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

Validation Study report

E. coli and *Salmonella* spp.
Inter-laboratory study

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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 Validation Study report on the 6 Horizontal Hygiene draft standards for *E. coli* and *Salmonella* spp. to be monitored in EU in sludges, treated biowastes and organic fertilisers. It describes the results of the Validation Study corresponding to the last step to achieve in the frame of the Horizontal-Hygiene project and realised by fourteen European participating laboratories from May to July 2007.

The main objectives of the Validation Study were:

- ✓ Applying of the 6 Horizontal-Hygiene draft standard methods (*E. coli* and *Salmonella* spp.) (Version of April 2007, **Annexes 1-6**) in the 14 participating laboratories involved in the validation inter-laboratory trial ;

- ✓ Complete statistical evaluation of the performances of the 6 methods in terms of accuracy of measurement;

- ✓ Check the fit for purpose of those methods on the 7 selected matrices representing sludge, treated biowastes and organic fertilisers.

Accuracy of measurement methods is divided into two components according to ISO 5725-1: trueness and precision.
In the specific context of the study, the first component must be interpreted in terms of relative trueness of the methods, i. e. relative bias between draft methods.
As for the relative trueness of the draft methods, the precision can be assessed using its two main components: repeatability and reproducibility.

Thanks to the results of the Validation Study, the 6 Horizontal-Hygiene draft standards will be amended while including some improvement issues from the results obtained and from the exchanges and decisions taken during two technical meetings : Technical Meeting 6 (2nd October 2007).
Then, the new versions of the Horizontal-Hygiene draft standards will be proposed for the second and last CEN consultation to progress towards the final step of the standardisation process.

3 Participating laboratories

The fourteen selected participating laboratories have been chosen for their expertise in sludges, soils and/or treated biowastes microbiology. Most of them are Reference European laboratories (3 laboratories being routine laboratories accredited ISO 17025).

Seven European laboratories, all involved in the HORIZONTAL-HYG project as partners participated to the Validation Study, in addition to the Suitability Study (the previous step). They represented 6 European countries, including two new European countries (Estonia and Hungary) (**Table 1**).

Table 1: The 7 European partner laboratories involved in the Validation Study

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

Seven additional European laboratories were also implied in the Validation Study (**Table 2**). They represented 7 additional European countries.

Table 2: The 7 additional European participating laboratories involved in the Validation Study

Laboratories	Town	Country
TEI - Central Public Health Laboratory	Athens	Greece
HSE Public Analyst's Laboratory	Dublin	Ireland
Instituto Nacional de Saude Dr Ricardo Jorge	Lisbon	Portugal
Laborex 2000 SRL	Bucharest	Romania
Institute of Public Health	Prague	Czech Republic
Environmental Centre of Helsinki	Helsinki	Finland
LSPG	San Sebastian	Spain

The 14 participating laboratories were all already trained for the methods to be tested during the Training Session of July 2005.

The technical preparation and organisation of the Validation Study were in charge of IPL, helped by the 3 expert partners ALcontrol, Anjou and UHOH, each involved in the preparation of two of the 6 standards.

Each of the 7 partners was in charge of preparing the sub-samples to be analysed by all the 14 participating laboratories.

The whole coordination of the Validation Study was in charge of IPL.
The data processing was supported by IPL and AGLAE, sub-contractant of IPL, in charge of the statistical analysis.

4 Materials and methods

A 3 months European inter-laboratory Validation Study was organised from May 2007 to July 2007.

4.1 Laboratory equipment and consumables

Each participating laboratory was in charge of preparing material and ordering consumables suitable to the implementation of the Validation Study. A list of materials and consumables corresponding to each method to be tested for validation was prepared by expert partners (**Annexes 7** & **8**).

Equipment (balance, homogeniser, incubators, filtration devices, central vacuum pump, freezers and refrigerators) and consumables (culture media, diluents, filters, …) used during the Validation Study should have been tested before the Validation Study for quality control.

4.2 Matrices and samples to be tested

The Validation Study work was based on a matrix-based approach. Seven matrices were analysed to evaluate the fit for purpose of the 6 selected methods (three methods per parameter) (**Table 3**).

Table 3: Selected matrices and their repartition between the partners

Matrix	Short name	Partner in charge of the preparation
Mesophilic anaerobic digested (MAD) sewage sludge	MAD	Partner 7
Anaerobic treated biowaste	ATB	Partner 6
Pelletised air-dried sludge	PADS	Partner 4
Digested sewage sludge presscake	DSSP	Partner 2
Composted sewage sludge	CSS	Partner 5
Composted green waste	CGW	Partner 1
Composted biowaste	CBW	Partner 3

The seven matrices were distributed between the 7 partners participating to the Validation Study, in charge of their preparation.

For feasibility reason and to achieve the statistical objectives, all the Validation Study was realised with analysis of spiked samples only, to be sure of the availability of positive samples of each matrix.

Two batches of the same matrix were spiked and prepared by a partner before being sent as spiked sub-samples to the 14 participating laboratories.
Each set of samples was analysed 3 times by every laboratory, with each of the 3 methods per parameter, at the same time, so that comparative data were obtained.

4.2.1 Sampling and sample handling

According to the repartition of the matrices, each partner with direct routine access to such kind of samples was in charge of sampling and handling the matrix-batch.

The requirements sheet for sampling and sample handling had circulated during the previous Suitability Study, to determine the best conditions of sampling and sample handling to be followed. Thus, the partners already involved in this study were well aware of them (Suitability Study report).
The requirements were based on the "sample handling protocols for sludges and treated biowaste for microbiological analysis" desk study realised in the frame of the Horizontal-hygiene project by the UREAD expert partner (work-package WP1).

4.2.2 Preparation of the spiked sub-samples

The preparation of the spiked sub-samples was done by each of the 7 partners in charge of providing the 14 participating labs with the spiked sub-samples.

The spiking has been agreed to ensure always positive results indispensable for the statistical data processing thanks to a determined bacteria concentration in the sub-samples sufficient to be detected.

The spiking protocol was based on the initial proposal of the UHOH expert partner during the Suitability Study and revised to ensure homogeneity and stability of the spiked sub-samples from all matrices. A procedure summarising the most important requirements had circulated before the Validation Study to ensure the well preparation of the batches by the partner laboratories (**Annex 9**).

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. The features of both reference materials are reported in **Table 4**.

Table 4: Reference materials used as spiking strains

Spiking strain	Collection reference	Supplier	Quantity/pastilles
Escherichia coli	RIVM WR1	BioReference Pastilles (IPL, France)	1.6×10^7
Salmonella Senftenberg	NCTC 9599	BioReference Pastilles (IPL, France)	1.4×10^6

The microbiological reference materials supplied by IPL as BioReferences pastilles were delivered to the 7 partners before the start of the Validation Study in order to make preliminary trials of spiking and to evaluate the homogeneity and stability of the prepared sub-samples.

4.2.3 Homogeneity and stability of the sub-samples

The 7 partners were also in charge of checking the homogeneity and stability to transport and storage conditions of the sub-samples they had to prepare, in order to avoid any transport/storage effect that could affect the analytical results of the Validation Study.
A time of preliminary trials was scheduled to determine the best conditions of sub-samples preparation and to assess their homogeneity and their stability to transport and storage up to 2 weeks. The 2 weeks simulated the time between the preparation of the sub-samples and the analyses to be done by the participating laboratories.

The indications to be followed for the realisation of those preliminary trials were given in the same document as the spiking protocol (**Annex 9**). They concerned:

- a procedure to prepare an homogeneous spiked batch;
- the requirements to evaluate the homogeneity of the spiked sub-samples;
- the requirements to determine the stability to transport and storage of the spiked sub-samples.

The partners were free to organise their preliminary trials before the start of the Validation Study as long as they respect the timetable of the sub-samples delivery to the 14 participating laboratories.

The homogenisation procedure should be optimised by the partner himself, according to the matrix features. The homogenisation step was applied on about 8.5 Kg of a spiked batch.
The contamination level of the prepared and spiked sub-samples was evaluated by the partner with the *E. coli* microtiter plate method and the *Salmonella* spp. filtration method analyses to check that it is adapted to the quantification range of the methods to be applied. It was expected to be within the range of 10^2-10^5 for both parameters.

A new batch was prepared according to the conditions optimised during the preliminary tests (spiking and homogenisation steps) in order to have sufficient homogeneous sub-samples to be delivered to the 14 participating laboratories. The instructions about the preparation of the sub-samples from the homogenised spiked batch were attached in **Annex 10**. They were sent to the 7 partners before the Validation Study.

4.2.4 Sub-samples packaging and sending

The shipments condition of each sub-samples set were arranged by the partners themselves. They were established following preliminary trials so as to maintain a temperature of (5 ± 3)°C during approximately 48h, the time to reach all destinations: Czech Republic, England, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Portugal, Romania and Spain).
Most of time, the two batches were sent at two different times, according to the timetable agreed by the partners.

A set of instructions and advices was delivered to the 7 partners at the start of the Validation Study to help them in the preparation of the parcels (**Annex 10**). The best parcel organisation was defined to:

- provide 6 sub-samples, containing the sufficient quantity of the spiked and homogenised matrix-batch to perform either the *E. coli* analyses or the *Salmonella* spp. analyses, three times;
- maintain the temperature during the holding time (using coolant packs);
- register the range of temperature during the transport with a temperature logger.

The partners were free to program their sending as long as it was during the indicated period of the Validation Study.

4.3 Validation Study work programme

The participating laboratories received a set of instructions before the start of the Validation Study to help them in the implementation of the technical work.

The work programme was set up from the repartition of matrices between partners and their availability to prepare the sub-samples. Constraints of national public holidays were also taking into account in the Validation Study timetable to be sure that each batch would be analysed by most participating laboratories.
The Validation Study timetable was agreed by the 7 partners to organise the sending of the matrices sub-samples (**Annex 11**). The defined timetable ensured that each week, one or two sets of sub-samples was (were) supplied to the 14 participants who all had to start the analyses on the first day of the week following the sending.

By following the instructions and respecting the timetable, fourteen set of analyses in total were expected to be done by each of the 14 participating laboratories between May and July 2007.

The technical work plan was well detailed in a document to clarify the work to be organised and realised by each participant of the validation study (**Annex 12**).
A list of all participating laboratories was joined to identify the role of each laboratory.

4.3.1 Methods to be applied

The 6 methods to be applied during the Validation Study are presented in **Table 5** for the 3 *E. coli* methods and in **Table 6** for the 3 *Salmonella* spp. methods.

Table 5: Methods applied for the detection and enumeration of *E. coli*

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
CEN Reference	prEN 15214-1	prEN 15214-2	prEN 15214-3
Short Title	Filtration method	Microtiter plate method	MPN macromethod
Validation Study Version	Amended version of Apr07	Amended version of Apr07	Amended version of Apr07

Table 6: Methods applied for the detection and enumeration of *Salmonella* spp.

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
CEN Reference	prEN 15215-1	prEN 15215-2	prEN 15215-3
Short Title	Filtration method	MPN macromethod	Presence/Absence method
Validation Study Version	Amended version of Apr07	Amended version of Apr07	Amended version of Apr07

The 6 applied protocols corresponded to the April 2007 Horizontal-Hygiene amended versions of the prEN standards edited in December 2004. These amended versions included editorial and technical improvements performed by the experts, since the start of the project. The April 2007 amended versions of the prEN 15 214-1, prEN 15 214-2, prEN 15 214-3 (*E. coli* methods) and prEN 15 215-1, prEN 15 215-2, prEN 15 215-3 (*Salmonella* spp. methods) are presented respectively in **Annexes 1**, **2**, **3, 4, 5** and **6**, with a synthetic scheme of the whole protocol.

4.3.2 Organisation of the Validation Study technical work

A work schedule detailed the technical Validation Study work to be achieved by the participating laboratories day by day, method by method and step by step (**Annex 13**).

A typical Validation Study week corresponded to the analysis of one set of samples:

- by starting the *Salmonella* spp. analyses on Monday (first steps of the 3 *Salmonella* spp. methods to be validated);
- by starting the *E. coli* analyses on Tuesday (first steps of the 3 *E. coli* methods to be validated, in parallel with the following of *Salmonella* spp. analyses).

The next and last steps of the 6 methods were achieved on the following days, to be all ended on Friday. The start of analyses of the whole set of sub-samples and corresponding to one parameter had to be performed on the same day and not spread over different days out.
In addition to the set of analyses, the partner in charge of delivering the spiked sub-samples should repeat the analysis on 7 sub-samples more, in parallel, to check the homogeneity of the provided spiked sub-samples, to avoid any bias.

Most of time, one set of sub-samples should be analysed per week. But, two sets of sub-samples were delivered on three different weeks, to be analysed at the same time so as to shorten the Validation Study work period.

4.4 Analyses of Validation Study results

The technical data and results of all analyses were reported on excel data sheets by the laboratories and returned by e-mail to IPL, technical coordinator, as soon as possible.

4.4.1 Data reporting

The fourteen participating laboratory were in charge of recording analytical results and technical data of each set of analyses, obtained with each of the 6 methods, on standard excel data sheets (**Annex 14**).

In addition, the seven partners in charge of preparing and sending the spiked sub-samples should report:

- results of their preliminary trials to evaluate the homogeneity and stability of the prepared and spiked sub-samples (**Annex 15**);
- results of the analyses to evaluate the homogeneity of the spiked sub-samples to be analysed by the 14 participating laboratories and conditions of the sub-samples delivery to the 14 participating laboratories (**Annex 16**).

4.4.2 Screening of analytical and technical results

During the validation Study, the 14 participating laboratories sent a completed reporting form (in a relevant Excel sheets) of analytical results and technical data method by method, per set of analyses, by e-mail to IPL, within 2 weeks following the analyses.

In order to avoid any error in data handling and calculation, results were collected and checked for completeness by IPL with each participant before sending a final version of collected data to AGLAE in charge of the statistical data processing.

IPL was also in charge of screening all technical data with the delivered procedures. Deviations from the procedure criteria were observed (medium, incubation time, and incubation temperature). Any doubtful results were marked or discarded.

4.4.3 Statistical data processing

The protocol used to carry out the analysis of the Validation Study was based on the statistical requirements of the assessment of accuracy.
The chosen experimental design stressed on the use of an appropriate number of replicates in order to assess properly the repeatability of the draft Horizontal-Hygiene standards; three repeated measurements per laboratory were considered as a suitable statistical design.
The participation of 14 laboratories in the interlaboratory testing was considered as a sufficient panel to enable the assessment of reproducibility.
As all participants analysed each sample with the 3 draft Horizontal-Hygiene methods per parameter, it was statistically possible to assess the relative bias of the methods.

All applied statistical terms are defined in **Annex 17**.
The procedure used to assess the accuracy of the draft standards methods was detailed in **Annex 18**. The main steps to be achieved were:

Assessment of Precision - for a given batch of a given matrix:

1. Calculated results from the collected intermediate values were used;
2. Result per replicate were expressed on a $\log_{10}$ scale;
3. A consensus value per method was calculated using a Log-Normal fitting model and statistical tests of detection of outliers;
4. An Analysis of Variance (ISO Guide 35) allowed us to distinctly evaluate the variability due to the mixed effect of sub-sampling and between-flask variance (assimilated to repeatability) and the variability due to Reproducibility.

Assessment of relative trueness – global assessment through all matrices:

1. The average results of the methods (consensus values) were plotted on a log scale;
2. The methods relative trueness was determined using paired mean comparisons;
3. 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;
4. A nonparametric test (Wilcoxon test - paired signed rank test) was used to determine whether the trend was statistically significant.

5 Validation study results

The 14 participants were able to perform the amount of work scheduled during the Validation Study 3-months (sample preparation, sample analysis and data sheets reporting).
The sampling data, the technical data and the results of the analysed samples (raw data) obtained by the participants are all annexed to this report.

5.1 Sub-samples and parcels data

The data recorded by the 7 partners in the sending report dealt with the sub-samples preparation, conditions of handling the prepared sub-samples to the 14 laboratories and of their analyses.

The features of the 14 matrix-batches prepared before being delivered as spiked sub-samples to the 14 participating laboratories are assembled in **Annex 19**.
Those data were collected for traceability and informative purpose, each partner being in charge of the preparation of two spiked batches sufficiently homogeneous and stable of the same matrix, taking into account the specificity of the matrix to adjust the spiking and homogenisation conditions.

The delivery conditions (parcels departure, receipt, temperatures recording) of the sub-samples sent by the 7 partners to the 14 participating laboratories are summarized in **Annex 20**. Information about the date of sub-samples analyses is also mentioned to identify any additional delay in the work schedule and to identify the participating laboratories.

5.2 Technical data related to the Validation analyses

All technical data registered by the participants are presented in **Annex 21**.
They were checked with the provided procedures. Deviations were most of time 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. 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 analysis

The results collected following the analysis of the 7 matrices with the 3 different *E. coli* methods in interlaboratory conditions are presented matrix by matrix, method by method in **Annex 22**.
Those data were the base of the statistical calculation, to determine the precision of the results of each method and the first assessment of their precision.

5.3.1 Precision of the results of the applied *E. coli* methods in interlaboratory conditions

5.3.1.1 Expression of repeatability and reproducibility

The values of repeatability and reproducibility are expressed in terms of maximal difference between two independent measurements on log scale, with a confidence level of 95%:

Example Assuming r_1 and r_2 two independent measurements observed for a given method in repeatability conditions with $r_1 > r_2$:

$$\log(r_1) - \log(r_2) \leq 0.9 \quad \text{(95\% of the cases)}$$

Another expression of repeatability and reproducibility is the maximal ratio between two independent measurements on natural scale (number of germs), with a confidence level of 95%:

Example $\log(r_1) - \log(r_2) \leq 0.9$ (95% of the cases)

then $r_1/r_2 \leq 10^{0.9}$

then $r_1/r_2 \leq 7.9$

thus r_1 is significantly higher than r_2 if $r_1/r_2 > 7.9$

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

The statistical results of the evaluation of the *E. coli* filtration method precision in term of repeatability and reproducibility of each matrix batch analysed by the 14 laboratories are presented in **Table 7**.

Table 7: Precision values of the *E. coli* filtration method observed during the validation study

Matrix – Batch	**Overall mean**	**Repeatability** (log scale)	**Repeatability** (ratio)	**Reproducibility** (log scale)	**Reproducibility** (ratio)
MAD 1	< 26.96 (1)	-	-	-	-
MAD 2	< 26.96 (1)	-	-	-	-
ATB 1	1 784 260	0.448	2.8	1.774	59.4
ATB 2	2 898 637	0.545	3.5	1.857	72.0
PADS 1	< 26.96 (1)	-	-	-	-
PADS 2	385 222	0.854	7.1	4.469	29 465.7
DSSP 1	6 632	0.508	3.2	3.875	7 499.5
DSSP 2	3 651	0.609	4.1	2.817	655.8
CSS 1	94 944 250	1.075	11.9	1.541	34.8
CSS 2	968 981	0.773	5.9	1.945	88.1
CGW 1	824 211	0.558	3.6	1.713	51.7
CGW 2	433 007	0.406	2.5	1.509	32.3
CBW 1	7 883 (2)	2.420 (2)	262.7 (2)	2.420 (2)	262.7 (2)
CBW 2	16 967 925	0.634	4.3	1.527	33.6
Expression of results	*g wet weight*	*Maximal difference between 2 independent measurements on log scale (95% of the cases)*	*Maximal ratio between 2 independent measurements on natural scale (95% of the cases)*	*Maximal difference between 2 independent measurements on log scale (95% of the cases)*	*Maximal ratio between 2 independent measurements on natural scale (95% of the cases)*

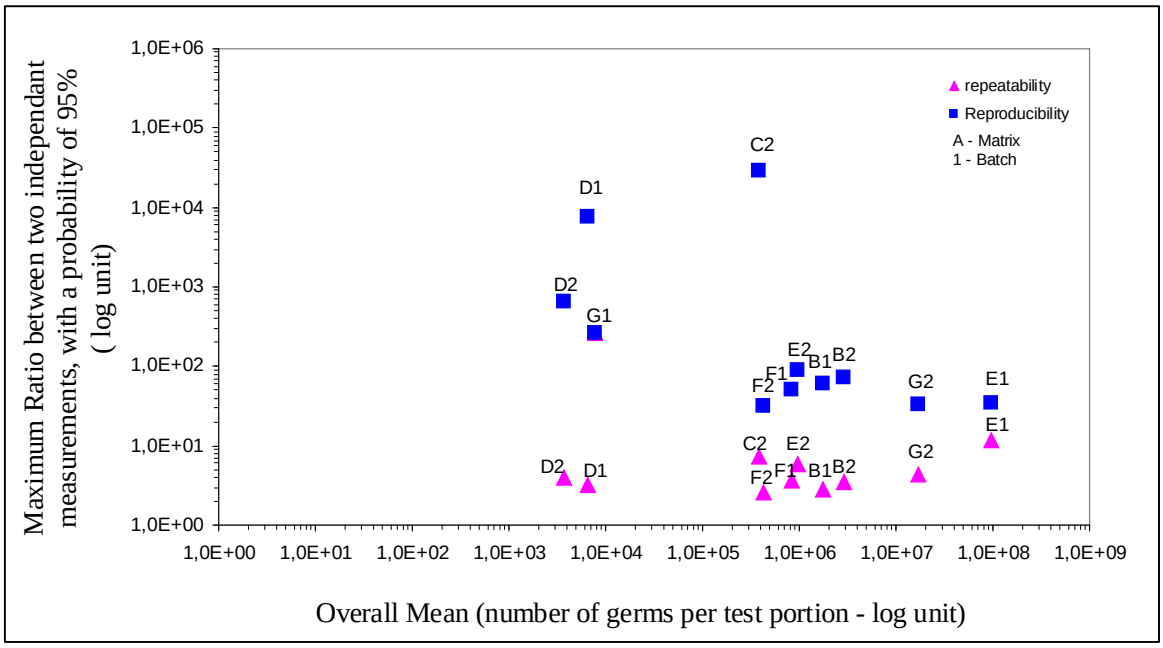
(1) Theoretical limit of detection with a probability of 95% calculated for the *E. coli* filtration method.
(2) Few data were finally available for data processing. The observed variance was only random variation (no significant laboratory bias). Estimation to be considered carefully.

In the experimental conditions of the interlaboratory testing, the repeatability and reproducibility could not be determined for 3 batches (MAD matrix-batches 1 and 2; PADS matrix-batch 1) due to the recording of results below the theoretical limit of detection of the *E. coli* filtration method (theoretical limit of detection: <26.96 cfu/g ww).
The estimation of repeatability and reproducibility of the CBW matrix-batch 1 should be considered with caution, since only few results were in the quantification range.

The observed repeatability of the *E. coli* filtration method was between 2.5 and 12 in term of maximal ratio (0.4 and 1.1 log in terms of maximal difference between log results), registered respectively for the CGW matrix-batch 2 and the CSS matrix-batch 1.

The observed Reproducibility of this method was between 32 and 656 in term of maximal ratio (1.5 and 2.8 log of maximal difference between log results), corresponding to the CGW matrix-batch 2 and DSSP matrix-batch 2. The observed Reproducibility depended on the microbial load.

The precision values of the *E. coli* filtration method calculated in repeatability and reproducibility conditions for each tested matrix are plotted in **Graph 1**.



Graph 1: Precision values obtained in repeatability conditions (pink triangle) and in reproducibility conditions (blue square) for the *E. coli* filtration method.

According to the **Graph 1**, the two Reproducibility results observed for DSSP matrix-batch 1 (D1) and PADS matrix-batch 2 (C2) were located out of the global pattern designed. These suspect results of Reproducibility were not taken into consideration in the global conclusion about the method precision.

5.3.1.3 *E. coli* microtiter plate method (prEN 15 214-2)

The **Table 8** summarizes matrix-batch by matrix-batch the statistical results of the evaluation of the *E. coli* microtiter plate method precision in term of repeatability and reproducibility.

Table 8: Precision values of the *E. coli* microtiter plate method observed during the validation study

Matrix - batch	**Overall mean**	**Repeatability** (log scale)	**Repeatability** (ratio)	**Reproducibility** (log scale)	**Reproducibility** (ratio)
MAD 1	< 67.40 [(1)]	-	-	-	-
MAD 2	< 67.40 [(1)]	-	-	-	-
ATB 1	1 378 339	0.377	2.4	1.019	10.5
ATB 2	2 470 799	0.721	5.3	1.580	38.1
PADS 1	97	1.397	25.0	1.875	74.9
PADS 2	724 659	1.023	10.5	4.749	56 115.6
DSSP 1	6 205	1.377	23.8	4.572	37 364.2
DSSP 2	2 099	0.645	4.4	3.674	4 718.9
CSS 1	30 307 023	1.075	11.9	1.075	11.9
CSS 2	2 036 471	0.855	7.2	1.332	21.5
CGW 1	1 809 954	0.651	4.5	0.651	4.5
CGW 2	396 173	0.618	4.2	1.042	11.0
CBW 1	16 868 [(2)]	3.270 [(2)]	1 863.6 [(2)]	3.270 [(2)]	1 863.6 [(2)]
CBW 2	14 915 052	0.545	3.5	1.257	18.1
Expression of results	*g wet weight*	*Maximal difference between 2 independent measurements on log scale (95% of the cases)*	*Maximal ratio between 2 independent measurements on natural scale (95% of the cases)*	*Maximal difference between 2 independent measurements on log scale (95% of the cases)*	*Maximal ratio between 2 independent measurements on natural scale (95% of the cases)*

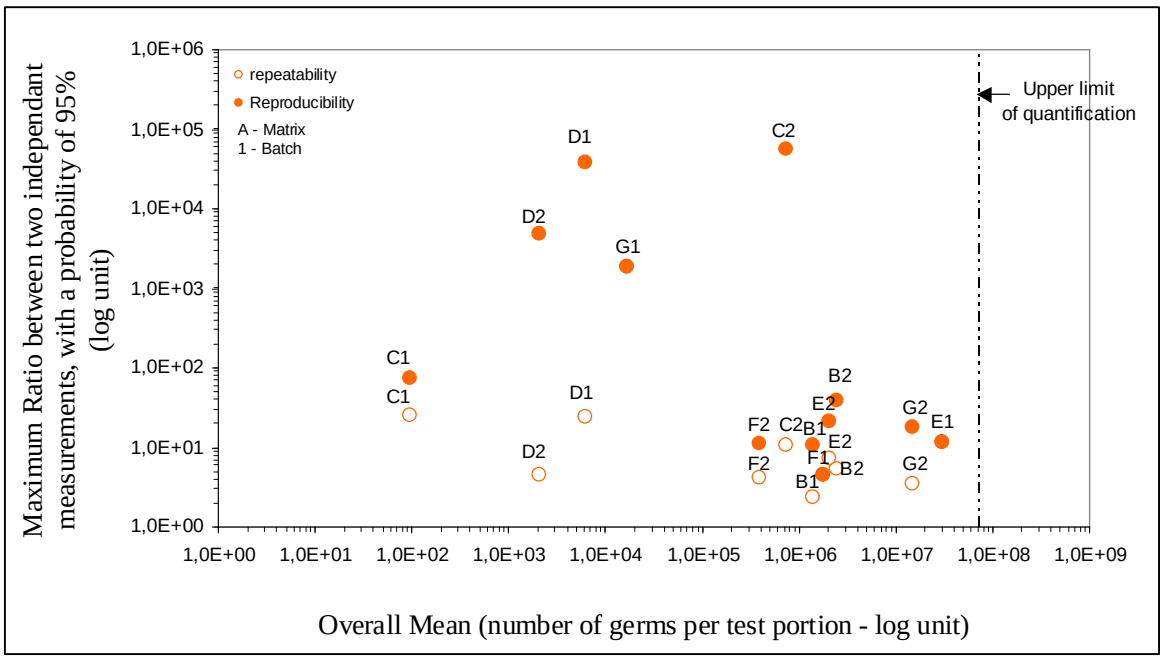
[(1)] Theoretical limit of detection with a probability of 95% calculated for the *E. coli* microtiter plate method.
[(2)] Few data were finally available for data processing. The observed variance was only random variation (no significant laboratory bias). Estimation to be considered carefully.

None repeatability and reproducibility values were calculated for the MAD matrix-batches 1 and 2 because all registered data were below the theoretical limit of detection of the *E. coli* microtiter plate method (theoretical limit of detection: <67.4 MPN/g ww).
Only few values in the quantification range were registered for the CBW matrix-batch 1, to estimate the repeatability and reproducibility that should in consequence be taken into account with caution.

The maximal ratio observed for the *E. coli* microtiter plate method repeatability was between 2.4 and 25 (0.4 and 1.4 log in terms of maximal difference between log results) registered respectively for the ATB and PADS matrices-batches 1.

The observed Reproducibility of this method varied from 4.5 to 4 719 in term of maximal ratio (maximal difference between log results: 0.7 and 3.7 log), data respectively registered with the DSSP matrix-batch 2 and the CGW matrix-batch 1. The observed Reproducibility depended on the microbial load.

The precision values obtained for each tested matrix-batch with the *E. coli* microtiter plate method are presented in **Graph 2,** according to repeatability and reproducibility conditions.



Graph 2: Precision values obtained in repeatability conditions (white circle) and in reproducibility conditions (orange circle) for the *E. coli* microtiter plate method.

According to **Graph 2** and as for the *E. coli* filtration method, the two Reproducibility results observed for DSSP matrix-batch 1 (D1) and PADS matrix-batch 2 (C2) were located out of the global pattern observed. These suspect results of Reproducibility were not taken into consideration in the global conclusion about the method precision.

5.3.1.4 *E. coli* MPN macromethod (prEN 15 214-3)

The statistical results of the evaluation of the *E. coli* MPN macromethod precision in term of repeatability and reproducibility are summarised matrix-batch by matrix-batch in **Table 9**.

Table 9: Precision values of the *E. coli* MPN macromethod observed during the validation study

Matrix - batch	Overall mean	Repeatability (log scale)	Repeatability (ratio)	Reproducibility (log scale)	Reproducibility (ratio)
MAD 1	< 8.99 (1)	-	-	-	-
MAD 2	< 8.99 (1)	-	-	-	-
ATB 1	832 837	0.847	7.0	1.410	25.7
ATB 2	1 547 828	1.033	10.8	1.791	61.9
PADS 1	< 8.99 (1)	-	-	-	-
PADS 2	394 368	0.963	9.2	3.420	2 632.7
DSSP 1	3 898	0.985	9.7	4.867	73 661.6
DSSP 2	1 288	1.304	20.1	3.545	3 505.1
CSS 1	> 4.65 x 10^6 (2)	-	-	-	-
CSS 2	833 659	1.273	18.8	1.931	85.3
CGW 1	818474	1.126	13.4	1.957	90.5
CGW 2	193497	0.930	8.5	1.696	49.6
CBW 1	1531 (3)	3.513 (3)	3 255.0 (3)	3.513 (3)	3 255.0 (3)
CBW 2	> 4.65 x 10^6 (2)	-	-	-	-
Expression of results	*g wet weight*	*Maximal difference between 2 independent measurements on log scale (95% of the cases)*	*Maximal ratio between 2 independent measurements on natural scale (95% of the cases)*	*Maximal difference between 2 independent measurements on log scale (95% of the cases)*	*Maximal ratio between 2 independent measurements on natural scale (95% of the cases)*

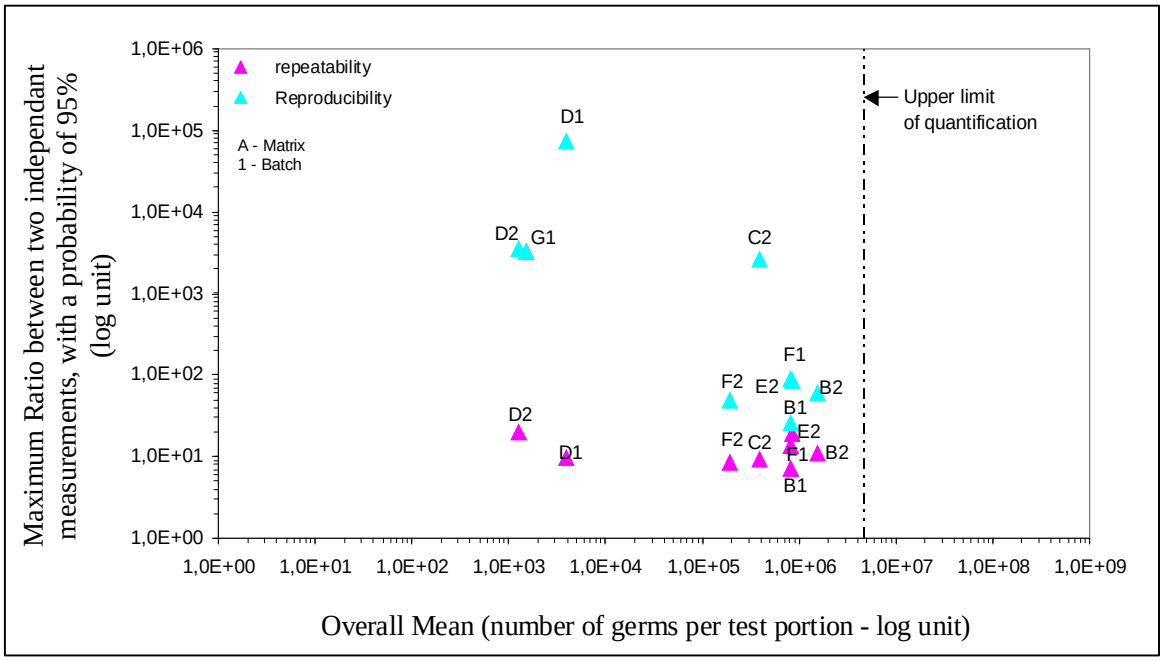
(1) Theoretical limit of detection with a probability of 95% calculated for the *E. coli* MPN macromethod.
(2) Theoretical upper limit of quantification with a probability of 95% calculated for the *E. coli* MPN macromethod.
(3) Few data were finally available for data processing. The observed variance was only random variation (no significant laboratory bias). Estimation to be considered carefully.

All data registered for the two MAD matrix-batches and for the PADS matrix-batch 1 were lower than the theoretical limit of detection of the *E. coli* MPN macromethod (theoretical limit of detection : <8.99 cfu/g ww).
The data sets of two matrices-batches were beyond the theoretical upper limit of quantification of the *E. coli* MPN macromethod (theoretical upper limit of quantification: >4.65 10^6 cfu/g ww): CSS matrix-batch 1 and CBW matrix-batch 2.
The calculation of the repeatability and reproducibility values was impossible for those five matrices-batches.

The maximal ratio of the observed *E. coli* MPN macromethod repeatability was between 7 and 20 (respectively 0.8 and 1.3 log in term of maximal difference between log results) corresponding to ATB matrix-batch 1 and DSSP matrix-batch 2.

The observed *E. coli* MPN macromethod Reproducibility was between 26 and 3 505 in terms of maximal ratio (1.4 and 3.5 log in terms of maximal difference between log results), respectively for ATB matrix-batch 1 and DSSP matrix-batch 2. The Reproducibility values depended on the microbial load.

The precision values corresponding to each matrix-batch analysed with the *E. coli* MPN macromethod are presented in **Graph 3** according to repeatability and reproducibility conditions.



Graph 3: Precision values obtained in repeatability conditions (pink triangle) and in reproducibility conditions (blue triangle) for the *E. coli* MPN macromethod.

According to **Graph 3** and as for the two previous methods, the two Reproducibility results observed for DSSP matrix-batch 1 (D1) and PADS matrix-batch 2 (C2) were not taken into consideration in the global conclusion about the method precision due to their location outside the global pattern observed.

5.3.1.5 Conclusions: precision of *E. coli* methods

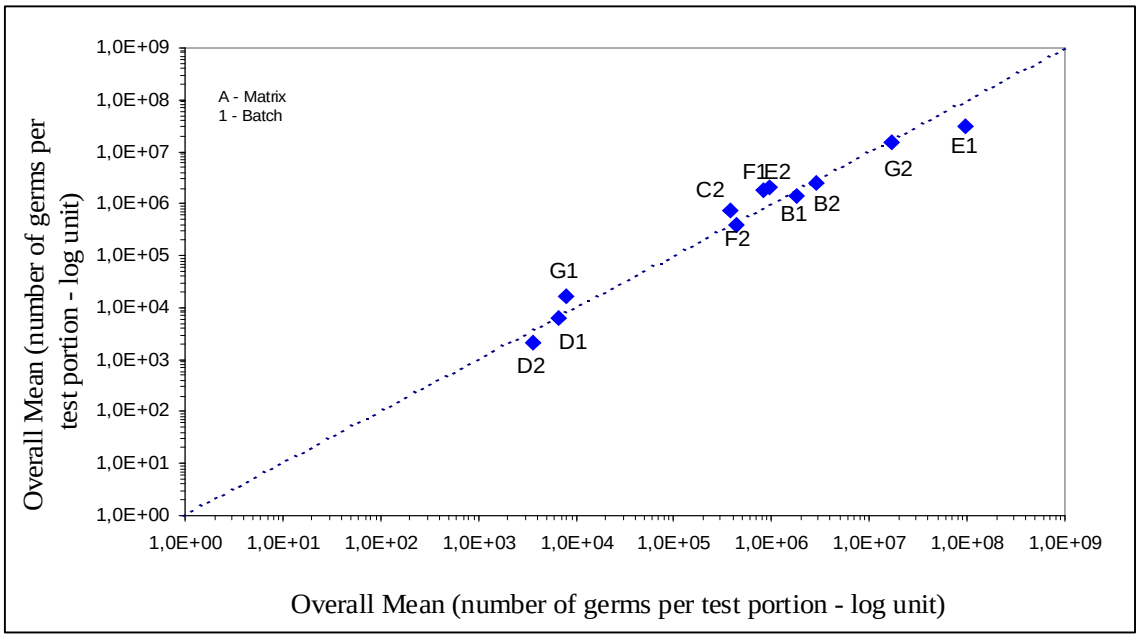
In repeatability conditions, it can be observed that the *E. coli* filtration method globally showed the lowest results (for 9 comparisons of matrix-batch data sets among the 11 comparisons including the *E. coli* filtration method and statistically tested).
The *E. coli* microtiter plate method is capable to give results as low as the *E. coli* filtration method for some matrices (ATB matrix-batch 1 (B1); DSSP matrix-batch 2 (D2); CSS matrix-batch 1 (E1); CBW matrix-batch 2 (G2)). However, the *E. coli* microtiter plate method showed the highest repeatability result for the two matrices for which sizeable reproducibility was also detected for all methods (PADS matrix-batch 2 (C2) and DSSP matrix-batch 1 (D1)).
As already seen for the intrinsic uncertainty of the method, the *E. coli* MPN macromethod is the one which globally showed the highest value of repeatability (for 7 comparisons of matrix-batch data sets among the 9 comparisons including the *E. coli* MPN macromethod and statistically tested).

In reproducibility conditions, the *E. coli* microtiter plate method showed the lowest results (for 7 comparisons of matrix-batch data sets among the 11 comparisons including the *E. coli* microtiter plate method and statistically tested). However, it can be noticed that the *E. coli* microtiter plate method gave the highest reproducibility values for two other matrices, in particular one giving sizeable precision assessment for all methods (PADS matrix-batch 2 (C2) and DSSP matrix-batch 2 (D2)).
The *E. coli* filtration method showed the lowest reproducibility for three matrices-batches (DSSP matrix-batches 1 and 2 (D1 and D2); CBW matrix-batch 1 (G1)) but the highest for five other matrices-batches (ATB matrix-batches 1 and 2 (B1 and B2); CSS matrix-batches 1 and 2 (E1 and E2); CBW matrix-batch 2 (G2)).
The *E. coli* MPN macromethod gave the lowest reproducibility for the CSS matrix-batch 2 (C2) (detected as one matrix-batch giving globally high precision results) and the highest for DSSP matrix-batch 1 (D1) (detected as the second matrix-batch giving globally high precision results), CGW matrix-batches 1 and 2 (F1 and F2).

5.3.2 Trueness of the results of the *E. coli* methods tested in interlaboratory conditions

The assessment of the relative bias between the 3 different *E. coli* methods leads to the evaluation of the trueness of the results obtained in interlaboratory conditions.

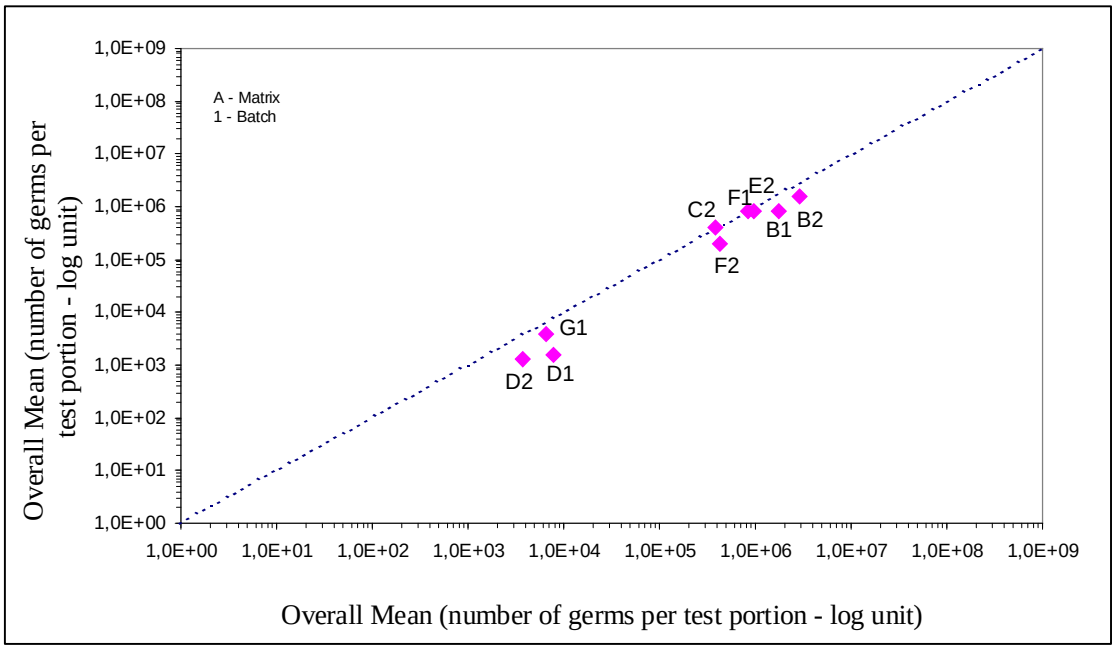
The values of the paired means comparisons between the *E. coli* filtration method and the *E. coli* microtiter plate method and corresponding to each tested matrix-batch are plotted on **Graph 4**.



Graph 4: Assessment of the relative bias between the *E. coli* filtration method (x axis) and the *E. coli* microtiter plate method (y axis) using paired means comparison.

According to the **Graph 4**, in interlaboratory conditions, no global significant difference was detected between the *E. coli* filtration method and the *E. coli* microtiter plate method. In terms of relative recovery, the two methods appear to be quantitatively comparable.

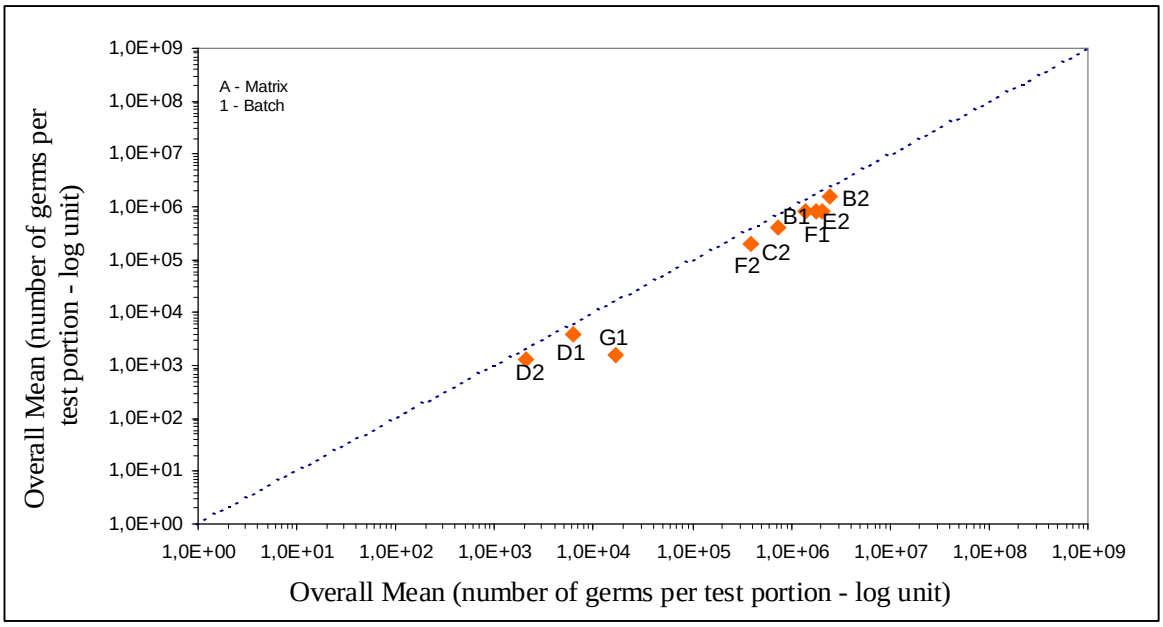
The same data processing was carried out with the paired means comparisons (between the *E. coli* filtration method and the *E. coli* MPN macromethod) corresponding to each tested matrix-batch (**Graph 5**).



Graph 5: assessment of the relative bias between the *E. coli* filtration method (x axis) and the *E. coli* MPN macromethod (y axis) using paired means comparison

The comparison of paired means results between the *E. coli* filtration method and the *E. coli* MPN macromethod showed a possible significant bias between the two methods. Indeed, the non parametric Wilcoxon test detected a significant difference at a 5% level of significance (not 1%) (**Graph 5**).
As a conclusion, the *E. coli* filtration method could give higher results than the *E. coli* MPN macromethod but the statistically suspect trend does not permit to be affirmative.

The paired means comparisons corresponding to each matrix batch analysed with the *E. coli* microtiter plate method and the *E. coli* MPN macromethod are plotted on **Graph 6**.



Graph 6: Assessment of the relative bias between the *E. coli* microtiter plate method (x axis) and the *E. coli* MPN macromethod (y axis) using paired means comparison.

From the results of the paired means comparison between the *E. coli* microtiter plate method and the *E. coli* MPN macromethod, a significant bias was observed at a 1% level of significance (**Graph 6**). The results of the *E. coli* microtiter plate method showed a clear trend to be higher than the ones obtained with the *E. coli* MPN macromethod.

5.3.3 Relation between *E. coli* validation results and evaluation of the prepared matrices-batches homogeneity and stability

The evaluation of the batch homogeneity and stability by the partner in charge of the preparation was an additional tool to judge the Validation Study results, precision of the methods in term of repeatability and reproducibility.

The preliminary trials conclusions to evaluate the stability and the results of homogeneity evaluation of the sub-samples are gathered in **Table 10**. The results of those additional trials are appended in **Annex 23**.

Table 10: Comparison of *E. coli* homogeneity and stability results versus analytical *E. coli* Validation results

	Additional results		**Validation Study result**	
	Stability	**Homogeneity**	**Repeatability**	**Reproducibility**
MAD 1	Significant contamination decrease	Verified	-	-
MAD 2	No data	No critical point	-	-
ATB 1	Contamination decrease	No critical point heterogeneity difficult to be assessed Many samples with high concentration	Normal	Normal, except for *E. coli* MPN macromethod: Good
ATB 2			Normal	Normal
PADS 1	-	No data	-	-
PADS 2	No data	No critical point	Normal	High
DSSP 1	Possible trend of contamination decrease	No critical point	Normal except for *E. coli* Microtiter plate method: slightly higher	High
DSSP 2	Significant trend of contamination decrease		Normal	Normal (no data for *E. coli* MPN macromethod)
CSS 1	Possible trend of contamination decrease	No critical point	Normal (no data for *E. coli* MPN macromethod)	Normal (no data for *E. coli* MPN macromethod)
CSS 2	No data		Normal	Normal
CGW 1	No critical point	No critical point	Normal	Normal
CGW 2	Slight instability	Satisfying	Normal (no data for *E. coli* MPN macromethod)	Normal (no data for *E. coli* MPN macromethod)
CBW 1	Significant trend of contamination increase	Heterogeneous batch	High	-
CBW 2	No data	No critical point	Normal (no data for *E. coli* MPN macromethod)	Normal (no data for *E. coli* MPN macromethod)

The stability and homogeneity studies were suitable to validate most of Validation Study result. They provided additional information that confirmed or explain the high repeatability and reproducibility results.

The decrease of the contamination in the sub-samples revealed through time with the preliminary stability trial was again encountered during the Validation Study. It could explain the lack of data in the quantification range.

Differences of the contamination level between the results registered by the participants and the control analyses are observed with the ATB matrix batches: many control analyses were upper than the quantification limit, whereas the participants reported validation results in the quantification range, suitable for precision parameters calculation. This was the consequence of the possible loss of bacteria already observed during preliminary trials.

The repeated measurements of the DSSP matrix-batch 1 and PADS matrix-batch 2 are suitable. The high interlaboratory error (reproducibility) was then not explained by the batches heterogeneity.

The heterogeneity of the CGW matrix-batch 1 was well established with the stability and homogeneity evaluation. The batch heterogeneity revealed by the high repeatability could interfere with the evaluation of the methods performances.

5.4 Data and statistics for Salmonella spp. analysis

The results collected following the analysis of the 7 matrices with the 3 different *Salmonella* spp. methods in interlaboratory conditions are presented matrix by matrix, method by method in **Annex 24**.
Those data were the base of the statistical calculation, to determine the precision of the results of each method and the first assessment of their precision.

5.4.1 Precision of the results according to the applied method in interlaboratory conditions

The repeatability and reproducibility are expressed in the same terms as for the 3 *E. coli* methods (5.3.1.1).

5.4.1.1 *Salmonella* spp. filtration method (prEN 15 215-1)

The statistical results of the evaluation of the *Salmonella* spp. filtration method precision in term of repeatability and reproducibility of each matrix-batch analysed by the 14 laboratories are presented in **Table 11**.

Table 11: Precision values of the *Salmonella* spp. filtration method observed during the validation study

Matrix - batch	**Overall mean**	**Repeatability** (log scale)	**Repeatability** (ratio)	**Reproducibility** (log scale)	**Reproducibility** (ratio)
MAD 1	14 (1)	0.759 (1)	5.7 (1)	2.014 (1)	103.3 (1)
MAD 2	18	1.206	16.1	2.421	263.9
ATB 1	390 886	0.709	5.1	0.962	9.2
ATB 2	259 263	0.283	1.9	2.045	111.0
PADS 1	< 2.70 (2)	-	-	-	-
PADS 2	191 740	0.713	5.2	3.853	7 121.3
DSSP 1	1 030	0.447	2.8	1.463	29.1
DSSP 2	6 384	0.514	3.3	2.199	158.2
CSS 1	1 850 642	1.050	11.2	2.455	285.4
CSS 2	19 616	0.543	3.5	2.299	198.9
CGW 1	40 327	0.807	6.4	0.996	9.9
CGW 2	13 302	0.737	5.5	2.514	326.5
CBW 1	1 046	2.344	221.0	3.892	7 794.8
CBW 2	1 399 819	0.865	7.3	1.476	30.0
Expression of results	*g wet weight*	*Maximal difference between 2 independent measurements on log scale (95% of the cases)*	*Maximal ratio between 2 independent measurements on natural scale (95% of the cases)*	*Maximal difference between 2 independent measurements on log scale (95% of the cases)*	*Maximal ratio between 2 independent measurements on natural scale (95% of the cases)*

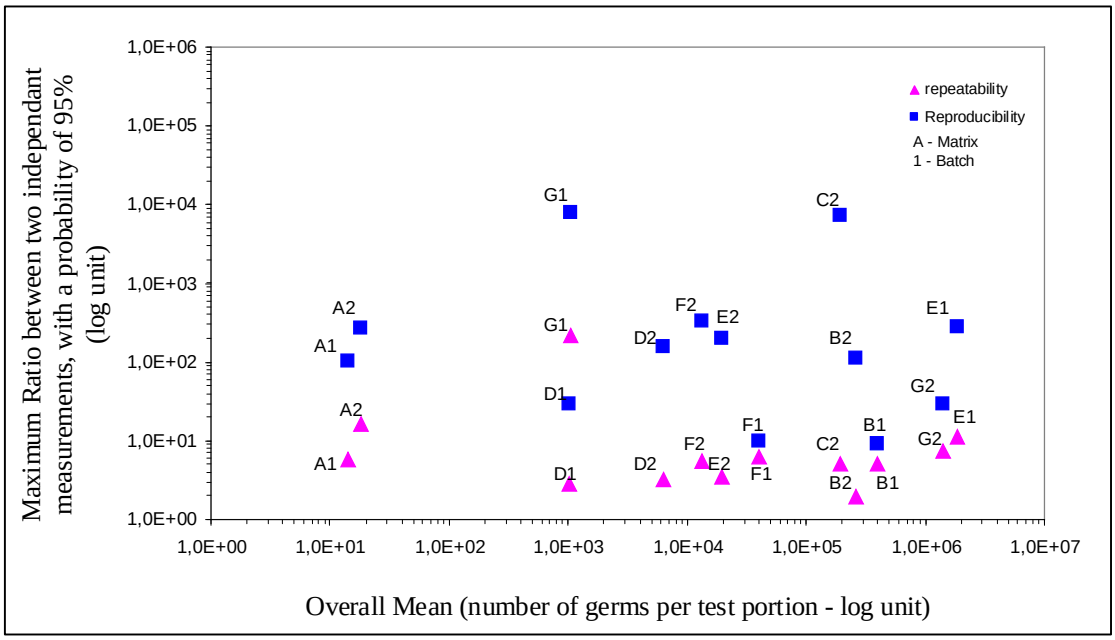
(1) Few data were finally available for data processing. Estimation to be considered carefully.
(2) Theoretical limit of detection with a probability of 95% calculated for the *Salmonella* spp. filtration method.

In the experimental conditions of the interlaboratory testing, the calculation of the repeatability and reproducibility values were possible for 13 of the 14 matrices-batches, all analytical results of the PADS matrix-batch 1 giving values lower than the theoretical limit of detection (limit of detection: <2.70 cfu /g ww).
Nevertheless, the calculation of repeatability and reproducibility values of the MAD matrix-batch 1 should be taken into account with caution, because of the low number of quantitative data registered.

The maximal ratio observed for the repeatability of the *Salmonella* spp. filtration method was between 1.9 (registered for the ATB matrix-batch 2) and 16.1 (registered for the MAD matrix-batch 2), corresponding to 0.3 and 1.2 log in terms of maximal difference between log results.

The observed reproducibility of the *Salmonella* spp. filtration method was between 9.2 and 326.5 in terms of maximal ratio, respectively calculated for the ATB matrix-batch 1 and the CGW matrix-batch 2 (maximal difference between log results corresponding to 1 and 2.5 log) depending on the microbial load.

The precision values corresponding to each tested matrix-batch with the *Salmonella* spp. filtration method are presented in the **Graph 7** according to repeatability and reproducibility conditions.



Graph 7: Precision values obtained in repeatability conditions (pink triangle) and in reproducibility conditions (blue square) for the *Salmonella* spp. filtration method.

According to the **Graph 7**, two Reproducibility results were located out of the global pattern observed: result of the PADS matrix-batch 2 (C2) and result of the CBW matrix-batch 1 (G1). These suspect results of Reproducibility were not taken into consideration in the global conclusion of the *Salmonella* spp. filtration method precision.

5.4.1.2 *Salmonella* spp. MPN method (prEN 15 215-2)

The statistical results of the evaluation of the *Salmonella* spp. MPN macromethod precision in term of repeatability and reproducibility are presented in **Table 12,** matrix-batch by matrix-batch.

Table 12: Precision values of the *Salmonella* spp. MPN macromethod observed during the validation study

Matrix - batch	**Overall mean**	**Repeatability** (log scale)	**Repeatability** (ratio)	**Reproducibility** (log scale)	**Reproducibility** (ratio)
MAD 1	9	1.395	24.8	2.020	104.8
MAD 2	43	1.973	94.1	2.604	401.4
ATB 1	> 4.65 x 10^4 (1)	-	-	-	-
ATB 2	> 4.65 x 10^4 (1)	-	-	-	-
PADS 1	- (2)	-	-	-	-
PADS 2	> 4.65 x 10^4 (1)	-	-	-	-
DSSP 1	1 559	1.433	27.1	2.236	172.0
DSSP 2	6 476	2.171	148.3	3.108	1281.1
CSS 1	> 4.65 x 10^4 (1)	-	-	-	-
CSS 2	48 693 (3)	0.647 (3)	4.4 (3)	0.647 (3)	4.4 (3)
CGW 1	9 244 (4)	1.493 (4)	31.2 (4)	5.331 (4)	214 388.3 (4)
CGW 2	12 589	1.148	14.1	1.908	81.0
CBW 1	136	3.951	8 936.7	5.011	102 600.0
CBW 2	> 4.65 x 10^4 (1)	-	-	-	-
Expression of results	*g wet weight*	*Maximal difference between 2 independent measurements on log scale (95% of the cases)*	*Maximal ratio between 2 independent measurements on natural scale (95% of the cases)*	*Maximal difference between 2 independent measurements on log scale (95% of the cases)*	*Maximal ratio between 2 independent measurements on natural scale (95% of the cases)*

(1) Theoretical upper limit of quantification with a probability of 95% calculated for the *Salmonella* spp. MPN macromethod.
(2) No statistical processing was carried out because numerous results were close to the theoretical limit of detection (with a probability of 95%) calculated for the *Salmonella* spp. MPN macromethod, i.e. < 0.9 g wet weight.
(3) Few data were finally available for data processing. The observed variance was only random variation (no significant laboratory bias). Estimation to be considered carefully.
(4) Few data were finally available for data processing. Estimation to be considered carefully.

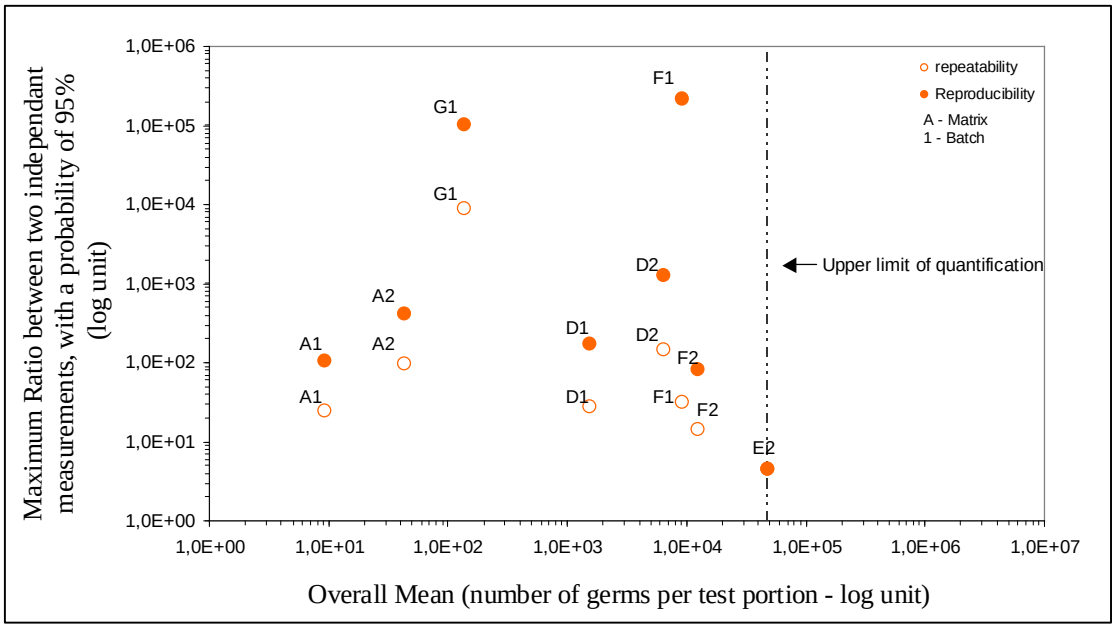
The calculation of the repeatability and reproducibility values were not processed for 6 of the 14 tested matrices-batches due to the lack of quantified results.
The analysis of five matrices-batches gave results upper than the theoretical limit of quantification (upper limit of quantification: > 4.65 x 10^4 cfu/g ww): ATB matrix-batches 1 and 2, PADS matrix-batch 2, CSS matrix-batch 1 and CBW matrix-batch 2.
The calculation of repeatability and reproducibility values was not processed for the PADS matrix-batch 1, since most of data were below the theoretical limit of detection (theoretical limit of detection: <2.70 cfu/g ww).

The estimation of the repeatability and reproducibility of the CSS matrix-batch 2 and CGW matrix-batch 1 should be considered with caution, as few data were finally available for the data processing.

The maximal ratio of the observed *Salmonella* spp. MPN macromethod repeatability was between 14.1 and 148.3 (respectively for the CGW matrix-batch 2 and DSSP matrix-batch 2) corresponding to maximal difference between log results of 1.1 and 2.2 log.

The observed *Salmonella* spp. MPN macromethod Reproducibility varied from 81.0 (CGW matrix-batch 2) to 1281.1 (DSSP matrix-batch 2) in terms of maximal ratio corresponding to a maximal difference between log results of 1.9 and 3.1 log. The results depended on the microbial load.

The precision values obtained for each tested matrix-batch with the *Salmonella* spp. MPN macromethod are presented in the **Graph 8** according to repeatability and reproducibility conditions.



Graph 8: Precision values obtained in repeatability conditions (white circle) and in reproducibility conditions (orange circle) for the *Salmonella* spp. MPN macromethod.

The results observed for CBW matrix-batch 1 (G1) (repeatability and reproducibility) and CGW matrix-batch 1 (F1) (reproducibility) were not taken into consideration in the global conclusion about the method precision (**Graph 8**). They were located out of the global pattern observed.
Moreover, the assessment of the precision on CSS matrix-batch 2 (E2) was based on few available data because the microbial load was close to the upper limit of quantification of the method. They have also not been included in the global conclusion.

5.4.1.3 Conclusion: precision of *Salmonella* spp. methods

In repeatability conditions, it can be observed that the *Salmonella* spp. filtration method showed always lower results than the *Salmonella* spp. MPN macromethod (7 comparisons of matrix-batch data sets, all giving enough quantitative results for a statistical processing). According to the respective intrinsic uncertainty of the two methods (results of the suitability

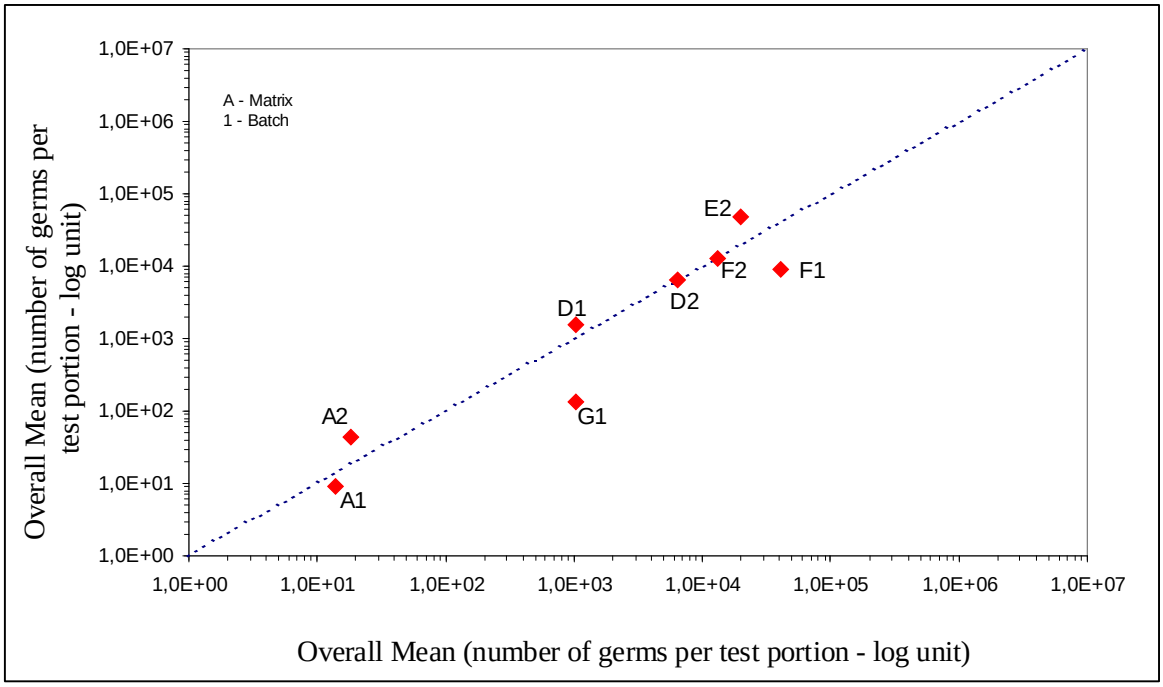
study), this pattern was expectable because the lowest intrinsic dispersion of outcomes had been given by the *Salmonella* spp. filtration method (lowest random variation).

In reproducibility conditions, the *Salmonella* spp. filtration method showed the lowest results for 4 comparisons of matrix-batch data sets among the 6 comparisons giving enough quantitative results for a statistical processing (MAD matrix-batch 2 (A2); DSSP matrix-batches 1 and 2 (D1 and D2); CBW matrix-batch 1 (G1)). The *Salmonella* spp. filtration method and the *Salmonella* spp. MPN macromethod gave comparable performances in reproducibility for MAD matrix-batch 1 (A1) for which the effective microbial load was the lowest statistically tested among all matrices. It can be noticed that the CGW matrix-batch 2 (F2) was the only one giving lower reproducibility values with the *Salmonella* spp. MPN macromethod than with the *Salmonella* spp. filtration method.

5.4.2 Trueness of the results of the *Salmonella* spp. methods tested in interlaboratory conditions

The assessment of the relative bias between the 2 different *Salmonella* spp. methods leads to the evaluation of the trueness of the results obtained in interlaboratory conditions.

The paired means comparisons between the *Salmonella* spp. filtration method and the *Salmonella* spp. MPN macromethod and corresponding to each tested matrix-batch are plotted on **Graph 9**.



Graph 9: Assessment of the relative bias between the *Salmonella* spp. filtration method (x axis) and the *Salmonella* spp. MPN macromethod (y axis) using paired means comparison.

In interlaboratory conditions, no global significant difference was detected between the filtration method and the MPN macromethod. In terms of relative recovery, the two methods appear to be quantitatively comparable.

5.4.3 Relation between *Salmonella* spp. validation results and the prepared sub-samples homogeneity and stability

The evaluation of the batch homogeneity and stability by the partner in charge of the preparation was an additional tool to judge the Validation Study results, precision of the methods in term of repeatability and reproducibility.

The stability results of preliminary tests and the homogeneity results of the batch prepared and analysed by the 14 participating laboratories are presented in **Annex 25**.
The conclusions of the preliminary stability trials and homogeneity of the sub-samples are summarised in **Table 13**.

Table 13: Comparison of *Salmonella* spp. Homogeneity and Stability results versus analytical *Salmonella* spp. Validation results

	Additional results		**Validation Study results**	
	Stability	**Homogeneity**	**Repeatability**	**Reproducibility**
MAD 1	Significant contamination decrease	No critical point Few sub-samples higher contaminated	Normal	Normal
MAD 2	No data		Normal	Normal
ATB 1	Significant instability	Variability of the contamination Maximal difference close to 1 Log unit between the sub-samples	Normal (except for the MPN method : > quantification limit)	Satisfying (except for the MPN method : > quantification limit)
ATB 2	Instability masked by the heterogeneity		Normal (except for the MPN method : > quantification limit)	Normal (except for the MPN method : > quantification limit)
PADS 1	No data	Variability of the contamination Maximal difference close to 1 Log unit between the sub-samples	< limit of detection	< limit of detection
PADS 2	No data	No data	-	-
DSSP 1	Significant trend of instability: contamination decrease	Homogeneous batches	Normal	Good with filtration method and normal with the MPN method
DSSP 2			Normal	Normal
CSS 1	Possible trend: contamination decrease	No critical point Homogeneous batch	Normal (except for the MPN method : > quantification limit)	Normal (except for the MPN method : > quantification limit)
CSS 2	No data	No data	-	-
CGW 1	Significant trend of contamination decrease	No critical point Few samples with higher contamination	Normal	Satisfying Except for MPN macromethod: very high reproducibility
CGW 2	No critical point	No critical point	Normal	Normal
CBW 1	Low results 3 weeks after batch contamination	No critical point Few samples less contaminated	Very high	Very high
CBW 2	No data	Homogeneous batch	Normal (no data for MPN method)	Normal (no data for MPN method)

The stability and homogeneity studies of most of matrices-batches were suitable to validate the result of the Validation Study. They gave additional elements to explain the results obtained with the analysis of PADS, CGW and CBW matrices.

Differences of the contamination level between the results registered by the participants and the control analyses are observed in two cases. All control analyses were in the range of quantification, whereas the participants reported lesser concentration than the detection limit in the PADS matrix-batch 1 and higher concentrations than the quantification limit in the CGW matrix-batch 1. Such variability was already observed during the preliminary trials.

The heterogeneity of the CBW matrix-batch 1 could partially explain the differences between results of one participant whatever were the methods followed. However, the variability was not important enough to explain the huge results difference that lead to very high repeatability and reproducibility.

5.5 Technical observations and proposed amendments

It was asked to each participating laboratory to report any technical remarks observed following each of the 6 methods during the validation study.
The main observations have been discussed during the 6th technical meeting in Paris on the 2nd October 2007 where the results of the Validation Study and their statistical analysis were presented. The amendments following the discussions, approved during the meeting will be included in the next amended prEN 15 214 and prEN 15 215 versions. The main observations and corresponding amendments are summarized here below.

5.5.1 Harmonisation of the 6 CEN draft standards

- ✓ All Titles will be revised to cancel mentions “soil improvers” and “growing media” following the demand of the convenor of the TC 223. Those mentions would be replaced by “organic fertilisers”, if agreed by the other TC’s.

- ✓ The “Foreword” will be modified:
  - The preparation of the standard involved the TC 308;
  - The table will be revised (soil improvers will be replaced by organic fertilisers) and completed.

- ✓ The “Scope” will be modified according to the modification of the titles to numerate all matrices tested during the Validation Study. All limits of detection (according the definition given in the ISO 13843) will be indicated. It will be specified that the quantification limit could be revised according to the dilution range used.

- ✓ The storage time indicated in all Horizontal-Hygiene draft standards will be modified. It will be specified that the samples should be analysed not later than 72h after receipt.

- ✓ The chapter “Performance data” will be revised to include statistical data obtained from the Suitability Ruggedness Study and the Validation Study.

5.5.2 Specific amendments of the 6 CEN draft standards

E. coli filtration method (prEN 15 214-1)

- ✓ The necessity of the prefiltration step was discussed since the last version of the method indicated this step as an option. All Validation Study results were obtained with analyses performed without prefiltration step. This step will be maintained as an option in the standard, with the mention that it is strongly recommended.

- ✓ The chapter "Expression of results" will be revised in order to make clearer how the number of confirmed colonies should be taken into account in the final result.

E. coli microplate method (prEN 15 214-2)

- ✓ The Validation Study was the opportunity to check the suitability of the incubation time of the microtiter plate by a first reading at 36h and a second reading at 72h, to reduce the time to get the results. According to the Validation Study results, the best conditions to read the microtiter plate is after an incubation time of 48h.

- ✓ The addition of a new step of confirmation was also tested to reduce false positive results. The results confirmed the importance of the indole test (addition of Kovacs reagent).
  The indole test will be included as a confirmation step on positive wells. It will be also described in the new version of the draft standard. The method results to be recorded will be then the number of fluorescent and indole positive wells.

E. coli MPN method (prEN 15 214-3)

- ✓ The standard should clearly specify that the range of dilution proposed has to be adapted according to the samples to be analysed. However, all statistical data reported in the standard correspond to the range of dilution indicated in the draft standard (conditions followed during the Suitability Study and the Validation Study.

Salmonella filtration method (prEN 15 215-1)

- ✓ The necessity of the prefiltration step was again discussed even if all Validation Study results were obtained without prefiltration step. It was agreed to keep this step as an option, but strongly recommended, in the draft standard.

Salmonella MPN method (prEN 15 215-2)

- ✓ The use of *Salmonella* Senftenberg H_2S- to spike the samples led to false results as the strain did not react as other *Salmonella* strains H_2S+ on XLD agar. A note in the new version of the Horizotnal-Hygiene draft standard will specify "if *Salmonella* Senftenberg H_2S- is present, the colonies have not the typical black colouration on XLD agar".

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

- ✓ The new version of the Horizontal-Hygiene draft standard will specify also that "if *Salmonella* Senftenberg H_2S- is present, the colonies have not the typical black colouration on XLD agar".

The six Horizontal-Hygiene draft standards were revised in the light of the Validation Study feedbacks, the analytical and statistical results and the conclusions of the Technical Meeting 6. The revision completed on January 2008 has led to the second and last CEN consultation of the updated Horizontal-Hygiene draft standards, from February 2008 to April 2008.
At receipt of the conclusions of the last CEN consultation, the 6 Horizontal-Hygiene draft standards will be a last time revised by the Horizontal-Hygiene experts.

6 Conclusion

The Validation Study was the opportunity to test the different draft procedures on complex matrices by the 14 European participating laboratories. It led to a complete statistical evaluation of the performances of the 6 Horizontal-Hygiene methods in terms of accuracy of measurement.

The results of the Validation Study, a part of the technical data and the results of the statistical data processing were presented and discussed between partners and additional participants during the 6th technical meetings (TM6, in Paris on 2nd October 2007. The conclusions of these meeting were included in the final results analyses.

General conclusions were favourable, last minor issues on the 3 Horizontal-Hygiene *E. coli* methods and the 3 Horizontal-Hygiene *Salmonella* spp. methods were resolved following the Validation Study thanks to the well documented data reporting and the discussions between the participants.
Participants have also reported the huge work performed for the realisation of the Validation Study.

The last amendments to be made on the Horizontal-Hygiene draft standards were also discussed with the partners thanks to the feedbacks received from the Validation Study, the statistical analyses of the results. Last minor changes were included in the updated versions of the methods, completed on January 2008 and proposed for a final CEN consultation so as to go on with the horizontal standardisation process.

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