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Use of enzymatic methods for rapid enumeration of coliforms in freshwaters

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I. GEORGE, M. PETIT AND P. SERVAIS. 2000. Rapid enumeration methods based on the enzymatic hydrolysis of 4-methylumbelliferyl-β-D-galactoside and 4-methylumbelliferyl-β-D-glucuronide were optimized for freshwaters. The enzymes β-D-galactosidase (GALase) and β-D-glucuronidase (GLUase) were shown to be already induced in freshwaters when tested, respectively, with the inducers isopropyl-β-D-thiogalactopyranoside and methyl-β-D-glucuronide. Both enzymatic activities were compared, respectively, with plate counts of total and faecal coliforms in freshwaters. Enzymatic methods and reference plate counts were significantly correlated in log–log plots. Moreover, the GLUase method allowed the detection of viable (presenting a detectable GLUase activity) but nonculturable *Escherichia coli.*

INTRODUCTION

Standard microbiological methods used to enumerate total and faecal coliforms are based on their ability to ferment lactose, producing gas, acid or aldehydes (American Public Health Association (1995). They necessitate cultivation of the bacteria, and thus require 24–96 h to complete. This considerable delay in the assay response makes it impossible to take sanitary measures immediately after a faecal pollution has occurred. Moreover, these methods do not seem well adapted to the enumeration of faecal bacteria in aquatic systems, since they overestimate the disappearance rate of these bacteria after their discharge in natural waters. When released in fresh or sea waters, faecal bacteria were indeed shown to lose rapidly their ability to form visible colonies on specific media, while preserving some metabolic activities (Colwell *et al.* 1985; Grimes and Colwell 1986; Roszak and Colwell 1987; Barcina *et al.* 1989; García-Lara *et al.* 1993; Davies *et al.* 1995; Pommepuy *et al.* 1996). According to some authors, these sublethally stressed bacteria, called 'viable but nonculturable' bacteria, can preserve their potential for virulence (Colwell *et al.* 1985; Grimes and Colwell 1986). The limitations of traditional methods led to the development of alternative methods of enumeration that could provide better and more rapid information on the abundance and the fate of faecal bacteria in aquatic environments.

Numerous works have reported the production of the enzymes β-D-galactosidase by total coliforms and β-D-glucuronidase by the main faecal coliform, *Escherichia coli* (Buehler *et al.* 1951; Dahlen and Linde 1973; Kilian and Bulow 1976). These properties have been exploited in a multitude of new tests based on the detection of hydrolysis products of fluorogenic or chromogenic substrates for both enzymes incorporated into culture media. Such tests are sensitive, simple to perform and do not require a confirmation step. They were applied to detect total coliforms and/or *E. coli* in food, drinking water, seawater, freshwater, sewage, and clinical and faecal samples (Bascomb 1987; Manafi *et al.* 1991; Frampton and Restaino 1993). However, they still often require 24 h to complete. More recently, the β-D-galactosidase and β-D-glucuronidase properties of total coliforms and *E. coli* were exploited in rapid assays without any cultivation step. Most studies were carried out on sewage or seawater samples (Müller-Niklas and Herndl 1992; Fiksdal *et al.* 1994; Apte *et al.* 1995; Davies *et al.* 1995; Davies and Apte 1996; Pommepuy *et al.* 1996; Tryland *et al.* 1998). Fiksdal *et al.* (1994) finalized a protocol based on fluorogenic substrates 4-methylumbelliferyl-β-D-galactoside (MUGal) and 4-methylumbelliferyl-β-D-glucuronide (MUGlu) for enzymatic detection of faecal coliforms in coastal waters in 30 min. The first objective of the present study was to optimize these rapid MUGal and MUGlu-based enzymatic

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assays for freshwaters in order to increase their sensitivity in these environments. The relationship between these methods and one of the current conventional methods for detection of coliforms in natural environments (plate counts) was subsequently investigated for freshwaters. Finally, experiments were performed to test the validity and the main advantages of the use of rapid enzymatic assays as a surrogate measure of coliform abundance in aquatic systems.

MATERIALS AND METHODS

Sampling

Samples were collected from ponds and rivers in Belgium and France in sterile bottles, maintained at in-situ temperature and analysed within 4 h.

Enumeration of culturable coliforms by plate count

Total coliforms (TC) and faecal coliforms (FC) were enumerated after membrane filtration (0·45 μm-pore-size, 47 mm diameter sterile cellulose nitrate filters; Sartorius,Goettingen, Germany) on lactose agar with Tergitol (Merck, Darmstadt, Germany; 0·095‰ wt/vol final concentration) and triphenyl 2,3,5-tetrazolium chloride (TTC) (0·024‰ wt/vol final concentration) (Anon 1985). Plates were incubated at 37 °C for 48 h and at 44 °C for 24 h, respectively. Orange to red (but not purple) colonies producing a yellow halo under the membrane after incubation at 37 °C for 48 h were considered TC colonies. Orange colonies producing a yellow halo under the membrane after incubation at 44 °C for 24 h were considered FC colonies. Galactosidase producing coliforms that were unable to produce acid from lactose were disregarded.

Enzyme assays

β-D-galactosidase (GALase) assay. The assay was a modification of the protocol described by Fiksdal *et al.* (1994). All the steps performed in the enzyme assay were optimized for freshwaters (see Results and Discussion) in order to finalize the following protocol. Freshwater samples (100 ml) were filtered through 0·2 μm pore size, 47 mm diameter polycarbonate filters (Nuclepore, Corning Costar, Badhoevedorp, The Netherlands). The filters were placed in 200 ml sterile Erlenmeyer flasks containing 20 ml of sterile phosphate buffer (pH 7·2) and 10 mg sodium dodecyl sulphate. Ten milligrams of MUGal (Sigma Chemical Co., St Louis, MO, USA) was added to each flask (final concentration: 500 mg l^{-1}) and the flasks were incubated in a shaking water bath at 37 °C. Every 5 min for 25 min, a 2·9 ml aliquot of the 20 ml was poured in a quartz cell with 100 μl of 1 mol l^{-1} NaOH solution to obtain a pH greater than 10. The fluorescence intensity of the aliquot was measured with an SFM 25 spectrofluorometer (Kontron AG, Zürich, Switzerland) at an excitation wavelength of 362 nm and emission wavelength of 445 nm. The 100% of fluorescence intensity of the fluorometer was calibrated using standards of known methylumbelliferone (MUF) concentrations from 50 to 12500 nmol l^{-1}; this procedure allowed the study of a wide range of enzymatic activities by changing only the fluorometer calibration. The total production rate of MUF (picomoles of MUF liberated per min for 100 ml of sample filtered), was determined by least-squares linear regression when plotting MUF concentration versus incubation time. The autohydrolysis rate of the substrate in the same assay conditions but in the absence of bacteria (no freshwater sample was filtered on the 0·2 μm pore size filter) was substracted from the total production rate of MUF. The remaining production rate corresponded to the release of MUF by bacterial enzymatic activity.

β-D-glucuronidase (GLUase) assay. The GLUase assay was performed similar to the GALase assay, except that the filters were aseptically placed in 17 ml of sterile phosphate buffer (pH 6·9). Three milliliters of MUGlu solution [50 mg of MUGlu (Sigma Chemical Co.) and 20 μl of Triton X-100 in 50 ml of sterile water] was added to each flask (final concentration: 150 mg l^{-1}). The temperature of incubation was 44 °C. One hundred microliters of 2 mol l^{-1} NaOH solution was added to each 2·9 ml aliquot to obtain a pH greater than 10 before the fluorescence measurement.

Induction of environmental GALase and GLUase enzymes

The effect of potential inducers of the GALase and GLUase enzymes was examined in several natural aquatic systems. The inducers used were, respectively, isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co.) and methyl-β-D-glucuronide (MetGlu) (Sigma Chemical Co.). IPTG is known to be a noncompetitive inducer, i.e. nonhydrolysable substrate by β-D-galactosidase (Herzenberg 1959) but the possible effect of MetGlu on GLUase activity needed to be tested. This was done by comparing GLUase activities of two natural subsamples: one was treated according to the standard procedure and the other was treated with MetGlu immediately before the enzyme assay was performed (0·02 g of MetGlu was added with MUGlu into the flask containing the phosphate buffer). Because no significant difference was observed between the two assay responses (data not shown), MetGlu was considered a noncompetitive inducer. To test the impact of inducer addition to natural freshwaters, the water collected from various freshwater systems was divided into two sets of three or four subsamples. One set was supplemented with inducer and both sets were incubated at 20 °C in the dark from 0–6 h. At successive

times, enzyme activity was measured simultaneously in two subsamples, including one supplemented with inducer.

Specificity of enzymatic activity measurements

An experiment was performed in order to determine if GALase-positive non-TC bacteria and GLUase-positive non-FC bacteria potentially present in natural waters (i.e. false-positive bacteria) could interfere in the GALase and GLUase assays. The principle was to determine enzymatic activity after adding increasing numbers of noncoliform bacteria to natural water containing a constant number of target organisms (TC or FC). A culture of noncoliform 'autochthonous' bacteria was prepared as follows. A natural freshwater sample without coliforms was supplemented with yeast extract (1–2 mg l^{-1}) and incubated for 4–5 d in aerobic conditions at 20 °C in the dark. Cell counts on specific medium were performed all along the incubation period to make sure of the nondevelopment of coliform bacteria. After incubation, the culture of 'autochthonous' bacteria was collected onto a 0·2 μm pore size 47 mm diameter polycarbonate filter (Nuclepore) and concentrated by suspension into a small volume of sterilized natural water. Increasing concentrations of this bacterial suspension were inoculated to aliquots (100 ml) of natural receiving waters containing coliforms [water 1: TC: 4000 per 100 ml and total number of cells (numerated by epifluorescence microscopy): $5{\cdot}5 \times 10^8$ cells per 100 ml; water 2: FC: 760 per 100 ml and total number of cells (numerated by epifluoresence microscopy): $5{\cdot}5 \times 10^7$ cells per 100 ml], so that the total bacterial abundance in these receiving waters was multiplied by a factor ranging from 1 to 20 (cell counts determined by epifluorescence microscopy). The GALase or GLUase assays were performed after each inoculation to determine a possible interference due to the enzymatic activity of false-positive bacteria.

Preparation of induced and noninduced *E. coli* cultures

E. coli (ATCC 25922) was grown in tryptic soy broth without dextrose (TSB) (Oxoid, Basingstoke, UK). This medium supplemented with 0·2 g l^{-1} of MetGlu was used to grow β-D-glucuronidase-induced cells. The cultures were grown overnight at 37 °C in the dark and bacteria were subcultured in fresh medium (TSB with or without MetGlu) until the cell count (determined by standard epifluorescence microscopy) was approximately 10^8 cells per ml (stationary phase, after 1–3 d). All measurements were performed using cell suspensions of bacteria from the stationary phase. Induced *E. coli* cells were used in all experiments with pure cultures unless otherwise specified.

Microcosms

The survival of *E. coli* in freshwater was studied in two microcosms consisting of 2-l sterile bottles containing 1·5-l subsamples of sterile (filtered through a 0·2 μm pore size Nuclepore filter and autoclaved) river water (Marne river, France). Induced *E. coli* cells were harvested from the culture broth after 3 d, centrifuged (3000 r.p.m for 15 min) and washed four times with sterile Ringer solution. *E. coli* cells were added to the microcosms to obtain a final concentration of 10^6–10^7cells per ml. Microcosms were incubated with gentle stirring for 12 d at 20 °C, one in the dark and the other under a suspended illumination system consisting of two 'daylight' lamps (Osram, Munich, Germany) and simulating a diurnal cycle with 8 h of 'daylight' ($0{\cdot}4 \times 10^{16}$ Quanta s^{-1} cm^{-2} at the surface of the water in the microcosms). The microcosms were sampled immediately after inoculation and afterwards at regular intervals. At each sampling time, an enumeration of the total number of cells by epifluorescence microscopy, a GLUase assay and plate counts were carried out.

Enumeration by epifluorescence microscopy

The total number of bacteria in pure culture and natural samples was estimated by epifluorescence microscopy (Leica, Wetzlar, Germany; equipped with a HBO-50 mercury lamp at a magnification factor of $\times$1250) after DAPI staining (Porter and Feig 1980). Before counting, *E. coli* cells in pure culture were harvested by centrifugation (3000 r.p.m., 15 min), washed four times in sterile Ringer's solution and blended at high speed in a vortex to break up cellular aggregates.

RESULTS AND DISCUSSION

Optimization of the enzymatic assays conditions

Parameters relating to the fluorescence intensity were first tested with MUF solutions. The excitation and emission wavelengths were found to be optimal at 362 nm and 445 nm, respectively. The fluorescence was maximal when the pH of the solution in the quartz cell was greater than 10 (data not shown).

Optimization of the parameters relating to the enzymatic reactions was carried out on several freshwater samples variously contaminated by coliforms. For each sample, the enzymatic activities were measured at various pH levels or temperatures and expressed as a percentage of maximal activity (Figs 1 and 2). The Gauss curves fitted to the experimental data showed maximal GALase activity at pH 7·2 and 37 °C, and maximal GLUase activity at pH 6·9 and 44 °C (Figs 1 and 2). GALase and GLUase activities were also

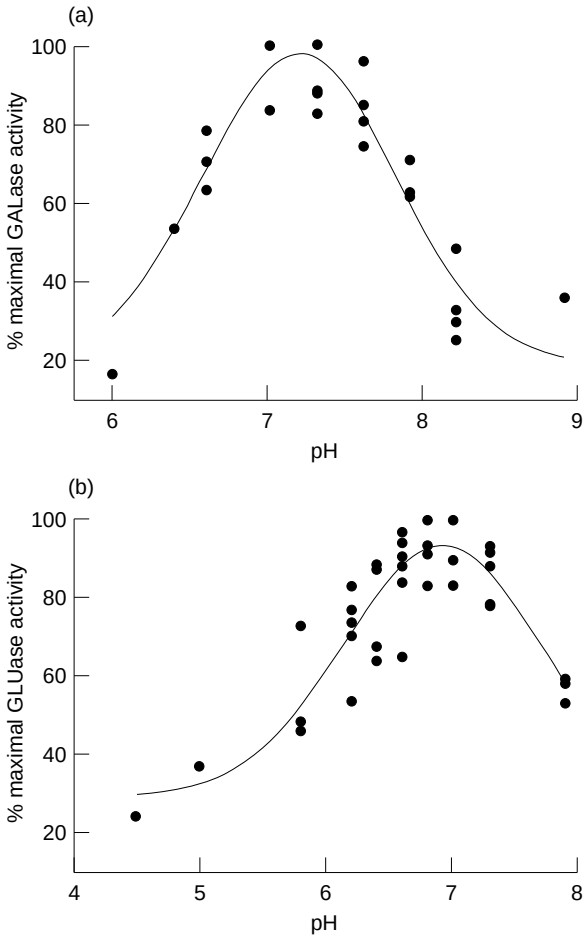

Fig. 1 Impact of pH on (a) β-D-galactosidase (GALase) and (b) β-D-glucuronidase (GLUase) activities measured on different natural freshwater samples. Data are expressed as a percentage of maximal activity for each sample. The solid line represents the fitting of the experimental data by a Gauss curve

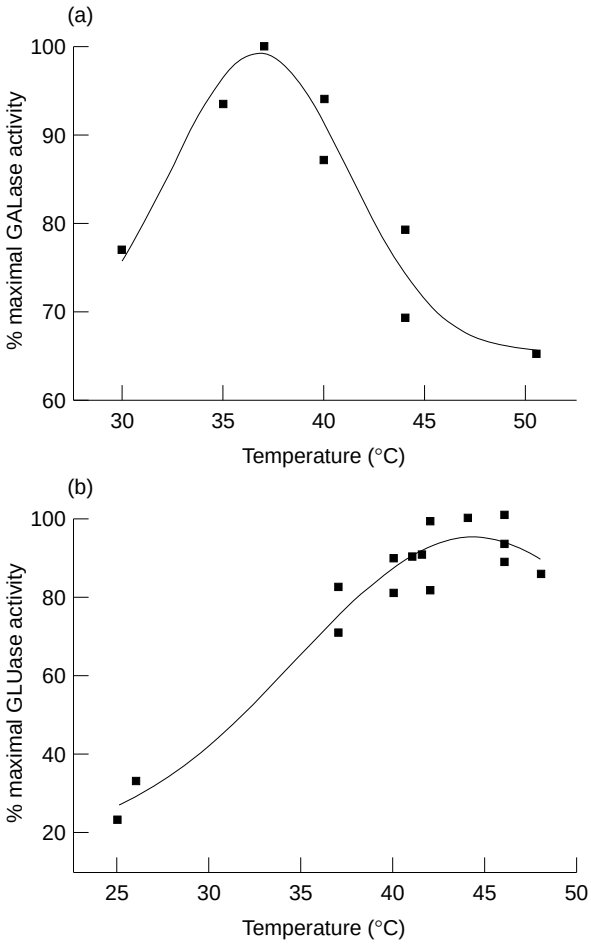

Fig. 2 Impact of temperature on (a) β-D-galactosidase (GALase) and (b) β-D-glucuronidase (GLUase) activities measured on different natural freshwater samples. Data are expressed as a percentage of maximal activity for each sample. The solid line represents the fitting of the experimental data by a Gauss curve

measured at various concentrations of MUGal and MUGlu, respectively, on two samples, one rich in coliforms and the other poor. As expected Michaëlis–Menten relationships were obtained (Fig. 3). Routine substrate concentrations were fixed at 500 mg MUGal per l and 150 mg MUGlu per l. These concentrations allowed the measurement of enzymatic activities close to the maximum (whether the water was rich in coliforms or not) whilst avoiding the waste of substrate.

Induction of enzymatic activities in natural samples

β-D-galactosidase and β-D-glucuronidase are well known to be inducible enzymes (Herzenberg 1959; Pardee and Prestidge 1961). In all natural freshwaters we tested, there was no obvious change in the level of β-D-galactosidase activity after the addition of inducer IPTG in concentrations highly greater than those of sugars in natural waters (Fig. 4). This strongly suggested that the β-D-galactosidase was already induced not only in highly but also in weakly polluted waters. Similarly, the addition of similar concentrations of inducer MetGlu had no apparent effect on β-D-glucuronidase activity. Therefore, we considered that both enzymes were already induced in freshwaters and that our standard procedure could be applied without adding any inducer whatever the abundance of coliforms in the water analysed.

Enzymatic assays versus reference plate counts

Freshwater samples representing a wide range of faecal contamination were analysed using both enzymatic methods and enumerations on selective medium. Log-transformed enzy-

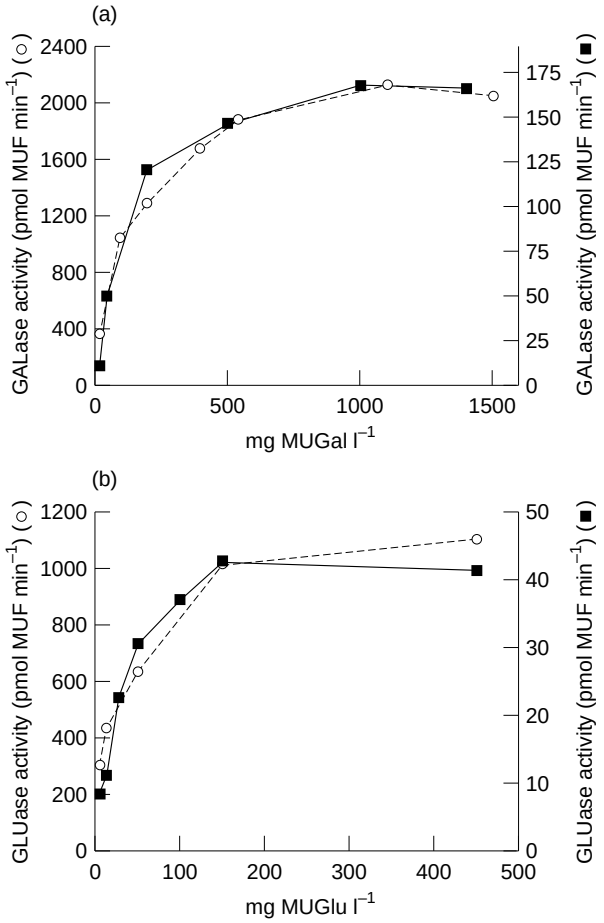

Fig. 3 Relationship between (a) β-D-galactosidase (GALase) and 4-methylumbelliferyl-β-D-galactoside (MUGal) concentration, and (b) β-D-glucuronidase (GLUase) activities and 4-methylumbelliferyl-β-D-glucuronide (MUGlu) concentration. Enzymatic activities were measured in one sample weakly (■) and one sample strongly (○) contaminated by coliforms

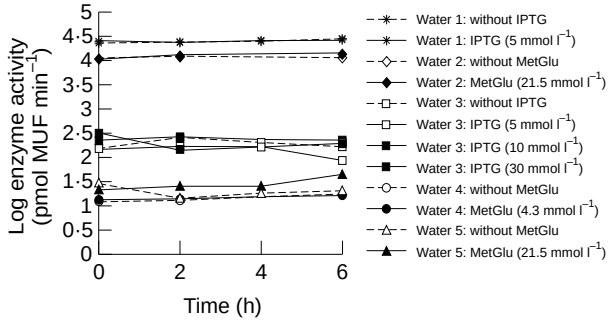

Fig. 4 Impact of different concentrations of inducers [isopropyl-β-D-thiogalactopyranoside (IPTG) for β-D-galactosidase activity and methyl-β-D-glucuronide (MetGlu) for β-D-glucuronidase activity] on enzymatic activities after various incubation times in the presence of inducers. Measurements were performed on natural freshwater samples differently contaminated by coliforms

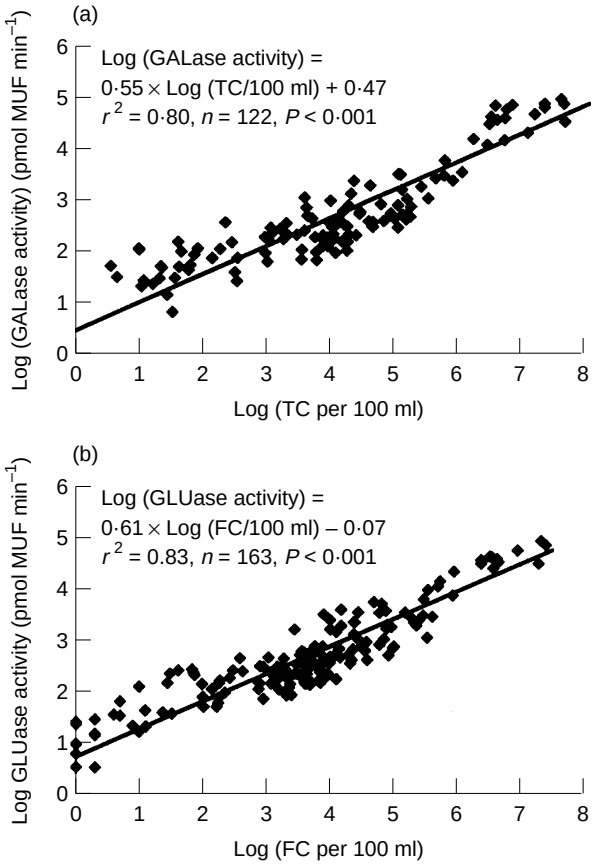

Fig. 5 Relationships between (a) β-D-galactosidase (GALase) activity and total coliforms (TC) enumerated by plate counts and (b) β-D-glucuronidase (GLUase) activity and faecal coliforms (FC) enumerated by plate counts for a large range of natural freshwater samples

matic activities and log-transformed plate counts were significantly correlated (Fig. 5a,b). Enzymatic methods could thus prove useful to monitor microbiological quality of natural waters. For each correlation, the slope and the intercept of a linear model II regression (reduced major axis regression) between both random variables (enzymatic activities and plate counts) was calculated (Fig. 5a,b).

However, further experiments in this work were mostly focused on GLUase activity, because the main FC (*E. coli*) is a better indicator of faecal contamination than TC (a very heterogeneous group of bacteria with some strains of aquatic or tellurian origin). *E. coli* is considered one of the two best indicators of faecal pollution in recreational freshwaters in

US bacterial water quality standards (US Environmental Protection Agency 1986).

Enzymatic activity per coliform

The slopes of the linear log–log data regressions presented in Fig. 5 were lower than one. This means that the activity per culturable coliform increased as the number of culturable coliforms decreased, instead of being constant regardless of the number of coliforms in the samples. Fiksdal *et al.* (1994) achieved the same trend with coastal samples in other assay conditions.

Several hypotheses might explain these results: (1) the enzymatic activity per coliform could really be higher in slightly polluted waters than in highly contaminated ones. This hypothesis was not tested during the course of this work, and to our knowledge there are no arguments likely to support it so far. (2) The enzymatic activity of coliforms could be overestimated in weakly contaminated waters or underestimated in heavily polluted ones, which means that the activity measured per coliform would depend on the number of coliforms present during the enzymatic assay. (3) The higher activity per culturable coliform in waters containing few coliforms could be due to a large contribution of GALase-positive or GLUase-positive noncoliform bacteria ('false-positive' or 'nontarget' bacteria) to rapid enzymatic assays in these waters. This interference of false-positive strains would be revealed only in weakly polluted waters if the proportion of false-positive noncoliforms versus coliforms is higher in weakly contaminated waters than heavily polluted ones. (4) The abundance of coliforms may be significantly underestimated by plate counts in weakly contaminated waters because of the existence in these environments of many active but nonculturable (ABNC) coliforms, i.e. cells presenting a detectable GALase or GLUase activity but unable to produce visible colonies on cultivation media. The last three hypotheses were investigated by several experiments.

Possible influence of the number of coliforms present during enzymatic assays on the activity measured per coliform

GLUase assays and plate counts of heavily contaminated FC were performed in four diluted samples (decimal dilutions) of a natural water containing 2×10^6 FC per 100 ml. The following relationship was obtained when plotting in log units the GLUase activity versus FC of the various dilutions:

$$\text{Log GLUase activity} = 0{\cdot}99 \log \text{FC} - 2{\cdot}37$$

$$(r^2 = 0{\cdot}995,\ n = 4)$$

The slope was very close to 1 showing that the enzyme activity and the number of faecal coliforms were linearly correlated. Thus, the activity measured per culturable coliform does not depend on the number of cells present during the enzymatic assay. It is worth noting that this experiment did not prove the absence of ABNC faecal coliforms in heavily contaminated waters but that, if they were present, their proportion in the population of active faecal coliforms was constant in the various dilutions.

Possible interference of nontarget bacteria to rapid enzymatic methods

Both enzymes have been found in several noncoliform strains (Frampton and Restaino 1993; Ley *et al.* 1993; Palmer *et al.* 1993; Davies *et al.* 1995; Van Poucke and Nelis 1997; Tryland and Fiksdal 1998). False-positive bacteria could affect these methods since they are lacking in any selective growth step. Indeed, the interference of nontarget bacteria in rapid enzymatic assays without cultivation step is a function of the level of enzymatic activity of target and nontarget microorganisms, and of the proportion of bacteria from each of the two groups (Tryland and Fiksdal 1998).

In the present study, the level of GLUase activity of *E. coli* cells from a pure culture was first measured using our standard procedure. It was found to be 1·77 pmol MUF-min^{-1} (log units) for 10^7 *E. coli*, i.e. 2·49 log units inferior to the activity that would be measured in natural samples at a similar abundance of faecal coliforms (Fig. 6). However, this difference in activity level was considerably reduced when

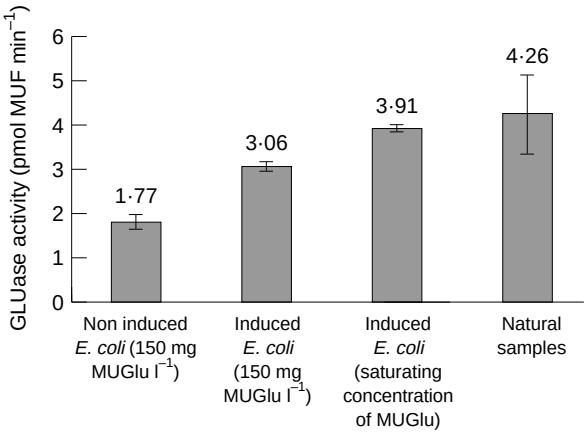

Fig. 6 Comparison of β-D-glucuronidase (GLUase) activities of noninduced cultured *E. coli* measured at a 4-methylumbelliferyl-β-D-glucuronide (MUGlu) concentration of 150 mg l^{-1}, induced cultured *E. coli* measured at a MUGlu concentration of 150 mg l^{-1}, induced cultured *E. coli* estimated at saturating concentration, and faecal coliforms (FC) from natural samples (according to the linear regression equation of Fig. 5b). In each case, GLUase activities were measured or estimated for 10^7 cells

the growth medium was supplemented with an inducer, as reported by Tryland and Fiksdal (1998). The GLUase activity of MetGlu-induced *E. coli* cells was indeed 1·20 log units inferior to the activity of environmental samples (Fig. 6). The response of induced *E. coli* was probably more representative of the enzymatic activity of FC in natural samples than the response of noninduced *E. coli,* as GLUase activity was found to be fully induced in natural samples (Fig. 4). The GLUase activity per culturable coliform remained higher in natural systems, which could be partly due to the contribution of nontarget bacteria to enzymatic assays. However, the MUGlu concentration used in GLUase assays on natural samples (150 mg l^{-1}) proved to be insufficient to reach maximal activity with the induced pure culture, as revealed by the Michaëlis–Menten relationship obtained between the GLUase activity of 10^7 induced *E. coli* cells and MUGlu concentration: V_{max} = 8183 pmol MUF-min^{-1}; half-saturation concentration = 509 mg MUGlu l^{-1}. This V_{max} value was approximately sevenfold superior to the activity measured at the routine concentration of 150 mg MUGlu l^{-1} and of the same order of magnitude as the corresponding activity in natural environments (Fig. 6). Thus, at the concentration of coliforms studied, it appeared that there was no overestimation of enzymatic activities in natural waters due to the presence of nontarget bacteria.

Tryland and Fiksdal (1998) tested the induction of GALase by IPTG and GLUase by MetGlu for different strains of nontarget and target bacteria isolated from natural waters. When the enzymes were induced, which seems to be the case in natural environments according to the results presented in Fig. 4, the activity per cell of the nontarget bacteria was generally several orders of magnitude lower than the activity of TC and FC. The authors concluded that most of these false-positive bacteria must be in correspondingly higher concentrations than those of target bacteria to interfere in rapid enzymatic assays. Fiksdal *et al.* (1997) proposed to estimate the ratio of nontarget versus target bacteria in aquatic environments by bacterial counts on nonselective agar incubated at 20 °C and supplemented with chromogenic or fluorogenic substrates for β-D-galactosidase and β-D-glucuronidase. GALase and GLUase-positive bacteria were found to be much more abundant than TC or FC recovered on selective medium at 35 and 44·5 °C. These results, similar to others previously reported (Van Poucke and Nelis 1997), strongly suggested the presence of high numbers of nontarget bacteria in natural environments. However, the authors showed that the difference between the two plate counts was mainly the result of high incubation temperatures with selective media that probably prevented the recovery of many injured coliforms. Moreover, in river samples, 87% of the GALase-positive bacteria enumerated on nonselective agar supplemented with chromogenic substrate had gram and oxidase reactions characteristic of coliforms. It is thus plausible that the proportion of nontarget bacteria in rapid assays was overestimated when evaluated by enumerations on nonselective agar at 20 °C.

Another experiment was proposed to evaluate the specificity of rapid enzymatic methods. Increasing concentrations of noncoliform bacteria grown in a culture made from a natural sample containing no coliforms were added to several subsamples of the same freshwater. The receiving freshwater was moderately contaminated by coliforms. This experiment did not reveal any significant contribution of false-positive bacteria to these methods, since neither GALase nor GLUase activities in the subsamples were modified, even when the total number of bacteria in the subsamples was multiplied by 17–20 (Fig. 7). These results might demonstrate the specific nature of enzymatic methods, if one assumes that the sample from which the culture was made was representative of most natural aquatic environments and that the culture did not select some GALase-or GLUase-negative strains that were

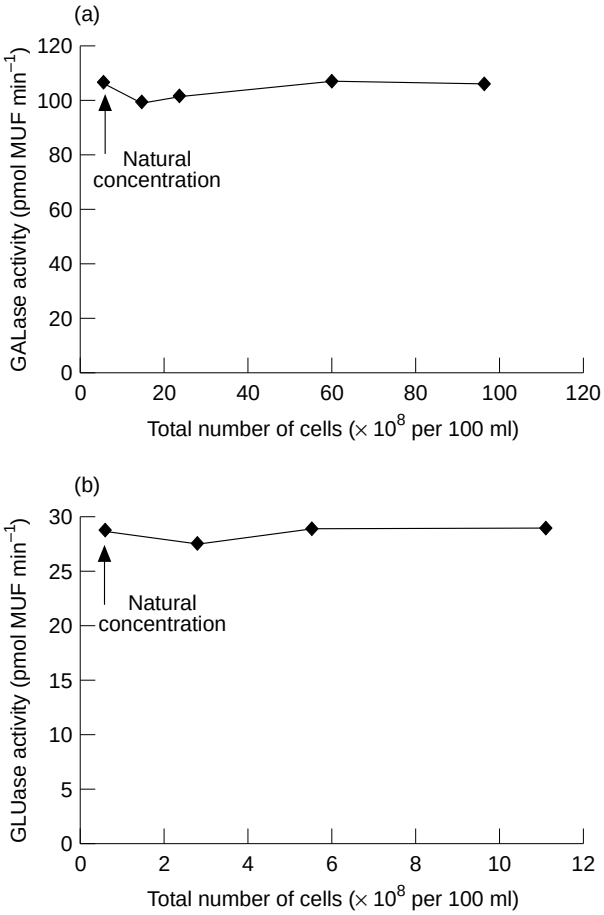

Fig. 7 Impact of the addition of noncoliform bacteria to (a) β-D-galactosidase (GALase) activity and (b) β-D-glucuronidase (GLUase) activity of a natural water sample

not dominant in the original sample. Both hypotheses could be checked by repeating these experiments on many natural freshwaters. In conclusion, to our knowledge, there is no experimental evidence so far of a significant contribution of nontarget bacteria to rapid enzyme assays that invalidates these methods. Moreover, at the present time, there is no information available in the literature on an increased ratio of nontarget versus target bacteria in weakly contaminated waters compared to that in heavily polluted ones.

Contribution of active but nonculturable coliforms to enzymatic activities

A possible explanation for higher enzymatic activity per culturable coliform in less contaminated waters could be a stronger underestimation of the number of coliforms when enumerated by plate count due to a higher proportion of active (cells with a detectable GALase or GLUase activity) but nonculturable (ABNC) coliforms in these waters. Indeed, several studies have shown that bacteria could be metabolically active even if they were not detected by the cultivation techniques commonly used (Colwell *et al.* 1985; Grimes and Colwell 1986; Roszak and Colwell 1987; Barcina *et al.* 1989; García-Lara *et al.* 1993; Davies *et al.* 1995; Pommepuy *et al.* 1996). In this study, cultured *E. coli* cells were discharged in sterile river water to test if stress conditions could lead to the appearance of ABNC coliforms. (Fig. 8). The total count (morphologically intact cells) remained stable throughout the light and dark experiments (12 d). In the dark microcosm, GLUase activity was stable while the number of FC determined by plate counts progressively decreased, which indicated that cells were reaching a ABNC state. The first-order decay rate of plate counts (k_d) calculated by linear regression was equal to $1{\cdot}7 \times 10^{-3}\,h^{-1}$. So far in the literature, the appearance of ABNC *E. coli* (as defined above) has only been shown in seawater by using the GALase assay (Davies *et al.* 1995; Pommepuy *et al.* 1996), and was the result of osmotic stress (Pommepuy *et al.* 1996). In this study, it was probably due to nutritional stress. The loss of culturability due to nutritional stress has indeed been mentioned in studies of survival of *E. coli* in natural waters (Colwell *et al.* 1985; Roszak and Colwell 1987; Dupray *et al.* 1993; García-Lara *et al.* 1993; Duncan *et al.* 1994). Light had an effect on enzymatic activity as shown by the loss observed in the light microcosm ($k_d = 4{\cdot}4 \times 10^{-3}\,h^{-1}$). However, the loss of culturability increased in comparison with the dark experiment ($k_d = 9 \times 10^{-3}\,h^{-1}$ versus $1{\cdot}7 \times 10^{-3}\,h^{-1}$) so that the difference between the decay rates of enzyme activity and plate counts was higher in the light experiment, showing that the transition from a culturable to a ABNC state can be enhanced by a light effect. The role of light in the transition to an active but nonculturable state for *E. coli* cells has already been reported by other authors with the following criteria of activity: GALase activity (Pommepuy *et al.* 1996) and electron transport system activity (Barcina *et al.* 1989). The experiments performed in microcosms showed that the enzymatic methods could detect GLUase activity of bacteria that had become nonculturable after nutritional and light stresses. These two factors are likely to have stronger effects in natural waters weakly contaminated by faecal coliforms because, in such systems, nutrient availability and water turbidity, which can reduce light effect (Davies-Colley *et al.* 1994), are generally lower than in strongly contaminated waters. Therefore, the proportion of ABNC coliforms would be more important

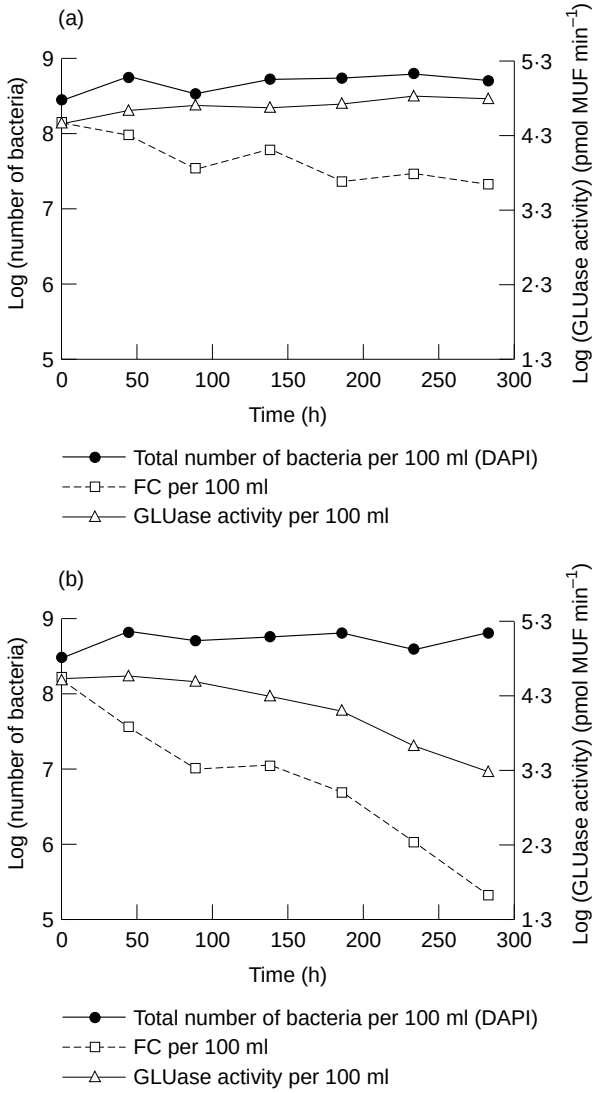

Fig. 8 Survival of *E. coli* discharged in microcosms containing sterile river water. and incubated at 20 °C in the (a) dark and (b) light. *E. coli* cells were studied by epifluorescence microscopy, culturable counts and β-D-glucuronidase (GLUase) assays

in less contaminated environments, which may partly explain the slope of less than 1 in Fig. 5.

In conclusion, according to the results presented in this work and those reported elsewhere (Davies *et al*. 1995; Pommepuy *et al*. 1996), both rapid enzymatic activities permit the detection of ABNC coliforms, which should lead to a better estimation of their abundance in natural waters. Moreover, their rapidity (response in less than half an hour, no need of time-consuming dilutions) gives them an indisputable advantage for the detection of faecal pollution. These tests could thus prove to be very useful tools for monitoring sewage pollution in natural waters.

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