Horizontal Standards on Hygienic Microbiological parameters for Implementation of EU Directives on Sludge, Soil and Treated Biowastes.

Critical review on: feasibility of horizontal standard rapid method for detection of *Clostridium perfringens* and enterococci in Sludges, Soil, Soil Improvers, Growing Media and Biowastes

(HOR-HYG WP 2/2)

Authors: Louise Weaver, C. William Keevil and Sarah L. Warnes

Environmental Healthcare Unit, Department of Microbiology, School of Biological Sciences, University of Southampton, Boldrewood Campus, Bassett Crescent East, Southampton. SO16 7PX. UK.

Correspondence address: cwk@soton.ac.uk

Final version: 28th October 2005

Project acronym: HORIZONTAL-HYG
Project full tile: Horizontal Standards on Hygienic Microbiological parameters for Implementation of EU Directives on Sludge, Soil and Treated Biowastes.
Project/Contract nº. : SSPI-CT-2004-513660

Acknowledgement

This work has been carried out with financial support from the European Commission in the frame of the FP6 STREP HOR-HYG project.

CONTENTS

List of figures

List of tables

0 GENERAL INTRODUCTION

The European STREP "HORIZONTAL-HYG" project to develop "Horizontal Standards on Hygienic Microbiological parameters for implementation of EU Directives on sludge, soil and treated biowaste" started on 1st December 2004. This project is carried out under the umbrella of the main project HORIZONTAL "Development of horizontal standards for soil, sludge and biowaste".

The strategic objectives of this HORIZONTAL-HYG project focus on the development of reliable and harmonised European standards for sampling and hygienic microbiological parameters in the field of sludge, soil and treated biowastes and similar matrices. These methods are of fundamental importance to properly evaluate the environmental problem they may pose and to facilitate regulation of these parameters related to different uses and disposal governed by EU Directives. The Working document on revision of the Sewage Sludge Directive (86/278/EEC; draft April 2000) and the Working Document on Bio-waste (draft February 2001) called for standards on sampling, and analysis of hygienic and biological parameters, inorganic parameters and organic pollutants.

This project is concentrated only on the development of horizontal standards (if possible) for **microbiological parameters**, including **sampling and sample handling** taking into account the limited stability of microbiological parameters. Defining test organisms and test methods for the validation of safe treatment processes (biotechnological, chemical and physical treatment) forms part of the project.

Besides sampling and sample handling (WP 1) and process control and process validation (WP3), the central work package (WP 2) deals with methods by which microbiological parameters describing the microbiological quality of the final product or applicable for the re-isolation of test organisms applied in validation procedures shall be determined in a reliable way :

For *Salmonella* spp. and *Escherichia coli* (SubWP2/1) drafted CEN standards are available and therefore a co-normative work will be performed consisting in the validation of those methods (performance data). This work will consist in three main steps : (i) a training in a

central laboratory of 16 EU laboratories for methods to be validated, (ii) an intralaboratory suitability study of methods to be validated (fit for purpose on the nine different matrices that are to be targeted) and finally (iii) an interlaboratory round robin test with selected laboratories to validate the methods.

For enterococci and *Clostridium perfringens* (SubWP2/2), **viable helminth ova** (SubWP2/3) and **bacteriophages** (SubWP2/4), all relevant from the point of view of human and animal health as well as plant protection and environmental safety, only a pre-normative work will be performed (no validation study). This will consist in two main steps : (i) a critical review including an European workshop with experts first leading to a decision if and for which substrates standards shall be drafted and (ii) an intralaboratory suitability study of identified draft standards (fit for purpose on the nine different matrices that are to be targeted).

For plant pathogens (SubWP2/5), only a 12 months desk study will be performed.

This report corresponds to the Critical review report on methods for enterococci and *Clostridium perfringens* to be monitored in EU in sludges, soil and treated biowastes that should be produced in the frame of the SubWP2/2. This report includes the conclusions of the European Horizontal-Hyg Workshop on this topic held in Lille (France) in April 2005. This report identifies draft horizontal methods for the targeted parameter to be studied for fit for purpose on sludge, soil and treated biowastes in the frame of an intralaboratory suitability study (pre-normative work).

1 INTRODUCTION

In May 2004 a desk study was conducted in the frame of the Horizontal project (Work Package 3, Task 4) by Partner 13 (University of Southampton) titled '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'. The outcomes of this study were that although methods exist for the analysis of *Clostridium perfringens* and enterococci in water and food matrices, there is a paucity of methods for sludge samples. If any are present they have not been fully validated and as such could not be immediately incorporated into regulatory analysis without further validation.

Also as the sample matrices to be tested are likely to have high particulate matter and presence of multiple organism species the use of membrane filtration may be limited. Massa *et al.* (2001) evaluated the use of membrane filtration (MF), most probable number (MPN) and pour plate (PP). Their statistical analysis showed that the three methods were equally valid for enterococci analysis in polluted water samples. Overall, the MPN method was more sensitive compared with MF and PP but account needs to be taken of the MPN procedure in terms of time and materials. They concluded that the pour plate offered an economical alternative for enumeration of high densities of enterococci in polluted waters. A problem with the pour plate method may be in the case of bacterial colonies growing within the agar requiring confirmation. This may be over come if rapid confirmatory media could be used.

2 UPDATE OF CURRENT METHODOLOGIES AVAILABLE.

2.1 *Clostridium perfringens*

There has been little further development for analysis of Clostridium perfringens probably due to a lack of interest in the water industry as few countries have regulations for C. perfringens levels at present.

2.1.1 CHROMagar Clostridium perfringens (Chromagar, France)

Dr Alain Rambach at Chromagar has been developing a media specific to *C. perfringens* for identification in clinical samples. It may be possible to develop this media for use on sludge and biowaste samples. Correspondence is underway to obtain samples of the media but at present it is still in development.

2.2 Enterococci

2.2.1 CHROMagar Orientation (Chromagar, France)

Chromogenic media is able to detect amongst other species, Enterococci species. Agar produces coloured colonies on plates for identification of *E. coli* (pink/ beige colonies), Enterococcus sp. (blue/turquoise colonies), group-B Streptococcus (light blue colonies, very small), Enterobacter sp. (metallic blue, ± pink halo), Proteus sp. (beige, brown halo). The media has been developed for use in clinical analysis of urine predominantly so further assessment of its use on sludge samples is required. Problems may arise due to the number of colonies on the plate may cause blending of colonies and distinguishing variations in colour and size of colonies may prove difficult. Also it has been noted (Aspevall *et al.*, 2002) that colonies of Lactobacillus and Enterococci may appear similar and further tests may be required to distinguish them. This may not be too problematic however, as it would only require gram staining or wet mount analysis.

2.2.2 CHROMagar Enterococcus (Chromagar, France)

In addition to the CHROMagar Orientation, a new agar is being developed to identify Enterococcus species in samples. Again it may be possible to develop this media for use on sludge and biowaste samples.

2.2.3 Pathatrix and Colortrix capture system

Matrix Microscience Ltd have developed systems for the rapid detection of food-borne pathogens. The Pathatrix system can specifically purify and detect pathogens in complex food matrices within hours. Colortrix system is a rapid screening system based on immunomagnetic colorimetric assay (IMCA) and can be combined with the Pathatrix system for easy to read and visual results in under 15 minutes.

Pathatrix relies on antibody coated paramagnetic particles to selectively bind and purify the target organism from the sample matrix. The whole sample can be analysed by re-circulation of the sample through a capture phase where the antibody coated magnetic beads are immobilised. If heat is applied to the system target organisms can be cultured and captured simultaneously. Once concentration further analysis is necessary to identify and possible enumerate organisms by Colortrix, fluorescence microscopy, PCR, ELISA and/or DNA probes.

Colortrix provides a means of rapid presumptive positive screening tool. The test takes less than 15 minutes to perform and a positive result is indicated by a blue colour. If positive, further analysis is required to confirm the result by biochemical or molecular analysis.

Both the Pathatrix and Colortrix have been developed for analysis of *E. coli* and *Salmonella*. It may be possible to develop methods for identification of enterococci and *C. perfringens* but none are presently available. The limiting factor at present is the cost of development, €1,500 for development of the beads not including any costs for antibodies required. The system could hold promise in the future but due to time scales and the costs involved it may not feasible in this research.

2.3 Molecular techniques

Biotype AG has developed a molecular method for the identification of *Clostridium perfringens* in DNA samples. As the method is developed for analysis of DNA, extensive pre treatment of the samples would be required prior to molecular analysis. If the target DNA is only present in very low amounts the sensitivity of the method may be brought into question as other organisms may be present. The method could be developed for confirmation after conventional analysis as results can be obtained quickly: 4-6 hours. The method uses PCR technology so additional equipment would be required: thermocycler, electrophoresis system and UV lamp or transilluminator for identification of bands present in gels.

Biochemical fingerprinting for characterisation of enterococci in sewages using an automated microplate technique has been evaluated and provided a means of identifying the source of pollution from the specific enterococci populations present (Manero and Blanch, 1999; Manero *et al.*, 2002). It would not however be of use in this study as the analysis takes up to 60 hours after initial incubation on selective agar. It may be a useful method after initial studies have identified presence of enterococci in pollution incidences but is not suitable in this research.

Frahm and Obst (2003) evaluated the application of the fluorogenic probe technique (TaqMan PCR) for the detection of enterococci in water samples. They developed a genus specific assay based on 23S rRNA sequence. Samples were prepared by filtering 100 ml aliquots through 0.45 µm pore size filters and incubating the filters in 10 ml non-selective peptone broth at 37ºC for at least 18 hours. From this enrichment broth 100 µl were analysed using the TaqMan PCR. During amplification PCR products were detected by measuring the increase in fluorescence against a baseline level. The threshold cycle (C_T) was defined as the number of cycles taken before the level of fluorescence first exceeds the background fluorescence (baseline) (Frahm and Obst, 2003). From this a calibration graph was extrapolated allowing a maximum C_T level to be set (27 cycles). By including an enrichment step the method was able to identify viable enterococci present. This enrichment also allowed detection of low numbers of enterococci present in samples to the limits set by drinking water regulations (<1 cfu/ 100ml). The research was able to demonstrate comparability with conventional microbiological methods (96% agreement). They concluded that using this method could provide a rapid methodology able to produce confirmed results within 25 hours.

The method described by Frahm and Obst (2003) could provide a rapid method for the analysis of sludges and biowaste samples. The method of enrichment could be adapted for sludges by taking aliquots or dilutions of samples and spiking into the enrichment broth without filtration. The presence of compounds such as humic acids and fumic acids in wastewaters and sludges could affect the efficacy of the TaqMan PCR especially if samples are not filtered or pre-treated prior to PCR. Further work is necessary to establish the usefulness of this method for analysis of sludges and biowastes for identification of Enterococci. Also an assessment of the initial cost of equipment would have to be made as this may be prohibitive to routine analysis. If the method does prove useful however, probes and primers could be developed for a similar approach to be used for identification of *Clostridium perfringens*.

3 METHODS FOR ASSESSMENT

3.1 Enterococci

3.1.1 Slanetz and Bartley or m-Enterococcus agar, BS EN 7899-2 method.

These media are the standard media used in most regulatory laboratories. Improvements in the confirmation step has allowed for transfer of the whole membrane from presumptive plates onto bile aesculin agar for rapid confirmation in 1-3 hours (Figueras *et al.*, 2000). Confirmation of intestinal enterococci is colouration of the surrounding medium brown to black. In addition the colony should be catalase negative.

3.1.2 Chromocult agar (Merck)

Filter aliquots or dilutions of samples through membrane filters (e.g. Pall Gelman GN-6, 0.45 µm pore size). Transfer filters onto Chromocult agar, face upwards using sterile forceps. Incubate aerobically at 36 ± 1.0ºC for 24 (± 4) hours. If a negative result is obtained incubate for a further 24 (± 4) hours. Enterococci will appear as red colonies with a diameter of 0.5 – 2 mm. Non-enterococci appear as colourless, blue/ violet or turquoise colonies.

There is a lack of research available using this media for the detection of enterococci species. Miranda *et al*. (2005) evaluated its use on faecal samples from broiler chickens. The research showed the media to be highly specific (98%) and the colonies, although small were easily

distinguished from other non-target colonies on plates. The incubation time used in this research was longer than recommended by Merck and further research is necessary to assess the specificity and recovery of enterococci after 24 hour incubation as well as its use for sludge and biowaste samples.

3.1.3 CHROMagar (Chromagar) Enterococcus

Filter aliquots or dilutions of samples through membrane filters (Pall Gelman GN-6, 0.45 μm pore size). Transfer filters onto CHROMagar, face upwards using sterile forceps. Incubate aerobically at 35 ± 1.0ºC for 18 (± 6) hours. Enterococci appear as turquoise blue colonies. Lactobacilli may appear similar and if suspected present confirmation is required by Gram staining or wet mount identification.

To our knowledge the use of either CHROMagar media (Orientation and Enterococcus) have not been evaluated for use on sludges and biowaste samples. Previously, research has used these media for the analysis of organisms present in urinary tract samples in clinical microbiology. In this research (Merlino *et al.*, 1996; Skulnick *et al.*, 2004) Orientation media was found to be useful for the identification, amongst other organisms, enterococci species. One problem using this media is the high numbers of other organisms present in sludges and biowastes could prove distinguishing of enterococci difficult.

3.1.4 USEPA Approved Method 1600 (mEI agar).

Filter aliquots or dilutions of samples through membrane filters (Pall Gelman GN-6, 0.45 μm pore size). Transfer filters onto mEI agar, face upwards using sterile forceps. Incubate aerobically at 41 ± 0.5ºC for 24 hours. All colonies with a blue halo surrounding the colony, regardless of colour of the colony itself are regarded as enterococci. Confirmation may be required by subbing onto BAA plates (bile aesculin agar). Intestinal enterococci colour the surrounding medium tan to black.

This media has been adapted from the original USEPA method (Anon., 1995) using a two step procedure of differential media (mE) followed by confirmation on bile aesculin azide after 48 hours. Dufour (1980) first adapted this method into a one step 24 hour method by substituting indoxyl-β-D-glucoside with aesculin in mE media, naming it mEI media. This meant the second step confirmation was eliminated.

Rhodes and Kator (1997) evaluated this media with regard to specificity and influence of sub-lethal stress on Enterococci recovery. They reported a significant increase in the number of enterococci recovered on mEI media if the incubation time was extended from 24 hours to 48 hours. This indicated the presences of sub-lethally stressed organisms in the samples (lake water). As previously papers reported the original media obtained high false negative results (73% - 80% in sewage and shellfish samples) Rhodes and Kator (1997) evaluated the number of false negative results obtained in river samples. Their research found 77% non-target organisms were confirmed as enterococci even after the extended incubation time. They suggested an improvement in the recovery could be obtained by adjusting the concentration of media components such as TTC (triphenyl-tetrazolium chloride).

A comparison between the original mE media and the modified mEI media containing a reduced concentration of TTC (0.02g/litre compared with 0.15g/litre in original) was conducted by Messer and Dufour (1998). They concluded that both media produced comparable results and so the modified media could be substituted for mE media. The mEI media was found to be specific as 94% of target organisms confirmed as enterococci and 6.5% of non-target organisms confirmed as enterococci. This meant that a confirmation was not necessary and thus could reduce the analysis time considerably (24 hours compared to over 48 hours with the original media).

3.1.5 Oxolinic acid-aesculin-azide medium (OAA)

A modification of kanamycin-aesculin-azide agar, replacing the kanamycin with oxolic acid (5 ml/L) and increasing the concentration of sodium azide to 0.4 g/L (Audicana *et al.*, 1995 and Figueras *et al.*, 1998). Typical colonies appear brown to black surrounded by a black halo. Audicana *etal* . (1995) modified kanamycin aesculin azide agar to improve the selectivity and sensitivity in detecting enterococci species in drinking water and sea water samples. They concluded that oxolinic acid aesculin azide (OAA) agar was more sensitive and selective in detecting enterococci species when compared with m-Enterococcus agar (Slanetz and Bartley) and KF Enterococcus agar. Also, the need for confirmation tests was eliminated thus reducing analysis time to 48 hours.

Figueras *et al.* (1998) evaluated OAA agar for use in a routine monitoring programme of bathing waters for faecal streptococci. The paper reports on the use of OAA agar as an

alternative to m-Enterococcus agar (Slanetz and Bartley) for detection of faecal streptococci in seawater and freshwater samples. The researchers analysed samples using membrane filtration onto both media and incubated samples at 35ºC for 48 hours.

3.2 *Clostridium perfringens*

3.2.1 m-CP (Sigma)

Membrane filter samples onto m-CP agar (Fluka) and incubate anaerobically at 44 ± 1ºC for 21 ± 3 hours.

Opaque yellow colonies that turn pink/ red on exposure to ammonium hydroxide vapours are *C. perfringens*. Problems may arise due to false positive and negative samples in highly contaminated samples (Sartory, D., pers comm.). Araujo *et al.* (2004) evaluated the use of m-CP agar (as stipulated in European Directive 98/83, 1998) compared with Tryptose Sulphite Cycloserine agar (TSC), fluorocult supplemented TSC agar and Sulphite Polymyxin Sulphadiazine (SPS). Using m-CP agar, spore counts were significantly lower compared with other media tested ($p < 0.05$). The findings in this research and previous research (Araujo *et al.*, 2001) suggest that m-CP agar is only suitable for use when there are a high number of vegetative cells present. Sartory *et al*. (1998) also found m-CP to be less efficient at recovery of vegetative and spore counts in river water samples and recorded a high incidence of false positives using m-CP compared with TSC media. This is of significance as our research has found low numbers of vegetative cells in all sludge samples tested (raw, thickened, mesophilic anaerobic digested, post digested, returned liquor, and pelleted sludge) with the exception of caked sludge, indicating although this media is recommended in European Directive (1998) for water analysis it may not be suitable for sludge analysis.

3.2.2 mTSC (Oxoid)

C. perfringens metabolises 4-methylumbelliferyl phosphate (MUP) using the enzyme acid phosphatase to produce 4-methylumbelliferone, which fluoresces when placed under long-wavelength (365-nm) ultraviolet light.

Previous research by Araujo *et al.* (2004) reported a significantly higher recovery of *Clostridium perfringens* in groundwater samples using a fluorogenic TSC media compared

with m-CP media. They also found that the fluorescent TSC media reacted comparable, with no significant difference with TSC and SPS media. Of the media tested fluorogenic TSC offered the highest specificity for *Clostridium perfringens* in groundwater samples. When confirmations were conducted 88.5% of presumptive *C. perfringens* confirmed and 10.3% of non *C. perfringens* confirmed positive. Also, the research conducted did not use an overlay method meaning results can be obtained without the extra cost of media and labour time using the overlay method. The anaerobic conditions could be more important if overlay is not used.

3.3 Broth media

3.3.1 Enterococci

Chromocult (Merck) and HiCrome (Fluka) have been developed as presence absence methods for the detection of enterococci in samples.

Up to 1 ml samples are pipetted into single strength Chromocult broth and samples up to 10 ml into double strength broth. Incubate at 36 ± 1.0ºC or 44 ± 0.5ºC for 24 (± 4) hours. To confirm negative result incubate for a further 24 (± 4) hours.

The presence of a strong blue-green colour indicates presence of enterococci and D-streptococci. Observed growth turbidity may be weak. There is a lack of research using these media available to date.

The Enterolert™ (Idexx) is being marketed as an alternative to the US standard methodology. A new method, Enterolert-E™ (Idexx) is being marketed as an alternative to the European standard methods. Enterolert-E™ has only been available for two years and so there is a paucity of knowledge on the applicability of the method compared to the standard methods. The medium contains a defined substrate consisting of 4-methyl-umbelliferyl Beta-D-glucoside. When this substrate is hydrolyzed by the Beta-D-glucosidase produced by the enterococci, it produces 4-methyl umbelliferone as a blue fluorescent hydrolysis product, as well as the D-glucose product that can be used as a substrate for bacterial growth.

Studies have shown the Enterolert-E™ method to be comparable with conventional methods (membrane filtration and most probable numbers) in bathing waters, drinking waters and wastewaters (Eckner, 1998; Abbott, 1998; Idexx, 2001). The research did not however,

always confirm the results obtained and a recent study by Adcock and Saint (2001) has brought to light some confirmation problems with the Enterolert system. If incubation for 24 hours is used the numbers of enterococci obtained were a significant underestimation of the numbers present. Also, if incubation was extended to 36 hours detection was improved but a number of false positives were obtained. Research by Kinzelman *et al.* (2003) also suspected the occurrence of false positives as not all Enterolert positive wells could be confirmed on bile aesculin agar. This could mean that the identification of enterococci using Enterolert may require confirmations which would extend the analysis time. A pre-enrichment step may also improve the detection within 24 hours, thereby reducing the chance of identification of non target organisms. Further evaluation of this method would be of use for analysis of sludges and biowastes, especially introducing an enrichment step prior to incubation with the Enterolert system as the methodology is rapid and interpretation of results is straightforward.

3.3.2 *Clostridium perfringens*

There is a lack of new methods for the analysis of *Clostridium perfringens* using broths or MPN systems.

3.3.2.1 MUP-ONPG (Adcock and Saint, 2001)

Adcock and Saint (2001) examined the use of a combined method for the confirmation of *Clostridium perfringens* in samples using two properties of *C. perfringens*. The first is detection of the enzyme acid phosphatase, enabling the metabolism of 4-methylumbelliferyl phosphate (MUP) to produce 4-methylumbelliferone, which fluoresces when placed under long wavelength (365-nm) ultraviolet light. Additionally, *C. perfringens* ferments lactose to acid and gas, utilizing *b*-galactosidase in the process. *b*-Galactosidase activity has been used successfully for the confirmation of coliforms by detecting hydrolysis of *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG), which yields the chromogenic product *ortho*-nitrophenol. Colonies growing on TSC agar are sub-cultured into MUP-ONPG in microtitre plate wells. Confirmation of positive *Clostridium perfringens* is colour change of media (clear to yellow) or fluorescence in wells after 4 hours incubation at 37ºC. Acid phosphatase alone was found to be unreliable as a method of confirmation by incorporating MUP into TSC (Tryptose) agar as many false positives were observed. In addition the fluorescence was difficult to establish

in plates and where high numbers of colonies were present the fluorescence merged between colonies.

In the research conducted by Adcock and Saint (2001) the MUP-ONPG assays were able to confirm 164 out of 333 isolates from wastewater samples as *Clostridium perfringens*. This was higher than the standard method results of 153 out of 333. Using Vitek analysis the 12 samples not confirmed by standard methods were identified as *C. perfringens*. Using MUP-ONPG gave a high level of sensitivity (99.3%) but specificity was reduced when compared with standard methods (7.1%). On statistical analysis no significant difference was detected. Adcock and Saint (2001) concluded that using MUP-ONPG improved confirmation compared with standard methods and yielded results within 4 hours compared with 72 hours for conventional methods. Another advantage of the method is that the confirmations do not need to be incubated anaerobically and aliquots of the assay can be stored frozen until required for up to 3 months. It may be possible to use this media as an MPN system if a pre-enrichment culture is used. Further research would be necessary to establish detection limits and the feasibility of this with sludges and biowaste samples.

3.3.2.2 Modified TSC broth

As there was a lack of novel broth media available we tested a broth version of the TSC media developed by Oxoid. Here, we made up a base broth containing tryptone, cycloserine and MUP, omitting metasulphite.

Initial studies have produced promising results and the broth can be shown to detect *Clostridium perfringens* in sludge samples in low numbers (~ 66 cfu per gram wet weight). Further analysis is planned to improve the detection limit so the media could be of use in a presence absence method. At present the method is useful as a MPN method and presence absence only if the limit is set at above 66 cfu per g wet weight. The limits therefore need to be set in order to finalise the method for use as a standard for *Clostridium perfringens* analysis. Also, the selectivity needs to be studied further to ensure the percentage of false positives and negatives obtained are within acceptable limits (below 5%) for all types of sludges.

3.4 Enrichment methods

The presence of Enterococci and *Clostridium perfringens* in sludges and biowaste samples may be masked by the fact that the organisms are sub-lethally stressed *i.e.* still viable but not able to be cultured using conventional media. In this case an additional step may be required to resuscitate the stressed bacteria present before growing on or in selective media.

The SCA (2003) Part 2 recommends if target organisms may be sub-lethally stressed after treatments such as heating, drying or chemical, the recovery may be enhanced if liquid culture methods are used. If this is not possible the use of a pre-enrichment media should be incorporated into the method.

The incorporation of a pre-enrichment step to culture sub-lethally stressed microorganisms has been studied previously (Cole *et al.*, 1993; Bogosian *et al.*, 1996; Kang and Siragusa, 1999 and 2001; Mascher *et al.*, 2000; Liao and Shollenberger, 2004 and Stevenson *et al.*, 2004). There is an agreement that the use of selective media fails to detect these sub-lethally injured cells but brief incubation (1-4 hours) in non-selective enrichment media can significantly increase the number of target cells recovered (Kang and Siragusa 1999 and 2001). Cole *et al* (1993) reported that resuscitation in liquid enrichment media was faster (less than 2 hours) compared with plate resuscitation but it precluded the ability to enumerate cells present. Most methods available either transfer filters onto selective media or overlay/underlay agar with selective media following pre-incubation on non-selective enrichment media (Ewald and Eie, 1992; Kang and Siragusa 1999; Bogosian *et al.* 2000). Recent research by Kang and Siragusa (2001) suggest that a liquid enrichment medium incorporated into microtitre plate counting technique using 2 fold dilutions (2FD). Here samples were diluted and incubated in 100µl of buffered peptone water (BPW) for 3 hours at 37ºC before equal amounts of selective medium were added to the wells and re-incubated. In this research the presence of E. coli was tested in spiked cows' faeces using 100µl double strength violet red broth (mVRBB). It is not clear however, if the significant increase in the counts obtained was due to the recovery of sub-lethally stressed cells or the non-selective growth of non-target organisms as it is not clear if confirmations were undertaken. This method could be very useful if proved effective for incorporation into already existing methods such as the miniaturised MPN method for Enterococci analysis (ISO 7899-1) and could be adapted to a similar method for *Clostridium perfringens* analysis.

The presence of viable but not culturable (VBNC) cells of microorganisms has been debated heatedly with the regard to 'real' samples (Bogosian and Bourneuf, 2001). The majority of research has been conducted on laboratory simulations and not on samples taken from the environment with naturally occurring microorganisms. There is limited research available in the area but those available suggest that non-culturable bacteria do not persist and may be predated by indigenous microbes (Bogosian *et al.*, 1996; Mascher *et al.*, 2000).

The important question has still to be answered fully is that are these VBNC cells a threat to public health? If this were the case would we not expect to have seen high numbers of infections occurring when samples are absent of target organisms? Few papers have reported the ability of VBNC cells to infect and those available failed to infect mice with VBNC cells (Smith *et al.*, 1999; Forsman *et al.*, 2000). Further research is required in this field to establish the importance of the presence of VBNC cells in regard to the threat to public health. In regard to the future work on analysis of sludges and biowastes for enterococci and *Clostridium perfringens*, the relevance of obtaining counts for these organisms may be brought into question if they are not infective but at present it may be prudent to establish methods including pre-enrichment to obtain counts for the number of cells including those sub-lethally injured.

4 PRELIMINARY TRIALS ON SLUDGE MATRICES

From the results of preliminary trials undertaken at University of Southampton it can be seen that *Clostridium perfringens* and enterococci are present in sludge samples. Samples tested were:

1. Raw sludge
2. Thickened sludge
3. Mesophilic anaerobic digested sludge
4. Digested sludge (post 3 MAD digesters)
5. Returned liquor (not sludge but the liquor returned from the pressed sludge to wastewater treatment stream)
6. Pelleted sludge (fertiliser)
7. Limed sludge

Samples were analysed as 25 g aliquots unless stated otherwise. The homogenisation protocols, medium used, incubation time and temperature are stated in the following sections.

Both *Clostridium perfringens* and enterococci were detected in raw and treated sludge samples, with the exception of limed sludge which showed an absence of cells in all but one occasion when miniaturised MPN gave a positive result for enterococci (27/g wet weight). Numbers of enterococci were lower than *C. perfringens* in all samples tested indicating *C. perfringens* is more resistant to sludge treatment compared with enterococci. When *C. perfringens* were heated prior to analysis the numbers were not significantly reduced with the exception of digested sludge and caked sludge samples. This indicates spores of *C. perfringens* are predominant in sludge samples except where sludge is heated as in mesophilic anaerobic digestion. Here more vegetative cells were present probably due to the increase in temperature causing spores to produce vegetative cells.

4.1 Pre-treatment of samples

Improvement to recovery of *Clostridium perfringens* and enterococci in sludges was evaluated using pre-treatment methods hand shaking, stomaching or pulsifying compared with no pre-treatment.

Samples of 25 g were weighed into stomacher bags and made up to 25 g (*i.e* a 1:10 ratio) with quarter strength Ringers (QSR). Samples were homogenised by one of the above methods for two minutes. In the case of no pre-treatment the samples were briefly shaken before analysed. Dilutions series were made where necessary in QSR as 1:10 ratios. All samples were

analysed by membrane filtration of 1 ml replicates onto tryptose sulphite cycloserine (TSC) agar for *C. perfringens* and Slanetz and Bartley and Bartley agar (SBA) for enterococci analysis. Incubation temperatures and times were as follows: *C. perfringens* samples 44 (± 1)ºC for 24 (± 4) hours and enterococci 37 (±1)ºC for 4 (± 2) hours followed by 44 (±1)ºC for 44 (± 4) hours.

Significant improvements in recoveries were only achieved on mesophilically digested (MAD) sludge samples when using hand shaking as a pre-treatment method for *Clostridium perfringens* analysis ($p = 0.004$) (Figure 4.1). A significantly lower result was obtained for thickened sludge samples when using hand shaking as a pre-treatment method for *Clostridium perfringens* analysis ($p = 0.013$). With respect to enterococci analysis, all samples showed reduced recoveries (only significantly lower in thickened ($p = 0.013$) and return liquor ($p = <0.001$) samples), with the exception of caked and pelleted sludges where an increase in numbers obtained were found (not significantly higher) (Figure 4.2).

Using a Stomacher as a method of pre-treatment of samples overall increased the numbers of enterococci and *Clostridium perfringens* seen, with the exception of MAD sludge samples for *C. perfringens* and limed sludges for both. The only significant increase in counts was seen in caked sludge samples for *C. perfringens* analysis ($p = <0.001$).

The Pulsifier® was found to reduce the numbers of enterococci and *C. perfringens* obtained in all sludge samples apart from caked and pelleted sludges. However, the results were only significantly different for *C. perfringens* analysis on thickened sludge samples ($p = <0.001$).

When comparing the pre-treatment methods, it appeared the method of choice was dependent on the sample type being analysed. So, although overall stomaching increased recovery of enterococci and *C. perfringens*, the type of sample should be taken into consideration when analysing samples. For most samples the differences were not found to be significant when analysed, using ANOVA and Tukey's pairwise comparisons. The exceptions being, thickened and MAD sludge where Pulsifying samples gave significantly lower numbers ($p = <0.05$); and return liquor samples where hand shaking gave significantly higher numbers ($p = <0.05$). We propose only using pre-treatment of samples where the percentage dry matter is high (as in caked and pelleted sludges) and for other samples rigorous shaking of the sample

by hand should be sufficient, however an evaluation of the method to be used should be conducted, prior to incorporation into routine analysis.

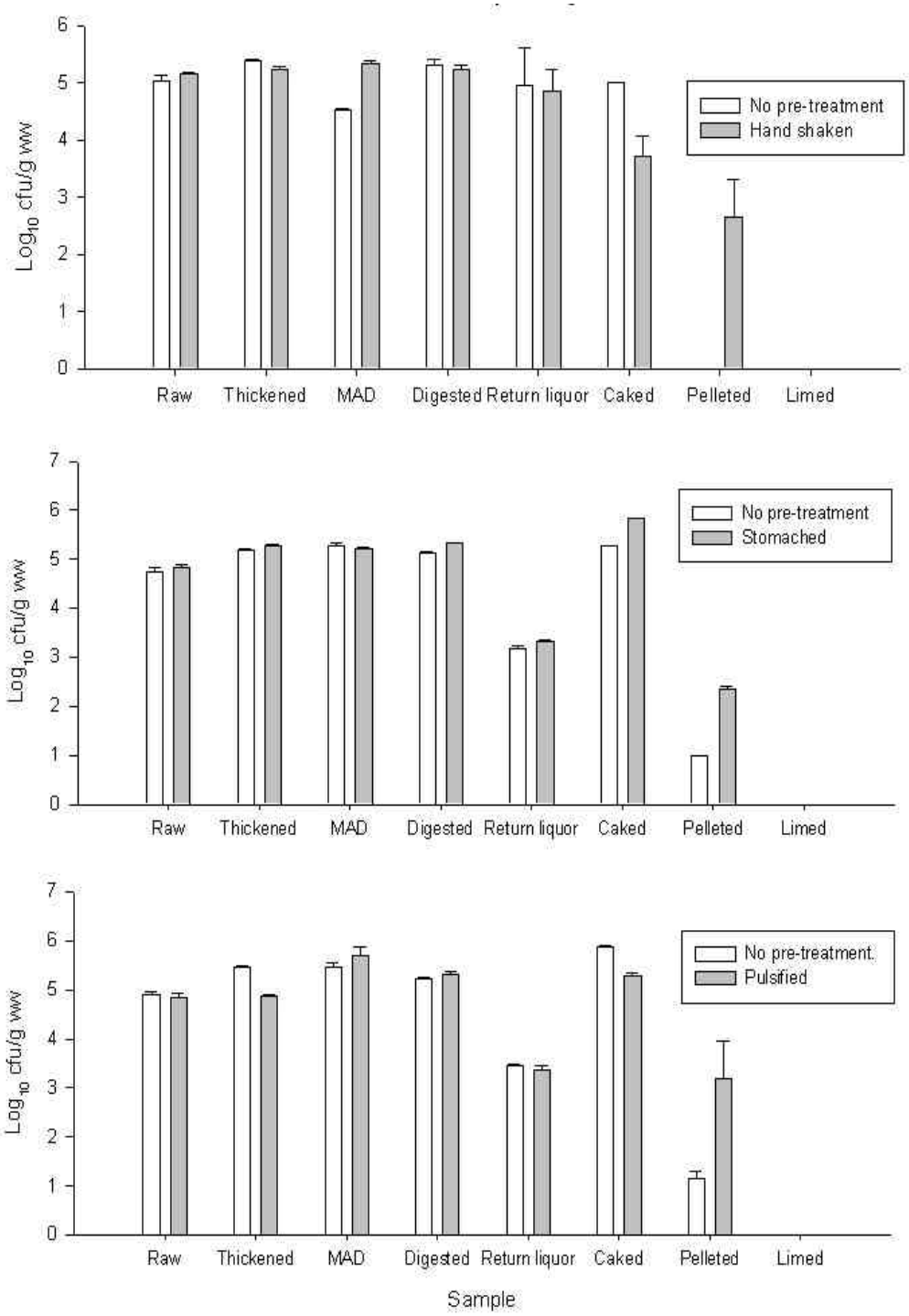

Figure 4.1: *Clostridium perfringens* counts (log10 cfu/g) for pre-treatments. Each compared against no pre-treatment. Lines indicate standard errors from the mean.

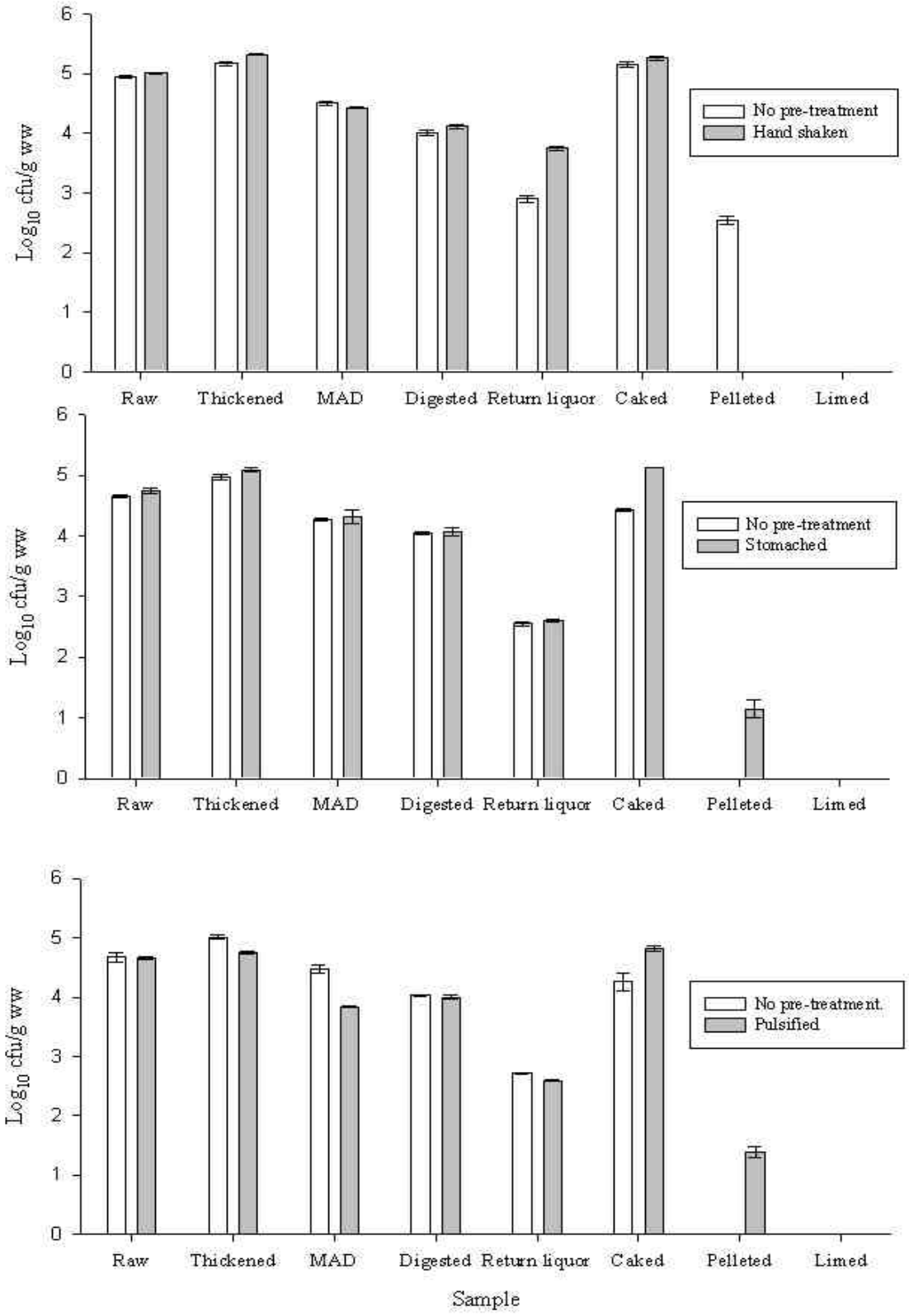

Figure 4.2: Enterococci counts (log10 cfu/g) for pre-treatments. Each compared against no pre-treatment. Lines indicate standard errors from the mean.

4.2 Diluents

Compared to deionised water the following diluents were tested for their improvement in numbers of enterococci and *Clostridium perfringens* obtained from raw and pelleted sludge samples:

Quarter strength Ringers (QSR)
Phosphate buffered saline (PBS)
(0.1%) Tween 20 in PBS (TPBS)
Maximum recovery diluent (MRD)
Synthetic sea water (SSW).

Samples of 25 g were weighed into stomacher bags and made up to 25 g (*i.e* a 1:10 ratio) with one of the above diluents. All samples were homogenised by 2 minutes stomaching. Dilutions series were made where necessary in the same diluent as 1:10 ratios. All samples were analysed by membrane filtration of 1 ml replicates onto tryptose sulphite cycloserine (TSC) agar for *C. perfringens* and Slanetz and Bartley and Bartley agar (SBA) for enterococci analysis. Incubation temperatures and times were as follows: *C. perfringens* samples 44 (± 1)ºC for 24 (± 4) hours and enterococci 37 (±1)ºC for 4 (± 2) hours followed by 44 (±1)ºC for 44 (± 4) hours.

All the diluents gave similar numbers of enterococci and *C. perfringens* when compared against deionised water. In raw sludges QSR gave the highest numbers of both enterococci and *C. perfringens* recovered (Figure 4.3 **and** Figure 4.4) and the numbers were found to be significantly higher for enterococci ($t = 11.92$, $p = 0.007$). SSW was found to lower the numbers recovered in raw sludges when detecting enterococci, although the difference compared with deionised water was not significant ($p = 0.120$) (**Figure 4.3**). For pelleted sludges, all diluents improved the numbers of enterococci obtained (QSR, TPBS and SSW significantly: $p = 0.003, 0.015, 0.033$ respectively for t-test). However, all diluents reduced the numbers of *C. perfringens*, although none were significantly different from deionised water (Figure 4.4).

Overall, the most applicable diluent for analysis of sludge samples for enterococci was QSR and for *C. perfringens* SSW, although there is no significant difference between these media so one could be used for both bacteria.

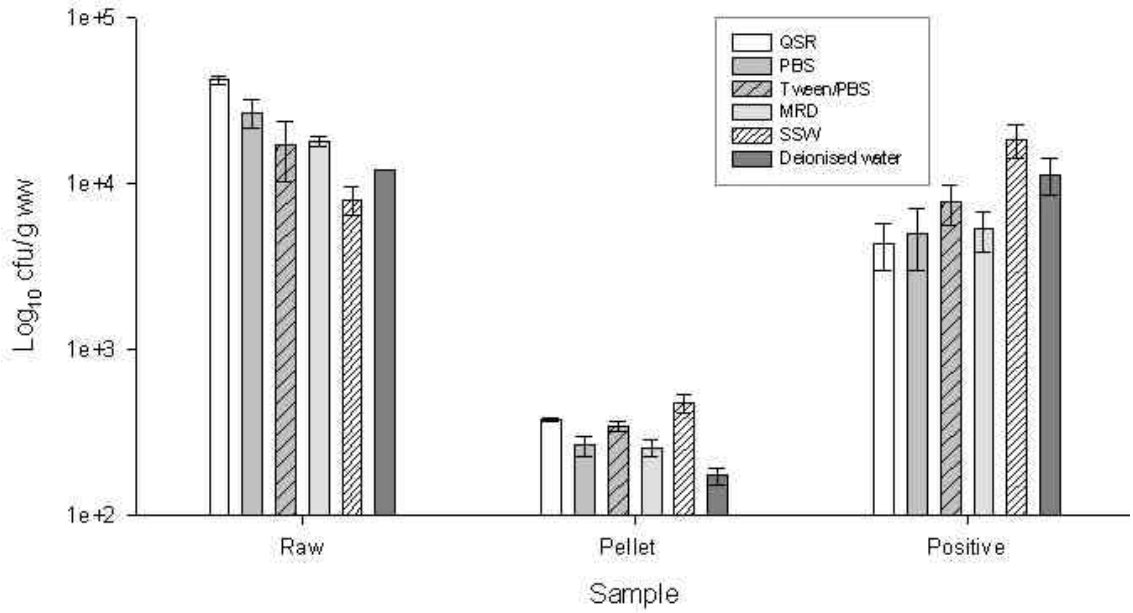

Figure 4.3: Log_{10} enterococci (cfu/g ww) for sludge samples using various diluents. Lines indicate standard error of the mean.

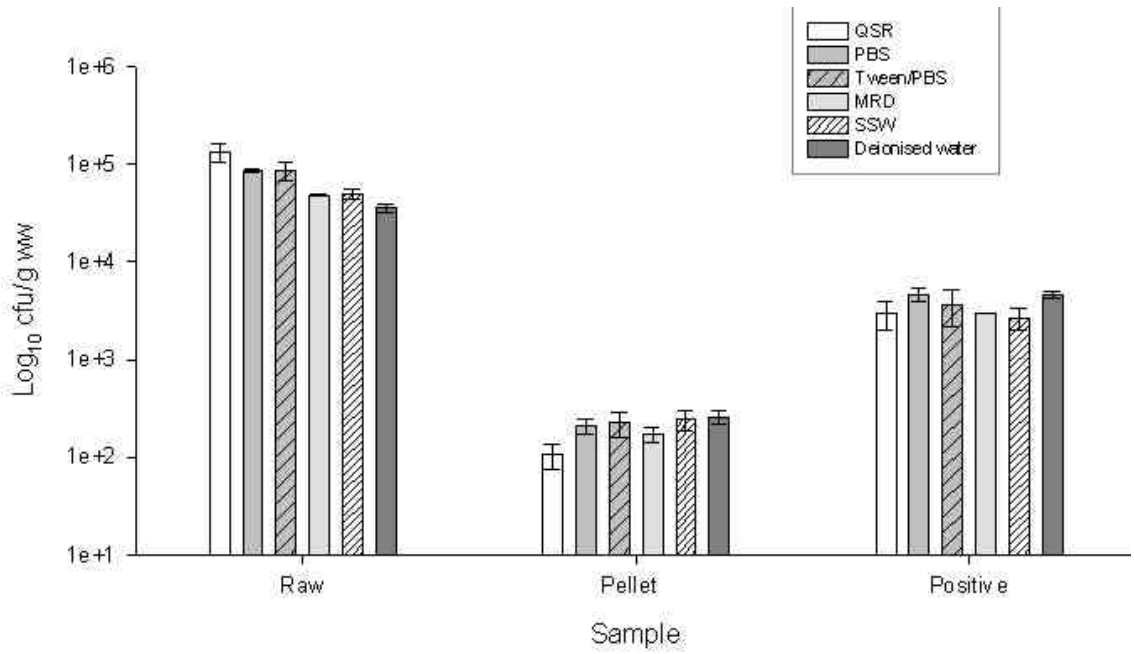

Figure 4.4: Log_{10} *C. perfringens* (cfu/g ww) for sludge samples using various diluents. Lines indicate standard error of the mean.

4.3 Membrane filtration

Samples of 25 g were weighed into stomacher bags and made up to 25 g (*i.e* a 1:10 ratio) with quarter strength Ringers (QSR). All samples were homogenised by 2 minutes stomaching. Dilutions series were made where necessary in QSR as 1:10 ratios. Samples were analysed by membrane filtration of 1 ml replicates or spread plating of 0.1 ml and 1 ml replicates onto tryptose sulphite cycloserine (TSC) agar for *C. perfringens* and Slanetz and Bartley and Bartley agar (SBA) for enterococci analysis. Incubation temperatures and times were as follows: *C. perfringens* samples 44 (± 1)ºC for 24 (± 4) hours and enterococci 37 (±1)ºC for 4 (± 2) hours followed by 44 (±1)ºC for 44 (± 4) hours.

On comparison of spread plates with membrane filtration no significant difference was seen ($p = > 0.05$). Membrane filtration offered a quicker analysis time as plates did not have to be allowed to dry prior to inversion and incubation. In addition plates were easier to read on membrane filters as less spreading of colonies occurred, compared with spread plates. Confirmation of enterococci presumptive colonies was also aided by analysis on membrane filters as it eradicated the need to pick off colonies as the whole filter can be transferred to confirmation media (bile aesculin agar). This also reduced the time for confirmation to between 1-4 hours compared with 24 hours. One major disadvantage of the membrane filtration system is the increased cost of analysis due to cost of filters and filtration system required. As the results obtained are comparable the use of membrane filters is not essential and laboratories should be able to use spread plating if the cost of membrane filtration is prohibitive.

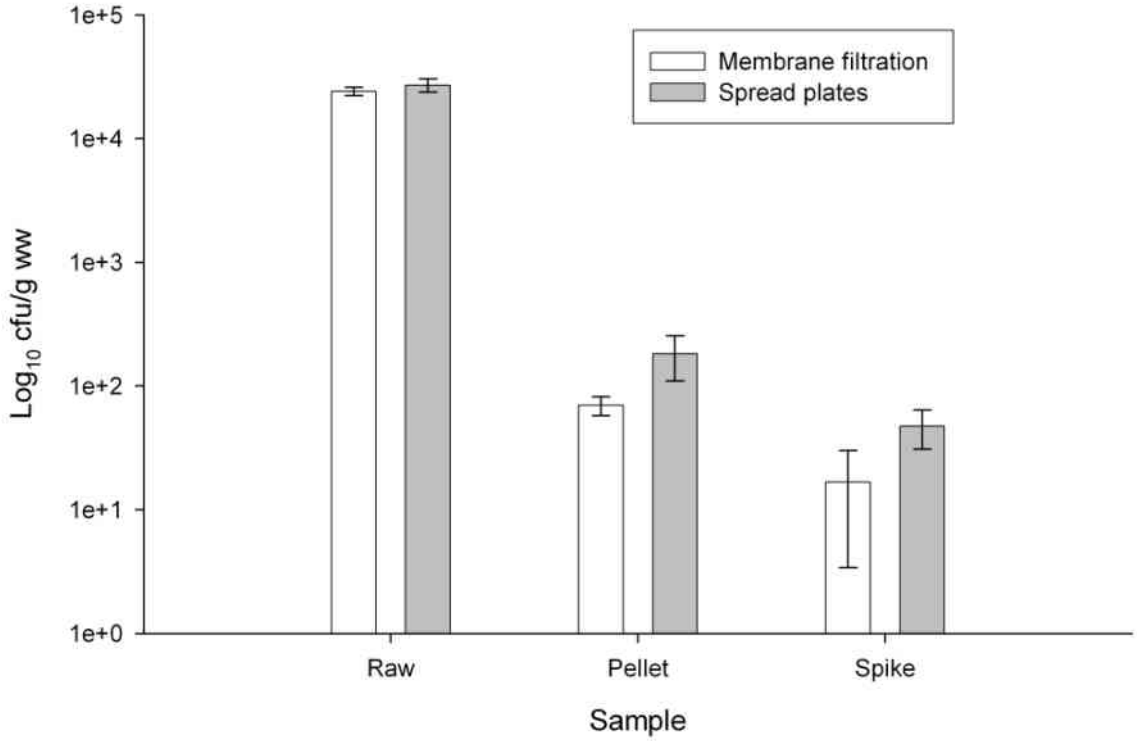

Figure 4.5: Membrane filtration results compared with spread plate counts on OAA media.

4.4 Most probable number technique (MPN)

Another method of analysis showing potential is the most probable number technique (MPN).

4.4.1 Enterococci

Numerous media and methods are available both as ready made media and in-house media. The EU recommends the use of a miniaturised MPN system for the analysis of enterococci in water samples, including bathing waters (ISO 7899-1). Although the method gives a recipe for the preparation of the MPN microtitre plates, in our experience it would be more prudent to use the ready made media available on the market (*e.g*. Biorad Enterococci microtitre plates, MU/SF and Biokar Enterococci microtitre plates). The reason for this is that some constituents (thallium I acetate, N, N-dimethylformamide and nalidixic acid) of the media are highly toxic and harmful to both the operator and the environment and special precautions are necessary *i*.e. working in a fume cupboard, with face masks and gloves. Also the media once prepared took three days to dehydrate in the microtitre wells.

On analysis of sludge samples using the method (ISO 7899-1), enterococci were identified in all the sludges tested, including limed sludge (27 MPN/g wet weight). This appeared to be an improvement on the colony counts obtained using m-Enterococcus agar, where enterococci were absent in all limed sludge samples tested. When statistically analysed there was no significant difference between counts obtained using MUD/SF media (miniaturised MPN's) or m-Enterococcus agar (p = > 0.05).

MUD/SF media was also compared with OAA spread plates and defined substrate MPN system for analysis of raw and pelleted sludges (Table 4.1). Statistical analysis showed significantly higher numbers of enterococci were obtained with OAA media compared to MUD/SF or MPN (p = 0.002, OAA vs MUD/SF and p = 0.015, OAA vs MPN). To investigate the significance of these results spiked samples were also tested and the results were found not to be significantly different (ANOVA, p = >0.05).

Table 4.1: miniaturised MPN results (MUD/SF) compared with plate counts on OAA agar.

Sample	Media	Mean cfu/g (OAA) or MPN/g (MUD/SF) ww	Min cfu/g (OAA) or MPN/g (MUD/SF) ww	Max cfu/g (OAA) or MPN/g (MUD/SF) ww	St. dev. cfu/g (OAA) or MPN/g (MUD/SF)	p-value (t-test) *(Mann Whitney Rank sum)
Raw	**MUD/SF**	26,325	24,048	28,090	2,069	
	OAA	84,000	64,000	96,000	17,436	0.005
Pelleted	**MUD/SF**	20,142	3,342	49,520	25,530	
	OAA	1,600	800	2,800	1,058	0.277
Positive control	**MUD/SF**	37,100	27,828	53,676	14,389	
	OAA	32,667	22,000	40,000	9,452	0.679
Negative control	**MUD/SF**	0	0	0		
	OAA	0	0	0		
Blank	**MUD/SF**	0	0	0		
	OAA	0	0	0		

Key: cfu colony forming unit
MPN most probable number
ww wet weight
n number
OAA oxolinic aesculin azide agar
Min minimum
Max maximum
St. dev. standard deviation

The advantage of the MPN system is that where low counts are present a positive result is obtained thus reducing false negatives obtained using membrane filtration. This is due to the

filters masking colonies present in lower dilutions and the filters becoming blocked. When MPN positive wells were confirmed all were positive with the exception of caked sludge, pelleted sludges and limed sludges (50%, 0% and 56% respectively). There was no improvement in the confirmed counts and the number of false positives obtained was higher, 33% compared with 4% using m-Enterococcus agar. One possible explanation for this is the presence of other organisms in the wells. It was noted that an improvement in the number of confirmations was obtained if the bile aesculin plates were incubated for 24 hours instead of 4 hours as used for membrane filter confirmation.

Thus in considering the suitability of the miniaturised MPN for analysis of sludge samples for enterococci the following needs to be considered further:

- Preparation time for the media is prolonged and involves handling toxic reagents
- Easier identification of low numbers of enterococci in sludges
- Numbers of false positive results appears to be increased
- Extended confirmation time is required, extending the overall analysis time to 60 hours compared to 50-52 hours using membrane filtration.

Overall the use of miniaturised MPN for analysis of enterococci in sludges could be useful, especially if the numbers present are low. If pre-prepared plates are used the labour time is reduced significantly and the risk to the staff is reduced. This may have implications however, if money costs are important to the laboratory as the pre-prepared plates are expensive (170 euros per 25 plates from Biorad, 300 euros per 25 plates from Biokar) if daily analysis is required. Further research is required to assess the use of pre-prepared plates with in-house prepared plates.

Other methods of analysis using MPN techniques are available, though most are pre-prepared media such as the Idexx Enterolert system. In our trials we found the results obtained using the Enterolert system was comparable with membrane filtration and offered an easy methodology due to reduced dilutions necessary, especially using the quantitray 200 system. Further research is necessary to establish the sensitivity of this method and compare it with the miniaturised MPN system and other MPN media available for all sludge matrices.

4.4.2 *Clostridium perfringens*

The appears to be a lack of MPN methods for the analysis of *Clostridium perfringens* and only one media was tested in preliminary trials, differential reinforced clostridia media (DRCM, Merck). The results obtained indicated MPN could provide a useful method of analysis of sludges, although if the criteria will be presence absence in 1 g the detection limit may not be adequate as our trials have found it to be around 50 cfu per g wet weight at present.

As there was a lack of media for MPN or presence/absence of *Clostridium perfringens* a modified TSC broth was made in our laboratory utilising a TSC broth with the addition of 4-methylumbelliferyl phosphate (MUP). Preliminary trials have been able to detect *Clostridium perfringens* in raw and pelleted sludge samples with numbers as low as 66 cfu per g wet weight. Although low, this not sensitive enough if the proposed method for *Clostridium perfringens* is set at absence of *Clostridium perfringens* in one gram of sample. The method gives a rapid (24 hours) method which may be improved to be sensitive enough for incorporation into a protocol for either presence/absence method or an MPN based method.

4.5 Selective media

4.5.1 Enterococci

25 g samples of sludges were made up to 250 g with QSR (1:10 dilution) and homogenised for 2 minutes in a Stomacher. Dilution series were performed as necessary in QSR and samples were either spread plated as 0.1 or 1 ml replicates or filtered through 0.45µm pore size nitro-cellulose filters as 1 ml replicates. Incubation times and temperatures were followed according to the manufacturer's instructions for the media. OAA media was tested at 37 (±1)ºC, 42 (±1)ºC and 44 (±1)ºC for 24 (±4) and 44 (±4) hours as the literature varied on these conditions.

When membrane filtration or spread plate media were tested with sludge samples, there was no significant difference between the following media types tested and all gave high recovery percentages using mesophilic anaerobic digested sludge:

Media	% recovery
• CHROMagar Enterococcus (CHROMagar)	100
• HiCrome Enterococcus agar Rapid (Fluka)	>100
• CHROMagar Orientation	>100
• mEI (modified Enterococcus identification media) (Difco)	>100
• Slanetz and Bartley agar (Oxoid)	>100
• m-Enterococcus (Slanetz and Bartley equivalent agar) (Merck)	91
• Oxolinic aesculin azide agar	100

As most of the media gave recoveries of over 100% this indicated false positives were being obtained. Although the CHROMagar Orientation offers a potentially specific media and if using the orientation media allows identification of other organisms present such as *E. coli* and Salmonella species these media were difficult to read as merged colonies did not show a definite colouration. As the use of Slanetz and Bartley media (or m-Enterococcus) is commonplace and the OAA agar gave the potential of 24 hour confirmed result we decided to concentrate on these media primarily.

The numbers of enterococci obtained in raw and pelleted sludges using Slanetz and Bartley agar and OAA agar were compared. The results obtained were similar for both media, with OAA agar giving slightly higher results. Using t-test statistical analysis, no significant difference was shown between the two media except if 44ºC incubation is used when OAA was significantly higher.

As there were few differences using these media the choice may be down to ease of use and cost of analysis. OAA media offers the advantage of a result obtained within 24 hours and confirmation is not necessary. The only draw back of OAA media is when using membrane filters for analysis the precipitation into the media can be masked or difficult to read. As there is no difference using membrane filtration or spread plates it may be easier to use spread plates for analysis using OAA media. Also, the length of incubation using OAA can be important because if left too long the whole plate turns brow/black preventing the enumeration of colonies producing brown/black pigmentation in media.

4.5.2 *Clostridium perfringens*

Comparing membrane filtration media for analysis of *Clostridium perfringens* in sludges the following media were tested:

- TSC (tryptose sulphite cycloserine) media (Merck)
- Modified TSC media (Oxoid) with and without egg
- mCP media (Fluka)
- DCA media (Merck)

All media produced typical colonies both using positive controls (*C. perfringens* Type A) and sludge samples (mesophilic anaerobic digested). There was no significant difference between the media types with the exception of DCA media which gave significantly lower numbers on all samples matrices (Table 4.2). The other media tested showed little variation in the colonies produced but using TSC media the colonies were not always black and when confirmed even some pale grey colonies confirmed as *C. perfringens*. The use of the modified TSC media (fluorogenic) offered easier enumeration of the colonies but problems arose when high numbers were present on plates as the fluorescence diffused across the plate. This was overcome by analysing lower dilutions but may be problematic if the expected numbers are not known in samples leading to analysis of many dilutions. Further research is being undertaken to assess the number of false positive and negative colonies obtained using this media. Comparing the addition of egg into modified TSC media only improved the counts obtained slightly and not significantly. The morphology of the colonies was not improved either so was omitted from further analysis.

Table 4.2: comparison of media for analysis of *C. perfringens* in MAD sludge samples.

Sample	Media	Trial 1 Mean cfu/g ww (n = 3)	Trial 2 Mean cfu/g ww (n = 3)	Mean cfu/g ww (n = 6)
Raw	TSC	206,667	180,000	193,334
	fTSC	310,000	290,000	300,000
	m-CP	283,333	230,000	256,667
	DCA	143,333	133,333	138,333
Negative	TSC	0	0	0
	fTSC	0	0	0
	m-CP	0	0	0
	DCA	0	0	0
Blank	TSC	0	0	0
	fTSC	0	0	0
	m-CP	0	0	0
	DCA	0	0	0

Key:

cfu	colony forming unit
ww	wet weight
TSC	tryptose sulphite cycloserine
fTSC	MUP-tryptose sulphite
m-CP	cycloserine
DCA	membrane clostridium
n	perfringens

It was noted that the colour of colonies obtained on TSC plates was dependent on anaerobic conditions. When conditions were not fully anaerobic not all colonies confirmed as *C. perfringens* produced typical black colonies on TSC media.

4.6 Confirmation

4.6.1 Enterococci

The use of bile aesculin agar and catalase tests was found to be comparable with API confirmations. This offers the advantage of confirmation in less than 24 hours and if membrane filters are transferred directly onto bile aesculin agar confirmation can be within 4 hours.

4.6.2 *Clostridium perfringens*

Adcock and Saint (2001) evaluated a new method for confirmation of *Clostridium perfringens* present in samples using a rapid tube method (results obtained in 4 hours) compared with traditional methods. The media contained MUP and ONPG to give a combined confirmation as previous research had indicated false negatives when using MUP or ONPG alone. Their results showed the confirmation method could be used for confirmation of presumptive colonies on TSC media. The advantages of this confirmation method were:

- the ease of use: transfer 100µl to pre-prepared microtitre plates containing 100µl MUP-ONPG,
- rapid results: within 4 hours
- elimination of anaerobic conditions
- long shelf life of the prepared media: frozen for up to 4 months

This method holds potential as a rapid easy to use confirmation procedure for *C. perfringens* and we are presently testing its use on sludge samples for both colony counts and MPN presumptive counts. Initial results indicate the method is robust for both plate counts and MPN analysis confirmations. Comparison with API system gave 100% agreement both with positive confirmations and negatives.

5 WORKSHOP LILLE 18TH-20TH APRIL 2004

5.1 Introduction

This workshop was part of a critical review work which consisted of pre-normative research that should lead to a first draft of a horizontal standard on *Clostridium perfringens*, intestinal Enterococci, viruses including bacteriophages. This work should contribute to future implementation of the Directive on the use of sewage sludge in agriculture (86/278/EEC) and on biowaste (draft February 2001).

More specifically, the consecutive sessions of the workshop focused on:

Bacteriophages: suitable target phages (somatic coliphages, F-specific RNA phages and/or phages infecting *Bacteroides fragilis*) and methods for testing and extracting phages were identified and proposed. Their relevance for different sludges, soils and treated biowastes matrices was also evaluated.

***Clostridium perfringens* and intestinal Enterococci**: the aim was to identify and propose the most fit for purpose method(s) applicable to sludges, soils and treated biowastes. Including identification of appropriate standard control organisms for validation trials.

Viable helminth ova: the aim was to identify the more suitable target organisms (Helminths or only *Ascaris* or *Taenidae* ova,) regarding risk assessment in EU countries, and to evaluate the performances of the available methods in terms of viability, organisms to be identified and most fit for purpose to sludges, soils and treated biowastes.

5.2 Overview and discussion sessions

The agenda of this two-day and a half workshop was prepared by the different organising partners (‘expert partners’) and coordinated by the Institut Pasteur de Lille (IPL): “Bacteriophages” session (half a day) by the University of Barcelona (ES), “Clostridium perfringens and intestinal Enterococci” session (one day) by the University of Southampton (UK) and the “Viable helminth ova” session (one day) by the Hungarian National Institute for Environmental Health (HU).

These three expert partners are in parallel in charge of the production of a critical review on the corresponding parameters (deliverable of the HOR-HYG project).

A questionnaire was also prepared and sent out to participants prior to the workshop to collect information about national regulations in the field of microbiological Quality Control of sludge, soil, biowastes and similar matrices, microbiological parameters monitored and found (level) and the associated methods used.

It was decided that the chairman of each session would present the synthesis of the collected information of the questionnaires and the preliminary conclusions of the critical review (at the stage of progress) during his session.

Participants were also invited to present their experience in the field of microbiological analysis of sludges, soils and treated biowastes. Eleven presentations were selected (by the organising partners and IPL) on the basis of the submitted abstracts.

The presentations of both experts and participants then provided information for a solid basis of discussions.

Many participants were identified by the members of the consortium of Horizontal-Hygiene project.

Besides, invitations were also sent through various ‘channels’: Eionet (public site of Horizontal projects: www.ecn.nl/horizontal), BT TF 151, secretariat of CEN TC 308 (characterisation of sludges), TC 223 (soil improvers and growing media), TC 345 (characterisation of soil), TC 292 (characterisation of waste) and various standards institutions.

A total of 47 participants from 14 EU countries (including 3 New EU countries: Cyprus, Czech Republic, Hungary) and 1 participant from South Africa attended the workshop, which took place at the Institut Pasteur de Lille.

5.3 Conclusions of *Clostridium perfringens* and intestinal enterococci session

Prior to the workshop a questionnaire was sent to laboratories across Europe. The following responses were received.

5.3.1 Responses

14 countries responded to the questionnaire:

Cyprus
Czech Republic
Denmark
Estonia
Finland
France (3)
Germany (2)
Hungary
Italy
Portugal
Slovakia
South Africa
Spain
UK (4)

Firstly, the questionnaires asked if legislation was already required for analysis of *Clostridium perfringens* and enterococci in sludges, soils and biowastes. Five countries already have a legislative requirement and the methods used are summarised in Table 5.1 below.

Table 5.1: Standard methods required as part of legislation including a brief summary of method

Country	**Standard method**		**Summary of methods**	
	Clostridium perfringens	**Enterococci**	***Clostridium perfringens***	**Enterococci**
Czech Republic	ES 1774/2001	ES 1774/2001	Spread plate of 1 ml onto DCA agar (Weenk). Overlaid with DCA agar. Incubation at 37°C for 24 hours under anaerobic conditions. Confirmation: Biomerieux API 20A kit.	Spread plate of 0.2 ml onto Slanetz and Bartley agar. Incubation at 37°C for 48 hours. Confirmation aesculin agar.
Denmark		DS 2401		DS 2256
Hungary		Equivalent to MSZ 318-27:1986		Dilution series into Litsky-Mallmann media. Incubation at 37°C for 24 hours (sludge) or 48 hours (soil). Positive samples inoculated onto Slanetz and Bartley media and incubated at 37°C for 24 hours.
Slovakia		None		Incubation on Slanetz and Bartley media.
UK	None	None	10 ml of dilutions filtered onto media.	

The proposed methods would include maximum limits to the number of organisms permissible in samples. The questionnaire indicated at present there are few countries with limits in place. Of the countries where maximum limits are in place the acceptable amounts varied widely, from absence in 150-250 g (Hungary) to less than 10^5 per gram dry weight (France). The values permissible were for sanitised sludge and compost samples. With the exception of Denmark and Hungary the values given were for dry weight of samples, indicating calculations should be made to allow dry weight values to be reported in the proposed methods.

Most countries analysed samples within 24 hours (ranging from 6 to 48 hours) and refrigerated samples in transport and storage prior to analysis.

The quantities of samples taken initially for analysis varied widely, from 5 g to 250 g or 0.01 ml to 10 ml (Table 5.2). The majority of responses used 10 g wet weight sample quantities. The current EC standard method for the analysis of *Salmonella* in Sludges states absence of *Salmonella* in 25 g samples. At present only Spain analyses this quantity. Most countries analysed wet weight quantities, probably due to the rapid turnaround required means dry weight is not possible at time of analysis.

Table 5.2: wet weight of samples used in different countries

Amounts taken as wet weight (except where specified differently)							
0.01 ml – 100 ml	**1 g**	**5 g**	**10 g**	**20 g**	**25 g**	**50 g**	**250 g**
Cyprus	Hungary (x5)	Hungary	Czech Republic	Germany	Spain	Portugal	Denmark
Portugal			Finland				
			France				
			Slovakia				
			South Africa (d.w.)				
			UK				

Homogenisation of samples was conducted on samples in most participants in the questionnaire. Stomaching of samples was the most popular method, with seven of the participants using this method (Czech Republic, France (group 5), Italy (group 1), UK (groups 1-4). The method of stomaching was similar with a 1:10 ratio of sample to diluent used. Variation occurred in the amount of sample taken and the time of stomaching, 1-2 minutes, although not all gave length of stomaching time.

The time given for analysis varied widely due the various methods used and whether confirmation was undertaken (Appendix 2). Where confirmation was conducted, various methods have been employed. Most used the API system (Biomerieux) or nitrate motility and lactose gelatine liquefaction for identification of *C. perfringens* and bile aesculin azide hydrolysis for enterococci.

Standard control cultures were used in eleven of the participants but there was a lack of a common source of these control organisms meaning comparisons difficult to make between laboratories.

Some interesting comments were made regarding the analysis of *Clostridium perfringens*. Some of the *C. perfringens* strains have been found to be negative for sulphite reduction; *C. botulinum* gives a positive reaction for sulphite reduction, producing typical black colonies; and the media for *C. perfringens* analysis needs to be freshly made as it degrades quickly leading to false negative reactions. These findings bring into question the specificity of the conventional media used for *C. perfringens* when sulphite reduction is the confirmatory reaction used. Research into new media using other confirmatory reactions for *C. perfringens* needs to be investigated further, such as metabolism of 4-methylumbelliferyl phosphate (MUP) to produce 4-methylumbelliferone, which fluoresces under ultraviolet light. or detecting hydrolysis of *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG), which yields the chromogenic product *ortho*-nitrophenol.

5.4 Conclusions of the session:

The following table of conclusions summarizes the issues and agreements reached during the session:

ISSUE	QUESTIONS / PROPOSALS	AGREEMENTS
Identification of suitable target bacteria	*C. perfringens*- vegetative and/or spores? Enterococci- all species or faecal?	*C. perfringens* vegetative cells and spores. (No pasteurisation). Enterococci.
Analysis	Presence/absence? Quantitative?	*C. perfringens* – P/A Enterococci – Quantitative
Sample preparation	10-20g (wet matter)? Estimation ?	Take 500-1000 g sample to lab and analyse 20 g of wet weight (if agar culture for *C. perfringens*); otherwise 1 g for P/A (Biowaste Directive).
Sample storage and transport (conservation)	Temperature: From 5 ± 3 ºC Storage: within 6h or longer?	Temperature: From 5 ± 3 ºC Storage: analyse within 24h; for chemical treatment (lime) neutralise immediately, following recommendations of working groups.
Treatment of the sample before extraction (?)	Separation or not of solids (applies to non-dewatered sludges)? Heat pretreat for vegetative cells (but damage spores)?	No separation treatment required

ISSUE	QUESTIONS / PROPOSALS	AGREEMENTS
Extraction of bacteria	Suspend solid/high DM samples in media/buffer? (which and ratios ?) Different degrees of homogenisation of the suspension (which homogenisation and time of contact ?)	Suspend high DM samples in media/buffer Homogenisation
Eluting solution	Medium or PBS ? Which pHs ?	Medium or PBS subject to laboratory testing pH 7
Ratio sample: eluent (w/v)	Ratio 1:10 ?	Ratio 1:10
Homogenisation	The same homogenisation as for bacteriophage? Time of homogenization-elution?	The same homogenisation as for bacteria: stomacher (to be compared to orbital or wrist shaking?). Comparable method. Maximum 2 minutes.
Detection methods	*C. perfringens* – broth and/or agar Enterococci –MPN or agar	*C. perfringens*: broth media but need to assess the sensitivity. Enterococci: MPN, miniaturised MPN, agar media
Analytical method and results for bacteria	*C. perfringens* – P/A Enterococci – MPN or Quantitative	See above

ISSUE	QUESTIONS / PROPOSALS	AGREEMENTS
Expression of results - Sludge	Referred to dry weight ? Referred to 1, 10, 25 or 100 g ?	*C. perfringens* absence in 1 g dry matter (Biowastes Directive) EN 1774-2 European Standard. Enterococci – cf *E. coli* <5 x 10^2 cfu/g wet wt (Sludge Directive).
Samples to test	Spiked "versus" non spiked? The most varied possible, and containing enough amount of naturally occurring bacteria.	Spiked for *C. perfringens* Non-spiked for enterococci? Sludge, dewatered sludge, compost, contaminated soils with sewage
Is it feasible to prepare reference materials for quality control?	Agree strains e.g. Sartory collection? Each lab prepares own inocula or central lab sends out lenticules/pastilles	Agree strains Prepare lenticules/pastilles for EU postage and spiking. Await results from *E. coli*, Salmonella Horizontal study.
Presence/absence?	Valid results – If absent or present what is the meaning of the results?	Might be more appropriate to use enumeration. If used only for treatment efficacy possible for P/A.

In conclusion:

- Omit pasteurisation for analysis of *C. perfringens*.
- Sampling of 500-1000g/l.
- Analysis of 20g of sample was suggested in the workshop but since it has been realised the methods already in place use 25g, therefore we now recommend using 25g for consistency,
- Samples to be stored at 5±3ºC prior to analysis and in transport.
- Samples to be analysed as quickly as possible but within 24 hours. Limed sludges should be neutralised as soon as the sample is taken. Awaiting recommendations of the sampling working group involved in this project.
- Various diluents to be assessed for suitability – MRD (maximum recovery diluent), QSR (quarter strength ringers), PBS (phosphate buffered saline), SSW (synthetic sea water).
- Various homogenisation methods to be tested – stomaching, Pulsifying, mechanical shaking, manual shaking.
- Media to test should include MPN techniques including miniaturised MPN and agar plate counts either by membrane filtration or spread plates.
- Results should be expressed as both wet weight and dry weight (post analysis calculation).
- Spiked samples to be analysed for *C. perfringens* and unspiked for enterococci. This may be changed as the numbers of enterococci are sometimes very low, especially in treated sludge samples.
- Sample matrices to be tested should be the same as for the *E. coli* and Salmonella trials but include raw sludges, dewatered sludges, composted sludges and soils amended with sludge.
- There is still a need to agree on the strains to use for analysis.
- At present it has been suggested that a presence/absence test is sufficient for *C. perfringens* but this may be altered to include a quantitative analysis if treatment efficiencies are to be assessed. It was recommended to enumerate enterococci.

6 FINAL CONCLUSIONS OF CRITICAL REVIEW

The previous desk review identified potential methods available for the analysis of enterococci and *Clostridium perfringens* in sludge samples. This update to the desk review has identified the following methods we recommend to be evaluated further for possible inclusion in an intra-laboratory trial:

6.1 Pre-treatment

- Stomaching samples for caked, pelleted or limed sludges.
- The use of either quarter strength Ringers or synthetic sea water as diluent.

  Note: further trials are necessary on all types of sludges to be used for the laboratory trials.
- Pre-enrichment in brain heart infusion broth, Tryptone Soya Agar (TSA) or buffered peptone water (BPW) and the underlay method (Kang and Siragusa 1999).

6.2 Media

Clostridium perfringens	Enterococci
TSC agar)	m-Enterococcus (Slanetz and Bartley
fTSC (fluorescent) agar and broth	OAA agar
Miniaturised MPN using MUP-ONPG or TSC based media	MU/SF MPN (in-house and Biorad) Differential MPN media

Note: further trials are necessary to assess TSC and fTSC agar for C. perfringens analysis and m-Enterococcus (Slanetz and Bartley agar) and OAA agar for all sludges when using membrane filtration method.

6.3 Methods

Both the use of MPN and membrane filtration should be further evaluated for sludges and biowaste samples but initial studies indicate there is no significant difference between these methods so it may depend on the cost analysis for a laboratory and ease of use. In our preliminary studies there appears to be an advantage in using MPN methods when low numbers of target bacteria are present in sludges, as when using membrane filtration blocking of the membrane occurs and colonies are masked by high particulate matter present.

6.4 Discussion

The use of MPN and membrane filtration should be further evaluated for analysis of enterococci and *Clostridium perfringens* in sludges and biowaste samples but initial studies indicate there is no significant difference between these methods so it may depend on the cost analysis for a laboratory and ease of use. In our preliminary studies there appears to be an advantage in using MPN methods when low numbers of target bacteria are present in sludges, as when using membrane filtration blocking of the membrane occurs and colonies are masked by high particulate matter present.

At present ongoing trials are taking place in our laboratory to establish the best methods for pre-treatment, enrichment and the diluent suitable for the intra-laboratory trial. It is hoped that a simple method of pre-treatment and pre-enrichment can be established for analysis of both enterococci and *Clostridium perfringens* thus allowing preparation of one sample for analysis of both microorganisms.

It must be said that many novel media tested in our preliminary trial have shown no significant difference with established media such as Slanetz and Bartley and TSC media but have not been included in the conclusions for further study due to extended analysis or incubation times necessary or the ease of use to the laboratory personnel in regards to preparation time and ease of identification of target organisms.

In conclusion we propose methods for analysis of enterococci and *Clostridium perfringens* should include the use of membrane filtration for low percentage dry solids samples, where high numbers of target organisms are present or treatment efficiency is being evaluated; MPN

methods for low concentrations of target organisms or high percentage dry solids and a presence/absence method for analysis if sufficient. The use of specific media for membrane filtration methods needs further evaluation but will include TSC and fluorescent TSC for *Clostridium perfringens*; Slanetz and Bartley (or m-Enterococci) and OAA media for enterococci analysis. A method of presence absence evaluation using fluorescent TSC broth for *C. perfringens* analysis will be tested The use of MPN media will be further assessed but will include the use of MUD/SF microtitre plates and defined substrate MPN for enterococci analysis.

The methods identified for each of both parameters (Appendix 1) will be further trialled in USOU laboratory and amended as appropriate. Then the proposed methods will be tested for their fit for purpose in the frame of the suitability study (ruggedness trial) scheduled for June to December 2006 in the frame of the HOR-HYG WP2/2.

7 APPENDIX 1

Draft proposed methods for suitability (ruggedness) study June – December 2006

7.1 *Isolation and enumeration of enterococci using membrane filtration onto selective agar*

7.1.1 Introduction

Sludges, soils, soil improvers, growing media and biowastes can contain microorganisms pathogenic to Man *e.g*. *Salmonella* spp. Most occur in the intestinal tract of humans and animals and can be transmitted through faecal contamination. Outbreaks of infection could be caused by use of the pathogen contaminated materials in agriculture due to the production of contaminated food and animal feed, or transmission to wild animals. As a consequence of this there is a need to monitor levels in these materials and application levels to land.

WARNING – "Waste and sludge samples may contain hazardous and inflammable substances. They may contain pathogens and be liable to biological action. Consequently it is recommended that these samples should be handled with special care. The gases which may be produced by microbiological activity are potentially inflammable and will pressurise sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided where possible. National regulations should be followed with respect to microbiological hazards associated with this method".

7.1.2 Scope

This draft standard method specifies a membrane filtration procedure for the quantitative analysis, by culture of individual colonies on selective agar, of enterococci species in sewage sludge, compost and biowaste samples. The user should, prior to analysis, validate the method for the particular type of sample they wish to analyse: sludges, soils, soil improvers, growing media (e.g. compost) and biowastes.

This method is of particular use if determination of treatment efficiency is required as outlined in the revision of Directive 86/278/EEC (3rd draft, CEN/TC 308-doc 525).

The method has a limit of detection of approximately 10 cfu/g wet weight, dependent of the solids content which at high concentrations (>20% (w/v)) can block the filtration of the

sample volume through the membrane if not diluted prior to filtration. Therefore if the sludge sample has or is expected to have high solids content this method may not be suitable.

7.1.3 Normative references

The following referenced documents were referred to extensively and offer indispensable advice for the application of this method.

EN 12880:2000. Characterisation of sludges – determination of dry residue and water content

EN ISO 5667-13:1997. Water quality – Sampling – Part 13: Guidance on sampling of sludges from sewage and water treatment works.

ISO 8199:2001. Water quality – General guide to the enumeration of microorganisms by culture.

7.1.4 Terms and definitions

For the purposes of this document the following terms and definitions apply.

Enterococci

Enterococci are gram-positive, catalase-negative, facultative anaerobic cocci that grow in pairs (diplococci) or in short chains, and possess Lancefield's Group D antigen. The organisms can be differentiated from other catalase-negative gram-positive cocci by the ability to grow in the presence of bile salts and sodium azide, 6.5% sodium chloride, at 44°C, reduce 2,3,5-triphenyltetrazolium chloride (TTC) to formazan and produce pyroglutamateaminopeptidase (*i.e.* PYR reaction).

Method definition

Reduction of TTC to formazan producing red/maroon to pink colonies when grown on Slanetz and Bartley (m-Enterococcus) agar.

Hydrolysis of aesculin to produce dextrose and osculating in the presence of oxolinic acid when grown on oxolinic aesculin azide agar. The aesculetin combines with ferric citrate in the media producing a black/brown precipitate in the media.

cfu, colony forming unit

Growth of an individual bacterial cell into visible colonies on Agar media, including membrane filters overlaying media.

Dry matter

The dry mass portion of the material tested after the specified drying process, expressed as percent or grams per kilogram (EN 12880:2000, 3.1).

7.1.5 Apparatus

With the exception of equipment supplied sterilised all should be sterilised before use in accordance with ISO 8199.

In addition to usual microbiological laboratory equipment:

Apparatus for sterilisation either dry heat (oven) or steam (autoclave).

Thermostatic incubator(s) either one cyclic regulated at 37 (±1)ºC and 44 (±1)ºC or two one regulated at 37 (±1)ºC and one at 44 (±1)ºC.

Homogeniser (e.g. Stomacher, Seward Laboratories or equivalent).

Sterile homogeniser bags 250ml volume with or without integrated mesh to remove large particles (e.g. Seward Laboratories 6041, 6041STR or equivalent).

pH meter with accuracy of ± 0.1.

Membrane filtration manifolds (e.g. Sartorius 13430-0475, Pall Gelman or equivalent).

Membrane filter units 150ml capacity either disposable (e.g. Millipore or equivalent) or sterilisible (e.g. Pall Gelman or equivalent).

Membrane filters 47 mm diameter, cellulose nitrate based, gridded 0.45µm nominal pore size.

Sterile forceps

Bunsen burner or working within a Class II safety cabinet

Boiling water bath (if sterilisible filter units are used such as Pall Gelman).

Pipettors capable of dispensing 100 µl and 1 ml

Graduated pipettes capable of dispensing 2-10 ml

Sterile tips.

7.1.6 Sampling hazards

Take samples of at least 100g wet weight and transport to laboratory as quickly as possible, chilled at 5±3ºC.

General

As samples are liable to ferment and contain pathogenic microorganisms, it is of paramount importance to adhere to national and international regulations relating to bio hazardous samples when handling and transporting samples.

It is essential to keep samples away from food or drink, and to protect any cuts.

See also Warning note in introduction of this method.

Storage

Do not store these samples on an open bench in the laboratory. If samples are not to be analysed immediately, store them at 5±3ºC in well labelled containers, preferable plastic. Samples can be stored for a maximum period of 36 hours.

Handling

Good laboratory practice and cleanliness is essential. When handling sludge samples it is necessary to wear gloves, face and eye protection, and sufficient body protection to protect against spillages or bottles bursting. The gas evolved when opening sludge samples is flammable and so should be carried out away from naked flames and all equipment should be flame proof.

See also the Warning note in the introduction.

7.1.7 Reagents, diluents and culture media

To ensure reproducible results, prepare culture media and diluents using either constituents of uniform quality and chemicals of recognised analytical grade, or a dehydrated diluent or complete medium prepared following the manufacturer's instructions. Prepare the media and reagents with demineralised or distilled water free from substances capable of inhibiting growth under test conditions. If the media are not used immediately, they should be stored in the dark at 5±3ºC for up to one month in conditions avoiding any alteration of their composition.

NOTE the use of chemicals of other grades is permissible providing that they are shown to be of equivalent performance in the test.

Quarter Strength Ringers solution

Ringer's solution composition per litre:

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride $6H_2O$	0.12 g
Sodium bicarbonate	0.05 g

Ringer's is commercially available in tablet form where one tablet is added to 500 ml water for quarter strength ringers. Solution is steam sterilised (autoclaved) at 121±3ºC for 15±1 minutes. pH 7.0±0.2.

Slanetz and Bartley agar (m-Enterococcus agar)

Per litre:

Tryptose	20.0 g
Yeast extract	5.0 g
Glucose	2.0 g
Di-potassium hydrogen phosphate	4.0 g
Sodium azide	0.4 g
Tetrazolium chloride	0.1 g
Agar	10.0 g

Add reagents to one litre of water and heat to just boiling DO NOT AUTOCLAVE. Once cooled to 50ºC pour into Petri dishes (do not store media to re-heat). pH 7.2±0.2.

NOTE media is commercially available in powder form or as pre-prepared plates (e.g. Oxoid, CM0377, PO0271).

Oxolinic aesculin azide agar (OAA)

Per litre:

Kanamycin aesculin azide agar base (Oxoid)	42.6 g
Sodium azide	2.5 ml (stock 1 g per 10 ml)

1 g + 50 ml 0.1N NaOH to dissolve then 50 ml deionised water. Filter sterilised.

Add kanamycin aesculin azide base to one litre of water and heat to boiling. Add sodium azide and sterilise at 121°C for 15 minutes. Once cooled to 50ºC add 0.5 ml oxolinic acid solution. Pour into 90 mm diameter Petri dishes (do not store media to re-heat). pH 7.2±0.2.

NOTE: oxolinic acid solution can be prepared in advance and stored at < 20°C in aliquots for up to 3 months.

Bile Aesculin agar

Per litre:

Peptone	8.0 g
Bile salts	20.0 g
Ferric citrate	0.5 g
Aesculin	1.0 g
Agar	15.0 g

Add reagents to one litre of water and heat to boiling and steam sterilise (autoclave) at 121±3ºC for 15±1 minutes. Media can be stored as described (7.1.7) above and then re-heated to dissolve. pH 7.1±0.2.

NOTE media is commercially available in powder form or as pre-prepared plates (e.g. Oxoid, CM0888, PO0169).

3% Hydrogen peroxide solution (catalase test)

Per litre:

Hydrogen peroxide	3 ml
Deionised water	100 ml

Prepare aseptically and store at room temperature for one week.

7.1.8 Procedure

Sample preparation

Determination of dry matter content

The dry matter content is measured using the method described in EN 12880:2000.

Suspension preparation

Mix sample and weigh out a representative sub-sample of 25 g (wet weight) into a homogeniser bag with an integrated mesh to remove large particles, if required.

Add a volume of quarter strength ringers (QSR) so the final volume is 250 ml (dilution A).

For lime treated sludges adjust the pH to 7.0± 0.5 with 1 mol/l hydrochloric acid.

NOTE 1: If the pH drops below 4.5 whilst neutralising the sample, a new sample should be prepared.

NOTE 2: If other chemical treatment is used on sludge samples to be tested a suitable neutralisation procedure should be adopted.

Place sample in a homogeniser (7.1.5) and homogenise for 2 minutes.

Preparation of dilutions

Prepare serial dilutions from dilution A in QSR as appropriate for the expected concentration of enterococci in sample. For example raw sludges may have up to 10^6 enterococci per g but treated sludges may only require dilutions to 10^{-1}-10^{-2}.

Mix the primary suspension (1/10 dilution) and using a sterile pipette aseptically transfer 1 ml into a sterile tube containing 9 ml QSR. Mix this dilution and using a new sterile pipetted transfer 1 ml of this dilution to a tube containing 9 ml QSR.

Continue this procedure until the required number of dilutions has been prepared.

7.1.9 Analysis

Filter 1 ml aliquots of each diluted sample through a 0.45 µm pore size cellulose nitrate filter. To ensure even distribution of the sample over the filter surface, prior to addition of the sample add approximately 10-20 ml of diluent (QSR or equivalent) to the membrane housed in the filter housing.

NOTE keep the vacuum in the off position until the sample has been added.

As soon as the sample has filtered through, using a low vacuum pressure (not exceeding 65 kPa 500 mm of mercury), switch off the vacuum so as little air as possible passes through the filter.

Resuscitation and enumeration of colonies on selective agar

Remove the filters from the filter housing using sterile tweezers, holding the filter at the edge of the filter. Place the filters face upwards onto 50 mm diameter plates containing pre-dried selective media (0 or 0). Use a 'rolling' action to prevent the formation of air bubbles under the media which would prevent the media coming into contact with the membrane. Invert the plates and incubate Slanetz and Bartley (m-Enterococcus) agar plates initially at 37 (± 1) °C for 4 (± 0.5) hours, then increase the temperature to 44 (±1) °C for 44 (± 4) hours, oxolinic aesculin azide agar should be incubated at 44 (±1) ºC for 16 (± 4) hours.

If using Slanetz and Bartley agar, enumerate maroon/pink colonies (including pale pink colonies) over 0.5 mm diameter that are smooth and convex in shape by eye.

Note: some species of *Bacillus*, *Aerococcus* and *Staphylococcus* can also grow on Slanetz and Bartley (m-Enterococcus). *Bacillus* spp. produce pink colonies but are usually rough, flat and sometimes spreading. *Staphylococcus* species produce red colonies but are catalase positive. *Aerococcus* species produce red colonies, are catalase negative but usually PYR (pyroglutamateaminopeptidase) negative.

For oxolinic aesculin azide agar, enterococci will appear as cream/brown colonies with a brown/black halo around the colony, enumerate by eye.

Note: some *Bacillus* species may also form a brown/black halo around colony but are catalase positive (usually) and are endospore forming.

The maximum number of colonies that should be counted on a single membrane is approximately 80 cfu. If no other plates are available with less colonies present an estimate of the number present can be made.

Confirmation

If confirmation is required membrane filters can be transferred onto bile aesculin agar (BAA, 0) and incubating for 1-4 hours at 44 (± 1) °C. Enumerate all colonies producing a brown blackening of the agar. All confirmed colonies on BAA should also be confirmed as catalase negative by picking a BAA positive colony on a loop and suspending in 3% hydrogen peroxide. An absence of bubbles indicates a negative reaction. Confirmed colonies are bile aesculin positive, catalase negative.

NOTE: if using oxolinic aesculin azide agar no confirmation is necessary but the colonies can be assessed for catalase activity.

Additionally, a biochemical tests can be applied such as Rapid ID32STREP (Biomérieux) test strips or equivalent can be used.

7.1.10 Expression of results

Calculation of the number of enterococci present (per g wet weight of the original sludge sample) is by multiplying the number of maroon/pink colonies on the filter by the dilution factor.

i.e. a/bd = c

where:
a = volume filtered through each membrane
b = initial dilution factor of the sludge in QSR (normally 10)
c = original concentration of enterococci per g
d = dilution factor for the serial dilutions used

If dry weight numbers are required the following equation if used:

a/bde = c

where:
e = % dry mass of the original sludge sample

7.1.11 Annex A

(Informative)

Performance data

To assess the performance of the media using the above described method sludge samples were analysed on Slanetz and Bartley (m-Enterococcus) agar and oxolinic aesculin azide agar using membrane filtration. Samples were analysed alongside a positive control of enterococci (*Enterococcus faecalis* NCTC 775) and a negative control and a blank (diluent only).

Confirmation of presumptive positive colonies was conducted on all colonies if less than ten were present and at least ten colonies if more were present.

The results showed a significantly higher number of enterococci were observed using OAA media for raw sludge ($t = 4.78$, $p = < 0.001$) (**Error! Reference source not found.** and **Error! Reference source not found.**). Pelleted sludge and positive control samples showed no significant differences between OAA and SBA ($p = > 0.05$).

When confirmed using bile aesculin hydrolysis and catalase reaction 100% colonies confirmed as enterococci on OAA and SBA for all sample types.

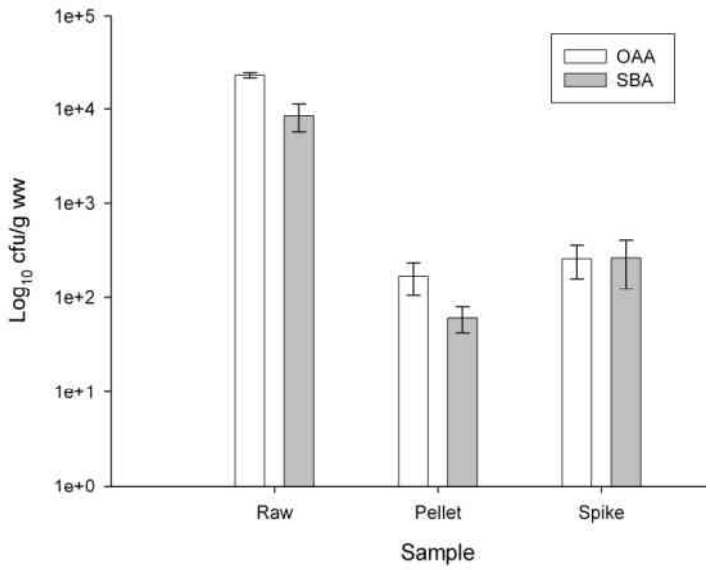

Figure 7.1: Comparison of OAA media and SBA media for detection of enterococci in sludges by membrane filtration. Lines indicate the standard error of the mean.

Table 7.1: Mean numbers of enterococci on OAA and SBA media using membrane filtration.

Sample	Media	Trial 1 Mean cfu/g ww (n = 3)	Trial 2 Mean cfu/g ww (n = 4)	Trial 3 Mean cfu/g ww (n = 3)	Mean cfu/g ww (n = 10)	p-value (t-test) *(Mann Whitney Rank sum)
Raw	**OAA**	21,333	26,260	21,000	23,204	
	SBA	17,667	1,238	9,460	8,541	< 0.001
Pelleted	**OAA**	97	50	400	169	
	SBA	123	45	0	61	0.144
Positive control	**OAA**	433	17	400	17	
	SBA	567	35	NR	35	0.435*
Negative control	**OAA**	0	0	0	0	
	SBA	0	0	0	0	
Blank	**OAA**	0	0	0	0	
	SBA	0	0	0	0	

Key: cfu	colony forming unit
ww	wet weight
n	number
NR	nor result
OAA	oxolinic aesculin azide agar
SBA	Slanetz and Bartley and Bartley agar

7.1.12 References

Audicana, A., Perales, I., Borrego, J. J. (1995). Modification of kanamycin-esculin-azide agar to improve selectivity in the enumeration of faecal streptococci from water samples. *Applied and Environmental Microbiology* **61**(12): 4178-4183.

Chuard, C., Reller, L. B. (1998). Bile-esculin test for presumptive identification of Enterococci and Streptococci: effects of bile concentration, inoculation technique, and incubation time. *Journal of Clinical Microbiology* **36**(4): 1135-1136.

Figueras, M. J., Inza, I., Polo, F., Guarro, J. (1998). Evaluation of the oxolinic acid-esculin-azide medium for the isolation and enumeration of faecal streptococci in a routine monitoring programme for bathing waters. *Canadian Journal Of Microbiology* **44**: 998-1002.

Standing Committee of Analysts (2002). The Microbiology of Drinking Water (2002) - Part 5 - A method for the identification and enumeration of enterococci by membrane filtration. *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

7.2 Enterococci analysis using miniaturised method (most probable number) by inoculation in liquid media

7.2.1 Introduction

Sludges, soils, soil improvers, growing media and biowastes can contain microorganisms pathogenic to Man *e.g*. *Salmonella* spp. Most occur in the intestinal tract of humans and animals and can be transmitted through faecal contamination. Outbreaks of infection could be caused by use of the pathogen contaminated materials in agriculture due to the production of contaminated food and animal feed, or transmission to wild animals. As a consequence of this there is a need to monitor levels in these materials and application levels to land.

WARNING – "Waste and sludge samples may contain hazardous and inflammable substances. They may contain pathogens and be liable to biological action. Consequently it is recommended that these samples should be handled with special care. The gases which may be produced by microbiological activity are potentially inflammable and will pressurise sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided where possible. National regulations should be followed with respect to microbiological hazards associated with this method".

7.2.2 Scope

This draft standard method describes a modified most probable number (MPN) method for the quantitative analysis, of enterococci species in sewage sludge, compost and biowaste samples. The user should prior to analysis validate the method for the particular type of sample they wish to analyse: sludges, soils, soil improvers, growing media (e.g. compost) and biowastes.

This method is of use if determination of treatment efficiency is required as outlined in the revision of Directive 86/278/EEC (3rd draft, CEN/TC 308-doc 525).

The method can be used for material with dry matter content of more than 10% in particular.

7.2.3 Normative references

The following referenced documents were referred to extensively and offer indispensable advice for the application of this method.

EN 12880:2000. Characterisation of sludges – determination of dry residue and water content

EN ISO 5667-13:1997. Water quality – Sampling – Part 13: Guidance on sampling of sludges from sewage and water treatment works.

ISO 8199:2001. Water quality – General guide to the enumeration of microorganisms by culture.

7.2.4 Terms and definitions

For the purposes of this document the following terms and definitions apply.

Enterococci

Enterococci are gram-positive, catalase-negative, facultative anaerobes that grow as diplococci in short chains. They can be differentiated from other catalase-negative gram-positive cocci by their ability to hydrolyze aesculin to produce dextrose and aesculetin in the presence of 40% bile salts, growth in 6.5% sodium chloride at 45°C, reduce 2,3,5-triphenyltetrazolium chloride (TTC) to formazan and produce pyrrolidonylarylamidase (*i.e.* PYR reaction).

Method definition

The medium contains a defined substrate consisting of 4-methyl-umbelliferyl Beta-D-glucoside. When this substrate is hydrolyzed by the Beta-D-glucosidase produced by the enterococci, it produces 4-methyl umbelliferone as a blue fluorescent hydrolysis product, as well as the D-glucose product that can be used as a substrate for bacterial growth.

Dry matter

The dry mass portion of the material tested after the specified drying process, expressed as percent or grams per kilogram (EN 12880:2000, 3.1).

7.2.5 Apparatus

With the exception of equipment supplied sterilised all should be sterilised before use in accordance with ISO 8199.

In addition to usual microbiological laboratory equipment:

Apparatus for sterilisation either dry heat (oven) or steam (autoclave).

Thermostatic incubator(s) at 44 (±1)ºC.

Tunnel drier or vertical laminar flow cabinet (Class II preferably).

Homogeniser (e.g. Stomacher, Seward Laboratories or equivalent).

Sterile homogeniser bags 250ml volume with or without integrated mesh to remove large particles (e.g. Seward Laboratories 6041, 6041STR or equivalent).

pH meter with accuracy of ± 0.1.

Ultraviolet observation chamber (Wood's Lamp, 366 nm).

WARNING – UV light can damage eyes and skin. Use protective goggles and gloves.

Bunsen burner.

Pipettors capable of dispensing 100 µl and 1 ml

Graduated pipettes capable of dispensing 2-10 ml

Adjustable or pre-set 8 channel multi-pipette for measuring and distributing 200 µl per well.

Sterile tips for multi-pipette.

Sterile microtitre plates – 96 well, 350 µl, flat bottomed, non-fluorescent.

Sterile adhesive cover strips for sealing microtitre plates.

Sterile Petri dishes – 90 mm diameter.

7.2.6 Sampling hazards

Take samples of at least 100g wet weight and transport to laboratory as quickly as possible, chilled at 5±3ºC.

General

As samples are liable to ferment and contain pathogenic microorganisms, it is of paramount importance to adhere to national and international regulations relating to bio hazardous samples when handling and transporting samples.

It is essential to keep samples away from food or drink, and to protect any cuts.

See also Warning note in introduction of this method.

Storage

Do not store these samples on an open bench in the laboratory. If samples are not to be analysed immediately, store them at 5±3ºC in well labelled containers, preferable plastic. Samples can be stored for a maximum period of 36 hours.

Handling

Good laboratory practice and cleanliness is essential. When handling sludge samples it is necessary to wear gloves, face and eye protection, and sufficient body protection to protect against spillages or bottles bursting. The gas evolved when opening sludge samples is flammable and so should be carried out away from naked flames and all equipment should be flame proof.

See also the Warning note in the introduction.

7.2.7 Reagents, diluents and culture media

To ensure reproducible results, prepare culture media and diluents using either constituents of uniform quality and chemicals of recognised analytical grade, or a dehydrated diluent or complete medium prepared following the manufacturer's instructions. Prepare the media and reagents with demineralised or distilled water free from substances capable of inhibiting growth under test conditions. If the media are not used immediately, they should be stored in the dark at 5±3ºC for up to one month in conditions avoiding any alteration of their composition.

NOTE the use of chemicals of other grades is permissible providing that they are shown to be of equivalent performance in the test.

Special diluent (SD)

per litre:

Synthetic sea salt	22.5 g
Deionised water	1 l

Salt is dissolved by heating and stirring. Solution is then steam sterilised (autoclaved) at 121±3ºC for 15±1 minutes.

NOTE: SD is available commercially from several suppliers (e.g. BioRad).

Culture medium: MUD/SF medium

Solution A

Tryptose	40 g
KH_2PO_4	10 g
D(+)-galactose	2 g
Polyoxyethylenesorbitan monooleate (or Tween 80)	1.5 ml
Water (deionised)	900 ml

Add Tryptose, $KH._2PO_4$, galactose and Tween 80 to 900 ml water, whilst gently heating with stirring. Then bring to the boil until completely dissolved. Allow to cool.

Solution B

$NaCO_3$	4 g
Naladixic acid	250 mg
Water (deionised)	50 ml

Add reagents to 50 ml water, whilst gently heating with stirring. Allow to cool.

Solution C

Thallium (I) acetate	2 g
2,3,5-triphenyltetrazolium chloride	0.1 g
Water (deionised)	50 ml

WARNING: Thallium (I) acetate is toxic. Harmful by inhalation, contact with skin. Use a chemical fume hood, wear gloves and eye protection.

Add reagents to 50 ml water, whilst gently heating with stirring. Allow to cool.

Solution D

MUD (4-methylumbelliferyl-β-D-glucoside)	150 mg
N,N-dimethylformamide (TOXIC USE FLAME HOOD)	2 ml

WARNING: *N,N*-dimethylformamide is toxic. Harmful by inhalation, in contact with skin and swallowing. May cause cancer. Use a chemical fume hood, wear eye protection and gloves.

Complete medium

Mix together solutions A, B, C and D. Adjust pH to 7.5 ± 0.2. Filter sterilise. Distribute 100 µl to each well of microtitre plates and immediately dehydrate in tunnel drier or laminar air cabinet.

7.2.8 Procedure

Sample preparation

Determination of dry matter content

The dry matter content is measured using the method described in EN 12880:2000.

Suspension preparation

Mix sample and weigh out a representative sub-sample of 25 g (wet weight) into a homogeniser bag with an integrated mesh to remove large particles, if required.

Add a volume of special diluent (SD) so the final volume is 250 ml (dilution A).

For lime treated sludges adjust the pH to 7.0± 0.5 with 1 mol/l hydrochloric acid.

NOTE 1: If the pH drops below 4.5 whilst neutralising the sample, a new sample should be prepared.

NOTE 2: If other chemical treatment is used on sludge samples to be tested a suitable neutralisation procedure should be adopted.

Place sample in a homogeniser (7.1.5) and homogenise for 2 minutes.

Preparation of dilutions

Dependant on the sample dilutions need to be made to the samples prior to analysis. (see Table 7.2).

The first dilution is made by adding 9 ml of sample (dilution A, homogenised) to 9 ml of special diluent (1:2 dilution). For subsequent dilutions transfer 1 ml of the first dilution (1:2) into 9 ml of special diluent.

Table 7.2

Origin of sample	No. of dilutions	No. wells/dilution	Measurement limits bacteria/100 ml
Bathing water	2	64 wells at 1:2 dilution 32 wells at 1:20 dilution	15 to $3.5x10^4$
Other surface water	4	24 wells at 1:2 dilution 24 wells at 1:20 dilution 24 wells at 1:200 dilution 24 wells at 1:2000 dilution	40 to $3.2x10^6$
Wastewater and treatment plants	6	16 wells at 1:2 dilution Up to 16 wells at 1:200000 dilution	60 to $6.7x10^8$

7.2.9 Analysis

Transfer contents of the first tube of dilution to an empty sterile Petri dish (90 mm). Distribute 200 µl to the wells of a microtitre plate. In accordance with

. For subsequent dilutions operate in the same way, changing the Petri dish and pipette tips.

Cover the microtitre plate and seal with tape.

Incubate @ 44 ± 0.5ºC for a minimum of 36 hours and maximum of 72 hours.

Resuscitation and enumeration of enterococci in microtitre plates

Observe each covered microtitre plate in the UV observation chamber. Consider all wells in which a blue fluorescence is observed as being positive.

For each dilution, note the number of positive wells,
e.g.

Dilution	Positive wells
1/20	16 of 16
1/200	12 of 16
1/2000	1 of 16

Confirmation

The media used in the described method does not require confirmation but biochemical tests can be applied such as Rapid ID32STREP (Biomérieux) test strips or equivalent.

7.2.10 Expression of results

Calculation of MPN

Use software in EN ISO 7899-1:1998 to calculate the MPN or refer to appropriate MPN tables such as De Mann (1975).

If none of the well are positive record the result as < n/ 100 ml where n is the MPN for 1 positive well according the dilution.

7.2.11 Annex A

Typical ion composition of synthetic sea salt

Major ion	% total weight
Chloride (Cl^-)	47.470
Sodium (Na^+)	26.280
Sulphate (SO_4^{2-})	6.602
Magnesium (Mg^{2+})	3.230
Calcium (Ca^{2+})	1.013
Potassium (K^+)	1.015
Bicarbonate (HCO_3^-)	0.491
Borate (B)	0.015
Strontium (Sr^{2+})	0.001
Water	13.88

7.2.12 Annex B

(Informative)

Performance data

Samples of raw and pelleted sludge alongside positive controls (Enterococci faecalis NCTC 775) were analysed using the methods described herein. To compare the results samples were also analysed by spread plating onto OAA (oxolinic aesculin azide) agar.

Although MUD/SF is designed as a confirmed media and as such no further confirmation should be necessary, in this research wells were confirmed on bile aesculin agar and analysed for catalase activity. Also some wells were analysed using the API system. Confirmation of presumptive positive colonies was conducted on all wells if less than ten were present and at least ten colonies if more were present.

The results found that the numbers of enterococci counted on OAA agar were significantly higher than the MPN results obtained using MUD/SF for raw sludges ($t = 5.\ 69$, 4 d.f., $p = 0.005$) (**Error! Reference source not found.**). Numbers for pelleted sludge samples and positive control samples were not significantly different between the two methods ($p = 0.277$ (4 d.f.) and $p = 0.679$ (4 d.f.) respectively) (**Error! Reference source not found.**). This

method was found to be particularly useful if the dry solids are high but the number of target bacteria present are low as the presence of high solids masked colonies on membrane filters.

Confirmation of the positive wells gave 100% agreement with both BAA/ catalase test and API confirmations.

Table 7.3 Numbers of enterococci in sludge samples using MUD/SF (MPN/g) or OAA (cfu/g).

Sample	Media	Mean cfu/g (OAA) or MPN/g (MUD/SF) ww	Min cfu/g (OAA) or MPN/g (MUD/SF) ww (n = 4)	Max cfu/g (OAA) or MPN/g (MUD/SF) ww	St. dev. cfu/g (OAA) or MPN/g (MUD/SF) ww	p-value (t-test) *(Mann Whitney Rank sum)
Raw	**MUD/SF**	26,325	24,048	28,090	2,069	
	OAA	84,000	64,000	96,000	17,436	0.005
Pelleted	**MUD/SF**	20,142	3,342	49,520	25,530	
	OAA	1,600	800	2,800	1,058	0.277
Positive control	**MUD/SF**	37,100	27,828	53,676	14,389	
	OAA	32,667	22,000	40,000	9,452	0.679
Negative control	**MUD/SF**	0	0	0		
	OAA	0	0	0		
Blank	**MUD/SF**	0	0	0		
	OAA	0	0	0		

Key: cfu colony forming unit
MPN most probable number
ww wet weight
n number
OAA oxolinic aesculin azide agar
Min minimum
Max maximum
St. dev. standard deviation

7.2.13 References

De Mann J. C. (1975) The probability of most probable numbers. European Journal of Applied Microbiology **1**: p. 67-78.
EN ISO 7899-1 (1998) Water quality – Detection and enumeration of intestinal enterococci – Part 1: Miniaturised method (Most Probable Number) for surface and wastewater.

7.3 *Enterococci analysis using presence/absence and enumeration by a defined substrate broth most probable number technique*

7.3.1 Introduction

Sludges, soils, soil improvers, growing media and biowastes can contain microorganisms pathogenic to Man *e.g*. *Salmonella* spp. Most occur in the intestinal tract of humans and animals and can be transmitted through faecal contamination. Outbreaks of infection could be caused by use of the pathogen contaminated materials in agriculture due to the production of contaminated food and animal feed, or transmission to wild animals. As a consequence of this there is a need to monitor levels in these materials and application levels to land.

WARNING – "Waste and sludge samples may contain hazardous and inflammable substances. They may contain pathogens and be liable to biological action. Consequently it is recommended that these samples should be handled with special care. The gases which may be produced by microbiological activity are potentially inflammable and will pressurise sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided where possible. National regulations should be followed with respect to microbiological hazards associated with this method".

7.3.2 Scope

This draft standard method describes a modified most probable number (MPN) method for the quantitative analysis, of enterococci species in sewage sludge, compost and biowaste samples. The user should prior to analysis validate the method for the particular type of sample they wish to analyse: sludges, soils, soil improvers, growing media (e.g. compost) and biowastes.

This method is of use if determination of treatment efficiency is required as outlined in the revision of Directive 86/278/EEC (3rd draft, CEN/TC 308-doc 525).

The method can be used for material with dry matter content of more than 10% in particular.

7.3.3 Normative references

The following referenced documents were referred to extensively and offer indispensable advice for the application of this method.

EN 12880:2000. Characterisation of sludges – determination of dry residue and water content

EN ISO 5667-13:1997. Water quality – Sampling – Part 13: Guidance on sampling of sludges from sewage and water treatment works.

ISO 8199:2001. Water quality – General guide to the enumeration of microorganisms by culture.

7.3.4 Terms and definitions

For the purposes of this document the following terms and definitions apply.

Enterococci

Enterococci are gram-positive, catalase-negative, facultative anaerobes that grow as diplococci in short chains. They can be differentiated from other catalase-negative gram-positive cocci by their ability to hydrolyze aesculin to produce dextrose and aesculetin in the presence of 40% bile salts, growth in 6.5% sodium chloride at 45°C, reduce 2,3,5-triphenyltetrazolium chloride (TTC) to formazan and produce pyrrolidonylarylamidase (*i.e.* PYR reaction).

Method definition

The medium contains a defined substrate consisting of 4-methyl-umbelliferyl Beta-D-glucoside. When this substrate is hydrolyzed by the Beta-D-glucosidase produced by the enterococci, it produces 4-methyl umbelliferone as a blue fluorescent hydrolysis product, as well as the D-glucose product that can be used as a substrate for bacterial growth.

Dry matter

The dry mass portion of the material tested after the specified drying process, expressed as percent or grams per kilogram (EN 12880:2000, 3.1).

7.3.5 Apparatus

With the exception of equipment supplied sterilised all should be sterilised before use in accordance with ISO 8199.

In addition to usual microbiological laboratory equipment:

Apparatus for sterilisation either dry heat (oven) or steam (autoclave).

Thermostatic incubator(s) at 44 (±1)ºC.

Homogeniser (e.g. Stomacher, Seward Laboratories or equivalent).

Sterile homogeniser bags 250ml volume with or without integrated mesh to remove large particles (e.g. Seward Laboratories 6041, 6041STR or equivalent).

pH meter with accuracy of ± 0.1.

Ultraviolet observation chamber (Wood's Lamp, 366 nm).

WARNING – UV light can damage eyes and skin. Use protective goggles and gloves.

Bunsen burner.

Pipettors capable of dispensing 100 µl and 1 ml

Graduated pipettes capable of dispensing 2-10 ml

Sterile tips.

Sterile measuring cylinder capable of measuring 100 ml.

Sterile 100 ml pots.

Enumeration trays e.g. Quantitray™ 2000 trays (Enterolert) – only necessary if enumeration is required (Idexx WQT-2K).

Tray sealer – e.g. Quantitray™ sealer – only necessary if enumeration is required (Idexx WQTS2X-230).

Tray sealer insert e.g. Quanti-Tray® /2000 Rubber Insert – only necessary if enumeration id required (Idexx WQTSRBR-2K).

7.3.6 Sampling hazards

Take samples of at least 100g wet weight and transport to laboratory as quickly as possible, chilled at 5±3ºC.

General

As samples are liable to ferment and contain pathogenic microorganisms, it is of paramount importance to adhere to national and international regulations relating to bio hazardous samples when handling and transporting samples.

It is essential to keep samples away from food or drink, and to protect any cuts.

See also Warning note in introduction of this method.

Storage

Do not store these samples on an open bench in the laboratory. If samples are not to be analysed immediately, store them at 5±3ºC in well labelled containers, preferable plastic. Samples can be stored for a maximum period of 36 hours.

Handling

Good laboratory practice and cleanliness is essential. When handling sludge samples it is necessary to wear gloves, face and eye protection, and sufficient body protection to protect against spillages or bottles bursting. The gas evolved when opening sludge samples is flammable and so should be carried out away from naked flames and all equipment should be flame proof.

See also the Warning note in the introduction.

7.3.7 Reagents, diluents and culture media

To ensure reproducible results, prepare culture media and diluents using either constituents of uniform quality and chemicals of recognised analytical grade, or a dehydrated diluent or complete medium prepared following the manufacturer's instructions. Prepare the media and reagents with demineralised or distilled water free from substances capable of inhibiting growth under test conditions. If the media are not used immediately, they should be stored in the dark at 5±3ºC for up to one month in conditions avoiding any alteration of their composition.

NOTE the use of chemicals of other grades is permissible providing that they are shown to be of equivalent performance in the test.

Quarter Strength Ringers solution

Ringer's solution composition per litre:

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride $6H_2O$	0.12 g
Sodium bicarbonate	0.05 g

Ringer's is commercially available in tablet form where one tablet is added to 500 ml water for quarter strength ringers. Solution is steam sterilised (autoclaved) at 121±3ºC for 15±1 minutes. pH 7.0±0.2.

Enterolert-E® medium (Idexx, WENTE020)

The medium is a commercially available dehydrated formulation provided in sachets sufficient for single 100 ml sample analysis. The medium is a chemically defined formula containing minimal nutrients and substrates for the specific detection of enzyme Beta-D-glucosidase.

7.3.8 Procedure

Sample preparation

Determination of dry matter content

The dry matter content is measured using the method described in EN 12880:2000.

Suspension preparation

Mix sample and weigh out a representative sub-sample of 25 g (wet weight) into a homogeniser bag with an integrated mesh to remove large particles, if required.

Add a volume of quarter strength ringers (QSR) so the final volume is 250 ml (dilution A).

For lime treated sludges adjust the pH to 7.0± 0.5 with 1 mol/l hydrochloric acid.

NOTE 1: If the pH drops below 4.5 whilst neutralising the sample, a new sample should be prepared.

NOTE 2: If other chemical treatment is used on sludge samples to be tested a suitable neutralisation procedure should be adopted.

Place sample in a homogeniser (7.1.5) and homogenise for 2 minutes.

Preparation of dilutions

Prepare serial dilutions from dilution A in QSR as appropriate if the expected concentration of enterococci in sample is over 20,000 per g. For example raw sludges may have up to 10^6 enterococci per g but treated sludges may only contain 10^1-10^2 enterococci per g.

Mix the primary suspension (1/10 dilution) and using a sterile pipette aseptically transfer 10 ml into a sterile pot containing 90 ml QSR. Mix this dilution and using a new sterile pipetted transfer 10 ml of this dilution to a tube containing 90 ml QSR.

Continue this procedure until the required number of dilutions has been prepared.

7.3.9 Analysis

Pour 100 ml of required dilutions of samples into sterile sample pots. Add one sachet of Enterolert-E™ media to each sample pot, shake and leave to dissolve (approximately 2 minutes). Once dissolved, if enumeration is required pour the contents of the pot into a Quantitray® 2000 tray and seal using the Quantitray® sealer. In this procedure the Quantitray® is placed in the rubber insert and fed into the Quantitray® sealer (previously switched on to warm up for approximately half an hour prior to analysis). The sealer passes the tray through heated rollers which seal the tray.

Incubate 100 ml sample pots or Quantitray® 2000 trays at 44 ± 1ºC for 24 ± 4 hours.

Resuscitation and enumeration of enterococci in microtitre plates

Observe each sample pot or Quantitray® 2000 in the UV observation chamber. Consider all pots or wells in which a blue fluorescence is observed as being positive.

If using the Quantitray® 2000 for each dilution, note the number of large and small positive wells. Refer to MPN tables supplied with the Idexx media.

Confirmation

The media used in the described method is confirmatory but if required biochemical tests can be applied such as Rapid ID32STREP (Biomérieux) test strips or equivalent.

7.3.10 Expression of results

Calculation of MPN

Use software in EN ISO 7899-1:1998 to calculate the MPN or refer to appropriate MPN tables supplied with the Idexx Quantitray® 2000.

To calculate the MPN and confidence intervals (C.I.) of enterococci per g wet weight:

Look up the MPN value from the number of large and small positive wells and refer to the MPN tables.

Take into account any dilutions used:

e.g. if a 1:10 dilution was made on the sample prior to analysis the MPN would be MPN from the table multiplied by 10.

This gives a result per 25 g in 100 ml of sample.

To calculate the result per g,

MPN/g = MPN (adjusted for dilution factor) x 100 / 25

If a result per g dry weight is required,

MPN/g d.w. = MPN (adjusted for dilution factor) x 100 / (25 x % dry matter)

If none of the well are positive record the result as < n/ g where n is the MPN for 1 positive well according the dilution. If all the wells are positive record the result as > n/ g where n is the MPN lower limit for all positive wells under the dilution conditions employed.

7.3.11 Appendix A

IDEXX Quanti-Tray®/2000
MPN Table (per 100mL) with 95% Confidence Limits

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
0	0	<1	0.0	3.7
0	1	1.0	0.0	3.7
0	2	2.0	0.3	5.6
0	3	3.0	0.6	7.3
0	4	4.0	1.1	8.9
0	5	5.0	1.7	10.5
0	6	6.0	2.3	12.1
0	7	7.0	2.9	13.7
0	8	8.0	3.7	15.3
0	9	9.0	4.5	15.8
0	10	10.0	5.2	16.9
0	11	11.0	5.9	18.5
0	12	12.0	6.9	20.1
0	13	13.0	7.8	21.2
0	14	14.1	8.6	21.9
0	15	15.1	9.0	23.4
0	16	16.1	9.6	24.9
0	17	17.1	10.5	25.7
0	18	18.1	11.5	26.9
0	19	19.1	12.5	28.6
0	20	20.2	13.2	29.3
0	21	21.2	13.9	30.5
0	22	22.2	14.5	31.8
0	23	23.3	15.7	33.1
0	24	24.3	16.4	34.2
0	25	25.3	17.6	35.2
0	26	26.4	18.3	36.5
0	27	27.4	19.5	37.7
0	28	28.4	19.7	38.6
0	29	29.5	21.0	39.9
0	30	30.5	21.7	41.2
0	31	31.5	22.5	42.3
0	32	32.6	23.9	43.4
0	33	33.6	24.6	44.4
0	34	34.7	25.4	45.7
0	35	35.7	26.2	46.8
0	36	36.8	27.7	48.0
0	37	37.8	28.5	49.0
0	38	38.9	29.2	50.3
0	39	40.0	30.0	51.2
0	40	41.0	30.8	52.8
0	41	42.1	31.6	53.7
0	42	43.1	33.3	54.7
0	43	44.2	34.1	56.1
0	44	45.3	34.9	57.1

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
25	0	33.6	22.0	48.9
25	1	35.0	22.9	51.2
25	2	36.4	23.8	52.6
25	3	37.9	25.5	54.0
25	4	39.3	26.5	55.9
25	5	40.8	28.3	57.3
25	6	42.2	29.3	59.0
25	7	43.7	30.3	60.7
25	8	45.2	31.3	62.5
25	9	46.7	33.3	64.2
25	10	48.2	34.4	66.0
25	11	49.7	35.4	67.3
25	12	51.2	36.5	69.0
25	13	52.7	37.6	70.7
25	14	54.3	39.7	72.4
25	15	55.8	40.9	74.0
25	16	57.3	42.0	75.9
25	17	58.9	43.1	77.6
25	18	60.5	45.5	79.5
25	19	62.0	46.7	81.2
25	20	63.6	47.8	83.0
25	21	65.2	49.0	84.6
25	22	66.8	50.2	86.2
25	23	68.4	51.5	87.4
25	24	70.0	54.0	89.5
25	25	71.7	55.3	91.6
25	26	73.3	56.6	93.9
25	27	75.0	57.8	94.6
25	28	76.6	59.1	96.1
25	29	78.3	60.4	98.6
25	30	80.0	61.7	101.0
25	31	81.7	64.6	101.6
25	32	83.3	65.9	103.6
25	33	85.1	67.3	106.2
25	34	86.8	68.6	107.3
25	35	88.5	70.0	109.1
25	36	90.2	71.4	111.4
25	37	92.0	72.8	112.8
25	38	93.7	74.2	114.9
25	39	95.5	77.4	116.4
25	40	97.3	78.9	118.3
25	41	99.1	80.3	120.4
25	42	100.9	81.8	121.9
25	43	102.7	83.2	124.2
25	44	104.5	84.7	126.0

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
0	45	46.3	35.7	58.1
0	46	47.4	37.5	59.5
0	47	48.5	38.3	60.7
0	48	49.5	39.2	61.6
1	0	1.0	0.1	5.5
1	1	2.0	0.3	5.9
1	2	3.0	0.6	7.3
1	3	4.0	1.1	8.9
1	4	5.0	1.7	10.5
1	5	6.0	2.3	12.1
1	6	7.1	3.0	13.7
1	7	8.1	3.7	15.3
1	8	9.1	4.3	16.2
1	9	10.1	5.2	17.2
1	10	11.1	6.0	18.5
1	11	12.1	6.8	20.1
1	12	13.2	7.6	21.7
1	13	14.2	8.7	22.2
1	14	15.2	9.4	23.6
1	15	16.2	9.7	25.1
1	16	17.3	10.6	26.4
1	17	18.3	11.6	27.0
1	18	19.3	12.6	28.6
1	19	20.4	13.3	30.0
1	20	21.4	14.0	30.7
1	21	22.4	14.7	32.3
1	22	23.5	15.8	33.1
1	23	24.5	17.0	34.5
1	24	25.6	17.2	35.7
1	25	26.6	18.4	37.1
1	26	27.7	19.2	38.1
1	27	28.7	20.5	39.2
1	28	29.8	21.2	40.6
1	29	30.8	22.0	41.6
1	30	31.9	22.7	42.7
1	31	32.9	23.5	44.0
1	32	34.0	24.9	44.9
1	33	35.0	25.7	46.4
1	34	36.1	26.4	47.2
1	35	37.2	27.9	48.8
1	36	38.2	28.7	49.6
1	37	39.3	29.5	51.0
1	38	40.4	30.4	52.0
1	39	41.4	31.2	53.1
1	40	42.5	32.8	54.5
1	41	43.6	33.6	55.4
1	42	44.7	34.5	56.6
1	43	45.7	35.3	58.0
1	44	46.8	36.1	58.9

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
25	45	106.3	86.2	128.0
25	46	108.2	89.8	130.2
25	47	110.0	91.3	132.1
25	48	111.9	92.9	133.7
26	0	35.5	23.2	52.0
26	1	36.9	24.9	53.7
26	2	38.4	25.9	55.4
26	3	39.9	26.9	56.5
26	4	41.4	27.9	58.6
26	5	42.8	29.7	60.1
26	6	44.3	30.7	61.8
26	7	45.9	31.8	63.5
26	8	47.4	32.8	65.4
26	9	48.9	34.9	66.6
26	10	50.4	36.0	68.5
26	11	52.0	37.1	70.4
26	12	53.5	38.2	72.2
26	13	55.1	40.4	73.7
26	14	56.7	41.5	75.7
26	15	58.2	42.7	77.6
26	16	59.8	43.8	79.2
26	17	61.4	45.0	80.8
26	18	63.0	46.2	82.7
26	19	64.7	48.6	84.2
26	20	66.3	49.8	85.4
26	21	67.9	51.1	87.3
26	22	69.6	52.3	89.5
26	23	71.2	55.0	91.6
26	24	72.9	54.8	93.9
26	25	74.6	57.5	94.6
26	26	76.3	58.8	96.2
26	27	78.0	60.1	98.6
26	28	79.7	61.5	101.0
26	29	81.4	62.8	102.2
26	30	83.1	64.1	103.6
26	31	84.9	67.1	106.2
26	32	86.6	68.5	108.0
26	33	88.4	69.9	109.4
26	34	90.1	71.3	111.8
26	35	91.9	72.7	113.5
26	36	93.7	74.1	115.1
26	37	95.5	75.6	117.5
26	38	97.3	78.9	119.2
26	39	99.2	80.4	121.2
26	40	101.0	81.9	123.1
26	41	102.9	83.4	125.0
26	42	104.7	84.9	127.1
26	43	106.6	86.4	128.9
26	44	108.5	90.1	130.6

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
1	45	47.9	37.0	60.1
1	46	49.0	38.7	61.3
1	47	50.1	39.6	62.5
1	48	51.2	40.5	63.6
2	0	2.0	0.3	7.1
2	1	3.0	0.7	7.4
2	2	4.1	1.2	9.0
2	3	5.1	1.6	10.6
2	4	6.1	2.3	12.1
2	5	7.1	3.0	13.7
2	6	8.1	3.7	15.3
2	7	9.2	4.4	16.9
2	8	10.2	5.3	17.8
2	9	11.2	6.0	18.6
2	10	12.2	6.8	20.1
2	11	13.3	7.7	21.7
2	12	14.3	8.5	22.9
2	13	15.4	9.4	24.0
2	14	16.4	9.8	25.1
2	15	17.4	10.4	26.8
2	16	18.5	11.4	27.5
2	17	19.5	12.4	28.8
2	18	20.6	13.5	30.4
2	19	21.6	14.1	31.2
2	20	22.7	14.8	32.6
2	21	23.7	16.0	33.8
2	22	24.8	17.2	35.0
2	23	25.8	17.9	36.1
2	24	26.9	18.1	37.1
2	25	27.9	19.4	38.5
2	26	29.0	20.7	39.7
2	27	30.0	21.4	40.6
2	28	31.1	22.2	42.0
2	29	32.2	23.6	43.4
2	30	33.2	24.4	44.4
2	31	34.3	25.1	45.7
2	32	35.4	25.9	46.6
2	33	36.5	26.7	48.0
2	34	37.5	28.2	48.9
2	35	38.6	29.0	50.3
2	36	39.7	29.9	51.2
2	37	40.8	30.7	52.8
2	38	41.9	31.5	53.7
2	39	43.0	32.3	54.9
2	40	44.0	34.0	56.2
2	41	45.1	34.8	57.1
2	42	46.2	35.7	58.6
2	43	47.3	36.5	59.7
2	44	48.4	37.4	60.8

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
26	45	110.4	89.5	132.4
26	46	112.3	93.2	135.0
26	47	114.2	94.8	137.0
26	48	116.2	96.4	138.7
27	0	37.4	24.5	54.5
27	1	38.9	26.2	55.9
27	2	40.4	27.3	57.4
27	3	42.0	28.3	59.7
27	4	43.5	29.3	60.8
27	5	45.0	31.2	63.0
27	6	46.5	32.3	64.7
27	7	48.1	33.3	66.1
27	8	49.6	35.4	67.8
27	9	51.2	36.5	69.7
27	10	52.8	37.6	71.7
27	11	54.4	38.8	73.4
27	12	56.0	41.0	75.0
27	13	57.6	42.2	77.2
27	14	59.2	43.3	78.6
27	15	60.8	44.5	80.6
27	16	62.4	45.7	82.3
27	17	64.1	46.9	83.7
27	18	65.7	49.4	85.3
27	19	67.4	50.7	87.3
27	20	69.1	51.9	89.5
27	21	70.8	53.2	91.6
27	22	72.5	55.9	93.9
27	23	74.2	55.8	94.6
27	24	75.9	58.5	96.2
27	25	77.6	59.9	98.6
27	26	79.4	61.2	101.0
27	27	81.1	62.6	102.5
27	28	82.9	63.9	103.8
27	29	84.6	65.3	106.2
27	30	86.4	68.4	108.3
27	31	88.2	69.8	109.6
27	32	90.0	71.2	111.8
27	33	91.9	72.7	113.8
27	34	93.7	74.1	115.4
27	35	95.5	75.6	118.0
27	36	97.4	77.1	119.8
27	37	99.3	80.5	121.8
27	38	101.2	82.0	123.7
27	39	103.1	83.5	125.9
27	40	105.0	85.1	127.6
27	41	106.9	86.6	129.4
27	42	108.8	88.2	132.0
27	43	110.8	89.8	133.7
27	44	112.7	91.4	135.3

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
2	45	49.5	38.2	61.8
2	46	50.6	40.0	63.0
2	47	51.7	40.9	64.2
2	48	52.8	41.8	65.4
3	0	3.1	0.7	8.9
3	1	4.1	1.2	9.1
3	2	5.1	1.7	10.6
3	3	6.2	2.3	12.1
3	4	7.2	3.0	13.7
3	5	8.2	3.6	15.3
3	6	9.2	4.4	16.9
3	7	10.3	5.1	18.3
3	8	11.3	6.1	18.8
3	9	12.4	6.9	20.2
3	10	13.4	7.7	21.8
3	11	14.5	8.6	23.4
3	12	15.5	9.5	24.1
3	13	16.5	10.2	25.3
3	14	17.6	10.5	26.9
3	15	18.6	11.5	28.2
3	16	19.7	12.5	29.2
3	17	20.8	13.6	30.4
3	18	21.8	14.3	31.8
3	19	22.9	15.0	32.7
3	20	23.9	15.7	34.2
3	21	25.0	16.8	35.2
3	22	26.1	18.1	36.5
3	23	27.1	18.8	37.7
3	24	28.2	19.6	39.1
3	25	29.3	20.9	40.2
3	26	30.4	21.6	41.3
3	27	31.4	22.4	42.7
3	28	32.5	23.2	43.6
3	29	33.6	24.6	44.9
3	30	34.7	25.4	46.2
3	31	35.8	26.2	47.2
3	32	36.8	27.0	48.6
3	33	37.9	27.8	49.5
3	34	39.0	29.3	51.0
3	35	40.1	30.2	52.0
3	36	41.2	31.0	53.2
3	37	42.3	31.8	54.5
3	38	43.4	32.6	55.4
3	39	44.5	34.3	57.0
3	40	45.6	35.2	58.0
3	41	46.7	36.0	59.0
3	42	47.8	36.9	60.2
3	43	48.9	37.8	61.5
3	44	50.0	38.6	62.6

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
27	45	114.7	95.2	137.8
27	46	116.7	94.6	140.1
27	47	118.7	98.5	142.1
27	48	120.7	100.2	144.2
28	0	39.5	25.9	57.0
28	1	41.0	27.7	58.8
28	2	42.6	28.7	60.7
28	3	44.1	30.6	62.5
28	4	45.7	30.8	63.8
28	5	47.3	32.8	65.9
28	6	48.8	33.9	67.5
28	7	50.4	35.0	69.1
28	8	52.0	37.1	71.0
28	9	53.6	38.2	73.0
28	10	55.2	39.4	74.4
28	11	56.9	41.7	76.4
28	12	58.5	41.7	77.9
28	13	60.2	44.1	80.0
28	14	61.8	45.3	81.5
28	15	63.5	46.5	83.4
28	16	65.2	47.7	85.3
28	17	66.9	50.3	87.3
28	18	68.6	51.6	89.5
28	19	70.3	52.8	91.6
28	20	72.0	54.1	93.9
28	21	73.7	55.5	94.6
28	22	75.5	56.8	96.2
28	23	77.3	59.6	98.6
28	24	79.0	61.0	101.0
28	25	80.8	62.4	102.8
28	26	82.6	63.7	103.9
28	27	84.4	65.1	106.2
28	28	86.3	66.5	108.8
28	29	88.1	68.0	110.3
28	30	89.9	71.1	112.2
28	31	91.8	72.6	114.7
28	32	93.7	74.1	116.4
28	33	95.6	75.6	118.3
28	34	97.5	77.1	120.4
28	35	99.4	78.6	121.9
28	36	101.3	80.1	124.2
28	37	103.3	83.7	126.0
28	38	105.2	85.3	128.5
28	39	107.2	86.9	130.6
28	40	109.2	88.5	132.4
28	41	111.2	90.1	135.0
28	42	113.2	91.7	137.0
28	43	115.2	95.6	138.7
28	44	117.3	95.0	140.7

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
3	45	51.2	40.5	63.8
3	46	52.3	41.4	65.0
3	47	53.4	42.2	66.1
3	48	54.5	43.1	67.3
4	0	4.1	1.7	9.5
4	1	5.2	1.8	10.8
4	2	6.2	2.4	12.2
4	3	7.2	2.9	13.7
4	4	8.3	3.6	15.3
4	5	9.3	4.5	16.9
4	6	10.4	5.2	18.5
4	7	11.4	5.9	19.5
4	8	12.5	6.9	20.5
4	9	13.5	7.8	21.8
4	10	14.6	8.7	23.4
4	11	15.6	9.6	24.6
4	12	16.7	10.3	25.7
4	13	17.8	10.9	26.9
4	14	18.8	11.6	28.6
4	15	19.9	12.6	29.3
4	16	21.0	13.7	30.7
4	17	22.0	14.8	32.2
4	18	23.1	15.1	33.1
4	19	24.2	15.8	34.5
4	20	25.3	17.0	35.7
4	21	26.3	18.3	37.1
4	22	27.4	19.0	38.1
4	23	28.5	19.8	39.2
4	24	29.6	20.5	40.6
4	25	30.7	21.9	41.8
4	26	31.8	22.6	42.8
4	27	32.8	23.4	44.2
4	28	33.9	24.9	45.6
4	29	35.0	25.7	46.5
4	30	36.1	26.5	48.0
4	31	37.2	27.3	48.8
4	32	38.3	28.1	50.3
4	33	39.4	29.7	51.2
4	34	40.5	30.5	52.8
4	35	41.6	31.3	53.7
4	36	42.8	32.2	55.2
4	37	43.9	33.0	56.2
4	38	45.0	34.7	57.4
4	39	46.1	35.6	58.6
4	40	47.2	36.4	59.8
4	41	48.3	37.3	60.9
4	42	49.5	38.2	62.5
4	43	50.6	40.0	63.5
4	44	51.7	40.9	64.7

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
28	45	119.3	99.0	143.0
28	46	121.4	98.4	145.4
28	47	123.5	102.5	147.9
28	48	125.6	104.2	149.8
29	0	41.7	28.1	59.8
29	1	43.2	29.1	61.4
29	2	44.8	30.2	63.4
29	3	46.4	31.3	65.4
29	4	48.0	33.3	67.2
29	5	49.6	34.4	68.5
29	6	51.2	35.5	70.7
29	7	52.8	36.6	72.3
29	8	54.5	38.8	74.1
29	9	56.1	40.0	75.9
29	10	57.8	41.2	77.8
29	11	59.5	42.4	79.5
29	12	61.2	44.8	81.4
29	13	62.9	46.0	83.3
29	14	64.6	47.3	85.3
29	15	66.3	48.6	87.3
29	16	68.0	51.2	89.5
29	17	69.8	51.1	91.6
29	18	71.5	53.8	93.9
29	19	73.3	55.1	94.6
29	20	75.1	56.5	96.2
29	21	76.9	57.8	98.6
29	22	78.7	60.7	101.0
29	23	80.5	62.1	103.3
29	24	82.4	63.5	104.7
29	25	84.2	65.0	106.4
29	26	86.1	66.4	109.0
29	27	87.9	67.8	111.1
29	28	89.8	69.3	112.8
29	29	91.7	72.6	114.9
29	30	93.7	74.1	116.9
29	31	95.6	75.6	119.2
29	32	97.5	77.2	121.2
29	33	99.5	78.7	123.1
29	34	101.5	80.3	125.0
29	35	103.5	81.8	127.3
29	36	105.5	83.4	129.1
29	37	107.5	87.1	131.7
29	38	109.5	88.8	133.7
29	39	111.6	90.4	135.3
29	40	113.7	92.1	138.1
29	41	115.7	93.8	140.4
29	42	117.8	95.5	142.2
29	43	120.0	99.6	144.2
29	44	122.1	99.0	146.3

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
4	45	52.9	41.8	66.0
4	46	54.0	42.7	67.3
4	47	55.1	43.6	68.4
4	48	56.3	44.5	69.4
5	0	5.2	2.3	11.9
5	1	6.3	2.5	12.7
5	2	7.3	2.9	13.9
5	3	8.4	3.7	15.3
5	4	9.4	4.3	16.9
5	5	10.5	5.2	18.5
5	6	11.5	6.0	20.1
5	7	12.6	6.8	21.2
5	8	13.7	7.6	21.9
5	9	14.7	8.5	23.4
5	10	15.8	9.4	25.1
5	11	16.9	10.4	26.4
5	12	17.9	11.0	27.0
5	13	19.0	11.7	28.6
5	14	20.1	12.4	30.0
5	15	21.2	13.4	31.2
5	16	22.2	14.6	32.3
5	17	23.3	15.7	33.8
5	18	24.4	16.0	35.0
5	19	25.5	17.2	36.1
5	20	26.6	17.9	37.5
5	21	27.7	19.2	38.5
5	22	28.8	20.0	39.9
5	23	29.9	20.7	41.2
5	24	31.0	22.1	42.3
5	25	32.1	22.9	43.4
5	26	33.2	23.7	44.9
5	27	34.3	24.4	45.8
5	28	35.4	25.9	47.2
5	29	36.5	26.7	48.5
5	30	37.6	27.6	49.5
5	31	38.7	28.4	51.0
5	32	39.9	29.2	52.0
5	33	41.0	30.8	53.3
5	34	42.1	31.7	54.5
5	35	43.2	32.5	55.7
5	36	44.4	33.4	57.0
5	37	45.5	35.1	58.0
5	38	46.6	36.0	59.3
5	39	47.7	36.8	60.7
5	40	48.9	37.7	61.8
5	41	50.0	38.6	63.0
5	42	51.2	39.5	64.2
5	43	52.3	41.4	65.4
5	44	53.5	42.3	66.6

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
29	45	124.2	103.1	148.9
29	46	126.4	104.9	151.7
29	47	128.6	106.7	153.5
29	48	130.8	108.6	155.9
30	0	43.9	29.6	62.6
30	1	45.5	30.7	64.5
30	2	47.1	31.8	66.6
30	3	48.7	33.8	68.0
30	4	50.4	34.9	70.1
30	5	52.0	36.1	72.2
30	6	53.7	38.3	74.0
30	7	55.4	39.5	75.8
30	8	57.1	40.7	77.6
30	9	58.8	41.9	79.5
30	10	60.5	43.1	81.4
30	11	62.2	45.6	83.3
30	12	64.0	46.8	85.3
30	13	65.7	48.1	87.3
30	14	67.5	49.4	89.5
30	15	69.3	50.7	91.6
30	16	71.0	52.0	93.9
30	17	72.9	54.8	94.6
30	18	74.7	56.1	96.2
30	19	76.5	57.5	98.6
30	20	78.3	58.9	101.0
30	21	80.2	60.3	103.6
30	22	82.1	63.3	105.0
30	23	84.0	64.8	106.4
30	24	85.9	66.2	109.0
30	25	87.8	67.7	111.4
30	26	89.7	69.2	112.9
30	27	91.7	70.7	115.1
30	28	93.6	74.1	117.5
30	29	95.6	75.6	119.2
30	30	97.6	77.2	121.8
30	31	99.6	78.8	124.2
30	32	101.6	80.4	126.0
30	33	103.7	82.0	128.2
30	34	105.7	83.6	130.6
30	35	107.8	87.4	132.1
30	36	109.9	89.1	135.0
30	37	112.0	88.6	137.0
30	38	114.2	92.5	138.7
30	39	116.3	94.3	141.6
30	40	118.5	96.0	144.0
30	41	120.6	97.8	145.9
30	42	122.8	99.6	148.2
30	43	125.1	101.4	150.3
30	44	127.3	105.7	152.6

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
5	45	54.6	43.2	67.8
5	46	55.8	44.1	69.0
5	47	56.9	45.0	70.4
5	48	58.1	45.9	71.7
6	0	6.3	2.9	13.7
6	1	7.4	3.2	14.4
6	2	8.4	3.7	15.3
6	3	9.5	4.4	16.9
6	4	10.6	5.3	18.5
6	5	11.6	6.0	20.1
6	6	12.7	6.8	21.7
6	7	13.8	7.7	22.2
6	8	14.9	8.6	23.6
6	9	16.0	9.5	25.1
6	10	17.0	10.5	26.8
6	11	18.1	11.1	27.5
6	12	19.2	11.8	28.8
6	13	20.3	12.5	30.4
6	14	21.4	13.6	31.8
6	15	22.5	14.7	32.6
6	16	23.6	15.9	34.2
6	17	24.7	16.6	35.2
6	18	25.8	17.4	36.5
6	19	26.9	18.1	37.8
6	20	28.0	19.4	39.1
6	21	29.1	20.2	40.2
6	22	30.2	20.9	41.6
6	23	31.3	22.3	42.7
6	24	32.4	23.1	44.2
6	25	33.5	23.9	45.2
6	26	34.7	24.7	46.5
6	27	35.8	26.2	47.9
6	28	36.9	27.0	48.8
6	29	38.0	27.9	50.3
6	30	39.2	28.7	51.2
6	31	40.3	29.5	52.8
6	32	41.4	31.1	53.8
6	33	42.6	32.0	55.4
6	34	43.7	32.9	56.2
6	35	44.8	33.7	57.6
6	36	46.0	35.5	58.9
6	37	47.1	36.4	60.1
6	38	48.3	37.2	61.3
6	39	49.4	38.1	62.5
6	40	50.6	39.0	63.6
6	41	51.7	39.9	64.8
6	42	52.9	40.8	66.1
6	43	54.1	42.8	67.5
6	44	55.2	43.7	68.7

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
30	45	129.5	107.5	155.5
30	46	131.8	109.4	157.6
30	47	134.1	111.3	159.8
30	48	136.4	113.2	162.7
31	0	46.2	31.1	65.5
31	1	47.9	32.3	67.5
31	2	49.5	34.4	69.3
31	3	51.2	35.5	71.7
31	4	52.9	36.7	73.7
31	5	54.6	37.9	75.6
31	6	56.3	39.1	77.6
31	7	58.1	41.4	79.5
31	8	59.8	42.6	81.4
31	9	61.6	43.9	83.3
31	10	63.3	46.4	85.3
31	11	65.1	47.7	87.3
31	12	66.9	49.0	89.5
31	13	68.7	50.3	91.6
31	14	70.5	51.7	93.9
31	15	72.4	53.0	94.6
31	16	74.2	55.8	96.2
31	17	76.1	57.2	98.5
31	18	78.0	58.6	101.0
31	19	79.9	60.1	103.6
31	20	81.8	61.5	105.7
31	21	83.7	63.0	107.0
31	22	85.7	66.1	109.1
31	23	87.6	67.6	111.8
31	24	89.6	69.1	113.8
31	25	91.6	70.7	115.4
31	26	93.6	72.2	118.0
31	27	95.6	73.8	120.4
31	28	97.7	75.4	121.9
31	29	99.7	78.9	124.7
31	30	101.8	80.5	127.1
31	31	103.9	82.2	128.9
31	32	106.0	83.9	131.5
31	33	108.2	85.6	133.7
31	34	110.3	87.3	135.3
31	35	112.5	89.0	138.7
31	36	114.7	93.0	140.4
31	37	116.9	94.7	142.7
31	38	119.1	96.5	145.4
31	39	121.4	98.4	147.9
31	40	123.6	100.2	150.0
31	41	125.9	102.1	152.3
31	42	128.2	103.9	154.7
31	43	130.5	105.8	156.6
31	44	132.9	110.3	159.8

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
6	45	56.4	44.6	69.8
6	46	57.6	45.5	71.0
6	47	58.7	46.5	72.4
6	48	59.9	47.4	73.7
7	0	7.5	3.6	14.9
7	1	8.5	3.9	15.6
7	2	9.6	4.4	16.9
7	3	10.7	5.1	18.5
7	4	11.8	6.1	20.1
7	5	12.8	6.9	21.7
7	6	13.9	7.8	22.9
7	7	15.0	8.7	24.0
7	8	16.1	9.6	25.3
7	9	17.2	10.6	26.9
7	10	18.3	11.6	28.2
7	11	19.4	12.3	29.3
7	12	20.5	12.6	30.5
7	13	21.6	13.7	31.9
7	14	22.7	14.9	33.1
7	15	23.8	15.6	34.5
7	16	24.9	16.8	35.7
7	17	26.0	17.5	37.1
7	18	27.1	18.3	38.1
7	19	28.3	19.6	39.7
7	20	29.4	20.4	40.6
7	21	30.5	21.2	42.0
7	22	31.6	21.9	43.4
7	23	32.8	23.4	44.4
7	24	33.9	24.2	45.7
7	25	35.0	25.0	47.2
7	26	36.2	26.5	48.3
7	27	37.3	27.3	49.5
7	28	38.4	28.2	51.0
7	29	39.6	29.0	52.0
7	30	40.7	30.6	53.5
7	31	41.9	31.5	54.5
7	32	43.0	32.4	55.9
7	33	44.2	33.2	57.1
7	34	45.3	34.1	58.3
7	35	46.5	35.0	59.7
7	36	47.7	36.8	60.8
7	37	48.8	37.7	61.9
7	38	50.0	38.6	63.4
7	39	51.2	39.5	64.7
7	40	52.3	40.4	66.0
7	41	53.5	41.3	67.3
7	42	54.7	43.3	68.5
7	43	55.9	44.2	69.7
7	44	57.1	45.1	70.8

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
31	45	135.3	112.3	162.4
31	46	137.7	114.3	163.9
31	47	140.1	116.3	167.8
31	48	142.5	118.3	168.8
32	0	48.7	32.8	69.0
32	1	50.4	33.9	70.9
32	2	52.1	36.1	73.0
32	3	53.8	37.3	75.0
32	4	55.6	38.5	77.2
32	5	57.3	39.7	79.1
32	6	59.1	42.1	81.2
32	7	60.9	43.4	83.3
32	8	62.7	44.7	85.3
32	9	64.5	46.0	87.3
32	10	66.3	48.6	89.5
32	11	68.2	49.9	91.6
32	12	70.0	51.3	93.9
32	13	71.9	52.6	94.6
32	14	73.8	54.0	96.2
32	15	75.7	56.9	98.5
32	16	77.6	58.4	101.0
32	17	79.5	59.8	103.6
32	18	81.5	61.3	106.2
32	19	83.5	62.8	108.0
32	20	85.4	64.3	109.4
32	21	87.5	65.8	111.8
32	22	89.5	69.0	114.7
32	23	91.5	70.6	116.4
32	24	93.6	72.2	118.3
32	25	95.7	73.8	121.2
32	26	97.8	75.4	123.1
32	27	99.9	77.0	125.1
32	28	102.0	78.7	127.6
32	29	104.2	82.4	130.5
32	30	106.3	84.1	132.1
32	31	108.5	85.8	135.0
32	32	110.7	87.6	137.0
32	33	113.0	89.4	139.5
32	34	115.2	91.1	142.1
32	35	117.5	95.2	144.2
32	36	119.8	94.8	146.2
32	37	122.1	99.0	148.9
32	38	124.5	100.9	151.7
32	39	126.8	102.8	154.0
32	40	129.2	104.7	156.2
32	41	131.6	106.7	159.5
32	42	134.0	111.3	161.3
32	43	136.5	113.3	163.7
32	44	139.0	115.4	167.2

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
7	45	58.3	46.1	72.2
7	46	59.4	47.0	73.4
7	47	60.6	48.0	74.4
7	48	61.8	48.9	75.9
8	0	8.6	4.5	16.9
8	1	9.7	4.5	17.2
8	2	10.8	5.2	18.6
8	3	11.9	5.9	20.2
8	4	13.0	6.7	21.8
8	5	14.1	7.8	23.4
8	6	15.2	8.7	24.6
8	7	16.3	9.7	25.7
8	8	17.4	10.4	26.9
8	9	18.5	11.4	28.6
8	10	19.6	12.4	30.0
8	11	20.7	13.1	30.7
8	12	21.8	13.9	32.3
8	13	22.9	14.6	33.8
8	14	24.1	15.7	35.0
8	15	25.2	17.0	36.1
8	16	26.3	17.7	37.7
8	17	27.4	18.5	38.5
8	18	28.6	19.2	40.2
8	19	29.7	20.6	41.3
8	20	30.8	21.4	42.7
8	21	32.0	22.2	44.0
8	22	33.1	23.6	44.9
8	23	34.3	24.4	46.5
8	24	35.4	25.2	47.8
8	25	36.6	26.1	48.8
8	26	37.7	27.6	50.3
8	27	38.9	28.5	51.2
8	28	40.0	29.3	52.8
8	29	41.2	30.2	53.8
8	30	42.3	31.8	55.4
8	31	43.5	32.7	56.4
8	32	44.7	33.6	58.0
8	33	45.9	34.5	59.0
8	34	47.0	35.4	60.2
8	35	48.2	36.3	61.6
8	36	49.4	38.1	63.0
8	37	50.6	39.0	64.2
8	38	51.8	39.9	65.4
8	39	53.0	40.9	66.6
8	40	54.1	41.8	67.8
8	41	55.3	43.8	69.1
8	42	56.5	44.7	70.4
8	43	57.7	45.7	71.7
8	44	59.0	46.6	73.0

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
32	45	141.5	117.4	168.3
32	46	144.0	119.5	172.4
32	47	146.6	121.7	174.4
32	48	149.1	123.8	177.0
33	0	51.2	34.5	72.3
33	1	53.0	36.7	74.3
33	2	54.8	38.0	76.4
33	3	56.5	39.2	78.6
33	4	58.3	40.5	80.6
33	5	60.2	42.9	83.0
33	6	62.0	44.2	85.1
33	7	63.8	45.5	87.2
33	8	65.7	46.8	89.2
33	9	67.6	49.5	91.6
33	10	69.5	50.9	93.9
33	11	71.4	52.3	94.6
33	12	73.3	53.7	96.2
33	13	75.2	55.1	98.5
33	14	77.2	58.1	101.0
33	15	79.2	59.5	103.6
33	16	81.2	61.0	106.2
33	17	83.2	62.6	108.3
33	18	85.2	64.1	110.3
33	19	87.3	65.6	112.2
33	20	89.3	67.2	114.9
33	21	91.4	70.5	117.5
33	22	93.6	72.2	119.2
33	23	95.7	73.8	121.8
33	24	97.8	75.5	124.2
33	25	100.0	77.2	126.0
33	26	102.2	78.8	128.9
33	27	104.4	80.6	130.6
33	28	106.6	84.4	133.7
33	29	108.9	86.1	135.3
33	30	111.2	88.0	138.7
33	31	113.5	89.8	140.5
33	32	115.8	91.6	143.0
33	33	118.2	95.8	145.9
33	34	120.5	95.3	148.2
33	35	122.9	99.6	150.9
33	36	125.4	101.6	153.0
33	37	127.8	103.6	155.9
33	38	130.3	105.6	158.7
33	39	132.8	107.6	160.7
33	40	135.3	109.7	163.7
33	41	137.8	111.7	167.2
33	42	140.4	113.8	168.3
33	43	143.0	118.7	172.4
33	44	145.6	120.9	174.4

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
8	45	60.2	47.6	74.3
8	46	61.4	48.5	75.7
8	47	62.6	49.5	77.0
8	48	63.8	50.5	77.9
9	0	9.8	4.7	18.4
9	1	10.9	5.6	19.5
9	2	12.0	6.0	20.3
9	3	13.1	6.8	21.8
9	4	14.2	7.6	23.4
9	5	15.3	8.5	25.1
9	6	16.4	9.5	26.4
9	7	17.6	10.5	27.0
9	8	18.7	11.5	28.6
9	9	19.8	12.6	30.4
9	10	20.9	13.3	31.2
9	11	22.0	14.0	32.6
9	12	23.2	14.7	34.2
9	13	24.3	15.9	35.2
9	14	25.4	17.1	36.5
9	15	26.6	17.9	38.1
9	16	27.7	18.7	39.2
9	17	28.9	19.5	40.6
9	18	30.0	20.8	41.8
9	19	31.2	21.6	43.2
9	20	32.3	22.4	44.4
9	21	33.5	23.2	45.7
9	22	34.6	24.7	47.2
9	23	35.8	25.5	48.0
9	24	37.0	26.3	49.5
9	25	38.1	27.9	51.0
9	26	39.3	28.8	52.0
9	27	40.5	29.6	53.7
9	28	41.6	30.5	54.5
9	29	42.8	32.2	56.1
9	30	44.0	33.1	57.1
9	31	45.2	34.0	58.6
9	32	46.4	34.9	59.8
9	33	47.6	35.8	61.2
9	34	48.8	36.7	62.5
9	35	50.0	38.6	63.6
9	36	51.2	39.5	64.7
9	37	52.4	40.4	66.1
9	38	53.6	41.3	67.5
9	39	54.8	42.3	69.0
9	40	56.0	44.3	70.3
9	41	57.2	45.3	71.5
9	42	58.4	46.2	72.5
9	43	59.7	47.2	74.0
9	44	60.9	48.2	75.4

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
33	45	148.3	123.1	177.0
33	46	150.9	125.3	180.8
33	47	153.7	127.5	182.1
33	48	156.4	129.8	186.4
34	0	53.9	37.4	75.8
34	1	55.7	38.6	77.9
34	2	57.6	39.9	80.0
34	3	59.4	41.2	82.2
34	4	61.3	43.7	84.6
34	5	63.1	45.0	86.9
34	6	65.0	46.4	89.2
34	7	67.0	47.7	91.5
34	8	68.9	50.5	93.8
34	9	70.8	50.5	94.6
34	10	72.8	51.9	96.2
34	11	74.8	54.8	98.5
34	12	76.8	56.2	101.0
34	13	78.8	57.7	103.6
34	14	80.8	60.8	106.2
34	15	82.9	62.3	108.8
34	16	85.0	63.9	111.1
34	17	87.1	65.5	112.8
34	18	89.2	67.1	115.1
34	19	91.4	68.7	118.0
34	20	93.5	70.3	120.4
34	21	95.7	73.8	121.9
34	22	97.9	75.5	125.0
34	23	100.2	77.3	127.3
34	24	102.4	79.0	129.4
34	25	104.7	80.8	132.1
34	26	107.0	84.6	135.0
34	27	109.3	84.3	137.0
34	28	111.7	86.1	139.5
34	29	114.0	90.2	142.2
34	30	116.4	92.1	145.0
34	31	118.9	94.0	147.9
34	32	121.3	96.0	150.0
34	33	123.8	97.9	152.6
34	34	126.3	102.4	155.5
34	35	128.8	101.9	158.2
34	36	131.4	106.5	160.1
34	37	134.0	108.6	163.7
34	38	136.6	110.7	166.5
34	39	139.2	112.9	168.0
34	40	141.9	115.0	172.4
34	41	144.6	117.2	174.4
34	42	147.4	119.4	177.0
34	43	150.1	124.6	180.8
34	44	152.9	126.9	182.1

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
9	45	62.1	49.1	76.4
9	46	63.4	51.4	77.8
9	47	64.6	51.1	79.3
9	48	65.8	53.4	80.6
10	0	11.0	5.7	20.1
10	1	12.1	6.5	21.1
10	2	13.2	7.1	22.0
10	3	14.4	7.7	23.6
10	4	15.5	8.6	25.1
10	5	16.6	9.6	26.8
10	6	17.7	10.6	27.5
10	7	18.9	11.6	28.8
10	8	20.0	12.7	30.4
10	9	21.1	13.4	31.8
10	10	22.3	14.1	33.1
10	11	23.4	14.9	34.5
10	12	24.6	16.1	35.7
10	13	25.7	16.8	37.1
10	14	26.9	18.1	38.5
10	15	28.0	18.9	39.7
10	16	29.2	19.7	41.2
10	17	30.3	21.0	42.3
10	18	31.5	21.8	43.4
10	19	32.7	23.3	44.9
10	20	33.8	23.5	46.5
10	21	35.0	25.0	47.5
10	22	36.2	25.8	48.8
10	23	37.4	26.6	50.3
10	24	38.6	28.2	51.2
10	25	39.7	29.1	52.8
10	26	40.9	30.0	54.1
10	27	42.1	30.9	55.4
10	28	43.3	31.7	56.8
10	29	44.5	33.5	58.0
10	30	45.7	34.4	59.1
10	31	46.9	35.3	60.7
10	32	48.1	36.2	61.8
10	33	49.3	37.1	63.0
10	34	50.6	39.0	64.5
10	35	51.8	40.0	66.0
10	36	53.0	40.9	67.3
10	37	54.2	41.8	68.5
10	38	55.5	42.8	69.7
10	39	56.7	43.7	71.0
10	40	57.9	45.8	72.3
10	41	59.2	46.8	73.7
10	42	60.4	47.8	75.0
10	43	61.7	48.8	76.3
10	44	62.9	49.8	77.6

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
34	45	155.7	129.3	187.0
34	46	158.6	131.6	188.5
34	47	161.5	134.0	192.5
34	48	164.4	136.5	194.9
35	0	56.8	39.4	79.5
35	1	58.6	40.7	81.5
35	2	60.5	42.0	83.7
35	3	62.4	43.3	86.2
35	4	64.4	44.6	88.6
35	5	66.3	47.3	90.4
35	6	68.3	48.7	93.0
35	7	70.3	50.1	94.6
35	8	72.3	51.5	96.4
35	9	74.3	53.0	98.8
35	10	76.3	55.9	101.2
35	11	78.4	57.4	103.6
35	12	80.5	59.0	106.2
35	13	82.6	60.5	109.0
35	14	84.7	62.1	111.4
35	15	86.9	65.3	113.8
35	16	89.1	67.0	115.4
35	17	91.3	68.6	118.3
35	18	93.5	70.3	121.2
35	19	95.7	72.0	123.1
35	20	98.0	73.7	126.0
35	21	100.3	75.4	128.2
35	22	102.6	79.2	130.6
35	23	105.0	81.0	133.7
35	24	107.3	82.8	135.3
35	25	109.7	84.7	138.7
35	26	112.2	86.5	140.7
35	27	114.6	88.4	144.0
35	28	117.1	92.6	146.2
35	29	119.6	94.6	148.9
35	30	122.2	96.6	152.0
35	31	124.7	98.7	154.7
35	32	127.3	100.7	157.0
35	33	129.9	102.8	159.9
35	34	132.6	107.5	163.6
35	35	135.3	109.7	165.7
35	36	138.0	111.9	168.0
35	37	140.8	114.1	172.4
35	38	143.6	116.4	174.4
35	39	146.4	118.6	177.0
35	40	149.2	121.0	181.4
35	41	152.1	123.3	182.9
35	42	155.0	125.7	187.3
35	43	158.0	128.1	190.0
35	44	161.0	130.5	192.8

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
10	45	64.2	50.7	79.2
10	46	65.4	53.0	80.0
10	47	66.7	54.0	81.4
10	48	67.9	55.1	83.2
11	0	12.2	6.8	21.4
11	1	13.4	7.4	22.3
11	2	14.5	7.8	23.6
11	3	15.6	8.7	25.3
11	4	16.8	9.7	26.8
11	5	17.9	10.7	28.2
11	6	19.1	11.4	29.3
11	7	20.2	12.4	30.7
11	8	21.4	13.6	32.3
11	9	22.5	14.3	33.8
11	10	23.7	15.5	35.0
11	11	24.8	15.8	36.1
11	12	26.0	17.0	37.7
11	13	27.2	18.3	39.1
11	14	28.3	19.1	40.2
11	15	29.5	19.9	41.6
11	16	30.7	20.7	42.7
11	17	31.9	22.1	44.2
11	18	33.0	23.6	45.7
11	19	34.2	23.7	46.8
11	20	35.4	25.2	48.0
11	21	36.6	26.1	49.5
11	22	37.8	26.9	51.0
11	23	39.0	28.6	52.0
11	24	40.2	29.4	53.7
11	25	41.4	30.3	54.6
11	26	42.6	31.2	56.2
11	27	43.8	32.1	57.4
11	28	45.0	33.9	58.9
11	29	46.3	34.8	60.1
11	30	47.5	35.7	61.3
11	31	48.7	36.6	62.6
11	32	49.9	38.5	64.2
11	33	51.2	39.5	65.4
11	34	52.4	40.4	66.6
11	35	53.7	41.4	67.8
11	36	54.9	42.4	69.2
11	37	56.1	43.3	70.7
11	38	57.4	45.4	72.2
11	39	58.6	45.2	73.4
11	40	59.9	47.4	74.5
11	41	61.2	48.4	75.9
11	42	62.4	49.4	77.6
11	43	63.7	50.4	78.6
11	44	65.0	51.4	80.0

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
35	45	164.0	136.1	195.8
35	46	167.1	138.7	198.9
35	47	170.2	141.3	202.9
35	48	173.3	143.9	205.9
36	0	59.8	41.4	83.4
36	1	61.7	42.8	85.4
36	2	63.7	44.1	87.8
36	3	65.7	45.5	90.3
36	4	67.7	46.9	92.7
36	5	69.7	49.7	95.3
36	6	71.7	51.1	97.5
36	7	73.8	52.6	99.5
36	8	75.9	54.1	101.4
36	9	78.0	55.6	103.8
36	10	80.1	58.7	106.4
36	11	82.3	60.3	109.1
36	12	84.5	61.9	111.8
36	13	86.7	63.5	114.7
36	14	88.9	66.9	116.4
36	15	91.2	68.6	119.2
36	16	93.5	70.3	121.8
36	17	95.8	72.0	124.2
36	18	98.1	73.8	127.1
36	19	100.5	75.5	129.1
36	20	102.9	77.3	132.1
36	21	105.3	81.2	135.0
36	22	107.7	83.1	137.0
36	23	110.2	85.0	140.4
36	24	112.7	87.0	142.7
36	25	115.2	88.9	145.4
36	26	117.8	93.2	148.2
36	27	120.4	95.2	151.7
36	28	123.0	94.9	154.0
36	29	125.7	99.4	156.6
36	30	128.4	101.6	159.8
36	31	131.1	103.7	163.1
36	32	133.9	105.9	165.2
36	33	136.7	110.8	168.0
36	34	139.5	110.4	172.4
36	35	142.4	115.4	174.4
36	36	145.3	117.8	177.0
36	37	148.3	120.2	181.9
36	38	151.3	122.6	183.1
36	39	154.3	125.1	187.3
36	40	157.3	127.5	190.0
36	41	160.5	130.1	193.1
36	42	163.6	135.8	197.7
36	43	166.8	138.4	200.4
36	44	170.0	141.1	203.5

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
11	45	66.3	52.4	81.4
11	46	67.5	54.7	83.0
11	47	68.8	55.8	83.7
11	48	70.1	56.8	85.3
12	0	13.5	7.8	23.4
12	1	14.6	8.2	24.6
12	2	15.8	8.8	25.7
12	3	16.9	9.4	27.0
12	4	18.1	10.4	28.6
12	5	19.3	11.5	30.0
12	6	20.4	12.6	31.2
12	7	21.6	13.7	32.6
12	8	22.8	14.9	34.2
12	9	23.9	15.7	35.2
12	10	25.1	15.9	36.5
12	11	26.3	17.2	38.1
12	12	27.5	18.0	39.2
12	13	28.6	19.3	40.6
12	14	29.8	20.1	42.0
12	15	31.0	21.5	43.4
12	16	32.2	22.3	44.9
12	17	33.4	23.2	46.2
12	18	34.6	24.7	47.2
12	19	35.8	24.8	48.8
12	20	37.0	26.4	50.3
12	21	38.2	27.3	51.2
12	22	39.5	28.9	52.8
12	23	40.7	29.8	54.4
12	24	41.9	30.7	55.4
12	25	43.1	31.6	57.0
12	26	44.3	32.5	58.0
12	27	45.6	33.4	59.7
12	28	46.8	35.2	60.8
12	29	48.1	36.1	62.5
12	30	49.3	37.1	63.6
12	31	50.6	38.0	64.7
12	32	51.8	40.0	66.1
12	33	53.1	40.9	67.7
12	34	54.3	41.9	69.0
12	35	55.6	42.9	70.4
12	36	56.8	43.9	71.7
12	37	58.1	44.8	73.0
12	38	59.4	47.0	74.4
12	39	60.7	48.0	75.8
12	40	62.0	49.0	77.2
12	41	63.2	50.0	78.6
12	42	64.5	51.0	79.5
12	43	65.8	52.1	81.4
12	44	67.1	53.1	82.7

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
36	45	173.3	143.8	206.2
36	46	176.6	146.6	210.2
36	47	179.9	149.4	213.8
36	48	183.3	152.2	217.5
37	0	62.9	43.6	87.4
37	1	65.0	45.0	89.6
37	2	67.0	46.5	92.0
37	3	69.1	47.9	95.6
37	4	71.2	50.7	98.3
37	5	73.3	52.2	100.1
37	6	75.4	53.8	102.6
37	7	77.6	55.3	104.5
37	8	79.8	56.9	107.0
37	9	82.0	60.0	109.4
37	10	84.2	61.7	112.2
37	11	86.5	63.3	114.9
37	12	88.8	65.0	117.5
37	13	91.1	66.7	120.2
37	14	93.4	70.3	121.9
37	15	95.8	72.0	125.0
37	16	98.2	73.8	127.6
37	17	100.6	75.7	130.6
37	18	103.1	77.5	133.7
37	19	105.6	79.4	135.3
37	20	108.1	83.4	138.7
37	21	110.7	83.2	142.1
37	22	113.3	87.4	144.2
37	23	115.9	89.4	147.9
37	24	118.6	91.5	150.0
37	25	121.3	93.6	152.6
37	26	124.0	95.7	155.9
37	27	126.8	97.8	159.5
37	28	129.6	102.5	162.7
37	29	132.4	104.8	164.6
37	30	135.3	107.0	168.0
37	31	138.2	109.3	172.4
37	32	141.2	111.7	174.4
37	33	144.2	114.1	177.0
37	34	147.3	116.5	181.9
37	35	150.3	118.9	184.2
37	36	153.5	124.4	187.3
37	37	156.7	127.0	191.9
37	38	159.9	129.6	194.0
37	39	163.1	132.2	198.0
37	40	166.5	134.9	200.4
37	41	169.8	137.6	205.3
37	42	173.2	140.4	209.3
37	43	176.7	146.7	212.3
37	44	180.2	149.6	215.7

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
12	45	68.4	54.1	83.7
12	46	69.7	56.5	85.3
12	47	71.0	57.6	86.9
12	48	72.4	58.6	87.7
13	0	14.8	8.5	25.1
13	1	16.0	9.2	26.4
13	2	17.1	9.9	27.4
13	3	18.3	10.5	28.8
13	4	19.5	11.6	30.4
13	5	20.6	12.7	31.8
13	6	21.8	13.4	33.1
13	7	23.0	14.6	34.5
13	8	24.2	15.8	35.7
13	9	25.4	16.6	37.1
13	10	26.6	17.4	38.5
13	11	27.8	18.2	39.9
13	12	29.0	19.5	41.3
13	13	30.2	20.3	42.7
13	14	31.4	21.8	44.2
13	15	32.6	22.6	45.6
13	16	33.8	23.4	46.6
13	17	35.0	25.0	48.0
13	18	36.2	25.8	49.5
13	19	37.5	26.7	51.0
13	20	38.7	27.6	52.0
13	21	39.9	28.5	53.7
13	22	41.2	30.1	54.7
13	23	42.4	31.1	56.2
13	24	43.6	32.0	57.6
13	25	44.9	32.9	58.9
13	26	46.1	34.7	60.2
13	27	47.4	35.6	61.6
13	28	48.6	36.6	63.0
13	29	49.9	37.5	64.4
13	30	51.2	38.5	66.0
13	31	52.5	39.4	67.3
13	32	53.7	41.4	68.5
13	33	55.0	42.4	69.7
13	34	56.3	43.4	71.0
13	35	57.6	44.4	72.4
13	36	58.9	45.4	74.0
13	37	60.2	46.4	75.7
13	38	61.5	48.6	76.9
13	39	62.8	49.6	77.9
13	40	64.1	50.7	79.5
13	41	65.4	51.7	81.2
13	42	66.7	52.8	82.3
13	43	68.0	53.8	83.4
13	44	69.3	54.9	85.3

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
37	45	183.7	152.5	219.3
37	46	187.3	155.5	222.7
37	47	191.0	158.5	227.0
37	48	194.7	161.6	229.7
38	0	66.3	46.0	91.7
38	1	68.4	47.5	96.2
38	2	70.6	48.9	98.5
38	3	72.7	51.9	101.0
38	4	74.9	53.4	102.9
38	5	77.1	55.0	105.7
38	6	79.4	56.6	107.7
38	7	81.6	58.2	110.3
38	8	83.9	59.8	112.8
38	9	86.2	63.2	115.4
38	10	88.6	64.9	118.3
38	11	91.0	66.6	121.0
38	12	93.4	68.4	123.1
38	13	95.8	70.2	126.0
38	14	98.3	73.9	128.9
38	15	100.8	75.8	132.1
38	16	103.4	77.7	135.0
38	17	105.9	79.7	137.0
38	18	108.6	81.6	140.4
38	19	111.2	83.6	143.0
38	20	113.9	85.6	146.2
38	21	116.6	90.0	148.9
38	22	119.4	92.1	152.3
38	23	122.2	94.3	155.5
38	24	125.0	96.5	158.7
38	25	127.9	98.7	161.3
38	26	130.8	103.5	163.9
38	27	133.8	105.9	168.0
38	28	136.8	105.6	172.4
38	29	139.9	110.7	174.4
38	30	143.0	113.1	177.0
38	31	146.2	115.6	181.9
38	32	149.4	118.1	185.4
38	33	152.6	120.7	187.3
38	34	155.9	123.3	192.2
38	35	159.2	126.0	194.9
38	36	162.6	131.8	198.9
38	37	166.1	134.6	203.5
38	38	169.6	137.5	206.2
38	39	173.2	140.4	209.3
38	40	176.8	143.3	213.8
38	41	180.4	146.3	218.1
38	42	184.2	149.3	221.8
38	43	188.0	152.3	226.4
38	44	191.8	159.2	229.7

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
13	45	70.7	55.9	86.7
13	46	72.0	58.4	87.4
13	47	73.3	59.4	89.5
13	48	74.7	60.5	90.8
14	0	16.1	9.3	26.8
14	1	17.3	10.3	28.2
14	2	18.5	11.0	29.2
14	3	19.7	11.7	30.7
14	4	20.9	12.4	32.3
14	5	22.1	13.6	33.7
14	6	23.3	14.8	35.0
14	7	24.5	16.0	36.1
14	8	25.7	16.8	37.7
14	9	26.9	17.6	39.1
14	10	28.1	18.4	40.6
14	11	29.3	19.7	41.8
14	12	30.5	20.6	43.4
14	13	31.7	22.0	44.4
14	14	33.0	22.9	45.8
14	15	34.2	23.7	47.2
14	16	35.4	24.6	48.8
14	17	36.7	26.1	50.3
14	18	37.9	27.0	51.2
14	19	39.1	27.9	52.8
14	20	40.4	28.8	54.5
14	21	41.6	30.5	55.4
14	22	42.9	31.4	57.1
14	23	44.2	32.4	58.6
14	24	45.4	33.3	59.8
14	25	46.7	35.1	61.3
14	26	48.0	36.1	62.5
14	27	49.3	37.0	64.0
14	28	50.5	38.0	65.4
14	29	51.8	39.0	66.6
14	30	53.1	41.0	67.9
14	31	54.4	42.0	69.6
14	32	55.7	43.0	70.9
14	33	57.0	44.0	72.3
14	34	58.3	45.0	73.7
14	35	59.6	46.0	75.0
14	36	60.9	47.0	76.4
14	37	62.3	49.3	77.8
14	38	63.6	50.3	79.5
14	39	64.9	51.4	80.8
14	40	66.3	52.4	82.1
14	41	67.6	53.5	83.3
14	42	68.9	54.5	85.3
14	43	70.3	55.6	86.3
14	44	71.6	56.7	87.4

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
38	45	195.7	162.4	234.0
38	46	199.7	165.7	238.0
38	47	203.7	169.1	243.1
38	48	207.7	172.4	245.9
39	0	70.0	48.5	98.5
39	1	72.2	50.0	101.1
39	2	74.4	53.0	103.6
39	3	76.7	54.6	106.2
39	4	78.9	56.3	108.8
39	5	81.3	57.9	111.4
39	6	83.6	59.6	113.8
39	7	86.0	61.3	115.5
39	8	88.4	63.0	119.2
39	9	90.9	66.6	121.6
39	10	93.4	68.4	124.2
39	11	95.9	70.2	127.6
39	12	98.4	72.1	130.6
39	13	101.0	74.0	133.7
39	14	103.6	75.9	135.3
39	15	106.3	77.9	138.7
39	16	109.0	82.0	142.2
39	17	111.8	84.0	145.4
39	18	114.6	86.1	148.2
39	19	117.4	88.3	151.7
39	20	120.3	92.8	154.7
39	21	123.2	95.0	158.2
39	22	126.1	94.9	160.7
39	23	129.2	99.6	163.7
39	24	132.2	102.0	168.0
39	25	135.3	104.4	172.4
39	26	138.5	106.8	174.4
39	27	141.7	109.3	177.0
39	28	145.0	114.7	181.9
39	29	148.3	117.3	185.5
39	30	151.7	120.0	187.8
39	31	155.1	122.7	192.8
39	32	158.6	125.4	196.8
39	33	162.1	128.2	200.4
39	34	165.7	131.1	203.8
39	35	169.4	134.0	208.0
39	36	173.1	140.3	212.3
39	37	176.9	143.4	215.7
39	38	180.7	146.5	219.3
39	39	184.7	149.7	223.6
39	40	188.7	152.9	228.5
39	41	192.7	156.2	233.7
39	42	196.8	159.5	236.1
39	43	201.0	166.9	242.5
39	44	205.3	170.4	245.0

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
14	45	73.0	59.2	89.5
14	46	74.4	60.3	90.7
14	47	75.7	61.4	91.6
14	48	77.1	62.5	93.8
15	0	17.5	10.1	28.6
15	1	18.7	10.8	30.0
15	2	19.9	11.9	30.9
15	3	21.1	12.6	32.6
15	4	22.3	13.7	34.1
15	5	23.5	14.9	35.2
15	6	24.7	15.7	36.5
15	7	25.9	17.0	38.1
15	8	27.2	17.8	39.7
15	9	28.4	18.6	41.2
15	10	29.6	19.4	42.3
15	11	30.9	20.8	44.0
15	12	32.1	21.6	44.9
15	13	33.3	23.1	46.5
15	14	34.6	24.0	48.0
15	15	35.8	24.9	49.5
15	16	37.1	26.4	51.0
15	17	38.4	27.3	52.0
15	18	39.6	28.2	53.7
15	19	40.9	29.1	55.2
15	20	42.2	30.9	56.2
15	21	43.4	31.8	58.0
15	22	44.7	32.8	59.0
15	23	46.0	33.7	60.7
15	24	47.3	35.6	61.8
15	25	48.6	36.5	63.5
15	26	49.9	37.5	64.7
15	27	51.2	38.5	66.1
15	28	52.5	39.5	67.8
15	29	53.8	40.5	69.0
15	30	55.1	41.4	70.4
15	31	56.4	43.5	72.0
15	32	57.8	44.6	73.4
15	33	59.1	45.6	74.7
15	34	60.4	46.6	75.9
15	35	61.8	47.7	77.6
15	36	63.1	48.7	79.2
15	37	64.5	51.0	80.6
15	38	65.8	52.1	81.5
15	39	67.2	53.1	83.3
15	40	68.5	54.2	85.1
15	41	69.9	55.3	86.2
15	42	71.3	56.4	87.3
15	43	72.6	58.9	89.5
15	44	74.0	60.0	90.5

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
39	45	209.6	174.0	251.4
39	46	214.0	177.6	254.1
39	47	218.5	181.3	260.5
39	48	223.0	185.1	264.5
40	0	73.8	51.2	103.6
40	1	76.2	52.8	106.2
40	2	78.5	56.0	109.0
40	3	80.9	57.7	111.7
40	4	83.3	59.4	114.6
40	5	85.7	61.1	117.2
40	6	88.2	62.9	120.2
40	7	90.8	66.5	123.1
40	8	93.3	66.5	125.4
40	9	95.9	70.2	128.9
40	10	98.5	72.2	132.1
40	11	101.2	74.1	135.0
40	12	103.9	76.1	137.0
40	13	106.7	78.2	140.4
40	14	109.5	80.2	144.0
40	15	112.4	84.5	147.9
40	16	115.3	86.7	150.0
40	17	118.2	88.9	154.0
40	18	121.2	91.1	156.6
40	19	124.3	95.9	159.9
40	20	127.4	95.8	163.7
40	21	130.5	98.1	168.0
40	22	133.7	103.2	172.4
40	23	137.0	105.7	174.4
40	24	140.3	108.2	177.0
40	25	143.7	110.8	181.9
40	26	147.1	113.5	186.9
40	27	150.6	119.2	190.0
40	28	154.2	119.0	193.1
40	29	157.8	124.8	197.7
40	30	161.5	127.8	200.4
40	31	165.3	130.8	205.9
40	32	169.1	133.8	209.3
40	33	173.0	136.9	213.8
40	34	177.0	140.0	219.3
40	35	181.1	143.2	222.4
40	36	185.2	146.5	227.0
40	37	189.4	153.5	230.9
40	38	193.7	157.0	235.5
40	39	198.1	160.6	242.2
40	40	202.5	164.2	244.1
40	41	207.1	167.8	251.4
40	42	211.7	175.7	254.1
40	43	216.4	175.4	260.5
40	44	221.1	183.6	264.5

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
15	45	75.4	61.1	91.6
15	46	76.8	62.3	93.8
15	47	78.2	63.4	94.6
15	48	79.6	64.5	96.1
16	0	18.9	11.3	30.4
16	1	20.1	12.4	31.8
16	2	21.3	12.7	32.6
16	3	22.6	13.9	34.5
16	4	23.8	14.6	35.8
16	5	25.0	15.9	37.1
16	6	26.2	17.2	38.5
16	7	27.5	18.0	40.2
16	8	28.7	18.8	41.6
16	9	30.0	19.6	42.7
16	10	31.2	21.0	44.4
16	11	32.5	21.9	45.7
16	12	33.7	23.4	47.2
16	13	35.0	24.3	48.8
16	14	36.3	25.2	50.3
16	15	37.5	26.8	51.2
16	16	38.8	27.7	52.8
16	17	40.1	28.6	54.5
16	18	41.4	29.5	55.9
16	19	42.7	30.4	57.1
16	20	44.0	32.2	58.6
16	21	45.3	33.2	60.1
16	22	46.6	34.1	61.5
16	23	47.9	35.1	63.0
16	24	49.2	37.0	64.2
16	25	50.5	38.0	66.0
16	26	51.8	39.0	67.3
16	27	53.2	40.0	68.5
16	28	54.5	41.0	70.3
16	29	55.8	43.1	71.7
16	30	57.2	44.1	73.0
16	31	58.5	45.2	74.4
16	32	59.9	46.2	75.9
16	33	61.2	47.3	77.6
16	34	62.6	48.3	78.9
16	35	64.0	49.4	80.0
16	36	65.3	50.4	81.4
16	37	66.7	52.8	83.3
16	38	68.1	53.9	84.8
16	39	69.5	55.0	85.9
16	40	70.9	56.1	87.3
16	41	72.3	57.2	89.5
16	42	73.7	58.3	90.3
16	43	75.1	60.9	91.6
16	44	76.5	62.0	93.8

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
40	45	226.0	187.6	270.3
40	46	231.0	191.7	274.9
40	47	236.0	195.9	281.0
40	48	241.1	200.2	285.6
41	0	78.0	54.1	109.0
41	1	80.5	55.8	111.8
41	2	83.0	57.5	114.9
41	3	85.5	60.9	118.0
41	4	88.0	62.8	121.0
41	5	90.6	64.6	124.1
41	6	93.3	66.5	126.3
41	7	95.9	68.4	130.5
41	8	98.7	72.3	133.7
41	9	101.4	74.3	136.1
41	10	104.3	76.4	139.3
41	11	107.1	78.5	142.7
41	12	110.0	80.6	146.2
41	13	113.0	82.8	149.1
41	14	116.0	87.2	152.6
41	15	119.1	89.6	156.1
41	16	122.2	91.9	159.8
41	17	125.4	94.3	163.7
41	18	128.7	94.2	168.0
41	19	132.0	99.2	172.4
41	20	135.4	101.8	174.4
41	21	138.8	107.1	177.0
41	22	142.3	107.0	181.9
41	23	145.9	109.7	187.2
41	24	149.5	115.3	190.1
41	25	153.2	118.2	194.0
41	26	157.0	121.1	198.9
41	27	160.9	124.1	203.5
41	28	164.8	130.4	206.2
41	29	168.9	133.6	212.3
41	30	173.0	133.4	215.7
41	31	177.2	140.1	221.8
41	32	181.5	143.5	226.4
41	33	185.8	147.0	229.7
41	34	190.3	150.5	235.5
41	35	194.8	154.1	240.7
41	36	199.5	161.7	243.4
41	37	204.2	165.5	251.4
41	38	209.1	169.5	254.1
41	39	214.0	173.5	260.5
41	40	219.1	177.6	266.0
41	41	224.2	186.1	270.3
41	42	229.4	186.0	277.9
41	43	234.8	194.9	282.2
41	44	240.2	194.7	290.1

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
16	45	77.9	63.2	94.6
16	46	79.3	64.3	96.1
16	47	80.8	65.5	98.4
16	48	82.2	66.6	98.9
17	0	20.3	12.1	32.2
17	1	21.6	12.9	33.7
17	2	22.8	14.0	35.0
17	3	24.1	14.8	36.5
17	4	25.3	16.1	37.7
17	5	26.6	16.9	39.2
17	6	27.8	18.2	40.6
17	7	29.1	19.0	42.0
17	8	30.3	20.4	43.4
17	9	31.6	21.3	44.9
17	10	32.9	22.2	46.5
17	11	34.1	23.7	48.0
17	12	35.4	24.6	49.5
17	13	36.7	25.5	51.0
17	14	38.0	26.4	52.0
17	15	39.3	28.0	53.7
17	16	40.6	28.9	55.4
17	17	41.9	29.9	56.4
17	18	43.2	30.8	58.0
17	19	44.5	32.6	59.7
17	20	45.9	33.6	60.8
17	21	47.2	34.6	62.5
17	22	48.5	35.5	63.6
17	23	49.8	37.5	65.4
17	24	51.2	38.5	66.6
17	25	52.5	39.5	68.4
17	26	53.9	40.5	69.7
17	27	55.2	41.5	71.0
17	28	56.6	42.6	72.4
17	29	58.0	44.7	74.0
17	30	59.3	45.8	75.7
17	31	60.7	46.8	77.2
17	32	62.1	47.9	78.6
17	33	63.5	49.0	79.8
17	34	64.9	51.3	81.4
17	35	66.3	51.1	83.3
17	36	67.7	53.5	84.6
17	37	69.1	54.6	85.4
17	38	70.5	55.8	87.3
17	39	71.9	56.9	89.4
17	40	73.3	58.0	90.3
17	41	74.8	59.1	91.6
17	42	76.2	60.3	93.9
17	43	77.6	62.9	94.6
17	44	79.1	64.1	96.1

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
41	45	245.8	204.0	294.4
41	46	251.5	208.7	302.0
41	47	257.2	213.5	306.9
41	48	263.1	218.4	314.5
42	0	82.6	57.3	115.4
42	1	85.2	59.1	118.3
42	2	87.8	60.9	121.9
42	3	90.5	62.8	125.0
42	4	93.2	66.5	128.0
42	5	96.0	68.4	131.7
42	6	98.8	70.4	135.3
42	7	101.7	72.5	138.2
42	8	104.6	74.6	142.1
42	9	107.6	78.8	145.4
42	10	110.6	81.0	148.8
42	11	113.7	83.3	151.7
42	12	116.9	85.6	155.9
42	13	120.1	87.9	159.8
42	14	123.4	92.8	163.7
42	15	126.7	95.3	168.0
42	16	130.1	95.3	172.4
42	17	133.6	97.8	174.4
42	18	137.2	103.1	177.0
42	19	140.8	105.9	182.0
42	20	144.5	108.7	187.2
42	21	148.3	111.5	192.3
42	22	152.2	114.4	195.0
42	23	156.1	120.5	200.4
42	24	160.2	123.6	205.3
42	25	164.3	126.8	209.3
42	26	168.6	130.0	213.8
42	27	172.9	133.4	219.3
42	28	177.3	136.8	222.7
42	29	181.9	140.3	228.5
42	30	186.5	147.5	234.0
42	31	191.3	151.3	238.0
42	32	196.1	155.1	243.1
42	33	201.1	159.1	251.4
42	34	206.2	163.1	254.1
42	35	211.4	167.2	260.5
42	36	216.7	175.7	268.2
42	37	222.2	175.7	270.9
42	38	227.7	184.6	279.8
42	39	233.4	189.2	283.1
42	40	239.2	193.9	290.4
42	41	245.2	198.7	296.2
42	42	251.3	208.6	303.3
42	43	257.5	208.7	310.9
42	44	263.8	219.0	315.5

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
17	45	80.5	65.3	98.6
17	46	82.0	66.5	99.0
17	47	83.5	67.7	101.0
17	48	84.9	68.8	102.5
18	0	21.8	13.4	33.9
18	1	23.1	14.2	35.7
18	2	24.3	15.4	37.1
18	3	25.6	15.7	38.4
18	4	26.9	17.1	39.8
18	5	28.1	18.4	41.3
18	6	29.4	19.2	42.7
18	7	30.7	20.7	44.2
18	8	32.0	21.6	45.7
18	9	33.3	22.4	47.2
18	10	34.6	23.3	48.8
18	11	35.9	24.9	50.3
18	12	37.2	25.8	51.2
18	13	38.5	26.7	52.8
18	14	39.8	27.6	54.5
18	15	41.1	29.3	56.1
18	16	42.4	30.3	57.4
18	17	43.8	31.2	58.9
18	18	45.1	33.0	60.2
18	19	46.5	34.0	61.8
18	20	47.8	35.0	63.4
18	21	49.2	36.0	64.7
18	22	50.5	38.0	66.1
18	23	51.9	39.0	67.8
18	24	53.2	40.0	69.1
18	25	54.6	41.1	70.7
18	26	56.0	42.1	72.2
18	27	57.4	43.1	73.7
18	28	58.8	45.3	75.1
18	29	60.2	46.4	76.6
18	30	61.6	47.5	77.9
18	31	63.0	48.6	79.5
18	32	64.4	49.7	81.4
18	33	65.8	50.8	83.0
18	34	67.2	53.2	84.2
18	35	68.6	53.0	85.3
18	36	70.1	55.4	87.3
18	37	71.5	56.6	89.2
18	38	73.0	57.7	90.0
18	39	74.4	58.9	91.6
18	40	75.9	60.0	93.9
18	41	77.3	61.2	94.6
18	42	78.8	63.9	96.1
18	43	80.3	65.1	98.6
18	44	81.8	66.3	99.2

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
42	45	270.3	219.1	325.8
42	46	276.9	229.8	328.6
42	47	283.6	235.4	340.8
42	48	290.5	241.2	343.5
43	0	87.6	60.7	123.1
43	1	90.4	62.7	126.0
43	2	93.2	64.6	129.1
43	3	96.0	68.5	132.1
43	4	99.0	70.5	136.7
43	5	101.9	72.7	140.4
43	6	105.0	74.8	143.9
43	7	108.1	77.0	147.2
43	8	111.2	79.3	151.7
43	9	114.5	81.6	155.5
43	10	117.8	86.3	158.2
43	11	121.1	88.7	163.3
43	12	124.6	91.2	167.8
43	13	128.1	93.8	172.1
43	14	131.7	96.5	174.4
43	15	135.4	99.2	177.0
43	16	139.1	101.9	182.0
43	17	143.0	107.5	187.2
43	18	147.0	110.5	192.9
43	19	151.0	113.5	197.7
43	20	155.2	116.7	200.4
43	21	159.4	119.9	206.2
43	22	163.8	123.1	212.3
43	23	168.2	126.5	215.7
43	24	172.8	133.3	221.8
43	25	177.5	133.5	227.0
43	26	182.3	140.7	233.7
43	27	187.3	144.5	238.0
43	28	192.4	148.4	243.1
43	29	197.6	152.4	251.4
43	30	202.9	160.5	254.1
43	31	208.4	160.8	260.5
43	32	214.0	169.3	269.7
43	33	219.8	173.9	273.3
43	34	225.8	178.6	281.0
43	35	231.8	187.9	287.7
43	36	238.1	188.3	294.4
43	37	244.5	198.2	302.0
43	38	251.0	198.6	306.9
43	39	257.7	208.9	315.5
43	40	264.6	214.5	322.8
43	41	271.7	220.2	328.6
43	42	278.9	226.0	340.8
43	43	286.3	232.0	343.5
43	44	293.8	238.1	356.3

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
18	45	83.3	67.5	101.0
18	46	84.8	68.7	102.8
18	47	86.3	69.9	103.8
18	48	87.8	71.1	106.2
19	0	23.3	14.4	36.1
19	1	24.6	15.1	37.6
19	2	25.9	16.4	39.1
19	3	27.2	17.3	40.2
19	4	28.5	18.1	41.8
19	5	29.8	19.5	43.4
19	6	31.1	20.9	44.9
19	7	32.4	21.8	46.5
19	8	33.7	22.7	48.0
19	9	35.0	23.6	49.5
19	10	36.3	25.2	51.0
19	11	37.6	26.1	52.0
19	12	39.0	27.0	53.7
19	13	40.3	27.9	55.4
19	14	41.6	29.7	57.0
19	15	43.0	30.7	58.0
19	16	44.3	31.6	59.8
19	17	45.7	32.6	61.3
19	18	47.1	34.5	62.6
19	19	48.4	35.5	64.2
19	20	49.8	36.5	66.0
19	21	51.2	37.5	67.3
19	22	52.6	39.5	69.0
19	23	54.0	40.6	70.4
19	24	55.4	41.6	71.7
19	25	56.8	42.7	73.4
19	26	58.2	43.7	75.0
19	27	59.6	44.8	76.4
19	28	61.0	47.1	77.8
19	29	62.4	48.2	79.5
19	30	63.9	49.3	81.2
19	31	65.3	50.4	82.7
19	32	66.8	51.5	83.7
19	33	68.2	54.0	85.3
19	34	69.7	53.7	87.3
19	35	71.1	56.3	89.1
19	36	72.6	57.4	89.8
19	37	74.1	58.6	91.6
19	38	75.5	59.8	93.9
19	39	77.0	60.9	94.6
19	40	78.5	62.1	96.1
19	41	80.0	64.9	98.6
19	42	81.5	66.1	99.5
19	43	83.1	67.3	101.0
19	44	84.6	68.6	103.3

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
43	45	301.5	244.4	360.0
43	46	309.4	256.8	372.4
43	47	317.4	263.5	378.5
43	48	325.7	270.3	388.2
44	0	93.1	64.6	130.6
44	1	96.1	66.6	135.3
44	2	99.1	68.7	138.7
44	3	102.2	70.9	142.2
44	4	105.4	75.1	145.4
44	5	108.6	77.4	150.0
44	6	111.9	79.8	154.0
44	7	115.3	82.2	158.1
44	8	118.7	84.7	162.7
44	9	122.3	87.2	167.2
44	10	125.9	92.2	172.0
44	11	129.6	95.0	174.4
44	12	133.4	95.1	177.9
44	13	137.4	97.9	182.9
44	14	141.4	103.5	187.8
44	15	145.5	106.6	193.2
44	16	149.7	109.7	199.0
44	17	154.1	115.9	203.5
44	18	158.5	119.2	209.3
44	19	163.1	122.7	213.8
44	20	167.9	126.2	219.3
44	21	172.7	129.9	226.4
44	22	177.7	133.6	229.7
44	23	182.9	137.5	235.5
44	24	188.2	141.5	243.1
44	25	193.6	145.6	251.4
44	26	199.3	153.7	254.1
44	27	205.1	158.2	260.5
44	28	211.0	162.8	270.3
44	29	217.2	167.5	274.9
44	30	223.5	172.4	282.2
44	31	230.0	177.5	290.4
44	32	236.7	187.3	296.2
44	33	243.6	188.0	305.1
44	34	250.8	198.4	314.5
44	35	258.1	204.1	320.9
44	36	265.6	210.1	328.6
44	37	273.3	221.5	340.8
44	38	281.2	222.5	343.5
44	39	289.4	234.6	359.4
44	40	297.8	235.5	364.3
44	41	306.3	248.3	378.5
44	42	315.1	255.4	382.7
44	43	324.1	262.7	398.6
44	44	333.3	270.2	401.7

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
19	45	86.1	69.8	103.9
19	46	87.6	71.0	106.2
19	47	89.2	72.3	107.6
19	48	90.7	73.5	109.1
20	0	24.9	15.8	38.1
20	1	26.2	16.6	39.7
20	2	27.5	17.5	41.2
20	3	28.8	18.3	42.7
20	4	30.1	19.7	44.2
20	5	31.5	20.6	45.7
20	6	32.8	22.1	47.2
20	7	34.1	23.0	48.8
20	8	35.4	23.9	50.3
20	9	36.8	25.5	51.2
20	10	38.1	26.4	52.8
20	11	39.5	27.4	54.5
20	12	40.8	28.3	56.2
20	13	42.2	30.1	57.6
20	14	43.6	31.1	59.0
20	15	44.9	32.0	60.7
20	16	46.3	33.0	62.5
20	17	47.7	34.9	63.6
20	18	49.1	36.0	65.4
20	19	50.5	37.0	66.6
20	20	51.9	38.0	68.5
20	21	53.3	40.1	69.7
20	22	54.7	41.1	71.5
20	23	56.1	42.2	73.0
20	24	57.6	43.3	74.4
20	25	59.0	44.4	75.9
20	26	60.4	45.5	77.6
20	27	61.9	47.8	79.3
20	28	63.3	48.9	80.8
20	29	64.8	50.0	82.3
20	30	66.3	51.1	83.4
20	31	67.7	52.3	85.3
20	32	69.2	53.4	87.3
20	33	70.7	55.9	88.7
20	34	72.2	57.1	89.6
20	35	73.7	58.3	91.6
20	36	75.2	59.5	93.9
20	37	76.7	60.7	94.6
20	38	78.2	61.9	96.1
20	39	79.8	63.1	98.6
20	40	81.3	64.3	99.5
20	41	82.8	65.5	101.0
20	42	84.4	68.4	103.3
20	43	85.9	69.7	104.4
20	44	87.5	70.9	106.2

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
44	45	342.8	277.8	418.7
44	46	352.4	285.7	423.0
44	47	362.3	293.7	439.5
44	48	372.4	301.8	446.2
45	0	99.3	68.8	140.4
45	1	102.5	71.1	144.2
45	2	105.8	73.4	148.9
45	3	109.2	75.7	152.6
45	4	112.6	78.1	156.6
45	5	116.2	80.6	160.7
45	6	119.8	83.1	165.2
45	7	123.6	85.7	170.1
45	8	127.4	90.8	176.4
45	9	131.4	93.6	180.4
45	10	135.4	96.5	184.0
45	11	139.6	99.5	190.0
45	12	143.9	102.6	195.0
45	13	148.3	105.7	199.9
45	14	152.9	112.0	206.2
45	15	157.6	115.4	212.3
45	16	162.4	119.0	215.7
45	17	167.4	122.6	222.7
45	18	172.6	126.4	228.5
45	19	178.0	130.3	235.5
45	20	183.5	134.4	243.1
45	21	189.2	142.3	251.4
45	22	195.1	146.7	254.1
45	23	201.2	151.3	260.5
45	24	207.5	156.1	270.3
45	25	214.1	161.0	279.8
45	26	220.9	170.4	283.1
45	27	227.9	175.8	294.4
45	28	235.2	176.8	302.0
45	29	242.7	187.2	310.9
45	30	250.4	193.2	318.2
45	31	258.4	199.4	328.6
45	32	266.7	205.8	340.8
45	33	275.3	212.4	343.5
45	34	284.1	219.2	360.0
45	35	293.3	232.0	365.5
45	36	302.6	233.5	378.3
45	37	312.3	247.0	388.2
45	38	322.3	254.9	401.7
45	39	332.5	263.0	413.9
45	40	343.0	278.0	423.0
45	41	353.8	279.9	439.5
45	42	364.9	295.7	446.2
45	43	376.2	297.6	467.0
45	44	387.9	314.4	472.5

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
20	45	89.1	72.2	108.0
20	46	90.7	73.5	109.1
20	47	92.2	74.8	111.4
20	48	93.8	76.1	112.8
21	0	26.5	16.8	40.1
21	1	27.9	18.2	41.3
21	2	29.2	18.5	43.1
21	3	30.5	19.4	44.8
21	4	31.8	20.8	46.4
21	5	33.2	21.7	47.9
21	6	34.5	23.3	49.5
21	7	35.9	24.2	51.0
21	8	37.3	25.8	52.0
21	9	38.6	26.8	53.7
21	10	40.0	27.7	55.4
21	11	41.4	28.7	57.1
21	12	42.8	30.5	58.6
21	13	44.1	31.5	60.1
21	14	45.5	32.5	61.6
21	15	46.9	33.5	63.0
21	16	48.4	35.4	64.7
21	17	49.8	36.5	66.1
21	18	51.2	37.5	67.8
21	19	52.6	38.5	69.2
21	20	54.1	39.6	70.9
21	21	55.5	41.7	72.4
21	22	56.9	42.8	74.0
21	23	58.4	43.9	75.8
21	24	59.9	45.0	77.6
21	25	61.3	46.1	79.2
21	26	62.8	48.5	80.6
21	27	64.3	49.6	81.7
21	28	65.8	50.7	83.4
21	29	67.3	51.9	85.3
21	30	68.8	53.1	87.2
21	31	70.3	54.2	88.6
21	32	71.8	56.8	89.5
21	33	73.3	58.0	91.6
21	34	74.9	59.2	93.9
21	35	76.4	60.4	94.6
21	36	77.9	61.7	96.1
21	37	79.5	62.9	98.6
21	38	81.1	64.1	99.8
21	39	82.6	65.4	101.0
21	40	84.2	68.2	103.6
21	41	85.8	69.5	104.7
21	42	87.4	70.8	106.2
21	43	89.0	72.1	108.3
21	44	90.6	73.4	109.4

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
45	45	399.8	316.2	495.6
45	46	412.0	334.0	498.7
45	47	424.5	344.1	524.9
45	48	437.4	354.5	526.2
46	0	106.3	71.6	151.7
46	1	109.8	76.1	155.9
46	2	113.4	78.7	159.9
46	3	117.2	81.3	163.9
46	4	121.0	83.9	168.3
46	5	125.0	86.7	176.8
46	6	129.1	89.5	181.9
46	7	133.3	92.4	186.9
46	8	137.6	98.1	192.3
46	9	142.1	101.3	196.8
46	10	146.7	104.6	201.1
46	11	151.5	108.0	207.8
46	12	156.5	111.6	215.4
46	13	161.6	115.2	220.6
46	14	167.0	119.0	227.9
46	15	172.5	123.0	235.5
46	16	178.2	130.5	243.1
46	17	184.2	134.9	251.4
46	18	190.4	135.7	254.1
46	19	196.8	144.2	260.8
46	20	203.5	149.1	270.3
46	21	210.5	154.2	281.0
46	22	217.8	159.5	290.4
46	23	225.4	169.5	296.2
46	24	233.3	175.4	306.9
46	25	241.5	176.9	315.5
46	26	250.0	188.0	328.6
46	27	258.9	194.7	340.8
46	28	268.2	201.6	343.5
46	29	277.8	208.9	360.0
46	30	287.8	216.4	372.2
46	31	298.1	230.0	382.9
46	32	308.8	232.2	398.6
46	33	319.9	246.8	405.3
46	34	331.4	255.7	423.0
46	35	343.3	264.8	439.5
46	36	355.5	281.2	446.2
46	37	368.1	284.0	471.2
46	38	381.1	294.0	476.1
46	39	394.5	312.1	498.1
46	40	408.3	315.0	515.2
46	41	422.5	334.2	526.2
46	42	437.1	337.2	555.5
46	43	452.0	357.6	556.7
46	44	467.4	369.8	586.6

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
21	45	92.2	74.7	111.8
21	46	93.8	76.0	112.9
21	47	95.4	79.2	114.9
21	48	97.1	78.7	116.4
22	0	28.2	17.9	42.3
22	1	29.5	18.8	44.0
22	2	30.9	19.6	45.5
22	3	32.3	21.1	47.2
22	4	33.6	22.0	48.8
22	5	35.0	23.6	50.3
22	6	36.4	24.5	51.3
22	7	37.7	26.2	52.9
22	8	39.1	27.1	54.5
22	9	40.5	28.1	56.2
22	10	41.9	29.1	58.0
22	11	43.3	30.9	59.7
22	12	44.8	31.9	60.8
22	13	46.2	32.9	62.5
22	14	47.6	33.9	64.2
22	15	49.0	35.0	66.0
22	16	50.5	37.0	67.3
22	17	51.9	38.0	69.0
22	18	53.4	39.1	70.4
22	19	54.8	40.2	72.2
22	20	56.3	41.2	73.7
22	21	57.8	43.5	75.7
22	22	59.3	44.6	77.2
22	23	60.8	45.7	78.6
22	24	62.3	46.8	80.0
22	25	63.8	47.9	81.5
22	26	65.3	50.4	83.3
22	27	66.8	51.5	85.3
22	28	68.3	52.7	87.0
22	29	69.8	53.9	88.2
22	30	71.4	55.1	89.5
22	31	72.9	56.3	91.6
22	32	74.5	58.9	93.9
22	33	76.1	60.2	94.6
22	34	77.6	61.4	96.1
22	35	79.2	62.7	98.6
22	36	80.8	63.9	100.1
22	37	82.4	65.2	101.0
22	38	84.0	66.4	103.6
22	39	85.6	69.4	105.0
22	40	87.2	70.7	106.4
22	41	88.9	72.0	108.8
22	42	90.5	73.4	110.0
22	43	92.1	74.7	111.8
22	44	93.8	76.0	113.8

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
46	45	483.3	382.3	600.6
46	46	499.6	404.9	618.9
46	47	516.3	408.4	648.2
46	48	533.5	422.0	652.5
47	0	114.3	79.3	163.7
47	1	118.3	82.0	168.0
47	2	122.4	84.8	177.0
47	3	126.6	85.3	181.9
47	4	130.9	88.2	187.2
47	5	135.4	91.3	192.7
47	6	140.1	97.2	198.8
47	7	145.0	100.5	204.8
47	8	150.0	104.0	211.8
47	9	155.3	107.7	218.7
47	10	160.7	111.4	226.4
47	11	166.4	115.4	234.0
47	12	172.3	119.5	242.2
47	13	178.5	123.8	250.5
47	14	185.0	131.9	256.3
47	15	191.8	136.7	264.5
47	16	198.9	141.8	273.3
47	17	206.4	147.1	283.1
47	18	214.2	152.7	294.4
47	19	222.4	158.5	303.3
47	20	231.0	169.2	315.5
47	21	240.0	175.8	328.6
47	22	249.5	177.9	340.8
47	23	259.5	185.0	343.5
47	24	270.0	197.7	360.0
47	25	280.9	205.8	378.3
47	26	292.4	214.2	388.4
47	27	304.4	217.0	401.8
47	28	316.9	232.1	423.0
47	29	330.0	248.2	439.5
47	30	343.6	251.7	446.2
47	31	357.8	262.0	471.2
47	32	372.5	280.1	495.6
47	33	387.7	299.1	498.7
47	34	403.4	295.5	526.2
47	35	419.8	315.6	555.5
47	36	436.6	336.9	556.7
47	37	454.1	341.4	586.6
47	38	472.1	355.0	618.9
47	39	490.7	378.6	623.8
47	40	509.9	393.4	652.5
47	41	529.8	398.4	687.9
47	42	550.4	424.6	700.1
47	43	571.7	441.1	724.5
47	44	593.8	458.1	763.6

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
22	45	95.5	77.4	115.1
22	46	97.1	78.7	117.3
22	47	98.8	82.0	118.7
22	48	100.5	83.4	120.4
23	0	29.9	19.0	44.4
23	1	31.3	20.5	46.0
23	2	32.7	21.4	47.7
23	3	34.1	22.3	49.2
23	4	35.5	23.9	51.0
23	5	36.8	24.8	52.2
23	6	38.3	25.8	53.8
23	7	39.7	26.7	55.4
23	8	41.1	28.5	57.1
23	9	42.5	29.5	58.9
23	10	43.9	30.5	60.2
23	11	45.4	32.3	61.8
23	12	46.8	33.4	63.6
23	13	48.3	34.4	65.4
23	14	49.7	35.5	66.6
23	15	51.2	37.5	68.5
23	16	52.7	38.6	70.3
23	17	54.2	39.7	71.7
23	18	55.6	40.8	73.4
23	19	57.1	41.9	75.0
23	20	58.6	43.0	76.4
23	21	60.2	45.2	77.9
23	22	61.7	46.4	79.5
23	23	63.2	47.5	81.4
23	24	64.7	48.7	83.3
23	25	66.3	51.1	85.3
23	26	67.8	51.0	86.9
23	27	69.4	53.5	87.8
23	28	71.0	54.7	89.5
23	29	72.5	56.0	91.6
23	30	74.1	57.2	93.9
23	31	75.7	58.4	94.6
23	32	77.3	61.2	96.1
23	33	78.9	62.4	98.6
23	34	80.5	63.7	100.4
23	35	82.2	65.0	101.2
23	36	83.8	66.3	103.6
23	37	85.4	67.6	105.6
23	38	87.1	68.9	106.4
23	39	88.7	70.2	109.0
23	40	90.4	73.3	110.3
23	41	92.1	74.6	112.2
23	42	93.8	76.0	114.0
23	43	95.5	77.4	115.4
23	44	97.2	78.8	117.5

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
47	45	616.7	487.8	786.4
47	46	640.5	494.2	804.8
47	47	665.3	513.2	847.5
47	48	691.0	546.6	886.5
48	0	123.9	83.5	181.9
48	1	128.4	86.5	187.2
48	2	133.1	89.7	192.9
48	3	137.9	92.9	199.0
48	4	143.0	99.2	206.3
48	5	148.3	102.8	212.4
48	6	153.9	106.7	222.4
48	7	159.7	110.7	229.7
48	8	165.8	114.9	238.0
48	9	172.2	119.4	245.0
48	10	178.9	124.0	257.8
48	11	186.0	125.3	268.8
48	12	193.5	130.4	279.5
48	13	201.4	135.7	284.0
48	14	209.8	145.5	301.1
48	15	218.7	151.7	314.5
48	16	228.2	158.2	323.1
48	17	238.2	165.2	340.8
48	18	248.9	172.6	350.2
48	19	260.3	175.4	365.2
48	20	272.3	183.5	382.9
48	21	285.1	197.7	398.8
48	22	298.7	207.1	423.2
48	23	313.0	217.0	439.5
48	24	328.2	233.9	448.0
48	25	344.1	245.3	472.5
48	26	360.9	257.3	498.7
48	27	378.4	262.4	526.2
48	28	396.8	275.1	555.5
48	29	416.0	296.6	556.7
48	30	436.0	310.9	586.6
48	31	456.9	334.6	618.9
48	32	478.6	341.2	652.5
48	33	501.2	357.3	687.9
48	34	524.7	374.1	700.1
48	35	549.3	402.3	724.5
48	36	574.8	432.2	763.6
48	37	601.5	440.6	804.5
48	38	629.4	448.7	847.5
48	39	658.6	482.4	886.5
48	40	689.3	518.3	892.6
48	41	721.5	542.6	941.5
48	42	755.6	553.4	993.9
48	43	791.5	579.7	1048.9
48	44	829.7	623.9	1108.7

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
23	45	98.9	80.2	119.2
23	46	100.6	81.6	121.2
23	47	102.4	83.0	123.1
23	48	104.1	86.4	124.7
24	0	31.7	20.7	46.6
24	1	33.1	21.7	48.1
24	2	34.5	23.3	50.1
24	3	35.9	24.2	51.9
24	4	37.3	25.2	53.3
24	5	38.8	26.1	54.7
24	6	40.2	27.1	56.4
24	7	41.7	28.9	58.0
24	8	43.1	29.9	59.8
24	9	44.6	30.9	61.3
24	10	46.0	32.8	63.0
24	11	47.5	33.9	64.7
24	12	49.0	34.9	66.1
24	13	50.5	36.0	67.8
24	14	52.0	38.1	69.7
24	15	53.5	39.2	71.0
24	16	55.0	40.3	73.0
24	17	56.5	41.4	74.4
24	18	58.0	42.5	75.9
24	19	59.5	44.8	77.8
24	20	61.1	45.9	79.5
24	21	62.6	47.1	81.4
24	22	64.2	48.3	83.3
24	23	65.8	49.4	85.1
24	24	67.3	50.6	86.3
24	25	68.9	53.2	87.7
24	26	70.5	54.4	89.5
24	27	72.1	55.6	91.6
24	28	73.7	56.9	93.9
24	29	75.3	58.1	94.6
24	30	77.0	60.9	96.1
24	31	78.6	62.2	98.6
24	32	80.3	63.5	100.6
24	33	81.9	64.8	101.5
24	34	83.6	66.1	103.6
24	35	85.2	67.4	105.7
24	36	86.9	68.8	107.0
24	37	88.6	70.1	109.0
24	38	90.3	71.4	111.1
24	39	92.0	74.6	112.2
24	40	93.8	76.0	114.7
24	41	95.5	77.4	116.4
24	42	97.2	78.8	118.0
24	43	99.0	80.2	120.1
24	44	100.7	81.7	121.8

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
48	45	870.4	654.5	1153.8
48	46	913.9	705.0	1174.6
48	47	960.6	703.6	1245.4
48	48	1011.2	740.6	1323.5
49	0	135.5	88.7	203.4
49	1	140.8	92.1	209.3
49	2	146.4	98.7	219.3
49	3	152.3	102.6	228.4
49	4	158.5	106.8	235.5
49	5	165.0	111.2	243.4
49	6	172.0	115.9	260.5
49	7	179.3	120.8	270.3
49	8	187.2	126.1	281.0
49	9	195.6	128.0	292.9
49	10	204.6	137.9	306.9
49	11	214.3	140.2	320.9
49	12	224.7	147.0	343.5
49	13	235.9	159.0	360.0
49	14	248.1	162.3	371.9
49	15	261.3	170.9	398.5
49	16	275.5	185.7	416.8
49	17	290.9	190.4	446.1
49	18	307.6	195.3	471.2
49	19	325.5	206.6	498.1
49	20	344.8	218.9	520.7
49	21	365.4	231.9	555.5
49	22	387.3	245.9	567.0
49	23	410.6	260.6	618.9
49	24	435.2	276.2	650.0
49	25	461.1	292.7	687.9
49	26	488.4	310.0	721.5
49	27	517.2	338.4	763.6
49	28	547.5	358.2	804.5
49	29	579.4	379.1	847.2
49	30	613.1	401.2	879.2
49	31	648.8	424.5	941.5
49	32	686.7	449.3	974.4
49	33	727.0	475.7	1048.9
49	34	770.1	549.0	1094.0
49	35	816.4	550.1	1174.6
49	36	866.4	583.8	1245.4
49	37	920.8	620.5	1282.0
49	38	980.4	660.6	1410.2
49	39	1046.2	705.0	1509.0
49	40	1119.9	754.6	1614.0
49	41	1203.3	810.8	1750.7
49	42	1299.7	850.4	1896.6
49	43	1413.6	924.9	2101.6
49	44	1553.1	1016.2	2353.1

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
24	45	102.5	83.1	123.4
24	46	104.3	86.6	125.1
24	47	106.1	86.0	127.3
24	48	107.9	89.6	128.9

Positive Large Wells	Positive Small Wells	MPN	95% Confidence Lower Limit	95% Confidence Upper Limit
49	45	1732.9	1167.7	2709.5
49	46	1986.3	1222.0	3300.2
49	47	2419.6	1630.4	4716.1
49	48	>2419.6	1439.5	infinite

7.3.12 Annex B

Performance data

(Informative)

Samples of raw and pelleted sludge were analysed using the described method and compared with the results obtained using membrane filtration onto oxolinic aesculin azide (OAA) agar and miniaturised MPN (MUD/SF plates).

Raw sludge samples analysed using the described method gave comparable results when compared with miniaturised MPN ($p = 0.099$) but the results for OAA media were significantly higher than the MPN method ($p = 0.015$) (**Error! Reference source not found.**).

Pelleted sludge results were not obtained for this MPN described as the results were over the readable range (> 9678/ g). Further analysis is necessary to obtain readable data for this matrix.

Positive control samples were also analysed and all media and methods tested gave comparable results ($f = 2.290$, $p = 0.182$).

This method could prove of use for a presence absence method but further analysis of the detection limits is required. Also it may be useful for samples with low numbers of target bacteria present but high dry solids content.

Table 7.4 enterococci counts (per g ww) for MPN and OAA media.

Sample	Media	Mean cfu/g (OAA) or MPN/g (MUD/SF) ww (n = 3)	Min cfu/g (OAA) or MPN/g (MUD/SF) ww (n = 3)	Max cfu/g (OAA) or MPN/g (MUD/SF) ww (n = 3)	St. dev. cfu/g (OAA) or MPN/g (MUD/SF) ww (n = 3)	p-value (t-test) *(Mann Whitney Rank sum)
Raw	**MPN**	48,293	42,000	52,880	5,637	
	OAA	84,000	64,000	96,000	17,436	0.028
Pelleted	**MPN**	>9678.4	>9678.4	>9678.4		
	OAA	1,600	800	2,800	1,058	
Positive control	**MPN**	37,100	17,820	21,870	2,128	
	OAA	32,667	22,000	40,000	9,452	0.090
Negative control	**MPN**	0	0	0		
	OAA	0	0	0		
Blank	**MPN**	0	0	0		
	OAA	0	0	0		

Key: cfu	colony forming unit
MPN	most probable number
ww	wet weight
n	number
OAA	oxolinic aesculin azide
Min	agar
Max	minimum
St. dev.	maximum

7.3.13 References

U.S. Environmental Protection Agency. 2004. Guidelines Establishing Test Procedures for the analysis of Pollutants Under the Clean Water Act; National Primary Drinking Waters Regulations; and National Secondary Drinking Water Regulations; Analysis and Sampling Procedures; Proposed Rule. *U.S. Federal Register* - 40 CFR Part 122 Vol. 69, No.66 (excerpt)

Enterolert™ procedure. http://www.idexx.com/water/refs/060215005ENT.pdf

Yakub, G. P. C., D. A.; Stadterman-Knauer, K. L.; Tobin, M. J.; Blazina, M.; Heineman, T. N.; Yee, G. Y.; Frazier, L. (2002). "Evaluation of Colilert and Enterolert Defined Substrate Methodology for Wastewater Applications." *Water Environment Research* **74**(2): 131-135.

7.4 Method for isolation and enumeration of *Clostridium perfringens* by membrane filtration onto selective agar

7.4.1 Introduction

Sludges, soils, soil improvers, growing media and biowastes can contain microorganisms pathogenic to Man *e.g*. *Salmonella* spp. Most occur in the intestinal tract of humans and animals and can be transmitted through faecal contamination. Outbreaks of infection could be caused by use of the pathogen contaminated materials in agriculture due to the production of contaminated food and animal feed, or transmission to wild animals. As a consequence of this there is a need to monitor levels in these materials and application levels to land.

WARNING – "Waste and sludge samples may contain hazardous and inflammable substances. They may contain pathogens and be liable to biological action. Consequently it is recommended that these samples should be handled with special care. The gases which may be produced by microbiological activity are potentially inflammable and will pressurise sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided where possible. National regulations should be followed with respect to microbiological hazards associated with this method".

7.4.2 Scope

This draft standard method specifies a membrane filtration procedure for the quantitative analysis, by culture of individual colonies on selective agar, of enterococci species in sewage sludge, compost and biowaste samples. The user should prior to analysis validate the method for the particular type of sample they wish to analyse: sludges, soils, soil improvers, growing media (e.g. compost) and biowastes.

This method is of particular use if determination of treatment efficiency is required as outlined in the revision of Directive 86/278/EEC (3rd draft, CEN/TC 308-doc 525).

The method has a limit of detection of approximately 1 cfu/g wet weight, dependent of the solids content which at high concentrations (>20% (w/v)) can block the filtration of the sample volume through the membrane if not dilution prior to filtration.

7.4.3 Normative references

The following referenced documents were referred to extensively and offer indispensable advice for the application of this method.

EN 12880:2000. Characterisation of sludges – determination of dry residue and water content

EN ISO 5667-13:1997. Water quality – Sampling – Part 13: Guidance on sampling of sludges from sewage and water treatment works.

ISO 8199:2001. Water quality – General guide to the enumeration of microorganisms by culture.

7.4.4 Terms and definitions

For the purposes of this document the following terms and definitions apply.

Clostridium perfringens

Clostridium perfringens are anaerobic (or micro-aerobic) gram-positive bacillus (rods), spore forming, non-motile, reduces sulphite, reduces nitrates to nitrites, produces acid and gas from lactose, metabolises 4-methylumbelliferyl phosphate (MUP) to 4-methylumbelliferone and liquefies gelatin within 48 h.

Method definition

C. perfringens metabolises 4-methylumbelliferyl phosphate (MUP) using the enzyme acid phosphatase to produce 4-methylumbelliferone, which fluoresces when placed under long-wavelength (365-nm) ultraviolet light.

cfu, colony forming unit

Growth of an individual bacterial cell into visible colonies on Agar media, including membrane filters overlaying media.

Dry matter

The dry mass portion of the material tested after the specified drying process, expressed as percent or grams per kilogram (EN 12880:2000, 3.1).

7.4.5 Apparatus

With the exception of equipment supplied sterilised all should be sterilised before use in accordance with ISO 8199.

In addition to usual microbiological laboratory equipment:

Apparatus for sterilisation either dry heat (oven) or steam (autoclave).

Thermostatic incubator(s) regulated at 37 (±1)ºC.

Homogeniser (e.g. Stomacher, Seward Laboratories or equivalent).

Sterile homogeniser bags 250ml volume with or without integrated mesh to remove large particles (e.g. Seward Laboratories 6041, 6041STR or equivalent).

pH meter with accuracy of ± 0.1.

Membrane filtration manifolds (e.g. Sartorius 13430-0475, Pall Gelman or equivalent).

Membrane filter units 150ml capacity either disposable (e.g. Millipore or equivalent) or sterilisible (e.g. Pall Gelman or equivalent).

Sterile forceps

Bunsen burner

Pipettors capable of dispensing 100 µl and 1 ml

Graduated pipettes capable of dispensing 2-10 ml

Sterile tips

Anaerobic condition generators anaerobic jars (e.g. Oxoid HP0011) with anaerobic packs (e.g. Oxoid ANN0035) or anaerobic incubator.

Note: Indicator strips e.g. Oxoid BR0055 should be included to ensure anaerobic conditions have been achieved.

Ultraviolet observation chamber (Wood's Lamp, 366 nm).

WARNING – UV light can damage eyes and skin. Use protective goggles and gloves.

7.4.6 Sampling hazards

Take samples of at least 100g wet weight and transport to laboratory as quickly as possible, chilled at 5±3ºC.

General

As samples are liable to ferment and contain pathogenic microorganisms, it is of paramount importance to adhere to national and international regulations relating to bio hazardous samples when handling and transporting samples.

It is essential to keep samples away from food or drink, and to protect any cuts.

See also Warning note in introduction of this method.

Storage

Do not store these samples on an open bench in the laboratory. If samples are not to be analysed immediately, store them at 5±3ºC in well labelled containers, preferable plastic. Samples can be stored for a maximum period of 36 hours.

Handling

Good laboratory practice and cleanliness is essential. When handling sludge samples it is necessary to wear gloves, face and eye protection, and sufficient body protection to protect against spillages or bottles bursting. The gas evolved when opening sludge samples is flammable and so should be carried out away from naked flames and all equipment should be flame proof.

See also the Warning note in the introduction.

7.4.7 Reagents, diluents and culture media

To ensure reproducible results, prepare culture media and diluents using either constituents of uniform quality and chemicals of recognised analytical grade, or a dehydrated diluent or complete medium prepared following the manufacturer's instructions. Prepare the media and reagents with demineralised or distilled water free from substances capable of inhibiting growth under test conditions. If the media are not used immediately, they should be stored in the dark at 5±3ºC for up to one month in conditions avoiding any alteration of their composition.

NOTE the use of chemicals of other grades is permissible providing that they are shown to be of equivalent performance in the test.

Quarter Strength Ringers solution

Ringer's solution composition per litre:

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride $6H_2O$	0.12 g
Sodium bicarbonate	0.05 g

Ringer's is commercially available in tablet form where one tablet is added to 500 ml water for quarter strength ringers. Solution is steam sterilised (autoclaved) at 121±3ºC for 15±1 minutes. pH 7.0±0.2.

7.4.8 4-methylumbelliferyl (MUP) disodium salt

4-methylumbelliferyl (MUP) disodium salt	100 mg
Distilled/ deionised water	5 ml

Add MUP to water and filter sterilise through a 0.2 µm millipore filter.

7.4.9 D-cycloserine supplement

D-cycloserine	400 mg
Distilled/deionised water	5 ml

Add D-cycloserine to water and filter sterilise through a 0.2 µm Millipore filter.

NOTE both supplements are commercially available as a single supplement in vial form (Merck 1.00888.0001). One vial is suspended in 5 ml sterile water and the contents added to 500 ml media.

7.4.10 Modified Tryptose sulphite cycloserine agar (mTSC agar)

Per litre

Tryptose	15 g
Soytone	5 g
Yeast extract	5 g
Ammonium iron (III) citrate	1 g
Water	1 L

Add reagents to one litre of water and heat to boiling. Autoclave at 121°C for 15 minutes. Once cooled to 50ºC to 500 ml of media add 2.5 ml of TSC supplement (Oxoid SR0088) or 200 mg D-cycloserine and swirl to mix and 50 mg 4-methylumbelliferyl phosphate disodium salt (Sigma M8168).

NOTE alternatively add one vial of Perfringens supplement (Merck 1.00888.0001) per 500 ml media.

Pour into Petri dishes (do not store media to re-heat). pH 7.2±0.2. This media should be prepared fresh before use and only stored for a maximum of 4 days in dark at 5 ± 3°C prior to use.

7.4.11 Procedure

Sample preparation

Determination of dry matter content

The dry matter content is measured using the method described in EN 12880:2000.

Suspension preparation

Mix sample and weigh out a representative sub-sample of 25 g (wet weight) into a homogeniser bag with an integrated mesh to remove large particles, if required.

Add a volume of quarter strength ringers (QSR) so the final volume is 250 ml (dilution A).

For lime treated sludges adjust the pH to 7.0± 0.5 with 1 mol/l hydrochloric acid.

NOTE 1: If the pH drops below 4.5 whilst neutralising the sample, a new sample should be prepared.

NOTE 2: If other chemical treatment is used on sludge samples to be tested a suitable neutralisation procedure should be adopted.

Place sample in a homogeniser (7.1.5) and homogenise for 2 minutes.

Preparation of dilutions

Prepare serial dilutions from dilution A in QSR as appropriate for the expected concentration of enterococci in sample. For example raw sludges may have up to 10^6-10^7 *Clostridium perfringens* per g but treated sludges may only require dilutions to 10^{-1}-10^{-2}.

Mix the primary suspension (1/10 dilution) and using a sterile pipette aseptically transfer 1 ml into a sterile tube containing 9 ml QSR. Mix this dilution and using a new sterile pipetted transfer 1 ml of this dilution to a tube containing 9 ml QSR.

Continue this procedure until the required number of dilutions has been prepared.

7.4.12 Analysis

Filter 1 ml aliquots of each diluted sample through a 0.45 µm pore size cellulose nitrate filter. To ensure even distribution of the sample over the filter surface, prior to addition of the sample add approximately 10-20 ml of diluent (QSR or equivalent) to the membrane housed in the filter housing.

NOTE keep the vacuum in the off position until the sample has been added.

As soon as the sample has filtered through, using a low vacuum pressure (not exceeding 65 kPa 500 mm of mercury), switch off the vacuum so as little air as possible passes through the filter.

Resuscitation and enumeration of colonies on selective agar

Remove the filters from the filter housing using sterile tweezers, holding the filter at the edge of the filter. Place the filters face upwards onto 50 mm diameter plates containing pre-dried selective media (7.4.10). Use a 'ro lling' action to prevent the formation of air bubbles under the media which would prevent the media coming into contact with the membrane. Invert the plates and place either in anaerobic gas generating jars with appropriate gas generator or in anaerobic incubator and incubate at 37 (±1) °C for 24 (± 4) hours. Enumerate light blue fluorescent colonies under UV by eye.

Confirmation

If confirmation is required biochemical tests can be applied such as Rapid ID32A (Biomérieux) test strips or equivalent.

7.4.13 Expression of results

Calculation of the number of *Clostridium perfringens* present (per g wet weight of the original sludge sample) is by multiplying the number of fluorescent blue colonies on the filter by the dilution factor.

i.e. a/bd = c

where:
a = volume filtered through each membrane
b = initial dilution factor of the sludge in QSR (normally 10)
c = original concentration of *Clostridium perfringens* per g
d = dilution factor for the serial dilutions used

If dry weight numbers are required the following equation if used:

a/bde = c

where:
e = % dry mass of the original sludge sample

7.4.14 Annex A

(Informative)

Performance data

Samples of raw sludge were analysed by membrane filtration using modified (fluorescent) TSC agar (MUP-tryptose sulphite cycloserine) and TSC agar.

When the results were compared the numbers of enterococci recovered on fTSC media were significantly higher than on TSC media (t = -4.573, d.f. = 10, p = 0.001) (**Error! Reference source not found.**).

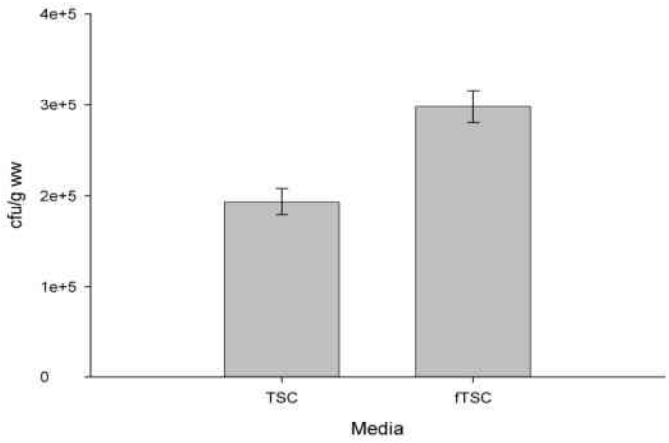

Figure 7.2: Numbers of enterococci enumerated from raw sludge samples on TSC and fTSC media. Lines represent the standard error of the mean.

Table 7.5: numbers of enterococci in raw sludge samples using fTSC and TSC agars.

Sample	Media	Trial 1 Mean cfu/g ww (n = 3)	Trial 2 Mean cfu/g ww (n = 3)	Mean cfu/g ww (n = 6)	p-value (t-test)
Raw	TSC	206,667	180,000	193,334	
	fTSC	310,000	290,000	300,000	0.001
Negative	TSC	0	0	0	
	fTSC	0	0	0	
Blank	TSC	0	0	0	
	fTSC	0	0	0	

Key:

cfu	colony forming unit
ww	wet weight
TSC	Tryptose sulphite
fTSC	cycloserine agar
n	MUP-tryptose sulphite

7.4.15 References

Adcock, A. W., Saint, C. P. (2001). Rapid confirmation of *Clostridium perfringens* by using Chromogenic and Fluorogenic substrates. *Applied and Environmental Microbiology* **67**(9): 4382-4384.

Araujo, M., Sueiro, R. A., Gomez, M. J., Garrido, M. J. (2001). Evaluation of fluorogenic TSCagar for recovering *Clostridium perfringens* in groundwater samples. *Water Science and Technology* **43**: 201-204.

Standing Committee of Analysts (2004). The Microbiology of Drinking Water (2004) - Part 6 - Methods for the Isolation and enumeration of sulphite reducing clostridia and *Clostridium perfringens* by membrane filtration. In: *Methods for the Examination of Waters and Associated Materials*. Environment Agency.

7.5 *Clostridium perfringens analysis using presence/absence by inoculation into selective liquid media*

7.5.1 Introduction

Sludges, soils, soil improvers, growing media and biowastes can contain microorganisms pathogenic to Man *e.g*. *Salmonella* spp. Most occur in the intestinal tract of humans and animals and can be transmitted through faecal contamination. Outbreaks of infection could be caused by use of the pathogen contaminated materials in agriculture due to the production of contaminated food and animal feed, or transmission to wild animals. As a consequence of this there is a need to monitor levels in these materials and application levels to land.

WARNING – "Waste and sludge samples may contain hazardous and inflammable substances. They may contain pathogens and be liable to biological action. Consequently it is recommended that these samples should be handled with special care. The gases which may be produced by microbiological activity are potentially inflammable and will pressurise sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided where possible. National regulations should be followed with respect to microbiological hazards associated with this method".

7.5.2Scope

This draft standard method specifies a membrane filtration procedure for the quantitative analysis, by culture of individual colonies on selective agar, of enterococci species in sewage sludge, compost and biowaste samples. The user should prior to analysis validate the method for the particular type of sample they wish to analyse: sludges, soils, soil improvers, growing media (e.g. compost) and biowastes.

This method is of particular use if determination of treatment efficiency is required as outlined in the revision of Directive 86/278/EEC (3rd draft, CEN/TC 308-doc 525).

The method has a limit of detection of approximately 1 cfu/g wet weight, dependent of the solids content which at high concentrations (>20% (w/v)) can block the filtration of the sample volume through the membrane if not dilution prior to filtration.

7.5.3Normative references

The following referenced documents were referred to extensively and offer indispensable advice for the application of this method.

EN 12880:2000. Characterisation of sludges – determination of dry residue and water content

EN ISO 5667-13:1997. Water quality – Sampling – Part 13: Guidance on sampling of sludges from sewage and water treatment works.

ISO 8199:2001. Water quality – General guide to the enumeration of microorganisms by culture.

7.5.4Terms and definitions

For the purposes of this document the following terms and definitions apply.

Clostridium perfringens

Clostridium perfringens are anaerobic (or micro-aerobic) gram-positive bacillus (rods), spore forming, non-motile, reduces sulphite, reduces nitrates to nitrites, produces acid and gas from lactose, metabolises 4-methylumbelliferyl phosphate (MUP) to 4-methylumbelliferone and liquefies gelatin within 48 h.

Method definition

C. perfringens metabolises 4-methylumbelliferyl phosphate (MUP) using the enzyme acid phosphatase to produce 4-methylumbelliferone, which fluoresces when placed under long-wavelength (365-nm) ultraviolet light.

Dry matter

The dry mass portion of the material tested after the specified drying process, expressed as percent or grams per kilogram (EN 12880:2000, 3.1).

7.5.5Apparatus

With the exception of equipment supplied sterilised all should be sterilised before use in accordance with ISO 8199.

In addition to usual microbiological laboratory equipment:

Apparatus for sterilisation either dry heat (oven) or steam (autoclave).

Thermostatic incubator(s) regulated at 37 (±1)ºC.

Homogeniser (e.g. Stomacher, Seward Laboratories or equivalent).

Sterile homogeniser bags 250ml volume with or without integrated mesh to remove large particles (e.g. Seward Laboratories 6041, 6041STR or equivalent).

pH meter with accuracy of ± 0.1.

Bunsen burner

Pipettors capable of dispensing 100 µl and 1 ml

Graduated pipettes capable of dispensing 2-10 ml

Sterile tips

Anaerobic condition generators anaerobic jars (e.g. Oxoid HP0011) with anaerobic packs (e.g. Oxoid ANN0035) or anaerobic incubator.

Note: Indicator strips e.g. Oxoid BR0055 should be included to ensure anaerobic conditions have been achieved.

Ultraviolet observation chamber (Wood's Lamp, 366 nm).

WARNING – UV light can damage eyes and skin. Use protective goggles and gloves.

7.5.6Sampling hazards

Take samples of at least 100g wet weight and transport to laboratory as quickly as possible, chilled at 5±3ºC.

General

As samples are liable to ferment and contain pathogenic microorganisms, it is of paramount importance to adhere to national and international regulations relating to bio hazardous samples when handling and transporting samples.

It is essential to keep samples away from food or drink, and to protect any cuts.

See also Warning note in introduction of this method.

Storage

Do not store these samples on an open bench in the laboratory. If samples are not to be analysed immediately, store them at 5±3ºC in well labelled containers, preferable plastic. Samples can be stored for a maximum period of 36 hours.

Handling

Good laboratory practice and cleanliness is essential. When handling sludge samples it is necessary to wear gloves, face and eye protection, and sufficient body protection to protect against spillages or bottles bursting. The gas evolved when opening sludge samples is flammable and so should be carried out away from naked flames and all equipment should be flame proof.

See also the Warning note in the introduction.

7.5.7Reagents, diluents and culture media

To ensure reproducible results, prepare culture media and diluents using either constituents of uniform quality and chemicals of recognised analytical grade, or a dehydrated diluent or

complete medium prepared following the manufacturer's instructions. Prepare the media and reagents with demineralised or distilled water free from substances capable of inhibiting growth under test conditions. If the media are not used immediately, they should be stored in the dark at 5±3ºC for up to one month in conditions avoiding any alteration of their composition.

NOTE the use of chemicals of other grades is permissible providing that they are shown to be of equivalent performance in the test.

Quarter Strength Ringers solution

Ringer's solution composition per litre:

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride $6H_2O$	0.12 g
Sodium bicarbonate	0.05 g

Ringer's is commercially available in tablet form where one tablet is added to 500 ml water for quarter strength ringers. Solution is steam sterilised (autoclaved) at 121±3ºC for 15±1 minutes. pH 7.0±0.2.

7.5.8 4-methylumbelliferyl (MUP) disodium salt

4-methylumbelliferyl (MUP) disodium salt	100 mg
Distilled/ deionised water	5 ml

Add MUP to water and filter sterilise through a 0.2 µm millipore filter.

7.5.9 D-cycloserine supplement

D-cycloserine	400 mg
Distilled/deionised water	5 ml

Add D-cycloserine to water and filter sterilise through a 0.2 µm Millipore filter.

NOTE both supplements are commercially available as a single supplement in vial form (Merck 1.00888.0001). One vial is suspended in 5 ml sterile water and the contents added to 500 ml media.

7.5.10 Modified Tryptose sulphite cycloserine broth (mTSC broth)

Per litre

Tryptose	15 g
Bacteriological peptone	5 g
Nutrient broth	5 g
Yeast extract	5 g
Water	1 L

Add reagents to one litre of water and heat to boiling. Autoclave at 121°C for 15 minutes. Once cooled to 50ºC add 2.5 ml of TSC supplement (Oxoid SR0088) or 200 mg D-cycloserine and swirl to mix and 50 mg 4-methylumbelliferyl phosphate disodium salt (Sigma M8168).

NOTE alternatively add one vial of Perfringens supplement (Merck 1.00888.0001).

7.5.11 Procedure

Sample preparation

Determination of dry matter content

The dry matter content is measured using the method described in EN 12880:2000.

Suspension preparation

Mix sample and weigh out a representative sub-sample of 25 g (wet weight) into a homogeniser bag with an integrated mesh to remove large particles, if required.

Add a volume of quarter strength ringers (QSR) so the final volume is 250 ml (dilution A).

For lime treated sludges adjust the pH to 7.0± 0.5 with 1 mol/l hydrochloric acid.

NOTE 1: If the pH drops below 4.5 whilst neutralising the sample, a new sample should be prepared.

NOTE 2: If other chemical treatment is used on sludge samples to be tested a suitable neutralisation procedure should be adopted.

Place sample in a homogeniser (7.1.5) and homogenise for 2 minutes.

7.5.12 Analysis

Add 10 ml aliquots of each sample to 90 ml TSC broth. Swirl to mix and replace lid, loosely.

Resuscitation and enumeration of colonies on selective agar

Place either in anaerobic gas generating jars with appropriate gas generator or in anaerobic incubator and incubate at 37 (±1) °C for 24 and 44 (± 4) hours. Positive reaction is the presence of light blue fluorescence under UV light.

Confirmation

The media used in the described method is confirmatory but if additional confirmation is required biochemical tests can be applied such as Rapid ID32A (Biomérieux) test strips or equivalent.

7.5.13 Expression of results

Clostridium perfringens are deemed present if the samples are fluorescent under UV light. As 10 ml of the 1:10 diluted sample (25 g sample in 225 ml QSR) is analysed this is equivalent to presence or absence in 1 g of sample.

7.5.14 Annex A
(Informative)

Performance data

Samples of raw and pelleted sludge were analysed using the described method. Alongside samples were analysed on TSC (tryptose sulphite cycloserine) agar to give an estimate of number of enterococci present.

Samples were seen to have clear fluorescence in raw and pelleted sludges. Comparing with the estimate counts on plates the limit of detection could be as low as 7 cfu/ g wet weight.

Table 7.6: Presence/absence results for raw and pelleted sludge samples compared with numbers counted.

Sample	Presence/absence (+/-) (n = 3)	Mean cfu/g ww (n = 6)
Raw	+	4.4×10^5
Pelleted	+	7
Positive	+	8.2×10^6/ml
Negative	-	
Blank	-	

Key:
cfu colony forming unit
ww wet weight
n number

7.5.15 References

Adcock, A. W., Saint, C. P. (2001). Rapid confirmation of *Clostridium perfringens* by using Chromogenic and Fluorogenic substrates. *Applied and Environmental Microbiology* **67**(9): 4382-4384.

Araujo, M., Sueiro, R. A., Gomez, M. J., Garrido, M. J. (2001). Evaluation of fluorogenic TSCagar for recovering *Clostridium perfringens* in groundwater samples. *Water Science and Technology* **43**: 201-204.

Standing Committee of Analysts (2004). The Microbiology of Drinking Water (2004) - Part 6 - Methods for the Isolation and enumeration of sulphite reducing clostridia and *Clostridium perfringens* by membrane filtration. In: *Methods for the Examination of Waters and Associated Materials*. Environment Agency.

8 APPENDIX 2

Questionnaires received prior to Workshop.

		Cyprus 1	Czech Republic 1	Denmark 1	Estonia 1	Finland 2	France 4	France 5	France 6
Regulation		P3 (on treated biowastes only)	P4&5	P6	P7	N5	P12	C1	C5
Required by legislation?	*Clostridium perfringens*	No	Yes	No	No	No	Yes	No	No
	Intestinal Enterococci	No	Yes	Yes	No	No	Yes	No	No
Standard method	*Clostridium perfringens*	No	No				Not to her knowledge (only for food)	SLUDGES : no. COMPOST: NF V 08-056 (required in NF U44-095:2002 French legislation from 18 March 2004)	
	Intestinal Enterococci	No	Yes	Yes – DS 2401			Not to her knowledge (only for water)	SLUDGES : no. COMPOST: NF-EN ISO 7899-1: 1999 (required in NF U44-095:2002 French legislation from 18 March 2004)	
Maximum level	*Clostridium perfringens*		ES 1772/2001					COMPOST: < 10^2/g wet weight (compost for market gardening). < 10^3/g wet weight (compost for farming except market gardening)	
	Intestinal Enterococci		10^3 CFU.g^{-1} dry weight for sludge, 10^2 CFU.g^{-1} dry weight for the first compost class	Yes - <100 cfu/g wet weight after sanitation				COMPOST: < 10^5/ g de wet weight (any compost)	
Sample storage and transport									
Max length of time and temperature of storage		No	48 h	Yes – DS standard: max . length of time is 24 hours. Temperature at storage 0-5 degree		No regulations but in practise the analyses are started within 36 hours (EN 13040).	Not to her knowledge	No regulation. Clostridium perfringens : (5±3)°C, before 24h. Intestinal enterococci : (5±3)°C, before 24h.	No. Storage of the samples below 10°C before analysis.
Preparation of sample									
Preparation of sample	Amount	Depends on the type of treated effluent: from 0,01 to 100mL	10g wet weight for *C. perf*, enterococci and fecal bact (50g wet weight for *Salmonella* sp.)	250 g		10 g wet weight	<10 grams (wet weight)	10 g wet weight for both parameters (results are given by g wet weight)	10 G of wet weight
	Method to homogenize	For treated effluent, sample shaken on a mechanical shaker for up to 30 min.	10g wet weight + 90ml phosphate buffer vigourously shaken for 2 min (Stomacher)	Described in the method.		The whole sample (4 – 7 litres) is sieved according to EN 13040 if possible.	*C. perfringens*: 10g of sample + 90ml of sterile diluant. Int.enterococci: 2 g of the sample + 18 mL of a sterile diluant. After hand shaking for 1 to 2 min, the samples are then serially diluted before inoculation.	*SLUDGES: 10 g wet weight sludge + Tryptone salt diluent to 100mL final volume. Homogenization in a stomacher® for 1 to 2 minutes. COMPOST: 10 g wet weight sludge + Tryptone salt diluent to 100mL final volume. Homogenization of the stomacher® bag containin*	Rotative homogeneisation during 2 min in tryptone-salt.
	pH checked		Yes for lime treated sludge: pH adjusted to 7,0 with 1N H_2SO_4			No	No	No	No

		Cyprus 1	Czech Republic 1	Denmark 1	Estonia 1	Finland 2	France 4	France 5	France 6
Analytical method and results									
Method	*Clostridium perfringens*		1 ml of diluted sample is inoculated on plate with differential Clostridial Agar (DCA) according to Weenk and then the plate is overlaid by the same medium. The dishes are cultured under anaerobic conditions 24 hours at 37°C in an Anaerostat. For make sur	DS 2256		ISO 7937, modified. Samples are inoculated into TSC agar using pour plate technique. The plates are incubated under anaerobic conditions at 37°C for 20 h. Typical colonies are isolated and inoculated onto sheep blood agar plates.	V08-056: Spores of *C. perfringens* are counted according to the AFNOR method V08-056 modified by the Pasteur Institut of Lille. Ten grams of each soil sample are suspended in 90 mL of tryptone salt broth and shaken. After a thermal shock (20 min. at 80°C),	COMPOST - NF V 08-056 : 1994. Pour plate enumeration : Dilution: 100 to 10-4 in Tryptone salt diluent. Pour on Tryptose-sulfite-cycloserine agar medium. incubation at (37±1)°C for (20±4)h in anaerobic conditions. Confirmation step : Transfer black (sulfit	Semi-quantitative enumeration in liquid media (thioglycolate broth), Confirmation in lactose sulfite broth
	Intestinal Enterococci	ISO 7899-2-200	0,2 ml of diluted sample is inoculated on plate with Slanetz – Bartlez medium. The dishes are cultured 48 hours at 37°C. Confirmation of Enterococci is performed by: pyrrolidinpeptidase test, test on possibility of hydrolysis Esculin	DS standard 2401		Nordic Committee on Food Analysis NMKL 68, modified. Samples are plated out on Slanetz and Bartley medium and incubated at 44°C for 48h.	XP T90-432.: Two grams of the sample are added to 18 mL of a sterile special microtitration plate diluant. After hand shaking for 1 min, the samples are then serially diluted (10^{-1}, 10^{-2} and 10^{-3}) in the diluant and 200 µL of each dilution are inoculated	COMPOST - NF EN ISO 7899-1 : 1999. Miniaturised MPN method : Dilution: 2 to 2.10-5 in special diluent. Inoculate a microtitre plate of 96 wells containing MUD medium (4-methyl-umbeliferyl-ß – D–glucoside). Incubation at (44±0.5) °C from 36h to 72h. Determ	Incubation of several dilutions of sample on BEA agar plates. Confirmation using catalase and Gram tests.
Analysis time	*Clostridium perfringens*		26h without API 20A test	48h		2 days	48h	3 days	48 hours
	Intestinal Enterococci	48h	55h	48h		2 days	36h	1 day	48 hours
Media	*Clostridium perfringens*		ready made	Ferrosulfit agar		TSC, Tryptose-sulfite-cycloserine agar. Perfringens agar base, Oxoid CM 587 and cycloserine, Oxoid SR88.	ready made: thioglycolate broth: Biokar, France. D-cycloserine: Sigma, France. lactose-sulphite broth: Biokar, France	COMPOST : SC : Tryptose-sulfite-cycloserine agar (agar base ready made). Thioglycolate broth (prepared in-house). Lactose sulfite broth (prepared in-house). SLUDGES :Thioglycolate broth (prepared in-house). Lactose sulfite broth (prepared in-house).	Thioglycolate broth (ready to use). Lactose sulfite (prepared in-house).
	Intestinal Enterococci	Confirmed	ready made	Slanetz agar		Slanetz and Bartley agar, Oxoid CM 377	ready made. Diluant and microplates MUD/SF : Bio-Rad, Marnes-la-Coquette, France	COMPOST : Microtitre plate Enterococcus (ready made). SLUDGES : D-Coccosel agar (DCO-D) (prepared in-house).	BEA broth (ready to use)
Results	*Clostridium perfringens*		Confirmed	Presumptive counts		Confirmed	Not confirmed	Confirmed	Confirmed
	Intestinal Enterococci		Confirmed	Presumptive counts		Confirmed	Yes, but not systematically.	Confirmed	Confirmed

		Cyprus 1	Czech Republic 1	Denmark 1	Estonia 1	Finland 2	France 4	France 5	France 6
Method if confirmation	*Clostridium perfringens*		API 20A test for five CFU			Haemolytic colonies are confirmed by Gram staining and API 20 A or API Rapid ID 32.		COMPOST : included in the method. SLUDGES : included in the method. (gas production and H_2S+).	Use of lactose media
	Intestinal Enterococci	ISO 7899-2-200 Confirmation on bile-aesculin-azide agar	For five CFU, pyrrolidinpeptidase test, test on possibility of hydrolysis Esculin			Confirmation of red colonies includes Gram staining, catalase test and API 20 Strep.	Sometime, given a false fluorescence, wells are inoculated on Bile Esculin Azide and incubated at 44 °C for 24 hours. Gram and catalase are performed on esculin positive colonies	COMPOST : included in the method (MUD hydrolysis at 44°C). SLUDGES : Gram +, Catalase -, Esculine +	Catalase and Gram tests
Comments	*Clostridium perfringens*		-						Homogenisation of the samples
	Intestinal Enterococci		-				Problem of false positive wells due to a fluorescence not associated with enterococci. This phenomenon was never observed for sludge and swine manure but for treated manure, composted sludge and soil.		Homogenisation of the samples
Typical range of levels									
Type of sludge where higher frequency	*Clostridium perfringens*	Sludges and soils not tested	Sludges but also growing medium, waste from hospitals			Treated biowastes and compost	Sludges, soils and also swine manure	Sludges, soils and also sea sediment	No comparative data
	Intestinal Enterococci		Sludges, but also growing medium, waste from hospitals, playground sand			Treated biowastes and compost	Sludges and also swine manure	Sludges, soils and also sea sediment	
Frequency of analysis	*Clostridium perfringens*		<10 in soils, treated biowastes and other / 10-50 in sludges			<10 in treated biowastes and compost	<10 in all matrices (numbers irregular. Depend on contracts)	10-50: sludge, <10 in soils, <10 sea sediment	<10 in sludges
	Intestinal Enterococci	10-50 in treated biowastes	<10 in soils, treated biowastes and other / 10-50 in sludges			<10 in treated biowastes and compost	<10 in all matrices (numbers irregular. Depend on contracts)	10-50: sludge, <10 in soils, <10 sea sediment	<10 in sludges
Quality control									
Standard control strains		Lenticules from HPA, *Enterococcus faecalis* and negative controls	CCM 4224 *Enterococcus faecalis* (Czech republic), RM *Enterococcuc faecium* WR 63 (RIVM,Niederland), RM *Clostridium perfringens* D 10 (RIVM, Niederland), Quanti-cult plus *Clostridium sporogenes* (Oxoid)			For testing of culture media and reagents *C. perfringens*, *E. faecalis*, and *E. coli* strains are used.	No	Not yet	No

		Germany 1	Germany 2	Hungary 1	Italy 1	Portugal 1	Slovakia 1	South Africa 1
Regulation		P15 (P16, P17, P18)	P19	C8	C9	P21	N9&10	P22
Required by legislation?	*Clostridium perfringens*	No			No	No	No	No
	Intestinal Enterococci	No		Yes	No	No	Yes	No
Standard method	*Clostridium perfringens*	No	unknown					
	Intestinal Enterococci	No		According to the Hungarian Standards, With a comparable result of the MSZ 318-27:1986 (x)			No	
Maximum level	*Clostridium perfringens*	-	unknown					
	Intestinal Enterococci	-		150-250 g of sample			$2x10^6$ in sludges what is used as fertilizer in agriculture	
Sample storage and transport								
Max length of time and temperature of storage		No	unknown	Sludge : max 24 hours time allowed (stored and handled at 4°C). Soil : max 48 hours before the analysis.	24h, 5±3°C	No	No	For research purposes: 05-10°C within 24 hours of collection
Preparation of sample								
Preparation of sample	Amount	20g wet weight	Wet weight 20 g	The data are converted to the 1 g dry weight amount. The dilution series is made from 5x1 g or 5 g quantity.	20 g wet weight	10 ml of the washing water (50g/500ml)	10 g wet weight from sample	For research purposes: 10 g dry weight
	Method to homogenize	Placing 20 g of sample into 180 ml 0,9% sterile NaCl solution. Shaking at a minimum of 150 rpm for up to 20 h at 6 °C.	Ball mill. Beaker, lid and balls to be autoclaved beforehand. 20 g sample + 30 g buffer solution.	The homogenisation is made with a vortex or a shaker.	Dilution 1 :10 of the sample in sterile 0.85% saline water. Homogenize in disposable sterile bag using a stomacher.	The sample is homogenised with distilled water in a rotative mixer for 30 minutes.	5 min. shaking, around 160 RPM	For research purposes: Vortex mixer for 5 min
	pH checked	Yes. For disinfectant (e.g. lime, peracetic acid) treated sludges, a suitable pre-treatment for neutralising the disinfecting agent is required. For lime treated materials adjust the pH to 7.0 ± 0.5 with 1 mol /l hydrochloric acid. For other relevant chem	Not always		No	No	No	No

		Germany 1	Germany 2	Hungary 1	Italy 1	Portugal 1	Slovakia 1	South Africa 1
Analytical method and results								
Method	*Clostridium perfringens*	Cultural method. Most Probable Number technique	Dilution, ELISA, toxin-ELISA, standard agar plates	Dilution series inoculated to Litsky-Mallmann media. Incubation for 24 hours at 37°C (sludge) 48 hours (soil). Positive tubes are inoculated to E 67 or Slanetz media and incubated for 24 hours at 37 centigrade. The result of the last three positive tubes	Inclusion in selective agarized medium of a dilution of the sample. Incubation at 44°C for 24+24 h in anaerobiose.			Methods described by US Standard Methods and ISO
	Intestinal Enterococci	Cultural method. Most Probable Number technique			Filtration through a membrane of a dilution of the sample. Put the membrane onto a selective agarized medium. Incubation at 36±1°C for 40-48 h.Colony confirmation for esculin hydrolysis.	10 ml of washing water + 90 ml of distilled water. Enterolert® method or ISO 7899-2	Cultivation method on Slanetz-Bartley agar	Methods described by US Standard Methods and ISO
Analysis time	*Clostridium perfringens*	3 days	Not determined. According to number, sample type, preparation time, cleaning-up, sterilisation, etc.	2 days	48 h			48 h
	Intestinal Enterococci	5 days			50 h	Enterolert – 24 h. MF – 48 h.	48 h	48 h
Media	*Clostridium perfringens*	Sodium chloride (manufact.) / Calcium chloride (manufact.) / Fluid thioglycollate medium (manufact.)/ Blood dextrose agar (in house)	RCM (Merck), normally prepared by ingredients in houseSometimes "Perfringes agar" ready made by manufacturer	Litsky-Mallmann. Slanetz. E67. Own preparation of the media.	ISO/CD 6461-2 made from the manufacturer			Ready made commercial media
	Intestinal Enterococci	Sodium chloride (manufact.) / Calcium chloride (manufact.) / Azide dextrose broth (manufact.) / Kanamyczin esculin azide agar (manufact.)			ISO 7899-2: 2000 made from the manufacturer	Manufacturer	media ready made from the manufacturer	Ready made commercial media
Results	*Clostridium perfringens*	Confirmed	Presumptive	MPN calculation	Considered confirmed			Presumptive
	Intestinal Enterococci	Confirmed			Confirmed	1- presumptive 2- confirmed	Confirmed	Presumptive

		Germany 1	Germany 2	Hungary 1	Italy 1	Portugal 1	Slovakia 1	South Africa 1
Method if confirmation	*Clostridium perfringens*	morphological, biochemical (API 20A)						Standard Methods and ISO
	Intestinal Enterococci	morphological, serological			Colony confirmation for esculin hydrolysis on BEA	BEA	Confirmation by hydrolysis of aesculin	Standard Methods and ISO
Comments	*Clostridium perfringens*	-	There are *C. perfringens* strains with negative sulfit reaction. C. botulinum will also give positive "black" colonies. Inhibition of growth by chemical and biological contents of the sample. PCR normally not working with sludge etc.		Medium must be prepared at the moment of the use			No
	Intestinal Enterococci	-						No
Typical range of levels								
Type of sludge where higher frequency	*Clostridium perfringens*	Sludges and slurry	Biogas sludge		sludges, soils, treated biowastes and sands			Sludges
	Intestinal Enterococci	Sludges and slurry		Sludges	Sludges		Sludges	Sludges
Frequency of analysis	*Clostridium perfringens*	<10 in soils, 10-50 in sludges and treated biowastes	<10 in sludges, soils, treated biowastes and other		<10 in sludges, soils, treated biowastes			
	Intestinal Enterococci	<10 in soils, 10-50 in sludges and treated biowastes		<10 in sludges	<10 in sludges, soils, treated biowastes	<10 in soils	<10 in sludges and treated biowastes	
Quality control								
Standard control strains		*Enterococcus faecalis, Clostridium perfringens*	Nil. We have a collection of approx. 500 *C. perfringens* strains, which might come in use for research	*Enterococcus faecalis* 80 1171	No	Yes. HPA lenticules	Yes, *Enterococcus faecalis* CCM 4224	Analyses applied only for research purposes, no routine monitoring

		Spain 1	UK 1	UK 2	UK 3	UK 4
Regulation		P23	C2	C3	P26	N14
Required by legislation?	*Clostridium perfringens*	No	Yes	No		No
	Intestinal Enterococci	No	Yes	No		No
Standard method	*Clostridium perfringens*		No			No
	Intestinal Enterococci		No			No
Maximum level	*Clostridium perfringens*					No
	Intestinal Enterococci					No
Sample storage and transport						
Max length of time and temperature of storage			Yes, has to be stored at 5°C ±3 and be tested within 6 hours.	6 hours		Refrigerated at 2-8ºC, in the dark and analysed as soon as possible, always within 24 hours of sampling.
Preparation of sample						
Preparation of sample	Amount	25g wet weight	10g wet w eight C. perfringens, intestinal enterococci and also E. coli (and 25g w et w eight Salmonella).	Dependant on type of sludge but at least 5 g, normally 10 g.		Most samples start from 10g w et w eight. Regulatory Salmonella 5 replicates of 2g 'dry w eight equivalent' In practice this means a variable w et w eight dependent on the typical dry w eight of the sludge matrix under study to ensure 2g dry equivalent is analy
	Method to homogenize	25 g sample + 225 g Buffer Peptone Water then homogenize with beads in Vortex 3 min.	Use of a stomacher/homogeniser, 10g of sample and 90ml of MRD for *C.perfringens* , intestinal enterococci and for *E. coli* (and 25g of sample and 225ml of PBS for Salmonella).	Trialing numerous methods e.g. settlement, stomaching, pulsifying, dilution series.	Stomaching gives the best recoveries in our hands	In most cases sub-samples are diluted w ith MRD and Stomached for 2 minutes at slow speed before dilution for analysis. For some matrices – thermally dried sludge a period of rehydration is applied – 30 minutes at room temperature – before stomaching. We a
	pH checked		Yes the pH is checked whilst in the stomacher bag and adjusted as required to the correct level.	Yes, no special treatment.	No, lime has not been used in the treatment	Limed sludges are neutralised with hydrochloric acid after stomaching and thoroughly mixed before analysis.

		Spain 1	UK 1	UK 2	UK 3	UK 4
Analytical method and results						
Method	*Clostridium perfringens*	25 g sample + 225 ml Buffer Peptone Water then homogenize with beads in Vortex. Ten-fold dilutions in Peptone Saline Solution. Inoculate 1 ml in TSC agar with supplements: 44 ºC 24 - 48 h, anaerobically.	Membrane filtration method, homogenise the sample, centrifuge the sample, dilute the sample and filter the 10ml dilutions of the sample.	Trialing numerous methods including standard methods (ISO and Blue book).		Membrane filtration and anaerobic incubation
	Intestinal Enterococci	25 g sample + 225 ml Buffer Peptone Water then homogenize with beads in Vortex. Ten-fold dilutions in Peptone Saline Solution. Inoculate 100 ml KAA agar: 42 ºC 48 h.		Trialing numerous methods including standard methods (ISO and Blue book).	Enterococcus agar (Oxoid). 34 hours at 37oC and 44 hours at 44°C. Membrane filtration.	Membrane Filtration
Analysis time	*Clostridium perfringens*	30 min without confirmation steps	24 Hours	Not calculated as yet		48 hours presumptive, 96 hrs confirmed
	Intestinal Enterococci	30 min without confirmation steps		Not calculated as yet	50 hours including confirmation	48 hours presumptive, 72 hrs confirmed
Media	*Clostridium perfringens*	Prepared in house	TSC without egg yolk commercially prepared by Oxoid.	Numerous media both in-house and pre-prepared. E.g. TSC, modified TSC, DRCM, DCA agar		Perfringens agar (Oxoid) prepared in house
	Intestinal Enterococci	Prepared in house		Numerous methods both in-house and pre-prepared e.g. Ready cult (Merck), SBA, Enterolert system, CHROMagar.	Oxoid Slanetz and Bartley agar made in house	Slanetz & Bartley agar (mEnterococcus agar) prepared in house.
Results	*Clostridium perfringens*	Presumptive counts	24 hrs presumptive and a further 48 hrs for confirmation of result.	Presumptive and confirmed		Usually presumptive.
	Intestinal Enterococci	Presumptive counts		Presumptive and confirmed	No	Usually presumptive

		Spain 1	UK 1	UK 2	UK 3	UK 4
Method if confirmation	*Clostridium perfringens*	Buffer nitrate-motility medium and Lactose gelatine medium: 36 ºC 24h, anaerobically	Nitrate Motility medium and Lactose Gelatin Medium, commercially prepared media from the Southern Group.	Standard methods as listed in Blue book method (motility test, nitrate reduction, lactose fermentation, gelatin liquifaction.		Crossley's Milk Medium
	Intestinal Enterococci	Bile Aesculine Azide Agar: 44 ºC 24h. Catalase test.		Standard methods as listed in Blue book method (Bile aesculin agar)	Kanamycin aesculin azide agar	Kanamycin Aesculin Azide Agar
Comments	*Clostridium perfringens*					Now very infrequent – more commonly in the past
	Intestinal Enterococci				Failure of many isolates to confirm	Now very infrequent – more commonly in the past
Typical range of levels						
Type of sludge where higher frequency	*Clostridium perfringens*	Not analysed at the moment	Sludges			Sludges and soils.
	Intestinal Enterococci				Cattle slurries (10 - 10^3)	Sludges and soils.
Frequency of analysis	*Clostridium perfringens*		51-100			<10 for sludges and soils
	Intestinal Enterococci				20 in cattle slurries	<10 for sludges and soils
Quality control						
Standard control strains		HPA lenticules	*Clostridia perfringens* NCTC 10240	*Enterococcus faecalis* (NCTC), *Enterococcus faecium* (Severn Trent Water Ltd), *Cl. perfringens* type A (NCTC 1265), *Cl. perfringens* Type B (NCTC 3110), *Cl. perfringens* Type C (NCTC 8081), *Cl. perfringens* Type D (NCTC 8503), *Cl. perfringens* Type E (NCTC 8084)	*NCTC 00755 Enterococcus faecalis*	Yes, *Clostridium perfringens* NCTC 8237 and for internal analysis QC a maintained wild type strain specific to Wessex Water. *Enterococcus faecium* NCTC 7171

9 REFERENCES

Abbott, S., Caughley, B., Scott, G. (1998). "Evaluation of Enterolert™ for the enumeration of Enterococci in the marine environment." New Zealand Journal of Marine and Freshwater Research **32**: 505-513.

Adcock, A. W., Saint, C. P. (2001). "Development of glucosidase agar for the confirmation of water-borne Enterococcus." Water Research **35**(17): 4243-4246.

Adcock, A. W., Saint, C. P. (2001). "Rapid confirmation of *Clostridium perfringens* by using Chromogenic and Fluorogenic substrates." Applied and Environmental Microbiology **67**(9): 4382-4384.

Araujo, M., Sueiro, R. A., Gomez, M. J., Garrido, M. J. (2001). "Evaluation of fluorogenic TSCagar for recovering *Clostridium perfringens* in groundwater samples." Water Science and Technology **43**: 201-204.

Araujo, M., Sueiro, R. A., Gomez, M. J., Garrido, M. J. (2004). "Enumeration of *Clostridium perfringens* spores in groundwater samples: comparison of six culture media." Journal of Microbiological Methods **57**: 175-180.

Aspervall, O., Osterman, B., Dittmer, R., Sten, L., Lindback, E., Forsum, U. (2002). "Performance of four chromogenic urine culture media after one or two days of incubation compared with reference media." Journal of Clinical Microbiology **40**(4): 1500-1503.

Audicana, A. P., I.; Borrego, J. J. (1995). "Modification of Kanamycin-Esculin-Azide Agar to Improve Selectivity in the Enumeration of *Faecal Streptococci* from Water Samples." Applied and Environmental Microbiology **61**(12): 4178-4183.

Bogosian, G., Aardema, N. D., Bourneuf, E. V., Morris, P. J. L., O'Neil, J. P. (2000). "Recovery of hydrogen peroxide sensitive culturable cells of *Vibrio vulnificus* give the appearance of resuscitation from a viable nonculturable state." Journal of Bacteriology **182**: 5070-5075.

Bogosian, G., Bourneuf, E. V. (2001). "A matter of life and death." EMBO Reports, European Molecular Biology Organisation **21**(9): 770-774.

Bogosian, G., Sammons, L. E., Morris, P. J. L., O'Neil, J. P., Heitkamp, M. A., Weber, D. B. (1996). "Death of *Eschericia coli* K-12 strain W3110 in soil and water." Applied and Environmental Microbiology **62**: 4114-4120.

Cole, M. B., Davies, K. W., Munro, G., Holyoak, C. D., Kilsby, D. C. (1993). "A vitalistic model to describe the thermal inactivation of *Listeria monocytogenes*." Journal of Industrial Microbiology **12**: 232-239.

Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. *Official Journal of the European Communities*, 5.12.98, L330/32-L330/53

Eckner, K. F. (1998). "Comparison of membrane filtration and multiple-tube fermentation by the Colilert™ and Enterolert™ methods for detection of waterborne coliform bacteria, *Eschericia coli*, and Enterococci used in drinking and bathing water quality monitoring in Southern Sweden." Applied and Environmental Microbiology **64**(8): 3079-3083.

EPA, U. (1997). Method 1600: Membrane filter test methods for enterococci in water. EPA-821/R-97/004, Office of Water, Washington D. C.

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

Ewald, S., Eie, T. (1992). "The effect of resuscitation and the incubation temperature on recovery of uninjured, injured and freeze injured Enterococci." International Journal of Food Microbiology **15**(1-2): 177-184.

Figueras, M. J. I., I.; Polo, F.; Guarro, J. (1998). "Evaluation of the oxolinic acid-esculin-azide medium for the isolation and enumeration of faecal streptococci in a routine monitoring programme for bathing waters." Canadian Journal of Microbiology **44**: 998-1002.

Forsman, M., Henningson, E. W., Larsson, E., Johansson, T., Sandstrom, G. (2000). "*Francisella tularensis* does not manifest virulence in viable but non-culturable state." FEMS Microbiological Ecology **31**(217-224).

Frahm, E., Obst, U. (2003). "Application of the fluorogenic probe technique (TaqMan PCR) to the detection of Enterococcus spp. and *Escherichia coli* in water samples." Journal of Microbiological Methods **52**(1): 123-131.

Hara-Kudo, Y., Ikedo, M., Kodaka, H., Nakagawa, H., Goto, K., Masuda, T., Konuma, H., Kojima, T., Kumagai, S. (2000). "Selective enrichment with a resuscitation step for isolation of freeze-injured *Eschericia coli* 0157:H7 from foods." Applied and Environmental Microbiology **66**(7): 2866-2872.

Idexx cited: Rex, A. C., Wong, L. L., Gofshteyn, M. (2001). "The 24-hour method (EPA Method 1600) significantly undercounts Enterococcus in chlorinated wastewater effluent, compared to 48-hour m-Enterococcus method." American Society for Microbiologists Annual Convention.

ISO (1999a). Water quality - detection and enumeration of Intestinal Enterococci in surface and wastewater - Part 1: Miniaturised method (Most Probable Number) by inoculation in liquid medium. *International Organisation for Standardisation*, Geneva.

Kang, D. H., Siragusa, G. R. (1999). "Agar underlay method for recovery of sub lethally heat-injured bacteria." Applied and Environmental Microbiology **65**(12): 5334-5337.

Kang, D. H., Siragusa, G. R. (2001). "A rapid two fold dilution method for microbial enumeration and resuscitation of injured and sub lethally injured bacteria." Letters in Applied Microbiology **33**: 232-236.

Kinzelman, J., Ng, C., Jackson, E., Gadus, S., Bagley, R. (2003). "Enterococci as indicators of lake Michigan recreational water quality: comparison of two methodologies and their impacts on public health regulatory events." Applied and Environmental Microbiology **69**(1): 92-96.

Liao, C.-H., Shollenberger, L. M. (2004). "Enumeration, resuscitation and infectivity of the sub-lethally injured *Erwinia* cells induced by mild acid treatment." Phytopathology **94**(1): 76-81.

Manero, A., Blanch, A. R. (1999). "Identification of *Enterococcus* spp. with a biochemical key." Applied and Environmental Microbiology **65**(10): 4425-4430.

Manero, A., Vilanova, X., Cerda-Cuellar, M., Blanch, A. R. (2002). "Characterisation of sewage samples by biochemical fingerprinting of Enterococci." Water Research **36**: 2831-2835.

Mascher, F., Hase, C., Moenne-Loccoz, Y., Defago, G. (2000). "The viable but nonculturable state induced by abiotic stress in the biocontrol agent *Pseudomonas fluorescens* CHA0 does not promote persistence in soil." Applied and Environmental Microbiology **66**: 1662-1667.

Massa, S., Brocchi, G. F., Peri, G., Altieri, C., Mammina, C. (2001). "Evaluation of recovery methods to detect faecal streptococci in polluted waters." Letters in Applied Microbiology **32**: 298-302.

Merlino, J., Siarakas, S., Robertson, G. J., Funnell, G. R., Gottleib, T., Bradbury, R. (1996). "Evaluation of CHROMagar Orientation for differentiation and presumptive identification of Gram-negative Bacilli and *Enterococci* species."

Messer, J. W. and A. P. Dufour (1998). "A Rapid, Specific Membrane Filtration Procedure for Enumeration of Enterococci in Recreational Water." Applied and Environmental Microbiology. **64**(2): 678-680.

Rhodes, M. W., Kator, H. (1997). "Enumeration of Enterococcus sp. using a modified mE method." Journal of Applied Microbiology **83**: 120-126.

Sartory, D. P. (2005). Telephone conversation on 22nd February 2005.

Sartory, D. P., Field, M., Curbishley, S. M., Pritchard, A. M. (1998). "Evaluation of two media for the membrane filtration enumeration of *Clostridium perfringens* from water." Letters in Applied Microbiology **27**: 323-345.

Standing Committee of Analysts (2003). "The Microbiology of Sewage Sludge (2003) - Part 2 - Practices and procedures for sampling and sample preparation." Methods for the examination of waters and associated materials. Environment Agency, UK.

Skulnick, M., Mitchell, B., Mazzulli, T., Small, G. W., Low, D. E., Cruz, R. H. (2004). "Cost effectiveness of BBL™ CHROMagar™ Orientation medium for routine urine cultures". 104th General Meeting of the American Society of Microbiology, New Orleans, L.A., USA.

Smith, R. J., Newton, A. T., Proctor, G. P., Harwood, C. R., Barer, M. R. (1999). "Active but non-culturable cells of *Salmonella typhimurium* are not infective in BALB/c mice". 99th General Meeting of the American Society for Microbiology, American Society for Microbiology, USA.

Stevenson, B. S., Eichorst, S. A., Wertz, J. T., Schmidt, T. M., Breznak, J. A. (2004). "New strategies for cultivation and detection of previously uncultured microbes." Applied and Environmental Microbiology **70**(8): 4748-4755.

Warnes, S. L., Keevil, C. W. (2004). "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". Horizontal Hygiene Project. European Union.