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An operational method for the real-time monitoring of *E. coli* numbers in bathing waters

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Abstract

The aim of this study was to investigate the potential application of the β-D-glucuronidase (GLUase) activity measurement for the routine detection and quantification of *E. coli* in marine bathing waters. GLUase activity was measured as the rate of hydrolysis of 4-methylumbelliferyl-β-D-glucuronide. Culturable *E. coli* were quantified by the most probable number (MPN) microplate method. Both methods were applied to a large set of seawater samples. Significant correlation was found between the log of GLUase activity and the log of culturable *E. coli*. The mean coefficient of variation (CV) of the GLUase activity was less than 15% at concentrations around the current standards of International regulations whereas the CV of the microplate method was around 30%. When samples were stored at 4 °C and 20 °C, the mean CV of the GLUase activity remained below 15% up to 6 hours after sample collection whereas the range of variation of the microplate method varied between 10 and 50%. We concluded that the GLUase activity is an operational, reproducible, simple, very rapid and low cost method for the real-time enumeration of *E. coli* in bathing waters and should be preferred to the microplate method. The GLUase activity method should be routinely applied to the rapid enumeration of *E. coli* in recreational waters and recommendations for its application were suggested to water quality managers.

Keywords: *E. coli*; Bathing waters; Real-time monitoring

1. Introduction

The quality of waters in coastal areas is a subject of special interest during the summer season. In Europe, more and more bathing beaches are classified at the end of the high season based on a large set of criteria. One of the most important criteria is the microbiological quality of waters. Although the classification of beaches into quality classes was and remains useful, it does not preserve users of recreational waters from the risk of bacterial or pathogen-induced disease. The protection of bathers from possible health risks requires fast, sensitive, simple and quantitative methods for the real-time monitoring of fecal pollution. Rapid methods are also essential to water quality managers to determine the source of pollution when it occurs and more generally, to the operational management of recreational waters.

Fecal coliforms (FC) are used universally as microbiological indicators of water quality and are commonly used to determine the quality of bathing waters. For instance, bathing beaches in the European community (EC) have a guideline compliance limit of 100 FC/100 ml and a maximum allowable concentration of 2000 FC 100 ml^{-1} (European Community Council Directive, 1975). Although FC have traditionally been regarded as good indicators of fecal contamination of

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waters, recent reviews suggested *Escherichia coli* to be a better indicator (Edberg et al., 2000; Fewtrell and Bartram, 2001). The detection of *E. coli* is actually commonly used in France as a current standard instead of FC. Until future changes of the EC regulations, *E. coli* are enumerated in bathing waters in France and their levels checked against the guidelines for FC, although *E. coli* represent only 80 to 95% of the FC counts. The choice of *E. coli* as an indicator organism of fecal pollution is already accepted by various world organizations (APHA, 1995; WHO, 2001). The compliance and maximum allowable values fixed by the future EC regulations should be 250 and 500 *E. coli* 100 ml^{-1}, respectively. These values are more related to those actually recommended by the US Environmental Protection Agency (US EPA, 1999).

The current culture-based test method (m-TEC agar) or the most probable number (MPN) microplate method take about 24–48 h to provide a result on *E. coli* numbers. Although culture-based tests to enumerate *E. coli* were improved in terms of specificity and rapidity by incorporating chromogenic and fluorogenic substrates (Manafi, 2000), they still require 18–24 h to complete. Thus, there is always a minimum one day delay between sample collection and analytical results. The closure of beaches based upon the data of the sample collected the previous day is not appropriate for an efficient protection of bathers. It also causes delay in reopening the beaches, which is not in the public's best interest. The need for a faster method that provides real-time results of *E. coli* concentrations has been widely recognized among responsible health departments. The desirable testing method should meet the following criteria: it should be fast, sensitive, simple, quantitative, portable and be well correlated with the current methods.

Several rapid assays have been developed for enumerating *E. coli*. They include a polymerase chain reaction (PCR)-based method (Iqbal et al., 1997), a fluorescent in situ hybridization (FISH) with a specific fluorescent rDNA 16S probe (Regnault et al., 2000), an immunofluorescence method (Zaccone et al., 1995), the detection of β-D-glucuronidase (an enzyme specific of *E. coli*) positive cells by solid phase cytometry (Van Poucke and Nelis, 2000) and more recently, an ATP bioluminescence assay associated with immunomagnetic separation (IMS) (Lee and Deininger, 2004). Most of these methods are specific enough but can only be performed in a well-equipped laboratory and require well-trained staff. The bioluminescence assay associated with IMS is fast and the equipment is portable but the IMS apparatus is expensive and the recovery of cells is never 100%. The PCR-based assays without any culture step have limitations in the quantification and discrimination of viable micro-organisms present at low concentrations (below 5 cells per ml) in the natural environment and are not yet appropriate for routine application. The FISH approach, although it is the most specific method, is time-consuming and it is often difficult or impossible to count cells in surface waters at concentrations below 10^{3-4} in 100 ml by epifluorescence microscopy (Garcia Armisen and Servais, 2004). Similar limitations apply to the immunofluorescence assay.

Some studies have proposed to estimate the β-D-glucuronidase (GLUase) activity of *E. coli* in rapid assays performed without any cultivation step as a surrogate of *E. coli*. Good correlations in log-log plot were generally found in natural waters between GLUase activity and FC or *E. coli* levels (Fiksdal et al., 1994; George et al., 2000, 2001, 2004; Farnleitner et al., 2001, 2002; Caruso et al., 2002). In these studies, GLUase activity was estimated by fluorometry as the production of fluorescent methylumbelliferone (MUF) resulting from the hydrolysis of the substrate 4-methylumbelliferyl-β-D-glucuronide (MUGlu). The aim of this study was to evaluate if the GLUase activity measurement can be routinely used for the monitoring of *E. coli* concentrations in coastal seawater samples in regards to the present regulations and to compare this method and its reproducibility to the normalized microplate method actually used in France for the routine monitoring of recreational waters during the summer season. The effect of sample storage at 4 °C and 20 °C between collection and microbiological analysis was also investigated. Preliminary results of the first application of GLUase activity measurement to the monitoring of bathing waters are also presented.

2. Methods

2.1. Samples collection

A total of 256 seawater samples were collected from different beaches along the French Catalan Mediterranean coast. Two beaches were analyzed daily (working days) from 15th of June to 1st of July 2004. Additional samples were sometimes collected near the release of treated wastewater in order to get samples within a wide range of *E. coli* concentrations. All samples were collected in sterile 2 l bottles, kept at 4 °C and analyzed within 2 h. When appropriate, samples were stored in the dark at room temperature (20 °C ± 3 °C) for storage experiments (storage time up to 24 h).

2.2. E. coli enumeration

A standardized miniaturized MPN method (ISO 9308-3) using microplates (Bio-Rad) was used for the enumeration of *E. coli*. In this method, based on the defined substrate approach (Edberg and Edberg, 1988), 200 µl of the several dilutions (1/2, 1/20, ...) of

the sample were added in each of the 96 wells of the microplate containing the substrate 4-methylumbelliferyl-β-D-glucuronide (MUGlu) in dehydrated form. This substrate is hydrolyzed by the β-D-glucuronidase that releases the fluorescent compound methylumbelliferone (MUF), which can be detected under ultraviolet light. The microplates were incubated for 36–48 h at 44 °C and the presence of *E. coli* was evaluated in each well by detection of fluorescence under UV light. The number of positive (fluorescent) wells allow the calculation of *E. coli* abundance using a statistical analysis based on Poisson's law.

2.3. β-D-glucuronidase activity measurements

β-D-glucuronidase (GLUase) activity measurements were performed following the protocol proposed by George et al. (2000) but slightly modified. Seawater samples (100 ml) were filtered through 0.2 μm-pore-size, 47 mm-diameter polycarbonate filters (Nuclepore). The filters were placed in 200 ml-sterile Erlenmeyer flasks containing 10 ml of sterile phosphate buffer (pH 6.9) and then, 2 ml of MUGlu solution (50 mg of MUGlu (Sigma, St. Louis, MO) and 20 μl of Triton X-100 in 50 ml of sterile water) was added to each flask (final concentration of 167 mg l^{-1}). The flasks were incubated in a shaking water bath at 44 °C. Every 5 min for 30 min, a 2 ml aliquot of the 12 ml was poured into a quartz cell with 30 μl of 2 M NaOH solution to obtain a pH between 10.5 and 11 (corresponding to the maximum of fluorescence of the MUF). The fluorescence intensity of the aliquot was measured with a Fluorescence Spectrophotometer (F-2500 Hitachi) at an excitation wavelength of 362 nm and emission wavelength of 445 nm. The fluorometer was calibrated using standards of known MUF concentrations from 0 to 1000 nM. The production rate of MUF (picomoles of MUF liberated per minute for 100 ml of sample filtered), expressing the enzymatic activity, was determined by least-squares linear regression when plotting MUF concentration versus incubation time. All the data of GLUase activities presented in this paper are expressed in picomoles of MUF liberated per minute for 100 ml of sample filtered.

2.4. Statistical analysis

The relationships between enzymatic activities and *E. coli* concentrations were determined by linear regression models. A parametric two-way analysis of variance (ANOVA) was used to determine the effect of temperatures and storage on the results provided by the two methods. All statistical analyses were performed using the Excel software (Microsoft) operating on a Macintosh G4 computer.

3. Results

To study the relationship between the GLUase activity and *E. coli* concentration in the samples from the various investigated seawater beaches, regression analysis was undertaken. In Fig. 1, log-transformed GLUase activities were plotted against log-transformed *E. coli* abundance. *E. coli* numbers below the detection limit of the microplate method (<15 *E. coli* 100 ml^{-1}) were not considered in this regression. The linear correlation between both variables in log units was highly significant ($r^2 = 0.81$, $n = 256$, $p < 0.001$).

The reproducibility of both methods was investigated by determining the coefficient of variation (CV) from four or five replicated samples covering a wide range of *E. coli* concentrations. In Fig. 2, the CV of the GLUase activity was generally below 10% but increased at the lowest activities, mainly below 6 pmoles min^{-1} 100 ml^{-1}. For the microplate method, CVs ranged between 8% and 68% for a wide range of concentrations and strongly increased below 300 *E. coli* 100 ml^{-1} (Fig. 3).

Both GLUase activity and *E. coli* concentration determined by the microplate method were proportional to the dilution factor (Fig. 4a,b). In both cases, the regression coefficient was highly significant ($n = 5$, $p < 0.001$) (Fig. 4a,b).

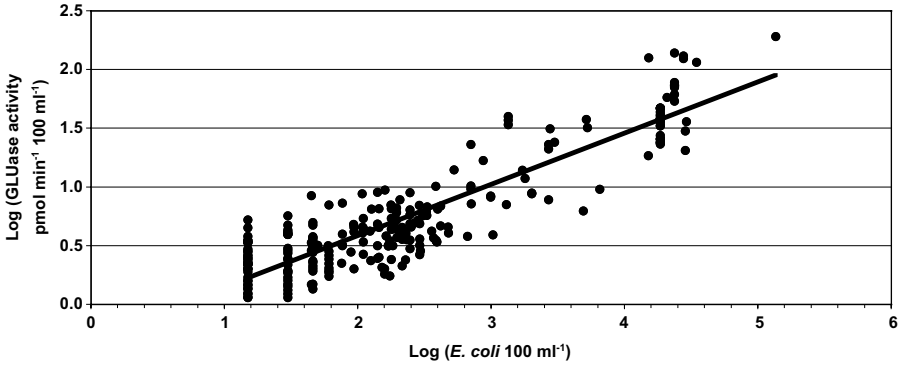

Fig. 1. Log–log linear regression between GLUase activity and *E. coli* concentrations in seawater samples: Log (GLUase act. pmoles min^{-1} 100 ml^{-1}) = 0.436 Log (*E. coli* 100 ml^{-1}) −0.818 ($r^2 = 0.81$, $n = 256$, $p < 0.001$).

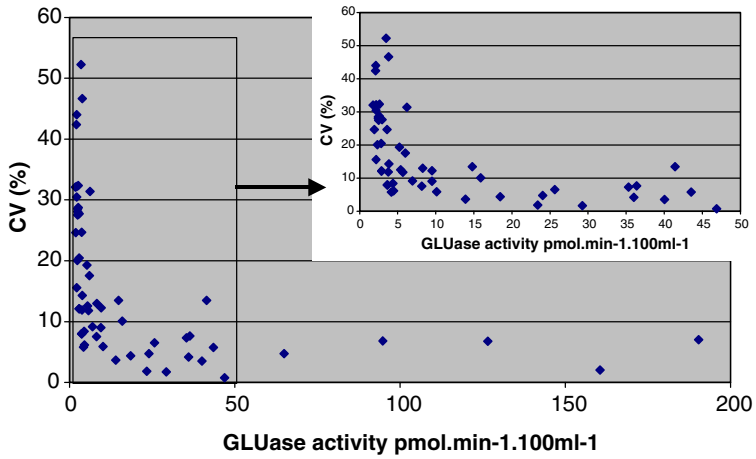

Fig. 2. Coefficient of variation (CV%) determined from 4 or 5 replicated samples versus GLUase activities.

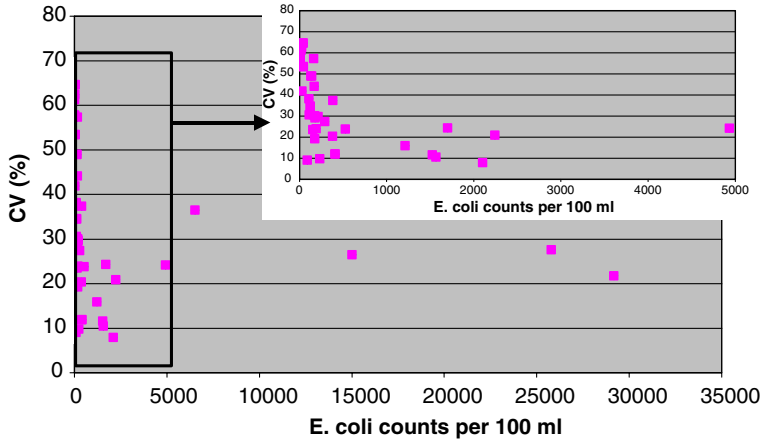

Fig. 3. Coefficient of variation (CV%) determined from 4 or 5 replicated samples versus *E. coli* concentrations.

For both methods, the effect of storage after sampling was investigated at two temperatures (4 and 20 °C) and at different times. At each temperature, subsamples were analyzed after 0, 2, 4 and 6 h of storage. Four replicates were analyzed at each time and each temperature. The experiment was reproduced on three different beaches corresponding to different contamination levels ranging from 15 to 1000 *E. coli* 100 ml^{-1} (as determined from the microplate method). In all cases, differences among temperatures were not significant (except one case for the enzymatic method) but the time of storage had sometimes a significant effect on the results obtained by both methods. In all cases, the variability associated to the measure itself was very high, the highest for the microplate method and much more important than variations due to the time and temperature of storage (Table 1).

Both methods were applied to the monitoring of two beaches between 15th June and 1st July 2004 (Fig. 5). *E. coli* concentrations were determined from GLUase activity measurement (mean from three replicates) using the regression model of Fig. 1. The predicted values were then compared to the observed ones determined by the microplate method (one microplate per sample). The absence of replicates for the microplate method was decided to mimic the strategy actually applied by official laboratories in France for the monitoring of *E. coli* on beaches. Therefore, the standard deviation reported on the histograms was determined from the relationship between SD and *E. coli* concentrations which was obtained from the data used in Fig. 3 (data not shown). For the enzymatic method, the standard deviation was determined from the three replicated samples. In some cases, *E. coli* concentrations calculated from the enzymatic activity were higher than those determined by the microplate method. For 12 samples among 30, *E. coli* concentration determined by the enzymatic activity was significantly higher than that determined from the microplate method. Although the opposite result was found in a few cases (i.e, days 1, 8, 10, 11, 13 and 14

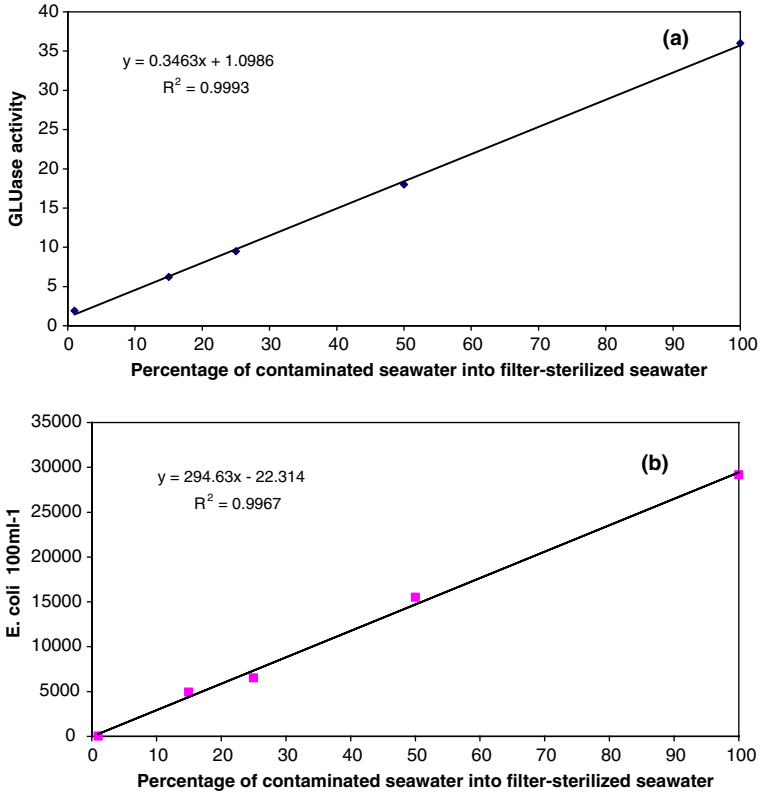

Fig. 4. GLUase activity (a) and *E. coli* concentration (b) versus theoretic values determined from dilution factors of a contaminated seawater into sterile seawater.

Table 1
Two-way ANOVA test to analyse variations in GLUase activity and *E. coli* concentrations associated to the temperature of storage (4 and 20 °C), time of storage (0, 2, 4 and 6 h), interaction between both and measures (four replicates)

	GLUase activity						*E. coli* concentration (microplate)					
	Beach 1		Beach 2		Beach 3		Beach 1		Beach 2		Beach 3	
T (°C)	2.6	NS	4.2	NS	34.3	S***	0.3	NS	1.2	NS	0.4	NS
Time	22.6	NS	30.6	S*	18.2	S**	24.8	NS	32.7	S*	1.3	NS
Interaction	13.3	NS	11.2	NS	23.2	S***	7.9	NS	6.6	NS	9.3	NS
Measure	61.5		54.0		24.3		67.0		59.5		89.0	

Results are given as percentages of total variance.
NS: non-significant difference.
S: significant difference.
* $p < 0.01$.
** $p < 0.05$.
*** $p < 0.001$.

for beach 2), differences were not significant due to an important standard deviation (data not shown).

4. Discussion

4.1. Relationships between methods

Data from Fig. 1 are congruent with previous reports showing good correlations in log-log plot between GLUase activity and *E. coli* concentrations estimated by plate counts using Chromocult medium in river waters (Farnleitner et al., 2001) or by immunofluorescence and fluorescence microscopy in seawaters (Caruso et al., 2002) and between GLUase activity and culturable FC (Fiksdal et al., 1994; George et al., 2000, 2004). The slope of the log-log linear equation between GLUase activity and *E. coli* abundances was lower than 1 (0.51) in agreement with previous studies (Caruso et al., 2002). This indicates that the GLUase activity per cultured *E. coli* cell decreased as the number of *E. coli* increased. This was already discussed by George et al. (2000) and explained by an underestimation of culturable *E. coli* cells in the less contaminated waters due

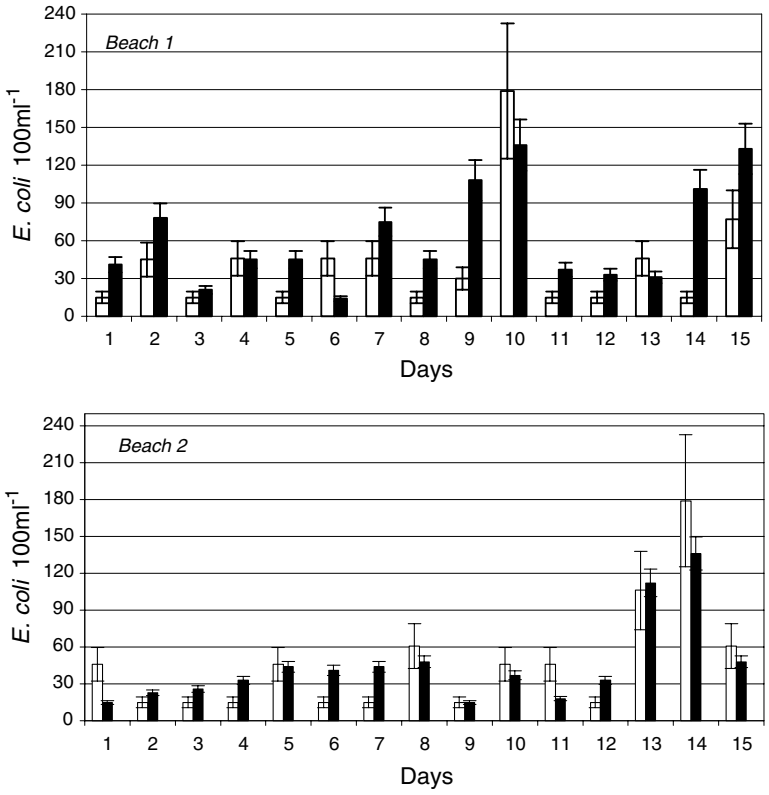

Fig. 5. Daily monitoring of *E. coli* concentrations determined by the GLUase activity method (black bars) and the MPN method (white bars) at two different beaches along the French Catalan coast.

to the presence of active but non-culturable (ABNC) cells which maintain a GLUase activity. Therefore, *E. coli* concentrations may be underestimated at low concentrations when determined by culture-based methods such as the microplate method used in this study. With the spectrofluorometer used in this study, the detection limit of the enzymatic assay was about five *E. coli* per 100 ml but the coefficient of variation was more than 30% at this very low concentration. The detection limit for the microplate approach is 15 cells per 100 ml.

4.2. Reproducibility of both methods

The coefficient of variation determined from replicated samples was less than 15% for the GLUase method for values higher than 6 pmol min^{-1} 100 ml^{-1}. This enzymatic activity corresponds to 283 *E. coli* per 100 ml when determined from the regression equation (see above). Therefore, the reproducibility of this method is good enough in the range of values fixed by the Council of European communities for bathing waters in the present (2000 FC/100 ml) and future (500 *E. coli*/100 ml) regulations. The method is also applicable in regards to the present guidelines for recreational waters (236 *E. coli* per 100 ml as a single sample maximum allowable density for freshwater bathing areas) fixed by the US Environmental Protection Agency (US EPA, 1999). The coefficient of variation of plate count standard techniques based on selective medium is around 25% (deduced from Zaccone et al., 1995). Thus, the mean CVs reported for the enzymatic method are lower than those found with the most commonly used standardized method. For the microplate method, the mean CV is higher and around 30% for a wide range of *E. coli* concentrations. It may partly explain the important dispersion of values around the regression straight line in Fig. 1. Values up to 40% of variation were found for concentration of 400 *E. coli* per 100 ml. This concentration is in the range of values that will be applied in the future EC regulation for bathing waters. This result means that a single measurement of *E. coli* concentration in seawater cannot provide a confident result. At least three replicate samples should be analyzed to increase this confidence. Actually the analysis of seawater beaches by National Agencies in France is based on a single analysis and the interpretation of results is made difficult in regards to the low reproducibility of the method. Increasing the number of samples per beach is difficult because of the large number of samples to be analyzed per day but also because of handling and costs since the microplate method is an expensive method. In contrast, the enzymatic method is not only rapid, more reproducible and easy to perform but also cheap and allows the

analysis of triplicate samples. Triplicate samples analyzed by the enzymatic method will remain less expensive than a single microplate analysis. Furthermore, the method has a great potential for automation.

In most cases, the temperature at which samples were stored until analysis had no significant effect on results. It means that there is no need to cool the samples after collection when analyses are performed within 6 h. The significant effect of the temperature reported in one case for the enzymatic method means that this source of variation was important for this sample but the total variance was low and consequently, the temperature effect had little significance. For two beaches and both methods, significant differences were found during storage. It means that the time of storage may have an effect on *E. coli* concentration, although this effect was not observed for one beach. Therefore, samples should be analyzed as soon as possible after collection. However and for both methods, the most important source of variability was associated with the measurement techniques themselves and the percentage of variance associated with the measurement technique was up to 89% for the microplate method. This is congruent with results reported in Figs. 2 and 3 and suggests that replicate measures should be performed. Therefore and considering the high variations associated with *E. coli* concentrations determined by the microplate method, the correlation between both methods should be considered with caution when used to relate any enzymatic activity to an *E. coli* concentration. In other words, when enzymatic activities are transformed into *E. coli* concentrations using the log–log relationship reported in Fig. 1, the *E. coli* concentration has little significance, particularly for low concentrations because of an increased variability (below 500 cells per 100 ml). Therefore, it would be better to determine guidelines for enzymatic activities using other relationships. One way to do that should be to determine the relationship between enzymatic activities and different culture method in environments where particles are not abundant since attached bacteria may result in an underestimation of *E. coli* concentrations when determined from plating methods (see below).

4.3. Application of both methods to the monitoring of two beaches

The two methods were applied to the daily monitoring of two beaches in June 2004. Although the results clearly show that both methods provide similar trends, differences were sometimes reported and *E. coli* concentrations were significantly higher when calculated from the enzymatic measurement using the log-log relationship. The higher concentration was not due to any autofluorescence signal since fluorescence was not detectable when the sample was incubated in the absence of substrate. However, the fluorescence signal was generally significantly reduced after filtration of the water sample through 2 μm porosity polycarbonate membranes (data not shown). It means that some *E. coli* cells were probably attached to particles and were not correctly enumerated by the microplate approach. In the microplate technique, only a 6.7 ml volume of the initial sample is inoculated into the 96 wells of each microplate and the effect of attached-bacteria may strongly underestimate the number of *E. coli* into 100 ml. This is probably one of the most important drawbacks of the method and this small volume also contributes to the low reproducibility of this method. Since the presence and concentration of attached-bacteria in natural coastal waters is submitted to important variations due to the hydrodynamic of coastal waters, this is probably why differences between the two methods are also submitted to variations from beach to beach. Further analyses including the determination of suspended matter concentrations and the monitoring of weather conditions (i.e, wind intensity and rain) should be helpful to validate these hypotheses.

4.4. Beaches monitoring schedule and rules for beach closure

The GLUase activity method is operational and should be now validated on a large diversity of recreational waters. In a first step, the application of this method will require the comparison with the current growth based method to determine if both methods provide similar results and if not, in which conditions differences can be observed? Why they occur? and what is the most appropriate method? This comparison of methods on different sites and coastal areas is important because if differences are found and provide different conclusions in regards to regulation limits, this may have important consequences for environmental managers and mayors. In France and because environmental managers would like to implement this new method, a regular beach monitoring based on the application of both methods was decided for the coming years by a set of cities located along the French Mediterranean, Atlantic and Channel coasts. As an example, the rules provided below are those suggested and agreed by all mayors of the concerned cities located along the French Mediterranean Catalan coast. These rules should be extended to other sites by those cities who would like to implement this method.

Samples are collected in the morning between 8:00 AM and 10:00 AM and stored at 20 °C until analysis within 2 h after collection. Results on *E. coli* concentrations determined by the enzymatic method are provided within one hour after the arrival of the samples to the laboratory. The results of the daily analyses are reported on the web site of each city before noon on the day of sampling. The web site of each city is in free access in each tourist information bureau. When *E. coli*

concentrations are higher than the maximum allowable concentration actually fixed at 2000 cells per 100 ml, three additional samples are collected within 3 h after the first sample was collected and if the mean value is above 2000, the mayor of the city closes the beach (early in the afternoon) until a new daily mean concentration determined from triplicate samples is below this value. During beach closure, triplicate samples are collected twice a day (morning and afternoon) and analyses are performed within 1 h. When rain events occur, the daily control is made in order to further understand the temporal fluctuations in *E. coli* concentrations but beaches are not closed since bathers are informed that water quality may be degraded under raining conditions and therefore, bathing is strongly inadvisable. All samples are also analyzed using the microplate method until we determine if one method can be used alone. This comparison of methods is made to further investigate the relationship between both methods, to validate the interest of the enzymatic approach for end users and to define recommendations for the application of the method in the future.

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