A review of new technologies for rapid coliform assay

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Statement of Use
This review contains information regarding current and future technology for the detection and enumeration of faecal indicators in environmental waters. The information is for use by Environment Agency staff and others involved in the assessment of microbiological water quality.

Keywords
Bathing water, characterisation, coliform, *E.coli*, enterococci, real-time, screening.

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EXECUTIVE SUMMARY

The quest for real-time monitoring of bathing water quality is currently driven by the revision of the Bathing Water Directive in the European Union. A move to the monitoring of faecal indicators more directly indicative of health risk, concentrating on intestinal enterococci for marine waters and *E. coli* for freshwaters, is planned. The need for rapid techniques to indicate water quality in near-real time is identified in the EU commission proposal (CEC, 2000). This would enable the provision of up-to-date information for the general public regarding beach water quality and activate rapid Environment Agency response to pollution events.

'As soon as the rapid tests, presently under development, for in situ measurements are considered robust and reliable, the commission will evidently encourage and support their use'
Developing a New Bathing Water Policy (COM(2000)860).

There are limitations to the application of rapid analytical methods to environmental waters, including the relatively low concentrations of target organisms present in contaminated waters. The water matrix in which faecal organisms are detected presents a number of difficulties involving non-target micro-organisms, native flora and fauna and particulate matter. The environmental stress imposed upon enteric micro-organisms, such as *E. coli* and enterococci, may affect organism viability to such an extent that it may not be culturable or even detectable by more complex means.

The water industry requires a method that will be equal to, or superior in sensitivity (detection limit of 1 cell per unit volume), and specificity (detection of the target organism only) compared with reference methods. The method should produce results within a working day, thus leaving time for pollution control action if required. The technique should also be user-friendly and portable, allowing analysis to be undertaken, or at least initiated, in the field. Finally, the cost should be comparable to standard methods. A large initial capital cost may be considered when the benefits are substantial and the costs are recoverable through reduction in consumables and staff costs.

A range of technologies for the detection of *E. coli* and enterococci are reviewed here, under the framework of a three-tiered screening – quantification – characterisation hierarchy. Technologies reviewed include rapid enzyme assays, the use of enzymatic substrates in cultivation; impedance; bioluminescence; microcolony cultivation; fluorescence, immunological and molecular cell labelling methods; flow cytometry, laser scanning; Polymerase Chain Reaction and biosensors. The future of diagnostic technology for the monitoring of bathing waters will be discussed, in addition to the recommendation for further research.

Consideration of a large range of practical and technical parameters required for a rapid method suitable for Environment Agency use has been undertaken. Screening of variable water quality may be achieved by rapid enzyme assays. Future development may lead to the use of specific bioluminescence assays or biosensors for the *in situ* detection of faecal indicators. Quantitative detection of faecal bacteria is unlikely to digress from the familiar cultivation techniques, therefore the detection of microcolony growth by instrumentation such as a laser scanner or high sensitivity camera is considered attractive. The use of enzymatic substrates by the MPN method is also a suitable alternative. Phenotyping of isolates by a high

throughput method is described, reducing the number of biochemical tests involved to enable more isolates per test at a reasonable cost. High cost genetic typing methods are commercially available for the identification of isolates to sub-species level.

The main conclusions and recommendations arising from this review are summarised below:

- Real-time detection and enumeration of faecal indicators in water is achievable with current technology. Limitations exist, which may be optimised through validation programmes. Future technological advancements may also provide further optimisation of current methods.

- The ideal of an instantaneous colorimetric dipstick providing *in situ* real time data is currently over the horizon. Further research is recommended to optimise a biosensor for the portable quantification of faecal indicators.

- A hierarchy of monitoring enables cost effective beach water quality management. Screening of samples can reduce the number of low contamination samples arriving at the laboratory.

- Screening of bathing waters allows rapid response to pollution within hours of sampling.

- Identification and characterisation of bacterial isolates enables rapid pollution response. Differentiation of contamination sources is essential to determine the factors influencing beach water quality.

The major limitation to the introduction of an alternative method is that it may recover the viable but non-culturable cells that are currently underestimated by reference methods, suggesting deterioration in water quality. One must consider the local, national and international implications of such a situation before applying one or more of the huge variety of technologies available.

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1. INTRODUCTION

Current methods for the enumeration of indicator bacteria are time consuming, may underestimate bacterial indicator load and produce retrospective results, which pose a significant health risk to bathers. Results are required in a more rapid time frame to inform the public of poor water quality to reduce the health risk. Current and novel technology providing more rapid and specific results exists commercially and within the biotechnological research field. The technology provides an opportunity for regulators such as the Environment Agency to improve upon response to public health risks in bathing waters. Rapid technology may also be used to reduce the loss of revenue experienced due to beach closure as the time for a return to acceptable conditions will be assessed in a shorter period than with standard methods.

The technologies and applications described in the following review are based on the best available information at the time of production. An attempt has been made to include all technologies with current or future application to the water industry. If any technology or application is excluded, it is unintentional and does not imply that the method is unsuitable for uses described here.

1.1 Existing Microbiological Legislation in Europe

Legislation requiring microbiological analysis includes the Bathing Water Directive (76/160/EEC), Shellfish Waters Directive (79/923/EEC) and Surface Waters Abstraction Directive (75/440/EEC; 79/869/EEC).

The European Community (EU) Directive concerning the quality of bathing water specifies that Member State designated beaches must be monitored by a competent authority, which is the Environment Agency in England and Wales, on at least fortnightly inspections throughout the bathing season from May to September. The EU Directive was implemented in the UK under the Bathing Waters (Classification) Regulations 1991. 472 coastal beaches are currently designated in the UK as bathing beaches “traditionally used by large numbers of bathers". Water samples are tested for a number of physico-chemical and microbiological parameters and are analysed on a pass or fail basis, with 95% compliance rate for mandatory total and faecal coliform standards and 80% for guideline standards (Council for European Communities, 1976). The microbiological parameters are shown in table 1.1.

The Shellfish Waters Directive requires quarterly monitoring for presumptive and confirmed faecal coliforms. The standards were translated into the Surface Waters (Shellfish) Regulations 1997. The Environment Agency is responsible for monitoring surface waters to ensure the health of shellfish for consumption. The faecal coliform guideline standard is < 300 per 100ml in shellfish flesh and intervalvular liquid. The Surface Water Abstraction Directive requires monthly sampling for total and faecal coliforms, faecal streptococci and salmonella. The guideline standards for microbiological parameters were translated into The Surface Waters (Abstraction for Drinking Water) (Classification) Regulations 1996. Guideline standards depend upon the population being served and what treatment the water will receive prior to entering the drinking water distribution system.

Table 1.1: Microbiological requirements for bathing water quality as required by the EU Bathing Waters Directive - 76/160/EEC (CEC, 1976).

	Fixed Standard values and % compliance		
Microbiological Parameter	**Sample volume (ml)**	**Mandatory standard**	**Guideline standard**
Total Coliforms	100	10 000 (95%)	500 (80%)
Faecal Coliforms	100	2 000 (95%)	100 (80%)
Faecal Streptococci	100	-	100 (90%)
Salmonella	1000	0 (95%)	-
Enteroviruses	10 000	0 (95%)	-

1.2 Limitations of the current Bathing Water Directive

Since its adoption in 1976, the Bathing Water Directive (BWD) has undoubtedly led to an improvement in beach water quality, and has also heightened the profile of environmental issues within the public domain. A number of limitations associated with the Directive have been raised throughout its lifetime, however, including a lack of harmonisation of sampling procedures, analysis methodologies and reporting throughout member states in the European Community. This makes comparison of water quality between EU countries relatively difficult. An evaluation of the BWD is presented by Figueras *et al*, 1997.

The BWD presents ambiguous definitions of 'bathing water' and bathing season' and 'abnormal weather waiver', which are subject to a range of different interpretations throughout the EU. The current faecal indicators and their statistical manipulation are misleading. Total and faecal coliforms have no conclusive dose-response relationship with risk of gastro-enteritis and other symptoms such as ear, nose and eye infections. The measure of total coliforms is redundant if thermotolerant coliforms are additionally analysed. As *E. coli* are one of the only truly faecal members of the coliform group it appears sensible to replace the faecal coliform parameter. Bacterial concentrations should be regarded as logarithmic normal distributions, not as percentage compliance as it is currently assessed in the Directive, as bacterial populations are generally not normally distributed. The use of geometric mean or median values should be considered.

1.3 Proposal for the revision of the Bathing Water Directive

The World Health Organisation (WHO) produced the draft Guidelines for Safe Recreational Environments: Coastal and Freshwaters in 1998 (WHO, 1998). The guidelines propose the use of enterococci for marine (and freshwaters) following epidemiological work by Kay *et al*, 1994. *Escherichia coli* was also proposed for freshwater monitoring following work undertaken by Van Asperen *et al*, 1997.

In 1999, WHO and the US Environmental Protection Agency (EPA) met in Annapolis, United States, to discuss the adequacy of recreational water monitoring and assessment (WHO, 1999). The resulting document (the Annapolis Protocol) has driven the formulation of final

WHO guidelines to be produced in 2001 and will ultimately influence the revision of the Bathing Water Directive in Europe.

A classification scheme was proposed to identify bathing waters as very poor, poor, fair, good or excellent. Initial classifications would result from a primary inspection of the bathing water based on a sanitary survey of potential contaminating sources to the beach zone and a microbiological assessment of water quality. Frequency of monitoring would depend upon the initial classification of the beach following primary inspections. The classification of a beach could then be improved or downgraded depending upon water quality and management practices.

Advances in scientific research and innovative technology have driven the Council of European Communities to update the Bathing Water Directive. An attempt at revision of the BWD occurred in 1994, although wide disagreement between member states precluded any change. The production of the definitive report from WHO will provide a strong base to propose significant changes to the legislation. In December 2000, the EU produced a consultation document (COM(2000)860) to introduce the proposal for revision of the directive and invite constructive comments (CEC, 2000). The initial consultation will be discussed in late April in Brussels, following which the final proposal will be produced by June/July 2001.

New definitions for the concept of 'bathing', 'bathing water' and 'bathing zone' have been proposed to remove ambiguity arising from the 1976 Directive. The revised definition of 'bathing' appears to include watersports, although makes no move to modify the definition of bathing seasons. The Environment Agency does not routinely monitor recreational waters. The Directive also seeks to provide the means to de-designate bathing waters which are no longer in public use.

The revision seeks to move towards a culture of active management of bathing waters, with emphasis upon challenging water quality standards for updated 'health' parameters and a requirement for relevant authorities to undertake prompt preventative or responsive actions to deterioration in water quality. The Commission of European Communities has proposed the following standards for coastal and freshwater bathing areas. These are likely to be refined with the publication of the World Health Organisation: Guidelines for Safe Bathing in spring 2001.

Table 1.2: Proposed standards for the protection of health in recreational waters COM(2000)860 (CEC, 2000).

Parameter	Bathing Medium	Proposed Standard
Escherichia coli	Freshwater	400 per 100ml
Intestinal Enterococci	Coastal/Estuarine water	50 per 100ml

The Commission of European Communities has stated that:

'It is more than ever necessary to have good quality information in near-real time about the bathing area'
(COM(2000)860), CEC, 2000.

The authors proposed the use of divergence from 'normal' pH in freshwaters and from 'normal' salinity in coastal waters as instant indicators of the entrance of unusual water. Although these indicators are cheap and simple to undertake, they are not explicitly indicative of faecal contamination and could therefore cause alarm in certain circumstances unrelated to sewage pollution.

As this review will show, current science and technology advances may soon lead to superior rapid, portable and simple monitors of specific indicator bacteria. The directive proposal states that:

'As soon as the rapid tests, presently under development, for in situ measurements are considered robust and reliable, the commission will evidently encourage and support their use'
(COM(2000)860), CEC, 2000.

The possibility of updating elements of the Directive in response to technical and scientific progress may be possible by using a management committee following proposed guidelines in the recent decision on procedures for the exercise of implementing powers 1999/468/EC (CEC, 1999).

1.4 Global Recreational Water Management

United States

Guidelines produced by the USEPA in 1986 (Ambient Water Quality Criteria for Bacteria) proposed the use of *Escherichia coli* for freshwater monitoring and enterococci for marine (and freshwater) monitoring. Revised methodologies for enumeration of *E. coli* and enterococci by the mEI and modified mTEC methods respectively, were published in March 2000. In 1999, when the EPA produced their BEACH Action Plan, only one third of all states had implemented these guidelines. Widespread use of faecal coliforms and total coliforms as indicators of water quality exists within the US EPA. The Office of Water has led the development of the BEACH Action Plan, 1999. The Office of Research and Development undertakes much of the research and development supporting the document. Target areas for research have been outlined as the following;

- Real or near-real time monitoring
- Indicators to distinguish human and non-human sources of pollution
- Novel techniques for pathogen analysis
- Indicators for non-enteric diseases
- Indicators for pollution in tropical climates

The focus on real time monitoring has driven research into the use of novel and innovative techniques for bacterial enumeration. The USEPA and USGS have been experimenting with the use of flow cytometry (Whitman R, personal communication), Polymerase Chain Reaction (PCR), bioluminescence and fibre-optic sensors for surface water quality analysis (Dufour A, personal communication). The Action Plan calls for development of a colour change dipstick instrument to enable *in situ*, real time monitoring for bacteria.

Canada

Health Canada guidelines were published in 1992 recommending enterococci and *E. coli* for marine and freshwaters respectively. Faecal coliforms were also recommended for freshwaters. The use of mTEC media for *E. coli* and mE agar for enterococci was proposed. Management practices and monitoring programmes are similar to those proposed in the Annapolis Protocol. Beaches may be posted or closed if a significant health hazard is suspected, particularly following heavy rainfall. Sampling frequency is determined by prior inspection to identify risk of contamination.

Australia

The Australian and New Zealand Environment and Conservation Council (ANZECC) published water quality guidelines for freshwater and marine waters in 1992. The National Water Quality Management Strategy (NWQMS) group has revised water quality guidelines, the draft released in 1999 and final document published in 2000. In New South Wales, bacteriological indicators for water quality are faecal coliforms and enterococci.

New Zealand

The Ministry of Health is responsible for recreational water quality in New Zealand. The 1999 Recreational Water Quality guidelines proposed a three tier management framework involving routine monitoring, investigations and actions depending upon compliance of running median values with guideline standards. Waters for bathing and watersports are monitored weekly for enterococci (marine) and *E. coli* (freshwater). Recommended analysis methodologies include Enterolert® or EPA method 1600 for enterococci enumeration in 24 hours and Colilert® or EPA method 1103.1 for *E. coli* enumeration in 18 - 24 hours.

2. TECHNOLOGY OVERVIEW

2.1 Indicators of Faecal Pollution

An ideal indicator of faecal contamination is one that is exclusively faecal in origin, unable to grow outside the intestine of humans or animals, and emulates the persistence of pathogens with respect to environmental and water treatment processes. Indicator organisms also need to be easy to identify, isolate and enumerate and their presence should be restricted to when pathogens also occur, and in greater abundance than that of the pathogens. *Escherichia coli* is considered to meet most of the above criteria at least within temperate regions of the world.

Members of the coliform group have been practically defined by their method of detection (Standing Committee of Analysts, 1994). Coliforms characteristically ferment lactose at 37^0C to produce acid and gas. This fermentation step requires the possession of the enzyme β-D-galactosidase to cleave lactose, producing glucose and galactose. Coliforms assumed to be of faecal origin are capable of growth, acid and gas production from lactose fermentation at 44^0C. *Escherichia coli* presence is confirmed from faecal coliform cultures by production of indole from tryptophan, an inability to utilise citrate and possession of β-D-glucuronidase. *E. coli* has been universally recommended as a suitable indicator for temperate freshwaters. The drawbacks of *E. coli* as a faecal indicator include reports of the indicator in waters free from faecal contamination (Ashbolt *et al,* 1997) and their poor persistence in the aquatic environment (Borrego *et al*, 1983).

Faecal streptococci have been widely reported as superior indicators for temperate marine and freshwaters due to their close relationship with symptoms of gastro-enteritis (Cabelli *et al* 1982, 1983., Dufour, 1984., Kay *et al* 1994., WHO, 1998). Although they are sometimes not as ubiquitous as coliforms in water (Borrego *et al*, 1982), they have greater resistance to environmental stresses and therefore more closely mirror the persistence of enteric viruses. The faecal streptococci group is comprised of two genera; enterococcus and streptococcus. In practice, the terms faecal streptococci, enterococci, intestinal enterococci and *Enterococcus* can be considered synonymous (Figueras *et al*, 2000). Criteria used to select for enterococci include the ability to grow at 45^0C, resistance to 60^0C for 30 minutes, growth at 9.6 pH and at 6.5 % NaCl and the ability to reduce 0.1% methylene blue.

Sulphite-reducing Clostridia or spores of *Clostridium perfringens* have been recommended as primary and secondary indicators of faecal contamination in temperate tropical marine and freshwaters (WHO, 1998). They are always present in sewage and particularly dog faeces, although are not generally indicators of non-sewage or animal faecal contamination. Clostridia tend to be relatively persistent in the aquatic environment and appear not to reproduce in water or sediment (Davies *et al*, 1995).

Alternative indicators of faecal contamination include bacteriophages, which are viruses whose survival characteristics are similar to human enteric viruses. Coliphages are specific viruses of the coliform group. F-specific RNA Bacteriophage targets the f-pilli of *E. coli* cells. Bacteriophages targeting *Bacteroides fragilis* have also been proposed as faecal indicators. Faecal sterols such as coprostanol, cholestanol and etylcoprostanol have been suggested as chemical indicators of pollution. They may also be of use in distinguishing

human from animal sources of faecal contamination using ratios obtained in water samples (see Section 6).

2.2 Concept of Viability

The cultivation of micro-organisms is commonly regarded as a measure of the number of viable cells in the sample under investigation. Research has shown, however, that significant proportions of cells in a typical cultivation test are not detected despite their viability (Xu *et al*, 1982). These are termed viable but non-culturable (VBNC) cells. Viable cells may become non-recoverable due to stress induced by time spent in the aquatic environment and by the selectivity of the culture medium (see Section 5.1.1). The former factor has an overwhelming influence on the applicability of many of the techniques presented here (Table 2.1). Sub-lethal injury may be attributed to one or more of a number of factors, including time and temperature of exposure to water, level of treatment (UV, chlorine or ozone), strain of organism, concentration of nutrients, predation by marine micro-organisms and presence of heavy metal ions (McFeters *et al*, 1982).

Table 2.1: Stress responses of enteric micro-organisms exposed to the aquatic environment and the implications for their detection method. Modified from Edwards *et al*, 1996

Stress Response	Effect upon detection procedure
Cells become viable but non-culturable (Table 2.2)	Not isolated from plate culture
Growth ceases although cell division continues	Ultramicrocells produced which are difficult to detect and identify (Torrella and Morita, 1981)
rRNA content decreases	Decreased sensitivity of rRNA probes
Altered surface components - antigenicity altered	Serotyping not possible
Long cell cycle times	Heterogeneity in cell cycles more pronounced
Resistance to UV light, heat and autolysis	Altered cell properties

Table 2.2 describes the criteria used in plate culture for the detection of viable cells (first column). Viability in this case is determined by the detection of reproductively active cells. A requirement for reproductive ability is for the cell to be metabolically active and possess intact cell membrane and cellular components. An intact, metabolically active cell may not necessarily grow, however, due to non-lethal injury. The cell remains viable, but is not detected in a plate culture. Metabolically active cells (second column) can be detected by a number of functions that they continue to express despite lacking growth. These include synthesis of nucleic acids and proteins, active membrane pump controls and enzymatic activity. The activities of enzymes such as esterase or β-D-galactosidase can be detected by their conversion of culture substrates (see Section 5.1.2).

An intact cell with nucleic acid damage due to water treatment or environmental damage, for example, may have lost its ability to be metabolically active, although is considered viable if the cell has retained its membrane integrity. DNA stains can be used to determine the

integrity of cellular membrane by stain uptake or exclusion (see Section 5.3.1). A dead or permeabilised cell will indiscriminately take up DNA stains. These dead cells are classified as non-viable.

It can be seen that current plate cultivation techniques for the enumeration of bacterial indicators may underestimate the viable bacterial load in a water sample. It remains an important consideration that an alternative methodology may select cells falling in to the category of metabolically active but not growing, or those viable cells possessing full membrane integrity, but show no signs of growth or metabolic activity. Such a method would result in enumeration of a greater number of cells compared to the reference culture method. This has consequences for the method of evaluation and validation of alternative tests, and wider implications for the reporting of water quality data which may suggest deterioration in quality.

Table 2.2: Concepts of viability in micro-organisms. Kindly reproduced and modified from Nebe-von-Caron *et al*, 2000

FUNCTIONAL CELL STATUS	INTACT CELLS			PERMEABILISED (DEAD) CELLS
	METABOLICALLY REPRODUCTIVE GROWING CELLS	ACTIVE CELLS		
TEST CRITERIA	CELL DIVISION	METABOLIC ACTIVITY	MEMBRANE INTEGRITY	MEMBRANE PERMEABILITY
DETECTION METHOD	**CELL COUNTING** FIXED VOLUME COUNT TIME INTEGRATION RADIOMETRIC COUNTING **CELL TRACKING** INTRACELLULAR OR MEMBRANE LABELLING	***ENERGY DEPENDENT*** **BIOSYNTHESIS** DNA/PROTEIN SYNTHESIS, CELL ELONGATION UNDER ANTIBIOTIC PRESSURE **PUMP ACTIVITY** pH CONTROL, DYE EFFLUX **MEMBRANE POTENTIAL** ACCUMULATION OF CATIONIC DYES, DISSIPATION OF ANIONIC DYES ***ENERGY INDEPENDENT*** **ENZYME ACTIVITY** SUBSTRATE CONVERSION	**SELECTIVE MEMBRANE PERMEABILITY** EXCLUSION OF MEMBRANE IMPERMEABLE DNA STAINS UPTAKE OF SUPRAVITAL DNA STAINS	INDISCRIMINATE UPTAKE OF CYTOPLASMIC STAINS
		CELLS WITH NUCLEIC ACID DAMAGE		

2.3 Guidelines for the Evaluation and Validation of Alternative Technologies

Evaluation should encompass the entire system of method change from the decisions considered in method selection to its implementation for routine use. In the US

Pharmaceutical Industry, the use of an Equipment Qualification Model is recommended (PDA, 2000). This covers considerations of the installation, operational and performance qualification aspects of alternative technologies. The model is loosely summarised below, with consideration of its application to the regulation of the water industry.

2.3.1 Method and Manufacturer Requirements

The operator must consider the specifications for an alternative method, including what the assay is capable of achieving and what attributes are required by the manufacturer of the instrument. Primarily the cost of the new method should be considered. If greater costs are incurred compared to current methods, the operator must calculate when the investment will be returned. Additional considerations include the speed and simplicity of analysis, sample throughput, instrument versatility and method specificity and sensitivity. These factors are evaluated in Table 2.3.

An important consideration for the validation of a method is the requirement for confirmation of presumptive results. Although confirmation may not be desired for routine purposes, it is essential to gain confidence in a new method prior to its translation to routine use. If the analytical technique is inherently destructive to test cells, confirmation is not possible.

A novel technology may give more accurate, specific and sensitive results than the current standard methods. Validation criteria will need to reflect that higher recovery of target organisms may occur. The operator must also consider the implications that a more sensitive method may have upon regulatory compliance and attitudes of the customer base (general public).

Considerations upon selection of a suitable manufacturer include the long-term economic viability of that business, support and training services offered, possession of accreditation documents and relevant qualifications and an extensive customer base within the relevant industry.

2.3.2 Evaluation and Validation Planning

A number of preparation and planning stages are required following the choice of an alternative method before practical evaluation can begin. A validation programme must be prepared and acceptance criteria established prior to embarking upon practical evaluation. A proof-of-concept phase may be considered as an opportunity to ensure that the application is feasible. Installation of the instrument, in collaboration with the manufacturer, is an important stage in the preparation to ensure health and safety and suitable operating conditions.

Table 2.3: Evaluation criteria for the selection and evaluation of analytical methods for the detection and enumeration of faecal indicators in bathing water.

Parameter of Interest	Environment Agency Requirement
Analytical Specifications	
Speed of Analysis	Confirmed quantitative result within one working day; including time for sampling and action response. Screening results required within 2 hours.
Sensitivity	The limit of detection of the method must be at least 1 cell in a unit volume of water.
Specificity	The method must be able to detect the required organisms from a complex sample matrix within a background of non-specific micro-organisms.
Viability	Method should distinguish between viable and non-viable cells.
Repeatable	Method needs to be repeatable with duplicate samples.
Reproducible	Method must be reproducible within the laboratory and between laboratories nationally and internationally.
Applicability in Different Environments	Method must be applicable to a range of aquatic environments globally (particularly within the EU).
Measurement Level	*Screening* - qualitative or semi-quantitative analysis for investigation purposes. *Quantitative* - Accurate count of target cells required for compliance reporting to public/DETR/EU. *Characterisation* – identification of target bacteria for pollution tracking.
Practical Specifications	
Instrument Cost	Capital cost of analytical instrument must be shown to be recoverable through labour time and other costs.
Cost per sample	Cost per sample (includes reagent, media and preparation costs) must be reasonably low and equivalent to standard method costs. If costs are not equivalent, then alternative benefits should be stated.
Instrument Through-put	Sample throughput may be high in certain laboratories e.g., 200 - 500 samples in one day.
Size	Large instruments would require additional bench-top space. A portable instrument reduces lab staff time and enables quick results to be obtained by minimally trained staff in the field.
Availability of Reagents	Reagents must be available for order by internal or external laboratories in bulk order.
Availability of Method	Analysis by the method should be available at external laboratories for Environment Agency regions currently requiring this service.
Sample handling	Samples should be safe to handle throughout preparation, analysis and disposal.
Ease of Performance	Method should be of low complexity to reduce risk of operator error.
Interpretation of Results	Results should be easy to interpret by operator or include software for complex analyses.
Training of Analyst	Manufacturer should provide analyst training. Skill level required for test should match that of present staff.
Service Provision	Support and service provision from manufacturer required for breakdown repairs.
Quality Control/Analysis	Analytical method must be subject to thorough evaluation and validation prior to approved use by the Environment Agency. Stringent quality control and quality analysis procedures must be implemented for selected technologies.
Disposal	Disposal pathways should either be in existence or simple to set up with contractors. Cost of disposal should be low. Disposal methods should strictly adhere to Environment Agency waste disposal policies.

Operation Qualification is the stage requiring evaluation of the test method in its operating environment and assurance that the method performs within the defined limits stated by the manufacturer. Verification of data supplied by the manufacturer regarding specificity, sensitivity, linearity and quantification may be conducted at this stage. Computer systems must also be validated, especially in automated systems.

Performance Qualification involves testing the system to confirm it will perform and meet requirements for regulatory standards. A protocol recording Standard Operating Procedures, contingency plans for changes to the test and instrument, validation tests following upgrading or maintenance of the system and a log of training for users should be established before implementation and kept up to date throughout the lifetime of the instrument. The performance of a new method should be periodically reviewed.

Implementation of the new method by comparison with standard reference methods should be the final step. Table 2.3 lists the criteria necessary to evaluate an alternative or novel technology for application to the monitoring of faecal indicators in bathing waters. Evaluation of new methods published in the scientific literature invariably state the accuracy as the percentage recovery of organisms and precision as the coefficient of variation between replicates. Equivalence is determined with reference methods in addition to the specificity, sensitivity and correlation coefficient. The limit and range of detection, raggedness and robustness of the method is also evaluated. Consideration of these requirements is employed in the evaluation of each technology presented within this review.

Table 2.5 summarises the technologies currently available for the detection of faecal indicators, such as *E. coli* and enterococci, including the consideration of speed of analysis, capital instrument and reagent costs (classification shown in table 2.4) and the reported sensitivity of each method.

Table 2.4: Classification of estimated costs for the purchase of analysis instruments and reagents.

Estimated Cost of Detection Instrument	Cost Symbol	Estimated Cost per Sample
<£1,000	£	< £1
£1,000 - £10,000	££	£1 - £5
£10,000 - £50,000	£££	£5 - £7.50
£50,000 - £100,000	££££	£7.50 - £10
>£100,000	£££££	>£10

Table 2.5: A summary of technologies currently available for the detection and enumeration of faecal indicator bacteria.

Technology	Speed of Detection	Limit of Detection	Instrumental Cost	Cost per Sample
Screening				
Enzyme Assay	<1- 12 hours	1 cell per sample	££ - £££	££
Bioluminescence	1 minute - 48 hours	$10^3 – 10^4$	£	£ - ££
Biosensors	<1 min - 30 mins	$10^4 - 10^9$	£ - £££	Depends upon labelling technique
Quantitative				
Plate Culture	24 - 72 hours	1 cell per sample	£	££
Enzyme (MF)	18 - 24 hours	1 cell per sample	£	££
Enzyme (MPN)	18 - 24 hours	1 cell per sample	£	££
Impedance	6 - 48 hours	$10^5 - 10^6$	£££	£
Pre-enrichment				
Plate Culture	2 - 4 hours	-	£	££
Immuno-magnetic Separation	15 - 30 minutes	-	£	££
PCR	10 minutes - 1 hour +	-	££ - £££	£ - ££
Labelling Technology				
Staining	Few minutes - 2 hours depending upon stain	1 cell per sample	Depends upon detection method	£ - ££
Immunoassay	Few minutes (flow injection) 2 -3 hours (solid phase)	$10^4 - 10^5$		£ - ££
Nucleic Probe	<1 hour depending upon probe	$10^4 - 10^5$		£ - ££
Detection Instruments	**Omitting pre-enrichment and labelling**			
Flow Cytometry	3 - 10 minutes	$10 - 10^2$	££ - £££££	Depends upon cell labelling method (see above)
Laser Scanning	3 minutes	1 cell per sample	££££	
Camera	2 - 5 minutes	1 cell per sample	£££	
Epifluorescence Microscope	Depends upon speed of operator	1 cell per sample	£££	
Luminometer	< 1 minute	$10^2 - 10^4$	££	£ - ££

3. MONITORING HIERARCHY

A monitoring programme with a tiered system of analysis methodologies is proposed to provide a framework for the discussion of technologies available (Figure 3.1). The framework is later used to suggest suitable analytical methods that may improve the effectiveness of the Environment Agency response to incidence of faecal pollution. The protocol may aid the distribution of resources more effectively depending upon the requirement of bacteriological analysis at each stage.

Screening (qualitative or semi-quantitative measure) can be used as a more rapid driver for response than the 1-day presumptive result. Screening systems may be fully automated or portable, providing detection of faecal contamination to a threshold level, such as the mandatory bathing water standard, within a few hours of collection.

Screening would be useful for frequent monitoring of bathing waters, both routinely and for investigative purposes, and could also offer future cost savings benefits by the selective quantification of poor water quality samples only.

The second tier of bacterial enumeration is quantification. The current standard methods would fit into this category, as methods giving a quantitative measurement of bacterial concentrations. Methodologies in this category must be thoroughly validated as data would be required for reporting to the DETR and the EU for Bathing Water and Shellfish Water compliance purposes. The data would also need to be robust in legal situations when the Environment Agency may bring prosecutions against polluters or when beach closures are to be defended in a court of law.

The third tier of monitoring is the identification and characterisation of target micro-organisms. Directive obligations do not require the identification of bacteria below the species level. Whilst in the past, the ratio between faecal coliform and faecal streptococci concentrations was used to determine a human or animal source, this has been widely dismissed as inaccurate (see Section 6). Alternative indicators, such as faecal sterols, may be used to pinpoint contamination source. Methods for the identification and characterisation of indicator bacteria by typing are now widely available. Differentiation between human and animal sources of enterococci and *E. coli* is of use when determining the cause of a water quality failure at a beach. Characterisation may also be used to determine whether a sample at one point is related to one at another. The more related the samples are, the more likely they have come from the same source. This aids detection of a source from a number of possible causes of pollution.

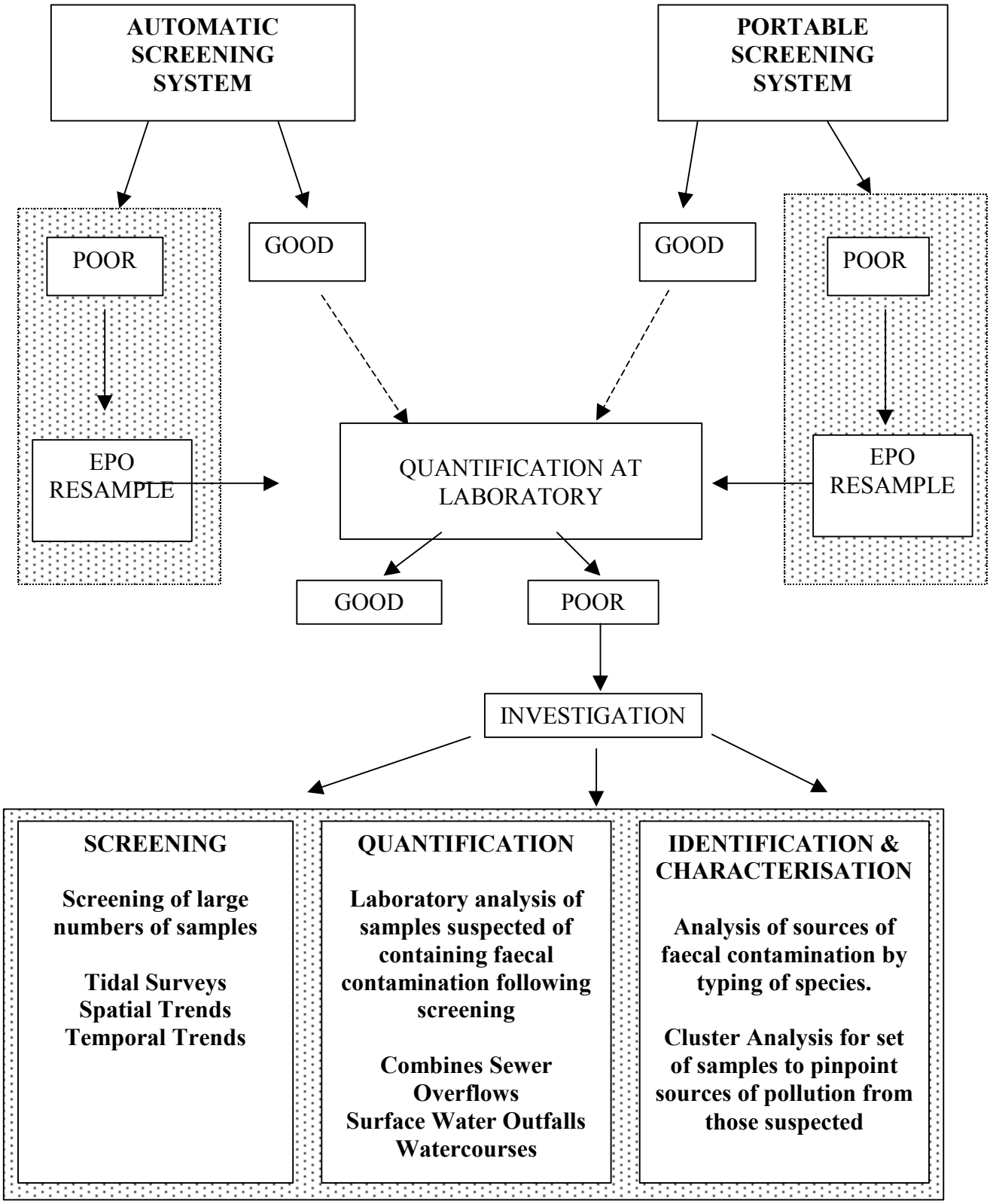

Figure 3.1: Schematic description for the application of a three-tier monitoring system. Elements in shaded boxes indicate improved Environment Agency response to pollution events. Dotted pathways indicate a cost saving for the Environment Agency if removed.

4. SCREENING TECHNOLOGIES

A screening system should provide the operator with an accurate estimation of water quality within a short time period. A number of technologies and commercial systems fall into the category of screening instruments, and many may additionally be used for enumeration. Technologies classified here involve the detection of a fluorescent, colorimetric, electrical or optical signal that is proportional to the concentration of target organisms present. The higher the concentration of faecal indicators in a sample, the quicker a threshold signal intensity is achieved. A threshold level may be set, for example using the mandatory bathing water standard, enabling notification of an exceedance prior to the full enumeration of the sample. Enumeration is possible by calibration of the procedure using standard methods to compare CFU measurements with signal intensity. Immediate remedial measures can therefore be initiated within hours of a sample being taken, rather than days. Confirmation of the exact magnitude may be obtained from allowing the assay to continue overnight, or re-sampling the bathing water and continuing enumeration by standard methods

Rapid enzyme assays and bioluminescence reactions may be performed in the field providing near-real time results. Biosensors are included in this section as they currently provide opportunities for estimation of water quality in highly contaminated situations. Biosensors are not currently optimal for the enumeration of samples containing low and moderate contamination, however, although future development may grant this option. A number of biosensors, such as impedance systems, are not portable and require substantial power inputs and software appliances. These systems remain of use as screening tools, however, as a rapid indication of poor water quality is obtainable in hours, enabling responsive action whilst the enumeration of the sample continues.

A screening system should be relatively cheap to use and maintain, portable or automated and simple to use and prepare. Screening is likely to be undertaken by staff in the field that have little or no relevant training, therefore these factors are of the absolute importance. Systems including notification of a sample exceeding the compliance threshold by email, text message or pager contact is of additional value.

4.1 Enzyme Assays

The detection and enumeration of enteric bacteria using the metabolic activity of cellular enzymes is a well-developed and applied technique. The working definitions for faecal indicator bacteria have been modified to accept the possession of the enzyme β-D-galactosidase, β-D-glucuronidase and β-D-glucosidase by coliforms, *E. coli* and enterococci respectively (SCA, 1994). Substrates encouraging the expression of these enzymes are used as culture media for target bacteria. Hydrolysis of the substrate by the constituent enzyme releases a fluorophore or chromophore, providing a signal for detection. Wide ranges of enzyme substrates are commonly used, as indicated in Table 4.1.

Table 4.1: Examples of enzyme substrates used for the detection and enumeration of coliforms, E. coli and Enterococci

Parameter	Enzymatic Substrates
Coliforms	4-methylumbelliferyl-β-D-galactosidase (MUGal) ortho-nitrophenyl-β-D-galactosidase (ONPG) para-nitrophenol -β-D-galactopyranoside (PNPG)
Escherichia coli	4-methylumbelliferyl-β-D-glucuronidase (MUG) Indoxyl-β-D-glucuronide (IBDG) Para-nitrophenyl-β-D-glucuronide (PNPG) 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG or XGLUC) Phenolphthalein-β-D-glucuronide (PHEG) 5-bromo-6-chloro-3-indolyl--β-D-glucuronide (magenta-glc) 6-chloro-3-indolyl-β-D-β-D-glucuronide (salmon-glc)
Enterococci	4-methylumbelliferyl-β-D-glucoside (MUD or MUST) 3,4-cyclohexenoesculetin-7-β-D-glucoside

Berg and Fiksdal, 1988, developed a rapid (15 minute) fluorogenic enzyme assay for the detection of coliforms using 4-MU-β-D-galactoside substrate. A semi-automated screening system has been developed by Colifast based upon this substrate. Presence-absence enzyme assays produced by manufacturers such as IDEXX are not discussed here, as their incubation time of 22 – 24 hours is considered too long for a screening method. For a review of presence-absence methods, see PHLS, 1996. The Colilert® total coliform and *E. coli* media has been developed in to a fully automated screening system by SERES. These screening systems are discussed below, and compared in table 10.1 (Appendix A).

Apte and Batley (1992) from the CSIRO (New South Wales, Australia) have developed a portable, rapid screening system for the detection of coliforms or *E. coli* in 1 hour from natural waters, using methylumbelliferyl-β-D-galactosidase or methyl-umbelliferyl-β-D-glucuronide respectively. Rapid detection for coliforms was achieved through modification of the media by the addition of buffers and nutrients. For rapid detection of enzyme activity, it is important that the production of target enzymes is stimulated, not hindered, by the media and that conditions are optimal for hydrolysis of the substrate. Unpublished data (Davies and Apte, 2000) suggests that an *E. coli* assay using 4-methylumbelliferyl-β-D-glucuronide can be performed in a similar way.

Fluorescence is measured immediately following incubation using a portable fluorimeter at an excitation wavelength of 370nm and emission wavelength of 465nm. Fluorescence intensity is proportional to coliform concentration in the water sample (Davies and Apte, 1999). Sample incubation involves a small dry block heater that can be run from a 12V-car battery.

The limit of detection of method is not limited by instrument sensitivity, but by the rate of hydrolysis of the substrate. The current limit of detection is 300 FC CFU per 100ml, which is sufficient for the detection of significant contamination. Davies and Apte, 1999 evaluated the portable version of the test system along the New South Wales coastline with 200 beach water samples. The authors reported a 98% success rate in predicting whether the FC concentration will be greater or less than 300 per 100ml (guideline standard). Less than 2 % false positives

and no false negatives were recorded. Laboratory evaluation (Davies and Apte, 1996) using the same procedure produced 92.8% agreement with prediction of concentrations less than or greater than 300 FC per 100ml. False positives were recorded as 4.7% and false negatives were 2.5%. The higher false detection rates were considered to be due to the seasonal variation in β-D-galactosidase activity in coliforms (Davies and Apte, 1999).

The Colifast Analyser (Colifast, Norway) is a semi-automated screening instrument for the detection and enumeration of total and thermotolerant coliforms, *E. coli* and faecal streptococci in water. Colifast media for coliform detection incorporates the medium 4-methylumbelliferyl-β-D-galactoside, which gives rise to fluorescent 4 -methyl-umbelliferone (MU) upon substrate hydrolysis by β-D-galactosidase. The media contains compounds inhibitory to non-target organisms and sodium lauryl sulphate to enhance the transfer of the substrate and enzyme across the outer membrane of cells. Table 10.1 (Appendix A) displays the specifications of the Colifast method.

The instrument can detect coliforms and *E. coli* to the detection limit of 1 CFU per sample volume. The instrument is aimed at bathing waters in particular, due to the difficulty of achieving the 0 – 1 CFU required for drinking water quality assessment. The speed of detection depends upon the level of contamination within the sample.

Results are given as concentrations of the fluorescent 4-MU, which can be converted to equivalent colony forming units by the use of calibration curves specific to the water type. A good correlation between log β-galactosidase and log faecal coliform plate counts has been noted by several authors (Fiksdal *et al*, 1994., Davies and Apte, 1996., George *et al*, 2000). Semi-quantitative information can be obtained by determining the Time To Detection (TTD), when fluorescence intensity reaches a certain threshold, or by the rate of hydrolysis, determined from the slope of the linear increase observed during cultivation (Samset, Hermansen and Berg, 2000). Calibration is performed during analysis by blanks and samples of known concentration to correct fluorescence readings. A base level is also set to disregard fluorescence intensity below a certain level, to filter out background fluorescence.

The Colifast Analyser has been applied as an early warning operational tool (Tryland *et al*, 2000)., in MPN format (Samset, 2000)., P/A format (Samset, Hermansen and Berg, 2000) and direct addition or membrane filtration (Angles d'Auriac *et al*, 2000). Angles d'Auriac *et al*, 2000 evaluated the Colifast system in the UK for faecal contamination of bathing water samples using the presence-absence (P/A) format and compliance with a threshold level of 100 CFU/100ml (the faecal coliform BWD guideline standard). The P/A format gave 83.9% agreement with mLSB standard membrane filtration method. Agreement with the 100 CFU threshold was 94.6%. False positives (2.9%) were recorded due to the presence of *Shigell*a spp., *Aeromonas hydrophila*., *Vibrio alginolyicus*., *Vibrio* spp., *Pseudomonas* spp., *Acinetobacter* spp. Interference from background fluorescence was found to be low, except when *A. hydrophila* was present at high concentrations. False negatives (7.8%) were recorded due to slow growing target bacteria, especially in samples with low contamination.

The Colifast Analyser can also be used to detect enterococci using 4-methyl-umbelliferyl-β-D-glucoside. Colifast developed Faecal Streptococcus - Enterococcus (FS-E) medium from the initial composition proposed by Little and Hartman, 1983, although modified the constituents to suppress growth of *E. coli* and *Enterobacter aerogens*. The medium has very high specificity for enterococci. Current sensitivity for the FS-E Colifast system is less than 100 CFU per sample volume within 20 hours.

The French company SERES and IDEXX have coordinated to produce a fully automated coliform detection system called the Colilert 3000. The system uses the Colilert® media (ONPG or MUG) for the detection of total coliforms or *E. coli*. The instrument is fully automated, holding up to 35 samples in a carousel that allows up four analyses per day. Samples are automatically collected, incubated for up to 17 hours, and the system thoroughly cleansed prior to the next sample. Specifications of the Colilert 3000 system are in Table 10.1 (Appendix A).

The instrument can detect 1 coliform per 100ml within 18 hours in potable water. Bathing beach monitoring can be undertaken for *E. coli* where 2000 CFU or more can be detected in 100ml within 8 hours. High faecal pollution will be detected in a shorter time frame, due to the greater fluorescence intensity produced. Recent applications include the remote detection of *E. coli* in seawater in the South of France (Biarritz). No published data is available to evaluate the system performance against standard methods, although the abundant literature regarding the Colilert® media is mentioned in Section 5.1.2. Use of the Colilert® MUG (for *E. coli* detection) substrate has not been validated at 44^0C, however, therefore there is uncertainty concerning the accuracy of *E. coli* enumeration by the automated system (Morris, S; personal communication).

In 1994, Fiksdal *et al*, reported a 25 minute enzyme assay using 4-MU-β-D-galactopyranosidase and 4-MU-β-D-glucuronidase substrates for the detection of coliforms and *E. coli* respectively. Caruso *et al*, 1998, found this assay produced insufficient fluorescence intensity for the detection of *E. coli* in marine waters. Doubts have also been directed at ultra-rapid enzyme assays by Van Poucke and Nelis, 1997. Figure 4.1 summarises the advantages and limitations of rapid enzyme assays.

Robertson *et al*, 1998 have developed a 6 hour membrane filtration method based on the detection of β-D-glucuronidase and β-D-galactosidase production by *E. coli* and FC by exposure to dioxetane glucuronide or dioxetane galactoside respectively. Following a 6 hour incubation at 44.5 ^{0}C, the dioxetane produced from substrate hydrolysis was detected using a luminometer. Quantitative results were generated from standard curves produced by parallel comparison to standard methods.

The method was used to determine whether beach samples collected from the Lake Huron, Canada complied with, or exceeded the *E. coli* objective of a geometric mean of 100 CFU per 100ml. When compared with standard membrane filtration procedures, the *E. coli* screen correlated well ($r = 0.89$) with 84.6% agreement, and the faecal coliform correlation was reasonable ($r = 0.79$) with similar agreement (85.9%). A false positive rate of 4.1% and false negative rate of 11.3% was recorded for the *E. coli* assay. The authors suggested that an overall 90% correct prediction rate of failure or compliance with the water quality standard (including false positives as an extra safety margin) was advantageous compared to a retrospective result by standard methods, which was correct only 65% of the time.

Snyder *et al*, 1991a, proposed a rapid method for the semi-quantitative enumeration of *E. coli* based upon the principle of detection of the vapours arising from the reaction between β-D-galactosidase and ONPG. The ion mobility spectrometer (Airborne Vapour Monitor, Graseby Ionics, UK) is portable and hand-held, detecting vapour produced by cells captured on a membrane filter. The detection limit was reported as approximately 200 cells per ml following a 15 - 30 minute incubation. Vapour detection by the same instrument from the

headspace of a broth culture produced a detection limit of 3300 cells per ml in 15 minutes (Snyder *et al*, 1991b)

Advantages

- Cost effective
- Rapid
- Easy to use
- Portable and/or Automated
- Potential for on-line control
- Detection of viable, but non-culturable organisms

Limitations

- Physiological condition of cells due to non-lethal injury may give variable non-expression of enzymes.
- A number of *E. coli* strains (including 0157:H7) do not express the β-D-glucuronide enzyme.
- Poor sensitivity due to autofluorescence of substrate, cell free enzymes and non-target micro-organisms (Tryland and Fiksdal, 1998).
- False positives - non-target bacteria such as *Aeromonas hydrophila*, *Vibrio cholerae* and *Kluyvera* spp. express β-D-galactosidase or β-D-glucuronide. Interference is low at high temperatures (Davies *et al*, 1995).
- Freshwater and marine plants and algae shown to express β-D-galactosidase or β-D-glucuronide activity (Davies *et al*, 1994). Significant when high algal or plant biomass is observed in the water.
- False negatives - slow growth of target organisms as a result of non-lethal injury and selectivity of the growth medium.

Box 4.1: Evaluation of enzymatic screening methods for the rapid detection of faecal indicators in surface water

4.2 Bioluminescence

Bioluminescence is the natural light given off by certain living organisms, such as the firefly. An enzyme known as luciferase generates the light. The firefly *Photinus pyralis* possesses luciferase in its tail. Free ATP in living cells catalyses the reaction between luciferin and the luciferase enzyme to generate light. Detection of bioluminescence can therefore be used as a measure of the presence of living organisms. Results are given as Relative Light Units, which are proportional to the concentration of cells present in the sample. The sensitivity of ATP bioluminescence reactions is approximately $10^3 - 10^4$ bacterial cells. Specificity is poor, however, as the test detects all living cells in the sample. An ATPase may be added, however, which hydrolyses non-bacterial ATP in the sample (Van der Zee and Huis in't Veld, 1997).

Bioluminescence reactions require detection of fluorescence by a luminometer (Section 5.4.6) or high sensitivity camera (Section 5.4.4). Swab samples can be analysed for total ATP content using a number of dipstick methods on the market (Luminometer K, Charm Sciences., Unilite Xcel, Biotrace., Lightning, IDEXX). The test is very rapid, although detects all living

matter which contains ATP, therefore would be of little use in environmental waters. These tests have application in the food industry to control general hygiene. Tests carried out by Colquhoun *et al*, 1998 showed that the IDEXX Lightning method produced reproducible, sensitive results for this application.

The sensitivity and specificity of the bioluminescence reaction has been improved by researchers at MAFF and DERA in the UK. Squirrel and Murphy, 1997 at DERA developed a bioluminescence reaction involving cellular adenylate kinase (AK) as a cell marker, rather than ATP. The authors found that the AK marker reaction test produced a 10 to 100-fold increase in the sensitivity of the ATP bioluminescence reaction.

Squirrell and Murphy, 1997 found that less than 10^2 *E. coli* cells could be detected in 5 minutes. Longer incubation time (35 minutes) for ATP generation produced a more sensitive assay. The authors suggest that the detection of a single cell may be possible through increasing incubation time and reducing sample volumes.

The specificity of the AK bioluminescence method can be improved by the selectivity of immunomagnetic beads (Sellick-Harmon *et al*, 2000) or specific bacteriophage-lysis (Blasco *et al*, 1998). Immuno-magnetic bead capture (see Section 5.2.2) of target *E. coli* 0157:H7 prior to the AK bioluminescence reaction gave a detection limit below 10^2 cfu in a 100 µl sample in 10 minutes. Researchers at MAFF have developed the use of specific bacteriophages to select target bacteria and lyse their hosts to release ATP, based on work conducted by Kodikara *et al*, 1991. Blasco *et al*, 1998 found that by combining phage-mediated specific lysis and the AK marker for ATP bioluminescence, *E. coli* detection could be undertaken in under 1 hour, with a detection limit of less than 10^4 ml^{-1}. The procedure has been commercialised as the AK Phage ^{TM} test, coupling the AK bioluminescence test with the specific ATP extraction by bacteriophages.

The employment of an AK assay in a dipstick format would provide faster assay times with greater sensitivity. Experimentation with the AK bioluminescence method has revealed a possible link between the thermo-stability and detergent resistance of cellular enzymes. Environmental cells could be differentiated from enteric cells by the concept of optimisation of the thermal conditions in each type of cell (Squirrell, D, personal communication). If such a theory was to be further investigated, the possibility to modify bioluminescence dipsticks to differentiate enteric bacteria may be realised. This may be the closest one could get to a colour-change dipstick. Dipsticks can be immediately read on a portable luminometer to obtain a presence – absence reading.

Advantages:

- Instantaneous to rapid, depending upon pre-enrichment
- Low Cost
- Portable and/or automated
- Good limit of detection if following an incubation period
- Measure of viability
- May be specific to enteric bacteria by using bacteriophage lysis

Limitations:

- Poor sensitivity
- Inhibition by non-specific matter in sample

No pre-enrichment:

- Non-specific unless modified.
- Low ATP in bacterial cells to produce a detectable signal
- High background ATP - poor signal differentiation

Cultivation Step:

- Slow result
- Time consuming preparation

Box 4.2: Evaluation of bioluminescence reactions for the detection of faecal indicators in surface water

4.3 Biosensors

Biosensors are analytical devices, incorporating a biological recognition element in intimate contact with a physical or chemical transducer, which yield a measurable signal proportional to the concentration of micro-organisms in the sample. Biosensors can provide direct detection of a biological reaction by measuring physical changes in pH, potential difference, oxygen consumption, ion concentrations, current, resistance or optical properties occurring as a direct result of the analyte-receptor complex formation on the transducer surfaces. Indirect detection of products of an initial biochemical reaction may also be detected by a biosensor.

These biosensors can be used intermittently (batch sensors) or continuously (automatic monitoring). The biological components can be divided into the catalytic group (whole cells, enzymes, mitochondria and tissue) and affinity reaction group (antibodies, cell receptors and nucleic acids). The biological receptor is immobilised in close proximity to the transducer. The analyte selectively binds to the biological material with an amount and speed that is proportional to the concentration of the analyte in the sample. The transducer detects and quantifies the binding reaction between the receptor and target cell, giving a measurable signal.

Transducers can be classed in one of four groups (Tothill and Turner, 1998., Pérez, 1998., Ivnitski, 1999):

- Electrochemical

  - fixed current (amperometry)
  - zero current (potentiometry)
  - conductivity changes
- Optical
- Calorimetric (heat sensitive sensors)
- Mass (piezometric or surface acoustic wave devices)

An ideal biosensor would enable *in situ*, real-time detection of viable target cells, at concentrations as low as 1 – 10 cells in 100ml, by relatively untrained staff. With few exceptions, the current biosensor market is generally geared towards the detection of high concentrations of target organisms. The detection limits of most biosensors are in the range of 10^4 - 10^6 CFU, restricting their application to extremely poor water quality. Biosensors including a cultivation step, such as impedance methods, enable the detection of lower contamination, although a time delay is introduced. A general evaluation of biosensors is considered in figure 4.5. There is a wide range of biosensors available on the market and undergoing evaluation. A number are mentioned here, with consideration to their potential application for bacterial detection in aquatic environments. For a thorough review of biosensors for microbial detection, see Ivnitski *et al*, 1999 and Hobson *et al*, 1996. Developments in the industry are continually advancing, therefore suitable instruments may be available in the future. Expenditure may have to be offered by interested users to develop a specific aquatic application.

Optical Sensors - fibre optics, ellipsometry, surface plasmon resonance (SPR), resonance mirror (RM), interferometry and monomode dielectric waveguides. Also includes natural fluorescence.

Bioluminescence - encoding luciferase genes into bacteriophage, who confer phenotype to host bacteria. Detect ATP or Adenylate Kinase bioluminescence (see Section 4.2).

Piezoelectric Biosensors - Crystals coated with antibodies specific to target organism - binding causes increase in crystal mass and reduction in resonance frequency.

Electrical Impedance - Malthus, Bactometer, RABIT

Fluorescent Labeled Immunosensor - Antibodies tagged with fluorochromes to aid detection with fluorimeter. Also include immunomagnetic labeling of target cells (see Section 5.3.2).

Microbial Metabolism Sensors - Measures electrical signal generated by metabolic oxidoreductase reactions. Flow Injection system can reduce assay time (see Section 5.4.3).

Electrochemical immunosensors - Light Addressable Potentiometric Sensor (LAPS), amperometric detection, immunomagnetic bead system with amperometric detection increases selctivity.

Flow Immunosensors - Enzyme linked flow immunoassay (ELIFA) (Clark et al, 1993), immunomagnetic assay with flow injection, flow injection amperometric immunofiltration system (Abdel-Hamid *et al*, 1999).

Genosensors - nucleic acid probes, radiolabeled probes, PCR - gene probe combination sensor, direct and indirect (labelled) methods available eg BIAcore and IAsys optical biosensors. See Section 5.3.3.

Electronic nose - Sensor array for odour and odourless volatile compunds produced by microbial metabolism. Detection of hydrocarbons, alcohols, aldehydes, acids and ammonia in system headspace. Neural network analysis applied for statistical analysis.

Box 4.3: Summary of the range of biosensors developed for the detection and enumeration of micro-organisms.

4.3.1 Electrometric Biosensors

Impedance

Impedance is not a new technology. First mentioned in the British Medical Association Journal in 1898, it was not until the 1970's that this method began to receive considerable attention through work by Ur and Brown (1975) and Cady (1975). Cady's work led to the development of the Bactometer (Biomerieux, France). Later work by Richards *et al* (1978) developed the technology, resulting in the Malthus system (IDG, UK). The RABIT arrived on the impedance instrumentation market in 1989 (Don Whitley Scientific Ltd, UK). The Bactrac (Sy-Lab, Austria) is a similar instrument, with limited distribution in the UK.

Impedance is the resistance to a flow of alternating current through a conducting medium. Microbial growth results in electrochemical changes in the conducting medium. As microorganisms grow they generally change weakly or uncharged substrates into highly charged end products of their metabolism, increasing the conductivity of the medium. Proteins are converted to amino acids, carbohydrates to lactate and lipids to acetate (Fristenberg-Eden and Eden, 1984). Metal electrodes placed into the conducting medium behave as resistor and capacitor in series. Ions migrate within the medium if an electric field is imposed. Anions move towards the positive anode electrode; cations to the negative cathode. This movement of ions constitutes the flow of current between the electrodes (Fristenberg-Eden and Eden, 1984).

The resultant current is dependent on the impedance of the medium. Impedance is a function of its resistance, capacitance and applied frequency. A flow of electric current generally encounters resistance, leading to a drop in voltage and conversion of electrical energy to heat. Conductance is the reciprocal to resistance. Capacitance is an element which stores energy in an electric field, but does not dissipate it. Microbial metabolism therefore increases conductivity and capacitance causing a decrease in impedance and increase in admittance (Fristenberg-Eden and Eden, 1984).

An instrument measures impedance once sufficient growth of microorganisms has occurred to enable the detection of change in the electrical current within the medium. The Time To Detection (TTD), from initial inoculation to detection of electrical changes, is found to be inversely proportional to the original micro-organism concentration in the sample. The method can be semi-quantitative when detection of time to a certain threshold is used. The method can be quantitative when response signal curves are compared to calibration curves to give a colony forming unit measure. Most Probable Number techniques can also be used. Impedance can be measured directly or indirectly.
Impedance is affected by temperature (a 1^0C change in temperature can lead to an increase of 0.9% in capacitance and a 1.8% increase in conductance (Fristenberg-Eden and Eden, 1984)), concentration and generation time of micro-organisms, electrode type and the concentration of the growth medium.

Direct Impedance

In the direct impedance system microbial metabolism causes a change in the conductance of the bulk metabolite in direct contact with the system electrodes. Irving and Easton, 1994 investigated the direct use of the Malthus, Bactometer and RABIT systems for the detection and enumeration of coliforms, *E. coli* and faecal streptococci. Specifications for three instruments are shown in Table 10.2 (Appendix A). The authors found that overall; the use of electrometric instruments did not constitute a particular cost saving. The main benefit for these instruments is for laboratories with a high throughput, where results can be monitored and recorded automatically.

The authors, however, did find a particular time saving for the enumeration of faecal streptococci. They used the direct impedance version of RABIT and Bactometer, obtaining results as sensitive as 1 CFU ml^{-1} in 19 - 28 hours (RABIT) and 18 - 33 hrs (Bactometer). This is a considerable reduction in time from the 48 hour conventional membrane filtration test. Higher concentrations (43 - 360 CFU ml^{-1}) were detected in 14.5 - 20.1 hours with the RABIT and 9.5 - 13.6 hours with the Bactometer (Irving and Easton, 1994).

Indirect Impedance

Owens *et al*, 1989 pioneered the indirect method of impedance at the University of Reading. The growing microorganisms and culture medium do not come into direct contact with the electrodes. Carbon dioxide produced by the metabolising organisms is absorbed by a strong alkali solution or agar, such as potassium hydroxide, and is converted to carbonate at pH values above 11 (Equation 4.1).

$CO_2 + 2OH^- \rightarrow CO_3^{2-} + H_20$
Equation 4.1

The resulting decrease in conductivity, a consequence of the high molar conductivity of hydroxide ions, is measured by electrodes within the alkali solution or agar (Owens *et al*, 1989). An increase in impedance, therefore occurs, which is the reverse of direct techniques.

The advantage of indirect conductimetry is that target organisms can be grown on standard culture media, often with a high salt content, without prior modification. The high conductance of these media put them outside the range of the direct impedance assay (Bolton, 1990). Stripping of CO_2 from the system did not seem to have an inhibitory effect on bacterial growth (Owens *et al*, 1989). A disadvantage is the extra time required and complexity of preparation of the cells. The method is suitable for all organisms producing CO_2 as a metabolic product. The technique can also be applied to conductimetric assays of other volatile metabolites including H_2S. Preliminary studies showed good absorbency of H_2S, produced by sulphite reducing bacteria, by KOH (Owens *et al*, 1989).

Timms *et al*, 1996 investigated the use of indirect impedance technology for the detection of *E. coli* in water. They used the Bactrac 4100 instrument (Sy-Lab, Austria) with membrane lauryl sulphate broth as a culture medium at 44^0C. Results were compared with the Colilert® Quanti-Tray™ technique. They found 93.3% agreement between the two methods, with 1 CFU *E. coli* detection in 15 hours with the Bactrac compared with 18 - 24 hrs with Colilert. The fully automated Bactrac 4100 hold up to 20 samples in reusable glass cells. Measurements are taken every 10 minutes throughout the 24 hour maximum incubation period, after which a printout of each cells impedance patterns is produced. Impedance signal response is representative of each bacterial species. Twenty eight strains of *E. coli* produced the same response signal using the Bactrac (Timms *et al*, 1996). The method showed good specificity for *E. coli* when screened against a range of organisms from pure culture and the environment (Timms *et al*, 1996). No false positives were recorded.

The sensitivity of the indirect impedance method appeared to be less than that of the Colilert® assay, leading to the possibility of false negatives occurring. A number of instances occurred where *E. coli* was detected by Colilert, but not by the MLSB indirect impedance method. This was found to only occur in samples with 4 or less *E. coli* CFU present. This may be due to lower recovery of stressed cells in the absence of a 4 hour 'ramping' period which is integral in conventional membrane filtration methods. Another suggestion is that the MLSB medium contains compounds inhibitory to the recovery and growth of chlorine stressed cells, particularly impurities from phenol red (Hay *et al*, 1994

The RABIT (Don Whitley Scientific, UK) is an automated instrument for direct and indirect impedance analysis. Pridmore, 1994 evaluated the RABIT system in comparison with membrane filtration for the enumeration of coliforms and enterococci in sewage effluent

samples. Coliforms were analysed using the direct impedance method. Enterococci were enumerated using the indirect method. Strong correlations were found to exist between each method comparison (-0.877 for total coliforms: -0.938 for enterococci). The majority of total and faecal coliform results were obtained within 7 hours. Most enterococci results were available within 11 hours, representing a significant time saving from standard methods. The sample preparation time for the membrane filtration method greatly exceeded that for the RABIT (approximately 2 hours work compared to 10 - 15 minutes for analysis of two samples, each for three target organisms). The RABIT uses re-usable test cells, thus reducing waste and consumables cost considerably. Cost per sample is determined by the cost of the culture media. Small quantities are required (1 - 10ml), therefore cost per sample may be extremely low.

The RABIT and BacTrac systems are very similar in design and function, although cover different markets worldwide (Table 10.2, Appendix A). Disadvantages of the Bactometer and Malthus systems are the potential for poor temperature control during incubation. The Bactometer is heated by fan convection and the Malthus in a water bath. Both may experience greater fluctuations in temperature than in thermally heated aluminium blocks such as those in the RABIT and BacTrac. A second disadvantage of the Bactometer is that a user may not access single cells without disturbing the incubation of others. Access to cells during incubation may be required if the system indicates that a cell is not working, or if an aliquot of a sample is required from a cell for further confirmation. The Malthus has similar inaccessibility to single cells as the electrodes are located at the top of the cell, therefore the impedance process would be disrupted.

Advantages:

- High throughput
- Optical clarity not important
- Reduction in time to result compared with standard methods
- Reduction in sample preparation time
- Automation
- Low cost reagents
- Little training required

Limitations:

- High capital costs
- Specificity only as good as medium used
- Non-portable
- Significant false detection
- Reliance on stable temperatures
- Speed of detection dependent on generation time and initial concentration of organism
- Comparison to standard method difficult as impedance is a measurement of metabolic change rather than growth.
- No simultaneous detection of *E. coli* and coliforms

Box 4.4: Evaluation of impedance as a method for the detection and enumeration of faecal indicators in surface water.

Amperometric and potentiometric measurements

Potentiometric analysis involves the measurement of the potential generated at an electrode with no external potential applied (Hobson *et al*, 1996). Microbial growth is detected by the effect of metabolism upon the medium redox-potential. The method is simple and cheap, although a time delay is introduced, as sufficient microbial growth is required. Amperometry measures the current that is produced at a working electrode, poised at a potential that oxidises the mediator at the electrode surface. Mediator reduced by microbial metabolism is re-oxidised at the electrode, producing a signal that is proportional to the concentration of target organisms (Hobson *et al*, 1996).

A portable cellobiose dehydrogenase (CDH) biosensor has been evaluated for the detection of *E. coli* in water based upon amperometric detection of 4-aminophenol (4-AP) which is released upon the cleavage of the substrate 4-aminophenyl-β-D-galactopyranoside (4APGal) by β-galactosidase (Osvik, 2001). Bacteria concentrated by membrane filtration were incubated in a water bath at 44.5^0C for up to 11 hours. Amperometric measurements of 4-AP enabled detection of *E. coli* of 10^4 CFU $100ml^{-1}$ and greater within a working day. The speed of this biosensor is not sufficient for screening, however, and therefore requires additional optimisation. Similarly Perez, 1998 found amperometric detection of 4-APgal lacking in sensitivity.

DNA hybridisation biosensors utilise the high specificity of hybridisation of an immobilised DNA layer with target nucleic acid sequence, which can produce an electrochemical signal response (Wang *et al*, 1997). *Cryptosporidium* detection was achieved using a microfabricated carbon strip and hand held chronopotentiometric analyser (Wang *et al*, 1996). Incorporation of a portable amplification (PCR) system would enable the rapid, *in situ* detection of low contamination levels of pathogens in water (Wang *et al*, 1997).

4.3.2 Optical biosensors

The BIAcore system (Biacore, Stevenage, Herts.) utilises Surface Plasmon Resonance (SPR) for the detection of biological molecules. Binding of molecules to immobilised targets on the sensor surface causes changes in the refractive index at the surface. These targets may be covalently bound ligands or streptavidin for the capture of biotinylated peptide, proteins and DNA. SPR causes a reduction in the intensity of light reflected from the sensor surface. Changes in the SPR are proportional to the mass adsorbed to ligands on the sensor surface (Malmqvist, 1993). The BIAcore has been applied within the clinical and pharmaceutical industries for rapid and automated analysis. The high expense and large size of the instrument would preclude routine use for the monitoring of environmental waters.

There are a number of ways of improving SPR analysis including the use of a metal surface layer to effect dielectrophoretic concentration of cells (Hughes & Morgan, 1998). The evanescent field close to the SPR surface can be used to generate light scattering from particles which may be clearly observed against a dark background. DERA have developed an instrument that employs light scattering detection in combination with surface plasmon resonance (SPR) detection of changes in refractive index (Perkins and Squirrell, 2000). This has been applied to the detection of *Bacillus subtilis* var. *niger* in comparison to the market leading SPR BIAcore instrument. The instrument gave detection limits of $10^5 – 10^6$ CFU per unit volume. Reasons for this poor sensitivity include the size of bacterium, inefficient antibody capture and low rate of diffusion by bacterial cells. Detection of *E. coli* 0157:H7

using SPR and antibody capture has previously resulted in poor sensitivity of 5 – 7 x 10^7 CFU per ml (Fratamico *et al*, 1998).

The Raptor® (Research International, Woodinville, WA) is a portable, rapid optical waveguide sensor. The instrument was developed for military use, enabling four simultaneous 3 - 10 minute assays for target micro-organisms. The instrument detects fluorescence on four optical waveguide sensors mounted on a disposable coupon. Immunoseparation steps may be required for turbid samples with high background interference. A sandwich ELISA procedure has been described where the primary antibodies are immobilised upon a waveguide surface and the sample introduced for 1.5 – 7 minutes. Secondary antibodies, tagged with a fluorochrome such as Cy5, are introduced for 1.5 minute incubation (Anderson *et al*, 2000). Evaluation of the Analyte 2000, a first generation optical waveguide instrument from the same manufacturers, was undertaken at the University of South Florida for the enumeration of *E. coli* 0157:H7 in beef and apple juice using a sandwich ELISA immunoassay (DeMarco *et al*, 1999). A detection limit of 3 CFU ml^{-1} was achieved in a 30 minute assay.

A fibre optic detection system is under evaluation by the USEPA (Dufour A, personal communication).

4.3.3 Colorimetric Biosensor

Scientists at Lawrence Berkeley National Lab in the US have developed a biosensor which turns blue to red on detection of *E. coli* 0157:H7 toxin (Charych *et al*, 1993, 1996). The artificial polydiacetylene polymer membrane has a backbone of linked lipid molecules supporting specific receptors mimicking those found in the human intestine wall. The *E. coli* enterotoxin binds to these receptors, breaking the linkage in the lipid backbone, causing an immediate chromatic transition to a magenta red colour that may be determined visually or by an absorption spectrophotometer. The colorimetric response increases with increasing concentration of target molecules. The developers hope the 'litmus' film could be used in the lids of food and drink products to detect the presence of toxic *E. coli*. Such a cheap film could have uses as a rough indicator of *E. coli* 0157:H7 in water, although the low concentrations found in water may not be sufficient for detection.

The incorporation of a membrane filter and flow injection system into the biosensor instrument can be used to pre-concentrate the sample and introduce target cells to the sensor at the optimal rate to ensure capture (see Section 5.4.3). Flow immunofiltration systems have been described which may enable accelerated binding kinetics between antibody and antigen, increasing speed and sensitivity (Morais *et al*, 1997). Bouvrette and Luong, 1995 evaluated a flow immunosensor for the detection of *E. coli* in food. The system combined an immuoassay with fluorescence determination from expression of the β-D-glucuronidase enzyme. The detection limits was found to be inadequate at 5 x 10^7 CFU ml^{-1}. The sensitivity of the system was also limited by the inadequacy of wash steps following each assay. The speed of the assay was rapid however, detecting *E. coli* within 30 minutes, and the system was re-usable.

A rapid flow amperometric immunofiltration system was also recently developed by Abdel-Hamid *et al*, 1999. Amperometric detection of immuno-captured *E. coli* 0157:H7 cells enabled detection of as low as 50 cells ml^{-1} in a 40 minute assay. Total *E. coli* detection was achieved in 35 minutes to the same detection limit.

4.3.4. Gene Chip Sensors

'Lab-on-a-chip' sensors have developed from the natural advancement of DNA hybridisation on beads, membranes and microtitre plates, to miniaturised surfaces, densely packed with an array of immobilised oligodeoxynucleotide probes for the rapid detection of target nucleic acid sequences (O'Donnell-Maloney *et al*, 1996). DNA chips may also be manufactured with immobilised DNA upon the sensor surface. Applications for DNA chips include gene expression and discovery studies, detection of mutations and polymorphisms and mapping genomic DNA clones (Ramsay, 1998).

Sample DNA is extracted and amplified prior to hybridisation with the microarray of specific probes. Detection of hybridised sequences involves direct analysis using radioactively or fluorescently labeled probes, or indirect detection via enzyme labeled probes such as horseradish peroxidase, enabling colorimetric detection (O'Donnell-Maloney *et al*, 1996). Radiolabeled probes may be detected using an autoradiograph or CCD camera. Conjugates labeled with selenium may be detected using optical waveguide sensors in conjunction with a high sensitivity camera or visual analysis. Detection of fluorescent signals is commonly undertaken by scanning instruments. The GeneChip (Affymetrix, Santa Clara, CA) involves the hybridisation of specific nucleic acid sequences, labeled with a fluorescent reporter, to the immobilised sensor surface. Detection of fluorescence is accomplished using a scanning instrument.

Advantages:

- Cost effective
- Portable, automatic and on-line systems
- Biosensor surfaces may be re-usable
- Provide rapid detection of target organisms, especially at high concentrations
- Certain methods are not limited by optical clarity of sample e.g., impedance
- Wide range of target cell capture methods available for specific biosensor development
- Flow injection and filtration may reduce limitations associated with poor diffusion of target cells and aid sample concentration
- Often requires simple operation only

Limitations:

- Lower specificity compared with plate cultivation methods
- Low sensitivity
- Interference from sample matrix – non-specific organisms and suspended solids
- Methods incorporating pre-enrichment suffer additional time delay to result
- Maintaining sterilisation may be a problem
- Certain systems are expensive and non-portable
- Certain methods may not indicate viability of target organisms e.g. Fibre optic systems employing antibody labelling

Box 4.5: Evaluation of biosensors for the rapid detection of faecal indicators in surface water.

5. Quantitative Technologies

5.1 Cultivation - Based methods

5.1.1 UK Reference Methods

Microbiological examination methods used by the Environment Agency are based upon guidelines stated in Bacteriological Examination of Drinking Water (1984), BSI 6068 (1989), ISO 9308-1 (1990), Microbiology of Water: Part 1(1994) and Microbiology of Recreational and Environmental Waters (2000). Table 5.1 outlines the UK standard methods utilised by the Environment Agency. *Escherichia coli* and confirmed faecal streptococci analysis is not routinely carried out for bathing and shellfish water quality monitoring.

Table 5.1: Environment Agency standard methods for the enumeration of coliforms, *Escherichia coli* and faecal streptococci in surface waters by membrane filtration.

Target Organism	Presumptive Test	Confirmation
Total Coliforms	Incubation on Membrane Lauryl Sulphate medium at 30^0C $\pm$ 1^0C for 4 hrs; then 37^0C $\pm$ 0.5^0C for 14 hrs.	Isolated yellow colonies produce acid and gas in Lactose Peptone Water at 37^0C $\pm$ 0.5^0C for 40 to 48 hrs.
Faecal Coliforms	Incubation on Membrane Lauryl Sulphate medium at 30^0C $\pm$ 1^0 C for 4 hrs; then 44^0C $\pm$ 0.5^0 C for 14 hrs.	Isolated yellow colonies produce acid and gas in Lactose Peptone Water at 44^0C $\pm$ 0.5^0C for 18 to 24 hrs.
Escherichia coli	Incubation on Membrane Lauryl Sulphate medium at 30^0C $\pm$ 1^0 C for 4 hrs; then 44^0C $\pm$ 0.5^0 C for 14 hrs.	Isolated yellow colonies produce acid and gas in Lactose Peptone Water at 44^0C $\pm$ 0.5^0C for 18 to 24 hrs. Addition of Kovács' reagent produces red colour indicating presence of indole.
Faecal Streptococci/ Enterococci	Incubation on Slanetz and Bartley agar, containing TTC at 37^0C for 4 hrs; then 44^0C for 44 hrs	Isolated maroon, red or pink colonies transferred to bile aesculin azide agar at 44^0C for 2 hrs. Hydrolysis indicated by black or brown colour in or around presumptive colonies.

Limitations of Standard Methods

The stress imposed upon intestinal bacteria on entering to the aquatic environment, as discussed in Section 2.2, can pose substantial difficulties for the enumeration of organisms, particularly by cultivation (Xu *et al*, 1982). The use of selective media, in the initial stages of cultivation in particular, may have an inhibitory effect upon organism recovery (Bissonnette *et al*, 1977., McFeters *et al*, 1982). The recovery efficiency of non-lethally injured cells on rich, non-selective broth was found to be superior prior to cultivation on selective media (Bissonnette *et al*, 1975). The presence of inhibitory compounds such as impurities in phenol

red in MLSB medium has also been proposed as a cause of low chlorine injured bacterial recovery (Hay *et al*, 1994).

The application of membrane filtration imposes additional stress upon the bacteria. Problems associated with membrane filter use include inhibition of growth on filter grid-lines, abnormal spreading of colonies, hydrophobic areas, poor colony sheen, brittleness, decreased recovery and wrinkling (Brenner and Rankin, 1990). Shipe and Cameron, 1954 suggested the build up of toxic compounds upon filters lead to poor recovery of cells and that stressed cells may recover more rapidly in a broth medium, rather than on a filter. Different brands of membrane filters also produce discrepancies in the enumeration of *E. coli* from water (Presswood and Brown, 1973., Brenner and Rankin, 1990), possibly determined by surface pore morphology (Sladek *et al*, 1975). Turbidity in water samples may preclude the use of filtration as high sediment concentration on the membrane surface may interfere with colony growth.

The presence of high densities of non-target bacteria may suppress coliform numbers, by overcrowding of membrane filters and substrate limitation, resulting in underestimation (false negative results) of target bacterial concentrations. A low percentage (1 - 10%) of viable bacteria are generally recovered by the membrane filtration method (Pickup, 1991). False negative results are also common where strains of lactose-fermenting negative coliforms are missed during tests, as they do not fit to the working definition of a 'coliform' requiring acid and gas production on the fermentation of lactose. Overestimation, known as a false positive result, can occur when bacteria possessing similar characteristics to coliforms are detected during tests, although high incubation temperatures and inhibitory substances in the media reduce background growth.

Advantages of the current methods, however, have outweighed the limitations for decades. The low cost per sample and low complexity of the procedure makes the techniques universally applicable to laboratories of all sizes with low academic requirements for staff. Sample preparation time is short, enabling a high throughput of samples per day in the busiest of laboratories. The selectivity of the media commonly used and the time for confirmed results remain major limitations. Considerable research has been aimed at improving these issues to enable continued confidence in plate cultivation for microbiological analysis.

Revision of plate culture methods

An improvement to m-Enterococcus (equivalent to Slanetz and Bartley) method was suggested by Figueras *et al* in 1996. The media has been shown to be specific, Dionisio and Borrego, 1995, but results may be erroneous due to the procedure of randomly selecting 10% presumptive colonies from the membrane filter for confirmation. A rapid confirmation step involving transfer of the entire membrane to Bile Esculin Agar (BEA) medium for 4 hours demonstrated 60% confirmation of colonies in evaluation studies compared with only 47% by standard confirmation. The authors also found the improved method to be less time consuming and costly. This improvement has been recognised in the recent revision of the ISO membrane filtration method for enterococci (Table 5.2).

Table 5.2: Revision of methods for the enumeration of *E. coli* and enterococci as recommended by the International Organisation for Standardisation (ISO)

Method step	Enterococci	Escherichia coli and coliforms	Rapid E. coli Test
Name of method **Method type** **Media Substrate**	**ISO 7899-2** Membrane filtration Slanetz and Bartley	**ISO 9308 - 1** Membrane filtration Lactose TTC	**ISO 9308 - 1** Membrane filtration TSA medium
Incubation	Incubate at 3± 2^{0}C for 44 ± 4 hours.	Incubate at 36 ± 2 ^{0}C for 21± 3 hours	Incubate at 36 ± 2 ^{0}C for 4 - 5 hours. Place membrane on TBA medium and incubate at 44 ± 0.5 ^{0}C for 19 - 20 hours. Double layer method may be used.
Presumptive positive	All red, maroon or pink colonies - positive	Yellow colonies are lactose positive.	
Confirmation	Transfer membrane to Bile-aesculin-azide agar, which has been preheated to 44^{0}C. Incubate at 44 ± 0.5 ^{0}C for 2 hours.	Subculture to nonselective agar at 36 ± 2 ^{0}C for 21 ± 2 hours and tryptophan broth at 44 ± 0.5 ^{0}C for 21 ± 3 hours	Place membrane on a filter pad soaked in indole reagent and irradiate with UV light for 10 - 30 mins.
Confirmed positive	Colonies with a tan to black colour in the surrounding medium are positive.	Deep blue purple colonies in oxidase test within 30 seconds - positive - on nonselective agar method. Class as coliform bacteria if negative. Cherry-red colour development indicating indole production in subculture to tryptophan broth. Class as *E. coli* if negative oxidase and indole positive.	Red colour development - Confirmed E. coli

A modification of Kanamycin-Esculin-Azide (KEA) agar, commonly used for the enumeration of enterococci, was proposed by Audicana *et al*, 1995. Previous research observed low specificity (80.5%) with KEA agar (Dioisio and Borrego, 1995). The modified agar, Oxolinic acid-Aesculin-Azide agar (OAA) has improved specificity, sensitivity and recovery of enterococci in a shorter time frame due to the absence of a confirmation step. Figueras *et al*, 1998 confirmed the superiority of the modified media over standard media, such as m-Enterococcus, for routine marine and freshwater monitoring.

5.1.2 Enzyme Metabolism

Enzyme substrates can be incorporated into traditional growth media for use in membrane filtration procedures or direct addition to MPN format. Examples of such substrates are given in Section 4.1. The USEPA have recommended the incorporation of enzyme substrates to their established growth media for the improved selectivity and reduced incubation time for analysis of E. coli and enterococci. The MPN format of Colilert® and Enterolert® are recommended for drinking water analysis in the United States (Federal Register, 1989), and recreational water analysis in Australia and New Zealand. A miniaturised enzyme based MPN method has recently been proposed as an ISO method in Europe (ISO, 1998, 2000).

Enzyme substrates to be used in membrane filtration methods

5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) is a chromogenic substrate used to detect and enumerate *E. coli*. Insoluble indigo is produced within target cells, giving a greenish blue colour to colonies. The substrate was initially incorporated into direct plating methods by Ogden and Watt in 1991. Sartory and Howard, 1992, evaluated the medium, known as membrane Lactose Glucuronide Agar (m-LGA), for simultaneous *E. coli* and coliform enumeration in drinking water samples. The authors incorporated 0.05 % sodium pyruvate into the medium to aid recovery of chlorine stressed organisms. The evaluation produced m-LGA true positives for E. coli as 98.6%. False negatives of approximately 5% were thought to be due to overcrowding of the membrane suppressing β-D-glucuronide production. Beaumont, 1997, used similar procedures except for use of a broth m-LGB culture. The m-LGB was compared with m-LSB media for the enumeration of *E. coli*, total and faecal coliforms. The m-LGB gave lower recovery for *E. coli*, but higher recovery for faecal coliforms. Underestimation (high false negatives) were considered to be due to overcrowding of the membrane, substrate limitation and inhibition by acidic conditions on the membrane as a result of lactose metabolism. Despite this underestimation, the m-LGB medium was cheaper per test as a result of no confirmatory consumables required. The improved time to result, improved faecal coliform recovery and ease of interpretation were seen as advantages of the substrate (Beaumont, 1997). The USEPA modified the mTEC media commonly used for enumeration of *E. coli* and coliforms in water by incorporation of BCIG to produce 'modified mTEC' (see Table 5.3) (USEPA, 2000).

Chromagar *E. coli* and ECC (Mtech) involve incorporation of BCIG into an agar or pad for one step membrane filtration for *E. coli* alone, or *E. coli* and coliforms respectively. Chromagar ECC was found to be superior to mLSB-UA for *E. coli* sensitivity (99.1%) and specificity (96.9%) by Ho and Tam, 1997. The enzyme substrate gave 95% true positives, compared to only 66.9% by m-LSB-UA. The authors also praised the Chromagar method preparation, which was found to be 1.2 minutes quicker than the m-LSB-UA preparation. Although the substrate was more expensive, timesavings in labour and test results in addition to more reliable results proved advantageous. Chromagar *E. coli* was evaluated by Alonso *et al*, 1996. The substrate, incorporating BCIG and MUG, was compared with m-LSB and mFC. The enzyme substrate was found to be equally as sensitive to the standard methods, with 2.5% false positives and 12.4% false negatives. False negatives were reportedly higher in samples from Spain, however, which was considered to be due to physiological differences of cell expression of enzyme activity.

Table 5.3: Modified membrane filtration methods for the detection and enumeration of *E. coli* and enterococci in recreational waters (USEPA, 2000).

Method step **Name of method** **Method type**	**Enterococci** **mEI** Membrane filtration	**Escherichia coli** **modified mTEC** Membrane filtration
Media Substrate	Original mE agar plus indoxyl-β-D-glucoside	Original mTEC agar plus 5-bromo-6-chloro-3-indoyl-β-D-glucuronide
Incubation	Incubate membrane filter on mEI agar at 41 $\pm$ 0.5^{0}C for 24 hours.	Incubate membrane filter on modified mTEC agar. Close dish, invert and incubate at 35 $\pm$ 0.5^{0}C for 2 hours. Transfer plate to Whirl-Pak bag and with plate inverted place in test tube rack in a water bath at 44.5 $\pm$ 0.2 ^{0}C for 22 - 24 hours.
Presumptive positive	Colonies with a blue halo, regardless of colour.	Count number of red or magenta colonies with lens/microscope
Confirmation	At least 10 isolates in BHIB tube for 24 hrs and BHIA slant for 48 hrs at 35 $\pm$ 0.5^{0}C. Loopful from each onto BHIB for 48 hrs at 45 $\pm$ 0.5^{0}C; BEA for 48 hrs at 35 $\pm$ 0.5^{0}C and BHIB plus 6.5% NaCl for 48hrs at 35 $\pm$ 0.5 ^{0}C	At least 10 isolates added to nutrient agar plates or slants and to Trypticase Soy Broth. Incubate at 35 $\pm$ 0.5^{0}C for 24 hours. Remove growth from nutrient agar with platinum loop onto filter paper saturated with Cytochrome oxidase reagent. Remove growth from Soy Broth to Simmons Citrate agar, Tryptone Broth. Incubate at 35 + 0.5 0C for 48 hours. Add Kovacs Indole reagent to Tryptone Broth. Also add growth from Soy Broth to EC Broth fermentation tube at 44.5 $\pm$ 0.2 ^{0}C in a water bath for 24 hours.
Confirmed positive	Gram positive cocci that grow and hydrolyse aesculin on BEA (black or brown precipitate) and grow on BHIB at 45 + 0.5^{0}C and BHIB plus 6.5% NaCl at 35 $\pm$ 0.5^{0}C are enterococci	Colony turns purple within 15 seconds on Cytochrome Oxidase filter. E. coli is gas positive, indole positive, oxidase negative and does not use citrate (medium remains green).
False positive rate **False negative rate** **Reference**	6% 6.5% Messer and Dufour, 1998	<1% 4% Dufour *et al*, 1981; USEPA, 1985

The incorporation of indoxyl-β-D-glucuronide (IBDG) into isolation media has been investigated in the United States by Brenner *et al*, 1993 and Haines *et al*, 1993. 93% true positives for *E. coli* was recorded by Haines *et al*, 1993 compared with MUG media. Brenner *et al*, 1993 incorporated IBDG and MUGal into culture agar (known as MI agar) for the enumeration of *E. coli* and coliforms, and compared membrane filtration methods with m-Endo, mTEC and MUG agar. The MI recovered 1.8 times more total coliforms than the standard methods, although there was no significant difference in recovery *of E. coli*. MI agar showed 95.7% specificity for *E. coli* with 4.3% false negative and positive detections. High false positive results, found in similar media caused by *Flavobacterium* spp. and *Aeromonas* spp. presence, were avoided in the MI agar as their growth was suppressed by cefsulodin antibiotic. Interference was caused on overcrowded plates, although high dilutions of heavily contaminated samples can be prepared. The medium was not affected by turbidity in the sample. IBDG was considered cheaper than many other chromogenic and fluorogenic substrates (Brenner *et al*, 1993). Grant, 1997, evaluated m-ColiBlue24 broth in comparison with m-Endo, mTEC and MUG agar. Recovery *of E. coli* was superior with mColiBlue24 for

sensitivity, specificity, false detections and overall agreement. *Serratia* spp., *Aeromonas* spp. and *Vibrio fluvialis* caused false positives for total coliforms.

A new substrate (cyclohexenoesculetin-β-D-glucoside) has been proposed as an alternative for aesculin for the detection of enterococci (James *et al*, 1997). Hydrolysis of aesculin by β-D-glucoside produces -β-D-glucose and esculetin. The latter compound is commonly detected in tests for enterococci as brown or black complex in the presence of iron salts. This complex tends to diffuse through media rapidly (James *et al*, 1997). The advantage of CHE-β-D-glucoside is that the black complex formed with iron is insoluble and results in discrete black colonies, which may be easily differentiated and counted.

Enzyme substrates for liquid MPN methods

Enzyme substrates may also be used in a multi-well Most Probable Number format. The statistical calculation of CFU arises from the number of coloured and/or fluorescent wells showing positive reaction with the defined substrate. A number of commercial products are available utilising this format for the detection and enumeration of coliforms, *E. co*li and enterococci. IDEXX (Chalfont-St Peter, Buckinghamshire, UK) produce defined substrate media for the simultaneous detection of total coliforms and E. coli, and also for enterococci quantification. The products enable microbiological analysis within 18 - 24 hours at laboratories with minimal equipment. Little training is required to perform the procedure and sample preparation time is reduced.

IDEXX Colilert®

The Colilert® defined substrate medium was developed by Edberg *et al*, in 1988. The medium is available as a presence-absence format, or most probable number (Quanti-Tray™) format. The system enables simultaneous detection and enumeration of total coliforms and *E. coli* within 18 - 24 hours. Sample preparation is minimal, requiring direct addition of the sample (diluted to required enumeration range) to the powdered medium. The Quanti-Trays is heat-sealed and the liquid sample equally distributed throughout the tray prior to incubation. Yellow coloured wells (Quanti-Tray™) are considered a positive reaction for total coliforms, whereas a yellow well that fluoresces blue under UV light is considered confirmation of *E. coli* presence. Colilert® Quanti-Tray™ is approved for the enumeration of total coliforms in drinking water by the USEPA, and for recreational water quality monitoring in New Zealand.

Colilert® has been evaluated by a large number of authors since its commercialisation. (Fricker *et al*, 2000., Anon, 1998., Eckner, 1998., Fricker *et al*, 1996., Fricker and Fricker, 1995., Palmer *et al*, 1993., Gale and Broberg, 1993., Schets *et al*, 1993., Berger, 1991., Clark, 1991, and Covert *et al*, 1989). Although the method clearly provides a reduction in the sample preparation and analysis time for water samples, doubts have been expressed concerning the adequacy of *E. coli* enumeration. The existence of β-D-glucuronidase negative *E. coli* strains (including the enteropathogenic 0157:H7) and β-D-glucuronidase positive non-*E.coli* organisms (Figure 5.1) may impose limits on the confidence that can be placed upon the confirmed result from the 18-hour incubation.

IDEXX Colisure®

Colisure® provides a quantitative measure of total coliforms and *E. coli* in 24 - 48 hours. The substrate contains CPRG, β-D-galactopyranoside conjugated with a chlorophenol red marker. Hydrolysis of the substrate by β-galactosidase produces a magenta colour. This end point colour is much more distinctive than the yellow produced by ONPG hydrolysis. *E. coli* enumeration is the same principle as with the Colilert® Quanti-Tray™ system. Colisure® is approved by the USEPA for analysis of coliforms and *E. coli* drinking waters.

McFeters *et al*, 1995 evaluated the use of Colisure® for the detection and enumeration of total coliforms and *E. coli* in chlorine treated samples. Superior recovery of total coliforms and *E. coli* was found by Colisure, with a specificity of between 96 and 100%. False positives for *E. co*li were 4.3% and false negatives less than 2.5%. The authors found that Colisure® was superior to accepted reference methods for the detection of chlorine-stressed coliforms and *E.coli*.

IDEXX Enterolert®

Enterolert Quanti-Tray™ is an MPN method for the detection and enumeration of enterococci in 24 hours. The test uses the defined substrate 4-methylumbelliferyl-B-D-glucoside. The 97 well Quanti-Tray™ enables a statistical estimate of enterococcal CFU to be calculated depending upon the number of blue fluorescent wells under UV light.

Studies in New Zealand and the US compared Enterolert® against the standard membrane filtration method using mE agar with further incubation on EIA agar to produce positive pink-red colonies. Statistical analysis of these comparisons gave strong linear correlations of r = 0.927 (Abbott *et al*, 1998) and total r = 0.97 (r = 0.99 for marine samples; r = 0.76 for freshwater samples) (Budnick *et al*, 1996). No significant difference between the two methods was found. False positives were found to be 2.4% in the New Zealand study (Abbott *et al*, 1998) and 5.1% in the US (Budnick *et al*, 1996). False negatives were 0.3 % and 0.4% respectively. Sample results in the New Zealand study ranged from 0 – 2500 CFU per 100ml. Enterolert sensitivity and specificity were found to be 99.8% and 97% respectively.

Fricker and Fricker, 1996 evaluated the use of Enterolert® for raw, partially treated potable and effluent waters and fully treated potable water in the UK. Enterolert® was compared to the standard membrane filtration method for enterococci enumeration involving incubation on Slanetz and Bartley agar with confirmation of presumptive colonies on bile aesculin agar. A strong correlation was found between the methods of r = 0.91. The range of counts were 0 – 200 CFU per 100ml. The Enterolert® technique gave false positives on 4.5% compared to 6.2 % by standard methods.

An activity-costing analysis revealed that Enterolert® cost per sample was less overall than the MF technique. This also applies to the Colilert® and Colisure® preparations. Consumable costs were higher, but savings in labour time were significant with the Enterolert® method for reagent and sample preparation, incubation and reading of results (Abbott *et al*, 1998). Time for sample preparation before incubation for Enterolert® was estimated at 3 minutes compared to 8 – 12 minutes for membrane filtration. The method requires little skill to perform and interpretation of the results is easier and less prone to error, especially for high colony concentrations, than from a membrane filter plate. The shelf life of the reagents are 18 times longer than for batch prepared agar medium plates. The Enterolert®

trays do take up more room inside an incubator, however. One tray has the equivalent space of 8 MF plates (Abbott *et al*, 1998). Although a cost saving was identified in the New Zealand evaluation, it was pointed out that a laboratory using Enterolert® would need to purchase a Quanti-Tray^{TM} heat sealer, at a price of approximately £2,100.

96 well microtitre plate

4-methylumbelliferyl β-glucuronidase and 4-methylumbelliferyl β-glucoside are used in a 96 well microtitration plate for the enumeration of *E. coli* and enterococci respectively (Hernandez *et al*, 1991). The *E. coli* and enterococci tests are not simultaneous, however. 200 µl of sample is added to each well, and the plate incubated for 36 - 40 hours at 44^0C. Fluorescent wells at 366nm are recorded as positive for target bacteria.

Table 5.4: Summary procedure for the enumeration of *E. coli* and enterococci using the miniaturised MPN method (ISO 1998., 2000., Hernandez *et al*, 1991)

Method step **Method type**	**Enterococci** MPN	**Escherichia coli and coliforms** MPN
Media Substrate	4-metylumbelliferyl-β-D-glucoside in selective media	4-metylumbelliferyl-β-D-glucuronide in A1 broth
Preparation	Produce dilution series for each sample depending upon source water	Produce dilution series for each sample depending upon source water
Incubation	Inoculate 200µl diluted sample to each microtitre well. Incubate at 44 $\pm$ 0.5 ^{0}C for 36 - 72 hours.	Inoculate 200µl diluted sample to each microtitre well. Incubate at 44 $\pm$ 0.5 ^{0}C for 36 - 72 hours.
Confirmed positive	Wells with blue fluorescence under UV light wavelength 366 nm is positive for intestinal enterococci. Number of positive wells and sample dilution are used to produce Most Probable Number result.	Wells with fluorescence under UV light wavelength 366 nm is positive for E. coli. Number of positive wells and sample dilution are used to produce Most Probable Number result.

An evaluation of the test with marine waters by Hernandez *et al*, 1991 exhibited recovery equal to, or superior to standard MPN and membrane filtration methods. The test was found to be more specific for *E. coli* than the membrane filtration method. A 100% confirmation rate was observed using the microtitre plates for the enumeration of enterococci. Hernandez *et al*, 1995 further evaluated the method in a Europe-wide study. The method has recently been recommended by the ISO (Table 5.4), and may be incorporated into the revised Bathing Water Directive as a recommended method.

Advantages:

- Cost effective by reducing preparation time
- Results available in 18 - 24 hours
- Presence-absence, colony count or MPN formats available
- Enumeration of viable, but non-culturable organisms
- Simple and rapid sample preparation
- Non-skilled procedure
- Enumeration of turbid samples not affected by interference from suspended solids

Limitations:

- Incorporation of water-soluble substrates such as MUG is disadvantageous for the membrane filteration format, as the colour tends to diffuse from the cells, making colony differentiation problematic.
- Physiological condition of cells due to non-lethal injury may give variable non-expression of enzymes.
- A number of *E. coli* strains (including 0157:H7) do not express the β-D-glucuronide enzyme.
- False positives - non-target bacteria such as *Aeromonas hydrophila*, *Vibrio cholerae* and *Kluyvera* spp. express β-D-galactosidase or β-D-glucuronide. Interference is low at high temperatures (Davies *et al*, 1995).
- Freshwater and marine plants and algae shown to express β-D-galactosidase or β-D-glucuronide activity (Davies *et al*, 1994). Significant when high algal or plant biomass is observed in the water.
- False negatives - slow growth of target organisms as a result of non-lethal injury and selectivity of the growth medium.

Box 5.1: Evaluation of enzymatic substrate systems for the detection and enumeration of faecal indicators in surface waters.

5.1.3 Microcolony Growth

The advantages provided by the current cultivation approach (simplicity, low expense and high throughput) can be retained in a method that performs adequate analysis within one working day. The growth of a microcolony demonstrates viability and suggests that if growth were to continue, the microcolonies would form positive, macrocolonies. Microcolony growth may be detected by eye, although a laser scanner or high sensitivity camera can detect earlier microcolony growth providing a sufficient signal is generated (see Section 5.4).

A seven hour microcolony growth procedure was developed by Reasoner *et al* in 1979 for the detection of faecal coliforms by a membrane filtration procedure with a buffered lactose based medium (m-7hr FC). The test was unsatisfactory in enumerating faecal coliforms in marine water, although it was recommended for use in freshwater. Berg and Fiksdal, 1988 developed a six hour enzymatic assay for the detection of faecal coliform microcolonies on an agar medium incorporating methylumbelliferyl-β-D-galactoside. The assay achieved a sensitivity

of 1 CFU per 100ml. The results correlated well with the seven hour membrane filtration test proposed by Reasoner *et al*, 1979.

Sartory *et al*, 1999 suggested a microcolony approach for the detection of *E. coli*, which allows high throughput of samples with results within six hours using a portable, field filtration system with incubator and cultivation on modified mLSB containing 4-methylumbelliferyl β-D-glucuronide (MUG). After 5.5 hours incubation, the membrane is removed and placed on a pad soaked in CTC and incubated for a further 30 minutes. Microcolony detection is then performed with an epifluorescence microscope, with confirmation of *E. coli* by a fluorescence signal at 366nm under UV light. The authors are currently working to semi-automate this system to increase the benefits afforded by a reduction in labour time.

A six hour membrane filtration method combining Colicult (Nelis, 1999), 4-trifluoro-methylumbelliferone-β-D-glucuronidase (TFMUG) and enzyme inducers was developed by Van Poucke and Nelis, 2000a in an attempt to reduce analysis time below 4 hours. Faint *E. coli* microcolonies could be detected by a UV lamp after 6 hours, although the addition of membrane permeabilisers, such as polymyxin B, to the enzyme substrate enhanced the signal. Manual enumeration with a hand held UV lamp was considered time consuming, although CCD camera detection showed no improvement. The authors also suggested the chemiluminescent detection of microcolonies using phenylgalactose or glucuronic acid-substitued adamantyl 1,2-dioxetane derivatives as enzyme substrates for β-D-galactosidase and β-D-glucuronidase respectively in the presence of polymyxinB. Alkaline enhancer was added prior to detection of luminescence using X-ray film or a CCD camera within 5.5 hours.

The ChemScan® RDI laser scanner has been evaluated to detect and enumerate fluorescent *E. coli* microcolonies within 4 hours. A two step fluorescence assay developed by Van Poucke and Nelis, 2000b involved the cultivation of microcolonies in 3 hours followed by a 30 minute enzyme labelling step. Evaluation is described in section 5.4.2.

Microcolonies can also be detected with antibody labelling, viability dyes such as 5-cyano-2,3-ditolyl terazolium chloride (CTC), DNA binding stains such as acridine orange and DAPI and nucleic probes (see Sections 5.3.2., 5.3.1 and 5.3.3 respectively). Rodrigues and Kroll, 1988, detected microcolonies of pure culture *E. coli* labelled with acridine orange in 3 hours, whilst Binnerup *et al*, 1993 used a similar method to determine viable but non-culturable cells of *Pseudomonas fluorescens*. Pyle *et al*, 1995 detected *E. coli* 0157:H7 using monoclonal antibodies against the *E. coli* antigen with CTC viability dye. Meier *et al*, 1997 labelled enterococci microcolonies with fluorescently labelled rRNA targeted DNA probes, which enabled detection within 4 – 5 hours (discussed further in section 5.3.3). Kodikara *et al*, 1991 detected *E. coli* in food using *lux*-recombinant bacteriophage within 1 hour.

Advantages:

- Results within a working day (6 hours)
- Detection of viable, but non-culturable cells
- Simple, traditional procedure
- Procedure can be commenced in the field
- Incorporates greater specificity than standard methods by incorporating enzyme selectivity, if used.
- Further confirmation is possible as the test is non-destructive to cells
- Equivalency to standard methods established (Sartory and Howard, 1992).

Disadvantages:

- Limitations associated with membrane filtration (see Section 5.1.1)
- Particulate matter interference on membrane affecting microscopy.
- Limitations associated with inhibiting compounds in standard media (see Section 5.1.1).
- A 6 hour method may not be rapid enough to enable sampling and pollution action to be undertaken in a working day.
- Manual microscopic procedure is tedious and time-consuming. Reduced by automated, instrumental detection.
- Cost of modified medium and viability stain
- Associated limitations of enzymatic substrates, if incorporated (see Figure 5.1)

Box 5.2: Evaluation of the microcolony approach for the detection and enumeration of faecal indicators in surface water.

5.2 Pre-Enrichment Procedures

Contaminated environmental waters contain low to moderate concentrations of target bacterial indicators in a matrix of high background non-target organisms, algae and plant matter and suspended particles. This complex matrix precludes the use of many analytical techniques due to interference and suppression by non-specific entities. Membrane filtration is universally used as a pre-enrichment step prior to cultivation on a specific nutrient medium. Filtration has its limitations, (see Section 5.1.1) and captures micro-organisms on a surface which precludes techniques such as flow cytometry, for example. There are a number of non-selective, semi- selective and specific techniques to concentrate, select or amplify micro-organisms or their cellular components in order to achieve a higher sensitivity and specificity in the analytical test of choice.

5.2.1 Polymerase Chain Reaction (PCR)

PCR is utilised to amplify target nucleic acid sequences to increase the chance of detection of low numbers of organisms within a complex mixture of micro-organisms and particulate material. The replication process involves purification and extraction of cellular DNA, followed by melting of the DNA to break down double stranded DNA to single strands. Oligonucleotide primers (commonly *Taq*) hybridise to regions of the DNA flanking the target sequence using DNA polymerase in the presence of free deoxynucleotide triphosphates, resulting in duplication of the target sequence (Steffan and Atlas, 1991). This procedure is repeating over a number of cycles to exponentially increase the quantity of target DNA. High

temperature cycles, to melt the DNA, are alternated with cool cycles, which provide the optimal temperature for hybridisation. PCR amplification time is limited by the length of time required for heating and cooling cycles. A number of second generation PCR instruments have utilised rapid heating and cooling to reduce PCR cycling times. PCR can be used to amplify target nucleic acid sequences, enumerate and identify amplified products. PCR can therefore be used as a screening, quantitative or characterisation technique. PCR is reviewed for the application to environmental microbiology by Steffan and Atlas, 1991.

The tagging of primers with fluorescent labels, enzymes such as horseradish peroxidase or alkaline phosphatase, or biotins may be utilised to enable rapid detection of amplification products (Steffan and Atlas, 1991). Labels can also be incorporated into the amplified product by hybridisation on the presence of labeled deoxyribonucleotides. PCR products can be detected by agarose gel electrophoresis, southern blot or dot-plot procedures.

Multiplex PCR utilises a number of primer pairs to amplify several DNA target sequences. Simultaneous detection of *E. coli* and coliforms in environmental waters was evaluated by Bej *et al*, 1990, using multiplex PCR and gene probe technology. Primers for the *lacZ* and *lamB* genes for coliforms *and E. coli* respectively, enabled detection limits of 1 – 5 viable bacteria per 100ml of water. The *lacZ* primer amplified nucleic acids for *E. coli* and *Shigella*. Results were later shown to be statistically equivalent to standard methods and defined substrate technology, approved in the US. The *lamB* primer amplified genetic material form *E.coli*, *Salmonella* and *Shigella*, which is not specific enough for application in environmental waters.

A primer for the detection of the *uidA* gene, which forms the basis for expression of β-D-glucuronidase enzyme was also evaluated (Bej, *et al* 1991). The PCR amplification allowed the detection of β-D-glucuronidase negative strains of *E. coli*, such as the enteropathogenic 0157:H7, when the enzyme was not expressed with substrate based enzymatic assays. Fricker and Fricker, 1994 evaluated the use of PCR amplification with primers for the *lacZ* and *uidA* gene for the detection of coliform and *E.coli*, respectively, in surface waters. The methods suggested by Bej *et al*, 1990 and 1991 were used. The *lacZ* coliform primer lacked specificity, correctly identifying only 70% of the coliforms present. The *uidA E. coli* primer was more successful, identifying all *E. coli* cells, and a number of non-*E. coli* coliforms.

PCR applied to the detection of enterococci in clinical samples has been investigated by Ke *et al*, 1999, targeting the *tuf* gene. The PCR assay was found to detect most enterococcal strains with excellent sensitivity and good specificity. The authors also observed sequence variations within the genus to enable the design of specific primers to allow strain differentiation. Other target genes used to detect enterococci include *ddl*, which encodes for D-alanine-D-alanine ligase) for *E. faecium* and *E.faecalis* detection (Satake *et al*, 1996). The *PBP5* gene, which encodes for penicillin binding protein, has also been utilised by Robbi *et al*, 1990 for the detection of *E. faecalis*.

A user-friendly PCR instrument, known as the BAX® system is available from Du Pont Qualicon. The system is automated and reagents can be specifically developed for operator specifications, available as tablets to reduce preparation time. Potential operator error or cross contamination is reduced by a closed cap system of the automated PCR. A throughput of 96 samples is possible per batch amplification, completed within 4 hours, with results of screenings automatically stored in complementary software. The system has been used to

detect *E. coli* 157:H7 and *Crytptosporidium* in water, although application to faecal indicators is yet to be achieved (Durbin, C: personal communication).

Quantification of PCR products

Quantitative PCR procedures are hampered by the fact that the exponential amplification of nucleic acid sequences may also amplify even the smallest parameters that affect the efficiency of the replication (Steffan and Atlas, 1991). Quantitation of PCR products can be undertaken by adding known quantities of competitive DNA or by Most Probable Number PCR. Second generation PCR instruments utilise fluorescence to quantify amplified DNA product.

Higuchi *et al*, 1992, 1993, developed the concept of enumeration of PCR products in real time. Ethidium bromide was added in each thermal cycle, binding to double stranded DNA. Irradiation of the PCR products with UV light emitted a detectable fluorescent signal. Development of the quantitative PCR field has lead to the utilisation of DNA stains, such as SYBR Green I, and fluorogenic probes, such as Taqman®. A CCD array is commonly used to detect fluorescent emission. A number of instruments are on the market offering quantification of PCR products in real-time (GeneAMP® and ABI PRISM® series, Applied Biosystems, CA., Light Cycler, Roche diagnostics).

The portable sized GeneAMP® 2700 system allows enumeration of PCR products by using SYBR Green I dye, which binds to double stranded DNA, or the Taqman® fluorogenic probe, which anneals to complementary target sequences. Cleavage by *Taq* DNA polymerase releases a reporter dye for detectable fluorescence.

Lightcycler (Idaho Technologies, now Roche Diagnostics) have developed a quantitative PCR instrument employing SYBR Green I DNA binding dye for the enumeration of PCR products. Rapid air heating and cooling is employed to substantially reduce cycling time. PCR can be performed in less than 30 minutes (30 – 40 cycles). Glass capillaries are used for effective heat transfer permitting rapid heating and cooling, although are vulnerable to breakage. The instrument holds up to 32 samples in a carousel. Analysis is driven and controlled by software supplied by the manufacturer. Characterisation of cells by melting curve analysis detecting base pair mutations can be achieved by the addition of oligonucleotide hybridisation probes

A rapid, portable, quantitative PCR instrument has been developed at DERA, Porton Down and awaits forthcoming commercialization (Squirrrell, D; personal communication). The kit incorporates the automatic PCR procedure with fluorimeter for the final quantification of PCR products and incorporated LCD screen to view results. The development of a novel heating system and advancement in fluorescence chemistries has enabled the full PCR test to be undertaken in 10 minutes. The limit of detection is a single cell in a 50 μl sample, thus approximately 20 cells per ml. The instrument employs an electrically conducting polymer to provide the heat source that drives the PCR reaction. The DNA solution can therefore be heated and cooled more quickly than in conventional instruments, thus reduces the overall thermal cycling times.

The briefcase-size instrument can be used in the field, powered from a car battery. Sample preparation is minimal and is designed to be conducted by untrained staff. Further analysis may be performed following the initial PCR to enable typing of detected strains. Additional

primers are required for this procedure, although detection time is again approximately 10 minutes. This could enable bacterial detection, quantification and characterization in a single visit to a bathing beach, within a few hours.

Advantages:

- Rapid
- Sensitive – improves sensitivity of further analytical techniques
- Specific – amplification of target organisms only
- Simultaneous detection of multiple target organisms
- Doesn't require gene expression or cultivation - detection of strains that possess, but don't express a specific gene
- Automation
- Simple technique
- Flexible
- Isolation of nucleic acids relatively easy from water samples (Steffan and Atlas, 1991)

Limitations:

- Can not distinguish live cells from dead.
- Formation of chimeric rDNA sequences (Liesack *et al*, 1991)
- Humic acid contamination
- Amplification of parameters affecting the efficiency of the PCR

Box 5.3: Evaluation of the Polymerase Chain Reaction (PCR) for the amplification of cellular components of target bacteria in surface waters.

5.2.2 Magnetic Separation

The use of immunomagnetic separation (IMS) as an enrichment step prior to rapid detection has become increasingly popular, enabling capture of target cells from a background of non-target organisms. The separation method provides a more selective alternative to membrane filtration, and a cheap and simple alternative to PCR. A review of magnetic separation procedures is offered by Safarík *et al*, 1995.

Commercial magnetic beads are available from Dynal (Oslo, Norway) or Beckton-Dickenson (MACS). Dynal beads, 2 - 4μm diameter, conjugated with specific antibodies, are mixed with the sample within a simple tube and magnetised by a rare earth magnet. Target cells captured by the magnetically linked antibodies are held against the magnet whilst the waste sample can be poured away. MACS beads are slightly more expensive, capturing magnetically labelled target cells within a steel wool matrix. The target cells may be eluted from the column once the magnetic field is removed. Losses from the sample by washing may be significant, and interference may occur in exceptionally turbid samples (Vesey *et al*, 1994).

Magnetic separations may also be conducted using lectins as labels for faecal indicator capture (Porter *et al*, 1998). Concanavalin A (conA), labelled with fluorescein, showed considerable potential for the separation of enteric bacteria from river water. Problems with the reproducibility of lectin-labelled beads have been reported (Payne *et al*, 1992) and the recovery rate of target organisms has been shown to vary (Payne *et al*, 1992., Porter *et al*,

1998). The separation procedure is similar to that described above for the antibody labelled beads. Following magnetic separation, bound target cells may be detected by ELISA, PCR, flow cytometry, impedance or cultural techniques.

5.3 Labelling Methodologies

5.3.1 Coloured or fluorescent compounds

Stains are commonly used in microbiology to determine the viability of cells, label cellular components and differentiate between species. A suitable stain should have a high extinction coefficient (light absorbed at a given wavelength) close to the spectral line of the light source to detect even the smallest concentrations of dye within cells. The quantum yield and photostability of the dye will determine the intensity and longevity of the emitted fluorescence (Davey and Kell, 1996).

Some reagents may be membrane impermeable, therefore cells need to be fixed or permeabilised prior to staining, using ethanol, formaldehyde or glutaraldehyde. Disadvantages, however, include that viability of the cell is lost and no further cultivation for confirmation may be conducted. Permeabilised cells will also leak their contents affecting the size and intensity of the detection signal.

Table 5.5 gives examples of a wide range of the most commonly used stains in microbiology. The catalogues produced by Molecular Probes (Eugene, OR) (Haugland, 1996) display the huge diversity of stains, conjugate dyes and molecular probes available on the market today. New novel dyes from the BOBO/POPO/TOTO/YOYO dimer family based on benzothiazolium-4-pyridinium and benzoxazolium-4-pyridinium stain single and double stranded DNA and RNA. The stains are exceptionally stable and provide excellent fluorescence signal upon binding, although can only stain impermeabilised cells.

The Green Fluorescent Protein

Shimomura *et al*, 1962 first discovered the green fluorescent protein (GFP) in the jellyfish *Aequorea victoria*. The protein converts blue chemiluminescence from the aequorin photoprotein to green fluorescent light (Errampalli *et al*, 1999). The GFP cDNA was first cloned by Prashar *et al*, 1992, and expression of the cloned gene produced green fluorescence in a number of organisms, including *E. coli* (Chalfie *et al*, 1994). In recent years, the GFP has become established as a marker of gene expression and protein in cells (Tsien, 1998).

Detection of the GFP in cloned cells requires no exogenous substrate, complex medium or equipment. Basic requirements include a hand-held blue or near-UV lamp, detecting fluorescence from the labeled cells. Detection is also possible by laser scanning, flow cytometry, epifluorescent microscope, luminometer and CCD camera (Errampalli *et al*, 1999). The wild type GFP originally cloned produced insufficient fluorescence intensity for bacterial detection by microscope (Errampalli *et al*, 1997). A number of mutant strains have been developed which not only produce a highly fluorescent signal, but also have a range of excitation wavelengths, enabling multiple organism identification (Errampalli *et al*, 1999).

Advantages of the GFP include its stability, persistence at high temperatures and pH, resistance to formaldehyde (thus enabling detection of fixed cells), ease of detection, possibility for on-line detection and the lack of background GFP fluorescence. The drawback

for use of the GFP for environmental monitoring is the insertion of the GFP plasmid, the unknown effect of environmental processes on GFP expression and the lifetime of GFP fluorescence extending beyond the life of viable cells. Further advancement of the technology may enable the GFP to be used as marker for faecal indicators in the assessment of water quality.

Table 5.5: Examples of the main viability and nucleic acid stains currently used for the detection or micro-organisms (Davey and Kell, 1996; Edwards, Diaper *et al*, 1996; Haugland, 1996).

Determinand	Dye	Application	References
Nucleic Acids	Ethidium Bromide	Dye exclusion from viable bacteria, yeast and protozoa	Pinder *et a*l, 1990 Campbell *et al*, 1992
	Propidium Iodide	Dye exclusion from viable bacteria, yeast and protozoa; membrane integrity	Williams *et al*, 1998 Campbell *et al*, 1992
	Chromomycin A3	Affinity for G-C rich DNA	Chrissman and Tobey, 1990
	Olivomycin Mithramycin	Dual staining of mithramycin and EB	Steen *et al,* 1994
	DAPI	Dye exclusion from viable bacteria, yeast and protozoa; affinity for A-T rich DNA	Campbell *et al*, 1992 Otto, 1990
	Hoechest 33342/33258	% G-C content; virus detection	Sanders *et al*, 1990.
	Acridine Orange	Differential staining of double and single stranded DNA and RNA	Francisco et al, 1973
	Symmetric cyanine dimers (BOBO/POPO/ TOTO/YOYO dimer family)	Stain single and double stranded DNA and RNA	Rye *et al*, 1993
	Monometric cyanine dyes (TO-PRO series)	Dead cell stain; cell impermeable	Haugland, 1996
	SYTO series	Green fluorescent live cell stain; cell membrane permeable	Haugland, 1996
	SYBR Green series	High sensitivity gel staining	Haugland, 1996
	Ethidium homodimer	Stain single and double stranded DNA and RNA	Vesey *et al*, 1994
Protein	FITC	Identification	Miller and Quarles, 1990
	Rhodamine 101 (Texas Red)	FCM and microscopic detection – long wavelength	Haugland, 1996
Lipids	Nile Red	Poly-β-hydroxy butyrate detection	Müeller *et al*, 1995
Membrane Potential	Dihexyloxacarbocyanine	FCM viable bacteria	Diaper et al, 1992
	Rhodamine 123	FCM and microscopic detection of viable bacteria	Diaper et al, 1992 Pinder et al, 1993
Esterase Activity, Membrane Integrity	Fluorescein diacetate (FDA)	FCM and microscopic detection of viable bacteria, fungi and algae	Chranowski et al, 1984 Bercovier et al, 1987
	Carboxyfluorescein diacetate (CFDA)	FCM viable bacteria	Diaper and Edwards, 1993
	BCECF-AM	FCM viable bacteria	Diaper and Edwards, 1993
	Calcein acetoxymethyl ester	FCM viable gram positive bacteria	Diaper and Edwards, 1993
Bacterial enzyme activity, Membrane Integrity	ChemChrome B	FCM viable bacteria	Diaper and Edwards, 1993., Reynolds *et al*, 1997.
β-galactosidase activity, membrane integrity	Fluorescein di-β-D-galactopyranoside	FCM sorting of viable bacteria and yeasts	Nir *et al*, 1990
Respiratory Chain	CTC	FCM and microscopic detection of viable bacteria	Reynolds *et al,* 1997 Rodriguez *et al*, 1992
pH	BCECF		Grogan and Collins, 1990 Haugland, 1996
Fluorescent brighteners	Tinopal AN		Mason *et al*, 1995

Abbreviations used; CTC – 5-cyano-2,3-ditolyl tetrazolium chloride., BCECF – 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein.., BCECF-AM – 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester., DAPI – 4,6-diamidino-2-phenylindole., FITC – Fluorescein isothiocyaninate.

5.3.2 Immunological Labelling

An immunological response is triggered when an antibody (Ab) recognises an antigen (Ag) molecule and a binding reaction takes place. An antigen may possess a number of epitopes, or determinants, which are specifically recognised by antibodies. Antibodies can be raised against the antigen of interest using the principle of the immune response to foreign compounds. Polyclonal antibodes are raised to recognise a range of antigen epitopes. Monoclonal antibodies are more specific, recognising a single epitope, and therefore produce a more reproducible and standardised immunoassay response.

Immunoassays may be competitive or non-competitive, based on the principle of antibody occupancy (Ekins, 1997). Residual unoccupied binding sites are measured in competitive immunoassays, whereby an overabundance of antibodies is added to the non-competitive assay and the occupancy of sites is determined.

Common techniques for the labelling of antibodies include the conjugation of a fluorescent dye, magnetic or fluorescent bead, secondary enzyme-linked antibody, or radioactive label, increasing the signal for detection of fluorescence, size, magnetism or enzyme activity (Figure 5.4). It is not essential to label the primary, specific antibody as a secondary antibody binding to the initial antibody may be labelled instead, saving time and reagents (Carter and Lynch, 1996). Kfir and Genthe, 1993, present a thorough review of the application of immunological techniques to water.

There are three main types of immunological reactions:

1) Direct Labelling – Primary antibody specific to target organism is conjugated with a fluorescent tag. Shapiro, 1990 reported that up to three fluorescein molecules could be added to an antibody before its specificity is affected, therefore the fluorescence signal may not be enough for detection of the cell.

2) Indirect Labelling – Primary antibody specific to target organism is recognised and bound to be a secondary antibody conjugated with fluorescence tags. Up to 6 antibodies may bind to the primary antibody, therefore between 10 – 20 fluorescein tags may be present, producing a stronger signal for detection.

3) Biotin-Avitin system – A biotinylated primary antibody specifically bound to the target cell attracts avidin, which may be conjugated with a fluorochrome.

- **Agglutination** - binding reaction between antibody and antigen produces visible clumps; often aided by latex bead agglutination. Mainly used for clinical applications

- **Radio-Immuno Assay (RIA)** - radioisotope tagged antibodies bind to specific target antigen for detection of mainly viruses in water. Detection instrumentation expensive and considerable radioactive waste is produced.

- **Enzyme Linked Immuno-Sorbent Assay (ELISA)** - Antigens or antibodies tagged with enzyme conjugate for detection in solid phase. Enzyme - substrate reaction produces colour that may be determined visually or by optical density. The colour formed by substrate catalysis is proportional to the concentration of the antigen present. Commonly used enzymes include horseradish peroxidase, alkaline phosphatase and β-D-galactosidase. A sandwich ELISA involves the detection of target cells between two layers of antibodies. Antibodies attached to the surface of microtitre plates initially capture target cells. Secondary, enzyme-linked antibodies allow detection of these cells by signal amplification.

- **Immuno-Electron Microscopy (IEM) -** Metal conjugation with antibodies to enable detection by electron microscope. Gold or platinum is commonly utilised. Mainly used to determine antigen locality upon target cell rather than detection.

- **Immuno-Fluorescent Assay (IF)** - Covalent binding of fluorescent dyes, such as fluorescein isothiocyanate, to antibodies to allow direct detection of target cells under microscope or by fluorescence measurement. Indirect IF involves the labelling of a fluorescent secondary antibody which binds to the non-fluorescent capture antibody. Immunofluorescent labelling of intracellular antigens may require permeabilisation of cells without allowing the cell contents to leak out.

Box 5.4: Summary of the range of immunological methods available for the enumeration of faecal indicators in surface waters

The sensitivity and specificity required for water quality assessment and the abundance of non-specific matter and background organisms producing false detections limit the routine application of immunoassay to water. Reduction of non-specific binding to particulate matter in the sample may be achieved by the addition of bovine serum albumin, which saturates protein-binding ligands (Edwards *et al*, 1996). The application for the detection of coliforms, *E. coli* and enterococci is further limited by the lack of commercially available antibodies specific for the required target. The *E. coli* group contains a number of strains, each of which requires a separate antibody for specific detection. An alternative is to raise an antibody specific to an antigen expressed by the whole target group.

An all purpose antibody for the entrerobacteriacae group is available for detection of the Enterobacteriaceae Common Antigen (ECA), first identified by Kunin *et al*, 1962. Obst *et al*, 1989 used a sandwich ELISA utilising monoclonal antibodies raised against the ECA. A pre incubation step was required to increase the number of cells per sample volume, although this lead to a reduction in sensitivity due to increased stress upon the target organisms. Evaluated for the analysis of drinking waters in 1992, the ELISA produced 98% agreement with a standard lactose-peptone broth method in a 24 hour assay (Hübner *et al*, 1992). The ELISA

produced 0.8% apparent false positives and 1.2% false negatives due to slow growers. The lowest detection limit for the test was recorded as 3.9 x 10^5 cfu/ml.
Joret *et al*, 1989 raised monoclonal antibodies against an external protein on the outer membrane of *E. coli* (porin 0mpF) and against an enzyme (alkaline phosphatase) in the periplasmic space. Polyclonal antibodies against the porin membrane protein showed cross reactivity with non-*E. coli* members of the enterobacteriaceae group. Monoclonal antibodies also showed cross-reactions, and an *E. coli* porin 0mpf negative strain was observed. One monoclonal antibody raised against the alkaline phosphatase enzyme in *E. coli* showed high specificity in an indirect immunofluorescence test in under 3 hours, and despite detecting *Shigella*, recognised no other enterobacteriaceae.

An alternative is to use antibodies raised against each specific antigen for *E. coli* detection. Porter *et al*, 1993, detected *E. coli* in lake water and sewage using polyclonal antibodies raised against the K12 strain, plus a fluorescent phycoerythrin immunoglobulin G secondary antibody. Fluorescence was measured using flow cytometry and cells were then sorted. 10^3 – 10^4 *E. coli* cells were detected within a background of 10^5 – 10^6 non-target organisms. Sorting of cells, confirmed by API (bioMérieux, Basingstoke, UK) identification, successfully separated *E. coli* from non-target organisms. The *E. coli* 0157:H7 strain is commonly detected using immunoassay techniques, as commercial, specific antibodies against the target antigen are readily available.

Aydintug *et al*, 1989 observed variable cross - reactivity with monoclonal antibodies raised against *E. coli* J5 lipopolysaccharide depending upon the physical state of the target cell and the specificity of the antibody. Pollack *et al*, 1989 found similar cross-reactivity of monoclonal antibodies against the Lipid A region of *E. coli* and *Salmonella* lipopolysaccharide.

Caruso *et al*, 2000 used polyclonal antibodies (Behring Serum test Coli anti OK pools A, B and C) for *E. coli* detection in marine waters by a two-hour immuno-fluorescence assay. A detection limit of 10^2 cells $100ml^{-1}$ was achieved, with no significant difference in quantitative results when compared to standard plate methods and enzymatic assay using a MUG substrate. The authors noted that the dilution of antiserum and labelled immunoglobulin is of considerable importance to ensure a maximum fluorescent signal.

The utilisation of monoclonal antibodies may increase the specificity and provide a reliable and standardised line of antibodies raised against the specific antigen. Monoclonal antibodies raised against heat killed *E. coli* in mice, however, were found to be limited in their specificity and affinity for environmentally isolated bacteria (Kfir *et al*, 1993). Pyle *et al*, 1995 detected *E. coli* 0157:H7 using monoclonal antibodies against the *E. coli* antigen with CTC viability dye. The disadvantage of using such specific antibodies to recognise bacteria from environmental samples is that starvation and stress suffered by the cells may have caused changes in antigenicity at the cell surface, resulting in failure to detect certain cells (Table 2.2) (Edwards *et al*, 1996., Davey and Kell, 1996).

Advantages:

- Simple
- Reduces detection time
- Less labour intensive
- MAb utilisation provides a continuous, standardised antibody for reactions with high quality assurance.
- May be combined with alternative detection techniques for increased sensitivity, such as flow cytometry, laser scanning or cultural methods.

Limitations:

- No distinction between viable and non-viable cells
- Lack of antibody availability to detect the universal *E. coli* group.
- Reactions with non-specific matter
- Cross reactivity with non-target organisms
- Not all cells will express target antigen
- Slow if detection by microscope
- Poor sensitivity - cannot detect 1 organism in 100ml.
- Only small volumes can be used for detection, therefore pre filtration required.
- Suspension in water and disinfection may damage cell walls to reduce chance of antibody affinity
- Radioactive waste (RIA)
- Binding of antibody or antigen to solid surface (ELISA and RIA)
- Unstable fluorochromes (IF)
- Cost of reagents and/or detection instruments (RIA and IEM-immunogold)

Box 5.5: Evaluation of immunological labelling methods for the detection and enumeration of faecal indicators in surface water.

5.3.3 Molecular Labelling

Oligonucleotide probes may be used to detect specific DNA or RNA sequences in target micro-organisms. Probes can be labeled with fluorescent markers, biotin or reporter enzymes such as alkaline phosphatase. *In situ* hybridisation of probes with target sequences may be conducted by fluorescent or chemiluminescent detection of bound target sequences. The incorporation of current cultivation methods with gene probe technology has resulted in the development of fluorescent microcolony hybridisation (FMH).

Fluorescent *in situ* hybridisation (FISH) technique involves the use of fluorescently labeled specific nucleic acid probes, which enter morphologically intact cells and bind to complementary sequences. Hybridisation at an optimal temperature allows the amplification of the fluorescent signal to enable detection by flow cytometry or epifluorescent microscope. The intensity of the fluorescent signal is assumed to indicate the viability of the cell. Advantages of FISH include the opportunity to design specific and inexpensive probes for the target organism (Vesey *et al*, 1994). The intensity of the fluorescent signal and interference from background fluorescence remains a problem to overcome.

Chemiluminescent *in situ* hybridisation (CISH) involves the binding of enzyme tagged probes with complementary target nucleic acid sequences. Enzyme reporters commonly used are horseradish or soybean peroxidase and alkaline phosphatase. Chemi-luminescent detection of marked sequences following substrate catalysis with the enzyme reporters is possible on x-ray film or high sensitivity camera.

Detection of rRNA sequences

The detection of ribosomal RNA (rRNA) sequences in target organisms involves the detection of small sub units such as 16S rRNA and large sub units such as 23S rRNA The technique has been widely evaluated for the detection of *E. coli* and enterococci from water samples.

The commercially available Gene-Trak (Merck Eurolab Ltd., Dorset, UK) assay kit was modified by Gale and Broberg, 1991 for the MPN enumeration of *E. coli* and *Salmonella* from water samples. Following an incubation period of 48 hours, a 2.5 hour, paired, FITC labelled oligonucleotide probe assay for a 16S rRNA specific sequence was undertaken on up to 28 samples per batch. Anti-fluorescein antibody labelled with horseradish peroxidase binds to the hybridised probes, enabling chromogenic detection of marked sequences (Covert *et al*, 1995). Comparison with UK standard methods reported an 85% detection of *E.coli*, but a lack of sensitivity of the gene probe assay. Amplification of target genetic material is required to achieve detection of 1 cell per 100ml. Confirmed results within two working days does not represent a considerable improvement over standard methods, considering the wide range of more rapid, alternative technologies. Evaluation by the USEPA (Covert *et al*, 1995) where Gene-Trak was initiated from LTB gas positive tubes, showed 87% detection of environmental *E. coli*. Limitations of the pre-enrichment media may be the source of the detection error. Sensitivity of the Gene-Trak system has been reported as 5×10^5 CFU ml^{-1}(Hsu *et al*, (1991).

Meier *et al,* 1997 evaluated the use of FISH and Fluorescent Microcolony Hybridisation (FMH) for the detection of enterococci in drinking water. Desoxyoligonucleotide probes were used to target rRNA (16S and 23S). Enc131-FLUOS and Efs129-CT are recommended for the detection of the four major enterococcal species of sanitary importance; *E.faecalis, E.faecium, E.hirae* and *E. durans.* The FMH procedure incorporates traditional membrane filtration and culture methods for sample concentration and the growth of viable cells. The authors used polycarbonate membrane filters (Meier *et al*, 1997) and incubation for 4 – 20 hours. DNA staining (DAPI) was used to label all organisms present, whilst FISH targeting rRNA was used for specific detection. Fluorescent cells were detected visually by epifluorescence microscopy, although automation would increase sample throughput. The FMH assay produced results in 6 –7 hours at the quickest, although assays of contaminated water took up to 20 hours due to slow growing enterococci. An improvement to this speed may be carried out by incubation on non-selective medium. Rapid ID 32 STREP (bioMérieux, Basingstoke, UK) identification of isolates mostly revealed agreement with the rRNA method.

Advantages:

- Highly conserved sequences
- rRNA abundant in active cells (10,000 – 100,000)
- Quantitative fluorescence detection should indicate the growth rate of target cells from intensity of signal.
- Can block non-specific binding by the addition of competitive probes (Manz *et al*, 1992., Flandaca *et al*, 1999)

Disadvantages:

- Low signal intensity.
- *E. coli* and *Shigella* possess identical specific rRNA sequences.
- Tedious and difficult examination of fluorescent cells by microscope.
- Lack of accessibility to target cells rRNA.
- Slow growing cells may be difficult to detect due to low rRNA content.
- Probes vary in their binding efficiency.
- Precise quantification is impossible as cells contain varying amounts of rRNA (Emblem and Stackebrandt, 1996).
- Stressed or starved organisms low rRNA content (Table 2.2)
- RRNA may not be able to assess viability of cells (Prescott and Fricker, 1999)

Box 5.6: Evaluation of rRNA target labelling for the detection and enumeration of faecal indicators in surface water.

Peptide nucleic acid probes

Peptide nucleic acid probes are DNA mimics with an uncharged pseudo-peptide backbone, to which individual nucleobases are attached (Nielsen and Egholm, 1999). PNA probes hybridise complementary nucleic acid targets with high specificity, stability and the uncharged nature of the backbone enables rapid binding kinetics (Isacsson *et al*, 2000). PNA probes can be labelled with the same signal enhancing markers as oligonucleotide probes. PNA probes are considered superior to other oligonucleotide probes.

Prescott and Fricker, 1999 utilised PNA oligonucleotide probes for the *in situ* hybridisation of *E. coli* in water within 3 hours. A biotinylated PNA oligonucleotide probe was targeted at the V_1 region of the 16S rRNA region of *E. coli*. The biotin label was detected following hybridisation using a streptavidin-horseradish peroxidase and tyramide signal amplification (TSA) system. The authors also evaluated the use of rRNA hybridisation assays for chlorinated water samples. Chlorine killed cells were detected by the assay, but not by the defined substrate Colilert® method. This casts doubt over the use of rRNA to determine the viability of cells. The incorporation of a viability stain, such as CTC, may improve the rRNA assay ability to detect viable cells.

A PNA CISH procedure has been proposed by Stender *et al*, 2001a enabling the simultaneous detection, enumeration and identification of viable *E. coli* from wastewater within a working day. Filtered wastewater was incubated on Typtic Soy Agar at 37^0C for 5 hours prior to *in situ* hybridisation of microcolonies with soybean peroxidase labelled PNA probes, targeting

the 16S rRNA *E. coli* specific sequence. Substrate hydrolysis by the enzyme label enables chemiluminescent light spots to be detected by X-ray film of a high sensitivity camera such as the MicroStar (Millipore) (see Section 5.4.4). The Eco16S07C probe detected *E. coli*, *Shigella* and *Pseudomonas aeruginosa* (only at concentrations greater than 10^3 cfu) from water samples. *P.aeruginosa* binding could be blocked by the addition of PNA probes specifically for the organism. Non-specific background chemiluminescence was reduced by the addition of 1% yeast extract. 92 – 95% of the light spots detected later confirmed as viable colonies on extended cultivation. The simultaneous detection of *Shigella* spp. is unavoidable as the 16S rRNA sequence is identical to that of *E. coli*. A probe designed to specifically detect *E. coli* and not *Shigella* would detect other members of the enterobacteriaceae (Tsen *et al*, 1998).

A modification of the above assay has been developed by Stender *et al*, 2001b using a PNA FISH assay for the enumeration and identification of *E. coli* using an array scanner. PNA probes for the 16S rRNA *E. coli* specific sequence are labelled with Cy-3 dye, and probe microcolonies following a 5 hour incubation period. Fluorescent spots, detected by an array scanner, correlate with viable colonies grown by standard methods. Membrane filters containing greater than 100 colonies were more effectively enumerated by the scanner than by eye, due to the discrimination of neighbouring colonies.

Advantages;

- Sensitive
- Robust
- Accessibility to environments not enabled by oligonucleotides due to their hydrophobic nature (Nielson and Egholm, 1999).
- Rapid binding due to neutrally charged backbone – reduces hybridisation time to 30 minutes (Prescott and Fricker, 1999)
- Labelling as with other oligonucleotides
- Binds to DNA and RNA.
- Hybridisation independent of salt concentration (Egholm *et al*, 1993)
- Resistant to nuclease and protease attack (Demidov *et al*, 1994).

Box 5.7: Advantages of the application of Peptide Nucleic Acid (PNA) probes to the labelling of faecal indicator in surface water.

5.4 Detection Instruments

5.4.1 Flow Cytometry

Flow cytometry measures the physical (size and length) and biochemical (DNA, photosynthetic pigments and proteins) characteristics of individual cells as they pass through a sharply focused, high intensity light beam derived from an arc lamp or laser (Shapiro, 1990). To ensure that cells pass through the beam in single file, they are delivered by hydrodynamic focusing. Cells are held in suspension in a fluid medium, which is delivered to the flow cell by a delivery tube. The inlet restricts the flow to a minimum, allowing a small but continuous flow of the sample solution into the flow cell. The core stream of sample flows into the cell surrounded by a fluid stream (sheath) flowing at a higher velocity. This flow impinges upon the flow in the core, confining the fluid to the central region of the sheath, thus delivering cells individually to the detection zone (Shapiro, 1995). As cells pass through the beam, scattered light is detected by photomultipler tubes and emitted fluorescence by detectors.

Vesey *et al*, 1994, Shapiro, 1995, Davey and Kell, 1996, and an entire issue of the Journal of Microbiological Methods no. 42 (2000) provide excellent reviews of flow cytometry, the first in particular focuses on environmental water analysis.

Cells can be detected by the effect of their physical status upon light scatter, or by fluorescence naturally emitted by cellular photosynthetic pigments or other fluorescent compounds conjugated to cell markers. The instrument detects cell presence when the signal strength exceeds a threshold set by the operator, which triggers a measurement. A constant, known flow rate enables the user to obtain an absolute count of cell number per unit volume injected. This is achieved by a constant sheath flow rate, maintained by pressure or pumps (Shapiro, 1995).

Measurements of physical, structural characteristics of cells are determined by light scatter. This may be measured as the forward scatter (Forward Angle Light Scatter FALS) or side scatter (Side Angle Light Scatter SALS). Forward scatter provides information upon the size of the cells, whilst side scatter indicates the surface and internal properties of the cell (Vesey *et al*, 1994). One must be aware, however, that a number of artefacts such as cell orientation may affect the light scatter from a cell in addition to its size. Although it is assumed that measurements result from the detection of single cells, aggregation may result in a single signal from a group of cells, and thus underestimate their presence in a sample. Clumping of cells in the flow cell can be avoided by sonication prior to analysis.

Measurement of intrinsic (cellular derived) or extrinsic (surface marker or stain derived) fluorescence is achieved by detection of emission spectra from each cell. Autofluorescence of non-target cells may be due to pyridine or flavin nucleotides (mammalian cells), chlorophyll and phycobiliproteins (plants and algae). Although autofluorescence enables the detection and enumeration of algal cells without addition of fluorocromes, it may also pose severe limitations to the analysis of non-fluorescing organisms due to interference. Flow cytometry relies upon the strength of the signal for detection; therefore most target bacterial cells are labeled with fluorescent stains (section 5.3.1), antibodies (section 5.3.2), lectins or nucleic probes (section 5.3.3). Stains can be used to identify cell viability and taxonomic identification to some extent, although antibody labelling and nucleic probe and in situ hybridisation provide a more specific identification method.

A wide range of flow cytometers allow the simple detection of light scatter with one or two fluorescence parameters, up to those with three to four lasers allowing up to 10 fluorescence parameters to be measured. This allows multiparameter analysis of samples, involving gating which uses one or more signal parameters to isolate certain signal patterns characteristic of the target organisms. Data generated by cell signals are displayed on isometric plots of light scatter against fluorescence.

Multivariate analysis by classification of cells can be undertaken by unsupervised learning (cluster analysis, principal component analysis) or supervised learning (discriminant functions, genetic algorithms and neural network analysis) (Davey and Kell, 1996). Neural network analysis, where the system is trained to recognise and classify signal characteristics from previous data and apply to new data sets, has been applied to the characterisation of phytoplankton populations (Frankel *et al*, 1989, 1996., Boddy *et al*, 1994, 2000 and Wilkins *et al*, 1996, 1999). A number of neural networks have been developed, including radial and asymmetric basis function networks (RBF and ARBF), Learning Vector Quantization (LVQ) and Multilayer Perceptron (MLP). RBF and ARBF have been reported to exhibit superior performance to other methods (Wilkins *et al*, 1996, Boddy *et al*, 2000). Evaluation of ANN for phytoplankton population analysis has revealed reasonably correct identification rates. Boddy *et al*, 1994 used MLP to correctly identify 75% of 40 species of phytoplankton. 98% of 6 cyanobacterial strains were correctly identified with back propagation (Frankel *et al*, 1996). 92% of 34 species were correctly identified with ARBF (Wilkins *et al*, 1999) and 70% of 72 species of phytoplankton were also identified by ARBF (Boddy *et al*, 2000).

Neural networks offer potential real-time analysis of multiparameter data. The systems are capable of handling non-linear variations in data and are adaptable to changes in populations analysed. A number of systems are also capable of rejecting input patterns from truly novel classes not previously learned. Misclassifications may occur due to overlapping populations, although teaching input of a wide range of population parameters should reduce this risk. ANN has not yet been applied to bacterial populations. The procedure may have an important application for the automation and advancement of flow cytometry in aquatic microbiology in the future (see section 8).

Flow cytometers may be used for analysis of sample heterogeneity only or combine analysis with cell sorting to provide purified samples of target organisms. Cell sorting is achieved by the discrimination of target cells by their electrical charge, which enables deflection from charged plates into a collecting vessel. Electrical charge is assigned to cells depending upon target size or fluorescence from a marker. Experiments conducted by Porter *et al*, 1993 showed sorting to achieve greater than 95% purity from a sample that contained the target organism as only 0.32% of the total population.

A simple, analysis only flow cytometer is more likely to be of use for routine water quality monitoring, using labelling of cells for specificity of quantification. Sorting may increase the cost of the instrument and requires skilled technicians, but provides isolated target organisms for further confirmation, cell identification and characterisation. The application for a sorting flow cytometer may therefore be of an advanced or research level of microbiology. The application of flow cytometry for the detection and enumeration of indicator bacteria is limited (Porter *et al*, 1993., Davey and Kell , 2000). As the use of immunofluorescence for the E. coli group is currently limited by antibody specificity, the application of nucleic probes and in situ hybridisation is possible.

The first generation of flow cytometers were designed specifically for clinical use, by manufacturers such as Becton Dickenson (B-D) and Coulter (now Beckman Coulter) and Ortho. B-D entered the market in the mid-1970's with the FACS (Fluorescence Activated Cell Sorter) instrument. The FACS series now contains the FACS Vantage and FACScalibur. B-D also markets the bench-top LSR system. Coulter entered the market with the TPS-1, which was later replaced by the EPICS series. The EPICS Elite is a widely used system that has been modified by a number of workers to accommodate microbiological flow cytometry. Beckman Coulter also manufactures the EPICS XL and EPICS ALTRA.

These instruments are expensive, large, complex to operate and are designed for larger cell analysis, often with a sorting component. A flow cytometer for routine use at a number of laboratories across England and Wales for the Environment Agency would need to be simple to use, relatively cheap and possibly portable for field staff utilisation. The instruments mentioned above therefore will not be discussed further, although information regarding these systems and a number of others may be found from contacts listed in Appendix B. A number of portable, automated systems are discussed below which have been specifically developed for use in the microbiology field.

RBD 2000

The Rapid Bacterial Detection system (AATI, Ames, IA) is a new generation flow cytometer specifically developed for the detection and enumeration of single bacterial or yeast cells. Specifications of the instrument are shown in table 10.3, Appendix A. The RBD 2000 may be used for direct flow cytometric detection of target cells, or following pre-enrichment and/or immunomagnetic capture of target organisms prior to flow cytometry. This reduces background interference from the sample matrix and from non-target cells in highly turbid or contaminated samples. Red laser excitation is used by the instrument to reduce background signal. The sensitivity of the instrument is estimated at 5 counts per injection, which is equivalent to 100 CFU $100ml^{-1}$ (Appl. Note no. 0250). Calibration of the instrument is achieved using known concentrations of fluorescent spheres injected into the flow cytometer at appropriate intervals (App. Note no. 0220).

The instrument has been evaluated for the detection of *E. coli* 0157:H7, non-enteropathogenic *E. coli* and *Cryptosporidium*. Direct flow cytometric detection using polyclonal E. coli 0157:H7 specific antibodies produced a correlation of $r^2 = 0.985$ compared with plate counts on TSA medium (Appl. Note no. 0250). Approximate analysis time per sample is 1 - 2 hours for immunomagnetic capture and labelling plus 10 minutes for flow cytometric analysis.

Work conducted by the USEPA (Smith and Rice, 2000) detected *E. coli* 0157:H7 in drinking water, successfully utilised the RBD 2000 after pre-enrichment and immunomagnetic separation (IMS). Polyclonal antibodies specific to the enteropathogenic E. coli strain were conjugated with Protein G coated MAC beads for simultaneous capture and labelling and cyanine-5 dye to enhance the fluorescent signal. Filtered drinking water samples were pre-incubated for 3 hours prior to the IMS procedure, to enhance the signal response. USEPA and USGS work evaluating the RBD 2000 for the enumeration of non-enteropathogenic *E. coli* in surface waters has suffered setbacks due to the lack of availability of group-specific antibodies for immuno-fluorescent labelling of target organisms (Whitman R: personal communication).

The RBD 2000 has been installed at Procter & Gamble (Ohio), producing robust and reproducible results for the enumeration of *E. coli* 0157:H7, *Salmonella cholerasius*, *Pseudomonas aeruginosa* and *Klebsiella terrigena*. Immunomagnetic capture and Syto 62 DNA stain are used for the enumeration of microbial load reduction in the pharmaceutical industry (Langworthy, 2001).

Microcyte®

The Microcyte® (Optoflow, Norway) is a portable, battery operated flow cytometer designed for the enumeration of micro-organisms. The diode laser emits light at 635nm (red), which reduces interference from autofluorescence of non-target organisms and particles, whilst photodetectors detect light scattering (particle size 0.4 – 15 μm) and fluorescence at 650 – 900 nm. Flow cytometric analysis is completed in under 10 seconds at a fixed flow rate, therefore absolute cell numbers per unit volume are obtained (Davey and Kell, 2000). The detection limit is approximately 10 - 100 cells/ml, although 10^4 – 10^6 cells are required for optimal signal detection. The instrument requires little training for successful utilisation and no operator alignment is required.

Applications include the analysis of microorganisms in river and drinking water, soil, industrial starter cultures, algae, urine, viable bacteria, yeast (Davey and Kell, 2000) and mammalian cell counts (Harding *et al*, 2000). A number of DNA stains, fluorescent stains and antibodies may be used to label target organisms for detection. The specification of the instrument are shown in table 10.3, Appendix A.

The instrument has been extensively evaluated at Aberystwyth University, particularly for the application to biowarfare. Davey and Kell, 2000 used a number of fluorescent stains as a semi-specific procedure for the enumeration of lab cultured *E. coli, Bacillus subtilis* var. *niger* and *Micrococcus luteus*. Davey *et al*, 2000 evaluated the Microcyte against the more expensive, lab-based Coulter Epics Elite. Whilst the Microcyte can measure light scatter and one fluorescence signal, the Epics Elite has four lasers and is capable of multiparameter discrimination, making target cell detection highly specific.
The Microcyte provided the opportunity to screen biological from non-biological particles using fluorescent dyes. The simplicity, low cost and portability of the instrument are definite advantages when compared to the large-scale flow cytometers.

CyFlow

Partec (Denmark) have developed a similar portable, affordable flow cytometer for microbiological purposes called the CyFlow. The PC sized instrument can be powered by a car battery and is robust, requiring no operator alignment. The instrument can detect forward and side scatter simultaneously and up to 3 fluorescence parameters, providing a more specific analysis. Red, green and blue laser diodes are available for fluorescence detection. As with the Microcyte, the instrument provides absolute cell counts per fixed unit volume. The specifications for the CyFlow are shown in table 10.3 in Appendix A.

Advantages:

- High sample throughput
- Automation
- High speed analysis - thousands of cells analysed per second
- Opportunity to sort cells
- Multiparameter data acquisition and multivariate analysis

Limitations:

- Requires 100 – 1000 cells for optimal detection; limit of detection depends upon fluorescence background and signal-to-noise ratio.
- May require pre-enrichment of samples with low target organisms.
- Size of bacterial cell – low signal
- Shape of bacterial cells may produce light scattering artifacts
- Autofluorescence of non-target cells
- Abiotic particles
- Non-specific dye binding
- High cost, although diode-laser based and non-sorting instruments may be cheaper
- Skilled technicians required, particularly for sorting procedures
- Optimal room conditions – space, clean atmosphere, adequate refrigeration systems for high powered lasers
- Controls and validations required.
- Some instruments have a variable flow rates, therefore will not give absolute cell counts.
- Large size
- Aerosols created from flow cell.

Box 5.8: Evaluation of flow cytometry for the detection and enumeration of faecal indicators from surface waters

5.4.2 Laser Scanning

The ChemScan® RDI (Chemunex, France) is a laser-scanning instrument developed for the detection and enumeration of microorganisms on a membrane filter in approximately 3 minutes. Microcolonies are displayed on a screen map and may be visually confirmed by epifluorescence microscope. The ChemScan® has been developed specifically for the detection of *Cryptosporidium*, *Giardia*, coliforms or *E. co*li. A three and a half-hour assay is required for the induction and labelling of target cells prior to laser scanning. Specifications of the instrument are shown in Table 10.4, Appendix A.

The assay described by Van Poucke and Nelis, 2000b involves initial sample filtration followed by a two step method of enzymatic labelling (patented by Nelis, 1999). A three hour induction phase at 37^0C, where stressed cells are resuscitated and optimal enzyme activity is ensured, is followed by a 30 minute incubation at 0 - 1^0C on fluorescein di-β-D-glucuronide enzyme substrate. At this stage, the substrate is cleaved by β-D-glucuronidase to release the fluorescent product, which is kept within the cell cytoplasm due to the low incubation temperature (Nolan *et al*, 1988). The laser scanner detects and locates the position of all

fluorescent microcolonies within 3 minutes. Up to 20 discriminants, such as colour ratio, signal shape and light intensity are applied to distinguish background interference from genuine labelled *E. coli* fluorescence. The instrument enables automatic positioning of an epifluorescence microscope to confirm the identity of labelled cells.

The ChemScan® *E. coli* assay with pure cultures showed equivalency with TSA + BCIG agar membrane filtration method and Colilert-18 MPN format (Van Poucke and Nelis, 2000b). When environmental samples were quantified using the laser scanner and compared m-FC and Chromocult membrane filtration methods, ChemScan® showed 90.6% and 91.7% agreement respectively and superior specificity in a number of cases. 3 - 6% 'apparent' false negatives were recorded, and a number of 'apparent' false positives (13.7% and 13.5% respectively) were observed, although likely to be due to enhanced sensitivity rather than true false detection. Enumeration of chlorine injured cells by the laser scanner was highly sensitive and accurate compared with m-FC, Chromocult and non-selective tryptone lactose yeast agar. The assay time of under 4 hours would enable approximately 50 - 75 tests to be carried out within a working day (Van Poucke and Nelis, 2000b). Confirmation of cells following scanning is possible providing a suitable resuscitation medium is used as some cellular damage may be afflicted by the laser scanning procedure (Pyle, B; personal communication)

Validation tests have been performed in the pharmaceutical, food and water industries. Data reported by Johnson, 2001 at GlaxoSmithKline demonstrated very good correlation with both pure and routine water samples analysed using the ChemScan® compared with R2A membrane filtration method ($r^2 = 0.998$; $r^2 = 0.9975$ respectively). Regulatory approval from the Parental Drug Association (PDA) is awaited.

Pyle *et al*, 1995 detected *E. coli* in ground beef using immunomagnetic separation (see Section 5.2), followed by labelling with CTC (viability) and FITC (green fluorescence) stains and laser scanning using the ChemScan® RDI.

Compucyte

The Laser Scanning Cytometer (LSC) manufactured by Compucyte measures 4 colour fluorescence and light scatter; providing greater detail than a flow cytometer (position and time of measurement of cells) and faster analysis compared with an image analyser. Cells are analysed from a microscope slide. The analyser can detect and enumerate cells marked by surface colour markers or fluorescent DNA probes (FISH). Stains to determine viability, such as Propidium Iodide, can't enter live cells, but stains dead cells red. SYTO 16 used to stain nucleic acids of live cells green. The Compucyte analyser produces plots similar to those from a flow cytometer, but the instrument can take you to a view of target cells for confirmation, such as the ChemScan® RDI. Applications using the Compucyte have been recorded for Cryptosporidium, Giardia, algae and the identification and counting of faecal indicator organisms in environmental waters.

Advantages:

- Automated
- Rapid scanning
- Software driven
- Confirmation by epifluorescence microscope
- Single viable cells or microcolonies detected.

Limitations:

- Limitations inherent in enzyme assays (Figure 4.1)
- Scanning of one sample at a time
- Background interference
- False positives at low concentrations

Box 5.9: Evaluation of laser scanning for the detection and enumeration of faecal indicators in surface water

5.4.3 Flow Injection Analysis

Flow injection analysis (FIA) systems offer on-line monitoring for environmental, bioprocess control, and clinical applications. The system offers rapid, sensitive, stable, reproducible and accurate detection of target organisms (Wang *et al*, 2000) with a selection of complimentary detection methods. The flow through procedure allows optimal target organism contact with the detection system at a defined and controlled rate. Immunoassay techniques, for example, benefit from the controlled flow through of target cells to improve antibody capture efficiency.

FIA has been used in conjunction with ultrasound filtration and CCD camera detection for batch control of mammalian cell culture (Wang *et al*, 2000) and is under development for the enumeration of *E. coli* 0157:H7 in water (Wang, personal communication). Pérez *et al*, 1998, utilised FIA in conjunction with immuno-magnetic sorting and an amperometric procedure for the detection of viable *E. coli* 0157:H7. Abdel-Hamid *et al*, 1999 used a similar FIA-amperometric-immunofiltration system for the detection of *E. coli* 0157:H7. The portable immunosensor developed detected greater than 100 *E. coli* 0157:H7 cells in 30 minutes.

5.4.4 TV Camera

High sensitivity, photon-counting TV cameras are used for the detection and enumeration of naturally fluorescent and fluorescently labelled bacterial cells. Masuko *et al*, 1991 reported the use of a photon-counting camera with image analyser to detect the fluorescent *Photobacterium* over a wide field (membrane filter). This method, known as the wide-field weak emitter counting method, showed efficient detection of fluorescent cells in 50 minutes. Most bright spots (>70%) were seen after a 10 minute assay. Confirmation was possible by further cultivation of bright spots.

Miyamoto *et al*, 1998 used a camera to detect bioluminescent cells of *E. coli* from a membrane filter following a 6 hour incubation (Section 4). The authors achieved a correlation

coefficient of 0.82 for the bioluminescent method when compared with standard 48 hour plating methods in the range of 1 - 100 CFU *E. coli*. Conducting the method without prior enrichment yielded a detection limit of 2 x 10^4 CFU. A 6 hour incubation period (nutrient broth plus 0.5% NaCl) was required to detect 1 CFU E. coli on the membrane filter. Problems observed with the method included the spread of ATP molecules over the membrane, causing an inability to visualise individual light spots. The inability of stressed organisms to grow within the 6 hour incubation was also cited as a reason for the underestimation of bacteria at levels less than 10^3 CFU.

Tanaka *et al*, 1997 proposed an ATP bioluminescence test where a high intensity, CCD camera is used to detect even weak bioluminescence from living cells. Problems encountered included the suppression of bioluminescence by the pre-enrichment medium, designed to reduce non-target organism. The test was modified for the detection of coliforms by the suppression of *Pseudomonas* spp. and fungi in a 6 - 7 hour anaerobic method. *E. coli* detection was achieved by the same method in 6 hours using a modified medium.

The MicroStar (Millipore) uses a Charge Couple Device (CCD) camera for the detection and enumeration of bioluminescent or labelled microorganisms from the surface of a specialised membrane. Bacterial cells, following a brief incubation, can be detected by this system, which includes all membrane filters and reagents required. The CCD camera detects total photon emission within 2 minutes. An image intensifier discriminates against low, background fluorescence before transferring the image to computer. The computer conducts automated counting and discrimination of non-target fluorescence. Application of the MicroStar includes analysis in the pharmaceutical, food and beverage industries. The system may be applicable for use in the water industry providing a selective media is used for organism incubation, and validation reveals a sufficiently sensitive detection limit.

5.4.5 Epifluoresence Microscope

The epifluorescence microscope is used widely for the detection and enumeration, by operator eye, of fluorescent, viable bacterial colonies. The advantage of using microscopic technology for bacterial quantification includes the link between human eye and brain. Colonies can be directly seen and confirmed by microscope. This increases operator confidence in the analysis results. Alternative detection techniques, such as flow cytometry involve counting blind to the operator.

Disadvantages include the cost of the instrument, operator error due to the tedious nature of analysis and slow throughput of samples. Many microscopic fields may need to be observed to produce an accurate result. The technique may also require skilled sample preparation and observation.

The epifluorescence microscopy technique can be used to detect cells labelled by fluorescent compounds, such as immunofluorescence assays, bioluminescent assays, fluorescent nucleic probe assays and cells labelled with viability stains. The ChemScan laser-scanning instrument includes an optional epifluorescence microscope for the confirmation of cells scanned by the instrument

5.4.6 Luminometer

Luminometers are required for the detection of fluorescence from ATP bioluminescence reactions. They utilise photomultiplier tubes, photodiodes or avalanche photodiodes, available at low cost from a number of manufacturers (including Celsis, Cambridge, UK., Biotrace, Bridgend, UK., Gelman, Ann Arbor, MI., Turner Designs, Sunnyvale, CA., bioMérieux, Basingstoke, UK). A number of automated bioluminescence systems are available off-the-shelf.

The Celsis RapiScreen^{TM} (Cambridge, UK) system enables a total viable cell count within 24 - 48 hours, including an incubation step. A selective media may be used to select for enteric bacteria allowing improved specificity. The Gelman BioProbe Luminometer (Ann Arbor, MI) allows instantaneous detection of viable cells on a membrane filter pad. The instrument is portable and easy to use. The detection limit is approximately 10^2 cells and greater for *E. coli* (Osak *et al,* 2000).

6. POLLUTION SOURCE IDENTIFICATION

Investigation of faecal contamination of a bathing water body is aided by information regarding the source of the polluting matter. Technologies are available that distinguish human from animal sources, identify specific strains of faecal indicators or offer a measure of similarity between samples collected from different sites.

Initially the ratio of faecal coliform to faecal streptococci concentrations was used as an indication of human or animal pollution. A ratio of greater than 4 suggested an animal source, whereas a low ratio indicated human faecal pollution (Geldrich, 1976). This method may be inaccurate, however, due to the differential survival times of the two indicators.

The detection of micro-organisms and compounds specific to either human or animal pollution has also been suggested. Micro-organisms such as *Streptococcus bovis* (animal), *Rhodococcus coprophilus* (animal), Bifidobacteria (human), *Enterococcus faecalis* (human), *Pseudomonas aeruginosa* (human), *Bacteriodes fragilis* spp.(ratio with E. coli determines source) and *Bacteriodes fragilis* bacteriophage (human) may be monitored (Irving *et al*, 1993). Chemical indicators of faecal pollution investigated include coprostanol (human) and other faecal sterols (Leeming *et al*, 1994, 1996), uric acid (no differentiation), synthetic oestrogen hormones (human) and caffeine.

A number of reviews of microbiological techniques for the differentiation of human and animal pollution have been published (Leeming *et al*, 1994., Irving *et al*, 1993). A thorough review of the subject is not given here. The scope of this section is to update on technologies available on the market or undergoing development to differentiate strains of *E. coli* or enterococci. Characterisation of faecal indicator species may be undertaken by analysis of isolate phenotype (expression of genomes) attributes or their genetic profile (genome possession).

6.1 Phenotyping

6.1.1 Substrate Utilisation

Biochemical fingerprinting of faecal indicator strains is a commonly used technology with a wide range of products available on the market. Identification can provide a species name for a colony isolate, or a coefficient of similarity based upon other samples analysed.

A number of products are available for the identification of species by their utilisation of a variety of substrates. These include the API system (bioMérieux Basingstoke, UK), Microlog (Biolog Inc, CA), Vitek (bioMérieux, Basingstoke, UK) and BBL Crystals (BD Biosciences, New Jersey). Sample analysis is expensive with these systems, however, especially when a large number of isolates must be identified.

API strips are widely used for the identification of micro-organisms, including enterobacteriaceae and enterococcus (price per isolate is £2.60 - £4.00). The Microlog and Vitek systems are now available in automated format, providing isolate identification for approximately £4.40 and £3.45 per test.

A biochemical fingerprinting system, known as PhenePlate™, has been developed in Sweden for the analysis of isolate similarity and bacterial diversity within samples (Kühn *et al*, 1991). The tests are to be brought onto the market by Don Whitley Scientific (Shipley, UK) in late 2001. Biochemical tests per plate have been reduced to allow a higher sample throughput with less labour and materials required (Kühn and Möllby, 1993). PhenePlate™ microplates, which contain 24 (for 4 isolate tests per plate) or 11 (for 8 isolate tests per plate) biochemical tests, have been developed and evaluated for the characterisation of coliforms in environmental water samples. Test plate reading, cluster and diversity analysis and reporting is automated with complementary software. Price per plate (4 - 8 tests) is approximately £5.00.

The PhenePlate (PhP) system was used to investigate faecal pollution sources to a river by identification of coliform sub-types from factory outlets and surface waters (Kühn *et al*, 1997). Coliform subtypes from the factory outlet were not identified in surface water samples, although sub-types within the river showed similarity suggesting a diffuse pollution source. Evaluation of the PhP for sub-typing of enterococci revealed a high discriminatory power, comparing well with discrimination by ribotyping and pulsed field gel electrophoresis (see Section 6.2) (Kühn *et al,* 1995). In conclusion, the PhP plates enable highly discriminatory, automated, simple and low cost analysis of bacterial diversity and similarity within and between samples.

6.1.2 Antibiotic Resistance

Antibiotic resistance analysis of faecal coliforms and faecal streptococci has been developed to determine the sources of faecal contamination in surface waters. The selective pressure imposed upon gastrointestinal bacteria such as coliforms and enterococci by antibiotic use results in patterns of resistance to those antibiotics (Antibiotic Resistance Patterns (ARP's)), therefore enabling the phenotypic analysis of faecal contamination in water and food.

Similarities in the patterns revealed by bacterial strains reflect the use of a wide range of antibiotics in humans and animals. This has lead to reports of poor specificity and reliability with antibiotic resistance analysis. The use of Multiple Antibiotic Resistance (MAR) analysis of faecal streptococci improves upon the procedure, correctly classifying resistance patterns from streptococci isolates as originating from known or unknown sources based on a database of ARP's (Wiggins *et al,* 1999). Multivariate discriminant analysis of ARPs in surface waters in South Florida revealed the ability to differentiate animal and human sources of faecal contamination (Harwood *et al*, 2000). 62.3% faecal streptococci were correctly classified to the correct sources, whilst 63.9% faecal coliforms were correctly classified. A technique correctly identifying over 50% of isolates when five or more pollution sources are probable, is considered useful by regulatory agencies in the United States (Harwood *et al*, 2000). Correct classification of greater than 95% faecal streptococci strains from rural Virginia enabled determination of diffuse faecal pollution sources within the local watershed (Hagedorn *et al*, 1999). High correct classification rate is likely to have been achieved due to the low variability in human isolates, which were obtained from individual septic tanks (Harwood *et al*, 2000).

Limitations of this method include the expense and time consumed in creating a database of ARP's. Databases can be composed of data from a geographically limited area, or larger, depending upon the required use of the data. Antibiotic resistance patterns follow the pattern

in use of antibiotics; therefore changes in the ARP's of faecal indicators are likely to occur with the emergence of new diseases or resistant strains of micro-organism.

6.2 Genotyping

6.2.1 rep-PCR

Figure 6.1 illustrates a number of genotyping methods used to discriminated between species of micro-organisms. Repetitive Extragenic Palindromic (Rep) Polymerase Chain Reaction (Rep-PCR) is a DNA fingerprinting method involving the amplification of the highly conserved repetitive DNA sequences, by universal rep-PCR primers. The distributions of these patterns are unique to each sub-species, therefore providing considerable discriminatory power. Bacterial BarCodes Inc (Houston, Texas) provide the reagents for a rep-PCR kit and extensive database to enable identification of the PCR fingerprint. Costs per test are reasonably low (£6 - £7), with all reagents supplied with the PCR kit. Identification should be completed within 15 to 18 hours (Taylor, 2001).

6.2.2 RAPD and AFLP

Tseng *et al*, 2001, have utilised Randomly Amplified Polymorphic DNA (RAPD) markers for the differentiation of *E. coli* isolates in water from human and animal sources. PCR amplification of genome polymorphisms produces strain specific products, which can be identified by gel electrophoresis. Commercially available Ready-To-Go RAPD analysis beads (Amersham Pharmacia Biotech, Buckinghamshire, UK) enables simple, cheap (£2.40 per test excluding electrophoresis) and reproducible discrimination of *E. coli* strains with high sample throughput.

Turner *et al*, 1997 used RAPD as a molecular marker for *E. coli* strains in rural surface waters from a human origin. Multiplex PCR was used for the detection of *E. coli* (targeting the *gusA* gene) from sewage effluent and sampling sites upstream and downstream from the sewage works. Marker positive strains were found in the raw sewage, treated effluent and downstream for the STW. No marked strains were found upstream of the STW, however, despite high faecal contamination. A GeneScan system (Applied Biosystems, CA) was also used to semi-automate and provide sensitive detection of fluorescent PCR products. The study revealed the potential of this PCR technique for differentiation of human sources of faecal contamination. The RAPD marker used, however, showed limitations as it is restricted to a small sub-set of human *E. coli* strains. Further investigation is required to select other markers that can be used in conjunction to detect a wider set of human *E. coli* strains.

Amplified Fragment Length Polymorphism (AFLP) is a similar PCR fingerprinting procedure amplifying DNA restriction fragments, although to all knowledge, has not yet been applied to the differentiation of bacteria in the aquatic environment.

6.2.3 Pulsed Field Gel Electrophoresis (PFGE)

Pulsed Field Gel Electrophoresis (PFGE) has been applied to the differentiation of *E. coli* strains in Florida (Parveen, 1998). A pulsing electric field drives the separation of DNA fragments and production of a fingerprint for each isolate. PFGE profiles did not correlate well with actual faecal sources for *E. coli* in estuarine water (Parveen, 1998). Multiple Antibiotic Resistance and ribotyping studies correlated well with pollution origin.

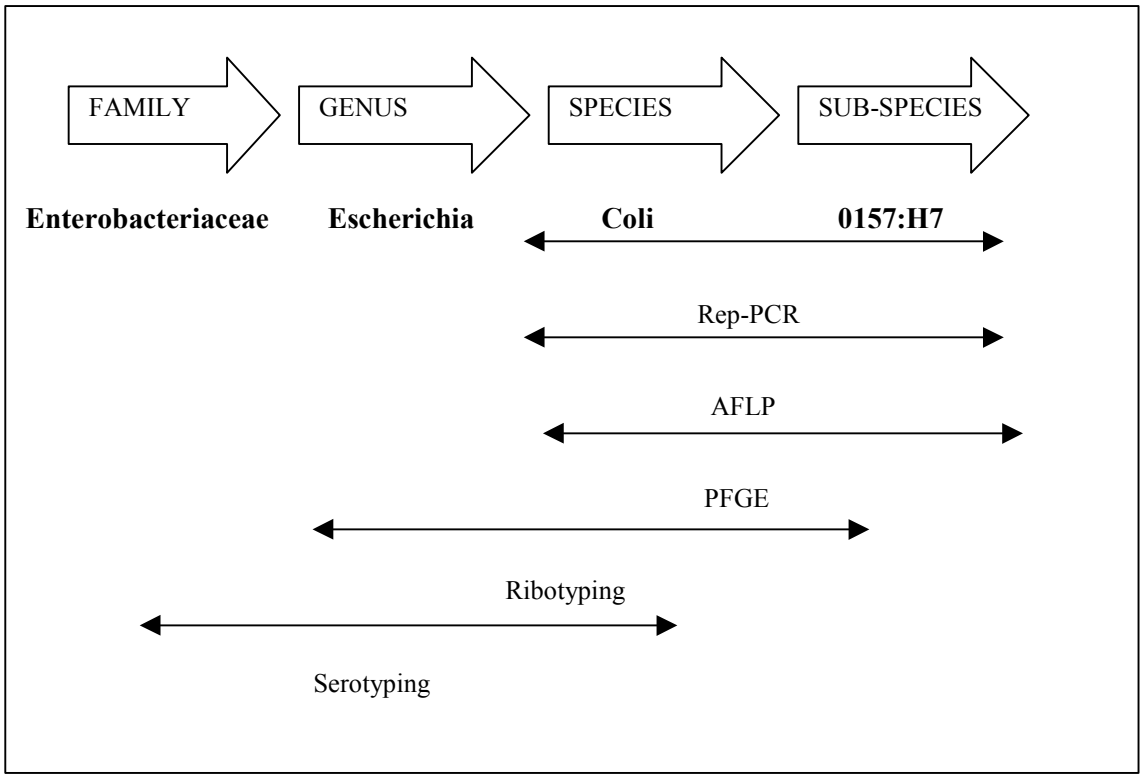

Figure 6.1: Diagram to illustrate the discrimination power of a number of typing methods

6.2.4 Ribotyping

Ribotyping produces a genetic fingerprint of an isolate organism, which can be used to identify strains of micro-organisms for pollution tracking and source identification. The RiboPrinter® is a commercial instrument from Du Pont Qualicon, which enables genetic fingerprinting using restriction enzymes within 8 hours, in a user-friendly, automated format. Fingerprints produced are compared with a large database (greater than 3000 entries) to accurately identify the sub-species isolated. Information regarding the similarity of an analysed isolate to a previous isolate, may aid in pollution tracking. The system was developed mainly for the pharmaceutical industry, with applications including tracking of the spoilage organism *Staphylococcus epidermis*, from a sterility failure to a technicians glove, saving $20,000 in batch release on time (McLean, 2001). The instrument also has applications for environmental monitoring, particularly for enterococci (Durbin, C: personal communication). The instrument is expensive, however, costing in the range of £80 - £95,000.

7 TECHNOLOGY ON THE HORIZON AND RECOMMENDATIONS FOR FUTURE RESEARCH

The USEPA stated in the recent publication of the BEACH Action Plan (EPA, 1999) the aim to develop a 'colour change' dipstick for the detection of pollution in water. The technology is available for such a technique for the detection of high concentrations of micro-organisms, such as *E. coli* 0157:H7, which may be rapidly labelled by specific antibodies on a biosensor chip (see Section 4.3). DNA chips, utilising microarrays of immobilised DNA or oligonucleotide probes specific to nucleic acid sequences in target organisms, are undergoing development and optimisation but would be limited to the detection of serious faecal pollution (10^6 CFU and greater). PCR enrichment for moderately polluted waters is therefore required prior to detection, but reduces the practicality for use in the field.

Increased automation of microbiological analyses is sought to reduce sample preparation and therefore labour and time required. Automation therefore offers substantial cost benefits. Current plating techniques can be automated with the use of robotics for media pouring and sample filtration. Colony counting is automated by the use of commercially available plate counters. Automation of alternative, rapid technologies is also continuing, including automatic samplers (Colilert 3000), automated PCR (Bax® system), impedance (Bactometer and RABIT) and flow cytometers (Microcyte® and RBD 2000). Automation and portability of detection instruments is likely to advance over the next few years.

Flow cytometric analyses of water samples is a highly advantageous technology, providing signal intensity from target organisms is sufficient. Evaluation of a specific and fluorescently labelled oligonucleotide probe, such as a PNA probe for rRNA target sequence detection for use with flow cytometry is recommended. The continuing development of artificial neural networks for the detection and classification of target organisms may provide a further step towards the goal of automated sample analysis. Currently applied to the detection of phytoplankton in marine waters, neural network analysis utilises the learning capability of powerful software with multiparameter analysis of samples to achieve rapid and specific analysis of samples (see Section 5.4.1). Classification of species in water by this method may provide simultaneous enumeration of a number of indicators per sample and identification below species level to aid pollution tracking.

Further development of the AK bioluminescence assay (see Section 4.2) with possible specification for target enterobacteriaceae is a promising candidate for a rapid, field-based assay, requiring only a dipstick and cheap, portable luminometer. A proof-of-concept study should be undertaken to evaluate the efficacy of such a technique.

8 CONCLUSIONS

Technological advancements are gathering speed to produce the ultimate rapid microbiological analysis system. The ideal technique for the enumeration of faecal indicators from water should be portable, simple to prepare and use, provide results within a working day, provide analysis equal to, or superior to that of standard methods and provide cost benefits through time and labour savings. The current market for rapid analysis technologies provides a number of instruments which may fit into one or more of these categories, although convey drawbacks which restrict their application to monitoring of the aquatic environment.

The detection and enumeration of faecal indicators in water is critically limited by a number of factors, including the low abundance of indicators in faecal contaminated waters (generally 10^0 - 10^4 CFU $100ml^{-1}$) and the complex matrix which water provides as a sample medium. Non-specific micro-organisms, plant and algal matter and suspended sediments impose a medium from which extraction of target organisms such as coliforms, *E. coli* and enterococci is relatively difficult. Membrane filtration has largely solved the problem of sample concentration, although imposes a number of limitations upon subsequent organism growth. Detection of indicators from within the matrix itself poses problems with the introduction of false organism detection. Enzymatic assays are particularly affected by the incidence of β-D-galactosidase and β-D-glucuronidase positive non-specific organisms and *E. coli* strains that do not express the specific enzyme.

When intestinal organisms enter the aquatic environment, they are affected to a varying degree by processes and organisms within those surroundings. Stress and starvation of bacterial cells can lead to their lack of reproductive activity, resulting in underestimation in cultivation-based methods, lack of surface antigens for the recognition of specific antibodies and a reduction in rRNA, reducing the efficiency of nucleic acid-based technologies. The detection of viable faecal indicators is of importance as a general guide to the level of faecal pathogens present in the water, particularly following disinfection treatment. Technologies which detect living and dead cells, such as PCR, immunological and nucleic acid assays, require additional viability markers to offer a suitable use for the monitoring of environmental waters. Multiparameter analysis by flow cytometry in this case would allow detection of cells marked for specificity and viability simultaneously.

The enrichment of bacterial presence in water samples is a pre-requisite for analysis by the majority of techniques reviewed here. Cultivation is the method of choice, although imposes a substantial time delay before obtaining a result. Pre-enrichment may be conducted by rapid techniques such as PCR or sorting and purification by immuno-magnetic beads (see Section 5.2). The utilisation of portable, enrichment instruments will enable samplers to present samples with a high target bacteria-to-low water volume at the laboratory, which may be analysed in a considerably shorter time frame.

The introduction of bathing water screening, particularly at sites expected to exhibit poor water quality, should form the first step of implementation in the spirit of the revised Bathing Water Directive. There are currently two commercially available products that may be applied to monitoring water quality; the Colifast Analyser and Colilert 3000 (see Section 4.1). Neither instrument will offer an instantaneous result, but may indicate a pollution problem of considerable magnitude within a short time frame. Both instruments convey the drawbacks of

enzymatic assays involving non-specific organism expression of enzymes and non-expression by certain *E. coli* strains, and the growth rate of organisms limit the time to detection. The optimisation of biosensors and developments in the field of bioluminescence assays may grant instantaneous detection.

Quantification of faecal indicators in water is currently achieved by membrane filtration and cultivation upon selective agar, producing results within 1 - 3 days. The revision of the Bathing Water Directive may proceed to suggest or impose a standard EU-wide method, in a similar manner as the revised Drinking Water Directive. Standard methods are most likely to be those proposed by the ISO, as described in Section 5.1. The most sensible alternative method for quantitative measurement would be the detection of microcolonies. This method embraces the familiar method of plate cultivation with the specificity of nucleic acid stains or probes and the sensitivity of detection by instrument (camera or laser scanner) rather than by the human eye. Detection of 1 cell per unit volume of sample has been shown to be achieved by this method within a working day (Sartory *et al*, 2000., Van Poucke and Nelis, 2000b). Enzyme substrates incorporated into an MPN format, such as the Colilert® or the 96 well microtitre plate (Section 5.1.2), may also be suitable alternatives. The procedures are simple and cost effective although do not enable analysis within one working day.

A number of alternative methods offer a range of advantages. Flow cytometry represents a sensitive technique for the rapid detection of single cells, particularly those that are not cultivable, but remain viable. Flow cytometry is a versatile technique, which may also be applied to the detection and identification of algae. The development of learned multivariate analysis (neural networks) may provide a powerful tool for rapid detection and identification of cells. The abundance of organism in the sample, however, limits the intensity of the detection signal, necessitating a pre-enrichment step. Impedance technology provides a cheap and automated method for the detection of faecal indicators, although does not represent a significant time saving compared to standard methods.

Identification and characterisation of faecal indicators provides an optional step in the framework for bathing water management. Investigation of pollution inputs to bathing waters are improved by the ability to either ascertain the source of contamination to a human or animal origin, or pinpoint contamination by the characterisation of different samples, assessing similarity of microbial composition. Many investigations do not require information regarding the sub-species composition of a sample. The tracking of pollution may only require the identification of a point or diffuse source, from a number of possibilities, by its high similarity to the polluted water body at a beach. The PhenePlate system represents a cheap, simple and effective method for high throughput screening of isolates to assess the similarity of target bacterial strain. More expensive genetic techniques for the detection of faecal indicators may be required for complex investigations where below-species level identification is required, providing information regarding the likely source of contamination. Antibiotic resistance patterns (ARP) may be of potential use in small catchments where a database of ARP's from each potential source of contamination can be built up for rapid use when a pollution incident occurs.

Suitable technologies for the three stages of recreational water management described in the hierarchy (Section 3) may therefore be summarised. Rapid enzyme assays provide a relatively cheap and simple method for screening of surface waters, although the development of ATP bioluminescence assays may provide an ultra-rapid, specific 'dipstick' that may be used in the field. A reduction in the analysis time of standard methods may be afforded by the

detection of microcolonies within a 4 - 6 hours. Microcolonies, by their growth alone suggest viability, and in conjunction with specific fluorescent or coloured markers enable sensitive detection by camera or laser scanner.

The characterisation of isolates from cultivation procedures can be conducted using phenotypic tests, based upon biochemical indicators of substrate utilisation. The PhenePlate® method represents a cheap, high throughput phenotypic method for investigations that do not require the specific identification of isolates for pollution tracking. Ribotyping or advanced PCR methods provide superior genetic fingerprinting techniques for sub-species identification of isolates, but at considerable expense.

The drive to optimise many of the technologies presented here continues, and in time we may see the development of bioluminescent dipsticks, specific antibodies for the *E. coli* group, robotics and artificial intelligence within the laboratory and biosensors with the sensitivity to detect a few bacterial cells in water. It is therefore essential to remain open-minded to the possibility of highly sensitive and specific techniques that will revolutionise microbiological analysis within the water industry.

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List of Acronyms

ATP	Adenalyse TriPhosphate
AK	Adenylate Kinase
BWD	Bathing Water Directive (76/160/EEC)
CFU	Colony Forming Unit
CCD	Charge Couple Device
DERA	Defence, Evaluation and Research Agency
DETR	Department of the Environment, Transport and Regions
DNA	Deoxyribonucleic Acid
EA	Environment Agency
EU	European Union
FC	Faecal Coliforms
ISO	International Organisation for Standards
MAFF	Ministry of Agriculture, Fisheries and Food.
MF	Membrane Filtration
MPN	Most Probable Number
P/A	Presence - Absence format
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
TC	Total Coliforms
US EPA	United States Environmental Protection Agency
USGS	United States Geological Survey
VBNC	Viable But Non-Culturable
WHO	World Health Organisation

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Appendix A. Comparison of two automated systems for rapid coliform screening using enzymatic activity as a measure of colony forming unit

Parameter	Colifast Analyser	Colilert 3000
Manufacturer	Colifast (distributed by Oi Analytical in US/Canada)	Seres (IDEXX)
Target Organism	TC and FC (*E. coli* and Enterococci optional)	Total Coliforms and *E. coli*
Technology	Fluorescent activity of methyl umbelliferae	Colour development from orthonitrophenol (ONP) release
Sample Preparation	Membrane Filtration or Direct Addition	Direct Addition
Speed of Analysis	TC: <1hr 1000 - 10,000 cfu FC: <1hr 10,000 cfu 1-2hr 100 cfu 1-1.5 hr 1000 cfu 5-7 hr 10 cfu 2.5-5.5 hr 100 cfu 6-11 hr 1 cfu 6.5 - 8 hr 10 cfu 7.5 - 13 hr 1 cfu	TC: 7 - 10 hrs 1000 cfu 10 - 13 hrs100 cfu
Sample frequency	Every hour	Every 6 hours (can be modified)
Instrument Cost	£20,000 - £30,000	£17,350 + £50/month cleaning solutions + £80/yr tubings
Reagent cost per sample	£1 - £1.50	£5.95
Quantitative or P/A	Presence/Absence or semi-quantitative (MPN method).	Presence/Absence
Volume of Sample	variable	100ml
Lower Detection Limit	1 cfu (<100 cfu for FS-E medium)	1 cfu/100ml
Upper Detection Limit	>10,000 cfu	>100,000 cfu
Specificity	False negatives at concentrations <10 cfu- "late growers" 7.8% false negatives; 2.9 % false positives. High specificity with FS-E medium.	No published work to date
Agreement with Standard methods	Total Coliforms: Faecal Coliforms:	No published work to date
Risk of cross contamination	Tubings rinsed with 0.5M HCl and 0.1% Triton X-100 between each sample.	Sterilisation achieved using Sodium hypochlorite and Chlorhydric acid
Viability	VBNC cells detected	VBNC cells detected
Repeatable		No published work to date
Reproducible	Not yet tested	No published work to date
Applicability to Different Environments	Tests completed in Wales (UK)(FW; E; M), Gothenburg (Sweden) (FW), Ostfold (Norway) (FW; M), Oslo (Norway) (FW; E), Boerum County (Norway) (FW; M)	France Colilert medium used world-wide with approval from US EPA for Total Coliforms

Parameter of	Colifast Analyser	Colilert 3000
Size (H x D x W) in cm	Incubator - 50.8 x 52.1 x 41.9 Analyser - 28.9 x 45.7 x 47.3	60 x 40 x 70
Weight	Incubator - 31 lbs; Analyser - 24 lbs	35 kg
Automation	Semi-automated - sample analysis only. Software package co-ordinated process control, data handling and reporting. Remote warning system to internet/mobiles/pagers.	Fully automated - sampling (semi-automation optional), analysis and reporting. Fully remote operation with data transmission to pagers etc.. offered
Availability of Reagents	Granular Colifast-6 medium available from manufacturers - reconstituted at site of analysis. TC additive, media developer and calibrant also available.	Reagents available from manufacturer
Shelf-life of reagents	Liquid Colifast-6 medium can be stored for up to 14 days. Up to 5 days with TC additive. Calibrant liquid can be stored for up to 28 days	6 months
Training of Analyst	Colifast offer 1 week traning course in Norway prior to sale of instrument	Training course and service contract offered by manufacturer.
Software		RS232 and 4-20 mA output
Interpretation of Results	Results in fluorescence activity of MU (ppb) WinFLOW v 4.1 software produces peak plot, calibration plot, peak results table and calibration results table.	Screen display of results. May be linked to a laptop computer.
Ease of Performance	Automated analysis and presentation of results	Fully automated
Sample Handling	Simple and safe sample preparation	Fully automated
Instrument Through-Put	Up to 100 samples per day	4 samples per day automated. Carousel contains 35 reagent vials.
Practicality	Sampling undertaken by hand - bankside, beach or boat. Instrument can be based at the smallest of labs/offices. Not practically portable, however.	Fully automated. Requires secure, covered storage and direct access to sampling point. Problematic for marine samples.
QA/QC	Baseline & calibration samples	Base line set by instrument (auto zero) for each analysis. Alarms indicate if base line calibration cannot be achieved.
Disposal of Finished products/wastes	Autoclave and Incinerate	Waste products from monitor stored in a container (10L/week volume). Manufacturer can organise licensed waste collection and disposal.

Note: Abbreviations: FW = Freshwater., E = Estuarine., M = Marine.

Appendix B. Specifications for instruments for the measurement of impedance.

Parameter	Bactometer	RABIT	BacTrac 4100/4110
Manufacturer	**BioMerieux**	**Don Whitley Scientific**	**Sy - Lab**
Methods accommodated	Direct Impedance only	Direct and Indirect	Impedance Splitting
Test signals	Capacitance and conductance	Conductance and capacitance	Conductance and capacitance
Target Organism	Faecal streptococci and *E. coli* /FC (Irving and Easton, 1994)	Coliforms and enterococci (manufacturer) Faecal streptococci and *E. coli* /FC (Irving and Easton, 1994)	*E. coli* (Timms *et al,* 1996)
Speed of Analysis	6 - 14 hrs coliforms (manufacturer) 9.5-13.6 hrs FS (43-360 $cfuml^{-1}$) 18-33 hrs FS (1-13 cfu ml^{-1})	Up to 7 hrs for $>10^3$ coliforms 5.5 - 6.5 hrs (direct) FC sediments 6.2 - 7 hrs (indirect) FC sediments 14.5-20.1 hrs FS (43-360 cfu ml^{-1}) 19 - 28 hrs FS (1-13 cfu ml^{-1})	15 hours - 1 *E. coli* CFU
Measurement Level	Quantitative/Semi-quantitative Option of MPN technique	Quantitative/Semi-quantitative	Quantitative/Semi-quantitative
Sensitivity	-	1 CFU per sample volume	1 CFU per sample volume
Reproducibility	-	Yes. Bolton, 1990 - Whitley Impedance broth with glucose.	No data
No. of samples per batch	16 (up to 512 samples)	32 (16 modules = 512)	40 (4100) and 20 (4110)
Volume of sample	2ml	2 - 10 ml	10 and 20 ml direct up to 10ml indirect
Interpretation of results	-	Traffic light pass/fail system. Print out of time to detection (x) against conductance (y). Print out of equivalent cfu from calibration curve.	Traffic light pass/fail system. Print out of time to detection (x) against % impedance change (y) Print out of equivalent cfu from calibration curve.
Availability of reagents	Suitable media available from manufacurer	All reagents and consumables available from manufacturer. Suitable media can also be purchased from other manufacturers	Usual laboratory suppliers of standard media
Sample Preparation	May require pre-incubation for low	Membrane filtration or direct addition	Membrane filtration or direct addition

	level sensitivities		
Parameter	**Bactometer**	**RABIT**	**BacTrac 4100/4110**
Electrode Type	Stainless steel	Stainless steel	Stainless steel
Electrode Position	Bottom	Bottom	Bottom
Incubator	Fan convected air incubator	Heated aluminium blocks	Heated aluminium blocks
Suitable Media	Optimised Biomeriux media eg.	Whitley Impedance Broth Other media eg. Colifast medium, Colilert etc..	Large range incl. Standard media eg. MLSB
Frequency of measurements	Every 6 minutes	Every 6 minutes	Every 10 minutes
Size **(W x H x D)**	-	400 x 600 x 400 mm	4100 - 300 x 230 x 580 mm 4110 - 400 x 235 x 540 mm
Weight	-	Incubator - 35 kg 75.5 kg (total module)	4100 - 21 kg 4110 - 31 kg
Cost	£309.59 for 40 disposable cells Media approximately £1 per bottle	Full system with 2 modules = £40,000 Approximate cost per sample - depending on media = 15p £8-10 for replacement electrodes. Full Service contract £2,500 per year	-
Software	Computer and printer supplied by manufacturer	RABIT for WINDOWS™ Computer supplied by manufacturer Remote access & network/pager /mobile/modem connections	Any up to date computer configuration with inkjet/LaserJet printer
Training	-	Regular courses based in Shipley, West Yorkshire	No distribution in UK
Customer Service & support	-	Full support & can modify individual modules for customers needs	No distribution in UK - support unlikely
Disposal	Autoclave and dispose or incinerate	Autoclave and dispose or incinerate	Autoclave and dispose or incinerate
Power supply	-	230V ± 10% 15 min power backup in event of power failure	115 V or 230 V

Appendix C. Evaluation of portable flow cytometers developed specifically for the enumeration of micro-organisms.

Parameter of Interest	RBD 2000	Microcyte	CyFlow
Manufacturer/ Distributor	Advanced Analytical Technologies Inc (AATI)	Optoflow	Partec
Analyser	✓	✓	✓
Cell Sorter	✗	✗	✗
Laser Excitation	635 nm	635 nm	635 nm (red) 532nm(green) 490 nm (blue)
No. Colour parameters	1	1	Up to 5
Light Source	Red diode laser	15 nW laser diode	Red diode laser (635nm), Green diode (532nm) or Blue diode (490nm)
Automation	✗	✗	✗
Functions	Fluorescence	Light Scatter/ Fluorescence	Forward scatter; side scatter & 3 fluorescence channels
Cell Sizes	bacterial	0.5 - 10um (scatter) or 1000 - 100000 dye molecules per cell (fluorescence)	0.2 um - 200 um
Sensitivity	1 cfu	<10 cfu	Data not available at present
Lower Detection Limit	1 - 10 cfu	10 cfu per 1ml	
Upper Detection Limit	>100,000 cfu	> 10,000,000	
Cost	£47,000	£21,000	
Analysis time	8 mins 100µl 12 mins 500µl	10 secs - 3 minutes	10,000 events/ second
Sample volumes	100µl, 250µl, 500µl, 1000µl	1, 10 or100 µl	Up to 1500 µl; 200µl for precision counting
Size	18 (W) x 20 (D) x 19 (H) inches	430 x 160 x 330 mm	43 (L) x 37 (W) x 16 (H) cm
Weight	65 Lbs	12 kg	-
Training	Training available in US, UK distributor sought	Training in UK available	-
Interpretation of results	NT workstation analyser incorporated	Software included in purchase	FloMax® software using any laptop computer
Service support	✓ No UK distributer	✓ UK	✓ 1-3 yr service contracts
Sample Through-put	7 - 8 per hour	Up to 20 per hour	-
Power supply	-	12V car battery	12 V Car battery

Appendix D. Specifications of the ChemScan RDI and Compucyte laser scanning instruments

Parameter of Interest	ChemScan RDI	Compucyte
Manufacturer	Chemunex (France)	Compucyte (Cambridge, MA, United States)
Speed of Analysis (excluding sample preparation)	3 minutes	<10 minutes
Scanning wavelengths	3 scanning wavelengths	4 scanning wavelengths plus measurement of light scatter
Sensitivity	1 CFU per sample volume	-
Specificity	3 - 6% False Negatives; 13.7% 'apparent' false positives (Van Poucke and Nelis, 2000b)	-
Viability	Detects viable cells only	Detects viable cells depending upon staining
Applicability in different environments	Widespread use in water and pharamceutical industry; water - potable and surface	-
Measurement Level	Quantitative	Quantitative
Instrument Cost	£75,000 - £100,000	
Cost per sample		
Sample throughput	12 - 15 per hour average	
Size (W x D x H) in cm	-	91.4 x 71.1 x 45.7
Weight	-	118 kg
Availability of reagents	-	Use a selection of widely available viability and specific stains
UK Distributor	UK distributor in Cambridge	UK Distributor: GRI Ltd, Braintree, Essex
Ease of performance	Non complex procedure; no special training required	-
Interpretation of results	Results ploted on a scan map; instrument moves epifluorescence microcope for cell confirmation	Instrument moves epifluorescence microcope for cell confirmation
Training and service provision	Fast Track Validation Package avaialable to cover customer service and training requirements	-

Appendix E. List of world wide web links of interest

General Interest

American Society for Microbiology - www.asmusa.org
American Water Works Association - www.awwa.org
Centre for Research into Environment and Health (CREH) www.creh.org
DERA - www.dera.gov.uk
EU - europa.eu.int/water/index-en.html
Foundation for Water Research - FWR - www.fwr.org
Health Canada - www.hc-sc.gc.ca/ehp/ehd/index.html
Journal of Microbiological Methods - Special Issue on Single Cell Analysis, 2000 www.elsevier.com/inca/publications/store/5/0/6/0/3/4/
Microbial Underground - www.medmicro.mds.qmw.ac.uk/underground/microbio.html
Molecular Biology Jump Station - www.highveld.com/micro.html
Molecular Probes - www.probes.com
PNA Jump Station - www.horizonpress.com/gateway/pna.html
SEPA - www.sepa.org.uk
SNIFFER - www.sniffer.org.uk
Society for General Microbiology - www.sgm.ac.uk
US EPA - www.epa.gov
Microbiology - www.epa.gov/nerlcwww/index.html or www.epa.gov/microbes
BEACH program - www.epa.gov/ost/beaches
USGS - www.usgs.gov
Water Research Centre - www.wrcplc.co.uk
World Health Organisation - www.who.int

Commercial Contacts

Enzymatic Substrates

IDEXX - www.idexx.com
SERES - www.seres-france.com
Colifast - www.colifast.no
Chromagar - www.chromagar.com

Bioluminescence

Celsis - www.celsis.com
Biotrace - www.biotrace.com

Impedance

RABIT - www.dwscientific.co.uk
Bactometer - www.biomerieux.fr
BacTrac - www.sylab.com

PCR

GeneAmp - www.appliedbiosystems.com
Lightcycler - www.biochem.roche.com/lightcycler

Probes

Molecular Probes - www.probes.com
Boston Probes - www.bostonprobes.com

Flow Cytometry

Becton Dickenson - www.bd.com
Beckman Coulter - www.coulter.com
AATI (RBD 2000) - www.aati-us.com
Partec - www.partec.de
Compucyte - www.CompuCyte.com

Chemunex - www.chemunex.com

Typing

Du Pont Qualicon - www.qualiconweb.com
PhenePlate - www.phplate.se
Amersham Pharmacia BioTech - www.apbiotech.com

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