Work Package 3

Task 3B:

Desk studies on feasibility of horizontal standard rapid methods for detection of *E. coli* (including *E. coli* O157) and *Salmonella*

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SUMMARY

The emerging methods becoming available for the rapid detection and enumeration of *E. coli* (including *E. coli* O157) and *Salmonella* in sludges, soil and treated biowastes have been evaluated with a view to possible future standardisation. The main methods that are available for the detection and enumeration of *E. coli* (including *E. coli* O157) and *Salmonella* have been developed largely for analysis of food and water and can be broadly divided into four groups. Proprietary Quantitray® technology, equivalent to the 5-tube most probable number (MPN) technique, employing disposable plastic trays for enumeration of *E. coli* and *Salmonella*. Immunological, involving a short or overnight pre-enrichment of the target organism followed by specific detection of cellular antigen in either a lateral flow device or following immunomagnetic capture. Molecular, involving PCR amplification of target DNA sequences from low numbers of cells, or preferably following a short pre-enrichment of the organism to amplify numbers and demonstrate viability prior to molecular detection. Physico-chemical, involving techniques such as measurement of impedance changes during enrichment and growth in appropriate media. The merits of each are described, in relation to their suitability for use with sludge, soil and biowastes. Since the majority of agar and MPN broth techniques take between 24-96 hours for identification and enumeration, we define "rapid" as any technique that detects, and if possible, enumerates the target organism in under 24 hours.

All of the methods described have strengths and weaknesses, dependent on not only the Regulators' types of requirements for sludge, soil and biowaste analysis but also their sensitivity, specificity, speed and cost. It is unlikely therefore that there can be only one methodology applicable to both *E. coli* (and *E. coli* O157) and *Salmonella* detection. Nevertheless, it is considered feasible to formulate horizontal standards to cover rapid analysis of *E. coli* and *Salmonella* in sludge, soil, soil improvers, growing media, and biowaste. None of the methods have been extensively evaluated for sewage sludge, soils or biowastes. As such, there is an urgent need for their modification and evaluation as part of the next phase of the Project Horizontal.

HORIZONTAL : WP3 – Hygienic parameters / Desk Studies 3 to 6

0. GENERAL INTRODUCTION

This report is one of the five Project Horizontal desk study reports that attempt to assess hygienic parameters (WP 3), which may be needed to assure the sanitation of sludges, soils, soil improvers, growing media and biowastes. The five desk study reports highlight draft potential methods for the hygienic parameters likely to be included in future sludge and biowaste Directives :

Desk study report 3A "Feasibility of horizontal standards for *Escherichia coli* and *Salmonella* in sludges, soils, soil improvers, growing media, and biowastes" deals with *Escherichia coli* and *Salmonella* spp.,

Desk study report 3B "Rapid Methods for detection of *E. coli* (including *E. coli* O157) and *Salmonella* in sludges, soils, soil improvers, growing media, and biowastes", deals with rapid methods available for *E. coli* (including *E. coli* O157) and *Salmonella*,

Desk study report 4 "Feasibility of Horizontal standard methods for detection of *Clostridium perfringens* and *Enterococci* in sludges, soils, soil improvers, growing media, and biowastes" deals with *Clostridium perfringens* and *Enterococci*,

Desk study report 5 "Feasibility of horizontal standards for the enumeration of viable helminth ova in sludges, soils, soil improvers, growing media, and biowastes", deals with viable helminth ova,

Desk study report 6 "Literature review on levels of pathogens and their abatement in sludges, soils, soil improvers, growing media, and biowastes" deals with the occurrence of pathogens and their abatement.

It is not only necessary to make methods available to determine specific micro-organisms, but also to provide a detailed protocol for sampling heterogeneous matrices such as sludges, soils, soil improvers, growing media, and biowastes to obtain fit for purpose results. Results are needed for validating plant performance (percentage pathogen reduction) and end product specification in terms of hygienic microbiological parameters (e.g. EU 2000). This will include co- and pre-normative research, including consideration of carrying out method validation for complementary bacterial indicators (e.g. *Enterococci* and *Clostridium perfringens*) and viable helminth ova (cestodes and nematodes). For parameters likely to be included in future Directives (i.e. *E. coli* and *Salmonella* spp.), the selected methods will be assessed in large Europe-wide interlaboratory trials involving many European countries. For other parameters, there is a need to develop preliminary standards in order to carry out the relevant research. In the Sludge and Biowaste draft directives (EU 2000 and EU 2001), *E. coli* and S*almonella* are specifically mentioned. This leads to the logical choice to start the work on these organisms as one of the parameters in phase 1 of project Horizontal. For the other parameters, Project Horizontal desk studies 4 and 5 of WP3 to prepare draft potential protocols for CEN and ISO discussion are also being prepared.

1. INTRODUCTION

1.1 Scope of the Report

This Desk Study Report 3B deals with an assessment of rapid methods for detection of *Escherichia coli* (including *E. coli* O157) and *Salmonella* in sludges, soils, soil improvers, growing media, and biowastes. Desk Study Report 3A reviews horizontal standards for *E. coli* and *Salmonella* spp. in sludges and treated biowastes**,** dealing extensively with general aspects of sampling requirements and performance of inter-laboratory trials. Therefore these aspects will not be considered here.

Appendix 1 quotes some relevant sections of the draft sludge and biowaste directives to give some indication of the type of measurements and microbiological species that are to be covered and the likely analysis limits of detection and specified log reductions to be assessed. The methods reviewed are mainly adapted from standard methods for the examination of food and water. In order to ensure that fit for purpose microbiological results can be obtained for a wide range of sludge and treated biowaste materials, Project Horizontal has to carry out co-normative research work to develop suitable international standards. The validation of these standards will be achieved by carrying out interlaboratory trial(s) with participation of a number of experienced European laboratories. Such validation requires application of the draft standards to a wide range of real sludge and biosolid samples.

1.2 General introduction to indicator and pathogen detection

Land application is recognised as the Best Practicable Environmental Option for using sewage sludge. Indeed, this practice has been strengthened in EU maritime countries following the ban on sea dumping of sewage sludge which came into force in the UK in 1998 as a result of the EC Urban Waste Water Treatment Directive (1991; incorporated into UK law 1994). Sewage sludge and agricultural wastes are recycled to soil with the aim of improving soil condition and fertility (Nicholson *et al.*., 2000). However, untreated sewage sludge may contain a range of microorganisms pathogenic to man, including bacteria (e.g. *Salmonella*, *Campylobacter*, *Listeria* and various strains of *E. coli*), virus particles (e.g. Polio and Hepatitis), protozoa (e.g. *Cryptosporidium*) and other intestinal parasites (e.g. Helminths) (Table 1).

Without suitable treatment, there is potential for pathogens present in sludge recycled to land to wash into adjacent surface waters, contaminate crops (fresh produce is of particular concern), or spread directly to man or farm and domestic animals using the land. Application of human sewage sludge currently represents a small proportion of waste applied to agricultural land; by far the greatest amounts are contributed by a variety of animal wastes including compost, faecal slurries, poultry litter, *etc*. (Table 2). The availability of suitable detection methods to facilitate understanding of the survival of potential human and animal pathogens in these wastes, before and after storage and treatment, and after application to land, is critical to delivering safe agricultural products to the market place.

Table 1 Principle microorganisms in organic wastes pathogenic to animals or man

Genera	**Principal species or strain**
Escherichia	*coli* (O157:H7; O26, O103, O111, O145)

Salmonella	*enteritidis* PT4, *typhimurium* DT104
Shigella	*dysenteriae, sonnei*
Campylobacter	*jejuni, coli*
Listeria	*monocytogenes*
Cryptosporidium	*parvum* (genotype 1 and 2)
Giardia	*lamblia*
Cyclospora	*cayetanensis*
Ascaris	
Enterovirus	
HeptitisA	

Table 2 Sludge, agricultural and food processing (e.g. abattoir) wastes recycled to land in the UK

Waste	**Amount recycled p.a.**
Sewage sludge	>1m tonne post 1998
Cattle slurry	40m tonne
Cattle manure	31m tonne
Pig manure	5.4m tonne
Poultry manure	4.5m tonne
Abattoir waste	0.4m tonne

EU Council Directive 86/278/EEC regulates sewage sludge applications to agricultural land throughout the EU. The Directive has been implemented in the UK by statutory instrument The Sludge (Use in Agriculture) Regulations 1989 (SI 1989, No 1263, as amended SI 880 1990; (HMSO, 1990), regarding the application of raw and treated sewage sludge to agricultural land. The regulations are supported by the DOE Code of Practice for Agricultural Use of Sewage Sludge 1989 (revised 1996; DOE). These strictly limit how the sewage sludge is to be applied, under what conditions and to which crops, reducing as far as possible occasional contact with animals and man. The Code of Practice was developed in the 1970s from the data available at the time, and before the emergence of highly infectious pathogens such as *E. coli* O157. The regulations or guidelines for sludge treatment vary world-wide and are under review. In the USA, the EPA Part 503 Sludge Regulation stipulates that sludge is acceptable for use on land if it contains less than 1000 faecal coliforms per g dry weight of sludge and less than 3 salmonellae, 1 virus and 1 viable helminth per 4 g. In the UK, the regulations are shortly to be amended following consultation of the UK Draft Sludge (Use in Agriculture) (Amendment) (England and Wales) 2002 (Appendix 1). These stringent regulations have highlighted the need

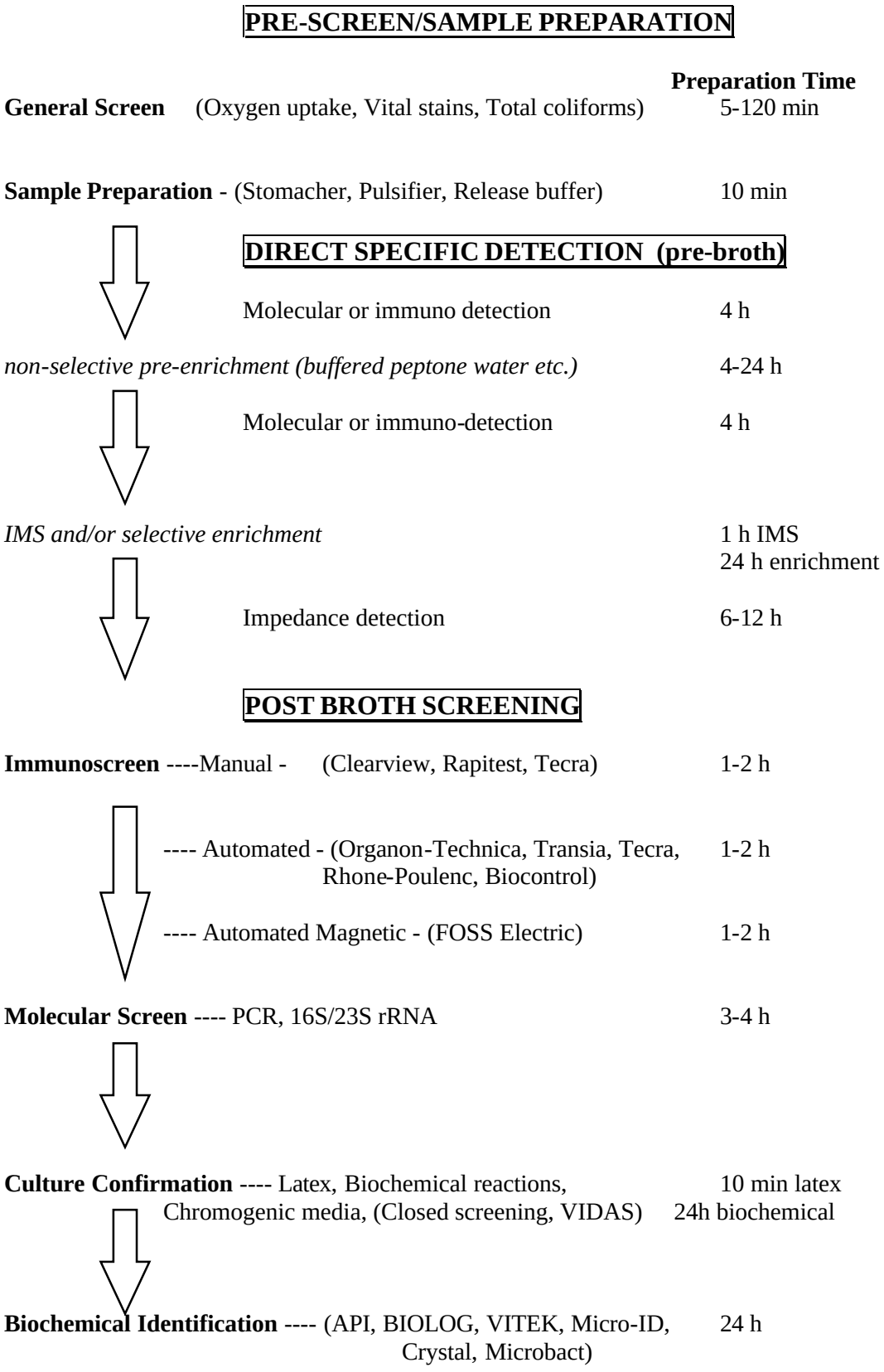
for better methods of detection for each type of indicator or pathogenic microorganism in sludge.

The challenge for analysts is to adapt some of the current or emerging methods, developed for water and food, or develop new methods, to provide an evaluation of the pathogen load in treated sludge, soil and biowastes. This problem has been exacerbated by the realisation that some pathogens may be sub-lethally damaged, but possibly still capable of causing disease, due to the storage and treatment processes and might be missed using conventional culture recovery techniques. Accordingly, some research has focused on appropriate resuscitation techniques involving either presence/absence or the newer quantitative methodologies.

Many thousands of untreated and treated waste samples recycled to land will need to be tested each year. This will demand mass screening techniques, ideally at low cost. Due to the low infectious dose of some of the pathogens, the rapid detection methods must be sensitive and specific, yet robust for the complex matrices involved. Different approaches are required for the type of monitoring required; for example, the Regulator may require accurate quantification of pathogen numbers for assessing process control of treatment methods, using specific culture, immunological and molecular techniques. However, where the efficiency of specific treatment effect has to be demonstrated (e.g. a 6-$\log_{10}$ kill) then the culture techniques are mandatory because the molecular approaches cannot truly discriminate between viable and dead cells. Conversely, it may be more suitable for an operator applying sludge or biowastes to land to rapidly screen them for their biological activity and stability, using respiration, enzyme assay, dipsticks or most probable number techniques. Recent developments in some of these areas will be presented in relation to detection of *E. coli* and *Salmonella*. Since the majority of agar and MPN broth techniques take between 24-96 hours for identification and enumeration, we define "rapid" as any technique that detects, and if possible, enumerates the target organism in under 24 hours.

The methods to be employed will ultimately depend on time, cost, and throughput of samples per day. The flow diagram (Figure 1) illustrates the possible integration of various isolation/detection technologies into protocols for each bacterial indicator or pathogen. The most promising of these technologies will be discussed in this report.

Figure 1 Integration of possible isolation and detection techniques



1.3 *E. coli* introduction

The use of indicator organisms, in particular the coliform group, as a means of assessing the potential presence of pathogens in environmental samples that could result in the contamination of human water and food supplies has been pivotal in protecting public health.

Coliform bacteria belong to the family *Enterobacteriaceae* and are found in many environmental samples. Several species are found in large numbers in the digestive tract of mammals and these are used as indicators of faecal pollution. They are Gram negative, non-spore forming rods which are capable of aerobic and facultative aerobic growth in the presence of bile salts or other surface active agents which are inhibitory to many other microorganisms. Coliforms usually ferment lactose at 37°C (or 44°C for faecal coliforms) within 48 hours as they possess β-galactosidase. They are oxidase negative.

E. coli is a coliform bacterium which occurs in the faeces of all mammals, often in very high numbers up to 10^9 per gram faeces. It is the only biotype of *Enterobacteriaceae* that is exclusively faecal in origin. It is believed to be the primary indicator of faecal contamination of food and water supplies. Most strains of *E. coli* are capable of fermenting lactose or mannitol at 44°C (usually within 24 hours) and produces indole from tryptophan. These strains possess the enzyme β-glucuronidase which can be detected with specific fluorogenic and chromogenic substrates (discussed later).

Detection of *E. coli* is relatively simple and easy and, as a result of this, there are many methods developed for the isolation of this faecal coliform from environmental samples and foodstuffs. This is due in part to their large numbers in the environment and availability of suitable selective media for this organism. There may be occasions where *E. coli* is not a suitable indicator of microbial contamination e.g. disinfected surface waters which may contain resistant and potentially viable microorganisms, in particular protozoan oocysts of the Coccidian parasite, *Cryptosporidium parvum*.

Some strains of *E. coli* can cause serious diarrhoeal disease. Several of these have been defined by the possession of virulence factors, in particular those strains that produce Verocytotoxin(s) (VTEC), also known as Shigatoxin-producing *E. coli* (STEC). One of the most important of these VTEC is serogroup O157, whilst other important serotypes such as O111 and O45 are now recognised to be increasingly associated with foodborne outbreaks. Infection with *E. coli* O157 results in clinical symptoms ranging from mild diarrhoea and fever to severe life threatening bloody diarrhoea and cramps. In 10-15% of cases this can also result in haemolytic uraemic syndrome and kidney failure. The young, old and immunocompromised are mostly at risk. Alarmingly, the infectious dose is very low, and ingestion of as few as 10 organisms are believed to result in disease. The majority of outbreaks have resulted from faecal contamination of contaminated food, especially undercooked meat, and water supplies following a failure in the treatment process. For example, the Walkerton outbreak in Ontario in 2000 caused 2000 people to become infected and 7 died. Untreated and private water supplies also carry a higher risk of *E. coli* O157 contamination (Keevil, 2000), possibly due to inadequate chlorination or UV disinfection (like other strains of *E. coli* the O157 serogroup is susceptible to concentrations of chlorine used routinely in water treatment plants). This, coupled with survival data suggesting this organism can survive several months in untreated faeces and soil (Maule *et al*., 1997, Keevil *et al*., 1999) demonstrate the huge importance of the availability of reliable and rapid detection methods.

VTEC may not be isolated or recognised by normal analytical methods for *E. coli*, for example VTEC are generally •-glucuronidase negative and may be difficult to grow above 37°C; they therefore may need specific methods for detection. The general consensus of opinion is, however, that if *E. coli* is found to be present in environmental samples than it can be assumed that VTEC could also be present. Conversely, if no *E. coli* is detected using approved methods, then it is unlikely that viable VTEC will be present.

1.4 *Salmonella* introduction

Members of the genus *Salmonella* are responsible for an estimated 2 to 4 million cases of the foodborne gastroenteritis and several hundred deaths per year in the United States. Similar statistics are found in Europe. There are over 2000 serotypes that infect a range of species including man, birds, domestic animals and rodents. A few are specific to one or two hosts causing severe systemic disease e.g. infection with *S. typhi* and *S. paratyphi* results in typhoid and paratyphoid fever, respectively, in man; *S. gallinorum* and *S. pullorum* are important pathogens in chickens. The human diseases are of particular importance in areas of the world with poor sanitation and no access to clean drinking water. Fortunately, a vaccine is available for these parts of the world.

Many serotypes can colonise the gut without invasion to give typical symptoms of food poisoning e.g. diarrhoea, and it is these serotypes, in particular *S. typhimurium* and *S. enteritidis*, that are responsible for the majority of *Salmonella* infections in western societies. These usually result from human or animal faecal contamination of the food and water chain. Main routes are through contaminated poultry, eggs, shellfish and raw meat, especially pork. Of concern is the emergence of multiple antibiotic resistant strains: for example, *Salmonella typhimurium* DT 104 is primarily associated with cattle but it has spread to a range of food animals, including pigs, sheep and poultry. There are now often no effective antimicrobials for use in cases where a veterinary surgeon considers it necessary to treat a clinical infection by *Salmonella typhimurium* DT 104. This pathogen is anticipated to become more widespread in the environment and hence throughout the food chain. Infection through the routes described is of huge commercial importance, involving morbidity and mortality, days off work and expensive hospitalisation and product recall costs.

2. EXISTING STANDARDS OR DRAFT STANDARDS

2.1 Current methods for the detection of *E. coli* and *E. coli* O157

There are many methods that have been devised for the detection and enumeration of *E. coli* in water, food and sludge. Some of these are detailed in Table 3 and primarily involve multiple tube fermentation (MTF) for turbid water samples and complex matrices, and membrane filtration (MF) for low turbidity drinking waters. Both of these methods use selective media which usually involve the detection of acid and gas production as a result of fermentation of lactose which is present in the medium along with inhibitory bile salts. Acid production is detected by inclusion of indicators e.g. phenol red or bromocresol purple in the medium. Often incubation is at a raised temperature (44^{o}C) for *E. coli* selection from other *Enterobacteriaceae*. Many selective media incorporate chromogenic and/or fluorogenic substrates which are cleaved by enzymes present in the microorganism. The two main enzymes utilised for the detection of *E. coli* are β-galactosidase and β-glucuronidase. Ortho-nitrophenyl-β-D-galactopyranoside (ONPG) chromogenic substrate is cleaved by β-galactosidase and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG or X-Glu) chromogenic substrate is cleaved by β-glucuronidase to give a yellow and blue/green end product respectively of sufficient intensity to colour the developing colonies. 4-methylumbelliferyl-β-D-glucuronide (MUG) is a fluorogenic substrate which is metabolised also by β-glucuronidase to result in colonies that emit a blue fluorescence visible under UV light. VTEC O157 does not possess β-glucuronidase and does not ferment sorbitol. Selection for this serotype is often made by distinguishing sorbitol fermenting and non-fermenting colonies on agar containing sorbitol following an immunomagnetic separation (IMS) isolation step. Following initial growth on selective media further incubations and confirmation tests (e.g. oxidase, indole production and/or latex agglutination) are usually required.

The Petrifilm system (3M) consists small, sealable, ready to use agar plates, containing bile salts, lactose and Violet red, with a grid to facilitate counting. They are approved for use by AFNOR and AOAC for use in food analysis.

There is a bewildering array of commercially available methods for the detection of *E. coli* and *E. coli* O157 (Table 3). These methods incorporate many technologies including immunological, molecular and impedimetric techniques.

Traditional culture methods are usually time consuming due to lengthy incubations followed by confirmation tests. Continuing improvements in media formulations may increase specificity but not necessarily reduce the time taken to get a result. Advances in molecular methods appear promising for the development of rapid methods if there are sufficient numbers of organisms of interest in the test sample and specificity can remain high.

A brief description of the advantages and disadvantages of each methodology is discussed:

2.2 Multiple tube fermentation (MTF)

This is a simple method to perform and expresses results as a most probable number (MPN) of bacteria in the original samples based on statistical analysis of the number of tubes/dilutions demonstrating growth and fermentation of specific substances in the media. It does not give true enumeration. However Seidler *et al.* (1998) demonstrated non-specific fermentation of lactose which resulted in inaccurate MPN values. There is also a risk of inhibition from contaminating organisms. This test is useful for complex and turbid matrices that cannot be filtered. For a simple presence/ absence test single incubations are performed (Standing Committee of Analysts water method E, Gray *et al.*, 2002 method for food).

Table 3 Isolation and detection of *E. coli* and *E. coli* O157

Origin of method	Matrix method devised for:	Sample size	Summary of method	Selective medium used:Presence of enzyme and use of defined substrate:	Comments/ validated etc.
SCA (EA) UK 2002:	All for water:				
Method A Coliforms and *E. coli* 2 membrane filtration	Drinking water (low turibity)	2 samples: one for coliforms and one for E. coli	2 filter onto pads soaked with MLSB: one 30^{oC} 4 h coliforms; one 44^{oC} 14h E.coli Subcut LPW 37^{oC}, 6 h followed by further 24 h in LPW (acid prodn.)and TW(Indole test) for E. coli	MLSB:Lactose and phenyl red indicator. Yellow cols indicate acidity (Enz. β-galactosidase)	NOTE:Some *Bacillus* and *Staphlococcus* spp. also give yellow colonies
Method B Single membrane filtration (Coliforms and *E. coli*)	Drinking water – including source waters of moderate turbidity		Filtration of appropriate diln, fliter onto MLGA ,4 h 30^{oC},14h 37^{oC} Green cols E.coli.Confirm with acid from lactose,-ve oxidase,indole from tryptophan.	MLGA with BCIG chromogenic substrate .E.coli cols. Green (Enz. β-glucuronidase)	
Method C multiple tube MPN (Coliforms and *E. coli*)	Water- high turbidity.Recommended for sludges		Diln in Ringers or MMG,37^{oC},18-24 h(acidity) Leave until 48h for development of growth. Confirm subcut MA incubate 440C E.coli	MMG contains lactose and bromocresol purple indicator.Yellow cols. Indicate acidity. (Enz. β-galactosidase)	
Method D Defined substrate MPN (Colilert® IDEXX)	Low tubidity water	Diln in pouches of defined media 18-22 h 37^{oC}		Detection of enz. β-galactosidase (chromogenic substrate ONPG- yellow cols) and β-glucuronidase(fluorogenic substrate MUG)	*Aeromonas* may give false positives
Method E Presence/absence	Simple modified MPN with only 1 tube 100mls water sample + 100ml medium	MMG broth Detect acid production after 24 h and growth after 48 h			
Method F O157 selective enrichment and IMS	Moderate turbidity Not suitable for high turbidity (filter blockage)	Membrane filtration (poss with Filter Aid) Selective enrichment in BPW or mTSB 24 h.Followed by IMS (x2:6 h, 24 h) Beads onto CT-SMAC Confirmation-serological/biochemical tests		CT-SMAC contains sorbitol and indicator Neutral Red. Positive cols do **not** ferment sorbitol and are colourless/pale orange.	May get inhibition by contaminating bacteria. There are some atypical strains O157 that do ferment sorbitol. Does not detect serotypes other than O157 that produce verocytotoxin.
EA/SCA 2003 update? Part 2 *E. coli* + O157					

Origin of method	Matrix method devised for:	Sample size	Summary of method	Selective medium used:Presence of enzyme and use of defined substrate:	Comments/ validated etc.
PHLS SOP UK W2 *E. coli* + coliforms	Water	100 ml or 200ml bottled	Filter onto 2:1 for coliforms, onto MLSB soaked pad 30^{oC} 4 h 37^{oC} 15 h; 1 for E.coli 30^{oC} 4 h 44^{oC} 15h. Confirm sucut oxidase and indole	MLSB:Lactose and phenyl red indicator. Yellow cols indicate acidity (Enz. β-galactosidase)	
W16 O157 only	Water	1000 ml	Filter mTSB filter aid (turbid samples)= IMS then sel agar CT-SMAC Confirm latex agg and biochemical	CT-SMAC contains sorbitol and indicator Neutral Red. Positive cols do **not** ferment sorbitol and are colourless/pale orange.	
W18 *E. coli* and O157 IDEXX	Water	100ml , not for bottled water	IDEXX colilert 18 quantitray mpn colour + fluorescence 22 hs to result	Detection of enz. β-galactosidase (chromogenic substrate ONPG-yellow cols) and β-glucuronidase(fluorogenic substrate MUG)	Method same as EA Method D
F17 *E. coli*	Food	25 g	Stomach in mTSB then incubate41.5^{oC} 22h IMS- sel agar (CT-SMAC) Confirm latex agg	CT-SMAC contains sorbitol and indicator Neutral Red. Positive cols do **not** ferment sorbitol and are colourless/pale orange.	
F20 *E. coli* direct enumeration	Food	1/10 diln	Straight onto BCIG plate 30^{oC} 4h 44^{oC} 18h Do not need to confirm	MLGA with BCIG chromogenic substrate .E.coli cols. Green/blue (Enz. β-glucuronidase)	Limit 10 cfu per gram cook/chill and ready to eat foods
? O157	Food				
F23 Enterobacteriaceae colony count	Food	1/10 diln	Diln + broth VRBGA 15 min 45^{oC} + med + solid overlay 37^{oC} 24 h Purple cols oxidase, ferm. tests		
D4 Coliforms	Dairy	1 ml?	1ml 3 petri+ molten VRBA agar 30^{oC} 24h red cols No confirm		
D5 Coliforms + presumptive *E. coli*	Dairy	1/10 diln?	Dilns + LTMUG broth 30^{oC} 48h Gas prodn. Subcut + NaOH observe under UV	Detection of β-glucuronidase(fluorogenic substrate MUG) Positive blue fluorescence under UV	
Soton/CAMR *E. coli* + O157	Sludge, blood, abbatoir waste	25 g	Stomacher PBS Filter dilns through glass fibre Enrichment TSB (+ novobiocin) Chromagar for O157 Latex agg	MLGA with BCIG chromogenic substrate .E.coli cols. Green/blue (Enz. β-glucuronidase)	

Origin of method	Matrix method devised for:	Sample size	Summary of method	Selective medium used:Presence of enzyme and use of defined substrate:	Comments/ validated etc.
CEN TC 308 WG1 TG5 E.coli Membrane filtration for the characterisation and quantification of sludges Part 1 sept 2002 draft	sludge	10g	Stomacher Filter dilns MLGA plate 30^{oC} 4 h 44^{oC} 14 h give green cols API	MLGA with BCIG chromogenic substrate .E.coli cols. Green/blue (Enz. β-glucuronidase)	
E.coli Miniturised MPN in liquid medium Detection and enumeration of *E. coli* from sewage sludge Part 2 Based on ISO 9308-3	sludge	10g dry matter	Make up to 100ml Homogenise in tryptone salt diluent Dilns Microplate 36 h 44^{oC}	Detection of β-glucuronidase(fluorogenic substrate MUG) Positive blue fluorescence under UV	Refer to ISO 9308-3 Inter lab trials sampling errors
E.coli Macromethod MPN liquid Detection and enumeration ofE.coli from sewage sludge Part 3 draft	Sludge	20 g wet weight	Make up to 200ml Homogenise salt buffer Shake 20 h 4-8 ^{oC} Dilns + MUG fluorescent lauryl sulphate broth 40 h 44^{oC} + NaOH Observe under UV	Detection of β-glucuronidase(fluorogenic substrate MUG) Positive blue fluorescence under UV	
Thames UK/CEN Coliforms and *E. coli*	Sludge		IDEXX + Colilert® 18 Homogenise Dilns UV	Detection of enz. β-galactosidase (chromogenic substrate ONPG- yellow cols) and β-glucuronidase(fluorogenic substrate MUG)	*Aeromonas* may give false positives

Origin of method	Matrix method devised for:	Sample size	Summary of method	Selective medium used:Presence of enzyme and use of defined substrate:	Comments/ validated etc.
ISO 9308-1 *E. coli* Std (coliforms) and rapid (*E. coli*)	Water	100ml or 250 ml bottled	STD (coliform) membrane filtration onto 2 filters,one for standard test,one for rapid test Lactose-TTC med then oxidase indole confirmation tests Takes 2-3 days Rapid; Membrane filtration Casein trytic digest med (TSA) + bile 4 h Then TBA med 20 h Whole membrane onto filter pads with indole see red cols under UV	Lactose fermentation.:Positive yellow cols Production of indole from tryptophan in the medium (red cols)	21 h
ISO 9308-2 Liquid enrichment method					
ISO 9308-3 Miniturised method (MPN) for the detection of E.coli in surface and waste water					

Media abbreviations:

MLSB Membrane lauryl sulphate broth
LPW Lactose peptone water
MA MacConkey agar
NA Nurient agar
TW Tryptone water
TSB Tryptone soya broth(and modified TSB)
MLGA Membrane lactose glucuronide agar with chromogenic substrate BCIG
BCIG 5-bromo-4-chloro-3-indolyl-β-D-glucuronide chromogenic substrate
ONPG ortho-nitrophenyl-β-D-galactopyranoside chromogenic substrate
MMG Minerals modified glutamate medium
MUG 4-methylumbelliferyl-β-D glucuronide fluorogenic substrate
LTMUG modified MLSB with MUG fluorogenic substrate
BPW Buffered peptone water
CT-SMAC Cefixime tellurite sorbitol MacConkey agar
TTC 2,3,5-triphenyl tetrazolium chloride
TSA tryptone soy agar
TBA tryptone bile agar

2.3 Membrane filter method

Membrane filtration of the sample is included in the method of choice for isolation and detection of *E. coli* from water in many approved methods. This method allows visualisation of resultant colonies and subsequent enumeration. The method is limited by the turbidity of the sample to be tested because very turbid samples can block the filter. To overcome this a range of serial dilutions (possibly containing a reducing agent e.g. cysteine hydrochloride to limit damage to bacterial cells from oxygen and free radicals) may be filtered. The UK Standing Committee of Analysts method for detection of O157 from water utilises Filter Aid. This is a solution of diatomaceous earth which when used with a sterile absorbent pad acts as a coarse filter. Also in development are methods which agitate the membrane filter when filtering turbid solutions but the concern is that the bacterial cells may be damaged in this process (R. Shepherd, personal communication)

In the approved methods utilising membrane filtration the sample is filtered through 0.45 • m membrane filter to trap the bacteria and the filter incubated on selective medium. Many different selective media have been used following membrane filtration. In the USA, the American Public Health Association (APHA) recommend mEndo agar for enumeration of coliforms in water, waste water and foods. The nutrients in this medium are casein, peptone, yeast extract and lactose. Lactose-fermenting colonies appear red with a metallic sheen due to the production of aldehydes. ISO 9308-1(1988) recommends the use of Lactose TTC in the membrane filtration method for detection and enumeration of *E. coli* . The medium contains Tergitol (sodium heptadecylsulphate) and positive lactose-fermenting colonies appear yellow/orange: yellow due to acid production detected by the indicator, Bromothymol blue, and orange due to weak reduction of 2,3,5-Triphenyltetrazolium chloride (TTC).

Problems have occurred if the organisms being recovered are stressed or sub-lethally injured, for example during water treatment processes. Sartory (1995) suggested that the addition of 0.01-0.1% (w/v) sodium pyruvate could reduce this effect.

More recently, methods in the UK for the analysis of drinking water have used membrane lauryl sulphate broths and agars (SCA methods A and B, and PHLS methods for water) (See Part 3A of Project Horizontal for a more detailed description). However, there is currently no universal medium for the isolation of *E. coli* from different environmental samples.

2.4 Chromogenic and fluorogenic substrates

Chromogenic and fluorogenic substrates produce colour and fluorescence, respectively, upon specific enzyme cleavage and are widely used in selective culture media. The principal substrates and the 2 major enzymes involved in the detection of *E. coli* have been described previously (see above). The inclusion of these substrates, together with the selective nature of the media (reducing the number of background microflora), have resulted in increased sensitivity and rapidity (Gaudet *et al.*,1996). It may be easier to distinguish specific colonies earlier using these media. Observation of fluorescent colonies is relatively easy using a basic a UV illumination chamber. Dogen *et al.* (2002) describe effective use of fluorogenic broths for the detection of *E. coli* in foods (MPN).

As with any enzymic reaction, conditions of pH and temperature must be optimal for the enzyme and substrates to function and these conditions must be specified in the protocol. For example, fluorogenic substrates are usually quenched at low pH and require neutral conditions in the medium. In the detection of clostridia using fluorogenic substrates which are cleaved by acid phosphatase neutral pH also results in activation of alkaline phosphatase giving false positive results (D.Sartory, personal communication).

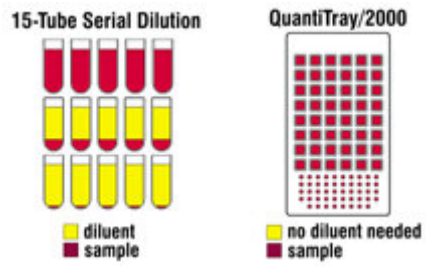
2.4.1 Agar media and broths

There are many commercially available chromogenic and fluorogenic media available as agar plates or broths e.g. Colisure, m-Coliblue, ColiComplete. Colisure® broth (IDEXX) simultaneously detects coliforms and *E. coli* in water utilising their ability to hydrolyse chlorophenyl red β-D-galactopyranoside (CPRG; sample turns yellow to red/magenta) or MUG (sample fluoresces). The manufacturer suggests that Colisure® can detect coliforms and *E. coli* at 10 cfu in100 ml within 24 hours when incubated at (35±0.5)ºC. It can also be used with the Quanti-Tray MPN system (see later). EPA included Colisure® in its proposal to update analytical methods for biological pollutants in ambient water, however the manufacturer declined to conduct the study and the product has therefore not been approved in the final rule (Federal Register, 2003). This may be because the product is being superceded by Colilert®.

2.4.2 Colilert®/Quantitray® technology

The Colilert® assay system (IDEXX Laboratories) has been accepted by the US EPA under their 40 CFR part 136 final rule for ambient water (Federal Register, 2003). Colilert® can be used for presence/absence samples utilising chromogenic (ONPG) and fluorogenic (MUG) substrates to simultaneously detect total coliforms and *E. coli* after incubation at 35ºC for 24 h. However, its strength lies in being adaptable to a semi-automated MPN method: this involves incubation of sample and defined substrate media in proprietary multiwell plates rather than tubes. The technology is based on the IDEXX Quanti-Tray and Quanti-Tray/2000 formats to provide easy, rapid and accurate counts of coliforms, *E. coli* and enterococci. The IDEXX Quanti-Tray and Quanti-Tray/2000 are semi-automated quantification methods based on the Standard Methods Most Probable Number (MPN) model. The Quanti-Tray® Sealer automatically distributes the sample/reagent mixture into separate wells. After incubation, the number of positive wells is converted to an MPN using a table provided. Quanti-Tray uses 51 wells and provides counts from one to 200 per 100 ml. The medium formulation is suggested to suppress up to 2 million heterotrophs per 100 ml; this could pose a challenge for analysing *E. coli* in sludge, soil and biowaste samples with high microbial background flora.
Quanti-Tray/2000 uses 97 wells of two different sizes and counts from one to 2,419 per 100 ml, with a far better 95% confidence limit than a 15-tube serial dilution (Fig. 2). IDEXX considers the technology to be superior to MPN and at least as good a performance as MF, with a greater counting range (Table 4). Total hands-on time is less than one minute per test. We consider that the if the Colilert® system is to be use for sludge, soil, and biowaste analysis then the Quanti-Tray/2000® format should be the preferred option because of its wider counting range.

Figure 2 Comparison of 15-Tube Serial Dilution vs. Quanti-Tray/2000



Colilert® is claimed to be able to detect 1 *E. coli* /100 ml, provide a less subjective interpretation, compared to counting colonies on agar, and identify 50% fewer false positives and 95% fewer false negatives than the standard membrane filtration (MF) method. The multiple well format gives greater precision than conventional 5-tube:3 dilution MPN methods with a MPN of <1 giving a range of lower and range at 95% confidence limits of 0 and 3.7

bacteria. Colilert® has 75% lower equipment cost than membrane filtration and is claimed to be 25-50% less expensive than traditional methods. The reagent packs have up to an 12-month shelf life and the comparatively rapid 24-hour test saves incubator space. IDEXX claim to be able to detect coliforms and/or *E. coli* in drinking water in under 24 hours.
IDEXX also sell Colilert-18® which has an enhanced formulation to detect coliforms and *E. coli* in 18 hours, improving workflow in large laboratories by reading afternoon samples the next morning. Samples need to be pre-warmed for presence/absence samples, if they are not already at 33-38ºC, but not for Quanti-Tray® or Quanti-Tray®/2000 samples. The sample should be placed in a 35ºC waterbath for 20 minutes or a 44.5ºC waterbath for 7-10 minutes. This pre-warming time is part of (not in addition to) the 18-hour incubation period for Colilert-18®.The company claim that the Colilert-18® / Quanti-Tray®/2000 technology has 95% less equipment costs than membrane filtration (MF). Colilert-18® is the only US EPA-approved 18-hour test and is included in the US Standard Methods for Examination of Water and Wastewater.

Table 4 Comparison of Quantitray, MPN and MF techniques

Method	Lower Counting Range/100 ml	Upper Counting Range/100 ml
Quanti-Tray	<1	200
Quanti-Tray/2000	<1	2,419
5-Tube MPN	<1.1	16
10-Tube MPN	<1.1	23
Membrane Filtration	<1	80

The Colilert® technology has been accepted in the USA for ambient water testing i.e. “any fresh, marine, or estuarine water used for recreation, propagation of fish, shellfish, or wildlife; agriculture, industry; navigation; or as a source water for drinking water facilities (US Federal Register, 2003). The US EPA recommends for testing for *E. coli* and enterococcal indicators in place of total and faecal indicators since “*E. coli* and enterococci show a direct correlation with swimming associated gastrointestinal illness rates, while faecal coliforms do not”.
Colilert® has been compared to the conventional MTF method using lauryl tryptose broth and brilliant green lactose bile broth and gave equivalent results. Schets *et al.* (1993) and Landre *et al*. (1998) found a significant number of false positive results caused by the presence of *Aeromonas* spp. in the latter medium. Fricker *et al.* (1997) found higher recoveries of coliforms from potable water samples using Colilert® compared to membrane filtration but no difference in counts of *E. coli.* A French study (De Roubin *et al*., 2000) found Colilert® to be equivalent to the French membrane filtration method (NF T90-414) and both were superior to the French 96 well microplate MPN method (AFNOR XP T90-433). Colilert’s false positive and false negative rates were found to be 2.4% and 3.85%, respectively.

Although the Colilert® assay was originally devised for water testing, it has applicability to monitoring *E. coli* (and coliforms if required) in sludge, soil and biowastes. Importantly, however, the US EPA has not yet approved *E. coli* (nor enterococci) methods for the analysis of wastewater samples because they have yet to be validated. This is now under way and the US EPA expects to propose test methods for wastewater by end of 2004.

Of relevance to this Part 3B report for Project Horizontal, Kramer and Liu (2002) compared the Colilert® method for sludge analysis with MTF. At the time of the study MTF was accepted in the U.S. for the enumeration of waste activated solids (WAS), and membrane filtration was tentatively viewed as acceptable. The MTF analysis followed the procedure which is outlined in the draft standards: CEN TC308/WG1/TG5 Detection and enumeration of *E. coli* from sewage sludge. Part 2: Miniaturised method (MPN) in liquid medium and CEN TC308/WG1/TG5 Detection and enumeration of *Escherichia coli* from sewage sludge Part 3: Macromethod (MPN) in liquid medium. Kramer and Liu's study (2002) with raw and pasteurised WAS described numerous comparisons that have been made between Colilert® / Quantitray® system and all existing standards and concluded that the Colilert® system is effective at detecting and enumerating coliforms and *E. coli* from WAS samples of varying bacterial content. There was no significant difference between the Colilert® method and the US-EPA multiple tube fermentation technique for WAS samples. However, it was noted that studies indicated that false positives did occur which were caused by the presence of *Aeromonas* spp.
The methodology developed for the Colilert® analysis of WAS used preparation techniques similar to all the membrane filter and MPN methods that have been developed for water and are might be considered for sludge, soil and biowaste. The sample is homogenised and added to buffered diluent i.e. water. From this stage the sample is diluted into dilutions A, B, and C. The dilutions are made up by adding 50g of WAS to 450 ml of sterilised buffered water which is then mixed thoroughly (0.1g of original sample per ml of mixture). From this mixture dilution A (0.001g of original sample per ml), dilution B (0.00001g of original sample per ml), and dilution C (0.0000001g of original sample per ml) are set up. From this stage 100 ml of each dilution is mixed with the Colilert® media and added to the Quantitray® package. It is then sealed and incubated at 35°C for 24 hours.
The Colilert® method is included in the Standard Methods for the Examination of Water and Wastewater and in UK Standing Committee of Analysts and PHLS methods for *E. coli* and *E. coli* O157. It is included as one of the three UK draft SCA methods (SCA 2003c) and is routinely employed by a number of laboratories in the UK for both potable water, sludge, soil, and biowaste analysis.

The advantages of the Colilert® system include:

- Sensitivity to concentrations of coliforms and *E. coli* as low as 1 cfu / 100 ml
- Results in 18-24 hours
- No confirmation tests needed
- Specific *E. coli* identification – no other tests needed
- Low cost
- Long shelf life of prepared media
- Configuration as either P/A or MPN tests
- Equal utilisation by small and large treatment plants

Disadvantages include the possibility that environmentally stressed and viable but non-culturable (VNC) organisms may not be recovered using narrowly defined substrate media and cross reactivity of contaminants resulting in false positives. However, this method should perhaps be considered for a larger trial for use as a standard European method for the rapid detection of *E. coli* in sludge, soil and biowaste. The test can distinguish *E. coli* from other coliforms. If knowledge of the latter is not required for sludge, soil and biowaste analysis, then perhaps the test could be made cheaper by asking the manufacture to removing the coliform substrate?
In the revised Desk Study Report 3A, the authors have included an Appendix 7 which gives results from a recent comprehensive interlaboratory trial with seven laboratories on the Colilert® method involving five types of sludge (spiked and unspiked) and vitroid reference materials. One conclusion of the trial is that the Colilert® test method does not involve a laboratory in any media or reagent preparation and the variations observed in the results should

solely relate to the laboratory personnel, the sample pre-treatment step (e.g. homogenisation step) and any variations in the supplied samples and / or *E. coli* reference materials. The initial interpretation of the data is that the Colilert® method did not respond to the presence of *Klebsiella* and has shown that it is able to detect *E. coli* consistently in original 'real' sludge samples and spiked sludge samples without significant interference from the sludge matrix. There were some discrepancies in some of the results reported from the trial which may have been due to laboratory error or dilution mis-calculations on the part of the participating laboratories. The Colilert® method has also illustrated that it can recover the target organism equally, when compared to other methods such as membrane filtration methods MLSB and MLGA, respectively.

2.5 Conventional methods to detect *Salmonella* species

A diverse range of methods have been developed over the years to detect *Salmonella*, primarily in foodstuffs and water supplies both as a routine monitoring of food and water quality and in the event of an outbreak detection of the contamination source. Because the infectious dose is very low in humans, the sensitivity of the methods used have to be high. This is further complicated by the fact that numbers of *Salmonella* in contaminated water, food and wastes are usually greatly outnumbered by other organisms of faecal origin e.g. *E. coli* and enterococci, and organisms naturally occurring in the environment including *Citrobacter* and *Proteus* spp. Any *Salmonella* that are present may be sub-lethally stressed and require incubation in a highly nutritious non-selective medium (enrichment) prior to further processing.
A range of methods have been developed to detect *Salmonella* in food and water which rely on standard culture methods and biochemical confirmation tests. A number of these have been standardised and are used routinely (Table 4). They all rely on the principal 4 stages outlined below:

2.5.1 Sampling and release of the bacterium from the matrix

Usually a 10-30g sample of food or waste is diluted approximately 1/10 in a buffer containing surfactant (e.g. non-ionic detergent) and mixed in a homogeniser, Stomacher or more recently Pulsifier (Microgen Bioproducts Ltd.) which employs combining shock waves and intense stirring. All these processes are used to ensure that the bacteria are completely released from the matrix and adequately dispersed to prevent clumping before the pre-enrichment stage.

2.5.2 Pre-enrichment

This stage allows small numbers of potentially environmentally stressed bacteria to recover and grow before the use of selective media. Because non-selective, highly nutritious media are used at this stage, often Buffered Peptone Water (BPW), there is a risk of overgrowth of other contaminants which could actually inhibit the growth of any *Salmonella*. The timing and temperature are therefore important at this stage to ensure the *Salmonella* can still be recovered. Usually an 18-24 hour incubation at 37^{o}C is adequate. In spite of these difficulties it is not advisable to use selective media from the outset because if the *Salmonella* cells are sub-lethally stressed they would not recover on selective media and produce a false negative test result. This has practical difficulties for developing a rapid assay taking less than 24 hours (see later).

2.5.3 Selective enrichment

Usually samples from the pre-enrichment broths are inoculated into selective broths. There is considerable dispute as to which selective agents give the best recovery and the choice depends on the matrix (food type e.g. meat; water or sludge), conditions of sampling and the species of *Salmonella* under investigation. The selective medium specified for use by the food industry on

highly contaminated foods in the USA and for food and water samples in the UK is Rappaport-Vassiliadis (RV) medium. Broths are incubated at a higher temperature of 41.5ºC for 24 and also 48 hours.
Yanko *et al.* (1995) however described the use of Tetrathionite Brilliant Green Broth recovering more *Salmonella* from activated sludge, compost and anaerobically digested biosolids. Bown and Keevil (2000) described a method for detection of *Salmonella* in human sludge, cattle and pig slurries which omits the pre-enrichment broth but after filtration of the sample onto 0.45 • m cellulose nitrate membranes the latter are incubated on filter pads soaked in Tetrathionate broth (Oxoid, European formulation) prior to growth on selective solid media. The inclusion of novobiocin is very important to suppress background competitors such as *Proteus* spp. which can reduce tetrathionate and impair the value of the medium to grow Salmonella. Novobiocin is included in the enrichment media specified in several of the draft MF and presence/absence CEN methods for detecting *Salmonella* in sludge (Table 5).

2.5.4 Agar media

In the majority of methods the RV broths are then used to inoculate solid selective media, for example desoxycholate citrate agar (DCA), xylose lysine desoxycholate citrate agar (XLD), Brilliant green Agar (BGA), bismuth suphite agar (BSA), Salmonella-Shigella agar (SS), or mannitol lysine crystal violet brilliant green (MLCB). Many of these contain selenium salts, brilliant green and malachite green to inhibit the growth of other *Enterobacteriaceae* and detect the production of hydrogen sulphide (although some serotypes are negative) by the *Salmonella.*
More recently used formulations are XLT4 (Tate, 1990) which contains xylose, lysine, lactose, sucrose, phenol red and the surfactant, Tergitol, which has given improved recoveries of *Salmonella* from meat from poultry farms.
Rambach agar (1990) uses a new phenotypic characteristic which is the formation of acid from propylene glycol and hydrolysis of X-Gal to differentiate *Salmonella* species from other *Enterobacteriaceae.*
Rainbow agar (Biolog) can isolate and differentiate the widest range of *Salmonella* species, including *S. typhi,* and can detect the weakest to the strongest hydrogen sulphide producers.
A combination of 2 or 3 of the above media is used routinely by UK PHLS and SCA published methods for the detection of *Salmonella* in water and food. (See Table 4). The membrane filtration methods for sludge and wastes recommended by CAMR/ University of Southampton and CEN 308 method 2 , and CEN 308 method 1 (liquid enrichment) used Rambach agar.
Other novel media are being developed all the time and those that incorporate a combined pre-enrichment/selective enrichment or result in reducing the time necessary to obtain a result (i.e become more rapid) are described later.

2.5.5 Biochemical and serological confirmation

To confirm that the resultant colonies on selective agars are *Salmonella* a range of biochemical and serological tests are usually performed e.g., urease test, growth on iron sugar medium, phage typing and latex bead agglutination (LBA).

The immunolabelling methods fall into several broad categories, summarised as immunofluorescence assay (IFA), enzyme immunoassay (EIA: also commonly referred to as enzyme-linked immunosorbent assay; ELISA) and latex bead agglutination (LBA). IFA can be used for the direct specific **detection** of original samples, providing the assay is sufficiently sensitive for the concentration of target organism present, and after sample concentration (filtration or immunocapture) or amplification (pre-enrichment) steps. EIA (ELISA) and LBA are used more often for rapid post-broth and agar colony **screening** to confirm the identity of the target organism, augmented by conventional serotyping (see later).
IFA offers the ability to specifically detect pathogens *in situ*, particularly where sub-lethally damaged or VNC bacteria are suspected. Once the antibody has been produced, the method is quick and inexpensive. The IFA has been combined with CTC-detected respiration to

determine the identity and physiological status of *E. coli* O157 in water (Pyle *et al*., 1995). Fluorescently-labelled antibodies have been used to screen wastewater for *Salmonella* (Desmonts *et al*., 1990). The method is rapid but requires that there is no cross-reactivity with other species, that the target epitope is expressed and conserved in the test environment and there are sufficient number of cells for observation by microscopy or cell cytometry (see later).

2.6 Conclusions for existing *Salmonella* methods

The traditional culture methods for *Salmonella* detection described above can take up to 6 days for an accurate confirmation. The worldwide incidence of salmonellosis is increasing and with increasing pressures on the food industry bound by law to produce safe foodstuffs there is a great need for new, rapid and sensitive methods for detection of *Salmonella*. The majority of methods for the detection of *Salmonella* have been devised to isolate the organism from food and water. These methods can be adapted to a different matrix such as soil and biosolids but modifications may have to be made. For example, Yanko *et al.* (2001) preferred a modified semi-solid RV (MSRV) medium rather than the conventional RV enrichment broth in spiked compost samples.

Table 5 Isolation and detection of *Salmonella*

Origin of method	Matrix method devised for:	Sample size	Summary of method	Comments/ validated etc.
EA/SCA UK 2002	Water	1000 ml treated-	Filter the pre enrich on BPW 37oC 24 hr Select. Enrichment on Rapp. Vass. Med 41.5oC 24 hr and 48 hr. Subcut at each time point to sel agars XLD, BGA, flagellar test, iron sugar etc	
PHLS SOP UK W7	Water	1000 ml	Filter the pre enrich on BPW 37oC 24 hr Select. Enrichment on Rapp. Vass. Med 41.5oC 24 hr and 48 hr. Subcut at each time point to sel agars XLD, BGA, MLCB flagellar test, iron sugar etc	
F13	Food	25g	Stomach in Buffered Peptone Water leave 18 hr (pre enrich) Selective enrich in Rapp-Vass + selenite cys 22 hr Subcut to sel agar XLD, BGA, MLCB	
Soton/CAMR	Sludge?	25g	Stomacher Dilns filtered and placed on resus pad soaked in tetrathionite broth + iodine Filter onto rambach agar	
CEN TC 308 Method 1 DIN Liquid enrichment	Sludge	10 g DM	10g + 90ml tryp Homogenise 2 mins Primary enrichment in sel cys 36oC 20 hr (dilns) Secondary enrich Rapp Vass Subcut to Rambach XLD Confirm urea, indole MPN	Validated
Method 2 UK? Pt 1 Membrane filtration 6 log drop	Sludge	25g wet weight	Stomacher MTSB (Novobiocin)* Fuller dilns Resus Tetrathionite broth 36oC 16 hr Rambach, spry and UV	
Method 3	Sludge , soil, slurry	20 g wet weight	20 g in 200ml sterile Na Cl Shake 20 hr 2 oC Dilns onto Rapp Vass 20 hr 42oC then subcut onto XLD,BPLA – Most Prob No. test	
ISO 6579 (2002)	Food		Muller Kauffman tetrathionate novobiocin broth, RVS broth then XLD agar	

3. EVALUATION OF DRAFTI NG A HORIZONTAL STANDARD

3.1 Emerging rapid methods for *E. coli* and *E. coli* O157

3.1.1 Fluorogenic substrates

Colifast (Colifast Systems,Oslo) is an automated system utilising fluorogenic substrates but does have an enrichment step for the recovery of stressed organisms and measures the fluorescence directly. This is usually recommended for bathing waters.
Suwansonthichai *et al.* (2001) describe enumeration of *E. coli* from frozen black tiger shrimp using conventional MPN and rapid methods Chromocult, Fluorocult and Petrifilm plates.

3.1.2 Cell cytometry

Cell cytometry relies on a stream of liquid flowing as discrete microdroplets through a laser beam. Optical signals are detected whenever a particle, either unlabelled or fluorescently labelled, passes through at rates exceeding 10,000 per second. The types of information available include size, shape, labelled RNA, DNA and surface antigen content. The data are collected for comparison of parameters such as size versus fluorescent intensity. Incorporation of a fluorescently activated cell sorter (FACS) allows gates to be set of say size versus fluorescence and each particle which gives a positive signal within the gate can be deflected to a collector to provide a specific separation and quantification procedure. The specificity of fluorescently labelled antibodies has been exploited to detect and purify microorganisms such as *E. coli*, *L. pneumophila*, spores of *Bacillus anthracis* and oocysts of *Cryptosporidium parvum* by flow cytometry.[50, 51] The coupling of flow cytometry with the use of 16S or 18S rRNA fluorescent probes has been advocated to facilitate the quantification of specific microorganisms from environmental samples (Phillips and K. L. Martin, 1988; Vesey et al., 1998).

3.1.3 Laser scanning

A system that addresses the need for rapid detection and identification of microorganisms from environmental samples has been developed by Chemunex (Maisons Alfort, France). The ChemScan RDI is based on direct fluorescent labelling of viable organisms trapped on a 25 mm diameter membrane, coupled with an ultra-sensitive laser scanning and counting system. The high level of sensitivity of the solid phase cytometer means that a single cell on a membrane can be detected. The use of fluorescently-labelled antibodies, enzyme substrates or nucleic acid probes provides the specificity for ChemScan to identify and enumerate target microorganisms without the need for enrichment. FITC-labelled *C. parvum* oocysts can be counted within 3 minutes before visual observation of the presumptive positives by epifluorescence microscopy (Reynolds et al., 1999). Of concern is that non-culturable cells can be detected by this method. Viability can be assessed by incubating with fluorochrome esters which fluoresce when the substrate is actively taken up by viable cells and intracellular esterases release the fluorochrome. The technology is now ready for application to untreated and treated wastes, provided good fluorescent antibody and oligonucleotide reagents are available, and trapped non-target cells do not interfere. The latter may be unlikely when looking for low numbers of a pathogen against a high background in sewage sludge, unless IMS or selective enrichment is undertaken first. Reynolds *et al.* (1999) observed that one advantage of isolating target organisms on a membrane was that interfering substances such as clay particles could be washed away before incubation with antibody reagents.

3.1.4 Immunological methods

These methods are based on the interaction between specific antibodies (polyclonal or monoclonal) and antigens and it is this very specificity which can limit the effectiveness of the method. The method usually takes the form of antibodies fixed to a solid phase such as a multiwell plate (ELISA), flow through grid, and dipstick or magnetic beads as in the case of immunomagnetic separation (IMS). The antibodies capture the specific bacterial cells or surface antigens and positive binding is detected by either growth in culture media or addition of further antibodies conjugated to enzymes. The addition of the enzyme substrate, usually chromogenic, results in a detectable colour change in ELISA and dipstick. In complex matrices including sludges, immunological methods may be inhibited by large numbers of contaminating organisms and large amount of debris in the samples.

IMS is used primarily to isolate the bacterium of choice from a complex matrix especially where there are low numbers in the sample or after an enrichment step. However, IMS is the method of choice for the isolation of *E. coli* O157 from moderate turbidity water supplies in the UK Standing Committee of Analysts (method F) following selective enrichment in buffered peptone water or modified tryptone soya broth. The selectivity of the antibodies used dictates the recovery efficiency and this method recommends Dynal Dynabeads or their equivalent e.g. Aureon Biosystems. The method is effective because it results in the isolation of the O157 serotype from the high numbers of contaminating other serotypes of *E. coli*. Once the organisms have been isolated they are plated onto selective media. This method is expensive but effective for complex matrices and more competitors are now available which could eventually affect the price. Recent evidence (Haro-Kudo *et al.*, 2000) does suggest that false negatives can be reported due to the loss of surface antigen in stressed serotype O157 especially in environmental samples where there are starvation conditions.

PATHIGEN (Igen) is a commercially available assay for *E. coli* O157 in food and environmental samples. Like Dynabeads, magnetic beads are coated with antibodies to *E. coli*. the bacteria in the sample bind to the beads and a second antibody which hasa fluorescent tag binds also. Positive samples are detected by flow cytometry. The product was evaluated by Norpath laboratories UK who claim it was 100 times more sensitive than selected dipstick and ELISA methods.

Tu *et al.* (2001) captured *E. coli* O157 using antibody conjugated to alkaline phosphatase. This complex was then immunomagnetically captured and the degree of enzyme plus substrate catalysis measured.

There are several commercially available ELISA including TECRA screen for serotype O157 and Assurance EIA (BioControl Laboratories) which is an enzyme immunoassay detecting O157 in food and environmental samples. Bio Control have also developed EHEC8 enrichment medium for isolation of serotype O157 from beef. VIDAS produce automated enzyme linked immunofluorescence systems. Itoh *et al.* (2002) described a filtration ELISA where bacterial cells were directly filtered into a 96 well tray. SafePath is an ELISA test for O157 serotype.

Other immunological tests include latex agglutination tests for the confirmation of colonies isolated by conventional culture techniques (microgen Bioproducts,Oxoid,Unipath,Meridian diagnostics). IFA details?

Oxoid have a EIA to detect the heat stable enterotoxin (ST) which uses a synthetic peptide toxin analogue and monoclonal antibodies.

PATHATRIX (Matrix Microscience) is a novel method for detection of a range pathogenic bacterium in food samples. The PATHATRIX system is a patented technology that relies on the use of antibody coated paramagnetic particles to selectively bind and purify the target organism from a comprehensive range of complex food matrices. It is unique in that it is the only microbial detection system that can analyse the entire 225ml + 25g sample simultaneously by re-circulating the sample through a “capture phase” where the antibody coated magnetic beads are immobilised. By providing heat to the system the organisms can be cultured and captured simultaneously, thus increasing the method sensitivity. Once captured and concentrated the sample is now ready for use with a variety of detection methods: either direct plating onto the appropriate selective media and incubated or tested using one of the following; COLORTRIX;

FLURATRIX (fluorescence microscopy); serology; PCR; ELISA; and/or DNA probe. There are two available formats to provide maximum flexibility and sample throughput to match customers specific requirements. The “3 Hour” format is intended for same day sample processing (no pre-incubation of sample required) whilst the “30 minute” format is intended for high throughout sample processing (following overnight incubation). Data from internal and external validation studies e.g. AOAC trials have demonstrated that PATHATRIX system is significantly more sensitive than many of the current standard methods, at low spike levels 1-10 cfu/25g sample. The company claim to have launched the world's fastest commercially available method for the detection of *E. coli* O157 in food samples.

For 25g samples, the test can be completed, from start to finish, in just over 5 hours. For 375g samples, the test can be completed in 6¾ hours. The new test combines two of Matrix's proprietary technologies, PATHATRIX and COLORTRIX. The PATHATRIX system is designed for the rapid detection and positive identification of microbiological food contaminants, while COLORTRIX is a screening system, which provides presence/absence results within 15 minutes. The PATHATRIX/COLORTRIX method, which is capable of detecting a single cfu in a 25g sample, is proving particularly popular with the beef market, where accurate, rapid testing can significantly enhance productivity and is critical for QA. To undertake the test, a 25g food sample is homogenised with 225ml of growth media in a stomacher and incubated for 4½ hours. PATHATRIX capture reagent, which consists of *E .coli*-specific antibody coated magnetic particles, is then added directly to the sample. The sample is loaded onto the PATHATRIX workstation, connecting the sample to the circulatory system in preparation for the Capture-Culture step. Once loaded, PATHATRIX is pre-programmed to run for 30 minutes and on completion of the run, the *E. coli* cells are bound onto the phase by the capture reagent. Residual debris and non-specific binding are removed during a single wash step. The captured pathogen complexes are then concentrated into a small volume. i.e., 200 •l using a magnetic rack. A COLORTRIX antibody/enzyme is then added to the concentrate for 5 minutes before being diluted with 1 ml of wash buffer and magnetic removal of the of the bead/bacteria complexes. After a further two washes, half the concentrate is removed and added to a second reagent. The sample is then left for 5 minutes to develop colour. A blue colour indicates a 'presumptive positive,' while a clear sample is recorded as a 'presumptive negative.' Should a positive result be recorded, the sample remaining in the wash vessel is plated on the appropriate agar media, while a negative indicates that no further action is required.

A recently announced product is the GridCount for the enumeration of bacteria in sewage sludges and other complex materials (MicroScience Technologies Ltd., Edinburgh). This is a quantitative immunoassay that uses polymer grid bars coated, on one side only, with millions of micron-sized dots containing antibody. The grid is placed in a screw cap tube with sludge and shaken. Bacteria present are recognised by the specific antibody, in this case *E. coli* O157, and become attached. The open grid design prevents fouling with suspended solids and ensures an even coating. Thus the bacteria are captured in a two-dimensional array, which facilitates further interrogation. The simplest method is to culture the grid 'upside down' on an agar plate. Within two hours, daughter cells from the fixed bacteria transfer on to the plate. The grid is then removed (and can be kept for further analysis, or disposed of). After overnight incubation the bacterial colonies, arrayed in grid formation, are counted. This can be done automatically using, for example, the Synbiosis Acolyte machine. The method can be used in conjunction with selective or chromogenic media, although this is not necessary since the antibody on the grid acts as a selective reagent. Alternatively, the grid can be stained and scanned directly using, for example, the Chemunex ChemScan RDI. The capacity of the grid used in culture mode is around 500 colonies. The capacity of the grid in scan mode is 500,000 colonies. The lower resolution in culture mode arises from the need to allow space for growth of the colonies. New versions of GridCount for total *E. coli* and *Salmonella* are planned shortly.

3.1.5 Lateral flow devices and dipsticks

There have been rapid advances with lateral flow devices, including Visual Immunoprecipitate assay (VIP; BioControl System Inc.) and gold labelled immunosorbent assay (GLISA) technologies such as the Merck Singlepath range for *E. coli* O157, *Salmonella* etc. The *Salmonella* test is carried out after a selective enrichment of the sample from one selective enrichment culture (taking 24-48 h) The end result is thus available two days sooner saving material and labour costs. A VTEC test for O157 is available, and yields a yes /no result in 20 minutes following overnight enrichment culture. It therefore provides the required detection in under 24 hours. Another development of the technology is the Duopath range which is used to detect the VT1 and VT2 verocytotoxins produced by VTEC. The technologies are claimed to be:

- **Fast:** Simple to handle, definite results from culture within 20 minutes.
- **Easy to use:** Simply apply the sample and read off the yes/no result.
- **Safe:** Definite test results with an additional positive control. Specially adapted enrichment media guarantee precise and reliable testresults.
- **Economic:** Rapid results help save laboratory costs and investments in automation; a faster product release gives you a head start in the marketplace.

Bown and Keevil (2000) evaluated two lateral flow devices for their suitability for rapid immunological detection of O157 in sludge: the Morningstar O157 7 hour test and the BioControl VIP. Initially, pure cultures of *E. coli* O157 were grown to test the response of the Morningstar kit. Following overnight growth 200 µl of the culture was applied to the well region of the device. A strong positive reaction was elicited in the observation window after 10 minutes incubation at room temperature. Spiked samples (equivalent to 10 cells of *E. coli* O157 per gram of sludge) were stomached and then enriched for 6 hours before the immunoprecipitation assays. At 4 hours enrichment no signal was detectable, however at 6 hours a strong signal was present. When this experiment was repeated using the VIP assay only a weak signal was detectable after 6 hours enrichment. If samples were enriched for 7 hours then the signal was stronger. The sensitivity varied for each kit detecting $>10^6$ and 10^3-10^4 of enriched cells in the VIP and Morningstar kits, respectively. A survey of 5 treatment works in one region of the UK was undertaken to assess the device performance. When tested by the VIP assay all samples were negative. Repeat of the experiment using Morningstar kits indicated a very strong positive in the Works A sample and a very weak positive in the Works B sample, all other samples were negative. These analyses were repeated with fresh samples over several days. This suggested the value of the Morningstar kit for same day screening of routine sludge samples for this important pathogen. Another flow device finding favour to detect E. coli O157 meat, milk and juices is the SAS *E. coli* O157 (SA Scientific, distributor M-Tech Diagnostics). Consequently, flow device technology shows promise and may be applicable for rapid detection of Salmonella (see later). However, a full parallel study comparing culture methods with the flow devices should be considered as considered as part of a true evaluation of a range of flow devices and dipstick types.

There are several dipstick rapid tests available for use in water and food matrices. Quix Rapid *E. coli* O157 strip test (distributor M-Tech Diagnostics) has been advocated for the detection of serotype O157 in human faeces and will be available soon. The literature suggests that this should be a promising test, claiming to give results in 5 minutes and is applicable to field testing. Further claims are that the detection level is 3.4×10^4 cfu/ml with 99% specificity and 100% sensitivity for all strains of *E. coli* O157. This company also just released a Quix immuno-chromatographic assay for analysis of enrichment broths.

3.1.6 Nucleic Acid based methods

Rapid advances in molecular biology and molecular taxonomy are making it clear that many microorganisms exist in the environment, including potable water and wastewater, which cannot presently be cultured. Research has indicated that only approximately 1% of the bacteria in potable water can be cultured (Staley and Konopka, 1985), yet they appear metabolically active (Roszak and Colwell, 1987). This non-culturability is presumed to be because:

- they are of previously unidentified genera/species whose physiology and growth requirements are not understood (implying that conventional laboratory growth media contain inappropriate nutrients), or
- they are environmentally stressed due to nutrient limitation, extremes of temperature, pH, redox, osmolarity etc., or to the presence of disinfectants such as chlorine, and may be termed viable but nonculturable (VNC) using routine laboratory media.

Knowledge of these non-culturable species is important because in the environment and the built environment they play a role in biofilm formation (causing biofouling, heat loss or corrosion of pipework, but making important contributions to wastewater treatment processes) and might also provide a shelter for bacteria causing infectious diseases (Keevil *et al*., 1995). In particular, sub-lethally damaged or VNC pathogens such as VTEC, *Salmonella*, *Shigella* or *Campylobacter* spp. may be present in untreated and treated wastes and, although possibly remaining capable of causing infection, are undetectable by routine culture (Xu *et al*., 1982; Roszak and Colwell, 1982; Roszak *et al*., 1984; Colwell *et al*., 1985; Rollins and Colwell, 1986; McFeters *et al*., 1995).

Where microorganisms can be cultured from low nutrient environments on specialised media, such as low nutrient R2A media (Reasoner and Geldreich, 1985), there is frequent disagreement over their identity when characterised using commercial API, BIOLOG and VITEK biochemical databases (as discussed previously). Even then, they make take 7-10 days to grow before identification.

However, 16S and 23S rRNA sequences provide a unique signature for each prokaryotic species. Phylogenetic analysis of the rRNA sequences can be used to identify recovered bacteria in relation to well characterised strains, or the creation of new genera (e.g. within the α, β, or γ subclasses of the Proteobacteria or Eubacteria) The development of *in situ* hybridisation with rRNA-targeted oligonucleotide probes (Amann *et al*., 1990b, 1994) has allowed rapid identification of bacteria within their natural habitat. Furthermore, where species are non-culturable, strain specific rRNA probes can be produced (using conserved primers as original templates to amplify the variable regions for sequence analysis; Weisberg *et al*., 1991) to determine their abundance *in situ.*

Fluorescence *in situ* hybridisation (FISH) relies on the presence of sufficient rRNA to bind to the labelled oligonucleotides and produce a bright fluorescence. Early studies correlated the ribosome content and growth rate of *S. typhimurium* (Schaechter *et al*., 1958) and this data has been extrapolated to, or found to be reproducible with, data obtained from other species. The detection of cells is dependent therefore on the number of ribosomes and, hence, their physiological state. In microorganisms with a low rRNA concentration, including VNC, ribosome content can be increased by pre-incubating samples in a nutrient medium such as yeast extract or R2A medium, in the presence of a DNA gyrase inhibitor such as pipemidic acid to inhibit cell division (Kogure *et al*., 1979; Kalmbach *et al*., 1997). Fidelity and specificity of probe binding also can be problematic, requiring incubation with a specific concentration of formamide to maintain stringency. However, the recent advent of protein nucleic acids may solve this problem since these molecules are more flexible than conventional nucleic acids and bind better to curves and hairpin loops in the rRNA.
Detection of hybridisation may be by direct or scanning microscopy or flow cytometry (see later). There may be limitations if the sample is taken from a nutrient starved environment

which affects the ribosomal content. These methods are useful, even if it is detection of rRNA cannot be truly correlated to viability.

Several rRNA probes are now commercially available (GeneTrak). However, the search for a specific rRNA probe sequence for *E. coli* remains elusive. *E. coli* has been detected in freshwater biofilms with FISH using a 23S rRNA probe (Szewzyk *et al*., 1994). The probe sequence (Gam 42a) is complementary to a selected region in the 23S rRNA of the bacteria grouped in the gamma-subclass of *Proteobacteria*, and is therefore not specific for *E. coli* or its serotypes (Manz *et al*., 1992). This group also contains other enterobacteria, *Acinetobacter* and *Pseudomonas* spp. To improve speicificity Regnault *et al*. (2000) constructed a 24-mer oligonucleotide probe (termed "Colinsitu"), complementary to a piece of the *E. coli* 16S rRNA, They tested its sensitivity by and specificity by visualizing *E. coli* cells by in situ hybridization and epifluorescence microscopy. The fluorescent dye-labeled probe was able to stain cells of E. coli, Shigella spp. and E. fergusonii. Shigella spp. are known to belong to the E. coli genomospecies and E. fergusonii is the nomenspecies closest to E. coli by DNA-DNA hybridization. The probe did not stain any strain of 169 other genomospecies of the family Enterobacteriaceae or of a few other species frequently encountered in the environment. Revivification without cell division allowed the visualization of *E. coli* cells in contaminated water. They concluded that ISH using the Colinsitu probe is a potential tool for the confirmation of (atypical) *E. coli* in reference centers and the rapid (3-6 h) detection and enumeration of E. coli in urine specimens, contaminated water and food. More work is needed to include ISH in the routine laboratory.

The development of rapid methods, and methods which requires enrichment and culture, is limited by how short incubation times can be before limits of detection drop to unacceptable levels. The use of molecular methods which could be used instead of lengthy culture incubations appears to be a promising direction to take in the development of rapid methods. However, these methods are only going to be efficient if the specificity of the genetic probes are high enough, especially in samples with a high bacterial load and the method is not hampered by inhibitory substances in complex environmental samples. It is also very difficult to quantify molecular methods as the amount of genetic material generated by the method has to be calibrated back to bacterial cell numbers. Recently the use of real time PCR which can be related to the true bacterial cycle and gives some degree of quantification. This method has been improved by the introduction of fluorescent gene specific probes. Results from molecular methods unlike culture methods do not assess whether the bacteria are viable in the samples tested. The method may be applied after an enrichment step but the result still cannot determine if the organisms are alive.

3.1.7 Polymerase chain reaction (PCR)

Individual genes have been targeted as specific for *E. coli* in the development of PCR methods (*malB ,lamb, uidA, phoE*). Bej *et al*. (1991) reported better sensitivity of the PCR method involving amplification of the gene encoding β-glucuronidase compared to MUG based defined substrate methods. A combination of PCR and ELISA was used for the detection of *E. coli* in milk (Daly *et al*. 2002).
Recently multiplex PCR methods which simultaneously amplify several sets of specific genes with multiple primers have been attempted often following a concentration step e.g. filtration. Campbell *et al*. (2001) used a multiplex PCR to detect *E. coli* O157 in soil and water samples following two enrichment stages. They claimed the method was sensitive (1 cfu/ml drinking water and 2 cfu/g soil) and results could be obtained within 1 working day. Kong *et al*. (2002) used a multiplex PCR in marine waters to detect 6 different bacterial pathogens simultaneously. Iberkwe *et al*. (2003) used a real time PCR in the analysis of soil, manure and faeces for the presence of serotype O157. The method utilised the amplification of *stx*1 and *eae* genes (Sharma *et al*., 1999) and specific fluorogenic probes which avoids the need for time consuming

agarose gel visualisation. They reported a highly sensitive and specific enumeration assay which was conducive with screening large numbers using automated PCR amplification and detection of products. A 16 hour enrichment step was used and results could be obtained within a working day. A trial of this method should perhaps be considered in comparison with improved culture techniques.
The BAX system (Qualicon) for *E. coli* O157 and *Salmonella* is an automated PCR system following sample enrichment which has been approved for use in Brazil (see later).

3.2 Summary of rapid methods for *E. coli* O157

The range of rapid test kits available for *Escherichia coli* on the international market in 2003, recognised by the Association of Analytical Communities (AOAC), is listed in Table 6.

Culture approved methods for the detection of *E. coli* O157 are based on the fact that this serotype does not ferment sorbitol or possess •-glucuronidase. Possession of the latter, in conjunction with fluorogenic substrates, is the basis of many methods for the detection of non toxic strains of *E. coli.* Most of the methods have been devised for water testing and often have a filtration step. When the methods have been used for more complex matrices of food and water filtration of dilutions of sludge may be done, e.g. Standing Committee of Analysts uses FilterAid to help prevent filter blockage. After an initial enrichment stage the organisms are concentrated out of the matrix by IMS followed by growth on selective media.
In the simpler method devised by CAMR/Southampton University samples of sludge were mixed in a stomacher prior to filtration. The trapped bacteria are retained on the filter and placed straight onto a enrichment media (TSB) which is selective because it contains novobiocin to inhibit the growth of other strains of *E. coli*. The filter with resulting growth is then transferred to selective Chromagar. The method is being included in the draft SCA methods for sludge analysis.
Recently developed media which allow simultaneous detection and confirmation of colonies have reduced the time taken to get a result. However any culture method still requires sufficient time to allow colonies to grow to a detectable level.
Can culture methods be used in the development of a rapid test? What is a ‘rapid test’? A consensus of opinion is perhaps a test that can be completed in a working day or 2 days at the most.
Molecular methods can work within this timescale but have to overcome problems of specificity, detection of viability and enumeration. The results from trials of any molecular methods should really be compared to conventional culture methods used for the same samples which has not been done in many reported cases. IMS used in conjunction with culture is described above. The use of IMS and immunofluorescence (PATHIGEN,Igen) may be promising and should be subject to validation for large scale laboratories.
Methods involving automated detection of fluorescently labelled bacteria trapped on a membrane using laser scanning should be considered. Pyle *et al.* (1995) suggested the use of respiratory indicator cyanoditolyl tetrazolium chloride (CTC) in combination with a specific fluorescent antibody as an indication of viability.
For truly rapid presence/absence testing lateral flow devices and dipsticks should be evaluated e.g. Quix SAS, as should a selection of EIA, for example the automated system VIDAS. Hamada *et al.* (2002) used this system and claimed to obtain results within 1 hour after 18 hour incubation.
Real time PCR using a defined set of primers (multiplex) should also be subject to trial. However, it is not clear that a commercial mix of primers, suitable for target organisms in sludge, soil or biowastes, exists at this time. Research may be required to develop an appropriate set.

Table 6 AOAC (Association of Analytical Communities) list of internationally available rapid test kits for *E. coli* in 2003

ANALYTE	COMPANY	KIT NAME	METHOD TYPE	RECOGNITION	PRIMARY MATRICES
E. coli	3M Microbiology Products	Petrifilm *E. coli* Count Plate		**AOAC *Official Method* 991.14, 998.08;** Association Francaise de Normalisation (AFNOR) Cert. No. 3M 01/4-09/92; FDA-BAM 8th ed., Rev. A; Health Protection Branch-CANADA MFHPB-34; NMKL 147.1993; USDA-FSIS: Pathogen/HACCP Final Rule	foods, meat, poultry, seafood
E. coli	BioControl Systems, Inc.	ColiComplete		**AOAC *Official Method* 992.30**	foods
E. coli	BioControl Systems, Inc.	ColiTrak		**AOAC *Official Method* 966.24**	foods
E. coli	BioControl Systems, Inc.	ColiTrak +		**AOAC *Official Method* 988.19**	foods
E. coli	BioControl Systems, Inc.	SimPlate coliform/*E. coli*		EMMAS	foods with some specific limitations
E. coli	bioMerieux	Bactometer			food, aseptic packaging, environmental samples, and pharmaceuticals
E. coli	Biopath Inc.	*E. coli* Identification Swabs			environmental swabs
E. coli	Don Whitley Scientific	RABIT			food and biologic samples
E. coli	Foss Electric A/S	MicroFoss			food and feed
E. coli	Microgen Bioproducts Ltd.	RAPID Tube *E. coli* Test			pure culture
E. coli	Neogen Corporation	GENE TRAK *Escherichia coli* Assay			food and environmental samples
E. coli	Neogen Corporation	ISO-Grid			
E. coli	Scil Diagnostics	BACIdent *Escherichia coli* DNA Detection System			food
E. coli	Sy Lab	BacTrac 4100			food, beverages, hygiene swabs
E. coli O157	ANI Biotech OY	Biocard EHEC			pure culture
E. coli O157	Antex Biologics	VeroTest			
E. coli O157	Becton Dickinson	Difco EZ Coli			
E. coli O157	Binax, Inc	NOW *E. coli* O157 and O157:H7			
E. coli O157	BioControl Systems, Inc.	Assurance EHEC EIA		**AOAC *Official Method* 996.10**	foods
E. coli O157	BioControl Systems, Inc.	VIP for EHEC		**AOAC *Official Method* 996.09**	foods
E. coli O157	Bioline	Immunocapture Device			food
E. coli	bioMerieux	VIDAS *E. coli* O157			food, ingredients

O157		(ECO)			
E. coli O157	bioMerieux	VIDAS Immunoconcentration System			food
E. coli O157	Celsis Ltd.	PATH-STIK			food
E. coli O157	Denka Seiken	*E. coli* O157, O111, O26 IMS Seiken			
E. coli O157	Denka Seiken	*E. coli* O157-CD			
E. coli O157	Denka Seiken	VTEC-Screen "Seiken"			
E. coli O157	Diffchamb Ltd	Transia Card *E. coli* O157			meat, dairy, and other food products; water and environmental samples
E. coli O157	Diffchamb Ltd	Transia Plate *E. coli* O157			meat, fish, dairy and other food products; water
E. coli O157	Dynal Ltd.	Dynal Anti-O157		AFNOR Cert. no. DYN 16/2-0696 FDA-BAM 8th edition Health Canada Compendium of Methods	food, feed
E. coli O157	Eichrom Technologies, Inc.	Eclipse *E. coli* O157:H7			food products and ingredients, water
E. coli O157	Foss Electric A/S	EiaFoss *E. coli* O157			food
E. coli O157	GEM Biomedical	EC Lite			food
E. coli O157	IDG, Lab M Ltd.	Captivate O157			milk and dairy products, meats, fruit juices, slaughterhouse environments, animal feces
E. coli O157	IGEN International	PATHIGEN *E. coli* O157 Test			food
E. coli O157	Kalix	*E. coli* Rapitest			food, ground beef
E. coli O157	Merck KGaA	Single Path *E. coli* O157			
E. coli O157	Meridian Diagnostics	Immunocard STAT			food
E. coli O157	Meridian Diagnostics	Premier EHEC			food
E. coli O157	Microgen Bioproducts Ltd.	Microscreen *E. coli* O157			pure culture
E. coli O157	Molecular Circuitry	Detex System MC-18 for *E. coli* O157 including H7		***Performance Tested Method*** 000301	raw beef and raw poultry
E. coli O157	Morningstar Diagnostics, Inc.	*Escherichia coli* O157 Antigen Detection Test			
E. coli O157	Neogen Corporation	ISO-Grid + SD39 Agar		**AOAC *Official Method***	food
E. coli O157	Neogen Corp.	Alert for *E. coli* O157			ground beef, beef cubes, lettuce, apple cider, other foods and environmental swabs
E. coli O157	Neogen Corp.	REVEAL 8 for *E. coli* O157		AOAC *Official Method 2000.13*	ground beef, beef cubes, lettuce and other foods
E. coli O157	Neogen Corp.	REVEAL for *E. coli* O157		AOAC *Official Method 2000.14*	ground beef, beef cubes, lettuce, apple cider, other

					foods and environmental samples
E. coli O157	Organon Teknika	EHEC-Tek			food
E. coli O157	Oxoid Ltd.	Dryspot *E. coli* O157			food
E. coli O157	Oxoid Ltd.	*E. coli* O157 Latex			food
E. coli O157	Oxoid Ltd.	VTEC-RPLA			food
E. coli O157	PE Biosystems	TaqMan *E. coli* O157:H7 Detection Kit			pure cultures
E. coli O157	PE Biosystems	TaqMan *E. coli* STX1 and STX2 Detecton Kit			pure cultures
E. coli O157	Pro-Lab Diagnostics	Prolex *E. coli* O157 Kit			
E. coli O157	Qualicon	BAX for *E.coli 0157:H7*		*Performance Tested Method* 990701 (note: Adobe Acrobat Reader is needed to view attached .pdf file link)	meat, environmental swabs
E. coli O157	r-Biopharm GmbH	RidaScreen Verotoxin			food
E. coli O157	REMEL Inc.	RIM *E. coli* O157:H7 Latex test			pure culture
E. coli O157	S A Scientific	SAS *E. coli* O157:H7 and *E. coli* O157 Test			pure culture
E. coli O157	Sanofi Diagnostics Pasteur	PROBELIA *E. coli* O157:H7			
E. coli O157	Sun International	C QUIC Plus *E. coli* O157 Test			
E. coli O157	TECRA Diagnostics	TECRA *E. coli* O157 Immunocapture			food and related samples
E. coli O157	TECRA Diagnostics	TECRA *E. coli* O157:H7 VIA		***Performance Tested Method 001101***	food and related samples
E. coli O157	umedik Inc.	DIA/PRO FAST-Q™ *E. coli* O157:H7			foods
E. coli O157	Xenith BioMed	BioGem			
E. coli O26	Bioline	*E. coli* 026 Immunocapture Device			

3.3 Conclusions for *E. coli*

There are a substantial number of commercial products available (Table 6).
Many of these are just modifications of MPN technologies e.g. ColiTrak system (BioControl Systems) which give results in 24-48 hours. GENETRAK (Gene Trak systems) is a nucleic acid hybridisation probe method which takes also 24-48 hours to deliver results and cannot therefore be considered rapid.
The lateral flow devices/dip sticks, following same day or overnight enrichment, appear attractive but are not quantitative and are therefore best suited for presence/absence studies e.g. with *Salmonella* (see later) or enterococci (see report Part 3C).
The most promising candidate appears to be the IDEXX Colilert® system as a quantitative system delivering results within 24 hours. This should form the basis of an interlaboratory trial as part of the next phase of Project Horizontal.

3.4 Potential for the emergence of a rapid method for the detection of *Salmonella* in sludges, soil and biowastes

The numbers of pathogens in waste waters and biosolids varies according to treatment. The numbers of *Salmonella* in raw sludge range from 7 – 8000 per 100 ml (Feachem *et al.*. 1983). In activated sludge, digested sludge or soil the cell numbers range from none detected to a most probable number detected of 400 cells per gram dry weight solids (Yanko *et al.*., 1995).
To be able to develop rapid methods for the detection of *Salmonella* in these matrices there are many factors to be taken into consideration. Obviously sensitivity, specificity and reliability are paramount but also how cost effective is the method in terms of equipment and reagents - in other words is the procedure to be used in smaller laboratories handling relatively few samples or is it to be used in a large scale treatment works where automation may be an option. Also how labour intensive is the method. Is the selected procedure required to be quantitative for viable bacterial cells and/or does it take into account dead and viable but non-culturable (VNC) cells i.e is the method for use before and/or after treatment of sludge which might damage the cells sublethally.

There are two main obstacles to the development of rapid methods:

- Low numbers of *Salmonella* in test samples
- Whether any *Salmonella* present are alive or dead - does the method have to distinguish between the two?

There are 3 routes to the development of a rapid method:

- Modification of the existing culture methods for food and water possibly reducing incubation times, which may occur because of the development of more effective selective media, and development of more accurate confirmation assays. This could involve the combination of existing steps e.g. the S.P.R.I.N.T. (Oxoid): this method employs a combination of pre-enrichment and selective enrichment steps which results in a reduced procedure time. Salmocyst broth (Merck) is also a combined pre-enrichment/enrichment medium. Bacteria grown from these enrichment procedures can then be cultured on conventional selective agars e.g. Rambach agar.
- Direct detection of contamination *in situ*, which would be the ideal method providing sensitivity and specificity are high enough.
- Novel methods for detection employing available new technologies e.g. laser scanning of fluorescently labelled bacterial cells in a flow cytometer or on a membrane (e.g. Chemscan) or scanning fluorescence microscope.

There is an increasing number of molecular, immunological and biochemical tests on the market at this time, many of which need to be validated in comparison with existing methods. These are designed to be used on their own or to be used in conjunction with culture methods for the final confirmatory tests.

3.4.1 Modification of existing culture methods

These usually include a pre-enrichment step in BPW or TSB and may also include a selective enrichment step. The modifications are devised to improve specificity at the end of procedure and may not result in reduced procedure time.

Although rapid end-point tests (e.g. lateral flow devices) enable *Salmonella* to be detected in only a few minutes (see later), even the most sensitive of them requires at least 10^4 cells ml^{-1} of broth. Most naturally contaminated foodstuffs or environmental samples contain far fewer stressed cells ml^{-1}, making the initial enrichment phase essential. To enable stressed *Salmonella*

to be isolated from food in under 48 hours, Oxoid has recently launched the S.P.R.I.N.T *Salmonella* kit which combines pre-enrichment and selective enrichment in a single incubation stage, so that the time taken to complete this step is reduced by half. The kit uses plastic bags, containing slow release capsule of selective enrichment ingredients, and is therefore suitable for use with samples first homogenised in a Stomacher or Pulsifier. When used with an endpoint test such as Salmonella Chromogenic Medium, BAX Salmonella or Oxoid Salmonella Rapid Test, a result can be achieved within 48hours. In a similar way, Merck have introduced Salmosyst broth which is a 2 step pre-enrichment and selective enrichment procedure. Pignato *et al*.[86] used Salmosyst broth as a combined pre-enrichment/selective enrichment broth and Rambach agar for isolation. They found that in artificially contaminated ground beef *S. enteritidis* was detected at a concentration of 10 cfu per 25 g.

3.4.2 Immunomagnetic separation

Latterly, researchers have attempted to overcome the problems of interference from the background matrix, lack of sensitivity of detection and the long process of enrichment by using selective separation with antibodies liganded to magnetic particles. There are 2 principal companies involved in immunomagnetic separation (IMS) of pathogens, Dynal and IDG, and both supply kits which require pre-enrichment of samples in broth culture before capture on superparamagnetic polystyrene beads linked to antibodies. The beads are designed to replace the use of selective enrichment broths, and produce about the same degree of enrichment within 30 minutes as opposed to 24 hours. At the appropriate time, powerful magnets draw the beads to one side of the incubation tube allowing the supernatant containing unwanted material to be aspirated. The beads can then be washed before further analysis of the captured pathogens by PCR, ELISA, staining and microscopy, or culture.

The technology involved in coupling monoclonal or polyclonal antibodies to magnetic beads for the IMS techniques is well established and has been used to detect salmonellae in food (Luk and Lindberg, 1991), and biotoxoids and bacterial spores (Gatto-Menking *et al*., 1995). More recently it has been advocated for the detection of low numbers of *C. parvum* and *Giardia lamblia (intestinalis)* in potable water, post-filtration, and forms the basis of UK and US EPA Methods 1622 and 1623. IMS has also found favour for the selective detection of *E. coli* O157 in food and water (Okrend *et al*., 1992) and faeces (Chapman *et al*., 1996); the detection limit was 1-2 cfu g^{-1} sample. Cubbon *et al*. (1996) found that IMS detection of O157 in faecal samples was more sensitive than culture and compared well with PCR. The main problem when using the IMS technique is the number of sorbitol non-fermenters other than *E. coli* O157 that adhere non-specifically to the magnetic beads. Recovery of the pathogen from enrichment broth is enhanced by using antibody-coated magnetic beads and non-specific binding of other organisms is reduced by washing beads with phosphate buffered saline containing 0.002-0.05% Tween 80 (Anon, 1994).

A new approach to molecular labelling without a PCR step is molecular labelling, using for example DNA oligonucleotide probes linked to biotin (e.g. LightOn Salmonella, Aureon, Vienna). The assay is performed very similarly to a regular ELISA procedure. The detection and confirmation is done with luminescence labelled reagent rather than colorimetric. The nucleic acid based hybridization yields the specificity required for immediate confrmation. Once the probes hybridise to the overnight culture of target cells, they can be labelled with streptavidin-linked to an enzyme producing fluorescence or light for sensitive detection. The sensitivity by light detection is about 100-fold more sensitive than the colorimetric detection in ELISA procedures. The sample is read by the 96 well Mediators PhL, an ultrasensitive luminometer, in less than 2 minutes. No target amplification is necessary. The assay is suitable for the detection and confirmation of all *Salmonella* spp., either as picked colonies from agar culture, or directly from enriched samples treated with Salmonella A-*Beads*. The *Salmonella* A-*Beads*™ are polydisperse 1.5µm cluster type paramagnetic particles covalently coupled with

antibodies raised against surface epitopes of *Salmonella.* They are designed for the fast and specific isolation of *Salmonella* from food and environmental samples. Any food, feed and environmental samples that contain a minimum of 100 *Salmonella* per ml of sample will yield a positive result. A-*Beads*™ coupled with an antibody to *Salmonella* will bind specifically to *Salmonella* in a mixed flora sample matrix. Using MagnetOn™, the target bacteria is isolated from the sample matrix and is ready for subsequent detection. The recovery of *Salmonella* from the sample is signficantly increased and yields almost pure *Salmonella* bacteria for optimal detection signal. Following enrichment, the *Salmonella* A-*Beads*™ will detect 1 viable organism in a 25g sample if present. The bacterial RNA is released from the bacteria upon lysis and is captured by an immobilized DNA-oligonucleotide. The captured RNA is hybridized with a fluorescein-labelled detection probe. This probe is detected by an alkaline phosphatase conjugated anti-fluorescein antibody. The action of alkaline phosphatase on the fluorescein substrate causes decomposition of a chemiluminescent intermediate and the energy released is emitted as light, which can be measured in the 96 well microplate luminometer. The system is also applicable to detection of *E. coli* O157.

Several methods have used an immunomagnetic separation (IMS) technique to concentrate the cells from a complex matrix, usually food, before proceeding with molecular or immunological steps. Wang *et al*.. (1999) used an 18 hour pre-enrichment broth followed by IMS and flow cytometry on washings of chicken carcasses.

The PATHATRIX system described earlier for *E. coli* O157 detection is also suitable for *Salmonella.* MATRIX MicroScience Ltd. has launched a unique rapid detection and positive identification system, which simultaneously tests for *Listeria* spp and *Salmonella* spp contamination in food samples. Previously, tests for each pathogen have had to be conducted separately.Giving completed test results in just 40 hours, the new PATHATRIX Dual test, has received AOAC* RI Validation after an extensive evaluation process at Campden & Chorleywood Food Research Association (CCFRA). MATRIX's PATHATRIX system has also received AOAC accreditation for the individual testing of *E. coli* O157, *Listeria* spp and *Salmonella* spp. As a result of the Dual test, laboratories will no longer have to conduct two separate tests, weigh both sets of samples or prepare two sets of selective media.

The single sample requires only addition of Buffered Peptone Water and the process achieves significant savings in terms of both equipment and consumables. Utilising the proven PATHATRIX technology, the method requires less than two minutes hands-on time per test. Viable cultures are produced during the test allowing full and detailed analysis of any positive results. A standard 25g food sample is homogenised with 225ml of growth media in a stomacher and is incubated overnight. PATHATRIX capture reagent, which consists of antibody coated magnetic particles specific to the target pathogen, are then added directly to the sample. The sample is loaded onto the PATHATRIX workstation using a MATRIX proprietary consumable pack, connecting the sample to the circulatory system in preparation for the Capture-Culture step. Once loaded, the PATHATRIX workstation is pre-programmed to run for 30 minutes at the desired incubation temperature. Upon completion of the run, the target microorganisms are bound onto the phase by the capture reagent. Residual debris and non-specific binding are removed during a single wash step. The capture phase is disconnected from the system and the capture reagent/pathogen complexes are eluted by washing the phase into a vessel. The captured pathogen complexes are then concentrated into a small volume. i.e., 200 •l using a magnetic rack. The sample can be plated directly onto selective media and incubated overnight for visualisation the following morning. In the case of the new Dual test, the single sample is simply split over two plates, each containing the appropriate media for the target pathogen. The standard PATHATRIX test enables colonies to be viewed within 40 hours from point of sample without the interference from other non-target organisms that are seen in conventional tests.

3.4.3 Molecular probes

There is a confusing array of *Salmonella* primers for use in PCR techniques currently available. Lofstrom *et al.*. used PCR to detect *Salmonella* in animal feeds DeMedici *et al.* (2003) compared using PCR and ELISA after a pre-enrichment step in meat samples and were able to detect 1-10 cells per 25g. Gado *et al.* (2000) used 2 primers (INVA 1/INVA) in a PCR, following pre-enrichment and selective broths, but experienced sensitivity problems.

The Bax system (DuPont) is the first commercially PCR-based approved method for food (AOAC Certified USA). The pre-enrichment has to produce at least 1000 cells per ml of culture. A lysate is made of this suspension followerd by PCR with specific primers. The manufacturers claim this test is 10 to 100 times more sensitive than immunoassays. It takes 4 h to obtain a result. The BAX Salmonella test is now commercially available (e.g. UK distributor is Oxoid) for food and may be applicable to sludge, soil and biowastes.

The GENE-TRACK nucleic acid hybridisation assay has been compared to conventional culture (Meckes and MacDonald, 2003) in the detection of *Salmonella* spp. in biosolids. The method has been used before successfully on foods. All samples were homogenised, pre-enriched in BPW followed by incubation in RV broth. Samples from these broths were tested by the molecular probe or sub-cultured onto selective agars or broths. There was some discrepancy between the two testing laboratories as each had slightly different protocols and the original samples were different (one tested poultry farm washings, the other biosolids before and after treatment. They concluded, however, that both methods gave equivalent results but the probe yielded results in 52 hours as compared to 120 hours for the culture methods. There was no indication of sensitivity and they did not use spiked samples.

Unfortunately *Salmonella* cannot be detected at the species or genus level using rRNA probes as there are no suitable oligonucleotide probes published to discriminate amongst the other coliforms of the δ subclass of Proteobacteria. Lin and Tsen (1995) confirmed this lack of specificity using 3 probes for the V3 to V6 region of the 16S rRNA gene of *S. enteritidis* and the cross reactivity with other coliforms. This lack of specificity was confirmed by Perry-O'Keefe et al., (2001). However, Nordentoft *et al.* (1997) selected an 18-mer oligonucleotide probe nn the basis of the 23S rRNA gene sequences representing all of the S. enterica subspecies and S. bongori. The specificity of the probe was tested by in situ hybridization to bacterial cell smears of pure cultures. Forty-nine of 55 tested Salmonella serovars belonging to subspecies I, II, IIIb, IV, and VI hybridized with the probe. The probe did not hybridize to serovars from subspecies IIIa (S. arizonae) or to S. bongori. No cross-reaction to 64 other strains of the family *Enterobacteriaceae* or 18 other bacterial strains outside this family was observed. The probe was tested with sections of formalin-fixed, paraffin-embedded tissue from experimentally infected mice or from animals with a history of clinical salmonellosis. In these tissue sections the probe hybridized specifically to Salmonella serovars, allowing for the detection of single bacterial cells. The development of a fluorescence-labelled specific oligonucleotide probe makes the FISH technique a promising tool for the rapid identification of S. enterica in bacterial smears, as well as for the detection of S. enterica in histological tissue sections. Similarly, it could have value as aprt of a rapid method for detecting Salmonella in sludge, soil and biowastes.

3.4.4 Immunoscreening

A large proportion of immunolabelling is used as culture confirmation of species identity following enrichment and agar culture, using enzyme immunoassay (EIA) (also called enzyme linked immunosorbent assay; ELISA) or latex bead agglutination (LBA) augmented with serotyping and phage typing. However, these techniques can also be used without the agar

culture step to speed up the recovery/detection time. A typical format for EIA or ELISA involves the coating of rabbit polyclonal or mouse monoclonal antibody to the wells of microtitre plates followed by introduction of the test sample. Protein or polysaccharide (lipopolysaccharide) antigens present in the sample are bound immunologically by the antibody. After washing to remove unbound material, enzyme-conjugated affinity-purified antibody specific to the target antigen is added. Following a second washing step to remove unbound enzyme-conjugated antibody, enzyme substrate is added and the incubation proceeds until stopped e.g. by addition of acid or alkali which also helps develop the product colour. Typical enzymes used include alkaline phosphatase (with p-nitrophenyl phosphate as substrate) and horseradish peroxidase (with 3,3',5,5'-tetramethylbenzidine (TMB) as substrate and hydrogen peroxide) Most EIA kits have a sensitivity of approximately 10^6 organisms ml^{-1}, and therefore usually require a concentration step (filtration, IMS and/or pre-enrichment). Pre-enrichment in appropriate medium may be obligatory if there are concerns that epitope expression, e.g. flagellar antigen, is affected by the environment.

Many rapid manual immunoscreening assays are commercially available to detect *E. coli* O157 and *Salmonella* spp. These tests are performed on heat-killed culture broth after 24 hours for *E. coli* O157 and 40-48 hours for *Salmonella* spp. Microtitre well-based ELISAs (e.g. Microgen Salmonella ELISA) and dipstick ELISAs (e.g. Lumac Salmonella Path-Stick) are also commercially available for these pathogens and are recommended for wastewater treatment. Organon Teknika have gone a step further by introducing an ELISA for *E. coli* O157 incorporating immuno-capture beads (EHEC-Tek). Recently, the manual ELISA tests have been adapted for use in automated instruments and greatly increase the capacity of a laboratory to perform up to 100,000 tests per annum on one instrument. ELISA has been used to detect the presence of enterotoxigenic *E. coli* in water[76] and *S. enteritidis* in raw sewage, sludge and wastewater (Brigmon *et al*., 1992).[77] ELISA technology is maturing rapidly and can be included in 96-well plates for automated reading and software manipulation. However, a major disadvantage of the technique is the lack of sensitivity. A minimum of 10^5 *S. enteritidis* ml^{-1} are required to generate a clear signal against the background.

LBA provides the least technically demanding method and, as the name suggests, relies on the agglutination of microscopic latex beads which are liganded with a specific polyclonal or monoclonal antibody to an epitope expressed by the microorganism. The preparations become cloudy or clump, which can be seen against a dark background. Sensitivity of detection varies from 10^2 - 10^6 cells ml^{-1}, depending on the avidity of the antigen-antibody reaction. For example, *E. coli* O157 can be detected with latex bead agglutination using antibodies raised against the lipopolysaccharide O157 antigen, reversed passive latex agglutination and passive haemagglutination. Isolates can also be serotyped with antisera and phage typed, as demonstrated successfully by Rahn *et al*.[78] in a detailed study of *E. coli* O157:H7 persistence in human and animal faeces.

However, Brehm-Stecher and Johnson (Food Research Institute) have developed a simplified DNA based FISH protocol that yields bright genus-specific hybridisation results in 10 minutes. They also demonstrated the compatability of FISH with the use of the respiratory substrate 5-cyano-2,3-ditolyl tetrazodium chloride (CTC), a red fluorescent viability indicator. They used flow cytometry and fluorescence microscopy to evaluate the fluorescent cells. This work gave simultaneous detection and viability determination of *Salmonella* in food. As with any molecular method the specificity of the probes is the key but if this method could be properly validated it could potentially be of great value in the screening of food, environmental and medical samples for salmonella.

3.4.5 Lateral flow devices

As discussed for *E. coli*, there have been rapid advances with lateral flow devices, including Visual Immunoprecipitate assay (VIP; BioControl System Inc.) and gold labelled

immunosorbent assay (GLISA) technologies such as the Merck Singlepath range for *E. coli* O157, *Salmonella* etc. The *Salmonella* test is carried out after a selective enrichment of the sample from one selective enrichment culture (taking 24-48 h) and yields a yes /no result in 20 minutes. The end result is thus available two days sooner saving material and labour costs.

Techra Salmonella Visual Immunoassay (VIA) kit includes a single selective enrichment step and takes 22 hours to get results. This method has undergone large scale trials in food industry and is now an AOAC official method

3.4.6 Biochemical testing profile

Improvement is biochemical screening do not really reduce the length of the total testing protocol but they do improve the sensitivity and specificity. The major tests available are the API (BioMerieux), Microbact (med Vet, supplied by Microgen Bioproducts) and the Automated VITEK.
These systems are used predominantly to identify isolated colonies of bacteria and yeasts by comparing biochemical activities under defined growth and assay conditions i.e. detecting the biochemical phenotype. The results are compared to databases of standard strains but, because of market forces, have concentrated on identification of human pathogens from a clinical environment rather than a wide range of microorganisms from the environment at large.

The API systems (bioMerieux, Basingstoke) measure activities based on species characteristics known for many years, including catalase, oxidase, nitrate reduction, urease, indole production, and sugar fermentation activities to produce coloured reactions. The API 20E Enterobacteriaceae system comprises 20 tests including ornithine decarboxylase, arginine dihydrolase, melibiose and inositol fermentation, and citrate utilisation. A 10 test system is also available. The API 20E and the similar Sensititre autoidentification system were both poor in discriminating between species of the genera *Klebsiella*, *Enterobacter* and *Serratia* (Barr *et al*., 1989). A later study showed that the API 20E identified 77% of *Enterobacteriaceae* strains correctly at the end of the initial incubation and subsequently identified >95% correctly when the additional tests were performed, as recommended by the manufacturer (O'Hara *et al*., 1993). The system was particularly good at identifying *E. coli* strains, as well as most *Salmonella enteritidis* and *Shigella* spp. The API 20E system has been shown to identify many yellow colonies of atypical *E. coli* isolated on m-FC medium from stream water; many of these strains were ONPG-positive and some produced gas in lactose medium at 44.5°C (Rychert and Stephenson, 1981). However, comparing fatty acid methyl ester (FAME) analysis with the use of API 20E gave good identification of approx. 30% of coliforms isolated on MacConkey agar from river water (Brown and Leff, 1996). Microgen Bioproducts now market the Microbact 12E and 24E Gram-negative identification system from Medvet (Mugg and Hill, 1981).

Automated systems are also produced; they include the Rapid ID 32E for *Enterobacteriaceae*, which gives results in 4 hours, and the VITEK AutoMicrobic system which uses a series of comparison cards for either detection, identification or susceptibility testing. The VITEK GNI card gave good identification (93%) of members of the *Enterobacteriaceae* within 4 to 18 hours (O'Hara et al., 1993) while the VITEK EPS card gave good discrimination of *Salmonella*, *Shigella* and *Yersinia* spp. (99.5%) after 4-8 hours incubation (Imperatrice and Nachamkin, 1993). This card is composed of 3 sections, with 10 wells in each section, allowing the user to test 3 different colonies on each card.

By contrast, the BIOLOG identification system (Don Whitley) employs a redox dye, tetrazolium violet, as an indicator of substrate utilisation. The cell's metabolism of the test substrate results in the formation of NADH, which, in order to be reoxidised, passes electrons to the dye via an electron transport chain to produce a purple formazan. The system is thus able to detect the ability to metabolise a range of carbohydrates, amino acids, peptides, and carboxylic

acids incubated in a 96 well format for automated reading. Active growth in the wells is not required. The GN Microplate system has been used to identify over 600 Gram negative strains, including each of the 4 *Shigella* species (47-93% identification), *E. coli* (80% identification) and a range of *Salmonella* spp. (7-100% identification) (Holmes *et al*., 1994). For greater discrimination, BIOLOG introduced the ES Microplate system to characterise and/or identify different strains *of E. coli* and *Salmonella* spp. It is up to the user to build their own database, as described for *Legionella* spp. using the GN Microplate system (Mauchline and Keevil, 1991).

The metabolic phenotype of microorganisms is subject to the variability of growth and assay conditions. A trial organised by Yorkshire Environmental circulated 23 isolates to 40 participating UK and European laboratories to assess reproducibility between laboratories for biochemical identification of coliform bacteria. Only 3 isolates were consistently identified by all of the participants (Anon, 1998). Indeed, up to 9 different genera were reported for a single sample.

3.4.7 Genotypic profile

The poor performance of phenotypic identification in this study and elsewhere prompted Qualicon, Inc to develop the RiboPrinter® system for DNA pattern identification of each isolate. This system analyses the genotype of a strain using *Eco*R1 restriction endonuclease to produce a characteristic fingerprint and is therefore not dependent on phenotype variability due to culture and assay conditions. RiboGroups are created by the system when patterns fall within specific similarity thresholds. Any pattern that does not meet this threshold will form a new RiboGroup. The original system was supplied with a database of 69 *Escherichia*, 97 *Salmonella*, 80 *Listeria* and 252 *Staphylococcus* pattern types, and has now been extended to over 600 patterns at the Cornell University Laboratory of Molecular Typing, including *E. coli* 0157. During the Yorkshire Environmental trial, two pairs of duplicate strains were circulated among 36 laboratories; a surprising 56% of the laboratories could not tell that each pair of strains were the same. The RiboPrint patterns showed unequivocally that the samples were duplicates. Some the DNA of some important species, such as *Campylobacter*, does not cut efficiently with *Eco*R1 but this can be substituted for other restriction endonucleases to produce specific patterns for comparison with type strains.

3.4.8 Impedance

Impedance technology is a rapid, automated qualitative technique which measures in a medium the conductance change induced by bacterial metabolism (Silley and Forsythe, 1996). The detection time is a function of both initial microorganism concentration and growth kinetics in a given medium. Specificity is incorporated into the technique by including either selective agents into the incubation broth and/or specific substrates. Thus, Easter and Gibson (1985) described an impedance technique in which changes in electrical conductance due to reduction by salmonellae of trimethylamine-N-oxide were monitored. By contrast, Bullock and Frosham (1989) pre-enriched salmonellas from contaminated confectionery in skimmed milk before 24 hour impediometry in lysine-iron-cystine-neutral red broth in a Bactometer 123 system (Bactomatic Ltd., Henley). The authors found that the inclusion of novobiocin (0.15 µg per well) eliminated false positive results due to *Citrobacter freundii* or *Enterobacter cloacae*. Pridmore and Silley (1998) used the Rapid Automated Bacterial Impedance Technique (RABIT, Don Whitley, Shipley) to detect total coliforms, thermotolerant coliforms and enterococci in domestic sewage and 70% industrial sewage from 2 wastewater treatment works. The coliforms were detected in Whitley MacConkey broth at 37°C and 44°C using the direct impedance technique. The majority of faecal coliform results were obtained within 7 hours (10^3 cfu ml^{-1}) compared to 24 hours using membrane filtration on MLSB, and without the need for serial dilution of samples and manual reading of plates.

The indirect impedance technique allows the use of components inappropriate in the direct method on account of high basal conductance. This method is based on the detection of carbon dioxide released by microorganisms into the culture medium, and which is absorbed in an alkaline solution in contact with the electrodes of the tubes. Blivet *et al*. (1998) proposed a new medium named KIMAN (Whitley Impedance Broth basal medium supplemented with 3 selective components: potassium iodide, malachite green and novobiocin,). This medium supported the growth of *Salmonella* serotypes, while inhibiting non-salmonella strains in pure culture, and was appropriate for the indirect impedance technique. As mentioned previously, the use of novobiocin is very important to suppress background competitors such as *Proteus* spp. and is included in the enrichment media specified in several of the draft MF and presence/absence CEN methods for detecting *Salmonella* in sludge.

3.5 Conclusions about the possibility of producing a standardised rapid method for the detection of *Salmonella* species in sludge

3.5.1 Possible routes of analysis

The possible routes of analysis for detection of *E. coli* and *Salmonella* in sludge, soil and biowastes are summarised in Figure 3. Four main paths are described, involving:

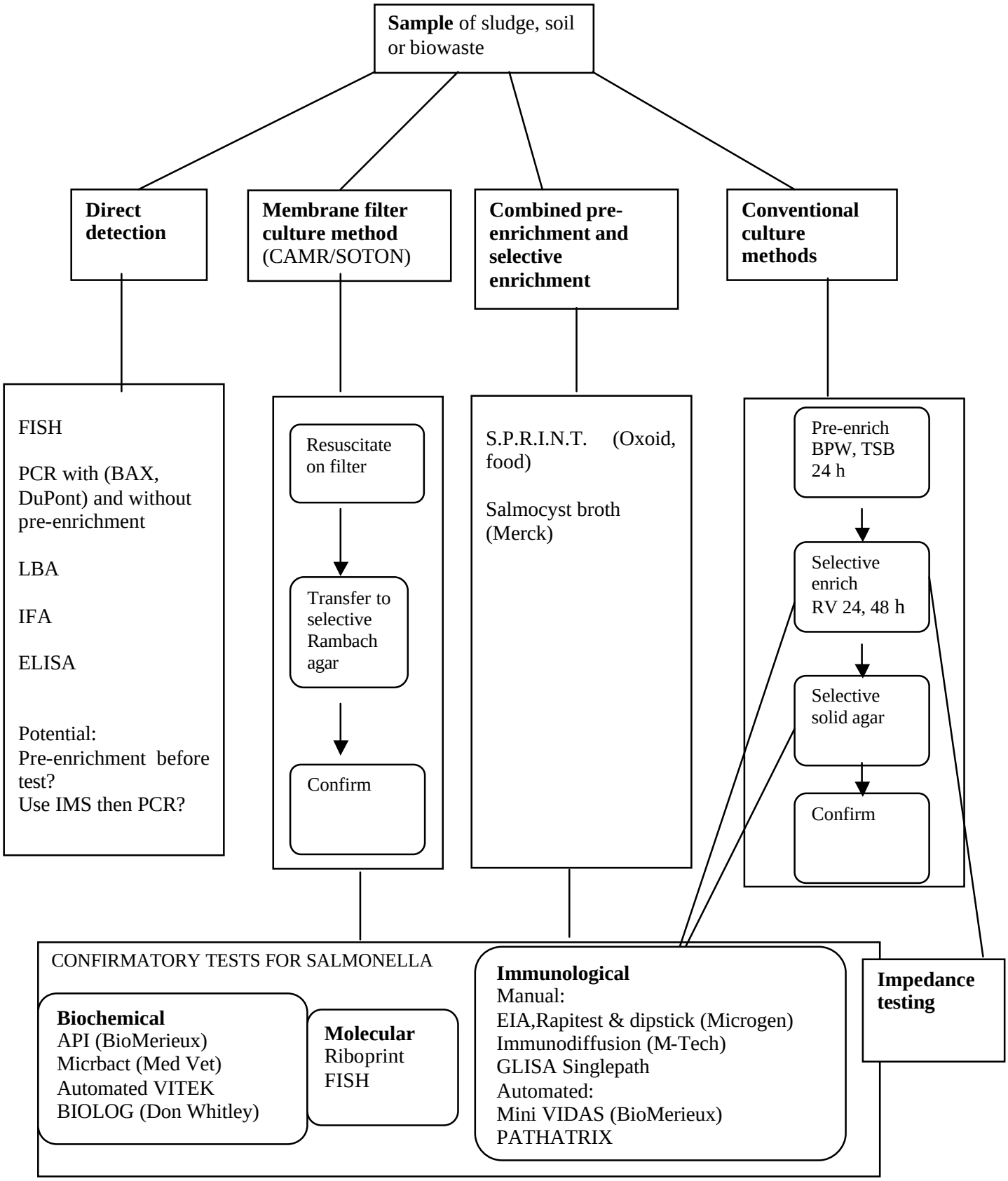
- Conventional culture
- Membrane filter resuscitation
- Combined pre-enrichment and selective enrichment
- Direct detection

The conventional culture and membrane filter resuscitation procedures have been reviewed extensively in Part 3A of Project Horizontal and, in any case, they take at least 48 hours to deliver a result. Consequently they will not be discussed further.

Pre-enrichment and/or enrichment steps for *Salmonella* take 24-72 hours but there may be scope for improvement. For example, the PATHATRIX recirculating IMS assay enriches *Salmonella* and delivers the result in 40 hours. The Singlepath GLISA is suggested to deliver a result in 24-48 hours. Therefore, perhaps either assay could have their enrichment step optimised in readiness for a robust assay procedure subject to inter-laboratory trial. In this regard, it is noteworthy that the PATHATRIX automated system requires a capital outlay of approximately 60,000 euros whereas the manual Singlepath system requires only consumable costs. Ultimately, there will need to be a comparison of performance (specificity and sensitivity) versus overall cost of analysis (including staff time).

It is still not apparent that the molecular and immunological methods available for direct detection are suitable for detection of *Salmonella* in sludge, soil and biowastes at less than 1 cell per g. Perhaps this situation could be improved by using immunological clean-up recovery (e.g. IMS or recirculating IMS (PATHATRIX) without pre-enrichment) followed by a sensitive PCR procedure not affected by an inhibitory background. At this point, the robustness of the detection primers will then need to be evaluated during inter-laboratory trial.

Figure 3 Routes for analysis of *Salmonella* in sludge, soil and biowastes



4. CRITICAL POINTS AND RECOMMENDATIONS

4.1 Sampling

Part 3A of Project Horizontal has considered sampling, health concerns during handling, storage and transport in great detail and does not need reiterating other than that the Horizontal sampling study should start to give an idea of the magnitude of the uncertainties of sludge, soil, and biowaste sampling with respect to chemical and physical analysis. It is essential that this key aspect of sampling for microbiological analysis is also addressed. Typical uncertainties associated with sampling for *E. coli* and *Salmonella spp.* and their analysis in various sludge, soil, and biowaste matrices need to be estimated. SCA (1977) has published procedures for sampling and initial preparation of sewage and waterworks' sludges, soils, sediments and plant materials prior to analysis. However, this publication only discusses chemical and physical testing. There is little published on protocols for microbiological sampling of sludge, soil, and biowastes. It is important that this key area is properly addressed.

4.2 Evaluation of Potential Methods

This report has concentrated on rapid methods i.e. typically able to detect the target organisms in a complex, heterogeneous organic matrix in less than 24 hours. There are many potential rapid methods available but not all are suitable for the requirements of sludge, soil and biowaste analysis. In particular, there are two requirements demanded in the draft standards (Appendix 1):

- A quantitative requirement to detect less than so many *E. coli* per weight of material, as well as being able to demonstrate process efficiency through a 6-log drop for endogenous *E. coli* and a spike organism such as *S. senftenberg* in some draft standards.

- A sensitive presence/absence requirement to show absence of *Salmonella* per weight of material

The first requirement may appear to be met for *E. coli* using the Colilert® or Colilert-18® system in under 24 hours, and this should be a priority for inter-laboratory evaluation in the next phase of Project Horizontal. The requirement for quantification of *Salmonella* is more difficult because there is not an equivalent of the Colilert® technology available for these pathogens. The current draft CEN and SCA methods take at least several days (and see below).

For the second requirement, it is still not apparent that the molecular and immunological methods available for direct detection are suitable for detection of *Salmonella* in sludge, soil and biowastes at less than 1 cell per g. Perhaps this situation could be improved by using immunological clean-up recovery (e.g. IMS and recirculating IMS: PATHATRIX) followed by a sensitive PCR procedure not affected by an inhibitory background. Consequently, the second requirement might have to be met in under 24 hours if there is a reliable enrichment procedure for *Salmonella*. The problem is that many methods using pre-enrichment/enrichment media specify at least 48 hours, partly because of the slow growth rates of some of the serotypes, particularly if they have been environmentally stressed and become sub-lethally damaged. Further work will be required to try and accelerate the enrichment process and select more optimal growth conditions. For now, there are various procedures which reliable enrich *Salmonella* in 48 hours, including the draft CEN presence/absence method for sludge and commercial systems such as the S.P.R.I.N.T pre-enrichment/enrichment (Oxoid). Once grown

out, the *Salmonella* can be reliably detected in minutes using lateral flow devices such as the GLISA Singlepath (Merck). These are much faster than PCR techniques, simpler to use in a busy laboratory and arguably cheaper.

It is recommended that the Singlepath or other devices be evaluated in Project Horizontal. At least this approach will give a result in 48 hours, possibly 24 hours, unlike present methods taking up to 4 or 5 days.

5. DRAFT STANDARD (CEN TEMPLATE)

5.1 Future rapid methods

Many of the methods described in this report usually take 24-72 hours to accomplish, and cannot therefore be considered rapid. As mentioned previously, there are advances in molecular biology involving PCR and 16S rRNA FISH analyses, but these are not reliably quantitative. Currently, they are best used for culture confirmation.

Similarly, there have been rapid advances with IMS (Areon LightOn) and recirculating IMS (PATHATRIX), and lateral flow devices and gold labelled immunosorbent assay (GLISA) technologies such as the Merck Singlepath range for *E. coli* O157 and *Salmonella* etc., discussed earlier. With appropriate development for sludge, soil and biowaste, they may have great potential for the future.

5.2 Conclusions as to the development for a method for *E. coli* in sludge/soils

This report has highlighted that many of the methods available have been developed for low turbidity water and sometimes food. The challenge for sludges, soil and biowastes is to develop methods capable of handling high turbidity and dry matter, complex matrices. There are strengths and weaknesses for both the membrane filtration and multiple tube MPN broth techniques, which have been summarised for their ability to analyse coliform bacteria (Table 7; WHO, 1997) as follows:

Table 7 Comparison of MPN and MF methods

Most probable number method	**Membrane filtration method**
Slower: requires 48 hours for a negative or presumptive positive result	Quicker: quantitative results in about 18 hours
More labour intensive	Less labour intensive
Requires more culture medium	Requires less culture medium
Requires more glassware	Requires less glassware
More sensitive	Less sensitive
Result obtained indirectly by statistical approximation (low precission)	Result obtained directly by colony count (high precision)
Not readily adaptable for use in the field	Readily adaptable for use in the field
Applicable to all types of water	Not applicable to turbid waters
Consumables readily available in most countries	Consumables costly in many countries
May give better recovery of stressed or damaged organisms under some circumstances	

Inevitably, the method requirement will be based on regulatory considerations. Should there be demonstrable process control procedures involving, for example, demonstrating a 6 $\log_{10}$-decrease on treatment or should there be merely a requirement for absence in 10, 25 or 50 g wet weight or dry weight of sample?

The current drafts of the UK and EC sludge and biowaste standards require effectively a quantitative method to satisfy that the treatment process shall achieve at least a 6 Log_{10}

reduction in *Escherichia coli* to less than 500 cfu / g (EC draft Sludge Directive, 2000) or that a set of samples shall contain no more than 1000 *E. coli,* {i.e. cfu /g dry weight) (UK draft Sludge Amendment, 2002).

- This suggests, given the strengths and weaknesses described in Table 7, that MF techniques are preferable. However, as described here and in Part 3A of Project Horizontal, these take 24 hours for *E. coli* and least 48 hours for *Salmonella*. Consequently, they are not truly rapid. It is also noteworthy that the conclusions of the WHO (1997), when comparing MF and MPN techniques, did not include an assessment of the Quanti-tray technology which was just becoming available. If Regulators accept that the low errors reported of this technology are satisfactory then this technology may eventually gain the upper hand for quantifying the presence of E.coli in sludge, soil and biowastes.

5.3 Conclusions as to the development for a rapid method for *Salmonella* in sludge, soils and biowastes.

The current drafts of the UK and EC sludge and biowaste standards require that there may be 2 types of analysis, presence/absence and quantitative to demonstrate a 6-log decrease of spiked cells during treatment :

There is some possibility of developing a rapid presence/absence test that would meet the requirements of being specific, sensitive and preferably cheap (including labour costs). This will require a 40-48 hour pre-enrichment and/or enrichment (but ideally 24 hours if this can be optimised) followed by detection involving by immunological capture and detection (IMS, recirculating IMS or lateral flow), ISH or PCR.

Any of these could be made semi-quantitative, by running serial dilutions, for example in microtitre plate format, confirming positive wells using the detection technology and applying look-up tables to calculate the MPN. However, this would increase cost and time to deliver the result. In any case the requirement for a rapid assay appears more pressing to confirm absence in a sample before the bulk waste is released for recycling, rather than verify process treatment efficiency which can be a more leisurely procedure. The draft CEN and SCA methods already cover this aspect. In reality, the enrichment and detection procedures such as lateral flow come into their own for presence/ absence determination e.g. no *Salmonella* in 10 or 50 g wet weight sample. This approach would overcome problems with having to disperse and filter a complex, fibrous matrix such as soil or biowaste for quantitative analysis whilst giving a specific identification of the live organism without further tedious, expensive confirmation tests.

5.4 General Conclusion

It is clear from the above that there are a variety of methods available, reliant on either pre-enrichment culture (followed by immunological capture and detection, ISH or PCR), membrane agar culture, test tube MPN broth or Quantitray® technologies. They all have strengths and weaknesses, dependent on not only the Regulators' types of requirements for sludge, soil and biowaste analysis but also their sensitivity, specificity, speed and cost. It is unlikely therefore that there can be only one methodology applicable to both *E. coli* (and *E. coli* O157) and *Salmonella* detection. None of the methods described have been extensively evaluated for sewage sludge, soils or biowastes. As such, there is an urgent need for their modification and evaluation as part of the next phase of the Project Horizontal.

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