ON-LINE REAL-TIME ENZYME DIAGNOSTIC SYSTEM FOR THE DETECTION AND MONITORING OF FAECAL CONTAMINATION OF WATER INTENDED FOR DRINKING PURPOSES

B Pletschke • C Togo • V Wutor

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Water Research Commission



ON-LINE REAL-TIME ENZYME DIAGNOSTIC SYSTEM FOR THE DETECTION AND MONITORING OF FAECAL CONTAMINATION OF WATER INTENDED FOR DRINKING PURPOSES

by

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EXECUTIVE SUMMARY

BACKGROUND AND MOTIVATION:

Untreated sewage is increasingly being channelled into marine and freshwater catchments and riverine systems. This increases the risk of a potential collapse of the national drinking water supply, and the outbreak of water borne diseases such as cholera, dysentery, typhoid, shigellosis, etc. Current tests to detect indicator microorganisms for the potential presence of pathogens in water intended for drinking purposes are in place (i.e. total coliform tests, *E. coli,* etc.), but these require often laborious and time-consuming procedures.

The concept of indicator microorganisms is an attempt to predict the possible presence of pathogenic microorganisms in the environment under consideration. It relies on the fact that some non-pathogenic microorganisms occur in the faeces of all warm-blooded animals. Thus the presence of these non-pathogenic microorganisms in water indicates the potential presence of pathogenic microorganisms of faecal origin. The presence/absence of pathogens in water is routinely assessed using indicator organisms because it is not feasible to detect and/or identify every pathogen in the water sample.

During the course of 2000 to 2002, unique enzymatic profiles present in primary sewage sludge were established by Pletschke and co-workers, one of the first studies of its kind in the world. This work was supported by a previous WRC grant, and results from this study have already been presented and accepted in internationally peer-reviewed scientific journals. It was then subsequently proposed that results from that very important study now paved the way for the development of a novel enzymatic diagnostic assay for the detection of faecal contamination in water intended for drinking purposes.

AIMS AND OBJECTIVES:

The ultimate aim of this work was to develop an on-line real-time enzyme diagnostic system for the detection and monitoring of sewage levels in drinking water, using suitable marker enzymes. This was accomplished by addressing the aims (originally stated in the contract for project K5/1446), which were to:

a) to identify suitable marker enzymes in sewage
b) to develop a suitable diagnostic enzyme assay kit/strip or assay procedure
c) to engineer a suitable on-line, real-time biosensor system for the detection of sewage in water systems
d) to establish a resource base in the enzymology of sewage and industrial wastewater treatment processes and to support cognate research areas in South Africa
e) to empower disadvantaged and rural sectors of the population, and increase the state of public health in general
f) to promote student training and corporate technology collaboration to enhance wastewater management in South Africa
g) to correlate the findings of this work with known established parameters for determining water pollution/contamination

All the aims stated above were achieved throughout the course of this study, with the exception of aim c). This aim was not achieved within the two year funding cycle due to the large amount of preliminary work that first had to be performed. However, this will be the focus of the follow-on WRC Project K5/1603, which has already been approved. Furthermore, proof of concept was established for the design of a suitable electrochemical biosensor for the rapid detection of faecal matter in water intended for drinking purposes, and this is discussed in chapter 8 of this report.

RESULTS AND CONCLUSIONS:

Research for detecting faecal pollution of water intended for drinking purposes in the past has been concentrated on developing selective and differential media for microbial growth. One of the most promising alternative approaches to faecal microbial detection (by performing direct assays for marker enzymes of these indicator microorganisms) was selected. Detection via marker enzymes was successfully achieved within 24 h although the aim is to reduce this time to less than nine hours.

A number of stages were involved in confirming that the concept of direct enzyme assays can indeed be applied to the direct *in situ* detection of faecal contamination in water samples. Beta-D-galactosidase (GAL) and beta-D-glucuronidase (GUD) (two marker enzymes, indicative of coliform bacteria and *E. coli*, respectively) were used. Polluted

river water was used to perform *in situ* GAL and GUD assays. Chromogenic substrates (chlorophenol red β-D-galactopyranoside (CPRG) and *p*-nitrophenyl-β-D-galactopyranoside (PNPG)) were selected over fluorogenic substrates due to their robustness in environmental enzyme assays and the lack of interference or quenching. Cell permeabilisation was shown not to be necessary for the *in situ* assays, thus saving on the costs involved and time required for analyses.

Physico-chemical characterisation of the enzymes in faecally polluted environmental samples showed inhibition by cadmium, ferric chloride and sodium hypochlorite. The presence of such compounds in water may therefore interfere with the developed method at very high concentrations, and a reference cell should be employed to account for this enzyme inhibition. pH and temperature optima of the enzymes and their assays were close to that of natural water environments, allowing for the execution of these enzyme assays at room temperature (20 ± 2 °C).

Attempts were made to concentrate the marker enzymes (originally present in very low concentrations) in order to facilitate bioinformatic profiling and identification of the source from which these markers enzymes had originated. Protein profiling and bioinformatics studies, supported by literature, confirmed the origin of GAL and GUD as being from *E. coli*, and not from plant or algal origin. In addition, studies were also performed to establish whether or not enzyme concentration could accelerate the assay reaction (thus decreasing the time required for analysis and increasing the sensitivity of the test), but this approach proved not to be feasible.

After enzyme assay optimisation, a liquid bioprobe was established. Field evaluation of the bioprobe showed the same degree of sensitivity as conventional membrane filtration methods with a maximum detection time of 24 h. Attempts to immobilise the substrates on a solid support were successful with modified nylon providing the best support. However, the use of the strips had limitations in the environmental samples due to substrate desorption with prolonged times of immersion in samples with low enzyme concentration. To implement real-time assaying, electrochemical detection of the enzyme breakdown products was investigated and proof of concept was established.
Real time monitoring of faecal pollution proved promising though direct electrochemical detection and development of a sequential flow injection analysis (SFIA) system.

RECOMMENDATIONS FOR FUTURE RESEARCH:

Based of the information gained in our study, it was established that the cheapest and most rapid way to develop an on-line monitoring system is through use of a sequential flow injection analysis (SFIA) system with a spectrophotometric detector or in combination with an electrochemical detector. A customised SFIA system for the simultaneous monitoring of GAL and GUD was proposed in this study. However, this SFIA system may not be as portable and applicable for the concept of "on-line, real-time" detection. The design of an electrochemical biosensor for the rapid on-line real-time detection of GUD and GAL is the focus of the follow-on WRC Grant K5/1603, which has already been approved. This design constitutes a high priority for future research and is based on the proof of concept that was established during the course of this study which is discussed in Chapter 8. At this point, a provisional patent is being applied for, based on results obtained in this and the follow-on study.

RESEARCH PRODUCTS

Capacity building: students trained
(postgraduate student competencies and degrees)

Post Doctoral Fellows:

Dr I. Foster (2003) (white female)
Thesis: Not applicable

PhD students:

C.A. Togo (Current) (black male)
Provisional title of thesis: "B-GUD as a suitable marker enzyme for *in situ E. coli* detection and biosensor design"

V. C. Wutor (Current) (black male)
Provisional title of thesis: "B-GAL as a suitable marker enzyme for *in situ* coliform detection and biosensor design"

MSc students:

S. Oyekola (Graduated 2003) (black male)

Title of thesis: "The enzymology of sludge solubilisation under biosulphidogenic conditions: isolation, characterisation and partial purification of endoglucanases"

BSc Honours students:

C. Carr (Graduated 2004) (white female)

Title of thesis: "Inhibition studies of coliform B-GAL and B-GUD enzymes during chlorination, UV treatment and at high salt concentrations."

D. Ridgway (Graduated 2004) (white female)

"Purification and identification of GUD from *E. coli* contaminated river Sources"

S. Watson (Graduated 2003) (white male)

Title of thesis: "Enzymes used for the rapid detection and enumeration of indicator micoorganisms in water-a pilot study"

The ratios of students trained for the duration of this project was as follows: black/white ratio = 3:4 and male/female = 3:4. Links with the local Grahamstown community was encouraged via the Rhodes University Experimental field station (EBRU) situated at the Grahamstown Municipal Sewage Works, Grahamstown. An academic and industrial resource base in the field of environmental and wastewater enzymology was established, with new knowledge contribution in this field.

More effective and rapid assays for the detection of sewage were designed, potentially improving the standard of living and public health for those communities living in rural parts of South Africa. Communities and public health officials should shortly have recourse to a novel, rapid and effective detection method for possible sewage contamination of potable water, thereby increasing hygiene and public health.

Patents

A provisional patent will be applied for after this report has been finalised. Ms S. Kalan from the WRC has kindly offered her assistance in this regard.

Papers

This project should culminate in the submission of at least three papers in internationally peer-reviewed scientific journals once the intellectual property has been secured and protected via a provisional patent (see above).

Technology transfer

Conference presentations were limited to those held locally in the region and Department, and were reports on general considerations already reported in literature, giving due concern to the protection of the intellectual property specifically inherent to this project:

1. Carr C., Wutor V.C. and Pletschke B.I. (2004) Inhibition studies of coliform B-D-galactosidase and B-D-glucuronidase during chlorination, ultraviolet treatment and at high salt concentration. 14th Annual Meeting of the Eastern Cape Division of the SASBMB, University of Port Elizabeth, Port Elizabeth, 26 November 2004. p 17.

2. Togo C.A. and Pletschke B.I. (2003) A rapid enzyme-based method for the detection of *E. coli* in water: beta-D-glucuronidase. 13th Biochemistry Symposium of the Eastern Cape. CEC, Rhodes University, Grahamstown. December 2003.

Other outputs

The design of a suitable bioprobe strip for the rapid detection of faecal contamination was recognized by the American Water Works Research Foundation (AwwaRF) as one the ten emerging technologies currently under development in South Africa through the "Emerging Technologies XI" programme for 2004.

Archiving of data generated during project

Data generated during the course of this project will be archived at Rhodes University, Grahamstown, South Africa in both hard and electronic format. All theses produced at the Masters and Doctoral level will be archived in the Rhodes University Library.

ACKNOWLEDGEMENTS

We would like to thank the Water Research Commission for the financial assistance provided during the course of this study, and the Steering Committee for Project K5/1446 for their valuable input and guidance. In particular, we would like to thank the chairperson Ms APM Moolman (WRC), and the following members of the Steering Committee for their valuable input: Prof P Jagals (University of Johannesburg), Ms B Genthe (CSIR, Environmentek), Mr IW Bailey (WEHcc), Dr SN Venter (University of Pretoria) and Dr K Whittington-Jones (Rhodes University), We would further like to thank our co-workers: Professor PD Rose, Dr WD Leukes and Dr JL Limson for their valuable input and contribution throughout the course of this study. Lastly, the Project Leader (Dr BI Pletschke) would like the following Project Team members for their contribution: Dr I Foster, Mr CA Togo, Mr VC Wutor, Mr S Oyekola, Ms C Carr, Ms D Ridgway and Mr S Watson.

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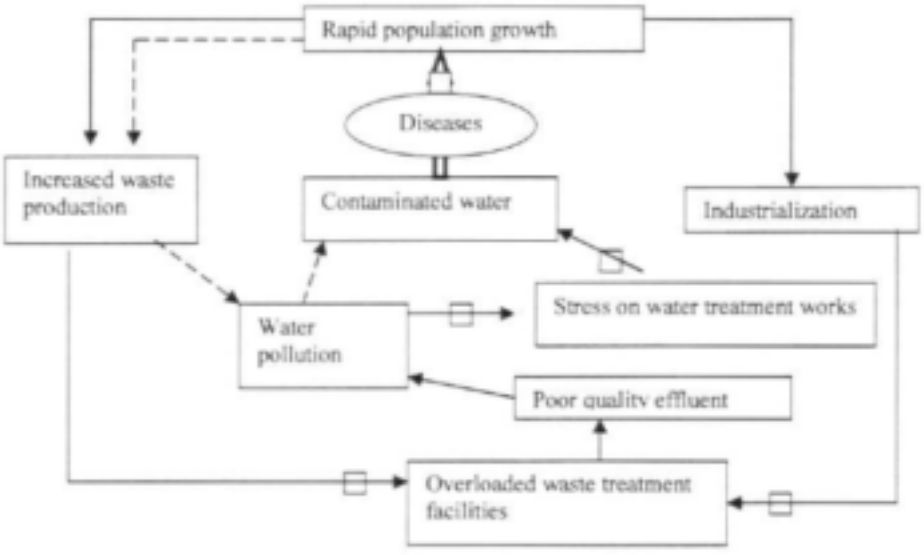
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1 BACKGROUND AND IDENTIFICATION OF MARKER ENZYMES

1.1 Waterborne Diseases

Waterborne diseases are caused by the ingestion of water contaminated by faecal material, especially from human or animal origin or urine that contains pathogenic microorganisms. Millions of people die annually worldwide because of waterborne diseases and outbreaks that include cholera, dysentery, typhoid and shigellosis. These diseases are still causing problems both in developing and developed countries (Berg and Fiksdal, 1988; Gleick, 2002; Ivnitski et al., 1999; Leonard et al., 2003; Venter, 2000). Aggravating such outbreaks is the lack of continuous water monitoring methods, slow and inefficient wastewater treatment, and expensive and laborious methods of detection for the supposed early warning system. In developing countries, the problem is exacerbated by rapid population growth that does not match the available water and wastewater treatment facilities. Above all, due to the AIDS pandemic, economies in developing countries are becoming overburdened to such an extent that improvements in wastewater handling facilities in order to match population growth are not always possible.

An increase in population has led to an increase in waste generation that overloads wastewater treatment facilities. This in turn has led to the deterioration in effluent quality released into the water bodies. This has triggered a cycle of problems that adversely affect the population (Fig. 1.1). Such a cycle calls for rapid, easy to use and sensitive water quality monitoring methods for an efficient early warning system. There is active research being performed in a bid to curb this growing water problem.



KEY: → Urban set up, - - → Rural set up

□ Possible water quality monitoring points

Figure 1.1: Cyclic water problems caused by rapid population growth without matching facilities and possible monitoring points to break the cycle.

1.2 The Problem of Water Quality

Public and environmental health protection agencies require safe drinking water. This means that drinking water must be free of pathogenic microorganisms, as well as other chemicals and substances considered pollutants or indicators of the possible presence of pollutants. Common water pollutants include microorganisms such as *Cryptosporium* spp, *Escherichia coli* and *Gardia* spp, lead, arsenic, chlorine, copper, asbestos, cadmium, mercury, toluene, pesticides, herbicides and their metabolites. All these pollutants are of primary health concern.

Coliforms are the group of bacteria used by the water industry to assess the microbiological quality of both drinking and recreational water. These bacteria, though not generally pathogenic (disease-causing) themselves, serve as indicators of the potential presence of organisms which may be pathogenic. Most coliforms are present in large numbers among the intestinal flora of human and other warm-blooded animals, and are thus found in faecal wastes. As a consequence, coliforms detected in higher

concentrations than pathogenic bacteria are used as an index of the potential presence of entero-pathogens in the water environment (Rompré et al., 2002).

Drinking water is not the natural environment for coliforms, thus their presence may indicate treatment inefficiencies, loss of disinfectant, breakthrough intrusion of contaminated water into the potable water supply or re-growth problems in the distribution system (Rompré et al., 2002) and this condition requires urgent attention. The conventional microbiological analysis of drinking water for coliforms requires 24 to 72 hours to complete (Berg and Fiksdal, 1988). This time elapse is obviously inappropriate when the need to provide prompt information about the potability of drinking water is required (Venter, 2000). Other limitations of these methods include antagonistic microorganisms, lack of specificity to the coliform group and a weak level of detection of slow-growing or stressed coliforms. Thus the need for efficient techniques for the detection of faecal pollution in aquatic environments is crucial (Farnleitner et al., 2001; Rompré et al., 2002).

1.3 Water Quality Assessment: The Concept of Indicator Organisms

The concept of indicator microorganisms is an attempt to predict the possible presence of pathogenic microorganisms in the environment under consideration. It relies on the fact that some non-pathogenic microorganisms occur in the faeces of all warm-blooded animals. Thus the presence of these non-pathogenic microorganisms in water indicates the potential presence of pathogenic microorganisms of faecal origin (Venter, 2000).

The presence/absence of pathogens in water is routinely assessed using indicator organisms because it is not feasible to detect and/or identify every pathogen (Rompré et al., 2002; Stevens et al., 2001; Tryland and Fiksdal, 1998; Venter, 2000) *Giardia* and *Cryptosporidium* are amongst the waterborne pathogens that are difficult to detect and take long (up to seven days) for confirmed results (Leonard et al., 2003; Sartory and Watkins, 1999; Taguchi et al., 2005) by which time the water would already have been used. Viral testing takes even longer, up to a few weeks (Venter, 2000).

According to Manafi (1998) and Stevens et al. (2001) an ideal indicator organism should have the following characteristics: be useful for all types of water, be present whenever enteric pathogens are present, have a reasonable longer survival time than the hardiest

enteric pathogen, not grow in water, be detectable using an easy to perform method, the density of the indicator organism should have some direct relationship to the degree of faecal pollution and should be a member of the intestinal microflora of warm-blooded animals.

Because of such properties, when these indicators are detected they signal the potential presence of pathogens and consequently a health hazard. The coliform group is among several microorganisms employed as indicators. This group includes *Escherichia coli*, *Citrobacter*, *Enterobacter* and *Klebsiella* (Frampton and Restaino, 1993; Rompré et al., 2002; Stevens et al., 2001). Specifically, all aerobic and facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that produce gas upon lactose fermentation in prescribed media in 48 hours at 35 °C fall under the coliform group (Frampton and Restaino, 1993; Rompré et al., 2002). It has been observed that the absence of coliforms in 100 ml of drinking water ensures the prevention of bacterial waterborne disease outbreak (APHA, 1986).

Stevens et al. (2001) discussed some deficiencies in the use of coliform bacteria as indicators of water quality. All members of the coliform group were observed to re-grow in natural surface and drinking water distribution systems. The amount and type of organic matter and the temperature of the water environment affected the concentration of the coliforms. Thus eutrophic tropical waters will contain high concentrations of coliform bacteria. Biofilm formation along the distribution system pipes (even in the presence of free chlorine) may give a false indication of faecal contamination. Coliforms are more resistant to free chlorine when attached to a surface than as free cells in water (LeChevallier et al., 1987; Ridgway and Olson, 1982). This regrowth and chlorine resistance gives a false positive indication of the potential presence of pathogens. The presence of large numbers of heterotrophic bacteria may mask the growth of coliforms in selective media (LeChevallier and McFeters, 1985). This in turn gives a false negative result, leaving the population prone to pathogen consumption under false security of the absence of indicator organisms.

1.4 Methods of Indicator Organism Detection

Various methods for determining and quantifying the presence of indicator organisms exist. These can be classified into (a) cultural or classical, (b) enzymatic and (c)

molecular methods (De Boer and Beumer, 1999; Frampton and Restaino, 1993; Rompré et al., 2002; Venter, 2000). The classes; however, overlap and improvements have seen them combined to increase the sensitivity and reduce time required for the assaying. The various methods (and combinations of these) have been listed in summarised form in Table 1.1. Table 1.1 also highlights the inherent advantages and disadvantages of each method.

1.4.1 Culture based methods

Membrane filtration (MF) and multiple tube fermentation (MTF) or the most probable number (MPN) techniques have been used. Membrane filtration involves the concentration and entrapment of microorganisms by a 0.45 or 0.22 μm (pore diameter) filter paper. The paper is then layered on selective media agar plates, incubated at 44.5 °C. Typical traditional media include MacConkey agar and m-Endo-type agar (Rompré et al., 2002). The agar and incubation conditions will allow growth of thermophilic coliforms. Colonies are observed after 18 – 24 hours (Brenner et al., 1993; Davies et al., 1994; Farnleitner et al., 2001; Manafi, 1998; Van Poucke and Nelis, 2000). On m-Endo-type agar, coliforms are observed as "red colonies with a metallic sheen" (Rompré et al., 2002). Further traditional confirmation as to whether they are *Escherichia coli* may require a further 24 – 48 hours (Sartory and Watkins, 1999).

Improvements of the MF technique, by incorporating enzyme substrates that release a colour product (chromogenic substrates) or that release a fluorochrome (fluorogenic substrates) into the media, have eliminated the confirmatory stage. Both enumeration and confirmation are therefore carried out within the same culturing period (16 – 24 hours) (Sartory and Watkins, 1999). Examples of such media include m-FC agar (Merck) and CM1046 (Oxoid).

Table 1.1. A summary of the various methods available for the detection and quantification of indicator microorganisms.

Method		Advantages	Disadvantages
a) Classical or cultural	Membrane Filtration (MF); Multiple tube fermentation (MTF); Most probable number (MPN).	Established; Good selectivity for microbial growth.	Time consuming-long time required for analysis; Laborious; Underestimation of microbial populations because of VBNC cells.
b) Enzymatic	Fluorogenic; Chromogenic	More rapid and sensitive; Less time-consuming compared to traditional methods; Can detect VBNC cells; Can be used for real-time monitoring.	Require high initial enzyme or microbial concentrations; Sensitive fluorogenic substrates prone to interference.
c) Molecular	Immuno-based; Nucleic acid (NA) based.	Reduction of analysis time required-rapid; Sensitive; Selective/specific; Can detect VBNC cells.	Cross-reactivity can lead to false positive results; Susceptible to background interference; Disadvantageous for routine use; Expensive; Requires dedicated lab space and equipment.

In the MTF technique, samples are inoculated in a serial dilution fashion in tubes with the media such as lauryl sulphate broth, or with additional selective compounds for coliforms that include cefslodin and bile acids (Alonso et al., 1996; Sartory and Watkins, 1999). These samples are incubated at 35 °C. Growth and gas production within 48 hours is regarded as presumptively positive indication for coliforms. Further confirmatory tests such as growth in brilliant green broth at 44.5 °C for 24 hours or an indole test are performed. The total analyses time will therefore vary between 48 and 96 hours (Davies and Apte, 2000; Ercole et al., 2002; Geissler et al., 2000; Leonard et al., 2003; Manafi 1998; Rompré et al., 2002). The microbial numbers are estimated as the most probable number based on the growth patterns at different dilutions.

The MTF method suffers the same shortcomings as the MF method in terms of the long analyses time required. Improvements to eliminate confirmatory tests involve incorporation of fluorogenic and chromogenic substrates for marker enzymes (see section 1.4.2). The use of these substrates illustrates an overlap of culture-based and enzyme-based methods of detection, combining the two methods for improved methodological design.

Although there is no doubt in the viability of the detected organisms (Venter, 2000), culture based techniques may lead to underestimation of microbial populations because of occurrence of viable but non culturable (VBNC) cells that cannot be detected using these techniques. Cells undergo such a condition because of nutrient stress due to the oligotrophic nature of many potable water bodies and injury suffered during treatment (George et al., 2002; Leonard et al., 2003; Rompré et al., 2002; Venter, 2000). Culture-based techniques are labour intensive and time consuming.

Unlike MF, MTF cannot allow the examination of large volumes of water. Generally speaking, the MF and MTF methods offer good selectivity in respect of microbial growth. Despite improvements in these methods, there is still the requirement for a culturing stage, as microbial growth cannot be accelerated (Venter, 2000). Such a growth period has led to retrospect results. This is very costly to human health, since water is in continuous demand and would already have been used before the microbial quality has been established. Direct targeting of the enzymes that act on the chromogenic and fluorogenic substrates can be an option for a reduction in the time required for analyses.

1.4.2 Enzyme based methods

It is difficult to single out these methods as free standing methodologies, because improvements in culturing methods are also enzyme based and molecular methods can also make use of enzymes. Therefore, direct enzyme assays of samples are discussed in this section.

The unique biochemical pathways (used in selective media design) that characterise each genera, species or strain are based on unique key enzymes that participate in such metabolisms. These enzymes are referred to as marker enzymes. It is therefore useful to assay directly for the activity of these enzymes. Since enzyme reactions are faster, this can potentially reduce the assay time. Beta-D-glucuronidase (GUD) is used as a marker enzyme for *E. coli* while β-D-galactosidase (GAL) is used for total faecal coliforms (Rompré et al., 2002; Sartory and Watkins, 1999). GAL catalyses the breakdown of lactose-based substrates to glucose and galactose (Davies and Apte, 1999, George et al., 2002, www.brenda.uni-koeln.de). The application of direct enzymatic detection of coliforms by monitoring β-D-galactosidase activity has been demonstrated by several authors including Davies and Apte (1999) and George et al. (2002). George et al. (2002) employed enzymatic reactions to detect pollution within 25-30 minutes. GUD catalyses the hydrolysis of glucuronide based substrates to corresponding aglycons and D–glucuronic acid (Rompré, et al., 2002). Substrates made up of glucuronide and galactose linked to a chromogen or fluorochrome (Table 1.2) have been designed for the detection of these enzymes (Frampton and Restaino, 1993).The use of enzymatic reactions to detect coliforms provides a rapid, sensitive and less expensive alternative to the more traditional methods.

Enzyme substrates are added to the samples in question and incubated under optimum conditions for maximum enzyme activity. Colour change or fluorescence is then scored (Frampton and Restaino, 1993). The principle is based on the hydrolysis of artificial chromogenic or flourogenic substrates, which are specifically split off by the enzyme under consideration. Chromogenic enzyme substrates are mainly phenolic. They are water soluble, heat stable, specific, show no diffusion (indolyl derivatives) and a wide range with different colours are available (Manafi, 1998). Examples of chromogenic substrates for GAL include *o*-nitrophenyl-β-D-galactopyranoside (ONPG), chlorophenol red β-galactopyranoside (CPRG), *p*-nitrophenyl-β-D-galactopyranoside (PNPG), 5-

bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 6-bromo-3-indolyl-β-D-galactopyranoside (Salmon-Gal), 2-(2-(4-(β-D-galactopyranosyloxy)-3-methoxyphenyl)-vinyl)-3-methylbenzothiazolium toluene-4-sulphonate (VBzTM-Gal), 2-(2-(4-(β-D-galactopyranosyloxy)-3-methoxyphenyl)-vinyl)-1-methylquinolilium iodide (VQM-Gal) among others (Manafi, 1998). 4-methylumbelliferone β-D-galactose, 4-methylumbelliferone β-D-heptanoate and 4-methylumbelliferyl β-D-galactopyranoside are amongst the most common fluorogenic substrates employed in the assay of β-galactosidases.

Table 1.2: Fluorogenic and chromogenic substrates for GUD (Adapted from Frampton and Restaino, 1993)

Substrate	Products of hydrolysis	Colour	Optimum pH for detection
	Glucuronic acid +		
Phenolphthalein-β-D-glucuronide (PHEG)	Phenolphthalein	Red	Alkaline
p-nitrophenol-β-D-glucuronide (PNPG)	*p*-nitrophenol	Yellow	7.5-8.5, >10
5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GLUC, BCIG)	Indoxyl→indigo	Blue	Alkaline
Indoxyl-β-D-glucuronide (IBDG)	Indoxyl→ indigo	Blue	Alkaline
4-methylumbelliferyl-β-D-glucuronide (MUG)	4-methylumbelliferone	Blue fluorescence	10.3

Colilert® has introduced a range of substrate based methods that include the quantitray (Berger, 1994; Sartory and Watkins, 1999) and the more advanced stand alone equipment, Colifast At-Line Monitor (CALM) with the ability to send continous data to the laboratory from a remote location (www.colifast.no). The latter makes use of

fluorogenic substrates. These methods however still need long incubation times (12 to 24 hours). In some cases, pre-enrichment through culturing is required, thus prolonging the time for analyses. ROSCO tablets® also use the marker enzyme technique and require about four hours incubation (Pérez et al., 1986).

The disadvantage with the enzyme-based methods is that they require high initial enzyme or microbial concentrations, which may not always be present in the samples. In addition, use of more sensitive fluorogenic substrates is prone to interference by inherent fluorochromes present in the water samples. Most of the techniques have been limited to laboratories with the use of pure water. Seeding of such water with microorganisms or sewage does not present the ideal environmental system (Sartory and Watkins, 1999) and this has therefore presented a tough obstacle in extrapolating such methods to the environment.

1.4.3 Molecular based methods

Molecular based methods can be broadly classified into immuno– and nucleic acid (NA) - based methods. The former entails using antibody–antigen interactions in techniques such as enzyme linked immunosorbent assay (ELISA), immunofluorescent assay (IFA) and immunoblotting. Nucleic acid based methods comprise of NA hybridisation, polymerase chain reaction (PCR) and the use of genetically modified bacteriophages (De Boer and Beumer, 1999; Rompré et al., 2002; Venter, 2000). As with direct enzyme assaying, molecular–based methods are aimed at reducing analysis time through the elimination of time-consuming culturing/growth stage.

1.4.3.1 Immuno–based techniques

Genera–and species– specific proteins and protein–based structures (e.g. cell receptor surfaces) can be taken advantage of in the selective detection of indicator organisms. Antibodies specific to such structures can be raised and conjugated to enzymes that will catalyse the breakdown of chromogenic or fluorogenic substrates. Examples of such enzymes include horseradish peroxidase, alkaline phosphatase, urease and GAL (Crowther, 2001). When enzyme labelled antibodies bind to the antigen, they remain on the support during washing of the unbound molecules. This is followed by the addition of the chromogenic/fluorogenic substrate to the experimental sample (Crowther, 1995). Occurrence of colour or fluorescence that signifies substrate breakdown will indicate the

presence of the microorganism in question, since its antigen has retained the enzyme labelled antibody.

Antigens that have been used for coliform and *E. coli* detection include enterobacterial common antigen (ECA) (Hübner et al., 1992; Levasseur et al., 1992), GUD, GAL and glutamate decarboxylase (Kaspar et al., 1987). Use of ECA monoclonal antibodies showed a 98 % similarity of results with MTF technique (Hübner et al., 1992), which makes it a good alternative technique to MF and MTF for indicator organisms' detection.

Immunological techniques are sensitive, selective/specific and rapid (Leonard et al., 2003; Taguchi et al., 2005; Venter, 2000). However, a major problem is cross-reactivity that can lead to false positive results. In addition, these techniques are susceptible to background interference when microbial population is below the threshold level of the limit of detection (LOD). Generally, the microbial population must constitute at least one percent of the total microbial load for less background interference (Mansfield and Forsythe, 2000; Rompré et al., 2002). Immunomagnetic separation (Mansfield and Forsythe, 2000) can be used prior to ELISA to reduce background interference. Use of such techniques to enhance results would imply more costs and requirement of skilled personnel, thus limiting its use for samples of high economic value like pharmaceuticals. In the end, such techniques will not be economically feasible to local authorities for the routine use in public services.

In a bid to improve sensitivity, Abuknesha and Darwish (2005) devised a tandem technique to immunologicallly monitor *E. coli*. The LOD was between 10^1 and 10^5 cells.ml^{-1} detected within two hours. Burestedt et al. (2000) attempted to link an immunoaasay technique to a biosensor for signal amplification.

Immunoassay techniques can be used to determine VBNC organisms, but they do not provide information about the physiological state of cells (Leonard et al., 2003; Rompré et al., 2002). Immuno–based methods can also be employed to detect the actual toxins of interest that cannot be detected by either culturing or genetic analysis (Iqbal et al., 2000; Leonard et al., 2003). However, they cannot be used for real-time detection (Leonard et al., 2003).

1.4.3.2 Nucleic acid (NA) – based techniques

Instead of targeting the genera– or species– specific gene products, gene sequences coding for these products can be targeted using nucleic acid probes. A probe can be defined as a molecule that interacts with a specific target and has a traceable means of detection after such an interaction occurs (Keller and Manak, 1989). In such a case, a labelled DNA sequence is used as a probe. The labelling can be radioactive (^{32}P), fluorometric or enzymatic (Fricker and Fricker, 1994; Joux and Lebaron, 2000; Keller and Manak, 1989; Köhler et al, 2000). The basis of NA methods relies on the complementarity that exists between sequences and the existence of some unique sequences in each species (Rompré et al, 2002).

Samples with target sequences can be fixed on membranes or on gel after electrophoresis, followed by the introduction of a probe. If whole cells are under investigation, they are permeabilised to expose the DNA and then incubated, in the presence of the probe, under conditions that allow strand annealing. Unbound probes are washed off and tracing of the probes on the sample follows. Tracing could either be through autoradiography, fluorescence or addition of a chromogenic substrate to the enzyme labelling the probe (Feng et al., 1991; Rompré et al., 2002). Signal detection will imply the presence of the target sequence or existence of the microbe with the target sequence in the case of whole cell samples.

Target DNA may occur in very low concentration and therefore has to be amplified using PCR (Rompré et al., 2002) or the cells are concentrated using filtration (Bej et al., 1991[a]). Primers flanking the target sequence are used for selective amplification after which the product is electrophoresed and/or membrane bound and hybridised (Keller and Manak, 1989). Some target sequences that have been used include the *uidA* (GUD gene), *uidR* (GUD regulatory gene) and the GAL gene (Bej et al., 1990; 1991[b]; Fricker and Fricker, 1994; Iqbal et al., 1997).

Nucleic acid methods are advantageous in that they can detect genes from VBNC bacteria. In addition, they can detect GUD negative E. coli O157:H7 that possesses the *uidA* sequence while lacking the enzyme (Feng et al., 1991; Fricker and Fricker, 1994). Use of PCR increases sensitivity, allowing detection of one cell per 100 ml sample (Bej et al., 1990; 1991[a]), which is the same sensitivity observed for improved culturing

techniques. Despite this sensitivity, nucleic acid methods can give false positives and negatives. For example, some strains of *Hafnia alvei* and *Serratia odorifera* showed the presence of the *uidA* during the detection of *E. coli*. This sequence is known to be absent in these non–*E. coli* strains (Fricker and Fricker, 1994). *Shigella* species were noted to interfere with the *E. coli* detection using *lamB* that codes for an *E. coli* surface protein targeted by the lambda phage (Bej et al., 1990). Therefore, in the presence of such strains one would expect false positive results.

The PCR technique can be inhibited due to environmental contaminants (Leonard et al., 2003; Rompré et al., 2002). Under such circumstances, the sample would need to be purified. Sample purification and contaminant removal will translate to prolonged assay time, the need for skilled labour and increased costs, all of which are undesirable for large routine sample processing.

Although NA techniques are sensitive, they can neither be used for microbial quantification nor the determination of the microbial physiological state (Leonard et al., 2003). The presence of genetic material does not necessarily imply the presence of the microbes or toxins (Taguchi et al., 2005; Tryland and Fiksdal, 1998). Therefore, extrapolating the presence of target sequences to the presence of viable microbes can lead to false positive results. When radio-labelling is employed, visualisation may take two to three days (Rompré et al., 2003) which compromises the objective of an early warning system.

Above all, molecular based methods are disadvantageous for routine use, as they require skilled manpower, are too expensive to run and demand dedicated laboratory space and equipment (Ivnitski, et al., 1999; Leonard et al., 2003; Rompré et al., 2002; Venter, 2000). The NA methods cannot be used for real-time monitoring in the environment, despite efforts by Heid et al. (1996) to monitor PCR products in real-time.

Real-time and on-line monitoring are key factors to be considered for any current method development for continuous indicator organism assessment to meet early warning requirements. However, all of the discussed techniques cannot afford real-time monitoring. To address this, research has focused on biosensor development for indicator organisms in order to achieve continuous monitoring (Leonard et al., 2003).

Other biomarkers like bile acids, sterols and aminoacetone have been found to be useful indicators of faecal contamination. These offer an added advantage of providing information about the actual origin of the faecal material. However, these materials are commonly associated with sediments, occur at low levels that are difficult to extract and detect (Bull et al., 2002; Fitzsimons et al., 1995; Truong et al., 2003), thus making them undesirable for routine use.

1.5 Biosensor Developments

The need for rapid, automated, real-time and on-line monitoring methods to do away with retrospect results and effectively implement early warning systems has resulted in many attempts to develop biosensors for indicator microorganisms (Leonard et al., 2003; Paitan et al., 2003). Biosensor detection entails the conversion of a signal (e.g. from previously discussed methods) to an electrical output. Signals can be optical, mass or electrochemical. A biosensor is made up of a bioreceptor surface, transducer and output display unit (Fig. 1.2) (Leonard et al., 2003; Purohit, 2003; Scouten et al., 1995; Thévenot et al., 2001).

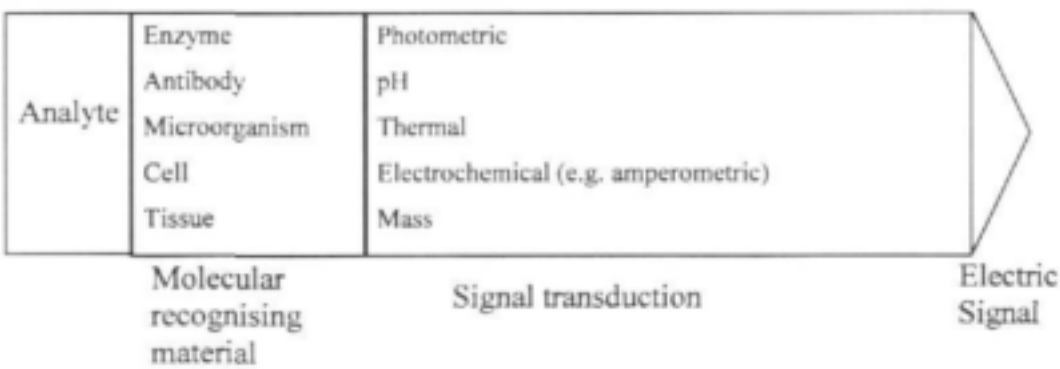


Figure 1.2: Schematic presentation of biosensor components

The bioreceptor surface selectively recognises the analyte. Receptor surfaces are constructed from different molecules that may be antibodies, enzymes, synthetic membranes and whole cells (Scouten et al., 1995; Thévenot et al., 2001). Strictly speaking, any device whose receptor surface is not derived from an organism cannot be called a biosensor (Thévenot, 2001) since there will be no biocomponent. Transducers convert signals generated at the receptor surface to an electrical form, and transfer them

to the output unit (Scouten et al., 1995; Thévenot et al., 2001). Different modes of signal transduction exist and can be used for biosensor classification. These can be electrochemical, mass, thermal or optical (Ivnitski, et al., 1995; Leonard et al., 2003). Electrochemical transducers, especially amperometric ones, are the most preferred owing to the advantages they offer. They are cheap and not subject to interference by turbid solutions (Scouten et al., 1995).

To the end user, a biosensor should preferably be an instrument where a probe is dipped into the sample, a button is pushed and a reading is obtained. However, to the developer, the process of developing such a device requires selection of an appropriate receptor surface, an appropriate method of signal detection and transduction, display format, definition of result range and optimisation of the function of the equipment.

Selective detection by biosensors is one of the reasons why they are preferred. They also produce results rapidly and can be used in real-time and on-line monitoring (Ivnitski et al., 1999; Leonard et al., 2003). When fully optimised, they are easy to use and do not need skilled manpower, they are not labour intensive and sometimes can be left in the field while capturing and transmitting data to the laboratory.

Several researchers have developed biosensors for *E. coli* and coliforms in laboratories. However, employing these biosensors in the field is met with a variety of challenges which include a small market to offset the development costs, low sensitivity when compared to currently existing methods, failure to meet requirements after rigorous tests in different environments, narrow scope of environmental applicability and the bureaucratic nature of legislation for them to be approved (Rogers, 1995).

Pyun et al. (1998) developed an *E. coli* biosensor using a flexural plate wave transducer. The minimum LOD was above 10^3 colony forming units (CFU) ml^{-1} although the time of analysis was less than an hour. If this is to be used for the purpose of monitoring drinking water it will not be sensitive enough to detect *E. coli* since the generally maximum acceptable CFU per 100 ml of drinking water is 1 CFU/100 ml.

Pérez et al. (2001) developed an amperometric culture-based biosensor for *E. coli* detection using the GAL substrate 4-aminophenyl-β-D-galactopyranoside (4APG). The

substrate was broken down to 4-aminophenyl (4AP) which was then determined electrochemically. Assay time for environmental samples varied between seven and ten hours, depending on the initial microbial load present in the sample. This enzyme, however, is widespread amongst the coliform group (Stevens et al., 2001) and can therefore be used for the detection of coliforms. Nistor et al. (2002) amplified the 4AP signal 4.5 fold using cellobiose dehydrogenase and detected more than 10^4 CFU 100 ml^{-1} in less than an hour. Reliance on a pre-culturing step is not desirable for the minimisation of the time required for analysis. Since the use of this biosensor involves a culturing stage, VBNC organisms may also not be detected.

Ercole et al. (2002) developed a biosensor based on a potentiometric (electrochemical) transducer for *E. coli*, but used it on vegetable samples. The LOD was 10 CFU ml^{-1} and minimum detection time was about one and half hours (Ercole et al., 2003). This LOD is not sensitive enough for the purpose of monitoring drinking water where no single CFU is expected in 100 ml of water. The use of such a biosensor for adequate monitoring of water samples would leave the population potentially vulnerable.

1.6 Project Justification

Despite the great strides that have been made in the improvement of indicator organism detection methods and efforts for real-time detection, there are still no satisfactory methods (in terms of short analysis time, low cost, improved sensitivity and ease of use) on the market for real-time monitoring. Although detection and confirmatory times have been considerably reduced, it is still imperative to reduce the detection time even further in order to enable sampling and results to be obtained in a single working day (Leonard et al., 2003; Pyun et al., 1998; Rompré et al., 2002; Sartory and Watkins, 1999; Venter, 2000). Taking advantage of the rapid detectability of enzyme activities (Holme and Peck, 1998), it is therefore our aim to assist and contribute to this improvement through the use of rapid direct enzyme assay using appropriate substrates and methods of detection. Such a contribution would greatly compliment efforts to implement the much-required early warning system of water supplying authorities for the enhanced protection of public health.

Most developments and improvements have remained excellent laboratory protocols that only work with pure cultures and seeded pure water samples. The major reasons for this

are the challenges met in translating such methods to the real field scenario (Rogers, 1995; Sartory and Watkins, 1999). Because of the differences between the two environments, it was decided to employ *in situ* assays, using typical polluted river water samples for our study. This would ensure faster and easier application of the methods in the field. In addition, this approach also allows proof of concept and field optimisation in one step.

1.7 Hypothesis

The *E. coli* GUD and coliform GAL enzymes can be found in the extracellular environment from which they hydrolyse glucuronide and lactose-based substrates, respectively. There is a positive linear relationship between the activity of these enzymes and *E. coli,* as well as coliform counts (Farnleitner et al., 2001; 2002). Enzyme catalysed reactions occur very rapidly (Holme and Peck, 1998). It is against this background that it was hypothesised that GUD and GAL can be used in the proof of concept for the development of an alternative, affordable, rapid and real-time *in situ* method for the efficient monitoring of water pollution by faecal material.

1.8 Aims

The ultimate aim of this work was to develop an on-line real-time enzyme diagnostic system for the detection and monitoring of sewage levels in drinking water, using suitable marker enzymes. This was accomplished by addressing the aims originally stated in the contract for project K5/1446, which were to:

a) to identify suitable marker enzymes in sewage
b) to develop a suitable diagnostic enzyme assay kit/strip or assay procedure
c) to engineer a suitable on-line, real-time biosensor system for the detection of sewage in water systems
d) to establish a resource base in the enzymology of sewage and industrial wastewater treatment processes and to support cognate research areas in South Africa
e) to empower disadvantaged and rural sectors of the population, and increase the state of public health in general
f) to promote student training and corporate technology collaboration to enhance wastewater management in South Africa

g) to correlate the findings of this work with known established parameters for determining water pollution/contamination

All the aims stated above were achieved throughout the course of this study, with the exception of aim c). This aim was not achieved within the two year funding cycle due to the large amount of preliminary work that had to be performed. However, this will be the focus of the follow-on WRC Project K5/1603, which has already been approved. Furthermore, proof of concept was established for the design of a suitable electrochemical biosensor for the rapid detection of faecal matter in water intended for drinking purposes, and this is discussed in Chapter 8.

1.9 Research Approach and Organisation

Execution of this work was divided into a fundamental study and an applied study. The insight gained from the fundamental study was used to support an applied study in order to establish likely alternative methods for GUD and GAL monitoring in polluted water. *In situ* assays were performed using appropriate samples from running and standing water sections of a typically polluted river. Where necessary, commercial GUD and GAL assays were run in parallel to serve as controls.

The work is presented in nine chapters as follows:

a) Chapter 1 contains the general project literature review
b) Chapters 2 and 3 deal with *in situ* assay optimisation using chromogenic and fluorogenic substrates, respectively
c) Chapter 4 comprises enzyme profiling studies
d) Chapter 5 contains work on interference studies by selected compounds on the enzymes and an investigation of the occurrence of some of the compounds found in water samples
e) Chapters 6 and 7 discuss the applied aspects and contain data about liquid and strip probes, respectively
f) Chapter 8 explores possibilities of electrochemical detection using GUD and GAL substrate breakdown products
g) Chapter 9 discusses the overall project conclusions and recommendations

2 CHROMOGENIC SUBSTRATE ASSAY OPTIMIZATION

2.1 Introduction

Determination of microbial water quality is a central activity in the control of total water quality. Efficient techniques for the detection of faecal pollution in aquatic environments are crucial for water monitoring programmes (Farnleitner et al., 2001; Rompré et al., 2002; Venter, 2000). The most widely used culture based methods are time consuming and tedious (Davies and Apte, 2000; Farnleitner et al., 2001; Rompré et al., 2002). Direct enzyme assays, in contrast, offer promising alternatives.

In order to establish an enzyme assay a number of steps are implemented to ascertain optimal conditions under which the enzyme will operate maximally. This process is called assay optimisation. Assay optimisation is usually based on previously published assay procedures and general characteristics of closely related enzymes. When an established protocol of a purified enzyme is in place, it can be used as a basis for the assay method of the same enzyme from different sources and in different environments. Parameters usually considered during optimisation of *in situ* assays include enzyme concentration, assay volume, assay time, pH and temperature. The relative location and abundance of the enzyme in the natural environment also has to be determined before the optimal assay conditions can be established.

The volumes of assay components, including enzyme/sample volumes and/or concentrations of enzymes required have to be designed in such a manner to obtain a linear, stable and consistent reaction over time while rapid reactions that are inconsistent are difficult to monitor are avoided. Once these parameters are established, scaling up and scaling down of assay procedures is easily performed.

Assay time can be a function of enzyme and substrate concentration, and pH and temperature. In order to accurately determine enzyme activity, one requires a linear range of maximum product accumulation. Such a range can be obtained from progress curves (a graphical plot of product formation versus time). Different enzymes and those from different environments have varying shapes of progress curves with some having lags, bursts and plateaus (Tipton, 2002). The determination of the characteristic shape of a

progress curve of the enzyme in question is therefore vital. When the assay requires a longer period, or when enzyme concentration is very low, an interval that shows a linear relationship between product formation and time must be obtained in order to save time for subsequent assays of the same enzyme. Readings are then confined to such time intervals, and valid extrapolations are then considered.

pH plays an important factor in the activity of enzymes. It affects the structural stability and solubility of, as well as the charge and charge distribution on both enzymes and substrates (Holme and Peck, 1998). These factors, in turn, alter bonds and bonding patterns, ultimately determining the rate of enzyme-catalysed reaction.

Temperature also influences the rate of molecular collision and bond vibrations. An increase in temperature (usually up to 40 °C) increases the number of collisions between substrates and enzymes, hence increasing the reaction rates. Increased bond vibrations due to an increase in temperature leads to bond disruptions that can facilitate easy and rapid substrate breakdown. However, this bond disruption will also cause denaturation of the enzymes themselves. As a result, optimum temperature will be a compromise between maximum activity and enzyme denaturation (Holme and Peck, 1998).

Each enzyme has a characteristic optimum pH and temperature. Similar enzymes, but from different environments can have different optimum assay conditions. Wakabayashi and Fishman (1961) noted variations in optimum pH of GUD from different sources using Phenolphthalein-β-D-glucuronide (PHEG) as a substrate. In other instances, an enzyme from a similar source can have different assay optimum conditions dictated by the type of substrate used.

Knowledge of the relative location and abundance of the enzymes to be assayed *in situ* is required for the design of a more accurate assay protocol. Enzymes in aquatic environments can be free floating or particulate bound, intracellular or extracellular (Chróst, 1991). A number of techniques can be employed to ascertain the location of these enzymes. Particulate bound and free-floating enzymes can be separated through filtration (Chróst, 1991; Farnleitner at al., 2002) or centrifugation. Chemical and mechanical cell permeabilisation methods (Chróst, 1991; Hsuchen and Feingold, 1973; Roe, 2001; Rotman, 1956; Woldringh and Van Iterson, 1972) can be employed in studies

to determine the relative cellular location of enzymes. However, special caution must be taken to use permeabiliser concentrations far below those which will inhibit the enzyme in question.

The major objective of the work covered in this chapter was to establish optimum assay conditions for GUD and GAL using the best chromogenic substrates available based on a previous pilot study performed by Watson and Pletschke (unpublished). The specific objectives were to:

a) determine appropriate sample volumes to be used in each enzyme assay,
b) ascertain the time period that produces a linear but maximal reaction,
c) determine the pH and temperature optima for GUD and GAL, and
d) investigate the location and abundance of GUD and GAL in the river samples.

2.2 Materials and Methods

2.2.1 Collection site

The Bloukrans river, which flows through the City of Grahamstown (Eastern Cape Province, South Africa), was selected for *in situ* assays. Previous water analyses from this river gave high coliform counts (Whittington–Jones, pers. comm). Three sampling points were initially selected as examples of 1) stagnant/standing water, 2) moderately flowing water (later called running water) and 3) fast flowing water.

2.2.2 Sampling

Water collection was performed in accordance with the standard procedures outlined in APHA (1986). Water samples were collected aseptically in 250 ml sterile pyrex glass bottles (Schott Duran, Germany), placed on ice and transported immediately to the lab and analyzed within an hour. The temperature was measured on site, and on arrival in the lab, and the pH was determined. Samples were collected in triplicate for studies in each case. All samples were collected at 08h30 in the morning.

2.2.3 Enzyme Assays

2.2.3.1 GAL

The GAL assay was performed according to the protocols of Seeber and Boothroyd (1996) and Pelisek et al. (2000) with modification. Chlorophenol red β-D-galactopyranoside (CPRG) (80 µg in 20 µl water) was added to 90 µl 0.1 M sodium phosphate buffer (pH 7.8), and 90 µl of environmental (water) sample was added to initiate the reaction. The change in absorbance was determined at 575 nm on a PowerWave$_x$ (Bio-Tek Instruments, USA). Enzyme activity was then calculated from the mean of triplicate results.

2.2.3.2 GUD

p-Nitrophenyl-β-D-galactopyranoside (PNPG) (10 mM) was prepared in the assay buffer. The assay buffer consisted of 0.1 M Tris–HCl, pH 8.0 containing 0.6 mM $CaCl_2$. Commercial GUD (EC 3.2.1.31) (Sigma, G7396) was dissolved in distilled water to produce a standard concentration of 500 $\mu g ml^{-1}$ GUD).

The standard PNPG assay was adapted from Fisher and Woods (2000). Assay buffer (110 µl) and 30 µl enzyme solution were mixed. The reaction was initiated by the addition of 110 µl PNPG. Kinetic readings (10 minutes at 30 seconds intervals) were performed versus a substrate blank, at 405 nm and 25 ^{o}C using a Power Wave$_x$ (Bio Tek Instruments, USA). This standard assay was used as a starting point for optimisation of the assay for use in the environment (water).

In all the environmental assays performed, two sets of controls were set up; an enzyme control and a substrate control. All readings were taken against distilled water. The control that gave a highest absorbance value was subtracted from all the test samples. All assays were performed in triplicate, unless otherwise stated. All results were reported as means ± standard deviations.

2.2.4 Linearity over time

Since enzymes in the river water are bound to be more dilute and behave differently due to presence of contaminants, it was necessary to establish a fixed time assay and to

determine the best time to monitor the assay reaction in order to work within the linear range of the readings. This was performed using the standard assays, substituting the pure commercial enzyme with the environmental/river water sample. Absorbance readings were taken at 15 min intervals for the first three hours and then subsequently at 2 h for the remaining 21 h. From this data, the optimal reading time for detecting the absorbance values in the linear section was deduced.

2.2.5 Volume optimisation

Volumes of river water varied between 30 µl and 90 µl, with corresponding adjustments to buffer volumes in order to obtain a fixed total end volume of 140 µl. Assays were performed at room temperature (22 ± 2 °C). The volume that gave reproducible results and showed the highest activity was used in subsequent assays.

2.2.6 pH optimisation

GUD and GAL activities were assayed at various pH values (5 – 11) using different buffers (Table 2.1) of constant (0.1 M) ionic strength. "Bracketing" of buffers at border pH values was employed to account for change in enzyme activity due to the various buffer components.

Table 2.1: Buffer systems used for pH studies

Buffer name	pH values
Sodium acetate	5 & 6
Sodium phosphate	6 & 8
Tris–HCl	7 – 9
Carbonate/bicarbonate	9 - 11

2.2.7 Temperature optimisation

GUD and GAL assays in river water samples were performed at temperatures of 4, 12, 20, 25, 30, 35, 40, 45 and 50 °C, respectively. The optimum volume and pH optimum were used to perform the assays as they were established in the above sections.

2.2.8 Fractionation studies

To determine the location of the enzymes, fractionation studies were performed on river water samples and effluent from the Rhodes University Environmental Biotechnology Research Unit (EBRU)'s facultative pond.

The two sets of samples were centrifuged at 6 000 *g* at 4 °C for 10 min to pellet the cells and particulate matter. Supernatant (designated supernatant 1) was separated from the pellet (pellet 1) and retained for sonication and enzyme assay. The pellet was washed in distilled water and centrifuged. The pellet wash was retained for enzyme assay. The washed pellet (pellet 2) was resuspended in the corresponding assay buffers for GUD and GAL, respectively. One millilitre aliquots each of supernatant 1 and pellet 1 were sonicated for two minutes [(at 25 W (RMS), 4 °C, 30 seconds sonication period with 15 seconds resting intervals, in ice)] using a Vibra Cell™ Sonicator (Sonics & Materials, USA).

GUD and GAL activities for the six fractions (whole fraction, supernatant 1, pellet wash, pellet 1, sonicated supernatant and sonicated pellet) were determined.

2.2.9 Permeabilisation studies

To investigate the effects of different cell permeabilisation methods on GUD and GAL activities, five methods of cell permeabilisation were employed. Stagnant/standing and moderately flowing (running) water samples were used.

2.2.9.1 Sodium dodecyl sulphate (SDS)

To each 1 000 µl of river water, 250 µl of 2 % (w/v) SDS (BDH laboratories, England) was added to give a final concentration on 0.4 % (w/v). This was briefly vortexed and placed on ice for five minutes (Roe, 2001) before performing the enzyme assays.

2.2.9.2 Sonication

One milliliter of each sample was sonicated as described in 2.2.8 and kept on ice before assaying for enzyme activity.

2.2.9.3 Triton - X100

A final concentration of 0.5 % (v/v) Triton–X100 was achieved by adding 250 µl of 2.5 % (v/v) Triton–X 100 to 1 000 µl of sample. This mixture was briefly vortexed and left at room temperature (20 ± 2 °C) for 45 minutes (Roe, 2001).

2.2.9.4 Toluene

Toluene (100 µl) was added to 1 000 µl of water sample and incubated at 37 °C for 10 min (Roe, 2001) to allow for cell lysis.

2.2.9.5 Polymyxin B (PxB) sulphate

The method was adapted from Van Poucke and Nelis (1997). A final concentration of 100 $\mu g ml^{-1}$ PxB sulphate (ICN Biomedicals, Ohio) in the water sample was attained. The mixture was briefly vortexed and incubated for 45 min at 37 °C before enzyme activity assays were performed.

2.3 Results

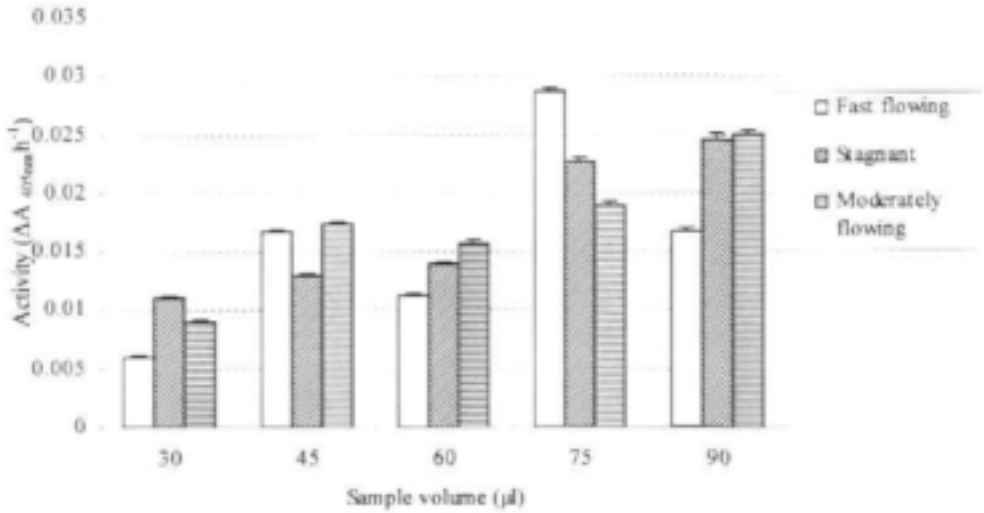
2.3.1 Linearity over time

Linearity was confirmed over a period of 24 h for the GAL assay (data not shown). However, variability existed over the length of the lag period for the GUD assay with some samples not having any lag phase whatsoever, while in others the lag phase was as long as 3 h (graph not shown). After the lag phase, a linear increase in activity up to 24 h was observed. As a result of the variations in the lag phases a two point reading for every sample in the GUD assays was not possible. Readings were taken at hourly intervals to get the maximum rate of *p*-nitrophenol production over time.

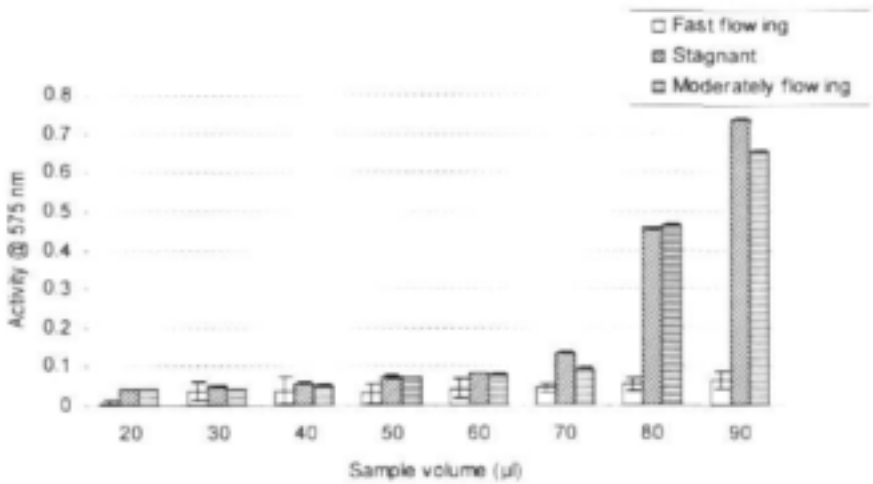
2.3.2 Volume optimisation

There was an increase in GUD and GAL activity with an increase in environmental water volume to 90 µl for both stagnant and running water samples (Figs. 2.1a and b). However, the trend for the fast flowing water was inconsistent for GUD (Fig. 2.1a) and almost consistently low for GAL with very high standard deviations as compared with the moderately flowing and stagnant samples (Fig. 2.1b). From these results, 90 µl was

selected for subsequent assays of the stagnant and moderately flowing water. The fast flowing water sample was omitted in subsequent studies.



(a)



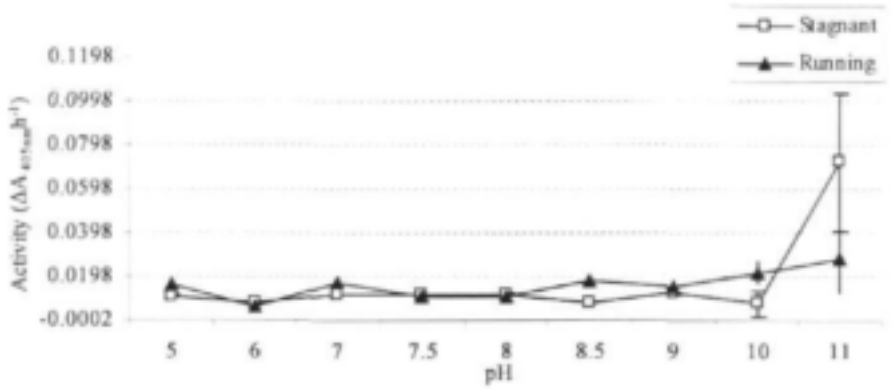
(b)

Figure 2.1: Effects of varying sample volume on GUD (a) and GAL (b) assays. All values are reported as mean ± SD (n=3).

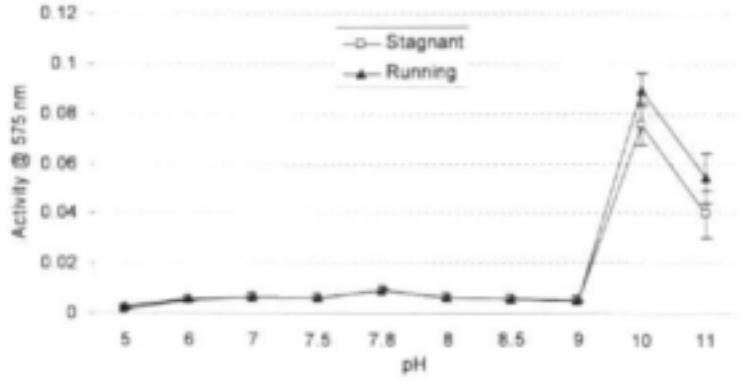
2.3.3 pH optimisation

Generally, GUD and GAL activities were constant between pH 5.0-9.0, with a sudden increase at pH 10-11. The increase at alkaline pH was coupled with reduced reproducibility as illustrated by the increase in error bars (Figs. 2.2a and b). A peak in GAL activity was observed at pH 7.8 for both stagnant and running water samples. A variability in GUD activity versus pH in standing and running water samples, respectively, was observed with the two samples having consistent results at pH 5-8 and 9 (Fig. 2.2a). GAL and GUD assays were performed at pH values of 7.8 and 8,

respectively, for the remainder of the study. Water intended for drinking purposes rarely has a pH far removed from neutrality and therefore performing GAL and GUD assays at these respective pH values is not anticipated to present any problem. For GAL, a second peak at pH 10 was observed. However, this was not reproducible and gave a high standard deviation.



(a)



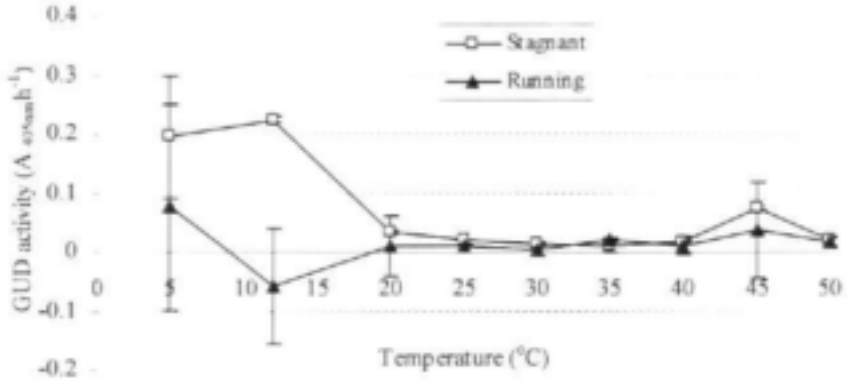
(b)

Figure 2.2: Effects of pH on GUD (a) and GAL (b) activity in stagnant and running water samples. All values represent the means ± SD (n=3).

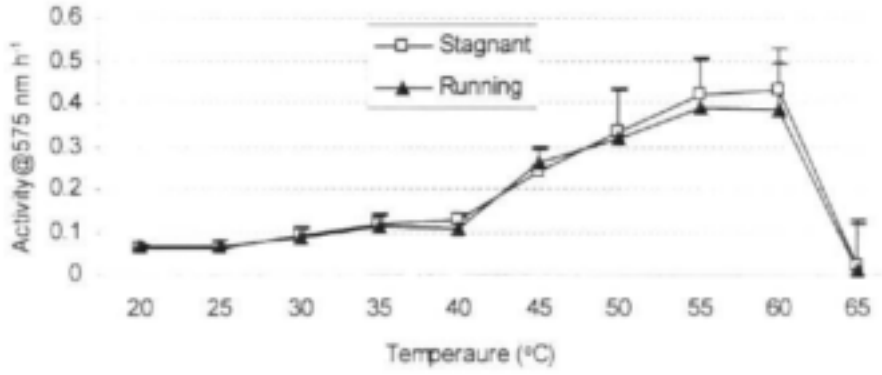
2.3.4 Temperature optimisation

As observed with pH, GUD response to exposure to extreme temperatures for stagnant and running water was variable (Fig. 2.3a). GUD activity for both samples was insignificantly different ($P > 0.05$) from 25 to 50 °C (Fig. 2.3a). A more consistent pattern was observed for GAL with an increase observed in the activity in both samples up to 60 °C and a sharp decline at 65 °C to almost zero (Fig. 2.3b). Reproducibility at higher temperatures decreased with GAL activity being more consistent (Fig. 2.3b). Room temperature (20 ± 2 °C) was selected for all further enzyme assays, and care should be taken when using enzyme assays to detect the indicator microorganisms as the assay

and subsequent bioprobe/biosensor implementation will be dependent on the ambient temperature.



(a)

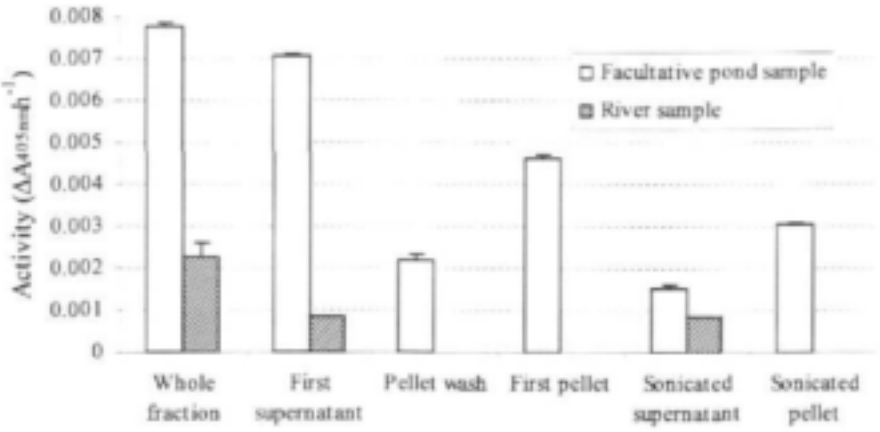


(b)

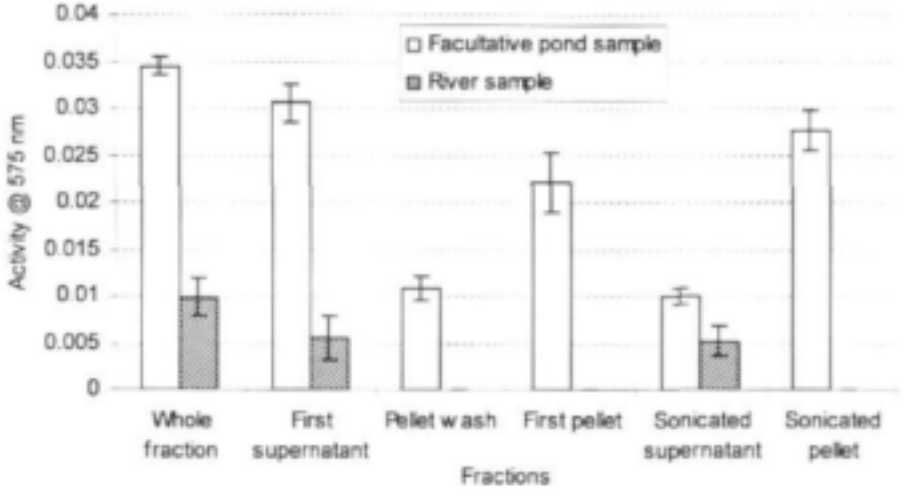
Figure 2.3: Effects of temperature on (a) GUD and (b) GAL activities in different water samples. All values represent the means ± SD (n=3).

2.3.5 Fractionation studies

There was no pellet in the river water, and therefore the centrifuged sample was regarded as the supernatant 1 and sonicated supernatant. The facultative pond sample yielded all six fractions. For both sets, the whole fractions (RS) had the highest enzyme activity (Figs. 2.4a and b). Supernatant 1 (first supernatant) of the facultative pond sample showed the highest activity for both GUD and GAL amongst the amended fractions followed by the pellet 1 (first pellet) and then the sonicated pellet (Fig. 2.4a and b).



(a)

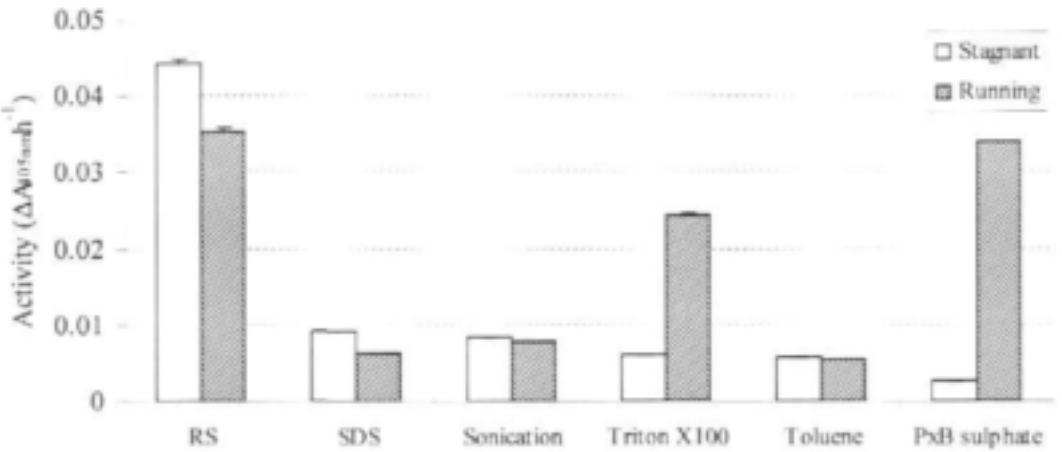


(b)

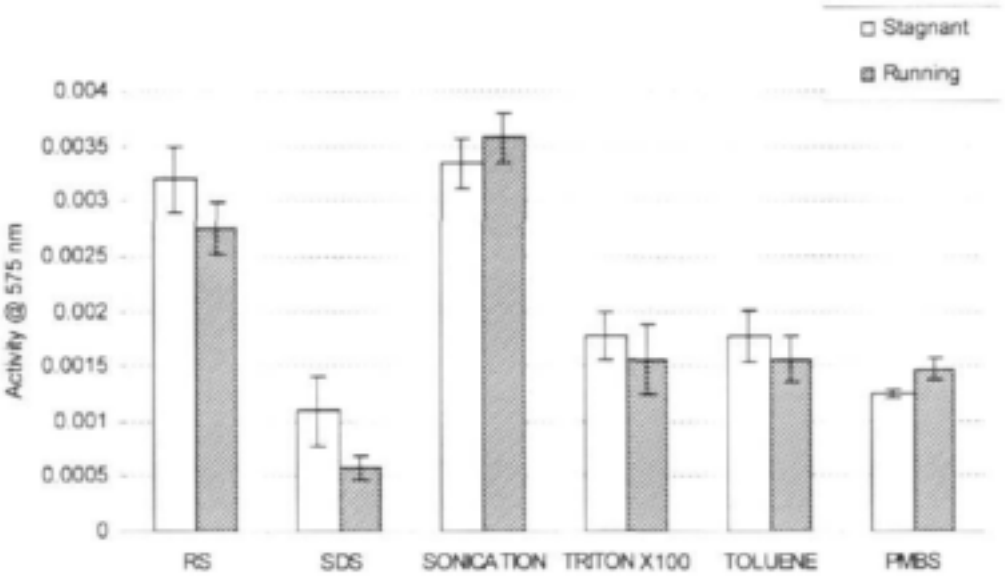
Figure 2.4: Activity of GUD (a) and GAL (b) in different fractions of aquatic samples All values represent the means ± SD (n=3).

2.3.6 Permeabilisation effects

Different permeabilisers inhibited GUD activity except for PxB sulphate in the running water sample (Fig. 2.5a). Polymyxin B sulphate was the most potent inhibitor of GUD in stagnant water. A similar pattern was noted for GAL, except that sonication led to an insignificant increase ($P > 0.05$) in activity (Fig. 2.5b).



(a)



(b)

Figure 2.5: Effects of different permeabilisation methods on GUD (a) and GAL (b) activity. All values represent the means ± SD (n=3).

2.4 Discussion and Conclusion

2.4.1 Discussion

The variation in the lag phases can be explained by the occurrence of different concentrations of inhibiting compounds in the polluted water (Tipton, 2002; Wetzel, 1991). Another possible explanation could be the occurrence of a more preferred substrate in the water samples (Tien and Kirk, 1988). GUD and GAL could be more

adapted to the natural substrates in the polluted water samples. Under such situations, the enzymes will cleave the natural substrates first (during the lag phase) before catalysing the breakdown of PNPG and CPRG. Prolonged lag phases noted when samples were concentrated (see chapter 4) support the occurrence of protein inhibitors, because concentration techniques employed later in the study also increased the concentration of protein inhibitors as well as the natural substrate concentrations. The unpredictability of the lag phases highlighted one of the difficulties that are faced when executing *in situ* assays. Although such difficulties mean more labour intensive techniques, *in situ* assaying remains the most viable option to minimise the time required for the development and simplification of an environmental adaptation of a method.

An increase in enzyme activity with an increase in river sample volume suggests the increase in enzyme concentration with increase in sample volume, as expected. Enzyme dilution, denaturation and inhibition by the pollutants inherently found in the water can possibly account for the lower activity observed in diluted river samples. The use of substrate dissolved in the assay buffer assisted in maintaining the buffering capacity of the reaction mixture since an increase in water samples was accompanied by a corresponding decrease in the assay buffer.

The lack of response of GUD and GAL to a wide pH and temperature range could be as a result of changes to the enzymes due to exposure to the stressful environmental conditions associated with polluted water. Protection by other pollutants can cause such a characteristic behaviour. Meyer–Reil (1991) noted a similar trend with temperature in different marine enzymes. The observed behaviour of GUD and GAL was different from that of similar enzymes in purified form. The presence of GUD and GAL from different sources can also give a lack of response due to occurrence of different pH and temperature optima for each of the two classes of enzymes.

A broad operational temperature and pH range works to the advantage of bioprobe and biosensor development, as this will save costs in temperature maintenance during enzyme assays. For GAL, an insignificant variation in activity was noted between 20 and 40 °C, above which there was poor reproducibility, which necessitated the use of ambient temperature in future studies. However, problems experienced with GUD assays at very low temperature may imply the limitation of using such a direct enzyme assay method in

low temperature environments or during cold seasons. The activity pattern of GUD at the lower temperatures and decreased GAL reproducibility at higher temperatures could be a result of chemical interference by pollutants.

Fractionation results illustrated the abundance of the enzymes in the extracellular environment. GUD is exported to the extracellular environment when cells reach stationary phase (Doyle et al., 1955). Since conditions in the natural environments are often stressful (George et al., 2002; Leonard et al., 2003; Venter, 2000), cells are forced to be in a stationary phase metabolic state. The presence of GUD in extracellular environments is therefore not unusual. The presence of natural substrates and competitors (Adams et al., 1990) can stimulate GUD production and exportation to the extracellular environment. The occurrence of a cocktail of detergents and other cell lysing factors in wastewater and polluted water can also alter cell permeability thus releasing the enzyme to the external environment.

Availability of the enzyme outside the cell provides a valuable advantage to the desired method of direct enzyme assay, as this will allow the assay of unamended (i.e. no permeabilisation procedures required) water samples. This, in turn, reduces processing costs and labour. Extracellular location was also confirmed by the results of the permeabilisation studies, where none of the permeabilising methods offered a significant degree of improved activity over the whole unamended samples. Fractionation studies results obtained were comparable to results of Farnleitner et al. (2002) who observed insignificant difference in GUD activity between filtrate and whole samples of lowly polluted waters.

Inhibition of the enzyme activities by permeabilisation makes these permeabilisers undesirable for use with the polluted Bloukrans river water samples. Such an inhibition suggests the presence of either similar pollutants or other pollutants that produce a synergistic inhibitory effect on the enzymes, because permeabiliser concentrations used were below enzyme inhibition threshold values (Clausell et al., 2003; Plummer, 1978; Roe, 2001). Inhibition of the enzyme activities were in agreement with the results obtained by Chróst (1991) when 25–92 % inhibition was noted in selected marine enzymes using Triton - X100, EDTA, toluene and SDS. The reduction in GUD activity after sonication was not expected. This could be accounted for by the disruption of

stabilising structures that are formed in environments or exposure of the enzyme to protease action after sonication.

Further studies will entail the use of fluorogenic substrates based on the insight gained on location, optimum temperature and pH optima of the enzymes. The substrates which produce the best results will then be used in further method development for subsequent bioprobe/biosensor design.

2.4.2 Conclusions

From these fundamental studies with chromogenic substrates, the following conclusions about GUD and GAL were drawn:

a) enzymes in the polluted environmental occur in lower concentrations and larger sample volumes have to be employed to show a reasonable level of activity,
b) physical and kinetic properties of environmentally exposed enzymes are different from that of enzymes in the purified state and enzymes in pure cell culture with defined media,
c) cell permeabilisation is not an essential step in direct enzyme assay for moderately polluted water samples such as that of the Bloukrans River for example.
d) regardless of cell growth phase, GUD and GAL occur extracellularly in polluted water environments, and as such could give rise to false positives,
e) GUD and GAL, if too stable or recalcitrant in the environment could have implications for analysis, as the enzyme activity would be present long after the *E. coli* or coliform cells had lost viability,

3 FLUOROGENIC SUBSTRATES STUDIES

3.1 Introduction

Photometric enzyme assays can be classified as either absorbance, turbidimetric or fluorometric, which involve measurement of light absorbance or transmittance, turbidity and fluorescence, respectively. Measurement of absorbed or transmitted light in enzyme assays mainly involves coloured compounds in the visible light range (400–750 nm). This technique suffers a lack of sensitivity at low compound concentrations due to the fact that there is little difference between incident and transmitted light (John, 2002). However, this technique is cheap and easy to execute.

Turbidity takes into consideration the clarity of a solution. Measurement of turbidity is based on light scattering (as opposed to absorbance) by a solution. Turbidimetry is employed when the enzyme substrates are insoluble polymers (John, 2002).

Fluorescence enzyme monitoring methods, unlike absorbance methods, take advantage of compounds that emit light energy (as opposed to heat) when returning to the ground state after excitation. As in absorbance spectroscopy, different compounds are excited and emit energy at distinct wavelengths (Gordon, 2001; John, 2002). An advantage of fluorimetric assays over absorbance is that they have a higher sensitivity. The latter is made possible because the fluorescence comparison is made with complete absence of light in the presence of the fluorochrome. However, fluorimetric techniques are more expensive than absorbance spectroscopic methods due to high substrate and instrumentation costs (John, 2002). Compounds detected through fluorescence are usually colourless.

In this chapter, the monitoring of the hydrolysis of two fluorogenic substrates, MUG and MuGAL, by GUD and GAL respectively, was attempted. Our main objective was to increase the LOD using the high sensitivity that fluorimetric assays afford.

3.2 Materials and Methods

3.2.1 GAL assay

Beta – galactosidase activity was measured according to the method elucidated by Apte and Batley (1994) with some modification. Briefly stated, the assay involved the addition of pure 4-methylumbelliferyl- ß-D-galactoside (MuGAL) substrate (0.0625 g of MuGAL in 259 ml of distilled water) and 0.1 M sodium phosphate buffer (pH 7.2) to 5 ml of sample and incubated for 60 min at room temperature (20 ± 2 °C). Activity was terminated with calcium carbonate buffer (pH 10) and the fluorescence was measured using a Hitachi F-2500 Fluorescence Spectrophotometer at 465 nm (emission slit width 20 nm) after excitation at 375 nm (slit width 10 nm).

3.2.2 GUD assays

The assay buffer was 0.1 M sodium phosphate (pH 7) and the stop buffer was made up of 0.2 M $NaCO_3$ (pH 10.4). A concentration of 7.1 mM MUG was prepared in distilled water.

The GUD assay was adapted from Farnleitner et al. (2001). Briefly stated, 500 µl of MUG was added to 450 µl assay buffer and left standing at room temperature (22 ± 2 °C) for 30 min, after which, 50 µl GUD or sample was added. This was briefly vortexed and 200 µl aliquots were withdrawn at suitable intervals into separate test tubes with 1 000 µl stop buffer. Fluorescence emission was measured at 444 nm (after excitation at 365 nm) using a Hitachi F-2500 Fluorescence Spectrophotometer. The excitation and emission slits were both set at 10.0 nm.

For river sample volume optimisation, the reaction mixtures were proportionally adapted with various volumes of water sample as depicted in Figure 3.1.

3.2.3 The effect of pH

The above assay procedures and selected volumes were used in an investigation to assess the effect of pH on the hydrolysis rates of river water GUD and GAL on their fluorogenic substrates. The buffer systems were as outlined as indicated in Chapter 2.

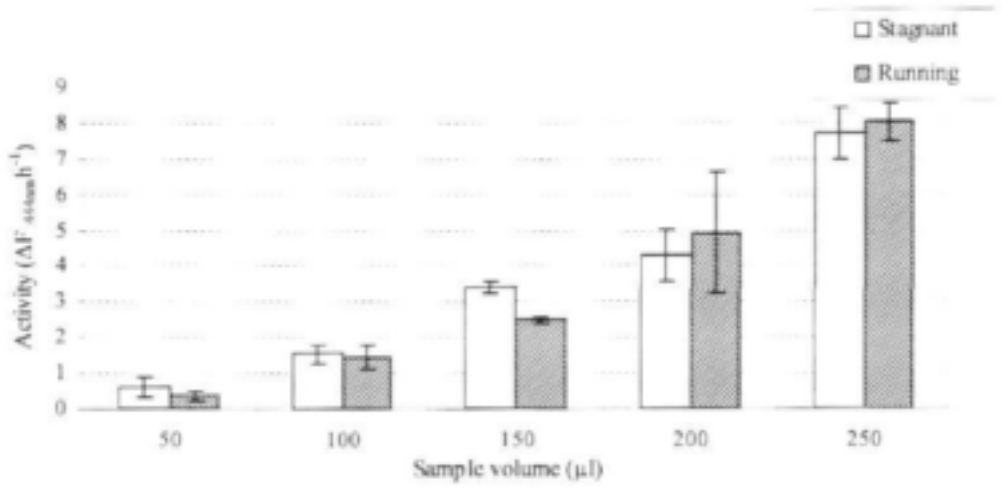
3.2.4 UV observations

Sample tubes containing the enzyme reaction mixtures, substrate and enzyme blanks were also visualized under UV illumination to detect any noticeable change in fluorescence due to the GUD and GAL activities present in the water samples.

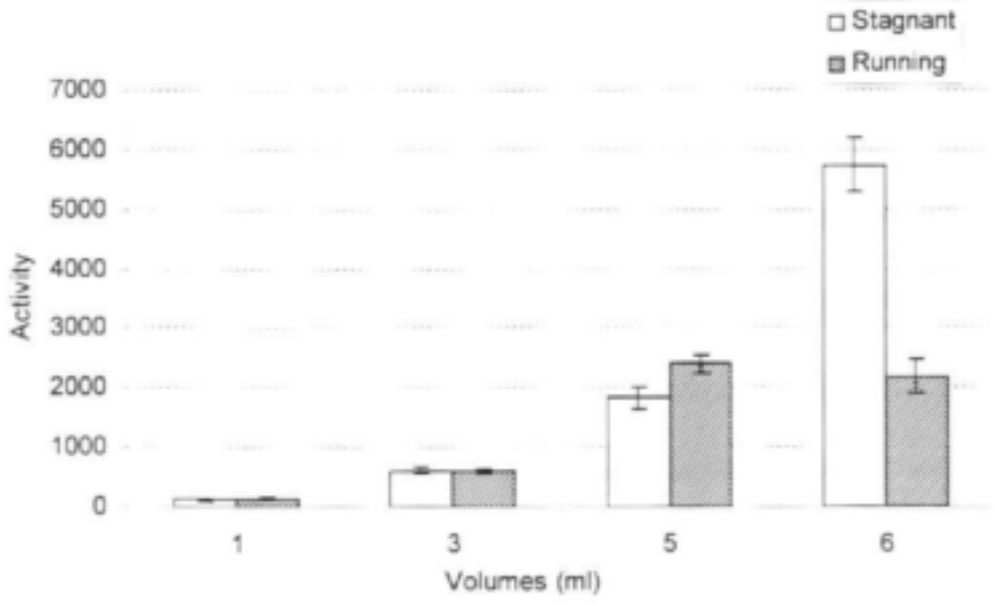
3.3 Results

3.3.1 Volume optimisation

There was a generally direct relationship between GUD and GAL activities and the volume of water samples (50 – 250 µl for GUD and 1 – 6 ml for GAL) added (see Figs. 3.1a and b). However, the errors were observed to be quite large at the higher volumes of water sample added [200 µl of running water giving the highest error (34.26 %) in the GUD assay (MUG)].



(a)



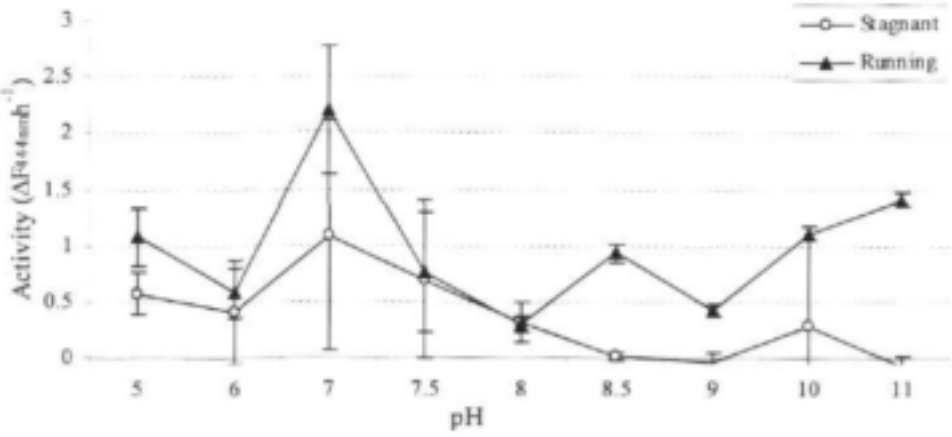
(b)

Figure 3.1: Effects of changes in river water sample volume on a) MUG and b) MuGAL. All values represent the means ± SD (n=3).

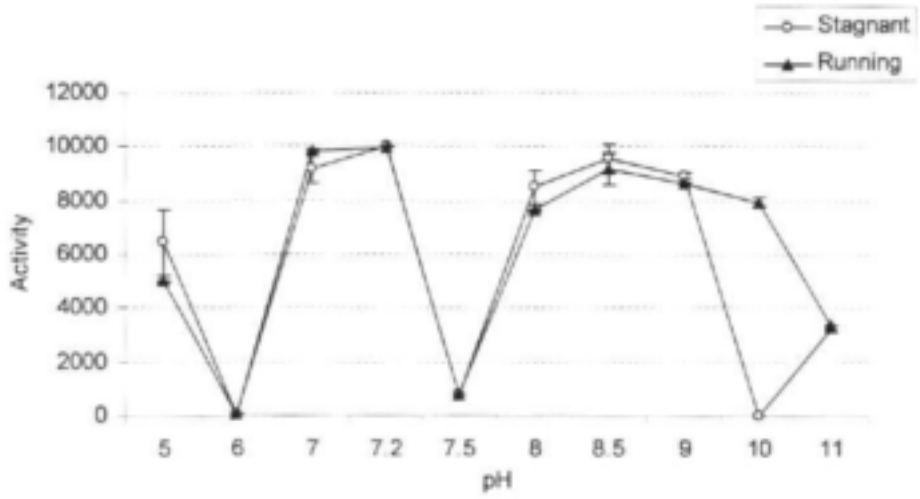
3.3.2 pH optimisation

Enzyme activity fluctuated with changes in pH. The highest GUD activity was obtained at pH 7.0 while for GAL the highest activity was recorded at pH 7.2 (see Figs. 3.2a and b). Increases in enzyme activity were mostly accompanied by concomitant increases in

error. Several samples were analysed in an attempt to reduce the margin for error for the GUD assays but this was not successful.



(a)



(b)

Figure 3.2: Effects of pH on a) GUD and b) GAL activities on MUG and MuGAL, respectively. All values represent the means ± SD (n=3).

3.3.3 UV visualisation

Both the enzyme reaction mixture and the uncatalysed substrate fluoresced under UV illumination. No visual distinction could be made between their fluorescence intensities.

3.4 Discussion and Conclusion

3.4.1 Discussion

The high values of error suggested an unreliability of the technique due to interference by a wide variety of environmental compounds. Fluorescent assays are very prone to interference by the naturally occurring fluorogenic components in the environments. Davies and Apte (1999) suggested that non-target bacteria, other cell-free enzymes and cell-free substances are potential contributory interfering agents.

Although fluorogenic assays are sensitive, the interference makes them less desirable for application in the environment. In addition, fluorogenic substrates are more expensive than chromogenic ones. This gives the PNP and CPRG an advantage over MUG and MuGAL, respectively. Fluorescence of the substrate under UV could be a result of high substrate concentration. However, a reduction in substrate concentration could potentially reduce the rate of the enzyme reaction. When assaying enzymes *in situ*, the substrate concentration must be at least ten times the K_m to get 91 % of the maximum enzyme activity (Holme and Peck, 1998).

3.4.2 Conclusion

Sensitivity was not obtained, and the analyses were affected by severely affected by quenching and interference. It was decided not to implement fluorogenic substrates for this project or product development in the light of these significant shortcomings that will adversely affect the use and applicability of these compounds to the environment. Focus in subsequent work was therefore placed on the chromogenic substrates PNPG and CPRG.

4 ENZYME CONCENTRATION AND PROFILING TO IDENTIFY THE SOURCE OF MARKER ENZYMES

4.1 Introduction

It was possible that the markers enzymes GUD and GAL could also have originated from other sources, such as those sources from plant or algal origin. Attempts were therefore made to concentrate these marker enzymes (originally present in very low concentrations) in order to facilitate bioinformatic profiling and identification of the source from which these markers enzymes had originated. In addition, studies were also performed to establish whether or not enzyme concentration could accelerate the assay reaction (thus decreasing the time required for analysis and increasing the sensitivity of the test).

Proteins often occur at very low concentrations in environments or may be contaminated by impurities. Under such circumstances there is a need to either concentrate the proteins or to remove the impurities. This enables the study of the protein without any source of interference. When a protein of interest in known, its unique properties can be used to selectively concentrate and isolate it (Dennison, 2003; Doonan and Cutler, 2004). After protein concentration further characterisation can be performed in the form of electrophoresis in order to determine the molecular weight and subunit composition. For enzymes, activity assays will assist to monitor the presence of the required protein throughout the various stages of isolation. Determination of the origin of a protein through peptide mass finger printing can also be employed. In order to minimise denaturation of the protein, concentrating techniques are often performed at 4 ^{o}C. However, some proteins are stable at higher temperatures and such a characteristic may be utilised as a positive selective advantage to isolate the protein at high temperatures. This would selectively denature unwanted and temperature sensitive protein contaminants.

4.1.1 Protein concentration

A range of techniques can be used to concentrate proteins. These include either precipitation with salts, organic solvents and organic polymers, or by freeze drying the protein solution.

Salt precipitation can be achieved either by salting in or out. Salting in is based on a reduction in protein solubility at low salt concentrations. This leads to protein aggregation and hence precipitation. The precipitate may then be removed by centrifugation. Salting in depends on the charge and polar interactions with solvents (Doonan, 2004; Harris 1994; Scopes, 1984). Globular proteins are mainly isolated and purified in this manner (Scopes, 1984).

In salting out, there is a decrease in protein solubility with an increase in salt concentration. The technique depends on the hydrophobicity of a protein. As the salt concentration is increased, there is a removal of water around the proteins because of the water solvating the ions. This, in turn, exposes the hydrophobic regions on proteins, leading to protein aggregation and precipitation (Harris, 1994; Scopes, 1984). Ammonium sulphate is commonly used in salting out for a number of reasons. It is cheap, increases protein stability and allows for easy pellet formation by the precipitate. However, it increases solution acidity slightly, which may have a negative impact on the protein structure (Dennison, 2003; Harris, 1994; Scopes, 1984).

The principle behind the use of organic solvents in protein precipitation is that of reduction in water activity that in turn decreases solvating power of water for charged proteins (Scopes, 1984). This will lead to a decrease in protein solubility causing them to aggregate and precipitate. Organic solvents are used at sub-zero temperatures thus preventing enzyme denaturation. Another advantage of using organic solvents is that of high volatility which allows them to be easily removed from the protein sample (Dennison, 2003; Scopes, 1984).

Organic polymers such as polyethylene glycol (PEG) 20 000 and Sephadex G25 can be employed in protein concentration. These agents draw water from the protein solution. Usually the solution to be concentrated is placed in dialysis tubing with a molecular weight cut off limit that retains proteins while allowing water and other smaller molecules to exit the tubing. An advantage of using the organic polymers is the concomitant removal of smaller soluble contaminants with the water during the concentration process, thus eliminating the subsequent step required for precipitant removal from the protein solution

Proteins can also be concentrated by freeze drying of the protein solution. Freeze drying concentrates all molecules in the solution (unlike organic polymer concentration). The resuspended powder has subsequently to be dialysed versus water or buffer to reduce the concentrated contaminants. Freeze drying can denature the proteins of interest. Techniques like ultrafiltration can also be used in protein concentration (Dennison, 2003; Schratter, 2004).

4.1.2 Analyses of concentrated proteins

Once the proteins have been concentrated or purified, they are subjected to further analyses to obtain information that include an assessment of purity, mass, structure and origin if these are not known. Electrophoresis is employed to ascertain purity, molecular weight and subunit composition of a protein (Walker, 2002).

Protein electrophoresis may be performed under non-denaturing or denaturing conditions (Bollag et al., 1996). Non-denaturing protein electrophoresis (e.g. polycramide gel electrophoresis (PAGE)) is used when the native protein properties require investigation. Denaturing PAGE is used when the molecular weight and subunit composition of the proteins are to be determined. Sodium dodecyl sulphate (SDS) and 2-mercaptoethanol may be used to denature the protein.

Determination of molecular weight and subunit composition may not be sufficient. Additional information about the origin of a protein from a mixed source environment is required. Peptide mass finger printing is used to identify the source of a protein in the environment. Electrophoresed protein is excised from the gel and digested by a selected enzyme and exposed to mass spectrometry (MS). Examples of enzymes that may be used in the digestion of a protein band include trypsin, V8 protease and chymotrypsin. Mass spectrometers used may be either matrix assisted laser desorption and ionisation time of flight (MALDI ToF) or electrospray ionisation (ESI) (Liebler, 2002). Once the peptide masses are obtained, they can be used in conjunction with known protein databases for the search and match of fragment masses of reference proteins (in the data base), digested with same enzyme under identical conditions. Once a match is found, the protein origin can be confirmed. MALDI ToF MS is sensitive and provides good resolution. However, the success depends on sample purity (Liebler, 2002).

The major objective of the work in this chapter was to concentrate GUD and GAL in stagnant and running water as well as to ascertain the origin of these enzymes. This was achieved through the use of different concentrating techniques, electrophoresis and MALDI ToF MS.

4.2 Materials and Methods

4.2.1 Protein concentration

One denaturing (acetone precipitation) and four non–denaturing concentration techniques were adapted from Bollag et al. (1996) and used. All concentration steps were performed at 4 °C.

4.2.1.1 Acetone precipitation

Five hundred millilitres of ice cold acetone were added to 100 ml of water sample, gently mixed and kept at -20 °C for three hours. This was followed by centrifugation at 27 000 g for five minutes at 4 °C. The pellet was air dried, re-suspended in minimal volume of water, aliquoted in eppendorf tubes and stored at -20 °C (Ó'Fágáin, 2004) for further analyses.

4.2.1.2 Ethanol precipitation

To 100 ml of pre-chilled river water samples, an equal volume of cold ethanol (-20 °C) was slowly added with gentle stirring (Dennison, 2003). The stirring was continued for a further 15 min. The solution was then centrifuged at 10 000 g for ten minutes and the pellet was resuspended in 1 ml of distilled water and stored as in 4.2.1.1.

4.2.1.3 PEG 20 000 concentration

Dialysis tubing with a molecular weight cut off of 12 KDa was prepared as outlined by the manufacturer (Sigma) and 100 ml of the water samples were added. Dialysis was performed until the remaining volume in the tubing was about five millilitres. This was dialysed overnight versus 10 mM Tris–HCl buffer (pH 7.5) and stored as outlined in 4.2.1.1.

4.2.1.4 Ammonium sulphate precipitation

To 100 ml of chilled water samples, 56.8 g of ammonium sulphate was added slowly with gentle stirring. Stirring was continued for 30 min after salt dissolution. The solution was centrifuged at 10 000 g for 10 min at 4 °C, and the pellet was resuspended in 3 ml of water and dialysed as in 4.2.1.3 with three buffer changes. Dialysis tube contents were stored as in 4.2.1.1.

4.2.1.5 Freeze drying

Water samples (100 ml aliquots) were poured into freeze drying flasks that were swirled in liquid nitrogen. After the contents were frozen, the flasks were applied to the freeze dryer and left over night. The powder was resuspended in 5 ml distilled water, dialysed and stored as in 4.2.1.4.

4.2.2 Protein concentration determination

Protein quantification was carried out using the Bradford assay adapted from Kruger (2002). Bovine serum albumin (BSA) was used to construct a standard curve. A BSA stock of 2 $mg.ml^{-1}$ was prepared from which dilutions of 0.4, 1.8, 1.2, 1.6 and 2 $mg.ml^{-1}$ were used for the BSA standard curve. To each BSA concentration and 5 µl of sample from 4.2.1, 250 µl of Bradford reagent was added, mixed, left for 5 min and the absorbance was read at 595 nm.

4.2.3 Activity gels

Polyacrylamide gels (6 %) were prepared for native protein electrophoresis, and the samples were prepared and loaded as described by Bollag et al. (1996). Each gel was overlaid (separately) by PNPG and CPRG solutions and any colour changes were observed.

4.2.4 SDS PAGE

Denaturing gel electrophoresis was performed as described by Bollag et al. (1996) using the BioRad mini protean III system. Eight percent resolving and 5 % percent stacking gels were prepared. Samples (20 µl) were mixed with 5 µl of 5x sample buffer, heated at 100 °C for five minutes (Bollag et al., 1996) and then 20 µl of each treated sample was

applied to the gel. Rainbow molecular weight markers were also applied to the gel. Electrophoretic separation was achieved at a constant voltage of 120 V, after which, the gel was Coomassie stained and the bands presumed to be GUD and GAL excised for MALDI ToF MS.

4.2.5 MALDI ToF MS

Excised bands from SDS-PAGE were sent to the School of Molecular Sciences MALDI ToF MS facility (University of Cape Town, South Africa) for tryptic digest mass mapping. The resultant masses were corrected for trypsin self digestion fragments. Expasy tool (PeptideSearch) was used to search and match the peptide mass to ascertain the origin of the enzymes (http://au.expasy.org/tools/).

4.3 Results

4.3.1 Protein concentration methods

Ethanol, ammonium sulphate and acetone precipitation methods achieved very little and insignificant ($P>0.05$) concentration while PEG 20 000 provided the highest degree of protein concentration (Fig. 4.1). There was more protein in stagnant than running water although more GUD activity was obtained in concentrated running water (Fig. 4.2a). The GAL activity was higher in stagnant than running water (Fig. 4.2b). The highest GUD (1 511.1 % fold increase) and GAL (3 484.9 %) activities were found in PEG 20 000 concentrated samples (Figs. 4.2a and b). Freeze dried and ammonium sulphate precipitated samples gave lower GUD activity than the whole water fraction. Progress curves for PEG 20 000 concentrated and freeze dried samples exhibited a longer lag phase. The PEG 20 000 concentrated samples were therefore selected for gel electrophoresis and tryptic mass mapping.

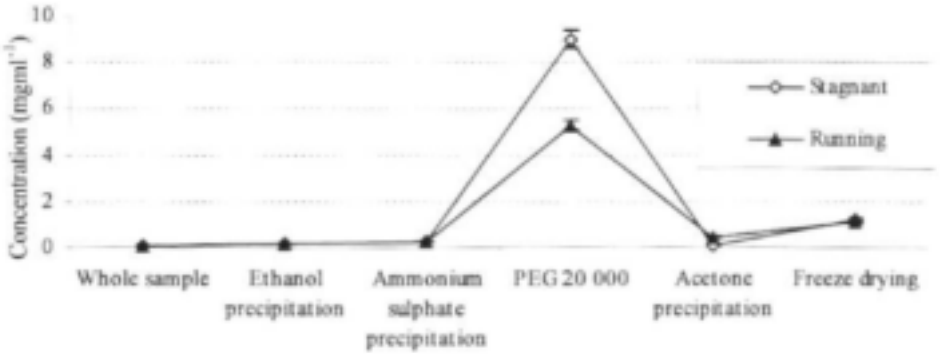
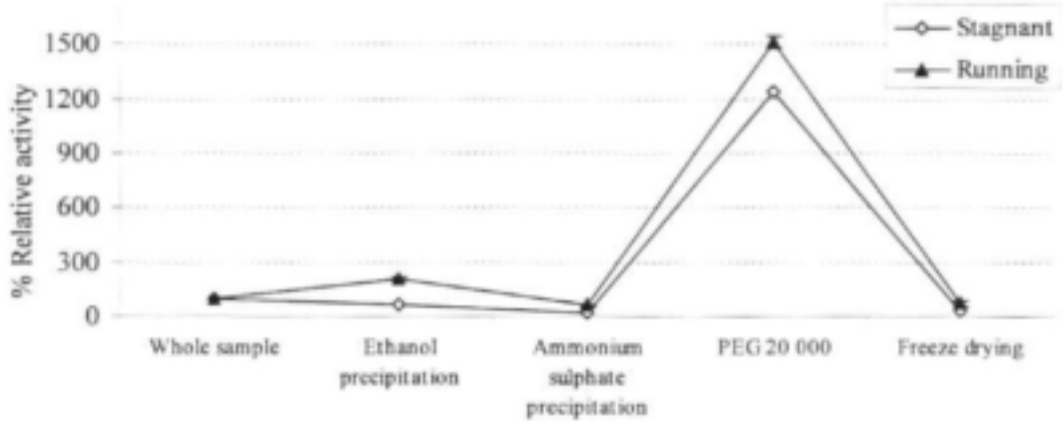
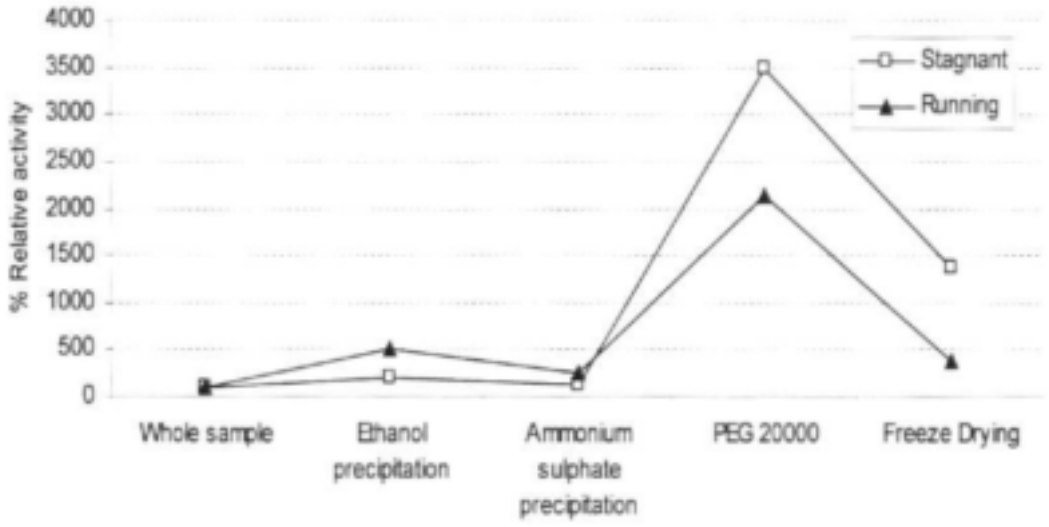


Figure 4.1: Protein concentration ($mg.ml^{-1}$) achieved by different methods of protein concentration for stagnant and running water. All values represent the means ± SD (n=3)



(a)



(b)

Figure 4.2: GUD (a) and GAL (b) activities in whole sample and concentrated fractions using non-denaturing methods. All values represent the means ± SD (n=3).

4.3.2 Activity gels

The colours from incubation of PNPG and CPRG rapidly diffused over the gel and into the solution from the specific regions of enzyme location.

4.3.3 SDS PAGE and MALDI ToF MS peptide mass matching

Several identical bands for stagnant (S) and running (R) water samples were observed (Fig. 4.3). Bands of interest were the presumed GAL (A) at 116 KDa and GUD at 68 KDa (Fig. 4.3). These were excised and sent for MALD ToF MS.

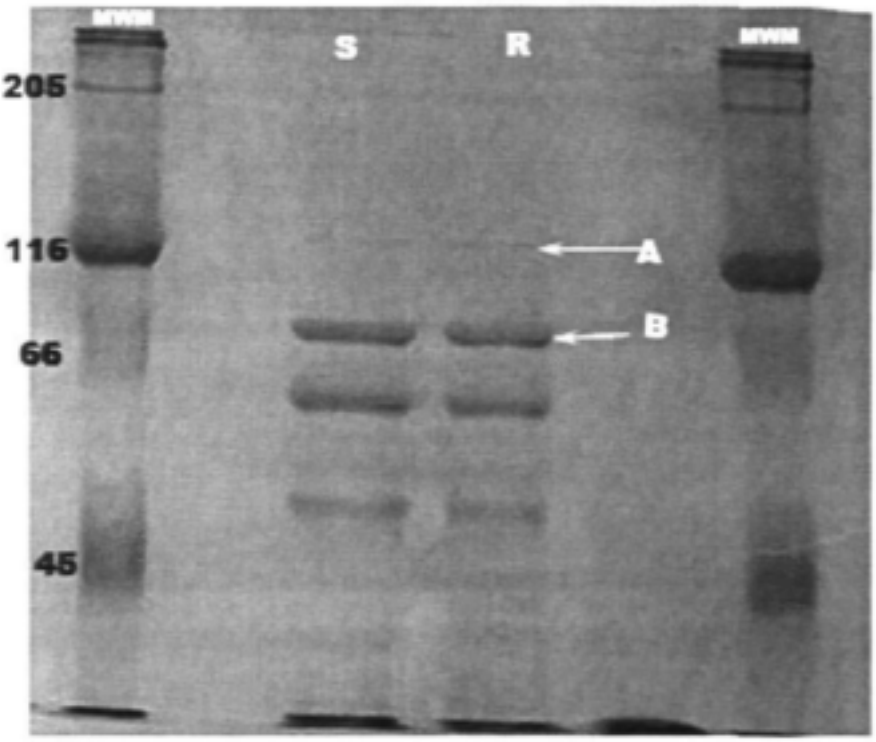


Figure 4.3: Presumed GAL (A) and GUD (B) bands obtained after SDS-PAGE. S and R are stagnant and running water samples, respectively, MWM is the rainbow molecular weight marker with numerical values in kDa.

Peptide mass matching confirmed band A (Fig. 4.3) to be *E. coli* GAL but failed to do so for GUD. Instead, reducing the level of accuracy to two daltons gave results indicating that some fragments in B are from *E. coli* GAL. Theoretical digestion of *E. coli* GUD sequence and mass matching require 26 fragments matches at 0.1 Da accuracy to be picked up as an *E. coli* GUD.

4.4 Discussion and Conclusion

4.4.1 Discussion

Ammonium sulphate precipitates protein in solution if the protein concentration is above 1 $mg.ml^{-1}$. In this case, the water sample protein concentration was less than this threshold values hence no protein concentration was achieved. Instead enzyme inhibition was noted. In other studies with enzymes in environmental water samples, inhibition of the enzymes by ammonium sulphate was noted (Ngwenya and Whiteley, unpublished). Similarly, failure to concentrate proteins by other precipitation methods could be attributed to low protein concentration and interaction of precipitants with interfering compounds inherent in the water (Scopes, 1984). Increases in enzyme activities with increase in protein concentration by PEG 20 000 suggest successful GUD and GAL concentration. However, the lower GUD activity in PEG 20 000 concentrated stagnant water, with a concomitant higher protein content, suggested the presence of a high proportion of non-GUD proteins in the sample. More GUD was found to be present in running water, most likely because of the turbulence that mixes flowing water while there could be vertical stratification of the enzyme in stagnant water. Lower activities in freeze-dried samples may be the result of enzyme denaturation during the freeze-drying process or a concomitant increase in inhibitor concentration. Pronounced lag phases in concentrated samples are more likely to result from increased protein inhibitor concentrations, especially in freeze-drying (Dennison, 2003).

The presence of GAL fragments in the presumed GUD band could be as a result of protein digestion in the natural environment before concentration. In addition, the 26 fragment matches required at 0.1 Da was high in relation to the 22 fragments from the MALDI ToF MS. However, the correlation of GUD and *E. coli* observed in this study and other studies by Farnleitner et al. (2001; 2002) support GUD activity to be mainly attributed to *E. coli*. In addition, the occurrence of the B band at 68 kDa position is in agreement with the molecular weight of this *E. coli* enzyme. Current emerging technologies, like the colifast at line monitoring (CALM) (www.colifast.no) use GUD activity as a surrogate indicator for *E. coli*. Therefore, the continued use of GUD in this project is justifiable.

4.4.2 Conclusion

From this chapter, it may be concluded that:

a) the best method of concentrating proteins was with the use of dry PEG 20 000,
b) hydrolysis of PNPG and CPRG was as a result of enzyme activity as observed from the activity gels,
c) the GAL enzyme in this study originated from *E. coli.*
d) GUD and GAL can be used for the indication of *E. coli* and coliforms, respectively.

In summary then, it appeared as if enzyme concentration was not a feasible option for accelerating the enzyme reaction rates and decreasing the times required for analysis. It has been stated in literature (Davies et al., 1994) that GUD and GAL enzymes originating from algal matter may interfere with this type of analysis, generating false positives and implicating biosensor studies in this field. On a positive note; however, it was shown in this study that the GAL enzyme had indeed originated from *E. coli*. The effect of algal matter on enzyme activity will be further investigated in the follow-on WRC grant K5/1603.

5 INTERFERENCE STUDIES AND ANALYSIS OF WATER COMPONENTS

5.1 Introduction

A wide variety of metal ions are found in the environment. Metal ions play important roles in the biological function of many enzymes and can have a variety of effects on enzyme systems. Enzymes function best in dilute aqueous solutions under limited conditions of temperature, pH and salt concentration (Berg et al., 1986; Singh et al., 1990). In this chapter, the effect of a wide variety of ions, commonly found in water intended for drinking purposes, on the activity of the marker enzymes was assessed.

The various modes of metal-protein interaction include metal-, ligand-, and enzyme-bridge complexes. Metals can also serve as electron donors or acceptors (Tryland et al., 1997). For some enzymes, the presence of metal ions is crucial and required for activity. Some enzymes require the assistance of metal ions in order to perform catalysis. Even in those cases where metals are required, very high metal concentrations or the incorrect metal can inhibit the enzyme's activity (Den Blanken, 1985). A large number of enzymes have been found to be dependent on alkali metal ions for activity. Of the alkali metal ions, sodium and potassium are commonly found in living systems. Metal ions such as Zn^{+2}, Mg^{+2}, Mn^{+2}, Fe^{+2}, Cu^{+2}, K^{+}, and Na^{+} can be employed as cofactors of enzymes (Den Blanken, 1985).

A number of substances may lead to a reduction in the rate of an enzyme's activity. Some of these (e.g. urea) are non-specific protein denaturants. Others, which generally acting in a fairly specific manner, are known as inhibitors. Loss of activity may be either reversible, where activity may be restored by the removal of the inhibitor, or irreversible, where the loss of activity is time dependent and cannot be recovered during the time period of interest. If the inhibited enzyme is totally inactive, irreversible inhibition behaves as a time-dependent loss of enzyme concentration. Heavy metal ions such as mercury and lead should generally be prevented from coming into contact with enzymes, as they usually lead to such irreversible inhibition by binding strongly to the amino acid backbone (Den Blank, 1985; Ridgway and Olson, 1982; Singh et al., 1990).

An environmental water sample is likely to contain ions/salts in quantities far above the acceptable limits. At the pH of most natural waters (pH 5-8), a large percentage of carboxylic acid groups will be ionized and many cations such as calcium will chelate

organic acids, therefore reducing protein-organic acids complex formation leading to increased enzyme activity. Divalent cations also adsorb organic acids and other substances (Wetzel 1991). Calcium carbonate precipitates dissolved organic acids in an aqueous environment that may also inhibit enzyme activity (Wetzel, 1991).

This chapter investigates the effects of a wide range of potential ions/salts in varying concentrations on GUD and GAL. Although GUD and GAL have been employed in differential media for detecting and enumerating coliforms and *E. Coli* over a long period, there is little information, if any at all, regarding the effect of most ions/salts on these assays. The effect of the river water sample on commercial GAL and GUD was also studied.

5.2 Materials and Methods

5.2.1 Water pollutant analyses

Water samples were collected as described in chapter 2 and submitted to the Nelson Mandela Municipality Scientific Services Department (Eastern Cape, South Africa) for analysis. Table 5.1 shows the results of these analyses and highlights the compounds that were present in the water samples.

5.2.2 Interference studies

The effects of various compounds found in water and those used in water treatment on GUD and GAL activity were investigated. Stock solutions of 5 000 $mg.l^{-1}$ of sulphate, chloride, carbonate, cadmium, magnesium, calcium, ferric chloride, potassium, ferulic acid, EDTA, sodium chloride, phosphate nitrate and 700 parts per million sodium hypochlorite were prepared. With the exception of sodium hypochlorite, an enzyme, buffer and effector concentration of ranges between 0 and 1000 $mg.l^{-1}$ were pre-incubated at room temperature (20 ± 2 °C) for 30 min, after which the reaction was initiated by the addition of the respective substrate. Controls for each effector concentration were also prepared. All assays were performed in triplicate.

5.2.3 Effects of environmental water samples on commercial GUD and GAL

Different percentages (v/v) of water from the two sampling points were added to commercial GUD and GAL solutions, pre-incubated for 30 min at room temperature (20 ± 2 °C) and the enzyme reaction was initiated by the addition of the respective substrates. The activities were compared with those of the respective enzymes diluted with distilled water.

5.3 Results

5.3.1 Water pollutant 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 (www.klmcs.co.za/html/waterguidelines.html) water quality guidelines (Table 5.1). Calcium carbonate and chloride were present in the highest concentration amongst the components analysed (Table 5.1). Differences in some of the compounds (e.g. sodium, carbonate and zinc) concentrations between stagnant and running water samples were observed. The units of the results depicted in Table 5.1 from the sample analyses are in $mg.l^{-1}$ except for mercury ($\mu g.l^{-1}$)

Table 5.1: Water samples analysis report from the Nelson Mandela Municipality Scientific Services Department and the SABS 241 limits

Sample identification	Stagnant sample	Running sample	Max. acceptable limits mgl^{-1}
Total alkalinity as $CaCO_3$	296	296	
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 SO4	126	125	600
Nitrate plus Nitrite as N	2.6	2.6	20
Phosphorus (soluble)	1.1	0.86	
Total Sulphides as H_2S	<1	<1	
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 (ugl^{-1})	<0.3	<0.3	5
Nickel as Ni	<0.005	<0.005	350
Zinc as Zn	0.024	0.014	10

5.3.2 Interference studies

Where similar trends of ion effects on GUD and GAL activity were observed, one typical graph for either enzyme was presented.

Metal ions had varied effects on GAL and GUD. Carbonate ions progressively increased the activity of GAL (Fig. 5.1) with an increase in concentration. A similar trend was observed for GUD. However, there was also an observed increase in pH as the carbonate concentration increased (Table 5.2). Cadmium inhibited the activities of both GAL and GUD in a similar trend (Fig. 5.2), although it inhibited more than 90% of GUD activity at as low a concentration as $50mg.l^{-1}$ and completely inhibited GAL activity at the same concentration.

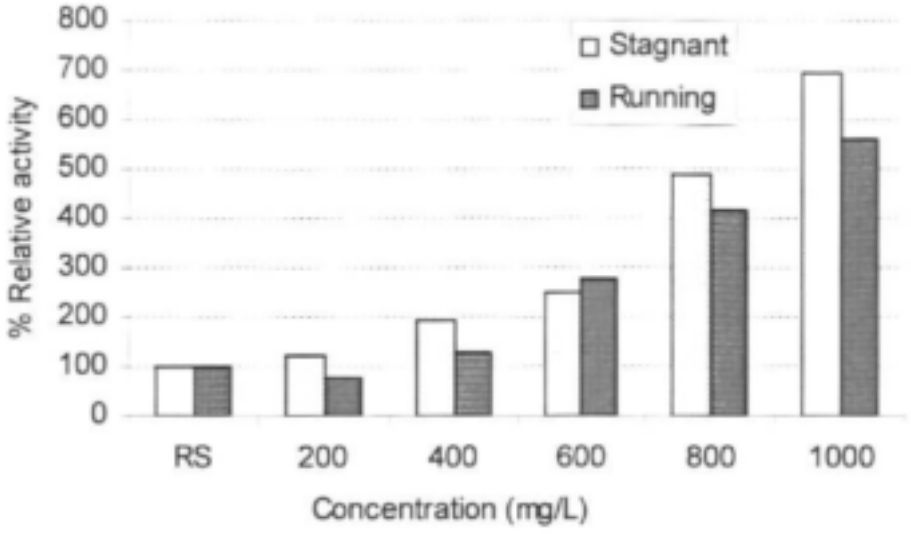


Figure 5.1: A typical graph showing an increase in GAL activity in the presence of different concentrations of carbonate ions. All values represent the means ± SD (n=3).

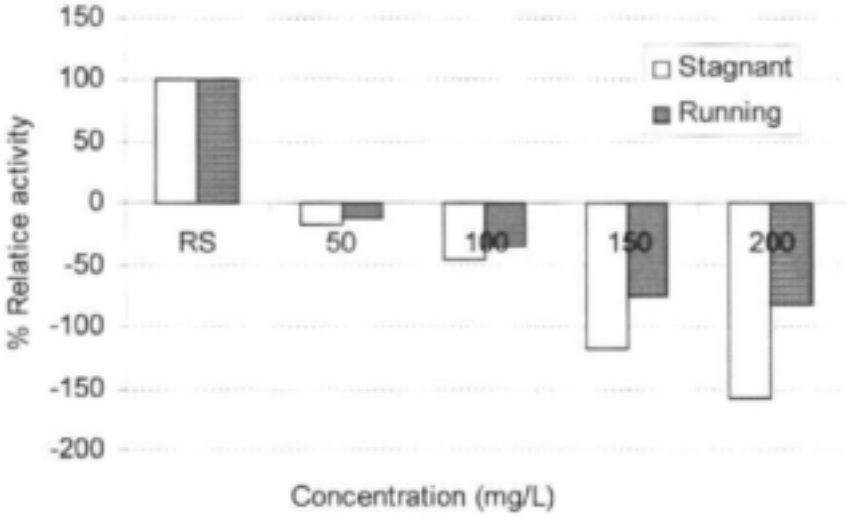
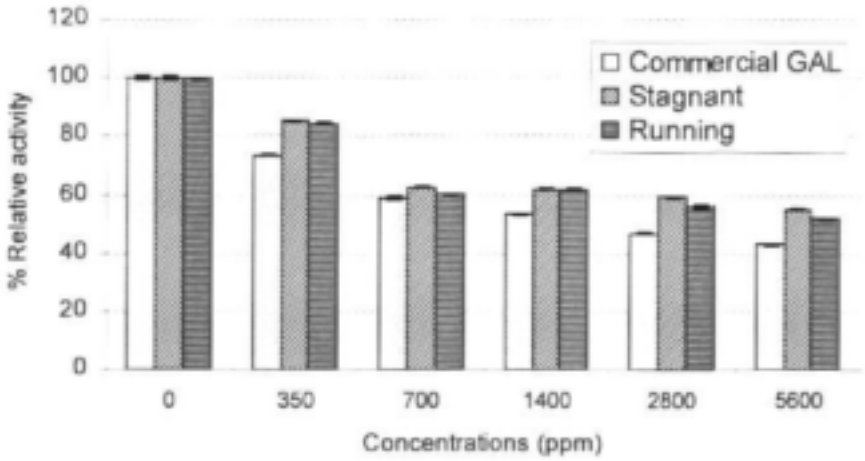


Figure 5.2: Inhibition of GAL activity by cadmium. All values represent the means ± SD (n=3).

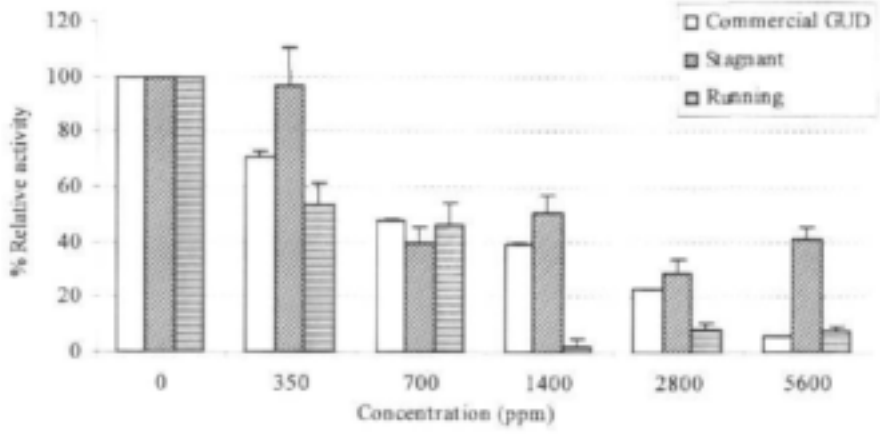
Sodium hypochlorite, which is normally used as a disinfectant, especially in remote areas, reduced the activities of GAL (Fig. 5.3 a) and GUD (Fig. 5.3 b) as did EDTA (Fig. 5.4 a) and ferrulic acid (Fig. 5.4 b). However, the degree of susceptibility of GUD and GAL to sodium hypochlorite was different; GUD was more affected. The commercial GUD was

less subject to inhibition by EDTA, when compared to the environmental enzymes (Fig. 5.4 a).

Ferric chloride reduced GAL activity between 200 mg l^{-1} and 400 mg l^{-1} and strongly inhibited it above 800 mg l^{-1}. Ferric chloride led to the hydrolysis of PNPG and a strong reduction in pH (Table 5.2), thus preventing an accurate assessment of its effect on GUD.

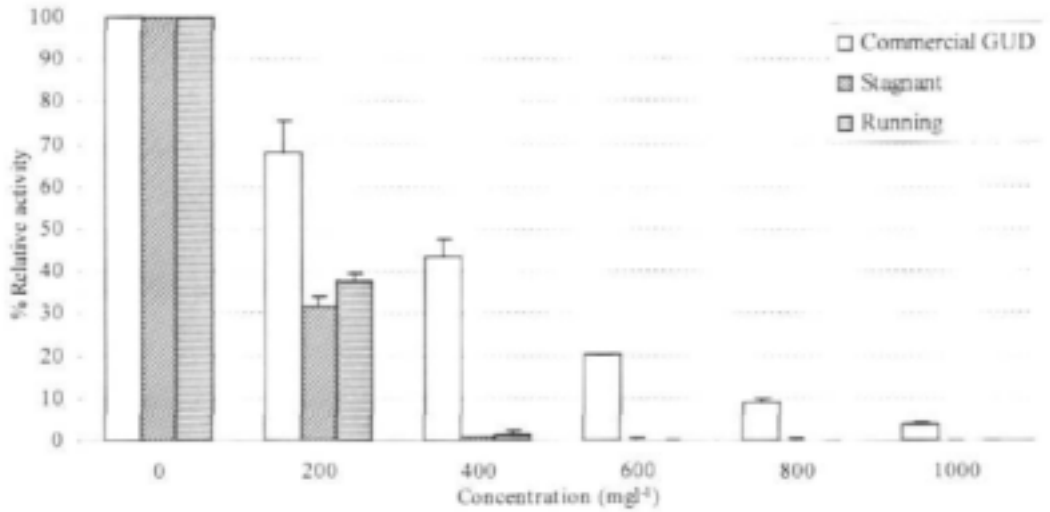


(a)

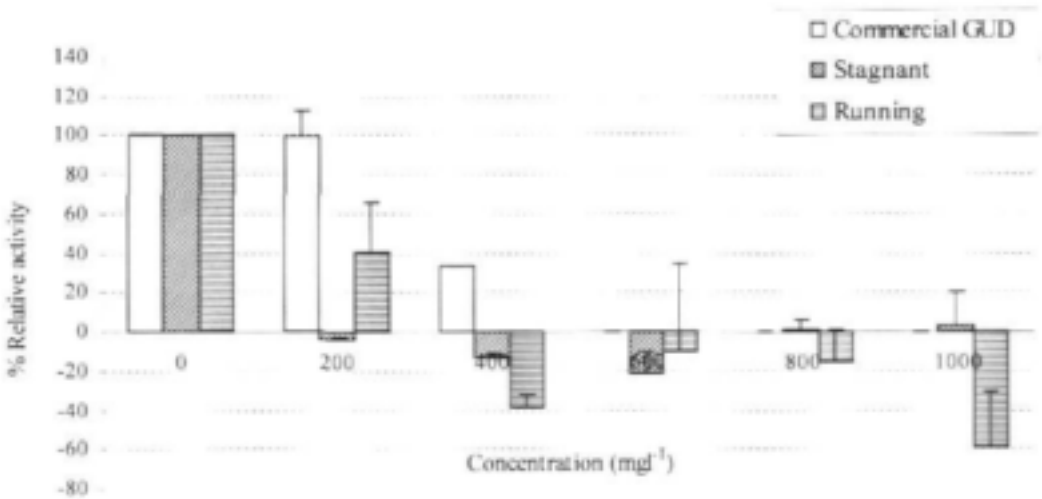


(b)

Figure 5.3: Inhibitory effect of sodium hypochlorite on (a) GAL and (b) GUD. All values represent the means ± SD (n=3).



(a)

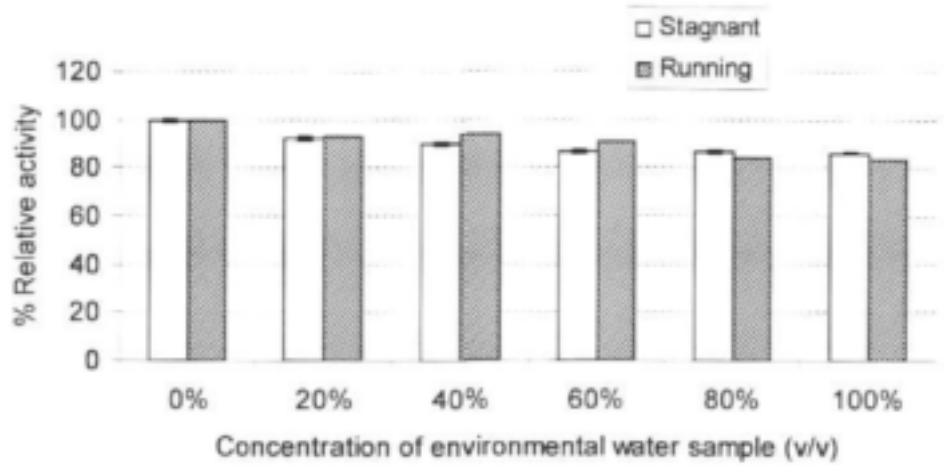


(b)

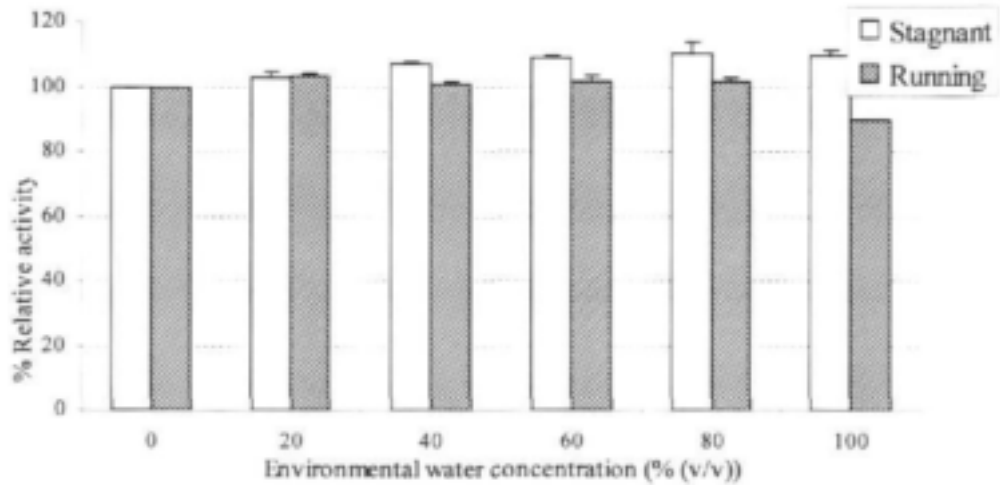
Figure 5.4: Effects of a) EDTA and b) ferulic acid on commercial and environmental GUD activities. All values represent the means ± SD (n=3).

5.3.3 Effects of environmental water samples on pure GUD and GAL

It was clear from the results on the effect of environmental water samples on commercial GAL and GUD, that GAL activity (Fig. 5.5a) is reduced by about 15% while the level of GUD activity is slightly increased in the stagnant water but reduced in the running water sample (Fig. 5.5b).



(a)



(b)

Figure 5.5: Effect of different concentrations of environmental water sample on commercial a) GAL and b) GUD. All values represent the means ± SD (n=3).

Table 5.2: Summarised effects of different ions on (a) GUD and (b) GAL activities

(a)

Effector	Effect on GUD activity	Effect on pH
Cadmium	Very strong inhibition, more than 90 % at 50 mgl^{-1} in environmental sample and 100 % in pure (commercial) GUD	Reduction from 8.02 to 7.31 at 1000 mgl^{-1}
Ferulic acid	Strong inhibition, less than 50 % enzyme activity at 400 mgl^{-1} for pure GUD, strongly inhibited GUD in stagnant as opposed to running water.	Strongly reduced pH (4.62 in pure GUD, 6.27 in river water)
Phosphate	Potentiated commercial GUD, weak inhibition at higher concentrations	No effect
Nitrate	Weak inhibitor for pure GUD. At $\geq$ 600 mgl^{-1} reduced activity in both environmental samples by more than 50 %	No effect
Potassium	Weak inhibitor for pure GUD that retained 80 % activity at 1 000 mgl^{-1}, increased activity in stagnant water up to 200 mgl^{-1}, inhibiting from 400 mgl^{-1} for both samples	No effect
Sodium chloride	Activated all GUD activity up to 200 mgl^{-1}, after which, environmental GUD was gradually inhibited. No reduction in pure GUD activity.	No effect
Sodium hypochlorite	Strong inhibitor	Slight increase
EDTA	Strong inhibitor, more on environmental samples	Reduced
Sodium	Moderate inhibition	No effect
Carbonate	Inhibited all at above 200 mgl^{-1}, but greater effect on pure GUD, slight potentiation below 200 mgl^{-1}.	Raised pH to more than 10
Calcium	Weak inhibitor at above 400 mgl^{-1}. commercial GUD least affected.	Slight reduction
Sulphate	Potentiated activity in running water up to 200 mgl^{-1}.weak inhibitor for environmental samples	No effect
Sulphite	No effect on pure GUD but potentiated activity for both environmental samples	Slight increase to 8.15
Magnessium	Moderate inhibition with more effect on pure GUD	No effect
Chloride	Potentiated activity in running water up to 600 mgl^{-1}, moderately inhibited pure GUD and stagnant water	No effect
Ferric Chloride	Hydrolysed the substrate	Strongly reduces pH

(b)

Effector	Effect on GAL activity	Effect on pH
Calcium	Potentiation of enzyme activity at 0.2 gl^{-1}	Strong reduction
Carbonate	Consistent activation with increasing concentration	Very strong increase
Magnesium	Increase in activity over 100 fold beyond 0.4 gl^{-1}	Slight reduction
Sodium Chloride	Inconsistent effect on activity	Slight increase
Potassium	No significant effect	No effect
Chloride	Moderate reduction at 0.2 gl^{-1} and a very strong inhibition beyond 0.5 gl^{-1}	Very strong reduction
Sodium hypochlorite	Inhibition on activity	Slight increase
Potassium Nitrate	No significant effect	Slight increase
Phosphate	No effect at lower concentrations (up to 0.4 gl^{-1}) after which an extensive reduction occurred	Slight reduction
Sulphite	Increase from 0.2 gl^{-1} to 0.6 gl^{-1} beyond which there was inhibition	No effect
Silver	Inconsistent effect	Slight reduction
Ferric chloride	Reduction between 0.2 gl^{-1} and 0.4 gl^{-1} but strong inhibition above 0.8 gl^{-1}	Strong reduction
Cadmium	Very strong inhibition at even low concentrations (below 50m gl^{-1})	Slight decrease
Sodium	Potentiation of activity	Very strong increase
EDTA	Same degree of reduction in activity between 0.2 and 1.0 gl^{-1}	Slight reduction
Sulphate	Slight increase at lower concentrations, then a weak inhibition	No effect

5.4 Discussion and Conclusion

5.4.1 Discussion

Although the permissible limit for total alkalinity was not provided, the pH value was within the 6 and 9 range, thus suggesting that the total alkalinity was acceptable.

Ferulic acid is a phenolic compound produced during lignin degradation, and its inhibition of the enzyme activities would imply that the use of the direct enzyme assays in

eutrophic water bodies may yield a false negative result. In such water bodies, there will be heavy decomposition of plant matter releasing this phenolic compound.

Enhancement of enzyme activities by carbonates could point to the existence of phenolic compounds in the environmental water. Carbonates chelate the phenolic compounds, thereby preventing their interaction with enzymes (Wetzel, 1991). Therefore, the impact of phenolic compounds in hard waters may be countered by the carbonates. The pattern of activity observed with carbonate and ferulic acid may be explained by this fact that carbonates are able to bind to the phenolics. However, the increase in pH caused by carbonate can also lead to an exaggerated positive result when phenolic compounds are absent in the water samples.

The presence of cadmium arrested the activity of the enzymes, thereby interfering with this enzymatic technique. However, the toxic effect of cadmium on living systems will also result in the absence of the microbes. Ferric chloride, which is used in water treatment, may present serious problems when a direct enzyme assay method is adopted. Spontaneous PNPG hydrolysis can result in a false positive if this compound is present in the water supply system. However, the reduction in pH would also suggest that ferric chloride can inhibit the enzyme activities.

Tryland et al. (1997) observed that low concentrations of chlorine and divalent cations such as magnesium increased GAL activity. In this study, however, the addition of sodium hypochlorite resulted in an inhibition of the activity of both environmental and commercial GUD and GAL. Sodium chloride at the studied concentrations did not have any noticeable effect on GAL, but potentiated the activity of GUD up to 200 mgl^{-1}, after which it inhibited the enzyme activity in the environmental water sample. Magnesium, however, increased the activity of GAL over 100 fold at a concentration of 0.4 gl^{-1} and above.

The weak inhibitory effect of the common compounds found in water, like potassium and sulphate, helps to explain why the environmental water did not strongly inhibit the commercial enzyme activity. However, the activity of GAL is underestimated by about 15 %, due the slight inhibitory effect it has on the commercial enzyme, while the GUD activity obtained in stagnant water represents the true GUD activity of the water sample.

5.4.2 Conclusion

Several compounds in the environment can potentially affect GAL and GUD activities. Results should therefore be stated with caution, especially when working with water, which could potentially contain any of the studied metal ions. There may be a need to remove some of these compounds prior to assaying for GAL and GUD. The possibility of obtaining false positive or negative readings in the enzymatic detection of GUD or GAL in contaminated water should be cautiously anticipated and verified using more traditional microbiology assays. The possibility of using an internal reference or standard (i.e. commercial GAL or GUD) should also be explored in order to assess and correct for chemical (ion) interference.

6 LIQUID BIOPROBES AND MONTHLY (SEASONAL) VARIABILITY

6.1 Introduction

Correlations between faecal coliforms, GUD and GAL activities have previously been reported (Farnleitner et al., 2001, 2002). This linear correlation provides the basis for the establishment of a liquid bioprobe where direct enzymatic assay of the sample in question is performed. However, the extent of the linear relationship varies with location and degree of pollution of a water body. It was therefore necessary to confirm this linearity so as to ascertain the applicability of the direct chromogenic substrate assay to our particular situation.

Enzyme activity and quantity in aquatic environments varies by depth (Hoppe, 1991), time of the day and season. Depth variations are influenced by the nature of occurrence of the enzyme. Some enzymes are immobilised onto sediments and settle on the river bed while others may be free floating, hence found uniformly throughout the water column.

Major determinants in daily and seasonal variations are temperature and volume of runoff. Warm temperatures enhance microbial growth, increasing enzyme production and activity. In addition, increases in temperature speed up degradation of organic compounds thus providing nutrients for the microbes leading to more enzyme activity. Therefore, warm seasons may exhibit high enzyme activity and quantity. Increases in temperature allow the mixture of stagnant water bodies by convection currents. During storms, runoff introduces allochthonous material into the water bodies. Depending on the quality and quantity of such material, enzyme activity and quantity may be enhanced or reduced. Such a variation has bearing on the time of sampling and determination of faecal material. Human activities along the course of the river (e.g. washing and bathing) can also potentially affect the quantity and rate of enzyme activity.

The work in this chapter is aimed at establishing a liquid bioprobe and investigating the monthly (seasonal) trends in activity of the two enzymes. The establishment of bioprobes was achieved through a combination of information from the previous chapters, confirmation of linearity, as well as determination of the limits of detection (LOD).

6.2 Materials and Methods

6.2.1 Regression studies and comparison with the MF technique

Water samples were collected from different environments in the Eastern Cape area (river mouths of Kariega, Bushmans and Sundays rivers, a marshy area around the Swartkops estuary near PE, final effluent from Port Elizabeth waste water treatment plant, marine environment and freshwater bodies around Grahamstown). Microbial counts by membrane filtration (MF), as outlined by Farnleitner et al. (2001), were adapted and performed on Oxoid CM 1046 and m-FC agar (Biolab Diagnostics, South Africa). Direct GUD and GAL assays for identical samples were also performed. Regression analyses were performed for microbial counts on enzyme activities. From these data sets, a comparison of the direct enzyme assays method and MF CFU determination was performed to determine the efficiency and applicability of each of these two methods.

From the regression curves, the LOD was determined. Commercial GUD and GAL were diluted and their visual and spectrometric limits of detection were established. The visual limit of detection was scored as the lowest enzyme concentration that gave a visible colour change within 24 h, while the spectrometric detection limit was the lowest enzyme concentration that gave a measurable change in absorbance in the same time period.

6.2.2 Monthly trends in GUD and GAL activities at the sampling points

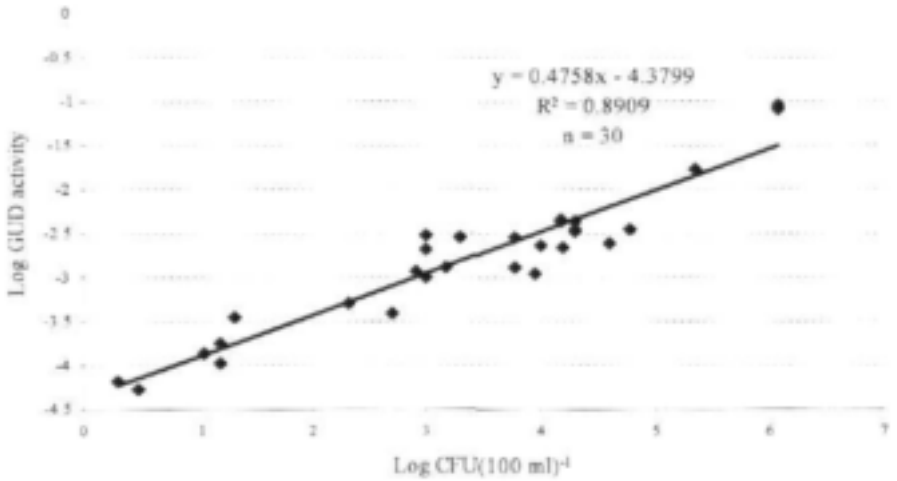
The activities of the two enzymes (GUD and GAL) from the two sampling points were assayed weekly for a period spanning over a year. Monthly results were averaged and a graph of activity versus month of the year was constructed.

6.3 Results

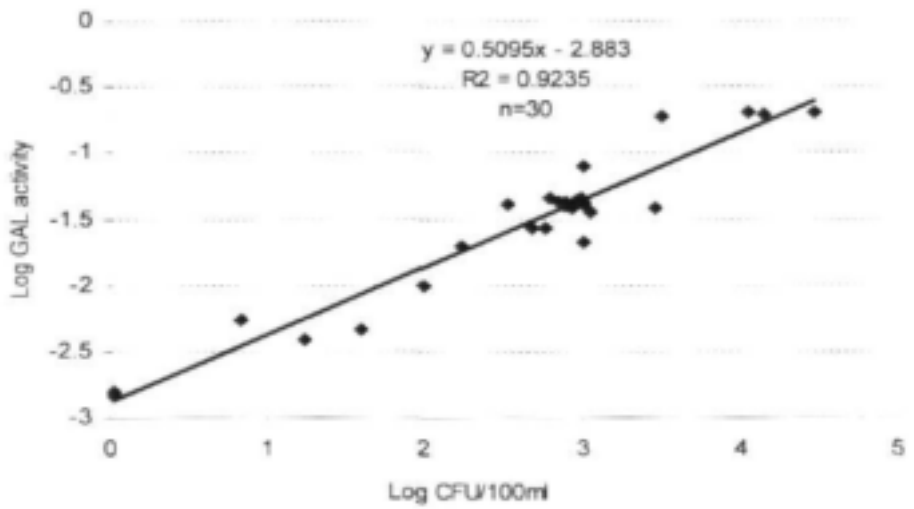
From the studies performed thus far, a liquid bioprobe can be established based on the chromogenic substrates hydrolysis by GUD and GAL. The ingredients of the bioprobe are (a) assay buffer, (b) substrate, and (c) the water sample to be examined. The detection can be achieved within 24 h with the limits of detection outlined as follows:

6.3.1 Regression studies and detection limits

There was a positive correlation between *E. coli* counts and GUD activity ($R^2 = 0.8909$), and faecal coliforms and GAL ($R^2 = 0.932$ for CM 1046 media) (Figs. 6.1a and b), even though the CFUs were not evenly distributed. From the different river mouth samples and marine environments, both GUD and GAL activities were detected together with microbial growth observed on the plates. The spectrometric LOD for *E. coli* and total coliforms were 2 CFU/100 ml and 1 CFU/ 100 ml respectively.



(a)



(b)

Figure 6.1: Regression of a) *E coli* counts vs GUD and b) faecal coliform counts vs GAL activities found in different water samples (n = 30).

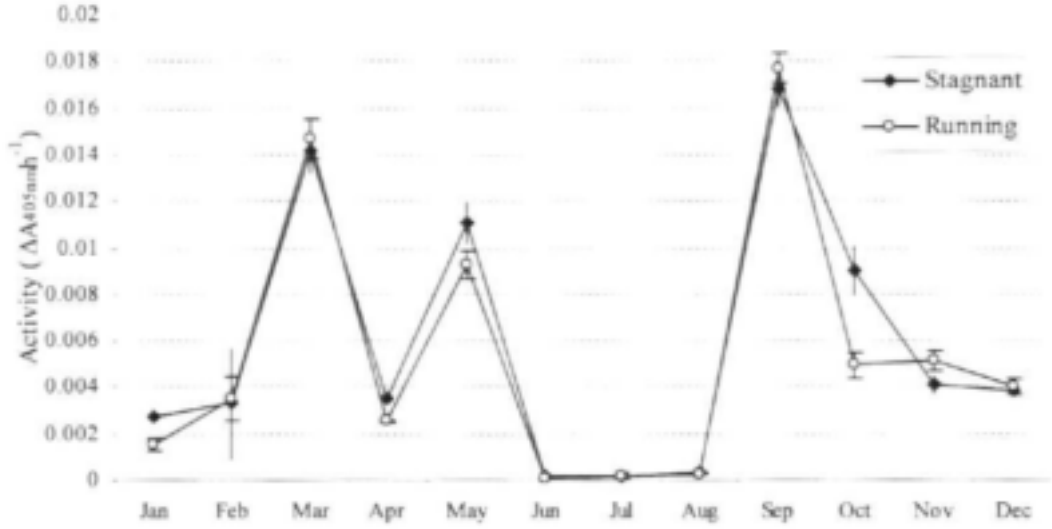
The least detectable level of commercial GUD activity was observed at 0.05 and 0.02 µg ml^{-1} , visually and spectrometrically, respectively. The former was detected after 5.5 h and the latter after 3 h (Table 6.1). Below these GUD concentrations, no change was detected, even after 24 h. The limit of detection for commercial GAL was at a value 0.01 $\mu g.ml^{-1}$ in 24 h, both spectrometrically and visually (Table 6.1). Therefore the concentrations of GUD and GAL can safely be determined above 0.02 µg ml^{-1} and 0.01 µg ml^{-1} respectively.

Table 6.1: Visual and spectrometric detection limits for GUD and GAL activities

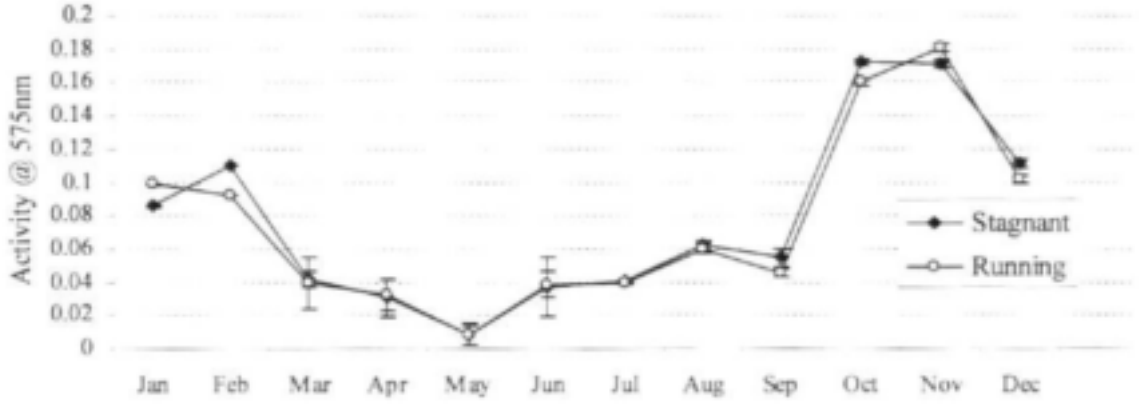
GUD			GAL	
Concentration (μgml^{-1})	**Spectrophotometric detection**	**Visual colour change**	**Concentration (μgml^{-1})**	**Visual and spectrophotometric**
60	Detectable	Instant	10	Instant
40	Detectable	Instant	1	10 minutes
20	Detectable	Instant	0.5	30 minutes
10	Detectable	Instant	0.2	6 h
5	Detectable	Instant	0.143	12 h
2	Detectable	Instant	0.1	12 h
0.5	Detectable	20 minutes	0.05	24 h
0.2	Detectable	40 minutes	0.025	24 h
0.05	Detectable	5.5 h	0.0166	24 h
0.02	3 h	No change after 24 h	0.0125	24 h
0.005	No change after 24 h		0.01	24 h

6.3.2 Monthly (Seasonal) trends

Generally, the period of June to August (i.e. the winter months) was found to show low enzyme activities with June and July having the lowest GUD activity (Fig. 6.2a). The lowest activity for GAL was recorded in May, while highest activity was in November (Fig. 6.2b). The highest GUD activity was observed in September. The GUD trend exhibited three peak periods (March, May and September) while GAL showed two peak periods (Fig. 6.2a) and GAL also exhibited two (October – November, and February).



(a)



(b)

Figure 6.2: Monthly GUD (a) and GAL (b) activity variations at the two study sites. All values represent the mean ± SD (n=3).

6.4 Discussion and Conclusion

6.4.1 Discussion

The 24 h period of detection allows enough time for noticeable changes in environmental enzyme activity that can initially be inhibited by the different pollutants. While increased temperatures can give more rapid results, such temperatures have been found to increase interference by pollutants (data not shown). The observed detection time compares well with the improved conventional methods (Rompré et al., 2002; Venter, 2000) and the Colilert methods that make use of fluorogenic substrates (Berger, 1994; Cowburn, 1994;

Fricker and Fricker, 1996; Grant, 1997; Sartory and Watkins, 1999), while the LOD is in our assays is comparable to the conventional methods of culturing.

The observed correlation for both enzymes agrees with the results ($0.73 < R^2 > 0.96$) obtained by Farnleitner et al. (2001). Generally, it should be difficult to obtain a high correlation in different environments, considering the VBNC bacteria and their degree of viability that may vary with water pollutants and nutrient concentrations. However, correlation coefficient of above 0.8 suggests positive correlation between the organisms in question and the activity of their respective marker enzymes. The finding that GUD and GAL activities were present in all samples highlighted the ability of direct enzyme assaying methods to detect enzyme activity of VBNC cells. This makes the technique more favourable and potentially more applicable to marine environments. While results of 1 and 2 CFUs 100 ml^{-1} elucidate an extremely sensitive technique, one should cite them with caution because it does not necessarily mean that the 2 or 1 CFU are entirely responsible for the observed activity. Low LOD could also be as a result of enzyme activity from non-target sources. Van Poucke and Nelis (1997) highlighted the paradoxical nature of this concept of sensitivity of direct enzyme assay to the level as low as 1 CFU per 100 ml water sample. They argued that an increase in sensitivity is coupled with a concomitant increase in susceptibility to interference from non-target sources.

The correlation between microbial counts and enzyme activities illustrates the degree of accuracy of the MF technique and the direct enzyme assays. If the two methods gave the same results with the same degree of accuracy then the correlation coefficient would be equal to one. However, the issue of detection of VBNC bacterial enzyme activity could potentially account for the deviation of R^2 from 1. The poor correlation between enzyme activity and CFUs in marine samples highlights the possible limitation of this technique to fresh water samples.

The enzyme activity is partly a function of the amount of enzyme present and partly of inhibitor concentration. Low enzyme activity may also be attributed to a high level of enzyme dilution. Higher activity obtained during warm periods may be explained by high microbial activity under such temperatures and mixing aided by convection currents.

6.4.2 Conclusion

It can safely be concluded that the concept of direct enzyme assay, using the chromogenic substrates PNPG and CPRG for GUD and GAL assays, respectively, is a feasible alternative method for the detection of faecal pollution. However, there is a need to investigate the possibility of immobilising the substrates on a solid support to enable ease of handling and prolonged shelf life for bioprobe design.

7 SUBSTRATE IMMOBILISATION

7.1 Introduction

The concept of immobilizing proteins, enzymes and ligands to insoluble supports has received lots of attention over the years. A number of membrane-based rapid chromographic devices are readily available. Major contributing factors to the high rate of development of these chromographic devices include improvements in conjugate technology and a growing understanding of the principles behind the immobilisation procedures. The amine groups on proteins are the most frequently used moieties used for covalently immobilisation. In most cases, pre-activation of these amines is not required, as they are readily available on the surfaces of the proteins.

Aldehydes, glutaraldehydes, epoxy, cyanogen bromide, tosyl are but a few of the reactive groups employed in the immobilisation of proteins. These reactive groups are able to yield highly stable enzyme-membrane bonds. However, a disadvantage of using these reactive groups is their instability to alkaline pH's.

Immobilisation strategies include covalent attachment with or without reactive groups, entrapment in gels, adsorption on materials such as zirconium, ion exchange resins, acrylic polymers, emulsion membrane reactors, nylon fibers, silica (Bayramoglu et al., 2004) and nitrocellulose (Steinitz and Tamir, 1995). A particularly convenient immobilisation matrix is nylon. It is inexpensive, inert, non-toxic, readily available and can be obtained in a number of forms (Isgrove et al., 2000).

The availability of a large number of support materials and methods of enzyme immobilisation leave virtually no bioactive species without a feasible route of immobilisation (Bayramoglu et al., 2004). Unmodified nylon surfaces are hydrophobic and can lead to substantial non-specific hydrophobic binding of ligands or proteins, which may lead to reduced purification efficiencies or leakage into products, hence contamination, as well as reduced bioreactor performance (Isgrove et al., 2000)
The stability and activity of immobilised compounds e.g. enzymes, are determined by the changes in chemical nature/conformation of the immobilised compound that are influenced by the reaction schemes involved and the microenvironment provided by the

supporting material (Su et al., 2005). Generally speaking, immobilisation improves stability and catalytic efficiency of the immobilised molecules.

While vast accounts of protein immobilisation protocols exist, there is limited information on non-protein based molecule immobilisation. Bearing in mind the advantages offered by immobilising molecules, this chapter explored attempts to immobilise GUD and GAL substrates on a solid support for a colour strip development.

7.2 Materials and methods

7.2.1 Physical adsorption on nitrocellulose and nylon membranes

Briefly stated, nitrocellulose strips measuring approximately 30 mm x 10 mm were incubated in CPRG and PNPG overnight. Different concentrations of commercial GAL and GUD were then applied to the immobilised strips and colour changes monitored over time.

7.2.2 Immobilisation onto Nylon

CPRG and PNPG were immobilised on nylon according a modification of the protocol described by Isgrove et al. (2000). Nylon film (with a thickness of approximately 50 μm) was cut into 10 mm x 10 mm segments, partially degraded by incubation in 2.9 M HCl for 2 h at 37 °C, washed thoroughly in water followed by 0.1 M Na phosphate buffer, pH 7.8 (for CPRG) or 0.1M Tris HCl (for PNPG) buffer (pH 8.0). The strips were then incubated separately in CPRG and PNPG solutions overnight at 4 °C and air-dried. Varying concentrations of commercial GAL and GUD were then applied to the finished strips and the colour changes were observed over time.

7.2.3 Colour scoring

Colour scoring with Adobe Photoshop 8.0 (Adobe Systems Incorporated, USA) was performed on the immobilised strips after reaction with GAL and GUD. Colour units were taken on the RGB colour scoring chart on per the display screen. The difference between the reading on a control strip and the coloured test strip was taken as the colour score.

7.3 Results

7.3.1 Physical adsorption

Colour changes were observed after addition of enzyme. However, there was rapid diffusion of the chromogen from the support material upon addition of the enzyme solutions.

7.3.2 Immobilisation on partially degraded nylon membrane

A noticeable visual colour change was observed within 20 min upon the addition of a drop of 5 μg ml^{-1} concentration of commercial GUD (Fig. 7.1a). Higher concentrations of commercial GUD produced instant colour changes. In the case of GAL, the 10 μg ml^{-1} solution resulted in an instant colour change from yellow to red while concentrations at 2.5×10^{-2} μg ml^{-1} resulted in colour changes within 24 h (Fig. 7.1a and Table 7.1). The visual limit of detection (LOD) for GUD was 5 μg ml^{-1} in a period of 20 min, while for GAL it was 0.143 μg ml^{-1} in 1 h (Table 7.1). Very limited colour diffusion from the support material was noted after addition of enzyme solutions (Fig. 7.1).

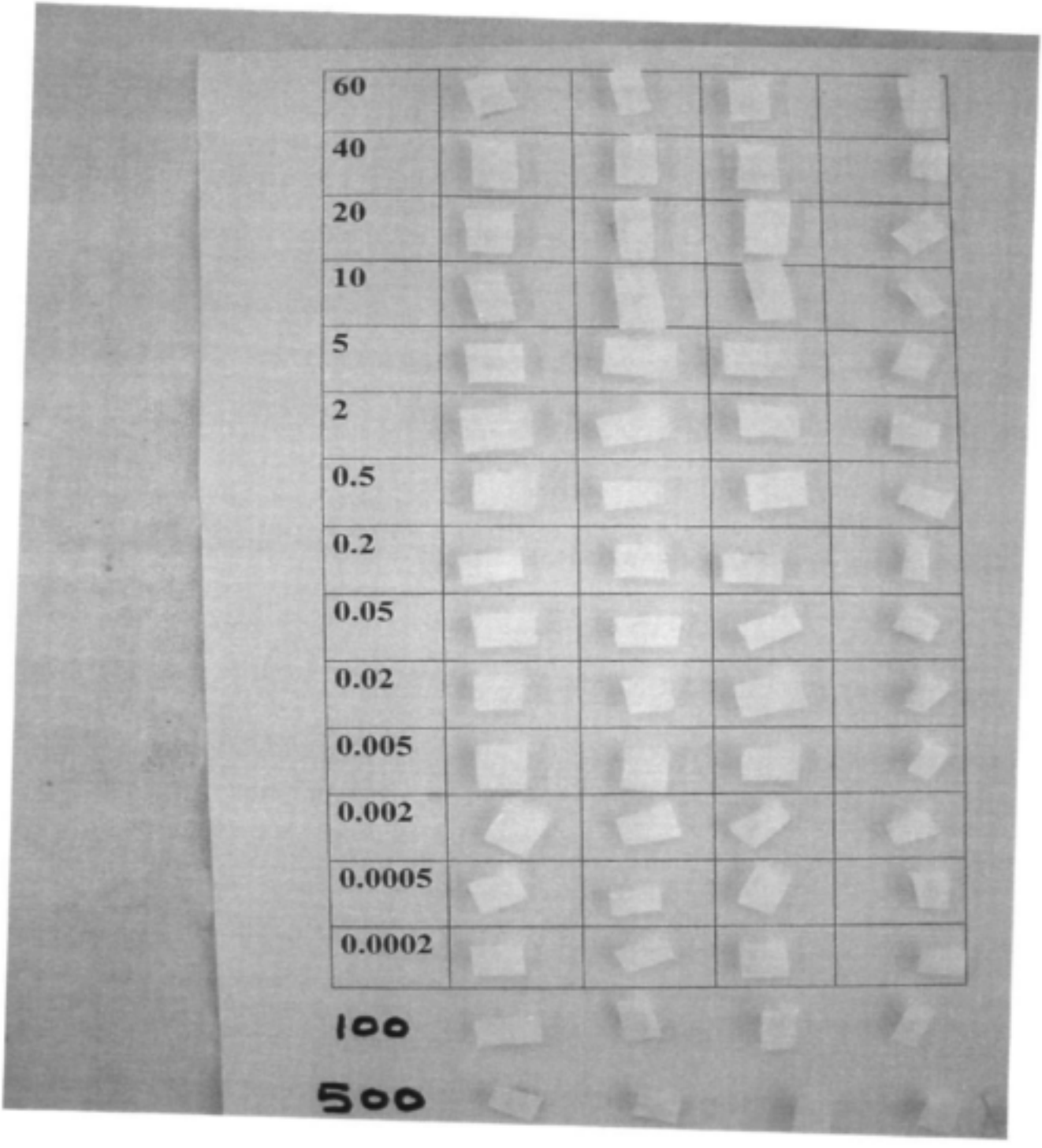


Figure 7.1a: Colour changes on immobilised PNPG hydrolysis by different respective commercial enzyme concentrations ($\mu g\ ml^{-1}$)

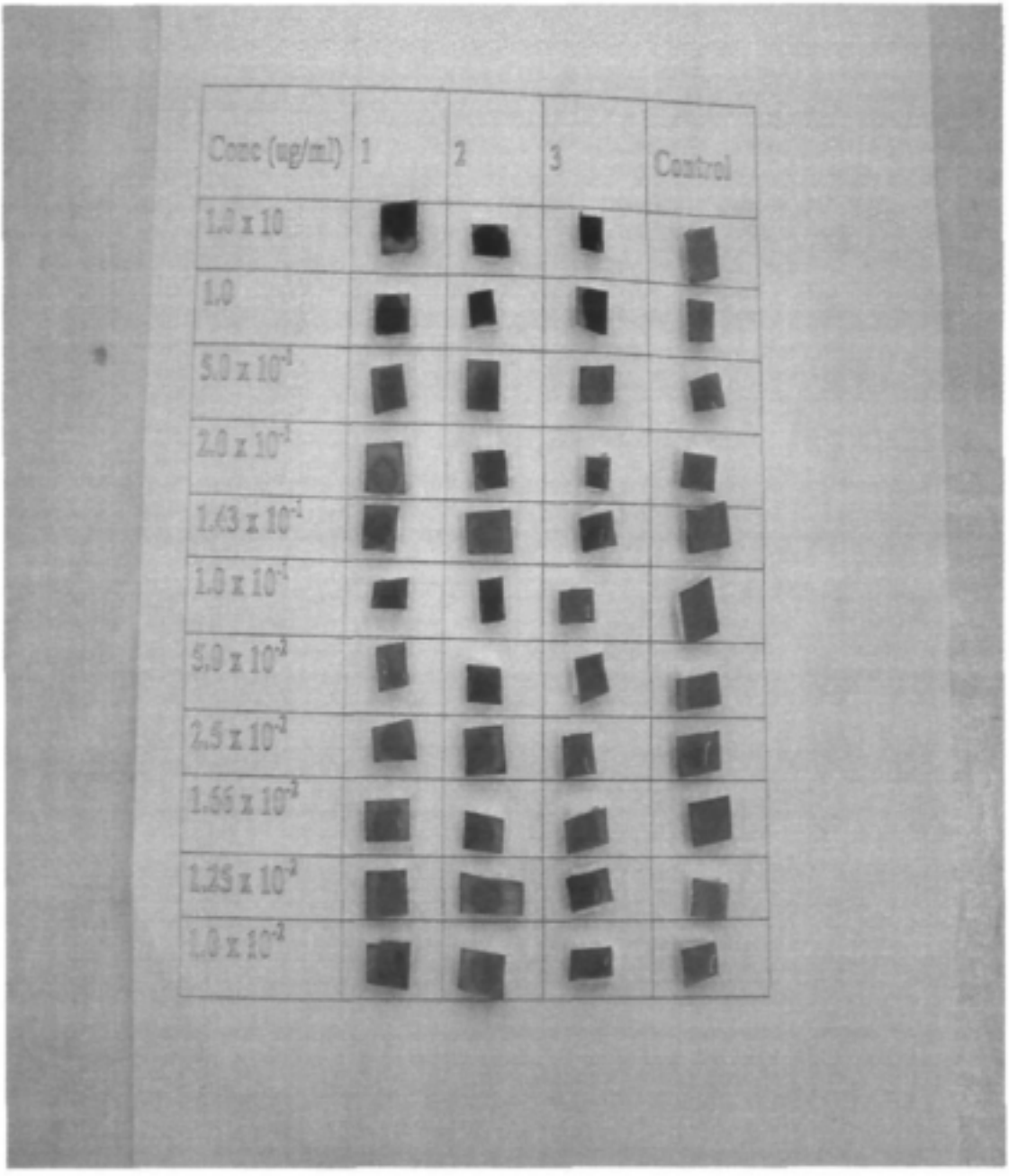


Figure 7.1b: Colour changes on immobilised CPRG hydrolysis by different respective commercial enzyme concentrations

Table 7.1: Detection times of GAL on CPRG immobilised strips.

Conc ($\mu g\ ml^{-1}$)	0 min	10 min	30 min	1 h	3 h	6 h	12 h	24 h
1.0×10	x							
1.0		x						
5.0×10^{-1}				x				
2.0×10^{-1}				x				
1.43×10^{-1}				x				
10×10^{-1}							x	
5.0×10^{-2}							x	
2.5×10^{-2}								x
1.66×10^{-2}								x
1.25×10^{-2}								x
1.0×10^{-2}								x

7.3.3 Adobe colour scoring

The limit of detection with the Adobe Photoshop colour scoring for GUD was 0.05 $\mu g\ ml^{-1}$. A significant ($P<0.05$) correlation ($R^2 = 0.9594$) was observed between the colour change and the concentration of GUD (Fig. 7.2). The range of linearity of GUD concentration and colour change extended from 0.05 to 100 $\mu g\ ml^{-1}$.

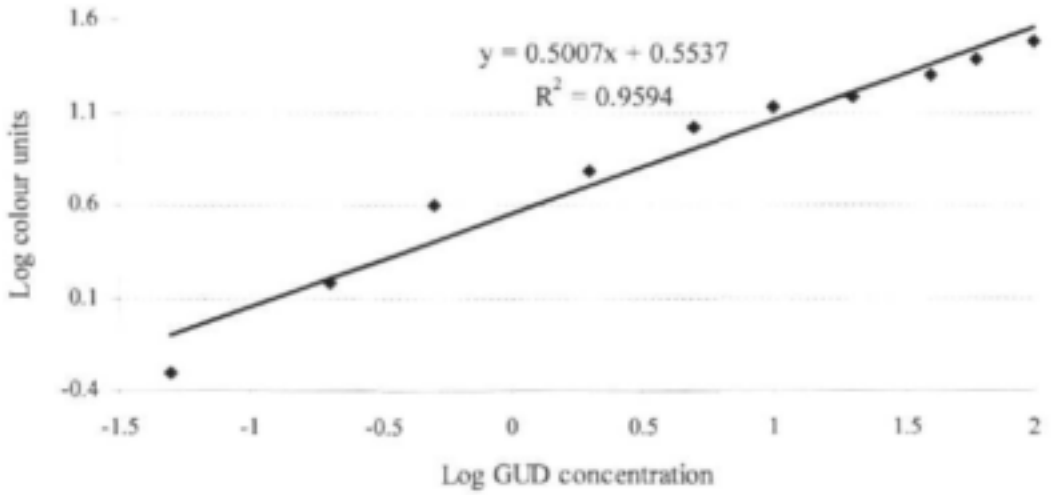


Figure 7.2: Double log plot for Adobe colour units versus GUD concentration on hydrolysis of PNPG immobilised on partially degraded nylon membrane

7.4 Discussion and Conclusion

7.4.1 Discussion

The extent of stability depends on the structure of a compound, the immobilisation method and the type of support (Bayramoglu et al., 2004). From our results, it is clear that the type of membrane greatly influenced immobilisation. The membranes, although resulting in a reasonable degree of immobilisation, allowed the diffusion of the CPRG and PNPG upon addition of the respective enzymes. It is therefore evident that physical adsorption is not desirable for PNPG and CPGR immobilisation. The observed diffusion in colour of the chromogen does not allow for accurate colour observation on the strip. In addition, this strip will require a limited exposure time to the enzyme solutions, which may not be adequate to allow enzyme hydrolysis of the substrates in environments characterised by low enzyme levels. Desorption is common in physically adsorbed molecules (Isgrove et al., 2000).

The nylon strips yielded good results with very limited diffusion that can be attributed to saturation of the functional groups on the membrane. The partial degradation of the nylon exposed functional groups that react with both the substrates and their chromogenic products yielding more stable bonds as opposed to physical adsorption alone. Additional modifications of the nylon membrane can produce highly stable bonds with the substrates enabling the combination of the two substrates on the same strip.

7.4.2 Conclusion

Immobilisation of the CPRG and PNPG substrates on *modified* nylon membrane is feasible. However, physical adsorption on both nylon and nitrocellulose membranes is however not feasible due to desorption of the substrates from the solid support.

8 PROOF OF CONCEPT: ELECTROCHEMICAL DETECTION AND BIOSENSOR DESIGN

8.1 Introduction

Based on the findings of chapters 1 to 7, the idea of using an electrochemical biosensor for the rapid detection of faecal contamination via the marker enzymes (GUD and GAL) of the indicator microorganisms *E. coli* and coliform bacteria appeared to be feasible. Enzyme-based analyses also afford more rapid detection. Chromogenic enzyme substrates were better suited for use in the environment compared to their fluorogenic counterparts. Results in Chapter 2 showed that cell permeabilisation was not necessary for *in situ* assays, allowing for the development of a direct biosensor-based assay in the environment. Optimum pH (pH 7-8) and temperature values for the enzymes were close to that of water intended for drinking purposes, further supporting the feasibility of using a biosensor for the rapid detection of faecal contamination in this water. In chapters 6 and 7, the feasibility of using liquid or immobilised bioprobes for the rapid detection of faecal contamination was indicated, finally paving the way forward to the development of an appropriate electrochemical biosensor for the rapid detection of faecal matter in water intended for drinking purposes. This chapter discusses this electrochemical detection and biosensor design, and establishes proof of concept.

Enzyme activity can be monitored using different techniques following either substrate disappearance or product accumulation. Examples of major categories of enzyme assay techniques include photometric, radiometric, chromatographic, electrophoretic and electrochemical monitoring. Electrochemical monitoring involves different methods, which include voltammetry, that are based on the interplay between the chemistry of molecules of interest and electricity.

Voltammetry consists of a group of methods that measure current as a function of applied voltage/potential (Skoog et al., 1996; Wang, 1994). Potential is applied to the sample using the working and reference electrodes. The resultant current is measured using the working and a counter/auxiliary electrode. The analyte is either oxidised or reduced at the working electrode, whose potential is altered linearly with the course of the experiment. Working electrodes can be made up of different materials and in this work a

glassy carbon electrode (GCE) was used. A counter electrode conducts electricity from the source through the solution to the working electrode, while the reference electrode has a constant potential throughout the experiment (Skoog et al., 1996).

Voltammetry is one of the most common techniques used in electrochemistry. Classification of voltammetric techniques is based on the mode of potential application. Examples of the classes include linear sweep, stripping and cyclic voltammetry (Skoog et al., 1996; Wang, 1994). In cyclic voltammetry, the potential is changed as a linear function of time from start to final potential and then reversed to the start potential (Skoog et al., 1996). The current generated depends on the number of electroactive groups on the molecule and is directly proportional to the concentration of the analyate. This technique is similar to spectrometry in that the potential can be equated to the wavelength and current to absorbance. Each electroactive species has a characteristic potential where it produces a maximum current. This is equivalent to maximum absorbance at a certain wavelength by each solution.
Most biosensor signal transductions are based on electrochemical techniques. Electrochemical transduction is preferred because it can be performed with simple, compact and easy to transport equipment. The techniques are readily adaptable for on-line monitoring, rapid and amenable to miniaturisation (Paitan, et al., 2003; Skoog et al., 1996). Electrochemical assays are known to be highly sensitive and reproducible. Another advantage of electrochemical monitoring is that results are not easily affected by solution turbidity as opposed to photometric assays (Scouten et al., 1995). Simultaneous measurements of several samples can be achieved through the use of electrochemical methods. Above all, cheap disposable electrodes may also be used.

In view of the highlighted advantages of electrochemical monitoring, this chapter deals with an investigation of the potential for employing these techniques for GUD and GAL monitoring to improve sensitivity, speed of assay and reduce interferences. In order to achieve this, it was necessary to confirm detection of substrate breakdown products (PNP and CPR) at the pH values used for enzyme assays and investigate any interference by their respective substrates (PNPG and CPRG). Attempts to eliminate substrate interference on PNP and CPR detection were performed using a Nafion® membrane.

8.2 Materials and Methods

8.2.1 Electrochemical detection of substrates and substrate products

The electrolyte solution was made up of the buffer used in the respective enzyme assays i.e. 0.1 M Tris–HCl (pH 8) (for PNP and PNPG), Na_2PO_4 (pH 7.2) (for CPR, CPRG and the compounds of interest) and made up to a final volume of 10 ml. A GCE was used against a $Ag/AgCl_2$ reference electrode with platinum as a counter electrode. After an appropriate concentration of substrate or chromogen was added, nitrogen was bubbled in for 10 minutes to remove oxygen and the electrochemical measurements (using cyclic voltammetry) were performed using a computer assisted Autolab pontentiostat at the scan rate of $100mv.s^{-1}$. The measurements were performed in both oxidation (0 to 1.5 V) and reduction (0 to -1.5 V) windows for all solutions.

To study the effects of the substrates on the detection of their breakdown products, mixtures of PNP and PNPG, CPR and CPRG were made, each analyate present at a final concentration of 1 000 µM in the 10 ml solution. Voltammetric measurements were performed and the voltammograms compared with those for individual pure solutions as shown in Figure 8.1.

8.2.2 Nafion® studies

To evaluate the feasibility of using Nafion® to eliminate interference by the substrates, the GCE was coated with different Nafion® concentrations (1 – 5 %, v/v) and used in the electrochemical measurements. The Nafion® was diluted in organic solvent (1:1; water: methanol) and 25 µl of the solution dropped on the GCE surface. This was allowed to dry at room temperature (20 ± 2 °C) (for 20 – 30 min) before using the working electrode. Voltammetric measurements were performed on separate substrates, products and the mixed solutions. A new coating was applied on the working electrode in each case. Results were compared to those obtained using unmodified electrodes.

Investigation in the durability of the coating when exposed to repeated electrochemical measurements was performed by running the experiment using the same electrode without changing the coating.

8.3 Results

8.3.1 Linearity ranges and interferences

PNP was detected both by oxidation (ca 0.95 V) and reduction (ca -0.77 V), although the reduction peaks were about four times greater than the oxidation ones. PNPG was detected by reduction at around -0.90 V and could not be detected by oxidation. Electro-reduction of the PNP-PNPG produced a synergistic peak at about -0.89 V (Fig. 8.1). Detection was linear from 100 to 1000 μM for both PNP and CPR. The CPR was oxidised at around 0.72 V, CPRG at 0.69 V and CPR mixed with CPRG at 0.71 V. Reduction of CPR and CPRG gave multiple peaks that were closer to one another. Both PNPG and CPRG had peak currents at the same values as their breakdown products (chromogenic) under reduction (Data for PNPG and PNP shown in Figs. 8.1 and 8.2). Synergistic peaks were also observed with CPR and CPRG. However, under oxidation PNPG did not produce the peak but in a mixture it slightly reduced the PNP peak height.

8.3.2 Attempts to eliminate interferences

Nafion® partially eliminated PNPG and CPRG interferences, but reduced the overall sensitivity of the PNP and CPR detection (Figs. 8.2, 8.3 and 8.4). PNP, PNPG and the mixture were reduced by 52.47, 96.48 and 65.83 %, respectively, during electro reduction when 5 % Nafion® was used (Fig. 8.3a). CPR and CPRG peaks were also reduced during oxidation (Fig. 8.3b). Diluting the Nafion® to improve sensitivity also increased the interference by the substrates (Fig. 8.3).

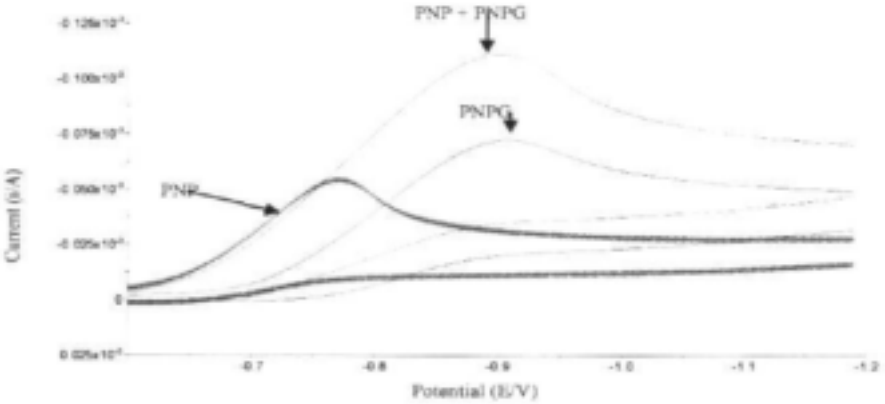


Figure 8.1: Voltammogram illustrating typical detection interference of the product (PNP) by the substrate (PNPG) during reduction

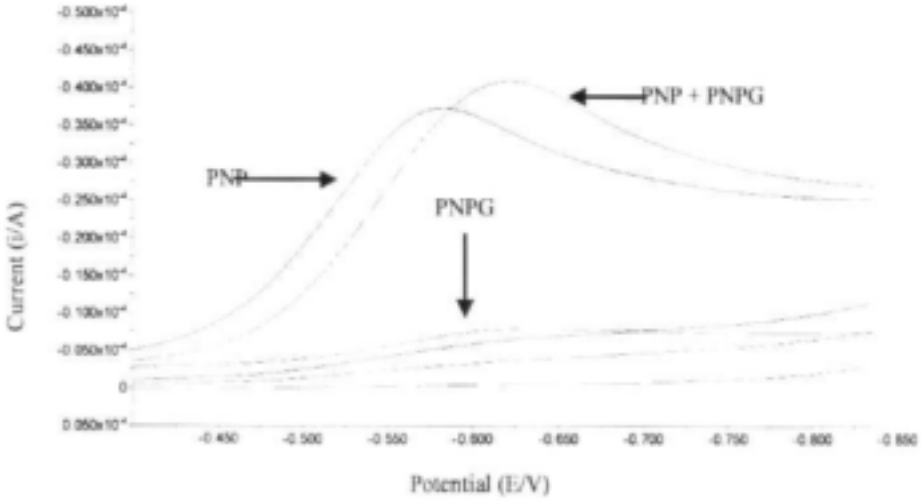
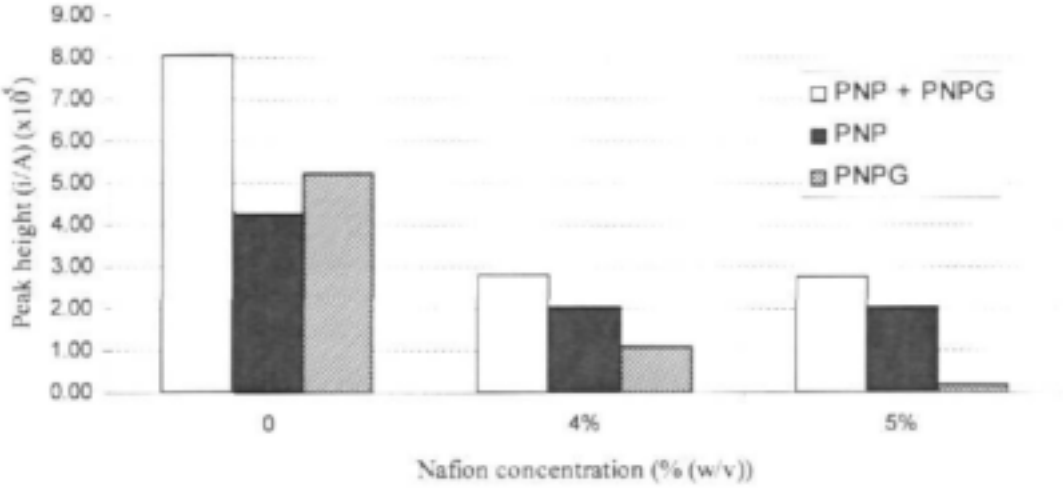
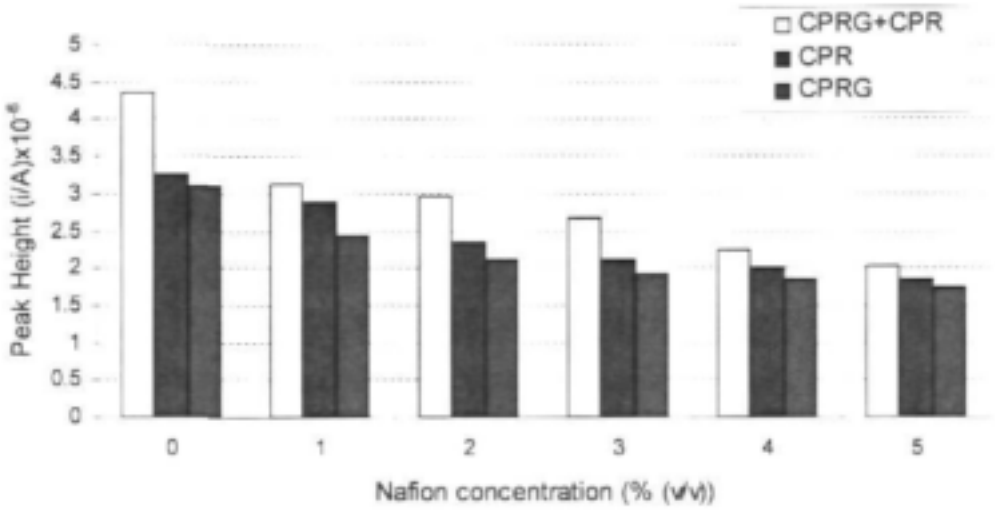


Figure 8.2: Typical voltammogram showing of sensitivity reduction and PNPG partial elimination by Nafion®



(a)



b)

Figure 8.3: Reduction in peak sizes (sensitivity) in PNP, PNPG and PNP-PNPG mixture reduction (a) and oxidation of CPR, CPRG and CPR-CPRG mixture (b) before and after coating the GCE with Nafion®

Nafion® membrane fouled chemically with repeated measuring cycles. The huge drop in current was observed between the first and second reading, after which the reduction was almost constant (Fig. 8.4).

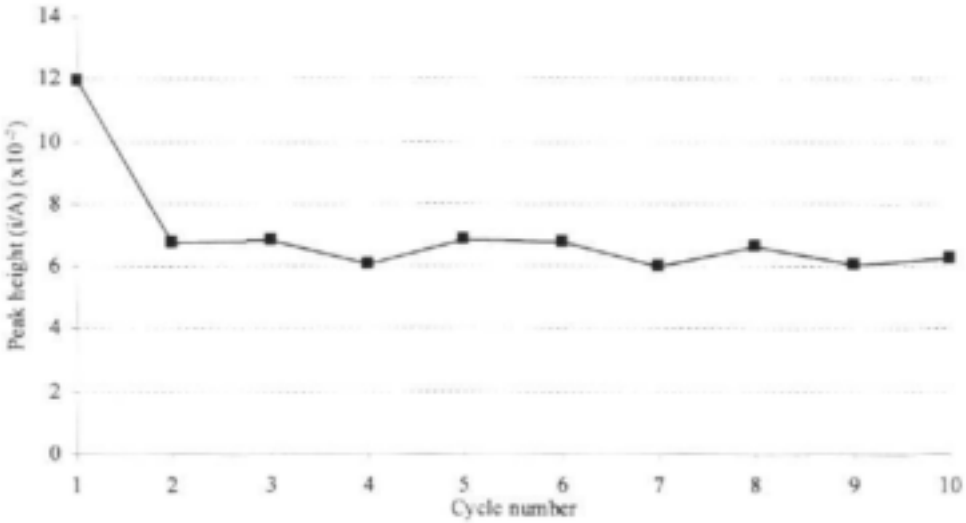


Figure 8.4: Progressive reduction in peak heights due to Nafion® fouling

8.4 Discussion and Conclusion

8.4.1 Discussion

Potential for peak currents in oxidation of PNP differed from the 0.3 V reported by Lindgren et al. (1999), while that for reduction was within the range observed in literature. Reduction potentials for PNP were reported as -0.78 V (Hu et al., 2001), -0.76 V (Calvo-Marzal et al., 2001) and -0.7 V (Luz et al., 2004). Differences in the potentials can be attributed to the pH of the electrolyte, buffer type, electrode modifications and the types of electrodes used. The LOD for PNP was higher than the 1.44 µM reported by Hu et al. (2001) using a chemically modified GCE. To our knowledge this constitutes a first report on electrochemical detection of CPR.

Differences in peak heights for the same compound between reduction and oxidation, as observed for PNP, is attributed to differences in the electro active groups involved and the number of electrons transferred. During reduction of PNP the nitro group is the electroactive component and is reduced to an amine with PNP becoming a *p*-aminophenol. This reaction is coupled with a transfer of six electrons (Calvo-Marzal, et al., 2001; Cheah et al., 1965). During PNP oxidation, the hydroxyl group becomes electroactive and is oxidised to produce a phenoxide or quinoline like group. The oxidation involves transfer of one electron (Zvimba, personal communication). Thus the more electrons are involved, the greater the peak size. Therefore, reductive determination

of PNP is more sensitive than the oxidative determination. Multiple peaks and interference noted in CPR and CPRG reduction makes it difficult to monitor the activity by reduction. Therefore, oxidation will be more desirable due to the simplicity of result interpretation and reduced interference.

Substrate interference with product detection is undesirable. Nafion® is a cationic membrane and acts both by charge repulsion and size exclusion (Calvo-Marzal, et al., 2001). Calvo-Marzal et al. (2001) successfully eliminated the interference of *p*-nitrophenyl phosphate on PNP detection using 5 % Nafion®. However, the attempts in our studies were not met with the same success. Reduction in sensitivity to PNP detection by Nafion® was also noted by Calvo-Marzal et al. (2001), but not to the extent that was observed in this study. Decaying of the Nafion® with each progressive measurement will translate to high costs and an inefficient system as the coating has to be performed for each measurement. This makes the implementation of Nafion® for the elimination of interference problematic, unless the sensitivity can be improved. Even without using the Nafion®, the 50 µM PNP that was not detected by electrochemical measurement showed a change in absorbance of 0.65 at 405 nm. Therefore further exploration of methods for the improvement of sensitivity is inevitable in order to maximally exploit this technique.

8.4.2 Conclusion and SFIA design

It is possible to detect GUD and GAL activity electrochemically, but the improvement of sensitivity will increase the feasibility of the technique greatly. From the Nafion® studies it can be concluded that the membrane excludes more substrate than products, but reduces the overall sensitivity of the products. Separation was therefore achieved at the expense of chemical fouling. Detection of both PNP and CPR can be performed by oxidation without use of Nafion® but the sensitivity still needs to be improved.

On-line and real-time monitoring for these enzyme activities may probably be best performed using a sequential flow injection analysis (SFIA) system (Fig 8.5). This system has the advantages of simultaneous detection of the two marker enzymes and a possibility of using both electrochemical and photometric detectors simultaneously. Based on the information obtained in this study, a design for a potential SFIA system (Fig. 8.5) has

been drafted for this type of analysis. In our customized design there is an added advantage of optional pre-concentration and inhibitor removal before assaying.

The specifications of our customized design are as follows:

1. Six inlet ports
2. Filtration Unit (labelled C)

   Must have a tube that retains components on molecular weight above 60 000 Da (60 KDa). It is supposed to take approximately 100 ml and after filtration must retain about 0.5 ml and the tubing should not allow protein adsorption. Waste solution must be continuously removed from the unit (possibly by suction). The filtration unit must be detachable
3. Label O

   Reaction between pre-concentrated sample and solution from tube 4 takes place in O. Mixing should take place with a temperature control option

   Entry and exit in O must be time controlled
4. Label D

   Split up of the reaction mixture into variable volumes to tubes T and V.
5. A and B
   a. Entry into reactor (F) (between A and B) must be time controlled
   b. Reactor F must be temperature controlled (20 to 60 ^{o}C)
   c. Reactants must be mixed
6. Reactant products (from 2 reactors) are taken to the spectrometer detector for simultaneous detection

The specifications of sequence of operation of system are as follows:

a) As soon as B valve outlet opens into the detector, A valve inlet should open to allow another reaction mixture into F.
b) Processing in tubes 3 and 4 through O should be independently timed such that there is always a ready sample at D to flow into T and V.
c) Inlet ports 1, 2, 5 and 6 must be individually and separately regulated (opening and closing) to allow baseline determination or correction of absorbance due to reactants from D mixed with solution from any of the four inlet ports.
d) The detection is supposed to be continuous; inlets must always contain the respective volumes of solutions.

e) There is the option of operating the 2 final reactors (labelled F) separately or simultaneously.

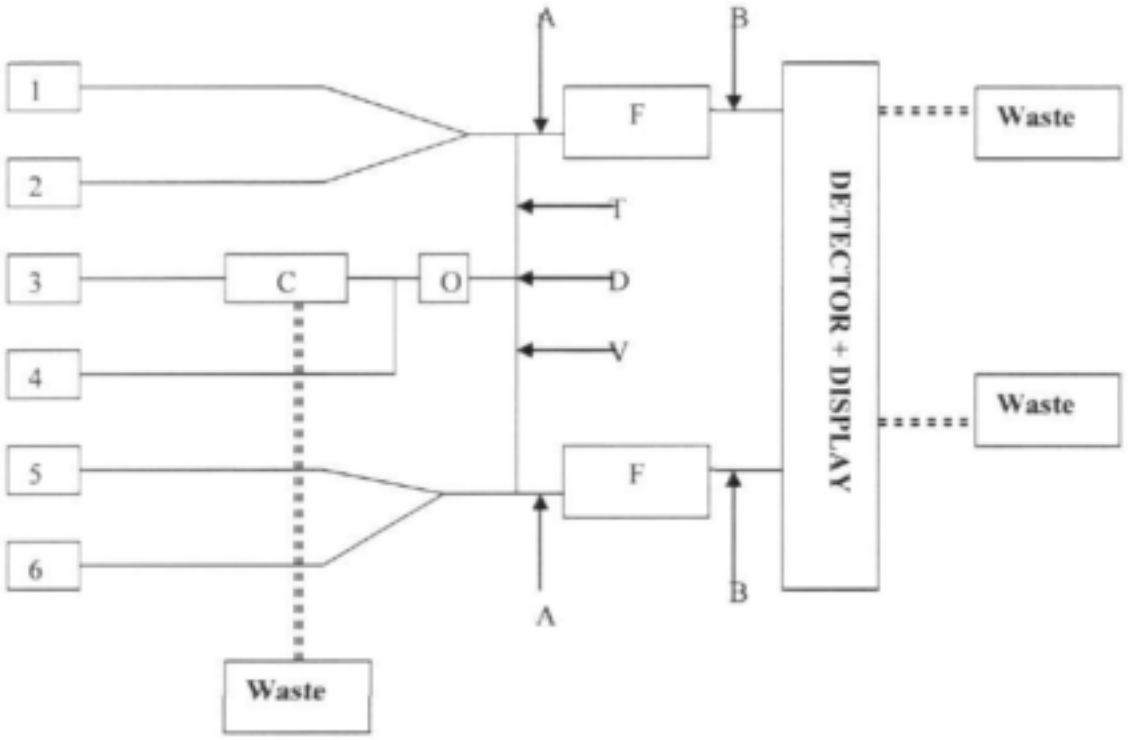


Figure 8.5: Sequential Flow Injection Analysis Equipment design

9 GENERAL DISCUSSION AND CONCLUSION

The aim of this study was to identify suitable marker enzymes, which could then be used for the detection of sewage material (faecal contamination) in water intended for drinking purposes. These enzymes had to fulfill certain key criteria in order to qualify as suitable biomarkers for this type of contamination from either human or animal origin. These criteria were as follows: a) sensitivity, b) accuracy, c) precision, d) reproducibility and e) feasibility.

An extensive literature review was conducted to investigate the current state of information in the field. A number of pathogenic organisms can be found in water. Coliform bacteria, although not pathogenic in themselves, are used to assess the microbiological quality of water, as they are present in large numbers among the intestinal flora of human and other warm-blooded animals and therefore in faecal wastes. Coliforms, and more specifically *E. coli*, are thus used as an index of the potential presence of entero-pathogens in faecally contaminated water. Enterococci, as well as *Clostridium perfringens,* can also be used to test for faecal contamination. *E. coli* and the coliform bacteria have been well characterised at the molecular and microbiological level. The genetic and enzymatic aspects are also well understood. From the literature, certain key enzymes appeared to be feasible for this study. ß-D-galactosidase (specific for coliform bacteria) and ß-D-glucuronidase (specific for *E. coli*) are two enzymes that have been identified as suitable markers of faecal contamination in water. A number of other enzymes have been shown to be implicated in studies of this kind, e.g. urease, ß-glucosidase and glutamate oxidase, but these have proved to be unlikely candidates for the reliable detection of coliforms and *E. coli* in water. The use of enzyme assays to detect coliforms presents a rapid, sensitive and less expensive alternative to the conventional microbiological analysis of drinking water, which requires laborious and time-consuming procedures. Other limitations of the conventional techniques include lack of specificity and an underestimation of the presence of VNBC (viable but non-culturable) organisms.

It was clearly evident that the two enzymes ß-D-galactosidase and ß-D-glucuronidase were the most suitable candidate markers for the detection and monitoring of coliforms

and *E. coli* in water. Suitable enzyme assays for these two indicator enzymes were therefore designed and performed. The enzyme assays were initially optimised using commercial enzymes. Results for both enzymes appeared promising and further work was undertaken to extrapolate and extend the applicability and relevance of these assays for use in the environment with river samples known to be faecally contaminated.

Assay optimisation studies were performed using a variety of chromogenic and fluorogenic substrates. The most suitable substrates were those which were relatively inexpensive, readily (commercially) available, and specific to either ß-D-galactosidase or ß-D-glucuronidase. Furthermore, chromogenic and fluorogenic substrates were used instead of natural substrates, which were relatively non-specific. Permeabilisation and fractionation experiments with pure *E. coli* cultures showed that both marker enzymes were predominantly intracellular, and were liberated into the surrounding medium upon sonication. Sonication was more effective as a permeabiliser than polymyxin B sulphate, toluene, cetyltrimethylammonium bromide, Triton X-100 or sodium lauryl sulphate. This was initially perceived as being a potential obstacle for the design of a bioprobe or biosensor (which requires the presence of free enzyme in the medium). However, subsequent experiments with contaminated river samples showed that these enzymes are spontaneously released into the environment.

The enzyme assays were optimised using pH and temperature optima experiments (physico-chemical characterisation). Temperature stability experiments were also performed at various temperatures (room temperature, cold storage at 4 °C and at the observed temperature optima) to further optimise the assays for increased sensitivity, accuracy and reproducibility.

Studies were performed to determine the effect of a wide variety of compounds on ß-D-galactosidase and ß-D-glucuronidase in environmental samples. Studies performed on commercial enzymes and pure *E. coli* cultures showed that cadmium may be an important inhibitor in these enzyme assays. Studies were also performed using environmental samples from contaminated river sources. Of the many effectors studied, cadmium, ferric chloride and sodium hypochlorite at high concentration were found to strongly inhibit the enzyme activity while calcium carbonate increased the observed activity. Nitrate, phosphate, and sulphate did not have any significant effect on the enzyme activity.

A correlation between colony forming units (CFU/ml) and the enzyme activities was established. In some cases, enzyme activities were observed in the absence of any colonies. This indicated to the obvious advantage that these enzyme assays can be used to detect VBNC (viable but non–culturable) bacterial cells

Bioprobes were also constructed by immobilising the substrates on colour strips. Various immobilization methods, including physical adsorption, were attempted on nitrocellulose, cellulose acetate and nylon membranes. A variety of parameters; including temperature, time of incubation of the substrate and support material, substrate (CPRG and PNPG) concentration, as well as the use of glutaraldehyde were investigated. Enzyme activity on the immobilised membrane was assayed using colour change as an indicator of activity. Among the different supports studied, modified nylon membranes provided the best support. The colour change on the membrane disks was instant upon addition of the commercial enzymes.

The origin of enzymes in the water samples was established using protein concentration techniques and bioinformatic studies. The presumed GAL band on SDS-PAGE was confirmed to be of *E. coli* origin. However, the presumed GUD band (at 68 kDa) did not match *E. coli* GUD. Instead some fragments were matching *E. coli* GAL.

It is possible to detect GUD and GAL activity electrochemically, but improving the sensitivity will greatly increase the feasibility of the technique. From studies performed with Nafion® it can be concluded that the membrane excluded more substrate than products, but reduced the overall sensitivity of the products. Separation was therefore achieved at the expense of chemical fouling. Detection of both PNP and CPR can be performed by oxidation without use of Nafion® but the sensitivity still needs to be improved.

Based of the information gained in our study, it was established that the cheapest and most rapid way to develop an on-line monitoring system is through use of a sequential flow injection analysis (SFIA) system with a spectrophotometric detector or in combination with an electrochemical detector. A customised SFIA system for the simultaneous monitoring of GAL and GUD was proposed in this study. However, this

SFIA system may not be as portable and applicable for the concept of "on-line, real-time" detection. The design of an electrochemical biosensor for the rapid on-line real-time detection of GUD and GAL is the focus of the follow-on WRC Grant K5/1603, which has already been approved. This design constitutes a high priority for future research and is based on the proof of concept that was established during the course of this study which is discussed in Chapter 8.

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- Assess the survival of general water quality indicator bacteria in biofilms within water distribution systems
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