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ORIGINAL ARTICLE

# Rapid enumeration of *Escherichia coli* in marine bathing waters: potential interference of nontarget bacteria

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**Keywords**
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## Abstract

**Aims:** To compare the *Escherichia coli* quantification given by the 'Coliplage®' assay, based on the direct measurement of the β-D-glucuronidase (GLUase) activity and the reference Most Probable Number (MPN) method from seawater sites and investigate the possible interference of non-*E. coli* strains in the GLUase activity measurement.
**Methods and Results:** Comparison performed from 69 French coastal bathing sites (1401 samples) showed nonconcordance between both methods, only for 8% of samples. Non-*E. coli* 4-methylumbelliferyl-β-D-glucuronide (MUG+) were isolated from nonconcordant samples. Phylogenetic analysis showed that *Gammaproteobacteria* were dominants and mainly represented by *Vibrio* species, which displayed GLUase activities on the same order of magnitude and sometimes much higher as *E. coli* reference strains.
**Conclusions:** The 'Coliplage®' assay is a rapid method for the quantification of *E. coli* showed few discordances with the standard MPN method. Some *Vibrio* species could interfere on the direct GLUase activity measurement of *E. coli*.
**Significance and Impact of the Study:** Data present the first qualitative investigation on disagreement between Coliplage® and the MPN results. If the interference of *Vibrio* species is confirmed *in situ*, appropriate treatments should be developed to remove the interfering signal.

## Introduction

Monitoring programmes for the microbiological quality of bathing waters are based on counts of bacterial indicators of faecal pollution (coliforms, *Escherichia coli*, enterococci). Current methods of enumerating these indicators are all culture based; with such methods, the minimum delay between sampling and result is 24 h. Because of this processing time lag, it is impossible for authorities to decide rapidly a beach closure for protecting public health in case of sudden microbiological contamination, and conversely, it can also delay reopening a beach. The delay can also negatively affect tracking the origins of the contamination. The development of faster methods for evaluating water microbiological quality is a priority for both scientists (Noble and Weisberg 2005) and end-users today. In addition to be rapid, the ideal method to estimate microbiological quality of bathing sites should also be sensitive, simple, quantitative, specific and well correlated with the current methods.

Total coliforms, faecal (also called thermotolerant) coliforms and faecal enterococci have been used for more than a century as bacterial indicators of faecal water contamination; they were used in the first European Bathing Water Quality Directive (EEC 1975) to control the microbiological water quality. But, recent reviews suggested *E. coli* as a better faecal coliform indicator of sanitary risks associated with bathing (Edberg *et al.* 2000; Fewtrell and Bartram 2001; Garcia-Armisen *et al.* 2007). Consequently, *E. coli* enumeration is today substituted for coliforms enumerations in the new European Bathing

Water Quality Directive (EEC 2006). Some authors have proposed using the measurement of an enzymatic activity specific to *E. coli* [β-D-glucuronidase (GLUase)] as an estimate of *E. coli* abundance (Fiksdal *et al.* 1994; George *et al.* 2000) because this enzymatic activity can be estimated in rapid assays without an intervening cultivation step. Good correlations in log–log plots were found between GLUase activity and *E. coli* concentrations estimated by a culture-based method both for coastal seawater (Lebaron *et al.* 2005) and freshwater (Farnleitner *et al.* 2001; Garcia-Armisen *et al.* 2005; Servais *et al.* 2005). In these studies, the direct measurement of GLUase activity was done using the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG), and the activity was measured as the rate of production of fluorescent methylumbelliferone (MUF) (estimated by fluorometry), resulting from the hydrolysis of the substrate. This type of direct activity measurement can be done in as little as half an hour; it was thus proposed as a possible surrogate to culture-based methods for rapid detection of faecal pollution in bathing waters (Lebaron *et al.* 2005; Servais *et al.* 2005). When the GLUase activity measurement is used as a surrogate of *E. coli* enumeration, it is first measured and then converted to *E. coli* concentration using a standard curve, with the final abundance expressed in *E. coli* per 100 ml.

Since 2005, Veolia Water has commercialized in France a test called Coliplage® based on the measurement of GLUase activity to control the quality of bathing waters and to track sources of faecal contamination (Henry *et al.* 2008). With this test, samples are collected early in the morning and results are made available before noon. The water quality of numerous beaches in France was evaluated by this approach during the last two bathing seasons (2006 and 2007) because the authorities in charge of bathing areas management consider the rapidity of the Coliplage® method as a considerable advantage with regard to the classical approaches. When the Coliplage® data are compared to data obtained by the reference culture-based method presently in use (the standardized miniaturized Most Probable Number (MPN) method (ISO 9308-3 1998), some discrepancies were found between the estimates of *E. coli* numbers. In a few cases, the *E. coli* abundance estimated by the Coliplage® method significantly exceeded that estimated by the culture-based method. Different hypotheses have been proposed to explain these discrepancies, among which is the presence of nontarget cells (non-*E. coli* cells) having a GLUase activity that can result in an overestimated *E. coli* abundance using the Coliplage® procedure. Several authors have already suggested the possible interference of nontarget bacteria in the direct GLUase activity measurement (Palmer *et al.* 1993; Fiksdal *et al.* 1997; Van Poucke and Nelis 1997); however, this hypothesis requires more investigations.

The objectives of the present paper were (i) to investigate the potential overestimation of *E. coli* concentration estimated by the Coliplage® method in regard to the reference MPN method and (ii) to determine from some samples, with an *E. coli* enumeration overestimated by Coliplage®, whether the interference on the GLUase activity measurement could be attributed to nontarget bacteria present in the water.

## Materials and methods

### Sample collection

Samples were collected from 14 sites located on the French coastline during two summer bathing seasons (from 15 June to 15 September, 2006 and 2007). The number of beaches per site ranged between 1 and 12. A total of 72 beaches were studied (1401 samples total analysed). All samples were collected in sterile 0·5 l bottles stored at 4°C and analysed within 4 h.

The interference of nontarget bacteria on the GLUase activity measurement was investigated at a group of French coastal bathing sites (both lagoonal and open coastal sites) sampled between 2005 and 2007.

### *Escherichia coli* enumeration

*MPN miniaturized method*

A standardized, miniaturized MPN method (ISO 9308-3 1998) using microplates (Biokar Diagnostics, Beauvais, France) was used for the enumeration of *E. coli.* In this method, based on the defined substrate approach (Edberg and Edberg 1988), 200 μl aliquots of different dilutions (1/2, 1/20, …) of each sample were added to microplate wells containing the substrate, MUG, in a dehydrated form. This substrate is hydrolysed by GLUase releasing the fluorescent compound MUF that is detectable under UV light. The microplates were incubated for 36–48 h at 44°C, and the MUF fluorescence was measured for each well under UV light. *Escherichia coli* abundance was based on the number of positive wells and a statistical analysis using Poisson's law. The detection limit of this method is 15 culturable *E. coli* per 100 ml. The expected coefficient of variation between replicates using this method is about 30% (Lebaron *et al.* 2005; Prats *et al.* 2008).

*Coliplage® method*

GLUase activity measurements were taken following the protocol proposed by George *et al.* (2000), but slightly modified. Seawater samples (100 ml) were filtered through 47-mm-diameter polycarbonate 0·2-μm-pore-size

membranes (GTTP; Millipore), and the membranes were placed in 100-ml sterile Erlenmeyer flasks containing 10 ml of sterile phosphate buffer (pH 6·9). Next, 2 ml of MUG stock solution [55 mg of MUG (Sigma, St Louis, MO, USA) and 20 $\mu$l of Triton X-100 in 50 ml of sterile water] was added to each flask (final MUG concentration of 0·6 mmol $l^{-1}$). The flasks were incubated in a water shaker bath at 44°C. Every 5 min for 20 min, a 2-ml aliquot was removed and added to a quartz cell and pH adjusted to between 10·5 and 11 by adding 40 $\mu$l of 2 mol $l^{-1}$ NaOH (this pH corresponds to the fluorescence maximum for MUF).

The fluorescence intensities were measured with a Fluorescence Spectrophotometer (F-2500 Hitachi or RF 1501 Shimadzu) at an excitation wavelength of 362 nm and emission wavelength of 445 nm. The fluorometer was calibrated using a set of MUF standard solutions from 0 to 1000 nmol $l^{-1}$. 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 *vs* incubation time. The GLUase activities, expressed in picomoles of MUF liberated per minute for 100 ml of sample filtered, were converted into *E. coli* concentrations by straight-line regressions in log–log plot between *E. coli* concentrations estimated by the MPN method and GLUase activities, as the one established for seawater samples by Lebaron *et al.* (2005).

### Isolation of non-*Escherichia coli* GLUase positive (MUG+) bacteria

Non-*E. coli* MUG+ bacteria was investigated on the marine bathing water samples collected from the Vendean and Mediterranean coasts, for which the Colilage® method gave a higher *E. coli* estimation than the MPN method. Cultivable bacteria were recovered from seawater samples by spreading 100 $\mu$l of sampled water onto culture media supplemented with 70 mg $l^{-1}$ of MUG and incubating for 24–72 h at room temperature. Four different culture media were used for isolation: marine agar (Difco, BD, France), R2A agar (Difco) supplemented with sterile seawater (70%), Mineral salts Medium (MMO) agar, MMO (Stanier *et al.* 1966) supplemented with sterile seawater (70%) and nutrient agar without glucose (15·0 g peptone, 3·0 g yeast extract, 6·0 g NaCl, 12·0 g agar). MUG+ bacteria were isolated by randomly picking fluorescent colonies from the agar plates. The isolates were purified onto new agar plates. Each isolate was confirmed as nonfaecal coliform strains after spreading onto tergitol agar medium. The 16S rDNA genes of the 108 bacterial strains isolated were partially sequenced.

### Nucleic acid extraction, 16S rDNA gene amplification and sequencing

DNA was extracted from colony using a thermal lysis protocol. A sample of each colonies was added to 50 $\mu$l of ultra pure water and incubated for 10 min at 94°C. The 16S rDNA genes were amplified from DNA by PCR using the primers SAdir (5′-AGAGTTTGATCATGGCT CAG-3′) and S17 rev (5′-GTTACCTTGTTACGACTT-3′), which are specific for the 1·5-kb region of eubacteria 16S rDNA. The 50 $\mu$l PCR mixture contained 5 $\mu$l of lysis product, 0·2 $\mu$l of each primer (50 $\mu$mol $l^{-1}$), 5 $\mu$l of PCR buffer 10×, 1 U of Super Taq DNA polymerase (HT Biotechnology Ltd, Cambridge, England) and 0·5 $\mu$l deoxynucleoside triphosphate mixture (20 mmol $l^{-1}$). The PCR programme consisted of 35 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 2 min followed by a final extension step at 72°C for 10 min. Amplification products were examined using agarose gel electrophoresis. A partial sequence (*E. coli* nucleotide numbering 28 and 905) for each isolate was obtained using the primer 907r (5′-CCG TCAATTCCTTTGAGTTT-3′). Sequencing reactions were performed by Macrogen (Seoul, Korea).

### Phylogenic analyses

All nucleotide sequences were assembled, analysed and manually edited using BioEdit Sequence Alignment Editor Software (ver. 5.0.9; NC State University, Raleigh, NC, USA) (Hall 1999) and compared to sequences within the NCBI database (http://www.ncbi.nlm.nih.gov/) using Basic Local Alignment Search Tool – 2 sequences (Blast, http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) (Tatusova and Madden 1999).

### $\beta$-D-glucuronidase activity measurements on MUG+ bacterial strains

In order to estimate the GLUase activity of the isolated MUG+ bacterial strains, they were cultivated in liquid medium. They were first grown overnight in Luria Bertoni broth without dextrose (10·0 g tryptone, 10·0 g NaCl, 10·0 g yeast extract, per litre), at 37°C for the *E. coli* strains and at 30°C for the non-*E. coli* strains. Cultures were centrifuged at 2795 ***g*** for 10 min; pellets were resuspended in sterile physiological water (9·0 g NaCl per litre) and homogenized. One per cent of the suspension was then grown in tryptic soy broth without dextrose (17·0 g biotrypcase, 3·0 g biosoyage, 2·7 g $K_2HPO_4$, 5·0 g NaCl, per litre, pH 7·3) supplemented with 0·4 g $l^{-1}$ of methyl-$\beta$-D-glucuronide (MetGlu) (Sigma) added as an inductor of GLUase activity (Tryland and Fiksdal 1998). The cultures were grown at 37°C for the *E. coli* strains

and at 30°C for the non-*E. coli* strains until stationary phase was reached (6 h for *E. coli* strains, several days for strains growing slowly). Induced cultures were centrifuged and resuspended and then homogenized in sterile physiological water, as described above. Aliquots of these suspensions were fixed with formaldehyde (2% final concentration), and total cell concentrations in the induced suspensions were determined by flow cytometry using the SybrGreen I procedure (Lebaron *et al.* 1998). GLUase assays were performed on aliquots of $10^6$ cells added to 200-ml sterile Erlenmeyer flasks containing sterile phosphate buffer and MUG solution as described previously.

According to the result of an experiment conducted to determine the saturating MUG concentration for pure strains, a 1·8-mmol $l^{-1}$ MUG concentration was used to investigate the GLUase activity of the isolated MUG+ strains.

## Results

### *Escherichia coli* enumeration by the Coliplage® and the reference MPN methods: analysis of nonconcordance

The maximum allowable concentration of faecal coliforms is 2000 FC per 100 ml for bathing beaches in the European Union (EEC 1975). In France, however, *E. coli* are enumerated in place of faecal coliforms, but using the same guidelines as those established for faecal coliforms.

The Coliplage® and the reference standardized miniaturized MPN methods were used on 1401 samples from 72 beaches. We evaluated whether or not the *E. coli* concentration obtained by each method gave the same result with respect to the water quality threshold defined in the guidelines (2000 *E. coli* per 100 ml). The number of samples for which *E. coli* concentrations obtained by the Coliplage® method and the MPN method were not in agreement is listed in Table 1. Based on our evaluation criteria, nonconcordance between methods occurred 113 times, representing 8% of the total analysed samples. Per site, the percentage of nonconcordance ranged from 0% (five sites) to 41% (site J).

### Diversity of non- *Escherichia coli* GLUase positive bacteria

The investigation into the diversity of non-*E. coli* GLUase positive bacteria was performed on samples from French coastal bathing sites. A total of 108 non-*E. coli* MUG+ strains were isolated from nonconcordant samples**.** The phylogenetic results are reported in Table 2. The isolated strains were affiliated with the following phylogenic classes: *Gammaproteobacteria* (66·7%), *Bacteroidetes* (32·4%) and *Actinobacteria* (0·9%). Most of the *Gammaproteobacteria* isolates (87·5%) were affiliated with 11 *Vibrio* species Four of these species (*Vibrio harveyi*, *Vibrio splendidus*, *Vibrio chagasii* and one unidentified) were highly represented (86% of the *Vibrio* isolates). Two GLUase positive *Bacteroidetes* species were also frequently

**Table 1** Comparison of *Escherichia coli* concentrations measured with the maximum allowable level for bathing waters (2000 per 100 ml) as estimated by both the Coliplage® and the Most Probable Number (MPN) methods. Samples collected during the summer bathing seasons 2006 and 2007 on 72 beaches at 14 different sites on French coastlines

| Site | Regional location of site | Number of beaches | Number of samples analysed each by Coliplage® and MPN methods | Number of samples for which *E. coli* (Coliplage®) >2000 100 $ml^{-1}$ and *E. coli* (MPN) <2000 100 $ml^{-1}$ | Percentage of samples for which *E. coli* (Coliplage®) >2000 100 $ml^{-1}$ and *E. coli* (MPN) < 2000 100 $ml^{-1}$ |
|---|---|---|---|---|---|
| A | Picardy coast | 2 | 217 | 7 | 3·23 |
| B | Picardy coast | 5 | 41 | 0 | 0·00 |
| C | Picardy coast | 1 | 164 | 2 | 1·22 |
| D | Picardy coast | 5 | 145 | 5 | 3·45 |
| E | Normandy coast | 12 | 350 | 16 | 4·57 |
| F | South-britanny coast | 6 | 6 | 0 | 0·00 |
| G | Vendean coast | 5 | 56 | 6 | 10·71 |
| H | Mediterranean coast | 6 | 29 | 0 | 0·00 |
| I | Mediterranean coast | 4 | 44 | 0 | 0·00 |
| J | Mediterranean coast | 2 | 133 | 55 | 41·35 |
| K | Mediterranean coast | 6 | 32 | 8 | 25·00 |
| L | Mediterranean coast | 6 | 33 | 0 | 0·00 |
| M | Mediterranean coast | 8 | 85 | 1 | 1·18 |
| N | Mediterranean coast | 4 | 66 | 13 | 19·70 |
| Total | | 72 | 1401 | 113 | 8·06 |

 

**Table 2** List of non-*Escherichia coli* 4-methylumbelliferyl-β-D-glucuronide+ strains isolated from seawater samples collected on the Vendean and Mediterranean coasts and their phylogenetic affiliation

| Bacterial species | Phylogenetic class | Number of isolates | Isolation culture media |
|---|---|---|---|
| *Vibrio harveyi* | *Gammaproteobacteria* | 16 | MA, NA |
| *Vibrio* sp.* | *Gammaproteobacteria* | 16 | MA, R2AS |
| *Vibrio splendidus* | *Gammaproteobacteria* | 13 | MA, R2AS |
| *Vibrio chagasii* | *Gammaproteobacteria* | 11 | MA |
| *Zobellia russellii* | *Bacteroidetes* | 9 | MA, R2AS, MMOS |
| *Maribacter dokdonensis* | *Bacteroidetes* | 5 | MA, R2AS |
| *Gramella echinicola* | *Bacteroidetes* | 4 | MA, R2AS |
| *Pseudoalteromonas* sp.* | *Gammaproteobacteria* | 4 | MA, MMOS |
| *Alteromonas macleodii* | *Gammaproteobacteria* | 2 | MA, R2AS |
| *Cytophaga* sp.* | *Bacteroidetes* | 2 | MA |
| *Flexibacter* sp.* | *Bacteroidetes* | 2 | MA, R2AS |
| *Taxeobacter gelupurpurascens* | *Bacteroidetes* | 2 | NA |
| *Vibrio pomeroyi* | *Gammaproteobacteria* | 2 | MA |
| *Zobellia laminariae* | *Bacteroidetes* | 2 | MA, MMOS |
| *Alteromonas addita* | *Gammaproteobacteria* | 1 | MA |
| *Arenibacter* sp./*Flexibacter* sp.* | *Bacteroidetes* | 1 | R2AS |
| *Brachybacterium conglomeratum* | *Actinobacteria* | 1 | NA |
| *Cellulophaga pacifica* | *Bacteroidetes* | 1 | R2AS |
| *Maribacter forsetii* | *Bacteroidetes* | 1 | MA |
| *Pedobacter cryoconitis* | *Bacteroidetes* | 1 | NA |
| *Pibocella ponti* | *Bacteroidetes* | 1 | MA |
| *Polaribacter dokdonensis* | *Bacteroidetes* | 1 | MA |
| *Polaribacter irgensii* | *Bacteroidetes* | 1 | MA |
| *Salegentibacter flavus* | *Bacteroidetes* | 1 | R2AS |
| *Vibrio alginolyticus* | *Gammaproteobacteria* | 1 | MA |
| *Vibrio calviensis* | *Gammaproteobacteria* | 1 | R2AS |
| *Vibrio fisheri* | *Gammaproteobacteria* | 1 | MA |
| *Vibrio fortis* | *Gammaproteobacteria* | 1 | MA |
| *Vibrio lentus* | *Gammaproteobacteria* | 1 | R2AS |
| *Vibrio midae* | *Gammaproteobacteria* | 1 | NA |
| *Vibrio parahaemolyticu* | *Gammaproteobacteria* | 1 | MA |
| *Winogradskyella* sp.* | *Bacteroidetes* | 1 | R2AS |

MA, marine agar; NA, nutrient agar; R2AS, R2A agar supplemented with sterile seawater (70%); MMOS, Mineral salts Medium supplemented with sterile seawater (70%).
*Identification was restricted to the genus level.

isolated and identified as *Zobellia russellii* (8·3% of total isolates) and *Maribacter dokdonensis* (4·6%).

### β-D-glucuronidase activity of marine isolated bacteria

George *et al.* (2000) reported that the GLUase activity depends on substrate concentration (MUG) according to a Michaëlis–Menten relationship. Different MUG concentrations (ranging from 0 to 3·6 mmol $l^{-1}$) were tested on four *E. coli* and non-*E. coli* strains (*E. coli* ATCC25922, *E. coli* ATCC10536, *V. harveyi* type CM37 isolate and *Vibrio parahaemolyticus* type M3110B isolate) to optimize the MUG concentration and therefore the GLUase activity (Fig. 1). The highest GLUase activity for the different strains was obtained at a MUG concentration of 1·8 mmol $l^{-1}$. This concentration was used for testing other pure strains.

The specific GLUase activity of two *E. coli* and 20 nontarget (non-*E. coli* MUG+) strains was measured after induction by MetGlu (Fig. 2). Three GLUase negative strains (*Proteus vulgaris* type A232, *Enterobacter aerogenes* type ENT134 and *Bacillus subtilis* type BA 5.1) were also tested as a control. No detectable GLUase activity (below 7 pmol $min^{-1}$ per $10^6$ cells) was found for these strains.

The *E. coli* strains were successfully induced by 0·4 g $l^{-1}$ of MetGlu. The activity for these strains ranged from 269 pmol $min^{-1}$ per $10^6$ cells for *E. coli* ATCC 25922 to 615 pmol $min^{-1}$ per $10^6$ cells for *E. coli* ATCC 10536.

For nontarget strains, the activity was determined for the most abundant *Vibrio* strains and also for the two dominant *Bacteroidetes* species (*Z. russellii* and *M. dokdonensis*). Surprisingly, *Vibrio* strains were successfully


 

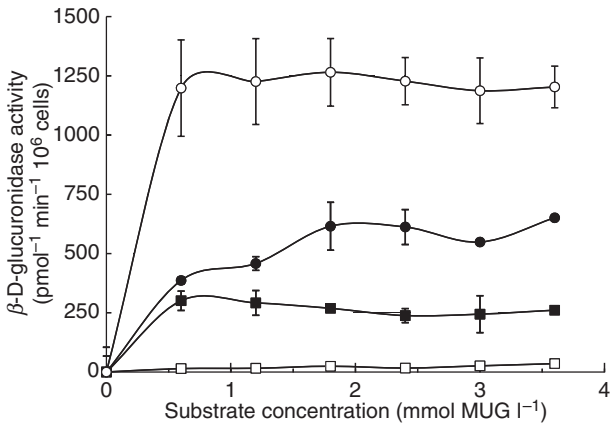


**Figure 1** Mean β-D-glucuronidase activities of two *Escherichia coli* strains (●) *E. coli* ATCC25922; (■) *E. coli* ATCC10536 and two non-*E. coli* 4-methylumbelliferyl-β-D-glucuronide (MUG)+ strains (○) *Vibrio harveyi* type CM37 isolate; (□) *Vibrio parahaemolyticus* type M3110B isolate plotted against MUG concentrations.

induced by adding MetGlu to the growth medium; the reported GLUase activities were also highly variable.

Within the 14 *Vibrio* strains tested in this study, seven strains belonging to five different species (*V. chagasii*, *V. harveyi*, *V. splendidus*, *Vibrio fortis* and *Vibrio midae*) displayed activities on the same order of magnitude (between 304 pmol min$^{-1}$ per 10$^6$ cells and 570 pmol min$^{-1}$ per 10$^6$ cells) as those of *E. coli* strains. Interestingly, two *V. harveyi* strains exhibited much higher activities (more than 1150 pmol min$^{-1}$ per 10$^6$ cells) than even the most active *E. coli* strains. The other nontarget strains showed very low activities.

## Discussion

The development of rapid methods for evaluating water microbiological quality allowing real-time management of bathing waters is a high-priority demand among stakeholders. In Europe, the new Bathing Water Quality Directive published in 2006 introduced the concept of active management of bathing water and recommended the evaluation of any potential sources of contamination of each bathing site (EEC 2006). The Coliplage® method has been used in France since 2005 for daily monitoring programmes because it offers the advantage of providing results in <1 h. However, using this method for closing or re-opening a beach after contamination requires a good agreement with the standardized culture-based method presently used in France [the standardized miniaturized MPN method (ISO 9308-3 1998)].

In our study, 8% of the samples analysed by Coliplage® estimated *E. coli* concentrations above the guideline of 2000 *E. coli* per 100 ml when the value obtained by the MPN method (for the same sample) was below the threshold. These differences may correspond to artefacts of the Coliplage® method or to *E. coli* cells that are not detected by the standardized method.

At least three hypotheses could explain the observations:

i The presence of cells with a detectable GLUase activity but unable to grow in the microplate. Garcia-Armisen *et al.* (2005) strongly suggested that the GLUase activity measurement took into account the activity of some nonculturable *E. coli* that could be present in marine waters. However, the quantification of active but nonculturable

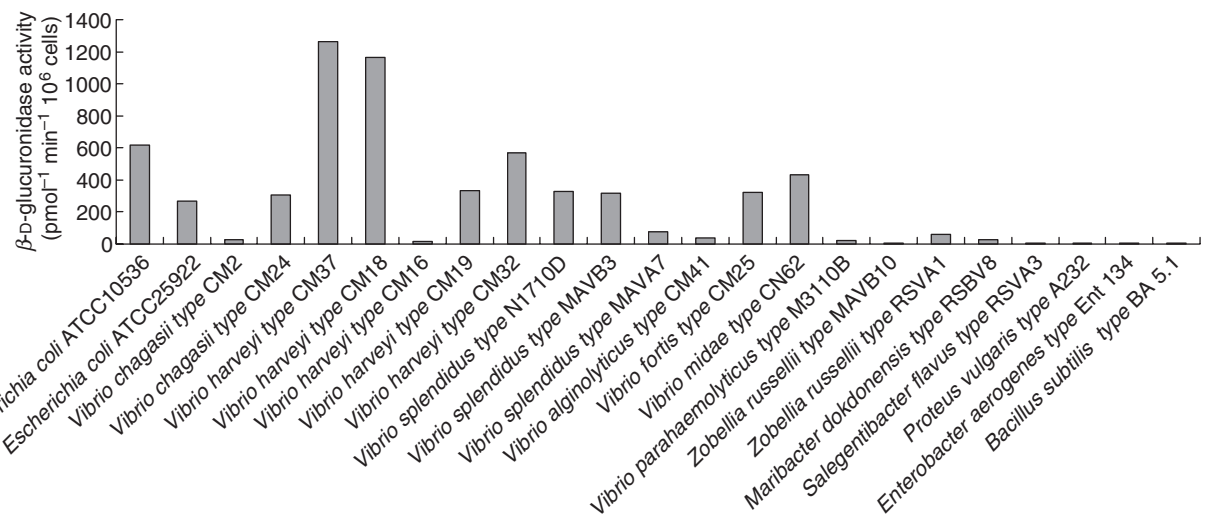


**Figure 2** β-D-glucuronidase activities of *Escherichia coli* strains and of non-*E. coli* 4-methylumbelliferyl-β-D-glucuronide+ strains isolated from seawater samples collected on the Vendean and Mediterranean coasts.

 

*E. coli* cells having a GLUase activity is impossible because of a lack of appropriate methods, but taking into account this population may be important for risk assessment.

**ii** The presence of a high proportion of aggregated *E. coli* or that attached to suspended solids can cause an underestimation of their numbers when evaluated by the MPN method, which is not the case when evaluated by the Coliplage® method. In the MPN method, several *E. coli* cells aggregated or attached to the same particle will be considered as a single *E. coli* cell, and thus the total abundance is underestimated. In this case, the Coliplage® method would be better for risk assessment purposes; however, this is not likely to explain the differences sometimes reported for samples with a low particle concentration.

**iii** The presence of nontarget cells (non-*E. coli* cells) with a GLUase activity can overestimate the *E. coli* concentration determined by the Coliplage® procedure. These non-*E. coli* cells presenting a GLUase activity are not accounted for by the MPN as they do not grow in the culture medium.

The third hypothesis has been poorly investigated in the marine environment. The samples collected on the French coasts (from lagoons and different coastal sites) also showed the most important disagreement between the two methods. Therefore, these sites were selected for the isolation of strains. Of the non-*E. coli* GLUase positive strains that were isolated, most of the selected strains clustered in only two phylogenetic bacterial classes : *Gammaproteobacteria* and *Bacteroidetes*. There was a high proportion of *Gammaproteobacteria* that were dominated by *Vibrio* species. Some reports also suggested that the GLUase enzyme could be present in non-*E. coli* cells. Davies *et al.* (1994) mentioned the possibility of an interference by aquatic plant and algal cells when present in high numbers for the test based on GLUase activity measurements. Other authors showed the presence of the enzyme GLUase in various non-*E. coli* bacterial strains and suggested a possible interference of such nontarget bacteria in the direct GLUase activity measurement because this procedure lacks a selective growth step (Palmer *et al.* 1993; Fiksdal *et al.* 1997; Van Poucke and Nelis 1997). Finally, Tryland and Fiksdal (1998) demonstrated that some isolates from aquatic systems (*Bacillus* spp. and *Aerococcus viridans*) had a level of glucuronidase activity similar to an *E. coli* strain. The potential interference of autochthonous bacteria as *Vibrio* on GLUase activity measurement was previously reported by Pisciotta *et al.* (2002) for marine samples using the Colilert-18 assay (incubation of the sample for 18 h in a defined substrate medium containing MUG). Our results show that some *Vibrio* species have similar and even higher specific glucuronidase activity than *E. coli* strains. In our study, five different species (*V. chagasii*, *V. harveyi*, *V. splendidus*, *V. fortis*, *V. midae*) were observed within the GLUase positive strains, and the first three species were also the most abundant.

The *Vibrio* genus is often reported as a dominant phylogenetic group in clone libraries, particularly in the marine environment (Thompson *et al.* 2004). These ubiquitous species include some that are pathogenic for humans or fishes (Dumontet *et al.* 2000). They can survive under stressful conditions (Colwell 1996, 2000), and many species are positively correlated to seawater temperature (Blackwell and Olivier 2008). Unfortunately, data on the abundance of *Vibrio* species in the natural environment are scarce because of the lack of methods for their quantification. Quantitative PCR methods for the enumeration of *Vibrio* species are difficult to develop because many species are very similar (Fukui and Sawabe 2008). Our results suggest that the specific GLUase activity of *Vibrio* species can vary between species and even between strains for a given species. Similar variations have also been reported for *E. coli* but more importantly, the specific activity of some *Vibrio* species was much greater than that of the most active *E. coli* cells tested in this study. Although we have no data on the concentration of *Vibrio* cells in the samples, we can assume that the concentration of each different species was at least 1000 cells in every 100 ml and probably even higher because each colony was detected after spreading 0·1 ml of natural seawater on the plate. Considering their GLUase activity, it means that these viable and culturable *Vibrio* cells may generate false-positive *E. coli* counts at concentrations very close to or above the maximum allowable concentration for bathing waters (2000 *E. coli* per 100 ml). The three most abundant *Vibrio* species (*V. chagasii*, *V. harveyi* and *V. splendidus*) were also among the non-*E. coli* MUG+ isolates in the Mediterranean waters tested, and some strains of *V. harveyi* in particular, were more active than *E. coli*. The GLUase activities of other non-*E. coli* and non-*Vibrio* species were very low; based on our results, they probably create little interference with the *E. coli* signal.

In conclusion, we reported a first qualitative investigation on disagreement between Coliplage® and the MPN results and showed that some *Vibrio* species can potentially interfered on the direct measurement of *E. coli* when the GLUase activity is targeted. Nevertheless, further studies are now required to provide additional information, about the circumstances under which marine *Vibrio* species could be the source of a significant overestimation of the *E. coli* concentration by direct GLUase activity measurement. If the interference is confirmed, appropriate treatments should be developed to remove the interfering signal in future work.

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