African Journal of Biotechnology Vol. 5 (22), pp. 2338-2344, 16 November, 2006
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2006 Academic Journals

Full Length Research paper

Properties of *in situ Escherichia coli* β-D-glucuronidase (GUS): evaluation of chemical interference on the direct enzyme assay for faecal pollution detection in water

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Accepted 20 October, 2006

A study of the activity of *Escherichia coli* β-D-glucuronidase (GUS) in polluted stagnant and running water samples was performed with an objective of assessing the viability of a direct marker enzyme assay as a suitable alternative to membrane filtration for the indication of faecal pollution in water intended for drinking purposes. The effects of temperature, pH and the presence of different ions on enzyme activity were investigated. GUS exhibited optimal activity at 40°C over a broad pH range (5-9). In general, CO_3^{2-} (as Na_2CO_3), Cl^- (NaCl) and NO_3^- (KNO_3) increased GUS activity, while ferric chloride ($FeCl_3$), OCl^- (as NaOCl) and ferulic acid were inhibitory. However, the enzymatic response to these effectors differed with the source of the sample. Therefore, when these compounds are present in water intended for drinking purposes they can either exaggerate or give false negatives with regards to GUS activity. Environmental GUS properties are different from those of the commercially available (and pure) *E. coli* GUS. This helps to explain difficulties encountered in applying laboratory methods (developed through seeding of pure water samples with pure enzymes and cultures) to environmental analyses.

Key words: Coliform, faecal, *p*-nitrophenyl-β-D-glucuronide (PNPG), p-nitrophenol, waterborne.

INTRODUCTION

Outbreaks of waterborne diseases remain a major challenge to public health providers; claiming millions of lives annually worldwide (Gleick, 2002; Rompré et al., 2002; Leonard et al., 2003). Developing countries are the most vulnerable since the majority of their population is rural based and lack appropriate sanitary conditions. For example, cholera outbreaks were reported in the 2005 – 2006 seasons in Mozambique, South Africa and Zimbabwe. In addition, the fight against the AIDS pandemic and other natural disasters has burdened economies of developing countries leading to a diluted attention to the provision of safe drinking water. This is exacerbated by the unavailability of real-time and continuous monitoring methods for faecal pollution to enable implementation of the much required early warning system (Rompré et al., 2002) to prevent the use of contaminated water. There is therefore a great need for research to help address this consistent threat of waterborne diseases in developing countries. Current methods of microbial water quality monitoring involve classical/culturing techniques that are time consuming, provide retrospective results and are labour intensive (Manafi, 1998; Caruso et al., 2002; Rompré et al., 2002). One way to circumvent the time consuming culturing stage is by performing a direct assay of the marker enzyme activity in the water samples.

β-D-Glucuronidase (GUS), also known as β-D-glucuronide glucuronoso-hydrolase (EC 3.2.1.31), is used as a marker enzyme for *Escherichia coli* detection in microbiological water quality control (Frampton and Restaino, 1993; Rompré et al., 2002). *E. coli* belongs to the *Enterobacteriaceae* family and is used as an indicator for faecal contamination (Stevens et al., 2001; Tallon et al., 2005). Defined substrate technology has seen major improvements in culturing methods because of the existence of these marker enzymes. Incorporation of synthe-

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tic chromogenic and fluorogenic substrates for GUS into the culturing media has led to a reduction in the time required to obtain confirmed positive results for *E. coli* presence from 96 to between 16 and 24 h (Berg and Fiksdal, 1988; Frampton and Restaino, 1993; Sartory and Watkins, 1999; Davies and Apte, 2000). When the *E. coli* cells grow on such media, GUS cleaves the substrate leading to localisation of the chromogen or fluorochrome around the colonies. The substrates incorporated into the media include *o*-nitrophenol glucopyranoside and methylumbelliferone-β-D-glucuronide (Frampton and Restaino, 1993; Berger, 1994; Tallon et al., 2005). Examples of media which make use of this technique include CM 1046 (Oxoid, Hampshire, England) and MFc media (Merck, Darmstadt, Germany). Despite the considerable reduction in the time required for confirmed positive results, the culturing methods are time consuming and labour intensive (Manafi, 1998; Sartory and Watkins, 1999). Therefore, there is still a need to reduce the time of analysis for early warning systems in public health protection (Manafi, 1998; Leonard et al., 2003).

Development of enzyme-based methods in laboratories through seeding of sterile water by pure enzymes and axenic cultures is not representative of the ideal environmental conditions (Sartory and Watkins, 1999). This approach faces challenges in applying the laboratory-based protocols to enzymes in polluted environments because of differences in the two environments that may lead to changes in enzymatic properties. Our hypothesis was that a cocktail of chemical pollutants exists in water that can give rise to unpredictable enzyme properties. Variation in pollutant levels between stagnant and running water environments also presents another potential factor that can alter enzymatic behaviour. To the best of our knowledge, there is no reliable information on the properties of GUS in polluted water in literature. Therefore, the aim of this study was to investigate the physico-chemical properties of *E. coli* GUS directly in the polluted environment. Information from this study should also assist in optimising direct *in situ* assay procedures for marker enzymes in environmental water samples.

MATERIALS AND METHODS

Study site and sampling

The Bloukrans River water, which flows through the City of Grahamstown (Eastern Cape Province, South Africa), was selected for fundamental *in situ* assays. Previous water analyses from this river gave high coliform counts. Two sampling points were selected as examples of stagnant/standing water and flowing/running water. Water samples were collected (at 08h30) aseptically in 250 ml sterile pyrex glass bottles (Schott Duran, Germany), placed on ice, transported immediately to the lab and analysed. Samples were collected in triplicate in each case.

GUS assays

Three sets of enzyme samples were used; commercial GUS [EC 3.2.1.31 (Sigma, Steinheim, Germany)], and environmental GUS in stagnant and running water. The commercial GUS assay was performed as described by Fisher and Woods (2000) and Aich et al. (2001) with alterations to volumes and the buffer used. The total assay volume was 250 µl, in flat-bottomed 96-well microplates, and 0.1 M Tris-HCl buffer, pH 8.0 (containing 0.6 mM $CaCl_2$) was used as the assay buffer. The substrate was 10 mM *p* nitrophenol β-D-glucuronide (Calbiochem/Merck, Darmstadt, Germany) in the Tris-HCl buffer. In commercial GUS assays, the ratio of volumes of enzyme solution:buffer:substrate was 0.12:0.44:0.44, while in the environmental assays was 0.36:0.2:0.44, respectively. The reaction was initiated by addition of the substrate to the enzyme-buffer mixture and absorbance readings at 405 nm were recorded, at 30 s intervals for 10 min for commercial GUS and hourly for environmental GUS, using a Power $wave_x$ microplate reader (BioTek Instruments, USA). Triplicate assays for each effector were performed and the mean values and standard deviations were reported.

pH and temperature optimisation

The effects of pH and temperature on GUS activity were investigated. In pH optimisation the buffers used were: sodium acetate for pH 5.0 and 6.0, sodium phosphate for pH 6.0 – 8.0, Tris-HCl (pH 7.0 – 9.0) and carbonate/bicarbonate (pH 9.0 and 10.0). The bracketing of pH values was performed to take into account changes in GUS activity due to differences in buffer components. PNPG was dissolved in their respective buffer systems at the corresponding pH during pH optimisation. Using the pH optimum, effects of temperature on GUS activity were evaluated at 4, 12 and 20 – 50°C for the two samples and commercial GUS up to 60°C.

Water analyses and chemical effects on GUS

The environmental water samples were analysed for different chemicals by the Nelson Mandela Metropolitan Municipality Scientific and Engineering Department, Port Elizabeth, South Africa to assess the nature and extent of water pollution. The effects of ions (commonly occurring in polluted river water environments) on GUS activity were investigated. These included: SO_4^{2-} (as Na_2SO_4), SO_3^- (Na_2SO_3), CO_3^{2-} (Na_2CO_3), Cl^- (NaCl), Ca^{2+} ($CaCl_2$), Mg^{2+} ($MgSO_4$), Cd^{2+} ($CdSO_4$), NO_3^- (KNO_3), K^+ (KCl), ferric chloride ($FeCl_3$), EDTA [all supplied by Merck (Darmstadt, Germany)], ferulic acid (Sigma) and OCl^- [NaOCl (Savemore, Pinetown, South Africa)]. Stock solutions of these compounds were added to the enzyme reaction mixture to final concentrations ranging mainly between 0 and 200 mg/l, except for NaOCl (350 – 5 600 [parts per million (ppm)] and $CdSO_4$ (1 – 10 ppm). These were incubated at 20 ± 2°C for 30 min after which the substrate was added and kinetic readings executed as described above.

Statistical analyses

Analysis of variance (ANOVA) was performed using Microsoft Excel 2003 statistical tool at 5 % level of significance.

RESULTS

Preliminary work in our research group confirmed that the GUS activity orginated from *Escherichia coli* in the environmental samples. While a wide range of GUS substrates and assays exist, PNPG was selected on the basis of cost effectiveness and availability of equipment.

In addition, PNPG was selected instead of the fluorogenic substrate methylumbelliferyl-β-D-glucuronide (MUG) because the latter yielded unreliable results due to environmental sample interference.

Table 1. Water samples analysis report from the Nelson Mandela Municipality Scientific Services Department and the SABS 241 limits. The units of the results depicted in Table 1 from the sample analyses are in mgl^{-1}.

Sample component	Stagnant sample	Running sample	Max. acceptable limits
Total alkalinity as $CaCO_3$	296	296	Not given
Magnesium as CO_3	214	140	100 (as Mg)
Sodium as Na	272	222	400
Potassium as K	18	12	100
Chloride as Cl	291	292	600
Sulphate as SO_4	126	125	600
Nitrate plus Nitrite as N	2.6	2.6	20
Phosphorus (soluble)	1.1	0.86	Not given
Total Sulphides as H_2S	<1	<1	Not given
Cyanide as HCN	<0.1	<0.1	70 (as CN)
Total Iron as Fe	0.48	0.54	2 000
Cadmium as Cd	<0.001	<0.001	20
Copper as Cu	0.008	<0.005	2 000
Mercury as Hg	<0.3	<0.3	5
Nickel as Ni	<0.005	<0.005	350
Zinc as Zn	0.024	0.014	10

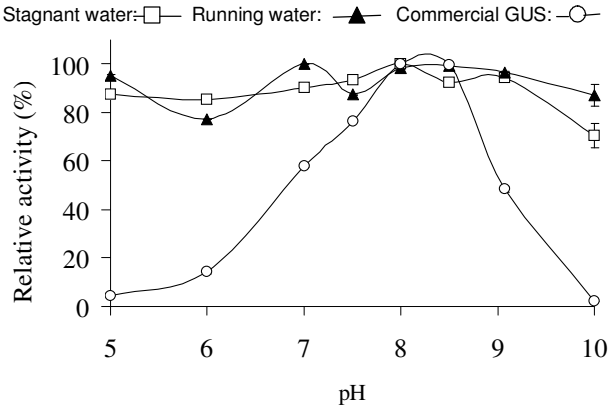

Figure 1. Effects of pH on *in situ* GUS activity. All values represent the means ± SD (n=3).

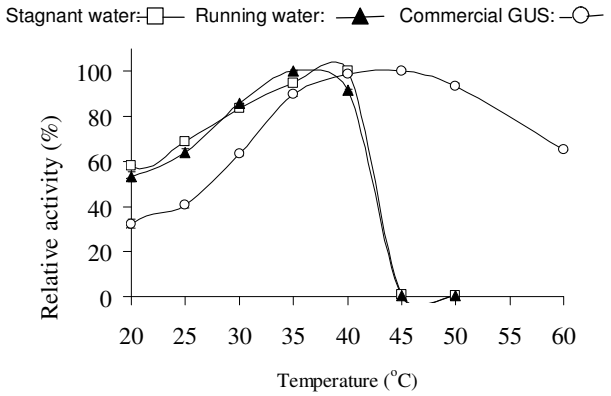

Figure 2. *In situ* GUS activity at different temperature values. All values represent the means ± SD (n=3).

Temperature and pH

In general, environmental (stagnant and running water) GUS activity was constant between pH 5 and 9 as opposed to the optimum pH of between 8.0 and 8.5 for commercial GUS (Figure 1). The pH values for river water samples fluctuated between 7.5 and 8.8. Insignificant ($P>0.05$) differences in activity were observed between the stagnant and running river water samples at different pH values (Figure 1). The subsequent GUS assays were performed at a pH of 8.0, as river water and water intended for drinking purposes are generally between pH 7.0 and 8.0.

Precipitation in the assay mixture occurred at temperatures of 4 and 12°C using environmental samples hence GUS activity assays at these low temperatures were regarded as unreliable. The temperature optimum for the environmental GUS assays was 40°C, followed by a sharp decline at 45°C (Figure 2). Commercial GUS had a temperature optimum of 45°C. However, all subsequent assays were performed at ambient temperature (20 ± 2°C) to circumvent the requirement of heating baths and other expensive equipment in the field.

Water analyses

All the components, with the exception of phosphorus, magnesium, total sulphide and total alkalinity, were below the maximum permitted limits according to the SABS 241 water quality guidelines (Table 1). Calcium carbonate and chloride were present in the highest concentration amongst the components analysed (Table 1). Significant ($P <$

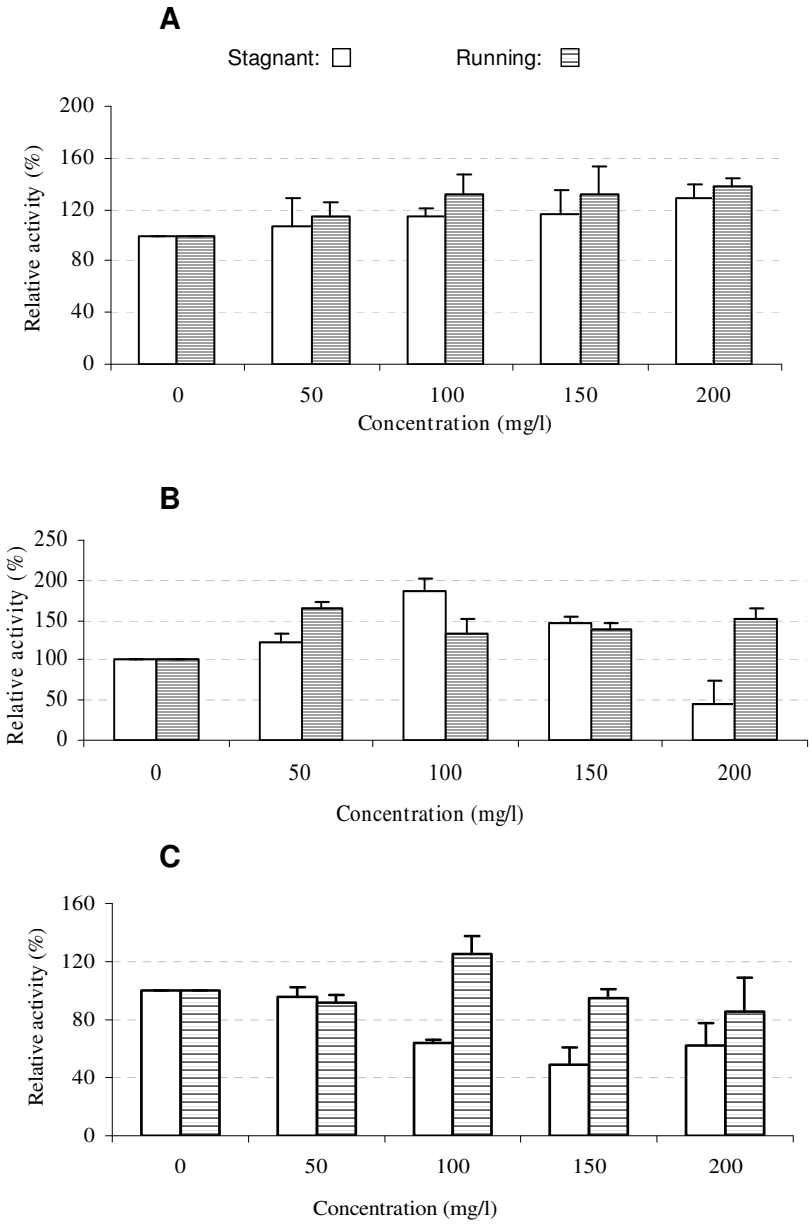

Figure 3. Effects of NO_3^- (A), PO_4^{3-} (B) and Cl^- (C) on GUS. All values represent the means ± SD (n=3).

0.05) differences in some of the compounds (e.g. sodium, carbonate and zinc) concentrations between stagnant and running water samples were observed (Table 1).

Interference studies

In general, NO_3^-, PO_4^{3-} and Cl^- ions increased the activity of GUS of the two environments (Figures. 3A-C). PO_4^{3-} gave the highest percentage activity increase for GUS in stagnant water at 100 mg/l (Figures 3B and C) while the running water GUS activity remained just above 100% of that of the unamended samples. Cl^- only increased the activity of running water (up to 100 mg/l), while inhibiting that of stagnant water (Figure 3C). Ferric chloride completely inhibited GUS activity in the two environmental samples at 30 mg/l, the concentration used in flocculation during water purification (Chow et al., 1998) (Figure 4A). However, the activity of the positive control (commercial GUS) was above 70% at 30 mg/l, while there was no activity in the environmental samples. Running water GUS was more sensitive to the addition of $FeCl_3$ than the stagnant water GUS (Figure 4A). Higher $FeCl_3$ concentrations (above 100 mg/l) led to spontaneous substrate hydrolysis and a drastic reduction in the pH of the assay solution. Hypochlorite; commonly used as a disinfectant in remote areas (approximately 1 cap in 1 bucket of water

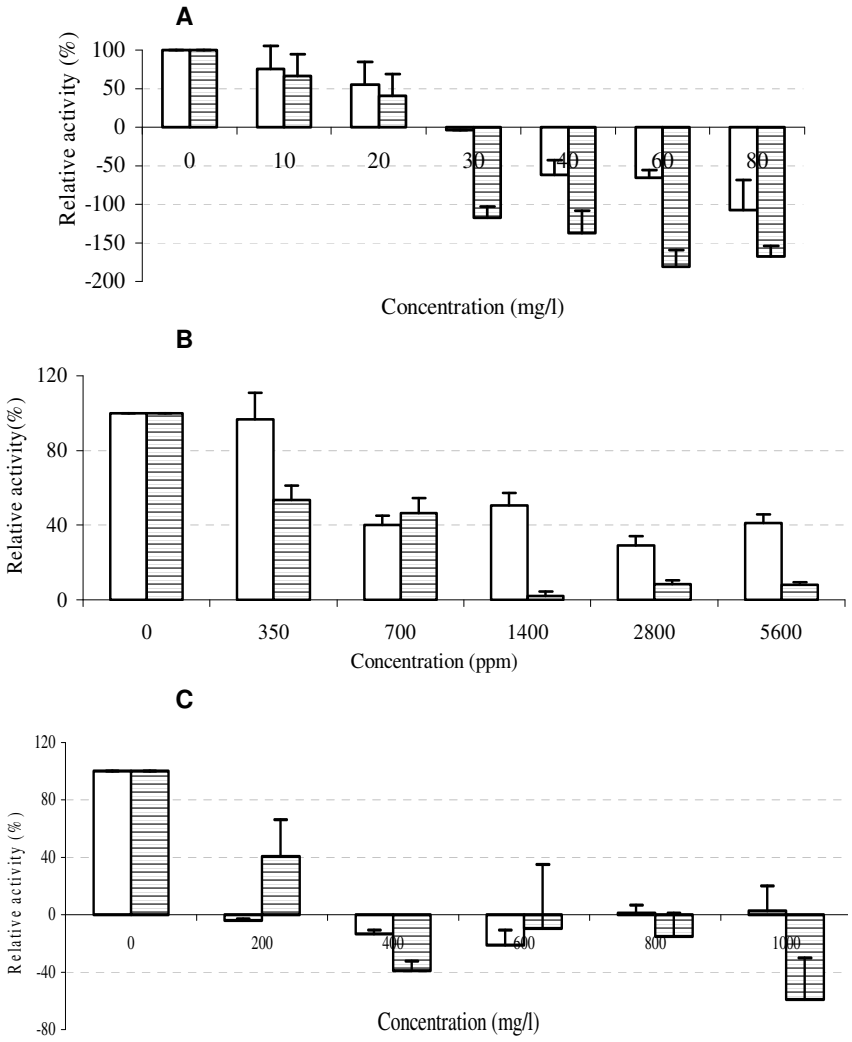

Figure 4. Effects of $FeCl_3$ (A), OCl^- (B) and Ferulic acid (C) on GUS. All values represent the means ± SD (n=3).

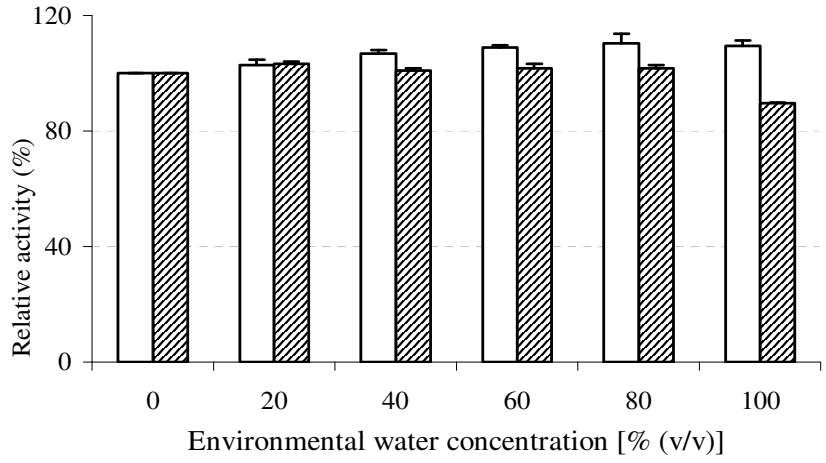

Figure 5. Effect of different concentrations of environmental water sample on commercial GUS. All values represent the means ± SD (n=3).

which is equivalent to 700 ppm), reduced GUS activity (Figure 4B)–as did ferulic acid (Figure 4C). The sensitivity of the enzyme in different environments to hypochlorite varied. GUS in running water was more susceptible, followed then by that in stagnant water (Figure 4B).

Other effectors had different effects on the environmental GUS enzyme. There was a direct relationship between carbonate concentration and GUS activity in running water while inhibition was observed in stagnant water GUS. A similar trend was observed for SO_4^{2-}, SO_3^- and Cl^-. EDTA and Mg^{2+} inhibited GUS in both samples while the reverse was true for K^+. Total environmental GUS activity inhibition by Cd+ was noted at 4 ppm while the same degree of inhibition in the positive control was observed at 10 ppm.

Effects of environmental water samples on comercial GUS

Stagnant water sample insignificantly ($P > 0.05$) increased commercial GUS activity while the running water sample inhibited GUS activity (Figure 5).

DISCUSSION

The temperature optimum of commercial *E. coli* GUS is around 45°C. The observed sharp decrease in environmental GUS activity above 40°C could be a result of acelerated enzyme denaturation because of potential presence of detergents in polluted water. This observation was supported by results from a follow up study in our laboratory where environmental GUS inhibition by detergents concentrations normally used for cell permeabilisation was observed. The same detergent concentrations did not inhibit either commercial GUS or axenic *E. coli* culture GUS activity. Water intended for drinking purposes has a pH around neutrality and therefore GUS assays at pH 8.0 will not be compromised during *in situ* assays. It is worth noting that PNP colour development is pH dependent. Hence at lower pH values (e.g. pH 5.0) the low activity in commercial GUS could be a result of both low colour development and low GUS activity.

GUS activity is not inhibited by anionic enzyme inhibittors (Doyle et al., 1955) and this was observed with SO_3^- and NO_3^-, especially in the running water samples. Carbonates chelate phenolic compounds thereby preventing enzyme inhibition (Wetzel, 1991). Since the substrate (PNPG) used in this study is phenol based, the chelation may help in explaining the observed inhibition in comercial and stagnant water GUS activities. However, the observed increase in activity in the presence of carbonate (up to 200 mg/l) in running water sample could be due to existence of other forms of phenolic compounds that readily bind to carbonate rather than PNPG. Research in our laboratory is currently involved in the identification of the different phenolic compounds in these environments in an attempt to explain the observed phenomenon. Inhibition of GUS activity by ferulic acid suggests that the use of the direct enzyme assay method in water bodies with decomposing plant matter may yield a false negative result.

Cadmium inhibited GUS because it is able to bind to the sulphydryl groups of the protein (Vallee and Ulmer, 1972). However, the toxic effects of cadmium in living systems at parts per billion concentrations will not considerably reduce the GUS activity. Although the primary concern is not to detect such inhibitors, direct enzymatic assay technique can be unreliable if enzyme activity is detected at metal concentrations that kill the organism, thus giving false positive results.

Hypochlorite alters proteins by reacting with amine groups. For example, tyrosine undergoes ring chlorinetion to give 3-chlorotyrosine and lysine gives chlorolysine in presence of OCl^- (Hawkins and Davies, 1999). Tyrosine is one of the three most important active site residues for GUS (Wong et al., 1998; Islam et al., 1999; Matsumura and Ellington, 2001). Lysine is important for the quaternary structure of GUS (Matsumura et al., 1999). Thus, changes in these amino acids will impair GUS activity.

The presence of a diverse range of ions and particulate matter in the environmental water samples aided flocculation by ferric chloride, thus making the enzyme unavailable for detection. For this reason, the positive control (commercial GUS) was less affected by the flocculant. The observed results support the reliability of GUS assay because removal of the enzyme will be coupled to the removal of the microbes from the water. However; the presence of ferric chloride above 100 mg/l can give rise to false positive results as was noted with PNPG hydrolysis, while reduced pH values decrease GUS activity. This will limit use of direct GUS assay as an indicator for presence of *E. coli* during flocculation with $FeCl_3$.

Fujisawa et al. (2000) observed that NaCl only increased GUS activity in *E. coli* culture through an increase in membrane permeability and there was no increase in GUS activity in sonicated cells. Therefore, the inhibition of GUS by Cl^- in stagnant water could be a result of high NaCl concentration in the polluted sample. Inhibition of GUS by EDTA was possibly a result of the synergistic effects of detergents in the environment. Other varying effects by the effectors can be attributed to different water pollutants in the two environments of stagnant and running water.

The failure of environmental water to strongly inhibit commercial GUS cannot be wholly attributed to the absence of inhibitors in the water, but it could be argued that irreversible inhibitors were bound to GUS in water and

therefore could not interact with the commercial GUS. Alternatively, the weak inhibitory effect of the common compounds found in water, like K^+ and SO_4^{2-}, may also help to explain why the environmental water did not strongly inhibit the commercial enzyme activity. Furthermore, it could be possible that the observed activity was a net effect of inhibition and potentiation cancelling one another.

Conclusions

Several chemical compounds in the environment can potentially affect *in situ* GUS activity; therefore, results of such assays should be stated with caution. There may be a need to remove some of these compounds prior to assaying for GUS. The possibility of obtaining false positive or negative readings in the detection of GUS activity in contaminated water should be anticipated. Further verification may therefore be required through more traditional microbiology assays. Since the pollutant composition of the water varies with site and time, use of an internal reference or standard (commercial GUS) when employing *in situ* assays is recommended to assess and correct for the effect of potential pollutants. Future studies will entail the determination of annual (seasonal) trends in GUS activity and attempts to detect GUS activity electrochemically.

ACKNOWLEDGEMENTS

The authors would like to extend their gratitude to the Water Research Commission (WRC) of South Africa, the National Research Foundation (NRF), Rhodes University (JRC) and Canon Collins Trust for their financial support during the course of this study.

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