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Development of Novel Disposable Metal/Coliform Biosensor Device for Simultaneous Monitoring of Chemical and Microbial Quality of Drinking Water and Urine

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Doctor of Philosophy (PhD)

Edinburgh, 2019

Declaration

I hereby declare that this thesis sums up research carried out by me and supervised by Prof. Chris E French, and unless otherwise stated, all the results present herein are my own work. This work has not been submitted for any other degree or personal qualification.

…… Date: 30th January, 2019

Ndubuisi Christopher Nwankwo

Layman's Abstract

The toxicity of heavy metals and coliform contamination of drinking water pose serious threats to life and there are numerous associated health risks. These molecules and cells are generally detected based on chemical reactions or using complex machinery, which may also contribute to environmental problems as many of such tests are hazardous, unrealistic and too expensive for practical use. Most of these modern techniques also require skilled personnel for result interpretation, facilities and laboratory conditions which are inadequate in many developing countries. Consequently, the major aim is to develop a system that can accurately and sensitively detect heavy metals and biological contaminants simultaneously in a single format. To achieve this, novel whole-cell bacterial sensors were developed which can sense specific metals of interest, such as arsenic, copper, zinc, cadmium, lead and mercury. This is based on using synthetic biology tools to insert genes which are responsive to the specific metals they sense. Also investigated was the biomedical potential of the arsenic biosensor for clinical samples such as urine, since arsenic is excreted in urine as a function of exposure to arsenic in drinking water. The systems work by allowing growth in specially formulated sensor media with lactose as the sole carbon source and makes use of a pH indicator for easy visual result measurement. Following incubation at a specified temperature and time, lactose in positive samples is degraded by the bacterial sensors which leads to a change in the colour of the system from blue to yellow. When tested in samples spiked with the metals of interest, the systems accurately detected those metals at the safe limits recommended by the World Health Organisation. A technique was also designed for incorporating testing of

biological contaminants (coliforms), associated with faecal pollution of water, into the system. The system worked as well as standard techniques, but is cheaper, and saves time. However, there is still room for improvement as it is important to further reduce the time for output giving room for more samples to be analysed. Also incorporated was a technique of preserving the sensor cells so they can be mass produced and easily distributed to other regions. The idea was to make the systems available where they are lacking so that they can be utilised by the users themselves. Nonetheless, the issue of regulation as a result of genetic modification of organisms, which has restricted whole-cell sensors to laboratory testing hindering their use in the field, was also taken into consideration. However, the system does not contain any foreign DNA as only native genes were used with the hope that it will be licensed for field use. The need for safe disposal of the sensor device and lifetime environmental impact will also be considered through consultations with designers, social scientists etc.

Abstract

The main sources of human exposure to heavy metals are water and food. Metals play significant roles in the growth and development of living organisms. However, they can be lethal at elevated concentrations due to their interference with normal biological processes, although some metal species are toxic even at low concentrations (e.g. Hg, Pb). The traditional laboratory-based analytical investigations for environmental contaminants are expensive and usually require trained staff and sophisticated facilities. This study sought to develop a sensitive, specific, accurate, rapid, cheap and portable novel disposable biosensor device for the detection of arsenic and other heavy metals in drinking water and investigate its biomedical potential for testing arsenic in urine samples. A pre-existing arsenic biosensor consisting of the *E. coli* chromosomal *ars* promoter, *arsR* repressor gene and *lacZ'α* gene was tested. The expression of *lacZ'α* allows the fermentation of lactose with production of acid, changing the coloured pH indicator bromothymol blue from blue to yellow. Our results showed that the biosensor responded reliably to arsenate concentrations in water and urine samples below the recommended World Health Organisation limit of 10 ppb arsenic. Novel zinc and copper biosensors were developed using promoter elements regulated by endogenous zinc-binding and copper-binding transcription factors, ZntR and CueR respectively, fused to *lacZ'α*, and a suitable test medium, ZBM3, was developed. Initial designs showed high background activity. The constructs were redesigned using either weaker ribosome binding sites (RBS), low copy number plasmids or promoters with lower activity. The redesigned Zn biosensor accurately detected zinc, cadmium, lead and mercury

concentrations (3 mg/L, 0.003 mg/L, 0.01 mg/L and 0.001 mg/L respectively) below the recommended WHO limits. Similarly, copper, silver and gold levels (2 mg/L, no guidelines for silver and gold respectively) were detected by the novel copper biosensor, below the limits recommended by the WHO. The stability of the sensor cells was independently tested by; air drying, freeze drying, or immobilised on paper, each within a re-sealable system that can then be stored and distributed, hence eliminating the need for routine culture and minimizing variation between different batches of cells. Sensor bacteria were successfully revived after 120 days storage at room temperature or 37^0C. Data obtained showed approximately 0.2% viable cells at the aforementioned conditions after initial inoculations of 1.3×10^8 CFU / mL of cells, an indication of reduced viability as a result of rigorous cell preparations and incomplete drying particularly air dried cells. However, data also obtained showed that dried cells with such survival rate were still effective in the assay.

These growth-based biosensors supported growth on lactose medium which allows use of the same format for detection of both metals and biological contaminants (coliforms) in a single unit. Coliforms were readily detected based on lactose fermentation using a variant of the same growth medium used for biosensor organisms, allowing for easy generation of a combined coliform/metal sensor device. In conclusion, metal contaminants in environmental samples can be reliably and accurately detected at the safe limits set by the regulatory authorities by the use of biological methods of testing. These testing techniques, together with the simultaneous testing of biological contaminants, have the potential of reducing the high costs of physico-chemical methods, save time as well as make sample testing available to areas lacking modern testing facilities. However, the systems need to be improved to allow for a reduction in time needed to obtain reliable results.

Dedication

Ekene dili Chukwu!

(To God be the Glory)

Acknowledgements

To Prof. Chris E French, my PhD supervisor, my sincere gratitude goes to you for giving me guidance, mentorship and direction without which this research would not have been possible. My appreciation also goes to Prof. Alistair Elfick, Drs. Garry Blakely and Jon Marles-Wright for your amazing inputs towards the completion of my project. I learnt a lot from your contributions during those thesis committee meeting we had and when I did my poster presentation and symposium presentation. I owe a huge debt of thanks to my sponsor, the Federal Government of Nigeria, through the Presidential Special Scholarship Scheme for Innovation and Development (PRESSID) for providing finance without which my masters' degree programme and this project here in Edinburgh would not have been borne.

Many thanks to Dr. David Radford, my bench supervisor, for providing me with a solid base when I started my project in the laboratory. I would also like to thank Margaret and the media kitchen team for providing all the necessary ingredients I needed to carry out my experiments. I deeply appreciate the contributions of my laboratory colleagues; Jan Othmann, Alejandro Salinas, Chao-Kuo Liu, Felipe Aguilera Millacura, Marcos Valenzuela Ortega, Dariusz Abramczyk, Maria Trubitsyna.

Special thanks to my lovely wife, Peace and my wonderful kids, Victor and Jeremy, for your inspiration, encouragement and reassurance particular when I was weighed down by the enormity of the work involved in this project. I would not forget all the sumptuous meals you used to package for me like a pupil going to the nursery. To my parents, siblings and the rest of the family, I owe you a lot for being there for me when I needed you most. To friends I met here and those I knew before coming to Scotland, thank you for making my stay here very enjoyable.

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Abbreviations

Abbreviation	Meaning
^{0}C	Degree Celcius
µg	Microgram
µL	Microlitre
µM	Micromolar
AAS	Atomic absorption spectroscopy
ABM6	Arsenic biosensor medium
AUM	Artificial urine medium
bp	Base pairs
BS	Bile salt
BSA	Bovine serum albumin
CIP	Calf intestinal alkaline phosphatase
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotidetriphosphate
EDTA	Ethylenediaminetetraacetic acid
et al.	*et alii*
fw/for	Forward
g	Gram
GFAAS	Graphite furnace atomic absorption spectroscopy
GMOs	Genetically modified organisms
GFP	Green fluorescent protein
GlpF	Aquaglyceroporin
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
iGEM	International Genetically Engineered Machine competition
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani medium
MFC	Microbial fuel cell
MFT	Membrane Filtration Technique
MPN	Most Probable Number
OD	Optical density
ONPG	o-nitrophenyl-β-D-galactopyranoside
p	Promoter
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNK	Polynucleotide kinase
ppb	Parts per billion
RBS	Ribosome binding site
rcf	Relative centrifugal force
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
T	Teepol
TAE	Tris acetate EDTA
U	Unit
UV	Ultraviolet
V	Voltage
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZBM3	Zinc biosensor medium 3

Chapter 1: Introduction and Literature Review

1.1 Introduction

The identification, detection and analysis of chemical and biological substances have been of great interest and significance since the beginning of science. The industrial revolution has not only made life on earth enjoyable and promising, but has also introduced a plethora of issues which make life unbearable and uncomfortable. As the complexity of the world we live in continues to increase, it calls for commensurate efforts and ideas towards sensing and checkmating those activities that make life difficult and intolerable. More often than not, such efforts are quite demanding particularly when it involves the detection of small inorganic molecules, such as heavy metals, and biological contaminants like coliforms. Heavy metal toxicity and coliform contamination have been proven to be a major threat to life and there are several health risks associated with them. Traditionally, these molecules and cells are detected on the basis of chemical reactions or using complex machinery, most of which also can be extremely polluting, unrealistic and basically costly (Nieman *et al*., 1998). Consequently, the spotlight of this thesis has been on whole-cell biosensors for the detection of heavy metals and biological contaminants in environmental samples.

1.2 Heavy metals: occurrence, toxicity and detection

Heavy metals are one of a conceivably misleading concepts as it has no universally accepted definition despite several definitions put up by scientists (Duffus, 2002).Toxicological importance, relative high density, formation of positive ions upon solubilisation, high molar mass or high periodic table number are among the features that basically define heavy metals (Sheppard, 1993; Duffus, 2002). The heavy metals discussed in this work include: arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg), zinc (Zn), gold (Au) and silver (Ag).

Whereas heavy metals exist as natural elements found throughout the earth's crust, the majority of human exposure and environmental contamination are caused by anthropogenic activities like mining and smelting, industrial production and use, as well as the use of metals and metal-containing compounds for domestic and agricultural purposes (Tchounwou *et al*., 2012). The metal and geographical location relatively affect the ratio between these two sources. Basically, the dramatic increase in the outputs from anthropogenic sources is largely due to the industrial revolutions over the last two centuries, whereas increased exposure to natural sources is caused by global population explosion and colonisation of previously uninhabited areas (Callender, 2003; Thevenon *et al*., 2011). The pH is of major significance when water comes into contact with any metal containing minerals. All metals of importance analysed in this work are solubilised from rocks and mineral deposits at significantly increased rates by acidic water (Bradl *et al*., 2005). Listed in table 1.1 are heavy metals of importance in this study together with their common sources and natural occurrence.

Table 1.1: Common sources and natural occurrence of heavy metals of importance in this study. Values are given in parts per billion (ppb) (which for aqueous solutions is equivalent to the same numerical value in µg/L), and parts per million (ppm) (which is equivalent to the same numerical value in mg/L) to reflect concentrations in solid minerals (Siegel, 2002; Bradl *et al*., 2005; National Health and Medical Research Council, 2011).

Metal	Natural source	Anthropogenic origin
Arsenic (As)	13 ppb in ocean clay and shale, 2 ppb in streams	Pesticides, paints, herbicides, electronic parts, wood preservatives, mining, coal combustion, insecticides
Cadmium (Cd)	300 ppb in shale and limestone, 0.01 ppb in streams	Batteries, coal combustion, pigments, mining, metal coatings, plastic stabilisers
Copper (Cu)	Magmatic rocks, 250 ppm in ocean clay, 94 ppm in basalt, 7 ppb in streams	Conductors, roofing, pipes, kitchenware, mining, pigments, herbicides
Gold (Au)	About 2 ppb in streams, below 100 ppb in groundwater	Electronics, jewellery, mining
Lead (Pb)	Magmatic rocks, 30 ppm in ocean clay, 20 ppm in shale, 1 ppb in streams	Batteries, glassware, plastic, pigments, solder, pipes, mining
Mercury (Hg)	0.4 ppb in shale, 0.07 ppb in streams	Metal extraction, fungicides, electronics, solder, mercury vapour lamps, gold mining
Silver (Ag)	Up to 500 ppb in soils, 0.3 ppb in streams	Mining, tableware, electronics, disinfectants, chemical industry, preservatives
Zinc (Zn)	Magmatic rocks, 200 ppm in ocean clay, 118 ppm in basalt, 20 ppb in streams	Anti-corrosion coatings, cans, PVC, batteries, rubber industry, silver purification, paints, solder, coal combustion

Some heavy metals are essential micronutrients that play important roles in many biological processes in microorganisms, plants and animals. For example, zinc is involved in many enzymatic reactions, as it is required in transcription, cell signalling and regulation of pH (Lemire, 2008). Adverse effects ranging from mild symptoms like skin problems to severe conditions of anaemia and immune deficiency are observed when the uptake of these trace elements are deficient (Keen & Gershwin, 1990). However, at elevated concentrations, all heavy metals become toxic as they inhibit the normal biological processes in living organisms.

The main sources of human exposure to heavy metals are water and food. Various types of public health problems ranging from liver and kidney malfunction, cancer, cardiovascular diseases, diarrhoea, etc., have been involved in recurrent exposure to heavy metals (WHO, 2000). Arsenic is a major health problem globally. The WHO recommends 10 µg/ L (10 ppb) limit of arsenic in drinking water (WHO, 1996). More than 77 million people globally are estimated to be at risk of drinking water contaminated with arsenic, in the form of arsenite (AsO_3^{3-}) with As as As(III) or arsenate (AsO_4^{3-}) with As as As(V) ions, which is widespread in South and South East Asia, particularly in West Bengal and Bangladesh (Guha Mazumder *et al*., 1998).

The toxicity of arsenic is based on its chemical form. It is generally accepted that inorganic forms of arsenic are of greatest potential concern to human health as compared to organic forms. Long-term exposure (over many years or decades) to high levels of inorganic arsenic is known to contribute to the risk of certain human cancers and can affect the gastrointestinal tract, kidneys, liver, lungs and epidermis. Short term exposure (over multiple days or weeks) to very high levels of inorganic arsenic can also cause various health issues including skin lesions, nausea, diarrhoea, vomiting and numbness in hands and feet (Joshi *et al*., 2009).

Toxic heavy metals such as arsenic, cadmium, lead, and mercury, can accumulate in living tissues because they are not biodegradable, with potentially causing death or severe health problems (Sheppard, 1993). These metals are often discharged downstream leading to accumulation in clays and adsorption onto algae as a result of their solubility in water. Once accumulated in the food chain, they expand their devastating effects on human and animal health (Bradl, 2005).

Based on these, this work sought to discuss the occurrence of some heavy metals of importance in drinking water, their toxicity and general methods of detection. This work could not discuss the array of remediation procedures like chemical, biological and technological methods which are available for heavy metal polluted solids and liquids because it is beyond its scope (Bradl & Xenidis, 2005). Heavy metal detection using biosensors is discussed (section 1.3.2), after a brief introduction into biosensors in general (section 1.3) and metalloregulatory proteins in bacteria (1.3.5). Table 1.2 shows an overview of the limits set by various national and international agencies for heavy metals in drinking water samples.

Table 1.2: Heavy metal limits in drinking water as set by international authorities.

Where no values are given signifies no set guidelines by the respective national bodies. Values indicated as * have been set for personal care products (PCPs). The approximate molar range is given for the various national and international standards. No limits have been set for Au. (World Health Organisation, 1996; European Commission, 2001; National Health and Medical Research Council, 2011; Health Canada, 2012; Environmental Protection Agency, 2016).

Metal	WHO	Canada	EU	USA	Australia	Molar range
Ag	n.a.	50 µg/L	n.a.	n.a.	100 µg/L	0.46 – 0.92 µM
As	10 µg/L	10 µg/L	10 µg/L	10 µg/L	10 µg/L	0.13 µM
Cd	3 µg/L	5 µg/L	5 µg/L	5 µg/L	2 µg/L	0.018 – 0.046 µM
Cu	n.a.	1 mg/L	2 mg/L	1.3 mg/L	2 mg/L	15.7 – 31.5 µM
Hg	6 µg/L	1 µg/L	1 µg/L	2 µg/L	1 µg/L	0.005 – 0.010 µM
Pb	n.a.	10 µg/L	10 µg/L	15 µg/L	10 µg/L	0.048 – 0.072 µM
Zn	n.a.	5 mg/L *	n.a.	n.a.	3 mg/L	45.9 – 76.5 µM

1.2.1 Arsenic, occurrence and toxicity

Arsenic (As) is the 20th most abundant element on earth and its inorganic forms such as arsenite [As (III)] or arsenate [As (V)] anions are poisonous to the environment and living creatures (Jaishankar *et al*., 2014). Arsenic contamination of potable water, in the form of arsenite or arsenate, has been reported as being a global problem (Stocker *et al*., 2003; Diesel *et al*., 2009; de Mora *et al*., 2011; Siegfried *et al*., 2015). This is particularly relevant for populations in remote and less developed regions where

contaminated water is widely used as a source of drinking water and is the main source of arsenic exposure (Rahman *et al*., 2001; Smedley and Kinniburgh, 2002; Ravenscroft *et al*., 2009). Natural waters contaminated by arsenic are of particular concern because arsenic is known to be toxic and carcinogenic (National Research Council, 1999; Smith *et al*., 2000). More than 100 million people exposed to arsenic poisoning due to arsenic in water supplies resulting from naturally occurring arsenic contamination of groundwater have been reported in recent years from China, South- and Southeast Asia, particularly Bangladesh, Vietnam, India and Cambodia, as a result of inadequate geological prospecting prior to tube well drilling in developmental aid programmes of the past (Guha Mazumder *et al*., 1998; Smith *et al*., 2000; Argos *et al*., 2010; Fendorf *et al*., 2010; Murcott, 2012). Similarly, about 14 million people are estimated to live in regions with elevated arsenic water concentration in Latin America (Smedley et al., 2002; Bundschuh *et al*., 2012).

The maximum safe limit set by the World Health Organisation is 10 µg/L (10 ppb or approx. 0.13 µM) for arsenic, which is now recognised as the most serious inorganic contaminant in drinking water (de Mora *et al*., 2011; World Health Organisation, 2011). Some older standards set this limit at 50 ppb (50 µg/L or approx. 0.67 µM) (Smith *et al*., 2000). Serious disorders, including vascular diseases, irritations of the skin and mucous membranes, keratosis, dermatitis and melanosis are known to be caused by systemic and chronic exposure to arsenic. Inorganic arsenic is carcinogenic to humans with ingestion leading to increased risk of developing cancer of the bladder, skin, kidney and liver (Abernathy *et al*., 1999). Reduction in arsenic exposure and provision of specific drugs for recovery or circumventing disease progression have been the treatment for arsenicosis (Stocker et al., 2003).

As suggested by the WHO, more than 1 in 10 people will die as a result of arsenic related cancers when they continuously consume drinking water with 500 ppb arsenic, approximately 1 in 100 for a contamination of 50 ppb as well as about 6 in 10,000 for 10 ppb. Hence, arsenic contamination of drinking water in Bangladesh alone has been reported as the largest poisoning of a population in history (Smith *et al*., 2000; WHO, 2013). It is imperative to continuously test for arsenic in drinking water as the arsenic contamination level can oscillate due to factors such as the time of the year, amount of rain, recent flooding and changes in topography (Berg *et al*., 2001). Therefore, it would be useful to develop cheap and simple tools (devices) for the local population to be testing arsenic contamination of drinking water sources. In any case, during the design of such devices, efforts should be geared towards minimizing the risks associated with false negative results due to operating errors.

Figure 1.1: Symptoms of chronic arsenic poisoning in Bangladesh and India which include nails rotting, dark spots, bleeding sores, swelling, large warts and a form of gangrene (Adapted from SOS-arsenic.net)

Four oxidation states of arsenic exist, two of which are bioavailable - arsenite (AsO_3^{3-}) and arsenate (AsO_4^{3-}), with arsenite being the more toxic form (Eisler, 1988). Monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) are formed when inorganic arsenic undergoes bio-activation through sequential reduction and methylation reactions in the body – in blood and urine. It is reported that most arsenic is in the form of DMA (Mester and Pawliszyn, 2000). These MMA and DMA are damaging to genes and in some cases more effective than arsenite at causing DNA damage (Agency for Toxic Substances and Disease Registry, 2007).

1.2.2 Cadmium, occurrence and toxicity

As a heavy metal with potential as a human carcinogen, cadmium (Cd) is of considerable environmental and occupational concern. Its wide distribution in the earth's crust at an average concentration of about 0.1 mg/ kg coupled with its frequent use in industrial activities such as production of alloys, pigments, and batteries makes it easily accessible to drinking water (Bradl *et al*., 2005; Tchounwou *et al*., 2012). Humans are exposed to cadmium via inhalation of cigarette smoke, and ingestion of food. Working in cadmium-contaminated work places, employment in primary metal industries, emissions from industrial activities, presence in trace amounts in foods like liver and kidney, leafy vegetables, grains and seeds, potatoes, etc., all are potential sources of cadmium exposure to humans (Tchounwou *et al*., 2012).

Cadmium is a serious pulmonary and gastrointestinal irritant, and is lethal if inhaled or ingested. Symptoms of acute ingestion include but are not limited to abdominal pain, burning sensation, nausea, vomiting, muscle cramps, loss of consciousness and convulsion which normally occur within 15 to 30 minutes (Baselt & Cravey, 1995).

Chronic exposure to cadmium leads to a harsh effect on serotonin, acetylcholine and norepinephrine levels (Singhal *et al*., 1976).

In natural surface waters, cadmium normally occurs largely in the divalent form, made up of various organic and inorganic compounds. An increase in pH and alkalinity causes a decrease in the solubility of dissolved cadmium. Many natural waters contain low background levels of cadmium. A number of aquatic animals accumulate cadmium, with bioconcentration factors in the range of 100 to 100,000. The safe limit of cadmium in drinking water has been set at between 2 µg/ L and 5 µg/ L (approx. 18 – 44 µM) by various national and international regulatory agencies (Table 1.2) to ensure that humans have below the critical amount of 200 mg/ kg cumulative uptake in their lifetime (Reeder *et al*., 1979; French 1986; Fridberg *et al*., 1992; World Health Organization, 1996; National Health and Medical Research Council, 2011; Environmental Protection Agency, 2016).

1.2.3 Copper, occurrence and toxicity

Some unique properties possessed by copper (Cu) have made it a very prominent material for a multitude of purposes. Its use in domestic water pipes is as a result of its corrosion resistance. Also copper is the most widely used conductor in local electricity installations and modern electronics due to its high conductivity. Copper is also used for household goods like pans and pots in a traditional setting. Following tap water installation in the home, prevention of algal growth in water storage systems has been made possible by the controlled use of $CuSO_4.5H_2O$ (World Health Organisation *et al*., 1996; National Health and Medical Research Council, 2011). Natural copper deposits are found in many minerals and in regions with such minerals, it has been

reported that Cu concentrations higher than 5 mg/ L and up to 22 mg/ L are found in water from tube wells or springs, a concentration high enough to pose potential health risks (Bradl *et al*., 2005; National Health and Medical Research Council, 2011).

Whereas copper is an essential trace element required as a cofactor for several enzymes, its high level uptake can cause serious cell damage via free radical formation (Hynninen and Virta, 2009). According to reports, about 2 – 3 mg daily uptake of copper is required by adult humans to stay healthy. There is no widely accepted level of Cu uptake that pose health problems to humans. No effects of Cu uptake in the concentration of up to 2 mg/L in drinking water was found in a study involving 3-month old infants. In other studies, several gastrointestinal symptoms including pain, nausea and vomiting have been implicated in the consumption of drinking water containing between 3 – 5 mg/ L copper (Pizarro *et al*., 1999).

Serious Cu poisoning, like liver cirrhosis in children, has been linked to the use of copper kitchenware (Tanner, 1998). The World Health Organisation has set 0.5 mg/ kg body weight as the maximum tolerable daily copper intake for humans. It's also reported that some national and international agencies have used the above as guidelines for setting their limits which range between 1 – 2 mg/ L (approx. 16 to 31 µM) (World Health Organisation *et al*., 1996; National Health and Medical Research Council, 2011; Environmental Protection Agency, 2016).

Copper is excreted in the body through faeces and urine, predominantly in faeces, which may take several days. Although copper does not seem to pose much of a toxicological concern overall and is not classified by the EPA as a human carcinogen, it is included in this thesis because of the use of copper compounds in Agriculture to treat plant diseases, like mildew, particularly in developing countries. These

compounds are capable of leaching into surface water bodies and can cause stomach cramps, vomiting, nausea, or diarrhoea, when such waters are consumed. High concentrations of copper, when intentionally consumed, can lead to liver and kidney damage, and even death. It is therefore important to include copper testing particularly in areas where qualitative health care services are not adequate and symptoms emanating from high dose of copper can lead to death when not properly detected and treated.

1.2.4 Gold, occurrence and toxicity

Gold (Au) is mainly used in electronics, dentistry, jewellery, and the aerospace industry. It is an essential tool in gene therapy as it serves as a vehicle for gene delivery (Merchant, 1998). It has been reported that Au abound in streams at an average of 2 µg/L and below 100 µg/L in groundwater (Rice *et al*., 2012). The environment is polluted through gold mining when toxic elements from gold tailing and other mine wastes are released into the environment (Ngole-Jeme and Fantke, 2017) with soils in Korea (Lee *et al*., 2005), USA (Straskraba and Moran, 1990), Sultanate of Oman (Abdul-wahab and Marikar, 2012), Ghana (Armah *et al*., 2013), Canada (Percival *et al*., 2014), and South Africa (Gzik *et al*., 2003) heavily contaminated with heavy metals from gold mining activities. Human toxicity has been implicated in poisonous compounds containing gold thiosulfate (Merchant, 1998). No limits exist for Au in drinking water due to the minimal health implications.

1.2.5 Lead, occurrence and toxicity

Lead (Pb) is one of the most abundant heavy metal in the earth's crust (Bradl *et al*., 2005, Table 1.1). Lead is mined and consumed in large quantities as it is used in roofing and solder as well as plumbing in historical buildings. Lead is also used in construction, for battery and accumulator production, as gasoline additives, as cable coatings, in ammunition and others (Sheppard, 1993), all of which release lead to the environment. It is reported that in the USA alone, more than 100 to 200,000 tonnes of lead per year is being released from vehicle exhausts. The high Gross Domestic Product (GDP) of countries like Afghanistan, Algeria, Iraq, Myanmar, North Korea and Yemen are oil-dependent. They were still using tetraethyl lead (TEL) till early 21st century when it was phased out due to economic consequences arising from the high cost of changing their leaded refineries to unleaded ones (Jaishankar *et al*., 2014; Sawe, 2017). Some of the released lead is taken up by plants, some adsorbs to the soil and flows into water bodies. The general population is exposed to lead either through food or drinking water (Jaishankar *et al*., 2014), while children are mainly poisoned through contact with dust contaminated by lead based paint (Brad *et al*., 2005).

Lead is an extremely toxic metal whose extensive use has caused considerable environmental contamination and health problems globally. Unlike other metals such as copper, zinc and manganese, lead does not play any biological functions in living cells (Jaishankar *et al*., 2014). The body of healthy adults absorbs 35 to 50% of ingested lead (≥ 50 % in children) most of which is deposited in the bones (Sheppard, 1993; Jaishankar *et al*., 2014). Although the blood lead level can reduce in weeks or months following lead exposure, the skeleton functions as a lead depository, capable

of releasing lead back into the blood over decades hence setting a half-life of bone lead practically between 7 and 27 years (Rabinowitz, 1991; Sheppard, 1993).

Acute and chronic lead exposure causes devastating effects on the cells and nervous system (Ercal *et al*., 2001). Depression, excitement and irritability are some of the early symptoms of lead exposure. Later effects include, but are not limited to, brain damage, kidney damage, headaches and gastrointestinal problems followed by nervous system malfunctions which diminishes nerve conduction and general mental performance (Sheppard, 1993; Chen *et al*., 2012; Jaishankar *et al*., 2014).

It is imperative that lead levels in drinking water be reduced as low as possible. This is on the premise of a report that lead can be carcinogenic to the kidney (Spector *et al*., 2011). Also, lead levels in human blood are reported to be routinely close to the baseline for lethal effects apart from additional dietary uptake or other additional exposure (Flegal & Smith, 1992). It is essential that local authorities particularly in developing countries should take adequate measures aimed at reducing lead levels if the lead limit of 15 ppb (approx. 0.072 µM) set by the EPA is crossed (Environmental Protection Agency, 2016). A limit of 10 ppb (approx. 0.048µM) has been set by the UK Drinking Water Inspectorate (HKSAR Government, 2015), although the USA Environmental Protection Agency has also stipulated that there should be no lead contaminant in drinking water based on the public health angle (Environmental Protection Agency, 2016). Millions of people around the world are still being affected by lead poisoning as the set limits are still usually exceeded in spite of the pragmatic approaches being taken (Olson *et al*., 2016).

1.2.6 Mercury, occurrence and toxicity

Mercury (Hg) is a heavy metal with unique characteristics in that it exists or occurs naturally in the metallic element, inorganic salts, and organic compounds forms, with each possessing its own toxicity and bioavailability profile (Clarkson *et al*., 2003). Elemental Hg exists at room temperature as a liquid with a high vapour pressure, and is released as Hg vapour into the environment. Mercury also exists as a cation with oxidation states of +1 (mercurous) or +2 (mercuric) (Jaishankar *et al*., 2014). The volatile form, methylmercury, is toxic and is formed when the inorganic (mercuric) forms of Hg are methylated in the presence of sulphides by microorganisms found in soil and water (Dopp *et al*., 2004).

Mercury is a toxin and is also very bioaccumulative. The marine environment is adversely affected when Hg is present, hence many studies are geared towards Hg distribution in water environment. Humans are exposed to all forms of mercury mainly through anthropogenic activities like agriculture, dental care, preventive medical practices, mining, municipal wastewater discharges, incineration, and discharges of wastewater of industrial origin (Chen *et al*., 2012).

The volatile methyl mercury $(CH_3Hg)^+$ is absorbed almost completely by the body. It is very toxic and can settle in the aquatic food chain (Harada, 1995; World Health Organisation, 2013). Several cases of Hg poisoning exist since the 1950s as a result of contaminated food and seafood, which ended up in the mass poisonings of Minamata, Japan and the Basra poison grain disaster (Takeuchi *et al*., 1962; Harada, 1995). The Minamata poisoning was as a result of the Hg entering the local rivers over three decades due to industrial activities which culminated in a high concentration of Hg in the aquatic food chain in rivers and local coastline. This led to the local

population developing a wide range of neurological symptoms from numbness to insanity, coma and death (Takeuchi *et al*., 1962; Harada, 1995). The Basra poison grain disaster occurred due to the feeding of livestock with Hg treated seed grains and its use in direct preparation of food. Bakir (1973) reported that symptoms similar to those observed in Japan were seen as a result of mass poisoning which affected thousands of the population.

1.2.7 Silver, occurrence and toxicity

Despite being used universally as a disinfectant and a preservative for drinking water, high levels of silver (Ag) are generally unacceptable. Akin to the side effects of antibiotics, the health implication of the antimicrobial effects at very high silver concentrations is not well understood. At prolonged exposure over an extended period, lower levels of Ag can still lead to negative effects. A well-known example is the argyria or argyrosis which is a condition caused by excessive exposure to chemical compounds of silver, or to silver dust. The most characteristic symptom of argyria is that the skin turns purple or purple-grey (Hill & Pillsbury, 1939). According to the EPA, 0.1 mg/L (approx. 0.93 µM) is the recommended limit for Ag in drinking water (Environmental Protection Agency, 2016). Very low concentrations of Ag (0.2 to 0.3 µg/L) are generally seen in natural water sources. These levels can be elevated to about 50 µg/L (approx. 0.46 µM) due to its extensive use as a disinfectant, and as a pollutant from industrial activities (Nordberg et al., 2007). Also the levels in food can rise to between 10 and 100 µg/kg (Gibson & Scythes, 1984). As a result of these, it is estimated that the safe limit for human consumption is a daily dose of 0.4 mg (approx. 3.7 µmol) (National Health and Medical Research Council, 2011).

1.2.8 Zinc, occurrence and toxicity

Zinc (Zn) is an essential metal that is actively imported to satisfy cellular Zn requirements. However, at high concentrations, Zn inhibits the aerobic respiratory chain, and it can be significantly toxic (Hynninen and Virta, 2009). Much more widespread is Zn deficiency. However, zinc toxicity occurs when Cu uptake is reduced as a result of the interfering effects of a high Zn diet. Symptoms of acute Zn exposure include cell death in the brain (Plum *et al*., 2010). Zn is widely used in many industrial processes, as it is relatively innocuous. In addition to being a common component in paints, rubber and cosmetics, Zn is widely used in the galvanising of steel to prevent corrosion leading to frequent contamination of potable water supplies. The decay of galvanised iron and steel is aided by unfavourable pH and other pollutants in drinking water. When water is stored in galvanised water tanks, the concentration of Zn can reach between 2 - 11 mg/L (approx. 30 – 168 µM). With no known health implication limits for Zn concentration in drinking water, the setting of limits of between 3 – 5 mg/ L (approx. 30 µM and 76 µM respectively) was to preserve the aesthetic properties of drinking water. Unpleasant taste and appearance of drinking water have been implicated in higher and lower Zn concentrations (World Health Organisation, 1996; National Health and Medical Research Council, 2011).

1.2.9 Current detection methods and their limitations

For a sustainable introduction of treatment technologies against health problems associated with heavy metal contamination, a countrywide system based on monitoring the water quality of treatment systems together with public and private wells should be used as an indicator (Siegfried *et al*., 2015). Among others, current methods

of arsenic testing in drinking water include: inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma optical emission spectrometry (ICP-OES), atomic absorption spectroscopy (AAS) and chemical test kits based on the Gutzeit method (Kinniburgh and Kosmus, 2002; Hung *et al*., 2004; Kabir, 2005). Generally, graphite furnace atomic absorption spectroscopy (GFAAS) has been the standard method for arsenic detection (Rice *et al*., 2012) and produces detection limits usually around 5 µg/L (approx. 66.7 nM) (National Health and Medical Research Council, 2011). For GFAAS, an atomization chamber having an inert atmosphere is required for the vaporization of the sample off a graphite tube (furnace) through extreme heat. These tubes need to be replaced after making about 50 to 100 measurements as they have a limited lifetime. Also each measurement requires standard curves as linear measurement range is minute and individual measurements can behave differently as a result of layout and component-wear.

However, GFAAS has its disadvantages as it is not suitable for field use, cannot measure complex samples and it requires skilled manpower and extreme laboratory conditions. GFAAS can suitably detect As, Cd, Cu, Pb, and Ag, and also has limited precision for Au and Hg (Rice *et al*., 2012). An alternative to GFAAS is hydride generation atomic absorption spectroscopy, which has a somewhat greater sensitivity (Health and Medical Research Council, 2011). In this method, As(V) has to be reduced to As(III), followed by conversion to its volatile hydride by sodium borohydride. The influence of many contaminants in the sample can be eliminated through the removal of the hydride, although Ag, Au, Cu, Pb, and some other metals are capable of suppressing the response of the hydride (Rice *et al*., 2012).

The ICP-MS incorporates a high temperature ICP (Inductively Coupled Plasma) source with a mass spectrometer. The ICP source functions by converting the atoms

of the elements in the sample to ions, which are then separated and detected by the mass spectrometer. Like the ICP-MS, the ICP-OES makes use of the emission spectra of the sample to identify and quantify the elements present. Here, samples are introduced into the plasma through a process which desolvates, ionises, and excites them. The constituent elements are then identified by their characteristic emission lines, and quantified by the intensity of the same lines. Although highly accurate results are obtained from ICP-MS, ICP-OES, AAS and other spectrometric methods, they are very expensive, require installation infrastructure (gas, chemical supplies) and highly skilled personnel for correct analysis and result interpretation, and they are not available in resource limited regions of the world (Tanner and Baranov, 1999; Elliott *et al*., 2004).

The chemical field test kits based on the Gutzeit method is currently the basic method of arsenic assay, and they are cheap and simple to handle (Crawford & Tavares, 1974). However this method adds to the public health problem as the toxic arsine gas (AsH_3) produced by the reduction of arsenite and arsenate reacts with hazardous chemicals, mercuric salts ($HgBr_2$) to generate a coloured compound (Rahman *et al*., 2002; Kabir, 2005). It is also reported that the method yields sensitivity limit of between 10 – 50 µg/L arsenic detection, but large field studies have demonstrated that even field testers, although instructed in a week-long training session, still generated a significant amount of false positive and false negative results (Jakariya *et al*., 2007). The available field test kits are also reported to be quite expensive in many regions of Asia with about 100 tests costing from around £95 (Hach, 2013).

GFAAS has been the standard method for cadmium detection in drinking water, amongst others. The limit of detection is around 5 µg/L (approx. 44 nM) (Rice *et al*., 2012). Also, GFAAS and other versions of atomic absorption spectroscopy can be

used to detect copper. Copper can be detected by these methods below the limits set for drinking water analysis, nonetheless the limitations posed by cost and complexity still persist (Rice *et al*., 2011). Traditionally, Au is detected by physical methods using variants of atomic absorption spectroscopy in small concentrations (usually µg/L, sub-µM) (Rice et al., 2012).

For lead, graphite furnace atomic absorption spectroscopy (GFAAS) has been the standard technique for detection in drinking water, detecting accurately at a limit of about 5 µg/L (approx. 24.1 nM) (National Health and Medical Research Council, 2011). However, GFAAS is unsuitable for field use as it cannot measure complex samples and it requires skilled manpower and extreme laboratory conditions for operation. The dithizone method can be used for lead detection and this is considerably simple to use. It takes the photometric determination of the cherry-red lead dithizonate which is a product of a chemical reduction reaction as well as a chloroform extraction (Snyder, 1947). This procedure is not suitable for field use as it requires chloroform and other conceivably harmful reagents. Also the need for a spectrophotometer and laboratory conditions add to its being unsuitable for use in the field.

The cold-vapour atomic absorption spectrometry (CVAAS) has generally been used by the Standard Methods for Water and Wastewater as the preferential method for the detection of mercury. This is because CVAAS is simple, highly sensitive and relatively free from interferences. Also, mercury is usually present at low levels in natural waters, hence sensitive methods are required for detecting such low concentrations (Manzoori *et al*., 1998). This technique, like all atomic absorption methods, needs large and costly equipment, highly skilled labour and laboratory conditions (Rice *et al*., 2012).

The graphite furnace atomic absorption spectroscopy (GFAAS) and the dithizone technique amongst other methods have been used for detecting silver. The GFAAS detects Ag to a limit of 2 µg/L (approx. 0.02 µM), while 10 µg/L (approx. 0.09µM) is the detection limit for dithizone method (World Health Organisation, 1996). Zinc is basically detected by atomic absorption spectrometry (AAS) with a detection limit of about 20 µg/L (approx. 0.3 µM) (Rice *et al*., 2012). Although the above physical methods are quantitative and reliable (Aggett and Aspell, 1976), there is still the problem of getting water samples from contaminated locations to laboratories with testing facilities which adds to cost.

1.3 Sensing of heavy metals by living organisms

Living organisms find themselves in chemical environments the issue of which evolution had been trying to solve for some 4 billion years (Bell *et al*., 2015). To be able to adapt in a variety of habitats in which they find themselves, microorganisms have developed the ability to sense and regulate a wide range of chemicals (Morita *et al*., 1999). ‟The immensely powerful molecular recognition properties of living systems'' (Budak *et al*., 2013) have presented man with great potential to utilise them in solving environmental and health problems that confront him. This concept can be regarded as the basis of biosensors. By definition, ‟A biosensor is an analytical tool which incorporates a biological recognition element with a physical transducer. The aim is to produce a quantitative signal which is proportional to the concentration of analytes (either a specific chemical or set of chemicals)'' (Turner *et al.*, 1987; Eggins, 2002). Biosensors have been comprehensively studied and widely applied in different fields, including food safety, public health, environmental monitoring, homeland security, since the first biosensor for glucose detection was developed in 1962 by Clark and

Lyon (Kissinger, 2005; Patolsky *et al*., 2006; Lazcka *et al*., 2007). Generally, the biological recognition elements are basically categorised into two groups: the biocatalysts, including enzymes, tissue material and whole microorganisms; and the bio-ligands, like trans-regulatory elements, antibodies, nucleic acids and lectins (Castillo *et al*., 2004; French *et al*., 2015). Biological recognition element is one of the features of classifying biosensors as shown in Figure 1.2.

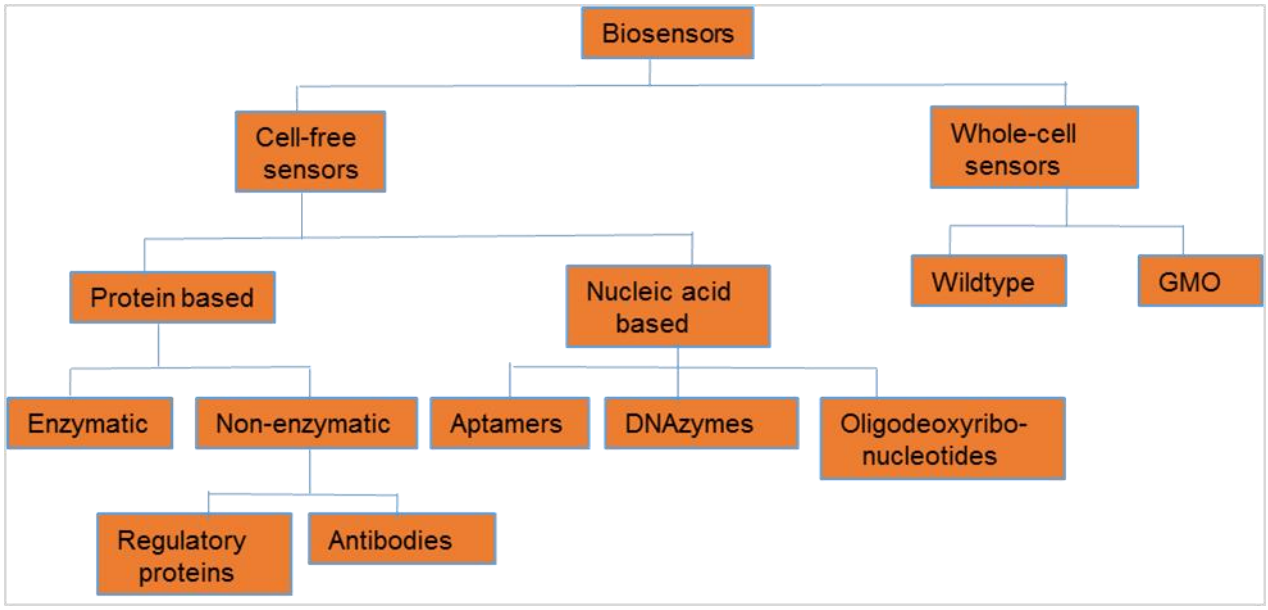


Figure 1.2: Biosensor classification by common biological recognition elements (modified after Verma & Singh, 2005).

It is noted that several criteria are considered when choosing a biological element, one of which is dependent on the properties of the analyte(s) of interest. For instance, it is impossible for any recognition system exclusively present inside the cell to sense analytes that cannot penetrate into the cell. Biological recognition elements offer major advantages in their use in biosensors because of their good specificity (detected metals), sensitivity (detectable concentrations) and portability and short detection

times. In addition, biological systems require miniaturized and inexpensive systems unlike chemical and physical analyses (Hynninen and Virta, 2009; Yáñez-Sedeño *et al*., 2014).

Different approaches to biosensors, for instance, the Edinburgh Arsenic biosensor, have expanded the relatively tight earliest definition of transducer as an element for generating digital electronic signal (Clark & Lyon, 1962; de Mora *et al*., 2011). Such approaches put into consideration the infrastructure, costs, workability under adverse conditions and availability of trained personnel by simplifying components while maintaining their specificity and sensitivity. Transducers generally used convert biological responses into quantifiable signals which are measured mechanically, electrochemically, calorimetrically, optically, or electronically (Cunningham, 1998; Eggins, 2002; Wilson, 2005). Although the biological recognition element is significant for the specificity, the sensitivity of the biosensor is largely influenced by the transducer used (Castillo *et al*., 2004).

For a biosensor to be accurate, its output must be quantifiable and proportional to the amount of analyte tested. The blood glucose biosensor has been the most successful biosensor since its development in 1962. This biosensor alone accounts for about the majority of the total biosensor sales (Clark & Lyon, 1962; Abrevaya *et al*., 2015). In this system, β-D-glucose is oxidised by glucose oxidase when flavin adenine dinucleotide (FAD) is present to generate $FADH_2$. The FADH2 then reacts with oxygen to produce hydrogen peroxide, which is oxidised by the transducer – a platinum electrode- and the signal (easily recognisable flow of electrons) is proportional to the amount of glucose molecules. However, modern systems make use of soluble mediators to replace oxygen. (Yoo & Lee, 2010). A wide range of biosensors have been developed, some of which are regarded as ‘’one-shot biosensors’’ as they are

used for single measurements unlike the original glucose sensors which are designed for continuous sensing (Turner, 2000; Giedroc & Arunkumar, 2007). Although biosensors are generally highly specific and sensitive, introducing a biological component into a technical system often creates problems of its own such as durability and shelf-life (Rawson *et al*., 1989).

1.3.1 Cell-free biosensors

In general, generation of cell-free biosensors involves growing organisms that house sensing elements followed by some purification steps. One major advantage of this system is that analytes can readily access the sensing elements faster and more easily since they are not hidden inside a living cell (Harms *et al*., 2006). The intricate surrounding medium for the recognition element is greatly reduced due to lack of metabolism. This also reduces any further interactions with chemicals, nucleic acids and proteins. However, enzymatic reporters have their disadvantages. Although purified enzymes are commonly used in biosensors because of their high specificity and sensitivity, the purification process is difficult, expensive and time-consuming (D'Souza, 2001; Yagi, 2007). Also the wide use of enzyme-based biosensors in the fields of environmental sciences and biotechnology has been hindered by the unstable nature of enzymes resulting in a decrease of enzyme activity (Byfield and Abuknesha, 1994).

1.3.2 Whole-cell biosensors

The employment of whole-cell biosensors using recombinant DNA technology can provide a possible alternative for simple, robust, accurate and cheap assay of heavy

metals. This involves the use of genetically modified bacteria to generate signals which are detectable when exposed to the analyte(s) of interest. Whole-cell biosensors have many advantages in that they are sensitive, specific, accurate, simple, cheap and portable (Tauriainen *et al*., 1997).

The interest in biosensor development has seen different biological recognition elements such as enzymes, cofactors, tissues, organelles, microorganisms, antibodies, and cells from higher organisms being utilized (Lei *et al*., 2006). A novel approach for constructing genetically modified microorganisms (GMMs) has been offered by recent developments in molecular and synthetic biology, which provides a new direction in manipulating the sensitivity and selectivity at the DNA level of microbial biosensors (Belkin, 2003; Paul *et al*., 2005; Urgun-Demirtas *et al*., 2006). For heavy metal detection, the construction of recombinant bacteria involves the fusion of a promoter responsive to the target heavy metal ion, with the genes for a reporter protein introduced in a host bacterium. The heavy metal ion induces synthesis of the reporter protein resulting in the production of a detectable signal proportional to the concentration of the ion (Castillo *et al*., 2004). Bacterial (lux) or firefly (luc) luciferase, which emits a bioluminescent signal, or β-galactosidase (*lacZ*), which can be detected electrochemically or via chemiluminescence, are common enzymes used as reporters (Tauriainen, *et al*., 1997; Ramanathan *et al*., 1998; Biran *et al*., 2000; Tauriainen *et al*., 2000; Ivask *et al*., 2002; Aleksic *et al*., 2007; Siegfried *et al*., 2015). Green fluorescent protein (GFP) is another reporter protein but it contains a covalently bound chromophore, hence does not need cofactors or substrates for producing a signal. Whole cell biosensors commonly produce signals which cause the surrounding media to change and are then read as outputs. Common examples include: colour change, change in pH, emission of light, or other changes in the chemical composition of the

intracellular or extracellular environment (Bousse, 1996). Comparatively, a long incubation period (several hours) is necessary to produce the signals which are derived from chemical compounds or enzymes secreted by the bacterial cell.

Most functions usually carried out by silicon based semiconductors have been incorporated into biological systems leading to a higher degree of integration (Simpson & Sayler, 2001). This is as a result of work being put in place to process various inputs and create logic gates by simulating semiconductor tools within a microbial cell (Bonnet *et al*., 2013). The task of developing these kind of biosensors by molecular engineering of cells is a complex one. To date, there is no complete understanding of gene functions and parts of the metabolism even though the genomes of many microorganisms have been completely sequenced.

Bacteria are the most common microorganisms used for whole-cell biosensor development although mosses (Ramos, *et al*., 1993), algae and yeasts (Lehmann *et al*., 2000) as biosensors have been reported. Several advantages of bacterial cells as the biological recognition component over purified enzymes include: the existence of various kinds of microorganisms in the natural environment and their maintenance in culture collections, giving room for the choice of an appropriate strain for a given purpose (Su *et al*., 2011); the rapid proliferation of bacteria under aerobic and anaerobic conditions in relatively cheap media (Yagi, 2007); and the ability to perform multi-step processes since all reactions are conveniently packaged within the cell and hence, efficiently carried out (Castillo *et al*., 2004]. Whole tissues or cells are a source of a large quantity of enzymes in their natural environment where enzymatic pathways are already optimized and all cofactors, substrates and reactants are available. Also, the stability of the enzyme is not compromised by purification steps, which can be expensive and decrease the biological activity of the protein (O'Connor *et al*., 2000).

Although the metabolism of bacteria is not specific, undesired metabolic pathways can be blocked or the desired metabolic pathways induced in order to achieve highly selective whole-cell biosensors (Su *et al*., 2011). The bacterial cells can also be adapted to the desired substrate of interest (target) via selective cultivation. The ability of bacterial cells to utilise multiple stages like transcription/translation or other complex multi-step reactions (all of which are efficiently carried out in the cells) as sensing mechanism make all these advantages more prominent (Castillo *et al*., 2004).

To design and construct whole-cell biosensors, it is important to understand the metabolism of microorganisms which is not as easy as usually claimed (Su *et al*., 2011). Hence, there is an introduction of some degree of undesirable ambiguity and complications in the development of whole-cell biosensors as a ‟biological package'' that supports, replicates and maintains the sensing components (Castillo *et al*., 2004). Another constraint for ‟biological package'' as biosensors is the issue of shelf life. Usually the life span of any biological or cell-free package is undoubtedly shortened when such package is preserved or stored under wrong environmental conditions (Bjerketorp *et al*., 2006). However, cells require a regular supply of nutrients, trace elements, sometimes oxygen and other substances essential for their growth as they replicate. It is also necessary to remove waste products from the environment they live. Stagnation in growth and eventually cell death occur when nutrients are not supplied or wastes removed. Hence, some of the solutions are to store the cells in an inactive form or to maintain the whole-cell biosensors in a continuous culture with a steady and effective control of growth conditions. This may not possibly be the best solution as it can lead to culture contamination and is difficult to maintain. Variation between different batches of cells can also be induced by routine culture, resulting in

biosensor failure due to a dysfunction in the sensing element or failure of signal transduction (Pooley *et al*., 2004; Endo *et al*., 2010).

In some cases, the penetration of molecules to be analysed into sensing cells poses a problem. Diffusion, nonspecific uptake or active transport are the major routes through which analytes penetrate into cells. When membrane permeability for an analyte is not adequate, a transport mechanism needs to be incorporated to import the analyte into the cell in adequate concentrations. The recognition element can also be specifically allocated to the outside of the cell. All these methods have their major drawbacks. It is therefore necessary to carefully manipulate the microbial membranes to avoid overcrowding or risks associated with disrupting innate membrane functions (Minton *et al*., 2000; Katzen *et al*., 2009).

Much as most whole-cell biosensors developed have not been licensed for field use because of regulatory issues with transgenic organisms, bacterial sensors can be made cisgenic, a system of innovative rewiring of the native chassis circuits with a natural gene coding for a desired trait, from the organism itself or from the relative strain. In this regard, no foreign DNA is introduced into the final organism. Although the genome is modified, it does not contain any DNA sequences particularly protein-coding sequences, from sources outside the host species or genus.

1.3.3 Whole-cell natural biosensors for heavy metals

Suffice to say that whole-cell biosensors which make use of organisms that are not modified are also available. As those organisms used are non-pathogenic, those non-engineered whole-cell systems, unlike the modified systems, are not subject to legislative regulations which is an added advantage. A biosensor which works by restricting the effect copper ions have on bacterial growth has been reported. The

growth of bacteria isolated from a Portuguese lagoon and cultured on a piezoelectric quartz crystal was measured by monitoring the frequency of the crystal. The bacterial growth was markedly reduced when copper [Cu(II)] added to the growth medium was above certain concentrations. This system can accurately measure copper concentrations as low as 18 ppm (Yamasaki *et al*., 2004), however it can respond to anything else that reduces growth.

Despite the relative advantages of microbial electrodes as sensor systems, they have some setbacks as they are vulnerable to contamination or interference since they work based on approximately indefinite toxicity or accumulation processes. This restraint can be partly overcome by using sensors based on genetically modified microorganisms (GMMs) by particularly generating signals that can be quantified (Verma & Singh, 2005).

1.3.4 Transcriptional regulatory protein families

Metals play a fundamental role in the growth and development of microorganisms, but they become toxic to microorganisms at high concentrations (Beveridge and Murray, 1976; Cohen *et al*., 1991; Bruins *et al*., 2000). This occurs by displacement of essential metals from their native binding sites or through ligand interactions (Poole and Gadd, 1989; Bruins *et al*., 2000). Toxicity also results from changes in the conformational structure of nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance (Poole and Gadd, 1989). To maintain their metal homeostasis, microorganisms have developed a variety of chromosomal, transposon, and plasmid-mediated resistance systems (Bruins *et al*., 2000; Lloyd and Lovley, 2001; Hynninen and Virta, 2009).

Generally, metal biosensors measure the bioavailability of metals in different environmental samples. The sensitivity (detectable concentrations) and specificity (detected metals) of the whole-cell biosensor are determined by the sensing element. Although the sensitivity and specificity are influenced by the binding capacity (metal species and sensed concentration) of the transcription factor used, they also depend greatly on the microbial metal homeostasis/resistance systems (Hynninen and Virta, 2009). In this regard, metal transporters are important for maintaining homeostasis of essential metals as well as conferring resistance to toxic heavy metals. These transporters potentially determine the intracellular concentration of metals that is basically available for detection by the biosensors (Blencow and Morby, 2003; Hynninen and Virta, 2009).

Copper homeostasis, for example, is required for the evolution of aerobic metabolism considering the toxicity of copper even at low concentrations. This is as a result of copper being a redox-active transition metal. Anaerobically, copper seems to convert from the Cu(II) to the Cu(I) oxidation state , becoming much more toxic, which could be as a result of the diffusion of Cu(I) through the cytoplasmic membrane as seen in Figure 1.3 (Beswick *et al*., 1976; Outten *et al*., 2001). The mechanisms of copper transport and homeostasis in bacterial cells are only partially understood, and their functions are listed in Table 1.3 (Rensing and Grass, 2003). *E. coli* is equipped with multiple systems to ensure proper maintenance of copper in changing environmental conditions. MerR-like activators regulate genes involved in copper homeostasis, and these activators are responsive to cytoplasmic Cu(I) or two-component systems sensing periplasmic Cu(I). Excess Cu(I) is removed from the cytoplasm by the Cu(I)-translocating P-type ATPase CopA which is the central component in copper homeostasis. The periplasmic space is kept safe from copper-induced toxicity by the

multi-copper oxidase CueO and the multi-component copper transport system CusCFBA. Genes involved in the detoxification of periplasmic copper is encoded by the *pco* determinant, although the mechanism is still unknown (Outten *et al*., 2000; Rensing & Grass, 2003; Solioz & Stoyanov, 2003).

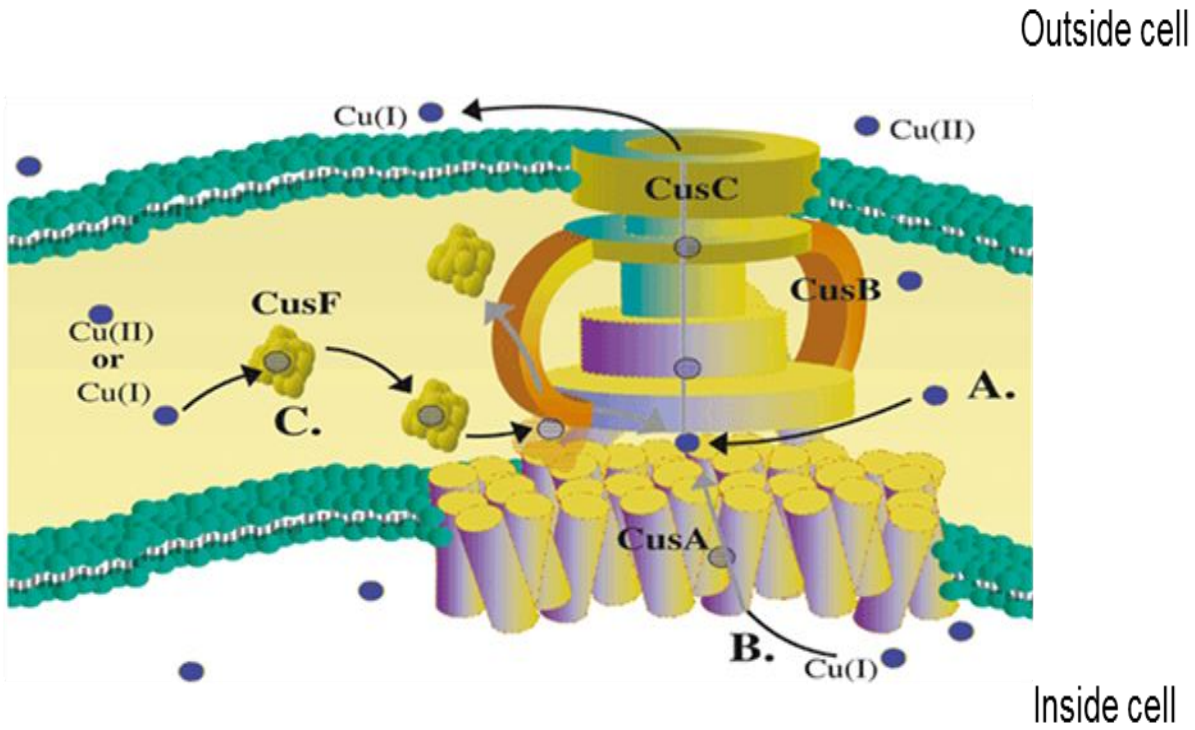


Figure 1.3: Functional model of the Cus efflux complex. The four-part Cus complex consists of the inner membrane pump CusA, the periplasmic protein CusB and the outer membrane protein CusC forming a channel bridging the periplasmic space. Entry of copper may occur from the periplasm (A), from the cytoplasm (B) or via the copper-binding chaperone CusF from the periplasm (C) (Rensing and Grass, 2003).

Table 1.3: Elements of Cu homeostasis in *E. coli* (Adapted from Rensing and Grass, 2003)

Homeostatic Mechanism	Regulated by	Function
CopA	CueR (sensing cytoplasmic Cu[I]) and CpxR (sensing cell envelope stress)	Efflux of cytoplasmic Cu(I)
CusCFBA	CusRS (two-component regulation system, sensing cytoplasmic Cu[I])	Efflux of periplasmic (and possibly cytoplasmic Cu[I])
CueO	CueR (sensing cytoplasmic Cu[I])	Activates efflux pumps
PcoABCD	PcoRS, (two-component regulation system, sensing periplasmic Cu[I]) (and CusRS)	Activates efflux pumps
PcoE	CusRS (PcoRS)	Periplasmic copper chaperone, copper binding

Bacteria can use different mechanisms to maintain Zn homeostasis processes, together with zinc import and export, intracellular zinc binding and zinc-sensing (Figure 1.4). The levels of presence of the importer and exporter proteins control the fluctuation of Zn^{2+} in and out of the cell. In bacteria, these levels are predominantly controlled at the transcriptional level, where several types of zinc-responsive transcription factors sense intracellular Zn^{2+} when available. These transcription factors include members of the ferric uptake regulator (Fur) family (Zur) and the MarR/SlyA family (AdcR) which up-regulate zinc import, and also members of the MerR (ZntR) and ArsR/SmtB families (SmtB, ZiaR, and CzrA) which increase zinc export and/or intracellular sequestration (Mikhaylina *et al*., 2018).

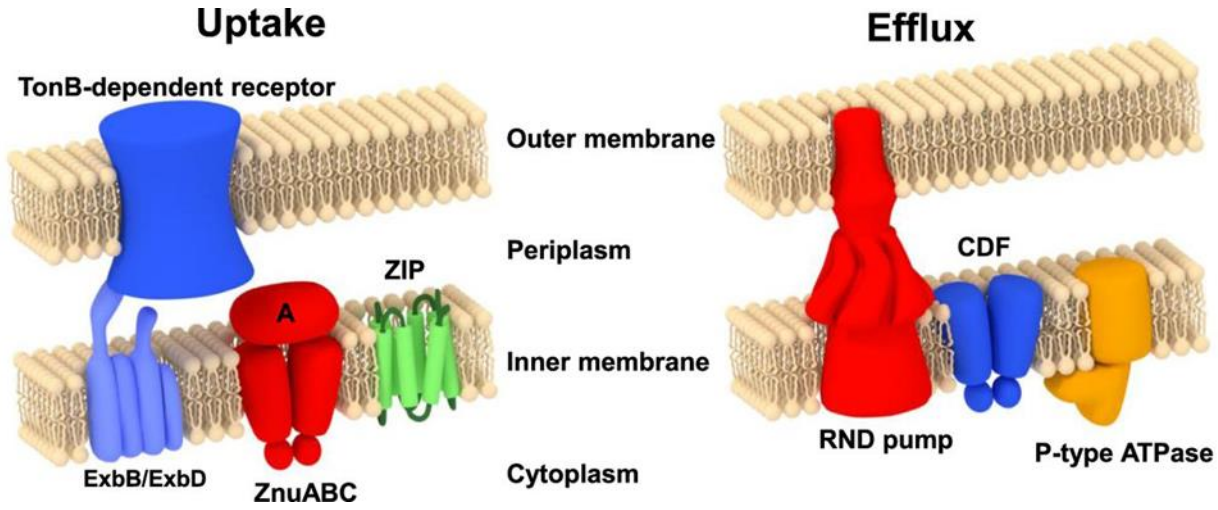


Figure 1.4: Overview of the major players in bacterial zinc uptake and efflux, illustrated for a Gram-negative bacterium. Proteins for import include members of the ZIP (zinc-iron permease) family and members of the ATP-binding cassette (ABC) superfamily. The latter systems consist of a membrane-bound permease, an ATPase, and a protein that is periplasmic in Gram-negative bacteria or on the cell surface in Gram-positive bacteria. These systems are usually named ZnuABC (Gram-negative bacteria) or AdcABC (Gram-positive bacteria), although this distinction is not consistently adhered to. A third label used frequently for such zinc importers is TroABC. Exporters include P-type ATPases, members of the cation–diffusion facilitator (CDF) family, and tripartite RND (root–nodulation–cell division) systems. Regulatory proteins and further processes are explained in the main text (Mikhaylina *et al*., 2018).

In prokaryotes, well-regulated processes of metal import and export across the cytoplasmic membrane, and metal ion sequestration by metallo-chaperones are basic mechanisms of maintaining cellular concentrations (Blencow and Morby, 2003). This is achieved via specific transcription factors, which sense excess concentrations of metal ions within the cell. The expression of genes involved in uptake mechanisms is firstly repressed which leads to a decrease in gene expression (Hynninen and Virta, 2009). The cell is activated to export excess metal caused by further increases in intracellular metal concentration, which after reaching a certain level, induces expression of efflux transporters (Rouch, *et al*., 1995). The promoters of these

transporters are controlled by the transcription factors and this forms the basis of most sensor elements in biosensors (Hynninen and Virta, 2009).

Biosensors can be constructed using transcription factors that regulate toxic metal resistance mechanisms based on the cell's ability to detect very low intracellular metal ion concentrations (Nies, 2003). Resistance operons to heavy metal ions are stably inserted into some bacterial chromosomes. Others are located on endogenous plasmids or transposons that usually have multiple resistance or detoxification operons (Silver, 1992; Silver and Phung, 1996; Taghavi *et al*., 1997). Resistance operons encode proteins including cytoplasmic or periplasmic metal transport proteins, metal reductases, metal-specific efflux pumps, membrane-bound transporters, or metal-sequestering proteins. In addition, they encode at least one trans-acting metal-responsive transcriptional regulator for the operon (Silver, 1992; Rosen, 1996; Xu *et al*., 1996). The transcriptional regulators can act as activators or repressors, and both types of sensor proteins have been reported in Gram-positive and Gram-negative bacteria (O'Halloran, 1993; Alonso *et al*., 2000). Two classes of transcriptional regulatory proteins exist, namely; the MerR and SmtB/ArsR families. Although different metals can be sensed by members of the same family, members of different families can also sense a specific metal (Osman & Cavet, 2010). There is no clear understanding of how metal specificity is accomplished and they can differ between the families (Osman & Cavet, 2010).

1.3.5 The SmtB/ ArsR family of metalloregulatory proteins

In 1992, Kaur & Rosen reported a tightly-controlled system for detoxifying arsenic which was firstly described in *Escherichia coli*. Bacterial cells accumulate arsenic through nonspecific transport via the active phosphate import system (Eisler, 1988). Resistance to arsenic is determined by specifically exporting arsenite (AsO_3^{3-}) and arsenate (AsO_4^{3-}) (Silver *et al*., 1981; Nies, 2003, see section 1.1.1 for more details on the toxicity and chemistry of arsenic). Although *E. coli* is best used for its study, the mechanism is generally similar in most microorganisms (Carlin *et al*., 1995; Moore & Helmann, 2005). The *ars* operon of *E. coli* consists of five genes – *arsRDABC* – which are controlled by the promoter, P_{ars} (Figure 1.5). The genes *arsA*, *arsB* encode for the two subunits of arsenite efflux pump, while *arsR* and *arsD* encode regulatory proteins (Busenlehner *et al*., 2003; Chen & Rosen, 2014). *ArsC* encodes arsenate reductase which reduces arsenate, As(V) to arsenite, As (III) which can then be transported by the arsenite efflux pump (Chen & Rosen, 2014).

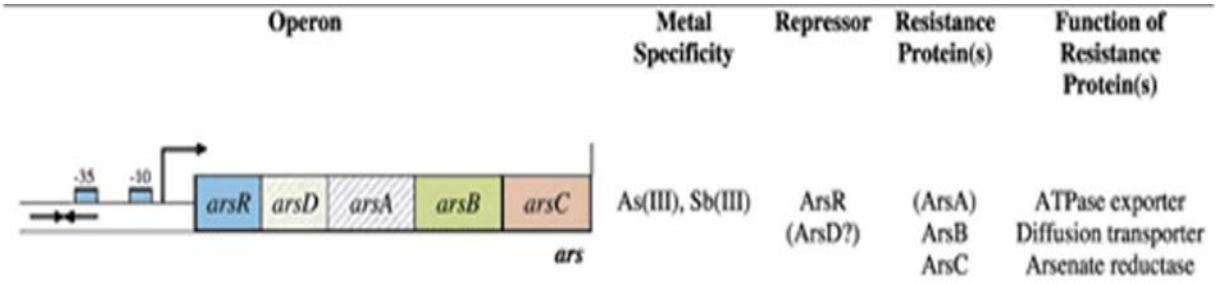


Figure 1.5: *ars* operon regulated by ArsR and general operon structures which confer resistance to metal in bacteria. Not all operons contain the coding genes, *arsD* and *arsA*, hence the proteins are given in bracket. (Busenlehner *et al*., 2003, copyright 2003 Federation of European Microbiological Societies).

The SmtB/ ArsR family of metal-responding regulators are repressors that act to prevent RNA polymerase binding by binding to the operator region. In the presence of metal ion, the metal ion binds to the repressor, releasing it from the DNA, hence allowing transcription to occur (Silver and Phung, 2005). ArsR is active as a homo-dimer. When arsenite binds to ArsR, the binding affinity of ArsR dimer is reduced to its specific binding site downstream of the -10 region in the promoter-operator (P_{ars}) of the *ars* operon via an allosteric conformational change. The *ars* operon is transcribed when ArsR is dissociated from the DNA. In the P_{ars} sequence, the site of ArsR binding is an imperfect 12-2-12 inverted repeat as illustrated in figure 1.6. Members of this family include: *E. coli* ArsR, *S. aureus* p1258 CadC, and *Synechococcus* sp. SmtB (Xu *et al*., 1996; Busenlehner *et al*., 2003).

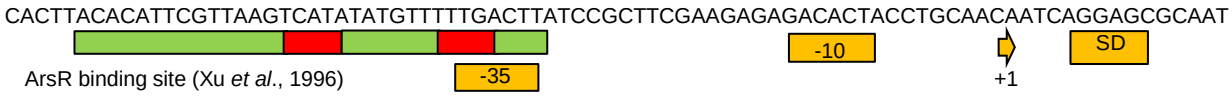


Figure 1.6: Regulatory region of the *E. coli* chromosomal *ars* operon. The *shaded green box* illustrates the binding site for ArsR as defined by DNase I foot printing. Within this site *shaded red* shows the contact points between ArsR and DNA. With *orange boxes*, the transcription start site is indicated as +1, the corresponding promoter elements are marked as -10 and -35, and the most likely Shine-Dalgarno sequence marked as SD (Xu *et al*., 1996). The sequence is reduced in front of the ArsR binding site to boost visibility, while the non-reduced total sequence length is 118 bp.

In *E. coli*, ArsR is a small cytosolic protein made up of 117 amino acids. It is an extended dimer having a two-fold axis of symmetry made up of five α-helices and two β-strands in a conserved α1-α2-α3-αR-β1-β2-α5 structure. ArsR binds DNA via a helix-turn-helix motif (α3-turn-αR) and specific residues on αR. The SmtB structure which is related to ArsR has been determined as shown in Figure 1.7.

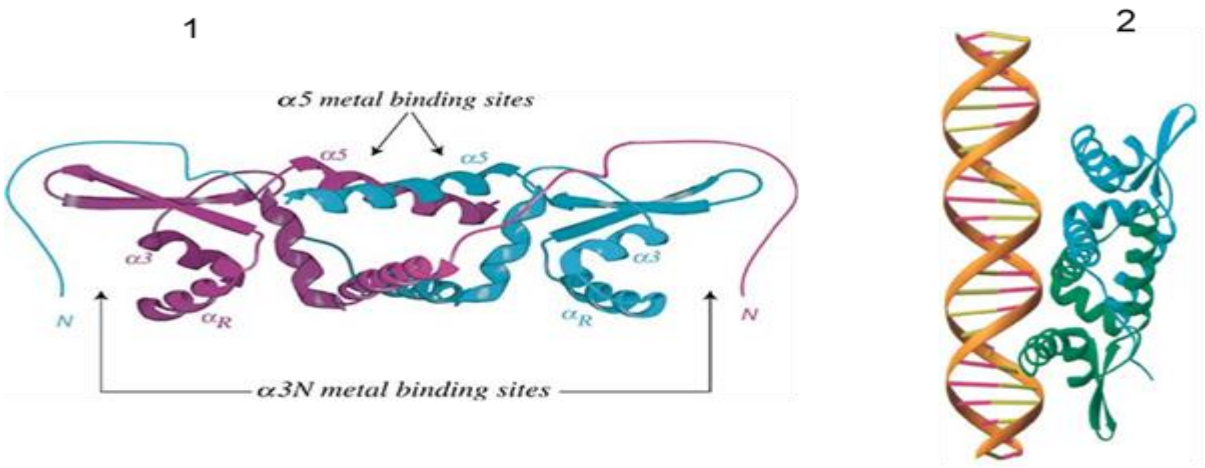


Figure 1.7: Crystallographic structure of the SmtB homologue of ArsR from *Synechococcus* PCC7942 and model of SmtB binding to DNA. (1) A ribbon depiction of the crystal structure of the Apo-SmtB homodimer with one monomer shaded *purple* and the other *blue*. It should be noted that ArsR does not have an N-terminal tail or α5 metal binding sites, but instead has 21 extra AAs at the C-terminal sequence end (Adapted from Busenlehner *et al*., 2003, copyright 2003 Federation of European Microbiological Societies). (2) Model of SmtB binding to DNA based on the similarity to the HNF-3 /*forkhead* DNA recognition motif and its resolved crystal structure (Clark *et al*., 1993). Monomers of the SmtB homodimer are shaded *blue* and *green* and DNA shown is a hypothetical β-DNA model (Cook *et al*., 1998, copyright 1998 Academic Press Limited).

This DNA binding mechanism has a strong structural resemblance to other bacterial transcriptional regulators such as CAP (catabolite activator protein), DtxR (the Fe(III)-regulated diphtheria toxin repressor), as well as MerR, the prototypical Hg(II)-responsive transcriptional regulator of the MerR family. Binding of metals occurs at a highly conserved ^{30}ELCVCD^{35} motif, otherwise known as the 'metal binding box' which was initially identified in members of the SmtB/ArsR family. It was determined that this motif is made up of residues which take part in metal coordination and are therefore directly involved in metal ion sensing (Busenlehner *et al*., 2003).

It is believed that metals are sensed by ArsR in a different way to other members of the SmtB/ArsR family. Busenlehner *et al*., 2003 proposed a "theme and variations''

model which describes the α3 site [ELCV(C/G)D or metal binding box] or α3N site (if the metal binding box interacts with residues on an N-terminal arm) including α5 site having its own binding motif. For members of the SmtB/ArsR family, one or both of these sites can be significant for metal binding. For *E. coli* ArsR used in this work, the N-terminal arm and the α5 site are lacking, hence only the α3 is important for metals to bind (Busenlehner *et al*., 2003). *E. coli* ArsR binds to trivalent arsenic [As(III)] and antimony [Sb(III)] the specificities of which are conferred basically by their thiophilic properties which make them perfect binding partners for sulphur ligands such as the cysteines in the metal binding box. This specificity is increased by the spatial orientations of the box and ligands (Chen & Rosen, 2014).

1.3.6 The MerR family of metalloregulatory proteins

The MerR family members are homodimeric protein activators that generally bind to RNA polymerase-binding operator regions irrespective of the presence of a bound metal ion. When activated by metal binding, the DNA undergoes a conformational switch that changes a sub-optimal promoter into an active one (Ansari *et al*., 1995; Brown *et al*., 2003, Hynninen A, Virta, 2009). Members of this family include: MerR (Shewchuk *et al*., 1989); ZntR (Outten *et al*., 1999) and CoaR (Rutherford *et al*., 1999) named the mercury-, zinc- and cobalt-inducible transcriptional activators in *E. coli* and *Synechocystis*, respectively. Also included in this group are the recently characterized Cu(II)-activated *E. coli* CueR (Outten *et al*., 2000; Stoyanov *et al*., 2001) and Pb(II) sensor *Ralstonia metallidurans* PbrR (Borremans *et al*., 2001).

As transcriptional regulators, the MerR family are made up of proteins that are characterised by their sequence similarity within the first 100 – 110 N-terminal amino

acids. The helix-turn-helix motif formed at this region specifically binds regulatory regions in promoter DNA with imperfect inverted repeats (Stoyanov *et al*., 2001; Brown *et al*., 2003). Unlike SmtB/ ArsR proteins, the MerR family of proteins mainly act as activators whereby they realign the operator DNA into a more optimal conformation for transcription initiation via a characteristic DNA distortion mechanism. This happens as a result of the controlled promoters being made suboptimal because of longer spacing between the -35 and -10 elements (19 – 20 bp, Figures 1.8 & 1.9) unlike the optimal 17±1 bp, in which case these elements are placed on opposite faces of the DNA double helix causing inefficiency in σ^{70}-factor recognition. When the MerR protein family are bound to their effectors, they partially unwind this region on the promoter DNA, which enhances the transcription efficiency of the controlled genes. This results because the -35 and -10 elements are brought onto the same face of the DNA molecule as well as into greater proximity (Frantz & O'Halloran, 1990; Philips *et al*., 2015). Although most of the recently known regulatory proteins confer activation or repression functionality on DNA when bound or unbound, members of the MerR family are capable of staying bound to the palindromic binding site within the promoter region irrespective of binding effectors. As recently reported, this is as result of the MerR family having an additional repression activity as they bend DNA, further forming an unfavourable conformation which prevents polymerase from binding when no effector is bound (Philips *et al*., 2015).

In cells, the MerR family is well known to control stress responses to different kinds of effectors ranging from oxidative stress to antibiotic and metal ions exposure. Hence their C-terminal sensing regions are markedly characterised with great variations (Couñago *et al*., 2016). Most of the work done in this project is based on the metal-responsive MerR family members. Although they selectively sense different metals,

these MerR protein family members share a high identity of C-terminal sequence (Brown *et al*., 2003). Even though there is no clear understanding of the metal binding specificity of these MerR protein families, the assumption is that the metal-coordinating residues at the C-terminal domain play an important role. It is reported based on mutational studies that specific metal binding and regulator activity is based on the correct positioning and orientation of the residues (Hobman *et al*., 2005; Philips *et al*., 2015). The crystallographic structure of CueR, a representative of the MerR family of metalloregulatory proteins, and an amino acid alignment of CueR and ZntR are represented in Figures 1.8 & 1.9. It is observed in the amino acid alignment that the DNA binding helix-turn-helix motif has a high sequence identity. Also, the similarity in the overall sequence between the three metal regulatory proteins; CueR, MerR and ZntR, as well as the conserved cysteine residues believed to be critical for metal binding, is demonstrated (Outten *et al*., 2000; Philips *et al*., 2015).

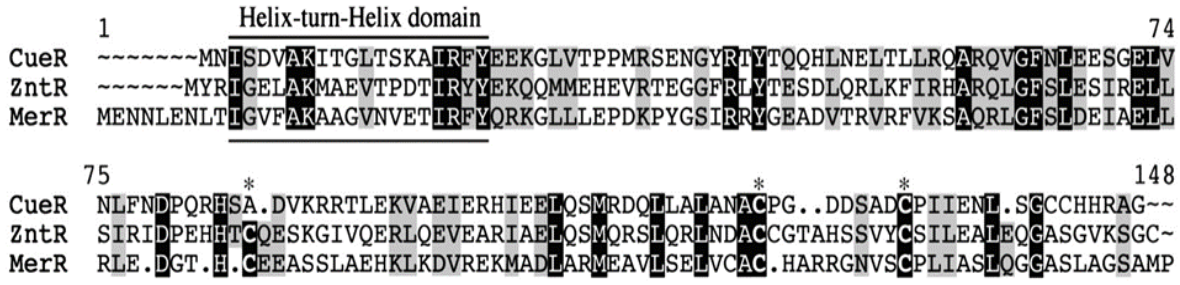


Figure 1.8: Amino acid alignment of CueR and ZntR from *Escherichia coli* and MerR from Tn501. Residues that are identical are highlighted in *black*, while similar residues are in *grey*. The conserved cysteine residues are shown with *asterisk* (Outten *et al*., 2000, copyright 2000 American Society for Biochemistry and Molecular Biology).

1.3.7 The ZntR

Formerly named YhdM, ZntR was the first *E. coli* metal-dependent MerR-like transcription regulator (Brocklehurst *et al*., 1999). Similar to MerR, ZntR is a protein with 141 amino acids (Christie *et al*., 1994). Based on DNA distortion similar to that of MerR and CueR, ZntR functions as an activator/repressor in its dimeric form (Outten *et al*., 1999; Philips *et al*., 2015). ZntR binds to P_{zntA}, which includes the usually elongated sequence between -35 and -10 motifs observed in MerR-family proteins. This spacer is 20 bp long, unlike $P_{merTPAD}$ and $P_{copA,}$ which are both 19 bp long (Brocklehurst *et al*., 1999; Outten *et al*., 1999). The foregoing implies that P_{zntA} is deformed by ZntR to a larger extent than that caused on $P_{merTPAD}$ and P_{copA} by their respective trans elements, since these promoters are all recognised by the σ^{70} subunit of the RNA polymerase (Hobman *et al*., 2005; Philips *et al*., 2015). Though still unclear, it is believed that the metal specificity of ZntR is based on five cysteine residues in principle similar to those seen in MerR (Outten *et al*., 1999; Khan *et al*., 2002; Figure 1.7).

The expression from ZntR-regulated promoters *in vivo* is activated by Zn(II), Cd(II) and Pb(II). However, Brocklehurst *et al*., 1999 reported that faster induction happens to be strongest by Zn(II), which was shown over a range of 10^2 – 10^3 µM. P_{zntA} is activated by Zn(II) – $ZntR_2$ with a Hill coefficient of 3.2, which signals a hypersensitive biological switch (Brocklehurst *et al*., 1999; Brown *et al*., 2003). ZntR transcription regulator controls the gene, *zntA* which encodes an ATPase transporting Zn(II), Cd(II) and Pb(II) out of the cell (Brocklehurst *et al*., 1999; Outten *et al*., 1999). Although there has been no report about direct ZntR repressing function, it is possible that ZntR works in a similar way to the recently characterised CueR, another member of the MerR family (Hobman *et al*., 2005).

1.3.8 The CueR

In *E. coli*, the activity of P_{copA} and P_{cueO} promoters have been reported to be controlled by CueR in response to copper at cellular levels, prompting the name CueR, which stands for Cu efflux regulator (Outten *et al*., 2000; Stoyanov *et al*., 2001; Figure 1.9).

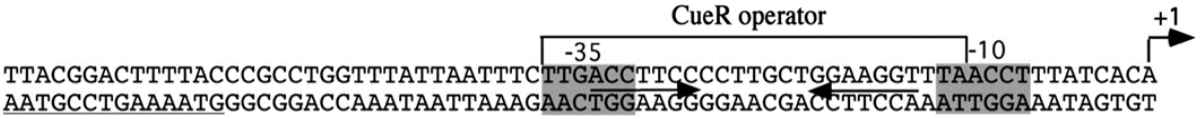


Figure 1.9: Sequence of *copA* promoter

The palindrome is indicated with *arrows* while the -35 and -10 regions of the promoter are highlighted in *grey*. A cornered arrow and +1 mark the transcriptional start. The binding region of the CueR operator is marked by a corresponding labelled brace above the sequence (Outten *et al*., 2000).

The mechanism of allosteric transcriptional regulation has recently been resolved. The DNA distortion mechanism in the MerR family proteins has previously been described with CueR shown having its activator and repressor state being bound to PcopA core region. Similar to MerR and ZntR, CueR is actively dimeric, and when it interacts with the DNA promoter and RNA polymerase, it acts as a regulator of expression. When CueR is in its repressor state, the DNA is bent, preventing contact between the polymerase and the -10 region of the promoter. The resulting bend is resolved in its metal bound form causing the distorted DNA to present the -35 and -10 regions to the polymerase in a perfect conformation (Philips *et al*., 2015; Figure 1.10). The above evidence shows that an active repressor activity as well as the generally accepted activator activity is not only found in the MerR member of the MerR protein family. Nonetheless, Philips *et al*., (2015) have reported that CueR proteins remain bound to the DNA with same affinities in spite of the presence of metal, but the MerR affinity for

its binding site in $P_{merTPAD}$ is reduced by the addition of mercury (Brown *et al*., 2003; Taniguchi *et al*., 2014).

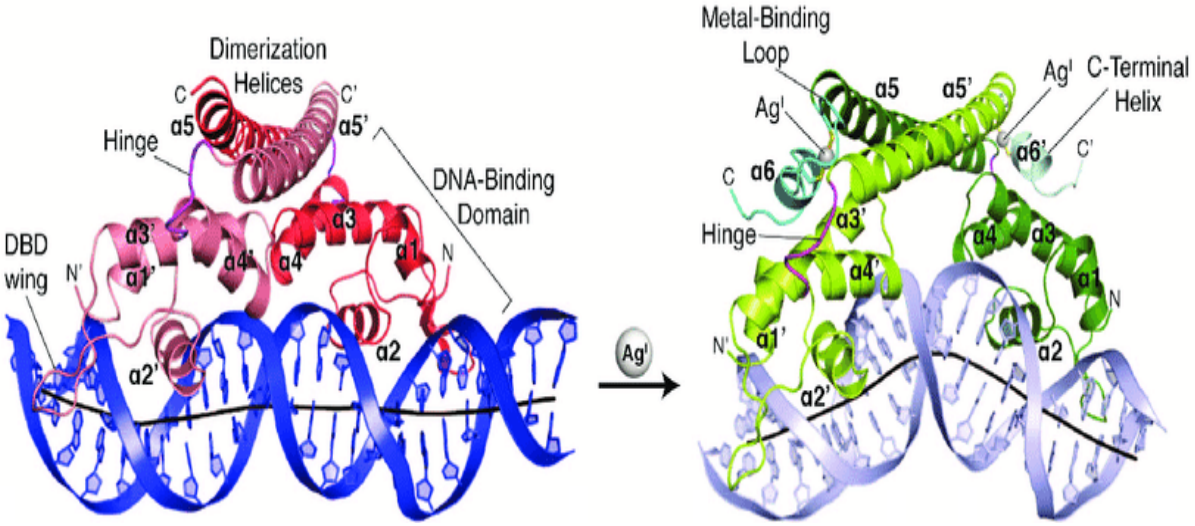


Figure 1.10: Crystallographic structure of the repressor and activator complex of CueR. Shown are the core region of the promoter DNA with the activator (*green*) and repressor (*red*) states of CueR bound. The secondary structures, characteristic functional domains of CueR and the binding sites of Ag(I) to the protein are labelled appropriately (Philips *et al*., 2015, copyright 2015 American Association for the Advancement of Science).

It has been shown that CueR can interact with copper (Cu), silver (Ag) and gold (Au) (Outten *et al*., 2000; Stoyanov *et al*., 2001; Changela *et al*., 2003). Results obtained in *in vitro* tests showed that Cu(I) binds to CueR with very high affinity, which is an indication of a hypersensitive response with half-maximal transcription obtained at 0.7 ± 0.2 µM total Cu^+, 0.013 ± 0.009 µM total Ag^+ and 0.6 ± 0.3 µM total Au^+ (Changela *et al*., 2003). It is postulated that there is a probability of such high binding affinity being of great importance for metal sensing in the cell, since metal regulatory proteins have

to efficiently outcompete other intracellular metal ligands (Hobman et al., 2005; Giedroc & Arunkumar, 2007).

A plasmid-borne *lacZ* under the control of P_{copA} in defined media having levels of $CuSO_4$ above 1 µM was found to have an increased *in vivo* expression. It was also observed that highest expression levels of *lacZ* were obtained when copper concentrations reached about 500 µM. However, such induction profile was not obtained under equivalent concentrations of Zn or Hg (Outten *et al*., 2000). Similar experiments were conducted by Stoyanov *et al*, and in contrast to findings by Outten *et al*., they observed that much lower concentrations of about 50 µM copper are needed for maximum induction (Stoyanov *et al*., 2001). This will not be unconnected with differences in experimental conditions. While Outten and group used a single copy vector construct carrying P_{copA} (Outten *et al*., 2000), Stoyanov and group made use of a low-copy number plasmid carrying the promoter sequence and a medium-copy number plasmid carrying the CueR regulatory protein (Stoyanov *et al*., 2001). Also, there are great difference in the total levels of expression of the *lacZ* gene used in both studies. There was also no *in vivo* induction by Cd, Hg, Ni, Pb, or Zn (Stoyanov *et al*., 2001).

1.3.9 Biosensors for heavy metals

The employment of whole-cell biosensors using recombinant DNA technology can prove possible alternative for simple, sensitive, specific, robust, accurate, portable and cheap assay of heavy metals in environmental samples. This involves the use of genetically modified bacteria to generate signals which are detectable when exposed to the analyte(s) of interest. The knowledge of synthetic biology has made possible

the design of biosensors using reporter genes that encode proteins as mechanisms for easy detection without requiring addition of any reagents. The development of biosensors utilising bacterial and insect luciferases by measuring chemiluminescence in a reaction catalysed by the enzymes have been reported (Meighen, 1991; Viviani, 2002). Though these biosensors gave quantifiable results, limiting expression from the lux operon to only luxAB requires the addition of an aldehyde substrate, a manipulation that can be technically difficult due to the high vapour pressure of the aldehyde (Lampinen *et al*., 1995). Similarly, the addition of the substrate, D-luciferin, to generate a signal is required in the application of insect luciferase as a reporter protein (Hynninen and Virta, 2009). Conversely, fluorescent proteins such as green fluorescent proteins (GFP) from *Aequorea victoria* do not require cellular activity, and are basically fluorescent once produced and correctly folded (Tsiens, 1998). However, dead cells could be fluorescent, thereby potentially compromising test results (Hynninen and Virta, 2009).

Notably, a fluorescence-based arsenic biosensor is available commercially (http://www.aboatox.com/environmetal_analysis. html#mercury), while a luminescence-based system has undergone field trial in Vietnam (Trang *et al*., 2005). These reporter systems work optimally under laboratory conditions, however, the need for a luminometer, spectrophotometer, or fluorimeter to quantify the response makes them unsuitable for developing a cheap and simple field test kit (Joshi *et al*., 2009).

A chromogenic system based on X-gal as an alternative for a qualitative response has been described (Stocker *et al*., 2003), but X-gal is relatively costly and requires refrigeration. An ideal biosensor should be inexpensive, sensitive, specific, accurate, portable and simple to use. A pH-based biosensor has been reported which mimics the metabolic pathways of wild-type strains of *E. coli* by fermenting lactose and other

sugars with the production of large quantities of acid products, the ‘mixed acid’ fermentation (Aleksic *et al*., 2007). The biosensor was constructed by the University of Edinburgh team in the 2006 International Genetically Engineered Machine competition (iGEM) using *lacZ* reporter gene (encoding β-galactosidase), which allows growth on lactose medium. Modular DNA components known as BioBricks consisting of the *E. coli* chromosomal ars promoter and arsR (Diorio *et al*., 1995; Cai and DuBow, 1996) and the 5' end of the *lacZ* gene were fused to give the final construct designated BBa_J33203, which was tested using the plasmid vector pSB1A2 in *E. coli* JM109 (Aleksic *et al*., 2007). Induction of the biosensor showed increased acidification of the biosensor medium in the presence of arsenic (Joshi *et al*., 2009).

The reasons presented above make a growth-based biosensor an excellent candidate in developing a new class of biosensor in which growth is used as the output, rather than a reporter gene, by allowing growth on lactose medium. This class of biosensor can be made cisgenic, a system of innovative rewiring of the native chassis circuits with a natural gene coding for a desired trait, from the organism itself or from the relative strain thereby avoiding the introduction of new material. Although this approach is apparently inappropriate for such applications as production of novel or non-native products through generation of hybrid metabolic pathways, it is certainly capable of dramatically improving the production of native products, or taking advantage of native analyte-sensing capabilities to generate whole-cell biosensors. It can also greatly simplify regulatory issues associated with transgenic systems.

Based on their actualization at the time of writing and relevance for this work, a brief collection of ideal biosensors for heavy metals developed through multiple procedures will be outlined.

1.4 Whole-cell biosensors based on genetically modified microorganisms

1.4.1 Arsenic biosensor based on pH

For arsenic biosensor, the *ars* promoter (P_{ars}), which usually functions in bacteria by activating an arsenic detoxification system, is fused adjacent to a reporter gene which generates a detectable signal. The *arsR* gene encodes the ArsR repressor protein which controls the *ars* promoter. In the absence of arsenic, ArsR binds to the promoter, hence inactivating it, but ArsR binds to arsenite when present, prompting its dissociation from the promoter and leading to expression of the arsenic detoxification system, or to the expression of the reporter gene in the case of whole-cell biosensors (Tauriainen *et al*., 1997).

Several arsenic biosensor systems have been reported using different reporter genes such as; *lacZ*, encoding β-galactosidase, which generates a blue pigment by acting on the chromogenic substrate X-gal; *luc*, encoding firefly luciferase, which produces a strong luminescent signal; *luxAB* or *luxCDABE*, encoding bacterial luciferase, which generates a luminescent signal; or gfp, encoding Green Fluorescent Protein (Daunert *et al*., 2000; Belkin, 2003). One example is the Edinburgh Arsenic Biosensor (Aleksic *et al*., 2007; Joshi *et al*., 2009; de Mora *et al*., 2011) which is based on a change in pH in the surrounding medium when β-galactosidase in *E. coli* is expressed following arsenite induction of arsenic promoter (P_{ars}) and subsequent lactose fermentation. Over a period of time, this sensor has been made sensitive enough to sense below 10 ppb (10 µg/L or approx. 0.13 µM) levels of arsenate when incubated overnight due to improvements in media formulation and screening methods. Hence this sensor is fulfilling the requirements needed for drinking water quality (de Mora *et al*., 2011).

For field use, the *Bacillus subtilis* spore-based sensor for arsenic in drinking water is an example of a biosensor currently in the advanced level of development. With the intrinsic problems of shelf-life and regulatory issues concerning the use and release of transgenic organisms, it has been reported that a *Bacillus subtilis* sensor system based on RFP has been adopted over the last 5 years. In this system, a chromosomal copy of the sensing mechanism is incorporated in the sensor in a knock-out mutant which makes survival in the wild impossible for a prolonged period of time. As a spore former, an increase in the shelf life of the sensor from a few weeks to years makes *B. subtilis* an excellent chassis (de Mora *et al*., 2011).

With the increase in development of biosensors, there is urgent need to develop biosensors that have increased shelf life and are cisgenic to be granted filed use, while being more rapid, robust, cheap, simple, sensitive and specific for analysis of toxic heavy metal contamination of drinking water and biomedical samples.

1.4.2 A luminescence sensor for copper, silver and gold

A biosensor based on P_{copA} and the metal-responsive regulator CueR that senses Cu, Ag and Au levels in *E. coli* has been reported (Stoyanov *et al*., 2003). The *Vibrio fischeri lux* gene cluster was placed under the control of P_{copA} in a plasmid having high copy number to measure the intracellular levels of Cu, Ag and Au. A knock-out *E. coli* mutant without a functional Cu and Ag efflux pump CopA was shown to be more sensitive to levels of metals in the surrounding medium based on their initial results. This system detected between 0.01 – 2 µM, 0.5 – 250 µM, and 10 - 60 µM levels of Ag, Cu and Au respectively (Stoyanov *et al*., 2003). However, this system might be limited for use as an actual sensor because luciferase based systems are susceptible

to severe changes in output due to variations in experimental timing and ambient temperature (Stoyanov *et al*., 2003).

1.5 Synthetic biology approach in biosensor construction

The emerging field of synthetic biology aims to adopt engineering approaches, like standardization of parts and assembly methods, for construction of novel biological systems. Currently, efforts are being focused on the use of modular genetic parts such as BioBricks (Knight, 2003), for easy assemblage via the use of a combination of restriction sites. This approach generates more complex multi-part systems, which are subsequently introduced into a host organism or 'chassis'. The BioBrick assembly standard is diagrammatically represented in Figure 1.11 (French, 2009). In order to design and construct genetic systems, numerous BioBricks such as ribosome binding sites (RBSs), promoters, coding sequences and terminators, are openly available at the Registry of Standard Biological Parts (Registry of Standard Biological Parts, 2009). Moreover, the choice of a suitable chassis organism is necessary for the support of the newly introduced pathways, as well as providing background processes such as replication and translation. In this regard, well-characterized microbes, including *E. coli*, *S. cerevisiae*, and *B. subtilis*, have been extensively utilized (French, 2009).

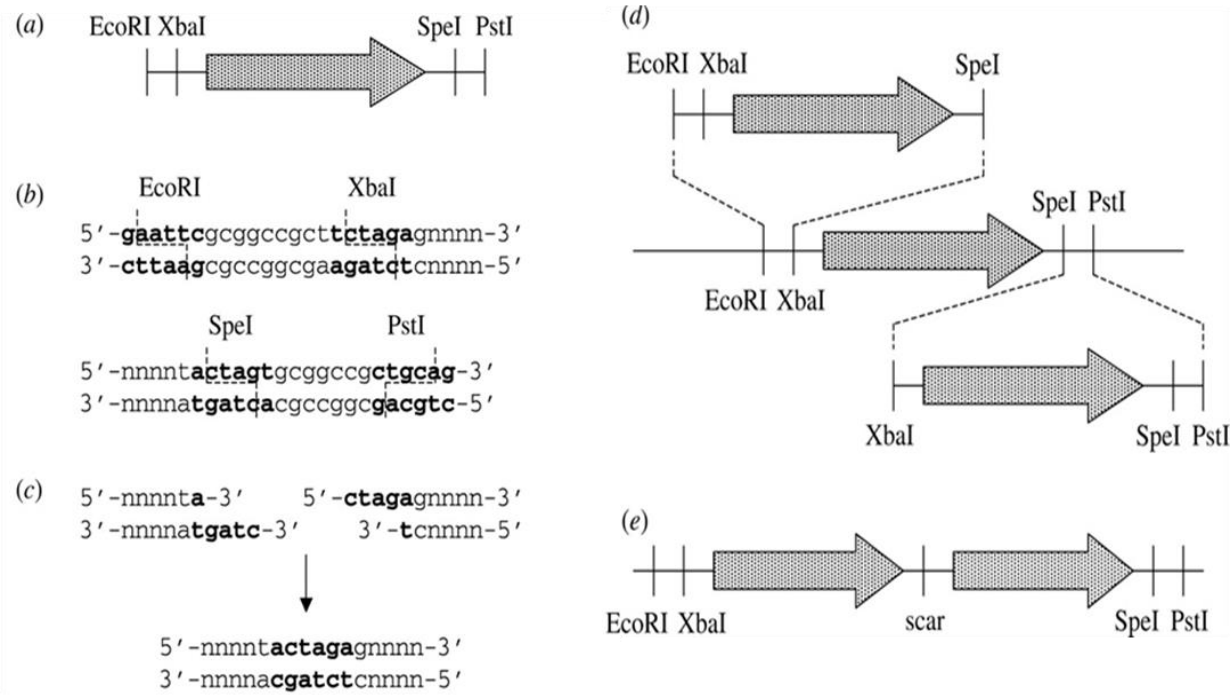


Figure 1.11: Diagrammatic sketch of the BioBrick 1.0 assembly standard. (a) Each BioBrick comprises a genetic component, such as ribosome binding sites, promoters, coding sequences, terminators or any combination among them, flanked by EcoRI and XbaI restriction sites on the upstream end, and by SpeI and PstI on the downstream end. (b) Standard prefix and suffix sequences for BioBricks. Recognition sites are in bold while dashed lines signify the cuts made by each enzyme. (c) Pattern of 'scar' formation after the ligation of two BioBricks cut by a specific restriction enzyme pair. The enzymes used for the generation of the cohesive ends do not recognize the scar. (d) Two BioBrick assembly. Any BioBrick can be inserted upstream or downstream of any other BioBrick depending on the restriction enzymes used. (e) The assembly of any two BioBricks always results to another BioBrick, which can be used for other assemblies, allowing the generation of complex systems composed of several genetic components (French, 2009).

1.6 Coliforms

There are different kinds of biological contaminants, such as bacteria, viruses, protozoa, and fungi in drinking water sources (Al-Gabr *et al*., 2014). Certain bacteria inhabiting the digestive tracts of animals are fundamental for obtaining nutrients from digested food. Together with faecal wastes, a great number of these naturally occurring microorganisms are ejected from the body, including pathogenic organisms if present (Bartram & Pedley, 1996; Szewzyk *et al*., 2000). Human diseases such as cholera (*Vibrio cholerae*), typhoid fever (*Salmonella typhi*), shigellosis (*Shigella*), salmonellosis (*Salmonella*), and gastroenteritis (*Campylobacter jejuni*, *Escherichia coli*, *Giardia lamblia*) can be transmitted by municipal and rural water supplies. The threat posed by such disease transmission is directly proportional to an increase in population density as more sewage pollutes public water supplies, carrying with it human intestinal pathogens (Szewzyk *et al*., 2000).

Epidemiological studies have shown that isolating or detecting the pathogenic organism itself in raw and drinking water is not always essential. Direct testing of water for pathogens can be laborious, costly and even hazardous, hence the need for indicator organisms to assess the possibility of faecal contamination. Often used as indicators are faecal coliform bacteria, members of the family *Enterobacteriacae*, which include *Escherichia coli*, *Citrobacter*, *Enterobacter* and *Klebsiella* species (Bartram & Pedley, 1996; Szewzyk *et al*., 2000; Rompre´ *et al*., 2002). These Gram negative bacilli (rod shaped bacteria) are found in the intestinal tracts of all warm-blooded animals. Although most are not pathogenic, because they are eliminated with faeces, they are associated with pathogens such as *Vibrio cholera* or Hepatitis A virus.

1.6.1 Coliform detection

Coliform detection in foods and water is indicative of possible contamination by fecally transmitted pathogenic bacteria or food-poisoning bacteria. Therefore, coliform tests are regarded as essential in health administration and are conducted under specific regulations in all countries. Lactose fermentation is the indicative property of coliforms, and β-galactosidase is the enzyme. Hence, the detection of β-galactosidase in cultured bacteria indirectly indicates the presence of coliforms (Masuda-Nishimura *et al*., 1999). The problem of arsenic in drinking water was mainly created by the drilling of tube wells to provide clean drinking water, since surface water is often contaminated by coliforms and waterborne pathogens. To avoid replacing one problem with another, it is important to test drinking water for both toxic metals and pathogenic bacteria along those lines.

Coliforms and associated faecal pathogens pose a serious threat to public health. An estimated 23,000 children die annually from diarrhoeal disease in Bangladesh resulting from pathogen-infected water (WHO, 2000). According to WHO (1996) guidelines, safe drinking water should not contain any coliform bacteria in a 100 ml sample. The Most Probable Number (MPN) and Membrane Filtration Technique (MFT) are the standard methods currently employed in coliform detection. These current detection methods need laboratory and technical support, are time consuming, expensive and inadequate, but biosensors can address these problems, hence the need for biosensor development. Also, since the whole-cell biosensors of interest in this project work by allowing growth on lactose medium (Aleksic *et al*., 2007; de Mora et al., 2011), they can also easily be adapted to additionally detect the growth of other bacteria, such as coliforms, so the same format of sensor detects both toxic metals and coliforms in a single unit.

1.7 Aims and objectives

Among major threats to public health are heavy metals and coliforms. Hundreds of millions of the public have been exposed to heavy metals and coliforms in drinking water and food since the era of industrial revolution and technological advancements. For arsenic, there is no known cure and the problem is still continuing. Several remedies have been proffered such as the availability of comparatively cheap filters for purification of samples contaminated by arsenic, yet delicate maintenance and control of such filters are required to ensure their continued efficiency. Reduction in arsenic exposure and provision of specific drugs for recovery or circumventing disease progression have been the treatment as there is no effective therapy for arsenicosis. Hence, the only practical approach existing currently against arsenic public health crisis involves frequent assessment of the water quality of individual tube well. With the number of tube wells increasing particularly in resource limited countries where access to good quality water is low, it is expedient that a simple, cheap, sensitive, selective and accurate test that can be used by each tube well user be designed. Such test should be suitable for field use and free from legislative restrictions (Smith *et al*., 2000; de Mora *et al*., 2011). One major aim of this work is to develop a sensor of such principle.

For testing water quality in a variety of situations, outside exclusive arsenic sensing, developing a multiplexed whole cell sensor able to detect cadmium, copper, gold, lead, mercury, silver and zinc can prove to be beneficial provided they have adequate low detection limits (Chen & Rosen, 2014). Developing multiple sensors that work together can elucidate the specific metals being detected since various metalloregulatory proteins detect specific metals.

The aims of this work include developing a simple, disposable device which can easily be mass produced and adapted to detect heavy metals and additionally detect the growth of other bacteria, such as coliforms, so the same format of sensor detects both toxic metals and coliforms in a single unit. The sensors must be cheap, simple to use, accurate and do not require sophisticated equipment for measurement and suitable for use particularly in developing economies as required by the sponsors. There is also a need to consider safe disposal of the sensor device and lifetime environmental impact through consultations with designers, social scientists etc.

The key objectives of the work presented in chapter 3 were:

1) To design and construct proof of concept biosensors for Ag, Au, Cd, Cu, Hg, Pb and Zn on the basis of previously introduced metal responsive promoters P_{copA}, P_{zntA} and their regulatory protein associates. This was to develop the use of biological methods in metal testing as physicochemical methods are expensive, laborious and require skilled personnel.
2) To assess the activity of whole-cell expression systems. This was to test the ability of the developed sensors in the detection of specific metals.
3) To investigate the probability of tuning Cu and Zn biosensors using weak ribosome binding sites. This was performed to evaluate the potential of using weak ribosome binding sites to reduce the high background activity of sensor cells.
4) To investigate the toxicity of constructs in different media. This was carried out in ascertaining the media suitable for the optimum growth and activity of the cells.
5) To investigate the effects of chelating agents on metal bioavailability. Chelating agents have effects on the availability of metals to cells when they react together

to form complexes. This was performed to assess the availability of metals to cells when chelating agents are present in samples.

The key objectives of the work demonstrated in chapter 4 were:

1) To assess the activity of a previously developed arsenic biosensor system in water. This system has been reported to detect 10 ppb arsenic in water samples and some of the principles of design would be employed to develop other novel biosensors.
2) To test the biomedical importance of the same arsenic biosensor in clinical samples. Real samples were not used as they could not be transported from areas with reported arsenic pollution due to some logistic problems. However, synthetic urine medium (AUM) spiked with arsenic was used as arsenic contaminated urine from arsenic hit regions could not be made available due to logistic challenges.
3) To evaluate the stability of dried biosensor cells under different storage conditions. This was to reduce the need for routine culturing and preparation of biosensor cells which can introduce variation between different batches of cells. This enables the maintenance of long-term viability and activity of the sensor bacteria, as well as the distribution of sensor cells to other regions.
4) To test the activity of dried biosensor cells. Cell viability was reduced during drying techniques and prolonged storage at environmental conditions, hence the need to test the effectiveness of the cells after drying and storage.

The key features of the work presented in chapter 5 were:

1) To design an approach adapted to additionally detect the growth of other bacteria, such as coliforms. This was aimed at determining the efficiency of this technique in comparison with the standard methods of coliform testing.

2) To quantitatively compare the novel approach with modern coliform detection techniques. This is necessary to ensure the accuracy and effectiveness of the newly developed method.

3) To evaluate the activity of lyophilised media and whole-cells in a single format. This was done to assess viability and efficacy of cells when prepared and dried together in media, and also enable simultaneous testing of metals and biological contaminants in a sample.

The key features of the work presented in chapter 6 were:

1) To investigate the possibility of using Lamda Red Recombineering technique to insert the newly designed sensor cassettes onto *E. coli* bacterial genome. This was to eliminate the use of antibiotics involved in maintaining sensor plasmids which has been of major regulatory issues.
2) To test the stability of these plasmids on different substrates. Since the antibiotic resistance gene which acts to maintain these plasmids had been deleted, it is necessary to test how stable these sensor plasmids are without the antibiotic resistance gene.
3) To evaluate the metal detection capabilities of the inserted cassettes of environmental samples. Metals need to be detected to assess the detection capabilities of the sensors developed through this technique.

Chapter 2: Materials and methods

2.1 List of laboratory suppliers

All materials used were obtained from the following suppliers, unless stated otherwise. Given in table 2.1 are their abbreviated names which will be used throughout this work for references.

Table 2.1: Checklist of laboratory consumable suppliers

Abbreviated name	Full supplier name	Source, Location
Sigma	Sigma-Aldrich	Gillingham, UK
Thermo Fisher	Thermo Fisher Scientific	Paisley, UK
Promega	Promega UK	Southampton, UK
Invitrogen	Thermo Fisher Scientific	Paisley, UK
NEB	New England Biolabs	Ipswich, MA, USA
Merck	Merck Serono	Feltham, UK
Merck	Merck Chemicals	Beeston, UK
EMD Millipore	EMD Millipore (U.K.)	Feltham, UK
Bio-Rad	Bio-Rad Laboratories	Hemel Hempstead, UK
BMG	BMG Labtech	Ortenburg, Germany
GE Healthcare	GE Healthcare	Little Chalfont Bucks, UK
Brand	BrandTech Scientific	Essex, CT, USA
Greiner	Greiner bio-one	Stonehouse, UK
Expedeon	Expedeon	Swavesey, UK
Tocris Bioscience	Tocris Bioscience	Bristol, UK

2.2 Bacterial strains, plasmids and oligonucleotides

Listed in table 2.2 are bacterial strains used in this work

Table 2.2: List of bacterial strains

Strain	Details	Use	Source
	Escherichia coli		
DH5α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ$^-$	General cloning, other than constructs containing streptomycin or ampicillin resistance genes.	Lab Stock generated from NEB commercial competent cells.
DH10β	F$^-$ *endA1 deoR*$^+$ *recA1 galE15 galK16 nupG rpsL* Δ(*lac*)*X74* φ80*lacZ*ΔM15 *araD139* Δ(*ara*, *leu*)*7697 mcrA* Δ(*mrr-hsdRMS-mcrBC)* Str^R λ(DE3 [l*acI lacUV5-T7p07 ind1 sam7 nin5*])	General cloning, other than constructs containing streptomycin or ampicillin resistance genes.	NEB commercial competent cells used to generate Lab Stock.
JM109 (DE3)	*endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB*$^+$ Δ(*lac-proAB*) *e14-* [F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] *hsdR17* ($r_K^-m_K^+$) λ(DE3[*lacI lacUV5-T7p07 ind1 sam7 nin5*])	General cloning for constructs containing streptomycin or ampicillin resistance genes.	Lab Stock
MG1655	K-12 F$^-$ λ$^-$ *ilvG*$^-$ *rfb-50 rph-1*	Genomic DNA preparations and as PCR template	Lab Stock
	Other bacterial strains		
Salmonella enterica serovar Typhimurium LT2	Non-coliform bacterium and a leading cause of human non-typhoidal gastroenteritis	Negative control for coliform detection assay	Lab Stock

Listed in table 2.3 are plasmids used in this work.

Table 2.3: List of plasmids

Plasmid	Length	Important features	Source
pSB1A2	2079 bp	High copy number, contains ampicillin resistance	Registry of standard biological parts
pSB1C3	2070 bp	High copy number, contains chloramphenicol resistance	Registry of standard biological parts
pSB4C5		Low copy number, chloramphenicol resistance	Registry of standard biological parts
pSB4K5		Low copy number, kanamycin resistance	Registry of standard biological parts
pSC101-BAD-gbaA		Tetracycline resistance, important plasmid for recombineering	GeneBridges
PCN1	2436 bp	High copy number plasmid containing chloramphenicol resistance gene cloned with zinc-responsive promoter and *lacZ'α*	This study
PCN1a		PCN1 with weaker RBS	This study
PCN1b		PCN1 with weaker RBS	This study
PCN1c		PCN1 with weaker RBS	This study
PCN2	2780 bp	High copy number plasmid containing chloramphenicol resistance gene cloned with copper-responsive promoter and *lacZ'α*	This study
PCN2a		PCN2 with weaker RBS	This study
PCN2b		PCN2 with weaker RBS	This study
PCN2c		PCN2 with weaker RBS	This study
PCN2d		PCN2 with weaker RBS	This study

Listed in table 2.4 are oligonucleotides used in this work

Table 2.4: Sequence of oligonucleotides used

Name	Sequence 5′ - 3′	Notes
A_P_{zntA}_f2	cggtcacttcctgatcgtc	For zinc biosensor constructs
A_P_{zntA}_r2	caggagtcgacatggcatc	
B_P_{zntR}_f1	actggaattgaagctgcg	
B_P_{zntR}_r1	agctcaccaatgcgatac	
PcueR-f1	catctctctgtgcgcagtac	For copper biosensor constructs
PcueR-r1	gctacatcgctgatgttc	
PcopA-F1	cctgatgcaaatcgagccg	
PcopA-R1	ccgtccagggtcaggtcg	
PcopA-F2	cggacttttacccgcctgg	
PcopA-R2	ccgcctgctcaacatccg	
PcueR-F1	catctctctgtgcgcagta	
PcueR-R1	cgataatcgggcagtcggc	
PcueR-F2	ctgtgcgcagtacttcctg	
PcueR-R2	ccgtctcgtcttaatcaccc	
PcueR-F3	ctgtattattgtggtggcg	
PcueR-R3	cgggcagtcggcgctgtc	
PcueR-F4	ggaggcgttgcgcgaacgatga	
PcueR-R4	ccgtctcgtcttaatcaccctg	
PcopA-F1	ttacggacttttacccgcc	
PcopA-R1	ggacaggccgtccaggg	
PcopA-F2	ctttacggacttttacccgc	
PcopA-R2	ggacaggccgtccagggtc	
PcopA-F3	ttacggacttttacccgcc	
PcopA-R3	gggtcaggtcgatagtttg	
CF1_P_{zntA}_RBS	tccccgaggttctatgtcgactcctggaggaaac	Weak ribosome binding site
CF2_P_{zntA}_RBS	taagagagttccaatgtcgactcctggaggaaac	
CF3_P_{zntA}_RBS	agaaggagctacaatgtcgactcctggaggaaac	
CF4_P_{zntA}_RBS	agaaagaagcacgggatgtatcgcattggtgagc	
CF5_P_{zntA}_RBS	ggaacctacctttaaatgtatcgcattggtgagc	
CF6_P_{zntA}_RBS	atcaagttaagaacgatgtatcgcattggtgagc	
CR1_P_{zntA}_RBS	taagtttttctcattaaccgaaggatacactctgg	
CR2_P_{zntA}_RBS	tatcgttgattttgtccaacaacttgtcagc	
CF1_P_{copA}_RBS	tcagagagctaaaatgtcacaaactatcg	
CF2_P_{copA}_RBS	ctaaacattttccggatgtcacaaac	
CF3_P_{copA}_RBS	ggcataagtagagcagatatgtcacaaac	
CF4_P_{copA}_RBS	ggcttacaaacggactaaatgtcacaaac	
CF5_P_{copA}_RBS	caagacggaactcgtatgtcacaaactatcgacc	
CF6_P_{copA}_RBS	acaagcgtccctaatgtcacaaactatcgacctgac	
CF7_P_{copA}_RBS	gcaaaagccgcccctatgtcacaaactatcgacc	
CR1_P_{copA}_RBS	aagacagttttgactggctgtgataaagg	
CR2_P_{copA}_RBS	gacagttttgactggctgtgataaagg	
CR3_P_{copA}_RBS	agttttgactggctgtgataaaggttaaac	
CR4_P_{copA}_RBS	gacagttttgactggctgtgataaaggttaaacc	
CR5_P_{copA}_RBS	aagacagttttgactggctgtgataaaggttaaacc	
PcopA/ZntA-F1	gaggaattccttctagagctggaaggtttatcctttatcacagccag	Switch of -35 and -10 sites
PcopA/ZntA-R1	gtactgcagctactagtacaaggggaaagtcaagaaattaataaacc	

P_{zntA}-F1	cggtcacttcctgatcgtc	Cassette insertion into genome
P_{zntA}-R1	gtcgttggcgttctgtaccgtggttagcggtttgaacgcagcaaattgaggtcactccagccagctttccg	
Pcas-zntA-F1	tcatatcgcattggtgagctggcaaaaatggcggaagtaacacccgacacgattcgttattacgaaaaac	
Pcas-zntA-R1	cggtttgaacgcagcaaattgaggggctttcttgccgtgattgtcaggagttcactccagccagctttcc	
P_{zntR}-F1	actggaattgaagctgcg	
P_{zntR}-R1	ctgtttttcgtaataacgaatcgtgtcgggtgttacttccgccatttttgctcactccagccagctttccg	
P_{copA}-F1	catctctctgtgcgcagta	
P_{copA}-R1	tttcgctgcgcatcggcggcgtcaccagccccttctcttcatagaagcgatcactccagccagctttccg	
Pcas-copA-F1	tcaccctgcccgatgatgacagcagccggagagattttcgataatcgggcagtcggcgctgtcatcgcc	
Pcas-copA-R1	cagtgaccgcaggacaggccgtccagggtcaggtcgatagtttgtgacattcactccagccagctttccg	
P_{arsR}-F1	ccaactcaaaattcacacc	
P_{arsR}-R1	gttgcactccagccgatgcctaaacctttcggctgccagataaccaatactcactccagccagctttccg	
pSBNX3insf2	aaataggcgtatcacgaggc	Sequencing primers
pSBNX3insr2	cagtgagcgaggaagcctgc	

2.3 Molecular weight markers and DNA ladders

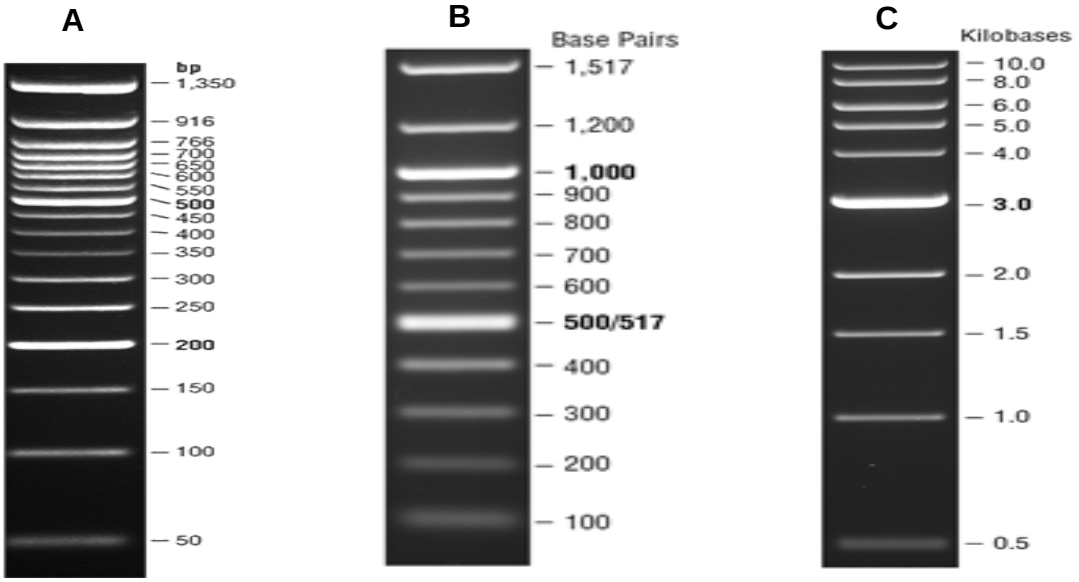


Figure 2.1: DNA ladders used in this work. (A) 50 bp DNA ladder, ethidium bromide staining on a 3% TBE agarose gel (New England Biolabs). (B) 100 bp ladder, ethidium bromide staining on a 1.3% TAE agarose gel (New England Biolabs). (C) Quick-load 1 kb ladder, ethidium bromide staining on a 0.8% TAE agarose gel (New England Biolabs).

2.4 DNA sequencing

Sanger DNA sequencing (Sanger *et al.,* 1977) was carried out by Edinburgh Genomics, Edinburgh, UK. General BioBrick sequencing primers (pSBNX3insf2 and pSBNX3insr2) were used for sequencing inserts in BioBrick constructs in pSB1C3, pSB4C5 or pSB4K5. Primers used in this study are listed in table 2.4.

2.5 Enzymes

All restriction enzymes used in this work were purchased at NEB and used following manufacturer's guidelines.

Listed in table 2.5 are DNA polymerases used in this work.

Table 2.5: DNA Polymerases used

Name	**Notes**	**Source**
GoTaq® Flexi	Direct to gel amplification buffer	Promega
KOD Hot Start	Proof reading, high performance in high GC amplifications	EMD Millipore
NEB Taq	Very cost effective	NEB
Phusion High-Fidelity	Proof reading	Thermo Fisher
Q5® High-Fidelity	Proof reading	NEB

2.6 Growth media

Unless otherwise stated, all growth media used in this work were autoclaved at 121^0C for 15 minutes and allowed to cool prior to use.

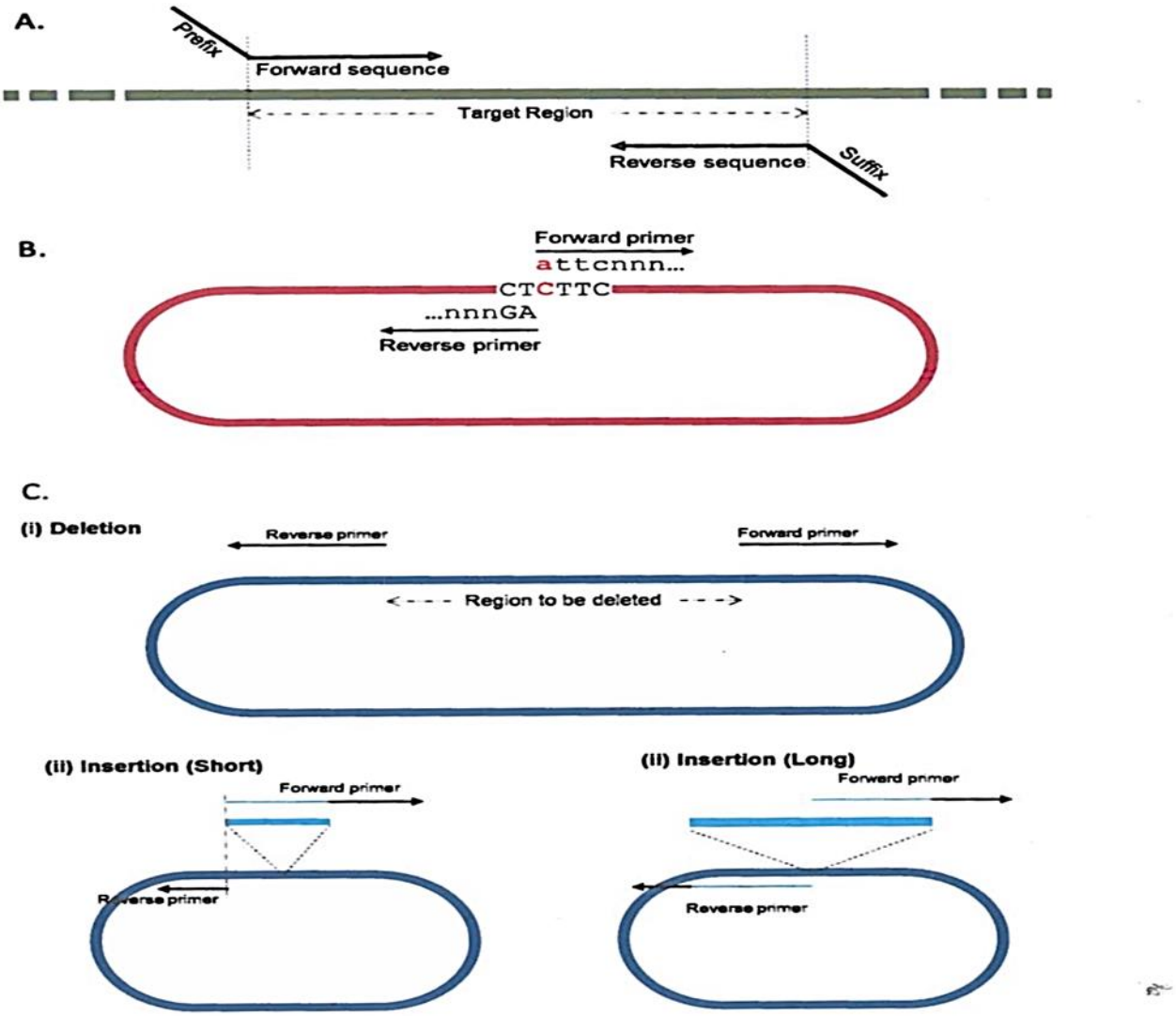


Figure 2.2: Primer design principles and strategies

(A) The principle of designing primers for amplification of parts for synthetic biology cloning. The standard prefix and suffix are included in the forward and the reverse primers respectively. (B) Designing primers for the removal of unwanted restriction sites. The forward primer, in the example shown above, is designed such that the EarI recognition site is removed. Supposing the sequence for the recognition site is in the frame, the third ′C′ (CTC – Leu) will be replaced with an ′A′ to maintain it as a codon for Leu (CTA). (C) i. For the deletion of a region within a plasmid, primers are designed such that they are divergent and exclude the unwanted region. ii. For a short sequence insertion (such as his-tag), the additional sequence is included as a tail to just one primer. iii. For the insertion of longer sequences (e.g. an 18 amino acid long linker to fuse two proteins), involves splitting the additional sequence into two, each half tailing the forward and reverse primers.

Listed in table 2.6 is the arsenic biosensor medium and its composition.

Table 2.6: Arsenic biosensor medium (ABM6) after de Mora *et al*., 2011.

Ingredient	**Amount**
Peptone	2 g
Yeast Extract	0.1 g
K_2HPO_4	0.3 g
$NaHCO_3$	2.1 g
Bromothymol blue	0.1 g
Lactose	10 g
ddH_2O	to 1 L

Listed in table 2.7 is the LB growth medium and its composition.

Table 2.7: Luria-Bertani broth after Bertani 1951.

Ingredient	**Amount**
Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Agar (if required)	15 g
ddH_2O	to 1 L

Table 2.8: M9 medium and its composition after Sambrook *et al*., 1989.

Ingredient	**Amount**
Na_2HPO_4	6 g
KH_2PO_4	3 g
NaCl	0.5 g
NH_4Cl	1 g
Glucose 20% (w/v) stock solution	20 mL
ddH_2O	to 1 L

Listed in table 2.9 is the artificial urine medium and its composition.

Table 2.9: Artificial urine medium (AUM) after Brooks and Keevil 1997.

Ingredient	**Amount**
Peptone	1 g
Yeast Extract	0.005 g
Lactic Acid	0.1 g
Citric Acid	0.4 g
$NaHCO_3$	2.1 g
Urea	10 g
Uric Acid	0.07 g
Creatinine	0.8 g
$CaCl_2.2H_2O$	0.37 g
NaCl	5.2 g
$FeSO_4.7H_2O$	0.0012 g
$MgSO_4.7H_2O$	0.49 g
$Na_2SO_4.10H2O$	3.2 g
K_2HPO_4	0.95 g
KH_2PO_4	1.2 g
NH_4Cl	1.3 g
ddH_2O	to 1 L

Table 2.10: Novel biosensor medium, ZBM3 and its composition

Ingredient	**Amount**
Peptone	2 g
Yeast Extract	0.1 g
K_2HPO_4	10 g
Bromothymol blue	0.1 g
Lactose	10 g
Agar (if required)	15 g
ddH_2O	to 1 L

Table 2.11: List of antibiotics used and working concentrations

Antibiotic	Solvent	Stock concentration	Working concentration
Ampicillin	ddH_2O	50 mg/mL	50 µg/mL
Carbenicillin	ddH_2O	100 mg/mL	100 µg/mL
Chloramphenicol	Ethanol	40 mg/mL	40 µg/mL
Kanamycin	ddH_2O	50 mg/mL	50 µg/mL

2.7 Buffers

Buffers used in this work are stated following their respective applications.

2.8 Manipulation of DNA

2.8.1 Polymerase chain reaction

Using a range of polymerases as listed in Table 2.4, PCR amplification of DNA was carried out using different programmes and reaction set ups suited for individual reaction requirements. Generally, glycerol was added to amplifications of high GC templates and annealing temperatures optimised to suit the lowest melting temperature (T_m) as obtained using the SnapGene software. The resulting PCR products were purified by agarose gel purification (see section 2.8.4). Where minimal template carry over was expected, 1 µL of restriction enzyme DpnI was added to the PCR reactions and incubated at 37^0C for 1 hour, followed by deactivation at 80^0C for 20 minutes before gel purification.

Table 2.12: Standard PCR reactions.

Reaction component	**GoTaq Flexi, GoTaq G2 Flexi, NEB Taq**	**Phusion HF**	**Q5 HF**	**KOD Hot Start**
DNA template	20 -200 ng	20 – 200ng	20 – 200ng	20 – 200 ng
Primer (10 mM)	0.5 µL each	1	2.5	1.5
Buffer	5 µL	10	10	5
$MgCl_2$	1.25 µL	-	-	3 µL $MgSO_4$
dNTPs (10 mM each)	0.5 µL	1	1	5
Polymerase	0.05 µL	0.5	0.5	1
Glycerol 50% (w/v) (optional)	-	-	-	10 µL
ddH_2O to	25 µL	50 µL	50 µL	50 µL

Table 2.13: KOD Hot Start PCR cycle protocol.

Stage	**Step**	**Duration**	**Temperature**
1.	Initial denaturation	2 min	95^0C
2.	20 – 40 cycles		
	a. Denaturation	20 sec	95^0C
	b. Annealing	10 sec	Lowest T_m
	c. Elongation	25 sec/kb	70^0C
3.	Final elongation	2 min	70^0C

Table 2.14: Q5 High-Fidelity PCR cycle protocol.

Stage	Step	Duration	Temperature
1.	Initial denaturation	30 sec	98^0C
2.	25 - 35 cycles		
	a. Denaturation	10 sec	98^0C
	b. Annealing	30 sec	Lowest T_m
	c. Elongation	30 sec/kb	72^0C
3.	Final elongation	2 min	72^0C

Table 2.15: GoTaq Flexi and GoTaq G2 Flexi PCR cycle protocol.

Stage	Step	Duration	Temperature
1.	Initial denaturation	2 min	95^0C
2.	33 cycles		
	a. Denaturation	30 sec	95^0C
	b. Annealing	45 sec	Lowest T_m
	c. Elongation	1 min/kb	72^0C
3.	Final elongation	10 min	72^0C

Table 2.16: NEB Taq cycle protocol.

Stage	Step	Duration	Temperature
1.	Initial denaturation	2 min	95^0C
2.	33 cycles		
	a. Denaturation	30 sec	95^0C
	b. Annealing	30 sec	Lowest T_m
	c. Elongation	1 min/kb	68^0C
3.	Final elongation	5 min	68^0C

Table 2.17: Phusion PCR cycle protocol.

Stage	Step	Duration	Temperature
1.	Initial denaturation	2 min	98^{0}C
2.	30 - 33 cycles		
	a. Denaturation	15 sec	98^{0}C
	b. Annealing	30 sec	Lowest T_m – 5^{0}C
	c. Elongation	30 sec/kb	72^{0}C
3.	Final elongation	10 min	72^{0}C

2.8.2 DNA restriction digests and ligations

DNA restriction enzymes were used to cut DNA. All enzymes used were supplied by NEB and used according to the manufacturer's guidelines. Following spectrophotometric determination of DNA at 260 nm, all ligations were carried out using NEB T4 DNA ligase with an appropriate ratio of vector to insert of 1:4, and the reactions incubated for 1 hour at room temperature or overnight at 16^{0}C.

Table 2.18: Standard ligation set up.

Component	Amount / concentration
Insert DNA	4 parts
Vector DNA	1 part
T4 Ligase Buffer	1 µL
T4 DNA Ligase	0.5 µL
ddH_2O	to 10 µL final volume

2.8.3 Removal of phosphate groups from linearized DNA

The phosphate groups from the 5′ end of DNA strands can be dephosphorylated to prevent self-ligation of linearized vectors and other restriction digested DNA fragments. Prior to column purification, Calf Intestinal Alkaline Phosphatase (CIP) reactions (table 2.19) were incubated at 37^0C for 20 minutes.

Table 2.19: CIP reaction mix.

Component	**Amount / concentration**
Linearized DNA	50 – 250 ng
CutSmart Buffer (10x)	2 µL
CIP	1 µL
ddH_2O	to 20 µL final volume

2.8.4 DNA agarose gel electrophoresis

Following Johansson 1972, agarose gel electrophoresis was carried out in BioRad gel tanks at voltages between 40 and 90 V based on expected sizes of fragments and accuracy. Gels were post stained using SafeView (NBS biologicals) following manufacturer's guidelines and visualised under blue light. 50 bp, 100 bp and 1 kb DNA ladders were used as markers.

Listed in table 2.20 is TAE buffer and its components for agarose gel electrophoresis.

Table 2.20: TAE-Buffer.

Component	**Concentration**
Tris base / HCl	40 mM
Glacial acetic acid	20 mM
EDTA	1 mM
pH 8 (NaOH / HCl)	as required

2.8.5 Gel extraction and purification of DNA

To purify DNA from Agarose gels, QIAquick gel extraction kit from QIAGEN was used following manufacturer's instructions and DNA eluted in volumes between 30 and 50 µL. The QIAquick PCR purification, QIAprep spin miniprep, or DNeasy blood & tissue kits from QIAGEN were used to purify genomic, plasmid and linearized DNA samples following manufacturers' instructions.

2.8.6 Mutagenesis with blunt-end ligation (MABEL)

The MABEL protocol was developed by Prof. Christopher E. French of the University of Edinburgh. The protocol as illustrated in figure 2.3 involves the following steps: (a) Removal of unwanted restricted sites by changing a base, (b) single or multiple (continuous) nucleotide deletions, and (c) insertion of up to 64 bp of DNA. e.g., as weaker ribosome binding sites. Insertion of small single locus sequence changes into circular DNA plasmids usually involves a simple technique called mutagenesis with blunt-end ligation (MABEL). In this work, this technique was used to insert a weak ribosome binding site (RBS) into circular DNA constructs with high basal activity. This was carried out by amplifying a circular DNA template in a standard PCR reaction with two divergent non-overlapping primers (forward and reverse). The primers were designed with a non-complementary tail at the 5′ end to introduce the desired sequence changes in relation to the template. The resulting PCR reactions were supplemented with 1 µL of the restriction enzyme DpnI to reduce template carry over, and the reaction mix incubated at 37^0C for 1 hour and purified by gel electrophoresis (refer to protocol). The blunt end PCR product was self-ligated by introduction of

phosphate groups to the purified DNA using T4 Polynucleotide Kinase (PNK) and ligation carried out with T4 DNA Ligase following manufacturer's instructions.

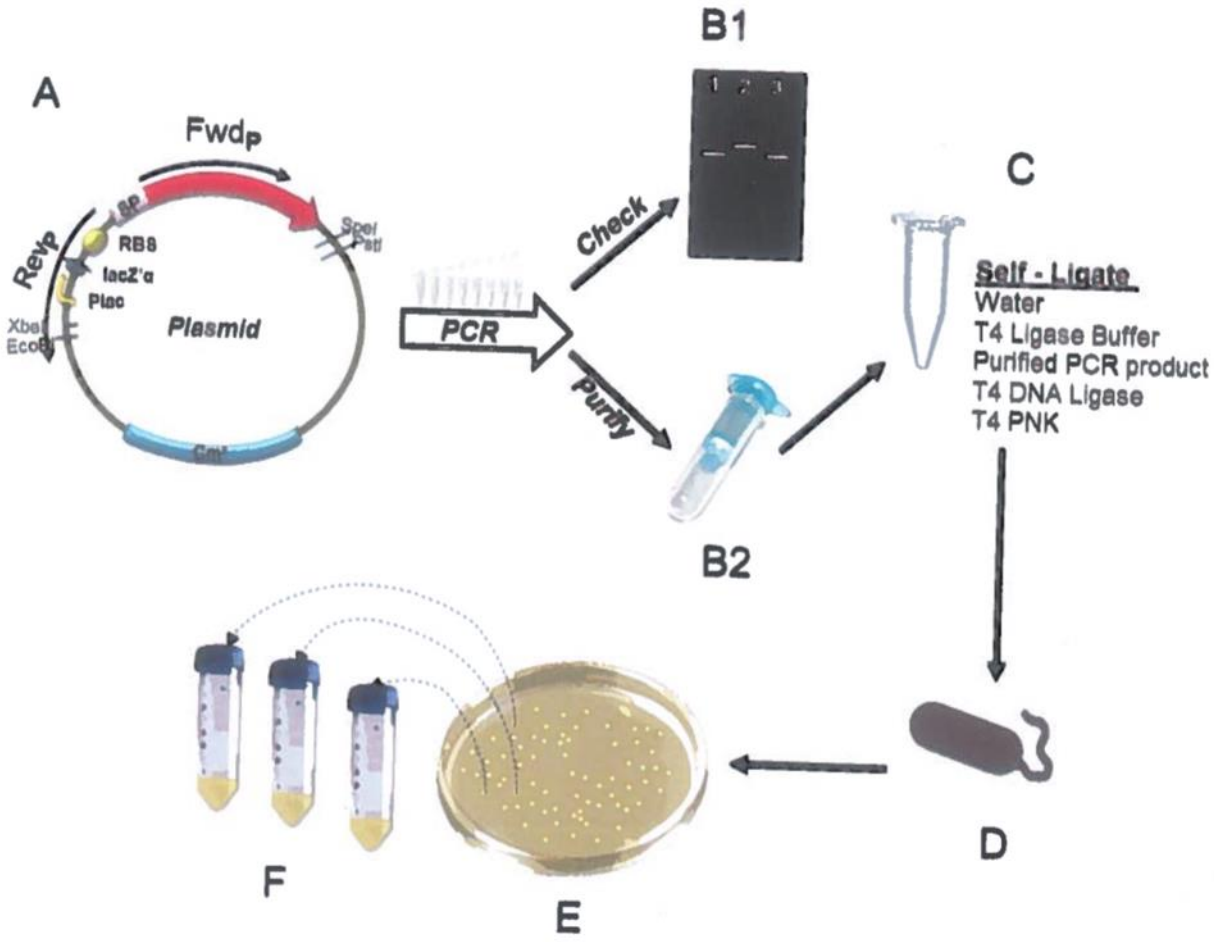


Figure 2.3: Applications of the methods of mutagenesis with blunt end ligation.

The MABEL protocol is employed to demonstrate a deletion of a piece of DNA (SP) in a plasmid using divergent non-overlapping primers (Fwd_p and Rev_p) for PCR. An aliquot of the PCR product is checked on a gel (B1) and the remaining product is purified (B2). The purified product is then self-ligated (C) and used for the transformation of *E. coli* competent cells (D). Colonies are selected on agar plates, miniprep purified (F) and confirmed by restriction digest and sequencing.

2.9 Microbiological techniques

2.9.1 Microbial cultivation

Using sterile inoculation loops, 5 ml, 25 mL or 50 mL Luria-Bertani broth (LB) medium in flasks were inoculated from agarose plates to prepare overnight culture, unless stated otherwise. The cultures supplemented with appropriate antibiotics were incubated at 37^0C for 16 – 20 hours in an orbital shaker at 200 rpm. Also, LB agar plates were grown with appropriate antibiotics at 37^0C, but at static incubation. Overnight cultures for storage at prolonged periods were mixed with 50% (v/v) glycerol in a 7:3 ratio of culture to glycerol in microcentrifuge tubes and stored at -80^0C.

2.9.2 Transformation

The modified method of Chung *et al*., (1989) was followed to prepare competent *Escherichia coli* cells. Fresh cultures of desired strains were prepared by streaking cells from -80^0C stock onto LB-agar plates without antibiotics and incubated overnight at 37^0C. A tiny colony from a fresh plate was inoculated into 50 mL sterile LB in a 250 mL Erlenmeyer flask and incubated at 37^0C with shaking at 200 rpm until an optical density at 600 nm (OD_{600}) reached 0.2 – 0.5. The flask was immediately chilled in ice water prior to centrifugation in Falcon tubes at 4,500 RCF at 4^0C for 15 minutes. The supernatant was discarded and cell pellets resuspended in 5 mL sterile newly prepared ice-cold transformation and storage solution (TSS, table 2.21). The suspension was transferred into pre-chilled 1.5 µL microcentrifuge tubes in 100 µL aliquots and the tubes immediately transferred to a -80^0C freezer for future use.

During transformations, the required tubes of competent cell aliquots were removed from the -80^0C freezer and allowed to thaw on ice for some time. Based on the DNA used for transformation, 1 µL of column purified supercoiled plasmid DNA or 10 µL of

ligation reaction (Table 2.18) were gently mixed with the competent cells and incubated on ice for 30 minutes. The tubes were heat shocked at 42^0C for 90 seconds and quickly transferred back to ice for a further 90 seconds incubation. 900 µL of room temperature LB medium was added to each tube and cells incubated at 37^0C with shaking at 200 rpm for 60 (ampicillin and carbenicillin resistance) to 90 minutes (all other antibiotic resistances). The transformants were centrifuged at 4,000 RCF for 4 minutes after which about 800 µL of supernatant was discarded. The cell pellets were resuspended in the remaining supernatant and was immediately plated onto LB-Agar plates with corresponding antibiotics for overnight incubation.

Table 2.21: Transformation and storage solution (TSS).

Component	**Concentration**
Lysogeny broth	17 mL
PEG 3350 (40% w/v) (For the attachment of DNA, also acts on cell membranes to increase transformation efficiency)	5 mL
$MgCl_2$ (induces the ability of the cells to take up DNA by altering the permeability of the membranes)	1 mL
DMSO (To permeabalise the cell membrane)	1 mL

2.9.3 Blue-white screening

After transformation, blue-white screening and selection were employed to screen for and select colonies that contained the plasmids and the inserts. Distinct colonies from

LB agar transformation plates were streaked on LB-Agar plates containing appropriate antibiotics and IPTG (isopropyl *β*-D-1-thiogalactopyranoside), x-gal (5-bromo-4-chloro-3-indolyl-*β*-D-galacto-pyranoside) for induction of the *lac* operon. The plates were incubated at 37^0C overnight and the plates placed at 4^0C for a few hours after the initial overnight incubation to increase pigment precipitation. Colonies with plasmids that lacked the inserts remained the whitish-cream colour of standard *Escherichia coli*, while those colonies with intact β-galactosidase produced insoluble blue pigment (4-chloro-3-brom-indigo) from x-gal were turned blue and hence selected for further confirmation. For complete confirmation of transformants, tiny blue colonies were inoculated into 5 mL of LB medium in McCartney bottles with appropriate antibiotics and incubated at 37^0C at 200 rpm for 16 hours. Column purification of plasmid DNA was carried out using QIAprep spin miniprep following the manufacturer's instructions and the extracted DNA sequenced in order to confirm the presence of the plasmids and the inserts.

2.9.4 β-galactosidase assay

Distinct blue colonies from transformed LB-Agar plates were inoculated into 50 mL sterile LB medium in 250 mL capacity flasks using sterile inoculating wire loops and incubated overnight at 37^0C at 200 rpm. 100 µl of overnight cultures were inoculated into 1 mL arsenic biosensor medium (ABM6) or zinc biosensor medium (ZBM3) in 1.5 mL microcentrifuge tubes in triplicates and supplemented with appropriate antibiotics and calculated amount of appropriate heavy metal stocks. The tubes were incubated statically for 18 hours at 37^0C, optical density at 600 nm measured and tubes centrifuged on a table top microcentrifuge at maximum speed for 10 minutes. For all

constructs, cell pellets (stored at 4^0C overnight) were thawed on ice and re-suspended in 800 µL Z-buffer containing 10 mg/ml lysozyme and 0.0025% *w/v* SDS. The reaction mixture was incubated at 37^0C for 45 minutes and after incubation, 200 µL ONPG (4 mg/ml in 0.1 M sodium phosphate buffer) was added and incubated at 37^0C for 10 minutes. The reaction was stopped by adding 0.5 ml stop buffer (Na_2CO_3; 1 M), centrifuged for 10 minutes and absorbance measured at 420 nm which corresponds to yellow colour production.

Listed in table 2.22 is Z-buffer and its composition.

Table 2.22: Z – buffer composition

Component	**Concentration**
Na_2HPO_4	8.52 g
NaH_2PO_4	5.52 g
KCl	0.75 g
$MgCl_2.7H_2O$	0.246 g
β-mercaptoethanol	2.7 mL
ddH_2O	to 1 L

Listed in table 2.23 is sodium phosphate buffer and its composition.

Table 2.23: Sodium phosphate buffer (pH 7.0)

Component	**Concentration**
Na_2HPO_4	3.05 ml (0.2 M)
NaH_2PO_4	1.95 ml (0.2 M)
ddH_2O	to 10 mL

2.9.5 Optical density measurement

Following microbial culturing, optical density at OD600 was measured by pipetting a 100 µL of the culture into a cuvette and adding 900 µL of phosphate buffered saline (PBS). Subsequently, the cell-PBS suspension was mixed by upward and downward pipetting. The Spectrophotometer was blanked with 1 mL PBS and the OD of the cultures measured.

2.9.6 Viable cell counts

The quantification of viable cells was conducted as colony forming units (cfu) per millilitre of culture sample. This was performed by serially diluting cultures in sterile 15 mL capacity disposable centrifuge tubes and spotted onto LB agar plates. 10 µL of each dilution was spotted in quadruplicate on plates using the map in Figure 2.4.

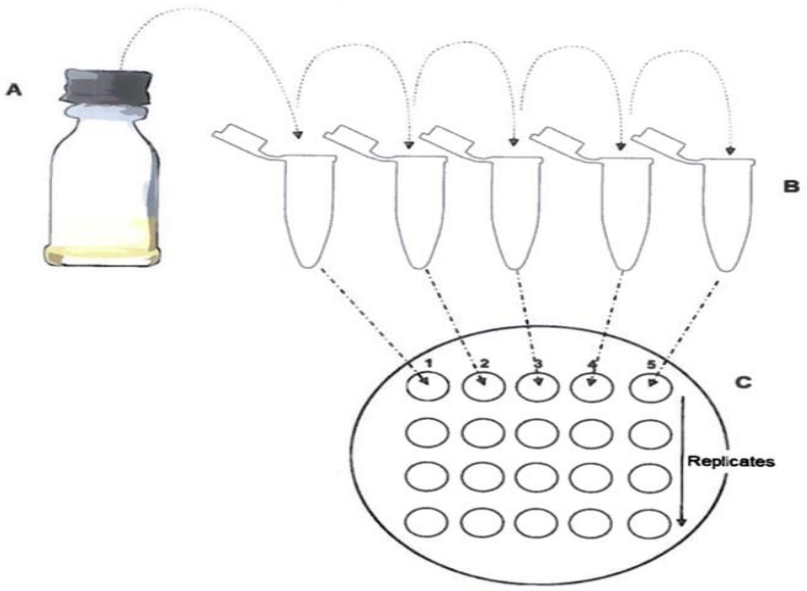


Figure 2.4: Schematic representation for colony count and replicates

Serial dilutions of the culture (A) were made 15 mL capacity disposable centrifuge tubes and spotted onto LB agar plates using 10 µL replicates. Plates incubated overnight at 37^0C.

Chapter 3: Development of novel whole-cell bacterial sensors for zinc and copper detection

3.1 Abstract

Novel zinc and copper biosensors developed using endogenous zinc-binding and copper-binding transcription factors, ZntR and CueR respectively, each fused to the reporter gene, *lacZ'α* were expressed in *Escherichia coli* JM109 strain and respectively tested for zinc and copper in newly formulated medium, ZBM3. Initial designs showed that the constructs had high background activity as there was false positive response to samples without metal supplements. To correct these, the constructs were redesigned using either weaker ribosome binding sites (RBS), low copy number plasmids or promoters with lower activity. Results obtained showed that the novel Zn biosensor accurately detected zinc, cadmium, lead and mercury concentrations (3 mg/L, 0.003 mg/L, 0.01 mg/L and 0.001 mg/L respectively) below the recommended WHO limits. Similarly, copper, silver and gold levels (2 mg/L, no guidelines for silver and gold respectively) were detected by the novel copper biosensor below the limits recommended by the WHO. By using two metal ligands which can alter the concentrations of biologically available metals, it has also been demonstrated that these bacterial sensor strains have the potential to differentiate between bioavailable from total metal in the assay.

3.2 Background

For normal growth and function of cells in prokaryotes and eukaryotes, heavy metals including Zn and Cu are very essential because they are constituents of many enzymes and other proteins (Hall, 2001). Still, growth inhibition and toxicity symptoms can occur due to elevated concentrations of both essential and non-essential metals (Hall, 2001). Most of these symptoms may be due to some extents of heavy metal interactions at cellular or molecular level. Toxicity may also result due to metal binding to sulphydryl groups in proteins, which leads to disruption of structure or inhibition of activity. Toxicity could also result from the displacement of an essential element which can cause deficiency effects (Van Assche and Clijsters, 1990). Also, high levels of heavy metals may trigger the formation of free radicals and reactive oxygen species (ROS), thus leading to oxidative stress (Dietz *et al*., 1999). However, living organisms have evolved a range of cellular mechanisms involved in the detoxification of heavy metals and hence, tolerance to metal stress (Hall, 2001). Investigations have shown that tolerant microbes, plants and animals exhibit enhanced avoidance and homeostatic mechanisms to prevent the onslaught of stress (de Vos *et al*., 1991; Dietz *et al*., 1999).

Nigeria is one of the developing countries of the world with large deposits of mineral resources. Heavy metals are released to the environment through anthropogenic activities such as mining of these solid minerals. It has been reported that $CuSO_4.5H_2O$ (commonly known as blue vitriol) is a component of herbicides, fungicides and pesticides ignorantly used by farmers. Copper compounds and other heavy metals leach into rivers, lakes, ponds and other surface waters (sources of drinking water in most remote areas in developing countries) during rainfall. Dating back to the colonial era, the northern part of Nigeria is popular for mining activities

both legal and illegal (due to poverty and ignorance). After crushing, ores are essentially processed in water bodies like lakes and rivers through floatation. It was reported that in 2010, at least 400 children died and more than 3,500 children were affected by lead poisoning in Zamfara State, Nigeria (NNP, 2011). In those affected areas, it was observed that children washed their sheep, water their flocks of sheep and herds of cattle, bathe and washed their clothes in ponds highly contaminated with lead from ore-processing, thus creating additional routes of lead exposure.

Figure 3.1: Illegal gold mining site in Bagega, Zamfara State, Nigeria. Men and children work in the mines located outside Bagega. When deeply dug and found, the rocks are then crushed to find the gold ore. Deposited inside the rock are other metals, such as lead, which has poisoned many of the children in the area.

Underground water has persisted as the main water source to the vast population of the people in the rural areas in most developing countries of the world. This entails that water quality assurance is a corporate responsibility. The oxidative stress and the production of reactive oxygen species (ROS) have been implicated in the toxicity and carcinogenicity of metals such as cadmium (Tchounwou *et al*., 2001), lead (Yedjou &

Tchounwou, 2008), and mercury (Sutton & Tchounwou, 2002; Sutton *et al*., 2002).These elements together with arsenic and chromium rank among the preference metals of great public health importance due to their high degree of toxicity (ATSDR, 2008; Tchounwou *et al*., 2012). Cadmium which is a by-product of zinc production is exposed to humans and animals at work or in the environment. It accumulates inside body throughout life once absorbed by humans, where it exerts adverse effects on the enzymatic systems of cells, oxidative stress as well as inducing nutritional deficiency in plants (Irfan *et al*., 2013).

Cadmium is also capable of binding with cysteine, glutamate, histidine and aspartate ligands which can lead to iron deficiency (Castagnetto *et al*., 2002). Having the same oxidation state with zinc, cadmium can replace zinc present in metallothionein, hence triggering inhibition of zinc from acting as a free radical scavenger within the cell (Tchounwou *et al*., 2012).

Mercury is essentially toxic and exceptionally bioaccumulative. Because of its adverse effects on the marine environment, majority of studies are directed towards its distribution in water environment (Chen *et al*., 2012). Mercury are widely distributed in water bodies such as rivers, lakes and oceans. When absorbed by microorganisms, they become methylated which later results in biomagnification leading to critical disturbance to aquatic lives (Trasande *et al*., 2005). In 1997, about 3, 596 cases of acute heavy metal poisoning was attributed to mercury by the American Association of Poison Control Centres. The brain is reported to be the target orange for mercury, although it is believed to impair any organ causing malfunctioning of nerves, kidneys and muscles (Patrick, 2002).

Lead is a highly toxic metal and its widespread use has significant effects on the environment and public health. Unlike other metals like copper, zinc and magnesium, there is no biological functions associated with lead (Tchounwou *et al*., 2012).

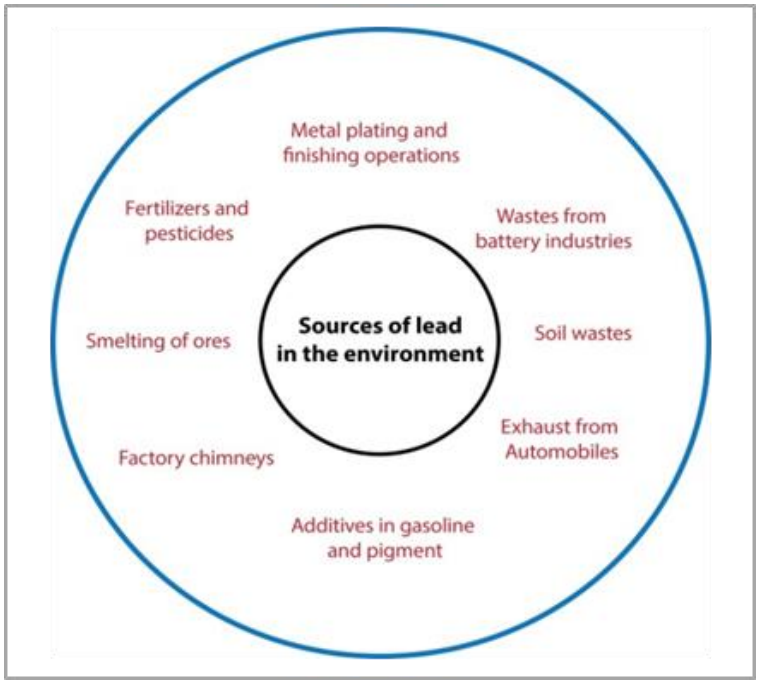


Figure 3.2: Various sources of lead pollution in the environment (Sharma & Dubey, 2005).

Metallic lead causes toxicity in cells through ionic mechanism and that of oxidative stress. At elevated concentrations, cells, proteins, nucleic acids, membranes and lipids are structurally damaged by ROS resulting in a stressed situation at cellular level (Matthew *et al*., 2011). The ability of metallic lead ions to replace other bivalent cations such as Ca^{2+}, Mg^{2+}, Fe^{2+} and monovalent cations like Na^{+} is caused by the ionic mechanism of lead toxicity, which basically disrupts the biological mechanism of the cell (Flora *et al*., 2008)

Zinc and other essential micronutrients are required by most organisms for growth, development and repair of tissues, but at high levels, zinc becomes toxic as it

interferes with normal biological processes including inhibition of the growth of most bacteria (Rainbow, 2002; Wang, 2010). In North America and Europe, the trace metal chemistry of surface water has been altered by acidification (Newman and McIntosh, 1919) by: (1) increasing the total concentration of zinc and other trace metals (e.g. Fe, Al, Pb, Cd, etc.), (2) shifting the speciation of dissolved metals toward free aqua ions, usually the species most toxic to aquatic biota, and (3) reducing particulate metal concentrations in favour of higher dissolved levels (Adriano, 2001). All these contribute to potential risks for all organisms (Aktar, 2011). Zinc production is found in many regions of the world including India, China, Canada, Australia and the United States. China is one of the top countries producing Zn, with about 950 000 tons of Zn found near smelting area sites in 2001 (Wang, 2009). Of serious concern is the pollution of aquatic ecosystem by Zn which is released from both natural and anthropogenic sources (Aktar, 2011). Exposure to high levels of Zn can disrupt acid-base regulation, possibly as a result of inhibition of carbonic anhydrase (CA) activity, which has effects of excretion of CO_2 (Santore, 2002). Similarly, exposure to high levels of Zn causes increased adverse respiratory response in fish resulting from acute inflammatory reaction which may likely destroy the bronchial surface (Satore, 2002).

In humans, various dysfunctions which can lead to impairment of growth and reproduction could be caused by excess Zn. Symptoms of Zn toxicity are bloody urine, vomiting, diarrhoea, anaemia, kidney failure, icterus (yellow mucus membrane) and liver failure (Fosmire, 1990). Excess Zn also perturbs lots of energy pathways including glycolysis, electron transport chain and tricarboxylic acid cycle (TCA), which causes reduction in energy production (Dineley, 2003). High concentration of Zn also causes the accumulation of ROS and peroxidation of lipids in neurons (Dineley, 2003).

Presently, heavy metal detection in Nigeria is based on physical methods such as; Energy Dispersive X-Ray Fluorescence (XDXRF) which operates under quantitative X-ray Analysis System (QXAS), flame Atomic Absorption Spectrophotometer, Atomic Fluorescence Spectroscopy and Mass Spectrometry, all of which are quite expensive and require skilled personnel for operation and result interpretation. Similarly, samples are required to be prepared and taken to areas where these facilities are situated which add to transportation cost and may take a long period of time to get back the results. Consequently, these problems can be solved using biosensors (see section 1.3.2).

3.2.1 Objectives of work presented in this chapter

The key features of the work presented in chapter 3 were:

1) To design and construct proof of concepts biosensors for Ag, Au, Cd, Cu, Hg, Pb and Zn on the basis of previously introduced metal responsive promoters P*copA*, P*zntA* and their regulatory protein associates.

2) To assess the activity of whole-cell expression systems.

3) To investigate the possibility of tuning Cu and Zn biosensors using weak ribosome binding sites.

4) To investigate the toxicity of heavy metals to constructs in different media.

5) To investigate the effects of chelating agents on metal bioavailability.

3.3 Materials and methods

3.3.1 Materials

Media were obtained from the media kitchen of the University of Edinburgh, unless otherwise stated. The following chemicals and related reagents were used in this study: $ZnSO_4$, $CdSO_4$, $Pb(NO_3)_2$ and $HgCl_2$, $Au(NO_3)_3$, $AgNO_3$ and $CuSO_4.5H_20$, EDTA were from Merck (Darmstadt, Germany), Na_2S was from Fluka, antibiotics were from Sigma. Restriction endonucleases and other enzymes for DNA manipulations, synthetic oligonucleotides (IDT) and DNA modifying enzymes were purchased from New England Biolabs. All enzymes were used as recommended by manufacturers or donors.

3.3.2 Bacterial strains and expression vectors

Plasmid transformations and β-galactosidase assays were performed in *E. coli* strain JM109 (K-12, *endA1 gyr96 hsdR17* Δ(*lac-proAB*) *recA1 relA1 supE44 thi-1* F'[$lacI^q$ *lacZΔ*M15 *proAB+ traD36*] (Casali and Preston, 2003), and *E. coli* MG1655.

3.3.3 Culture conditions

Bacterial cells were cultivated at 37^0C with shaking at 200 rpm for liquid cultures in Luria-Bertani (LB) medium or M9 medium supplemented with casamino acids and thiamine as described by Sambrook *et al*., (2001). M9 medium used for metal induction assays involving silver salts was devoid of sodium chloride to circumvent Agcl precipitation (Stoyanov *et al*., 2001). The antibiotics used: chloramphenicol (Cml, 40 mg/ml) and kanamycin (Kan, 50 mg/ml) were stored as concentrated solutions and required amounts added to the media. For induction and selection, 0.5 mM IPTG and 40 µg/ml X-gal were used when the constructs contained P*zntA*-*lacZ'α* and P*copA*-*lacZ'α*, respectively. Zinc and copper biosensor medium (ZBM3) was formulated

following the protocol of de Mora *et al*., (2011), except that $NaHCO_3$ was omitted and the concentration of K_2HPO_4 increased to 10% due to toxicity of ABM6 to the cells.

3.3.4 DNA preparations

Large scale DNA preparations were prepared by lysis of overnight cultures of *E. coli* MG1655 strain using DNeasy kit and following manufacturer's protocols. All DNA preparations were stored in Buffer EB at 4^0C.

3.3.5 DNA manipulations

Standard DNA manipulations were performed following the protocols as described by Sambrook *et al*., (1989). Oligonucleotides were designed using the SnapGene software and synthesized by Integrated DNA Technologies. For the construction of P*zntA_lacZ'α*, *E. coli* MG1655 genomic DNA was prepared by the lysis and purification of overnight culture strain using DNeasy Blood & Tissue Kits from QIAGEN. The *zntA* promoter region on the genomic DNA was PCR amplified using modified upstream primer (see table 2.4 for more of the primers used) (5'−GAGGAATTCCTTCTAGAGCGGTCACTTCCTGATCGTC−3') and downstream primer (5'−GTACTGCAGCTACTAGTACAGGAGTCGACATGGCATC−3') that created *Eco*RI and *Pst*I sites (underlined sequences) at positions -126 and +37, respectively with respect to start codon. The resulting PCR product and pSB1C3_RFP (a gift from French Lab) were digested with *Eco*RI and *Pst*I and ligated. The purified product was transformed into *E. coli* JM109 and plasmid DNA purified through Miniprep. The purified plasmid was digested with *Pst*I and *Spe*I and ligated into *Xba*I-*Pst*I digested *lacZ'α* to yield the final sensor plasmid P*zntA_lacZ'α* (PCN1). Similar procedure was followed to generate the plasmid PCN2, a pSB1C3-based vector containing the PCR-amplified promoter/operator of *copA* using the following primers: 5′− GAGGAATTCCTTCTAGAGCCGTCCAGGGTCAGGTCG−3′ and

5′−GTACTGCAGCTACTAGTACGGACTTTTACCCGCCTGG−3′. All cloned products were sequence verified by the Gene Pool sequencing service of the University of Edinburgh.

3.3.6 β-galactosidase assays

For all constructs, overnight shake cultures grown at 37^0C in LB medium in the presence of chloramphenicol or kanamycin were inoculated in ZBM3 in 1.5 mL microcentrifuge tubes and supplemented with appropriate amounts of metal salts. The tubes were incubated statically overnight at 37^0C. Optical density was measured and cells pelleted. The cell pellets were immediately chilled on ice and re-suspended in 800 µL Z-buffer containing 10 mg/ml lysozyme and 0.0025% *w/v* SDS. The reaction mixtures were incubated at 37^0C for 45 minutes. After incubation, 200 µL ONPG (4 mg/ml in 0.1 M sodium phosphate buffer) was added and incubated at 37^0C for 10 minutes. The reaction was stopped by adding 0.5 ml stop buffer, cells were immediately harvested by centrifugation for 10 minutes and the β-galactosidase activity of each construct was measured as described by Miller (1972).

3.3.7 Metal detection assays

For each construct, distinct colonies were inoculated into LB medium supplemented with appropriate antibiotics and incubated at 37^0C overnight at 200 rpm shaking. Overnight cultures with inoculum size of 100 µL was inoculated into 1 mL ZBM3 in 1.5 ml microcentrifuge tubes in triplicates and supplemented with calculated amount of metal salts. The tubes were incubated statically in an upright position at 37^0C for 24 hours and observed for a development of colour change from blue to yellow.

3.3.8 Treatment of metals with EDTA and Na_2S

Two chelating compounds, EDTA and Na_2S were used to investigate chromogenic response of *E. coli* JM109/pSB1C3_P*zntA*_*lacZ'α* and *E. coli* JM109/pSB1C3_P*copA*_*lacZ'α* on metal bioavailability both in the presence and absence of metals. Metal concentrations in the range suitable to ensure the elicitation of toxic responses and a nearly total inhibition of bacterial sensor pH colour change were used as follows: 100 µM $ZnSO_4$; 10 µM $CdSO_4$; 10 µM $Pb(NO_3)_2$; 0.5 µM $HgCl_2$; 100 µM $CuSO_4$; 5 µM $AgNO_3$; and 5 µM $AuCl_3$. Metal/chelate molar ratios in the range of 0 to 2 were prepared by diluting EDTA and Na_2S into each metal solution. Suitable amounts were added into a suspension of 1 mL ZBM3 and 100 µL overnight culture of sensor in 1.5 mL microcentrifuge tubes in triplicates and incubated statically at 37^0C overnight for a pH colour change from blue to yellow. The tubes were centrifuged at maximum speed on table top centrifuge for 5 minutes and absorbance of supernatants measured at 625 nm for the disappearance of blue colour.

3.4 Results

Listed in table 3.1 are the promoters used in this study, together with the proteins that regulate them and the metals they respond to.

Table 3.1: Summary of metal responsive promoters, regulatory proteins and metals they are responsive to.

Promoter	Regulatory protein	Responsive to
P_{copA}	CueR	Cu, Ag, Au
P_{zntA}	ZntR	Zn, Pb, Cd, Hg

3.4.1 Design of zinc and copper biosensors

The gel electrophoresis of *zntA* and *copA* PCR-amplified promoters showed the correct band sizes as illustrated in figure 3.4(a). The pSB1C3-RFP plasmid, having the red fluorescence protein, was CIP-digested and ran on gel (figure 3.4b). The upper band (2.07 kb), representing the plasmid, was excised and gel-purified. The plasmid DNA and purified DNA of each of the promoters were ligated together with *lacZα* gene (the reporter) and then transformed into competent *E. coli* JM109. The plasmid maps of the constructs are shown in Figure 3.5, and all constructs were confirmed by sequencing (Figure 3.3).

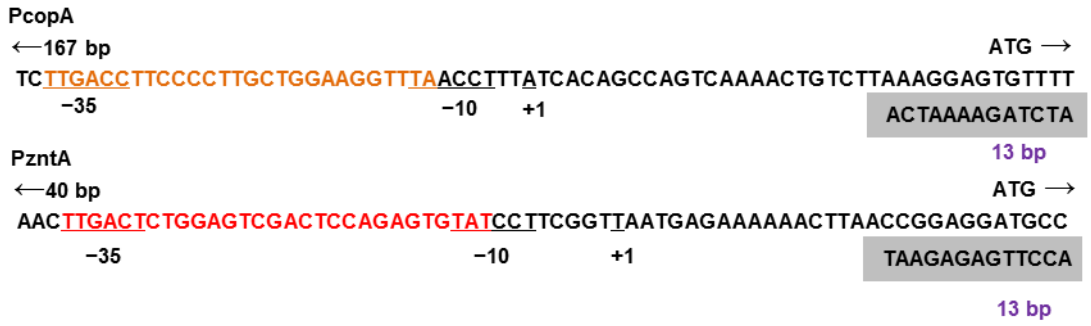


Figure 3.3: Partial promoter sequences of P_{copA} and P_{zntA}. The sequences are abbreviated as indicated above the corresponding sequence with a left point arrow (←). The areas of protein-DNA interaction published in the according literature are highlighted for all sequences. The -35 and -10 promoter regions together with transcription start (+) are underlined and labelled accordingly. Sequences of the weaker strength synthetic RBS are shown below the native sequences in purple. All sequences end before the start codon of the controlled genes, marked ATG and an arrow on the right (Xu *et al*., 1996; Brocklehurst *et al*., 1999; Outten *et al*., 2000; Brown *et al*., 2003)

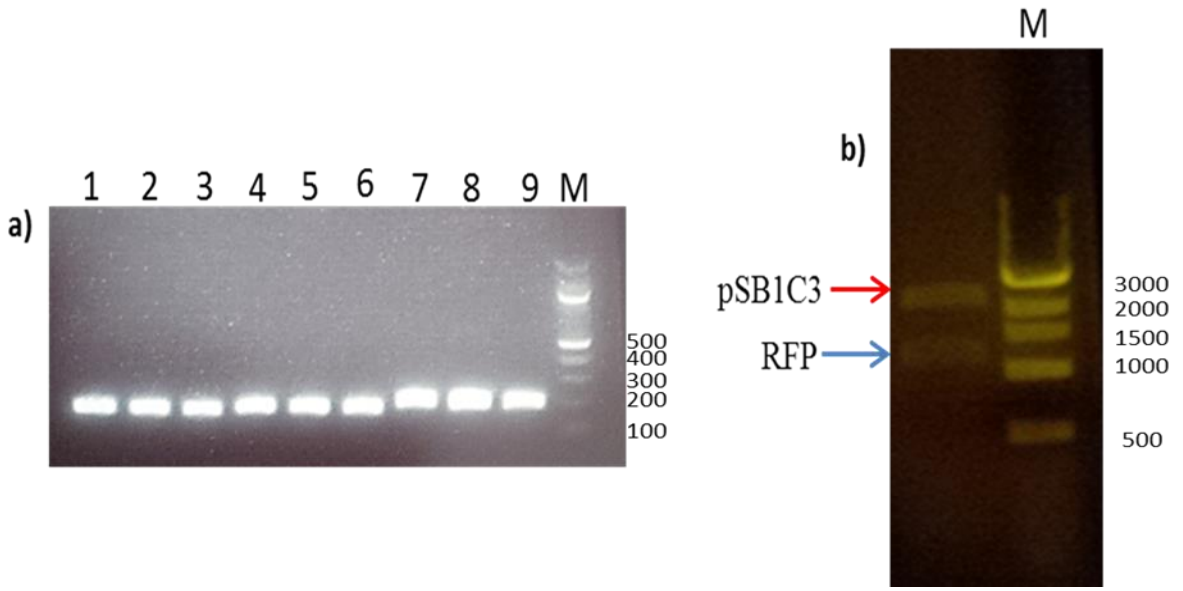


Figure 3.4: Gel electrophoresis. a) PCR-amplified promoters. Sample identification: 1-6: *zntA* (159 bp). 7-9: *copA* (180 bp). M= 100 bp ladder. b) Digested plasmid. Plasmid size= 2.07 kb, RFP= 1.06 bp. M= 1 kb ladder.

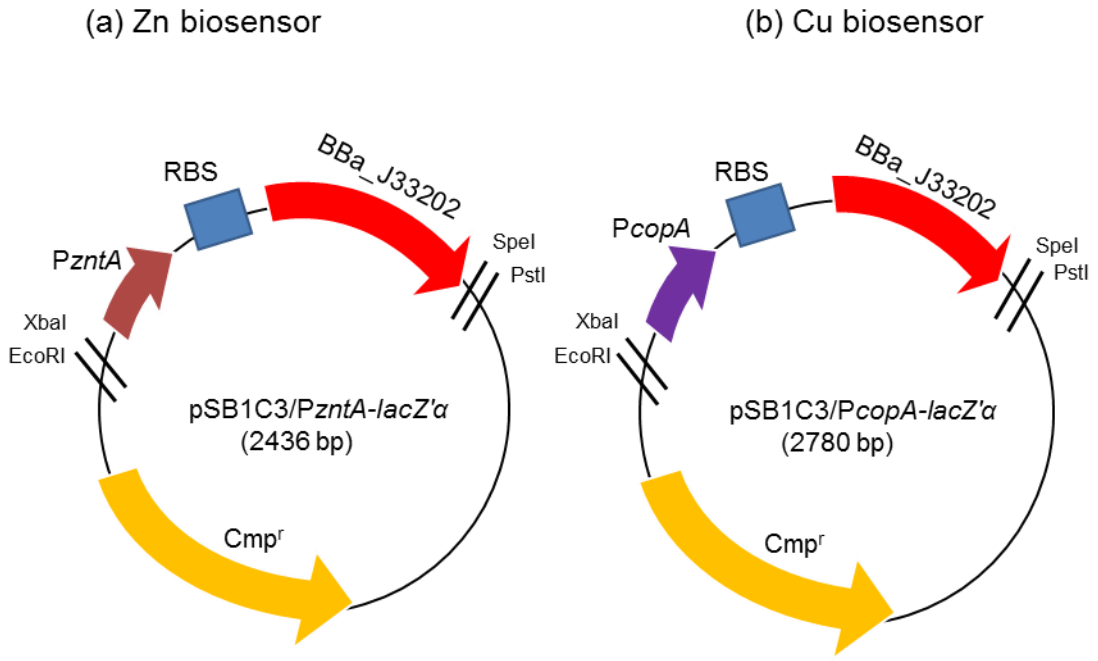


Figure 3.5: Plasmid maps for copper and zinc biosensors. The constructs were cloned into pSB1C3. Restriction enzyme recognition sites are indicated. Cmp^r: chloramphenicol resistance gene. Diagrams are not to scale.

3.4.2 Induction and selection of recombinant *E. coli* JM109

E. coli JM109 cells transformed with plasmids for the induction of Zn and Cu were grown on LB plates and incubated at 37^0C overnight. The laboratory strain *E. coli* JM109 was used as a negative control. Distinct colonies were subcultured on LB plates containing 0.5 mM IPTG, 40 µg/mL X-gal, 40 µg/mL chloramphenicol, 0.3 mM $CuSO_4.5H_2O$ or 2.0 mM $ZnSO_4.7H_2O$, respectively for selection of blue colonies. The plates were incubated at 37^0C overnight. As shown in figure 3.6, the appearance of blue colonies which is caused by cleavage of the chromogenic lactose complement 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) by β-galactosidase in the presence of IPTG and induction of Zn and Cu showed that the recombinant *E. coli* JM109 contained *lacZ'α* gene and was expressed.

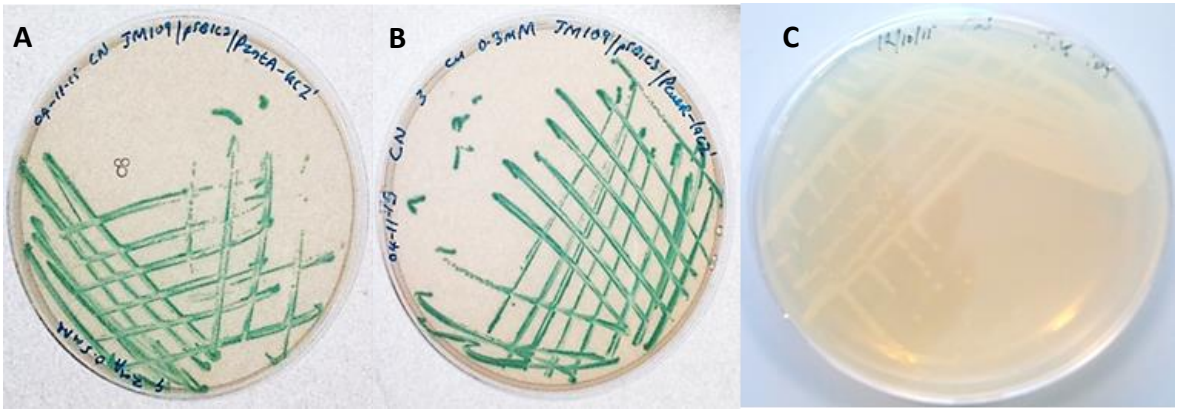


Figure 3.6: Growth of recombinant *E. coli* JM109 in selective media. **A)** Recombinant *E. coli* JM109/pSB1C3_P*zntA*_*lacZ'α*. **B)** Recombinant *E. coli* JM109/pSB1C3_P*copA*_*lacZ'α*. **C)** *E. coli* JM109 (negative control) which contains the *lacZΔ*M15 mutation which requires the *lacZ'α* gene for functional complementation.

3.4.3 Testing toxicity of constructs in different media

In order to select media for growth assay of Zn and Cu constructs, toxicity test was carried out in different media. Overnight cultures of Zn and Cu biosensors, and *E. coli* JM109 (used as control) were inoculated into 1 ml of LB and arsenic biosensor medium ABM6 (no colour indicator) in 1.5 ml microcentrifuge tubes in triplicates. The tubes were spiked with different concentrations of Zn or Cu and incubated at 37^0C for 24 h. The cell density of each sample was measured at 600 nm.

For both copper and zinc biosensors as well as JM109, growth in LB was very high in the presence of Cu and Zn supplements but Zn became toxic to the cells at 1.5 mM (*purple* bars). Cu^{2+} and Zn^{2+} ions in ABM6 medium were found to be toxic to the cells as there were significant reductions in growth of both copper and zinc biosensors, and *E. coli* JM109 (control) (*blue* and *green* bars). This was evident in the tubes without copper or zinc supplements (0 mM) as the cells possibly grew to optical density of approximately 0.8 and 0.5 nm respectively. Consequently, Cu^{2+} and Zn^{2+} ions in ABM6 were found to be toxic to cells at all concentrations tested and hence ABM6 is not a suitable medium for the assay (Figure 3.7). The results obtained also showed that the biosensor constructs have high background activity. This is evident in the tubes without metal supplements turning yellow (Figure 3.9), also the high β-galactosidase activity in same tubes (Figure 3.8).

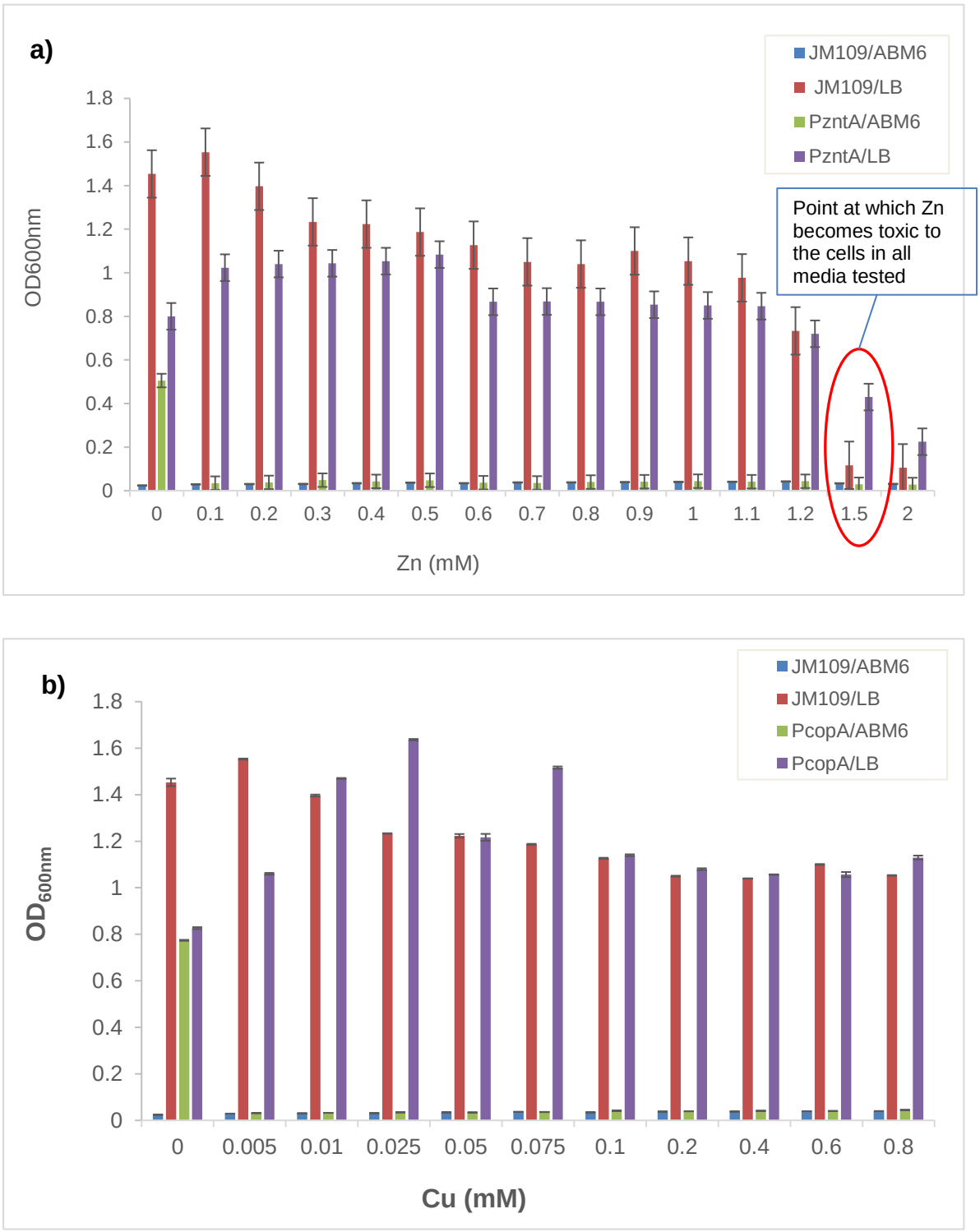


Figure 3.7: Testing the toxicity of biosensor constructs (a) Recombinant *E. coli* JM109/pSB1C3_P*zntA_lacZ'α* and (b) Recombinant *E. coli* JM109/pSB1C3_P*copA_lacZ'α* in different media. The tests were conducted in LB and ABM6 media in triplicates and incubated at 37^{0}C for 24 hours followed by cell density measurement at 600 nm. The error bars indicate the standard error calculated from three replicates.

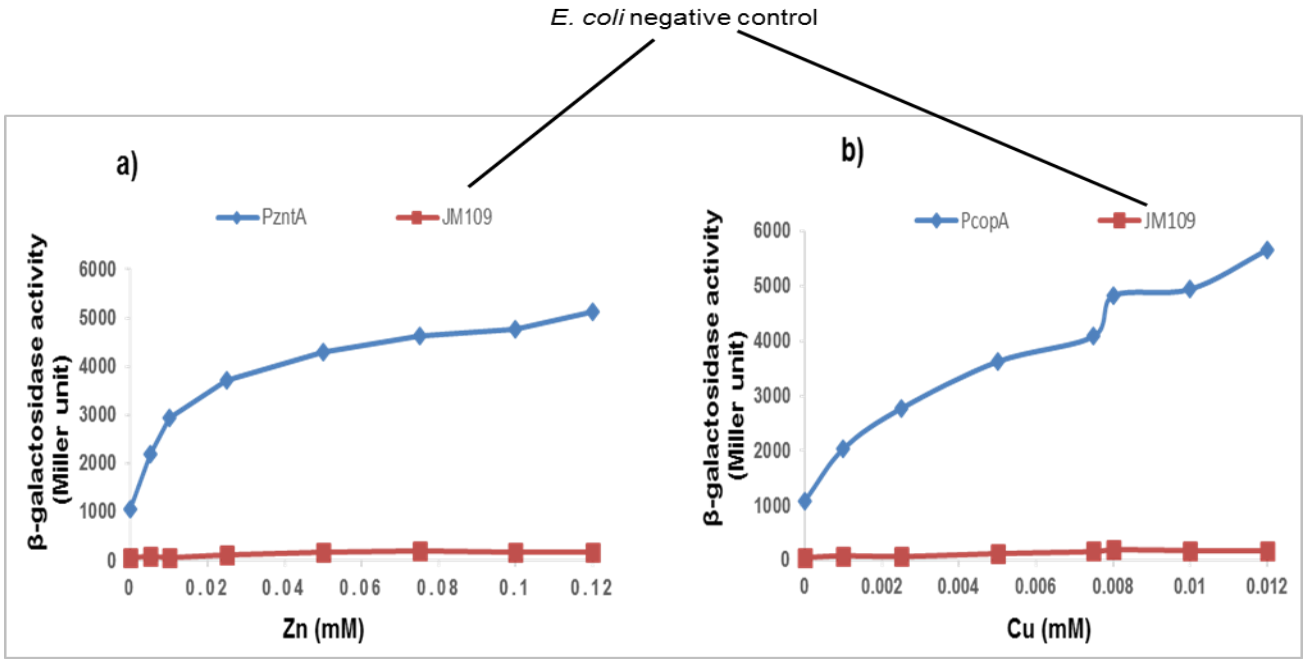


Figure 3.8: β-galactosidase (Miller assay) activity at increasing metal ion concentrations in M9 medium. a). Recombinant *E. coli* JM109/pSB1C3_P*zntA*_*lacZ'α* b). Recombinant *E. coli* JM109/pSB1C3_P*copA*_*lacZ'α*. The *error bars*, which show the standard error calculated from three replicates, are smaller than the symbols.

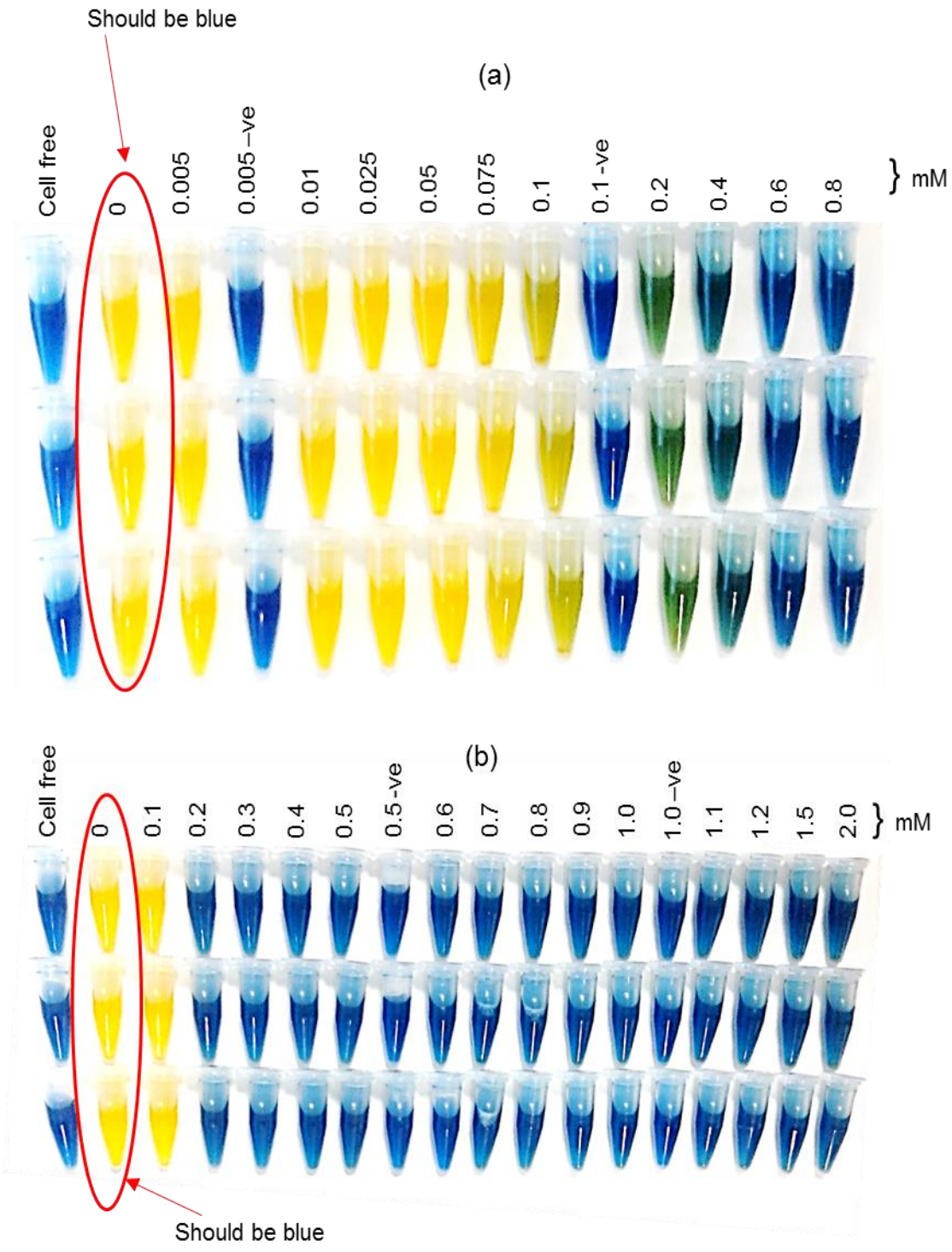


Figure 3.9: Whole-cell biosensor assay for (a) copper and (b) zinc in ABM6 medium using bromothymol blue as a pH indicator. Each assay was conducted in triplicate and tubes statically incubated at 37^0C for 24 hours. Samples shown are tubes having 100 µL inoculum size.

To assess media suitable for growth assays of Cu and Zn biosensor constructs, M9 medium was supplemented with lactose (1%) and 0.2% thiamine. 20% w/v lactose was filter-sterilized and added to the medium after autoclaving the mineral base. The toxicity test was conducted as in section 3.4.3.

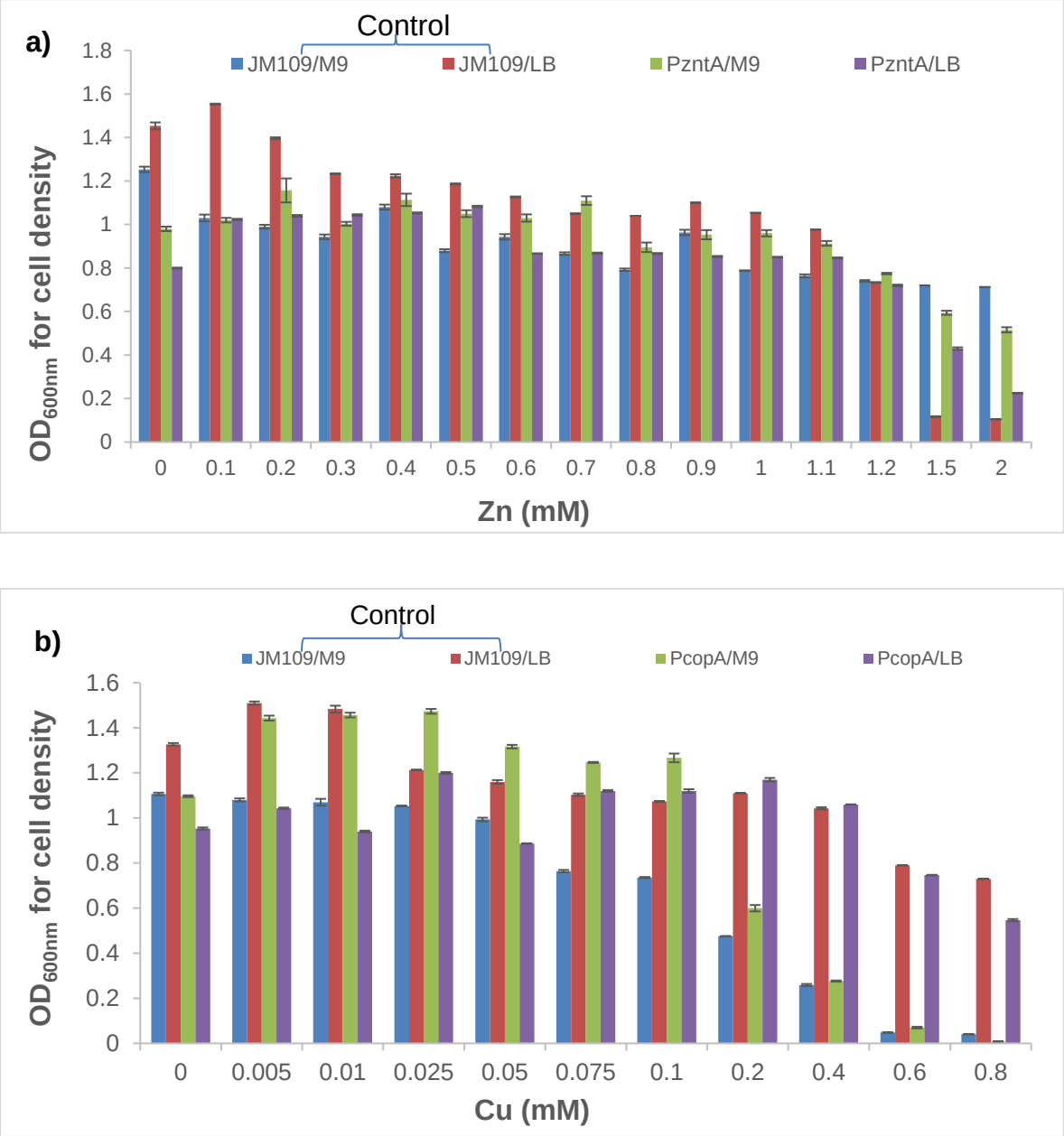


Figure 3.10: Toxicity test of constructs in LB and M9 media. (a) Recombinant *E. coli* JM109/pSB1C3_P*zntA_lacZ'α.* (b) Recombinant *E. coli* JM109/pSB1C3-P*copA-lacZ'α*. The error bars indicate the standard error calculated from three replicates. Cell density measure at 600 nm after 24 hour incubation at 37^0C.

As observed in Figure 3.10, M9 minimal medium supported the growth of the designed copper and zinc biosensors, and *E. coli* JM109. It was found that cells were able to grow in the presence of zinc but at elevated concentration at 1.5 mM, it became toxic to the cells as shown by a decrease in cell growth (Figure 3.10a). For the Cu biosensor, M9 medium supported cell growth up to 1.0 mM Cu^{2+} after which the increase in Cu concentration led to a decrease in cell growth due to metal toxicity to the cells. Hence, M9 minimal medium appeared to be suitable for conducting Zn and Cu growth assays, but only for a certain maximum concentration for these metals. However, samples of unknown metals need to be diluted before conducting the assay.

3.4.3.1 Assay procedures in M9 medium

The repeated Zn and Cu assays in M9 showed that the developed biosensors were able to produce *lacZ'α* under the conditions tested as indicated by the change of pH colour indicator from blue to yellow. However, the β-galactosidase activity of constructs explained why the systems did not work well under the same conditions as the arsenic biosensor, as shown by the high activity of the systems in tubes lacking metal supplements. As observed in negative control *E. coli* JM109 lacking *lacZ'α* gene, there was no activity of β-galactosidase enzymes (Figure 3.10). Zn biosensors grew in the presence of Zn up to 2.0 mM (Figure 3.11a), while the highest growth limit for Cu was 0.1 mM as the metal became toxic to the cells after this level. The change of colour in 0 mM tubes and the lower absorbance values for tubes with lower levels of added copper and zinc (Figure 3.10b & d) showed that designed biosensors have overall high background activity and needed to be improved by reducing the activity using either weaker RBS, low copy number plasmids or promoters with reduced

strengths. Similarly, there was interference of bromothymol pH indicator with components of M9 medium, hence colour change of positive tubes to pink which is a deviation from the *yellow* colour expected as output and established by the manufacturer. Consequently, M9 medium was found to be unsuitable for the assay which led to the formulation of another medium for the assay.

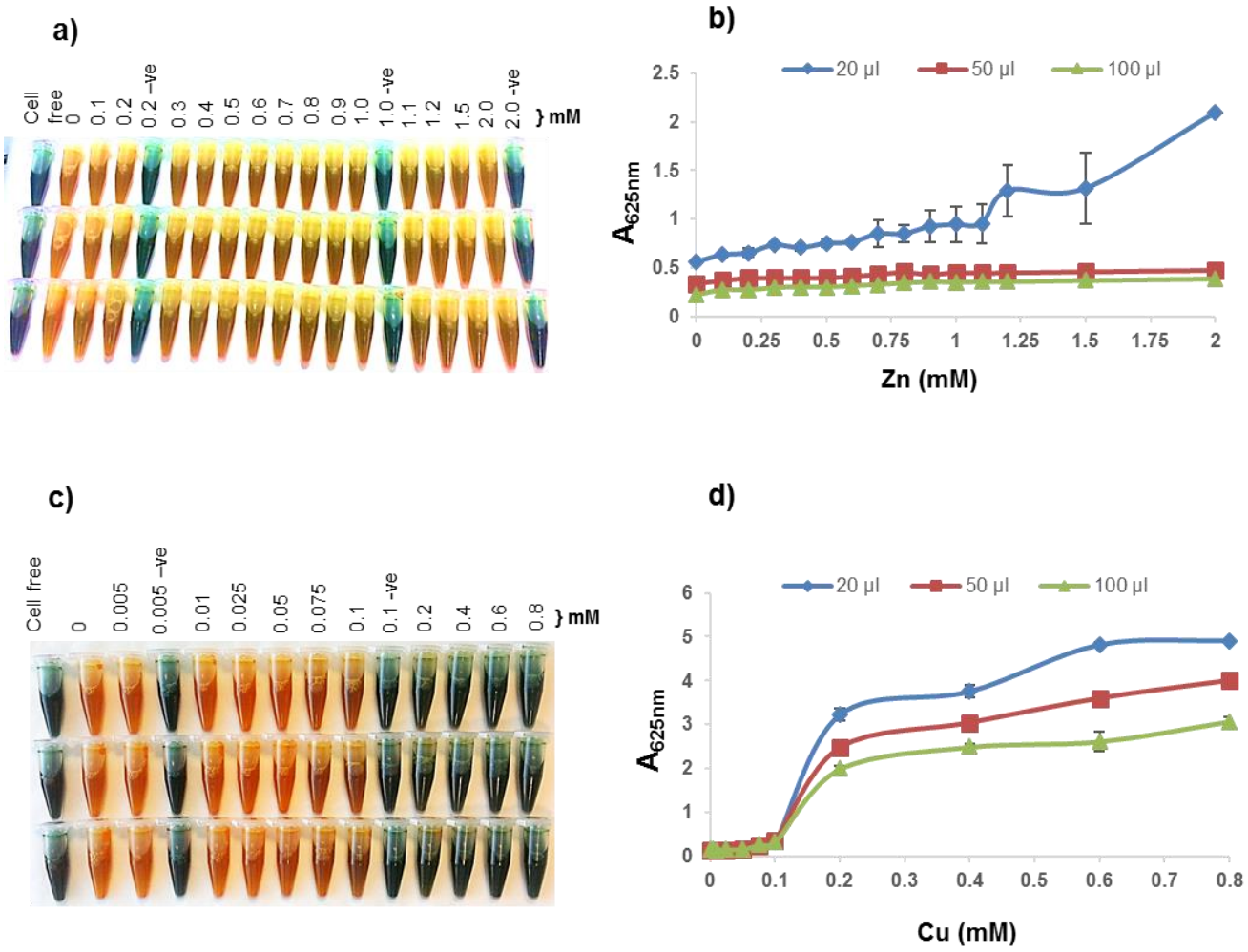


Figure 3.11: Whole-cell biosensor assays for (a) zinc and (c) copper determination in M9 medium using bromothymol blue as a pH indicator. Each assay was conducted in triplicate and tubes statically incubated at 37^0C for 24 hours. Samples shown are tubes having 100 µL inoculum size. (b & d) shows absorbance values of the disappearance of blue colours of supernatants measured at 625 nm following centrifugation of samples. The *error bars* show the standard error calculated from three replicates.

3.4.4 Tunable control of the translation initiation rates (TIRs) of novel constructs

The translation initiation rates (TIRs) of the constructs were determined using the RBS Calculator v2.0 Software developed by the Salis Lab at the Pennsylvania State University. Results obtained showed that the first generation of each of the constructs had predicted translation initiation rates (TIR) of 19574 and 16263 arbitrary unit (au) for the original sequences of copper and zinc respectively (Salis *et al*., 2009; Espah Borujeni *et al*., 2014) in their most recent iteration (2.0) at the time, which were somewhat high. Several attempts were made by employing the design features of the RBS calculator to design synthetic RBSs with reduced translation initiation rates for each promoter (Table 3.2). The oligonucleotides of the redesigned RBS sequences were synthesized and PCR performed using purified plasmid DNA constructs as templates. The PCR products were digested with DpnI enzyme and gel purified following protocols. The purified plasmid DNA was self-ligated, followed by transformation into *E. coli* JM109 (Figure 3.12), screening and selection. Plasmid DNA was prepared following protocols and plasmid DNA constructs were later verified by Sanger sequencing.

Table 3.2: Redesigned sensor plasmids and the transcription initiation rates of their synthetic weak ribosome binding site

Plasmids	Transcription Initiation Rate (au)	RBS sequence	Construct	Sensor
PCN1	16262.70	ACCGGAGGATGCC	Initial	Zinc
PCN1a	1576.16	CTCCGCACAAAATAGTTAACGGAAAACTTAA	New	
PCN1b	644.23	TTCAATATCGAAAGAACAAGACCAA	New	

PCN1c	447.03	ATAAATCAAAAGATTAAAAGGATATCGATATTATA	New	
PCN2	19574.00	AAAGGAGTGTTTT	Initial	Copper
PCN2a	654.23	ACTAAAAGATCTA	New	
PCN2b	379.31	TATACAGCACTAGGT	New	
PCN2c	694.21	CAAGACGGAACTCGT	New	
PCN2d	671.16	ACAAGCGTCCCTA	New	

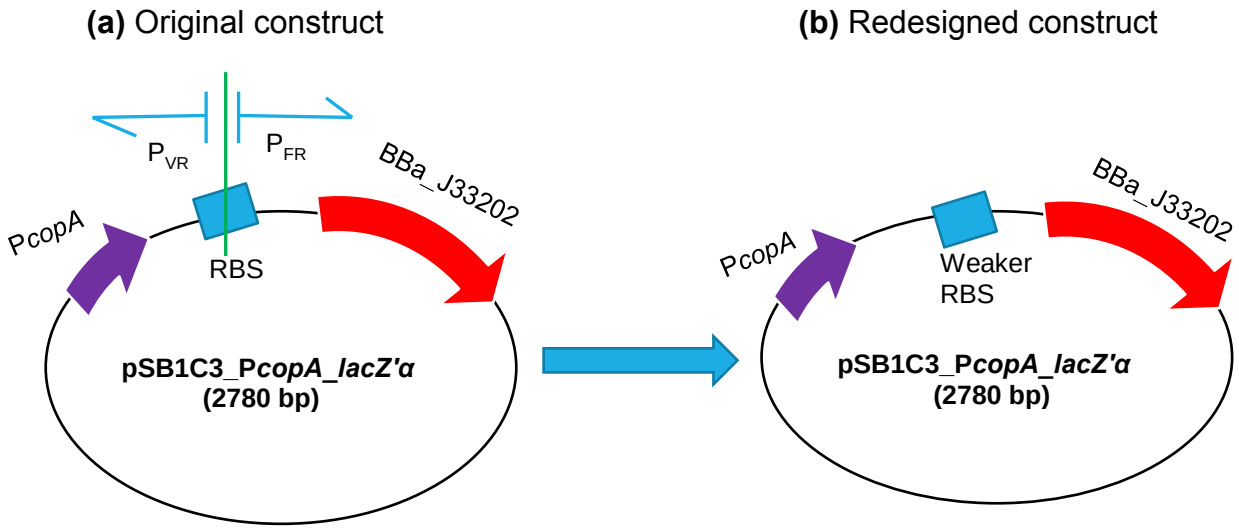


Figure 3.12: Insertion of synthetic weak RBS into constructs. Tuning of novel whole-cell biosensors. a) First generation of constructs with high translation initiation rate (TIR). (b) Redesigned construct with weaker ribosome binding site (RBS). The design features of RBS calculator was employed to design synthetic RBS having lower translation initiation rates. The synthesized oligonucleotide sequences of the RBS were used as primers to conduct PCR using the original construct plasmids (a) as templates. Following digestion and ligation, the resulting plasmid (b) was transformed into *E. coli* JM109, plasmid DNA purified and sequenced to confirm the correct inserts.

3.4.5 Design of zinc biosensor medium 3 (ZBM3)

To formulate a medium suitable for the novel biosensors, one of the possible causes which caused low growth or high death rate of cells in ABM6 was the reaction of sodium bicarbonate ($NaHCO_3$) in the medium with some metal ions. To evade this, variants of the medium were prepared without sodium bicarbonate. Sensor cells cultivated in the new medium showed appreciable growth patterns. Unfortunately, metal detection assays conducted in the medium with bromothymol blue pH indicator and metal supplements showed that there was persistent cell death and no positive response in all the assays tested (data not shown). In the third trial, it was decided to modify the concentration of potassium hydrogen phosphate, K_2HPO_4, the buffering agent in the medium. Its concentration in arsenic biosensor medium, ABM6 is 0.03%, so several variants of the new medium were produced with K_2HPO_4 buffer concentrations ranging from 0.01 – 2.0%. Preliminary assays were conducted with several screened colonies of biosensor cells with/or without metal supplements in either 1.5 mL microcentrifuge tubes in duplicates (static incubation at 37^0C for 24 hrs) or 96 Microtitre well plates (shaking incubation at 37^0C for 24 hrs).

After sequencing, it was found that high background activity still existed in wells and tubes (samples 1, 4, and 5 were screened colonies from low copy number plasmids, while samples 7 & 8 were screened from promoters with weaker strength). These samples with/and without metal supplements had pH colour change from blue to yellow. Samples 2, 3 & 6 were selected as being successful constructs as it could be confirmed that there was no colour change of the pH indicator in tubes and wells without metal supplements whereas these samples turned yellow with added metals after incubation (figure 3.13). Glycerol stocks of the sensors were stored at -80^0C. Also it was observed that the medium version with 1% K_2HPO_4 buffer concentration best

supported the positive results obtained whereas it could be seen that the same samples nearly remained blue in medium (figure 3.13b) with 2% K_2HPO_4 concentration which could probably be as a result of the interference of the disappearance of blue colour of pH indicator by high concentration of K_2HPO_4. Hence, the formulated medium with 1% K_2HPO_4 was adopted as the best suitable for the novel biosensor and was named ZBM3 (zinc biosensor medium 3) indicating three attempts at obtaining the right medium for the sensors.

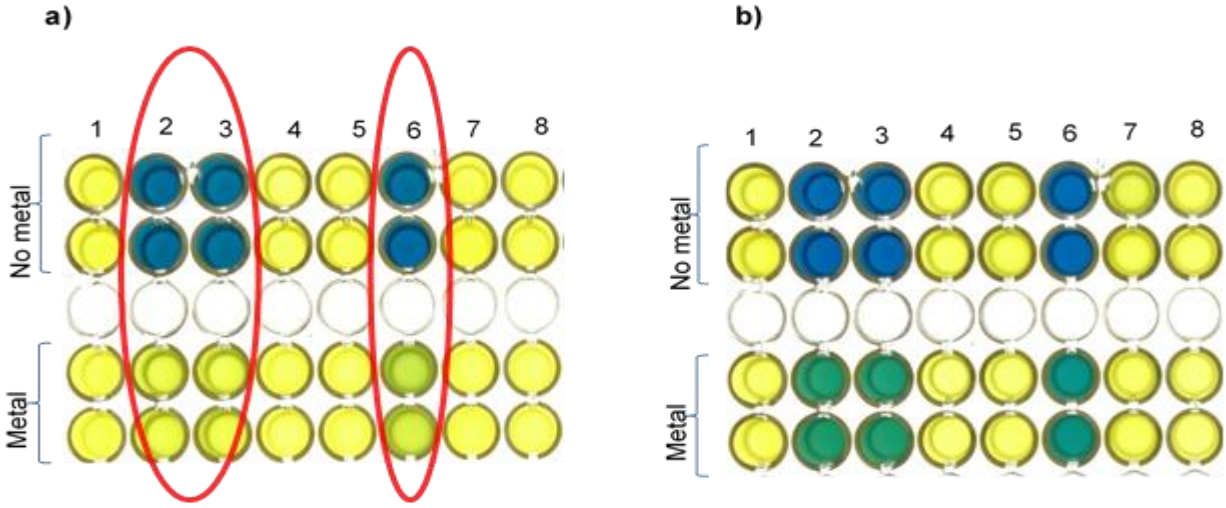


Figure 3.13: Preliminary metal detection assays in newly formulated medium variants having (a) 1% K_2HPO_4 and (b) 2% K_2HPO_4 in duplicates in 96 Microtitre well plates. Incubation at 37^0C for 24 hours with shaking. Sample identification: (**1, 4, 5=** screened colonies from low copy number plasmids; **2, 3, 6**= colonies from weaker RBS; **7, 8**= colonies from promoters with weaker strength). Assay was conducted using 0.08 mM $ZnSO_4$ and Zinc biosensors.

3.4.6 Induction of the tuned copper and zinc biosensors by heavy metals

The tuned and redesigned biosensors were induced as described in section 2.9.4. The *copA* promoter was tested in the presence of increasing concentrations of copper, gold and silver, while *zntA* promoter was tested in the presence of increasing concentrations of different metal ions such as cadmium, lead, mercury and zinc

(Figure 3.14). Metal concentrations that induced highest β-galactosidase activity were 1 µM of $Au(NO_3)_3$, $HgCl_2$, $CdSO_4$ and $Pb(NO_3)_2$, and 30 µM of $AgNO_3$ and $CuSO_4.5H_20$. When exposed to higher levels of metal concentrations, the enzyme activity rapidly declined, an indication of reaching a toxic metal level in the assay. In the assay, the enzyme activity still maintained highest level at 100 µM of $ZnSO_4.7H_2O$, suggesting that a toxic metal level had not been reached. In contrast to *copA* where enough responses to metals were triggered at 3 nM, sufficient responses of *zntA* to Hg(II), Zn(II), Pb(II) and Cd(II) were triggered at 10 nM. No induction was observed in Cu and Zn plasmids (negative controls) which have copper and zinc promoter respectively, but lacked *lacZ'α* gene.

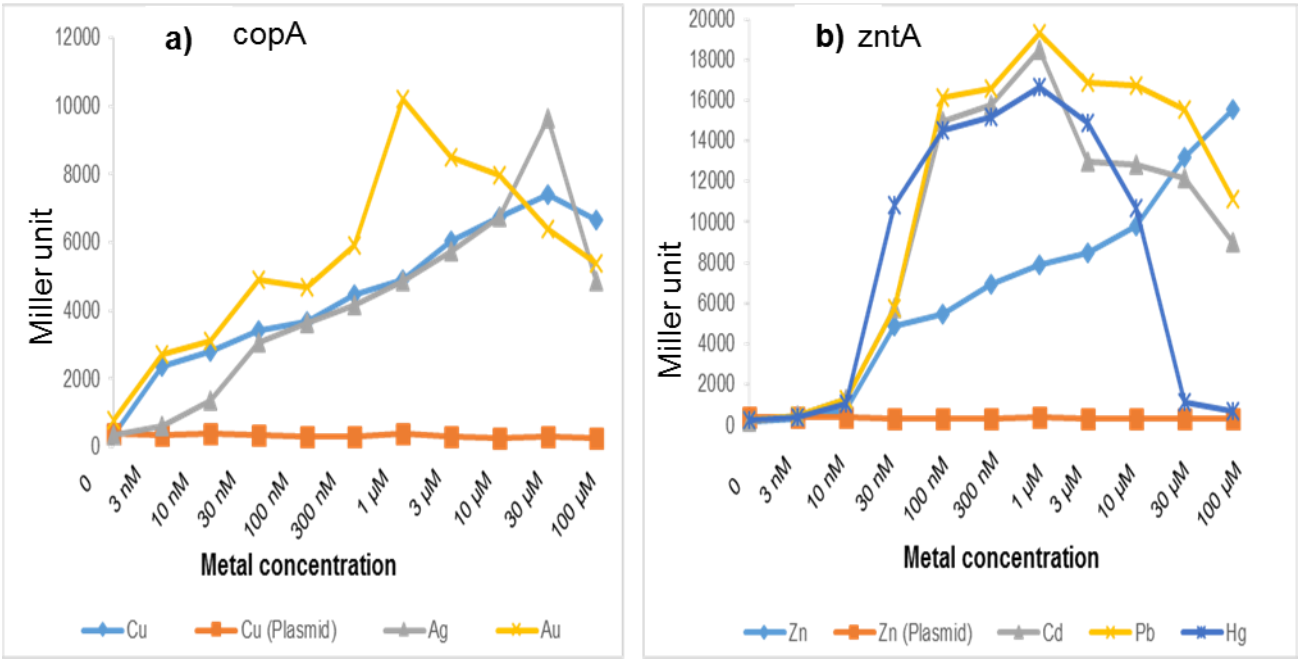
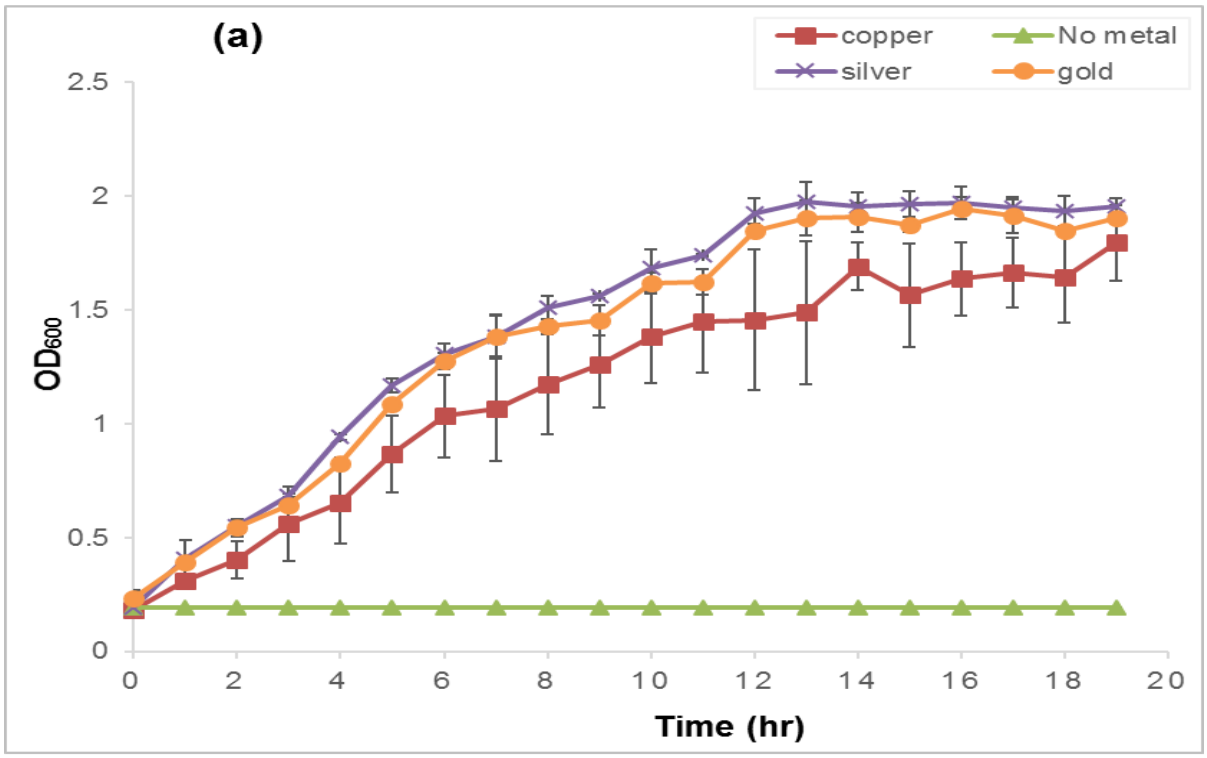


Figure 3.14: β-galactosidase activity of redesigned copper and zinc biosensors with weak RBS in the presence of different metals. The response of **(a)** *E. coli* JM109/pSB1C3_P*copA_lacZ'α* with $CuSO_4.5H_2O$ (*blue diamonds*), $AgNO_3$ (*grey triangles*) and $Au(NO_3)_3$ (*yellow asterisks*), **(b)** E. *coli* JM109/pSB1C3_P*zntA_lacZ'α* with $ZnSO_4.7H_2O$ (*blue diamonds*), $HgCl_2$ (*blue asterisks*), $CdSO_4$ (*grey triangles*) and $Pb(NO_3)_2$ (*yellow asterisks*). Negative control copper and zinc plasmids (*orange square*). **Key:** Cu (plasmid) and Zn (plasmid) lacked *lacZ'α* genes.

3.4.7 Testing growth and function of novel biosensors in ZBM3

Cells were tested for growth and function in the newly formulated sensor medium before conducting metal assays. In this regard, bacterial sensor glycerol stocks from -80^{0}C were streaked on LB agar plates supplemented with calculated amount of appropriate metal, chloramphenicol, IPTG and x-gal stocks and incubated overnight at 37^{0}C. Distinct colonies were inoculated into LB broth supplemented with calculated amount of chloramphenicol in 250 mL conical flasks and incubated for 18 hours with shaking at 37^{0}C. Aliquots of sterile sensor medium, ZBM3 with no bromothymol blue pH colour indicator were dispensed in 96 well plates, inoculated with calculated inoculum volume of overnight cultures and supplemented with calculated amounts of corresponding metal stock solutions. The plates were incubated in Microtitre plate reader at 37^{0}C with shaking for 20 hours and readings taken.

As observed in figure 3.15, novel bacterial sensor cells were able to grow in the newly formulated ZBM3 medium in the presence of all the metals tested in K_2HPO_4 buffer, utilising lactose as the sole carbon source. However, growth did not occur in the absence of metals. For copper sensor, cells were seen to be able to grow in the presence of copper, gold and silver, while there was substantial growth of zinc sensor cells with added zinc, cadmium, lead and mercury. It could be seen that the biosensors react to multiple metals. This was possibly because resistance to toxic lead and cadmium ions, and homeostasis of zinc in *E. coli*, is mediated, at least in part, by the *zntA* gene. This gene which encodes an integral membrane protein of the CPx-type ATPase family is shown to be responsible for the export of Zn(II), Cd(II) and Pb(II) ions from the cell. Similarly, ZntR transcription factor has a weak affinity and can form an active complex with Cd, Pb, and Hg.



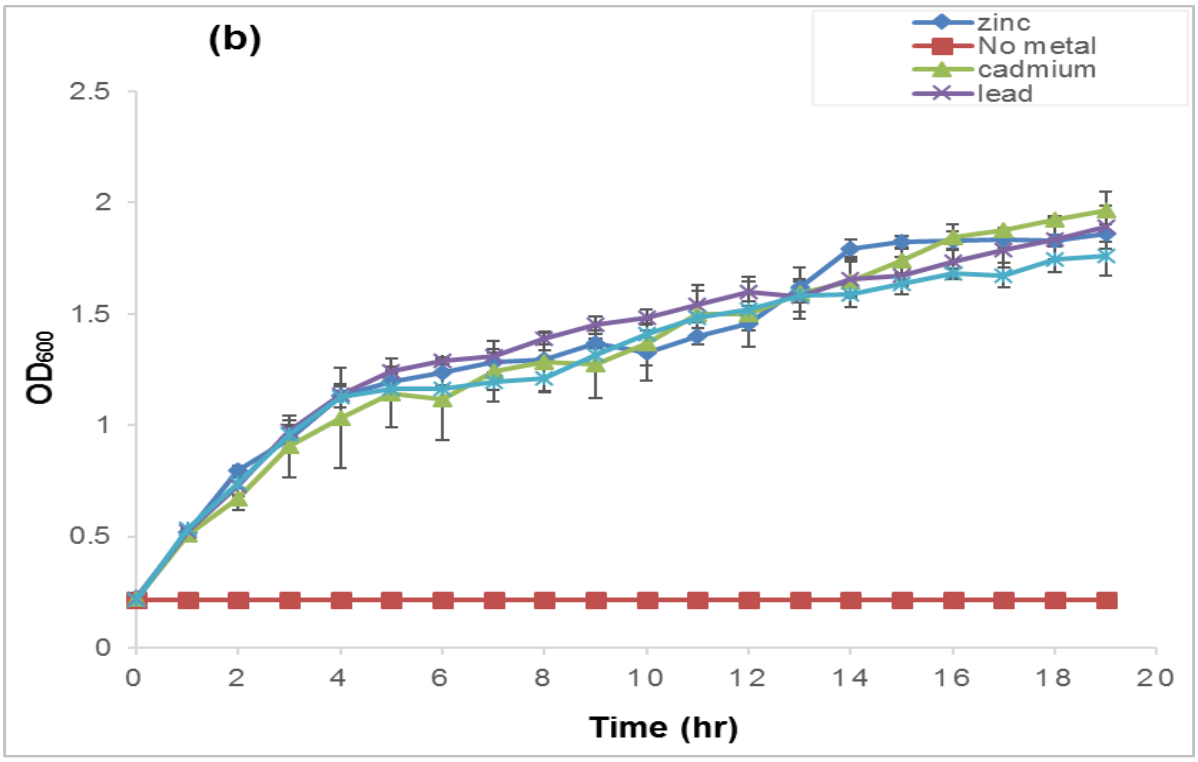


Figure 3.15: Testing growth and function of novel whole-cell (a) copper and (b) zinc biosensors in Zinc biosensor medium (ZBM3) with added 40 µg/mL chloramphenicol. Cell density measured over time for 19 hours, the *error bars* represent the standard error of triplicate tests.

3.4.8 Metal detection assays

Same procedures outlined in section 3.4.5 were followed to prepare cells for metal detection assays. However, assays were conducted in ZBM3 medium (which according to tests conducted best supported the growth and function of the sensors) with bromothymol blue pH colour indicator in either 96 well Microtitre plates, 1.5 mL microcentrifuge tubes, or Bijou bottles by inoculating calculated inoculum volumes in testing apparatus supplemented with corresponding metal stock solutions and incubated at 37^0C with shaking (well plates and Bijou bottles) or statically (microcentrifuge tubes) for 24 hours.

The novel zinc biosensor detected zinc (3 mg/L, approx. 0.046 mM, Figure 3.16a), cadmium (0.003 mg/L, approx. 3 ppb, Figure 3.16c), lead (0.01 mg/L, approx. 10 ppb, Figure 3.16b), and mercury (0.001 mg/L, approx. 1 ppb, Figure 3.16d) concentrations below the limits set by the World Health Organisation (WHO) for the respective metals. This was evident in the colour change of the pH indicator from blue to yellow in such tubes, an indication of contamination by metals. Tubes with green colour as safe levels as described by the manufacturers of the indicator, while cell-free (medium only) and negative control (medium and inoculum) tubes remained blue. The tubes were centrifuged at maximum speed on table top microcentrifuge and disappearance of blue colour of supernatants measured on spectrophotometer at 625 nm, an indication of a drop in pH as shown by the appearance of yellow colour (Figure 3.16e). In cases where readings were above 0.8, samples were diluted 10-folds with ZBM3 medium lacking pH indicator, corresponding metal added, readings taken and multiplied by a factor of 10. Blue tubes showed high absorbance value, while lower values were obtained in tubes that changed to yellow colour, an indication of a drop in pH and presence of metals at levels higher than the safe limits as recommended by the WHO.

No induction was observed when zinc sensor was tested with copper, and vice versa as all tubes remained blue (data not shown).

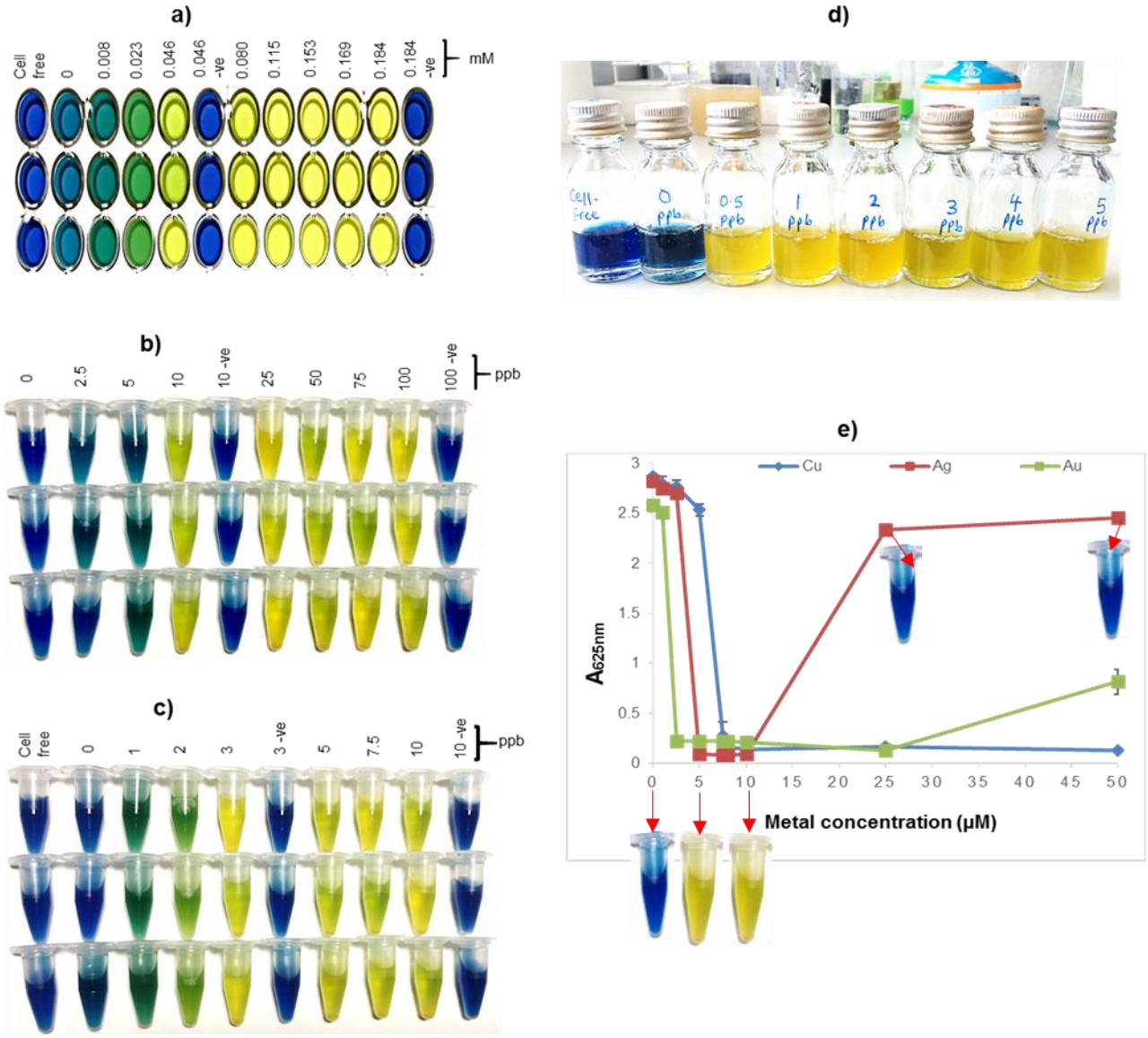


Figure 3.16: Metal detection assay in ZBM3 medium. (**a**) Zinc detection assay in 96 well plate. (**b, c**) Lead and cadmium detection assay respectively in 1.5 mL microcentrifuge tubes. (**d**) Mercury detection assay in Bijou bottles. (**e**) Absorbance measurement of copper, silver and gold detection assays. The *error bars* show the standard error calculated from three replicates. All assays incubated at 37^0C for 24 hours. Assays in 96 well plate and Bijou bottles were done at shaking incubation, while tubes were incubated statically.

3.4.9 The effects of chelating agents on metal bioavailability

Two chelating compounds, EDTA and Na_2S were used to investigate the chromogenic response of *E. coli* JM109/pSB1C3_P*zntA*_*lacZ'α* and *E. coli* JM109/pSB1C3_P*copA*_*lacZ'α* on metal bioavailability both in the presence and absence of metals. This was aimed at assessing the ability of the two biosensors to differentiate between available and immobilised forms of metals. Metal concentrations in the range suitable to ensure the elicitation of toxic responses and a nearly total inhibition of bacterial sensor pH colour change were used as follows: 100 µM $ZnSO_4.7H_2O$; 10 µM $CdCl_2$; 10 µM $Pb(NO_3)_2$; 0.5 µM $HgCl_2$; 100 µM $CuSO_4.5H_2O$; 5 µM $AgNO_3$; and 5 µM $AuCl_3$. Metal/chelate molar ratios in the range of 0 to 2 were prepared by diluting EDTA and Na_2S into each metal solution. Suitable amounts were added into a suspension of 1 ml ZBM3 and 100 µl overnight culture of sensor cells in 1.5 ml microcentrifuge tubes in triplicate and incubated statically at 37^0C overnight for a pH colour change from blue to yellow. The tubes were centrifuged at maximum speed on table top centrifuge for 5 minutes and absorbance of supernatants measured at 625 nm. The addition of EDTA caused a dramatic decrease in chromogenic response in an experiment conducted with $CdCl_2$, $Pb(NO_3)_2$ and $ZnSO_4.7H_2O$ concentrations that caused a colour change in a metal detection assay with *E. coli* JM109/pSB1C3_P*zntA*_*lacZ'α* (5 ppb, 20 ppb and 0.05 Mm, respectively) (not shown). It was observed that EDTA on its own, however, had a strong negative effect on the chromogenic response of zinc sensor, possibly as a result of the chelation of essential trace elements in the sensor medium used. To explain this, the assays were repeated following same procedures but with high, toxic, metal concentrations. Expectedly, the addition of EDTA reduced the metal toxicity to the two bacterial sensor strains, as shown by the drop in absorbance value of supernatants when the tubes turned from

blue to yellow. These were true for all the metals tested, unfortunately, there were deviations in relation with Ag, Cd, Cu and Zn as the EDTA/metal molar ratios increased (Figure 3.17a & b). Higher EDTA/metal ratios, nonetheless, caused a near total shut down of pH colour change in the assay. There was no colour change response in the absence of the addition of metal/chelating agent solution, an indication that the bacterial sensor cells were switched off in such conditions.

Unlike EDTA, it was found that Na_2S did not necessarily reduce chromogenic response of the two bacterial strains (Figure 3.17c & d), except Ag which remained blue after 1 Na2S/metal molar ratio (Figure 3.17d) possibly due to its high solubility in water resulting from its high solubility constant (K_{sp}).

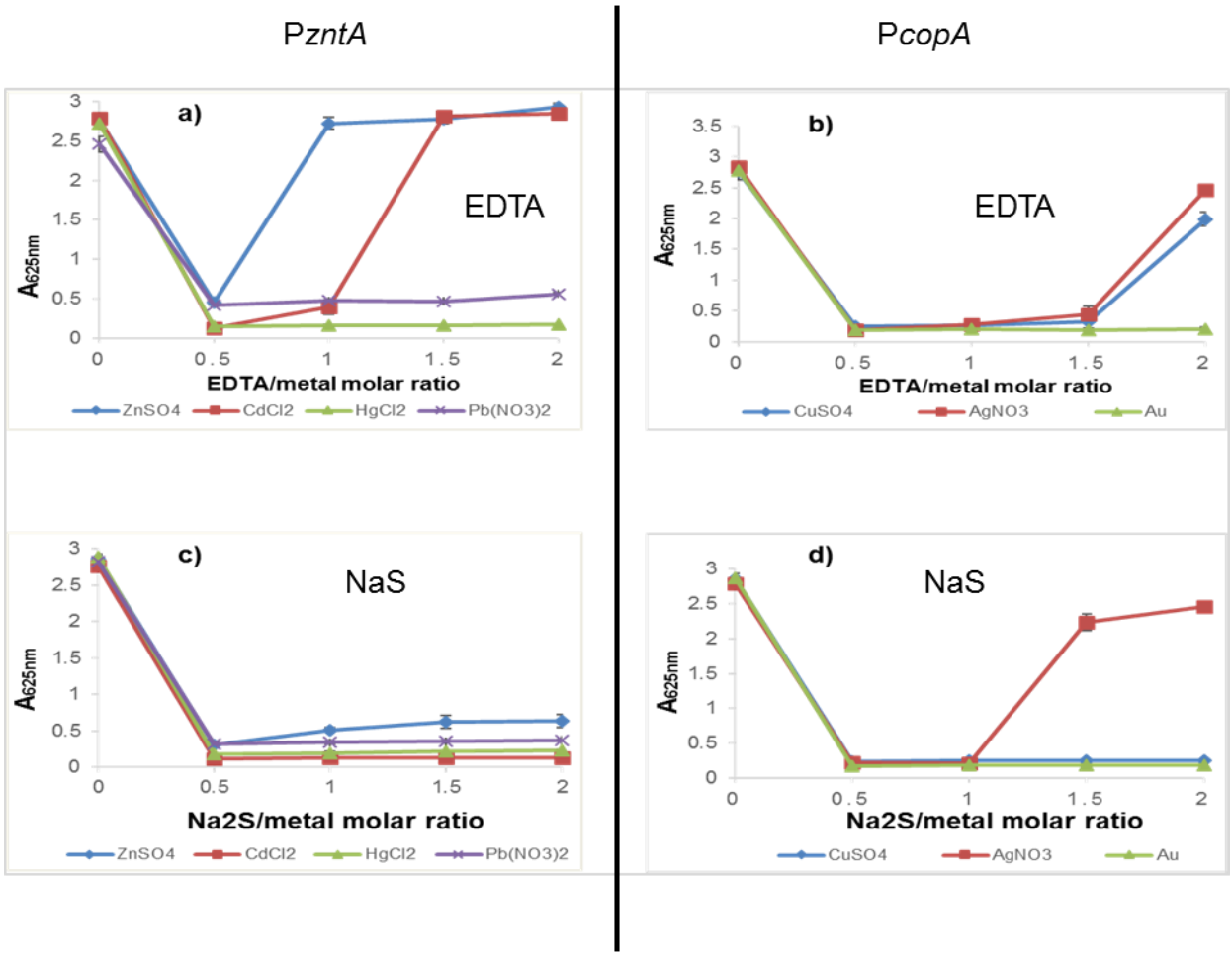


Figure 3.17: Effect of two metal complexing agents on the chromogenic response of *E. coli* JM109/pSB1C3_P*zntA*_*lacZ'α* (**a** & **c**) and *E. coli* JM109/pSB1C3_P*copA*_*lacZ'α* (**b** & **d**). Effect of increasing concentrations of **a** & **b**) EDTA and **c** & **d**) Na_2S on the colorimetric

response of copper biosensor exposed for 24 hours to 100 µM $CuSO_4.5H_2O$ (*blue diamond*), 5 µM $AgNO_3$ (*red square*), 5 µM $AuCl_3$ (*green triangle*) and zinc biosensor exposed to 100 µM $ZnSO_4.7H_2O$ (*blue diamond*), 0.5 µM $HgCl_2$ (*green triangle*), 10 µM $CdCl_2$ (*red square*) and 10 µM $Pb(NO_3)_2$ (*purple asterisks*).

3.5 Discussion

Metals perform basic functions in the life processes of living organisms (Hughes and Poole, 1989; Poole and Gadd, 1989; Ji and Silver, 1995; Bruins *et al*., 1999), but metals are toxic to microorganisms at high concentrations (Hughes and Poole, 1989; Poole and Gadd, 1989). Current metal detections depend generally on physical methods (Kinniburgh and Kosmus, 2002; Rahman *et al*., 2002; Hung *et al*., 2004; Kabir, 2005) most of which are expensive and insufficient in resource limited countries. Bacterial species have evolved several genetically-determined systems to deal with toxic metals (Ji and Silver, 1994; Bruins *et al*, 2000; Lloyd and Lovley, 2001; Silver and Phung, 2005). These living microbial cells can be utilised sensitively and effectively to sense environmental factors in which they live (Tauriainen *et al*., 2000).

Globally, the potential for human exposure to metals beyond the safe limits recommended by regulatory authorities is alarming. The worst hit are the developing countries which lack sufficient facilities for accurate and rapid metal testing. Through transcriptional fusions between a reporter gene and a regulated promoter, bacterial sensors are generally developed which are used for the detection of environmental pollutants and of industrial waste products (Rouch *et al*., 1994). As earlier stated, the principal aim of this study was to develop cheap, simple to use, accurate, sensitive and potable bacterial sensor systems for metal detection. The whole-cell biosensors of interest in this project work by allowing growth on lactose medium with a pH bromothymol blue colour indicator which changes to yellow following fermentation

when metals are present (Aleksic *et al*., 2007; de Mora *et al*., 2011). Using synthetic biology techniques, novel zinc and copper biosensors were developed using promoter elements regulated by endogenous zinc-binding and copper-binding transcription factors, ZntR and CueR respectively, fused to *lacZ'α*, and a suitable test medium, ZBM3, was developed. Data obtained from metal assays conducted with the initial designs of our systems showed false-positive results. Both samples with and without metals changed colour from blue to yellow and the analysis of the systems showed that they had high background activity. To correct these, it was decided to redesign the systems by using either weaker ribosome binding sites (RBS), low copy number plasmids or promoters with lower activity. Based on the analysis of the sensors using Salis Lab Software® to determine the translation initiation rates (T.I.R) of the systems, it was found that copper and zinc biosensor constructs had high translation initiation rates of 19574 and 16263 arbitrary units, respectively. It was decided to design synthetic RBS with lower T.I.Rs (see Table 3.1), which were used together with low copy number plasmids (pSB4C5 and pSB4K5) and promoters with weaker strengths to redesign the systems. The redesigned systems were confirmed through PCR and sequencing, following which Miller assays and subsequently metal assays were conducted. Data obtained showed that only the redesigned systems with weaker RBS was successful with lower values for tubes with lower metal concentrations while others still had higher values (Miller assay), exhibiting background activity. The new systems were also tested for growth and function in the formulated biosensor medium and were observed to growth in the presence of corresponding metals with no growth when metals were not added (Figure 3.15). In zinc biosensor PCN1b (pSB1C3_P*zntA*_*lacZ'α*), the expression of *lacZ'α* was placed under the control of ZntR and the promoter of *zntA* which originated from *E. coli* chromosomal DNA. The

transcription of *lacZ'α* is induced when Zn^{2+} is present and change of bromothymol blue pH indicator from blue to yellow can be observed. Nonetheless, Cd^{2+}, Hg^{2+} and Pb^{2+} co-induced chromogenic response, in addition to Zn^{2+}, in the Zn biosensor system. Hence, the Zn biosensor accurately detected zinc, cadmium, lead and mercury concentrations (3 mg/ L, 0.003 mg/ L, 0.01 mg/ L and 0.001 mg/ L respectively) below the recommended WHO limits. Ivask *et al*., (2002) termed their sensor as "zinc – cadmium sensor", since their investigation corresponded with Binet and Poole (2000) who reported that the *znt* resistance system (ZntR and *zntA* promoter) is also induced with Cd^{2+} and Hg^{2+} in addition to Zn^{2+}. It has also been reported that Zn^{2+} as well as other two-valent ions such as Cd^{2+}, Co^{2+}, Ni^{2+} and Pb^{2+} are exported from the cells by the ZntA protein of *znt* resistance system (Beard *et al*., 1997; Gatti *et al*., 2000). The expression of *zntA* is mediated by ZntR, belonging to the MerR family of transcriptional regulators, which binds the *zntA* promoter, thereby activating transcription when Zn^{2+} is present (Outten *et al*., 1999). Hence, the induction of the zinc system by Hg^{2+}, Cd^{2+} and Pb^{2+} is not too astonishing. It is also important to note that the Zn^{2+}, Cd^{2+}, Pb^{2+} and Hg^{2+}concentrations required to induce pSB1C3_P*zntA*_*lacZ'α* are very different (Figure 3.14b). The peak induction of the system by Pb^{2+}, Cd^{2+} and Hg^{2+} was observed at 1 µM concentrations of each of the metal which drastically reduced when the concentrations of the metals reached 100 µM, indicating metal toxicity of the system at this level. Unlike Pb^{2+}, Cd^{2+} and Hg^{2+}, Zn^{2+} concentration at 100 µM was not toxic to the cell as the induction profile was still increasing. Ivask *et al*., (2002) reported that the interrelationship between the sensitivity and toxicity of metals is not surprising since the 'sensing' elements of the current biosensors are derived from living organisms.

In copper sensor PCN2a (pSB1C3_P*copA*_*lacZ'α*), *lacZ'α* was expressed under the control of CueR and *copA* promoter. Data obtained in the study also showed that in addition to Cu^{2+}, Ag^{2+} and Au^{2+} also induced the *cue* resistance system (CueR and promoter of *copA*). The expression of *copA* is reported to be regulated by CueR, also a member of the MerR family, which interact with Cu^{2+} and the region of copA promoter (Outten *et al*., 2000). Data obtained are in conformity with previous reports that copper, silver and gold ions induce *copA (*Rensing *et al., 2000)*. Similarly, copper, silver and gold levels (2 mg/ L, no guidelines for silver and gold respectively) were detected by the novel copper biosensor, below the limits recommended by the WHO. Data obtained from testing the reaction of both copper and zinc biosensors against each metal ions showed that the novel copper bacterial sensor did not react/or detect zinc metal and vice versa. To date, recognition of metal and transduction of signal by both ZntR and CueR on molecular basis is yet to be clarified. Exploitation of information on the metal binding site of regulators of the MerR family will go a long way in generating reporter strains having new metal specificities important in monitoring environmental metals. Preferably, the concurrent use of bacterial sensors having different response specificities, according to Riether *et al*., (2001), has the potential of allowing each metal in a mixture to be qualitatively detected.

It is reported that chelating agents of natural (humic and fulvic acids) or anthropogenic (EDTA or polyphosphates) sources can alter the concentrations of biologically available metals (Riether *et al*., 2001). Microbial processes can also alter metal solubility, for instance, metal sulphide precipitation and immobilization in sediments are mediated by sulphide produced by sulphate-reducing bacteria. This is widely used in bioremediation procedures (Gadd, 2000). By using two metal ligands, we have shown that our bacterial sensor strains have the potential to differentiate between

bioavailable from total metal in the assay. By reducing availability (and toxicity) and gradual restoration of chromogenic response of our biosensor systems when exposed to different metal concentrations at toxic levels proves that our systems support the hypothesis of complexation of metal ions. Our results are in agreement with Riether *et al*., (2001), Campbell *et al*., (2000) and Tauriainen *et al*., (2000), where luminescent reporter sensors were used to test EDTA effect on either metal toxicity or bioavailability. We also observed that sulphide from Na_2S has effect on metal bioavailability to *E. coli*, although with less effect than EDTA chelation, possibly through the formation of insoluble metal sulphides difficult to penetrate the cell.

Chapter 4: Arsenic problem, detection techniques, and preservation of biosensor cells for storage and distribution

4.1 Abstract

The main sources of human exposure to heavy metals are water and food. Metals play significant roles in the growth and development of living organisms but can be lethal at elevated concentrations due to their interference with normal biological processes. The traditional laboratory-based analytical investigations for environmental contaminants are expensive and usually require trained staff and sophisticated facilities. This study sought to develop sensitive, specific, accurate, rapid, cheap and portable novel disposable biosensor device for the detection or arsenic in drinking water and test its biomedical importance through testing for arsenic in urine samples. A pre-existing arsenic biosensor was developed by Prof Chris French and group by fusing modular DNA components known as BioBricks consisting of the *E. coli* chromosomal *ars* promoter, *arsR* repressor gene and the 5' end of the *lacZ'α gene* in a plasmid vector pSB1A2 to give the final construct designated BBa_J33203 (Aleksic *et al*., 2007). The expression of the *lacZ'α* allows the fermentation of lactose with production of acid, changing the coloured pH indicator bromothymol blue from *blue* to *yellow*. The plasmid DNA was transformed into *Escherichia coli* JM109 and tested in arsenic sensor medium and artificial urine medium spiked with concentrations of sodium arsenate (as arsenic) ranging from 0 to 500 ppb following overnight static incubation. Data obtained showed that the tested arsenic biosensor responded reliably to arsenate concentrations in water and urine samples below the recommended WHO

limit of 10 ppb arsenic. Bacteria are able to sense their environment and this can be utilized in the measurement of the bioavailability and toxicity of environmental pollutants. However, the development of cell-based tools for environmental monitoring require that both the viability and activity of the sensor cells be effectively maintained for a prolonged period of time. In this study, the developed sensor cells were air-dried, freeze-dried and immobilised on paper within a re-sealable system that can then be stored and distributed, hence eliminating the need for routine culture and minimize variation between different batches of cells. Following this method, sensor bacteria were successfully revived after 120 days storage at room temperature and 37^0C. Data obtained showed approximately 0.2% viable cells at the aforementioned conditions after initial inoculations of 1.3×10^8 CFU / mL of cells, an indication of reduced viability as a result of rigorous cell preparations and incomplete drying particularly air dried cells. Although the survival rate was not high, data also obtained showed that such survival rate was enough for the biosensors to still work as the dried cells were still effective in the assay under the conditions tested.

4.2 Background

More than 77 million people globally are estimated to be at risk of drinking water contaminated with arsenic, in the form of arsenite or arsenate ions, which is widespread in South and South East Asia, particularly in West Bengal and Bangladesh (Guha Mazumder *et al*., 1998). Due to limitation in decentralised water infrastructure, developing economies are worst hit by arsenic epidemic as a vast number of the population is exposed to arsenic contaminated drinking water from tube wells (Jakariya *et al*., 2007). At lower concentrations, arsenic has chronic effects but becomes rapidly toxic at elevated levels (~60, 000 ppb) (ATSDR, 2007; French *et al*., 2012). A maximum safe limit of 10 ppb (10 µg/ L) has been set by the World Health Organisation, although 50 ppb (50 µg/ L) is still considered by many regions as a safe goal (de Mora *et al*., 2011). Victims of inorganic arsenic poisoning can exhibit symptoms such as nausea, diarrhoea, vomiting, stomach and intestinal irritation, when they are exposed to concentrations ranging from 300 to 30,000 ppb (ATSDR, 2007). Prominent among clear signs of arsenic poisoning includes skin lesions with feet, torso and hands being the worst hit. If left untreated, these lesions can ultimately lead to skin cancer, although social banishment is the most prominent effect of arsenicosis as most people perceive it to be contagious (de *Mora et al*., 2011; Hasan *et al*., 2005).

Generally, epidemiological studies depend on measurement of arsenic in drinking water as a means of estimating exposure. Using such studies to evaluate the relationship between assess and dose has been disputed as it does not guarantee the accuracy of quantitative measurement of arsenic in water supplies and in the remodelling of exposure (Brown and Chen, 1995; Goessler *et al*., 1997). Excretion of urine is the major route through which arsenic compounds are eliminated from the body, therefore evaluating arsenic in urine is essential for the assessment of exposure

through occupation (Chana and Smith, 1987). However, there are still scepticism about the relationship between arsenic exposure and its clearance in urine as an indicator of exposure to arsenic in drinking water (Calderon *et al*., 1999). Following entry into the body, inorganic arsenic is possibly biomethylated to form the monomethyl and dimethyl species (Braman and Roeback, 1973). It is reported that DMA is the major arsenic metabolite found in urine, although its concentration in each individual varies (Buchet *et al*., 1981). Normally, less than 100 ppb total arsenic concentration is found in urine samples (Buchet and Lauwerys, 1981; Chana and Smith, 1987; UriChem Urine Chemistry Control Data Sheet, 1988). This low level of arsenical urine as well as the spectra of toxic behaviour patterns in each individual make the speciation of arsenic in urine by HPLC - ICP-MS of great interest (Heitkernper *et al*., 1989).

Physical methods particularly the atomic absorption spectroscopy (AAS) is generally carried out as a current arsenic testing procedure in worst hit regions. Although a quantitative and reliable technique, this method has its disadvantages since water samples must be transported to testing laboratories from contaminated areas (Aggett & Aspell, 1976). There are other physical and chemical methods of arsenic testing each with its own merits and demerits (see section 1.2.9).

Biosensors appear to present the only solution to heavy metal pandemic since physical and chemical methods available today are expensive, inadequate and require skilled labour particularly in resource limited countries (Jakariya *et al*., 2007). Several arsenic biosensors have been characterised, but not many are available for field use and/or give rapid pragmatic outputs (Kaur *et al*., 2015). Faced with this predicament, the concept of a biosensor that is simple to use has been developed. Based on Edinburgh arsenic biosensor, it has been demonstrated that the local population of

areas plagued by heavy metal contaminations do not necessarily desire the quantitative responses most sensors are yearning for, rather they prefer sensors so simple to categorically tell them which drinking water sources to ‘’drink’’ or ‘’not to drink’’ (David S. Radford, personal communication). For practical use, a sensor to follow such principles has to additionally meet some of such requirements like; being easy to use, not requiring additional devices or technology, giving a clear feedback at relevant arsenic concentrations, should be cheap, and have sufficient shelf life under local ambient conditions.

Although cell free systems have the potential of producing rapid responses, still they require additional devices for output measurement. It is common knowledge that whole-cell biosensors require some time to produce an output, however, they can be improved in order to optimize the sensitivity, selectivity and robustness. Whole-cell biosensors have the benefits of low cost and improved stability compared to enzymes or other proteins. Tedious purification associated with enzyme biosensors could be avoided and cells can be massively produced through a simple cell culturing step and also the necessary cofactors are already present inside the cells. Moreover, microbes are easy to manipulate and have better stability under harsh environments (Lagarde & Jaffrezic-Renault, 2011; Su *et al*., 2011; Park *et al*., 2013).

Taking this into consideration, this work tries to assess a previously developed arsenic biosensor system in drinking water and urine. For this purpose, assays were conducted based on induction and expression of arsenic-responsive promoter, P_{ars} and generation of output by *lacZ'α* reporter gene. This system has been reported as an effective method for determining this characteristic, being more rapid, robust, cheap, simple, sensitive and specific tests than the current methods such as ICP-MS, ICP-OES, AAS, and chemical test kits based on the Gutzeit method (de Mora *et al*., 2011).

Although some biosensors have been field-tested, little information is available for biosensor detection of toxic chemicals in biological samples, including urine (Roda *et al*., 2001). Through improvement in whole-cell biosensor development, a new class of biosensor has been designed in which growth is used as the output rather than a reporter gene (French *et al*., 2012). Since there is no heterologous reporter gene, regulatory issues can be greatly simplified by making this class and other novel biosensors cisgenic.

The ability to preserve and store bacterial sensors is important to eliminate the need for routine culture, minimize variation between different batches of cells and maintain long-term viability and activity of the sensor bacteria. This is particularly important for resource-limited countries which lack sophisticated equipment and trained manpower, hence may depend on the supply of preserved cells. These demands can be met via various techniques of bacterial preservation such as air drying (Bjerketorp *et al*., 2006; Hays *et al*., 2005); freeze drying (Tang and Pikal, 2004; Jennings, 1999); continuous cultivation (Pooley *et al*., 2004; Lee and Gu, 2005); vacuum drying (Manzanera et al., 2002; Manzanera *et al*., 2004); and immobilisation in biocompatible polymers of organic or inorganic origin (Kim *et al*., 2003; Nielsen *et al*., 2004; Morgan *et al*., 2006).

Water is essential for all life processes and its reactions. However, a good method of preserving live cells in a condition of arrested metabolism is to dehydrate them to the point of total desiccation. The arrested metabolism is restarted after rehydration. Since living organisms including microorganisms, invertebrates and plants generally employ arrested metabolism (anhydrobiosis) to survive harsh periods of drought, such concept can be used to preserve and store whole-cell biosensors for future use. Practically, the accumulation of compatible solutes (e.g. lactose) in true anhydrobionts is generally understood to act as metabolically inert chemical chaperones. Although there are

arguments on the molecular functions of compatible solutes, they are believed to be involved in protecting proteins and lipid membranes from damage induced by desiccation (Crowe *et al*., 2001; Yancey, 2004; Bjerketorp *et al*., 2006).

For decades, desiccation has been the preferred choice for preservation of microorganisms for long term storage. Many industrial applications utilize preserved aliquots of microorganisms for microbiological testing. In this sense, positive control samples of reference microbial strains are used in quality control of this testing (Morgan *et al*., 2006).

Freeze drying or lyophilisation technique in relation to the preservation of microorganisms for long term storage almost always involves cell suspensions that have greater than 10^8 cells/mL. This is as a result of the majority of cells dying when they are stored for a prolonged period. It has been demonstrated that survival of 0.1% of the original cell population is sufficient to enable the continuation of the strain (Bozoglu *et al*., 1987). However, it is argued that comparing literature on the survival of viable cells when high initial cell concentrations are used can be quite difficult as figures can be given a misleading positive misrepresentation when percentage loss of viability is reported on actual arithmetic counts in contrast to log cell counts. This poses a major disadvantage when high initial cell concentrations are used, suggesting that conceivably validation of drying methods should aim at using low cell numbers to provide a clearer understanding of survival rates.

Generally, any drying process should aim to allow long term storage of microorganisms and at the same time preserve cell viability. While several different methods of drying microorganisms exist, there are certain common phases involved in all methods of drying as illustrated in Figure 4.1.

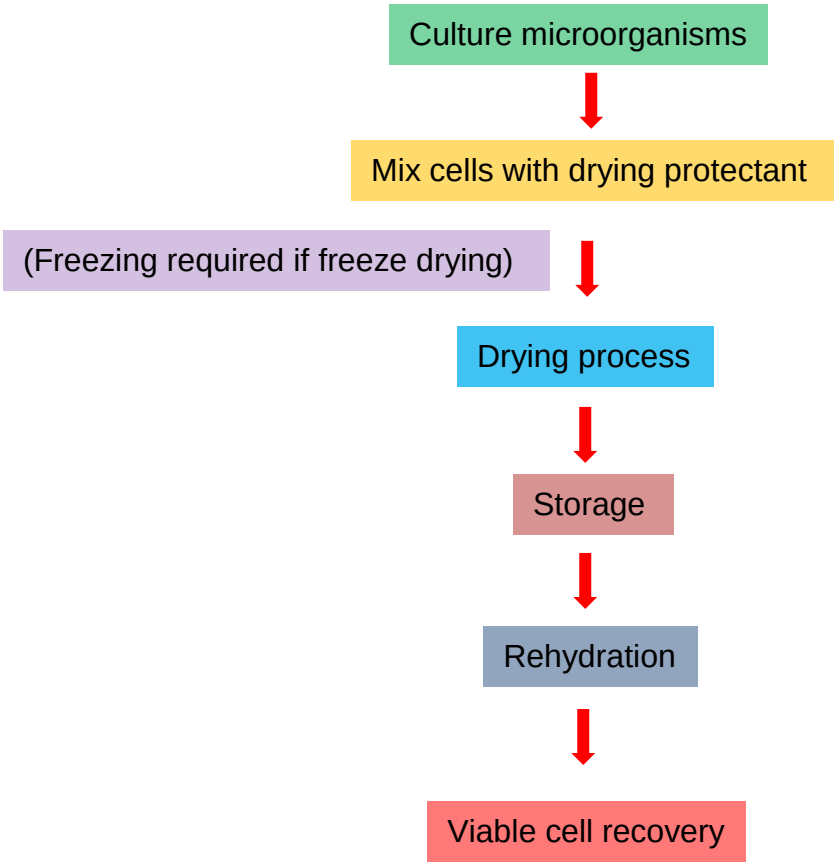


Figure 4.1: General drying process outline (Morgan *et al*., 2006).

Preserving and stabilising the developed biosensor bacteria by drying helps to preserve formulation structure, establish proven industrial performance record and produce easily rehydrated product. It may be costly and require complex techniques particularly in resource limited countries, and the product is sensitive to moisture. However, this method of preservation would allow for the availability and use of developed bacterial sensors in developing economies when produced and distributed to such areas.

4.2.1 Objectives of work presented in this chapter

Most of the work presented in this project is based on the visualisation of colour change of bromothymol blue (a pH colour indicator) from blue to yellow when the

carbon source lactose is fermented through a mixed acid fermentation. Also, the concept of preserving the developed whole-cell biosensors in this project is based on the fact that the needed resources and techniques for biosensor development are limited in developing countries. Consequently, the following aims were defined:

1) To assess the activity of a previously developed arsenic biosensor system in water.
2) To test the biomedical importance of the same arsenic biosensor in clinical samples.
3) To investigate the stability of dried biosensor cells under different storage conditions.
4) To test the activity of dried biosensor cells.

4.3 Methods

4.3.1 Cloning of arsenic sensor organism

A fresh culture of the BioBrick arsenic assay construct, pSB1A2-BBa_J33203 which consists of the parts BBa_J33201 (arsenic-responsive promoter) and BBa_J33202 (*lacZ'α* reporter gene) (Aleksic *et al*., 2007) a kind gift of Prof Chris E. French, streaked on LB agar plates supplemented with appropriate amount of ampicillin antibiotic. Following overnight plate incubation at 37^0C, a single colony of the sensor bacteria was inoculated into 5 mL LB broth in Bijou bottle, supplemented with 5 µg/mL ampicillin and incubated at 37^0C for 16 hours with shaking. Purified plasmid DNA was prepared using QIAprep spin miniprep kit following manufacturer's instruction. The purified plasmid DNA was digested with enzymes according to the restriction sites to allow for the visualisation and confirmation of correct plasmid and insert sizes when ran on

agarose gels at 80 V for 40 minutes. Small aliquots of column purified supercoiled plasmid DNA or 10 µL of ligation reaction (see table 2.18) were transformed into *E. coli* JM109 following protocols as described in section 2.9.2.

4.3.2 β-galactosidase activity of bacterial sensor organisms

The β-galactosidase activity of the arsenic biosensor was performed in ABM6 lacking bromothymol blue pH colour indicator following protocols as illustrated in section 2.9.4.

4.3.3 Arsenic assay testing in ABM6

A single colony of arsenic biosensor cells from freshly prepared plates was inoculated into 50 mL LB broth in 250 mL capacity Erlenmeyer flask and supplemented with 50 µL of ampicillin stock solution for selection, and incubated at 37^0C overnight with shaking. 1 mL of arsenic biosensor medium, ABM6, was added to 1.5 mL microcentrifuge tubes in triplicates and seeded with different inoculum sizes (20, 50 and 100 µL) of overnight cell cultures. The desired concentrations of arsenic (as sodium arsenate) dissolved in water were added to the tubes and incubated statically at 37^0C for 24 hours. The absorbance of each sample was measured at 625 nm after centrifugation.

4.3.4 Measuring of arsenic in laboratory-made urine medium

4.3.4.1 Testing of growth and function of biosensor in artificial urine medium

For arsenic assay in urine to be investigated, the arsenic biosensor was initially tested for growth and function in an artificial urine medium, AUM (Table 2.9). Cell pellets (from 0.5 mL overnight culture in LB broth medium with ampicillin antibiotic) were resuspended in 0.5 mL AUM and inoculated into 50 mL AUM supplemented with 50

µg/mL of ampicillin stock. Cells were incubated at 37^0C with shaking at 200 rpm and cell density at 600 nm measured over time.

4.3.4.2 β-galactosidase activity of bacterial sensor organisms in AUM

The β-galactosidase activity of the arsenic biosensor was performed in AUM without pH colour indicator following protocols as illustrated in section 2.9.4.

4.3.4.3 Arsenic assay testing in AUM

This was conducted following protocols as illustrated in section 4.3.3 but in synthetic urine, AUM.

4.3.5 Cultivation of whole-cell biosensors

Fresh cultures of newly developed whole-cell biosensors were prepared by streaking cells from -80^0C stock onto LB agar plates with appropriate antibiotics and incubated overnight at 37^0C. A single colony from a fresh plate was inoculated into 50 mL sterile LB medium supplemented with appropriate antibiotics in a 250 mL Erlenmeyer flask and incubated at 37^0C with shaking at 200 rpm overnight for 16 – 18 hrs. The overnight cultures were then used to prepare variants of dried cells.

4.3.6 Preparation of air-dried cells

For all constructs, overnight cell cultures (initial volume of 20 to 100 µL per 1.5 ml microcentrifuge tube) were air-dried with open lids overnight at 37^0C from the suspension containing 20% w/v lactose until the cell-lactose mixtures were dried to a glassy consistency. The tubes were tightly capped and stored on laboratory benches at room temperature for days.

4.3.7 Preparation of freeze-dried cells

To prepare freeze-dried cells, cell pellets (centrifuged at 5000 x g for 10 minutes) were resuspended in 20% w/v lactose, dispensed into 1.5 ml microcentrifuge tubes (section 5.3.2) and frozen at -80^{0}C overnight. The cells were then dried with open lids in an Edwards Modulyo freeze dryer for 15 hours, tightly capped and stored at room temperature for days.

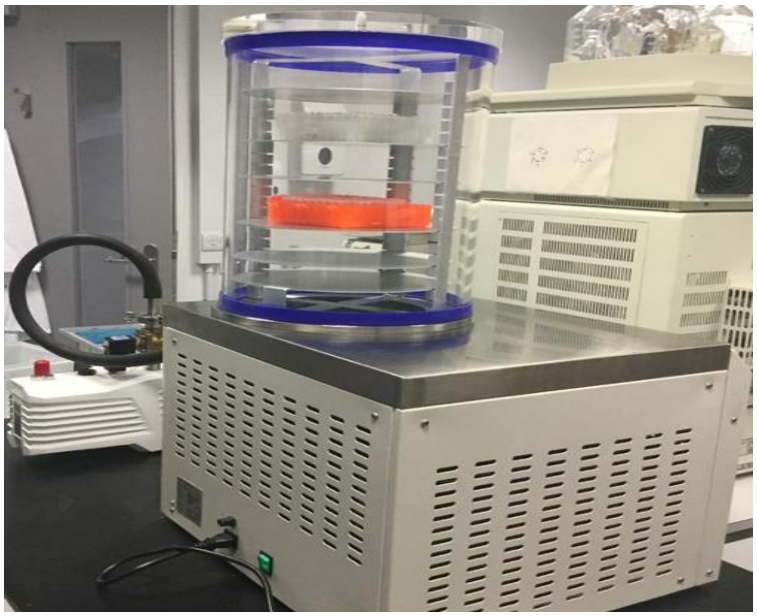


Figure 4.2: An Edwards Modulyo freeze dryer loaded with samples for freeze-drying.

4.3.8 Immobilisation of cells on paper

For paper-based storage of cells, overnight cultures of whole-cell biosensors were mixed with equal volume of 20% sterile lactose solution and 500 µL cell-lactose suspension immobilised on 2 cm x 2 cm sterile Gel Blot paper and dried at 37^{0}C for 5 hours. The immobilised cells were capped in sterile containers and stored at room temperature.

4.3.9 Investigation of survival rates of stored cells

To investigate the viability and durability of stored cells, all dried cells were resuspended in phosphate buffered saline (PBS) and serially diluted in 15 mL capacity disposable centrifuge tubes using sterile distilled water. Several dilutions were plated on LB agar medium supplemented with appropriate antibiotics and incubated at 37^0C overnight for colony count.

4.4 Metal sensing using dried whole-cell biosensors

To conduct metal sensing assay using dried cells, tubes of air- and freeze-dried cells were resuspended with 1 mL of ZBM3 medium in triplicate and supplemented with calculated amount of metal salts from stock solutions. The tubes were incubated statically at 37^0C for 24 hours and observed for a development of colour change from blue to yellow. For paper-based cells, 5 ml of ZBM3 medium was added to dried cells in McCartney bottles and metal salt supplements added. The bottles were incubated at 37^0C for 24 hours with shaking at 200 rpm for colour change from blue to yellow.

4.5 Results

4.5.0 Cloning of arsenic sensor organism

A previously developed arsenic biosensor was assessed to evaluate the correct band sizes of the inserts for onward transformation into *E. coli* JM109. As illustrated in agarose gel performed (Figure 4.3), the expected band sizes of 2.3 kilo base pairs (kbp) and 800 base pairs were observed for the high copy number plasmid pSB1A2,

having the ampicillin resistance marker, and the arsenic responsive promoter having the *lacZ'α* reporter gene respectively, as demonstrated by the designers. This was further confirmed by the sequencing of the plasmid DNA.

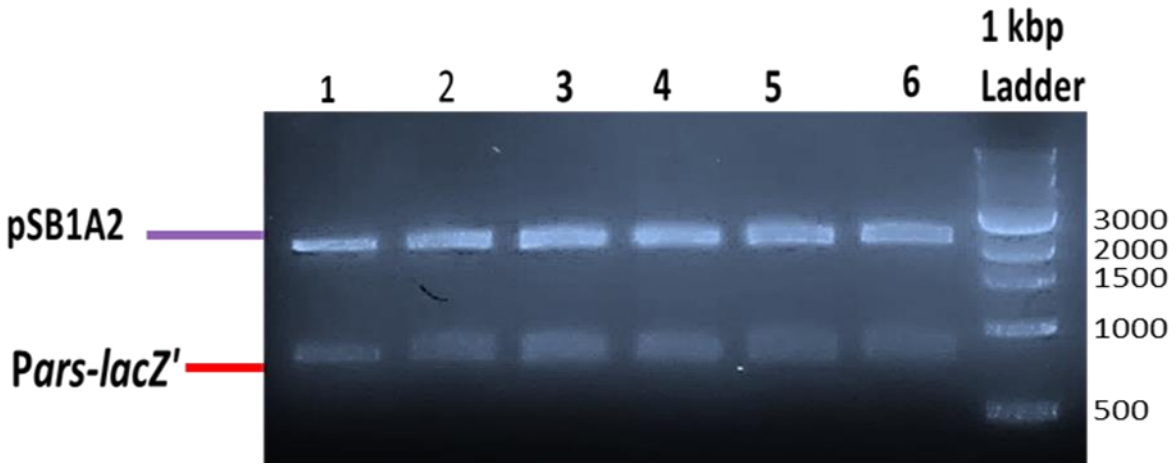


Figure 4.3: Agarose gel electrophoresis of *E. coli* JM109/pSB1A2-P*ars-lacZ'α* digest. Lanes (1-6) showed expected band sizes of ~ 0.8 kbp for the inserts and pSB1A2 (2.3 kbp) after digestion with EcoRI/PstI.

4.5.1 β-galactosidase activity of arsenic biosensor

In *E. coli*, β-galactosidase is encoded by the *lacZ* gene of the lac operon. The enzyme cleaves lactose to glucose and galactose which are used as carbon/energy sources by the cells. The enzyme, β-galactosidase also recognises the synthetic compound o-nitrophenyl-β-D-galactoside (ONPG) as a substrate, which is cleaved to yield galactose and o-nitrophenol which has a yellow colour. The deletions in both genomic and episomal copies of the *lacZ* gene lead to lack of β-galactosidase activity in JM109 (Miller, 1992). The deletion in the episomal (F' factor) copy of the *lacZ* gene (*lacZ*ΔM15) can be complemented by addition of functional α-peptide encoded by a plasmid or phagemid vector. The production of yellow colour can be used to determine enzyme concentration when ONPG is in excess over the enzyme in a reaction, in which case,

the production of o-nitrophenol per unit time is proportional to the concentration of β-galactosidase (Miller, 1992).

Assays were performed following procedures as outlined in section 2.9.4. In the absence of arsenate, ArsR binds to the promoter, hence inactivating it, but ArsR binds to arsenite when present, prompting its dissociation from the promoter and leading to the expression of the reporter gene, *lacZ'α*. An increase in arsenate concentration caused a corresponding increase in the activity of β-galactosidase (Figure 4.4). At 500 ppb, it was observed that high activity of the enzyme was not affected by the high arsenate concentration, hence arsenate was not toxic to cells at that level. In terms of inoculum size, there was no significant difference at lower arsenate levels. However at increased arsenate concentration, the amount of inoculum played a significant role in the activity of β-galactosidase enzymes.

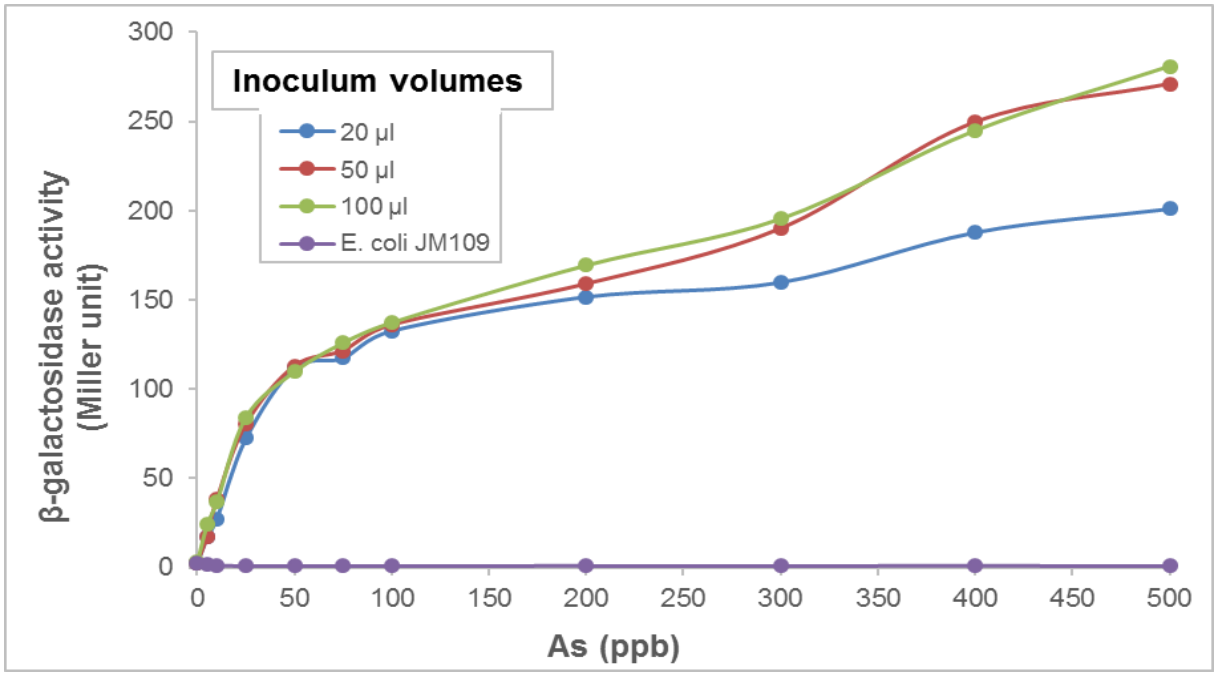


Figure 4.4: β-galactosidase (Miller assay) activity at increasing concentration of arsenic as arsenate. The e*rror* bars, which may be smaller than the symbols, show the standard error of three replicates using different inoculum volumes in 1 mL of ABM6 with no pH colour indicator. *E. coli* JM109, which lacked P_{ars}_*lacZ'α*, was used as control

4.5.2 Arsenic testing in water

Arsenic assay was carried out as described in section 4.3.3 in ABM6 with bromothymol blue as pH indicator using different concentrations of arsenic (as sodium arsenate) dissolved in water. In the first test conducted, there was a pH colour change from blue to yellow in all the tubes including 0 ppb which showed a false positive result (data not shown). This was probably due to the degradation of lactose in ABM6 medium to glucose and galactose during autoclaving.

To clarify this development, 20% *w/v* lactose was prepared, filter-sterilised and added to the medium aseptically to a final concentration of 1% *w/v*. Considering the results of the repeated assay, the arsenic biosensor was highly sensitive with the lowest detection limit (LDL) of 10 ppb as observed in colour change (Figure 4.5) of pH indicator from blue (safe level of arsenic) to yellow (contamination) while the shades of green (Figure 4.5c) are the transition region as specified by the suppliers of bromothymol blue (Sigma).

The 10 ppb tubes in Figure 4.5a could not turn yellow after 24 h, indicating that smaller inoculum sizes needed longer time to produce a quantitative result. Thus, it appears that larger inoculums caused more rapid response, this could be seen as only the 100 µL inoculum volume tubes had the LDL of 5ppb, but at prolonged incubation periods, the amount of initial inoculum made little difference to pH colour change over the range tested (data not shown). The absorbance measurements of the disappearance of blue colour of the pH indicator in each sample were carried out in a Jenway Spectrophotometer 7300 / 7305 at 625 nm after centrifugation. For samples without arsenic or with small concentration to be detected by the sensor, as well as the control experiments, the tubes remained blue which caused the absorbance measurement to be above 0.8. The supernatants from such tubes were diluted 10 fold with sterile

distilled water before measuring the absorbance and the results multiplied by 10 (Figure 4.6).

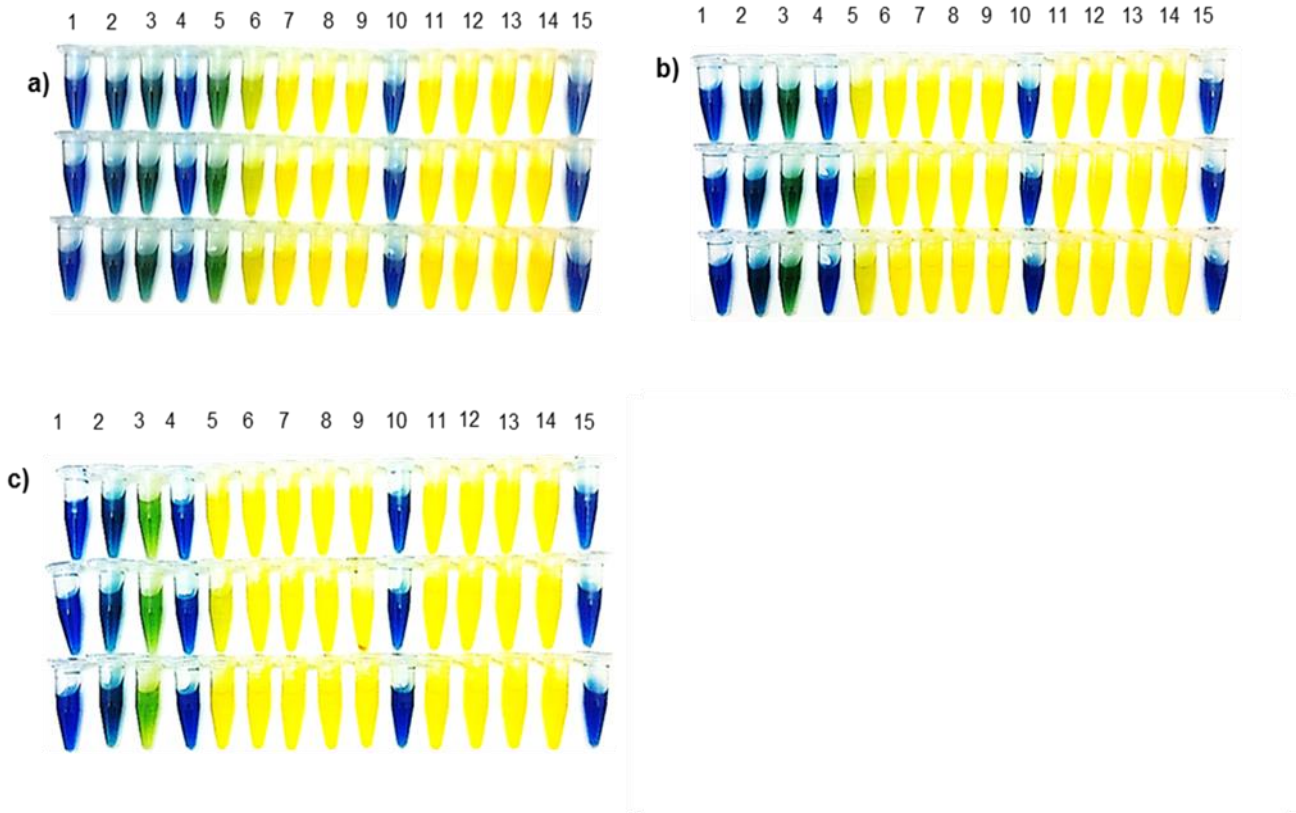


Figure 4.5: Arsenic assay in ABM6 medium at increasing arsenate concentrations using bromothymol blue as a pH indicator. **a)** 20 µL inoculum volume. **b)** 50 µL inoculum volume. **c)** 100 µL inoculum volume. The assay was carried out in triplicate for each arsenic concentration in 1.5 mL microcentrifuge tubes and incubated at 37^0C for 24 hours without shaking. Sample identification: ***1*=** Cell-free control. ***2*=** 0 ppb arsenic. ***3*=** 5 ppb. ***4*=** 5 ppb cell-free control. ***5*=** 10 ppb. ***6*=** 25 ppb. ***7*=** 50 ppb ***8*=** 75 ppb. ***9*=** 100 ppb. ***10*=** 100 ppb cell-free control. ***11*=** 200 ppb. ***12*=** 300 ppb. ***13*=** 400 ppb. ***14*=** 500 ppb. ***15*=** 500 ppb cell-free control.

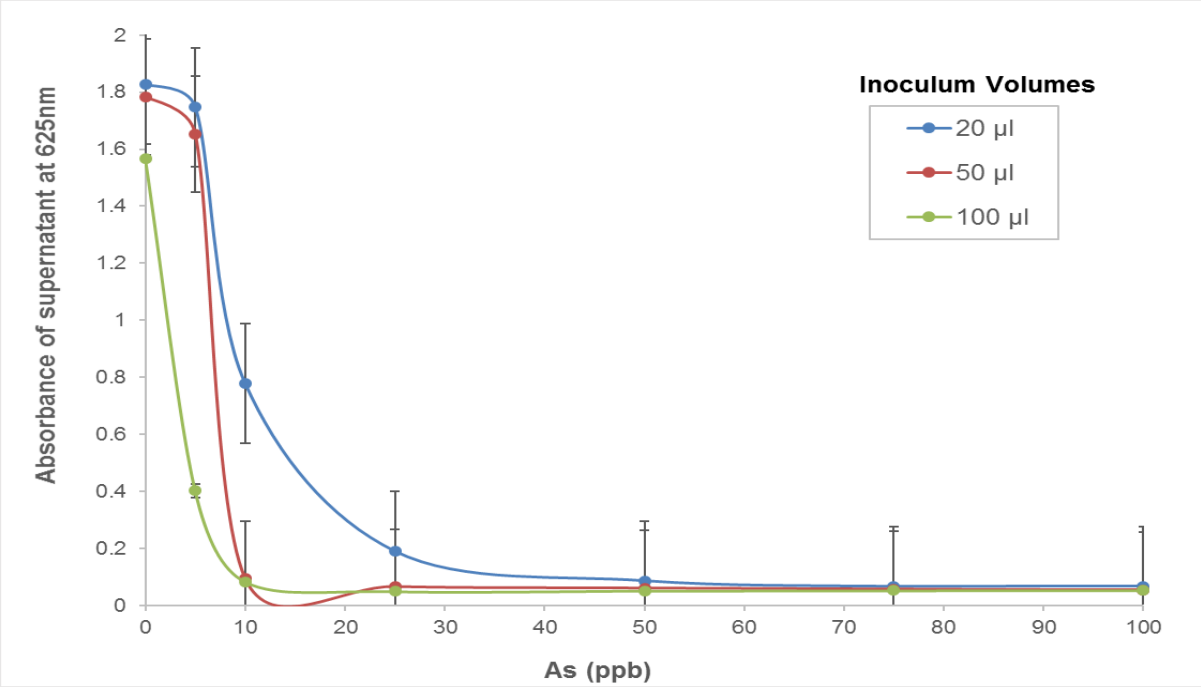


Figure 4.6 Spectrophotometric measurement of blue colour of supernatant at 625 nm. The assay was carried out in triplicate for each arsenic concentration in 1 mL ABM6 in 1.5 mL microcentrifuge tubes using different inoculum volumes and incubated at 37^0C for 24 hours without shaking. Measurement of supernatants taken following centrifugation to pellet the cells. The e*rror* bars indicate the standard error calculated from three replicates. Data >100 ppb were cut in order to expand the figure so that differences in lower arsenic levels could be clearly observed.

4.5.3 Arsenic testing in synthetic urine

4.5.3.1 Testing growth and function of arsenic biosensor in AUM

Arsenic is excreted in urine as a function of exposure to arsenic in drinking water. To develop a biosensor for detecting arsenic in urine samples, the biosensor organism was investigated for growth and function in artificial urine medium (AUM). This broth medium provides conditions similar to that found in human urine, and supports growth at concentrations of up to 10^8 CFU/mL similar to normal urine (Roda *et al*., 2001).

For arsenic assay in urine to be investigated, the arsenic biosensor was initially tested for growth and function in an artificial urine medium, AUM (Table 2.9) following protocols as described in section 4.3.4.1. As observed in Figure 4.7, the arsenic biosensor was capable of growing in artificial urine medium although growth was not vigorous when compared to growth in LB medium. Hence AUM can be used for testing arsenic concentrations in urine samples from clinical environments.

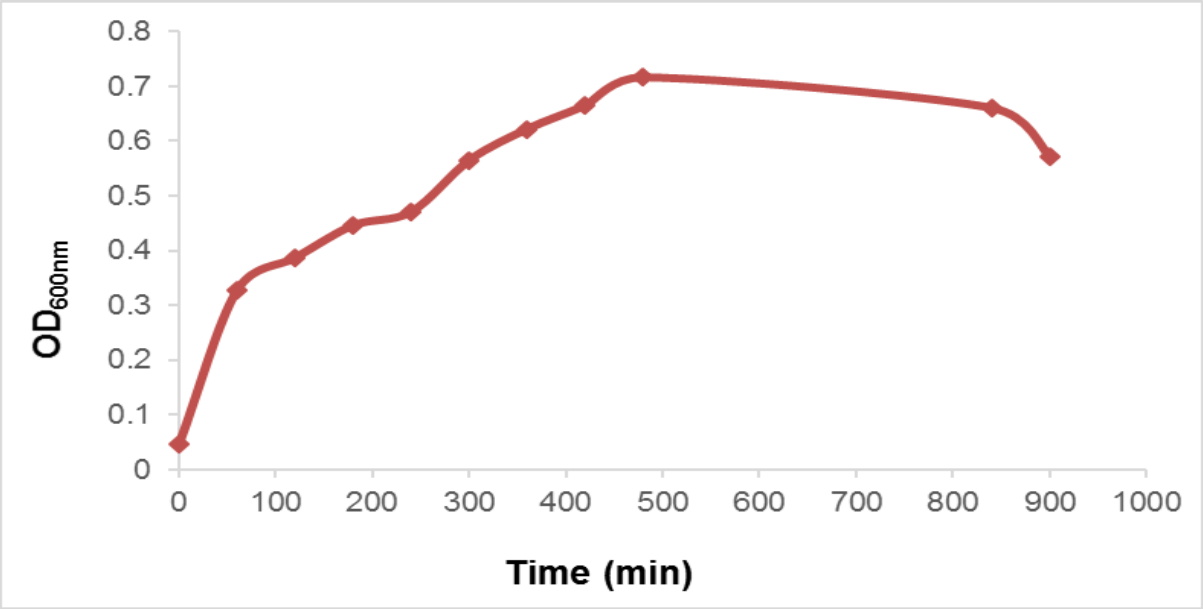


Figure 4.7: Testing growth and function of arsenic biosensor construct in artificial urine medium (AUM).

4.5.3.2 β-galactosidase activity of arsenic biosensor in AUM

The β-galactosidase activity was carried out as described in section 2.9.4. The result obtained showed that there was a significant increase in the activity of β-galactosidase as arsenic concentration increased (Figure 4.8). There was no observable toxic effect on the cells at high arsenic concentration as the activity of β-galactosidase enzymes

was not affected at the highest arsenic level tested. However, it was observed that the inoculum volume has a significant effect on enzyme activity at high level of arsenic.

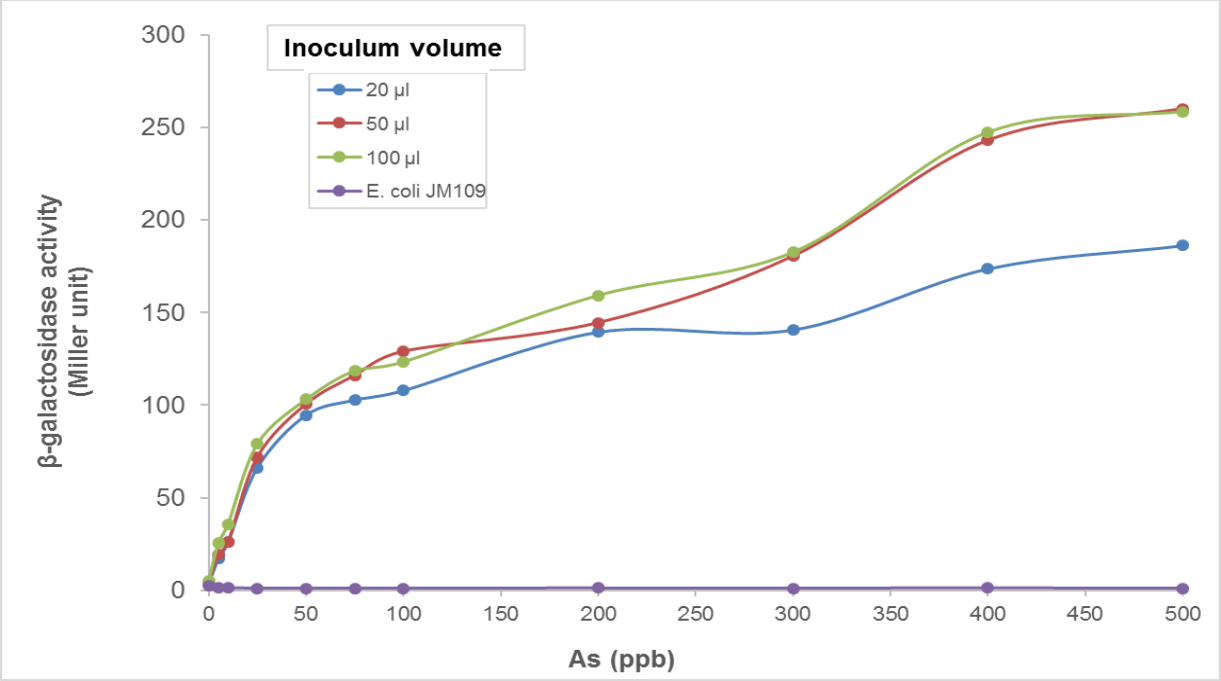


Figure 4.8: β-galactosidase (Miller assay) activity of arsenic biosensor in 1:1 mixture of single strength AUM and double strength ABM6 at increasing concentration of sodium arsenate as arsenic. The *error* bars, which may be smaller than the symbols, show the standard error calculated from three replicates using different inoculum volumes in 1 mL of 11 ABM6 with no pH colour indicator.

4.5.3.3 Arsenic assay in AUM

Development of this assay for use in biomedical samples will require that assays be tested in artificial urine, a broth medium which has been proved to provide conditions similar to that found in human urine. To conduct arsenic assay in urine medium, the same procedure (section 4.3.3) was adopted for the assay, but 1:1 ABM6 and AUM was used (these were initially prepared at 2x concentration of ABM6 and 1x

concentration of AUM, and equal volume of both mixed together). The artificial urine medium was prepared and filter-sterilized to prevent precipitation of components during autoclaving. Visible colour change of the pH indicator from blue to yellow was observed in the tubes contaminated with arsenic with lowest detection limit of 10 ppb (Figure 4.9). In the absence of arsenic, there was no colour change (tubes 2), while the 10 ppb tubes (20 µL inoculum) still required longer incubation periods to give a positive result. Also, the 100 µL inoculum tubes here, when compared to the test conducted in ABM6, had LDL of 10 ppb. Nevertheless, the amount of initial inoculum made little difference to pH colour change over the range tested.

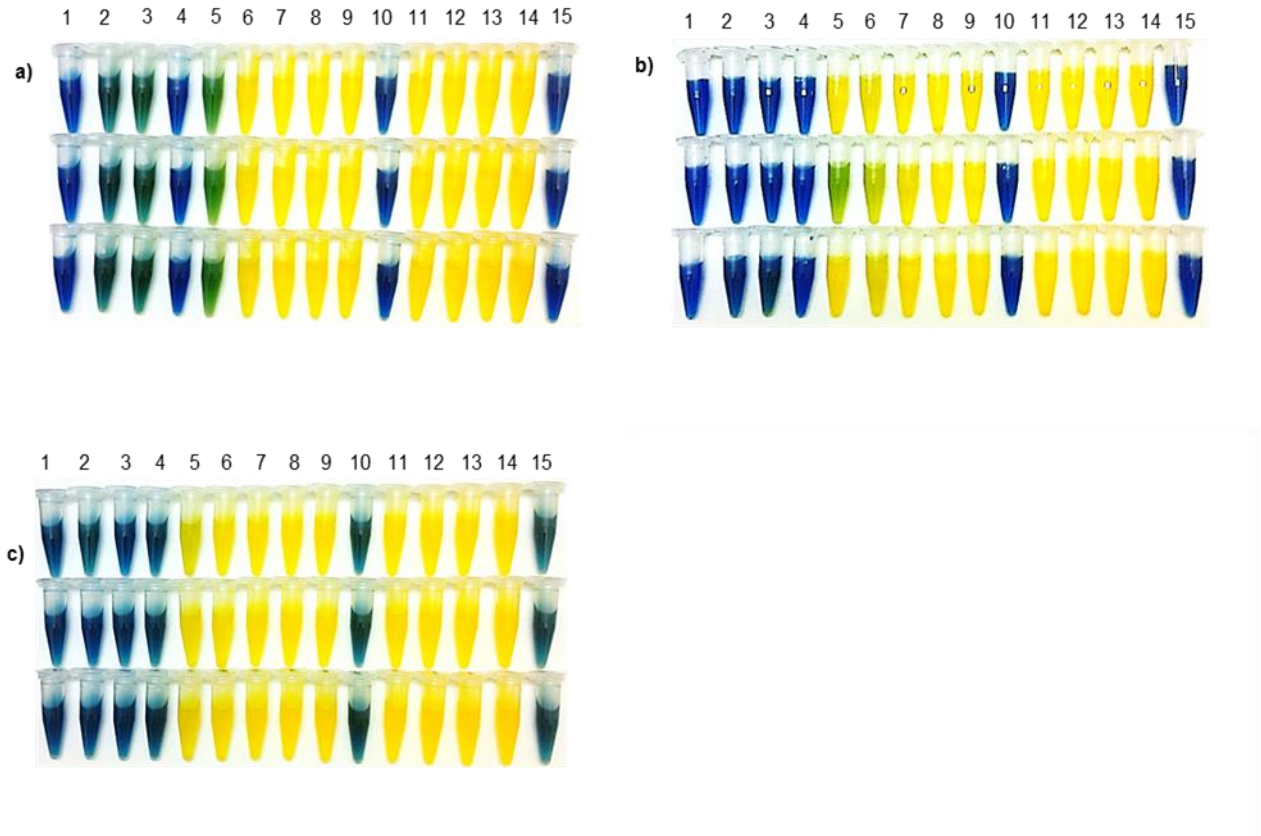


Figure 4.9: Arsenic assay in 1:1 mixture of single strength AUM and double strength ABM6 medium at increasing arsenate concentrations using bromothymol blue as a pH indicator. **a)** 20 µL inoculum volume. **b)** 50 µL inoculum volume. **c)** 100 µL inoculum volume. The assay was carried out in triplicate for each arsenic concentration in 1.5 mL microcentrifuge tubes and

incubated at 37^{0}C for 24 hours without shaking. Sample identification: ***1*****=** Cell-free control. ***2*****=** 0 ppb arsenic. ***3*****=** 5 ppb. ***4*****=** 5 ppb cell-free control. ***5*****=** 10 ppb. ***6*****=** 25 ppb. ***7*****=** 50 ppb ***8*****=** 75 ppb. ***9*****=** 100 ppb. ***10*****=** 100 ppb cell-free control. ***11*****=** 200 ppb. ***12*****=** 300 ppb. ***13*****=** 400 ppb. ***14*****=** 500 ppb. ***15*****=** 500 ppb cell-free control.

As with assay in drinking water, samples without arsenic or with small concentration to be detected by the sensor, as well as the control experiments remained blue and had to be diluted 10 fold using sterile distilled water, when the absorbance measurement was above 0.8, before measuring the final absorbance values and the results multiplied by 10.

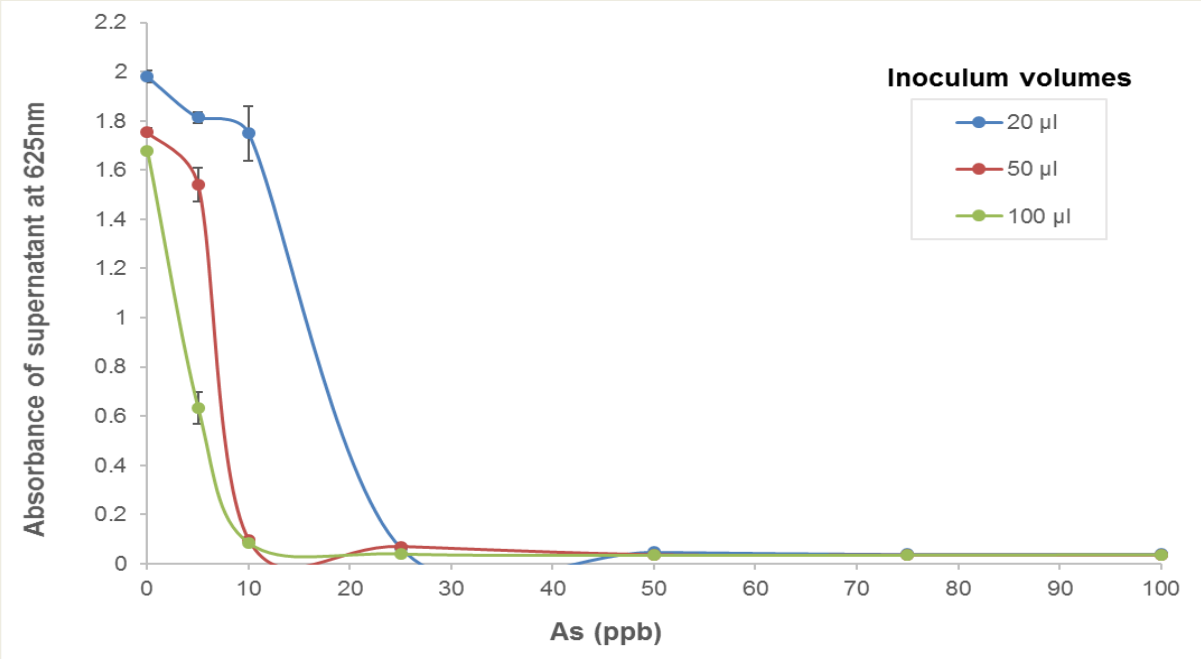


Figure 4.10: Spectrophotometric measurement of blue colour of supernatant at 625 nm. The assay was carried out in triplicate for each arsenic concentration in 1.5 mL microcentrifuge tubes containing 1 mL of 1:1 mixture of single strength AUM and double strength ABM6 medium at increasing arsenate concentrations using bromothymol blue as a pH indicator. Different inoculum volumes were added and tubes incubated at 37^{0}C for 24 hours without shaking. Measurement of supernatants taken following centrifugation to pellet the cells. The *error* bars indicate the standard error calculated from three replicates. Data >100 ppb were cut in order to expand the figure so that differences in lower arsenic levels could be clearly observed.

4.5.4 Dried cell formats

For storage and distribution of bacterial sensors for field use, the cells need to be dried and preserved. This is usually achieved by the presence of compatible solutes, in this case, lactose which has been reported as an excellent cryoprotectant for air- and freeze-drying of cells. For this protocol, lactose is also provided which is needed for the assay. Dried cells were prepared as described in section 5.3. The survival of the dried and stored cells from initial volumes of 20, 50 or 100 µL of cell suspension is shown in Figures 4.11 and 4.12. For air-dried cells, there was more rapid die-off of cells in the tubes containing 100 µL of cells which originally showed higher survival. This may possibly be due to higher moisture content as a result of insufficient drying. For freeze-dried cells, there was a sharp decrease in the mass of dried cells in the tubes containing 20 µL cells which may be caused by over-drying of cells in the dryer and ultimately led to sharp decline in cell viability (Figure 4.12).

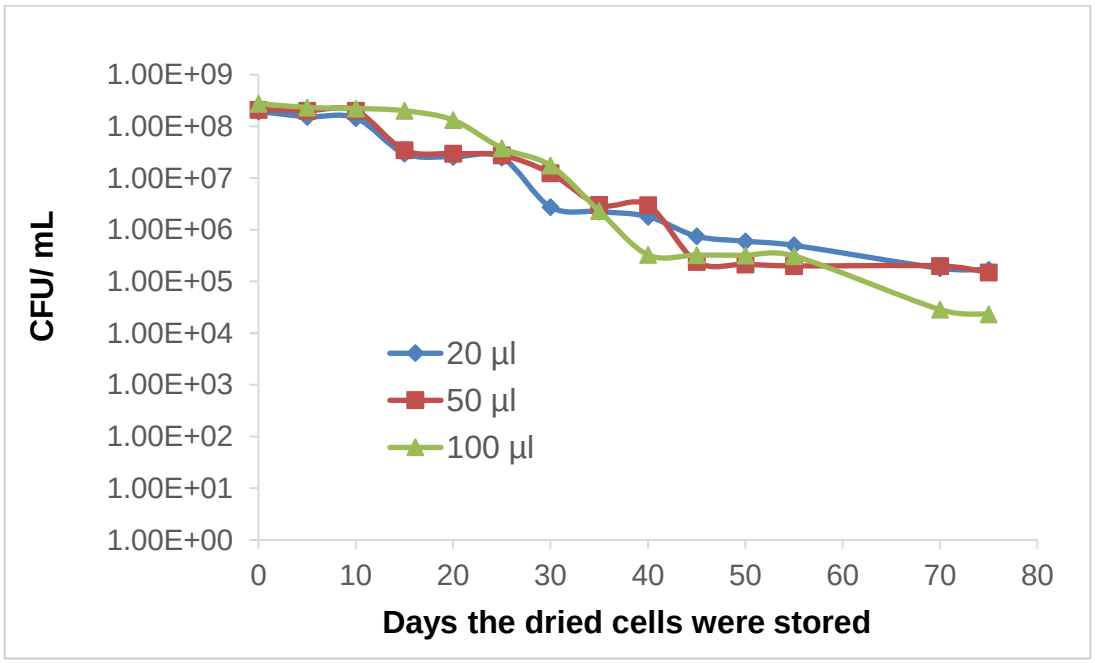


Figure 4.11: Survival of air-dried arsenic biosensor cells from initial volumes of 20, 50 or 100 µL of cell suspension. Tubes containing 100 µL of cells initially demonstrated higher rate of

survival but later showed rapid die-off possibly due to less effective drying and higher moisture content when sealed.

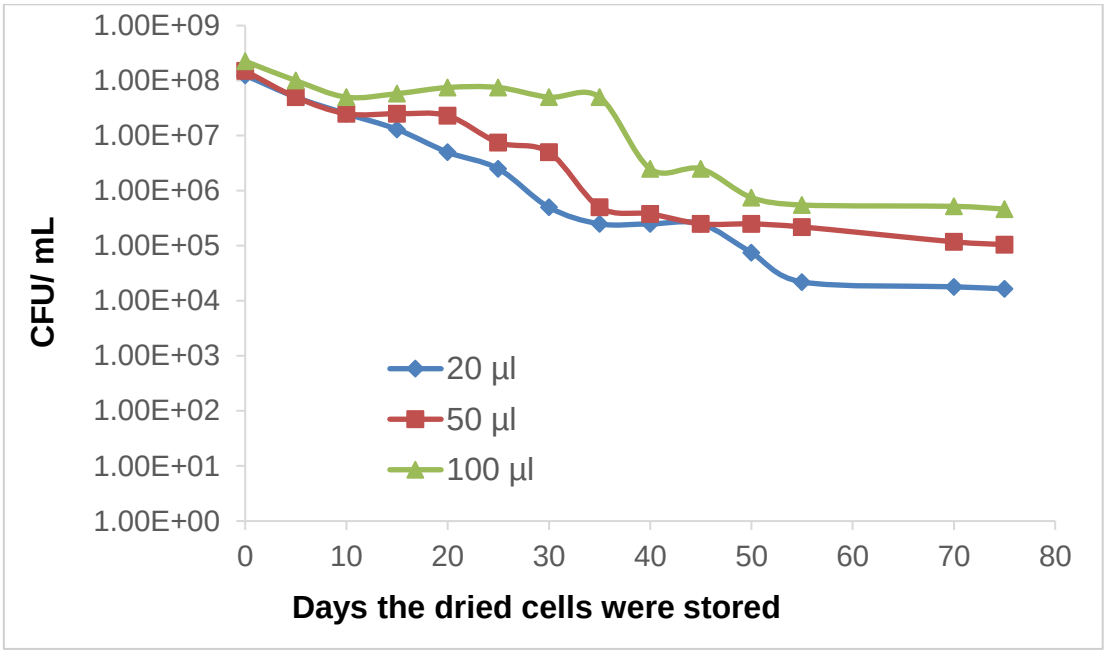


Figure 4.12: Survival of freeze-dried arsenic biosensor cells from initial volumes of 20, 50 or 100 µl of cell suspension.

For the assays conducted with both air- and freeze-dried cells, the tubes containing 50 and 100 µL cell-lactose mixture detected arsenate up to 10 ppb lowest detection limit as demonstrated by pH colour change of the bromothymol blue indicator from blue to yellow after 120 days storage (Figure 4.13). The 10 ppb sample of the 20 µL tubes did not change colour after 24 hours, but did at prolonged incubation periods, indicating that the amount of initial inoculum made little difference to pH colour change over the range tested. There was no change in colour in the tubes without arsenic. Similar results were obtained in assays conducted in artificial urine medium (data not shown). Using freshly-grown cells as controls, it could be seen that with 100 µL inoculum volume, both dried and freshly-grown cells detected arsenic at 10 ppb.

Conversely, there was remarkable difference between dried and freshly-grown cells when 20 µL inoculum volume was used as dried cells could detect arsenic at 20 ppb under similar conditions tested. Hence, the need to put volumes of inoculum into consideration when such assays are conducted.

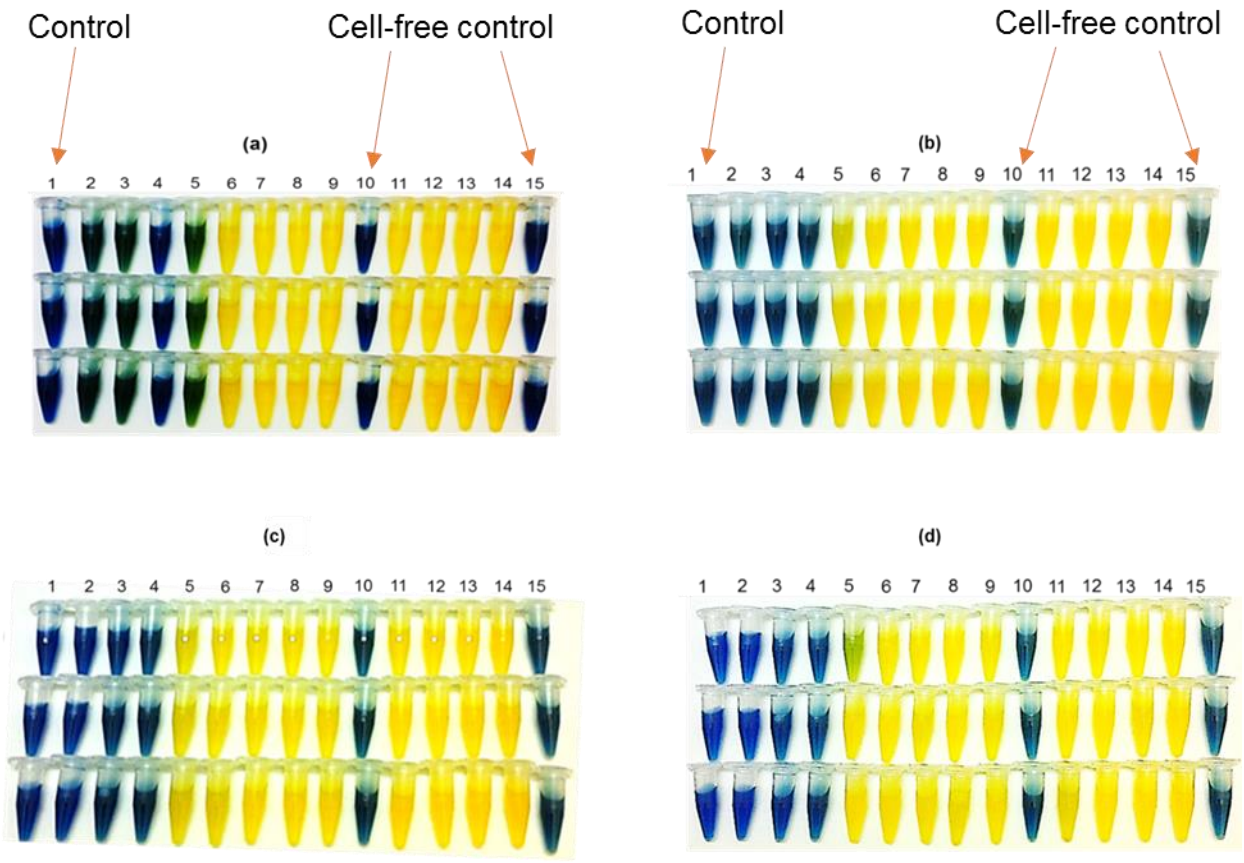


Figure 4.13: Arsenic assay using dried cells stored for 120 days. Assay was conducted in ABM6 medium at increasing arsenate concentrations using bromothymol blue as a pH indicator. **a)** 20 µL inoculum volume. **b)** 100 µL inoculum volume. (c) & (d) with inoculum volumes of 20 and 100 µL respectively, were conducted using freshly-grown cells as controls. The assay was carried out in triplicate for each arsenic concentration in 1.5 mL microcentrifuge tubes and incubated at 37^{0}C for 24 hours without shaking. Sample identification: ***1*=** Cell-free control. ***2*=** 0 ppb arsenic. ***3*=** 5 ppb. ***4*=** 5 ppb cell-free control. ***5*=** 10 ppb. ***6*=** 25 ppb. ***7*=** 50 ppb ***8*=** 75 ppb. ***9*=** 100 ppb. ***10*=** 100 ppb cell-free control. ***11*=** 200 ppb. ***12*=** 300 ppb. ***13*=** 400 ppb. ***14*=** 500 ppb. ***15*=** 500 ppb cell-free control.

Results obtained showed that regardless of reduction in cell viability, air- and freeze-dried arsenic biosensor cells showed accurate response to arsenate for at least 120 days when tested after storage. This was the same for both zinc (Figure 4.14) and copper (data not shown) biosensor cells stored at room temperature and tested with their respective metal stock solutions.

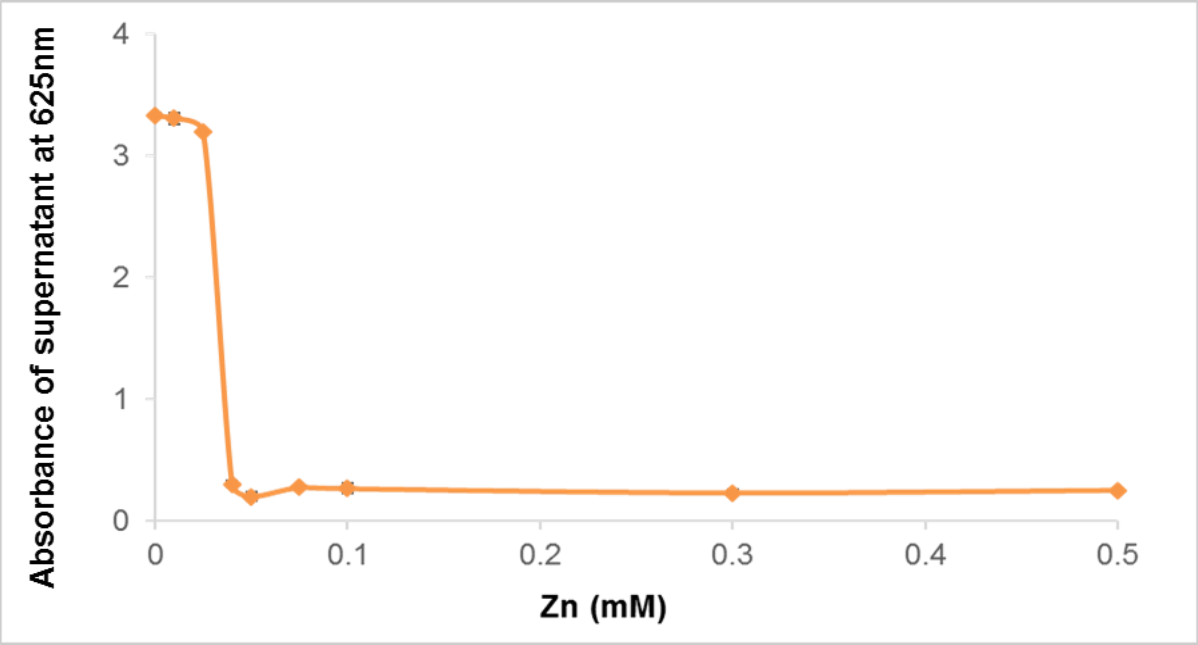
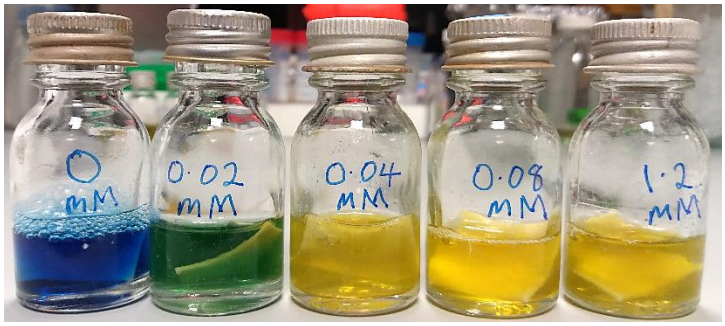


Figure 4.14: Zinc assay using dried cells after 120 days storage at room temperature. Despite reduced viability, dried cells are still effective in the assay. *Error bar* represents standard error of assay performed in triplicate.

Metal detection assays were also conducted using cells immobilised on Gel Blot paper and stored at room temperature for few weeks. Data obtained showed that the dried cells were still efficient in detecting the corresponding metals tested (Figure 4.15).

(a) As



(b) Zn

(c) Hg

Figure 4.15: Arsenic assay **(a)** zinc assay **(b)** and mercury assay **(c)** using whole-cell arsenic and zinc biosensor cells dried for 15 hrs on 2 cm x 2 cm Gel Blot paper and stored at room temperature for 5 days. Assay conducted in ABM6 (a) and ZBM3 (b & c) with shaking incubation overnight at 37^0C. Assay results showed blue (no metal), *green* (safe level) and yellow (heavy metal contamination) colours.

4.6 Discussion

Generally, whole-cell bacterial sensors for heavy metal detection are constructed by the fusion of a promoter, responsive to a specific heavy metal ion, with a reporter gene incorporated in a host bacterium. The presence of the heavy metal ion induces the synthesis of the reporter protein resulting in the emitting of a detectable signal which is proportional to the ion concentration (Daunert *et al*., 2000; Belkin, 2003; Castillo *et al*., 2004). Several biosensor systems have been reported for arsenate and arsenite detection (Stocker *et al*., 2003; French *et al*., 2012). These arose from the need for an ideal biosensor that is cheap, easy to use, sensitive and accurate assay for rapid monitoring of arsenic in environmental samples. Such biosensor should also not demand costly equipment and should be easily reproduced (de Mora *et al*., 2011; Chen and Rosen, 2014).

Arsenic assays were conducted in drinking water and urine using a pre-existing arsenic biosensor. This growth-based biosensor supports growth on lactose medium by fermenting lactose with production of acid, changing the coloured pH indicator bromothymol blue from blue to yellow (de Mora *et al*., 2011). Data obtained showed that lactose is unstable when sterilised by autoclaving, hence the need for lactose to be filter-sterilised and added separately. In arsenic assays conducted, our results showed that the biosensor responded reliably to arsenate concentrations in water below the recommended World Health Organisation limit of 10 ppb arsenic. Similarly, data obtained indicated that higher arsenate concentrations of up to 500 ppb were not toxic to the cells as viable counts were not affected up to that level. Miller (1992) reported that β-galactosidase concentration is proportional to the production of o-nitrophenol per unit time, the yellow colour produced when β-galactosidase enzyme

cleaves o-nitrophenyl-β-D-galactoside (ONPG) substrate. An increase in the concentration of arsenate resulted in excess yellow colour production due to the high activity of β-galactosidase enzymes caused by high inoculum volumes. At 500 ppb arsenate, it was observed that high activity of the enzyme was not affected by the high arsenate concentration, hence arsenate was not toxic to cells at that level. In terms of inoculum size, there was no significant difference at lower arsenate levels. However, at increased arsenate concentration, the amount of inoculum played a significant role in the activity of β-galactosidase enzymes as high inoculum volume produced high number of enzymes which corresponded to the high amount of O-nitrophenol production (as seen by denser yellow colour) and hence higher absorbance value. It was also demonstrated that the arsenic biosensor organism was capable of active growth in artificial urine medium. This broth medium has been reported to provide conditions similar to that found in human urine, and supports growth at concentrations of up to 10^8 CFU/mL similar to normal urine (Roda *et al*., 2001). Arsenic is excreted in urine as a function of exposure to arsenic in drinking water (Chana and Smith, 1987; Fricke, 1989; Morton and Leese, 2011). Sensor results obtained in artificial urine medium are the same compared to arsenic sensor medium, ABM6. This proves that the bacterial sensor can reliably measure arsenic in urine samples.

For decades, different drying methodologies have been employed in the preservation of microorganisms. Extensive research efforts have been made to merge these bacterial preservation strategies with the construction of sensor cell collections on platforms such as biochips or optic fibres. It is with great hope that these will lead to practical miniaturised whole-cell biosensor systems (Bjerketorp *et al*., 2006; Morgan *et al*., 2006). In terms of storage and distribution, it has been demonstrated that adding lactose as a cryoprotectant to cells prior to air- and freeze-drying can aid the survival

of biosensor cells tested. Air-dried cells showed to remain very stable than the freeze-dried cells for the first 10 days of storage. This can be better explained to be as a result of the formation of glassy consistency in air-dried tubes, resulting in a tight packing and a firm top layer which may provide better protection against damage cause by oxygen. However, the tubes of air-dried cells containing 100 µL of cell-lactose suspension originally showed higher survival but there was more rapid die-off. This may be possibly due to higher moisture content in air-dried tubes after drying due to insufficient drying.

It has also been demonstrated that whole-cell biosensor cells can be preserved on Gel Blot paper. Despite reduced viability in all the dried formats following several weeks of storage at room temperature, dried cells are still effective in the assays conducted. Although these have not been tested on the field, tests conducted with seeded positive metal stock solutions demonstrated successful detection of arsenic, cadmium, copper, lead, mercury, and zinc.

Chapter 5: Coliforms and the need for cheaper detection methodologies

5.1 Abstract

Drinking water available to the growing world population is constantly contaminated by natural and anthropogenic sources which pose serious threat to human lives. The aim of this study is to develop a system that can be easily adapted to incorporate a coliform assay into biosensor devices to allow simultaneous monitoring of microbial and chemical quality of water. We tested seeded positive samples, bottle water and surface water samples from a variety of sources within Edinburgh, using novel biosensor ZBM3 medium, thin film agar and a semi-quantitative most probable number (MPN) ZBM3 test. Coliforms were readily detected based on lactose fermentation using a variant of the same growth medium used for biosensor organisms to allow for easy generation of a combined coliform/metal sensor device. Following a 24 hour incubation, ZBM3 test had both sensitivity and positive predictive value using water samples with *Escherichia coli* levels below 100 colony forming units (CFU) per 100 ml. Similarly, when incubated for less than 24 hours, heavy metals such as arsenic (10 ppb), copper (2 mg/l) and zinc (3 mg/l) were detected in thin film sensor agar medium below the safe limits recommended by the World Health Organisation. Hence the test can be used for faecal contamination in drinking water in the field where laboratory facilities are inadequate.

5.2 Background

Water is a fundamental resource for the sustenance of life, and it has to be available in a safer form to safeguard public health (Atherholt *et al*., 1998). Because the availability of safe drinking water is still of a global concern, authorities maintain considerable efforts concerning water quantity and water quality assurance. Microbiological hazards are generally involved in the deterioration of water quality as microbial contamination is implicated in the majority of apparent water-related health problems (Zamxaka *et al*., 2004).

Drinking water quality is tested for *Escherichia coli* as an indicator of faecal contamination, using a standard membrane filtration protocol (World Health Organisation, 2004). Skilled personnel, supporting materials and facilities in a microbiology laboratory are requirements for the standardised procedures available for the detection of faecal contamination of drinking water. In most resource limited economies, the water quality surveillance is inadequate for regular testing of drinking water resource (Hirulkar and Tambekar, 2006). Samples need to be collected aseptically and carefully transported on ice to laboratories that are well equipped. This process is time-consuming, expensive and must be performed by trained microbiologists (Gupta *et al*., 2007). These drawbacks have made this process impractical for regular field use, particularly in environments that are inaccessible due to some factors like floods. Qualitative or semi-quantitative methods use ultraviolet fluorescence for *E. coli* detection as an alternative to standard membrane filtration. These methods are still costly and incubation at a set temperature for at least 24 hours is still required.

Consequently, a reliable, cheap and portable field test is needed for effective monitoring of drinking water and water resource by users themselves (Hirulkar and Tambekar, 2006). The H_2S test, which is based on hydrogen sulphide production by bacteria that are implicated in faecal contamination, has been reported to be more sensitive, cheaper, and simple to use (Manja *et al*., 1982; Gupta *et al*., 2007). However, it needs various modifications in medium composition, incubation period and temperature in different tropic and temperate regions (Castillo *et al*., 1994; Genthe *et al*., 1999; Hirulkar and Tambekar, 2006). Also, the H_2S test is time consuming as 18 to 48 hour incubation is generally required for positive results (Pillai *et al*., 1999).

The whole-cell biosensors of interest in this project work by allowing growth on lactose medium (Aleksic *et al*., 2007; de Mora *et al*., 2011). We seek to develop a system that can be easily adapted to additionally detect the growth of other bacteria, such as coliforms, so that the same format of sensor detects both toxic metals and biological contaminants in a single unit. Our system is also cheap, simple and sensitive, and can work effectively in the ambient temperature in different tropic and temperate regions. The system is also economical as both coliforms and heavy metals can be detected simultaneously in a single system. Similarly, biosensor lyophilisates can be distributed enabling rapid testing and also removing the need for continuous cell cultivation which can introduce variation in the system.

5.2.1 Objectives of work presented in this chapter

The key features of the work presented in chapter 5 were:

1) To design an approach adapted to additionally detect the growth of other bacteria, such as coliforms.

2) To quantitatively compare the novel approach with modern coliform detection techniques.

3) To evaluate the activity of lyophilised media and whole-cells in a single format.

5.3 Methods

5.3.1 Cultivation and harvesting of bacteria

To test for growth and function of coliforms in ZBM3 sensor medium, distinct colonies of freshly prepared wild type *E. coli* MG1655 and non-coliform *Salmonella enterica* serovar Typhimurium LT2, which acts as a negative coliform control, were inoculated into 50 mL LB broth in 250 mL conical flasks and incubated for 18 hours with shaking at 37^0C. Cell pellets of the overnight cultures were washed in PBS and resuspended in 50 mL ZBM3. Thereafter, 0.025 mL of cell-medium suspension was inoculated into 0.25 mL ZBM3 medium without pH colour indicator in 48 well Microtitre plates. The plates were incubated in BMG Labtech - SPECTROstar Nano Microtitre plate reader at 37^0C with shaking for 20 hours and optical density readings taken.

5.3.2 Coliform assay in zinc biosensor medium 3 (ZBM3)

Assay media were prepared by dispensing 2.5 ml each of bile salt solution (at increasing concentrations) and double strength ZBM3 medium with/without bromothymol blue pH indicator into a set of sterile McCartney bottles. Similarly, 5 ml of ZBM3 medium with/without pH colour indicator, supplemented with various concentrations of Teepol, were dispensed into another set of sterile bottles. Inoculum volume of 50 µl (from 10^{-5} dilution) each of overnight cultures of *E. coli* MG1655 and *Salmonella enterica* serovar Typhimurium LT2 was inoculated into the prepared media

and incubated overnight at 37^0C with shaking. Following centrifugation of 1 mL of each sample in a table top microcentrifuge at maximum speed, absorbance of supernatants (for those cultured in ZBM3 with pH colour indicator) was measured at 625 nm to determine the disappearance of blue colour of the pH indicator as a result of fermentation by coliforms when present, while cell density (for those without colour indicator) was measured at 600 nm in a spectrophotometer.

For assays without colour indicator, colony count was performed by serial dilution of 1 ml of overnight culture and plating on LB agar. For the assay with colour indicator, 1 ml of culture was centrifuged at 5000 x g for 10 minutes, cell pellets washed in 1 ml PSB, centrifuged again and resuspended in 1 ml PSB. The cells were serially diluted, inoculated on LB agar plates and incubated at 37^0C overnight. This was necessary to measure the possible bactericidal effect of Teepol and Bile salt, which preferentially inhibit the growth of non-coliforms, on coliforms.

5.3.3 Coliform detection assay on thin film ZBM3 agar

To prepare thin film agar, the ZBM3 sensor medium broth was mixed with appropriate volume of sterile water agar (15 g/L agar) and 1 mL of ZBM3 agar medium dispensed aseptically to form thin films and allowed to gel. Thereafter, 0.1 mL of serially diluted overnight culture of *E. coli* MG1655 from 10^0 to 10^{-6} were aseptically spread over the films, covered with clear resealable cellophane bags to avoid desiccation and incubated at 37^0C for pH colour change as an indication of the presence of coliform in the samples tested.

5.3.4 Design of approach for co-detection of metal and biological contaminants

For coliform detection, viable count and most probable number (MPN) techniques were adopted.

5.3.4.1 Viable count

An appropriate set of dilutions of samples were prepared in sterile water in test-tubes. For bottled water from supermarket, 10^0 to 10^{-2} were used, while 10^{-1} to 10^{-5} were used for surface water. Then four pour-plates from each of the dilutions were prepared by dispensing 1 mL in the bottom of each petri dish and approximately 20 mL of molten LB agar from a 45^0C water bath poured on top of each sample and gently swirled. Following solidification, two plates from each dilution were incubated at 22^0C while the remaining two plates were incubated 37^0C overnight after which the plates were counted for colonies. Bacterial count in bottled water was performed as a control since such water was produced from an industry and has passed through quality control and assurance, hence should not contain any coliform bacteria. Conversely, surface waters are prone to coliform contamination through indiscriminate sewage disposal, breakage of sewage pipes, open defecation and ruminant grazing.

5.3.4.2 Most Probable Number (MPN) for coliforms

For each water sample, five double-strength and ten single-strength (established protocol for MPN test) ZBM3 broth medium were prepared in test-tubes then 10 mL of each water sample poured into each of the five double-strength medium, 1 mL of each sample poured into each of the five single-strength medium and 0.1 mL of each sample poured into each of the remaining five single-strength medium. Each of the tubes had an inverted Durham tube. The tubes were incubated statically in an upright position at 37^0C and examined after 24 hours for fermentation as shown by acid and gas

production. Coliform confirmatory tests, as described below, were carried out for the presumptive positive tubes, however, the negative tubes were re-incubated for a further 24-hour period to check for gas production from fermentable lactose in the ZBM3 medium (Seidler *et al*., 1981; Rompré *et al*., 2002).

For coliform confirmatory test, one loopful of broth from each presumptive positive tubes was transferred into test-tubes each containing 10 mL sterile Brilliant Green Bile Lactose Broth (peptone = 10 g/L, lactose = 10 g/L, ox-bile dried = 20 g/L, Brilliant green = 0.0133 g/L, final pH = 7.2 ± 0.2 at 25^0C) with inverted Durham tubes. The subculture tubes were incubated statically at an upright position for 24 hours at 44 ± 0.5 ^{0}C in a water bath and examined for growth and the presence of gas production in the Durham tubes (Seidler *et al*., 1981; Rompré *et al*., 2002).

For coliform complete test, one loopful of broth from each of the positive confirmatory tubes showing gas production was transferred to 10 mL Tryptone Water warmed to 45^0C and incubated in a water bath at 45^0C for 24 hours. Subsequently, 0.1 ml Kovacs reagent was added to each tube, gently mixed and examined after 1 minute for indole production which is confirmation of *E. coli* (Seidler *et al*., 1981; Rompré *et al*., 2002).

5.3.4.3 Metal and coliform assays using surface water samples

Metal and coliform parallel detection tests were performed using water samples at two different locations on the same water source (the Braidburn) in Edinburgh: a stream near Braidburn Valley Park, Oxgangs (sample B), and another stream near Cameron toll shopping complex (sample C). For metal assay, the same procedure outlined in section 4.3.3 was used. However, copper or zinc stock was mixed with surface water samples and increasing concentrations added to 1 mL single strength ZBM3 medium

supplemented with 40 mg/ L chloramphenicol (to inhibit the growth of coliform bacteria) in 1.5 ml microcentrifuge tubes in triplicate. Metal stock solutions were added to establish their presence in the surface water samples since there was no available information about their presence in the samples. Antibiotic was also added to maintain the plasmids in the sensor organisms and suppress the growth of coliforms, ensuring that only each of the specific metal was being detected to avoid false positive results. The tubes were inoculated with 0.1 mL of overnight culture of either copper or zinc biosensor in triplicate. For coliform test, 0.1 mL of each surface water sample was inoculated into 1 mL of ZBM3 medium in 1.5 mL microcentrifuge tubes with and without 40 mg/ L chloramphenicol to assess the bactericidal effect of the antibiotic. The tubes were incubated statically in an upright position at 37^0C for 24 hours. Absorbance of supernatants was measured at 625 nm in a spectrophotometer following centrifugation on a table top centrifuge at 13,000 x g and 17,000 rpm for 5 minutes.

5.3.4.4 Lyophilisation of media and whole-cells in a single format

For biosensor lyophilisate preparation, biosensor cells were cultivated (see section 4.3.5). Following cultivation, cells were pelleted at 7,500 x g for 5 minutes and resuspended with equal volume of 20% sterile lactose. To each 2 mL microcentrifuge tube of either ZBM3 or ABM6, 0.2 mL of either arsenic, copper or zinc biosensor cell-lactose suspension was added and frozen at -80^0C. Similarly, 2 mL ZBM3 medium was dispensed in 2 mL microcentrifuge tubes and frozen at -80^0C overnight for coliform assay. The tubes were freeze-dried with open lids in Edwards Modulyo freeze drier for 24 hours, tightly capped and stored at room temperature for days (some tubes were stored at 37^0C as a prototype for environmental temperature in the tropics).

5.3.4.5 Metal and coliform assays using designed format

For metal assay, the same procedure as outlined in section 5.3.6 was used. Here, the dried tubes were rehydrated with 2 mL of surface water samples and supplemented with suitable antibiotic solution and corresponding metal stock solution at increasing concentrations. For coliform assay, 2 mL from each surface water sample was added to the freeze-dried ZBM3 medium in microcentrifuge tubes and resuspended. The tubes were mixed by inversion and incubated statically in an upright position at 37^0C for 24 hours. The absorbance of supernatants was measured at 625 nm following centrifugation at 13,000 x g for 5 minutes.

Metal and coliform parallel detection tests were also performed on thin film agar. See section 6.3.3 for coliform thin film agar procedure. For metal assay, 0.1 mL overnight culture of each biosensor was mixed with suitable antibiotic solution and corresponding metal stock solution at increasing concentrations, aseptically spread over the films, covered to avoid desiccation and incubated at 37^0C for change of the bromothymol blue pH colour from blue to yellow.

5.4 Results

5.4.1 Testing growth and function of coliforms in ZBM3

To optimise conditions for coliform assay in biosensor medium, single colonies of wild-type *E. coli* MG1655 and wild-type non-coliform bacterium *Salmonella enterica* serovar Typhimurium LT2 were cultured in ZBM3 medium. An investigation of the bacterial growth in the sensor medium with lactose as carbon source and without bromothymol blue pH colour indicator showed that *E. coli* MG1655 had more rapid growth than *Salmonella enterica* serovar Typhimurium LT2 (Figure 5.1). Although it is a non-coliform and does not ferment lactose, there was an appreciable growth of *Salmonella enterica* serovar Typhimurium LT2 in the medium. It was found that both bacteria grew more in LB medium than in the sensor medium (data not shown) as observed in their cell density measurement.

The assay of coliforms in ZBM3 was performed as described in section 5.3.3. It was observed that the coliform *E. coli* MG1655 grew and utilized the sensor medium more than the non-coliform *Salmonella enterica* serovar Typhimurium LT2 (Figure 5.2). The coliform bacterium *E. coli* MG1655 unlike the non-coliform *Salmonella enterica* serovar Typhimurium LT2 , fermented lactose to acid, causing a reduction in pH as indicated by colour change from blue to yellow (Fig. 5.2 a & c). The green colour in Figure 5.2(b) was due to Teepol supplement (greenish in colour). It was found that the addition of different concentrations of Teepol and bile salt preferentially inhibit the growth of the non-coliform bacterium *Salmonella enterica* serovar Typhimurium LT2, while *E. coli* MG1655 growth was not inhibited (Fig. 5.2 e-h) (Pillai *et al*., 2000; Hirulkar and Tambekar, 2006). However, the optical density of cell cultures at 600 nm showed that *E. coli* MG1655 growth slowed down slightly as the concentration of Teepol and Bile salt increased, an indication that high concentration of inhibitors of non-coliform

bacteria could have slight negative effect on coliform growth (Figure 5.3b). The decrease in the CFU/mL of *Salmonella enterica* serovar Typhimurium LT2 showed that Teepol and Bile salt are efficient bacteriostatic agents against some non-coliform bacteria, Teepol being more effective than bile salt (Figure 5.3 c & d).

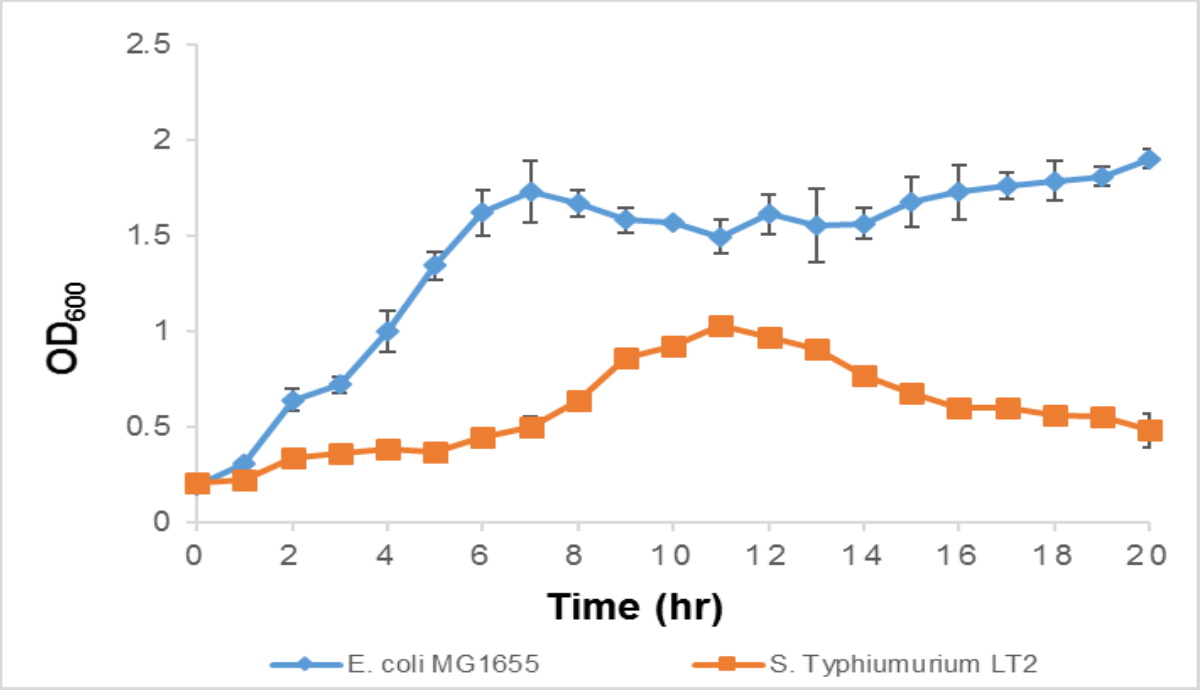


Figure 5.1: Testing growth and function of coliforms in ZBM3. Cell pellets of overnight cultures of wild type *E. coli* MG1655 (*diamond*) and non-coliform *Salmonella enterica* serovar Typhimurium LT2 (*square*) grown in LB medium were resuspended in ZBM3, inoculated into ZBM3 medium and incubated at 37^0C with shaking and optical density measured. The *error bars* indicate the standard error of 3 replicates.

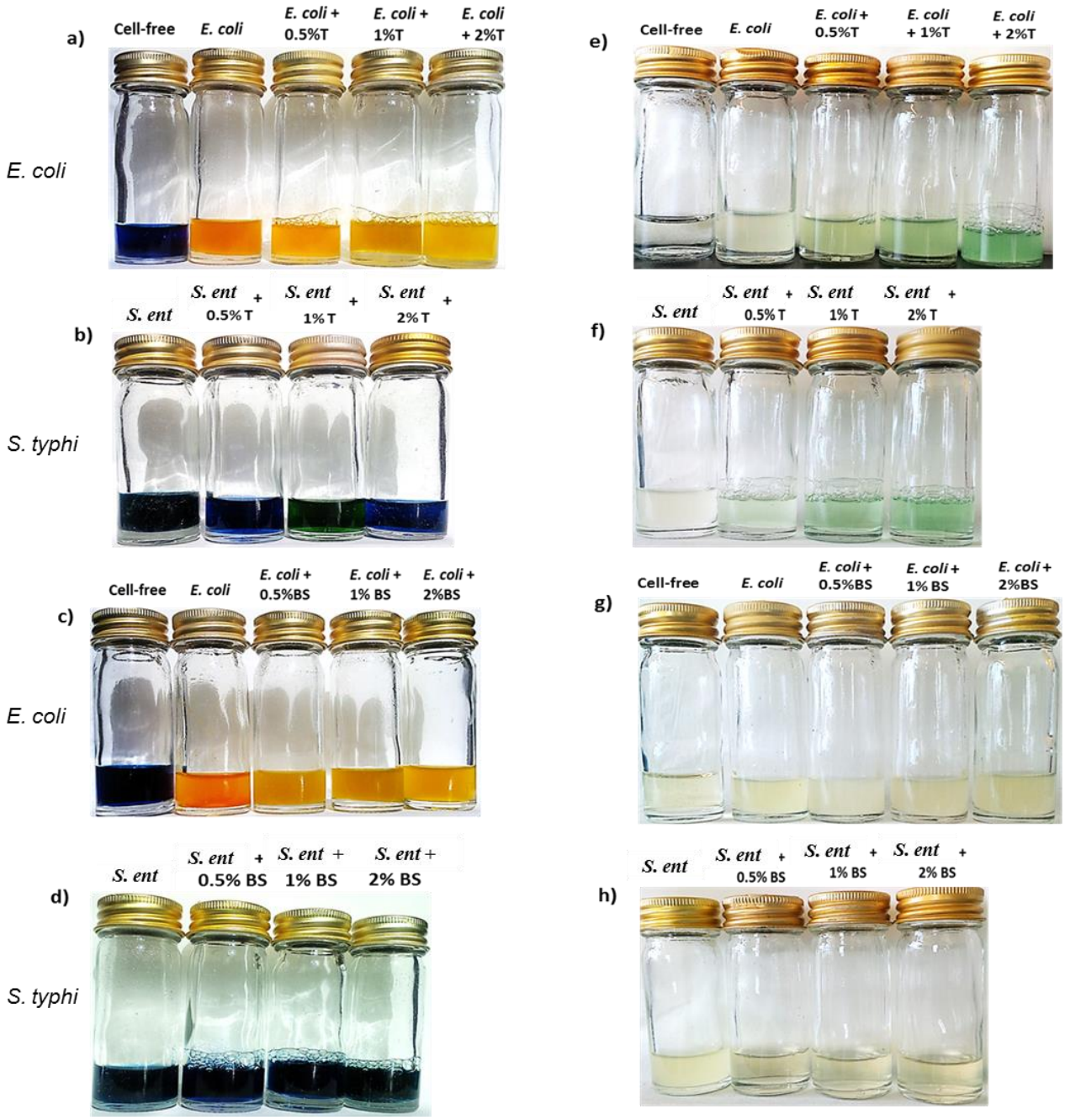


Figure 5.2: Coliform detection assay in ZBM3. **a & b)** Colour indicator with Teepol. **c & d)** Colour indicator with bile salt. **e & f)** Teepol, no colour indicator. **g & h)** Bile salt, no colour indicator. Cell cultures were incubated at 37^{0}C for 24 hours with shaking. The coliform bacterium *E. coli* MG1655 (a & c) unlike the non-coliform *Salmonella enterica* serovar Typhimurium LT2 (b & d), fermented lactose with acid production, causing a reduction in pH as indicated by colour change from blue to yellow. Teepol and Bile salt added at increasing concentrations. The green colour in (e & f) was due to the reaction between medium and Teepol supplement, an inhibitor of non-intestinal bacteria. Key: T= Teepol, BS= Bile salt.

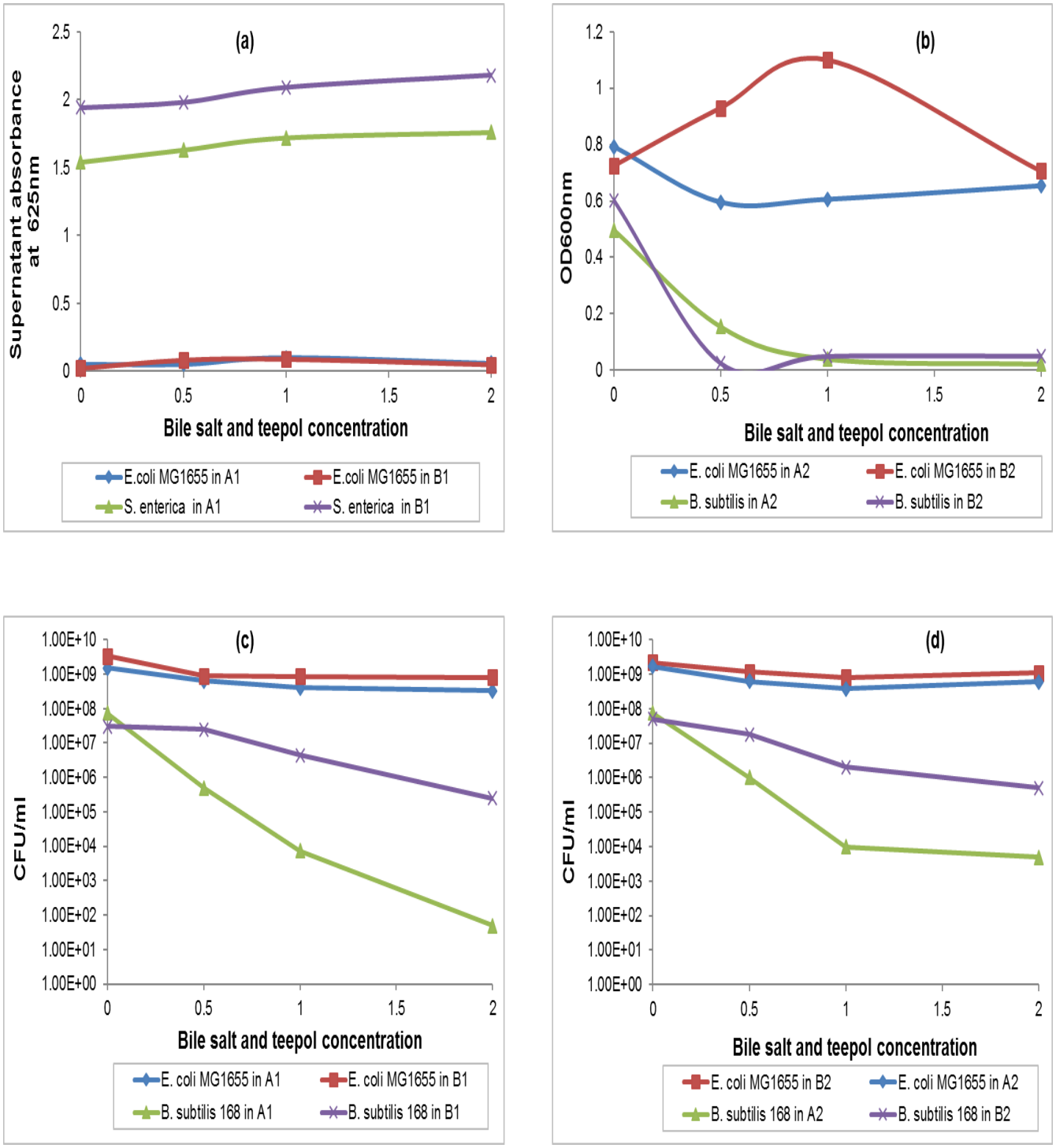


Figure 5.3: Coliform assay continued. **a)** Absorbance of supernatant at 625 nm. **b)** OD at 600 nm. **c & d)** Colony count. Sample identification: **A1** single strength medium with colour indicator and Teepol. **B1** double strength medium with colour indicator and Bile salt. **A2** single strength medium with Teepol, no colour indicator. **B2** double strength with Bile salt, no colour indicator. In plot (a), the *E. coli* curves are almost zero since the disappearance of the blue colour of the pH indicator was being measured, indicating fermentation of lactose by the coliform, hence a drop in pH. Conversely, the high absorbance value of the *S. typhi* curves

shows that the non-coliform could not ferment lactose causing the medium to remain blue indicating high pH or alkaline state. Similarly, the high CFU/mL value for *E. coli* in plot (c) is an indication that the coliform was able to grow, ferment and utilise lactose in the medium and there was little or no effect of Bile salt or Teepol on the bacterium. However, the decline in *S. typhi* curves is an indication that its growth was preferentially inhibited by the increasing concentrations of bile salt and Teepol. This is supported by the 0 tubes (lacking non-coliform inhibitors) with a high CFU/mL value. Graphs (a) and (b) were measured at different wavelengths because the disappearance of blue of the pH indicator (a) is always measured at 625 nm following manufacturer's guideline, while (b) was measuring cell density at 600 nm of cells grown at overnight at 37^0C with shaking.

5.4.2 Coliform assay on thin film ZBM3 agar

Following the procedure described in section 5.3.3, wildtype *E. coli* MG1655 was used to test for coliforms in the formulated sensor agar film. It was observed that coliforms were detected in the film as indicated by the change of bromothymol blue pH indicator from blue to yellow. No visible colour was observed after 3 h incubation, but the development of yellow colour was observed after 9 h which was more prominent when checked at 12 h incubation. Positive results were also obtained at 10^6 dilutions. Serial dilution experiment was conducted for the surface water samples to assess the possibility of the technique detecting small load of coliforms if present in the sample. The films were overcrowded, hence lack of visible colonies, but small colonies were seen on the edges indicating too numerous cells to count. There was no colour change in the cell-free and non-coliform (negative control) films, an indication of aseptic procedure as well as inability to ferment lactose in the ZBM3 medium (Figure 5.4). The colour change development in this format is faster than in liquid assay where colour change was observed at 18 h incubation.

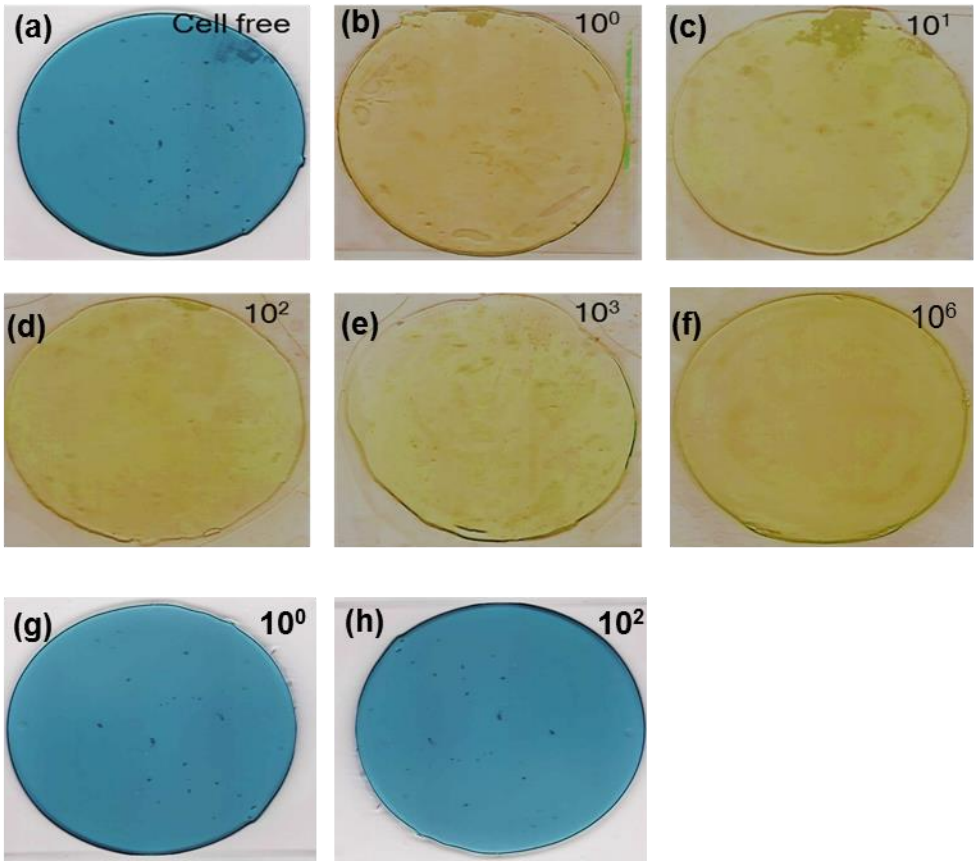


Figure 5.4: Rapid coliform detection in thin film ZBM3 agar. Coliform was rapidly detected in parallel with metals in thin film ZBM3 agar sensor medium within 12 hrs of incubation at 37^0C, a confirmation of lactose fermentation as shown by colour change from blue to yellow. Sample identification: **(a)** = Cell-free medium, **(b – f)** = dilutions of overnight cultures of *E. coli* MG1655, **(g – h)** = dilutions of overnight cultures of *S. typhi*, a non-coliform as negative control.

5.4.3 Complete test for coliforms in drinking and surface water

The complete test microbiological technique for coliforms involving presumptive, confirmatory and complete tests as outlined in the guidelines for drinking-water quality was carried out (section 5.3.4). Water samples purchased from a supermarket and surface water collected from streams were used for the assay. Coliforms were detected from water samples using the novel sensor medium. This was confirmed by the production of acid and gas in both the sensor and Brilliant Green Bile Lactose

media (indicative of fermentation of lactose) as shown in (Figures 5.5 & 5.6), and the production of indole in Tryptone Water (Figure 5.7). The most probable number obtained showed that there were no coliforms in the bottled water used with MPN of 0, while the surface water samples collected from two different locations showed high presence of coliforms as expected with MPN of 550 and 900 respectively (Table 5.1).

For the co-detection of metals and coliform in a single system, assays were conducted in ZBM3 sensor medium with added antibiotic, chloramphenicol (see section 5.3.4.3 for protocol). Metal stock solutions were added to establish their presence in the surface water samples since there was no available information about their presence in the samples. Antibiotic was added to maintain the plasmids in the sensor organisms and suppress the growth of coliforms, ensuring that only each of the specific metal was being detected to avoid false positive results. The results obtained showed that the system worked very well by accurately detecting metals below the safe limits as recommended by the WHO (Figure 5.8 a & b, left-hand sides). The absorbance measurements showed that there was a decrease in the pH of the supernatants, indicative of lactose fermentation to acid as observed by the change of the pH indicator from blue to yellow after incubation. This was evident for zinc (Figure 5.8 a & b, right-hand side) and copper (data not shown) tested. For coliform test in the surface water samples, assay was conducted as prescribed in (section 5.3.4.3). Two versions of the assay for each surface water sample were prepared, with antibiotic added to one to kill any coliform bacteria present and ensure that only metallic ions were detected, while the other was left without any antibiotic to test for the presence of coliforms. Following incubation, there was no growth and colour change in tubes with antibiotic while there was pH colour change in those without antibiotic, indicating coliform presence (Figure 5.8 c).

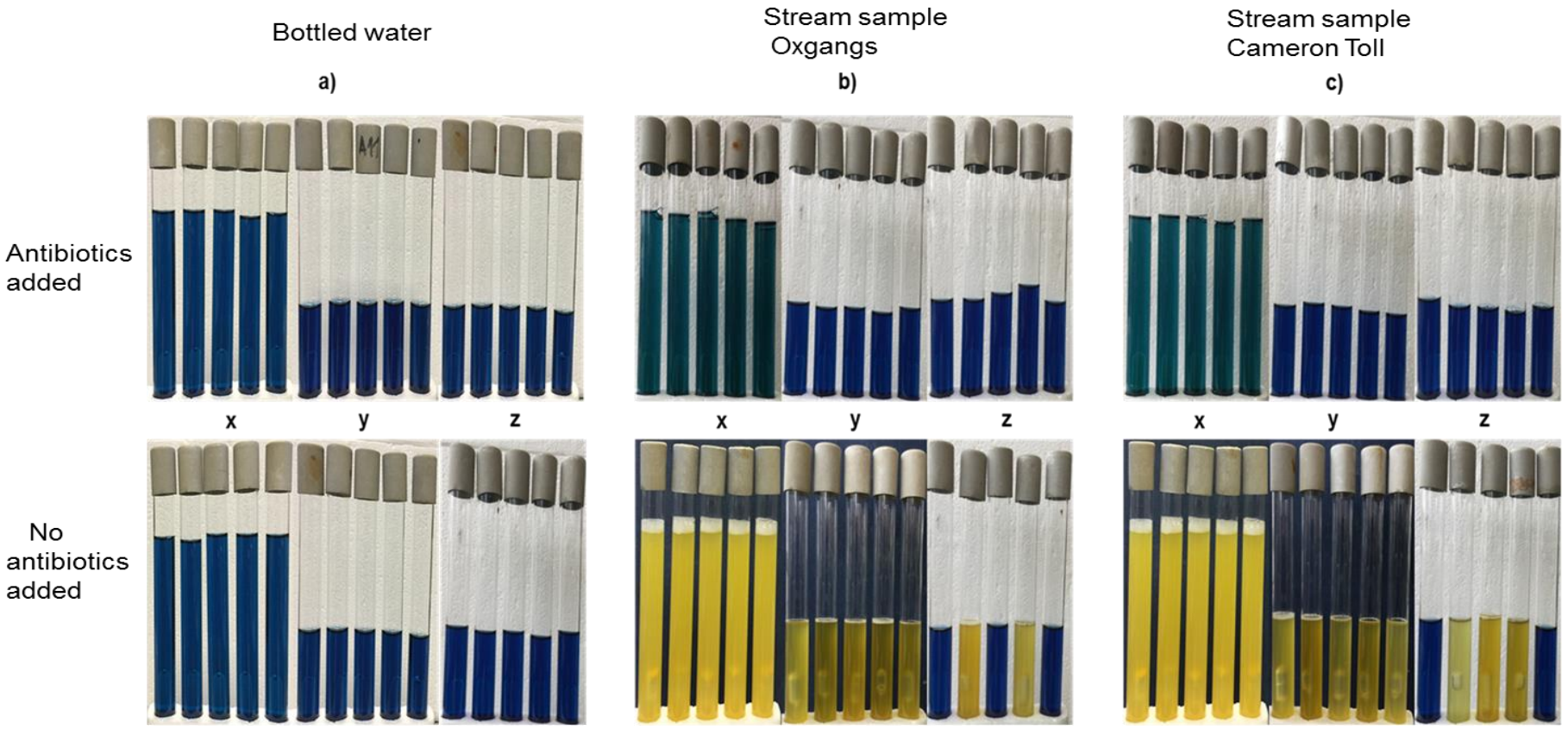


Figure 5.5: Coliform Assay: Presumptive test (a) bottled water purchased from a supermarket (b) Braidburn Valley Park stream, Oxgangs, Edinburgh (c) Stream near Cameron Toll shopping complex, Edinburgh. Tubes were incubated statically upright at 37^0C for 24 hours. Fermentation by coliforms occurred in stream samples (b & c, bottom), an indication of acid and gas production in inverted Durham tubes as shown by change of pH colour indicator from blue to yellow. Sample identification: Upper row= 40 mg/L chloramphenicol added; Lower row= No antibiotic added; x= 5 tubes of 10 mL water sample in 10 mL double strength ZBM3 (4% bile salt); y= 5 tubes of 1 mL water sample in 5 mL single strength ZBM3 (2% bile salt); 5 tubes of 0.1 mL water sample in 5 mL single strength ZBM3 (2% bile salt). Dark background in b & c (lower row) based on where picture was taken.

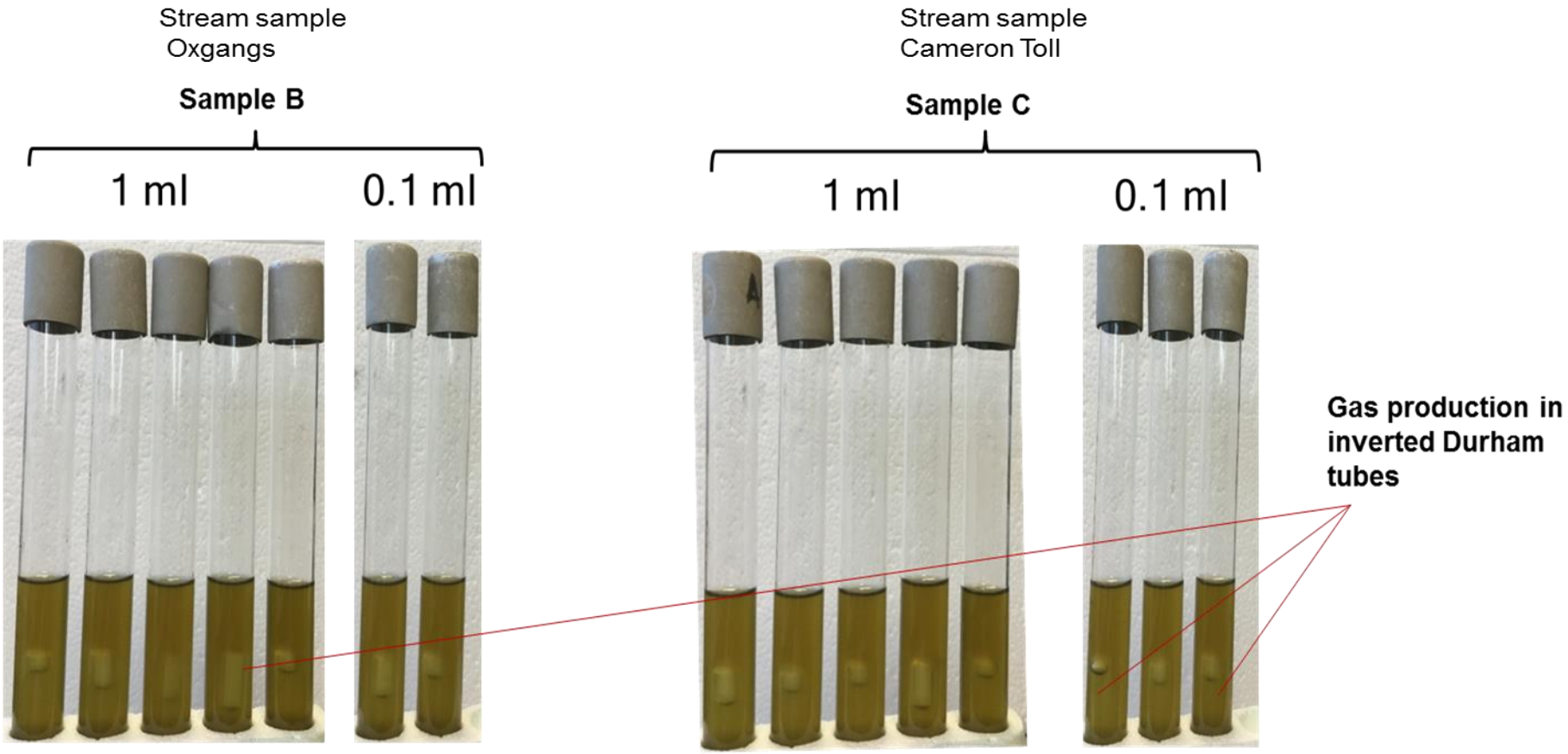


Figure 5.6: Coliform confirmatory test in Brilliant Green Bile Lactose Broth. Subculture tubes from presumptive positive tubes incubated statically at upright position for 24 hours at 44 ± 0.5 ^{0}C. Positive tubes showed growth and presence of gas in the inverted Durham tubes.

Sample identification: 1 ml = 5 positive y tubes from the presumptive test; 0.1 ml = 2 or 3 positive z tubes from the presumptive test all from Figure 5.5.

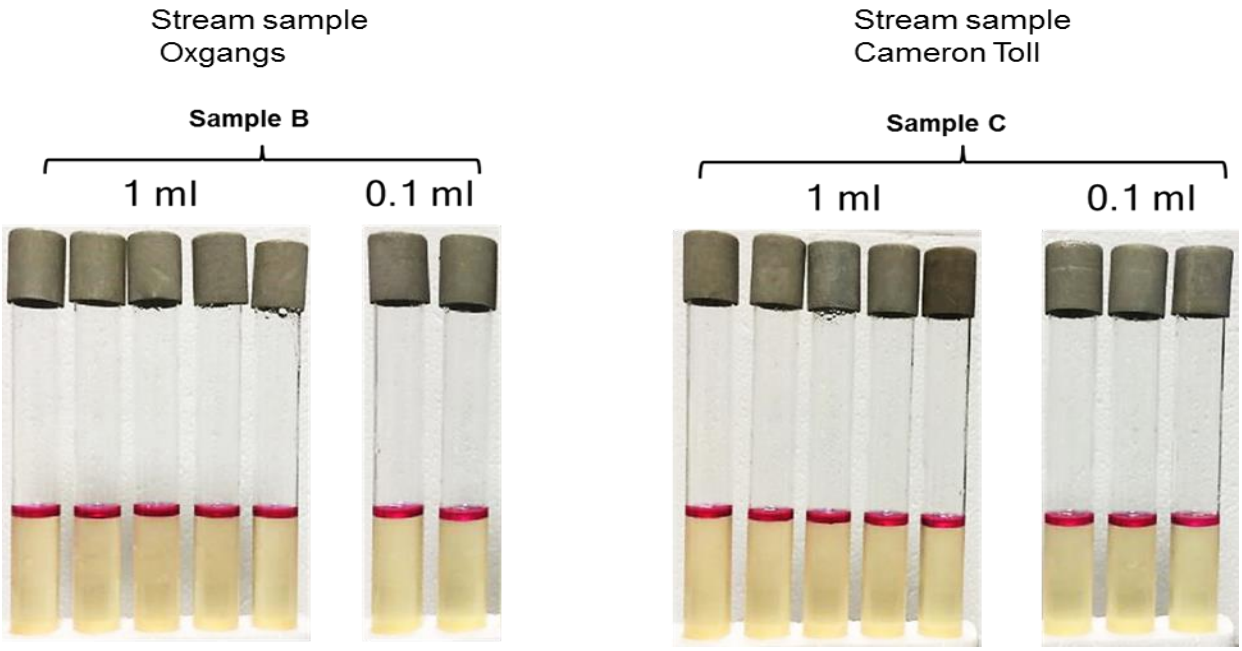


Figure 5.7: Complete test in Tryptone water. Tubes incubated at 37^{0}C for 24 hours after inoculation of one loopful of each tube of confirmatory tubes into Tryptone water warmed to 45^{0}C. Formation of ''Red Ring'' at the top of the tubes by addition of 0.1 ml Kovacs reagent in Tryptone Water culture indicative of indole production, hence coliform presence. Sample identification: 1 ml = 5 positive tubes from the confirmatory test; 0.1 ml = 2 or 3 positive tubes from the confirmatory test, all from Figure 5.6.

Table 5.1: M^{c}Crady's Tables for 5 tubes

This is a 5-tube MPN table used to determine the most probable number of positive organisms per 1 mL of inoculum.

Quantity of water added in each tube	10 ml	1 ml	0.1 ml	Probable number of coliform organisms in 100 ml of the original water	Sample identification
Number of tubes used	5	5	5		
	Number of tubes giving positive reaction (acid and gas)				
	0	0	0	0	Bottle water
	5	5	2	550	Stream sample Oxgangs
	5	5	3	900	Stream sample Cameron Toll

5.4.4 Metal and coliform assays in surface water samples

Metal and coliform tests were performed as described in section 5.3.4.3 in parallel to test the possibility of designing a tool that can co-detect both contaminants in a single format. The presence of metal contaminants in the water samples tested could not be established, hence metal stock solutions were prepared by dissolving zinc and copper compounds in sterile distilled water and aseptically added to the experimental tubes. To maintain the plasmids in the sensor organisms and suppress the growth of coliforms, appropriate amounts of chloramphenicol antibiotic solution were also added to the tubes to ensure that only each of the specific metal was being tested to avoid false positive results. According to data obtained, the bacterial sensors accurately detected zinc at 0.046 nm after 18 hour incubation at 37^0C (Figure 5.8a & b). It could be seen that the 0.025 nm tubes have changed from blue to green indicating that at prolonged incubation were capable of turning to yellow. The change of the pH indicator from blue to yellow was as a result of the expression of the zinc responsive promoter in the sensor when zinc ion was present causing the repressor protein to be released from the promoter, resulting in the production of a detectable signal (yellow colour) proportional to the concentration of the zinc ion. The measurement of the disappearance of the blue colour of the indicator was indicated by a reduction in the absorbance value as the zinc ion concentration increases. Similarly, it was observed that the growth of coliforms was adequately inhibited by the chloramphenicol antibiotic added. The coliform test conducted showed that no coliform was found in bottled water sample, while the tubes of samples from surface water indicated the presence of coliforms which fermented the lactose in the medium leading to acid production as could be seen by a reduction in pH supported by the change of the bromothymol blue from blue to yellow (Figure 5.8c).

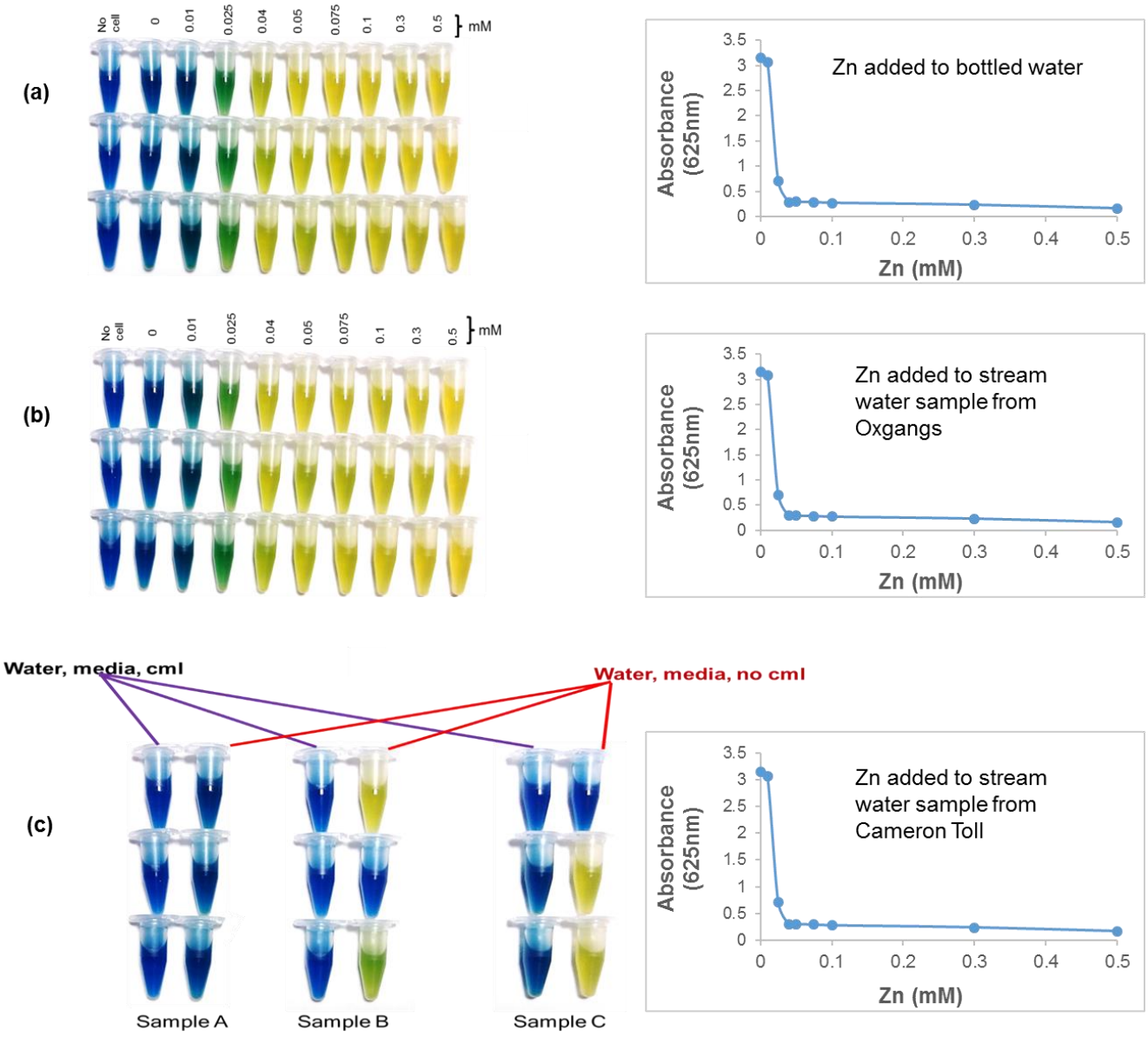


Figure 5.8: Zinc and coliform assay in surface water samples. Zinc stock solution added to (a) bottled water from supermarket; freshwater samples from (**b**) Braidburn Valley Park stream, Oxgangs, Edinburgh and (**c**) stream near Cameron Toll shopping complex, Edinburgh, in 1.5 ml microcentrifuge tubes in triplicates. Assay was conducted in single strength ZBM3 medium supplemented with chloramphenicol. Coliform test of fresh water samples in ZBM3 with/without chloramphenicol antibiotic (**c**). Tubes incubated statically in upright position at 37^0C for 24 hours. Absorbance of supernatants measured at 625 nm in a spectrophotometer. The *error bars* (which may be smaller than the symbols) represent standard error of three replicates. **Key:** cml= chloramphenicol.

5.4.5 Metal and coliform assays using designed tool for field use

For field use, sensor cells were dried in media and stored at room temperature for days (see section 5.3.4.4). Metal assays were conducted following procedures as outline is section 4.4. Results obtained after incubation showed that the concept worked effectively well as the metals tested were accurately by their respective bacterial sensor cells below the limits as recommended by the WHO (Figure 5.9 a-c). There was colour change from blue to yellow of the pH colour indicator in the sensor media used and a drop in pH as indicated by the absorbance measurement of the supernatants. For coliform assay, freeze-dried sensor medium was used (see section 5.3.4.4). Following the resuspension of the freeze-dried medium tubes with diluted surface water samples (10^0 to 10^6) and incubation, results obtained showed that coliform was detected in the water samples. Tubes showed colour change from blue to yellow indicating growth and lactose fermentation and a drop in pH as indicated by absorbance measurement of the supernatants (Figure 5.9d). It was also observed that the dilutions (10^0 to 10^6) showed positive results with colour change, lower absorbance value ranging between 0.2 to 0.5 and higher CFU/ml (38 and 20 colonies) respect. Higher dilutions (10^2 to 10^6) yielded negative results as the tubes remained blue with high absorbance values (4.83 to 5.11) with no colonies found on the plates.

Similarly, metal assays conducted on thin film sensor agar films using surface water samples mixed with appropriate concentrations of metals in increasing levels and respective sensor cells showed that metals in surface water can be detected at the safe limits as set by the World Health Organisation (WHO, 1996) on thin film sensor agar media used (Figure F.10a – c). Coliform was also detected from the samples on thin film sensor agar media but at lower dilutions, hence the system could only detect

coliform in samples with heavy contamination but failed at lower contamination levels (Figure 5.10d).

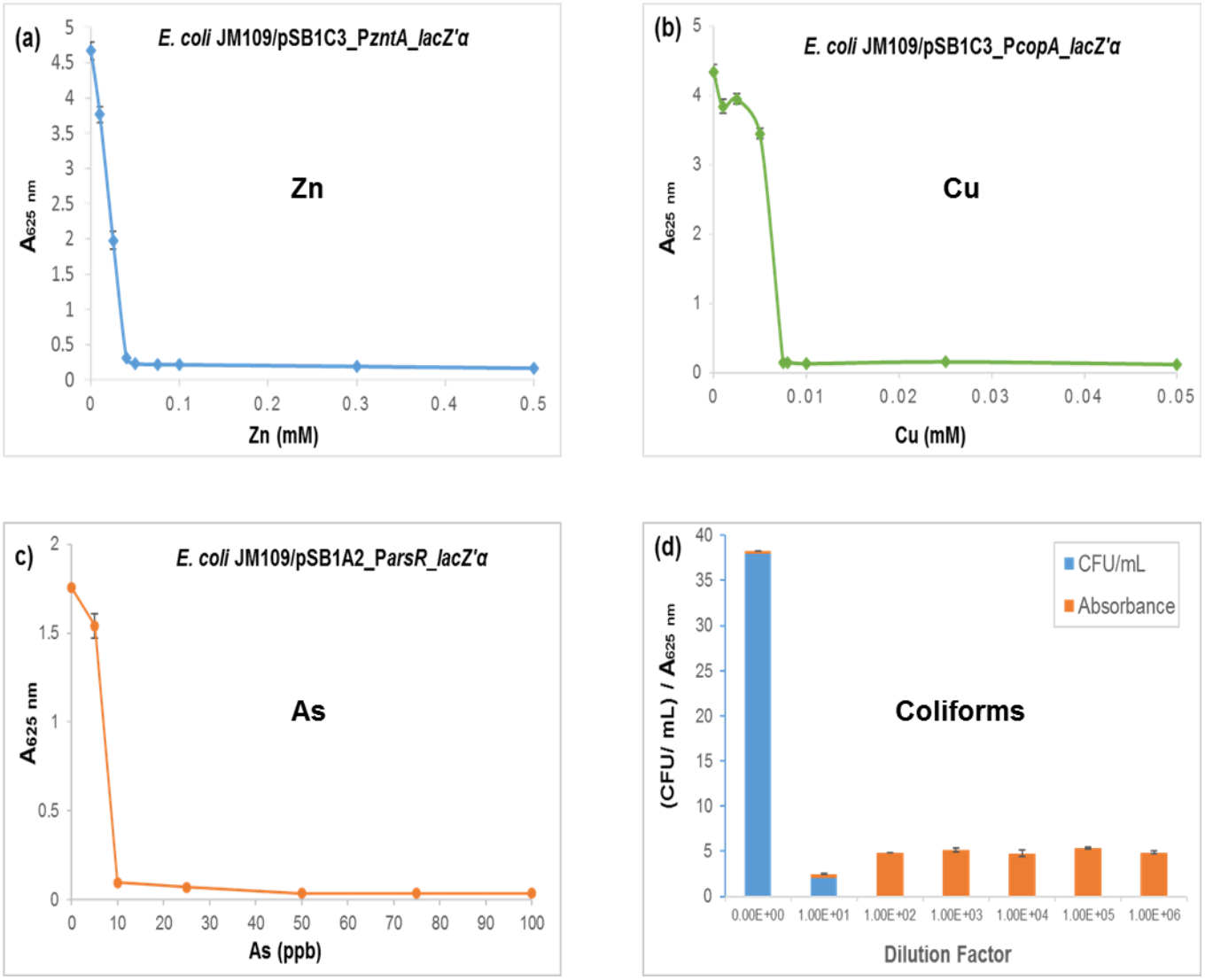


Figure 5.9: Metal and coliform assay using biosensor lyophilisates. (**a**) Zinc (**b**) copper and (**c**) arsenic sensor cells frozen and dried in sensor medium, resuspended with surface water samples and supplemented with suitable antibiotics and appropriate metal solution, (**d**) coliform assay in freeze-dried sensor medium. Tubes incubated statically in upright position at 37^{0}C for 24 hours. Absorbance of supernatants measured at 625 nm in a spectrophotometer. The *error bars*, which may be smaller than the symbols, represent standard error of three replicates.

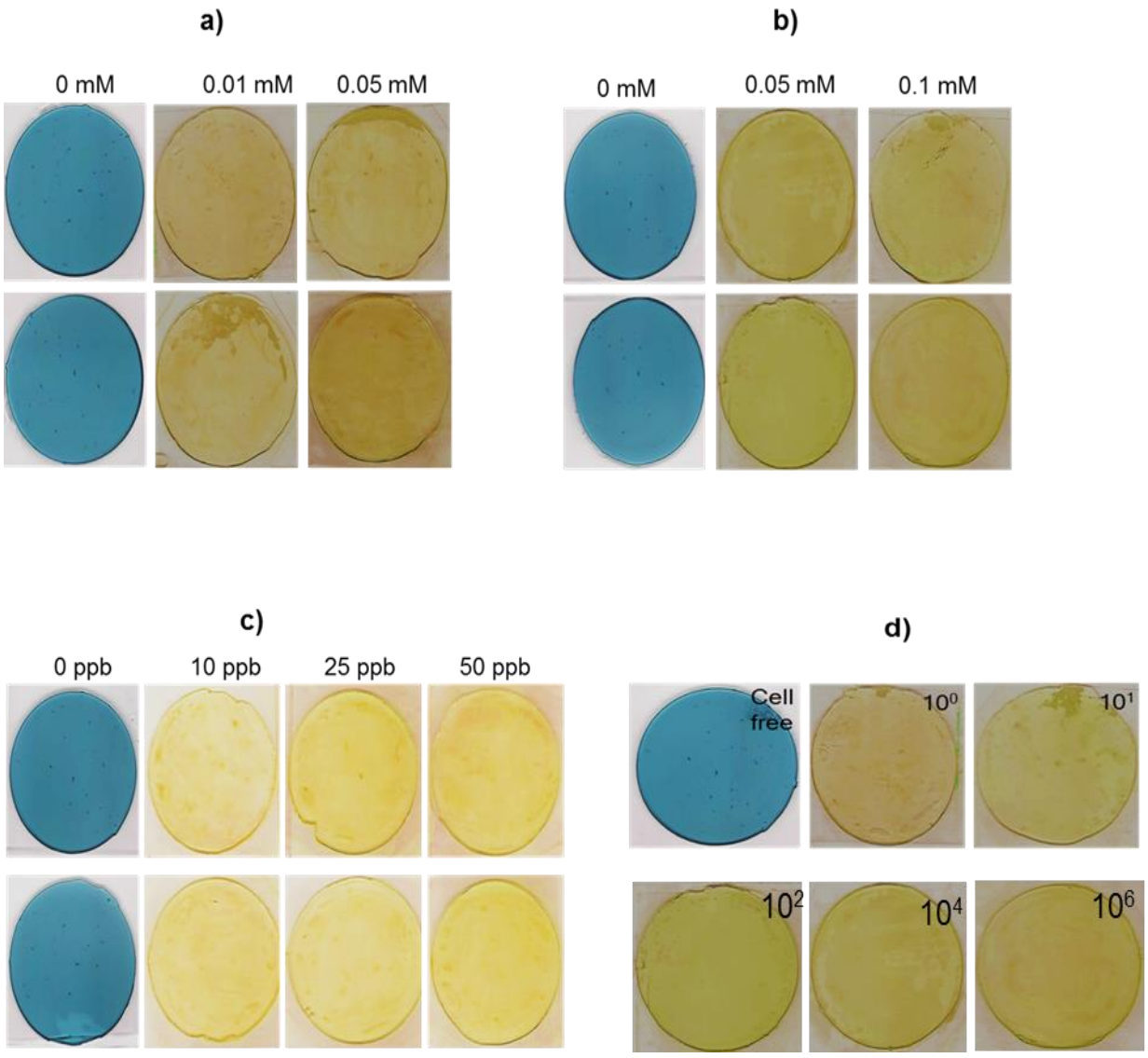


Figure 5.10: Rapid metal (**a**) copper (**b**) zinc (**c**) arsenic and (**d**) coliform detection on thin film ZBM3 agar. The system rapidly co-detected metals and coliform in surface water in thin film ZBM3 agar sensor medium within 12 hrs of incubation at 37^0C, a confirmation of lactose fermentation as shown by colour change from blue to yellow higher dilutions (0-6). For the coliform kits, the films were overcrowded, hence lack of visible colonies, but small colonies were seen on the edges indicating too numerous cells to count.

5.5 Discussion

Water is an essential commodity for the sustenance of life and drinking water needs to be safe as a basic necessity for human existence. The problem of coliforms and associated faecal pathogens to public health is of a serious concern and to ensure the availability of safe drinking water, greater attention is being paid to water quantity and water quality assurance. Modern techniques for coliform detection need laboratory and technical support, are time consuming, expensive and inadequate. Most developing countries are the worst hit as these techniques are either lacking or quite expensive thereby contributing to health challenges to the growing population. One of the major aims of this work was to develop novel whole-cell bacterial sensors for heavy metal detection in drinking water. The whole-cell biosensors of interest in this project work by allowing growth on lactose medium (Aleksic *et al*., 2007; de Mora *et al*., 2011). We therefore considered it essential to develop a system that can be easily adapted to additionally detect the growth of other bacteria, such as coliforms, so that the same format of sensor detects both toxic metals and biological contaminants in a single unit. To achieve this, a concept was developed such that contaminated water samples would be divided into two compartments: one for metal detection assay using the developed biosensors, while the other would be tested for coliforms. For metal detection assays, chloramphenicol antibiotic was added to suppress coliform growth which could ultimately interfere with the results since they grow in and ferment lactose leading to acid production and change of pH colour indicator from blue to yellow. To test for coliforms, different concentrations of Teepol and Bile salt were added to the assay to inhibit the growth of non-coliform bacteria.

The ability of coliform bacteria to grow in the formulated ZBM3 biosensor medium was tested. It was found that the coliform bacterium, *E. coli* MG1655, grew more rapidly

than the non-coliform counterpart *Salmonella enterica* serovar Typhimurium LT2, in the medium. Although a non-coliform which does not ferment lactose, *Salmonella enterica* serovar Typhimurium LT2 was found to grow appreciably in the medium. But compared to their growth in LB medium, it was found that both bacteria grew more in LB medium than in the sensor medium. As a result of this, it was necessary to add non-coliform inhibitors such as Bile salt or Teepol in the test to preferentially inhibit the growth of non-coliforms which could produce false positive results in the system.

Following a 24 hour incubation, the ZBM3 sensor medium test used showed positive predictive values for water samples heavily contaminated by 10^8 CFU/mL coliforms, which gives a promising alternative for water quality assessment under field conditions. This test proves to be cheap, portable and easy to use. This was evident by the fermentation of lactose in the medium to acid, causing a reduction in pH as indicated by colour change from blue to yellow. The system showed to be devoid of contamination by non-intestinal bacteria when used in the field as Teepol and Bile salt proved to be efficient bacteriostatic agents against those bacteria. However, higher concentrations of these agents could decrease the sensitivity of the system by decreasing the growth of coliform bacteria (Sobsey and Pfaender, 2002).

There are several conventional bacteriological methods of coliform detection in water although H_2S has been reported to be more sensitive, simple, and cheaper with various modifications in medium composition, incubation period and temperature in different tropic and temperate regions (Castillo *et al*., 1994; Genthe *et al*., 1999; Hirulkar and Tambekar, 2006). However caution is given based on standardisation of formulation and testing protocol to allow for comparison across regions. An example is the NGO Forum which can produce about 100 H_2S kits per day. It was recommended that the specificity of this product be improved upon with a cap that will

retain sterility and prevent leakage since inner plastic cap of the product is clumsy to remove (Gupta *et al*., 2007). Also, the H_2S test is time consuming as 18 to 48 hour incubation is generally required for positive results (Pillai *et al*., 1999) as compared to our test which yields positive results within 12 hours.

This system is also cheap, simple and sensitive and can work effectively in temperature in different tropic and temperate regions. Compared to MPN test using seeded positive and surface water samples, results obtained were more favourable than the MPN test and at a shorter time. The use of thin film sensor agar is also favourable as it saves space for incubation, hence more samples can be tested at a time. Also, it is favourable in the tropics as incubation can be done at room temperature. The system is also economical as both coliform and heavy metals can be detected simultaneously in a single system. Similarly, biosensor lyophilisates can be distributed enabling rapid testing and also removing the need for continuous cell cultivation which can introduce variation in the system.

Chapter 6: Insertion of biosensor cassettes onto bacterial genome using lambda red recombineering

6.1 Abstract

Whole-cell biosensor recombination systems were developed in which plasmids are maintained without antibiotics or antibiotic resistance genes. This approach involved the insertion of the sensor plasmids onto bacterial genome in which PCR products were generated by using primers with 50-nt extensions that are homologous to the regions adjacent to the genes to be inactivated. The PCR products were *DpnI*-digested, gel-purified and plasmid DNA inserted onto *E. coli* genome using λ-Red recombination approach. Plasmids with or without antibiotic resistance genes were tested for stability following a 7-day incubation at 37^0C without antibiotics. The presence of heavy metals in water samples was also tested using the recombineered systems. Results obtained showed that plasmids can maintained without antibiotics or antibiotic resistance genes. Although there were some appreciable loss in plasmids, data obtained showed that the plasmids were still effective in the assay. Similarly, the system detected heavy metals but with high background activity. Our previous data have shown that this can be corrected using weaker ribosome binding site (RBS).

6.2 Background

One of the most important tools in the development of molecular biology is the use of antibiotics and their resistance genes. When present on recombinant plasmids, antibiotic resistance genes allow these plasmids to be effectively selected after transformation of competent bacterial cells. The maintenance of these plasmids subsequently relies on the presence of the selective antibiotic in the growth media to kill plasmid-free transformants. However, regulatory authorities have restricted their use in many areas of biotechnology making the presence of these genes and the antibiotics themselves become undesirable (Hanak and Cranenburgh, 2001; Glenting and Wessels, 2005; Vidal *et al*., 2008).

In gene therapy and DNA vaccine applications, DNA is directly delivered into patients to replace faulty gene functions or kill tumours. It is reported that these DNA vaccines consist of plasmid-encoded antigens with the function to induce an immune response. Many have feared the risk of transforming a patient's microflora and the spread of resistance genes when antibiotic selection markers that confer resistance to antibiotics in vaccine plasmids are used. Hence, the antibiotic must be knocked out from the final product in recombinant protein production for therapeutic use (Sato *et al*. 1996; Hanak and Cranenburgh, 2001). According to the 'Guidance for Human Somatic Cell Therapy and Gene Therapy' published by the Food and Drug Administration (FDA) in the USA (Murphy 1998), "it is recommended that penicillin and other β–lactam antibiotics be avoided during production, due to the risk of serious hypersensitivity reactions in patients". FDA guidelines also state that: "it is preferable not to use selection markers which confer resistance to antibiotics in significant clinical use, in order to avoid unnecessary risk of spread of antibiotic resistance traits to environmental microbes".

In genetic engineering to create transgenic organisms, serious concerns have grown about the presence of antibiotic resistance genes in genetically modified organisms (GMOs) having the potential of being transferred to wild-type microorganisms in the environment or the intestines of animals and humans, including the pathogenic organisms (Hanak and Cranenburgh, 2001; Vidal *et al*., 2008). Wadman (1996) reported that the Advisory Committee on Novel Foods and Processes in the UK declared that "the presence of a gene conferring resistance to β–lactam antibiotics in a strain of genetically modified maize posed an "unacceptable risk" due to the chance that bacteria in the gut could take up and express the gene".

It is also of concern that horizontal gene transfer can occur between soil bacteria and transgenic crop plants. Several antibiotic resistance genes have been employed the most common being *nptII* (kanamycin, neomycin and gentomycin resistance) which is present in over 30 transgenic plants (Dröge *et al*. 1998). Research has shown that some species of soil bacteria maintain their natural competence during the whole of or at certain stages in their life cycle. Similarly, DNA is capable of persisting in the soil through adsorption to particles of sand and clay, which enhances their capability of becoming more resistant to nucleases up to 100 to 1000 folds as well as maintaining the ability to transform competent bacteria for several weeks (Davidson 1999).

Hanak and Cranenburgh (2001) has reported that the risk of such horizontal gene transfer taking place is highly small, and no transfer has been detected by studies looking for evidence of horizontal gene transfer. Questioning the use of *nptII* in transgenic plants, Dröge *et al*. (1998) following their review on horizontal gene transfer arrived at the conclusion that: "effort should be put into the development of new strategies to remove antibiotic resistance genes from transgenic constructs". In spite of small possibility of horizontal gene transfer, it is advocated that since there is no

function of resistance gene in the GMOs but is an antique of the earlier cloning process, its presence is not necessary. Hence the likelihood of legislation restricting the use of antibiotic resistance genes in GMOs is being increased by this fact along with public concern over genetic engineering and the availability of alternative plasmid selection systems (Hanak and Cranenburgh 2001).

Firstly published under the name "ET cloning'', lambda red recombination (''recombineering'') has been used as a powerful tool for making precisely defined insertions, deletions, and point mutations in *E. coli*, requiring as few as 35 bp of homology on each side of the desired alteration (Zhang *et al*., 1998; Mosberg *et al*., 2010).

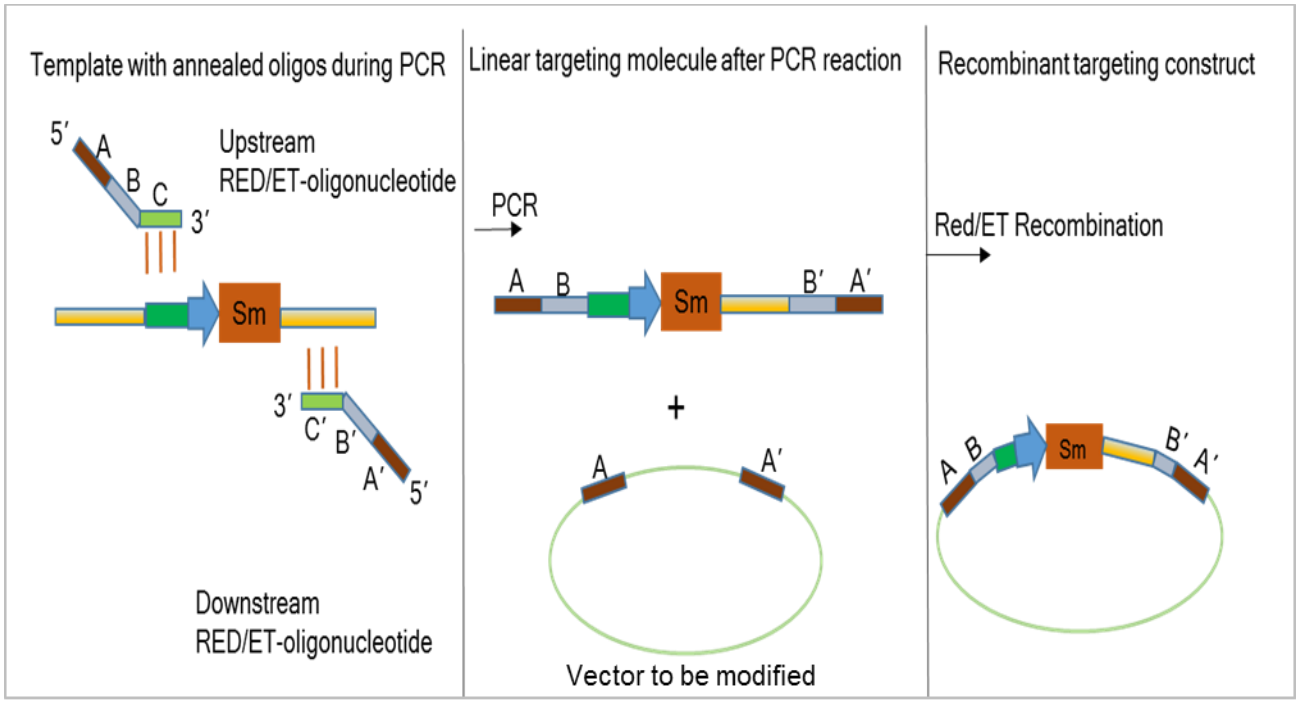


Figure 6.1: Practical steps involved in Red/ET illustrating the principle for modifying episomes such as bacterial artificial chromosomes (BACs). *Sm*, selectable marker; the *small blue arrow* indicates a prokaryotic promoter. (Adapted from Muyrers *et al*., 2001) copyright 2001 *TRENDS in Biochemical Sciences*).

The Red/ET recombineering technique can be employed in inserting whole-cell biosensor cassettes onto *E. coli* chromosome. For the chromosome at the site(s) of choice to be targeted, incorporate short homology regions need to be attached into the functional cassette carrying the selectable marker “Sm”. These selectable markers, which are the genes or elements that allow the selection of transformants, are not always the same as those used for plasmid maintenance. Consequently, two oligonucleotides have to be ordered for PCR amplification (Figure 6.1). Each oligonucleotide contains two parts: (a) required Part A (A′ for the second oligonucleotide) which is the homology region shared by the target molecule and the linear molecule. The homology regions are the 50 bp directly adjacent to either side of the insertion site; (b) optional Part B (B′ for the second oligonucleotide) which is the oligonucleotide part allowing the insertion of useful sequences like His-tags, HA-tags, restriction sites, multiple cloning sites, etc., and this part can be excluded if the introduction of such operational sequences is not desired; (c) required Part C (C′ for the second oligonucleotide) which is normally 18 – 24 nucleotides long and primes the PCR amplification of the selectable marker from the template provided (Hanak and Cranenburgh 2001; Gene Bridges, 2014).

The nature of this system gives us the opportunity to remove antibiotic selection markers, since organisms with the *lacZ′* gene can be selected based on their ability to growth with lactose as the main carbon source (since the hosts lack this ability, as was demonstrated).

6.2.1 Objectives of work presented in this chapter

The key features of the work presented in chapter 6 were:

4) To investigate the possibility of using lambda red recombineering technique to insert the newly designed sensor cassettes onto *E. coli* bacterial genome.
5) To test the stability of these plasmids on different substrates.
6) To evaluate the metal detection capabilities of the inserted cassettes of environmental samples.

6.3 Methods

6.3.1 Design of oligonucleotide for Red/ET recombineering

We designed primers flanking the insertion construct (20 bp of overlap with the insertion construct), and added 50 bp of the homology matching the *E. coli* K-12 genome flanking the region in which the new gene will be inserted (the overhang part) (see Figure 6.3a). The strategy was to insert *lacZ'α* gene onto the chromosome adjacent to the native metal-inducible promoter. The lengths of the annealing regions of the primer were chosen so that they have a similar (within 1^0C) melting temperature for the PCR reaction. Table 6.1 shows the PCR reaction set up employed.

Table 6.1: PCR reaction used

Component	Reaction volume (µL)
10x KOD Buffer	5
25 mM $MgSO_4$	3
dNTPs	5
KOD DNA Polymerase	1
Forward primer	1.5
Reverse primer	1.5
Miniprep DNA (construct)	0.25 or 0.5
PCR Grade water	To 50 µL

Table 6.2: Oligonucleotide sequence used for PCR amplification of the target cassettes

Here, it is not necessary to add 50 bp homology to the forward primers, since the promoter region in each case already matches chromosomal DNA; only the reverse primer, which matches *lacZ'* sequence, needs to have chromosomal homology added.

Primers	Oligonucleotide sequence	Cassettes
Forward primer	5′–CCAACTCAAAATTCACACC–3′	Arsenic
Reverse primer	5′–TTGCACTCCAGCCGATGCCTAAACCTTTCGGCTGCCAGATAACCAATACTCACTCCAGCCAGCTTTCCG–3′	
Forward primer	5′–CATCTCTCTGTGCGCAGTA–3′	Copper
Reverse primer	5′–TTTCGCTGCGCATCGGCGGCGTCACCAGCCCCTTCTCTTCATAGAAGCGATCACTCCAGCCAGCTTTCCG–3′	
Forward primer	5′–CGGTCACTTCCTGATCGTC–3′	Zinc
Reverse primer	5′–GTCGTTGGCGTTCTGTACCGTGGTTAGCGGTTTGAACGCAGCAAATTGAGGTCACTCCAGCCAGCTTTCCG–3′	

6.3.2 PCR to get the desired product

The PCR was carried out using the primers in (Table 6.2) using miniprep DNA from PCN2 and PCN1, the original versions of copper and zinc biosensor constructs with strong RBS as templates and KOD DNA polymerase following protocols (see Table 6.1). The PCR products were *DpnI*-digested and ran on an agarose gel, and then either gel extracted (when there where side products) or PCR purified (when the reaction ran clear) and eluted in molecular grade water.

6.3.3 Competent cell preparation

Glycerol stocks of *E.coli* DH5α, DH10B and JM109 strains having the inserted arabinose-inducible lambda red plasmid, pSC101_BAD_gbaA with *lacZ* gene deletions and tetracycline resistance gene marker were streaked on M9 agar plates and incubated at 30^0C in the dark to avoid antibiotic degradation. Distinct colonies were inoculated into 50 mL LB broth in 250 mL capacity flasks and supplemented with 1.5 µL tetracycline stock (3 µg/mL), flasks incubated at 30^0C overnight with shaking to maintain the plasmid. 100 µL of overnight cultures of each strain were inoculated into fresh 50 LB broth in 250 mL capacity flasks supplemented with 3 µg/mL tetracycline solution and incubated at 30^0C with shaking for 6 hours until optical density of 0.250, 0.447 and 0.367, respectively were obtained. Following this, 1.75 mL 10% L-arabinose was added to each flask to induce the expression of the Red/ET recombination proteins and cells were incubated at 37^0C for 1 hour with shaking. Subsequently, competent cells were prepared by chilling the flasks in ice-water slurry for 30 minutes to lower cell density. Cells were spun down in pre-chilled 50 mL Falcon tubes at 4500 x g rev for 10 minutes at 4^0C and supernatant discarded. Pre-chilled 1 x TSS (transformation and storage solution) added to each tubes and cell pellets resuspended. Aliquots of cell suspension (0.1 mL) dispensed into pre-chilled 1.5 mL microcentrifuge tubes, allowed to stand in ice for 30 minutes and stored at -80^0C.

6.3.4 Transformation of cassettes

Prior to transformation, the recipient *E. coli* strains (DH5α, DH10B and JM109 DE3 competent cells) were tested for growth in M9-glucose broth and failure to grow in M9-lactose broth since these strains contain the *lacZΔ*M15 mutation which requires the

lacZ'α gene for functional complementation. Distinct colonies of each *E. coli* strain was inoculated into 4 different variants of M9 broth medium; M9/lactose, M9/lactose with thiamine, M9/glucose, and M9/glucose with thiamine, and incubated at 37^0C with shaking for 24 hours for optical density measurement.

Transformation of *E. coli* DH10B competent cells using 1 µL of purified PCR of cassettes was performed following protocols (see section 2.9.2). Tubes were incubated at 37^0C with shaking for 70 minutes. Subsequently, 0.1 and 0.2 mL inoculum volumes were plated on M9/lactose plates supplemented with calculated amounts of corresponding metals and incubated at 37^0C overnight. For screening and selection of successful transformants, distinct colonies were streaked on LB agar plates supplemented with IPTG, x-gal and corresponding metals, and also on LB agar plates with IPTG, x-gal, metals and chloramphenicol and incubated at 37^0C overnight. Blue colonies were selected for colony PCR for confirmation of successful insertion. There was no growth on plates with chloramphenicol (for copper and zinc) and carbenicillin (for arsenic).

For colony PCR, oligonucleotide primers for each locus (Table 6.3) which lay outside the region of insertion were designed. PCR was conducted following protocol, purified and PCR products sequenced.

Table 6.3: Oligonucleotide sequence for colony PCR

Primers Name	Oligonucleotide sequence
Pars-F13	CGTCCTGATTCAGACCTCCTTTCAAATGAATAG
Pars-R13	GCGCCTGCCAGTAACATAATGCC
PcopA-F01	GGCAATGGTCATGAAGCGC
PcopA-R01	CGTGTAGGTGCGATAACCG
PzntA-F01	CGCTTCCGTGCGGATAAC
PzntA-R01	GAGTTGGCGTGCTGGAAC

6.3.5 Plasmid Stability

Plasmid stability was evaluated using two engineered bacteria sourced from Prof French Lab: one with ampicillin resistance gene (*E. coli* JM109/pSB1A2_P*ars*_*lacZ'α*) and the other lacking antibiotic resistance gene (*E. coli* JM109/pSB1_P*ars*_*lacZ'α*). Fresh cultures were prepared by streaking each recombinant on LB agar plates supplemented with IPTG, X-gal and ampicillin (for recombinant with ampicillin resistance gene) stock solutions and incubated at 37^0C overnight. Distinct colonies from each plates were inoculated into 50 mL LB broth in a 250 mL capacity flask and incubated with shaking at 37^0C for 24 hours. Following incubation, cells were pelleted, washed in PBS and inoculated into LB and M9 broth media supplemented with 10 ppb arsenic (see Table 6.3) and incubated at 37^0C with shaking. No antibiotic was added as it would maintain the plasmid and result affected. Cell density was measured for 7 days continuous incubation.

Following each cell density measurement, aliquots were washed in PBS, serial dilution performed and inoculated onto variants of agar media plates (Figures 6.4 & 6.5). Blue and white colonies were counted after 24 hour incubation at 37^0C.

6.3.6 Metal detection assays using recombineered bacterial sensors

Metal detection assays were performed in ZBM3 (but arsenic tests were done in ABM6 rather than ZBM3) medium having bromothymol blue pH colour indicator in 1.5 microcentrifuge tubes in triplicate by inoculating 0.1 mL inoculum of overnight cultures and supplemented with corresponding metal stock solutions and incubated at 37^0C statically in an upright position for 24 hours. Following incubation, cells were pelleted and absorbance of supernatants was measured.

6.4 Results

6.4.1 Testing growth and function of *E. coli* strains in M9 broth medium

E. coli strains used for transforming biosensor cassettes were tested for growth and function in variants of M9 broth medium (see section 6.3.4). Results obtained showed that there was no significant growth in media with lactose as carbon source whereas tremendous growth was observed in media having glucose (Figure 6.2). This demonstrated that the cells which failed to utilise lactose but grew in glucose lacked *lacZ* genes, an indication that they contain the *lacZΔ*M15 mutation which requires the *lacZ'α* gene for functional complementation. Similarly, *E. coli* DH10B strain was found to grow best in all media variants with glucose, although all the three *E. coli* strains were selected as chassis for the transformation of the sensor plasmids.

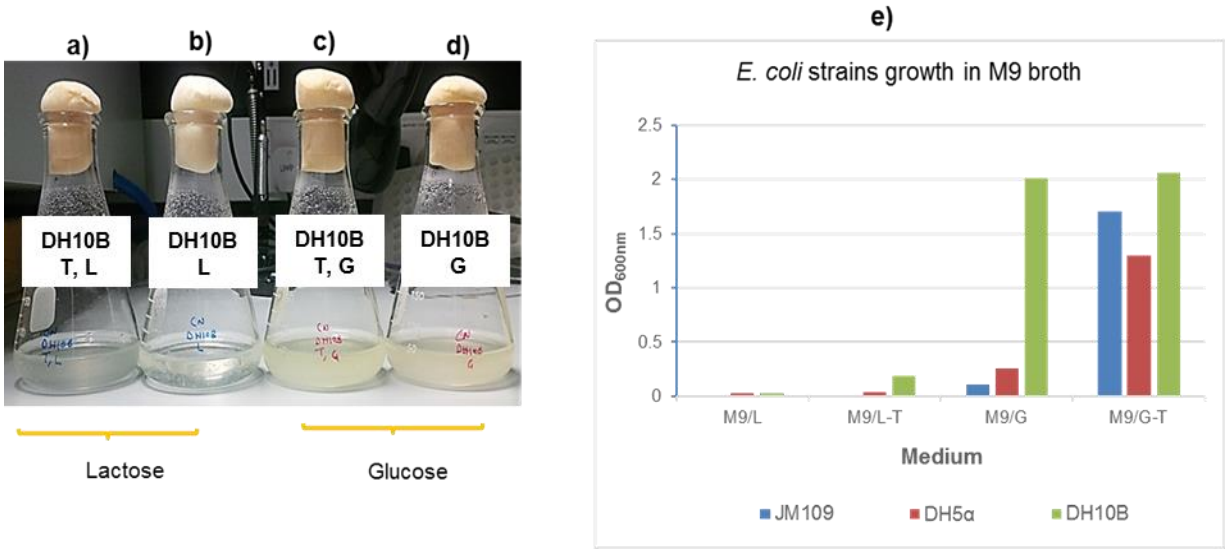


Figure 6.2: Testing growth and function of *E. coli* strains in M9 broth medium. Shown above was *E. coli* DH10B/ pSC101-*BAD-gbaA* grown in M9 broth medium supplemented with **a**) thiamine and lactose only; **b**) lactose; **c**) thiamine and glucose; **d**) glucose only. The cells were incubated at 37^0C for 24 hours with shaking.

6.4.2 PCR and gel assay

Following PCR and transformation of sensor cassettes onto *E. coli* genome, insert sizes were confirmed through colony PCR and miniprep following protocols. Results obtained when both products were ran on gel showed that the band sizes matched the correct number of nucleotide sequences inserted (Figure 6.3 b-d). The constructs were further confirmed through sequencing.

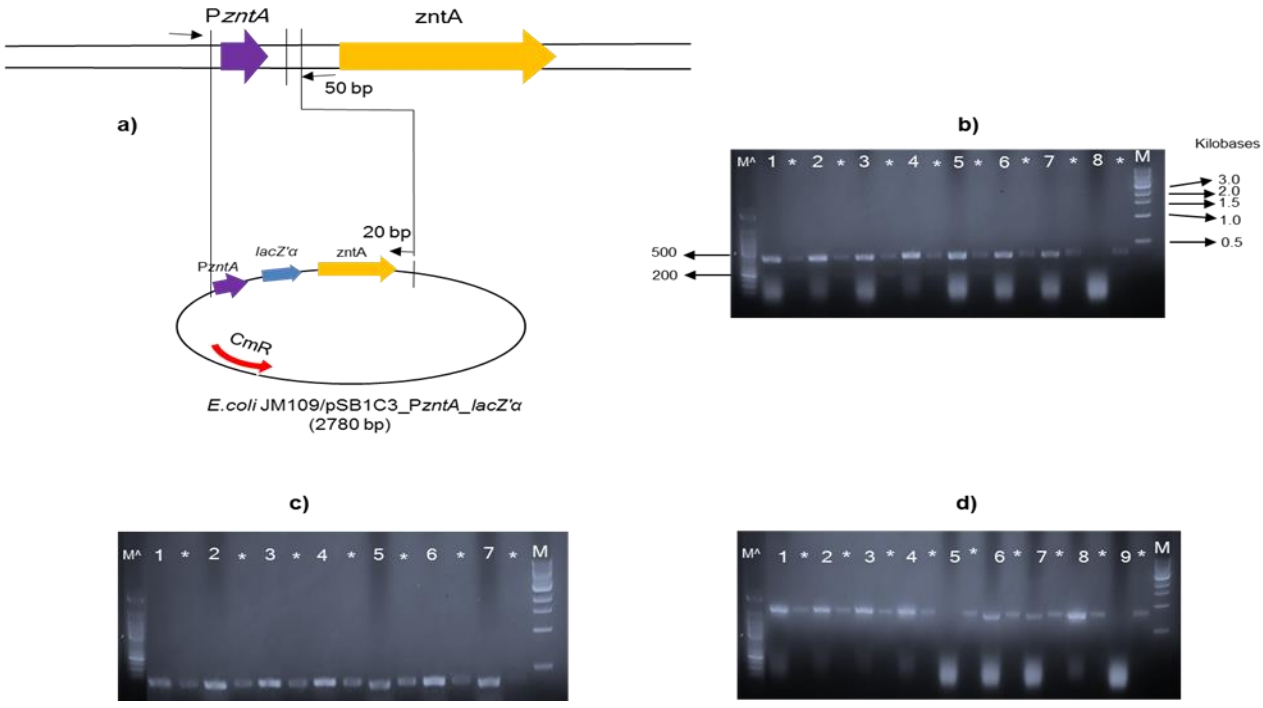


Figure 6.3: Lambda Red recombineering. **a**) Design of oligonucleotides for insertion of bacterial sensor cassettes onto *E. coli* genome. Firstly, primers flanking the insertion construct (20 bp of overlap with the insertion construct), with added 50 bp of the homology matching the *E. coli* K-12 (MG1655) genome flanking the region in which the new gene will be inserted (the overhang part) was designed. PCR conducted using plasmid DNA from respective biosensor construct as template. Agarose gel electrophoresis of **b**) zinc (498 bp); **c**) copper (491 bp); and **d**) arsenic (876 bp) biosensor cassettes following colony PCR. Key: 1 – 9 = samples screened through colony PCR; asterisks= samples screened through miniprep; M^= 50 bp DNA Ladder; M= 1 kb DNA Ladder; Cm^R= Chloramphenicol; (→ = forward primer); ← = reverse primer).

6.4.3 Plasmid loss determination

The stability of plasmids with /and without antibiotic resistance genes was tested (see section 6.3.5). Optical density obtained showed that the cells grew more rapidly in LB and M9/glucose media within 48 hours than in media variants with lactose (Table 6.4). However, there were decline in growth rate in the former from 72 hours possibly due to utilisation of available sugar and production of wastes leading to cell death. Meanwhile, media variants with lactose supported growth throughout the 7 days testing period.

Table 6.4: Optical density of cells to determine plasmid stability

Time (days)	pSB1A2				pSB1_			
	LB	M9/Glu	M9/Lac	M9/Lac/As	LB	M9/Glu	M9/Lac	M9/Lac/As
1	3.69	2.69	0.128	0.11	3.48	2.26	0.122	0.116
2	3.08	2.45	2.00	1.98	3.03	2.34	1.67	1.30
3	2.96	1.99	3.56	3.67	2.86	1.74	3.02	3.19
4	2.84	1.95	3.36	3.63	2.74	1.74	2.79	2.96
5	3.01	1.94	3.49	3.99	2.65	1.66	2.76	3.32
6	2.78	1.95	3.73	4.10	2.66	1.75	2.74	3.32
7	3.00	2.17	3.72	4.13	2.73	1.68	3.57	3.13

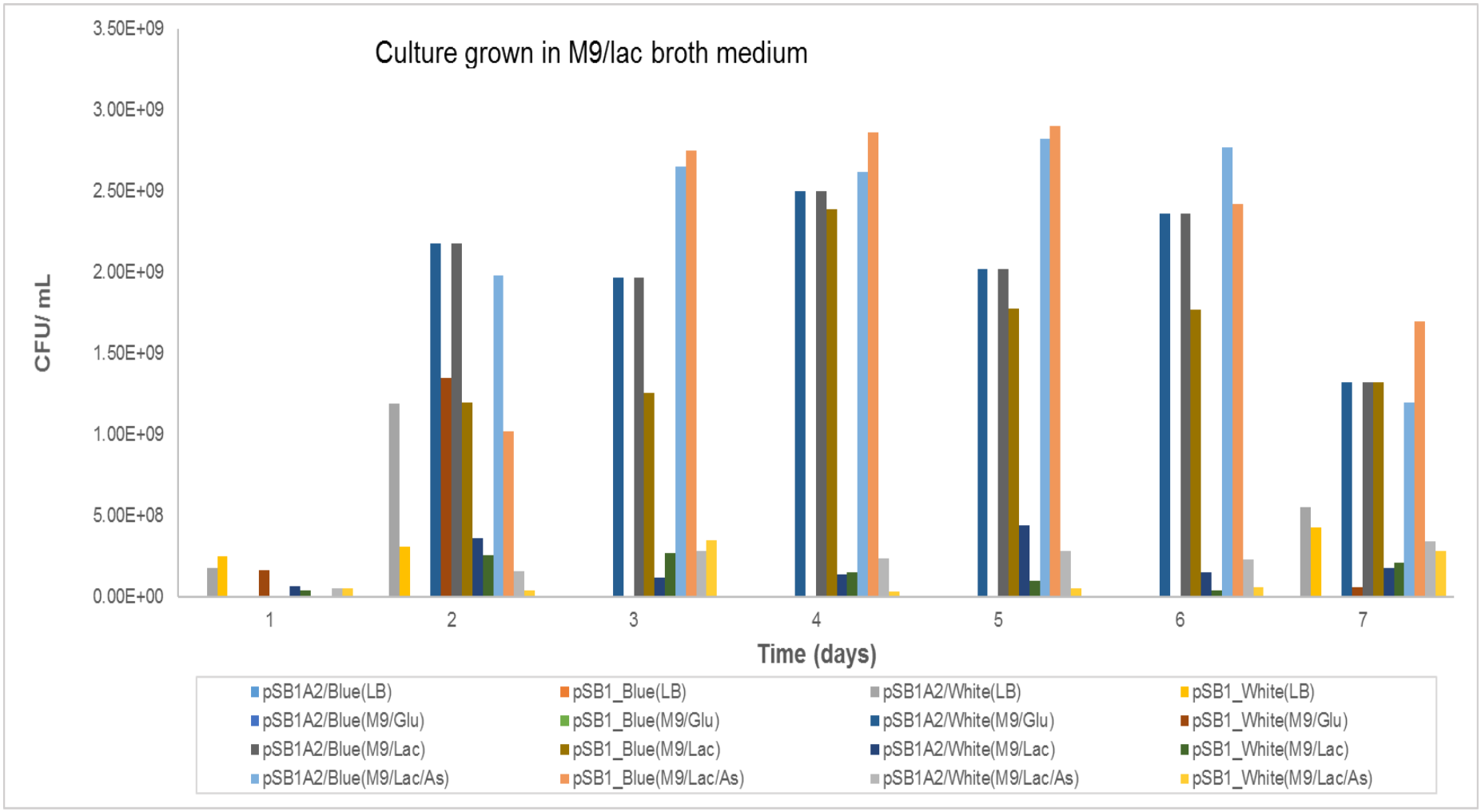


Figure 6.4: Plasmid loss determination using pSB1A2_J33202 and pSB1_J33202 in different agar plate media. Cell cultures in M9/lactose incubated for 7 days while optical density was measured each day. Aliquots were serially diluted and plated on agar media. Plates were incubated at 37^0C for 24 hours and blue and white colonies counted. **Key:** As= arsenic. Glu= glucose. Lac= lactose

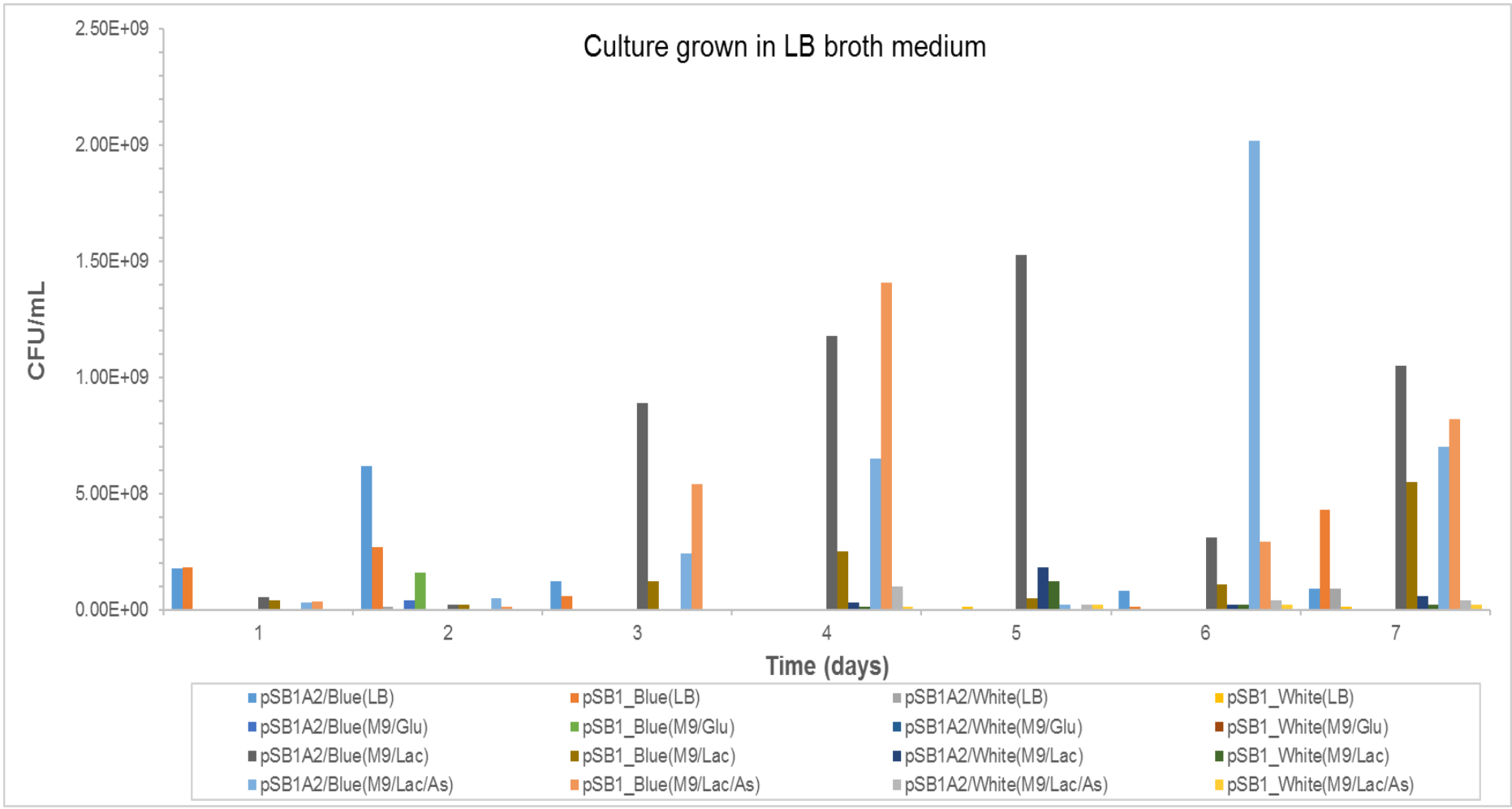


Figure 6.5: Plasmid loss determination using pSB1A2_J33202 and pSB1_J33202 in different agar plate media. Cell cultures in LB incubated for 7 days while optical density was measured each day. Aliquots were serially diluted and plated on agar media. Plates were incubated at 37^0C for 24 hours and blue and white colonies counted. Key: As= arsenic. Glu= glucose. Lac= lactose

The colony count of the two constructs showed that the plasmids were stable and maintained over the period tested. Multiple blue colonies of the plasmid with antibiotic resistance gene as well as the plasmid without antibiotic gene were observed in plates with all variants of agar media from day 2 to day 7 (Figure 6.4). This was particular with cultures grown in M9/lactose broth, diluted and plated on M9/lactose agar media but more in plates supplemented with arsenic. However, little or no blue colonies were found on M9/glucose plates as colonies found were mostly white throughout the days tested. Although white colonies were found from both plasmids plated on variants of agar media particularly those with carbon source other than lactose which indicated plasmid loss, it could be deduced that plasmids can be maintained without antibiotics.

Similar results were obtained with cultures cultivated in LB broth medium before dilution and plating. However, the cells did not grow well when plated on LB and M9/glucose agar media following dilution as little or no blue or white colonies were observed (Figure 6.5). Hence it is important to culture the plasmids in broth media having lactose as carbon source since they contain *lacZ* gene for the fermentation of lactose.

6.4.4 Metal detection assays using recombineered bacterial sensors

Metal detection assays were performed in ZBM3 medium as described in (section 3.4.8). This was done using the recombineered genome integrated strains, and not the plasmid strains. Results obtained showed that all the tubes turned yellow (false positive results), except the negative control (medium and metal) and cell-free tubes (Figure 6.6 left). The weakness of our method could be that by selection for *lacZ*+ strains (with strong X-Gal activity), we may be selecting for those with high background

activity, hence the need to use weaker ribosome binding sites (RBS), or promoter with weaker strength. As expected, there was no colour change in positive control tubes conducted with non-transformed *E. coli* DH10B strain (Figure 6.6 right) as indicated by the high supernatant absorbance values.

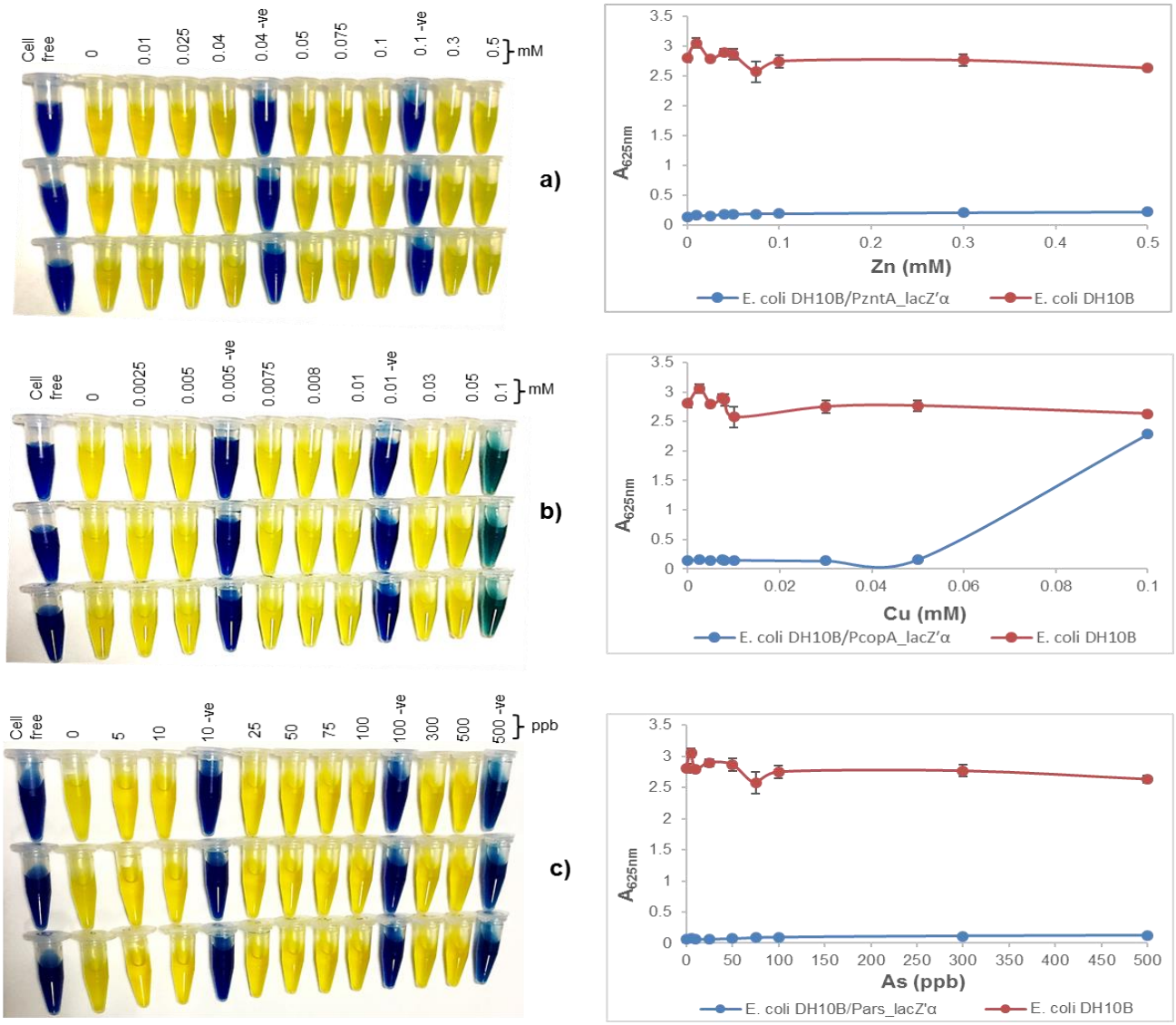


Figure 6.6: Metal detection assay using biosensors lacking antibiotic resistance genes. **a**) Zinc biosensor. **b**) Copper biosensor. **c**) Arsenic biosensor. Assay conducted in ZBM3 with bromothymol blue as a pH indicator. Each assay was conducted in triplicates and tubes statically incubated at 37^0C for 24 hours. Samples shown are tubes (left-hand sides) having 100 µL inoculum size while absorbance values of supernatants (right-hand sides) were obtained following centrifugation of samples. The *error bars* show the standard error calculated from three replicates.

6.5 Discussion

The use of antibiotics and antibiotic resistance genes in engineering recombinant proteins and producing therapeutic DNA come with many drawbacks. There are small but avoidable risks of transferring antibiotic resistance genes genetically engineered into transgenic organisms to environmental microorganisms. There are also regulations hindering the field use of recombinant microorganisms containing antibiotic resistance genes, hence restricting their use to the laboratory. These drawbacks can be highly mitigated by the use of alternative systems which circumvent the use of antibiotic resistance genes (Yu *et al*., 200; Vidal *et al*., 2008).

In this study, the characterisation of homologous recombination between linear DNA and the bacterial chromosome based on the functions of λ recombination with very short homologies has been demonstrated. We have shown that the novel biosensor cassettes can be successfully integrated onto *E. coli* chromosome through the recombineering technique. The use of antibiotic selection marker to prevent loss of plasmid can be replaced by this promising alternative. Yu *et al*., (2000) and Muyrers *et al*., (2004) reported the efficiency of this system in which 30- to 50-bp homologies were recombined *in vivo* with several advantages. This technique eliminates multiple steps generally involved in making recombinant DNA molecules, DNA fragments are generated without restriction enzyme digests, also different DNA fragments at novel junctions are joined without DNA ligase reactions.

We also observed that there was variability of Red/ET recombination efficiency between the strains used. Overall number of colonies were less for DH5α and JM109 *E. coli* strains in all experiments conducted. However, it was observed that the number of colonies and percentage of clones with correct insertion were higher in *E. coli*

DH10B. The plasmid stability test conducted showed that there was no remarkable difference in the number of blue colonies observed in both plasmids within the period tested, but little or no colonies were observed in media plates with glucose as carbon source. Where present, most of those colonies were basically whitish which probably indicated loss of plasmid. Heavy metal testing using the systems showed that specific metals were detected by the corresponding recombinant. Nonetheless, all the tubes turned yellow including those without metals indicating false positive results. This could possibly be as a result of the system exhibiting high background activity which could possibly be corrected using weaker ribosome binding sites.

Chapter 7: Discussion and conclusion

Water and food are essential commodities needed for the sustenance of human life. However, these resources are basically the source of human exposure to metal and biological contaminants. Various health challenges have been the result of prolonged and recurrent exposure to heavy metals which have been implicated in an array of human health disorders, like kidney and liver dysfunctions, various kinds of cancer, skin lesions, various kinds of cancer, etc. In many countries of the world, the authorities involved have been responsible for making available safe drinking water which has been a global challenge. Metals play significant roles in the growth and development of living organisms but can be lethal at elevated concentrations due to their interference with normal biological processes. There has been an increase of awareness suggesting that heavy metal toxicity heavily depend on their chemical form, which had led to increasing interests in the quantitative measurement of individual species. One of the significant features of global research efforts is life quality improvement. Hence, accurate and rapid monitoring of toxins, environmental pollutants, traces of hazardous chemicals, hormones, and/or pathogens is a critical task in environmental management, health care and homeland security, as a result of their significant impact on human life and the environment.

The present study focused on the development of novel recombinant chromogenic bacterial sensors for the detection of heavy metals, and incorporation of novel techniques for simultaneous detection of biological contaminants, particularly coliforms, in environmental samples. These whole-cell biosensors work by allowing growth on lactose medium with a pH bromothymol blue colour indicator which changes to yellow following fermentation when metals are present. For *lacZ'α* gene to be

expressed in zinc biosensor PCN1b (pSB1C3_P*zntA*_*lacZ'α*), the gene was placed under the control of ZntR and the promoter of *zntA* which originated from *E. coli* chromosomal DNA. When Zn^{2+} is present, *lacZ'α* transcription is induced, causing a change of the bromothymol blue pH indicator from blue to yellow which can easily be seen. However, in addition to Zn^{2+}, colour change was also induced by Cd^{2+}, Hg^{2+} and Pb^{2+} in the Zn biosensor system. Consequently, the Zn biosensor accurately detected zinc, cadmium, lead and mercury concentrations (3 mg/ L, 0.003 mg/ L, 0.01 mg/ L and 0.001 mg/ L respectively) below the recommended WHO limits. This phenomenon was supported by the work of Ivask *et al*., (2002), who termed their sensor as "zinc – cadmium sensor", since their investigation corresponded with Binet and Poole (2000) who also reported that the *znt* resistance system (ZntR and *zntA* promoter) is also induced with Cd^{2+} and Hg^{2+} in addition to Zn^{2+}. It has also been reported that Zn^{2+} as well as other two-valent ions such as Cd^{2+}, Co^{2+}, Ni^{2+} and Pb^{2+} are exported from the cells by the ZntA protein of *znt* resistance system (Beard *et al*., 1997; Gatti *et al*., 2000). The expression of *zntA* is mediated by ZntR, belonging to the MerR family of transcriptional regulators, which binds the *zntA* promoter, thereby activating transcription when Zn^{2+} is present (Outten *et al*., 1999). Therefore, it is not surprising that the developed zinc system was also induced by Hg^{2+}, Cd^{2+} and Pb^{2+}. It is also important to note that the Zn^{2+}, Cd^{2+}, Pb^{2+} and Hg^{2+}concentrations required to induce pSB1C3_P*zntA*_*lacZ'α* are very different. The peak induction of the system by Pb^{2+}, Cd^{2+} and Hg^{2+} was observed at 1 µM concentrations of each of the metal which drastically reduced when the concentrations of the metals reached 100 µM, indicating metal toxicity of the system at this level. Unlike Pb^{2+}, Cd^{2+} and Hg^{2+}, Zn^{2+} concentration at 100 µM was not toxic to the cell as the induction profile was still on the increase. This suggests that the interrelationship between the sensitivity and toxicity of metals

is not surprising since the ‘sensing’ elements of the current biosensors originated from living organisms (Ivask *et al*., 2002).

In copper sensor PCN2a (pSB1C3_P*copA*_*lacZ'α*), *lacZ'α* was expressed under the control of CueR and *copA* promoter. Data obtained in the study also showed that in addition to Cu^{2+}, Ag^{2+} and Au^{2+} also induced the *cue* resistance system (CueR and promoter of *copA*). The expression of *copA* is reported to be regulated by CueR, also a member of the MerR family, which interact with Cu^{2+} and the region of copA promoter (Outten *et al*., 2000). Data obtained are in conformity with previous reports that copper, silver and gold ions induce *copA (*Rensing *et al., 2000)*. Similarly, copper, silver and gold levels (2 mg/ L, no guidelines for silver and gold respectively) were detected by the novel copper biosensor, below the limits recommended by the WHO.

Data obtained from testing the reaction of both copper and zinc biosensors against each metal ions showed that the novel copper bacterial sensor did not react/or detect zinc metal and vice versa. To date, recognition of metal and transduction of signal by both ZntR and CueR on molecular basis is yet to be clarified. Exploitation of information on the metal binding site of regulators of the MerR family will go a long way in generating reporter strains having new metal specificities important in monitoring environmental metals. Preferably, the concurrent use of bacterial sensors having different response specificities, according to Riether *et al*., (2001), has the potential of allowing each metal in a mixture to be qualitatively detected. Copper proteins take part in various biological processes, and disease states or pathophysiological conditions normally occur when these enzymes are insufficient, or there are changes in their activities. Since copper is highly toxic even at low concentrations, there is the need to maintain copper homeostasis which is required for the evolution of aerobic metabolism. This is partly because copper is a redox-active

transition metal which is possibly responsible for the generation of superoxide or other reactive oxygen species in the cell. Copper also appears to change from the Cu(II) to the Cu(I) oxidation state under anaerobic conditions and becomes highly toxic, probably as a result of its diffusion via the cytoplasmic membrane. Hence, copper need to be accurately and rapidly detected to maintain the intracellular copper concentrations within very thin limits.

It is reported that chelating agents of natural (humic and fulvic acids) or anthropogenic (EDTA or polyphosphates) sources can alter the concentrations of biologically available metals (Riether *et al*., 2001). Microbial processes can also alter metal solubility, for instance, metal sulphide precipitation and immobilization in sediments are mediated by sulphide produced by sulphate-reducing bacteria. This is widely used in bioremediation procedures (Gadd, 2000). By using two metal ligands, we have shown that our bacterial sensor strains have the potential to differentiate between bioavailable from total metal in the assay. By reducing availability (and toxicity) and gradual restoration of chromogenic response of our biosensor systems when exposed to different metal concentrations at toxic levels proves that our systems support the hypothesis of complexation of metal ions. Our results are in agreement with Riether *et al*., (2001), Campbell *et al*., (2000) and Tauriainen *et al*., (2000), where luminescent reporter sensors were used to test EDTA effect on either metal toxicity or bioavailability. We also observed that sulphide from Na_2S has effect on metal bioavailability to *E. coli*, although with less effect than EDTA chelation, possibly through the formation of insoluble metal sulphides difficult to penetrate the cell.

In terms of storage and distribution, it has been demonstrated that adding lactose as a cryoprotectant to cells prior to air- and freeze-drying can aid the survival of biosensor cells tested. Air-dried cells showed to remain very stable than the freeze-dried cells

for the first 10 days of storage. This can be better explained to be as a result of the formation of glassy consistency in air-dried tubes, resulting in a tight packing and a firm top layer which may provide better protection against damage cause by oxygen. However, the tubes of air-dried cells containing 100 µL of cell-lactose suspension originally showed higher survival but there was more rapid die-off. This may be possibly due to higher moisture content in air-dried tubes after drying due to insufficient drying. It has also been demonstrated that whole-cell biosensor cells can be preserved on Gel Blot paper. Despite reduced viability in all the dried formats following several weeks of storage at room temperature, dried cells are still effective in the assays conducted. Although these have not been tested on the field, tests conducted with seeded positive metal stock solutions demonstrated successful detection of arsenic, cadmium, copper, lead, mercury, and zinc.

In summary, the future of using β-galactosidase gene fusion technology in the monitoring of environmental contamination by metals has been proved by the designed systems. Though simple to use, cheap, sensitive and portable, the novel developed biosensors can alternatively be used in place of expensive spectrometric devices or other high-end laboratory equipment, and they also have the potential of evaluation of bioavailable portion of metals. One of the novel idea in this work is to develop a simple, disposable device which can easily be mass produced and adapted to co-detect the growth of other bacteria, such as coliforms, so the same format of sensor detects both toxic metals and coliforms in a single unit. In this sense, designers will be instructed to produce a simple and portable kit with two compartments; one of which will house dried bacterial sensor cells and ZBM3 medium, while the other compartment will contain only dried medium. Water samples containing metal and coliform contaminants will be divided into two portions. One part with added

chloramphenicol will be used to rehydrate the former compartment to test for metals, while the other part without antibiotic will be used to rehydrate the latter compartment housing only dried ZBM3 medium to test for coliforms following incubation at 37^0C for 24 hours. The need for safe disposal of the sensor device and lifetime environmental impact will also be considered through consultations with designers, social scientists etc.

However, the developed systems work within 24 hours and possibly need optimization for rapid response. This work has also contributed in steps towards actualizing the use of reporter strains of organisms in monitoring of environmental samples contaminated by metals. Currently, there are various attractive and innovative techniques for precise immobilisation of bacterial biosensors. However their successful implementation in actual devices continued to be impeded by problems in maintaining the survival and activity of the cells (Bjerketorp *et al*., 2006). Interestingly, regarding different preservation methods, high variability exists between different bacteria strains and species. Following dehydration, it is also important to note the physiological condition of biosensor cells regarding their viability and activity after rehydration. Belkin (2003) reported that the sensing promoter of bacterial biosensors can be activated by the stress exerted by the preservation protocol which could possibly make the reporter cells ineffective even when their viability is maintained.

This work has demonstrated that heavy metals and coliform can be accurately and sensitively detected simultaneously in a single system. This is particularly important for resource limited economies which lack basic facilities and skilled personnel needed for modern detection techniques. However, there is still room for improvement to make the system more rapid for the detection of metal and biological contaminants in drinking water. Further investigation should also be carried out to ascertain the

efficiency of cells dried with sensor media and stored for a prolonged time at room temperature. There is also the need to interpret the results within the larger context of drinking water risk assessment as with all water quality tests (World Health Organisation, 2004).

Overall, it has been demonstrated that the engineered antibiotic-free system that carry the genetic constructs on their chromosome can serve as a promising alternative approach to avoid incorporation of an antibiotic resistance gene and increase the genetic stability of the bacterial sensor cells. It was also shown that the genetic constructs were successfully integrated onto bacterial chromosome. Although the system was not successful as confirmed by false positive results obtained, further investigations should be focussed on employing weaker ribosome binding sites (RBS) or the use of promoters with weaker strength. Previous results obtained in this study showed that these approaches are tenable and can be incorporated into the system to reliably detect heavy metals of interests.

Chapter 8: References

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