

**DEVELOPMENT OF AN *IN SITU*  $\beta$ -D-GLUCURONIDASE  
DIAGNOSTIC *MORAXELLA*-BASED BIOSENSOR FOR  
POTENTIAL APPLICATION IN THE MONITORING OF WATER  
POLLUTED BY FAECAL MATERIAL IN SOUTH AFRICA**

A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

of

RHODES UNIVERSITY

by

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November 2006

## ABSTRACT

The prevention of outbreaks of waterborne diseases remains a major challenge to public health service providers globally. One of the major obstacles in this effort is the unavailability of on-line and real-time methods for rapid monitoring of faecal pollution to facilitate early warning of contamination of drinking water. The main objective of this study was to develop a  $\beta$ -glucuronidase (GUD)-based method that could be used for the on-line and real-time monitoring of microbial water quality. GUD is a marker enzyme for the faecal indicator bacteria *Escherichia coli*. This enzyme breaks down the synthetic substrate *p*-nitrophenyl- $\beta$ -D-glucuronide (PNPG) to D-glucuronic acid and *p*-nitrophenol (PNP), which turns yellow under alkaline pH. The enzymatically produced PNP was used to detect GUD activity. *In situ* GUD assays were performed using running and stagnant water samples from the Bloukrans River, Grahamstown, South Africa. The physico-chemical properties of environmental GUD were determined, after which a liquid bioprobe and a microbial biosensor modified with *Moraxella* 1A species for the detection of the enzyme activity were developed. In order to determine the reliability and sensitivity of these methods, regression analyses for each method versus *E. coli* colony forming units (CFU) were performed. The storage stabilities of the bioprobe and biosensor were also investigated. The physico-chemical properties of *in situ* GUD were different from those of its commercially available counterpart. The temperature optimum for the former was between 35 and 40 °C while for the latter it was 45 °C. Commercial (reference) GUD had a pH optimum of 8.0 while the environmental counterpart exhibited a broad pH optimum of between pH 5.0 and 8.0. The liquid bioprobe had a limit of detection (LOD) of GUD activity equivalent to 2 CFU/100 ml and a detection

time of 24 h. The method was less labour intensive and costly than the culturing method. The liquid bioprobe was stable for at least four weeks at room temperature ( $20 \pm 2$  °C). The biosensor was prepared by modifying a glassy carbon electrode with PNP degrading *Moraxella* 1A cells. The biosensor was 100 times more sensitive and rapid (5-20 min) than the spectrophotometric method (24 h), and was also able to detect GUD activity of viable but non-culturable cells. Thus it was more sensitive than the culturing method. Furthermore, the biosensor was selective and cost-effective. The possibility of using a *Pseudomonas putida* JS444 biosensor was also investigated, but it was not as sensitive and selective as the *Moraxella* 1A biosensor. The *Moraxella* biosensor, therefore, offered the best option for on-line and real-time microbial water quality monitoring in South African river waters and drinking water supplies.

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## LIST OF ABBREVIATIONS

$\leq x$ :	less than or equal to x (where x is a given number)
%:	percent
$\mu\text{A}$ :	microampere
$\mu\text{g}$ :	microgram
$\mu\text{l}$ :	microlitre
$\mu\text{m}$ :	micrometre
$\mu\text{M}$ :	micromolar
$A_{405\text{nm}}$ :	absorbance at 405 nm
$\text{Ag}/\text{AgCl}$ :	silver/silver chloride
AIDS:	acquired immuno-deficiency syndrome
APHA:	American Public Health Association
$\text{Ca}^{2+}$ :	calcium
$\text{CaCl}_2$ :	calcium chloride
CALM:	Colifast At-Line Monitor
$\text{Cd}^{2+}$ :	cadmium
$\text{CdSO}_4$ :	cadmium sulphate
CFU:	colony forming unit
Cl:	chloride
$\text{CO}_3^{2-}$ :	carbonate
Da:	Dalton
DCPA:	direct current potential amperometry
ECA:	enterobacterial common antigen
EDTA:	ethylenediaminetetraacetic acid
ELISA:	enzyme linked immunosorbent assay
FAD:	oxidised flavin adenine dinucleotide
$\text{FeCl}_3$ :	ferric chloride
GAL:	$\beta$ -galactosidase
GCE:	glassy carbon electrode
GUD:	$\beta$ -D-glucuronide glucuronosyl-hydrolase or $\beta$ -D-glucuronidase
h:	hour
HCl:	hydrochloric acid
IFA:	immunofluorescent assay
IUPAC:	International Union of Pure and Applied Chemistry
$\text{K}^+$ :	potassium
KCl:	potassium chloride
kDa:	kilodalton
$\text{KNO}_3$ :	potassium nitrate
l:	litre
LOD:	limit of detection
M:	molar
MF:	membrane filtration
mg:	milligram
$\text{Mg}^{2+}$ :	magnesium
$\text{MgSO}_4$ :	magnesium sulphate
min:	minute

ml:	millilitre
mm:	millimetre
mM:	millimolar
MTF:	multiple tube fermentation
MUG:	4-methylumbelliferyl- $\beta$ -D-glucuronide
mV:	millivolt
nA:	nanoampere
NA:	nucleic acid
Na <sub>2</sub> CO <sub>3</sub> :	sodium carbonate
Na <sub>2</sub> HPO <sub>4</sub> :	di-sodium hydrogen phosphate
Na <sub>2</sub> SO <sub>3</sub> :	sodium sulphite
Na <sub>2</sub> SO <sub>4</sub> :	sodium sulphate
NaCl <sub>2</sub> :	sodium chloride
NADPH:	reduced nicotinamide adenine dinucleotide phosphate
NaOCl:	sodium hypochlorite
nm:	nanometre
nmol/h/ml:	nanomoles per hour per millilitre
NO <sub>3</sub> <sup>-</sup> :	nitrate
OCl <sup>-</sup> :	hypochlorite
PAGE:	polyacrylamide gel electrophoresis
PCR:	polymerase chain reaction
pK <sub>a</sub> :	pH at which an acid is half dissociated
PNP:	<i>p</i> -nitrophenol
PNPG:	<i>p</i> -nitrophenyl- $\beta$ -D-glucuronide
PO <sub>4</sub> <sup>3-</sup> :	phosphate
ppm:	parts per million
rpm:	revolutions per minute
rRNA:	ribosomal ribonucleic acid
SD:	standard deviation
SDS:	sodium dodecyl sulphate
s:	second
SFIA:	sequential flow injection analysis
SO <sub>3</sub> <sup>2-</sup> :	sulphite
SO <sub>4</sub> <sup>2-</sup> :	sulphate
sp.:	species
TIM:	triose-phosphate isomerase
Tris:	TRIS(hydroxymethyl) aminomethane
v/v:	volume per volume
VBNC:	viable but non-culturable
w/v:	weight per volume
WHO:	World Health Organisation
ZAR:	South African Rand

## **ACKNOWLEDGEMENTS**

The author would like to thank the following:

My supervisor (Dr B.I. Pletschke) and co-supervisor (Dr J.L. Limson) for their inspiring suggestions and discussions during the course of the research.

My wife (Mucha), kids (Tanya and Anesu) and family members for their endurance during my absence and support.

Friends (Abraham Chawanji, Addmore Shonhai, Cecil Mutambanengwe and Victor Wutor) for their support.

The Water Research Commission (WRC) of South Africa and the Canon Collins Trust for their financial support.

## LIST OF OUTPUTS

### A Technical report

Pletschke, B.I., **Togo, C.A.** and Wutor, V.C. (2006) On-Line Real-Time Enzyme Diagnostic System for the Detection and Monitoring of Faecal Contamination of Water Intended for Drinking Purposes. *Technical Report for Water Research Commission Report No. 1446/1/06*. Water Research Commission (ISBN No: 1-77005-463-4, South Africa.

This is a peer reviewed technical report for Water Research Commission (WRC) project No. 1446/1/05. My main role in this report was drafting the overall report outline, writing of chapters 1, 2, 3, 4, 6 and 8, methodologies for the whole report (except for the GAL assay protocol and CPR voltammetry for Mr Wutor). Mr Wutor contributed with results from the enzyme he was studying (GAL) while I did the same with the enzyme GUD for the whole report. Dr Pletschke was the project leader and supervisor, and he wrote the executive summary, overall project discussion, edited the report and effected corrections suggested by the reviewers.

### B Publications in peer-reviewed scientific journals

**Togo, C.A.**, Wutor, V.C. Limson, J.L. and Pletschke, B.I. Novel detection of *Escherichia coli*  $\beta$ -D-glucuronidase activity using a microbially modified glassy carbon electrode and its potential for faecal pollution monitoring. *Biotechnology Letters*. DOI: 10.1007/s10529-006-9282-5.

This paper resulted from Water Research Commission (WRC) project No. K5/1603 for which Dr Pletschke was the grant holder and principal investigator (main supervisor), while Dr Limson was co-supervisor. My role in the preparation of this paper was to design and execute experiments, analyse data and write the paper. Dr Pletschke and Dr Limson offered expert advice and constructive criticism. Dr Pletschke, Dr Limson and Mr Wutor all assisted by editing the manuscript.

Wutor, V.C., **Togo, C.A.**, Limson, J. and Pletschke, B.I.. A novel biosensor for the detection and monitoring of  $\beta$ -D-galactosidase of faecal origin in water. *Enzyme and Microbial Technology*. DOI:10.1016/j.enzmictec.2006.10.039.

This paper resulted from Water Research Commission (WRC) project No. K5/1603 for which Dr Pletschke was the grant holder and principal investigator (main supervisor), while Dr Limson was co-supervisor. My role in the preparation of this paper was to contribute ideas to the design of experiments, data analysis and editing of the paper. Mr Wutor performed the experiments and wrote the paper. Dr Pletschke and Dr Limson offered expert advice, constructive criticism and edited the manuscript.

Wutor, V.C., **Togo, C.A.** and Pletschke, B.I. (2007). Seasonal variations in coliform  $\beta$ -D-galactosidase and *Escherichia coli*  $\beta$ -D-glucuronidase activities, temperature and pH in environmental water samples from the Eastern Cape of South Africa. *Water SA*. **33**: 107-110.

This paper resulted from the WRC project No. 1446/1/05 for which Dr Pletschke was the grant holder and principal investigator. My role in the preparation of this paper was to design and execute the experiments, analyse the data and write part of the paper for the  $\beta$ -D-glucuronidase section, while Mr Wutor contributed in a similar way to the  $\beta$ -D-galactosidase section of the paper. Dr Pletschke contributed by providing expert advice and criticism as well as editing the manuscript.

**Togo, C.A.**, Wutor, V.C. and Pletschke, B.I. (2006). Properties of *in situ* *Escherichia coli* beta-D-glucuronidase (GUS): evaluation of chemical interference on the direct enzyme assay for faecal pollution detection in water. *African Journal of Biotechnology*. **5**: 2338-2344.

This paper resulted from WRC project No. 1446/1/05 for which Dr Pletschke was the grant holder and principal investigator (supervisor). My role in the preparation of this paper was to design and execute experiments, analyse data and

write the paper. Dr Pletschke offered expert advice at all stages of the work. Both Dr Pletschke and Mr Wutor assisted in the editing of the manuscript.

Wutor, V.C., **Togo, C.A.** and Pletschke, B.I. The effect of physico-chemical parameters and chemical compounds on the activity of  $\beta$ -D-galactosidase ( $\beta$ -GAL), a marker enzyme for indicator microorganisms in water. *Chemosphere*. **(Submitted)**

This paper resulted from WRC project No. 1446/1/05 for which Dr Pletschke was the grant holder and principal investigator (main supervisor). My role in the preparation of this paper was to play a major role in the design of experiments, helping with data analysis and editing of the paper. Mr Wutor performed the experiments and wrote the paper. Dr Pletschke provided expert advice, positive criticism and edited the paper.

### **C Provisional patent**

**Togo, C.A.**, Wutor, V.C., Limson, J.L. and Pletschke, B.I. *Biosensor*. South Africa (ZA) Patent No. 2006/04442.

The provisional patent resulted from WRC project No. K5/1603 for which Dr Pletschke was the principal investigator, grant holder and main supervisor. The inventors are listed above. Dr Limson was the co-supervisor and offered expert advice on electrochemical aspects. During the design of the biosensor, two main methods were used. Mr Wutor contributed to the construction of the  $\beta$ -D-galactosidase based sensor while I contributed to the construction of the  $\beta$ -D-glucuronidase based sensor. Dr Pletschke and Dr Limson contributed by providing expert advice and critical input.

# **1 LITERATURE REVIEW**

## **1.1 Water Pollution**

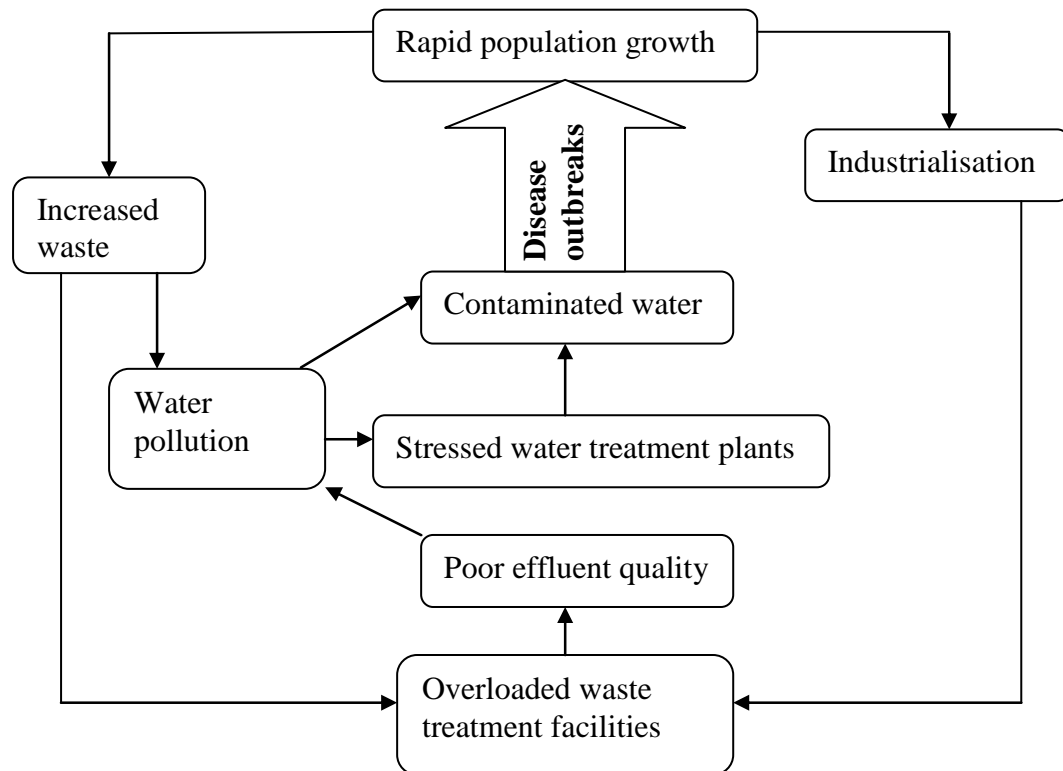
Provision of safe drinking water is one of the major concerns for governments in the world. However, in as much as public service providers desire to supply clean water to their communities, they have been unable to meet their targets due to practical and economic limitations (Gleick, 2002). Water pollution is a major problem worldwide and has great negative and far reaching consequences to both aquatic and terrestrial life. Sources of water pollution include agricultural, industrial and municipality wastes. One of the biggest threats to human health is faecal contamination from municipality wastes. This has consistently remained a major challenge to public service providers, both in developing and developed countries, because of waterborne diseases.

## **1.2 Waterborne Diseases**

Waterborne diseases result from the ingestion of water contaminated by faecal material or urine (especially of mammalian origin) that contains pathogenic microorganisms. Waterborne diseases like cholera, dysentery, typhoid and shigellosis are major killers with millions dying annually worldwide (Berg and Fiksdal, 1988; Ivnitski *et al.*, 1999; Venter, 2000; Gleick, 2002; Leonard *et al.*, 2003). Infants and poor communities are the most vulnerable. Exacerbating such outbreaks is the lack of efficient continuous water monitoring methods, overloaded wastewater treatment systems, and expensive and laborious methods of detection for the supposedly early warning system. In developing countries, the problem is further exacerbated by rapid population growth that does not match the available water and wastewater treatment facilities. Furthermore, due to a focus on the acquired immuno-deficiency syndrome (AIDS) pandemic, public health budgets for developing countries are limited to such an extent that improvements in wastewater handling facilities, in order to match increasing demands, are not always possible (Pletschke *et al.*, 2006).

The problem of waterborne diseases is cyclical (Fig. 1.1). When a population increases, there is a corresponding increase in domestic waste generation. Industrialisation is coupled to population growth and adds more waste to the system. The upsurge in waste exerts strain on the waste treatment plants (Fig. 1.1). This subsequently leads to insufficient waste treatment, and as a result raw effluent is dumped into the natural water bodies. In addition, facilities are overburdened resulting in sewage pipe bursts. This compromises the treatment process, leading to water pollution. In rural areas, the prevalence of poor sanitary conditions also leads to pollution of the water bodies by raw sewage. When polluted water is passed through the treatment plants, inefficient treatment (and sometimes treatment failures) can occur. The huge demand for water from an increase in the population is not always met by public service providers, forcing consumers to tap water directly from polluted water bodies. Consequently, the population is supplied with contaminated water without proper quality (real-time or on-line) assessment. This leads to outbreaks of waterborne diseases (Fig. 1.1). Under these circumstances, sensitive and user-friendly water quality monitoring methods, as well as efficient routine early warning systems are necessary.

Major advances in research have been made to achieve the real-time continuous monitoring of water for detecting chemical pollutants from industries and agricultural practices. This has seen the development of a variety of single analyte and broadband sensors for herbicides, pesticides, metals and other industrial chemicals (Evans *et al.*, 1986; Barzen *et al.*, 2002; Shitinda *et al.*, 2005). However, as further discussed, the same cannot be said for faecal pollution and microbial water quality. There is therefore a need for research that addresses the demands of this public health threat.



**Figure 1.1** Illustration of events and problems caused by population pressure on inadequate water facilities.

### 1.3 Microbial Water Quality Assessment

Water intended for drinking purposes has to meet certain quality guidelines that are put in place by governments and other organisations with an interest in public health. Amongst such standards is the World Health Organisation (WHO) stipulation that, for drinking water, faecal coliform counts must be zero per every 100 ml of water sample tested (Tallon *et al.*, 2005).

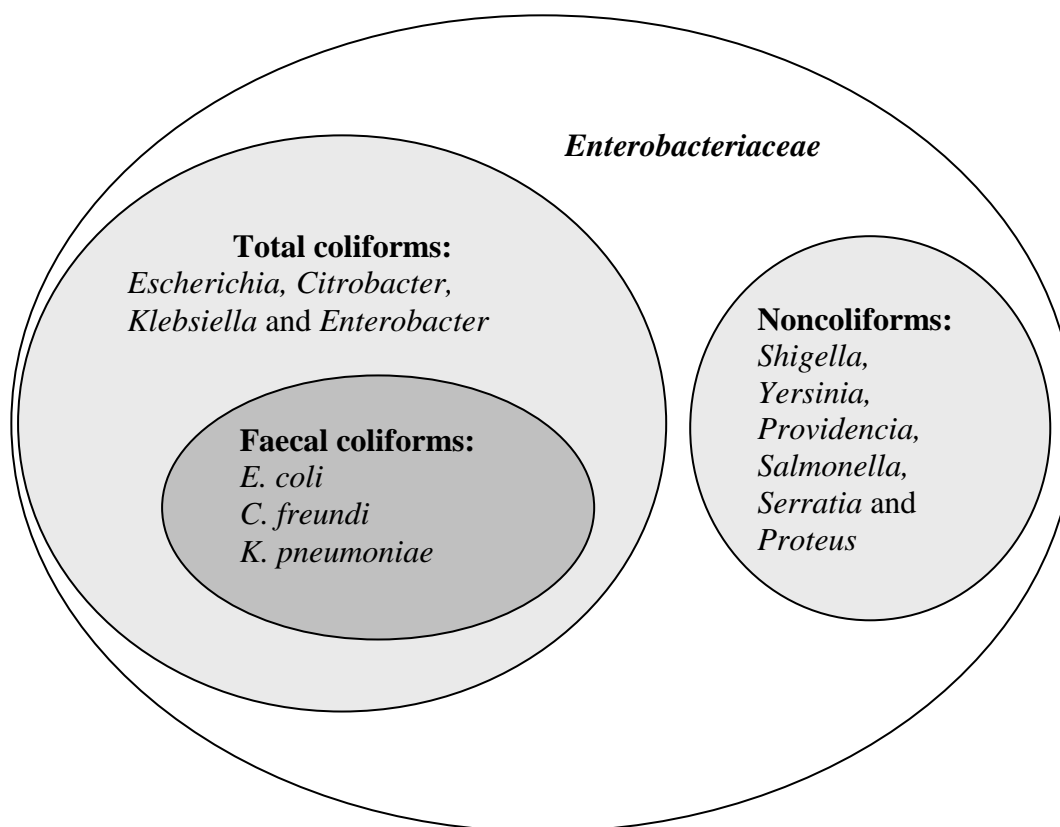
#### 1.3.1 The concept of indicator organisms

Indicator organisms are routinely used for the assessment of the potential presence/absence of pathogens in water because most pathogens occur in very small numbers and are extremely difficult to culture (Grabow, 1996; Tryland and Fiksdal, 1998; Venter, 2000; George *et al.*, 2001; Stevens *et al.*, 2001; Rompré *et al.*, 2002;

Cabral and Marques, 2006). Examples of such pathogens include *Bacillus dysenteriae*, *Giardia* sp., *Cryptosporidium* sp. and viruses. In addition, a long time (up to weeks) may be required to obtain confirmed positive results for such pathogens (Sartory and Watkins, 1999; Venter, 2000; Leonard *et al.*, 2003; Taguchi *et al.*, 2005), by which time the water would already have been used.

An ideal indicator organism for faecal contamination must be present whenever enteric pathogens are present and should have a longer survival time than most enteric pathogens. In addition, it should not proliferate in natural water, and must be easily, reliably and cheaply detectable. The density of the indicator organism should have a direct relationship with the degree of faecal pollution and must be a member of the intestinal microflora of warm-blooded animals (Grabow, 1996; Manafi, 1998; Stevens *et al.*, 2001; Pletschke *et al.*, 2006). Because of such properties, when these indicators are detected they signal the potential presence of pathogens and consequently a health hazard.

Microorganisms of the total coliform group are widely employed as faecal indicators since they exhibit most of the above mentioned properties. This group comprises e.g. of genera *Escherichia*, *Citrobacter*, *Enterobacter* and *Klebsiella* (Fig. 1.2) (Frampton and Restaino, 1993; Stevens *et al.*, 2001; Rompré *et al.*, 2002). Total coliforms are a subset of the family *Enterobacteriaceae* within which species like *E. coli*, *C. freundii* and *K. pneumoniae* are found (Fig. 1.2). The members from the non-coliform group are not used as indicator organisms although they belong to the same *Enterobacteriaceae* family as total coliforms (Fig. 1.2). The coliform group is made up of aerobic and facultative anaerobic, gram-negative, non-spore forming, rod-shaped bacteria that produce gas upon lactose fermentation within 48 hours at 35 °C (Frampton and Restaino, 1993; Rompré *et al.*, 2002). The presence of coliforms in drinking water can be attributed to inefficient treatment systems, loss of disinfectant potency and breakthrough intrusion of contaminated water into the potable water supply or re-growth problems in the distribution system (Rompré *et al.*, 2002).



**Figure 1.2** Relationship between indicator organisms of the family *Enterobacteriaceae* (adapted from Stevens *et al.*, 2001).

### 1.3.2 Limitations to using total coliforms as indicators

Despite being a widely accepted indicator system, the use of total coliforms has some limitations. Most members of the coliform group regrow on natural surface and drinking water distribution systems. The physico-chemical conditions (e.g. amount and type of organic matter and the temperature) of the water environment affect the concentration of the coliforms (Fujioka *et al.*, 1999; Stevens *et al.*, 2001). Thus, eutrophic tropical waters will contain high concentrations of coliform bacteria. Formation of biofilms in the storage containers (Jagals *et al.*, 2003) and the water distribution pipes may give a false indication of faecal contamination because immobilised coliforms are more resistant to chlorine than the free cells in water (LeChevallier *et al.*, 1987). 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, consequently exposing the public to waterborne diseases (Pletschke *et al.*, 2006).

#### 1.4 Methods of Indicator Organism Detection

A variety of techniques for the determination and quantification of indicator organisms exist. These techniques can be subdivided into (a) cultural, (b) molecular and (c) enzymatic methods (Frampton and Restaino, 1993; De Boer and Beumer, 1999; Venter, 2000; Rompré *et al.*, 2002). These classes tend to overlap and improvements have led to combinations of methods in different classes to increase the sensitivity, and reduce the amount of labour and time required for water sample analyses.

##### 1.4.1 Culture based methods

Membrane filtration (MF) and multiple tube fermentation (MTF) or the most probable number (MPN) techniques are the commonly used culture based methods. Membrane filtration entails the use of a 0.45 or 0.22  $\mu\text{m}$  (pore diameter) membrane filter to concentrate and entrap bacteria. The membrane filter is then layered on selective agar, incubated at 35 to 37 °C for total coliforms to grow. The coliform colonies are observed after 18 – 24 h (Brenner *et al.*, 1993; Davies *et al.*, 1994; Grabow, 1996; Manafi, 1998; Van Poucke and Nelis, 2000; Farnleitner *et al.*, 2001). Examples of traditional media include MacConkey agar and m-Endo-type agar. On m-Endo-type agar, the coliforms are observed as “red colonies with a metallic sheen” (Rompré *et al.*, 2002). Further culturing at  $44.0 \pm 0.5$  °C to establish if the colonies are thermotolerant coliforms or presumptive *Escherichia coli* may be required taking a further 24 – 48 h (Grabow, 1996; Sartory and Watkins, 1999).

Elimination of the confirmatory stage has been achieved by addition of specific synthetic enzyme substrates in the media. The substrates can be chromogenic (releasing a colour product upon hydrolysis) or fluorogenic (releasing a fluorophore/fluorochrome after hydrolysis). Use of such media allows concomitant

microbial quantification and species confirmation within the 16 – 24 h culturing period (Sartory and Watkins, 1999). Examples of such media include m-FC agar (Merck, Darmstadt, Germany) and CM1046 (Oxoid, Hampshire, England).

In the MTF technique, tubes with media (e.g. lauryl sulphate broth), or with additional selective compounds for coliforms (e.g. sodium azide, cefsulodin and bile acids) are inoculated with the samples in a serial dilution pattern and incubated between 35 and 37 °C (Alonso *et al.*, 1996; Fricker and Fricker, 1996<sup>a</sup>; Sartory and Watkins, 1999). Presumptively coliform positive tubes exhibit growth and gas production within 48 h. Confirmatory tests, for thermotolerant coliforms, such as allowing the cultures to grow in brilliant green broth (at  $44.0 \pm 0.5$  °C within 24 h) or an indole test are performed. The most probable number tables are used to estimate the microbial population based on the presence/absence of growth at different dilutions. The total analysis time will therefore vary between 48 and 96 h (Berg and Fiksdal, 1988; Grabow, 1996; Manafi 1998; Davies and Apte, 2000; Geissler *et al.*, 2000; Ercole *et al.*, 2002; Rompré *et al.*, 2002; Leonard *et al.*, 2003). Confirmatory stages in MTF can be eliminated by the incorporation of synthetic substrates for marker enzymes in the broth. Such improvements illustrate the overlap of culturing/classical and enzyme based methods.

The major limitation of these culturing methods is the long time required for sample analysis. This time lapse is inappropriate when prompt information about the safety of drinking water is required (Venter, 2000). Other limitations of culturing methods include unreliability in the presence of antagonistic microorganisms and a weak level of detection of slow-growing or stressed coliforms. Thus the need for efficient and timely techniques for the detection of faecal pollution in aquatic environments is crucial (Farnleitner *et al.*, 2001; Rompré *et al.*, 2002).

Culturing techniques are very sensitive and selective (Lazcka, *et al.*, 2006), allowing microbial viability to be determined accurately (Venter, 2000). However, the methods may underestimate microbial counts since they cannot detect viable but non culturable (VBNC) cells. Cells become non-culturable or fail to grow due to stress

induced by lack of nutrients in most water bodies and injury suffered during treatment (Venter, 2000; George *et al.*, 2002; Rompré *et al.*, 2002; Leonard *et al.*, 2003). Culture-based techniques are also time consuming and labour intensive.

The disadvantage of the MTF technique, as opposed to MF, is that it does not allow for the analysis of large volumes of water and sample numbers. Turbid samples may prevent filtration of large volumes of water leading to limited sensitivity and sample volumes to be analysed (Niemi, pers. comm.). A major obstacle in the improvement of culturing methods is that the microbial growth cannot be accelerated during culturing (Venter, 2000). Consequently, retrospective results are inevitable when using these methods. This presents a health hazard, since the water would already have been used before the microbial quality is established. Assaying for the enzymes responsible for the break down of the synthetic substrates can therefore be a viable option for reducing the time required for microbial water quality analyses.

#### **Hydrogen sulphide test: simplified culturing**

Hydrogen sulphide (H<sub>2</sub>S) test strips (or media) are used to detect H<sub>2</sub>S-producing bacteria associated with faecal contamination (Sobsey and Pfaender, 2002; Hirulkar and Tambekar, 2006). H<sub>2</sub>S production is detected by formation of black iron sulphide precipitate on the strips or in the medium where the samples are incubated (18-24 h). Different versions and media for H<sub>2</sub>S tests exist and the method is not standardised world wide.

The H<sub>2</sub>S method is cheaper than the conventional culturing methods, does not require trained staff and can be applied in the field (Sobsey and Pfaender, 2002; Hirulkar and Tambekar, 2006). However, the time of analysis between 18 and 24 h does not allow the required early warning system. In addition, the method is not specific to human coliforms and therefore unreliable when used in other water bodies that have other sources of coliforms e.g. decomposing organic matter (Sobsey and Pfaender, 2002).

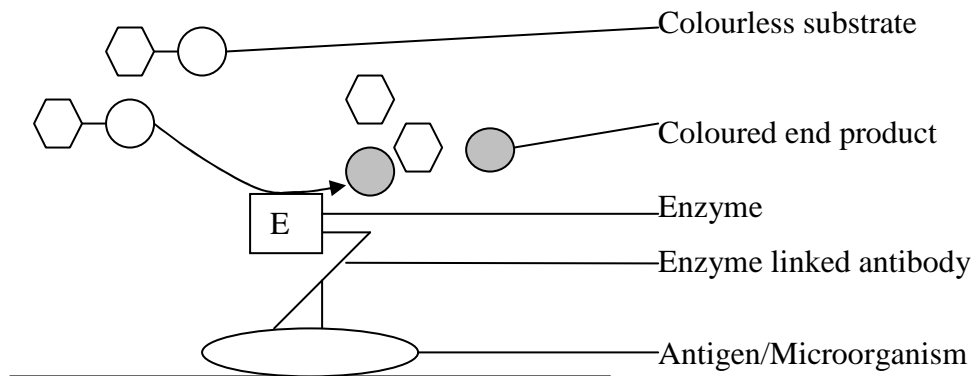
### 1.4.2 Molecular based methods

Molecular based methods are aimed at reducing analysis time through the elimination of the time-consuming culturing/growth step. They can be classified into immuno- and nucleic acid (NA)-based methods. Immuno-based methods take advantage of the specificity of antibody-antigen interactions in techniques such as enzyme linked immunosorbent assay (ELISA), immunofluorescent assay (IFA) and immunoblotting. Nucleic acid based methods comprise NA hybridisation, polymerase chain reaction (PCR) and the use of genetically modified bacteriophages [that express genes (e.g. *lux*) in specific hosts in the environment] (De Boer and Beumer, 1999; Venter, 2000; Rompré *et al.*, 2002; le Roux *et al.*, 2004).

#### Immuno-based techniques

Selective detection of indicator organisms is possible through targeting of genera- or species-specific proteins. Antibodies for these targets can be raised and conjugated to enzymes that will catalyse the breakdown of chromogenic or fluorogenic substrates. Enzymes that can be used in conjunction with the antibodies include horseradish peroxidase, alkaline phosphatase, urease and  $\beta$ -D-galactosidase (GAL) (Crowther, 2001).

Various forms of ELISA namely: direct [direct labelled antibody (Ab)/antigen (Ag)]; indirect; sandwich (direct/direct); competition (direct/indirect Ab competition and direct/indirect Ag competition) exist (Crowther 1995; 2001). Direct labelled Ab ELISA will be described to illustrate the principle and stages of ELISA. Enzyme-labelled antibodies are bound to the antigen immobilised to a support material and will remain attached to the antigen during washing of the unbound antibodies (Fig. 1.3). This is followed by the addition of the chromogenic/fluorogenic substrate to the experimental sample (Crowther, 1995). Occurrence of colour or fluorescence (signifies substrate breakdown) indicates the presence of the microorganism in question, as it would have retained the conjugated antibody during washing (Fig. 1.3).



**Figure 1.3** Illustration of a direct labelled antibody ELISA (adapted from Crowther, 1995).

Hübner *et al.* (1992) and Levasseur *et al.* (1992) used the enterobacterial common antigen (ECA) for coliform and *E. coli* detection, while Kaspar *et al.* (1987) reported the use of  $\beta$ -D-glucuronidase (GUD), GAL and glutamate decarboxylase in the immunological detection of *E. coli*. The use of ECA monoclonal antibodies was comparable to that of the culturing methods, as the immunoassay results were 98 % similar to the results obtained with the MTF technique (Hübner *et al.*, 1992). Thus, the ECA can provide a reliable alternative method to MF and MTF for indicator organism detection.

Immunological techniques are sensitive, specific and rapid (Venter, 2000; Leonard *et al.*, 2003; Taguchi *et al.*, 2005). However, the disadvantages of immunological methods include cross-reactivity that can lead to false positive results and susceptibility to background interference under low microbial populations [below the threshold level of the limit of detection (LOD)]. For negligible background interference, the target population must be at least one percent of the total microbial load in the sample in question (Mansfield and Forsythe, 2000; Rompré *et al.*, 2002). When the target microorganisms occur in low numbers they can be selectively concentrated using techniques such as immuno-magnetic separation (Mansfield and Forsythe, 2000; Müller *et al.*, 2003), before analyses are performed. However, employing such techniques for improved results will translate into extra costs and

requirement for skilled personnel. Hence the techniques will not be economically feasible to local authorities for routine use.

In a bid to improve sensitivity, Abuknesha and Darwish (2005) coupled the enzymatic and immunological techniques to monitor *E. coli*. The LOD was between 10 and 10<sup>5</sup> cells/ml detected within two hours. Burestedt *et al.* (2000) attempted to link an immunoassay technique to a biosensor for signal amplification. The limitations to field implementation of such techniques are the high costs involved.

Although the immunoassay techniques are capable of detecting VBNC organisms, they do not provide information about the physiological state of cells (Rompré *et al.*, 2002; Leonard *et al.*, 2003). Efforts to determine the microbial physiological state were made by Rockabrand *et al.* (1999). These entailed the use of proteins: DnaK (heat shock protein 70; a molecular chaperone), Dps (highly conserved 19-kDa DNA binding protein important for stationary phase physiology) and Fis (an 11-kDa DNA binding protein that plays a role in rRNA synthesis), expressed at different life stages and growth conditions. If this technique is used in the environment, there will be a requirement of three antibodies to these antigens, thus further complicating the assay method.

Immuno-based methods are advantageous, over both culturing and NA based methods, as they can be used to detect the toxins of interest (Iqbal *et al.*, 2000; Leonard *et al.*, 2003). However, they are not feasible for real-time detection (Leonard *et al.*, 2003).

#### **Nucleic acid (NA)-based techniques**

Gene sequences coding for genera-or species-specific proteins can be detected using nucleic acid probes. According to Keller and Manak (1989), a probe is a molecule that interacts with a specific target and has a traceable means of detection after such an interaction. Radioactive (<sup>32</sup>P), fluorometric or enzymatic labelling of the NA probes are commonly used (Keller and Manak, 1989; Fricker and Fricker, 1994; Joux and Lebaron, 2000; Köhler *et al.*, 2000). The complementarity between NA base

sequences and the existence of some unique sequences in each species form the basis of NA methods (Rompré *et al.*, 2002).

Performing a typical NA based method for indicator organisms detection involves fixation of test samples on membranes (or in the gel after electrophoresis), and then introduction of a probe. When samples contain whole cells, they are permeabilised to expose and linearise the NA, and incubated in the presence of the probe under conditions that allow strand annealing between the probe and target NA. Unbound probes are washed off followed by tracing of the probes on the sample using different techniques that include autoradiography, fluorescence or colour monitoring (Feng *et al.*, 1991; Rompré *et al.*, 2002). The presence of the target sequence or the microbe possessing the target sequence is confirmed by detection of the signal.

In general, target DNA occurs at low concentrations and sometimes in the presence of interfering compounds in the environment, consequently purification and PCR amplification is required before further analysis (Rompré *et al.*, 2002). Microorganisms can be concentrated using filtration (Bej *et al.*, 1991<sup>a</sup>). Selective amplification of the DNA of interest is enabled by use of primers for the target sequence, after which the PCR products are separated by electrophoresis and hybridised with the probe (Keller and Manak, 1989). Target sequences that have been used for faecal indicator organisms include the *uidA* (GUD gene), *uidR* (GUD regulatory gene) and the GAL gene (Bej *et al.*, 1990; 1991<sup>b</sup>; Fricker and Fricker, 1994; Iqbal *et al.*, 1997).

Advantages of using NA methods include the ability to detect genes from VBNC bacteria and the detection of GUD negative *E. coli* O157:H7 (that contains the *uidA* sequence) (Feng *et al.*, 1991; Martins *et al.*, 1993; Fricker and Fricker, 1994; Monday *et al.*, 2001). In addition, NA methods are as sensitive as improved culturing methods when PCR is used (Bej *et al.*, 1990; 1991<sup>a</sup>). However, NA methods can give both false positive and negative results. Fricker and Fricker (1994) showed that strains of *Hafnia alvei* and *Serratia odorifera* have the *uidA* sequence that is used as a target sequence in the detection of *E. coli*. Bej *et al.* (1990) noted that *Shigella* species

interfered with *E. coli* detection using *lamB* that codes for an *E. coli* surface protein targeted by the lambda phage. Therefore, in the presence of such strains and absence of *E. coli*, false positive results would be expected. Once a method proves to be sensitive, but gives false positives, commercial enterprises are reluctant to adopt such techniques as these false positives will impact negatively on the companies' production and quality (Sartory and Watkins, 1999).

PCR is susceptible to inhibition by (and give false positive results due to) environmental contaminants (Rompré *et al.*, 2002; Leonard *et al.*, 2003). Environmental samples will, therefore, require purification before analyses. This sample pre-treatment translates into a prolonged assay time, the requirement for skilled labour and more costs, all of which are unattractive for large routine sample processing.

Despite the advantage of high sensitivity, NA methods cannot be employed for microbial quantification and the determination of the microbial physiological state (Leonard *et al.*, 2003; Lazcka, *et al.*, 2006). The presence of specific NA does not necessarily imply the presence of the microorganisms or toxins they produce (Tryland and Fiksdal, 1998; Taguchi *et al.*, 2005). Consequently, the detection of target sequences is not indicative of the presence of viable microorganisms in the sample (false positive results). Furthermore, signal visualisation may require prolonged processing times (2 – 3 days) especially when using radio-labelling (Rompré *et al.*, 2002), thus compromising the objective of establishing an early warning system.

Molecular based methods will remain, for quite some time, undesirable for routine use because they require skilled manpower, are costly and demand dedicated laboratory space and equipment (Ivnitski, *et al.*, 1999; Venter, 2000; Rompré *et al.*, 2002; Leonard *et al.*, 2003). In addition, these methods are not feasible for real-time monitoring in the environment with contaminated samples.

Other biomarkers like bile acids, sterols and aminoacetone have been found to be potentially 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 and occur at low levels that are difficult to extract and detect (Fitzsimons *et al.*, 1995; Bull *et al.*, 2002; Truong *et al.*, 2003), rendering them undesirable for routine purposes.

### 1.4.3 Enzyme based methods

The enzyme based methods are difficult to single out as free standing techniques, because developments in culturing and molecular methods are mainly enzyme based. For example, improvements in defined media technology are based on enzyme catalysed microbial biochemical pathways. The existence of genera- or species-specific metabolic pathways is due to certain key marker enzymes that participate in such metabolism. Therefore, the direct assaying of marker enzymes can be a viable alternative for indicator organism detection, bearing in mind that most enzyme mediated reactions are rapid and time saving.

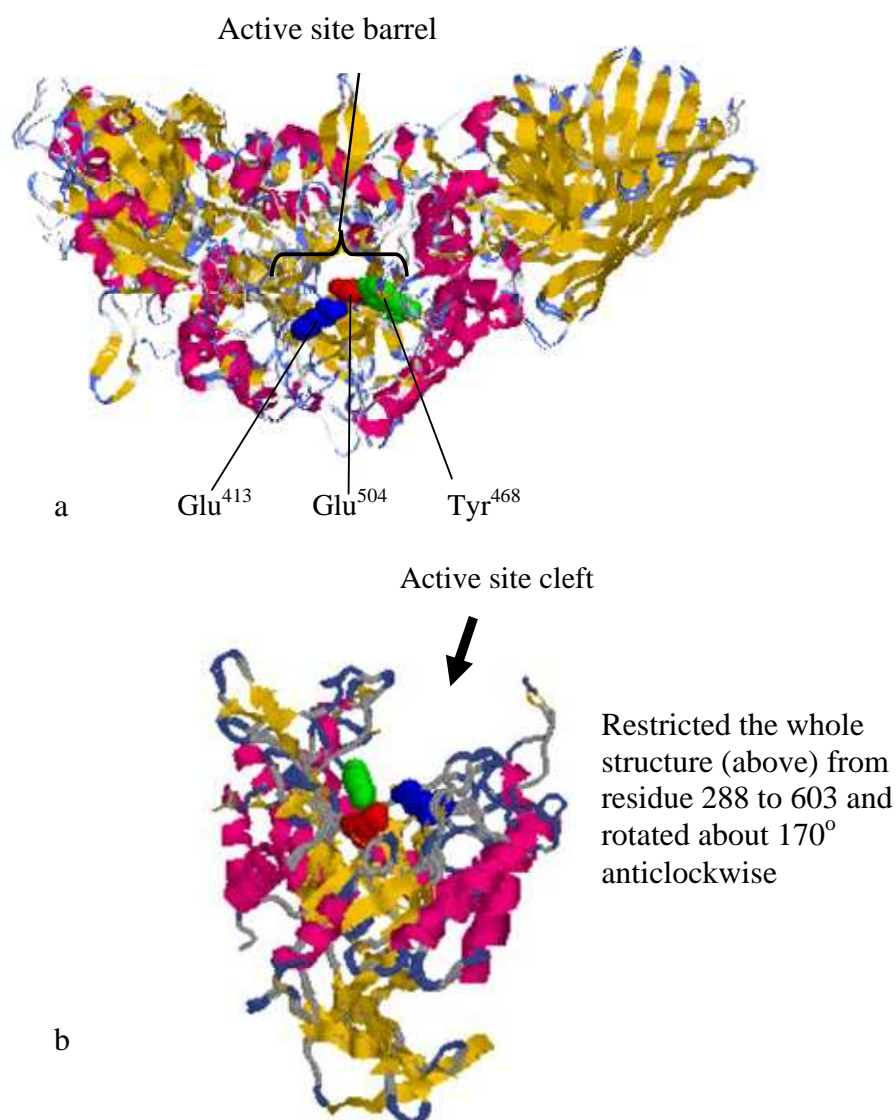
Beta-D-glucuronidase [formally  $\beta$ -D-glucuronide glucuronosyl-hydrolase (EC 3.2.1.31)] abbreviated as GUD or GUS, is used as a marker enzyme for *E. coli* and  $\beta$ -D-galactosidase (GAL) for total coliforms (Martins *et al.*, 1993; Gilissen *et al.*, 1998; Sartory and Watkins, 1999; Rompré *et al.*, 2002; Garcia-Armisen *et al.*, 2005; Cabral and Marques, 2006; Momba *et al.*, 2006).

#### **GUD**

*Escherichia coli* GUD is synthesised from *gusA* (formerly *uidA*) gene (Matsumura *et al.*, 1999; Geddie and Matsumura, 2004). It is a globular protein (Fig. 1.4a), active as a homotetramer and made up of 68 kDa monomers of 603 amino acids. It is an exohydrolase and belongs to the family 2 of hydrolases that include galactosidases and mannosidases (Henrissat, 1991; Wong *et al.*, 1998). The *E. coli* GUD does not require cofactors, is very specific, has broad pH optimum (5.0 – 7.5), exhibits a weak GAL activity (Gilissen *et al.*, 1998; Matsumura and Ellington, 2001) and has approximately 50 % amino acid sequence identity to human GUD. The active site residues of the enzyme are highly conserved (Matsumura *et al.*, 1999; Geddie and

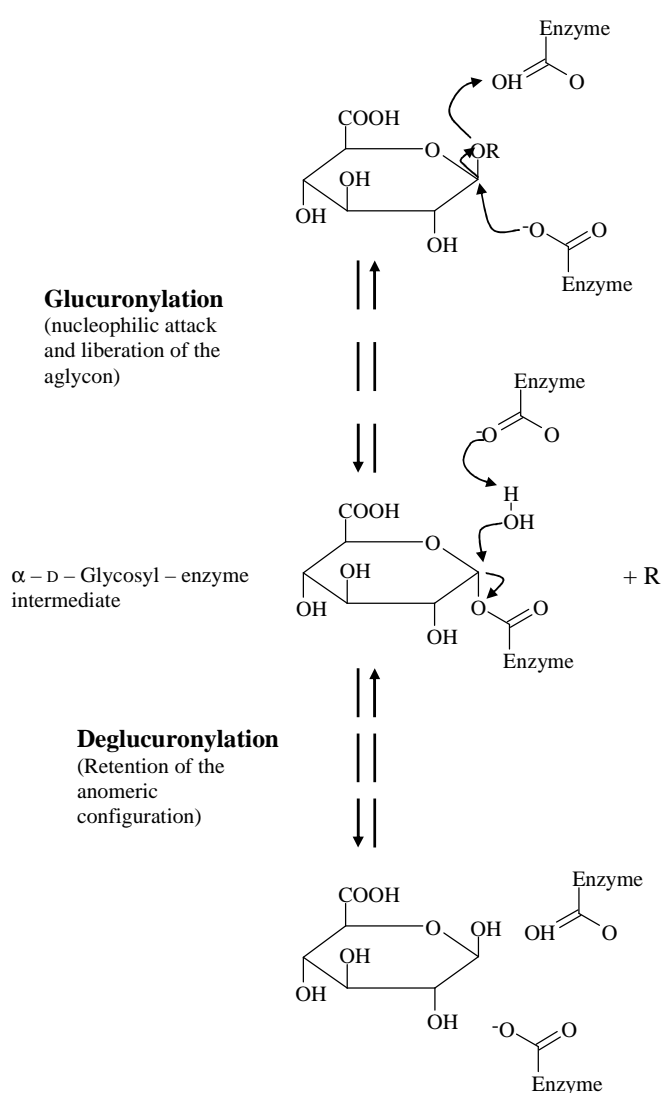
Matsumura, 2004). Substrate specificity of GUD from *E. coli* and human is identical (Matsumura and Ellington, 2001). In addition, *E. coli* contributes significant amounts of the GUD required in the human intestines (Gilissen *et al.*, 1998). GUD catalyses the hydrolysis of  $\beta$ -D-glucuronides based substrates to corresponding aglycons and D-glucuronic acid (Wong *et al.*, 1998; Rompré, *et al.*, 2002). The enzyme retains the anomeric configuration of the glucuronic acid and is appropriately termed a retaining hydrolase (Islam *et al.*, 1999).

The enzyme has three important residues that participate in the breaking down of the glucuronides. It breaks down the O-glycosyl bond by nucleophilic attack with the nucleophilic residue being E504 or Glu<sup>504</sup> (equivalent to Glu<sup>540</sup> in humans). The acid-base residue is Glu<sup>413</sup> (equivalent to human Glu<sup>451</sup>) while the residue Tyr<sup>468</sup> (Tyr<sup>504</sup> in humans) has been proven important but its role is not clear (Wong *et al.*, 1998; Islam *et al.*, 1999; Matsumura and Ellington, 2001). Figure 1.4b shows the relevant locations of these residues in the active site cleft, in the C-terminal triose-phosphate isomerase (TIM) barrel. Matsumura and Ellington (2001) modelled the *E. coli* GUD against the human crystal structure and proposed that seven conserved residues (Asp<sup>163</sup>, Tyr<sup>468</sup>, Glu<sup>504</sup>, Tyr<sup>549</sup>, Arg<sup>562</sup>, Asn<sup>566</sup> and Lys<sup>568</sup>) form eight-intermolecular hydrogen bonds with the substrate. This bonding is thought to confer the typical specificity of GUD to  $\beta$ -D-glucuronide based substrates.



**Figure 1.4** Illustration of the globular nature and packing of a homotetramer *E. coli* GUD showing the active site barrel/cleft, the nucleophilic Glu<sup>504</sup> (red), acid-base Glu<sup>413</sup> residue (blue) and Tyr<sup>468</sup> residue (green) (a); a restricted version to show the housing of the three residues in the cleft (b). The pdb file (modelled against 1bhg.pdb) was obtained from Swiss-Prot (<http://ca.expasy.org/sprot>) after submitting GUD sequence (accession code P05804) and modelled using RasWin Molecular Graphics version 2.7.2.1.1 ([www.bernstein-plus-sons.com/software/rasmol/](http://www.bernstein-plus-sons.com/software/rasmol/)). Active site residue information was obtained from Matsumura and Ellington (2001).  $\alpha$ -helices are coloured red,  $\beta$ -sheets yellowish, loops in blue and grey.

The actual mechanism of GUD catalysis entails a two-step process, glucuronylation and deglucuronylation steps as illustrated in Figure 1.5. The first step comprises a nucleophilic attack on the sugar anomeric centre leading to the release of an aglycon. This is aided by acid catalysis. The aglycon departure causes formation of an  $\alpha$ -D-glycosyl-enzyme intermediate. The second step entails hydrolysis of the  $\alpha$ -D-glycosyl-enzyme intermediate by “base-catalysed attack of water at the anomeric centre”. This results in the retention of the anomeric configuration. These stages are reversible (Wong *et al.*, 1998).



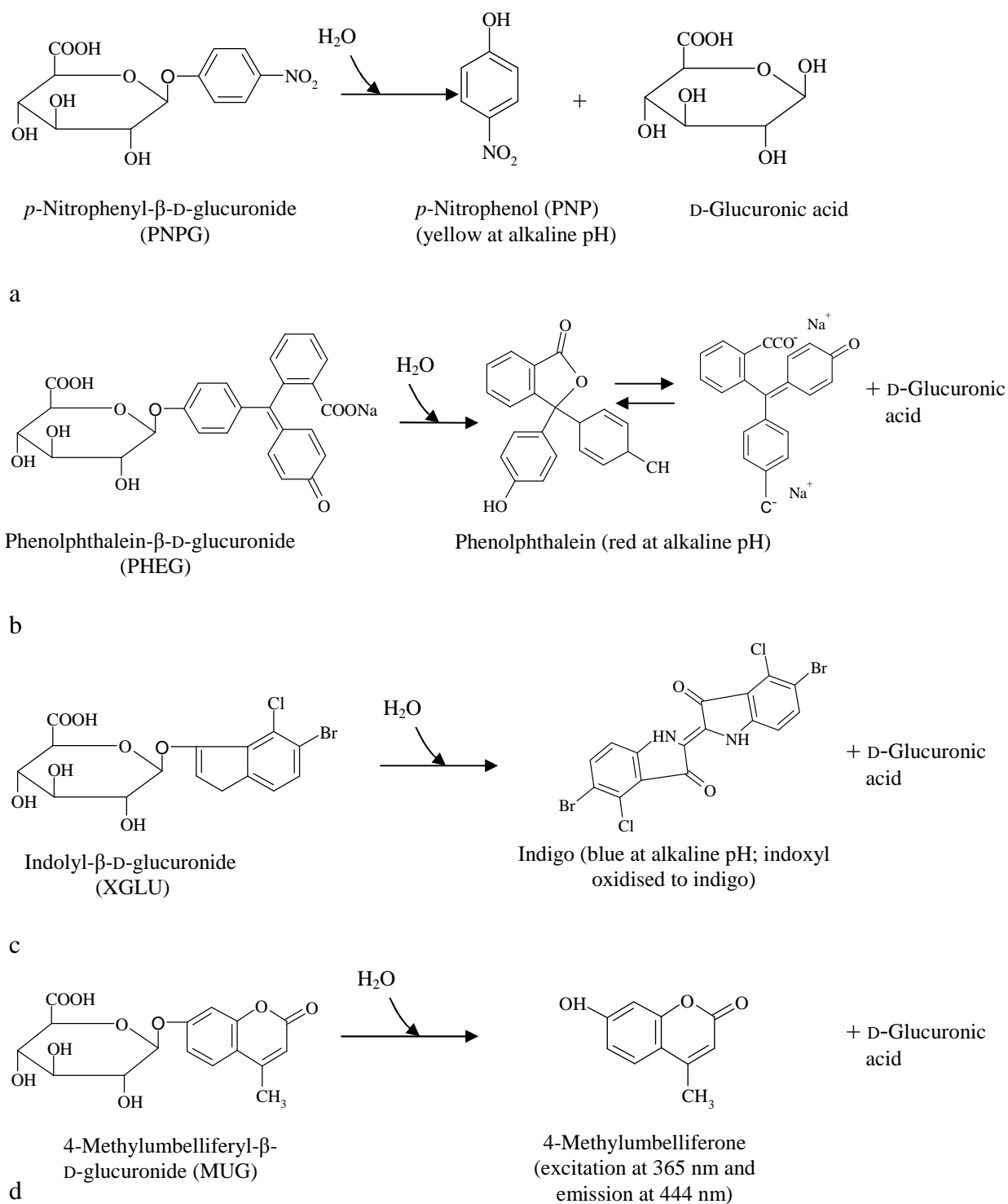
**Figure 1.5** The mechanism of GUD catalysis: nucleophilic attack and retention of the anomeric configuration (adapted from Wong *et al.*, 1998). R: aglycon.

### Synthetic substrates

Substrates made up of glucuronide linked to a chromogen or fluorochrome (Fig. 1.6) have been designed for the detection of GUD (Frampton and Restaino, 1993). The use of enzymatic reactions to detect *E. coli* can provide a rapid, sensitive and less expensive alternative to the more time consuming traditional methods. Enzyme substrates can be added to the samples in question and incubated under optimum conditions for maximum enzyme activity. Colour formation or fluorescence due to the hydrolysis of the synthetic substrates can be scored. In general, chromogenic substrates are phenol-based, water soluble, heat stable, specific and occur in a wide range of different colours (Manafi, 1998). Examples of common GUD synthetic substrates and their hydrolysis products are shown in Figure 1.6. The conditions under which each hydrolysis yields colour or fluoresces are stated in Figure 1.6. Most of the chromogenic substrates produce the characteristic colour under alkaline pH.

A range of chromogenic and fluorogenic substrate-based methods are commercially available, for example, the Colilert<sup>®</sup> techniques including the Quantitray<sup>®</sup> (Berger, 1994; Sartory and Watkins, 1999) and more advanced stand alone instruments such as the Colifast At-Line Monitor (CALM) that can be remotely linked to the laboratory ([www.colifast.no](http://www.colifast.no)). The CALM makes use of fluorogenic substrates, and like other conventional methods, it requires culturing before analysis. Culture enrichment before analyses prolongs the total analyses time, which is undesirable.

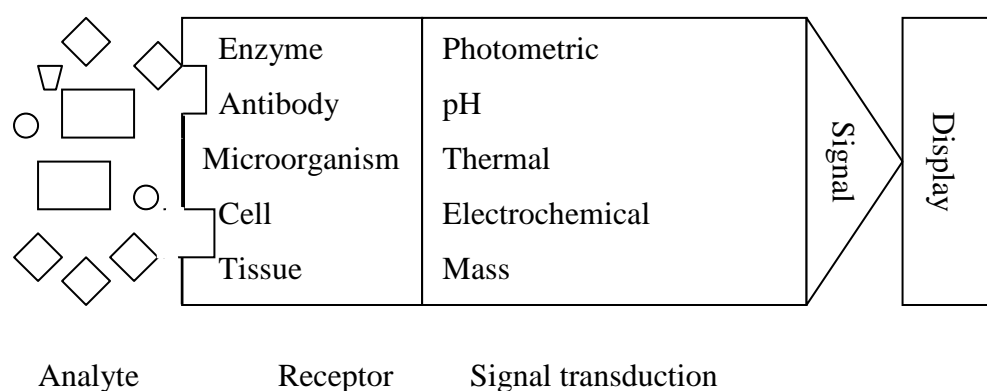
Enzyme-based methods may require high initial enzyme or microbial concentrations, which may not always be the case for environmental samples. Use of more sensitive fluorogenic substrates is prone to interference by fluorochromes inherently present in the water samples (Pletschke *et al.*, 2006). Another disadvantage of enzyme based techniques is poor environmental performance because seeded pure water samples are usually used for protocol development. Seeding of water with microorganisms or sewage is not representative of an ideal environmental system (Sartory and Watkins, 1999) and poses a problem when the same methods are extrapolated to the environment.



**Figure 1.6** Examples of synthetic substrates for GUD and their chromogenic (a-c) and fluorogenic (d) breakdown products (adapted from Frampton and Restaino, 1993).

## 1.5 Biosensors

The need for rapid, automated, real-time and on-line monitoring methods to circumvent retrospect results and effectively implement early warning systems has seen many attempts to develop biosensors for indicator microorganisms (D'Souza, 2001; Leonard *et al.*, 2003; Paitan *et al.*, 2003). According to the International Union of Pure and Applied Chemistry (IUPAC), an electrochemical biosensor is a “self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element” that is in direct contact with an electrochemical transducer (Thévenot *et al.*, 2001). Biosensor detection entails the conversion of a signal (e.g. microbial growth, enzyme activity or antibody-antigen reaction) to a measurable output. Signals can be optical, mass, thermal or electrochemical. A biosensor is made up of a bioreceptor surface, transducer and output display unit as shown in Figure 1.7 (Göpel and Heiduschka, 1995; Scouten *et al.*, 1995; D'Souza, 2001; Thévenot *et al.*, 2001; Leonard *et al.*, 2003; Purohit, 2003; Kissinger, 2005).



**Figure 1.7** 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; Ziegler and Göpel, 1998; Thévenot *et al.*, 2001). Strictly speaking, any device whose receptor surface is not derived from an organism may not qualify to be called a biosensor (Thévenot *et al.*, 2001) since

there will be no “bio”-component. 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.*, 1999; Leonard *et al.*, 2003; Deobagkar *et al.*, 2005). Electrochemical transducers, especially amperometric, are the most preferred due to the advantages they offer. They are sensitive, cost-effective and offer portability (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 pushed and a reading obtained. However, to the developer, the process of attaining that level of operation requires selection of an appropriate receptor surface, method of signal detection and transduction, display format, definition of result range and optimisation of the function of the equipment.

Good selective detection by biosensors is one of the reasons why they are preferred. They also produce results rapidly and can be miniaturised for use in real-time and on-line monitoring (Ivnitski *et al.*, 1999; D’Souza, 2001; Leonard *et al.*, 2003). When fully optimised, they are easy to use and do not need skilled manpower, they are not labour intensive and can be programmed to operate remotely, capturing and transmitting data to the laboratory.

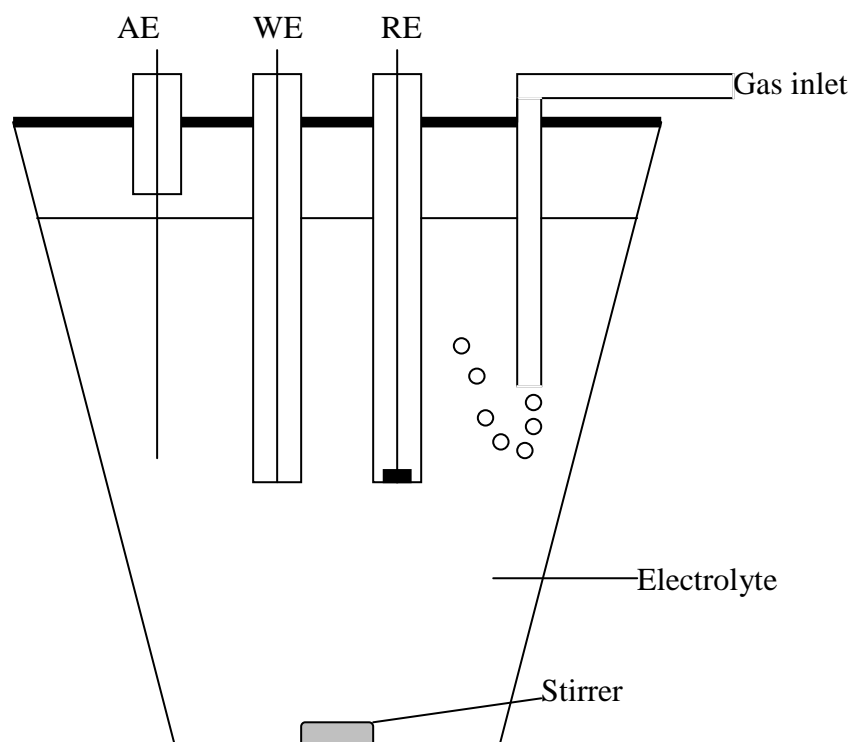
### ***1.5.1 Electroanalysis: foundation of biosensor technology***

Monitoring of enzyme breakdown products can be performed using optical, fluorometric or electroanalytical/electrochemical methods. Electrochemical methods are advantageous in that they are not prone to solution turbidity, as is the case with spectrophotometric analyses. The instrumentation for electrochemical analysis is cheap, portable, highly sensitive, selective and offer linearity over a wider range than spectrophotometric assays. Furthermore, electrochemical signals are relatively easy to integrate when constructing a biosensor (Brainina, 1987; Wang, 1994; Skoog *et al.*,

1996; D'Souza, 2001). Therefore the biosensor advantages are directly derived from the advantages of using electrochemical methods.

Electrochemical analysis is based on the interplay between voltage and molecules of interest (Skoog *et al.*, 1996). Electroactive groups on molecules are capable of gaining or losing electrons thus producing a current during such a reaction. The ability of a molecule to be oxidised or reduced depends on the number of electroactive groups, their positions, number, types, pH value of the electrolyte and temperature (Hu *et al.*, 2001; Stanca *et al.*, 2003; Pedrosa *et al.*, 2004). Such differences help in imparting the selective nature of electroanalytical methods.

Electrochemical analyses such as voltammetry or amperometry require an electrolytic electrochemical cell (Fig. 1.8) and a potentiostat (potential generating and current measuring instrument). The electrochemical cell contains substances that can either be oxidised or reduced (electroactive substances) after applying appropriate potential to produce a measurable current. The current produced is proportional to the concentration of the electroactive compound. Electrochemical cells may comprise of two or three electrodes and the analyte (electrolyte). The three electrodes are: the working, reference and auxiliary electrodes (Fig. 1.8).



**Figure 1.8** A schematic illustration of a typical three-electrode electrochemical cell (adapted from Wang, 1994). AE: auxiliary electrode; WE: working electrode; RE: reference electrode.

### Electrodes

Electrochemical measurements are based on the processes occurring at the working electrode either through control of its potential (potentiostatic) or the current that the working electrode passes (galvanostatic). A good working electrode must not be easily affected by background noises (interference) and should generate reproducible results. It must withstand the potential at which the electroactive species are analysed. Carbon, boron-doped diamond and metals like gold, mercury and platinum can be used in the construction of working electrodes (Skoog *et al.*, 1996; Zhang *et al.*, 2000; Pedrosa *et al.*, 2004). Selection of material for working electrode construction is based on the desired properties of the electrode.

Carbon working electrodes are amongst the most popular because they tolerate a broader potential window (-1.5 to + 1.5 V), and are cheap, robust and inert. Carbon electrodes tend to have a shortfall of slow electron transfer rate on their surfaces.

However, surface modification can be performed to circumvent this (Murray *et al.*, 1987; Baldwin and Thomsen, 1991; Zhang *et al.*, 2000). Variants of carbon electrodes include the glassy-carbon electrode (GCE), carbon-paste electrode (CPE) and carbon-fibre electrode (CFE). The GCE has an additional advantage of possessing excellent mechanical properties (Wang, 1994; Skoog *et al.*, 1996; Zhang *et al.*, 2000).

A reference electrode maintains a constant potential, independent of the electrolyte, against which the potential of the working electrode is compared, or referenced. The constant potential is attributed to a “constant composition of both forms of its redox couple” (Wang, 1994). Examples of reference electrodes are the silver/silver chloride (Ag/AgCl) electrode, hydrogen electrode and mercury/mercury chloride ( $\text{Hg}_2/\text{Hg}_2\text{Cl}_2$ ) electrode. The Ag/AgCl electrode is normally used because it is cheap, robust, it tolerates high temperatures (100 °C) and is not toxic as opposed to the  $\text{Hg}_2/\text{Hg}_2\text{Cl}_2$  electrode.

The auxiliary electrode acts as a source or sink of electrons, completing the circuit (Wang, 1994). An auxiliary electrode is normally made up of inert metals like platinum.

### **Gas inlet**

Dissolved oxygen can be reduced leading to interference if the reduction potential of the analyte is closer or equivalent to that of oxygen. Therefore, there is a need to purge solutions with inert gases such as nitrogen or helium to flush out oxygen from the electrolyte. During the experiment an inert gas blanket is maintained above the electrolyte to ensure oxygen free conditions. However, some reactions may require oxygen (Lei *et al.*, 2003; Mulchandani *et al.*, 2005) and the same port can be used to introduce oxygen.

### **Classification of electrochemical cells and techniques**

Electrochemical cells can be electrolytic or galvanic (voltaic). In the latter, the chemical reaction produces electrical energy and occurs when the cell circuit is closed. The voltage is determined by the difference between the two half reactions

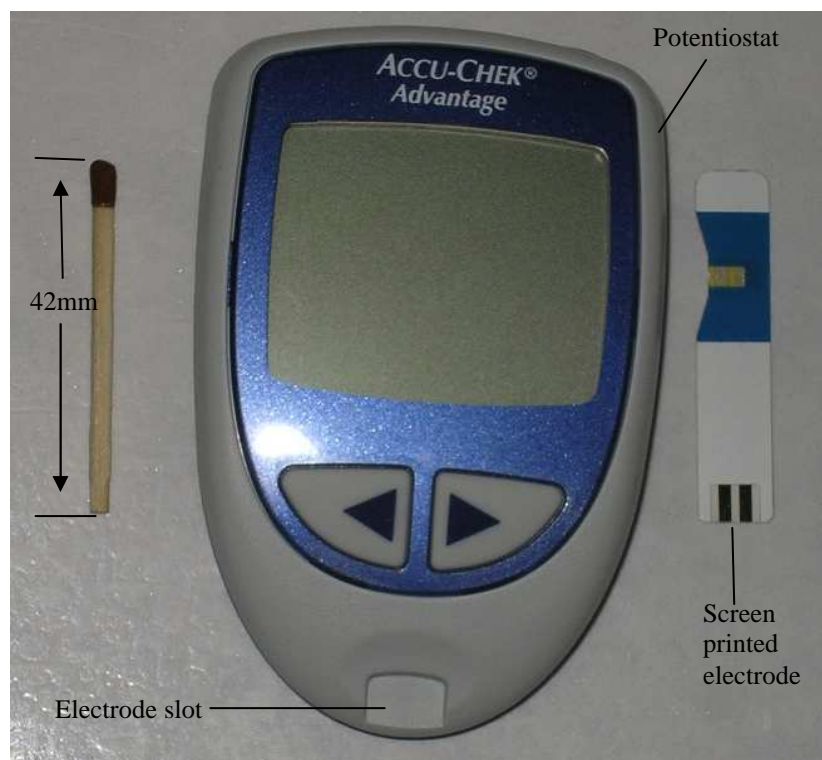
(Wang, 1994; Skoog *et al.*, 1996). Electrolytic cells are common in biosensor development. Techniques that use galvanic cells are called potentiometric and those that use electrolytic cells are called potentiostatic. Potentiometric techniques (zero-current methods) are used for generating information about sample compositions through establishment of potential across membranes. Different membranes possess unique ion recognition properties. This forms the basis of selectivity in potentiometric biosensors.

Potentiostatic techniques (controlled-potential) are based on dynamic systems. The electrode potential drives redox reactions on species of interest and the resultant current is measured (Wang, 1994; Skoog *et al.*, 1996). The current generated is proportional to the concentration of an electroactive species. Amongst the potentiostatic techniques are cyclic voltammetry, linear sweep voltammetry and amperometry. Potentiostatic measurements were performed in this work.

The cyclic voltammetric technique entails a linear increase of potential to a particular switch potential where the voltage is changed back linearly to the starting value. A diagram of current generated versus the potential (voltammogram) can be plotted and used to identify the redox potential of the electroactive components and whether the redox reactions are reversible (Skoog *et al.*, 1996). These techniques help in understanding the behaviour of a molecule and form the basis of selection of the type of transducer for the compound. Once the redox potential of a species has been established, the potential may be fixed for further studies of that species. The fixed potential technique is called amperometry (Skoog *et al.*, 1996). Most biosensors have amperometric transducers.

The advent of miniaturisation has brought tremendous advancement to electroanalysis (Göpel and Heiduschka, 1995; Zhang *et al.*, 2000; D'Souza, 2001). This has led to development of portable biosensors using screen printed electrodes and portable battery operated potentiostats. Screen printed electrodes comprise of thin films of the three electrodes (sometimes two) embedded on a non-conducting surface (Zhang *et al.*, 2000). One unit can measure 5 x 2 cm (Fig. 1.9). Construction of screen printed

electrodes is highly cost effective. These are mass produced and can be disposed after a single use. They can measure microlitre quantities of analyte. An example of a biosensor based on screen printed electrodes and portable potentiostat is the blood glucose biosensor (Fig. 1.9).



**Figure 1.9** A portable potentiostat and screen printed electrode (miniaturised biosensor). A matchstick (42 mm long) gives an indication of the relative size of the instrument.

### 1.5.2 Developments in biosensors for *E. coli* detection

Several researchers have developed biosensors for *E. coli* and coliforms in laboratories. However, employing these biosensors in the water sector has been met with a variety of challenges which include a poor market to offset the developmental costs, low sensitivity when compared to 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; Kissinger, 2005; Lazcka *et al.*, 2006).

Pyun *et al.* (1998) developed an *E. coli* biosensor using a flexural plate wave transducer. The minimum LOD was above  $10^3$  CFU/ml although the time of analysis was less than an hour. This is not applicable to drinking water due to the high LOD. High LOD yields false negatives since the generally maximum acceptable limit is a single CFU per 100 ml of water intended for drinking purposes (Tallon *et al.*, 2005).

Pérez *et al.* (2001) developed an amperometric culture-based biosensor for *E. coli* detection using the GAL substrate 4-aminophenyl- $\beta$ -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 that can be of non-faecal origin (Stevens *et al.*, 2001), and thus, may not accurately detect *E. coli*. Nistor *et al.* (2002) amplified the 4AP signal 4.5 fold using cellobiose dehydrogenase and detected about  $10^4$  CFU/100 ml in less than an hour. Reliance on a pre-culturing step is not desirable for 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 improved LOD of the biosensor was ten CFU/ml 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. The use of such a biosensor for adequate monitoring of water samples would leave the water consumers potentially vulnerable. These biosensors with high LOD are more applicable to food samples that are rich in nutrients to support the rapid growth of microbes.

LaGier *et al.* (2005) designed a biosensor for *E. coli* detection based on nucleic acid hybridisation. However, the LOD is too high ( $10^7$  cells) for reliable use in drinking water. Sun *et al.* (2006) developed a piezoelectric quartz crystal (PQC)/DNA biosensor that has an *E. coli* LOD of 23 cells/100 ml. The LOD allows reliable usage

of the biosensor in recreational water and bathing water, but not in drinking water (Tallon *et al.*, 2005; Sun *et al.*, 2006).

Tang *et al.* (2006) developed a bienzyme biosensor for *E. coli* (using pure cultures) with a LOD of  $9.7 \times 10^2$  cells/ml and a detection time of 3 h. The LOD is too high considering the microbial standards for drinking water (Tallon *et al.*, 2005). In addition, the biosensor signal is based on polyphenolic compounds, which may present selectivity problems and background interferences in the environment.

## 1.6 Project Justification

Ability to rapidly test microbiological quality of drinking water is a powerful tool for meeting water quality standards and guidelines, risk assessment and management systems. It can also be important for educational purposes especially in outreach programmes for sanitary awareness (Sobsey and Pfaender, 2002). The current water quality assessment is based on the end-point monitoring. The world health organisation (WHO) stressed the need to revise such an approach and use hazard analysis and critical points (HACCP) for preventative risk management (Fewtrell and Bartram, 2001; Howard, 2003; WHO, 2004). Plans to implement these have been underway in different countries, for example, the drinking water quality framework of South Africa (Sinclair and Rizak, 2004; Hodgson and Manus, 2006). However, the goals can be easily achieved in presence of continuous or semi-continuous on-line methods of microbiological water monitoring.

Real-time and on-line monitoring are key factors for consideration in current method developments for continuous indicator organism assessment to meet early warning requirements and water safety plans. However, all of the discussed techniques cannot afford real-time monitoring for early warning system(s). To address this, research has focused on biosensor development for indicator organisms (Leonard *et al.*, 2003; Lazcka *et al.*, 2006).

While great innovations have been made in a bid to improve methods of indicator organism detection, attention has now focused on methods that try to satisfy the market needs which are: real-time detection, short analysis time, low cost, high sensitivity and ease of use. The total analysis time per water sample has indeed been considerably reduced, but further reduction in the time of analysis to obtain results within a working day or even shorter time is still desirable (Pyun *et al.*, 1998; Sartory and Watkins, 1999; Venter, 2000; Rompré *et al.*, 2002; Leonard *et al.*, 2003). Based on the fact that enzyme mediated reactions are rapid (Holme and Peck, 1998), the aim of this work was, therefore, to contribute to the improvement of faecal indicator organisms detection using direct enzyme assays with an appropriate substrate. This contribution will hopefully complement efforts to implement the much required early warning system and the water quality framework for enhanced public health protection in South Africa (Hodgson and Manus, 2006) and the Southern African region as a whole.

Most developments and improvements have remained excellent laboratory protocols for cultures and seeded pure water samples. The major reasons for this are the challenges met in applying such methods to the heterogeneous environment (Rogers, 1995; Sartory and Watkins, 1999; Kissinger, 2005). Since variation between the controlled laboratory and open field environments exists, *in situ* assays using typical South African polluted river water samples were employed in this study. This will ensure rapid application of the methods in the field, as well as concomitant proof of concept and field optimisation.

## 1.7 Hypothesis

The *E. coli* GUD can be found in the extracellular environment from which it hydrolyses glucuronide-based substrates. There is a positive linear relationship between the activity of this enzyme and *E. coli* counts as observed by Farnleitner *et al.* (2001; 2002) and Garcia-Armisen *et al.* (2005). Enzyme catalysed reactions occur rapidly (Holme and Peck, 1998). It is against this background that the hypothesis for this work, which is: GUD 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 in South Africa river waters, was postulated.

## 1.8 Objectives

The ultimate objective of this work was to prove the concept of an alternative faecal pollution detection method, using GUD, and to evaluate possible avenues for its implementation in (South) African river waters. This was accomplished by addressing the broad specific objectives, which were to:

- (a) optimise an *in situ* GUD assay and select a suitable substrate,
- (b) perform enzyme concentration and bioinformatic studies,
- (c) evaluate the effects of compounds commonly occurring in water and those used in water treatment on GUD,
- (d) investigate the use of liquid and strip probes, and
- (e) explore possibilities of electrochemical biosensor detection of GUD activity.

## 1.9 Research Approach and Organisation

Execution of this work was divided into a fundamental study and an applied study. Insight gained from the fundamental study was used to support an applied study in order to establish likely alternative methods for GUD monitoring in polluted water. *In situ* assays were performed using appropriate samples from running and stagnant water sections of a typically polluted river in (South) Africa. Where applicable, commercial (reference) GUD assay was run in parallel to serve as a positive control.

The work is presented in nine chapters as follows:

Chapter 1 contains the general project literature review, Chapter 2 deals with *in situ* assay optimisation using chromogenic and fluorogenic substrates, while Chapter 3 comprises enzyme profiling studies. Chapter 4 contains work on interference studies

by selected compounds on the enzymes and an investigation of the presence of some of these compounds in the water samples. Chapters 5 and 6 deal with applied aspects and contain data about liquid and strip probes, respectively. Chapter 7 explores possibilities of electrochemical detection of a GUD substrate breakdown product (PNP). Chapter 8 attempts to improve sensitivity of the electrochemical detection through working electrode modifications. Chapter 9 contains the overall discussion, conclusions of the thesis and future perspectives. The experimental chapters (2 to 8) are further subdivided into introduction, materials and methods, results, and discussion and conclusions sections.

## 2 SUBSTRATE SELECTION AND ASSAY OPTIMISATION

### 2.1 Introduction

In order to establish an enzyme assay, a number of steps are implemented to determine the conditions under which the enzyme will operate optimally. This process is called assay optimisation. Optimising an assay for a new enzyme is based on previously published assay procedures and general characteristics of closely related enzymes. Once the established protocol for a purified enzyme is in place, it can be used as a basis for an *in situ* assay method of the same enzyme in the environment. Parameters considered during optimisation of *in situ* assays include enzyme concentration, assay volume and time, pH and temperature. The relative location and distribution of the enzyme in the natural environment has to be determined before the optimal assay conditions can be established. The assay components should allow a linear, stable and consistent reaction over time to avoid rapid and inconsistent reactions that are difficult to monitor. Once these parameters are established, scaling up and down of assay volumes can be performed with ease.

#### 2.1.1 Assay period

The total assay time for an enzyme reaction is a function of the interplay of factors such as enzyme and substrate concentrations, pH and temperature. Accurate determination of enzyme activity requires the establishment of a linear range of maximum product accumulation using information obtained from progress curves (a graphical plot of product formation versus time). The shape of progress a curve can be used to gain an insight in the mechanism of the enzyme reaction involved. Enzymes from different sources or from the same source operating in different environments exhibit varying shapes of progress curves with some having lag phases, bursts and plateaus (Tipton, 2002). When enzyme concentration is low (leading to slow product accumulation) or when long lag phases are observed, an interval that exhibits a linear relationship of product accumulation over time must be established to limit the readings to such time periods in subsequent assays.

### 2.1.2 Roles of pH and temperature in enzyme assays

pH plays an important role in the activity of enzymes because it is responsible for the ionisation of active site residues. The pH profile of an enzyme can be explained in terms of the  $pK_a$  values of crucial residues at the active site (Stryer, 1988). An enzyme is expected to have at least 50 % activity between the pH values equivalent to the  $pK_a$  values of the crucial amino acid residues at the active site. Thus the closer the  $pK_a$  values of the residues, the narrower the pH range of operation, and vice versa (Engel, 1977). In addition, pH affects the structural stability and solubility, the charge and charge distribution on both the enzyme and substrate (Holme and Peck, 1998). These factors, in turn, alter bonds and bonding patterns, ultimately determining the rate of an enzyme-catalysed reaction.

Temperature dictates the frequency of molecular collisions and bond vibrations. An increase in temperature (usually up to 40 °C) increases the frequency of collisions between substrates and enzymes, hence increasing the rate of an enzyme catalysed reaction. Increased bond vibrations with an increase in temperature lead to bond disruptions that can facilitate easy and rapid substrate breakdown. However, high temperatures also lead to enzyme denaturation due to excessive bond vibrations that alter the enzyme's functional structure. Thus, the temperature optimum of an enzyme's activity is a compromise between the enzyme's stability and maximum activity (Holme and Peck, 1998).

Enzymes have unique pH and temperature profiles. Similar enzymes obtained from different environments can require different optimum assay conditions. Wakabayashi and Fishman (1961) noted variations in the pH optimum of GUD from different sources using phenolphthalein- $\beta$ -D-glucuronide (PHEG) as a substrate. Diez and Cabezas (1979) noted different pH and temperature optima for two molecular forms of GUD from a mollusc *Littorina littorea* L. These characteristic features can be used in setting conditions that allow the selective detection of enzyme activity from a specific source in the environment. In other instances, an enzyme from a similar source can have different optimum conditions dictated by the type of substrate used.

### 2.1.3 Enzyme location

Designing an accurate *in situ* enzyme assay protocol requires knowledge of the relative location and abundance of the enzyme. Enzymes in aquatic environments can be free floating or particulate bound, intracellular or extracellular (Chróst, 1991). Different techniques such as filtration and centrifugation can be employed to determine the location of particulate bound and free-floating enzymes in the environment (Chróst, 1991; Farnleitner *et al.*, 2002). Chemical and mechanical cell permeabilisation methods (Rotman, 1956; Woldringh and Van Itersen, 1972; HsuChen and Feingold, 1973; Chróst, 1991; D'Souza, 2001; Roe, 2001) can be employed in the determination of the relative cellular location of enzymes. However, caution must be exercised to avoid use of excessively high permeabiliser concentrations which will inhibit the enzyme in question.

### 2.1.4 Photometric enzyme assays

Photometric enzyme assays can be subdivided into absorbance or turbidimetric (involving measurement of light absorbance or transmittance) and fluorometric assays (which involve measurement of fluorescence). Measurement of absorbed or transmitted light involves coloured compounds in the visible light range (400–750 nm). Although absorbance techniques are cheap and easy to execute, they are not sensitive at low compound concentrations, due to the small difference between incident and transmitted light. Turbidity is based on light scattering (as opposed to absorbance) by a solution and takes into consideration the clarity of the solution. Turbidimetry is favoured when the enzyme substrates are insoluble polymers (John, 2002).

Fluorescence enzyme monitoring methods are based on the emission of light energy when excited compounds return to their ground states. As with absorbance spectroscopy, most compounds are excited and emit energy at distinct wavelengths (Gordon, 2001; John, 2002). Fluorometric techniques are more sensitive than the absorbance methods, because the fluorescence comparison is made in the complete

absence of light in the presence of the fluorochrome. However, fluorometric techniques are relatively more expensive than absorbance spectroscopic methods due to the high costs of fluorogenic substrates and instrumentation (John, 2002). Furthermore, they are subject to interference by natural fluorochromes in the environment (Pletschke *et al.*, 2006) and attempts to remove the contaminants will translate to high costs.

There is a paucity of information on the properties and assay conditions of environmental GUD. Therefore, the major objectives of the work covered in this chapter were to establish optimum assay conditions for GUD using the best chromogenic substrates available based on a previous pilot study performed by Watson and Pletschke (unpublished data) and to evaluate the suitability of using a fluorogenic substrate with environmental samples. The specific objectives were to:

- (a) determine appropriate sample volumes to be used in enzyme assay,
- (b) determine the time period that exhibited a linear, but maximal, reaction,
- (c) determine the pH and temperature optima for environmental GUD,
- (d) explore the feasibility of using fluorogenic substrate for GUD assay and
- (e) investigate the location and abundance of GUD in the river samples.

## 2.2 Materials and Methods

### 2.2.1 Sampling site

The Bloukrans river, which flows through the city of Grahamstown (Eastern Cape, South Africa), was selected for *in situ* assays. Previous water analyses from this river showed 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 (Fig. 2.1).



**Figure 2.1** The running (moderately flowing) water (a), stagnant water (b) and fast flowing water (c) sampling sites. X shows the exact site where the water samples were collected from.

### 2.2.2 Sampling

Water collection was performed in accordance with standard procedures outlined in *Standard Methods* (2005). Water samples were collected (between 08:00 and 08:30) aseptically in 250 ml sterile Pyrex glass bottles (Schott Duran, Germany), placed on

ice, transported immediately to the lab and analysed within an hour. The temperature was measured both on site and on arrival in the lab. Sample pH was also determined. Samples were collected in triplicate for studies in each case.

### 2.2.3 GUD assays

The fluorogenic substrate was used only in volume and pH studies, as the fluorogenic assays were subsequently discontinued due to their lack of feasibility.

#### Chromogenic substrate assay

Ten millimolar PNPG (Calbiochem – Merck, Darmstadt, Germany) was prepared in the assay buffer (0.1 M Tris-HCl, pH 8.0 containing 0.6 mM CaCl<sub>2</sub>). Commercial *E. coli* GUD (EC 3.2.1.31, Sigma, Steinheim, Germany) was dissolved in 20 mM sodium phosphate buffer, pH 6.8, to give a stock standard concentration of 500 µg/ml.

The standard PNPG assay was adapted from Fisher and Woods (2000) and Aich *et al.* (2001<sup>a</sup>). Assay buffer (110 µl) and 30 µl enzyme solution were mixed in a 96-well, flat-bottomed microtitre plate (Appendix A). The reaction was initiated by the addition of 110 µl PNPG. Kinetic readings (10 min at 30-sec intervals) were performed against a substrate control (buffer + substrate), at 405 nm and 25 °C using a spectrophotometer (Power Wave<sub>x</sub>, BioTek Instruments, USA). This standard assay was used as a starting point for optimisation of the assay for use in the environment (water). An illustration of calculation of the activity is given in Appendix B.

In all the environmental assays performed, two sets of controls were set up; an enzyme control (buffer + enzyme) and a substrate control (buffer + substrate). All readings were taken against milli Q water (Millipore Corporation, USA). 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. Standard deviation values were used for error bars on the graphs that were plotted using the mean values.

**Fluorogenic substrate assay**

An assay buffer of 0.1 M sodium phosphate (pH 7.0) was used and the stop buffer was made up of 0.2 M NaCO<sub>3</sub> (pH 10.4). A concentration of 7.1 mM 4-Methylumbelliferyl- $\beta$ -D-glucuronide [MUG (Fluka, Steinheim, Germany)] was prepared in milli Q water.

The GUD assay was adapted from Farnleitner *et al.* (2001). Briefly stated, 0.5 ml of MUG was added to 0.45 ml assay buffer and left standing at room temperature ( $22 \pm 2$  °C) for 30 min (to equilibrate), after which 50  $\mu$ l GUD or sample was added. This was briefly vortexed and 0.2 ml aliquots were removed at suitable intervals into separate test tubes with 1 ml of 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 slit widths were set at 10 nm.

**2.2.4 Linearity over time**

As enzymes in river water were anticipated to be more dilute and to behave differently due to the presence of pollutants, it was necessary to establish a fixed time assay and to determine the most appropriate time for monitoring the enzyme reaction, in order to work within the linear range of the assay (Pletschke *et al.*, 2006). This was performed by substituting the commercial enzyme with the environmental/river water sample in the standard spectrophotometric assay described above. Absorbance readings were taken at 15 min intervals for the first 3 h and then subsequently at 2 h intervals for the remaining 21 h.

**2.2.5 Volume optimisation**

For the spectrophotometric assay, volumes of river water samples were varied between 30  $\mu$ l and 90  $\mu$ l, with corresponding adjustments to buffer volumes in order to obtain a fixed total volume of 140  $\mu$ l (enzyme + buffer). Assays were performed at room temperature ( $20 \pm 2$  °C). The river sample volume that yielded the highest and most reproducible activity was used in all subsequent assays.

In the fluorometric assay, the reaction mixtures were proportionally adapted, varying river water volumes from 50 to 250  $\mu\text{l}$  at 50  $\mu\text{l}$ -intervals. The volumes of the buffer were adapted in accordance with the volume of environmental sample added.

### 2.2.6 UV observations

Sample tubes containing the enzyme reaction mixtures, substrate and enzyme blanks were visualised under UV illumination to detect any noticeable change in fluorescence due to the GUD activity in the water samples.

### 2.2.7 pH optimisation

GUD activity assays for both chromogenic and fluorogenic substrates were performed at different pH values using different buffers at 0.1 M (Table 2.1). “Bracketing” of buffer systems at “border” pH values was employed to account for change in enzyme activity due to the buffer system used.

**Table 2.1** Buffer systems used for pH studies in GUD assays.

Buffer	pH values
Sodium acetate	5.0 & 6.0
Sodium phosphate	6.0 & 8.0
Tris-HCl	7.0 – 9.0
Carbonate/bicarbonate*	9.0 – 11.0

\* Fluorometric assays were performed up to pH 9.0 only.

### 2.2.8 Temperature optimisation

GUD assays in river water samples were performed at temperatures of 4, 12, 20, 25, 30, 35, 40, 45 and 50  $^{\circ}\text{C}$ , respectively. The reaction volumes were increased to 10 ml. Samples were withdrawn at suitable time intervals for absorbance readings. The

optimum values for pH and volume established in the previous sections (sections 2.2.5 and 2.2.7) were used.

### **2.2.9 Fractionation studies**

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

Particulate matter and cells were pelleted by centrifugation at 16 000 g at  $4 \pm 1$  °C for 10 min. Supernatant (first supernatant) was retained for sonication and enzyme assay, while the pellet (first pellet) was washed in milli Q water and centrifuged as described above. The pellet wash was retained for enzyme assay. The washed pellet was resuspended in assay buffer. One millilitre aliquots each of first supernatant (designated sonicated supernatant after sonication) and washed pellet (later called sonicated pellet) were sonicated for 2 min at 25 W intensity and  $4 \pm 1$  °C (30 s sonication period with 15 s resting intervals, on ice) using a Vibra Cell™ Sonicator (Sonics & Materials, USA). GUD activities for the six fractions (whole fraction, first supernatant, pellet wash, first pellet, sonicated supernatant and sonicated pellet) were determined.

### **2.2.10 Permeabilisation studies**

Five methods of cell permeabilisation were employed to investigate the effects of different cell permeabilisation methods on GUD activity in stagnant/standing and moderately flowing (running) water samples.

#### **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 of 0.4 % (w/v). This was briefly vortexed and placed on ice for 5 min (Roe, 2001), after which enzyme assays were performed.

**Sonication**

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

**Triton-X100**

A final concentration of 0.5 % (v/v) Triton-X100 was achieved by adding 250  $\mu$ l of 2.5 % (v/v) Triton-X100 to 1 000  $\mu$ l of sample. This mixture was briefly vortexed and left at room temperature ( $20 \pm 2$  °C) for 45 min before assaying for GUD activity (Roe, 2001).

**Toluene**

Toluene (100  $\mu$ l) was added to 1 000  $\mu$ l of water sample and incubated at 37 °C for 10 min (Roe, 2001) for cell lysis to occur, after which the enzyme activity was determined.

**Polymyxin B (PxB) sulphate**

The PxB sulphate permeabilisation method was adapted from Van Poucke and Nelis (1997). A final concentration of 100  $\mu$ g/ml PxB sulphate (ICN Biomedicals, Ohio, USA) in the water samples was used, briefly vortexed and incubated for 45 min at 37 °C before performing enzyme assays.

**2.2.11 Statistical analyses**

Mean and standard deviation calculations, and comparison of data sets (using analysis of variance (ANOVA)) performed with Microsoft Excel 2003 statistical tools. The ANOVA was performed at 5 % level of significance.

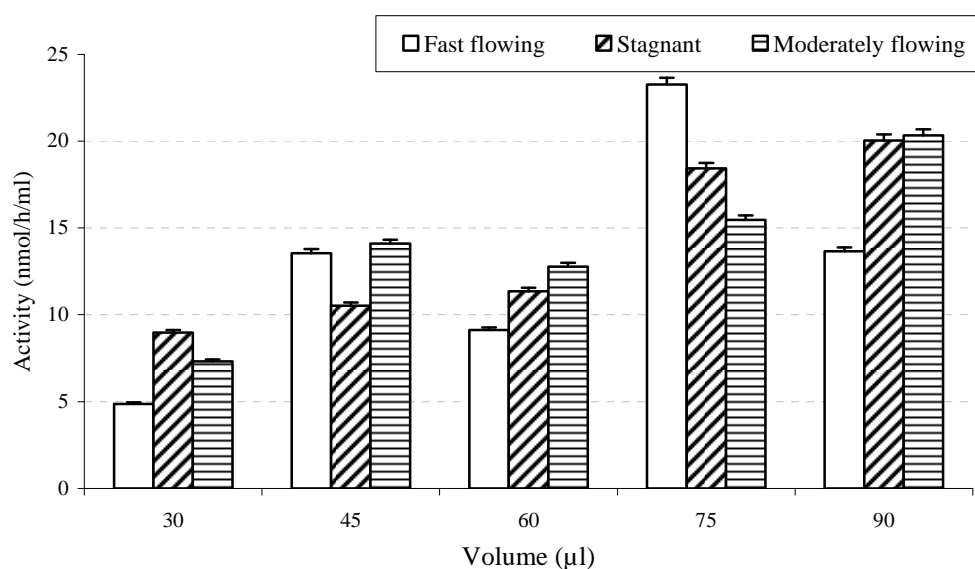
**2.3 Results****2.3.1 Linearity over time**

Linearity was confirmed over a period of 24 h. However, there was variation with regards to the length of the lag phases. Some samples did not have any lag phase,

while in others the lag phase was as long as 5 h (Appendix C). 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 simple end point reading for every sample in the GUD assays was not possible. Hence, readings were taken at hourly intervals to get an interval for the maximum rate of *p*-nitrophenol production over time.

### 2.3.2 Volume optimisation

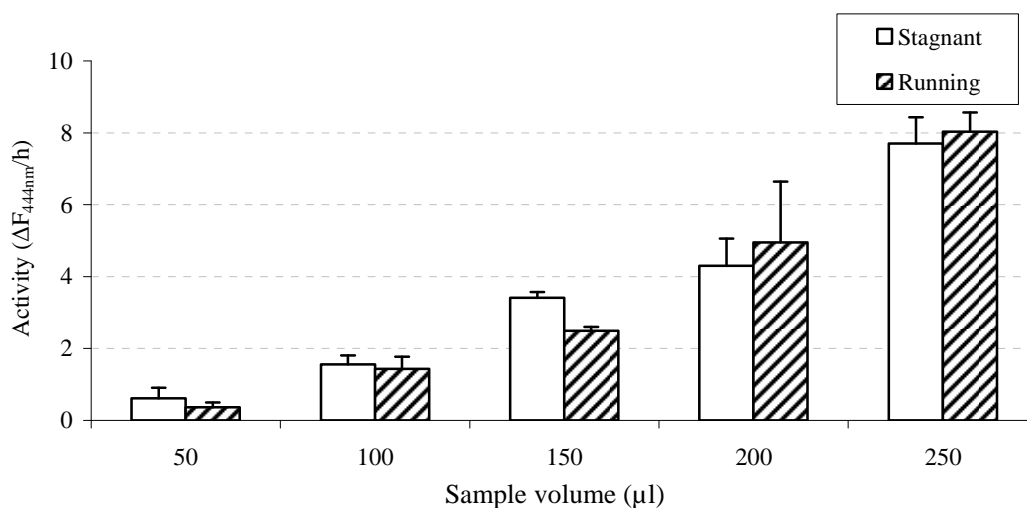
There was an increase in GUD activity with an increase in environmental water volume to 90  $\mu\text{l}$  for both stagnant and running water samples (Fig. 2.2). However, the trend for the fast flowing water was inconsistent (Fig. 2.2). From these results, 90  $\mu\text{l}$  was selected for all subsequent assays of the stagnant and moderately flowing water. The fast flowing water sample was omitted in subsequent studies, based on the inconsistent results obtained and the fact that most drinking water in rural Africa is obtained in stagnant to slow flowing pools.



**Figure 2.2** Effects of varying sample volume on GUD assay. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

There was a direct relationship between GUD activity and the volume of water samples added in fluorometric assay (Fig. 2.3). However, the degree of error increased with an increase in volume of water sample added to the assay mixture (Fig.

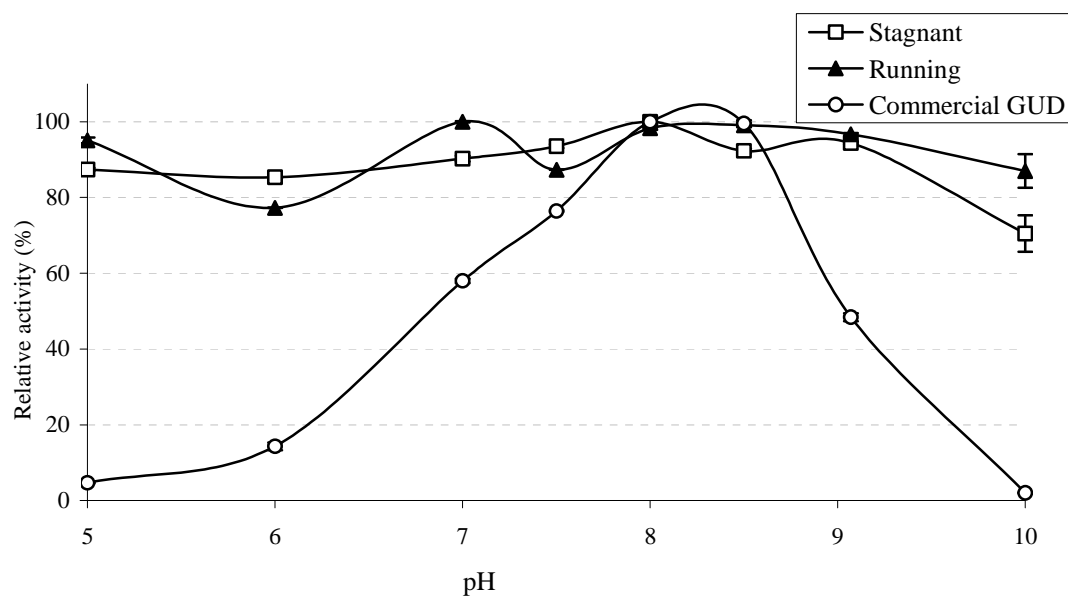
2.3). For the determination of the effect of pH on GUD in fluorometric assay, the 250  $\mu\text{l}$  sample volume was employed.



**Figure 2.3** Effect of river water sample volume on MUG hydrolysis. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

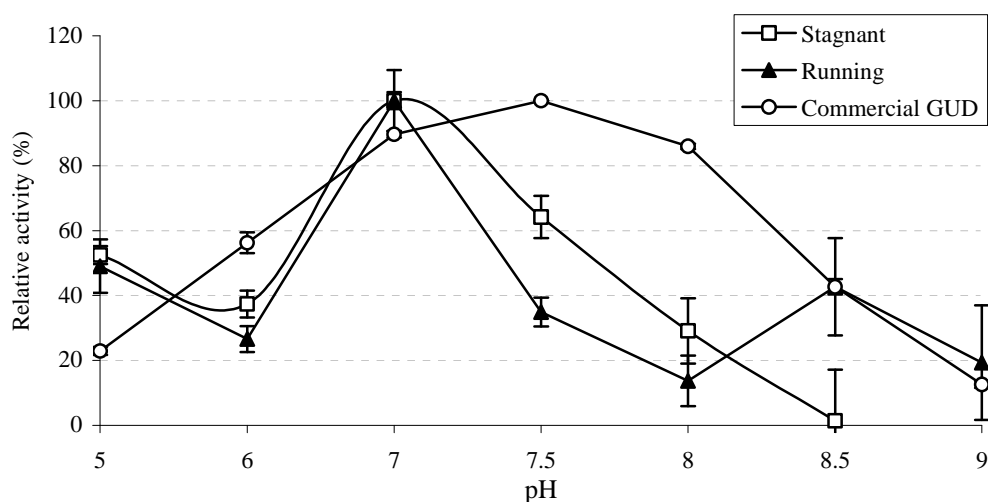
### 2.3.3 pH optimisation

For spectrophotometric assays, GUD activity for river samples was constant between pH 5.0 and 10.0 (Fig. 2.4). The carbonate buffer (at pH 11) led to inflated values due to spontaneous PNPG hydrolysis. This was accompanied by increased error, and hence results were not regarded as reliable. Insignificant ( $P > 0.05$ ) variability in GUD activity with changes in pH in standing and running water samples was observed. The two samples showed similar profiles for GUD activity from pH 5.0 to 9.0 (Fig. 2.4). All subsequent GUD assays were performed at pH 8.0 for the remainder of the study. Water intended for drinking purposes rarely has a pH far removed from neutrality and therefore performing GUD assays at pH 8.0 is appropriate. The pH optimum for the commercial GUD was also around 8.0, but the enzyme exhibited a narrower range of pH optima (Fig. 2.4).



**Figure 2.4** Effects of pH on the activities of commercial GUD, and GUD in stagnant and running water samples in the spectrophotometric analysis. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

In fluorometric GUD assays, the enzyme activity fluctuated with changes in pH. The highest GUD activity for stagnant and running water samples was obtained at pH 7.0 (Fig. 2.5). Increases in enzyme activity were mostly accompanied by concomitant increases in error. Several samples were analysed in an attempt to reduce the error margin for the assays but this was unsuccessful. The commercial GUD exhibited a pH optimum of 7.5 (Fig. 2.5).



**Figure 2.5** Effects of pH on MUG hydrolysis by commercial, stagnant and running water GUD in the fluorometric analysis. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

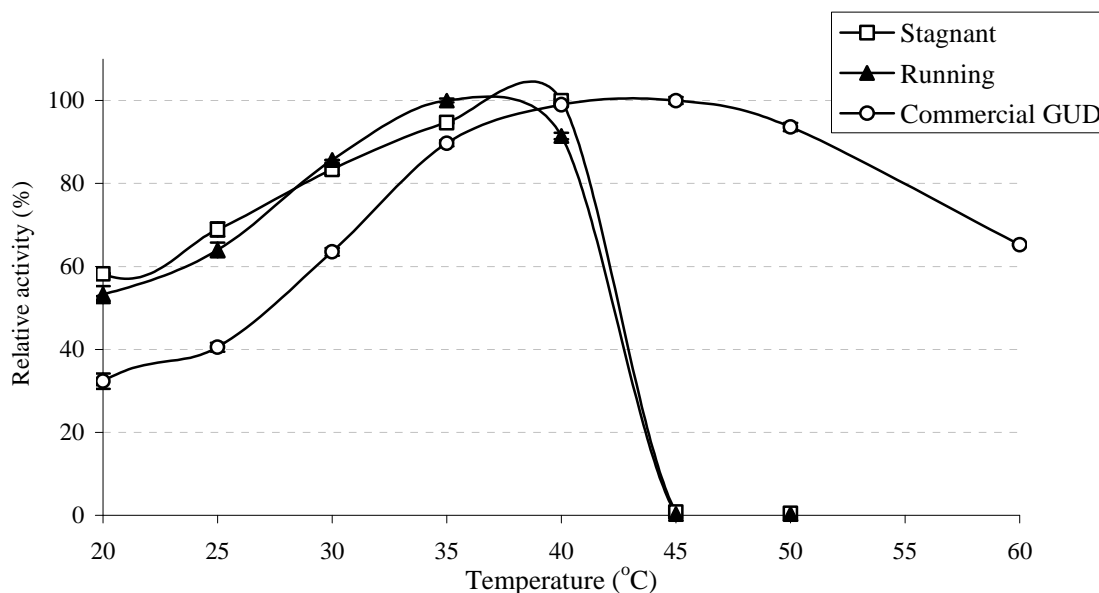
### 2.3.4 UV visualisation

Both the enzyme reaction mixture and the uncatalysed substrate fluoresced under UV illumination. No visual distinction could be made between their fluorescence intensities. On the basis of the inconsistencies in pH optimisation in section 2.3.3 (above), and the absence of visual distinction between MUG and the reaction it was decided to discontinue the use of fluorometric assays in this study.

### 2.3.5 Temperature optimisation

The response of GUD to low temperatures (4 and 12 °C) was unreliable and there was precipitate formation observed in the assay mixture at these low temperatures. GUD activity in running and stagnant water samples increased from 20 °C to 35 and 40 °C, respectively (Fig. 2.6). The differences in GUD activity between the two environmental samples (between 20 and 40 °C) was not significantly different ( $P > 0.05$ ) (Fig. 2.6). There was also no activity observed for both samples above 45 °C while the temperature optimum for commercial GUD was 45 °C (Fig. 2.6). Room temperature ( $20 \pm 2$  °C) was selected for further enzyme assays as there was sufficient

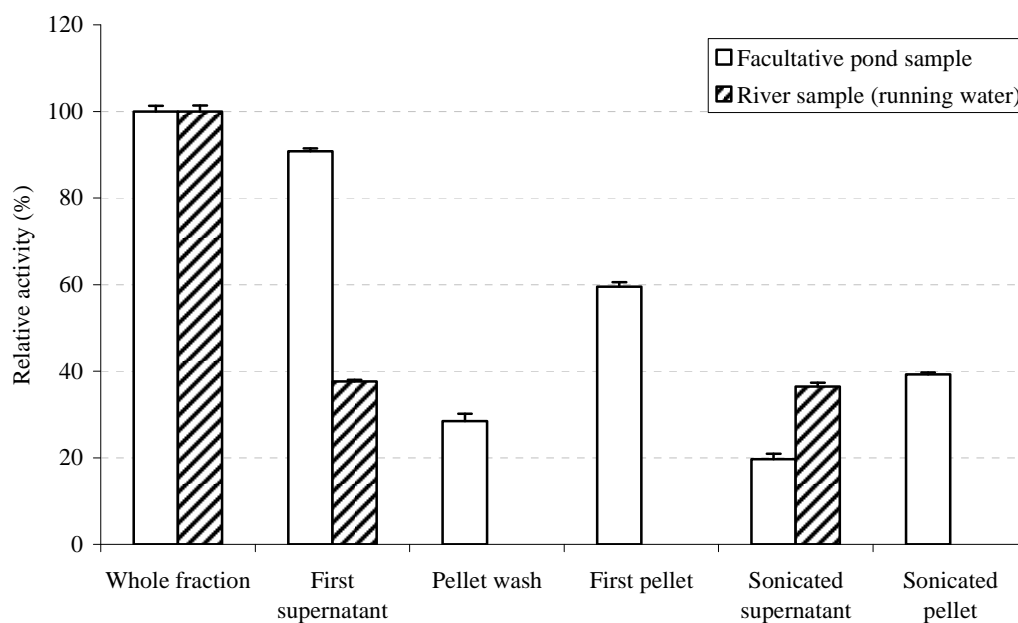
activity at this temperature, and to circumvent the requirement of heating baths and other expensive equipment in remote/rural areas.



**Figure 2.6** Effects of temperature on activities of commercial, stagnant and running water GUD. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

### 2.3.6 Fractionation studies

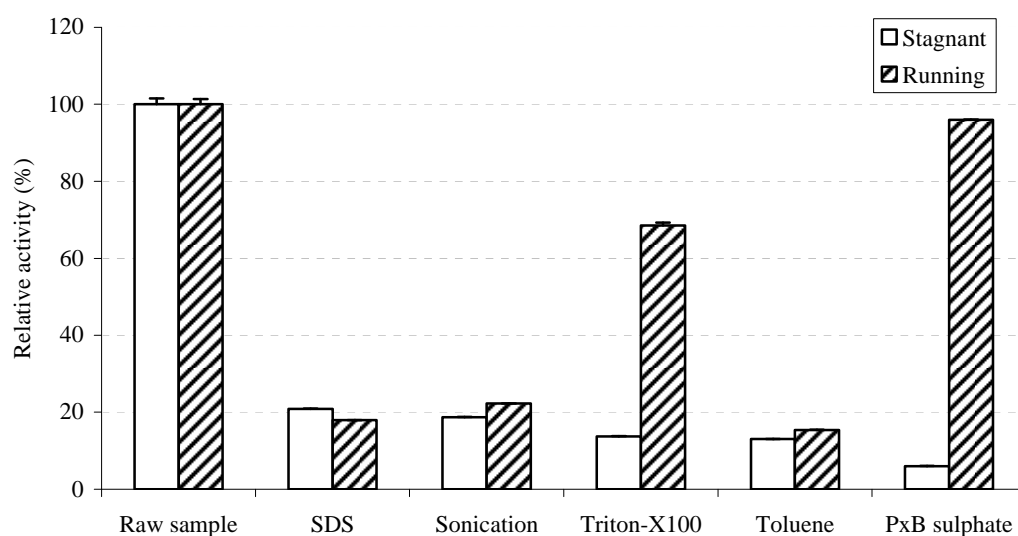
There was no pellet observed for both stagnant and running river water samples and hence one (running) environmental water sample was used. Therefore, a portion of the sample was stored as first supernatant and the remainder sonicated (Fig. 2.7). In contrast, the facultative pond sample yielded all six fractions. The whole fractions exhibited the highest GUD activity (Fig. 2.7). Supernatant 1 (first supernatant) of the facultative pond sample showed the highest activity among the treated fractions, followed by the pellet 1 (first pellet) and then the sonicated pellet (Fig. 2.7).



**Figure 2.7** Relative activity (%) of GUD in different fractions of aquatic samples. All data points represent the mean  $\pm$  SD (n = 3).

### 2.3.7 Permeabilisation effects

The different permeabilisers investigated inhibited GUD activity except for polymyxin B (PxB) sulphate in the running water sample (Fig. 2.8). However, polymyxin B sulphate was the most potent inhibitor of GUD in stagnant water. The extent of inhibition by Triton-X100 differed in the two samples, with the enzyme in stagnant water being more susceptible (showing 86.27 % reduction in activity) to Triton-X100 addition than that in running water (4.04 % reduction in activity). Sonication, SDS and toluene had a similar inhibitory effect on GUD activity during treatment (Fig. 2.8).



**Figure 2.8** Effects of different permeabilisation methods on GUD activity. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

## 2.4 Discussion and Conclusions

### 2.4.1 Discussion

The variation in the lag phases can be explained by the possible presence of inhibiting compounds in the polluted river water samples. Some enzyme inhibition can be reversible over time, especially due to inhibitor decomposition. The duration of the lag phase can be directly proportional to the period of inhibition reversal or inhibitor compound deterioration with time, as is the case with phenols (Wetzel, 1991; Tipton, 2002). Therefore; when inhibitor concentration is high, the time required for reversal is prolonged, thus leading to prolonged lag phases. Prolonged lag phases were noted when samples were concentrated (Chapter 3; Appendix C) further supporting the presence of protein inhibitors, as the concentration techniques employed later in the study increased the concentration of the potential protein inhibitors present in the samples.

Another possible explanation for lag phases could be the presence of a more preferred natural substrate in the water samples. In the presence of more preferred natural substrates, like glucuronides, the enzyme will cleave such substrates first (during the

lag phase) before catalysing the breakdown of the synthetic substrate (PNPG) (Tien and Kirk, 1988). Thus the period of the lag phase can be directly proportional to the amount of the natural substrates available in the sample. The unpredictability of the lag phases highlighted one of the difficulties faced when executing *in situ* assays. However, *in situ* assays remained the most viable option for minimising the time required for the development and environmental adaptation of a rapid method for the detection of indicator organisms.

An increase in enzyme activity with an increase in river sample volume suggested an increase in enzyme concentration with increase in sample volume, as expected. Enzyme denaturation and inhibition by the pollutants inherently found in the water, and dilution by the river water can possibly account for the lower activity observed in river water 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 sample volumes was accompanied by a corresponding decrease in the volume of the assay buffer.

Glutamic acid and tyrosine are important active site residues for GUD (Chapter 1). Glutamic acid has a  $pK_a$  value of 4.3, while the  $pK_a$  value for tyrosine is 10.1 (Garrett and Grisham, 1999). Therefore, GUD is expected to operate at least between the pH values (4.3 and 10.1) equivalent to the  $pK_a$  values of the important active site residues (Engel, 1977). The optimum pH for *Escherichia coli* GUD ranges between 5.0 and 8.0 (Gilissen *et al.*, 1998; Aich *et al.*, 2001<sup>b</sup>). The observed wider range (pH 5.0 to 9.0) is not unusual because the river samples used were not pure. In addition, the polluted environment probably increased the enzyme resistance to a change in pH. Furthermore, protection by other pollutants could also have caused such a characteristic behaviour. The presence of a high concentration of carbonates in the polluted environment (Chapter 4) might have aided the buffering capacity of water (as the pH of the samples was always around 8.0), leading to a generally uniform enzyme activity over a range of pH values. The presence of GUD from different sources could also have accounted for the observed wide activity of GUD, due to presence of different GUD enzymes with different pH optima. However, GUD from plants and

other aquatic organisms have been reported to operate optimally at acidic pH values below 5.0 (Diez and Cabezas, 1979; Gilissen *et al.*, 1998; Aich *et al.*, 2001<sup>b</sup>). The assay method used (whether continuous or discontinuous) leads to different optimum pH values (Aich *et al.*, 2001<sup>a</sup>). In this study, a continuous assay method was used. The broad pH optimum range observed is advantageous to the process of subsequent bioprobe and biosensor development, since there will be no need to adjust the pH of water intended for drinking purposes, which is usually between pH 7.0 and pH 8.0.

However, it is important to note that the pH profile can also be affected by the fact that PNP colour development is pH dependent. The yellow colour that absorbs at 405 nm develops at alkaline pH. Therefore, the low activity in commercial GUD at lower pH values could be partially explained by lack of PNP yellow colour development at these pH values.

The temperature optimum for *E. coli* GUD is reported to be 45 °C (Tryland and Fiksdal, 1998). However, this was not the case for the environmental GUD in this present study. The presence of different pollutants and detergents in the environment could have contributed to rapid GUD denaturation above 40 °C. Extra equipment is required for temperature maintenance at higher assay temperatures. These imply greater costs for the proposed method. Biosensor analyses usually work with small samples that can evaporate quickly at 40 °C before the required signal is generated. Costs for maintaining high temperatures are not desirable, especially when a product is targeted for routine purposes by budgetary strained service providers. In addition, maintenance of high assay temperatures are not feasible for use in rural communities. For these above reasons, room temperature was selected for future studies using an assay volume of 250 µl.

The precipitation observed when assaying GUD at low temperatures (5 and 12 °C) may imply the lack of feasibility of using direct enzyme assay method in low temperature environments and during cold seasons. The activity pattern of GUD at lower temperatures could also have resulted from chemical interference by pollutants that formed precipitates.

The high values of error observed in fluorometric assays suggested inconsistencies and a lack of reproducibility in the technique. This can be attributed to interference by a wide variety of environmental compounds. Fluorescent assays are also prone to interference by naturally occurring fluorogenic components in the environments (Marxsen and Witzel, 1991). Although fluorogenic assays are sensitive, the interference makes them less desirable for application in the environment. In addition, fluorogenic substrates are more expensive than their chromogenic counterparts. Therefore, PNPG is a more feasible substrate and is the least expensive amongst chromogenic substrates (Aich *et al.*, 2001<sup>a</sup>). The fluorescence of the substrate under UV could have been as a result of high substrate concentration. However, a reduction in substrate concentration during *in situ* assays would limit the rate of the enzyme reaction (Meyer-Reil, 1991). The substrate concentration during an enzyme assay should be at least 10 X (ten times) the  $K_m$  to achieve 91 % of the maximum enzyme activity (Holme and Peck, 1998).

Fractionation results illustrated the abundance of the enzymes in the extracellular environment. This is not surprising, because GUD is exported to the extracellular environment when cells reach stationary phase (Doyle *et al.*, 1955). Since conditions in natural environments are often stressful (Roszak and Colwell, 1987; Roberto *et al.*, 1993; Venter, 2000; George *et al.*, 2002; Leonard *et al.*, 2003), cells are forced into stationary phase. The presence of GUD in extracellular environments is therefore not totally unexpected. In addition, the presence of natural substrates and competitors can stimulate GUD production and exportation to the extracellular environment (Adams *et al.*, 1990). The presence 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 (Pletschke *et al.*, 2006). Availability of the enzyme outside the cell provides an advantage to the desired direct enzyme assay method, as this will allow the assay of untreated (i.e. no permeabilisation procedures required) water samples. This, in turn, will reduce process costs and labour.

Results from the permeabilisation studies confirmed the extracellular location of GUD, as none of the permeabilising methods yielded a significant degree of improvement in the amount of GUD activity released. Results from fractionation studies were comparable to results of Farnleitner *et al.* (2002), who observed an insignificant difference between GUD of the filtrate and whole samples in lowly polluted waters. While the extracellular location of the enzyme is advantageous in reducing costs of the methods to be developed, it can also give rise to false positive results of microbial presence when the GUD activity persists long after cell lysis.

Inhibition of the enzyme activities by permeabilisation makes these permeabilisers undesirable for use with the polluted Bloukrans River samples. Such an inhibition could suggest 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 (Plummer, 1978; Roe, 2001; Clausell *et al.*, 2003). Inhibition of the enzyme activities were in agreement with the results obtained by Chróst (1991) when 25–92 % inhibition was noted in other 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 enzyme stabilising structures that are formed in environments (Meyer-Reil, 1991) or exposure of the enzyme to protease action from other lysed organisms in the water samples.

#### **2.4.2 Conclusions**

From these fundamental studies the following conclusions were drawn:

- (a) GUD in the polluted environment occurs at lower concentrations than in pure *E. coli* in defined media and larger sample volumes are required to achieve a reasonable level of activity,
- (b) physico-chemical properties of environmentally exposed GUD are different from those of the commercial GUD, or GUD in pure cell culture with defined media (Watson, pers. comm.),

- (c) regardless of cell growth phase, GUD occurs extracellularly in polluted water environments,
- (d) cell permeabilisation is not a required step in the direct enzyme assay for moderately polluted water samples such as that of the Bloukrans River, and
- (e) implementation of a fluorogenic substrate assay for this project or product development will adversely affect the feasibility for use in the environment, due to the observed limitations.

The next chapter will investigate the concentration of the GUD from the environment in an attempt to identify the source of GUD from the environment.

### **3 GUD CONCENTRATION AND PROFILING**

#### **3.1 Introduction**

Proteins often occur at low concentrations in the environment, and this should be addressed before further analyses can be performed. When the properties of a protein of interest are known, its unique properties can be used to selectively concentrate the protein (Dennison, 2003; Doonan and Cutler, 2004). After protein concentration, further characterisation can be performed. Enzyme activity assays assist in monitoring the presence of the required protein throughout the various stages of concentration or isolation. Subsequent determination of the origin of a protein can be achieved through peptide mass finger printing (Liebler, 2002). The work in this chapter entailed concentration and profiling of GUD to determine whether its source was in fact *E. coli*.

##### **3.1.1 Protein concentration**

Different techniques (which include precipitation with salts, organic solvents and organic polymers and freeze drying) can be used to concentrate proteins (Harris, 1994). Salt precipitation is achieved by either salting in or salting out. Salting in is based on increase in protein solubility with an increase in salt concentration. Proteins will precipitate at pH values equivalent to their isoelectric points. The protein precipitate can be then removed by centrifugation. Salting in depends on the charge and polar interactions with solvents. Globular proteins are mainly isolated and purified by salting in (Scopes, 1984; Harris, 1994; Doonan, 2004).

The salting out 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 (Scopes, 1984; Harris, 1994). Ammonium sulphate is commonly used because it is generally cheap, increases protein stability and allows easy pellet formation by the precipitate.

However, it slightly increases solution acidity which may disrupt the structure of some proteins (Harris, 1994; Dennison, 2003).

The use of organic solvents in protein precipitation is based on the reduction in water activity; which, in turn, decreases the solvating power of water for charged proteins (Scopes, 1984). This leads to a decrease in protein solubility, leading to aggregation and precipitation. Organic solvents are used at sub-zero temperatures to prevent enzyme denaturation. Use of organic solvents is advantageous because of their volatility which allows them to be easily removed from the protein sample. Consequently, this method minimises time and costs for removal of the precipitant (Scopes, 1984; Dennison, 2003).

Organic polymers such as polyethylene glycol (PEG)-20 000 and Sephadex G25 are also used in protein concentration. The solution to be concentrated is placed in a dialysis tubing with a molecular weight cut off that retains proteins while allowing water and other smaller molecules to exit the tubing. The organic polymer draws out water and the smaller molecules, leaving a more concentrated protein solution in the tubing. The concomitant removal of smaller soluble contaminants with the water during the concentration process is desirable as this eliminates the subsequent steps required for precipitant removal from the protein solution.

Unlike organic polymer concentration, freeze drying of the protein solution concentrates all molecules in the solution. Hence the concentrated powder has to be subsequently dialysed to reduce the concentration of soluble contaminants. Freeze drying can denature the proteins of interest. In addition to freeze drying, techniques such as ultrafiltration can also be used in protein concentration (Dennison, 2003; Schratte, 2004).

### ***3.1.2 Analyses of concentrated proteins***

Following concentration or purification, proteins can be analysed for purity, mass, structure and origin. Electrophoresis can be used to confirm 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 electrophoresis [e.g. polyacrylamide gel electrophoresis (PAGE)] is used when the native protein properties require investigation while denaturing PAGE (e.g. SDS-PAGE) is used when the molecular weights and subunit compositions of the proteins are to be determined (Bollag *et al.*, 1996).

Information on a protein's size and subunit composition may be insufficient, and additional information about the origin of the protein from a mixed source may be required. Peptide mass finger printing can assist in identifying the source of a protein in the environment. Enzymes used in the digestion of a protein in mass finger printing include trypsin, V8 protease and chymotrypsin. Mass spectrometers used (to determine the masses from the digested peptide) may either be matrix assisted laser desorption and ionisation time of flight (MALDI ToF) or electrospray ionisation (ESI) based (Liebler, 2002). Once the digested peptide fragment masses are obtained, they can be used in conjunction with known protein databases for searching and matching of fragment masses to those of reference proteins in the database. Similarities in the peptide masses between the reference protein and the protein in question are then used to confirm the origin of the protein. However, success depends on sample purity (Liebler, 2002) and known protein modifications before and during isolation.

The major objective of work in this chapter was to concentrate GUD in stagnant and running water and then investigate the origin of this enzyme. This was attempted through the following specific objectives, which were to:

- (a) evaluate the effectiveness of different protein concentration techniques,
- (b) separate the GUD subunits from other proteins using SDS-PAGE,
- (c) perform MALDI ToF mass spectrometry (MS) on the electrophoresed GUD band, and
- (d) attempt to identify the origin of the GUD band through peptide mass matching.

## 3.2 Materials and Methods

### 3.2.1 Protein concentration

One denaturing (acetone precipitation) and four non-denaturing concentration techniques were used. All concentration steps were performed at  $4 \pm 1$  °C.

#### Acetone precipitation

Five hundred millilitres of ice cold acetone was added to 100 ml of water sample, mixed gently and kept at -20 °C for 3 h. This was followed by centrifugation at 27 000 g for 5 min at  $4 \pm 1$  °C. The pellet was air dried, resuspended in minimal volume of water, aliquoted in eppendorf tubes and stored at -20 °C (Ó'Fágáin, 2004) for further analyses.

#### Ethanol precipitation

To 100 ml of pre-chilled river water samples, an equal volume of cold ethanol (pre-cooled at -20 °C for 5 h) was added slowly with gentle stirring (Dennison, 2003) and stirring was continued for a further 15 min. The solution was then centrifuged at 10 000 g for 10 min and the pellet was resuspended in 1 ml of distilled water and stored as described under acetone precipitation.

#### Polyethylene glycol (PEG)-20 000 concentration

Dialysis tubing with a molecular weight cut off of 12 kDa was prepared as outlined by the manufacturer (Sigma, Steinheim, Germany). Briefly stated, the dialysis tubing was cut to required length and treated by washing in running water for 3 h to remove glycine, heated at 80 °C with 0.03 % (w/v) sodium sulphide to remove sulphur compounds and placed in hot water (60 °C) for 2 min. The tubes were then acidified by immersing in 0.2 % (v/v) sulphuric acid, washed in hot water and stored at  $4 \pm 1$  °C in 20 % (v/v) ethanol until use. The dialysis tubing was thoroughly rinsed with milli Q water prior to use. One hundred millilitres of each water sample were added in separate tubes and covered by dry PEG-20 000. Wet PEG was scrapped off the tubing surface at 30-min intervals until the water volume remaining in the tube was

about 5 ml. This was dialysed overnight against 10 mM Tris-HCl buffer (pH 7.5) and stored as outlined above (in acetone precipitation section).

#### **Ammonium sulphate precipitation**

To 100 ml of chilled water samples, 56.8 g of ammonium sulphate was added slowly with gentle stirring on ice and stirring was continued for 30 min after salt dissolution. The solution was centrifuged at 10 000 g for 10 min at  $4 \pm 1$  °C and the pellet was resuspended in 3 ml of milli Q water and dialysed as described in the PEG-20 000 concentration with three buffer changes. Dialysis tube contents were stored in the same way as acetone precipitated samples.

#### **Freeze drying**

Water samples (100 ml aliquots) were poured into freeze drying flasks and dried by swirling the flasks in liquid nitrogen. The flasks with dried samples were applied to the freeze dryer and left overnight. The powder was resuspended in 5 ml milli Q water, dialysed and stored as in outlined under acetone precipitation section.

### **3.2.2 Protein concentration determination**

The Bradford assay adapted from Kruger (2002) was used for protein quantification. Bovine serum albumin (BSA) was used to construct a standard curve (Appendix D). A BSA stock of 2 mg/ml was prepared, from which dilutions of 0.4, 1.8, 1.2, 1.6 and 2 mg/ml were prepared and used for the BSA standard curve. To each BSA standard and 5 µl of sample from 3.2.1, 250 µl of Bradford reagent was added, mixed, left for 5 min, after which the absorbance readings at 595 nm were taken.

### **3.2.3 Activity gel/zymogram**

PEG-20 000 concentrated samples were prepared and loaded on to a 6 % polyacrylamide for native protein electrophoresis as described by Bollag *et al.* (1996). The gel was overlaid by PNPG solution and colour changes were noted.

### 3.2.4 SDS-PAGE

Denaturing gel electrophoresis was performed as described by Bollag *et al.* (1996) using the Bio-Rad mini protean III system (Bio-Rad, California, USA). Eight percent resolving and 5 % stacking gels were prepared. Briefly stated, samples (20  $\mu$ l) were mixed with 5  $\mu$ l of five times (5X) sample buffer, heated at 100 °C for 5 min and then 20  $\mu$ l of each treated sample was applied to the gel. A rainbow molecular weight marker (Sigma, Steinheim, Germany) was loaded alongside the samples. Separation was achieved at a constant voltage of 120 V, after which, the gel was Coomassie stained and destained. A band that corresponded to GUD molecular weight and an *E. coli*  $\beta$ -galactosidase (GAL) band (positive control) from molecular weight marker lane were excised for MALDI ToF mass spectrometry (MS).

### 3.2.5 MALDI ToF MS

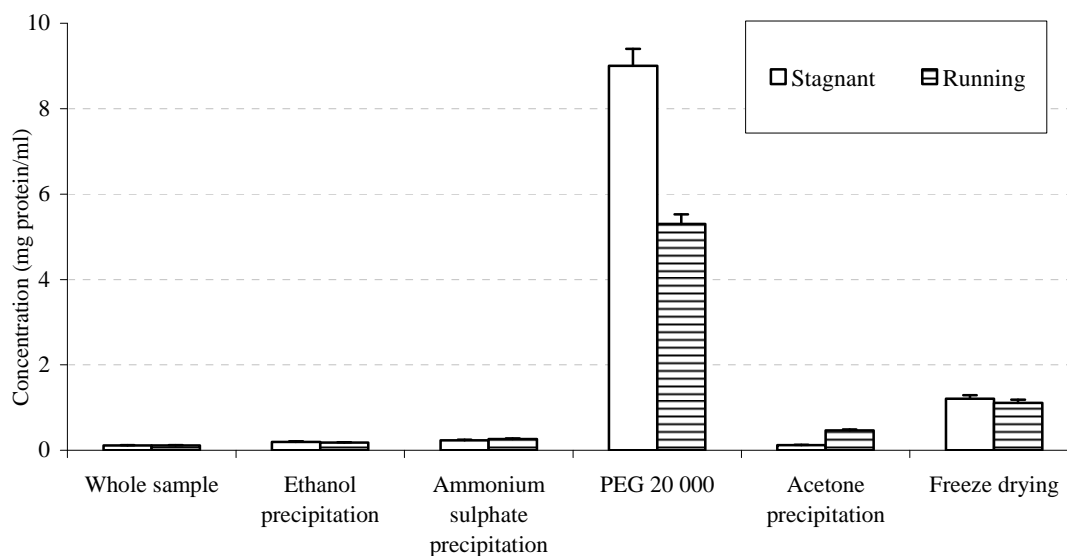
Excised bands (see section 3.2.4) were sent to the School of Molecular Sciences MALDI ToF MS facility (University of Cape Town, South Africa) for tryptic digest mass mapping (Appendix E). The resultant masses were corrected for trypsin self digestion fragments. ExPasy tool (PeptideSearch) (<http://au.expasy.org/tools/>) was used to search and match the peptide masses to ascertain the origin of the enzymes.

## 3.3 Results

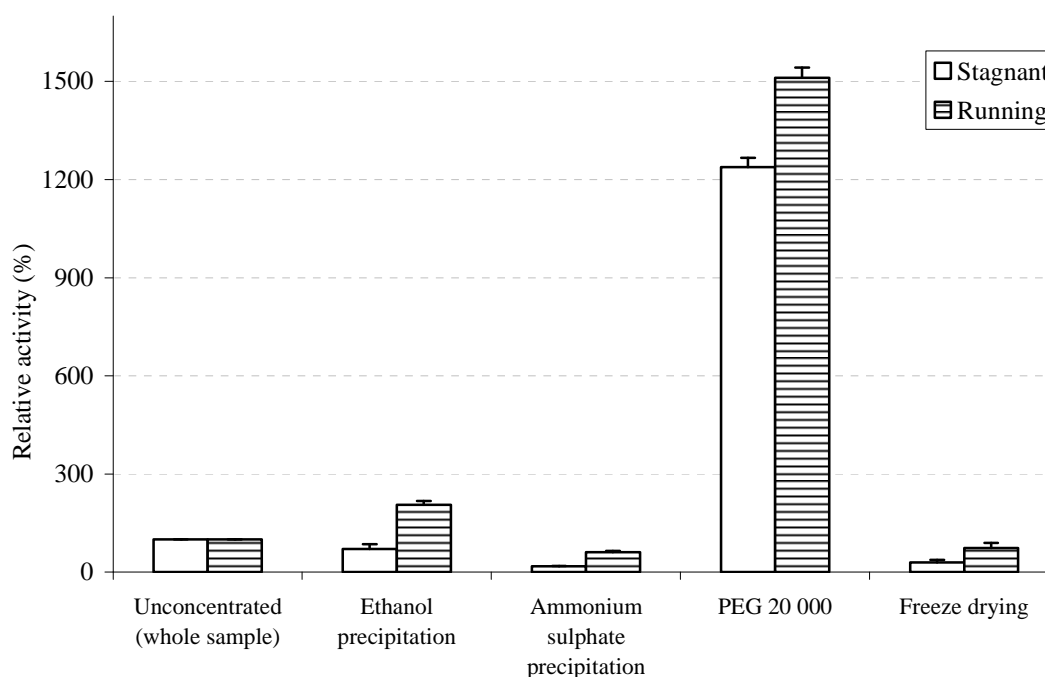
### 3.3.1 Protein concentration methods

Ethanol, ammonium sulphate and acetone precipitation methods achieved insignificant ( $P > 0.05$ ) protein concentration while PEG 20 000 provided the highest degree of protein concentration (Fig. 3.1). There was more protein in stagnant than running water and a similar trend in relative GUD activity was noted (Fig. 3.2). The maximum (100 %) GUD activity for stagnant and running water samples were  $41.06 \pm 0.41$  and  $13.70 \pm 0.05$  nmol/h/ml respectively. The GUD activity was 15-fold in the PEG-20 000 concentrated running water sample (Fig. 3.2). Freeze dried and ammonium sulphate precipitated samples gave lower GUD activity than the

unconcentrated (whole water) fraction. Ammonium sulphate could have also inhibited GUD activity. Progress curves for PEG-20 000 concentrated and freeze dried samples exhibited the longest lag phase. The PEG-20 000 concentrated samples were selected for gel electrophoresis and tryptic mass mapping because they contained both a high content of protein and enzyme activity.



**Figure 3.1** Protein concentration (mg/ml) achieved by different concentrating methods for stagnant and running water samples. All data points represent the mean  $\pm$  SD ( $n = 3$ ).



**Figure 3.2** Relative GUD activity (%) in whole sample and concentrated fractions using non-denaturing methods. All data points represent the mean  $\pm$  SD (n = 3).

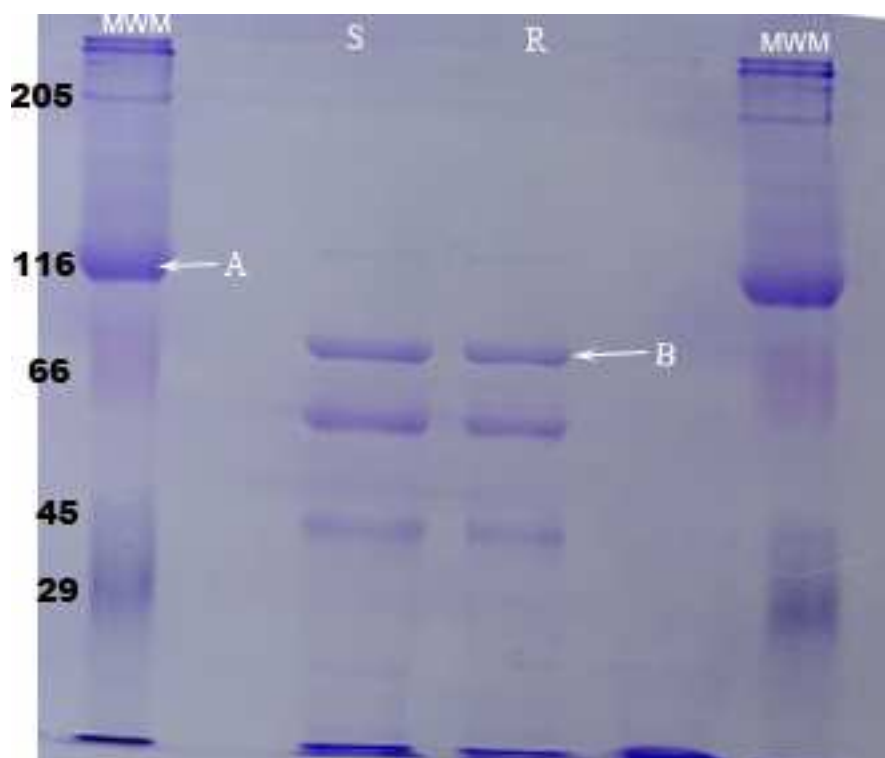
### 3.3.2 Activity gels

The yellow PNP diffused rapidly over the gel and into the solution from the specific regions of enzyme location upon addition of the PNPG, thus confirming enzymatic hydrolysis of PNPG and failure of the gel to entrap PNP.

### 3.3.3 SDS-PAGE and MALDI ToF MS peptide mass matching

Several identical bands were observed in the stagnant and running water samples (Fig. 3.3). The 68 kilodaltons (kDa) band and GAL (116 kDa) (Fig. 3.3; bands B and A respectively) were sent for MALDI ToF MS analyses. Peptide mass matching confirmed band A to be an *E. coli* GAL but the same conclusion was not drawn for the 68 kDa band (band B). Instead, reducing the level of accuracy to 2 Da confirmed that five fragments were similar to those of *E. coli* GAL. In addition, the total number of fragments generated from B was lower than the number required for a GUD positive match. Theoretical digestion of *E. coli* GUD sequence and mass matching

required 26 fragment matches at 0.1 Da accuracy to be identified as an *E. coli* GUD yet band B gave only 22 fragments (for peptide matching) from tryptic digestion (Appendix E).



**Figure 3.3** GAL (A) and presumed GUD (B) bands obtained after SDS-PAGE. S: stagnant water sample; R: running water sample; MWM: rainbow molecular weight marker; the numbers represent molecular weights (kDa). The 205 kDa protein is myosin; 116 kDa: GAL; 66 kDa: BSA; 45 kDa: egg albumin and 29 kDa: carbonic anhydrase.

### 3.4 Discussion and Conclusions

#### 3.4.1 Discussion

The attempt to concentrate protein using ammonium sulphate was unsuccessful because of the low protein concentration in the samples. Ammonium sulphate precipitates protein in solution only if the protein concentration is above 1 mg/ml (Scopes, 1984). The possibility of enzyme inhibition associated with the use of ammonium sulphate cannot be ruled out as this is one of the limitations of using ammonium sulphate (Harris, 1994; Ngwenya and Whiteley, pers. comm.). 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 polluted water samples (Scopes, 1984). Increases in enzyme activities with an increase in protein concentration by PEG-20 000 suggested successful GUD concentration (Figs. 3.1 and 3.2). More GUD activity was recorded in stagnant water probably because turbulence in running water resulted in thorough mixing of the water with the enzyme inhibitors and denaturants. Particulate matter tends to sediment in stagnant water, thus there could be some vertical distribution of both pollutants and GUD. 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 (Dennison, 2003).

The *E. coli* GUD and GAL diverged from a common ancestor and their active sites have about 25 % sequence homology (Matsumura and Ellington, 2001). In addition, these enzymes belong to the same family of hydrolases (Henrissat, *et al.*, 1995; Wong *et al.*, 1998). This might explain the existence of five fragments in the B band that had masses matching those of *E. coli* GAL. There could be slightly different enzymes produced by different *E. coli* strains or other bacterial species in the environment. Alternatively, the presence of GAL fragments in the presumed GUD band could be as a result of protein modification in the natural environment that could have changed the trypsin cleavage sites. Such modification can have a significant impact on cleavage site recognition consequently generating fragments that were not in the database. In addition, the 26 fragments required at 0.1 Da were higher than 22 obtained from MALDI ToF MS. The correlation between GUD activity and *E. coli* counts observed in this study (Chapter 5) and that reported by Farnleitner *et al.* (2001; 2002) strongly suggest that the GUD is of *E. coli* origin. 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](http://www.colifast.no)) use GUD activity as a surrogate indicator for *E. coli*. Therefore, the continued use of GUD in this project was justifiable based on confirmatory findings that were established in this work (Chapter 5).

### 3.4.2 Conclusions

From this chapter, it may be concluded that:

- (a) the best method of concentrating proteins is the use of dry PEG-20 000,
- (b) hydrolysis of PNPG in river water samples is not a chemical process but a result of enzyme activity,
- (c) trypsin cleavage pattern on GUD exposed to pollution differs from that of the pure and commercially available GUD, and
- (d) GUD can be used for indicating the presence of *E. coli*.

Results from Chapter 2 (changes in pH and temperature optima of GUD in environmental samples) and this chapter was followed by a comparative study on the behaviour of environmental and commercially available GUD to different polluting compounds commonly found in water environment in the subsequent chapter. Pollutant analyses of the water sample used were performed to obtain a better understanding of GUD behaviour *in situ*.

## 4 INTERFERENCE STUDIES

### 4.1 Introduction

Inadequately treated or polluted water is likely to contain ions/salts in concentrations above the generally maximum acceptable limits for drinking water set by regulatory authorities. Under such conditions, *in situ* enzyme assays are bound to produce trends different from those of similar enzymes in unpolluted environments. Metal ions play important roles in enzyme catalysed reactions. The effects of the ions vary from inhibition to enhancement of enzyme activity (Berg *et al.*, 1986; Singh *et al.*, 1990). Enzyme inhibition can be due to binding of the ions to the active site residues or other residues that alter the active site conformation (allosteric inhibition) (Holme and Peck, 1998). Enzyme inhibitors can also bind to the substrate thereby blocking the functional groups that interact with the active site residues. In either case, the normal enzyme-substrate recognition and interaction is disrupted. Enzyme inhibition can be reversible or irreversible. Examples of naturally occurring reversible inhibitors are phenolic compounds that decompose over time, thus allowing restoration of enzyme activity (Wetzel, 1991). Irreversible inhibitors include heavy metals like mercury and cadmium.

The presence of certain metal ions (e.g. potassium, sodium and iron) is essential for enzyme reactions to take place as they can serve as electron donors or acceptors in the reactions (Tryland *et al.*, 1998). However, excessive metal concentrations can inhibit the enzyme activity (Den Blanken, 1985). Metal ions such as  $Zn^{2+}$ ,  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Fe^{2+}$ ,  $Cu^{2+}$ ,  $K^{+}$ , and  $Na^{+}$  can be employed as cofactors of enzymes (Ridgway and Olson, 1982; Den Blanken, 1985). For example, Huber *et al.* (1979) observed that  $Mg^{2+}$  and  $Mn^{2+}$  activated the activity of  $\beta$ -galactosidase, a marker enzyme for faecal coliforms (Manafi, 1998; Stevens *et al.*, 2001).

Enzyme-ion interaction and extent of inhibition can be a function of pH and temperature. At the pH values of most natural water bodies (pH 5.0 – 8.0), a large percentage of carboxylic acid groups will be ionized and many cations such as

calcium will bind to organic acids. This will reduce protein-organic acid complex formation, leading to increased enzyme activity. Divalent cations also bind to organic acids and other substances, thus preventing enzyme inhibition through blocking of enzyme-organic acid complex formation (Wetzel, 1991).

Development of enzyme-based methods in laboratories through seeding of sterile water by pure enzymes and axenic cultures is not representative of the ideal environmental conditions (Sartory and Watkins, 1999). This approach faces challenges in applying the laboratory-based protocols to *in situ* enzymes, because of differences in the two environments that may lead to changes in enzymatic properties. Variation in the levels of pollutant between stagnant and running water environments presents another potential factor that can alter enzymatic behaviour. Although GUD has been employed in differential media for detecting and enumerating *E. coli* over a long period, there is no reliable information on the properties of GUD in polluted water in literature. Therefore, the main aim of the study in this chapter was to establish the effects of different ions on environmental GUD. Information from this study will aid in understanding and optimising *in situ* assay procedures for marker enzymes. In addition, this will allow for the accurate interpretation of results. The specific objectives were to:

- (a) determine the different chemical pollutants commonly occurring in the water samples used in this study,
- (b) investigate the effects of different ions on GUD activity, and
- (c) evaluate the effects of environmental water samples on commercial GUD activity.

## **4.2 Materials and Methods**

### **4.2.1 Water pollutant analyses**

Water samples were collected as described in Chapter 2, in 1 l volumes, and submitted to the Nelson Mandela Municipality Scientific Services Department

(Eastern Cape, South Africa) for analysis. The results are presented, together with the maximum permissible levels according to South African standards in section 4.3.1.

#### **4.2.2 Interference studies**

The effect of ions commonly occurring in polluted river water on GUD activity were investigated. These were:  $\text{SO}_4^{2-}$  (as  $\text{Na}_2\text{SO}_4$ ),  $\text{SO}_3^{2-}$  (as  $\text{Na}_2\text{SO}_3$ ),  $\text{CO}_3^{2-}$  (as  $\text{Na}_2\text{CO}_3$ ),  $\text{Cl}^-$  (as  $\text{NaCl}$ ),  $\text{Ca}^{2+}$  (as  $\text{CaCl}_2$ ),  $\text{Mg}^{2+}$  (as  $\text{MgSO}_4$ ),  $\text{Cd}^{2+}$  (as  $\text{CdSO}_4$ ),  $\text{NO}_3^-$  (as  $\text{KNO}_3$ ),  $\text{K}^+$  (as  $\text{KCl}$ ),  $\text{PO}_4^{3-}$  (as  $\text{Na}_2\text{HPO}_4$ ), ferric chloride ( $\text{FeCl}_3$ ), EDTA [all supplied by Merck (Darmstadt, Germany)], ferulic acid (Sigma, Steinheim, Germany) and  $\text{OCl}^-$  [as  $\text{NaOCl}$  (Savemore, Pinetown, South Africa)]. Stock solutions of these compounds were added to the enzyme reaction mixture to final concentrations ranging mainly between 0 and 200 mg/l, except for  $\text{OCl}^-$  (350 – 5 600 [parts per million (ppm); 1 ppm = 1 mg/l] and  $\text{Cd}^{2+}$  (1 – 10 ppm). These effector ions were separately pre-incubated with the river water and commercial GUD at room temperature ( $20 \pm 2^\circ\text{C}$ ) for 30 min after which the substrate was added and kinetic readings taken as described in Chapter 2.

#### **4.2.3 Effects of environmental water samples on commercial GUD**

Different ratios (% , v/v) of water from the two sampling points (Chapter 2) were added to commercial GUD solutions, pre-incubated for 30 min at room temperature and the enzyme reaction was initiated by the addition of the PNPG. Kinetic readings were performed as described in Chapter 2. The activities were compared with that of a positive control containing commercial GUD with milli Q water added instead of environmental water sample.

### **4.3 Results**

#### **4.3.1 Water pollutant analyses**

All the compounds, with the exception of phosphorus, magnesium, total sulphide and total alkalinity (whose maximum permitted limits were not given), were below the

maximum permitted limits according to the SABS 241 water quality guidelines (<http://www.klmcs.co.za/html/waterguidelines.html>; Table 4.1). Calcium carbonate and chloride were present in the highest concentration amongst the components analysed (Table 4.1). Significant ( $P < 0.05$ ) differences in some of the compounds (e.g. sodium, magnesium, carbonate and zinc) concentrations between stagnant and running water samples were observed (Table 4.1).

**Table 4.1** Results from water sample analyses and the South African maximum permissible limits for treated drinking water. All units are in mg/l.

Attribute/analyte	Stagnant sample	Running sample	Max. acceptable limits
Total alkalinity as CaCO <sub>3</sub>	296	296	Not given
Magnesium as CO <sub>3</sub>	214	140	100 (as Mg)
Sodium as Na	272	222	400
Potassium as K	18	12	100
Chloride as Cl	291	292	600
Sulphate as SO <sub>4</sub>	126	125	600
Nitrate plus Nitrite as N	2.6	2.6	20
Phosphorus (soluble)	1.1	0.86	Not given
Total Sulphides as H <sub>2</sub> S	<1	<1	Not given
Cyanide as HCN	<0.1	<0.1	70 (as CN)
Total Iron as Fe	0.48	0.54	2 000
Cadmium as Cd	<0.001	<0.001	20
Copper as Cu	0.008	<0.005	2 000
Mercury as Hg	<0.3	<0.3	5
Nickel as Ni	<0.005	<0.005	350
Zinc as Zn	0.024	0.014	10

#### 4.3.2 Interference studies

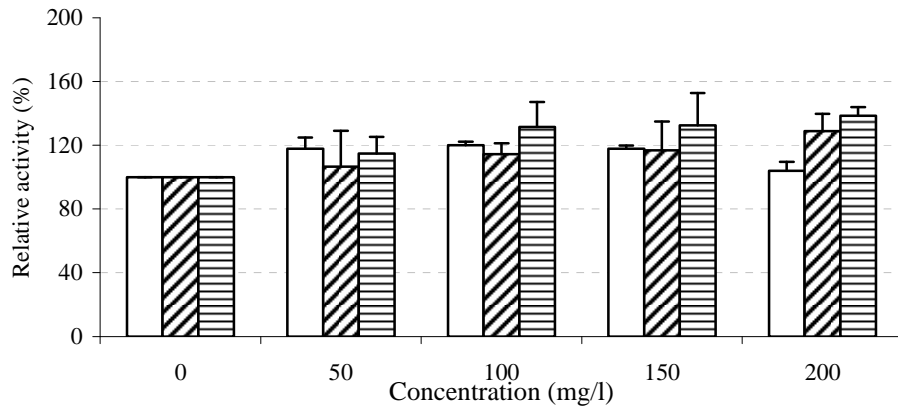
The average activity in unamended samples (i.e. samples where no effector had been added) for commercial GUD was  $11.57 \pm 1.02 \mu\text{mol/h/ml}$ , while for stagnant and

running water samples were  $15.31 \pm 2.27$  and  $15.51 \pm 2.10$  nmol/h/ml, respectively. In general,  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$  and  $\text{Cl}^-$  ions increased the activity of GUD of the three environments (Figs. 4.1a-c). At 100 mg  $\text{PO}_4^{3-}$ /ml the highest relative activity increase for GUD in stagnant water was observed (Figs. 4.1b and c), while the commercial and the running water GUD activity remained just above 100 % of that of the unamended samples.  $\text{Cl}^-$  only increased the activity of running water and commercial GUD (up to 100 mg/l), while inhibiting that of stagnant water (Fig. 4.1c)

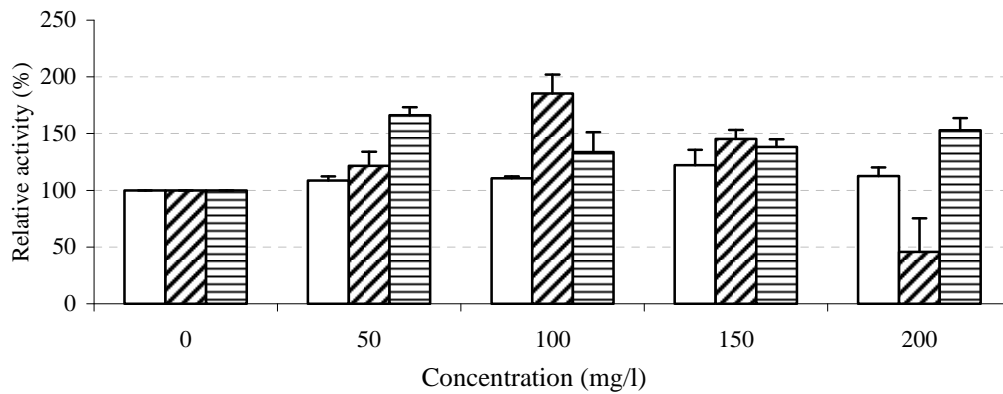
Ferric chloride completely inhibited GUD activity in the two environmental samples at 30 mg/l, the optimum concentration used in flocculation during water purification (Chow *et al.*, 1998) (Fig. 4.2a). However, the activity of commercial GUD was above 70 % at 30 mg/l. GUD from the running water sample was more sensitive to the addition of  $\text{FeCl}_3$  than the stagnant water GUD (Fig. 4.2a). However, a 30-min exposure of *E. coli* cells to 30 mg/l  $\text{FeCl}_3$  did not significantly ( $P > 0.05$ ) reduce their viability. Higher  $\text{FeCl}_3$  concentrations (above 100 mg/l) led to spontaneous substrate hydrolysis and a drastic reduction in the pH of the assay solution.

Hypochlorite ( $\text{OCl}^-$ ); commonly used as a disinfectant in remote areas (at concentrations of approximately 700 ppm), significantly ( $P < 0.05$ ) reduced GUD activity (Fig. 4.2b) – as did ferulic acid (Fig. 4.2c). The sensitivity of the enzyme in different environments to hypochlorite varied. GUD in running water was more susceptible, followed then by commercial GUD and that in stagnant water (Fig. 4.2b).

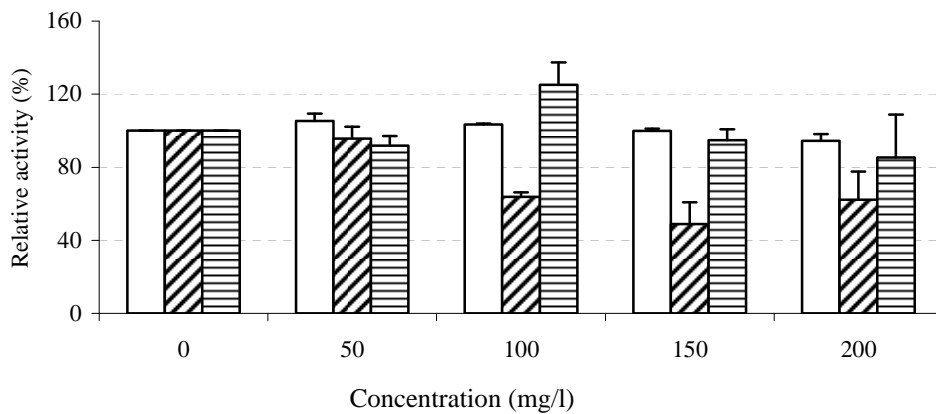
Other chemicals had different effects on the environmental and commercial GUD enzymes (Table 4.2). There was a direct relationship between carbonate concentration and GUD activity in running water while inhibition was observed in both commercial and stagnant water GUD. A similar trend was observed for  $\text{SO}_4^{2-}$ ,  $\text{SO}_3^{2-}$  and  $\text{Cl}^-$ . EDTA and  $\text{Mg}^{2+}$  inhibited GUD in all three samples while the reverse was true for  $\text{K}^+$  (Table 4.2).



a



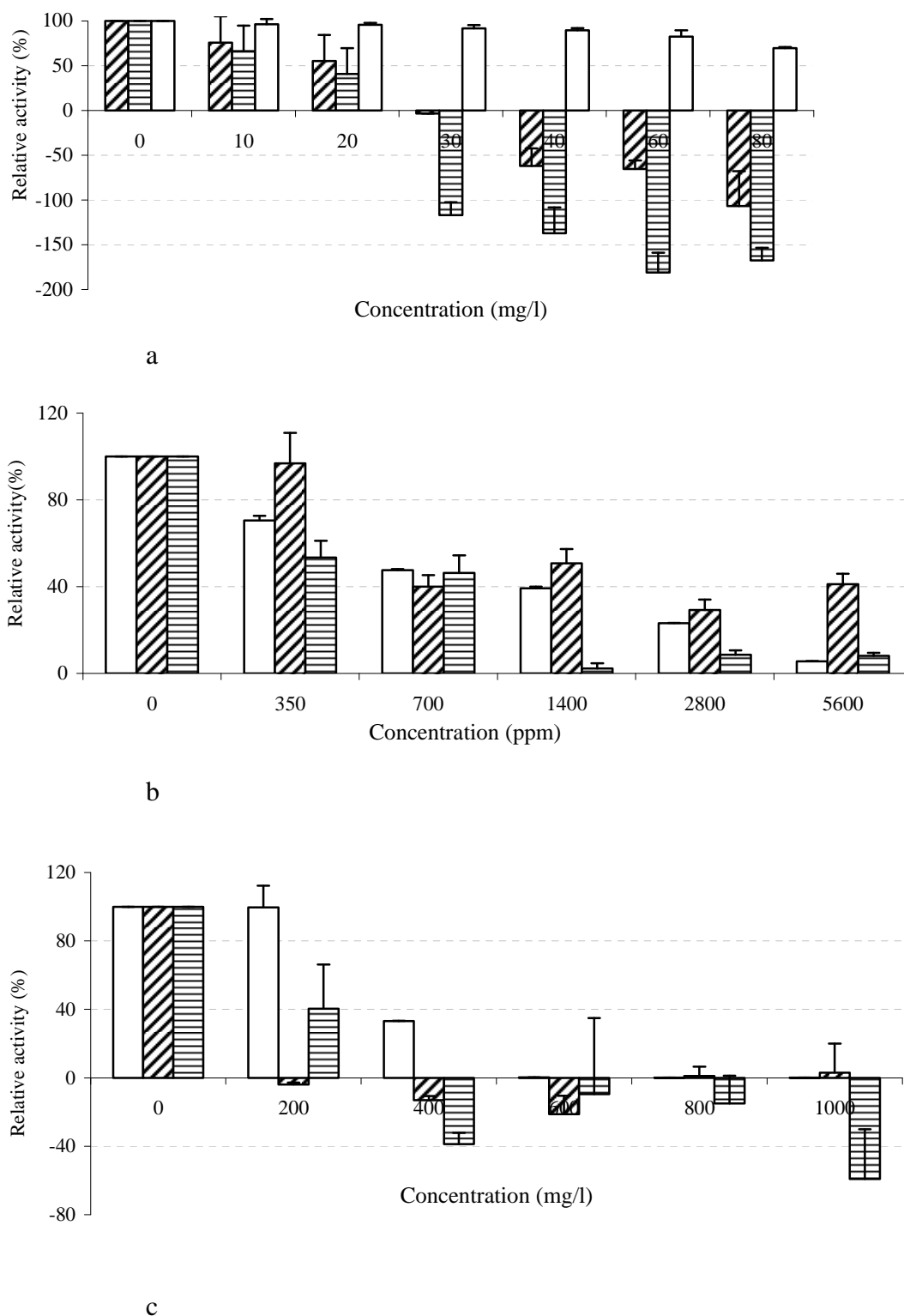
b



c

**Figure 4.1** Effects of  $\text{NO}_3^-$  (a),  $\text{PO}_4^{3-}$  (b) and  $\text{Cl}^-$  (c) on GUD activity. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

Commercial GUD:  Stagnant:  Running:



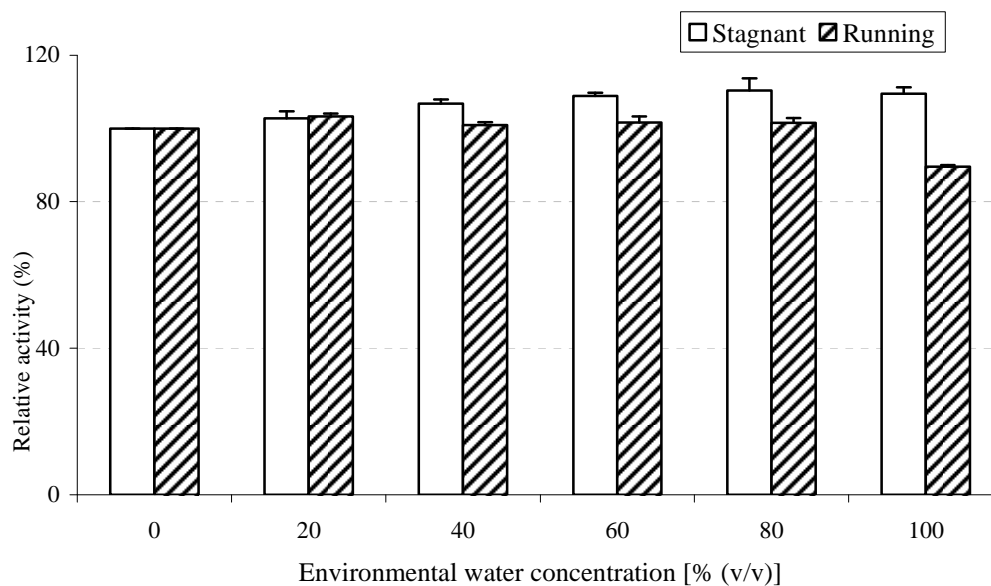
**Figure 4.2** Effects of  $\text{FeCl}_3$  (a),  $\text{OCI}^-$  (b) and Ferulic acid (c) on GUD activity. All data points represent the mean  $\pm$  SD ( $n = 3$ ). Commercial GUD:  $\square$  Stagnant  $\text{▨}$  Running:  $\text{▩}$

**Table 4.2** Summary of effects of different ions on GUD.

Effector	GUD samples		
	Commercial	Stagnant water	Running water
SO <sub>4</sub> <sup>2-</sup>	13 % inhibition at 120 mg/l	30 % inhibition at 120 mg/l	26 % increase at 120 mg/l; 6 % inhibition at 240 mg/l
SO <sub>3</sub> <sup>2-</sup>	No significant (P > 0.05) change in activity	Four- and nine-fold increase in activity at 200 and 1000 mg/l, respectively	Two- and six-fold increase at 200 and 1000 mg/l, respectively
CO <sub>3</sub> <sup>2-</sup>	6 and 36 % inhibition at 200 and 400 mg/l	3 and 77 % inhibition at 200 and 400 mg/l	36 % increase at 200 mg/l; 56 % inhibition at 400 mg/l
Cl <sup>-</sup>	12 and 17 % inhibition at 140 and 280 mg/l	23 and 47 % inhibition at 140 and 280 mg/l	45 and 94 % increase at 140 and 280 mg/l; 6 % inhibition at 560mg/l
Mg <sup>2+</sup>	76 % and 94 % inhibition at 200 and 400mg/l; total inhibition at 600 mg/l; synergistic effect with SO <sub>4</sub> <sup>2-</sup>	58 and 71% inhibition at 200 and 400 mg/l; 100 % inhibition at 600 mg/l	71 % and 74 % inhibition at 200 and 400 mg/l; total inhibition at 600mg/l
Cd <sup>2+</sup>	50 % inhibition between 2 and 4 ppm; total inhibition above 10 ppm	Total inhibition at 4 ppm	80 % inhibition at 2 ppm; total inhibition at 4ppm
K <sup>+</sup>	10 % increase at 90 mg/l to 3 % increase at 380 mg/l after which it inhibited	1.6-fold increase at 190 mg/l; 1.3-fold at 380 mg/l and inhibited above 380 mg/l	59 and 81 % increase at 90 and 380 mg/l, inhibited from 760 mg/l
EDTA	36 % inhibition at 200mg/l	68 and 99 % inhibition at 220 and 400 mg/l	62 and 92 % inhibition at 200 and 400 mg/l

### 4.3.3 Effects of environmental water samples on commercial GUD

Environmental water samples did not have a significant ( $P > 0.05$ ) impact on GUD activity although stagnant water showed a slight increase in the activity (Fig. 4.3).



**Figure 4.3** Effect of different concentrations of environmental water sample on commercial GUD activity. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

## 4.4 Discussion and Conclusions

### 4.4.1 Discussion

GUD activity is not inhibited by anionic enzyme inhibitors (Doyle *et al.*, 1955) and this was also observed in this present study with  $\text{SO}_3^{2-}$  and  $\text{NO}_3^-$ , especially in the running water sample. Edberg and Kontnick (1986) reported enhanced GUD activity in presence of  $\text{Na}_2\text{CO}_3$ . Carbonates bind to phenolic compounds, thereby preventing enzyme inhibition by organic acids (Wetzel, 1991). Since the substrate (PNPG) used in this study is phenol based, the chelation may help in explaining the observed inhibition in commercial and stagnant water GUD activities. However, an increase in activity in the presence of carbonate (up to 200 mg/l) in running water sample could

be due to the existence of other forms of phenolic compounds that have a higher affinity for carbonate than PNPG. Inhibition of GUD activity by ferulic acid suggests that the use of the direct enzyme assay method in water bodies polluted by phenols or with decomposing organic matter may yield a false negative result.

Cadmium inhibited GUD because it is able to bind to the sulphhydryl groups of the protein (Vallee and Ulmer, 1972). Sulphydryl groups are involved in the active site of GUD (Fernley, 1962). Therefore,  $\text{Cd}^{2+}$  may have led to distortion of the active site conformation. However, the toxic effects of cadmium in living systems at parts per billion concentrations will not considerably reduce the GUD activity. Although the primary concern is not to detect such inhibitors, direct enzymatic assay techniques can be unreliable if enzyme activity is detected at metal concentrations that kill the organism, thus giving false positive results. Other reported inhibitors for a wide variety of GUD include  $\text{Ag}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{CoCl}_2$ ,  $\text{MnCl}_2$  and  $\text{FeCl}_2$  (Fernley, 1962; Diez and Cabezas, 1979; Kim *et al.*, 1995). However, normal anionic enzyme inhibitors like cyanide were reported not to inhibit GUD (Fernley, 1962; Diez and Cabezas, 1979).

Hypochlorite changes proteins by interacting with the amine groups. Tyrosine undergoes ring chlorination to give 3-chlorotyrosine and lysine gives chlorolysine in presence of  $\text{OCl}^-$  (Domigan *et al.*, 1995; Kettle, 1996; Hawkins and Davies, 1999). Tyrosine is one of the three most important active site residues for GUD (Wong *et al.*, 1998; Islam *et al.*, 1999; Matsumura and Ellington, 2001). Lysine is important for the quaternary structure of proteins (Matsumura *et al.*, 1999). Hence changes in these crucial amino acids will impair GUD activity. Another possible mechanism by which  $\text{OCl}^-$  affects GUD activity is by allowing uncontrolled protease activity (Aruoma and Halliwell, 1987) that may lead to hydrolysis of GUD. This is possible considering that environmental water has a number of organisms that contribute these proteases upon exposure to  $\text{OCl}^-$ .

Hypochlorite strongly reduces survival of *E. coli* (McKenna and Davies, 1988). Hence GUD inhibition by  $\text{OCl}^-$  will be an accurate indication of *E. coli* viability.

However, if pathogens resistant to OCl<sup>-</sup> and chlorine (e.g. *Cryptosporidium* oocysts) (Nwachuku and Gerba, 2004) are present, direct GUD assay may leave the population vulnerable to pathogens. Under such circumstances,  $\beta$ -galactosidase (a marker enzyme for total faecal coliforms) can be used as it has been reported to be tolerant to chlorine (Tryland *et al.*, 1998).

The presence of a diverse range of ions and particulate matter in the polluted environmental water samples (Table 4.1) aided flocculation by ferric chloride (Leentvaar *et al.*, 1979), thus making the enzyme unavailable for detection. For this reason, commercial GUD was less affected by the flocculant. The observed results support the reliability of GUD assay in polluted water because removal of the enzyme will be coupled to the removal of most microbes from the water. However, the presence of FeCl<sub>3</sub> above 100 mg/l can give rise to false positive results as was noted with spontaneous PNPG hydrolysis, while drastic pH reduction inhibited GUD activity. This may limit the use of direct GUD assay as an indicator for presence of *E. coli* during flocculation with FeCl<sub>3</sub>.

Caution should be exercised in comparing the interference results because of the differences in concentration of commercial GUD, and GUD in stagnant and running water samples. Enzyme inhibition is also a function of the enzyme to inhibitor ratio. While 'within sample' comparisons are valid, the same may not be true for 'between sample' comparisons especially when the enzyme to inhibitor ratios differ between the samples. Therefore, where the ions had less inhibitory effect on commercial GUD than environmental GUD (as with FeCl<sub>3</sub>), the observed differences in inhibition patterns can be also explained by the differences in the enzyme to inhibitor ratio. In this case, it can be argued that FeCl<sub>3</sub> did not inhibit all GUD molecules in the commercial sample. However, the general trend in enzyme activity showed that FeCl<sub>3</sub> inhibited GUD activity.

Fujisawa *et al.* (2000) observed that NaCl only increased GUD activity in *E. coli* culture through an increase in membrane permeability and there was no increase in

GUD activity in sonicated cells. Therefore, the inhibition of GUD by  $\text{Cl}^-$  in stagnant water could be a result of high NaCl concentration in the polluted sample.

Since EDTA is a powerful chelator (Kari and Giger, 1996), an increase in GUD activity in the environmental samples would be expected by precipitation of a variety of ionic inhibitors present in the polluted water samples (Table 4.1). This was not observed because the chelation of ions may also depend on pH (Lim *et al.*, 2004). Therefore, the pH values of the water samples might not have been ideal for the chelation of these ions. Other varying effects by the effectors can be attributed to different water pollutants in the two environments of stagnant and running water.

The inability of environmental water to strongly inhibit commercial GUD cannot be wholly attributed to the absence of the inhibitors in the water, but it could be argued that irreversible inhibitors were bound to GUD in water and therefore could not interact with the commercial GUD. Alternatively, the weak inhibitory effect of the common compounds found in water, like  $\text{K}^+$  and  $\text{SO}_4^{2-}$ , may help to explain why the environmental water did not strongly inhibit the commercial enzyme activity. Furthermore, it could be possible that the observed activity was a net effect of enzyme inhibition and activation.

#### 4.4.2 Conclusions

- (a) Several chemical compounds in the environment can potentially affect *in situ* GUD activity; therefore results of such assays should be stated with caution.
- (b) In the presence of strong GUD inhibitors like  $\text{Cd}^{2+}$  there may be a need to remove some of these compounds prior to assaying for GUD.
- (c) The possibility of obtaining false positive or negative readings in the detection of GUD activity in contaminated water should be anticipated. Therefore, further verification maybe required through more traditional microbiology assays.

- (d) Since the pollutant composition of the water varies with site and time, use of an internal reference or standard (i.e. commercial GUD) when employing *in situ* assays is recommended to assess and correct for the effects of pollutants in the water samples.

Using the insight gained from previous chapters, a method for *E. coli* detection in water based on PNPG can be established. Therefore, studies in the following chapter involve the characterisation of a liquid probe and a study of annual GUD activity trends at the two sampling sites.

## 5 LIQUID BIOPROBE

### 5.1 Introduction

Correlation between *Escherichia coli* and GUD activity has been reported by Farnleitner *et al.* (2001; 2002) using the fluorogenic substrate, 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG). This linear relationship provides the basis for the establishment of a liquid bioprobe where direct enzyme assay of the sample in question is performed. However, the extent of the linear relationship varies with sample location, substrate used and the degree of pollution of a water body. It is therefore necessary to confirm linearity in this study to ascertain the feasibility of direct GUD assay using the substrate PNPG to indicate the presence/absence of *E. coli*.

#### 5.1.1 Product shelf life

Since water may be stored at room temperature or refrigerated (at  $4 \pm 1$  °C) before consumption (Jagals *et al.*, 1999), it is worthwhile to investigate the storage conditions and period that give reliable results or whether the storage conditions eliminate indicator organisms. This enables an estimation of the efficiency of the direct enzyme assay method on stored water samples. Standridge and Delfano (1983) reported an insignificant change in coliform counts in water stored at room temperature for 48 h from the time of sampling. However, it is not known whether this is also the case for GUD activity.

Storage stability of the substrate solution has a bearing on the feasibility of the liquid bioprobe method to be used for indicator organism detection. If the substrate solution is not stable at room temperature or requires refrigeration, this may attract an additional storage cost which is undesirable. In addition, power supply for refrigeration may not be always available in the rural areas. Thus a stable substrate at room temperature is the most preferred option.

### 5.1.2 Spatial and temporal enzyme variations

The abundance and activity of enzymes in aquatic environments varies with depth (Hoppe, 1991), time of the day and season (Jagals *et al.*, 1995; Jagals, 1997). 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 (Chróst, 1991; Hoppe, 1991).

The major determinants of daily and seasonal variations are temperature and volume of runoff. Warm temperatures enhance both microbial growth and death, increasing enzyme production and activity. In addition, increases in temperature accelerate degradation of organic compounds, consequently providing nutrients for some of the microbes, leading to high enzyme activity. However, some by-products of the degradation processes may inhibit the enzyme activity (Wetzel, 1991). Furthermore, increases in temperature encourages mixing in stagnant water bodies by convection currents. Temperature also affects the dissociation constants of pollutants and pH of the environment. These, in turn, influence enzyme activity and stability. Therefore, enzyme activity manifested in environmental samples can be a net result of the interplay of several factors.

During storms, runoff introduces allochthonous material into the water bodies. Depending on the quality and quantity of such material, enzyme activity and concentration may be enhanced or reduced (Venter, 2000). Such a variation has a bearing on the time of sampling and determination of faecal material. Understanding these variations will aid in accounting for results obtained using a specific method of analysis in different environments. Human activities along the course of rivers (e.g. washing and bathing) potentially affect the enzyme activity. Hence establishment of monthly GUD activity trends at sampling sites plays an important role in the interpretation of the results obtained.

The major aim of work in this chapter was to establish a liquid “bioprobe” for direct GUD assaying, based on results obtained in Chapters 2 to 4. This was achieved through the following specific objectives, which were to:

- (a) investigate the correlation between *E. coli* counts and environmental GUD activity,
- (b) determine the limit of detection (LOD) for the bioprobe,
- (c) establish suitable storage conditions for the PNPG solution for prolonged shelf life,
- (d) evaluate the maximum period water samples can be stored while giving reliable results for GUD activity, and
- (e) establish the annual trends for the environmental GUD enzymes at the two sampling points.

## 5.2 Materials and Methods

### 5.2.1 Regression studies and comparison with the MF technique

Water samples were collected from different environments in the Eastern Cape of South Africa (river mouths of Kariega, Bushman’s and Sunday’s Rivers, a marshy area around the Swartkops Estuary near Port Elizabeth (PE), final effluent from two PE wastewater treatment plants, marine environment and freshwater bodies around Grahamstown). *Escherichia coli* enumeration using the membrane filtration (MF) method was performed on CM 1046 agar (Oxoid, Hampshire, England) as outlined by Farnleitner *et al.* (2001) and *Standard Methods* (2005). Briefly stated, 100 ml of environmental water samples were filtered separately through a 0.22 µm membrane filter (Millipore Corporation, USA) to concentrate the microbes. The membrane filter was then layered (upside down) on the agar in petri dishes and cultured at 37 °C for 24 h. *Escherichia coli* colonies were purple in colour. Samples with high microbial density were serially diluted in sterile quarter strength Ringer solution (Oxoid) and the dilution factor taken into account when reporting the CFU/100 ml of the original sample.

Direct GUD assays for the plated samples were performed. Regression analyses were performed for microbial counts versus enzyme activities using Microsoft Excel 2003 statistical tools. From these data sets, a comparison of the direct enzyme assay method and MF CFU determination was performed to determine the reliability of direct enzyme assay. The experimental LOD was determined as the lowest number of CFU/100 ml that yielded detectable GUD activity.

### **5.2.2 Commercial GUD LOD**

The activity of a range of GUD concentrations (0.02 – 60 µg/ml) was assayed (as described in Chapter 2) to establish visual and spectrophotometric detection limits. The visual LOD was scored as the lowest enzyme concentration that produced a visible colour change within 24 h. Spectrophotometric LOD was the lowest enzyme concentration that produced a measurable change in absorbance within 24 h.

### **5.2.3 PNPG storage stability**

PNPG (10 mM) was prepared and 20 ml vials were kept under the following conditions (in triplicate):

- (a) -20 °C,
- (b)  $4 \pm 1$  °C,
- (c) room temperature ( $20 \pm 2$  °C), protected from light by aluminium foil, and
- (d) room temperature ( $20 \pm 2$  °C), exposed to light.

From each sample, 250 µl aliquots were drawn daily for absorbance reading at 405 nm to monitor accumulation of PNP. GUD assays using the same substrate samples were performed to ascertain the presence of PNPG. This was performed for 28 days.

### **5.2.4 Environmental GUD storage stability**

Water samples from the stagnant and running water sites were stored in triplicate at room temperature and  $4 \pm 1$  °C. GUD activity was determined daily for a week.

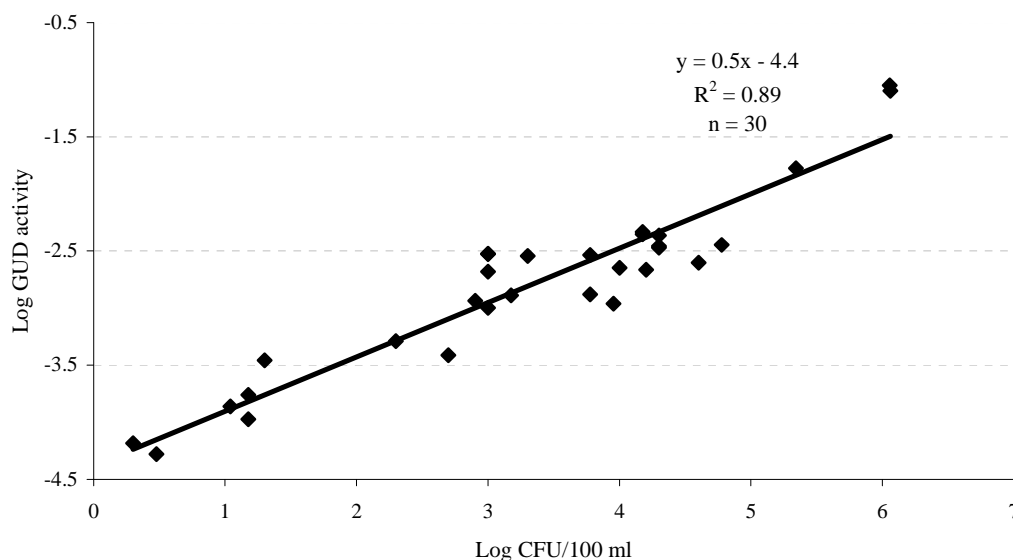
### 5.2.5 Monthly trends in GUD activity at the sampling points

The activity of GUD from the two sampling points was assayed weekly for a period of over a year. Mean monthly GUD activity values were used to construct annual trend graphs.

## 5.3 Results

### 5.3.1 Regression studies and LOD

There was a significant ( $P < 0.05$ ) positive correlation between log *E. coli* counts and log GUD activity ( $R^2 = 0.89$ ) (Fig. 5.1). The spectrophotometric LOD of GUD activity was equivalent to 2 CFU/100 ml of water sample determined by MF (Fig. 5.1).



**Figure 5.1** Regression of GUD activity versus *E. coli* counts in environmental water samples.

The lowest commercial GUD concentration that gave detectable activity was 0.05 and 0.02  $\mu\text{g/ml}$  visually and spectrophotometrically, respectively. The former was detected after 5.5 h and the latter after 3 h (Table 5.1). Neither visual nor

spectrophotometric changes were detected after 24 h below the enzyme concentrations of 0.05 and 0.02  $\mu\text{g/ml}$ , respectively.

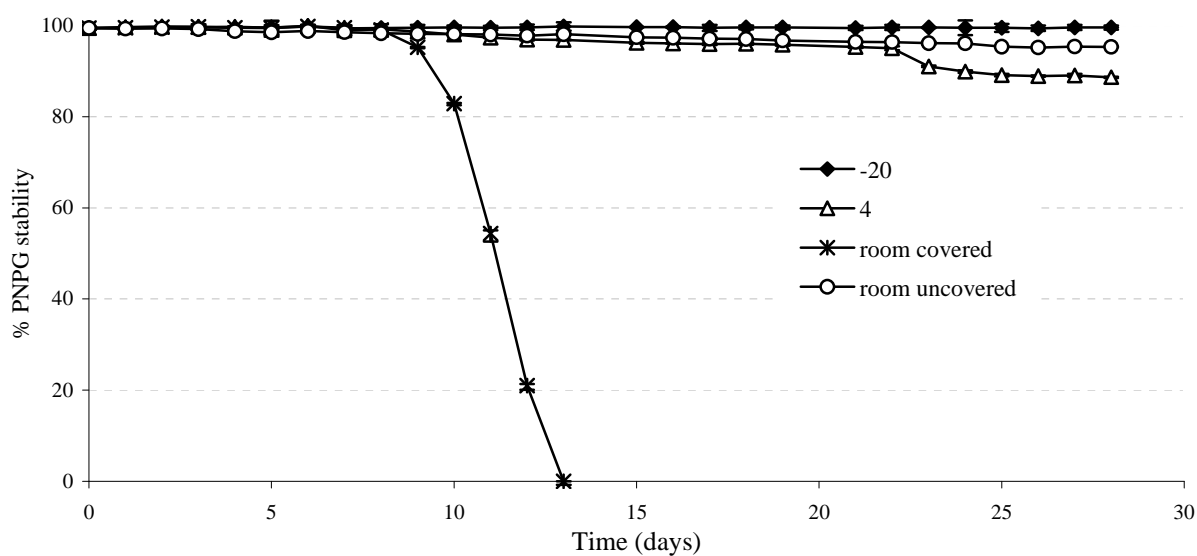
**Table 5.1** Visual and spectrophotometric detection limits for GUD activity.

Concentration ( $\mu\text{g/ml}$ )	Spectrophotometric	Visual colour change
60	+	+*
40	+	+*
20	+	+*
10	+	+*
5	+	+*
2	+	+*
0.5	+	+ (20 min)
0.2	+	+ (40 min)
0.05	+	+ (5.5 h)
0.02	3 h	No change after 24 h
0.005	No change after 24 h	

+ : detected; \* instant colour change; + (20 min): colour detected after 20 min.

### 5.3.2 PNPG storage stability

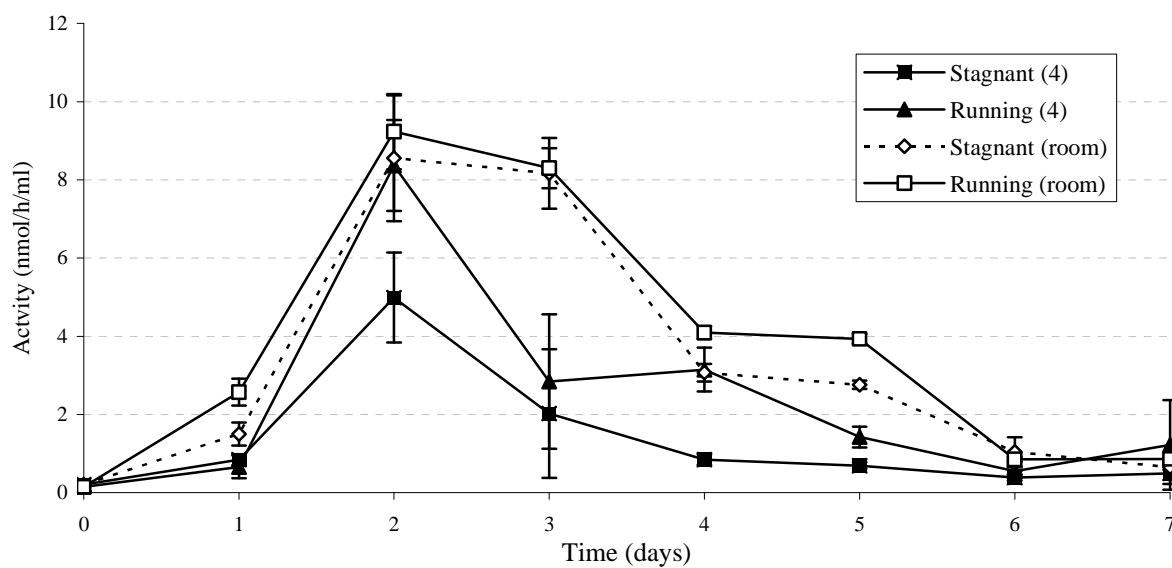
The highest stability was observed for PNPG stored at  $-20\text{ }^{\circ}\text{C}$  and uncovered PNPG stored at room temperature (away from direct sunlight and UV, but exposed to electric light). The covered PNPG at room temperature was the least stable and degraded rapidly from day 9 to 13 (Fig. 5.2). There was noticeable PNPG breakdown between day 22 and 23 at  $4 \pm 1\text{ }^{\circ}\text{C}$ . Therefore, the best recommended storage condition for PNPG is at  $-20\text{ }^{\circ}\text{C}$  or at room temperature.



**Figure 5.2** Storage stability of PNPG under different conditions. -20 and 4 represent temperature values ( $^{\circ}\text{C}$ ) at which samples were stored. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

### 5.3.3 Environmental GUD storage stability

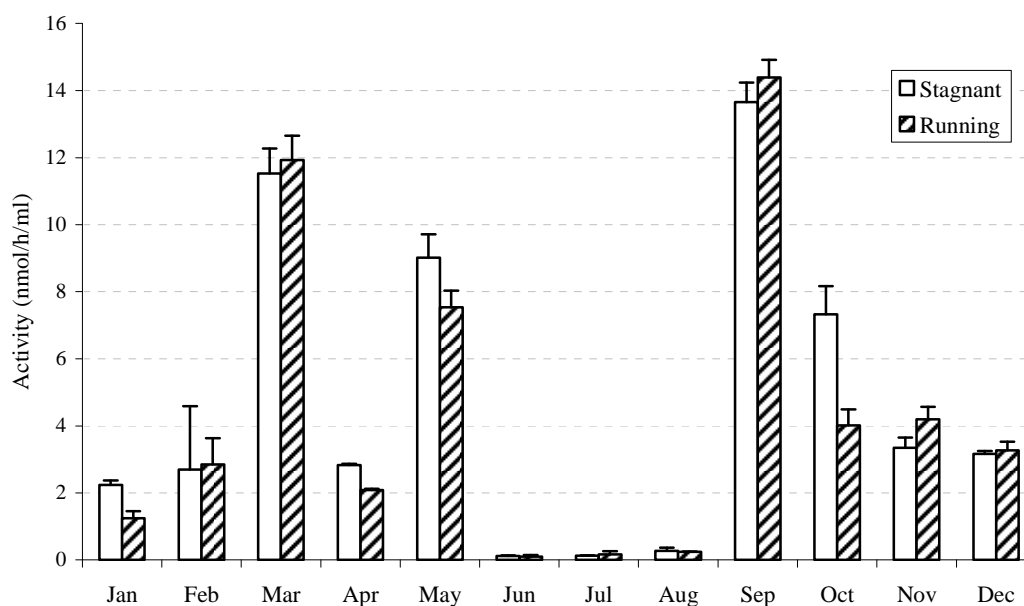
Interestingly, there was an increase in GUD activity in all samples from sampling time up to day two (Fig. 5.3). Samples at room temperature had consistently higher GUD activity than those stored at  $4 \pm 1$   $^{\circ}\text{C}$ . A similar trend was noted with *E. coli* counts that decreased after day two.



**Figure 5.3** GUD activity in stagnant and running water samples stored under different conditions. Stagnant (4): sample from stagnant water stored at  $4 \pm 1$  °C; Running (4): sample from running water stored at  $4 \pm 1$  °C. All data points represent the mean  $\pm$  SD (n = 3).

#### 5.3.4 Monthly trends

In general, the June to August period (winter months) exhibited low enzyme activity (Fig. 5.4). The highest GUD activity was observed in September. The GUD trend exhibited three peak periods, March, May and September (Fig. 5.4).



**Figure 5.4** Mean monthly GUD activity variations at the two study sites. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

## 5.4 Discussion and Conclusions

### 5.4.1 Discussion

The 24 h period of detection allowed enough time for noticeable changes in environmental enzyme activity that could initially have been inhibited by the different pollutants. Wetzel (1991) observed a reduction in phenolic compounds with time in water. Therefore the 24 h period will allow GUD to operate maximally after phenolic compound reduction especially in water polluted by wastes from textile and paint industries. While increased temperatures can yield more rapid results, such temperatures have been found to increase interference by pollutants and accelerate GUD denaturation (Chapter 2). The observed detection time and LOD compares well with the improved conventional methods (Venter, 2000; Rompré *et al.*, 2002) and the Colilert methods that make use of fluorogenic substrates for GUD. The time of analysis in these methods ranges between 16 and 24 h and can detect one culturable CFU per 100 ml (Berger, 1994; Cowburn *et al.*, 1994; Fricker and Fricker, 1996<sup>b</sup>; Grant, 1997; Sartory and Watkins, 1999).

The observed correlation coefficient ( $R^2 = 0.89$ ) is greater than the 0.73 reported by Farnleitner *et al.* (2001), but less than the 0.92 reported by Garcia-Armisen *et al.* (2005). In general, it is difficult to obtain a high correlation in different environments, considering the presence of VBNC bacteria that can potentially contribute to the GUD activity. The degree of viability may vary with water pollutants and nutrient concentrations, contributing to skewed correlation. However, correlation coefficient of above 0.8 in the environmental samples suggests a positive correlation between the *E. coli* and GUD activity. The observation that GUD activity was present in all samples highlighted the ability of direct enzyme assaying method to detect enzyme activity of VBNC cells. While a LOD of GUD activity equivalent to 2 CFU/100 ml may indicate a sensitive technique, it does not necessarily mean that the two CFU are entirely responsible for the observed activity. The activity could also be from GUD from VBNC bacteria and lysed cells. Furthermore, low correlation can be attributed to GUD from non-target sources (Tryland and Fiksdal, 1998; Pisciotta *et al.*, 2002).

When non-target microorganisms producing GUD occur in high numbers, a sensitive GUD detection technique may be susceptible to interference. Van Poucke and Nelis (1997) highlighted the paradoxical nature of sensitivity of direct enzyme assay to a level as low as 1 CFU/100 ml of water sample. They argued that an increase in sensitivity is coupled to a concomitant increase in susceptibility to interference from non-target sources. While this is true, GUD from different sources normally operate optimally at different pH values (Gilissen *et al.*, 1998). This will assist in reducing interference by GUD from non-target sources.

In addition, the correlation between microbial counts and enzyme activities illustrated the difference in the degree of accuracy of the MF technique and the direct enzyme assay. If the two methods provided 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 one. The trend of PNPG stability at room temperature suggests that light may be playing a role in the

substrate stability. Further investigation to fully explain the influence of light on the chemical structure of PNPG may be required.

Increase in GUD activity during water storage (Fig. 5.3) could be due to microbial growth (Jagals *et al.*, 2003) and reversal of enzyme inhibition (Wetzel, 1991). Therefore, the deterioration of microbial water quality during storage may give a false sense of safety when the monitoring of indicator organisms is terminated at the point of supply.

Contrary to the observation that a high level of faecal coliforms were observed after rainstorms (Venter *et al.*, 1997), results from these studies showed a decrease in enzyme activities that were positively correlated to the coliform counts. The enzyme activity is partly a function of the concentration of enzyme and partly of inhibitor present. Low GUD activity during months that received high rainfall may be attributed to a high level of enzyme dilution as the runoff would have carried away most faecal material (Jagals *et al.*, 1995; Jagals, 1997) and diluted the remaining GUD in the water before sampling was conducted. Higher GUD activity obtained during the warm and dry periods may be explained by high microbial activity under such temperatures and mixing aided by convection currents. Low runoff could be used to explain the high activity due to accumulation of faecal material in the course of the river and surrounding areas. In addition, levels of enzyme inhibitors could have been increased by runoff during the rainy months. Monthly assays help to explain the microbial dynamics and changes in GUD activity detected throughout the year.

### 5.4.2 Conclusions

From these studies, a liquid bioprobe could be established based on the hydrolysis of PNPG by GUD. The ingredients of the bioprobe were:

- (a) assay buffer (0.1 M Tris-HCl, 0.6 mM CaCl<sub>2</sub>, pH 8.0),
- (b) substrate (10 mM PNPG in assay buffer), and
- (c) the water sample to be examined.

The ratio of (a):(b):(c) in the assay mixture was 0.2:0.44:0.36, respectively.

The detection of GUD could be achieved within 24 h with a LOD of 2 CFU/100 ml water sample. Therefore, the concept of direct enzyme assay, using PNPG for GUD assay, was a feasible alternative method for the detection of faecal pollution in South African river water samples. However, there is a need to investigate the possibility of immobilising the substrates on a solid support to enable ease of handling, improved stability and prolonged shelf life of the substrate.

## 6 COLOUR STRIP DEVELOPMENT

### 6.1 Introduction

The concept of immobilising ligands to insoluble supports has received a lot of attention over the years (Krajewska, 2004). A number of membrane-based chromophoric devices are readily available. The major contributing factors to the high rate of development of these chromographic devices include advantages associated with immobilised ligands and improvements in conjugate technology. The amine groups on proteins are the most frequently exploited moieties in immobilisation. Reactive groups usually employed on the support material include aldehydes, cyanogen bromide, epoxy and tosyl. These reactive groups are able to yield stable enzyme-support material bonds. However, a disadvantage of using these reactive groups is their instability at alkaline pH (Hermanson *et al.*, 1992).

Immobilisation strategies include covalent attachment, gel entrapment, physical adsorption on materials such as ion exchange resins, acrylic polymers, emulsion membrane reactors, nylon fibres, silica and nitrocellulose (Hermanson *et al.*, 1992; Steinitz and Tamir, 1995; Bayramoğlu *et al.*, 2005). A particularly convenient immobilisation matrix is nylon. It is inexpensive, inert, non-toxic, readily available and can be obtained in a number of forms. Unmodified nylon surfaces are hydrophobic and can lead to substantial non-specific binding of proteins (Isgrove *et al.*, 2001). Generally, immobilisation improves stability and catalytic efficiency of the immobilised molecules (Krajewska, 2004; Su *et al.*, 2005).

While vast accounts of protein immobilisation exist (Krajewska, 2004); there is a paucity of information on non-protein based molecule immobilisation. For this reason, the main objective of this chapter was to attempt to immobilise PNPG on a solid support to obtain a GUD test strip. The specific objectives were to:

- (a) investigate the use of nitrocellulose and nylon membranes as support materials for PNPG,
- (b) score colour change (due to GUD activity) of the immobilised substrate,

- (c) establish the GUD LOD on the immobilised PNPG, and
- (d) investigate the storage stability of the test strips.

## 6.2 Materials and methods

### 6.2.1 Physical adsorption on nitrocellulose and nylon membranes

The support materials used were readily available and affordable. Nitrocellulose (Millipore, USA) and nylon (Boehringer Mannheim, USA ) membrane squares, measuring approximately 10 x 10 mm, were incubated in a solution of PNPG (1 mg PNPG/ml prepared in 0.1 M Tris-HCl buffer, pH 8.0) overnight and air dried at room temperature. Different concentrations of commercial GUD were then applied to the immobilised strips and colour changes monitored over time.

### 6.2.2 Immobilisation on partially degraded nylon

PNPG was immobilised on nylon using a protocol modified from Isgrove *et al.* (2001). Nylon membrane was cut into approximately 10 x 10 mm squares, partially degraded by incubation in 2.9 M HCl for 2 h at 37 °C, washed thoroughly with water and then with 0.1 M Tris-HCl buffer (pH 8.0). The strips were then incubated in PNPG (1 mg/ml) solution overnight at  $4 \pm 1$  °C and air-dried at room temperature. Varying concentrations of commercial GUD were applied to the dried membranes and the colour changes were scored.

### 6.2.3 Colour scoring

Colour scoring was performed, as stated by Russell and Burton (1999), using Adobe Photoshop 8.0 (Adobe Systems Incorporated, USA). The membranes were scanned and colour units read from the RGB colour scoring chart on the display screen. The difference between the reading on a substrate control strip and the coloured test strip was taken as the colour score in Adobe colour units.

### 6.2.4 Storage stability of substrate strips

PNPG immobilised on partially degraded nylon membrane strips was stored in petri dishes under conditions described in Chapter 5, for the liquid substrate. Colour scoring after addition of (a) commercial GUD solution to confirm presence on PNPG and (b) buffer to monitor accumulation of PNP was performed every other day for a month.

## 6.3 Results

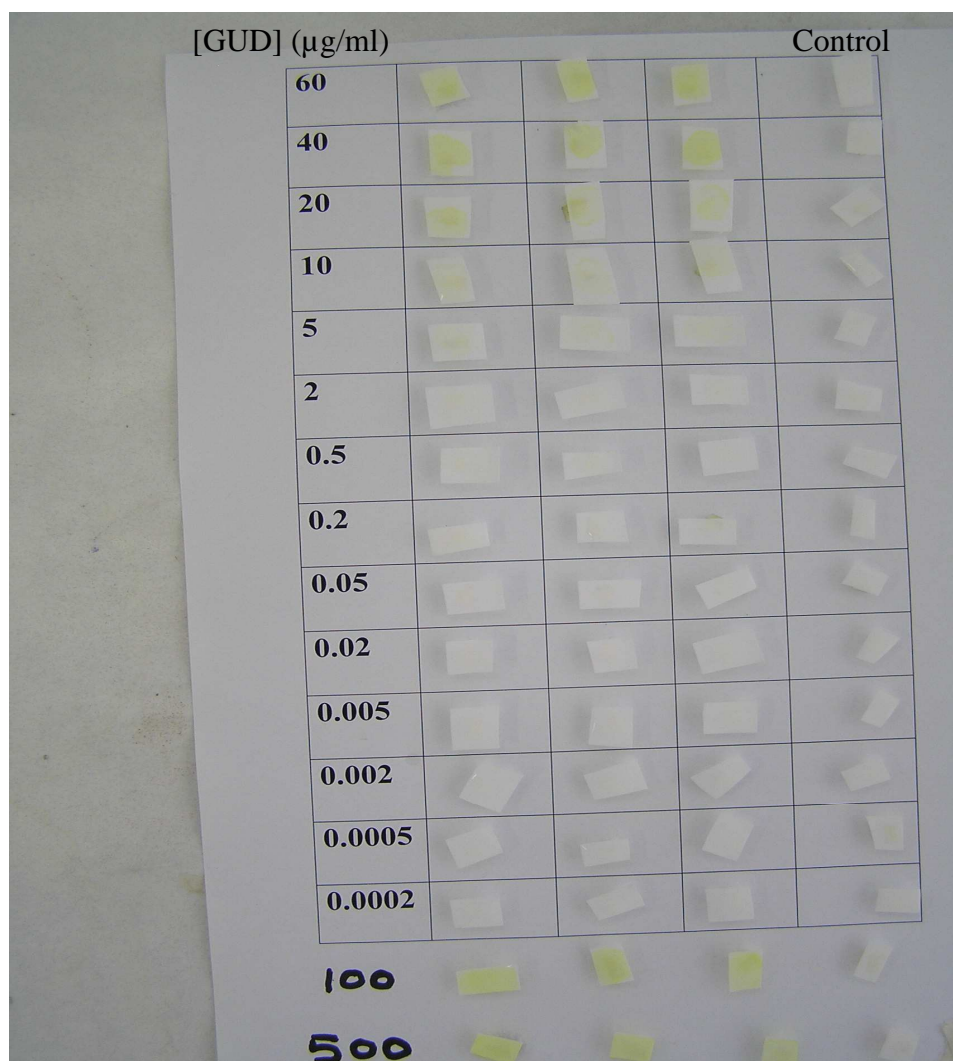
### 6.3.1 Strip colour changes

Colour changes were observed on the strips after addition of GUD. However, there was rapid diffusion of the PNP (yellow) from PNPG adsorbed to the unmodified nylon and nitrocellulose films. When different concentrations of GUD were added to the substrate immobilised on partially degraded nylon varying colour intensities were observed (Fig. 6.1). The time taken for colour to develop was inversely proportional to GUD concentration with high concentrations producing an instant yellow colour (Table 6.1). The visual limit of detection (LOD) for GUD was 5 µg/ml within 20 min (Table 6.1). Very limited colour diffusion from the support material was noted after addition of the enzyme solution.

**Table 6.1** Summary of observed colour changes and Adobe colour scoring LOD.

GUD concentration (µg/ml)	Rate of colour change (visual)	Detection by Adobe Photoshop colour scoring
10 – 500	+	+
5	+	+
2	Not detected	+
0.5	Not detected	+
0.2	Not detected	+
0.05	Not detected	+

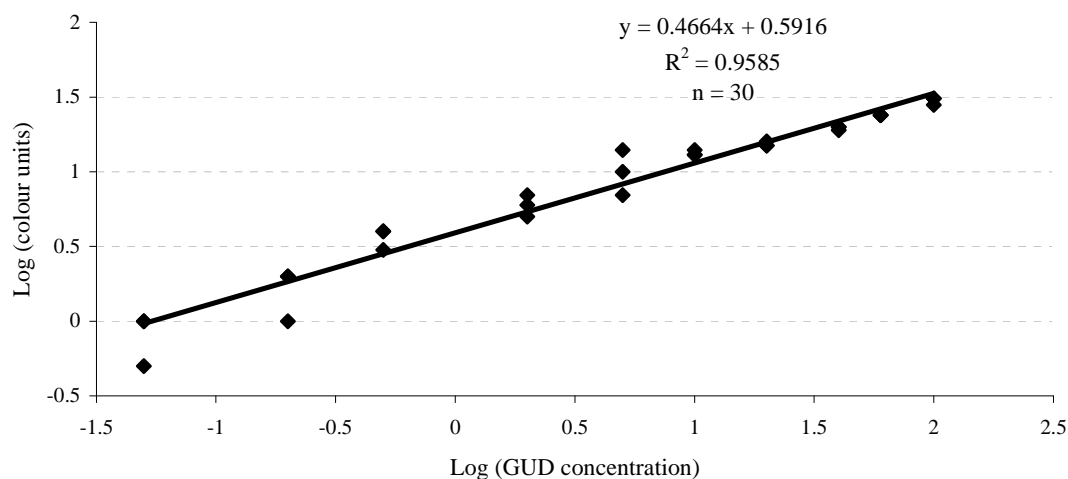
+: detected; +\*: instant detection; + (20 min): detected after approximately 20 min.



**Figure 6.1** Hydrolysis of PNPG immobilised on partially degraded nylon membrane by different concentrations of commercial GUD.

### 6.3.2 Adobe colour scoring

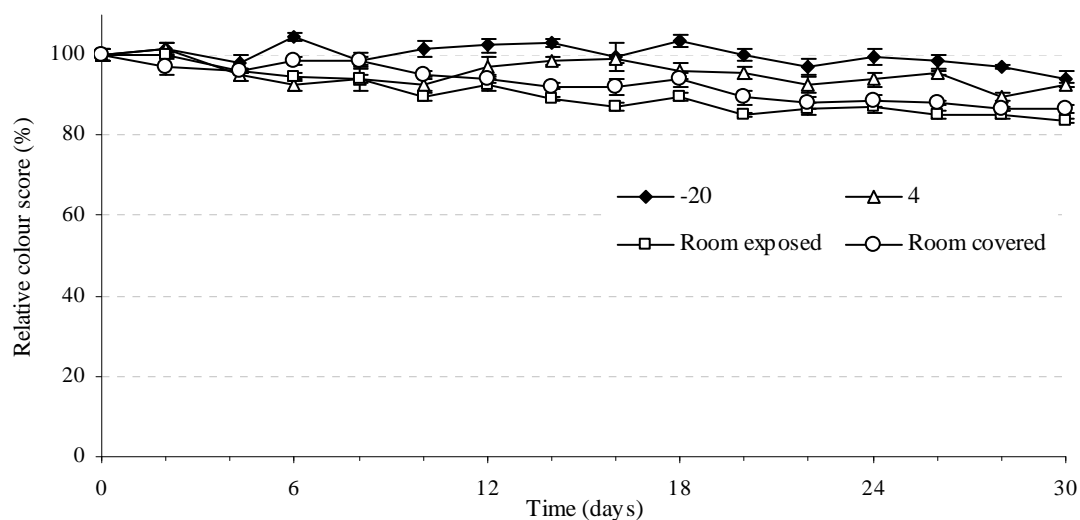
The GUD limit of detection with the Adobe Photoshop colour scoring was  $0.05 \mu\text{g/ml}$  (Table 6.1). A significant ( $P < 0.05$ ) correlation ( $R^2 = 0.9594$ ,  $n = 30$ ) was observed between the transformed colour units and GUD concentration (Fig. 6.2). The range of linearity of GUD concentration and colour change extended from  $0.05$  to  $100 \mu\text{g/ml}$ .



**Figure 6.2** Double log plot for Adobe colour units versus GUD concentration on hydrolysis of PNPG immobilised on partially degraded nylon membrane.

### 6.3.3 Strip storage stability

All the strips retained above 80 % of the initial activity for the whole month (Fig. 6.3). The strips stored at  $-20\text{ }^{\circ}\text{C}$  were the most stable; followed by those stored at  $4 \pm 1\text{ }^{\circ}\text{C}$ ; room temperature covered by aluminium foil; and lastly the uncovered strips (Fig. 6.3). In general, PNPG was more stable when immobilised than dissolved in the Tris-HCl buffer (Chapter 5).



**Figure 6.3** Storage stability of PNPG immobilised on the partially degraded nylon membrane. -20 and 4 represent temperature ( $^{\circ}\text{C}$ ). All data points represent the mean  $\pm$  SD ( $n = 3$ ).

## 6.4 Discussion and Conclusions

### 6.4.1 Discussion

The strength of bonds between the ligand and support material depends on the structure of a ligand, the method of immobilisation and type of support (Hermanson *et al.*, 1992; Bayramoğlu *et al.*, 2005). Results obtained in this study showed that the type of membrane and modification influenced PNP/PNPG immobilisation stability. The unmodified membranes, although resulting in some degree of adsorption, allowed the diffusion of the PNP upon addition of GUD. Therefore, physical adsorption of PNPG to unmodified nitrocellulose and nylon membranes is undesirable. The observed diffusion in colour of the chromogen does not allow for accurate colour observation on the strip. In addition, this strip requires a limited exposure time to the enzyme solutions, which may not be adequate to allow notable enzyme hydrolysis of the substrate using environmental samples characterised by low levels of enzyme. Desorption is a common phenomenon of physically adsorbed molecules (Isgrove *et al.*, 2001; Krajewska, 2004). The problem of desorption can be circumvented by

synthesising substrates with modified/additional functional groups for covalent bonding to the surface of interest without reducing the enzyme's affinity for the substrate. Support material can be designed to enable covalent attachment to the substrate. However, these options were beyond the scope of this study as they require the input of specialists in chemical synthesis.

The partial degradation of nylon membrane exposes carboxyl and hydroxyl groups that interact with carboxyl and nitro groups on PNPG, and hydroxyl and nitro groups on PNP (Alkorta *et al.*, 1996; Haydar *et al.*, 2003) yielding more stable bonds as opposed to physical adsorption alone. A reasonable explanation could be that the liberated *p*-nitrophenol formed stable bonds with the modified nylon membrane.

#### **6.4.2 Conclusions**

From the immobilisation attempts, the following could be concluded:

- (a) immobilisation of the PNPG substrates on modified nylon membrane is feasible, and
- (b) physical adsorption on both nylon and nitrocellulose membranes is however, not feasible, due to desorption of the substrates from the solid support.

Liquid bioprobe requires the same time for analysis as the conventional culturing methods. The test strip is not feasible in environmental samples with low GUD concentration and activity. With these points in mind, it was necessary to look at the electrochemical detection of GUD activity to improve sensitivity with the aim of developing either a continuous or semi-continuous technique for routine monitoring systems. This will contribute to the implementation of water safety plans and quality framework proposed by WHO (2004) (Hodgson and Manus, 2006). This forms the basis of the following two chapters.

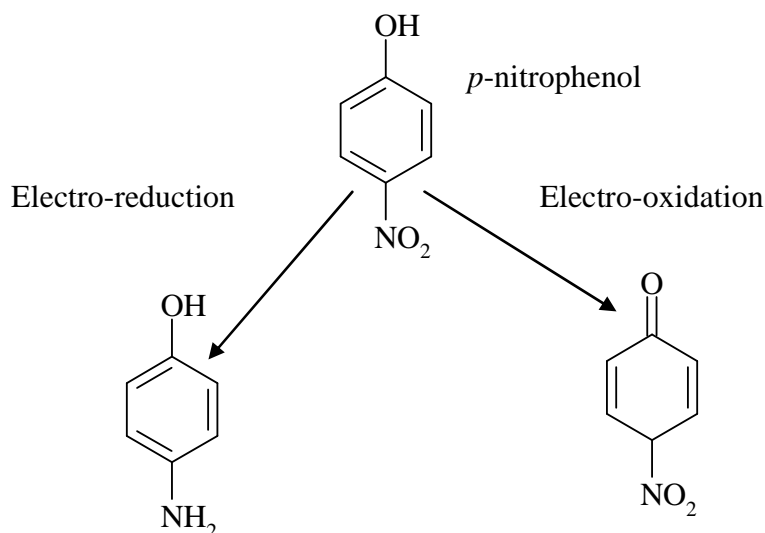
## 7 ELECTROCHEMICAL DETECTION: PNPG-PNP INTERFERENCE

### 7.1 Introduction

In previous chapters, it was concluded that the sensitivity of spectrophotometric assays of GUD is highly comparable to the membrane filtration method in detecting *E. coli*. However, the incubation time required for the enzyme assay at room temperature was 24 h. In addition, spectrophotometric methods are susceptible to interference by turbid solutions. Electrochemical detection is generally sensitive and not susceptible to the turbidity of the analyte. In addition, electrochemical methods are readily adaptable for on-line monitoring, reproducible and amenable to miniaturisation (Scouten *et al.*, 1995; Skoog *et al.*, 1996; Leonard *et al.*, 2003; Paitan *et al.*, 2003; Kissinger, 2005). Simultaneous measurements of samples can be achieved through the use of electroanalytical methods. The use of inexpensive disposable electrodes makes these methods even more attractive. For the reasons above, electrochemical detection of GUD activity was investigated based on the production of PNP from PNPG. It was important then to characterise the electrochemical analysis of PNPG and PNP.

Reports on detection of PNP and other phenolic pollutants mainly use electro-reduction methods (D'Souza, 2001; Sotomayor *et al.*, 2002; Stanca *et al.*, 2003; Pedrosa *et al.*, 2004). During reduction of PNP, the nitro group is reduced to an amine group (Fig. 7.1) liberating four to six electrons, depending on electrolyte conditions. The hydroxyl group is electroactive during oxidation. It is oxidised to a quinone (Fig. 7.1); under normal conditions this oxidation involves a transfer of one electron (Cheah *et al.*, 1966; Cosnier, 1999; Calvo-Marzal, *et al.*, 2001; Hu *et al.*, 2001; Borrás *et al.*, 2003; Luz *et al.*, 2004). The electrons transferred are directly proportional to current produced (Skoog *et al.*, 1996). Therefore, PNP detection by electro-reduction is more sensitive than electro-oxidation. Most reports on electrochemical PNP detection describe methods for monitoring environmental chemical pollution (D'Souza, 2001; Mulchandani *et al.*, 2002; Lei *et al.*, 2003;

Mulchandani *et al.*, 2005) but none have been used to detect enzyme activity for faecal pollution monitoring.

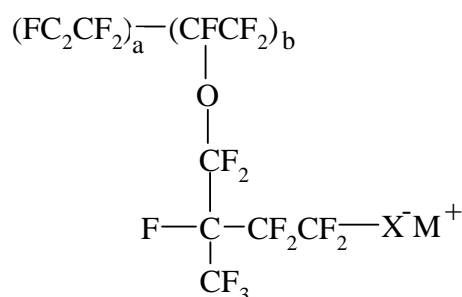


**Figure 7.1** Illustration of PNP electroactive groups and their products of electro-oxidation and electro-reduction.

PNP is optimally detected at acidic pH (Hu *et al.*, 2001), a factor that makes it difficult to monitor neutral or alkaline enzyme activity. In electrochemical enzyme assaying; Calvo-Marzal *et al.* (2001) monitored acid phosphatase activity by detecting PNP produced from the breaking down of *p*-nitrophenyl phosphate (PNPP) at pH 5.0, while Pérez *et al.* (2001) and Nistor *et al.* (2002) monitored *E. coli* using  $\beta$ -D-galactosidase (GAL) activity on 4-aminophenyl- $\beta$ -D-galactopyranoside (4APG). The breakdown product, 4-aminophenol (4AP), was then detected by electro-oxidation. However, the limits of microbial detection from these attempts were too high to be used for microbiological water quality monitoring. In addition, culturing was required to generate a reasonable signal thus prolonging the assay method. Furthermore, 4AP is not stable in aqueous solution (Thompson *et al.*, 1993) and this compromises the sensitivity of the technique.

It is important that the monitored product (PNP) in an enzyme assay be distinct from the substrate (PNPG). When a substrate interferes with the detection of its breakdown product the true enzymatic effect is masked, thus rendering the assay ineffective.

Strategies to mitigate the interference include coating electrodes using polymers that enable selective passage of the breakdown product to electrode surfaces, for example Nafion<sup>®</sup> (Calvo-Marzal *et al.*, 2001). Nafion<sup>®</sup> (Fig. 7.2) is a cationic exchanger membrane and screens molecules both by charge and size exclusion. It is preferred due to its good mechanical properties (Shi and Anson, 1997; Okada *et al.*, 1998; Singh and Shahi, 1998; Shi and Anson, 1998; Daniele *et al.*, 1999; Brown and Lowry, 2003; Wilson and Gifford, 2005).



**Figure 7.2** Nafion<sup>®</sup> perfluorinated ionomer structure (Adapted from James *et al.*, 2000; Rollet *et al.*, 2002). X: either a sulphonic or carboxyl group; M: metal cation in neutralised form or H<sup>+</sup> in acid form.

In view of the highlighted advantages of electrochemical monitoring, there is need to explore electroanalytical methods for indicator organisms detection. Therefore, the main objective of this chapter was to investigate the possibility of electrochemical detection of PNP in the presence of PNPG at pH 8.0. The specific objectives were to:

- establish redox potentials for PNP and PNPG,
- assess PNPG interference on PNP detection,
- reduce PNPG-PNP interference using Nafion<sup>®</sup>, and
- compare the redox currents for PNP and PNPG.

## 7.2 Materials and Methods

### 7.2.1 Cyclic voltammetry

The electrolyte solution was made up of Tris-HCl buffer (as described in Chapter 2) used in the spectrophotometric enzyme assay. A glassy carbon electrode (GCE) was used as the working electrode against a silver/silver chloride (Ag/AgCl) reference

electrode with platinum as a counter electrode. The electrolyte was degassed by flushing in nitrogen for 10 min. A nitrogen blanket was maintained above the electrolyte during the electrochemical analysis of the samples. Cyclic voltammetry was performed using an Autolab PGSTAT 30 potentiostat (Autolab, Utrecht, Netherlands) at a scan rate of 0.1 V/sec. The GCE was polished with 0.05  $\mu\text{m}$  alumina (aluminium oxide), rinsed thoroughly with ethanol, milli Q water and dried with an absorbent paper towel after each voltammetric cycle. The described voltammetric method was used for all subsequent analyses. The electrochemical measurements were performed at controlled room temperature ( $20 \pm 2$  °C). The limit of detection (LOD) was determined experimentally as the lowest enzyme concentration that produced a measurable peak current. Triplicate measurements were performed, and mean values and standard deviations were used to plot the graphs.

### 7.2.2 PNPG-PNP interference

To investigate the interference of PNPG on PNP detection a mixture of PNP and PNPG was prepared, with a final analyte concentration of 1 mM in 10 ml electrolyte. Cyclic voltammograms of PNP, PNPG and PNP in the presence of PNPG were analysed for the interference of PNPG on PNP detection.

### 7.2.3 Nafion<sup>®</sup> studies

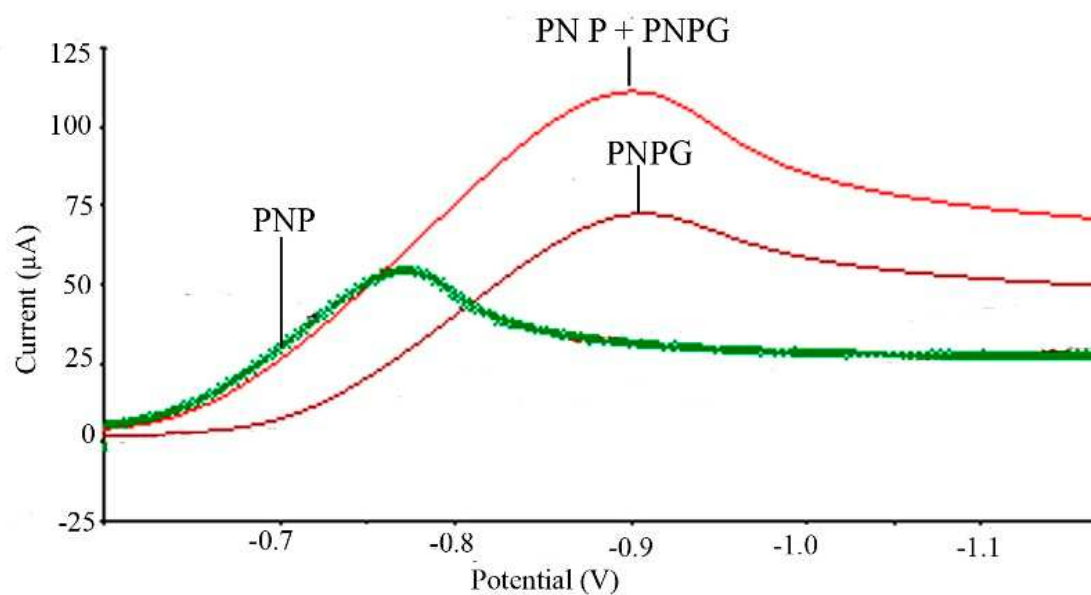
An attempt to eliminate interference between PNPG and PNP was made using Nafion<sup>®</sup> (Aldrich, Steinheim, Germany). The Nafion<sup>®</sup> was diluted in organic solvent (1:1; water: methanol) to obtain stocks of concentration between 1 and 5 % (v/v) (Daniele *et al.*, 1999). Twenty five microlitres of the diluted Nafion<sup>®</sup> was dropped on the surface of an upright GCE and allowed to dry at room temperature (Calvo-Marzal *et al.*, 2001; Brown and Lowry, 2003). After each voltammetric measurement, the electrode was polished as described above (section 7.2.1) and a new coating applied. Results were compared with those obtained using unmodified electrodes.

Investigation of the durability and fouling of the coating when exposed to repeated electrochemical measurements was performed by running the experiment using the same electrode without changing the coating. After each voltammetric cycle the electrode was rinsed with milli Q water and used with freshly prepared analyte.

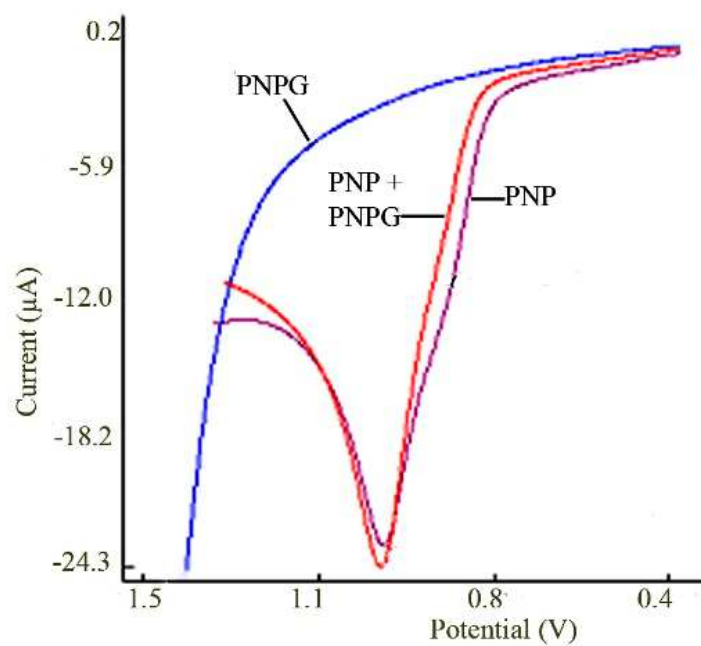
## 7.3 Results

### 7.3.1 Interference

PNP was detected both by oxidation (at +0.95 V) and reduction (at -0.77 V), although the reduction current peaks were about four times greater in magnitude than the oxidation peaks. PNPG generated one irreversible cathodic wave at -0.90 V but was not detected by oxidation (Fig. 7.3, Table 7.1). Electro-reduction of the PNP-PNPG mixture produced a synergistic peak at -0.89 V (Fig. 7.3a). The reduction current peaks for both PNP and PNPG were linear from 0.1 to 1 mM (Table 7.1).



a



b

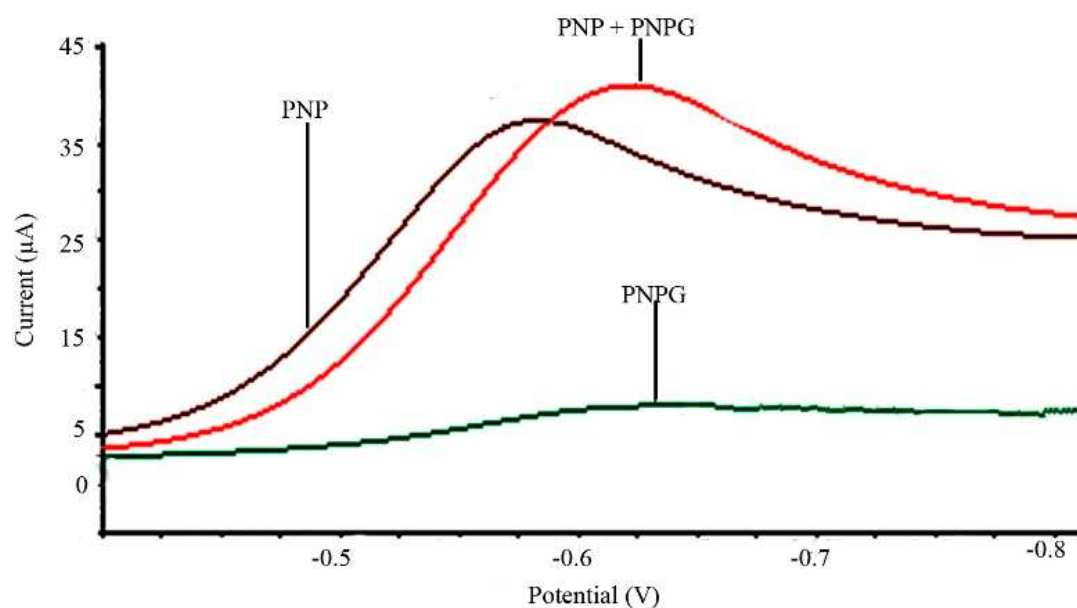
**Figure 7.3** Voltammograms illustrating interference of PNPG on PNP detection during reduction (a) and no interference during oxidation (b). [PNP] = 1 mM; [PNPG] = 1 mM, pH = 8.0 (Tris-HCl); scan rate (0.1 V/s). The return scans are not shown for the sake of clarity.

**Table 7.1** A summary of results of cyclic voltammetric characterisation of PNPG and PNP.

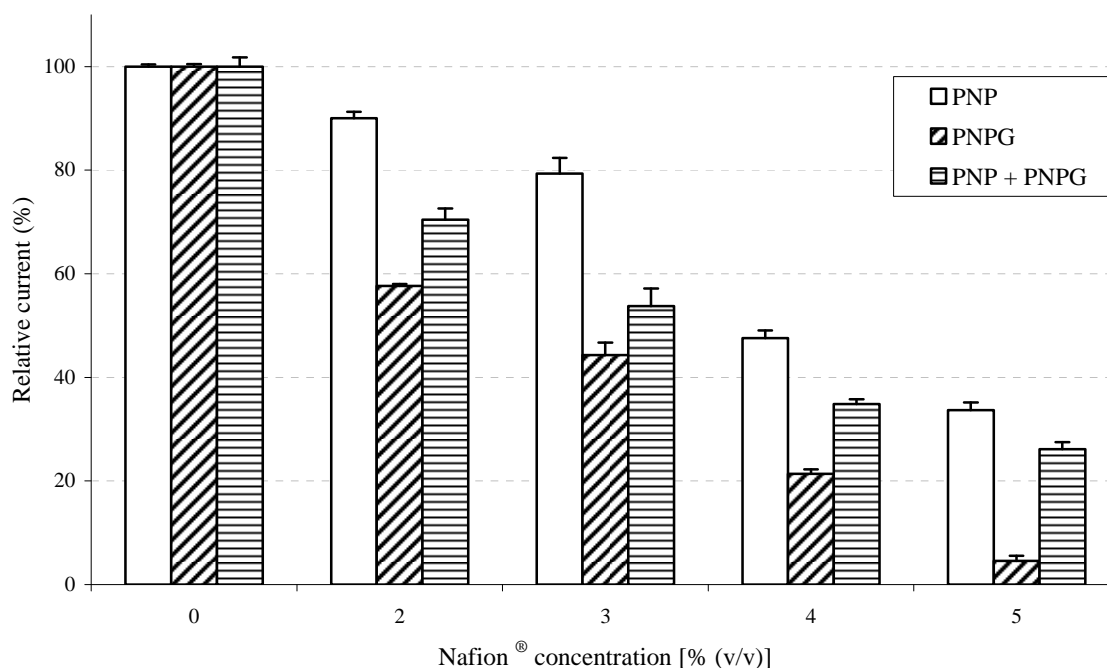
Attribute	Plain GCE		5 % Nafion <sup>®</sup> coated GCE
	Oxidation (V)	Reduction (V)	Reduction (V)
<b>Redox potential (V)</b>			
PNPG	Not oxidised	-0.90	-0.61
PNP	+0.95	-0.77	-0.57
PNP + PNPG	+0.95	-0.89	-0.61
<b>LOD (mM)</b>			
PNPG	N/A	0.05	0.1
PNP	0.1	0.05	0.1
<b>Linearity</b>			
PNP (mM)	0.1 – 1 $R^2 = 0.95$	0.05 – 1 $R^2 = 0.98$	Not determined
<b>Electrode fouling in PNP detection</b>	No peak observed after first run	Same as oxidation	Reduced fouling (Fig. 7.6)

### 7.3.2 Nafion<sup>®</sup> modification of the GCE

The current obtained using a plain GCE was assigned a value of 100 % against which current obtained at the Nafion<sup>®</sup> modified electrode was compared. Nafion<sup>®</sup> decreased PNPG interference (Fig. 7.4). However, the sensitivity for PNP analysis was decreased. The current response for PNP, PNPG and the PNP-PNPG mixture was lowered by 66.36, 95.42 and 73.90 %, respectively, when 5 % Nafion<sup>®</sup> was used (Fig. 7.4). There was a shift in reduction potential (to less negative values) of PNP and PNPG after electrode modification (Table 7.1). Diluting the Nafion<sup>®</sup> layer to improve sensitivity to PNP led to a concomitant increase in PNPG interference (Fig. 7.5). Nafion<sup>®</sup> allowed more PNP to pass through than PNPG at all concentrations.

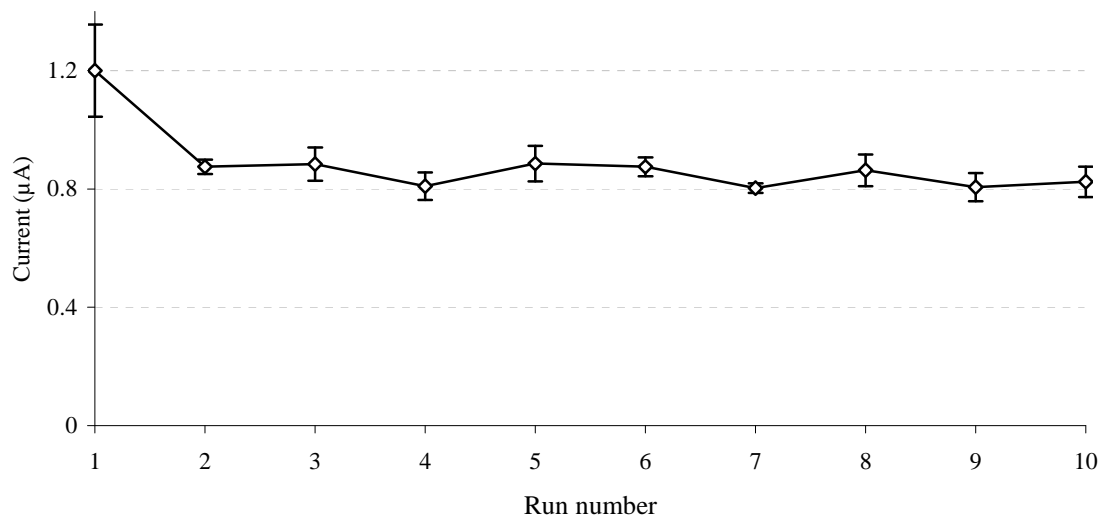


**Figure 7.4** Voltammograms illustrating PNPG interference on PNP detection by electro-reduction at a Nafion<sup>®</sup> coated GCE. [PNP] = 1 mM; [PNPG] = 1 mM, pH = 8.0; scan rate (0.1 V/s). The return scans are not shown for the sake of clarity.



**Figure 7.5** Comparison of current response for PNP, PNPG and the PNP-PNPG mixture detection before and after coating the GCE with Nafion<sup>®</sup>. pH = 8.0. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

Nafion<sup>®</sup> membrane increased resistance to fouling although it fouled with repeated voltammetric measurements for PNP. An abrupt drop in peak current (26.7 %) was observed between the first and second readings, after which the current remained constant (Fig. 7.6).



**Figure 7.6** Progressive decrease in peak heights of PNP (1 mM) due to Nafion<sup>®</sup> (5 %) fouling. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

## 7.4 Discussion and Conclusions

### 7.4.1 Discussion

Redox potentials for PNP vary with electrolyte conditions. For example, -0.73 V at pH 5.0 (Calvo-Marzal *et al.*, 2001), -0.82 V at pH 3.4 (Hu *et al.*, 2001), and -0.74 V at pH 4.0 (Luz *et al.*, 2004). The observed reduction potential for PNP using plain GCE was within the range of these values from literature. However, in literature there is no information pertaining to the reduction of PNPG. Therefore, this could be the first report on PNPG electroanalysis. The closeness in reduction potentials for PNP and PNPG suggests a reduction of the nitro group in both instances. The shift in reduction potential to a less negative value for PNP in the Nafion<sup>®</sup> coated electrode was not reported in literature when a similar electrode modification was performed for PNP detection (Calvo-Marzal *et al.*, 2001). The oxidation potential for PNP was within the

+0.80 – +1.15 V range observed by Pedrosa *et al.* (2004) and closer to the value of +0.90 V reported by Mulchandani *et al.* (2005). Shifts in redox potentials can be accounted for by temperature, pH, electrode modification, buffer system and the type of electrodes used.

PNP reduction is best detected at acidic pH (Hu *et al.*, 2001; Luz *et al.*, 2004) because of the abundance of “protons to form pre-protonated species to be reduced” (Calvo-Marzal *et al.*, 2001). Acidic pH was not employed in this study because GUD performed optimally at neutral to slightly alkaline pH (Chapter 2). Therefore, real-time PNP detection will be more favourable at pH 8.0 where GUD catalyses the PNPG breakdown maximally. Differences in peak heights for the PNP between reduction and oxidation (Fig. 7.3) confirms the differences in the electro active groups involved and the number of electrons transferred (Hu *et al.*, 2001; Borrás *et al.*, 2003; Pedrosa *et al.*, 2004).

Substrate interference with product detection is undesirable. Calvo-Marzal *et al.* (2001) successfully eliminated the interference of *p*-nitrophenyl phosphate (PNPP) with PNP using 5 % Nafion<sup>®</sup>. However, attempts to eliminate inference of PNPG in this study were not met with similar success. Reduction in sensitivity to PNP detection by Nafion<sup>®</sup> was also noted by Calvo-Marzal *et al.* (2001), but not to the extent observed in this study. These differences might be attributed to different pH values and substrates used in the assays. Calvo-Marzal *et al.* (2001) suggested that PNPP was repelled by negatively charged sulphonic acid groups on nafion membrane. In this study, the size of molecules seemed to be a major determining factor in exclusion of PNPG because of the trend noted in diluting Nafion<sup>®</sup> (Fig. 7.5). Increase in water content of Nafion is proportional to “hydrophilic domains through which ions move” and their size (Okada *et al.*, 1998). Therefore, as Nafion<sup>®</sup> concentration decreases, the channels become larger thus increasing access of both PNP and PNPG to the electrode surface.

Electrode fouling is a common problem encountered when working with phenolic compounds (Wang and Hutchins, 1985). Reduction in peak current with repeated use

of the modified electrode is a typical phenomenon of electrode fouling. The fouling is as a result of adsorption of reactants on the electrode surface, thus hindering conductivity (Wang and Hutchins, 1985).

Fouling of the Nafion<sup>®</sup> would translate into greater costs and an inefficient system. This is because the coating has to be performed for each measurement. This makes the choice of Nafion<sup>®</sup> for the elimination of PNPG interference difficult. Even without using Nafion<sup>®</sup>, electrochemical LOD of PNP (0.05 mM) at pH 8.0 gave an absorbance value of 0.65 at 405 nm. Thus, spectrophotometric detection at this level was more sensitive. If acidic pH is to be used for improved sensitivity, then a sequential flow analysis system (Chapter 9) would be more appropriate. Further exploration of other methods for the improvement of sensitivity is therefore required in order to maximally exploit this technique.

#### 7.4.2 Conclusions

The following conclusions were drawn from the work in this chapter:

- (a) detection of GUD activity on PNPG by electro-reduction is not feasible due to PNPG-PNP interference,
- (b) spectrophotometric detection of PNP at alkaline pH is more sensitive than electrochemical detection using a plain GCE,
- (c) detection of GUD by electro-oxidation is possible with no interference from substrate PNPG, but there is a need to improve the sensitivity of the method,
- (d) Nafion<sup>®</sup> reduced PNPG-PNP interference but there was a concomitant decrease in sensitivity, and
- (e) Nafion<sup>®</sup> modified GCE may be more suitable for a once off reading.

Nafion<sup>®</sup> coated GCE may require modification to increase the sensitivity to PNP detection. However, it is worthwhile to investigate possibilities of modifying the plain GCE to increase sensitivity for PNP detection by electro-oxidation. This will minimise the number of modification steps and simplify the detection process. The

next study (see chapter 8) was therefore focused on the modification of the GCE for the electro-oxidative detection of PNP.

## 8 MICROBIAL MODIFICATION OF GCE

### 8.1 Introduction

In the previous reductive voltammetric detection method of analysis, PNP-PNPG interference problems were encountered. This was accompanied by the need to purge oxygen. Interference is not desirable as it reduces the reliability of the proposed biosensor. The removal of oxygen adds to the costs and is thus not preferred. Attempts to eliminate the substrate and the breakdown product interference with the use of Nafion<sup>®</sup> led to a reduction in sensitivity (Chapter 7). Electro-oxidation showed no interference but reduced sensitivity was a problem. The major objective of the work in this chapter was to improve sensitivity of the detection of the PNP during electro oxidation. This was explored through microbial modification of the working electrode.

Electrode modification can be employed for a number of purposes that include: accelerated redox reactions, preferential analyte accumulation, selective permeability, improved electrode protection and stability (Murray *et al.*, 1987; Baldwin and Thomsen, 1991; Zhang *et al.*, 2000; Stanca *et al.*, 2003; Luz *et al.*, 2004). Electrodes can be modified with a range of biorecognition molecules that include enzymes and microbial cells (Mulchandani, 1998; Riedel, 1998; Stanca *et al.*, 2003). Pure enzymes are highly specific for their substrates, but disadvantageous because of the tedious, time-consuming, and costly purification and are sometimes unstable (D'Souza, 2001; Lei *et al.*, 2006). In addition, some enzyme electrode modification steps require multiple enzymes (Scheller *et al.*, 1998), which can further complicate the process. Microorganisms possess several enzymes that allow concomitant detection of a variety of compounds, are amenable to genetic modification, and tolerate extreme conditions (Riedel, 1998; D'Souza, 2001; Lei *et al.*, 2006). However, the ability to detect a wide range of compounds by microbial sensors may be undesirable when specificity is required. Most enzymes used in detection of phenolic compounds have broad specificity (Russell and Burton, 1999; D'Souza, 2001; Tang *et al.*, 2006), and thus may not be able to distinguish between PNP and PNPG. PNP degrading

microorganisms (e.g. *Moraxella*) (Spain and Gibson, 1991) have been reported to be highly specific and produce desirable electroactive intermediates (Mulchandani *et al.*, 2002; 2005). The approach used in this study took advantage of the ability of such microbes to degrade PNP, producing hydroquinone, which is more electroactive than PNP (Mulchandani *et al.*, 2005).

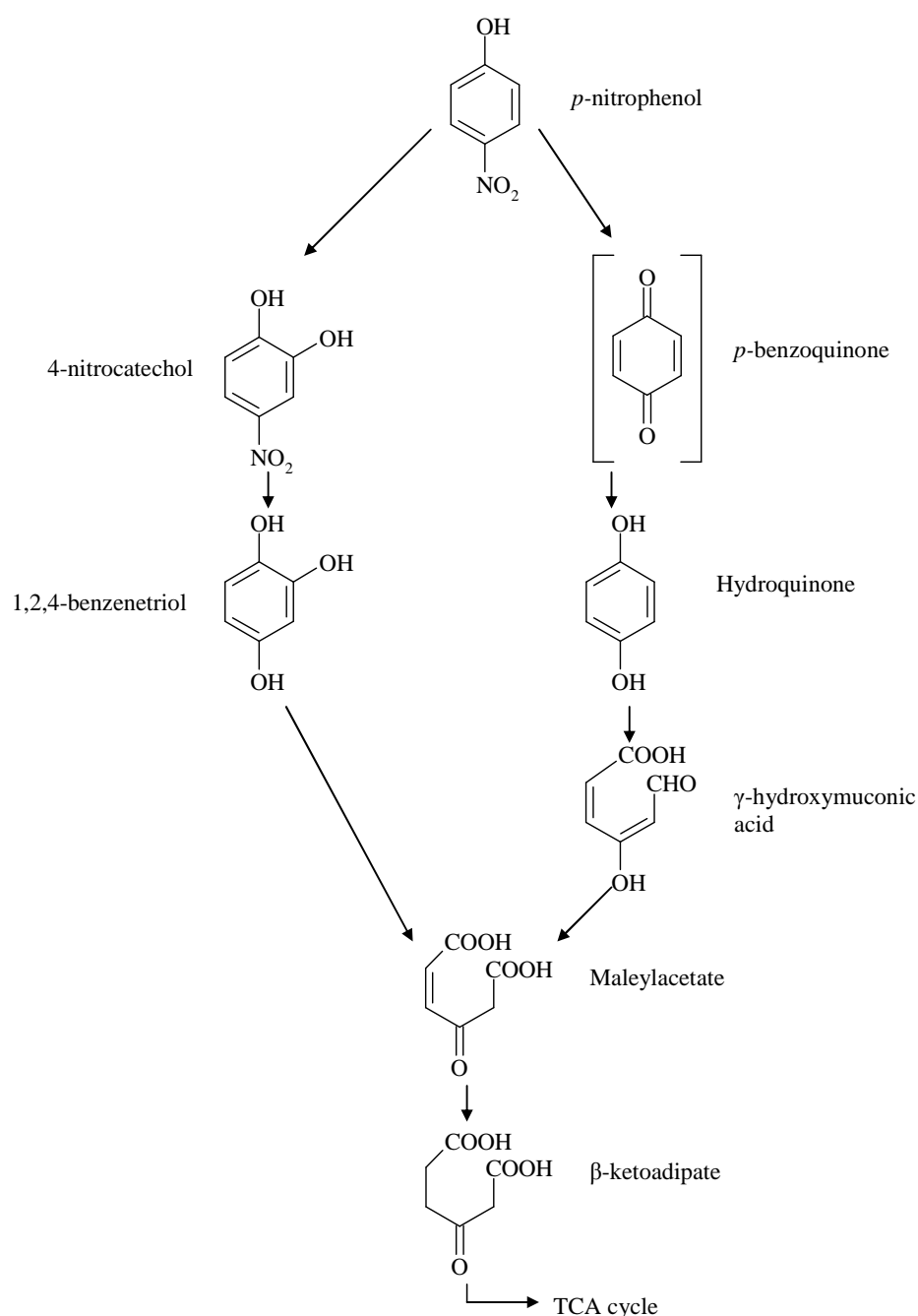
### 8.1.1 Microbial degradation of PNP

A number of bacterial species are known to degrade nitro-aromatic compounds either aerobically or anaerobically (Zeyer *et al.*, 1986; Spain and Gibson, 1991; Herman and Costerton, 1993; Nishino and Spain, 1993; Prakash *et al.*, 1996; Lei *et al.*, 2003; Leung *et al.*, 2005; Kulkarni and Chaudhari, 2006). During the degradation the microbes release nitrite or ammonia and channel other products to the tricarboxylic acid cycle (TCA) as shown in Figure 8.1 (Spain and Gibson, 1991; Nishino and Spain, 1993). *Pseudomonas* and *Moraxella* species are among those capable of using nitro-aromatic compounds like PNP as their sole source of carbon and nitrogen (Spain and Gibson, 1991).

The degradation pathways and intermediates differ between species. *Arthrobacter* species produce 4-nitrocatechol and 1,2,4-benzenetriol as early intermediates, hence the degradation pathway they use is known as the 1,2,4-benzenetriol pathway (Lei *et al.*, 2003) while *Moraxella* species produce 4-benzoquinone and hydroquinone in the hydroquinone pathway (Fig. 8.1) (Spain and Gibson, 1991). *Pseudomonas* species utilise the 1,2,4-benzenetriol and/or hydroquinone pathways in PNP degradation (Spain and Gibson, 1991; Prakash *et al.*, 1996). *Moraxella* species break down PNP via the hydroquinone route (Spain and Gibson, 1991). Figure 8.1 illustrates both pathways of PNP degradation. Regardless of the pathway taken, both produce maleylacetate which is converted to  $\beta$ -keto adipate. The latter is further broken down to provide reactants for the TCA cycle (Fig. 8.1).

The PNP – hydroquinone generation stage requires oxygen and NADPH; the latter is an electron acceptor in pathways of both *Pseudomonas* and *Moraxella* species (Zeyer,

*et al.*, 1986; Spain and Gibson, 1991). The specificity of the oxygenase and reductase enzymes involved in PNP degradation varies between species. Mulchandani *et al.* (2002) took advantage of the oxygen consumption stage to detect PNP using a *Moraxella* modified oxygen electrode. The analyte was saturated with oxygen and the oxygen consumption was monitored.

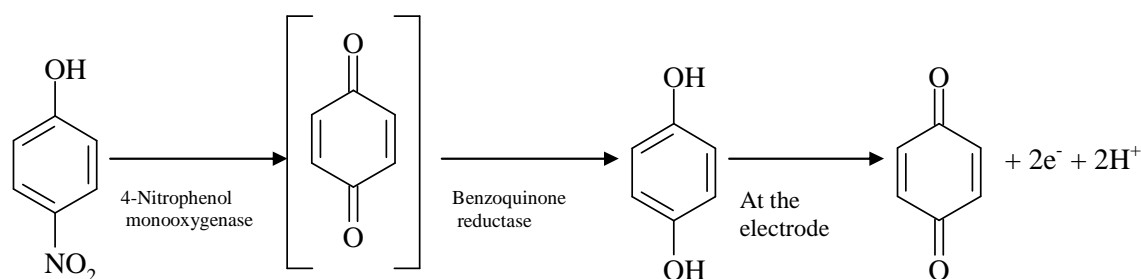


**Figure 8.1** Degradation pathways for PNP in bacteria (adapted from Spain and Gibson, 1991; Nishino and Spain, 1993; Mulchandani *et al.*, 2005).

### 8.1.2 Why bacterial modification of GCE?

During the early stages of PNP degradation benzoquinone generated is not oxidisable while the hydroquinone cannot be electro-reduced but is electro-oxidisable. The detection of hydroquinone by electro-oxidation is therefore preferred. Another advantage of hydroquinone electro-oxidation is that it is oxidised at relatively lower potential (300 mV) (Mulchandani *et al.*, 2005) than PNP (950 mV) (Chapter 7). This eliminates any possible interference between hydroquinone and PNP. The fact that hydroquinone is produced in the early stages of the degradation pathway allows for rapid detection and temporal separation with intermediates produced later in the pathway (after hydroquinone) that could interfere with the detection process. In addition, no studies have been reported that use monitoring of modified GCE for the detection of GUD activity on PNPG.

*Moraxella* 1A and *Pseudomonas putida* JS444 were selected for these studies. Both organisms degrade PNP and produce hydroquinone in the early stages of PNP degradation. *Moraxella* species specifically produces hydroquinone using a *p*-nitrophenol monooxygenase and benzoquinone reductase (Fig. 8.2). *Pseudomonas putida* JS444 degrades PNP faster than *Moraxella* 1A (Spain, pers. comm.), and should lead to more rapid generation of hydroquinone if it employs the same pathway as *Moraxella*. The *Moraxella* 1A species was therefore selected on the basis of hydroquinone production and *P. putida* JS444 on the basis of rate of PNP degradation.



**Figure 8.2** Detailed pathway for the early stages of PNP degradation by *Moraxella* sp. and reaction at the electrode under oxidative potential (adapted from Spain and Gibson, 1991; Mulchandani *et al.*, 2005).

The main objective of this chapter was to modify the GCE for improved sensitivity and selectivity to PNP, a product of PNPG hydrolysis by GUD. The specific objectives were to:

- (a) acclimatise cells for PNP degradation,
- (b) evaluate feasibility of different membranes for microbial entrapment,
- (c) investigate selectivity for PNP by *Moraxella* 1A and *P. putida* JS444 species,
- (d) compare sensitivity of the *Moraxella* 1A and *P. putida* JS444 biosensors,
- (e) investigate effects of cell disruption on biosensor response,
- (f) determine optimum pH, temperature, microbial load and volume of the enzyme solution for maximal biosensor performance,
- (g) perform field evaluation studies with the selected biosensor, and
- (h) determine the storage stability of the most sensitive biosensor.

## 8.2 Materials and Methods

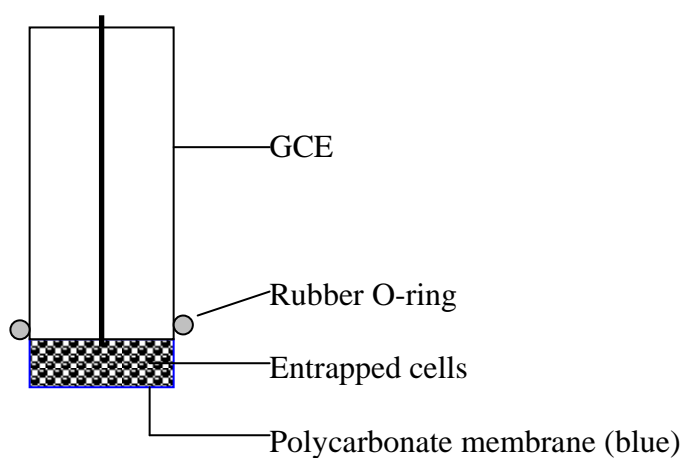
### 8.2.1 Culture revival, inoculum build-up and biomass scale-up

Dried cells (donated by Prof. J. C. Spain, Georgia Institute of Technology, Atlanta, USA) were resuspended in 1 ml 20 mM sodium phosphate buffer, pH 7.5. Two hundred microlitres were dispensed into flasks with 20 ml media (0.2 g/l Yeast extract powder (YEP) (Biolab, Midrand, South Africa), 50 mg/l *p*-nitrophenol (PNP) (Aldrich, Steinheim, Germany) and incubated at 30 °C with shaking at 250 rpm. Cells were transferred to 200 ml media and cultured as described above until the yellow PNP cleared (Appendix F). The cultures were transferred into 1.2 l media that contained 0.2 g/l YEP and 100 mg/l PNP, cultured at 30 °C, shaking at 100 rpm, in an orbital shaker. PNP was added twice after successive clearing of the PNP colour. The cells were harvested (when  $A_{600\text{nm}} = 1 \pm 0.15$ ).

### 8.2.2 Cell harvesting and immobilisation

The cultures were centrifuged at 15 300 g at  $4 \pm 1$  °C for 15 minutes, in pre-weighed tubes, washed twice in sodium phosphate buffer (20 mM, pH 7.5), weighed, and cells resuspended in the sodium phosphate buffer to give 100 mg/ml (wet mass/volume). These cells were stored at  $4 \pm 1$  °C with periodic supplementation of 15 mg PNP/l. Prior to use, the PNP was removed by centrifugation and the cells resuspended in phosphate buffer maintaining a concentration of 100 mg cells/l.

Cells were immobilised using a method adapted from Mulchandani *et al.* (2002; 2005) and Lei *et al.* (2003). The required volume of cell suspension corresponding to the desired wet weight was dropped onto a 25 mm diameter polycarbonate membrane, 50 nm pore diameter (Whatman, Kent, USA) and semi-dried under suction. The cells were then entrapped between the polycarbonate membrane and the glassy carbon electrode (GCE) with the former secured on the latter using a rubber O-ring and parafilm (Fig. 8.3).



**Figure 8.3** Schematic presentation of the microbially modified GCE.

### 8.2.3 Amperometric measurements

Amperometric measurements were performed using a Bioanalytical systems (BAS), USA, Epsilon voltammetric analyser with Epsilon EC 2000 software, version 1.50.59. All the experiments were carried out using a three electrode electrochemical cell (total

capacity 30 ml) with a working volume of 5 ml (2.95 ml buffer, 2 ml analyte (PNP), 25  $\mu$ l each of NADPH (CalBiochem/Merck, Darmstadt, Germany) and FAD (Sigma, Steinheim, Germany). The working electrode was a glassy carbon electrode (GCE), 3 mm diameter, reference electrode being Ag/AgCl and a platinum wire as an auxiliary electrode, all from BAS.

For fundamental studies using PNP, baseline was attained using a mixture of buffer and coenzymes (final concentrations of 0.15 mM NADPH and 0.03 mM FAD) after which PNP was added to a final concentration of 20  $\mu$ M. For the detection of GUD activity, PNPG (4.4  $\mu$ M final concentration) and coenzyme mixture were used for baseline, after which GUD was added. A steady-state current was noted using a constant potential of 0.3 V (Mulchandani *et al.*, 2005). Current generated was calculated as the difference between baseline and steady-state currents (Appendix G).

All experiments were performed in triplicate, unless otherwise stated. Each microbial load was used once, after which the GCE was polished (see section 7.2.1).

#### **8.2.4 Support material evaluation**

Before using the membranes to entrap cells it was necessary to determine if they allowed current flow and to assess their impact on sensitivity. This was achieved by employing a linear sweep voltammetry technique (from 0 to 1.4 V) using the BAS equipment described above. PNP and buffer were used as the electrolyte solution. Polycarbonate membrane and SnakeSkin<sup>®</sup> (Pierce, Rockford, USA) dialysis tubing (molecular weight cut off of 10 kDa) were used separately to cover the plain GCE and for entrapping the cells. The response of the four GCE modifications was compared to that of the plain GCE. The peak current and potential that yielded the peak current for each scan were noted. Repeated scans using the same working electrode were performed to assess the reusability of the modified electrode. Wet weights of 1 mg and 3 mg were used for microbial loading in order to evaluate the optimum mass of cells to employ in subsequent assays.

### 8.2.5 *Selectivity, sensitivity and sonication studies*

The effects of four phenolic compounds similar to PNP; 2-nitrophenol, 3-nitrophenol, 2-4-dinitrophenol and phenol (Aldrich, Steinheim, Germany), on the *Moraxella* 1A and *P. putida* JS444 modified GCE response were investigated. A working concentration of 20  $\mu\text{M}$  for each phenolic compound was used. A paired sample t-test was used to compare the responses of the *Moraxella* and *Pseudomonas* modified GCE at 5 % level of significance.

To investigate the effects of cell permeabilisation, 1 ml of each cell suspension of 100 mg/l was sonicated for 2 min (30 s sonication intervals) at 25 W using a Vibra Cell Sonicator<sup>TM</sup> (Sonics and Materials, USA). The sonicated material was immobilised onto the polycarbonate membrane using the procedure for whole cells (section 8.2.2).

### 8.2.6 *pH studies*

The effect of pH (ranging from 6.5 to 8.5) on the response of the electrode modified separately by *Moraxella* 1A and *P. putida* JS444, to 20  $\mu\text{M}$  PNP, was investigated. Tris-HCl and sodium phosphate (both 20 mM) buffer systems were employed for the two biosensors.

### 8.2.7 *Temperature studies*

Buffer, electrolyte cell and the PNP samples were preheated in water baths before being transferred into an insulated vessel with water circulating at the temperature under investigation. The amperometric response to 20  $\mu\text{M}$  PNP was noted for temperatures ranging from 4 to 45 °C.

### 8.2.8 *Microbial loading*

Different volumes of cell suspensions corresponding to the desired wet mass (1 – 8 mg) were loaded onto the polycarbonate membrane to immobilise the cells. The biosensor response to 20  $\mu\text{M}$  PNP was evaluated for each cell load.

### **8.2.9 Volume optimisation for GUD assay**

To determine the maximum volume of enzyme solution to be added to aid mixing of the analytes, a range of volumes of GUD stock solutions were added while maintaining a constant final concentration of the enzyme in every assay mixture at 60 µg/ml. The volume of the substrate (10 mM PNPG) was kept at 2.2 ml, and NADPH and FAD at 25 µl each. Buffer volume was adjusted accordingly. Baseline was generated using all assay components except GUD. The enzyme was added and current generated determined.

### **8.2.10 Commercial and environmental GUD assays**

To determine the most economical concentration of commercial GUD for assays, different GUD stocks were prepared to enable addition of a fixed volume (1000 µl) of enzyme solution to obtain different GUD concentrations in the assay mixture. The assay was performed as described above (section 8.2.9).

GUD activity in samples from different environments in the Eastern Cape of South Africa (Chapter 5) was detected as described in section 8.2.9. The membrane filtration (MF) technique was performed as described in Chapter 5 on CM1046 agar (Oxoid, Hampshire, England) to determine *Escherichia coli* colony forming units (CFU) counts in the water samples. The biosensor response was compared to the microbial CFU per 100 ml of each water sample.

### **8.2.11 Interference Studies**

The effects of sodium chloride, calcium chloride and sodium carbonate (compounds commonly occurring in polluted water) on the biosensor were evaluated at concentrations from 0 to 200 mg/l at 50 mg/l intervals. The modified electrode was preincubated (separately) for 20 min in each effector concentration, after which 20 µM PNP was added to determine the biosensor response.

### **8.2.12 Effect of environmental water samples on biosensor response to commercial GUD**

In order to determine the degree of under- or over-estimation of the GUD activity in the environmental samples, effects of the environmental samples on the commercial GUD were investigated. The different ratios of the environmental sample to commercial GUD solution were set up, incubated for 20 minutes, after which the biosensor response to the mixture was determined following addition of PNPG. This was compared to the response of the same concentration of a positive control that comprised of commercial GUD in Tris-HCl buffer.

### **8.2.13 Modified electrode and free cells storage stability**

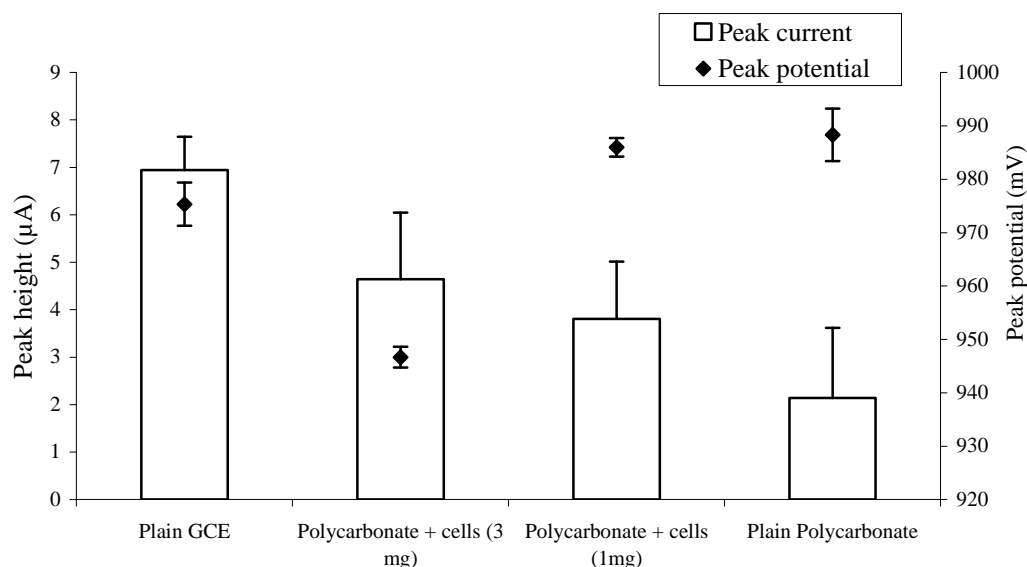
Daily PNP detection, for 10 days, using a *Moraxella* modified GCE, was performed, after which the electrode was washed with milli Q water and stored at  $4 \pm 1$  °C in 20 mM sodium phosphate buffer.

As a control, daily electrode responses by GCE modified using *Moraxella* cells stored at  $4 \pm 1$  °C in 15 mg/l PNP in 20 mM sodium phosphate buffer (pH 7.5) were determined to investigate the storage stability of non-immobilised cells.

## **8.3 Results**

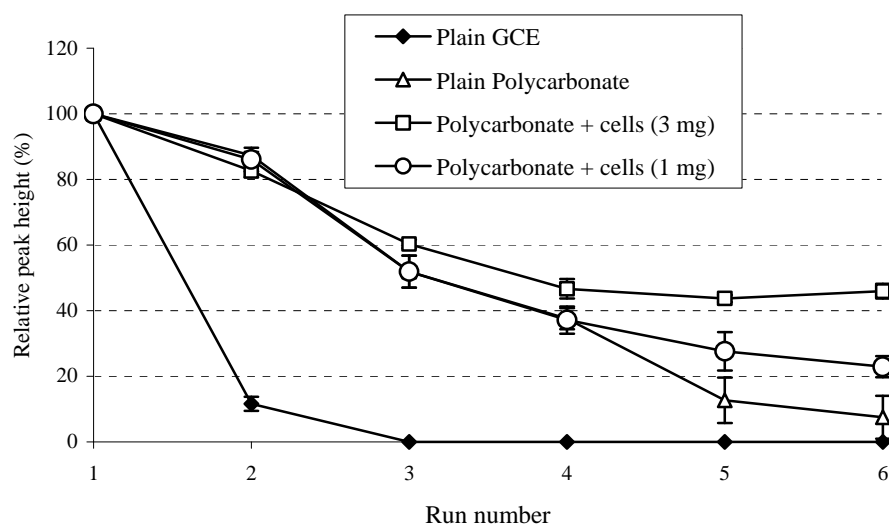
### **8.3.1 Effects of different membranes on voltammetric response**

No current for PNP detection was generated when the plain GCE was covered with both SnakeSkin<sup>®</sup> dialysis tubing and microbes entrapped by this membrane. Covering the electrode with plain polycarbonate membrane reduced the first current peak by 69.14 % relative to the first current peak height (6.94  $\mu$ A) obtained using unmodified GCE. However, current generated increased with cell load from 1 to 3 mg (Fig. 8.4). The cell loading led to a shift in the potential at which peak current was observed (Fig. 8.4).

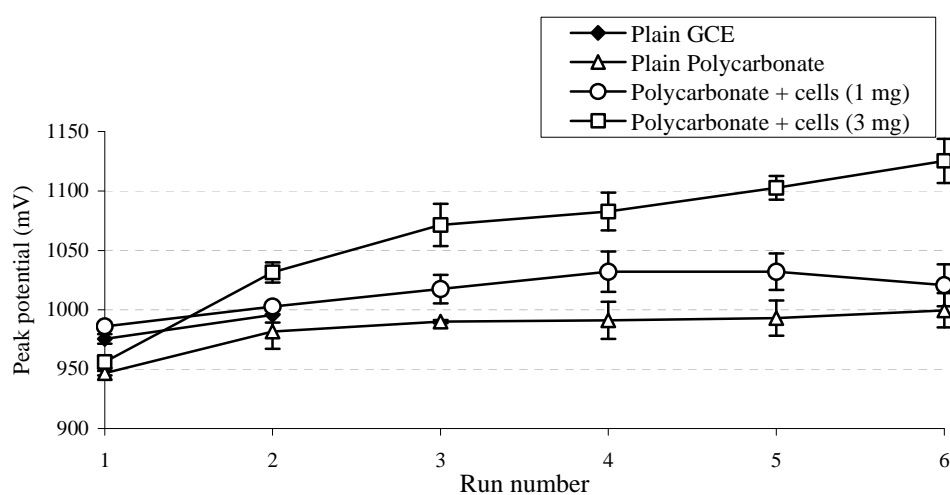


**Figure 8.4** Reduction in sensitivity (bars) and shift in potential giving peak current (data points) for different GCE modifications for PNP detection by linear sweep voltammetry. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

All electrodes fouled as the number of runs increased (Fig. 8.5). The plain GCE was the most sensitive to fouling, followed by the GCE covered by polycarbonate membrane only, then with 1 mg cell weight and lastly with 3 mg cell weight (Fig. 8.5). It appears therefore, that the cells offered some degree of protection to the electrode. However, there was a shift in the cathodic peak potential towards more positive values with successive voltammetric sweeps (Fig. 8.6). The GCE modified with 3 mg cells exhibited the largest shift as shown in figure 8.6. Thus while decreasing sensitivity, the membrane increased resistance to fouling while cell loading increased the level of protection against fouling.



**Figure 8.5** Relative reduction in peak current produced by modified and unmodified GCE with successive linear voltammetric sweeps for PNP detection by linear sweep voltammetry. All data points represent the mean  $\pm$  SD ( $n = 3$ ).



**Figure 8.6** Progressive shifts in potential, producing peak currents with number of linear voltage sweeps for modified and unmodified GCE for PNP detection. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

### 8.3.2 Selectivity, sensitivity and sonication studies

Unless stated, the results from this point onwards (in this chapter) were obtained from amperometric measurements.

None of the phenolic compounds tested produced a detectable response on the *Moraxella* 1A modified GCE. Phenol and 3-nitrophenol produced 54.61 and 31.65 % responses, respectively on the *P. putida* JS444 modified GCE with respect to the response to PNP ( $14.63 \pm 0.35$  nA; Table 8.1). There was no detectable response to 2-nitrophenol and 2-4-dinitrophenol by the *Pseudomonas* modified GCE (Table 8.1).

**Table 8.1** Sensitivity and selectivity comparison of PNP detection between *Moraxella* 1A and *P. putida* JS444 modified GCE.

	Mean current $\pm$ SD (nA) (n = 3)	
	<i>Moraxella</i> 1A	<i>P. putida</i> JS444
<i>p</i> -nitrophenol	$24.33 \pm 1.24$	$14.63 \pm 0.35$
Phenol	0	$7.99 \pm 0.06$
3-nitrophenol	0	$4.63 \pm 0.10$
2-nitrophenol	0	0
2-4-dinitrophenol	0	0

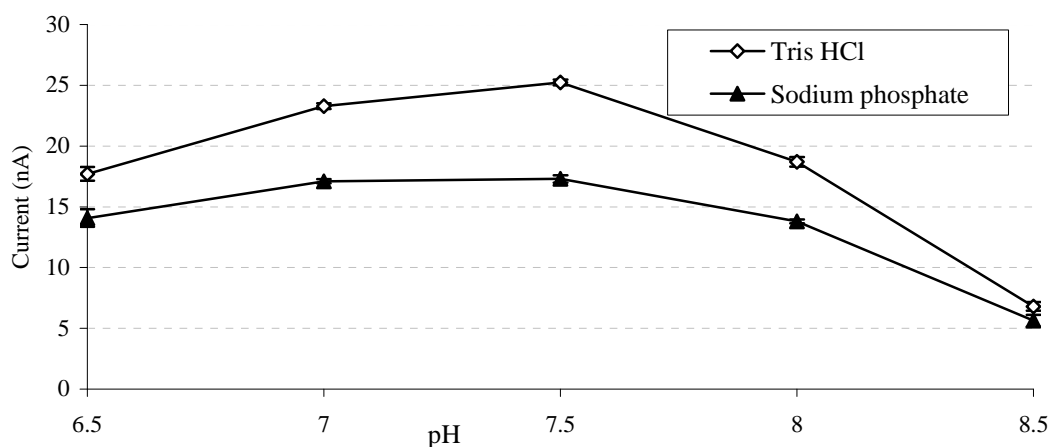
The current response by the *Moraxella* modified GCE was significantly higher ( $P < 0.05$ ) than that of the *Pseudomonas* modified GCE (Table 8.1). The former was therefore more sensitive than the latter (Appendix H). Linear response was noted between 5 and 25  $\mu$ M PNP for both biosensors (Appendix H), with an experimental LOD of 5  $\mu$ M.

There was no response to PNP when the GCE was modified by sonicated *Moraxella* 1A while the sonicated *P. putida* JS444 cells exhibited a 50 % reduction in current response for PNP (from  $14.63 \pm 0.04$  to  $7.33 \pm 0.02$  nA). Whole cells were therefore used in subsequent studies.

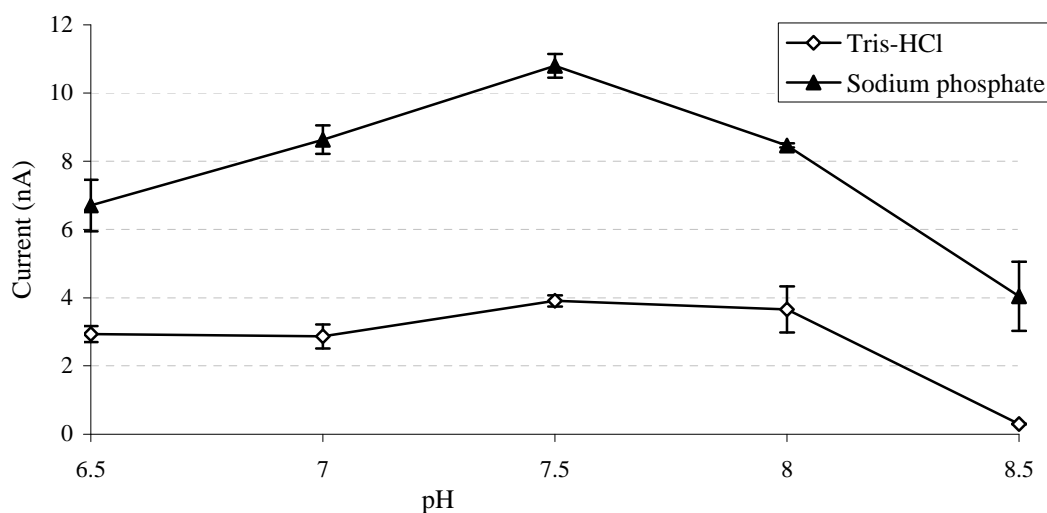
### 8.3.3 Effect of pH

The Tris-HCl buffer produced consistently a higher biosensor response than the sodium phosphate system in the *Moraxella* 1A biosensor (Fig. 8.7a). In contrast, the phosphate buffer gave a higher biosensor response in the *P. putida* JS444 biosensor

(Fig. 8.7b). However, in both cases the pH optimum was 7.5 for the two buffer systems. Based on the observed selectivity and sensitivity, all subsequent studies were confined to the *Moraxella* 1A modification of the GCE.



a

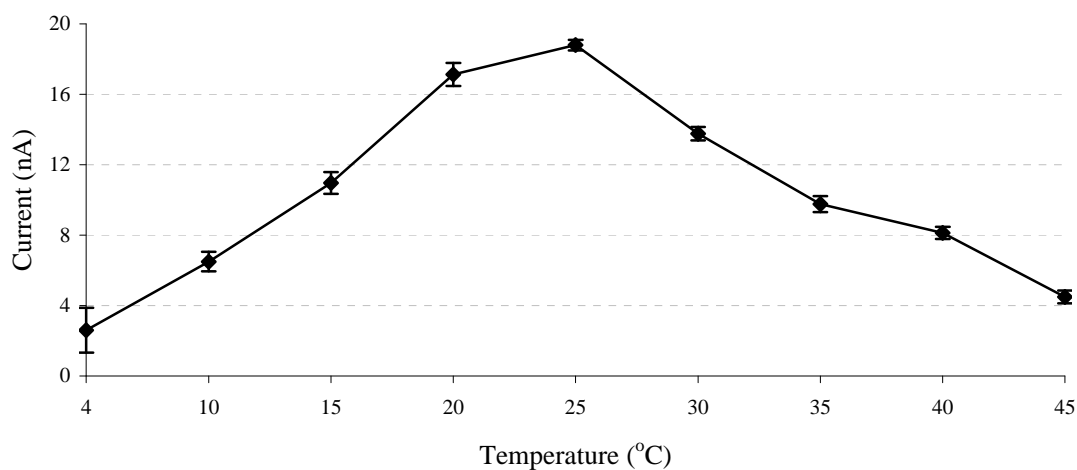


b

**Figure 8.7** Effects of pH and buffer systems on the detection of *p*-nitrophenol using (a) *Moraxella* 1A and (b) *P. putida* JS 444 modified GCE. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

### 8.3.4 Effect of temperature

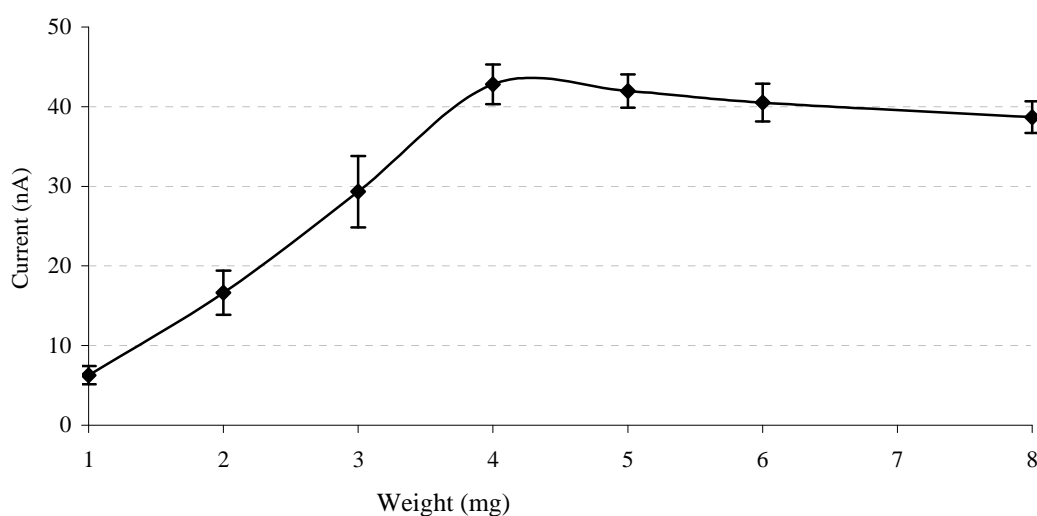
The *Moraxella* 1A biosensor response increased from  $2.6 \pm 1.3$  nA at  $4 \pm 1$  °C to  $18.8 \pm 0.3$  nA at 25 °C, after which it decreased to  $4.5 \pm 0.4$  nA at 45 °C (Fig. 8.8). Room temperature ( $20 \pm 2$  °C) was selected for further assays.



**Figure 8.8** Effect of temperature on PNP detection using a *Moraxella* 1A modified GCE. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

### 8.3.5 Effect of cell loading

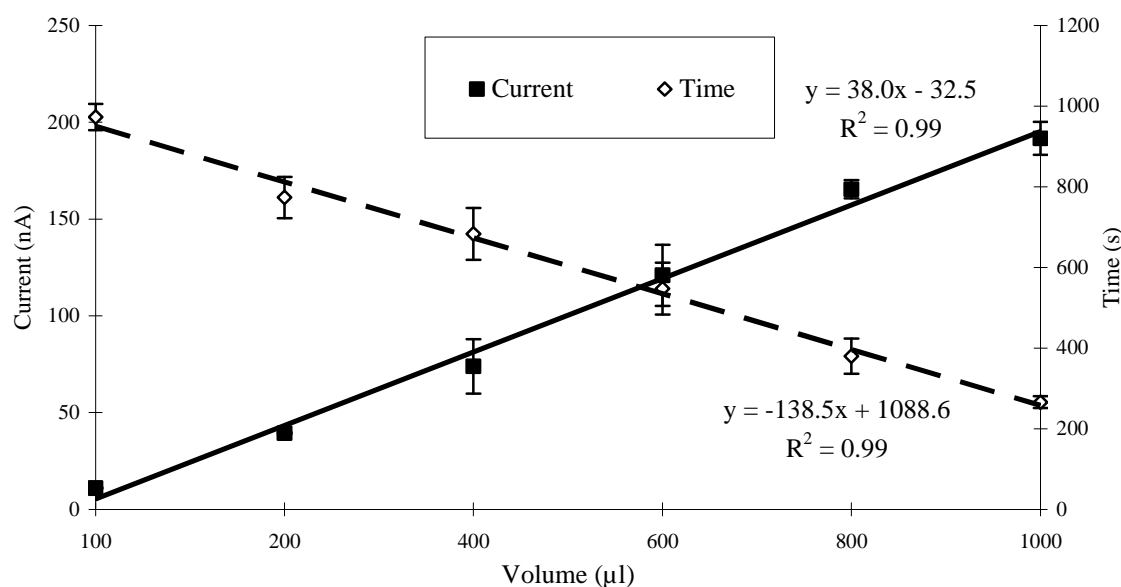
There was an increase in biosensor response up to 4 mg of *Moraxella* 1A cells (producing  $42.8 \pm 2.5$  nA), after which the signal reduced to  $38.8 \pm 2.0$  nA at 8 mg (Fig. 8.9). For this reason, 4 mg was used in subsequent studies.



**Figure 8.9** Effects of microbial loading on current generated by *Moraxella* 1A modified GCE. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

### 8.3.6 Volume optimisation

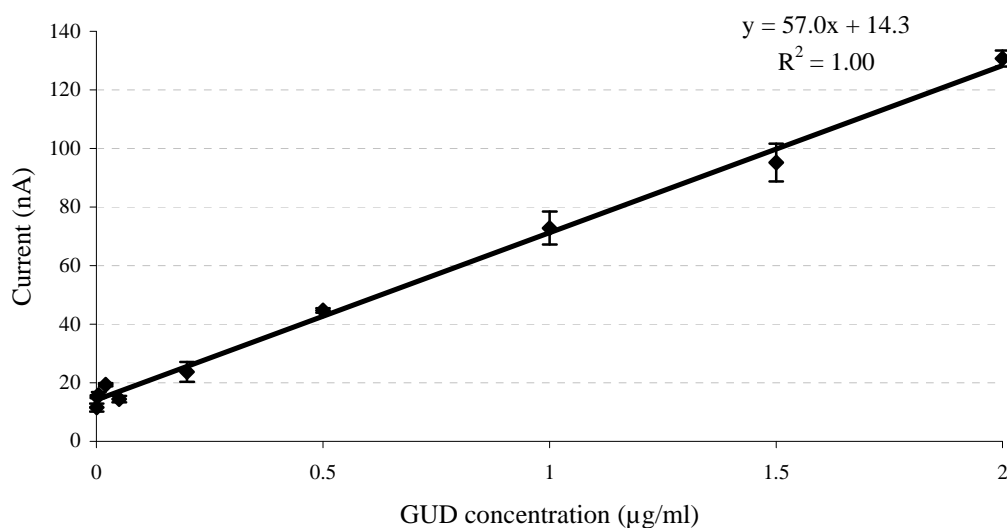
The biosensor response increased linearly with an increase in the volume of enzyme solution to 1 000  $\mu\text{l}$  (Fig. 8.10) ( $y = 38.0x - 32.5$ ;  $R^2 = 0.99$ ), above which it levelled off (not shown on graph, Fig. 8.10). The 1 000  $\mu\text{l}$  volume of enzyme solution was used in subsequent studies, the buffer volume was kept at 1 750  $\mu\text{l}$ , substrate volume was 2 200  $\mu\text{l}$  and cofactors 50  $\mu\text{l}$  (see 8.2.9). The time taken to reach a maximum steady-state current decreased linearly ( $y = -129.5x + 1\ 064.4$ ;  $R^2 = 0.99$ ) with an increase in volume of GUD solution added (Fig. 8.10).



**Figure 8.10** Relationship between GUD volume, biosensor response [current (nA)] and time required for maximum response for amperometric detection of PNP. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

### 8.3.7 GUD linearity and LOD

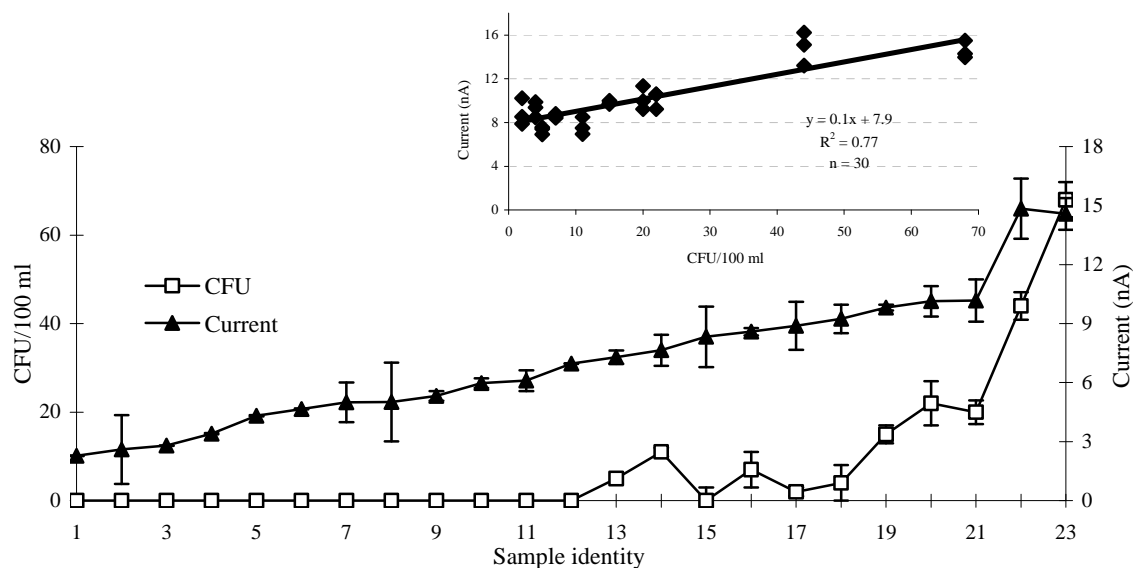
*Moraxella* 1A biosensor response was directly proportional to the enzyme concentration up to 2  $\mu\text{g/ml}$  (Fig. 8.11). Although the biosensor response was detected at lower enzyme concentration (0.0002  $\mu\text{g/ml}$ ), the linear relationship was poor (Fig. 8.11). However, the experimental LOD (lowest level detected) for GUD remains 0.0002  $\mu\text{g/ml}$ .



**Figure 8.11** Biosensor response with increase in commercial GUD concentration. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

### 8.3.8 Environmental GUD assays

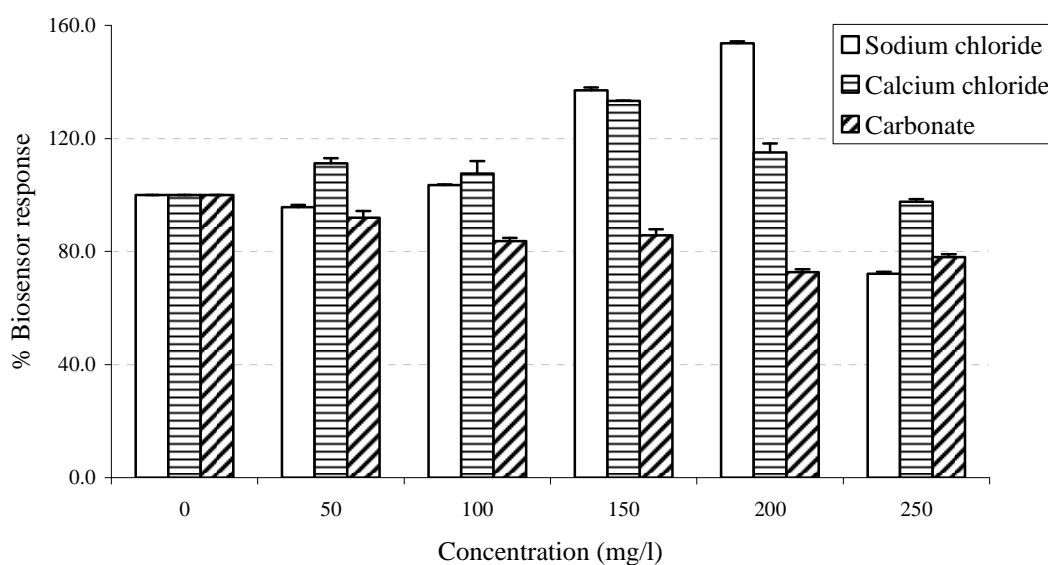
The *Moraxella* 1A biosensor response (current) was more sensitive than the MF CFU counting method. Hence, there was a weak correlation between CFUs and the biosensor response (Fig. 8.12). Of the 60 samples tested, the biosensor signal was positive for 23, and of these 23 the colony forming units were observed only in ten samples (Fig. 8.12 insert). Regression analysis of the 23 samples gave a low  $R^2$  value (current =  $0.2(\text{CFU}/100 \text{ ml}) + (5.7 \pm 0.5)$ ;  $R^2 = 0.68$ ). The *E. coli* LOD was 2 CFU/100 ml, which was matched by 8.89 nA generated at a *Moraxella* 1A biosensor by environmental sample GUD activity.



**Figure 8.12** Biosensor response (current) to environmental samples with different microbial counts. The insert shows a regression of biosensor response on CFU/100 ml for the 10 samples in triplicate. All data points in the main figure represent the mean  $\pm$  SD ( $n = 3$ ).

### 8.3.9 Interference Studies

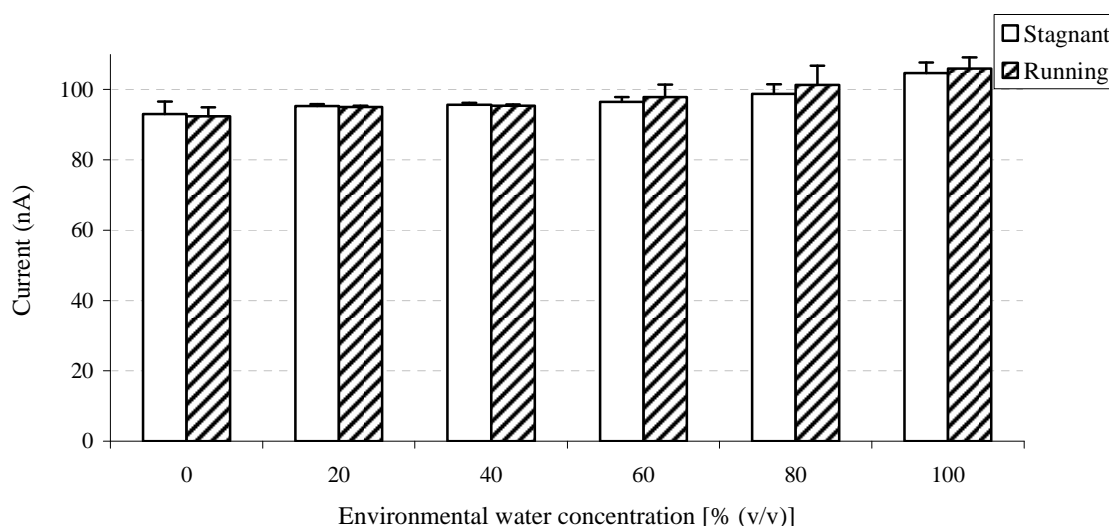
Sodium carbonate decreased the biosensor response (Fig. 8.13). In contrast, sodium chloride increased the biosensor response up to 200 mg/l. Calcium chloride increased biosensor responses up to 150 mg/l (Fig. 8.13). The chloride ions therefore, unlike carbonate ions, amplified the response.



**Figure 8.13** Effects of sodium chloride, calcium chloride and sodium carbonate on the PNP biosensor. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

### 8.3.10 Environmental sample effects on commercial GUD

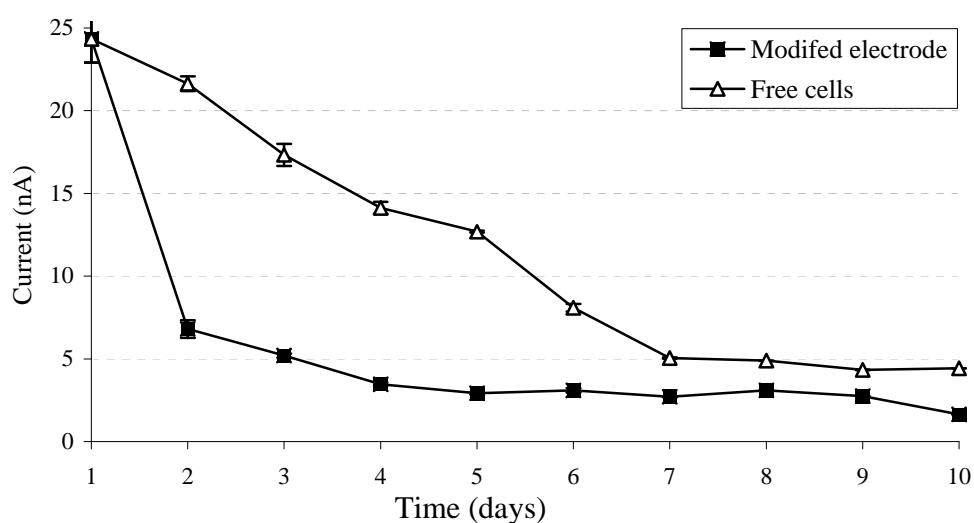
There was a slight increase in biosensor response when commercial GUD was mixed with the environmental samples (Fig. 8.14). Both stagnant and running water samples resulted in a similar biosensor response pattern (Fig. 8.14).



**Figure 8.14** Effects of environmental samples on biosensor response to commercial GUD activity. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

### 8.3.11 Storage Stability

The biosensor response decreased by 72.02 % by day two when the *Moraxella* 1A cells were entrapped on the electrode and stored, while there was a steady decrease in response when the cells were stored in buffer at  $4 \pm 1$  °C and applied daily. The 72.02 % reduction in signal for the free cells occurred between day one and seven (Fig. 8.15). From day seven to ten there was an insignificant ( $P > 0.05$ ) loss of response by both immobilised and free cells.



**Figure 8.15** Storage stability of *Moraxella* modified GCE and free *Moraxella* 1A cells at  $4 \pm 1$  °C. All data points represent the mean  $\pm$  SD ( $n = 3$ ).

## 8.4 Discussion and Conclusions

### 8.4.1 Discussion

The Snakeskin dialysis tubing could not be used as a support material for microbial entrapment, as it hindered signal detection. From the trend in current decay with voltammetric runs (Fig. 8.5), it is reasonable to suggest that the microbial biomass offered some degree of protection to the GCE against fouling. Electrode fouling occurs because oxidation of phenolic compounds leads to formation of free radical intermediates, that polymerise on the electrode surface, thereby limiting flow of

current (Wang and Hutchins, 1985; Wang and Li, 1989). The general reduction in sensitivity after electrode modification could be attributed to limited electron transfer.

The *Moraxella* 1A biosensor was more selective than the *P. putida* JS444 biosensor, because it only generated a measurable response to PNP and not to other phenolic compounds studied. Response to phenol and 3-nitrophenol by the *P. putida* JS444 biosensor indicated that the *Pseudomonas* may have other enzymes capable of degrading these nitro-aromatic compounds through the hydroquinone pathway. Alternatively, the enzymes involved in the PNP degradation pathway in this organism may not be specific to PNP and therefore differ from the enzymes in *Moraxella* 1A. However, the former seems to be the most likely scenario. Spain and Gibson (1991) highlighted differences in properties and locations of *Pseudomonas* and *Moraxella* enzymes involved in PNP degradation. *Pseudomonas* species degrade PNP through either the 1,2,4-benzenetriol-catechol and/or the hydroquinone pathway (Spain and Gibson, 1991; Ignatov *et al.*, 2002). The low sensitivity of the *P. putida* JS444 biosensor can therefore be attributed to low hydroquinone generated as the microbe could have been producing 1,2,4-benzenetriol in addition to (or instead of) hydroquinone.

Considering the rate at which the *P. putida* JS444 cells degraded PNP in the culture, these cells are expected to produce more hydroquinone and hence be more sensitive than the *Moraxella* 1A biosensor. Degradation of phenol by *Pseudomonas* species was also reported by Schmidt *et al.* (1987). High selectivity and sensitivity are some of the most desired attributes for a biosensor (Leonard *et al.*, 2003; Paitan *et al.*, 2003) and once these are absent, then the biosensor is no longer feasible. This was the reason why the *P. putida* JS444 biosensor was abandoned in this study.

Decrease in the current generated by the sonicated *P. putida* JS444 biosensor and absence of current in sonicated *Moraxella* 1A biosensor for PNP can be explained by either stability, location or failure in immobilisation of the enzymes. The oxygenase that participates in hydroquinone generation from PNP in *Moraxella* species is membrane bound (Spain and Gibson, 1991). Sonication could most likely have

disrupted the membrane stability and bonding, thus disrupting the catalytic efficiency of the enzyme. In *Pseudomonas* species, oxygenase is not membrane bound (Spain and Gibson, 1991) and thus may not have been affected by sonication to the same extent as the *Moraxella* 1A enzyme. Sonication was therefore not a viable option at this stage although one would expect an increased response with the release of enzymes, assuming that enzyme stability is not compromised.

Differences in enzyme properties between the two microorganisms can be used to explain variations in current generated with respect to buffer systems used (Spain and Gibson, 1991). Although Zeyer *et al.* (1986) noted an insignificant difference in PNP degradation by *P. putida* B2 between Tris-HCl and sodium phosphate buffer systems, the phosphate buffer in this study gave a consistently higher current for *P. putida* JS444 biosensor. This could be due to the different roles played by buffer ions in the enzyme systems of the microbes.

The GUD assay in environmental water did not vary significantly between pH 5.0 and 9.0 (Fig. 2.4). Therefore, the assay can be adapted to pH 7.5 to accommodate the pH optimum for the *Moraxella* 1A modified GCE without compromising sensitivity. The consistently higher current with Tris-HCl buffer is an advantage to the proposed method because phosphate buffer was shown to interfere with the *in situ* spectrophotometric GUD assays (Chapter 2). Wu *et al.* (1986) highlighted the fact that phosphate ions contribute to accelerated degradation of NADPH. Tris-HCl instead of the sodium phosphate buffer was therefore used in all subsequent assays.

A temperature optimum of 25 °C was below the expected range (35-40 °C). Since these microbes are associated with sludge (Spain and Gibson, 1991; Leung *et al.*, 2005), they are likely to be of enteric origin where they usually operate optimally at 37 °C. However, the stability of NADPH could play a role in defining the temperature optimum. NADPH is required for enzyme activity (Spain and Gibson, 1991) but is degraded with increased temperature (Wu *et al.*, 1986). Therefore, at higher temperatures, the coenzyme will be limiting to the hydroquinone generation by *Moraxella*. Solubility of oxygen, which is required for the enzymatic reaction, is

inversely proportional to temperature. Hence, lower temperatures will be favourable for both NADPH stability and oxygen availability to the enzymatic processes. In light of these observations, room temperature ( $20 \pm 2$  °C) was regarded as the most suitable since there would be no additional costs for temperature maintenance. The room temperature has been used for the GUD assay (Chapters 2 to 6); and therefore its use will allow effective comparison of the biosensor response to the GUD activity. Lower biosensor response at lower temperatures may imply reduced responses when water samples are tested early in the morning or in winter.

The observed increase in biosensor response with increase in enzyme volume can be explained by an improvement in mixing of the enzyme and substrate. This mixing encourages uniform distribution of the breakdown product (PNP) and thus rapid access to the electrode surface. Therefore, if the system is not stirred the enzyme volume must be considerable (not less than 20 % of the total volume of the electrolyte). It is important to ensure that none of the steps are limiting (with the exception of hydroquinone oxidation). GUD must therefore be abundant and uniformly distributed in the solution to generate highest possible levels of PNP.

Despite the poor linear response at very low GUD concentration, the PNP generated was detected (Fig. 8.11). The low  $R^2$  (0.6830) value, for all environmental samples that produced a signal detected with the biosensor, can be accounted for by the presence of viable but non-culturable (VBNC) cells with detectable GUD activity (Tryland *et al.*, 1998; George *et al.*, 2002; Rompré *et al.*, 2002) as well as persistent levels of GUD (Fig. 5.3). Some of the GUD activity detected could have been from non-target sources (Tryland and Fiksdal, 1998; Pisciotta *et al.*, 2002). In addition, when the enzyme concentration is low, PNP production becomes another limiting factor. The presence of more than one limiting factor compromises the linearity of the biosensor response. The poor linear relationship between biosensor response and CFU suggests that the sensor will be more appropriate as a presence/absence detecting device.

The spectrophotometric GUD assay LOD, 0.02  $\mu\text{g/ml}$ , is 100 X (one hundred times) less sensitive than that of electroanalysis. In addition, the time required to obtain a full response ( $\leq 5$  min with commercial GUD and  $\leq 20$  min with environmental GUD) was less than that required for spectrophotometric assays. To the best of our knowledge, this is the first time an electrochemical method of GUD activity detection using the substrate PNPG has been reported.

The observed direct relationship between biosensor response and cell load could be attributed to an increase in catalytic sites that produce hydroquinone. Limited electron transfer on the electrode surface at cell concentrations above 4 mg/ml reduced the biosensor response.

Chloride ions increase the conductivity of the electrolyte solution (Ebbing and Gammon, 1999). This led to the observed direct relationship between biosensor and chloride ions (Fig. 8.13). Carbonate increases pH, which, in turn, reduces the rate of PNP degradation (Spain and Gibson, 1991) leading to a reduced response because of low hydroquinone generated. Therefore, in salt water there can be an increased biosensor response while in hard water false negative results may be obtained due to the antagonistic nature of carbonates on monooxygenase and reductase activities in *Moraxella*. The increased biosensor responses when commercial GUD was mixed with the Bloukrans River samples suggest that there is little/no underestimation of the GUD activity. Thus the commercial GUD activity may not have been inhibited by the water samples. Similar results were obtained in spectrophotometric assays (Chapter 4).

The observed rapid decline in response by stored modified GCE implied poor storage stability of the electrode. This is in contrast to the 20 readings without a significant drop in signal obtained when oxygen and carbon paste electrodes were used for the detection of PNP (Lei *et al.*, 2003; Mulchandani, *et al.*, 2005). Therefore, the entrapment of *Moraxella* cells onto the GCE is appropriate for a once off reading at a disposable electrode. Free cells were more stable than immobilised cells because of the availability of nutrients.

### 8.4.2 Conclusions

From the studies performed in this chapter, the following conclusions were made:

- (a) the *Moraxella* biosensor is more sensitive and selective than the *Pseudomonas* biosensor,
- (b) sonication is not a desired method for producing a cell free extract for increasing sensitivity of the biosensor,
- (c) the *Moraxella* biosensor can be used for presence/absence monitoring of *E. coli* and when a certain response level (e.g. 8.89 nA) is reached, the water can be regarded as of unacceptable microbiological quality,
- (d) traditional colony counting methods can be simultaneously applied with the biosensor to establish the reliability,
- (e) electrochemical detection of GUD activity is more sensitive and rapid than spectrophotometric methods,
- (f) the impact of stimulating and inhibiting compounds in water should be considered in the assays, and
- (g) poor storage stability and requirement of culture maintenance make the proposed *Moraxella* biosensor undesirable. Use of dried cells and carbon paste electrodes may be the best viable option.

A summary of characteristics of the *Moraxella* biosensor is shown in Table 8.2.

**Table 8.2** Summary of characteristics of the *Moraxella* biosensor.

Characteristic	Value
pH optimum	7.5
Optimum temperature	25 °C
Optimal cell load	4 mg
LOD	≈ 2 CFU/100 ml
Detection time	20 min
Linearity	0.0002 – 2 µg/ml
Storage stability	Poor (< 1 day)

## 9 OVERALL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

### 9.1 Discussion

In order to adapt to stressful environments, microorganisms alter their metabolic pathways and gene expression (Groat *et al.*, 1986). Consequently, the properties of proteins will change and GUD is no exception. In addition, the protein changes may occur on the synthesised peptide (i.e. not at the gene level) when the peptide is exposed to an environment different from the one it usually operates in. These changes were also evident in the differences observed in the physico-chemical properties between environmental and commercial GUD enzymes. These differences favoured the method of *in situ* enzyme assay to detect *E. coli*. For example, the pH optima values (7.5 and 8.0) for GUD assays increase the accuracy of the *in situ* assay through minimisation of interference by acidic GUD from non-target sources (Gilissen *et al.*, 1998; Aich *et al.*, 2001<sup>b</sup>).

While other workers found pH 8.0 to be optimal for *E. coli* GUD (Gilissen *et al.*, 1998; Aich *et al.*, 2001<sup>a,b</sup>), the fact that PNP colour development is pH dependent (Frampton and Restaino, 1993), should not be overlooked. This is one of the limitations of colorimetric assays since most chromogens depend on pH for colour development (Frampton and Restaino, 1993). The enzyme activity can therefore be underestimated under conditions that do not allow maximum colour development, despite the fact that the enzyme would be active. Some pollutants (e.g. ferric chloride) may lead to false positive results through the chemical hydrolysis of PNPG. Others may lead to false negative results by inhibiting colour development of PNP. When such pollutants are suspected to be present, further confirmation of presence of indicator organisms by means of traditional culturing techniques is necessary. Electro-oxidation is also affected by pH, because the rate of PNP redox reactions is pH dependent (Calvo-Marzal *et al.*, 2001).

In interference studies, commercial GUD acted as a positive control to illustrate the relative effects of different ions. Although the concentration of the environmental

GUD was lower than that of commercial GUD, the results remain valid because the comparisons were made within each sample and not across the different samples. Attention should be paid to enzyme to inhibitor ratio when explaining the observed inhibition trends. It is also important to note that some ions could affect both the *E. coli* and GUD in environmental samples. However, since the studies confirmed the non-requirement of permeabilisers and extracellular location of GUD, the interference of ions on GUD activity due to effects on the *E. coli* cells *per se* is expected to be minimal.

The correlation between *in situ* GUD activity and *E. coli* counts in this study, supported by similar observations by Farnleitner *et al.* (2001; 2002) using MUG, showed that GUD activity is directly proportional to the amount of *E. coli* CFU. Hence, GUD activity can be used to extrapolate the presence/absence of *E. coli*. The correlation also served to illustrate that the observed GUD activity was not merely background activity. This was further confirmed by the enzymatic breakdown of PNPG in the activity gel study. Although the non-requirement of permeabilisers in environmental samples was in contrast with results obtained using pure *E. coli* K<sub>12</sub><sup>+</sup> cultures in a pilot survey prior to this study (Watson, pers. comm.), the extracellular location of GUD in polluted water agreed with the observations of Doyle *et al.* (1955) and Farnleitner *et al.* (2001; 2002). Cell permeabilisation prior to performing GUD assays is therefore not necessary. Furthermore, the liquid bioprobe can be used with minimum prior knowledge and skill. In spite of the observation that low enzyme concentrations led to prolonged assay times (equivalent to those of traditional culturing techniques), direct GUD assay is not labour intensive. Consequently, the liquid bioprobe provides a good alternative to the traditional culturing techniques (Table 9.1).

The presence of low GUD concentration in the environment led to prolonged assay times in the spectrophotometric technique (Table 9.1). However, the non-requirement of permeabilisers assisted in cost minimisation. The 24 h assay period for the liquid bioprobe compares well with other techniques, like the Colilert<sup>®</sup> methods (Olson *et al.*, 1991; Berger, 1994; Van Poucke and Nelis, 1997).

Inconsistencies in the fluorogenic assay were most likely due to the existence of natural fluorogenic substances in the environment as observed by Marxsen and Witzel (1991). Interference from these substances will compromise the sensitivity of the technique. Hence the technique may not be suitable for tropical regions with active biomass decomposition in water bodies that generate such substances or the background fluorescence has to be measured.

A colour test strip, where PNPG is immobilised on to a solid support, is a potential alternative to the traditional culturing techniques. The test strip is more suitable for use in samples with high GUD concentration, which is seldom the case with polluted environments (Table 9.1). Optimisation of immobilisation procedures is usually a time-consuming trial and error process (Krajewska, 2004).

**Table 9.1** Summary findings of the main techniques investigated for *E. coli* detection.

Technique	LOD	Assay time	Comments
Liquid bioprobe	2 CFU/100 ml	24 h	Both visual and spectrophotometric LOD.
	0.02 µg/ml (commercial GUD)	3 h	Spectrophotometric LOD.
Test strip	0.05 µg/ml (commercial GUD)	20 min	Detection by Adobe colour scoring. Technique not feasible for dilute environmental GUD due to PNPG desorption.
Electrochemical Reduction			
Plain GCE	0.05 mM PNP	Instant	Less sensitive than spectrophotometric detection of the breakdown product, hence not feasible for environmental GUD assay.
Nafion modified GCE	0.1 mM PNP	Instant	
Oxidation			
Plain GCE	0.1 mM PNP	Instant	Less sensitive than spectrophotometric assay.
<i>Moraxella</i> modified GCE	< 2 CFU/100 ml 0.0002 µg/ml commercial GUD	5-20 min 5 min	More rapid and sensitive than spectrophotometric detection.

Course of study

While Standridge and Delfano (1983) observed an insignificant difference in coliform CFU in water stored for 48 h, the same could not be said about GUD activity in this study. The results obtained showed an increase in activity, which could either be a

result of microbial metabolism and growth or reversal of enzyme inhibition (Wetzel, 1991). It is true therefore that the indicator organisms can proliferate in water if the nutritional status permits (Fujioka *et al.*, 1999; Stevens *et al.*, 2001; Jagals *et al.*, 2003). Hence the point that indicator organisms must not grow in water (Manafi, 1998) only holds for oligotrophic water bodies.

Electro-reduction of the nitro group on both PNPG and PNP led to interference when the two compounds were detected in a mixture. While Nafion<sup>®</sup> reduced the PNPG detection by 95 %, the synergistic peak was still detected and sensitivity to PNP was reduced (Table 9.1), which was undesirable. The *Moraxella* 1A conversion of PNP to hydroquinone and electro-oxidation of the hydroquinone at the modified GCE was rapid, specific and the most sensitive method for GUD detection (Table 9.1). In addition, this conversion was cost-effective and reproducible. The cost of reagents for a single *Moraxella* biosensor assay was about ZAR 0.50 as opposed to ZAR 18 – 31 for defined substrate culturing and less than ZAR 5.00 for the H<sub>2</sub>S test (Genthe and Franck, 1999). Availability of battery powered portable potentiostats could lead to lower power costs. Reproducibility was dependent on the consistency of acclimatisation and culturing conditions, as well as the cell load on the polycarbonate membrane. To minimise variations, cells may be preserved by freeze drying. Therefore, once fully automated, the electrochemical detection of PNP using a *Moraxella* 1A biosensor has the potential to provide the solution to on-line and real-time monitoring of microbial water quality. However, the automation to a “single-push-button machine” will require expertise in different scientific fields (section 9.3.3).

The presence of GUD activity in water samples that did not produce CFU on the CM1046 agar indicated that the direct *in situ* GUD assay was more sensitive than the culturing method. While biosensor response in absence of CFU may be attributed to background interference in the samples, the culturing methods have an inherent disadvantage of the inability to detect VBNC bacteria (Rompré *et al.*, 2002). The results therefore illustrate that loss of culturability does not necessarily imply an inability to produce active GUD.

As suggested by Van Poucke and Nelis (1997) the question of high sensitivity will always present a paradoxical argument about susceptibility to interference by GUD from non-target sources. However, when doubt about microbial contamination arises from the direct enzyme assay, confirmatory tests can always be performed to determine the presence/absence of pathogens. GUD can therefore effectively be used in preliminary faecal pollution monitoring.

The use of a resting cell suspension in *Moraxella* modification of the GCE is disadvantageous in that the cells' viability always has to be maintained, which increases labour demand. Freeze-drying and reconstitution of the cells prior to use could be a viable option.

## 9.2 Conclusions

From the results obtained in this study, the following major conclusions can be drawn:

- (a) physico-chemical properties of environmental GUD are different from those of either its commercial counterpart or GUD in pure *E. coli* K<sub>12</sub><sup>+</sup> culture,
- (b) GUD activity assay is a reliable, cheap and an easy-to-use alternative method for the detection of faecal pollution in water using a liquid bioprobe,
- (c) electrochemical detection of GUD activity using a *Moraxella* 1A modified GCE is rapid, cost-effective, sensitive and feasible for on-line and real-time monitoring of faecal pollution in water,
- (d) GUD activity of VBNC *E. coli* is detectable in *in situ* assays,
- (e) an electrochemical technique for *E. coli* detection has been developed, and
- (f) the results and techniques from this study contribute significantly towards meeting of the WHO and South Africa water quality framework, water safety plans and risk assessment.

Therefore GUD, in faecal polluted water, can be used for the development of an alternative method for on-line and real-time monitoring of microbial water quality.

### 9.3 Future Recommendations

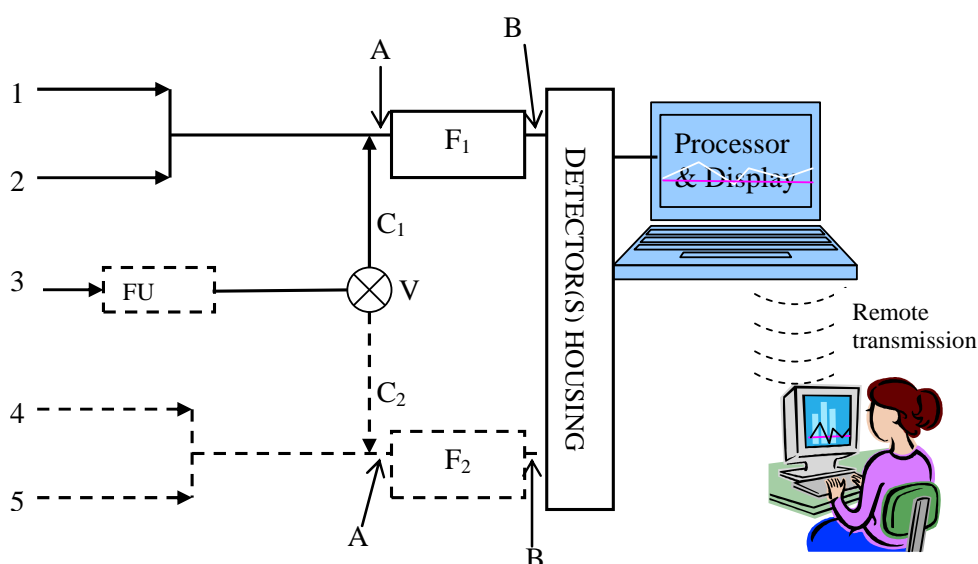
#### 9.3.1 Market considerations: from fundamentals to product

Most biosensor studies have remained laboratory protocols with no field applications (Kissinger, 2005; Lazcka *et al.*, 2006). In this study, the use of environmental samples for *in situ* GUD assays assisted in applying laboratory-developed protocols to the field. The developed electrochemical detection was successfully applied to rivers in the Eastern Cape of South Africa. The cost of a market-ready product is usually prohibitive to poorer communities. This is further complicated by the requirement of combining antagonistic attributes such as simplicity, cost-effectiveness and automation in designing biosensors (Ivnitski *et al.*, 1999). In the light of cost considerations in biosensor production, the *E. coli* biosensor from this study can be manufactured in two forms in order to meet both the low end (poor rural communities) and the high end (municipalities) markets. For the low end market, single-use disposable, electrodes and portable potentiostats can be manufactured, while for the high end market a more automated, continuous and on-line instrument can be designed based on a sequential flow injection analysis (SFIA) system.

#### 9.3.2 SFIA set up

Since the substrate is used to probe the presence of GUD in the environment, the best option for on-line monitoring of GUD activity is through liquid phase analysis using an SFIA as outlined in Figure 9.1. This system has the advantages of concurrent detection of the activity of more than one marker enzyme and the possibility of using both electrochemical and spectrophotometric technologies. This system requires no substrate immobilisation, thus circumventing difficulties in PNPG immobilisation. Furthermore, the customised design (Fig. 9.1) has an added advantage of optional pre-concentration and an inhibitor removal unit (FU; Fig. 9.1) before spectrophotometric detection. However, when the electrochemical method of detection is used, there is

no need for pre-concentration because the technique is sensitive and rapid. The sample from inlet 3 (Fig. 9.1) can then be distributed at V to tubes ( $C_1$  and  $C_2$ ) leading to different reactors ( $F_1$  and  $F_2$ ). In  $F_1$ , the environmental sample is mixed with the buffer and substrate (that enter the system through 1 and 2; Fig. 9.1). Entrance into the reactor (through tube A; Fig. 9.1) and exit (through tube B; Fig. 9.1) must be synchronised. This is achieved through use of programmable SIA Lab-on-Valves<sup>®</sup> (FIALab Instruments, USA). The reactor can be temperature controlled. The same system can be duplicated (dotted lines; Fig. 9.1) for parallel analyses where more than one marker enzyme activity can be detected. Under such circumstances the Lab-on-valves<sup>®</sup> enable easy control. In the case of a semi-portable device, the results can be sent to the laboratory from a remote monitoring point (Fig. 9.1)



**Figure 9.1** Proposed outline of the sequential flow injection analysis (SFIA) equipment. 1) and 2): buffer and substrate inlets; 3): water sample inlet; 4) and 5): optional inlets for substrate and buffer for other marker enzyme(s); FU): optional filtration unit; V): valve distributing the water samples to different reactor channels ( $C_1$  and  $C_2$ );  $F_1$ ) and  $F_2$ ): reaction chambers that allow mixing and have optional temperature control; A) and B): inlet and outlet tubes for the reactors; detector (s) housing will have the electrodes; - - - : optional/additional lines.

### 9.3.3 Future studies

While this study has shown that GUD in polluted water can be used for on-line electrochemical detection of faecal pollution, it has also stimulated other perspectives for further work. These include the automation of the proposed SFIA, investigation of the temporal distribution of GUD in water bodies, preferential binding of phenolic substrates to carbonates and extending the biosensor application to other organisms.

#### **Automation of the SFIA: Team work**

Collaborative partnerships with researchers from different backgrounds (including electronic engineers and mathematicians) should be established with regards to the production of a compact SFIA device.

#### **Temporal (time-dependent) vertical distribution of GUD in stagnant water bodies**

It would be desirable to perform depth profile studies of the GUD to determine whether there is a vertical distribution of the enzyme in the water body with daily temperature cycles and relate this to the microbial distribution. This can be used to recommend appropriate depth of sampling during a particular time of the day.

#### **Investigation of preferential binding of phenolic compounds to carbonates**

Following the trends in GUD activity in different environments subjected to elevated concentration of carbonates, it may be ideal to identify different phenolic compounds in the polluted environments. Once these have been determined, their binding patterns to carbonates can then be investigated and related to other phenol-based substrates. This will help in an interpretation of the *in situ* enzyme assays in presence of carbonates. Research by other students in our laboratory is ongoing to identify different phenolic compounds in these environments and their effect on the sensor response.

#### **Possible applications of the biosensor concept to other problematic organisms and their toxins**

This study has presented an opportunity for the possibility of detecting other organisms, beside *E. coli*, using the same concept. It could be of relevance to other researchers to apply this concept for the detection of algal and other microbial

contamination. However, the success will be dependent on the existence of suitable marker enzymes and/or other metabolic markers.

#### **Baseline analyses and inhibitor removal studies**

Investigations into baseline analyses of PNP and other pollutants in the environmental water samples using the biosensor can be pursued. These will assist in assessing possibilities of on-line inhibitor removal from the water samples in the SFIA system. For example, feasibility of heavy metal removal can be investigated using a method described by Di Nezio *et al.* (2004). Effects of the methods of inhibitor removal on the activity of GUD will also be worth investigating.

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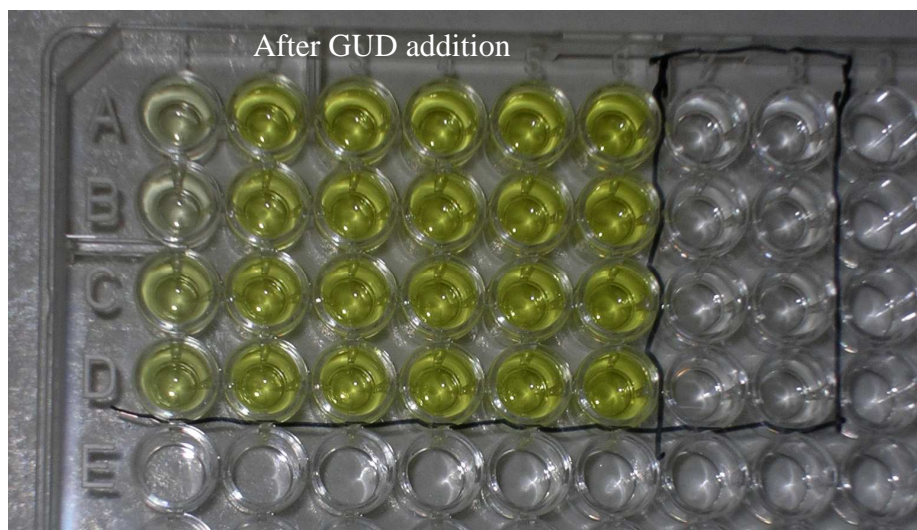
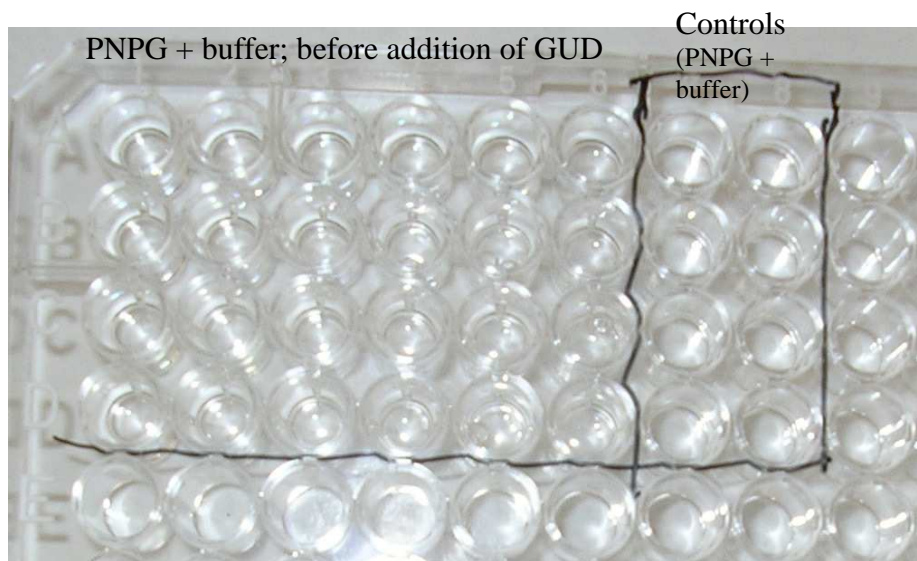
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## APPENDICES



**Appendix A** Illustration of the colour change during GUD catalysed hydrolysis of PNPB in microtitre wells.

**Appendix B: GUD activity calculation**

$$\text{Activity} = (\Delta A/\text{time}) \times \epsilon^{-1} \times l^{-1} \times \text{dilution factor}$$

$\epsilon$ : extinction coefficient;  $l$ : light path and  $\Delta A$ : change in absorbance

Extinction coefficient of PNP changes with pH and has to be corrected according to the formula given by John (2002):

$$\Rightarrow \epsilon_{\text{pH}} = 18\,000 \times [10^{-7.15}/(10^{-\text{pH}} + 10^{-7.15})] \text{ l/mol/cm}$$

$$\therefore \epsilon_{8.0} = 15\,773.022 \text{ l/mol/cm}$$

The light path was calculated using the formula;

$$\text{Volume} = \pi r^2 l$$

$$\Rightarrow 250 \mu\text{l} = (22/7) \times (7/2 \text{ mm})^2 \times l$$

$$\therefore l = 0.65 \text{ cm}$$

The dilution factor for the commercial GUD was (250/30) while for the environmental sample was (250/90).

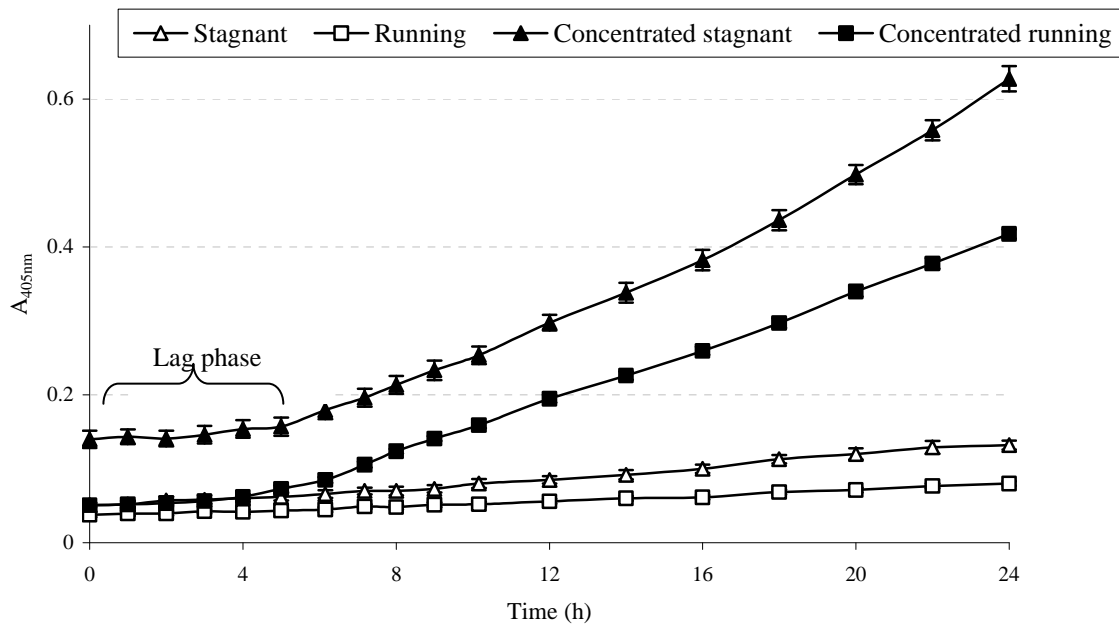
Therefore activity (A) for commercial GUD was given by:

$$A = (\Delta A/h) \times 0.01355 \mu\text{mol/min/ml}$$

$$\text{Activity for environmental GUD} = (\Delta A/h) \times 0.004516 \mu\text{mol/min/ml}$$

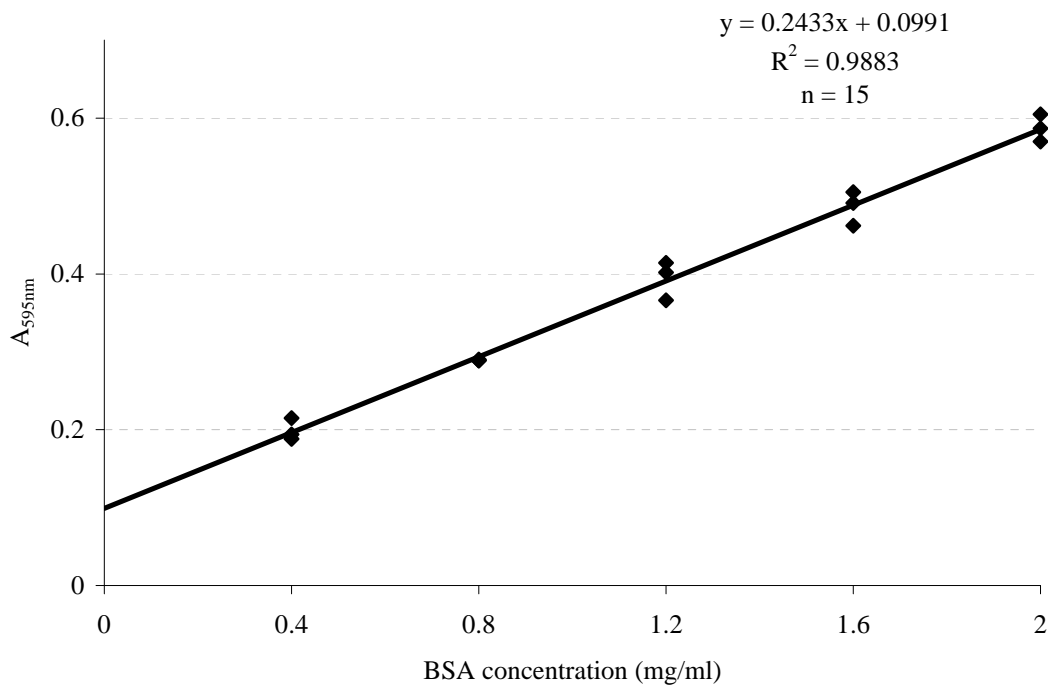
The activity can be expressed as nmol/h/ml by multiplying the above formulae with corresponding factors.

**Lag phase illustration**



**Appendix C** Illustration of typical lag phase of GUD activity in PEG-20 000 concentrated samples and non-concentrated samples. All data points represent the mean  $\pm$  SD (n = 3).

**BSA sample standard curve**

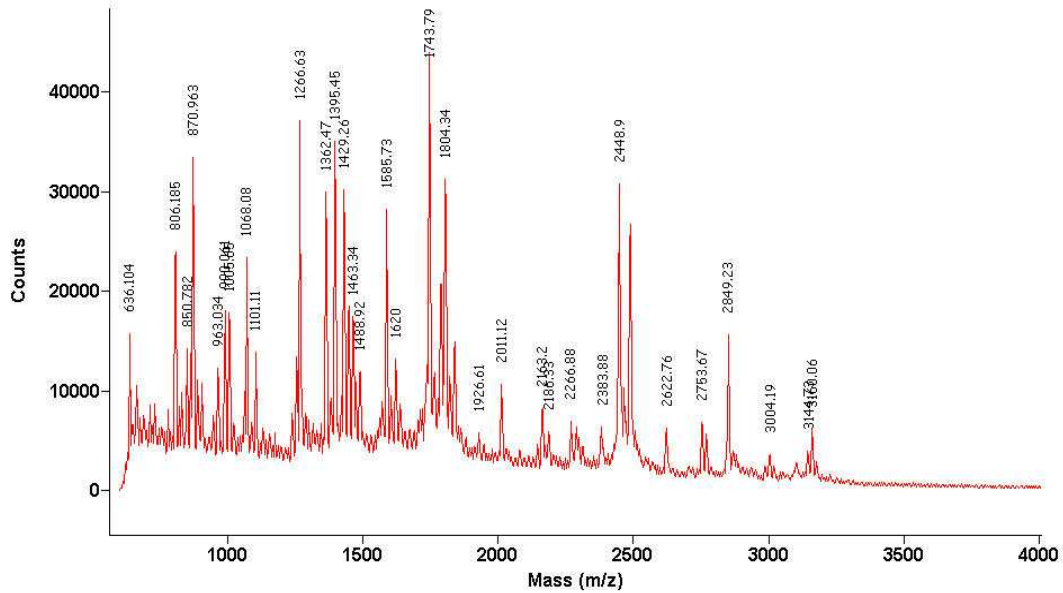


**Appendix D** The BSA standard curve.

**PerSeptive Biosystems**

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 Comment: chca

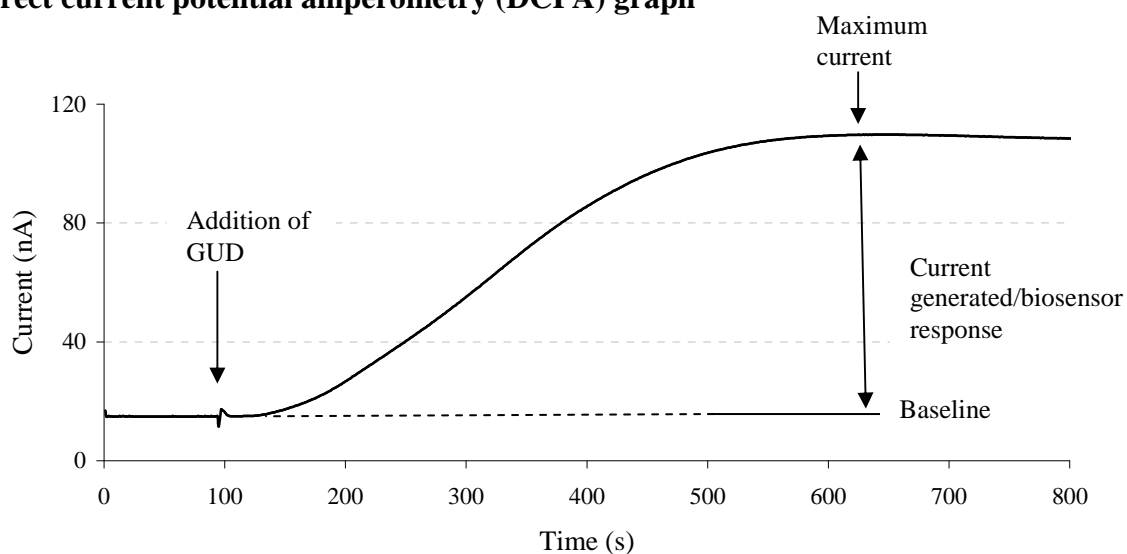
Method: HCD1002      Laser: 3000  
 Mode: Linear          Scans Averaged: 206  
 Accelerating Voltage: 20000      Pressure: 2.74e-07  
 Grid Voltage: 94.000 %      Low Mass Gate: 600.0  
 Guide Wire Voltage: 0.050 %      Timed Ion Selector: 24.9 OFF  
 Delay: 400 ON      Negative Ions: OFF  
 Sample: 24      Collected: 10/19/04 9:47 AM



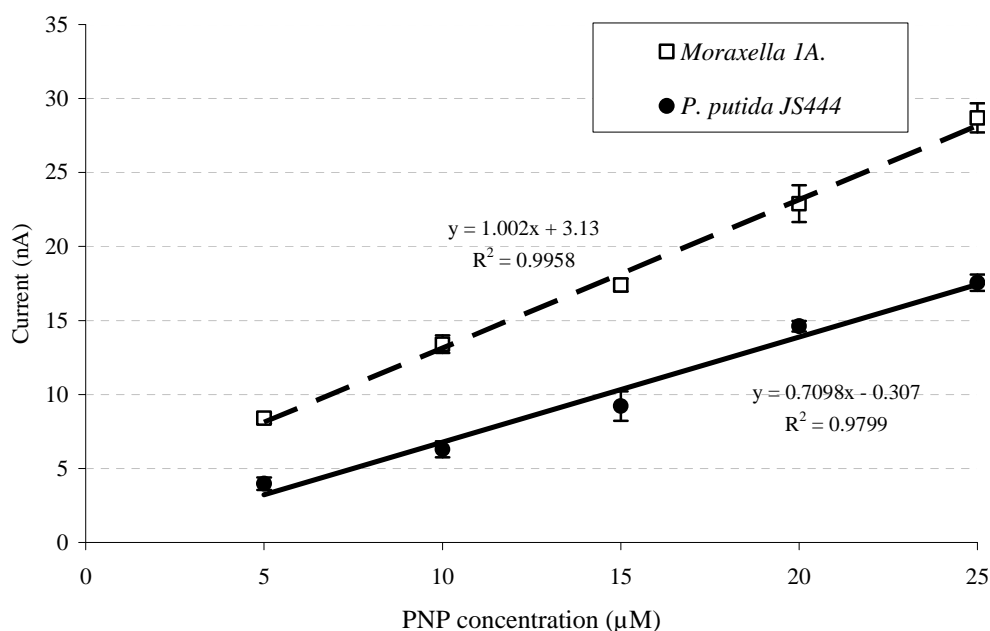
**Appendix E** MALDI ToF MS map for the presumed GUD band. NB: some fragments are from trypsin autolysis.



**Appendix F** Illustration of PNP degradation by *Moraxella* 1A, PNP before addition of *Moraxella* (a) and after culturing of *Moraxella* 1A(b).

**Direct current potential amperometry (DCPA) graph**

**Appendix G** Illustration of the typical DCPA graph and current generated (as the difference between base line and maximum current) at a *Moraxella* modified GCE. [PNPG] = 4.4 mM; pH 7.5; applied potential = 300 mV.

***Moraxella* 1 A and *P. putida* JS444 biosensors sensitivity and linearity to PNP**

**Appendix H** Illustration of linearity and higher sensitivity of the *Moraxella* 1A biosensor than the *P. putida* JS444 biosensor. [PNP] = 20  $\mu\text{M}$ ; pH = 7.5. All data points represent the mean  $\pm$  SD (n = 3).