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Validation of Colilert-18 Method for Detection of Escherichia Coli in Compost

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Abstract: European, as well as Czech legislation requires a microbiological quality control of treated biowaste, such as compost. *Escherichia coli* represents one of indicator organisms which is monitored. In this study a comparison of two quantification methods for *E. coli* was performed. The first one was a currently used method in the Czech Republic, consisting of direct plating of diluted compost sample on a membrane faecal coliform (mFC) agar with subsequent *E. coli* confirmation. The second method was Colilert-18, which was evaluated by this study as a suitable alternative to mFC agar for the analysis of compost and similar matrices for the presence of *E. coli* and should be considered as an alternative method in the context of revised regulations.

Key words: Escherichia coli, compost, Colilert-18, sludge

1. Introduction

Sewage sludge management is a huge task not only in the Czech Republic. Developed EU countries solve the problem of their disposal by burning them in incineration plants, however, there are countries which use the organic compounds in the sludge, including the nutrients, as a valuable fertilizer. The sludge cannot be used directly as a fertiliser due to its content of pathogenic microorganisms, which are often resistant to antibiotics, and content of certain organic pollutant residues. It has been proven that composting is one of the ways how to treat the sludge and also other waste because by composting the infectious and pathogenic microorganisms can be removed from the waste (sewage sludge, food waste, animal bedding, manure etc.). Efficiency of sewage sludge treatment is evaluated by microbiological analyses of the compost. According to the amendment of Czech Act No. 156/1998 Coll., on Fertilizers [1] it is also necessary to monitor, among others, the indicator organism E.coli. It is necessary to monitor microbiological parameters of treated biowaste also according to Decree No. 341/2008 Coll. [2], on particulars of biologically decomposable waste management, during which we also have to monitor the efficiency of hygienization of biowaste treatment (composting).

Currently, there are no unified European standards for detection of indicator organisms in compost and sewage sludge. Each country prefers a different detection method. In the Czech Republic, E.coli is detected in compost by the method of plating a diluted compost suspension on selective mFC agar with a subsequent biochemical confirmation of presumptive E. coli colonies [3].

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Ministry of Environment of the Czech Republic, a method of *E.coli* detection in the sewage sludge was modified and validated. Because this method, originally intended for potable water, was successful in its modification, Colilert 18 method was therefore modified and validated in cooperation with IDEXX-Laboratories, USA at the turn of 2017 and 2018 to detect *E. coli* in compost. 10 laboratories participated in this validation and 5 spiked samples in concentrations of 0 to 10^8 CFU/g d.w. of compost and one sample of a compost from a composting plant treating sewage sludge were analyzed.

Colilert®-18 and Quanti-Tray® are registered trademarks of IDEXX Laboratories, Inc. Westbrook Maine USA.

2. Experimental Work

2.1 Method

Three matrices (compost from sewage sludge, compost from green biowaste and digestate) were sterilised, split into 10 g sub-samples and inoculated with an *E. coli* suspension of known concentration. Analysis was performed on a number of matrices in several batches marked Z1–Z5. Several bacterial concentrations were used to inoculate the samples (0 to 10^6 CFU per gram of sample). Moreover one batch (Z6) was composed of unsterilised and un-inoculated samples with naturally occuring bacteria (Table 1).

Table 1 unit: CFU per gram; NA: not applicable as real samples were analysed, dilutions cannot be determined.

Sample	Matrix	A	B	C	D	E
Z1	Compost from WWTP sludge	10^0	10^1	10^2	10^4	10^6
Z2	Compost from green biowaste	10^0	10^1	10^2	10^4	10^6
Z3	Digestate	10^0	10^1	10^2	10^4	10^6
Z4	Compost from WWTP sludge	10^0	10^1	10^2	10^4	10^6
Z5	Compost from green biowaste	10^0	10^1	10^2	10^4	10^6
Z6	Compost from green biowaste	NA	NA	NA	NA	NA

Samples were produced by a central expert laboratory (Státní zdravotní ústav) and distributed to ten participating laboratories for analysis. Samples were shipped under controlled storage conditions to each participating laboratory with analysis taking place the following day.

All 10 laboratories participating in this study started the analysis of samples at the same time. Samples were analysed using both direct plating onto mFC agar according to standard procedures and Colilert®-18/ Quanti-Tray® according to ISO 9308-2:2012 [4]. Results were recorded in data collection sheets prepared by SZÚ. All data was sent electronically to SZÚ for collation and analysis.

2.2 Results and Statistical Analysis

The usable data generated by each laboratory were analysed graphically, using standard statistical tools and also according to methods specified in EN ISO 17994:2014 [5].

Data were omitted from the analysis when both methods returned values of zero or one of the methods gave a maximum value. Typically, in cases where one method gives a zero value and the alternate method returned a countable value, a value of "1" is added to each method as is allowable by EN ISO 17994 [5]. However, as the numbers generated were typically greater than 1×10^2, these data pairs were also excluded from the analysis to avoid the introduction of bias. There were fewer than 10 data pairs which were excluded for this reason.

The EN ISO 17994 [5] outputs were calculated using a maximum acceptable deviation (2L) set to 10, which represents limits on the expanded uncertainty of ±10%. Additionally, as the matrices being analyzed were composts, which typically contain high levels of bacteria, a maximum acceptable deviation of 20 (expanded uncertainty of ±20%) was also considered. This degree of acceptability is typically used with recreational waters as the level of contamination, and potential variation in performance, is greater than for

drinking water. It would appear valid to include composts and similar matrices in this category also.

To ensure a meaningful analysis of the data, the data generated for all six sample matrices at all dilutions were analyzed individually and also combined. All raw data, are included in Annex A with data used for statistical analysis included in Annex B.

A preliminary assessment of the data was made using a scatter plot to compare the performance of Colilert-18 to mFC agar. As the counts generated during the trial ranged from 1×10^2 to 1×10^8, the data were log transformed prior to plotting the chart (Fig. 1). The data compare very favourably with an R^2 coefficient of close to 1.

2.3 Preliminary Data Analysis

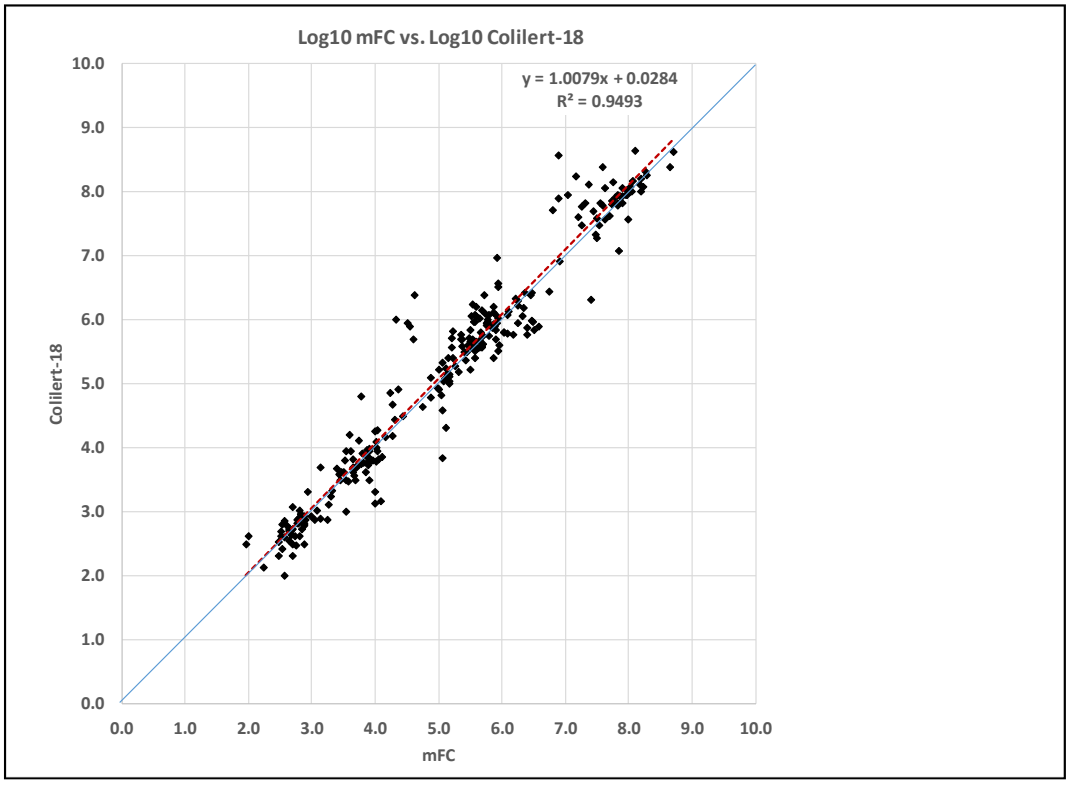

Fig. 1 Scatter plot of log transformed data from the comparison of Colilert-18 and mFC agar.

Subsequently, a Student's t-Test was employed to perform a statistical analysis of the data (Table 2). A one-tail analysis of the data returned a p-value of 0.027 which suggests that there is a statistically significant difference between the two methods whilst a two-tail analysis returned a p-value of 0.054 which suggests that there is no significant difference between the methods.

2.4 Statistical Analysis According to ISO 17994 [5]

Paired data from the trial were analysed according to guidelines specified in EN ISO 17994. The calculated parameters and the formulae used are summarised in Table 3.

The combined data from the trial were initially analysed to get an overall picture of how the methods compare. Data generated are summarised in Table 4 and the outputs of the analysis are summarised in Table 5. Data generated is represented graphically in Fig. 2. The lower and upper limits of the expanded uncertainty are both greater than zero, therefore indicating that the Colilert-18 method is significantly more sensitive than the mFC agar method.

Table 2 Student's t-Test – Colilert-18 vs. mFC. Data are statistically significant if P < 0.05.

Parameter	Colilert-18	mFC
Mean	1.99E+07	1.55E+07
Variance	3.29E+15	2.64E+15
Observations	276	276
Pearson Correlation	0.762	
Hypothesized Mean Difference	0	
df	275	
t Stat	1.939	
P(T<=t) one-tail	0.027	
t Critical one-tail	1.650	
P(T<=t) two-tail	0.054	
t Critical two-tail	1.969	

Table 3 Calculations used for analysis of data according to EN ISO 17994:2014 [5].

Parameter	Notation	Formula
Relative difference	X_i	$X_i = [\ln(a_i) - \ln(b_i)] \times 100$ Where: i: index X_i: relative difference between methods a and b for sample i a_i: result for the test method for sample i b_i: result for the reference method for sample i
Mean relative difference	$\bar{x}$	$\bar{x} = \Sigma X_i / n$ Where: ΣX_i : sum of all relative differences n: total number of samples
Standard deviation	S	$S = \sqrt{[\Sigma(X_i - \bar{x})^2 / (n-1)]}$
Standard uncertainty	$S\bar{x}$	$S\bar{x} = s / \sqrt{n}$
Half width of expanded uncertainty	W	$W = K \times S\bar{x}$ Where: K: coverage factor (typically 2)
Lower limit of expanded uncertainty	X_L	$X_L = \bar{x} - W$
Upper limit of expanded uncertainty	X_U	$X_U = \bar{x} + W$

Table 4 Statistical analysis of data performed according to EN ISO 17994:2014 [5].

EN ISO 17994 analysis	Symbol	Combined
Mean relative difference	$\bar{x}$	15.79
Standard deviation	S	0.91
Standard uncertainty	$S\bar{x}$	0.05
Half width of expanded uncertainty	W	10.94
Lower limit of expanded uncertainty	X_L	4.85
Upper limit of expanded uncertainty	X_U	26.73
Number of Samples	n	276

Table 5 outputs from statistical analysis according to EN ISO 17994:2014 [5]. Colilert-18 is the alternative (ALT) method with mFC being the current standard method.

Maximum acceptable deviation (2L)	EN ISO 17994 [5] output	
	2-sided	1-sided
10	DIFFERENT	ALT' HIGHER
20	DIFFERENT	ALT' HIGHER

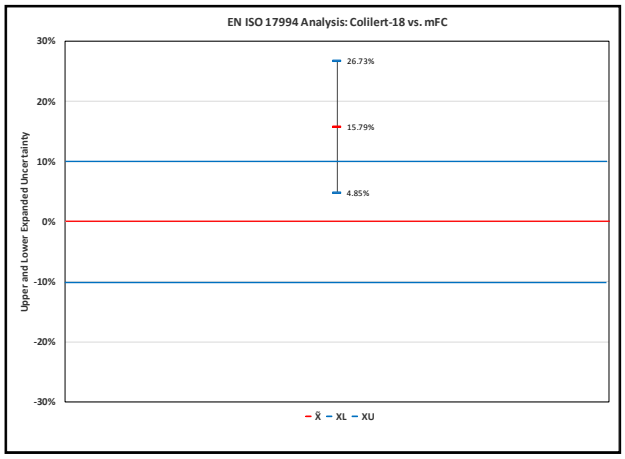

Fig. 2 Analysis of combined data according to EN ISO 17994 [5].

As the data generated were produced for several different (albeit related) matrices it was decided to analyze each set of data individually and in combination. In addition, several dilutions were used giving bacterial concentrations ranging from 1×10^2 to 1×10^8. It was therefore decided to analyse the data for individual dilutions also.

The data analysis for individual and combined matrices is summarised in Table 6 with analysis outputs for maximum confidence levels of 10% and 20% summarised in Table 7. Data is represented graphically in Fig. 3 and 4. The data analysis for individual dilutions along with the combined data are summarised in Table 8 analysis outputs for maximum confidence levels of 10% and 20% summarised in Table 9. Data is represented graphically in Fig. 5.

3. Discussion and Conclusions

A trial was performed to compare the performance of Coliert-18 and mFC agar methods for detection of *E. coli.* Samples of compost generated from sewage sludge and green bio-waste were analysed for presence of *E. coli* which was either artificially introduced to the samples or was naturally occurring.

Data generated during a trial were compared using a range of statistical methods including those specified in EN ISO 17994:2014 [5].

The analysis of the individual data sets by matrix type show some variation, however the general trend is

that the Colilert-18 method is more sensitive. When data for related matrices were combined, statistically significant differences between the methods are clearly evident. Analysis of the complete combined data set suggests that Colilert-18 is more sensitive that mFC agar for the detection of *E. coli* in the matrices analyzed.

Table 6 Statistical analysis of data, by matrix type, performed according to EN ISO 17994:2014 [5].

Stat'	Z1	Z2	Z3	Z4	Z5	Z6	Z1/Z4	Z2/Z5/Z6	All
$\overline{x}$	27.59	14.17	16.53	14.67	29.43	3.93	21.13	12.99	15.79
S	1.12	0.97	0.63	0.74	0.69	1.05	0.94	0.95	0.91
$S\overline{x}$	0.18	0.15	0.1	0.12	0.11	0.12	0.11	0.08	0.05
W	35.77	30.42	20.12	23.73	21.8	23.8	21.37	15.10	10.94
X_L	-8.18	-16.25	-3.59	-9.06	7.64	-19.87	-0.24	-2.12	4.85
X_U	63.36	44.59	36.64	38.39	51.23	27.74	42.5	28.09	26.73
n	39	41	39	39	40	78	78	159	276

Table 7 ISO 17994 [5] outputs for data generated by matrix type. Maximum confidence levels of 10% and 20% were both considered.

Matrix	ISO 17994 - 2L = 10%		ISO 17994 - 2L = 20%	
	2-Sided	1-Sided	2-Sided	1-Sided
Z1	INCONCLUSIVE	NO DIFFERENT	INCONCLUSIVE	NO DIFFERENT
Z2	INCONCLUSIVE	INCONCLUSIVE	INCONCLUSIVE	NO DIFFERENT
Z3	INCONCLUSIVE	NO DIFFERENT	INCONCLUSIVE	NO DIFFERENT
Z4	INCONCLUSIVE	NO DIFFERENT	INCONCLUSIVE	NO DIFFERENT
Z5	DIFFERENT	ALT' HIGHER	DIFFERENT	ALT' HIGHER
Z6	INCONCLUSIVE	INCONCLUSIVE	INCONCLUSIVE	NO DIFFERENT
Z1+Z4	INCONCLUSIVE	NO DIFFERENT	INCONCLUSIVE	NO DIFFERENT
Z2+Z5+Z6	INCONCLUSIVE	NO DIFFERENT	INCONCLUSIVE	NO DIFFERENT
ALL DATA	DIFFERENT	ALT' HIGHER	DIFFERENT	ALT' HIGHER

Table 8 Statistical analysis of data, by dilution, performed according to EN ISO 17994:2014 [5].

Stat'	A	B	C	D	E	All
$\overline{x}$	-6.33	-0.29	3.52	35.8	26.39	15.79
S	0.77	0.58	0.95	0.89	1.11	0.91
$S\overline{x}$	0.21	0.07	0.12	0.11	0.13	0.05
W	42.58	14.85	23.6	21.47	26.85	10394
X_L	-48.91	-15.15	-20.07	14.33	-0.46	4.85
X_U	36.25	14.56	27.16	57.28	53.24	26.73
n	13	62	65	68	68	276

Table 9 ISO 17994 [5] outputs for data generated by dilution. Maximum confidence levels of 10% and 20% were both considered.

Matrix	ISO 17994 - 2L = 10%		ISO 17994 - 2L = 20%	
	2-Sided	1-Sided	2-Sided	1-Sided
A	INCONCLUSIVE	INCONCLUSIVE	INCONCLUSIVE	INCONCLUSIVE
B	INCONCLUSIVE	INCONCLUSIVE	NO DIFFERENT	NO DIFFERENT
C	INCONCLUSIVE	INCONCLUSIVE	INCONCLUSIVE	INCONCLUSIVE
D	DIFFERENT	ALT'HIGHER	DIFFERENT	ALT'HIGHER
E	INCONCLUSIVE	NO DIFFERENT	INCONCLUSIVE	NO DIFFERENT
ALL DATA	DIFFERENT	ALT'HIGHER	DIFFERENT	ALT'HIGHER

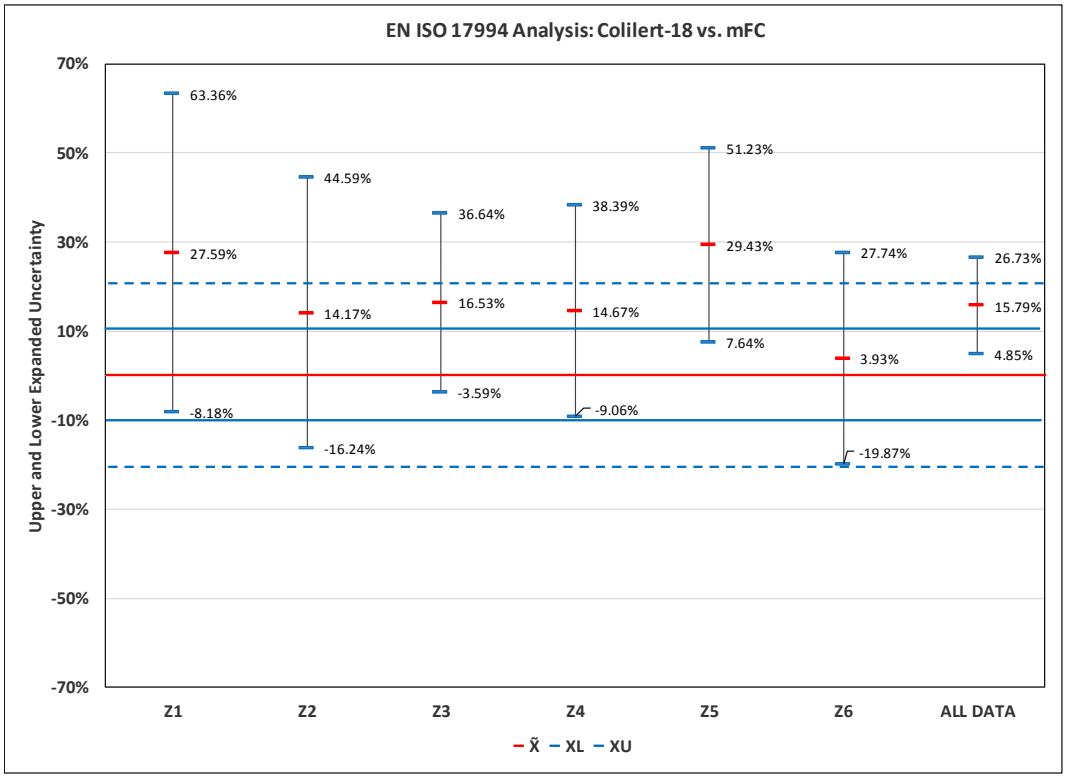

Fig. 3 Graphical representation of ISO 17994 [5] analysis of data generated for individual matrices.

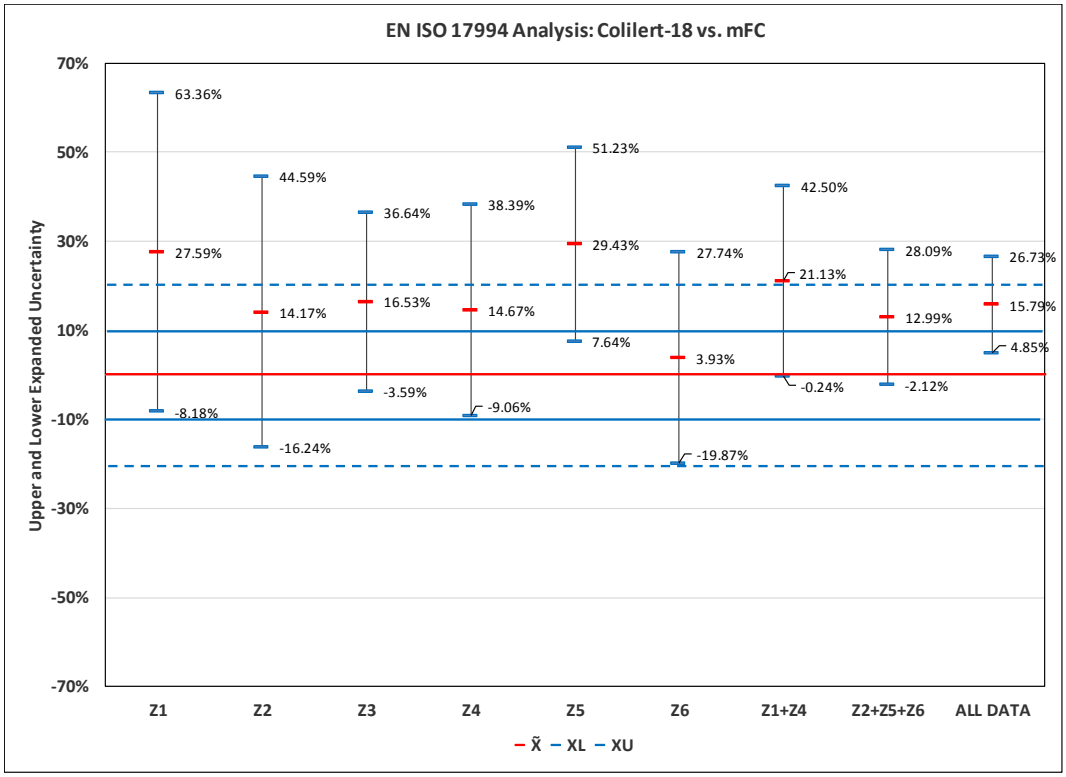

Fig. 4 Graphical representation of ISO 17994 [5] analysis of data generated for individual and combined matrices (combined by matrix type).

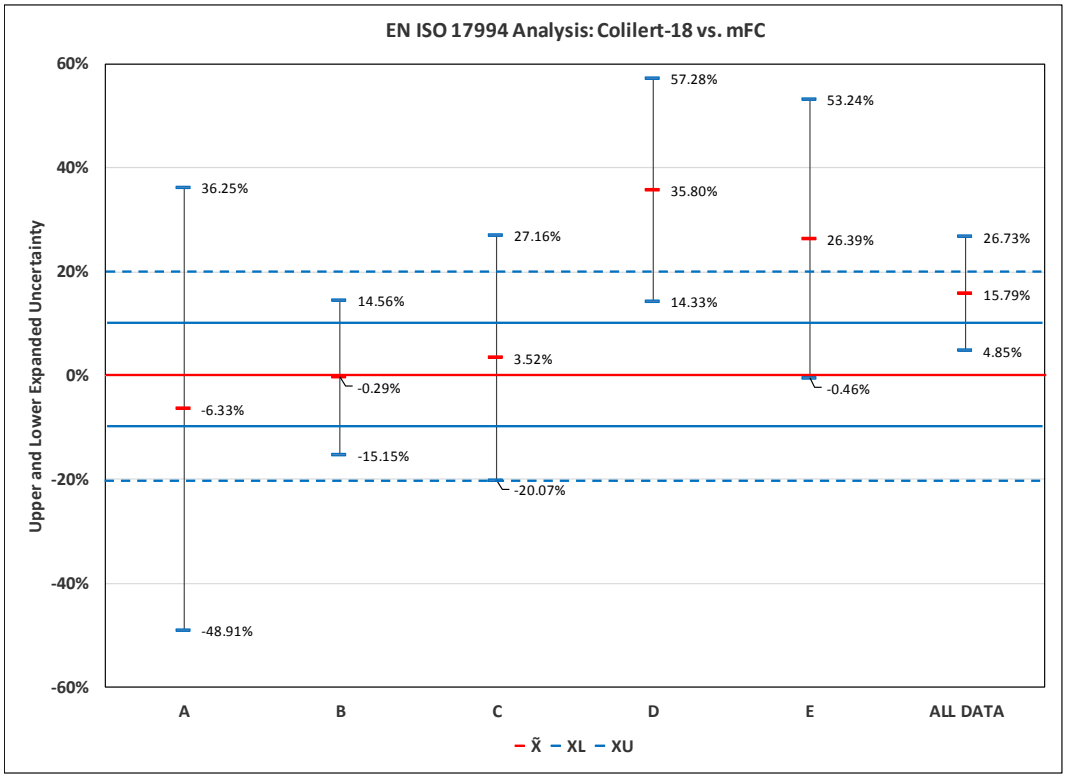

Fig. 5 Graphical representation of ISO 17994 [5] analysis of data generated for individual dilutions.

The analysis of individual data sets by bacterial concentration also shows some variation however, the general trend also suggests that Colilert-18 is more sensitive.

The ISO 17994 [5] outputs for a number of analyses gave an inconclusive result indicating that more samples need to be analysed to generate a conclusive statistical outcome. Combining the data increases the sample size and typically resulted in conclusive statistical outputs.

Overall, the data generated during the trial suggests that Colilert-18 is a suitable alternative to mFC agar for the analysis of compost and related matrices for the presence of *E. coli* and should be considered as an alternative method in the context of revised regulations.

References

[1] Czech Act No. 156/1998 Coll., on Fertilizers, Supplementary Soil Substances, Supplementary Plant Preparations and Substrates and on Agrochemical Testing of Agricultural Land (Act on Fertilizers).

[2] Decree No. 341/2008 Coll., on particulars of biologically decomposable waste management.

[3] Redakční Rada, V. Bencko, J. Mika, F. Rettich, J. Veselá and J. Volf, Metodický návod pro stanovení indikátorových organismů v bioodpadech, upravených bioodpadech, kalech z čistíren odpadních vod, digestátech, substrátech, kompostech, pomocných růstových prostředcích a podobných matricích, Acta Hygienica, Epidemiologica et Microbiologica, 2008.

[4] ISO 9308-2, Water quality — Enumeration of Escherichia coli and coliform bacteria Part 2: Most probable number method, 2012.

[5] ISO 17994, Water quality — Criteria for establishing equivalence between microbiological methods, 2014.