the pileup and identify a region that differentiates the sequence of the target organism from all others (*see* **Note 9**).
4. An oligonucleotide probe should be constructed using the criteria for selection of sequencing primers (**Subheading 3.2.1.**), although probes are commonly between 15 and 30 nucleotides in length (*see* **Note 10**).
5. Input the sequence selected for use as a probe into SEQED within GCG and perform a BLAST/FASTA search against any relevant databases (e.g., Genbank/EMBL) to check that the probe is specific to target taxa (*see* **Note 11**).

3.2.4. Phylogenetic Analysis

Many studies involving the analysis of DNA sequences from natural environments make use of phylogenetic analysis packages (**Table 1**) to infer relationships between cloned sequences and/or cultured strains by reference to public databases (**Table 1**). Details of phylogenetic analyses are given in Chapters 10 and 11.

The approaches used can be adapted to allow use of other packages, which may be more readily available in some locations. For users in the United Kingdom, access to SEQED at Daresbury provides a full range of tools for the analysis of DNA sequences.

There is an extensive and ever-increasing range of packages currently available for DNA sequence analysis, many of them free and easily accessible over the Internet. The ability to rapidly analyze sequence data and identify probe and primer target sites increases the rate at which molecular ecological studies can be performed. At the most basic level, these approaches can quickly and easily indicate the presence or absence of organisms, including slow-growing taxa and those thought to be uncultivable. If used judiciously, the techniques can lead to a greater understanding of the roles played by a target organism in its natural environment.

4. Notes

1. Studies on primers ME1 and ME2 have been previously published ***(16)***. Fragments ME4 and ME5 were sequenced from primers designed by reference to the sequences of fragments ME1 and ME2, respectively (Upton, M., unpublished data). For information on the design of sequencing primers, *see* **Subheading 3.2.1.**
2. The sequence files can also be converted to PC-compatible formats.
3. Chromatograms can be used solely for confirmation of sequence information by visual inspection, or sequence can be read directly from them and input manually into editing packages. Mistakes made by the automated base-calling software include incorrect assignment of bases obscured by high background, calling an incorrect number of bases to a series of peaks, and assignment of bases where there should be spaces in regions of compression of sequence data.
4. Results of a FASTA search can be used to confirm the identity of a sequence and will give an indication of the novelty of the cloned DNA. In addition, the sequences most closely related to the test sequence can be copied from the databases and used as reference strain data in phylogenetic analysis.
5. When using a single clone/sequence, the alignment is not possible, and primer design can be performed by visual examination of the single chromatogram or sequence file.
6. PCR primers can be selected by visually scanning the sequence for suitable regions. PCR primers are generally 18–21 nucleotides in length and should be designed under the criteria used for sequencing primer design (**Subheading 3.2.1.**). Care should be taken to avoid regions of complementarity both internally and between primers since this results in hairpin loop or primer dimer formation.
7. This screen is not necessary when designing primers for onward sequencing of cloned fragments, although obviously primers should have only one site in the target molecule.
8. For example, a 16S rRNA gene sequence from *Methanosarcina barkeri* would be aligned with 16S rDNA sequences from other members of the kingdom Archaea

(both euryarchaea and crenarchaea) and a representative of the Bacteria. This alignment allows regions of inter taxa variability to be easily identified.

9. Locating suitable areas can be assisted by using sequences from several organisms of the target group in the pileup. Target sites can also be selected using some of the software listed in **Table 1**.
10. Entire gene sequences can also be used as probes. Such probes can easily be produced by labeling PCR products using specific primers with digoxygenin-labeled (DIG; Boehringer Mannheim) dUTP added to the reaction mixture.
11. Databases are being updated on such a regular basis that it is sensible to screen a probe for mismatch sites even when using previously published probes. These may have been designed before homologous sequence from nontarget organisms was submitted to the database.

References

1. Wagner, M., Rath., G., Amann, R., Koops, H. P., and Schleifer, K.-H. (1995) In-situ identification of ammonia oxidising bacteria. *Syst. Appl. Microbiol.* **18,** 251–264.
2. Siering, P. L. and Ghiorse, W. C. (1997) Development and application of 16S rRNA-targeted probes for detection of iron- and manganese-oxidizing sheathed bacteria in environmental samples. *Appl. Environ. Microbiol.* **63,** 644–651.
3. Lacourt, I. and Duncan, J. M. (1997) Specific detection of *Phytophthora nicotinanae* using the polymerase chain reaction and primers based on the DNA sequence of its elicitin gene ParA1. *Eur. J. Plant Pathol.* **103,** 73–83.
4. Hoshina, S., Kahn, S. M., Jiang, W., Green, P. H. R., Neu, H. C., Chin, N., Morotomi, M., Logerfo, P., and Weinstein, B. (1990) Direct detection and amplification of *Helicobacter pylori* ribosomal 16S gene from gastric endoscopic biopsies. *Diagn. Microbiol. Infect. Dis.* **13,** 473–479.
5. Ho, S. A., Hoyle, J. A., Lewis, F. A., Secker, A. D., Cross, D., Mapstone, N. P., Dixon, M. F., Wyatt, J. I., Tompkins, D. S., Taylor, G. R., and Quirke, P. (1991) Direct polymerase chain reaction test for detection of *Helicobacter pylori* in humans and animals. *J. Clin. Microbiol.* **29,** 2543–2549.
6. Yamamoto, H., Hashimoto, Y., and Ezaki, T. (1993) Comparison of detection methods for Legionella species in environmental samples by colony isolation, fluorescent antibody staining and polymerase chain reaction. *Microbiol. Immunol.* **37,** 617–622.
7. Olsen, J. E., Aabo, S., Hill, W., Notermans, S., Wernars, K., Granum, P. E., Popovic, T., Rasmussen, H. N., and Olsvik, O. (1995) Probes and polymerase chain reaction for detection of food-borne bacterial pathogens. *Int. J. Food Microbiol.* **28,** 1–78.
8. Honerlage, W., Hahn, D., Zepp, K., Zeyer, J., and Normand, P. (1994) A hypervariable 23S rRNA region provides a discriminating target for specific characterisation of uncultured and cultured Frankia. *Syst. Appl. Microbiol.* **17,** 433–443.
9. Joshi, B. and Walia, S. (1996) PCR amplification of catechol 2,3-dioxygenase gene sequences from naturally occurring hydrocarbon degrading bacteria isolated

from petrolium hydrocarbon contaminated groundwater. *FEMS Microbiol. Ecol.* **19,** 5–15.

10. Fach, P., Hauser, D., Guillou, J. P., and Popoff, M. R. (1993) Polymerase chain reaction for the rapid identification of *Clostridium botulinum* type A strains and detection in food samples. *J. Appl. Bacteriol.* **75,** 234–239.
11. Butendieck, B., Morales, P., Figueroa, J., Concha, M., and Leon, G. (1995) Specific gene amplification as a means to detect *Renibacterium salmoninarum. Archivos De Medicina Venterinaria* **27,** 47–54.
12. Vesey, G., Narai, J., Ashbolt, N., Williams, K., and Veal, D. (1994) Detection of specific microorganisms in environmental samples using flow cytometry. *Methods Cell Biol.* **42,** 489–522.
13. Thomas, J. C., Desrosiers, M., St-Pierre, Y., Lirette, P., Bisaillon, J. G., Beaudet, R., and Villemur, R. (1997) Quantitative flow cytometric detection of specific microorganisms in soil samples using rRNA targeted fluorescent probes and ethidium bromide. *Cytometry* **3,** 224–232.
14. Assmus, B., Hutzler, P., Kirchhof, G., Amann, R., Lawrence, J. R., and Hartmann A. (1996) In situ localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labelled, rRNA targeted oligonucleotide probes and scanning confocal laser microscopy. *Appl. Environ. Microbiol*. **61,** 1013–1019.
15. Swofford, D. (1991) PAUP: phylogenetic analysis using parsimony, version 3.0. Computer program distributed by Illinois Natural History Survey, Champaign, IL.
16. Hales, B. A., Edwards, C., Ritchie, D. A., Hall, G. H., Pickup, R. W., and Saunders, J. R. (1996) Isolation and identification of methanogen-specific DNA from blanket bog peat using PCR amplification and sequence analysis. *Appl. Environ. Microbiol*. **62,** 668–675.

10

Fluorescent Polymerase Chain Reaction/Restriction Fragment Length Polymorphism Monitoring of Genes Amplified Directly from Bacterial Communities in Soils and Sediments

Kenneth D. Bruce and Mark R. Hughes

1. Introduction

There has been a growing acknowledgment of both the ecological and biotechnological importance of microbes in natural environments. Concerns about the nonrepresentative nature of traditional analytical methods, as a result of their requirement for prior cultivation, have led to the introduction of molecular biological approaches to these areas of study ***(1)***. Considerable effort has led to the development and application of a number of molecular procedures to profile the diversity of microbial sequences in environments.

For many of these molecular approaches, the first step is the amplification by the polymerase chain reaction (PCR) of specific target sequences present in nucleic acids extracted from environmental samples. These target sequences are frequently regions of ribosomal genes, used because of their established phylogenetic framework. However, other sequences are being increasingly used owing to the interest in tracking marked strains and following gene spread and evolution. The oligonucleotide primers used in PCR either can be made highly specific for a single target (e.g., to track the progress of a specific strain through an environment) or can access the widest range of sequence variants of a particular gene that are available (through the use of consensus regions of database variants). As examples, specific regions of 16S rRNA genes have detected ammonia-oxidizing bacteria of the genera *Nitrosospira* ***(2)*** and *Nitrobacter* populations ***(3)***, in comparison to the many studies which use "universal" ribosomal sequences. Describing the

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information contained within the resulting pool of PCR products, however, presents a different challenge.

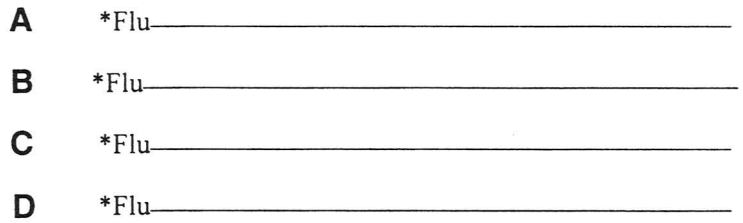
Several techniques have been devised to examine PCR products amplified from natural communities. One of the first approaches required individual amplification products to be cloned into plasmid vectors to form "libraries" prior to screening by oligonucleotide hybridization or direct sequencing. Although this method provides detailed information, the time-consuming nature and the potential for introducing cloning biases make it generally unsuitable for environmental monitoring. It was therefore important to develop methods that resolve the diversity of the amplified products more rapidly and preferably in a single electrophoretic run. A number of such methods have been developed including denaturing gradient gel electrophoresis (DGGE) ***(4,5,6)*** and those based on restriction fragment length polymorphism (RFLP) ***(7)***.

DGGE has been used to study bacterial community diversity in marine microbial mats and biofilms from wastewater treatment plants ***(4)***, and bacterial communities within a microbial mat ***(8)***. Subsequent studies have characterized the genetic diversity within species or functional groups of bacteria. For example, Wawer and Muyzer ***(6)*** found higher diversity for *Desulfovibrio* species in a natural microbial mat than those in an experimental bioreactor using DGGE of [NiFe] hydrogenase sequences. Similar DGGE-based studies have examined the diversity of sulfur-oxidizing bacteria in hydrothermal vents ***(5)*** and sulfate-reducing bacteria in a stratified marine water column ***(9)***. RFLP of amplified 16S rDNA has been used to profile the diversity of bacterial communities in soil ***(10)*** and in hypersaline waters ***(7)***.

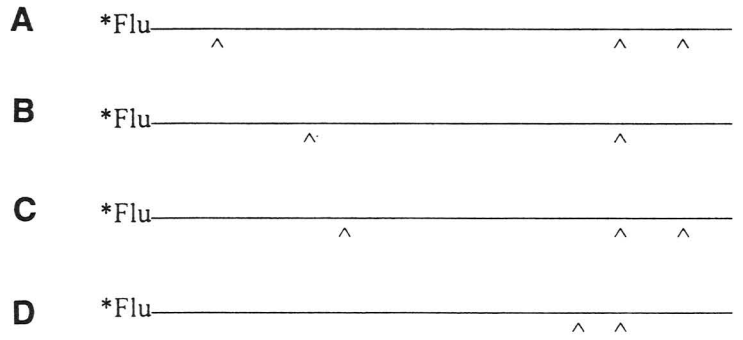
Fluorescent polymerase chain reaction/RFLP (FluRFLP) has been devised to profile variants of specific sequences amplified from natural environments ***(11)***. FluRFLP, shown diagrammatically in **Fig. 1**, differs from other RFLP studies of mixed-community PCR products in that the sole focus of the analysis is a single fragment, in contrast to the multiple fragments in a conventional RFLP. This fragment is generated by digestion of PCR products (amplified either from a single culture or from mixed-community DNA) using a specific restriction endonuclease. The restriction endonuclease chosen differentiates, at the level of discrimination required, the sequence variants of the target gene on the basis of the distance from the first restriction endonuclease site to the start of the PCR product. The different size variants generated by the restriction endonuclease are identified by the fluorescent label on the 5' end of one primer used in the PCR. When FluRFLP digests are electrophoresed, only those single-stranded products bearing the fluorescent label are detected using automated DNA sequencing technology. In this manner, only size variants are detected. Furthermore, through the use of computer software originally

1. Mixed community DNA (e.g. with sequence variants, A-D) is amplified using oligonucleotide primers designed to amplify the sequence of interest. One primer is fluorescently labelled (*Flu)

2. Amplified products carry the fluorescent label at one 5' end



3. The DNA sequence of each variant is used to identify the restriction endonuclease (sites marked by ^) which discriminates between the different sequence variants



4. On digestion with this restriction endonuclease, only the fragments from the fluorescent label to the first restriction endonuclease site will be detected by the automated DNA sequencer.

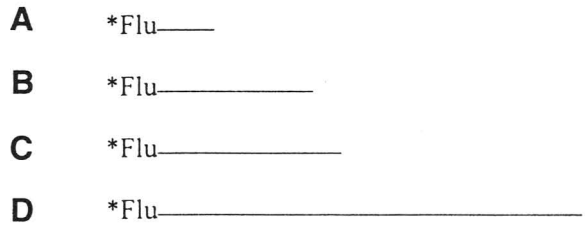


Fig. 1. Diagrammatic representation of the FluRFLP process.

designed for studies of microsatellites in eukaryotic populations ***(12)***, the size and relative abundance of each peak can be estimated accurately.

If the PCR product has been generated from one sequence variant, a single FluRFLP fragment will be identified. Knowledge of the varying sequence types can therefore be used to compile a list of different size variants. This can be used subsequently when analyzing DNA isolated from mixed communities. One of the major advantages of FluRFLP lies in the assessment and monitoring of subtypes of particular gene sequences in complex genetic backgrounds such as those found frequently in natural environments. Furthermore, FluRFLP can address quantification not only at the overall level of genes (loads or persistences), but also the quantification of the relative amounts of particular subtypes.

The drawbacks of the FluRFLP technique are those often identified with the use of PCR. Care must be taken in making inferences on the starting concentrations of genes derived from the final ratio of PCR products ***(13)*** owing to various potential biases, e.g., reannealing, degeneracy, and GC content in primers. Given the persistence of DNA in natural environments, it is possible that signals can be generated from material outside of cells ***(14)***. As with all PCR-based studies, care must be taken not to introduce "contaminating" DNA sequences during the DNA isolation or PCR amplification steps. In addition, it is possible that chimeric sequences can be generated from the coamplification of homologous genes ***(15)***, leading to the creation of apparently "novel" types. A strategy has been developed to try to obviate this potential problem in FluRFLP. In this strategy, two separate PCR reactions are carried out using each source of template DNA. One primer, the one bearing the fluorescent label, is common to both reactions, with the two other primers designed to different consensus regions. On amplification, two products of different length are generated. However, because they "started" from the same position, the digestion of either should produce the same fragment when amplified from a single sequence variant. It is hoped that this should assist in the discrimination between informative and spurious PCR products, since fragments generated from spurious products would be seen only in one digestion profile.

The present FluRFLP approach can be extended to incorporate known amounts of specific target sequences into the original PCR mix. These specific target sequences can be modified by altering the position of the restriction endonuclease sites within the PCR target (either by sequence modification to create a new restriction endonuclease site or by the insertion/deletion of 10 bases) so that they produce a FluRFLP size variant that is not observed in natural environments. This will provide absolute discrimination between the original subclasses and the "newly-created" variants. These can then be used both

in experiments to quantify the relative abundances and to monitor persistence and spread in natural environments and in a wider range of environmental monitoring studies. This chapter outlines the principles and use of FluRFLP to study bacterial sequence diversity and discusses its role in environmental monitoring.

2. Materials

Before performing PCR, preliminary work should be carried out (*see* **Note 1**).

2.1. Amplification of PCR Products from Test Sample

1. Reaction template, e.g., DNA extracted from environmental samples (*see* **Note 2**).
2. PCR reagents: typically 0.5-mL microfuge tubes or 96-well plates, *Taq* DNA polymerase and reaction buffer, deoxynucleotide triphosphates, primers (as appropriate), mineral oil (depending on PCR machine), sterile distilled water (*see* **Note 3**).
3. Agarose, electrophoresis buffer of choice, DNA stain (e.g., ethidium bromide), loading dye.
4. PCR machine.

2.2. Restriction Endonuclease Digestion of PCR Products

1. PCR products, generated by methods in **Subheading 2.1.**
2. Microcon-30 spin columns (Amicon, Beverly, MA) (*see* **Note 4**).
3. Restriction endonuclease of choice and reaction buffer (*see* **Note 5**).
4. 1.5-mL microcentrifuge tubes.
5. Water bath to incubate restriction endonuclease digests.

2.3. Electrophoresis of Restriction Endonuclease–Digested PCR Products

This method has been written for the Applied Biosystems 373A automated DNA sequencing machine.

1. Digested PCR products.
2. Size standards. For the 373A Genescan-500 or -2500, TAMRA (*N*, *N*, *N*', *N*'-tetramethyl-6-carboxyrhodamine) internal markers (Applied Biosystems) are appropriate (*see* **Note 6**).
3. IM NaOH in ethanol (*see* **Note 7**).
4. Sequagel-6 (National Diagnostics, Atlanta, GA) for a 6% denaturing polyacrylamide gel (*see* **Note 8**).
5. Ammonium persulfate.

2.4. Analysis of Banding Patterns

Recommended analysis software: Genescan (version 1.2.2-1) and Genotyper software (version 1.1) (PE Biosystems, Narrington, UK) running on Power Macintosh computers.

3. Methods

3.1. Amplification of PCR Products from Test Sample

1. Prepare a master mix of PCR reagents using amplification conditions established previously.
2. Divide master mix into wells/0.5-mL tubes and cover with oil if required.
3. Raise the temperature of the PCR machine to 95°C.
4. Add template DNA.
5. Following cycling, examine the products generated by agarose gel electrophoresis and confirm by DNA hybridization.
6. Store PCR products at 4°C until restriction endonuclease digestion.

3.2. Restriction Endonuclease Digestion of PCR Products

1. Add the selected restriction endonuclease (typically 5 µL, but varies according to manufacturer) to 200 µL of 1X restriction buffer supplied with the enzyme.
2. Centrifuge this mix for 5 min at room temperature through a Microcon-30 spin column at 13,000g in a microcentrifuge.
3. Invert the Microcon-30 column into a 1.5-mL microcentrifuge tube.
4. Spin this assembly at 6500g in a microcentrifuge for 30 s at room temperature.
5. Measure the amount of retained enzyme/buffer into a fresh tube and store at 4°C.
6. Digest the amplified PCR products with an appropriate volume of the retained enzyme/buffer and a final concentration of 1X restriction buffer using the reaction conditions recommended by the manufacturer.

3.3. Electrophoresis of Restriction Endonuclease Digested PCR Products

3.3.1. Casting Polyacrylamide Gels

1. Wash glass plates, comb, and spacers in warm water.
2. Align spacers and plates and clamp in position (following the instructions supplied in the user manuals).
3. Mix the desired amount of Sequagel-6 with TBE running buffer (following instructions).
4. Add 0.4 mL of 10% (w/v) ammonium persulfate for every 60 mL of gel solution.
5. Pour the gel avoiding the formation of air bubbles. If bubbles do appear, tap the glass plates to release them.
6. Insert and clamp the spacer to form a well.
7. Leave to set for a minimum of 2 hours at room temperature.
8. Unclamp the gel and wash all traces of acrylamide.

3.3.2. Gel Loading and Electrophoresis

1. Remove the spacer and flush with 1X TBE buffer.
2. Insert the comb (either 24- or 36-well).
3. Flush the wells with 1X TBE buffer.

4. Prior to loading samples, the gel is analyzed to ensure that no spurious fluorescent signals are being generated.
5. When using a 36-well comb, add 2 µL of dextran blue formamide denaturing loading buffer (as in PE Biosystems manual) and 0.5 µL of TAMRA markers to the digested PCR products (up to 6 µL of PCR products can be loaded; precipitate DNA if required).
6. Vortex and heat samples to 90°C for 2 min.
7. Store samples at 4°C.
8. Load the odd-numbered lanes and electrophorese the samples for 5 min. Electrophoresis is carried out with the voltage limited to 1150 V for 3.5 h on 12-cm wells to read plates.
9. Load the even-numbered lanes and complete the electrophoretic run.

3.4. Analysis of Banding Patterns

1. The resulting banding pattern can be analyzed using the Local Southern Method of size calling within Genotyper software. Other methods are available in the Genotyper Manual.
2. The position and area of individual peaks can be calculated using Genotyper software (version 1.1) lane by lane using the calibration provided by the TAMRA labeled markers.

3.5. Specific Example of FluRFLP

FluRFLP has been applied to study genes within the bacterial mercury resistance (*mer*) operon—a model system for many ecologic studies ***(16,17)*** owing to the well-characterized nature of its genetics and biochemistry ***(18)***. Using regions of consensus from database sequences, oligonucleotide primers were derived which amplify the *merRTΔP* region (approx 1 kb) that contains regulatory and transport genes of the major subclasses of archetypal Gram-negative *mer* operons (Tn*501* ***[19]***, Tn*21* ***[20]***, pKLH2 ***[21]*** and pDU1358 ***[22]***). *merR* DNA sequence data have been used to group these archetypal Gram-negative *mer* operons ***(23)***. Further analysis of *mer* sequences showed that six major subclasses of *mer* could be differentiated on the basis of the length from RX (the start of *merR*) to the first *Fok*I restriction endonuclease site (**Fig. 2**).

Preliminary experiments showed that individual *mer* sequence types present in cultures of mercury-resistant bacteria gave a single fragment of the same size as predicted by DNA sequence analysis. Each sample of soil and sediment community DNA tested, to date, has contained one or more of the fragment sizes identified in **Fig. 2**. Distinct profiles have been found for different sample locations. **Figure 3** shows the profile obtained for a sample taken from Fiddlers Ferry, on the river Mersey. Because the subclass type is known from the fragment size, any potential link between gene type and different physiochemical environments can be explored rapidly. This is potentially important for *mer*, because only certain subclasses carry the *merB* (organomercurial lyase) gene which effects resistance to organomercurial compounds

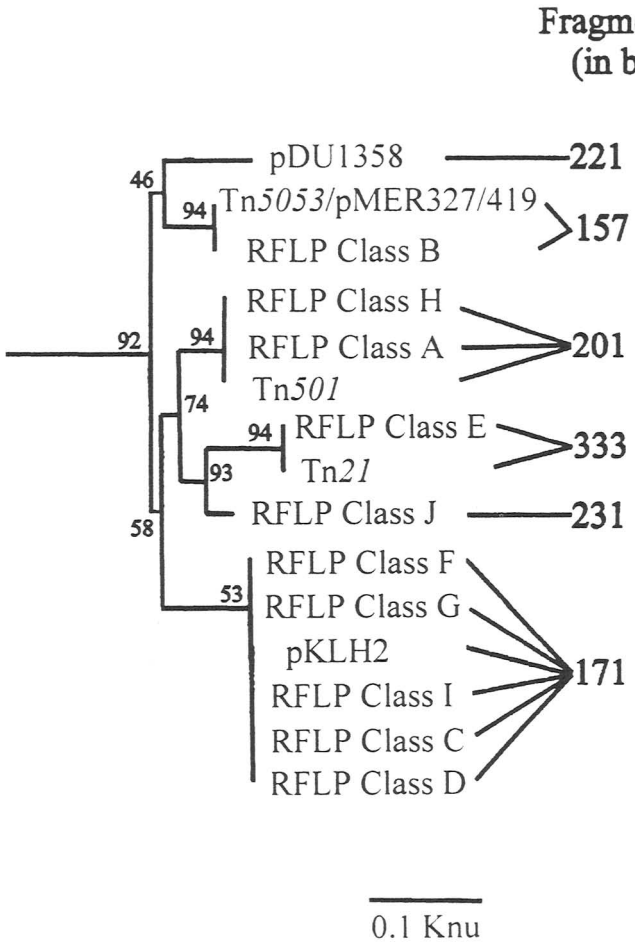


Fig. 2. Different FluRFLP fragment sizes generated by *Fok*I digestion of different *merRTΔP* sequences. The dendrogram on which the sites have been superimposed, derived from *merR* sequence data adapted from **ref. *23***, shows the major subclasses of the archetypal Gram-negative mercury resistance genes.

(22). In addition to the sizes in **Fig. 2**, additional fragment sizes were detected that were predicted on the basis of current DNA database entries. Subsequent studies will determine whether these represent novel sequences or were amplified from *mer* gene homologs.

4. Notes

1. Before using FluRFLP, preliminary work must be carried out. This involves using database manipulations of the sequences of interest, the design of oligonucle-

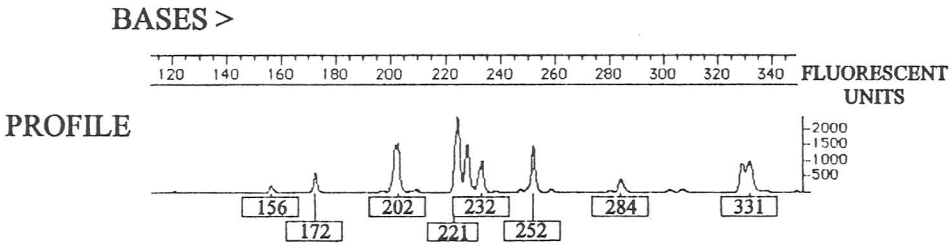


Fig. 3. Processed image of FluRFLP fragment produced by *Fok*I digestion of *merRTΔP* PCR products (FluRX to PX) amplified from DNA extracted from Fiddlers Ferry soil with the *mer* fragments shown in bases.

otide primers for PCR, and, crucially, the choice of restriction endonuclease. The choice of enzyme determines the resulting fragment profile and in turn the value of the information that can be derived from the digests. Computer programs that are useful in this process in the Genetics gcg package (Genetics Computing Group, Madison, WI) include FASTA and MAPSORT. Before incurring the expense of fluorescent oligonucleotide primers, it is advisable to check that the target sequence can be amplified using conventional PCR primers and to confirm this by DNA hybridization of the generated PCR products. Furthermore, although the automated DNA sequencer provides highly accurate sizing information, it is important, in practical terms, to select a restriction endonuclease that allows >10 bases between each size variant and over a region that can be sized by the automated DNA sequencer software.

2. Numerous protocols exist for DNA isolation. Here, the method used was as described in **ref.** ***24*** with the addition of a bead-beating step of 30 s using a Braun cell homogenizer (B. Braun Biotech, Germany) to ensure lysis of bacterial cells in soils and sediments.
3. Primers used here were taken from **ref.** ***24***. Incorporating a tetraethylene glycol molecule between the fluorescent moiety and the oligonucleotide ***(25,26)*** can be useful to balance the hydrophobicity. One oligonucleotide primer, FluRX, was labeled with the green fluorescent label TET (4, 7, 2', 7'-tetrachloro-6-carboxyfluorescein, Oswel Labs, University of Southampton, UK).
4. The use of Microcon-30 columns prevents the profound distortion caused by a compound present in the restriction endonuclease- possibly glycerol- observed in the original (prespin column) automated sequencer runs.
5. The choice of restriction endonuclease is case specific. *Fok*I (Boehringer Mannheim, Lewes, UK) was used here to profile the amplified *mer* genes.
6. The TAMRA-500 markers were used here since the sizing region was <500 bases.
7. This solution can be used to remove accumulated background fluorescence from sequencing plates, if required.
8. Other nonfluorescent acrylamide solutions can be used.

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References

1. Amann, R. I. ., Ludwig, W., and Schleifer, K. H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59,** 143–169.
2. Hiorns, W. D., Hastings, R. C., Head, I. M., McCarthy, A. J., Saunders, J. R., Pickup, R. W., and Hall, G. H. (1995) Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of Nitrosospiras in the environment. *Microbiology* **141,** 2793–2800.
3. Degrange, V. and Bardin, R. (1995) Detection and counting of *Nitrobacter* populations in soil by PCR. *Appl. Environ. Microbiol.* **61,** 2093–2098.
4. Muyzer, G., De Waal, E. C., and Uitterlinden, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction—amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59,** 695–701.
5. Muyzer, G., Teske, A., Wirsen, C. O., and Jannasch, H. W. (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch. Microbiol.* **164,** 165–172.
6. Wawer, C. and Muyzer, G. (1995) Genetic diversity of *Desulfovibrio* spp. in environmental samples analyzed by Denaturing Gradient Gel Electrophoresis of [NiFe] hydrogenase gene fragments. *Appl. Environ. Microbiol.* **61,** 2203–2210.
7. Martinez-Murcia, A. J., Acinas, S. G., and Rodriguez-Valera, F. (1995) Evaluation of prokaryotic diversity by restriction digestion of 16S rDNA directly amplified from hypersaline environments. *FEMS Microbiol. Ecol.* **17,** 247–256.
8. Ferris, M. J., Muyzer, G., and Ward, D. M. (1996) Denaturing Gradient Gel Electrophoresis profiles of 16S ribosomal RNA defined populations inhabiting a hot spring microbial mat community. *Appl. Environ. Microbiol.* **62,** 340–346.
9. Teske, A., Wawer, C., Muyzer G., and Ramsing, N. B. (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most probable number counts and Denaturing Gradient Gel Electrophoresis of PCR-amplified ribosomal DNA fragments. *Appl. Environ. Microbiol.* **62,** 1405–1415.
10. Porteous, L. A., Armstrong, J. L., Seidler, R. J., and Watrud, L. S. (1994) An effective method to extract DNA from environmental samples for Polymerase

Chain Reaction amplification and DNA fingerprint analysis. *Curr. Microbiol.* **29,** 301–307.

11. Bruce, K. D. (1997) Analysis of *mer* gene sub-classes within bacterial communities in soils and sediments resolved by fluorescent PCR/ restriction fragment length polymorphism profiling. *Appl. Environ. Microbiol.* **63,** 4914–4919.
12. Levitt, R. C., Kiser, M. B., Dragwa, C., Jeducka, A. E., Xu, J., Meyers, D. A., and Hudson, J. R. (1994) Fluorescence-based resource for semiautomated genomic analyses using mircosatellite markers. *Genomics* **24,** 361–365.
13. Suzuki, M. T., and Giovannoni, S. J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S ribosomal-RNA genes by PCR. *Appl. Environ. Microbiol.* **62,** 625–630.
14. Trevors, J. T. (1996) DNA in soil-adsorption, genetic transformation, molecular evolution and genetic microchip. *Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **70,** 1–10.
15. Wang, G. C. Y. and Wang, Y. (1996) The frequency of chimeric molecules as a consequence of PCR coamplification of 16S ribosomal RNA genes from different bacterial species. *Microbiology* **142,** 1107–1114.
16. Barkay, T., Liebert, C., and Gillman, M. (1989). Hybridisation of DNA probes with whole-community genome for detection of genes that encode microbial responses to pollutants: *mer* genes and Hg^{2+} resistance. *Appl. Environ. Microbiol.* **55,** 1574–1577.
17. Rochelle, P. A., Wetherbee M. K., and Olson, B. H. (1991) Distribution of DNA sequences encoding narrow- and broad- spectrum resistance mercury resistance. *Appl. Environ. Microbiol.* **57,** 1581–1589.
18. Hobman, J. L. and Brown, N. L. (1996) Bacterial mercury resistance genes, in *Metal ions in Biological Systems* (Sigel, H. and Sigel, A., eds.), Marcel Dekker, New York, pp. 527–568.
19. Brown, N. L., Ford, S. J., Pridmore, R. D., and Fritzinger, D. C. (1983) Nucleotide sequence of a gene from the *Pseudomonas* transposon Tn*501* encoding mercury resistance. *Biochemistry* **22,** 4089–4095.
20. Barrineau, P., Gilbert, P., Jackson, W. J., Jones, C. S., Summers, A. O., and Wisdom, S. (1984) The DNA sequence of the mercury resistance operon of the Inc FII plasmid NR1. *J. Mol. Appl. Genet.* **2,** 601–619.
21. Kholodii, G. Y., Gorlenko, Z. M., Lomovskaya, O. L., Mindlin, S. Z., Yurieva, O. V., and Nikiforov, V. G. (1993) Molecular characterisation of an aberrant mercury resistance transposable element from an environmental *Acinetobacter* strain. *Plasmid* **30,** 303–308.
22. Griffin, H. G., Foster, T. J., Silver, S., and Misra, T. K. (1987) Cloning and DNA sequence of the mercuric- and organomercurial resistance determinants of plasmid pDU1358. *Proc. Natl. Acad. Sci. USA* **84,** 3112–3116.
23. Osborn, A. M., Bruce, K. D., Strike, P., and Ritchie, D. A. (1995) Sequence conservation between regulatory mercury resistance genes in bacteria from mercury polluted and pristine environments. *Syst. Appl. Microbiol.* **18,** 1–6.

24. Bruce, K. D., Osborn, A. M., Pearson, A. J., Strike, P., and Ritchie, D. A. (1995) Genetic diversity within *mer* genes directly amplified from communites of non-cultivated soil and sediment bacteria. *Mol. Ecol.* **4,** 605–612.
25. Brown, D. J. S. and Brown, T. (1995) *PCR: Essential Data* (Newton, C. R., ed.), Wiley, New York, pp. 57–70.
26. Grzybowski, J., McPhilips, F., and Brown, T. (1995) *PCR: Essential Data* (Newton, C. R., ed.), Wiley, New York, pp. 93–98.

11

Recovery and Analysis of Ribosomal RNA Sequences from the Environment

Ian M. Head

1. Introduction

1.1. Historical Development of Ribosomal RNA Analysis of Microbial Populations

Since the 1980s the use of ribosomal RNA (rRNA) sequence-based analysis to characterize microbial populations (mainly bacterial and archaeal populations) has increased significantly. This increased use is in response to the recognition that culture-based methods grossly misrepresent the composition of microbial populations as they occur in nature ***(1)***. To circumvent the biases inherent in culture-dependent studies of microbial communities, it was suggested that, by extraction of nucleic acids directly from environmental samples, genes that were present in all taxa could be isolated and sequenced ***(2,3)***. Comparative analysis of sequences recovered from environmental samples with those from cultured isolates would permit phylogenetic relationships of the uncultured taxa to be determined ***(2,3)***. The universally distributed genes most commonly used for such analyses are the rRNA genes, particularly those encoding the small ribosomal subunit RNAs (16S and 18S rRNA). rRNA genes have many advantages over other candidate genes, including the following:

1. They are crucial components of ribosomes.
2. They possess a common, essential function in all cells.
3. Functional necessity constrains their primary and secondary structure and hence the degree of divergence in different taxa.
4. Their primary structure is a mosaic of conserved and variable tracts of sequence. This permits unambiguous alignment of homologous positions in

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an rRNA sequence and identification of universally conserved and taxon-specific sequence motifs.
5. There is little evidence of horizontal transfer of rRNA genes.
6. Extensive rRNA reference sequence databases exist.
7. A "tree of life" based on rRNA sequences provides a framework within which sequences recovered from natural samples can be accommodated.

Methodological constraints meant that initial studies were limited to analysis of bacterial communities of limited diversity using 5S rRNA sequences extracted and purified directly from environmental samples ***(2,3)***. 5S rRNA suffers from being only approx 120 nucleotides in length and, hence, permits only relatively low-resolution phylogenetic analyses. Methods were therefore developed for the recovery of larger and more informative rRNA sequences (16S and 23S rRNA in *Bacteria* and *Archaea*, 18S and 28S rRNA in *Eukarya*). It was initially suggested that shotgun cloning of DNA, extracted from environmental samples, in phage λ vectors could be used for the recovery of 16S rRNA genes ***(2,3)***. Initial attempts resulted in recovery of 16S rRNA genes in approx 0.2–0.3% of clones in a lambda library ***(2)***. To obtain a single 16S rRNA sequence using this approach would require screening of around 300–500 clones. Thus, even to determine the composition of a microbial community containing no more than a few dominant species would be an extremely laborious task.

However, the development of procedures to selectively recover rRNA sequences from environmental nucleic acids as cDNA or using the polymerase chain reaction (PCR) ***(4–6)*** has made rRNA analysis of microbial populations relatively commonplace.

Broadly speaking, there have been two main approaches adopted for the study of natural microbial populations based on the PCR for amplification of rRNA sequences: PCR amplification of rRNA genes followed by cloning and comparative analysis of the cloned rRNA sequences, or PCR using specific primers with or without post-PCR analysis using oligonucleotide probes. Cloning and sequencing allow the microbial population as a whole or a specific subgroup of the population to be characterized, but this is still relatively laborious. By contrast, the use of the PCR utilizing specific primers allows rapid identification of particular members of the microbial community. Refinement of these basic approaches (e.g., the application of denaturing gradient gel electrophoresis [DGGE]) has extended the scope of both methodologies and facilitated more rapid analysis of multiple samples.

This chapter outlines the basic approaches used in rRNA sequence-based analysis of natural microbial populations, and the limitations and applications of rRNA-based environmental analysis are discussed. DGGE and whole-cell hybridization procedures are presented in Chapters 12 and 15.

1.2. Principles

1.2.1. Recovery of rRNA Genes Using the PCR

Since its invention the PCR has found application in almost every realm of the biological sciences, and environmental microbiology is no exception. The sensitivity of the method has led to its use for the detection of bacteria present in very low numbers in environmental matrices (e.g., *see* **refs. *7*** and ***8***), and characterization of PCR-amplified rRNA genes is increasingly the method of choice for determining the composition of microbial communities.

The starting point for the majority of PCR-based analyses of microbial populations is a nucleic acid preparation extracted from an environmental sample. A variety of techniques have been developed to do this, and any particular method will have inherent biases. Extraction of nucleic acids from environmental samples is often problematic. Difficulties encountered include coextraction of materials inhibitory to the DNA polymerases used in the PCR. In addition, an extraction technique developed for one particular sample type may not be directly transferable to different samples. A detailed discussion of nucleic acid extraction techniques is given in Chapter 7.

Standard PCR conditions are generally adequate for the amplification of rRNA genes though techniques such as hot start ***(9)*** and touchdown PCR ***(10)*** can reduce mispriming and increase specificity of amplification.

1.2.2. Amplification of 16S rRNA Genes Using Broad Specificity Primers

The use of PCR primers targeting conserved regions of the rRNA molecule has probably been the most frequently adopted approach to the characterization of microbial communities (e.g., *see* **refs. *11–13***). Primers have been designed that allow the selective amplification of rRNA genes from the *Archaea, Bacteria,* or *Eukarya*. These primers, in principle, allow amplification of rRNA sequences from all members of a particular phylogenetic Domain. In reality there are few positions in the rRNA molecule that are absolutely conserved in composition ***(14)***. Consequently, so-called domain-specific or universal primers can exert some selectivity on the sequences they amplify. Using relatively low annealing temperatures in the PCR can reduce this selectivity.

1.2.3. Amplification of 16S rRNA Genes Using Specific Primers

The presence of variable regions in the rRNA primary structure permits the design of oligonucleotides that can be used as diagnostic hybridization probes and PCR primers. Oligonucleotides targeting hypervariable regions of the rRNA sequence can be made sufficiently specific to allow detection and identi-

fication at the species and in some cases, subspecies level. Furthermore, regions of the rRNA with more conserved sequence make it possible to design probes with genus, subdivision, and phylum specificity. However, the rules governing design of such probes are not universal, and in some lineages (e.g., the round-spore–forming bacilli) high rRNA sequence similarity may be observed even between phenotypically well-defined species ***(15)***. In addition, with the rapid expansion of the database of rRNA sequences ***(16)*** it is becoming apparent that a single oligonucleotide signature may not be sufficient to unequivocally identify a specific organism ***(17)***. Nonetheless, the ability to "tune" the specificity of rRNA sequence-based PCR for the identification of particular taxa in natural assemblages is a significant advantage over many other approaches.

1.2.4. Reverse Transcriptase PCR

The most straightforward format for PCR-dependent characterization of microbial populations is direct amplification of rRNA genes from "genomic" DNA extracted from a natural sample. This provides a snapshot of the total microbial population that is present but may also confuse matters by the amplification of naked DNA, indicating the presence of organisms that may be no longer present ***(18)***. Furthermore, the metabolically active members of a microbial community are often the ones of most interest, and these organisms cannot be distinguished from those that are inactive at any given time using analyses based on purified DNA. The rRNA content of cultured bacteria has been found to correlate well with specific growth rate ***(19–21)***. RNA is also considerably less stable than DNA, and RNase enzymes are both ubiquitous and robust; the half-life of RNA in the environment should therefore be considerably shorter than that of DNA. rRNA clone libraries obtained from purified RNA should provide a profile of the active members of a microbial population at the time of sampling.

A consequence of the relative instability of RNA is that its extraction from natural samples is more problematic than DNA isolation. This is particularly true of soil and sediments and only recently have methods been developed for purification of RNA from these environments in a form amenable to reverse transcription ***(22,23)***. Direct probing of rRNA extracted from natural samples with taxon-specific oligonucleotides has been used to assess the relative abundance of different sequence types present in natural samples (e.g., *see* **refs. *24–26***), but reverse transcription of the RNA and detailed characterization of the active fraction of the microbial population has only rarely been attempted (***22,27***; Miskin, I. P., et al., unpublished data).

1.3. Analysis of PCR-Amplified rRNA Sequences

rRNA sequences amplified from nucleic acids extracted from natural samples can be analyzed by a number of techniques. Rapid, low-resolution

characterization of sequences from a particular organism(s) is possible by probing blotted PCR products with specific oligonucleotide probes, and an overall indication of the diversity of the microbial population can be achieved using DGGE (*see* Chapter 12). Cloning of the heterogeneous population of rRNA sequences separates single sequences into individual *Escherichia coli* clones in a clone library. The cloned rRNA fragments can be sequenced from all, or a selection, of the clones, and a detailed picture of the sequence types present in a particular environment can be achieved.

1.3.1. Oligonucleotide Probing

Probing, of blotted PCR-amplified rRNA gene fragments, with specific oligonucleotide probes can be used to detect the presence of rRNA sequences characteristic of particular microorganisms or groups of organisms in an environmental sample (e.g., *see* **refs.** ***27–29***). Oligonucleotide probing has the advantage that it is rapid and a large number of samples can be screened simultaneously. Furthermore, the same rRNA gene fragments can be challenged with a range of different probes to provide a rapid characterization of the important sequence types represented. However oligonucleotide probes that target particular organisms are often designed from comparative analysis of rRNA sequences from cultured taxa, and the presence of target sequences in uncharacterized, unrelated taxa cannot be discounted. Consequently, inferences, from such studies, about the presence of particular microbial groups should be treated with caution. The degree of confidence is, however, increased substantially if the PCR is conducted with specific primers and confirmation of the identity of the sequences amplified is obtained by probing with a third specific oligonucleotide ***(28)***.

Relative quantitation of particular sequence types is also possible ***(30)*** using oligonucleotide probing, although this is subject to a number of caveats relating to potential biases exerted by the PCR amplification and the occurrence of multiple rRNA operons in some organisms (**Subheading 1.4.2.**).

1.3.2. Cloning and Sequencing

In contrast to oligonucleotide probing, cloning and sequencing of PCR products with subsequent comparative analysis to determine the relationship of environmental sequences to cultured isolates can be laborious. It does, however, allow identification of novel taxa and their phylogenetic placement in relation to cultured isolates and sequences recovered from other environments. DGGE also offers the opportunity to obtain sequence data from novel taxa by excision of bands, followed by reamplification and direct sequencing ***(31,32)***. The DGGE approach, however, is limited in the size of rRNA gene fragment that can be analyzed, and in complex environments in which DGGE can

generate large numbers of bands, it can be problematic to obtain single bands, free from contamination with other rRNA gene fragments, that can be sequenced directly.

1.4. Limitations

The development of molecular biological techniques to study microbial populations as they occur in nature has permitted culture-independent determination of the dominant microbial types present. Although the considerable limitations and biases inherent in culture-based techniques are circumvented by this approach, the molecular methods, too, have limitations and intrinsic biases. In the context of rRNA sequences recovered from environmental samples, differential lysis of microbial cells during DNA extraction procedures can result in cells that are more resistant to lysis being overlooked in molecular inventories of microbial diversity. This is discussed in Chapter 7. However, several additional factors also confound attempts to infer accurately the diversity of natural microbial populations.

1.4.1. Sampling and Coverage in Clone Libraries

An issue that is frequently overlooked in studies of microbial diversity based on the analysis of rRNA sequences is sampling. This is in part owing to the expense and labor-intensive nature of some of the approaches used. Cloning of rRNA gene sequences obtained from independently obtained replicate samples and sequencing of large clone libraries obtained from each sample is not normally feasible on the basis of cost and time. Sequence data obtained from single clone libraries, by their nature, represent a snapshot of the dominant members of the microbial population present in space and time and tell us nothing about temporal or spatial variation. For this reason, single rRNA gene clone libraries may tell us little about what sequence types might represent "typical" bacteria from a particular environment. However, the discovery of related rRNA gene clusters recovered independently from different locations by different groups using a variety of methods has allowed the identification of novel bacterial taxa, known only from rRNA sequences, that are apparently globally distributed ***(33–35)***.

The introduction of DGGE has permitted variation between samples to be investigated. Comparison of DGGE band patterns of rRNA gene fragments from replicate samples and samples taken over time are now being used to assess the temporal and spatial variation in microbial communities. For instance, DGGE analyses have demonstrated that considerable stability exists in the dominant bacterial populations of hot spring microbial mat communities ***(36)*** and in wastewater treatment plants (Craine, N. G. and Curtis, T. P., personal communication). This work implies that few samples need to be charac-

terized to obtain representative information about the microbial populations present. The environments mentioned are slightly unusual in that the former is relatively stable in terms of the physical and chemical conditions and the latter is well mixed. It seems unlikely that the spatial and temporal stability observed in these environments will be the rule when more heterogeneous environments such as soils and sediments are examined with similar rigor. There is, therefore, a need for a more systematic approach to sampling in the molecular characterization of microbial communities. Subjecting replicate samples to DGGE analysis to determine the degree of variability in the microbial population as a whole, followed by careful selection of subsets of the original samples for more detailed investigation, may be a sensible way to proceed.

Another important sampling issue in analysis of rRNA sequences cloned from environmental samples is the estimation of how much of the actual diversity in a natural sample is sampled in a clone library ***(37)***. Methods to estimate coverage in clone libraries have been borrowed from studies of populations of macroorganisms ***(38)***. Coverage (C) is determined using a simple equation:

$$C = 1 - (n_1/N) \qquad (1)$$

where n_1 is the number of sequence types from a clone library that are encountered only once, and N is the total number of clones analyzed. Hence, if there is a large proportion of unique sequences recovered in a clone library, n_1/N tends toward unity and coverage is small. To simplify the derivation of a value for coverage, it has been suggested that sequences >97% in similarity should be considered identical ***(37)***. This is based on the observation that organisms with 16S rRNA sequence homologies below 97% are unlikely to exhibit genomic DNA homology >80% (indicative of a relationship at the species level; ***[39]***). If anything, this may underestimate species diversity since a number of organisms known to have rRNA sequence homology of >99% are clearly distinct species based on DNA-DNA reassociation experiments and phenotypic data (e.g., *see* **refs. *15*** and ***40***). Adopting a 97% cutoff for operationally defining what constitutes a single sequence type will lead to an underestimation of the diversity if organisms with high rRNA sequence homologies that are genetically distinct are present. However, this conservative approach is justified since heterogeneity of different rRNA operons within a single organism can be significant ***(41,42)***.

Calculations from published data indicate that in clone libraries from different environments, coverage can range from as little as 4% ***(43)*** to >80% ***(37)***. This implies that in clone libraries where coverage is low, considerable undiscovered diversity exists. With coverage values as low as 4%, the clone library to be analyzed would have to be enormous in order to catalog the majority of the diversity in a sample. Consequently, in such diverse environ-

ments it will be difficult to identify "key" uncultured taxa that may be environmentally important.

1.4.2. Quantitative and Qualitative Discrepancies in PCR-Generated rRNA Clone Libraries

The PCR is an immensely powerful technique. There are, however, important limitations to what can be achieved using the PCR. In particular, quantitative inferences from PCR-amplified rRNA sequences derived from environmental samples should not be accepted uncritically. Quantitative discrepancies in PCR-derived data sets fall into two primary categories: those that are a consequence of the properties of rRNA sequences themselves, and those that are brought about by mechanistic features of the PCR.

1.4.2.1. Intrinsic Biases

Anomalies that are a consequence of features of rRNA sequences include selective amplification of some sequences over others ***(44)*** leading to overrepresentation in clone libraries; more efficient amplification of rRNA sequences that are clustered on the genome ***(45)***; overrepresentation of sequences from organisms with multiple rRNA operons ***(45)***; and overestimation of population diversity owing to heterogeneity in rRNA operons within a single organism ***(41,42)***. Furthermore, qualitative and quantitative anomalies can be the result of primer selection. Even when "universal" primers are used, it is possible that a low level of mismatch between the primer and target sequence can result in preferential amplification of certain rRNA gene sequences. Introduction of degeneracy into primer sequences can minimize this, but degenerate primers, essentially a mixture of similar but not identical primers, also have the potential to cause biases in PCR amplification. This can result from differences in the annealing temperatures of oligonucleotides in a degenerate mixture. Also, exhaustion of the primer sequence corresponding to the most abundant sequence types in a sample may result in a skewed distribution of sequence types recovered in a clone library because amplification of less abundant sequences is favored toward the end of the amplification cycle ***(37)***.

1.4.2.2. Mechanistic Biases

Competition between primer annealing and template reannealing has recently been recognized as a further cause of potential bias in the PCR amplification of rRNA genes ***(46)***. Using defined mixtures of rRNA gene templates, it was demonstrated that some primer pairs gave a strong correlation between the ratio of genes in the starting mix and the ratio in the final PCR product ***(46)***. This was not consistently observed with all primer pairs used. In instances in which the starting ratio of rRNA genes was not reflected in the final ratio

obtained in the PCR product mixture, it was found that the ratio was generally close to 1:1 and that this was independent of the starting ratio of the two genes. Increasing the number of cycles in the PCR reaction encouraged this effect. A kinetic model was developed to explain this phenomenon. The model predicted the observed PCR bias and indicated that the cause was preferential reannealing of the template DNA (**Fig. 1**). The explanation for a tendency toward a 1:1 ratio of products regardless of the initial ratio of genes present was that in a mixture of two rRNA genes with one present in excess, as the PCR proceeds, the concentration of the most abundant template reaches a critical concentration. Once this concentration of template is attained, reannealing is favored over primer annealing and amplification of this template decreases. Thus, the originally less dominant template becomes more effectively amplified in the later cycles of the PCR until it too reaches a concentration at which template reannealing outcompetes primer annealing (**Fig. 1**). This did not occur with all primer pairs used because when a primer pair amplified with low efficiency, the critical concentration for template reannealing was never reached. However, it was argued that the phenomenon may not be a serious problem when amplifying from environmental DNA because it would harbor a variety of templates, all at relatively low concentrations. Any single template, therefore, would be unlikely to reach a concentration at which reannealing would be favored over primer annealing ***(46)***.

It has also been noted that cloned PCR products generated using different primers resulted in significantly different composition of clone libraries ***(47)***. Furthermore, the same batch of PCR product cloned using either blunt-end or sticky-end cloning procedures gave different results. However, it is not clear how internal restriction enzyme cleavage affected the results since the clone libraries were screened by dot-blot hybridization procedures and the size of the insert DNA in the screened clones was not reported.

No DNA polymerase is totally accurate in reproducing DNA. Consequently, errors occur during replication of PCR-amplified genes. The frequency of nucleotide misincorporation varies for different thermostable DNA polymerases used in the PCR. Enzymes such as *Pyrococcus furiosus* DNA polymerase (*Pfu* DNA polymerase) that have a 3'–5' proofreading function have very low rates of nucleotide misincorporation. More commonly used enzymes such as *Thermus aquaticus* (*Taq* DNA polymerase) lack a proofreading exonuclease activity and hence have higher error rates. However, the degree of error resulting from misincorporation of bases during the PCR is generally relatively small (fractions of 1%) compared to differences in rRNA sequences between bacterial species (approx 2 to 3% ***[39]***). Nonetheless, this does not always hold, and many well-defined species show much lower differences in their rRNA sequences, close to the levels of misincorporation reported for

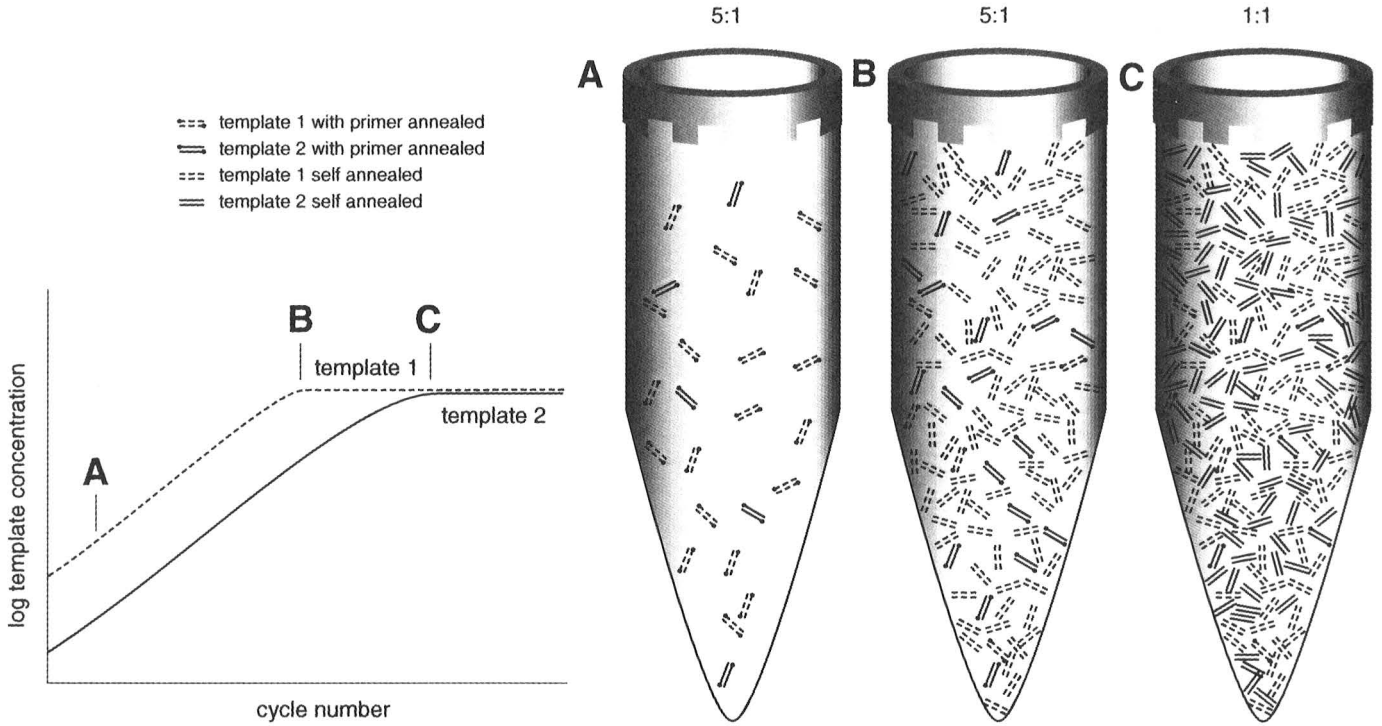


Fig. 1. Schematic of the effect of preferential template annealing on the PCR amplification of a mixed template. **(A)** Template 1 is originally more abundant than template **2,** both amplify at the same rate. **(B)** Template 1 has reached a concentration at which template annealing is favored over primer annealing. The rate of amplification of template 1 is greatly reduced. Template 2 remains at a concentration at which primer annealing is favored and continues to be amplified efficiently. **(C)** Template 2 reaches a concentration at which template annealing is favored and amplification is diminished.

some thermostable DNA polymerases ***(15)***. However, the ability of rRNA sequence analysis to resolve relationships at the species level is limited under these circumstances.

A further problem associated with analysis of microbial diversity using PCR-amplified rRNA genes is the formation of chimeric PCR products ***(48)***. Chimeric genes result from the incomplete synthesis of an rRNA gene fragment during amplification. If the incomplete fragment anneals to a homologous rRNA gene fragment forming a heteroduplex, it can be extended to full length. This results in an rRNA gene fragment that has been replicated from two (or more) different templates and, thus, represents a complete rRNA sequence that does not exist naturally in a living organism (**Fig. 2**). The occurrence of chimeric molecules can best be detected by conducting phylogenetic analyses on opposite ends of the rRNA sequence. If the sequence is chimeric, then the trees generated from the two fragments will be incongruent. If the sequence is genuine, the trees generated independently from different regions of the rRNA molecule should be identical, or at least very similar. Several computer programs have now been developed to identify chimeric sequences (e.g., *see* **ref. *49***), but these have difficulties in recognizing chimeric molecules in which the "parent" sequences have >85% homology. These programs should, therefore, only be used as a guide, and the occurrence of chimeras should be confirmed by careful analysis of secondary structure interactions and independent phylogenetic analyses with different regions of the molecule. The frequency of chimera formation has been determined to be up to 30% when PCR has been conducted with mixtures of similar templates ***(50)***. Occurrence of chimeric sequences in clone libraries from natural samples has been reported to be slightly lower (e.g., *see* **ref. *37***). Nonetheless, it is advisable to test PCR-derived sequences for their possible chimeric nature since they can lead to overestimation of the microbial diversity present in a particular sample.

1.5. Quantitation using PCR

PCR-dependent methods for characterizing microbial communities or identifying particular organisms present in an environmental sample are invaluable. The extreme sensitivity of the PCR lends it to sensitive qualitative analysis of specific organisms when presence/absence data are required. Obtaining quantitative data using the PCR is more problematic. The biases outlined in **Subheading 1.4.2.1.** and **1.4.2.2.** all confound attempts to use the PCR for accurate quantitation of microorganisms in nature. Although elegant solutions have been developed that allow quantitation based on the use of internal standards as competitive templates (e.g., *see* **ref. *51***), they rely on the assumption that all templates in a heterogeneous mixture are amplified equally efficiently. And, although this may often hold ***(51,52)*** this is not always a valid assumption

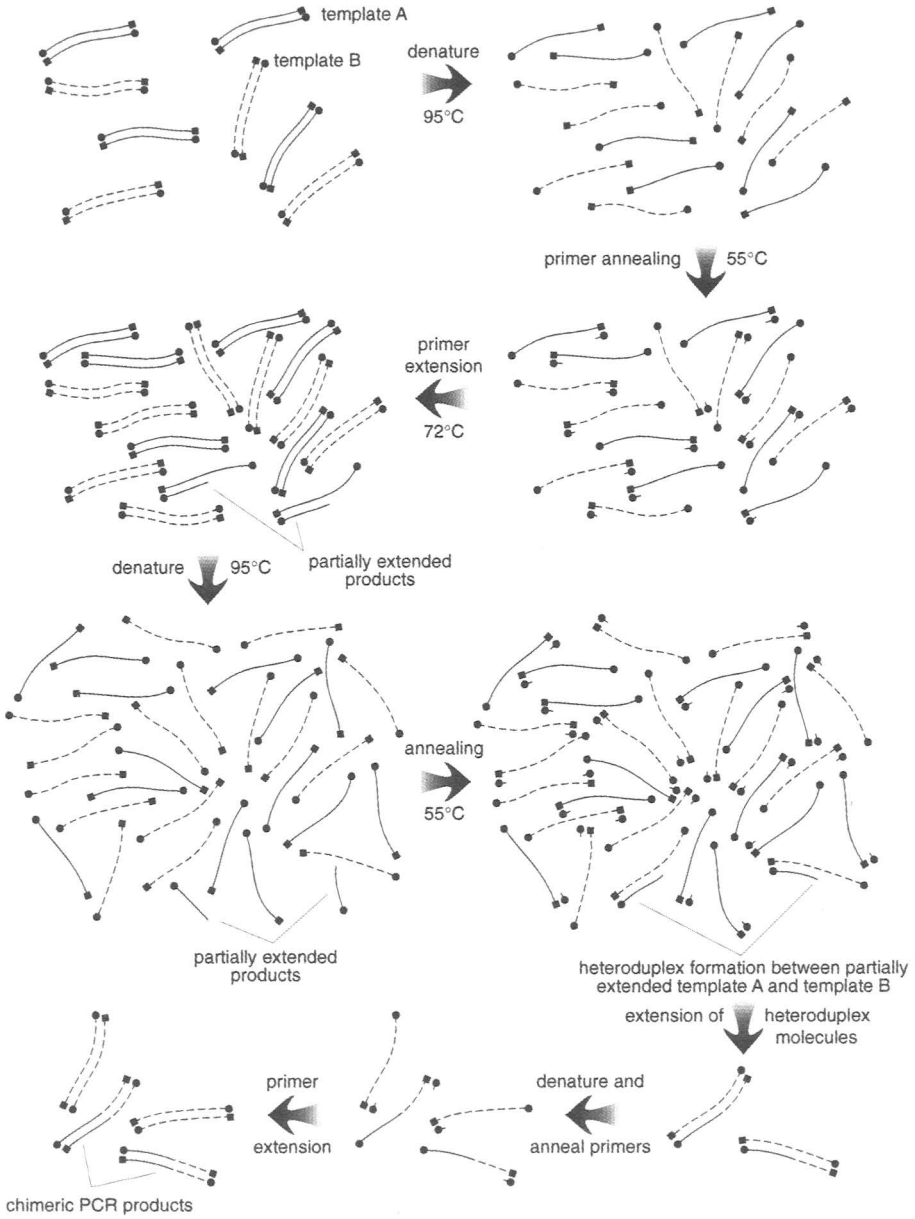


Fig. 2. Schematic diagram of chimeric product formation during the PCR.

(48) and thus absolute quantitation of specific rRNA sequences based on the PCR must be considered with care. However, quantitative competitive PCR using an internal standard that can be shown empirically to amplify with the

same efficiency (or at least at a reproducible and measurable efficiency relative to the target sequence) as the target sequence holds promise. Quantitative, competitive PCR using primers specific for a particular group of organisms has been demonstrated to work well for the quantitation of rRNA sequences from microbial taxa recognized only from environmentally recovered rRNA sequences ***(52)***. This was possible by the careful design and use of a competitor template that was shown to amplify with the same efficiency as the target template. However, conversion of the abundance of a particular rRNA sequence to a value for cell numbers or biomass remains problematic.

Determination of the relative abundance of particular organisms using hybridization with specific oligonucleotides to quantify specific sequence types in a PCR-amplified mixture is also feasible in some circumstances. Instances in which this may be used validly include time-series data and depth distributions of particular sequence types (e.g., *see* **ref. *53***). In these situations, it is the relative abundance of a particular sequence type at different points in space or time that can be determined. It would be impossible to convert this measure to a figure for the proportion of the total microbial population that this represents unless all templates present were known to amplify with the same efficiency. Likewise, an absolute number of cells cannot be inferred without information on the size of the genome and the rRNA gene copy number for all of the organisms present. This approach is suited principally to autoecological studies, and useful information on the particular niche occupied by uncultivated microorganisms has been obtained by this approach ***(30,33,53)***. With this type of information about the physical and chemical conditions found in which an organism is most abundant ,one can begin to formulate strategies for isolating that organism in culture and determining its metabolic activities of biogeochemical relevance.

Complementary to this approach is the relative quantitation of rRNA extracted directly from environmental samples rather than rRNA genes ***(24–26)***. Like relative quantitation of rRNA gene sequences in PCR-amplified mixtures, this does not provide a value that can be converted to cell numbers or biomass, but since the rRNA content of a cell is proportional to its growth rate ***(54)*** it can give an indication of the relative activity of specific organisms rather than their relative abundance.

In conclusion, it seems wise to consider recovery and analysis of rRNA sequences from environmental samples, not as a definitive cataloging exercise but as the first step toward identifying relatively abundant, uncultured members of the microbial population. The cloned sequences provide markers that permit the presence of particular taxa to be determined, and relative changes in the uncultured populations in time and space in relation to changing environmental conditions can be studied. They also provide the information required

to embark on the targeted isolation of taxa that have not yet been cultivated but potentially catalyze important biogeochemical functions. It is only by isolation of the organisms that the sequences represent that their true role in the environment can be discovered.

1.6. PCR Amplification of rRNA Genes from Environmental DNA Practical Considerations

The major issue regarding amplification of rRNA genes, and any other gene, from environmental samples is overcoming inhibition of the PCR by substances coextracted with the nucleic acids. Details of procedures designed to remove inhibitory substances are given in Chapter 7. Essentially the procedures involve differential precipitation of the contaminating substances with ammonium acetate, physical separation using gel permeation techniques, and adsorption of inhibitory materials onto ion-exchange matrices and adsorbents such as polyvinypolypyrollidone. A simple method that often yields successful PCR is dilution of the environmental DNA preparation to reduce the levels of the inhibitory contaminants to below the level at which the PCR is inhibited.

Assuming that nucleic acids of sufficient quality have been purified from the environmental sample of interest, the PCR of rRNA genes is quite straightforward.

As with any application of the PCR, it is desirable to have a dedicated work area and instruments (pipets, and so forth). Reagents and PCR products should be stored remote from template DNA preparations. When amplifying rRNA sequences using universal primers or primers targeting very broad phylogenetic groupings, such as all bacterial rRNA genes, contamination can be a serious problem. For example, many thermostable DNA polymerase preparations contain sufficient DNA from the producing organism to yield PCR products in negative controls containing no added DNA. However, treatment of enzyme preparations with DNase I has been proven to remove DNA effectively from enzyme preparations ***(55)***. Bovine serum albumin (BSA) in PCR buffers can harbor bacterial contamination. Poor-quality water can also be a source of contaminating DNA, and even high-quality water purification systems, if not regularly cleaned and maintained, can carry sufficient DNA to allow amplification of PCR product in negative control tubes. In addition, mineral oil overlays can be an important source of contaminating DNA. All reagents should be prepared with filtered, sterile distilled or deionized water of high quality. If contamination with exogenous bacterial DNA is a persistent problem, exposure to ultraviolet (UV) radiation either on a UV transilluminator or using a UV crosslinker will also help reduce DNA contamination.

Primer selection is a crucial consideration and will depend on the particular application and whether rRNA genes from a wide range of organisms (e.g., all members of the domain *Bacteria*) or a specific group of organisms are being

targeted. It has also been found that some primer pairs may amplify environmental DNA successfully, whereas others do not ***(56)***. The protocol described here works well for the amplification of bacterial rRNA genes from environmental samples. Most of the components of the PCR mixture can be obtained commercially from a variety of suppliers, and many companies provide an economical service for the synthesis of custom-designed oligonucleotide primers. Purchase of high-quality reagents from reputable suppliers is recommended because this ensures reproducibility between batches of reagents and also reduces the risk of contamination of reagents with low levels of DNA during preparation, thereby saving time and effort.

1.7. Analysis of PCR-Amplified rRNA Sequences: Practical Considerations

1.7.1. Use of Diagnostic Primers and Oligonucleotide Probing

The most straight-forward and rapid means to analyze PCR-amplified rRNA sequences relies on the use of diagnostic oligonucleotides. The oligonucleotides are used either as hybridization probes or as PCR primers, and amplification of a product of the correct size under stringent conditions is generally indicative of the presence of a particular organism or group of organisms (e.g., *see* **ref.** ***57***). Greater confidence in the source of the amplified rRNA gene fragment can be obtained by probing the PCR product in either a Southern blot or dot-blot format (e.g., *see* **ref.** ***28***). PCR products amplified using universal primers can also be screened with diagnostic oligonucleotides ***(28,30)*** and some degree of quantitation is offered by this approach ***(30)***. When combined with DGGE, the PCR-probe approach can provide useful qualitative information on the composition of microbial populations ***(27)***.

1.7.2. Cloning and Sequencing PCR-Amplified rRNA Sequences

The most widely adopted approach to characterizing a microbial population in detail involves sequencing of cloned rRNA gene fragments amplified from environmental DNA. It is also possible to sequence distinct rRNA gene fragments resolved on DGGE gels (*see* Chapter 12). The cloning approach, however, generally facilitates the sequencing of larger fragments. This permits phylogenetic analysis at greater resolution and offers more scope for the design of diagnostic oligonucleotides. The three main approaches to cloning PCR products that vary depending on the ligation method used are sticky-end, blunt-end, and TA-cloning.

1.7.2.1. Sticky-End Cloning

Sticky-end cloning or forced cloning as it is sometimes termed requires the addition of restriction sites to the 5' end of the amplification primers (e.g., *see*

refs. ***56*** and ***58***) It has the advantage that if different restriction sites are incorporated in each primer, then double digestion can be carried out, preventing recircularization of the cloning vector, hence improving cloning efficiency. However, cleaving restriction sites at the termini of PCR products can be problematic ***(59)***. Furthermore, restriction endonuclease cleavage at sites within amplified rRNA gene products can result in the recovery of truncated rRNA sequences in clone libraries. This can be overcome to some extent, by the use of rare cutting restriction endonucleases such as *Not*I ***(50)***.

1.7.2.2. Blunt-End Cloning

Blunt-end ligation procedures are less efficient than sticky-end ligation. Nonetheless cloning of PCR products using blunt-end cloning of rRNA fragments amplified from environmental samples has been widely adopted (e.g., *see* **refs.** ***60–62***). Since there is no need for restriction digestion, full-length PCR products can be cloned. However, thermostable DNA polymerases that lack a 3'–5' proofreading function (e.g., *Taq* DNA polymerase) have terminal deoxynucleotide transferase activity and add a template-independent deoxyadenosine residue to the 3' ends of the PCR product ***(63)***. This substantially reduces the efficiency of blunt-end ligation procedures. Consequently, the PCR products must be modified to produce blunt ends. This is normally achieved using a DNA polymerase that has a 3'–5' proofreading function (e.g., T4 DNA polymerase or *Pfu* DNA polymerase). It has been reported that *Pfu* DNA polymerase (Stratagene, Cambridge, UK) generally gives better blunt-end cloning efficiency than does T4 DNA polymerase. Alternatively, PCR products amplified with thermostable DNA polymerases with a proofreading exonuclease activity (e.g., *Pfu* DNA polymerase) obviate this treatment since they do not produce PCR products with a single nucleotide 3' extension ***(61,62)***. Recently improved blunt-end cloning procedures have been developed. These maintain a high level of linear blunt-ended vector by inclusion of the rare-cutting blunt-end restriction endonuclease *Sfi*I in the ligation mixture.

1.7.2.3. TA-Cloning

Although blunt-end cloning is compromised by the addition of 3' overhanging nucleotides by nonproofreading DNA polymerases, this factor is exploited for the efficient cloning of PCR products in "T-vectors". T-vectors are plasmids that when linearized have single deoxythymidine residues at the 3' ends. This can be conveniently achieved by cleavage with restriction endonucleases that produce blunt ends. 3' dT overhangs can then be generated by incubation with *Taq* DNA polymerase and dTTP. Vectors generated in this way can be obtained commercially (e.g., pGEM-T, Promega, Southampton, UK). These

vectors allow sticky-end ligation of PCR products generated by nonproofreading thermostable DNA polymerases without the need for restriction digestion.

1.8. Screening rRNA Clone Libraries

To reduce the number of clones that require sequencing in a 16S rRNA gene library from environmental samples, it is desirable to screen the library to identify similar or identical rRNA sequences. This can be done using colony hybridization procedures with oligonucleotide probes of defined phylogenetic resolution (e.g., *see* **ref. *61***). However, the specificity of the probe used is critical. If a probe is of too great a specificity, clones containing sequences of interest can be overlooked. Conversely, it is possible to discount unique clones if they contain the target site for the oligonucleotide probe, but are otherwise quite different. Isolation of plasmids from individual clones and digestion with frequently cutting restriction endonucleases can also be used to group related sequences (e.g., *see* **refs. *60*** and ***62***). Alternatively, colony PCR using, e.g., sequencing primers with priming sites that flank the insert DNA can be used as a rapid screening procedure to detect cloned PCR products. These are digested with restriction endonucleases to identify related sequences in the clone library.

Alternatively, single-lane sequencing (t-tracking) can also be done to allow higher resolution screening ***(64)***. If manual sequencing is used, this can be an effective way of identifying similar clone sequences without the requirement to carry out all four sequencing reactions. However, with the more widespread availability of rapid automated DNA sequencers, sequencing of clones using a single primer can be relatively cost-effective and provides sufficient information for basic phylogenetic analysis.

1.9. Characterization of Uncultured Microorganisms

The application of rRNA sequence analysis to the characterization of microbial populations is extremely powerful. In the relatively short time since the inception of these techniques, much has been discovered about as-yet uncultivated microorganisms in natural populations. Consequently, this approach is becoming routine in many research laboratories. Although the technology is well established, characterization of microbial populations by the PCR-clone–sequence approach remains labor intensive and time-consuming. It is therefore unlikely to be used as a routine environmental monitoring tool. It is, however, the method of choice for detailed culture-independent characterization of microbial populations and provides the framework for subsequent studies using methods more amenable to comprehensive sampling and rapid analysis (e.g., PCR and probing, whole-cell *in situ* hybridization and DGGE analysis). Several novel, globally-distributed microbial taxa ***(25,34,52)*** have been uncovered

by this approach and would likely have remained unknown otherwise. In addition to the discovery of novel phylotypes, cloned rRNA sequences have provided the information necessary to design oligonucleotide primers and probes that have facilitated autoecological studies of uncultured taxa ***(17,30,33,35)***. Using this approach it has been possible to determine the distribution and temporal population dynamics of uncultured microorganisms ***(30,33,35)***.

Sequence data can also be obtained from DGGE gels by excision of individual bands reamplification and direct sequencing of the PCR product obtained. This can, however, be difficult if complex communities are analyzed since obtaining pure bands of a single sequence type may require several rounds of purification and DGGE under different denaturing conditions, to obtain purified PCR product and good quality sequence data.

1.10. Detection of Specific Groups of Organisms

PCR utilizing specific primers or diagnostic oligonucleotide probing of rRNA sequences amplified using broad specificity primers probably offers the most promise for the development of rapid techniques to monitor specific microbial populations. The technique is sensitive: detection limits as low as 70 cells/g of soil (equivalent to less than a single cell per PCR assay) have been claimed ***(57,65)***. Detection limits of the order of 10^3 to 10^4 cells/g of soil are probably more realistic, even when using nested PCR ***(66)*** or subsequent oligonucleotide probing to increase sensitivity ***(67)***. In the cited examples, environmental samples were inoculated with known amounts of cultured bacterial cells, and detection limits for indigenous populations may be slightly higher.

This approach has been used to detect bacterial inoculants with specific catabolic activities in environmental matrices (e.g., *see* **refs. *66*** and ***67***) and particular groups of indigenous pollutant-degrading microorganisms ***(57)***. Biogeochemically significant organisms have also been detected using PCR amplification either with or without oligonucleotide probing ***(28,68,69)***. The presence of pathogens such as *Legionella* ***(70)*** has also been detected in water samples using PCR amplification of rRNA gene fragments. In the case of *Legionella* spp. and *L. pneumophila,* this has been developed into a commercial diagnostic kit for environmental monitoring (EnviroAmp™, PE Applied Biosystems, Warrington, UK). The use of diagnostic PCR amplification of rRNA sequences is, however, much more widespread than this and, in addition to strictly environmental applications, has many potential clinical applications. It is equally suited to the detection of pathogens and spoilage organisms in the food and agriculture industries (e.g., *see* **refs. *71*** and ***72***).

To date, most applications of PCR amplification of rRNA sequences to environmental monitoring have been at the level of presence/absence testing.

This has stemmed largely from the difficulties in reliably quantifying the amount of a particular rRNA gene sequence present in template DNA extracted from environmental samples, using the PCR. However, the development of genuinely quantitative PCR assays, particularly when specific oligonucleotide primers are used ***(52)*** holds considerable promise for the future. It may be possible to determine accurately the starting concentration of a particular template gene using carefully controlled competitive PCR. However, without knowing of the size of an uncultivated organisms genome or the number of copies of the rRNA gene per genome, it will not be possible to convert this to cell numbers or biomass. This problem remains a key issue in PCR-dependent quantitation of uncultured microbial taxa.

1.11. Autoecological Studies of Uncultured Bacteria

Routine absolute quantitative assays based on the PCR may be slightly inaccurate and require careful validation, but robust methods that allow relative quantitation of specific rRNA sequences are available (*see* **Subheading 1.5.**). These assays are suited to autoecological studies of organisms whether or not they can be cultivated. Useful information on the relative abundance of taxa recognized only from environmentally isolated rRNA sequences has been obtained using this method. For example, the relative abundance of a novel bacterial lineage (SAR406) related to *Chlorobium* was investigated in surface water of the western Sargasso Sea over a 30-mo period ***(30)***. The relative abundance of SAR406 exhibited strong seasonal variations and correlated well with chlorophyll *a* concentration in the same samples. Furthermore, SAR406 sequences were found to be most abundant at a depth in the water column that was just below the deep chlorophyll maximum ***(30)***. Similar studies have revealed that different members of the SAR11 environmental rRNA gene cluster may exhibit niche partitioning with some SAR11-like sequences being most abundant in deeper water and others being more abundant towards the surface of the water column ***(33)***.

This technique along with whole-cell *in situ* hybridization (*see* Chapter 15) provide useful means to monitor specific microbial populations in natural environments, relatively rapidly. The limitations of these procedures have been explored (**Subheading 1.4.** and **1.5.**). Nonetheless, they do provide the means to monitor particular microorganisms independent of the requirement for cultivation. Prior to the development of molecular biological monitoring methods this was not possible.

1.12. Changes in Microbial Community Structure

The autoecological studies outlined provide information on the behavior and distribution of discrete microbial populations (either specific organisms or

groups of related organisms). It is also possible to monitor variation in microbial populations at a more gross level. Two primary approaches have been used to achieve this with amplified rRNA sequences: DGGE, and amplified ribosomal DNA restriction analysis (ARDRA).

DGGE allows rapid comparisons of microbial communities (subject to the caveats above, **Subheading 1.4.**). It permits temporal and spatial changes in microbial populations to be monitored, and if conducted using specific primers or coupled with the use of oligonucleotide probing, the dynamics of specific groups of organisms can be monitored.

The recolonization of hot spring cyanobacterial mats following physical disturbance has been studied using DGGE of PCR-amplified 16S rRNA genes ***(73)***. This study demonstrated that rapid recolonization of the disturbed mats occurred, but not all of the original cyanobacterial sequence types originally detected were present even after 40 d, and novel cyanobacteria were responsible for much of the initial recolonization. Longer-term monitoring indicated that the bacterial populations present in the same hot spring environment were rather stable over a 10-mo period and that there was a succession of bacterial types along the thermal gradient from the source pool of the hot spring ***(36)***. DGGE analysis also demonstrated that populations of ammonia-oxidizing bacteria varied across a sand dune system with sequences related to marine ammonia oxidizers found largely on the seaward side of the dunes. In addition, different populations of *Nitrosospira*-like organisms were, in some cases, associated with samples of different pH ***(32)***.

A further technique that has been introduced recently to characterize complex microbial communities based on PCR-amplified rRNA genes is ARDRA ***(74,75)***. This method is based on variations in the frequency and location of tetrameric restriction enzyme recognition sites in rRNA genes. Almost full-length rRNA genes are amplified from environmentally isolated DNA and digested with restriction endonucleases that recognize tetrameric sequences (e.g., *Alu*I, *Bst*UI, *Hae*III, *Hha*I, *Mbo*I, *Msp*I, *Rsa*I, and *Taq*I). Different rRNA genes produce different sized restriction fragments and hence can be distinguished on agarose gels ***(74,76)***.

Monitoring inoculant and indigenous groundwater bacteria in a fluidized bed reactor treating toluene-contaminated groundwater has been a useful application of ARDRA. *Pseudomonas putida* PaW1, *Burkholderia cepacia* G4, and *Burkholderia pickettii* PKO1 produce distinctive ARDRA band patterns that can be recognized on agarose gels ***(74)***. Following inoculation of these bacterial strains into a fluidized bed reactor, treating filter-sterilized groundwater containing toluene, changes in the population of the three bacteria were monitored using ARDRA. After an initial period of 53 d, unfiltered groundwater amended with toluene was used to feed the bioreactor. It was clear from

the ARDRA data and parallel, culture-dependent enumeration of the inoculant bacteria that strain PaW1 outcompeted the other two bacteria for toluene and, ultimately, excluded them from the bioreactor. Contamination with groundwater bacteria had occurred by d 38 of the experiment, and this was clearly evident from changes in the ARDRA band pattern. By d 58 a stable population of indigenous groundwater bacteria had developed, but low levels of strain PaW1 were still detectable in the reactor. This suggested that introduced inocula were unlikely to compete well against indigenous organisms utilizing the same carbon source, even if the inoculant strains had initially colonized the bioreactor.

ARDRA is a rapid and simple technique to fingerprint whole microbial communities. Unlike DGGE analysis, there is no requirement for relatively complex polyacrylamide denaturing gradient gels. However, the low resolution of agarose gels means that many similar but not identically sized bands will be seen as a single band, thus underestimating the true diversity. This is exacerbated by the fact that digestion with at least three restriction endonucleases is required to give maximal taxonomic resolution ***(76)***. Nonetheless, ARDRA band patterns obtained with replicate samples from a fluidized bed reactor using three different restriction endonucleases showed good agreement in the diversity observed ***(74)***. Furthermore, ARDRA is only likely to allow changes in the most abundant members of the population to be characterized. Consequently, it is likely to be of use in the study of microbial populations comprising a small number of dominant organisms. In the limited number of studies conducted, to date, using ARDRA for whole community analysis, the bands detected in ARDRA analyses have corresponded well with the ARDRA patterns obtained from the dominant culturable members of the bacterial community isolated by dilution enrichment techniques ***(74,75)***. These examples from biotreatment systems, devised for the decontamination of polluted groundwater, indicate the utility of combing ARDRA monitoring and cultivation techniques. The ability to use molecular techniques to target isolation of key species is significant. Physiological data from the isolated taxa, which can be demonstrated by molecular methods to be dominant members of the bacterial population, will be invaluable for informing mathematical models of biotreatment.

2. Materials

2.1. PCR Amplification of 16S rRNA Genes

1. 10 X PCR buffer: 100 m*M* Tris-HCl (pH 8.8 at 25°C), 500 m*M* KCl, 15 m*M* $MgCl_2$, 1% (v/v) Triton X-100 (*see* **Note 1**).
2. 100 m*M* deoxynucleotide triphosphate (dNTP) solutions (dATP, dCTP, dGTP, dTTP): Ideally these should be purchased from a reputable supplier of molecular biology reagents, e.g., Ultrapure™ dNTPs (Pharmacia Biotech, St. Albans, UK). Dilute aliquots of each dNTP (100 µL each) with 600 µL of sterile deionized

water. Dispense 10-µL vol of this 10 m*M* dNTP solution into sterile microcentrifuge tubes and store at –20°C. This prevents excessive degradation of the dNTPs owing to repeated cycles of freezing and thawing.

3. Oligonucleotide primers: Primers are dissolved in sterile, deionized water and quantified by their UV absorbance at 260 nm and diluted to 10 µ*M* (*see* **Note 2**). The diluted oligonucleotide solutions are stored frozen as small volume aliquots (e.g., 20 µL) and used as required to prevent degradation owing to repeated freezing and thawing. Many primers have been designed with a range of specificities for the amplification of small subunit rRNA genes. Primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pHr (5'-AAGGAGGTGATC CAGCCGCA-3') devised by Edwards *et al*. *(77)* for the amplification of near-complete rRNA genes from *Bacteria* have been used successfully with the procedure described.
4. Deionized water. Distilled water treated using a propriety deionizing system (e.g., Milli-Q, Millipore, Watford, UK) is filtered through a sterile 0.2-µm filter and autoclaved. If persistent DNA contamination is a problem, the water may be UV irradiated (e.g., 5 min exposure on a UV transilluminator).
5. Thermostable DNA polymerase (*see* **Note 3**). 2 U/µL of Dynazyme (Flowgen, Lichfield, UK). Dynazyme is isolated from a strain of *Thermus brockianus*. Although it does not have 3'–5' proofreading exonuclease activity, it is reported by the manufacturers to have a lower misincorporation rate than *Taq* DNA polymerase, and I have found that it performs better than *Taq* DNA polymerase with DNA templates isolated from environmental samples. If a final extension of >10 min is used, the enzyme also adds 3' dA overhangs that allow TA-cloning of the PCR product.

2.2. Sticky-End Cloning

1. Vector DNA: Many commercially available plasmid cloning vectors can be used to clone PCR-amplified rRNA genes. The procedure described used pUC18 (BCL, Lewes, UK).
2. PCR primers: The primers are identical to those described in **Subheading 2.1.** except that restriction sites (in italics) have been incorporated at the 5' end. pA (*Pst*I) 5'- GTG*CTGCAG*AGAGTTTGATCCTGGCTCAG-3', pHr (*Bam*HI) 5'-CAC*GGATCC*AAGGAGGTGATCCAGCCGCA-3'. The PCR is carried out as described in **Subheading 3.1.**
3. Restriction endonucleases: The particular enzymes used depend on the restriction sites incorporated into the primers. For the primers described here, *Bam*HI and *Pst*I are required.
4. 5X Ligation buffer: 250 m*M* Tris-HCl (pH 7.6), 50 m*M* $MgCl_2$, 5 m*M* adrenosine triphosphate (ATP), 5 m*M* dithiothreitol (DTT), 25% (w/v) polyethylene glycol-8000.
5. T4 DNA ligase: Many commercial suppliers provide T4 DNA ligase. The amount required for the ligation reaction should be determined with reference to the manufacturer's specifications. This protocol was developed with T4 DNA ligase supplied by Life Technologies (Paisley, UK).

6. Competent *E. coli* cells: competent cells (*see* **Note 4**) can be prepared in the laboratory. However, in my experience, PCR-amplified rRNA genes do not always clone efficiently and best results are obtained when commercially obtained high efficiency competent cells are used (e.g., XL-1 Blue, SURE2, Stratagene).
7. Isopropyl-β-D-thiogalactopyranoside (IPTG): 0.1 *M* IPTG is prepared by dissolving 0.24 g of IPTG in 10 mL of Milli-Q water. The solution is filter sterilized and stored at 4°C.
8. X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside): Dissolve 50 mg/mL X-gal in *N,N*-dimethyl formamide.
9. Ampicillin: Dissolve 50 mg/mL ampicillin in sterile distilled water.
10. Growth medium for selection of recombinants (LB/Ap/IPTG/X-gal agar): 2.5 g of yeast extract, 5 g of Tryptone, 2.5 g of NaCl, 7.5 g of Agar are dissolved in distilled water (500 mL) and adjusted to pH 7.5 with NaOH. The agar is sterilized by autoclaving. When the agar has cooled to 50°C the following are added aseptically. 0.5 mL of ampicillin solution (50 mg/mL), 0.5 mL of IPTG solution (0.1 *M*) and 0.4 mL of X-gal solution (50 mg/mL in dimethyl formamide). The medium should be stored at 4°C and used within 30 d. The plates should be dried for 30–40 min in an incubator set at 37°C prior to use. This removes surface liquid from the plates and allows discrete colonies to develop.

2.3. Blunt-End Cloning

The same materials are required for blunt-end cloning as for sticky-end cloning except that a restriction enzyme generating blunt-ended vector DNA must be used (e.g., *Sma*I) and primers containing restriction sites are not required. Production of PCR products with blunt ends requires polishing of the PCR products to remove 3' overhangs. The necessary reagents are as follows:

1. 10X T4 DNA polymerase buffer: 330 m*M* Tris-acetate (pH 7.9), 600 m*M* potassium acetate, 100 m*M* magnesium acetate, 5 m*M* DTT.
2. Bovine serum albumin: 1 mg/mL acetylated BSA.
3. dNTPs: 1 m*M* dATP, 1 m*M* dCTP, 1 m*M* dGTP, 1 m*M* dTTP. These should be prepared from concentrated stock solutions of Ultrapure (Pharmacia Biotech, St. Albans, UK) dNTPs (100 m*M*).
4. T4 DNA polymerase.
5. TE buffer: 10 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA.

2.4. TA-Cloning

All materials required for TA-cloning are described in **Subheading 2.2. and 2.3.** except the T-vector. A number of T-vectors are available commercially, but all operate on the same principle. The protocol detailed has been used with the pGEM–T–vector system (Promega, Southampton, UK) Most commercially available TA-cloning systems also include a control insert DNA. It is advisable that control ligations be carried out with this insert since exonuclease

activity can remove 3' overhangs from the vector or PCR product, reducing the ligation efficiency.

3. Methods

3.1. PCR Amplification of 16SrRNA Genes

It is convenient to prepare a bulk reaction mix for 5–10 PCR reactions of 50–100 µL vol each and to dispense these into 0.5- or 0.2-mL reaction tubes or 96-well microtiter plates designed for use with thermal cyclers. The following protocol provides sufficient reaction mix for 10X 50-µL reactions.

1. Prepare 500 µL of bulk reaction mix containing 50 µL of 10X PCR buffer, 10 µL of dNTP mix (10 m*M* each), 10 µL of forward primer (10 µ*M*), 10 µL of reverse primer (10 µ*M*), 355 µL of sterile distilled water, and 5 µL of thermostable DNA polymerase (2 U/µL).
2. After the bulk reaction mix has been prepared and carefully mixed, aliquots (49 µL) are dispensed into individual reaction tubes. To each of these, add 1 µL of DNA template (*see* **Note 5**). Add sterile distilled water (1 µL) to one of the tubes as a negative control.
3. Overlay all of the reactions with a few drops of mineral oil (if the thermal cycler is fitted with a heated lid, no oil is required).
4. Subject the samples to the following PCR cycling program: initial denaturation at 95°C for 4 min followed by 30 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 1 min. After the final extension hold the reaction at 72°C for 10 min to extend fully any incomplete PCR products (*see* **Note 6**).

Annealing temperature is of vital importance when amplifying rRNA gene sequences from environmental samples. If specific diagnostic primers are used, then the annealing temperature must be sufficiently high to allow amplification of the target sequence without amplifying other closely related sequences. Conversely, if universal primers are used, lower annealing temperatures will allow amplification of rRNA genes even when there is some mismatch between the primer and target site (**Fig. 3**). Lowering the annealing temperature too far can, however, result in the amplification of nonspecific PCR products. This is particularly problematic if the PCR fragment is to be cloned. The presence of multiple PCR products necessitates gel purification of the PCR-fragment of interest.

3.2. Sticky-End Cloning

1. PCR amplification of rRNA genes with primers containing restriction sites can be done using the protocol described in **Subheading 3.1.**
2. Purification of PCR product: The amplified PCR product should be purified prior to cloning to remove excess primers and dNTPs. If a single band is observed, this

can be done directly from the reaction mix using commercially available PCR product purification kits (e.g., QIAquick spin columns, Qiagen, Crawley, UK); if multiple bands are observed, the band of the correct size (approx 1.5 kb for the primers described) can be excised from the gel and purified using a gel extraction kit (QIAquick gel purification system, Qiagen, Crawley, UK).

3. Restriction digestion: Digest the purified PCR product and vector with *Bam*HI and *Pst*I as recommended by the enzyme supplier. The small fragment of polylinker can be removed from the restriction digest using QIAquick spin columns.
4. Ligation: Ligation reactions containing a range of molar ratios of vector to insert are prepared. Molar ratios of 3:1, 1:1, and 1:3 are usually adequate. The amount of PCR product and vector required to give a particular molar ratio can be calculated using the following equation.

   Nanograms of insert to give desired molar ratio =
   [(nanogram of vector × size of insert in kilobase)]/
   size of vector in kilobase]× desired molar ratio

   Thus, to obtain a 1:1 molar ratio of vector to insert of a 1.5-kb PCR product and 50 ng of pUC18, use the following equation:

   $$[(50 \times 1.5)/2.69] \times (1/1) = 27.9 \text{ ng (insert is required)}$$

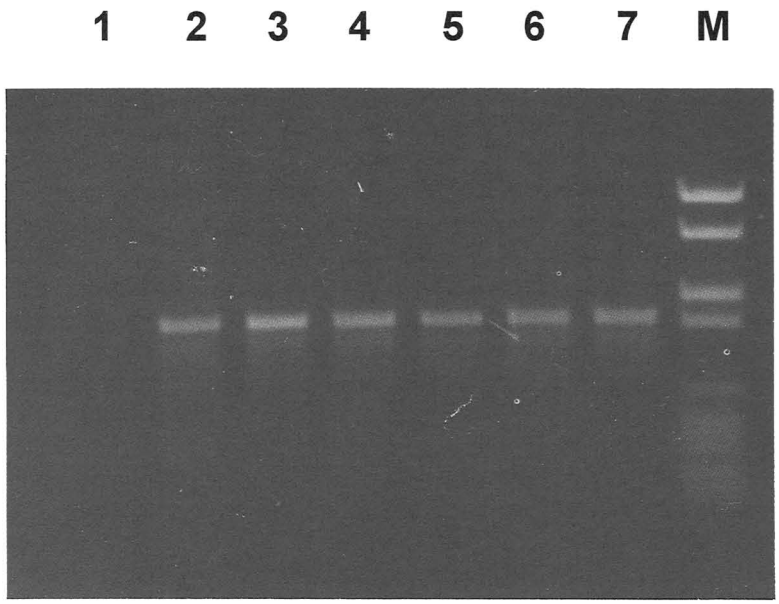
   Mix in a microcentrifuge tube 2.5 µL of digested pUC18 (20 ng/mL), 2 µL of 5X T4 DNA ligase buffer, and the appropriate volumes of digested PCR product, sterile distilled water, and T4 DNA ligase to give a final volume of 10 µL, and incubate at 4°C overnight.
5. Transformation: Thaw the high-efficiency competent cells on ice. Add 20 µL of the competent cells to a sterile 1.5-mL microcentrifuge tube on ice. Add 1 µL of ligation mix to the cells and tap gently to mix. Do not vortex the cells because competent cells can be rather fragile. Incubate on ice for 30 min. Heat shock the cells for exactly 40 s in a hot water bath set at 42°C. Incubate the cells on ice for 2 min. Add 80 µL of LB broth (supplemented with 12.5 m*M* $MgSO_4$, 12.5 m*M* $MgCl_2$, and 20 m*M* filter-sterilized glucose) to the cells. Incubate the cells at 37°C for 1 h. Plate out replicate 50-mL aliquots on LB/Ap/IPTG/X-gal agar and incubate overnight at 37°C.

   Using pUC18 and an appropriate host strain (*see* **Note 4**), blue/white colony selection is possible. White colonies can be picked and transferred to a fresh patch plate, and colony PCR using primers flanking the insert DNA allows screening of the putative recombinants for the presence of the appropriate sized fragment (**Fig. 4**). Positive clones can then be selected for subsequent sequence analysis.

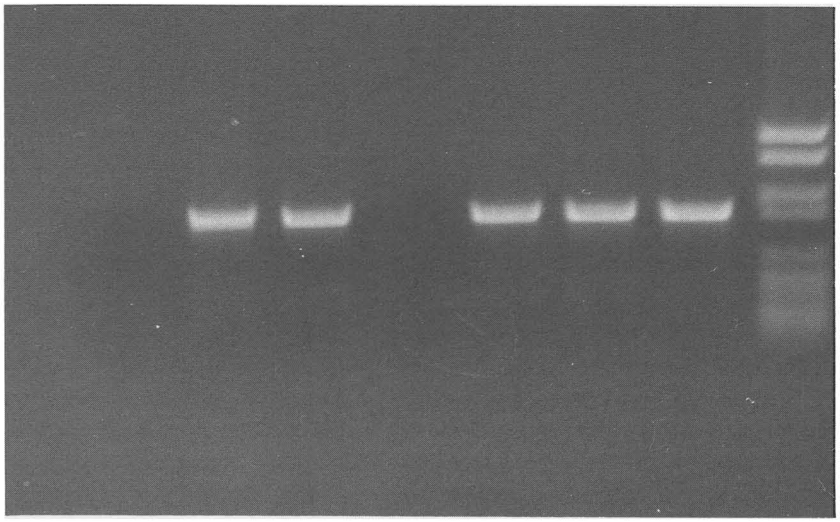
3.3. Blunt-End Cloning

1. Purify the PCR product using a QIAquick spin column (gel purify if required).
2. Add 2 vol of ice-cold absolute ethanol to the purified PCR product and precipitate at –20°C.

A



B



3. Dissolve the DNA pellet in 2 µL of 10X T4 DNA polymerase buffer.
4. Add 2 µL of dNTPs solution (*see* **Subheading 2.3., item 3**).
5. Add 2 µL of BSA solution.
6. Add 5 U of T4 DNA polymerase per microgram of PCR product.
7. Make up to 20 µL with sterile distilled water.
8. Incubate at 37°C for 5 min.
9. Inactivate the T4 DNA polymerase by heating at 75°C for 10 min.
10. Precipitate the DNA with 2 volumes of ice cold absolute ethanol and dissolve the pellet in 50 µL of TE buffer.
11. Ligation and transformation: ligation and transformation can be carried out as described for sticky-end cloning (*see* **Subheading 3.2., steps 4** and **5**).

3.4. TA-Cloning

1. Purify PCR product using a QIAquick spin column. Gel purification of the PCR product may be required, particularly if the PCR reaction does not produce a single, distinct DNA band.
2. Prepare ligation reactions as described for sticky-end cloning (*see* **Subheading 3.2., step 4**) with a range of vector to insert ratios with PCR product and control DNA.
3. Ligation and transformation are done as previously described (*see* **Subheading 3.2., steps 4** and **5**).

3.5. Summary

Rapid, simple, routine analyses are required for environmental monitoring applications. Although the PCR can take several hours to amplify a specific gene fragment, high-performance thermal cyclers are now available that are ideally suited to rapid throughput of samples ***(78)***. These thermal cyclers offer the possibility of running 30 cycles of the PCR in under 15 min if thin-walled glass capillaries are used, and they are well suited to routine analysis of large numbers of samples. Furthermore the technology has been developed to allow real-time monitoring of the PCR based on fluorescence technology ***(79)*** and

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Fig. 3. *(previous page)* Effect of annealing temperature on the amplification of rRNA gene fragments from genomic DNA of autotrophic ammonia-oxidizing bacteria using primers Nso190 (5'-CGATCCCCTGCTTTTCTCC-3') and Nso1225 (5'-CGCCATTGTATTACG TGTGA-3') ***(81)*** specific for ammonia-oxidizing bacteria of the β-*Proteobacteria*. **(A)** Primer annealing at 57°C. **(B)** Primer annealing at 58°C. Lane 1, no DNA control; lane 2, *Nitrosospira* sp. Nv141; lane 3, *Nitrosomonas europaea* Nm5**0; l**ane 4, *Nitrosomonas eutropha* Nm57; lane 5, *Nitrosospira* sp. NpAV; lane 6, *Nitrosospira multiformis* C-7**1; l**ane 7, *Nitrosospira* sp. 23.21. M, mol wt marker VI (BCL). The *N. eutropha* Nm57 16S rRNA gene has a single base mismatch with primer Nso190.

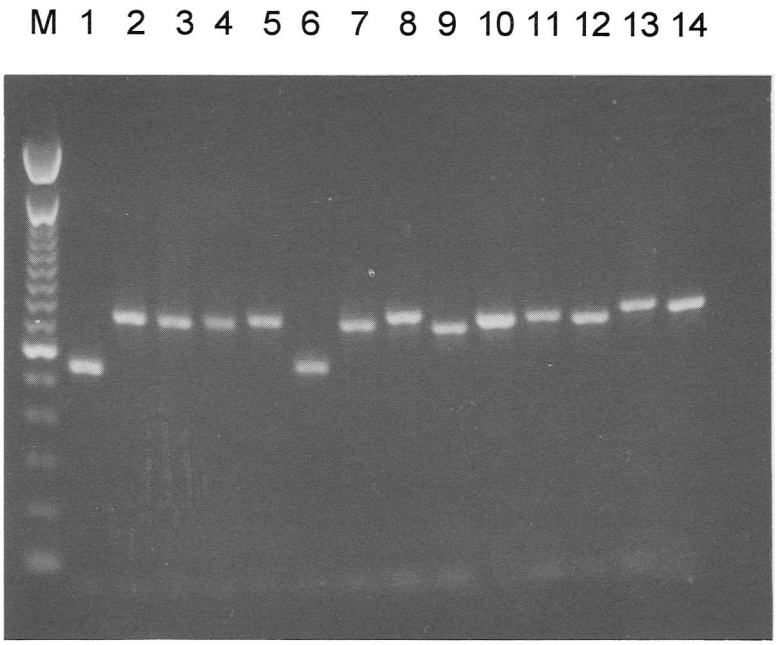

Fig. 4. Screening of pUC18 clones for the presence of rRNA gene inserts by colony PCR using pUC/M13 forward (5'-GTAAAACGACGGCCAGT-3') and reverse primers (5'-CAGGAAACAGCTATGAC-3'). The PCR product insert was approx 500 bp long. The products are approx 120 bp longer than this because the primers used amplify regions flanking the insert DNA. The size variation is consistent with length variation in rRNA genes from different taxa. The PCR products were cloned using a sticky-end cloning procedure. Lanes 1 and 6 show truncated inserts that contained internal restriction sites. M, 100-bp mol wt marker (Promega). The Intense band in the ladder is 500 bp in size.

instruments that can simultaneously monitor three fluorescence channels are commercially available ***(80)***. With this instrumentation, there is potential to monitor the amplification of different templates in real time by the inclusion of specific fluorescent hybridization probes within the PCR reaction mix ***(79)***. Thus, the amplification of an internal standard and competitor can potentially be measured rapidly, and problems such as preferential template reannealing can be identified. However, the instrumentation is expensive and a relatively recent development, and thus its value for many quantitative PCR applications has yet to be fully evaluated.

The ability to quantify specific groups of organisms, or at least the abundance of specific rRNA gene sequences using PCR, is essential if we are to realize the full potential of rRNA gene sequence-based environmental moni-

toring. For instance, it may be valuable to know that a specific organism capable of a particular catabolic function is present, but it is much more valuable to be able to determine how large the population of the organism is and, potentially, how active it is. It is now apparent that reliable PCR-dependent quantification of rRNA and rRNA genes is a real possibility.

Potential end users for the technology include the water industry and those involved in cleanup of polluted land, sediments, and water. To be widely adopted the techniques must be rapid, simple, cheap, and reliable. A major challenge will be to take the techniques from the research laboratory and to develop rapid, simple, analytical formats that can be used in a wide range of situations.

The use of sophisticated molecular techniques does not abrogate our responsibility to conduct rigorous science. It is therefore essential that when molecular techniques are used, suitable means to determine the quality of the data be adopted. Only recently have steps been taken to determine how reproducible and representative data obtained from molecular biological techniques are. Such considerations are critical if routine environmental monitoring using molecular biological techniques is to become a reality. This is not to say that the largely adopted more piecemeal approach to date is not valid, and it is still necessary to carry out baseline studies to determine what microbial diversity actually exists. In the context of environmental monitoring, however, a more focused outlook is required.

4. Notes

1. Buffers supplied with particular enzymes are likely to vary in their composition. The buffer described is recommended for use with Dynazyme (Flowgen, Lichfield, UK) DNA polymerase.
2. An A_{260} of 1 is equivalent to 20 µg/mL of oligonucleotide, and the mol wt of the oligonucleotide can be calculated using the mol wt of individual nucleotides (dA = 330.2, dC = 306.2, dG = 346.2, dT = 321.2) – 79 (98 for the 5' phosphate group, which is not present on synthetic deoxyoligonucleotides).
3. A wide range of thermostable DNA polymerases are available commercially for use in the PCR, including proofreading enzymes such as *Pfu* DNA polymerase (Stratagene) and UL*Tma* DNA polymerase (PE Applied Biosystems) which is a modified version of a *Thermotoga maritima* enzyme. These are often used to generate PCR products for preparation of 16S rRNA gene clone libraries by blunt-end ligation. They have a lower rate of misincorporation than enzymes such as *Taq* DNA polymerase and generate flush-ended products suitable for direct blunt-end ligation.
4. The choice of host strain used in the cloning of PCR-amplified rRNA genes is of considerable importance. All organisms contain rRNA sequences, and a high degree of sequence conservation is evident across a wide range of taxa. Consequently, the potential for homologous recombination between cloned rRNA

sequences and the *E. coli* host is significant. Therefore, recombination-deficient *E. coli* host strains must be used. Generally *recA* mutants have been used most successfully (e.g., JM109, XL-1 Blue strains). I have found that SURE2 competent cells (Stratagene) are also good hosts for cloned rRNA gene fragments. SURE2 cells are not *recA* mutants but are disabled with respect to *recB* and *recJ*. Together these two mutations confer a phenotype similar to *recA* mutants.

5. The amount of DNA added depends on a several factors, but typically 200 ng of template per reaction works well. However, smaller quantities can be used, particularly if dilution to reduce the amount of inhibitory contaminants is required. Using larger quantities of DNA, if of high enough purity, can allow detection of rRNA sequences that are present in low abundance.
6. This protocol has been used with an Omnigene thermal cycler (Hybaid Ltd., Ashford, UK) to amplify almost complete rRNA gene fragments (approx 1.5 kb) from a wide range of cultured bacteria and environmental samples using primers targeting the distal and proximal ends of bacterial 16S rRNA genes *(77)*. It has also enabled the amplification of cloned rRNA gene fragments using pUC/M13 primers to prepare templates for DNA sequencing.

References

1. Brock, T. D. (1987) The study of microorganisms *in situ*: progress and problems. *Symp. Soc. Gen. Microbiol.* **41,** 1–17.
2. Olsen, G. J., Lane, D. J., Giovannoni, S. J., and Pace, N. R. (1986) Microbial ecology and evolution: a ribosomal RNA approach. *Annu. Rev. Microbiol.* **40,** 337–365.
3. Pace N. R., Stahl D. A., Lane D. J., and Olsen G. J. (1986) The analysis of natural microbial populations by ribosomal RNA sequences. *Adv. Microb. Ecol.* **9,** 1–55.
4. Weller R. and Ward D. M. (1989) Selective recovery of 16S rRNA sequences from natural microbial communities in the form of cDNA. *Appl. Environ. Microbiol.* **55,** 1818–1822.
5. Giovannoni S. J., Britschgi, T. B., Moyer, C. L., and Field K. G. (1990) Genetic diversity in Sargasso Sea bacterioplankton. *Nature* **345,** 60–63.
6. Ward D. M., Weller R., and Bateson M. M. (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345,** 63–65.
7. Steffan, R. J. and Atlas, R. M. (1988) DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. *Appl. Environ. Microbiol.* **54,** 2185–2191.
8. Tsai, Y.-L. and Olson, B. H. (1992) Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. *Appl. Environ. Microbiol.* **58,** 754–757.
9. D'Aquila, R. T., Bechtel, L. J., Videler, J. A., Eron, J. J., Gorczya, P., and Kaplan, J. C. (1991) Maximizing sensitivity and specificity of PCR by preamplification heating. *Nucleic Acids Res.* **19,** 3749.
10. Don, R. H., Cox, P. T., Wainwright, B., Baker, K., and Mattick, J. S. (1991) "Touchdown" PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* **19,** 4008.

11. Borneman, J. and Triplet, E. W. (1997) Molecular microbial diversity in soils from Eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl. Environ. Microbiol.* **63,** 2647–2653.
12. Godon, J.-J., Zumstein, E., Dabert, P., Habouzit, F., and Moletta, R. (1997) Molecular microbial diversity of an anaerobic digestor as determined by small subunit rDNA sequence analysis. *Appl. Environ. Microbiol.* **63,** 2802–2813.
13. Ward, D. M., Bateson, M. M., Weller, R., and Ruff-Roberts A. L. (1992) Ribosomal RNA analysis of microorganisms as they occur in nature. *Adv. Microb. Ecol.* **12,** 219–286.
14. van de Peer, Y, Chapelle, S., and de Wachter, R. (1996) A quantitative map of nucleotide substitution rates in bacterial ribosomal RNA. *Nucleic Acids Res.* **24,** 3381–3391.
15. Fox , G. E., Wisotzkey, J. D., and Jurtshuk, P., Jr. (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int. J. Syst. Bacteriol.* **42,** 166–170.
16. van de Peer, Y., Vancanneyt, M., and de Wachter, R. (1996) Compilation of pseudomonad sequences present in a database on the structure of ribosomal RNA. *Systematic Appl. Microbiol.* **19,** 493–500.
17. Amann, R., Snaidr, J., Wagner, M., Ludwig, W., and Schleifer, K.-H. (1996) *In situ* visualization of high genetic diversity in a natural microbial community. *J. Bacteriol* **178,** 3496–3500.
18. Spring, S., Amann, R., Ludwig, W., Schleifer, K.-H., and Petersen, N. (1992) Phylogenetic diversity and identification of non-culturable magnetotactic bacteria. *Systematic Appl. Microbiol.* **15,** 116–122.
19. Rosset, R., Julien, J., and Monier, R. (1966) Ribonucleic acid composition of bacteria as a function of growth rate. *J. Mol. Biol.* **18,** 308–320.
20. Kjeldgaard, N. O. and Kurland, C. G. (1963) The distribution of soluble and ribosomal RNA as a function of growth rate. *J. Mol. Biol.* **6,** 341–348.
21. Neidhardt, F. and Magasanik, B. (1959) Studies on the role of ribonucleic acid in the growth of bacteria. *Biochim. Biophys. Acta* **42,** 99–116.
22. Felske, A., Engelen, B., Nübel, U., and Backhaus, H. (1996) Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. *Appl. Environ. Microbiol.* **62,** 4162–4167.
23. Miskin, I. P., Bruce, K. D. Farrimond, P., and Head, I. M. (1997) Rapid extraction of indigenous bacterial RNAs from freshwater sediments and reverse transcriptase-polymerase chain reaction amplification of ribosomal and messenger RNA, submitted.
24. DeLong, E. F. (1992) *Archaea* in coastal marine environments. *Proc. Natl. Acad. Sci. USA*. **89,** 5685–5689.
25. DeLong, E. F., Wu, K. Y., Prezelin, B. B., and Jovine, R. V. M. (1994) High abundance of *Archaea* in Antarctic marine picoplankton. *Nature* **371,** 695–697.
26. Fry, N. K., Fredrickson, J. K., Fishbain, S., Wagner, M., and Stahl, D. A. (1997) Population structure of microbial communities associated with two deep, anaerobic, alkaline aquifers. *Appl. Environ. Microbiol.* **63,** 1498–1500.

27. Teske, A., Wawer, C., Muyzer, G., and Ramsing, N. B. (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Appl. Environ. Microbiol.* **62,** 1405–1415.
28. Hiorns, W. D., Hastings, R. C., Head, I. M., McCarthy, A. J., Saunders, J. R., Pickup, R. W., and Hall, G. H. (1995) Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidising bacteria demonstrates the ubiquity of nitrosospiras in the environment. *Microbiology* **141,** 2793–2800.
29. Brooks, J. L., Moore, A. S., Patchett, R. A., Collins, M. D., and Kroll, R. G. (1992) Use of the polymerase chain reaction and oligonucleotide probes for the rapid detection and identification of *Carnobacterium* species from meat. *J. Appl. Bacteriol.* **72,** 294–301.
30. Gordon D. A., and Giovannoni S. J. (1996) Detection of stratified microbial populations related to *Chlorobium* and *Fibrobacter* species in the Atlantic and Pacific oceans. *Appl. Environ. Microbiol.* **62,** 1171–1177.
31. Muyzer, G., Teske, A., Wirsen, C. O., and Jannasch, H. W. (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch. Microbiol.* **164,** 165–172.
32. Kowalchuk, G. A., Stephen, J. R., DeBoer, W., Prosser, J. I., Embley, T. M., and Woldendorp, J. W. (1997) Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Appl. Environ. Microbiol.* **63,** 1489–1497.
33. Field, K. G., Gordon, D., Wright, T., Rappe, M., Urbach, E., Vergin, K., and Giovannoni, S. J. (1997) Diversity and depth-specific distribution of SAR11 cluster rRNA genes from marine planktonic bacteria. *Appl. Environ. Microbiol.* **63,** 63–70.
34. Mullins, T. D., Britschgi, T. B., Krest, R. L., and Giovannoni, S. J. (1995) Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. *Limnol. Oceanog.* **40,** 148–158.
35. Massana, R., Murray, A. E., Preston, C. M., and DeLong, E. F. (1997) Vertical distribution and phylogenetic characterization of marine planktonic *Archaea* in the Santa Barbara Channel. *Appl. Environ. Microbiol.* **63,** 50–56.
36. Ferris, M. J., and Ward, D. M. (1997) Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **63,** 1375–1381.
37. Giovannoni, S. J., Mullins, T. D., and Field, K. G. (1995) Microbial diversity in oceanic systems: rRNA approaches to the study of unculturable microbes, in *Molecular Ecology of Aquatic Microbes*, NATO ASI series G38 (Joint, I., ed.), Springer-Verlag, Berlin, pp. 217–248.
38. Good, I. J. (1953) The population frequencies of species and the estimation of the population parameters. *Biometrika* **40,** 237–264.

39. Stackebrandt, E. and Goebel, B. M. (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44,** 846–849.
40. Nakagawa, Y., Sakane, T., and Yokota, A. (1996) Emendation of the genus *Planococcus* and transfer of *Flavobacterium okeanokoites* Zobell and Upham 1944 to the genus *Planococcus* as *Planococcus okeanokoites* comb. nov. *Int. J. Syst. Bacteriol.* **46,** 866–870.
41. Mylvaganam, S., and Dennis, P. P. (1992) Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaebacterium *Haloarcula marismortui*. *Genetics*. **130,** 399–410.
42. Nubel, U., Engelen, B., Felske, A., Snaidr, J., Weishuber, A., Amann, R. I., Ludwig, W., and Backhaus, H. (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J. Bacteriol.* **178,** 5636–5643.
43. Borneman, J., Skroch, P. W., O'Sullivan, K. M., Palus, J. A., Rumjanek, N. G., and Triplett, E. W. (1996) Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl. Environ. Microbiol.* **62,** 1935–1943.
44. Reysenbach A., Giver L. J., Wickham G. S., and Pace N. R. (1992) Differential amplification of rRNA genes by polymerase chain reaction. *Appl. Environ. Microbiol.* **58,** 3417–3418.
45. Farrelly, V., Rainey, F. A., and Stackebrandt, E. (1995) Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl. Environ. Microbiol.* **61,** 2798–2801.
46. Suzuki M. T. and Giovannoni S. J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* **62,** 625–630.
47. Rainey, F. A., Ward, N., Sly, L. I., and Stackebrandt, E. (1994) Dependence on the taxon composition of clone libraries for PCR amplified, naturally occuring 16S rDNA on the primer pair and the cloning system used. *Experientia* **50,** 796,797.
48. 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 Ecol.* **21,** 191–198.
49. Kopczynski, E. D., Bateson, M. M., and Ward, D. M. (1994) Recognition of chimeric small subunit ribosomal DNAs composed of genes from uncultivated microorganisms. *Appl. Environ. Microbiol.* **60,** 746–748.
50. Wang G. C.-Y. and Wang Y. (1996) The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. *Microbiology* **142,** 1107–1114.
51. Blok, H. J., Gohike, A. M., and Akkermans, A. D. L. (1997) Quantitative analysis of 16S rDNA using competitive PCR and the QPCR™ System 5000. *Biotechniques* **22,** 700–704.
52. Lee, S. Y., Bollinger, J., Bezdicek, D., and Ogram, A. (1996) Estimation of the abundance of an uncultured soil bacterial strain by a competitive quantitative PCR method. *Appl. Environ. Microbiol.* **62,** 3787–3793.

53. Wright, T. D., Vergin, K. L., Boyd, P. W., and Giovannoni, S. J. (1997) A novel delta-subdivision proteobacterial lineage from the lower ocean surface layer. *Appl. Environ. Microbiol.* **63,** 1441–1448.
54. Kerkhof, L. and Ward, B. B. (1993) Comparison of nucleic acid hybridization and fluorometry for measurement of the relationship between RNA/DNA ratio and growth rate in a marine bacterium. *Appl. Environ. Microbiol.* **59,** 1303–1309.
55. Rochelle, P. A., Weightman, A. J., and Fry, J. C. (1992) DNase I treatment of *Taq* DNA polymerase for complete PCR decontamination. *Biotechniques* **13,** 520.
56. Rochelle, P. A., Will, J. A. K., Fry, J. C., Jenkins, G. J. S., Parkes, R. J., Turley, C. M., and Weightman, A. J. (1995) Extraction and amplification of 16S rRNA genes from deep marine sediments and seawater to assess bacterial community diversity, i*n Nucleic Acids in the Environment: Methods and Applications* (Trevors, J. T. and van Elsas, J. D., ed.), Springer-Verlag, Berlin, pp. 219–239.
57. Zhou, J., Palumbo, A. V., and Tiedje, J. M. (1997) Sensitive detection of a novel class of toluene-degrading denitrifiers, *Azoarcus tolulyticus*, with small subunit rRNA primers and probes. *Appl. Environ. Microbiol.* **63,** 2384–2390.
58. Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173,** 697–703.
59. Kaufman, D. L. and Evans, G. A. (1990) Restriction endonuclease cleavage at the termini of PCR products. *Biotechniques* **9,** 304–305.
60. Moyer, C. L., Dobbs, F. C., and Karl, D. M. (1994) Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S ribosomal RNA genes from a microbial mat at an active hydrothermal vent system, Loihi Seamount, Hawaii. *Appl. Environ. Microbiol.* **60,** 871–879.
61. Rheims, H., Sproer, C., Rainey, F. A., and Stackebrandt, E. (1996) Molecular biological evidence for the occurrence of uncultured members of the actinomycete line of descent in different environments and geographical locations. *Microbiology* **142,** 2863–2870.
62. Suzuki, M. T., Rappe, M. S., Haimberger, Z. W., Winfield, H., Adair, N., Strobel, J., and Giovannoni, S. J. (1997) Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. *Appl. Environ. Microbiol.* **63,** 983- 989.
63. Clark, J. M. (1988) Novel non-templated nucleotide reactions catalysed by procaryotic and eukaryotic DNA polymerases. *Nucleic Acids Res.* **18,** 9677–9686.
64. Ward D. M., Weller R., and Bateson M. M. (1990) 16S rRNA sequences reveal uncultured inhabitants of a well-studied thermal community. *FEMS Microbiol. Rev.* **75,** 105–116.
65. Tsai, Y.-L. and Olson, B. H. (1992) Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Appl. Environ. Microbiol.* **58,** 2292–2295.
66. Fantroussi, S. E., Manhillon, J., Naveau, H., and Agathos, S. N. (1997) Introduction of anaerobic dechlorinating bacteria into soil slurry microcosms and nested-PCR monitoring. *Appl. Environ. Microbiol.* **63,** 806–811.

67. Briglia., M., Eggen, R. I. L., de Vos, W. M., and van Elsas, J. D. (1996) Rapid and sensitive method for the detection of *Mycobacterium chlorophenolicum* PCP-1 in soil based on 16S rRNA gene-targeted PCR. *Appl. Environ. Microbiol.* **62,** 1478–1480.
68. Voytek, M. A., Ward, B. B., and Witzel, K.-P. (1995) Phylogenetic diversity of natural populations of ammonia-oxidizers investigated by specific PCR amplification. *Microbial Ecol.* **33,** 87–96.
69. Holmes, A. J., Owens, N. J. P., and Murrell, J. C. (1995) Detection of novel marine methanotrophs using phylogenetic and functional gene probes after methane enrichment. *Microbiology* **141,** 1947–1955.
70. Miyamoto, H., Yamamoto, H., Arima, K, Fujii, J, Maruta, K., Izu, K., Shiomori, T., and Yoshida, S.-I. (1997) Development of a semi-nested PCR method for detection of *Legionella* species and its application to surveillance of Legionellae in hospital cooling tower water. *Appl. Environ. Microbiol.* **63,** 2489–2494.
71. Wilson, K. H. (1994) Detection of culture-resistant bacterial pathogens by amplification and sequencing of ribosomal DNA. *Clin. Infect. Dis.* **18,** 958–962.
72. Wang, K.-F., Cao, W.-W., and Johnson, M. G. (1992) 16S rRNA-based probes and polymerase chain reaction method to detect *Listeria monocytogenes* cells added to foods. *Appl. Environ. Microbiol.* **58,** 2827–2831.
73. Ferris, M. J., Nold, S. C., Revsbech, N. P., and Ward, D. M. (1997) Population structure and physiological change within a hot spring microbial mat community following disturbance. *Appl. Environ. Microbiol.* **63,** 1367–1374.
74. Massol-Deya, A., Weller, R., Rios-Hernandez, L., Zhou, J.-Z., Hickey, R. F., and Tiedje, J. M. (1997) Succession and convergence of biofilm communities in fixed-film reactors treating aromatic hydrocarbons in groundwater. *Appl. Environ. Microbiol.* **63,** 270–276.
75. Fries, M. R., Hopkins, G. D., McCarty, P. L., Forney, L. J., and Tiedje, J. M. (1997) Microbial succession during a field evaluation of phenol and toluene as the primary substrates for Trichloroethene cometabolism. *Appl. Environ. Microbiol.* **63,** 1515–1522.
76. Moyer, C. L., Tiedje, J. M., Dobbs, F. C., and Karl, D. M. (1996) A computer-simulated restriction fragment length polymorphism analysis of bacterial small subunit ribosomal RNA genes: efficiency of selected tetrameric restriction enzymes for studies of microbial diversity in nature. *Appl. Environ. Microbiol.* **62,** 2501–2507.
77. Edwards, U., Rogall, T., Blöcker, H., Emde, M., and Böttger, E. C. (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.* **17,** 7843–7853.
78. Wittwer, C. T., and Garling, D. J. (1991) Rapid cycle DNA amplification—time and temperature optimization. *Biotechniques* **10,** 76.
79. Wittwer, C. T., Herrmann, M. G., Moss, A. A., and Rasmussen, R. P. (1997) Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* **22,** 130–138.

80. Wittwer, C. T., Ririe, K. M., Andrew, R. V., David, D. A., Gundry, R. A., and Balis, U. J. (1997) The LightCycler™: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* **22,** 176–181.
81. Mobarry, B. K., Wagner, M., Urbain, V., Rittman, B. E., and Stahl, D. A. (1996) Phylogenetic probes for analysing abundance and spatial organization of nitrifying bacteria. *Appl. Environ. Microbiol.* **62,** 2156–2162.

12

Application of Denaturing Gradient Gel Electrophoresis to Microbial Ecology

Richard Hastings

1. Introduction

A significant proportion of microbial ecology is now based on the description of community structure in naturally occurring bacterial assemblages. The development of molecular biological techniques has facilitated this task, primarily via the cloning and sequencing of microbial genes retrieved from the environment. However, the labor-intensive nature of a cloning procedure, as well as the biases that it can introduce, have generated the need for alternative laboratory methods that more accurately describe microbial community structure; denaturing gel electrophoresis is an approach largely adopted by molecular microbial ecologists to perform this function. Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are techniques based on the separation of polymerase chain reaction (PCR)-amplified gene fragments, not according to size, but owing to variation in the targeted nucleotide sequences. Nucleotide pair dissociation is mediated in denaturing gradient gels (DGGE) by the chemicals urea and formamide. These denaturants are incorporated into the gel in increasing concentrations to form the denaturing gradient. A so-called melting domain within the DNA fragment being analyzed loses its helical symmetry as it migrates through a linear denaturing gradient gel when it reaches its melting temperature (T_m) point ***(1)***. Loss of the double-stranded structure of DNA virtually halts migration. Sequence specificity of domains dictates that they have individual melting temperatures, so, in theory, similar-sized DNA fragments from closely related organisms are resolvable in denaturing gels.

TGGE uses urea and formamide in fixed concentrations to encourage but not mediate DNA dissociation; thus, PCR products are subjected to a linear

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increase in temperature as they migrate through the gel during electrophoresis. When a melting domain T_m is reached, dissociation occurs and migration ceases. It is usual for a GC-rich sequence of approx 40 nucleotides in length to be attached to one end of the DNA fragments being analyzed, acting as a high-temperature melting domain. This significantly increases the detection of base pair variants over identical but nonclamped fragments ***(2,3)***. GC-clamps can easily be incorporated into PCR products by the use of one oligonucleotide primer that contains the clamp sequence at its 5' end.

Gene cloning and denaturing gel electrophoresis techniques, applied to questions of microbial ecology, are both prone to the biases of PCR amplification ***(4–6)***, but the latter is believed to offer certain advantages over cloning techniques. Once optimized, denaturing gel electrophoresis is a relatively quick and easy way of analyzing genetic diversity within a microbial community. Also, a broader spectrum of environmental genetic diversity may be observable using denaturing gels, because the analysis of library clones can be a random process influenced by laboratory time and number of clones available.

DGGE is proving to be most useful in microbial ecology studies when combined with other molecular techniques. Electrophoretic profiles can give readily observable indications of community structure, but the probing of resolved bands with oligonucleotides and/or sequencing of excised bands are additions that can yield more meaningful information of microbial diversity. There are, however, limitations to these supplementary techniques. Bands that do not hybridize an oligonucleotide probe remain unidentified beyond the level of PCR primer specificity, and the quantity of sequence data yielded from excised bands for phylogeny studies is limited (16S rDNA V3 regions analyzed typically extend between 200 and 250 bases in length).

DGGE was originally used to analyze genetic mutation, but since its first microbiologic application ***(3)***, it has become increasingly popular with molecular microbial ecologists, and the list of reports detailing its use in numerous and different ecosystems continues to lengthen ***(7–10)***.

2. Materials

2.1. DGGE

1. Appropriate gel electrophoresis apparatus (*see* **Note 1**).
2. Dual-chamber gradient maker (*see* **Note 2**).
3. Gel reagents: Acrylamide stock solution 40% w/v (37.5:1, acrylamide:*bis*-acrylamide), formamide (deionized with AG 501-X8 mixed-bed resin [Bio-Rad, Hercules, CA]), Tris-acetate buffer (0.5X TAE: 20 m*M* Tris, 10 m*M* acetate, 0.5 m*M* Na_2EDTA, pH 7.4), molecular biology grade sterile distilled water, ultrapure urea (Gibco-BRL, Paisley, UK), and ammonium persulfate, *N,N,N′N′*-tetramethylethylendiamine (TEMED).

4. DNA stain (*see* **Note 3**).
5. Loading dye 2% (w/v) bromophenol blue.
6. PCR products for analysis (*see* **Note 4**).

2.2. TGGE

1. Appropriate gel electrophoresis apparatus (*see* **Note 1**).
2. Gel reagents. Acrylamide stock solution 40% (37.5 :1, acrylamide:*bis*-acrylamide), formamide (deionized with AG 501-X8 mixed-bed resin [Bio-Rad]), Tris-acetate buffer (0.5X TAE: 20 m*M* Tris, 10 m*M* acetate, 0.5 m*M* Na_2EDTA, pH 7.4), molecular biology grade sterile distilled water, ultrapure urea (Gibco BRL), glycerol, ammonium persulfate, TEMED.
3. DNA stain (*see* **Note 3**).
4. Loading dye: 2% (w/v) bromophenol blue.
5. PCR products for analysis (*see* **Note 4**).

3. Methods

3.1. Preparation of Gels

DGGE is performed in one of two ways, the option depending on whether electrophoretic parameters are to be optimized or genetic diversity is to be analyzed. Parameter optimization requires perpendicular DGGE whilst subsequent genetic analysis requires parallel DGGE.

3.1.1. Casting a Denaturing Gradient Gel

This protocol uses the example of a gel incorporating a denaturing gradient range of 0–100% (7 *M* urea, 40% v/v formamide) to a 20-mL volume.

Component	0% Denaturant	100% Denaturant
8% Acrylamide solution	9.8 mL	5.8 mL
50X Tris-acetate buffer	0.2 mL	0.2 mL
Urea	—	4.2 g
Formamide	—	4.0 mL

1. Assemble ethanol-cleaned glass plates.
2. Place the gradient maker on a magnetic stirrer approximately 5 cm higher than the assembled glass plates. Close the valve and seal its outflow tube with a small clamp.
3. Once the denaturing acrylamide solutions are prepared, pipet an appropriate volume of the higher concentration denaturing solution into the mixing chamber and briefly open the valve, allowing some acrylamide solution to pass through to the reservoir. With the valve closed, transfer this solution back to the mixing chamber. Remove traces of the denaturing solution from the reservoir by washing with fresh 8% acrylamide solution.
4. Begin rotation of the stirrer bar at a low rate of revolution (but sufficient to adequately mix acrylamide solutions when the valve is opened).

5. Pipet an equal volume of the lower concentration denaturing solution into the reservoir. Adjustment may have to be made for difference in volume between the two solutions as the volume of acrylamide in the mixing chamber will have been increased by insertion of the stirrer bar. This can be performed by the addition of a compensating bar to the reservoirs solution.
6. Add the polymerizing agents to each chamber. For an acrylamide volume of 10 mL, use 10 µL of TEMED and 100 µL of freshly prepared 10% (w/v) ammonium persulfate. Polymerizing agents added to the reservoir need to be mixed manually.
7. Position the outflow tube centrally on the glass plates, open the valve, and release the clamp, allowing pouring to begin. It may be necessary to initiate the flow by sucking solution along the tube with a pipetting action.
8. Insert comb.
9. Once the gel is poured, wash the gradient maker with distilled water to prevent tube blockage.
10. Allow the gel to set for at least 60 min. Gels can be made and stored overnight at 4°C after sealing with cling film.

3.1.2. Casting a Perpendicular Denaturing Gradient Gel

The direction of electrophoretic migration is perpendicular to denaturant concentration. The orientation of pouring, therefore, must be at right angles to the comb. A single-well comb that extends the length of the gradient is inserted into the gel allowing loading of DNA across the entire gradient range (*see* **Fig. 1A**).

3.1.3. Casting a Parallel Denaturing Gradient Gel

Because the direction of migration is parallel to denaturant gradient, the multiwell comb is inserted in the glass plates at the lower end of the denaturant range (*see* **Fig. 1B**). An adjustment to the denaturant range may have to be made since comb teeth (and therefore PCR products when loaded) extend a distance into the gradient.

3.1.4. Casting a Constant Denaturing Gradient Gel

The use of a gradient maker is unnecessary since gel component can be mixed in a glass container and poured by pipetting. A multiwell comb is used and can be easily inserted into the gel immediately after pouring without necessitating adjustment to the denaturant range.

3.2. Running a Perpendicular Denaturing Gradient Gel

1. Switch on the heating mechanism and allow the operating temperature to read 55–60°C.
2. Position the polymerized gel into the electrophoresis apparatus. This step will depend on the make of apparatus being used. Gel plates may have to be dismantled and removed (Multiphor II system, Pharmacia Biotech, Uppsala, Swe-

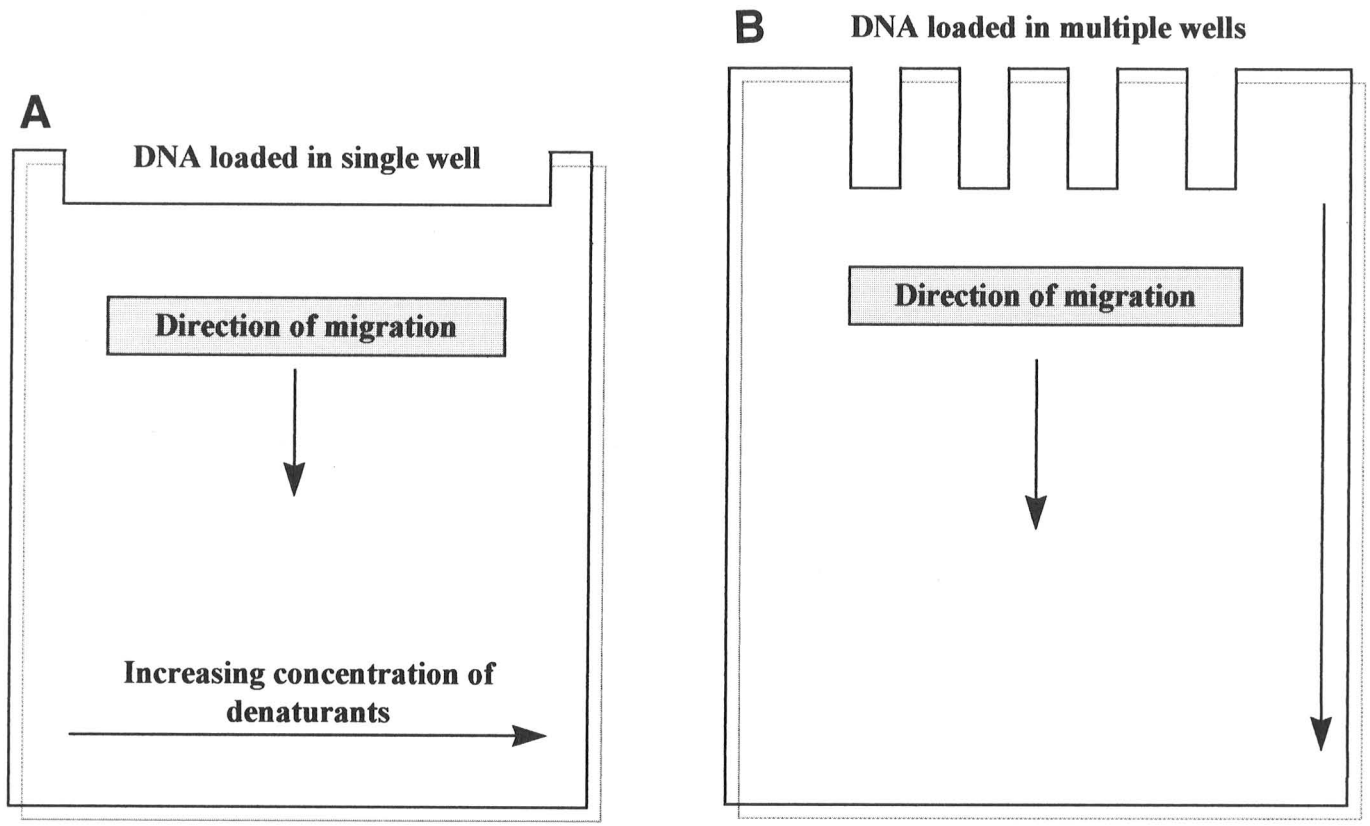

Fig. 1. **(A)** The principle of perpendicular denaturing gel electrophiresis. DNA is loaded across the length of the denaturant gradient and migrates into the gel at right angles to this gradient. **(B)** The principle of parallel denaturing gel electrophoresis. DNA is loaded at the lower denaturant concentration and migrates parallel with the increasing gradient of denaturant.

den) or, more usually, clamped into a supporting frame of the electrophoresis apparatus (Dcode™, Bio-Rad).
3. Clean away unpolymerized acrylamide solution from wells when the comb is removed with fresh tank buffer by a pipetting action (this step may be performed before the gel is attached to the electrophoresis apparatus, if more convenient).
4. Allow the gel time to equilibrate with the operating temperature of the heating mechanism.
5. Load an appropriate volume of DNA and dye in each sample well (usually 10 parts PCR poducts:1 part dye).
6. Connect the power supply to the apparatus electrodes and begin current flow. The usual voltage applied across denaturing acylamide gels is 150–200 V. The time of electrophoresis needs to be determined for individual PCR fragments (*see* **Subheading 3.3.2.**).

3.3. Optimization of DGGE Parameters

An essential component of denaturing gel electrophoresis is the determination of melting behavior of the DNA under investigation, which enables the optimization of electrophoresis parameters. Parameters applicable to DGGE include the range of the denaturant gradient and the duration of electrophoresis. These are determined empirically because it is important to establish optimal electrophoresis parameters for different PCR products. Determination of denaturant gradient range is best performed by perpendicular DGGE whilst determination of electrophoresis run time is performed by parallel DGGE.

3.3.1. Optimization of Denaturant Gradient Range

It is advisable to begin with a broad denaturant range and observe the characteristic sigmoid curve of DNA migration after electrophoresis through a perpendicular denaturing gradient gel. The region of interest in these gels is the point of DNA inflection at which discrete fragments are being resolved in the gel (**Fig. 2**). Denaturant concentrations flanking this region can be largely ignored, and subsequent gels are made spanning the gradient range of interest. The degree of fine-tuning in determining the precise gradient range depends mostly on the quality of band resolution. At its extreme, denaturant ranges can be optimized to a single point (constant denaturing gel electrophoresis, *see* **Subheading 3.1.4.**).

3.3.2. Optimization of DGGE Run Time

The denaturant gradients determined in **Subheading 3.3.1.** should be used to optimize electrophoresis run time. Replicate samples of DNA are loaded into adjacent lanes of a parallel denaturing gradient gel with specific time intervals between loading. Maximum band resolution is thus observed from a known run time.

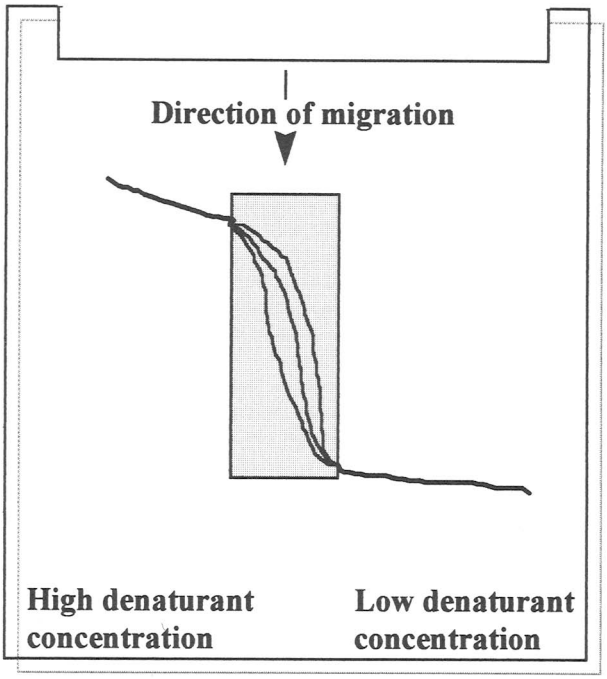

Fig. 2. Diagrammatic representation of perpendicular DGGE showing the characteristic "sigmoid" migration pattern of DNA. At high denaturant concentrations, DNA melts on entering the gel and migration is limited. At low denaturant concentrations, melting does not occur and migration is unimpeded. Point of inflection (shaded) indicates optimum denaturant gradient in which to resolve individual DNA fragments.

3.4. Casting a Temperature Denaturing Gel

This protocol describes a gel composition that is suitable for beginning the parameter optimization procedure for TGGE. As with DGGE, the TGGE procedure needs to be optimized to resolve band profiles at a usable clarity. Gel volume is 40 mL.

1. Mix components of gel (8% acrylamide solution, 1X Tris-acetate buffer, 20% deionized formamide, 7 *M* urea, 2% glycerol).
2. Polymerize gel by the addition of 40 µL of TEMED and 400 µL of freshly prepared 10% ammonium persulfate. Insert comb and allow gel to set for at least 60 min.
3. Switch on electrophoresis apparatus and set to the desired start temperature. Allow heating mechanism to reach this operating temperature.
4. Position the polymerized gel into the electrophoresis apparatus, and allow it to reach the operating temperature.

5. Clean away unpolymerized acrylamide solution from wells when the comb is removed with fresh tank buffer by a pipetting action (this step may be performed before the gel is attached to the electrophoresis apparatus, if more convenient).
6. Load 10 µL of PCR products and 2X loading dye, and electrophorese at 100 V with heating ramp rate and final temperature determined (*see* **Subheading *3.5.*)**.
7. Visualize band profiles with appropriate DNA stain (*see* **Note 3**).

3.5. Optimization of TGGE Parameters

TGGE parameters that need to be optimized include the temperature range over which electrophoresis proceeds and run time. A suitable temperature gradient to begin with would extend from 30 to 60°C. If a ramp rate of 2°C/h is used and electrophoresis is continued for 15 h, the gel would be divisible across its length into known temperature increments. After electrophoresis and staining, the region of gel (and therefore temperature gradient) across which all melting occurred is determined. Repeat this gel using the newly defined temperature gradient and adjusting the ramp rate to give a convenient run time. Typically, temperature gradients of 15°C are suitable, so a ramp rate of 1°C increase per hour gives a run time of 15 h.

3.6. Electroblotting of Band Profiles

1. Equilibrate the gel in electrophoresis buffer for 15 min.
2. Transfer band patterns to Hybond-N^+ nylon membrane (Amersham) using suitable electrotransfer apparatus (Transblot Cell™, Bio-Rad; Semi-Dry Electroblotter, Schleicher & Schuell, Dassel, Germany) by applying a current of 0.5 mA/cm^2 of gel for 45 min.
3. Denature the transferred DNA by placing the membrane on a piece of 3MM Whatman paper soaked in denaturing solution (0.4 *M* NaOH, 0.6 NaCl) for 10 min.
4. Neutralize by two rinses in 2.5X SSC (0.375 *M* NaCl, 0.038 *M* Na citrate).
5. Expose for 45 s to ultraviolet (UV) light (302 nm) to crosslink the DNA fragments to the membrane.
6. Membranes can be sealed in cling film and stored at –20°C if probe hybridization is delayed.

3.7. Oligonucleotide Probe Hybridization of Band Profiles

There are various hybridization protocols available using RNA and DNA probes that have been radiolabeled or nonradiolabeled. Given here is a typical example of probing a denaturing gradient gel band profile using a ^{32}P (γ ATP)-labeled DNA oligonucleotide (*see* **Note 5** for nonradioactive method).

1. Prehybridize blotted membrane in blocking solution (10% Blocking Reagent [Boehringer Mannheim], 25% 5X SSPE [20X is 3.6 *M* NaCl, 0.2 *M* Na phos-

phate, 0.02 *M* Na_2EDTA, pH 7.7], 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate [SDS], 20% deionized formamide) at 45°C for at least 60 min.
2. Incorporate ^{32}P (γ ATP) into the oligonucleotide (10–20 pmol) using a commercially available nucleotide kinase according to the manufacturer's instructions.
3. Remove blocking solution and rinse membrane with pre-warmed hybridization solution (25% 5X SSPE, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 20% deionized formamide). Immerse membrane in approx 20 mL of fresh, prewarmed hybridization solution and add labeled oligonucleotide probe. Hybridize overnight at the appropriate incubation temperature (*see* **Note 6**).
4. Remove probe solution and elute nonspecifically bound oligonucleotide from the membrane in a series of washes using fresh, prewarmed hybridization solution with gentle shaking. Continue washing until radioactivity is confined to the DNA bands suspected as being complimentary to probe sequence. Remove excess wash solution and wrap membrane in cling film.
5. Hybridization signals are detected using autoradiography (placing X-ray film against the membrane and allowing exposure for an appropriate period) or a Phosphor Imager SF system and associated software according to manufacturer's instructions (Molecular Dynamics, Sevenoaks, UK).

3.8. Excision of DNA from Gels

1. Remove the area of gel containing the band of interest with a suitable sterile tool (e.g., pipet tip or scalpel blade), and place in a sterile microfuge tube.
2. Add a volume of sterile distilled water to the tube, and allow the DNA to diffuse passively from the gel at 4°C overnight. (The quantity of water added is dependent on the volume of gel removed, but smaller volumes result in more concentrated template for subsequent PCR; a typical volume is 20 µL.)
3. Remove half the volume of water and use as template for PCR.

4. Notes

1. There are a variety of manufacturers that currently market apparatus for denaturing gel electrophoresis. Consideration should be given for the technique to be used (i.e., DGGE or TGGE), because not all apparatus is dual purpose. Available apparatus include the following: Hoefer Scientific (San Francisco, CA), SE600 system; Bio-Rad, Protean II and DCode™ systems; Diagen (Düsseldorf, Germany), TGGE system; CBS Scientific (Del Mar, CA), DGGE system; Pharmacia Biotech, Multiphor II system.
2. There are a variety of manufacturers that currently market gradient pouring devices, including Gibco and M.S.E.
3. There are currently three DNA staining methods applicable to denaturing gels. Choice of a particular method should be made after review of their advantages and disadvantages.
   a. Ethidium bromide is quick, inexpensive, and allows recovery of bands from the stained gel. It has a relatively low sensitivity, can give background fluorescence, and is highly toxic.

b. Silver staining is more expensive and takes longer to perform than ethidium bromide (typically 2 to 3 h) but may be automated (Pharmacia Biotech). It has a high sensitivity and does not produce background staining. DNA can not be retrieved from the gel after its prestaining fixation.
c. SYBR Green I (Molecular Probes, Eugene, OR) is also relatively expensive and has a limited life once used. It offers good sensitivity, a lack of toxicity, and no background staining. Bands can be observed under UV transillumination and recovered from the stained gel.

4. The majority of studies into microbial ecology by the application of DGGE or TGGE have targeted the 16S rRNA gene. Within this ribosomal gene, the variable V3 region is exploited the most because PCR primers are readily available for the eubacterial group of 16S rRNA genes that flank this sequence, and the fragment generated by amplification is of an appropriate size (216 bases) to electrophorese. Some studies have targeted alternative variable regions of the 16S rDNA gene ***(11)***, or other, functional genes ***(12)***. As with oligonucleotides used as probes, PCR primers must have their optimum annealing temperature determined empirically to provide amplification with the desired specificity (but it is not within the remit of this chapter to detail methodology for determination of this parameter of amplification). A touchdown protocol of thermal cycling is given here which is suitable for any primer pair used to generate GC- clamped PCR products for denaturing electrophoresis (13).
   a. Heat the DNA/reaction mix to 94°C for 5 min to denature double-stranded DNA molecules completely. Cool to 80°C and add *Taq* polymerase.
   b. Cool to 10°C above optimum primer annealing temperature and hold for 1 min.
   c. Heat to 72°C and hold for 1.5 min.
   d. Denature at 94°C for 1 min, anneal at 10°C above optimum primer annealing temperature again for 1 min, and extend primers at 72°C for 1.5 min.
   f. Continue thermal cycling but drop annealing temperature by 3°C after every two cycles until the optimum annealing temperature is reached.
   g. Perform another 15–20 cycles at this annealing temperature. Check PCR product yield by electrophoresis of 0.1 vol of reaction mix in 1.5% agarose gel.

   Primers suitable for PCR amplification of a 193 nucleotide sequence across the V3 region of Eubacteria 16S rDNA (14) include the following:

Primer 1 (forward)	5'-CCT ACG GGA GGC AGC AG-3' (*E. coli* position 341-358)
Primer 2 (reverse)	5'-ATT ACC GCG GCT GCT GG-3' (*E. coli* position 534-517)
GC-clamp	5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3'

5. Nonisotopic methods of probe labeling have proved to be popular owing to the lack of radioactivity and need for specialized containment facilities. In addition, nonradioactive probes are reusable longer than the half life of ^{32}P (14 d). A commonly used nonradioactive label is the DIG System (Boehringer Mannheim). The manufacturers claim a detection sensitivity comparable to that of radioactivity. The DIG System may be used with DNA, RNA, or oligonucleotide probes.

6. Oligonucleotide probes need to be hybridized to immobilized target DNA at a temperature at which one-half of the bound probe is released from the hybrid. This so-called temperature of dissociation (T_d) is determined by a temperature-graded wash series as previously described ***(15)***.

References

1. Fischer, S. G. and Lerman, L. S. (1983) DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondencee with melting theory. *Proc. Natl. Acad. Sci. USA* **80,** 1579–1583.
2. Sheffield, V. C., Cox, D. R.,Lerman, L. S., and Myers, R. M. (1989) Attachment of a 40-base pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc. Natl. Acad. Sci. USA* **86,** 232–236.
3. Muyzer, G., De Waal, E. C., and Uitterlinden, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59,** 695–700.
4. Liesack, W., Weyland, H., and Stackebrandt, E. (1991) Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. *Microbiol. Ecol.* **21,** 191–198.
5. Suzuki, M. T. and Giovannoni, S. J. (1996) Bias caused by template annealing in the amplification mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* **62,** 625–630.
6. Wintzingerode, F. V., Gobel, U. B., and Stackebrandt, E. (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* **21,** 213–229.
7. Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E., and Akkermans, A. D. L. (1997) Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soil. Microbiology **143,** 2983–2989.
8. Ferris, M. J. and Ward, D. M. (1997) Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **63,** 1375–1381.
9. Heuer, H., Krsek, M., Baker, P., Smalla, K., and Wellington, E. M. H. (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. **63,** 3233–3241.
10. Ovreas, L., Forney, L., Daae, F. L., and Torsvik, V. (1997) Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl. Environ. Microbiol. **63,** 3367–3373.
11. Nöbel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., Ludwig, W., and Backhaus, H. (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. Appl. Environ. Microbiol. **178,** 5636–5643.

12. Wawer, C. and Muyzer, G. (1995) Genetic diversity of *Desulphovibrio* spp. in environmental samples analysed by denaturing gradient gel electrophoresis of [NiFe] hydrogenase gene fragments. *Appl. Environ. Microbiol.* **61,** 2203–2210.
13. Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K., and Mattick, J. S. (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* **19,** 4008.
14. Edwards, U., Rogall, T., Blocker, H., Embe, M., and Bottger, E. C. (1989) Isolation and complete direct nucleotide determination of entire genes. Characterisation of a gene coding for the 16S ribosomal RNA. *Nucleic Acids Res.* **17,** 7843–7853
15. Raskin, L. Stromley, J. M., Rittman, B. E., and Stahl, D. A. (1994) Group-specific 16S rRNA hybridisation probes to describe natural communities of methanogens. *Appl. Environ. Microbiol.* **60,** 1232–1240.

13

Reporter Gene Expression for Monitoring Microorganisms in the Environment

James R. Firth

1. Introduction

1.1. Principles of Reporter Gene Expression

Reporter gene is a generic term that is used to describe genes whose expression is indicative of the presence of a particular cell, a specific event occurring within that cell, or a reflection of the expression of other, usually targeted genes of interest. For example, the presence of such a gene may make an organism standout to help in identification, as will be described in this chapter, or it could be that a gene is only expressed under certain conditions, e.g., during different developmental stages ***(1)***, environmental stresses ***(2,3)*** , or DNA repair as a result of induction of the SOS response to DNA damage ***(4)***. Naturally-occurring reporter genes can be used to avoid the need for genetic engineering by selecting a unique property of an organism and using it as the reporter. However, by far the commonest approach is to introduce a reporter gene into a cell to allow detection of a particular organism or to monitor its activity. The reporter gene can be located on a plasmid or to increase the stability of the gene within the cell it can be incorporated into the bacterial chromosome. There are a wide variety of reporter genes available, and all have particular properties that make them more or less useful depending on the application (**Table 1**). The choice of a particular reporter gene is made on the basis of the environment into which the organism is to be released, how the gene is to be detected, and what factors might interfere with the detection of the gene and/or its protein product.

Expression of the best reporter genes can be detected phenotypically, thus avoiding the need for molecular techniques. Most reporter genes code for an enzyme, so that expression of the gene can be either monitored directly or

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Table 1
Examples of Different Types of Reporter Genes

Method of detection	Source	Reporter gene	Gene product	Mode of detection	Reference
Resistance	Various plasmids	Antibiotic resistance genes, e.g., *catP amp*	For example, chloramphenicol acetyltransferase, β-lactamase	Selective culture in presence of antibiotic, herbicide or metal	***32***
	Pseudomonas putida EEZ15	Herbicide resistance	Unknown		***6***
	Various plasmids	Heavy metal resistance, e.g., *merA*	For example, mercuric reductase		***5***
Fluorescence	Fluorescent marine bacteria, e.g., *Vibrio fischerii, V. harveyi, Photobacterium* spp.	*lux*	Luciferase	Scintillation counter, luminometry, CCD (charge-coupled device) camera,	***3,4,9,19-26***
	Fire Fly	*luc*		X-ray film,	***33***
	Click beetle	*rluc*		microscopy	***1***
	Aequorea victoria (jellyfish) *Renilla reniformis* (sea pansy)	*gfp*	Green fluorescent protein (GFP)	fluorescent microscopy, flow cytometry,	***15-18***
	V. fischerii	*yfp*	Yellow fluorescent protein (YFP)	confocal microscopy	***38***
Chromogenic	*E. coli, Bacillus stearothermophilus*	*lacZY, BgaB*	β-Galactosidase	Enzyme assay in presence of	***2,7, 8,9 35***
	E. coli	*gusA, uidA*	β-Glucuronidase	appropriate substrate, e.g., X-Gal, catachol	***31***
	TOL plasmid pWWO of *Pseudomonas putida*	*xylE*	Catechol 2,3-dioxygenase	spectrophotometry, visually	***10–14***

monitored after addition of a substrate as enzyme activity. The substrate used normally produces colored products when reporter gene expression has taken place. When selecting reporter genes for detection of specific microorganisms, there are a number of factors that must be taken into consideration. The first is that the reporter genes has to be unique to the environment in which it is to be monitored. There has to be a means of clear selection of gene expression that distinguishes the target organism from the background microflora. There must be high sensitivity of detection so that large samples are not required. Ideally, detection of the reporter gene should be simple, making the process as quick and as easy as possible. An inexpensive system is obviously best as this allows more samples to be analyzed. The detection method for expression of the chosen gene may involve either destructive or nondestructive sampling and where population changes and community interactions are being studied such as in biofilms (**Chaps**. **19** and **20**), the nondestructive option may be essential. Ideally, the methods chosen would also allow *in situ* detection so that interactions in the actual environment can be examined.

1.2. Applications of Reporter Genes

1.2.1. Detection of Target Organisms

There are three different ways that reporter genes are used. The first, and the most common, is to distinguish a target cell from within a heterogeneous natural population. A commonly used marker system in the laboratory is antibiotic resistance markers, which enables an organism to survive and grow on a medium in the presence of a particular antibiotic. Any organisms not carrying the resistance marker will be unable to survive. The limitations here are that the environment contains an increasing number of microorganisms that have a natural resistance to a number of antibiotics. These microorganisms would not be screened out by the selective plates and would therefore make detection of a specific organism difficult if not impossible even if multiple antibiotic resistance genes are used. Recently, concerns about multiply-resistant pathogens and the difficulty in treating such organisms has rightly highlighted the potential risk of releasing antibiotic resistance genes into the environment. Alternative resistance genes have been employed encoding resistance to herbicides ***(5)*** and heavy metals ***(6)***, but, again, natural resistance limits the usefulness of such genes.

An alternative to resistance genes is the use of chromogenic reporter genes, which, when expressed, cause a color change to occur within the cell. Probably the most popular of these reporter systems is based on the *lacZY* genes of *Escherichia coli*. These genes encode β-galactosidase and lactose permease, and, once inserted into an organism, their expression can be detected by growth on a solid medium containing X-Gal (5-chloro-4-bromo-3-indolyl β-galactopyranoside). When X-Gal is cleaved by the enzyme, the colony appears blue–green. Hofte et al. ***(7)*** claimed

to be able to detect cells at densities of 10 per gram of soil using this method and detection limits of as low as 1 per gram of soil have also been claimed ***(8)***. This system has been used to monitor the colonization of biofilms in a drinking water supply line by enteric bacteria ***(9)***. Again with this system there is the problem of the indigenous microflora also possessing the *lac* genes, and this possibility must be taken into consideration before employing the technique and obviously the target organism must be *lac*-minus.

Another example is the *xylE* gene, which is found naturally on the TOL plasmid pWW0 of *Pseudomonas putida*. The gene codes for the enzyme catechol 2,3-dioxygenase (C23O) that converts catechol, which is colorless, to hydroxymuconic semialdehyde, which is yellow, and, allows cells carrying the gene to be distinguished. The *xylE* system has been used in both Gram-positive organisms, e.g., *Streptomyces lividans* ***(10)***, and numerous Gram-negative organisms ***(11, 12)*** as a reporter/marker gene. In the studies on Gram-negative species, the *xylE* gene was cloned into the broad host range, nonconjugative IncQ plasmid pKT230. The gene was expressed from either the p_L or p_R promoter of the lambda bacteriophage under the control of the temperature-sensitive lambda repressor cI857, which allows expression at 37°C but not at 30°C. This allows the gene to be switched off until detection is required, thus reducing the metabolic burden caused by the high level of expression of *xylE*. The system has since been used in IncP conjugative plasmids ***(13)***. Morgan et al. ***(14)*** found that such systems could be employed to detect recombinant *P. putida* at concentrations as low as 10^3 cells mL^{-1} of lakewater.

The other group of reporter genes are the bioluminescent and fluorescent reporter genes. These include the luciferases and the green and yellow fluorescent proteins, GFP ***(15–17)*** and YFP ***(18)*** (Table 1). These reporter genes, when expressed, produce light that can then be visualized in a number of ways (*see* **Note 1**). Since the source of these genes is eukaryotic organisms (with the exception of the *lux* system), there is no problem with background populations of microorganisms producing the same response. Even the *lux* system is very useful in soils and freshwater, since the *Vibrio spp*. in which it occurs naturally are marine organisms. The range of different bioluminescent reporter genes also produce light of different wavelengths, so that two or more can be used in conjunction to distinguish between different organisms or metabolic processes. One of the major problems with using GFP used to be its high stability, which meant that the protein remained within the cell long after the gene had been switched off, a problem when trying to investigate what factors might switch on particular processes. This problem has now been overcome by the development of more unstable GFPs ***(19),*** which can be degraded more easily by proteases within the cell giving the protein a half

life of minutes Much work has been carried out using luciferases to monitor the distribution and survival of numerous microorganisms in the environment ***(20–22)***. An interesting and novel method of detecting microorganisms in the environment is to use bioluminescent reporter genes in conjunction with species-specific phage. These phages are modified to contain the reporter gene, which , since viruses do not synthesize proteins, is not expressed. However, when the phage infects the host cell and inserts its DNA into the host chromosome, the reporter gene is expressed and the cell lights up. This has been used to detect a number of bacterial species including *Listeria* ***(23, 24),*** and *Mycobacteria* ***(25).***

1.2.2. Reporter Gene Fusions to Detect Gene Expression

Another way in which reporter genes are used is in gene fusions in which the reporter gene is linked to a gene of interest. Expression of the reporter gene is assumed to reflect the time of expression of the fused gene product. This kind of application is suited to the bioluminescent reporter genes. For example, *lux* gene fusions have been used to monitor the expression of the toluene dioxygenase enzyme in *P. putida* ***(26)***. Here the *lux* gene was fused to the gene encoding the enzyme, which not only allowed a correlation between the time of expression but also between the activity of the enzyme and the amount of bioluminescence. Taken further, such an approach can be used to monitor the concentrations of various pollutants in the environment. Heitzer et al. ***(27)*** used a *nahG-lux fusion* for naphthalene catabolism to investigate bioavailability of fuel hydrocarbons. Since bioluminescence appeared at the same time as enzyme expression, which, in turn, was proportional to the concentration of pollutant, bioluminescence showed a linear correlation with the amount of hydrocarbon pollution in solution.

1.2.3. Reporter Gene–Promoter Fusions to Monitor Environmental Conditions

Recently the trend has been to move away from fusing reporter genes to the structural genes of interest and instead fusing the reporter directly to the promoter region of the target gene. This approach has been used in a wide variety of applications. It has been used to construct plasmid vectors incorporating the regulatory *xylS xylR* genes of the TOL plasmid into which various reporter genes can be inserted to detect a number of hydrocarbon pollutants ***(28, 29)***. The GFP reporter system has been used to replace the coat protein gene of potato virus X so that when the virus infected a potato cell, GFP was synthesized to show which cells had become infected ***(30)***. This also showed that the coat protein that had been replaced was essential for cell-to-cell movement. The *luxAB* genes have been used to monitor circadian rhythms in cyanobacteria by fusing them to the promoter of *psbAI*, a gene thought to be influenced by

circadian rhythms ***(31)***. Promoter fusions are not limited to bioluminescent reporter genes. Flaherty et al. ***(32)*** used the β-glucuronidase reporter gene fused to the promoter of a gene responsible for the production of aflatoxin in *Aspergillus flavus*. This leads to expression of the reporter gene under conditions that would cause the production of the toxin. In a similar way, the antibiotic resistance gene *catP* has been fused to the promoter region of the alpha-toxin gene of *Clostridium perfringens* so that resistance to chloramphenicol is only inferred under conditions that would promote production of the toxin ***(33)***.

Reporter genes can also be used to demonstrate activity of cells. For example, *Mycobacterium tuberculosis* and *M. bovis* expressing the *luc* luciferase gene have been used to evaluate the activities of a number of antimicrobial compounds ***(34)***. The more effective the compound, the less light is emitted from the cells. This is also the basis of the commercially available Microtox assay ***(35)***. A similar approach has also been applied but using the *lacZ* reporter system ***(36)***.

Reporter genes can also be used to monitor the effect of microorganisms without the gene actually being present inside the cell. The pathogenic effect of *Erwinia amylovora* on tobacco or pear plants was investigated by introducing the luciferase not into the bacterial cells but into the plant cells ***(37)***. The pathogenic effect was then monitored by following the reduction of light being emitted by the plant cells as they were killed.

The take home message from all of this is that there is no perfect reporter gene that can be used to investigate all organisms and all metabolic processes in all environments. Each reporter gene has its own limitations, be they the need to extract samples before growth on selective plates, the presence of naturally occurring microorganisms carrying the gene, or the need for relatively high metabolic activity to allow detection of expression. However, by using a range of the reporter genes available, we can gather enormous amounts of information about the behavior of microorganisms in the environment

The example described here uses the plasmid pLV1013 (**Fig. 1**) to monitor the survival of *P. aeruginosa* introduced into sterile and nonsterile lakewater microcosms. In this plasmid the *xylE* gene is temperature regulated and expressed from the p_R promoter of the bacteriophage lambda. The plasmid also contains kanamycin- and streptomycin-resistance genes for an additional selection method.

2. Materials

2.1. Labeling P. aeruginosa *with the* xyl*E Reporter Gene*

1. *P. aeruginosa* recipient strain.
2. *E. coli* donor strain carrying the plasmid pLV1013.
3. *E. coli* CA60 carrying the mobilization plasmid pNJ5000.

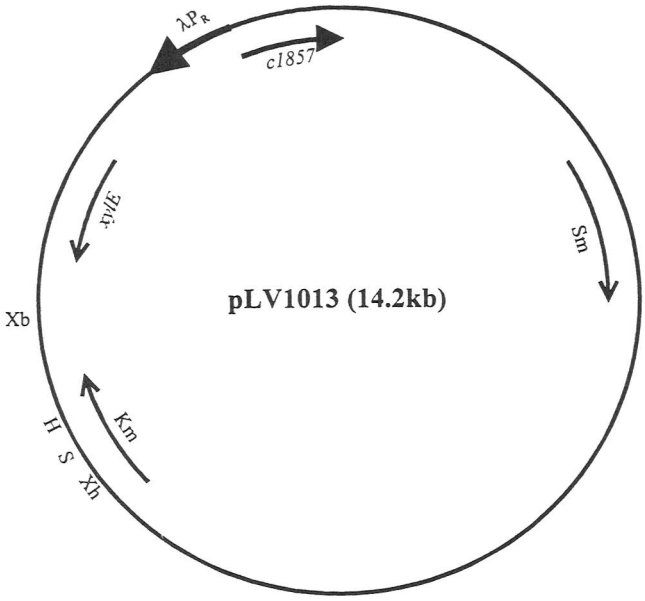

Fig. 1. Plasmid pLV1013 carrying the genes for kanamycin resistance (Km), streptomycin (Sm), and the *xylE* reporter gene. *Cutting sites for the restriction enzymes *Sma*I(s), *Xho*I(xh), *Hin*dIII(H), and *Xba*I(Xb) are shown

4. Simmons citrate agar.
5. Kanamycin stock solution (50 mg/mL).

2.2. Inoculation of Microcosms

1. *P. aeruginosa* carrying the reporter gene.
2. Fresh, nonsterile lakewater.
3. Autoclaved lakewater.
4. Spectrophotometer.
5. OD_{550} vs. colony forming units calibration curve.
6. Sterile distilled water.
7. Desk-top centrifuge.

2.3. Extraction and Detection of Marked Microorganisms from Microcosms

1. Sterile pipet.
2. Sterile 1 mL Eppendorf tubes.
3. 1% (w/v) catechol solution.
4. Volatilization chamber and aerosol.
5. Fume hood.
6. Glass spreader.

7. Pot of ethanol.
8. Bunsen burner.

3. Methods

3.1. Labeling the Organism with the xylE Reporter Gene

The experiment described here involves monitoring the survival of *P. aeruginosa* labeled with the *xylE* reporter gene. Other reporter gene systems will vary in the details of their use, but essentially the principles of the protocol described here will be the same for all culture-based detection systems (*see* **Note 1**).

1. Grow up overnight cultures of the recipient *P. aeruginosa*, *E. coli* CA60, and the donor *E. coli* strain in nutrient broth with the appropriate concentration of antibiotic (*see* **Note 2**).
2. Place a 10 µL drop of each organism on top of each other, on a nutrient agar plate containing no antibiotic and allow to dry.
3. Incubate the plate at 30°C overnight.

3.2. Screening of Plasmid Transfer

1. Resuspend one of the spots of bacterial growth in 0.5 mL of sterile distilled water.
2. Make a 10x dilution series of the suspension using sterile distilled water.
3. Spread 0.1 mL of each dilution onto separate plates of Simmons citrate agar ***(38)*** containing 50 µg/mL kanamycin, (*see* **Note 3**).
4. Incubate the plates at 30°C overnight.
5. Select a plate with approx 30 colonies on it and subculture in duplicate any colony that appears to be surrounded by a green coloration to a new Simmons agar plate containing 50 µg/mL of kanamycin and streak out.
6. Incubate one of each duplicate plate at 30°C and one at 37°C overnight.
7. Place all the plates in a fume cupboard and spray evenly with a light covering of 1% catechol solution. If the plasmid has been taken up successfully by the recipient *P. aeruginosa,* the colonies grown at 30°C will remain cream in color, while those grown at 37°C will turn a bright yellow.

3.3. Assessment of Plasmid Stability

1. Remove a single bacterial colony from Simmons agar plate and inoculate in triplicate into 100 mL of nutrient broth.
2. Incubate for 24 h at 30°C.
3. After 24 h transfer 50 µL from each culture to a fresh flask into nutrient broth and grow for a further 24 h at 30°C.
4. Take a further 1 mL sample from each flask and make a 10x dilution series of each sample.
5. Plate each dilution series onto nutrient agar with and without 50 µL/mL kanamycin.
6. Incubate these plates overnight at 30°C followed by a further 2 h at 42°C (*see* **Note 4**).
7. Spray the plates with catechol as described previously (**Subheading 3.2., step 7**).

8. Choose plates with between 30 and 300 colonies both with and without the kanamycin for each replicate and count the number of yellow colonies and white colonies on each (*see* **Note 5**).

3.4. Setting up of Sterile and Non-Sterile Microcosms

1. Take a sample of river or lake water and divide into 6x 500 mL batches in 500 mL conical flasks (*see* **Note 6**).
2. Autoclave three of the flasks to sterilize the water.
3. Take an overnight culture of the *P. aeruginosa* carrying the marker plasmid and pellet the cells by centrifuging 1 mL volumes for 3 min at 900*g* in a desktop centrifuge.
4. Pour off the supernatant and resuspend the cells in 1 mL of sterile distilled water.
5. Repeat the centrifugation and resuspension three times to prevent any carry-over of nutrients to the microcosms.
6. Take a reading of the optical density at 550 nm of the cell suspension using a spectrophotometer and calculate the cell density using an OD_{550} vs. colony forming U (cfu) calibration curve (*see* **Note 7**).
7. Inoculate each microcosm with the cell suspension to give the required cell density.

3.5. Extraction and Detection of xylE Carrying Organisms

1. After 24 h remove 1 mL volumes from each of the microcosms aseptically, using sterile pipets (*see* **Note 8**).
2. Make 10x dilution series of each sample in sterile distilled water.
3. Plate out and incubate the samples on nutrient agar containing 50 µL/mL kanamycin overnight at 30°C and then for a further 2 h at 42°C.
4. Spray the plates with the catechol solution as described previously (**Subheading 3.2., item 7**).
5. Choose plates with approx. 30–300 *xylE* positive colonies and make accurate counts (*see* **Note 9**).
6. Repeat this process at the required sampling times for the duration of the experiment.

4. Notes

1. As well as using culture based techniques, fluorogenic and chromogenic reporter genes can also be visualized and counted using other technologies such as flow cytometry (**Chap. 5**), confocal laser scanning microscopy (**Chap. 17**) and charge-coupled device cameras ***(22)***. Fluorescent reporter genes can also be monitored *in situ* by using X-ray film, which, when placed over or next to the sample in the dark, becomes exposed by the light being emitted by the cells. These different visualization methods have their own advantages and disadvantages.
2. *E. coli* CA60 carrying the conjugative plasmid pNJ5000 and the donor *E. coli* strain, in this case strain ED8654 carrying the plasmid pLV1013, are grown on agar or in nutrient broth containing 50 µg mL^{-1} of kanamycin as selection pressure to ensure the plasmid is maintained within the cells. The antibiotic and its concentration may vary depending on which plasmids are being used. Antibiotics should be filter steri-

lized and added to broth or agar after autoclaving. Agar should be below 60ºC before adding antibiotics. An indication that the agar is cool enough is when the bottle can be held comfortably in the hand without using gloves.

3. Simmons citrate agar ***(38)*** is a medium that contains citrate as the sole carbon source. Since *E.coli* cannot utilize citrate as a carbon source and *P. aeruginosa* can, only *P. aeruginosa* should be able to grow on the plates. Since kanamycin is also incorporated into the plates, only those *P. aeruginosa* cells that have taken up the pLV1013 plasmid should be present.
4. Since the expression of the reporter gene *xylE* is temperature regulated in the plasmid pLV1013, no expression occurs while the organisms are grown at 30°C. Therefore, in order to express the gene, the culture or plates containing the plasmid carrying cells can be incubated at 42°C for 2 h prior to spraying with catachol.
5. Plasmid stability is important as the reporter gene must be maintained within the cell and be inherited by subsequent generations in order for those cells to be detected. Stability is expressed as the percentage of the total number of cells (the total number of colonies) that show expression of the *xylE* gene. In practice any cells that have lost the plasmid will appear white when sprayed with catechol.
6. The usefulness of *xylE* as a marker gene is dependent on the gene not being present in the natural bacterial population. It is therefore essential to first plate out a sample of the uninoculated microcosm and spray with catachol to show that there are no naturally occurring *xylE* carrying organisms present.
7. A calibration curve of OD_{550} vs. colony forming units is constructed by making dilutions of a culture and taking the OD_{550} . Each dilution is then used to make a 10x dilution series, which is spread onto agar plates and incubated overnight. After counting colonies it is then possible to correlate each OD_{550} to a number of colonies, i.e., cells in the original sample.
8. It may be that 1 mL of water from the microcosm may not contain any of the introduced cells that may still be present but at lower concentrations. Under such circumstances larger vols can be extracted and concentrated up by centrifugation before resuspending the pellet of cells in a smaller volume of water. This concentration step must be taken into consideration when quantifying the target organism.
9. One problem often encountered when trying to count organisms carrying the reporter gene on a plate, especially when they are present in low numbers, is that they can be swamped by the natural microflora. Using the kanamycin in the plates will help to select against these background cells, although natural kanamycin resistance may still lead to swamping of the plates. An added advantage of the plasmid pLV1013 is that it also carries a gene for streptomycin resistance and this can be utilized as an extra selection pressure, which should further reduce the number of background cells growing on the plates.

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References

1. Srikantha, T., Klapach, A., Lorenz, W. W., Tsai, L. K., Laughlin, L. A., Gorman, J. A., and Soll, D. R. (1996) The sea pansy *Renilla reniformis* luciferase serves as a sensitive bioluminescent reporter for differential gene expression in *Candida albicans. J. Bact.* **178,** 121–129.
2. Schrogel, O. and R. Allmansberger (1997) Optimization of the *BgaB* reporter system: Determination of transcriptional regulation of stress responsive genes in *Bacillus subtilis*. *FEMS Microbiol. Lett.* **153,** 237–243.
3. Kragelund, L., Hosbond, C., and Nybroe, O. (1997) Distribution of metabolic activity and phosphate starvation response of *lux*-tagged *Pseudomonas fluorescens* reporter bacteria in barley rhizosphere. *Appl. Environ. Microbiol.* **63,** 4920–4928.
4. Volmer, A. C., Belkin, S., Smulski, D. R., Van Dyk, T.K., and La Rossa, R.A. (1997) Detection of DNA damage by use of *Escherichia coli* Carrying recA'-*lux*, uvrA'-*lux* or alkA'-*lux* reporter plasmids. *Appl. Environ. Microbiol.* **63,** 2566–2571.
5. Bale, M. J., Fry, J. C., and M. J. Day (1987) Plasmid transfer between strains of *Pseudomonas aeruginosa* on membrane filters attached to river stones. *J. Gen. Microbiol.* **133,** 3099–3107.
6. Ramos, J. L., Duque, E., and Ramos-Gonzalez, M. -I. (1991) Survival in soils of an herbicide-resistant *Pseudomonas putida* strain bearing a recombinant TOL plasmid. *Appl. Environ. Microbiol.* **57,** 260–266.
7. Hofte, M., Mergeay, M., and Verstraete, W. (1990) Marking the *Rhizopseudomonas strain* $7NSK_2$ with a mu d(*lac*) element for ecological studies. *Appl. Environ. Microbiol.* **56,** 1046–1052.
8. Drahos, D. J., Barry, G.F., Hamming, B.C., Brant, E. L., Kilne, E. L., and Skipper, H. D. et al. (1992) Spread and survival of genetically marked bacteria in soil, in *Release of Genetically Engineered and Other Micro–organisms,* (Fry, J. C., and Day, M. J., eds.) Cambridge University Press, Cambridge, UK, pp. 147–159.
9. Robinson, P. J., Walker, J. T., Keevil, C. W., and Cole, J. (1995) Reporter genes and fluorescent-probes for studying the colonization of biofilms in a drinking-water supply line by enteric bacteria. *FEMS Microbiol. Lett.* **129,** 183–188.
10. Wipat, A., Wellington, E. M. H., and Saunders, V. A. (1991) *Streptomyces* marker plasmids For monitoring survival and spread of streptomycetes in soil. *Appl. Environ. Microbiol.* **57,** 3322–3330.
11. Winstanley, C., Morgan, J. A. W., Pickup, R. W., Jones, J. G., and Saunders, J. R. (1989) Differential regulation of lambda *p*L and *p*R promoters by a *c*I repressor in a broad–host–range thermoregulated plasmid marker system. *Appl. Environ. Micro.* **55,** 771–777.
12. Morgan, J. A. W., Winstanley, C., Pickup, R. W., Jones, J. G., and Saunders, J. R. (1989) Direct phenotypic and genotypic detection of a recombinant pseudomonad population released into lake water. *Appl. Environ. Microbiol.* **55,** 2537–2544.
13. Winstanley, C., Morgan, J. A. W., Pickup, R. W., Jones, J. G., and 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. *Appl. Environ. Microbiol.* **57,** 1905–1913.
14. Morgan, J. A. W., Cranwell, P. A., and Pickup, R. W. (1991) Survival of *Aeromonas salmonicida* in lake water. *Appl. Environ. Microbiol.* **57,** 1777–1782.
15. Burlage, R. S., Yang, Z. K., and Mehlhorn, T. (1996) A transposon for green fluorescent protein transcriptional fusions: Application for bacterial transport experiments. *Gene* **173,** 53–58.
16. Josenhans, C., Friedrich, S., and Suerbaum, S. (1998) green fluorescent protein as a novel marker and reporter system in *Helicobacter* sp. *FEMS Microbiol. Lett.* **161,** 263–273.
17. Suarez, A., Güttler, A., Strätz, M., Staendner, L. H., Timmis, K. N., and Guzmán, C. A. (1997) Green fluorescent protein–based reporter systems for genetic analysis of bacteria including monocopy applications. *Gene* **196,** 69–74.
18. Lybarger, L., Dempsey, D., Patterson, G. H., Piston, D. W., Kain, S. R., and Chervenak, R. (1998) Dual–color flow cytometric detection of fluorescent proteins using single-laser (488–nm) excitation. *Cytometry.* **31,** 147–152.
19. Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjorn, S. P., Givskov, M., and Molin, S. (1998) New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl. Environ. Microbiol.* **64,** 2240–2246.
20. Rattray. E. A. S., Prosser, J. I., Killham, K., and Glover, L. A. (1990) Luminescence–based non–extractive technique for *in situ* detection of *E. coli* in soil. *Appl. Environ. Microbiol.* **56,** 3368–3374.
21. Rattray. E. A. S., Tyrrell, J. A., Prosser, J. I., Glover, L. A., and Killham, K. (1993) Effect of bulk density and temperature on wheat rhizosphere colonization by *lux*–modified *Pseudomonas fluorescens. Eur. J. Soil Biol.* **29,** 73–82.
22. Prosser, J. I., Killham, K., Glover, L. A., and Rattray, E. A. S. (1996) Luminescence-based systems for detection of bacteria in the environment. *Crit. Rev. Biotech.* **16,** 157–183.
23. Pearson, R. E., Jurgensen, S., Sarkis, G. J., Hatfull, G. F., and Jacobs, W. R. (1996) Construction of D29 shuttle plasmids and luciferase reporter phages for detection of *Mycobacteria. Gene.* **183,** 129–136.
24. Carriere, Riska, C. P. F., Zimhony, O., Kriakov, J., Bardarov, S., Burns, J., et al. (1997) Conditionally replicating luciferase reporter phages: Improved sensitivity for rapid detection and assessment of drug susceptibility of *Mycobacterium tuberculosis. J. Clin. Microbiol.* **35,** 3232–3239.
25. Loessner, M. J., Rees, C. E. D., Stewart, G. S. A. B., and Scherer, S. (1996) Construction of luciferase reporter bacteriophage *A511::luxAB* for rapid and sensitive detection of viable listeria cells. *Appl. Environ. Microbiol.* **62,** 1133–1140.
26. Applegate, B., Kelly, C., Lackey, L., McPherson, J., Kehrmeyer, S., Menn, F. M., et al. (1997) *Pseudomonas putida* B2: A *tod-lux* bioluminescent reporter for toluene and trichloroethylene co-metabolism. *J. Indust. Microbiol. Biotech.* **18,** 4–9.
27. Heitzer, A., Applegate, B., Kehrmeyer, S., Pinkart, H., Webb, O. F., Phelps, T. J., et al. (1998) Physiological considerations of environmental applications of *lux* reporter fusions. *J. Microbiol. Meth.* **33,** 45–57.

28. Blatny. J. M., Brautaset, T., Winther–Larsen, H. C., Haugan, K., and Valla, S. (1997) Construction and use of a versatile set of broad–host-range cloning and expression vectors based on the RK2 replicon. *Appl. Environ. Microbiol.* **63,** 370–379.
29. Blatny. J. M., Brautaset, T., Winther–Larsen, H. C., Karunakaran, P., and Valla, S. (1997) Improved broad–host–range RK2 vectors useful for high and low regulated gene expression levels in Gram–negative bacteria. *Plasmid.* **38,** 35–51.
30. Baulcombe, D. C., Chapman, S., and Cruz, S. S. (1995) Jellyfish green fluorescent protein as a reporter for virus–infections. *Plant. J.* **7,** 1045–1053.
31. Liu, Y., Golden, S. S., Kondo, T., Ishiura, M., and Johnson, C. H. (1995) Bacterial luciferase as a reporter of circadian gene-expression in cyanobacteria. *J. Bact.* **177,** 2080–2086.
32. Flaherty, J. E., Weaver, M. A., Payne, G. A., and Woloshuk, C. P. (1995) A beta-glucuronidase reporter gene construct for monitoring aflatoxin biosynthesis in *Aspergillus flavus. Appl. Environ. Microbiol.* **61,** 2482–2486.
33. Bullifent, H. L., Moir, A., and Titball, R. W. (1995) The construction of a reporter system and use for the investigation of *Clostridium perfringens* gene-expression. *Microbiol. Lett.* **131,** 99–105.
34. Arain, T. M., Resconi, A. E., Singh, D. C., and Stover, C. K. (1996) Reporter gene technology to assess activity of antimycobacterial agents in macrophages. *Antimic. Agents and Chemo.* **40,** 1542–1544.
35. Bundy, J. G., Wardell, J. L., Campbell, C. D., Killham, K., and Paton, G. I. (1997) Application of bioluminescence–based microbial biosensors to the ecotoxicity of organotins. *Lett. Appl. Microbiol.* **25,** 353–358.
36. Ulijasz, A. T., Grenader, A., and Weisblum, B. (1996) A vancomycin-inducible *lacZ* reporter system in *Bacillus subtilis*: Induction by antibiotics that inhibit cell wall synthesis and by lysozyme. *J. Bact.* **178,** 6305–6309.
37. Chang, J. R., and Geider, K. (1995) The Use of luciferase as a reporter for response of plant-cells to the fireblight pathogen *Erwinia amylovora*. *Plant Cells Rep.* **14,** 497–500.
38. Simmons, J. S. (1926) A culture medium for differentiating organisms of typhoid-colon aerogenes groups and for isolation of certain fungi. *J. Infect. Dis.* **39,** 209.

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Characterizing Microorganisms in the Environment by Fatty Acid Analysis

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1. Introduction

1.1. Fatty Acid Methyl Ester (FAME) Analysis as an Ecological Tool

Determining the taxonomic composition, biomass, and physiological status of microbial assemblages is still one of the greatest challenges facing microbial ecologists. There are many reasons why assessment of microbes in the environment is so demanding, not least their number, diversity, and limited size. Unlike eukaryotes, descriptions of the morphology of prokaryotes usually yields little or no information concerning the phylogenetic affiliation or ecological role of organisms. Consequently, although direct observations can provide an indication of biomass, they do not allow the investigator to distinguish among the many microbial populations present in samples. Classical approaches that utilize enrichment methods for the isolation of microorganisms from the environment continue to provide valuable information in biochemical, taxonomic, and autoecological studies. The primary limitations to such approaches are those of nonculturability (the active cellular component that cannot be grown in the laboratory on artificial media), and the problem of characterizing and identifying statistically relevant numbers of isolates necessary to gain insight into the population ecology and community diversity of any but the simplest of habitats. Furthermore, these approaches rarely provide information on microbial assemblages *in situ*. Modern molecular techniques may help to resolve some of these limitations and their application to environmental samples has allowed descriptions of microbial "species," and cellular location and activity in targeted communities ***(1)***. However, as

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with all techniques, there may be bias with regard to limited quantification and amplification of genetic material, which imposes inevitable selection for the sequences analyzed in the collected "representative" samples. Although this can, to some extent, be controlled, it is far from simple to routinely relate nucleic acid diversity to relative taxonomic abundance ***(2)***. Nevertheless, recent developments, such as denaturing gradient gel electrophoresis of polymerase chain reaction (PCR)-amplified products of isolates or whole community DNA ***(3)***, are beginning to reduce these difficulties.

Current efforts focusing on the molecular biology of nucleic acids may, to some extent, undervalue the enormous amount of information that can be derived by examining the chemistry of other cell constituents. The culmination of these data sets provides a combined understanding of both taxonomy and microbial ecology. Analysis of whole cells, e.g., by mass-spectrometry (MS)-pyrolysis, or cell wall constituents such as peptidoglycans, proteins, and lipids all provide valuable chemical data for the distinction of microbial groups, families, genera, species, and subspecies. Assessment of the cell wall composition of bacteria using differential reactions to the crystal/iodine complex of Gram's stain was one of the first methods that exploited chemical differences in microbial taxa. One group of cellular constituents, the lipids, have been widely and effectively used to provide taxonomic information for individual isolates and whole microbial assemblages. The wealth of information lipids provide about the identity, classification, biomass, and physiological state of microorganisms, together with technologic advancements in analyzes based on gas chromatography (GC) and computerized data handling, has significantly improved the understanding of environmental microbiology.

It is not intended here to cover all aspects of microbial fatty acids, but to provide some practical insight on how two of the most commonly used fatty acid profiling approaches, phospholipid fatty acid (PLFA) and fatty acid methyl esters (FAME), have been applied to the study of microbial diversity and function in the environment. PLFA has been widely exploited by microbial ecologists, but for further details of the principles, methodologies and application of this technique, the reader is referred White et al. ***(4)***. The introduction of semiautomated FAME analysis has revolutionized the study of cellular fatty acid components by making the approach available to the untrained user by combining speed without loss of sensitivity. In the following sections, we have outlined approaches for the use and application of FAME as an ecological tool and, where appropriate, have contrasted it with other methods of lipid analysis. Two other approaches to lipid analysis focus on the polar lipids and isoprenoid quinones, which are only briefly considered here. For further details on methods for the extraction, identification, and quantification of polar lipids and isoprenoid quinones, the reader should consult the reviews of Suzuki et al. ***(5)*** and Komagata and Suzuki ***(6)***.

1.2. Isoprenoid Quinones

Isoprenoid quinones are located in the cytoplasmic membrane of most prokaryotes, in which they play a role in electron transport systems. The variation in isoprenoid quinones is generally considered to provide useful taxonomic markers ***(5)***. Isoprenoid quinones have been widely applied to taxonomic studies and can distinguish members of the four rRNA subclasses (α, β, δ, and γ) of Proteobacteria.

1.3. Lipids

Lipids are amphipathic molecules, containing both a hydrophilic and a hydrophobic moiety, include phospholipids and glycolipids, and are located in bacterial membranes. Variation in the polar hydrophobic head has also been used as a taxonomic marker, although predominantly for distinguishing the actinomycetes ***(7,8)***. There are four main classes of lipid: long-chain fatty acids, polar, mycolic acids, and the isoprenoid quinones. Here we will concentrate on the fatty acids and polar lipids; other studies have confirmed the utility of the mycolic lipids and the isoprenoid quinones for differentiating different taxa ***(9,10)***.

1.4. Long-Chain Fatty Acids

Carboxylic acids with long hydrocarbon chains are the basic constituents of important lipids including the glycerides. Bacterial lipids range in chain length from simple 2-carbon atom backbones to those, as in the case of the mycolic acids, that contain over 90 carbons atoms. Taxonomically, fatty acids in the range of C_{11}–C_{24} provide the greatest information and are present in a diverse range of microorganisms. These cellular and structural fatty acids are distinct from lower weight compounds associated with metabolism that can also provide taxonomic information, but they are not considered in this review. Most fatty acids are located in the cytoplasmic membrane as constituents of polar lipids and glycolipids, in which they form the integral part of the lipid layer. In Gram-negative bacteria, fatty acids are also present in the outer membranes as part of the lipopolysaccharide. Fatty acids are properly named by the basic structure of their carbon skeleton, i.e., the number and position of double bonds, in the carbon chain, and the presence of functional groups ***(5)***. Diversity in fatty acids such as chain length, position of the double bonds and substituted groups associated with their highly regulated synthesis make them valuable biomarkers. For example, hydroxylated fatty acids having the –OH group in either position 2 or 3 can be found in most Gram-negative isolates. Fatty acids with branched alkyl chains predominate in Gram-positive genera.

1.5. Polar Lipids

Amphipathic polar lipids constitute the major part of bacterial membranes. They comprise a hydrophobic head group linked to two hydrophobic fatty acids. Variation in the polar or hydrophobic head group is not so marked as for the fatty acids but, nevertheless, provides general taxonomic information.

1.6. Nomenclature

Fatty acids are designated according to the number of carbon atoms, the number of double bonds, and the position of the double bond relative to the methyl terminus (ω) of the molecule. The configuration of the double bond is indicated by c (*cis*) and t (*trans*). The prefixes a and i indicate anteiso- and iso-branching, and br- indicates an unknown methyl branching position. For example, 10Me indicates a methyl group on the tenth carbon atom from the carboxyl end of the molecule and cy- refers to cyclopropane fatty acids (e.g., cy 17:0). The position of a hydroxyl group is numbered from the carboxyl end of the fatty acid, with OH as a prefix (e.g., 3-OH17:0; 3-hydroxy-heptadecanoate) ***(4,11)***.

1.7. Applications and Environmental Monitoring

1.7.1. Chemotaxonomy and Biomass

The wealth of information on the composition of microbial fatty acids has been widely used by microbiologists to improve understanding of community composition and dynamics in the environment. This has been achieved using two distinctive but complementary approaches: assessing community composition by extraction of the total fatty acid content of environmental samples and using signature fatty acids as biomarkers of specific populations, and culturing individual isolates and characterization by the fatty acid content.

PLFA analysis has been most commonly and effectively used to examine microbial community structure in whole environmental samples, since different subsets of a community have different PLFA patterns ***(29)***. This approach exploits the fact that distinctive microbial groups comprise characteristic PLFA patterns and has been sculpted in a broad range of habitats (**Table 1**). When determining community structure by using a signature fatty acid approach, it is essential to consider the environment from which the sample was retrieved ***(2,30)***. For instance, in a sediment two functional groups (defined by Findlay and Dobbs ***[2]*** as suites of microorganisms sharing biochemical characteristics) can be distinguished by PLFA analysis. First, the eukaryotes, composed of PLFA of both animal and plant or algal origin. These are distinguishable depending on the position of the first unsaturation being either in the ω6 (animals) or ω3 (plant) positions of the PLFA. The second functional group com-

Table 1
Application of PLFA for Monitoring Microbial Communities in the Environment

Microbial community investigated	References
Microbial biomass and community in soil	***(55,56)***
Soil bacterial response to presence of roots	***(57)***
Biomass arbuscular soil mycorrhizal fungi	***(58)***
Biomass and community structure, biofilms	***(59)***
Impact on soil microbial communities of pollution stress	***(11,56,59,60–62)***
Impact of land management practices	***(11,59,63–67)***
Microbial biomass, community structure, and physiologic state in deep-sea sediments	***(68–72)***
Biomass and community structure in rocks	***(73,74)***
Monitoring specific population (sulfate-reducing bacteria, methanotrophic bacteria) in soils and sediments	***(16,75,76)***
Quality assurance methods of sampling and storage methods (soils and sediments)	***(13,77,78)***

posed of prokaryotes can be further divided into bacteria utilizing anaerobic desaturase pathway, *Bacillus*-type Gram-positive bacteria and even specific genera such as *Desulfobacter*, according to the presence of specific marker PLFAs. Some PLFAs are even more specific markers and are highly diagnostic of specific groups. For instance, type II methane-oxidizing bacteria form monoenoic PLFA with the unsaturation in atypical positions, such as 18:1 ω8c ***(27)***. Unlike most other bacteria, this group contain more of the 18-carbon moiety (18:0) than the 16-carbon, a pattern normally characteristic of fungi. At a less specific level, general trends in PLFA can be observed within specific functional groups. Straight-chain PLFAs tend to be present in greater quantities in bacteria as the 16-carbon moiety (16:0), whereas microeukaryotes contain greater amounts of the 18-carbon form. Some distinctive groups share similar fatty acid constituents, and, consequently, interpretation of profiles must be undertaken with care and with the habitat characteristics in mind. For instance, terminally branched saturated PLFAs are common to Gram-positive bacteria but are also present in some Gram-negative anaerobic bacteria. Branched monoenoic PLFA are common in the anaerobic *Desulfovibrio*-type sulfate-reducing bacteria but are also found in some aerobic bacteria. Although PLFA profiles do not reveal species-level information directly, this approach provides a fingerprint of microbial diversity present.

The main application of FAME analysis has been in the identification and elucidation of the taxonomic relationships among cultures of microorganisms

(17). Improvements in GC techniques, computerization, and more efficient methods of FAME extraction have come together as part of the new generation of instrument-based systems, for rapid characterization of microorganisms. FAME analysis of bacterial cellular fatty acids is extensively used as either a primary or an adjunctive means for identification of clinical and phytopathogenic bacteria *(31–33)*. It is well established that fatty acid composition of microorganisms is an important taxonomic character *(34)* and that FAME data can be analyzed quantitatively to provide important taxonomic information at the species and subspecies levels *(35,36)*. Although FAME analysis utilizing the microbial identification system (MIS) has been commonly applied to bacteria, it has also been used to differentiate yeasts *(37)*, mycorrhizal fungi *(38,39)*, spirochetes *(40,41)* and iridescent viruses *(42)*. In many of these studies, the groupings obtained have proved to be highly congruent with other more labor-intensive methods such as genomic analysis by the direct restriction fragment comparison of total extracted DNA or PCR amplification of target regions. MIS-FAME analysis has also been applied to assess whole communities of complex substrates such as fecal stools *(43)*, soils *(44)* and model bacterial communities *(45)* (**Table 2**).

In addition to providing information on community composition and dynamics, PLFAs fulfill many of the criteria required to be effective chemical markers of microbial biomass. This includes their universal distribution in the cellular components of intact cells, but short residence time in detrital pools after death. In general, they are expressed at relatively constant levels within a community throughout the growth cycle. The following PLFAs are considered to be of predominantly bacterial origin: i15:0, 15:0, i16:0, and 16:1ω9.

1.7.2. Physiological Status

The fatty acid components of the individual membrane lipids are not fixed but vary with nutritional status and environmental conditions. By studying the changes of phospholipid fatty acid profiles, especially the presence of certain PLFA markers, it is possible to assess the physiologic status of the microbial community. For example, changes typically found in PLFA profiles when Gram-negative bacteria are starved include an increase in the ratio of saturated to unsaturated fatty acids *(15,46,47)*, and an increase in the ratio of the *trans*- to *cis*- monoenoic unsaturated fatty acid. By contrast, negligible changes in PLFA profiles are observed in Gram-positive bacteria *(48)*. The observed changes in the relative ratio of specific lipids at a gross level and the proportion of poly-β-hydroxyalkanoic acid (a storage lipid) in bacteria *(49)* relative to PLFA, provides a measure of nutritional-physiological status *(4)*. Bacteria in bulk soils that are characteristically low in available nutrients have PHA/PLFA ratios below 1. By contrast poly-β-hydroxyalkanoic acid PHA/PLFA ratios of

Table 2.
Application of FAME Analysis for Monitoring and Assessing Individual Isolates and Microbial Communities in the Environment[a]

Microbial community investigated	References
Bacterial diversity inaquatic ecosystems	***(79,80)***
Characterization of xenobiotic degrading bacteria	***(81,82,83)***
Characterization of mycorrhizal fungi	***(38,39)***
Phytosphere bacterial population dynamics	***(54,84–86)***
Bacterial community succession in necrotic plant tissue	***(87,88)***
Predicting biocontrol activity of environmental bacterial isolates on the indigenous microbial community	***(89,90)***
Impact of a genetically modified bacterium	***(91)***

[a]Since 1986 there have been at least 50 articles published in which the MIS has been used to characterize the FAME composition of individual isolates or total community profiles from environmental samples.

6 or more have been recorded for bacteria growing in the nutrient-rich conditions typical of the rhizosphere ***(50)***. Starvation, stationary-phase growth, and anaerobic metabolism in bacteria all lead to conversion of monoenoic PLFA to the cyclopropyl PLFA, which can also provide a useful indicator of physiological state ***(15)***.

Changes in the PLFA profiles of individual isolates grown in culture can be induced following exposure to potentially toxic substances, and have been used as effective indicators of pollution stress ***(51)***. For instance, increasing concentrations of organics such as phenol induces a reduction in the proportion of monoenoic to saturated PLFA, and an increase in the proportion of *trans*-unsaturated fatty acids of exposed bacteria unable to degrade the contaminant. Similar changes have been observed in a range of microorganisms exposed to different organic compounds and have been associated with altered cell permeability and specific resistance mechanisms ***(51–53)***. However, little is known of the mechanisms of solvent tolerance, although the recorded quantitative increase in fatty acids suggests that membrane repair mechanisms, involving rapid fatty acid synthesis, are involved. Not surprisingly, since cellular fatty acid composition is dependent on growth conditions, the membrane compositions of bacterial cells that can utilize pollutants as a nutrient source are altered, whereas, typically, increases in the proportion of saturated fatty acids are observed.

1.7.3. Environmental Monitoring: Method Application

Investigators have taken two basic approaches to the investigation and interpretation of community fatty acid profiles: first, examination of specific fatty

acid profiles known or assumed to be unique to a given functional or taxonomic group, and second, application of multivariate analysis to discriminate between composite profiles to assess habitat disturbance. As with any selected method for monitoring microbial communities present in environmental samples, PLFA and FAME analyzes have advantages and limitations.

The primary advantage of PLFA analysis is that microbial biomass and community structure can be assessed from the same sample. The results obtained integrate across the entire community, and thus avoid the inevitable selective pressures on the sampled community owing to the choice of media and growth conditions inherent to culture-dependent studies. Also, any weaknesses associated with enumerative studies, such as dislodging microbial cells from substrate (extraction), can be avoided and therefore reduce problems of representative sampling and direct cell visualization. This method is also applicable to a range of dense and solid substrates such as sediments, soils, sand, and rock, in which microscopic methods can be of limited value. Relative to molecular techniques, the approach is often more cost- and time-effective without the loss of the high precision or quality in the data obtained, although molecular methods are continuously becoming more reliable and less time consuming to undertake. Finally, sample extracts can be used to undertake further biochemical characterizations ***(2)***.

FAME analysis has been widely accepted in clinical microbiology as a primary or adjunctive means for identification of medically important bacteria ***(32)***. However, FAME analysis, using instrument-based systems, has not been as widely applied as a sensitive, reliable, and rapid method for microbial characterization in environmental microbiology. This may, in part, be owing to the capital cost of equipment. Nevertheless, FAME analysis compares well with other methods of strain characterization such as DNA hybridization or target nucleic acid amplification. Indeed, when fitted with an electron detector, femtomole (10^{-15}) quantities of fatty acids can be detected that may be more sensitive than for direct (nonamplified) DNA/RNA detection. For example, for highly specialized communities such as the phyllosphere, FAME data of individual pseudomonad isolates aligned well with genomic studies ***(54)***. Assurance of the reliability of FAME data overcomes the need for supporting characterization by traditional diagnostic biochemical methods that are labor-intensive and rely on the organisms being reactive. FAME-MIS is also reasonably rapid, easy to perform, and requires little specialized technical training.

Despite the need to consider the contribution of the active but unculturable component in any environmental sample, the challenge remains for the further development and exploitation of current techniques that enable large numbers of strains to be rapidly and reliably characterized. And although existing databases for environmental strains may be limited, preventing precise species iden-

tification for unknown isolates, large numbers of environmental isolates are being examined, which further improves the quality and precision of identification. There is certainly a need for a managed database, i.e., a Web site, for FAME and PLFA profiles that compare to the quality of databases developed for DNA sequence information

There are, of course, associated disadvantages. Analysis of PLFA and FAME requires special analytical apparatus such as a gas chromatogram, in the case of PLFA, with mass spectrometer. Solvents, reagents, and glassware must be scrupulously clean and rigor must be observed in sample handling and record keeping. All procedures should be stringent and careful attention paid to the inclusion of appropriate standards. In addition, specialized equipment is necessary and requires considerable understanding to optimize its application at the limits of sensitivity. Furthermore, interpretation of PLFA profiles is often complex and requires a thorough knowledge of a widely scattered literature. Finally, few genera or even functional groups have distinctive fingerprints, and with limited data sets, often discrimination at population level is not possible.

Although factors for converting phospholipids data to carbon content are established, uncertainties remain for quantifying phospholipid data in terms of cell numbers or biovolume. And although the fatty acid composition of many microbial taxa are known, it is difficult to convert PLFA data for the precise description of the composition of microbial communities. Because fatty acid composition is directly dependent on growth conditions, one of its strengths is that it can be used as a diagnostic tool, e.g., to describe pollution impact, habitat perturbation, or differentially regulated gene expression in response to local environmental changes (**Table 1**).

Future developments require investments appropriate to the application of current research objectives linked to automating and accelerating the speed of the analyzes. It is, nonetheless, realistic to believe that these developments will result in automated systems for signature lipid biomarker analysis that will be accomplished in a matter of hours, instead of the current time frame of days.

2. Materials

1. Four reagents are required to saponify the cells, esterify, extract, and base wash the fatty acids. All reagents should be prepared and stored in acid-washed, brown (light opaque) glass bottles fitted with volumetric plungers. Extraction should only be undertaken in glass tubes fitted with Teflon-coated screw-capped lids.
   a. Reagent 1 (saponification): 3.75 *M* NaOH in methanol/H_2O (50:50 by volume).
   b. Reagent 2 (methylation): 4.7 *M* HCl in methanol (325 mL 6M HCl, 275 mL methanol).
   c. Reagent 3 (hexane/methyl-tert butyl ether [MTBE]): 200 mL hexane, 200 mL MTBE.
   d. Reagent 4 (base wash): 0.3 *M* NaOH in deionized distilled water.

2. Water baths are required at 100°C, 80°C, and room temperature. A rotating mixing device should be used for test tubes.
3. All reagents should be of high-performance liquid chromatography analytical grade.
4. All procedures should be undertaken in an appropriate, ventilated facility or chemical fume hood. Reagents 1 and 4 are caustic and reagent is 2 acidic; they should be only handled by operators wearing safety goggles and gloves. MTBE and hexane are flammable; extinguish all flames and sources of heat before use.

3. Methods

Although the principles of PLFA and FAME analyzes are similar, the two methods are generally used for distinct purposes. PLFA is most commonly used to analyze the biomass and total community composition of environmental samples, and FAME to characterize isolates grown on defined laboratory media (*see* **Note 1**).

3.1. PLFA Extraction

Environmental samples must be handled with extreme care to limit disturbance to the microbial community. This can be achieved best by halting microbial activity by rapid freezing to –20°C or by lyophilization. Alternative methods of preservation should not be used since these can adversely affect certain lipids. Gas chromatographic analysis of PLFAs extracted from environmental samples generally requires nanomolar sensitivity. This necessitates the use of clean glassware washed in either 10% (v/v) HCl or Decon phosphate-free detergent (BDH[Merck] Ltd., Lutterworth, UK) and baked in an oven (450°C for 4 h). Plastic must be avoided at every step from sample collection and storage to eventual extraction and analysis. The extraction is usually undertaken at room temperature. All solvents and chemicals used must be of analytical grade.

Preparation of PLFAs consists of three steps: extraction of lipids, separation of phospholipids by column chromatography, and methylation of esterified fatty acids in the phospholipid fraction. There are many variations of the extraction procedure, each modified according to requirements. The procedure described by White et al. ***(4)*** is summarized next:

1. Transfer 1–3 g of humus or soil into centrifuge tubes equipped with Teflon-lined screw caps.
2. Extract in a single-phase chloroform-methanol mixture (1:2, v/v) the Bligh and Dyer mixture ***(12)***. Alternatively, a dichloromethane-methanol mixture (1:2 v/v) can also be used ***(13)***. For soils with high clay content, supplement the mixture with phosphate or citrate buffer (1:2:0.8, v/v/v) to increase PLFA recovery ***(11,14)***.

3. Centrifuge samples at 6000*g* for 30 min, remove the liquid, and shake with equal volumes of chloroform and distilled water (or buffer) to produce an emulsion that is allowed to stand overnight.
4. Remove the lipid-containing organic phase, filter, and collect the lipids by rotary evaporation at 37°C.
5. Dissolve the dried total lipid extract in chloroform, transfer to silica acid columns, and separate into neutral, glycolipid- and polar lipid fractions by elution with solvents of increasing polarity ***(15)***. The polar lipid containing the phospholipid is subjected to transesterfication by mild alkaline methanolysis ***(16)*** and the resulting FAME is separated, quantified, and tentatively identified by capillary GC.

3.2. Fatty Acid Methyl Esters

Preparation of cellular fatty acids consists of hydrolysis using sodium hydroxide to form sodium salts, and then methylation of the fatty acid esters to make them volatile in the gas chromatograph ***(17)***. There are various procedures that have been used to obtain the end product for GC analysis, and all involve acid or base hydrolysis followed by esterification with methanol ***(18–20)***. However, recent advances in the methods have optimized the recovery of fatty acids that formerly were difficult to identify reliably. This is owing, in large part, to the efforts of Miller ***(21)***, who developed a simple washing procedure with NaOH that removes free acids and prevents the tailing of hydroxyl acid peaks during GC analysis. This development, together with other refinements in the procedures ***(18,19)*** has led to a relatively simple four-step process for the preparation of samples ***(22)***.

1. Harvest approx 50 mg (wet wt) of cells from culture plates incubated for 24–48 h.
2. Saponify using a sodium hydroxide–methanol solution for 30 min at 100°C, to release fatty acids from cellular lipids.
3. Methylate with HCl in methanol at 80°C for 10 min and extract the FAME into a solution of hexane and MTBE for 10 min.
4. Wash the extract in aqueous NaOH for 5 min. This procedure is simple to perform and up to 120 samples can be processed in a day. Samples can be prepared from pure cultures or environmental samples.
5. The FAME profile is used to identify isolates against the Microbial Identification Software database (MIDI-MIS, Newark, Delaware). However, databases for any media or growth condition can be constructed to suit particular requirements such as growth conditions on a specific medium. Similarly, total extracts from directly sampled habitats, such as soil samples, can be made to profile the diversity of the microbial community.

3.3. FAME Analysis of Isolates

The following standard protocol has been developed for cultures prepared after exactly 24 h of growth, at 28°C in 85-mm Petri dishes containing 20 mL

of tryptone soy broth agar (TSBA) that is allowed to set and dry at 37°C for 16–20 h before use.

1. Spread a single colony of bacteria, using a loop, over a quadrant of a dried TSBA plate, sterilize the loop and spread a second quadrant from the edge of the first quadrant. Repeat this procedure until four reducing densities of inocula are present in the quadrants.
2. After exactly 24 h of incubation at 28°C, collect 50 mg wet wt of cells from the third quadrant using a loop and deposit at the bottom of a 10 × 1 cm Pyrex glass boiling tube with teflon-lined screw-capped lids (samples may be stored at –70°C for many months).
3. Add 1 mL of reagent 1, vortex to suspend the cells and incubate at 100°C for 5 min. Vortex the suspension and incubate at 100°C for a further 25 min.
4. Rapidly cool the saponified samples to room temperature by placing tubes in water.
5. Methylate with 2 mL reagent 2, and incubate at 80°C for 10 min. Cool to room temperature. This is the most critical step and should be undertaken exactly as described.
6. Add 1.25 mL of reagent 3, and mix by inversion on a rotating platform for 10 min at room temperature.
7. Allow phases to separate, and collect and discard the lower aqueous phase.
8. Wash organic layer with 3.0 mL of reagent 4 by mixing on the rotator for 5 min.
9. Allow the contents of the tubes to settle; add several drops of saturated NaCl solution to aid separation of phases.
10. Using a glass pipet, collect two-thirds of the top organic phase, transfer to a glass GC-vial, and seal with sample caps.
11. Store samples at –20°C for up to 4 wk and run on the gas chromatograph (*see* **Note 1***)*.

3.4. Statistical Analysis of Data

PLFA and other profiles are subjected to multivariate statistical applications to assess similarities between PLFA profiles. Dendrograms from hierarchical cluster analysis are usually constructed from arcsine-transformed PLFA mole percentage values, with similarities based on modified Euclidean distances. The use of two-dimensional plots generated from a principal analysis not only identifies the extent that specific PLFAs contribute to the plots but also reveals profile similarities ***(4,28)***.

Data analysis is rapid and efficient with the MIS. The retention time is used to calculate an equivalent chain length. The equivalent chain length is equal to the number of carbon atoms of a straight-chain saturated fatty acid or to a number that can be calculated by interpolation with a mathematic formula for other fatty acids. The amounts of fatty acid detected are calculated as a percentage of the total amount and printed together with the most likely identification

according to similarity to entries in the database ***(17,22)***. Multivariate statistical methods, resulting in pattern recognition, are used for interpretation of data, matching unknowns with database entries and resulting in an identification. Numerical analysis of fatty acid data can be applied for the construction of dendrograms. An unweighted pair-matching method can also be applied to determine the extent of similarity between isolates and the collection to the genus, species and subspecies level.

4. Notes

1. There have been two major advances since the 1950s that have brought routine fatty acid analysis to the laboratory. The first is the development and implementation of fused-silica capillary columns ***(19,23)***. In contrast to packed columns and others with a broad diameter, capillary columns allow reproducible recovery of hydroxy fatty acids and the ability to distinguish several isomers of fatty acids with the same carbon chain length. Parallel developments in other methods such as nuclear magnetic resonance spectrometry, infrared spectroscopy, and mass spectrometry have also been widely used to identify fatty acids ***(24)***. The second major advance is the development of microcomputers systems that enable efficient data processing ***(25)***.

   The resulting FAME obtained from the phospholipid as described above, or one of the many variants of this procedure, are separated on a gas chromatograph equipped with a flame ionization detector and phenylmethyl silicone capillary column ***(14)***. In the procedure described by Frostegård et al. ***(14)*** hydrogen is used as the carrier gas, and injection is made in a splitless mode. Tentative identification of fatty acids is based on retention time on the column as measured against calibration standards. Individual components can also be identified by mass spectrometric analysis. With this procedure, the GC conditions are identical to those used above, but helium is used as the carrier gas. Identification of FAMEs is based on comparison with spectra that are obtained either from standards ***(26)*** or by analysis of the dimethyl disulfide adducts ***(27)***.

   The fatty acid analysis of microorganisms is now so common that a commercially available GLC system is now available. This system was initially codeveloped by Hewlett-Packard and Microbial ID (Newark, Delaware) for the identification of aerobic bacteria, but more recently it has been used for the analysis of fungi, viruses, spirochetes, and the lipid content of total soil extracts. The system consists of a gas chromatograph (HP 5890 Series II) equipped with a flame ionization detector, 5% methylphenyl silicone fused-silica capillary column (25 m X 0.2 mm), autosampler (HP7673), integrator, computer and printer. Equipment designed and dedicated for the purpose of microbial identification is not a necessary requirement, but the MIS greatly facilitates the interpretation of results ***(17)***. Furthermore, an element of automation is added by the automatic sampler, which lets the operator run up to 100 samples without intervention.

References

1. Amman, R. I., Ludwig, W., and Schleifer, K.-H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59,** 143–169
2. Findlay, R. H. and Dobbs, F. C. (1993) Quantitative description of microbial communities using lipid analysis, in *Handbook of Methods in Aquatic Microb. Ecol.* (Kemp, P. F., Sherr, B. F., Sherr, E. B., and Cole, J. J., eds.), Lewis Publishers, Boca Raton, FL, pp. 271–284.
3. Heuer, H. and Smalla, K. (1997) Application of DGGE and TGGE for studying microbial communities, in *Modern Soil Microbiology* (van Elsas, J. D., Wellington, E. M. H., and Trevors, J. T., eds.), Marcel Dekker, New York, pp. 353–373.
4. White, D. C., Pinkart, H. C., and Ringleberg, D. B. (1997) Biomass measurements: biochemical approaches, in *Manual of Environmental Microbiology* (Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenbach, L. D. and Walter, M. V., eds.), ASM, Washington, DC, pp. 91–101.
5. Suzuki, K., Goodfellow, M., and O'Donnell, A. G. (1993) Cell envelopes and classification, in *Handbook of New Bacterial Systematics* (Goodfellow, M. and O'Donnell, A. G. eds.), Academic, London, pp. 195–250.
6. Komagata, K. and Suzuki, K. (1987) Lipid and cell wall analysis in bacterial systematics. *Meth. Microbiol.* **19,** 161–207.
7. Kroppenstedt, R. M. (1985) Fatty acid and menaquinone analysis of actinomycetes and related organisms, in *Chemical Methods in Bacterial Systematics* (Goodfellow, M. and Minnikin, D. E., eds.), Academic, London, pp. 173–199.
8. Goodfellow, M. 1989 Supragenic classification of actinomycetes, in *Bergey's Manual of Systematic Bacteriology* (Williams, S. T., Sharpe M. E., and Holt J. G., eds.) Williams and Wilkins, Baltimore, pp. 2333–2339.
9. Komagata, K. and Suzuki, K. (1987) Lipid and cell wall analysis in bacterial sytematics. *Methods Microbiol.* **19,** 161–207
10. Collins, M. D. (1985) Isoprenoid quinone analyses in bacterial classifications and identification, in *Chemical Methods in Bacterial Systematics* (Goodfellow M. and Minnikin D. E., eds.), Academic, London, pp. 267–287.
11. Frostegård, A., Tunlid, A., and Baath, E. (1993) Phospholipid fatty acid composition biomass, and activity of microbial communities from two soil types experimentally exposed to two different heavy metals. *Appl. Environ. Microbiol.* **59,** 3605–3617.
12. Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **31,** 179–184.
13. Petersen, S. O. and Klug, M. J. (1994) Effects of sieving, storage and incubation temperature on the phospholipid fatty acid profile of a soil microbial community. *Appl. Environ. Microbiol.* **60,** 2421–2430.
14. Frostegård, A., Tunlid, A., and Baath, E. (1991) Microbial biomass measured as total lipid phosphate in soils of different organic content. *J. Microbiol. Methods* **14,** 151–163.

15. Guckert, J. B., Hood, M. A., and White, D. C. (1986) Phospholipid, ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increase in *trans/cis* ratio and proportions of cyclopropyl fatty acids. *Appl. Environ. Microbiol.* **52,** 794–801.
16. Dowling, N. J., Widdel, F., and White, D. C. (1986) Phospholipid ester linked fatty acids biomarkers of acetate oxidising sulphate reducers and other sulphide forming bacteria. *J. Gen. Microbiol.* **132,** 1815–1825.
17. Welch, D. F. (1991) Applications of cellular fatty acid analysis. *Clin. Microbiol. Rev.* **4,** 422–438.
18. Lambert, M. and Moss, C. W. (1983) Comparison of the effect of acid and base hydrolysis and cyclopropane fatty acids in bacteria. *J. Clin. Microbiol.* **18,** 1370–1377.
19. Moss, C. W. (1981) Gas-liquid chromatography as an analytical tool in microbiology. *J. Chromatogr.* **203,** 337–347.
20. Moss, C. W., Lambert, M. A., and Lombard, G. L. (1977) Cellular fatty acids of *Peptococcus variabilis* and *Peptostreptococcus anaerobius*. *J. Clin. Microbiol.* **5,** 665–667.
21. Miller, L. T. (1982) Single derivatisation method for routine analysis of bacterial esters, including hydroxy acids. *J. Clin. Microbiol.* **16,** 584–586.
22. Sasser, M. (1990) Identification of bacteria by gas chromatography of cellular fatty acids. Tech. note 101. MIDI, Newark, DE.
23. Moss, C. W., Dees, S. B., and Guerrant, G. O. (1980) Gas-liquid chromatography of bacterial fatty acids with fused silica capillary column. *J. Clin. Microbiol.* **12,** 127–130.
24. Kaneda, T. (1991) Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiol. Rev.* **55,** 288–302.
25. Eerola, E. and Lehtonen, O. P. (1988) Optimal data processing procedure for automatic bacterial identification by gas-liquid chromatography of cellular fatty acids. *J. Clin. Microbiol.* **26,** 1745–1753.
26. Saranpaa, P. and Nyberg, A. (1987) Lipids and sterols in *Pinus sylvestris* L. sapwood and heartwood. *Trees* **1,** 82–87.
27. Nichols, P. D., Mancuso, C. A., and White, D. C. (1987) Measurement of methanotroph and methanogen signature phospholipids for use in assessment of biomass and community structure in model systems. *Organic Geochem.* **11,** 451–461.
28. Almeida, J. S., Sonesson, A., Ringleberg, D. B., and White, D. C. (1995) Applications of artificial neural networks to the detection of *Mycobacterium tuberculosis,* its antibiotic resistance and prediction of pathogenicity amongst *Mycobacterium* sp. based on signature lipid biomarkers. *Binary Comput. Microbiol.* **7,** 53–59.
29. Tunlid, A. and White D. C. (1992) Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil, in *Soil Biochemistry*, vol. 7 (Stotzky G. and Bollag J.-M., eds.), Marcel Dekker, New York.
30. White, D. C. (1993) Analysis of micro-organisms in terms of quantity and activity in natural environments, in *Microbes in Their Natural Environment* (Slater, J. H.,

Whittenbury, R., and Wimpenny, J. W. T. eds.), Society of General Microbiology Symposium 37, Cambridge University Press, Cambridge, UK.
31. De Boer, S. H. and Sasser, M. (1986) Differentiation of *Erwinia carotovora* ssp. *carotovora* and *Erwinia carotovora* ssp. *atroseptica* on the basis of cellular fatty acid composition. *Can. J. Microbiol.* **32,** 796–800.
32. Dees, S. B., Hollis D. G., Weaver R. E., and Moss, C. W. (1981) Cellular fatty acids of *Brucella canis* and *Brucella sius*. *J. Clin. Microbiol.* **14,** 111, 112.
33. Lambert, M. A., Patton, C. M., Barrett, T. J., and Moss, C. W. (1987) Differentiation of *Campylobacter* and *Campylobacter* like organisms by cellular fatty acid composition. *J. Clin. Microbiol.* **25,** 706–713.
34. O'Donnell, A. G. (1985) Fatty acid analysis in the identification of natural isolates: possibilities for *in situ* identification using multivariate pattern recognition, in *Recent Advances in Microb. Ecol.* (Hattori, T., Ishida, Y., Maruyama, Y., Morita, R., and Uchida A., eds.), Japan Scientific Societies Press, Tokyo, Japan, pp. 674–678.
35. Mukwaya, G. M. and Welch, D. F. 1989 Subgrouping of *Pseudomonas cepacia* by cellular fatty acid composition. *J. Clin. Microbiol.* **27,** 2640–2646.
36. Rainey, P. B., Thompson, I. P., and Palleroni, N. J. (1994) Genome and fatty acid analysis of *Pseudomonas stutzeri*. *Int. J. Syst. Bacteriol.* **62,** 93–101.
37. Vilojoen, B. C, Kock, J. L. F., and Lategan, P. M. (1986) Fatty acid comparison as a guide for the classification of selected genera of yeasts belonging to the Endomycetales. *J. Gen. Microbiol.* **132,** 2397–2400.
38. Graham, J. H., Hodge, N. C., and Morton, J. B. (1995) Characterisation of fatty acid methyl ester profiles for characterisation of Glomalean fungi and their endomycorrhizae. *Appl. Environ. Microbiol.* **61,** 58–64.
39. Bentivenga, S. P. and Morton, J. B. (1996) Congruence of fatty acid methyl ester profiles and morphological characters of arbuscular mycorrhizal fungi in Gigasporaceae. *Proc. Natl. Acad. Sci. USA* **93,** 5659–5662.
40. Livesley, M. A., Thompson, I. P., Bailey, M. J., and Nuttall, P. A. (1993) Comparison of the fatty acid profiles of *Borrelia*, *Serpulina* and *Leptispira* species. *J. Gen. Microbiol.* **139,** 889–895.
41. Livesley, M. A, Thompson, I. P., Gern, L and Nuttall, P. A. (1993) Diversity in fatty acid profiles of *Borrelia burgdorferi*. *J. Gen. Microbiol.* **139,** 1947–1957.
42. Williams, T. and Thompson, I. P. (1994) Fatty acid profiles of invertebrate iridescent viruses. *Arch. Virol.* **140,** 975–981.
43. Peltonen, R., Ling, W. H, Hanninen, O., and Eerola, E. (1992) An uncooked vegan diet shifts the profile of human fecal microflora: computerised analysis of direct stool sample gas-liquid chromatography profiles of bacterial cellular fatty acids. *Appl. Environ. Microbiol.* **58,** 3660–3666.
44. Cavigelli, M. A., Robertson, G. P., and Klug, M. J. (1995) Fatty acid methyl ester (FAME) profiles as measures of soil microbial community structure. *Plant Soil* **170,** 99–113.
45. Haack, S. K., Garchow, H., Odelson, D. A., Forney, L. J., and Klug, M. J. (1994) Accuracy, reproducibility and interpretation of fatty acid methyl ester profiles of model bacterial communities. *Appl. Environ. Microbiol.* **60,** 2483–2493.

46. Linder, K. and Oliver, J. D. (1989) Membrane fatty acid and virulence changes in the viable but non-culturable state of *Vibrio vulnificus*. *Appl. Environ. Microbiol.* **55,** 2837–2842.
47. Rice, S. A. and Oliver, J. D. (1992) Starvation response of the marine barophile CNPT–3. *Appl. Environ. Microbiol.* **58,** 2432–2437.
48. Kostiw, L. L., Boylen, C. W., and Tyson, B. J. (1972) Lipid composition of growing and starving cells of *Arthrobacter crystallopoietes*. *J. Bacteriol.* **94,** 1868–1874.
49. Nickels, J. S., King, J. D., and White, D. C. 1979. Poly-beta-hydroxybutarate accumulation as a measure of unbalanced growth of estuarine detrital microbiota. *Appl. Environ. Microbiol.* **37,** 459–465.
50. Tunlid, A., Baird, B. H., Trexler, M. B., Olsson, S., Findlay, R. H., Odham, G., and White, D. C. (1985) Determination of phospholipid ester-linked fatty acids and Poly-beta-hydroxybutarate for the estimation of bacterial biomass and activity in the rhizosphere of the rape plant *Brassica napus* (L.). *Can. J. Microbiol.* **13,** 1113–1119.
51. Sikkema, J., deBont, J. A. M., and Poolman, B. (1995) Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **59,** 210–222.
52. Heipieper, H. J., Diffenbach, J. B., and Kewelob, H. (1991) Microbial respiratory quinones in the environment: a sensitive liquid chromatographic method. *J. Microbiol. Methods* **5,** 243–254.
53. Pinkart, H. C., Wolfram, J. W., Rogers, R., and White, D. C. (1996) Cell envelope changes in solvent-tolerant and solvent-sensitive Pseudomonas putida strains following exposure to O-xylene. *Appl. Environ. Microbiol.* **62,** 1129–1132.
54. Rainey, P. B., Bailey, M. J., and Thompson, I. P. (1994) Phenotypic and genotypic diversity of fluorescent pseudomonads isolated from field grown sugar beet. *Microbiology* **140,** 2315–2331.
55. Frostegård, A. and Baath, E. (1996) The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol Fertl. Soils* **22,** 59–65.
56. Frostegård, A., Tunlid, A., and Baath, E. (1996) Changes in microbial community structure during long-term incubation in two soils experimentally contaminated with metals. *Soil Biol. Biochem.* **28,** 55–63.
57. Olsson, P. A., Baath, E., Jakobsen, I., and Soderstrom, B. (1996) Soil bacteria respond to presence of roots but not to mycelium of arbuscular fungi. *Soil Biol. Biochem.* **28,** 463–470.
58. Olsson, P. A., Baath, E., Jakobsen, I., and Soderstrom, B. (1995) The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. *Mycol. Res.* **99,** 623–629.
59. White, D. C., and Findlay, R. H. (1988) Biochemical markers for measurement of predation effects on the biomass, community structure, nutritional status and metabolic activity of microbial biofilms. *Hydrobiologica* **159,** 119–132.
60. Franzmann, P. D., Patterson, B. M., Power, T. R., Nichols, P. D., and Davis, G. B. (1996) Microbial biomass in a shallow, urban aquifer contaminated with aromatic hydrocarbons: analysis by phospholipid fatty acid content and composition. *J. Appl. Bacteriol.* **80,** 617–625.

61. Heipierer, H. J., Meulenbeld, G., van Oirschot, Q., and deBont, J. A. M. (1996) Effect of environmental factors on the trans/cis ratio of unsaturated fatty acids in Pseudomonas putida S12. *Appl. Environ. Microbiol.* **62,** 2773–2777.
62. Pennanen, T., Frostegard, A., Fritze, H., and Baath, E. (1996) Phospholipid fatty acid composition of soil microbial communities along two heavy metal polluted gradients in coniferous forests. *Appl. Environ. Microbiol.* **62,** 420–428.
63. Baath, E., Frostegård, A., Pennanen, T., and Fritze, H. (1995) Microbial community structure and pH response in relation to soil organic matter quality in wood ash fertilised, clear-cut or burned coniferous forest soils. *Soil Biol. Biochem.* **27,** 229–240.
64. Zelles, L., Bai, Q. Y., Rackwitz, R., Chadwick, D., and Beese, F. (1995) Determination of phospholipid and lipopolysaccharide derived fatty acids as an estimate of microbial biomass and community structures in soils. *Biol. Fertil. Soils* **19,** 115–123.
65. Jordan, D., Kremer, R. J., Bergfield, W. A., Kim, K. Y., and Cacnio, V. N. (1995) Evaluation of microbial methods as potential indicators of soil quality in historical agricultural fields. *Biol. Fertil. Soils* **19,** 297–302.
66. Wander, M. M., Hedrick, D. S., Kaufman, D., Traina, S. J., Stinner, B. R., Kehrmeyer, S. R., and White, D. C. (1995) The functional significance of the microbial biomass in organic and conventionally managed soils. *Plant Soil* 1**70,** 87–97.
67. Bardgett, R. D., Hobbs, P. J., and Frostegård, A. (1996) Changes in soil fungal-bacterial biomass ratios following reductions in the intensity of management of an upland grassland. *Biol. Fertil. Soils* **22,** 261–264.
68. Rajendran, N., Matsuda, O., Imamura, N., and Urushigawa, Y. (1992) Variation in microbial biomass and community structure in sediments of eutrophic bays as determined by phospholipid ester linked fatty acids. *Appl. Environ. Microbiol.* **58,** 562–571.
69. Parkes, R. J., Dowling, N. J. E., White, D. C., Herbert, R. A., and Gibson, G. R. (1993) Characterisation of sulphate-reducing bacterial populations within marine and estuarine sediments with different rates of sulphate reduction. *FEMS Microbiol. Ecol.* 1**02,** 235–250.
70. Rajendran, N., Matsuda, O., Urushigawa, Y., and Simidu, U. (1994) Characterisation of microbial community structure in the subsurface sediment of Osaka Bay, Japan, by phospholipid fatty acids lipid analysis. *Appl. Environ. Microbiol.* **60,** 248–257.
71. Kieft, T. L., Ringelberg, D. B., and White, D. C. (1994) Changes id ester linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous medium. *Appl. Environ. Microbiol.* **60,** 3292–3299.
72. Guezenne, J. and Fiala-Medioni, A. (1996) Bacterial abundance and diversity in the Barbados Trench determined by phospholipid analysis. *FEMS Microbiol. Ecol.* **19,** 83–93.
73. Amy, P. A., Halderman, D. L. Ringelberg, D., and White, D. C. (1994) Changes in bacterial recoverable from subsurface volcanic rock samples during storage at 4°C. *Appl. Environ. Microbiol.* **60,** 2679–2703.

74. Hirsch, P., Eckhardt, F. E. W., and Palmer, R. J. (1995) Methods for the study of rock-inhabiting micro-organisms: a review. *J. Microbiol. Methods* **23,** 143–167.
75. Brink, D. E, Vance, I., and White, D. C. (1994) Detection of *Desulfobacter* in oil field environments by non-radioactive DNA probes. *Appl. Microbiol. Biotechnol.* **42,** 469–475.
76. Sundh, I., Borga, P., Nilsson, M., and Svensson, B. H. (1996) Estimation of cell numbers of methanogenic bacteria in boreal peatlands based on analysis of specific phospholipid fatty acids. *FEMS Microbiol. Ecol.* **18,** 103–112.
77. Haldeman, D. L., Amy, P. S., Ringelberg, D., White, D. C., Garen R. E., and Ghiorse W. C. (1995) Microbial growth and resuscitation alter community structure after perturbation. *FEMS Microbiol. Ecol.* **17,** 27–38.
78. Lehman, R. M., Colwell, F. S., Ringelberg, D. B., and White, D. C. (1995) Microbial community level analysis based on patterns of carbon source utilization and phospholipid fatty acid profiles for quality assurance of terrestrial subsurface cores. *J. Microbiol. Methods* **22,** 263–281.
79. Leff, L. G., Kernan R. M., McArthur, J. V., and Skimkets, L. J (1995) Identification of aquatic Burkholderia (Pseudomonas) cepacia by hybridisation with species-specific rRNA gene probes. *Appl. Environ. Microbiol.* **61,** 1634–1636.
80. Brown, B. J., and Leff, L. G. (1996) Comparison of fatty acid methyl ester analysis with use of API 20NE and NFT strips for identification of aquatic bacteria. *Appl. Environ. Microbiol.* **62,** 2183–2185.
81. Mergaert, J., Webb, A., Anderson, C., Wouters, A., and Swings, J. (1993) Microbial degradation of poly-3-hydroxybutyrate. *Appl. Environ. Microbiol.* **59,** 3233–3238.
82. Ka, J. O, Holben, W. E., and Tiedje, J. M. (1994) Genetic and phenotypic diversity of **2,**4-dichlorophenoxyacetic acid (**2,**4-D) degrading bacteria isolated from **2,**4-D treated field soils. *Appl. Environ. Microbiol.* **60,** 1106–1115.
83. Tonso, N. L., Matheson, V. G., and Holben, W. E. (1995) Polyphasis chracterisation of a suite of bacterial isolates capable of degrading **2,** 4-D. *Microb. Ecol.* **30,** 3–24.
84 Thompson, I. P., Bailey, M. J., Ellis, R. J. & Purdy, K. J. (1993) Sub-grouping of bacterial populations by cellular fatty acid composition. FEMS Microbiol. Ecol. **12,** 75–84.
85. Kloepper, J. W., McInroy, J. A., and Bowen, K. L. (1992) Comparative identification by fatty-acid analysis of soil, rhizosphere, and geocarposphere bacteria of peanut (*Arachis-hypogaea* L). *Plant Soil* **139,** 85–90.
86. Lilley, A. K., Fry, J. C., Bailey, M. J., and Day, M. J. (1996) Comparison of aerobic heterotrophic taxa isolated from four root domains of mature sugar beet (*Beta vulgaris*). *FEMS Microbiol. Ecol.* **21,** 231–242.
87. Foster, J. L. M and Fogelman, J. C. (1993) Identification and ecology of bacterial communities associated with necroses of three cactus species. *Appl. Environ. Microbiol.* **59,** 1–6.
88. Foster, J. L. M., and Fogelman, J. C. (1994) Bacterial succession in necrotic tissue of agria cactus (*Strenocereus gummosus*). *Appl. Environ. Microbiol.* **60,** 619–625.

89. Frachon, E., Hamon, S., Nicolas, L., and de Barjac, H. (1991) Cellular fatty acid analysis as a potential tool for predicting mosquitocidal activity of *Bacillus sphaericus* strains. *Appl. Environ. Microbiol.* **57,** 3394–3398.
90. Ndowora, T. C. R., Kinkel, L. L., Jones, R. K., and Anderson, N. A. (1995) Fatty acid analysis of pathogenic and suppressive strains of Streptomyces species isolated in Minnesota. *Phytopathology* **86,** 138–143.
91. Thompson, I. P., Ellis, R. J., and Bailey, M. J. (1993) Autecology of a genetically modified fluorescent pseudomonad on sugar beet. *FEMS Microbiol. Ecol.* **17,** 1–14.

15

Fluorescent *In Situ* Hybridization and the Analysis of the Single Cell

Anthony G. O'Donnell and Andrew S. Whiteley

1. Introduction

1.1. Overview of the Method

The discovery that prokaryotic and eukaryotic cells could be made permeable to fluorescently labeled, sequence specific oligonucleotides makes possible the determinative probing of intact microbial cells ***(1)***. Thus, individual target cells can be identified and enumerated in heterogeneous populations (or even when present as endosymbionts of other organisms ***(2)*** without the need for direct isolation and culture of the organisms of interest. In microbial ecology, the primary targets for such procedures, referred to collectively as fluorescent *in situ* hybridization (FISH) techniques, have been the ribosomal RNAs (rRNAs). The rRNAs have proved exceptionally good targets for determinative probes for several reasons. First of all, despite being highly conserved biopolymers owing to their role in protein synthesis, they also exhibit regions of marked sequence variability. Thus, the rRNAs can be considered as mosaics of highly conserved and highly variable sequence. Regions of highly conserved sequence have remained virtually unchanged throughout evolution and provide ideal targets for so-called universal or consensus probes and for probes directed at higher levels of taxonomic rank. The variable regions, on the other hand, have evolved more rapidly and can be used to differentiate among species or even subspecies of bacteria. A second advantage is that they are present in high copy numbers in active cells (1000–10,000 ribosomes per cell) ***(3)***, thereby increasing the sensitivity of direct determinative examinations. A third advantage is associated with the generation of rDNA sequence in that sequencing of the rDNA operons, primarily the 16S and 18S rDNA (1.6 and 1.8-kb,

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respectively), is relatively straightforward and yields a great deal of information for evaluating phylogenetic relationships among organisms as well as for FISH probe design. In addition, sequence generation, and hence probe design, is applicable to both cultured and "unculturable" taxa using standard gene amplification, cloning and sequencing strategies.

Whole cell *in situ* hybridization with fluorescently labeled oligonucleotide probes was first used in microbial ecology in the late 1980s by De Long and colleagues ***(1)***. Since then, FISH procedures have become widely used in the analysis of microbial communities in natural environments, and an excellent review of their application in the detection and phylogenetic characterization of individual microbial cells is provided by Amann et al. ***(4)***.

1.2. FISH: Limitations and Potential Solutions

Although FISH techniques are relatively easy to perform and can be used to provide useful determinative information on microorganisms in natural environments, several methodological constraints do exist. As previously described, permeabilization remains an important limitation and whether cells will permeabilize cannot be easily predicted. Furthermore, it is has been shown that cells growing in natural environments such as soils may exhibit different permeabilities to oligonucleotide probes than those grown under laboratory conditions. Macnaughton et al. ***(5)*** have shown using *Rhodococcus fasciens*–infected root sections that although permeabilization by mild acid hydrolysis prior to FISH was necessary for successful probing in the laboratory, it was not needed for samples located at the surface of a growing root. Similar findings were reported by Hahn et al. ***(6)***, who demonstrated that the enzymatic permeabilization pretreatment used successfully to probe *Streptomyces scabies* in pure culture was not required to permeabilize the same organism when it was grown in nutrient-amended soil.

Even though cell permeabilization is assured, there is no guarantee that probe hybridization to rRNA within the cell will occur. This is thought to be owing to the target sequence being inaccessible because of strong interactions with ribosomal proteins or to highly stable secondary structure elements within the rRNA ***(7)***. The latter should be suspected if a strong hybridization signal can be obtained with a universal probe that is known to target a different, accessible site on the rRNA molecule. A list of successfully targeted sites for rRNA-directed FISH is available in an excellent review by Amann et al ***(4)***.

It is also important to consider the sensitivity of FISH techniques when used to study microorganisms in natural environments. Probes carrying a single fluorochrome will only give a strong signal if the cells are actively metabolizing. This makes such probes ideal for studying laboratory cultures, but for natural environments in which oligotrophic conditions prevail, alternative detection

strategies may be required. A number of approaches have been proposed to improve the sensitivity of *in situ* hybridization techniques, including the use of more than one probe in a single cell ***(8,9)***, the use of detection systems that allow for enzymatic signal amplification or enzymatic production of reaction products that allow discrimination of probe binding against background fluorescence ***(11)***. Another problem with the use of FISH approaches in natural environments is that even though the target cells are active, they may represent only a relatively small fraction of the indigenous population, making it necessary to search large numbers of microscopic fields in order to locate the target organisms. Realistically, this limits microscopic analysis of target populations to organisms present at more than 10^4 –10^5 mL^{-1}. Such problems might be overcome by cell sorting using flow cytometry (*see* Chapter 5) or by some form of enrichment prior to analysis.

With greater availability of sequences from diverse organisms, the problem of probe specificity and the design of diagnostic probes is becoming increasingly difficult; this has always been a potential limitation to the widespread application of FISH. As for all oligonucleotide-dependent techniques—not only for FISH—the probability of a specific 18-mer probe or primer targeting a variable region in an unrelated organism is $1:4^{18}$. However, even in variable regions the likelihood of finding 18 variable positions is limited, and it is more likely that the probe differs in only four or five base positions. Under these circumstances, the likelihood of encountering the same sequence in an unrelated organism falls to $1:4^5$ (for five base differences). It has been proposed that FISH techniques can be made more specific by using multiple specific probes, labeled with different fluorochromes and targeting different sites on the rRNA molecule. Thus, although a single oligonucleotide target sequence may be found in several related taxa, the probability is much lower that target sites for several specifically designed oligonucleotides are present in nontarget organisms.

Brock ***(25)***, in his 1966 book the *Principles of Microbial Ecology*, stated that "the cell is small and therefore its environment is also small." If one accepts this view, then to understand the role of microorganisms in their natural environments, one needs to study microbial cells at scales appropriate to their size and to the microniches they influence. This idea was reiterated by Brock over 20 yr later when he identified that a major factor limiting the use of microscopic studies in ecology is the difficulty in identifying microorganisms *in situ* and in using the microscope to measure cell activity. In the last 10 yr, the development of molecular microbial ecology and the introduction of FISH and cytochemical staining procedures have helped to resolve these problems. It is expected that over the next decade microbial ecologists will continue to harness advances in molecular biology and to use these developments to study, at the correct spatial scales, important, microbially mediated ecologic processes.

2. Materials

2.1. General

1. All reagents were obtained from Sigma (Poole, Dorset, UK) unless stated otherwise.
2. Fluorescence microscope with appropriate filter blocks.

2.2. Cell Fixation

Paraformaldehyde fixing solution is freshly made to a final concentration of 4% in phosphate buffered saline (PBS): 8 g NaC1, 0.24 g KH_2PO_4, 0.2 g KCl, 1.44 g Na_2HPO_4 dissolved in 800 mL distilled water, pH 7.4, with HCl and made to 1000 mL).

2.3. Cell Permeabilization

Cell permeabilization was carried out using a graded series (in water) of ethanol at 50%, 80%, and absolute.

2.4. Cell hybridization

Hybridization buffer (*see* **Subheading 3.4.1.**). Wash buffer: 0.9 *M* NaCl, 20 m*M* Tris HCl, pH 7.4, 0.1% v/v SDS. Negative controls treated with RNase I prior to probing.

2.5. Fluorescently Labeled Oligonucleotide Probes

Oligonucleotide probes were supplied 5' labeled with fluorescein or rhodamine from GENOSYS Biotechnologies, Cambridge, UK.

2.6. Fluorescence Microscopy

Standard fluorescence immersion oil and mountant.

2.7. Cytochemical Staining

2,3,5-tri-*p*-nitrophenyl-2H-tetrazolium chloride (TNTTC) 15 m*M* stock diluted to 1-5 m*M* synthesized by E. Seidler. Commercially available tetrazolium salts, 15 m*M* diluted to 1.5 m*M*, provided by Sigma

3. Methods

Fig. 1 provides a general overview of the approach used. Briefly, cells are fixed and made permeable by treatment with paraformaldehyde and detergents or alcohol. This helps to maintain the morphologic integrity of the cells, allows for passage of the probe through the cell envelope, and crosslinks the target rRNA, making it accessible to probe binding. Once fixed, cells are attached to gelatin or 3-aminopropyltriethoxysilane (APS) coated microscope slides and dehydrated prior to hybridization with a fluorescently labeled oligonucleotide

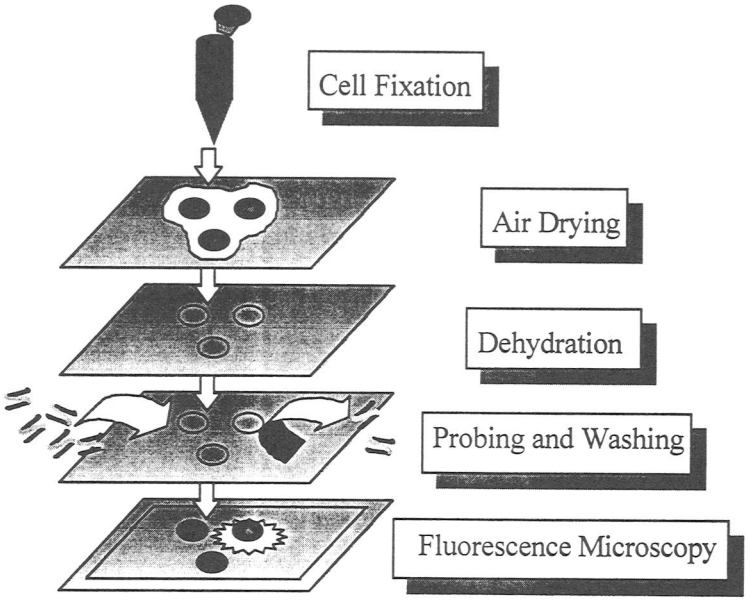

Fig. 1. Schematic diagram of the basic procedures for FISH protocols.

probe (15–25 nucleotides in length). Alternatively, fixed cells can be hybridized in suspension and then immobilized on slides prior to microscopic examination, or analyzed by flow cytometric techniques directly from suspension hybridizations (*see* Chapter 5).

3.1. Preparation of Gelatin-Coated Slides

1. WEARING GLOVES, prepare fresh wash solution by dissolving 50 g of KOH in 95% ethanol (500 mL).
2. Leave slides in the KOH/ethanol solution for 1 h.
3. Remove the slides and wash in distilled H_2O (×3). Place rack on filter paper and allow slides to air dry.
4. Dissolve gelatin (0.1% w/v) and KCr $(SO_4)_2$ (0.01% w/v) in 500 mL of hot distilled water. Keep dissolved gelatin in a glass beaker in a water bath at 70°C (*see* **Note 1**).
5. Put the slides into the gelatin for 1 min lifting up and down gently to coat them. Remove the slides and allow them to dry for 5 min. Repeat this four times. Allow the slides to air dry, and store them in the dark in a dust-free box prior to use.

3.2. Preparation of APS-Coated Slides

APS reacts with free hydroxyl groups on the glass surface, and the resultant covalently coated surface carries a positive charge at physiological pH (bacte-

rial cells tend to be negatively charged at this pH). APS treatment also leaves the surface more hydrophobic than the original glass. Bacterial cells can be deposited on the slide using either air drying or a cytospin apparatus.

1. Clean microscope slides by immersion in acetone, then distilled water and blot with tissue.
2. Lay the slides flat in a suitable container (glass dish or similar), and, working in a chemical fume hood, immerse the slides in a freshly prepared 2% (v/v) solution of APS in acetone, and leave at room temperature for between 16 and 24 h (*see* **Note 2**).
3. Remove the APS/acetone coating solution, and immerse the slides in acetone for 5 min to remove excess APS.
4. Remove the acetone and individually wash the slides by sequentially immersing them in two distilled water washes. Note that care must be taken to identify the upper (coated) surface (*see* **Note 3**).
5. Drain the slides and gently blot with tissue paper. Leave at 37°C for 1 h to air dry.
6. Store the slides for up to 1 mo in a clean, dry Petri dish (*see* **Note 4**).

3.3. Cell Permeabilization and Fixation

3.3.1. Paraformaldehyde Treatment

A good and widely utilized procedure that has proven to be reliable, particularly when permeabilizing Gram-negative bacteria, is based on that originally proposed by De Long et al. ***(1)***. Given that the intensity of fluorescence corresponds with the cellular rRNA content ***(12)***, FISH procedures are best carried out on actively growing, log-phase cells; however, this cannot be guaranteed for probings within natural assemblages.

1. Centrifuge cells (*see* **Note 5**) in a microfuge and resuspend in 0.4 mL, 0.1% (w/v) Nonidet P-40. Spin down and resuspend in 0.3 µL of PBS (*see* **Note 6**). Make up fresh a solution of 4% paraformaldehyde in PBS. Allow to cool and add 0.1 µL of the solution to the cells. Fix for between 3 and 16 h (overnight) at 4°C (*see* **Note 7**).
2. Centrifuge cells and resuspend in Nonidet P-40 (0.1% w/v) so that there are approx 10^4–10^5 cells in 5 µL of solution. Spot 5 µL onto a gelatin-subbed or APS-treated slide (*see* **Note 8**).
3. Allow to dry, then dehydrate using ethanol:H_2O (50% for 3 min, 80% for 3 min, and 96% for 3 min). Allow to dry (*see* **Note 9**).

3.3.2. Acid Pretreatment

Fixation using 4% paraformaldehyde and/or ethanol is the commonly used method for permeabilizing microbial cells and stabilizing rRNA prior to hybridization with fluorochrome-labeled oligonucleotides ***(4–6,8,12–15)***. Nevertheless, the failure to permeabilize many Gram-positive cells has been well documented ***(6,11,16,17)***. Attempts have been made to provide a general

method for permeabilizing all cell types including those with highly hydrophobic cell envelopes such as the actinomycetes . However, differences in cell wall structure between Gram-positive and Gram-negative organisms make it unlikely that any single method capable of permeabilizing all organisms can be easily achieved. The method described next is from Macnaughton et al. ***(17)*** and was proposed for the pretreatment of selected mycolic acid containing actinomycetes.

1. Use exponential phase cells for optimum results.
2. Immobilize cells on gelatin-subbed slides and dehydrate by immersion in ethanol (50, 80, and 95%, v/v) as described above.
3. Allow to air dry, then immerse slides in 1 *M* HCl at 37°C for between 30 and 50 min (for mycolic acid containing actinomycetes) (*see* **Notes 10** and **11**).
4. Wash three times in distilled water and air dry prior to fixation in ethanol/paraformaldehyde (**Subheading 3.3.1.**).

3.4. Hybridization

Once cells have been fixed and permeabilized, they are ready to be hybridized. Hybridization needs to be carried out in a securely sealed chamber to prevent loss of the hybridization buffer through evaporation. If the buffer evaporates and the preparation is allowed to dry out, then significant nonspecific binding will result. We routinely use a small airtight sandwich box as a hybridization chamber, but a 50-mL polypropylene tube can also be used.

3.4.1. Preparation of Hybridization Buffer

1. Hybridization buffer: 1.8 mL of 5 *M* NaCl, 0.2 mL of 100 mg/mL Ficoll, 0.8 mL of 25 mg/mL polyvinylpyrolidone, 0.4 mL of 50 mg/mL bovine serum albuorin, 1.0 mL of poly A (10 mg/mL), 2.5 mL of 0.2 *M* phosphate buffer, 0.5 mL of 20% (w/v) SDS, 0.1 mL of 0.5 *M* EDTA, 0.77 mL of formamide, 1.93 mL of H_2O for a total of 10.0 mL. Filter sterilize each of these components except the poly A. Store in 1-mL aliquots at –20°C until needed.
2. Spot 9 µL of hybridization buffer onto the fixed cell preparation (*see* **Note 13**) taking care to avoid air bubbles (*see* **Note 14**). Add 1 µL of probe solution (50 ng/µL of each probe prepared in sterile distilled water). Carefully place a coverslip onto the cells.
3. Line the incubation chamber (an airtight sandwich box) with Whatmann 3**M** filter paper and wet with 0.9 *M* NaCl. Place chamber in a water bath at 37°C and equilibrate for 30 min. Pour off excess salt solution. Place slides into chamber. Leave to hybridize for between 2–16 h (overnight) at a hybridization temperature appropriate for the probe (*see* **Notes 15** and **16**).
4. Prior to microscopic analysis, wash slides thoroughly at the hybridization temperature using 0.1% SDS and 20 m*M* Tris-HCl (pH 7.2) in 0.9 *M* NaCl wash buffer (wash stringency can be modified by lowering the NaCl concentration,

e.g., *see* **ref. *18***). This is most easily done using a washing chamber immersed in a water bath set at the hybridization temperature. Cover slips should float off. Remove slides from buffer and wash in distilled H_2O (×3). Blot slides on filter paper and allow to air dry. Mount in appropriate mountant (e.g., Citifluor™, Agar Scientific, Stansted, UK), and store at 4°C in the dark prior to examination by epifluorescence microscopy (*see* **Notes 17** and **18**).

3.5. Microscopic Analysis

Following hybridization each slide is mounted in antifadent (Citifluor) and examined using an epifluorescence microscope fitted with the required filter sets (e.g., fluorescein has an absorbance at 490 nm and emission wavelength of 520 nm whereas rhodamine has an absorbance at 550 nm and an emission at 610 nm). Fluorescein is the more sensitive stain and provides lower detection limits. However, it is also more prone to bleaching and can be difficult to detect against background fluorescence. For these reasons, it is good practice first to examine samples using phase contrast and to switch to epifluorescence only when the sample is located. Using epifluorescence microscopy to locate cells of interest can lead to premature bleaching and to a loss in fluorescence intensity below background levels. Similar precautions should be taken when using rhodamine.

For more detailed quantitative analysis of a range of fluorochromes and variations in their intensity, we recommend using a microscope with associated image analysis capabilities. We routinely use an inverted microscope that provides a stable platform for physiologic experiments, which can be coupled with FISH experiments ***(19)***, and there is no need to change the light path from the objective to the detector to obtain phase contrast, bright-field, or fluorescence images, a problem that can cause image misalignment when taking multiple exposures on conventional microscopes. Several systems are available for image processing, and a detailed discussion of the equipment, procedures, and protocols can be found in Whiteley et al. ***(20)***.

3.6. Colocalization of Phenotypic and Genotypic Characteristics in Individual Bacterial Cells

Cytochemical procedures combining tetrazolium salts with or without exogenous oxidizable substrates (substrate-enhanced reactions) can be used to study the pattern of oxidative metabolism in individual cells without the need to culture them ***(21)***. This has obvious implications for the analysis of microbial communities in many natural environments in which most of the organisms remain largely uncultured. However, to exploit the potential of this cytochemical information and to apply it to environmental samples requires clear determinative information on the individual cells present. Such informa-

tion is now readily available by combining FISH techniques with substrate-enhanced tetrazolium reduction since both methods have a common goal: the description of a cellular characteristic at the single-cell level without the need for culture. Thus, the substrate-enhanced tetrazolium reduction is used to assign a phenotype, since actively respiring cells convert tetrazolium salts to insoluble cell-localized precipitates, whereas the rRNA probing techniques (FISH) are used to colocate genotypes ***(19)***. An overview of the experimental protocol for this type of analysis, applicable to natural communities to assess respiratory activity within rRNA-probed cells, is provided next, utilizing the tetrazolium salt "TNTTC," a typical analysis is shown in **Fig. 2**. Protocols for more in-depth single-cell physiologic analyses using cytochemical techniques coupled with FISH is provided in Whiteley et al. ***(20)***.

1. Add 100 µL of 10 m*M* TNTTC (*see* **Note 19**) to 900 µL of cells (approx 10^6–10^7 cells/mL). Cells can be concentrated by filtration or centrifugation prior to analysis if cell concentration is low.
2. Incubate cells for up to 4 h with TNTTC (*see* **Notes 20** and **21**) at a temperature appropriate for the conditions from which the cells were derived.
3. Fix the reacted cells with 3 vol of freshly prepared 4% paraformaldehyde and proceed as in **Section 3.4.** for FISH protocols (*see* **Note 22**).

4. Notes

1. The gelatin is best added to the $KCr(SO_4)_2$/distilled water rather than adding the solution to the gelatin.
2. We routinely store the undiluted APS stock solution at 4°C for a maximum of 1 mo, because we find that activity declines rapidly with longer storage periods.
3. Mark the coated surface of the slide for subsequent analysis (i.e., the side facing upward in the acetone/APS solution). Marking the top left-hand corner with an indelible pen serves as a good reference.
4. Microscope slides or cover slips can be coated using the APS method. Cover slips tend to be used for physiological measurements of immobilized bacteria on inverted microscopes where the cover slip provides a thin focal pathway through which to view the cells from below while solutions can be placed over the cells above. APS has the advantage over gelatin for this procedure in that cells do not have to be dried to the slide but can be efficiently deposited by centrifugation onto the cover slip within a liquid medium.
5. To facilitate subsequent microscopic examination, we usually try to ensure that our starting cell concentration is about 10^8 cells · mL^{-1}. This can be determined by counting cells in a hemocytometer.
6. Note that when preparing the 4% paraformaldehyde in PBS, the paraformaldehyde dissolves only at between 70 and 80°C with stirring. Addition of a few drops of concentrated sodium hydroxide (2M) will aid the dissolution of the paraformaldehyde.

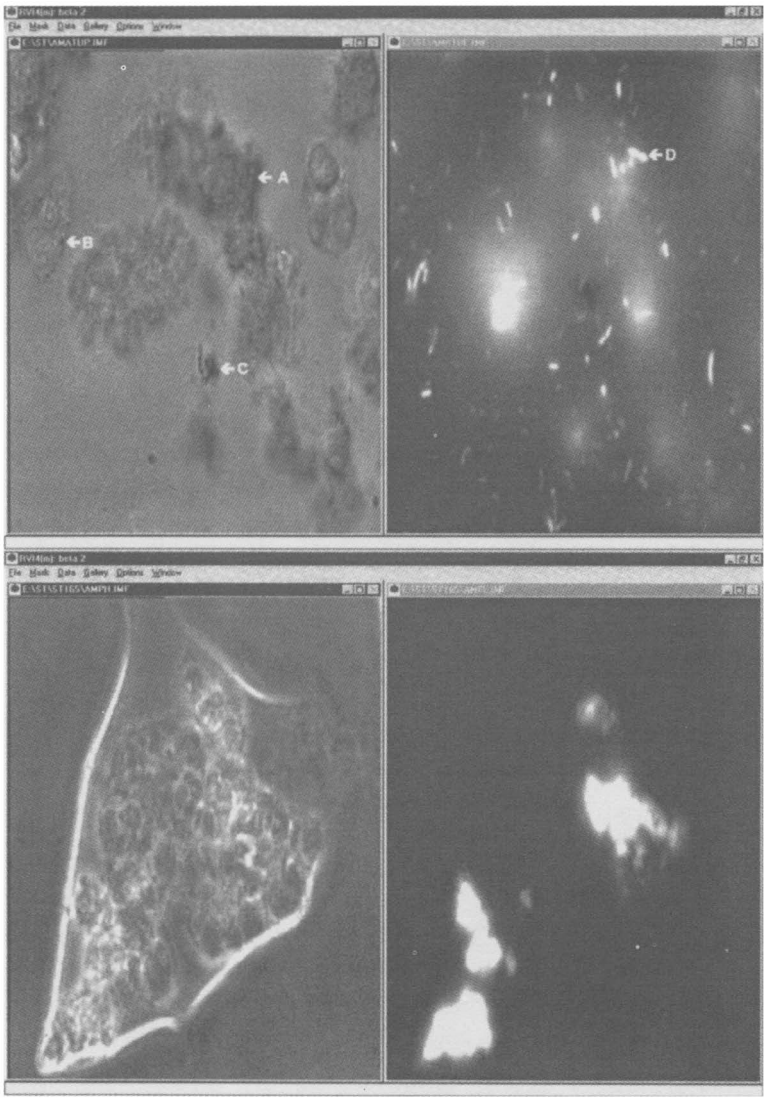

Fig. 2. Colocalization of TNTTC with rRNA probing as part of the FISH analyses of ammonia-oxidizer communities. **(Top left)** Ammonia-oxidizer communities reacted with TNTTC and viewed under bright-field microscopy. Note the distinct ammonia-oxidizer colonies **(A)** and the tetrazolium deposition indicating strong respiratory activity **(B)** and cells with an extremely high-respiratory activity **(C)**. **(Top right)** The same field as top left but viewed with epifluorescence microscopy after probing with EUB338, a eubacterial-specific probe. Note the diffuse fluorescence in the background owing to the depth of field and size of the ammonia-oxidizer colonies, indicating the

7. Prolonged storage in fixative reduces the quality and strength of the fluorescent signal. Fixed cells may be stored for up to 8 wk with only slight deterioration of signal ***(22)***. Glutaraldehyde or formaldehyde as a substitute for paraformaldehyde is a poorer choice since a high level of autofluorescence can be observed.
8. We routinely spot two samples for probing per conventional microscope slide however specific slides are available that allow up to 15 separate samples to be processed on one slide (e.g., ICN Chemicals, OH: Multitest slides).
9. Note that for some cells, fixation in 1% paraformaldehyde may not be necessary and that cells can be prepared for FISH simply by ethanol dehydration. Indeed, Braun-Howland et al. ***(23)*** suggest that some organisms (*Staphylococcus aureus, Micrococcus luteus*) may even respond adversely to fixation in 1% paraformaldehyde, resulting in lower *in situ* fluorescence when probed.
10. The length of treatment time in 1 *M* HCl differs according to the cell envelope structure of the organisms being probed. Mycolic acid containing organisms generally required between 30 and 50 min hydrolysis whereas cells such as *Bacillus subtilis* were rendered permeable in only 10 min.
11. For most of the actinomycetes tested, fluorescence intensity increased as the time the cells were immersed in 1 *M* HCl increased, and only decreased when immersion exceeded 50 min. A possible explanation for the decreased intensity is that longer exposure to acid caused degradation of the target rRNA.
12. The success of the procedures described in **Notes 10** and **11** seems to depend, to some extent, on the chain length of the mycolic acids. Organisms such as *Tsukamurella*, *Gordona*, and *Nocardia* in which mycolic acid chain lengths range from 47–76 carbon atoms, could not be permeabilized. However, *Mycobacterium fortuitum* and *Nocardia asteroides*, which possess longer chain mycolates (60–90 carbons and 46–60 carbons, respectively), were moderately permeable, suggesting that chain length alone does not explain the differences between cells. Strains of *Lactobacillus plantarum* could not be permeabilized using any of the procedures described.
13. When applying the hybridization buffer and the probe, it is useful to have marked previously the face of the slide to which the cells were added.
14. If bubbles do form when applying the hybridization buffer, they can be removed by "popping" with a fine syringe needle.
15. The optimal temperature for hybridization is a function of the base composition of the probe (dissociation temperature T_d) and the complementary target

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large colonies to be eubacterial in origin, as well as the presence of smaller cells, also eubacterial in origin **(D)** and closely associated with the large ammonia-oxidizer colonies. **(Bottom left)** The large colonies (phase contrast) were subsequently probed with a probe specific for ammonia oxidizers, assigning these organisms to this specific group **(bottom right)**. Note, however, the heterogeneous distribution of fluorescence within the colony, possibly indicating localized areas of cells with high ribosome content, or limited probe accessibility within the dense colony.

sequence. This should be determined empirically to minimize any nonspecific binding of the probe owing to mismatches between the probe and its target sequence ***(22)***. A rough guide for T_d calculation is 2°C for every A or T and 4°C for every G or C base within the oligonucleotide. An alternative to changing the temperature of hybridization, or if the hybridization temperature is too high based on the probe dissociation temperature (>55°C), is the addition of formamide in the hybridization buffer. Since formamide is a strong denaturant, it will disrupt higher-order structures within the rRNA molecule (e.g., hairpin loops) and increase probe accessibility to sequences while also allowing a reduction in the required hybridization temperature. In general, the addition of 1% formamide accounts for a 0.7°C reduction in hybridization temperature. A comprehensive discussion of methods for optimizing hybridization conditions is provided by Amann et al. (1995) ***(4)***.

16. Although procedures are available for the fluorescent labeling of oligonucleotide probes, these are often best obtained from commercial suppliers already labeled. To end-label probes an aminohexyl linker (Amino-Link 2, Applied Biosystems, Foster City, CA) is incorporated into the oligonucleotide at the 5' end during synthesis. The fluorescent dye (for microbial studies, this is usually fluorescein-isothiocyanate hydrochloride or tetramethyl-6-carboxy-rhodamine) is then reacted with the primary amino group according to the manufacturer's protocols. Any unincorporated fluorochrome is removed from labeled oligonucleotide using an oligonucleotide purification cartridge. Full-length, labeled oligonucleotides are then purified by thin-layer chromatography using a Sure-Pure oligonucleotide purification plate (Cambridge BioScience, Cambridge, UK). Labeled probes can then be dispensed and stored in sterile deionized water at –20°C until needed. We have kept probes prepared in this way for up to 2 yr.
17. When using FISH techniques, it is vital that proper controls be included since nonspecific binding and autofluorescence are common. We routinely include, in our analysis, both a positive and a negative control. For the positive control, if probing bacteria, we use the eubacterial probe EUB 338 (5'-GCTGCCTCCCGTAGGAGT- 3') to determine whether cells are permeabilized and suitable for probing. We also include in the analysis a cell preparation known to be permeable and one that will hybridize under the chosen reaction conditions. As negative controls, we use a probe with the same sequence as the target rRNA (no complementarity, therefore no binding) as well as fixed cells pretreated with RNase I (100 $\mu g \cdot mL^{-1}$, 1 h, 37°C) to assess nonspecific probe binding to other cellular constituents. For natural sample work, counterstaining of the bacterial cells with DNA-specific fluorochromes that have emission wavelengths outside those of the labeled oligonucleotides provide a means of locating cells within the field of view and, more important, provide a total cell count to which the fraction of probe-positive cells can be compared. The use of DNA-specific fluorochromes for locating cells becomes essential if oligonucleotide probing is performed on polycarbonate or cellulose nitrate filters ***(24)***.
18. We recommend examining preparations immediately, but when this is not possible slides can be stored at 4°C in the dark for 4–6 wk.

19. In our original paper we used INT (*p*-iodonitrotetrazolium violet)-formazan to measure substrate-enhanced reduction. However, INT-formazan and several other formazans, including CTC-formazan, are extracted by the ethanol used to dehydrate cells prior to FISH. This makes colocalization more difficult and requires image analysis to locate and record immobilized cells since the formazan is no longer deposited in the cells when they are subsequently probed using FISH. The use of TNTTC (which is not soluble in ethanol) or alternative permeabilization and dehydration strategies (possibly using reagents such as cold lysozyme treatment and polyethylene glycol) could circumvent this problem when using INT. Currently, TNTTC is not commercially available, and, therefore, its use is limited because it must be chemically synthesized.
20. Exogenous substrates can be added during the incubation with TNTTC to assess substrate enhanced tetrazolium reduction. In parallel, controls with only TNTTC addition should also be prepared.
21. The incubation period is critical to the analyses. Rapidly growing cells may only require an incubation period of the order of min before substantial deposition is observed. For more slowly growing cultures, or natural samples, incubation periods may have to be extended up to 4 h to ensure adequate tetrazolium deposition. The incubation period should be empirically determined by removing serial samples at distinct time points and microscopically checking for tetrazolium deposition. Substantial extracellular formazan deposition generally indicates cell lysis has occurred and that the reaction has been performed for a longer period than necessary.
22. Since FISH requires that cells are fixed and permeabilized prior to probing, it must be performed after the tetrazolium reduction assay.

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References

1. De Long, E. F., Wickham, G. S., and Pace, N. R. (1989) Phylogenetic stains: ribosomal RNA based probes for the identification of single microbial cells. *Science* **243,** 1360–1363.
2. Amann, R. I., Springer, N., Ludwig, W., Gortz, H. D., and Schleifer, K. H. (1991) Identification in situ and phylogeny of uncultured bacterial endosymbionts. *Nature* 351, 161–164.
3. Giovannoni, S. J., Delong, E. F., Olsen, G. J., and Pace, N. R. (1988) Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial-cells. *J. Bacteriol.* **170,** 720–726.
4. Amann, R. I., W. Ludwig, and K. H. Schleifer. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59,** 143–169.

5. Macnaughton, S. J., Booth, T., Embley, T. M., and O'Donnell, A. G. (1996) Physical stabilization and confocal microscopy of bacteria on roots using 16S rRNA targeted, fluorescent-labeled oligonucleotide probes. *J. Microbiol. Methods*. **26,** 279–285.
6. Hahn, D., Amann, R. I., Ludwig, W., Akkermans, A. D. L., and Schleifer, K. H. (1992) Detection of microorganisms in soil after *in situ* hybridization with rRNA-targeted fluorescently-labeled oligonucleotides. *J. Gen. Microbiol.* **138,** 879–887.
7. Frischer, M. E., Floriani, P. J., and Nierzwickibauer, S. A. (1996) Differential sensitivity of 16S rRNA-targeted oligonucleotide probes used for fluorescence *in situ* hybridization is a result of ribosomal higher-order structure. *Can. J. Microbiol.* **42,** 1061–1071.
8. Amann, R. I., Krumholz, L., and Stahl, D. A. (1990) Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental-studies in microbiology. *J. Bacteriol.* **172,** 762–770.
9. Lee, S. H., Malone, C., and Kemp, P. F. (1993) Use of multiple 16S rRNA-targeted fluorescent probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. *Marine Ecol. Prog. Ser.* **101,** 193–201.
10. Schonhuber, W., Fuchs, B., Juretschko, S., and Amann, R. I. (1997) Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. *Appl. Environ. Microbiol.* **63,** 3268–3273.
11. Amann, R. I., Zarda, B., Stahl, D. A., and Schleifer, K. H. (1992). Identification of individual prokaryotic cells by using enzyme-labeled, rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* **58,** 3007–3011.
12. Poulsen, L. K., Ballard, G., and Stahl, D. A. (1993) Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. *Appl. Environ. Microbiol.* **59,** 1354–1360.
13. Jurtshuk, R. J., Blick, M., Bresser, J., Fox, G. E., and Jurtshuk, P. (1992) Rapid *in situ* hybridization technique using 16S rRNA segments for detecting and differentiating the closely related gram-positive organisms *Bacillus polymyxa* and *Bacillus macerans*. *Appl. Environ. Microbiol.* **58,** 2571–2578.
14. Wagner, M., Amann, R. I., Kampfer, P., Assmus, B., Hartmann, A., Hutzler, P., Springer, N., and Schleifer, K. H. (1994) Identification and *in situ* detection of gram negative filamentous bacteria in activated sludge. *Systematic Appl. Microbiol.* **17,** 405–417.
15. Wallner, G., Erhart, R., and Amann, R. I. (1995) Flow cytometric analysis of activated-sludge with rRNA-targeted probes. *Appl. Environ. Microbiol.* **61,** 1859–1866.
16. Hahn, D., Amann, R. I., and Zeyer, J. (1993) Whole cell hybridization of *Frankia* strains with fluorescence labeled or digoxigenin labeled 16S rRNA- targeted oligonucleotide probes. *Appl. Environ. Microbiol.* **59,** 1709–1716.
17. Macnaughton, S. J., Odonnell, A. G., and Embley, T. M. (1994) Permeabilization of mycolic acid containing actinomycetes for *in situ* hybridization with fluorescently-labeled oligonucleotide probes. *Microbiology* **140,** 2859–2865.

18. Snaidr, J., Amann, R. I., Huber, I., Ludwig, W., and Schleifer, K. H. (1997) Phylogenetic analysis and *in situ* identification of bacteria in activated sludge. *Appl. Environ. Microbiol.* **63,** 2884–2896.
19. Whiteley, A. S., Odonnell, A. G., Macnaughton, S. J., and Barer, M. R. (1996) Cytochemical colocalization and quantitation of phenotypic and genotypic characteristics in individual bacterial-cells. *Appl. Environ. Microbiol.* **62,** 1873–1879.
20. Whiteley, A. S., Hunt, A., Grewal, R., and Barer, M. R. (1998) *Digital Image Analysis of Microbes* (Wilkinson, M. H. F. and Schut, F., eds.), Wiley, New York.
21. Gribbon, L. T. and Barer, M. R. (1995) Oxidative metabolism in nonculturable *Helicobacter pylori* and *Vibrio vulnificus* cells studied by substrate-enhanced tetrazolium reduction and digital image-processing. *Appl. Environ. Microbiol.* **61,** 3379–3384.
22. Stahl, D. A. and Amman, R. I. (1991) Development and application of nucleic acid probes in bacterial systematics, in Sequencing and Hybridisation Techniques in Bacterial Systematics (Stackebrandt, E. and Goodfellow, M., eds.), John Wiley, Chichester, England.
23. Braun-Howland, E. B., Danielsen, S. A., and Nierzwickibauer, S. A. (1992) Development of a rapid method for detecting bacterial cells *in situ* using 16S rRNA-targeted probes. *Biotechniques* **13,** 928.
24. Lim, E. L., Caron, D. A., and Delong, E. F. (1996) Development and field application of a quantitative method for examining natural assemblages of protists with oligonucleotide probes. *Appl. Environ. Microbiol.* **62,** 1416–1423.
25. Brock, T. D. (1966) *Principles of Microbial Ecology.* Prentice-Hall, Englewood Cliffs, NJ.

16

Specific Detection, Viability Assessment, and Macromolecular Staining of Bacteria for Flow Cytometry

Jonathan Porter

1. Introduction

Direct analysis of bacteria from natural environments is problematic. Direct examination of samples by microscopy is an essential technique for bacteriologists, but is prone to error, is time-consuming, and can be tedious. In many situations, the process can be automated using flow cytometry (FCM) ***(1,2)***. FCM can be considered an alternative and complementary technique to microscopy, and it can also extend the range and value of microscopical measurements, allowing quantitative analysis of thousands of bacterial cells, one at a time, every second. Data can thus be obtained on millions of cells, with useful information acquired for individual cells. The option of cell sorting also allows physical separation of specific cells. A detailed discussion of the physical basis of FCM and cell sorting, different instrument configurations, and some applications to environmental bacteriology are given in Chapter 5.

Successful application of FCM to environmental bacteriology generally requires a fluorescent label to differentiate events of interest (cells) from background events (environmental particulates, machine noise, or nontarget cells). Dyes or probes of general interest allow total cell enumeration (DNA staining), specific cell enumeration (antibody or oligonucleotide staining), viability assessment, and physiological assessment (staining cells for total nucleic acid and protein content). The wide range of samples that may be encountered within environmental bacteriology precludes a defined protocol for each analysis. However, the procedures outlined subsequently are robust and should provide a useful starting point for enumeration of total and specific cells, viability assessment, and macromolecular staining. Protocols will almost certainly need

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optimization for each environment, and can also be easily adapted (e.g., for different fluorochromes and for dual-labeling procedures). Additionally, the range of fluorescent probes available continues to expand rapidly *(3)*. This improves choice of dye for each application, but means that protocols may be rapidly outdated. It is worthwhile to spend time designing the experimental procedure carefully (**Fig. 1**), especially if dual-labeling techniques are to be used, to ensure that the assays and fluorochromes being used are suitable for the instrument, the environment from which the cells originate, and to ensure that spectral overlap does not hamper detection.

2. Materials

2.1. FCM

1. Flow cytometer with appropriate filter blocks.
2. Filter apparatus with 0.1- and 0.2-µm filters for both large and small volumes.
3. Sheath fluid, filtered at least three times through 0.1-µm filters (*see* **Notes 1** and **2**).
4. Filtered buffers and stock solutions (*see* **Notes 2** and **3**): Useful solutions and buffers include filtered distilled water, phosphate buffered saline ([PBS]; 8 g NaCl, 0.24 g KH_2PO_4, 0.2 g KCl, Na_2HPO_4 dissolved in 800 mL distilled water; pH to 7.4 with HCl and make up to 1 L), Tris buffer (Tris-[hydroxymethyl] aminomethane, e.g., 10 m*M* or 100 m*M*, dissolve in distilled H_2O and pH adjusted with HCl before making up to final volume), TE (10 m*M* Tris-HCl and 1 m*M* EDTA pH 8.0).
5. Environmental cell suspension (*see* **Note 4**).

2.2. Total Cell Enumeration

1. 4',6-diamidino-2-phenylindole ([DAPI], 1 mg/mL stock in distilled H_2O [dH_2O]) (*see* **Note 5**).
2. Filtered formalin (38–40% formaldehyde) (*see* **Note 6**).

2.3. Specific Cell Enumeration

2.3.1. Specific Cell Detection Using Fluorescent In Situ *Hybridization (FISH) with Oligonucleotide Probes*

1. Labeled oligonucleotide probes (*see* **Note 7**).
2. Freshly prepared, filtered paraformaldehyde (4% in PBS) (*see* **Note 6**).
3. Hybridization solution: 0.9 *M* NaCl, 20 m*M* Tris-HCl, pH 7.2, 0.1% sodium dodecyl sulfate (SDS) or Nonidet P-40.

2.3.2. Specific Cell Detection Using Antibodies

1. 3% Bovine serum albumin in PBS (BSA/PBS).
2. Antisera against target cells: If primary antisera are not labeled, then a fluorescently labeled secondary antibody is required (*see* **Note 7**).

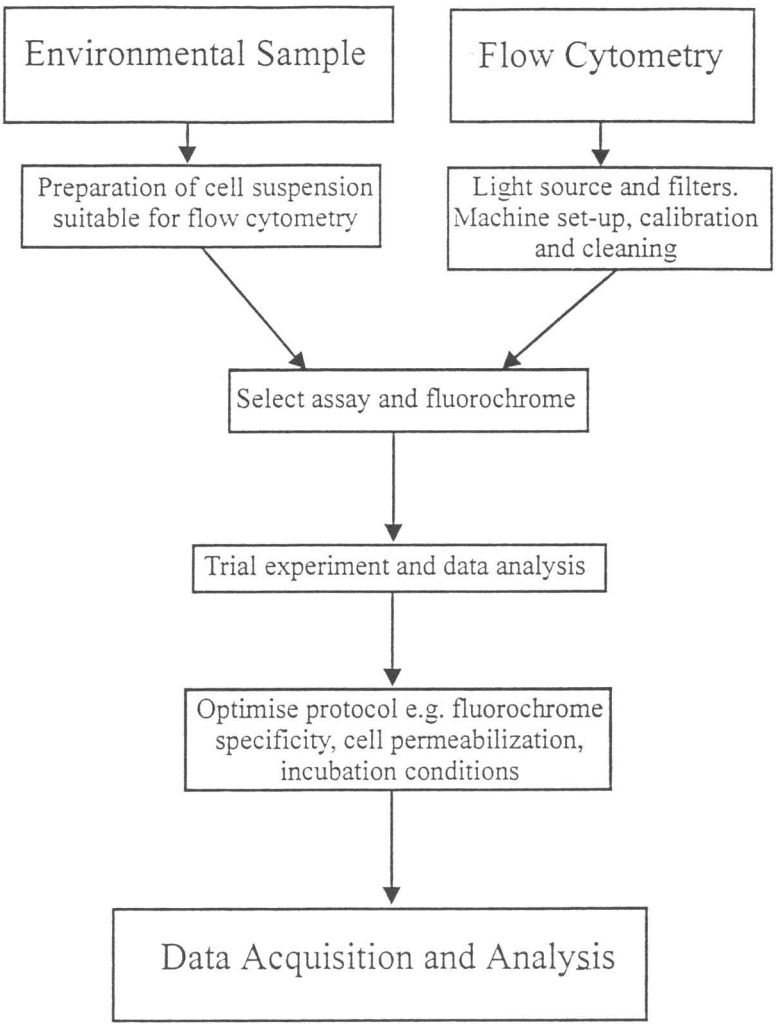

Fig. 1. Diagram showing aspects of the experimental work that must be considered carefully before successfully applying FCM to the study of environmental bacteria.

2.4. Viability Assessment

2.4.1. Membrane Potential Using Rhodamine 123 or a Cyanine Dye

1. Rhodamine 123 (Rh123) (stock 0.05 mg/mL in PBS or 1X TE buffer) or dihexyloxacarbocyanine ($DiOC_6$[3]; stock 0.57 mg/mL in dimethyl sulfoxide [DMSO] or absolute ethanol).

2. Valinomycin (stock 1 mg/mL in DMSO or absolute ethanol).
3. Gramicidin S (stock 1 mg/mL in DMSO).

2.4.2. Membrane Potential using Oxonol

1. *Bis*-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBac_4[3]$; Molecular Probes, Eugene, OR; stock 0.517 mg/mL in DMSO).
2. Heated control cell suspension (80°C for 10 min).

2.4.3. Intracellular Enzyme Activity and Membrane Integrity

1. 5 (6) Carboxyfluorescein diacetate ([CFDA], mixed isomers; stock 1 m*M* in acetone).
2. Chemchrome B (bacterial viability substrate from Chemunex SA, Maisons-Alfort, France).

2.4.4. Nucleic Acid Dye Exclusion Assays

1. Propidium iodide (stock 10 mg/mL in dH_2O) or 4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylidene]-1-(3'-trimethylammonium propyl)-pyridinium diiodide ([PO-PRO-3]; obtained from Molecular Probes as a 1 m*M* stock solution in DMSO) or BacLight viability testing kit (Molecular Probes).
2. Control cell suspension (*see* **Note 8**).

2.5. Cell Macromolecular Content Analyses

2.5.1. Ribosomal RNA Content

1. Propidium iodide (stock 10 mg/mL in dH_2O).
2. RNase A (10 mg/mL stock, boiled and filtered).
3. Fixative (formalin or 70% filtered ethanol).

2.5.2. Total Cell Protein Content

Fluorescein isothiocyanate (FITC) (stock 1 mg/mL in acetone).

3. Methods

3.1. Instrument Setup, Calibration, and Monitoring

1. Exact instructions will vary with each instrument. However, focusing and calibration must be achieved using standard particles (*see* **Note 9**).
2. Save calibration data each time the machine is set up, and confirm focusing periodically throughout a series of measurements. Often this may be achieved by inoculating samples with a known number of calibration beads, which helps both monitoring and counting.
3. Detection of bacteria requires stringent filtration of buffers and fluids to keep background particles to a minimum. Allowing growth of bacteria within the instrument tubing will also interfere with sensitive measurements.
4. Wash and flush the instrument fluid systems thoroughly. Rinse thoroughly with filtered distilled water after bleach or detergent cleaning. Have a standard clean-

ing and shutdown procedure that all users must follow. Monitor users to identify those that do not leave the system clean.
5. When analyzing cells, it is preferable to keep the rate of analysis at or below 2000 cells/s (although some machines may cope with far higher analysis rates, especially if upgraded). Rates such as this reduce the coincidence of two cells passing through the sensing region together.

3.2. Total Cell Enumeration

1. Dilute or concentrate sample until a concentration of approx 1×10^6 cells/mL is achieved (*see* **Notes 10** and **11**).
2. Fix sample if necessary (*see* **Note 6**).
3. Add dye from stock to a final concentration of 0.5 µg/mL (*see* **Note 5**).
4. Incubate in the dark for 1 h. (These conditions may be improved on by optimization on different environments, but should allow detection from the first run.)
5. Analyze by FCM.
6. Controls may be prepared by inoculating samples with known numbers of cultured cells prior to fixation.

3.3. Specific Cell Enumeration

3.3.1. *Fluorescent* In Situ *Hybridization*

1. Fix cell suspension for 16 h in 3 vol of freshly prepared paraformaldehyde in PBS.
2. Wash and resuspend cells in PBS to a final concentration of approximately 1×10^9 cells/mL (if possible).
3. Add an equal volume of absolute ethanol (the cells are now ready for FISH, but are stable and can be stored at –20°C for several months).
4. Add approx 1×10^6 fixed cells into 50 µL of prewarmed hybridization buffer in a water bath (actual temperature will depend upon the probe being used; for the general eubacterial probe, Eub338, hybridize at 46°C).
5. Add between 0.1 and 0.5 ng/µL probe and incubate for at least 6 h if no washing step is to be performed (*see* **Note 3**). If cells are to have posthybridization washes, add 1–5 ng probe/µL and incubate for 1 to 2 h (*see* **Note 12**).
6. Wash cells if necessary, and resuspend in ice-cold, filtered buffer. If no washing steps are to be performed, add 1 mL of ice-cold, filtered buffer and hold on ice until analysis.
7. Analyze by FCM if the oligonucleotide probe has a fluorescent label. If immunologic detection is required to detect bound probe (*see* **Note 7**), go to **Subheading 3.3.2.**
8. A very effective control is to substitute the probe complement (e.g., Eub338') in a parallel sample. Other probes known not to bind to the target cells may also be used as nonspecific staining controls. RNase treatment may also be used (*see* **Note 13**).

3.3.2. Immunofluorescence Detection

1. Prepare cell suspensions in BSA/PBS to approx 1×10^6 cells/mL. Surface labeling of antigens can be performed on fixed or live cells.

2. Add antibody to the cell suspensions to achieve a range of concentrations of active antibody of between 0.1 and 10 µg/mL. If the active concentration is unknown, test from 1/10 and 1/10,000 dilutions. If the primary antibody is directly labeled with the fluorochrome, it will be necessary to test a range of concentrations to find the optimal level. Too much antibody will give a high background whereas too little will mean poor detection.
3. Incubate for 30 min room temperature.
4. For directly labeled antibodies, wash and resuspend in BSA/PBS and analyze by FCM. If the background fluorescence is high, repeat the washing step up to three times until background is satisfactory.
5. If secondary detection is required, wash and resuspend cells at least twice before adding the secondary fluorescent antibody. Again, a range of dilutions will need to be tested, but as a general rule, try slightly higher dilutions than those tested for the primary (e.g., 1/10–1/500).
6. Incubate for 30 min room temperature.
7. Wash and resuspend once in BSA/PBS and analyze by FCM. Again, if background fluorescence is high, perform more washing steps.

3.4. Viability Assessment

3.4.1. Membrane Potential Using Rhodamine 123 or a Cyanine Dye

1. Resuspend cells in an appropriate buffer (*see* **Notes 3**, **11**, and **14**) to produce 5 mL of cell suspension in a particle-free 30-mL glass bottle (*see* **Note 2**).
2. Depending on dye used, add Rh123 to a final concentration of 5 µg/mL (50 µL stock), or add $DiOC_6(3)$ to a final concentration of 50 n*M* (prepare a 1 in 10 dilution of stock to produce a working solution, add 25 µL working solution to 5 mL).
3. Incubate for 15–30 min, with shaking (*see* **Note 15**).
4. Analyze by FCM (*see* **Note 16**).
5. Controls are prepared by incubating parallel samples with Gramicidin S (add 5 µL stock to 5 mL to give a final concentration of 0.1 µ*M* active ingredient). A further control is the use of valinomycin (add 25 µL stock to 5 mL, *see* **Note 17**).

3.4.2. Membrane Potential Using Oxonol

1. Prepare 1 mL of cell suspension in 5 m*M* Tris buffer, pH 7.5 (*see* Note 18).
2. Add oxonol to a final concentration of 1 µ*M* (5 µL stock).
3. Incubate at room temperature for 3–5 min.
4. Analyze by FCM.
5. Controls for oxonol labeling may be prepared using heated cells (e.g., 80°C for 10 min). Other investigators have used Gramicidin (1 µL stock/mL).

3.4.3. Intracellular Enzyme Activity and Membrane Integrity

1. Resuspend cells in appropriate buffer (*see* **Note 19**).
2. Add fluorogenic substrate (10 µL stock CFDA/mL or 10 µL Chemchrome B stock/mL (*see* **Note 20**).

3. Incubate for 10–30 min (*see* **Note 21**).
4. Analyze by FCM.
5. Controls may be prepared using formaldehyde-fixed cells or heated cells.

3.4.4. Nucleic Acid Dye Exclusion Assays

1. Resuspend cells in appropriate buffer (*see* **Note 22**).
2. For propidium iodide, add dye to a final concentration of 50 µg/mL (20 µL stock/mL suspension). For PO-PRO-3, add dye to 3 µ*M* final concentration. If using the BacLight kit, follow manufacturer's instructions (*see* **Note 23**).
3. Incubate for 10 min in the dark.
4. Analyze by FCM.
5. Controls may be prepared using formalin-fixed cells, heated cells, or including octanol (10 µL/mL) in the incubation mixture.

3.5. Cell Macromolecular Content

3.5.1. Ribosomal RNA Content

1. Fix cells in 70% ice-cold ethanol (*see* **Note 6** regarding fixation in ethanol).
2. Wash and resuspend twice in PBS.
3. Add propidium iodide to 50 µg/mL (if this results in high background staining, reduce the propidium iodide to 15 µg/mL rather than introducing a wash step).
4. Incubate in the dark for 45 min at 4°C.
5. Analyze by FCM.
6. Controls for this will include RNase digestion (*see* **Note 13**). Propidium iodide staining intensities have been correlated with biochemical determinations of RNA (*see* **Note 24**).

3.5.2. Total Cell Protein Content

1. Fix cells in 70% ice-cold, filtered ethanol (*see* **Note 6** regarding fixation in ethanol).
2. Wash and resuspend cells twice in 100 m*M* Tris-HCl, pH 9.0.
3. Add 2.5 µL/mL stock FITC solution.
4. Incubate in dark for 10 min.
5. Analyze by FCM.
6. Controls are difficult for an assay of this type, because enzymatic digestion would destroy the cells. Comparison of sample fluorescence intensities with cultured cell standards may be possible in some cases. This type of staining has also been correlated with biochemical determinations of protein content (*see* **Note 24**).

4. Notes

1. It may be helpful to aliquot sufficient sheath fluid for a day's work before autoclaving. Any remaining at the end of the day can be discarded after instrument cleaning. The flow cytometer will have an in-line filter somewhere between the sheath fluid tank and the sensing region. If it is necessary to fit a custom filter, a

large effective filtration area may be required to maintain sheath fluid pressure. If the solution used for sheath fluid is to be used for sorting, it will need to contain salt for the droplet charging process. The amount of salt necessary for successful sorting may well be substantially less than that recommended by the manufacturer. It is simple to try the required buffer in the instrument and check for satisfactory droplet deflection. Finally, it is best (if possible) to use the same buffer for sheath fluid and for the cell suspensions. The waste sheath and cells are collected in a separate tank, to which it may be possible to add concentrated disinfectant to treat biological and chemical hazards.

2. Routinely filter all buffers and sheath fluid before use. Some stock dyes may come in solution, or only a few milligrams may be purchased to prepare stock. Since often less than a microliter may be added to label cells, it is generally unnecessary to filter such stocks (although they should be prepared in filtered buffer). For cheaper dyes and larger volumes, filtration of stocks is worthwhile. When filtering stock buffers or solutions, pour an aliquot of filtrate from the first run into the final receiving container, cap, shake vigorously, and decant the solution to be filtered again. Repeat this once more. Thus, solutions are filtered three times, and the containers are rinsed in particle-free solution twice.
3. If absolute counting is critical for the experimental objectives, it will be preferable to limit cell pelleting/resuspension steps. Often this may be achieved by amending the samples with concentrated buffer, as well as by selecting a specific protocol that requires minimal washing steps (e.g., Hoechst or Chromomycin labeling of DNA, viability assessment using oxonol or fluorogenic esters, FISHs using low levels of oligoprobe and long hybridization times, using minimal levels of antisera for effective detection, and so on).
4. Obtaining a representative sample of bacterial cells in suspension may be problematic for some environments. In general, marine and freshwater samples are ideally suited to FCM, although crude filtration (e.g., 50–100-µm nylon mesh) may be preferred in some situations. The sample must not contain large particles that could clog the instrument fluid systems. Samples such as soil and sediment may be prepared (e.g., using blending/centrifugation steps) for FCM, but the user may risk criticism over the representativeness of the suspension. However, all investigators are faced with the same problems of representative cell extraction, for FCM, microscopy, or molecular biology, and for most applications a satisfactory cell suspension should be possible.
5. More specific labeling may be achieved using Chromomycin A_3 (G/C rich DNA; 1.2 mg/mL stock in dH_2O) or Hoechst 33342/33258 (A/T rich DNA; 0.5 mg/mL stock in dH_2O). The specificity of these dyes allows detection of discrete chromosomes in bacteria after rifampicin treatment. Control cells produced in this way ***(4)*** may help in estimation of DNA content of cells from environmental samples. Diaper and Edwards ***(5)*** correlated FCM measurements of Hoechst 33342 fluorescence with the diphenylamine method for biochemical determination of DNA ***(6)***. These dyes can be substituted for DAPI in the procedure but have a greater quantum yield (brightness). DAPI has often been used at higher

concentrations (e.g., 2.5 μg/mL) than the other dyes. Dye stocks can be aliquoted and stored frozen. SYBR Green I (Molecular Probes) allows specific measurements of DNA in marine samples, when the high ionic strength solution may inhibit binding of other dyes. Dyes such as acridine orange have a reputation for staining instrument tubing and contaminating subsequent samples. This can be overcome by bleach treatment, but in view of the range of DNA dyes available, it is probably best to avoiding acridine orange.

6. Optimal fixation conditions have been widely debated; however, fixation by the addition of formalin is quick, easy, and does not promote cell clumping as alcohol fixation can do. Often samples have to be fixed for storage prior to analysis. Aldehyde fixation in clean bottles followed by storage at 4°C should prove adequate in most situations. In many cases, fixation is performed at the time of sampling to allow analysis at a later date. Freshly prepared paraformaldehyde has been demonstrated to be an effective fixative in many situations (e.g., dual labeling for total cell enumeration combined with FISH) at a final concentration of between 1 and 4% w/v. Formaldehyde-based fixatives have been suggested to cause nonspecific binding of DAPI to cellular material, but this has not been reported for Hoechst or Chromomycin dyes. Nucleic acid staining should still prove effective if the fixation conditions are altered. If alcohol fixation is required, cell clumping can be avoided by injecting the sample gently into the centre of ice-cold, vortexing ethanol. Cell permeabilization after fixation can often be improved by inclusion of a detergent in the buffer (e.g., 0.1% SDS, Tween-20, or Nonidet P-40).
7. When choosing a label for specific detection, it is possible that a dual labeling technique may be useful (e.g., total cell enumeration, or viability assessment). The options available will depend on the light sources of the instrument in use. Dual-laser options enable use of dyes with no spectral overlap. Many viability dyes share the fluorescein excitation/emission wavelengths. Thus, single light source instruments would require a phycoerythrin label (or equivalent) for antibody labeling. Generally, immunofluorescence output from bacteria is dim (at least compared with mammalian systems), and thus the necessary machine settings may cause spectral overlap. It is possible to compensate for this, depending on the software available, but it is unlikely that the procedure will be as straightforward when applied to bacteria as it may appear to be from clinical cytometry research publications. FISH requires killed bacteria, which precludes dual labeling with a viability dye, although some viability dyes are fixable in place using an aldehyde fixation/crosslinking step. However, determining total and specific cell numbers using nucleic acid and oligonucleotide probing with a single light source instrument may still be possible by use of a hapten (e.g., digoxygenin or biotin) linked to the oligonucleotide probe instead of a directly conjugated fluorochrome. The procedure would then be to hybridize *in situ*, detect bound oligonucleotide hapten using (e.g., phycoerythrin) immunofluorescence, and detect total cells using a 488-nm excited nucleic acid dye (e.g., SYBR Green 1 from Molecular Probes). Autofluoresence from photosynthetic pigments should also

be considered when designing the fluorochrome combinations.

8. Controls for this type of assay involve permeabilizing cells to allow free dye passage across the membrane. This may be achieved by fixation in formalin, heating, or addition of octanol.
9. Clinical FCM often utilizes chicken red blood cells as standards. However, for bacteria it is probably preferable to use uniform fluorescent microspheres, which are available in a range of sizes and fluorescence intensities (numbers of fluorescein equivalents per bead). Using beads of approximately bacterial cell size will help instrument setup. Instruments that utilize high-numerical aperture objective lenses for light collection generally require precise focusing to allow very precise measurements, and small beads (e.g., 0.5 μm diameter) are suitable. However, for jet-in-air systems, it may be preferable to use larger beads. This allows for cells that follow slightly different paths through the sensing region to remain in focus.
10. Dilute cells in an appropriate diluent (e.g., if analyzing lake water samples, dilute cells in filtered lake water). Dilution steps are also an ideal way of altering salt concentrations, which may affect some dyes binding to DNA (*see* **Note 5**). In such cases, use an appropriate diluent. Other environments may require sample concentration (*see* **Note 11**).
11. When concentrating cells from some environmental samples by centrifugation, a fraction of them may be difficult to pellet. If centrifugation is to be used, it may be preferable to remove 90% of the volume by gentle pipetting from the surface layer to try to avoid losing cells that have concentrated near the bottom of the tube, but that are not firmly in the pellet. For larger volumes of sample, tangential flow filtration (described in Chapter 3) may be used.
12. When present at a concentration >0.1 μg/mL, the amount of oligonucleotide is not limiting (i.e., there is more probe than ribosomes providing the total number of cells does not exceed approx 1×10^6). Very high probe concentrations (e.g., 20 μg/mL) cause high background owing to nonspecific binding, possibly caused by reactivity of the fluorescent label. Detailed optimization experiments led Wallner et al. *(7)* to recommend intermediate probe concentrations and shorter hybridization times if samples are to be washed, and low probe concentrations with longer hybridization times if washing is to be omitted. This approach is strongly recommended (Porter, J., et al., unpublished data). Hybridization stringency can be increased by the addition of formamide in the buffer.
13. RNase digestion of ribosomal RNA inside intact, fixed cells has been reported by several investigators. However, other data (Porter, J., et al., unpublished data) suggest that effective and reproducible digestion requires larger amounts of enzyme and extended incubation periods. Treatment of fixed, washed cells with 1 mg/mL of final concentration enzyme for periods of 4–16 h at 37°C have been used (Porter, J., et al., unpublished data). Such extended treatments do not affect cell integrity, but have on occasion been noted to alter (increase) cell forward light scatter (Porter, J., et al., unpublished data).
14. Gram-positive bacteria generally label satisfactorily in PBS whereas Gram-negative bacteria require a permeabilization step. This can be achieved using 1 m*M* EDTA in

the buffer (e.g., TE). If EDTA is toxic to the cells of interest, EGTA has also been used at 0.1 m*M* ***(8)***. Gram-positive bacteria also label well in the presence of EDTA, so such a permeabilization step should be used for heterogeneous suspensions.

15. Cell labeling will generally occur within 15 min. The cyanine dyes work slightly more rapidly than Rh123, but this varies according to the buffer used. For Rh123, allow 30 min. For cyanine dyes, allow 15 min, but increase this to 30 min in a protein-containing suspension.
16. Using these conditions it should be possible to avoid a wash step. Labeling specificity can be confirmed using the controls to ensure that membrane potential is being measured. Some previous work has used higher concentrations of rhodamine ***(9)*** and relied upon the use of extensive wash steps to provide dye equilibrium. Other reports ***(8,10)*** have advocated lower concentrations, thus avoiding wash steps.
17. Valinomycin selectively transports K^+ across the membrane until membrane potential is a function of the K^+ gradient. Thus valinomycin will hyperpolarize cells in low-K^+/high Na^+ buffers and depolarize cells in low Na^+/high-K^+ buffers. Hyperpolarization can be achieved in PBS.
18. Labeling with oxonol has proven to be a robust method, and may be performed in many standard buffers, or directly in broth for cultures. Its robustness and lack of requirement for sample pretreatment has led to suggestions that it is the superior dye for viability assessment in some applications ***(11,12)***.
19. This approach is a very robust procedure, but some permeabilization step should be used. Amending lake water with Tween-20 (0.1% final concentration) allowed effective labeling of lake water bacteria ***(13)*** and maximized viable cell counts. However, a labeling buffer is provided with the Chemchrome B kit (Chemunex SA) and this also works well. The procedure uses excess dye, and it is possible to prepare the viability substrate in buffer and mix 1:1 (v/v) with the cell suspension ***(14)***. Samples can be heated to ensure dye access into the cell, although temperature effects may be minimal in some environmental samples ***(13,15)***. If samples are heated (e.g., 40°C), subsequent chilling on ice may be required before analysis to ensure dye retention (Porter, J., et al., unpublished data). The large advantage offered by these fluorogenic ester dyes is that they are non-fluorescent until cleaved inside a cell, thus ensuring low background signals. Labeling efficiency may, however, vary according to the growth phase of cells.
20. Using cocktails of the dyes does not increase viable cell counts, suggesting that the different dyes label the same bacterial cells in heterogeneous samples ***(13,15)***.
21. Extended incubation times do not generally improve the viable cell count and may cause nonspecific hydrolysis of the substrate.
22. Often it is possible to perform these assays in the original cell suspension, but some investigators may prefer to add concentrated, defined buffer.
23. It is often possible to scale down manufacturer's protocols to allow more assays from an expensive kit.
24. FCM measurements of protein have been correlated with biochemical determinations ***(5,16)***. These methods ***(6,17)*** require large numbers of cells, which may

lead to undue extrapolation to sample cell levels. Additionally, blocking nonspecific labeling may prove problematic in some samples, because proteinaceous blocking agents would not be applicable.

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References

1. Porter, J., Deere, D., Pickup, R., and Edwards, C. (1996) Fluorescent probes and flow cytometry: new insights into environmental bacteriology. *Cytometry* **23,** 91–96.
2. Porter, J., Deere, D., Hardman, M., Edwards, C., and Pickup, R. (1997) Go with the flow-use of flow cytometry in environmental microbiology. *FEMS Microbiol. Ecol.,* **24,** 93–101.
3. Haugland, R. P. (1996) *Handbook of Fluorescent Probes and Research Chemicals*, 6th ed., Molecular Probes, Eugene, OR.
4. Steen, H. B., Skarsted, K., and Boye, E. (1990) DNA measurements of bacteria. *Methods Cell Biol.* **33,** 519–526.
5. Diaper, J. P. and Edwards, C. (1994) Survival of *Staphylococcus aureus* in lakewater monitored by flow cytometry. *Microbiology* **140,** 35–42.
6. Herbert, D., Phillips, P. J., and Strange, R. E. (1971) Chemical analysis of microbial cells. *Methods Microbiol.* **5B,** 210–344.
7. Wallner, G., Amann, R., and Beisker, W. (1993) Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14,** 136–143.
8. Mason, D. J., Lopez-Amoros, R., Allman, R., Stark, J. M., and Lloyd, D. (1995) The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. *J. Appl. Bacteriol.* **78,** 309–315.
9. Diaper, J. P., Tither, K., and Edwards, C. (1992) Rapid assessment of bacterial viability by flow cytometry. *Appl. Microbiol. Biotechnol.* **38,** 268–272.
10. Kaprelyants, A. S., and Kell, D. B. (1992) Rapid assessment of bacterial viability and vitality by Rhodamine 123 and flow cytometry. *J. Appl. Bacteriol.* **72,** 410–422.
11. Mason, D. J., Allman, R. Stark, J. M., and Lloyd, D. (1994) Rapid estimation of bacterial antibiotic susceptibility with flow cytometry. *J. Microsc.* **176,** 8–16.
12. Jepras, R. I., Carter, J., Pearson, S. C., Paul, F. E., and Wilkinson, M. J. (1995) Development of a robust flow cytometric assay for determining numbers of viable bacteria. *Appl. Environ. Microbiol.* **61,** 2696–2701.
13. Porter, J., Diaper, J., Edwards, C., and Pickup, R. (1995) Direct measurements of natural planktonic bacterial community viability by flow cytometry. *Appl. Environ. Microbiol.* **61,** 2783–2786.

14. Deere, D., Porter, J., Pickup, R., and Edwards, C. (1996) Direct analysis of starved *Aeromonas salmonicida*. *J. Fish Dis.* **19,** 459–467.
15. Porter, J., Pickup, R. W., and Edwards, C. (1997) Evaluation of flow cytometric methods for the detection and viability assessment of bacteria in soil. *Soil Biol. Biochem.* **29,** 91–100.
16. Allman, R., Hahn, A. C., Phillips, A. P., Martin, K. L., and Lloyd, D. (1990) Growth of *Azotobacter vinelandii* with correlation of Coulter cell size, flow cytometric parameters, and ultrastructure. *Cytometry* **11,** 822–831.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193,** 265–275.

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Confocal Laser Scanning Microscopy of Environmental Samples

David Lloyd, Anthony J. Hayes, and James R. Ralphs

1. Introduction

1.1. Confocal Laser Scanning Microscopy for Microbiology

Enormous technical advances in imaging and data acquisition techniques, combined with a continuing increased scope for fluorescence labeling of specific constituents of living organisms, have brought about a revolution in approaches to biological problems. Whereas spatial organisation at an ultrastructural level would have seemed definitely the province of electron microscopy just a few years ago, now the new methods of light microscopy can offer not only a complementary approach, but can achieve more, especially in terms of noninvasive and real-time measurements. Confocal laser scanning microscopy (CLSM) is the most revolutionary development in optical microscopy since the early sevent eenth century. From the microbial ecologist's point of view, the availability of this powerful new technique, which has been driven primarily by the needs of the biomedical sciences to define *in situ* in cells the interactions of ions, molecules and macromolecules, with membranes with a minimum of perturbation, could not be more timely. The past century has seen great advances in microbial physiology and biochemistry, mostly with organisms grown in suspension on rich media and at high growth rates. More recently there is a growing awareness that surface growth, nutrient deprivation, or pulsatile nutrient sufficiency are more realistic modes of laboratory cultivation with respect to the natural environment. But the study of heterogeneity in space and time brings a host of methodological problems not encountered in the shake flask or continuously stirred tank reactor. In any case, most of the organisms responsible for biogeochemical processes, nutrient cycling, and environmental change cannot yet be studied in the laboratory by traditional microbiological procedures. CLSM provides the means

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whereby individual organisms can be identified and their activities probed in sampled ecosystems with little disturbance. Imaging in three dimensions enables elucidation of the spatial relationships of microorganisms with one another, and their location with respect to the substratum (surface, host plant, or animal). The technique has been reviewed extensively (e.g., *see* **refs. *1–3***), and three excellent books ***(4–6)*** provide many practical details. The 'bible' of fluorescent probes is published by Molecular Probes (Eugene, OR) ***(7)***. However, information on sample preparation of use to the microbial ecologist is still not widely available ***(8)***.

1.2. Principles and Methodology

The first commercially produced confocal laser scanning microscope became available in 1983. It has revolutionized microscopy in that it transforms the microscope from a device that resolves structures in two dimensions to one capable of reconstructing in three—in real-time CLSM, even in four dimensions. It is simple in principle (**Fig. 1**). The laser light source is projected onto the specimen (usually by the microscope objective lens) and imaged by the same lens, onto a pinhole (or aperture) in front of a photomultiplier detector. A complete image is obtained by rapid scanning of the area back and forth (in a raster scan) across the specimen, so that the image is built up point by point. Light from out-of-focus planes above and below the plane of the scan is widely spread at the pinhole and hence eliminated, so that defocused information contributes very little to the final image. A confocal microscope can thus form a very sharp image, even of thick objects. By stepping through a range of focal planes, a series of optical "sections" through the specimen can be acquired; these can be used to reconstruct a three-dimensional ***(30)*** image of the object of interest. This can then, if desired, be optimally displayed by selection of a preferred orientation, or can be viewed from all angles as the image is rotated. This is the basic process of image acquisition, construction, and display that is most often used for intensity analysis in conjunction with specific fluorescent probes. More recently, real-time confocal instruments have been produced ***(9)***. These allow study of events and processes as well as static structure.

The principle of confocal microscopy was established by Minsky ***(10)***; several different systems have been used including multiple-point scanning using a Nipkow disc, stage scanning, and beam scanning ***(2)***. Systems that scan the beam using one or two mirrors have become predominant since their introduction in the mid-1980's ***(11,12)***; although, in theory, one mirror is ideal, in practice it is easier to use two. Ideally suited to measurement of fluorescence, almost all instruments use either small air-cooled argon-ion, helium-neon, or krypton lasers as the excitation source. Appropriate excitation and emission filters are used, depending on the fluorescence characteristics of the fluorophores used.

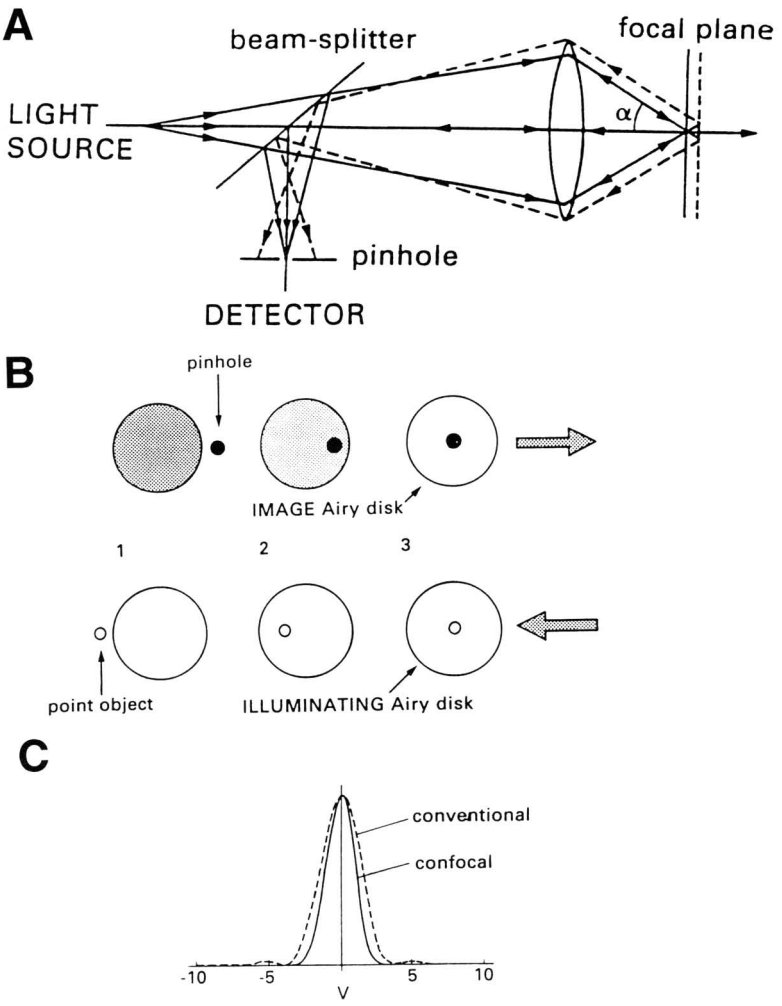

Fig. 1. **(A)** The layout of a typical confocal microscope. Illuminating light is focused to a point at one plane in the specimen. Fluorescent or reflected light from this point is focused on the pinhole and hence passes through to the detector. At a different plane (broken lines), the light illuminates a diffuse patch of the specimen and forms an even larger patch at the pinhole, and thus very little light passes through. **(B)** The basis of improved resolution in confocal microscopy. As the illuminating spot passes across a point object, the object is imaged as an Airy disk of progressively increasing intensity that scans across the detector pinhole. **(C)** The resulting intensity profile across the image of a point object in conventional and confocal microscopes. (Reproduced with permission from **ref. *2***.

1.3. Application of the Technique

Sampling of natural ecosystems for CLSM presents practical difficulties. As Caldwell et al. *(8)* have pointed out, bacteria operate on a scale one million times smaller than we do, and 1 mm^2 on that scale is equivalent to 1 km^2 on ours. Hence, looking for a bacterium on a 1 mm^2 surface is like looking for a single person in a 1-km^2 forest. The marked spatial heterogeneities of natural environments make the selection of sampling sites and depths difficult as only very small samples (e.g., 1 $mm^2 \times 0.2$ mm depth) can be studied in detail.

Samples of this size, containing individual organisms, microcolonies, and microbial communities attached to mineral surfaces in soils, sediments, or on the surfaces of plant or animal tissues, may be examined discretely. Optical sectioning enables successive fields to be acquired (e.g., 20 sections of 0.5 µm thickness with 1-µm intersection spacings).

Thicker samples must be physically sectioned first, and cryosectioning provides an excellent option. For those examples in which spatial associations are easily lost, enmeshing in agarose gel can prevent loss of structural information. Penetration of antibodies and of molecular probes into organisms necessitates prior fixation and sometimes treatment with solvents (e.g., formamide enables ribosomal-targeted oligonucleotides to reach their hybridization sites).

2. Materials

2.1. Some Suppliers of Confocal Laser Scanning Microscopes

1. Bio-Rad Microscience Ltd., Bio-Rad House, Maryland Avenue, Hemel Hempstead, Herts, HP2 7TD, UK (tel: 44-1442-232552; fax: 44-1442-2334434).
2. Carl Zeiss Ltd., P.O. Box 78, Woodfield Road, Welwyn Garden City, Herts, AL7 1LU (tel: 44-1707-871200, fax: 44-1707-871287.
3. Olympus Microscopes, 2-8 Honduras Street, London, EC1Y 0TX (tel: 44-171-250-0179, fax: 44-171-250-4678).
4. Nikon UK Ltd., Nikon House, 380 Richmond Road, Kingston, Surrey, KT2 5PR (tel: 44-181-541-4440, fax: 44-171-250-4678).

2.2. Materials Required for Analysis

Requirements for vital staining, activity, and 16S ribosomal RNA (rRNA) analysis are described elsewhere (*see* Chapters 11, 15, and 16).

3. Methods

3.1. Direct Examination of Samples After Vital Staining for in Situ Activities

The staining methods discussed here are described in detail in previous chapters (*see* Chapters 15 and 16). One of the most attractive and important advan-

tages of the application of CLSM in microbial ecology is the imaging of live microbes *in situ.* The quantitation of the relative contributions of individuals to overall processes may even be possible. Considerable population heterogeneity with regard to any measured activity (e.g. respiration) is evidenced even in well-mixed laboratory cultures; this can be resolved on many time scales ***(13)***. Flow cytometry (FCM) provides a powerful means of study ***(14)***.

In natural populations there are usually many different types of organisms crowded together. Using CLSM it is possible to evaluate spatial distributions of activities with minimal disturbance and thereby produce 3D maps of interactions (microbe-microbe, microbe-plant or microbe-animal). Organisms firmly attached to surfaces (leaves, roots, mucosal membrane, *etc.)* are viewed directly as small specimen blocks (1 $mm^2 \times 0.2$ mm thick). Natural samples that tend to disaggregate (e.g., from loosely aggregated sediments or soils) should first be embedded in 2% (w/v) agarose. The sample is impregnated with low-melting point (39°C) agarose solution; after solidification, it is sliced into small blocks before further treatment with fluorophores.

Organisms in liquid suspension (e.g., from various regions of the water column) present some problems, even if nonmotile. Two methods of preventing Brownian movement (as well as swimming movements) may be utilized. The first is to increase viscosity by use of 10% (w/v) methyl cellulose solution, and to compress them slightly by withdrawal of excess fluid from under the cover slip. Alternatively, slides can be precoated with a thin layer of the polyanion polylysine formed by drying a 2% (w/v) solution.

A wide variety (*see* **refs.** ***7*** and ***15*** as well as Chapter 16) of "activity"-indicating fluorophores are available (*see* **Note 1**). The propriety kits for "viability staining" that ideally enable differential counting of "live" and "dead" organisms (e.g., *Bactolite* and *Fungolite* from Molecular Probes) use undisclosed dye formulation; alternatively, a large number of well-understood staining methods are available. The most frequently used is the dual-staining combination of propidium iodide (red), and fluorescein diacetate (nonfluorescent). The nucleic acid intercalating agent can only penetrate damaged or dead organisms, whereas the esterase substrate locates active enzymes in live cells by fluorescein production (yellow-green) ***(16)***. "Improvement" of this classical protocol includes the use of the more highly permeable 6-carboxyfluorescein diacetate, Calcein acetoxymethyl ester (both from Sigma), or the Chemchrome dyes (Chemunex SA, Maisons-Alfort, France) ***(17,18)***. Some organisms have nonspecific efflux pumps ***(19)*** that can expel vital dyes ***(20)***. Only seldom has the validity of estimates of live organisms in natural samples been confirmed by fluorescence-activated cell sorting and subsequent culture ***(21)***. The complementary nature of information obtainable from the CLSM

and FCM techniques is likely to become increasingly evident in the future. Other vital activity stains include those for transmembrane electrochemical potential (the cyanine dyes and rhodamine 123, both cationic and actively taken up by a process of electrophoresis through any biological membrane with positive change on the outer face) and the anionic oxonols, excluded but seen as a "halo." Choice of a cyanine dye is based on hydrophobicity and hence permeability properties; $DioC_6(3)$ and $DiBaC_4(3)$ appear to be the most generally useful cyanine and oxonol dyes, respectively ***(22,23)***. Provided appropriate excitation is available, samples with marked autofluorescence are better stained with indodicarbocyanine or oxonol VI; both emit in the red ***(24)***.

A widely used activity strain is the tetrazolium salt CTC (5-cyano-2,3-ditolyl tetrazolium chloride), which reacts with the dehydrogenases of electron transport chains to produce an insoluble red fluorescent formazan ***(25)***; it produces extremely bright images of bacteria in CLSM (**Fig. 2**). Use of different electron donors (e.g., organic acids, H_2S, H_2) can be measured directly by image subtraction between tests and control after incubations with various substrates.

Other available argon-ion laser-excitable fluorophores ***(7)*** include many that indicate ion concentrations (e.g., fluor 2 for Ca^{2+}) or pH (BCECF or SNARF) as well as a host of fluorescently labeled antibodies for extracellular components. Before mounting under cover slips, material should be washed free of excess dyes and suspended in a solution of a free-radical scavenging agent (e.g., 2.5% DABCO [w/v] (1,4-diazabicyclo-2,2,2-octane), propylgallate, or phenylene diamine) to prevent photobleaching of the fluorophore (*see* **Note 2**).

3.2. Cryosectioning

An excellent background to the cryopreparation of biologic specimens ***(26)*** gives the theory and practice of this technique. Advantages include processing in native extracellular fluid environments, maintenance of activity of enzymes and antigens, and rapid (within 0.1–1 ms) immobilization of organisms. After placing the sample on the microtome chuck, it is placed on a bed of ethanol "slush" cooled to 77K in liquid N_2. When frozen the sample may be stored in liquid N_2, or transferred to a cryostat chamber at 253K, cut with a steel knife into 7-μm sections, and then allowed to thaw after collection on a microscope slide at ambient temperature. Alternatively, the section is allowed to freeze-dry overnight on the slide. Vital staining or fixation is also performed on the slide. Subsequent procedures can include scanning electron microscopy (SEM) or microprobe analysis for elemental distribution (*see* **Notes 3** and **4**).

3.3. Fixed Samples

Identification of microbes *in situ* requiring the use of rRNA-targeted oligonucleotides necessitates prior fixation and permeabilization ***(27)***. A potential

problem is the low copy number of rRNA molecules present in cells found in nutrient-limited environments, which can result in low levels of fluorescence in stained cells, making their discrimination difficult (*see* **Note 5**). Freshly hydrolyzed paraformaldehyde solution (4% w/v) in phosphate-buffered saline minimalizes problems of autofluorescence. Fixation is for 3 h at 4°C. Processing is conveniently carried out with agarose-embedded material or with material attached to slides. Clean slides (soaked in 10% KOH in ethanol for l h) rinsed thoroughly with 0.2 µm filtered distilled water and air dried are coated with gelatin by dipping them in a 0.1% gelatin, 0.01% chromium potassium sulfate solution at 70°C, then allowed to air dry in a vertical position.

Dehydration (3 min each in 50, 80, and 98% [v/v] ethanol) is followed by the *in situ* hybridization procedure. Hybridization buffer (0.9 *M* NaCl, 0.1% sodium dodecyl sulfate, 20 m*M* Tris-HCl at pH 7.2 in 0.2 µm filtered distilled water) can be supplemented with formamide, or the organisms can be subjected to other permeabilization procedures ***(28)***.

3.4. Applications of Methods

CLSM has been extensively used to study biofilms growing on teeth, submerged steel structures, silicone rubber catheters, and so on ***(8,29,30)***. In specially designed flow cells, repeated investigations enable studies of the buildup of the film and the successions of organisms, as well as providing information on pH and redox gradients thereby generated. Combined with microelectrode determinations (e.g., of O_2 and NO_3^-), studies of the distribution of 16S rRNA–targeted oligonucleotide probes provide detailed maps of microbial communities ***(31,32)***. We have found that CLSM gives invaluable data on the stratification of activities and identities of organisms in peat bogs (***33***, **Figs. 3**). Studies of rhizosphere organisms using rRNA-targeted oligonucleotide probes allows high-resolution analysis of spatial distributions of bacteria with respect to plant roots ***(34)***. Investigations of a denitrifying sand-filter ***(35)***, and of sewage sludge ***(36)*** have also used these methods. A method for the enumeration of soil bacteria has been elaborated ***(37)***. Diffusion coefficients for the migration of small molecules through gels have been determined by CLSM ***(38)***. The activity and distribution of toluene-degrading *Pseudomonas putida* has been evaluated in a multispecies biofilm ***(39)***. Intracellular bacteria are splendidly revealed by CLSM studies, even when their hosts are very large (e.g., 80 µm in the case of the rumen entodiniomorphid protozoon *Polyplastron multivesciculatum)* ***(40)***. The technique also finds important applications in the study of pathogenic *Legionellaceae* within the protozoon *Tetrahymena pyriformis* ***(41)***.

A neglected area of microbial ecology, the phagocytosis of bacteria by protozoa (they have a prodigious propensity for grazing and can turn over the

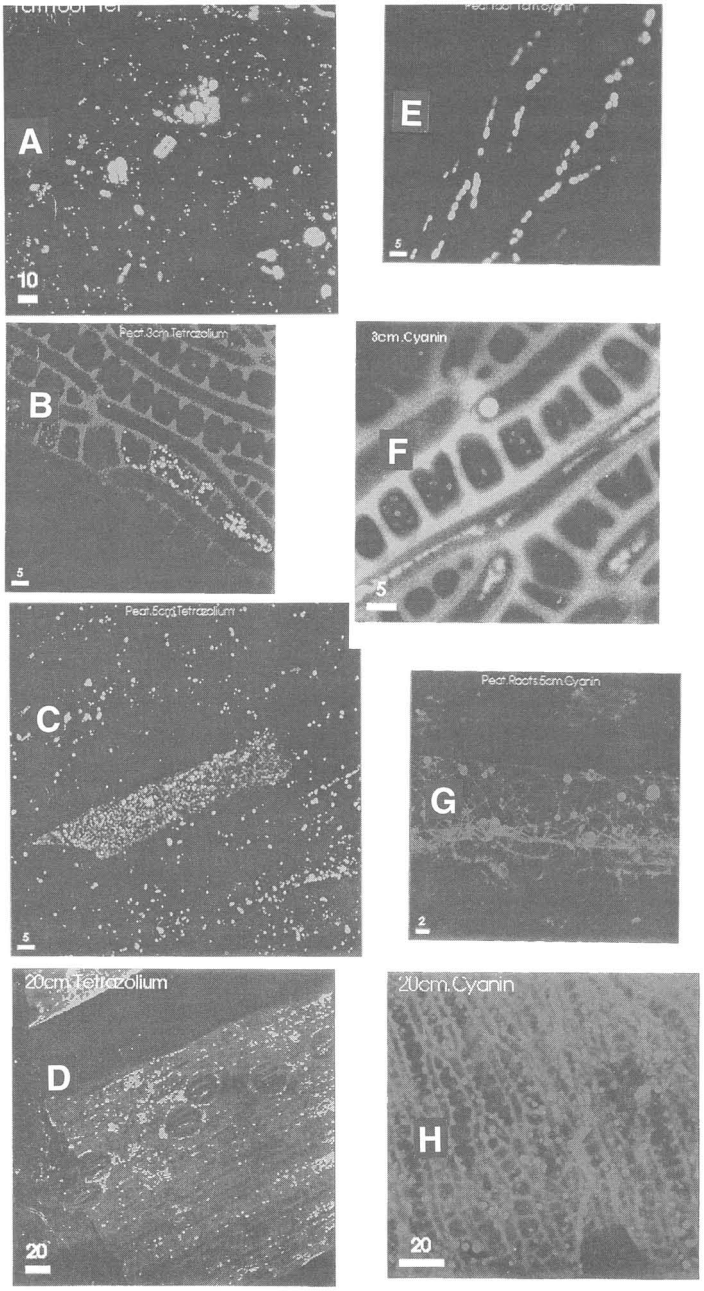

Fig. 2. Fluorescent images of plant-associated microorganisms from a peat core. These were obtained on optical sectioning of 1-mm^3 peat samples taken from known depths within an intact core and stained with either tetrazolium (CTC) **(A–D)** or cya-

entire bacterial population of a sediment within a few days ***[42]***), is conveniently studied by CLSM. Thus the kinetics of fluorescent bead uptake by *Acanthamoeba castellanii,* a common soil amoeba, can be measured quantitatively by FCM, and the complementary technique of CLSM is necessary to distinguish between ingested and surface-adsorbed particles ***(43)***. In marine and freshwater anaerobic environments, it is the major function of large ciliates (e.g., *Metopus* spp.) to carry out this process. These organisms lack mitochondria, and their hydrogenosomes can be revealed by the use of membrane potential–sensitive dyes ***(44)***. These organelles serve also as Ca^{2+} stores, and fluor-3 has been used to show this in CLSM images. Similar results have been obtained for the other hydrogenosomes of lower eukaryotes living in O_2-deficient or anaerobic environments: *Neocallimastix frontalis,* a chytrid fungus that inhabits the rumen ***(45)***, and *Trichomonas vaginalis* ***(46),*** a flagellated protozoon parasite of the human vagina (*see* **Note 6**).

4. Notes

1. Limited availability and economy of light sources and optics largely preclude the use of useful ultraviolet (UV) excitable dyes for most commercially available confocal laser scanning microscopes. However, reasonable longer wavelength alternatives, for the most part, are already in use and this range becomes extended daily.
2. In conventional epifluorescence microscopy, the image fails to represent 3D objects accurately. Stray light blurs this image, and because the entire object is illuminated, fluorescent probes may become bleached. In CLSM, rejection of all out-of-focus light gives a true optical section with a thickness as little as 0.2 µm; depth resolution is proportional to the square of the numerical aperture ***(2)***. Emission from a fluorophore can be quantified for the calculation of molecular concentration. Digital imaging of a sequence of optical sections obtained by stepping in the *z*-direction is followed by 3D reconstruction. The advantage of the confocal method is clearly evident in the images of intracellular bacteria within a rumen protozoon (**Fig. 3**). Software is available for rapid quantification of pixel (voxel) intensities with chosen areas (volumes) of the image. Rapid scanning of the object by the focused spot minimizes exposure to the potential photobleaching of the fluorophore.
3. The resolving power of the confocal laser scanning microscope is marginally improved (i.e., the minimal resolved distance is 0.7 of that of the conventional

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nine [$DiOC_6(3)$] **(E–H)** for 90 min. Depths of sampling were (A, E) 1 cm; (B, F) 3 cm; (C, G) 5 cm; (D, H) 20 cm. Samples from 1–5 cm were stained under an atmosphere of air, whereas those from 20 cm were stained anaerobically, under N_2 (d, h). Bars are calibrated in micrometers.

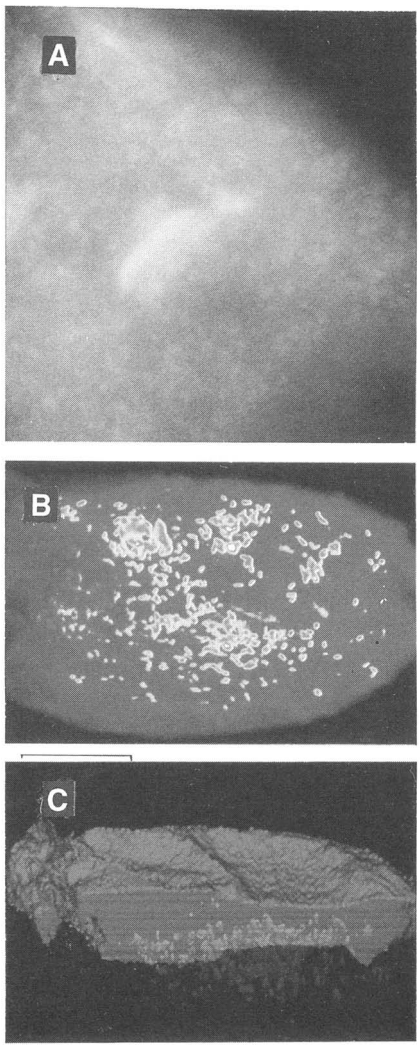

Fig. 3. *Polyplaston multivesiculatum,* a rumen ciliate protozoon containing bacterial endosymbionts labeled with fluorescein–16S rRNA probe by *in situ* hybridization. **(A)** Epifluorescence, image-diffuse, out-of-focus autofluorescence and overlap of bacterial images makes counting impossible, **(B)** confocal laser scanning of similar specimen a single section. **(C)** image as in (B), but reconstructed after latitudinal rotation and cut away to show surface features and internalized bacteria. (Reproduced from Lloyd et al. ***[40]***.)

light microscope ***[2]***). As well as enabling nondestructive enumeration of organisms in microassemblages, and thereby evaluation of associations, extracellular fluorescence can be determined as a measure of diffusible metabolites, pH varia-

tions or gradients of ions, or O_2 as indicators of metabolic activities. Direct visualization of gene expression is possible (e.g., specific promoter-controlled expression of green fluorescent protein or bioluminescent emission flux in photon counting mode).

4. Electron microscopy scanning (SEM) or transmission (TEM) electron microscopy gives much higher resolution, but almost always requires use of fixed and dehydrated material. Repeated examination of live samples by CLSM allows the dynamics of change to be investigated on rapid (milliseconds to seconds) or slow (hours to months) time scales. Thus, as well as having great potential for the studies of spatiotemporal oscillations (e.g., Ca^{2+} waves), the establishment and development of biofilms over extended periods of time have also been documented ***(38)***. Three-dimensional spatial reconstruction of TEM images by serial sectioning is an extremely time-consuming, operator-intensive task. Other techniques of scanning probe microscopy (scanning tunneling, atomic force, scanning ion-conductance) use probes that give information only on surface topography. An example of the complementarity of some of these methods for the examination of biofilm has been presented ***(47)***.
5. Further developments include enhancement of axial resolution to better than 0.05 μm by standing-wave excitation ***(48)***, and fluorescence lifetime imaging ***(49)***, enabling more reliable estimation of fluorophore concentrations. A new, Nipkow disc, real-time confocal microscope ***(50)*** shows advantages for weakly fluorescent objects. A microscope/macroscope enables examination of small and large specimens in a single device ***(51,52)***. The burgeoning list of available fluorophores includes new red dyes ***(53,54)*** that potentially give greater sensitivity of measurement at wavelengths longer than characteristic of the Raman scattering of water and those commonly encountered in autofluorescence (e.g., chlorophyll). Fast acquisition systems that allow 3D imaging of living microorganisms in their natural environments is an obvious target ***(55)***.
6. High-powered argon-ion lasers (lines between 330 and 364 nm) have to be watercooled and are very expensive: microscope lenses are not chromatically corrected into the UV. Thus, although UV-excitable dyes such as DAPI, Hoechst, DANSYL, fura-2, and indo-l cannot be used with most systems, derivatives of fluorescein, rhodamine, BOIDIPY, Texas red, cyanine, oxazole, thiazole, phenanthridine, and the phycobilins are excitable by small argon-ion lasers (488 and 514 mm) or mixed-gas Krypton-argon lasers (488, 568, and 647 nm). New fluorescent calcium indicators include fluor 3, Calcium green, Calcium orange, Calcium crimson, and Fura red. pH indicators used in confocal scanning applications are BCECF and carboxy SNARF. New red dyes show great promise ***(53,54)***.

References

1. Farkas, D. L., Baxter, G., DeBiasio, R. L., Gough, A., Nederlof, M. A., Pane, D., Pane, J. Patek, D. R., Ryan, K. W., and Taylor, D. L. (1993) Multimode light microscopy and the dynamics of molecules, cells and tissues. *Annu. Rev. Physiol.* **55,** 785–817.

2. Cox, G. (1993) Trends in confocal microscopy. *Micron* **24,** 237–247.
3. Boyde, A. (1990) Confocal optical microscopy, in *Modern Microscopies. Techniques and Applications* (Duke, P. J. and Michelle, A. G. eds.), Plenum, New York, pp. 185–204.
4. Wilson, T. and Sheppard, C. (1984) *Theory and Practice of Scanning Optical Microscopy.* Academic, New York.
5. Pawley, J., ed. (1995) *The Handbook of Biological Confocal Microscopy,* 2nd ed., Prenum, New York.
6. Stevens, J. K., Mills, L. R., and Trogadis, J. E., eds. (1994) *Investigation of Biological Specimens. Academic, San Diego.*
7. Haugland, R. P. (1996) *Handbook of Fluorescent Probes and Research Chemicals,* 6 ed., Molecular Probes, Eugene, OR 97402 and B. V. Leiden, The Netherlands.
8. Caldwell, D. E., Korber, D. R., and Lawrence, J. R. (1992) Confocal laser microscopy and computer image analysis in microbial ecology, vol. 12, *Advances in Microbial Ecology* (Marshall, K. C., ed.), Plenum, London, pp. 1–68.
9. Xiao, G. Q., Corle, T. R., and Kino, G. S. (1988) Real-time confocal scanning optical microscope. *Appl. Phys. Lett.* **53,** 716–718.
10. Minsky, M. (1988) Memoir on inventing the confocal scanning microscope. *Scanning* **10,** 28–138.
11. White, J. C., Amos, W. B., and Fordham, M. (1987) An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. *J. Cell Biol.* **103,** 41–48.
12. Carlsson, K. and Liljeborg, A. (1989) A confocal laser microscope scanner for digital recording of optical serial sections. *J. Microsc.* **153,** 171–180.
13. Lloyd, D. (1993) Flow cytometry, a technique waiting for microbiologists, in *Flow Cytometry in Microbiology* (Lloyd, D., ed.), Springer-Verlag, London, pp. 1–10.
14. Porter, J., Deere, D., Hardman, M., Edwards, C., and Pickup, R. (1997) Go with the flow—use of flow cytometry in environmental microbiology. *FEMS Microbiol. Ecol.* **24,** 93–101.
15. Lloyd, D. and Hayes, A. J. (1995) Vigour, vitality and viability of microorganisms. *FEMS Microbiol. Lett.* **133,** 1–7.
16. Schupp, D. G. and Erlandsen, S. L. (1987) A new method to determine *Giardia* cyst viability: correlation of fluorescein diacetate and propidium iodide staining with animal infectivity. *Appl. Environ. Microbiol.* **53,** 704–707.
17. Edwards, C., Diaper, J., and Porter, J. (1996) Flow cytometry for the targeted analysis of structure and function of microbial populations, in *Molecular Approaches to Environmental Microbiology* (Pickup, R. and Saunders, J. R., eds.), Eliss Harwood, Chichester.
18. Brailsford, M. and Gatley, S. (1993) Rapid analysis of microorganisms using flow cytometry, in *Flow Cytometry in Microbiology* (Lloyd, D., ed.), Springer-Verlag, London, pp. 171–180.
19. Paulsen, I. T., Brown, M. H., and Skurray, R. A. (1996) Proton-dependent multidrug efflux systems. *Microbiol. Rev.* **60,** 575–608.

20. Midgely, M. (1987) An efflux system for cationic dyes and related compounds in *Escherichia coli. Microbiol. Sci.* **4,** 125- 127.
21. Porter, J., Robinson, J., Pickup, R., and Edwards, C. (1995) Recovery of a bacterial sub-population from sewage using immunofluorescent flow cytometry and cell sorting. *FEMS Microbiol. Lett.* **133,** 195–199.
22. Lloyd, D., Moran, C. A., Suller, M. T. E., Dinsdale, M. G., and Hayes, A. J. (1996) Flow cytometric monitoring of rhodarnine 123 and a cyanine dye uptake by yeast. *J. Inst. Brewing* **102,** 251–259.
23. Willetts, J., Seward, R., Dinsdale, M. G., Suller, M. T. E., Hill, B., and Lloyd, D. (1997) Vitality of cider yeast grown micro-aerobically with added ethanol, butanol-ol or iso-butanol. *J. Inst. Brewing* **103,** 79–84.
24. Lloyd, D. (1993) Spectral characteristics of some fluorescent dyes and excitation sources. In *Flow Cytometry in Microbiology* (Lloyd, D., ed.), Spring-Verlag, London, pp. 181–184.
25. Lopez-Amoros, R., Mason, D. J., and Lloyd, D. (1995) Use of two oxonols and a fluorescent tetrazolium dye to monitor starvation of *Escherichia coli* in sea water by flow cytometry. *J. Microbiol. Methods* **22,** 165–176.
26. Roos, N. and Morgan, A. J. (1990) Cryopreparation of thin biological specimens for electron microscopy, in *Royal Microscopical Society Microscopy Handbooks*, Oxford University Press, Oxford, UK.
27. Amann, R. I., Krumholz, L., and Stahl, D. (1990) Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology. *J. Bacteriol.* **172,** 762–770.
28. Macnaughton, S. J., O'Donnell, A. G. O., and Embley, T. M. (1994) Permeabilization of mycolic-acid-containing actinomycetes for *in situ* hybridization with fluorescently labeled oligonucleotide probes. *Microbiology* **140,** *2859–2865.*
29. Singleton, S., Albiston, L., Treloar, R., Mahers, E., Hodgson, R., Watson, K., Schilling, K., and Allison, C. (1995) Optical imaging and characterisation of oral biofilm structures using vital stains and specific antibody probes, in *The Life and Death of Biofilm* (Wimpenny, J. W. T., Handley, P., Gilbert, P., and Lappin-Scott, H., eds.), Bioline, Cardiff, pp. 33–36.
30. Caldwell, D. E., Korber, D. R., and Lawrence, J. R. (1992) Imaging of bacterial cells by fluorescence exclusion using scanning confocal laser microscopy. *J. Microbiol. Methods* **15,** 249–261.
31. Zanyk, B. N. Korber, D. R., Lawrence, J. R., and Caldwell, D. E. (1991). 4-D visualization of biofilm development by *Pseudomonas fragi. Binary* **3,** 24–39.
32. Schramm, A., Larsen, L. H., Revsbech, N. P., Ramsing, N. B., Amann, R., and Schleifer, K. H. (1996) Structure and function of a nitrifying biofilm as determined by *in situ* hybridization and the use of microelectrodes. *Appl. Environ. Microbiol.* **62,** 4641–4647.
33. Lloyd, D., Thomas, K. L., Hayes, A., Hill, B., Hales, B. A., Edwards, C. A., Saunders, J. R., Ritchie, D. A., and Upton, M. (1997) Micro-ecology of methanogens in peat: minimally-invasive analysis using confocal laser scanning

microscopy, membrane inlet mass spectrometry and PCR amplification of methanogen-specific gene sequences. *FEMS Microbiol. Ecol.* **25,** 179–188.
34. Assmus, B., Hutzler, P., Kirchof, G., Amann, R., Lawrence, J. R., and Hastimann, A. (1995) *In situ* localization of *Azospirillum brasilense* in the rhizosphere of wheat. *Appl. Environ. Microbiol.* **61,** 1013–1019.
35. Neef, A., Zaglauer, A., Meier, H., Amann, R., Lemmer, H., and Schleifer, K.-H. (1996) Population analysis in a denitrifying sand-filter conventional and *in situ* identification of *Paracoccus* spp. in methanol fed biofilms. *Appl. Environ. Microbiol.* **62,** 4329–4339.
36. Rossello-Mora, R. A., Wagner, M., Amann, R., and Johleifer, K-H. *(1995) The abundance of Zoogloea ramigera* in sewage treatment plants. *Appl. Environ. Microbiol.* **61,** 702–707.
37. Bloem, J., Veninga, M., and Shepherd, J. (1995) Fully automatic determination of soil bacterium numbers, and frequencies of dividing cells by confocal laser scanning microscopy and image analysis. *Appl. Environ. Microbiol.* **61,** 926–931.
38. Lawrence, J. R., Wolfaardt, G. M., and Korber, D. R. (1994) Determination of diffusion coefficients in biofilms by confocal scanning laser microscopy. *Appl. Environ. Microbiol.* 60 1166–1173.
39. Moller, S., Pedersen, A. R. Poulsen, L. K., Arvin, E., and Molin, S. (1996) Activity and 3D distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assessed by quantitative *in situ* hybridization and scanning confocal laser microscopy. *Appl. Environ. Microbiol.* **62,** 4632–4640.
40. Lloyd, D., Williams, A. G. Amann, R., Hayes, A. J., Durrant, L., and Ralphs, J. R. (1996) Intracellular prokaryotes in rumen ciliate protozoa: detection by prokaryotes in rumen ciliate protozoa: detection by confocal laser scanning microscopy offer in *situ* hybridization with fluorescent 16S rRNA probes. *Europ. J. Protist* **32,** 523–531.
41. Manz, W., Amann, R., Szewzyk, R., Szewzyk, U., Stenstrom, T-A., Hutzler, P., and Schleifer, K.-H. (1995) *In situ* identification of *Legionellaceae* using 16S rRNA-targetted oligonucleotide probes and confocal scanning laser microscopy. Microbiology **141,** 29–39.
42. Fenchel, T. (1987) *Ecology of Protozoa.* Springer-Verlag Berlin.
43. Avery, S. V., Harwood, J. L., and Lloyd, D. (1995) Quantification and Characterisation of phagocytosis in the soil amoeba *Acanthamoeba castellanii* by flow cytometry. *Appl. Environ. Microbiol.* **61,** 1124–1132.
44. Biagini, G. A., Hayes, A. J., Suller, M. T. E., Winters, C., Finlay, B. J., and Lloyd, D. (1997) Hydrogenosomes of *Metopus contortus* resemble mitochondria. *Microbiology* **143,**1623–1629.
45. Biagini, G. A. van der Giezen, M., Hill, B., Winters, C., and Lloyd, D. (1997) Ca2+ accumulation in the hydrogenosomes of *Neocallimastix frontalis* L2: a mitochondrial-like physiological role. *FEMS Microbiol. Lett.* **149,** 227–232.
46. Humphreys, M. Ralphs, J., Durrant, L., and Lloyd, D. (1994) Hydrogenosomes in trichomonads are Ca^{2+} stores and have a transmembrane electrochemical potential. *Biochem. Soc. Trans.* **22,** 3245.

47. Surman, S. B., Walker, J. T., Goddard, D. T;, Moston, L. H. G., Keevil, C. W., Weaver, W., Skinner, A., Hanson, K., Caldwell, D., and Kurtz, J. (1996) Comparison of microscope techniques for the examination of biofilms. *J. Microbiol. Methods* **25,** 57–70.
48. Bailey, B., Farkas, D. L., Taylor, D. L., and Lanni, F. (1993) Enhancement of axial resolution in fluorescence microscopy by standing-wave excitation. *Nature* **366,** 44–48.
49. Buurman, E. P., Sanders, P., Draaijer, A., Gertitsen, H. C., van Veen, J. J. F., Houtpt, P. M., and Levine, Y. K. (1992) Fluorescence lifetime imaging using a confocal laser scanning microscope. *Scanning* **14,** 155–159.
50. Juskaitis, R., Wilson, T., Neil, M. A. A., and Kozubek, M. (1996) Efficient real-time confocal microscopy with white light sources. *Nature* **383,** 804–806.
51. Damaskinos, S., Dixon, A. E., Ellis, K. A., and Dlehl-Jones, W. L. (1995) Imaging biological specimens with the confocal scanning laser microscope/macroscope. *Micron* **26,** 493–502.
52. Kwon, Y. H., Wells, K. S., and Hoch, H. C. (1993) Fluorescence confocal microscopy: applications in fungal cytology. *Mycologia* **85,** 721–733.
53. Miller, J. N. (1993). Long wave fluorescence microscopy. *Spectroscopy Europ.* **5,** 34–38.
54. Whitaker, J. E., Haugland, R. P., Ryan, D., Hewitt, P. C., and Prendergast, F. G. (1992) Fluorescent rhodol derivatives; versatile photostable labels and tracers. *Analyt. Biochem.* **207,** 267–279.
55. Draaijer, A. and Houpt, P. M. (1993) High scan-rate confocal laser scanning microscopy, in *Electronic Light Microscopy* (Shotton, D., ed.), Wiley-Liss, New York.

18

Monitoring Microbial Activities Using Membrane Inlet Mass Spectrometry

James R. Firth and Clive Edwards

1. Introduction

1.1. Principles of the Technique

Monitoring microbial activities in the environment is difficult owing to the lack of suitable methods. For a technique to be useful for monitoring *in situ* activities, it must possess the following properties: sensitivity, selectivity, stability, the ability to make continuous real-time measurements, and be noninvasive or perturbing to the microorganisms or to the environment being studied. Currently used methods include manometric techniques, microsensors, chemical assays, gas chromatography and high-performance liquid chromatography, but all have their limitations and usually require substantial disruption to the environment being studied. The principles of membrane inlet mass spectrometry (MIMS) have been described elsewhere ***(1,2)***, and are summarized in detail here. Although MIMS allows measurements of numerous gases to be made in both the liquid and gas phases, only liquid phase measurements are detailed in this example.

Essentially the method involves the ionization of the gas or volatile molecules followed by the separation and selection of the required ion on the basis of its mass/charge ratio (*m/z*). **Figure 1** shows the basic components of the system. Gases diffuse continuously from the environment being studied across a silicon rubber membrane located at the tip of the stainless-steel dissolved species probe. Alternative membranes can be used such as Teflon, depending on the properties of the gas or volatile being measured. The tip of the probe has an external diameter of 0.7 mm and contains a number of fine slits to allow the gases to pass through. The system described here has a manifold with four such probes that can sample sequentially by programming the mass spectrometer.

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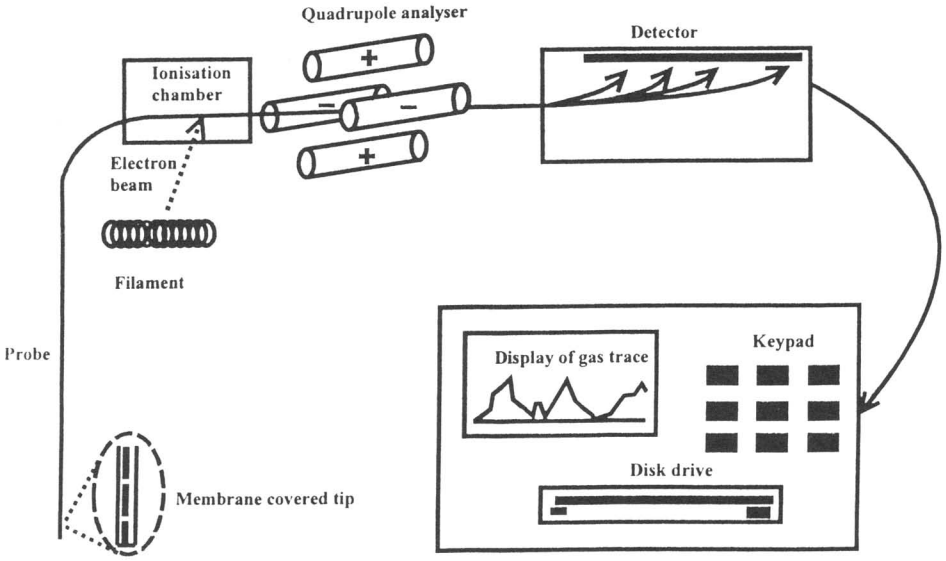

Fig. 1. A schematic diagram of the workings of the quadrupole membrane inlet mass spectrometer

Opening of valves and switching between probes is carried out pneumatically, and it therefore requires a gas cylinder of inert gas, e.g., air or nitrogen, to obtain the required pressure (6 bar). Once inside the probe, the gases are drawn along by the high vacuum produced by the mass spectrometer's turbo molecular pump. On reaching the ionization chamber, the gas molecules are bombarded by a stream of high-energy electrons generated from a heated, thoriated iridium filament. This causes the ionization of the gas molecules. The vacuum continues to draw the ions along into the quadrupole analyzer. This consists of four metal rods, usually steel or molybdenum, with a potential difference and radio frequency applied across each pair. The number of positive ions passing through the analyzer can be quite large, and each of these is deflected to differing degrees by the magnetic field between the rods. The differences in the degree of deflection are dependent on the atomic mass and charge of each ion, i.e., their *m/z*. Many ions will be deflected onto the rods and neutralized, but by scanning through a range of DC voltages and radio frequencies, a number of *m/z* channels can be examined and the ions of interest focused onto the detector. As each deflected ion comes into contact with the detector, the signal is amplified, giving a readable current. Since the number of ions detected is proportional to the concentration of the gas present in the environment (given that the

Table 1
An Example of a Mass Spectra Cracking Pattern[a]

Gas	2	12	18	28	30	32	34	40	44	46	64
N				100							
NO_2					100					37	
N_2O				11	31				100		
NO		6			100						
CO_2				11					100		
O						100					
SO_2						10					100
H_2S						44	100				
H_2O	1		100								
H	100										
Ar								100			

[a]These data are of amplitudes relative to the major peak (100%).

ionization source remains the same), it is therefore possible to quantify the concentration of that gas.

Because different gases are ionized simultaneously, they may produce ions of the same *m/z* ratios; e.g., some *m/z* channels may contain contributions from a number of different gases. To overcome this problem, a mass spectral cracking pattern is used (**Table 1**). For example, when studying denitrification, the gases of interest present major peaks at *m/z* channels 28 (nitrogen), 30 (nitric oxide [NO]), 32 (oxygen), and 44 (nitrous oxide [N_2O] and carbon dioxide [CO_2]). Therefore, some peaks are potentially made up of contributions from other gases, e.g., channel 44 consists of the major peaks of both [N_2O] and CO_2. It is therefore necessary to study additional *m/z* channels to measure various minor peaks to establish what contribution each gas makes to a major peak. To distinguish between the two gases, CO_2 can be measured at *m/z* channel 12, at which it causes a peak corresponding to 6% of its contribution at channel 44 (*see* **Table 1**). From this minor peak, it is therefore possible to calculate what fraction of the channel 44 peak is owing to CO_2 with N_2O making up the remainder.

The Hal quadrupole gas analyzer (Hiden Analytical, Warrington, UK) has the ability to measure and record up to 16 *m/z* channels simultaneously. Because of the need to carry out multiple calculations, the data are saved to computer disk by the mass spectrometer in the form of a spreadsheet. The spreadsheet is then imported into Excel 5.0 (Microsoft), where a macro program can be designed to perform the necessary calculations. Most of the experiments carried out involve the sequential use of all four of the dissolved

species probes, and hence this means the data for all four experiments are initially located in a single spreadsheet. To analyze the data, it is therefore necessary to place each data set into a separate spreadsheet corresponding to the different experiments. Once the data has been processed each probe is calibrated against a known concentration of gas to convert the mass spectrometer units into molar concentrations.

1.2. Applications of MIMS

This chapter describes the use of MIMS to study denitrification in environmental samples. This is an important research area because of the global importance of the denitrification process regarding to the greenhouse effect and water quality, and the fact that so many of the compounds involved are gases, i.e., O_2, CO_2, N_2, NO, and N_2O. The ability to monitor oxygen alongside the products of denitrification has lead to many studies using MIMS reporting the existence of aerobic denitrification ***(3–6)***. The method has demonstrated an enormous variation in both rates and products of denitrification between different denitrifying isolates under different conditions ***(7,8)***. This is also reflected in the environment ***(9)***.

The technique is useful for real-time monitoring of any microbial process that has gaseous or volatile end products. It has been used to study various microbial processes both in the laboratory and in the environment as reviewed by Degn et al. (10). One of the first processes studied with the technique was nitrogen fixation and its relationship with hydrogen cycling ***(11,12)***. Hydrogen production by nitrogenase in the light is inhibited by acetylene, but production is restored in the presence of carbon monoxide, which inhibited hydrogen uptake by the hydrogenase enzyme. Jensen et al. ***(13)*** monitored nitrogen fixation in cyanobacteria and found that at high light levels, an increase in nitrogen uptake occurred, which corresponded to a rise in hydrogen concentration. Later work found that during light conditions, a concentration of hydrogen is reached which actually inhibits nitrogenase activity (14), again showing the close relationship between nitrogenase and hydrogenase in nitrogen fixation.

Another microbial system that has been studied using MIMS is fermentation, and it has been shown that both aerobic respiration and anaerobic fermentation of glucose are inhibited by ethanol and that higher alkanols exert an increasingly inhibitory affect on CO_2 production ***(15)***. Degn ***(2)*** proposed the industrial application of MIMS in process monitoring and when Bohátka et al. ***(16)*** put the system to work as a device for monitoring fermentation they found that they could distinguish changes in gas concentrations of <0.02% vol. Gaseous exchanges in the rumen ecosystem both in vitro and in vivo have been examined ***(17)***, giving an insight into the complex relationship between organisms in a fluctuating environment. The properties of MIMS make it an excel-

lent tool for monitoring pollution ***(18)***, and the fact that it is so easily transported means it can be used to sample numerous sites ***(19)***. A more novel approach, however, has been used by Cristea and Langer ***(20)***. Here MIMS was coupled to the water plant *Elodea canadensis* by inserting the probe into the plant stalk. From the stalk, oxygen and CO_2 were monitored, and the effect of various pollutants on the levels of these gases were recorded. A further process studied using MIMS is methanogenesis. Benstead and Lloyd ***(21)*** studied methanogenesis in peat cores and found that in the surface layers, aerobic decay of organic matter led to methane oxidation to CO_2 by methanotrophic bacteria. At greater depths, at which anaerobic conditions predominated owing to water saturation of the peat, methanogenesis could occur.

The advantages of MIMS are that it is continuous, sensitive to the level of parts per trillion ***(22)***, and virtually noninvasive. In addition, it can be used to measure numerous gases and volatiles simultaneously in either the liquid or gas phase ***(23)***, and is very stable, requiring calibration only every few months. Furthermore, response times can be as short as a few seconds depending on the length of the probe being used, meaning that the results obtained are almost instantaneous.

The MIMS's relatively small size enables it to be easily mobilized, and thus, with a generator and gas bottle the entire system can be setup out in the field, next to a river or lake, e.g. After the initial expenditure on the apparatus, the cost of the technique is extremely cheap, with no continuous cost of consumables required.

The primary limitation of the technique seems to be that detecting a change in gas concentration is dependent on the rate at which it is produced or consumed. For example, when gases that occur naturally within a given environment are measured, e.g., N_2, diffusion out of solution may occur at a comparable rate to that of production. Under such circumstances, no net change in concentration would be seen. In nutrient-rich laboratory media, this inability to detect a change in gas concentration is unlikely to be a problem because processes such as denitrification will be occurring almost to their maximum potential; in the environment, however, this would be problematic.

2. Materials

2.1. General Consumables for Membrane Inlet Mass Spectrometry

1. Membrane inlet mass spectrometer (e.g., Hal series mass spectrometer, Hiden Analytical).
2. Mixtures of gases to be measured, at known concentrations.
3. A cylinder of an inert gas capable of producing a pressure of 6 bar to operate the valve system of the mass spectrometer.

4. A PC-formatted floppy disk to record the data produced.
5. Environmental sample.
6. In the example described here, the environmental sample was transferred to a benchtop 2-L fermentor with stirrer, temperature, pH, and aeration control in order to characterize the denitrification process under controlled conditions.

2.2. Cleaning and Sterilization of Dissolved Species Probe and Replacement of Membranes

1. 70% (v/v) Ethanol.
2. Replacement platinum-cured silicon rubber membrane, internal diameter 0.63 mm (Merck Magna Park, Lutterworth, UK) (*see* **Note 1**).
3. Scalpel.
4. Chloroform.
5. Steel forceps.

2.3. Calibration of the Dissolved Species Probes

1. Sterile distilled water.
2. Thermometer.
3. Gas mixtures of known concentrations.
4. Gas solubility tables.

2.4. Data Processing.

1. A PC capable of supporting a spreadsheet package.
2. A spreadsheet package (Microsoft Excel 5.0.) (*see* **Note 2**).
3. Mass spectral cracking pattern.

3. Methods

3.1. Programming the Mass Spectrometer

The instrument described here is a Hal series quadrupole mass spectrometer supplied by Hiden Analytical (Warrington, England). Other systems will vary in the details of their use, but essentially the principles of the protocol described here will be the same for all.

1. Select MID mode to program the mass spectrometer to record the appropriate *m/z* channels. For example, channels 12, 18, 28, 30, 32, 34, 40, 44, 46, and 64 are selected for denitrification. These allow concentrations of the gases N_2, N_2O, NO, CO_2, and O_2 to be calculated by subtracting contributions from other gases such as hydrogen sulfide (H_2S) and sulfur dioxide (SO_2) that contribute to channel 32, where oxygen is measured.
2. Set the period setting, i.e., the length of time between recorded scans to 900 s (*see* **Note 3**). This ensures that despite scanning the range of *m/z* channels continuously, the system only records its designated range of *m/z* channels every 900 s.
3. Insert a floppy disk and select the disk option (*see* **Note 4**).

3.2. Sterilization of the Dissolved Species Probes

Applications of the technique to pure cultures will require the dissolved species probe to be sterile. This is carried out as follows:

1. Insert the probe into a 10-mL glass pipet to avoid damage, and wrap the exposed end in aluminum foil.
2. Sterilize the probe by autoclaving at 121°C for 15 min (*see* **Note 5**).
3. Oven dry the probe at 60°C for 2 h before reconnection to the mass spectrometer. This prevents moisture from entering the mass spectrometer.
4. Attach the probe to the mass spectrometer before inserting into any liquid, to ensure that full vacuum is reached (*see* **Note 6**).

3.3. Preparing the Sample

The example used here is measurement of denitrification by aquatic bacteria in river water samples taken from the river Mersey, Merseyside, UK.

1. Place the river water sample in a suitable vessel, e.g., a fermentor pot, and stir at 200 rpm, sufficient to keep the cells in suspension but avoiding mixing too much air into the solution.
2. Incubate the sample at a fixed temperature; in this example we used 30°C to increase the rate of denitrification.
3. Supplement with 15 m*M* of $NaNO_3$ and 50 m*M* of sodium acetate in order to stimulate the denitrifying population.
4. Insert the probe into the water sample once vacuum has been achieved, keeping it clear of the stirrer baffles.
5. Switch the probe from "standby" to "on" at the manifold, and the mass spectrometer to begin recording data.

3.4. Calibration of the Dissolved Species Probes

Once the experiment has ended, the probe is switched to "standby" on the manifold and removed from the river water.

1. Immerse the probe tip in distilled water after gently wiping away any debris that may have accumulated on its outer surface.
2. Switch the probe on at the manifold.
3. Bubble the gases of interest through the water in turn until saturation is reached (*see* **Note 7**).
4. Calculate the gas solubility at a known water temperature from data tables ***(24)***. By dividing the saturation concentration of the gas at a given temperature by the reading recorded by the mass spectrometer, a molar concentration can be assigned to each mass spectrometer unit.

3.5. Data Processing

1. Remove the disk from the mass spectrometer once the experiment is finished and transfer to a PC (*see* **Note 2**). The data are saved by the mass spectrom-

eter as a text file that can be accessed as a spreadsheet using Excel 5.0 (Microsoft).
2. Opening the file header gives information about the setup of the mass spectrometer, which is displayed above the recorded data. The header information is deleted and the columns of data labeled with *m/z* channel numbers. Data are displayed with the smallest recorded *m/z* channel on the left up to the highest on the right, i.e., 12, 18, 28, 30, 32, 34, 40, 44, 46, and 64.
3. Using data from the cracking pattern (*see* **Table 1**), the CO_2 value is calculated by multiplying the data from channel 12 by 16.667. This is converts the CO_2 reading as recorded to its actual concentration.
4. Subtract these values from those in channel 44, where CO_2 and N_2O both contribute to the peak value. The remainder is owing to N_2O.
5. Apply these principles to the rest of the data, again using the cracking pattern to calculate the value of each gas.
6. Use the calibration data to convert these values into gas concentrations.
7. Recording these calculations as a macro program in the Excel package allows subsequent data to be processed at the push of a button, as long as the data are first located in the same place in the spreadsheet.
8. Plot the data as a graph (**Fig. 2**). Concentrations of nitrogen, oxygen, and N_2O measured over 48 h are presented showing the initial drop in oxygen concentration to a low steady state as the microorganisms carry out respiration using the added succinate. Subsequently, an increase in N_2O is detected followed by nitrogen production. This is the result of denitrification, as the denitrifying organisms begin to reduce the added nitrate to a gaseous form, again utilizing the succinate present.

3.6. Maintenance

Wear and tear means that the system requires some low level maintenance as described here (*see* **Note 8**).

3.6.1. Cleaning the System

1. Clean the probe and replace the membrane.
2. Immerse the tip of the probe in 70% (v/v) ethanol and continuously sample with the mass spectrometer. This speeds up the passage of dirt through the system and therefore returns the peak profiles to their normal "bell" shape.

3.6.2. Cleaning the Dissolved Species Probe

1. After extensive use, an accumulation of dirt may appear on the surface of the steel probe, beneath the membrane.
2. Remove the membrane by scoring with a scalpel and gently peeling away from the probe.
3. Wipe the steel probe clean using an ethanol-soaked lens tissue (*see* **Note 9**).
4. Apply a new membrane to the clean probe.

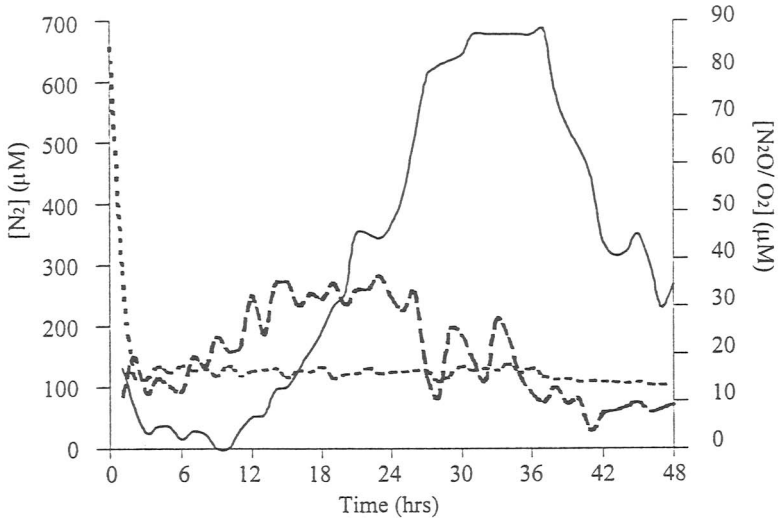

Fig. 2. A typical result obtained by using MIMS to meadure denitrification in river water supplemented with 15 m*M* nitrate and 50 m*M* sodium succinate. The gases shown are nitrogen ——, nitrous oxide – – –, and oxygen ------.

3.6.3. Membrane Replacement

1. Soak the membrane in chloroform for 30 s, causing it to soften and expand.
2. Slide the expanded membrane onto the probe ensuring that no creases or bubbles are present.
3. Rinse the membrane with water to allow it to shrink back to size, ensuring a tight seal is formed at both ends.
4. Seal the tip of the membrane by gently but firmly squeezing with hot steel forceps to ensure that no liquid can enter along the length of the probe (*see* **Note 10**). A tight fit at the top of the membrane is also essential to prevent gases from traveling down between the probe and membrane, which tends to give aberrant readings (*see* **Note 11**).

4. Notes

1. As an alternative to silicon rubber, Teflon, polypropylene, or polyethylene, membranes can also be used depending on the circumstances involved. These allow diffusion of small, nonpolar gas molecules but are less permeable to water and polar molecules, which is why silicon rubber was used in this work.
2. Although the description given here uses a spreadsheet software package to process the data, this machine and others may be used in conjunction with specific, dedicated software provided with the machine. Such software may or may not be preferred.

3. The period setting used here was chosen because it produced enough readings to give a good representative curve when plotted as a graph. If the period is too long, important periods of activity may be missed, and if it is too short, the enormous number of results generated means that long experiments will produce too much data to fit onto the floppy disk. Such data will therefore be lost.
4. Although this example uses data saved to a floppy disk, some systems can save data to a small internal hard drive or linked PC hard drive. The use of a floppy disk enables the data to be easily transferred to other PCs.
5. Silicon rubber is resistant to repeated dry and wet heat sterilization, and, hence, generally the probes can be repeatedly sterilized without causing damage. However, heating and cooling may slightly affect the permeability of the silicon to gases, making calibration essential after each experiment.
6. Water and other liquids possess a great potential for expansion when converted to a gaseous form. This increase in volume prevents the attainment of the required vacuum within the mass spectrometer.
7. Saturation of the calibration gases is achieved when no further increase in concentration is detected by the mass spectrometer. This will vary at different temperatures, and therefore temperature must also be considered. Calibration should also be carried out under conditions as similar to the experiment as possible. For example, discrepancies can occur if the sample is static but the calibration solution is agitated since a zone of gas depletion can occur around the inlet under static conditions.
8. When membrane integrity is breached or built-up dirt from the surface of the probe enters the mass spectrometer, performance is reduced dramatically. This reduced performance manifests itself as aberrant split peak profiles owing to the ionization of the contaminating matter. Occasionally this dirt may be drawn into the mass spectrometer, leading to a dramatic loss in performance. To prevent this the probes are cleaned whenever discoloration becomes apparent.
9. Ethanol is used to clean the probes to prevent water from entering the system on reconnection, because water can lead to damage of the turbo molecular pump bearings.
10. If this is not done, it is possible that a leak in the membrane may allow a substantial amount of liquid to be drawn into the system.
11. If the membrane does not have a tight seal at the top of the probe, samples can be contaminated with gases traveling from the atmosphere down the length of the probe between steel and membrane to the tip. This can manifest itself as unusually stable readings of atmospheric gases at atmospheric levels. Such readings may also indicate a leak in the system elsewhere.

Acknowledgments

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References

1. Lloyd, D. and Scott, R. I. (1983) Direct measurement of dissolved gases in microbial systems using inlet mass spectrometry. *J. Gen. Microbiol. Methods* **127,** 313–328.

2. Degn, H. (1992) Membrane inlet mass spectrometry in pure and applied microbiology. *J. Microbiol. Methods* **15,** 185–197.
3. Davies, K. J. P., Lloyd, D., and Boddy, L. (1989) Effect of oxygen on denitrification in *Paracoccus denitrificans* and *Pseudomonas aeruginosa. J. Gen. Microbiol.* **135,** 2445–2451.
4. Van Niel, E. W. J., Robertson, L. A., Cox, R. P., and Kuenen, J. G. (1992) Inhibition of denitrification and oxygen utilization by *Thiosphaera pantotropha. J. Gen. Appl. Microbiol.* **38,** 553–558.
5. Lloyd, D., Boddy, L., and Davies, K. J. P. (1987) Persistence of bacterial denitrification capacity under aerobic conditions: the rule rather than the exception. *FEMS Microbiol. Ecol.* **45,** 185–190.
6. Lloyd, D. (1993) Aerobic denitrification in soils and sediments: from fallacies to facts. *Trends Ecology Evol.* **8,** 352–356.
7. Thomas, K. L., Lloyd, D., and Boddy, L. (1994) Effects of oxygen, pH and nitrate concentration on denitrification by *Pseudomonas* species. *FEMS Microbiol. Lett.* **118,** 181–186.
8. Thomsen, J. K., Geest, T., and Cox, R. P. (1994) Mass spectrometric studies of the effect of pH on the accumulation of intermediates in denitrification by *Paracoccus denitrificans. Appl. Environ. Microbiol.* **60,** 536–541.
9. Thomas, K. L. and Lloyd, D. (1995) Measurement of denitrification in estuarine sediment using membrane inlet mass spectrometry. *FEMS Microbiol. Ecol.* **16,** 103–114.
10. Degn, H., Cox, R. P., and Lloyd, D. (1985) Continuous measurement of dissolved gases in biological systems with the quadrupole mass spectrometer. *Methods Biochem. Anal.* **31,** 185–197.
11. Jouanneau, Y., Kelley, B. C., Berlier, Y., Lespinat, P. A., and Vignais, P. M. (1980) Continuous monitoring by mass spectrometry of H_2 production and recycling in *Rhodopseudomonas capsulata. J. Bacteriol.* **143,** 628–636.
12. Berlier, Y. M. and Lespinat, P. A. (1980) Mass spectrometric kinetic studies of the nitrogenase and hydrogenase activities in in-vivo cultures of *Azospirillum brasilense Sp.7. Arch. Microbiol.* **125,** 67–72.
13. Jensen, B. B., Cox, R. P., and Degn, H. (1981) Mass spectrometric measurements of steady-state kinetics of cyanobacterial nitrogen fixation by monitoring dissolved N_2 in an open system. *FEMS Microbiol. Lett.* **12,** 37–40.
14. Jensen, B. B. and Cox, R. P. (1988) Measurement of hydrogen exchange and nitrogen uptake by mass spectrometry. *Methods Enzymol.* **167,** 467–474.
15. Carlsen, H. N., Degn, H., and Lloyd, D. (1991) Effects of alcohols on the respiration and fermentation of aerated suspensions of Baker's Yeast. *J. Gen. Microbiol.* **137,** 2879–2883.
16. Bohátka,. S., Futó, I., Gál, I., Gál, J., Langer, G., Molnár, J., Paál, A., Pintér, G., Simon, M., Szádai, J., Székely, G., and Szilágyi, J. (1993) Quadrupole mass spectrometer system for fermentation monitoring. *Vacuum* **44,** 669–671.
17. Lloyd, D., Ellis, J. E., Hillman, K., and Williams, A. G. (1992) Membrane inlet mass spectrometry: probing the rumen ecosystem. *Journal of Applied Bacteriology Symposium Supplement 1992* **73,** 155S–163S.

18. Virkki, V. T., Ketola, R. A., Ojala, M., Kotiaho, T., Komppa, V., Grove, A., and Facchetti, S. (1995) On-site environmental analysis by membrane inlet mass spectrometry. *Analytical Chemistry* **67,** 1421–1425.
19. Harland, B. J. and Nicholson, P. J. (1993) Continuous measurement of volatile organic chemicals in natural waters. *Sci. Total Environ.* **135,** 37–54.
20. Cristea, O. and Langer, G. (1992) Gas metabolism measurements of aquatic living structures by membrane inlet mass spectrometry for water pollution detection. *Sensors and Actuators B* **7,** 518–521.
21. Benstead, J. and Lloyd, D. (1994) Direct mass spectrometric measurement of gases in peat cores. *FEMS Microbiol. Ecol.* **13,** 233–240.
22. Lauritsen, F. R. and Gylling, S. (1995) On-line monitoring of biological reactions at low parts-per-trillion levels by membrane inlet mass spectrometry. *Analytical Chemistry* 67:1418–1420.
23. Lloyd, D., Davies, K. J. P., and Boddy, L. (1986) Mass spectrometry as an ecological tool for *in situ* measurement of dissolved gases in sediment systems. *FEMS Microbiol. Ecol.* **38,** 11–17.
24. Wilhelm, E., Battino, R., and Wilcock, R. J. (1977) Low pressure solubility of gases in liquid water. *Chem. Rev.* **77,** 219–230.

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Experimental Biofilms and Their Applications in the Study of Environmental Processes

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1. Introduction

1.1. Why Study Biofilms?

The trend in research in recent years has been to extrapolate results from studies of planktonic bacteria into environmental systems. This method of studying planktonic bacteria under in vitro conditions has undoubtedly yielded important data in a wide range of areas; however, the examination of several environmental habitats, extreme or otherwise, such as a drinking water pipeline has revealed only relatively low numbers of planktonic cells. In aquatic systems the biofilm bacterial count per square centimeter of surface has been estimated to be approx 1000-fold higher than the corresponding planktonic count per cubic centimeter ***(1)***. Surface colonization by microorganisms was first recognized as significant as early as 1943 ***(2)***, and there is now a realization that we need to study microorganisms not only as biofilms but also in the context of the biofilm interactions with their immediate surroundings and the influences they exert on the environment. The environment has a significant effect on the metabolic activities of bacteria, and studies of biofilm bacteria represent the best tool for examining growth in natural and pathogenic ecosystems ***(3)***. The study of biofilms is relevant to a wide range of areas, and a multidisciplinary approach is the most productive route forward in the quest to understand the interactions occurring not only between the cells and the surfaces to which they adhere, but between the microcolonies that coexist within multispecies biofilms ***(4)***.

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1.2. What is a Biofilm?

A microbial biofilm is essentially microbial cells immobilized at an interface, covered with a microbially produced exopolysaccharide layer. The initial colonization of surfaces and subsequent growth as a biofilm is the bacterial survival response to environmental stimuli such as low nutrient levels ***(5)***, and may occur as a response to the nutrient accumulation that is thought to occur at air-liquid or liquid-solid interfaces ***(6,7)***. Adhesion to a submerged surface by starved *Vibrio* cells resulted in the cells regaining their normal morphology and growth characteristics ***(5)***. However, attachment has also been observed in systems with an increased dissolved organic carbon content ***(8)***, and higher nutrient and substrate concentrations alone are insufficient to explain the overall effects of surfaces in terms of bacterial activity ***(9)***. Benefits of the attached mode of growth include increased protection against antimicrobial agents ***(10–13)*** and the body's defense mechanisms—phagocytosis, opsonization, and so on. Initially the biofilm was viewed as a homogeneous distribution of cells in a confluent, blanket-like exopolysaccharide matrix ***(3)*** but confocal scanning laser microscopy (CLSM) has been the driving force behind altering our understanding of the processes and the structures within the biofilm. This form of nondestructive visualization has allowed the three-dimensional and real-time visualization of hydrated biofilms. Biofilms are now modeled as microcolonies or clusters of cells enclosed within a hydrated matrix, with pores or channels throughout the nonconfluent biofilm ***(14,15)***. The pores and channels facilitate transport of oxygen and nutrients to the microcolonies and removal of waste and secondary products, but the biofilm matrix or exopolysaccharide has a postulated role in antimicrobial resistance, possibly acting as an ion exchange resin ***(11)*** or ionically hindering the inward diffusion of cationic molecules ***(16)***. Far from being a random structure, the biofilm represents an optimized arrangement of cells to facilitate maximal nutrient diffusion to enable the establishment of microbial consortia allowing metabolic exchange and recycling of essential nutrients ***(17)***, and to facilitate the transfer of plasmids enclosing drug and heavy metal resistance as a result of the close proximity of the cells within the biofilm ***(17,18)***. The biofilm has been likened to a primitive eukaryotic tissue, with homeostatic control mechanisms and a high level of physiological cooperativity ***(19)***.

1.3. The Role of Biofilms in Understanding Environmental Processes

Our understanding of environmental systems and processes has largely been obtained through the extrapolation of data obtained from laboratory systems involving planktonic cells; but this is increasingly inapplicable to real ecosystems ***(3,15)***. Direct observations using some of the techniques described in this

chapter have confirmed the dominance of biofilm bacteria, both numerically and metabolically, in nutrient-sufficient environments ***(1,15)***. Data that can be viewed as unequivocal, owing to its acquisition using nondestructive *in situ* techniques, has demonstrated that biofilm bacteria possess a wide range of phenotypical differences in contrast to their planktonic counterparts ***(15)***. Biofilms have enormous potential to be utilized in the biotechnology industry, because they exhibit a number of capabilities such as the ability to localize a specific biological response (biosensors), production of specific metabolic compounds, and an increased level of performance in a reactor system compared to planktonic bacterial cultures ***(20).*** Wastewater treatment systems commonly utilize trickling filter and porous/nonporous fluidized beds in a number of areas such as denitrification, xenobiotic detoxification, and heavy metal removal from water. Biofilm reactors have been developed and utilized for toluene degradation, removal of uranium, and the degradation of hydrocarbons from wastewater systems ***(20)***. Biofilms play an important role in the biodegradation of organic compounds and the transformation of inorganic compounds, subsequently acting to minimize the buildup of pollutants ***(21)***. Most sections of the human and animal gastrointestinal tract are colonized by bacteria that form tissue-protective biofilms, preventing adhesion by foreign bacteria ***(22)***.

1.4. Detrimental Effects of Biofilms

The uncontrolled and undesirable accumulation of biofilms in biomedical and engineering systems has three primary effects: physical damage, e.g., corrosion and tooth decay; reduction in proper function of the surface, e.g., reduced efficiency of heat exchangers ***(7)*** and turbine power losses in hydroelectric pipelines ***(23)***; and the creation of a reservoir of potential pathogens. Biofouling has been defined as damage to surfaces or the environment as a direct result of surface-associated microbial growth ***(23)***.

1.4.1. Physical Damage and Reduction in Surface Efficiency

Metal corrosion of ships, pipelines, and oil rigs is an expensive problem, with biofilm formation occurring rapidly following immersion of the surface. Marine macrobiofilms on ships act to increase drag and frictional forces, resulting in increased fuel consumption; an 18% difference in power consumption was observed in trials to determine the effect of biofilm removal from ship hulls ***(8)***. The physical thickness of the biofilm reduces the pipe diameter in industrial heat exchanger systems, affecting flow, and the exchange of heat between the liquid and the cooling surface is reduced ***(24)***, with a subsequent estimated cost of £500 million annually ***(23)***. Anaerobic zones are formed within the biofilm as depletion of oxygen occurs by the aerobic microorganisms present in the microbial consortium, favoring the growth of primary corro-

sion organisms such because the sulfate-reducing bacteria (SRB) ***(23)***. Physical damage occurring as a result of biofilm formation can also be observed in the body. Dental caries are an indirect consequence of the formation of a multispecies oral biofilm ("plaque") on the enamel surface of the tooth. Demineralization of the enamel occurs as a result of by-products of the bacterial metabolism, such as organic acids, which become trapped at the tooth surface ***(19)***. The oral microflora utilize biofilm formation not only as a mechanism to avoid the antimicrobial action of salivary components such as lysozyme and mechanical removal, but also to facilitate the optimal utilization of the abundant nutrient supply.

1.4.2. Creation of a Reservoir of Pathogens

The primary concern of the food, water, and medical industries is to determine the potential of the biofilm to act as a pathogen reservoir and to develop effective control strategies ***(6,23,25)***. In most cases, planktonic cell counts do not accurately represent the extent to which biofilm formation is occurring. The contamination of food products may occur following contact with potentially detrimental bacteria sequestered within surface-associated biofilms ***(6)***. The accumulation of coliform bacteria in biofilms in water distribution systems may act to mask the presence of indicator organisms occurring as a result of deficiencies in the treatment processes ***(25,26)***. *Legionella pneumophila* has been demonstrated to be harbored within biofilms that would be present in cooling towers and water systems ***(27)***. The recent increase in the use of indwelling medical devices and advances in intravenous therapy can be correlated with a corresponding increase in nosocomial infection ***(28)***. Extensive bacterial biofilms, formed in the presence of optimal growth conditions provided by the nutrient-rich body fluids, have been observed on sutures, cardiac catheters, central venous lines, pacemakers, heart valves, and prosthetic hip joints ***(7)***, and these biofilms may act as sites for further dissemination of infection. The inherent resistance of the bacteria to phagocytosis and antibiotic chemotherapy may result in the surgical removal of infected devices, in order to dispel chronic device-associated infections ***(24)***.

1.4.3. Strategies for Biofilm Control

Treatment regimes against biofilm-associated infections are normally developed using data that measure measures the efficacy of an antimicrobial agent against planktonic organisms, subsequently resulting in ineffectual eradication of the biofilm ***(29–31)***. Biofilm control can be divided into two areas: the prevention of initial colonization and subsequent biofouling, and the development of removal/control strategies against the established biofilm.

1.4.3.1. Prevention of Biofouling/Adhesion

The efficacy of several different antifouling coatings and repellents against marine biofilms has been examined ***(32)***, but there must be a balance between efficiency against biofilms and the level of toxicity to other marine life. The use of impregnated or coated catheters has been examined as a method of reducing the incidence of catheter-associated urinary tract infections; inhibition of primary adhesion to silver-coated latex catheters has been observed in vitro ***(33)***. The incorporation of biocides such as 10,10-oxybisphenoxyarsine (OBPA) has been shown to reduce adhesion to polyvinyl chloride ***(34)***. However, it appears that regardless of the surface roughness, charge, hydrophobicity, or incorporated antimicrobial agents, bacteria will eventually adhere to any surface, and the search for a completely effective antifouling/antiadhesion surface is ongoing.

1.4.3.2. Sanitization/Removal Strategies

In industrial systems, chemical biocides represent the primary strategy for biofilm control. Chlorine, in four different forms—monochloramine, hypochlorous acid, hypochlorite, or chlorine dioxide—is the most commonly used biocide for chemical treatment of water. Monochloramine has been found to be the most effective in the inactivation of biofilm bacteria ***(35)***. Environmental factors, such as nutrient loading, shear stress, and physiologic properties of the bacteria (e.g., growth rate and metabolic status), will affect the overall properties of the biofilm, subsequently affecting biocide efficiency. The nonuniform pattern of microbial respiratory activity that occurs following monochloramine treatment ***(36)*** is suggestive of variations in antibiotic penetration rates ***(36,37)*** and the presence of distinctive biocide gradients within the biofilm ***(38)*** suggests that local differences occur within biofilms in terms of resistance to chlorine and other disinfectants. The food industry uses sanitizers or disinfectants following detergent treatment; commonly used chemical disinfectants include chlorine, iodine, and ammonium-based compounds. Antibiotic treatment of device-associated infections is largely dependent on the organism, or organisms, involved. However, the inherent resistance of the bacterial biofilms ***(29)*** may result in the surgical removal of the infected device in order to dispel chronic device-associated infections ***(24)***. There is currently a trend toward the development of methods that will enable the testing of the susceptibility of the organism of interest as a biofilm.

2. Materials and Equipment for Studying Biofilms

2.1. Model Systems for Establishing Experimental Biofilms

The complexity of biofilms and the need to study them under laboratory conditions has led to the development of model systems for the establishment

Table 1
Experimental Variables and Parameters for the Investigation of Biofilms Using Laboratory Model Systems

Variables	Parameter
Physical	Temperature, surface composition, surface charge, surface roughness
Chemical	pH, substrate concentration, dissolved oxygen concentration
Biological	Organism type, organism concentration

and study of experimental biofilms. Model systems enable the testing of hypotheses and the extrapolation of data under defined, controlled conditions. There are two main types of experimental biofilm models ***(39)***: replicative, which encompass a wide range of complex environmental variables, and investigative which are generally simpler and enable the control of a variety of influencing factors. Most laboratory systems are of the latter type and tend to examine biofilm formation at solid/liquid interfaces utilizing fixed surfaces. **Table 1** lists several variables and parameters that can be examined using laboratory model systems, and **Fig. 1** lists some analytical methods for the measurement of biofilm parameters. This section describes only a number of the most common laboratory model systems in use; for further information on other systems, *see* **refs. *4***, ***7***, ***15***, and ***40***.

2.1.1. The Robbins Device

The Robbins device was developed at the University of Calgary to examine biofouling in industrial pipelines *in situ* ***(41)***. Initially composed of brass or stainless steel, it was later modified for use in examining medical device–associated biofilms. The modified Robbins device (MRD) is a rectangular Perspex block 44 cm long, 2 cm high, and 2.5 cm wide, with a 2 mm high by 1 cm central lumen and a series of removable studs placed along its length to which different surfaces can be fitted. The system is sterilized using ethylene oxide gas since high temperature/pressure results in warping of the Perspex. This system allows the examination of a range of physical, chemical, and biological parameters on biofilm formation and analysis of the response of biofilms to antibiotic and biocide treatment.

2.1.2. Continuous Culture Flow Cell

The continuous flow of media prevents the accumulation of waste and metabolic products and the depletion of oxygen and nutrients ***(42–44)*** and subsequently enables the control of the bacterial growth conditions ***(44)***. Several different designs of continuous culture flow cell are currently in use, utilizing

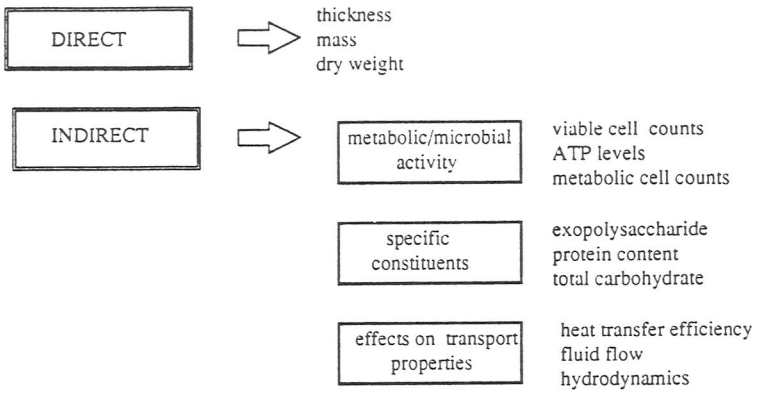

Fig. 1. Experimental variables and parameters for the investigation of experimental biofilms established using laboratory model systems.

materials such as glass and Perspex and ranging from relatively simple, such as two, sealed, glass cover slips with silastic rubber tubing in and outlets, to the more complex. The limitations with the use of flow cells are primarily due to the restriction on the use of transparent surfaces, but when used in conjunction with CLSM, microscopy, image analysis, and metabolic stains, flow cells can provide a great deal of information concerning initial attachment, biofilm structure and functional organization within single and multispecies biofilms.

2.1.3. Perfused Biofilm Fermenter

The perfused biofilm fermenter system was developed to enable distinction between the effects of growth rate and adhesion by selecting synchronous bacterial populations ***(45)***. Midexponential phase bacterial cultures were filtered onto a cellulose acetate membrane; the impregnated membrane was removed and inserted upside down in a continuous fermentation apparatus ***(46)***. Fresh medium was then perfused from below through the filter, with the numbers of eluted cells reaching a steady state after approx 2 h—a situation similar to that of bacterial surface infections of soft tissues. This system has applications for use in examining the effects of antibiotic therapy on soft tissue infections.

2.1.4. Rototorque

Also known as the annular reactor, the rototorque system is composed of two concentric cylinders with a number of removable slides in a continuous culture system. Rotation of the inner cylinder creates a shear field independently of the medium flow ***(47)***. The system is highly sensitive to changes in fluid frictional resistance and is capable of varying fluid shear and stress and

residence times independently. The surface area of the slides are exposed to uniform shear stress values and complete mixing of the liquid in the system enables the analysis of a range of biofilm processes.

2.1.5. Constant Depth Film Fermenter

The constant depth film fermenter is an enclosed fermenter that has been used to examine a river water community ***(48)*** and a *Pseudomonas aeruginosa* metalworking fluid biofilm ***(49)***. It contains a rotatable steel or polytetrafluoroethylene (PTFE) turntable, with a series of removable film pans, each containing six removable plugs. The biofilm is maintained at a constant depth by a scraper blade and, based on protein levels, viable counts, dry weight measurements, and carbohydrate levels, is considered to be "quasi steady-state" ***(40)***. Biofilm formation can be controlled, is reproducible, and is easily sampled under specified nutrient and gas conditions.

3. Methods

3.1. Microscopy

The ability to visualize the biofilm is important in defining the architecture of biofilms and the interactions occurring between the cells and the surfaces. Microscopy has been widely used for the direct visualization of initial attachment and subsequent biofilm formation ***(44,50–53)*** and phenotypic changes following adhesion ***(15,54)***.

3.1.1. Electron Microscopy

The major advantage of electron microscopy is its ability to resolve objects that cannot be seen using light microscopy; the resolution of electron microscopes is approx 0.5 nm compared with the 0.2-µm resolution of differential light absorption microscopes ***(55)***. In electron microscopes, the heating of a tungsten filament generates an electron beam that is focused by a series of magnetic lenses under high vacuum onto the specimen.

3.1.1.1 Scanning Electron Microscopy (SEM)

Unlike conventional bright-field and phase contrast microscopy, SEM does not require a transparent surface. Scanning of the specimen surface by the electron beam causes the emission of secondary electrons that enter the detector and strike a scintillator, generating light flashes, which are converted to an electrical current by the photomultiplier. Subsequent amplification and transmission to a cathode ray tube produce a raster display image ***(55)***. The number of electrons detected is dependent on the surface topography; the presence of depressions causes electrons to be trapped, and consequently, the area appears darker compared to raised and therefore lighter areas. Sample preparation

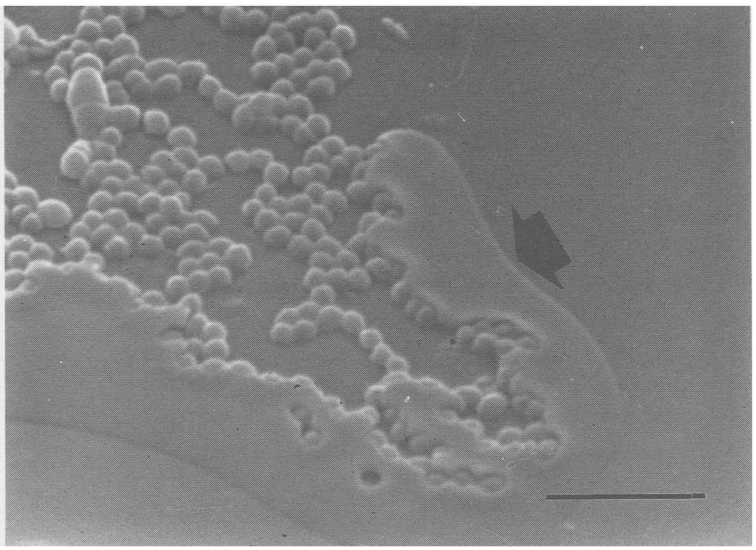

Fig. 2. Laminar flow biofilm (flow rate of 0.8 mL · min^{-1} formed on glass surfaces in a flow-through culture MRD and visualized using SEM. The *Enterococcus faecium* biofilm was 72-h old; microbially produced exopolysaccharide is visible as a blanket-like layer (black arrow), revealing underlying coccoid bacterial cells. Scale bar = 5 μm.

involves the fixation of the surface and attached biofilm using glutaraldehyde or formaldehyde, followed by dehydration and either air or critical-point drying. The specimen is coated with a fine layer of metal particles, placed in the microscope chamber, subjected to a vacuum, and bombarded with electrons ***(56)***. **Figure 2** shows a biofilm on glass visualized by SEM.

3.1.1.2. Electroscan SEM (ESEM)

ESEM, a modified form of SEM enables imaging of hydrated specimens ***(57)*** by placing the specimen in a chamber at pressures exceeding 20 torr—the saturated partial pressure of water at room temperature ***(58)***. This enables the visualization of hydrated specimens under high magnification, with minimized shrinkage and generation of artifacts compared to conventional SEM techniques. However, as is the case with standard SEM, the electron beam damages the specimen in a relatively short period of time.

3.1.1.3. Transmission Electron Microscopy (TEM)

In TEM the electrons are scattered as they pass through the specimen, then focused by magnetic lenses to form an image on a fluorescent screen. TEM has

been used to produce information on biofilm thickness and on the interactions occurring at a cellular level among members of a biofilm. It enables detailed analysis of the spatial arrangements and cellular structure of cells present within the biofilm.

3.1.1.4 Atomic Force Microscope (AFM)

The AFM is a scanning probe microscope, in which variations in voltage occur owing to deflection of the electron cloud at the AFM tip by surface atoms ***(59)***. When a sample is scanned in a raster pattern, variations in the surface topography cause undulations of the cantilever to which a silicon nitride tip is attached. A laser measures this movement and feeds back a signal to the piezoscanner, causing the cantilever deflection to be kept at a constant level. The voltages applied to the piezo scanner can then be converted to an artificially colored image, which consequently mimics the topography of the surface at a constant rate of deflection ***(57)***.

3.1.2 Light and Phase Contrast Microscopy

Studies using bright-field and phase contrast microscopy coupled with image analysis have examined colony development, effects of nutrient concentration on attachment, and so on ***(53,60)***. Phase contrast microscopy has been used to demonstrate reversible and irreversible attachment of marine bacteria to glass surfaces ***(53)***. However, most bright-field and phase contrast microscopy is heavily reliant on the use of transparent surfaces, severely limiting their application to the study of biofilms on opaque materials, except in situations in which stains such as acridine orange and 5-cyano-2, 3-ditolyltetrazolium chloride (CTC) can be used and then visualized using epifluoresence microscopy.

3.1.3. Differential Interference Contrast (DICM) Microscopy

DICM has a marked level of superiority compared to phase contrast microscopy, allowing the observation of biological samples without the generation of artifacts. The DICM microscope is a conventional light microscope with ultraviolet fluorescence, which has undergone reconfiguration of the epifluorescence and episcopic DICM sections to above the microscope stage. These and other adaptations allow the visualization of opaque specimens, and the light intensity can be enhanced by mirrors present in the mercury lamp casing ***(51)***. DICM can provide details of the surface topography of the biofilm and allow visualization of the biofilm exopolysaccharide (EPS) ***(57)***.

3.1.4. Confocal Laser Scanning Microscopy

In CLSM, penetration into thick biofilms is made possible owing to the use of a krypton/argon laser, which excites fluorophore dyes present within the

sample. The resulting fluorescence is detected by photomultiplier tubes and a digital image is obtained. Alteration of the focal (*z* plane) depth and the subsequent collection of the *x*-*y* plane images (parallel to the surface) enables the collection of a series of optical sections that can then be computer processed using image analysis software to create a 3D image ***(61)*** . CLSM is an effective tool for the study of a wide range of biofilm features, including physiologic profiles and structural heterogeneity (*see* also Chapter 17). Because of its ability to allow the *in situ* study of intact, fully hydrated biofilms; the measurement of pH, oxygen, and nutrient profiles and microcolonies using microelectrodes; the analysis of velocity and diffusional processes and a number of other features, CLSM represents a technique of major importance in the study of medical, industrial, and environmental biofilms ***(62)***.

3.1.5 Metabolic/Vital Stains

Laboratory techniques utilized in the enumeration of planktonic bacteria, such as viable cell counts, possess an inherent tendency to underestimate the total number of viable bacteria present owing to the presence of viable but nonculturable cells or a biofilm. Direct microscopic techniques coupled with the use of vital stains represent a more accurate technique for the enumeration and visualization of such bacteria. Metabolic stains such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and INT (2-[*p*-iodophenyl]-3-[*p*-nitrophenyl]-5-phenyltetrazolium chloride) have been used to detect metabolically active bacteria present in water samples ***(63)***, on pipelines, and in disinfected biofilms ***(64)***. In the presence of an active electron transport chain, CTC undergoes reduction, resulting in the formation of an insoluble purple CTC-formazan crystal that fluoresces red when excited with a certain wavelength of epifluorescent light. CTC has the advantage over the related compound INT in that it allows the visualization of actively respiring cells on membrane filters and other optically opaque surfaces such as wood, metal, and plastic ***(65)***. Other fluorogenic compounds that have been used to assess biofilm physiological activity include rhodamine, which determines membrane potential ***(66)***, and 4,6-diamidino-2-phenylindole (DAPI), which stains living and dead cells.

3.2. Additional Techniques for Studying Biofilms

3.2.1. Attenuated Total Reflection Fourier Transform Infrared Spectrometry (ATR/FTIR)

In the study of biofilms, ATR-IR radiation is directed through an internal reflectance element (germanium or zinc selenide crystals) to which bacteria are attached. IR radiation is absorbed by a molecule when the energy of the radiation is equal to that required to put the molecule in an excited, vibrational state. This absorption only occurs at discrete frequencies, and the number of

molecules present is proportional to the amount of radiation absorbed. This frequency-dependent absorption produces a unique absorbance pattern of the spectrum that is defined by the structure of the molecule. In biofilms this spectrum is the composite of the spectral signatures of each of the biomolecules present. The frequency at which a molecule absorbs radiation is determined by the presence of specific groups of atoms within the molecule. The group frequency is defined as the individual wave number range at which a specific group of atoms absorbs radiation. Differences in molecular structure can subsequently be identified and quantified using tables of characteristic frequencies to identify specific IR absorbance bands ***(52)***.

3.2.2. Cryoembedding

Cryoembedding is applicable to biofilms of variable thickness on an assortment of opaque or transparent surfaces, and involves the fixation of a biofilm using a cryoembedding compound that contains a number of water-soluble polymers to maintain the intact biofilm structure ***(64)***. The embedding compound is placed onto the biofilm while it is still attached to the surface. This process is carried out on dry ice in order to freeze the sample rapidly, avoiding the formation of ice crystals. The embedded biofilm is removed from the surface and the opposing side embedded, so that the frozen biofilm is sandwiched between the embedding compound ***(66)***. Cross sections of variable thickness can then be cut using a cryostat and imaged using microscopy. Minimal sample disruption ensures that individual cells, microcolonies, and water channels all remain visible; physiologic gradients of metabolic activity, such as those present following antibiotic treatment, can be observed using a combination of metabolic dyes and fluorescence microscopy.

3.3. Model Systems and Experimental Biofilms

Experimental biofilms established using model systems represent a useful tool for the laboratory-based study of sanitization and disinfection strategies, metabolic processes, nutrient utilization, gene transfer, and biodegradation. They allow the examination of a wide range of hypotheses or the determination of those parameters that have a role in influencing biofilm formation, architecture, and functional characteristics. The production of reproducible biofilms under laboratory conditions represents an important factor in the study of biofilms, with particular relevance to environmental processes.

3.4. Applications of Biofilms to Study Industrial Systems

Despite the applicability of experimental biofilms and associated techniques for the study of environmental processes, it should be recognized that biofilms are the site where the majority of environmental processes occur, rather than

simply a tool to facilitate their study. The study of biofilms encompasses a wide range of disciplines and has many important applications in furthering our understanding of key environmental processes. The primary use of biofilms, from an industrial point of view, is in the control of unwanted biofilms, e.g., in the development of antifouling coatings or surfaces that will reduce or prevent microbial adhesion.

3.4.1 Influence of Surface Type on Adhesion and Biofilm Formation

SEM (**Subheading 2.2.1.1.**) has been used to examine the attachment mechanisms utilized by marine-fouling bacteria to glass, plastic, and antifouling painted surfaces ***(67)***. The MRD (**Subheading 2.2.1.**) enables the testing of a wide range of surface types in batch or continuous culture and is a good model for studying flow system biofilms ***(68)***, although it does not allow distinction between factors attributable to growth rate and those owing to adhesion. Mild-steel surfaces exhibited a 10-fold difference in the number of colonized heterotrophic bacteria relative to polycarbonate surfaces when examined using the annular reactor (**Subheading 2.2.4.**) in a study of the persistence of coliforms in mixed-population biofilms ***(69)***.

3.4.2. Physiological Effects of Biocides

To evaluate a particular antimicrobial agent for utilization in treatment regimes, it is necessary to determine the effects on the biofilm in terms of alterations to the physiology or metabolic activity of the bacterial cells. Biofilms that form in heat exchangers, pipelines, and drinking water systems ***(26,38)*** are notably resistant to chlorine which is frequently the main disinfectant of choice. This poses several questions; e.g.: Is this the result of an inability of the biocide to penetrate the biofilm as a result of the presence of an EPS matrix or owing to transport or diffusional processes? Are all of the cells within a biofilm equally affected by the treatment? The use of chlorine microelectrodes and CLSM for the visualization of chlorine penetration into a mixed *P. aeruginosa* and *Klebsiella pneumoniae* biofilm grown in a rototorque revealed the presence of reaction-diffusion interactions, which resulted in limited chlorine penetration into the biofilm ***(38)***. The gradients of physiologic activity within a biofilm following biocide treatment that have been visualized using cryoembedding and image analysis have shown a nonuniform loss of respiratory activity within the biofilm ***(36)***. Cryoembedding has also been used to visualize the physiological responses of bacteria in biofilms to treatment with chlorine ***(37,64)***. To date, there seems to be no single factor that can be identified as being solely responsible for the observed recalcitrance of biofilms. One thousand to 10,000-fold higher concentrations of antimicrobial agents may be required to cause levels of killing equivalent to those observed with planktonic

cells ***(29)***. It is postulated that a combination of altered antibiotic permeability with regard to the cell envelope, binding of antibiotic molecules or modification of molecular targets by EPS ***(29)***, altered physiological status of the cells at different sites within the biofilm ***(36,37)***, and growth rate ***(12)*** all influence the effectiveness of a particular treatment strategy.

3.4.3 Biocorrosion and Pitting

The biofilms existing in the majority of natural ecosystems are present as complex mixed communities, which possess complementary metabolic functions, resulting in the formation of several localized microenvironments. Biofilms are recognized as playing an important role in biocorrosion, and this role can be attributed to a number of features of the biofilm ***(70)***. The heterogeneity inherent of many biofilms ***(47,71–73)*** results in the establishment of localized corrosion cells; anaerobic zones created by the utilization of oxygen by the aerobic and facultatively aerobic organisms favor the growth and activity of SRB. Under optimal conditions, the SRB are important contributors in corrosion. Enhancement of their activity may occur as a result of the EPS, which is capable of acting both as a metal binder and in the retention of corrosion products. AFM (**Subheading 3.1.1.4.**) has been used to examine a bacterial biofilm on a copper surface (previously assumed to be toxic to microorganisms) and has shown that the organism tested was directly associated with the pitting corrosion of copper ***(59)***. AFM does not require sample dehydration and can provide information on the association between the cells, the EPS produced and the surfaces to which they attach. Positioning of a microelectrode tip (<10 μm) in relation to microcolonies and water channels has been used to examine the pH and dissolved oxygen levels in biofilms present at metal/artificial seawater interfaces ***(74)***. In a *P. aeruginosa* biofilm, the levels of dissolved oxygen decreased as the microelectrode was moved away from the biofilm interface and deeper into the less aerobic central zones of the microcolony ***(3)***.

3.4.4 Fluid Flow Systems

Laboratory studies involving experimental biofilms are relevant to industrial and natural systems. Knowledge concerning the effects of factors such as flow rate, hydrodynamics, and shear stress is applicable not only to the undesirable biofilm causing a reduction in the flow rate of a pipeline but also to the aquatic biofilms on rocks in fast-flowing rivers or streams. Biofilm accumulation in pipelines can affect the hydrodynamics of the system, with consequences for heat and mass transfer properties. Even under conditions of turbulent flow, which are common in both natural and engineered systems, a laminar flow sublayer probably exists in the vicinity of the pipe wall ***(68)***.

Variations in the flow rate will influence diffusion rate and nutrient availability and, subsequently, colonization levels. Liquid flow velocity in model systems is an important factor in predicting the effect of a biofilm on system hydrodynamics. **Figure 3** shows a biofilm developed under turbulent conditions and visualized using CLSM. The MRD has been used in an examination of the relationship between biofilm formation and laminar flow conditions ***(68)***. By tracking fluorescently labeled latex beads through the biofilm present on the surface of a flow cell using CLSM, it is possible to link flow velocity with various physical parameters such as biofilm structural heterogeneity ***(71)***. The structural heterogeneity of a biofilm may correspond with heterogeneity in some physiologic parameters such as dissolved oxygen gradients. Transport processes occurring within biofilms will influence the supply of oxygen and nutrients and the overall efficiency of biocides such as chlorine.

3.4.5. Food and Water Treatment Industries **(75)**

Biofilms possess the potential to act as reservoirs for potentially harmful microorganisms capable of affecting the quality of the finished product ***(25)***. This is highly undesirable and there is a need to determine the extent to which existing strategies used in cleaning and sanitization are effective in eradication of these microorganisms ***(6)***, and to develop further strategies for the prevention of bacterial adherence, e.g., by polymer surface modification ***(75)***. The use of a combination of two fluorogenic compounds and cryoembedding to examine gradients in respiratory activity in a mixed culture biofilm following disinfection with monochloramine revealed a nonuniform loss of respiratory activity within the biofilm following monochloramine treatment ***(36)***. The highest loss of activity occurred at the surface of the biofilm near the biofilm and bulk fluid interface, leaving underlying organisms relatively unaffected in terms of their respiratory and metabolic activity. Biofilm bacteria may directly affect water quality by entering the bulk phase liquid or may represent a site for the sequestration of viruses ***(76)***, coliform bacteria ***(69)***, or pathogens such as legionella. Since these organisms will not be detected during standard sampling procedures, this will consequently mask the true quality of the finished water in terms of microbial load. The indirect effects of the presence of biofilm bacteria may include changes in taste or odor, and discoloration of the finished water owing to microbial biodegradation. Fluorescence microscopy of a laboratory-grown drinking water biofilm stained with a β-galactosidase probe showed the presence of *Escherichia coli* (containing a l*ac*Z reporter gene) ***(25)***, demonstrating the ability of coliforms such as *E. coli* to become situated within a biofilm. Coliforms acclimatized to oligotrophic conditions similar to those inherent in water distribution systems were found to be successful in colonization of the mild steel and polycarbonate surfaces of an annular reactor ***(69)***. Differential interference contrast microscopy (DICM) (**Subheading 2.2.3.**) combined with fluorescein

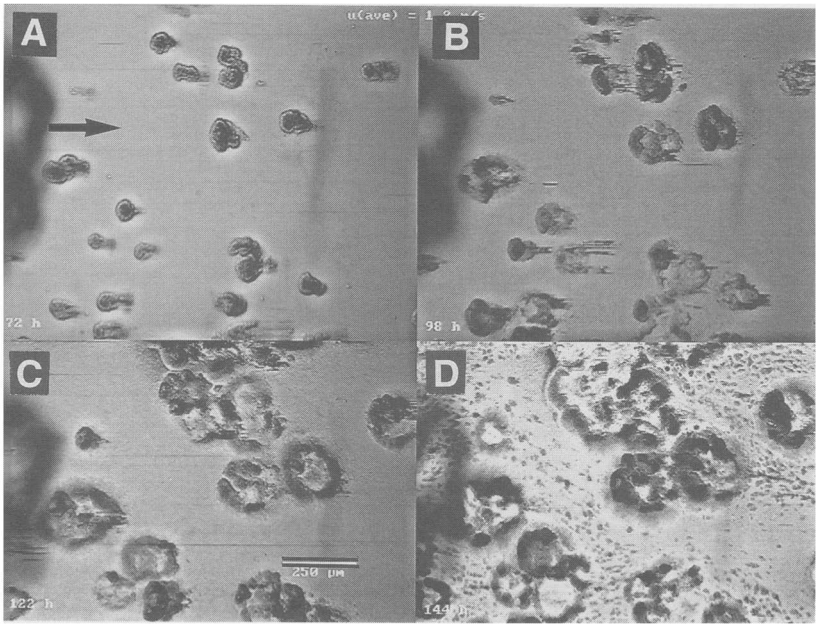

Fig. 3. Biofilm developing under turbulent flow conditions on a glass cover slip in a polycarbonate flow channel at 72 h **(A)**, 98 h **(B)**, 122 h **(C)**, and 144 h **(D)** using CLSM in transmitted light mode. The biofilm was composed of *P. aeruginosa*, *Pseudomonas fluorenscens*, and *K. pneumoniae*. The average flow velocity was 1.8 ms^{-1}, the flow direction is indicated by the arrow in (A). The large black mark on the left edge of each panel is a relocation mark drawn on the outside of the cover slip. Scale (C) = 250 µm. (Image supplied by Paul Stoodley, University of Exeter.)

immunolabeling has demonstrated the presence of *L. pneumophila* within a multispecies tap water biofilm grown on plumbing material surfaces ***(77)***.

3.5. Medical Environments

Any foreign implant introduced into the body, such as a catheter, an artificial joint, or heart valve, represents a potential site for biofilm formation owing to the absence of the normal host defence mechanisms associated with, e.g., the mucous layer ***(78)***. The physical damage/disruption of tissues or organs by invasive techniques may result in a loss of the protective antiadhesion coatings maintained by the body and the establishment of an opportunistic infection ***(79)***.

3.5.1. Development of Antifouling/Adhesion Coatings

The development of antiinfective devices such as catheters is desirable due to the high incidence of associated nosocomial infection and primary septice-

mia ***(80)***. The MRD represents a good in vitro model system for testing the efficacy of various incorporated antimicrobial agents ***(80)***. A combination of minocycline and rifampin were found to be effective against Gram-positive cocci, Gram-negative bacilli and yeasts such as *Candida albicans*.

3.5.2. Evaluation of Antibiotic Susceptibility

Current tests for the determination of antibiotic sensitivity on which treatment strategies are based, such as the disk diffusion assay and minimum inhibitory concentration tests ***(30,81)***, rely heavily on the use of planktonic cells. The role of biofilms in clinical disease is well documented ***(7,28)***, and recent increases in the use of indwelling medical devices such as catheters and artificial joints have been closely shadowed by an increased incidence of implanted device-associated infections ***(82)***. Coagulase-negative staphylococci account for more occurrences of device-associated infection than any other microorganism ***(83)***. The MRD represents a good in vitro model for studying this colonization with a view to determining effective concentrations of antibiotics, either for treatment of the infection or as a coating on the catheter surface to decrease initial attachment of the cells ***(80)***. ATR/FTIR has been used to study the effects of biofilms on substrate and in the examination of biofilm composition ***(37,52,84)***, and also in a medical context to investigate the penetration of ciprofloxacin into a *P. aeruginosa* biofilm; the penetration of the antibiotic from the bulk fluid to the surface was significantly reduced by the presence of the biofilm ***(37)***. The perfused biofilm fermenter closely mimics the situation which occurs with soft-tissue infections, and represents a good model for examining the role of cell growth rate in antibiotic resistance by biofilms ***(12)***.

3.6. Natural Systems

Biofilms were first studied with particular relevance to aquatic systems ***(79)***, and research into this ubiquitous mode of bacterial growth has subsequently expanded into all natural environments. Growth as a biofilm enables exploitation of the nutrients which may be concentrated at a surface ***(5,9)***, protects against desiccation and changes in the pH, temperature, or osmolarity of the environment, and may offer increased protection from grazing predators such as amoebae and protozoa ***(85)***.

3.6.1 Gene Transfer/Exchange

The requirement to understand gene transfer, as it occurs in terms of the ability of the natural population to uptake exogenous DNA, has been prompted by concerns about the ability of indigenous populations to uptake genetic sequences from engineered organisms. Transformation occurring in the river epilithon ***(86)*** has been suggested to represent a possible mechanism by which

resistance genes could be spread through natural populations; the transfer of mercury-resistant plasmids from epilithic communities to *Pseudomonas pudita* recipients ***(17)*** has been demonstrated. Further studies of genetic transfer between microbial communities in aquatic and terrestrial environments should account for the existence of the majority of microorganisms, as biofilm communities and experimental systems should be designed accordingly.

3.6.2. Influence of External Factors on Microbial Biofilm Formation

Environmental forces such as temperature and nutrient concentration exert an effect on microbial behavior in the natural environment. Lawrence and Caldwell ***(43)*** used light microscopy and continuous-flow slide cultures to demonstrate a number of colonization maneuvers shown by bacteria from a natural stream community. Computer-enhanced microscopy has been used to examine colony development on surfaces ***(43)***, examine the behavior of bacterial stream populations within the hydrodynamic surface layers of microenvironments ***(43)*** and to look at the effects of different concentrations of organic nutrients on bacterial colonization ***(5)***.

3.7. How Representative Are Experimental Biofilms?

Experimental biofilms represent a compromise between two extreme types of models; holistic, which involves a study of the complete systems, and reductionistic, which enables wider predictions to be made following the study of individual elements of the system, but which often does not take into account community interactions ***(87)***. Most model systems and experimental biofilm studies fall somewhere in the middle of these two classifications. The use of holistic/replicative models for producing experimental biofilms represents the best system in terms of their relative similarity to environmentally occurring biofilms, but the variety and number of ecologic niches means that these types of models can vary widely ***(39)***. Reductionistic/investigative models simplify the environment, producing reproducible biofilms for the examination of features common to different systems. These enable a wider degree of control over specified experimental factors that have a postulated role in influencing structural and functional processes ***(87)***.

The biofilms present in the environment, on riverbeds, oil rigs, or ship hulls represent a consortia of bacteria, fungi, algae, and protozoa; biofilms in the body are composed of a number of different bacterial species. For example, in the case of dental plaque the primary plaque formers are the oral streptococci, which are then followed by secondary formers such as fusobacterium ***(88)***. Monoculture (single species) biofilms are widely used in laboratory studies but are more usually only present in soft-tissue infections such as endocarditis. Biofilm characteristics are reflective of their growth environment ***(89)*** and are

influenced by nutrition, fluid dynamics, species composition, and physicochemical properties. The laboratory environment differs significantly from the external environment in terms of fluctuations in nutrient supply and demand, and growth conditions should therefore attempt to mimic those observed in vivo ***(29)***. The nature of the growth-limiting nutrient is important in influencing the phenotypic characteristics of the cells ***(90)*** and, consequently, must be considered prior to the extrapolation of data from in vitro models to *in situ* biofilms ***(39)***. In the environment, biofilms represent dynamic systems, with complex interactions such as predator-prey relationships, e.g., the grazing of protozoa on biofilms ***(85)***. These relationships and others, such as specific changes in the physicochemical properties of the surface or microenvironment and the interactions occurring between complex microbial communities, can often prove difficult to model under a laboratory environment. For example, in the perfused biofilm fermenter model, the nutrient concentration will be similar for most of the cells; however, in a naturally occurring biofilm, nutrient gradients and subsequent differences in cell physiology exist owing to the spatial distribution of the cells ***(85)***. The modified Robbins device (MRD) allows no distinction to be made between features occurring as a result of adhesion and those owing to growth rate ***(40,45)***.

3.8. Visualization of the Biofilm

Since CLSM, light, and phase contrast microscopy are limited to transparent surfaces, this restricts the variety of substrates that can be examined. Although electron microscopy enables a variety of surfaces to be examined, dehydration of the sample prior to examination severely condenses the hydrated glycocalyx ***(58)***, destroying the complex architecture of the biofilm (with the exception of environmental SEM ***[58]***). Structural components of the biofilm are often lost during preparation for SEM, sampling is sacrificial, and artifacts are common—a cell may appear present in a pit, suggesting breakdown of the surrounding substratum, but the pit may actually be the condensed residue of the dehydrated glycocalyx ***(7)***. Despite its applicability with the study of biofilms, there are some of disadvantages with CLSM: the use of autofluorescing environmental samples may cause problems when used in conjunction with a fluorescent stain in visualizing objects within a biofilm; shadowing may sometimes occur owing to the presence of objects that are not penetrated by the laser beam, and extremely thin specimens may be difficult to find ***(91)***. ATR/FTIR only examines the base layer (approx 1 μm) and averages the picture to apply to all of the exposed area ***(52)***.

3.9. Sampling Techniques

Primary methods for the enumeration of bacterial viability and activity are largely dependent on the ability of the bacteria to form colonies on laboratory

media. Sublethal injury following exposure to antimicrobial agents, reduced culturability, and the varied microbial composition of biofilm communities may hamper the accurate assessment of surface-associated bacteria ***(92)***. Physical removal of the adherent cells results in changes in the physiologic characteristics of the cells. The spatial distribution of the cells and interspecies interactions may be important in influencing the biodegradative efficiency of a biofilm ***(93)*** or in determining the effects of antimicrobial agents on biofilm processes ***(92)***. There is, therefore, a need to develop techniques that enable the nondestructive analysis of biofilms, as opposed to destructive sacrificial procedures such as colony counts, total cell counts, and SEM. Metabolic stains such as CTC ***(65)*** and rhodamine 123 ***(94,95)*** have been coupled with microscopic visualization and used for the *in situ* study of bacterial metabolic activity ***(96)***.

3.10. Future Applications for Biofilms in the Study of Environmental Processes

It is now clear that biofilms represent the primary tool in the processes of gaining a clearer understanding of a wide range of environmental processes that have previously relied on the extrapolation of data obtained from planktonic microbial cultures ***(15)***. The study and use of biofilms in environmental processes is applicable to a wide range of areas; general areas of future interest may include the following:

1. The further determination of important biofilm structural and metabolic processes that will enable the development of a model of structure/architecture applicable to both high- and oligotrophic nutrient environments.
2. The development of systems to enable the more accurate assessment of the efficacy of biocides and antimicrobial agents; i.e., less reliance on the use of planktonic cell systems in the assessment of antibiotic efficacy prior to the treatment of biofilm-associated infections or in the development of sanitization strategies in the food industry.
3. A more detailed understanding of both cell-cell and cell-interface interactions to enable the development of antifouling surfaces/coatings for use across a wide range of industries, coupled with an understanding of the mechanisms/signaling processes involved in defining biofilm structure/architecture.
4. A study of the underlying genetic processes that influence biofilm formation, such as the expression of genes related to alginate or EPS production; the production of cell signaling factors, and the genetic characteristics that account for the observed physiologic differences between the planktonic and biofilm cells.
5. The determination of the degradation rates of pollutants that occur in the environment by biofilm bacteria rather than owing to planktonic cells. For example, the adsorption of organic pollutants and surfactants onto sediments present in

soils or rivers may act to stimulate attachment, resulting in accelerated biodegradation and depletion of the absorbed surfactant ***(97)***.

6. An understanding of the interactions that occur between the bacterial populations present in a multispecies biofilm in terms of nutrient exchange and recycling, utilization of oxygen, and subsequent effects on metabolic activity, cell distribution, and interspecies cooperation.

3.11. Biofilms and Environmental Monitoring

A model system is essentially a smaller scale reproduction or simplification of a complex system, which allows calculations to be made, along with the testing of hypotheses and predictions. The choice of the system to be used for the production of an experimental biofilm is a process that involves an analysis of the ultimate end-point requirements: Do we require qualitative (SEMs, AFM, light microscopy images) or quantitative (viable counts, metabolic counts, total carbohydrate levels) data? Are we interested in biological, physical, or chemical parameters? How accurate a representation of the natural environment do we require? Once determined, we can then balance the desirable features against the disadvantages of the system of interest. No single model will produce a biofilm capable of examining all areas of interest simultaneously. Whether looking at the chemical factors influencing biofilm formation, the effects of antimicrobial treatment regimes, or the influence of structure on degradative ability, an awareness of the inherent problems connected with the experimental model systems can allow the selection of a system with optimized applications for the area of interest being investigated. A knowledge of the movement of particles and fluids, physiological conditions within the biofilm, the presence of chemical and physical gradients, the spatial arrangement of cells, and diffusional and transport processes occurring within biofilms is important in furthering our understanding of dynamic processes such as nutrient transport and the diffusion of antimicrobial agents. Biofilms are ubiquitous and represent the site for the majority of environmental process. As such, they therefore represent an essential experimental tool in the quest to understand those environmental systems and processes of interest to us.

References

1. Costerton, J. W., Nickel, J. C., and Ladd, T. I. (1986) Suitable methods for the comparative study of free-living and surface-associated bacterial populations, in *Bacteria in Nature, vol.* **2,** (Poindexter, J. S. and Leadbetter, E. R., eds.), Plenum, New York, pp. 49–84.
2. Zobell, C. E. (1943) The effect of solid surfaces on bacterial activity. *J. Bacteriol.* **46,** 39–56.
3. Costerton, J. W., Lewandowski, Z., deBeer, D., Korber, D., and James, G. (1994) Biofilms: the customised microniche. *J. Bacteriol.* **176(8),** 2137–2142.

4. Characklis, W. C. and Marshall, K. C. (1989) Biofilms: a basis for an interdisciplinary approach, in *Biofilms*, (Characklis, W. C. and Marshall, K. C., eds.), Wiley Interscience, New York, NY, pp. 3.
5. Kjellerberg, S. and Hermansson, N. (1984) The effect of interfaces on small starved marine bacteria. *Appl. Environ. Microbiol.* **48,** 497–503.
6. Zottola, E. A. and Sasahara, K. C. (1994) Microbial biofilms in the food processing industry—should they be a concern? *Int. J. Food Microbiol.* **23,** 125–148.
7. Costerton, J. W., Cheng, K.-J., Geesey, G. G., et al. (1987) Bacterial biofilms in nature and disease. *Ann. Rev. Microbiology* **41,** 435–464.
8. Bohlander, G. S. (1991) Biofilm effects on drag: measurements on ships, in *Polymers in a Marine Environment*. Marine Management (Holdings), p. 135.
9. van Loosdrecht, M. C., Lyklema, J., Norde, W., and Zehnder, A. J. B. (1990) Influence of interfaces on microbial activity. *Microbiological Rev.* **54(1),** 75–87.
10. Anwar, H., Dasgupta, M. K., and Costerton, J. W. (1990) Testing the susceptibility of bacteria in biofilms to antibacterial agents. *Antimicrob. Agents Chemother.* **34(11),** 2043–2046.
11. Anwar, H., Strap, J. L., and Costerton, J. W. (1992) Establishment of ageing biofilms: possible mechanisms of bacterial resistance to antimicrobial therapy. *Antimicrob. Agents Chemother.* **36(7),** 1347–1351.
12. Brown, M. R. W., Allison, D. G., and Gilbert, P. (1988) Resistance of bacterial biofilms to antibiotics: a growth related effect? *J. Antimicrob. Chemother.* **22,** 777–780.
13. Nickel, J. C., Ruseska, I., Wright, J. B., and Costerton, J. W. (1985) Tobramycin resistance of cells of *Pseudomonas aeruginosa* growing as a biofilm on urinary catheter material. *Antimicrob. Agents Chemother.* **27,** 619–624.
14. Marshall, K. C. (1994) Microbial adhesion in biotechnological processes. *Curr. Opin. Biotechnol.* **5,** 296–301.
15. Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., and Lappin-Scott, H. M. (1995) Microbial biofilms. *Annu. Rev. Microbiol.* **49,** 711–745.
16. Hoyle, B., Jass, J., and Costerton, J. W. (1990) The biofilm glycocalyx as a resistance factor. *J. Antimicrob. Chemother.* **26,** 1–6.
17. Bale, M. J., Fry, J. C., and Day, M. J. (1988) Transfer and occurrence of large mercury resistance plasmids in river epilithon. *Appl. Environ. Microbiol.* **54,** 972–978.
18. Lappin-Scott, H. M., Costerton, J. W., and Marrie, T. J. (1992) Biofilms and biofouling, in *Encyclopaedia of Microbiology*, Academic, pp. 277.
19. Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D., and Lappin-Scott, H. M. (1995). Microbial Biofilms. *Ann. Rev. Micro.* 4**9,** p713.
20. Bryers, J. D. (1994) Biofilms and the technological implications of microbial cell adhesion. *Colloids Surfaces B: Biointerfaces* **2,** 9–23.
21. Marshall, K. C. (1992) Biofilms: an overview of bacterial adhesion, activity and control at surfaces. *ASM News* **58(4),** 202–207.
22. Geesey, G. G., Stupy, M. W., and Bremer, P. J. (1992) The dynamics of biofilms. *International Biodeteriation and Biodegradation* **30,** 135–154.

23. Lappin-Scott, H. M. and Costerton, J. W. (1989) Bacterial biofilms and surface fouling. *Biofouling* **1,** 323–342.
24. MacLeod, F. A., Guiot, S. R., and Costerton, J. W. (1995) Electron microscopic examination of the extracellular polymeric substances in anaerobic granular biofilms. *World J. Microbiol. Biotechnol.* **11,** 481–485.
25. Robinson, P. J., Walker, J. T., Keevil, C. W., and Cole, J. (1995) Reporter genes and fluorescent probes for studying the colonisation of biofilms in a drinking water supply line by enteric bacteria. *FEMS Microbiol. Lett.* **129,** 183–188.
26. LeChevallier, M. W., Babcock, T. M., and Lee, R. G. (1987) Examination and characterisation of distribution system biofilms. *Appl. Environ. Microbiol* **53(12),** 2714–2724.
27. Walker, J. T., Rogers, J., and Keevil, C. W. (1993) An investigation of the efficacy of a bromine-containing biocide on an aquatic consortium of planktonic and biofilm microorganisms including *Legionella pneumophila*. *Biofouling* **8,** 47–54.
28. Goldmann, D. A. and Pier, G. B. (1993) Pathogenesis of infections related to intravascular catheterisation. *Clin. Microbiol. Rev* . **6(2),** 176–192.
29. Anwar, H. and Costerton, J. W. (1992) Effective use of antibiotics in the treatment of biofilm-associated infections. *ASM News* **58(12),** 665–668.
30. Jones, R. N., Barry, A. L., Gavan, T. L., and Washington, J. A. (1985) Susceptibility tests: microdilution and macrodilution broth procedures, in *Manual of Clinical Microbiology* (Lennette, E. H., Balows, A., Hausler, W. J., and Shadomy, H. J., eds.), ASM, Washington, DC, pp. 972–977.
31. Amabile-Cuevas, C. F., Cardenas-Garcia, M., and Ludgar, M. (1995) Antibiotic resistance. *American Scientist* **83,** 320–329.
32. Eighmy, T. T., Arwa, J., deRome, L., et al. (1992) Controlled release of antifouling coatings. II. The effects of controlled release of **2,**4-dinitrophenolate and benzoate on marine biofilm development and metabolic activity. *Biofouling* **6,** 147–163.
33. Gabriel, M. M., Mayo, M. S., May, L. L., Simmons, R. B., and Ahearn, D. G. (1996) In vitro evaluation of the efficacy of a silver-coated catheter. *Curr. Microbiol.* **33,** 1–5.
34. Jones, C. R., Handley, P. S., Robson, G. D., Eastwood, I. M., and Greenhalgh, M. (1996) Biocides incorporated into plasticised polyvinylchloride reduce adhesion of *Pseudomonas fluorescens* BL146 and substratum hydrophobicity. *J. Appl. Bacteriol.* **81,** 553–560.
35. Yu, F. P., Pyle, B. H., and McFeters, G. A. (1993) A direct viable count method for the enumeration of attached bacteria and assessment of biofilm disinfection. *J. Microbiol. Methods* **17,** 167–180.
36. Huang, C. T., Yu, F. P., McFeters, G. A., and Stewart, P. S. (1995) Non-uniform spatial patterns of respiratory activity within biofilms during disinfection. *Appl. Environ. Microbiol.* **61(6),** 2252–2256.
37. Suci, P. A., Mittelman, M. W., Yu, F. P., and Geesey, G. G. (1994) Investigation of ciprofloxacin penetration into *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* **38(9),** 2125–2133.

38. deBeer, D., Srinivasan, R., and Stewart, P. S. (1994) Direct measurement of chlorine penetration into biofilms during disinfection. *Appl. Environ. Microbiol.* **60(12),** 4339–4344.
39. Gilbert, P. (1995) The value of in-vitro models to the study of biofilms, in *The Life and Death of Biofilm* (Wimpenny, J., Handley, P., Gilbert, P., and Lappin-Scott, H. M., eds.), Cardiff, BioLine, Cardiff, UK, pp. 13–16.
40. Gilbert, P. and Allison, D. G. (1993) Laboratory methods for biofilm production, in *Microbial biofilms: formation and control* (Denyer, S. P., Gorman, S. P., and Sussman, M., eds.), Blackwell Scientific, Oxford, UK, pp. 29.
41. McCoy, J. C., Bryers, J. D., Robbins, J., and Costerton, J. W. (1981) Observations in fouling biofilm formation. *Can. J. Microbiol.* **27,** 910–917.
42. Schalkowsky, S. and Hunt, L. G. (1995) Assessment of therapeutic potential by means of a probability model of antimicrobial action. *J. Antimicrob. Chemother.* **35,** 31–52.
43. Lawrence, J. R. and Caldwell, D. E. (1987) Behaviour of bacterial stream populations within the hydrodynamic boundary layers of surface microenvironments. *Microb. Ecol.* **87(14),** 15–27.
44. Caldwell, D. E., and Lawrence, J. R. (1995) Study of attached cells in continuous-flow slide culture, in *Handbook of Laboratory Model Systems for Microbial Ecosystems*, pp. 117–138.
45. LeChevallier, M. W., Cawthon, C. D., and Lee, R. G. (1988) Inactivation of biofilm bacteria. *Appl. Environ. Microbiol.* **54(10),** 2492–2499.
46. Gilbert, P., Allison, D. G., Evans, D. J., Handley, P. S., and Brown, M. R. W. (1989) Growth rate control of adherent bacterial populations. *Appl. Environ. Microbiol.* **55,** 1308–1311.
47. Gjaltema, A., Arts, P. A. M., van Loosdrecht, M. C. M., Kuenen, J. G., and Heijnen, J. J. (1994) Heterogeneity of biofilms in rotating annular reactors: occurrence, structure and consequences. *Biotech. Bioeng.* **44,** 194–204.
48. Peters, A. C. and Wimpenny, J. W. T. (1988) A constant depth laboratory film fermenter. *Biotech. Bioeng.* **32,** 263–270.
49. Kinniment, S. L. and Wimpenny, J. W. T. (1990) Biofilms and biocides. *International Biodeterior.* **26,** 181–194.
50. Geesey, G. G. and White, D. C. (1990) Determination of bacterial growth and activity at solid-liquid interfaces. *Annu. Rev. Micro.* **44,** 579–602.
51. Keevil, C. W. and Walker, J. T. (1992) Normarski DIC microscopy and image analysis of biofilms. *Binary* **4,** 92–95.
52. Nivens, D. E., Palmer, R. J., and White, D. C. (1995) Continuous nondestructive monitoring of microbial biofilms: a review of analytical techniques. *J. Ind. Microbiol.* **15,** 263–276.
53. Marshall, K. C., Stout, R., and Mitchell, R. (1971) Selective sorption of bacteria from seawater. *Can. J. Microbiol.* **17,** 1413–1416.
54. Davies, D. G., Chakrabarty, A. M., and Geesey, G. G. (1993) Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* **59(4),** 1181–1186.

55. Prescott, L. M., Harley, J. P., and Klein, D. A., eds. (1996) The study of microbial structure: microscopy and specimen preparation, in *Microbiology*. Wm. C. Brown, Oxford, UK, pp. 20–39.
56. Beveridge, T. J., Popkin, T. J., and Cole, R. M. (1994) Electron microscopy, in *Methods for General and Molecular Bacteriology* (Gerhardt, P., Murray, R. G. E., Wood, W. A., and Krieg, N. R, eds.), ASM, Washington, DC., pp. 42–70.
57. Surman, S. B., Walker, J. T., Goddard, D. T., *et al.* (1996) Comparison of microscope techniques for the examination of biofilms. *J. Microbiol. Methods* **25,** 57–70.
58. Sutton, N. A., Hughes, N., and Handley, P. S. (1994) A comparison of conventional SEM techniques, low temperature SEM and the electroscan wet scanning electron microscope to study the structure of a biofilm of *Streptococcus crista* CR3. *J. Appl. Bacteriol.* **76,** 448–454.
59. Bremer, P. J., Geesey, G. G., and Drake, B. (1992) Atomic force microscopy examination of the topography of a hydrated bacterial biofilm on a copper surface. *Curr. Microbiol.* **24,** 223–230.
60. Lauvvik, T. and Bakke, R. (1994) Biofilm thickness measurements by variance analysis of optical images. *J. Micro. Methods* **20,** 219–224.
61. Caldwell, D. E., Korber, D. R., and Lawrence, J. R. (1993) Analysis of biofilm formation using 2D verses 3D digital imaging. *J. Appl. Bacteriol.* **74S,** 52S–66S.
62. Stewart, P. S., Camper, A. K., Handran, S. D., Tuang, C.-T., and Warnecke, M. (1997) Spatial distribution and coexistence of *Klebsiella pneumonia* and *Pseudomonas aeruginosa* in biofilms. *Microb. Ecol.* **33,** 2–10.
63. Schaule, G., Flemming, H.-C., and Ridgway, H. F. (1993) Use of 5-Cyano–2, 3-Ditolyl Tetrazolium chloride for quantifying planktonic and sessile respiring bacteria in drinking water. *Appl. Environ. Microbiol.* **59(11),** 3850–3857.
64. Yu, P. F. and McFeters, G. A. (1994) Physiological responses of bacteria in biofilms to disinfection. *Appl. Environ. Microbiol.* **60(7),** 2462–2466.
65. Rodriguez, G. G., Phipps, D., Ishiguro, K., and Ridgway, H. F. (1992) Use of a fluorescent redox probe for direct visualisation of actively respiring bacteria. *Appl. Environ. Microbiol.* **58(6)**, 1801–1808.
66. Yu, F. P., Callis, G. M., Stewart, P. S., Griebe, T., and McFeters, G. A. (1994) Cryosectioning of biofilms for microscopic examination. *Biofouling* **8,** 85–91.
67. Dempsey, M. J. (1981) Marine bacterial fouling: a scanning electron microscope study. *Marine Biol.* **61,** 305–315.
68. Brading, M. G., Boyle, J., and Lappin-Scott, H. M. (1995) Biofilm formation in laminar flow using *Pseudomonas fluorescens* EX101. *J. Ind. Microbiol.* **15,** 297–304.
69. Camper, A. K., Jones, W. L., and Hayes, J. T. (1996) Effect of growth conditions and substratum composition on the persistence of coliforms in mixed-population biofilms. *Appl. Environ. Microbiol.* **62(11),** 4014–4018.
70. Hamilton, W. A. (1995) Biofilms and microbially influenced corrosion, in *Microbial Biofilms* (Lappin-Scott, H. M., and Costerton, J. W., eds.), Cambridge University Press, Cambridge, UK, pp. 171–182.

71. Stoodley, P., deBeer, D., and Lewandowski, Z. (1994) Liquid flow in biofilm systems. *Appl. Environ. Microbiol.* **60(8),** 2711–2716.
72. deBeer, D., Stoodley, P., and Lewandowski, Z. (1993) Effects of biofilm structures on oxygen distribution and mass transport. *Biotech. Bioeng.* **43,** 1131–1138.
73. Lawrence, J. R., Korber, D. R., Hoyle, B. D., Costerton, J. W., and Caldwell, D. E. (1991) Optical sectioning of microbial biofilms. *J. Bacteriol.* **173(20),** 6558–6567.
74. Lewandowski, Z., Lee, W. G., Characklis, W. G., and Little, B. (1987) Dissolved oxygen and pH microelectrode measurements at water-immersed metal surfaces. *Corrosion Sci.* **45(2),** 92–98.
75. Jansen, B. and Kohnen, W. (1995) Prevention of biofilm formation by polymer modification. *J. Ind. Microbiol.* **15,** 391–396.
76. Quignon, F., Sardin, M., Kiene, L., and Schwartzbrod, L. (1997) Poliovirus-1 inactivation and interaction with biofilm: a pilot-scale study. *Appl. Environ. Microbiol.* **63(3),** 978–982.
77. Jiang, H.-Q., Chen, Y.-F., Li, A. N., and Li, Z. D. (1994) Clinical burn wound infection caused by L-forms of *Staphylococcus aureus*. *Burns* **20(1),** 83–84.
78. McLean, R. J. C., Nickel, J. C., and Olson, M. E. (1995) Biofilm associated urinary tract infections, in *Microbial Biofilms* (Lappin-Scott, H. M. and Costerton, J. W., eds.), Cambridge University Press, Cambridge, UK, pp. 261–273.
79. Costerton, J. W., and Lappin-Scott, H. M. (1995) Introduction to microbial biofilms, in *Microbial Biofilms* (Lappin-Scott, H. M. and Costerton, J. W., eds.), Cambridge University Press, Cambridge, UK, pp. 1–11.
80. Raad, I., Darouiche, R., Hachem, R., Sacilowski, M., and Bodey, G. P. (1995) Antibiotics and prevention of microbial colonisation of catheters. *Antimicrob. Agents Chemother.* **39(1),** 2397–2400.
81. Thornsberry, C. and Sherris, J. C. (1985) General considerations, in *Manual of Clinical Microbiology* (Lennette, E. H., Balows, A., Hausler, W. J., and Shadomy, H. J., eds.), ASM, Washington, DC, pp. 959–966.
82. Maki, D. (1994) Infections caused by intravascular devices used for infusion therapy: pathogenesis, prevention and management, in *Infections Associated with Indwelling Medical Devices* (Bisno, A. L. and Waldvogel, F. A., eds.), American Society for Microbiology, Washington, DC, pp. 155–212.
83. Christensen, G. D., Baldassarri, L., and Simpson, W. A. (1994) Colonisation of medical devices by coagulase-negative staphylococci, in *Infections Associated with Indwelling Medical Devices* (Bisno, A. L. and Waldvogel, F. A., eds.), American Society for Microbiology, Washington, DC, pp. 45–78.
84. Bremer, P. J. and Geesey, G. G. (1991) An evaluation of biofilm development utilising non-destructive attenuated total reflectance fourier transform infrared spectroscopy. *Biofouling* **3,** 89–100.
85. Rogers, J. and Keevil, C. W. (1995) Species diversity in developing freshwater biofilms, in *The Life and Death of Biofilm* (Wimpenny, J., Nichols, W., Stickler, D., and Lappin-Scott, H., eds.), Bioline, Cardiff, UK, pp. 77–82.

86. Williams, H. G., Day, M. J., Fry, J. C., and Stewart, G. J. (1996) Natural transformation in river epilithon. *Appl. Environ. Microbiol.* **62(8),** 2994–2998.
87. Wimpenny, J. W. T. (1995) On the nature and validity of models, in *The Life and Death of Biofilm* (Wimpenny, J., Nichols, W., Stickler, D., and Lappin-Scott, H., eds.), BioLine, Cardiff, pp. 1–8.
88. Addy, M., Slayne, M. A., and Wade, W. G. (1992) The formation and control of dental plaque—an overview. *J. Appl. Bacteriol.* **73,** 269–278.
89. Busscher, H. J. and Weerkamp, A. H. (1987) Specific and non-specific interactions in bacterial adhesion to solid substrata. *FEMS Microbiol. Rev.* **46,** 165–173.
90. Brown, M. W. R. and Gilbert, P. (1995) Some perspectives on preservation and disinfection in the present-day. *Int. Biodet. Biodeg.* **36(3–4),** 219–226.
91. Caldwell, D. E., Korber, D. R., and Lawrence, J. R. (1992) Confocal laser microscopy and digital image analysis in microbial ecology, in *Advances in Microbial Ecology* (Marshall, K. C., ed.), Plenum, New York, pp. 1–67.
92. McFeters, G. A., Yu, F. P., Pyle, B. H., and Stewart, P. S. (1995) Physiological methods to study biofilm disinfection. *J. Ind. Microbiol.* **15,** 333–338.
93. Wolfaardt, G. M., Lawrence, J. R., Robarts, R. D., and Caldwell, D. E. (1994) The role of interactions, sessile growth and nutrient amendments on the degradative efficiency of a microbial consortium. *Can. J. Microbiol.* **40,** 331–340.
94. Ronot, X., Benel, L., Adolphe, M., and Mounolou, J. (1986) Mitochondrial analysis in living cells: the use of rhodamine 123 and flow cytometry. *Biol. Cell* **57,** 1–8.
95. Matsuyama, T. (1984) Staining of living bacteria with rhodamine 123. *FEMS Microbiol. Lett.* **21,** 153–157.
96. Pyle, B. H., Broadway, S. C., and McFeters, G. A. (1995) Factors affecting the determination of respiratory activity on the basis of Cyanoditolyl Tetrazolium Chloride reduction with membrane filtration. *Appl. Environ. Microbiol.* **61(12),** 4304–4309.
97. White, G. F., Russell, N. J., Marchesi, J. P., and House, W. A. (1993) Surfactant adsorption, bacterial attachment and biodegradation in river sediment: a three-way interaction, in *Bacterial Biofilms and Their Control in Medicine and Industry* (Wimpenny, J., Nichols, W., Stickler, D., and Lappin-Scott, H., eds.), BioLine, Cardiff, pp. 121–126.

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Establishment of Experimental Biofilms Using the Modified Robbins Device and Flow Cells

Luanne Hall-Stoodley, Joanna C. Rayner, Paul Stoodley, and Hilary M. Lappin-Scott

1. Introduction

1.1. Properties of Biofilms

Recent studies have shown that biofilms (a complex organization of bacterial cells present at a surface or interface, which produces a slime-like matrix) represent the principal form of bacterial growth in all environments studied to date *(1)*. There are numerous advantages to bacteria growing in biofilms. These include extended protection against environmental changes, antimicrobial agents such as chemical disinfectants and antibiotics *(2)* and grazing predators such as amebae *(3)*, as well as providing increased access to limited nutrients *(4)*. Biofilms are of interest in medical, industrial, and natural environments for several reasons. For example, they can act as reservoirs from which the dissemination of pathogens may occur. *Legionella pneumophila* has been shown to be harbored within biofilms formed within drinking water pipelines *(5)*. Similarly, it is well established that biofilms can colonize numerous types of medical implants *(6)*. In industrial systems, detrimental effects may occur following biofilm growth such as reductions in heat-transfer efficiency and flow capacity. Biofouling may also markedly increase corrosion *(7)*. Finally, biofilms represent a bacterial architecture that may support genetic transfer, nutrient utilization, and biodegradation *(8)*.

1.2. Establishing Experimental Biofilms

A major problem associated with the investigation of environmental systems is the inherent degree of complexity within a system. To facilitate the study of biofilms in the laboratory, simplified model systems have been devel-

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oped that enable the growth of biofilms, along with the analysis of several defined parameters, under conditions that can be replicated. There are several model systems in current use (*see* Chapter 19). Among these, the modified Robbins device (MRD) and flow cells have many advantages and are readily adaptable to individual experimental systems. The major advantage is that they allow the study of biofilms under flowing conditions with controlled hydrodynamics.

1.2.1. Modified Robbins Device

The MRD was initially developed to study biofouling in industrial pipelines ***(8)***. It has since been modified and used to investigate biofilms from a number of environmental habitats. The primary advantage associated with the system is the number of colonized sampling ports available for analysis. This allows for several samples to be taken simultaneously as well as sampling more than a single time point in the development of the biofilm. Quantification of several aspects of the biofilm, such as viable and total cell counts, and total protein and carbohydrate content is therefore possible. Microscopic analysis is possible using conventional staining techniques of slide-mounted samples or electron microscopy of the colonized surfaces. The MRD is also relatively inexpensive. It can be used in both batch (recirculating) and flow-through culture systems and can be connected to a chemostat if close monitoring of growth conditions is required. Disadvantages of the MRD system include the inability to visualize the biofilm *in situ*, the possibility of nutrient gradients existing along the length of the device, and the possibility of compromised hydrodynamics around the sampling stud. Finally, the MRD is prone to the drawback shared by many systems utilized in the study of biofilms. For quantitative analysis of the biofilm to be carried out, destructive sampling techniques are required. Conventional techniques such as viable cell counts, total cell counts, and total protein or carbohydrate content analysis usually all involve disruption of the biofilm.

1.2.2. Flow Cells

The continuous and nondestructive monitoring of biofilms is essential in understanding biofilm processes ***(9)***. There are several different types of flow cells suitable for many different experiments ***(10)***. Flow cells can also overcome many of the drawbacks of the MRD. Firstly, they allow *in situ* visualization of the biofilm in its hydrated form when used in association with computer-enhanced image analysis or a television camera. This is particularly advantageous because alteration of the biofilm by fixation or desiccation, which may have unknown effects on biofilm structure, are avoided. Also, using image analysis, accumulation rates can be calculated by comparing captured images with those at the outset of the experiment, allowing quantification of the growth

kinetics of the biofilm. Qualitative information regarding surface colonization is also possible. Second, the flat plate reactor (developed at the Center for Biofilm Engineering, Bozeman, Montana) can accommodate various surfaces so that they may be compared. The surface can be removed at the end of the experiment to enable either quantification of the biofilm by scraping and/or sonication of the surface, as in the MRD, or analysis using scanning electron microscopy (SEM).

A disadvantage of the flat plate reactor is that it is constrained by the channel thickness. This type of flow cell must necessarily be thin owing to the limitations of the working distance of the microscope objectives. Thus, there is a tradeoff between magnification and hydrodynamics ***(11)***. Another type of flow cell, the square glass tube reactor, overcomes this problem by facilitating higher magnification of the biofilm, allowing the flow cell to be viewed from above, and the channel depth is not restricted. This flow reactor was designed as a model to study biofilm biofouling in industrial pipelines. Most flow reactors that allow direct microscopic observation operate at low, laminar flow rates. However, this system can be operated at high, fully turbulent flow rates which are often more industrially relevant. The system can be operated using two parallel flow cells through which the flow rates can be independently controlled, allowing the influence of flow on biofilm structure and biofouling to be determined. The hydrodynamics of the square tube reactors have been well characterized using the relationship between the friction factor and the Reynolds number and fit well to established equations describing laminar and turbulent flow through a smooth pipe ***(12)***. They are also easy to make and adapt to particular experimental conditions. However, larger bore tubing requires thicker glass, thereby restricting magnification. Nevertheless, depending on the experimental conditions, there are several flow devices that permit analysis of biofilms.

2. Materials

2.1. Modified Robbins Device

1. Ethylene-oxide gas sterilized MRD (**Fig. 1A**) fitted with removable studs to which the surfaces of choice have been fitted.
2. Sterile replacement studs.
3. Bacterial culture reservoir (usually a glass flask) with an outflow connector and filtered air inlet.
4. Sterile medium reservoir with an outflow connector and filtered air inlet (*see* **Note 1**).
5. Sterile flask for waste collection.
6. Sterile silicon rubber tubing for connection of the MRD to the medium reservoir, bacterial culture reservoir, and waste flasks (*see* **Note 2**).

7. Peristaltic pump calibrated to give required flow rate (*see* **Note 3**).
8. Sampling equipment consisting of sterile scalpel blades, buffer solution, waste jar containing bleach or disinfectant, sterile test tubes containing diluent (e.g., 0.9 mL sterile buffer solution), forceps, scalpel blade holder, 70% alcohol, 5-mL pipet tips, and 5-mL Gilson pipet.

2.2. Flow Cells

2.2.1. Flat Plate Reactor

1. Sterile closed channel reactor with an observation window consisting of a 24 mm × 60 mm glass cover slip held in place by a rubber gasket and metal flange (*see* **Note 4**).
2. Sterile flask with outflow connector, filtered air inlet, tubing, flow breaks, and connectors for attachment of the flow cell.
3. Sterile waste reservoir including inflow connector and filtered air outlet, tubing with flow break, and connectors for attachment to the flow cell.
4. Peristaltic pump calibrated to desired flow rate.
5. Water bath or heating or cooling units, if necessary, to keep test cultures at temperatures other than room temperature.
6. Microscope.
7. Camera (*see* **Note 5**).
8. Computer with Framestore board (*see* **Note 6**).
9. Image analysis software (*see* **Note 7**).

2.2.2. Square Glass Tube Reactor

1. Flow cells made from sections of square glass tubing (S-103 Camlab, Cambridge, UK) 3 mm wide and 3 mm deep and 20 cm long (**Fig. 1C** ***[13]***).
2. Sterile nutrient reservoir.
3. Peristaltic pump delivered with a recycle flow rate (*see* **Note 8**) controlled with a vane head pump (Masterflex, Cole-Parmer, Niles, IL).
4. Flow meters (McMillan Flo-sensor model 101T #3724 and 3835 supplied by Cole-Parmer).
5. Pressure transducers (RS Components, Corby, Northants, UK, model 286-686).
6. Waste reservoir.
7. Polycarbonate holder mounted on the stage of an upright microscope with epifluorescence capabilities. By positioning the flow cells on the holder, the biofilm can be imaged *in situ* without interrupting flow.
8. Camera (*see* **Note 5**)
9. Computer with Framestore board (*see* **Note 6**).

2.3. Suppliers

All of the described flow devices can be found on the following Web pages: for information on MRDs, contact Environmental Microbiology Research Group at Exeter University at http:\\www.ex.ac.uk/biology/

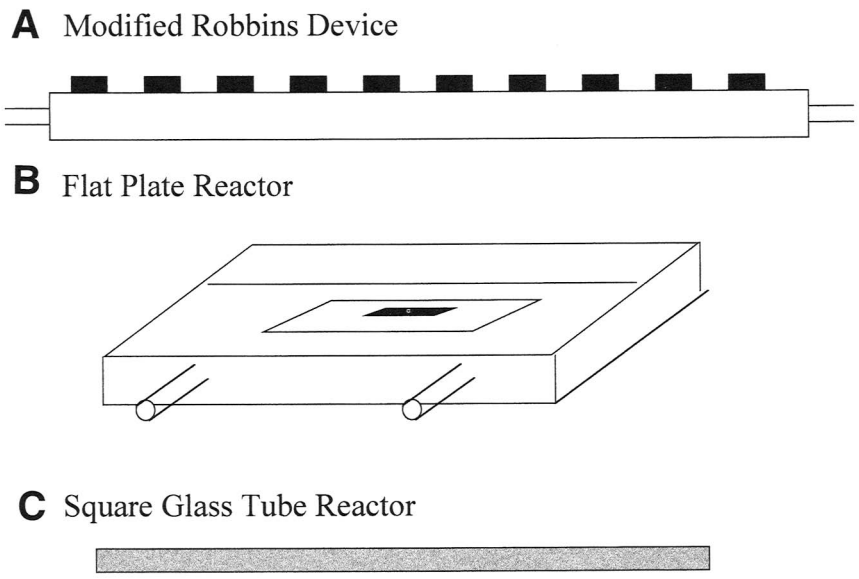

Fig. 1. Three types of devices to study biofilms under flow conditions.

resrch.html#DrHMLappin-Scott; for information on flat plate reactors, contact BioSurface Technologies Corp. at http://www.imt.net/~mitbst/flowcell.html.

3. Methods

3.1. Modified Robbins Device

3.1.1. Preparation of the MRD

1. Cut silastic rubber, black backing discs using a 0.85-mm cork borer.
2. Attach the surfaces, e.g., silastic rubber, glass, or plastic, of a known diameter to the black backing discs using a strong adhesive or waterproof sealant (*see* **Note 9**).
3. Wipe the fitted surfaces with 70% alcohol solution and lint-free tissue and allow to air dry.
4. Fit the studs into the MRD so that the surfaces for colonization lie flush with the central lumen.
5. Wipe the MRD with 70% alcohol and seal in gas-permeable bags.
6. Package 25 replacement studs in batches of approx 4 studs per bag to prevent contamination during the course of the experiment.
7. Sterilize the MRD using ethylene oxide gas (*see* **Note 10**).

3.1.2. Inoculation of Surfaces and Biofilm Treatment

1. Remove the MRD from the gas-permeable bag and check for loose surfaces.
2. Remove any surfaces, that have become detached during sterilization.

3. Connect the MRD to the culture, medium, and waste reservoirs using wide-bore, sterile silastic rubber tubing (*see* **Fig. 2** for experimental setup).
4. Inoculate the culture reservoir to give an appropriate planktonic viable cell count, e.g., 3% v/v exponential bacterial broth culture and incubate for 18 h or overnight.
5. Inoculate the MRD surfaces. Turn on the peristaltic pump and ensure that the inoculated culture is moving through the MRD system and into the waste jar. After the initial biofilm has been formed (e.g., by inoculating the surfaces for 24 h), quickly switch the system to flowthrough with only the sterile medium and control agent (antibiotic or biocide), by changing the open and closed clamps.
6. Maintain a low rate of flow during the switchover to prevent backflow of liquid through the system.
7. Remove any air bubbles from the MRD by turning it upside down and tilting at a 45° angle for a few minutes while under the normal flow conditions.

3.1.3. Sampling Colonized Surfaces

1. Switch off the pump at the appropriate time period, and clamp the silastic tubing at either end of the MRD.
2. Remove a stud (determine using random number tables) and immediately replace it with a sterile replacement stud.
3. Hold the removed stud above a pot containing disinfectant or bleach solution. Rinse to remove any nonadherent bacteria by pipetting 10 mL of sterile buffer solution gently onto the side of the stud, so that the flow is not directed at the immediate colonized surface (*see* **Note 11**).
4. Place both the scalpel blade and the scraped surface into a test tube containing sterile buffer solution (for techniques for the analysis of biofilm formation *see* **Subheading 3.2.**).
5. Sonicate the scalpel blade and surface for approx 5 min to disperse the biofilm and any clumps of cells.
6. Place the used MRD stud into the pot of disinfectant.
7. Repeat for appropriate number of samples.
8. Spray the MRD with 70% alcohol after sampling, wipe, and remove the clamps.
9. Switch the pump back on and turn the MRD upside down to remove any air bubbles formed during sampling.

3.1.4. Final Procedure of the Experiment

1. Empty the tubing and the MRD by tipping the reservoirs while maintaining the pump rate so that no liquid is taken up into the system.
2. Spray the tubing with 70% alcohol and disconnect from the reservoirs and the MRD.
3. Seal all open ends of tubing with aluminum foil and autoclave.
4. Soak the MRD in bleach or disinfectant for approx 12–24 h (increase the time of disinfection depending on thickness/viscosity of biofilm). Do not soak for longer than 48 h.

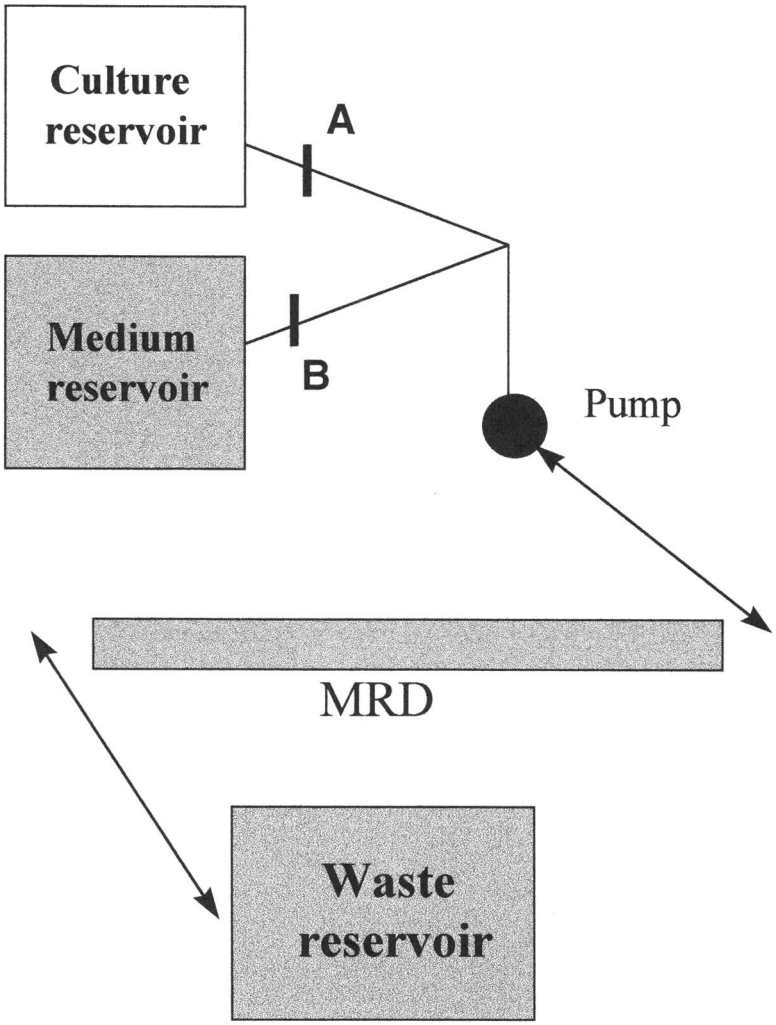

Fig. 2. Schematic diagram of MRD experimental setup. **(A)** and **(B)** indicate clamps: B closed = inoculation of surfaces, and A closed = flowthrough with only steril medium.

5. Connect to a tap and rinse in cold, running water for 8–12 h to remove residual disinfectant.
6. Soak the used studs in bleach or disinfectant for approx 8 h and then rinse in continuous running water for a further 8-12 h.
7. Allow the MRD and studs to dry prior to reassembling.
8. Autoclave all medium, waste, and inoculum reservoirs as appropriate (increase autoclaving time for large volumes).

3.2. Experimental Measurements

3.2.1. Viable Cell Counts

1. Vortex the tube containing the surface and scalpel blade for 15–20 s after sonication (**Subheading 3.1.3.**).
2. Serially dilute in buffer and plate out on an appropriate solid growth medium.
3. Calculate the numbers of viable cells per square centimeters of surface using the following equation:

$$\text{Number of bacterial colonies} \times 10 \text{ (biofilm removed is assumed to constitute 0.1 mL)} \times Df \times 1/As = \text{bacteria/cm}^2$$

where Df = dilution factor and As = area of surface in square centimeters (*see* **Note 12**).

3.2.2. Scanning Electron Microscopy

1. Fix the surface at room temperature for 2 h or overnight at 4°C (**Subheading 3.1.3.**).
2. Remove from the buffer and use a successive ethanol series to dehydrate the sample: start with a concentration of 30% and work through 50, 70, and 100% ethanol for 3 min.
3. Remove the solution after 3 min and discard, then replace with the next solution in the series.
4. Place the stud onto an SEM mount, coat with silver using a palladium catalyst, and view using SEM (*see* **Note 13**).

3.2.3. Total Cell Count

1. Scrape one to three surfaces into glutaraldehyde cacodylate buffer (*see* **Note 11**).
2. Fix at room temperature for 2 h or overnight at 4°C. Samples may also be frozen and processed at a later date.
3. Serially dilute the sample in buffer solution and filter 3–5 mL onto a black 0.2-µm polycarbonate membrane. Apply and then release the vacuum.
4. Stain with 1 to 2 mL (enough to cover membrane) of a 0.1 mg/mL acridine orange solution (in phosphate buffer) for 3 min.
5. Reapply vacuum and while running destain with 1.5 mL of isopropyl alcohol.
6. Remove filter and air dry on filter paper, view using a ×100 oil immersion lens under epifluorescence microscopy, with a calibrated eyepiece graticule, a mercury lamp and acridine orange filter block (emission wavelength of 488–514 nm).
7. Count the fluorescently stained cells in approx 10 fields of view and average to calculate the total cell count.

3.3. Flat Plate Reactor

1. Calibrate pump to desired flow rate by volumetric displacement prior to autoclaving all tubing and connectors.

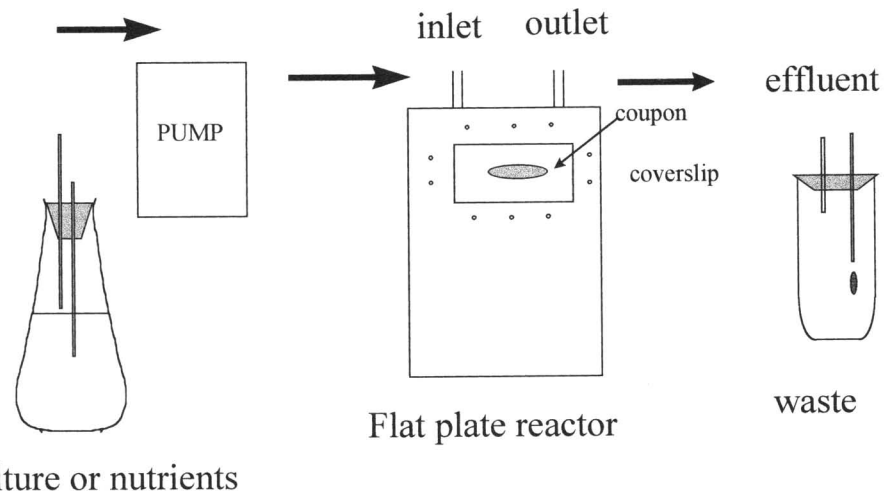

Fig. 3. Example of flat plate reactor system with nutrients and waste reservoir.

2. Autoclave the flow cell (**Fig. 1B**) after fitting with the coupon or test material, and cover with a rubber gasket, glass cover slip, and metal covering.
3. Grow the bacterial culture to the desired density and attach to pump, flow cell, and waste reservoir via sterile silicon rubber tubing (*see* **Fig. 3**).
4. Initiate flow (*see* **Note 14**) and monitor flow cell, tubing, and connectors for leaks. Tighten seals if necessary.
5. Monitor the biofilm at various time points depending on the experimental design (*see* **Note 15**).
6. Autoclave the tubing, reservoirs, and flow cell at the end of the experiment, and rinse well in running water to remove any biofilm residue.
7. Replace any tubing if necessary.
8. Clean the flow cell with 70% ethanol to remove any remaining residue, and fit with a new surface before autoclaving in preparation for the next experiment.

3.4. Square Glass Tube Reactor

The square glass tube reactor flow system (*see* **Fig. 4**) was designed to have laminar flow in one flow cell and turbulent flow in the other.

1. Measure the flow rate through each of the flow cells (Q_f) using flow meters controlled independently by tightening or loosening clamps on the inlet tubing. The average flow velocity (u) is calculated from:

$$u = Q_f/\text{CSA} \tag{1}$$

where CSA is the cross sectional area (in this case 9×10^{-6} m^2). The Reynolds number is found from:

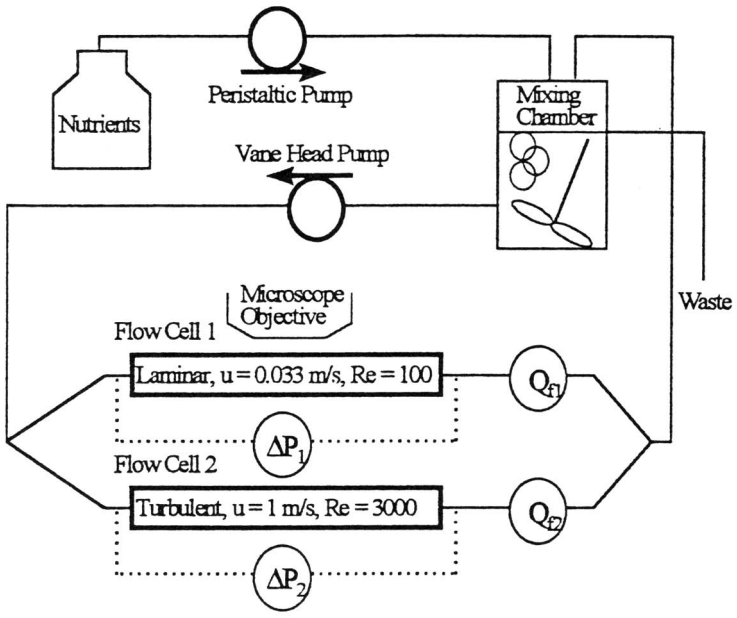

Fig. 4. Example of biofilm reactor system consisting of parallel flow cells in a recycle loop attached to a mixing chanber. The mixing chamber was aerated and the level maintained by overflow to waste.

$$\mathrm{Re} = uD_h/\nu \tag{2}$$

where ν is the kinematic viscosity of the media (for low-nutrient media the value for water can be used), D_h is the characteristic length, which in this case is the hydraulic diameter calculated from:

$$D_h = 4\mathrm{CSA}/\mathrm{WP} \tag{3}$$

WP is the wetted perimeter of the flow cell, 2(width + depth). For these flow cells, $D_h = 3 \times 10^{-3}$ m. The Reynolds number is a dimensionless number commonly used by engineers to characterize flow conditions. It is particularly useful because it predicts whether flow will be laminar or turbulent and can be used as a comparative parameter for a diverse range of flow systems.

2. Determine the pressure drop (ΔP) across each flow cell using differential pressure transducers. ΔP can be used to find the Fanning friction factor (f):

$$f = (\Delta P \times D_h)/2l_p\rho_w u^2 \tag{4}$$

where ρ_w is the density of liquid media and l_p is the distance between pressure ports ***(14)***. f is also a dimensionless number and can be used as an indicator of biofouling. The predicted f for laminar flow through a smooth (clean) pipe (from the Hagen–Poiseuille equation) is:

$$f = 16/\mathrm{Re} \quad (5)$$

and in the turbulent region f is predicted from the Blasius formula:

$$f = 0.0791/\mathrm{Re}^{0.25} \quad (6)$$

The relationship between Re and f for 20-cm long flow cells showed that the transition between laminar and turbulent flow occurred at Re = 1200 (Q_f = 3.15 cm^3/s). To increase the sensitivity of the ΔP measurement, the flow cells can be lengthened.

3. At the end of the experiment, biofilms can be fixed with 1% paraformaldehyde (30 min) and stained with nucleic acid stains such as propidium iodide (0.4%, at 25°C for 30 min).
4. Biofilms can be imaged *in situ* using confocal laser scanning microscopy, transmitted light microscopy, or epifluorescent ultraviolet microscopy, all of which can be used in conjunction with image analysis.
5. Biofilm accumulation can be routinely monitored (e.g., by obtaining surface area coverage data) and related to changes in pressure drop.
6. Metabolic activity of the biofilms can be examined using the metabolic stains such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) in which flow cells are removed from the reactor system and stained with CTC (0.04 % w/v) for 30 min at 25°C in a shaker incubator.

4. Notes

1. Antibiotics or biocides may be added to the medium reservoir after autoclaving if testing susceptibility of biofilms to antimicrobial agents.
2. Insert metal or thick-walled glass tubing into rubber bungs and place them into the sterile medium, waste, and inoculation reservoir flasks. After sterilization, the silastic rubber tubing can then be attached to the reservoirs via the tubing.
3. Precalibrate the pump to the required flow rate by using a nonsterile system with water in place of the culture or growth medium.
4. Certain designs allow a desired surface to be fitted with various test materials.
5. We use a COHU 4612-5000 charge-coupled device (Cohu, San Diego, CA).
6. We use a Scion VG-5 PCI (Scion, Frederick, MD).
7. We use the NIH-Image 1.59 program from the National Institutes of Health, available from the Internet by anonymous FTP from zippy.nimh.nih.gov or floppy disk from the National Technical Service, Springfield, VA, part no. PB95-500195GEI.
8. The volume (V) of the mixing chamber and recycle loop, including the flow cells, was approx 175 mL. The nutrient influent flow rate (Q_n) was 4.3 mL/min, giving a resulting residence time ($\theta = V/Q_n$) of 40 min.
9. When cutting surfaces, ensure that the diameter of the surface does not exceed that of the sample port; otherwise, this may interfere with removal of the stud from the MRD and the surfaces may become detached.
10. Autoclaving and chemical disinfectants damage the MRD and O-rings used to ensure a tight seal where the studs are fitted into the MRD (*see* **Fig.1A**). Check

for leaks on the MRD and in the areas where the tubing is joined to the MRD. Spray with 70% alcohol, wipe, and then seal with a quick-drying waterproof aquarium sealant where necessary.

11. If sampling for SEM, place the rinsed surface in 3–5% glutaraldehyde buffer (25% SEM grade glutaraldehyde diluted in 0.1 *M* cacodylate buffer). If sampling for viable cell counts, total cell counts, total protein, or carbohydrate, use a sterile scalpel blade to scrape the biofilm from the colonized surface (7–10 times should be sufficient to remove the adherent cells).
12. The planktonic viable cell count in the system can also be monitored in order to check sterility and cell growth by removing 0.1-mL samples, carrying out serial dilutions, and plating out on appropriate growth medium.
13. Alternatives to the use of the ethanol series include critical point and air drying of the sample. Once dehydrated and coated, samples can be stored for 1 to 2 wk until required.
14. Determine planktonic cells at the initiation of flow. A sampling port near the effluent interrupted by a flow break to reduce the possibility of contamination allows easier access.
15. For example, initial colonization events may be monitored in the first 24 h, or a biofilm of a certain thickness may be grown before examination. Biofilm thickness may be measured microscopically by focusing on the substratum of the cell cluster and then on the surface of the cell cluster and noting the difference on calibration on the fine focus adjustment ***(12)***. It is important to determine the optimal working distance between the microscope objective and the flow cell and to use the appropriate objective lens. Once this is established, surface area, as well as heights and areas of cell clusters can be compared to previous images. The appropriate software allows for images to be linked and provides a virtual record documenting changes over time. Length and width of cells may be measured, and all the assays outlined for the MRD are possible, but at only one time point. Focusing on a single area of the biofilm enables a cell cluster or groups of clusters to be monitored with time. Such images may be animated to provide a real-time record of cell attachment, aggregation, and sloughing, as well as the evolution of the biofilm with time.

    The flat plate reactor flow cell is easily disassembled at the end of the experiment and the coupon can be removed and subjected to the same experimental measurements and quantitative sampling used in the MRD, that is rinsing and sonication of the coupon resulting in disruption of the sessile organisms to yield viable cell counts (*see* **Subheading 3.1.**) However, viable cell determination of the colonized surface and SEM is available only at the final time point to maintain sterility.

References

1. Costerton, J. W., Nickel, J. C., and Ladd, T. I. (1986) Suitable methods for the comparative study of free-living and surface-associated bacterial populations, in

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