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An early warning method to detect faecal contamination of river waters

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Abstract - Faecal coliforms (FC) and *Escherichia coli* enumeration is classically used to monitor the microbiological quality of surface waters. Traditionally, the methods used for the enumeration of these indicator bacteria are culture-based (in liquid or on solid culture media) requiring long incubation times; this makes them unable to rapidly detect faecal pollution. In this study, an early warning method to detect faecal contamination of river waters was investigated. This method is based on the measurement of the β-D-glucuronidase (an enzyme specific of *E. coli*) activity (GLUase activity) without any cultivation step using the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG). The GLUase activity measurement can be performed within 30 min. Significant linear regressions in log-log plots between the glucuronidase (GLUase) activity and, on one hand, FC number estimated by plate count and, on the other hand, *E. coli* abundance evaluated by the most probable number microplate method were found during the present study. Based on experimentally determined regressions, FC and *E. coli* abundance were calculated from GLUase activity data. The use of this early warning method was first positively tested to evaluate the microbiological quality in raw water of a drinking water treatment plant located in an urban area. The method proved to be able to rapidly and accurately estimate the spatial distribution of the microbiological contamination in a large river. Due to its rapidity, reproducibility (better than that of the culture-based methods) and relatively low cost, the GLUase activity measurement appears to be a very useful tool for the monitoring of microbiological pollution in freshwaters.

Key words*:* early warning system, *Escherichia coli*, faecal coliforms, microbiological quality, river water, β-D-glucuronidase activity.

INTRODUCTION

The monitoring of microbiological water quality is usually based on the enumeration of indicator bacteria as total coliforms (TC), faecal coliforms (FC), *Escherichia coli* and intestinal enterococci (APHA, 1995; WHO, 2001). Traditionally, the methods used for the enumeration of these indicator bacteria are culture-based (in liquid or on solid culture media). These methods require long incubation times (18 to 48 h) and are thus unable to rapidly detect faecal pollution. Therefore, detection of faecal pollution with these culture-based methods is of little value as a decision-making tool for preventive protection of public health. Rapid fluctuations of rivers microbiological water quality can be observed in the cases of heavy rainfall, combined sewer overflow or accidental pollution. In this case, there is a real need for methods that allow a rapid estimation of microbiological quality of river waters, particularly when they are used for producing drinking water or for recreational purposes. Thus, rapid detection of a faecal pollution event allows, for example, to adapt the treatment in drinking water treatment plant or to restrict bathing in suddenly contaminated areas.

To increase the specificity and decrease the response time of classical culture-based methods used to enumerate TC and *E. coli*, enzymatic properties (presence of β-D-galactosidase for TC and β-D-glucuronidase for *E. coli*) have been exploited in a multitude of new tests based on the assumption that both enzymes are selective markers for TC and *E. coli*. In these tests fluorogenic (4-methylumbelliferyl derivatives) or chromogenic (nitrophenol or indoxyl derivatives) substrates incorporated into culture media are hydrolysed by the β-D-galactosidase and/or β-D-glucuronidase enzymes, with subsequent detection of the fluorescent or coloured hydrolysis products, respectively (Manafi *et al*., 1991; Frampton and Restaino, 1993; Manafi, 2000). Enzyme-based tests are sensitive, simple to perform and in most cases do not require a confirmation step. They have been applied to enumerate TC and/or *E. coli* in food, drinking water, seawater, freshwater, sewage, and clinical samples. However, these culture-based tests still often require 18 to 24 h to complete.

More recently, the β-D-galactosidase and β-D-glucuronidase activities of TC and *E. coli* have been exploited in rapid assays performed without any cultivation step in seawaters (Fiksdal *et al*., 1994; Davies *et al*., 1995; Caruso *et al*., 2002), in freshwaters (George *et al*., 2000; Farnleitner *et al*., 2001, 2002) and in wastewaters (Apte *et al*., 1995, George *et al*., 2001a). George *et al*. (2000) optimised a protocol for measuring the β-D-glucuronidase activity (GLUase) in river waters using the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) in a period as short as 30 min. Using

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similar types of protocol, several studies have shown good correlations in log-log plot between the GLUase activity and FC enumerated on plate counts in various types of aquatic systems (Fiksdal *et al*., 1994; George *et al*., 2000; Farnleitner *et al*., 2002). A significant correlation has been also recently found between GLUase activity and *E. coli* abundance estimated by plate counts in polluted rivers (Farnleitner *et al*., 2001). Therefore, this rapid enzymatic measurement had a great potentiality to evaluate faecal pollution in river waters. The objective of the present study was to test the GLUase activity as an early warning method to detect faecal contamination of freshwaters.

In the present work, we tested the ability of the direct measurement of β-D-glucuronidase to rapidly and accurately detect microbiological contamination in river waters. The GLUase method was used to monitor temporal fluctuation of faecal contamination in a river located in an urban area and used as resource for drinking water production. The ability of the enzymatic method to give a rapid overview of the spatial distribution of faecal contamination along a large river stretch was also tested. In order to be able to convert GLUase activity data into *E. coli* or FC counts, linear regressions between enzymatic activity and both culture-based methods were established by measurements in river water samples covering a large range of faecal contamination. Most Probable Number (MPN) estimation of *E. coli* by the microplate method and plate count of FC on Tergitol medium were used as culture-based methods as they are normalised in France (AFNOR, 2001). Tests on the reproducibility of the GLUase activity measurement were also performed.

MATERIALS AND METHODS

Samples collection. In order to compare measurements of GLUase activity with enumerations of faecal indicators (*E. coli* and FC), water samples were collected from different rivers of the Seine river hydrographical network (France) showing a large range of faecal contamination levels, including small rivers upstream any domestic wastewater discharge and highly contaminated rivers. All samples were collected in 2 litres sterile bottles, kept at 4 °C and analysed within 6 h. These freshwater samples were analysed using three techniques in parallel: GLUase activity, *E. coli* enumeration by MPN microplate method and FC numbers estimated by plate counting.

GLUase activity measurement used as an early warning method for faecal pollution was first tested on river water used as raw water in a drinking water treatment plant located in the Parisian area. GLUase activity measurements were performed on raw water of the plant several times (3 to 6) per week during a two months period, in parallel with FC enumeration by plate count.

In order to test the ability of the GLUase activity measurement to correctly describe the spatial fluctuation of the microbiological pollution in a river, water samples were collected in April 2003 along a longitudinal profile from 10 km downstream from Paris to the entrance into the Seine estuary at Poses Dam. Sampling stations as well as the location of the large Achères treatment plant which releases its treated effluents in the studied stretch of the Seine river are indicated in Fig. 1. *Escherichia coli* numbers were estimated by the MPN microplate method; GLUase activity was also measured.

***Escherichia coli* enumeration.** A standardized miniaturized MPN method (AFNOR, 2001) 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; Edberg *et al*., 1990), 200 µl of the water sample and of several decimal dilutions of the sample were added in each of the 96 wells of the microplate containing the substrate MUG in a dehydrated form. This substrate is hydrolysed by the β-D-glucuronidase and the fluorescent

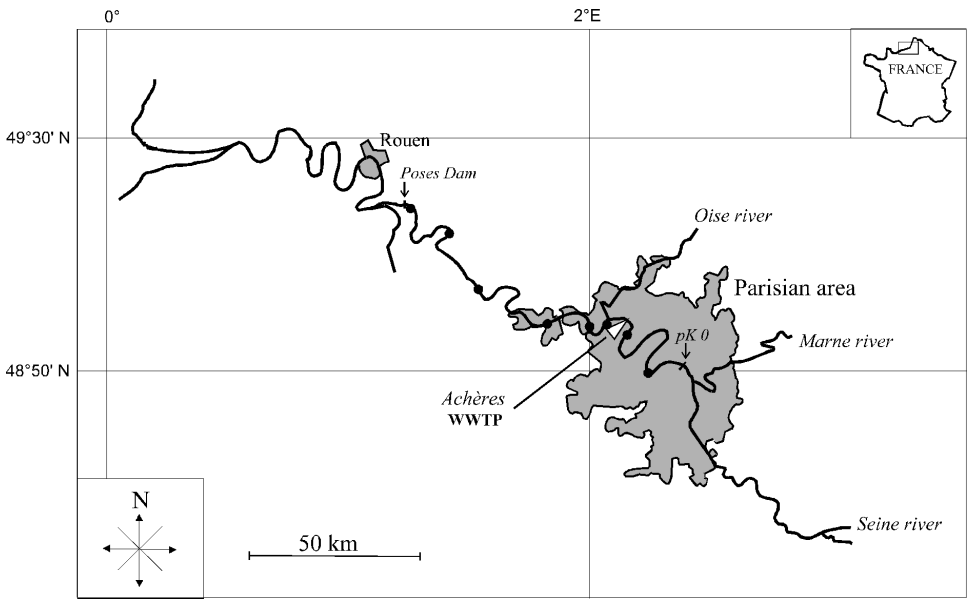

FIG. 1 – Map of the Seine river from Paris to the mouth of its estuary showing the location of the Achères wastewater treatment plant (WWTP) and the sampling stations along the river (•). PK is a kilometric unit used by the "Service de la Navigation de la Seine", which is set to zero at "Pont Marie" in downtown Paris and increases to the estuary.

compound methylumbelliferone (MUF) released can be detected under ultraviolet light. The microplates were incubated for 36 to 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 allows calculating the *E. coli* abundance using a statistical analysis based on Poisson's distribution. *Escherichia coli* enumeration data were expressed in number of *E. coli* per 100 ml.

Fecal coliform enumeration. Faecal coliforms (FC) were enumerated by plate count after membrane filtration (0.45 µm-pore-size, 47-mm-diameter sterile cellulose nitrate filters, Sartorius) and incubation on lactose agar with Tergitol (0.095‰ w/v final concentration) and triphenyl 2,3,5-tetrazolium chloride (TTC) (0.024‰ w/v final concentration) according to the French standards (AFNOR, 2001). Orange colonies producing a yellow halo under the membrane after incubation for 24 h at 44 °C were considered FC colonies. FC counts were expressed as colony-forming units (CFU) per 100 ml of sample.

β-D-glucuronidase activity measurements. β-D-glucuronidase (GLUase) activity measurements were performed following the protocol proposed by George *et al*. (2000) slightly modified. River water 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 17 ml of sterile phosphate buffer (pH 6.9) and 3 ml of MUG solution (55 mg of MUG (Biosynth, Switzerland) and 20 µl of Triton X-100 in 50 ml of sterile water) was added to each flask (final concentration: 165 mg l^{-1}). The flasks were incubated in a shaking water bath at 44 °C. Every 5 min for 30 min, a 2.9 ml aliquot of the 20 ml was put in a quartz cell with 110 µl of 1 M NaOH solution to obtain a pH of 10.7 (corresponding to the maximum of fluorescence of the methylumbelliferone (MUF)). The fluorescence intensity of the aliquot was measured with a 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 MUF concentrations from 50 to 3000 nM; this procedure allowed us to study a wide range of enzymatic activities by changing only the fluorometer calibration. The production rate of MUF (picomoles of MUF released 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. A blank (autohydrolysis of the MUG substrate) was subtracted to each GLUase activity; it was estimated by performing the measurement as described above but no water was filtered on the 0.2 µm-pore-size membrane before adding the filter in the sterile phosphate buffer. All the data of GLUase activities presented in this paper are expressed in a similar way. For comparison with data of FC or *E. coli* enumeration by culture-based techniques, GLUase activity data were converted by the following equations based on the log-log linear regressions presented in Fig. 2 and 3: *E. coli* = $10^{[(\log \text{GLUase act} - 0.012)/0.521]}$, FC = $10^{[(\log \text{GLUase act} - 0.013)/0.48]}$

Statistical analysis. In order to compare the data of GLUase activity measurements with FC and *E. coli* numbers, linear regressions were calculated after log transformation of the values using the SPSS statistical analysis program.

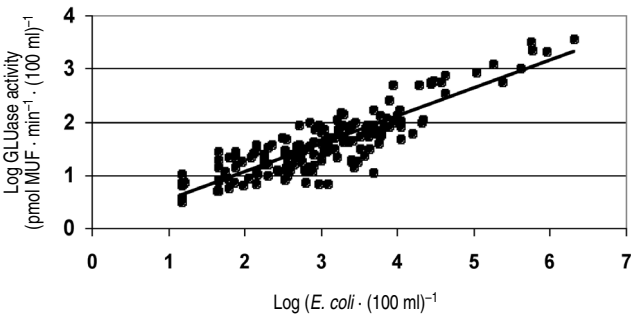

FIG. 2 – Log-log linear regression between GLUase activity and *Escherichia coli.* in river water samples: Log GLUase act. (pmoles MUF·min^{-1}·100 ml^{-1}) = 0.521 Log (*E. coli*·100 ml^{-1}) + 0.012 (r^2=0.76, n=166, p<0.0001).

RESULTS AND DISCUSSION

The use of the GLUase activity measurement as an early warning system to detect faecal contamination requires the conversion of GLUase activity data into FC or *E. coli* counts to be compared with guideline levels provided by regulations on microbiological water quality. Therefore, relationships between GLUase activity and *E. coli* and FC abundances were established in the first part of this study. In Fig. 2, log-transformed GLUase activities were plotted against log-transformed *E. coli* abundance. Some samples for which the *E. coli* number was below the detection limit of the MPN method (15·100 ml^{-1}) were not considered in this comparison. The linear regression between both variables in log units was highly significant (n=166, r^2=0.7648, standard error=0.2892, F=533.13, p<0.0001). This agrees with previous results obtained in polluted rivers, by Farnleitner *et al*. (2001), when plotting log-transformed GLUase activities versus log-transformed *E. coli* numbers estimated by plate count on Chromocult agar medium.

In Fig. 3, log-transformed GLUase activities were plotted against log-transformed FC numbers. The linear regression between both variables in log units was also highly significant (n=152, r^2=0.7228, standard error=0.3127, F=393.51, p<0.0001). These data confirm previous results showing good linear regressions in log-log plot between GLUase activity and faecal coliforms abundance estimated by plate counts for natural freshwater (George *et al*., 2000, 2004; Farnleitner *et al*., 2002) and wastewater (George *et*

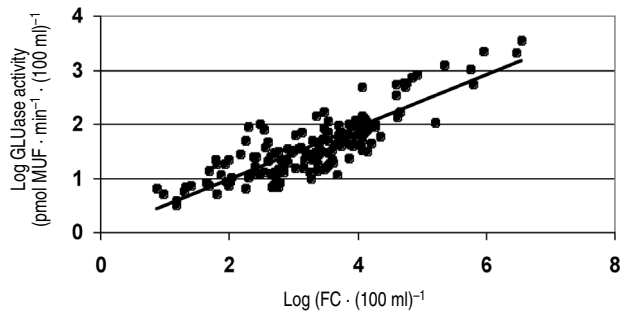

FIG. 3 – Log-log linear regression between GLUase activity and FC in river water samples: Log GLUase act. (pmoles MUF·min^{-1}·100 ml^{-1}) = 0.480 Log (FC·100 ml^{-1}) + 0.013 (r^2=0.72, n=152, p<0.0001).

al., 2001a) samples. The fact that the slope of the regression straight line between GLUase activity and FC abundance was lower than 1 was fully discussed by George *et al*. (2000). They suggested that it was due to an underestimation of FC by plate counts in low contaminated waters because of the existence in these environments of many active but non-culturable (ABNC) coliforms, i.e. cells presenting a detectable GLUase activity but unable to produce visible colonies on agar media. Less contaminated waters are likely to promote the transition from culturable to ABNC due to higher light penetration and lower nutrient availability. Recently, comparing *E. coli* enumeration by the microplate MPN method and by fluorescent *in situ* hybridisation (FISH) coupled with the direct viable count technique, Garcia-Armisen and Servais (2004) confirmed the presence of a large fraction of non-culturable *E. coli* in low contaminated river waters.

The comparison of Fig. 2 and 3 showed that the Y intercept of both linear regressions were similar but the slope of the regression between GLUase acivity and *E. coli* (slope=0.521, see legend of Fig. 2) was higher than that of the regression between the enzymatic activity and FC (slope=0.480, see legend of Fig. 3). This means that a given GLUase activity corresponds to a higher FC number than an *E. coli* number. This is consistent with the fact the *E. coli* is a part of the FC. In the present study, *E. coli* numbers were in average 73% of the FC numbers. The lower significance of the liner regression between FC numbers and GLUase activity data (r^2=0.72) compared with that calculated between *E. coli* numbers and GLUase activity data (r^2=0.76) is not surprising as GLUase is an enzyme specific of *E. coli* (Hartman, 1989). These regressions allowed calculating *E. coli* or FC abundances on the basis of GLUase activity measurement data.

The accuracy (reproducibility) of the GLUase activity measurement has been compared to the accuracy of MPN determination of *E. coli* and FC enumeration on plate counts for a variety of contaminated freshwater samples. Five replicates were performed with each method. The mean, the standard deviation and the coefficient of variation (CV) were calculated and shown in Table 1. The precision of enzymatic activity assays was good with CV ranging from 8 to 15% whereas the CV of both culture-based method were higher. This range of calculated CV for the GLUase method was similar to that found by George *et al*. (2001a). The CV of culturable FC estimated by plate counts was quite similar for the three samples (23 to 28%). A precision around 25% is quite usual for plate count methods. The CV of statistical MPN determinations of *E. coli* were higher, from 31 to 105%. From these data, we can conclude that the reproducibility of the GLUase method is better than the culture-based methods used for routine evaluation of microbiological water quality. Therefore, a part of the dispersion observed in Fig. 2 can be attributed to the low reproducibility of the MPN method.

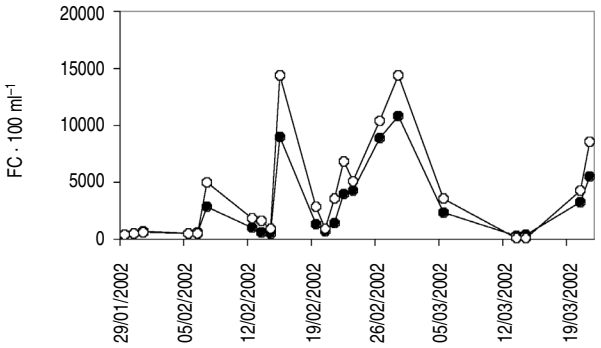

FIG. 4 – Temporal fluctuation of FC in the raw water of the Choisy-le-Roi drinking water treatment plant. FC were enumerated on plate counts (•) and calculated on the basis of the GLUase activity (o) using the regression presented in Fig. 3.

According to the data presented here above showing (i) good correlations between GLUase activity and the abundance of two of the main faecal indicators (*E. coli* and FC) and (ii) the good reproducibility of the GLUase assay, we suggest the use of the GLUase activity as an alternative to the culture-based methods to evaluate faecal pollution in freshwater systems; it allows to define the level of microbiological contamination in less than one hour in the laboratory.

In the second part of this work, we have tested the performance of the GLUase activity measurement as an early warning method for faecal pollution in river waters. The data obtained during these tests were of course not included in the set of data used to establish the correlations presented in Fig. 2 and 3.

Concerning the temporal fluctuations of FC abundance, Fig. 4 shows the distribution of their values obtained by plate counts. The data of GLUase activities were transformed into FC numbers using the regression from Fig. 3 and plotted in Fig. 4. This figure shows that the general pattern of

TABLE 1 – Accuracy of GLUase activity measurement, *Escherichia coli* abundance estimated by the miniaturized MPN method and FC plate counts in three different contaminated river water samples

Sample	GLUase activity		*Escherichia coli*		FC	
	(pmoles MUF·min^{-1} ·100 ml^{-1})[a]	CV[b]	(N·100 ml^{-1})[a]	CV[b]	(N·100 ml^{-1})[a]	CV[b]
1	6.7 ± 0.5	8	1218 ± 381	31	540 ± 142	28
2	80 ± 12	15	7880 ± 8259	105	14040 ± 3179	23
3	590 ± 87	15	29540 ± 15853	54	52000 ± 12981	25

The three measurements were repeated 5 times on each sample and the mean, standard deviation, coefficient of variation were calculated.
[a] Mean ± standard deviation; [b] the coefficient of variation was calculated as the standard deviation divided by the mean and multiplied by 100.

the FC temporal fluctuations was similar when evaluated by the plate count and the enzymatic method. The peaks of microbiological pollution observed in the river were recorded by both methods. Sometimes, the FC values calculated using the GLUase activity measurement data were higher the FC enumerated by plate count; this can be due to the presence of viable but non-culturable FC cells detected by the enzymatic measurement and not by the culture based method. The temporal fluctuations of the microbiological water quality at the sampling station were mainly due to combined sewers overflows into the river, occurring during heavy rain events, some kilometres upstream the drinking water treatment plant. In addition, due to works in the wastewater network of the Parisian suburb, some release of untreated wastewaters into the river upstream the drinking water treatment plant also occurred during the studied period. These events were responsible for the several peaks of pollution observed in Fig. 4. All the samples collected during the period under study showed FC abundances lower than the European guidelines (20000 FC·100 ml^{-1}) for raw water used to produce drinking water in a treatment line including two stages of filtration as it is the case in the studied drinking water treatment plant.

Even if the FC numbers calculated from the GLUase activity measurements did not always exactly correspond to the FC abundances estimated by the traditional plate count method, the data from Fig. 4 clearly indicates that the enzymatic method is able to detect all the microbiological pollution events affecting the microbiological quality of the raw water of the plant. This demonstrates the potential importance of the GLUase activity measurement as a tool to rapidly detect a problem of microbiological water quality allowing possible preventive actions as the adaptation of the treatment processes to maintain the microbiological quality of the produced water.

Concerning the assessment of microbiological pollution along a spatial scale, *E. coli* abundances were calculated on the basis of the GLUase activity measurements using the regression line reported in Fig. 2 and *E. coli* numbers were also estimated by the MPN microplate method; both estimations were plotted in log units in Fig. 5. The agreement between data obtained by both methods confirmed the ability of the GLUase activity to be an alternative to culture-based methods to rapidly screen the spatial distribution of microbiological contamination level of aquatic systems. Both estimates of *E. coli* abundance in the Seine river showed a similar general pattern along the longitudinal profile from Paris to the beginning of the estuary. *Escherichia coli* numbers increased drastically downstream PK 63 where the river receives an important flow (around 27 m^3 sec^{-1}) consisting of the treated effluents from the large Achères wastewater treatment plant (WWTP) (6.5×10^6 inhabitant-equivalents from the Parisian area, Fig. 1). Actually, the abundance of faecal bacteria in raw wastewater entering WWTP is so high that, even if an important part of the microbiological pollution is removed by the treatment (primary settling followed by activated sludge) at the WWTP (between one and two log removal as estimated by George *et al*. (2002) for FC at the Achères WWTP), the outfalls of the Achères WWTP obviously damage the microbiological quality of the receiving river. Downstream from Achères, *E. coli* abundance decreased in the Seine river, partly due to the dilution of the highly contaminated Seine water by the less contaminated Oise river (confluence 9 km downstream from Achères). Downstream from the Oise river confluence, a great reduction of *E. coli* abundance was observed along the 150 km between the Achères WWTP outfall and the Poses Dam. Similar longitudinal patterns were previously observed in this stretch of the Seine river for total and faecal coliforms by George *et al* (2001b).

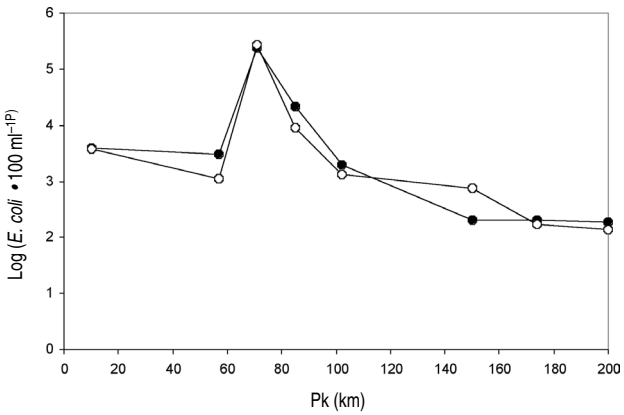

FIG. 5 – Spatial distribution of *Escherichia coli* in the Seine river from Paris to the entrance of its estuary. *Escherichia coli* were enumerated by the MPN microplate method (•) and calculated on the basis of the GLUase activity (o) using the regression presented in Fig. 2. PK is a kilometric unit used by the "Service de la Navigation de la Seine", which is set to zero at "Pont Marie" in downtown Paris and increases to the estuary.

CONCLUSION

In conclusion, rapid β-D-glucuronidase-based assay was tested in this work as alternative to plate counts and to standardized MPN methods for estimating faecal contamination in river waters. Significant linear regressions were observed between the enzymatic method and traditional culture-based techniques for *E. coli* and FC. The ability of the GLUase method as an early warning method for detecting the level of microbiological pollution was tested in two different situations: to follow the water quality in the raw water of a drinking water treatment plant and to rapidly estimate the spatial distribution of the microbiological contamination in a river. In both cases, the GLUase activity was able to detect pollution peaks and the conversion of the GLUase activity using the regression straight lines calculated in this paper allowed an accurate estimation of the abundance of FC, in one case, and of *E. coli,* in the other case.

The precision of the enzymatic method was higher than that of the culture-based methods. The cost in terms of consumables of a GLUase assay is approximately the same than the cost of a culturable coliforms enumeration by membrane filtration and is far less than a microplate MPN assay. The short time required to know the level of contamination (30 min instead of more than 18 h) seems the main advantage of the GLUase measurement as it allows a fast and more or less "real time" warning in case of pollution. Therefore, GLUase activity seems to be a very useful tool for the monitoring and management of microbiological pollution.

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