DEVELOPMENT OF A NOVEL *IN SITU* CPRG-BASED BIOSENSOR AND BIOPROBE FOR MONITORING COLIFORM β-D-GALACTOSIDASE IN WATER POLLUTED BY FAECAL MATTER

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

DOCTOR OF PHILOSOPHY

(BIOTECHNOLOGY)

of

RHODES UNIVERSITY

by

VICTOR COLLINS WUTOR

MSc (Applied Microbiology), B.Pharm (Hons)

NOVEMBER, 2006

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DEDICATION

To my loving wife

- Valerie -

**You are the most beautiful miracle I have experienced,
and continue to every day!**

I love you with all my heart, mind, body and soul now and forever! You have gone through a very tough time and carried most of the burden so I could concentrate on my studies. There are no words to express my appreciation!

Thank you very much, my angel!

ABSTRACT

The ultimate objective of this work was to develop a real-time method for detecting and monitoring β-D-galactosidase as a suitable indicator of the potential presence of total coliform bacteria in water environments. Preliminary comparison of the chromogenic substrate, chlorophenol red β-D-galactopyranoside and the fluorogenic substrate, MuGAL, revealed unreliable results with the fluorogenic technique due to interference from compounds commonly found in environmental water samples. Thus, the chromogenic assay was further explored. Hydrolysis of the chromogenic substrate chlorophenol red β-D-galactopyranoside by β-D-galactosidase to yield chlorophenol red was the basis of this assay. Fundamental studies with chlorophenol red β-D-galactopyranoside showed that β-D-galactosidase occurs extracellularly and in low concentrations in the polluted water environment.

A direct correlation between enzyme activity and an increase in environmental water sample volume, as well as enzyme activity with total coliform colony forming unit counts were observed. Spectrophotometric detection was achieved within a maximum period of 24 h with a limit of detection level of 1 colony forming unit 100 ml^{-1}. This enzyme also exhibited physical and kinetic properties different from those of the pure commercially available β-D-galactosidase. Cell permeabilisation was not required for releasing enzymes into the extracellular environment. PEG 20 000 offered the best option for concentrating β-D-galactosidase. The source of β-D-galactosidase in the polluted environmental water samples was confirmed as *Escherichia coli* through SDS-PAGE, tryptic mapping and

MALDI-TOF, thus justifying the further use of this method for detecting and/or monitoring total coliforms.

Several compounds and metal ions commonly found in environmental water samples (as well as those used in water treatment processes) did have an effect on β-D-galactosidase. All the divalent cations except Mg^{2+}, at the concentrations studied, inhibited the relative activity of β-D-galactosidase in both commercial β-D-galactosidase and environmental samples. Immobilisation of chlorophenol red β-D-galactopyranoside onto a solid support material for the development of a strip bioprobe was unsuccessful, even though the nylon support material yielded some positive results. A monthly (seasonal) variation in β-D-galactosidase activity from the environmental water samples was observed, with the highest activity coinciding with the highest monthly temperatures.

Electro-oxidative detection and/or monitoring of chlorophenol red was possible. Chlorophenol red detection was linear over a wide range of concentrations (0.001-0.01 $\mu g\ ml^{-1}$). Interference by chlorophenol red β-D-galactopyranoside in the reduction window affected analysis. A range of phthalocyanine metal complexes were studied in an attempt to reduce fouling and/or increase the sensitivity of the biosensor. The selected phthalocyanine metal complexes were generally sensitive to changes in pH with a reduction in sensitivity from acidic pH to alkaline pH. The tetrasulphonated phthalocyanine metal complex of copper was, however, more stable with a minimum change of sensitivity. The phthalocyanine metal complexes were generally stable to changes in temperature. While only two consecutive scans were possible with the

unmodified glassy carbon electrode, 77 consecutive scans were performed successfully with the CuPc-modified glassy carbon electrode.

Among the phthalocyanine metal complexes studied, the CuPc-modified glassy carbon electrode therefore provided excellent results for the development of a biosensor. The CuPc modified-glassy carbon electrode detected 1 colony forming unit 100 ml^{-1} in 15 minutes, while the plain unmodified glassy carbon electrode required 6 hours to detect the equivalent number of colony forming units. CoPc, ZnPc and CuTSPc required 2, 2.25 and 1.75 h, respectively, to detect the same numbers of colony forming units. The CuPc-modified glassy carbon electrode detected 40 colony forming units 100 ml^{-1} instantly.

In general, a direct correlation between colony forming units and current generated in the sensor was observed (R^2=0.92). A higher correlation coefficient of 0.99 for 0-30 coliform colony forming units 100 ml^{-1} was determined. Current was detected in some water samples which did not show any colony forming units on the media, probably due to the phenomenon of viable but non-culturable bacteria, which is the major disadvantage encountered in the use of media for detecting indicator microorganisms.

This novel biosensor therefore presents a very robust and sensitive technique for the detection and/or monitoring of coliform bacterial activity in water.

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LIST OF ACRONYMS COMMONLY USED IN THE THESIS

A	Amps
ABNC	Active but non-culturable
AIDS	Acquired Immunodeficiency Syndrome
BSA	Bovine serum albumin
CFU	Colony forming units
CoPc	Cobalt phthalocyanine
CuPc	Copper phthalocyanine
CoTSPc	Cobalt tetrasulphonated phthalocyanine
CuTSPc	Copper tetrasulphonated phthalocyanine
CPR	Chlorophenol red
CPRG	Chlorophenol red β-D-galactopyranoside
CV	Cyclic voltammetry
DMF	Dimethyformamide
EDTA	Ethylene diamine tetraacetate
FC	Faecal coliform
GCE	Glassy carbon electrode
B-GAL	β-D-galactosidase
GUL	β-D-glucuronidase
HPC	Heterotrophic Plate Count
ISO	International Organization for Standardisation
LoD or LOD	Limit of detection
MF	Membrane filtration
MPc	Metallophthalocyanine
MPN	Most probable number
MTF	Membrane tube filtration
MTSPc	Tetrasulphonated metallophthalocyanine
mV	Millivolt
NiPc	Nickel phthalocyanine
^{o}C	Degree Celcius

OH	**Hydroxyl free radical**
Pc	**Phthalocyanine**
PCR	**Polymerase chain reaction**
PEG 20000	**Polyethylene glycol 20000**
pH	**Logarithm of the reciprocal of the hydrogen ion concentration**
R	**Running (water sample)**
S	**Stagnant (water sample)**
S/FIA	**Sequential /flow injection analysis**
SD	**Standard deviation**
SDS	**Sodium duodecyl sulphate**
TC	**Total coliform**
µA	**Microamp**
V	**Volt**
v/v	**Volume per volume**
v/w	**Volume per weight**
VBNC	**Viable but non-culturable cells**
ZnPc	**Zinc phthalocyanine**

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Cogito ergo sum, I think therefore I am!

We are not Human Beings Having a Spiritual Experience,
We are Spiritual Beings Having a Human Experience!

RESEARCH PRODUCTS

PATENT

SOUTH AFRICAN PROVISIONAL PATENT APPLICATION NO 2006/04442: "Biosensor" In the name of: The Water Research Commission. Inventor(s): CA TOGO, VC WUTOR, JL LIMSON, BI PLETSCHKE. Date 31 May 2006.

PUBLICATIONS FROM THE PROJECT

1. Pletschke, B.I., Togo, C.A. and Wutor, V.C. (2006) Technical report (Project 1446/1/05) for the Water Research Commission (WRC) of Southern Africa: *Online real-time enzyme diagnostic system for the detection and monitoring of faecal contamination of water intended for drinking purposes*.

[I co-compiled and wrote this technical report together with Mr. Togo. Dr Pletschke, principal investigator and grant holder revised the drafts and final report].

2. Wutor, V.C., Togo, C.A. and Pletschke, B.I. (2007). Comparison of the direct enzyme assay method with the membrane filtration technique in the quantification and monitoring of microbial indicator organisms – seasonal variations in the activities of coliforms and *E. coli*, temperature and pH. Water SA, (2007) Volume 33 No 1 pp 107-110

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3. Wutor, V.C., Togo, C.A., Limson, J.L. and Pletschke, B.I. A novel biosensor for the detection and monitoring of β-D-galactosidase of faecal origin in water. Enzyme and Microbial Technology, 40 (2007) pp1512-1517

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4. Wutor, V.C., Togo, C.A. and Pletschke, B.I. The effect of physico-chemical parameters and chemical compounds on the activity of β-D-galactosidase (B-GAL), a marker enzyme for indicator microorganisms in water. Chemosphere, 68 (2007) pp 622-627.

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5. Togo, C. A., Wutor, V.C. and Pletschke, B.I. (2006). Properties of *in situ Escherichia coli* β-D-glucuronidase (GUS): evaluation of chemical interference on the direct enzyme assay for faecal pollution detection in water. African Journal of Biotechnology (2006) 5: (22), pp. 2338-2344

[I assisted with sampling, experimentation and data analysis. Mr. Togo compiled and wrote the paper. Dr Pletschke, principal investigator and grant holder, provided assistance with the experimental design and revision of the draft and final manuscript].

6. Togo, C.A., Wutor, V.C., Limson, J.L. and Pletschke B.I. (2007). Novel detection of Escherichia coli β-D-glucuronidase activity using a microbially-modified glassy carbon electrode and its potential for faecal pollution monitoring. Biotechnology Letters, (2007) Vol 29 (4), pp 531-537

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CHAPTER ONE

INTRODUCTION

The question in life is not whether you get knocked down. You will. The question is, are you ready to get back up, And fight for what you believe in!

Access to safe drinking water is a basic human right and essential to people's health (Hodgson and Manus, 2006). In developing countries such as South Africa, most of the rural communities are poverty-stricken, lack access to potable water supplies and rely mainly on streams, rivers, well and pond water sources for their daily water needs (Nevondo and Cloete, 1999; Obi *et al.*, 2002). As a result, rural communities are exposed to water borne diseases (Schalekamp, 1990; Grabow, 1996).

Microbial pollutants of water are a major source of health and economic problems the world over. Even in developed countries, the number of outbreaks reported in the last decade demonstrates that the transmission of pathogens by drinking water remains a significant problem and that, despite substantial advances in recent years, access to safe drinking water is still a major challenge to public health.

In recent years, waterborne microbial diseases have been more common in many African countries, emerging in Congo, Cote d'Ivoire, Madagascar, Mozambique, Somalia, Zambia and Zimbabwe, among others. Zambia's 1999 outbreak was among the most serious, recording more than 11 000 cases and at least 390 deaths. In 2001, an outbreak of cholera that lasted for nearly a year in South Africa, killed more than 200 people and infected more than 100 000 of the rural population in the eastern part of South Africa that relies on rivers and streams for their water (Dufuor *et al*., 2003; Elettra, 2003). The United Nations Office for the Coordination of Humanitarian Affairs (OCHA) reported that statistics provided by Medecins Sans Frontieres stated a cholera outbreak in Cote d'Ivoire's main city, Abidjan, with 40 cases confirmed, including four deaths, since the beginning of July 2006 United Nations (OCHA), 2006). These are just a few examples illustrating the need for reliable techniques in detecting microorganisms involved in waterborne diseases.

High levels of microbial contamination often make the water unfit for domestic purposes, recreational or irrigation of crops eaten raw (Jagals, 2000; Griesel, 2001). Since it is not feasible to test water supply for all pathogens related to water-borne diseases due to the complexity of the testing, time and cost, indicator organisms are often used to assess the

microbiological status of water (Hazen, 1988; Lehloesa and Muyima, 2000; Grabow, 2001).

Indicator bacteria have long been used in water quality studies to assess the level of faecal contamination in water bodies (Mill *et al.*, **2006). The presence of these microorganisms is also used to estimate the potential health risk of other pathogenic organisms of faecal origin to users (Dadswell, 1993). Faecal contamination is usually detected by testing water for the presence of certain faecal-derived indicator bacteria such as coliforms, faecal coliforms,** *Escherichia coli* **and enterrococci (Leclerc** *et al.*, **1996, 2001; Huertas** *et al.*, **2003; Pletschke** *et al.*, **2006). Analysing for total coliform bacteria and** *Escherichia coli* **is the most common method used to test the hygienic quality of drinking water (Pitkanen** *et al.*, **2006).**

Recently, there have been criticisms questioning the suitability of bacterial indicators, especially total coliforms, as markers of microbial water safety (Gofti *et al.*, **1999; Leclerc** *et al.*, **2001; Rose and Grimes, 2002; Hörman and Hänninen, 2006). Advances in the bacteriology of total coliforms reveal that they can have environmental origin as well as originate from non-faecal sources thus this may not be suitable indicators of faecal contamination (Leclerc** *et al.*, **2001).** Figure 1.1 **shows the classification of coliforms, which justifies the concern of the use of these indicator microorganisms as markers of faecal contamination. Despite improvements in the Standard methods, coliforms are still being used in national and international guidelines for water microbiological quality assessment (Grabow** *et al.*, **1999; Noble** *et al.*, **2003a and b; Horman** *et al.*, **2004; Byamukama, 2005).** *Escherichia coli* **and enterococci have been shown to be much better indicators of faecal contamination (Leclerc** *et al.*, **1999; 2001).**

The future of water quality monitoring is likely to rely on a matrix approach placing less emphasis on any one single parameter (Ashbolt *et al.*, **2001; Haveleer** *et al.*, **2001; Niemelä** *et al.*, **2003; Van Heerden** *et al.*, **2005).**

Several chromogenic and fluorogenic media based on the detection of β-D-galactosidase (B-GAL) and β-D-glucuronidase activities are available and widely used in water microbiology to monitor coliform and *Escherichia coli* **presence and/or absence respectively (Perez** *et al.***, 1986; Manafi, 2000, 1996; Rompre** *et al.***, 2002, Pitkanen** *et al.***, 2006). B-GAL, an enzyme produced by total coliforms, catalyses the breakdown of lactose into galactose and glucose and has been used mostly for enumerating this group of bacteria. Chromogenic and fluorogenic substrates produce color and fluorescence upon breakdown by specific enzymes. Bacterial diagnosis based on enzymes has attracted extensive research over the years. The chromogene chlorophenol red β-D-galactopyranoside (CPRG) (yellow in colour) can be broken down by the enzyme β-D-galactosidase into chlorophenol red (CPR) (red colour) (Pelisek** *et al.***, 2000; Wutor** *et al.***, 2007a, 2007b, 2007c).**

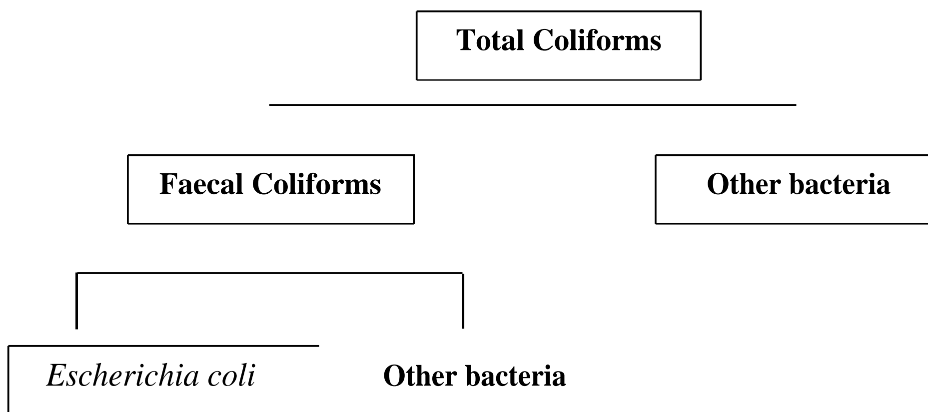


Figure 1.1**: Classification of Coliforms**

PROJECT JUSTIFICATION

Progress has been made in improving the detection and confirmatory times required for indicator organism detection. However, there are still no satisfactory methods (in terms of short analysis time, low cost, improved sensitivity and ease of use) on the market for real-time monitoring of these indicator microorganisms. The need for methods to detect and confirm the presence/absence of indicator microorganims within a working day is urgent (Holme and Peck, 1998; Pyun *et al.***, 1998; Sartory and Watkins, 1999; Venter, 2000; Rompré** *et al.***, 2002; Leonard** *et al.***, 2003).**

HYPOTHESIS

Coliform B-GAL can be found in the extracellular environment in which coliforms hydrolyse lactose-based substrates. There is a positive linear relationship between the activity of this enzyme and coliform counts (Farnleitner *et al.***, 2001, 2002). Enzyme catalysed reactions occur very rapidly (Holme and Peck, 1998). We therefore hypothesise that B-GAL can be used in the development of an alternative, affordable, rapid and real-time** *in situ* **method for the efficient detection and monitoring of water pollution by faecal material. This technique will employ, among others, metallophthalocyanine complexes (especially copper) in the development of a novel biosensor.**

RESEARCH OBJECTIVES

The aim of this study was to exploit the rapid detectability of enzyme activities and to develop direct *in situ* **enzyme assay protocols by employing appropriate chromogenic and/or fluorogenic substrates. Such a contribution will greatly complement efforts in the development of the much-required early warning system for water supplying authorities in protecting the public.**

The ultimate objective of this work was to develop an alternate method for detecting and monitoring B-GAL as a suitable indicator of the presence of total coliform bacteria in the water environment and to evaluate possible avenues for its implementation. This was accomplished by addressing the broad specific objectives stated below;

- **optimisation of enzyme assays and selection of suitable substrates,**
- **enzyme concentration and bioinformatic studies,**
- **evaluation of the effects of compounds commonly occurring in water (and those used in water treatment) on B-GAL activity,**
- **investigation of the use of liquid and strip probes,**
- **design and development of an electrochemical biosensor for the detection and monitoring of B-GAL activity.**

RESEARCH APPROACH

This study employed *in situ* **assays, using typically polluted river water samples, to ensure more rapid and easier application of the method in the field.**

This study was divided into two major parts; a fundamental study and an applied study. Insight gained from the fundamental study guided the applied study. *In situ* **assays were performed using appropriate samples from running and standing water sections of a typically polluted river in the Eastern Cape Province of South Africa. When necessary, commercial B-GAL and microbial plate assays on CM1046 and mEndo media were run in parallel to serve as positive controls. Chapters 3-6 deal mainly with the enzymological aspects of the study while chapter 7 deals solely with the electrochemical aspects.**

Chapter one is the introduction while chapter two is the literature review on the research topic. The major objective of Chapter three was to compare a chromogenic and a fluorogenic substrate through optimisation of reaction parameters with specific objectives to determine appropriate sample volumes to be used in each enzyme assay, ascertain the time period that produced a linear but maximal reaction, determine the effect of the

environmental water samples on B-GAL activity, determine the pH and temperature optima for B-GAL, investigate the location and abundance of B-GAL in the river samples, and select the most suitable assay (chromogenic or fluorogenic) for further studies.

The major objective in chapter four was to concentrate B-GAL in the stagnant and running water samples as well as to ascertain the origin of these enzymes. This was achieved through the use of different concentration techniques, electrophoresis and MALDI ToF MS. Chapter five investigated the effects of a wide range of potential ions/salts in varying concentrations on both commercial and environmental B-GAL.

Chapter six was aimed at developing a suitable liquid bioprobe and to determine the monthly (seasonal) trends in the activity of B-GAL *in situ*. **The major aim of chapter seven was to investigate the potential of employing electrochemical techniques by the use of a range of metallophthalocyanine (MPc) complexes to detect and/or monitor CPR as a sensitive measure of B-GAL activity by total coliform contamination of water. The specific objectives in this chapter were to confirm the electrochemical detection of CPR, optimise parameters such as pH and temperature, modify glassy carbon electrode (GCE) with MPc in an attempt to increase sensitivity and/or reduce fouling and investigate the potential interference by chemicals which are commonly found in the water environment. Chapter eight composes of general discussion, conclusions and future recommendations.**

CHAPTER TWO

LITERATURE REVIEW

One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality - Albert Einstein

2.1 WATERBORNE DISEASES

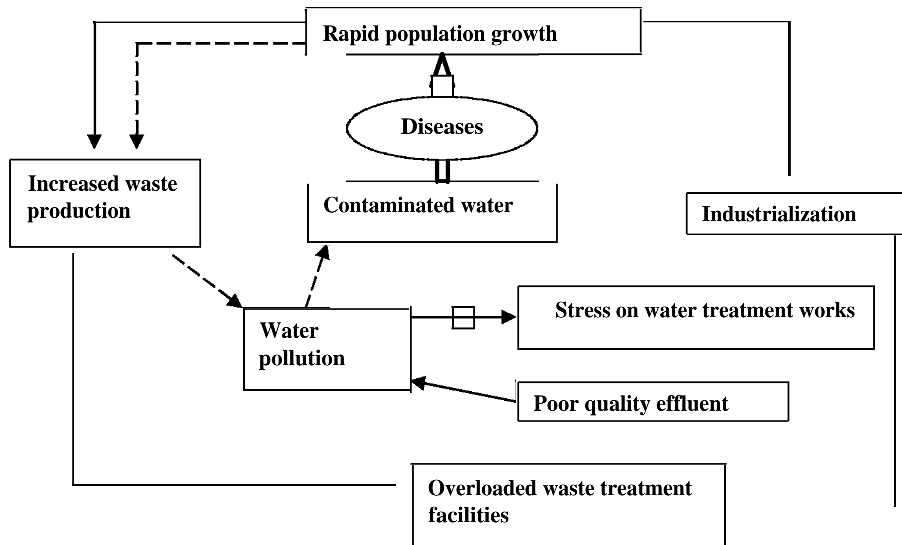
The ingestion of water contaminated by faecal material that contains pathogenic microorganisms might cause waterborne diseases. The most important bacterial waterborne diseases are cholera, salmonellosis and shigellosis. This accounts for millions of deaths worldwide. Both developed and developing countries suffer from these waterborne diseases (Berg and Fiksdal, 1988; Ivnitski *et al*., 1999; Venter, 2000; Gleick, 2002; Leonard *et al*., 2003). It must however be stated that, these diseases are much more problematic in the developing countries. Aggravating outbreaks of these diseases is the lack of continuous water monitoring methods, slow and inefficient wastewater treatment, improper treatment of drinking water and expensive and laborious methods of detection for the supposed early warning system. Developing countries are affected most by these problems as a result of rapid population growths that do not match the available water and wastewater treatment facilities.

Increases in populations lead to increasing demand for drinking water and hence increase in waste generation that overload wastewater treatment facilities. These, in turn, lead to the deterioration in effluent quality released into water bodies. This triggers a cycle of problems that adversely affects the population (Figure 2.1). Such a cycle calls for a rapid, easy to use and sensitive water quality monitoring method to act as an efficient early warning system. This growing water problem has resulted in an active research area over recent years.

2.2 THE PROBLEM OF WATER QUALITY

Public and environmental health protection agencies require the provision of safe drinking water. This means that drinking water must be free of pathogenic microorganisms, as well as other chemicals and substances considered pollutants or indicators of the possible presence of pollutants. Common water pollutants include microorganisms such as *Cryptosporium* spp, *Escherichia coli, Giardia* spp, *Vibrio* spp., *Salmonella* spp. and *Shigella* spp., metals such as lead, arsenic, copper, cadmium, and

mercury, organic pollutants such as toluene, pesticides, herbicides and their metabolites as well as chlorine based agents and asbestos (Yang *et al*, 2005; Zawala *et al*, 2007). All these pollutants are of primary health concern.



KEY: Urban set up Rural set up

Possible water quality monitoring points

Figure 2.1: Cyclical water problems caused by rapid population growth without matching facilities and possible monitoring points to break the cycle..

Coliforms are a group of bacteria used by the water industry to assess the microbiological quality of both drinking and recreational waters. These bacteria, though not generally pathogenic themselves, serve as indicators of the potential presence of organisms which may be pathogenic. Most coliforms are present in large numbers among the intestinal flora of human and other warm-blooded animals, and are thus commonly found in faecal wastes. As a consequence, coliforms detected in higher concentrations than pathogenic bacteria are used as an index of the potential presence of entero-pathogens in the water environment (Rompré *et al*., 2002). Bacteria as diverse as *Escherichia coli*, *Citrobacter*, *Klebsiella*, *Enterobacter*, *Serratia*, *Erwinia* and *Yersinia* are all positive for the traditional coliform test (Standard Methods, 1998). Among the afore-mentioned bacteria, only

Escherichia coli **without any ambiguity is of faecal origin (Gofti *et al*, 1999; Rompré *et al.* 2002; Schraft and Watterworth, 2005).**

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

2.3 WATER QUALITY ASSESSMENT: THE CONCEPT OF INDICATOR ORGANISMS

The main goal of microbiological water analysis is the detection of epidemiologically relevant microbial contamination as early as possible (Hattenberger *et al*., 2001). However, these microorganisms are not routinely searched for because of reasons such as high cost in their detection, and also because their presence in the environment are sporadic and erratic (Howard *et al*, 2004; Lucena *et al,* 2004). The concept of indicator microorganisms is an attempt to predict the possible presence of pathogenic microorganisms in the environment under consideration. It relies on the fact that some non-pathogenic microorganisms occur in the faeces of all warm-blooded animals. Thus the presence of these non-pathogenic microorganisms in water indicates the potential presence of pathogenic microorganisms of faecal origin (Venter, 2000) and consequently a health hazard.

Indicator microorganisms are routinely employed to detect the presence/absence of pathogens in water as it is not feasible to detect and/or identify every pathogen (Tryland

and Fiksdal, 1998; Venter, 2000; Stevens *et al*., 2001; Rompré *et al*., 2002; Health Canada, 2006). Some microorganisms are very difficult to identify and detect (Sartory and Watkins, 1999; Leonard *et al*., 2003; Taguchi *et al*., 2005). Viral testing techniques take even longer, up to a few weeks and are more difficult (Venter, 2000).

According to Bitton *et al*. (1995), Manafi (1998), Stevens *et al*. (2001) and Bitton (2005), an ideal indicator organism should have the following characteristics:

- **be useful for all types of water,**
- **be present whenever enteric pathogens are present and absent in uncontaminated samples,**
- **should have a reasonable longer survival time than the hardiest enteric pathogen,**
- **not grow in water,**
- **should be at least equally resistant as the pathogen(s) to environmental factors and to disinfection in water and wastewater treatment plants,**
- **should not multiply in the environment,**
- **the density of the indicator organism should have some direct relationship to the degree of faecal pollution (more appropriate if present in greater numbers than the pathogens),**
- **should be a member of the intestinal microflora of warm-blooded animals and**
- **should be detectable using an easy, rapid and inexpensive method.**

It must be stated that, even though the coliform group of bacteria has been the cornerstone of water regulation in many countries for a long time and is used by many in the water supply industry as a criterion of operational parameters, some dissatisfaction on the reliance of this group of bacteria as indicators has been reported (Leclerc *et al*., 2001; Stevens *et al*., 2001). These include their occurrence in the absence of documented waterborne morbidity in the community, and outbreaks where coliforms have not been found (McFeters and Camper, 1983; McFeters *et al*., 1986). The former situation represents a complex unresolved problem of increasing dimensions that is frequently

described as regrowth within the distribution system. The latter may be due to currently accepted methods that lead to underestimations in the detection of waterborne coliforms (Hudson *et al*., 1983; McFeters and Camper, 1983; McFeters *et al*., 1986).

All members of the coliform group are known to re-grow in natural surface and drinking water distribution systems. The amount and type of organic matter and the temperature of the water environment affects the concentration of the coliforms. Thus, eutrophic tropical waters will contain high concentrations of coliform bacteria amongst others. Biofilm formation along distribution system pipes (even in the presence of free chlorine) may give a false indication of faecal contamination. Coliforms are more resistant to free chlorine in biofilms or when attached to surfaces than as free cells in water (Ridgway and Olson, 1982, LeChevallier *et al*., 1987). This regrowth and chlorine resistance gives a false positive indication of the potential presence of pathogens. It has also been reported that the presence of large numbers of heterotrophic bacteria may mask the growth of coliforms in selective media (LeChevallier and McFeters, 1985). This, in turn, may give a false negative result, which then leaves the population prone to pathogen consumption.

LeChevallier *et al*. (1987) and LeChevallier and McFeters, (1985), reported that a number of chemical and physical factors common to drinking water systems are known to cause a form of sub-lethal and reversible injury that is responsible for the failure of waterborne coliforms to grow on accepted media such as m-Endo media used in the analysis of drinking water. Amongst the factors found in drinking water that can cause injury include chlorine and other biocides, low concentration of metals such as copper and zinc, extremes of temperature and pH, and interactions with other bacteria (McFeters and Camper, 1983; McFeters *et al*., 1986).

In the spite of the potential interferences and disadvantages with the use of the coliform bacteria as indicators, significant correlation (log-log linear relations) between coliforms and the various assays have been found in many aquatic environments (Farnleitner *et al*., 2002), thus highlighting the approach as a potentially useful monitoring technique. The

coliforms are still considered as an imperfect but useful criterion of drinking water quality.

The 'Annapolis Protocol' (1999) as adopted by the World Health Organisation (WHO) lists procedures which could be employed in the determination of the presence/absence of indicator microorganisms in recreational water (Table 2.1).

2.3.1 Review of indicator microorganisms

Coliforms - the coliform group is among several microorganisms employed as indicators. This group includes *Escherichia coli*, *Citrobacter*, *Enterobacter* and *Klebsiella* (Frampton and Restaino, 1993; Stevens *et al*., 2001; Rompré *et al*., 2002; Health Canada, 2006). Specifically, all aerobic and facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that produce gas upon lactose fermentation in prescribed media in 48 hours at 37 ^{o}C (total coliforms) and at 44.5 ^{o}C (faecal or thermotolerant bacteria) fall in the coliform group (Frampton and Restaino, 1993; Rompré *et al*., 2002;). It has been observed that the absence of coliforms in 100 ml of drinking water ensures the prevention of a bacterial waterborne disease outbreak (Standard Methods, 1998)

Faecal Streptococci - this group comprises *Streptococcus faecalis, S. bovis, S. faecium, S. gallinarium, S. equines* and *S. avium. S. faecalis and S. faecium* commonly inhabit the intestinal tracts of humans and warm-blooded animals, hence their use in the detection of faecal contamination in water. Although members of this group survive longer than other bacteria indicators, they do not survive in waters for a long time and also do not reproduce in the environment. This group has been suggested as potentially useful for indicating the presence of viruses, particularly in biosolids and marine environments.

Faecal streptococci/enterococci can be detected using selective growth media in most probable numbers or membrane filtration formats. Enzyme based methods are available as well. Faecal streptococci can be detected by incorporating fluorogenic substrates such

as 4-methylumbelliferone-β-D-glucosidase (MUD) or chromogenic substrates such as indolyl- β-D-glucosidase into selective media.

Manafi and Sommer (1993) reported that the enterococci group can be easily detected via fluorogenic or chromogenic enzymatic assays based on two specific enzymes namely pyroglutamyl aminopeptidase and β-D-glucosidase Pourcher *et al*. (1991). Hernandez *et al*. (1990) succeeded in using microplates and MUD to selectively detect the enterococci in environmental samples.

Table 2.1 Possible sewage contamination indicators and their functions

Indicator/use	**Functions** **Demerits**	**Merits**
Faecal *Streptococci/ Enterrococci*	**Marine and potentially freshwater human health indicator** **More persistent in water and sediments than coliforms** **Faecal streptococci may be cheaper than enterrococci to assay**	**May not be valid for tropical waters due to potential growth in soils**
Thermotolerant coliforms	**Indicator of recent faecal contamination**	**Possibly not suitable for tropical waters due to growth in soils and waters** **Compounded by non-sewage sources e.g. *Klebsiella spp* in pulp and paper wastewater**
E. coli	**Potentially a freshwater human health indicator** **Indicator of recent faecal contamination** **Potential for typing *E. coli* to aid in sourcing faecal contamination** **Rapid identification possible if define as β-glucuronidase producing bacteria**	**Possibly not suitable for tropical waters due to growth in soils and waters**
Sanitary plastics	**Immediate assessment can be made for each bathing day** **Can be categorized** **Little training of staff required**	**May reflect old sewage contamination and of little health significance** **Subjective and prone to variable description**
Preceding rainfall (12, 24, 48 and 72 h)	**Simple regression may account for 30-60% of the variation in microbial indicators for a particular beach**	**Each beach catchment may need to have its rainfall response assessed.** **Response may depend on the period before the event**

Sulphite-reducing clostridia / *Clostridium perfringens*	**Always in sewage impacted waters Possibly correlated with enteric viruses and parasitic protozoa Inexpensive assay with H_2S production**	**May also come from dogs May be too conservative an indicatorEnumeration requires anaerobic culture**
Somatic coliphages	**Standard method well established Similar physical behavior to human enteric viruses**	**Not specific to sewage May not be as persistent as human enteric viruses May grow in the environment**
F-specific RNA phages	**Standard ISO method well established More persistent than some coliphages Host does not grow in environmental waters below 30 ^{o}C**	**Not specific to sewage WG49 hot may lose plasmid (although F-amp more stable) Not as persistent in marine environment**
***Bacteroides fragilis* phage**	**Appears to be specific to sewage ISO method recently published More resistant than other phages in the environment and similar to hardy human enteric viruses**	**Requires anaerobic culture Numbers in sewage are lower than other phages, and most humans do not excrete this phage (hence no value for small population)**

Faecal sterols	Coprostanol largely specific to sewage Coprostanol degradation in water similar to die-off of thermotolerant coliforms Ration of 5β/5α stanols >0.5 is indicative of faecal contamination. i.e coprostanol/5-cholestanol>0.5 indicates human faecal contamination; while $_{C29}$ 5β (24-ethylcoprostanol)/ 5 stanol ratio >0.5 indicates herbivore faeces. Ratio of coprostanol:24-ethylcoprostanol can be used to indicate the proportion of human faecal contamination, which can be further supported by ratios with faecal indicator bacteria (Leeming *et al*., 1996)	Requires gas chromatographic analysis and is expensive (USD 100/sample) Requires up to 10 L of sample to be filtered through glass filter (Whatman) to concentrate particulate stanols
Caffeine	May be specific to sewage, but unproven to date Could be developed into a dip-stick assay	Yet to be proven as a reliable method
Detergents	Relatively routine methods available	May not be related to sewage (e.g. industrial pollution)
Turbidity	Simple, direct and inexpensive assay available in the field	May not be related to sewage, correlation must be shown for each site type
Cryptosporidium (animal sources pathogens)	Required for potential zoonoses, such as *Cryptosporidium spp* where faecal indicator bacteria may have died out, or not present	Expensive and specialized assay (e.g. method 1622, USEPA) Human/animal speciation of serotypes not currently defined

Sourced from the WHO, Health-based monitoring of recreational waters; 'The Annapolis Protocol' (1999).

Anaerobic bacteria – among the anaerobic bacteria that are an important part of faecal flora are *Clostridium perfringens, Bacteroides spp* and *Bifidobacteria*. Although *Clostridium perfringens* are mostly opportunistic pathogens, clostridia have been

implicated in human diseases such as gas gangrene (*C. perfringens*), tetanus (*C. tetani*), botulism (*C. botulinum*) or acute colitis (*C. difficile*) (Payment *et al*., 2002). *C. perfringens* is an anaerobic gram-positive, endospore-forming, rod-shaped, sulphite-reducing bacterium found in the colon of and represents approximately 0.5 % of the faecal microflora. It is a member of the Sulphite Reducing Clostridia (SRC) group and is commonly found in human and animal faeces and in wastewater. SRC have traditionally been employed in Europe as an indicator of water quality. European Union regulations specifically consider *C. perfringens* as the indicator of choice and require 0/100 ml of drinking water (European Union, 1998). A disadvantage in the use of this microorganism is the hardy spores which make it too resistant to wastewater-treatment practices and very persistent in the environment (Venczel *et al.,* 1997).

Thus anaerobic bacteria could be useful as indicators of past pollution and as a tracer to follow the fate of pathogens. Payment and Franco (1993) recommended their use as an indicator of the presence of viruses and parasitic protozoa in water treatment plants and as an indicator of the quality of recreational waters (Fujioka, 1997).

***Bacteriodes spp* - these non-spore forming obligate anaerobic bacteria occur in the intestinal tract at concentrations in the order of 10^{10} cells per gram of faeces. They represent about one-third of the human faecal bacteria, outnumbering the faecal coliform bacteria (Bitton, 2005). Strict anaerobic bacteria are desirable indicators because they are restricted to warm-blooded animals and do not survive long once deposited in waters (Meays *et al*., 2004). However, these bacteria are more difficult to grow in the laboratory than coliforms or enterococci.**

Bifidobacteria* - these are anaerobic, non-spore forming, gram-positive bacteria that live in the guts of humans. They have long been suggested as faecal indicators. *Bifidobacterium* is the third most common genus found in the human gut. Since some of these bacteria (such as *B. bifidum, B. adolescentis, B, infantis, B. dentium*) are primarily associated with humans, they may serve as another tool to differentiate human from animal faecal contamination sources (Bitton, 2005). The human isolates of *Bifidobacteria

have the ability to ferment sorbitol and can be selectively detected in sorbitol agar (Rhodes and Kator, 1999). The major disadvantage with the use of *Bifidobacteria* as indicators is their sensitivity to environmental factors and the difficulty to grow on common laboratory culture media and conditions.

Bacteriophages – these microorganisms have a basic structure similar to animal viruses and infect a wide range of bacteria. They initiate a lytic cycle, which results in the production of phage progeny and the destruction of the bacterial host cells. Phage detection in environmental samples consists of concentrating the sample using one of several published procedures, decontaminating the concentrate and carrying out a phage assay by the double or single-layer method (Primrose *et al*., 1982; Yates *et al*., 1985; Berger and Oshiro, 2002). A wide range of bacteria host cells have been used, as some are more efficient than others in hosting phages. Somatic coliphages can be assayed on an *E. coli* C host, while the assay of male-specific phage requires the use of specific host cells such as *Salmonella typhimurum* strain WG49 or *Escherichia coli* strain HS[pFamp]R, but may be complicated by the growth of some somatic phages. Three groups of phages have been considered as indicators; somatic coliphages, male-specific RNA coliphages (FRNA phages) and phages infecting *Bacteriodes fragilis* (Leclerc *et al*., 2000; Berger and Oshiro, 2002).

Bacterial Spores - aerobic spores are non pathogenic, ubiquitous in aquatic environments, occur at much higher concentrations that the parasitic protozoan cysts, do not grow in environmental waters, and their assay is simple, inexpensive and relatively quick. Bacillus spores may serve as good surrogates to assess the removal efficiency of *Cryptosporidium oocysts* or *Giardia cysts* and disinfection efficiency in water and wastewater treatment plants (Nieminski *et al*., 2000; Chauret *et al*., 2001; Nieminski, 2002; Radziminski *et al*., 2002). The detection of bacterial spores is relatively simple and consists of pasteurizing the sample at 60 ^{o}C for 20 min and passing it through a membrane filter which is incubated on nutrient agar supplemented with 0.005% bromothymol blue (Francis *et al*., 2001).

Heterotrophic Plate Count (HPC) - this represents the aerobic and facultative anaerobic bacteria that derive their carbon and energy from organic compounds. This group includes gram-negative bacteria belonging to the following: *Pseudomonas, Aeromonas, Klebsiella, Flavobacterium, Enterobacter, Citrobacter, Serratia, Acinebacter, Proteus, Alcaligenes, Moraxella* and nontubular *mycobacteria*. Some of these are opportunistic pathogens (e.g. *Flavobacterium, Aeromonas*), but little is known about the effects of high numbers of HPC bacteria on human health. Segments of the population particularly at risk of infection with opportunistic pathogens are newborn babies, the elderly and the sick (Grabow, 1996; Rustin *et al.*, 1997; Pavlov *et al.*, 2004). HPC levels in drinking water should not exceed 500 organisms/ml. Numbers above this limit generally signal a deterioration of water quality in distribution systems (Bitton, 2005). The HPC is useful to water treatment plant operators with regard to the following: assessing the efficiency of various treatment processes (including disinfection), in a water treatment plant; monitoring the bacteriological quality of the treated water during storage and distribution and determining the potential for regrowth of aftergrowth in treated water in distribution systems (Reasoner, 1990). The HPC test is included in water quality specifications and recommendations in South Africa. The South African Bureau of Standards specifies a HPC limit of 100 CFU/100ml for drinking water (Pavlov *et al.*, 2004).

2.4 METHODS OF INDICATOR ORGANISM DETECTION

The detection and quantification of indicator microorganisms have been achieved through different methods, which can be categorised into (a) cultural or classical, (b) enzymatic and (c) molecular methods (Frampton and Restaino, 1993; De Boer and Beumer, 1999; Venter, 2000; Rompré *et al*., 2002). It must be noted that some of these categories may overlap in an attempt to improve on the selectivity and/sensitivity of the method.

2.4.1 Culture based methods

Major sub-categories of the culture based methods are membrane filtration (MF) and the multiple tube fermentation (MTF), which have different sensitivities (MF technique

could theoretically detect 1 bacterium in 1000 ml water while MFT may not detect 1 bacterium in 1 ml of water). A typical membrane filtration technique will involve the concentration and entrapment of microorganisms on a 0.45 or 0.22 µm (pore diameter) filter paper. The paper is then layered onto a selective media agar plate and incubated at an appropriate growth temperature. Thermophilic coliforms, for example, will grow on MacConkey agar and m-Endo-type agar (Rompré *et al*., 2002) at 45 ^{o}C within 18 – 24 hours (Brenner *et al*., 1993; Davies *et al*., 1994; Manafi, 1998; Van Poucke and Nelis, 2000; Farnleitner *et al*., 2001). m-Endo media at 37 ^{o}C detects coliforms as red colonies with a metallic sheen (Rompré *et al*., 2002). A further 24 – 48 hours may be required to confirm whether the microorganisms under observation are *Escherichia coli* or not (Sartory and Watkins, 1999).

Due to the importance of selectivity and/or sensitivity in these techniques, the MF technique has been improved by incorporating chromogenic or fluorogenic enzyme substrates into the media. Examples of such media include m-FC agar (Merck), mEndo (Merck) and CM1046 (Oxoid).

The MTF technique involves inoculation of a sample into tubes containing appropriate media in serially diluted tubes. There may be a need to add selective compounds for coliforms (Alonso *et al*., 1996; Sartory and Watkins, 1999). A total of 48 to 96 hours is required for the MFT technique. (Manafi 1998; Davies and Apte, 2000; Geissler *et al*., 2000; Ercole *et al*., 2002; Rompré *et al*., 2002; Leonard *et al*., 2003). The microbial numbers are presented as the most probable number based on the growth patterns at different dilutions.

The MTF method suffers from the same shortcomings as the MF method in terms of the long analyses time required. Improvements to eliminate confirmatory tests involve incorporation of fluorogenic and or chromogenic substrates for marker enzymes (see section 2.4.2). The use of these substrates illustrates an overlap of the culture-based and enzyme-based methods of detection, combining the two methods for improved methodological design. The most probable number (MPN) is a statistic technique used to

estimate concentrations using dilutions and an all-or-none response (Mubyana-John *et al.*, 2007).

The major disadvantage of this technique is the inability to detect viable but non culturable (VBNC) bacteria. Various reasons including nutrient stress, solar radiation, injury during water treatment and salinity may account for cells undergoing this phenomenon (Venter, 2000; George *et al*., 2002; Rompré *et al*., 2002; Leonard *et al*., 2003). Secondly, the culture-based techniques are labour intensive and time consuming. Also, they do not allow for the examination of large volumes of water. The MF and MTF techniques generally have high levels of selectivity for microbial contamination.

2.4.2 Enzyme based methods

These methods are based on the fact that each genera, species or strain of microorganism has enzymes that participate in unique biochemical pathways. These unique enzymes are referred to as marker enzymes and could be employed in the design of selective assays. β-D-galactosidase (B-GAL) is a marker enzyme for total coliforms and β-D-glucuronidase (GUD) for faecal coliforms, specifically *E. coli* (Sartory and Watkins, 1999; Rompré *et al*., 2002). B-GAL catalyses the breakdown of lactose-based substrates to galactose and glucose-based products (Davies and Apte, 1999; George *et al*., 2002).

B-GAL from *E. coli* is a tetrameric metalloenzyme which performs two reactions with its disaccharide substrate lactose and exhibits broad specificity. The hydrolysis reaction produces the monosaccharides galactose and glucose. The transglycosylation reaction produces the disaccharide allolactose, which acts to induce the production of more β-galactosidase. By this approach, *E. coli* both responds to and utilizes lactose as a food source. The specificity of the enzyme is confined to the sugar moiety and the anomeric character of the linkage but not to a particular aglycan. Synonyms include lactase, β-D-galactosidase galactohydrolase. It is a tetramer with a monomeric molecular weight of 116.4 kDa. Crystallographic studies show that each monomeric unit is constructed from five distinct domains organised around a central alpha/beta barrel. The active site is

shaped from residues of four of the domains into a pocket which complements the relatively small size of lactose.

The application of direct enzymatic detection of total coliforms by monitoring R-D-galactosidase activity has been demonstrated by several authors including Davies and Apte (1999) and George *et al*. (2002). George *et al*. (2002) employed enzymatic reactions to detect pollution within 25-30 minutes. GUD catalyses the hydrolysis of glucuronide based substrates to corresponding aglycan and D–glucuronic acid (Rompré, *et al*., 2002). Substrates made up of glucuronide and galactose linked to a chromogen or fluorochrome have been designed for the detection of these enzymes (Frampton and Restaino, 1993). The use of enzymatic reactions to detect coliforms provides a rapid, sensitive and less expensive alternative to the more traditional methods (George *et al*. (2002).

Enzyme substrates are added to the samples in question and incubated under optimum conditions for maximum enzyme activity. Colour change or fluorescence is then scored (Frampton and Restaino, 1993). This assay principle is based on the hydrolysis of artificial chromogenic or fluorogenic substrates, which are specifically split off by the enzyme under consideration. Chromogenic enzyme substrates are mainly phenolic. They are water soluble, heat stable, specific, show no diffusion (indolyl derivatives) and a wide range with different colours are available (Manafi, 1998). Examples of chromogenic substrates for B-GAL include *o*-nitrophenyl-R-D-galactopyranoside (ONPG), chlorophenol red R-galactopyranoside (CPRG), *p*-nitrophenyl-R-D-galactopyranoside (PNPG), 5-bromo-4-chloro-3-indolyl-R-D-galactopyranoside (X-Gal), 6-bromo-3-indolyl-R-D-galactopyranoside (Salmon-Gal), 2-(2-(4-(R-D-galactopyranosyloxy)-3-methoxyphenyl)-vinyl)-3-methylbenzothiazolium toluene-4-sulphonate (VBzTM-Gal), 2-(2-(4-(R-D-galactopyranosyloxy)-3-methoxyphenyl)-vinyl)-1-methylquinolilium iodide (VQM-Gal) among others (Manafi, 1998). 4-methylumbelliferone R-D-galactose, 4-methylumbelliferone R-D-heptanoate and 4-methylumbelliferyl R-D-galactopyranoside are

amongst the most common fluorogenic substrates employed in the assay of β-galactosidases.

Merck has introduced several substrate based products such as Colilert® and Colifast At-Line Monitor (CALM) on the market (Berger, 1994; Sartory and Watkins, 1999). CALM employs a fluorogenic substrate and has the ability to send continuous data to the laboratory from a remote location (Clark *et al*., 2002; State *et al*., 2004). These products, however, do require long incubation times ranging between 12 and 24 hours. Pre-enrichment through culturing may be required in some cases.

ROSCO tablets® also use the marker enzyme technique and require about four hours incubation to determine β-glucuronidase activity (Pérez *et al*., 1986).
A major disadvantage of the enzyme-based method is the high levels of microbial contamination, hence enzyme, required to give activity, which may not be present in the samples under consideration and also the unspecific nature of the enzyme source. In addition, the use of more sensitive fluorogenic substrates is prone to interference by inherent compounds such as chemicals, cations and anions which may be present in the water samples. This has limited most of these techniques to laboratories for use on pure water samples.

Coliform enzyme activity has been determined by different methods, by (i) either measuring the activity in the water sample itself by addition of the respective substrates or (ii) collecting the cells by filtration, followed by respective enzyme assays with the resuspended cells (Fiksdal *et al*., 1994; George *et al*., 2000). Either of these approaches will affect the results, since the enzymatic reaction conditions and sample manipulations will vary in each case. It is however, expected that the former situation will yield results close to what pertains in the environments, thus being more representative.

2.4.3 Molecular based methods

As with direct enzyme assays, molecular–based methods are aimed at reducing analysis time through the elimination of the time-consuming culturing/growth stage.

2.4.3.1 Immuno–based techniques

These techniques entail using antibody–antigen interactions such as those in enzyme linked immunosorbent assay (ELISA), immunofluorescent assay (IFA) and immunoblotting (IB). Genera–and species– specific proteins and protein–based structures (e.g. cell receptor surfaces) are the basis of the selective detection of indicator organisms. Antibodies specific to such structures can be conjugated to enzymes such as horseradish peroxidase, alkaline phosphatase, urease and B-GAL (Crowther, 2001) to catalyse the breakdown of chromogenic or fluorogenic substrates. Enzyme labelled antibodies bind to antigens so strongly that they remain on the support, while the unbound molecules are washed off. This is then followed by the addition of the chromogenic/fluorogenic substrate to the experimental sample (Crowther, 2001) and the presence of colour or fluorescence then signifies the presence of the microorganism in question.

Hübner *et al*. (1992), Levasseur *et al*. (1992) and Fisher and Woods (2000) reported the use of enterobacterial common antigen (ECA) to detect coliform and *E. coli.* A correlation of 98 % between the ECA monoclonal antibodies and the MTF technique was shown (Hübner *et al*., 1992), which suggested that this could serve as a good alternative technique to MF and MTF for the detection of indicator organisms.

Even though immunological techniques are rapid, sensitive and selective (Venter, 2000; Leonard *et al*., 2003; Taguchi *et al*., 2005), cross-reactivity can lead to false positive results. Background interference when the microbial population is below the threshold level of the limit of detection (LOD) may also be a disadvantage. A microbial population at least one percent of the total microbial load is required for minimum background interference (Mansfield and Forsythe, 2000; Rompré *et al*., 2002). This method is also expensive and requires skilled personnel.

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

While immunoassay techniques can detect VBNC organisms, they do not provide information about the physiological state of cells (Rompré *et al*., 2002; Leonard *et al*., 2003). Immuno-based techniques have the advantage of being able to determine the actual toxin of interest over both the culturing or genetic analysis methods (Iqbal *et al*., 1997, 2000; Leonard *et al*., 2003).

2.4.3.2 Nucleic acid (NA) – based techniques

Nucleic acid based methods comprise of NA hybridisation, polymerase chain reaction (PCR) and the use of genetically modified bacteriophages (De Boer and Beumer, 1999; Venter, 2000; Rompré *et al*., 2002).

Gene sequences coding for genera– or species– specific gene products are targeted using nucleic acid probes in this technique. DNA sequence labelled with a radioactive (^{32}P), fluorometric or enzymatic component could be used as the probe (Keller and Manak, 1989; Fricker and Fricker, 1994; Joux and Lebaron, 2000; Köhler *et al*., 2000).

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

Target DNA occurring in low concentrations has to be amplified using the polymerase chain reaction (PCR) technique (Rompré *et al*., 2002) or the cells are concentrated using filtration (Bej *et al*., 1991a, 1991b). Primers flanking the target sequence are used for selective amplification after which the product is electrophoresed and/or membrane bound and hybridised (Keller and Manak, 1989). Nucleic acid methods are very sensitive and capable of detecting genes from VBNC bacteria. However, false positive/negative results have been reported using these methods.

The PCR technique can be inhibited due to environmental contaminants (Bej *et al*., 1990; Rompré *et al*., 2002; Leonard *et al*., 2003). Under such circumstances, the sample needs to be purified. Sample purification and contaminant removal will translate to prolonged assay times, the need for skilled labour and increased costs, all of which are undesirable for large routine sample processing. Comparing the conventional nested PCR with nested hex-PCR for the detection of human adenoviruses (HAds) in water samples in South Africa, Van Heerden *et al* (2005) reported a more sensitive first round hex-PCR than the conventional PCR. Methods based on real-time polymerase chain reaction (RT-PCR), conventional nested PCR and DNA microarray technology offer a format for analyzing multiple pathogen species in a single assay (Pommepuy and Le Guyader, 1998; Toze, 1990; Lucchini *et al.*, 2001; Van Heerden *et al.*, 2005).

Although NA techniques are sensitive, they can neither be used for microbial quantification nor for the determination of the microbial physiological state (Leonard *et al*., 2003) since the presence of genetic material does not necessarily imply the presence of the microbes or toxins (Tryland and Fiksdal, 1998; Taguchi *et al*., 2005). Heid *et al.* (1996) reported that the NA methods cannot be used for real-time monitoring in the environment.

In general, molecular based methods are expensive and require skilled manpower (Ivnitski, *et al*., 1999; Venter, 2000; Rompré *et al*., 2002; Leonard *et al*., 2003).

2.4.3.3 Chemical targets

Faecal sterols - biochemical tracers such as sterols offer a potentially viable approach for distinguishing between faecal samples from human and animal sources. Faecal materials contain sterols and the breakdown product, stanol. In the gastrointestinal tract (GIT), cholesterol is degraded to coprostanol in humans and epicoprostanol in animals. Plant-derived 24-ethylcholesterol is reduced in the gut of herbivores to 24-ethylcoprostanol and 24-ethylepicoprostanol, and reduced in the environment to 24-ethylcholesterol (Leeming *et al*., 1996). Coprostanol appears to be the main stanol in human sewage where its concentration is much higher than in animals. Isobe *et al*. (2004) reported a good relationship between levels of *E. coli* and coprostanol concentrations in tropical regions (R^2 varying from 0.81 and 0.92), but this result was affected by temperature. Temperature variation could affect *E. coli* activity and/or mechanism of action of the coprostanol. These compounds, however, are known to persist in the environment, thus making it difficult to gain information about recent faecal contamination (Pitt, 2001; Isobe *et al*., 2002). The measurement of microbial and chemical indicators in three rivers from New Zealand showed that the ratios of faecal sterols to stanols (coprostanol:24-ethylcoprostanol and coprostanol:epicoprostanol) increased downstream from the faecal pollution sources, thus indicating human pollution (Gilpin *et al*., 2002). Noble *et al*. (2004) employed faecal sterols to trace the faecal contamination of the surf zone at Huntington Beach to bird faecal input and not to sewage input.

Bile acids - bile acids such as deoxycholic and lithocholic acids are associated with human faecal contamination. They are more resistant to degradation than coprostanol and can help in distinguishing between human and animal contamination sources (Elhmmali *et al*., 1997, 2000).

Caffeine - Caffeine is found in several beverages such as coffee, tea and soft drinks, and was proposed as a suitable indicator of human sewage pollution. A significant correlation was found between watershed scale land-use and the presence of caffeine and consumer

product fragrance materials in wastewater treatment plant effluents (Standley *et al*., 2000).

Fluorescent whitening agents and other chemicals - wastewater from human sources often contains fluorescent whitening agents (FWA), which are included in detergents and washing powders. They have been used to indicate contamination from septic tanks or gray water discharge (Close *et al*., 1989). Sodium tripolyphosphate and linear alkylbenzene sulphonates (LAS) have also been under consideration for source tracking (Sinton *et al*., 1996, 1998). FWA are detected by HPLC with fluorescence detection (Kramer *et al*., 1996; Poinger *et al*., 1996). FWA adsorb to particles and are not readily biodegradable in aquatic environments (Poinger *et al*., 1998).

Boron, a major ingredient of laundry detergents, was proposed as a tracer of sewage pollution. However, boron may not be suitable due to changes in formulations in modern laundry detergents (Pitt, 2001).

Pharmaceuticals such as aspirin and ibuprofen (non-steroidal anti-inflammatory agent), and clofibric acid (from cholesterol lowering drugs) can also help identify sewage contamination (Pitt, 2001).

Biomarkers like bile acids, sterols and aminoacetone offer an added advantage of providing information about the actual origin of the faecal material. The major disadvantage with the use of these markers is that they 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), thus making them undesirable for routine use.

Real-time monitoring is a key factor for consideration for any current method development for indicator organism assessment for early warning. However, all of the previously discussed techniques cannot afford real-time monitoring. To address this,

research has focused on biosensor development for indicator organisms in order to achieve real-time monitoring methods (Leonard *et al*., 2003).

2.5 BIOSENSOR DEVELOPMENTS

The need for rapid, real-time, on-site and potentially on-line monitoring methods to avoid retrospect results and effectively implement early warning systems has resulted in many attempts to develop biosensors for indicator microorganisms (Leonard *et al*., 2003; Paitan *et al*., 2003). Biosensor detection entails the conversion of a signal (e.g. from previously discussed methods) to an electrical output. Signals can be optical, mass or electrochemical. A biosensor is made up of a bioreceptor surface, transducer and output display unit (Figure 2.2) (Scouten *et al*., 1995; Thévenot *et al*., 2001; Leonard *et al*., 2003; Purohit, 2003).

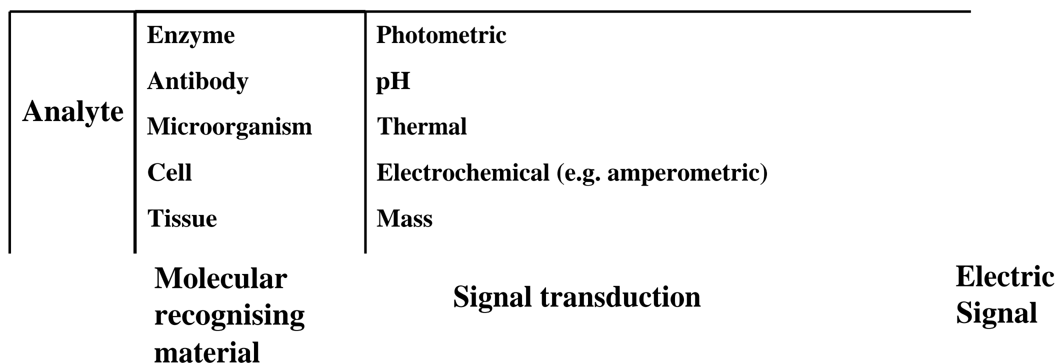


Figure 2.2: Schematic presentation of biosensor components

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

modes of signal transduction exist and can be used for biosensor classification. These can be electrochemical, mass, thermal or optical (Ivnitski *et al*., 1999; Leonard *et al*., 2003). Electrochemical transducers, especially amperometric ones, are the most preferred due to the advantages they offer. They are cheap and not subject to interference by turbid solutions (Scouten *et al*., 1995).

To the end user, a biosensor should preferably be an instrument where a probe is dipped into the sample, a button is pushed and a reading is obtained. However, to the developer, the process of developing such a device requires selection of an appropriate receptor surface, an appropriate method of signal detection and transduction, display format, definition of result range and optimisation of the function of the equipment.
Selective detection by biosensors is one of the reasons why they are preferred. They also produce results rapidly and can be used in real-time and on-line monitoring (Ivnitski *et al*., 1999; Leonard *et al*., 2003). When fully optimised, they are easy to use and do not need skilled manpower. Automation of sensors to capture and transmit data to a research centre or laboratory will provide an ideal situation but this is hindered by the cost of automation, software, engineering and hardware requirements. As a result, disposable on-site analysis using disposable screen-printed electrodes for once of testing and data capture have become more popular (Tymecki *et al*., 2005).

Several researchers have developed biosensors for *E. coli* and coliforms in laboratories. However, employing these biosensors in the field is met with a variety of challenges which include a small market to offset the development costs, low sensitivity when compared to currently existing methods, failure to meet requirements after rigorous tests in different environments, narrow scope of environmental applicability and the bureaucratic nature of legislation for them to be approved (Rogers, 1995, Kissinger, 2005). Another limiting factor is the time and sensitivity of these sensors.

Pyun *et al*. (1998) developed an *E. coli* biosensor using a flexural plate wave transducer. The minimum LOD was above 10^3 colony forming units (CFU) ml^{-1} although the time of analysis was less than an hour. If this is to be used for the purpose of monitoring

drinking water it will give false negatives since the generally maximum acceptable CFU 100 ml^{-1} of drinking water is 1.

Pérez *et al*. (2001) developed an amperometric culture-based biosensor for *E. coli* detection using the B-GAL substrate 4-aminophenyl-β-D-galactopyranoside (4APG). The substrate was broken down to 4-aminophenyl (4AP) which was then determined electrochemically. Assay times 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 total coliform group (Stevens *et al*., 2001) and can therefore be used for the detection of total coliforms. Nistor *et al*. (2002) amplified the 4AP signal 4.5 fold using cellobiose dehydrogenase and detected more than 10^4 CFU 100 ml^{-1} in less than an hour. Reliance on a pre-culturing step is not desirable for the minimisation of the time required for analysis. Since the use of this biosensor involves a culturing stage, VBNC organisms may not also be detected.

Ercole *et al*. (2002) developed a biosensor based on a potentiometric (electrochemical) transducer for *E. coli,* but used it on vegetable samples. The LOD was 10 CFU ml^{-1} and the minimum detection time was about 1.5 hours (Ercole *et al*., 2003). This LOD is not sensitive enough for the purpose of monitoring drinking water where no single CFU is expected in 100 ml of water. The use of such a biosensor for adequate monitoring of water samples would leave the population potentially vulnerable.

2.5.1 Electrode modification

In the preparation of an electrochemical biosensor, one of the most important steps is to modify the working electrode. Electrode modification may result in minimisation of interaction, lowering of overpotential, increase in electron transfer kinetics, preferential deposition of analytes onto surface functionalities and improvements in stability, sensitivity and selectivity, which are all advantages in biosensor development (Cullen *et al.,* 1990). Hence, modification of electrodes adds a powerful new dimension to biosensor development.

In general, there are four techniques, namely: physical entrapment, crosslinking, covalent bonding and physical adsorption.

Physical entrapment: This technique can either involve entrapment of the recognition molecule in a gel, a polymeric network, or behind a semi-permeable membrane (Schmidt *et al.*, 1992). It is expected that the pores of the polymeric network are large enough to allow free diffusion of substrates and products, but should retain the enzyme within the network (Palmisano *et al.*, 2000). The major obstacle of this method lies in the difficulty in controlling the pore sizes.

Crosslinking: This technique is often used in conjunction with adsorption or entrapment. Crosslinking of enzymes using intermolecular covalent linkages can increase sensor stability and improve electron transfer. Bifunctional crosslinking agents such as glutaraldehyde, dialdehyde, poly(ethylene glycol) ether and diisothiocyanates have been used in various applications (Schmidt *et al.*, 1992; Larsson, 2001).

Covalent coupling: This technique leads to strong binding between the immobilised substance and the working electrode surface. Primary reactive groups on both the enzyme and support material are required for binding. Functional groups such as hydroxyl, carboxyl, thiol and amino moieties can be treated with suitable reagents to allow binding with amine, thiol and hydroxyl groups present in the amino side chains of enzymes (Turner *et al*., 1987; Schmidt *et al.*, 1992).

Physical adsorption: This is the most commonly used technique, in which no reagents are required and only a minimal activation may be necessary (Schmidt *et al.*, 1992) The interactions are mainly due to hydrogen bonding, hydrophobic and van-der-Waals interactions, or electrostatic adhesion (Schmidt *et al.*, 1992). Conformational changes, denaturation of enzymes, desorption due to pH changes, ionic strength and temperature may however, be major challenges.

2.5.2 Detection or measurement modes

There are several electrochemical methods for detecting analytes of interest including surface charge using field effect transistors (FETs), conductometry, potentiometry, amperometry and voltammetry (Thevénot *et al.*, 1999, 2001; Hu *et al*., 2001), the last two being of importance in this study.

2.5.2.1 Amperometry

This technique is based on the measurement of the current resulting from the electrochemical oxidation or reduction of an electroactive species. Amperometry is performed by maintaining a constant potential at a platinum, gold or carbon based working electrode or an array of electrodes with respect to a reference electrode, which may also serve as the auxiliary (counter) electrode, if currents are low (from 10^{-9} to 10^{-6} A). The resulting current is directly correlated to the bulk concentration of the electroactive species or its production or consumption rate within the adjacent biocatalytic layer. As biocatalytic reactions rates are often chosen to be first order dependent on the bulk analyte concentration, such steady-state currents are usually proportional to the bulk analyte concentration (Thevénot *et al.*, 2001).

2.5.2.2 Voltammetry

Voltammetry is one of the most commonly used techniques in electrochemistry and consists of a group of methods that measure current as a function of applied voltage/potential at a working electrode surface (Wang, 1994; Skoog *et al*., 1996; Wang *et al.,* 2004a, 2004b). A counter electrode conducts electricity from the source through the solution to the working electrode, while the reference electrode has a constant potential throughout the experiment (Skoog *et al*., 1996).

Classification of voltammetric techniques is based on the mode of potential application. Examples of these classes include linear sweep, stripping and cyclic voltammetry (Wang,

1994; Skoog *et al*., 1996; Hu *et al*., 2001; Wang *et al.,* 2004a, 2004b). In cyclic voltammetry, the potential is changed as a linear function of time from start to final potential and then reversed to the start potential (Skoog *et al*., 1996). The current generated depends on the number of electroactive groups on the molecule and is directly proportional to the concentration of the analyte. Each electroactive species has a characteristic potential where it produces a maximum current. This technique is thus both quantitative and qualitative.

2.5.3 Phenolic detection by electrochemical methods

Electrochemical analysis has been successfully employed in the detection and/or monitoring of some phenolic compounds in water (Clement *et al*., 1995; Mafatle and Nyokong, 1997; Lei *et al*., 2003; Mulchandani *et al*., 2005). According to Mafatle and Nyokong (1997), Niwa *et al*. (1990, 1991), Marko-Varga *et al*. (1995), Lindgren *et al.* (1999), Luz *et al*. (2004), Gomes *et al.* (2004) and Mena *et al*. (2005), most phenolic compounds are readily oxidised at accessible potentials.

In general, electrooxidation of phenolic compounds results in the formation of radical intermediates which dimerise or polymerise at electrode surfaces. These oxidation products are problematic as they lead to the passivation of the electrode (Ežerskis and Jusys, 2001; Ureta-Zañartu *et al.*, 2003). The main disadvantage then of the use of voltammetry for the determination of phenolic compounds is the fouling of the electrode by oxidation products (Mafatle and Nyokong, 1997; Abdollah *et al.*, 2000; Ežerskis and Jusys, 2001). Fouling causes a reduction in the detection of the phenolic compounds on the second and subsequent scans. Many researchers have attempted to find solutions to this fouling problem (Manafi, 2000). Among the several methods used, include modification of the electrode surface with metallophthalocyanine (MPc) complexes and laccase enzymes.

Increased sensitivity in the detection of phenols and lowering of the oxidation potential as well as a lowering of the fouling of the electrode can be obtained through the use of

catalysts such as metallophthalocyanine (MPc) complexes immobilized at glassy carbon electrodes (Mafatle and Nyokong, 1997) as well as through the use of enzymes such as laccase which have a broad catalytic activity towards phenols.

2.5.4 Chemical modification

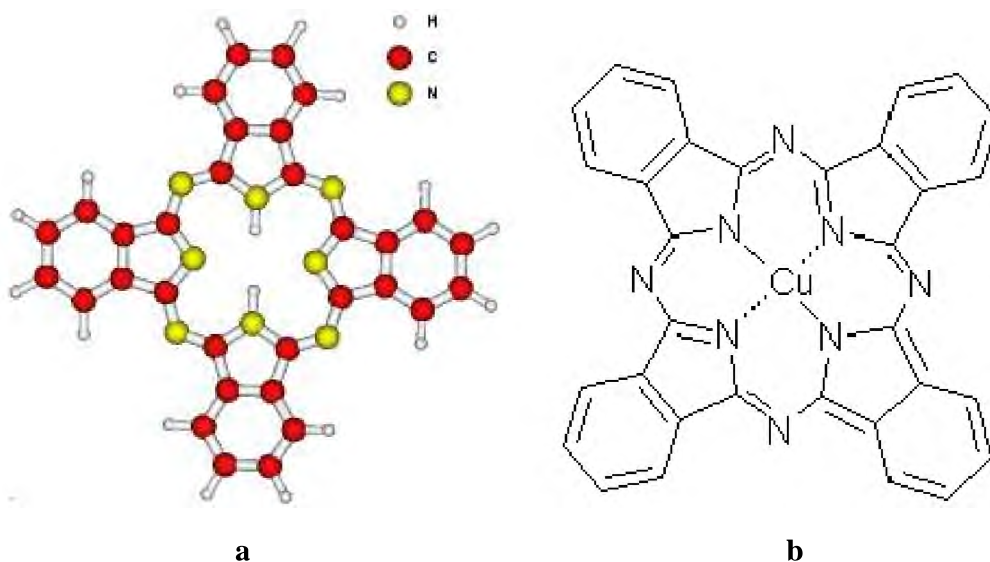
The use of chemically modified electrodes with transition metal complexes provides a strategy for improving the performance of solid electrodes by incorporating catalytic sites at the electrode/solution interface. Metalloporphyrins, metallophthalocyanines (MPc), metal Schiff bases and metal tetraazamacrocyclic complexes, amongst others, have been immobilised on different electrode surfaces and show catalytic activity for a great variety of electrodic reactions (Mafatle and Nyokong, 1997; Ureta-Zañartu *et al.*, 2003).

The fundamental constitution of phthalocyanines are similar to chlorophyll and the heme group of hemoglobin which exist in the natural world, and consists of very large conjugated double bonds of tetrabenzotetraazaporphine constitution (Figure 2.3). They are composed of a ring system made up of four isoindole units, at the center of which various metals can be inserted. They are stable to acid and alkali, and heat resistant. There are various crystal forms in the same chemical structure and each one has different characteristics towards light and electrons. These characteristics also vary depending on the metal in the center of the molecule. The catalytic activity of MPc complexes resides in the electroactive central metal centre. It has been reported by several authors that MPc complexes that contain electroactive central metals such as copper and iron show higher catalytic activity than those MPc complexes with ring-based redox processes such as nickel and zinc. Because of their intense colour, redox activity and high thermal stability, MPcs have found many applications in photoconductive surfaces, optical information storage media and electrochromic devices. In all these devices, the redox properties of the MPcs define their utility (Mho *et al.*, 1995; Mafatle and Nyokong, 1997, Limson, 1998; Jiang and Kucernak (2000, 2001a, 2001b; Caro *et al*., 2003).

2.5.5 Biological modification

Enzyme based biosensors are sensors based on enzymatic biorecognition elements. The consumption of a substrate or the generation of a product can be used as a measure of the enzyme activity. Major categories of enzyme assay techniques include photometric, radiometric, chromatographic, electrophoretic and electrochemical methods (Janata, 1990; Bier, 1998).

The design of enzyme electrodes is such that the current or potential measured is proportional to the rate limiting step in the overall reaction. For reactions described by Michaelis-Menten kinetics, a leveling off of calibration curves is expected at high substrate concentrations. The signal may also be dependent on the pH of the water sample or its heavy-metal content. The need to modify enzyme-based biosensors to reach lower detection limits is very important (Wollenberger *et al.*, 1994; Xie *et al.*, 2005).



a **b**

Figure 2.3: Backbone structure of metal free phthalocyanine (a) and chemical structure of copper phthalocyanine (b).

Among the 19 congener chlorophenols, 2-chlorophenol; 2, 4-dichlorophenol; 2, 4, 6-trichlorophenol and pentachloride are listed in the Priority Pollutant List of the US Environmental Protection Agency (USEPA). Electrocatalytic methods of assaying chlorophenols using enzymatic reactions have been employed by several researchers due to their high selectivity and sensitivity. Direct electrocatalytic methods using laccase have been reported for the determination of phenolic compounds which can easily be oxidiszed, such as catechol, hydroquinone, p-cresol, 4-chlorophenol, and 4-amino-4-chlorophenol. (Yaropolov *et al*., 1994; Zhao and Luong, 1996; Gianfreda *et al.,* 1999; Maheshwari *et al*., (2000); Fernández-Sánchez *et al*., 2002; Shleev *et al*. 2005). Specificity of laccase is however rather poor and many phenolic and non-phenolic compounds can be detected (Jarosz-Wilkolazka *et al.*, 2002, 2004).

CHAPTER THREE

CHROMOGENIC AND FLUOROGENIC SUBSTRATES STUDIES

Conscience never deceives us; she is the true guide of man, it is to the soul what instinct is to the body; he who obeys his conscience is following nature and he need not fear that he will go astray - Jean-Jacques Rousseau

3.1 INTRODUCTION

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

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

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

Temperature and pH play important roles in enzyme activities. pH affects the structural stability and solubility of, as well as the charge and charge distribution on both enzymes and substrates (Holme and Peck, 1998). These factors; in turn, alter bonds and bonding patterns, ultimately determining the rate of enzyme-catalysed reaction.

Temperature influences the rate of molecular collision and bond vibrations. Increasing temperature (usually up to 40 ^{o}C) increases the number of collisions between substrates and enzymes, thus increasing the reaction rate.

An increase in temperature may also result in increased bond vibrations which could lead to bond disruptions, hence enhancing substrate breakdown. The bond disruption may, however, denature the enzymes under consideration. As a result, optimum temperature will be a compromise between maximum activity and enzyme denaturation (Holme and Peck, 1998). Each enzyme has a characteristic pH and temperature optimum.

Enzyme and substrate concentrations, pH and temperature affect assay times. In order to accurately determine enzyme activity, a linear range of maximum product accumulation from progress curves of product formation or substrate consumption versus time is required. Tipton (2002) reported that different enzymes and those from different environments have varying shapes of progress curves. It is thus important to determine the characteristic shape of a progress curve of the enzyme in question.

It is necessary to determine the location of the enzyme in question in order to design an accurate protocol. A number of techniques can be employed to ascertain the location of these enzymes. In aquatic environments, enzymes can be free floating or bound to particulate matter, intracellular or extracellular (Chr6st, 1991). Particulate bound and free-floating enzymes can be separated through filtration (Chr6st, 1991; Farnleitner *et al*., 2002) or centrifugation. The relative cellular location of enzymes can be determined by chemical and mechanical cell permeabilisation methods (Chr6st, 1991; Roe, 2001).

Photometric enzyme assays include measuring turbidity, absorbance or fluorescence. Measurement of absorbed or transmitted light in enzyme assays mainly involves coloured compounds in the visible light range (400–750 nm). Although this technique is inexpensive and easy to execute, it has the disadvantage of lacking sensitivity at low compound concentrations (John, 2002). Turbidity measurement is based on the light

scattering ability of a solution and is employed when the enzyme substrates are insoluble polymers (John, 2002).

Fluorescence is based on the fact that some compounds emit light energy when returning to the ground state after excitation. Different compounds are excited and emit energy at distinct wavelengths (Gordon, 2001; John, 2002). Although fluorimetric assays are very sensitive, they are more expensive than absorbance spectroscopic methods (John, 2002) and easily affected by various compounds.

3.2 MATERIALS AND METHODS

3.2.1 Collection site

The Bloukrans river, which flows through the City of Grahamstown in the Eastern Cape Province of South Africa, was selected for *in situ* assays. This selection of this river was based on the fact that high coliform levels had been detected previously by colleagues from the Department. Three sampling points were initially selected as examples of 1) stagnant/standing water, 2) moderately flowing water (called running water) and 3) fast flowing water (Appendices C1, C2 and C3).

Chlorophenol red β-D-galactopyranoside (CPRG), chlorophenol red (CPR), disodium hydrogen phosphate, dihydrogen sodium phosphate, sodium hydroxide, potassium chloride, sodium sulphate, sodium carbonate, sodium chloride, calcium chloride, sodium acetate, Triton X-100, tris(hydroxymethyl)aminomethane, glacial acetic acid, sodium bicarbonate, sulphuric acid were all supplied by Merck (Darmstadt, Germany) while 4-methylumbelliferyl-β-D-galactoside (MuGAL), β-D-galactosidase (EC 3.2.1.23) (B-GAL) and toluene were supplied by Sigma-Aldrich (Munich, Germany), UV spectrophotometer by Shimadzu (UK) and pH Meter (high-precision 780) supplied by (Metrohm, South Africa (Pty) Ltd).

3.2.2 Sampling

Water samples were collected in triplicates in accordance with the standard procedures outlined in Standard Methods (1998). Briefly, water samples were collected aseptically in 250 ml sterile Pyrex glass bottles (Schott Duran, Germany). These were placed on ice and transported immediately to the laboratory for analysis within an hour. The temperature was measured on site and the pH determined upon arrival in the laboratory. All samples were collected in triplicate between 08:00 and 08:30 on each sampling day.

In all the environmental assays performed (for both fluorogenic and chromogenic assays), two sets of controls were set up; an enzyme control (containing sample and substrate only) and a substrate control (containing substrate and buffer only). These controls were used to assess the effects of inherent compounds which may have an effect on the reaction components. All readings were taken against distilled water. The control that gave the 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 and unless otherwise stated, all statistical analyses were performed with analysis of variance (ANOVA) using Microsoft Excel 2003 statistical tool at 5 % level of significance.

3.2.3 Fluorogenic substrate

3.2.3.1 B-GAL Enzyme Assay

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

3.2.3.2 Volume optimisation

In order to optimise the sample volume, varying volumes of 1, 3, 5 and 6 ml were reacted with the substrate and buffer (as described in *3.2.3.1*). Buffer was used to adjust the volumes so as to maintain constant final reaction volume.

3.2.3.3 Effect of pH

The above assay procedures and selected volumes were used in an investigation to assess the effect of pH on the hydrolysis rates (of river water B-GAL) on the selected fluorogenic substrate. The buffer systems employed are as outlined in *3.2.4.4*. Controls to account for the spontaneous chemical degradation of the MuGAL substrate at the different pH values were run accordingly.

3.2.3.4 UV observations

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

3.2.4 Chromogenic substrate

3.2.4.1 B-GAL Enzyme Assay

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

3.2.4.2 Linearity over time

Enzymes in river water samples are expected to occur in low concentrations due to the large levels of dilution, and to behave differently from the pure and commercially available counterparts. It was thus necessary to determine the best time for monitoring the assay reaction based on the linear range of the reaction. A modified CPRG assay with was employed (*3.2.4.1*). The pure commercial enzyme was substituted with the environmental/river water sample and absorbance readings taken at 15 min intervals for the first three hours and then subsequently at 2 h intervals for the remaining 46 h (49 h in total). From this data, the optimal reading time for detecting the absorbance values in the linear section was deduced.

3.2.4.3 Volume optimisation

Volumes of the water samples were varied between 20 µl and 90 µl, with corresponding adjustments in the buffer volumes to maintain a constant total end volume of 200 µl (including 20 µl of substrate). Assays were performed at room temperature (20 ± 2 ^{o}C). The volume of water sample that gave reproducible results and showed the highest activity was used in subsequent assays.

3.2.4.4 pH optimisation

B-GAL activity was assayed at various pH values (5 – 11) using the following buffer systems: pH 5.0-6.0, sodium acetate; pH 6.0-8.0, sodium phosphate; pH 7.0-9.0, Tris-HCl and pH 9.0-11.0, carbonate-bicarbonate. All buffers in the assay solution had a final concentration of 0.1 M. "Bracketing" of buffers at border pH values was employed to account for changes in enzyme activity due to the various buffer components.

3.2.4.5 Temperature optimisation

B-GAL assays in river water samples were performed at temperatures of 4, 12, 20, 25, 30, 35, 40, 45, 50, 55 and 60 ^{o}C. The optimum volume, temperature and pH as obtained from the above sections were employed in all subsequent assays.

3.2.4.6 Effect of environmental water samples on B-GAL activity

In order to establish the effect of environmental water samples on the assay due to the presence of inherent materials such as metal ions, different volumes (0-50% of the total assay volume) of the water samples were pre-incubated with the commercial B-GAL for approximately 30 min, after which residual enzyme activity was determined. In order to maintain constant total assay volumes, the volumes of the buffer were adjusted appropriately.

3.2.4.7 Fractionation studies

In order to determine the relative location of B-GAL, fractionation studies were performed on river water samples and effluent from the Rhodes University Environmental Biotechnology Research Unit (EBRU)'s facultative pond.

The two sets of samples were centrifuged at 6 000 g at 4 ^{o}C for 10 min to pellet the cells and particulate matter. The supernatant (labelled supernatant 1) was separated from the pellet (labelled pellet 1) and retained for sonication and enzyme assay. The pellet was then washed in distilled water and centrifuged. The pellet wash was also retained for enzyme assay. The washed pellet (labelled pellet 2) was re-suspended in the corresponding assay buffers for B-GAL. For each supernatant 1 and pellet 1 samples, 1 ml aliquots were sonicated for two minutes [(at 25 W (RMS), 4 ^{o}C, 30 seconds sonication period with 15 seconds resting intervals, on ice)] using a Vibra CellTM Sonicator (Sonics & Materials, USA).

B-GAL activities in the six fractions (whole fraction, supernatant 1, pellet wash, pellet 1, sonicated supernatant and sonicated pellet) were subsequently determined.

3.2.4.8 Permeabilisation studies

In order to investigate the effects of different cell permeabilisation methods on B-GAL activity, five different methods of cell permeabilisation (below) were employed. Stagnant/standing and moderately flowing (running) water samples were analysed.

3.2.4.8.1 Sodium dodecyl sulphate (SDS)

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

3.2.4.8.2 Sonication

One milliliter of each sample was sonicated as described in *3.2.4.7* **and kept on ice before assaying for enzyme activity.**

3.2.4.8.3 Triton - X100

To 1 000 µl of sample, 250 µl of 2.5% (v/v) Triton–X 100 was added to make a final concentration of 0.5% (v/v) Triton–X100. This mixture was briefly vortexed and left at room temperature (20 ± 2 ^{o}C) for 45 minutes (Roe, 2001) before performing the enzyme assay.

3.2.4.8.4 Toluene

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

3.2.4.8.5 Polymyxin B (PxB) sulphate

This method was adopted from Van Poucke and Nelis (1997). A final concentration of 100 µg ml^{-1} PxB sulphate (ICN Biomedicals, Ohio) in the water sample was used. The

mixture was briefly vortexed and incubated for 45 min at 37 °C before enzyme activity assays were performed.

3.3 RESULTS AND DISCUSSION

3.3.1 Fluorogenic substrate

3.3.1.1 UV observation

It was observed that both the enzyme reaction mixture and the uncatalysed substrate fluoresced under UV illumination. No visual distinction could be made between their fluorescence intensities. Fluorescence of the substrate under UV could be a result of high substrate concentration. However, a reduction in substrate concentration could potentially reduce the rate of the enzyme reaction. According to Holme and Peck (1998), when assaying enzymes *in situ*, the substrate concentration must be at least ten times the K_m to get 91 % of the maximum enzyme activity.

A lack of reproducibility with the environmental water samples was observed with the fluorogenic substrate. These high values of error suggested an unreliability of the technique due to interference by a wide variety of environmental compounds (Figure 3.1). Davies and Apte (1999) suggested that non-target bacteria, other cell-free enzymes and cell-free substances are potential contributory interfering agents.

Although the fluorogenic assay was very sensitive, interferences from various compounds and chemicals make it less desirable for application on environment samples. Also, the adjustment of pH to alkaline conditions in this assay introduces more labour and increase in cost. In addition, fluorogenic substrates are more expensive than chromogenic ones.

3.3.1.2 Volume optimisation

As expected, there was generally a direct relationship between B-GAL activity and the volume of water samples (1 – 6 ml) added (Figure 3.1). However, the standard deviations observed at higher water sample volumes were quite large.

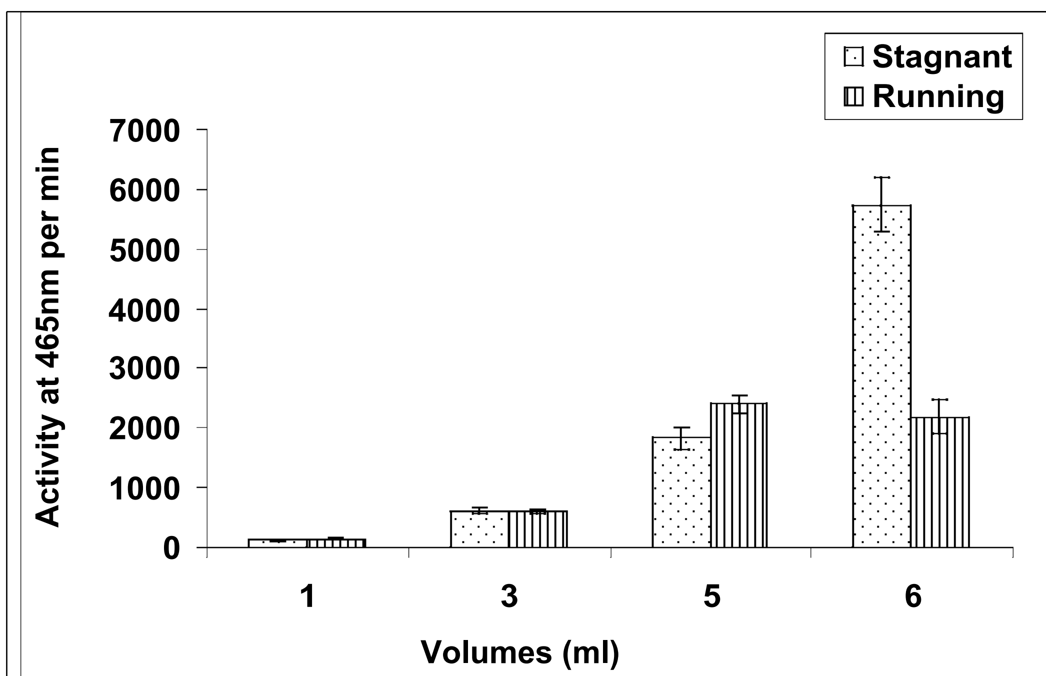


Figure 3.1: Effects of changes in river water sample volume on MuGAL hydrolysis. All values represent the means ± SD (n=3).

3.3.1.3 pH optimisation

Enzyme activity fluctuated with changes in pH. The highest B-GAL activity was recorded at pH 7.2 (Figure 3.2). Increases in enzyme activity were mostly accompanied by concomitant increases in standard deviation (SD). Several samples were analysed in an attempt to reduce the value of SD for the B-GAL assay, but this was unsuccessful.

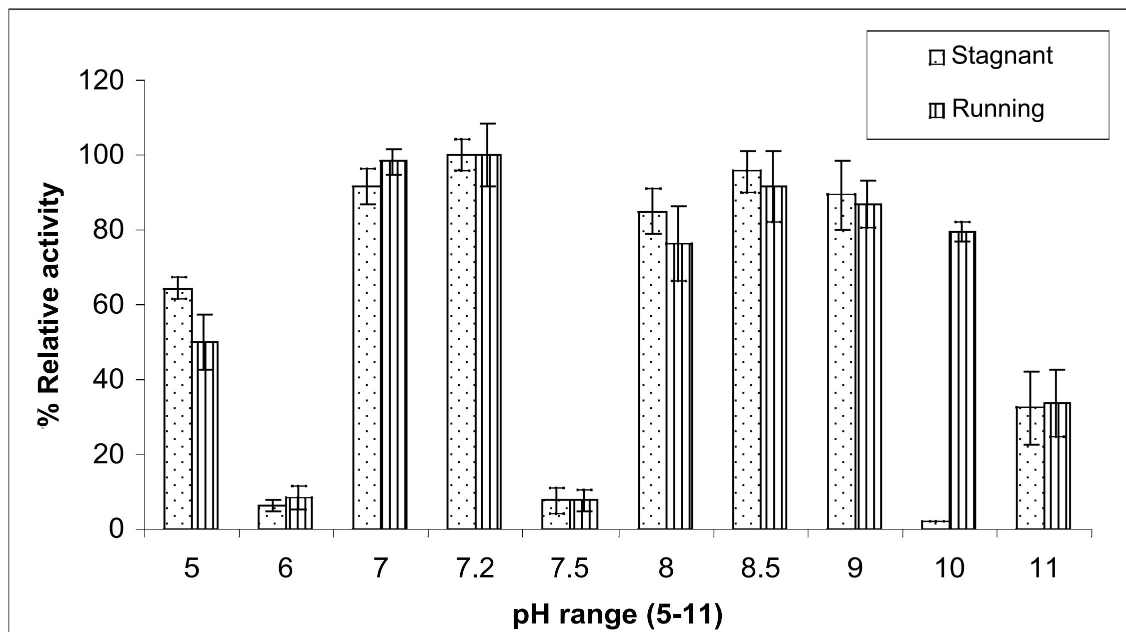


Figure 3.2: Effects of pH on B-GAL activity on MuGAL. All values represent the means ± SD (n=3).

3.3.2 Chromogenic substrate studies

Figure 3.3 shows a microtitre plate result for a typical spectrophotometric B-GAL assay. CPR and CPRG are monitored at different wavelengths (Appendix B2).

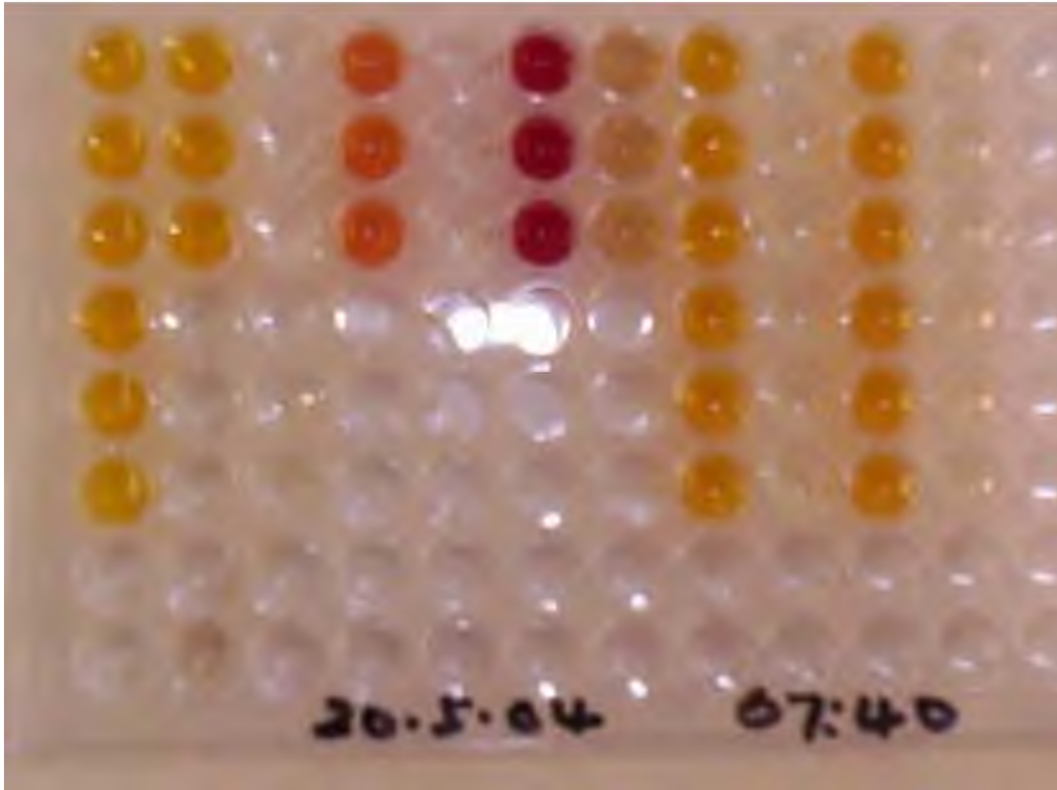


Figure 3.3: Micro titre plate showing a typical assay result; CPRG (yellow), CPR (red), partially-hydrolysed CPRG (light red), controls (colourless).

3.3.2.1 Linearity over time

As observed from Figure 3.4, the most linear part of the graph was between 15 and 29 h. This region also gave the minimum standard deviations. For this reason, 24 h was selected as an appropriate time for further assays. It must be stated, however, that depending on the level of contamination, the assay times could in fact have been either be shorter or longer.

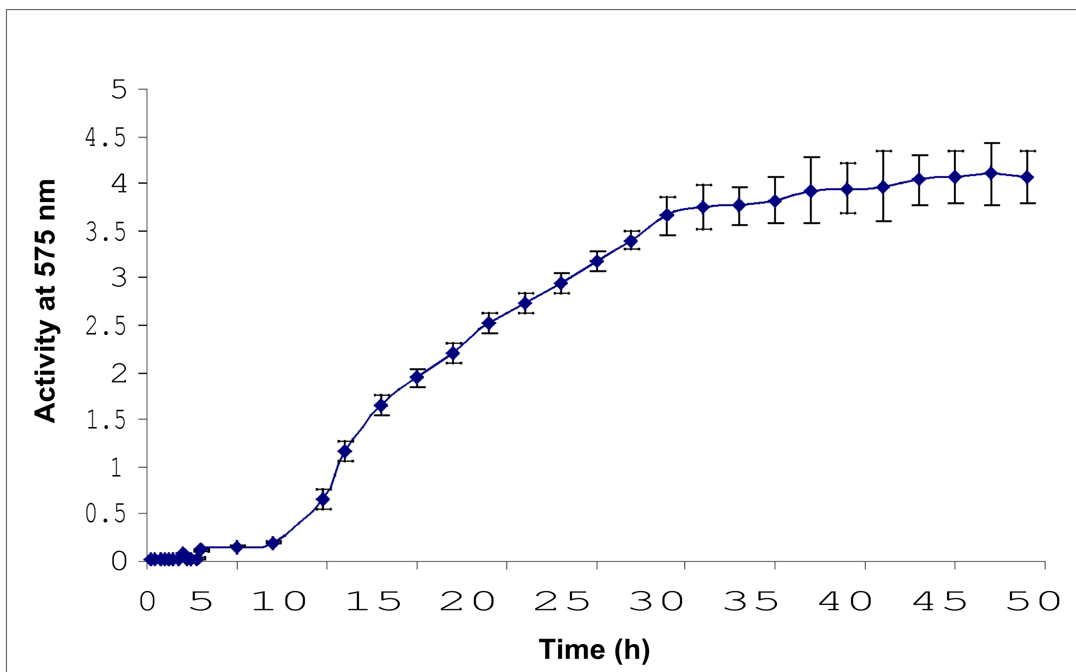


Figure 3.4: Linearity of B-GAL activity over time. All values are reported as mean ± SD (n=3).

3.3.2.2 Volume optimisation

There was a general increase in B-GAL activity with an increase in environmental water volume. However, 90 µl was selected so as not to compromise the total reaction volume of 200 µl (containing 90 µl buffer, 90 µl sample and 20 µl substrate) (Figure 3.5). However, the activity of B-GAL for the fast flowing water sample was consistently low (activity of less than 0.1 at 575 nm) with very high standard deviations as compared with the moderately flowing and stagnant samples (Figure 3.5). From these results, 90 µl was selected as being most appropriate for subsequent assays of the stagnant and moderately

flowing water sample. The fast flowing water sample was not used for further studies as the activity was very low and because water is normally collected at sites where the flow is slow or stagnant.

Enzyme dilution, denaturation and inhibition by pollutants inherently found in the water can possibly account for the lower activity observed in diluted river samples (Pletschke *et al*, 2006). The use of substrate dissolved in the assay buffer assisted in maintaining the buffering capacity of the reaction mixture since an increase in water samples was accompanied by a corresponding decrease in the assay buffer.

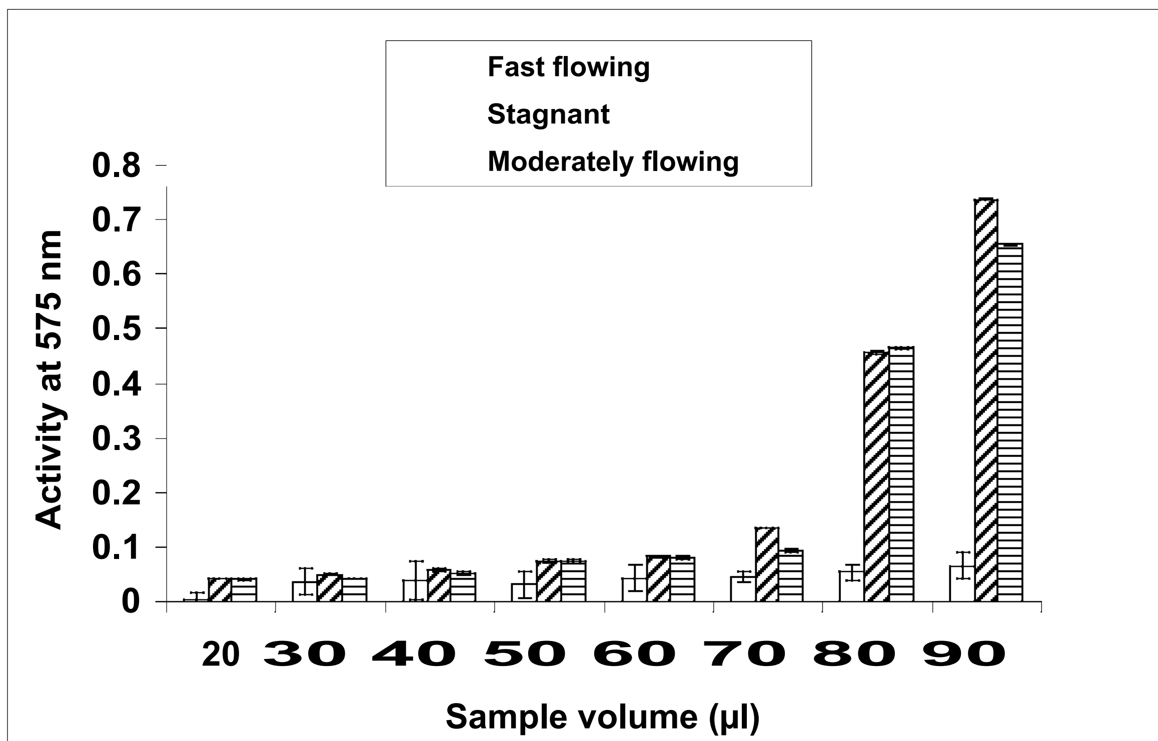


Figure 3.5: Effects of varying sample volume on B-GAL assay. All values are reported as mean ± SD (n=3).

3.3.2.3 pH optimisation

A gradual increase in B-GAL activity for the stagnant and running samples was observed between pH 5.0 and 7.5 after which a sharp rise appeared at 7.8. Increase in activity (about 40%) occurred when the pH was increased from 7.5 to 7.8 and a similar level of

reduction occurring between 7.8 and 8.0 (Figure 3.6). A similar trend was observed with both stagnant and running water samples. The pH optimum for the commercial enzyme was at pH 8.0 (Figure 3.6). This does not deviate much from the optimum value (pH 7.8) determined for the environmental water samples. Changes in the activity as a result of pH variation could be due to structural stability and solubility, charge and charge distribution on both enzymes and substrates.

All subsequent B-GAL assays were thus performed at pH 7.8 for the remainder of the study. Water intended for drinking purposes rarely has a pH far removed from neutrality and therefore performing B-GAL assay at this pH was appropriate.

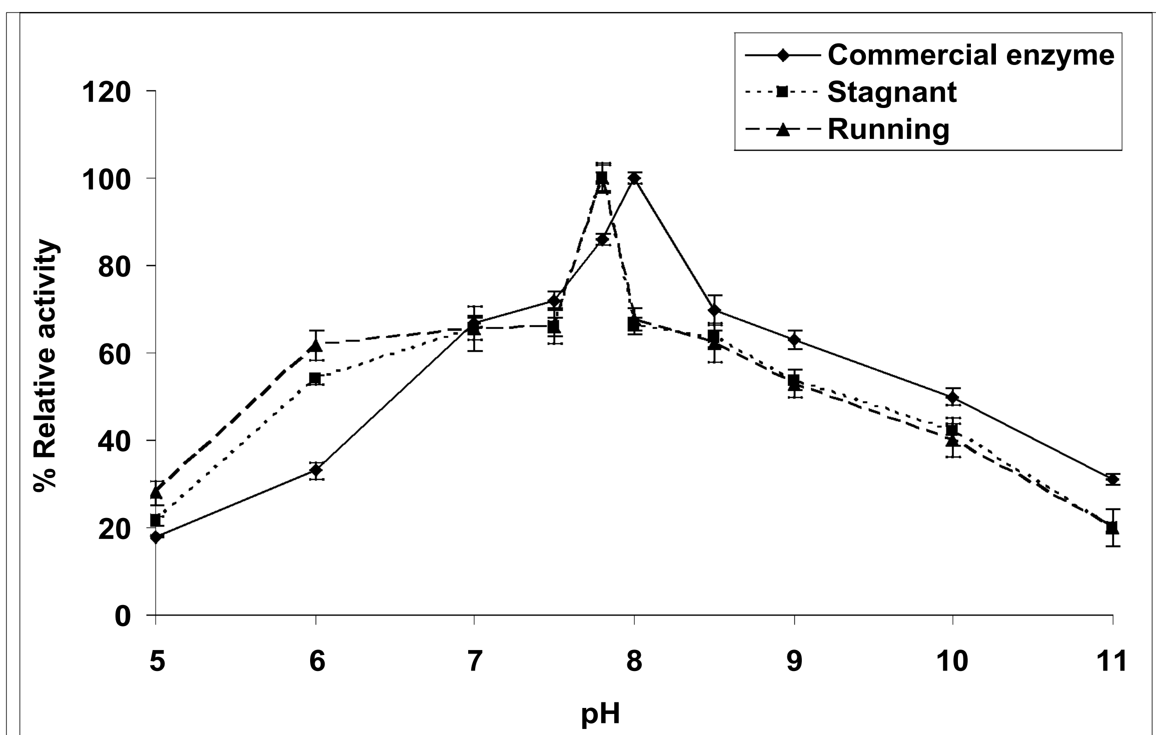


Figure 3.6: Effects of pH on B-GAL activity in commercial, stagnant and running water samples. All values represent the means ± SD (n=3).

3.3.2.4 Temperature optimisation

The temperature optimisation data presented a very interesting result (Figure 3.7). A consistent increase in activity with increase in temperature was observed for

environmental B-GAL up to 35 ^{o}C. Two peaks at 35 and 55 ^{o}C were observed. A sudden drop in activity at 40 ^{o}C for both samples was unexpected. Increasing the temperature from 20 to 35 resulted in about 30% increase in activity. Room temperature (20 ± 2 ^{o}C) was selected for all further B-GAL enzyme assays, as there was a sufficient level of B-GAL activity present, thus circumventing the necessity for heating equipment for mainly higher temperatures for detecting and monitoring the indicator microorganisms. The temperature optimum for the commercial enzyme was observed to be 37 ^{o}C.
Two temperature optima at 35 and 55 ^{o}C were observed with the environmental water samples. This could be due to the presence of two groups of microorganisms classified as coliform.

The lack of response of B-GAL to a wide pH and temperature range could be a result of changes to the enzymes due to exposure to the stressful environmental conditions associated with polluted water. Protection by pollutants could cause such a characteristic behaviour. A broad operational temperature and pH range is advantageous to bioprobe and biosensor development, as this will save costs in temperature maintenance during enzyme assays. An increase of 30% in activity was noted between 20 and 35 ^{o}C. Above 35 ^{o}C, reproducibility was poor, thus ambient temperature (20 ± 2 ^{o}C) was adopted for future studies. The reproducibility pattern of B-GAL activity at higher temperatures could be a result of chemical interference by pollutants or chemicals in the water sample. Meyer–Reil (1991) noted a similar trend with temperature by different marine enzymes.

Different species or groups of microorganisms classified as coliforms have been identified with different pH and temperature (Krulwich *et al*, **1978; Pisani** *et al*, **1990; Matzke** *et al*, **2000; Nagy** *et al*, **2001; Haki** *et al*, **2003; Gul-Guven** *et al*, **2006) pH ranging from 2.0 to 8.5 and temperature ranges of 15-65 ^{o}C. It is thus possible that there were more than one species/classes or groups of coliform bacteria in the water samples.**

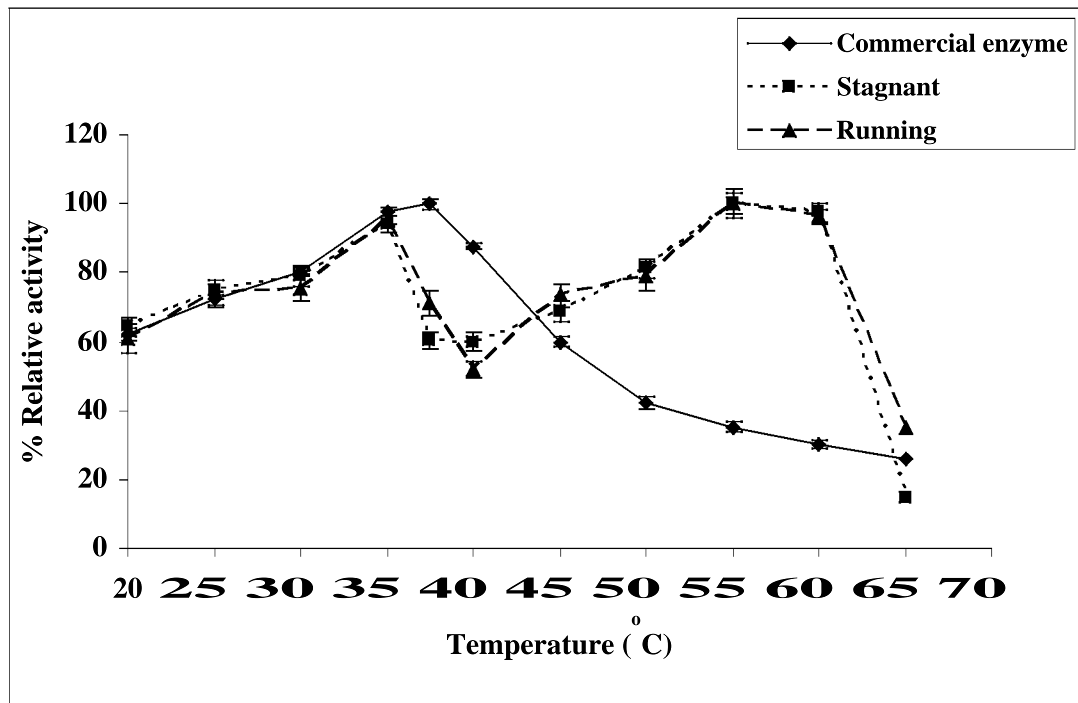


Figure 3.7: Effects of temperature on B-GAL activity in commercial, stagnant and running water samples. All values represent the means ± SD (n=3)

3.3.2.5 Effect of environmental water sample on commercial B-GAL activity.

In order to establish the total contribution of compounds present in the environmental water samples on the enzyme assay, varying volumes ranging between 0-50 percent of the total assay volume were pre-incubated with commercial B-GAL assay for 30 min prior to determining the enzyme activity. At a 50% volume level, the environmental water samples reduced the activity of B-GAL about 15% (Figure 3.8). This implied that there was some level of underestimation of the amount of B-GAL determined *in situ.*

The reduction of B-GAL activity by the environmental water samples could be due to the presence of metal ions or other pollutants which inhibit the activity of the enzyme or substrate or both. An underestimation of 15% at about 50% of the total assay volume was observed. Since the volume of the water samples in the assay is 45% (90:90:20 in µl) of the total volume, there may be a need to account for this loss in activity if quantitative results are required.

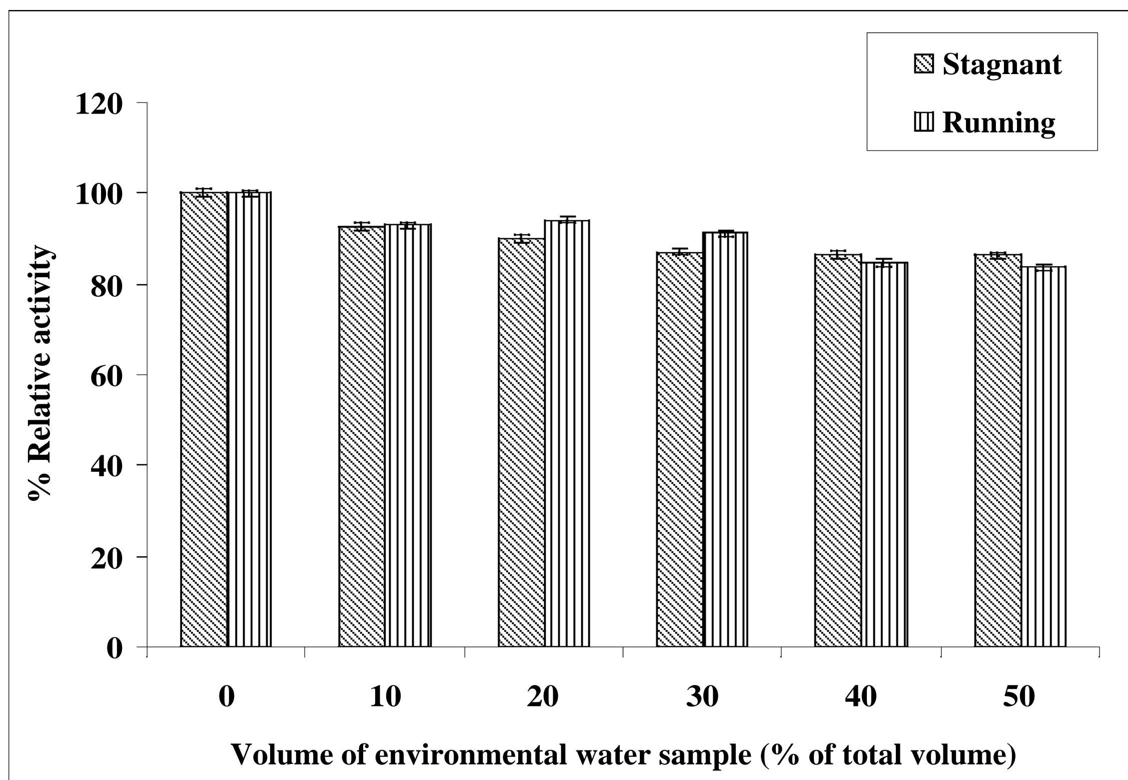


Figure 3.8: Effect of environmental water sample on commercial B-GAL activity. All values represent the means ± SD (n=3).

3.3.2.6 Fractionation studies

No pellets were obtained from the river water samples, and therefore the centrifuged sample was regarded as the supernatant 1 (see section 3.2.4.7). The facultative pond sample yielded all six fractions. For both sets of samples (see section 3.2.4.7), the whole fractions (RS) had the highest enzyme activity (Figure 3.9). Supernatant 1 (first supernatant) of the facultative pond sample exhibited the highest activity for B-GAL among the treated fractions followed by pellet 1 (first pellet) and then the sonicated pellet (Figure 3.9).

It was observed from the fractionation results that B-GAL occurred abundantly in the extracellular environment. Multimeric nature is common among β-galactosidases. The enzyme has a homotetrameric form in *E coli, Bacillus macerens* **and** *Lactobacillus*

helveticus **(Steers and Cuatrecases, 1974; Nadder** *et al***, 1983; Gargova** *et al***, 1995). The enzyme is mainly extracellular (Wildmer and Leuba, 1979; Gargova** *et al***, 1995; Rogalski and Lobarzewski, 1995; Shaikh** *et al***, 1999; Nagy** *et al* **2001) but intracellular forms also occur (Fisher** *et al***, 1995; Diaz** *et al***, 1996).**

Doyle *et al.* **(1995) reported that enzymes are exported to the extracellular environment when cells reach stationary phase. Since conditions in the natural environments are often stressful (Venter, 2000, George** *et al***., 2002; Leonard** *et al***., 2003), it was possible that the cells were forced into the stationary phase. This factor could thus explain the availability of the enzyme in the extracellular environment. The presence of natural substrates and competitors (Adams** *et al***., 1990) as well as other compounds, cations and anions in the water can also stimulate B-GAL production and export to the extracellular environment. The availability of the enzyme extracellularly provides a valuable advantage to the direct enzyme assay method, as this will allow the assay to proceed without further modification. This, in turn, will reduce running costs and labour.**

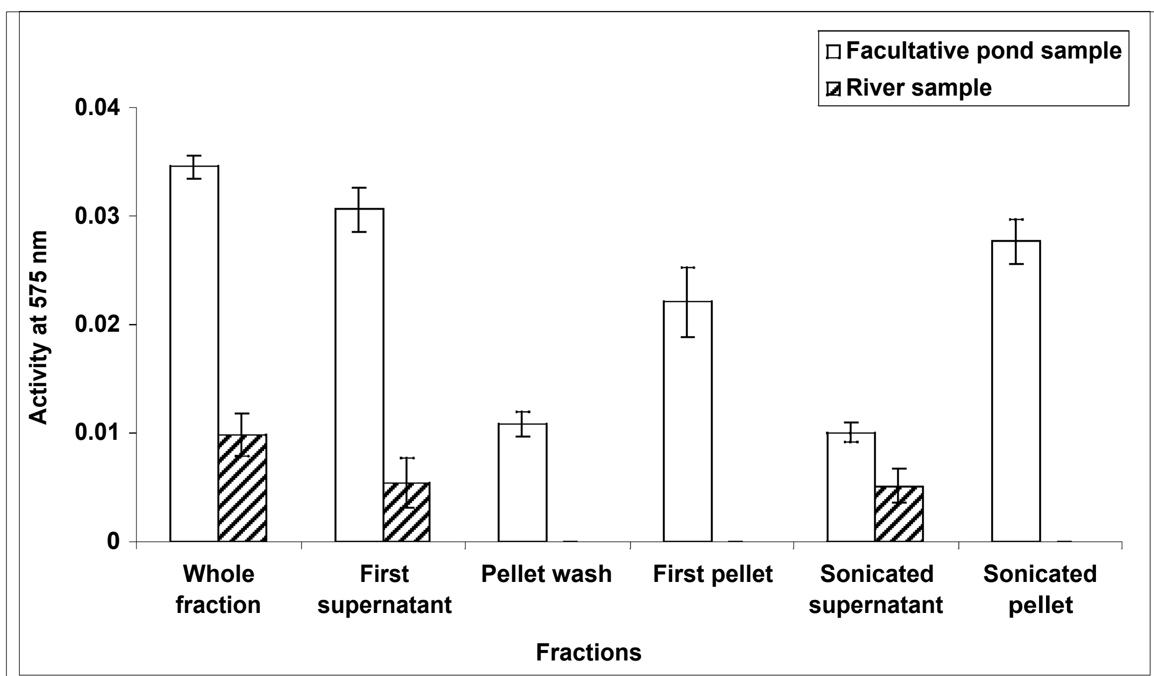


Figure 3.9: Activity of B-GAL in the different fractions of aquatic samples tested. All values represent the means ± SD (n=3).

3.3.2.7 Permeabilisation effects

All the permeabilisation methods inhibited B-GAL activity, except for sonication in both running and stagnant water samples (Figure 3.10). Sonication increased B-GAL activity by 30% in the running water sample and 10% in the stagnant sample as compared to the untreated samples (RS). SDS was the most potent inhibitor of B-GAL activity in the water samples and inhibited about 70% of the enzyme activity. With the exception of SDS, higher activity was observed in all the running water samples as compared to the stagnant water samples.

Extracellular location of B-GAL was also confirmed by the results of the permeabilisation studies, where none of the permeabilising methods offered a significant degree of improved activity over the whole unamended samples (RS). Fractionation study results obtained were comparable to results of Farnleitner *et al*. (2002) who observed an insignificant difference in the activity between filtrate and whole samples of lowly polluted waters.

Inhibition of the enzyme activities by permeabilisation (except sonication) makes these permeabilisers undesirable for use with the polluted Bloukrans river water samples. The inhibition could have been compounded by pollutants or other chemicals present in the water samples 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 selected marine enzymes using Triton - X100, EDTA, toluene and SDS.

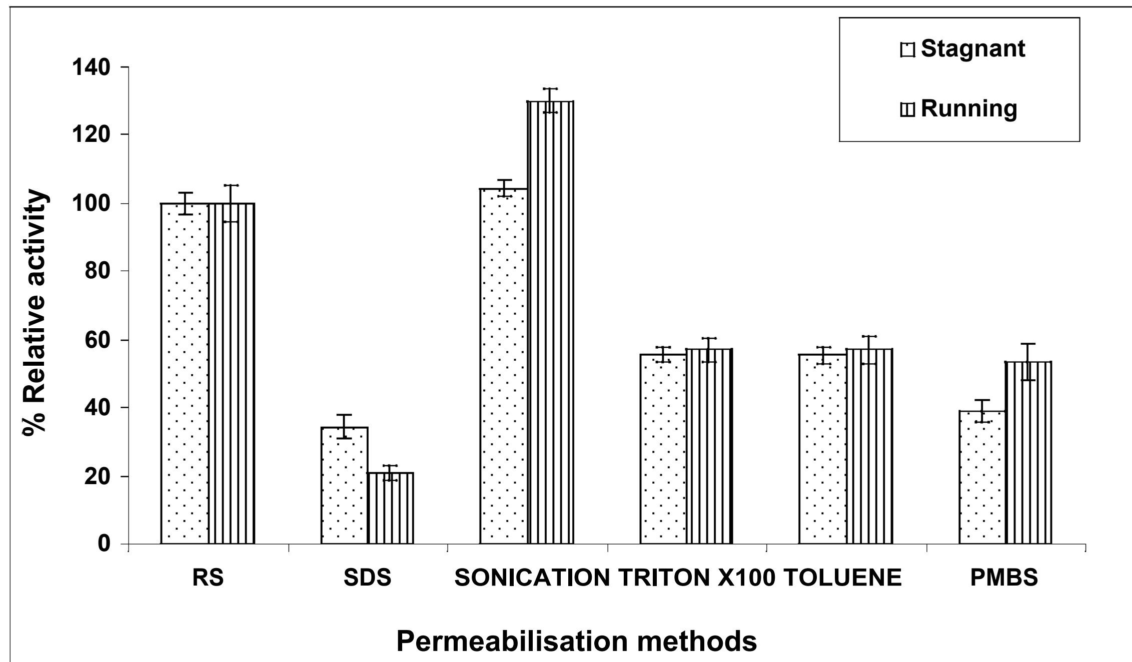


Figure 3.10: Effects of different permeabilisation methods on B-GAL activity. All values represent the means ± SD (n=3).

3.4 CONCLUSIONS

The above results clearly limit the use of the fluorogenic assay on environmental water samples. This implies that the sensitivity, selectivity, applicability and adaptability of a MuGAL assay product for environmental water samples in South African river samples would be greatly compromised.

The following conclusions about B-GAL were drawn from the fundamental studies with CPRG (the chromogenic substrate):

(a) enzymes in the polluted environmental occur in lower concentrations and larger sample volumes are required to detect a reasonable level of activity,
(b) physical and kinetic properties of environmentally exposed B-GAL enzymes are different from their pure and commercially available counterparts
(c) B-GAL occurs extracellularly in polluted water environments,
(d) cell permeabilisation is not a required step in direct enzyme assay for moderately polluted water samples such as that of the Bloukrans River, and
(e) environmental water samples do reduce the activity of pure B-GAL activity by about 15% at 50% of total assay volume.

In view of these conclusions, it was decided that the fluorogenic assay was not the best way to proceed for this study or product development. As a result, all subsequent work focused solely on the chromogenic substrate CPRG.

CHAPTER FOUR

ENZYME SOURCE IDENTIFICATION THROUGH PROTEIN CONCENTRATION AND ENZYME PROFILING

No one is free whose mind is not like a door with a double-acting hinge swinging outward to release their own ideas and inward to receive the worthy thoughts of others – Validivar.

4.1 INTRODUCTION

From the previous study, it was observed that the concentrations of the enzyme in the environmental water samples were low. This necessitated the need to concentrate the enzyme for faster activity. Since other organisms such as algae are also known to produce B-GAL, identifying the source of the enzyme of interest was also important.

In the environment, proteins often occur at very low concentrations or may be contaminated by impurities. It is therefore necessary to either concentrate the protein or remove the impurities in order to study such proteins in isolation without interference. When a protein of interest is known, its unique properties can also be used to selectively concentrate and isolate it (Dennison, 2003; Doonan and Cutler, 2004). After protein concentration, further characterisation can be performed to determine the molecular weight and subunit composition. For enzymes, one may employ activity assays to monitor the presence of the required protein throughout the various stages of isolation. It is also possible to determine the origin of a protein through peptide mass finger printing.

Concentration techniques are normally performed at 4 ^{o}C in order not to denature the protein of interest. In the case of proteins that are stable at high temperatures, the appropriate temperature conditions have to be utilised in concentrating them. This will selectively denature and remove unwanted and temperature sensitive proteins.

4.1.1 Protein concentration

Several techniques are available for concentrating proteins. These include: precipitation with salts, organic solvents and organic polymers, and freeze drying.

In salt precipitation, one can either employ salting in or out. Salting in is based on increasing protein solubility at low salt concentrations. This then leads to protein aggregation, hence precipitation. The precipitate may then be removed by centrifugation. Salting in depends on surface charge and polar interactions with solvents (Scopes, 1984;

Harris 1994; Doonan, 2004). Globular proteins are mainly isolated and purified in this manner (Scopes, 1984).

Salting out is based on decreasing protein solubility with an increase in salt concentration. This process exploits the hydrophobicity of a protein. As the salt concentration increases, there is a concomitant removal of water around the proteins. The hydrophobic regions on the proteins are thus exposed, leading to protein aggregation and precipitation (Scopes, 1984; Harris, 1994). Ammonium sulphate is commonly used in salting out for reasons such as cost, increased protein stability and allowing for easy pellet formation by the precipitate. However, it increases solution acidity slightly, which may have a negative impact on some protein structures (Scopes, 1984; Harris, 1994; Dennison, 2003).

In using organic solvents in protein precipitation, a reduction in water activity is expected which, in turn, decreases the solvating power of water for charged proteins (Scopes, 1984). This will then lead to a decrease in protein solubility causing them to aggregate and precipitate. Organic solvents are used at sub-zero temperatures and due to their high volatility, are easily removed from the protein sample (Scopes, 1984, Dennison, 2003).

Organic polymers such as polyethylene glycol (PEG) 20 000 and Sephadex G25 can be employed in concentrating protein. These agents draw water from the protein solution. More often than not, the solution to be concentrated is placed in dialysis tubing with a molecular weight cutoff limit that retains proteins but allows water and other smaller molecules to exit the tubing. The use of organic polymers causes the concomitant removal of smaller soluble contaminants with the water during the concentration process, thus eliminating the subsequent step required for precipitant removal from the protein solution.

Freeze drying (lyophilisation) can also be used to concentrate proteins in solution. Freeze drying concentrates all molecules in the solution and the resuspended powder is then

dialysed against water or buffer to reduce the concentration of contaminants. Freeze drying can potentially denature the proteins of interest.

Other techniques such as chromatography, affinity purification and ultrafiltration can be employed for enzyme purification (Dennison, 2003; Schratter, 2004; Gallo *et al.,* 2005; Najafi and Kembhavi, 2005). While chromatography and affinity purification require complex instrumentation, give low yield, are expensive and suitable for producing small quantities, ultrafiltration (UF) is a cost-effective method, giving a high yield and reasonable product purity. UF is also easier to scale-up in comparison to chromatography and electrophoresis (Li *et al.,* 2006).

4.1.2 Analyses of concentrated proteins

After concentration or purification, proteins can be subjected to further analyses to obtain information about their mass, structure and origin. One such method is electrophoresis, which can be employed to determine purity, molecular weight and subunit composition of a protein (Walker, 2002). Protein electrophoresis can be performed under non-denaturing or denaturing conditions (Bollag *et al*., 1996).

Non-denaturing protein electrophoresis (e.g. polycramide gel electrophoresis (PAGE)) is used for investigating the native protein properties while denaturing PAGE is necessary for studying the molecular weight and subunit composition of the protein subunits.

Polyacrylamide gel electrophoresis separates molecules in complex mixtures according to size and charge. During electrophoresis there is an intricate interaction of samples, gel matrix buffers, and electric current resulting in separate bands of individual molecules. Hence the variables that must be considered in electrophoresis are gel pore size, gel buffer systems, and the properties of the molecule of interest (McLellan, 1982; Chramback and Jovin, 1984; Bollag *et al*, 1996).

Empirically the pore size providing optimum resolution for proteins is that which results in a relative mobility (R_f) value between 0.55–0.6. R_f values for specific proteins are calculated as follows;

$$R_f = \frac{\text{Distance migrated by the protein of interest}}{\text{Distance migrated by the ion front}}$$

Separation of Proteins under Denaturing conditions

Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone - and SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length – i.e.: the denatured polypeptides become "rods" of negative charge cloud with equal charge or charge densities per unit length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with 2- mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight (McLellan, 1982; Chramback and Jovin, 1984; Bollag *et al*, 1996).

Determination of Molecular Weight

This is done by SDS-PAGE of proteins - or PAGE or agarose gel electrophoresis of nucleic acids - of known molecular weight along with the protein or nucleic acid to be characterised. A linear relationship exists between the logarithm of the molecular weight of an SDS-denatured polypeptide, or native nucleic acid, and its *Rf*. The Rf is calculated as the ratio of the distance migrated by the molecule to that migrated by a marker dye-front. A simple way of determining relative molecular weight by electrophoresis (*M*r) is to plot a standard curve of distance migrated vs. log10MW for known samples, and read

off the log*Mr* of the sample after measuring distance migrated on the same gel (McLellan, 1982; Chramback and Jovin, 1984; Bollag *et al*, 1996).

Gel Buffer System

The buffer system determines the power requirements and affects separation. The buffer system is composed of the buffer used in the gel and the running buffer. There are continuous and discontinuous buffer systems. In continuous buffer systems, the same buffer ions are present, at constant pH and concentration throughout the system. The gel is typically made of one continuous %T and the sample is loaded directly into the part of the gel where separation will occur. The band width is determined in part by the height of the sample load in the well, so samples should be concentrated and volumes small for best results. In discontinuous buffer systems, different buffer ions are present in the gel and electrode reservoirs. By using different buffers in the gel and in the electrode solutions and adding a stacking gel to the resolving gel, samples are compressed into a thin starting band and individual proteins are finely resolved and separated. The most popular discontinuous system employed is the SDS-PAGE buffer system by Laemmli (1970). The Laemmli buffer system is a discontinuous buffer system that incorporates SDS in the buffer. In this system, proteins are denatured by heating them in buffer containing sodium dodecyl sulfate (SDS) and a thiol reducing agent such as 2-mercaptoethanol (βME.). The resultant polypeptides take on a rod-like shape and a uniform charge-to-mass ratio proportional to their molecular weights. Proteins separate according to their molecular weight, making this system extremely useful for calculating molecular weights (McLellan, 1982; Chramback and Jovin, 1984; Bollag *et al*, 1996).

Further information about the origin of a protein sample may be necessary, especially if it is located in a mixed environment. Peptide mass finger printing can be used to identify the source of a protein. In this process, an electrophoresed protein is excised from the gel and digested with enzymes such as trypsin, V8 protease or chymotrypsin and exposed to mass spectrometry (MS). Matrix assisted laser desorption and ionisation time of flight (MALDI ToF) or electrospray ionisation (ESI) can be used (Liebler, 2002). MALDI ToF

MS is sensitive and provides good resolution; success however, depends on sample purity (Liebler, 2002). Once the peptide masses are obtained, they can be used in conjunction with known protein databases under the same working conditions to determine the origin of a protein.

4.2 MATERIALS AND METHODS

Bradford reagent, bovine serum albumin, glycine, gycerol, bromphenol blue, beta-mercaptoethanol, Coomassie brilliant blue, acrylamide gel, methanol, standard mixture for molecular weights 30,000-200,000 (rainbow marker), ethanol, ammonium persulphate, APS, TEMED, acetone, PEG 20000 and ammonium sulphate were all supplied by Sigma-Aldrich (Munich, Germany); Chlorophenol red β-D-galactopyranoside (CPRG), acetic acid and Tris(hydroxymethyl)aminomethane by Merck (Darmstadt, Germany); Beckman Coulter Avanti J-E centrifuge by (Beckman, South Africa) and sodium duodecyl sulphate (SDS) by BDH laboratories, (England).

4.2.1 Protein concentration

One denaturing (acetone precipitation) and four non–denaturing concentration techniques were adapted from Bollag *et al*. (1996) and modified to suit our study. All concentration steps were performed at 4 ^{o}C, unless stated otherwise.

4.2.1.1 Acetone precipitation

To 100 ml of water sample, 500 ml of cold acetone (stored at -20 ^{o}C) was added. This was gently mixed and kept at -20 ^{o}C for 3 h, followed by centrifugation at 27 000 g for 5 minutes at 4 ^{o}C. The pellet was then air dried, re-suspended in 5 ml volume of water, aliquoted in eppendorf tubes and stored at -20 ^{o}C (Ó'Fágáin, 2004) for further analyses.

4.2.1.2 Ethanol precipitation

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

4.2.1.3 PEG 20 000 concentration

Dialysis tubing with a molecular weight cut off of 12 kDa was prepared as outlined by the manufacturer (Sigma) and 100 ml volumes of the water samples were added. Dialysis was performed until the remaining volume in the tubing was about 5 ml. This was dialysed overnight against 10 mM Tris–HCl buffer (pH 7.5) and stored as outlined in *4.2.1.1.*

4.2.1.4 Ammonium sulphate precipitation

To 100 ml of chilled water samples, 56.8 g of ammonium sulphate was slowly added in small amounts with gentle stirring on ice. Stirring was continued for 30 min after salt dissolution and left to stand for about 15 min to ensure complete precipitation. The solution was centrifuged at 10 000 g for 10 min at 4 ^{o}C, and the pellet resuspended in 3 ml of water and dialysed as in *4.2.1.3* **with three buffer changes. Dialysis tube contents were stored as in** *4.2.1.1.*

4.2.1.5 Freeze drying

Water samples (100 ml aliquots) were poured into freeze drying flasks and swirled in liquid nitrogen. After freezing the content, the flasks were freeze dried overnight. Prior to attaching the samples, the vacuum pump was evacuated and allowed to warm up (30 min) before the condensers were cooled. The condenser was then switched on and allowed to cool to approximately -60 ^{o}C. The resulting powder was removed and immediately re-suspended in 5 ml distilled water. This was then dialysed and stored as in *4.2.1.4.*

4.2.2 Protein concentration determination

The Bradford (1976) assay adapted from Kruger (2002) was employed in quantifying the protein. Bovine serum albumin (BSA) was used to construct a standard curve. A BSA stock solution of 2 mg ml^{-1} was prepared from which dilutions of 0.4, 1.8, 1.2, 1.6 and 2 mg ml^{-1} were used for the assay to construct the standard curve (Appendix A). To each BSA concentration and 5 µl of sample from *4.2.1*, 250 µl of Bradford reagent was added, mixed, left for 5 min and the absorbance read at 595 nm.

4.2.3 Activity gels

Polyacrylamide gels (6 %) were prepared for native protein electrophoresis. The samples were prepared and loaded as described by Bollag *et al*. (1996). Each gel was overlaid by a CPRG solution and any colour changes were noted.

4.2.4 SDS-PAGE

A denaturing SDS-PAGE analysis was performed as described by Bollag *et al*. (1996) using the BioRad Mini Protean III system. The molecular weight was determined by SDS-PAGE using the standard markers; Carbonic anhydrase (Bovine erythrocytes, 29,000 Da), Albumin, (Egg ovalbumin, 45,000 Da), Albumin (Bovine plasma, 66,000 Da), Phosphorylase B, (Rabbit muscle 97,400 Da), β-D-Galactosidase, (*Escherichia Coli*, 116,000, Da) and Myosin, (Rabbit muscle 205,000 Da). Eight percent (8%) resolving and 5 % percent stacking gels were prepared. Samples (20 µl) were mixed with 5 µl of 5 x sample buffer, heated at 100 ^{o}C for 5 min (Bollag *et al*., 1996) and then 20 µl of each treated sample was applied to the gel. Rainbow molecular weight markers were also applied to the gel. Electrophoretic separation was achieved at a constant voltage of 120 V, after which the gel was stained with Coomassie dye and the bands presumed to be B-GAL excised for MALDI ToF MS analysis.

Information on separating gel buffer, stacking gel buffer, separating gel solution, stacking gel solution, SDS solution, 2X sample buffer, electrode buffer, fixative solution, staining

reagent, preparation of samples, molecular weight makers, electrophoresis gel, electrophoresis, staining and destaining solutions and interpretation of results were adopted from the Sigma Tech. Bulletin No. MWS-877L (7-88), (See appendix D for reagents and protocols).

4.2.5 MALDI ToF MS analysis

The excised bands from the SDS-PAGE (see section 4.2.4) were sent to the School of Molecular Sciences MALDI ToF MS facility at the University of Cape Town, South Africa for tryptic digest mass mapping. The resultant masses were corrected for trypsin self digestion fragments and Expasy tool (PeptideSearch) used to search and match the peptide mass to ascertain the origin of the enzymes (http://au.expasy.org/tools/).

4.3 RESULTS AND DISCUSSION

Identification of the source of the enzyme as well as results of protein concentration methods are presented below.

4.3.1 Protein concentration methods

PEG 20000 provided the highest degree of protein concentration followed by freeze drying (Figure 4.1), followed by freeze drying. The highest percentage relative B-GAL activity was observed with PEG 20 000 concentrated samples (Figure 4.2). Increases in enzyme activities with an increase in protein concentration by PEG 20 000 suggested successful B-GAL concentration. Hence PEG 20 000 can be employed when necessary.

It was observed that ethanol, ammonium sulphate and acetone precipitation methods yielded very little and insignificant ($P>0.05$) concentration. B-GAL activity was observed to be higher in stagnant than running water samples using PEG 20000 (Figure 4.2).

Ammonium sulphate precipitates protein in solution if the protein concentration is above 1 mg ml^{-1}. In this case, the water sample protein concentration was less than this threshold value, hence no protein concentration was achieved. Instead, enzyme inhibition was noted. In other studies with enzymes in environmental water samples, inhibition of the enzymes by ammonium sulphate was noted (Ngwenya and Whiteley, pers. communication). Similarly, the failure to concentrate proteins by other precipitation methods could be attributed to low protein concentration and interaction of precipitants with interfering compounds inherent in the water samples (Scopes, 1984).

All the methods of precipitation employed exhibited higher B-GAL activities than the unconcentrated raw samples (RS). The PEG 20 000 concentrated samples were subsequently selected for gel electrophoresis and tryptic mass mapping.

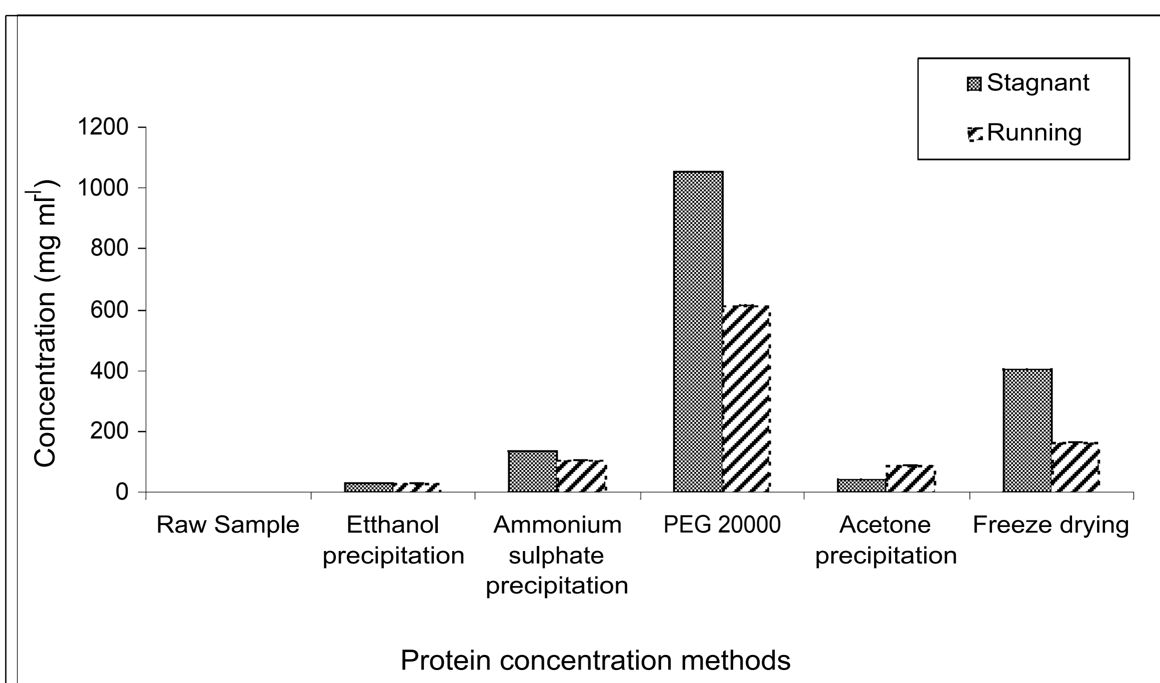


Figure 4.1: Protein concentration (mg ml^{-1}) achieved by different methods of protein concentration for stagnant and running water. All values represent the means ± SD (n=3)

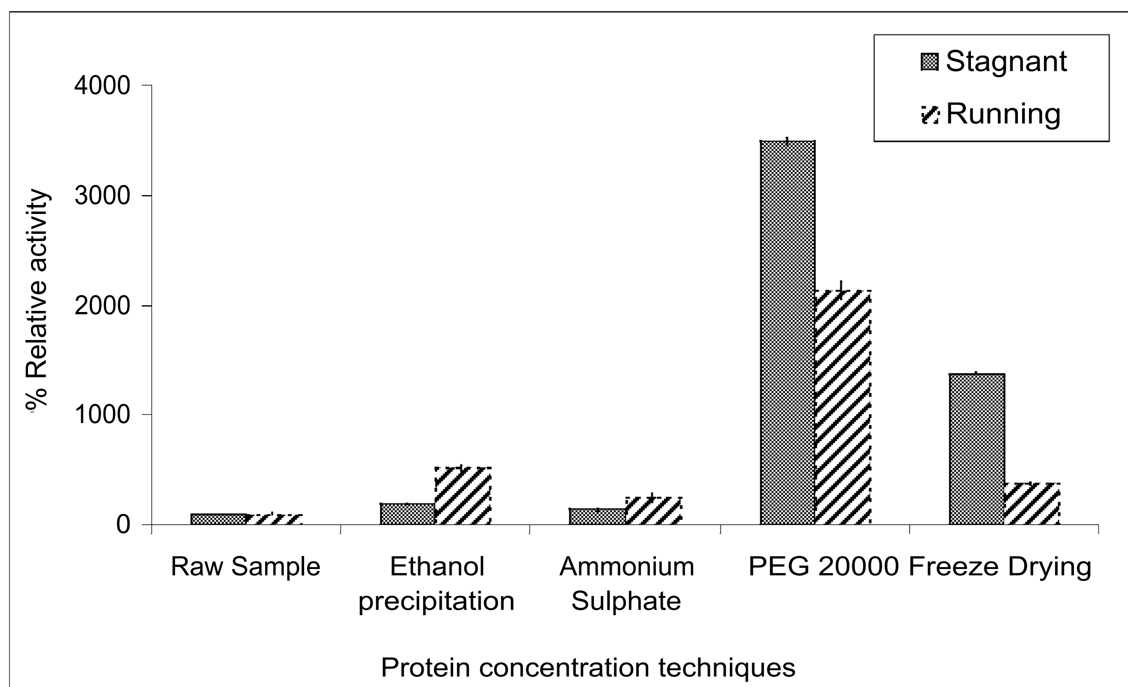


Figure 4.2: B-GAL activity in whole raw sample and concentrated fractions using non-denaturing methods. All values represent the means ± SD (n=3).

4.3.2 Activity gels

Activity gels did not provide much information as the CPR substrate completely diffused over the gel. This made it impossible to observe any bands clearly.

4.3.3 SDS-PAGE and MALDI ToF MS peptide mass matching

The stagnant (S) and running (R) water samples gave several identical bands (Figure 4.3). The presumed band of interest for B-GAL was 116 kDa (Figure 4.3). This was excised and sent for MALDI ToF MS analysis (Appendices D1, D2, D3, D4, D5 and D6).

Band A was confirmed from the peptide mass matching (Figure 4.3) as β-D-galactosidase from of *E. coli* origin. Reducing the level of accuracy to two Daltons gave results indicating that some fragments in B were from *E. coli* B-GAL. The lower two bands were

not further investigated because of their molecular mass sizes which fall way out of the theoretically stated values of the enzyme of interest. The presence of faecal coliform, *E. coli* in the water samples was determined concurrently by chromogenic enzyme activity (PNPG) and mFC media (Togo *et al*, 2007). Subsequent confirmation of the excised B-GAL as of *E. coli* origin thus validated faecal contamination of the water samples. The use of SDS-PAGE and MALDI-ToF provided an accurate technique in the identification of *E. coli*. This technique has been used successfully in the biochemical field for the analysis of proteins, peptides, glycoproteins, oligosaccharides, and oligonucleotides. It is capable of measuring masses to within 0.01% of the molecular mass of the sample (Takach et al, 1997; Whitelegge, 2003; Kammerera et al, 2005).

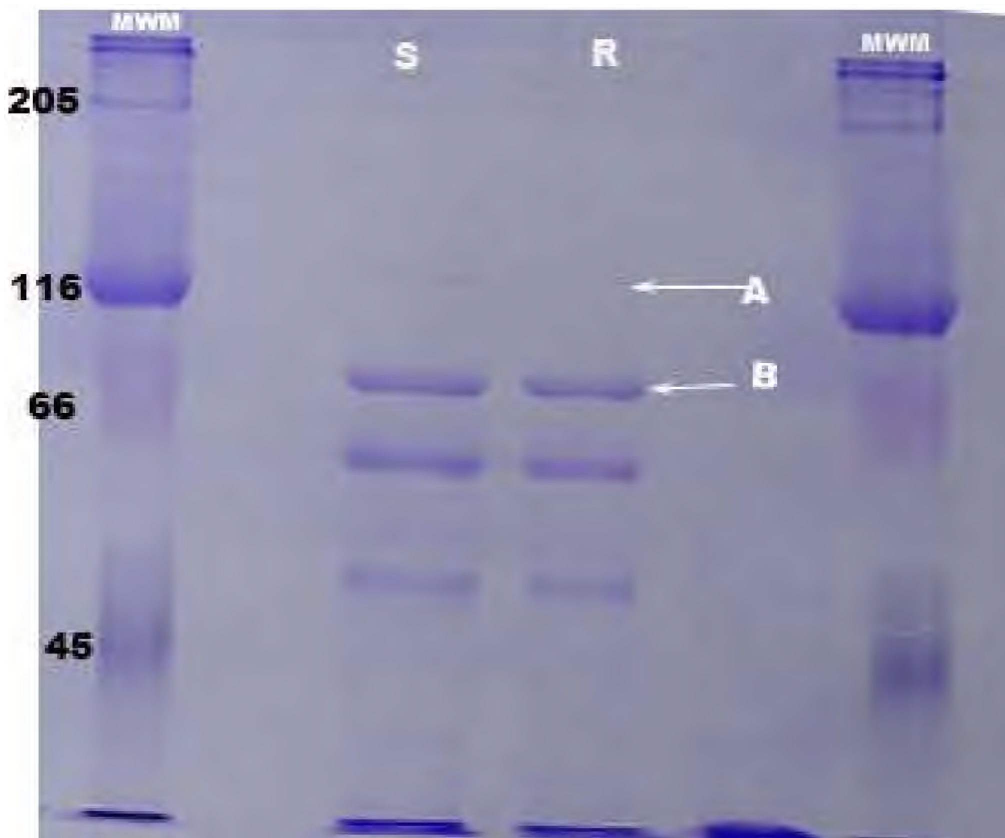


Figure 4.3: Presumed B-GAL bands obtained after SDS-PAGE. S and R are stagnant and running water samples, respectively, MWM is the rainbow molecular weight marker (Molecular weights in kDa).

4.4 CONCLUSIONS

The following conclusions could be drawn from this chapter;

(a) the best method of concentrating B-GAL from the environment samples was through the use of dry PEG 20 000,

(b) all precipitating methods employed yielded better results than the raw sample

(c) protein concentration in the water samples was below 1 mg ml^{-1}.

(d) hydrolysis of CPRG was as result of enzyme activity, as observed from the activity gels,

(e) the B-GAL enzyme in this study originated from *E. coli,* thus

(f) the use of B-GAL as an indicator of coliforms is justified.

CHAPTER FIVE

INTERFERENCE STUDIES AND WATER ANALYSIS

The most beautiful and most profound emotion we can experience is the sensation of the mystical. It is the sower of all true science. So to whom this emotion is a stranger, who can no longer wonder and stand rapt in awe, is as good as dead - Albert Einstein

5.1 INTRODUCTION

Although B-GAL has been employed in differential media for detecting and enumerating coliforms for a long time, there is little information, if any, regarding the effect of most ions/salts on this enzyme in situ in river samples.

A wide variety of metal ions are found in the environment. Metal ions play important roles in the biological function of many enzymes and can have a variety of effects on enzyme systems. Enzymes in dilute aqueous solutions function best under limited conditions of temperature, pH and salt concentration (Berg *et al*., 1986; Singh *et al*., 1990).

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

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

usually lead to such irreversible inhibition by binding strongly to the amino acid backbone (Ridgway and Olson, 1982, Den Blanken, 1985, Singh *et al*., 1990).

An environmental water sample is likely to contain ions/salts in varying concentrations. At the pH of most natural waters (pH 5-8), a large percentage of carboxylic acid groups will be ionized and many cations such as calcium will chelate organic acids, therefore reducing protein-organic acids complex formation, leading to increased enzyme activity. Divalent cations also adsorb organic acids and other substances (Wetzel 1991). Calcium carbonate precipitates dissolved organic acids in an aqueous environment, which may also inhibit enzyme activity (Wetzel, 1991).

5.2 MATERIALS AND METHODS

Sodium hypochlorite was supplied by Savemore (Pinetown, South Africa); Sodium sulphate, ferric chloride, potassium nitrate, cadmium sulphate, calsium sulphate, chlorophenol red β-D-galactopyranoside (CPRG), chlorophenol red (CPR), disodium hydrogen phosphate, dihydrogen sodium phosphate, ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, potassium chloride, sodium sulphate, sodium carbonate, citric acid, ferulic acid, magnesium chloride, copper sulphate, sodium chloride, calcium chloride, sodium acetate, triton X-100, tris(hydroxymethyl)aminomethane, glacial acetic acid, disodium sulphite, sodium bicarbonate, sulphuric acid were all supplied by Merck (Darmstadt, Germany). β-D-galactosidase (EC 3.2.1.23) (B-GAL) and toluene were supplied by Sigma-Aldrich (Munich, Germany), UV spectrophotometer by Shimadzu (UK) and high-precision 780 pH Meter (Metrohm South Africa (Pty) Ltd).

5.2.1 Water pollutant analyses

Water samples (100 ml) were collected as described in Chapter 3 and submitted to the Nelson Mandela Municipality Scientific Services Department (Eastern Cape, South Africa) for SOLUTE ANALYSIS. Table 5.1 shows the results of these analyses and highlights the compounds that were found to be present in the water samples.

5.2.2 Interference studies

The effects of various compounds found in water and those used in water treatment on B-GAL activity were investigated. Stock solutions of 500 mg l^{-1} of sulphate, chloride, carbonate, cadmium, magnesium, calcium, ferric chloride, potassium, ferulic acid, ethylenediaminetetraacetic acid (EDTA), sodium chloride, phosphate nitrate and 700 parts per million sodium hypochlorite were prepared. With the exception of sodium hypochlorite, an enzyme, buffer and effector concentration of a range between 0 and 200 mg l^{-1} were pre-incubated at room temperature (20 ± 2 ºC) for 30 min, after which the reaction was initiated by the addition of the respective substrate. Suitable substrate and enzyme controls for each effector concentration were also prepared to assess the background chemical rate of hydrolysis. All results were reported as means ± standard deviations and all statistical analyses were performed with analysis of variance (ANOVA) using a Microsoft Excel 2003 statistical tool at 5 % level of significance. Unless otherwise stated, a 1 X 10^{-2} µg ml^{-1} concentration of commercial B-GAL enzyme (CE) was employed in all assays (Wutor *et al*., 2007b).

5.3 RESULTS AND DISCUSSION

The effects of a wide range of potentially interfering ions/salts in varying concentrations on both commercial and environmental B-GAL were reported below.

5.3.1 Water pollutant analyses

Table 5.1 shows the water samples analysis report from the Nelson Mandela Municipality Scientific Services Department and the South African Bureau of Standards (SABS) 241 limits. All the components, with the exception of phosphorus, magnesium, total sulphide and total alkalinity, were below the maximum permitted limits according to the SABS 241 water quality guidelines (www.klmcs.co.za/html/waterguidelines.html) (Table 5.1). Calcium carbonate and chloride were present in the highest concentration amongst the components analysed (Table 5.1). Differences in some of the compound concentrations (e.g. sodium, magnesium (carbonate) and zinc) between stagnant and

running water samples were observed. The units of the results depicted in Table 5.1 from the sample analyses are in mg l^{-1} except for mercury (µg l^{-1}).

Table 5.1: Results of SOLUTE ANALYSIS of water samples collected from the Bloukrans River, as conducted by the Nelson Mandela Municipality Scientific Services Department according to the SABS 241 Guidelines (2001).

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

5.3.2 Interference studies

The effect of various cations, anions and compounds on commercial and environmental B-GAL over a concentration range of 0 – 200 mg l^{-1} is presented in Table 5.2. Direct

comparison of commercial B-GAL with coliform B-GAL from the environment could be biased since the amount of commercial B-GAL was much higher than that in the water samples and also, the chemical pollutants could have had an effect on either or both the coliform cells and/or the coliform B-GAL, thus presenting a more complicated situation. In an attempt to minimise this, the commercial B-GAL was diluted so as to obtain activity similar to the activity that was recorded in the environmental water samples. Also, the traditional coliform culturing method was done to confirm the presence of coliforms.

Monovalent sodium cations at 50 mg l^{-1} increased the relative activity of the commercial enzyme (139 %) while reducing the relative activity of the environmental enzymes (87.6 and 91.8 %) for stagnant and running water samples, respectively. At 200 mg l^{-1} concentration, the relative activity of B-GAL in both commercial and environmental samples was lower than that observed at 50 mg l^{-1} (Figure 5.1).

Potassium ions had a stronger activation effect at lower concentrations than did sodium (Figure 5.2). Potassium cations had a positive effect on the enzyme activity over the concentrations studied even though their effect on enzyme activity at higher concentrations were less than those observed at lower concentrations.

Silver ions (from AgCl) strongly inhibited the relative activity of B-GAL in both commercial and environment samples at concentrations between 50-150 mg l^{-1}, while yielding an unexpected increase in relative activity of B-GAL at 200 mg l^{-1} from both environmental and commercial sources (Figure 5.3). A level of 150 mg l^{-1} resulted in the largest inhibition in the relative activity of the commercial enzyme (Table 5.2 and Figure 5.3).

SEE ATTACHED TABLE 5.2 IN LANDSCAPE.

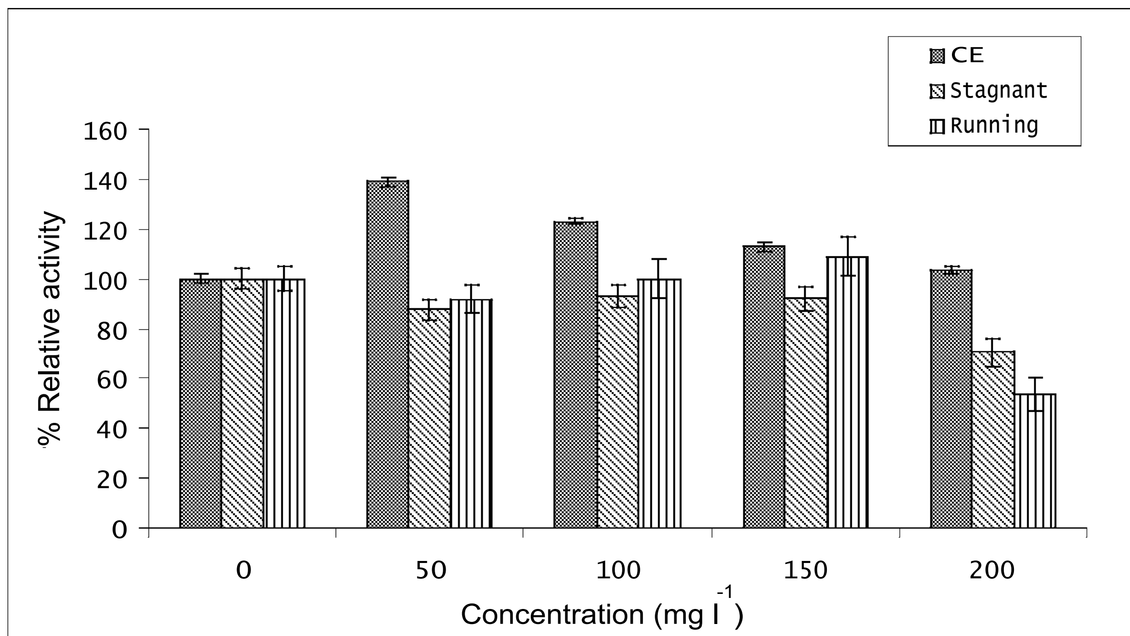


Figure 5.1: Effect of sodium (sodium chloride) on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

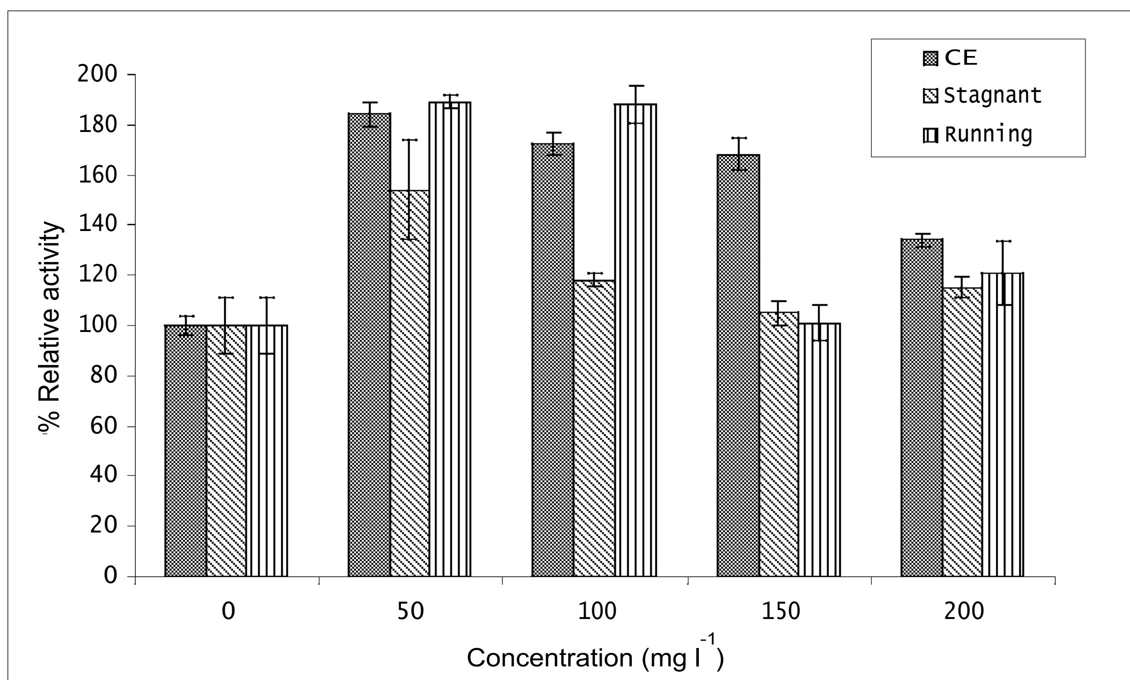


Figure 5.2: Effect of potassium (potassium chloride) on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

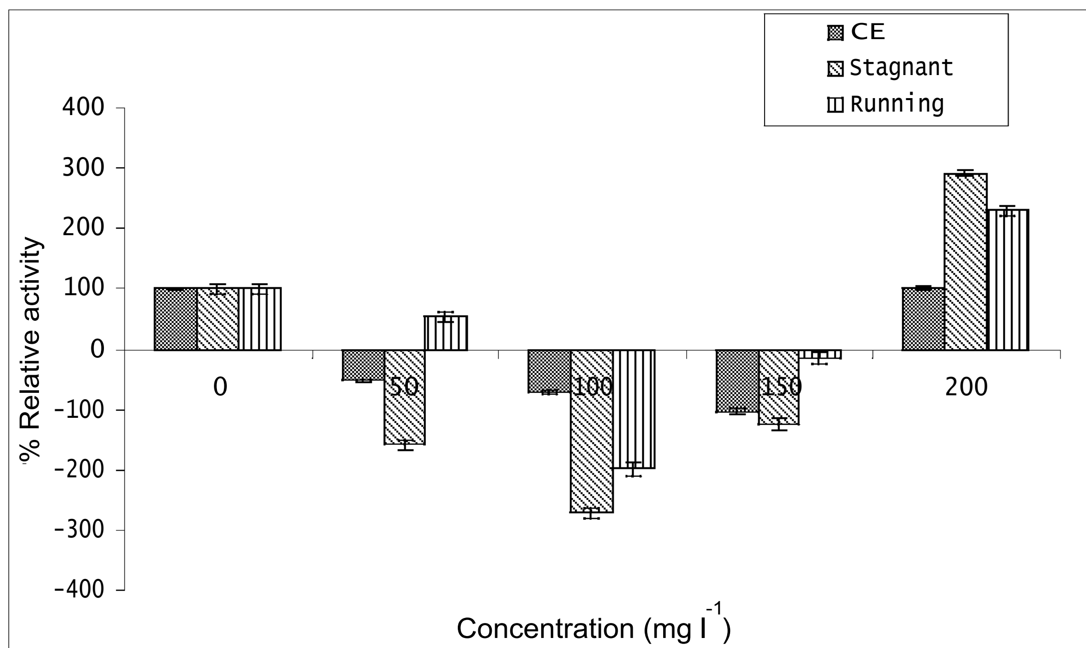


Figure 5.3: Effect of silver (silver chloride) on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

With the exception of Mg^{2+}, divalent cations generally inhibited the relative enzyme activity at higher concentrations (Table 5.2 and Figure 5.4). A gradual reduction in the relative activity of commercial B-GAL with an increase in concentration was observed with magnesium ions.

Ferrous ions increased enzyme activity between 50 and 100 mg l^{-1} but inhibited the activity at 150 mg l^{-1} and above (Table 5.2 and Figure 5.6). The addition of ferrous ions also resulted in a strong decrease in pH (data not shown). Calcium ions at 200 mg l^{-1} inhibited B-GAL activity, while activation was observed at lower concentrations (50 – 150 mg l^{-1}) (Figure 5.5). A similar inhibitory effect at same concentration was observed with copper ions (Table 5.2 and Figure 5.7).

Cadmium at all the studied concentrations inhibited B-GAL activity (Table 5.2 and Figure 5.8). It must, however, be noted that the toxic effect of cadmium on living cells will also result in the absence of the microbes which produce the enzymes. The presence of cadmium arrested the activity of the enzymes, thereby interfering with this enzyme

assay. Further dilutions (0 – 1 µg ml^{-1}) of cadmium showed a similar inhibitory trend on B-GAL activity. Magnesium ions, however, increased the activity of B-GAL in the running and stagnant water samples respectively, at a concentration of 150 mg l^{-1}. Ferrous chloride increased the relative activity of all three B-GAL enzyme samples at 50 mg l^{-1}, beyond which a reduction in relative activity was observed. Cadmium has no known biological function and is a highly toxic metal to aquatic organism and occupies a special place among the most abundant non-essential elements due to its immense usage in various industrial applications (Aina *et al*, 2007; Hou *et al*., 2007; Luchese *et al*, 2007; Zhang *et al*, 2007).

Mg^{2+} is important for maximum activity of *Escherichia coli* B-GAL (Sutendra *et al*, 2006). High resolution structure of B-GAL indicates that there are several other metal binding sites (Juers *et al*, 2000) and Mg^{2+} can bind to this side via a direct bond with the main chain carbonyl of Ans-597 and indirectly via water molecules with Glu-797 (Sutenda *et al*, 2006) to increase the activity B-GAL (Table 5.2 and Figure 5.4).

Tryland *et al*. (1997) observed that low concentrations of chlorine and divalent cations such as magnesium increased B-GAL activity. While their observation with magnesium is in agreement with our results, the differences in observation with the chlorine ions may be attributed to the complexity of the environmental water samples employed in this study.

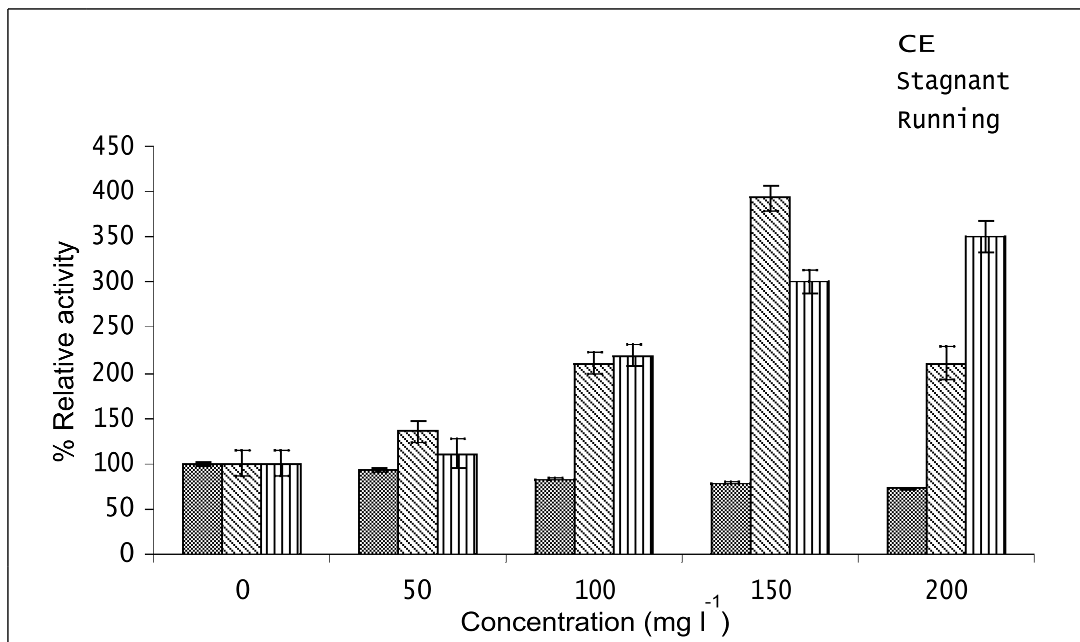


Figure 5.4: Effect of magnesium (magnesium chloride) on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

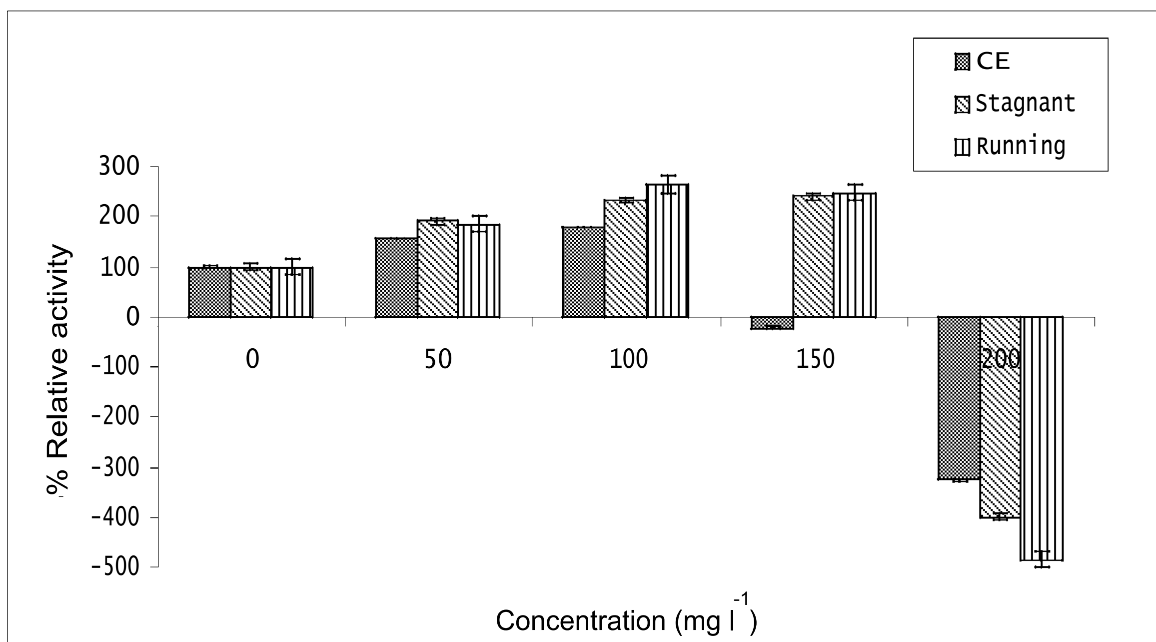


Figure 5.5: Effect of calcium (calcium chloride) on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

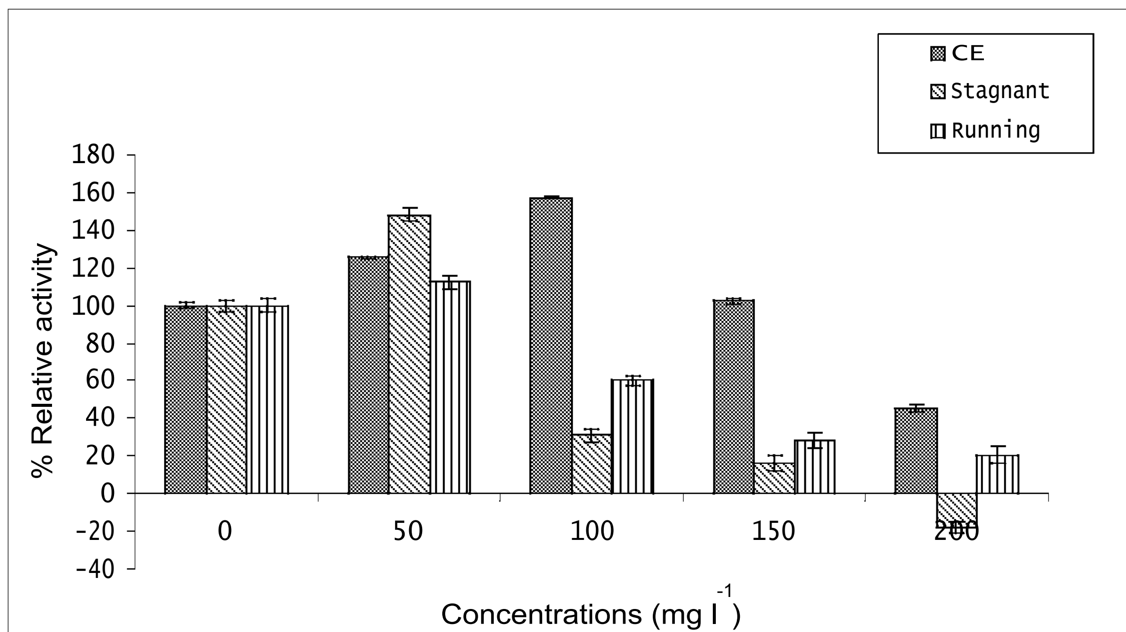


Figure 5.6: Effect of ferrous ions (ferrous chloride) on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

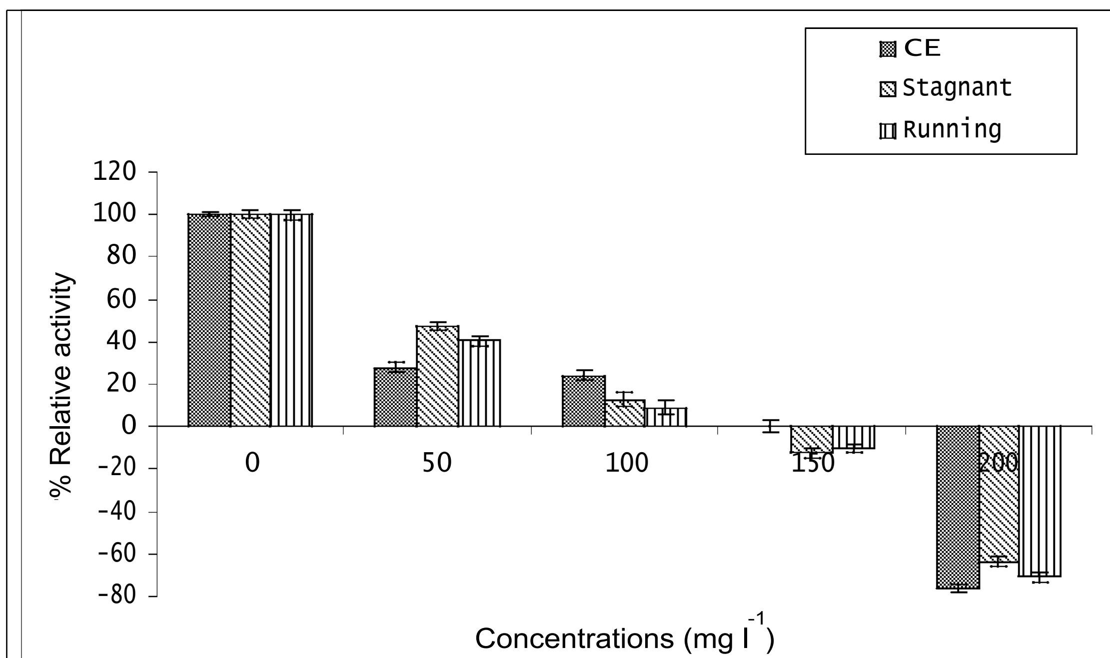


Figure 5.7: Effect of copper (copper sulphate) on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

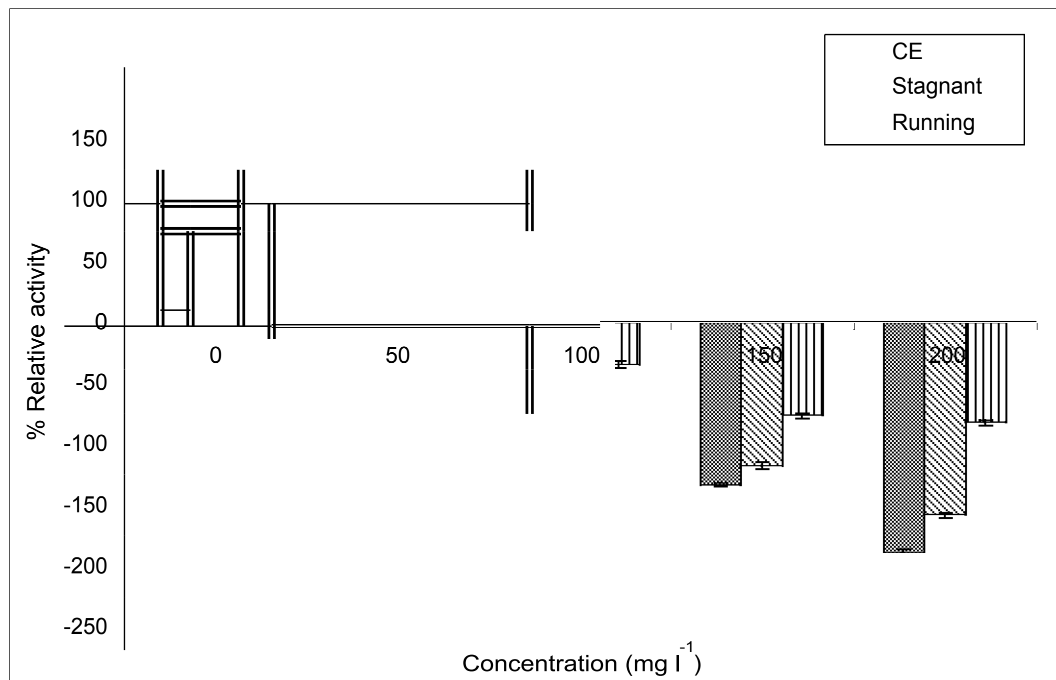


Figure 5.8: Effect of cadmium (cadmium chloride) on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

Figures 5.9, 5.10 and 5.11 show the effect of anions on B-GAL activity. Sulphate anions resulted in an increase in relative activity with an increase in concentration in B-GAL from the stagnant water sample. An increase in relative activity, though much less pronounced than that observed for the stagnant water sample, was observed with the commercial B-GAL (Table 5.2 and Figure 5.9).

Carbonate ions generally inhibited B-GAL activity at all concentrations studied. However, at a concentration of 200 mg l^{-1}, the relative activity was almost restored in both commercial and environmental B-GAL samples (Figure 5.10). An increase in pH was also observed as the carbonate concentration increased (data not shown).

In general, chloride anions resulted in a reduction in relative activity of the environmental B-GAL samples between 50 and 150 mg l^{-1}, with an increase in activity observed at 200 mg l^{-1}. A reduction in relative activity up to 100 mg l^{-1} followed by an increase in relative activity in commercial B-GAL was observed (Table 5.2 and Figure 5.11).

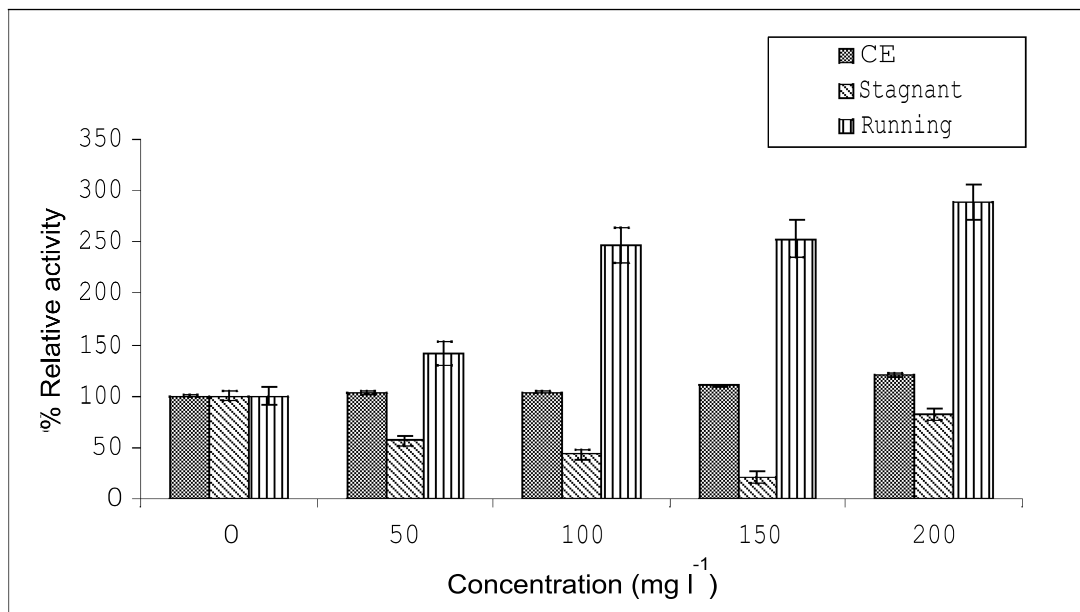


Figure 5.9: Effect of sulphate (sodium sulphate) on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

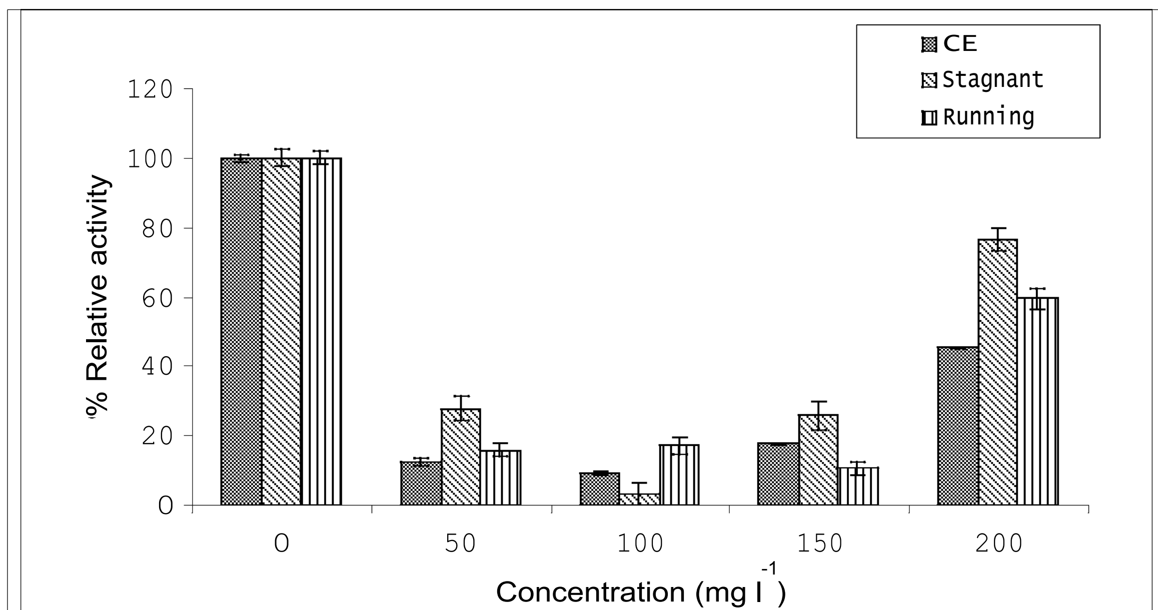


Figure 5.10: Effect of carbonate ions (sodium carbonate) on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

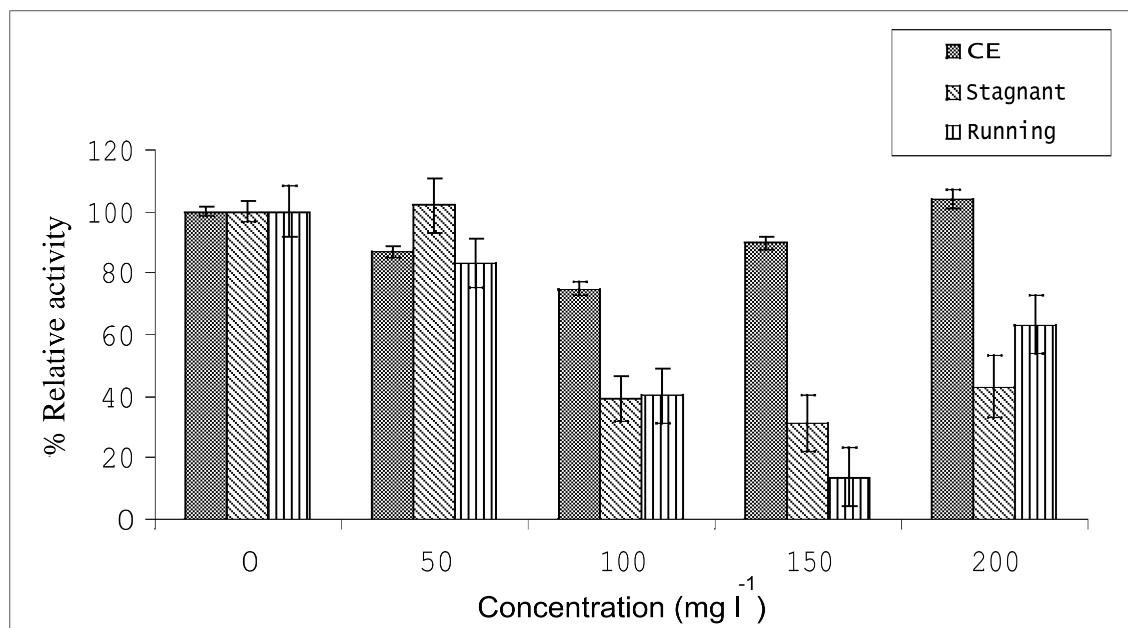


Figure 5.11: Effect of chloride ions (sodium chloride) on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

An increase in relative activity at lower concentration (50 mg l^{-1}) followed by a reduction in relative activity at higher concentrations (100 – 200 mg l^{-1}) was observed with citric acid on B-GAL from the environmental samples (Table 5.2 and Figure 5.12). An increase in the concentration of this compound, however, resulted in a gradual increase in relative activity with increase in concentration with the commercial enzyme (Table 5.2 and Figure 5.12). Citric acid or 2-hydroxy-1, 2, 3-propanetricarboxylic acid also known as tricarboxylic acid is responsible for the tart taste of various fruits in which it occurs, e.g., lemons, limes, oranges, pineapples, and gooseberries. It is obtained also by fermentation of glucose with the aid of the mould *Aspergillus niger.* **Citric acid is implicated in the tricarboxylic acid (TCA) cycle, which is important in all living cells. This cycle is involved in metabolic pathways to generate energy in aerobic organisms. Thus, this compound is readily available in the environment and may affect the activity of B-GAL.**

EDTA exhibited a marked inhibitory effect on the environmental B-GAL enzymes as compared with a gradual reduction in relative activity on commercial B-GAL (Figure 5.13).

Ferulic acid, a phenolic compound, reduced the relative activity in both commercial and environmental B-GAL over the concentrations studied (Figure 5.14). Ferulic acid is a phenolic compound produced during lignin degradation and its ability to inhibit the enzyme activities could imply that the use of direct enzyme assays in eutrophic water bodies may yield a false negative result. In such water bodies, there will be heavy decomposition of plant matter leading to the release of this phenolic compound. Carbonates chelate phenolic compounds, thereby preventing their interaction with enzymes (Wetzel, 1991). Even though the impact of phenolic compounds in hard waters may be countered by the presence of carbonates, the presence of carbonates in water samples could also yield false negative results.

Sulphite ions (as disodium sulphite) caused an increase in the enzyme activity at all concentrations studied, except at 200 mg $^{-1}$ on B-GAL from the running water sample from the environment, where a relative decrease in activity was observed (Figure 5.15). A general increase in relative activity of B-GAL was also observed with nitrate ions (comparing potassium nitrate and potassium chloride) (Table 5.2 and Figure 5.16). Nitrate ions were more potent than chloride anions in activating B-GAL activity (Table 5.2, Figures 5.2 and 5.16).

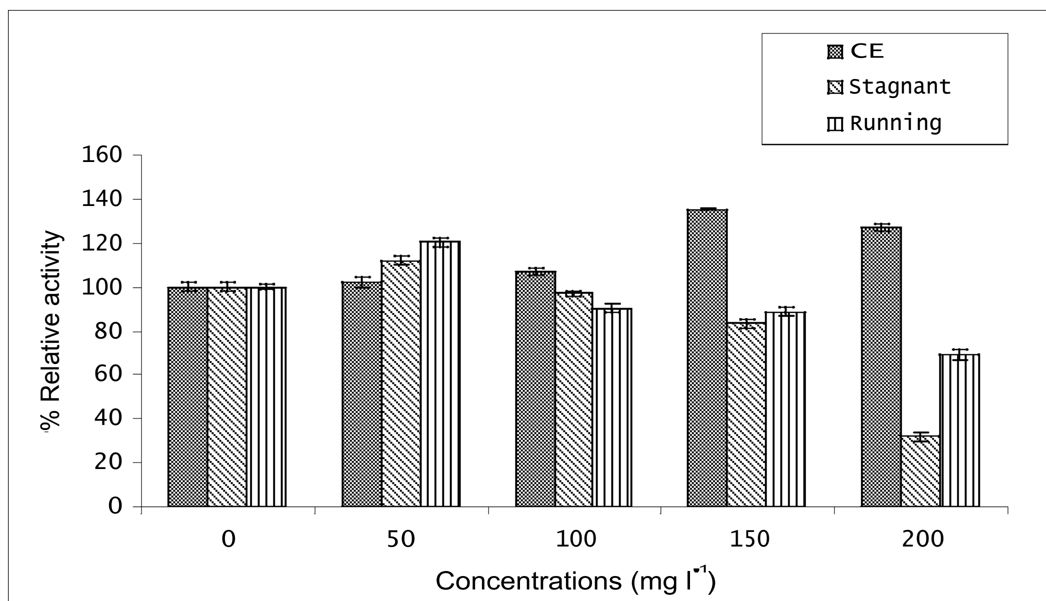


Figure 5.12: Effect of citric acid on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

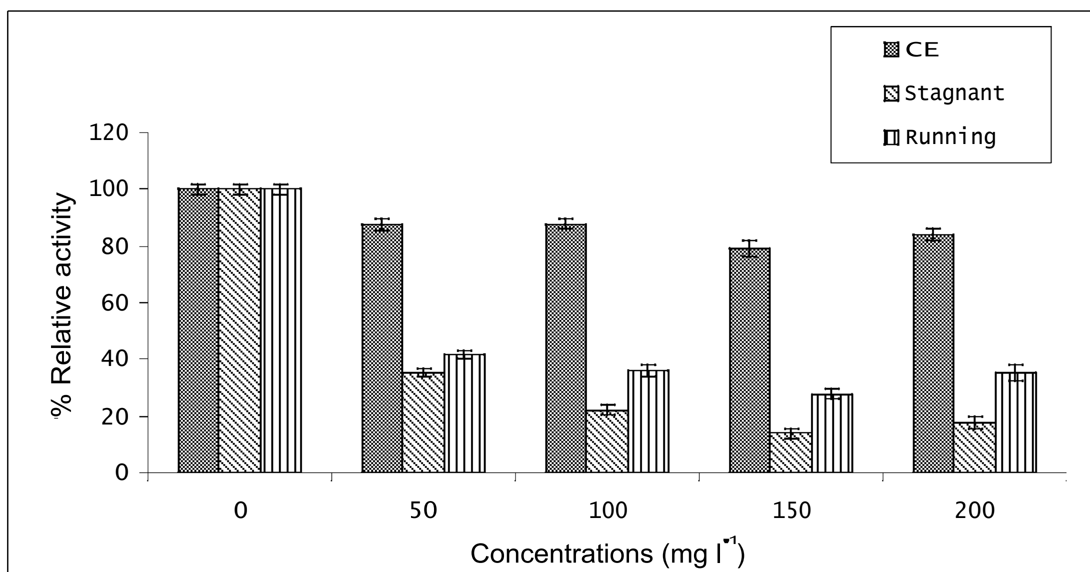


Figure 5.13: Effect of EDTA on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

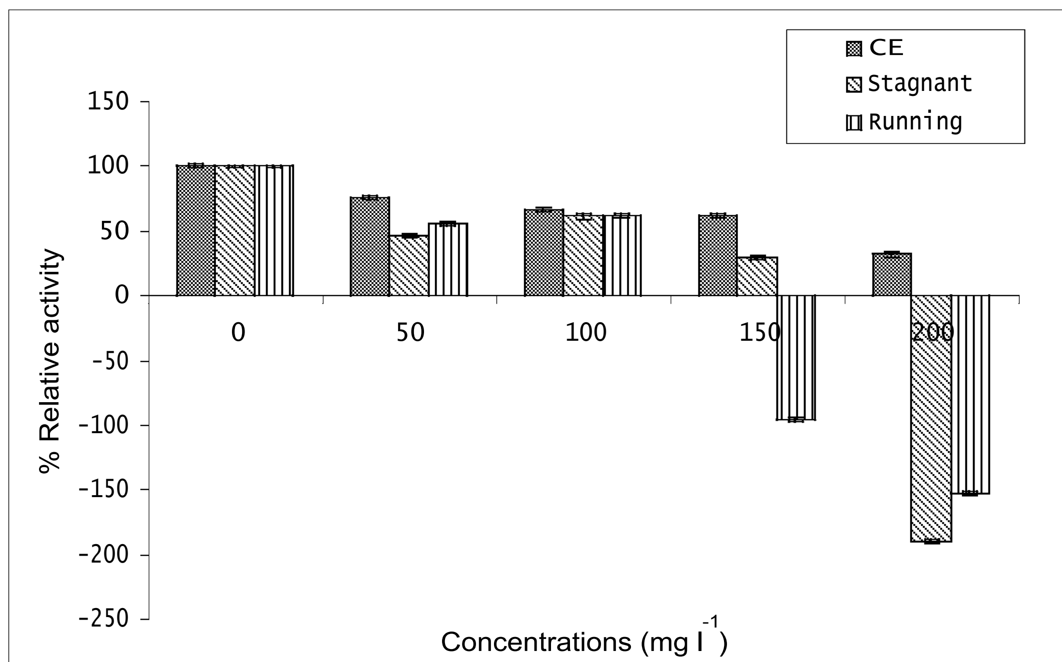


Figure 5.14: Effect of ferulic acid on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

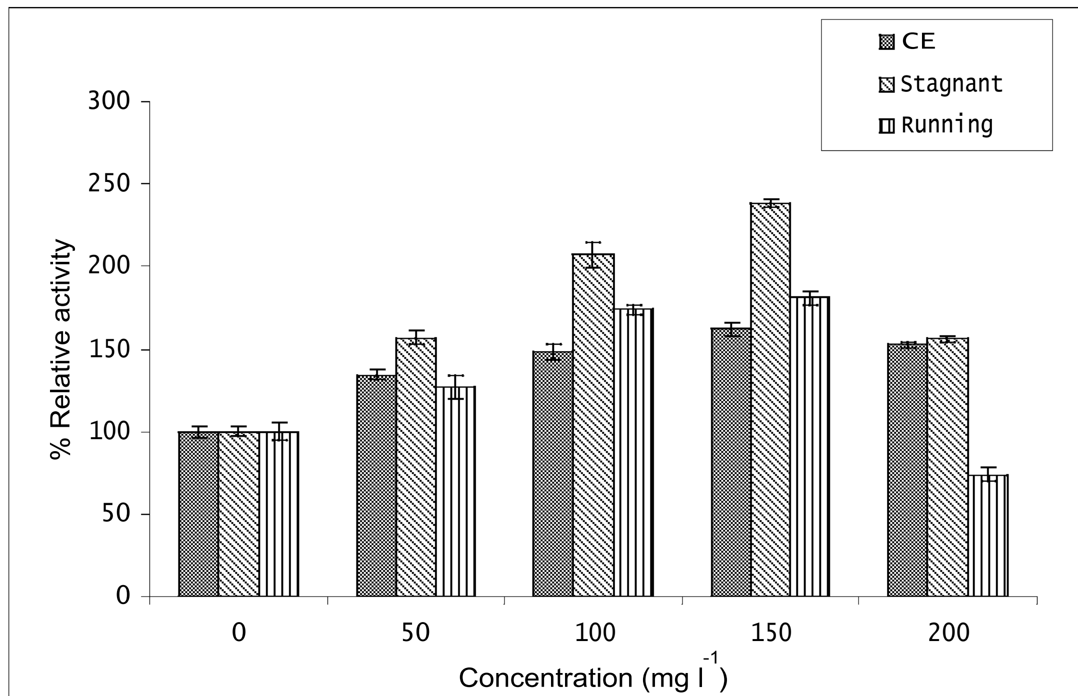


Figure 5.15: Effect of disodium sulphite ions on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

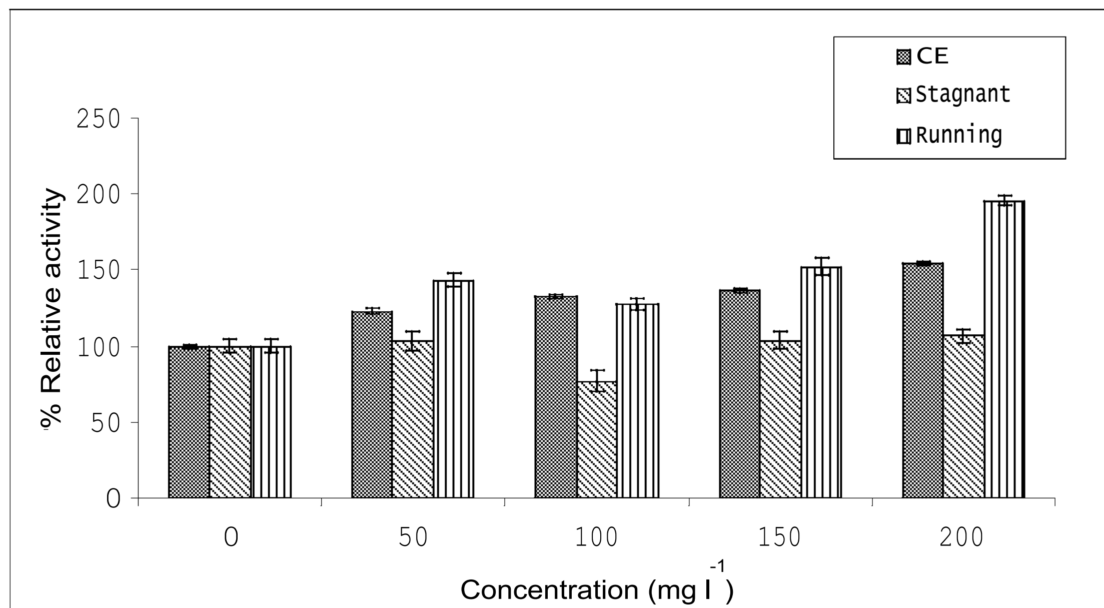


Figure 5.16: Effect of nitrate ions (potassium nitrate) on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

Sodium hypochlorite, which is normally used as a disinfectant, especially in remote rural areas, reduced the activities of the B-GAL enzymes (Figure 5.17). In this study, the addition of sodium hypochlorite resulted in an inhibition of the activity of both environmental and commercial B-GAL enzymes. Commercial sodium hypochlorite solutions sold for use as a disinfectant generally contain 10 – 14 % available chlorine. This product is diluted to achieve a target concentration of 2 mg l^{-1} (2 ppm) in the water supply. In emergency cases, 8 drops of chlorine bleach (containing 5.25-6.0 % sodium chloride active ingredient) can be added to 2 litres of water to be disinfected. Concentrations of 100 parts per million (ppm) available chlorine for routine disinfection or 500 ppm for emergency post-flood disinfection using sodium hypochlorite have been employed. The bactericidal effect of sodium hypochloride is based on the penetration of the chemical and its oxidative action on essential enzymes in the cell (Byun *et al*., 2006).

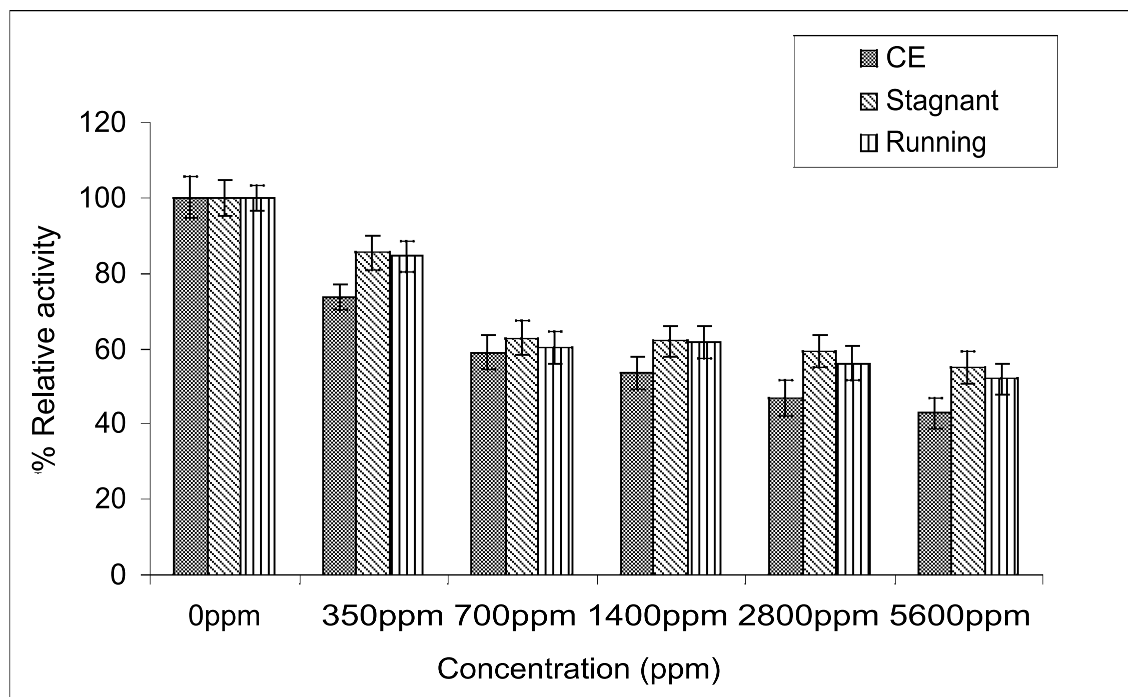


Figure 5.17: Inhibitory effect of sodium hypochlorite on environmental and commercial B-GAL (CE) activity. All values represent the mean ± SD (n=3)

The weak inhibitory effect of the common compounds found in water, like potassium and sulphate, helps to explain why the environmental water sample inhibited the commercial enzyme activity as observed in Chapter 3, Figure 3.8 (15% inhibition at 50 % of total reaction volume). The activity of B-GAL is therefore underestimated by about 15 %, due to the slight inhibitory effect the water sample had on the commercial enzyme.

5.4 CONCLUSIONS

Several compounds commonly found in the environment (such as carbonates, sulphates, chlorides) were able to affect B-GAL activity in the study. The results of enzymatic quantification of coliforms in polluted waters should therefore be stated with caution, especially when working with water samples which could potentially contain any of the metal ions studied. It may be prudent to remove some of these compounds (especially those which have adverse effect on the assay such as ferulic acid and copper) prior to assaying for B-GAL.

Sodium chloride at the concentrations studied generally reduced the activity of B-GAL. This observation has direct consequences and implications for use of this enzyme assay in marine environments; possibility of getting false negative results in marine environments.

The possibility of obtaining false positive or negative readings in the enzymatic detection of B-GAL in contaminated water should be cautiously anticipated and verified using more traditional microbiological methods. The direct enzyme assays; however, still serve as an excellent early warning sign for the potential presence of faecal material in water. The possibility of using an internal reference or standard (i.e. commercial B-GAL) should also be explored in order to assess and correct (calibrate) for chemical (ion) interference when testing environmental samples.

Table 5.2: Summary of the effect of different cations, anions and compounds on commercial and environmental B-GAL activity expressed as percentage relative activity*. Results presented in triplicate without SD values.

Ions	50 mg l^{-1}			100 mg l^{-1}			150 mg l^{-1}			200 mg l^{-1}		
	CE	S	R	CE	S	R	CE	S	R	CE	S	R
Na^{+} (NaCl)	139.0	87.6	91.8	123.0	92.9	100.0	112.8	91.9	108.9	103.5	70.5	53.6
K^{+} (KCl)	183.9	154.1	189.0	172.3	118.2	188.0	168.1	105.1	100.9	134.4	115.2	121.1
Ag^{+} (AgCl)	-52.0	-159.3	53.3	-72.0	-271.2	-198.1	-102.9	-124.7	-15.9	102.3	292.0	230.1
Mg^{2+} ($MgCl_2$)	193.9	136.0	111.4	183.0	210.8	218.7	178.1	392.9	301.4	173.0	210.2	351.0
Ca^{2+} ($CaCl_2$)	156.1	190.6	184.5	178.0	232.1	262.8	-21.4	239.6	248.3	-326.1	-400.0	-484.5
Fe^{2+} ($FeCl_2$)	125.3	148.3	112.2	157.2	31.3	60.0	102.3	16.4	28.5	45.3	-17.9	20.7
Cu^{2+} ($CuSO_4$)	27.9	47.3	40.3	23.9	12.9	9.0	0.14	-12.4	-10.3	-75.9	-63.5	-71.0
Cd^{2+} ($CdSO_4$)	-20.2	-17.5	-12.0	-53.2	-45.0	-34.0	-132.5	-117.5	-76.0	-187.9	-157.5	-82.0
Ca^{2+} ($CaSO_4$)	102.3	-101.3	-102.4	120.9	-104.3	-121.9	137.9	-183.7	-147.8	-127.9	33.7	-62.5
SO_4^{2-} (Na_2SO_4)	103.2	156.6	141.2	04.2	143.4	247.1	110.3	121.1	252.9	120.7	182.9	288.2
CO_3^{2-} (Na_2CO_3)	12.5	27.9	15.9	9.3	3.2	17.2	17.7	25.9	10.6	45.4	76.6	59.6
Cl^{-} (NaCl)	86.8	101.9	83.4	74.8	39.2	40.1	89.8	31.4	13.7	104.2	43.1	63.4
Citric acid	102.0	112.1	120.5	107.2	96.8	90.3	135.2	83.4	88.6	127.3	32.1	69.3
EDTA	87.8	35.0	41.7	87.8	22.2	36.1	79.0	13.9	27.8	83.9	17.6	35.2
Ferulic acid	76.3	46.9	55.6	66.2	61.3	61.9	62.1	29.2	-95.2	32.1	-190.0	-152.4
$Na_2S.9H_2O$	134.2	156.9	127.0	148.2	207.1	174.0	162.0	238.3	181.0	152.8	156.1	73.9
NO_3^{-} (KNO_3)	123.0	103.6	143.2	132.3	76.8	127.3	136.5	103.6	152.3	154.3	106.6	195.5

*For each ion, 100 percentage relative activity is when there is no effector in the reaction mixture. CE -commercial enzyme, R- running water sample, S - stagnant water sample.

CHAPTER SIX

CORRELATION STUDIES, SEASONAL VARIATION AND SUBSTRATE IMMOBILISATION

The spiritual life does not remove us from the world but leads us deeper into it - Henri J. M. Nouwen

6.1 INTRODUCTION

Possible sources of microbial contamination of river water sources include human and animal faeces or introduction of microorganisms by birds and insects (Paul *et al*, 1995; Nevondo and Cloete, 1999; Lehloesa and Muyima, 2000). Correlations between total coliforms and B-GAL activity have previously been reported (Farnleitner *et al*., 2001, 2002, Villarino *et al.,* 2003). This linear correlation 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 location and degree of pollution of a water body. It was therefore necessary to confirm this linearity so as to ascertain the applicability of the direct chromogenic substrate assay to our particular situation.

A good correlation was observed between β-glucuronidase and B-GAL activity and the number of *E. coli* and faecal coliform bacteria detected by cultivation (Villarino *et al*., 2003). Noble *et al*. (2003a) analysed water from beaches and areas near freshwater outlets in the southwest coast of the USA and found significant correlation between total coliform (TC) and faecal coliform (FC) in all seasons. Espigares *et al*. (1996) studying the microbiological characteristics of several water samples along the Guadalquivir river in Spain also found very significant correlations between FC and TC. Martins *et al.* (1995) observed high correlation between FC and TC in swimming pool waters.

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

The concept of immobilising proteins, enzymes and ligands to insoluble supports has received lots of attention over the years. A number of membrane-based rapid chromographic devices are readily available. Major contributing factors to the high rate of development of these chromographic devices include improvements in conjugate

technology and a growing understanding of the principles behind the immobilisation procedures. The amine groups on proteins are the most frequently used moieties used for covalent immobilisation. In most cases, pre-activation of these amines is not required, as they are readily available on the surfaces of the proteins.

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

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

The availability of a large number of support materials and methods of enzyme immobilisation leave virtually no bioactive species without a feasible route of immobilisation (Bayramoglu *et al*., 2004). Unmodified nylon surfaces are hydrophobic and can lead to substantial non-specific hydrophobic binding of ligands or proteins, which may lead to reduced purification yields or leakage into products, hence contamination, as well as reduced bioreactor performance (Isgrove *et al*., 2000)

The stability and activity of immobilised compounds, e.g. enzymes, are determined by the changes in chemical nature/conformation of the immobilised compounds that are influenced by the reaction schemes involved and the microenvironment provided by the supporting material (Su *et al*., 2005). Generally speaking, immobilisation improves stability and catalytic efficiency of the immobilised molecules.

While vast accounts of protein immobilisation protocols exist, there is limited information available on non-protein based molecule immobilisation. Bearing in mind the advantages offered by immobilising molecules, this chapter explored the possibility of immobilising CPRG on a solid support for colour strip development.

6.2 MATERIALS AND METHODS

Chlorophenol red β-D-galactopyranoside (CPRG), chlorophenol red (CPR), disodium hydrogen phosphate, dihydrogen sodium phosphate, hydrochloric acid, and sodium hydroxide were all supplied by Merck (Darmstadt, Germany). β-D-galactosidase (EC 3.2.1.23) (B-GAL); UV spectrophtometer by Shimadzu (UK); high–precision 780 pH Meter (Metrohm South Africa (Pty) Ltd) and nylon, Whatman filtration membrane (0.45 µm) and nitrocellulose membrane by Millipore (South Africa),

6.2.1 Regression studies and comparison with the MF technique

Water samples were collected from different environments in the Eastern Cape area (river mouths of Kariega, Bushmans and Sundays Rivers, a marshy area around the Swartkops estuary near Port Elizabeth, final effluent from Port Elizabeth waste water treatment plant, marine environment and freshwater bodies around Grahamstown). Microbial counts by membrane filtration (MF), as outlined by Farnleitner *et al*. (2001). Briefly, water sample volumes of 100 mℓ were filtered through membrane filters (Whatman 0.45 µm pore size) and placed on CM 1046 media (selective *E. coli*/Coliform chromogenic medium) (Oxoid) and mEndo-agar (Total coliforms) (Merck) and incubated at 37°C for 24 h, after which colonies were counted. All colonies which developed a pink colour were counted as other coliforms while those that developed a purple colour were counted as *E. coli* (CM 1046 media) (the sum of both pink and purple colony counts gave the total coliforms load of the water sample under consideration); Red colonies with metallic reflections were counted as total coliforms (m-Endo agar). These were then expressed as coliform colony-forming units (CFU) per 100 mℓ (Wutor *et al*., 2007a). Direct B-GAL assays for identical samples were also performed. Regression analyses (using Microsoft Excel) were performed for enzyme activities on microbial counts. From these data sets, a

comparison of the direct enzyme assays method and MF CFU determination was performed to determine the relationship between these two methods. Change of 0.72 in absorbance (nm) over 24 h was equivalent to 3.2 x 10^{2} CFU/100 ml.

From the regression curves obtained, the limit of detection (LOD) was extrapolated. Commercial B-GAL was diluted and their visual and spectrometric LODs were established. The visual LOD was scored as the lowest enzyme concentration that produced a visible colour change within 24 h, while the spectrometric detection limit was the lowest enzyme concentration that produced a measurable change in absorbance in the same time period.

6.2.2 Monthly trends in B-GAL activities at sampling points

B-GAL activity from the two sampling points (stagnant and running) was determined weekly for a period of over a year. Monthly results were averaged and a graph of activity versus month of the year was constructed.

6.2.3 Physical adsorption on nitrocellulose and nylon membranes

Briefly stated, nitrocellulose strips measuring approximately 30 mm x 10 mm were incubated in CPRG overnight. Varying concentrations of commercial B-GAL (1 x 10^{1} – 1.0 x 10^{-2} µg ml^{-1}, Figure 6.4) were then applied to the immobilised strips and colour changes monitored over time.

6.2.4 Immobilisation onto nylon

CPRG was immobilised on nylon according to the protocol described by Isgrove *et al.* (2000) with modification. Nylon film (with a thickness of approximately 50 µm) was cut into 10 mm x 10 mm segments, partially degraded by incubation in 2.9 M HCl for 2 h at 37 ^{o}C, washed thoroughly in water followed by 0.1 M Na phosphate buffer at pH 7.8. The strips were then incubated in CPRG solutions overnight at 4 ^{o}C and air-dried. Varying concentrations of commercial B-GAL (10 – 1.0 x 10^{-2} µg ml^{-1}) were then applied to the treated strips and the colour changes were observed over time.

6.2.6 Adobe colour scoring

Colour scoring with Adobe Photoshop 8.0 (Adobe Systems Incorporated, USA) was performed on the immobilised strips after reaction with B-GAL.

All assays were performed in triplicate. All results were reported as means ± standard deviations and unless otherwise stated; all statistical analyses were performed with analysis of variance (ANOVA) using Microsoft Excel 2003 statistical tool at 5 % level of significance.

6.3 RESULTS AND DISCUSSION

The 24 h period *(3.2.2.1)* **of detection allowed enough time for noticeable changes in environmental enzyme activity, which could initially have been inhibited by the different pollutants present in the water environment. While increased temperatures can give more rapid results, such temperatures have been found to increase interference by pollutants. The observed detection time (24 h) compared well with the improved conventional methods (Venter, 2000; Rompré** *et al***., 2002) and the Colilert methods that make use of fluorogenic substrates (Berger, 1994; Cowburn** *et al.***, 1994; Fricker and Fricker, 1996; Grant, 1997; Sartory and Watkins, 1999). However, occasional discrepancies between rapid enzymatic assays and cultivation methods have been observed (George** *et al***., 2001, Tryland** *et al***., 2002). In many estuary field trials, rapid assays yielded a high enzymatic response with a low recovery on cultivation methods (false positive results) (George** *et al***., 2001, Tryland** *et al***., 2002).**

From the studies performed, a liquid bioprobe could be established based on the hydrolysis of the chromogenic substrate CPRG by the enzyme beta-galactosidase. The ingredients of the bioprobe are (a) assay buffer, (b) substrate, and (c) the water sample to be examined.

6.3.1 Regression studies and detection limits

There was a positive correlation between total coliforms and B-GAL ($R^2 = 0.941$, r = 0.9566 for CM 1046 media, and $R^2 = 0.7051$, r = 0.8397 for m-Endo-media) (Figures 6.1 and 6.2, respectively), even though the CFUs were not evenly distributed especially with the m-Endo media. Both correlations were highly significant ($P < 0.001$). B-GAL activity and microbial growth were observed on the plates in samples from river mouths and marine environment. The spectrometric LOD for total coliforms was 1 CFU/100 ml^{-1} using CM 1046 (Oxoid). The observed correlation for B-GAL agreed with the results ($0.73 < R^2 > 0.96$) obtained by Farnleitner *et al*. (2001) with the CM 1046 media ($R^2 = 0.941$). The m-Endo media however gave a lower correlation ($R^2 = 0.7051$). Villarino *et al*. (2003) observed a good correlation between B-GAL activity and the number of faecal coliform bacteria detected by culture.

The presence of these viable but non-culturable (VBNC) bacteria sometimes referred to as active but non-culturable (ABNC) bacteria in the environment could be important from a sanitary point of view as some authors have stated (Colwell *et al*., 1985).

In general, it can be difficult to obtain a high correlation in different environments, considering the VBNC bacteria and their degree of viability that may vary with water pollutants and nutrient concentrations. However, a correlation coefficient of above 0.8 suggests positive correlation between the organisms in question and the activity of their respective marker enzymes. The observation that B-GAL activity was present in all samples highlighted the ability of direct enzyme assaying methods to detect the presence of VBNC cells. This makes the technique more favourable and potentially more applicable to marine environments. While a result of 1 CFU 100 ml^{-1} indicates an extremely sensitive technique, these results should be stated with caution, because it does not necessarily mean that 1 CFU is entirely responsible for the observed activity. Very low LOD could also be as a result of enzyme activity from non-target sources. Van Poucke and Nelis (1997) highlighted the paradoxical nature of this concept of sensitivity of direct enzyme assay to a level as low as 1 CFU per 100 ml water sample. They argued

that an increase in sensitivity is coupled with a concomitant increase in susceptibility to interference from non-target sources. The correlation between microbial counts and enzyme activities illustrates the degree of accuracy of the MF technique and the direct enzyme assays. If the two methods provided the same results with the same degree of accuracy then the correlation coefficient would be equal to one. However, the issue of detection of VBNC bacterial enzyme activity could potentially account for the deviation of R^2 away from 1. The poor correlation between enzyme activity and CFUs in marine samples highlights the possible limitation of using this technique only with fresh water samples.

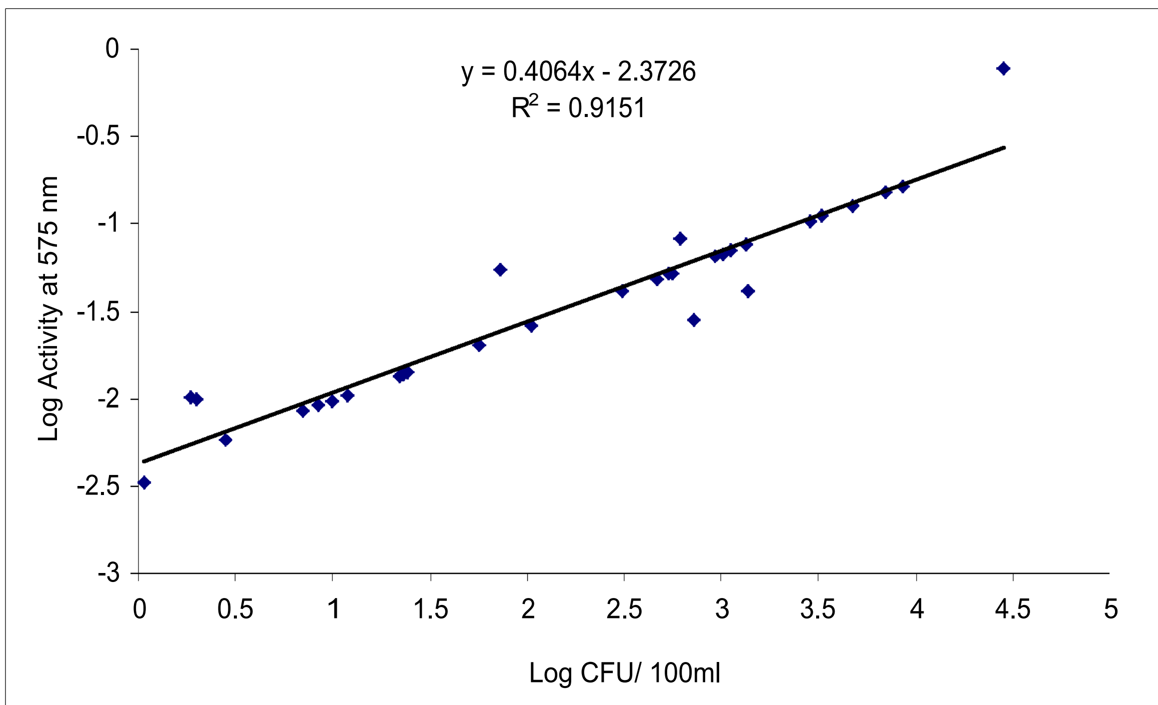


Figure 6.1: Regression of total coliform CFUs on CM 1046, Oxoid media as observed against B-GAL activity found in different water samples (n = 35).

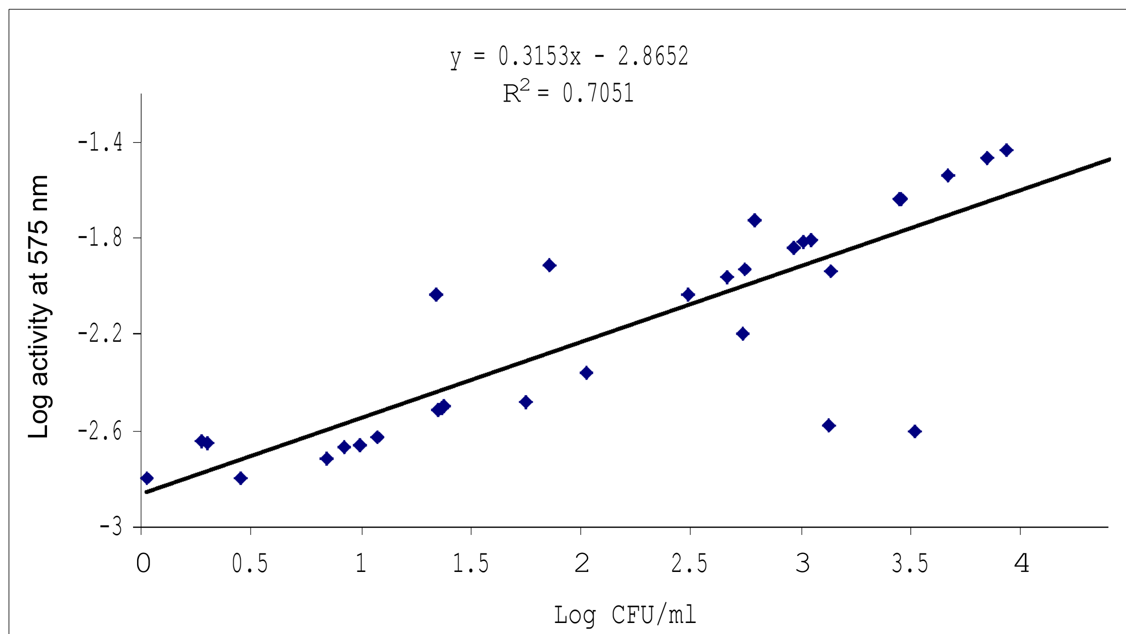


Figure 6.2: Regression of total coliform CFUs on m-Endo media as observed against B-GAL activity found in different water samples (n = 35).

The limit of detection for commercial B-GAL was at a value 0.01 µg ml^{-1} in 24 h, both spectrometrically and visually (Table 6.1). Therefore the concentrations of B-GAL can safely be determined above 0.01 µg ml^{-1}.

Table 6.1: Limit of detection of commercial B-GAL visually and spectrophotometrically

B-GAL Concentration (µg ml^{-1})	Visual and spectrometric color change
10	Instant color change
1	10 min
0.5	30 min
0.2	6 h
0.143	12 h
0.1	12 h
0.05	24 h
0.025	24 h
0.0166	24 h
0.0125	24 h
0.01	24 h

6.3.2 Monthly (seasonal) trends

The lowest activity for B-GAL was recorded in May (2.46 x 10^{2} CFU/100 ml). , while highest activity was in November (6.0 x 10^{2} CFU/100 ml) (Figure 6.3a and 3b). February yielded activity close to what was determined in September. This observation compares fairly well with winter and summer seasons with low and high water temperatures, respectively. Sampling periods immediately after rainfall mostly yielded lower enzyme activities. A possible reason for this observation may be that rainfall initially increases counts because of 'washoff' but dilutes the concentrations of coliform in the water once "washoff" is completed. Sampling directly after a rain storm and sampling days after a rain storm could therefore influence results. Contrary to the observation that a high level of faecal coliforms are 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. Sampling periods immediately after rainfall mostly yielded lower enzyme activities. A possible reason for this observation may be that rainfall initially increases counts because of 'washoff' but dilutes the concentrations of coliform in the water once "washoff" is completed. Jagals *et al.* **(1995) recorded faecal coliform counts of up to 4 400 000 100 ml^{-1} (levels equivalent to what is recorded in many raw sewage effluents) from stormwater run-off from a low socio-economic informal settlement in South Africa. FC counts exceeding safety margins in guidelines for recreational water have also been recorded for stormwater run-off from well developed urban structures in South Africa (Jagals, 1997).**

When released in natural waters, faecal bacteria were shown to lose their ability to grow on culture media while preserving some metabolic activities (Colwell *et al***., 1985, Grimes and Colwell, 1986, Roszak and Colwell, 1987, Barcina** *et al***., 1989, Garcia-Lara** *et al***., 1993, Davies** *et al***., 1995, Pommepuy** *et al***., 1996, George** *et al.***, 2000 and 2001). Loss of culturability could be due to osmotic (when released in seawater), nutritional, sunlight, toxins and competition with autochthonous flora (Anderson** *et al***., 1983, Palmer** *et al***., 1984, Barcina** *et al***., 1989, Garcia-Lara** *et al***., 1993). Among the various stresses to which enterobacteria are subjected to when discharged into aquatic systems, solar radiation seems to be the most significant in reducing coliform numbers (Davies-Colley** *et al***., 1999) probably acting in synergy with salinity (Solic and Krstulovic, 1992). The effect of simulated solar radiation on** *E. coli* **cells was mainly attributed to UV-B (290 – 320 nm) which causes loss of culturability and generation of cells considered in a viable but non-culturable state (Muela** *et al***., 2000). Most false positives results appear in summer (Tryland** *et al***., 2002).**

The enzyme activity is partly a function of the amount of enzyme present and partly of inhibitor concentration. Low enzyme activity may also be attributed to a high level of enzyme dilution. Higher activity obtained during warm periods may be explained by high microbial activity under such temperatures and mixing aided by convection currents. Significant and positive correlation between microbiological activity and water temperature was reported by Martins *et al***. (1995) in swimming pool waters.**

The monthly (seasonal) changes in the enzyme activity could be due to factors ranging from changes in domestic, agricultural and social habits. Livestock activities could also affect enzyme activity. The highest B-GAL activity coincided with the highest temperature recorded. A pH range of less than two units was observed throughout the year (Figure 6.3a). Major determinants in daily and seasonal variations are temperature and volume of runoff. Warm temperatures enhance microbial growth, thereby increasing enzyme production and activity. In addition, increases in temperature speed up degradation of organic compounds, thus providing nutrients for the microbes, leading to more enzyme activity. Therefore, warm seasons may exhibit high enzyme activity. Increases in temperature also allow the mixture of stagnant water bodies by convection currents.

Also, during storms, runoff introduces allochthonous material into the water bodies. Depending on the quality and quantity of such material, enzyme activity and quantity may be enhanced or reduced. Such a variation has bearing on the time of sampling and determination of faecal material.

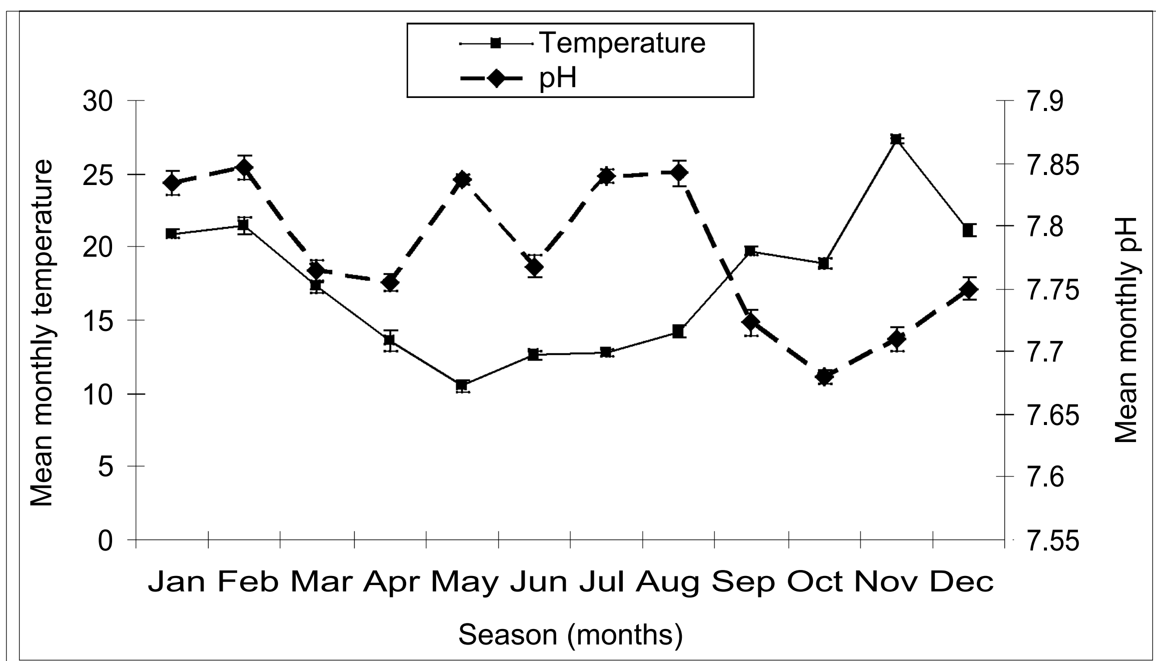


Figure 6.3a: Seasonal (monthly) pH and temperature variations at the two study sites. All values represent the mean ± SD (n=3).

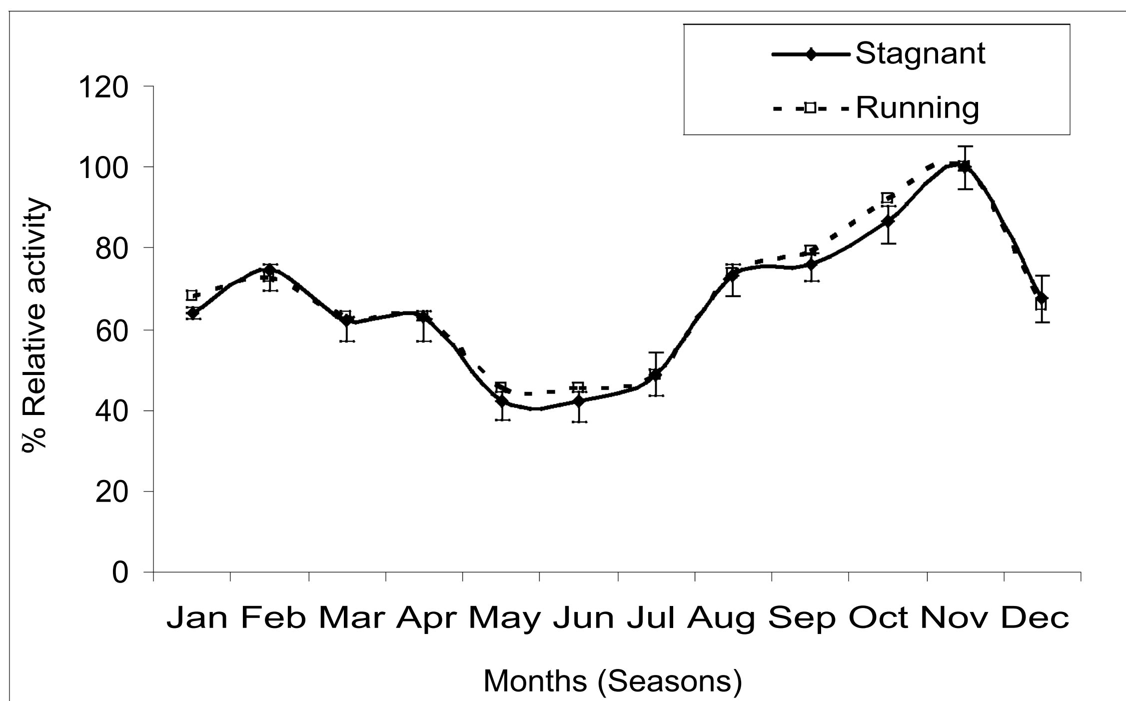


Figure 6.3b: Seasonal (monthly) variations in B-GAL activity at the two study sites. All values represent the mean ± SD (n=3).

6.3.3 Physical adsorption

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

6.3.4 Immobilisation on partially degraded nylon membrane

A 10 µg ml^{-1} solution of B-GAL resulted in an instant colour change from yellow to red, while concentrations at 1.0 x 10^{-2} µg ml^{-1} and above resulted in a colour change within 24 h (Table 6.2 and Figure 6.4). The visual limit of detection (LOD) for B-GAL in 1 h was 0.143 µg ml^{-1} (Table 6.2). Very limited colour diffusion from the support material was noted after addition of the enzyme solutions (Figure 6.4). The extent of stability of immobilised compounds depends on the structure of a compound, the immobilisation method and the type of support (Bayramoglu *et al*., 2004). From the results obtained, it is clear that the type of membrane greatly influenced immobilisation. The membranes,

although resulting in a reasonable degree of immobilisation, resulted in diffusion of the CPRG upon addition of enzyme. It was therefore evident that physical adsorption alone was not desirable for immobilisation of CPRG. Isgrove *et al*. (2000) reported that desorption commonly occurs in physically adsorbed molecules. The observed diffusion in colour of the chromogen did not allow for accurate colour observation on the biostrips. In addition, this strip will require a limited exposure time to the enzyme solution, which may not be adequate to allow enzyme hydrolysis of the substrates in environments characterised by low enzyme levels.

The nylon strips yielded better results than their nitrocellulose counterpart. The limited diffusion observed could be attributed to saturation of the functional groups on the membrane. The partial degradation of the nylon exposed functional groups that reacted with the substrate, yielding more stable bonds as opposed to physical adsorption alone.

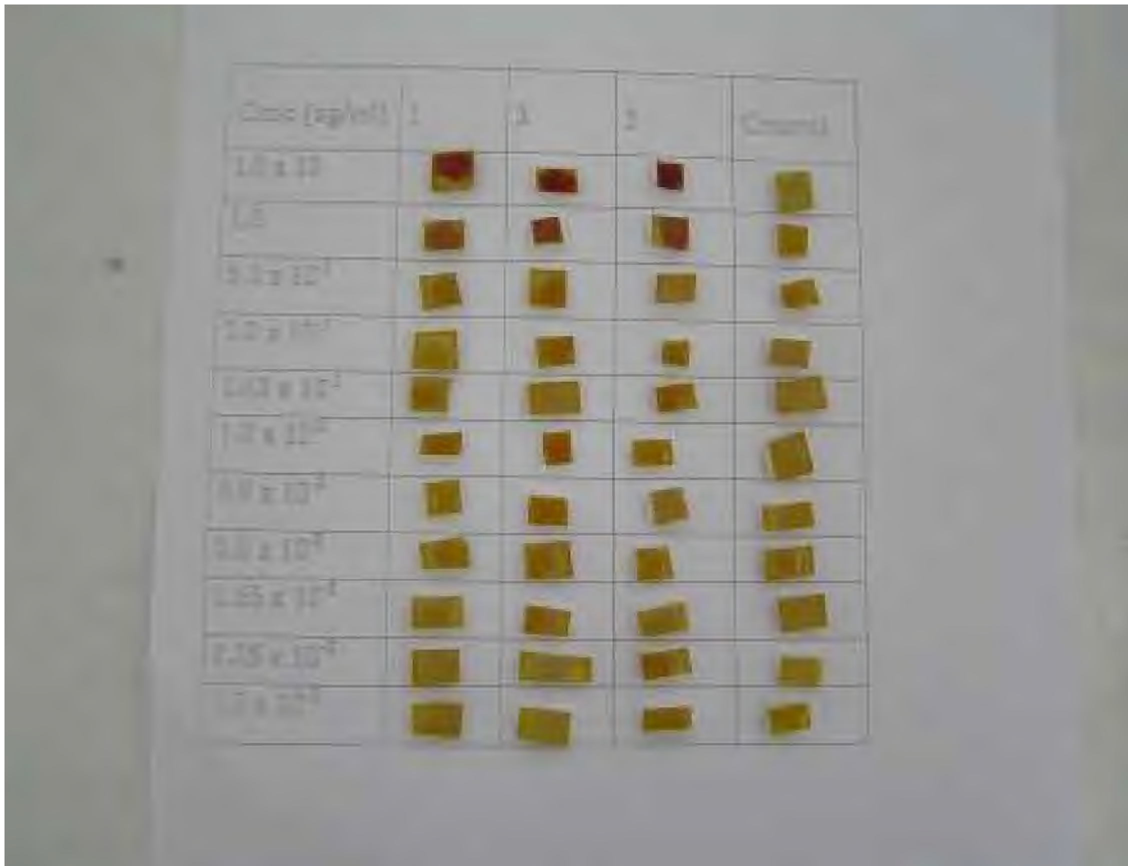


Figure 6.4: Colour changes due to immobilised CPRG hydrolysis by different commercial B-GAL concentrations.

Table 6.2: Detection of B-GAL on CPRG immobilised on partially degraded nylon membrane strips over a 24 h time period.

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

6.3.5 Adobe colour scoring

The limit of detection with Adobe Photoshop colour scoring for B-GAL was 0.05 $\mu g\ ml^{-1}$. A significant ($P<0.05$) correlation ($R^2 = 0.9824$) was observed between the colour change and the concentration of B-GAL (Figure 6.5). The range of linearity of B-GAL concentration and colour change extended from 0.05 to 100 $\mu g\ ml^{-1}$.

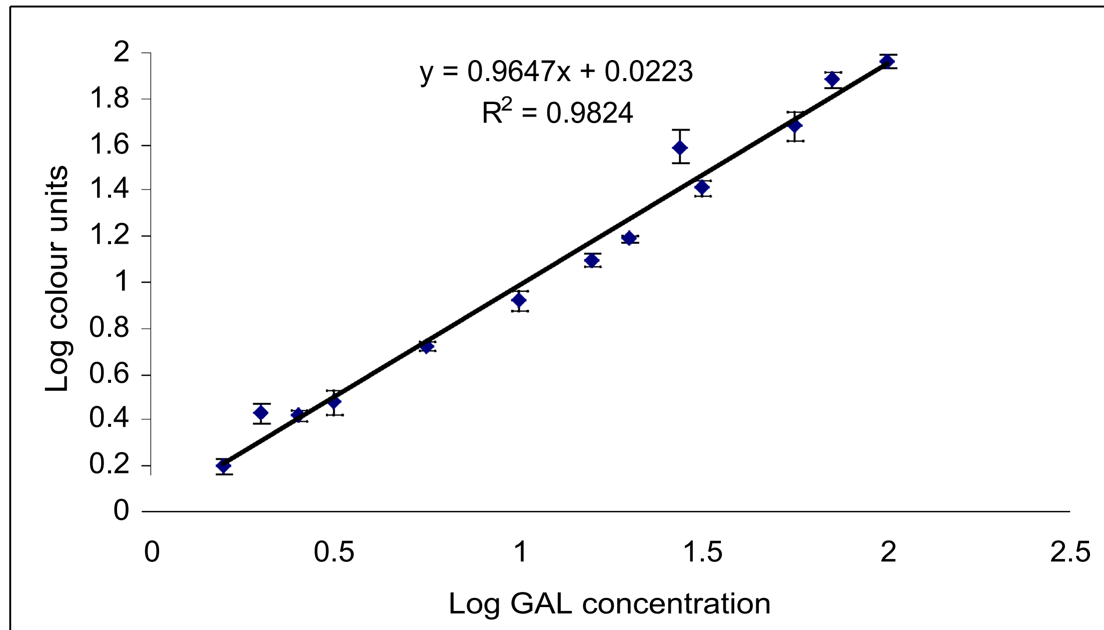


Figure 6.5: Double log plot for Adobe colour units versus B-GAL concentration on hydrolysis of CPRG immobilised on partially degraded nylon membrane.

6.4 CONCLUSIONS

The concept of direct enzyme assay, using the chromogenic substrate CPRG for B-GAL assay is a feasible alternative method for the detection of faecal pollution in South African river waters. However, there was a need to investigate the possibility of immobilising the substrates on a solid support to enable ease of handling and prolonged shelf life for solid bioprobe design.

B-GAL solution of concentration 10 µg ml^{-1} and above resulted in an instant visible colour change, while concentrations of 2.5 x 10^{-2} µg ml^{-1} and above yielded a colour change within 24 h.

Immobilisation of the CPRG substrate on modified nylon membrane was therefore a feasible approach. Physical adsorption on both nylon and nitrocellulose membranes was, however, not feasible due to rapid desorption of the substrates from the solid support.

CHAPTER SEVEN

ELECTROCHEMICAL DETECTION AND BIOSENSOR DESIGN

And the shifting of the winds and the clouds, pressed into service betwixt heaven and earth, are signs to people who understand them - Koran

7.1 INTRODUCTION

A practical sensor for direct or indirect microbial detection which can satisfy market requirements, such as short analysis time, cost-effective instrumentation, high sensitivity and suitability to on-line monitoring of industrial processes and environmental surveying is not readily available (Cullen *et al*., 1990; Scheller *et al*., 1998; Ercole *et al*., 2002).

As mentioned previously, β-D-galactosidase (B-GAL), an enzyme produced by coliforms, catalyses the breakdown of lactose into galactose and glucose and has been used mostly for enumerating the coliform group of bacteria (Frampton and Restaino, 1993; Davies and Apte, 1999; George *et al.*, 2002). Chromogenic and fluorogenic substrates produce colour and fluorescence upon breakdown by specific enzymes. Bacterial diagnosis based on enzymes has attracted extensive research over the years (Seeber and Boothroyd, 1996). Several chromogenic substrates are available for such purposes (Manafi, 2000). The chromogene chlorophenol red β-D-galactopyranoside (CPRG) (yellow in colour) can be broken down by the enzyme β-D-galactosidase into the phenolic molecule chlorophenol red (CPR) (red colour) (Appendices B1, B2 and B3) (Seeber and Boothroyd, 1996; Pelisek *et al*., 2000b), the detection of which provides a premise for the development of sensor technology for monitoring of total coliform contamination.

Studies have reported on the use of electrochemical biosensors and chemosensors for the detection and/or monitoring of microbial contamination (Calvo-Marzal *et al*., 2001; Leonard *et al*., 2003, Paitan *et al*., 2003).

In principle, biosensors use biological materials, such as enzymes, antibodies or whole cells immobilized on a physical transducer such as electrochemical, optical, thermometric, piezoelectric or magnetic systems. The transducer produces an electrical signal in recognition of the analyte under investigation (Covington, 1994; Kutner *et al*., 1998; Thévenot *et al*., 1999, 2001). Chemosensors are similar except that the recognition agent is synthetic in origin. Most biosensor and chemosensor signal transductions are based on electrochemical techniques.

Electrochemical transduction is preferred because it can be performed with simple, compact and easy to transport equipment, it is inherently sensitive and also offers specificity through the introduction of chemical or biological recognition molecules. The techniques are readily adaptable for on-line monitoring, rapid and amenable to miniaturisation (Skoog *et al*., 1996; Paitan, *et al*., 2003) and automation (Freire *et al.*, 2001, 2002). Another advantage of electrochemical monitoring is that the results are not as easily affected by solution turbidity as are photometric assays (Scouten *et al*., 1995). Simultaneous measurements of several samples can be achieved through the use of electrochemical methods. Above all, cheap disposable electrodes may also be developed for once-off testing (Marrazza *et al.*, 1999a, 1999b; Freire *et al.*, 2002, 2003). Biosensors can make ideal sensing systems to monitor the effects of pollutants on the environment due to their biological basis, ability to operate in complex matrices, short response time and small size (Freire *et al.*, 2001, 2003).

There are several requirements for the development of chemical and biological sensing technology including sensitivity, selectivity (for the analyte in a complex matrix), reproducibility of the sensor response and stability (in terms of shelf-life). When tested in a real sample, additional factors are considered in terms of the stability of the sensor in the presence of fluctuating conditions such as pH and temperature as well as the presence and effect of interferents. Selectivity and sensitivity are important factors which help to establish the ability of the product to detect compound(s) of interest from others. This is often achieved through electron mediators or catalysts (such as metallophthalocyanines) or electrode modifiers such as enzymes which show specificity for the target analyte.

Electrochemical sensors are subject to fouling which affects the reproducibility and hence reusability of the sensor. The effect of fouling then determines to a large extent the type of detection system to be developed; be it a disposable or continuous electrode.

Additional considerations for the development of sensor technology are factors such as minimal or no sample preparation, prospect of portability, low cost per product, no or low risk to the user which may also be assessed to a limited extent at the initial research stage.

7.2 MATERIALS AND METHODS

7.2.1 Materials

Sodium phosphate buffer (0.1 M, pH 7.2) prepared with Milli-Q system (18 MΩ cm) (Millipore, Milford, CT, USA) purified water was used for all electrochemical analyses. Phthalocyanine metal complexes of cobalt (II) (CoPc), copper (II) (CuPc), zinc(II) (ZnPc), nickel(II) (NiPc), tetrasulphonated metal complexes (MTSPc) of copper(II) (CuTSPc) and cobalt(II) (CoTSPc), chlorophenol red β-D-galactopyranoside (CPRG) and chlorophenol red (CPR) were all purchased from Sigma-Aldrich (Deisenhofen, Germany). All the reagents were of the highest analytical grade.

7.2.2 Methods

7.2.2.1 Cyclic voltammetry and the construction of MPc-modified GCE

Cyclic voltammetry experiments were performed using a BAS Epsilon EC-200-XP (Bio-Analytical Systems, Inc., USA). All measurements were carried out in a glass cell with a conventional three-electrode configuration at a scan rate of 100 mVs^{-1}. A glassy carbon working electrode (GCE) with a surface diameter of 3 mm was used together with a platinum wire counter electrode and a silver/silver chloride (3 mol dm^{-3} KCl) reference electrode. Prior to modifying the GCE with MPc complexes, the GCE was polished with alumina on a Buehler felt pad, rinsed with ethanol, followed by water, soaked in dilute nitric acid (0.5 M) and a further rinse in distilled water.

7.2.2.2 Electrode pre-treatment

Solutions of phthalocyanine complexes (1 mM) were prepared in dimethylformamide (DMF) (HPLC grade). The tetrasulphonated MPcs were dissolved in distilled water to make 1 mM solutions. Onto the surface of the GCE, 10 µl of each sample was dropped and allowed to air dry at room temperature for about 30 min. Cyclic voltammograms of

standard CPRG, CPR and the phenolic compounds tested were collected in the presence and absence of the MPc catalysts examined. Triplicate results were obtained in each case unless stated otherwise.

7.2.2.3 Electrochemical detection of substrate and product

Unless stated otherwise, the electrolyte solution consisted of 0.1 M sodium phosphate buffer (pH 7.0) and 0.004 % chlorophenol red (CPR) solution. A GCE was used against Ag/AgCl reference electrode with platinum as a counter electrode. After an appropriate concentration of substrate or chromogen was added, nitrogen was bubbled through for 10 min to remove oxygen and the electrochemical measurements (using cyclic voltammetry) were performed at the scan rate of 100 mV s^{-1} as described in Wutor *et al*., 2007c.

7.2.2.4 Linearity

Linearity of the biosensor response was studied by monitoring current generation with an increase in the concentration of CPR. The final volume was maintained at 10 ml in each case by varying the volume of the buffer solution accordingly.

7.2.2.5 Effect of temperature and pH on the MPc-modified GCE

The effect of temperature on the analysis of CPR was investigated by recording cyclic voltamograms (CVs) at different temperatures between 5 ^{o}C and 60 ^{o}C. The reaction cell was maintained at the appropriate temperature for about 15 min, after which a CV was recorded. pH dependency of the sensor was also evaluated within the pH range of 4.0 and 10.0, using sodium citrate buffer (pH 3.0-6.0, 0.1 M), sodium phosphate buffer (pH 7.0-8.0, 0.1 M) and carbonate bicarbonate buffer (pH 9.0-11.0, 0.1 M). pH ranges were 'bracketed' to check the effect of the buffer.

7.2.2.6 Effect of environmental water samples on the sensitivity of the sensor in detecting CPR

In order to determine the effect of compounds (which may be in the environmental water sample) on the sensor response for CPR detection, different volumes (0 - 50 % of the total reaction volume) were analysed for electrochemical detection. The volume differences were adjusted with the buffer to maintain a constant final volume.

7.2.2.7 Effect of fouling on the CuPc-modified GCE

In order to determine the effect of electrode fouling on the sensor response, consecutive CV scans of CPR were recorded with the CuPc-modified GCE and unmodified GCE.

7.2.2.8 Stability of the MPc-modified GCE

Single daily readings were taken with the unmodified and MPc-modified GCEs for 30 days. After each scan, the electrodes were rinsed with water and stored at 4 ^{o}C. The electrodes were allowed to reach room temperature (20 ± 2 ^{o}C) for about 15 min before a CV was recorded.

7.2.2.9 Selectivity and sensitivity of the sensor to some phenolic compounds

The selectivity and sensitivity of the sensor in detecting phenolic compounds (which may be present in the environment) were evaluated. Equimolar concentrations of chlorophenol red; 4-chloro-3-methyl phenol; 2, 4-dichlorophenol; phenol; 2-nitrophenol; 3-nitrophenol; 2, 4-dinitrophenol; 2, 4, 6-trinitrophenol and 2-chlorophenol were studied.

7.2.2.10 Correlation of CFU/100 ml and electrochemical detection based on the least time required for detection

Polluted water samples were collected from and around Grahamstown, Eastern Cape, South Africa for this study. A 100 ml water sample of each was filtered through a membrane (Whatman, 0.45 µm pore size) and placed on CM 1046 (Oxoid media) and incubated at 37 ^{o}C for 18-24 h, after which the number of colony forming units (CFUs) were counted. Aliquots of the corresponding water samples (4 ml of water sample, 5 ml of phosphate buffer and 1 ml of the CPRG) were mixed in the glass cell for electrochemical analysis (*7.2.2.3*). Readings were taken every min for the first 15 min, after which 5 min time intervals were allowed between subsequent readings.

7.2.2.11 Correlation between CFU/100 ml and biosensor detection of CPR

A total number of 35 water samples were collected from various sources in the Eastern Cape, South Africa for analysis. CFUs and electrochemical analysis were determined as described in *7.2.2.10.*

7.2.2.12 Mode of transport

The mode of transport of CPR to the electrode was studied by varying the scan rate and plotting a graph of the peak current against the square root of the scan rate.

7.3 RESULTS AND DISCUSSION

In this chapter results on the feasibility of a biosensor incorporating metallophthalocyanine catalysts for detection of chlorophenol red generated from the enzymatic degradation of CPRG is examined.

To the best of our knowledge, this is the first report on the electrochemical detection of CPR in detecting and monitoring microbial contamination of water. No reports in literature were found on the electrochemical detection of CPR as a measure of coliform activity.

Results obtained with plain GCE at 20 ± 2 ^{o}C were considered as 100 % and all other results related to this value in the definition of relative sensitivity.

7.3.1 Detection of CPR at MPc-modified electrodes

Multiple peaks and interference were noted during voltammetric reduction of CPR and CPRG, therefore making it difficult to monitor the conversion of CPRG to CPR. CPR and CPRG were reduced at -0.52 and -0.50 V respectively. However, the oxidation window allowed the determination of CPR without any interference in the presence of CPRG. Thus the oxidation window was adopted for subsequent analysis.

An oxidation wave at 0.72 V was observed for standard CPR as well as CPR generated as a result of the enzymatic breakdown of CPRG by B-GAL. The oxidation wave attributed to CPR was observed with no interference from CPRG and the enzyme at the GCE as shown in Figure 7.1. Increases in current response for detection of CPR were observed at CuPc modified electrodes compared to a bare GCE (Figure 7.2).

The sensor response was dependent on the metal, as well as the substituents on the phthalocyanine ring, with increase in the detection of CPR, relative to the bare GCE, ranging from 21 % for NiPc to 147 % for CuTSPc, in the following order: CuTSPc >

ZnPc> CoTSPc> CuPc> NiPc (data presented in Table 7.1). The modification of electrode surfaces with NiPc has been reported as a way to design selective and sensitive sensors for the electrochemical detection of certain biological analytes such as nitric oxide (Lantoine *et al.*, 1995; Bedioui *et al.*, 1996; Caro *et al.*, 2003).

One of the most important observations of MPcs is their natural tendency to adsorb to electrode surfaces, effectively producing a stable chemically modified electrode at which catalytic oxidation can occur at lowered potentials. It has been reported that each MPc exhibits a slightly different voltammetric response related to its mode of interaction with the electrode and the substrate under investigation (Mho *et al.*. 1995).

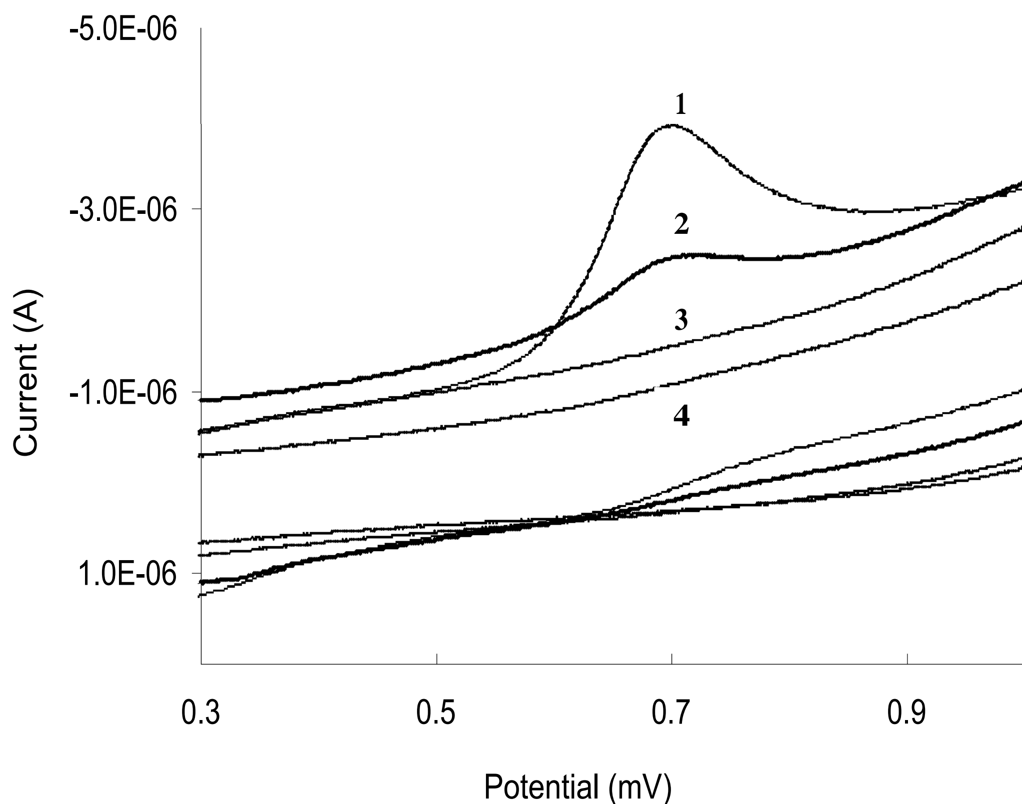


Figure 7.1 Comparative cyclic voltammograms (CVs) of (1) standard CPR, (2) CPRG and B-GAL in buffer (peak due to partial breakdown of CPRG to CPR), (3) standard CPRG, and (4) sodium phosphate buffer (0.1 M, pH 7.2).

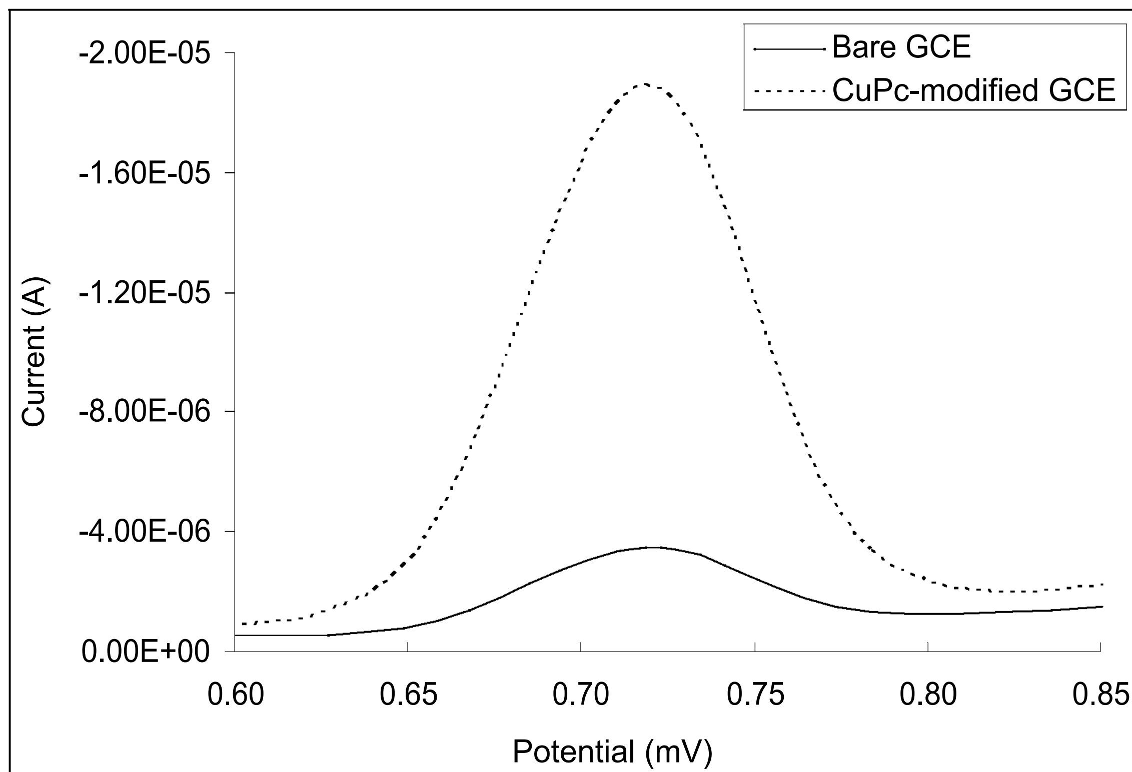


Figure 7.2 Comparative cyclic voltammograms (CVs) of standard CPR at a bare electrode and at a CuPc-modified GCE.

7.3.2 Linearity of biosensor detection of CPR

CPR detection was linear up to 0.006 µg ml^{-1} (standard error of deviation range of 0.002-0.230) with wide margins of deviation thereafter (standard error of deviation range of 0.321-0.576) (Figure 7.3). A concentration of 0.004 µg ml^{-1} was thus selected for all further studies requiring the use of commercial CPR.

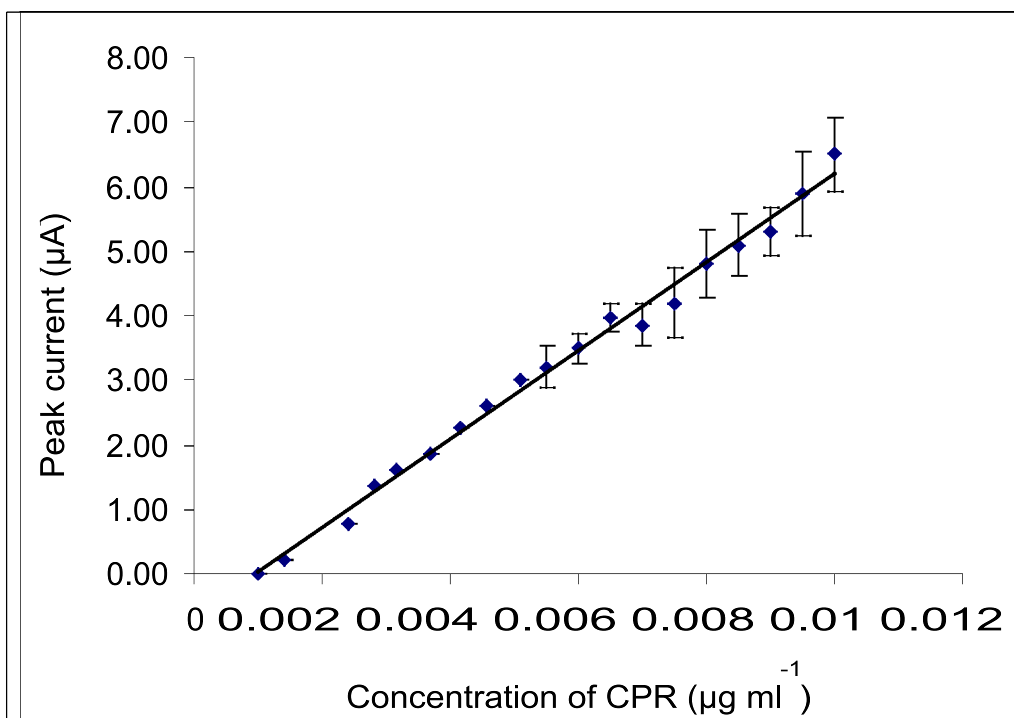


Figure 7.3: Linearity of biosensor detection of CPR on bare GCE. All values are reported as means ± SD (n=3).

7.3.3 Effect of temperature and pH on the MPc-modified GCEs

Electrodes modified with MPcs were generally sensitive to changes in pH for the detection of CPR. A reduction in sensitivity from acidic pH (pH 4) to alkaline pH (pH 10) was observed. The CuTSPc, however, was more stable with minimum change of sensitivity (about 17 %) as compared with ZnPc, where the relative sensitivity changed from 73 % at pH 10.0 to 125 % at pH 4.0 (Figure 7.4).

The selected MPcs were variable in their responses to temperature fluctuations. ZnPc was the most susceptible to temperature change, while CuTSPc was the most stable (Figure 7.5). A 7 % change in sensitivity (for ZnPc) between 15 and 60 ^{o}C was not significant, thus the MPc sensors were not subject to sharp changes in sensitivity with temperature variation (Figure 7.5).

MPcs tend to aggregate in solutions due to intermolecular interactions and the lability of axial ligand for water molecules. The ionic strength, temperature and concentration of the reacting media are important factors in the aggregation phenomenon. Previous research from pH studies of CoTSPc adsorbed at GCE showed that higher polymerisation occurs at low pH, high concentrations and ionic strength (Zecevic *et al.*, 1995). The aggregation of PCs could lead to the reduction of sensitivity of the sensor.

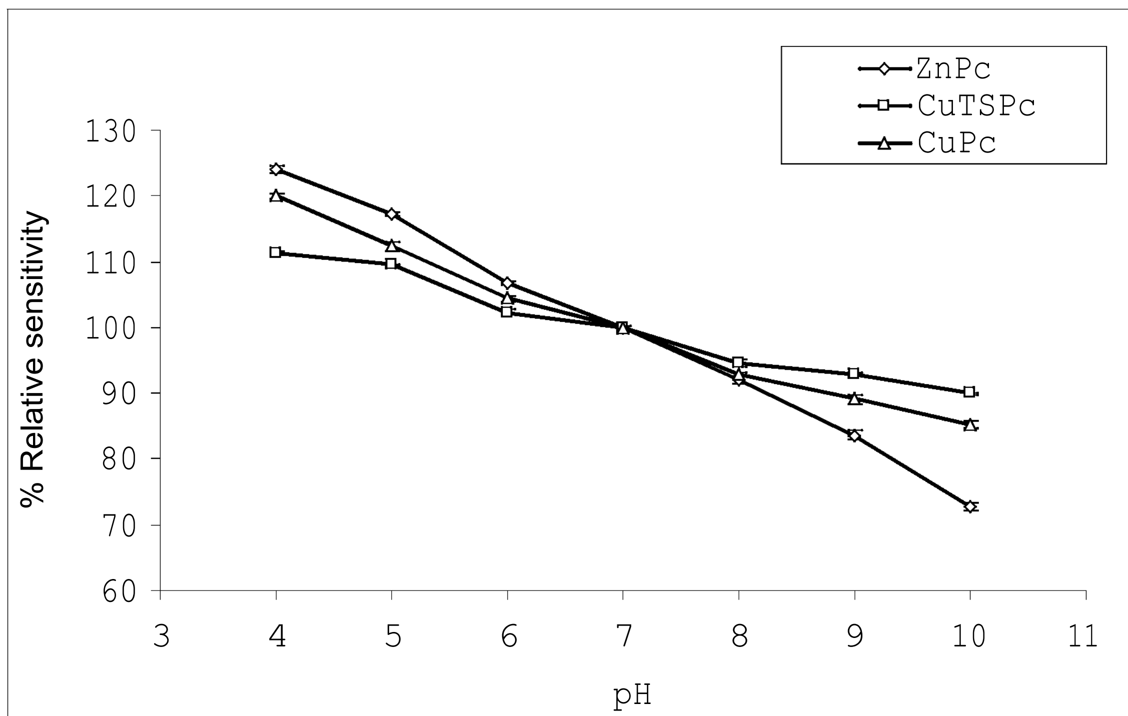


Figure 7.4: The stability of selected MPc modified GCEs to varying pH (4-10) for the detection of CPR (0.004 µg ml^{-1}). All values are reported as means ± SD (n=3).

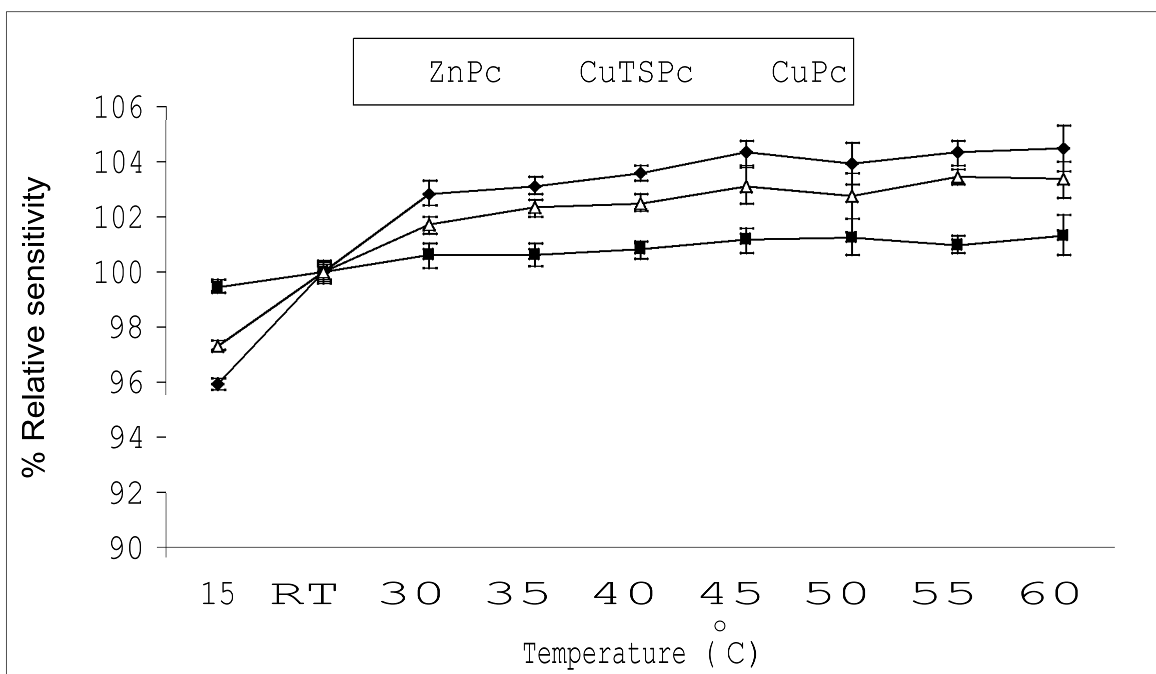


Figure 7.5: The effect of temperature on selected MPc modified GCEs for the detection of CPR (0.004 µg ml^{-1}). All values are reported as means ± SD (n=3).

7.3.4 The effect of environmental water samples on the sensitivity of the electrochemical detection of CPR

Figure 7.6 shows the effect of environmental water samples on the sensitivity of CPR detection electrochemically at a CuPc modified GCE. There was no significant change in sensitivity observed with volumes up to 50 % of the total assay volume. This study was aimed at determining the influence of the environmental water samples (containing pollutants) on the biosensor. Thus, there may not be a need to apply a correction factor in the use of the biosensor on environmental water samples, unless there is cause to believe that the water sample under investigation contains high levels of compounds which may potentially interfere with the B-GAL assay.

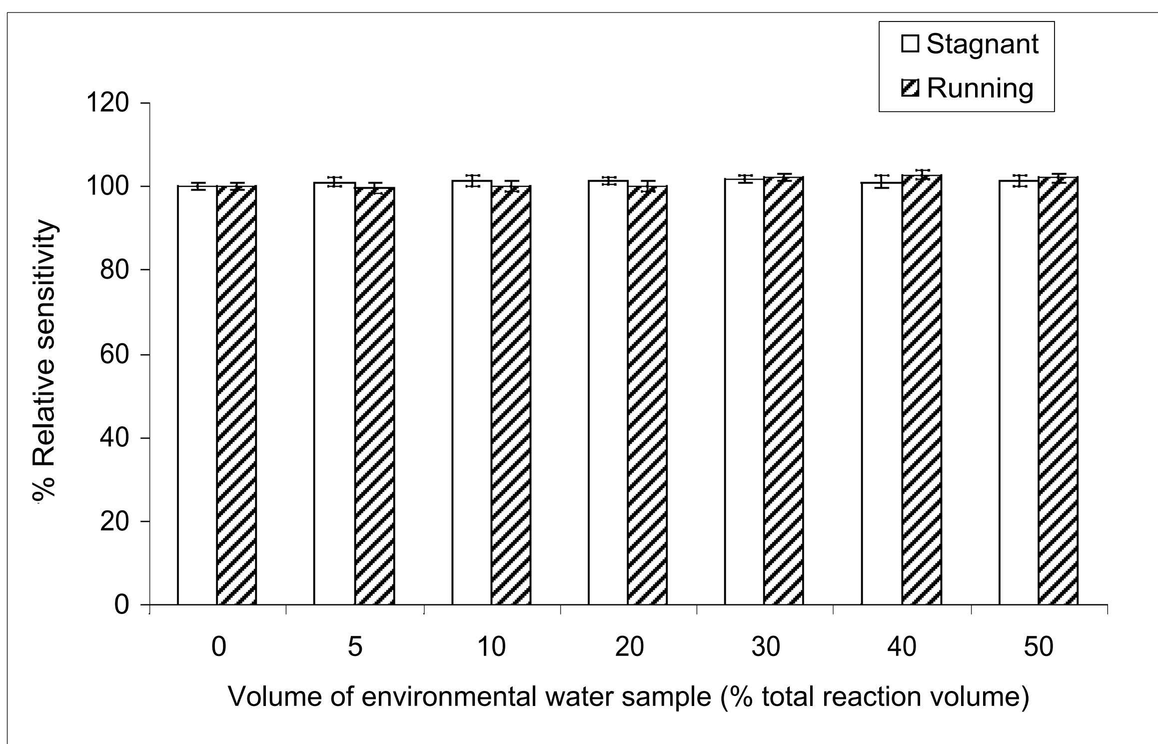


Figure 7.6: The effect of environmental water samples on CPR detection (0.004 µg ml^{-1}) on the CuPc modified GCE biosensor. All values are reported as means ± SD (n=3).

7.3.5 Effect of fouling on the MPc-modified GCEs

Electrodes modified with MPc complexes all showed a decrease in fouling for CPR detection relative to the unmodified electrode as indicated by the number of successive scans possible using the same electrode surface (Table 7.1).

The first 10 CV scans for detection of CPR at a CuPc modified GCE maintained 100 % sensitivity, after which there was a subsequent reduction in sensitivity up to the 50th scan when the results became inconsistent (Figure 7.7). Beyond the 50th scan, a fluctuation in the peak currents was observed.

In general, electrooxidation of phenolic compounds results in the formation of radical intermediates which dimerise or polymerise at electrode surfaces. These oxidation products are problematic as they lead to the passivation of the electrode (Ežerskis and Jusys, 2001; Ureta-Zeñartu *et al***., 2002). Increased sensitivity in the detection of phenols, as well as a lowering of the fouling of the electrode can be obtained through the use of catalysts such as metallophthalocyanine (MPc) complexes (Mafatle and Nyolkong, 1997).**

It has been suggested that the decrease in fouling observed in the presence of the MPc catalyst is due to the minimisation of the adsorption of the oxidation products onto the electrode as result of steric hindrance caused by the MPc (Mafatle and Nyokong, 1997). This may be the case in our study, given the size of the CPR compared to phenols examined. However, the absence of fouling within the first ten successive scans using the CuPc modified GCE, suggested that other factors may increase the resistance to fouling. It was possible that intramolecular rearrangement may have occurred following hydrogen abstraction without the formation of complex polymeric structures which is believed to foul the electrode (Wutor et al, 2006). However, further analysis by spectroscopic methods of the oxidation products formed is required for accurate characterisation of these products.

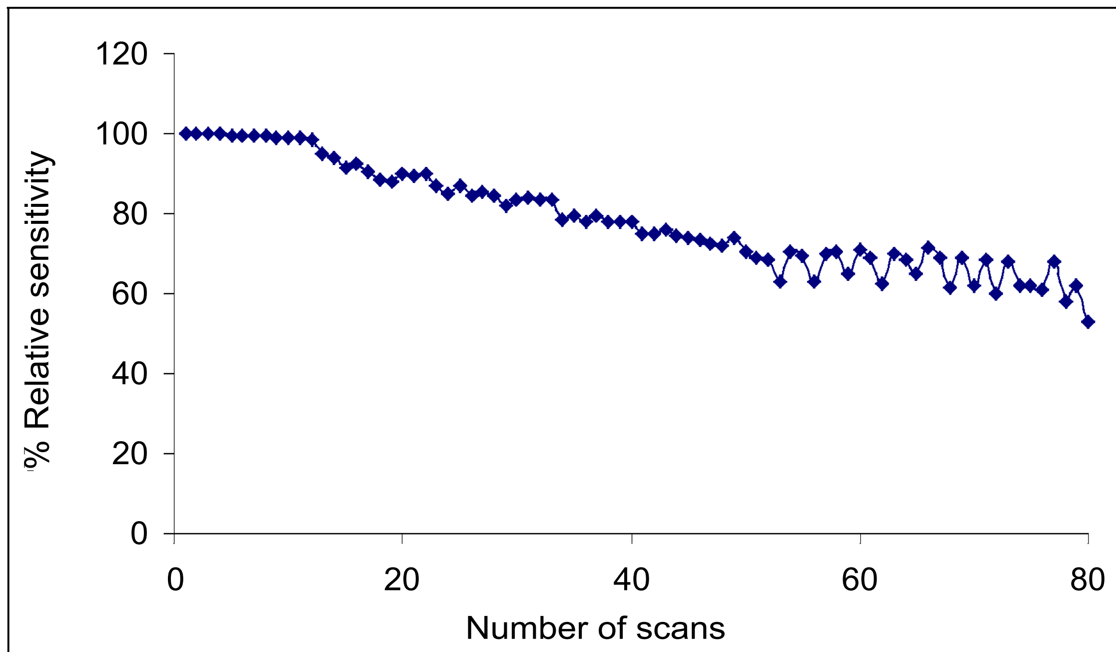


Figure 7.7: Successive voltammetric runs with the CuPc-modified GCE, (2 minute time intervals between successive readings were allowed).

7.3.6 Stability of the MPc-modified GCEs in the detection of CPR

The stability of the selected MPc-modified GCE over time for the determination of CPR was illustrated by studying the daily variation of the current responses. As observed in Figure 7.8, the CuPc-modified electrode for CPR detection was more stable than the other electrodes, maintaining 40 % of its sensitivity over a 30 day period. There was minimal change in sensitivity over the first 5 (five) days of scanning at this electrode. The least stable electrode was the unmodified GCE, experiencing an 80 % loss of sensitivity in seven days. The CuTSPc modified electrode lost 58% sensitivity after 13 days while the ZnPc modified electrode lost 60 % sensitivity after 6 days.

These results compare well with the performance of the electrode in terms of resistance to fouling where a similar trend was observed, with CuPc modified electrode being most resistant to fouling followed by CuTSPc and ZnPc. The stability of the electrodes over time may thus be linked to an extent with the resistance to fouling offered by MPc modification of the electrodes.

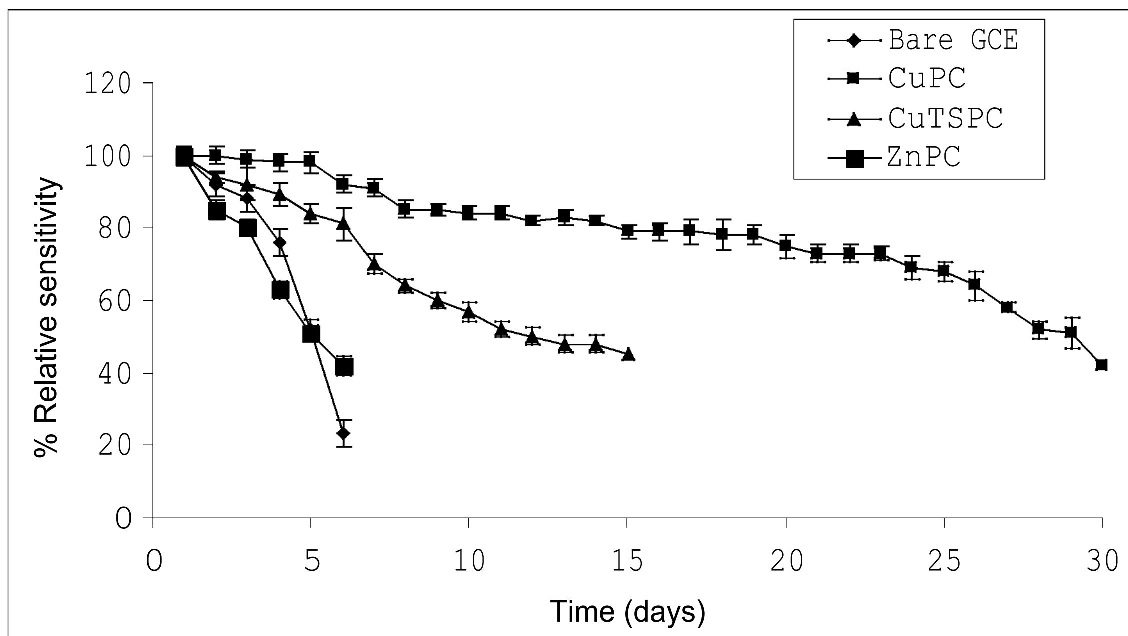


Figure 7.8: Stability of the MPc-modified and unmodified GCE over a 30 day period. All results reported as means ± SD (n=3).

Table 7.1 shows the results of the cost benefit analysis of Pcs which detected CPR over a wide concentration range, comparing cost with sensitivity and resistance to fouling Cobalt, manganese, magnesium and iron Pc modified GCEs did not detect CPR and thus were not presented in the table.

The CuPc modified-GCE thus not only increased sensitivity, but also reduced fouling compared to the bare GCE. The tetrasulphonated complex of copper increased sensitivity over 100% relative to the plain GCE and permitted 10 consecutive scans in comparison with the plain unmodified GCE (Table 7.1). From the results obtained above and cost considerations, CuPc, CoTSPc and ZnPc were selected for further studies.

Table 7.1: Cost benefit analysis of phthalocyanine metal complexes studied

MPc	%Relative sensitivity	# of scans	Cost/g
Plain GCE	100	2	
Nickel Pc	121.2	5	ZAR 328.60
Copper Pc in DMF	137.3	77	ZAR 788.90
Cobalt TSPc in DMF	151.8	10	ZAR 570.00
Zinc Pc	158.8	3	ZAR 89.70
Plain metal free Pc	128.0	3	ZAR 448.0
Copper TSPc in H_2O	247.0	10	ZAR 337.50

7.3.7 Selectivity and sensitivity of the sensor to phenolic compounds commonly found in polluted waters

Out of the phenolic compounds studied, 4-chloro-3-methylphenol and 2, 4-dichlorophenol were detected by the CuPc-modified sensor with higher sensitivity than chlorophenol red (Table 7.2). However, these phenolic compounds oxidized at different potentials, thus making it possible to differentiate between them. NiPc was the most selective of the studied MPcs, in that oxidation waves were only recorded for CPR at the concentrations studied.

SEE ATTCHED TABLE 7.2 IN LANDSCAPE.

7.3.8 Correlation between CFU/ 100 ml and electrochemical detection based on the least time required for detection.

While at the unmodified GCE 1 CFU/ 100ml was detected after 8 h, (based on detection of CPR generated) only 15 min was required for the detection of the same at a CuPc-modified electrode (Table 7.3). All the Pc-modified GCEs were more sensitive than the unmodified GC in detecting CPR. Electrodes modified with cobalt, zinc and the TSPc of cobalt required 2:00, 2:15 and 1:45 h respectively to detect the same numbers of CFUs. Water samples containing 40 CFU/100 ml were instantly detectable with the CuPc-modified GCE (Table 7.3).

Perez *et al.* **(2001) detected 100 CFU 100 ml^{-1} in less than 10 h electrochemically. Paitin** *et al.* **(2003) demonstrated the electrochemical detection of enteric bacteria in general and** *E. coli* **specifically. Amperometric detection of 1000 CFU 100 ml^{-1} within 60-75 min was achieved and pre-incubating the samples for 5-6 h further increased the sensitivity to as low as 1 CFU 100 ml^{-1} within 6-8 h (Paitan, 2003). Mittelmann** *et al.* **(2003) succeeded in amperometrically detecting an** *E. coli* **concentration of 100 CFU/100 ml within a working day. These results are in agreement with our system which detected 1 CFU 100 ml^{-1} within 6 h (unmodified GCE). However, the results achieved in our studies with the CuPc modified GCE is the most sensitive reported to date in the literature (1 CFU 100 ml^{-1} in 15 min).**

Table 7.3: Correlation between CFU/ 100 ml and electrochemical detection based on the least time required for detection.

CFU 100 ml^{-1}	Peak current (nA)	Plain GCE	MPc-modified GCE			
			Cu	Co	Zn	CoTSPc
1	24.52	8 h	15 min	2 h	2: 15 h	1:45 h
5	34.52	5:30 h	7 min	1:15 h	1: 45 min	2:30 min
10	225.17	1:20 h	6 min	50 min	1 h	1:15 h
20	594.85	35 min	2:50 min	30 min	40 min	45 min
40	854.81	16: 40 min	instant	13 min	21:50 min	30 min

7.3.9 Correlation between CFU/100 ml and electrochemical detection of CPR

Generally, a direct correlation between CFUs and current generated in the sensor was observed (Figure 7.9a and 7.9b). Anodic waves of CPR indicated the presence of coliform enzyme, B-GAL, which hydrolysed the substrate CPRG to CPR. Current was detected in some samples notably 32, 46 and 47 which did not show any colony forming units on the media. It is possible that the current generated in such instances was due to the phenomenon of viable but non-culturable bacteria, the major disadvantage in the use of media in detecting microorganisms. It must be stated that non-faecal B-GAL sources could also have contributed towards the current generated.

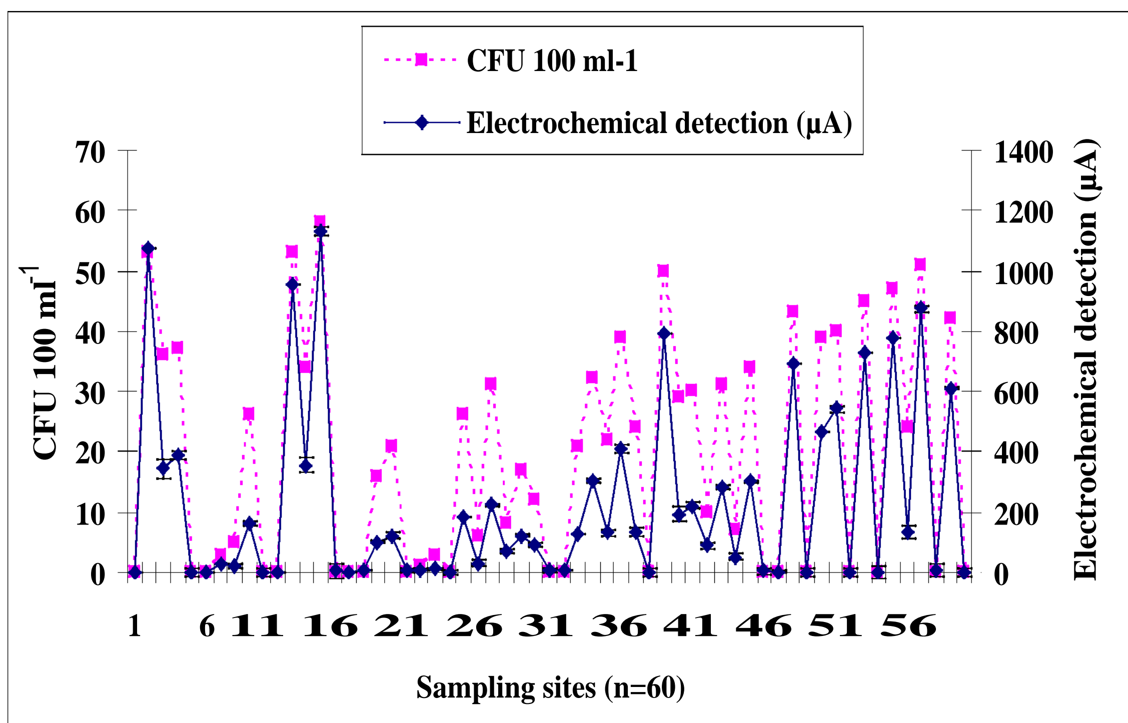


Figure 7.9a: Graph showing the relationship between CFU 100 ml^{-1} and electrochemical detection of CPR. All values reported as mean ± SD (n=3).

A highly significant correlation (R^2 = 0.9199, r = 0.959, P < 0.001) between current response and CFU 100 ml^{-1} in the range of 0 - 60 was observed (Figure 7.9b).

There appears to be two phases in the correlation between CFU 100 ml^{-1} and current generated in Figure 7.9b; the first from 0 - 30 CFU 100 ml^{-1} and the second from 30 - 60 CFU 100 ml^{-1} with correlation coefficients of 0.99 and 0.96 respectively. The high correlation of 0.99 at lower concentration ranges of CFUs is beneficial in terms of the requirement for sensitivity and reliability. The gradient at the second phase is greater than the first phase. This could be due to the fact that larger amounts of CFUs yield greater current as a result of the higher ionic concentration.

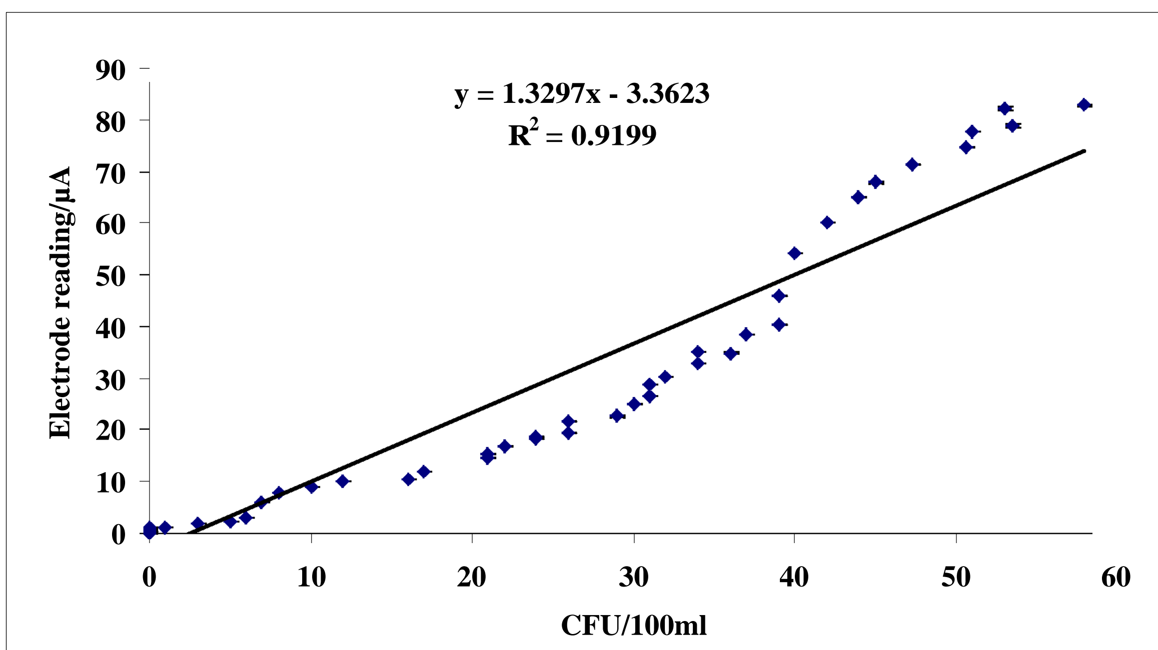


Figure 7.9b Graph showing correlation of CFU/100ml and electrode readings (µA)

7.3.10 Mode of CPR transport to the electrode

The square root of the scan rate (mV/s) was directly proportional to the current generated (Figure 7.10). A plot of the square root of the scan rate (mV/s) against the peak current (µA) gave a straight line graph with an R^2 value of 0.9875. This linearity reflected a diffusion-controlled mass transport of CPR to the electrode.

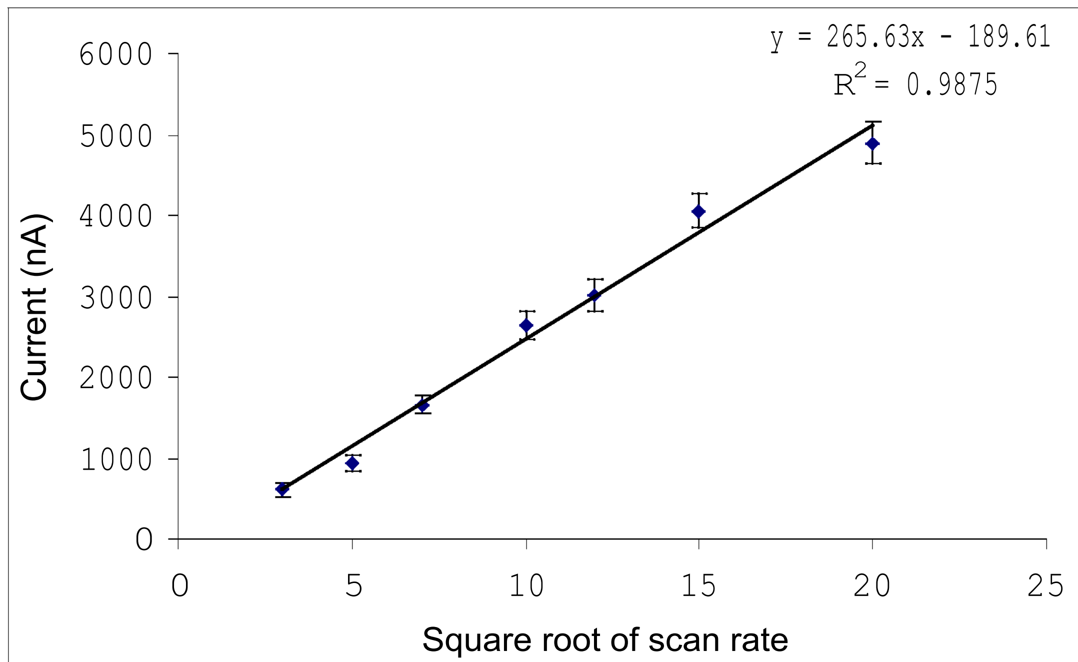


Figure 7.10: Peak current versus the square root of the scan rate of CPR. All values were reported as mean ± SD (n=3).

7.4.2 CONCLUSIONS

In conclusion, electrooxidative detection of CPR as a measure of coliform B-GAL activity was possible. The sensitivity of the sensor was highly improved upon modification of the GCE with phthalocyanine metal complexes especially that of copper.

The sensitivity of the biosensor was dependent on the metal, as well as the substituents on the phthalocyanine ring. MPc-modified electrodes were generally sensitive to changes in pH in the detection of CPR with a reduction in sensitivity from acidic pH to alkaline pH. The CuTSPc, however, was more stable with minimum change of sensitivity (about 17 %) as compared with ZnPc, where the relative sensitivity changed from 73 % at pH 10.0 to 125 % at pH 4.0. The selected MPc modified electrodes were variable in their responses to temperature fluctuations. ZnPc was the most susceptible to temperature change with a 7 % change in sensitivity between 15 and 60 ^{o}C, while CuTSPc was the most stable. Considering the pH and temperature ranges of the study, the sensor would be applicable in environments under different conditions of temperature and pH.

The first 10 CV scans for detection of CPR at a CuPc modified GCE maintained 100 % sensitivity, after which there was a subsequent reduction in sensitivity up to the 50th scan when the results fluctuated. CuPc-modified electrode was the most stable over the 30 day study period, maintaining 60 % of its sensitivity over the period studied. There was virtually no change in sensitivity over the first 5 days of scanning. The least stable electrode was the unmodified GCE, with an 80 % loss of sensitivity detected in 7 days. Unlike with the unmodified GCE, where only a maximum of 5 consecutive scans could be determined, it was possible to perform 80 consecutive scans upon immobilisation of the GCE with CuPc. Immobilisation of the GCE with phthalocyanine metal complexes thus markedly reduced fouling of the electrodes.

The phenolic compounds 4-chloro-3-methylphenol and 2, 4-dichlorophenol were detected by the CuPc-modified sensor with higher sensitivity than chlorophenol red. However,

these phenolic compounds oxidized at different potentials, thus not interfering with the detection of CPR.

While the unmodified GCE detected 1 CFU/ 100ml after 8 h, only 15 min was required for the detection of the same number of CFUs at a CuPc-modified electrode. All the Pc-modified GCEs were more sensitive than the unmodified GC in detecting CPR. Water samples containing 40 CFU/100 ml were instantly detectable with the CuPc-modified GCE. A direct relationship between CFU an electrode reading was observed. A strong correlation factor of 0.92 was observed between CFU/100 ml and the electrode readings.

This novel biosensor thus provides a sensitive method in the detection of coliform contamination of water, thus solving a major problem of the length of time required for confirming the potability or lack thereof in the microbiological water quality industry. The sensor has the potential of counteracting false negative results obtained due to the phenomenon of viable but non-culturable bacteria (VBNC), a major disadvantage in the use of media in detecting microorganisms, by detecting both culturable and unculturable coliforms.

Based on the sensitivity, rapidness of response, stability and selectivity for the detection of CPR in the presence of the phenolics examined, MPc electrodes, in particular CuPc, represent a promising alternative to traditional methods for rapid detection of total coliforms and has the potential of being employed in both once-off (disposable) and continuous systems. Although non-faecal sources of B-GAL could result in a false positive using this electrochemical sensor approach, the strong correlation observed between current and CFU, coupled with the rapidness of response time, represents a distinct advantage over other approaches as an early warning indicator.

Table 7.2: Selectivity and sensitivity of biosensor towards some phenolic compounds

		Peak current (µA)				
	Voltage (mV)	Plain	CuPc	ZnPc	NiPc	CoTSPc
Chlorophenol red	692	1.325	4.033	2.103	1.605	2.011
4-chloro 3-methyl phenol	608	8.901	13.03	1.012	-	9.329
2, 4-dichlorophenol	614	6.202	9.027	-	-	8.563
Phenol	-	-	-	-	-	-
2-nitrophenol	-	-	-	-	-	-
3-nitrophenol	-	-	-	-	-	-
2, 4-dinitrophenol	-	-	-	-	-	-
2, 4, 6-trinitrophenol	-	-	-	-	-	-
2-chlorophenol	-	-	-	-	-	-

CHAPTER EIGHT

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE RECOMMENDATIONS

When we think of the most noble sentiments that we are capable of feeling and expressing, such as wonder, compassion, love, and so on—we cannot doubt that humanity is innately divine and capable of transcending itself for the greater good!

8.1 GENERAL DISCUSSION AND CONCLUSIONS

The strength of the research relies on the use of polluted real water samples as sources of enzyme and the demonstrated correlation of the enzyme activity with total coliform colony forming units, as well as in the development of an efficient transduction approach in the form of a biosensor. The study also shed more light on potential interfering compounds found in polluted waters which are often underestimated in many research papers published in literature. Furthermore, to the best of our knowledge, the use of metal phthalocyanine-modified electrodes to improve the kinetics of several electron transfer reactions and also to decrease fouling of electrode surface is the first time that this approach has been used to detect the presence of total coliforms in polluted waters.

While *E. coli* is unequivocally of faecal origin, preliminary/pilot studies established a correlation between total coliforms (TC) and *E. coli* (FC) (R^2=0.89; range: 0.85-0.93) in the water samples for the studies. Two parallel Doctoral studies were carried out in our laboratory; one targeting *E. coli* (β-glucuronidase) and the other, total coliforms TC (β-galactosidase) on the same water samples. Togo *et al*., 2006 and 2007 and Pletschke *et al.,* 2006 are some products emanating from the use of FC (β-glucuronidase) in this study.

This study successfully showed that the concept of using direct enzyme assays to detect the presence of total coliforms with the chromogenic substrate CPRG is a viable approach in the determination of the microbiological quality status of water.

Spectrophotometric detection was achieved within a maximum required period of 24 h. More polluted water samples gave results in less time. While higher temperatures resulted in higher enzyme activity, results at such high temperatures were non-reproducible and were assumed to increase the effect of interfering compounds on the assay. The observed detection time compared well with the improved conventional methods (Venter, 2000; Rompré *et al*., 2002) and the Colilert® methods that make use of

fluorogenic substrates (Berger, 1994; Fricker and Fricker, 1996; Grant, 1997; Sartory and Watkins, 1999).

A direct correlation between enzyme activities and increase in environmental water samples was observed. A positive correlation between enzyme activity and CFU counts was also observed. Dilution of the enzyme by the large volume of water in the environmental (river) water sources may account for the low enzyme activities observed. The observed direct correlation between B-GAL activity and CFU counts agreed well with the results ($0.73 < R^2 > 0.96$) obtained by Farnleitner *et al*, (2001). A R^2 value of 0.941 and 0.7051 was determined with CM 1047 and mEndo media, respectively, in this study.

The good degree of correlation between microbial counts and enzyme activities illustrated the degree of accuracy of the MF technique and the direct enzyme assays. However, the possible detection of VBNC bacterial enzyme activity could potentially account for the deviation of R^2 from 1. A poor correlation between enzyme activity and CFUs in marine samples was observed and this may limit the technique for use with fresh water samples only.

Contrary to the observation that a high level of faecal coliforms are observed after rainstorms (Venter *et al*., 1997), results from this study showed a decrease in enzyme activities that were positively correlated to the coliform counts. The enzyme activity is partly a function of the amount of enzyme present and partly of inhibitor concentration. Low enzyme activity may also be attributed to a high level of enzyme dilution. Higher activity obtained during warm periods may be explained by high microbial activity under such temperatures and mixing aided by convection currents.

A spectrophotometric LOD of 1 CFU 100 ml^{-1} was obtained from this study. While this indicated a very sensitive technique, caution should be exercised when stating such results, since non-target sources were observed by Van Poucke and Nelis (1997) to interfere at such low levels of sensitivity.

The monthly (seasonal) changes in the enzyme activity could be due to factors ranging from changes in domestic, agricultural and social habits. Livestock activities could also have affected enzyme activity. The highest levels of B-GAL activity coincided with the highest temperatures recorded. Warm temperatures enhance microbial growth, thereby increasing enzyme production and activity. In addition, increases in temperature speed up the rate of degradation of organic compounds, thus providing nutrients for the microbes, leading to higher enzyme activity. Therefore, warm seasons are expected to exhibit higher levels of enzyme activity.

The environmental water sample inhibited commercial B-GAL activity to about 15 % at a 1:1 ratio (of environmental water sample to CPRG and buffer). The presence of inhibitory pollutants and compounds in the environmental sample may be responsible for this observation. The environmental enzyme samples appeared to exhibit different physico-chemical properties from those of their commercial counterpart.

Broad pH and temperature optima for the enzyme activities were very significant, as this made the use of the enzyme assay more applicable to different environments and therefore more versatile. The direct enzyme assay was less laborious and simple to perform. The assay could be performed at room temperature (20 ± 2 ^{o}C). The enzyme was readily available in the extracellular environment, and thus there was no need for fractionation, permeabilisation and/or concentration of the enzyme prior to performing the assay. This observation may be explained by the stressed conditions the microorganisms encounter in the environment. George *et al*. (2002) and Leonard *et al.* (2003) both expressed the view that stressful conditions in the water environment forces the cells into secreting their enzyme extracellularly.

The fluorogenic substrate MuGal assay proved to be very sensitive when compared to the chromogenic CPRG assay. However, a high degree of unpredictability with the fluorogenic assay was observed. The low threshold to interference from a wide range of compounds in the environment made the use of this substrate impossible. This

observation was in agreement with the observation of Davies and Apte (1999). From the above results, the chromogenic substrate rather than the fluorogenic counterpart, was selected for further studies. B-GAL was successfully concentrated by PEG 20 000 and the source of the enzyme confirmed as *Escherichia coli* through SDS-PAGE, tryptic mapping and MALDI-TOF. The use of B-GAL as a suitable indicator of coliform contamination was thus confirmed and justified.

An extensive study of the effect of compounds commonly found in the environment (as well as those used in water treatment processes) showed that several of these compounds did in fact affect B-GAL activity. This implies that results of enzyme activity in environmental water samples must be stated with caution. The possibility of using an internal reference or standard (i.e. commercial B-GAL) should also be explored in order to assess and correct (calibrate) for chemical interference when testing environmental samples.

The next step in the study was to explore the possibilities of immobilising the substrate on a solid support (strip bioprobe) to enable ease of handling and increase shelf life. Nylon and nitrocellulose membranes were studied for this purpose. However, although these membranes resulted in a reasonable degree of immobilisation, diffusion of the substrate upon addition of the water drops on the strip was observed. The observed diffusion of the chromogen did not allow for accurate colour quantification on the strips. This observation agrees with Isgrove *et al*. (2000) in that desorption commonly occurs in physically adsorbed molecules.

To our knowledge, this is the first report on the electrochemical detection of CPR in the detection and monitoring of microbial contamination of water. It was possible to indirectly detect B-GAL activity electrooxidatively by measuring CPR accumulation as a breakdown product of CPRG. A wide range of phthalocyanine metal complexes was studied in an attempt to reduce fouling and/or increase the sensitivity of the product. One of the most important observations of MPcs is their natural tendency to adsorb to electrode surfaces, effectively producing a stable chemically modified electrode at which

catalytic oxidation can occur at lowered potentials. It has been reported that each MPc exhibits a slightly different voltammetric response related to its mode of interaction with the electrode and the substrate under investigation (Jiang and Kucernak (2000, 2001a, 2001b).

Of all the MPcs studied, the CuPc-modified GCE provided the best option for developing a biosensor for the detection of CPR. The stability of the GCE was enhanced by coating with MPcs (especially CuPc). The reduction in fouling could have been due to inhibition of the formation of dimeric or polymeric oxidation products that are known to poison the electrode or due to some steric hindrance caused by the CuPc species, thus minimising the adsorption of the oxidation products on the Cu-Pc modified electrode. The CuPc modified-GCE biosensor detected 1 CFU 100 ml^{-1} in 15 minutes, while it took the plain unmodified GCE six hours to detect the same number of CFUs. Mittelmann *et al*. (2003) succeeded in amperometrically determining *E. coli* within a working day, while Neufeld *et al*. (2003) increased the sensitivity of their biosensor to as low as 1 CFU 100 ml^{-1} within 6-8 hours by pre-incubating their samples for 5-6 h.

The results obtained in this study showed that immobilisation processes have an important influence on biosensor sensitivity. This biosensor provides a very robust and sensitive approach for the detection and monitoring of coliform activity in drinking and recreational waters.

There are essentially two main areas for consideration in the development of chemical and biological sensing technology. The first may be viewed as the laboratory based research phase in which the sensor is optimized under ideal conditions, the second in which the sensor is examined in real samples. The next stage entails product development (Taylor, 1996).

One of the critical stages in the development of chemical and/or biological sensors involves laboratory-based testing and trouble-shooting of the sensing approach which is the scope of this study (Taylor, 1996).

Bringing a new product from the laboratory to the market requires interdisciplinary skills and experience. Technical expertise in microbiology, biochemistry, electronics, software engineering, quality control, quality assurance, regulatory requirements, manufacturing and process experience in scale-up, technology transfer, packaging, marketing and distribution knowledge may all be required in the process (Deshpande, 1996; Taylor, 1996; Wild, 2001).

Further studies are required beyond the research stage before the product finally gets on the market. It is critical to assess and decide on the design of a new product early in the commercialization process. These considerations should include a survey of markets for the product as well as an assessment of other competitive products already on the market. These analyses allow projections of both potential market share and return on investment (Taylor, 1996; Wild, 2001).

Firstly, if the new product or system is a competitor to an already established product, it must show improved performance; for example, lower cost per assay, greater efficiency, and, if possible, lower capital equipment investment. There is also the need to perform accelerated shelf-life testing on the product and its individual components to determine realistic shelf life, storage conditions, and usage conditions. This information is also critical to assessing the overall quality control for the product and the instructions for its use. Secondly, if the product claims new diagnostic capabilities, these must address long-standing needs (Deshpande, 1996; Taylor, 1996; Wild, 2001).

There are two major types of electrodes; continuous use and disposable. The main advantages of the disposable electrodes (screen printed) include simplicity, versatility, modest cost, potability, ease of operation, reliability, small size and mass production capabilities, leading to its development in various applications in the electroanalytical chemistry field. Disposable electrodes have the advantage of circumventing the problem of fouling as well as the decrease in sensor response over time as observed in this study. Additionally, environmental variables which alter the electrochemical signal generated such as temperature and pH (as shown in this study) can be compensated for through the use of buffered media within defined temperature ranges. Alternative approaches include

the development of standard curves over a wide pH range, requiring the inclusion of pH measurements as well as current generated.

Continuous electrodes (S/FIA systems) are exposed to flowing buffer and possibly other solvents and solutes. Consequently, there is generally significant current loss and therefore the lifetime of the sensor will be considerably reduced due to fouling of the electrode surfaces. Such systems are associated with very high cost particularly when coupled to automated monitoring of physical and chemical variants which can affect the sensor response.

While disposable electrodes are more appropriate for one once-off testing offering more practical low cost sensing solutions in rural settings (where a critical need exists in this area in developing countries such as South Africa), the continuous electrodes (S/FIA) may be more suitable for water treatment facilities where the cost of the equipment and maintenance could be easily borne. In as much as this sensor has the potential of targeting both markets, further research is required in the development of screen printed electrode and S/FIA solutions

8.2 FUTURE RECCOMMENDATIONS

We propose an on-line and real-time monitoring of B-GAL by employing a sequential flow injection assay (SFIA). This system has the advantage of detecting two or more marker enzymes and employing both electrochemical and photometric detectors simultaneously. Figure 8.1 is our drafted design for an SFIA system. In this customised design, there is an added advantage of an optional pre-concentration and inhibitor removal step before assaying. The specifications of the customised design are as follows:

The designed SFIA should take in approximately 100 ml and after filtration retain about 0.5 ml and the tubing should not allow protein adsorption. The waste solution must be continuously removed from the unit (maybe by suction). The filtration unit must be detachable. Reaction between pre-concentrated sample and solution from tube 4 takes

place in O. Mixing should take place with a temperature control option. Entry and exit in O must be time controlled. D splits up the reaction mixture into variable volumes to tubes T and V. Entry into reactor (F) (between A and B) must be time controlled while F is temperature controlled (20 to 60 ^{o}C). There is then a reaction of the water sample with buffer and substrate in F and finally the products of substrate hydrolysis by enzyme (s) are taken to the spectrophotometer and/or another detection technique (e.g. electrochemical) for simultaneous detection.

The specifications of the sequence of operation of the system are as follow:

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

FIAlab Instruments, Inc. (www.flowinjection.com), a company in the US has been contacted and is willing to develop this design.

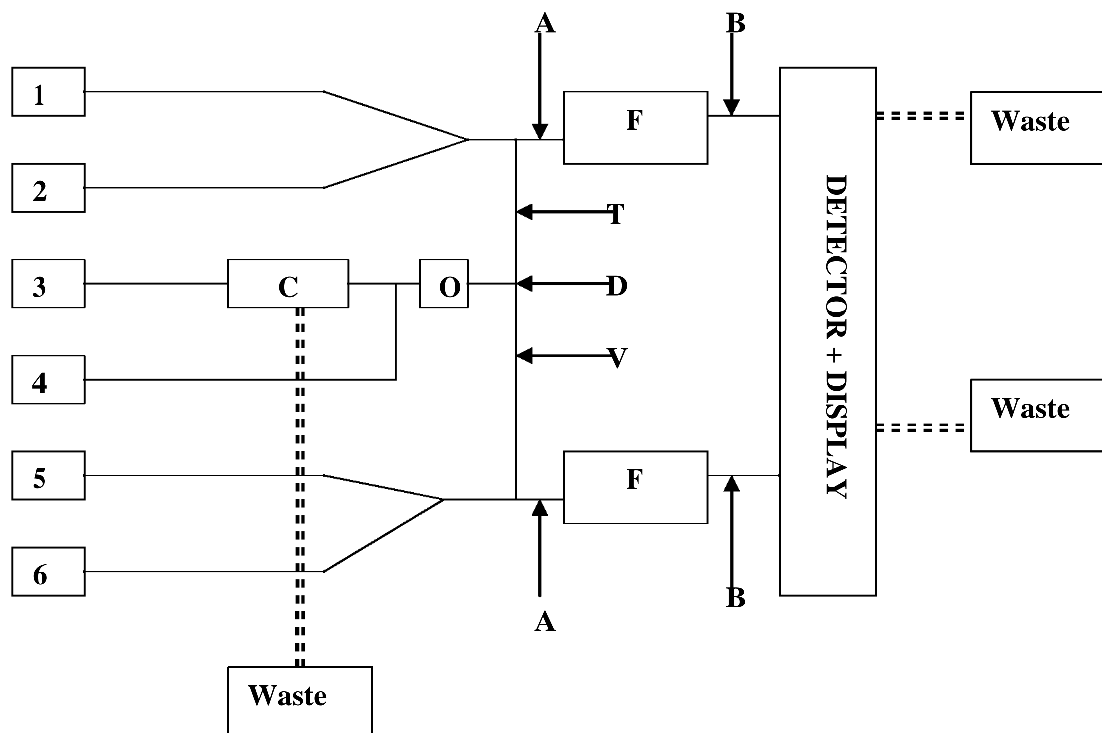


Figure 8.1: Sequential Flow Injection Analysis (SFIA) equipment design

It is also recommended that further studies are carried out to immobilise the chromogenic substrate on a suitable support material to yield a strip bioprobe. Miniaturisation of the biosensor into low cost disposal screen-prints should also be exploited for ease of handling and portability. The CoPc-modified GCE should be further studied for the development of single use biosensors, since it gave a very high first scan result, but fouled readily with subsequent scans.

This study therefore provides a reliable electrochemical technique for determining the presence of coliforms in water, thus making a large contribution towards solving a major public health problem, especially in developing countries like South Africa.

The next step in the practical development of these systems is the collaboration of tertiary education based research and industry with access to in-house expertise in market research, costing, materials research, software design and mechanical engineering.

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Great spirits have always encountered violent opposition from mediocre minds. The mediocre mind is incapable of understanding the man who refuses to bow blindly to conventional prejudices and chooses instead to express his opinions courageously and honestly - Albert Einstein

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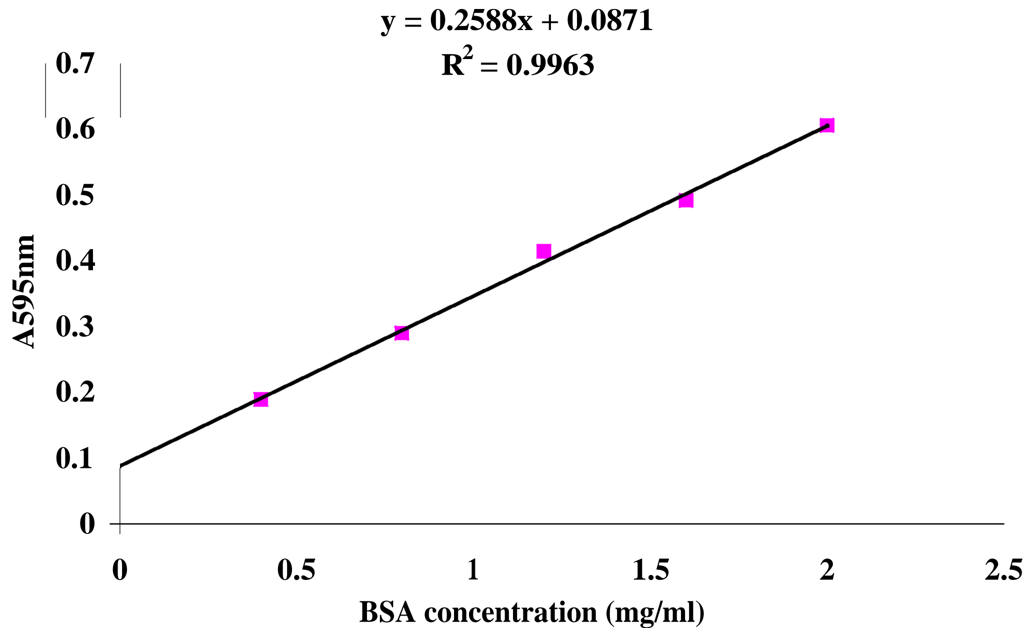
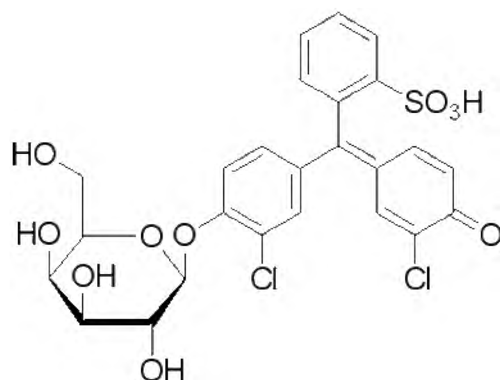
APPENDICES

The future belongs to those who believe in the beauty of their dreams - Eleanor Roosevelt

A: Standard curve for protein determination

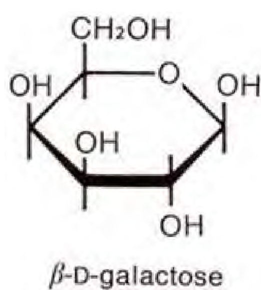
Protein concentration was determined using the Sigma reagent for protein determination according to the method of Bradford (1976). This method is rapid, convenient and produces an equivalent absorbance change for many proteins. When mixed with protein solution, a protein-dye complex is formed, which causes a shift in the dye absorbance maximum from 465 to 595 nm. The dye is called Brilliant blue G-250, and the amount of absorbance produced is proportional to the protein concentration. Bovine Serum Albumin (BSA) was used as a suitable standard.

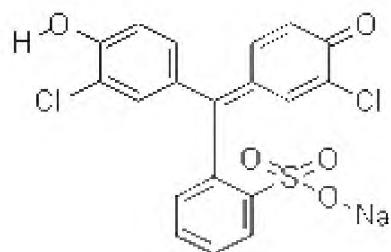




Chlorophenol red β-D-galactopyranoside (Yellow)

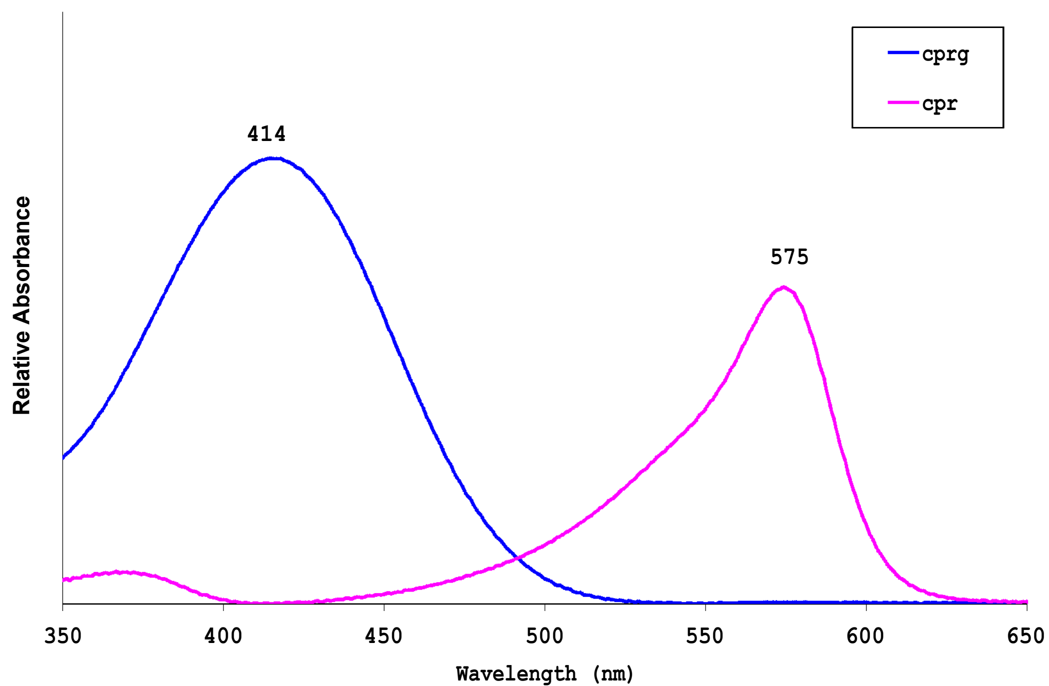
β-D-galactosidase (B-GAL)



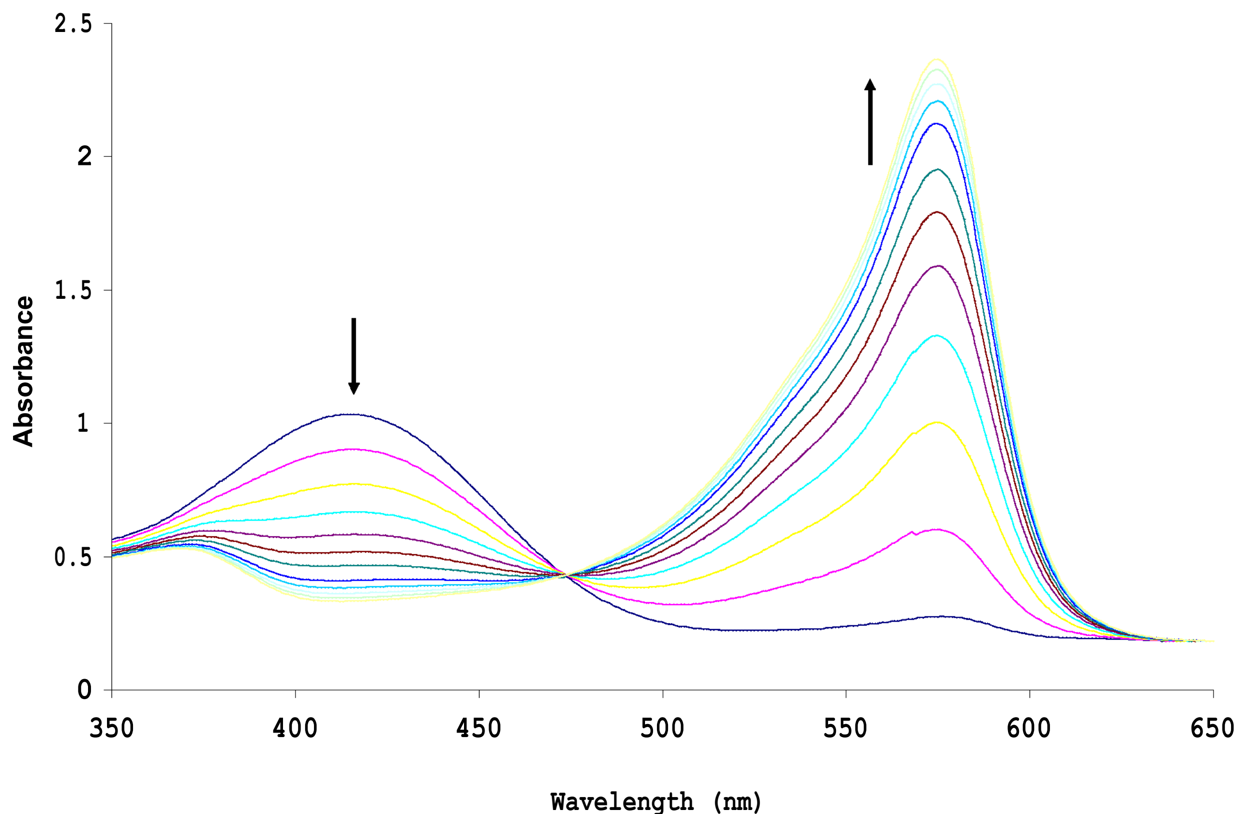


Chlorophenol red (Red)

B1. Hydrolysis of chlorophenol red β-D-galactoctosidase (CPRG) to chlorophenol red (CPR) by B-GAL



B2. UV-Vis Spectra of CPR (575 nm) and CPRG (414 nm)



B3. Electronic Absorption Spectra of CPRG conversion to CPR showing the conversion of CPRG to CPR upon addition of B-GAL enzyme. [B-GAL]=1.0 x 10 mg ml^{-1}, [CPRG] =0.004 %. Arrows show decreasing and increasing levels of CPRG and CPR respectively.

C1. Bloukrans River (sampling site) after a moderate rainfall period

C2. Bloukrans River (running water sample site)

C3. Bloukrans River (standing water sample site)

Appendix D (Protocol as stipulated in the BioRad III Protean Manual)

Volumes Required Per Gel

The volumes listed are required to completely fill a gel cassette. Amounts may be adjusted depending on the application (with or without comb, with or without stacking gel).

Gel Thickness (mm)	Volume (ml)
0.5	2.8
0.75	4.2
1.0	5.6
1.5	8.4

Note: 10 ml of monomer solution is sufficient for two stacking gels of any thickness.

SDS-PAGE (Laemmli) Buffer System

Stock Solutions and Buffers

Acrylamide/Bis (30% T, 2.67% C)

87.6 g acrylamide	**(29.2 g/100 ml)**
2.4 g N'N'-bis-methylene-acrylamide	**(0.8 g/100 ml)**

Make to 300 ml with deionized water. Filter and store at 4 °C in the dark (30 days maximum.)

10% (w/v) SDS

Dissolve 10 g SDS in 90 ml water with gentle stirring and bring to 100 ml with deionized water.

1.5 M Tris-HCl, pH 8.8

27.23 g Tris base (18.15 g/100 ml)

80 ml deionized water

Adjust to pH 8.8 with 6 N HCl. Bring total volume to 150 ml with deionized water and store at 4 °C.

0.5 M Tris-HCl, pH 6.8

6 g Tris base

60 ml deionized water

Adjust to pH 6.8 with 6 N HCl. Bring total volume to 100 ml with deionized water and store at 4 °C.

Sample Buffer (SDS Reducing Buffer)

3.55 ml deionized water

1.25 ml 0.5 M Tris-HCl, pH 6.8

2.5 ml glycerol

2.0 ml 10% (w/v) SDS

0.2 ml 0.5%(w/v) bromophenol blue

9.5 ml Total Volume

Use: Add 50 µl β-mercaptoethanol to 950 µl sample buffer prior to use. Dilute the sample at least 1:2 with sample buffer and heat at 95 °C for 4 minutes. Store at room temperature.

Staining of Gel

Coomassie Brilliant Blue

Make up stain: 0.2% CBB in 45:45:10 % methanol:water:acetic acid. Cover gel with staining solution, seal in plastic box and leave overnight on shaker (RT) or for 2 to 3 hours at 37 c also with agitation.

Destain with 25% 65% 10% methanol: water: acetic acid mix, with agitation.

5x Sample Buffer

10% w/v SDS

10 mM Dithiothreitol, or beta-mercapto-ethanol

20 % v/v Glycerol

0.2 M Tris-HCl, pH 6.8

0.05% w/v Bromophenolblue

10x Electrode (Running) Buffer, pH 8.3 (makes 1 L)

30.3 g Tris base

144.0 g Glycine

10.0 g SDS

Dissolve and bring total volume up to 1,000 ml with deionized water. Do not adjust pH with acid or base. Store at 4 °C. If precipitation occurs, warm to room temperature before use.

10% APS (fresh daily)

100 mg ammonium persulfate

Dissolved in 1 ml of deionized water.

Gel Formulations (10 ml)

Prepare the monomer solution by mixing all reagents except the TEMED and 10% APS. Degas the mixture for 15 minutes.

Immediately prior to pouring the gel, add:

For 10 ml monomer solution:

Resolving Gel:

50 µl 10% APS and 5 µl TEMED

Stacking Gel:

50 µl 10% APS and 10 µl TEMED

Swirl gently to initiate polymerization.

Note: Prepare any desired volume of monomer solution by using multiples of the 10 ml recipes. The volumes of APS and TEMED must be adjusted accordingly.

PerSeptive Biosystems

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Guide Wire Voltage: 0.050 %
Delay: 400 ON
Sample: 24

Laser : 3000
Scans Averaged: 206
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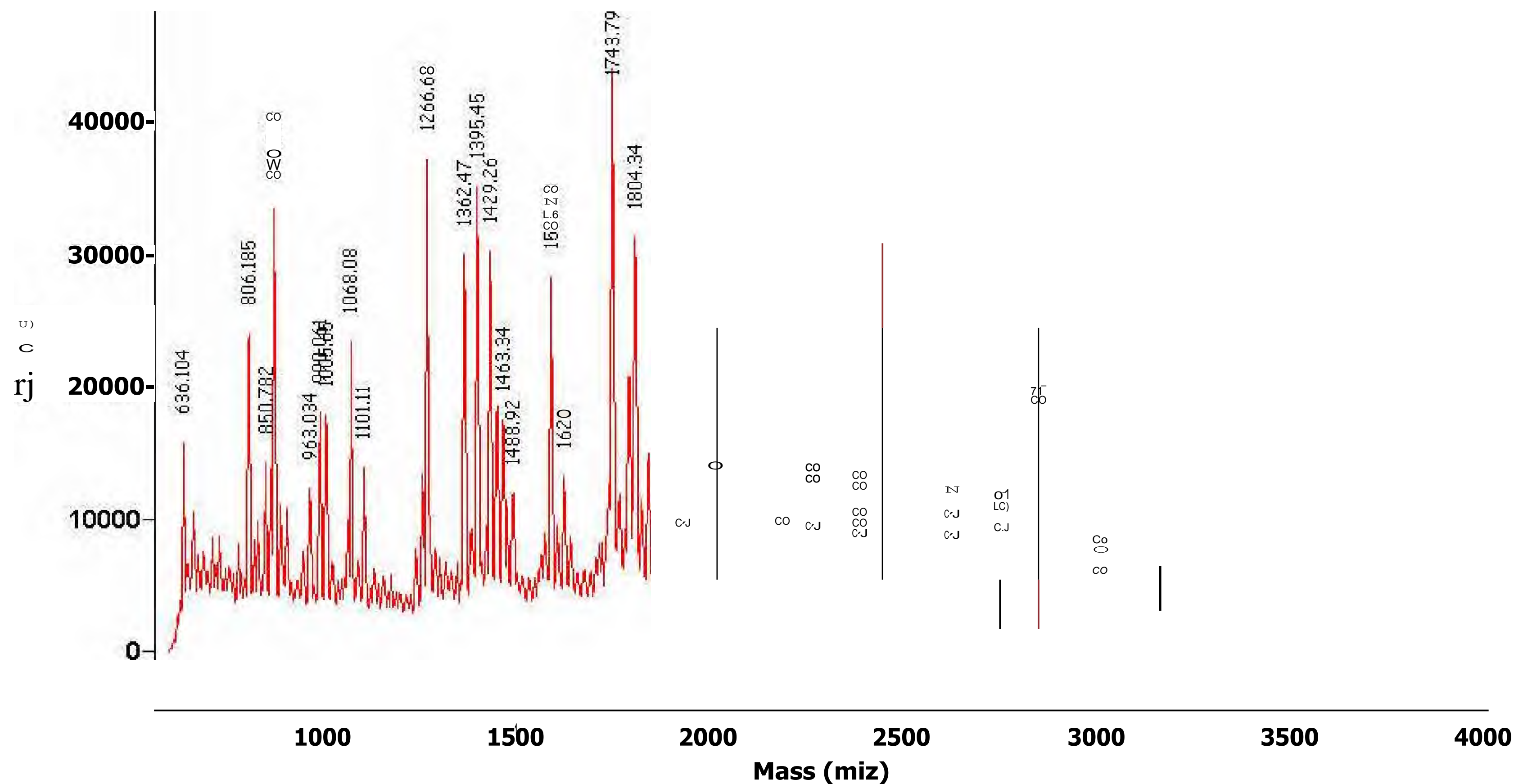

D 1 MALDI ToF analysis

PerSeptive Biosystems

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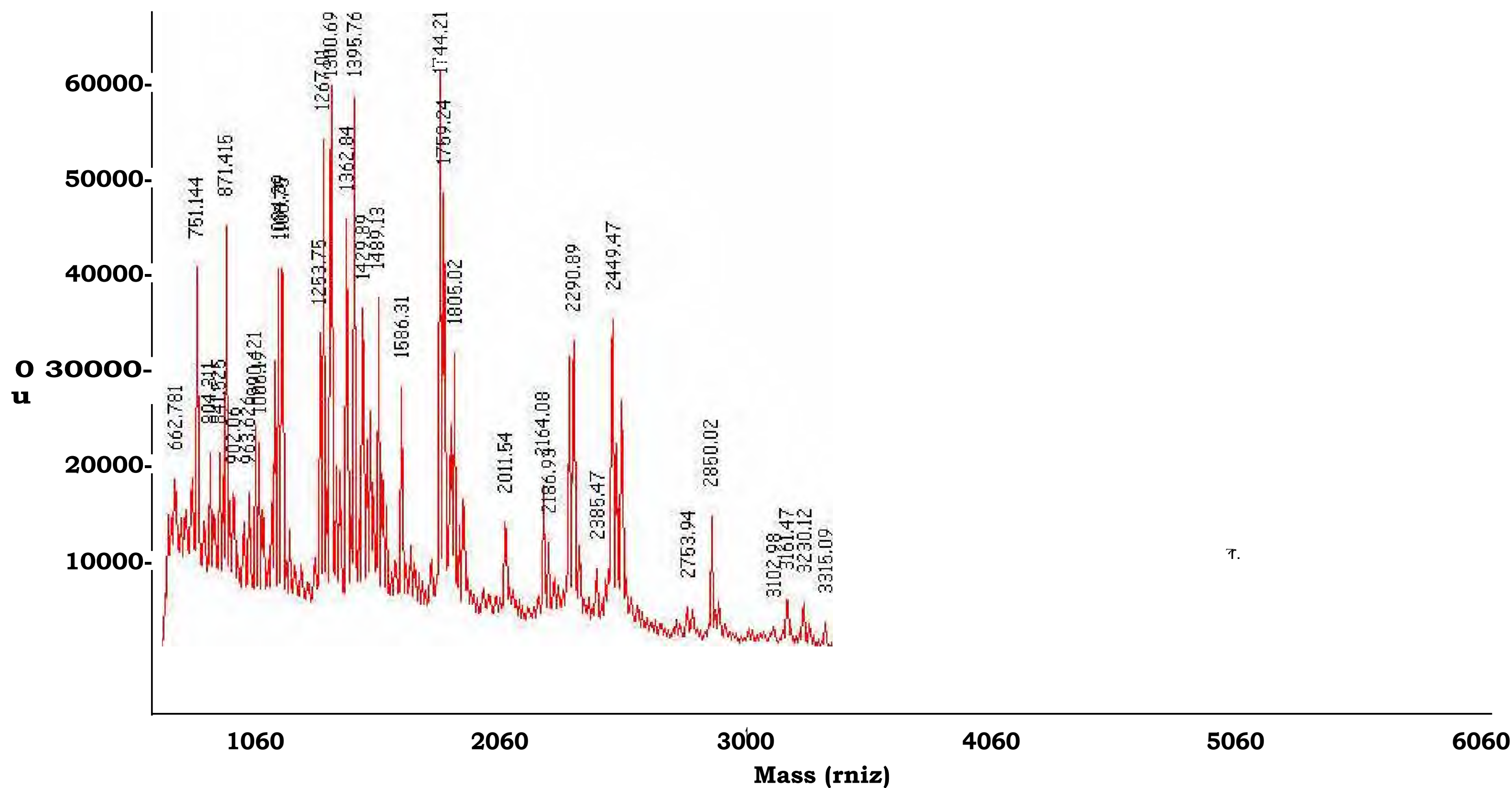

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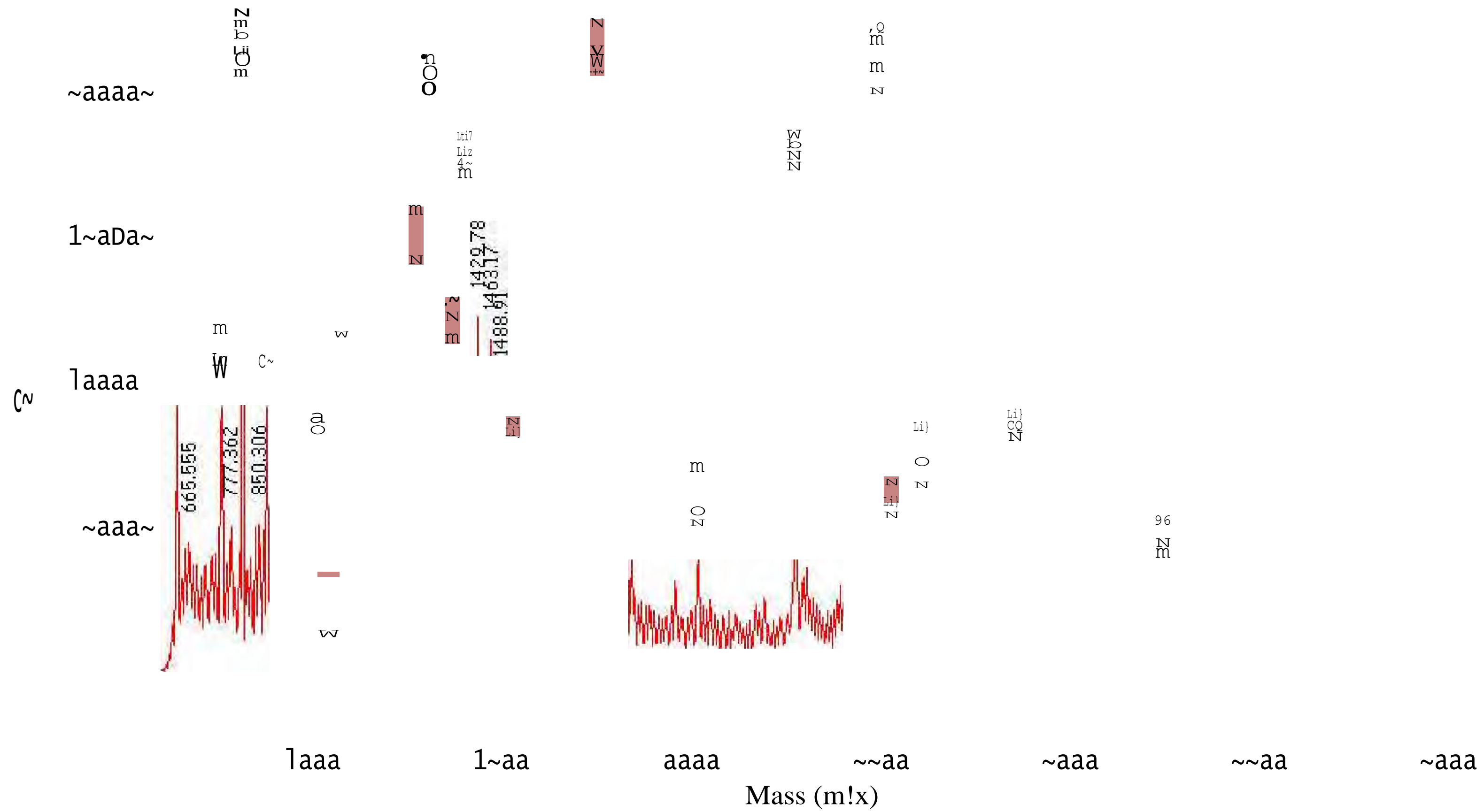

D 3 MALDI ToF analysis

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Comment: call.+2dhh

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Mode: Linear
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Grid Voltage: 94.000 %
Guide Wire Voltage: 0.050 %
Delay: 400•ON
Sample: 72

Laser: 3000
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Pressure: 1.45e-07
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Negative Ions: OFF
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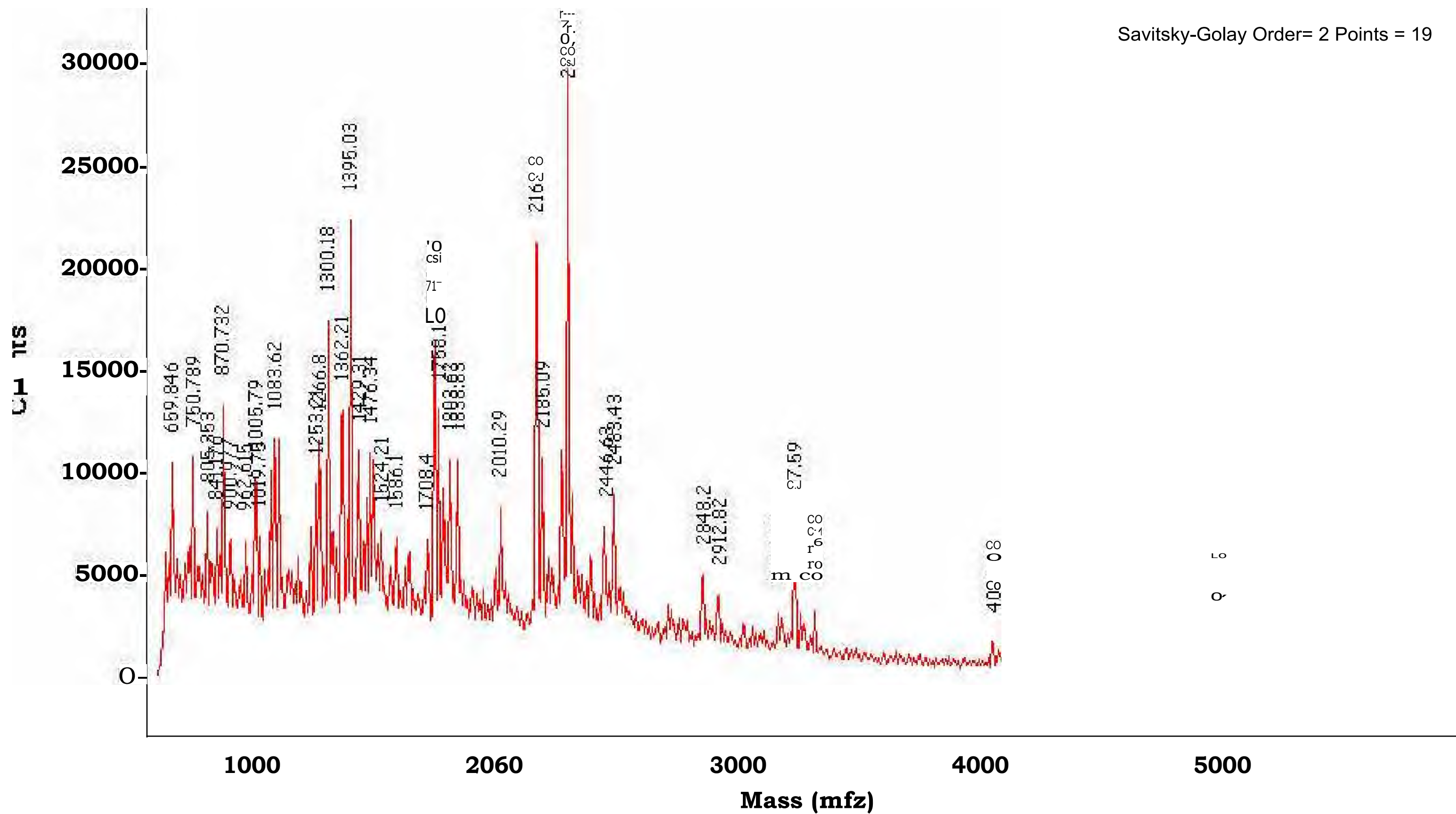

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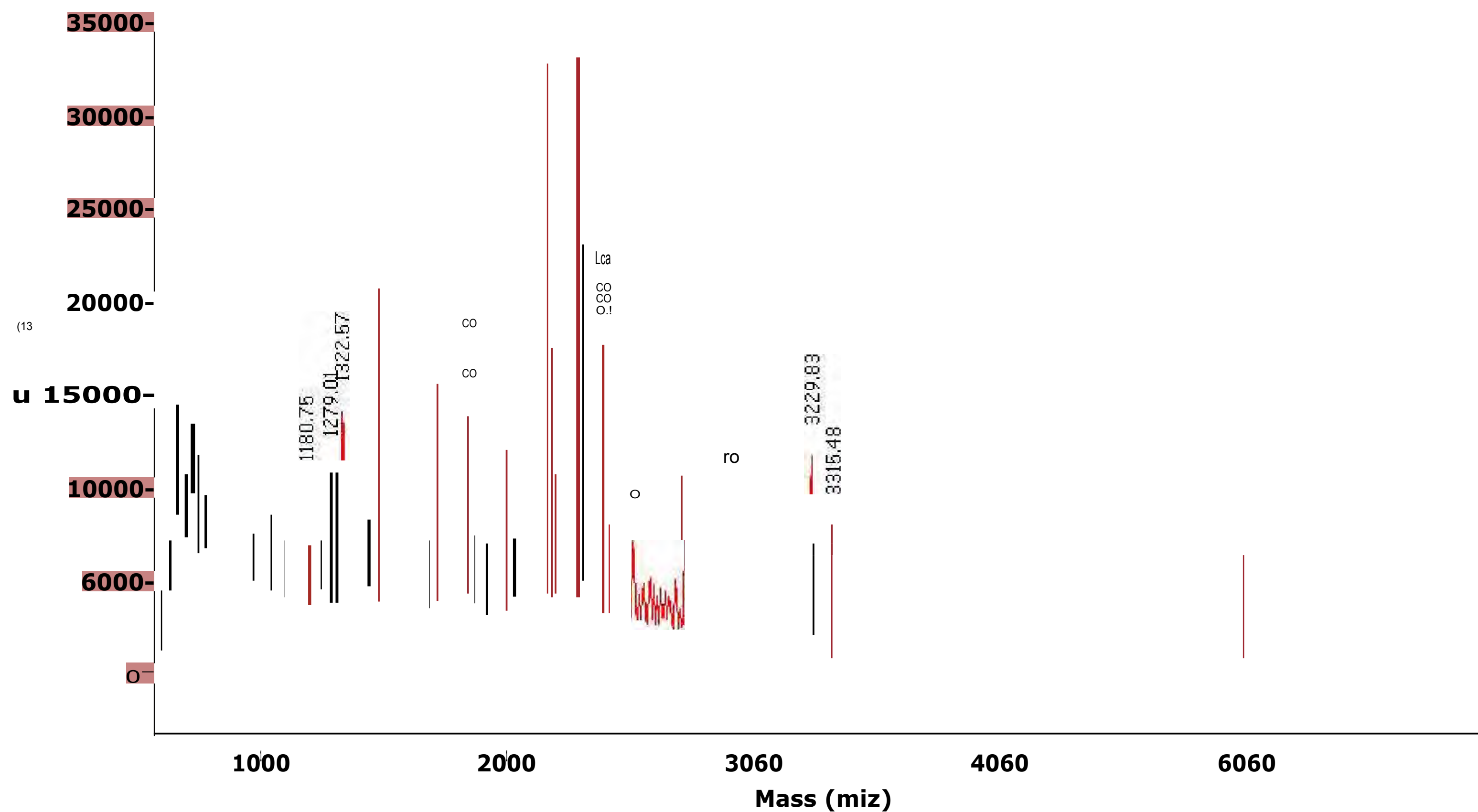

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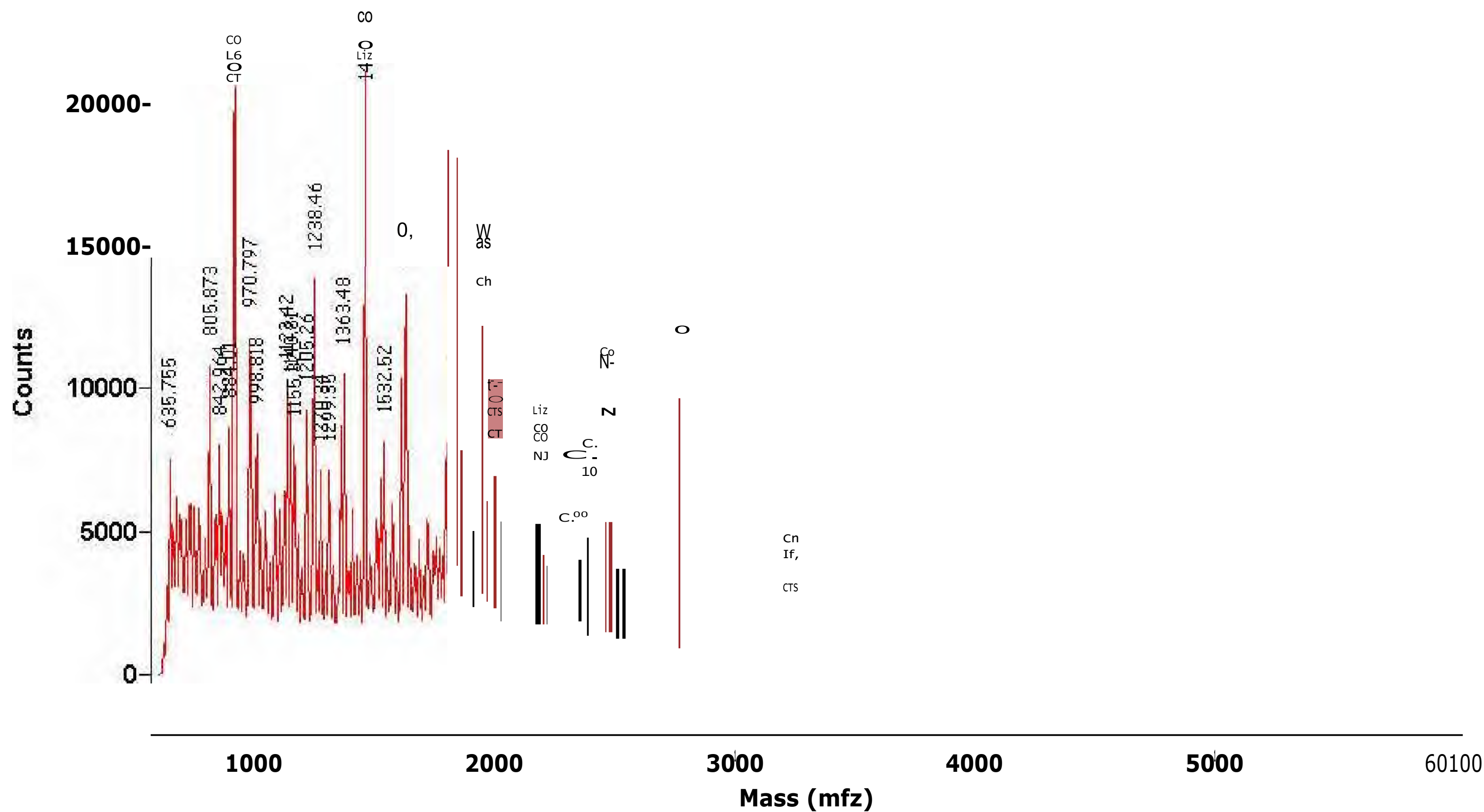

D 6 MALDI ToF analysis