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**HORIZONTAL - HYG**

**HORIZONTAL STANDARDS ON HYGIENIC PARAMETERS FOR IMPLEMENTATION OF EU DIRECTIVES ON SLUDGE, SOIL AND TREATED BIO-WASTE**

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# Suitability Study Report
# E. coli and Salmonella spp. Intra-lab study (D2/1.5)

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# Suitability Study report

# *E. coli* and *Salmonella* spp. Intra-lab study

# (DL 2/1.5)

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# Abbreviations and symbols

BPLS: Brilliant green-Phenol red-Lactose-Sucrose
Cfu: colony forming units
IPL: Institut Pasteur de Lille
LRM : liquide reference material
MPN: Most Probable Number
MRD: Maximal Recovery Diluent
MRS: Matrix reference sample
MTSB: Modified Tryptone Soya Broth
QC: quality control
Sub WP: Sub Work package
WP: work package
XLD: Xylose Lysine Deoxycholate

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# 1 GENERAL INTRODUCTION

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

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

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

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

**For *Salmonella* spp. and *Escherichia coli*** (SubWP2/1) drafted CEN standards are available and therefore a co-normative work will be performed consisting in the validation of those methods (performance data). This work will consist in three main steps: (i) a training in a central laboratory of 16 EU laboratories for methods to be validated, (ii) an intralaboratory suitability study of methods to be validated (fit for purpose on the nine different matrices that are to be targeted) and finally (iii) an interlaboratory round robin test with selected laboratories to validate the methods.

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

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

# 2 Introduction

This report corresponds to the Suitability Study report on the 6 CEN draft standards for *E. coli* and *Salmonella* spp. to be monitored in EU in sludges, soil and treated biowastes that should be validated in the frame of the SubWP2/1. This report describes the results of the Suitability Study realised by the 8 European participant-partner laboratories from January to May 2006.

The Suitability Study has three main objectives:

- ✓ Applying of the 6 CEN draft standard methods (prEN 15214 for *E. coli* and prEN 15215 for *Salmonella* spp.) (Version of November 2005, **Annexes 1-6**) in the 8 partners laboratories involved for the suitability intra-laboratory ruggedness trial;
- ✓ First statistical evaluation of the performances of the 6 methods;
- ✓ Check the fit for purpose of those methods on 9 matrices representing sludge, soil and treated biowastes.

As a result of the suitability study, the 6 CEN draft standards will be amended as Horizontal draft standards while including some improvement issues from the results obtained and from the exchanges and decisions taken during two technical meetings : Technical Meeting 4 ($21^{st}$ June 2006) and Technical Meeting 5 ($5^{th}$ September 2006).
The first horizontal draft standards will be then proposed for the CEN consultation.
According to the CEN conclusions, the performances of the standards will be evaluated on the selected matrices representing sludge, soil and treated biowastes during the Validation interlaboratory Study.

# 3 Participant laboratories

Eight European laboratories, all involved in the HORIZONTAL-HYG project as partners participated to the Suitability Study. They represented 6 European countries, including two new European countries (Estonia and Hungary) (**Table 1**).
These selected participating laboratories have been chosen for their expertise in sludges, soils and/or treated biowastes microbiology and are for most of them Reference European laboratories (3 laboratories being routine laboratories accredited ISO 17025).

**Table 1** The 8 European participant-partner laboratories involved in the suitability study

| Laboratories | Town | Country |
|---|---|---|
| Institut Pasteur de Lille (IPL) | Lille | France |
| ALcontrol | - | United-Kingdom |
| University of Hohenheim (UHOH) | Stuttgart | Germany |
| Veolia (CAE) | St Maurice | France |
| University of Southampton (USOU) | Southampton | United-Kingdom |
| University of Tartu (UT) | Tartu | Estonia |
| Instituto Superiore di Sanita' | Roma | Italy |
| Research Institute Soil Science and Agricultural Chemistry of Hung. Acad. Sci. (RISSAC) | Budapest | Hungary |

The technical preparation and organisation were in charge of IPL, helped by 3 partners (Alcontrol, Veolia, and UHOH), each involved for the preparation of the standards as "expert" for one method for *E. coli* and one method for *Salmonella* spp. (respectively for part 1, part 2 and part 3 of both prEN 15214 and prEN 15215 methods ).
The whole coordination of the Suitability Study was in charge of IPL.
The processing of all results was supported by IPL and AGLAE, sub-contractant of IPL, in charge of the statistical analysis.

# 4 Materials and methods

A 5 months intra-laboratory (ruggedness) Suitability Study was organised from January 2006 to May 2006.

## 4.1 Laboratory equipment and consumables

Each participating laboratory was in charge of preparing material and ordering consumables according to a list of materials and consumables for each method prepared by experts staff (**Annexes 7** & **8**).

Preparation and quality controls of diluents, reagents and media were also assumed by each participating laboratory.

Equipment used during the Suitability Study (scales, homogeniser, shaking apparatus, incubators, filtration devices, central vacuum pump, freezers and refrigerators) and consumables (culture media, diluents, filters) should have been tested before the Suitability Study for quality control.
Incubating times and incubating temperatures were both reported in the technical data sheet corresponding to each set of analyses for all methods.

## 4.2 Matrices and samples to be tested

The Suitability Study work was based on a matrix-based approach. Nine matrices were analysed to evaluate the fit for purpose of the 6 selected methods (three methods per parameter) (**Table2**).

**Table 2**: The 9 matrices to be tested

| Matrix |
|---|
| Raw sewage sludge |
| Mesophilic anaerobic digested (MAD) sewage sludge |
| Anaerobic treated biowaste |
| Pelletised air-dried sludge |
| Digested sewage sludge presscake |
| Lime-stabilised sewage sludge |
| Composted sewage sludge |
| Composted green waste (composted biowaste) |
| Sludge amended "nutrient weak" soil |

The analysis of one matrix was performed by 2 or 3 labs to reduce the risk of masking the tested “matrix effect” by any “laboratory effect”, and to facilitate the providing of samples by the participants.
The matrices were distributed between participants taking into account the availability of sampling sites in function of matrices.

The answer to an enquiry circulating on October 2005 on which matrices could be sampled by each of the 8 participants, has lead to set up the work programme. The repartition of matrices between the participants and the corresponding number of expected data (positive results) per lab and per matrix to be obtained are presented in **Annex 9**: Each participant has worked on 3 different types of matrices out of the 9 to be tested.

Twenty to twenty one different positive samples fully representing each type of matrix should be tested for all the six methods. For feasibility reason (availability of positive samples), the 20-21 positive samples were distributed in natural samples and spiked samples: the higher the number of positive natural samples was, the lesser the number of spiked samples was in order to obtain at least a total of 20-21 positive samples.

Each analysis was carried out in duplicate with the 6 methods in parallel so that comparative data have been obtained.

### 4.2.1 Sampling and sample handling

Natural samples were sampled by each participant partner with direct routine access to such kind of samples (corresponding to a given matrix to be analysed).
The sampling included the matrix samples to be used for the spiked samples.

A requirements sheet for sampling and sample handling has circulated before the Suitability Study start to determine the best conditions of sampling and sample handling to be followed by all the participants. This document is appended as **Annex 10**.
The requirements were based on the "sample handling protocols for sludges and treated biowaste for microbiological analysis" desk study realised by UREAD team (HOR-HYG partner implied in the work-package WP1).

The number of samples and sites to be sampled depended on the results obtained following the 3 methods tested for each parameter. If the results of each of the 3 methods applied were negative, an additional sample should be then analysed so as to obtain 7 to 10 positive results per lab for the same matrix (a positive result obtained for one of the 3 methods was sufficient to validate the result as a positive one, a negative result corresponded then to the obtaining of negative result following each of the 3 methods).

### 4.2.2 Spiking of prepared natural samples

The spiking has been used to ensure that 20 positive results were available for the statistical analyses. It was applied according to the procedure proposed by the UHOH expert partner and sent to all participants (**Annex 11**).

The spiking procedure consisted in several steps to achieve:

- preparation of the natural sample;
- preparation of a spiking bacterial suspension;
- inoculation of the prepared samples with the spiking suspension.

Commercially available microbiological reference materials were used to prepare the spiking suspension of each parameter to be detected (**Table 3**).

**Table 3**: Reference materials used as spiking strains

| Spiking strain | Reference | Origin | Initial concentration |
|---|---|---|---|
| *E. coli* | RIVM WR1 | BioRéférence Pastilles (IPL, France) | $1.25 \times 10^6$ cfu |
| *Salmonella* Senftenberg $H_2S$- | NCTC 9599 | Vitroids (CDP, UK) | $1.082 \times 10^7$ cfu |

The *E. coli* strain was provided by IPL as BioReferences pastilles and the *Salmonella* Senftenberg strain by CDP (UK) as Vitroids.

### 4.2.3 Analyses quality controls

Two types of quality controls were performed per analysis set, parameter and method:

- one solid quality control (matrix reference samples, MRS),
- one liquid quality control (liquid reference material, LRM).

Both quality controls were realised in duplicate for each set of analyses and for each parameter.

#### 4.2.3.1 Matrix reference samples - MRS

Natural raw sewage sludge from a wastewater treatment plant near Lille (France) was used as solid quality control. The preparation of the raw sewage sludge samples was done by IPL in charge of providing the 8 partners participants with those MRS.

*Homogeneity and stability of the batch to transport*

IPL was in charge of checking the homogeneity and stability to transport and storage conditions of the MRS batch, in order to assess in which conditions these MRS could be used as a quality control samples for the trial, to detect any “laboratory effect”.
According to preliminary trials, the homogeneity and the stability to transport of the MRS batch were ensured up to 5 weeks, up to the development of an interfering flora like fungi on the top of the sub-samples which could interfere with the analyses to be performed (**Annex 12**).
For homogeneity and stability reasons of the MRS batch, IPL was in charge of providing the 8 partners participants with MRS prepared from the same batch and suitable for use during 4 consecutive analyses weeks.

*Preparation of the month batch and the month-MRS*

On the first week of January and on every 4-weeks during the Suitability Study, raw centrifuged sewage sludge was sampled and stored at IPL at (5±3) °C, before being prepared to constitute a month-batch to be used as month-MRS.

The contamination level of the MRS batch was determined each time to check that it is adapted to the 6 methods.
Preliminary analyses were performed on a batch sample to check the presence of *E. coli* and *Salmonella* spp. populations in the batch. Methods used for the enumeration of the *E. coli* and *Salmonella* spp. were respectively *E. coli* Microtiter plate method (prEN 15214-2) and *Salmonella* spp. MPN method (prEN 15215-2).
The **Table 4** reports the results obtained for each MRS month-batch for both parameters.

**Table 4**: Contamination level of each MRS month-batch from January to June

| MRS month-batch | Date | | | Dry content | *E. coli* (MPN/g) | *Salmonella* spp. (MPN/g) |
|---|---|---|---|---|---|---|
| | sampling | analysis | sending | | | |
| January | 21/12/05 | 27/12/05 | 03/01/06 | 19.4 % | $1{,}10 \times 10^5$ | 0.43 |
| February | 19/01/06 | 23/01/06 | 31/01/06 | 21.5 % | $4{,}10 \times 10^5$ | 2.4 |
| March | 16/02/06 | 20/02/06 | 28/02/06 | 21 % | $1{,}70 \times 10^5$ | 2.4 |
| April | 16/03/06 | 20/03/06 | 28/03/06 | 17.5 % | $1{,}50 \times 10^5$ | 2.4 |
| May | 13/04/06 | 18/04/06 | 25/04/06 | 19.1 % | $5{,}20 \times 10^5$ | 2.4 |
| June | 11/05/06 | 15/05/06 | 24/05/06 | 19.5 % | $1{,}60 \times 10^5$ | 0.43 |

The first estimation of the *E. coli* and *Salmonella* spp. concentrations ranged from $1.1 \times 10^5$ MPN/g to $5.2 \times 10^5$ MPN/g, and from 0.43 MPN/g to 2.4 MPN/g respectively.

This optimised homogenisation procedure described in **Annex 12** was performed on 21.5 Kg of the month-batch, on the day before the shipment of the MRS.
Following this homogenisation step, 140 sub-samples of 150g of sludge were weighted from the homogenised month-batch and distributed into 140 sterile plastic flasks labelled month-MRS. The flasks were capped and stored at (5±3) °C until their packing on the following day.

*Packaging and sending*

Six sets of shipments were organised by IPL to send the 16 month-MRS to each of the 8 participants. One set of shipments corresponded to the sending of between 2 and 16 parcels, with 2 parcels per participating laboratory.
The transport conditions were those established following preliminary trials so as to maintain a temperature of 5±3°C during approximately 48h, the time to reach all destinations (England, Germany, France, Italy, Estonia, and Romania). The results of the preliminary trials are presented in **Annex 12**.

Each parcel then contained:

- 8 flasks containing 150 g of MRS;
- 5 ice packs;
- Polystyrene partitions to avoid freezing of MRS by contact between flasks and ice packs;
- One small electronic temperature logger to register the range of temperature during the transport.

The **Figure 1** shows the inner organisation of a month-parcel (top view and front view).

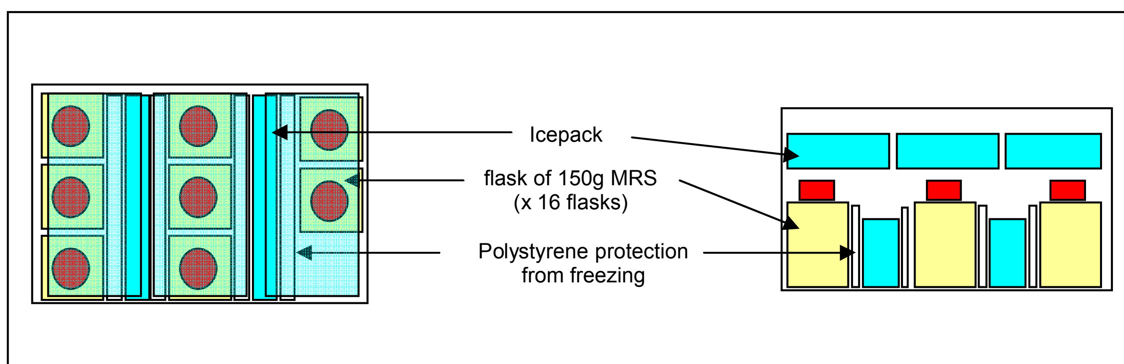



**Figure 1**: Scheme of a month-parcel with MRS and LMR quality controls. On the left: Top view; on the right: front view.

The first parcels sent on January were slightly different, as they contained in addition to the MRS, the LRM (see **Figure 2**, paragraph 4.2.3.2.).

### 4.2.3.2 Liquid reference material - LRM

The liquid reference material (LRM) is added to the MRS to ensure at least one quality control to detect any "laboratory effect" in the whole time of the study since the MRS were different from month to month (homogeneity and stability verified up to 5 weeks).
The LRM correspond to a reference material recovered as liquid sample in a given tryptone salt diluent volume. *E. coli* LRM was provided by IPL as BioReferences pastilles and *Salmonella* spp. LRM by CDP (UK) as Vitroids. The features of those both reference materials and the conditions of their use are reported in **Table 5**.

**Table 5**: Reference strains used as LRM

| Reference strain | Initial concentration | Final concentration | |
|---|---|---|---|
| *E. coli* (RIVM WR1) | 1.25 x $10^6$ cfu | 1 pastille in 50 mL tryptone salt diluent | 2.5 $10^4$ cfu/mL |
| *Salmonella* Senftenberg $H_2S$- (NCTC 9599) | 1.082 x $10^7$ cfu | 1 disc in 150 mL tryptone salt diluent | 7.2 $10^4$ cfu/mL |

*Homogeneity and stability of the batch to transport*

IPL was in charge of checking the homogeneity and stability to transport and storage conditions of the *E. coli* LRM batch, before the start of the Suitability Study.
According to preliminary trials, the homogeneity and the stability of the *E. coli* LRM were observed during 5 weeks before the start of the trial. They were also checked during the whole period of the Suitability Study. The results are presented in **Annex 12**.
From the preliminary trials to the end of the Suitability Study, the concentration of *E. coli* LRM ranged from 5.6 x $10^2$ MPN/mL to 4.2 x $10^4$ MPN/mL.

The concentration of *Salmonella* Senftenberg LRM was certified homogeneous and stable by the supplier. The range of concentration and the stability to transport were checked. The results were presented in **Annex 12**.
The concentration of *Salmonella* Senftenberg LRM was evaluated at more than $10^5$ *Salmonella* Senftenberg cfu /mL, as expected.

*Packaging and sending*

The microbiological reference materials were sent by IPL to the 8 participant partners at the beginning of the trial respecting the conditions determined following preliminary trials to maintain the lower temperature without reaching O°C degree, during the transport (**Annex 12**).

Both LRM were sent with the January MRS. Then, in addition to the elements composing the parcel described for the MRS, each January parcel included one envelop for each parameter with in 4 bags of 5 tubes of LRM (**Figure 2**).

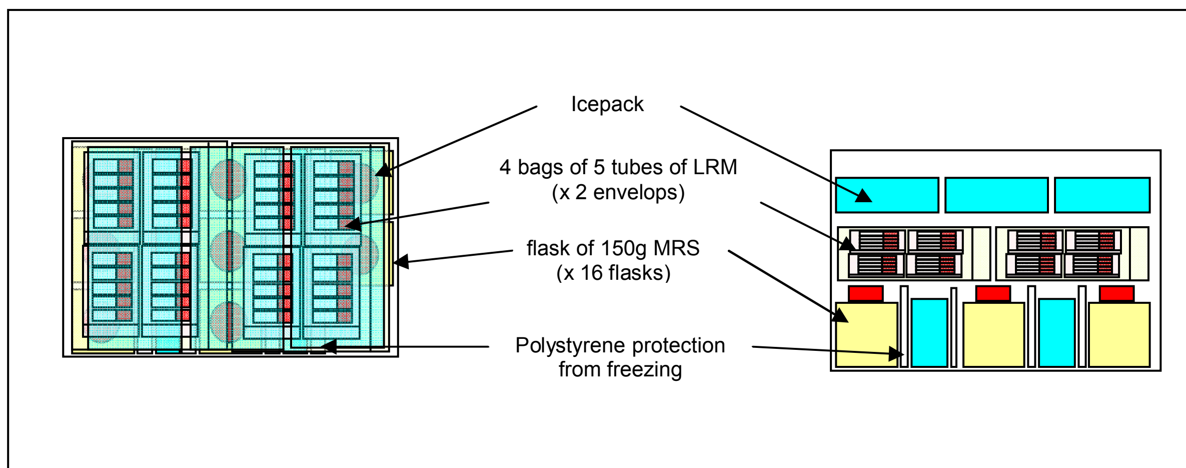



**Figure 2** : Scheme of the January parcel with MRS and LMR quality controls. On the left: Top view; on the right: front view

The instructions for the use of the LMR and MRS during the whole Suitability Study were those defined in **Annex 13**.

## 4.3 *Suitability Study work program*

### 4.3.1 Methods to be applied

The 6 methods to be applied during the suitability study are presented in **Table 6** for the 3 *E. coli* methods and in **Table 7** for the 3 *Salmonella* spp. methods.

**Table 6** Methods applied for the detection and enumeration of *Escherichia coli*

| Reference | **prEN 15214-1** | **prEN 15214-2** | **prEN 15214-3** |
|---|---|---|---|
| CEN draft title | Detection and enumeration of *Escherichia coli* in sludges, soils, soil improvers and biowastes – **Part 1**: Membrane filtration method for quantification | Detection and enumeration of *Escherichia coli* in sludges, soils, soil improvers and biowastes – **Part 2**: Miniaturised method (Most Probable Number) by inoculation in liquid medium | Detection and enumeration of *Escherichia coli* in sludges, soils, soil improvers and biowastes – **Part 3**: Macromethod (Most Probable Number) in liquid medium |
| Short Title | Filtration | Microtiter plate | MPN |
| Version used for the Suitability Study | Amended version of Nov05 | Amended version of Nov05 | Amended version of Nov05 |

**Table 7** Methods applied for the detection and enumeration of *Salmonella* spp.

| Reference | **prEN 15215-1** | **prEN 15215-2** | **prEN 15215-3** |
|---|---|---|---|
| CEN draft title | Detection and enumeration of *Salmonella* spp. in sludges, soils, soil improvers and biowastes – **Part 1**: Membrane filtration method for quantification | Detection and enumeration of *Salmonella* spp. in sludges, soils, soil improvers and biowastes – **Part 2**: Liquid enrichment method in selenite-cystine medium followed by Rappaport-Vassiliadis for semi-quantitative Most Probable Number (MPN) determination | Detection and enumeration of *Salmonella* spp. in sludges, soils, soil improvers and biowastes – **Part 3**: Presence/Absence method by liquid enrichment in peptone-novobiocin medium followed by Rappaport-Vassiliadis |
| Short Title | Filtration | MPN | Presence/Absence |
| Version used for the Suitability Study | Amended version of Nov05 | Amended version of Nov05 | Amended version of Nov05 |

The protocols applied for each of the 6 methods were the November 2005 HOR-HYG amended versions of the prEN standards edited in December 2004. These amended versions included editorial and technical improvements performed by the experts on each of the 2 methods they were in charge, following the training session of July 2005. The November amended versions of prEN 15214-1, prEN 15214-2, prEN 15214-3, prEN 15215-1, prEN 15215-2 and prEN 15215-3 are presented respectively in **Annexes 1**, **2**, **3**, **4**, **5** and **6**, with a synthetic scheme of the whole protocol.

### 4.3.2 Work program

Each partner participant received a set of instructions before the start of the Suitability Study to help them in the implementation of the technical work (**Annex 14**).

From January to May 2006, the 16 participants should perform between 20-27 set of positive analyses in total according to the instructions. The total number of analyses to be performed by the participating laboratories depended on the final result and of the matrices to be analysed.

The participants were free to organise their work programme (sampling and analyses) as long as it was performed in the indicated period, according to the Suitability Study time table (**Annex 15**). They were also free to plan their analyses weeks, but most of them followed the proposed work schedule (**Annex 16**) by starting one set of analyses with:

- the *Salmonella* spp. analyses with each of the 3 methods to be tested on Monday.
- the *E. coli* analyses with each of the 3 methods to be tested on Tuesday, in parallel to the following of *Salmonella* spp. methods.

The other days, the following and/or the end of each of the 6 methods were realised.

For each of the two parameters, the set of analyses to perform with each of the 3 methods by a participant was composed of:

- at least 1 natural or spiked sample, in replicate;
- 2 month-MRS;
- 2 LRM.

The whole analyses corresponding to one parameter had to be performed on one day and not spread out over different days. It was permitted to analyse more than one sample per set.

## *4.4 Analyses of results*

The results of each set of analyses had to be reported on the corresponding excel data sheets and returned by e-mail to the IPL coordinator within 2 months following the analyses.
The IPL coordinator checked the results for completeness and sent them to AGLAE for the statistical processing. The IPL coordinator was also in charge of screening all technical data.

Technical data and results of statistical data processing were presented and discussed in two central technical meetings with the partners (TM 3 on the 21$^{st}$ June 2006 and TM 4 on the 5$^{th}$ September 2006). The conclusions of these meetings were used for the final analyses.

### 4.4.1 Screening of technical results

The 8 participants sent a completed reporting form (in a relevant Excel sheets) of technical data per set of analyses per method to the IPL coordinator (**Annex 17**).
The IPL coordinator checked all technical data with the prescribed procedures. Deviations from the procedure criteria were observed (medium, incubation time, and incubation temperature). Any doubtful results were marked or discarded.
During the Technical Meeting 3 (on the 21$^{th}$ June 2006), first technical observations were discussed to evaluate possible effects on the results.

### 4.4.2 Statistical data processing

The 8 participants had also to note their results per set of analyses per method in the relevant Excel reporting sheets (**Annex 17**). The Excel file had to be sent by e-mail to the IPL coordinator.
The IPL coordinator checked the results for completeness and whether they were correctly entered into the Excel sheets. Once the data forms well registered, they were sent to AGLAE for statistical analyses.
During the Technical Meeting 4 (on the 5th September 2006), statistical results were presented and discussed with partners.

*Quality controls*

Liquid reference materials (LRM) were the only analysed samples extracted from a single batch. Although results on these samples give a partial prediction of the analytical protocols, they enabled to evaluate the proficiency of laboratories in order to remove outliers.

Matrix reference samples (MRS) were more representative of the complete analysis. Several batches have been used all along the study due to the stability of the material, but all laboratories carried out analyses on the same MRS batch at the same period of time. Data from these samples were used to check the reliability of the analysis for every draft method.

The aim of the quality controls data processing was to detect abnormal data (outliers) in order to carefully consider the connected results on tested matrices.

The data processing was composed of several steps:

1. The results to be used were calculated from the collected intermediate values;
2. The results per replicate were expressed on a $\log_{10}$ scale;
3. A consensus value and an objective Standard Deviation were calculated using a Log-Normal fitting model;
4. The comparison of mean values of laboratory results was done using an Analysis of Variance (ISO Guide 35). Moreover, this statistic allowed us to distinctly evaluate the variability due to the mixed effect of subsampling and between-flask variance and the variability due to Reproducibility;
5. A z-score was calculated for each laboratory result on MRS or LMR (in relation to a given set of analysis on natural samples). This z-score represents a standardized position of the laboratory result in relationship to the consensus value and the objective standard deviation, according to the formula:

$$\text{z-score} = (x - m) / s_z$$

where: x is the mean of the 2 duplicates ($\log_{10}$ scale),
m is the overall mean (consensus value - $\log_{10}$ scale),
$s_z$ is the standard deviation ($\log_{10}$ scale).

Finally, all quality controls results greater than **2** or less than **-2** in terms of z-score were considered as a signal that an anomaly could have occurred. The corresponding data observed on natural sample were carefully taken into consideration for further statistical analysis.

*Relative performance of the methods*

The experimental plan used to carry out the analysis of the Suitability Study had been decided upon the statistical requirements of the methods comparison. The best organisation was to apply the different draft methods to the same samples within laboratories. In order to avoid a potential bias of a laboratory, it was statistically important that several laboratories analysed samples of the same matrix.
The following procedure was used to compare the relative performance of the draft methods:

1. The results calculated from the collected intermediate values were used;
2. Calculations of the final result per sample and confidence intervals were carried out;
3. The methods results were plotted on a log scale;
4. The methods relative accuracy was determined using paired comparisons;
5. Graphically, the position of the dots on the biplot in relationship to the straight line of equivalence allowed us to detect relative bias between methods;
6. A nonparametric test (Wilcoxon test - paired signed rank test) was used to determine whether the trend was statistically significant;
7. The paired differences were computed (method A results - method B results) and the median difference was used to give an order of magnitude of the significant bias.

*First assessment of the precision of the different methods:*

At this stage of the study, it was not possible to evaluate every component of the methods precision. The Suitability Study planning was based especially on the detection of the bias between the methods for each of the 9 tested matrices.
As a first step, it was possible to calculate the variability inherent in every method. It does not depend on the experimental data but only on the design of the measurement protocol (random variation).
The organisation of the validation study will be rigorously planned in order to assess repeatability and other components like interlaboratory Reproducibility.
The statistical concepts used for the data processing were:

- the Poisson distribution, which is the random distribution of the number of particles (germs) at the moment of sub-sampling a perfectly homogenised suspension;
- the limit of detection, which is defined as the number of particles (germs per test portion) when the probability of a negative result is 5% (superior limit of the confidence interval of the null result);
- the relative variance $U^2$ (Relative standard deviation squared) corresponding to the Ratio of the standard deviation squared and the mean squared as:

$$U^2 = s^2 / m^2$$

where: s is the standard deviation,
m is the arithmetic mean of a series of measurement (natural scale).

This statistic is commonly used to express dispersion or uncertainty of microbiological test results (ex: ISO 13843 Guidance on validation of microbiological methods).

# 5 Suitability study results

The 8 participants were able to perform the amount of work scheduled during the Suitability Study 5-months (sample preparation, sample analysis, plates reading and data sheets reporting).
The results of the analyses of the 9 matrices to be tested were reported by the participating partners on standard sheets for the 6 procedures applied (**Annex 17**).
In order to avoid errors in data handling and calculation, results were collected and verified by the IPL coordinator with each participant before sending a final version to AGLAE.
The sampling data, the quality controls data, the technical data and the results of the analysed samples and quality controls obtained by the participants are all annexed to this report.

## *5.1 Sampling and parcels data*

All data recorded by the participants dealing with the sampling and the samples handling are assembled in **Annex 18**.

Information about the parcels (receipt, temperatures recording, MRS dry matter content, storage of MRS and LRM) sent during the whole Suitability Study are also gathered in **Annex 19**.

Those data were not included in the data processing; they were collected for traceability and informative purpose.

## *5.2 Technical results*

All technical data registered by the participants are presented in **Annex 20**.
They were checked with the prescribed procedures. Deviations were well documented by participants and eventually further discussed with them. In case it was expected that deviations could have influenced the results, it was decided not to use these results for further analyses.

The verifications of temperature incubation, time of incubation, media expiry date were satisfying. The quality control verifications of incubators and apparatus were in charge of the participant laboratories
No data was also eliminated according to this information.

No relation between results and media or apparatus manufacturers combination has been studied.

## *5.3 Data and statistics for E. coli analyses (prEN 15215)*

The results collected following the analysis of the 9 matrices with the 3 different *E. coli* methods are presented matrix by matrix, method by method in **Annex 21**.
Those data were the base of the statistical calculation, taking into account the results of the quality controls in a first time, to determine the relative performances of the methods and the first assessment of their precision.

### 5.3.1 Quality control

The quality control results of quantitative methods (prEN 15214-1/-2/-3) are presented in **Annex 22**, **Annex 23**, and **Annex 24**, for each method respectively. In these **Annexes**, for a given batch of MRS (corresponding to a month of analysis), a first **Table** gives details of the laboratory results and the obtained z-scores. The laboratory results for the analysis of the LRM and the corresponding z-scores are presented in a second **Table**. A last **Table** shows the estimated inter-laboratory precision calculated for each draft method.

The precision is defined as the closeness of agreement between independent results obtained under stipulated conditions.

According to the statistical planning of the quality control (1 analysis of 2 samples extracted from the same batch in repeatability conditions), the estimated components of precision that were evaluated were:

- A combined variance of the repeatability and the between-flask dispersion called "r"
- A combined variance of an intralaboratory Reproducibility and inter-laboratory Reproducibility called "R" (several results of a laboratory and several results of several laboratories were available for a given month).

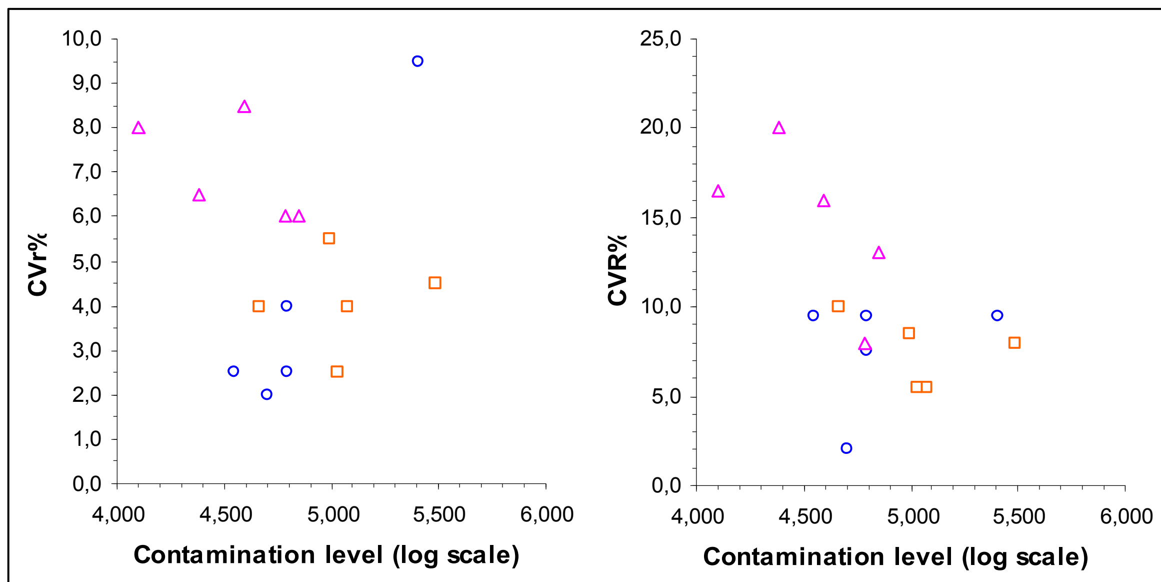
The range of the precision values obtained for each draft method is presented in the synthetic **Table 8**. The presented precision values correspond to the lower value and the upper value observed through the statistical analysis of the available data from the different batches of MRS (different month apart from June). The data processing was carried out after a $\log_{10}$ transformation of data.

**Table 8**: Range of precision values of *E. coli* methods (prEN 15214) tested on MRS

| **Evaluated methods** | **prEN 15214-1** | **prEN 15214-2** | **prEN 15214-3** |
|---|---|---|---|
| **Range of observed value of CVr%** (Variance due to repeatability and between-flask dispersion) | 2% to 9.5% | 2.5% to 5,5% | 6% to 8,5% |
| **Range of observed value of CVR%** (mixed variance due to intra-laboratory and inter-laboratory Reproducibility) | Not significant[(1)] to 9.5% | 5.5% to 10% | 8% to 20% |

[(1)]The observed variance in Reproducibility conditions was reduced to the observed variance in repeatability conditions (random variation).

The variances values obtained for each *E. coli* method are presented in the **Graph 1** according to the repeatability conditions and reproducibility conditions.




**Graph 1**: Precision values obtained in repeatability conditions (CVr%) and in reproducibility conditions (CVR%) for the 3 *E. coli* methods on MRS. Blue circle: Filtration method (prEN 15214-1); orange square: Microplate (prEN 15214-2); pink triangle: MPN macromethod (prEN 15214-3).

The first assessment of CVr% and CVR% observed for the filtration method and the Microplate method appears to be quantitatively comparable. Both values observed for the MPN macromethod CVr% and CVR% are globally higher.

### 5.3.2 Relative performance of the *E. coli* methods (prEN 15214)

The results of the relative performance of the 3 *E. coli* methods (prEN 15214) are presented matrix by matrix in **Annex 25**.

The **Table 9** summarizes the observed statistical trends.

**Table 9**: Statistical trends of relative performances of the 3 *E. coli* methods

| | Matrix | prEN 15214-1 & prEN 15214-2 | | prEN 15214-1 & prEN 15214-3 | | prEN 15214-2 & prEN 15214-3 | |
|---|---|---|---|---|---|---|---|
| | | Relative performance | Ratio | Relative performance | Ratio | Relative performance | Ratio |
| 1: | **Raw sewage sludge** | 214-1 **<** 214-2 | 6,3 (6,3) | 214-1 = 214-3 | | 214-2 **>** 214-3 | 3,3 (2,8) |
| 2: | **Mesophilic anaerobic digested** | 214-1 **<** 214-2 | 3,0 (2,8) | 214-1 = 214-3 | | 214-2 **>** 214-3 | 2,6 (2,1) |
| 3: | **Anaerobic treated biowaste** | 214-1 **<** 214-2 | 2,3 (2,3) | 214-1 **<** 214-3? | 2,1? | 214-2 = 214-3 | |
| 4: | **Pelletised air dried sludge** | 214-1 = 214-2 | | 214-1 **>** 214-3? | 16,6 (13,0)? | 214-2 **>** 214-3 | 11,3 (12,1) |
| 5: | **Digested sewage sludge presscake** | 214-1 **<** 214-2 | 4,2 (3,2) | 214-1 >= 214-3? | | 214-2 **>** 214-3 | 10.3 (6.4) |
| 6: | **Limed stabilised sewage sludge** | No data processing | | No data processing | | No data processing | |
| 7: | **Composted sewage sludge** | 214-1 **<** 214-2 | 4,5 | 214-1 **<** 214-3 | 1,8 (1,9) | 214-2 **>** 214-3? | 2.6 (2.0)? |
| 8: | **Composted green waste** | 214-1 = 214-2 | | 214-1 >= 214-3? | | 214-2 **>** 214-3 | 2.1 (2.5) |
| 9: | **Sludge amended "nutrient weak" soil** | 214-1 **<** 214-2? | 4,1 (60,6?) | 214-1 <= 214-3? | | 214-2 **>** 214-3 | 2.3 (4.2) |

Relative performance: conclusion in agreement with the Wilcoxon test significance: **<** or **>**: 1% significant trend; **?**: only 5% significant trend (not 1%); **=**: no significant trend. Ratio: order of magnitude of the difference expressed on natural scale; (between brackets) calculated ratio with suspect quality control data excluded.

Globally, on natural or spiked samples, two obvious trends can be noticed:

- the results obtained with the microplate method showed a significant trend to be higher than the ones obtained with the filtration method (ratio from 2.3 to 6.3);
- the results obtained with the microplate method showed a significant trend to be higher than the ones obtained with the MPN macromethod (ratio from 2.1 to 11.3);

The comparison of the paired results observed for the filtration method and the MPN macromethod showed no obvious trend for any of the two methods: the trend was different from one matrix to another.

The same data processing was carried out with results obtained on the Matrix Reference Samples (**Annex 25**). The **Table 10** summarizes the observed statistical trends for the MRS analysis.

**Table 10**: Statistical trends of relative performances of *E. coli* methods with MRS

| Month-MRS batch | prEN 15214-1 & prEN 15214-2 | | prEN 15214-1 & prEN 15214-3 | | prEN 15214-2 & prEN 15214-3 | |
|---|---|---|---|---|---|---|
| | Relative performance | Ratio | Relative performance | Ratio | Relative performance | Ratio |
| **January MRS** | 214-2>214-1 | 1.8 | 214-1=214-3 | | 214-2>214-3 | 1.6 |
| **February MRS** | 214-2=214-1 | | 214-1=214-3 | | 214-2>214-3 | 2.4 |
| **March MRS** | 214-2=214-1 | | 214-1>214-3 | 2.4 | 214-2>214-3 | 4.5 |
| **April MRS** | 214-2=214-1 | | 214-1=214-3 | | 214-2>214-3? | 1.5 |
| **May MRS** | 214-2=214-1 | | 214-1>214-3 | 1.7 | 214-2>214-3 | 2.1 |

Relative performance: conclusion in agreement with the Wilcoxon test significance; **<** or **>**: 1% significant trend; **?**: only 5% significant trend (not 1%); **=** no significant trend. Ratio: order of magnitude of the difference expressed on natural scale; (between brackets) calculated ratio with suspect quality control data excluded.

The same tendency was observed with the MRS results showing higher results for the microplate method than for the MPN macromethod (ratio from 1.5 to 4.5).

On the other hand, no global significant difference was detected between the results of the microplate method and the ones obtained with the filtration method (only one MRS batch among the five MRS batches used showed higher results with the microplate method).

The comparison of the paired results for the filtration method and the MPN macromethod did not show obvious trend through the different batches of MRS used (equivalence for three MRS batches and greater results with the filtration method for the two other MRS batches).

### 5.3.3 *First assessment of the precision of the 3 E. coli prEN 15214 methods*

The results of the variability inherent in each *E. coli* method are presented in **Annex 26**.
The values of relative variance ($u^2$) calculated in repeatability conditions for each tested matrix are plotted in relationship to the intrinsic variability of each method.
These first results should be carefully taken into consideration because few data obtained in repeatability conditions were available (only two replicates per sample were systematically analysed).

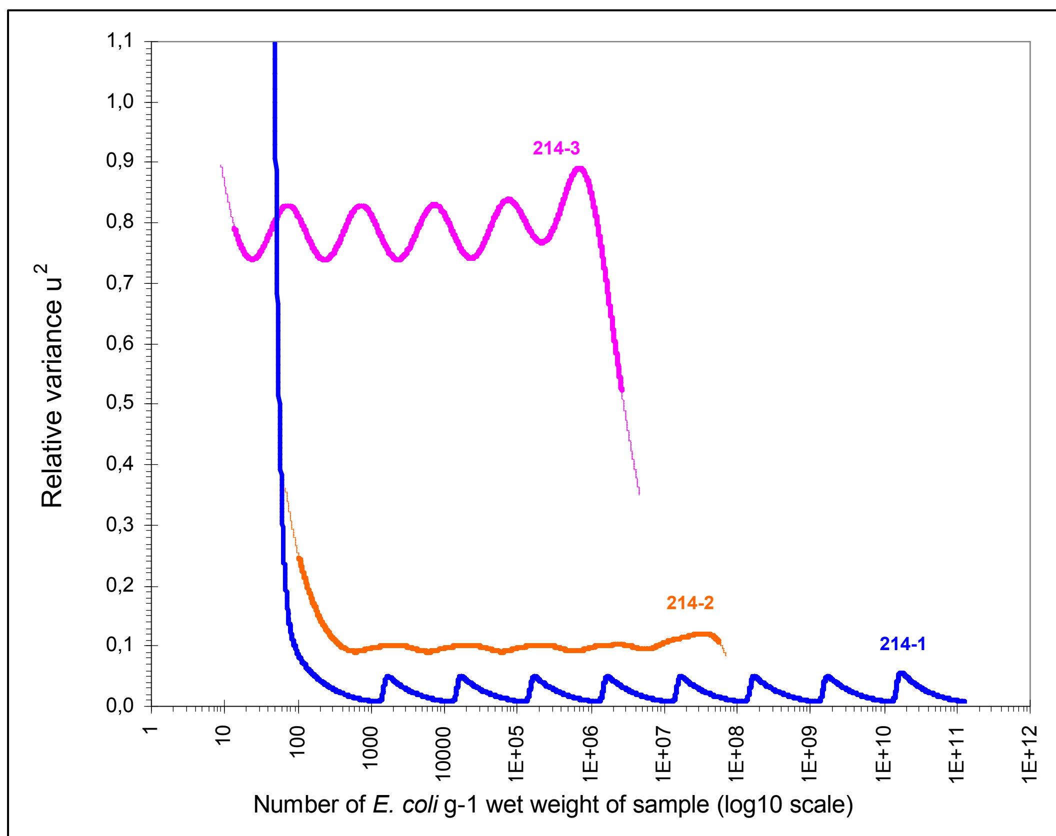
The **Table 11** summarizes the important information about the first assessment of the precision for each of the 3 evaluated methods.

**Table 11**: First assessment of the precision of *E. coli* methods (prEN 15214)

| Method | Limit of detection (5%) | Upper limit of quantification (5%) | Range of quantification | Results of dispersion U² |
|---|---|---|---|---|
| **prEN 15214-1*** | 26.96 | $1.32 \times 10^{11}$ | 9.7 | < 0.05 |
| **prEN 15214-2** | 67.40 | $7.07 \times 10^{7}$ | 6.0 | 0.1 |
| **prEN 15214-3** | 8.99 | $4.65 \times 10^{6}$ | 5.7 | 0.8 |
| **Expression of Results** | *E. coli* per g wet weight | *E. coli* per g wet weight | Log 10 unit | - |

*The assumption of a possible quantification within the range of 1-150 colonies had been chosen to carry out the calculations for the filtration method (prEN 15214-1)

The **Graph 2** represents the relative variance ($u^2$) versus the mean of the 3 draft methods.




**Graph 2** Relative variance ($u^2$) versus the mean of the 3 *E. coli* methods to be evaluated: Filtration method (prEN 15214-1, blue curve); microplate method (prEN 15214-2, orange curve); MPN macromethod (prEN 15214-3, pink curve).

Whatever is the *E. coli* method, the oscillations of the curves represent the change of dilution level. The boundaries of the two MPN methods curves and the left-extremity of the filtration method curve should not be taken into consideration. Indeed, in these areas, the increase or decrease of the relative variance correspond to the lack of variability of the possible results, in other words: a deterioration of uncertainty. Optimal working conditions on these methods are located in the middle part of the curves where the relative variance is quite stable.

Due to the well-known properties of the MPN calculations, the intrinsic uncertainty of the macromethod appears to be higher than the one of the microplate. Actually, the number of inoculations per dilution level plays a major role in the intrinsic variability of the MPN methods: Three tubes are thus inoculated per dilution level for the MPN macromethod, while 16 wells for the microplate method.
Besides, thanks to the number of wells per dilution, it can be noticed that the uncertainty of the microplate method is really close to the uncertainty of the filtration method. Generally, enumerative methods are the ones which show the lower intrinsic dispersion of outcomes (Poisson distribution).

## *5.4 Data and statistics for Salmonella spp. analyses (prEN 15215)*

The results collected following the analysis of the 9 matrices with the 3 different *Salmonella* spp. methods are presented matrix by matrix, method by method in **Annex 27**.
Those data were the base of the statistical calculation, taking into account in a first time the results of the quality controls, to determine the relative performances of the methods and the first assessment of their precision.

### 5.4.1 Quality control

The quality control results of the two quantitative methods (prEN 15215-1 and prEN 15215-2) are presented in **Annex 28** and **Annex 29** respectively. In these **Annexes**, for a given batch of MRS (corresponding to 4 weeks of analysis), a first **Table** gives details of the laboratory results and the obtained z-scores. The laboratory results for the analysis of the LRM and the corresponding z-scores are presented in a second **Table**. A last **Table** shows the estimated inter-laboratory precision calculated for each draft method.

The precision is defined as the closeness of agreement between independent results obtained under stipulated conditions.

According to the statistical planning of the quality control (1 analysis of 2 samples extracted from the same batch in repeatability conditions), the estimated components of precision that were evaluated were:

- A combined variance of the repeatability and the between-flask dispersion called "**r**";
- A combined variance of an intralaboratory Reproducibility and interlaboratory Reprobucibility called "**R**" (several results of a laboratory and several results of several laboratories were available for a given month).

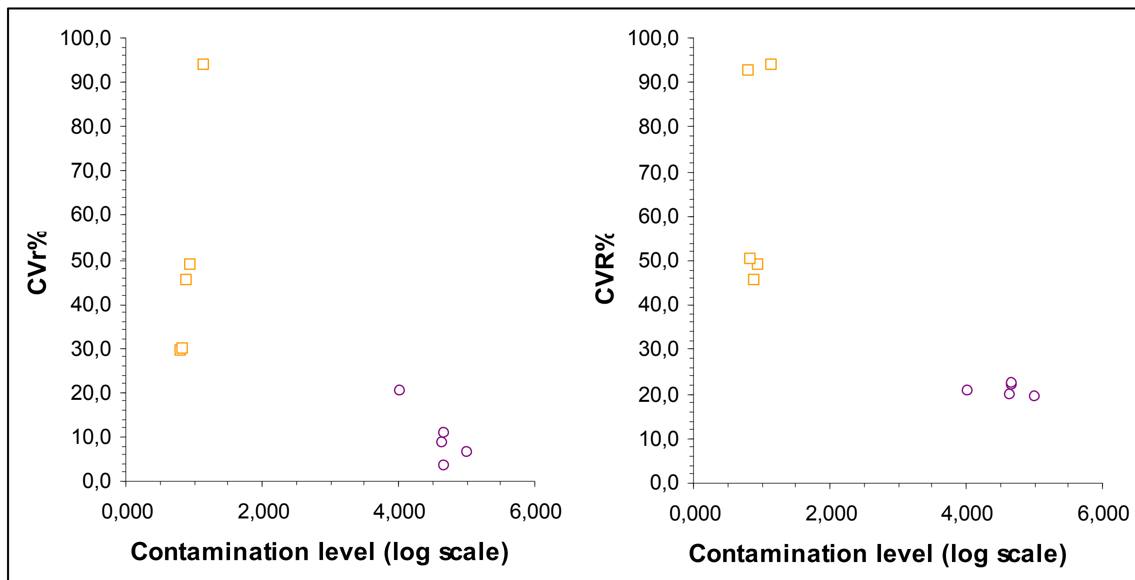
The range of the precision values obtained for each draft method is presented in the synthetic **Table 12**. The presented precision values are the lower value and the upper value observed through the statistical analysis of the available data from the different batches of MRS (different month apart from June). The data processing was carried out after a $\log_{10}$ transformation of data.

**Table 12**: Range of precision values of *Salmonella* spp. methods (prEN 15215) tested on MRS

| Evaluated methods | prEN 15215-1 | prEN 15215-2 | prEN 15215-3 |
|---|---|---|---|
| **Range of observed value of CVr%** (Variance due to repeatability and between-flask dispersion) | 3,5% to 20,5% | 29,5% to 94% | No assessment (qualitative method) |
| **Range of observed value of CVR%** (mixed variance due to intralaboratory and interlaboratory Reprobucibility) | Not significant[(1)] to 22,5% | Not significant[(1)] to 92,5% | No assessment (qualitative method) |

[(1)]The observed variance in Reproducibility conditions was reduced to the observed variance in repeatability conditions (random variation).

The variances values obtained for each *Salmonella* spp. method are presented in the **Graph 3** according to the repeatability conditions and reproducibility conditions.




**Graph 3**: Precision values obtained following the 2 *Salmonella* spp. quantitative methods (prEN 15215) in repeatability conditions (CVr%) and in reproducibility conditions (CVR%) on MRS. Purple circle: prEN 15215-1 - Filtration method; orange square: prEN 15215-2 - MPN macromethod.

The orders of magnitude of the contamination level quantified by the two methods are totally different. This particularity is due to a difference of trueness, i.e. a difference in terms of closeness of the agreement between the average value obtained from a large series of test results and an accepted reference value.
As a consequence, it is not possible to compare the evaluated values of CVr% and CVR%. Indeed, these values are not independent of the contamination level.

### 5.4.2 Relative performance of the *Salmonella* spp. methods (prEN 15215)

The results of the relative performance of the *Salmonella* spp. methods (prEN 15215) are presented matrix by matrix in **Annex 30**.
The **Table 13** summarizes the observed statistical trends

**Table 13**: Statistical trends of relative performance of the two quantitative *Salmonella* spp. methods: Filtration method (prEN 15215-1) & MPN macromethod (prEN 15215-2)

| Matrix | | Relative performance | note |
|---|---|---|---|
| **1:** | **Raw sewage sludge** | 215-1 = 215-2 | - |
| **2:** | **Mesophilic anaerobic digested** | 215-1 > 215-2? | No significant trend with suspect QC data excluded |
| **3:** | **Anaerobic treated biowaste** | **?** | 2 different groups of results: low results for 214-2 include numerous improbable MPN |
| **4:** | **Pelletised air dried sludge** | 215-1 = 215-2 | 2 different groups of results: group of low results for 214-2 include numerous suspect QC data |
| **5:** | **Digested sewage sludge presscake** | 215-1 > 215-2 | low results for 214-2 include numerous suspect QC data |
| **6:** | **Limed stabilised sewage sludge** | No statistical processing | - |
| **7:** | **Composted sewage sludge** | 215-1 = 215-2? | 2 different groups of results (statistical processing on group 1 only - sufficient number of data) |
| **8:** | **Composted green waste** | 215-2 > 215-1? | 2 different groups of results (statistical processing on group 1 only - sufficient number of data) |
| **9:** | **Sludge amended "nutrient weak" soil** | 215-2 > 215-1 | - |

Relative performance: conclusion in agreement with the Wilcoxon test significance. **<** or **>**: 1% significant trend; **?**: only 5% significant trend (not 1%) or doubt due to a particularity; **=**: no significant trend.

On natural or spiked samples, the main comment on the paired comparison of the two quantitative methods is the presence of two different groups of results. This anomaly did not permit to evaluate properly the relative performance of the methods.
Nevertheless, for the MPN macromethod, it can be noticed that some data that belong to the lower group of results appear to be unlikely MPN, i.e. estimation corresponding to a low probability of occurrence (**Annex 27**).
This intrinsic defect of the method is a major drawback because unreliable outcomes were detected for 13% to 37% of the available data per matrix.
Assuming independence of results and random distribution of micro-organisms, an improbable result should not be used as the basis of the microbial concentration estimation. When this kind of result occurs, it is usually an indication that something is wrong with the experimental technique (ex: problem with the dilution experiment, inadequate mixing) or the medium used (ex: overgrowth).

For the presence / absence method, few negative results following the analysis of natural or spiked samples were observed whenever the two other methods gave positive results (**Annex 30**).

The same data processing was carried out with results obtained on the month-MRS batches (**Annex 30**). The **Table 14** summarizes the observed statistical trends for the analysis of MRS.

**Table 14**: Statistical trends of relative performance of the 2 quantitative *Salmonella* spp. methods (prEN 15215-1 & prEN 15215-2) on MRS batches

| MRS batches | Relative performance | Ratio |
|---|---|---|
| **January MRS** | 215-1 **>** 215-2 | 1351,6 |
| **February MRS** | 215-1 **>** 215-2 | 1974,0 |
| **March MRS** | 215-1 **>** 215-2 | 1231,1 |
| **April MRS** | 215-1 **>** 215-2 | 1037,2 |
| **May MRS** | 215-1 **>** 215-2 | 4070,6 |

Relative performance: conclusion in agreement with the Wilcoxon test significance. **<** or **>**: 1% significant trend; **?**: only 5% significant trend (not 1%) or doubt due to a particularity; **=**: no significant trend.

According to the results of the MRS analysis, 13% of the results were very improbable estimates. The assessment of the relative performance of the two quantitative methods could be carried out without any major difficulty (absence of sub-group of results).
A large difference of recovery (trueness) between the two quantitative methods was observed: the filtration method gave higher results with an order of magnitude of 3.0 to 3.6 log than the MPN method.

Only one measurement among the 163 analysis in duplicate was negative for the presence / absence method.

### 5.4.3 First assessment of the precision of the 3 *Salmonella* methods (prEN 15215)

The results of the variability inherent in every *Salmonella* spp. method are presented in **Annex 31**.
The values of relative variance ($u^2$) calculated in repeatability conditions for each tested matrix are plotted in relationship to the intrinsic variability of each method.
These first results should be carefully taken into consideration because few data obtained in repeatability conditions were available (only two replicates per sample were systematically analysed).

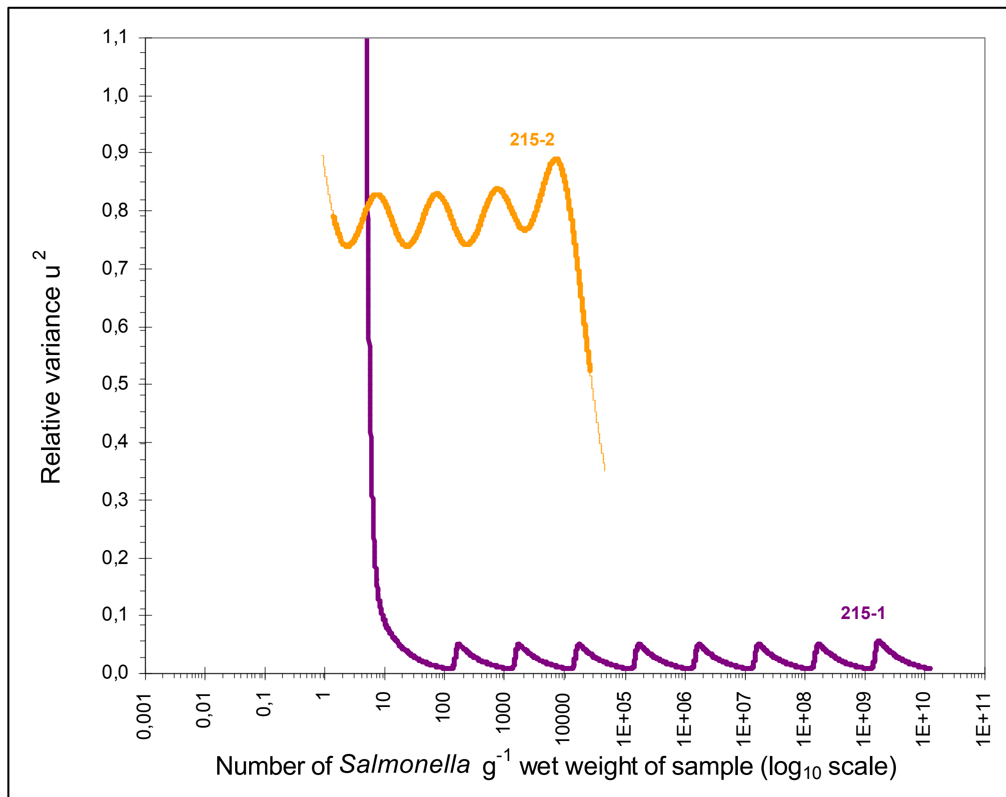
The **Table 15** summarizes the important information about the first assessment of the precision of the quantitative *Salmonella* spp. methods.

**Table 15**: First assessment of the precision of the 2 quantitative *Salmonella* spp. methods (prEN 15215)

| Relative performance | Limit of detection (5%) | Upper limit of quantification (5%) | Range of quantification | Results of dispersion U² |
|---|---|---|---|---|
| **prEN 15215-1** | 2.70 | $1.32 \times 10^{10}$ | 9.7 | < 0.05 |
| **prEN 15215-2** | 0.90 | $4.65 \times 10^{4}$ | 4.7 | 0.8 |
| **Expression of results** | *Salmonella* per g wet weight | *Salmonella* per g wet weight | Log 10 unit | - |

The assumption of a possible quantification within the range of 1 to 150 colonies for the filtration method (prEN 15215-1) had been chosen to carry out the calculations.

The **Graph 4** represents the relative variance ($u^2$) versus the mean of the 2 draft quantitative methods.




**Graph (4)** Relative variance versus the mean of the 2 quantitative *Salmonella* spp. methods: violet curve: relative variance of the filtration method (prEN 15215-1); orange curve: relative variance of the MPN macromethod (prEN 15215-2).

The filtration method showed a reduced intrinsic uncertainty in relation to the Poisson variability.
The MPN macromethod intrinsic uncertainty is highly greater because of the statistical reduced number of inoculation per dilution level.

## 5.5 *Technical observations and proposed amendments*

It was asked to each laboratory participant to report any technical remarks observed during the implementation of each of the 6 methods.
The main observations have been discussed during the 3rd technical meeting in Brussels on the 21st June 2006 where the first results of the Suitability Study were presented and during the 4th technical meeting in Lille on the 5th September 2006 where the statistical analysis results of the Suitability Study were presented. The amendments following the discussions, approved during these both meetings, will be included in the next amended prEN 15214 and prEN 15215 versions (D2/1.6 - November 2006).
The main observations followed by the amendments linked to the discussions are summarized here below, method by method.

### 5.5.1 Harmonisation of the 6 CEN draft standards

- ✓ The sample preparation step is often described in standards as being the addition of an exact volume of a specific diluent to a specify weight of sample to maintain the 1/10 proportion. It was agreed to modify "add **… mL** of diluent" by "add diluent **qsp** …" for an easier practical.

- ✓ Specific diluents used in draft standards prEN 15214-1, 15214-2, 15215-1 and 15215-2 appeared to have the same composition: tryptone salt, MRD (maximal recovery diluent). Peptone is also a synonym.
  The proposed common name to harmonise the methods will be peptone saline solution, as in ISO standards.

- ✓ To be in accordance with the ISO requirements, temperatures and times of incubation will be revised in each of the 6 prEN standards (for example: change (37±1)°C in (36±2)°C, change (48±2) hours in (46±4) hours).

- ✓ Two different compositions of Rambach® agar are given in the standards prEN 15215-1 and prEN 15215-2. The composition will be adapted in order to be equivalent between them. Otherwise a note will be added to let a degree of liberty in the composition of this media to avoid that the laboratories use different forms of the same product to respect the indicated composition in each standard.

- ✓ Each of the two standards prEN 15215-2 and prEN 15215-3 gives its description of typical colonies on XLD. The description will be harmonised between the two methods to avoid confusion for the observer since the colour of typical colonies depend of the features of the strain: $H_2S$+ strains produce black colonies on XLD.

- ✓ By comparing the 3 *E. coli* methods, the results could be different according to the confirmation step followed and consequently according to the features of present *E. coli* strains. The target to be detected will be better defined in each CEN draft standard according to the confirmation step followed, to make easier the choice of the method to apply.

- ✓ The useful of performing both serological and biochemical confirmations was discussed to achieve the confirmation step of prEN 15215-2 and prEN 15215-3. It will be notice in the two standards that a serological test will be performed first. If the result is not relevant, then a biochemical test using for example API 20E will be realised.

- ✓ The fixed lower and upper limits of the range for colonies quantification for prEN 15214-1 and prEN 15215-1 led sometimes to non quantitative results. It will be thus added in the CEN draft standards that these limits can be ignored if no plate in this range can be take into account to obtain a quantitative result.

- ✓ The MPN was determined by the participants using only the 3 digits according to the standards prEN 15214-2, prEN 15214-3 and prEN 15215-2, whereas statistical calculation was based on the full dilution range. The new versions of the standards will mention as usually the use of only 3 digits, the whole number of digits being useful only for precision of the calculation, for statistical studies.

- ✓ The importance of determining the lower and upper limits in addition to the mean value was reminded to take care of the range of quantification. It will be specified in the CEN draft standards that those limits correspond to tolerance limits related to the precision of results for MPN methods. However, it will be advised to keep the mean value as a final result of the analyses.

- ✓ According to the Suitability Study results, the low limit of detection of each standard was revised following the statistical calculation, the high limit of detection depending on the dilution step performed. This low limit will be corrected in each standard.

### 5.5.2 Specific amendments for each of the 6 CEN draft standards

*E. coli Filtration method (prEN 15 214-1)*

- ✓ The colour of presumptive typical *E. coli* colonies should be better defined as being only dark green colonies, in the standard.
  A sentence like "other Gram negative bacteria could be detected with *E. coli*" (for example *Citrobacter*) will be also added.

- ✓ The way to determine the final result was discussed. It will be maintained to test only 2 presumptive colonies for the confirmation step. However, the result of this step should be taken into account in the final result which will be better detailed in the standard.

*E. coli Microplate method (prEN 15 214-2)*

- ✓ The need to confirm more than the fluorescence criteria as for the MPN macromethod was discussed. The percentage of error in terms of false positive was approximately estimated at 1% for the analyses on MRS with the standard, compared with the same procedure applied by adding the indole test confirmation step using Kovacs reagent.
  The indole test will be included as a confirmation step on positive wells during the Validation Study so as to evaluate the percentage of false positive wells found with this method. This additional step will be also described in the new version of the draft standard

to be used for the Validation Study. The number of fluorescent positive wells and fluorescent + indole positive wells will be both registered.

*Salmonella filtration method (prEN 15 215-1)*

- ✓ Reliable results with the MU-Cap test confirmation step were difficult to obtain, as variable intensity and variable colour of the fluorescence were obtained according to analysed samples. But, the change of confirmation step will be a major technical change which can not be processed before the Validation Study to be included in the updated version of the method.
  So, no change of confirmation step will be envisaged for the new version of the standard which will be used for the following of the project.

- ✓ However, a requirement will be added in the standard following the observations of partners: letting the Mu-Cap dry on the plates few minutes in order to make easier the fluorescence reading.

*Salmonella MPN method (prEN 15 215-2)*

- ✓ The confirmation of 2 colonies from each of both media will be still required in the new version of the draft standard, so at last 4 colonies to be tested to confirm the MPN result.

- ✓ The final result was sometimes difficult to report due to the fact that two different media are used in the standard. It will be then mentioned that the observer should only keep the highest characteristic number (CN) obtained from the two media to determine the MPN value.

*For the Salmonella spp. Presence/Absence method (prEN 15 215-3)*

- ✓ The standard should specify the number of colonies to test for confirmation. At least 2 presumptive colonies should be tested but if results are negative as many colonies as possible should be tested to confirm the presence of *Salmonella* spp.

The six draft standards will be revised in the light of the Suitability Study feedbacks and discussions of the Technical Meeting 3, and according to the analytical results and the conclusions of the discussion during the Technical Meeting 4 as well.
Updated prEN HOR standards will be completed by the end of November 2006 before being proposed for CEN consultation as first draft CEN standards (Deliverable D2/1.6).
A mention will be added to each procedure to precise that the analytical procedure will be followed as described in the draft until the conclusions of the full Validation trial are obtained.

# 6 Conclusion

General conclusions was favourable, a number of critical issues on the 6 prEN 15214 *E. coli* and prEN 15215 *Salmonella* spp. methods were resolved following the Suitability Study thanks to the well documented data reports and the discussions between the participants. Participants have also reported the huge work performed for the realisation of the Suitability Study.

The Suitability Study was the opportunity to test the different draft procedures on complex matrices by the 8 partners who will participate to the Validation Study of 2007. It led to determine relative performance and first assessment of the precision of the 6 methods.

The results of the Suitability Study were presented and discussed with the participants during two technical meetings (TM3, in Brussels on 21st June 2006, and TM4, in Lille on 5th September 2006).
The point on the amendments to be made on the draft standards was also discussed with the partners thanks to the feedbacks received from the Suitability Study, the Technical Meeting 3 the statistical results and the Technical Meeting 4 as well. A number of critical issues on the 6 prEN *E. coli* and *Salmonella* spp. methods were resolved, with only minor changes to be included in the updated versions of the methods, so as to keep the first statistical data which will be used to validate results of the next trial.

Updated standards as horizontal-prEN standards will be completed by November 2006 (D 2/1.6) and will be proposed for CEN consultation before being evaluated during the Validation Study.

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