Work Package 3

Task 4:

Desk studies on feasibility of horizontal standard rapid methods for detection of *Clostridium perfringens* and enterococci in Sludges, Soil, Soil Improvers, Growing Media and Biowastes

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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. At least one report has suggested that the use of m-CP agar medium, which is used in the reference method in the European Union, is not suitable for recovering *C. perfringens* spores from groundwater. This questions its possible use as a method for detecting *C. perfringens* in sludge, soil, soil improvers, growing media, and biowaste.

Indeed, all of the methods described for detection of *C. perfringens* and enterococci 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. Nevertheless, it is considered feasible to formulate horizontal standards to cover analysis of *C. perfringens* and enterococci in sludge, soil, soil improvers, growing media, and biowaste. However, none of the methods have been extensively evaluated for these waste types. 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 4 deals with an assessment of the feasibility of horizontal standard methods for detection of *Clostridium perfringens* and *enterococci* 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 sampling and interlaboratory trial aspects will not be considered here.

Appendix A 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. *Clostridium perfringens* is specifically mentioned in the draft Biowaste Directive (EU 2001). 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 pre-normative and 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 Clostridia 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 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. Their main value is an indicator for presence of faecal material and the efficiency of the treatment process. *Clostridium* spp. (e.g. *C. perfringens*) are common in raw sludge and are resilient to environmental stress in the spore form. *C. perfringens* is a not

only a pathogen but also an important indicator for the potential presence of other dangerous spore-forming clostridia such as *C. botulinum* that produce harmful toxins if they survive waste treatment processes and then vegetate (Strauch, 1998). The US EPA (1999) has tabulated a list of pathogens that may be found in sludge of faecal origin; this includes not only enterococci and clostridia but also the spore-forming *Bacillus anthracis* (Table 1).

Table 1 Pathogenic agents potentially present in organic waste from urban origin

Bacteria	Parasites	Yeast
Bacillus anthracis *Campylobacter spp.* ***Clostridium* spp.** *Chlamydia psittaci* *Escherichia coli* ***Enterococci*** *Erysipelothrix rhusiopathiae* *Leptospira spp.* *Listeria monocytogenes* *Mycobacterium spp.* *Pasteurella spp.* *Pseudomonas aeruginosa* *Salmonella sp* *Serratia spp* *Shigella sp* *Staphylococcus spp* *Vibrio cholerae* *Yersinia enterocolitica* *E. coli* O157 and other enterohemorrhagic *E. coli*	Protozoa *Entamoeba histolytica* *Giardia intestinalis* *Toxoplasma gondii* *Sarcocystis spp* *Cryposporidium spp* Cestodes *Taenia saginata* *Taenia solium* *Diphyllobothrium latum* *Echinococcus granulosus* *Hymenolepis spp* Nematodes *Ascaris lumbrocoides* *Ankylostoma duodenale* *Toxocara canis spp* *Toxocara cati* *Trichuris trichura*	*Candida albicans* *Candida krusei* *Candida tropicalis* *Candida guillermondii* *Cryptococcus neoformans* *Trichosporon* Fungi *Aspergillus spp.* *Aspergillus fumigatus* *Phialophora richardsii* *Geotrichum candidum* *Trichophyton spp.* *Epidermophyton*
Viruses		Phytopathogens
Adenovirus (49 types) Astrovirus (7 types) Coronavirus Norovirus Sapovirus Reovirus (3 types) Rotavirus (3 groups) HAV (1 type)	Enterovirus : - Poliovirus (3 types) - Coxsackievirus A (23 types) - Coxsackievirus B (6 types) - Echovirus (32 types) - Enterovirus 68 - 71 (4 types)	*Botrytis cinerea* *Clavibacter michiganensis pv. sepedonicus* *Globodera pallida* *Globodera rostochiensis* *Phytophtora ramorum* *Polymyxa betae* *Polymyxa graminis* *Pythium spp.* *Ralstonia solanacearum* *Rhizoctonia solani* *Sclerotinia sclerotiorum* *Sclerotium cepivorum* *Synchytrium endobioticum* Tobacco mosaic virus (TMV) Potato spindle tuber viroid (PSTV)

In Spain, clostridia are tested not just as indicator organisms but as pathogens in their own right. Therefore, a method to detect spore-forming clostridia in treated wastes is important to show that these wastes are safe. *E. coli* or helminth ova detection/viability methods cannot replace this. Perhaps surprisingly, there has been little published data on the quantification of clostridia in wastes. Table 2 shows some values found in the literature, mostly for yard wastes.

Table 2 Concentration of pathogenic micro-organisms in organic wastes from urban origin

Sewage sludge	Municipal solid wastes
Campylobacter : 0 - 10^6 / 100 mL	*E. coli* : 0 - 10^9 CFU/g
Thermotolerant coliforms: 10^4 - 10^8 / g	*Mycobacterium spp.* : 120 – 5 10^4 / g
E. coli : 10^4 - 10^7/g	*Salmonella* : 0 - 10^4 /g
Listeria spp : 2743 / g	**Enterococci : 10^8 - 10^9 /g**
Mycobacterium spp : 0 – 3173 / g	Viable *Ascaris* ova : present in 50g
Salmonella : 0 - 10^5 /g	
Enterococci : 10^1 - 10^8 /g	Yard wastes
Giardia cysts: 0 - 3,1 10^5 / 10 g	***Clostridium perfringens* : 30 – 125 / g**
Viable *Ascaris* ova : 0 -20 /10g	Thermotolerant coliforms: 80 /g
Taenia ova: 0 - 10^2 / 10 g	*E. coli* : 12 – 10^4 /g
Toxocara ova : 0 - 23 /10 g	*Salmonella* : 0 in 25 g
Hymenolepis ova : 0 - 200 / 10 g	**Enteroccoci : 1,4 x 10^4 / g**
Enteroviruses : up to 250 / g	
Rotavirus : up to 500 / g	

(Results per g dry weight except for yard wastes)

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 are 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*., 1985), 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. Denmark intends to introduce routine testing of water using the draft ISO 6461-2 (2003) (see later).

1.3 Enterococci introduction

Intestinal enterococci are lactic acid bacteria and 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 (Table 2). By contrast, although there has been little published data on the quantification of enterococci in wastes, they occur in high numbers in municipal solid wastes (Table 2).

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.

2. EXISTING STANDARDS OR DRAFT STANDARDS

2.1 Current methods for the detection of clostridia

2.1.1 Growth media

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, such as other anaerobic species and lactobacilli, so some media now contain inhibitors and other selective agents. Sulphide and an iron source are usually used as indicators. The clostridia reduce the sulphite to sulphide 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.

Indeed, sodium sulphite and ferric citrate are added to RCM to become differential RCM (DRCM) which has been recommended for the detection of sulphite reducing clostridia in drinking water (UK SCA, The Microbiology of Water, 1994, pt.1) and is specified in the ISO standard 6461-1(1986) liquid enrichment method for water. The blackening of the colonies due to sulphite reduction can present problems in agar media. By contrast, Mead (1992) has described advantages of the test in relation to its use in liquid media for Most Probable Number determinations, similar to the ISO 6461-1 (1986) methodology. An MPN approach would offer a relatively simple assay with increased sensitivity compared to membrane filtration (see Table 9), ideal for the presence/absence requirement of the draft Biowaste Directive stipulating that *C. perfringens* should be absent in 1 g dry matter (Appendix 1).

An alternative to RCM agar is Perfringens Agar Base supplemented with egg yolk emulsion and either kanamycin sulphate (12mg/litre) and polymyxin B sulphate (30,000 IU/litre), to prepare Shahidi-Ferguson Perfringens (SFP) Agar, or D-cycloserine (400mg/litre) to prepare Tryptose Sulphite Cycloserine (TSC) Agar. Again, sodium metabisulphite and ferric ammonium citrate are used as an indicator of sulphite reduction by *C. perfringens* which produces black colonies in both media. Harmon *et al*. (1971a) showed that polymyxin B and kanamycin sulphate used in SFP Agar allow a greater recovery of both vegetative cells and spores of *C. perfringens* than either polymyxin B or sulphadiazine used in Sulphite Polymyxin Sulphadiazine (SPS) Agar, or neomycin, used in Tryptone Sulphite Neomycin (TSN) Agar. However, a greater number of non-specific colonies were found on SFP Agar. These authors went on to show that *Serratia marcescens* and *Streptococcus lactis* were the only facultative anaerobes to grow on TSC Agar, whereas SFP Agar also allowed the growth of *Enterococcus, Proteus* and *Enterobacter* strains, but allowed a slightly higher rate of recovery of C. perfringens than TSC Agar (Harmon *et al.*, 1971b). Both SFP Agar and TSC Agar permitted growth of other sulphite-reducing *Clostridium* spp. tested, with the exception of *C. sordellii* which was completely inhibited and *C. bifermentans* which was partially inhibited on TSC Agar. Both strains grew on SFP Agar. Some strains of *C. perfringens* produce an opaque zone around the colony due to lecithinase activity, but Hauschild and Hilsheimar (1973) did not consider this reaction to be universal for all *C. perfringens* strains after overnight incubation and both black lecithinase-positive and black

lecithinase-negative colonies should be considered as presumptive *C. perfringens* on TSC or SFP Agars and confirmatory tests carried out. Egg yolk positive facultative anaerobes may grow on SFP Agar to produce completely opaque plates thus masking the egg yolk reaction of *C. perfringens.* Conversely, Hauschild and Hilsheimar (1973) described an egg yolk-free TSC Agar which has the advantage that smaller colonies are formed. This can simplify the counting of plates with high numbers of colonies. Higher counts have been demonstrated by using it with a pour plate technique.

TSC was the medium proposed by Harmon *et al.* (1971) for detection of vegetative and spore forms of *C. perfringens* in foodstuffs and clinical specimens; the D-cycloserine causing non-clostridial colonies which develop to remain smaller. Subsequently, Harmon and Kautter (1987) showed that for the detection of *C. perfringens* in human faeces associated with an outbreak, TSC was superior to trypticase-soy-blood (TSB) agar, lactose-sulfite (LS) medium, and iron milk (IM) medium. Faecal samples were heat treated at 75ºC for 20 min and dilutions were plated directly onto TSB and TSC, and a 3-tube most probable number determination was made with each specimen in LS and IM incubated at 45ºC. Confirmed counts on TSC and TSB were similar for all specimens, but counts of 8 of 25 outbreak specimens were 2-4 log units lower in LS and IM than on plating media; spores in specimens associated with 2 of 5 outbreaks were intolerant of the elevated temperatures. These data showed that elevated temperature MPN methods in LS and IM are inappropriate for the examination of outbreak stools. TSC medium complies with the ISO draft method for analysis of meat (1978), DIN Norm 10165 and APHA recommendations for the examination of foods (1992). The medium is used in the standard method for isolation and enumeration of clostridia in food by the USA 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. These sulphite reducing organisms grow readily on SFP and TSN agar media as black colonies with a tendency to spread and obscure the whole surface of the medium. Some strains of enterococci will grow on OPSP as white colonies, easily distinguished from the large black colonies of *C. perfringens*.

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). The method exploits the lack of •-D-glucosidase activity (involved in cellobiose fermentation), and the fermentation of sucrose and production of acid phosphatase to differentiate presumptive *C. perfringens* colonies from other *Clostridium* spp. Lack of •-D-glucosidase activity means that *C. perfringens* does not cleave the chromogen, indoxyl •-D-glucoside, in the medium. Accompanying sucrose fermentation reduces the pH and changes bromocresol purple from purple to yellow. This results in characteristic opaque yellow *C. perfringens* colonies. Most other *Clostridium* spp. will appear as either purple colonies, due to the lack of sucrose fermentation, or blue/green colonies where the organism is still cleaving indoxyl •-D-glucoside and also fermenting sucrose (see Table 3). Presumptive positive *C. perfringens* colonies can be further tested for acid phosphatase activity by exposure to ammonium hydroxide vapour for 20 to 30 seconds. *C. perfringens* colonies turn pink or red as phenolphthalein diphosphate is cleaved by acid phosphatase. No colour change will be seen with colonies of organisms that do not posses acid phosphatase. It is important this further test is

carried out as there are a very small number of non-perfringens clostridia that produce yellow colonies. However, these colonies will remain yellow after exposure to ammonium hydroxide as they are acid phosphatase negative. D-cycloserine, polymyxin B and incubation at 44° C inhibit the growth of background flora such as Gram-negative organisms and staphylococci.

Table 3 Appearance of typical clostridial colonies on m-CP agar medium

Typical Colony Colour	Phenotype	Organism
Opaque Yellow	Sucrose positive/Glucosidase negative then pink/red after exposure to NaOH	*C. perfringens*
Blue/Green	Sucrose positive/Glucosidase positive	*C. baratii, C. paraputrificum, C. tertium*
Purple	Sucrose negative/Glucosidase positive or negative	*C. bifermentans, C. difficile, C. sporogenes*
Opaque Yellow	Sucrose positive/Glucosidase negative remain yellow after exposure to NH_4OH	Other clostridia

m-CP agar 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: the verification of typical colonies was 93% and the average recovery from inoculated filter-sterilised sea water suspensions was 90% (Bisson and Cabelli, 1979). m-CP has been recommended in European Council Directive 98/83/EC (1998) for testing the quality of water intended for human consumption.

Sartory *et al.* (1998) compared TSC and m-CP agar media for recovery of *C. perfringens* in environmental and part-treated drinking water. Samples were filtered through 0.45 •m membrane filters, placed on the agar media and incubated at 44ºC for 18-24 hours. For laboratory strains of *C. perfringens, C. baratii, C. chauvoei, C. paraputrificum* and *C. tertium*, m-CP was found more selective and specific, but markedly less efficient for the enumeration of both vegetative cells and spores. TSC also recovered significantly greater numbers of *C. perfringens* from river water and part-treated drinking water. In contrast to previous work reporting only a 2.0% false-negative rate (Bisson and Cabelli, 1979), these authors found a significant number of false presumptive positives and negative isolates on m-CP (only 47% confirmed as *C. perfringens* by the set criteria, compared to 86.7% on TSC; 16.5% of 65 atypical colonies from m-CP identified as *C. perfringens* while non of the 48 non-black isolates from TSC were so identified). The 86.7% recovery rate on TSC was in close agreement to the 92.6% reported previously (Sartory, 1986). Consequently, they concluded that TSC is a more suitable medium for the routine monitoring of water supplies for the presence of *C. perfringens*. The specificity of TSC for *C. perfringens* might be further improved by using a modification of TSC, Sulphite Cycloserine Azide agar, with incubation at either 37ºC or 44ºC (Eisgruber and Reuter, 1995).

An alternative chromogenic medium is Differential Clostridial Agar (DCA) for enumeration of sulphite reducing clostridia in dried foods (Weenk *et al.*, 1991). This medium contains sulphite, iron, starch to promote spore germination and Resazurin as a redox indicator, turning red at high redox potential indicating aerobic conditions. The manufacturers suggest that the sample is heated at 30ºC for 10 minutes 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 e.g. using reversed passive latex agglutination (RPLA) (Oxoid).

Although primary isolation on TSC agar takes 24 hours, the standard method of confirmation for presumptive *C. perfringens* can take up to 72 hours, and is tedious and expensive. Therefore, 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 fluorophore 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) described a rapid method utilising both of these chromogenic substrates for the detection of *C. perfringens* in river and surface storage water, and sewage effluent. In initial experiments, samples were filtered and the membranes incubated on TSC agar containing MUP, but this proved unreliable due to diffusion of the fluorescent product 4-methylumbelliferone and interference from particulate matter. Subsequent experiments transferred colonies from TSC agar to TSC-MUP agar for confirmation but this gave a high false-positive rate, indicating that reliance on acid phosphatase alone is unreliable. Combining detection of acid phosphatase with β-galactosidase activity using a MUP-ONPG liquid assay improved confirmation markedly, yielding results within 4 hours: *C. perfringens* demonstrated acid phosphatase activity (UV fluorescence) within 1 hour and β-galactosidase activity (yellow colouration) with 4 hours. This test does not need to be performed anaerobically, removing the need for prereduction of media and complex anaerobic incubations. The MUP-ONPG method confirmed 164 of 333 isolates as *C. perfringens*, compared to 153 of 333 isolates using the standard method. The MUP-ONPG assay demonstrated a high level of sensitivity (99.3%), although specificity (93.3%) was adversely affected by the number of false negative results (7.1%) obtained by the standard method of confirmation.

In Spain, Araujo *et al*. (2001) evaluated TSC agar, fluorogenic TSC (TSCF) 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 TSCF were significantly greater ($P = < 0.05$) than with m-CP, supporting the work reported earlier of Sartory and colleagues. Araujo *et al*. (2001) summarised their study by stating that the membrane filtration technique using TSCF agar showed the best performance characteristics of all three media tested, as judged by recovery efficiency and specificity in these water samples (85.3% of typical colonies and 82.8% of atypical colonies confirmed). The fluorogenic substrate is available commercially as a convenient additive to conventional TSC agar base (Fluorocult, Merck). Recently, Araujo *et al.* (2004) have extended their study of different recovery media to also include TSN agar, SPS agar and Wilson-Blair (WB) agar. Groundwater samples were pretreated by heating to 80ºC for 5 min. The *C. perfringens* spore counts on mCP agar were significantly lower ($P<0.05$) than the corresponding values of TSC, TSCF, SPS, and WB media. No statistically significant differences were found between *C. perfringens* spore counts on

TSCF compared with those of other methods used. On the other hand, the identification of typical and atypical colonies isolated from all media demonstrated that TSCF agar was the most specific medium for *C. perfringens* spore recovery in groundwater samples. The authors concluded that m-CP agar, which is used in the reference method in the European Union, is not a suitable medium for recovering *C. perfringens* spores from groundwater.

Table 4 summarises the media currently in use for the isolation of clostridia and Appendix B details the recipes for each of them. 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 of 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 macerates of food in buffered cysteine hydrochloride/peptone/saline, immediately over layered 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.

Table 4 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
SPS agar (Sulphite Polymyxin Sulphadiazine)	Food, clinical specimens		BBL
TSC agar (Tryptose sulphite cycloserine)	Food, clinical specimens		Merck; Oxoid
TSN agar (Tryptose sulphite neomycin)	Food, clinical specimens		Merck
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)	Water	More economical (Armon *et al*. 1988)	
DCA (Differential Clostridial Agar)	Dried foods		Merck
Clostridia impedance broth	Milk	No details available but false positives due to aerobic spore formers reduced in presence of Oxyrase (lower the redox)	SY-LAB

The inclusion of complex ingredients in the media, e.g. yeast extract or meat extract, may make absolute standardisation 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.

There is some dispute as to the superiority of one medium over another (e.g. Bisson and Cabelli, 1979; Weenk *et al*., 1995; Sartory *et al*., 1998; Adcock and Saint, 2001). In any case, 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.

2.1.2 Confirmation tests

As described previously, 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. In this regard, the MUP-ONPG liquid 4 hour liquid assay confirmation test already described shows some merit.

2.1.3 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 has 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. In the presence of a catalyst, 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 atmosphere.

2.2 Current standard methods for clostridia

Several national and internationally approved standard methods have been developed for the detection of clostridia. These address regulatory concerns with potable water and food quality and include:

Standing Committee of Analysts, The Microbiology of Drinking Water (2002) Part 6: Methods for the isolation and enumeration of sulphite–reducing clostridia and *Clostridium perfringens* by membrane filtration. *Methods for the Examination of Waters and Associated Material*, Environment Agency, UK.

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* (for food).

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 6461-2: 1993. Detection and enumeration of the spores of sulphite-reducing anaerobes (clostridia) - Part 2. Method by membrane filtration.

US FDA: Bacteriological Analytical Manual, 2001, 8th edition Revision A (1998).

These methods are summarised in Table 5.

Table 5 Isolation and detection of clostridia: methods available

Origin of method	Matrix 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^{o}C 20-44 h. Black colonies: no other confirmation done	No performance data in the standard (but see Araujo *et al*., 2001; 2004).
PHLS SOP W5	Water	20 ml or 50 ml bottled	Heat kill vegetative cells. Filter to collect spores. Put filter in TSCA medium at 40^{o}C, allow to set and incubate at 37^{o}C 20-44 hr. Black cols – can subculture	As above
PHLS SOP F14	Food	Serial dilutions	Dilns made, 1ml plus molten selective culture medium (TSCA) at 40^{o}C, allow to set then overlay 37^{o}C 20 h. Black colonies confirm by subculture, motility, nitrate, lactose gelatin medium.	Minimum detection limit of 10 cfu/g.
Draft method for toxin	Faecal specimens			Use commercial *C. perfringens*

				toxin tests (e.g. Unipath) sensitivity 2 ng/ml)
ISO 6461-1: 1986 Detection and enumeration of the spores of sulphite-reducing anaerobes (clostridia) Part 1:Method by enrichment in a liquid medium	All types of water, including turbid water	50 ml plus dilutions.	Heat kill vegetative cells (75^{o}C,15 min) Diln in media (DRCM, sodium sulphite, iron citrate) 37^{o}C, 44 hr 'blackening' i.e. reduction of sulphite taken as positive MPN	Positive growth indicated by reduction of sulphite and precipitation of iron (II) sulphide. MPN using 50 ml sample; presence/absence using 100 ml
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^{o}C, 15 min) Filter the samples, place membrane face down in dish add media (sulphite iron agar) at 50^{o}C. Once set incubate anaerobically 37^{o}C for 20 to 44 hrs Black cols positive	Report number of black colonies from 100 ml of sample. Use smaller volumes for polluted waters and sewage.
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^{o}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

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

The clostridia are a group of anaerobic bacteria that vary considerably in their biochemical and physiological properties. Not surprisingly, attempts to develop a single isolation medium for all species that occur in foods, faeces and other matrices have not been entirely successful, and the problem is compounded by the need to recover both vegetative cells and spores, some of the latter being unable to germinate without heat activation. Although the literature generally

supports the advantages of TSC agar-based methods, a comparison is required of the most promising selective media e.g. TSC, m-CP chromogenic and TSC-fluorogenic agars for sludges, soil and biowastes. 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 and should include an evaluation of the rapid MUP-ONPG 4 hour procedure. It should not be forgotten that the Draft Biowaste Directive (Appendix 1) specifies that clostridia should be absent per g dry matter. Clearly, validation studies should ensure that this sensitivity of detection is achievable for sludge, soil and biowastes. This requirement would support the arguments of Mead (1992) for liquid enrichment, albeit taking 48 hours, and includes methods such as ISO 6461-1.

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 acid phosphatase method of Ueno *et al*. (1970) and modified by Mead *et al*. (1981)(D. Sartory, personal communication).

2.4 Current 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 in a 2 step membrane filtration procedure: a primary isolation medium, membrane enteroccus agar (mEA), and aesculin iron agar (EIA). The incubation time required for the selective primary isolation mEA at 41ºC was 48 hours, followed by transfer of the membrane to the EIA substrate medium which is incubated at 41ºC for 20 minutes. Pink to red colonies on mEA that produce a brownish-black precipitate on EIA are identified as enterococci. The specificity, as reported for various environmental water samples, was 10% false-positive and 11.7% false negative (USEPA, 1985). This method was revised in 1997 (USEPA, 1997) and resulted in the use of a single medium, mEI agar, which was a modification of the original mEA by reducing the concentration of triphenyl tetrazolium chloride from 0.15 to 0.02 g/l and adding 0.75 g/l of a chromogenic cellobiose analogue, indoxyl-β-D glucoside. This improvement resulted in a reduced incubation time of 24 hours and a specificity of 6.0% false positives and 6.5% false negatives (Messer and Dufour, 1998).

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 suppresses 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 enterococci (*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 were 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 cleaves 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.

2.5 Current standard methods for enterococci

Several standard methods have been developed for the detection of enterococci. As with clostridia, these methods address regulatory concerns with potable water and food quality. Tables 6, 7 and 8 briefly describe the current approved methods for the isolation and detection of enterococci and details of the primary selective media and subculture media employed by these methods. This is followed by an assessment of the methods. Fuller details of the methods and the confirmatory tests used are described in Appendix 3.

Table 6 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.	1 cfu per 100 ml if not heavily polluted	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 Subculture to bile aesculin (BEA)	As above	Substrate TTC
EPA 1106.1	Water		Filter then 2-step: MEA 41.5ºC, 48 h then EIA (Esculin iron agar) 41.5ºC 30 min.	1 cfu per 100 ml if not heavily polluted.	Substrate TTC
EPA modified 1600	Water		Filter then 1-step: modified mEI 41.5ºC 24 h -blue colonies, no subculture.	1 cfu per 100 ml if not heavily polluted. 6.0% false positive; 6.5% false negative	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 1992	Food Has been used for compost		Dilute in peptone salt (NMKL 91), pour plate with tryptone soy agar at 45ºC-incubate 2hrs 37ºC. Pour EA at 41ºC over this 44ºC, 48h Subculture to tryptone soy agar 24 h then test catalase or grow in 6.5% NaCl or high pH for 3 days. Turbid culture = +ve		Substrate TTC
ISO 7899-1:1998 Detection and enumeration enterococci in	Water (fresh and brackish, sea water, waste water,		Dilns in saline or DW (depends on sample) tubes add 200• l to 100• l medium MUD/SF	Not suitable for < 15 per 100ml	Substrate 4-methylumbelliferyl-• -D-glucoside (MUD) in presence thallium acetate,

waste water Pt 1 (miniaturised, MPN)	treatment plants)		44ºC min 36 hrs max 72 h. Measure fluorescence under UV		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 (Slanetz & Bartley) 36ºC, 44 h. Subculture red colonies to BEA 44ºC 2 h, black cols	1 cfu per 100 ml if not heavily polluted.	Substrate TTC (and Aesculin hydrolysis on subculture)

Table 7 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 2mL (TOXIC) Mix A, B, C and D, adjust pH to 7.5, filter sterilise. Volume 1002 mL	ISO 7899-1

Table 8 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

The methods can be assessed as follows:

2.5.1 SCA Microbiology of Drinking Water (2002) Part 5

In the Standing Committee of Analysts approved membrane filtration 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. This is basically the method also described by the UK PHLS, now HPA.

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 47 mm 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.

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) for catalase and aesculin hydrolysis (enterococci are catalase negative and aesculin positive). 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.

2.5.2 US EPA Method 1600

The mEI agar method is a one-step membrane-filtration method that allows the detection of enterococci in 24 hours with incubation at 41°C. It is recommended for use in place of a previous enterococci method, the mE agar method (USEPA Method 1106.1), which is a two-step method that takes 48 hours to complete with 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, which 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. Confirmation 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. Gram positive cocci which grow at 35ºC in both bile aesculin agar and brain heart infusion broth (BHI) + 6.5% NaCl, and BHI Broth at 45ºC, and hydrolyze aesculin, are verified as enterococci.

As with all membrane filtration methods, water samples containing colloidal or suspended particulate materials can clog the membrane filter and prevent filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies. The specificity of the medium used in this method is 6.0% false positive and 6.5% false negative for various environmental water samples (Messer and Dufour, 1998). 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. The precision among laboratories for marine water and surface water was 2.2% and 18.9%, respectively.

2.5.3 ISO 7899-1 Part 1: Miniaturised method for surface and waste water.

This miniaturised (MPN) 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. Consequently, the formulation of any standard for enterococci in sludge should take this into account if the method is to be considered.

The method involves inoculation of 200 •l of the sample from a dilution series (using specified artificial sea water as diluent) in a row of microtitre plate wells containing 100 •l of medium. The plates are incubated at 44 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, nalidixic acid and 2,3,5-triphenyltetrazolium chloride (TTC) in the medium, to the methylumbelliferyl fluorophore. Wells observed with blue fluorescence under UV irradiation 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 the interlaboratory trials are shown in Table 9.

Table 9 Repeatability and reproducibility of ISO 7899-1 Part 1

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

The method appears appropriate for the detection of enterococci in naturally contaminated sewage samples. Of concern, however, 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 when they are being handled.

2.5.4 ISO 7899-1 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 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 materials. Azide compounds should be decomposed and rendered safe with excess sodium nitrite before disposal.

2.6 Emerging methods for enterococci

The conventional methods just described 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 (particularly 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 (see Section 3.2.1) 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.

2.7 Enterococci conclusions

Most of the validated methods for the detection of enterococci 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 choice of diluent (membrane filtration unless analysing greatly diluted samples or samples that have had heavy particulate material removed by coarse filtration or centrifugation; this may not be a viable option).

Much depends on what regulatory standard may be applied in the future. If it is similar to the clostridia requirement in the Draft Biowaste Directive (absent per g dry matter; Appendix A) then this sensitivity of detection for may require a presence/absence liquid enrichment test, as

specified by Mead (1992) for clostridia. This would include methods such as advocated by Stampi *et al.* (2001), inoculating dilutions of samples into azide dextrose broth and then subculturing; albeit this is a 2-step procedure taking 72 hours to accomplish. Alternatively, chromogenic broths such as Chromocult Enterococci broth and Readycult Enterococci broth (Merck) may be quicker but will need careful validation for sludge, soil and biowastes. Certainly, they produce only a few false positive results for enterococci in bathing waters compared to Slanetz and Bartley agar-based methods.

ISO 7899-1:1998 would be precluded because it is not suitable for less than 15 organisms per 100 ml sample (e.g. wastewater) and all of the other standards described involve membrane filtration which may or may not be capable of detecting 1 organism per g dry matter following dilution and homogenisation of the sample. These methods will therefore require modification and validation to assess their performance characteristics for detecting enterococci in sludge, soil and biowastes. A suitable compromise may be to use the Enterolert system (see Section 3.2.1). Enterolert is semi-automated MPN-based system 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.

On the other hand, if an enterococci standard followed that proposed for *E. coli* in sludge in the Draft Sludge Directive (less than $5 \cdot 10^2$ CFU/g; Appendix A), then all of the international standard methods may be suitable.

Clearly, further guidance is required from regulators as to the purpose of an enterococci standard and what the level of detection should be.

3. EVALUATION OF DRAFTING A HORIZONTAL STANDARD

3.1 Emerging methods for clostridia

3.1.1 Fluorogenic substrates

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). It may be feasible to include this in a simple, broth-based presence/absence assay or even a sensitive MPN-based method e.g. similar to the fluorogenic Colilert (see Part 3A of Project Horizontal) and Enterolert (see Section 3.2.1, this report) methods.

3.1.2 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. There have been no attempts to market an antibody-based assay for vegetative cells or spores of *C. perfringens* that could be developed for sludges and other wastes.

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.

3.2 Emerging methods for enterococci

3.2.1 Enterolert

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.

3.2.2 Rapid methods

The same can be said about rapid immunological or molecular methods for enterococci as described for clostridia. Moreover, 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. The development of lateral flow devices and gold labelled immunosorbent assay (GLISA) technologies should be encouraged.

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 clostridia and enterococci 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 Detection Methods

This desk study has evaluated the current existing methods available for the detection and enumeration of *Clostridium perfringens* and enterococci with a view to implementing horizontal standardisation. The main methods used 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 were described.

In particular, at least one report (Araujo *et al*., 2004) has suggested that the use of m-CP agar medium, which is used in the reference method in the European Union, is not suitable for recovering *C. perfringens* spores from groundwater. This questions its possible use as a method for detecting *C. perfringens* in sludge, soil, soil improvers, growing media, and biowaste. Rather the authors suggested that TSCF agar was the most specific medium for *C. perfringens* spore recovery in groundwater samples. However, a membrane filtration assay may not be appropriate for high solids content matrices (see next).

The 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 high dry matter content, complex matrices. There are strengths and weaknesses for both the membrane filtration and multiple tube MPN broth techniques, which have been summarised in Table 9 for their ability to analyse coliform bacteria (WHO, 1997).

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 of g dry 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 or microtitre plate 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 trialed and validated for various low turbidity samples and is now beginning to be used more for waste waters.

Table 10 Comparison of MPN and membrane filtration formats

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 precision)	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	

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 labeling, 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 labeled 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.

Overnight culture followed by rapid immunological or molecular detection would also overcome the risk of false positive reactions that have been described for all of the methods described in this report for clostridia and enterococci.

Consequently, we consider it feasible to formulate a horizontal standard to cover sludge, soil, soil improvers, growing media, and biowaste. However these are complex matrices compared to what the methods were originally designed for i.e. food and water. Consequently, there are several essential stages of method development that must ensure robustness and reproducibility in interlaboratory testing, utilising sample pre-treatment to overcome potential matrix problems of recovery and interference with the detection method. As such, there is an urgent need for their modification and evaluation as part of the next phase of the Project Horizontal.

We recommend that further work be undertaken with such an approach to simplify the assay procedure and produce a rapid, sensitive, robust, inexpensive methodology for the routine analysis of large numbers of sludges, soil, soil improvers, growing media and biowastes. This work should involve collaboration with existing diagnostics companies already familiar with the development of such assays for the clinical, food and environment markets.

5. DRAFT STANDARD (CEN TEMPLATE)

This report has identified that standard methods for water analysis exist for spores of *Clostridium* (EN/ISO 6461-1, Parts 1 and 2; SCA Microbiology of Water, Part 6) and intestinal enterococci (EN/ISO 7899-1, Parts 1 and 2; USEPA Method 1600; SCA Microbiology of Water, Part 5) which are applicable to contaminated water samples. It is clear from this desk study that there is a need for a complete assessment and suitable modification of these methods to make them applicable for sludge or bio-waste analysis. This requires pre-normative research and a proposal to include such work for *C. perfringens* and enterococci has been incorporated in the HORIZONTAL-HYG proposal recently submitted to the European Commission. This has been submitted by a consortium of partners who serve on CEN committees; if the application is successful, then these partners will undertake the necessary development work.

CEN CMC has officially endorsed project HORIZONTAL in a letter to DG ENV and to Member States (October 25th, 2002). The BT Task Force 151 has been installed by BT to manage the results of the HORIZONTAL research. The majority of the results will be presented to this task group, which will then have the full range of options on how best to use them. The decisions on how to develop standards from this research therefore rest entirely with the BT Task Force. It is the intention to produce standards for *C. perfringens* and enterococci applicable to sludges, soil, soil improvers, growing media and biowastes as appropriate to fulfil the required legislation.

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APPENDIX A 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"

APPENDIX B MEDIA CURRENTLY IN USE FOR THE ISOLATION OF CLOSTRIDIA

Table 11 Reinforced RCM medium

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 12 DRCM media

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 13 TSC medium

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 g l^{-1}) = SFP agar		

Table 14 Perfingens agar (OPSP)

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 15 Membrane *Clostridium perfringens* agar (m-CP)

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
	Make up to 1 l distilled water
Additives	

**As used by Ohio District Laboratory*

Table 16 Differential Clostridial agar (DCA)

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

APPENDIX C DETAILS OF APPROVED METHODS FOR THE DETECTION OF ENTEROCOCCI IN WATER

C.1 Standard Committee of Analysts, The Microbiology of Drinking Water (2002) Part 5: A method for the isolation and enumeration of enterococci by membrane filtration. Environment Agency, 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 may be required, depending on the degree of accuracy required by the test. If less than 10 red colonies, all should be subcultured, as follows:

C.1.1 Catalase test confirmation

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*).

C.1.2 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.

In addition, other differential tests may be applied, as follows:

C.1.3 Serology

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.

C.1.4 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.

C.1.5 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.

C.1.6 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.

C.1.7 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 (now HPA) is basically the same as above.

C.2 US EPA Method 1600 (2002).: Enterococci in water by membrane filtration using membrane-Enterococcus Indoyl-• -D-Glucoside Agar

The membrane-Enterococcus Indoyl-• -D-Glucoside Agar (mEI agar) method 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.

Verification Procedure

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.

C.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 ($0.22 – 1.5 \times 10^4$ / 100ml)	1.5	2.7
Sewage waters (4 samples; 9 labs)	Naturally contaminated ($0.19 – 51 \times 10^5$ / 100ml)	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.

C.4 ISO 7899-1 Water Quality- Detection and Enumeration of intestinal enterococci in surface and waste water. 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.