Are the defined substrate-based methods adequate to determine the microbiological quality of natural recreational waters?

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ABSTRACT

Monitoring the microbiological quality of water used for recreational activities is very important to human public health. Although the sanitary quality of recreational marine waters could be evaluated by standard methods, they are time-consuming and need confirmation. For these reasons, faster and more sensitive methods, such as the defined substrate-based technology, have been developed. In the present work, we have compared the standard method of membrane filtration using Tergitol-TTC agar for total coliforms and *Escherichia coli*, and Slanetz and Bartley agar for enterococci, and the IDEXX defined substrate technology for these faecal pollution indicators to determine the microbiological quality of natural recreational waters. ISO 17994:2004 standard was used to compare these methods. The IDEXX for total coliforms and *E. coli*, Colilert®, showed higher values than those obtained by the standard method. Enterolert® test, for the enumeration of enterococci, showed lower values when compared with the standard method. It may be concluded that more studies to evaluate the precision and accuracy of the rapid tests are required in order to apply them for routine monitoring of marine and freshwater recreational bathing areas. The main advantages of these methods are that they are more specific, feasible and simpler than the standard methodology.

Key words | Colilert®, comparison of methods, Enterolert®, faecal pollution, ISO methods, microbial indicators

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INTRODUCTION

Water is one of the most important sources of infectious microorganism transmission to humans, via ingestion, contact with contaminated water, aerosol or by consumption of contaminated shellfish (Amaral *et al.* 2003; WHO 2003; Hurst 2007). Under certain conditions, water may be adversely affected by faecal pollution from a variety of sources, including wastewater and raw sewage, private septic disposal systems, and storm water runoff (Casteel *et al.* 2006). The contamination of natural waters with untreated faecal material may result in an increased risk of transmission of human diseases (Sinton *et al.* 1993; Borrego & Figueras 1997). Therefore, it is necessary to adopt control measures based on the determination of microbiological water quality, especially regarding the detection and quantification of pathogenic microorganisms (Budnick *et al.* 1996; Figueras *et al.* 2000; Moe 2007). Many microbial pathogens are difficult to detect and/or quantify in water samples, and methods to detect most of them from environmental samples have not been developed yet (Koster *et al.* 2003). For these reasons, regulatory agencies and public health authorities have typically relied on the use of microbial indicators of faecal pollution to assess

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microbiological water quality and safety (NRC 2004; Savichtcheva & Okabe 2006).

Classically, total coliforms, *Escherichia coli* and enterococci have been used as indicators of faecal pollution, and as indexes of water quality deterioration (Toranzos *et al.* 2001). In addition, several studies have demonstrated that these microbial indicators provide a measure of health risk associated with ingestion of or contact with contaminated water (Moe *et al.* 1991; MacKenzie *et al.* 1994; Mariño *et al.* 1995*a*,*b*; Borrego & Mariño 1998; Kinzelman *et al.* 2003; WHO 2003).

There are two standard methods accepted by the EU (EU 2006) for the enumeration of indicator microorganisms from waters. The multiple tube fermentation technique, which provides a most probable number (MPN) of microorganisms after cell growth in broth, and the membrane filtration (MF) technique, which enumerates the colonies grown on the surface of an adequate solid media providing a colony-forming unit (CFU) count (*Standard Methods* 1998). Both methods require confirmation tests after the initial observation of a positive result. Consequently, a complete analysis can require an additional 24–72 h to obtain a final result, which constitutes the major limitation of these methods (Geissler *et al.* 2000; Toranzos *et al.* 2007). In addition, although these methods show a good recovery for coliforms and enterococci from natural and treated waters, the false-positive and false-negative percentages found in recreational waters may be higher than 10% (Budnick *et al.* 1996; Yakub *et al.* 2002). To overcome these shortcomings, two semi-automated most probable number methods, Colilert® and Enterolert®, have been developed for the enumeration of coliforms and enterococci, respectively. These methods require less quality control testing than the MF procedure (Budnick *et al.* 1996).

Two nutrient-indicators, ortho-nitrophenyl galactopyranoside (ONPG) and 4-methyl-umbelliferyl-glucuronide (MUG) are the major sources of carbon in Colilert®. These substrates can be metabolized by the coliform enzyme β-galactosidase and the *E. coli* enzyme β-glucuronidase, respectively. As coliforms grow in Colilert®, they use β-galactosidase to metabolize ONPG and change it from colourless to yellow. *E. coli* uses β-glucuronidase to metabolize MUG with the production of fluorescence under UV light (IDEXX 2001). The Enterolert® test utilizes a nutrient indicator substrate, 4-methylumbelliferone-β-D-glucoside, which fluoresces when it is metabolized by enterococci. Methylumbelliferyl derivatives are highly sensitive and specific, non-carcinogenic and easily detected under UV light (Budnick *et al.* 1996).

Several studies have been carried out to compare the standard methods and the defined substrate technology for the enumeration of indicator microorganisms in natural waters, such as total coliforms, *E. coli* and enterococci (Buckalew *et al.* 2006; Griffith *et al.* 2006; Horman & Hanninen 2006). However, the equivalence between both methodologies has not been deeply studied yet.

The aim of this work was to study the equivalence between the IDEXX technology and the standard methods used for the enumeration of total coliforms, *E. coli* and enterococci from natural recreational waters of southern Portugal. The results of this study should be applied to the national monitoring programme of the microbiological quality of recreational waters as alternative methods (EU 2006), since defined substrate-based technology shows higher efficiency and suitability, and is easier to apply compared with standard methods.

METHODS

Sampling and physicochemical parameters

Natural recreational water samples were collected using sterile plastic bottles from the subsurface layer (30 cm), and transported to the laboratory in cold boxes at approximately 4°C. Samples were processed within 6 h after collection. To determine the pH and salinity values, a Crison GLP22 pHmeter, and a Crison GLP32 conductimeter, respectively, were used.

Microbiological parameters

For the enumeration of total coliforms and *E. coli*, a total of 39 samples were analysed in parallel by the standard method membrane filtration described in the NF EN ISO 9308-1 standard (ISO 2000*a*), and the Colilert® defined substrate technology following the manufacturer recommendations (Idexx Laboratories, Westbrook, Maine).

ISO 9308-1 standard method involves the incubation of membrane filters (47 mm diameter and 0.45 μm pore-size) on Tergitol-TTC agar (Oxoid Ltd, Basingstoke, UK) at 36 ± 1°C for 21 ± 3 h for total coliforms and at 44.5 ± 0.5°C for *E. coli*. Colonies that showed negative oxidase reaction were considered as coliform bacteria. Colonies with negative oxidase reaction and positive indole reaction were considered as *E. coli*.

For the enumeration of enterococci, 46 samples were analysed by the reference method described in the NF EN ISO 7899-2 standard (ISO 2000*b*) and the Enterolert® defined substrate technology, according to the manufacturer recommendations. The reference method uses membrane filters (47 mm diameter and 0.45 μm pore-size) cultured on Slanetz & Bartley agar (Biokar Diagnostics, Pantin, France) incubated at 36 ± 1°C for 44 ± 4 h. Red, brown or rose colonies were considered as typical colonies of presumptive enterococci. The confirmation was performed by transferring the filters to Bile Esculin Azide agar (Biokar Diagnostics) according to Figueras *et al.* (1996) previously preheated at 44°C. Membrane filters were incubated at 44 ± 1°C for 2 h, and all the colonies with brown or black colour were considered as enterococci.

Statistical analyses

Equivalence of the methods was analysed as described in the ISO 17994:2004 standard (ISO 2004), which establishes the criteria for the equivalence of microbiological methods. The equivalence between techniques is based on a confidence interval determined for the expanded uncertainty around the relative mean difference. The relative difference (x_i) is given by: $x_i = [\ln(a_i) - \ln(b_i)] \times 100\%$, where a_i and b_i are the results obtained for the same sample i with the method in study (a) and the reference method (b). Samples that showed results of zero with both methods were excluded from the study. The relative difference for samples that showed results equal to zero when they were analysed by one of the methods used is calculated by: $x_i = \ln(a_i + 1) \times 100\%$ if the result was (a_i, 0); and by $x_i = -\ln(b_i + 1) \times 100\%$, if the result was (0, b_i). The relative mean difference (X) is given by the mean of the relative difference of all samples. The expanded uncertainty (U) is determined by: $U = ks/\sqrt{n}$, where k is 2 for a 95% confidence interval, s is the standard deviation of the relative mean difference, and n is the total number of samples. The confidence interval has a lower (x_L), and a higher (x_H) limit given by: $(X - U)$ and $(X + U)$, respectively. The methods are considered 'not different', if the relative mean difference is not significantly different from zero and the expanded uncertainty is not far away from the established level for the maximum acceptable deviation D. Or: $-D \leq x_L \leq 0$ and $0 \leq x_H \leq +D$. In this study we assumed D as 10%, which is the standard recommended value when analysing waters for human consumption. The methods are 'different' if: $x_L > 0$ or $x_H < 0$. The results are considered 'inconclusive' if: $x_L < -D$ and $x_H > 0$ or if $x_L < 0$ and $x_H > +D$. In this case, the analysis of a higher number of samples is recommended, and the estimation of that number is given by: $n = 4(s/y)^2$ where n is the number of samples; s is the standard deviation of the relative mean difference and y is the higher value of two: $y_1 = X$ and $y_2 = |X| - |D|$, both X and D are in percentage units.

Non-parametric statistical methods were used to test for correlations and statistical differences between methods. Non-parametric tests were used because faecal indicator bacteria data were not normally distributed. Spearman's rank correlation was used to determine whether results between methods were significantly different. The relationships between environmental variables and estimated microbial concentrations were determined applying the Pearson correlation analysis. In all tests, a P-value of 0.01 was employed and all analyses were conducted using STATISTICA version 6.

RESULTS

The values for pH and salinity are shown in Table 1. pH varied from 6.94 to 8.0, with a mean of 7.61, in the samples used for coliform and *E. coli* enumeration. In the samples used for enterococci analysis, the pH variation was between 7.23 and 8.38, and the mean value was 8.02. In samples used for coliform and *E. coli* enumeration, the salinity values varied from 33.8 to 36.0‰, with a mean value of 34.5‰. A maximum value of 35.6‰ and a minimum of 28.0‰ were determined in samples used for enterococci enumeration.

Table 1 | Values obtained for the pH and salinity of the natural samples analysed

	pH			Salinity (‰)		
Samples	**Minimum**	**Maximum**	**Mean**	**Minimum**	**Maximum**	**Mean**
Coliforms and *E. coli*	6.94	8.00	7.61	33.8	36.0	34.5
Enterococci	7.23	8.38	8.02	28.0	35.6	33.6

The results of the enumeration of total coliforms, *E. coli* and enterococci are shown in Table 2. Total coliforms were present in a concentration ranging from zero to 6.0×10^7 CFU/100 ml, whereas the concentration of *E. coli* was between zero and 3.6×10^7 CFU/100 ml using the standard method of membrane filtration. The Colilert® test showed a variation between zero and 2.4×10^6 MPN/100 ml for both total coliforms and *E. coli*. This interval was between zero and 1.1×10^4 CFU/100 ml for enterococci by the standard method, and between zero and 6.7×10^3 MPN/100 ml by the Enterolert® procedure.

Table 3 gives the percentage of the analysed samples that were below and above the European Bathing Water Directive (2006/7/EC) limit for microbiological quality of recreational waters (EU 2006). Water for recreational activities should not exceed 500 CFU/100 ml for *E. coli*, and 200 CFU/100 ml for enterococci. The results showed that, 52.5% of the samples analysed for *E. coli* by the standard method and 57.5% of the samples analysed by Colilert® were below the limit for good microbiological water quality. For the enterococci parameter, 38.6% and 50.0% of the samples analysed by the standard method and by the Enterolert® procedure, respectively, were below the limit. A lower percentage of the samples would not be adequate for recreational activities when analysed by the defined substrate-based procedures in comparison with the standard method, which suggests that the new technology is more restrictive than the ISO standard methods.

The relative differences between the standard method (MF) and the defined substrate technology are shown in Table 4. For total coliforms and *E. coli*, the 95% confidence interval determined $(X - U;\ X + U)$ was 109.98; 286.50 and − 78.34; 152.64, respectively. These results suggest that the methods under study are not equivalent for the enumeration of total coliforms, and more samples should be analysed to compare the enumeration of *E. coli* by both methods. For the enterococci, the 95% confidence interval was − 223.07; − 87.17, showing that these methods are not equivalent.

Table 2 | Enumeration of total coliforms, *E. coli* and enterococci in the natural water samples analysed

		Standard method			Defined substrate-based method		
Microbial parameters	**n**	**Minimum (CFU/100 ml)**	**Maximum (CFU/100 ml)**	**Mean ± SD (CFU/100 ml)**	**Minimum (MPN/100 ml)**	**Maximum (MPN/100 ml)**	**Mean ± SD (MPN/100 ml)**
Coliforms	39	0	6.0×10^7	$1.5 \pm 0.9 \times 10^6$	0	2.4×10^6	$1.8 \pm 5.3 \times 10^6$
E. coli		0	3.6×10^7	$8.8 \pm 5.6 \times 10^4$	0	2.4×10^6	$3.8 \pm 2.3 \times 10^5$
Enterococci	46	0	1.1×10^4	$1.5 \pm 0.3 \times 10^3$	0	6.7×10^3	1.7 ± 0.9

Table 3 | Percentage of samples below and above the water quality limit acceptable in the European Bathing Water Directive (2006/7/EC) for *E. coli* (500 CFU/100 ml) and enterococci (200 CFU/100 ml) for recreational waters

Bacteria	**Method**	**% Samples < limit**	**% Samples > limit**
E. coli	Standard (ISO 9308-1:2000)	52.5	47.5
	IDEXX (Colilert®)	57.5	42.5
Enterococci	Standard (ISO 7899-2:2000)	38.6	61.4
	IDEXX (Enterolert®)	50.0	50.0

Table 4 | Analysis of the relative differences between the standard method of membrane filtration and the defined substrate technology for the enumeration of total coliforms, *E. coli* and enterococci

Bacteria	*n*	Relative mean difference (*X*)	Standard deviation (*s*)	Expanded uncertainty (*U*)	*X* − *U*	*X* + *U*	Evaluation
Coliforms	39	198.24	278.90	88. 62	109.98	286.50	No equivalent
E. coli		37.15	364.94	115.49	−78.34	152.64	Inconclusive
Enterococci	46	−155.12	225.26	67.95	−223.07	−87.17	No equivalent

Spearman's correlation coefficients were significant ($P < 0.01$) among all methods. Correlation coefficients between MF (ISO method) and defined substrate-based methods were 0.754 for total coliforms, 0.676 for *E. coli* and 0.665 for enterococci. The Pearson correlation analyses indicated a direct and significant ($P < 0.01$) correlation between pH and salinity and between salinity and MF for samples used in the total coliform analysis (Table 5). In the case of *E. coli* analyses, only a significant correlation between pH and salinity was obtained (Table 5). In contrast, significant correlations were found between pH and salinity, pH and MF technique, pH and Enterolert® and between salinity and MF technique for the samples used for the enterococci determination (Table 5).

DISCUSSION

The European Water Directive defines reference methods for the enumeration of microbiological parameters in water. One of the standard methods for coliforms and *E. coli* is the membrane filtration technique on Tergitol-TTC agar confirmed with the oxidase and indole tests (EU 2006). However, several technical drawbacks of the procedure, as well as limitations regarding the taxonomy of these microorganisms, require the evaluation of alternative methods (Bernasconi *et al.* 2006). The Colilert® test is referred to in the *Standard Methods for the Examination of Water and Wastewater* (1998) as a method for the enumeration of *E. coli* with application to surface water samples. The detection limit of this procedure is 1 MPN/100 ml, and the precision is ± 20% relative percentage of deviation. Although calibration and normalization of this procedure have not been performed yet, the Colilert® procedure has already been approved or accepted for the analysis of drinking waters in several countries of the European Union (Schets *et al.* 2002; Niemela *et al.* 2003; Jeppesen 2007). In the case of natural waters, the US Environmental Protection Agency nominated and approved the Colilert® and the Enterolert® tests for the enumeration of *E. coli* and enterococci, respectively (USEPA 2003).

The IDEXX principle is based on enzymatic activities, which depend on the physiological state of the bacteria. Changes in irradiation, salinity, temperature, pH and nutrient concentration of the environment may cause stress on bacteria and, consequently, may hamper the bacterial recovery on culture media (Fiksdal *et al.* 1994). Under these adverse conditions, bacteria adopt a survival state known as viable but non-culturable (VBNC). Although in this state it is not possible to culture bacteria on conventional growth media, cells preserve their metabolic activities, such as respiration, membrane integrity and gene transcription, and pathogenic bacteria keep their virulence factors (Toranzos *et al.* 2007). In addition, VBNC bacteria can recover the division ability when favourable environmental conditions are restored (Lleò *et al.* 2001).

In the present study, Pearson's correlation coefficient between physicochemical parameters and bacterial enumeration methods showed a significant correlation

Table 5 | Matrix of correlation between the environmental variables tested and microbial parameters using the Pearson correlation analysis

	Coliforms			*E. coli*			Enterococci		
Variable	Salinity	MF	Colilert®	Salinity	MF	Colilert®	Salinity	MF	Enterolert®
pH	0.549*	0.016	−0.082	0.549*	−0.138	−0.114	0.539*	−0.699*	−0.696*
Salinity	1	0.409*	0.160	1	0.184	0.296	1	−0.406*	−0.274

*Significant at the 0.01 level.

(0.409 at a significance level of $P = 0.01$) only between the salinity and the standard method (MF) for the enumeration of total coliforms. However, no significant relationship was obtained between pH values and both enumeration methodologies. Coliforms possess an enzyme, β-D-galactosidase, that catalyses the breakdown of lactose in galactose and glucose. Several studies have demonstrated a gradual increase in environmental β-D-galactosidase activity when the pH increases from 5 to 7.5, and a maximal activity at pH values between 7.2 and 7.8 in freshwater samples (George *et al.* 2000; Wutor *et al.* 2007). An increase of pH from 7.8 to 9 resulted in a reduction of the β-D-galactosidase activity by the same percentage (40%) as the increase between pH 7.2 and 7.8 (Wutor *et al.* 2007). Significant correlations between the physicochemical parameters tested and the *E. coli* numbers were not obtained in the present study (Table 5). George *et al.* (2000) determined the activity of the enzyme β-D-glucuronidase produced by *E. coli* in natural waters, obtaining the maximal activity at pH 6.9 and 44°C. Our results showed a negative correlation between pH and the enterococci concentration obtained by both standard methodology (-0.699 at a significance level of $P = 0.01$) and Enterolert® (-0.696 at a significance level of $P = 0.01$). A negative and significant correlation between salinity and the enumeration of enterococci by the standard method (-0.406 at a significance level of $P = 0.01$) was also found, which can be explained by the discharge of contaminated freshwaters that possess lower salinity.

In 82.5% of the samples analysed for total coliform enumeration by the Colilert® test, the results showed higher values than those yielded using the standard method. Following the ISO 17994:2004 standard (ISO 2004) that states the criteria for the equivalence of microbiological methods, it can be concluded that these methods are not equivalent for total coliform enumeration (Table 4). Similar results were obtained by other authors studying natural and drinking waters (Fricker *et al.* 1997; Eckner 1998; Schets *et al.* 2002; Ribas *et al.* 2005), or wastewater samples (Eccles *et al.* 2004). The Colilert® procedure generally yields a higher number of total coliforms when compared with the membrane filtration method. Differences in the culture media, as well as in the substrate used by both procedures, can lead to the detection of different species within the coliform group, which can account for this different result obtained. *Standard Methods* (1998) states that the low bacterial enumeration obtained by membrane filtration methods might be caused by the poor identification of colonies in turbid samples, the presence of high numbers of non-coliforms or toxic substances in the sample. To grow on a solid culture medium, coliforms need the presence of two enzymes: β-lactate-permease, which transports the lactose inside the cell, and β-galactosidase, which metabolizes the lactose with the production of acid and gas. Coliforms without the coding gene for β-lactate-permease will not be differentiated on solid culture medium unless the lactose is present in high concentrations. Those coliforms will be enumerated in the defined substrate technology as they have the coding gene for the lactose metabolization (Ribas *et al.* 2005).

It seems that the existence of a higher coliform population encoding just for the β-galactosidase enzyme leads to higher enumerations of coliforms using the Colilert® method (Schets *et al.* 2001). Interference from non-coliforms does not appear to influence Colilert® results, since the medium contains two nutrient-indicators that are metabolized by coliform enzymes, which are absent in most non-coliform organisms (IDEXX 2001). However, it has also been demonstrated that the Colilert® procedure may detect and enumerate *Aeromonas* spp. and *Vibrio* spp. as false-positive coliforms (Davies *et al.* 1995; Landre *et al.* 1998). In addition, β-galactosidase has been found to be present in non-coliform strains, which makes the level of enzymatic activity a result of the proportion of target and non-target bacteria in rapid enzymatic assays without a cultivation step (Tryland & Fiksdal 1998).

In 54.8% of the samples analysed for the enumeration of *E. coli*, the Colilert® procedure showed higher values when compared with the standard method of membrane filtration. The results obtained in this study using the criteria defined in the ISO 17994:2004 standard (ISO 2004) are not conclusive, which means that more samples should be analysed (Table 4). Using the same standard for the estimation of the number of samples, 385 samples should be analysed in order to establish the equivalence of these methods. Similarly, Ribas *et al.* (2005) concluded that both methods are not equivalent when using the ISO 17994:2004 standard to establish that comparison. In contrast, other authors concluded that the standard and defined-substrate

methods were equivalent to analyse different types of water (Fricker *et al.* 1997; Eckner 1998; Yakub *et al.* 2002; Buckalew *et al.* 2006). However, since the Colilert® system requires short incubation periods and is easy to apply, it can be useful for enumerating coliforms from natural waters and wastewaters (Grasso *et al.* 2000; Kramer & Liu 2002; Eccles *et al.* 2004). Studies carried out with tropical marine and freshwater samples resulted in a disparity between the numbers estimated by Colilert® and membrane filtration (Chao *et al.* 2003). In these studies, the concentration of microorganisms estimated by the enzymatic technique was 1–2 orders of magnitude higher than that detected by the membrane filtration technique (Pisciotta *et al.* 2002; Chao *et al.* 2003). Chao (2006) found a high rate of false-positive for coliforms and *E. coli* after culture using Colilert®. A possible explanation of those findings could be the presence of aquatic strains belonging to *Vibrio* and *Aeromonas* species, which are lactose-fermenting and, therefore, may interfere in β-galactosidase-based assays producing significant false-positive reactions (Davies *et al.* 1995; Landre *et al.* 1998; Yakub *et al.* 2002).

In 82.6% of the samples analysed for the enumeration of enterococci, the standard method of membrane filtration showed higher enumerations when compared with the Enterolert® test. These methods gave results that were not equivalent to those yielded by the standard ISO 17994:2004 (ISO 2004) (Table 4). There are only a few reports about the comparison of the methods under study for the enumeration of enterococci (Budnick *et al.* 1996; Adcock & Saint 2001; Yakub *et al.* 2002; Kinzelman *et al.* 2003). Budnick *et al.* (1996), using marine and fresh recreational waters, concluded that the standard method of membrane filtration and the Enterolert® test were equivalent for a confidence interval of 95%. These authors obtained percentages of false-positive and false-negative of 5.1 and 0.4%, respectively, using the Enterolert® procedure. In contrast, Adcock & Saint (2001) found that Enterolert® significantly underestimated the number of enterococci in river water samples.

CONCLUSIONS

The results obtained in this study confirm that defined substrate-based procedures provide higher numbers of total coliforms and *E. coli* than those obtained by the standard method of membrane filtration, although they are less sensitive for the enumeration of enterococci in recreational water samples tested.

Owing to the fact that enterococci have been proved to be the most reliable predictors of health risk in recreational waters (Kay *et al.* 1994, 2004; Mariño *et al.* 1995*a*; Borrego & Mariño 1998), it is necessary to carry out more studies to improve the detection and enumeration of enterococci in natural waters using the defined substrate-based procedure.

The defined substrate-based methods tested might be included in monitoring programmes for microbial quality of recreational waters on the basis of their higher efficiency and suitability compared with standard methods and because they are easier to apply.

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