PERGAMON

Water Research 36 (2002) 975–981

WATER RESEARCH

www.elsevier.com/locate/watres

Hydrolysis of 4-methylumbelliferyl-β-D-glucuronide in differing sample fractions of river waters and its implication for the detection of fecal pollution

A.H. Farnleitner[a,*], L. Hocke[b], C. Beiwl[b], G.G. Kavka[b], R.L. Mach[a]

[a] *Institute of Biochemical Technology and Microbiology 172/5, Technical University of Vienna, Getreidemarkt 9, 1060 Vienna, Austria*

[b] *Institute of Water Quality, Federal Agency for Water Management, Schiffmühlenstrasse 120, 1220 Vienna, Austria*

Received 2 January 2001; received in revised form 22 May 2001; accepted 6 June 2001

Abstract

The hydrolysis rate of 4-methylumbelliferyl-β-D-glucuronide (MUG-HR) was determined in unamended samples, filtered samples, and in corresponding buffer resuspended filter residues of various river waters of slight to excessive fecal pollution covering a four orders of magnitude range. Regression analysis of the log MUG-HR of the unamended water samples versus the log MUG-HR of the filter residues revealed a highly significant linear relationship ($R^2 = 0.94$; $p < 0.001$). The median of the MUG-HR of the filtrated water samples was about 10% the MUG-HR of the corresponding unamended water samples. If MUG-HR determinations were used as a surrogate for estimating fecal coliform contamination, both the MUG-HR of the unamended water samples and the MUG-HR of the filter residues would have been equally adequate techniques at river sites of higher fecal pollution levels. However, at river locations of decreased fecal pollution, MUG-HR determination of filter residues appeared to be the more sensitive technique in order to estimate fecal coliform concentrations.

Keywords: 4-methylumbelliferyl-β-D-glucuronide hydrolysis; Rivers; Fecal pollution; Monitoring

1. Introduction

Efficient techniques for the detection of fecal pollution in aquatic environments are crucial for monitoring programmes as well as for watershed management. For this purpose, determination of microbial indicators by cultivation provide a sensitive way to estimate the degree of fecal pollution. However, cultivation-based detection of microbial cells demand considerable manipulation activities and time, although recent developments reduced incubation length procedures significantly [1]. In order to avoid cultivation, efforts were made to generate alternatives which directly detect microbial fecal indicators or related activities. Besides other potential alternatives [2,3], direct measurement of enzymatic activities in aquatic environments represents a promising approach. Furthermore techniques based on enzymatic activities bear a great potential for automation and on-line monitoring [4].

The application of direct enzymatic detection of coliforms (CF) and fecal coliforms (FC) by β-D-galactosidase (GAL) or β-D-glucuronidase (GLU) activity in differing aquatic environments has been demonstrated by various authors [5–8]. The principle is based on the hydrolysis of artificial chromogenic or fluorogenic substrates which are specifically split by the respective enzyme to be under the investigation. Substrates for the detection of GAL or GLU activities have frequently been used for the last 30 years in bacterial diagnostics and are well established in clinical

*Corresponding author. Tel.: +43-1-58801-17254; fax: +43-1-58-16266.

E-mail address: a.farnleitner@aon.at (A.H. Farnleitner).

0043-1354/02/$ - see front matter
PII: S0043-1354(01)00288-3

microbiology [9]. By applying these substrates, detection of FC or CF can be achieved within 25–30 min [5].

Enzymatic GAL or GLU activity is not only expressed by CF and FC, hence a number of different sources in aquatic environments may potentially interfere with this rapid enzymatic fecal detection approach. Possible interference was demonstrated from plants and algae [10], non-particle bound fractions [7], and bacteria other than CF and FC [11,12]. Erroneous results may also be caused by false GLU negative FC [13]. Despite those potential interference mechanisms, significant log–log linear relationships between FC and TC versus GAL and GLU have been observed for various aquatic environments [5,6,8], highlighting the approach as a potential useful monitoring technique. In addition, it was suggested that enzymatic activities permit the detection of active but non-culturable cells (ANBC) which might lead to a better abundance estimation of FC as with cultivation-based techniques in the environment [8,14].

Different methods have been used to determine the enzymatic activity, (i) either measuring it in the water sample itself by addition of the respective substrate [6] or (ii) collecting the cells by filtration followed by respective enzyme assays with the resuspended cells [5,8]. Both methods are likely to effect the observed GLU or GAL activities in different ways, since sample manipulations, enzymatic reaction conditions, and measured enzyme fractions (e.g. particle bound enzymes and/or dissolved free enzymes) are differing. No comparative investigations between those two approaches are available to date, although methodical differences may have further implications, for example on the design of on-line approaches. Furthermore, scarce information exists on the amount of particle bound versus non-particle bound enzymatic activities of GAL and GLU in natural aquatic environments.

Thus the aim of this study was (i) to examine the relationship between the measured hydrolysis of 4-methylumbelliferyl-β-D-glucuronide (MUG) directly in the water samples versus in the resuspended filter residues, (ii) to determine the amount of non-particle bound hydrolysis activity of 4-methylumbelliferyl-β-D-glucuronide and (iii) to analyse the activity relationships of the different fractions to FC concentrations in the investigated rivers. The fluorogenic MUG was used as a specific substrate for GLU activity determinations.

2. Materials and methods

2.1. Sampling and sampling locations

River water samples for microbiological and enzymatic analysis were collected aseptically in 250 cm^3 Pyrex glass bottles (Pyrex) from a water depth of 30 cm. Samples were immediately put in a 4°C cooling box and processed within 6 h after collection. All samples were taken within one year period from April 1998 to 1999 in temperate rivers of Lower Austria. From River Danube, samples were taken bimonthly from downstream locations of Vienna at Wildungsmauer, Wolfsthal, Devin, Karlova Ves, Bad Deutsch-Altenburg. Samples from Rivers Schwechat, Fischa, and March, and Danube Channel were taken from sampling sites before approaching River Danube on a monthly schedule. All chemophysical parameters were determined according to APHA [15].

2.2. Fecal coliform determination

For cultivation of FC the membrane filtration technique was used [16]. For filtration 0.45 μm pore size cellulose nitrate membrane filters (45 mm diameter, Sartorius) were used. Filters were applied to mFC agar plates (Biomerieux) and incubated for 24 h in a water bath at 44.5°C. All colonies which developed a blue colour were accounted as FC and expressed as colony forming units (CFU) per 100 ml of sample. It should be mentioned that the most appropriate designation for this parameter would be "thermotolerant coliforms" instead of FC, since some members of this group can be of non-fecal origin [17]. However, we used the term FC to correspond with previously published papers on this subject.

2.3. Determination of MUG hydrolysis rate (MUG-HR)

2.3.1. Preparation of fractions

All sample processing steps were performed using autoclaved equipment. For the unamended sample fractions a sample aliquot of 18 ml was given in sterile 25 ml glass flasks (Pyrex). For filtration-fractionations, 100 ml of the samples were filtered through 0.45 μm pore size cellulose nitrate membrane filters as used for FC determination; except for the highly polluted samples from the Danube Channel, where only 20 ml was used. Filters were placed into sterile 250 ml glass bottles (Pyrex) containing 18 ml of phosphate buffer adjusted to pH 6.4 [5]. Aliquots of 18 ml from the filtrated water samples were also put into 25 ml glass flasks (Pyrex). All flasks were closed and equilibrated to 37°C. From the procedure described above, three fractions resulted, including unamended and filtrated water samples and buffer resuspended filter residues (designated as water samples, filtrated samples and filter residues).

2.3.2. MUG-HR determination

To all prepared flasks 2 ml of MUG substrate solution (1 g $MUG\,l^{-1}$ supplemented with 1% Triton X-100 v/v) was added. The flasks were tightly closed again and

incubated at 37°C in a shaking incubator. The fluorescence intensity was measured after 10, 20 and 30 min incubation by adding 100 µl 0.1 M NaOH to the 2.5 ml sample in the cuvette to obtain a pH greater than 10. No substrate depletion was observed for the used concentration (100 mg MUG l^{-1}) throughout the study as observed for lower concentrations (data not shown). Shimadzu RF5000 spectrofluorometer operating at an excitation of 365 nm and detecting at an emission of 444 nm was used.

2.4. Calculations and statistics

The relative fluorescence increase was calculated by least-squares linear regression, and only slopes significantly differing from 0 were accounted. The selected significance level was $p \geqslant 0.95$ for water sample and filter residue fractions and $p \geqslant 0.90$ for filtrated samples. To convert relative fluorescence values to absolute concentrations, standard additions by using methylumbelliferone (MUF, Fluka) concentrations from 10 to 800 nM were performed. The MUG-HR was calculated as MUF production per time (nM MUF min^{-1}). MUG-HR from filter residue determinations were expressed per 20 ml to make it comparable with MUG-HR of other fractions. Regression analysis and non-parametric tests were performed by the statistical software package SPSS Version 7.5.

3. Results and discussion

Rivers of slight to excessive fecal pollution were selected for this study [18]. Table 1 represents an overview of the observed range of FC contamination and basic chemophysical parameters at the sampling locations during the one year investigation. The selected rivers represent sewage influenced aquatic environments, since there are a number of sewage treatment plants located alongside the river stretches. Pollution levels are regularly monitored by federal authorities [18].

Table 1
Water quality characteristics of the analysed freshwaters[a]

Parameters	n	Range	
		Minimum	Maximum
pH	51	7.3	8.3
Conductivity (µS cm^{-1})	51	316	748
BOD (mg O_2 l^{-1})	51	1.2	52
Dissolved oxygen saturation (%)	51	54	112
Fecal coliforms (CFU 100 ml^{-1})	98	6.7×10^1	1.6×10^7

[a] (n)sample numbers; (BOD) biochemical oxygen demand for 5 days.

The MUG-HR of the unamended water samples versus the filter residue fractions revealed comparable levels (Table 2). Although activities of the filter residue fractions were significantly higher (Wilcoxon Signed Ranks Test, $n = 98$, $p < 0.01$) showing an approximately 30% increased median value as compared to the water sample. Furthermore, the median of all calculated single ratios of MUG-HR between the filter residues and the corresponding water samples (i.e. MUG-HR filter residue per MUG-HR water sample) was 1.15, i.e. revealed also a 15% higher MUG-HR for the filter residues as for the corresponding water samples. According to recently published data about the pH response of GLU activities in freshwaters [8], the filter residue and the unamended water sample fractions in this study were determined at about 85% (i.e. pH 6.4 of phosphate buffer) and at 57% (i.e. pH 8.1 of river water median), respectively, of the 100% GLU pH optimum level. Assuming that the phosphate buffer solution did not significantly alter the pH response, the 30% increased median levels of the MUG-HR of the filter enriched fraction could simple be explained by pH differences. Regression analysis of log MUG-HR of the filter residues versus log MUG-HR of the unamended water samples revealed a perfect linear relationship (Fig. 1). A variation of 94% of the variable log MUG-HR filter residue was explained by the variable log MUG-HR water sample, underlining the strong relationship between those two parameters; the slope of the log–log function was close to 1 (Fig. 1).

The MUG-HR observed from the filtrated sample fractions accounted only for a small amount of the total MUG-HR of unamended water samples (Table 2). Only 58 samples out of 98 analysed filtrated water samples exerted detectable MUG hydrolysis. There was an apparent threshold level—samples having FC contamination higher than 10^5 CFU per 100 ml showed general detectable MUG hydrolysis, whereas samples with FC contamination below this value yielded either detectable or not detectable activities. The median value of the MUG-HR of the filtrated fraction was about 10% the activity level of the unamended water sample fractions, and the median of all calculated single ratios of MUG-HR between filtrated waters and the corresponding unamended water samples (i.e. MUG-HR filtrated water per MUG-HR unamended water sample) was 0.04. The results demonstrated that the particle bound GLU enzymes were retained by the filtration process and only a small part of free dissolved MUG-HR, most likely due to GLU enzymes, existed in the investigated river systems. In this study we applied the same 0.45 µm pore size cellulose nitrate membrane filters as standardised for the determination of fecal coliforms [16] in order to make the data on fecal coliform concentrations and the corresponding MUG-HR comparable. It should be mentioned that the distinction between the dissolved

Table 2
Hydrolysis of the 4-methylumbelliferyl-β-D-glucuronide in differing sample fractions of the analysed river waters[a]

Sample fraction	n	Substrate hydrolysis (nM MUF 30 min^{-1})		
		Median	Minimum	Maximum
Freshwater samples	98	5.7	0.04	2670
Suspended filter residues	98	8.1	0.09	2013
Filtrated freshwater samples	98	0.6	n.d.	219

[a](n.d.) not detectable, 30 samples out of 98 showed n.d. hydrolysis for filtrated freshwater samples.

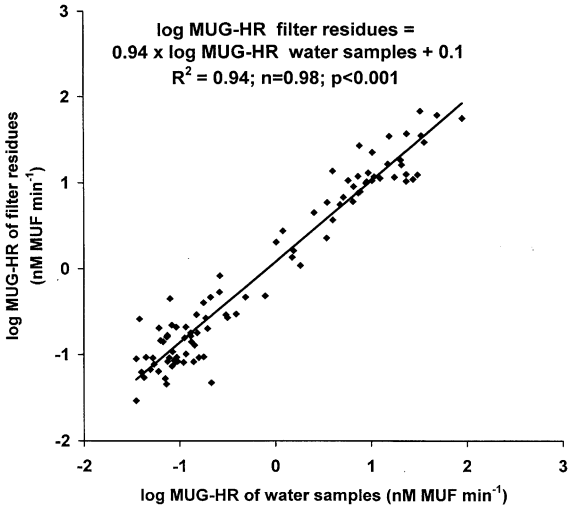

Fig. 1. Regression analysis of the log MUG-HR of the unamended water samples versus the MUG-HR of the resuspended filter residues.

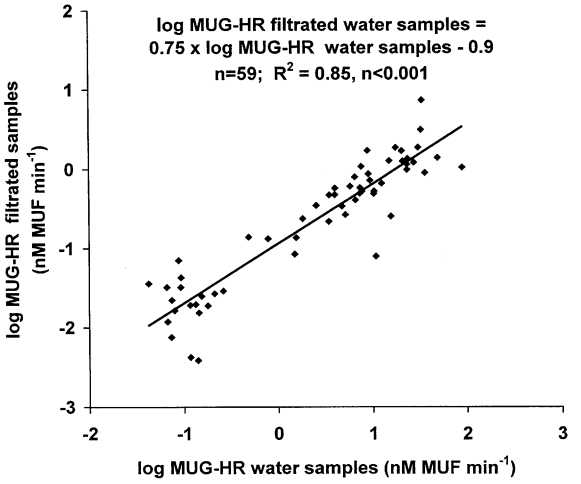

Fig. 2. Regression analysis of the log MUG-HR of the unamended water samples versus the MUG-HR of the filtered water sample.

and the particular fraction of organic material in aquatic environments is defined solely based on size (pore size usually between 0.5 and 0.7 μm) rather than on solubility or other physicochemical characteristics [19]. Former studies used 0.2 μm polycarbonate filters for the determination of MUG-HR of filter residues [5,8]. Differences between pore size as well as type of filtration process (surface versus deep-bed filtration) of the 0.2 μm polycarbonate and the 0.45 μm cellulose nitrate membrane (this study) might cause differences in the measured MUG-HR. Especially, the resuspension of filter residues from cellulose nitrate membranes might be less effective due to the catch of bacterial cells within the filter material. However, the comparison of the performance of filter types in terms of MUG-HR determination was not the aim of this study, nevertheless possible filter difference should be taken into account when MUG-HR are compared between studies.

Regression analysis for the 58 detectable MUG-HR of the filtrated samples versus the MUG-HR of the corresponding unamended water samples resulted in a linear log–log relationship (Fig. 2). Increased MUG-HR in the unamended water samples were associated with increased MUG-HR in the filtrated fraction, although the relationship showed a higher degree of non-explained variation ($R^2 = 0.85$) as compared to the regression in Fig. 1. The slope of the log–log linear regression was far below 1 (Fig. 2), indicating that the fraction of the MUG-HR in the filtrated water in relation to the MUG-HR of the total water fraction decreased as the GLU activity or the pollution of the rivers increased. Accordingly, to this log–log linear relationship, mean percentages of 22%, 13%, 7% and 4% of MUG-HR activities in the filtrated fraction at corresponding total MUG-HR (i.e. of the unamended water sample) of 0.1, 1, 10, and 100 nM MUF min^{-1}, respectively, were observed. This suggests that at freshwater locations of lower fecal pollution a higher fraction of dissolved GLU enzymes appeared. This might have happened either due to the lysis of microbial cells and the subsequent release of enzymes, smaller GLU bound particles at lower polluted freshwater sites which are not

retained by the filters, or due to a more pronounced interference from dissolved GLU enzymes from sources other than fecal origin [10].

Enzymes are known to be particularly sensitive against various substances, and are thus used for toxicological evaluations [20]. General competitive or reversible toxicological effects on the GLU activity from possible toxic substances released by the sewage effluents could not be detected at the various investigated rivers. Both MUG-HR of the buffer resuspended filter residues and the corresponding unamended water samples were comparable. The MUG-HR fraction differences (including or excluding the $<0.45\,\mu m$ part) accounted only for a small amount, and observed MUG-HR differences could be explained by pH effects (see above). If significant general competitive or reversible toxicological GLU effects were observed in the unamended water samples, they should have been out diluted at the filter residues resulting in MUG-HR differences between the compared fractions.

To study for relationships between the MUG-HR of the various investigated river water fractions and the observed fecal coliform concentrations, regression analysis was undertaken. All MUG-HR of the respective analysed fractions resulted in significant log–log linear regressions to fecal coliforms, although remarkable differences existed in detail (Fig. 3). The best curve fit was achieved from the log MUG-HR of the filter residues ($R^2 = 0.87$), followed by the log MUG-HR of the water samples ($R^2 = 0.83$), and the log MUG-HR of the filtrated water fraction ($R^2 = 0.79$). The fact that the variable MUG-HR of the filter residue fractions was the best explained fraction by the variable log FC seems reasonable, since the filter residues constituted the potential pool of FC cells which were applied to the cultivation process. Both regressions, the log MUG-HR of the filter residues (Fig. 3A), and the log MUG-HR of the water samples (Fig. 3B) yielded corresponding regression equations. Their slopes of the log–log linear equation (0.82) was in agreement with former studies from marine and freshwater environments which observed also slopes significantly smaller than one [5,8]. This means an increase of the GLU activity per culturable fecal coliforms as the number of culturable fecal coliforms decreased. A possible explanation for this observation was given by George et al. [8] who argued that higher enzymatic activities per culturable FC in less contaminated waters could be due to a stronger underestimation of the number of FC when enumerated by plate count due to a higher proportion of active but non-culturable cells (ABNC) of FC in these waters. Indeed it was shown by several studies that ABNC of FC can still be detected by enzymatic techniques [8,14,21]. Less contaminated waters are likely to promote the transition from culturable to ANBC due to higher light penetration and lower nutrient availability. Although the slopes of both log–log regressions for the whole data set of the investigated samples were exactly the same (Fig. 3A and B), partial regression analysis performed for the lower polluted range (from log FC 2.5 to 4.5) of the rivers revealed distinct results (Fig. 4). MUG-HR from filter residues (Fig. 4A) revealed a much higher slope (0.49 instead of 0.27) and R^2 (0.39 instead of 0.13) as compared to MUG-HR from the unamended water samples (Fig. 4B). The same partial analysis performed for the higher fecal polluted range did not reveal such a difference for the unamended water sample versus the filter residues (the slopes differed by 20%, data not shown). This trend is also indicated in Fig. 1, where the highest variation between the log MUG-HR from the filter residues and the MUG-HR from the unamended water samples was observed in the lower part of the regression (around -1 of the log MUG-HR). We suggest two main reasons principally responsible for this result. First, the $<0.45\,\mu m$ GLU fraction tended to be increased in lower polluted river sections (see discussion above), and thus likely interfering with the relationship of the log FC and log MUG-HR of the unamended water samples, as this fraction is not separated as performed for the MUG-HR filter residues. Second, determinations of MUG-HR in

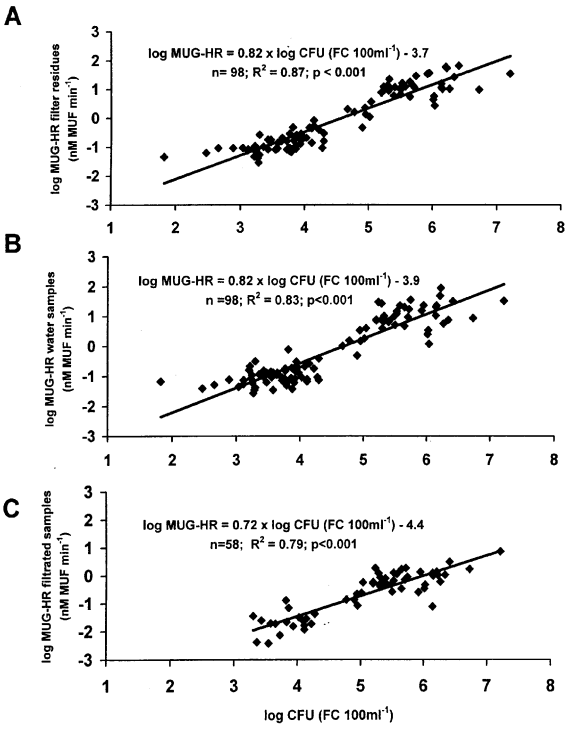

Fig. 3. Regression analysis of the log MUG-HR of different analysed river sample fractions versus the log of fecal coliform CFU.

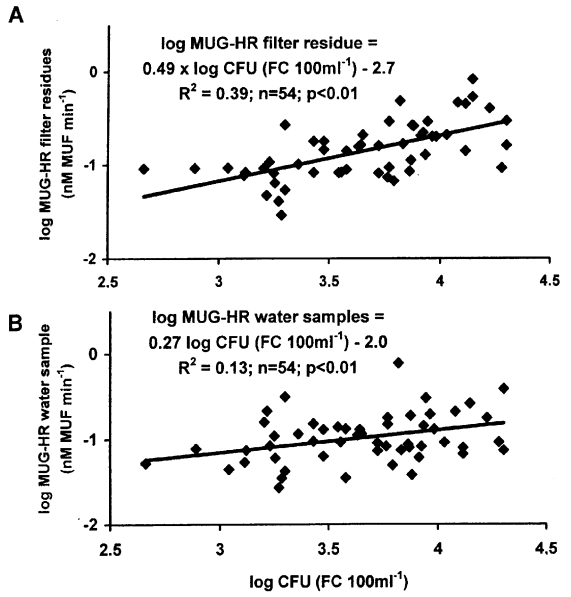

Fig. 4. Partial regression analysis of the log MUG-HR of the unamended water samples and the filter residues, respectively, versus the log of fecal coliform CFU at lower fecal pollution levels (log 2.5–4.5 FC CFU).

the unamended water samples, especially at lower activities, are more prone to be biased from interfering effects such as from background fluorescence, higher noise-to-signal ratios, etc., as compared to MUG-HR filter residue measurements. In our case, MUG-HR determinations of filter residues at lower polluted locations were performed by filtering 100 ml (but expressing it to 20 ml) of sample and resuspending it in 20 ml buffer solution. This procedure should have decreased background fluorescence as well as decreased noise-to-signal ratio as five-fold enrichments of FC cells were used for the activity determination. According to our data set, if MUG-HR determinations were used as a surrogate for estimating FC concentrations or the rapid detection of fecal pollution, both the MUG-HR of unamended water samples and the MUG-HR of filter residues would have been equally adequate techniques at higher fecal pollution levels of the investigated rivers. The use of the MUG-HR determination on unamended water samples was straightforward and created less costs and labour and furthermore it would be easier for possible automation as compared to filter techniques. However as the fecal pollution level decreased, MUG-HR determination of filter residues as a surrogate for FC concentrations was the more sensitive (i.e. higher slope of the partial regression) and adequate technique. The MUG-HR determination of unamended water samples was prone to be more biased by interfering effects. Selective optimisation of the MUG hydrolysis by FC originated GLU enzymes and/or the inhibition of GLU enzymes of non-fecal origin could decrease the limitation as argued by Davies and Apte [7]. Accelerated temperature cannot be used as a differential selective pressure on fecal GLU enzymes, as it was demonstrated that also GLU enzymes of non-target bacteria were stable at 44.5°C [12]. The regression from the log MUG-HR of the filtrated water and the log of the FC (Fig. 3C) revealed a worse relationship as compared to Fig. 3A and B and furthermore demonstrated a limited detection range of fecal pollution.

4. Conclusions

In this study, we examined the relationship between MUG-HR of different water sample fractions of slightly to excessively fecal polluted rivers. The results should have implications for further developments on the rapid enzymatic detection of fecal pollution in aquatic environments in order to provide efficient on-line monitoring tools in future. The present study has shown that:

- In fecal influenced rivers, a highly significant linear relationship between the log MUG-HR of unamended water samples versus the log MUG-HR of filter residues ($R^2 = 0.94$; $p < 0.001$) can be observed. However, the study site was restricted to temperate Austrian river systems and general conclusions to other habitats should only drawn with caution.
- At river sites of higher fecal pollution levels (logFC > 4.5 per 100 ml), both fractions, the unammended water samples and the filter residues can equally be used for MUG-HR determination in order to rapidly estimate FC concentrations
- At rivers sites of lower fecal pollution levels (log FC < 4.5 per 100 ml), determination of the MUG-HR from filter residues is the more sensitive, an accurate technique for rapid FC estimation.
- A higher relative MUG-HR in the filtrated water sample fraction seems to appear as fecal pollution levels of a river decreases. If this is caused by increased numbers of dissolved glucuronidase enzymes has to be investigated in the future. In addition the use of reference microorganism in microcosm experiments might be a useful tool on this subject, especially when low fecal polluted aquatic environments are under investigation.

Acknowledgements

We are very grateful to the Hochschuljubileumsstiftung der Stadt Wien, Austria, who funded this study (project number H-42/97).

References

[1] Van Poucke SO, Nelis HJ. Rapid detection of fluorescent and chemiluminescent total coliforms and *Escherichia coli* on membrane filters. J Microbiol Methods 2000;42:233–44.

[2] Farnleitner AH, Kreuzinger N, Kavka GG, Grillenberger S, Rath J, Mach RL. Simultaneous detection and differentiation of *Escherichia coli* populations from environmental freshwaters by means of sequence variations in a fragment of the β-D-glucuronidase gene. Appl Environ Microbiol 2000;66(4):1340–6.

[3] Van Poucke SO, Nelis HJ. A 210-min solid phase cytometry test for the enumeration of *Escherichia coli* in drinking water. J Appl Microbiol 2000;89:390–6.

[4] Font X, Caminal G, Gabarell J, Lafuente J, Vicent MT. On-line enzyme activity determination using the stopped-flow technique: application to laccase activity in pulp mill waste-water treatment. Appl Microbiol Biotechnol 1997;48:168–73.

[5] Fiksdal L, Pommepuy M, Caprais MP, Midttun I. Monitoring of fecal pollution in coastal waters by use of rapid enzymatic techniques. Appl Environ Microbiol 1994;60(5):1581–4.

[6] Davies CM, Apte SC. Rapid enzymatic detection of faecal pollution. Water Sci Technol 1996;34(7-8):169–71.

[7] Davies CM, Apte SC. An evaluation of potential interferences in a fluorimetric assay for the rapid detection of thermotolerant coliforms in sewage. Lett Appl Microbiol 2000;30:99–104.

[8] George I, Petit M, Servais P. Use of enzymatic methods for rapid enumeration of coliforms in freshwaters. J Appl Microbiol 2000;88:404–13.

[9] Manafi M, Kneifel W, Bascomb S. Fluorogenic and chromogenic substrates used in bacterial diagnostics. Microbiol Rev 1991;55:335–48.

[10] Davies CM, Apte SC, Peterson SM, Stauber JL. Plant and algal interference in bacterial beta-D-galactosidase and beta-D-glucuronidase assays. Appl Environ Microbiol 1994;60(11):3959–64.

[11] Fiksdal L, Tryland I, Nelis H. Rapid detection of coliform bacteria and influence of non-target bacteria. Water Sci Technol 1997;35(11-12):415–8.

[12] Tryland I, Fiksdal L. Enzyme characteristics of beta-D-galactosidase-and beta-D-glucuronidase-positive bacteria and their interference in rapid methods for detection of waterborne coliforms and *Escherichia coli*. Appl Environ Microbiol 1998;64(3):1018–23.

[13] Chang GW, Brill J, Lum R. Proportion of beta-D-glucuronidase-negative Escherichia coli in human fecal samples. Appl Environ Microbiol 1989;55(2):335–9.

[14] Fiksdal L, Tryland I. Effect of u.v. light irradiation, starvation and heat on *Escherichia coli* β-D-galactosidase activity and other potential viability parameters. J Appl Microbiol 1999;87:62–71.

[15] APHA. Standard methods for the examination of water and waste water, 19th ed. American Public Health Association,Washington, DC, 1995.

[16] ISO.Water quality—detection and enumeration of coliforms, thermotolerant coliform and presumptive *Escherichia coli*. International Organisation of Standardisation, Geneva, 1990.

[17] Edberg SC, LeClerc H, Robertson J. Natural protection of spring and well drinking water against surface microbial contamination II. Indicators and monitoring parameters for parasites. Criti Rev Microbiol 1997;23(2):179–206.

[18] IWG. Güteentwicklung der Donau: Rückblick und Perspektiven (water quality development of the River Danube: past and perspectives). Institute for Water Quality of the Federal Agency for Water Management, Vienna, Austria, 1997.

[19] Kepkay PE. Colloids and the ocean carbon cycle. In: Wangersky PJ, editor. Marine chemistry. Berlin: Springer, 2000. p. 35–56.

[20] Lee K, Tay KL. Measurement of microbial exoenzyme activity in sediments for environmental impact assessment. In: Wells PG, Lee K, Blaise C, editors. Microscale testing in aquatic toxicology. Boca Raton: CRC Press, 1998. p. 219–36.

[21] Pommepuy M, Fiksdal L, Gourmelon M, Melikechi H, Caprais MP, Cormier M, Colwell RR. Effect of seawater on *Escherichia coli* β-galactosidase activity. J Appl Bacteriol 1996;81:174–80.