Desk studies on feasibility of horizontal standard methods for detection of *Clostridium perfringens* and Enterococci

Sarah L. Warnes and C.William Keevil

University of Southampton

Acknowledgement

Summary

The existing methods currently available for the detection and enumeration of Clostridia and Enterococci in sludges and treated biowastes have been evaluated with a view to possible standardisation. The main methods used for the detection and enumeration of *Clostridium* and *Enterococcus* spp. have been developed largely for analysis of food and water and can be broadly divided into three groups. Quantification of colonies on agar media; most probable number (MPN) quantification in indicator broth using conventional test tube technology; and proprietary Quantitray® technology equivalent to the 5-tube MPN technique employing disposable plastic trays for enumeration of enterococci. The merits of each are described.

Scope of the Report

This report is one of five Project Horizontal reports that attempt to assess hygienic parameters, which may be needed to assure the sanitisation of sludge and treated biowaste. The five reports highlight draft potential methods for the hygienic parameters likely to be included in future sludge and biowaste Directives. Task 3A, *Horizontal Standards for* Escherichia coli *and* Salmonella *spp. in Sludges and Treated Biowastes*, deals extensively with general aspects of sampling requirements and performance of inter-laboratory trials. Therefore these will not be considered here.

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 and treated 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 helminth ova (cestodes and nematodes). For parameters likely to be included in future Directives (i.e. *E. coli, Salmonella spp.* and *Clostridium perfringens*), 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. ***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. In the Sludge and Biowaste draft directives (EU 2000 and EU 2001), *Clostridium perfringens* is specifically mentioned. This leads to the logical choice to start the work on this organism as one of the parameters in phase 1 of Project Horizontal. For the other parameters, desk studies to prepare draft potential protocols for CEN and ISO discussion are also being prepared.

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.

Clostridium introduction

The genus *Clostridium* comprises the Gram-positive, spore-bearing anaerobic bacilli. There are more than 100 species and some are able to grow slowly in trace amounts of air. Most species of this genus are saprophytes that normally grow in soil, water and decomposing organic material. Some species are commensal inhabitants of animal and human intestine, the most characteristic of which is *C. perfringens (C. welchii),* and are important after the hosts death in the decomposition of the corpse. A few species are opportunistic pathogens e.g. *C. perfringens* (gas gangrene), *C. tetani* (tetanus) and *C. botulinum* (botulism). With only a few exceptions the bacteria produce powerful exotoxins which result in clinical symptoms associated with disease resulting from clostridial infection. All clostridia produce resistant spores that enable

4

the organism to survive adverse conditions. These spores can survive for significantly longer periods than vegetative bacterial cells and may be resistant to levels of chlorination used in water treatment; therefore, testing for presence of clostridia can be indicative of the efficiency of the water treatment process or past faecal contamination. Because of their longevity, they have been regarded as indicating intermittent or remote contamination and are not recommended for routine monitoring of distribution systems (WHO, 1997). There is some dispute about the value of testing for clostridia in treated waters, as testing for oocysts of the protozoan parasite, *Cryptosporidium parvum*, may be of more value. Clostridial spores are similar in size to *Cryptosporidium* oocysts and have similar chlorine resistance properties. *C. perfringens* spores have been demonstrated to be useful surrogate indicators for monitoring water treatment processes for the removal not only of *Cryptosporidium* oocysts but also *Giardia* cysts and viruses (Payment *et al.*,1993).

Although large numbers of *C. perfringens* can produce a severe but self-limiting diarrhoea in humans, the low numbers that may occasionally be found in water supplies are not considered a health risk as the organism cannot grow in water to a significant degree and does not produce toxins in this environment.

Gastroenteritis caused by *C. perfringens* usually results from ingestion of uncooked meat or poultry or food that has been cooked, killing vegetative cells but not clostridial spores. If this food is stored for a length of time, even in a refrigerator, the spores may germinate and grow in the food to sufficient concentrations of organisms that could result in food poisoning.

As in the water treatment plants, there is huge commercial interest in the use of clostridial spores as an indicator of food processing efficiency by the food industry. New methods, often molecular, are continually being developed to isolate clostridia from food matrices. Broda *et al.* (2003) describe PCR detection of clostridia in 'blown pack' meat spoilage: *C. estertheticum* and *C. gasigenes* were the main species responsible. Other investigations have tested glass-bottled foods (Fugisawa *et al.*, 2000) and frozen foods (Cordoba *et al.*, 2001).

There is an increasing number of methods for the typing of clinical isolates. These methods usually involve detection of clostridial enterotoxin or the *cpe* gene that encodes it. There are many genotypic methods including plasmid analysis (Eisgruber *et al*., 1995), ribotyping (Kilic *et al*., 2002; Schalch *et al*., 1997,1999), PCR (Miwa *et al*., 1997; Fach *et al*., 1997; Lukinmaas *et al*., 2002; Song *et al*., 2002; Schoepe *et al*. 1998; Kim *et al*. 2000; Augustynowicz *et al*., 2002), HPLC (Harpold *et al*., YEAR), pulsed field gel electrophoresis (PFGE) (Maslanka *et al*., 1999) and amplified fragment length polymorphism (AFLP).

The basic culture media developed for the isolation of clostridia are constantly being revised and new media developed. The importance of testing for the presence of clostridia varies across Europe. In the UK many water authorities do test for clostridia but many also do routine testing for *Cryptosporidium* oocysts in preference, partly due to regulatory requirements. In Spain, clostridia are not used as indicator organisms but as pathogens in their own right. In Germany there is no routine testing for clostridia, only occasional testing of surface water and rivers.

Existing methods and culture media for Clostridia

Conventional methods for the detection of clostridia have traditionally incorporated heat killing of vegetative cells of clostridia and contaminating bacteria (to identify the presence or quantify the clostridial spores present). This is followed by the use of a nutritionally rich base medium, e.g. meat broth or blood agars, to promote spore germination. The addition of starch in many media is to facilitate germination and in some methods gentle heating of the sample prior to inoculation is recommended (de Jong *et al*. 2002)(e.g. DCA medium described later).

Reinforced Clostridial Medium (RCM) is based on a basic nutrient medium developed in the 1950s (Hirsch *et al*., 1954) Use of this medium may also result in non-selective growth of contaminating bacteria so some media now contain inhibitors and other selective agents. Sulphide and an iron source are usually used as indicators. The clostridia reduce the sulphide to sulphite which gives a black precipitate with the iron

present in the medium. Sulphite reducing clostridia are then enumerated as black colonies if solid media is used.

Sodium sulphite and ferric citrate may be added to RCM to become differential RCM (DRCM) which was recommended for the detection of sulphite reducing clostridia in drinking water (The Microbiology of Water, 1994, pt.1) and is specified in the ISO standard 6461-1(1986) liquid enrichment method for water.

Tryptose sulphite cycloserine agar (TSC) was a medium proposed by Harmon *et al.* (1971) for detection of vegetative and spore forms of *C. perfringens* in foodstuffs and clinical specimens, which contains cycloserine as an inhibitor of accompanying bacterial flora and causes the colonies which develop to remain smaller. This medium is used in the method for isolation and enumeration of clostridia in food by the FDA (with the addition of egg yolk to detect lecithinase production), the UK Environment Agency (EA) and the UK Public Health Laboratory Service (PHLS), now health protection Agency (HPA), Methods for Food and Water. There have been further modifications of this medium incorporating a fluorogenic substrate (described in detail later).

Subsequently, media were then developed to be more selective for *C. perfringens* and inhibitory to other species of clostridia. Oxoid Perfringens Agar (OPSP) is based on the formulation developed by Handford (1974) for the detection of clostridia in foods. In addition to incorporation of sulphite and iron, this medium utilises sulphadiazine, oleandomycin phosphate and polymyxin B sulphate to give a high degree of selectivity and specificity for *C. perfringens*. Other *Clostridium* species, e.g. *C. bifermentans* and *C. butyricum,* are inhibited.

Membrane Clostridium Perfringens (m-CP) agar is a selective and chromogenic medium for the presumptive identification of *C. perfringens* from water samples, first described by Bisson and Cabelli (1979). It was used for the rapid quantification of *C. perfringens* from a variety of waste water samples (seawater, potable water and sewage) and gave better recoveries than the Bonde pour tube method. m-CP has been

recommended in European Council Directive 98/83/EC (1998) for testing the quality of water intended for human consumption

C. perfringens does not cleave the chromogen, indoxyl β-D-glucoside, in the medium because the organism lacks β-D-glucosidase. Sucrose in the medium is fermented, causing the bromocresol purple indicator to change colour with reduced pH. Other *Clostridium* species appear purple or blue/green. Characteristic opaque yellow colonies of *C. perfringens* are seen which can be confirmed by exposure to ammonium hydroxide vapour for 20-30 seconds. *C. perfringens* colonies turn pink or red as phenolphthalein diphosphate is cleaved by acid phosphatase.

An alternative chromogenic medium is Differential Clostridial Agar (DCA) for enumeration of sulphite reducing clostridia in dried foods (Weenk, 1991). This medium contains sulphite, iron, starch to promote spore germination and Resazurin as a redox indicator. The manufacturers suggest that the sample is heated at 30°C prior to inoculation to facilitate spore germination.

There are a few commercially available test kits for the detection of clostridia. Biomedix have a presumptive test for *C. perfringens* using traditionally prepared media. The Neogen Corporation have an ISO-GRID most probable number (MPN) method for *C. perfringens* using a modified TSC agar. Commercial kits are also available to detect *C. perfringens* type A enterotoxin (Oxoid)

More recently media using fluorogenic and chromogenic substrates have been used (Manafi, 2000). The detection of acid phosphatases has been shown to be useful for the enumeration of *C. perfringens* (Ueno *et al.,*1970; Eisgruber *et al*., 2001). This organism can metabolise 4-methylumbelliferyl phosphate (MUP) using the enzyme acid phosphatase to produce 4-methylumbelliferone, which fluoresces when placed under long wavelength UV light (365 nm). The substrate can be bought as a supplement to conventional selective media. Methylumbelliferyl derivatives have the

advantage of being highly sensitive and specific, non-carcinogenic and easily detected with simple UV sources. The methylumbelliferyl flurorophore has also been used in the method for the isolation and identification of enterococci and is commercially available in kit format (Enterolert, IDEXX) (see later). *C. perfringens* also ferments lactose to acid and gas using β-galactosidase which in turn can hydrolyse *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) to the chromogenic product *ortho*-nitrophenol (Edberg *et al.,*1988). This substrate has also been used successfully in the methods for detection of coliforms.

Adcock *et al*. (2001) describe a rapid method utilising both of these chromogenic substrates for the detection of *C. perfringens* in river and surface storage water, and sewage effluent. Samples were filtered and incubated on TSC agar before incubation in a MUP-ONPG liquid medium. The authors tried to incorporate MUP into solid TSC agar but this resulted in too many false positives.

In Spain, Araujo *et al*. (2001) evaluated TSC agar, fluorogenic TSC agar and m-CP in a method for the detection of *C. perfringens* in groundwater samples. Variance analysis of the data showed no statistically significant differences in the counts obtained between all media used for this study. However the recovery efficiencies with TSC and fluorogenic TSC were significantly greater (P= < 0.05) than with m-CP. They summarised the study by stating that the membrane filtration technique using fluorogenic TSC agar showed the best performance characteristics of all three media tested as judged by recovery efficiency and specificity in these water samples. The fluorogenic substrate is available commercially as a convenient additive to conventional TSC agar base (Fluorocult, Merck).

Table 1 summarises the media currently in use for the isolation of clostridia and Table 2 details the recipes for each of them.

Table 1. Summary of some commercially available media for isolation of clostridia

Medium	Matrix designed for:	Details	Company
RCM agar (solid) (Reinforced Clostridial Agar)	Food, human and animal faeces		Oxoid
RCM (semi solid) (Reinforced Clostridial Medium)	Food, human and animal faeces	NOTE: Can add sodium sulphite and ferric citrate for differential version (DRCM) for drinking water	Oxoid
TSC agar (Tryptose sulphite cycloserine)	Food, clinical specimens		
Perfringens Agar(OPSP) (sulphadiazine,oleandomycin phosphate, polymyxin B sulphate)	Food		Oxoid
m-CP medium (Membrane Clostridium Perfringens)	Water and sewage	Differentiate *C. perfringens* from other *Clostridium* spp. Chromogenic	Oxoid
Modified m-CP medium (Membrane Clostridium Perfringens)		More economical (Armon *et al*. 1988)	
DCA (Differential Clostridial Agar)	Dried foods		Merck
Clostridia impedance broth		No details available	SY-LAB

The composition of the media used substantially affects the productivity of the isolation procedure. Weenk *et al*. (1995) reported that standardisation of reagents, preparation of media and methods are necessary. In their analysis of DCA agar, used in the method for analysis of dried food, they stressed the importance or rigorous standardisation of sulphite activity and ferrous iron concentration; tryptose was one of the most appropriate nitrogen sources and that the basal medium should be free of lactate and acetate (the latter is present in RCM and DRCM). They also described a variant method which used a bottom layer of mannitol/egg yolk/ polymyxin/

bromocresol purple agar inoculated with mascerates of food in buffered cysteine hydrochloride/peptone/saline, immediately overlayered with fresh DCA. Plates were incubated and read in tightly closed plastic bags with a low oxygen permeability coefficient, eliminating the need for anaerobic jars.

The inclusion of complex ingredients in the media, e.g. yeast extract or meat extract, may make absolute standarisation unachievable. Differences in batches of these complex constituents may have to be investigated. In addition, storage conditions necessary for individual media need to be specified in the methods or performance could be affected. Schneider *et al.* (1988) described the necessity for using freshly made DRCM for efficient performance.

Table 2: (i)

Reinforced RCM (Oxoid)	Quantity (g)
Yeast extract	3
'Lab lemco' powder	10
Peptone	10
Glucose	5
Soluble starch	1
Sodiun chloride	5
Sodium acetate	3
Cysteine hydrochloride	0.5
agar	15 (solid) or 0.5 (semi-solid)
(pH 6.8)	Make up to 1 litre distilled water

Table 2: (ii)

DRCM (As specified by ISO 6461-1: 1986, liquid enrichment)	Quantity (g)	(As specified by ISO 6461-2: 1993, Membrane filtration)	
Yeast extract	1.5		
Meat extract	10	3	
Peptone tryptic digest of meat	10	10	
Glucose	1		
Soluble starch	1		
Sodium chloride		5	
Hydrated Sodium acetate	5		
L-Cysteine hydrochloride	0.5		
Agar	Make up to 1 litre distilled water	15	Make up to 1 litre with distilled water
	Add Na_2SO_3 (to final concentration 0.04%) and iron citrate (final concentration 0.07%)		Add Na_2SO_3 (to final concentration 0.5%) and iron citrate (final concentration approx 0.4% - drops added)
			Note: alternative medium specified is TSC, no cycloserine

Table 2: (iii)

TSC medium	Specified by FDA in food method (Bacteriological analytical manual) Quantity (g)	Merck Quantity (g)	Specified in EA (UK) water method Quantity (g)	Specified in PHLS (UK) W 5, F 14 Quantity (g)
Tryptose	15	15	15	15
Yeast extract	5	5	5	5
Soytone (soymeal peptone)	5	5	5	5
Ferric ammonium citrate	1	1	1	1
Sodium metabisulphite	1	1	1	1
Agar	20	15	14	12
Distilled water	900 ml	Up to 1 l	Up to 1 l	Up to 1 l
Cycloserine added to autoclaved medium	0.4 g l^{-1}	0.4 g l^{-1}	0.4 g l^{-1}	0.4 g l^{-1}
Additives	Diln of food in TSC with egg yolk, allow to set, overlay in TSC no egg yolk	Polymyxin (0.003 g l^{-1}), Kanamycin 0.012 gl^{-1}) = SFP agar		

Table 2: (iv)

Perfringens agar (OPSP; Oxoid)	Quantity (g)
Tryptose	15
Yeast extract	5
Soya peptone	5
Liver extract	7
Ferric ammonium citrate	1
Sodium metabisulphite	1
Tris buffer	1.5
Agar	10
(pH 7.3)	Make up to 1 l distilled water
Additives	Can add sodium sulphadiazine, oleandomycin phosphate, polymyxin B

Table 2: (v)

Membrane Clostridium Perfringens agar (m-CP; Oxoid)*	Quantity (g)
Tryptose	30
Yeast extract	20
Sucrose	5
L- cysteine hydrochloride	1
Magnesium sulphate.7 H_2O	0.1
Bromocresol purple	0.04
Agar	15
	Up to 1l
Additives	

*As used by Ohio District Laboratory

Table 2: (vi)

Differential Clostridial agar (DCA; Merck)	Quantity (g)
Peptone from casein	5
Peptone from meat	5
Meat extract	8
Starch	1
D-glucose	1
Yeast extract	1
Cysteinium chloride	0.5
Resazurin	0.002
Agar	20
	Up to 1l
Additives	Heat treatment of the sample at $30^{o}C$ for 10 minutes to facilitate spore germination

There is some dispute as to the superiority of one medium over another (Weenk *et al.*, 1995). As the majority of media have been developed for detection of clostridia in food and water, modifications may have to be made in the development of a method for sewage sludge, soil and biowastes.

Confirmation tests

Confirmation tests on the sulphite reducing colonies can be time consuming, prolonging the time taken to process a sample to over 72 hours. Isolates may be subcultured onto blood agar (BA) for aerotolerance testing, purity check and Gram stain before inoculation into nitrate motility medium (NMM) to detect nitrate reduction and motility (*C. perfringens* is non-motile) and into lactose gelatin medium (LGM) to detect liquefaction of gelatin and lactose fermentation. Clinical isolates may be analysed by the API biochemical test system (Niculescu *et al.*, 1985).

These tests are usually very labour intensive and costly (a second generation of anaerobic conditions is required which demands significant workspace) and may be

prone to misreporting of results due to the selection of mixed cultures upon subculturing from TSC agar.

Generation of anaerobic conditions

Any methods devised for the isolation of clostridia are only as efficient as the availability of suitable anaerobic conditions for culture. Schneider *et al.* (1988) described reduced recovery efficiencies, possibly due to inadequate generation of the anaerobic atmosphere during validation of the method for water (DIN 38411, part 7). The source of anaerobic conditions therefore have to be included in the methodology. Generation of anaerobic conditions is considerably easier now than it used to be with the advent of gas pack sachets that can remove the oxygen in a known volume very rapidly. The first sachets contained a mixture of sodium borohydride, sodium bicarbonate and tartaric acid. The addition of water activates the pack and hydrogen plus carbon dioxide are produced. The oxygen in the atmosphere within the container combines with the hydrogen to produce water which takes approximately 30 minutes (Kit BR0038, Oxoid). Other kits contain ascorbic acid which results in absorption of the oxygen and simultaneous release of carbon dioxide (Anaerogen, Oxoid) without addition of water.

There is also some dispute (Weenk *et al.*, 1995) on the efficiency of different commercially available gas packs which needs to be addressed as part of a future study. A useful quality control procedure would be to include redox indicator strips and also monitor the time required for the procedure to remove all of the oxygen from the incubation atomosphere.

ISOLATION AND DETECTION OF CLOSTRIDIA: METHODS AVAILABLE

Origin of method	Matrix method devised for:	Sample size	Summary of method	Comments/ validated etc.
EA/SCA UK 2002	Water	100 ml processed water - less if polluted	Heat kill vegetative cells. Filter to collect spores onto solid TSCA medium 37°C 20-44 h. Black colonies: no other confirmation done	
PHLS SOP UK W5	Water	20 ml or 50 ml bottled	Heat kill vegetative cells.Filter to collect spores. Put filter in TSCA medium at 40°C, allow to set and incubate at 37°C 20-44 hr Black cols – can subcut	
F14	Food	Dilns	Dilns made, 1 ml plus molten selective culture medium (TSCA) at 40°C,allow to set then overlay 37°C 20 h. Black colonies confirm by subculture, motility, nitrate, lactose gelatin medium.	
Draft method for toxin	faecal specimens			Use commercial *C. perfringens* toxin tests (Unipath)
ISO 6461-1: 1986 Detection and	All types of water,	50 ml plus dilns.	Heat kill vegetative cells (75°C,15 min)	Positive growth indicated by

enumeration of the spores of sulphite-reducing anaerobes(Clostridia) Part 1:Method by enrichment in a liquid medium	including turbid water		Diln in media (DRCM,sodium sulphite,iron citrate) 37°C, 44 hr 'blackening' i.e. reduction of sulphite taken as positive MPN	reduction of sulphite and precipitation of iron (II) sulphide
ISO 6461-1: 1993 Detection and enumeration of the spores of sulphite-reducing anaerobes (Clostridia) Part 2:Method by membrane filtration	Water	100 ml for drinking water, spring water, mineral waters < 100ml for highly polluted water	Heat kill vegetative cells (75°C, 15 min) Filter the samples, place membrane face down in dish add media (sulphite iron agar) at 50°C. Once set incubate anaerobically 37°C for 20 to 44 hrs Black cols positive	
FDA: Bacteriological Analytical manual, 2001, 8th edition Revision A (1998)	Food	25 g	Homogenise in peptone buffer Dilutions onto TSC (no egg), allow to set then overlay more medium (no egg) Incubate anaerobically 35°C 20-24 h. Count black colonies. Possibly inoculate into chopped liver broth and Iron milk presumptive test.	As above, colonies have a opaque white halo as a result of lecithinase activity

Clostridia Conclusions

Most of the validated methods for the detection of *C. perfringens* have been developed for food and water matrices. Although they have occasionally been used in the analysis of sewage sludge a strict validation procedure is required to incorporate:

Sample preparation and diluent (membrane filtration unless analysing greatly diluted samples or samples that have had heavy particulate material removed; this may not be a viable option). Inclusion of a reducing agent e.g. cysteine hydrochloride in all diluents may reduce any damage on bacterial cells/spores from oxygen and free radicals (D. Sartory, personal communication)

A comparison is required of the most promising selective media e.g TSC, m-CP chromogenic and TSC-fluorogenic agars. The study should involve inclusion of standard media preparation, media storage conditions and standardisation of anaerobic atmospheres. If confirmatory tests are required, these should be standardised as part of Project Horizontal. It should not be forgotten that the Draft Biowaste Directive (Appendix 1) specifies that Clostridia should be absent per g dry weight. Clearly, validation studies should ensure that this sensitivity of detection is achievable for sludge, soil and biowastes.

The widespread use of chromogenic and fluorogenic media is a testament to their value in the improvement of many standard methods. The inclusion of these substrates, together with the defined and selective qualities of the base media to reduce the number of contaminants, have resulted in increased sensitivity and rapidity. It is easier to distinguish specific colonies earlier using these media. However, for any procedure relying on activity of enzymes, conditions of pH, temperature, *etc* have to be optimised. These conditions must be specified in the protocol. There is some concern about the use of fluorogenic substrates cleaved by acid phosphatase for the detection of clostridia. Fluorogenic substrates are usually quenched at low pH and require neutral conditions in the medium. At neutral pH alkaline phosphatase which is present in many strains of clostridia would also be detected and give false positive

results. If the pH is lowered to optimise for acid phosphatase this can be inhibitory for stressed clostridia on primary isolation. Work is currently in progress to develop a single medium based on the method of Ueno *et al*. (1970) and modified by Mead *et al*. (1981)(D. Sartory, personal communication)

Enterococci introduction

Intestinal enterococci are defined as Gram positive cocci that tend to form in pairs and chains. They are non-spore forming , oxidase –negative, catalase-negative, hydrolyse aesculin and possess Lancefield's group D antigen. This latter characteristic defined the so-called "faecal streptococci" whose members belong to the *Enterococcus* and *Streptococcus* genera. They can grow aerobically and anaerobically in the presence of bile salts, and in sodium azide solutions, concentrations of which are inhibitory to coliform bacteria and most Gram- negative bacteria.

Enterococci occur normally in faeces at lower concentrations than *E. coli* and rarely greater than 10^6 per gram human faeces. The presence of enterococci is considered to be a secondary indicator of faecal pollution and if coliforms and enterococci are present, but not *E. coli,* the coliforms are taken to be faecal in origin. The fact that enterococci are more resistant to environmental stress, e.g. desiccation and chlorination, than coliforms is used as an indicator of surface run off pollution and utilised to monitor the efficiency of water treatment procedures. Enterococci rarely multiply in water and can be found on plants which may not be as a result of faecal contamination.

There are two main groups of enterococci found in human and animal faeces: *Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae* and *Enterococcus durans* are all found in human and animal faeces; whereas *Streptococcus bovis, Streptococcus equinus and Streptococcus avium* are not found in humans but in cattle, horses and birds, respectively. *Enterococcus casseliflavus* and *Enterococcus mundtii* are non-faecal species which may be present in water samples due to the presence of plant material or some industrial effluents. They form yellow pigmented colonies on

non-selective agar. The possible interference of these non-faecal species in assay procedures should therefore not be overlooked.

Until recently enterococci were mainly considered as ordinary bowel commensals and of little clinical significance except for being the causative agent in rare cases of endocarditis and meningitis. In the last decade however, enterococci have been recognised as one of the major causes of nosocomial bacteraemia, surgical wound infection and urinary tract infection. In the USA the incidence of gastroenteritis from enterococci found in swimming pools is increasing. Enterococci have a naturally intrinsic and more recently acquired resistance to many antibiotics; in particular, vancomycin resistance is becoming a serious problem in the treatment of enterococcal infection.

In the farming community there is widespread use of enterococci, lactobacilli , bifidobacteria and yeasts as probiotics in animal feeds. It has been suggested that this and the use of antibiotic growth-promoters, in particular the drug avoparcin (an analogue of vancomycin), in animal feeds has contributed to the development and spread of antibiotic resistant enterococci.

Existing methods for the detection of enterococci

Methods for the detection of enterococci have usually been devised for water testing and usually rely on the resilient nature of the organisms to survive concentrations of sodium azide and bile salts that are inhibitory to coliform bacteria and most other Gram-negative bacteria, and at a temperature of 44 °C. Some species are resistant to heating at 60 °C for 30 minutes, to pH 9.6, and are able grow in nutrient broth containing 6.5 % (w/w) sodium chloride. Enterococci also hydrolyse the glycoside, aesculin, yielding dextrose and aesculitin: the latter combines with ferric citrate in the agar to form a dark brown/black complex.

Methods developed for water have usually incorporated a filtration step followed by transfer of the membrane onto selective media containing sodium azide and bile salts.

The original test method was developed by Levin *et al*. (1975; USEPA, 1985) and introduced in 1986 (USEPA, 1986b) using 2 media: a primary isolation medium, mE Agar, and aesculin iron agar (EIA). This method was revised in 1977 (USEPA, 1986b) and resulted in the use of a single medium, mEI Agar, which was a modification of the original mE Agar by reducing the concentration of triphenyl tetrazolium chloride and adding a chromogenic cellobiose analogue, indoxyl-β-D glucoside

The choice of media in a method for the detection of enterococci is complicated as commercial versions of the same culture medium may vary in recipe and /or performance from producer to producer (Reuter, 1992). Other media than the ones described above include citrate azide tween carbonate agar (CATC), kanamycin aesculin azide agar (KAA), aesculin bile azide agar (ABA) and thallous acetate tetrazolium glucose agar (TITG). No medium appears to be completely selective for all enterococci but some are highly selective for a single species e.g. *E. faecalis* commonly used as an indicator of human pollution.

Chromocult Enterococci broth and Readycult Enterococci broth (Merck) both contain the substrate X-GLU (5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside) which is cleaved, stimulated by selective peptones, by the enzyme β-D-glucosidase which is characteristic for enterococci. This results in the formation of bromochloroindigo, an intense blue/green colour. The sodium azide present suppress any non-enterococci, β-D-glucosidase-positive bacteria (Manafi and Windhager, 1997). The results obtained with pure cultures indicated 97% of strains, which gave positive results, were identified as enerococci (*E. faecalis, E. faecium, E. durans, E. casseliflavus* and *E. avium*). The false positive strains were *Lactococcus lactis, Leuconostoc* and *Aerococcus* spp. Amoros and Alonso (1996) compared Slanetz-Bartley agar with Enterococci broth containing X-GLU to detect enterococci in sea water. The agar medium showed decreased specificity in the sea water samples and there was a considerable number of false positives. More recently, Merck have introduced Chromocult Enterococci agar, a selective agar containing azide and ox bile as well as a mix of confidential chromogenic substrates. The enzyme activity of the enterococci cleave the chromogenic substrates, producing red coloured colonies; non-enterococci

produce colourless, blue/violet or turquoise colonies. The agar is inoculated the medium by the pour-plate-method or by spreading the sample material on the surface of the plates or the membrane-filter-technique. The type of membrane filter affects the performance of the medium (growth and colouration of colonies) and best results were obtained using membrane filters of cellulose-mixed-ester material, e.g. Gelman GN-6 (Ossmer *et al.*, 1999). The plates are incubated for 24 ± 4 hours at 35-37 °C and if there is neither visible growth nor colour change then the incubation should continue for up to 44 ± 4 hours.

More recently, fluorogenic media have been devised for the detection of specific microorganisms. As described earlier, Clostridia can metabolise 4-methylumbelliferyl phosphate (MUP) using the enzyme acid phosphatase to produce 4 methylumbelliferone which fluoresces when placed under long wavelength UV light (365 nm). Enterolert (IDEXX Laboratories) is a semi-automated, most probable number (MPN) commercial kit for the enumeration of enterococci in water samples utilising a fluorescent substrate. 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 provides counts from one to 200 per100 ml. Quanti-Tray/2000 counts from one to 2,419 per100 ml. Total hands-on time is less than one minute per test. The Enterolert system utilises the indicator substrate, 4-methylumbelliferyl-β-D-glucoside (MUD), which is metabolised by enterococci at 41ºC and has been evaluated in the USA compared to the standard membrane filter method (Budnick *et al.*,1996). The same substrate is used in ISO 7899-1:1998 miniaturised MPN method for the detection of enterococci in waste water (Table 1). Some of the species of enterococci that Enterolert can detect are: *faecalis, faecium, avium, gallinarum, casseliflavis,* and *durans*. Enterolert is claimed to be able to detect 1 enterococci/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. Enterolert has 75% lower equipment cost than membrane filtration, the reagent packs have up to an 18-month shelf life and the comparatively rapid 24-hour test saves incubator space. So far, Enterolert has been used on samples from fresh water and salt water beaches, shellfish areas, drinking water and waste water (Fricker *et al.*, 1995; Budnick *et al*, 1996; Eckner, 1998). It is approved by the American Society for Testing and Materials (ASTM) Committee on Water for use with drinking water, source water, recreational (fresh and marine) water, bottled water and waste water (ASTM D6503-99). The US EPA has recently approved the use of Enterolert 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". Importantly, the US EPA has not yet approved *E. coli* and 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.

Tables 1, 2 and 3 briefly describe current approved methods for the isolation and detection of enterococci and details of the primary selective media and subculture media employed by these methods.

Table 1. Isolation and detection of enterococci

Origin of method	Matrix method devised for:	Sample size	Summary of method	Sensitivity	Comments/ validated etc.
EA/SCA UK	Water	100 ml treated- less if polluted	Membrane filtration: Membrane Enterococci Agar (MEA). Potable water: 37ºC, 48 h. Untreated: 37ºC 4 h, then 44ºC 44 h.		Substrate triphenyl tetrazolium chloride (TTC) reduced to red formazan.
PHLS SOP UK W3	Water Faecal streptococci	100 ml or 250 ml bottled	Membrane filtration Membrane Enterococci Agar (MEA). 37ºC 4h then 44ºC 40 hr Subcut bile aesculin(BEA)		Substrate TTC
EPA 1106.1	Water		Filtration then MEA 41.5ºC,48 h then EIA (Esculin iron agar) 41.5ºC 30 min.		Substrate TTC
EPA modified 1600	Water		Filter then modified mEI 41.5ºC **24 h** -blue colonies, no subculture.		Results in 24 hours mEI medium contains reduced TTC and has substrate indoxyl • - D- glucoside that turns blue when cleaved with • - glucosidase present in enterococci
NMKL 68 Denmark	Food Has been		Dilute in peptone salt (NMKL 91),		Substrate TTC

1992	used for compost		pour plate with tryptone soy agar at 450C-incubate 2hrs 37ºC Pour EA at 41ºC over this 44ºC, 48h Subculture to typtone soy agar 24 h then test catalase or grow in 6.5% NaCl or high pH for 3 days. Turbid culture = +ve		
ISO 7899-1:1998 Detection and enumeration enterococci in waste water Pt 1 (miniturised, MPN)	Water (fresh and brackish, sea water, waste water, treatment plants)		Dilns in saline or DW(depend on sample) tubes add 200•l to 100•l medium MUD/SF 44ºC min 36 hrs max 72 h. Measure fluorescence under UV	Not detect < 15 per 100ml	Substrate 4-methylumbelliferyl-•-D-glucoside (MUD) in presence thallium acetate, nalidixic acid and TTC (Hazardous-dilutions in safety cabinet)
ISO 7899-2:2000 Detection and enumeration enterococci in waste water Pt 2 (membrane filtration)	Drinking water		Filter, transfer to EA (Slantz & Bartley) 36ºC, 44 h. Subculture red colonies to BEA 44ºC 2 h, black cols		Substrate TTC (and Aesculin hydrolysis on subculture)

Table 2 Inoculation media- Primary selective media

Medium	Recipe	Specified by method:
Membrane Enterococci Agar (MEA) (Slanetz and Bartley)	Tryptose 20g Yeast extract 5g Glucose 2g Dipotassium hydrogen phosphate 4g Sodium azide 400mg Agar 12g (8-18g in ISO 7899-2) 2,3,5-triphenyltetrazolium chloride (TTC) 10ml (1% m/v aqueous soln.). Distilled water 1000 ml	EA/SCA, PHLS UK, ISO 7899-2
mE agar	Peptone 10g Sodium chloride 15g Yeast extract 30g Esculin 1g Actidione (cycloheximide) 0.05g Sodium azide 0.15g Agar 15g Distilled water 1000mL Sterilise above constituents and add nalidixic acid, sodium hydroxide and 0.15g TTC	EPA Method 1160.1 (USA) ASTM D5259-10
mEI Agar	Peptone 10g Sodium chloride 15g Yeast extract 30g Esculin 1g Actidione(cycloheximide) 0.05g Sodium azide 0.15g Agar 15g Distilled water 1000mL Indoxyl β- D-glucoside 0.75g Sterilise above and add nalidixic acid, sodium hydroxide and 0.15g TTC	Modified EPA method 1600
MUD/SF	Solution A: Tryptose 40g, KH_2PO_4 10g, D(+) -galactose 2g, Tween 80 1.5 mL, Distilled water 900 ml Solution B: Sodium bicarbonate 4g, Nalidixic acid 250 mg, Distilled water 50ml Solution C: Thallium acetate 2g (TOXIC), TTC 0.1g, Distilled water 50mL Solution D: MUD 150mg, N,N-dimethylformamide	ISO 7899-1

2mL (TOXIC) Mix A,B,C and D, adjust pH to 7.5,filter sterilise. Volume 1002 mL	

Table 3

Subculture media recommended

Medium	Method recommending use:
Kanamycin aesculin azide agar Bile aesculin	EA and PHLS (UK). Latter uses slightly different agar concentrations
Esculin Iron agar Brain heart infusion broth (+/- 6.5% sodium chloride) Bile aesculin agar (different recipe to above)	EPA 1106-1 and 1600
Bile aesculin azide agar (different recipe)	ISO 7899-2

These conventional methods are usually based on phenotypic characteristics. In addition, there are an increasing number of molecular methods, in particular PCR, for the detection of clinically significant isolates (particulary vancomycin resistant strains) and specific hybridisation with specific probes based on both 16S and 23S rRNA genes (Manero *et al.,* 2002). Although research is proceeding into their use for direct quantification of enterococci in organic matrices, their sensitivity and inability to detect viable cells without a pre-culture step is problematic. Therefore, they are currently best employed at the end of the classic quantification procedures to accurately confirm the identify enterococci to species level.

Chromogenic agars, including Chromocult, and Enterolert have been compared in several studies for enumeration of enterococci in environmental water samples (Budnick et al.,1996; Heiber, 1998; Yakub *et al*., 2002; Kinzelman, 2003). For example, of 138 marine and freshwater recreation samples, Enterolert had a false

positive rate of 5.1% versus the mE membrane filtration's 10.0%, and a false negative rate of 0.4% versus 11.7% (Budnick *et al.*, 1996). Another comparison of the Enterococci agar and Enterolert methods with *E. coli* methods, analyzing 124 recreational water samples, yielded a poor correlation ($R^2 = 0.69$) between the two indicator bacteria and also the enterococci methods ($R^2 = 0.62$). This was further confounded by the frequent inability to verify enterococci from those samples producing fluorescence by the defined substrate test using conventional microbiological methods. Based on U.S. EPA bacterial indicator threshold levels of risk for full body immersion, using enterococci would have resulted in 56 additional unsafe-recreational-water-quality advisories compared to the total from using *E. coli* and the substrate-based methods. These results suggest that further research is necessary regarding the use of defined substrate technology interchangeably with the U.S. Environmental Protection Agency-approved membrane filtration test for the detection of enterococci from fresh surface water.

The conventional filtration/selective media methods devised for water testing have also been employed on more complex matrices such as raw sewage. Iverson *et al.* (2002) sampled raw sewage, treated sewage, surface water and hospital sewage in Sweden in a screening program for vancomycin-resistant enterococci (VRE). Samples were serially diluted before filtering and the filters transferred to brain heart infusion agar for 2 hours before transfer to Enterococcus agar (Becton Dickinson) for 48 hours at 37°C. This was followed by a short (2 h) incubation on bile aesculin agar where black colonies were tested for catalase activity.

In the evaluation of peracetic acid in the disinfection of sewage effluents, Stampi *et al.* (2001) inoculated dilutions of samples into azide dextrose broth (Oxoid) for 48 h at 35°C. Tubes with growth were subcultured onto Pfizer selective *Enterococcus* agar (PSE, Oxoid) at 35°C for 24 hours. Brown/black colonies with brown halos were taken as positive enterococci.

Details of approved methods for the detection of enterococci in water

1. Environment Agency (EA): Standard Committee of Analysts (SCA) UK

In the UK approved method from the Environment Agency, presumptive enterococci reduce triphenyltetrazolium chloride after incubation to insoluble red formazan to produce red, maroon or pink colonies on membrane enterococcus agar. Some strains may produce very pale colonies. Confirmation is based on the organism being catalase-negative and on the demonstration of aesculin hydrolysis on bile aesculin agar or kanamycin aesculin azide agar incubated at 44 °C for up to 18 hours, although some strains of *Streptococcus bovis* and *Streptococcus equinus* may fail to grow at this temperature.

The method has been developed for the water industry. The initial stages of the method involve filtration of the water sample using sterilised filtration apparatus housing 47mm diameter, 0.45 • m cellulose-based filters. This is not feasible for turbid matrices such as sludge and soils which would tend to block the membrane filter. This will limit the volume of sample that can be filtered. Accumulated deposit on the membrane filter may mask or inhibit the growth of indicator organisms. The method also allows some other species (for example, *Aerococcus viridans* and species of *Staphylococcus* and *Bacillus*) to grow. The maximum number of colonies that should be counted from a single membrane filter is approximately 100.

Confirmation tests
Depending on the degree of accuracy required by the test, subculturing of red colonies may be performed (if less than 10 colonies, all should be subcultured)

Catalase test
Enterococci are catalase-negative. Emulsify some of the isolated colony from the MEA in approximately 0.1 ml of quarter strength Ringer's solution. Add approximately 0.05 ml of 3 % hydrogen peroxide solution and replace the cap. The immediate appearance of bubbles (of oxygen) indicates catalase activity. An alternative procedure is to add the hydrogen peroxide to an overnight culture of an

isolate obtained from nutrient agar. The test should preferably not be performed on a slide because of the risk of aerosol formation. Commercial test kits for catalase testing can be used after validation procedures. On each occasion that catalase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example, *Staphylococcus aureus*) and one species is known to give a negative reaction (for example, *E. faecalis*).

Aesculin hydrolysis

From membrane enterococcus agar, subculture to bile aesculin agar or kanamycin aesculin azide agar and incubate at 44 °C for up to 18 hours. Enterococci should produce discrete colonies surrounded by a brown or black halo from aesculin hydrolysis. The development of this colour is usually evident within a few hours and should provide rapid confirmation. *Bacillus* species may produce some discoloration around the original inoculum site but should not develop discrete colonies.

Additional differentiation tests for enterococci

Although the possession of Lancefield's Group D antigen is referred to in the definition, serological methods of confirmation present many practical difficulties. Tolerance of 40 % bile is also characteristic of enterococci. Further tests with subcultures may be undertaken if necessary, partly as an aid to species differentiation. However, full identification depends on the demonstration of biochemical and other characteristics or by means of one of the multi-test differential systems now available. Commercial biochemical and serological methods can be used, following appropriate verification of performance at the laboratory. *Enterococcus* species are differentiated from other streptococci by their ability to grow in nutrient broth containing 6.5 % sodium chloride, and in glucose phenolphthalein broth modified to pH 9.6.

Bile tolerance

From an overnight culture on nutrient agar incubated at 37°C, sub-culture to a plate or tube of 40 % bile agar and incubate at 37 °C for 24 - 48 hours. Growth on this medium indicates tolerance of bile salts. Alternatively, use MacConkey agar to show growth in the presence of bile salts. Enterococci form small deep red colonies on

MacConkey agar.

Heat resistance

Transfer 1 ml of a nutrient broth culture incubated at 37 °C for 24 hours to a small test tube. Place the test tube in a water bath at 60°C for 30 minutes. Cool the tube rapidly and incubate at 37 °C for 24 hours. Subculture the broth to a blood agar plate or other non-selective medium. Incubate at 37°C and examine for growth.

Growth at pH 9.6

From a nutrient agar plate, inoculate into a tube of glucose phenolphthalein broth modified to pH 9.6 and incubate at 37°C for 24 hours. Tolerance of pH 9.6 is indicated by heavy growth and decolourisation of the medium.

Salt tolerance

From a nutrient agar plate, inoculate into a tube of nutrient broth containing 6.5% of sodium chloride and incubate at 37 °C for 24 - 48 hours. Examine for growth.

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *E. faecalis*) and non-target bacteria (for example, *Staphylococcus* species). Petri dishes should be incubated for 48 hours at 37 °C.

Note: The method used by the PHLS is basically the same as above.

2. EPA 1600

The mEI agar method (USEPA Method 1600) is a one-step membrane-filtration method that allows the detection of enterococci in 24 hours with an incubation at 41°C. It is recommended for use in place of the old enterococci method, the mE agar method (USEPA Method 1106.1), which is a two-step method that takes 48 hours to complete with an incubation at 35°C. Method 1600 can be done in the field or laboratory.

The mEI medium is similar to the mE medium except that it contains a reduced amount of triphenyltetrazaolium chloride (TTC) and contains a substrate, indoxyl-•-D-glucoside, that turns blue when cleaved by an enzyme present in enterococci (•-glucosidase). All colonies with any blue halo are recorded as enterococci, regardless of colony colour. Low power magnification with a dissecting microscope is used for counting to give maximum visibility of colonies.

Specificity - The specificity of the medium used in this method is 6.0% false positive and 6.5% false negative for various environmental water samples. The false positive rate was calculated as the percent of colonies which reacted typically, but did not verify as members of the enterococcus group. The false negative rate was calculated as the percent of all verified enterococcus colonies not reacting typically.

<u>Verification Procedure</u>
Colonies with any blue halo can be verified as enterococci. Verification of colonies may be required in evidence gathering, and is also recommended as a QC procedure upon initial use of the test and with changes in sample sites or lots of commercial media. The verification procedure follows.
Using a sterile inoculating needle, transfer cells from the centres of at least 10 well-isolated typical colonies into a brain heart infusion broth (BHI) tube and onto a BHI agar slant. Incubate broth tubes for 24 h and slants for 48 h at 35 ± 0.5ºC.
After 24 h incubation, transfer a loopful of material from each BHI broth tube to:
Bile Esculin Agar (BEA) and incubate at 35 ± 0.5ºC for 48 h.
BHI Broth and incubate at 45 ± 0.5ºC for 48 h.
BHI Broth with 6.5% NaC1 and incubate at 35 ± 0.5ºC for 48 h.

Observe for growth.
After 48 h incubation, apply the Gram stain to growth from each BHI agar slant. Gram positive cocci which grow in BEA, BHI Broth at 45ºC, and BHI Broth + 6.5% NaCl, and hydrolyze aesculin, are verified as enterococci.

3. ISO 7899-1 Water Quality- Detection and Enumeration of intestinal enterococci in surface and waste water. Part 1: Miniaturised method (Most Probable Number) for surface and waste water.

This miniaturised method for the detection and enumeration of major intestinal enterococci is applicable to all types of surface and waste waters, particularly those rich in suspended matter. The method is not suitable for drinking water and any other type of water for which the guideline count is less than 15 per 100 ml.

The method involves inoculation of 200 •l of the sample from a dilution series (using a specified artificial sea water as diluent) in a row of microtitre plate wells containing 100 •l of medium. The plates are incubated at (44 ± 0.5) ºC for a minimum of 36 h and a maximum of 72 h. Enterococci hydrolyse 4-methylumbelliferyl-•-D-glucoside (MUD), in the presence of thallium acetate, nalidixix acid and 2,3,5-triphenyltetrazolium chloride (TTC) in the medium, to the methylumbelliferyl fluorophore. Wells observed with blue fluorescence under UV irradiation (e.g. Woods Lamp, 366 nm) are considered positive. There are no confirmatory tests. The MPN is calculated as a statistical estimation of the density of the microorganisms, assumed to correspond to a Poisson distribution in the volumes inoculated. The repeatability (r) and reproducibility (R) calculated according to ISO 5725-2 (1994) as part of interlaboratory trials showed:

Sample	Inoculum level / 100 ml	r	R
Bathing water (1 sea water, 2 fresh waters; 100 labs)	100	3.6	5.2
	400	2.1	3.7
River water (4 samples; 9 labs)	Naturally contaminated $(0.22 - 1.5 \times 10^4 / 100\text{ml})$	1.5	2.7
Sewage waters (4 samples; 9 labs)	Naturally contaminated $(0.19 - 51 \times 10^5 / 100\text{ml})$	2.6	3.9

Of concern is that the method involves growth media containing thallium acetate and N,N-dimethylformamide, the latter used to initially dissolve the MUD as a stock solution. Both of the former are toxic and great care should be taken, for example when making up the solutions in a chemicals fume hood.

4. ISO 7899-1 Water Quality- Detection and Enumeration of intestinal enterococci in surface and waste wate . Part 2: Membrane filtration method.

This membrane filtration method for the detection and enumeration of major intestinal enterococci is suitable for the examination of drinking waters, including samples from all stages of treatment and distribution, and those waters of moderate turbidity. High turbidity samples tend to block the 47 mm diameter, 0.45 • m cellulose-based filter, limiting the volume of sample to be filtered.

Presumptive enterococci reduce TTC after incubation to insoluble red formazan to produce red, maroon or pink colonies on membrane enterococcus agar (Slanetz and Bartley, 1957). The agar plates are incubated at 37ºC for 48 hours, although some organisms resembling enterococci may also grow on this medium. Selectivity is better at 44ºC although lower counts of enterococci may be obtained. It may be more appropriate that membrane filters from samples of potable water are incubated at 37ºC for 48 hours, whilst membrane filters from untreated waters are incubated at 37ºC for 4 hours followed by 44ºC for 44 hours. Because the method may allow other species to grow, such as *Aerococcus viridans* and *Staphylococcus* and *Bacillus* spp., confirmation is required. This is based on the enterococci being catalase-negative and on the demonstration of aesculin hydrolysis on bile aesculin agar or kanamycin aesculin agar incubated at 44ºC for up to 18 hours. Nevertheless, some strains of *S. bovis* and *S. equinus* may fail to grow at this temperature. If the aim is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be sub-cultured if fewer than ten are present, or at least ten colonies should be sub-cultured if more than ten are present.

Additional differential tests for enterococci may utilise their ability to grow in nutrient broth containing 6.5% sodium chloride at 37ºC and in glucose phenolphthalein modified to pH 9.6 (to differentiate them from other streptococci). They can also grow on 40% bile agar at 37ºC and survive exposure in nutrient broth to 60ºC for 30 minutes.

Of concern is that the method involves growth media containing sodium azide which is both highly toxic and can form explosive compounds with metals such as copper and lead. Great care should be taken, for example when making up the solutions in a chemicals fume hood and disposing of waste material carefully into drains. These should ideally be made of non-metallic maerials. Azide compounds should be decomposed and rendered safe with excess sodium nitrite before disposal.

Future rapid methods

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. Although the enterococci are routinely described to possess Lancefield's Group D antigen, serological methods of confirmation present many practical difficulties and there have been no robust latex bead or ELISA based procedures competing in the market place.

This is surprising, since there have been rapid advances with lateral flow devices and gold labelled immunosorbent assay (GLISA) technologies such as the Merck Singlepath range for *E. coli* O157, *Salmonella* etc. discussed in detail in *Project Horizontal Task 3B: Desk studies on feasibility of horizontal standard methods, including potential rapid methods for detection of* E. coli *and* Salmonella.

Conclusions as to the development for a method for enterococci 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 (WHO, 1997) as follows:

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 of sample?

We are not aware of any requirements for a strictly quantitative method being required at this time, which suggests that multiple tube MPN or presence/absence methods should be satisfactory. Given that assays should be specific, sensitive and preferably cheap (including labour costs), then two strategies seem appropriate.

The first involves further development of the Enterolert system due to its relatively high, semi-quantitative, precision; convenience, without requiring a lot of equipment or staff time; and speed, producing results conveniently in 24 hours. The system has been trialled and validated for various low turbidity samples and is now beginning to be used more for waste waters.

The second involves development of overnight enrichment culture techniques followed by one of two technologies, either:

a) disposable lateral flow devices, similar to the Singlepath technology. A convenient overnight enrichment culture of the target organism can be prepared and then confirmed in only a few minutes serologically. The challenge for this test will be to find an appropriate antibody (specific and sensitive for, say, the Lancefield's group D antigen) capable of bulk production for large demand in the market place.

b) Molecular labelling, using for example DNA oligonucleotide probes linked to biotin (e.g. Aureon, Vienna). Once these 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.

Either 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 seems pointless, given the potential for the Enterolert system to do this already and probably more cheaply. In reality, the lateral flow devices come into their own for presence/ absence determination e.g. no enterococci 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.

Both of these approaches should also be applicable to Clostridia, where initial easy growth of the target organisms in an anaerobic environment is an important prerequisite.

General Conclusion

It is clear from the above that there are a variety of methods available, reliant on either pre-enrichment culture, membrane agar culture, test tube MPN broth or Quantitray® technologies. None 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.

REFERENCES

Adcock, P.W. *et al.* (2001). Rapid confirmation of *Clostridium perfringens* by using chromogenic and fluorogenic substrates. Appl. Environ. Microbiol. **67**; 4382-4384

Armon *et al.* (1988). A modified m-CP medium for enumerating *Clostridium perfringens* from water samples. Can. J. Microbiol. **34**; 78-79

Araujo, M. *et al.* (2001). Evaluation of fluorogenic TSC agar for recovery of *Clostridium perfringens* from groundwater samples. Water Sci. Technol. **43**(12);201-4

ASTM (2000). D6503-99: Standard test method for enterococci in water using Enterolert. Annual Book of ASTM Standards – Water and Environmental Technology. Section 11.02. West Conshohoken, PA: American Society for Testing and Materials. Issued April 2000.

Augustynowicz, E. (2002). Detection of enterotoxigenic *Clostridium perfringens* with a duplex PCR. J.Med. Microbiol. **51**(2);169-72

Beerens,H. (1978). Int. J. Food Microbiol **40**; 203-207

Bisson, J.W. *et al.* (1979). Appl. Environ. Microbiol. **37**; 55-66

Broda *et al.* (2003). PCR detection of psychrophilic *Clostridium* species causing 'blown pack' spoilage of vacuum-packed chilled meats. J.Appl.Micrbiol**. 94**; 515-522

Budnick *et al.* (1996). Evaluation of Enterolert for enumeration of enterococci in recreational waters. Appl. Environ. Microbiol. **62**;3881-4

Cordoba, M.G. *et al.* (2001). Differentiation of *Clostridium perfringens* and *Clostridium botulinum* from non toxigenic clostridia isolates from prepared and frozen foods by PCR-DAN based methods. Nahrung **45**; 125-8

De Jong, A.E. *et al.* (2002). Optimising sporulation of *Clostridium perfringens.* J. Food Protect. **65**(9); 1457-62

Drahovska *et al.* (2002) PCR based methods for identification of *Enterococcus* species. Folia Microbiol (Praha) **47**; 649-53

Dromigny *et al.* (1997). New media for detection and counting of clostridia in foods. Lett. Appl. Microbiol. **24**; 19-22.

Dungu, B. (2000). Evaluation of two PCR based procedures for typing *Clostridium perfringens*. J. S. Afr. Vet. Assoc. **71**;111-4.

E.C. (1998). 98/83/EC of Council of 3rd November 1998 on the Quality of Water Intended for Human Consumption. Off. J. Eur. Commun. L330; 32-54.

Eisgruber, H. *et al.* (1995). Use of plasmid profiling as a typing method for epidemiologically related *Clostridium perfringens* isolates from food poisoning cases and outbreaks. Lett. Appl. Microbiol. **20**; 290-294.

Eisgruber, H. *et al.* (2003). Evaluation of different methods for detection of *Clostridium perfringens* phosphatases. Int. J. Food Microbiol. **82**;81-86.

Environment Agency, UK (2002). The Microbiology of Drinking Water (2002) Part 5: A method for the isolation and enumeration of enterococci by membrane filtration.

Environment Agency, UK (2002). The Microbiology of Drinking Water (2002) Part 6: Method for the isolation and enumeration of sulphite –reducing clostridia and *Clostridium perfringens* by membrane filtration.

The Water Supply (Water Quality) Regulations (Scotland), 2000.

Fach, P *et al.* (1997). Detection of enterotoxigenic *Clostridium perfringens* in food and faecal samples with duplex PCR and slide latex agglutination test. Appl. Environ. Microbiol. **63**(11); 4232-6.

Federal Permit (2002). Authorisation to discharge under the national pollutant discharge elimination system. New Hampshire Dept. Resources and Economic Development.

Fontana *et al.* (2001). Rapid enumeration of clostridia spores in raw milk samples with an impedimetric method. SY-LAB documentation submitted to J. Rapid Meth. Automat. Microbiol.

Fugisawa *et al.* (2000). Occurrence of clostridia in glass bottled foods. Int. J. Food. Microbiol. **54**; l213-7.

Garcia-Fontan, M.C. (2002). Identification of enterococci isolated from cows milk cheese :Comparison of classical methods and API 20 STREP system. Acta Microbiol. Immunol. Hung. **49**; 119-28.

Haber *et al.* (1998). Comparison of four methods for the detection of faecal streptococci in water. Zentralbl Hyg. Umvelymed. **201**; 357-69

Hanford, P.M. (1974). J. Appl. Bacteriol. **37**; 559-570.

Harmon, S.M. *et al.* (1971). Comparison of media enumeration of *Clostridium perfringens*. Appl. Microbiol. **21**; 922-927

Harmon, S.M. (1976). Collaborative study of an improved method for the enumeration and confirmation of *Clostridium perfringens* in foods. J. AOAC **39**; 606-612.

Harpold, D.J. *et al.* (1985). Rapid identification of *Clostridium* species by high pressure liquid chromatography. J. Clin. Microbiol. **22**(6);962-7.

Hauschild, A.H.W. *et al.* (1974). Enumeration of faecal *Clostridium perfringens* in egg yolk-free tryptose-sulphite cycloserine agar. Appl. Microbiol. **27**; 527-530.

Heiber, I., Frahm, E., and Obst, U. (1998). Comparison of four methods for the detection of fecal streptococci in water. Zentralbl. Hyg. Umweltmed. 201, 357-369.

Hibiscus Coast Sewerage Strategy Review 2002 Briefing Paper 4.

Hill, R.T. *et al.* (1996). Distribution of sewage indicated by *Clostridium perfringens* at a deep water disposal site after cessation of sewage disposal. Appl. Environ. Microbiol. 1996; 1741-1746.

Hirsch,A. *et al.* (1954). J. Dairy Res. **21**; 101-110.

Huycke *et al* (date) Multiple drug resistant enterococci: The nature of the problem and an agenda for the future.   Journal?

ISO 5725-2: 1994. Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.

ISO 6461-2: 1986. Detection and enumeration of the spores of sulphite-reducing anaerobes (clostridia) - Part 2. Method by membrane filtration.

ISO 6461-1: 1986. Detection and enumeration of the spores of sulphite-reducing anaerobes (clostridia)- Part 1. Method by enrichment in a liquid medium.

ISO 7899-1: 1999 Water Quality-Detection and enumeration of intestinal enterococci in surface and waste water. Part 1: Miniaturised method (Most Probable Number) for surface and waste water.

ISO 7899-2: 1999 Water Quality-Detection and enumeration of intestinal enterococci in surface and waste water. Part 2: Membrane filtration method.

Iverson, A. (2002). High prevalence of vancomycin resistant enterococci in Swedish sewage Appl. Environ. Microbiol **68**; 2838-2842.

Killic, U. *et al.* (2002). Ribotyping of *Clostridium perfringens* from industrially produced ground meat. Lett. Appl. Microbiol. **34**; 238

Kim, S. *et al.* (2000). Inhibitory effects of collagen on the PCR for detection of *Clostridium perfringens.* Appl. Environ.Microbiol. **66**; 1213-5.

Kinzelman, J., Ng, C., Jackson, E., Gradus, S. and Bagley, R. (2003). Enterococci as indicators of Lake Michigan recreational water quality: comparison of two methodologies and their impacts on public health regulatory events. Appl. Environ. Microbiol. 69, 92-6.

Leclerc, B (date?). Poster presentation. Direct detection of vancomycin resistant enterococci from faecal samples duringa hospital outbreak using multiplex PCR and captive probe hybridisation (venue?).

Leuschner *et al.* (2002). A collaborative study of a method for the enumeration of probiotic enterococci in animal feed.   J. Appl. Microbiol. **93**; 781-86.

Lukinmaa,S. *et al.* (2002). Molecular epidemiology of *Clostridium perfringens* related to food borne outbreaks of disease in Finland from 1984-1999. Appl. Environ. Microbiol. **68**; 3744-49.

Manafi, M. (1993). Rapid identification of enterococci with a new fluorogenic-chromogenic assay. Water Sci.Technol. 27; 271-274.

Manafi, M. (2000). New developments in chromogenic and fluorogenic culture media. Int. J.Food Microbiol. **60**; 205-218.

Manero *et al.* (2002). Identification of *Enterococcus* species based on specific hybridisation with 16S rDNA probes. J. Micro. Methods **50**; 115-21.

Manero *et al* (2002). Characterisation of sewage waters by biochemical fingerprinting of enterococci. Water Res. **36**; 2831-5.

Manual of Clinical Microbiology. (2000). Enterococci (Chapter 18). 7th edition. ASM Press, Washington DC.

McNamara, A. *et al.* (1998).USDA/FSIS Microbiology Laboratory Guidebook 3rd Edition.

Maslanka, S.E. *et al.* (1999). Molecular subtyping of *Clostridium perfringens* by pulsed field gel electrophoresis to facilitate food-borne disease outbreak investigation. J.Clin. Microbiol. **37**; 2209-14.

Merck. Simple and Rapid Detection of Bacteria and Yeasts using Fluorogenic and Chromogenic culture media.

Miwa *et al.* (1999). Most probable numbers of enterotoxigenic *Clostridium perfringens* in intestinal contents of domestic livestock detected by nested PCR. J. Vet. Med. Sci. **59**; 557-60.

Niculescu, E. *et al.* (1905). Differentiation of clostridia. Zentralbl. Bakteriol. Mikrobiol. Hyg. 259; 307-16.

Nordic Committee on Food Analysis (1992) Enterococcus determination in foods No.68, 2nd Edition.

Nordic Committee on Food Analysis (2002). Sampling and pre treatment of foods and animal feedstuffs for quantitative microbiological examination. No. 91, 4th Edition.

Ohio District Microbiology Laboratory. Analysis of *Clostridium perfringens* in Environmental Water Samples,

Orth, D.S. (   ). Comparison of sulphite-polymyxin-sulphadiazine medium and tryptose-sulphite cycloserine medium without egg yolk for the recovery of *Clostridium perfringens.* Appl. Environ. Microbiol. **33**; 968-988

Ossmer, R., Schmidt, W., Mende, U. (1999). Chromocult[®] Coliform Agar – Influence of Membrane Filter Quality on Performance. – Poster presentation Congresso de la Sociedad, Espanola de Microbiologia, Granada, Spain

Perez-Hernandez *et al* (2002). A PCR assay for rapid detection of vancomycin resistant enterococci. Diagn. Microbiol. Infect. Dis. **42**; 273-7.

Pillai, S.D. *et al.* (1996). Occurrence of airborne bacteria and pathogen indicators during land application of sewage sludge. Appl. Environ. Microbiol. **62**; 296-99.

Public Health Laboratory Service (2002). Standard Operating Procedure W3: Enumeration of faecal streptococci by membrane filtration.

Public Health Laboratory Service (2002). Standard Operating Procedure W5: Enumeration of sulphite–reducing clostridia by membrane filtration.

Public Health Laboratory Service (2002). Standard Operating Procedure F14: Enumeration of *Clostridium perfringens*.

Reuter, G. (1992). Culture media for enterococci and Group D streptococci. Int. J. Food Microbiol. **17**;101-111.

Rhodehamel, E.J *et al,* (1998) Bacteriological Analytical Manual, 8th Edition, Revision A, Chapter 16.

Schalch, B. *et al.* (1997). Ribotyping for strain characterisation of *Clostridium perfringens* isolates from food poisoning cases and outbreaks. Appl. Environ. Microbiol. **63**; 3992-3994.

Schalch, B. *et al.* (1999). Molecular methods for the analysis of *Clostridium perfringens* relevant to food hygiene. FEMS Immunol. Med. Microbiol. **24**; 281-6.

Schneider, J. *et al.* (1998). Microbiological research methods of drinking water regulations in West Germany from 1986. Suitability of the specificity of DIN 38411, Pt.7 for the detection of sulphite reducing spore forming anaerobes. Zentralbl. Bakteriol. Mikrobiol. Hyg. **185**; 368-78.

Schoepe, H. *et al.* (1998). Controlled multiplex PCR of enterotoxigenic *Clostridium perfringens* strains in food samples. Mol. Cell Probes **12**; 359-65.

Skanavis, C. *et al.* (2001). *Clostridium perfringens* as a potential indicator for the presence of sewage solids in marine sediments. Marine Pollution Bulletin **42**; 31-35.

Slanetz , L.W. and Bartley, C.H. (1957). Numbers of enterococci in water, sewage and faeces determined by the membrane filter technique with an improved medium. J. Bacteriol. **74;** 591-595.

Song,Y. *et al.* (2002). Syst. Appl. Microbiol. **25** (4); 528-35.

Stampi *et al.* (2001). Evaluation of the efficiency of peracetic acid in the disinfection of sewage effluents. J.Appl. Microbiol. **91**; 833

Tyrrell *et al* (1997). Species identification of enterococci via intergenic ribosomal PCR. J. Clin. Microbiol. **35**; 2434.

Ueno *et al.* (1970). Acid phosphatase in *Clostridium perfringens*: a new, rapid and simple identification method. Jpn. J. Microbiol. **14**; 171-3.

US Federal Register (2003). Part III Environment Protection Agency. 40 CFR Part 136 Vol. 68, No.139, pp. 43272-43283.
US EPA Water Science (1986). Method 1600: Membrane filter test method for enterococci in water.

US EPA (2002). Method 1106.1: Enterococci in water by membrane filtration using membrane-Enterococcus-Esculin Iron Agar (mE-EIA). U.S. Environment Protection Agency, Office of Water, Washington, DC. EPA-821-R-02-021.

US EPA (2002). Method 1600: Enterococci in water by membrane filtration using membrane-Enterococcus Indoyl-• -D-Glucoside Agar (mEI). U.S. Environment Protection Agency, Office of Water, Washington, DC. EPA-821-R-02-022.

US EPA Water Science (2002). Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and *Escherichia coli* Methods 1106.1,1600

U.S. Food and Drug Administration (1992) Foodborne Pathogenic Microorganisms and Natural Toxins Handbook.

Van Dyke *et al.* (1995). Int. J. Food Microbiol. **27**; 185-200.

Van Dyke, M.I. *et al.* (2002). Appl. and Environ. Microbiol. **68**; 2049-2053.

Vilanova *et al.* (2002). The effect of sewage treatment plant effluent on the faecal coliforms and enterococci populations of the reception river waters. J. Appl. Microbiol. **92**; 210-4.

Weenk, G. *et al.* (1991). Selective enumeration of spores of *Clostridium* species in dried foods. J. Appl. Bacteriol. **70**; 135-143.

Weenk, G.H. *et al.* (1995). Modified methods for the enumeration of spores of mesophilic *Clostridium* species in dried foods. Int. J. Food Microbiol. **27**;185-200.

Wheeler, A. (2002). Potential of *Enterococcus faecalis* as a human faecal indicator for microbial source tracking. J. Environ. Qual. **31**;1286-93.

Yakub, G. P., Castric, D. A., Stadterman-Knauer, K. L., Tobin, M. J., Blazina, M., Heineman, T. N., Yee, G. Y. and Frazier, L. (2002). Evaluation of Colilert and Enterolert defined substrate methodology for wastewater applications. Water Environ. Res. 74, 131-135.

Appendix 1

Some Quotes from Relevant Sections of the Draft Sludge
and Biowaste Directives and UK Draft Sludge (Use in Agriculture) (Amendment)
(England and Wales) 2002 No. To be assigned.

Draft Sludge Directive (EU 2000)

"Advanced treatments (hygienisation)

The treated sludge shall not contain *Salmonella spp* in 50 g (wet weight) and the treatment shall achieve at least a 6 Log_{10} reduction in *Escherichia Coli* to less than $5 \cdot 10^2$ CFU/g.

The process shall be initially validated through a 6 Log_{10} reduction of a test organism such as *Salmonella senftenberg W 775*."

"Conventional treatments

Storage in liquid form at ambient temperature as a batch, without admixture or withdrawal during the storage period[*]. The sludge treatment shall at least achieve a 2 Log_{10} reduction in *Escherichia coli*."

Draft Biowaste Directive (EU 2001)

"Methods for analysis and sampling

Salmonella spp. number/50 g dm (i.e. absence)

Clostridium perfringens number/1 g dm" (i.e. absence)

UK Draft Sludge (Use in Agriculture) (Amendment) (England and Wales) 2002   No. Awaited

"For the purpose of this schedule-

a)   "units" of *E. coli* means colony-forming units of *Escherichia coli* expressed as units per gram (dry weight) of sludge and

Salmonella spp. shall be measured by reference to 2 grams (dry weight) of sludge."

"The sludge produced shall be sampled as follows: -

On each occasion a set of five samples shall be taken at random from a batch of sludge, each consisting of 100ml in the case of liquid sludge or 100g in the case of dried sludge

Each sample shall be analysed separately in accordance with paragraph 5"