Review of the Methodologies for the Extraction, Detection and Identification of Microorganisms in the Environment

Final Report

May 2000

WS Atkins Environment

Woodcote Grove, Ashley Road, Epsom, Surrey KT18 5BW
Tel: (01372) 726140 Fax: (01372) 740055

PREFACE

This study is part of a series of research reports published by the Department of Environment, Food and Rural Affairs (DEFRA). The work was carried out under the DEFRA's *Genetically Modified Organisms (GMO) Research Programme,* which aims to underpin Government policy on the environmentally safe use of genetically modified organisms.

The study is the result of two contracts let by DEFRA. The original work was conducted by the University of Liverpool. The original report has been substantially revised and updated to its current form through a contract with WS Atkins Environment, Woodcote Grove, Epsom, KT18 5BW.

This desk study reviews the methods available for the extraction, detection and identification of microorganisms (viruses, bacteria and fungi) in the environment (air, water, soil, food and human body).

Reports published from the DETR's GMO Research Programme are peer-reviewed prior to publication.

CONTENTS

PREFACE **I**

EXECUTIVE SUMMARY **IV**

1. INTRODUCTION **1-1**

Need to Monitor Microorganisms in the Environment 1-1
Common Problems with Monitoring Microorganisms in the Environment 1-4

2. REVIEW OF METHODS FOR THE EXTRACTION OF MICROORGANISMS FROM THE ENVIRONMENT **2-1**

Air 2-2
- Sedimentation Methods 2-2
- Centrifugal Samplers 2-3
- Impaction Methods 2-4
- Filtration Methods 2-4
- Impingement Methods 2-5
- Electrostatic Precipitation 2-5
- Sampling and Measurement Guidelines 2-5

Water 2-6
- Centrifugation 2-6
- Dielectrophoresis 2-7

Soil 2-8
Sediment 2-9
Food 2-10
Human Body 2-11

3. REVIEW OF METHODS FOR THE DETECTION AND/OR IDENTIFICATION OF MICROORGANISMS IN THE ENVIRONMENT **3-1**

Ex-situ Methods to Detect Microorganisms in the Environment 3-1
- Culture-based Techniques 3-1
- Biolog 3-6
- Fatty Acid Analysis 3-7

In-situ Methods to Detect Microorganisms in the Environment 3-9
- Microscope-based Techniques 3-10
- Flow cytometry 3-13

Colorimetric-based Techniques 3-14
Reporter Gene Based Techniques 3-16
Molecular-based Techniques - Introduction 3-19
Molecular-based Techniques - Nucleic Acid Based Methods 3-22
Molecular-based Techniques - Other Methods 3-27

4. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK 4-1

5. REFERENCES 5-1

List of Tables

Table 2.1 - Commercial sources of aerosol samplers (taken from Vanderzant and Splittstoesser, 1992). 2-3
Table 2.2 - Basic parameters for USEPA methods to extract microorganisms from air 2-5
Table 3.1 - Detection of human pathogenic viruses in the environment (adapted from Metcalf *et al.,* 1995). 3-3

EXECUTIVE SUMMARY

This report is a review of the methods for the extraction, detection and identification of microorganisms in the environment. The report was produced by WS Atkins Environment as part of the research programme of the Chemicals and Biotechnology Division of the Department of Environment, Food and Rural Affairs (DEFRA), and is a revised and updated version of a report originally prepared by the University of Liverpool.

The reason for the study is that genetically modified microorganisms may be released and we want to know how to monitor and detect them. The original methods for the detection and identification of microorganisms were culture-based techniques, where the microorganism was grown (cultured) on a medium containing substrates specific to its own nutritional requirements. Such techniques required the microorganism to be extracted from its environment before any identification could be made. The requirement for extraction is used as the principle differentiating factor between detection/identification techniques. Methods that require an extraction stage are described as *ex-situ*, and those that do not require the microorganism to be extracted from its environment are known as *in-situ.* However, *in-situ* methods may involve some extraction or sample clean-up step prior to the detection or identification method. *In-situ* methods are divided into microscope, colorimetric and molecular-based approaches.

Because of the diversity of microorganisms in the environment, and the heterogeneity of many environments at a microbial level, a large number of different methods have been developed to detect and/or identify as large a proportion of the microorganisms present as possible. However, due to the diversity of microorganisms, and the different niches in which they inhabit, no single method is applicable for the extraction, detection or identification of all the microorganisms in a particular sample (except for clinical samples which may be infected with a single species of microorganism). The detection and/or identification of the microorganisms present in many environmental samples is also restricted by the existence of many of the microorganisms in the environment in a viable but non-culturable state (VBNC). VBNC microorganisms cannot be isolated by culture-based methods, and require the application of *in-situ* methods.

1. INTRODUCTION

1.1 The purposes of this report are to provide a review of the methods available for the extraction, detection and identification of microorganisms in the environment, and to provide an assessment of the validity and efficacy of each of the methods for different microorganisms and environments. The microorganisms covered in this report are viruses, bacteria and fungi, and the environments of interest are air, water, soil and sediment, food and the human body.

1.2 This report has been divided into two main sections, covering the methods available for the extraction, and the detection and identification of microorganisms in the environment. Each section contains a brief description of the technique or method and the advantages and disadvantages of the method for the extraction of microorganisms from each environment. Each section is not intended to be exhaustive but has attempted to provide an assessment of the range of methods available for each type of microorganism and environment. Where methods have been identified that are applicable to more than one section, for example the use of fatty acid methyl ester (FAME) analysis for the detection and identification of microorganisms in terrestrial and aquatic environments, the description of the method has only been provided in one section. However, the relevant advantages and disadvantages of the method are discussed in both sections.

NEED TO MONITOR MICROORGANISMS IN THE ENVIRONMENT

1.3 The need to monitor microorganisms in the environment developed from the discovery that microorganisms were the causative agents of a wide range of diseases that affect humans, animals and plants (Metcalf *et al.,* 1995). In order to determine the role of a particular microorganism in the occurrence of a specific illness, infection or disease, it was necessary to be able to identify that virus, bacterium or fungus throughout the disease cycle. Identification and detection techniques were developed to determine the presence of a known pathogenic microorganism (one that is able to cause disease) in a particular environment, such as drinking water, and to confirm the existence of a particular disease.

1.4 In addition to their importance as pathogens, microorganisms, particularly bacteria and fungi, have also been found to have an important role in the function and maintenance of many environmental process including the turnover of organic and inorganic nutrients (Gsell *et al.*, 1997) and as a sink for various nutrients including pollutants (Schnürer *et al.*, 1985). Many of the microorganisms involved in environmental processes are non-pathogenic, and could not be detected and/or identified by the techniques developed for pathogenic microorganisms. The study of microorganisms of environmental importance therefore required the development of new detection and identification techniques (Akkermans *et al.,* 1994).

1.5 Recent developments in biotechnology and genetic engineering have led to the use of specially developed strains of microorganisms for the production of certain compounds, or the catalysis of specific biochemical reactions. Although these microorganisms are used predominantly in contained facilities such as fermenters and bioreactors, techniques are required to monitor the physiological state of the microorganisms in the process vessel, and also to determine whether any emissions of the microorganisms occur to the environment during the process. Emissions that may contain genetically modified microorganisms are required to be monitored to ensure that any releases of viable microbial cells that do occur are detected[1].

1.6 Techniques to monitor, detect and/or identify the presence of microorganisms in the environment do not always require the use of a method that can monitor the presence of the whole organism. The proposed goal of the monitoring technique should always be addressed when considering the method that needs to be employed. With pathogenic microorganisms, the goal of the monitoring technique is to determine whether exposure to a particular food or water sample is likely to cause disease. Selection of the technique used should include for example an assessment of whether the entire pathogen is required to cause the disease and whether the pathogen is required to be in a viable state. The pathogenicity of the bacterium *Clostridium botulinum* (causative agent of botulism) for example is a consequence of the toxin produced by the microorganism. *C. botulinum* that is unable to produce the toxin is non-pathogenic. Therefore in order to assess whether a particular food sample could cause botulism if consumed, a method to detect the presence of the toxin would be suitable.

1.7 The presence of some microorganisms in the environment is often determined by the detection of 'indicator' species. Such microorganisms are often relatively easy to monitor, and the techniques used a well-established. Monitoring for 'indicator'

[1] As specified under European Directive 90/219/EEC (to be replaced by Directive 98/81/EC after 5 June 2000).

strains is frequently applied for the detection of gastro-intestinal pathogens such as *Enterococcus sp*., *Shigella dysenteriae*, *Salmonella sp*. and *Campylobacter sp*. in drinking water. Such microorganisms are present in sewage and cause gastroenteritis. Examples of 'indicator' microorganisms are:

- *Escherichia coli* - indicator of the presence of other bacteria that reside in the gastro-intestinal tract, including the pathogenic strain *E. coli* O157, as well as *Enterococcus sp*., *Shigella dysenteriae*, *Salmonella sp*. and *Campylobacter sp*. (American Public Health Association, 1989).

- *Listeria monocytogenes* - heat resistance comparable to many other non-sporulating mesophilic[2] bacteria (Coote *et al.,* 1991), such as *Staphylococcus sp., Streptococcus sp*., *E. coli* and *Pseudomonas sp*. *Pseudomonas aeruginosa* (Gram negative bacterium[3]) and *S. aureus* (Gram positive bacterium) are recognised by STATT[4] as indicator microorganisms for all vegetative bacteria (including those resident of the gastro-intestinal tract).

- Feline calicivirus - closely related to Norwalk and Norwalk virus-like particles (NVLPs) which are important causes of gastroenteritis in humans. However, Norwalk and NVLPs cannot be cultured and little is known of the survival in the environment or resistance to heat or disinfection (Doultree *et al.,* 1999).

- Polio 2 or polio 3 and MS-2 bacteriophage - recognised as the indicator microorganisms for viruses in general (Turnberg, 1996). Viruses may be divided into lipophilic and hydrophilic viruses. Hydrophilic viruses such as poliovirus and adenovirus do not have an outer protein coat surrounding their central capsid structure. The absence of a protein coat confers a greater resistance of such viruses to thermal, chemical and UV inactivation. Enveloped or lipophilic viruses such as HIV, herpes simplex virus and hepatitis B virus are more sensitive to inactivation by thermal, chemical and UV treatments, and are therefore less likely to survive the treatment process. Polio 2 and polio 3 viruses are particularly resistant to thermal and chemical inactivation compared to other viruses. MS-2 bacteriophage is used as an

[2] Refers to bacteria that grow best at temperatures of 5-50ºC (Neidhart *et al*., 1990).
[3] Refers to the division of bacteria into two groups (Gram positive and Gram negative), according to their ability to retain a crystal violet-iodine stain when treated with organic solvents (alcohol or acetone). Those bacteria that retain the Gram stain are termed Gram positive, and those that lose it Gram negative. The two groups of bacteria differ physically in the structure of their cell wall and cell membrane (Neidhart *et al*., 1990).
[4] The State and Territorial Association on Alternate Treatment Technologies. A US based organisation which provides guidance on the treatment of waste by processes other than incineration. Turnberg WL. (1996). Biohazardous Waste - risk assessment, policy and management. ISBN 0 471 59421 0.

indicator virus as it is similar in terms of resistance to heat and disinfection to hepatitis viruses.

- *Mycobacterium terrae, M. phlei* and *M. bovis* - as indicator microorganisms for the mycobacteria (Turnberg, 1996). *M. terrae* has a similar resistance to disinfection as the pathogen *M. tuberculosis* (causative agent of tuberculosis) (Turnberg, 1996).

- *Candida albicans* and *Penicillium chrysogenum* - as indicator organisms for fungi, and yeasts and moulds in particular (respectively) (Turnberg, 1996).

- *Bacillus stearothermophilus* - used as an indicator for endospore forming bacteria (including *Bacillus sp*. and *Clostridia sp*.), and potentially also spore forming fungi (less resistant to inactivation than bacterial spores). Bacterial spores are recognised as the microbial structures most resistant to heat treatment and disinfection. *B. stearothermophilus* spores are used as the indicator for assessing the efficacy of wet heat sterilisation. Spores of *B. subtilus* are also recognised by STATT as suitable for assessing the efficacy of chemical, thermal and irradiation treatment systems. However, spores of *B. stearothermophilus* have a greater resistance to heat and disinfection (Turnberg, 1996).

1.8 Microorganisms that are used in bioreactors and fermentation processes often possess a specific characteristic by which they can be monitored. This may be the ability to catalyse a certain chemical reaction, or to synthesise a specific compound. Genetically modified microorganisms are often labelled with a specific marker during their development. If the purpose of the monitoring programme is to detect the presence of the microorganism, then a method can be used that can detect that specific marker. Such methods are however likely to provide a quantitative rather than a qualitative result.

COMMON PROBLEMS WITH MONITORING MICROORGANISMS IN THE ENVIRONMENT

1.9 One common problem with monitoring microorganisms in the environment (with the exception of some fungi) is that of size. Because of their small size, microorganisms, particularly viruses and bacteria are not visible to the naked eye. Viruses range in size from 25-300 nm, whereas bacterial cells are approximately 1-2 µm. Fungal spores are <5 µm in diameter. Microorganisms can only be observed without magnification either when they form larger colonies, for example on rotting food or vegetation; or

indirectly when they cause disease[5] such as discolouration on leaf surfaces or inflammation or pus around a skin wound.

1.10 Monitoring techniques for the detection and identification of microorganisms have therefore relied on one or more of the following groups of techniques. The choice of technique depends on the objective of the detection or identification process, the microorganism of interest and the environment in which that microorganism is present:

- magnification of the microorganism so that individual cells become visible to the naked eye (microscope-based techniques);
- culturing the microorganism so that the biomass becomes visible to the naked eye (culture-based techniques);
- the use of a compound that changes colour in the presence of the microorganism (colorimetric-based techniques); and
- the identification of a specific component of the microorganism, such as a gene, protein or particular cell structure (molecular techniques).

1.11 The objective of the detection or identification process can generally be divided into whether a qualitative or quantitative result is required. A qualitative result is defined as one in which the objective is to determine the presence or absence of microorganisms in an environment. A qualitative technique can be tailored to determine the presence or absence of a specific microbial taxon, but will usually not provide a value for the number of microorganisms present. This requires a quantitative approach.

1.12 The other common problem with monitoring microorganisms in the environment, is the environment in which the microorganism exists. Historically, microorganisms had to be extracted or isolated from their environment before any detection or identification process could take place. The problem with such *ex-situ* techniques is that unless all the microorganisms present in an environment can be extracted by a single technique, then the microorganisms that are isolated from the environment are unlikely to be representative of the community of microorganisms present in that environment, in terms of both numbers and diversity of taxa. This is applicable particularly to more complex environments such as soil and aquatic systems, where

[5] Viruses are only visible in this way without some form of magnification.

the microorganisms may be free-living in the water or water-filled pore spaces in the soil particles, or bound to the soil or sediment particles. Microorganisms that are bound tightly to soil or sediment particles are less likely to be extracted from the soil or aquatic environments.

1.13 This limitation with *ex-situ* techniques led to the development of *in-situ* techniques which do not require the microorganism to be extracted from the environment. In *in-situ* methods the presence or absence of a microorganism(s) can be determined using a microscope-based, colorimetric or molecular-based technique.

2. REVIEW OF METHODS FOR THE EXTRACTION OF MICROORGANISMS FROM THE ENVIRONMENT

2.1 The purpose of this section is to review the methods available for the extraction of microorganisms from the environment. The objective of all extraction methods is to remove or isolate the microorganisms from their environment into another environment in which they can be more easily detected and/or identified by *ex-situ* methods. The latter is usually a buffered solution such as phosphate buffer. The use of a buffered solution rather than water prevents the microorganisms from being damaged by osmotic changes.

2.2 The extraction of microorganisms from the environment is required as the initial stage in all *ex-situ* detection or identification techniques. For *in-situ* methods, the microorganisms are (by definition) not extracted from their environment (although some sample clean-up stage may be required to remove material that might contaminate the sample during a later stage of the process, for example organic matter or large particulate debris). The choice of extraction technique depends on:

- the environment that is being investigated - for example, the relatively low concentration of microorganisms in air means that the extraction of microorganisms from this environment requires the sampling of large volumes of air (up to 15 l), compared to samples of <10 ml which are required to extract microorganisms from water; and

- the characteristics of the microorganism that is being extracted - for example the extraction of microorganisms that are relatively resistant to desiccation (such as spore-forming bacteria and fungi), from soil and sediment, usually involves the soil or sediment sample being dried prior to extraction. Drying removes non-spore forming microorganisms, and reduces the potential contamination from these microorganisms during the later detection and identification stages.

AIR

2.3 Microorganisms exist in air as aerosols of either single unattached cells or clumps of cells. The microbial aerosols exist as either free-living organisms surrounded by a film of dried organic or inorganic material, or as cells attached to dust particles. The particulates in microbial aerosols are reported to range in size from <1-50 µm. Due to their lower tolerance to desiccation, vegetative bacteria are often only present in low numbers in a microbial aerosol, unless the relative humidity of the air is relatively high, or that the vegetative bacterium is enclosed in some form of protective medium. The predominant microorganisms in microbial aerosols are bacterial and fungal spores (Vanderzant and Splittstoesser, 1992).

2.4 The presence of viable microorganisms in air can be determined quantitatively by a number of methods (Blomquist *et al.,* 1987). However, no single sampling method is suitable for the collection and analysis of all types of bioaerosols, and no standardised protocols are available (Buttner *et al.,* 1997). The methods that are available include sedimentation, impaction on solid surfaces, filtration, centrifugation, electrostatic precipitation, impingement in liquids and thermal precipitation. With the exception of thermal precipitation, all these methods are used widely for the extraction of microorganisms from air. A range of commercial aerosol samplers that can be used for each of these methods are presented in Table 2.1.

Sedimentation Methods

2.5 Sedimentation methods rely on gravity and air currents to deposit airborne microorganisms on a selective solid growth medium. Results are obtained as colony forming units (cfu) or particles deposited per minute. Collation of airborne microorganisms by sedimentation is recommended to collect the microorganisms on a disc of selective growth medium of 90 mm in diameter and an exposure time of 15 minutes[6].

2.6 The advantages of using sedimentation methods are ease of use and low cost. The disadvantages of these methods are that they are only applicable to measuring fall-out onto a particular surface, and in general requires a relatively long sampling time. The results obtained from sedimentation methods for air sampling are reported to return different results to other quantitative methods, possibly because of the low numbers of cells likely to sediment onto the selective growth medium during the exposure period. The reliance on gravity means that the method is biased heavily towards large

[6] Standard Methods for the Examination of Dairy Products. American Public Health Association, 1985 (cited by Vanderzant and Splittstoesser, 1992).

particles. If the environment to be sampled is likely to experience disturbance and mixing of the air, then sampling by volumetric methods will be more effective than sedimentation based methods (Vanderzant and Splittstoesser, 1992).

Centrifugal Samplers

2.7 Centrifugal samplers use a centrifugal force to propel aerosol particles onto a collection surface (usually a solid selective medium). Because centrifugal samplers do not generate high velocity jet flows, the microorganisms are exposed to lower stresses during extraction (compared to impaction and impingement methods), and are therefore more likely to survive to the detection or identification stages. Centrifugal samplers are also easy to operate and may be less expensive than impactor based methods. They can also sample a large volume of air relatively rapidly (Vanderzant and Splittstoesser, 1992).

Table 2.1 - Commercial sources of aerosol samplers (taken from Vanderzant and Splittstoesser, 1992).

Sampler type	Commercial sampler	Manufacturer
Impinger	All-Glass Impinger 30 and Pre-Impinger	Ace Glass Inc, USA
	Midget Impinger with Personal Air Sampler	Supelco Inc, USA
	May 3-stage Glass Impinger	AW Dixon, UK
Impactor (slit type)	Casella Single-slit and Four-slit Sampler	BGI Incorporated, USA
	Mattson-Garvin Air Sampler	Matteson-Garvin Company, USA
	New Brunswick STA Air Sampler	New Brunswick Scientific Company, USA
Impactor (sieve type)	Anderson 6-stage, and 2-stage Samplers	Anderson Samplers Inc, USA
	Ross-Microban Sieve Air Sampler	Ross Industries, USA
	Personal Particulate, Dust, Aerosol Collector	SKC Inc, USA
Filtration	Millipore Membrane Filterfield Monitor	Millipore Corporation, USA
	Gelman Membrane Filter Air Sampler	Gelman Sciences Inc, USA
	MSF 37 Monitor	Micro Filtration Systems, USA
Centrifugal samplers	RCS Centrifugal Sampler	Folex-Biotest-Schluessner Inc, USA
Electrostatic precipitation	LVS Sampler	Sci-Med Environmental Systems, USA
	General Electric Electrostatic Air Sampler	General Electric Company, USA

Impaction Methods

2.8 Impaction methods also involve the collection of the airborne microorganisms on a solid selective medium. However, with impaction methods, the microorganisms are directed onto the solid medium with an air jet. This means that impaction methods achieve a higher particle recovery than sedimentation methods (Vanderzant and Splittstoesser, 1992). Impactors are of two types:

Slit sampler

2.9 Slit samplers direct the air onto the selective medium through a slit. Although the flow rate may be altered by changing the dimensions of the slit, slit samplers usually operate at a flow rate of 28.3 l min^{-1}. Slit samplers do not discriminate for the size of the airborne particles, and can be used to detect bursts of airborne contamination associated with specific activities at known times (Vanderzant and Splittstoesser, 1992).

Sieve sampler

2.10 Sieve samplers operate by drawing air through a large number of small evenly spaced holes in a metal plate positioned at a set distance above the solid medium. Sieve samplers can have one (single stage) or several (multistage) plates, with multistage samplers able to differentiate between particles of different sizes, and can also provide information on the profile of particulates in the microbial aerosol. The main disadvantages of multistage samplers is that they are cumbersome to handle and expensive to use (Vanderzant and Splittstoesser, 1992).

Filtration Methods

2.11 Filtration methods are used widely for aerosol sampling because of their low cost and simplicity of operation. Microorganisms are extracted from air by sucking the air through a filter of specific pore size. A limitation of filtration methods is the potential for the microorganisms to become dehydrated following their extraction onto the filter, although the shorter sampling times used in gelatin membrane filtration may reduce the potential stress incurred. Filtration methods are therefore more suitable for the enumeration of microorganisms that have a high tolerance to desiccation such as *Micrococcus* sp., *Bacillus* sp., *Corynebacterium* sp., *Neisseria* sp. and *Actinomyces* sp., and the fungi *Penicillium* sp., *Aspergillus* sp. and *Cladosporium* sp. (Hýsek *et al.,* 1991), rather than vegetative bacteria (Vanderzant and Splittstoesser, 1992).

Impingement Methods

2.12 Impingement methods involve passing the air through a liquid, and trapping the particulate matter (including any microorganisms present) in the liquid. Liquid impingers are described as low velocity or high velocity samplers. Low velocity impingement samplers are not suitable for trapping particles <5 µm in diameter, and are therefore not applicable for the extraction of single microbial cells from air. High velocity impingement samplers draw air through a small jet and direct it at a liquid surface. This method can extract particles of >1 µm in diameter, but because of the high velocities involved is liable to destroy some vegetative cells. Because the high velocity also disperses clumps of cells, the counts obtained are also usually higher than those obtained by other methods (Vanderzant and Splittstoesser, 1992).

Electrostatic Precipitation

2.13 Electrostatic precipitation samplers impart a uniform electrostatic charge to incoming airborne particles, which are then collected on an oppositely charged rotating disc. Such samplers have a relatively high throughput (up to 1000 L min^{-1}), but are complex and not straight-forward to handle. Little information is known on the effect of the process on the viability of the microorganisms extracted, and this may restrict the applications in which electrostatic precipitation may be used (Vanderzant and Splittstoesser, 1992).

Sampling and Measurement Guidelines

2.14 The United States Environmental Protection Agency (USEPA) has set up a number of standard methods for sampling mould spores, fungal spores, cell fragments and bioaerosols. The basic parameters of each method are presented in Table 2.2.

Table 2.2 - Basic parameters for USEPA methods to extract microorganisms from air

Microorganism	Sampling device	Volume of air sampled[a]	Sampling rate
Mould spores	Air-O-Cell cassette 225-9501	1.5-15 l	1500 ml min^{-1}
Fungal spores	Air-O-Cell cassette 225-9501	1.5-15 l	1500 ml min^{-1}
Cell fragments	Air-O-Cell cassette 225-9501	1.5-15 l	1500 ml min^{-1}
Bioaerosols	SKC Biosampler 225-9595	1.5-15 l	1500 ml min^{-1}

[a] Analysis of the sample following extraction is dependent on the nature of the sample.

WATER

2.15 Microorganisms can be extracted from aquatic environments simply by taking a sample of the water (either a volume of water, or a swab sample). Microorganisms can then be observed in the water sample with a microscope, or the water sample can be used to inoculate selective growth media (Section 3). In cases where the water sample is likely to contain macrooorganisms or particulate matter, the sample may require filtration to remove this material, prior to further extraction.

2.16 In environments where the numbers of microorganisms are low, for example cold freshwater lakes (oligotrophic environments), or where the microorganism of interest is known to makeup a small percentage of the total microbial community, the water sample may need to be concentrated prior to extraction. The most common method for concentrating microorganisms in water samples is centrifugation, although filtration with a small pore size filter can also be used. Filtration is however vulnerable to blockage by particulate matter, and is therefore more applicable to 'clean' environments, such as waste streams from fermenters or bioreactors. Analysis of water samples for viruses is usually performed by passing large volume water samples (>400 l) through special charge-modified, virus retaining filters to extract the viruses, which are usually only present at low levels (Metcalf *et al.,* 1995). The use of sephadex or chelex resin in the filter process helps remove humic acid compounds, and minimise their effect on subsequent molecular analyses.

Centrifugation

2.17 The use of centrifugation to extract microorganisms from water samples involves the sample being spun at high speeds. This imposes high centrifugal forces on suspended particles such as microorganisms, and separates the particles from the water on the basis of differences in weight. Speeds of 12096 x g for 15 min are usually sufficient to extract bacterial cells from aqueous samples. Due to their smaller size, the extraction of viral particles from aqueous samples requires higher speeds (often described as ultra- or hyper-centrifugation).

Density gradient centrifugation

2.18 In density gradient centrifugation, the sample containing the microorganisms is centrifuged in a medium such as sucrose, Percoll (Pharmacia, Sweden) or Nycodenz (Nyegaard and Co, Norway), rather than water or buffer (Lindahl and Bakken, 1995). The advantage of density gradient centrifugation, is that during spinning, the particles in the original sample are dispersed through the sucrose medium. After the sample

has been spun, the particles remain dispersed through the medium (usually in bands of similar weight), rather than in a single clump as in conventional centrifugation. Particles of different weights can then be extracted from the medium separately.

Dielectrophoresis

2.19 Dielectrophoresis is the lateral motion induced on particles (including microbial cells) by non-uniform (alternating current) electric fields (Markx *et al.,* 1994). Exposure to an electric field causes the particles present to become polarised. The non-uniformity of the electric field results in a non-uniform force distribution on the polarised particles causing them to move towards the region of highest field intensity.

2.20 Because differences in cell size and shape (spherical or rod-shaped), as well as differences in cell surface components (eg. proteins, polysaccharides and techoic acids) and intracellular components (eg. proteins, sugars, RNA and DNA), all contribute to the overall polarisation of an individual microbial cell, then dielectrophoresis can be used to separate specific types of cells from an environment. Because movement and therefore separation of the cells is best in an environment that is freely permeable to microbial cells, then dielectrophoresis is most suitable to aqueous environments such as water, food and blood (or other body fluids).

2.21 The movement of particles <1 μm in size within a non-uniform electric field is dominated by their surface properties. Dielectrophoresis is reported to be able to manipulate and spatially separate tobacco mosaic virus and herpes simplex virus (Morgan *et al.,* 1999).

2.22 Applications of dielectrophoresis have included the selective spatial manipulation and separation of mixtures of (Pethig and Markx, 1997):

- bacteria in aqueous environments such as water, food and blood;
- viable and non-viable cells;
- cancerous and normal cells; and
- red and white blood cells.

SOIL

2.23 At a microbial level, soil is a very heterogeneous environment consisting of an array of different sized particles surrounding air-filled and water-filled spaces, in which microbial cells are localised in microhabitats that may differ even over small distances (van Elsas and van Overbeek, 1993). Microorganisms reside throughout the soil profile and are present:

- in a free-living state in the water filled spaces between the soil particles;
- on the surface of the soil particles; or
- in pore spaces within the soil particles. Microorganisms are attached to soil particles by polysaccharide adhesions, slime and electrostatic interactions.

2.24 The extraction of microorganisms from soil is affected significantly by the heterogeneity of this environment, with the free-living microorganisms the most easily extracted, and those present within the soil particles the most difficult to remove. Sorption of microorganisms to soil particles by electrostatic interaction or by the production of extracellular polysaccharides will also adversely affect the extraction of microorganisms from such environments. Because of their high organic content, clay rich soils pose particular problems for extraction of microorganisms (Metcalf *et al.,* 1995).

2.25 All methods for the extraction of microorganisms from soil have two common stages:

- dispersion of the soil within an aqueous solution; and
- separation of the microbial cells and soil particles by centrifugation according to diameter or buoyant density or both.

2.26 Maximum dispersion of the soil is vital to release the microorganisms resident within the soil particles and aggregates. Dispersion is also required to assist in the dissociation of the microorganisms from the surfaces of the soil particles. Dispersion of the soil has been conducted by physical or chemical processes, or a combination of the two (Lindahl and Bakken, 1995).

2.27 Microorganisms can be extracted from soil simply by mixing a sample of soil in a buffered solution. The number of microorganisms isolated is positively correlated to

the intensity and duration of agitation, although only the free-living microorganisms and those that are bound weakly to the surface of the soil particles are likely to be removed. Extraction of microorganisms from soil can be improved by sonicating the soil in buffer. Sonication involves the use of high frequency sound waves to break up soil particles and to dislodge microbial cells. A sonication time of 2.5 min has been reported to extract the optimum number of attached microorganisms (Ellery and Schleyer, 1984, cited by Kepner and Pratt, 1994), although other reports have proposed longer sonication times but at a more gentle level of sonication (Kepner and Pratt, 1994). A limitation of sonication is that it is a destructive technique and may cause cell lysis. Damage to cells during extraction is though a limitation of all extraction techniques.

2.28 The dispersal and differential centrifugation technique (Hopkins *et al.,* 1991) combines centrifugation and sonication to remove microorganisms from soil. The extraction efficiency of 30-60 percent is greater than those incurred using just agitation, centrifugation and sonication on their own (<10 percent) (Hopkins *et al.,* 1991).

2.29 The dispersal and differential centrifugation technique works by progressively isolating more microorganisms from the soil through the use of centrifugation between each stage of the process. The stepwise isolation of microorganisms means that those microorganisms that are free-living within the soil matrix or are bound weakly to soil particles are extracted at an early stage in the process. These microorganisms are removed from the extraction process by centrifugation, and are therefore not exposed to the harsher and potentially more damaging extraction steps later in the process. Each individual stage of the process has been used before on its own to extract microorganisms from soil. The use of sodium cholate (MacDonald, 1986), Tris buffer (Niepold, 1979; cited by Hopkins *et al.,* 1991), and mild ultrasonication (Ramsay, 1984; cited by Hopkins *et al.,* 1991), all facilitate dissociation of the soil particles and cells. Agitation in water is incorporated into the process to attempt to reduce electrolyte concentration of the solution, thereby increasing the repulsive interaction that occurs between two "like" charged surfaces, (Rutter and Vincent, 1980; cited by Hopkins *et al.,* 1991).

SEDIMENT

2.30 Due to the similar physical characteristics between sediment and soil, the same techniques can be used to extract microorganisms from both environments. However, because sediment is predominantly an anaerobic environment (apart from the upper surface layer), the extraction of microorganisms from sediment should be conducted

under both aerobic and anaerobic conditions to ensure that the microorganisms extracted are representative of the microbial community present. A similar argument applies to the extraction of psychrotrophic ('cold-tolerant') microorganisms from the environment. The extraction of pyschrotrophic microorganisms under ambient conditions may be expected to extract different microorganisms from those extracted the process was conducted at 5 ºC with pre-cooled media.

2.31 Because of the role of sediment as a reservoir for microorganisms (particularly viruses) in aquatic environments (Metcalf *et al.,* 1995), this environment should be analysed as part of any assessment of the overlying water environment.

2.32 The location of sediment below water means that a method is required to obtain samples of sediment before extraction of the microorganisms can take place. There are two main types of device for sampling sediment:

- grab samplers - these samplers penetrate into the sediment to a depth of approximately 10 cm and extract a sample of sediment from an area of 0.1-0.2 m^2, depending on the size of the grab. The principal disadvantage of grab samplers is that as the sediment is mixed during extraction, analysis of the spatial arrangement of microorganisms through the sediment with depth is not possible. Examples of grab samplers include the Petersen grab, van Veen grab, Shipek grab, Ekman grab and the Smith-McIntyre grab; and

- core samplers - the simplest type of core sampler consists of a plastic or metal tube which is driven into the sediment by hand. This method is however restricted to extracting sediment that is covered in no more than 50 cm of water. Samplers such as the Makereth corer are pneumatically driven, with the sampling apparatus anchored to the surface of the sediment, and the corer tube driven down into the sediment by compressed air. Such samplers can extract sediment cores from a depth of up to 6 m. The use of frame mounted core samplers minimises compression of the sediment sample and disturbance of the fine upper layers of sediment. However, as the sediment sample is retained within the corer tube without mixing, the spatial distribution of the microorganisms through the sediment profile (which may be 30-40 cm in depth) is preserved during extraction.

FOOD

2.33 Microorganisms are extracted from food in the same way as described for the extraction of microorganisms from water and soil. The exact method used is likely to

be dependent on the characteristics of the food, particularly the solidity and water content. Solid foods such as meat may require a maceration stage prior to any extraction of the microorganisms present. The extraction of microorganisms from food should take into account the present of pyschrotrophic, thermotolerant, thermophilic ('heat-loving') and halophilic ('salt-loving') microorganisms, as well as mesophilic microorganisms.

HUMAN BODY

2.34 Microorganisms are extracted from the human body, body parts and tissue fluids in the same way as described for the extraction of microorganisms from water and soil. The exact method used is likely to be dependent on the characteristics of the sample, particularly the solidity and water content. Due to the high water content of the human body, body parts and tissue fluids, it is likely that those techniques identified for the extraction of microorganisms from water would be most suitable.

2.35 Methods for the extraction of microorganisms from air are applicable to the capture of microorganisms exhaled from the human respiratory system.

3. REVIEW OF METHODS FOR THE DETECTION AND/OR IDENTIFICATION OF MICROORGANISMS IN THE ENVIRONMENT

3.1 The purpose of this section is to review the methods available for the detection of microorganisms in the environment. As discussed in Section 1 of this review, detection methods can be divided into two groups, those that require an extraction stage prior to the detection of the microorganisms (*ex-situ* techniques) and those that can be performed in the absence of any previous extraction (*in-situ* techniques). Because of the differences in the environments inhabited by microorganisms, a method may be described as *ex-situ* and *in-situ* depending on the environment for which it is used. For example, the detection of microorganisms in soil with a microscope requires a pre-extraction process to separate the microorganisms from the soil particles, whereas the detection of microorganisms in water by the same method is less likely to need prior extraction of the microorganisms due to the presence of significantly less particulate matter.

EX-SITU METHODS TO DETECT MICROORGANISMS IN THE ENVIRONMENT

3.2 *Ex-situ* methods require the microorganism(s) to be extracted from the environment prior to any detection methodology being employed. *Ex-situ* methods consist largely of culture-based techniques, although other techniques often require a prior extraction stage, particularly if soil or sediment samples are being investigated.

Culture-based Techniques

3.3 Due to their simplicity and low cost, culture-based techniques are the most common method for detecting and identifying microorganisms extracted from the environment. The principle limitation with culture-based techniques, is that the microorganism to be detected or identified must be culturable. As <10 percent of the all mcroorganisms in the environment are reported to be culturable (Torsvik *et al.,* 1994), then culture-based techniques cannot be applied to the detection of all microorganisms in the

environment. Culture-based techniques are applicable to the detection and identification of viruses, bacteria and fungi.

3.4 Culture-based methods represent the earliest techniques used to detect microorganisms in the environment. The original methods involved the culturing of the microorganism on a food or other material, or the observation of disease in humans, animals or plants.

3.5 Initial methods for the detection of viruses in the environment involved the infection and subsequent observation of the action of a virus in laboratory animals (Metcalf *et al.,* 1995). These methods have largely been replaced by growing the virus in cultures of immortal cell lines. The exceptions are the hepatitis A and hepatitis E viruses, caliciviruses, rotaviruses and astroviruses which do not grow or are difficult to grow in cell culture, and therefore require the use of molecular-based techniques for their detection and/or identification (Metcalf *et al.,* 1995). The basic types of method used to detect and/or identify human pathogenic viruses in the environment are presented in Table 3.1.

Table 3.1 - Detection of human pathogenic viruses in the environment (adapted from Metcalf *et al.,* 1995).

Virus[1]	Virus detection by:	
	Cell culture[4]	Molecular-based technique
Enteroviruses		
Polio	+	+
Coxsackie A	+	+
Coxsackie B	+	+
Echo	+	+
Entero 68-71	+	+
Hepatitis A[2]	+/-	+
Caliciviruses		
Human calicivirus, Sapporo[3]	-	+
Norwalk[3]	-	+
Norwalk-like[3]	-	+
Hepatitis E[2]	-	+
Rotaviruses[3]	+/-	+
Enteric viruses	+	+
Astrovisures[3]	+/-	+
Coronaviruses	+/-	+

1 All these viruses contain an RNA genome, except enteric adenoviruses, which contain a double-stranded DNA genome. The RNA genome of rotavirus is double-stranded RNA, whilst the genomes of the other RNA viruses are single-stranded.
2 Causes hepatitis.
3 Causes acute gastroenteritis.
4 Enteroviruses are recoverable in primary and continous-type cultures of primate of human origin. Hepatitis A virus can sometimes be recovered in special cultures of primate or human origin. Coronaviruses are sometimes recoverable in tracheal organ cultures. Enteric adenoviruses are recoverable in Graham 293 cultures. Some rotaviruses and astroviruses can be recovered in cultures of primate or human origin with the help of tryptic enzymes.

3.6 Culture-based techniques involve the extracted microorganisms being grown (cultured) under specific conditions (primarily temperature, but also pressure and oxygen content) in a liquid or solid medium containing specific nutrients. Although some growth media such as Nutrient Agar are described as non-selective, the existence of a large number of non-culturable microorganisms in the environment means that by definition all growth media are selective. Some media are tailored to the nutrient requirements of specific microbial taxa, for example Pseudomonas Selective Agar (Oxoid, UK) which is designed for the culturing of pseudomonads

(Cartwright *et al.,* 2000), and are therefore more selective than a general medium such as Nutrient Agar.

3.7 Liquid and solid growth media are usually inoculated with a small volume (usually <100 µl) of liquid (water, buffer, saline) containing the microorganisms. The medium is then incubated under set conditions for a period of time. Different incubation temperatures will also select for different microorganisms:

- incubation temperatures of >55 ºC will select primarily for thermotolerant or thermophilic microorganisms;

- incubation temperatures of <10 ºC will select primarily for psychrotrophic microorganisms; and

- incubation temperature of 5-55 ºC will select primarily for mesophilic microorganisms. Temperature of around 37 ºC (body temperature) are likely to select for microorganisms that normally reside within the human body such as gastrointestinal bacteria, rather than those present in the wider environment. Temperatures of 37 ºC are usually required to select for pathogens of the human body. Microorganisms such as *Staphylococcus aureus* which are present on the surface of the skin, and can cause infections in skin wounds are usually cultured at temperatures lower than body temperature.

3.8 The length of incubation time is usually representative of the growth rate of the microorganisms of interest. For example fast growing bacteria such as *E. coli* and *Pseudomonas* sp. are incubated for up to 48 h, whereas slow growing species such as *Helicobacter pylori* and *Streptomyces* sp. are incubated for >5 d and for the streptomycetes for as long as 42 d.

3.9 After incubation of the growth medium, the microorganisms present can be detected on the basis of cell growth, and can be quantified by counting the number of colonies present. Quantification is only possible using solid growth media, and must be conducted whilst the microorganisms are visible as discrete colonies and before they have colonised the whole surface of the culture medium. Quantification of microorganisms by counting cfu assumes that each microorganism present in the original sample has grown to produce one colony. The microorganisms present can be identified by:

- the presence of growth. If the growth medium is specifically tailored for the nutrient requirements of a single taxon, then it is usually assumed that any microorganisms present are of that selected taxon. Microorganisms that are resistant to one or more antibiotics can also be selected, and therefore identified, by the addition of those antibiotics to the growth medium. If necessary further tests can be performed to confirm the identity of the microorganism, for example Gram stain[7], catalase[8] and oxidase[9] tests. Hattori *et al.* (1997) proposed that microorganisms could be categorised into taxonomic groups on the basis of kinetics of colony formation on solid media;

- colony morphology. Microorganisms such as the actinomycetes and the pseudomonads have a distinctive colony morphology that enables them to be identified when grown on solid growth media;

- the production of distinctive pigments, either in the colony, or within the surrounding growth medium. Applicable to bacteria such as the actinomycetes. The colour change is brought about either by the production of a specific pigment by the microorganism, or by providing the microorganism with a substrate which changes colour when utilised as a carbon or energy source. For example, the Coliert test for the rapid detection of coliform bacteria in water samples is a defined substrate-based test based on the detection of ß-galactosidase activity using the chromogenic substrate *ortho*-nitrophenyl- ß-D-galactopyranoside (ONPG). The MMO-MUG test involves the detection of ß-glucuronidase activity using the fluorgenic substrate MUG, and is used to detect *E. coli* in water samples (Bej *et al.,* 1991); and

- the production of antimicrobial compounds, such as antibiotics. Microorganisms known to produce compounds that are toxic to other microorganisms are often incubated on solid media in the presence of the

[7] Test used to divide bacteria into two groups (Gram positive and Gram negative), according to their ability to retain a crystal violet-iodine stain when treated with organic solvents (alcohol or acetone). Those bacteria that retain the Gram stain are termed Gram positive, and those that lose it Gram negative. The two groups of bacteria differ physically in the structure of their cell wall and cell membrane (Neidhart *et al*., 1990).

[8] Test used to differentiate bacteria on their ability to catalyze the conversion of hydrogen peroxide and superoxide into diatomic oxygen and water, by the production of catalase. The catalase test involves adding hydrogen peroxide to a culture sample. If the bacteria in question produce catalase, they will convert the hydrogen peroxide and oxygen gas will be evolved. The evolution of gas causes bubbles to form and is indicative of a positive test.

[9] Test used to distinguish bacteria on the basis of their ability to produce oxidase. Test involves the application of oxidase reagent (aqueous solution of 1 percent tetramethyl-p.phenylenediamine and ascorbic acid) to a colony of the microorganism grown on a solid medium. The development of a purple colour is a positive result.

sensitive strain. Inhibition of the sensitive strain indicates the presence of the anti-microbial compound producing strain.

3.10 Culture-based techniques can be used in the detection of bacteria, viruses and fungi extracted from air, water, soil, food and clinical samples in both a qualitative and quantitative manner. The advantages of culture-based techniques are that they are extremely easy to conduct, but are potentially time-consuming when a large number of growth media need to be inoculated and the counted.

Biolog

3.11 The Biolog system (Biolog Inc, USA) was developed originally for the detection and identification of specific microbial taxa (Bochner, 1989). The technique differentiates microbial taxa on the basis of their ability to utilise a range of substrates (Fulthorpe and Allen, 1994). Because of their different nutritional requirements, different microbial taxa will utilise a range of substrates at characteristic levels and rates. The Biolog system is however only applicable to the detection and identification of bacteria and non-filamentous fungi. Biolog systems have been developed specifically for Gram-positive bacteria, Gram-negative bacteria and yeasts.

3.12 In the original Biolog system microorganisms were inoculated into 96 wells of a microtitre plate. Ninety-five of the wells contained a different substrate (the sole source of carbon and nitrogen present), buffer and a tetrazolium salt. The ninety-sixth well was the control and only contained buffer and the tetrazolium salt. The purpose of the tetrazolium salt was to indicate the presence of microbial growth in the microtitre well (and therefore utilisation of the substrate). The tetrazolium salt is not reported to support microbial growth (Bochner, 1989). Metabolically active microbial cells take up the colourless tetrazolium salt and convert it to its reduced form, which is coloured and insoluble. Growth of the microorganisms on the substrate is therefore visible as a red clump at the base of the microtitre well (Guckert, 1996). The darker the red coloration, the greater the metabolic activity of the microbial cells present. The degree of red coloration is measured directly by visual observation, or spectrophotometrically by an automatic plate-reader.

3.13 The limitation of the Biolog system is that, because the technique is based on growth to provide a positive result, it is not applicable to the detection and identification of non-culturable microorganisms. However, because a liquid growth medium is reported to be a less stressful environment than the surface of a solid growth medium, more microorganisms are likely to be cultured in the Biolog system than on solid media.

3.14 The technique is also not suitable for the detection of filamentous microorganisms such as fungi. This is because such organisms can spread across a number of wells, and consequently produce false-positive results.

3.15 Garland and Mills (1991) reported the first use of Biolog plates for the characterisation of terrestrial and aquatic microbial communities, rather than single cultures of microorganisms. Although the technique requires the extraction of the microorganisms from the environment, the method is considerably less labour intensive and time consuming than multiple selective isolation plating (Buyer and Drinkwater, 1997), and has been found to be a reproducible method for assessing changes in microbial community function (Haack *et al.*, 1995). Because the assay is dependent on the growth of the microorganisms present in the microtitre wells, care should however be taken to ensure that the test conditions are standardised (particularly inoculum density) in order to compare a number of samples (Palojärvi *et al.,* 1997). A criticism of the Biolog system was that the substrates used in the plates were not relevant for environmental analysis. The choice of substrates was subsequently reduced to 31 substrates (Biolog Eco plates) (Insam, 1997) sufficient to distinguish soil microbial communities.

Fatty Acid Analysis

3.16 As with the Biolog system, the use of fatty acid analysis to detect and identify microorganisms on the basis of the types and relative amounts of fatty acid molecules present in their membranes, was originally developed for the identification of single cultures of microorganisms (Descheemaker and Swings, 1995). The technique works on the basis that individual fatty acids are discontinuously distributed between different microbial taxa, and that therefore individual taxa have a distinct fatty acid ‘fingerprint’. Fatty acid analysis is applicable to the detection and identification of bacteria and fungi.

3.17 Fatty acids consist of a chain of carbon atoms with a carboxyl group at one end and a methyl group at the other. Prokaryotic organisms almost exclusively produce saturated or monoenoic (one double bond) unsaturated fatty acids, whereas eukaryotic organisms such as fungi produce more polyenoic unsaturated fatty acids. Comparison of the ratio of monoenoic and polyenoic fatty acids is reported as an indicator of the relative abundance of bacteria and fungi in environmental samples.

3.18 The original use of fatty acid analysis for the detection and identification of microorganisms required the microorganisms to be extracted from their environment

and cultured prior to analysis. The method is therefore limited to culturable microorganisms.

3.19 Phospholipid fatty acid (PLFA) analysis and fatty acid methyl ester (FAME) analysis are both types of fatty acid analysis. Both methods rely on the extraction and identification of the fatty acid molecules present in the microorganism's cell membrane. FAME analysis includes all the fatty acids present in a microbial cell, whereas PLFA analysis only analyses the phospholipids present in cell membranes, and not those present in a cell as storage lipids (Palojärvi *et al.,* 1997). However, although different microbial taxa express different fatty acids (or the same fatty acids but in different ratios), fatty acid production is also affected by environmental conditions, particularly temperature.

3.20 Under conditions of ambient temperature the phospholipid molecules (comprising of a polar head group and two fatty acid chains) in the microbial membrane are in a semifluid state. Normal cellular function is dependent on the microorganism posessing a fluid state membrane (Melchior, 1982). Changes in temperature alter the degree of fluidity, and the microorganisms compensate for this by altering the composition of the fatty acids in their membrane (Diefenbach *et al.,* 1992). An increase in temperature causes an increase in membrane fluidity (Neidhardt, 1990), and microorganisms respond by replacing membrane fatty acids with ones that have a higher melting point, thereby reducing membrane fluidity (Melchior, 1982).

3.21 Therefore the analysis of fatty acid molecules in bacteria and fungi must be conducted under standardised conditions, as changes in temperature will influence the results and are likely to give incorrect results.

3.22 As with the Biolog system, fatty acid analysis has also been applied to the analysis of taxonomic changes in microbial communities (Bååth *et al.,* 1992; Rajendran *et al.,* 1994; Haack *et al.,* 1994; Pennanen *et al.,* 1996). Both PLFA analysis (Guckert *et al.*, 1985; Pennanen *et al.*, 1996) and FAME analysis (Thompson *et al.*, 1993; Cavigelli *et al.*, 1995; Cartwright *et al.,* 2000) have been used, although PLFA analysis is the more established approach. FAME analysis has however been shown to provide equivalent information to PLFA and has the advantage of automated analysis of samples using the Microbial Identification System (MIS) system allowing a greater throughput of samples (Haack *et al.*, 1994).

3.23 Although the technique is performed directly on the environmental sample, without any prior extraction, it is described in this section (rather than with the *in-situ* methods) for completeness.

3.24 Fatty acid analysis of whole environmental samples is sensitive to small changes in community structure due to the large number of fatty acids and combinations of fatty acids present in microbial lipids (Cavigelli *et al.,* 1995). Due to the catabolism of fatty acids after cell death, the fatty acids detected are part of intact cell membranes and therefore represent the viable microbial community (Tunlid and White, 1992 (cited by Palojärvi *et al.,* 1997); Zelles *et al.*, 1992).

3.25 Certain taxonomic groups of bacteria and fungi within the community are reported to be identified by the presence of specific 'biomarker' fatty acids (Dowling *et al.,* 1986; Haack *et al.*, 1994; Cavigelli *et al.*, 1995). Frostegård *et al.* (1993) used PLFA analysis to detect an increase in the ratio of Gram-positive to Gram-negative microorganisms, following an increase in soil pH due to the addition of lime or wood ash. Monoenoic and cyclopropane fatty acids were used as biomarkers for Gram-negative bacteria, and branched-chain fatty acids as a biomarker for Gram-positive microorganisms. Branched-chain fatty acids though have been detected in *Cytophaga* and *Flavobacterium* (Gram-negative) indicating that these biomarkers are not exclusive to Gram-positive organisms (Haack *et al.*, 1994). Fatty acid analysis has been used as a measure of microbial biomass (Zelles *et al.* 1995; Frostegård and Bååth, 1996), although the presence of lipid storage molecules may distort biomass determination by FAME analysis.

3.26 The expression of 'biomarker' fatty acids by a number of microbial taxa reduces their value as unique taxonomic signatures (Zelles *et al.,* 1995; Buyer and Drinkwater, 1997). Measurement of the biodiversity of microbial communities by fatty acid analysis should therefore be treated with care (Griffiths *et al.,* 1997). Fatty acid analysis has also been reported as a method of determining the nutrient status of the microbial community (Guckert *et al.,* 1985), as the fatty acids expressed by microorganisms are different during periods of environmental stress (Cartwright *et al.,* 2000).

IN-SITU METHODS TO DETECT MICROORGANISMS IN THE ENVIRONMENT

3.27 The advantage of *in-situ* methods for the detection of microorganisms in the environment, is that the microorganism(s) do not need to be extracted from the environment prior to detection or identification. Therefore, unlike culture-based *ex-situ* techniques, *in-situ* methods are able to detect and identify non-culturable microorganisms. As the microorganisms within this group are reported to constitute the majority of microorganisms present in the environment, particularly water, soil and sediment (Torsvik *et al.,* 1994), *in-situ* methods may be expected to detect and/or

identify a more representative proportion of the microorganisms present in an environment.

3.28 *In-situ* methods include microscope-based techniques, colorimetric-based techniques and molecular-based techniques.

Microscope-based Techniques

3.29 Microscope-based techniques are used commonly to enumerate microorganisms in environmental samples, without prior culturing (although the detection of microorganisms in soil by microscope usually requires some prior extraction of the cells into buffer). Enumeration of microorganisms directly is often referred to as direct-counting (Kepner and Pratt, 1994). Because direct-counting methods do not require the microorganisms to be cultured, such methods have been shown not to underestimate microbial numbers in a sample, a criticism that has been directed at culture-based techniques (Zobell, 1946; and Daley, 1979, both cited by Kepner and Pratt, 1994).

Brightfield and Phase-contrast Microscopy

3.30 Brightfield and phase-contrast microscopy are applicable to the enumeration of bacteria and fungi in environmental samples. Unless the microorganism has a distinctive characteristic cell morphology, both brightfield microscopy and phase-contrast microscopy are usually unable to identify the microorganism present due to the relatively low magnification of the sample.

Epifluorescence Microscopy

3.31 Epifluorescence microscopy is reported as the method of choice for the direct enumeration of microorganisms in environmental samples (Kepner and Pratt, 1994). Kämpfer *et al.* (1991) found that direct cell counts, as determined by epifluorescence microscopy, measured ten times more microorganisms than viable counts on selective media, indicating that the majority of soil microorganisms are viable but non-culturable.

3.32 In epifluorescence microscopy, the microbial cells present in the sample are labelled with a fluorescent marker (a fluorochrome). When the sample is viewed by epifluorescence microscopy with a specific filter then the labelled cells appear coloured, and are therefore more easily detected. Different fluorochromes are available for different types of cells, and also cells in different metabolic states.

Epifluorescence microscopy can be used, for example, to differentiate between viable and non-viable cells (irrespective of their culturability). Fluorochromes are available that indicate cell viability by differences in cytoplasmic redox potential, electron transport chain activity, enzymatic activity, cell membrane potential and membrane integrity (Kepner and Pratt, 1994).

3.33 The two fluorochromes that are most frequently used to enumerate microorganisms in environmental samples are acridine orange (3, 6-bis[dimethylamino]acridinium chloride) and DAPI (4',6-diamidino-2-phenylindole) (Kepner and Pratt, 1994). Both fluorochromes are nucleic acid stains and allow microorganisms to be distinguished on the basis of colour; and if the microscope is connected to an image analysis system, by size and shape of the microbial cells as well.

3.34 Acridine orange binds to both DNA and RNA. Single stranded nucleic acid emits an orange-red fluorescence and double stranded nucleic acid fluoresces green. DAPI fluoresces blue or bluish-white when bound to DNA and yellow when bound to non-DNA material (Kepner and Pratt, 1994). However, because DNA retains its staining properties even in non-viable cells, acridine orange and DAPI cannot be used to differentiate between dead, metabolically inactive but living, and living cells (Kepner and Pratt, 1994).

3.35 A limitation of epifluorescence microscopy is that with environmental samples such as soil and sediment, the fluorochrome may also bind to organic matter present, leading to an overestimation of the number of microorganisms present (Kämpfer *et al.*, 1991). If the sample contains too much organic matter, or too many microbial cells, then the sample is likely to appear as a mass of colour and the differentiation and enumeration of individual cells will not be possible. The potential for any binding of the same fluorochrome to organic particles and microorganisms should be addressed at the start of the method.

Immunofluorescence Microscopy

3.36 In immunofluoresence microscopy, the fluorochrome is attached to the microorganism by an antibody-antigen interaction. The antibodies used have a binding site for the fluorochrome (for example fluorescein isothiocyanate (FITC)) and also for a specific cell surface characteristic on the target microorganism. The use of monoclonal antibodies means that the fluorochrome can be targeted to a specific strain of microorganism. Labelled cells can then be detected by epifluorescence microscopy, or by flow cytometry.

3.37 The use of fluorescent labelled antibodies is limited as a method for the detection of microorganisms in soil, due to the non-specific adsorption of fluorescent labelled antibodies to soil particles (Lindahl and Bakken, 1995). This will lead to an overestimation of the numbers of microorganisms present in the soil sample.

3.38 Detection limits for immunofluoresence microscopy are reported to be 10^6 cfu g^{-1} soil. The specificity of the method is both an advantage in that the method may be targeted at individual strains or species of microorganisms, and also a disadvantage, as the antigens on the microorganisms may only be expressed under certain environmental conditions, such as nutrient limitation. Therefore, this technique is not suitable as a general monitoring tool, and is best applied in specific situations, where the microorganisms are likely to be present under similar environmental conditions, such as a bioreactor or fermenter.

3.39 Because of the expense and time required to generate antibodies, this technique is best applied to environments in which the target microorganism is known to be present. Other techniques are more suitable as a general screening method. Although monoclonal antibodies are usually used in preference to polyclonal antibodies to provide greater specificity, cross reactivity can still be a problem.

Confocal Microscopy

3.40 In confocal microscopy (also known as scanning confocal laser microscopy), the environmental sample is treated with one or more fluorochromes. A laser is then directed onto the sample causing the fluorochrome to fluoresce. By moving the laser across the sample, a three-dimensional picture of the fluorochrome labelled sample can be generated. The applications for confocal microscopy in the detection of microorganisms in environmental samples, include the analysis of the *in-situ* colonisation of soil particles by microorganisms and the *in-situ* structure of microbial biofilms (Bloem *et al.,* 1995; Hansen *et al.,* 1997; Neu and Lawrence, 1997)

Electron Microscopy

3.41 Both transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have been used in the detection and identification of microorganisms (viruses, bacteria and fungi). SEM is more suitable for determining the surface appearance of microbial cells and is used to analyse associations between cells, growth habits and in the study of biofilms. TEM however, has greater resolving power and is applicable therefore for the detection and identification of viruses. Rotaviruses were first detected by electron microscopy (Bishop *et al.,* 1973, cited by Metcalf *et al.,* 1995),

although immunological and nucleic acid-based techniques are now more widely used to detect this group of viruses (Metcalf *et al.,* 1995).

Flow cytometry

3.42 Flow cytometry is a technique for quantifying components or structural features of cells primarily by optical means (Muirhead *et al.,* 1985). The technique combines microscopy and cell biology into a single technique that has the ability to analyse many thousands of cells within seconds (Porter *et al.,* 1997). The method requires the cells to be in an aqueous suspension as single cells. Therefore the technique is only applicable as an *in-situ* process where the cells are present in this form, for example in water samples, some food samples, and blood and other body fluids. The application of flow cytometry to the quantification of microorganisms in soil and sediment samples, or in aqueous samples where the cells are present as aggregates or bound to particulate matter may require pre-extraction of the microorganisms present to get them in to a single cell (monodisperse) suspension). The application of flow cytometry is limited to the analysis of highly particulate matter such as soil (Porter *et al.,* 1997).

3.43 Once in suspension, the cells are passed single-file through a laser beam by continuous flow of a fine stream of the suspension. Each cell scatters some of the laser light, and the amount of light scattered is dependent on the size of the particle and the presence or absence of specific cell surface features. No staining is required for this process, although a fluorescent stain can be added to the cells to provide an additional detection marker. For the quantification of microorganisms in environmental samples, fluorescence is usually required, in the form of an autofluorescence signal, a general macromolecular dye, a fluorescent probe for a cellular function, or a specific label such as a fluorescent antibody or oligonucleotide probe (Porter *et al.,* 1997). The light scatter characteristics are not sufficient to differentiate cells in environmental samples (Porter *et al.,* 1997).

3.44 Flow cytometry is most applicable to the study of bacteria and yeast cells in aqueous samples. Viruses are too small to detect by conventional flow cytometry (Porter *et al.,* 1997). The application of flow cytometry to the detection of the protozoan parasites *Cryptosporidium* and *Giardia* in water is reported to be capable of detecting one oocyst in 10-100 l of water (depending on the turbidity of the sample) (Vesey *et al.,* 1994). Under optimum conditions, the automated process can count 1-2 x 10^3 cells s^{-1} (Davey and Kell, 1996).

3.45 The combination of flow cytometry with fluorescent *insitu* hybridisation (FISH) has the potential to quantify and/or sort cells on the basis of differences in nucleic acid sequences. FISH methods involve the labelling of specific nucleic acid sequences inside intact cells using so-called phylogenetic stains (DeLong *et al.,* 1989). Because the oligonucleotide probe conferring the fluorescence is correlated to the ribosomal RNA (rRNA) of the cell, then the method provides an indication of growth rate, cell activity and viability (DeLong *et al.,* 1989; Wallner *et al.,* 1993). However, in oligotrophic environments, low nutrient availability may result in the microorganisms present possessing too few ribosomes to permit sufficient oligonucleotide probe binding. This technique may therefore be limited in the analysis of such samples (Porter *et al.,* 1997).

3.46 Amplification of DNA sequences by polymerase chain reaction (PCR) prior to analysis by FISH has been proposed as a method for overcoming the potential detection limitations of FISH (Porter *et al.,* 1997). This approach has been developed for the detection of virus infected cells in tissue specimens, and should allow detection of cells using genes with a low copy number.

Colorimetric-based Techniques

3.47 Colorimetric-based techniques have applications for the *in-situ* detection of microorganisms in the environment. These techniques are based on the use of a compound which changes colour in the presence of the microorganism, or on the use of a coloured marker which binds to the target cell. Different compounds can be used to differentiate between different microbial taxa, and also microorganisms in different metabolic states.

Detection of Metabolic Activity

3.48 The compounds fluorescein diacetate (FDA) and 2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl tetrazolium chloride (INT) are commonly used to monitor the presence of the activity of bacteria and fungi in an environmental sample. Both compounds are taken up by the microbial cell, and are used by the microorganism as a terminal electron acceptor. If the microorganism is metabolically active the INT or FDA are reduced. With INT, the compound is reduced to iodonitrotetrazolium formazan (INTF) which is red and insoluble. The degree of reduction of the INT to INTF, and therefore the level of dehydrogenase activity (often used as a measure of metabolic activity) can be determined spectrophotometrically at 464 nm (Cartwright, 1998). Although the method will provide a measure of metabolic activity, the technique does not give a quantitative result for the number of microorganisms present in the sample.

The technique is also not suitable for the monitoring of viruses. Virus particles do not replicate outside their host organism, and are therefore not metabolically active in the environment.

3.49 The reduction of INT to INTF is also used as the basis of the dehydrogenase assay and the Biolog microtitre plate system (Bochner, 1989; Bauer *et al.*, 1991).

Most Probable Number Assays

3.50 Most probable number (MPN) assays are methods used to quantify the number of microorganisms in aqueous samples without direct counting (Makkar and Casida, 1987; Jones and Knowles, 1991). Serial dilution of the sample estimates the density of microorganisms present on the basis that one microorganism will produce a positive result after incubation. The application of MPN assays to soil samples does require the extraction of the microorganisms present into water or buffer.

3.51 MPN methods are proposed to be more reliable than growth on selective media for enumeration of microbial populations capable of degrading poorly soluble hydrophobic substrates such as long-chain hydrocarbons. Non-hydrocarbon degrading bacteria can grow on impurities present in even ultra-pure agar (Wrenn and Venosa, 1996), and homogeneous distribution of insoluble substrates in selective media is difficult to obtain (Haines *et al.*, 1996). MPN methods do not require direct counting and have been used widely for the enumeration of microorganisms (Makkar and Casida, 1987; Jones and Knowles, 1991).

Enzyme Linked Immunosorbant Assay

3.52 The enzyme linked immunosorbant assay (ELISA) method uses an antigen-antibody interaction to detect microorganisms in environmental samples. Antibodies are developed to bind to specific antigens on the surface of the target microorganism, and cause the microorganisms to become clumped together, and visible to the naked eye.

3.53 ELISA can be used quantitatively to detect the presence of bacteria, fungi and viruses in a range of environmental samples, including sewage, sediment, food and water samples (Nybroe *et al.,* 1990; Hübner *et al.,* 1992). The disadvantages with the ELISA technique is that it is not a straightforward method to perform, and with a detection limit of 10^5 organisms ml^{-1} is not as sensitive as other detection techniques such as PCR. As with immunofluoresence microscopy, the technique is dependent on the antigen antibody interaction. Therefore, the technique is not suitable as a general screening tool, and in environments where the antigens expressed by the target

microorganism are likely to change, for example in environments with a high level of competition for nutrients. Where the antigen is also expressed after cell death, the technique cannot be used to differentiate between viable and non-viable cells.

Reporter Gene Based Techniques

3.54 Reporter gene based techniques combine aspects of colorimetric-based and molecular-based methods for the detection of microorganisms in environmental samples. Although most reporter gene technology has focused on the detection of bacteria, reporter genes do exist for fungi. The term 'reporter gene' is used as a generic term to describe genes whose expression can be exploited to report on the occurrence of a specific event such as the expression of particular gene, or the presence of a particular cell, in an environmental sample.

3.55 Reporter genes can be used to distinguish a specific microorganism containing a known gene from the background microbial population that do not possess the gene, with a reported detection limit of approximately 100 cells g^{-1} soil or plant matter. Where the function of the reporter gene is likely to be influenced by environmental conditions, for example *lux* marked microorganisms which undergo starvation may lose fluorescence, then this should be taken into account when analysing the results. Reporter genes are usually inserted into the microorganism artificially, although genes indigenous to the microorganism may be used as reporter genes.

3.56 The selection of the reporter gene is dependent on the method used to detect the gene and the information that the reporter gene is intended to provide. No single reporter gene can provide all the information that is required in any environment. All reporter genes used must fulfil the following requirements:

- to encode something that can be measured, such as light or colour;
- to be unique to the environment that is monitored;
- to have a clearly determinable state of expression;
- to have a high sensitivity of detection, or at least a limit of detection sufficient to satisfy the needs of the application;
- ideally not to involve complex methodology, enabling any detection to be made as quickly as possible; and

- to be cheap to use, and to allow a large throughput of samples.

3.57 The best reporter genes are those which can be detected phenotypically. Most code for an enzyme which allow the expression of the reporter gene to be monitored directly, or indirectly by the addition of a substrate and the subsequent measurement of enzyme activity. Indirect measurements often use substrates which produce a coloured product when the reporter gene is expressed. Examples of reporter genes used in conjunction with substrates include the *lacZY* and *xylE* genes.

3.58 Although the detection method may involve either destructive or non-destructive sampling, the latter option may be essential in the analysis of population changes and community dynamics. Non-destructive sampling also allows *in situ* analysis of environmental samples.

Colour Marked Reporter Genes

3.59 The expression of colour marked reporter genes can be detected by the production of a coloured substrate. Examples include *xylE*, *gusA* and *lacZY.*

3.60 The *xylE* gene encodes for the compound catechol monooxygenase. Bacteria that express the *xylE* gene (either naturally or following genetic transformation) turn yellow when exposed to the colourless compound catechol. This system is useful to identify *xylE* labelled isolates growing on agar plates, as the catechol can be added as a spray across the whole isolation plate.

3.61 The disadvantages of the system are that the marked cells must be metabolically active to be identified, and that because the *xylE* gene is relatively widespread in soil bacterial species, its use as a selectable marker in the soil environment is limited.

3.62 The *gusA* gene encodes the enzyme β-glucuronidase, which in the presence of the substrate X-gluc results in the formation of a blue colour. This system has been used for the identification of marked cells on isolation plates and *in situ.*

3.63 The *lacZY* gene encodes the enzyme β-galactosidase, which in the presence of the substrate X-gal also results in the formation of a blue colour. The gene is more widely present in the environment than other colour marker genes, but is still useful as a reporter gene in some environments, and as a secondary marker gene.

Bioluminescent Reporter Genes

3.64 Bioluminescent reporter genes encode bioluminescent or fluorescent products. Examples include:

- *luc* - from *Photinus sp*. and *Phyrophorus sp*.;
- *Rluc* - from *Renila reniformis*;
- *lux* - from *Vibrio sp*. and *Photobacterium sp*.;
- *gfp* - from *Aequorea victoria* and *Renilla reniformis*; and
- *yfp* - from *Vibrio fischeri.*

3.65 The firefly's *luc* gene encodes a single 60.7 kDa polypeptide (luciferase). In the presence of the substrates D-luciferin and ATP, and the co-factors Mg^{2+} and O_2, the enzyme oxidises the luciferin with the reaction emitting light at a wavelength of 560nm. Because the quantum efficiency of the reaction is high, a relatively large amount of light is produced from the expression of a single copy of the *luc* gene.

3.66 The advantages of the *luc* gene system is that its expression places little additional metabolic burden on its host, which may be either prokaryotic or eukaryotic. The system works well at 37ºC, and is also suitable for use in marine systems. Because of the natural bioluminescence of a range of marine organisms, the expression of specific bioluminescent genes cannot always be distinguished from the background activity. However, as the light from the *luc* system is emitted at a different wavelength to marine bioluminescent organisms then expression of the *luc* gene can be measured without background interference.

3.67 The disadvantages of the *luc* gene system are that the substrate D-luciferin is expensive and is only freely permeable below pH 5, thereby restricting its use to acidic environments.

3.68 The luciferase (*Rluc*) gene isolated from the Click Beetle operates in a very similar manner to the firefly luciferase, and can use the same substrate. The advantage of the *Rluc* gene system over the *luc* system is that the luciferase gene isolated from different species of click beetle encode luciferase enzymes with different emission

wavelengths (574-593 nm). This enables an organism to be marked with a double luminescent marker if required.

3.69 The *Rluc* gene system was reported to have been used to mark the soil microorganism *Rhizobium meliloti* at the *recA* site on the chromosome. *Rhizobium sp.* marked with the *luc* gene was detected *in situ* in the root nodules of an alfalfa plant following the exposure of the nodules onto film using X-rays. The use of such *in situ* methods removes the requirement for extracting and culturing the microorganisms.

3.70 The bacterial luciferase (*lux*) differs from the insect luciferases in that the substrates are a long chain aldehyde and the reduced form of flavin mononulceotide ($FMNH_2$). Because the presence of this gene is rare in the environment, there is very little problem with background interference, and this makes the *lux* gene well suited for use in the environment. Because the luminescence of a particular cell is linked to its metabolic activity, the insertion of the *lux* gene into cells in nutrient limited environments enables their level of activity to be monitored.

3.71 *In situ* monitoring of *lux* marked *Pseudomonas syringae* and *Enterobacter cloccae* in the rhizosphere revealed that the highest cell density was in the area closest to the root.

3.72 The *gfp* gene encodes the single polypeptide green fluorescent protein (GFP). Although *gfp* is of eukaryotic origin, it can be used in bacterial systems. A property of GFP is that it is naturally fluorescent and does not require the presence of any co-factors to operate. This however, can be an advantage and a disadvantage. For example *gfp* marked bacteria can be detected following their ingestion into the food vacuole of a ciliate. However, as the GFP is relatively stable (and naturally fluorescent) is will be continued to be detected after the *gfp* marked bacterium is dead.

3.73 Because of this persistence, the production of GFP is a poor marker of gene expression, unless its production is monitored continuously.

Molecular-based Techniques - Introduction

3.74 Molecular-based techniques involve the detection and/or identification of a specific component of the microbial cell. In general molecular techniques usually refer to the analysis or detection of a specific gene or nucleic acid sequence. However molecular-based techniques also include the detection of specific proteins or characteristic cellular structures or compounds, such as lipid storage molecules and cell surface antigens (Akkermans *et al.,* 1994).

3.75 Although molecular-based techniques are described in this report as *in-situ* processes, as the methods are performed without the target microorganism being cultured, the methods used usually requires some form of extraction stage. Analysis of genes or specific nucleic acid sequences requires the DNA or RNA to be extracted from the microorganism prior to analysis. This is usually achieved by lysing the cell by a physical or chemical process. Cells may be lysed *in-situ* in the environmental sample, or may be extracted from the sample and then lysed (Leff *et al.,* 1995; Krsek and Wellington, 1999).

3.76 The extraction of 16S rRNA from environmental samples is reported to be preferable in certain circumstances over the extraction of DNA, as 16S rRNA is often present in the sample in larger quantities (Akkermans *et al.,* 1994). Methods based on RNA detection are also more relevant if information is required on the metabolic state of the microorganisms present in the sample. However, because RNA is chemically less stable than DNA and is more easily degraded by nucleases (present in large quantities in environmental samples), more elaborate extraction procedures are required to minimise loss of sample (Akkermans *et al.,* 1994). Analysis of the metabolic activity of microorganisms in marine sediments using an evaluation of the ratio of the amounts of RNA and DNA present is limited by the presence of substantial amounts of DNA associated with dead cells and/or absorbed to particulate matter (Dell'anno *et al.,* 1998).

3.77 Methods for the extraction of nucleic acid from the environment can be divided into physical and chemical based methods (Krsek and Wellington, 1999). The number of methods used to extract DNA from soil for example is an indication of the heterogeneity of soil and the microbial community within it, as well as the range of subsequent analysis techniques employed (Krsek and Wellington, 1999). The sensitivity and precision of the molecular methods used to study the nucleic acid are dependent on the efficiency of the techniques used to extract and purify the DNA and/or RNA from the microorganism (Haughland *et al.,* 1999). None of the available methods available are reported to be applicable as a universal analysis method (Akkermans *et al.,* 1994), and to date there has been no systematic survey and comparison of the efficiency and reliability of the available extraction methods (Krsek and Wellington, 1999):

- physical methods - include glass bead disruption, RiboLyser/FastPrep systems, freeze/thaw and hydrodynamic shear:

  - glass bead disruption - the environmental sample is shaken vigorously in a vessel containing buffer and small glass beads. The shear forces

generated during shaking fracture the cell membranes and release the nucleic acids. The vessel is often precooled to minimise detrimental heating of the sample during agitation. The method is applicable to the extraction of nucleic acids from bacteria and fungi from a range of environmental samples, including soil and water. Efficiency of disruption depends on cell structure, and the size and type of beads used can be varied to enhance extraction from particular target microorganisms (Ogram *et al.,* 1987).

- RiboLyser/FastPrep systems - both these systems extract nucleic acids by agitating the sample at high speed to lyse the cells. The Hybaid system which is used to extract RNA, lyses the cells with a matrix of silica and ceramic particles, with the released RNA stabilised by further reagents in the lysing matrix. Other matrices are available for the extraction of DNA. Both systems can be used to extract nucleic acids from microorganisms in a range of environmental samples, including soil and water. RNA extraction kits are available for bacteria, fungi and yeasts. Efficiency of disruption depends on cell structure, and the size and type of beads used can be varied to enhance extraction from particular target microorganisms.

- freeze/thaw - used for the extraction of high molecular weight DNA from environmental samples including soil and sediment. The disadvantage of the method is that it is an indiscriminate method for lysing cells, and is likely to release cell nucleases which will damage the released nucleic acid unless the lysed cells are treated quickly.

- hydrodynamic shear - involves the use of a French press (or French pressure cell) to lyse the cells as they are forced through a small orifice under pressure. This method is applicable to the extraction of nucleic acid from many bacteria, although some Gram-positive cocci and some archaebacteria are not easily lysed in this way. The advantage of this method compared to sonication (Section 2), is that lysis is virtually instanteous and the lysed cells are not subjected to additional shear forces.

• chemical methods - the addition of chemicals such as polyvinylpolypyrrolidone (PVPP) to the sample during extraction of nucleic acids can help to improve the purity of recovered DNA by removing humic acids which may interfere with subsequent analyses of the nucleic acid.

Because the strength of most bacterial cell walls is due to the presence of murein, destruction of this compound prior to cell lysis can improve the degree of lysis achieved. Murein can be damaged or destroyed by the addition of specific enzymes such as lysozyme, which will leave the cells osmotically sensitive (Tsai and Olsen, 1991). Such cells can then be lysed following exposure to a hypotonic solution. Exposure to lysozyme is reported to result in the extraction of DNA with less fragmentation compared to agitation with glass beads (Leff *et al.,* 1995). However, the yield of DNA was lower than that achieved with extraction using glass beads (Leff *et al.,* 1995). This method is not applicable to the archaebacteria as they posess a lysozyme-insensitive pseudomurein (rather than murein) in their cell walls.

The resistance of fungal spores and conidia to lysis should also be taken into account when extracting nucleic acid from such cells (Haughland *et al.,* 1999).

Molecular-based Techniques - Nucleic Acid Based Methods

3.78 Nucleic acid based methods involve the detection and/or identification of microorganisms on the basis of their genetic material (DNA or RNA). The advantage of nucleic acid based methods is that because the microorganisms do not have to be cultured or even extracted from the environmental sample prior to analysis, the methods are applicable to the analysis of a greater proportion of the microbial community. Information on the microorganisms present in an environmental sample can be inferred from the analysis of the genes present (Gottschal *et al.,* 1997). The use of the range of molecular-based methods now available, in particular those using rRNA targeted fluorescent molecular probes, enables the abundance and diversity of microorganisms present in an environmental sample to be comprehensively assessed (Gottschal *et al.,* 1997).

3.79 The extraction of DNA from soil has been used to assess the genetic diversity of microbial communities (Griffiths *et al.,* 1997), with good agreement with phenotypic diversity measurements (Torsvik *et al.*, 1990). DNA reassociation assays of the extracted DNA can indicate the species diversity present (Leung *et al.,* 1994). Heterogeneity of extracted DNA is a measure of the total number of genetically different bacteria in soil, and can be determined by thermal denaturation and reassociation kinetics (Torsvik *et al.*, 1990). The use of 16S rRNA-targeted oligonucleotide probes can detect the presence or absence of specific taxa, although this is limited by the choice of probe (Amann, 1995; Stahl, 1995).

Polymerase Chain Reaction

3.80 The polymerase chain reaction (PCR) is a method for amplifying a sequence of DNA using a heat-stable polymerase and two 20-base primers, one complementary to the (+)-strand at one end of the sequence to be amplified and the other complementary to the (-)-strand at the other end. Amplification of DNA in a sample by PCR is required if the target microorganisms are present in numbers of $<10^6$ ml^{-1} (Akkermans *et al.,* 1994).

3.81 Because the newly synthesised DNA strands can subsequently serve as additional templates for the same primer sequences, successive rounds of primer annealing, strand elongation, and dissociation, produce rapid and highly specific amplification of the desired sequence. Continuation of the process by 25 cycles is reported to lead to an approximate 1 million fold increase in the amount of DNA present. PCR can also be used to detect the existence of the defined sequence in a DNA sample, and the amplification capability means that even small samples of DNA in a sample can be increased to a sufficient level to allow them to be studied by other techniques.

3.82 PCR is applicable to the study of bacteria, fungi and viruses in a range of environmental samples, including soil, sediment, water, air and clinical samples (Haughland *et al.,* 1999). The use of the method though is limited with soil and sediment samples due to the presence of humic acids and metal ions which may inhibit the PCR process, although the development of new methods is reported to remove this problem (Gelsomino *et al.,* 1999). In theory, PCR is able to detect a single copy of a nucleic acid sequence. However, with environmental samples, this is unlikely to be achieved. Sensitivity can be improved by increasing the number of cycles, optimisation of the reaction conditions and concentration of the amount of target DNA in the sample. Steffan and Atlas (1988) reported that PCR could be used to detect a single cell of *Pseudomonas cepacia* in one gram of sediment, against a background of 10^{11} cell g^{-1}, although Bej *et al.* (1991) reported that a detection level of single cells in 100 ml was more likely in environmental samples. A further limitation of the technique is the inability to distinguish between culturable and non-culturable microorganisms (Buttner *et al.,* 1997).

3.83 Since the development of the original technique several variations have been developed, each with a number of advantages over the standard approach:

- nested PCR - the PCR cycle is performed 15-30 times with one primer set, and then a further 15-30 times with a second set of primers on an internal region of the first amplified DNA product. The advantage of this method is that because

non-specific primer annealing is avoided, the level of specificity and amplification efficiency is increased (Puig *et al.,* 1994);

- *in-situ* PCR - the reaction is performed within the intact microbial cell. This allows the product to be contained at the site of amplification. The advantage of this method is the ability to obtain spatial information on gene expression (Porter *et al.,* 1997);

- reverse transcriptase PCR - this is performed as an initial stage prior to the standard PCR. The reverse transcriptase is used to synthesise a complementary DNA strand (cDNA) to a target RNA sequence (Tsai *et al.,* 1993); and

- multiplex PCR - involves the use of multiple sets of primers and results in the production of multiple products (Bej *et al.,* 1991).

Restriction Fragment Length Polymorphism

3.84 Restriction fragment length polymorphism (RFLP) involves the exposure of the DNA to one or more restriction endocnucleases. These enzymes cleave the DNA at specific sequences into fragments ranging from 100 basepairs (bp) to 10 kbp in length.

3.85 The DNA fragments are then separated on the basis of size by gel electrophoresis. Polyacrylamide gels are used to separate fragments of <1000 bp, whereas more porous agarose gels are used to resolve mixtures of larger fragments (up to 20 kbp) (Stryer, 1988).

3.86 Following separation by gel electrophoresis, a fragment containing a specific base sequence can be identified by hybridising it with a labelled complementary DNA strand (DNA probe); a process known as Southern Blotting[10,11]. A mixture of restriction fragments are then separated on a second agarose gel, denatured to form single-stranded DNA and transferred to a nitrocellulose sheet. Autoradiography is used to identify the restriction fragment with a complementary sequence to that of the DNA probe.

[10] So named as it was devised by EM Southern (Stryer, 1988).

[11] A similar process, known as Northern Blotting is used to identify RNA fragments, and Western Blotting is the technique used to detect a particular protein by staining with a specific antibody. Southern, Northern and Western Blotting are also known as DNA, RNA and protein blotting (Stryer, 1988).

3.87 A limitation of RFLP analysis is that it can only be performed on single species of microorganism. Therefore in order to analyse mixed populations, the sample must first be separated, either by culture, PCR with specific primers, or by PCR and cloning techniques. The application of RFLP is therefore best suited to the analysis of single cultures of microorganisms, or samples with a low biodiversity, such as food or clinical samples.

Denaturing Gradient Gel Electrophoresis

3.88 Denaturing gradient gel electrophoresis (DGGE) is a technique that can be used to separate DNA fragments of the same length but differing in as little as a single base change. The basis of the technique is that DNA fragments that have different nucleotide sequences denature to differing degrees in the presence of a given concentration of denaturing chemicals (7M urea and 40 percent formamide, and temperature of 50-65 °C). The more denatured the DNA fragment, the lower its electrophoretic mobility in polyacrylamide gel.

3.89 Because DGGE is based on analysis of nucleic acid sequences, it is applicable to the study of bacteria, viruses and fungi in any environment from which DNA can be extracted (Ferris *et al.,* 1996; Kowalchuk *et al.,* 1997). The sensitivity of the technique depends on the quality of the DNA extracted from the sample, and is reported to be similar to that for PCR. As DGGE requires the use of PCR to amplify the DNA present it can only be described as semi-quantitative.

Temperature Gradient Gel Electrophoresis

3.90 Temperature gradient gel electrophoresis (TGGE) is a similar technique to DGGE. However, with TGGE the concentration of denaturing chemicals remains uniform whilst the temperature of the gel is increased gradually and uniformly, so that as the DNA passes down the gel, it encounters gradually increasing temperatures (Schafer *et al.,* 1993; Lu *et al.,*1995; Nubel *et al.,* 1996). The advantage of TGGE over DGGE, is that as no chemical gradient is required, rapid high-throughput screening of samples is possible. However, the specificity of TGGE is slightly lower than DGGE.

Nucleic Acid Probes

3.91 Nucleic acid probes enable the presence of specific microorganisms in an environmental sample to be detected by direct hybridisation with a specific probe. Nucleic acid probes are nucleotide sequences complementary to the sequences in the nucleic acid of the target microorganism (Akkermans *et al.,* 1994). The probes can be

either DNA or RNA and are usually labelled with some form of marker or reporter group. The probe signal that is obtained from the reporter group after hybridisation and washing away the excess unbound probe is proportional to the amount of target nucleic acid present in the sample (Akkermans *et al.,* 1994).

3.92 DNA probes used in the analysis of environmental samples may consist of either relatively long cloned fragments of plasmids or genes, or shorter (usually 20-30 bp) nucleic acid sequences. These shorter sequences are termed oligonucleotide probes (Akkermans *et al.,* 1994). 16S rRNA oligonucleotide probes have been used successfully for the detection of both culturable and non-culturable microorganisms. Because the sequence required for the probe can be determined readily by PCR, 16S rRNA probes can be developed relatively easily.

3.93 Although nucleic acid probes have been shown to be very useful in the analysis of microorganisms in environmental samples, the lack of sensitivity of the technique is a significant limitation on what can be studied. Microbial populations of $<10^6$ cells ml^{-1} are not detected accurately using this technique (Akkermans *et al.,* 1994).

Fluorescent In Situ Hybridisation

3.94 Fluorescent *in-situ* hybridisation (FISH) methods involve the labelling of specific nucleic acid sequences inside intact cells using so-called phylogenetic stains (DeLong *et al.,* 1989). Because the oligonucleotide probe conferring the fluorescence is correlated to the ribosomal RNA (rRNA) of the cell, then the method provides an indication of growth rate, cell activity and viability (DeLong *et al.,* 1989; Wallner *et al.,* 1993).

Gene Expression

3.95 All of the techniques discussed above involve the detection of microorganisms on the basis of rRNA or DNA target sequences. Although such methods are applicable to the detection and/or identification of particular microorganisms in an environmental sample, they do not provide information on the metabolic activity of the cell. This can be determined by measuring the expression of particular genes and can also provide information to differentiate between live and dead cells (Akkermans *et al.,* 1994). Gene expression can be detected by measuring the production of messenger RNA (mRNA). However the very short half-life of mRNA produced by microbial cells (compared to that in eukaryotic systems) does not make the assay easy to perform (Akkermans *et al.,* 1994).

Molecular-based Techniques - Other Methods

3.96 Non-nucleic acid based molecular techniques involve the detection and/or identification of microorganisms on the basis of specific physical cellular characteristics. Some of these techniques have been described previously in this report, such as fatty acid analysis, in which the detection of individual fatty acids expressed by microorganisms in their cell membrane is used to identify individual microbial taxa (to the species level), and also to detect changes in microbial community function and biodiversity. The presence of cell surface structures forms the basis of the immunological techniques described, including ELISA, and immunofluorescence microscopy.

3.97 Other methods that have not been discussed, include whole cell analyses in which all the characteristics of the microorganism are analysed as part of the identification process. Whole cell analyses can include the use of a battery of physiological and biochemical tests, but are more likely to describe a single process such as pyrolysis mass spectrometry (PyMS) which generate a 'chemical fingerprint' of the microorganism. Such techniques are *ex-situ* processes and are used primarily for identification of pure cultures of microorganisms (fungi and bacteria).

3.98 Pyrolysis is the thermal degradation of complex organic material in an inert atmosphere or vacuum. Thermal intramolecular vibrations cause molecules to cleave at their weakest point producing a mixture of low molecular weight, predominantly volatile organic compounds. These compounds are then separated by mass spectrometry on the basis of their mass to charge ratio, to produce a pyrolysis mass spectrum or pyrogram (essentially the 'chemical fingerprint' of the microorganism).

3.99 The advantage of PyMS compared to other identification procedures, such as the detection of specific chemical markers and the use nucleic acid probes, is that it is rapid with respect to both single and multiple samples. Results are obtained quickly, approximately 90 seconds per sample with up to 300 samples being analysed in one run. The results consist of quantitative information on the pyrolysis products. Although most bacteria generate similar pyrolysis products, the quantities, shown by the peak heights on the pyrogram, vary significantly and reproducibly from one strain to another. Their differences can be exploited to determine the relationships between organisms by comparing the relevant pyrograms. This is handled rapidly by commercially available statistical packages such as GENSTAT and SIMCA, which select the most reproducible and discriminatory peaks to separate the samples.

4. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

4.1 There are a wide variety of methods available for the extraction, detection and identification of microorganisms from the environment. Detection and identification methods are divided into *ex-situ* and *in-situ* techniques, with *in-situ* processes offering the potential to detect and/or identify a greater proportion of the microorganisms present in an environmental sample, particularly those in very heterogeneic environments such as soil and sediment, where a proportion of the microorganisms are likely to be bound to the soil or sediment particles.

4.2 Because of the variety of methods available for the extraction, detection and identification of microorganisms in the environment, the objective of the extraction, detection or identification process should be considered before any method is selected. Each of the methods available has a number of advantages and disadvantages, and these should be addressed as part of the selection process. The reasons for selecting a particular method include the cost, the time available, the environment to be sampled and the microorganism to be extracted, detected or identified. Although many culture-based methods are relatively cheap to use and require a relatively low level of technical expertise, some of the culture programmes require a long culture period (>10 days), and will therefore not provide the rapid result that may be required. Another point that should be considered is whether the information required from the detection or identification process needs to be quantitative, or whether a qualitative result is acceptable.

4.3 The development of methods for the extraction, detection and identification of microorganisms from the environment is ongoing continuously, with new methods, and new applications for existing methods constantly being developed and presented in the scientific literature. No particular areas have been identified in the production of this report that are proposed as requiring further research or investigation. However, it is recommended that due to ongoing developments in this field, that this report is updated periodically to reflect the changes that are likely to occur in the most suitable methods to extract, detect and/or identify microorganisms in the environment.

5. REFERENCES

Akkermans ADL, Mirza MJ, Harmsen HJM, Blok HJ, Herron PR, Sessitsch A, Akkermans WM (1994). Molecular ecology of microbes: a review of promises, pitfalls and true progress. *FEMS Microbiology Reviews*, 15,185-194.

Amann RI (1995). Fluorescently labelled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. *Molecular Ecology* 4, 543-554.

American Public Health Association (1989). Standards methods for the examination of water and wastewater, 17th ed. American Public Health Association, Washington DC, USA.

Bååth E, Frostegård A, Fritze H (1992). Soil bacterial biomass, activity, phospholipid fatty-acid pattern, and pH tolerance in an area polluted with alkaline dust deposition. *Applied and Environmental Microbiology* 58, 4026-4031.

Bauer E, Pennerstorfer C, Holubar P, Plas C, Braun R (1991). Microbial activity measurement in soil - a comparison of methods. *Journal of Microbiological Methods* 14, 109-117.

Bej AK, Mahbubani MH, Dicesare JL, Atlas RM (1991). Polymerase chain reaction-gene probe detection of microorganisms by using filter-concentrated samples. *Applied and Environmental Microbiology,* 57, 3529-3534.

Bloem J, Veninga M, Shepherd J (1995). Fully-automatic determination of soil bacterium numbers, cell volumes and frequencies of dividing cells by confocal laser scanning microscopy and image-analysis. *Applied and Environmental Microbiology*, 61, 926-936.

Blomquist G; Palmgren U; Ström G (1987). Methodological aspects of measurement of exposure to mould. *European Journal of Respiratory Diseases*, 154 (71), 29-36.

Bochner B (1989). Breathprints at the microbial level. *ASM News* 55, 536-539.

Buttner MP, Willeke K, Grinshpun SA (1997). Sampling and analysis of airborne microorganisms. In: Hurst CJ, Knudsen GR, McInerney MJ, Stetzenbach LD, Walter MV (eds) Manual of Environmental Microbiology, American Society for Microbiology, Washington DC, USA, pp 629-640.

Buyer JS, Drinkwater LE (1997). Comparison of substrate utilisation assay and fatty acid analysis of soil microbial communities. *Journal of Microbiological Methods* 30, 3-12.

Cartwright CD (1998). Biodegradation and impact of phthalate plasticisers on a soil microbial community. PhD Thesis, University of Kent at Canterbury, Canterbury, UK.

Cartwright CD, Thompson IP, Burns RG (2000). Degradation and impact of phthalate plasticizers on soil microbial communities. *Environmental Toxicology and Chemistry,* 19 (5), 1253-1261.

Cavigelli MA, Robertson GP, Klug MJ (1995). Fatty-acid methyl-ester (FAME) profiles as measures of soil microbial community structure. *Plant and Soil* 170, 99-113.

Coote PJ, Holyoak CD, Cole MB (1991). Thermal inactivation of *Listeria monocytogenes* during a process simulating temperatures achieved during microwave heating. *J Appl Bacteriol*, 70:6, 489-94.

Davey HM, Kell DB (1996). Flow cytometry and cell sorting of heterogenous microbial populations: the importance of single-cell analyses. *Microbiological Reviews*, 60 (4), 641-696.

Dell'Anno A, Fabiano M, Duineveld GCA, Kok A, Danovaro R (1998). Nucleic acid (DNA, RNA) quantification and RNA/DNA ratio determination in marine sediments: Comparison of spectrophotometric, fluorometric, and high performance liquid chromatography methods and estimation of detrital DNA. *Applied and Environmental Microbiology,* 64, 3238-3245.

DeLong EF, Wickham GS, Pace NR (1989). Phylogenetic stains: Ribosomal RNA-based probes for the identification of single cells. *Science*, 243, 1360-1363.

Descheemaker P, Swings J (1995). The application of fatty acid methyl ester analysis (FAME) for the identification of heterotrophic bacteria present in decaying Lede-stone of the St. Bavo Cathedral in Ghent. *The Science of the Total Environment* 167, 241-247.

Diefenbach R, Heipieper HJ, Keweloh H (1992). The conversion of *cis* into *trans* unsaturated fatty acids in *Pseudomonas putida* P8: evidence for a role in the regulation of membrane fluidity. *Applied Microbiology and Biotechnology* 38, 382-387.

Doultree JC, Druce JD, Birch CJ, Bowden DS, Marshall JA (1999). Inactivation of feline calicivirus, a Norwalk virus surrogate. *J Hosp Infect*, 41:1, 51-7.

Dowling NJE, Widdel F, White DC (1986). Phospholipid ester-linked fatty acid biomarkers of acetate-oxidising sulphate-reducers and other sulphide-forming bacteria. *Journal of General Microbiology* 132, 1815-1825.

Ferris MJ, Muyzer G, Ward DM (1996). Denaturing gradient gel-electrophoresis profiles of 16S ribosomal-RNA-defined populations inhabiting a hot spring microbial mat community. *Applied and Environmental Microbiology,* 62, 340-346.

Frostegård A, Bååth E, Tunlid A (1993). Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty-acid analysis. *Soil Biology and Biochemistry* 25, 723-730.

Frostegård A, Bååth E (1996). The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biology and Fertility of Soils* 22, 59-65.

Fulthorpe RR, Allen DG (1994). Evaluation of Biolog MT plates for aromatic and chloramatic substrate utilisation tests. *Canadian Journal of Microbiology* 40, 1067-1071.

Garland JL, Mills AL (1991). Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon- source utilization. *Applied and Environmental Microbiology* 57, 2351-2359.

Gelsomino A, Keijzer-Wolters AC, Cacco G, van Elsas JD (1999). Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis. *Journal of Microbiological Methods*, 38(1-2), 1-15.

Gottschal JC, Meijer WG, Oda Y (1997). Use of molecular probing to assess microbial activities in natural ecosystems. In *Microbial Communities: functional versus structural approaches*, pp. 10-18. Edited by Rangger, A. & Insam, I. Berlin: Springer.

Griffiths BS, Ritz K, Wheatley RE (1997). Relationship between functional diversity and genetic diversity in complex microbial communities. In *Microbial Communities: functional versus structural approaches*, pp. 1-9. Edited by Rangger, A. & Insam, I. Berlin: Springer.

Gsell TC, Holben WE, Ventullo RM (1997). Characterization of the sediment bacterial community in groundwater discharge zones of an alkaline fen: a seasonal study. *Applied and Environmental Microbiology* 63, 3111-3118.

Guckert JB, Antworth CP, Nichols PD, White DC (1985). Phosopholipid, ester linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. *FEMS Microbiology Ecology* 31, 147-158.

Guckert JB (1996). Toxicity assessment by community analysis. *Journal of Microbiological Methods* 25, 101-112.

Haack SK, Garchow H, Odelson DA, Forney LJ, Klug MJ (1994). Accuracy, reproducibility, and interpretation of fatty-acid methyl- ester profiles of model bacterial communities. *Applied and Environmental Microbiology* 60, 2483-2493

Haack SK, Garchow H, Klug MJ, Forney LJ (1995). Analysis of factors affecting the accuracy, reproducibility, and interpretation of microbial community carbon source utilization patterns. *Applied and Environmental Microbiology* 61, 1458-1468.

Haines JR, Wrenn BA, Holder EL, Strohmeier KL, Herrington RT, Venosa AD (1996). Measurement of hydrocarbon-degrading microbial populations by a 96-well plate most-probable-number procedure. *Journal of Industrial Microbiology* 16, 36-41.

Hansen M, Kragelund L, Nybroe O, Sorensen J (1997). Early colonisations of barley roots by *Pseudomonas fluorescens* studied by immunofluorescence technique and confocal laser scanning microscopy. *FEMS Microbiology Ecology*, 23, 353-360.

Hattori T, Mitsui H, Hattori R, Shikano S, Gorlach K, Kasahara Y, El-Beltagy A (1997). Analysis of the bacterial community according to colony development on solid medium. In *Microbial Communities: functional versus structural approaches*, pp. 229-235. Edited by Rangger, A. & Insam, I. Berlin: Springer.

Haugland RA, Heckman JL, Wymer LJ (1999). Evaluation of different methods for the extraction of DNA from fungal conidia by quantitative competitive PCR analysis. *Journal Of Microbiological Methods*, 37(2), 165-176.

Hopkins DW, Macnaughton SJ, O'Donnell AG (1991). A dispersion and differential centrifugation technique for representatively sampling microorganisms from soil. *Soil Biology and Biochemistry,* 23, 217-225.

Hübner I, Steinmetz I, Obst U, Giebel D, Bitter-Suerman D (1992). Rapid determination of members of the *Enterbacteriaceae* in drinking water by an immunological assay using a monoclonal antibody against enterobacterial common antigen. *Applied and Environmental Microbiology*, 58, 3187-3191.

Hýsek J, Fisar Z, Zizka Z, Kofronova O, Binek B (1991). Airborne microorganism monitoring: a comparison of several methods, including a new direct counting technique. *Zentralbl Mikrobiol*, 146:6, 435-43.

Insam H (1997). A new set of substrates proposed for community characterisation in environmental samples. In *Microbial Communities: functional versus structural approaches*, pp. 259-260. Edited by Rangger, A. & Insam, I. Berlin: Springer.

Jones AM, Knowles R (1991). Enumeration of *Flexibacter canadensis* in environmental samples by using a bacteriophage isolated from soil. *Applied and Environmental Microbiology* 57, 3043-3045.

Kämpfer P, Steiof M, Dott W (1991). Microbiological characterization of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria. *Microbial Ecology* 21, 227-251.

Kepner RL, Pratt JR (1994). Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. *Microbiological Reviews,* 58 (4), 603-615.

Kowalchuk GA, Gerards S, Woldendorp JW (1997). Detection and characterisation of fungal infections of *Ammophila arenaria* (Marram Grass) roots by denaturing gradient gel-electrophoresis of specifically amplified 16S rRNA. *Applied and Environmental Microbiology*, 63, 3858-3865.

Kresk M, Wellington EMH (1999). Comparison of different methods for the isolation and purification of total community DNA from soil. *Journal of Microbiological Methods,* 39, 1-16.

Leff LG, Dana JR, McArthur JV, Shimkets LJ (1995). Comparison of methods of DNA extraction from stream sediments. *Applied and Environmental Microbiology,* 61, 1141-1143.

Leung K, Strain SR, Debruijn FJ, Bottomley PJ (1994). Genotypic and phenotypic comparisons of chromosomal types within an indigenous soil population of *Rhizobium-leguminosarum bv trifolii*. *Applied and Environmental Microbiology* 60, 416-426.

Lindahl V, Bakken LR (1995). Evaluation of methods for the extraction of bacteria from soil. *FEMS Microbiology Ecology,* 16, 135-142.

Lu MJ, Funsch B, Wiese M, Roggendorf M (1995). Analysis of Hepatitis-C virus quasi-species populations by temperature-gradient gel-electrophoresis. *Journal of General Virology,* 76, 881-887.

MacDonald RM (1986). Sampling soil microfloras: optimisation of density gradient centrifugation in Percoll to separate microorganisms from soil suspensions. *Soil Biology and Biochemistry,* 18, 407-410.

Makkar NS, Casida LE (1987). Technique for estimating low numbers of a bacterial strain(s) in soil. *Applied and Environmental Microbiology* 53, 887-888.

Markx GH, Huang Y, Zhou X-F, Pethig R (1994). Dielectrophoretic characterisation and separation of microorganisms. *Microbiology,* 140, 585-591.

Melchior DL (1982). Lipid phase transitions and regulation of membrane fluidity in prokaryotes. *Current topics in membranes and transport* 17, 263-316.

Metcalf TG, Melnick JL, Estes MK (1995). Environmental virology: from detection of virus in sewage and water by isolation to identification by molecular biology - a trip of over 50 years. *Annual Reviews of Microbiology,* 49, 461-487.

Morgan H, Hughes MP, Green NG (1999). Separation of submicron bioparticles by dielectrophoresis. *Biophys J*, 77(1), 516-525.

Muirhead KA, Horan PK, Poste G (1985). Flow cytometry: Present and future. *Biotechnology,* 3, 337-356.

Neidhart FC, Ingraham JL, Schaechter M (1990). Physiology of the bacterial cell: a molecular approach. Sunderland: Sinauer Associates, Inc.

Neu TR, Lawrence JR (1997). Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy. *FEMS Microbiology Ecology,* 24, 11-25.

Nubel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, Ludwig W, Backhaus H (1996). Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature-gradient gel-electrohporesis. *Journal of Bacteriology,* 178, 5636-5643.

Nybroe O, Johansen A, Laake M (1990). Enzyme-linked immunosorbent assays for detection of *Pseudomonas fluorescens* in sediment samples. *Lett Applied Microbiology,* 11, 293-296.

Ogram A, Sayler GS, Barkay T (1987). The extraction and purification of microbial DNA from sediments. *J Microbiology Methods,* 7, 57-66.

Palojärvi A, Sharma S, Rangger A, von Lutzow M, Insam H (1997). Comparison of Biolog and phospholipid fatty acid patterns to detect changes in microbial community. In *Microbial Communities: functional versus structural approaches*, pp. 37-48. Edited by Rangger, A. & Insam, I. Berlin: Springer.

Pennanen T, Frostegård A, Fritze H, Bååth E (1996). Phospholipid fatty-acid composition and heavy-metal tolerance of soil microbial communities along 2 heavy metal-polluted gradients in coniferous forests. *Applied and Environmental Microbiology* 62, 420-428.

Pethig R, Markx GH (1997). Applications of dielectrophoresis in biotechnology. *Trends Biotechnol,* 15(10), 426-432.

Porter J, Deere D, Hardman M, Edwards C, Pickup R (1997). Go with the flow - use of flow cytometry in environmental microbiology. *FEMS Microbiology Ecology*, 24, 93-101.

Puig M, Jofre J, Lucena F, Allard A, Wadell G, Girones R (1994). Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. *Applied and Environmental Microbiology,* 60, 2963-2970.

Rajendran N, Matsuda O, Urushigawa Y, Simidu U (1994). Characterization of microbial community structure in the surface sediment of Osaka Bay, Japan, by phospholipid fatty-acid analysis. *Applied and Environmental Microbiology* 60, 248-257.

Schafer P, Braun RW, Mohring K, Henco K, Kang J, Wendland T, Kuhn JE (1993). Quantitative-determination of human cytomegalovirus target sequences in peripheral-blood leukocytes by nested polymerase chain reaction and temperature gradient gel-electrophoresis. *Journal of General Virology,* 74, 2699-2707.

Schnürer J, Clarholm M, Rosswall T (1985). Microbial biomass and activity in an agricultural soil with different organic matter contents. *Soil Biology and Biochemistry* 17, 611-618.

Stahl DA (1995). Application of phylogenetically based hybridisation probes to microbial ecology. *Molecular Ecology* 4, 535-542.

Steffan RJ, Atlas RM (1988). DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. *Applied and Environmental Microbiology,* 54(9), 2185-2191.

Stryer L, (1988). Biochemistry, 3rd edition. WH Freeman and Company, New York.

Thompson IP, Bailey MJ, Fenlon JS, Fermor TR, Lilley AK, Lynch JM, McCormack PJ, McQuilken MP, Purdy KJ, Rainey PB, Whipps JM (1993). Quantitative and qualitative seasonal-changes in the microbial community from the phyllosphere of Sugar-Beet (*Beta-vulgaris*). *Plant and Soil* 150, 177-191.

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

Torsvik V, Goksoyr J, Daae FL, Sorheim R, Michalsen J, Salte K (1994). Use of DNA analysis to determine the diversity of microbial communities. In: Ritz K, Dighton J, Giller KE (eds) Beyond the Biomass: compositional and functional analysis of soil microbial communities, John Wiley & Sons Ltd., Chichester, UK, pp 39-48.

Tsai YL, Olson BH (1991). Rapid method for direct extraction of DNA from soil and sediment. *Applied and Environmental Microbiology*, 57, 1070-1074.

Turnberg WL (1996). Biohazardous Waste - risk assessment, policy and management. ISBN 0 471 59421 0.

Vanderzant C, Splittstoesser D (1992). Compendium of Methods for the Microbiological Examination of Foods, 3rd edition. American Public Health Association, ISBN 0-87553-173-3.

Van Elsas JD, van Overbeek LS (1993). Bacterial responses to soil stimuli. In: Kjelleberg S (ed), Starvation in Bacteria, Plenum Press, New York, USA, pp. 55-79.

Vesey G, Hutton P, Champion A, Ashbolt N, Williams KL, Warton A, Veal D (1994). Application of flow cytometry methods for the routine detection of *Cryptosporidium* and *Giardia* in water. *Cytometry*, 16, 1-6.

Wallner G, Amann R, Beisker W (1993). Optimising fluorescent in situ hybridisation with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry,* 14, 136-143.

Wrenn BA, Venosa AD (1996). Selective enumeration of aromatic and aliphatic hydrocarbon-degrading bacteria by a most-probable-number procedure. *Canadian Journal of Microbiology* 42, 252-258.

Zelles L, Bai QY, Beck T, Beese F (1992). Signature fatty-acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. *Soil Biology and Biochemistry* 24, 317-323.

Zelles L, Rackwitz R, Bai QY, Beck T, Beese F (1995). Discrimination of microbial diversity by fatty-acid profiles of phospholipids and lipopolysaccharides in differently cultivated soils. *Plant and Soil* 170, 115-122.