Hygienic Parameters Feasibility of Horizontal Standards for *Escherichia coli* and *Salmonella spp.* in Sludges, Soil, Soil Improvers, Growing Media and Biowastes

K. Clive Thompson

Richard Shepherd

Andrew Hockin

ALcontrol Laboratories

Email – clive.thompson@alcontrol.co.uk

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The report has evaluated the current existing methods available for the detection and enumeration of *Escherichia coli* and *Salmonella* spp. with a view to implementing horizontal standardisation.

The formulation of a horizontal standard to cover sludge, soil, soil improvers, growing media, and biowaste is considered feasible, however there are several key areas which require resolution through ruggedness and interlaboratory testing.
The CEN TC308/WG1/TG5 group recommended the following matrices sludge, soil, soil improvers, growing media, and biowaste which are the specifically mentioned matrices in the six draft CEN microbiological standards be targeted. These six methods have now been submitted to CEN for acceptance as Technical Reports rather than draft international standards because they lack sufficient validation data. Without some additional funding, it will be impossible to validate these six methods across Europe

A key issue highlighted from the report is that it is much more difficult to assess the effectiveness and recovery rates achieved during sample preparation than it is during the detection stage. The first stage, which involves separating the bacteria from the solids present in the matrix, is much harder to evaluate. This should be addressed in the proposed FP 6 study.

With regard to the detection stage of the methods it is essential to have a stable reference material in order to obtain a microbiological standard to check the effectiveness of the methods. The MICROCRM conference recently held in Lille discussed the issue of commercially available microbiological reference materials. Also reference materials if prepared in a solid dry matrix simulating the matrix being tested could in theory be used for crudely assessing the effectiveness of the sample preparation stage. Some work is being carried out in the UK on this aspect. In our view, it is essential to have a Europe-wide validation of the methods. It is of no use to test the robustness of the methods on a small scale just with a few prestigious non-routine research institute type laboratories.

There has to be an improvement in the level and quality of the performance data attached to the methods. The advent of proficiency schemes for sludge, soil, soil improvers, growing media, and biowaste matrices should be actively encouraged. The setting up of a Europe-wide scheme would make it possible to assess the extent of variation in testing between laboratories.

It would be useful to arrange a large-scale round-robin exercise that would compare the commercially developed methods against the conventional six draft CEN methods. The main advantage of the commercial methods is that they do involve as many variables as the CEN standards and should be more robust. If it is found that they show significantly better performance data we should recommend them as an acceptable alternative approach. The more rapid analysis turnround time to a confirmed result for most of these commercial methods is another advantage.

It should be stated that the commercial methods have ***not*** been put forward for approval as standards, fully validated commercial methods should be incorporated as informative annexes. The commercial methods must be fully validated against the relevant final CEN standard(s)

before use in laboratory analysis. (See Appendix 5). A summary of commercial Escherichia coli and Salmonella test kits referenced in the document is given in Appendix 6

The rate of throughput for the methods varies between the rapid methods developed commercially by IDEXX, Matrix Micro-science, and Merck, which are able to generate confirmed results within 24 hours. The draft CEN standards use more traditional methods, which can take up to three to four days to provide a confirmed result.

The draft sludge directive quotes a *Salmonella spp.* detection limit of absence of any viable *Salmonella* spp. in 50g of received material. Of the three methods proposed, the presence/absence method has the best potential limit of detection of ~1 viable organism in 10g of received material. The membrane filtration methods is limited to thin sludges with less than 10 –20 % dry solids content, it has to be questioned whether or not the limit of detection specified in the directive is achievable for *Salmonella spp.*

An interesting thought is to question why the draft limit for sludge testing is absence in 50g and yet the limit in food microbiology is absence in 25g?

The membrane filtration Salmonella method is primarily concerned with log reduction measurement determination and only involves a temperature resuscitation step unlike the other two draft *Salmonella* methods.

The six draft CEN methods for *Salmonella* (3) and *Escherichia coli* (3) have been sent to CEN for approval as technical reports rather than draft standards due to the lack of sufficient validation data.
In order to take the methods through to horizontal standards it is essential that thorough testing of all the proposed the methods is carried out (**See Appendix 5**). The FP6 Horizontal-HYG project should hold the key to a lot of the questions raised in this desk study.

K. Clive Thompson
Richard Shepherd
Andrew Hockin

12th Feb 2004

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

0 GENERAL INTRODUCTION

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

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

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

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

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

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

It is not only necessary to make methods available to determine specific micro-organisms, but also to provide a detailed protocol for sampling heterogeneous matrices such as sludges, soils, soil improvers, growing media, and biowastes to obtain fit for purpose results. Results are needed for validating plant performance (percentage pathogen reduction) and end product specification in terms of hygienic microbiological parameters (e.g. EU 2000). This will include co- and pre-normative research, including consideration of carrying out method validation for complementary bacterial indicators (e.g. *Enterococci* and *Clostridium perfringens*), and viable helminth ova (cestodes and nematodes). For parameters likely to be included in future Directives (i.e. *E. coli* and *Salmonella* spp.), the selected methods will be assessed in large

Europe-wide interlaboratory trials involving many European countries. For other parameters, there is a need to develop preliminary standards in order to carry out the relevant research. In the sludge and Biowaste draft directives (EU 2000 and EU 2001), *E. coli* and S*almonella* are specifically mentioned. This leads to the logical choice to start the work on these organisms as one of the parameters in phase 1 of project Horizontal. For the other parameters, Project Horizontal desk studies 4 and 5 of WP3 to prepare draft potential protocols for CEN and ISO discussion are also being prepared.

1. INTRODUCTION

1.1 *Scope of the Report*

1.1.1 This report is one of four Project Horizontal reports that attempt to assess hygienic parameters, which may be needed to assure the sanitisation of sludges, soil, soil improvers, growing media and biowaste. The four reports highlight draft potential methods for the hygienic parameters likely to be included in future sludges, soil, soil improvers, growing media and biowaste directives. In relation to this report, CEN/TC308/WG1/TG5 has put together draft methods for the detection and enumeration of *Escherichia coli* and *Salmonella spp*. in sludges, soil, soil improvers, growing media and biowaste. The selected methods are mainly adapted from standard methods for the examination of water. In order to ensure that fit for purpose microbiological results can be obtained for a wide range of sludges, soil, soil improvers, growing media and biowaste materials Project Horizontal has to carry out co-normative research work to develop suitable international standards. The validation of these standards will be achieved by carrying out interlaboratory trial(s) with participation of a number of experienced European laboratories. Such validation requires application of the draft standards to a wide range of real sludges, soil, soil improvers, growing media and biowaste samples.

1.1.2 It is not only necessary to make methods available to determine specific micro-organisms, but also to provide a detailed protocol for sampling heterogeneous matrices such as sludges, soil, soil improvers, growing media and biowastes to obtain fit for purpose results. Results are needed for validating plant performance (percentage pathogen reduction) and end product specification in terms of hygienic microbiological parameters (e.g. EU 1 2000). This will include co- and pre-normative research, including consideration of carrying out method validation for complementary bacterial indicators (e.g. *Enterococci* and *Clostridium perfringens*), and helminth ova (cestodes and nematodes). For parameters likely to be included in future Directives (i.e. *E. coli*, *Salmonella spp.* and *Clostridium perfringens*), the selected methods will be assessed in large Europe-wide interlaboratory trials involving many European countries. For other parameters, there is a need to develop preliminary standards in order to carry out the relevant research. ***Appendix 2*** quotes some relevant sections of the draft sludge, soil, soil improvers, growing media and biowaste directives to give some indication of the type of measurements and microbiological species serovars that are to be covered and the likely analysis limits of detection and specified log reductions to be assessed.

1.1.3 In the sludge, soil, soil improvers, growing media and biowaste draft directives, [EU 2000 and EU 2 2001] *E. coli*, *Salmonella spp.*, and *Clostridium perfringens* are specifically mentioned. This leads to the logical choice to start the work on these parameters in phase 1. For the other parameters desk studies to prepare draft potential protocols for CEN and ISO discussion are prepared. *Clostridia perfringens* is outside the remit of this report and is been addressed in a desk study (No. 4.3.4) by Keevil and Warnes from Southampton University.

In this report, the methods currently available on assessing *Escherichia coli* and *Salmonella spp.* in sludges, soil, soil improvers, growing media and biowaste are evaluated. The identification of key points where possible differences exist between different methods are studied and are then evaluated to establish to what extent such perceived differences can be overcome and a draft standard prepared with annotations to facilitate horizontal standardisation.

1.2 Sample Matrices Considerations

1.2.1 It is assumed that a method should be fit for purpose for a range of sludges, soils, soil improvers, growing media and biowastes. The Horizontal-HYG FP6 project group have recommended that the following nine matrices should be included in the proposed validation trial: -

1.2.2 Manures; food wastes; paper wastes, slaughterhouse wastes and water works sludges would not be included

1.2.3 *E. coli* or *Salmonella spp.* in a soil invariably originate from added sludge, biowaste or from direct addition of animal or bird faecal material. It has been shown that the numbers of *E. coli* in sludge amended soil rapidly decline and are indistinguishable from natural background populations in soil within three months of applying sludge irrespective of the numbers initially present in the sludge, the time/season of application or environmental conditions and soil (Lang et al. 2003).

1.2.4 Agricultural soils are generally coarser and contain much less organic matter than sludges, composts or biowaste materials and any method capable of providing fit for purpose results on sludges, composts and biowastes should, almost certainly, be applicable to agricultural soil matrices. There is a wide range of soil types (e.g. sandy, clay, loam, peaty etc) and it would be difficult to justify inclusion of all potential soil

types in any standard (method) validation exercise.

1.2.5 Laboratories carrying out microbiological soil analysis should validate their method for the range of soil types analysed being sure to include "worst-case" soil matrices.

1.2.6 It is also contended that it will be impossible to carry out standard (method) validation studies to cover all potential sludge, compost, soil and biowaste matrices. A pragmatic approach where a number of "worst-case" matrices are selected should be adopted.

1.2.7 Untreated biowastes should also be amenable to any proposed standard (method) assuming suitable size reduction/homogenisation steps are employed followed by a suitable bacterial extraction and solids handling steps are incorporated into the method.

1.2.8 The final analytical procedure for culturing, identifying and enumerating the target bacteria should apply to all sample matrices (e.g. sludges, soil, soil improvers, growing media and biowaste) after suitable pre-treatment steps. The issue of how to rigidly define empirical pre-treatment protocols needs to be addressed.

1.2.9 This report does not cover the taking of representative samples for micro-biological analysis. This topic should be addressed by a future separate report on the sampling aspects for microbiological analysis of sludges, soils, soil improvers, growing media and biowastes. This appears to be a neglected area.

1.2.10 For this report it is assumed that the sub-sample received at the laboratory is representative of the bulk material being sampled.

1.2.11 It is important to appreciate that often not only are the bulk materials of interest heterogeneous with respect to the physical size of the solid matter present but also with respect to microbial content. Localised parts of a bulk material may have *E. coli* counts per g up to 10^8 times higher than closely adjacent parts of the bulk material (e.g. contamination by animal or bird faecal material). This can make representative sampling very difficult.

1.3 Equivalence of Method Considerations

1.3.1 *E. coli* or *Salmonella spp*. methods for sludges, soils, soil improvers, growing media and biowastes can be subdivided into four separate stages after receipt of the samples:-

(i) Particle size reduction (if required) and sample homogenisation

(ii) Extraction of the bacteria into the liquid phase

(iii) Removal of excess solids (if required) to allow the final bacterial measurement phase to function efficiently.

(iv) The final bacterial measurement stage.

Note: Losses of viable target organisms can occur during any of these four stages.

1.3.2 It is contended that subject to satisfactory performance of the first three stages, most robust analytical microbiological procedures for the final measurement stage should be fit for purpose for suitable pre-treated sludge, soil, soil improvers, growing media and biowaste samples.

1.3.3 It is important to appreciate that proficiency testing scheme analysis is carried out on homogenised samples that readily disintegrate on contract with water (or Maximum Recovery Diluent (MRD)). Consequently, only the final bacterial measurement stage of the method is actually tested. Thus the performance indicated by proficiency sample results (as described later in this report) is considered optimistic with respect to the situation with the real (heterogeneous) samples actually received by laboratories.

1.3.4 The larger the typical particle size and the more heterogeneous the received sample, the worse the repeatability/reproducibility of the expected results.

1.4 Particle Size Reduction and Sample Homogenisation Considerations

1.4.1 For non-labile conservative chemical parameters, the sludge, soil, soil improvers, growing media or biowaste sample is normally air-dried (30 – 105^{0}C) and then a representative portion is ground to less than 200 micron particle size. A sub-sample of the ground homogeneous sample is thus taken for analysis. This type of protocol has been shown to give consistent repeatable and reproducible results.

1.4.2 It is not practicable to dry samples or subject them to heat generating processes such as grinding for microbiological analysis as it will stress or kill the target bacterial. This, therefore, limits the range of pre-treatment techniques that can be used.

1.4.3 It is contended that sludge and biowastes closely resemble foods, thus it is recommended that well-established techniques used in food microbiological routine analysis should be adapted for use in sludge, (soil, soil improvers and growing media)

and biowaste analysis.

1.4.4 The most common method for particle size reduction sample homogenisation for foods is a Stomacher® device. The sample is added to a diluent in a 1 in 10 dilution. The device operates by using two rotating paddles which homogenise the food through a crushing motion. The Stomacher® is ideal for a food or sludge / biowaste matrices as it can be easily cleaned and sterilised to prevent cross-contamination.

Other suitable devices include the use of a Pulsifier®, and the use of a blender device with cutting blades. (See below.)

1.5 Extraction of the Bacteria into the Liquid Phase

1.5.1 Again the techniques employed by routine food microbiological laboratories are considered suitable for solid and semi-solid sludge, soil, soil improvers, growing media and biowaste materials.

1.5.2 The main potential techniques are considered to be:-

(i) Conventional mechanised agitation (e.g. Stomacher®).

(ii) Pulsifier® – a relatively new principle in the preparation of microbial suspensions. Instead of the familiar paddle action which crushes samples, a Pulsifier® beats the outside of the bag at high frequency, producing a combination of shockwaves and intense stirring which drives microbes into suspension, whilst causing minimal dispersion of the sample.

(iii) Blender – not usually used as a routine technique for bacteria extraction, the technique is occasionally used to breakdown tough and fibrous matrices such as meat. The sample is subjected to disintegration from corrosion resistant metal cutting blades.

1.5.3 For all these techniques, tests should be carried out to demonstrate that the mechanical action and any associated heat generation within the sample do not significantly affect the target microbial population especially with respect to stressed bacteria. Tests should

also be carried out to ensure that the pre-treatment processes are optimised with respect to agitation energy and time to achieve maximum recovery of the target bacteria.

1.6 ***Removal of Excess Solids from the Initial Sample Extract***

1.6.1 This is only normally required for membrane filtration methods.

Due to the high content of solids in the test matrices, membrane filtration becomes difficult as membrane filters become blocked by solid particles present in the filtered sample aliquot. The amount of excess solids in the sample can be reduced by allowing the sample to settle so that any solids present in the sample settle at the bottom of the bottle. Serial dilutions can also be used to decrease the amount of solids present in the sample; the level of solids will decrease with every dilution.

1.7 ***Validation of Sample pre-treatment Protocols for Relevant Matrices***

1.7.1 This is a complex area and it is important that the uncertainty associated with the sample pre-treatment stage can be differentiated from the final measurement stage. This will then ensure that both major components of the method (pre-treatment and final measurement stages) can be independently optimised.

1.7.2 The main problems with evaluating the full sample pre-treatment stage of a method are considered to be:-

(i) Recovery of stressed bacterial (viability issues).

(ii) Taking representative sub-sample liquids from the received often very inhomogeneous sample for analysis.

(iii) Ensuring lack of cross contamination from the apparatus used to prepare the samples (unlike most food sample some sludges, soils, soil improvers, growing media and biowastes can contain very significant numbers of *E. coli*).

(iv) Covering the wide range of sample types that the Horizontal project wishes to encompass.

(v) Covering all the major relevant serovars of the target organism.

(vi) Covering the inherent sample stability issues.

1.7.3 It can clearly be seen, that unlike chemical methods, it is very difficult to fully validate a microbiological method for a wide range of inhomogeneous materials. A compromise must be reached that allows a realistic validation study at an economic price to be carried out.

1.7.4 There appears to be very little data in the scientific literature on the variability (uncertainty) associated with the sample pre-treatment stage of microbiological analysis of sludges, soils, soil improvers, growing media and biowastes.

1.7.5 Realistically, the sample pre-treatment stage of a standard (method) can only validated by individual laboratories on a range of their typical samples by repeatability tests on bulk samples with some spiking recovery trials on samples containing low numbers of target organisms.

1.7.6 During development of the standard, tests should have been carried out on worst-case matrices to optimise the time and degree of mechanical action needed for stages (i) & (ii) of the sample pre-treatment stages. (See 1.3.1).

1.7.7 It is contended that it is only practicable to attempt to validate the final measurement stage of a standard (method) across a number of laboratories in different countries. Although thus is also true for chemical analysis, much more robust and vigorous sample homogenisation and pre-treatment stages can be employed for the vast majority of chemical methods leading to much less uncertainty with respect to the sample homogenisation pre-treatment stage of any proposed method.

1.7.8 It is worth reiterating the microbiological methods are empirical and the result to a large extent is dictated by the method rather than there being a fixed absolute number of viable, culturable target bacteria in a sample.

1.7.9 The proposed international standard (method) validation trial should be able to evaluate the final bacterial measurement stage and indicate whether any components extracted from the distributed homogeneous samples has a significant effect on the recovery of the target bacteria.

1.8 Method Selection

1.8.1 Following a meeting of the members of work package 3: Hygienic Parameters, it was decided that a letter outlining the project be sent to all member states. The aim of the

letter was to obtain feedback from the member states through TC308 regarding all accepted national or international standards. With regard to this report all methods for the detection and enumeration of *Escherichia coli* and *Salmonella spp.* in sludge, soil, soil improvers, growing media, and biowastes. Any validated standards for water and wastewater on *Escherichia coli* and *Salmonella spp*. were also requested. The member states were also asked if there were any promising new or rapid methods developed or undergoing development regarding *Escherichia coli* and *Salmonella spp.* detection and enumeration across the relevant matrices.

1.8.2 In order not to exclude the smaller European countries from the study, effort was also made to contact all those members listed on the CEN / ISO voting list. This enabled the any validated methods from those countries to be included in the report. The letter sent out to member states included an introduction to the project "Horizontal" so that it gave members an understanding of what the objectives were. The request for method information also included background on Work Package 3.

1.9 *Request for Information*

A copy of the letter sent out is given in ***Figure 1***

Figure 1 Letter Seeking Information and Outlining the Horizontal Project

ALcontrol Laboratories
Templeborough House
Mill Close
Rotherham
South Yorkshire, S60 1BZ
United Kingdom

8th March 2003

Ref: L250-GA14424

Dear Sir/Madam

Re: - Information needed for the Oslo meeting of TG5 27th March 2003 for the Horizontal Project

With respect to the project "HORIZONTAL", we are presently carrying out desk studies in order to gather the relevant information on existing data concerning microbiological parameters in sludges, soil, soil improvers, growing media and biowaste. You will find below in the appended Annex some information about the project and the specific objectives of Work Package 3: "Hygienic parameters".

Please could you kindly provide information on any or all of the following: -

1. Established validated national or international standards methods for *E. coli*, *Salmonella or Clostridium perfringens* in sludge, soil, compost and biowaste
2. Established validated national or international standards methods for *E. coli*, *Salmonella or Clostridium perfringens* in water/wastewater
3. Any methods for helminth ova (nematodes and cestodes) in waters, wastewaters, sludge, soil, compost or biowaste
4. Any established or novel rapid method (significantly more rapid than the relevant culture method) for *E. coli* or *Salmonella*
5. Any local or national study on the typical levels of pathogens or indicators in sewage sludge, soil compost or biowaste.
6. Laboratories (including appropriate contact) to take part in interlaboratory trials to validate the proposed *E. coli* and *Salmonella* standards for sludge, soil, compost and treated biowaste.

We would appreciate your E-mail response to the undersigned by Monday 24th March 2003, so that it can be discussed at our Oslo meeting of CEN/TC308/WG1 that will take place from March 26th to 28th.

Thanking you in anticipation

Yours faithfully

K Clive Thompson
Secretary TC 308/WG1/TG5

Marie-Renee de Roubin
Convenor TC 308/WG1/TG5

clive.thompson@alcontrol.co.uk

marie-renee.de-roubin@generale-des-eaux.net

1.10 *Responses to Request*

1.10.1 Responses were requested before the meeting of TC308 in Oslo on 29th March 2003 so that all submitted methods from the member states could be discussed at the meeting. From the letter sent out after the meeting requesting information on WP3; Hygienic Parameters for *Escherichia coli* and *Salmonella spp.*, there was a very limited response. The only responses we had were from Simon Cole; Chairman of the Standing Committee of Analysts – Sludge Microbiology Group, and David Sartory of Severn Trent Water. These responses are appended as ***Appendices 3 and 4.*** (David Sartory was also sent copies of the MF and MPN draft CEN methods to comment upon.)

1.10.2 The six draft CEN standards for *Escherichia coli* and *Salmonella spp.* were obtained through TC 308/WG1/TG5 and are covered in this report as they are the only potential non-commercial methods upon which some method validation has been or is likely to be performed in the near future. (See also Last para in Executive Summary).

1.10.3 The other methods made available for the study were therefore collated by contacting the relevant companies such as IDEXX Laboratories with regard to the Colilert® *E. coli* and coliforms method, and the Environment Agency, Standard Committee of Analysts (SCA) for copies of their draft methods for *Escherichia coli* and *Salmonella spp.* in sludges, soil, soil improvers, growing media and biowastes.

1.10.4 The Matrix MicroScience Ltd. Pathatrix® method for *Salmonella spp.* was also obtained through direct contact with the company. This method is in routine use in food microbiology and is thought to eminently transferable for use with sludges, soil, soil improvers, growing media and biowaste matrices. Data from internal and external validation studies e.g. AOAC trials have demonstrated that the Pathatrix® system is significantly more sensitive than many of the current standard methods, at low spike levels 1-10 cfu/25g sample. It is felt that this would be the only method that could potentially reach the absence of *Salmonella spp.* in 50g of as received sludge quoted in the third draft of the sludge directive. (EU 1 2000). The Pathatrix® system takes just 16 hours to identify *Salmonella spp.*, compared with between two and four days taken by existing conventional methods. Indications are that the method gives good recovery of stressed *Salmonella spp.* as harsh selective media are not required.

1.10.5 The Pathatrix® *Salmonella spp*. system has been reviewed under the AOAC Research Institute's Performance Tested Methods K Program, and found to perform as stated by the manufacturer. This certificate authorizes the manufacturer to display the AOAC Performance Tested K certification mark along with the statement - "This test kit's

performance was reviewed by AOAC research institute and was found to perform to the manufacturer's specifications"

1.10.6 One Canadian and one American method were also included in the report. The Canadian A-1 method and the U.S. EPA MTF method (EPA 1978) to detect and enumerate *Escherichia coli* from wastewater solids are described.

1.10.7 No other responses were received to date. (20th Jan 2004).

2. EXISTING STANDARDS OR DRAFT STANDARDS [1]

2.1 List of Methods – Escherichia coli

Following the letter that was distributed to member states and the two replies that were received, the methods listed in Table 1 were selected for consideration for *Escherichia coli* detection and enumeration standards. These methods are therefore included in this report to assess the feasibility of horizontal standards for *E. coli*.

Table 1: Draft CEN Sludge E. coli Methods

1.) CEN (2003a) Draft Standard: - Characterisation of sludges, soils, soil improvers, growing media and biowastes – Detection of *Escherichia coli* – Part 1: Membrane filtration method for quantification (MLGA)

2.) CEN (2003b) Detection and enumeration of *Escherichia coli* in sludges, soils, soil improvers, growing media and biowastes. Part 2:Miniaturised method (MPN) by inoculation in liquid medium

3.) CEN (2003c) Detection and enumeration of *Escherichia coli* in sludges, soils, soil improvers, growing media and biowastes. Part 3: Macromethod (MPN) in liquid medium.

Table 2: Other Membrane Filtration Water E. coli Methods

4.) ISO 16649-1: (2001) - Detection and enumeration of *Escherichia coli* – Membrane filtration method using Chromocult® agar

5.) SCA (2002) The Microbiology of Drinking Water – Part 4 : Detection and enumeration of *Escherichia coli* – Membrane filtration method using MLSA agar

6.) SCA (2002) The Microbiology of Drinking Water – Part 4 : Detection and enumeration of *Escherichia coli* – Membrane filtration method using MLSB Agar

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[1] ***New Zealand Standard E. coli Sludge Methods – late received information***
E. coli Part 9221E or Part 9223B Standard Methods for the Examination of Water and Wastewater (APHA, 1998)

Table 3: Other Non-membrane Filtration Sludge E. coli Methods

7.) Andrews and Presnell (1990): The A-1 Method Greater Vancouver Regional Council (GVRD): Multiple Tube Fermentation (MTF) technique to detect and enumerate *Escherichia coli* in biosolids

8.) Colilert®, IDEXX Laboratories Ltd.(2003), IDEXX Colilert® Method – Enumeration of Coliform and *Escherichia coli* bacteria in waste water solids using defined substrate technology

9.) APHA (1998), USEPA – MTF technique for the detection and enumeration of *Escherichia coli* in waste activated solids EPA-600/8-78-017.

Table 4 UK Standing Committee of Analysts (SCA) Sludge E. coli Methods

(Available on the web from http://www.environment-agency.gov.co.uk/science/219094/399393/)

10.) SCA (2003a) Microbiology of Sewage Sludge Part 3 Method A (2003) isolation and enumeration of *Escherichia coli* using a chromogenic membrane filtration technique.

11.) SCA (2003b) Microbiology of Sewage Sludge Part 3 Method B (2003) isolation and enumeration of *Escherichia coli* using a Multiple Tube MPN technique.

12.) SCA (2003c) Microbiology of Sewage Sludge Part 3 Method C (2003) The enumeration of *Escherichia coli* by a defined substrate MPN technique.

2.2 *Discussion of the E. coli Methods*

2.2.1 The existing standards for *Escherichia coli* put forward for inclusion in the report rely on four main methods; membrane filtration, multiple tube fermentation (MTF) miniaturised MPN method, MTF macro MPN method, and the commercially available Colilert® defined substrate method from IDEXX laboratories.

2.2.2 The membrane filtration method is useful for enumerating the target organism in this case *Escherichia coli* and *Salmonella spp.* through culturing colonies on the membrane and simply counting them. The use of serial dilutions enables the test material to pass

through the membrane at various diluted levels to obtain the optimum count number on the membrane. (20 – 300)

2.2.3 There are five membrane filtration (MF) methods to consider and all vary in terms of the media used to detect and enumerate the target organism. The MF method listed as the draft CEN standard uses MLGA (Membrane Lactose Glucuronide Agar) as a chromogenic agar to detect the *Escherichia coli* a member of the family *Enterobacteriaceae*, •-glucuronidase-positive and able to hydrolyse 5-bromo-4-chloro-3-indolyl-•-glucuronide (BCIG) when growing on an agar medium at the temperature of 44°C. This results in the *Escherichia coli* colony producing a blue / green pigment which distinguishes it from other coliforms present.

2.2.4 The Chromocult® method (Merck 2002) uses Chromocult® TBX (Tryptone Bile X-glucuronide) Agar which relies on a similar principle to MLGA, the presence of the enzyme β-D-glucuronidase differentiates *Escherichia coli* from other coliforms. *Escherichia coli* absorbs the chromogenic substrate (X-β-D-glucuronide, the growth of accompanying Gram-positive flora is largely inhibited by the use of bile salts and the high incubation temperature of 44°C. The *Escherichia coli* colonies are distinguished from the coliforms by a blue / green appearance on the membrane. Furthermore Chromocult® TBX Agar complies with the ISO/DIS Standard 16649 for the Enumeration of *E. coli* in food and animal feeding stuffs. (ISO 16649-1 (2001): and ISO 16649-2 (2001))

2.2.5 For the other two membrane filtration methods MLSA and Membrane Lauryl Sulphate Broth (MLSB), the samples are incubated (resuscitated) at 30°C for 4 hours and at 44°C for a further 14 hours. The media is not able to distinguish between coliform bacteria and *Escherichia coli* chromogenically. Therefore, confirmation of colonies coloured yellow is required to determine the total number of *E. coli* present in the sample. The confirmation techniques are the same for both MLSA and MLSB. The only difference between the methods is that one uses an agar and the other uses a broth. The target colonies are put through three confirmatory tests. The oxidase test determines whether the colony is oxidase positive or oxidase negative, *Escherichia coli* is oxidase negative. The second test is a lactose peptone tryptone water test, this should be strongly positive (+++) for *Escherichia coli*.

2.2.6 An API 20E® test available from Biomerieux™ can also be used to confirm the target organism in the sample. The latter above two methods are slower in providing a confirmed result for *Escherichia coli* due to the need for confirmatory tests to be carried

out. The MLGA and the Chromocult® methods are able to provide the laboratory with a confirmed result for *Escherichia coli* within 24 hours such is the specificity of the media used. The MLGA and Chromocult® methods were developed with this quicker timescale in mind and therefore have become the preferred method for *E. coli* / coliforms used in membrane filtration.

2.2.7 With regard to MTF analysis, there are also five methods included for discussion in this report. The two draft CEN standards for *Escherichia coli* detection and enumeration in sludges are included (CEN TC308/WG1/TG5 (2003 a and b)) Part 2: Miniaturised method (MPN) in liquid medium, and Part 3: Macro method (MPN) in liquid medium. There are also UK SCA versions of the two draft CEN standards. (SCA 2003 a and b). In addition there are standards included from Canada and the United States. The A-1 method from the GVRD (Greater Vancouver Regional District) uses MTF analysis to enumerate *Escherichia coli* in bio-solids. The U.S. method from the EPA-600/8-78-017, MTF technique for the detection and enumeration of *Escherichia coli* in waste activated solids. The methods included all rely on the same principle of MPN which can be used in several formats. ISO 8199 (2003) details protocol on working out MPN values and states this can be done by using Mathematical formulae, use of MPN tables, and computer programs. There are slight differences between the methods listed in terms of incubation times and ease of use for example. The A-1 method, currently in use in Canada, requires no enrichment step using LTB making it easier to use and quicker in terms of incubation times. The U.S. version of the MTF technique requires three phases of the test; the presumptive phase, the confirmed phase, and the completed phase. This makes the test longer both in terms of procedure and incubation times. The two draft CEN standards Part 2 and Part 3 use a primary suspension stage which precedes the serial dilution step. Part 2: the miniaturised method then uses a micro plate and incubates for 36 hours at 44°C, Part 3: the macro method splits the sample into 3 tubes containing MUG Fluorocult® LSB and incubates for 40 hours at 44°C. The five tests all differ in terms of speed of test, ease of use, cost of consumables, and accuracy.

2.2.8 The last method included in the report is the IDEXX Colilert® method for the detection and enumeration of coliform bacteria and *Escherichia coli* from waste water solids using Defined Substrate Technology® which is in routine use in a number of European laboratories. For this reason, this method is included in this desk study. The IDEXX Quantitray® and Quantitray/2000® provide easy, rapid and accurate counts of coliforms, *E. coli* and *Enterococci*. The IDEXX Quantitray® and Quantitray/2000® are semi-automated quantification methods based on the Standard Methods Most Probable

Number (MPN) model. The Quantitray® Sealer automatically distributes the sample/reagent mixture into separate wells. After incubation, the number of positive wells is converted to an MPN using a table provided. Quantitray® (50 wells) provides counts from one to 200/100 ml. Quantitray/2000® counts from one to 2,419/100 ml. Total hands-on time is less than one minute per test. The Quanti-Tray/2000® is the preferred option for sludges, soil, soil improvers, growing media and biowaste analysis because of its wider counting range.

2.2.9 The method relies on the use of commercially developed reagents and associated simple equipment to detect and enumerate the target organism. Colilert® is based on the Defined Substrate Technology® (DST). DST® utilises indicator nutrients which cause target microbes contained in the sample and incubated in the DST® reagent system to produce a colour change (or another change, e.g. expression of fluorescence), both indicating and confirming their presence. The indicator-nutrient is cleaved by the target microbe which metabolises the nutrient and frees the indicator to express a different colour or generate a fluorescence signal. The growth and reproduction process of the target microbe is fuelled by the specific carbon source nutrients. Colilert® is a specially designed reagent formulation of salts, nitrogen, and carbon sources that are specific to total coliforms. It provides specific indicator nutrients: ONPG (O-Nitrophenyl-β-d-galactopyranoside) and MUG (4-Methylumbelliferyl-β-d-glucuronide) for the target microbes, total coliforms and *Escherichia coli* respectively. As these nutrients are metabolised, yellow colour (from ONPG) and fluorescence (from MUG) are released confirming the presence of total coliforms and *Escherichia coli* respectively. Non-coliform bacteria are suppressed and cannot metabolise the two indicator nutrients. Consequently, they do not interfere with the specific identification of the target microbes during the test incubation period.

2.2.10 The method uses preparation techniques similar to all the methods whereby the sample is homogenised and added to buffered dilution water. From this stage the sample is diluted into dilutions A, B, and C. The dilutions are made up by adding 50g of Waste Activated Solid (WAS) to 450ml of sterilised buffered water which is then mixed thoroughly (0.1g of original sample per ml of mixture). From this mixture dilution A (0.001g of original sample per ml), dilution B (0.00001g of original sample per ml), and dilution C (0.0000001g of original sample per ml) are set up. From this stage 100ml of each dilution is mixed with the Colilert® media and added to the Quantitray® package. It is then sealed and incubated at 35°C for 24 hours. Kramer and Liu (2002) details how

the Colilert® method performed in trials against the US-EPA multiple tube fermentation (MTF) method in detecting and enumerating *Escherichia coli* from waste activated solids (WAS). The paper states there is no significant difference between the Colilert® method and the US-EPA multiple tube fermentation technique for WAS samples.

2.2.11 There are two other *Escherichia coli* methods which may be applied to a sludge, soil, soil improver, growing media or biowaste matrix. The methods are listed as international standard documents ISO 16649-1 and 16649-2 (2001) Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli*. The methods are split into Part 1: Colony count technique at 44°C using membranes and 5-bromo-4-chloro-3-indolylβ-D-glucuronide, and Part 2: Colony count technique at 44°C using 5bromo-4-chloro-3-indolylβ-D-glucuronide, but without a membrane filtration pre-step.

2.2.12 The two methods work on the same principle whereby duplicate plates of tryptone-bile-glucuronic medium (TBX) are inoculated with 1ml of the test sample and duplicate dilutions of the test sample. The dishes are incubated for between 18-24 hours at 44°C, the plates are then examined to detect the presence of colonies which exhibit characteristics of β-glucuronidase-positive *Escherichia coli*. This is defined as the formation of a typical blue colony on the TBX medium.

2.2.13 ISO 16649-1 Part 1 uses a membrane filtration pre-step not used in Part 2, 1ml of the test sample or initial suspension is inoculated onto cellulose membranes. The membranes are overlaid on mineral modified glutamate agar (MMGA), then incubated at 37°C for 4 hours. The principle then follows that of ISO 16649-2 Part 2 using TBX.

2.2.14 Although the methods are specific to the microbiology of food and animal feeding stuffs, the principle of the methods may be applied for possible use on a sludge, soil, soil improvers, growing media or biowaste matrix.

2.3 Review of E. coli Performance Data

2.3.1 The main point highlighted through analysing the current methods available for detecting and enumerating *Escherichia coli* in sludge is that though there are different techniques used, the level of performance data attached to illustrate the ability of the method is of a varying standard and depth.

2.3.2 Detection and Enumeration of *Escherichia coli* in sludges, soils, soil improvers, growing media and biowaste Part 1: Membrane filtration for quantification (CEN TC308/WG1/TG5(2003a)) has two pages of performance data attached to this standard. The performance data deals with an interlaboratory trial which took place in 1998, where a single bulk sample of a mesophilic anaerobic digested sludge was collected from a participating survey site. The bulk sample was split into individual samples of 200g and sent to each of the 11 participating laboratories by midday the next day. The trial report does not mention the storage conditions of the sample with regard to temperature, and any information about the variation in time of transit between the 11 laboratories. This could affect the viability of the study before the sample has arrived at the laboratories due to the changeable nature of the sludge matrix. The summary (Fig.2) of the trial looks at the Log_{10} *Escherichia coli* / 100g, and percentage (%) dry solids. The data for the trial has a SD of 0.66 for Log_{10} *Escherichia coli* and a SD of 0.14% for % dry solids; however the size of the trial is small. The trial data is considered inadequate and more detail (e.g. raw data) should be included. There is little point in giving just mean data for a routine sample (see Fig 2), and more information regarding geographical position of each participating laboratory could usefully be included. More information about the robustness of the standard could be realised from a larger trial encompassing more laboratories and more replicates. The fact that the data only deals with the analysis of one sample is only a snap shot of the capability of the method for a mesophilic digested sludge. Presscake, limed presscake, thermally dried and composted sludges were not included in this trial. A log_{10} SD of 0.66 would result in the 95% confidence limits covering a range of ~25 – 10,000 for a sample with a nominal concentration of 500 cfu/g of *E. coli*

2.3.3 Detection and Enumeration of sludges, soils, soil improvers, growing media and biowaste Part 2: miniaturised method (MPN) by inoculation in liquid medium (CEN TC308/WG1/TG5 (2003b) has two pages of performance data attached to the method. The performance data outlines an interlaboratory trial carried out in 2001 comparing the micro plate MPN technique with the enumeration on agar plates on Chromocult® media. Thirteen laboratories took part in the trial, working with five different sludge materials. The identity of the participating laboratories is not stated in the annex of the attached performance data. There is a summary of results (Fig.3) illustrating the quantification limits for each technique for the MPN method (after discarding some high results) 410 *Escherichia coli* per g dry matter (α = 5%), 762 *Escherichia coli* per g dry matter (α = 1%), the Chromocult® method has figures of 369 and 530 respectively. The data illustrates the miniaturised method is able to recover a higher

number of *Escherichia coli* per g dry matter than the Chromocult® at both the 5% and 1% significance level. Although the trial claims the Chromocult® method produces significantly lower results than that of the MPN, there are several key points missing from the data. There is no inclusion of the source, treatment, storage, or transportation of the samples used in the trial. More importantly there is no inclusion of the raw data from the study, outlier data rejected or is there any explanation of geographical position of any of the participants. The spread of results shown in Fig 3 appears significantly less than the previous *E. coli* method. For a sludge with 140,000 cfu/g the 95% confidence limits would cover a range of ~70,000 – 250,000 cfu/g. ***Appendix 5*** includes a draft CEN technical report outlining an interlaboratory protocol for carrying out chemical and microbiological analysis trials.

2.3.4 Detection and Enumeration of *Escherichia coli* in sludges, soils, soil improvers, growing media and biowastes Part 3: Macro method (MPN) in liquid medium (CEN TC308/WG1/TG5 (2003c)). Table 13 which is difficult to understand compares *Escherichia coli* counts between 20 compost samples and 20 sludge samples. Both were contaminated at three artificial contamination levels, the recovery rates cfu/g calculated the mean, confidence interval = 100* (1-alpha) % and the standard deviation. The data supplied only highlighted the results collated from the analysis and there is no explanation of the data supplied. There is no detailed discussion about the protocol used in the validation trial regarding sampling and participants. The data is comprehensive in terms of statistical analysis, but is difficult to understand in terms of comparing the participating laboratories. It would be useful to incorporate the raw data with the statistical analysis so that the figures are easier to compare. It would also appear that, in general, the standard deviations of the bacterial count were of the same order as the mean of the count. It would appear that the 95% confidence intervals relate to the standard error of the mean based on 20 results rather than to individual results.

2.3.5 The performance data attached to the IDEXX Colilert® method (Table 8) is taken from Kramer and Liu (2002) when a comparison between Colilert® and MTF (USEPA Method (APHA 1998)) was carried out. The performance data attached to the paper indicates that the Colilert® / Quantitray® system produces equal estimates of *Escherichia coli* populations for waste activated solids when compared with the Multiple Tube Fermentation (MTF) technique. Equal most probable numbers for *Escherichia coli* were obtained from waste activated solid samples of varying bacterial populations. However, these results considered only one waste activated solid sampling

site, and a greater variety of sludge types should be examined to illustrate the robustness of the method. Several investigations have compared the enzyme-specific media techniques against the traditional methods Grasso et. al (2000) compared lactose broth and Colilert® media in MTF enumeration of over 80 water samples and concluded that the Colilert® media accurately determined both coliform and *Escherichia coli* counts. Gale and Broberg (1993) compared Colilert® to the UK standard method and found no statistical difference between the two identification methods for coliform enumeration. However, Colilert® was found by Gale and Broberg (1993) to be less effective at detecting *Escherichia coli* at levels of one *E. coli* /100ml in the samples tested.

2.3.6 A summary of the results of a recent UK study using the Colilert® *E. coli* method are given in **Appendix 7**. Appendix 7 contains details of a comprehensive UK interlaboratory trial to test the proprietary Colilert® method. This involved seven laboratories and five different sludge matrices. In addition Vitroid *E. coli* reference material was supplied from CPD Ltd. This was used after reconstitution and dilution with MRD to assess recoveries in the absence of a sludge matrix. It was also used to spike each of the five supplied sludge samples at an appropriate level to assess the recovery for each sludge matrix. The results obtained from this trial were considered good and it would appear that the Colilert® method can be considered fit for purpose. However, a larger validation study involving more laboratories would be necessary to confirm this. It is felt that this information will prove useful when assessing the interlaboratory trial data of the three (final version) CEN *E. coli* methods. The Colilert® test method does not involve the laboratory in any media or reagent preparation and the variations observed in the results should solely relate to the laboratory personnel, the sample pre-treatment step (e.g. homogeniser) and any variations in the supplied samples and / or E. coli reference materials.

2.3.7 Another useful research paper of note has been published by the Environment Agency (SCA 1998) which looks at evaluation trials for the MLGA membrane filtration method against the Colilert® Quantitray® MPN broth based method. The test methods were compared against the reference method in this case the MLSB membrane filtration method. Although the paper refers to potable water samples as means for comparison it is still a useful technique to demonstrate the performance of each of the methods against each other. The outcome of the trials can be seen in Table 5 below.

Table 5: Evaluation trials for MLGA and Colilert® for the simultaneous detection and enumeration of *Escherichia coli* and coliform organisms (adapted from Methods for the Examination of Waters and Associated Materials)

Number (percentage) of *Escherichia coli* positive samples from non-raw water samples for MLSB and MLGA

Lab Code	Total Number of Non-raw Samples	Number (%) of colonies On MLSB by definition *	Number (%) of Colonies On MLGA by definition *
LAB 10 & 11	1551	5 (0.3)	3 (0.2)
LAB 20	2017	5 (0.2)	7 (0.3)
LAB 30	2105	9 (0.4)	5 (0.2)
LAB 40	2067	4 (0.2)	5 (0.2)
LAB 50	1948	0 (-)	2 (0.1)
LAB 70	2133	5 (0.2)	3 (0.1)
LAB 80	2025	35 (1.7)	34 (1.7)
Total	13846	63	59

Number of *Escherichia coli* organisms isolated by MLSB and Colilert ® Quantitray®

Lab Code	Number of *E. coli* isolated by MLSB AT 44°C	Number of *E. coli* isolated by Colilert® Quantitray ®
CLAB 1	2615	3089
CLAB 2	0	18
CLAB 3	8	7
CLAB 4	227	199
CLAB 5	17	5
CLAB 6	935	1677
CLAB 7	29	18
CLAB 8	11	8
CLAB 9	5	5
CLAB 10	1504	1162

* isolates obtained at 37°C and 44°C and confirmed as *Escherichia coli* by conventional tests (acid and gas from Lactose Peptone Water, and indole from Tryptone Water and oxidase negative), and confirmation as *Escherichia coli* on API 20E®

2.3.8 Thompson et al. (2002a) have carried out six-day recovery trials on the SCA Colilert® 2000 sludge *E. coli* method. A number of sludges were heated at 70°C for six hours and *E. coli* shown not to be detectable. Then 10g sample aliquots were spiked with a known concentration of an *E. coli* vitroid suspension and the percentage recovery was calculated. The results from this study are shown in Table 6. It can be seen that the

recoveries, down to 5-10 cfu/g *E. coli* as received sample, were good and the percentage recovery for 10-1000 cfu/g *E. coli* in the as received samples were also good.

Table 6: Recovery trials on the SCA Colilertâ 2000 sludge E. coli method.

***E. coli* MPN Recovery No.1 by Quanti-Tray*/2000 using *E. coli* NCTC 9001**					
Vitroids at 1000 cfu/disk and 10,000 cfus/disk					
Date 04.10.01					
No	**Nominal cfu**	**Actual cfu**	**Quantitray result**	**MPN cfu**	**% Recovery**
1	1	1	1, 0	1	100
2	1	1	0, 0	<1	0
3	2	2	0, 0	<1	0
4	5	5	5, 0	5.2	104
5	10	5.3	4, 0	4.1	77
6	50	26.5	18, 2	24.3	92
7	100	53	32, 4	56	106
8	100	53	31, 4	52.9	100
9	200	106	44, 10	126	118
10	500	265	49, 26	488.4	184
11	0	0	0, 0	0	na

***E. coli* MPN Recovery No.2 by Quanti-Tray*/2000 using *E. coli* NCTC 9001**					
Vitroids at 1000 cfu/disk and 10,000 cfus/disk					
Date 9.10.2001					
No.	**Nominal cfu**	**Actual cfu**	**Quantitray result**	**MPN cfu**	**% recovery**
1	1	1.3	0, 0	<1	0
2	1	1.3	0, 0	<1	0
3	1	1.3	3, 0	3.1	238
4	2	2.6	2, 0	2	115
5	5	6.5	3, 0	3.1	48
6	10	13	8, 2	10.8	83
7	50	65	21, 4	31.8	49
8	100	130	39, 8	88.4	68
9	200	260	46, 13	161.6	62
10	500	650	49, 22	387	60
11	1000	1299	49, 42	1299.6	100
12	0	0	0, 0	0	

***E. coli* MPN Recovery No.4 by Quanti-Tray*/2000 using *E. coli* NCTC 9001**					
Vitroids at 1000 cfu/disk and 10,000 cfus/disk					
Date 15.10.2001					
No.	**Nominal cfu**	**Actual No.**	**Quantitray result**	**MPN cfu**	**% Recovery(based on nominal cfu)**
1	100	n\a	20. 2	27.5	27.5
2	10		2, 0	2	20
3	1		0, 0	<1	0
4	1000		49, 24	435	43.5
5	100		20, 2	27.5	27.5
6	10		0, 1	1	10
7	0		0, 0	0	

***E. coli* MPN Recovery No.3 by Quanti-Tray*/2000 using *E. coli* NCTC 9001**					
Vitroids at 1000 cfu/disk and 10,000 cfus/disk					
Date 10.10.01					
No.	**Nominal cfu**	**Actual cfu**	**QuantitrayÒ result**	**MPN cfu**	**% Recovery(based on nominal cfu)**
1	20	n\a	17, 2	22.8	114
2	20		12, 1	14.6	73
3	50		27, 3	41.9	84
4	50		26, 3	39.9	80
5	100		38, 5	77.1	77
6	100		34, 8	68.9	69
7	100		35, 9	74.3	74
8	100		40, 6	88.2	88
9	500		49, 17	290.9	58
10	500		49, 31	648.8	129
11	1000		49, 36	866.4	87
12	1000		49, 34	770.1	77
13	2000		49, 47	2419.2	121
14	5000		49, 48	>2419.2	100
15	10000		49, 48	>2419.2	100
16	0		0, 0	0	

***E. coli* MPN Recovery No.5 by Quanti-Tray*/2000 using *E. coli* NCTC 9001**					
Vitroids at 1000 cfu/disk and 10,000 cfus/disk					
Date 16.10.2001					
No.	**Nominal cfu**	**Actual cfu**	**Quantitray result**	**MPN cfu**	**% Recovery**
1	100	60	38, 12	93.4	155
2	10	6	10, 1	12.1	200
3	1	0.6	0, 0	<1	
4	1000	1525	49, 44	1553	102
5	100	152	39, 10	93.3	61
6	10	15.2	11, 0	12.2	80
7	0	0	0	0	0

***E. coli* MPN Recovery No.6 by Quanti-Tray*/2000 using *E. coli* NCTC 9001**					
Vitroids at 1000 cfu/disk and 10,000 cfus/disk					
Date 24.10.2001					
No.	**nominal cfu**	**Actual No.**	**Quantitray result**	**MPN cfu**	**% Recovery**
1	100	67.5	38, 1	68.4	101
2	10	6.75	3, 1	4.1	61
3	1	0.68	1, 0	1	147
4	1000	1430	49, 41	1203	84
5	100	143	45, 6	119.8	84
6	10	14.3	6, 0	6.3	44
7	0	0	0, 0	0	0

***E. coli* MPN Recovery No.7 by Quanti-Tray*/2000 using *E. coli* NCTC 9001**					
Vitroids at 10,000 cfus/disk					
Date 8.11.01					
No.	**Nominal cfu**	**Actual cfu**	**Quantitray Result**	**MPN cfu**	**% Recovery**
1	10	16.5	11, 2	14.5	88
2	10	16.5	14, 1	17.3	105
3	100	165	39, 7	86	52
4	100	165	43, 5	109	66
5	500	825	49, 24	439	53
6	500	825	49, 25	461	56
7	1000	1650	49, 37	920	56
8	1000	1650	49, 44	1553	94
9	0	0	0, 0	0	na

Figure 2: Summary of E. coli results of interlaboratory comparison Part 1.

Parameter	Mean	Range	SD	Organiser's reference result
Log_{10} *E. coli*/100g	7.29	5.3-7.5	0.66	7.68
Dry solids %	4.42	4.07-4.6	0.14	4.45

The frequency distribution of results for both parameters was calculated and the results plotted in Figures 1 and 2.

Figure 1 Frequency distribution of *E. coli* counts

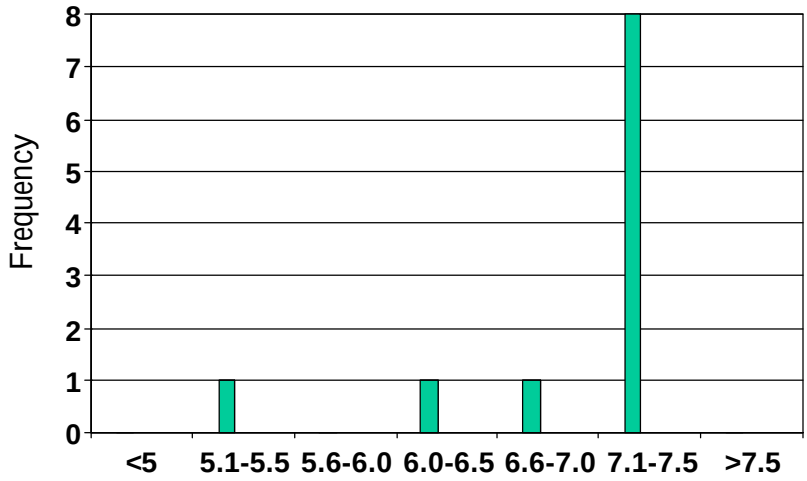
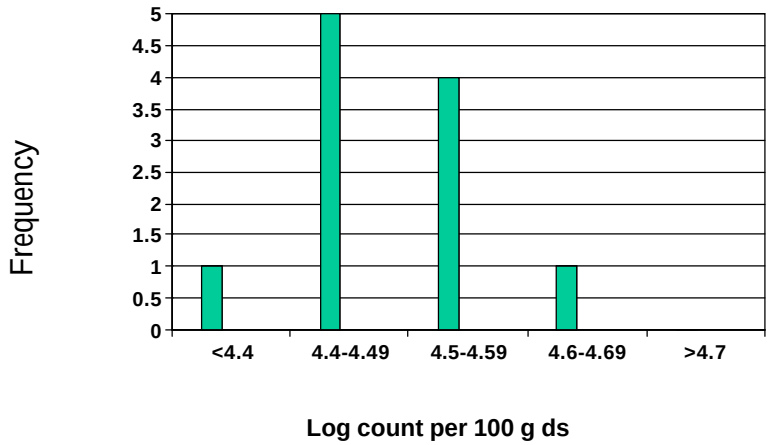


Figure 2 Frequency distribution of dry solids data



Discussion

The findings from this inter-laboratory exercise were encouraging. The results for the dry solids showed an approximate normal distribution, with the majority of laboratories (n=9) reporting results between 4.4% and 4.6% ds content. There was one result (4.07%) which could be described as an outlier.

Microbiological data are expected to be more variable than results of chemical analysis. This was evident in the exercise. Counts of micro-organisms tend to be log normally distributed and to account for this, values were log transformed. The range of counts spanned 2 logs, although a single outlier was responsible for this. The large majority of labs (n=9) reported results within one order of magnitude.

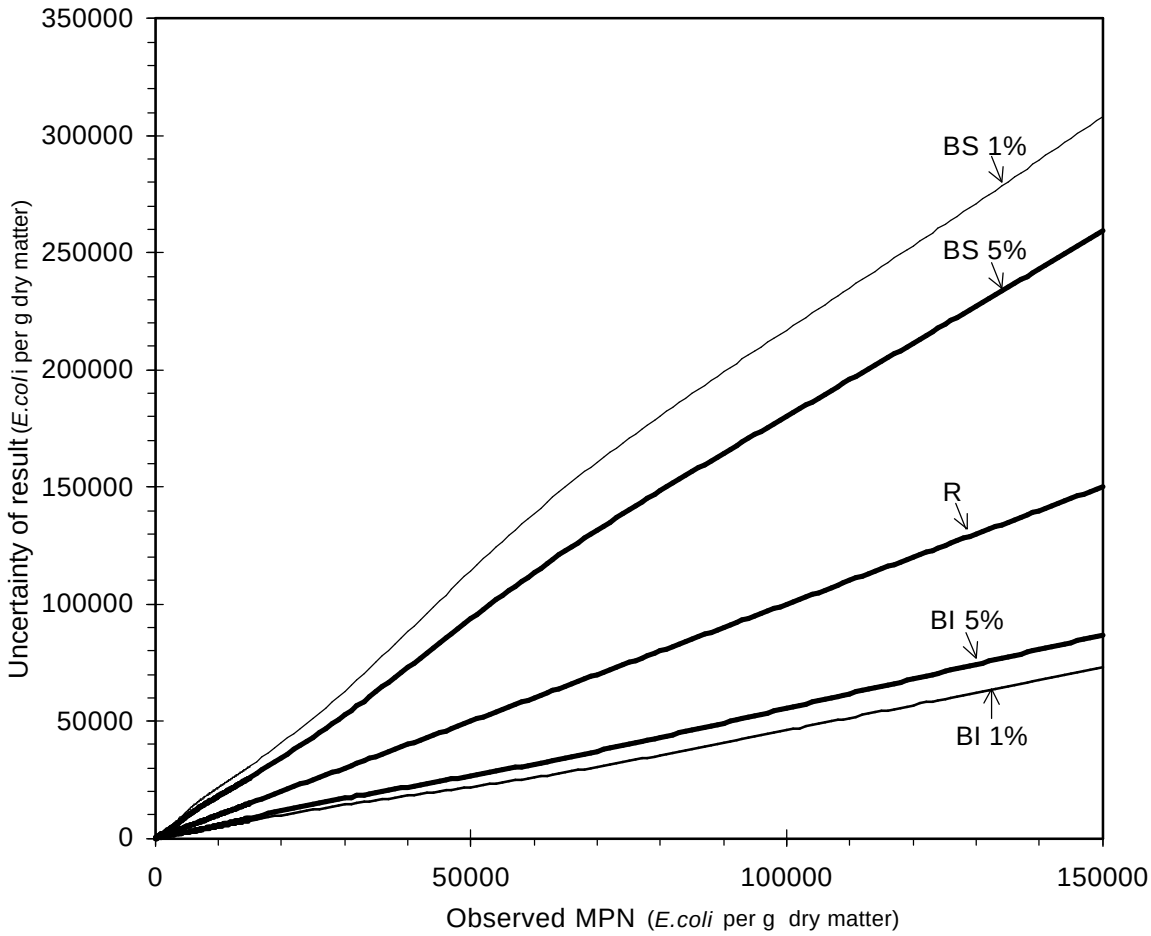
Overall, it is concluded that the variability between laboratories is no greater than expected for microbiological analysis.

Figure 3: Summary of E. coli results of interlaboratory trials Part 2.

If the "high" results can be explained and discarded, the method can be summarised as follows:

- quantification limit= 410 *E. coli* per g dry matter (α=5%), 762 *E. coli* per g dry matter (α=1%) (these values are slightly higher than that of Chromocult® method, which are 369 and 530 for α=5% and 1%, respectively)
- dispersion of results described by the Poisson distribution
- the uncertainty of the results are described on the graph

Enumeration of *E. coli* in sludge samples: uncertainty of results



BS 1% - Upper Limit 1%
BS 5% - Upper Limit 5%
R – Most Probable Number
BI 5% - Lower Limit 5%
BI 1% - Lower Limit 1%

Table 7: Summary of E. coli results of comparative study Part 3.

	Bacterial content (*E. coli*) of the compost samples		
	10^1	**10^2**	**10^3**
mean	**3,13E+01**	**1,96E+02**	**3,68E+03**
standard deviation	**2,85E+01**	**9,91E+01**	**2,88E+03**
95% confid. interv.	**1,8 – 4,46 E+01**	**1,49 - 2,42 E+02**	**2,33 – 5,02 E+03**

	Bacterial content (*E. coli*) of the sludge samples		
	10^1	**10^2**	**10^3**
mean	**1,45E+01**	**1,82E+02**	**9,92E+02**
standard deviation	**1,23E+01**	**1,44E+02**	**6,38E+03**
95% confid. interv.	**8,7E+00 – 2,02E+01**	**1,15 – 2,49 E+02**	**6,93E+02 – 1,29E+03**

The basis of the validation is 20 single samples providing data reflecting 10 experiments in two groups (each 10).
The calculation had been done by the standard Excel programme.

The data included in the annex is difficult to understand and explanation must be sought for the origin of this data. There is no explanation of the protocol of the trial, there is minimal information included which needs expanding.

Table 8: Escherichia coli data comparing IDEXX Colilert® method with USEPA accepted MTF technique (From Kramer and Liu (2002))

***E. coli* Data**

• – calculated mean

• – standard deviation

• – pooled, estimated, degree of freedom

t' – t-distribution estimate

t • /2 – t-distribution value at 99% confidence

	$\overline{X}$	s	n	t'	$t_{\alpha/2}$
Raw WAS MTF	1.28×10^6 (MPN/g)	5.09×10^5 (MPN/g)	32	2.337	2.750
Raw WAS Colilert®	9.87×10^5	2.35×10^5	32	2.337	2.750
15 min Pasteurized WAS MTF	28.9	11.4	35	2.634	2.727
15 min Pasteurized WAS Colilert®	36.6	6.40	35	2.634	2.727
30 min Pasteurized WAS MTF	ND	ND	~	~	~
30 min Pasteurized WAS Colilert®	ND	ND	~	~	~

ND = non-detect

2.4 List of Methods – Salmonella spp. [2]

Following the letter that was distributed as a request for information, the following methods were selected for consideration for *Salmonella spp*. detection and enumeration standards. These methods are therefore covered in the report to assess the feasibility of horizontal standards for *Salmonella spp*.

Table 9: CEN Draft Methods for Salmonella spp Parts 1, 2 and 3

1 CEN (2003d) Detection and Enumeration of *Salmonella spp* in sludges, soils, soil improvers, growing media and biowastes— Part 1: Membrane filtration method for quantitative resuscitation of sub-lethally stressed bacteria (to confirm efficacy of log drop treatment procedures)

2 CEN (2003e) Detection of *Salmonella spp*. in sludges, soils, soil improvers, growing media and biowastes -Part 2: Liquid enrichment method in selenite-cystine medium followed by Rappaport-Vassiliadis for semi-quantitative Most Probable Number determination

3 CEN (2003f) Detection of *Salmonella spp.* in sludges, soils, soil improvers, growing media and biowastes- Part 3: Presence/absence method by liquid enrichment in peptone-novobiocin medium followed by Rappaport-Vassiliadis

Table 10: UK Standing Committee of Analysts (SCA) Sludge Methods for Salmonella spp.

(Available on the web from http://www.environment-agency.gov.co.uk/science/219094/399393)

SCA (2003), Microbiology of Sewage Sludge - Part 4 - Methods for the detection, isolation and enumeration of *Salmonella spp.* .

4 SCA (2003f), Microbiology of Sludge Part 4 – The detection of *Salmonella spp.* Using a presence/ absence technique

5 SCA (2003g), Microbiology of Sludge Part 4 – Isolation and Enumeration of *Salmonella spp.* by a multiple tube MPN technique

6 SCA (2003h) Microbiology of Sludge Part 4 – The Enumeration of *Salmonella spp.* by membrane filtration with a chromogenic detection medium

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[2] ***New Zealand Standard Salmonella Sludge Methods – late received information***
Salmonella Part 9260D Standard Methods for the Examination of Water and Wastewater (APHA, 1998) or Detection and enumeration of *Salmonella* and *Pseudomonas aeruginosa* (Kenner and Clark, 1974 Journal of Water Pollution Control Federation 46 : 9, 2163-2177

7 Matrix MicroScience (2003), Detection of *Salmonella spp.* using Immuno-magnetic Separation using Pathatrix®

8 Merck (2002) Singlepath GLISA Salmonella® Lateral Flow Test.

2.5 *Discussion of the Salmonella spp. Methods*

2.5.1 The standards put forward for inclusion in the report rely on four different techniques for the detection and enumeration of *Salmonella spp.* from sludges, soil, soil improvers, growing media and biowaste matrices. The techniques included are membrane filtration, semi- quantitative MPN determination, Presence / Absence using liquid enrichment, and immunomagnetic separation using the commercially available Matrix MicroScience Pathatrix® technique.

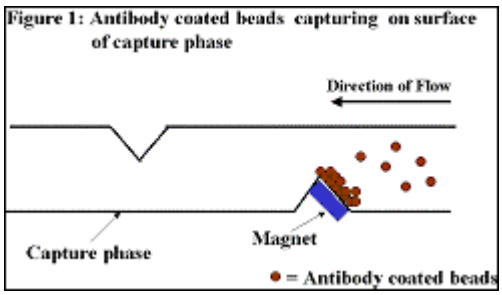
2.5.2 The membrane filtration method involves filtering a suitably diluted sample (1 - 6 log) through a 0.45µm membrane for detection and quantification. The membrane filtered sample is put into a resuscitation tetrathionate broth and incubated for 24 hours at 36°C. The membrane is then aseptically recovered and placed into another incubation stage on Rambach Agar for 24 / 48 hours at 36°C. Following this incubation, the colonies present are quantified by the indication of red colonies from the fermentation of propylene glycol. The membrane filtration technique is the most quantitative out of the existing methods for detection and enumeration of *Salmonella spp.*, as the colonies are able to be enumerated from the membrane. However the technique does rely on the accuracy of the serial dilution stage to prepare the sample so that it is able to pass through the filter membrane before the resuscitation step.

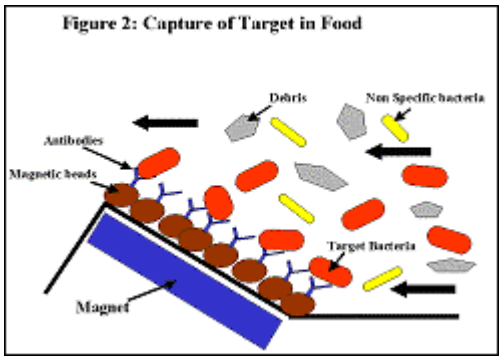
2.5.3 Part 2 and Part 3 of the methods for *Salmonella spp.* detection use selective liquid enrichment to minimise the effect of interfering bacteria from the sample, it does not inhibit the growth of the *Salmonella spp.*; Part 2 is able to detect and enumerate the *Salmonella spp.* through the MPN principle. Three series of three tubes containing selenite cysteine broth are set up with serial dilutions of the sample enabling the method to give an MPN figure for the sample. . It is; however, felt that the use of selenite cysteine broth in the context of the method is no longer favoured. It is recommended that alternative broths should be sought and validated as soon as possible. The MPN method is able to give an idea of the contamination rate for heavily contaminated materials such as sludge or biowaste which is a distinct advantage compared to the other methods. Part 3 is a presence / absence method and after the use of a selective medium buffered peptone water (BPW) supplemented with novobiocin, the sample is incubated in Rappaport Vassiliadis. A loop full of the sample is plated out onto XLD and brilliant

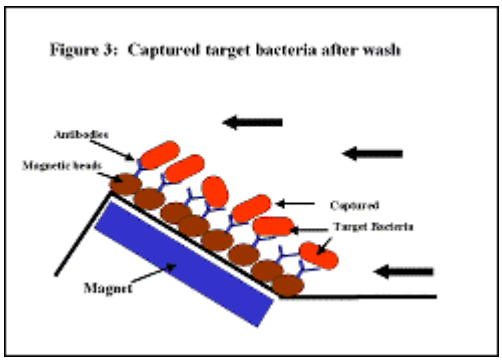
green-Phenol red-Lactose-Sucrose agar (BPLA) culture plates. The typical colonies are then sub-cultured on Standard 1 agar plates and the result "*Salmonella spp.*" is confirmed by microscopic slide agglutination with polyvalent O antisera. If necessary further agglutinations using monovalent O antisera can be used, biochemical identification (API 20E®) can also be performed if required. The presence / absence method is more appropriate for lightly contaminated matrices such as sanitised sludge or biowaste, as well as fertilisers and growing media (in 25g for instance).

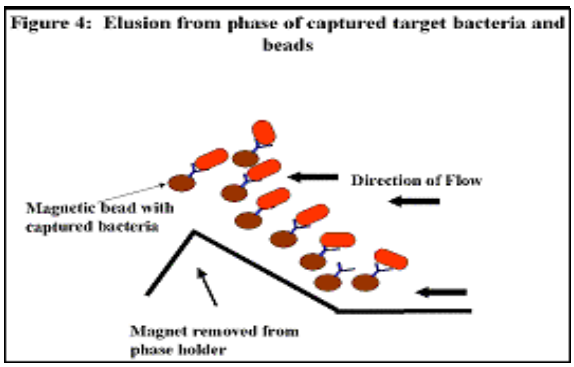
2.5.4 A commercial technique available in the field of *Salmonella spp.* detection and enumeration is available from Matrix MicroScience, Pathatrix® method. The company has developed a method that relies on the use of antibody coated paramagnetic particles to selectively bind and purify the target organisms from a comprehensive range of complex matrices. The minimum level of detection is quoted as of 1 viable *Salmonella spp.* per 25ml (liquid product) or 1 viable *Salmonella spp.* per 25g (of as received sample for other products). The Pathatrix® system takes just 16 hours to identify the *Salmonella spp.*, compared with between two and four days taken by existing systems. It is unique that it is the only microbial detection system that can analyse the entire 225ml plus 25g sample simultaneously by re-circulating the sample through a "capture phase" where the antibody coated magnetic beads are immobilised. By providing heat to the system the organisms can be cultured and captured simultaneously, thus increasing the method sensitivity. Once captured and concentrated the sample is now ready for use with a variety of detection methods: either direct plating onto the appropriate selective media and incubated or tested using one of the following; Colortrix; Fluoratrix (fluorescent microscopy); serology; polymerase chain reaction (PCR); enzyme linked immunosorbent assay (ELISA); and / or DNA probe. A schematic overview of the Pathatrix® system can be seen below.

Table 11: Potential Method for Salmonella spp. using Commercial Systems (Matrix MicroScience Pathatrix® Technique and Merck Singlepath GLISA Salmonella® Lateral Flow Test









Data from internal and external validation studies e.g. The Association of analytical communities (AOAC) trials have demonstrated that Pathatrix® system is significantly more sensitive than many of the current standard methods, at low spike levels 1-10 cfu/25g sample.

It is probably the only potential system that could approach the 1 in 50g requirement of the draft sludge directive for *Salmonella spp.*.

2.5.5 Another promising commercial endpoint confirmation technique is the Merck Singlepath *Salmonella*® lateral flow test. (Merck, 2002) This is an immuno-chromatographical disposable test device. It considerably simplifies the analysis protocol. An 18 – 24 hour pre-enrichment in BPW followed by 24 hour selective growth on Rappaport-Vassiliadis (RVS) broth is used. Then 1 – 2 ml of the RVS broth is heated in a boiling water bath to sterilise it and allowed to cool. Then 160•l is applied to the immuno-chromatographical disposable test device. There is a clear and distinct positive or negative test result within 20secs with a built-in positive control. The device is as sensitive as standard methods. Any positives can then be confirmed by streaking the RVS broth on to differential agars. No capital investment is required. Other commercial lateral flow devices for this devices are also available.

2.5.6 SCA methods for *Salmonella spp.* are included and rely on the principles put forward in the current draft CEN standards. Membrane Filtration principle - A sample of sludge is initially homogenised and then serially diluted. The diluted sludge is filtered through a membrane filter and incubated at a temperature of 36 ± 1 °C on a sterile glass fibre filter saturated with resuscitation medium comprising tetrathionate broth. After incubating for 24 hours, the membrane filter is further incubated at 36 ± 1 °C on chromogenic medium (Rambach agar). The membrane filters are then examined after 24 and 48 hours and positive colonies enumerated. Incubation for 48 hours is particularly important for the recovery of some serovars such as *Salmonella dublin*. The presence of *Salmonella spp*. is indicated by the presence of bright red colonies resulting from the fermentation of propylene glycol. Colonies of other members of the *Enterobacteriaceae* appear blue, green, violet or colourless due to their inability to ferment propylene glycol. Some produce ß-galactosidase which hydrolyses colourless X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) in the medium to produce a blue chromophore.

2.5.7 *Salmonella spp.* may be distinguished from the occasional false positives due to *Citrobacter spp.* by tests for C_8 esterase enzyme activity using a fluorogenic substrate

carried out in-situ on presumptive colonies on Rambach agar. *Salmonella spp.* colonies exhibit fluorescence when exposed to UV light at 366 nm.

2.5.8 Presence / Absence Method - Isolation and detection is based on appropriate homogenisation of sludge, followed by a pre-enrichment involving incubation in a non-selective medium (to recover environmentally-stressed organisms), and selective enrichment with subculture to a selective agar containing xylose with additional indicators of acidity and H_2S production. Characteristic colonies are confirmed by biochemical tests for example (API 20 E®) and serological tests which are based on slide agglutination.

2.5.9 MPN Method - Isolation and enumeration is based on appropriate homogenisation of sludge, followed by multiple tube pre-enrichment involving incubation in a non-selective medium (to recover environmentally-stressed organisms), and selective enrichment with subculture to selective agar containing lactose and an indicator of acidity. Characteristic colonies are confirmed by biochemical tests for example (API 20 E®) and by slide agglutination. The most probable number of organisms in the sample is estimated from the appropriate probability tables.

2.5.10 Another technique of note is in use in Canada, for the detection and enumeration of *Salmonella spp.* in a sludge matrix, the Greater Vancouver Regional District (GVRD) method. A selective medium, such as tetrathionate broth with brilliant green (TTBG) is used for enrichment because it is a highly sensitive enrichment method. Detection of *Salmonella spp.* is achieved by streaking a loop-full of top growth from an enrichment tube on to a selective diagnostic medium and after incubation looking for typical *Salmonella spp.* colonies. The GVRD method validation involves testing whether cells from a *Salmonella spp.* control culture such as *S. typhi* will grow when seeded to the enrichment medium at a low density of one or two cells per tube. If *Salmonella spp.* is successfully recovered and isolated, screened and serotyped, the sensitivity of the method is proven. If *Salmonella spp.* are not recovered from a bio-solids sample when the test is used, the *Salmonella spp.* are probably not present.

2.6 Review of Performance Data – Salmonella spp

2.6.1 CEN TC308/WG1/TG5, (2003d) Detection and Enumeration of *Salmonella spp.* In sludges, soils, soil improvers, growing media and biowastes— Part 1: Membrane filtration method for quantitative resuscitation of sub-lethally stressed bacteria (to confirm to efficacy of log drop treatment procedures). The performance trial of this

method states full validation data with a minimum of eight laboratories from three different countries will be produced once the proposed validation trials have been agreed, financed, and carried out. The data that, therefore, accompanies the method is preliminary data. There is only basic data collated for each *Salmonella spp.* tested. The data has only been produced from one laboratory and therefore the robustness and repeatability of the method requires further validation. The existing data looks at three serovars of *Salmonella*; *seftenberg*, *typhimurium*, and *enteriditis*. The method has tested the samples pre-treatment and post-treatment; the validation data shows that the treatment process works as no growth is indicated by the data after treatment of the sample. To provide a true representation of the performance of the method, the method needs to undergo further testing with more samples to provide higher rates of replication for the comparative purposes. The existing trial data only analysed two repetitions which is not considered adequate to provide statistical evidence supporting the suitability of the method. All existing concerns regarding the quality of the performance data for this method should be addressed in the full validation trials when they take place.

2.6.2 CEN TC308/WG1/TG5, (2003e) Detection and Enumeration of *Salmonella spp.* In sludges, soils, soil improvers, growing media and biowastes-Part 2: Liquid enrichment method in selenite-cystine medium followed by Rappaport-Vassiliadis for semi-quantitative Most Probable Number (MPN) determination. The performance data attached to this method requires further explanation. The data outlines the protocol for the trial but does not include any actual raw data. The results from the French intralaboratory and interlaboratory trials carried out in 2001 and 2002 by 14 laboratories on five different materials, need to be sought and included together with a summary of the findings. The suitability and workability of the method needs to be evident when assessing a method put forward as a draft European standard.

2.6.3 CEN TC308/WG1/TG5, (2003f) Detection and Enumeration of *Salmonella spp.* in sludges, soils, soil improvers, growing media and biowastes- Part 3: Presence/absence method by liquid enrichment in peptone-novobiocin medium followed by Rappaport-Vassiliadis. The performance data has been collated with a view to comparative validation using 112 waste water, and 130 compost samples. The data illustrates the detection percentages of various methods of enrichment and selective culture techniques. Although difficult to follow, the tabulated results do illustrate the ability of the method to detect the target organisms. However, more explanation of the procedure that was followed together with a summary of the findings would be a useful addition.

2.6.4 Thompson et al. (2002b) also carried out similar type trials for their presence/absence *Salmonella spp*. method based on the German standard methods for the examination of water, waste water and sludge, Sludge and sediments (group S), Detection of *Salmonella spp*. in disinfected sewage sludge (S13) DIN 38414 Part 13. The method followed used by Thompson et al. (2002b) is based around the CEN TC308/WG1/TG5, (2003f) Detection and Enumeration of *Salmonella spp*. in sludges, soils, soil improvers, growing media and biowastes- Part 3: Presence/absence method by liquid enrichment in peptone-novobiocin medium followed by Rappaport-Vassiliadis. Isolation is by pre-enrichment in a non-selective (BPW) medium followed by inoculation and incubation in a selective enrichment medium (Rappaport-Vassiliadis Enrichment Broth (Oxoid CM669)) and then plating on to selective XLD and BPLA agar culture plates which are incubated at 36± 1°C for 20 ± 2 hours. Typical colonies are sub-cultured onto Standard 1 agar plates, and characteristic colonies are confirmed by latex agglutination and biochemical tests (e.g. API 20E®) using 20g as received sludge sample aliquot, it was shown that *Salmonella goldcoast* could be detected down to about 2cfu per 20g aliquot. The results are summarised in Table 12 overleaf.

Table 12: Recovery trials on the Presence / Absence Detection of Salmonellae in disinfected sewage sludge method based on (S13) DIN 38414 Part 13.

Table 12 (a): Analysis from 1.8.01 – 4.8.01

***Salmonella* RECOVERY EXERCISE No.1 USING *S.goldcoast* nctc13175.**						
Vitroids at 30000 cfu. (batch 3701)						
DATE OF ANALYSIS. 1.8.01-4.8.01						
No.	**sample matrix**	**cfu spike**	**BPW**	**RVS**	**XLD**	**RESULT P\A (4.8.01)**
1	BPW only	10	1.8.01	2.8.01	3.8.01	PRESENT
2	BPW only	10	1.8.01	2.8.01	3.8.01	PRESENT
3	BPW only	50	1.8.01	2.8.01	3.8.01	PRESENT
4	BPW only	50	1.8.01	2.8.01	3.8.01	PRESENT
5	BPW only	100	1.8.01	2.8.01	3.8.01	PRESENT
6	BPW only	100	1.8.01	2.8.01	3.8.01	PRESENT
7	20g sludge (cond.cake)	0	1.8.01	2.8.01	3.8.01	ABSENT
8	20g sludge (cond.cake)	0	1.8.01	2.8.01	3.8.01	ABSENT
9	20g sludge (cond.cake)	10	1.8.01	2.8.01	3.8.01	PRESENT
10	20g sludge (cond.cake)	10	1.8.01	2.8.01	3.8.01	PRESENT
11	20g sludge (cond.cake)	50	1.8.01	2.8.01	3.8.01	PRESENT
12	20g sludge (cond.cake)	50	1.8.01	2.8.01	3.8.01	PRESENT
13	20g sludge (cond.cake)	100	1.8.01	2.8.01	3.8.01	PRESENT
14	20g sludge (cond.cake)	100	1.8.01	2.8.01	3.8.01	PRESENT
15	20g sterilised sludge	0	1.8.01	2.8.01	3.8.01	ABSENT
16	20g sterilised sludge	0	1.8.01	2.8.01	3.8.01	ABSENT
17	20g sterilised sludge	10	1.8.01	2.8.01	3.8.01	PRESENT
18	20g sterilised sludge	10	1.8.01	2.8.01	3.8.01	PRESENT
19	20g sterilised sludge	50	1.8.01	2.8.01	3.8.01	PRESENT
20	20g sterilised sludge	50	1.8.01	2.8.01	3.8.01	PRESENT
21	20g sterilised sludge	100	1.8.01	2.8.01	3.8.01	PRESENT
22	20g sterilised sludge	100	1.8.01	2.8.01	3.8.01	PRESENT

Table 12 (b): Analysis from 7.8.01 – 10.8.01

***Salmonella* RECOVERY EXERCISE No.2 USING *S.goldcoast* nctc13175.**							
Vitroids at 30000 cfu. (batch 3701)							
DATE OF ANALYSIS. 7.8.01-10.8.01							
No.	**sample matrix**	**cfu spike**	**BPW**	**RVS**	**XLD**	**RESULT P\A (10.8.01)**	**ESTIMATED CFU IN PRESENCE**
1	BPW only	0	7.8.01	8.8.01	9.8.01	ABSENT	
2	BPW only	0	7.8.01	8.8.01	9.8.01	ABSENT	
3	BPW only	5	7.8.01	8.8.01	9.8.01	PRESENT	1000+
4	BPW only	5	7.8.01	8.8.01	9.8.01	PRESENT	1000+
5	BPW only	10	7.8.01	8.8.01	9.8.01	PRESENT	1000+
6	BPW only	10	7.8.01	8.8.01	9.8.01	PRESENT	1000+
7	BPW only	20	7.8.01	8.8.01	9.8.01	PRESENT	1000+
8	BPW only	20	7.8.01	8.8.01	9.8.01	PRESENT	1000+
9	20g sludge (cond.cake)	0	7.8.01	8.8.01	9.8.01	ABSENT	
10	20g sludge (cond.cake)	0	7.8.01	8.8.01	9.8.01	ABSENT	
11	20g sludge (cond.cake)	5	7.8.01	8.8.01	9.8.01	PRESENT	4
12	20g sludge (cond.cake)	5	7.8.01	8.8.01	9.8.01	PRESENT	94
13	20g sludge (cond.cake)	10	7.8.01	8.8.01	9.8.01	PRESENT	400+
14	20g sludge (cond.cake)	10	7.8.01	8.8.01	9.8.01	PRESENT	100+
15	20g sludge (cond.cake)	20	7.8.01	8.8.01	9.8.01	PRESENT	200+
16	20g sludge (cond.cake)	20	7.8.01	8.8.01	9.8.01	PRESENT	2
17	20g sterilised sludge	0	7.8.01	8.8.01	9.8.01	ABSENT	
18	20g sterilised sludge	0	7.8.01	8.8.01	9.8.01	ABSENT	
19	20g sterilised sludge	5	7.8.01	8.8.01	9.8.01	PRESENT	1000+
20	20g sterilised sludge	5	7.8.01	8.8.01	9.8.01	PRESENT	1000+
21	20g sterilised sludge	10	7.8.01	8.8.01	9.8.01	PRESENT	1000+
22	20g sterilised sludge	10	7.8.01	8.8.01	9.8.01	PRESENT	1000+
23	20g sterilised sludge	20	7.8.01	8.8.01	9.8.01	PRESENT	1000+
24	20g sterilised sludge	20	7.8.01	8.8.01	9.8.01	PRESENT	1000+

Table 12 (c): Analysis from 14.8.01 – 17.8.01

***Salmonella* RECOVERY EXERCISE No.3 USING *S.goldcoast* nctc13175.**							
Vitroids at 30000 cfu. (batch 3701)							
DATE OF ANALYSIS. 14.8.01-17.8.01							
No.	**sample matrix**	**cfu spike**	**BPW**	**RVS**	**XLD**	**RESULT P\A (17.8.01)**	**ESTIMATED CFU IN PRESENCE**
1	BPW only	0	14.8.01	15.8.01	16.8.01	ABSENT	
2	BPW only	0	14.8.01	15.8.01	16.8.01	ABSENT	
3	BPW only	1	14.8.01	15.8.01	16.8.01	PRESENT	1000+
4	BPW only	1	14.8.01	15.8.01	16.8.01	PRESENT	1000+
5	BPW only	5	14.8.01	15.8.01	16.8.01	PRESENT	1000+
6	BPW only	5	14.8.01	15.8.01	16.8.01	PRESENT	1000+
7	BPW only	10	14.8.01	15.8.01	16.8.01	PRESENT	1000+
8	BPW only	10	14.8.01	15.8.01	16.8.01	PRESENT	1000+
9	20g sludge (cond.cake)	0	14.8.01	15.8.01	16.8.01	ABSENT	
10	20g sludge (cond.cake)	0	14.8.01	15.8.01	16.8.01	ABSENT	
11	20g sludge (cond.cake)	1	14.8.01	15.8.01	16.8.01	PRESENT	9
12	20g sludge (cond.cake)	1	14.8.01	15.8.01	16.8.01	**ABSENT**	
13	20g sludge (cond.cake)	5	14.8.01	15.8.01	16.8.01	PRESENT	1000+
14	20g sludge (cond.cake)	5	14.8.01	15.8.01	16.8.01	PRESENT	1000+
15	20g sludge (cond.cake)	10	14.8.01	15.8.01	16.8.01	PRESENT	30
16	20g sludge (cond.cake)	10	14.8.01	15.8.01	16.8.01	PRESENT	1000+
17	20g sterilised sludge	0	14.8.01	15.8.01	16.8.01	ABSENT	
18	20g sterilised sludge	0	14.8.01	15.8.01	16.8.01	ABSENT	
19	20g sterilised sludge	1	14.8.01	15.8.01	16.8.01	PRESENT	1000+
20	20g sterilised sludge	1	14.8.01	15.8.01	16.8.01	PRESENT	1000+
21	20g sterilised sludge	5	14.8.01	15.8.01	16.8.01	PRESENT	1000+
22	20g sterilised sludge	5	14.8.01	15.8.01	16.8.01	PRESENT	1000+
23	20g sterilised sludge	10	14.8.01	15.8.01	16.8.01	PRESENT	1000+
24	20g sterilised sludge	10	14.8.01	15.8.01	16.8.01	PRESENT	1000+

Table 12 (d): Analysis from 3.9.01 – 6.9.01

***Salmonella* RECOVERY EXERCISE No.4 USING *S.goldcoast* nctc13175.**							
Vitroids at 30000 cfu. (batch 3701)							
DATE OF ANALYSIS. 3.9.01-6.9.01							
No.	**sample matrix**	**cfu spike**	**BPW**	**RVS**	**XLD**	**RESULT P\A (6.9.01)**	**ESTIMATED CFU IN 10 PRESENCE**
1	BPW only	0	3.9.01	4.9.01	5.9.01	ABSENT	
2	BPW only	0	3.9.01	4.9.01	5.9.01	ABSENT	
3	BPW only	1	3.9.01	4.9.01	5.9.01	PRESENT	1000+
4	BPW only	1	3.9.01	4.9.01	5.9.01	**ABSENT**	
5	BPW only	5	3.9.01	4.9.01	5.9.01	PRESENT	1000+
6	BPW only	5	3.9.01	4.9.01	5.9.01	PRESENT	1000+
7	BPW only	10	3.9.01	4.9.01	5.9.01	PRESENT	1000+
8	BPW only	10	3.9.01	4.9.01	5.9.01	PRESENT	1000+
9	20g sludge (cond.cake)	0	3.9.01	4.9.01	5.9.01	ABSENT	
10	20g sludge (cond.cake)	0	3.9.01	4.9.01	5.9.01	ABSENT	
11	20g sludge (cond.cake)	1	3.9.01	4.9.01	5.9.01	PRESENT	1
12	20g sludge (cond.cake)	1	3.9.01	4.9.01	5.9.01	PRESENT	15
13	20g sludge (cond.cake)	5	3.9.01	4.9.01	5.9.01	PRESENT	15
14	20g sludge (cond.cake)	5	3.9.01	4.9.01	5.9.01	PRESENT	13
15	20g sludge (cond.cake)	10	3.9.01	4.9.01	5.9.01	PRESENT	30
16	20g sludge (cond.cake)	10	3.9.01	4.9.01	5.9.01	PRESENT	25
17	20g sterilised sludge	0	3.9.01	4.9.01	5.9.01	ABSENT	
18	20g sterilised sludge	0	3.9.01	4.9.01	5.9.01	ABSENT	
19	20g sterilised sludge	1	3.9.01	4.9.01	5.9.01	PRESENT	1000+
20	20g sterilised sludge	1	3.9.01	4.9.01	5.9.01	**ABSENT**	
21	20g sterilised sludge	5	3.9.01	4.9.01	5.9.01	PRESENT	1000+
22	20g sterilised sludge	5	3.9.01	4.9.01	5.9.01	PRESENT	1000+
23	20g sterilised sludge	10	3.9.01	4.9.01	5.9.01	PRESENT	1000+
24	20g sterilised sludge	10	3.9.01	4.9.01	5.9.01	PRESENT	1000+

Table 12 (e): Analysis from 13.9.01 – 16.9.01

***Salmonella* RECOVERY EXERCISE No.5 USING *S.goldcoast* nctc13175.**						
Vitroids at 30000 cfu. (batch 3701)						
DATE OF ANALYSIS. 13.9.01-16.9.01						
No.	**sample matrix**	**cfu spike**	**BPW**	**RVS**	**XLD**	**RESULT P\A (16.9.01)**
1	BPW only	0	13.9.01	14.9.01	15.9.01	ABSENT
2	BPW only	0	13.9.01	14.9.01	15.9.01	ABSENT
3	BPW only	1	13.9.01	14.9.01	15.9.01	PRESENT
4	BPW only	1	13.9.01	14.9.01	15.9.01	PRESENT
5	BPW only	5	13.9.01	14.9.01	15.9.01	PRESENT
6	BPW only	5	13.9.01	14.9.01	15.9.01	PRESENT
7	BPW only	10	13.9.01	14.9.01	15.9.01	PRESENT
8	BPW only	10	13.9.01	14.9.01	15.9.01	PRESENT
9	20g sludge (cond.cake)	0	13.9.01	14.9.01	15.9.01	ABSENT
10	20g sludge (cond.cake)	0	13.9.01	14.9.01	15.9.01	ABSENT
11	20g sludge (cond.cake)	1	13.9.01	14.9.01	15.9.01	PRESENT
12	20g sludge (cond.cake)	1	13.9.01	14.9.01	15.9.01	PRESENT
13	20g sludge (cond.cake)	5	13.9.01	14.9.01	15.9.01	PRESENT
14	20g sludge (cond.cake)	5	13.9.01	14.9.01	15.9.01	PRESENT
15	20g sludge (cond.cake)	10	13.9.01	14.9.01	15.9.01	PRESENT
16	20g sludge (cond.cake)	10	13.9.01	14.9.01	15.9.01	PRESENT
17	20g sterilised sludge	0	13.9.01	14.9.01	15.9.01	ABSENT
18	20g sterilised sludge	0	13.9.01	14.9.01	15.9.01	ABSENT
19	20g sterilised sludge	1	13.9.01	14.9.01	15.9.01	**ABSENT**
20	20g sterilised sludge	1	13.9.01	14.9.01	15.9.01	PRESENT
21	20g sterilised sludge	5	13.9.01	14.9.01	15.9.01	PRESENT
22	20g sterilised sludge	5	13.9.01	14.9.01	15.9.01	PRESENT
23	20g sterilised sludge	10	13.9.01	14.9.01	15.9.01	PRESENT
24	20g sterilised sludge	10	13.9.01	14.9.01	15.9.01	PRESENT

Table 12 (f): Analysis from 18.9.01 – 21.9.01

***Salmonella* RECOVERY EXERCISE No.6 USING *S.goldcoast* nctc13175.**							
Vitroids at 30000 cfu. (batch 3701)							
DATE OF ANALYSIS. 18.9.01-21.9.01							
No.	**sample matrix**	**cfu spike**	**BPW**	**RVS**	**XLD**	**RESULT P\A (21.9.01)**	**ESTIMATED CFU IN 10 PRESENCE**
1	BPW only	0	18.9.01	19.9.01	20.9.01	ABSENT	
2	BPW only	0	18.9.01	19.9.01	20.9.01	ABSENT	
3	BPW only	1	18.9.01	19.9.01	20.9.01	**ABSENT**	
4	BPW only	1	18.9.01	19.9.01	20.9.01	PRESENT	1000+
5	BPW only	5	18.9.01	19.9.01	20.9.01	PRESENT	1000+
6	BPW only	5	18.9.01	19.9.01	20.9.01	PRESENT	1000+
7	BPW only	10	18.9.01	19.9.01	20.9.01	PRESENT	1000+
8	BPW only	10	18.9.01	19.9.01	20.9.01	PRESENT	1000+
9	20g sludge (cond.cake)	0	18.9.01	19.9.01	20.9.01	ABSENT	
10	20g sludge (cond.cake)	0	18.9.01	19.9.01	20.9.01	ABSENT	
11	20g sludge (cond.cake)	1	18.9.01	19.9.01	20.9.01	**ABSENT**	
12	20g sludge (cond.cake)	1	18.9.01	19.9.01	20.9.01	PRESENT	45
13	20g sludge (cond.cake)	5	18.9.01	19.9.01	20.9.01	PRESENT	3
14	20g sludge (cond.cake)	5	18.9.01	19.9.01	20.9.01	PRESENT	200
15	20g sludge (cond.cake)	10	18.9.01	19.9.01	20.9.01	PRESENT	16
16	20g sludge (cond.cake)	10	18.9.01	19.9.01	20.9.01	PRESENT	300
17	20g sterilised sludge	0	18.9.01	19.9.01	20.9.01	ABSENT	
18	20g sterilised sludge	0	18.9.01	19.9.01	20.9.01	ABSENT	
19	20g sterilised sludge	1	18.9.01	19.9.01	20.9.01	**ABSENT**	
20	20g sterilised sludge	1	18.9.01	19.9.01	20.9.01	PRESENT	150
21	20g sterilised sludge	5	18.9.01	19.9.01	20.9.01	PRESENT	1000
22	20g sterilised sludge	5	18.9.01	19.9.01	20.9.01	PRESENT	1000
23	20g sterilised sludge	10	18.9.01	19.9.01	20.9.01	**ABSENT?**	
24	20g sterilised sludge	10	18.9.01	19.9.01	20.9.01	**ABSENT?**	

2.7 Existing Methods in UK Laboratories

2.7.1 The collation of information outlining the different methods in use in UK Laboratories is a useful guide to determine the method variation in sludges, soil, soil improvers, growing media and biowaste testing in the UK. The information will provide the ability to compare certain methods against each other in interlaboratory trials at certain laboratories using the relevant method as routine. Seven UK Laboratories were contacted and information on current methods was requested. Wessex Water are using the MLGA membrane filtration method for digested and caked samples, and the MPN LTB method for limed and thermally dried sludge's for *Escherichia coli*. The Colilert® method is currently under trial at Wessex with a view to replacing the MPN method.

2.7.2 For *Salmonella spp.* the method uses buffered peptone water (BPW) for pre-enrichment at 37°C for 24 hours, Rappaport Vassiliadis for selective enrichment for up to 48 hours at 41.5°C and plating on to xylose-lysin-deoxycholate (XLD) only. Confirmation is with Urea and TSI slopes, and poly O and H antisera, it should be noted that API 20E® commercial identification kits available from Biomerieux™ are occasionally used to resolve ambiguous results. This method can be used as either a presence / absence or MPN format. When dealing with a limed sludge the sludge is neutralised in MRD first and then transferred in an appropriate volume into double strength BPW. The methods that Wessex Water currently use relate to the methods outlined by the Environment Agency – SCA (2003)The Microbiology of Sewage Sludge (2003) - Part 4 - Methods for the isolation and enumeration of *Salmonella spp.* draft (March 2003) which are included in this report.

2.7.3 United Utilities incorporating North West Water use two methods to detect and enumerate *Escherichia coli,* the MLGA membrane filtration method, and an MPN method. In the membrane filtration method the sample is filtered onto MLGA and incubated at 30°C for 4 hours and at 44°C for a further 14 hours. The target colonies are identified chromogenically and enumerated from the membrane. Due to the specificity of the MLGA medium confirmation of blue / green isolates indicating *Escherichia coli* is not required. The laboratory also use an multiple tube fermentation (MTF) method which uses Lauryl Tryptose Broth (LTB) with Bromocresol Purple which is an MPN method, the samples are incubated at 37°C for 48 hours. Confirmatory tests are carried out using Brilliant Green Bile Broth and Tryptone Water.

2.7.4 United Utilities also carry out *Salmonella spp.* analysis on sludges, soil, soil improvers, growing media and biowaste samples, firstly the percentage of dry solids is determined,

and then a wet weight equivalent of 2g dry solids is used in multiples of 10g aliquots for a presence / absence result. The aliquots contain Buffered Peptone Water and the sample is incubated for 24 hours at 37°C. The next stage of the analysis involves using Rappaport Vassiliadis for 48 hours at 41°C. At the 24 hour stage the sample is plated out on to XLD agar and at the 48 hour stage the sample is plated out on to Brilliant Green Agar (BGA) both plates are incubated for a further 24 hours at 37°C. Confirmation of target organisms is achieved through the use of TSI and Urea (24 hours at 37°C). If Urea and H2S are negative, serology is used (poly O/H).

2.7.5 South West Water use the same methods at present as United Utilities (North West), they are however, looking into using the Colilert® method on sludge samples. The Colilert® method, if successfully trialled will eventually replace the existing methods for *Escherichia coli* namely MLGA and LTB respectively.

2.7.6 Anglian Water detect *Escherichia coli* using a chromogenic membrane filtration method. The method uses MLGA as the culture medium. Anglian Water take part in an EQA scheme and stated that the results submitted for the chromogenic *Escherichia coli* method were acceptable.

2.7.7 The method used for *Salmonella spp*. detection and enumeration follows the CEN TC308/WG1/TG5, (2003e) Characterisation of sludges- Detection of *Salmonella spp*. - Part 2: Liquid enrichment method in selenite-cystine medium followed by Rappaport-Vassiliadis for semi-quantitative Most Probable Number determination listed as a draft ISO standard. Due to the length of the current MPN method Anglian Water looked into the possibility of using chromogenics for *Salmonella spp*. testing, however, this did not compare favourably with the MPN method. Anglian Water is hoping to adapt the current MPN method to cut down the analysis time as results are required as quickly as possible.

2.7.8 The information received from UK laboratories highlights which methods are currently in use for *Escherichia coli* and *Salmonella* testing for sludges, soil, soil improvers, growing media and biowastes. The information is limited to the UK laboratories; however, every effort was made to contact laboratories throughout Europe. The response from the European laboratories was limited and therefore a plurinational approach has not been possible.

Table 13: Results of Salmonella spp. Performance Data Part 1.

The preliminary data for the membrane filtration method illustrates the effectiveness of the treatment process. The trial does however; only test two replicates for each of the *Salmonella spp*. samples. The full validation of the method should incorporate more laboratory samples and different types of sludges to test fully the robustness of the method under routine testing situations.

Table A.1 *Salmonella seftenberg*

Treatment	**Dilution**	**Rep 1 (n=3) cfu/g**	**Rep 2 (n=3) cfu/g**	**Mean cfu/g**
Pre-	10^4	1,42 x 10^6	1,18 x 10^6	1,30 x 10^6
	10^5	1,25 x10^6	1,60 x 10^6	1,43 x 10^6
Post-	Undiluted	< 1	< 1	< 1
	Undiluted	< 1	< 1	< 1
Inocula mean		1, 34 x 10^6	1, 39 x 10^6	1, 37 x 10^6

Table A.2 *Salmonella typhimurium*

Treatment	**Dilution**	**Rep 1 (n=3) cfu/g**	**Rep 2 (n=3) cfu/g**	**Mean cfu/g**
Pre-	10^3	2,65 x 10^4	2,90 x 10^4	2,78 x 10^4
	10^4	3,00 x10^4	2,50 x 10^4	2,75 x 10^4
Post-	Undiluted	< 1	< 1	< 1
	Undiluted	< 1	< 1	< 1
Inocula mean		2,83 x 10^4	2,70 x 10^4	2,77 x 10^4

Table A.3 *Salmonella enteriditis*

Treatment	**Dilution**	**Rep 1 (n=3) cfu/g**	**Rep 2 (n=3) cfu/g**	**Mean cfu/g**
Pre-	10^5	4,00 x 10^6	5,30 x 10^6	4,65 x 10^6
	10^6	1,00 x10^7	6,00 x 10^6	8,00 x 10^6
Post-	Undiluted	< 1	< 1	< 1
	Undiluted	< 1	< 1	< 1
Inocula mean		7,00 x 10^6	5,65 x 10^6	6,33 x 10^6

Figure 4: Summary of Performance Data for Part 2 method

Characterisation of *Salmonella* spp. enumeration method
Intra- and interlaboratory trials (14 French laboratories working on 5 different materials) have been carried out in 2001 and 2002.

The trials have been carried out with sludges from a French waste water treatment plant, after centrifugation. These sludges samples have been collected before lime treatment.
The sludges were not naturally contaminated with *Salmonella spp*. and therefore have been spiked with an environmental strain of *Salmonella spp*. before the trials have been carried out.
The quantitative technique described (Most Probable Number with 3 dilutions and 3 tubes per dilution) does not generate any significant error (either repeatability or reproducibility). The dispersion can be totally attributed to sampling (dispersion linked to the constitution of aliquot fractions, described by the Poisson distribution).

The method characteristics can be summarised as follows :
– quantification limit = 10 *Salmonella spp*. in 10 g of dry matter (α=•5 %), 14 *Salmonella* spp. (α• = 1%) ;
– higher limit of quantification: 465 *Salmonella spp*. in 10g de dry matter (α=•5 %),
263 *Salmonella spp*. (α=•1 %);

Table 14: Summary of Results of Performance Data Part 3(CEN 2003f)

Table 14 (a) — Comparative studies for examination of Salmonella spp.

Additions / enrichment (peptone water)	Enrichment	Selective medium	*Salmonella seftenberg* H2S+ 101, 103, 105					
			Set 0 Detection-qualitative					
			Sludge		Food waste		Liquid manure	
			37°C	43°C	37°C	43°C	37°C	43°C
Novobiocin	1. Rappaport	XLD/BPLA	+	+	+	+	+	+
	2. Tetrathionate with Novobiocin	XLD/BPLA	+	+	+	+	+	+
	3. Rappaport with Novobiocin	XLD/BPLA	+	+	+	+	+	+
			Salmonella seftenberg H2S- 101, 103, 105					
			Set 0 Detection-qualitative					
			Sludge		Food waste		Liquid manure	
			37 °C	43°°C	37°°C	43 °C	37°°C	43°°C
Novobiocin	1. Rappaport	BPLA	+	+	+	+	+	+
	2. Tetrathionate with Novobiocin	BPLA	+	+	+	+	+	+
	3. Rappaport with Novobiocin	BPLA	+	+	+	+	+	+
			Salmonella enteriditis 101, 103, 105					
			Set 0 Detection-qualitative					
			Sludge		Food waste		Liquid manure	
			37 °C	43 °C	37 °C	43 °C	37 °C	43 °C
Novobiocin	1. Rappaport	XLD/BPLA	+	+	+	+	+	+
	2. Tetrathionate with Novobiocin	XLD/BPLA	+	+	+	+	+	+
	3. Rappaport with Novobiocin	XLD/BPLA	+	+	+	+	+	+

XLD = Xylose - Lysine - Deoxycholate – Agar.

BPLA = Brilliant green – Phenol red - Lactose - Saccharose – Agar.

Table 14 (b) — Results of the comparative validation with several cultural methods of 112 waste water and 130 compost samples collected under practical conditions

Additions /enrichment (peptone water)	Enrichment	Selective medium	Material			
			Waste water		Compost	
			Detection		Detection	
			37°C	43°C	37°C	43°C
without	1. Rappaport	XLD/BPLA	53,95%	8,03%	72,81%	16,15%
	2. Tetrathionate with Novobiocin	XLD/BPLA		2,88%		23,43%
	3. Tetrathionate	XLD/BPLA	27,63%	13,39%	50,48%	22,31%
	4. Tetrathionate	XLT	0,00%	0,00%	6,41%	0,88%
Ferrioxamin E	1. Rappaport	XLD/BPLA	47,37%	5,36%	69,90%	13,85%
	2. Tetrathionate with Novobiocin	XLD/BPLA		3,85%		12,62%
	3. Tetrathionate	XLD/BPLA	17,10%	11,60%	43,69%	23,08%
	4. Tetrathionate	XLT	0,00%	0,00%	2,56%	1,77%
	5. Rappaport with Ferrioxamin E	XLD/BPLA	38,16%	5,36%	67,97%	12,30%
	6. Tetrathionate with Novobiocin and Ferrioxamin E	XLD/BPLA		2,88%		13,52%
	7. Tetrathionate with Ferrioxamin E	XLD/BPLA	17,10%	10,71%	47,58%	18,46%
	8. Tetrathionate with Ferrioxamin E	XLT	0,00%	0,00%	1,28%	0,88%
Sodium pyruvate	1. Rappaport	XLD/BPLA	47,37%	13,40%	76,70%	17,69%
	2. Tetrathionate with Novobiocin	XLD/BPLA		2,88%		13,51%
	3. Tetrathionate	XLD/BPLA	10,52%	12,50%	50,48%	20,00%
	4. Tetrathionate	XLT	0,00%	0,00%	6,41%	2,65%
Ferrioxamin E and sodium pyruvate	1. Rappaport	XLD/BPLA	47,37%	8,03%	71,84%	20,77%
	2. Tetrathionate with Novobiocin	XLD/BPLA		3,85%		18,92%
	3. Tetrathionate	XLD/BPLA	11,84%	10,71%	54,37%	20,00%
	4. Tetrathionate	XLT	0,00%	0,00%	6,41%	1,77%
Novobiocin	1. Rappaport	XLD/BPLA	47,37%	3,57%	74,76%	6,15%
	2. Tetrathionate with Novobiocin	XLD/BPLA		0.96%		1,80%
	3. Tetrathionate	XLD/BPLA	22,37%	0,89%	64,07%	11,54%
	4. Tetrathionate	XLT	0,00%	1,11%	6,41%	0,88%
			n=112		n=130	

XLD = Xylose - Lysine - Deoxycholate – Agar.

BPLA = Brilliant green – Phenol red - Lactose - Saccharose – Agar.

XLT = Xylose - Lysine - Tergitol 4 – Agar.

3. EVALUATION OF DRAFTING A HORIZONTAL STANDARD

3.1 Techniques Used - Escherichia coli

3.1.1 Of the *Escherichia coli* standards assessed, some used the membrane filtration technique and some used the Most Probable Number (MPN) technique. There are other commercial methods in use which rely on different principles such Colilert® with the Quantitray® system. However, in order for standardisation to be realised, a choice has to be made regarding the preferred technique of analysis. The standardisation of methods in the laboratory is important. International standard methods should be evaluated under local conditions before they are formally adopted by national surveillance programmes.

3.1.2 In the first membrane filtration method (MF) method **(**CEN TC308/WG1/TG5 (a)) 10g of sludge is added to 90ml of MRD and it is then diluted down to 10^7 for untreated sludge. The difference in the membrane filtration methods primarily differ in terms of the media used to grow the target organism. The report includes four membrane filtration methods for *Escherichia coli* in sludge, the chromogenic media, MLGA method, MLSB, MLSA, the environment agency SCA method (2003a), and the Chromocult® method.

3.1.3 In the first MPN method (part 2) 10g of dry weight matter is added to the tryptone diluent in a final volume of 100ml, this is the primary suspension. The primary suspension is then added to the special diluent. (ISO 9308-3, 7.2.1) The micro plate is then inoculated and incubated for 36 hours at 44°c.

3.1.4 The second MTF method (CEN TC308/WG1/TG5 (b)) again relies on MPN techniques where 20g wet weight is placed into 180ml of 0.9% sterile NaCl solution. A serial tenfold dilution is prepared (1ml PS + 9ml 0.9% NaCl) up to 10^7. All the tubes are then inoculated from each dilution step into 3 tubes containing 5ml MUG fluorocult lauryl sulphate broth and incubated for 40 hours at 44°c.

3.1.5 It is evident when assessing the procedures they differ in terms of analysis techniques. Very often the choice between multiple tube and membrane filtration methods will depend on national or local factors e.g. the equipment already available or the cost of certain consumables. The advantages and disadvantages of each method should be considered when a choice has to be made. (WHO: 1997). These differences are highlighted and summarised below.

Most Probable Number Method	Membrane Filtration Method
Slower: Requires 48h for a negative Or presumptive positive result	Quicker: Quantitative results in about 18 hours
More labour intensive	Less labour intensive
Requires more culture medium	Requires less culture medium
Requires more glassware	Requires less glassware
More Sensitive	Less sensitive
Result obtained indirectly by statistical approximation (low precision)	Result obtained directly by colony count (high precision)
Not readily adaptable for use in the field	Readily adaptable for use in the field
Applicable to all matrices	Not applicable to all matrices without serial Dilution
Consumables readily available in most Countries	Consumables costly in many countries
May give better recovery of stressed or damaged organisms under some circumstances	
No need for filtration	Filter may be blocked by the sludge / biowaste matrix

3.1.6 There are other methods available for the detection and enumeration of bio-solids particularly in the U.S. and Canada. The Greater Vancouver Regional District (GVRD) relies on MPN multiple tube fermentation (MTF) technique using A-1 medium to enumerate *Escherichia coli* in bio solids. The A-1 medium is used because it is reliable and efficient in recovering *Escherichia coli* in just 24 hours. For a number of years, the only recognised multiple tube fermentation technique involved the use of an enrichment step of the coliforms in LTB to obtain the optimum recovery of coliforms. The total time required for the procedure was 72 hours. The medium A-1 was developed in the early to mid 1990's; it can recover *Escherichia coli* in 24 hours and in greater numbers. The GVRD uses the A-1 method for monitoring *Escherichia coli* in both recreational water and in bio solids. All samples are conditioned in an incubator at 35°c for 3 hours before they are placed in a faecal water bath at 44.5°c.

3.1.7 There are other methods capable of detecting and enumerating *Escherichia coli* from sludges, soil, soil improvers, growing media and biowaste. For example the commercially available Colilert® system from IDEXX, combined with the Quantitray System® (both supplied by IDEXX) is able to detect and enumerate *Escherichia coli* from a biosolid matrix.

3.1.8 A research note detailing the use of this method (Kramer and Liu 2002) illustrates how the Colilert® method compares with multiple tube fermentation (MTF). At the time of the study multiple tube fermentation (MTF) was accepted in the U.S. for the enumeration of waste activated solids (WAS), and membrane filtration was tentatively viewed as acceptable. The MTF analysis followed the procedure which is outlined in the draft standards; CEN TC308/WG1/TG5 Detection and enumeration of *Escherichia coli* from sewage sludge. Part 2: Miniaturised method (MPN) in liquid medium and CEN TC308/WG1/TG5 Detection and enumeration of *Escherichia coli* from sewage sludge Part 3: Macromethod (MPN) in liquid medium.

3.1.9 The paper (Kramer and Liu 2002) outlines numerous comparisons that have been made between Colilert® / Quantitray® system and all existing standards and concludes that the Colilert® system is effective at detecting and enumerating coliforms and *Escherichia coli* from waste activated solids (WAS). However, it should be noted that studies indicated that false positives did occur which were caused by the presence of *Aeromonas* bacteria.

3.1.10 The results of the paper indicated that the Colilert® / Quantitray® system produces equal most probable number (MPN) numbers for *Escherichia coli* obtained from waste activated solids (WAS) samples of varying bacterial content.
It should be noted that the Colilert® / Quantitray® system is also under review by Prof. C.W. Keevil and S.L.Warnes of Southampton University as part of unit 4.3.2 of Work Package 3. The study of rapid methodology for the detection and enumeration of *Escherichia coli* is incorporated into that section of study. (However the Colilert® method (Kramer and Liu 2002) has also been covered in this report as it has been included as one of the three UK draft SCA methods (SCA 2003c) and is routinely employed by a number of laboratories in the UK for both potable water, sludges, soil, soil improvers, growing media and biowaste analysis.)

3.1.11 See ***Appendix 7*** for a summary of the results of a recent UK trial using Colilert Quantitray method

3.2 Salmonella spp. – Evaluation of techniques used

3.2.1 The detection and enumeration of *Salmonella spp.* uses four techniques, there are eight potential methods included in the report. The four techniques are membrane filtration, liquid enrichment miniaturised for MPN, Presence /Absence liquid enrichment, and the two commercially available systems relying on immunological techniques. (Matrix MicroScience Pathatrix® IMS system and the Merck Singlepath GLISA Salmonella® Lateral Flow Test.)

3.2.2 The membrane filtration method involves filtering a diluted sludge sample from a series of serial dilutions. The membrane is placed in a resuscitation tetrathionate broth for 24 hours at 36°c to recover the sub-lethally damaged *Salmonella spp*. After the resuscitation step the membrane is placed on to Rambach agar for 24 to 48 hours, the latter longer time period being required to recover the slower growing *S. dublin*. *Salmonella spp.* are indicated by the presence of red colonies resulting from fermentation of propylene glycol, some produce β-galactosidase which hydrolyses x-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to form a blue chromophore. The suitability of the membrane filtration technique for a sludge matrix has to be discussed; the solids content of the matrix requires serial dilution steps to enable the sample to pass through the membrane. The high serial dilution levels the sample is typically analysed at is an area where the accuracy of the method may be compromised. This method is included primarily as a method for determining log drop during sludge treatment. It is not considered suitable for demonstrating absence of *Salmonella spp.* at the low levels of interest.

3.2.3 There are certain techniques within the methods: CEN TC308/WG1/TG5, (2003d) Detection and Enumeration of *Salmonella spp* in sludges, soils, soil improvers, growing media and biowastes— Part 1: Membrane filtration method for quantitative resuscitation of sub-lethally stressed bacteria (to confirm to efficacy of log drop treatment procedures) and Microbiology of Sludge Part 4 (2003) – Isolation and Enumeration of *Salmonella spp.* membrane filtration method using resuscitation and chromogenic techniques. The membrane filtration methods are methods that require further discussion.

3.2.4 There are four methods in the report which use resuscitation steps or liquid enrichment stages as part of either a presence / absence method or most probable number (MPN) method. The nature of *Salmonella spp.* requires the resuscitation and enrichment steps to be sensitive to the target organism. The methods that have been studied use different resuscitation broths and liquid enrichment medium, as a result the potential for variation in results occurs depending on the sensitivity of these steps. Selenite is a good selective broth for *Salmonella spp.* as it is highly inhibitory to many of the competitor organisms. However a 'sub-lethally damaged organism' may not be recovered as efficiently and is not reliable for the detection of *S. typhi.* Tetrathionate broth is less inhibitory and is thought to be favoured for the recovery of enteric *Salmonella spp.* from clinical samples. Rappaport Vassiliadis is a favoured medium and it compares well against selenite for the recovery of all types of *Salmonella spp.* where they are or maybe in small numbers against a heavy competitive presence of other organisms. It would be advisable to use peptone saline or some suitable resuscitation means as a first step. If this is done then it is then safer to use selenite (but the risk of not picking up enteric *Salmonella spp.* remains).

3.2.5 The enumeration of *Salmonella spp*. by a membrane filtration technique with resuscitation and culture on a chromogenic detection medium SCA (2003h) is suitable for the examination of untreated, conventionally treated, and enhanced treated sludges. Samples of conventionally treated sludge may include mesophilic anaerobic digested sludges; enhanced treated sludges may include sludges derived from treatment processes such as, thermophilic digestion, lime stabilisation, and composting. Depending on the sludge matrix, different preparative techniques may be required prior to using this method (see 1.5.2).

3.2.6 The principle of the method involves homogenising and serially diluting the sample. The diluted sludge is filtered through a membrane filter and incubated at a temperature of 36°C on a sterile glass fibre filter saturated with tetrathionate broth resuscitation medium. After incubating for 24 hours, the membrane filter is further incubated at 36°C on chromogenic medium (Rambach agar). The membranes are examined after 24 and 48 hours and positive colonies are enumerated. The presence of *Salmonella spp.* is indicated by the presence of bright red colonies resulting from the fermentation of propylene glycol. Colonies of other members of the *Enterobacteriacae* appear blue, green, violet, or colourless due to their inability to ferment propylene glycol.

3.2.7 According to the SCA method (SCA 2003h), it is stated that there are certain limitations regarding the use of the method, sludge samples with high solids content (greater than 20% m/V) tend to block the membrane filter at minimal dilutions. The level of solids may also mask or inhibit the growth of the target organism. This will affect the sensitivity of the method and limit the level at which *Salmonella spp.* can be detected and enumerated. The growth of high numbers of non-target bacteria on the membrane may inhibit or obscure the growth of *Salmonella spp.*

3.3 Methodology Variables

3.3.1 Of the methods assessed in the trial there are obviously differences in methodology as they rely on different principles. When trying to evaluate the methods in terms of the feasibility of producing a horizontal standard it is logical to look at the variables in each method. What effect do these variables have on the performance of the method?

3.3.2 The apparatus used in the standards should be of recognised quality and conform to all the manufacturers' guarantees. The standard of the equipment used could have an effect on the ability of the method to detect and enumerate *Escherichia coli* and *Salmonella spp*. from the sludge matrix. With the exception of equipment supplied sterile, all apparatus should be sterilised in accordance with the guidelines outlined in ISO 8199 (2003).

3.3.3 The sample that arrives at the laboratory for analysis must do so under strict controlled conditions, the treatment of the sample will ultimately affect result the laboratory produces. A sample of 100g wet weight is taken from the sample site; this can vary between different standards. The sample is taken and transported to the laboratory at 5°C (±3°C) as quickly as possible, it is important that the same treatment is awarded to every sample analysed. It is important that there is proper control over the sampling and transportation of sludge, if this is not the case, differences in the methods will be insignificant due to the difference in the samples when they arrive at the laboratory.

3.3.4 The procedure of the method can also affect the outcome of the result; the nature of reagent the sample is exposed to during some procedures may affect the bacterial content of the sample. The use of an enrichment step in certain methods such as CEN TC308/WG1/TG5, (2003e) Detection and Enumeration of *Salmonella spp*. in sludges, soils, soil improvers, growing media and biowastes -Part 2: Liquid enrichment method in selenite-cystine medium followed by Rappaport-Vassiliadis for semi-quantitative Most Probable Number determination, the use of selenite cysteine may inhibit the

recovery of *Salmonella spp*. from the sample. The same method also requires the use of a glass fibre filter to decant the supernatant from the tubes to remove the fine debris can cause a lower enumeration figure depending on the pore size. The target organisms may become lodged on the glass fibre filter and result in lower numbers detected from the sample.

3.3.5 An integral part of any method, the media provides the target organism in this case *Escherichia coli* and *Salmonella spp.* with the nutrients it requires to grow. It is therefore important that the media in use across Europe complies to certain standards so that certainty regarding its performance can be assured.

3.3.6 In the CEN TC308/WG1/TG5 Draft Standard: - Detection and Enumeration of *Escherichia coli* in sludges, soils, soil improvers, growing media and biowastes– Part 1: Membrane filtration method for quantification (MLGA) the compound 5-bromo-4-chloro-3-indolyl-β-d-glucuronide (BCIG) is used as a chromogenic substrate to enumerate the target organism. The ingredients for membrane lactose glucuronide agar (MLGA) are listed in the method and instructions are given on how to make the media. However, MLGA is commercially available from Oxoid™. The question of whether or not making the media from the list of ingredients or buying the commercially available product would affect the method performance has to be discussed. This applies to all media used in this way.

3.3.7 In the same membrane filtration (MF) method two types of maximum recovery diluent (MRD) are listed, the first uses Bacteriological Peptone, NaCl, and Distilled Water. The second uses Oxoid MRD® (CM733), and Distilled Water. Does this affect the performance of the MRD?

3.3.8 If a viable, workable, standard is to be introduced everything in the method, including the media has to be consistent across the whole of Europe. If, for example, the commercially available MLGA performed better than the self-made media, discrepancies may occur if laboratories are using two different forms of MLGA. That is just one scenario possible in one method; there is therefore a need to reduce the variability in the media used if standardisation is to be a workable concept. A suggestion may be to follow the guidelines outlined in ISO 8199 to ensure all media meet certain standard requirements.

3.3.9 Variation between incubation times and temperatures between the existing draft CEN and other potential methods:

Part 1: 30°C 4 hrs / 44°C 14 hrs (CEN 2003a)

Part 2: 44°C 36hrs (CEN 2003b)

Part 3: 44°C 40± 4 hrs (CEN 2003c)

IDEXX Colilert®: 35°C 24 hrs (SCA 2003a)

A-1 Method: 35°c 3 hrs / 44.5°C 21 hrs (Andrews and Presnell 1990)

U.S. EPA MTF Method: 35°C 48 hrs / 44.5°C 24 hrs (APHA 1998)

In the Membrane Filtration method for quantification (MLGA), the target organism *Escherichia coli*, is defined as a member of the family *Enterobacteriaceae*, • -glucuronidase-positive and is able to hydrolyse 5-bromo-4-chloro-3-indolyl-• -glucuronide (BCIG) when growing on an agar medium at the temperature of 44°C. All the draft ISO standard methods state 44°C as the incubation temperature, whereas the U.S. methods for MTF and Colilert® have temperatures of 44.5°C and 35°C respectively.

3.3.10 A fundamental issue of any draft method is whether or not the method can work under routine test conditions. The method should be thoroughly tested for its robustness through vigorous validation performance tests. The performance data submitted with the majority of the draft standards does not test the methods thoroughly, and may not identify any potential problems. The protocol for the validation of performance data included in this report should ensure any validated standards are robust and fit for purpose. All standards should be easy to use under routine laboratory conditions when time is an important consideration. The importance of robustness and ease of routine use is a crucial consideration in the selection of any method.

3.4 *Implications of Uncertainty of Measurement*

3.4.1 The QWAS proficiency scheme of *E. coli* simulated effluent sludge has a target standard deviation of 0.50 (log_{10} basis) consequently the results are considered acceptable if they fall with +/- two standard deviations of the mean. For the two most recent exercises the following applies:-

Table 15: Review of QWAS proficiency scheme data April, June and October 2003

Exercise Date	*No. of labs*	*Assigned values*	**-2z**	**+2z**	*% of labs with results outside* **± 2z**	*Std Dev of log values*

April 2003	13	2939	294	29390	23	0.60
June 2003	15	1100	110	11,000	27	0.80
October 2003	17	648	64.8	6480	11	0.72

3.4.2 In the context of an absolute limit of 500 *E. coli*/g sludge DS these results would indicate that using existing *E. coli* methods, it would be pointless to try and enforce this limit. It is possible to imagine a scenario where a sludge producer sends its samples to a number of accredited laboratories and reports the lowest received result to the regulator.

3.4.3 It is thought the vast majority of laboratories taking part in the above scheme are all experienced water company laboratories with UKAS ISO 17025 accreditation.

3.4.4 With respect to the determination of log reductions during sludge treatment, the situation is even worse as the reduction is calculated from two measurements. Using the two standard deviations (0.80 and 0.60) of the April and June 2003 exercises would result in a log reduction (difference) standard deviation of 1.0. Thus for the 95% confidence limits for a calculated log reduction would be approximately the mean log reduction result ± 2.0. It is important that these issues are addressed.

3.4.5 It would appear that no matter what *E. coli* method is used, that regulatory action would not be appropriate on a single sample result. It is recommended that the minimum acceptable performance data for regulatory parameters should be specified in any legislation together with a recommended sampling protocol. Failure should be determined on the results from a number of samples.

3.4.6 Before any microbiological limit for a given parameter is set it is important that the existing state of the art analytical performance for that parameter is determined.

3.4.7 The UK approach for regulatory drinking water chemical analysis (SI 2000) and contaminated land analysis (Environment Agency 2003) where method performance standards are specified rather than fully prescribed methods could be considered. It is acknowledged that this is more difficult with microbiological methods.

3.4.8 The alternative approach, as adopted in the UK *Cryptosporidium* regulations (included within SI 2000), is to rigidly specify a method, run a compulsory proficiency scheme backed up by regulatory announced and unannounced audits. This latter approach has proved very successful for improving the accuracy and precision of regulatory

Cryptosporidium analysis in treated waters, but at a very high financial cost. These regulations also address the sampling aspects of the analysis which are fundamental in obtaining fit for purpose results.

3.4.9 The EU should seriously consider setting up a blind proficiency trial to assess the real state of regulatory analysis across Europe as a top priority in order to assess the current real situation with respect to routine sludge microbiological analysis. (See 4.5.4). Only laboratories currently carrying out routine regulatory sludge analysis should be included in this trial. Also the setting up of a compulsory EU wide sludge proficiency scheme(s) for laboratories carrying out routine regulatory sludge analysis should also be seriously considered. (See 4.5.6 - 4.6.10)

3.4.10 Efforts should be made to make reliable certified microbiological reference materials available at an affordable price so laboratories can ensure adequate QC to ensure that a method remains in control and can achieve the specified detection limit.

4. CRITICAL POINT AND RECOMMENDATIONS

4.1 ***Sampling***

4.1.1 The Horizontal sampling study should start to give an idea of the magnitude of the uncertainties of sludges, soil, soil improvers, growing media and biowaste sampling with respect to chemical and physical analysis. It is essential that this key aspect of sampling for microbiological analysis is also addressed. Typical uncertainties associated with sampling for *E. coli* and *Salmonella spp.* analysis in various sludges, soil, soil improvers, growing media and biowaste matrices need to be estimated. SCA (1977) has published procedures for sampling and initial preparation of sewage and waterworks' sludges, soils, sediments and plant materials prior to analysis. However, this publication only discusses chemical and physical testing. There is little published on protocols for microbiological sampling of sludges, soil, soil improvers, growing media and biowaste. It is important that this key area is properly addressed.

4.1.2 Appropriate Health and Safety guidelines for sampling sewage sludges, soil, soil improvers, growing media and biowaste that may contain pathogens must be followed.

4.1.3 All samples should be taken in an appropriate container (e.g. polyethylene or polypropylene) in a correct manner. Glass containers should be avoided owing to potential fermentation and gas pressure build up.

4.1.4 The storage and transport of the sample should be undertaken in a manner designed to minimise change, including deterioration, and avoid contamination. The maintenance of integrity through representative sub-sampling for analysis, accurate dilution and adequate homogenisation using appropriate aseptic technique all contribute to the reproducibility of microbiological sludge, compost, soil and biowaste results.

4.1.5 The UK approach to sampling (SI 2002) is recommended where the heterogeneous nature of sludge samples is addressed by a requirement that on each occasion a set of five samples shall be taken at random from each batch of sludge to be tested. Each sample consisting 100ml in the case of liquid sludge or grams in the case of a dried sludge. Each sample is individually analysed. Taking a single grab sample is not considered suitable for this type of analysis. Micro-organisms are unlikely to approach an even distribution in sludge, composts, soil, and biowastes.

4.1.6 The sample pre-treatment steps (sub-sampling, homogenisation etc.) must also be carefully considered and comprehensively documented.

4.1.7 The temperature variance in Europe can be as much as 60^{o}C. Northern Finland can routinely experience ambient temperatures of –20^{o}C whilst Southern Greece can routinely reach 40^{o}C. It is very important that once samples have been taken, the conditions for each sample should remain under the same specified conditions (e.g. 3 – 5^{o}C) until arrival at the analysing laboratory within a specified time period from the sampling.

4.2 Evaluation of Potential Methods

4.2.1 The draft CEN methods and alternative methods for the detection and enumeration of *Escherichia coli* and *Salmonella spp.* in a sludges, soil, soil improvers, growing media and biowaste matrix have been evaluated with a view to implementing horizontal standards across Europe.

4.2.2 There are three draft CEN standards put forward for the detection and enumeration of *Escherichia coli* and three draft CEN methods for *Salmonella spp.* The methods are evaluated on their ability to produce fit for purpose results in line with the proposed revised sludge directive (EU 2000). The methods drafted by CEN/TC308/WG1 are intended to provide fit for purpose microbiological results for a wide range of sludges, soil, soil improvers, growing media and biowaste matrices.

4.2.3 The draft *Salmonella spp.* membrane filtration method CEN TC 308/WG1/TG5 (2003d) for *Salmonella spp*. (Part 1: Membrane filtration for quantitative resuscitation of sub-lethally stressed bacteria (to conform to efficacy of 6-log drop treatment procedures) has been evaluated. This method is primarily for determining log reductions. The pore size of the glass filters is quoted in the method as being 2.7µ. The size of the pore on the glass fibre filter is much larger than that of the nominal membrane pore size and the typical dimensions of a *Salmonella spp.*. However, when dealing with some very high solid sludge matrices containing much colloidal material, this may significantly reduce the pore size of the filter. For these type of samples it is recommended that a recovery check is carried out.

4.2.4 The evaluation of the other CEN draft *Salmonella spp.* methods looked at CEN TC308/WG1/TG5 (2003e) Part 2: Liquid enrichment in selenite cysteine medium followed by Rappaport-Vassiliadis for semi-quantitative Most Probable Number

determination and CEN TC308/WG1/TG5 (2003f) Part 3: Presence / absence method by liquid enrichment in peptone-novobiocin medium followed by Rappaport-Vassiliadis. The evaluation of the methods highlights the presence / absence method has a greater sensitivity to that of the MPN method due to the sample weight analysed by each method. The presence / absence method has a potential minimum level of detection of 1 viable *Salmonella spp.* / 50g, whereas the MPN method has a potential minimum level of detection of 1 viable *Salmonella spp.* / 10g. There is no evidence that these levels could be detected. The current lower level of detection for the presence / absence method (CEN 2003c) is 1 viable *Salmonella spp.* / 10g. Hopefully the proposed method validation trials will give a better estimate of the true lower level of detection.

4.2.5 With regard to the MPN method (CEN 2003e), there is also an issue with use of selenite cysteine as an enrichment medium (***Appendix 4***). There are concerns about the use of selenite cysteine because it may cause the inhibition of sub-lethally damaged *Salmonella spp.* This is an issue which requires further investigation to determine the existence or extent of the inhibition. There are also concerns regarding the human toxicity and Sweden has made the comment that selenite is carcinogenic and that suitable warnings must be included in the method.

4.2.6 Thompson et al. (2002) have provided extensive validation data using a method based on CEN TC308/WG1/TG5 (f) Part 3: Presence / absence method by liquid enrichment in peptone-novobiocin medium followed by Rappaport-Vassiliadis with a 20g sample weight. The results illustrate the method is able to perform well at low levels of *Salmonella spp.* The evaluation of the *Salmonella spp.* draft methods concludes that the CEN TC308/WG1/TG5 (f) Part 3: Presence / absence method by liquid enrichment in peptone-novobiocin medium followed by Rappaport-Vassiliadis is able to provide the most accurate and fit for purpose results in line with the revised sludge directive. (EU 2000).

4.2.7 The *Escherichia coli* MPN methods CEN TC308/WG1/TG5 (b) Part 2: Miniaturised method (MPN) in liquid medium and CEN TC308/WG1/TG5 (c) Part 3: Macromethod (MPN) in liquid medium have been evaluated for possible standardisation. The conclusion from the evaluation is that the macromethod has a higher degree of sensitivity; the method has a minimum level of detection of 1 viable *Escherichia coli* / 20g compared with 1 viable *Escherichia coli* / 10g for the miniaturised method. The comparison between the two methods is made difficult by the lack of performance data

attached to the draft standards. There is a requirement to test the methods further in terms of robustness and ease of routine use; this will enable an informed decision to be made regarding the choice of the most suitable method. The conclusion would be at this time to accept the CEN TC308/WG1/TG5 (c) Part 3: Macromethod (MPN) in liquid medium as the preferred method for the analysis of *Escherichia coli* in a sludge matrix.

4.2.8 The alternative conventional membrane filtration and MPN *E. coli* and *Salmonella spp.* methods considered were not thought to be significantly better than the draft CEN methods.

4.2.9 The *E. coli* chromogenic membrane filtration method, SCA (2003a) is thought to have considerable potential as it minimises the time for confirmed results. The specificity of the medium (i.e. MLGA) for *E. coli* is such that, following performance verification within the laboratory, confirmation of green colonies as *E. coli* may not be required. The combination of the selectivity of temperature and specificity of β-glucuronidase are considered sufficient for most practical purposes.

4.2.10 The Colilert® SCA (2003c) defined substrate method is also thought to have considerable promise. It is robust, very simple to use and also does not require any confirmation. Results can be obtained in 18 hours. Although this is a commercial method, it should be given serious consideration. The fact that no media and minimal reagents are required should help to improve reproducibility across laboratories.

4.2.11 The Matrix MicroScience (2003) Pathatrix® IMS method is also thought to have potential for the detection of very low numbers of stressed *Salmonella spp*. It has a capital cost of 6,750 Euro with a test consumable cost of 8 Euro per sample. (See Table 17.)

4.2.12 The Merck Singlepath GLISA *Salmonella*® Lateral Flow Test is thought to be a potentially simple and robust test for routine laboratories

4.2.13 The evaluation has highlighted the requirement for further, more comprehensive validation across a wide range of matrices. Of the six draft standards proposed, there is a general lack of sufficient validation data attached to the methods. The methods require thorough validation using routine samples, obtained through interlaboratory exercises under the strict control of a pre-defined protocol to ensure accurate, fit for purpose results are achieved. Only then will the suitability of the method be illustrated.

4.2.14 There appears to be a reluctance for some countries to use membrane filtration techniques whilst others are reluctant to use MPN techniques. This conservative attitude needs to be addressed.

4.3 ***Performance Data and Validation Trials***

4.3.1 One area highlighted from the study of the potential methods was the general lack of fit for purpose comprehensive performance data. Also the range of countries that participated in all discussed *E. coli* and *Salmonella spp*. method validation studies all cases was limited. (Mostly limited to one country).

4.3.2 There is a need for more laboratories to take part in the validation of the standards. The more laboratories taking part the greater will be the confidence in the standard.

4.3.3 The range of matrices tested was limited to one or two for the reported performance studies. It is important that a representative range of sludge, soil, soil improvers, growing media and biowaste materials are tested.

4.3.4 If a laboratory wishes to apply an improved method to sludge, soil, soil improvers, growing media or biowaste matrices not listed in the standard (with appropriate performance data), then the laboratory shall be required to validate the method for these matrices. **(*see Appendix 5*)**. A recommended protocol for achieving this should be included in all relevant microbiological standards.

4.3.5 Presence/absence test methods (e.g. *Salmonella spp.)* where the result is simply expressed in terms of “detected” or “not detected” need to be validated using a range of typical sample matrices. The samples selected ideally should have a non-detectable level of the target organisms, but typical levels of competitive flora present. Then these samples should be spiked with the target organism close to the actual “not detected” statutory level (e.g. 2 – 10 organisms in the required statutory amount of sludge or associated dry solid). Also the same amount of organisms should be added to an equivalent weight of MRD. These latter samples act as a control. Replicate analysis of these three sets of samples (unspiked and spiked sludge, soil, and treated biowaste samples and MRD spiked samples) is needed to allow for the Poisson distribution of the added bacteria.

4.3.6 For quantitative microbiological test methods, (e.g. *E. coli*) the specificity, sensitivity, bias, repeatability, reproducibility at levels close to any statutory limits and limit of determination should be determined in the presence of typical levels of competing flora. (see 4.2.5). The differences due to the matrices must be taken into account when testing different types of samples. The results should be evaluated with appropriate statistical methods.

4.3.7 The issue of the range of target organism serovars that need to be tested needs to be agreed. (For instance *Salmonella spp.* has over 2000 different serovars and *E. coli* has a significant number of serovars.) For example, *S. dublin* is much more difficult to culture than the more common serovars such as *S. typhimurium* and *S. enteritidis.* This would be a good serovar for validating the *Salmonella spp.* methods.

4.3.8 The issue of the range of potential interfering competitive bacteria that needs to be tested also needs to be agreed.

4.3.9 Details of a UK interlaboratory trial to assess the Colilert® method on sludge and biowaste samples is given in Table 16 at the end of the report. ***Appendix 7*** illustrates the results of this study.

4.3.10 If an alternative simplified commercial method to the standard is proposed (e.g. Colilert® or Merck Singlepath GLISA *Salmonella*® Lateral Flow Test), suitable tests should be carried out to ensure that fit for purpose results are obtained relative to the CEN / ISO standard. A protocol for achieving this is outlined in **(*Appendix 5*)**.

4.3.11 An official approved scheme such as the AOAC International, Performance Tested Methods, Test Kit Database for food test method should be given serious consideration. (AOAC 2003)

In summary, in order to try and ensure that a standard method will give fit for purpose results the following issues need to be considered:-

- Matrices to be covered by the standard; the method should be tested for its robustness and repeatability with a variety of sludge, soils, soil improvers, growing media and biowastes;
- Typical levels of the target organisms and associated microbial flora likely to encountered;

- Statutory limit values (need to be able to detect 10% of any statutory quantitative limit);
- Suitable resuscitation steps need to be included to recover stressed and damaged organisms;
- Ensuring coverage of all relevant serovars of the target bacterium;
- Lack of false positives from potential interfering competitive bacteria;
- Lack of false negatives resulting from the sample matrix or adverse environmental conditions stressing viable bacteria;
- Robustness of method; it must be suitable for routine use by “normal” routine laboratory staff;
- Adequate method performance data (within and between batch data with adequate degrees of freedom);
- Suitable internal analytical control protocol to ensure that statutory limit monitoring results have acceptable uncertainty values:
- Suitable external analytical control (third party proficiency scheme). Any significant trend in the results that is detected must be investigated and rectified.

Feasibility of developing suitable microbiological reference materials needs to be investigated. If these could be developed, it should greatly assist in improving the accuracy of sludge microbiological analysis.

4.4 ***Standard Method Validation Protocol***

4.4.1 A draft protocol for carrying out chemical and microbiological analysis inter-laboratory trials has been circulated with TC308 for publication as a technical report (CEN 2003/WGI 2003) ***(Appendix 5).*** It is strongly recommended that this protocol should be adopted for validating any microbiological methods.

4.4.2 The issue of how to distribute homogeneous, stable sludges, soil, soil improvers, growing media and biowaste samples for method validation across Europe also needs to be addressed. Without this it will be very difficult to ensure that any proposed methods are fit for purpose. One major problem is that sending out sludge samples by air freight is virtually impossible due to the very stringent air transport safety regulations. One possible way forward is for each country to propose a lead laboratory, which would be responsible for distribution of the appropriate pre-tested sludges, soil, soil improvers, growing media and biowaste matrices within their country by courier, but all laboratories using the same spiking regime. (***Appendix 5***)

4.4.3 The various sludge samples would then be spiked via lenticules, pastilles or vitroid suspensions that contained a known amount of *E. coli* and *Salmonella spp.* prior to sample pre-treatment (e.g. homogenising). Also the same amount of the lenticules, pastilles or vitroids suspension would be added to 100ml MRD and treated in the same manner. This should give an idea of the effect of the matrix upon the analysis. It will not give any information on the efficiency of the pre-treatment process for release of the *E. coli* or *Salmonella spp*. from the solid material. (See also 4.3.5)

4.4.4 The UK has run a validation trial to assess the feasibility of the Colilert® method for *E. coli* detection in a range of five sludges/biowastes (Table 16) against the existing methods used by seven UK water company laboratories. Results from this trial are outlined in ***Appendix 7*** at the end of the report.

4.4.5 Another limited study is being set up to incorporate *E. coli* and *Salmonella spp*. in some dried sludge /biowaste products to attempt to assess the efficiency of the sample pre-treatment (e.g. homogenising) step in releasing *E. coli* and *Salmonella spp.* from solid material.

4.5 ***Relevant Proficiency Testing Schemes***

4.5.1 There is a UK-based proficiency scheme currently assessing analysis of sludge for *E. coli* and *Salmonella spp.* (QWAS 2003). QWAS operates a wide range of environmental and food proficiency schemes in over 55 countries.

4.5.2 The bacteria are incorporated into a (dried) simulated sludge matrix together with some coliform (non-*E. coli*) bacteria.

4.5.3 Homogeneity tests based on five random samples analysed in duplicate are carried out for each distribution.

4.5.4 No other commercial European scheme was found, although further efforts are being made to find other potential European proficiency schemes for *E. coli* and *Salmonella spp*. in sludges, soil, soil improvers, growing media and biowaste.

4.5.5 The QWAS scheme conforms to ISO 9001 (2000) and ISO (1977) ISO IEC Guide 43-1, Proficiency testing by interlaboratory testing, Part 1, Development and operation of proficiency testing schemes.

4.5.6 The data from the April 2003 *Salmonella spp.* distribution showed 13 laboratories participating in the exercise, the spiking level of the test sample was 77 cfu/g *Salmonella salford*, and the test was based purely on a presence / absence basis. Of the 13 laboratories, three laboratories reported a result of not detected which gave a 77% "detected" result from the participating laboratories. This was for a sample containing almost 4000 times the proposed *Salmonella spp.* limit of absence in a 50g received sample! (EU 2000). The standard of the analysis is quoted as: for *Salmonella spp.*; "Results were very good with 76% of participants correctly detecting *Salmonella spp.* in the test material"

4.5.7 The April 2003 *E. coli* proficiency sample had an assigned value of 2,939 cfu /g *E. coli*, and participants were judged between +2z scores and -2z scores (294 – 29,939 cfu/g) from the assigned value (2,939 cfu /g), of the sample. Of the 19 results 76% of participants reported a result within 2 z-scores of the assigned value, on a negative note the quantitative results for cfu /g ranged from 90 to 5,400 which is a large variation for a proficiency sample. For *E. coli;* "Results for the detection of *E. coli* were excellent" this is clearly not the case in relation to proposed statutory limits. (EU 2000).

4.5.8 The data from the June 2003 *Salmonella spp*. distribution showed 13 laboratories participating in the exercise, the spiking level of the test sample was 73 cfu/g *Salmonella montevideo,* and the test was based purely on a presence / absence basis. All 13 laboratories (29 actual results) detected *Salmonella spp.* which gave a 100% "detected" result from the participating laboratories.

4.5.9 The next *E. coli* exercise in June 2003 had an assigned *E. coli* value of 1,100 cfu /g *E. coli* and participants were judged between +2z scores and -2z scores (110 –11,000 cfu/g). Of the 35 results 71 % of participants reported a result within 2 z-scores of the assigned value, the quantitative results for cfu /g ranged from <3 to 4,600 cfu/g (ignoring one outlier of 110,000 cfu/g.) 14% of participants failed to detect the *E. coli*.

4.5.10 The QWAS samples were sent out as normal proficiency sample and therefore more attention is likely to be awarded to these samples over routine samples analysed at the laboratories. If the variation in results is so great between proficiency samples, what is the likely extent of variation between routine samples? Explanation as to the large variation in results submitted could be explained by sample variation between sites; ability of staff; differences in the sensitivity of the methods used and variations in resuscitation techniques used. However, the underlying fact is that there is a large variation in the quality of the analysis currently being produced by established and competent laboratories.

4.6 Further Development of Relevant Proficiency Testing Schemes

4.6.1 The performance data highlighted in this report clearly demonstrates the need for Europe wide compulsory proficiency scheme(s) for laboratories carrying out regulatory *E. coli* and *Salmonella spp.* in sludges, soil, soil improvers, growing media and biowaste. (also any other later regulated parameters would also have to be included). Due to the complex nature of the test material the nature of the matrix used to distribute the bacteria requires careful consideration when attempting to develop an accurate, robust, and representative external proficiency scheme for participating laboratories. There are many variables to consider when setting up and maintaining a Europe wide proficiency scheme. It is important that typical competing bacteria are considered. For the size of the likely European market, it is unlikely that more than two non-grant aided schemes could be supported

4.6.2 One way of achieving an accurate comparison between these participating laboratories is to send out sterile dry sludges to ensure that sample variation in transit is eliminated.

The inclusion of commercially available vitroids, lenticules or pastilles to provide the organisms for spiking could be a possible method that should be looked into. The vitroids, lenticules and pastilles should be from low pass generation NCTC cultures. They are useful for providing a test sample with a known concentration of organisms for detection and enumeration if required. However, sterilisation of the sludge damages the background organisms present in the matrix and does not provide a sample representative of a routine sample. Possible spiking with suitable levels of main competitor organisms may be a feasible option.

4.6.3 Recommendations of how to prepare a suitable sample for proficiency testing and interlaboratory method validation trial purposes should be discussed.

4.6.4 As has been seen with the QWAS proficiency data (4.4.6 – 4.4.9), there are discrepancies between the quality of the analysis between laboratories. The discrepancies exist on known proficiency samples which are likely to have been awarded more attention during analysis. It would therefore be an interesting exercise to devise a method for sending out parallel blind dry material proficiency samples as routine commercial samples to determine whether or not the quality of the results would differ from the existing proficiency scheme data. Although this would be expensive to set up, it will provide a true reflection of the standard of sludge analysis in Europe. It is strongly recommended that this exercise is carried out.

5. DRAFT STANDARD (CEN TEMPLATE)

5.1 TC308/WG1/TG5 has decided that all three draft *E. coli* methods (CEN 2003a, b and c) and all three *Salmonella spp.* methods (CEN 2003d, e and f) should be included in the final standard. Recommendations will be given as to the preferred method for specific applications and matrices. No single method will be suitable for all intended applications.

5.2 The six draft standards have been finalised and sent to CEN for approval as technical reports at this stage. The six standards are referred to as technical reports due to the lack of sufficient validation data attached to the standards. It is hoped that the proposed FP6 study will provide the performance data from which the six standards can be validated by CEN as international standards.

5.3 It is also hoped that relevant commercial methods found to give equivalent (or better) performance than the six standard methods in the proposed performance / validation trials can be included as informative annexes in the relevant standards. (e.g. Colilert® and Chromocult® for *E. coli*, Singlepath® or other similar lateral flow commercial systems and Pathatrix® for *Salmonella spp*.)

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Appendix 1: Summary

0.1 The existing methods currently available for the detection and enumeration of *Escherichia coli* and *Salmonella spp.* in sludges, soil, soil improvers, growing media and biowaste have been evaluated with a view to possible standardisation. The main methods used for the detection and enumeration of *Escherichia coli* can be assigned into three groups. Two of the groups include the draft CEN *E. coli* standards (CEN 2003a, b and c) these use two techniques; membrane filtration, and multiple tube fermentation, most probable number (MPN) determination. The third group uses a commercial product called "Colilert®" combined with the 97 well Quantitray® system to detect and enumerate *Escherichia coli* from a sludge matrix using defined substrate technology®.

0.2 The report includes twelve selected methods for *Escherichia coli,* five of which use membrane filtration techniques which differ in terms of the media used to give specific growth of *Escherichia coli.* The four types of media in the report included two chromogenic media: - membrane lactose glucuronide agar (MLGA), and Chromocult® agar and two non-chromogenic media: - membrane lauryl sulphate broth (MLSB) and membrane lauryl sulphate agar (MLSA). Five of the methods use multiple tube fermentation (MTF) analysis with most probable number (MPN) determination, and two use Defined Substrate Technology®.

0.3 The existing methods for the detection and enumeration of *Salmonella spp.* from sludge, soil, soil improvers, growing media and biowaste matrices can be broadly divided into five groups. There are six existing methods and two potential new methods included in the report. Three of the methods are draft CEN standards using membrane filtration, liquid enrichment (MPN) determination, and presence / absence using liquid enrichment and selective culture medium. There are also three Environment Agency, Standing Committee of Analysts (SCA) methods similar to the draft CEN standards included. The fourth technique is a commercial product called Pathatrix® available from Matrix MicroScience in Newmarket UK, which is based on the use of antibody coated paramagnetic particles to selectively bind and purify the target organism from a sludge, soil , soil improvers, growing media or biowaste matrix. A basic system costs 6750 Euro and can handle up to 60 samples per day at a unit test cost of 8 Euro. The fifth (antibody-based) technique the Merck Singlepath GLISA Salmonella® Lateral Flow Test. (Merck 2002) This is an immuno-chromatographical disposable test device. It considerably simplifies the analysis protocol. An 18 – 24

hour pre-enrichment in BPW followed by 24 hour selective growth on Rappaport-Vassiliadis (RVS) broth is used. Then 1 – 2 ml of the RVS broth is heated in a boiling water bath to sterilise it and allowed to cool. Then 160• l is applied to the immuno-chromatographical disposable test device. The unit cost of this disposable test device is ~7 Euro. Other commercial *Salmonella* spp. lateral flow devices are also available.

0.4 The existing methods differ in terms of the amount and quality of the associated performance data included in the method. It is felt that the amount and breadth of the performance data in general should be improved for CEN/ISO standards. Recommendations are made in the report regarding the reworking of method performance and validation.

0.5 Section 3.4 of this report deals with the implications of uncertainty of microbiological analysis of sludges. It clearly indicates a problem with very high uncertainties associated with *E .coli* and *Salmonella spp.* sludge analysis results. It is important that the wide variations in results between laboratories, currently observed, when analysing the same sludge samples are acknowledged and addressed before any legislation on microbiological limits in sludge and associated materials are enacted.

0.6 Regarding the *Escherichia coli* methods, there is a decision required on the preferred technique of analysis. The existing very limited performance data for a range of sludges, soil, soil improvers, growing media and biowaste is such that it is difficult to make any valid judgement selecting one of the three draft CEN methods over the other two methods. It is thought that each method may be the most suitable for a given type of analysis. Thus all three are likely to be included in the standard.

0.7 The *Salmonella spp.* methods included in the report differ in the levels of performance data included in the methods. One standard, (CEN 2003e) utilising liquid enrichment method in selenite cysteine medium for semi-quantitative MPN does not have any performance data included, although inter and intralaboratory trials have been carried out in 2001 and 2002 by 14 French laboratories working on five different materials. This data is being sought. It is imperative if any decision is to be made on the feasibility of standardisation that acceptable data is produced for all three existing methods. Efforts must be made to ensure fit for purpose method validation is carried out and the advent of a relevant sludge proficiency scheme to demonstrate that laboratories can monitor absence of *Salmonella spp*. at any specified regulatory compliance limit. There is little

benefit in proposing limits that cannot currently be achieved. (I.e. less than 1 in 50g as received sample for all sludges and biowastes).

0.8 The commercial kits now available in this field from developments at IDEXX Laboratories (Colilert®), Matrix MicroScience Ltd (Pathatrix®) and Merck (Singlepath®) have utilised modern techniques for *Escherichia coli* and *Salmonella spp.* detection and enumeration. Although they are not established methods the possibility of using these simple and robust methods should not be overlooked. The Matrix MicroScience Ltd. method for *Salmonella spp.* detection in particular uses antibody coated paramagnetic particles to selectively bind and purify the target organism. It is thought to be the most sensitive of the techniques considered.

0.9 Techniques such as PCR are much further away in development with respect to applying the technique to sludge, soil, soil improvers, growing media and biowaste matrices on a routine basis. They will be difficult to justify on cost grounds and require a skilled operator.

0.10 There are no reports outlining the use and performance of Pathatrix® and Singlepath® methods for *Salmonella spp.* in sludges, soil, soil improvers, growing media and biowaste. With the possibility of adequate funding to organise and run suitable interlaboratory method performance / validation, this will give an ideal opportunity to compare and contrast the methods. The possibility of including the Colilert®, Singlepath® and Matrix MicroScience Ltd Pathatrix® methods should be actively encouraged. This should then show whether simpler commercial methods could be reliably used for the detection and enumeration of *E. coli* and *Salmonella spp.* in sludge, soil, soil improvers, growing media and biowaste matrices.

0.11 Also the setting up of a Europe-wide regulatory sludge, soil, compost and biowaste proficiency scheme for laboratories carrying out regulatory routine analysis of these matrices would enable direct comparison to be made between both methods and laboratories. The scheme although difficult and expensive to set up and manage would provide an excellent overview of the quality of routine testing throughout Europe. It is difficult at this stage to know whether or not the quality of the work being produced is of an acceptable standard. The advent of a suitable proficiency scheme would provide the data to prove or disprove this theory.

0.12 The difficulty and high cost of setting up a suitable, workable, and adaptable proficiency scheme for sludges, soil, soil improvers, growing media and biowaste should not delay its inception. The proficiency scheme would highlight weaknesses in methods and in performance of laboratories. The main reason for standardisation is to make analysis comparable and of a higher quality, the proposed proficiency scheme would greatly aid this.

0.13 The standardisation of laboratories throughout Europe does not just rely on the standardisation of the methods. Laboratories are required to comply with certain standards and practices. Any given method is only as good as the laboratory staff that perform the analysis. The setting up of a standardised quality control for sludge testing is looked at and recommendations are made regarding a similar protocol to that followed in ISO 8199 (2003) for standardisation. More effort on routine quality control would improve consistency between laboratories. The use of lenticules (HPA), pastilles (Institute Pasteur) or vitroids (CDP) for quality control purposes is strongly recommended.

0.14 The methods existing for the detection and enumeration of *Escherichia coli* and *Salmonella spp.* in sludges, soil, soil improvers, growing media and biowastes utilise various analysis techniques. The lack of sufficient and comparative performance data in the six draft standards makes comparison of various methods very difficult. The limited data from the draft CEN standards and from related research papers indicates that each method can perform acceptably for specified matrices and levels of target bacteria. Only from organising and running interlaboratory method performance trials and setting up and running a Europe-wide proficiency scheme will it be possible to judge the relative performance of the various methods with all relevant matrices.

0.15 It is important to appreciate that, unlike most chemical methods, microbiological methods are empirical and the end result is very dependent upon the method used. This is especially true for treated sludge, soil, soil improvers, growing media and biowaste samples where many of the target bacteria will be highly stressed. Thus the method protocol and any associated resuscitation pre-treatment are crucial. Small changes (e.g. small variations in media, incubation temperatures and times etc.) can have a very significant effect upon the results obtained

0.16 It is also contended that it will be impossible to carry out standard (method) validation studies to cover all potential sludge, soil, soil improvers, growing media and biowaste

matrices. A pragmatic approach where a number of "worst-case" relatively homogeneous matrices are selected should be adopted.

0.17 Untreated biowastes and any other relevant non-homogeneous materials should also be amenable to any proposed standard (method) assuming suitable size reduction/homogenisation steps are employed followed by a suitable bacterial extraction and solids handling steps are incorporated into the method. Individual laboratories will have to carry out their own repeatability trials on these types of their typical samples.

0.18 The final analytical procedure for culturing, identifying and enumerating the target bacteria should be applicable to all sample matrices (e.g. sludges, soils, soil improvers, growing media and biowastes) after suitable pre-treatment steps. The issue of how to rigidly define empirical pre-treatment protocols needs to be addressed.

0.19 It is important to appreciate that proficiency testing scheme analysis is carried out on homogenised samples that readily disintegrate on contact with water (or MRD). Consequently, only the final bacterial measurement stage of the method is actually tested. Thus the performance indicated by proficiency sample results (as described earlier in this report) is considered optimistic with respect to the situation with the real routine (inhomogeneous) samples actually received by laboratories.

0.20 The larger the typical particle size and the more heterogeneous the received sample, the worse the repeatability/reproducibility of the expected results.

0.21 The EU should seriously consider setting up a blind proficiency trial to assess the ***real state*** of regulatory analysis across Europe as a top priority in order to assess the current actual situation with respect to routine sludge, soil, soil improvers, growing media and biowaste microbiological analysis. Only laboratories carrying out routine regulatory analysis should be included in this trial.

Prof K Clive Thompson
Richard Shepherd
Andrew Hockin
20th Jan 2004

Appendix 2
Some quotes from relevant sections of the draft sludge and biowaste directives and UK Draft Sludge (Use in Agriculture) (Amendment) (England and Wales) 2002 (Awaiting Number)

Draft Sludge Directive (EU 2000)

"Advanced treatments (hygienisation)

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

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

"Conventional treatments

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

DRAFT BIOWASTE DIRECTIVE (EU 2001)

"– *Salmonella spp* absent in 50 g of compost/digestate [under review]
– *Clostridium perfringens* absent in 1 g of compost/digestate [under review]"

"Methods for analysis and sampling
Salmonella spp. number/50 g dm
Clostridium perfringens number/1 g dm"

UK Draft Sludge (Use in Agriculture) (Amendment) (England and Wales) 2002

NO. AWAITED

For the purpose of this schedule-

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

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

Where analysis of samples over a continuous period of six months shows that none of them contains *Salmonella spp*. or more than 100 units of *E. coli*, the interval before next sampling may be increased to three months.

Where such analysis shows that none of a set of samples contains *Salmonella spp*. (i.e. no Salmonella spp. per 2 grams (dry weight) of sludge), or more than 1000 *E. coli*, {i.e. cfu /g dry weight) the batch of sludge in question shall be treated as satisfying the end product test for enhanced treated sludge.

The sludge produced shall be sampled as follows: -
On each occasion a set of five samples shall be taken at random from a batch of sludge, each consisting of 100ml in the case of liquid sludge or 100g in the case of dried sludge

Each sample shall be analysed separately in accordance with paragraph 5

Appendix 3
Response to circulated letter from Dr Simon Cole

Prof C. W. Keevil
Environmental Healthcare Unit
School of Biological Sciences
University of Southampton
Bassett Crescent East
Southampton
SO16 7PX

24 March 2003

Dear Bill

Re: EC Project HORIZONTAL Questions

I do apologise for the delay in responding regarding the above EC Questions. I am responding as a member of the UK Environment Agency-Standing Committee of Analysts Microbiology of Sewage Sludge Panel and Manager of a Water Company Microbiology Laboratory.

1. The SCA are shortly to publish national standard methods for *E. coli* and *Salmonella* in sludge. The methods for *E. coli* have been used in a large inter-laboratory exercise, managed by UKWIR Ltd, to establish levels of *E. coli* in different types of sludge. The defined substrate MPN method has been performance tested in individual laboratories. *Salmonella* methods are based on those that have been used routinely over many years in the UK. The membrane filtration methods described for *E. coli* O157 and *Salmonella* have been devised and performance tested as part of a UKWIR Ltd project. The *Salmonella* method is now a draft ISO standard.
I am unable to contribute a published method for *Clostridium perfringens*. In our laboratory we have used a membrane filtration method based on that applied to Drinking Waters using TSC supplement with *perfringens* agar base. The method includes a heat treatment step and is therefore primarily for the enumeration of spores.

2. These methods are detailed in the 'blue book' series Methods for the examination of water and associated materials published by the Environment Agency under the auspices of the Standing Committee of Analysts. Specifically the booklets: The Microbiology of Drinking Water (2002) Parts 1,2,3,4,6 and 9 are relevant (available through the environment agency/national laboratory service web page) and 'The microbiology of recreational and environmental waters (2000)'.

3. I am unable to help with methods for helminth ova, although I am aware that there are laboratories in the UK with experience in this area.

4. I am aware that the defined substrate method due to be published 2003 offers advantages for some types of sludge and that Anglian Water have some in house methods using chromogenic substrates for *E. coli*. For *Salmonella* I am aware of a paper by Lang N L et al. (2001, I think) entitled Methods for the enumeration of *Salmonella* serovars in sewage sludge and soil which may be of interest.

5. I refer again to recent surveys carried out for UKWIR in 1998/9 and 2000/1 on levels of *E. coli* and pathogens in sludge.

6. My own laboratory would have limited capacity for participation in inter-laboratory trials and other participants in the Standing Committee of Analysts may well wish to be included. I will raise this at the next meeting scheduled for 3rd April 2003.

I hope that this information is helpful at your CEN/TC308/WG1 meeting in Oslo.

Yours sincerely

Dr Simon Cole
Manager Microbiological Analysis,
SCA Sludge Microbiological Methods Panel

Appendix 4
Response to circulated letter from David Sartory

Clive,
I have had a chance to have a quick look through the two methods, not in detail, but just to get a feel for them and give some general comments. No time for significant editorial comment - maybe I can do that for you on later drafts.

Method 1 - MF procedure

Generally reasonably happy with this as it has some performance data. However, I have some questions:-

Is the use of Modified Tryptose Soya Broth simply for preparing dilutions really justified?

Are not PBS or Buffered Peptone Water (supplemented with novobiocin is necessary) simpler and just as suitable?

Additionally, is the addition of Bile Salts No3 to MTSB really necessary? I would have thought that the novobiocin would have been sufficient.

In the formulation the Tryptose ???' should be 'Soya peptone'.

I think you need to be much more specific about the type of glass fibre filters (grade etc.) that can be used for the tetrathionate resuscitation.

In 4 Principle - 'fastidious' is not really the right word for *S. dublin.* It is slower growing and less hardy than other *Salmonellas* (which makes it a good QA strain). Additionally in the following sentence it says 'other coliforms' in a way that implies that *Salmonella* are coliforms. Do you really mean 'Other *Enterobacteriaceae*' as the sentence goes on to refer to only some possessing beta-galactosidase (which is the diagnostic characteristic for coliforms)?

Method 2 - MPN procedure

I'll keep this simple - I don't like the proposed method and don't think it is really appropriate (sorry, but I have to be honest). I thought that selenite-cysteine medium was pretty well discredited for environmental samples. I would have thought that something along the lines of the UK method for environmental waters would have been more appropriate.

That's it. Not much (but then not much time!!). Any queries let me know.

Best regards,

David.

David Sartory
Company Advisor (Microbiology)
Quality & Environmental Services
Severn Trent Water Ltd
Welshpool Road

Shelton

Shrewsbury

Tel: +44 (0) 1743 265765
Fax: +44 (0) 1743 265043
Mobile +44 (0) 7880 788208
E-mail:
david.sartory@severntrent.

SY3 8BJ

Appendix 5: -Draft Protocol for Carrying out Chemical and Microbiological Analysis Interlaboratory Trials in CEN/TC 308/WG1 (Issue 5)

1. Introduction

1.1 This document specifically addresses the requirements of TC 308 "Characterisation of Sludges", but may be adapted for other sample types (e.g. waters; effluents; wastes and soils etc.).

1.2 For international harmonisation it is essential that a fit for purpose interlaboratory trial using a range of typical sludges with relevant analyte concentrations be carried out prior to proposing for publication any method as a European standard.

1.3 At least ten laboratories from three different countries should participate in this exercise.

1.4 Only experienced laboratories routinely carrying out sludge analysis should participate.

1.5 The organizer should aim for 12-15 laboratories to participate in each exercise in as many countries as possible in case of a non-return of results.

1.6 Efforts must be made to ensure that there is a minimum of intersample variation amongst the circulated samples.

1.7 It is recommended that each trial includes a range of sludges that is representative of the proposed scope of the method, as a minimum this should include an untreated (raw) sludge, a treated (an aerobically digested) sludge, a presscake and samples of any other sludges relevant to the method being tested. In addition a spiking solution and a calibration check standard will generally be distributed

1.8 If possible, samples shall be in the same form as would normally be submitted to a laboratory for analysis.

1.9 For sample preparation methods without a final measurement stage (e.g. Aqua regia digestion for metal analysis), see ***Appendix 1***

2. Planning Protocol

WG1 shall agree the planning protocol prior to carrying out the exercise.

The main exercise variables to be agreed are:

(i) Organising laboratory

(ii) The documented method to be tested

(iii) The number and nature of the samples

(iv) Statistical calculations including rejection of outliers

(v) Payment arrangements

(vi) Deadlines for sending out samples, receipt of results from participants and final exercise report from the organizer to WG1.

(vii) Spiking protocol to calculate percentage recovery

(viii) Analysis of a calibration check standard will also allow a check to be made on the comparative accuracy of the calibration standards used by all the participants. This can sometimes explain differences between laboratories)

3. Exercise Protocol Issues.

3.1 Exercise Organiser and dispatch of samples

It is essential that the bulk material is homogeneous. This should be checked using a suitable low cost parameter. (e.g. Cu, Zn)

3.2 The organizer will compile an exercise contact list with

(i) Contact name

(ii) Contact full address

(iii) Contact phone numbers (named contact and switchboard)

(iv) Contact fax number

(v) Contact E-mail address

3.3 The organizer will obtain a written undertaking **(see Appendix 2)** from each laboratory agreeing to participate, agreeing to follow the provided method exactly and meet the deadlines for commencement and completion of analysis and for submission of the results on the appropriate form.

3.4 An additional 20% of samples shall be prepared and held by the organizer at -18^{o}C or less. These can be used if any problems are encountered during the exercise. For microbiology this may not be appropriate.

3.5 Participating Laboratories.

Participating laboratories should be laboratories that routinely carry out sludge analysis.

3.6 Statistical Calculations.

An initial check should be made to confirm a normal distribution of results. If the distribution is not normal then appropriate robust statistics should be employed.

3.7 Assuming a normal distribution, the statistical calculations should be carried out according to ISO 5725 by a suitably experienced person for chemical parameters.

3.8 Suitable rejection procedures should be used for microbiological parameters.

3.9 Rejection of outliers should also follow ISO 5725. All Type B (reproducibility) outliers should be eliminated.

3.10 All Type C outliers from the mean of one lab (repeatability) should be included.

3.11 In addition the elimination of 'obvious' outliers by the statistical organizer (Type D) shall be allowed as long as they are fully documented with the reason for elimination.

3.12 Each sample shall be analysed in replicate (x times) on y separate days. (x and y to be agreed for each individual exercise.)

4. **Exercise Procedure**

4.1 The organizer shall procure and prepare the samples. These shall contain appropriate concentrations of the analyte.

4.2 The minimum sample mass for wet sludges shall be 1kg. For presscakes a minimum mass of 500g shall be sent. For special purposes (e.g. methods that only require a few grammes of sample) a smaller amount of sample will be circulated.

4.3 All samples will be sent out in polyethylene, polypropylene, stainless steel or aluminium bottles that will tolerate a slight increase in pressure. It is not thought necessary, or advisable, to use glass bottles. Each bottle will be clearly labelled with the type of sample, the analysis required and the dates by which the analysis should commenced and completed.

4.4 All sample containers will be packed in stout sealed polyethylene / polypropylene insulated containers.

4.5 Samples will be sent out at a temperature below [4^{o}C] with suitable cooling boxes/bags/packs to ensure that the temperature remains below [4^{o}C] for at least 48 hours.

4.6 Courier arrangements.

4.6.1 The organizer will arrange a suitable courier with a guaranteed 24-hour delivery that will accept sewage and other sludge samples.

4.6.2 The sample pick-up time will be agreed with the courier. The delivery time window for each participating laboratory by an authorized person will be agreed between the laboratory and the organising laboratory.

4.6.3 Each participant will be informed of the expected delivery time window and shall ensure that suitable sample reception facilities are available.

4.6.4 The courier will be provided with full addresses (including the postcode) and contact details for each participating laboratory.

4.6.5 A simple chain of custody protocol will be implemented for transport and receipt of the samples.

4.6.6 All participating laboratories will upon receipt immediately record the temperature of the samples. They shall then store the received samples at a temperature of less than 4^{o}C until commencement of analysis. This should be within two working day (they have had due warning) of receipt of the samples.

4.6.7 The organizer shall ensure that all appropriate sample transport of hazardous biological and chemical material regulations are complied with. (E.g. pathogens, dioxin or PCB standard solutions etc.) This will cover within and between country delivery of the samples.

4.7 Documentation to be sent with the samples will be:-

(a) Another copy of the agreed method

(b) The spiking solution/material if relevant

(c) The instructions for the exercise

(d) The form for the submission of result

(e) The analysis start date (Four days after dispatch.)

(f) The analysis completion date (not more than two weeks after the dispatch date)

(g) The deadline for return of results. (Within three weeks of sample dispatch)

(h) Full contact details of the organizer to allow for queries and for the return of results

5. Final Report of Exercise

5.1 The organizer shall provide a full report of the exercise to WG1 and all participants by the agreed deadline. This will include:

(a) A listing of all of the results

(b) The mean value for each tested parameter

(c) The calibration check solution result

(d) The reproducibility (absolute and relative)

(e) The repeatability (absolute and relative)

(f) The percentage recovery (if relevant)

(g) The names and countries of laboratories that returned valid results

(h) The total number of results returned

(i) The number of different countries that returned valid results

(j) The temperature of the samples upon receipt at the participating laboratories.

(k) The number of outliers from non-rejected laboratories

(l) Any exceptional circumstances/problems reported by any of the laboratories on an anonymous basis

(m) Summary of performance: - i.e. interpretation of figures and data

(n) A detailed description of the samples and of the interlaboratory trial.

6. Other Comments

6.1 Ideally each exercise should include a range of analyte concentrations. However, with the range of sludge types and the availability of 'contaminated' sludge samples, this may not always be possible.

6.2 A spiking solution of the analyte will normally be supplied, so a sample aliquot can be spiked with the analyte. This will allow a percentage recovery to be calculated. It is accepted that the 'spike analyte will be more easily recovered than the endogenous analyte. However, the results should indicate whether there is any significant bias in the final analysis step. It will also allow a check to be made on the comparability of the calibration standards used by each of the participants. (For microbiological parameters, it should be possible to utilize suitable 'lenticules' or 'pastilles' for this purpose.)

6.3 The spiking level ideally should be at least equal to the value of the analyte in the sample and should raise the concentration of the analyte to 75-95% of the calibration range of the method (without dilution of the sample).

6.4 All published results shall be anonymous with respect to originating laboratory.

7 Charges [in those cases where the work is performed under a financial mandate or where it is conducted within a funded project]

7.1 CEN should pay the organizer the costs incurred in:

(a) Collection and preparation of the samples
(b) Suitable homogeneity and chemical / microbiological stability checks
(c) Supply of sample cooling boxes, bags and packs
(d) All courier charges
(e) Final report preparation and statistical calculation work.

7.2 CEN should pay reasonable analysis charges to the participating laboratories.

Appendix 1 (of Appendix 5)

Proposed Protocol for Sample Preparation Methods

1. For methods which are only sample preparation methods without a final measurement stage (e.g. aqua regia digestion for metal analysis), the described protocol will not assess the errors of the preparation stage if all the participating laboratories analyse the final prepared digests. In this instance, the interlaboratory trial would estimate the errors associated with both the digestion and the final measurement stages.

2 To attempt to minimise this problem, it is recommended that all the resulting prepared sample extracts are promptly returned to one specified 'experienced' laboratory. This laboratory will then remove a known amount from each returned sample extract (e.g. 10% of the volume received) and make up a 'composite sample'.

3 This laboratory would then analyse all the received sample extracts, interposing composite extracts between each returned sample extract.

4 By then assessing the results obtained against the preceding and subsequent composite sample, it should be possible to assess the results with respect to the sample preparation stage. The variation in the composite sample results is attributable to the measurement stage in the experienced laboratory.

Appendix 2 (of Appendix 5)

Agreement to Participate in the …….

Sludge Method Interlaboratory Validation Trial

This signed form to be returned to the trial organizer
We agree to participate in the above trial and accept the following conditions: -

1 We agree to follow the supplied method exactly. Results from any other method used by the laboratory are **not** acceptable

2 Our laboratory has previous experience of this type of analysis

3 We agree to consult with the trial organiser if any minor deviations from the supplied method are thought desirable and to report them in detail.

4 We agree to report any exceptional circumstances on the results submission form.

5 We agree to have a suitable laboratory representative to be available to receive the samples within the agreed receipt time window.

6 We will immediately, upon receipt store all samples at less than $4^{o}C$.

7 We undertake to commence the analysis within two day of receiving the samples. (The dispatch day will always be a Monday).

8 We undertake to complete the analysis within two weeks of the dispatch of the samples.

9 We undertake to supply the results by the agreed deadline of three weeks from the date of dispatch of the samples.

10 We undertake to return the sample cooling boxes, bags and packs if required.

11 If we encounter any problem with this inter-laboratory trial we undertake to inform the organizer immediately.

12 It is understood that all published results will be on an anonymous basis.

13 We accept that (in the event of participating in work for which there is financial reimbursement) we shall forfeit the right to reimbursement in the event of failing to comply with this agreement.

Signed by: -…….Date……

Block capitals ……..

Company ……..

Address ……..

……

Postcode ……Country……

Contact phone number …….. Switchboard……

FAX number ……

E-mail address …….

Table 16: Proposed protocol of Escherichia coli trial in the UK for Colilert® method

Proposed Sludge *E. coli* Trial

Note: - Only samples 1 - 9 will be sent out. Participants will prepare samples 10 - 14 by spiking samples 5 - 9 with a specified amount of an *E. coli* vitroid suspension. A vitroid is a gel type tablet containing a known number of *E. coli*. These are being provided by CDP Ltd. (Controlled Dried Products, 213A, Western Way, Darras Hall, Ponteland, Newcastle, NE20 9ND, UK.)

Sample No	Matrix	*E. coli* cfu/g sludge	*E. coli* cfu/vitroid	Comments
1	Vitroid 1		10000 - 40000	Actual *E. coli* assigned level will not be given to participants, but should be within this range. These three samples do not require any analysis. They are for spiking
2	Vitroid 2		2000 - 8000	
3	Vitroid 3		50000 - 200000	
4	Vitroid 4	0	0	~50000 - 200000 coliforms (Klebsiella)
5	Digested sludge	~3000		%DS figures to be provided
6	Limed sludge presscake	~0		%DS figures to be provided
7	Typical presscake	~500		%DS figures to be provided
8	Thermally dried sludge	~0		%DS figures to be provided
9	Composted type sludge	low		%DS figures to be provided
10	Digested sludge 5	spiked with specified vitroid suspension		Prepared from distributed sample 5
11	Limed sludge presscake 6	spiked with specified vitroid suspension		Prepared from distributed sample 6
12	Typical presscake 7	spiked with specified vitroid suspension		Prepared from distributed sample 7
13	Thermally dried sludge 8	spiked with specified vitroid suspension		Prepared from distributed sample 8
14	Composted type sludge 9	spiked with specified vitroid suspension		Prepared from distributed sample 9
15 - 19	As 10 -14 but 10ml MRD instead of sludge	spiked with specified vitroid suspensions		These samples give the sludge matrix-free results equivalent to samples 10 -14
Notes: -				
1	Assume for samples 5 - 9 that 10 g as received sludge aliquot is diluted with MRD to make a final volume of 100 ml prior to stomaching			
2	Participants will spike a 10g aliquot of samples 5 - 9 to create samples 10 - 14. A specified vitroid will be reconstituted with 100 ml MRD. A specified amount of the vitroid suspension (5 - 25 ml) will be added to the 10g sludge aliquot and then this will be diluted with MRD to make a final volume of 100ml prior to stomaching. Spiking will aim to add from 50cfu (for sterile unspiked sludge samples) and up to four times the level of *E. coli* in the unspiked sludge samples.			

3	Participants should also repeat the spiking, but replacing the sludge aliquot with 10ml of MRD. **This will give equivalent sludge matrix-free results for samples 15 -19 mimicking samples 10 -14, but without any sludge present**
4	Dry solids (%) for all samples will be provided
5	Triplicate analysis of samples 5 - 14; the five MRD (no sludge) spiked samples 15 - 19 (see 2) and vitroid 4 (*Klebsiella*, see 7) is required **A total of 48 analyses.**
6	A results sheet will be provided. All sludge replicates use a new sludge sub-sample, but the same vitroid suspension is always used for spiking.
7	Also triplicate analysis of sample (vitroid 4) to 100ml with MRD; 20ml to be used plus 80 ml MRD (no sludge) .
8	Option for carrying out more replicates of vitroid samples 1 - 3 to establish precision of supplied vitroids. There is no requirement to analyse these vitroid samples
9	All samples and vitroids provided free of charge. Vitroids will be numbered 1 - 4
10	All Colilert® reagents to be provided by IDEXX
11	A total of 40 of each vitroid to be provided to each participant
12	The limed sludge presscake aliquots should be neutralised using the to be circulated Thames Water procedure
13	For samples 10 - 14 participants will be instructed which vitroid to use for spiking
14	All samples will be distributed at ~4oC on the same day (Tues 2nd Sept) for 10-00 hours delivery on the following day (Wed)
15	Analysis to commence on day of receipt (Wed), if at all possible. This must be recorded as well as delivery date and time. **The circulated draft SCA method C (ColilertÒ defined substrate) method) to be used. It is hoped to circulate an updated final version before the trial**
16	Detailed instructions for preparing the four vitroid suspensions will be provided.
17	Sample 4 to act as a control to show the effect of a lactose fermenting coliform. No *E. coli*, but *Klebsiella* will be present
18	ALcontrol will endeavour to establish the mean no of *E. coli* in each vitroid samples 1 -4 prior to sample distribution
19	ALcontrol will analyse all sludges prior to sending out to establish background levels of *E. coli*
20	Labs are asked if time permits to analyse 5 separate replicate vitroids 1 - 3 to try and establish the variation between different vitroids containing nominally the same number of *E. coli*. If necessary existing *E. coli* methods can be used for this if insufficient Colilert® pouches
21	A final set of instructions will also be sent out with the Excel results reporting sheet.

Appendix 6

Summary of commercial *Escherichia coli* and *Salmonella* test kits referenced in the document

In addition to the CEN draft standards and the Environment Agency SCA methods the report includes commercial methods for review. The commercial methods included for *Escherichia coli* and *Salmonella* detection and enumeration are summarised below.

1.) Colilert®, IDEXX Laboratories Ltd.(2003), IDEXX Colilert® Method – Enumeration of Coliform and *Escherichia coli* bacteria in waste water solids using defined substrate technology

2.) Matrix MicroScience (2003), Detection of *Salmonella spp.* using Immuno-magnetic Separation using Pathatrix®

3.) Merck (2002) Singlepath GLISA *Salmonella*® Lateral Flow Test.

The commercial methods are not proposed to be put forward for approval as validated standards they maybe included as informative annexes. If a laboratory uses a commercial test kit method it should be fully validated against the proposed standards reviewed in this report.

The use of commercial test kits in laboratories is ever increasing as analysis becomes increasingly time dependant. There is a need for commercial laboratories to assess the throughput of a particular method, in addition to cost, before the method is considered for use in the laboratory. With this viewpoint, a summary of the throughput of each of the methods is included below:

IDEXX Colilert® Method: Each individual test takes less than one minute to set up, the sealed quantitray® is incubated at 35°C for 24 hours. Due to the specificity of the test, confirmation of the tray is not required. The throughput time for the IDEXX Colilert method is 24 hours.

Matrix Micro-science Pathatrix® system: Each individual test prepared and incubated on the Pathatrix system at 37°C for 3 hours, the sample is incubated on selective medium at 37°C for 24 hours. It is possible to give an identification of *Salmonella* spp. within 16 hours of sample processing.

Merck Singlepath GLISA Salmonella® Lateral Flow Test: An 18 – 24 hour pre-enrichment in BPW followed by 24 hour selective growth on Rappaport-Vassiliadis (RVS) broth is used. Then 160• l is applied to the immuno-chromatographical disposable test device. There is a clear and distinct positive or negative test result within 20secs. The total throughput time is approximately 48 hours.

Appendix 7

Summary of the Colilertâ Interlaboratory Trial organised by ALcontrol Laboratories in 2003

1. General

1.1 The protocol followed during the running of the trial is listed in the report as part of **Table 16**, the table includes information on the spiking procedure, types of matrices used in the trial and the methodology to be followed in the trial. There are also some key notes included with the protocol to assist in the smooth running of the trial. ***Figure 1*** outlines the analysis protocol to be followed during the *E. coli* sludge trial.

Figure 1 (of Appendix 7): **Letter outlining the trial protocol to trial participants**

ALcontrol Laboratories
Templeborough House
Mill Close
Rotherham
S60 1BZ
Tel. 01709 841078
Fax. 01709 841011

2nd September 2003
Our ref: GA14519

Dear All

Analysis Protocol for E. Coli Sludge Trial

1 Samples

You should receive five sludge samples labelled 1A, 2A, 3A, 4A and 5A for analysis before 10-00 hours on 3rd Sept. ***(All analysis in this trial to be in triplicate)***

Sample	Description	Dry Solids %	Approx *E. coli* cfu/g as received ***
1A	Digested sludge*	1.20	300 – 500
2A	Limed sludge**	37.0	0
3A	Presscake	18.9	$3 \times 10^5 – 6 \times 10^5$
4A	Thermally dried sludge	94.0	0
5A	Composted sludge	77.2	<50

* Apologies for low dry solids, but this is what YWS provided.
** Neutralisation protocol previously sent. This must be applied to this extract.

*** Please note approx. *E. coli* levels are given as **cfu/g wet weight (as received**), <u>not</u> as dry weight. This will make dilution calculation easier

2 Vitroids

You will also receive a batch of 10 of each of the following CPD Ltd vitroids:-

Vitroid No. *	Bacteria	Approx *E. coli* level cfu/vitroid
2	E. coli	5,000 – 25,000
3	E. coli	25,000 – 125,000
4	*Klebsiella*	80,000 – 400,000

* Please note there is no vitroid Number 1.

3 Spiked Sample Preparation

To create the five spiked sludge samples 1B, 2B, 3B, 4B and 5B for analysis take the appropriate number of vitroid tablets (see below) and allow to disintegrate in 100ml MRD for 20 min with gentle stirring. Then add the indicated volume in ml of the vitroid suspension to the 10g aliquot of the appropriate sludge and then dilute to 100ml with MRD and mix well to make the primary dilution prior to any Stomaching® or other pre-treatment step. (See para C9.1 in the SCA method). This primary dilution solution is used for all relevant further dilutions. It is **<u>not</u>** analysed.

Preparation Details of the Five Spiked Sludge Samples

Sample	Sludge description 10g to be taken	Vitroid No.	Number of Vitroids to be added to 100 ml MRD	Vol (ml) of Vitroid suspension to be used	Vol (ml) MRD
1B	Spiked digested	3	1	25	65
2B	Spiked limed*	2	1	12	78
3B	Spiked presscake** diluted extract 1000 times	3	3	30	70
4B	.	2	1	10	80
5B	Composted	2	1	20	70

* It is essential that the neutralisation procedure is applied prior to spiking this extract.

** The presscake extract must be diluted 1000 times (10^3) prior to adding the vitroid spike. Report result as though this extract was obtained from the original sludge as ***cfu/g dry solid***. (I.e. As if the original 10 ml sludge aliquot had been spiked with 1000 times more *E. coli*. This avoids the use of vitroids containing very high levels of *E. coli* having to be sent out.

4 Preparation of the spiked MRD Samples (These mimic the above spiked sludge samples, but with no sludge matrix present). This should allow an estimate of the effect of the sludge matrix on the added *E. coli* to be made.

Report all results as per samples 1B – 5B (cfus/g dry solid)

Sample	Vitroid No.	Number of vitroids to be added to 100 ml MRD	Vol (ml) of vitroid suspension to be used	Vol (ml) MRD
1C	3	1	25	75
2C	2	1	12	88
3C	3	3	30	70
4C	2	1	10	90
5C	2	1	20	80
6C*	4	2	25	75

- *Klebsiella* breakthrough test assume 100% dry solids (10g original sample weight) for result calculation

Determination of number of *E. coli* / *Klebsiella* in the supplied vitroids (Optional analysis)

5. It would be appreciated if laboratories could find the time to analyse three of each of the supplied vitroid tablets in triplicate to give an indication of the reproducibility of the supplied vitroids for the *E. coli* (Vitroids 2 and 3) and *Klebsiella* (Vitroid 4). *This is purely optional*.

6. Other Comments

6.1 ***All analysis should be in triplicate.***

6.2 A results Excel Spreadsheet is appended for the results. There are four separate spreadsheets. Please fill in all relevant sheets including the comments and information final sheet. Please E-mail this file back to me.

6.3 Please use the supplied ***SCA Method C Colilert method*** and the Thames Neutralisation protocol for sample 2.

6.4 ***All results to be calculated as cfu/g dry weight*** using the % dry solids figures supplied.

6.5 Any results from other *E. coli* methods will be welcomed on spreadsheet 3

6.6 If you have any queries on this trial please ring Clive Thompson on 01709 841078 or 07767 616996.

Hoping for a successful trial.

Yours sincerely

Prof K Clive Thompson
Chief Scientist

1.2 The supplied vitroids had to be tested and enumerated in order to check their reproducibility. They were tested on site using three different methods; two membrane filtration methods (MLSB and MLGA) and the Colilert® method. The results for each of the vitroids used can be seen below. Two vitroids of each type were used in the pre-trial and analysis was performed in triplicate on each.

Table 1: **Results of the pre-trial for the Colilert® method**

Colilert® Method Results for Vitroids (Optional)				
		Results		
		No. of *E.coli* per Vitroid		
Vitroids	**Replicate tablet no.**	**1**	**2**	**3**
1	1	3250	2380	2990
1	2	2600	2990	2280
2	1	17300	12000	12000
2	2	14100	9800	14100
3	1	37800	72700	61300
3	2	64900	57900	54800
			***Kleb*. numbers**	
4	1	199000	242000	141000
4	2	>242000	242000	>242000

Table 2: **Results of the pre-trial for the MLSB method**

Membrane Filtration (MLSB) Method Results for Vitroids (Optional)				
		Results		
		No. of *E.coli* per Vitroid		
Vitroids	**Replicate tablet no.**	**1**	**2**	**3**
1	1	1700	1600	2900
1	2	1600	1800	1900
2	1	8300	8400	9500
2	2	7200	7700	9600
3	1	43000	48000	56000
3	2	39000	45000	46000
			***Kleb*. numbers**	
4	1	175000	159000	189000
4	2	166000	152000	171000

Table 3: **Results of the pre-trial for the MLGA method**

Vitroids For Sludge Trial (MLGA Results)				
Vitroid	Replicate Tablet no.	Results (cfu/vitroid) Replicate 1	Replicate 2	Replicate 3
1	1	1900	1700	2100
1	2	1800	1300	1500
1	3	2000	2200	2200
1	4	1900	1900	1800
1	5	1700	1900	1600
2	1	11100	8200	8500
2	2	9500	5600	5500
2	3	8500	8900	7800
2	4	7900	8900	9700
2	5	9800	9000	9900
3	1	48000	52000	58000
3	2	47000	48000	55000
3	3	59000	61000	52000
3	4	63000	53000	59000
3	5	60000	51000	57000
4	1	171000	210000	182000
4	2	116000	180000	173000
4	3	161000	204000	181000
4	4	169000	153000	178000
4	5	177000	184000	221000

Vitroids 1 to 3 - *E. coli*
Vitroid 4 - *Klebsiella*

1.3 Production of Vitroids

1.3.1 The vitroids are produced by a company called CDP Ltd., and can be produced for use as sludge controls using NCTC cultures in this case *E. coli* (NCTC ref. no.09001). Replicate counts were prepared using *E. coli* at 2000 and 10000 cfu on selective and non-selective media. Dilutions were made in Brain Heart Infusion (BHI) by placing one disc into 2ml and 10ml respectively. A volume of 67µl per suspension was spread on each to give a readily and accurately countable number. All media was freshly prepared

using Oxoid™ materials, there were three different types of media used; Blood Agar using a Colombia base (BA), Cystine Lysine Electrolyte Depleted agar (CLED), and Membrane Lauryl Sulphate Agar (MLSA).

1.3.2 In the first test run, one disc was suspended into 2ml of BHI and a 67µl volume was plated onto BA and CLED agar and incubated at 37°C. Three additional runs were analysed using different volumes (2ml and 10ml) of suspension and different incubation temperatures of 37°C and 44°C respectively. The results of the first run an be seen below in table 4:

Table 4: **Efficiency of Plating of vitroids derived from *E. coli* NCTC 09001**

	Colony Counts				Number of *E.coli* per Vitroid			
BA								
						2000/67 =	29.85075	
	57	58	58	59	1701	1731	1731	1761
	63	64	64	64	1881	1910	1910	1910
	65	66	77	78	1940	1970	2299	2328
and CLED								
	50	52	53	54	1493	1552	1582	1612
	55	56	59	62	1642	1672	1761	1851
	62	67	70	71	1851	2000	2090	2119

1.3.3 The conclusions which can be drawn from the test runs are firstly that the Efficiency of Plating (EOP) is not affected by the temperature of incubation between 37°C and 44°C, the latter being used for the isolation of *E. coli* in water, food and environmental samples. The efficiency of plating for CLED, a medium widely used for the isolation of *E. coli* and coliform organisms from clinical samples such as urine, was around 85%. The EOP of MLSA, a medium widely used in the water industry to check for the presence of *E. coli* gave an expected value of 67%. The colony counts gave quite a close range which illustrates there is little variation between either the runs or the samples.

1.4 Once all the samples had been analysed, the results were sent back to the lead laboratory for collaboration and statistical analysis. The samples were essentially analysed in three parts; the original sludge sample, the spiked sludge sample and the MRD matrix sample. In addition to the *E. coli* trial results, each participating laboratory analysed a *Klebsiella* suspension to ascertain the ability of the method to detect coliforms and differentiate between the two organisms. There were five types of sample to be analysed in each section; digested, limed, presscake, thermally dried and composted sludge. The results for each laboratory are listed in order, together with average mean results and standard deviation results for both individual laboratories and for each replicate for all seven laboratories.

1.5 The results received from the participating laboratories have been analysed using a simple mean and standard deviation. The data provide an overview of the results from the trial. A full statistical review of the data will take place after a proposed meeting of all participants to discuss the data and investigate if some suspected outlier results are due to dilution factor errors. The statistical data AVE (mean) and SD (standard deviation) respectively does not include less than or greater than values reported by participating laboratories. Where a less than or greater than value appears in all three replicates, there is no average mean or standard deviation value calculated. The occurrence of these values reported by the laboratories may in instances be due to the use of an inappropriate dilution factor during analysis.

***Table 5*: Data for Original Sludge Sample**

Lab No.	1A Digested sludge (* denotes suspect result omitted) (** inappropriate dilution)				
				AVE	**SD**
1	14400	13300	24300	17400	6000
2	17900	17900	10600	15400	4200
3	22000	11000	19000	17300	5680
4	9330*	>20200**	>20200**	-	-
5	14300	12100	16900	14400	2420
6	11500	16300	13900	13900	2420
7	17900	20700	32300	23600	7620
Mean	**15300**	**15200**	**19600**	**17000**	
Std dev	**4290**	**3760**	**8000**	**3540**	
RSD (%)	**28.0**	**24.5**	**40.0**	**20.8**	
	2A Limed sludge (* Denotes suspect result omitted) (inappropriate dilution)**			**AVE*** **	**SD*** **
1	0	0	0		
2	104*	0	3		
3	<1000**	<1000**	<1000**		
4	0	0	0		
5	<27.03**	<27.03**	<27.03**		
6	0	0	0		
7	<3	<3	<3		

***Due to the nature of the results, is has not been possible to perform meaningful statistical analysis on the data received from the participating laboratories. Therefore, there is no statistical data available for limed sludge samples.

	3A Presscake (** inappropriate dilution)				
				AVE	**SD**
1	1210000	1850000	1780000	1610000	353000
2	1250000	1070000	1080000	1130000	101000
3	1300000	2500000	2200000	2000000	624000
4	121000	878000	2300000	1460000	745000
5	666000	748000	572000	662000	88200
6	>128000**	>128000**	>128000**	-	-
7	803000	1300000	716000	941000	318000
Mean	**1070000**	**1390000**	**1440000**	**1300000**	
Std dev	**267000**	**668000**	**754000**	**485000**	
RSD (%)	**24.9**	**48.0**	**52.3**	**37.3**	
	4A Thermally dried sludge (inappropriate dilution)**				
1	0	0	0	***	***
2	0	0	0		
3	<1	<1	<1		
4	0	0	0		
5	<10.64**	<10.64**	<10.64**		
6	1	2	1		
7	<10**	<10**	<10**	-	-

***Due to the nature of the results, is has not been possible to perform meaningful statistical analysis on the data received from the participating laboratories. Therefore, there is no statistical data available for thermally dried sludge samples.

Table 5(cont.): Data for Original Sludge Sample

	5A Composted sludge (* denotes suspect result omitted) (** inappropriate dilution)				
				AVE	**SD**
1	110	116	121	116	5.5
2	3	1	3	2	0.8
3	<10**	<10**	<10**	-	-
4	12	7	11	10	2.9
5	<13.9**	<13.9**	<13.9**	-	-
6	29	14	<1.38	22	10.1
7	142	233	1070*	188	64.3
Mean	**59**	**74**	**300**	**67.6**	
Std dev	**62.7**	**101**	**514**	**81.5**	
RSD (%)	**106**	**135**	**171**	**82.9**	

2. Discussion of the Data for Original Sludge Samples

2.1 The data illustrates that the results from each of the participating laboratories is comparable across the different matrices. The column titled AVE is the best indicator of variation between laboratories because it takes into account all three replicates tested. The average result from each laboratory is compared against the total average for all the laboratories; it is able to highlight variation in performance between each of the laboratories. The SD column relates to the standard deviation of the three replicates from each laboratory. It is recognised that a more meaningful statistical evaluation needs to be carried out after a meeting of the participating laboratories has taken place.

2.2 The results for the digested sludge analysis had an average mean of 17,000 with a standard deviation of 3,540. The method has performed well on this type of sludge in all participating laboratories.

2.3 The results for the limed sludge should be consistently lower for this type of sludge. The majority of the participating laboratories achieved satisfactory results, 6 of the 7 laboratories reported either a less than value or zero. Laboratory two reported a result of 104* in replicate 1. Simple statistical evaluation of the data for the limed sludge with many less than results is not considered feasible.

2.4 The results reported for the presscake show a significant variation, this is to be expected when analysing a sludge of this nature with such a high content of the target organism present in the matrix. The participating laboratories reported an average mean result of 1,300,000 with a standard deviation of 485,000.

2.5 The results reported for the thermally dried sludge showed consistent analysis among the participating laboratories with only laboratory six reporting a result different from a less than or zero value. Laboratory six was the only laboratory to detect the target organism in the thermally dried sludge matrix. As for the limed sludge, simple statistical evaluation of the data for the thermally dried sludge with many less than results is not considered feasible

2.6 The results reported for the composted sludge show a large variation in the organisms detected from the matrix. The seven laboratories had an average mean of 67.6 with a standard deviation of 81.5, Laboratory one and laboratory seven (1070* omitted) reported significantly higher results than the other five participating laboratories.

***Table 6*: Data for Spiked Sludge Sample**

	1B Digested Sludge		(* denotes suspect result omitted)		
Lab No.				**AVE**	**SD**
1	116000	71700	64700	84200	28000
2	129000	118000	129000	126000	6710
3	89000	84000	54000	75700	18900
4	168000	190000	175000	178000	11400
5	96100	64300	96100	85500	18400
6	100000	144000	121000	122000	22100
7	121000	16300*	73500	97300	33600
Mean	**117000**	**98400**	**102000**	**110000**	
St. Dev.	**26500**	**57300**	**42800**	**35700**	
RSD (%)	**22.7**	**58.3**	**42.0**	**32.4**	
	2B Limed Sludge		(* denotes suspect result omitted) (** inappropriate dilution)		
Lab No.				**AVE**	**SD**
1	238	198	176	204	31.4
2	672	14*	607	-	46
3	<1000**	<1000**	<1000**	-	-
4	186	284	189	220	55.4
5	<27.0**	<27.0**	<27.0**	-	-
6	123	102	123	116	11.9
7	178	195	208	194	15.0
Mean	**279**	**159**	**261**	**184**	32
St. Dev.	**223**	**103**	**196**	**46.3**	
RSD (%)	**79.9**	**65.1**	**75.3**	**25.1**	

3. Discussion of the Data for Spiked Sludge Samples

3.1 The spiking protocol is outlined in the letter sent out to all participating laboratories at the start of the appendix. The spiking levels are outlined in the letter, regarding what type and number of vitroids to include in the samples for analysis. The results reported for the spiked digested sludge are satisfactory, with all seven laboratories reporting

average results close to the target range. The laboratories reported an average mean of 110,000 with a standard deviation of 35,700 .The only significant result was reported by laboratory seven which reported a significantly low result in replicate number two (16300*); this result was omitted from the mean and standard deviation.

3.2 For the spiked limed sludge a spike solution was made containing a vitroid containing approximately between 5,000-25,000 cfu/vitroid of which an appropriate dilution was made to give a target range of 625-3125. The seven laboratories reported an average mean of 184 with a standard deviation of 46.3 The majority of laboratories reported consistent results, however there were discrepancies in the results of laboratory two (rep. two; 14* omitted) and particularly laboratory five which reported a less than value in all three replicates.

3.3 The spiked presscake produced some consistent results from the majority of laboratories, only laboratory four failed to enumerate the sample accurately at such high levels reporting results of >12800 for all three replicates. Possibly a dilution mis-calculation. The laboratories reported an average mean of 13,400,000 with a standard deviation of 5,850,000.

3.4 The results for the spiked thermally dried sludge produced very consistent results from all participating laboratories. The participants reported an average mean of 70 with a standard deviation of 22.5.

3.5 The results for the composted sludge showed relatively consistent results between the laboratories, although laboratory three had a significantly higher standard deviation of 127 compared individually to the other participants. The laboratories reported an average mean of 321 with a standard deviation of 158.

***Table 6* (cont.): Data for Spiked Sludge Sample**

Presscake	**3B**	(* denotes suspect result omitted) (** inappropriate dilution)			
Lab No.				**AVE**	**SD**
1	17200000	16200000	17500000	17000000	642000
2	11100000	16600000	14600000	14100000	2740000
3	9900000	9400000	10500000	9930000	551000

4	>12800**	>12800**	>12800**	-	-
5	30700000	20500000	17200000	22800000	7000000
6	2820000	12800000	5350000	7000000	5190000
7	8630000	10100000	71600000*	9370000	1040000
Mean	**13400000**	**14300000**	**22800000**	**13400000**	
St.Dev.	**9640000**	**4260000**	**24400000**	**5850000**	
RSD (%)	**71.9**	**29.9**	**107**	**43.6**	
Thermally Dried Sludge	**4B**				
Lab No.				**AVE**	**SD**
1	121	71	91	94	25.2
2	75	74	94	81	11.3
3	29	41	31	34	6.4
4	81	66	94	80	13.8
5	116	55	79	83	30.6
6	55	80	79	72	14.0
7	50	33	46	43	8.9
Mean	**75**	**60**	**73**	**70**	16
St. Dev.	**34.0**	**17.7**	**25.0**	**22.5**	
RSD (%)	**45.2**	**29.4**	**34.1**	**32.4**	
Composted Sludge	**5B**				
Lab No.				**AVE**	**SD**
1	250	242	174	222	41.8
2	332	329	450	370	69.0
3	540	630	790	653	127
4	256	327	312	298	37.2
5	201	186	216	201	15.2
6	210	271	154	212	58.5
7	374	239	254	289	74.0
Mean	**309**	**318**	**336**	**321**	60
St. Dev.	**120**	**147**	**224**	**158**	
RSD (%)	**38.7**	**46.2**	**66.6**	**49.4**	

Table 7: Data for MRD Matrix Samples

Digested Sludge	**1C**				
Lab No.				**AVE**	**SD**
1	125000	82300	111000	106000	21800
2	108000	129000	11800	119000	10600
3	35000	35000	35000	35000	0.0

4	118000	108000	69200	98400	25800
5	72100	77800	73300	74400	2980
6	30500	57200	36300	41300	14100
7	84000	97700	59200	80300	19500
Mean	**81800**	**84000**	**71700**	**79200**	
St. Dev.	**38300**	**31600**	**32800**	**31800**	
RSD (%)	**46.8**	**37.7**	**45.7**	**40.1**	
Limed Sludge	**2C**	(* denotes suspect result omitted)			
Lab No.				**AVE**	**SD**
1	544	396	434	458	76.9
2	282	389	591	421	157
3	1000	6000*	1000	1000	0
4	392	268	284	314	67.6
5	111	327	257	232	110.3
6	318	245	331	298	46.4
7	188	156	232	192	38.2
Mean	**405**	**1110**	**447**	**416**	
St. Dev.	**297**	**2160**	**274**	**274**	
RSD (%)	**73.4**	**194**	**61.2**	**65.8**	
Presscake	**3C**	(* denotes suspect results omitted)			
1	25800000	29000000	23000000	26000000	2970000
2	9880000	11700000	8800000	10100000	1460000
3	140000*	166000*	158000*	Mis-calculation of the dilution factor suspected	13300
4	17200*	20500*	19300*	As above	1630
5	20500*	20500*	15400*	As above	2940
6	10200*	12100*	16300*	As above	3090
7	12100000	13300000	13100000	12800000	638000
Mean	**6860000**	**7740000**	**6440000**	**16300000**	
St. Dev.	**9850000**	**11100000**	**9020000**	**8500000**	
RSD (%)	**144**	**143**	**140**	**52.1**	
Thermally Dried	**4C**				
1	122	85	92	100	19.7
2	96	105	102	101	4.6
3	115	86	88	96	16.2
4	112	122	78	104	23.3
5	55	21	79	52	28.9
6	66	102	59	76	23.1
7	77	39	51	56	19.4
Mean	**92**	**80**	**78**	**83**	
St. Dev.	**26.2**	**36.7**	**18.1**	**22.3**	
RSD (%)	**28.5**	**45.9**	**23.0**	**26.8**	

Table 7 (cont.): Data for MRD Matrix Method

Composted Sludge	**5C**				
Lab No.				**AVE**	**SD**
1	306	339	243	296	48.8
2	381	343	315	346	33.1
3	196	388	345	310	100.8

4	284	248	229	253	28.1
5	242	202	237	227	21.8
6	297	275	218	263	40.6
7	137	176	210	174	36.5
Mean	**263**	**282**	**257**	**267**	
St. Dev.	**79.7**	**78.5**	**52.0**	**56.8**	
RSD (%)	**30.3**	**27.9**	**20.3**	**21.2**	

4. Discussion of Data for MRD Matrix Samples

4.1 The results collated from the MRD matrix samples illustrate the performance of the method in each of the participating laboratories without the affect of the various sludge matrices. If the performance of the vitroids is consistent, which they have been shown to be, the laboratories results should be comparable for all 5 types of MRD matrix. The first MRD matrix sample was designed to simulate a digested sludge, one vitroid at 5,000-25,000 cfu/vitroid was diluted into 100ml of MRD. Subsequently, 25ml of the vitroid solution was diluted further into 75ml of MRD to give the target range for the test. The results reported for the simulated digested sludge sample were consistent for all the participating laboratories. The average mean was 79,200 with a standard deviation of 31,800

4.2 The results for the simulated limed sludge are generally consistent with the exception of laboratory three which has reported a significantly high average mean compared with the remaining laboratories. The average mean for the simulated limed sludge was 416 with a standard deviation of 274. This figure does not include the result reported by laboratory three (6000*).

4.3 The results reported for the simulated presscake are difficult to interpret due to the wide variation in the results reported. The wide variation in results is illustrated by an average mean of 16,300,000 with a standard deviation of 8,500,000. The explanation of the wide margin of variation of results could be due to a dilution error or in some cases mis-calculation of the dilution factor (* results omitted).

4.4 The comparison between the participating laboratories for the data collated for the simulated thermally dried sludge is considered very good. All seven laboratories have reported results within the expected range and this is illustrated by the statistical data. The average mean for the simulated thermally dried sludge was 83 with a standard deviation of 22.3; the results highlight the ability of the method to be reasonably consistent across the laboratories at this level.

4.5 The results from the simulated composted sludge illustrate good comparison between the participating laboratories. There are consistent results across all three replicates for all seven laboratories. The average mean result is 267 with a standard deviation of just 56.8 which represents an average variation from of 21.2% from the mean result.

4.6 The *Klebsiella* samples were analysed as simulated sludge samples in an MRD matrix (see sample 6C in results table). The table below (***Figure 10***) outlines the results reported from all the participating laboratories for *E. coli.*

Table 8*: Results of MRD matrix samples for *E. coli

Laboratory No.	*E. coli* result for *Klebsiella* sample
1	0
2	0
3	<1000
4	0
5	0
6	0
7	<1000

5. Conclusion

5.1 The table below highlights the comparison between the results of the simulated MRD samples and the original sludge sample results subtracted from the spiked sludge samples. (i.e. 1C in comparison to 1B – 1A for the digested sludge etc.)

Sludge Type	Average Mean of MRD samples (C)	Average Mean of spiked sludge samples (B)	Average Mean of original sludge samples (A)	Comparison of recovery against MRD samples (C)
Digested	**79,200**	110,000	17,000	**93,000**

sludge				
Limed sludge	**416**	184	Invalid***	**184**
Presscake	**16,300,000**	13,400,000	1,300,000	**12,100,000**
Thermally Dried sludge	**83**	70	Invalid***	**70**
Composted sludge	**267**	321	67.6	**253**

5.2 The conclusions which can be drawn from the trial are encouraging. The Colilert® method is able to produce reasonably consistent results when analysing a variety of samples. The Colilert® method did not respond to *Klebsiella* and has shown that it is able to detect *E. coli* consistently in original 'real' sludge samples, spiked sludge samples and in simulated MRD samples without significant interference from the sludge matrix. The were some discrepancies in some of the results reported from the trial which may have been due to laboratory error or dilution mis-calculations on the part of the participating laboratories. The Colilert® method has also illustrated that it can recover the target organism equally, when compared to other methods such as membrane filtration methods MLSB and MLGA respectively. This is illustrated by the results of the pre-trial using the vitroids supplied by CDP Ltd. Further interlaboratory trials are planned involving a number of *E. coli* methods including Colilert®.

5.3 There is an interlaboratory trial planned for March 2004 organised by WRc-NSF to demonstrate that enumeration by chromogenic plate counting is a suitable alternative to chromogenic membrane filtration for the enumeration of *E. coli* in sludge. The objective of the trial is to assess the different variations of the SCA Blue Book method. The chromogenic plating method and the chromogenic membrane filtration method will also be assessed against in-house methods amongst the contributors.

The approach will see each participant using the following three methods;

1. **Trial method 1** - SCA Blue Book method A – The isolation and enumeration of *Escherichia coli* by a chromogenic (MLGA) membrane filtration technique.
2. **Trial method 2** – Replacing membrane filtration (MLGA) by plate counting (MLGA) in SCA Blue Book Method A.
3. **Trial Method 3** – Optional but will allow contributor to assess the performance of their method against the standard method in the SCA Blue Book.

The procedure for the trial will involve analysis of ten sludge samples which will be provided by WRc. The media required for the analysis in methods 1 and 2 will also be provided by WRc. Each sample will be pre-processed before being sent out to eliminate any inconsistencies that might arise from physical treatment between laboratories. The analysis of the trial samples will be carried out on a specific date agreed between all participants. Each test will require 5 dilutions to be examined in duplicate.

5.4 It is felt that this information will prove useful when assessing the interlaboratory trial data of the three (final version) CEN E. coli methods. The Colilert® test method does not involve the laboratory in any media or reagent preparation and the variations observed in the results should solely relate to the laboratory personnel, the sample pre-treatment step (e.g. Homogenisation step) and any variations in the supplied samples and / or E. coli reference materials.

Table 17: Comparison of *Salmonella spp.* Methods	Principle	Time to resolve	Confirmation	Approximate cost of test	Capital outlay	Typical Throughput	Robust-ness /10	Sensit-ivity	Competitive Flora	Solids
CEN TC308/WG1/TG5, (2003d) Part 1 Detection of *Salmonella* spp. - membrane filtration method for quantitative resuscitation of sub lethally stressed bacteria (log drop)	Diluted sludge sample filtered, and incubated at 36°c in tetrathinate broth to resuscitate for 24hrs Membrane recovered, incubated at 36°c on Rambach agar Membranes are examined after 24hrs and 48hrs (for *S.Dublin*) Positive colonies are quantified, *Salmonella* indicated by red colonies resulting from fermentation of propylene glycol, some produce b-galactosidase which hydrolyses x-gal to a blue chromophore	Incubate at 48 hrs or 72 hrs (*s.dublin*) at 36°C	Spraying colonies with 1mg/ml 4-methylumbelliferyl caprylate, fluoresecent colonies confirmed as Salmonella spp. Biochemical and Serological tests.	Tetrathionate broth €30 / 500g Rambach agar€ 40 / 500g Petri dishes €65 per box API 20E €125 for 25 strips **Approx. cost of disposables €260**	Manifolds €2000 Cup and Bases €1000 Incubators €3000 Homogeniser €1750 Pipettes €150 Boiling Bath €520 Vacuum Pump €750	Typically a sample can be processed in 48hrs and confirmed in 72hrs	5	+	+	+
CEN TC308/WG1/TG5, (2003e) Part 2 Liquid enrichment method in selenite cysteine medium followed by Rappaport Vassiliadis for semi quantitative MPN Determination.	Sample preparation suitable for a solid matrix A selective pre enrichment step to eliminate interfering bacteria Three series of three tubes with serial dilutions for MPN format enumeration Culturing of bacteria in primary selective medium Selective Enrichment Preparation of pure cultures inoculating special solid media	Culture tubes incubated at 42°C for 20hrs. incubated on XLD and Rambach agar at 36°C for 20 hrs	Identification by biochemical and serological tests	Selenite cysteine €20 /10ml (24) Rappaport-Vassiliadis €30 / 500g XLD €50 / 500g Rambach agar €40 /500g Culture tubes €50 per box API 20E® €125 for 25 strips **Approx. cost €315**	Incubator €3000 Pipettes €150 Homogeniser €1750	Typically 2 days for sample processing and a further day for confirmation	6	+	+ +	+ + +
CEN TC308/WG1/TG5, (2003f) Part 3										

Table 17: Comparison of *Salmonella spp.* Presence / Absence method by liquid enrichment in peptone novobiocin medium followed by Rappaport Vassiliadis	Four Stages of Detection Culturing of bacteria in a primary selective medium Enrichment in a selective medium which inhibits growth of other micro organisms but promotes Salmonella Preparation of pure cultures by innoculating special solid media with subcultures	Incubate in BPW with novobiocin at 36°C for 20 hrs Incubate on XLD at 36°C for 20 hrs	Identification by morphological, biochemical, and serological tests	BPW €20 / 90ml (10) Novobiocin €85 / 10ml (100) XLD €50 / 500g API 20E €125 for 25 strips Culture tubes €50 per box **Approx. cost €330**	Incubator €3000 Pipettes €150 Homogeniser €1750	Typically 2 days for sample processing and a further day for confirmation	8	+ + +	+ +	+ + +
SCA (2003f) The Microbiology of Sewage Sludge (2003) - Part 4 - Methods for the detection, isolation and enumeration of *Salmonella* - The detection of *Salmonella* spp. by use of a presence/absence technique.	Isolation and detection is based on appropriate homogenization of sludge, followed by a pre-enrichment involving incubation in a non-selective medium (to recover environmentally-stressed organisms), and selective enrichment with subculture to a selective agar containing xylose with additional indicators of acidity and H2S production.	Incubate in BPW with novobiocin at 36°C for 20 hrs Incubate on XLD at 36°C for 20 hrs	Characteristic colonies are confirmed by biochemical tests and serological tests based on slide agglutination	Tetrathionate broth €30 / 500g Rambach agar €40 / 500g Petri dishes €65 per box API 20E €125 for 25 strips **Approx. cost €270**	Incubator €3000 Pipettes €150 Homogeniser €1750	Typically 2 days for sample processing and a further day for confirmation	8	+ +	+ +	+ + +

Table 17: Comparison of *Salmonella spp.*	Isolation and enumeration is based on appropriate homogenisation of sludge, followed by multiple tube pre-enrichment involving incubation in a non-selective medium (to recover environmentally-stressed organisms), and selective enrichment with subculture to selective agar containing lactose and an indicator of acidity. The most probable number of organisms in the sample is estimated from the appropriate probability tables	Incubate in BPW with novobiocin at 36°C for 20 hrs Incubate on XLD at 36°C for 20 hrs	Characteristic colonies are confirmed by biochemical tests and serological tests based on slide agglutination	Selenite cysteine €20 /10ml (24) Rappaport-Vassiliadis €30 / 500g XLD €50 / 500g Rambach agar €40 / 500g Culture tubes €50 per box API 20E® €125 for 25 strips **Approx. cost €315**	Incubator €3000 Pipettes €150 Homogeniser €1750	Typically 2 days for sample processing and a further day for confirmation	7	+ +	+	+ +
SCA (2003h), The Microbiology of Sewage Sludge (2003) Part 4 - Methods for the detection, isolation and enumeration of *Salmonella*- The enumeration of *Salmonella* spp. by a membrane filtration technique with resuscitation and culture on a chromogenic detection medium	Diluted sludge sample filtered, and incubated at 36°c in tetrathinate broth to resuscitate for 24hrs Membrane recovered, incubated at 36°c on Rambach agar Membranes are examined after 24hrs and 48hrs (for *S.Dublin*) Positive colonies are quantified, salmonella indicated by red colonies resulting from fermentation of propylene glycol, some produce b-galactosidase which hydrolyses x-gal to a blue chromophore	Incubate at 48 hrs or 72 hrs (*s.dublin*) at 36°C	Salmonella colonies exhibit fluoresence when exposed to UV light at 366nm.	BPW €20 / 90ml (10) Novobiocin €85 / 10ml (100) XLD €50 / 500g API 20E €125 for 25 strips Culture tubes €50 per box **Approx cost 330E**	Manifolds €2000 Cup and Bases €1000 Incubators €3000 Homogeniser €1750 Pipettes €150 Boiling Bath €520 Vacuum Pump €750	Typically a sample can be processed in 48hrs and confirmed in 72hrs	5	+	+	+

Table 17: Comparison of *Salmonella spp.*										
atrix MicroScience (2003) Detection of *Salmonella* using Immunomagnetic Separation using Pathatrix®	Enrichment. A 25 -27g portion of the sample is weighed in a stomacher bag and Buffered Peptone Water added to make a 1 in 10 dilution. This processed using the stomacher for 30secs. To this is added antiserum coated beads, and the bag is incubated on the Pathatrix Equipment at 37°c for 3 hours. The beads are recovered and rinsed, and the concentrated bead suspension is plated on to the selective medium. This is incubated at 37°c for 24 hours.	Incubated on the Pathatrix system for 3hrs at 37°C Incubated on the selective medium for 24hrs at 37°C	Confirmed by flurotrix (fluorescent microscopy) and serology	BPW €20 / 90ml (10) Antiserum coated beads Matrix Micro-science Pathatrix System €6750 €8 per unit test	Pathatrix® System Gravimetric diluter Incubator €3000	Identification in 16 hrs	6	+ + +	+ +	+
N.B It should be noted that all methods will also require the use of an autoclave (steam steriliser) to make relevant media for use in the methods (approx. price €40,000) All prices quoted are in Euros Numbers in brackets are related to the pack size of the product										

Table 18 - *Escherichia Coli* Method	Principle	Time to resolve	Confirmation	Approximate cost of test	Capital outlay	Typical Throughput	Robust-ness /10	Sensi-tivity	Competitive Flora	Solids
CEN TC308/WG1/TG5(a) Characterisation of Sludges – Detection of *Escherichia Coli* Part 1: Membrane Filtration for Quantification	10g Sludge into 90ml MRD (PS) Serial Dilution 1ml PS - 9ml MRD TO 10-7 Membrane Filtration through 0.45mm membrane	Incubate on MLGA at 30°C 4hrs / 44°C for 14 hrs	Confirm on API 20E if requested, not usually required	Membranes 70 per box MLGA (Oxoid) €120 per tub /500g Petri dishes €65 per box (1620) **Approx. cost of disposables: €255**	Manifolds €2000 Cup and Bases €1000 Incubators €3000 Homogeniser €1750 Pipettes €150 Boiling Bath €520 Vacuum Pump €750	Dilutions to make up, filtering relatively quick 20 samples per hour. Confirmed result within 18 hrs of filtration	5	+ + +	+ +	+
CEN TC308/WG1/TG5 (b) Characterisation of Sludges – Detection and Enumeration of Sewage Sludges - Miniaturised method (MPN) in Liquid Medium	10g Dry Matter Sludge into a tryptone diluent final volume of 100ml (PS) Mix 2ml of PS with 18ml of Special Diluent Prepare Serial Dilutions in Special Diluent e.g. 1/10 - 1/200 000 Inoculate the microplate	Incubate at 44°C for 36hrs minimum and 72 hrs maximum	The presence of *E. coli* is indicated by a blue fluoresence resulting from the hydrolysis of MUG	Microplate €55 per plate Tryptone salt diluent €20 /500g Dehydrated culture medium Culture tubes €50 per box MUG Lauryl sulphate broth €75 / 500g **Approx. cost €200**	Incubators €3000 Homogeniser €1750 UV-lamp (366nm) €300 Laboratory shaker €1500 Pipettes €150	Samples take approximately 24 hrs to be processed Confirmed results within 48 hrs	7	+ +	+	+ + +

CEN TC308/WG1/TG5(c) Characterisation of Sludges - Detection and Enumeration of *Escherichia Coli* from Sewage Sludge Part 3: Macromethod (MPN) in Liquid Medium	Place 20g wet weight of sample into 180ml 0.9% sterile NaCl solution prepare serial tenfold dilution 1ml of PS + 9ml 0.9% NaCl upto 10-7 from each dilution step transfer 1ml per tube into 3 tubes containing MUG Fluorocult LSB each	Incubate for 40hrs at 44°c	To read add 0.5ml NaOH to each tube and observe for fluorescence with a 366nm UV light	Culture tubes €50 per box NaCl €10/ 500g NaOH €30/ 250g MUG Lauryl sulphate broth €75/ 500g **Approx. cost €165**	Incubators €3000 UV-lamp(366 nm) €300 Pipettes €150 Homogeniser €1750	Samples take approximately 24 hrs to be processed Confirmed results within 48 hrs	8	+ + +	+ +	+ + +
ISO 16649-2: Detection and enumeration of *Escherichia coli* - membrane filtration method using Chromocult® agar	10g Sludge into 90ml of MRD (Primary Suspension) Serial Dilutions of 1ml PS - 9ml MRD to 10-7 Membrane filtration through 0.45mm membrane Escherichia Coli colonies are blue - green (X-b-D glucuronide reaction)	Incubate at 30°C for 4hrs and then 44°C for 18-20 hrs	Confirmation of blue/ green colonies not required due to the specificity of the media	Chromocult® agar €120 per tub Membranes €70 per box Petri dishes €65 per box (1620) **Approx. cost €155**	Incubators €3000 Manifolds €2000 Cups and Bases €1000 Homogeniser €1750 Boiling bath €520 Vacuum Pump €750	Dilutions to make up, filtering relatively quick 20 samples per hour. Confirmed result within 20 hrs	5	+ +	+ +	+
SCA (2002) The Microbiology of Drinking Water - Part 4: Detection and enumeration of *Escherichia coli* -membrane filtration method using MLSA agar	10g Sludge into 90ml MRD (Primary Suspension) Serial Dilutions of 1ml PS - 9ml MRD to 10-7 Membrane filtration through 0.45mm membrane onto MLSA agar	Incubation at 30°C for 4hrs and then 44°C for 14 hrs	Confirmation through the production of acid from lactose, negative oixdase reaction and indole formation	MLSA agar €120 per tub /500g Membranes €70 box Petri dishes €65 per box (1620) **Approx. cost €155**	Incubators €3000 Manifolds €2000 Cups and Bases €1000 Homogeniser €1750 Boiling bath €520 Vacuum Pump €750	Dilutions to make up, filtering relatively quick 20 samples per hour. Confirmation within 72 hrs	4	+	+	+

SCA (2002) The Microbiology of Drinking Water - Part 4: Detection and enumeration of *Escherichia coli* - membrane filtration method using MLSB agar	10g Sludge into 90ml MRD (Primary Suspension) serial dilutions of 1ml PS - 9ml MRD to 10-7 Membrane filtration through 0.45mm membrane onto MLSB agar	Incubation at 30°C for 4hrs and then 44°C for 14 hrs	Confirmation through the production of acid from lactose, negative oxidase reaction and indole formation	MLSB €40 per tub /500g Membranes €70 per box Petri dishes €65 per box **Approx. cost €175**	Incubators €3000 Manifolds €2000 Cups and Bases €1000 Homogeniser €1750 Boiling bath €520 Vacuum Pump €750	Dilutions to make up, filtering relatively quick 20 samples per hour. Confirmation within 72hrs	4	+	+	+
Andrews and Presnell (1990): The A-1 method Greater Vancouver Regional Council (GVRD): Multiple Tube Fermentation (MTF) technique to detect and enumerate *Escherichia coli* in biosolids	Similar to MTF analysis (MPN) technique sample homogenised and serial dilutions taken to 10-7 uses A-1 media, no requirement for enrichment step using LTB	Samples conditioned at 35°C for 3hrs and placed in a faecal water bath at 44.5°C for 21 hrs	A-1 media does not require confirmation due to its specificity	A-1 Media €130 per box Culture tubes €50 per box **Approx. cost €180**	Incubators €3000 Water Bath €750 Homogeniser €1750 Pipettes €150	Samples take approximately 24 hrs to be processed Confirmed results within 24hrs	6	+ +	+ +	+ +
Colilert® IDEXX Laboratories Ltd. (2003) IDEXX Colilert® Method – Enumeration of coliform and *Escherichia coli* bacteria in waste water solids using defined substrate technology®	Sample Homogenised and 50ml added to 450ml of sterile, buffered dilution water Serial dilutions prepared - 20 replicates, dilution A (0.001), B (0.00001), C (0.0000001) Take 100ml of A,B,C mix with Colilert media, add to Quantitray package, seal and incubate	35°C for 24hrs	No confirmation required, colour change indicates presence of *E. coli*	Quantitray Packs 100ml containers Colilert® media **($5 per unit sample)** (approx. €5)	Incubators €3000 Quantitray sealer UV Lamp €300 Homogeniser €1750 Pipettes €150	Less than one minute per sample test Confirmed result within 24 hrs	8	+ + +	+ +	+ + +

U.S. EPA - MTF technique for the detection and enumeration of *Escherichia coli* in waster activated solids EPA-600/8-78-017.	Sample Homogenised and 50ml added to 450ml of sterile, buffered dilution water Serial dilutions prepared - 20 replicates, dilution A (0.001), B (0.00001), C (0.0000001) Presumptive Phase - 4 groups of 5 tubes incubated at 35°c Various dilution added to tubes containing LTB Confirmed Phase - using Brilliant Green LTB, Completed phase using EC-MUG media	Incubated at 35°for 48 hrs and then completed phase incubated at 44.5°C for 24hrs	Confirmation using brilliant green LTB and EC MUG media	Culture tubes €50 per box Lauryl Tryptone Broth €50 / 500g Brilliant Green LTB €60 / 500g EC-MUG Media €75 / 500g **Approx. cost € 235**	Incubators €3000 Homogeniser €1750 UV Lamp €300	72 hrs before a confirmed result	7	+	+ +	+ +
Microbiology of Sewage Sludge - Part 3 Method A (2003) isolation and enumeration of *Escherichia coli* using a chromogenic membrane filtration technique	Sample homogenised, serially diluted with MRD and filtered through membrane filter and placed onto an agar plate of MLGA media. Colonies that are β-glucuronidase positve and ferment lactose are considered as *E. coli*	Incubation at 30°C for 4hrs and then 44°C for 14 hrs	Confirmation not required	MLGA Media €70 per tube Petri dishes €65 per box Membranes €70 per box **Approx. cost €205**	Incubators €3000 Manifolds €2000 Cups and Bases €1000 Homogeniser €1750 Boiling bath €520 Vacuum Pump €750 Pipettes €150	Dilutions to make up, filtering relatively quick 20 samples per hour. Confirmed result within 18hrs of filtration	5	+ + +	+ +	+
Microbiology of Sewage Sludge - Part 3 Method B (2003) isolation and enumeration of *Escherichia coli* using a multiple tube fermentation MPN technique	10g sample homogenised in MRD and added to a series of tubes containing liquid enrichment broth. Positive tubes are sub-cultured onto confirmation media.	Selective enrichment at 36°C for 24 hrs and then at 44°C for 24hrs	Growth at 44°C in the presence of brilliant green with the production of gas from lactose and the formation of indole	Brilliant Green Bile Broth €55 / 500g Tryptone Water €30 / 500g Sodium Lauryl Sulphate Bromocresol purple Culture tubes €50 per box **Approx. cost €200**	Incubators €3000 Homogeniser €1750		7	+ +	+	+ + +

Microbiology of Sewage Sludge - Part 3 Method C (2003) isolation and enumeration of *Escherichia coli* using defined substrate technology for MPN determination	Samples are homogenised and serially diluted with MRD. The samples are incubated in a defined liquid medium containing specific substrates for the detection of the enzymes β-galactosidase and β-glucuronidase. The samples is then added to a Quantitray® pouch and incubated.	Incubate at 37°C for 18 hours	No confirmation required	Quantitray Packs 100ml containers Colilert® media	Incubators €3000 Quantitray Sealer Homogeniser €1750	Less than one minute per sample test	8	+ + +	+ +	+ + +
N.B It should be noted that all methods will also require the use of an autoclave (steam sterilser) to make relevant media for use in the methods (approx. price €40,000) All prices quoted are in Euros Numbers in brackets are related to the pack size of the product										