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Identification of the faecal indicator *Escherichia coli* in wastewater through the β-D-glucuronidase activity: comparison between two enumeration methods, membrane filtration with TBX agar, and Colilert®-18

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ABSTRACT

Escherichia coli (*E. coli*) is one of the most commonly adopted indicators for the determination of the microbiological quality in water and treated wastewater. Two main types of methods are used for the enumeration of this faecal indicator: membrane filtration (MF) and enzyme substrate tests. For both types, several substrates based on the β-D-glucuronidase activity have been commercialized. The specificity of this enzyme for *E. coli* bacteria has generated considerable use of methods that identify the β-D-glucuronidase activity as a definite indication of the presence of *E. coli*, without any further confirmation. This approach has been recently questioned for the application to wastewater. The present study compares two methods belonging to the above-mentioned types for the enumeration of *E. coli* in wastewater: MF with Tryptone Bile X-glucuronide agar and the Colilert®-18 test. Confirmation tests showed low average percentages of false positives and false negatives for both enumeration methods (between 4 and 11%). Moreover, the counting capabilities of these two methods were compared for a set of 70 samples of wastewater having different origins and degrees of treatment. Statistical analysis showed that the Colilert®-18 test allowed on average for a significantly higher recovery of *E. coli*.

Key words | coliforms, *Escherichia coli*, faecal indicator, methods, wastewater

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INTRODUCTION

Environmental and human health protection requires the treatment of municipal wastewater, aimed to limit the diffusion of potentially hazardous chemical and microbiological contaminants. In particular, the presence of pathogenic organisms of faecal origin has to be limited and adapted to the final destination of the reclaimed effluent. For this purpose, proper indicators of faecal contamination are commonly analysed in treated wastewater prior to discharge or reuse. The enteric bacterium *Escherichia coli* (*E. coli*) is considered a very good indicator of this type of contamination, since it is easily detectable, it is almost exclusively of faecal origin, and its presence is linked to the presence of pathogens. For these reasons, *E. coli* is used in most regulations regarding the microbiological quality of treated municipal wastewater (United States Environmental Protection Agency (USEPA) 2012).

The main accepted methods for the enumeration of *E. coli* in water and wastewater can be divided into three categories: (i) multiple tube fermentation (MTF) technique, which measures the gas produced in glucose or lactose broths; (ii) membrane filtration (MF) technique, which comprises the cultivation of bacteria, retained by a filter, on specific media; (iii) enzyme substrate test, which allows the detection of a particular enzymatic activity that can be associated with the target bacteria.

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MTF was the first method used to enumerate *E. coli* in water; MF, introduced about 50 years later, gradually replaced it, since it showed higher accuracy and allowed for cost and time saving. However, MF presents some limitations, as well. High turbidity samples with a low *E. coli* content (no need of dilution) may cause filter clogging, hence requiring a long filtration time or even making it impossible to perform the analysis. Moreover, MF may underestimate the *E. coli* count, because it detects only cultivable bacteria and also because the formation of *E. coli* colonies can be limited by the competition with other bacteria. On the other hand, colonies formed by other bacteria can be confused with *E. coli* colonies (false positives), therefore a confirmation test is normally required. To minimize these possible errors, several MF methods based on different agars, incubation temperatures, and confirmation tests, have been proposed (American Public Health Association (APHA) 2005). More recently, methods based on the enzymatic activity of the target bacteria have been developed to overcome some of the MF drawbacks. Unlike MTF and MF methods, which grow all aerobic bacteria and eliminate non-target bacteria with inhibitory chemicals, some enzyme substrate tests aim to feed only the target bacteria and for this reason this type of technique has also been called defined substrate technology (Edberg & Edberg 1988).

Among the various enzyme substrate tests that have been commercialized, one of the most used is the Colilert®-18 (IDEXX Laboratories Inc.). The enzymatic reaction evidenced by the Colilert®-18 test involves β-D-glucuronidase, which is known to be characteristic of *E. coli* and few other bacterial species (Kilian & Bulow 1976). Several media based on β-D-glucuronidase have been commercialized, both for MF and enzyme substrate tests, and they showed higher detection capability than most other common media (Niemela *et al.* 2003; Hörman & Hänninen 2006; Bonadonna *et al.* 2007; Maheux *et al.* 2014). Moreover, the high specificity of β-D-glucuronidase for *E. coli* may enable avoidance of further confirmation of presumptive *E. coli*. As a matter of fact, for the analysis of drinking and natural water the Colilert®-18 test presented very low false positive rates (0–5%) (Niemela *et al.* 2003; Bonadonna *et al.* 2007) and it has been certified as a viable method for the enumeration of *E. coli* by several international agencies (APHA 2005; ISO 9308-2:2012). Based on the same enzymatic principle as the Colilert®-18 test, the chromogenic agar Tryptone Bile X-glucuronide (TBX) has been further introduced for cultivation in MF methods and adopted as standard for food analysis with no need for confirmation tests (ISO 16649-1:2001).

However, the identification of *E. coli* through the β-D-glucuronidase activity showed some limits for wastewater samples, where a relevant presence of other bacteria of the Enterobacteriaceae family can cause high false positive rates (Yakub *et al.* 2002; McLain *et al.* 2011). Nevertheless, both the Colilert®-18 test and MF with TBX agar are widely used for the enumeration of *E. coli* in wastewater without any further confirmation (APAT & IRSA-CNR 2003; Mellado *et al.* 2006; Wen *et al.* 2009; O'Luanaigh *et al.* 2012; Haaken *et al.* 2014; Kitajima *et al.* 2014; Michael-Kordatou *et al.* 2015; Petousi *et al.* 2015).

The main objective of this paper is to assess the suitability of two methods based on the β-D-glucuronidase activity, the Colilert®-18 test and MF with TBX agar, for quantifying *E. coli* in wastewater samples, evaluating their applicability without any further confirmation. Samples of raw and treated wastewater, over a range of *E. coli* concentrations of more than six orders of magnitude, were analysed for the target faecal indicator through these two methods. In order to assess their robustness, three confirmation tests (cytochrome oxidase, lactose fermentation, and indole production) were used to estimate the percentages of false positives and false negatives. Moreover, this paper aims to evaluate the *E. coli* detection capability of the two methods. For this purpose, the two series of *E. coli* counts obtained through the Colilert®-18 test and MF with TBX agar were statistically compared.

MATERIALS AND METHODS

Wastewater samples

A total of 70 samples of wastewater, with different origins and degrees of treatment, were collected and analysed: 23 samples were collected at a wastewater treatment plant (WWTP) treating agro-industrial wastewater, 47 samples were collected at municipal WWTPs. In terms of degree of

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treatment, samples can be divided as follows: raw wastewater (16/70), secondary settled effluents (18/70), tertiary treated effluents (36/70). As for the latter, tertiary treatments included: cloth filtration (12/36), sand filtration (5/36), membrane filtration (7/36), and ultraviolet (UV) disinfection (12/36).

Wastewater samples were analysed for *E. coli* within 4 hours from the collection. Before the analyses, samples were diluted with tap water (*E. coli* free) according to the expected *E. coli* concentrations.

E. coli enumeration

For the enumeration of *E. coli* through MF, the following procedure was adopted: 100 mL of sample were filtered through 0.45 μm nitrocellulose membrane; then the membrane was placed on a plate on TBX agar (Oxoid) and incubated at 37 °C for 24 h; finally, the number of positive (blue-green) colonies on the plate was counted. The incubation temperature was chosen after comparing, for 10 wastewater samples, the following two options: (i) 37 °C for 24 h; (ii) 37 °C for 4 h, then 44.5 °C for 20 h. The first option was chosen because it maximized the recovery of *E. coli*. The result of MF analysis represents the number of *E. coli* present in the water that are able to form a colony during the incubation period, therefore it is expressed as colony forming units per 100 mL. MF is based on the assumption that each colony on the plate is formed by only one *E. coli* bacterium. This may be inaccurate for high bacterial counts, so in this study only counts lower than 80 were considered as valid. The dehydrated TBX medium was stored at 20 °C. The plates prepared with TBX agar were stored at 4 °C for 1–4 days before use.

The procedure of the Colilert®-18 (IDEXX Laboratories Inc.) test consists of the following steps: introducing 100 mL of sample and the dehydrated Colilert®-18 medium into a sterile plastic bottle and pouring the mixture into a tray (Quanti-Tray®/2000); sealing the tray and incubating it at 35 °C for 18–22 h; counting the positive wells (yellow wells that become fluorescent under 365 nm UV light indicate the presence of *E. coli*); finally, a matrix correlates the numbers of positive wells (small and big) with the most probable number (MPN) of *E. coli* present in the analysed sample. Therefore the result of this analysis is expressed as MPN per 100 mL. The maximum concentration of *E. coli* in the analysed sample that can be counted through this method is 2420/100 mL.

Confirmation tests

For part (12%) of the wastewater samples analysed, all presumptive *E. coli*-positive colonies and wells and some presumptive *E. coli*-negative colonies and wells were submitted to confirmation tests. For every colony/well analysed, three different confirmation tests were performed: (i) cytochrome oxidase, (ii) lactose fermentation, and (iii) indole production. Colonies were picked with sterile tip, resuspended in 0.3 mL of NaCl (0.8%) solution, and then portions of the resuspension were used for confirmation tests. As for the Colilert®-18 test, portions of the medium were directly picked from the wells. Oxidase disks (Bio-Rad) were used for the detection of cytochrome oxidase. DEV Lactose Peptone Broth (SIFIN) was used for the lactose fermentation test, which consisted of incubation at 37 °C for 21 h and the observation of a change of turbidity and colour (from purple to yellow) for positive reactions. For the indole test, incubation at 44 °C for 21 h in a tryptophan broth (SIFIN) was followed by the addition of a few drops of Kovacs Reagent (Bio-Rad) to check for positive reactions.

The colonies and wells that resulted as oxidase negative, lactose positive, and indole positive were considered as *E. coli* positive. When one of these conditions was not satisfied by a presumptive *E. coli*-positive colony/well, this was considered as a false positive. On the other hand, a presumptive *E. coli*-negative colony/well that satisfied all conditions was considered as a false negative.

Statistical analysis

For each sample of wastewater, both the MF and the Colilert®-18 methods were used for *E. coli* enumeration, obtaining two datasets (defined as TBX and Colilert®-18 datasets) composed of 70 observations each. To evaluate whether the differences between the TBX and the Colilert®-18 datasets were statistically significant, the paired t-test was used. This test requires two preliminary conditions: (i) normality of each dataset; (ii) homoscedasticity (variance homogeneity) between the two datasets. In order

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to meet these conditions, the data were firstly log-transformed, i.e. each observation x was replaced with the transformed value $\log_{10}(x)$. The normal distribution of each log-transformed dataset was graphically verified (with a confidence level of 95%) through the Q-Q plot test for normality. The F-test for variance comparison confirmed the homoscedasticity between the two log-transformed datasets (p-value = 0.49). Moreover, in order to evaluate the accuracy of the t-test in relation with the sample size, the absolute errors in the estimation of the means were determined (Bellera *et al.* 2012). For a confidence level α of 0.05, the absolute errors were 0.58 for both the TBX and the Colilert®-18 datasets.

Linear regression analysis was used to determine the best relationship between the *E. coli* counts obtainable with the two enumeration methods. Also, this process was applied to the log-transformed data. The normality of the residuals of the regression analysis was checked through the Chauvenet criterion, which resulted in 4 outliers being identified and discarded.

RESULTS AND DISCUSSION

Incubation temperature for MF analysis

In cultivation methods, the incubation temperature plays an important role to achieve optimal conditions for the formation of colonies. In order to choose the incubation temperature to be used for the MF method, the following two options were compared: (i) 37 °C for 24 h; (ii) 37 °C for 4 h, then 44.5 °C for 20 h. Indeed, the incubation at 44.5 °C favours the growth of thermo-tolerant coliform bacteria (such as *E. coli*) with respect to non-tolerant bacteria, limiting possible competitive effects, but at the same time it does not allow for the full recovery of injured bacteria, which is enhanced at temperatures close to that of the human body. For these reasons, some standards suggest incubation at 35–37 °C for a short period, followed by incubation at 44.5 °C for 20–24 h (ISO 16649-1:2001). Results of the preliminary analyses indicated that, in 8 cases over a total of 10, the *E. coli* detection capability was higher after incubation at 37 °C for 24 h. On average, this option allowed the recovery of 19% more presumptive *E. coli*. This result can be associated with both the characteristics of the samples and the type of agar used. Wastewater samples analysed in this study underwent processes such as pumping, filtration, and disinfection, that increased the probability of injuries on *E. coli* bacteria. On the other hand, the specificity of the TBX agar for *E. coli* cultivation reasonably minimized the competition with other bacteria which, on the contrary, can be relevant with other substrates used for MF methods.

Confirmation tests

Results of the confirmation tests are reported in Table 1. False positive rates were around 11% (11/97) and 4%

Table 1 | Results of confirmation tests performed on presumptive *E. coli*-positive and on presumptive *E. coli*-negative colonies/wells

	MF with TBX agar, incubation at 37 °C for 24 h		Colilert®-18 Quanti-Tray®/2000	
	Presumptive *E. coli*-positive	Presumptive *E. coli*-negative	Presumptive *E. coli*-positive	Presumptive *E. coli*-negative
Number of colonies/well analysed	97	30	92	79
Oxidase negative	97	30	92	79
Lactose positive	97	24	92	59
Indole positive	86	2	88	10
False positive[a]	11		4	
False negative[b]		3		9

[a]Number of presumptive *E. coli*-positive colonies/wells for which at least one confirmation test was not satisfied.
[b]Number of presumptive *E. coli*-negative colonies/wells for which all confirmation tests were satisfied.

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(4/92) for the TBX and the Colilert®-18 methods, respectively. The value related to the Colilert®-18 test is lower than those reported for wastewater samples and comparable to those observed for samples of natural water in previous studies (Yakub *et al.* 2002; Niemela *et al.* 2003; Bonadonna *et al.* 2007; McLain *et al.* 2011). This apparent discordance can be explained by considering the distribution of false positives among the samples analysed. Over a total of 15 false positives, 14 were obtained from just two samples (a raw wastewater and a secondary settled effluent) collected on the same day from the same municipal WWTP (WWTP1). Considering just the results related to these two samples, false positive rates were much higher: 28% (10/36) for TBX and 14% (4/29) for Colilert®-18. On the other hand, for the other eight samples submitted to confirmation, three of which were collected at the same WWTP1, the percentages of false positives were close to zero: 2% (1/61) for TBX and 0% (0/63) for Colilert®-18. These results highlight the strong variability of the microbial ecosystem of wastewater, both among different WWTPs and over time. Moreover, they suggest that possible interferences due to the presence of bacteria other than *E. coli* that are capable of β-D-glucuronidase activity cannot be predicted for wastewater samples.

False negative rates were 10% (3/30) and 11% (9/79) for TBX and Colilert®-18, respectively. The presence of false negatives was expected for both substrates, since it is known that some strains of *E. coli* are phenotypically β-glucuronidase negative. It is interesting also to notice that most (5/9) false negatives related to the Colilert®-18 test were observed in a raw wastewater sample that showed a high percentage of false positives as well. The concurrence of high percentages of false positives and false negatives for the same sample could be a coincidence, or it may indicate that a wider microbial composition of the wastewater would cause the simultaneous presence of both β-glucuronidase negative *E. coli* and other bacteria from the Enterobacteriaceae family. Further investigations on a large number of samples are required to clarify this aspect.

The low average percentages of false positives and false negatives observed indicate that methods based on the β-D-glucuronidase activity can be generally considered valid for the identification of the faecal indicator *E. coli* in wastewater samples. In particular, TBX agar showed better confirmation percentages than those reported in the literature for other agars commonly used for *E. coli* enumeration through MF (Niemela *et al.* 2003; Pitkänen *et al.* 2007; McLain *et al.* 2011). On the other hand, in some cases, both methods tested in this study may either overestimate or underestimate the *E. coli* content.

As regards the type of confirmation tests, looking at the data reported in Table 1 it is clear that the indole test was the only one suitable for the discrimination of *E. coli*-positive from *E. coli*-negative colonies/wells. The lactose fermentation was somehow useful to identify non-coliform bacteria. Indeed, 84% of presumptive coliform-positive wells (yellow non-fluorescent) had a positive reaction for lactose fermentation, whereas this percentage was much lower (27%) for presumptive coliform-negative wells (white). The oxidase test gave negative reactions for all the colonies/wells analysed, even when applied to presumptive coliform-negative wells. Therefore, the use of the oxidase test for the confirmation of presumptive *E. coli* in wastewater samples is recommended only in conjunction with other confirmatory tests.

Uncertain identifications

Besides the possible errors that can be made due to false negatives or false positives, confirmation tests are also necessary in case of unclear or doubtful indications. These are colonies with an unclear blue-green colour and wells with a weak fluorescence. Figure 1 shows some pictures of plates and wells as an example. As shown in Figure 1(a) and 1(e)–1(g), a few uncertain indications are normal for both the enumeration methods. In the tests reported here, the average percentages of unclear indications were very low: 5.3% for the MF with TBX agar, and 1.7% for the Colilert®-18.

However, in some cases it was not possible to achieve a univocal interpretation of the MF analysis, therefore these were excluded from the comparative analysis between the counting capabilities of the two enumeration methods (Figure 1(b)–1(d)). In Figure 1(b) it is possible to recognize several small blue-green colonies surrounded by a very large number of tiny yellow-green colonies (on the whole, looking like a background colour in the picture), which made the enumeration of presumptive *E. coli*-positive

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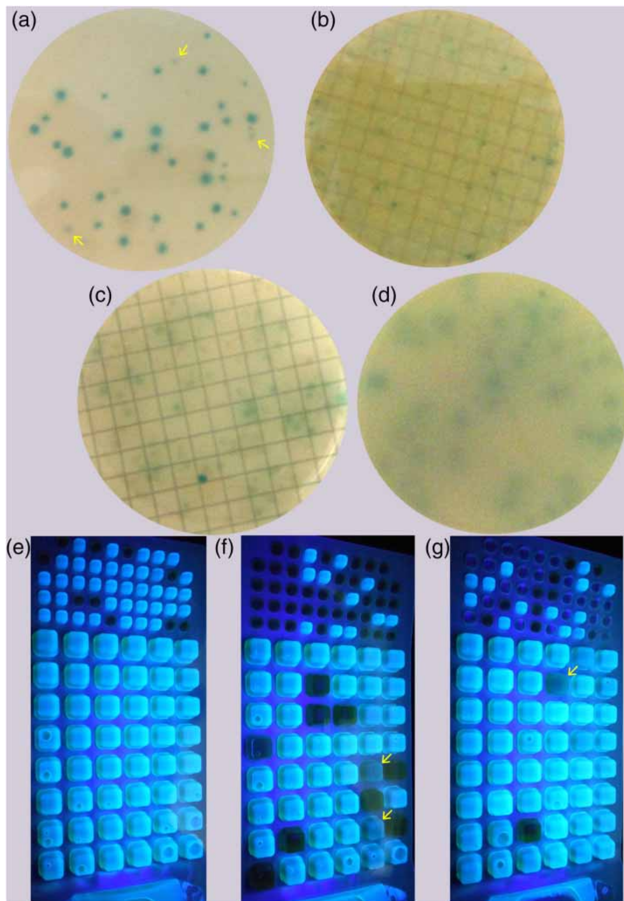

Figure 1 | Examples of results obtainable through the two enumeration methods. (a) Typical result of MF with TBX agar, with uncertain colonies indicated by yellow arrows. (b–d) Cases for which the results of MF with TBX agar are not easily interpretable. (e–g) Typical results of Colilert®-18 with uncertain wells indicated by yellow arrows. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wh.2016.119.

colonies impossible. This case, which refers to a sample of secondary settled wastewater stored for approximately one week in a tank, indicates that even for the TBX agar, generally considered very specific for *E. coli*, competition with other bacteria may be relevant. Also in the cases represented in Figure 1(c) and 1(d), both related to samples of treated wastewater disinfected by UV radiation, it was not possible to clearly identify presumptive *E. coli*-positive colonies. Indeed, the colonies observed on the plate were characterized by different colours and some of them also had undefined borders, so that it was difficult to distinguish blue-green colonies from the others (green-brown, green). In the three cases represented in Figure 1(b)–1(d), the enumeration through MF with TBX agar clearly needs a subsequent confirmation step. On the contrary, for the samples related to Figure 1(b) and 1(c), the enumeration of *E. coli* through the Colilert®-18 test had a clear interpretation and indicated the presence of presumptive *E. coli*. The sample related to Figure 1(d) was not analysed through the Colilert®-18 test.

Counting capability

The results of the comparative analysis between the counting capabilities of the two methods used in this study consist of two values of presumptive *E. coli* concentration for each wastewater sample: one refers to the MF with TBX agar ($E.\ coli_{\mathrm{TBX}}$), the other to the Colilert®-18 test ($E.\ coli_{\mathrm{Colilert}}$). These values are reported in Figure 2 (in log-log scale, to allow for a clear graphical representation), where each point represents one sample and its coordinates are the concentrations measured through the two enumeration methods. As can be noticed by comparing the location of each point in Figure 2 with respect to the quadrant bisector, most (79%) of the observations obtained with the Colilert®-18 test were higher than the corresponding ones obtained with MF. The ratio r between the values obtained with the two enumeration methods ($r = E.\ coli_{\mathrm{Colilert}}/E.\ coli_{\mathrm{TBX}}$) was also calculated for each sample. The distribution of the values of r, displayed in Figure 3, has a normal pattern with a peak between 1 and 3 (63% of the values belong to this interval), suggesting the existence of a functional relationship between the two counting methods.

In order to apply statistical techniques to compare the two enumeration methods, data were firstly log-transformed. Indeed, the original datasets had values that cover more than six orders of magnitude and, for this reason, their distributions were not normal and their variances were not homogeneous.

The *paired t-test* result validated, with a high degree of confidence (p-value $= 9.4 \times 10^{-10}$), the hypothesis of significant difference between the average values of the two log-transformed datasets. Therefore it can be stated that the values of *E. coli* concentration estimated by the MF and the Colilert®-18 methods were different on average.

Once this was verified, the existence of a possible functional relationship between the two counting methods was

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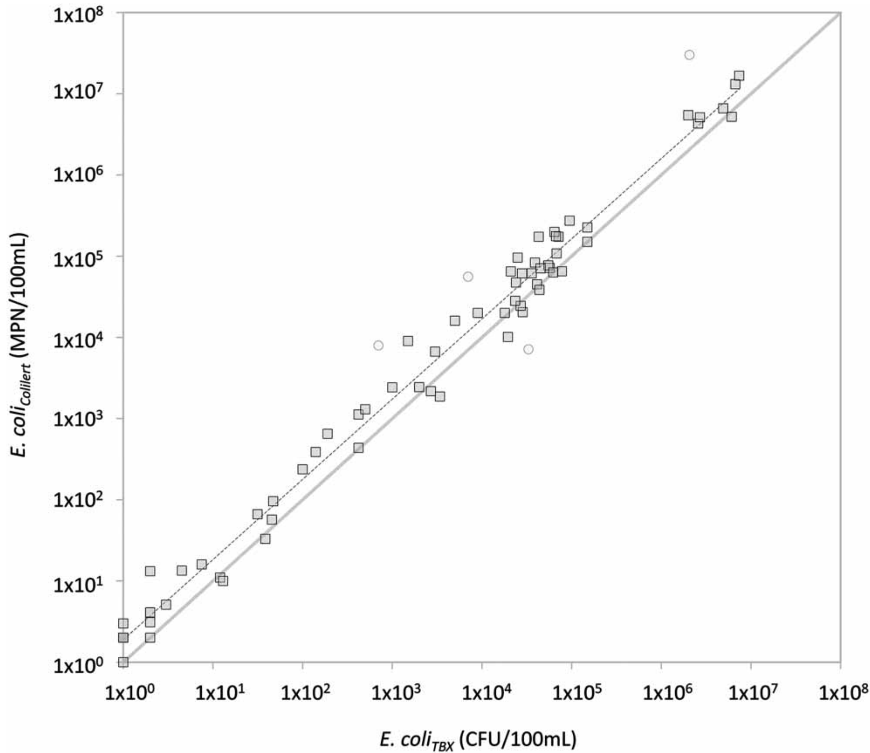

Figure 2 | Comparison between the MF (with TBX agar) and the Colilert®-18 methods for *E. coli* enumeration. Each point represents one sample and its coordinates are the estimated *E. coli* content obtained through the two enumeration methods. The dotted line represents the best fit determined through regression analysis. Grey circles identify outliers.

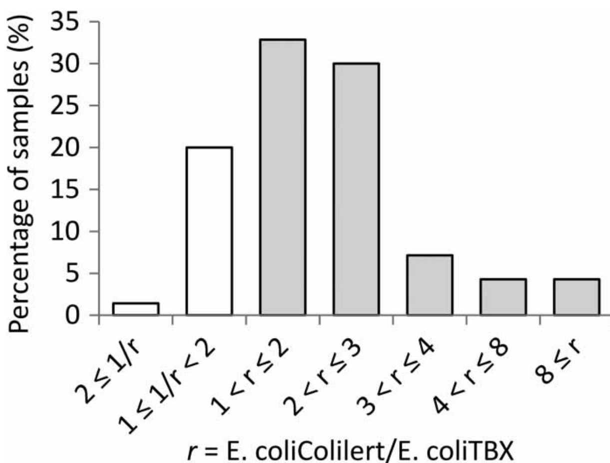

Figure 3 | Percentage distribution of the ratio *r* between the values obtained, for each sample, with the two enumeration methods.

investigated through regression analysis. This showed that a linear model fitted the log-transformed data ($\log_{10}(E.\ coli_{\text{Colilert}}) = 0.99 \cdot \log_{10}(E.\ coli_{\text{TBX}}) + 0.28$) with an adjusted R^2 of 0.985 and a highly significant *p*-value (2.4×10^{-61}). This log-log linear model (represented by the dotted black line in Figure 2) corresponds to a power relationship between the original observations ($E.\ coli_{\text{Colilert}} = 1.92 \cdot E.\ coli_{\text{TBX}}^{0.99}$). Moreover, the 95% confidence intervals (CIs) related to the regression analysis have been computed. Considering the general relationship $E.\ coli_{\text{Colilert}} = \text{A} \cdot E.\ coli_{\text{TBX}}^{\text{B}}$, the values of A and B associated with the 95% CIs resulted as $1.47 \le \text{A} \le 2.51$ and $0.96 \le \text{B} \le 1.02$. All the values contained in the CIs can be considered equally acceptable as estimates of the regression parameters.

Since the two enumeration methods compared in this study are based on the same enzymatic principle, the significant difference observed can be reasonably associated to the procedures, so it may be interesting to underline the main differences between them. Firstly, the media compositions are different. The selectivity of the medium to the target bacteria has a great influence on its recovery (Maheux *et al.* 2014). The Colilert®-18 test can be considered highly selective, because it employs substrates that can be used only by bacteria that are β-D-galactosidase or β-D-glucuronidase positive. Secondly, MF methods require the growth of

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colonies, whereas enzyme substrate tests require just target bacteria to proliferate enough for the signal to be visible. The lower growth needed by the Colilert®-18 test may have favoured the detection of injured bacteria. Finally, in MF analysis more bacteria can develop the same colony. This phenomenon is considered probable for high bacterial counts, but it may happen also for small counts, although it is not likely to have produced a large differential effect.

To the best of the authors' knowledge, no other studies have compared the Colilert®-18 and MF with TBX agar methods. However, it has been already reported that Colilert®-18 has a higher *E. coli* detection capability than common MF methods (with m-Tec, LTTC, and m-FC agars) for samples of natural and drinking water (Niemela *et al.* 2003; Hörman & Hänninen 2006; Bonadonna *et al.* 2007). The results presented here confirm that, when compared with MF methods, the Colilert®-18 method allows for a higher recovery of *E. coli* from wastewater samples as well.

CONCLUSIONS

Confirmation tests indicated a very good average correspondence between the β-D-glucuronidase activity, detected through both the TBX and the Colilert®-18 substrates, and the presence of *E. coli* in wastewater samples. The Colilert®-18 and the TBX gave false positive or false negative indications for 8% of wells and 11% of colonies analysed, respectively.

On the other hand, confirmation tests suggested the existence of situations (wastewater samples) in which both the TBX and the Colilert®-18 would make relevant errors in the enumeration of *E. coli* (up to 14% for the Colilert®-18 test and up to 28% for MF with TBX agar). However, probably only raw or partially treated wastewater have such a variable microbial population that generate high false positive and false negative rates. Further investigations are needed to clarify this aspect.

Moreover, for some wastewater samples, the results of the MF analysis were not easily interpretable, because most of the colonies were characterized by an unclear (slight) blue-green colour or due to the presence of a background colour. Therefore, when using the MF with TBX agar, a method for the confirmation of presumptive *E. coli* should be always available and used when uncertain indications are obtained.

The comparative analysis of MF (with TBX agar) and Colilert®-18 methods indicated that the values of *E. coli* concentration estimated through the Colilert®-18 method were on average significantly higher, approximately twice as big as those estimated through MF. It is interesting to notice that the underestimation that would result from using the MF with TBX agar followed by confirmation of presumptive *E. coli* (indole test) is on average higher than the possible error that could be made by applying the Colilert®-18 method without any further confirmation.

The reliability of the method selected for *E. coli* enumeration is very important for warranting human health protection. This concerns the monitoring executed by water quality control laboratories and the studies performed by technicians and researchers to assess the performance of disinfection processes and the fate of faecal contamination in the environment. The results presented here suggest that the Colilert®-18 method could be viable for the enumeration of *E. coli* in wastewater samples.

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