
Whole-Cell Bacterial Biosensors and the Detection of Bioavailable Arsenic

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FOREWORD

Arsenic can be found at most sites on the National Priority List and at the top of the Agency for Toxic Substances and Drug Registry's (ATSDR) 2001 CERCLA Priority List of Hazardous Substances based on its toxicity to human health and potential for human exposure (ATSDR 1993). Current risk assessments for arsenic are calculated based upon the total arsenic present. However, toxicity, solubility, and mobility can all vary depending upon which species of arsenic is present, thus affecting the bioavailability of the arsenic contamination. The bioavailable fraction is the portion of arsenic that is available for biological uptake. Risk assessments could be over or under-estimating the potential risk to the environment and human health by not considering the bioavailability of the arsenic at a contaminated site.

Methods have been developed to estimate the bioavailability of toxicants, including arsenic. Whole-cell bacterial biosensors are genetically engineered bacteria capable of measuring bioavailable arsenic. A reporter gene is coupled with arsenic resistant genes and inserted into a host bacteria strain. In the presence of arsenic, the biosensor emits light which can be measured and used to determine the bioavailable concentration of arsenic. The purpose of this paper is to provide a detailed analysis of whole-cell bacterial biosensors. It will investigate the state and practice of using whole cell bacterial sensors for measuring the bioavailability of arsenic.

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The report is available on the Internet at www.clu-in.org/studentpapers/.

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CONTENTS

	<u>Page</u>
1 Introduction	1
2 Arsenic	1
3 Assessing Arsenic Contamination	2
4 Whole-Cell Bacterial Biosensors	3
4.1 Reporter Genes	4
4.2 Contaminant-Sensing Components	5
5 Arsenic Sensing Biosensors	6
6 Methods For Using Biosensors	8
6.1 Measuring Luminescence/Fluorescence	9
6.1.1 Luminometer/Fluorimeter	9
6.1.2 Optical Fibers	9
6.2 Optimizing Results	10
6.3 Interpreting Results	10
6.4 Applying Results	11
7 Advantages and Disadvantages	12
8 Potential	14
9 Conclusions	15
10 Bibliography	15
Appendix A: Developed Biosensors	18
Appendix B: Commercially Available Kits	19

FIGURES

Figure 1. (A) Sequence of <i>ars</i> Operon (B) Arsenic Resistance Mechanisms	6
Figure 2: General Mechanisms of Biosensor	6
Figure 3. Plot of Induction	9
Figure 4: Example of Dose-Response Curve	11
Figure 5: Example of Dose-Response Curve	11

1 Introduction

Arsenic is the 20th most abundant element in the earth's crust (NRC, 1977) and is a common contaminant of sites on the National Priority List. Arsenic has the ability to change valence states and form many inorganic and organic compounds, which vary in their solubility, mobility, and toxicity. The current method for assessing the potential risk at an arsenic contaminated site involves the analysis of the total inorganic arsenic (EPA, 1989). Speciation techniques such as atomic absorption spectrometry and sequential extraction procedures are used to determine the form of arsenic present and estimate its bioavailability (Jain and Ali, 2000; Caussy, 2003). The ability of a contaminant to interact with organisms is termed its bioavailability. Bioavailability can be influenced by environmental properties such as pH, cation exchange capacity, soil iron content, and clay content (NRC, 2003). Techniques are being developed that will better estimate a contaminant's bioavailability without expensive equipment or the need for specialized training.

One such method, whole-cell bacterial biosensors, consists of genetically engineered bacteria containing a contaminant-sensing gene capable of detecting the presence of an analyte, coupled with a reporter gene capable of producing a detectable response. Bioavailable arsenic is able to penetrate the membrane of the bacterial biosensor and trigger the detectable response, luminescence, which can be measured. From this measurement, a concentration of bioavailable arsenic can be estimated (Biran et al., 2003; Tauriainen et al., 2000, Turpeinen et al., 2003; Daunert et al., 2000). Limited understanding of the biochemistry involved in the response of higher organisms to arsenic restrict the applicability of biosensors to higher organisms. However, the information gathered from biosensors can at least be qualitatively incorporated into risk assessments.

2 Arsenic

Arsenic occurs naturally in the environment, often released with the weathering of sulfide minerals, enargite, orpiment, and realgar. The dominant anthropogenic source of arsenic is the mining and smelting of ores. It is also associated with glass and chemical manufacturing and is used as a wood preservative in pressure-treated wood. Arsenic has been registered as a pesticide for weed control. The natural arsenic concentration varies across the country from 1 part per million (ppm) to 40 ppm but can reach much higher levels in contaminated areas surrounding mines or waste disposal facilities. Most water sources naturally have levels of arsenic around 1 parts per billion (ppb) but heavily contaminated waters can have levels as high as 1,000 ppb (ATSDR, 1993).

Arsenic's complex chemistry and ability to form many different compounds makes it a difficult element to understand. Arsenic (As) is not quite a metal but instead a metalloid because it generally can be found as an oxyanion. Arsenic is most commonly found in two valence states, As(III) and As(V). The most common inorganic As(III) compounds found are arsenic trioxide, sodium arsenate, and arsenic trichloride. As(V) inorganic compounds such as arsenic pentoxide, arsenic acid, and arsenates are also fairly common. Arsenic forms organic compounds and is methylated by microorganisms, but neither group is considered as toxic as inorganic As(III) and As(V) compounds (Klaassen, 2001).

Whole-Cell Bacterial Biosensors and the Detection of Bioavailable Arsenic

The toxicity of arsenic depends upon the valence state, form, and exposure duration, either acute or chronic. As(III) is more toxic than As(V) for acute or short exposures, but the body's ability to reduce As(V) to As(III) means chronic or long-term exposure to either can be equally toxic. While most organic arsenic compounds are not as toxic, chronic exposure to some can be as toxic as inorganic arsenic compounds. Acute exposure to high levels of inorganic arsenic can be fatal while acute exposure to lower levels can result in a sensation of "pins and needles" in hands and feet, vomiting, decreased production of red and white blood cells, abnormal heart rhythm, and damage to blood vessels (ATSDR, 1993). Skin lesions are a common sign of arsenic exposure (Klaassen, 2001). Chronic exposure is especially damaging to the liver, causing cirrhosis. It is also neurotoxic, damaging peripheral and central nervous systems. On a cellular level, arsenic interferes with the functions of the mitochondria and induces deletion mutations and chromosomal aberrations in DNA. Lastly, arsenic is a carcinogen associated with skin, lung, and liver cancer (Klaassen, 2001).

3 Assessing Arsenic Contamination

Current risk assessments for evaluating the potential ecological or human health risks associated with arsenic exposure rely on an analysis of the total arsenic content in the environment. However, because there are a great number of naturally occurring arsenic compounds that are easily transformed and a limited understanding of the individual toxicity of different compounds, a more detailed calculation of risk is difficult. The concentrations of each form of inorganic arsenic are not differentiated during risk assessments but remain combined instead. Organic and methylated arsenic compounds are not incorporated into the risk assessment (EPA, 1989). The ATSDR Toxicological Profile for arsenic does not provide detailed information on the toxicity of all forms of arsenic. According to ATSDR and risk assessment guidelines, inorganic compounds of arsenic have the same toxic endpoint, despite the greater toxicity of As(III) compounds compared to As(V) compounds (ATSDR 1993).

Differentiating between the forms of inorganic arsenic compounds is essential to an accurate risk assessment, because certain characteristics such as mobility and solubility can vary and ultimately effect the toxicity and bioavailability of the arsenic (Cullen and Reimer, 1989). The bioavailable fraction is the portion of the total quantity of the chemical present that is available for uptake by an organism (Klaassen, 2001). The bioavailability of arsenic is not considered in risk assessments or it is assumed that the arsenic is 100 percent bioavailable (EPA, 1989). However, it is becoming more evident that the behavior of arsenic, and therefore its bioavailability, is dependent upon its physical and chemical properties (Cullen and Reimer, 1989).

The differing arsenic compounds and their properties have been overlooked in part due to analytical limitations. Recent progress has improved the analytical procedures for detecting and differentiating between arsenic compounds (Caussy, 2003). Speciation analysis is used to estimate the bioavailability of arsenic in soil, water, sediment, or air samples by differentiating between the various forms of arsenic. Methods for arsenic speciation include atomic absorption spectrometry (AAS), inductively coupled plasma-atomic electron spectrometry (ICP-AES), gas chromatography, X-ray absorption fine structure spectroscopy (XAFS), and sequential extraction

Whole-Cell Bacterial Biosensors and the Detection of Bioavailable Arsenic

procedure (SEP) (Jain and Ali, 2000; Caussy, 2003). These methods offer a powerful analysis when combined but require expensive, bulky equipment, high-grade analytical reagents, and specialized training (Caussy, 2003).

Other methods are being developed that can identify the bioavailable fraction of arsenic to be incorporated into risk assessments (NRC, 2003). One such method uses genetically engineered bacteria that illuminates in the presence of bioavailable arsenic. These whole-cell bacterial biosensors are being developed to detect arsenic and provide at least qualitative information about the bioavailability of arsenic in the contaminated soil or water.

4 Whole-Cell Bacterial Biosensors

Bacteria can be used as biosensors to demonstrate the toxicity of a variety of environmental media including soil, sediment, and water by coupling bacteria to transducers that convert a cellular response into detectable signals (Biran et al., 2003). These bacterial biosensors are engineered by pairing a reporter gene that generates a signal with a contaminant-sensing component that responds to chemical or physical change, such as exposure to a specific analyte. When the biosensor is exposed to such a change, the sensing component stimulates the reporter gene through a biochemical pathway in the cell. The reporter gene then produces a measurable response, such as emitting visible light, which is indicative of the degree of chemical or physical change (Biran et al., 2003; Tauriainen et al., 2000, Turpeinen et al., 2003; Daunert et al., 2000). Several biosensors have been developed that indicate toxicity of any chemical or physical change; new biosensors are being developed to respond to particular analytes. Such biosensors have been developed for heavy metals and metalloids including arsenic, cadmium, mercury, and lead (NRC, 2003).

Biosensors measure the bioavailable concentration for the contaminant they are designed to detect (Tauriainen et al., 2000). To test the measurements made by biosensors, a chelating agent known to decrease bioavailability of lead was added to a lead solution. Measurements of the lead solution containing chelating agents were taken and compared to measurements of the lead-only solution. A decrease in the biosensor's luminescence matched a decrease in bioavailable concentration of lead in the solution. This demonstrates that biosensors are sensitive to the bioavailable fraction of the contaminant and their luminescence reflects the bioavailable concentration (Tauriainen et al., 2000).

Biosensors have been further tested by comparing their results with the results from chemical analysis of arsenic contaminated samples (Turpeinen et al., 2003). Chemical analyses such as sequential extraction procedure, can be used to determine the total arsenic, water-soluble arsenic, and acid-soluble arsenic content of the sample. The acid and water soluble contents are used to determine the mobility of arsenic, in an attempt to clarify true exposure potential. The results from a biosensor correlated moderately with the water-soluble arsenic determined by chemical analysis, but not with the acid-soluble or total arsenic content. Therefore, the acid-soluble or water-soluble arsenic content does not completely represent the bioavailable content of arsenic (Turpeinen et al., 2003).

4.1 Reporter Genes

A reporter gene encodes for a mechanism that produces a detectable cellular response. It determines the sensitivity and detection limits of the biosensor. Specific characteristics are needed for the reporter gene to be used in a biosensor. The gene must have an expression or activity that can be measured using a simple assay and reflects the amount of chemical or physical change. Also, the biosensor must be free of any gene expression or activity similar to the desired gene expression or activity that is being measured. Ensuring that the biosensor is free of any similar gene expression or activity prevents misinterpretation of the response and guarantees the measurement directly reflects the desired chemical or physical change (Daunert et al., 2000). Several reporter genes meet the necessary requirements and are frequently used (Turpeinen et al., 2003; Tauriainen et al., 2000; Petänen et al., 2001; Roberto et al., 2002).

The most commonly used reporter gene is the *luc* operon from the firefly *Photinus pyralis*. It has been widely used as a monitor of gene expression and a reporter in bacterial biosensors (Daunert et al., 2000). The *luc* operon produces the enzyme, luciferase, capable of generating the luminescence of the firefly. The activation of the gene results in the transcription or reading of the *luc* operon, which causes the cell to produce luciferase. The enzyme luciferase spurs a chemical reaction that produces CO₂ and visible light by catalyzing the oxidation of its substrate, D-luciferin, which binds to the enzyme's active site. The visible light produced can be measured with a variety of instruments including a luminometer and optical fibers. The reaction has a high sensitivity level and a broad dynamic range, but is complicated by requirements such as the addition of the substrate, an aerobic environment, and ATP as a source of energy. However, this reporter gene has great versatility and can be mutated to produce enzymes that express a range of colors from green to red, which can then be independently controlled for multianalyte assays (Daunert et al., 2000).

Other organisms have similar genes capable of emitting light. A close homolog to the firefly is the click beetle, *Pyrophorus plagiophthalmus*, whose luciferase is capable of producing a wide range of colors (Tauriainen et al., 1999). The click beetle gene has also been used in biosensors. However, the *luc* operon from the firefly has proven better suited for biosensors than the *luc* operon from bacteria such as *Vibrio harveyi* and *Vibrio fischeri*. The firefly *luc* operon is simply more efficient at converting chemical energy to light (Tauriainen et al., 1997).

A photoprotein emits light when excited with light of a specific wavelength range. A photoprotein, green fluorescent protein (GFP), and its encoding gene from the jellyfish *Aequorea victoria* have been used in biosensors (Roberto et al., 2002). The production of GFP in the jellyfish results in the emission of a green fluorescence that can be measured. Therefore, the GFP system can be used as a reporter gene for biosensors, although it has a lower sensitivity compared to the *luc* operon. The GFP system, however, allows real time detection without the addition of substrates and without disrupting the cell's metabolism (Tauriainen et al., 1999). Also, GFP does not rely on internal reducing equivalents being produced by the cell, which may mean that this reporter gene is not as sensitive to the growth or nutritional status of the biosensor (Roberto et al., 2002). The ease of detection and the minimal metabolic cost to the host cells will lead to an increase in the use of GFP in biosensors (NRC, 2003).

Whole-Cell Bacterial Biosensors and the Detection of Bioavailable Arsenic

Another advantage of GFP is its ability to alter its stability and spectral properties through structural alterations, and thus produce mutants with improved fluorescence intensity, thermo-stability, and chromophore folding (Daunert et al., 2000). GFP is a very stable protein, which means it can accumulate in the cell over time which results in background fluorescence. Mutants can be developed with less stable protein to improve the biosensor by reducing the background fluorescence (Tauriainen et al., 1999). Measurements can be taken of the number of proteins produced or their fluorescence if the background fluorescence is considered. Results are more stable if the number of proteins is measured instead of their fluorescent activity, because their activity, the number of proteins is not dependent on the performance of each protein (Sagi et al., 2003).

4.2 Contaminant-Sensing Components

Microorganisms have developed systems for detoxifying or excreting toxic substances. These systems can be used as the contaminant-sensing component of the biosensor by detecting the substance for which it is designed to detoxify or excrete. The contaminant-sensing component is combined with reporter genes to create biosensors that can identify toxic substances at very low levels (Tauriainen et al., 1999). When the contaminant-sensing component detects the substance, it triggers the reporter gene, which produces the luminescent enzyme.

The sensing component determines the specificity of the biosensor (Daunert et al., 2000). Some microorganisms have developed genes for heavy metal resistance, which tend to be specific towards a particular metal instead of a general mechanism for all heavy metals. Thus, biosensors can be developed that detect only one specific metal. Genes have been found for resisting lead, nickel, mercury, chromium, and arsenic.

The pathway developed by bacteria to manage arsenic is used as the contaminant sensing part of the biosensor. A gene located on a plasmid encodes the mechanisms of the pathway and has been cloned for use in biosensors. The gene is highly homologous in all bacteria studied, with only slight variation in the number of components involved in the mechanism (Silver, 1998). The general mechanism remains similar in all bacteria despite such slight variations in the gene.

In gram-negative bacteria, the arsenic resistance gene remains inactive with the absence of As(III) in the cell due to the binding of the *ars* operon repressor protein to the promoter region of the gene. As(III) activates the system by binding to the repressor protein and freeing the promoter region for transcription (Roberto et al., 2002). The freed promoter region is transcribed to produce various components of the mechanism such as *arsB*, an arsenite-translocating protein that serves as a transmembrane efflux channel. This protein functions either chemiosmotically, without an energy source, or by ATP hydrolysis when coupled with *arsA*, an arsenite-specific ATPase. *ArsC*, the enzyme arsenate reductase, is also transcribed to reduce As(V) to As(III), since As(V) cannot pass through the *arsB/arsA* pump. *ArsD* is a regulatory protein for additional control over the expression of the system and *arsR* is a transcriptional repressor (Figure 1) The mechanism varies slightly in gram-positive bacteria, which lack *arsA* and *arsD*. Understanding the mechanism for arsenic resistance is necessary in order to develop an appropriate biosensor and to better understand its response.

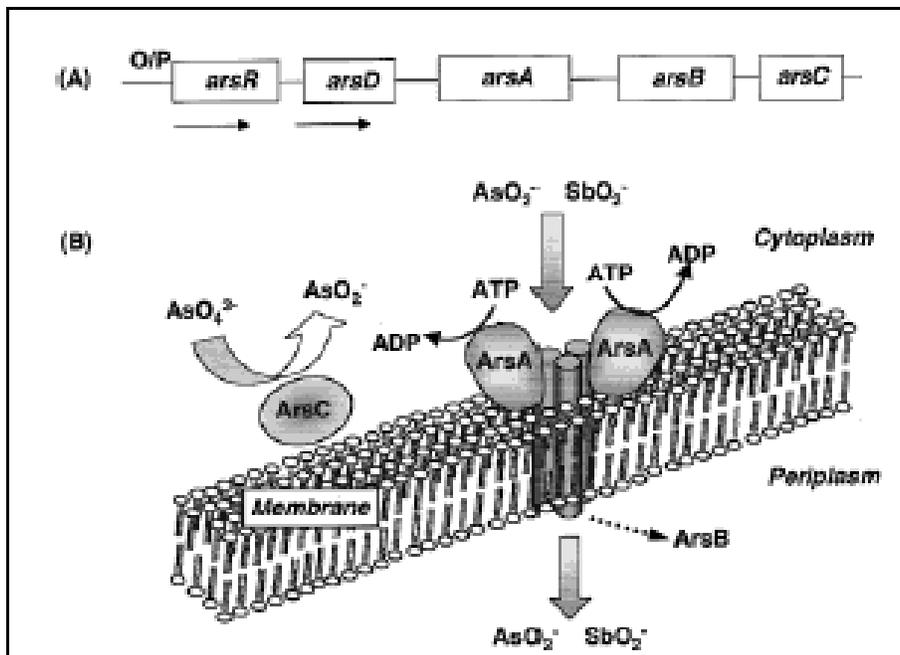


Figure 1. (A) Sequence of *ars* Operon (B) Arsenic Resistance Mechanisms (Daurert et al., 2000)

5 Arsenic Sensing Biosensors

Various biosensors have been developed and tested on a research level for detecting bioavailable arsenic (Ji and Silver, 1992; Corbisiera et al., 1993; Tauriainen et al., 1997; Petänen and Romantschuk, 2003; Roberto et al., 2002). To develop the biosensor, the arsenic resistant gene and the reporter gene are cloned and inserted onto one plasmid, which is then inserted into a host bacteria. All arsenic sensing biosensors are triggered by arsenic, the analyte, entering the biosensor and activating the transcription of the resistant gene, which is followed by the transcription of the reporter gene. The entire resistant gene is not needed, so many biosensors only use the beginning components such as the promoter. It is able to recognize the arsenic and begin the transcription of the plasmid that contains the reporter gene. The transcription of the reporter gene produces proteins, which glow in direct correlation to the amount of arsenic entering it (Figure 2).

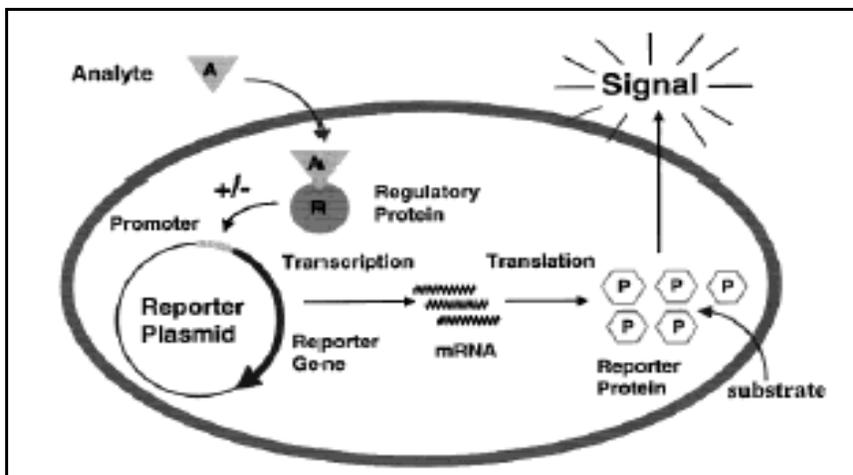


Figure 2: General Mechanisms of Biosensor (Daurert et al., 2000)

Both gram-negative and gram-positive arsenic resistance systems have been utilized in biosensors as the

Whole-Cell Bacterial Biosensors and the Detection of Bioavailable Arsenic

arsenic sensing component (Roberto et al., 2002). Constructed biosensors have used either luciferase or GFP as the reporter gene coupled with various combinations of arsenic resistance mechanism components. Various strains of bacteria have served as the host bacteria. Many combinations of reporter genes, resistance gene components, and host strains can be incorporated into a biosensor; studies have tried to determine which combination is most advantageous (Appendix A).

Ji and Silver (1992) investigated the regulation of the *ars* operon in *Staphylococcus aureus* plasmid p1258. The plasmid was inserted into various strains of bacteria to determine the pattern and causes of induction. Through gene fusion, Corbisier et al (1993) combined the *luxAB* gene encoding luciferase from *V. harveyi* and the arsenic operon from *Staphylococcus aureus* plasmid p1258, and achieved similar results. The recombinant plasmid was tested in two host strains, *Escherichia coli* and *S.aureus*, which differed in their patterns of induction. Both biosensors were induced by arsenite, arsenate, and bismuth while the *S.aureus* biosensors were also induced by antimonite. Arsenite was the strongest inducer for the *E. coli* biosensors and generated the maximum light emissions at a concentration of 10 μM (Corbisiera et al., 1993).

Another arsenic biosensor was created by Tauriainen et al. (1999) using the *luc* operon from the firefly, which is more efficient at converting chemical energy to light energy, making its luminescence more intense. The *luc* operon was coupled with the *ars* promoter and the *arsR* gene from *E. coli* plasmid R773 and inserted into two strains of *E. coli*. It demonstrated greater sensitivity than its predecessor, pTOO21, which used the *ars* operon from *S.aureus* plasmid p1258 (Tauriainen et al., 1997). Unlike the previous plasmid, the recombinant plasmid pTOO31 is able to detect both As(III) and As(V) with equal strength (Tauriainen et al., 1999). One strain of *E. coli*, MC1061, proved to be a better host for the plasmid pTOO31. It has a detection range of 33nm to 1mM for As(III) and a range of 33 μM to 33mM for As(V). Luminescence can also be moderately induced by antimony and cadmium (Tauriainen et al., 1999). *E. coli* MC1061 (pTOO31) was used to test the bioavailability of arsenic in contaminated soil samples. The sensor reacted to As(III) at concentrations 100 times lower than As(V). The sensitivity was 0.1 μM for As(III) and improved to 0.5 μM for As(V) when cultivated in the nutrient-rich Luria Bertani medium (Turpeinen et al., 2003).

S.aureus, *B.subtilis*, *E. coli*, and *P.fluorescens* all perform equally well as the host strain when testing for As(III) using either plasmid pTOO31 or pTOO21. Overall, *E. coli* tends to be the most sensitive; it detects levels as low as 100 nm and works well for both As(III) and As(V) (Tauriainen et al., 2000). However, *E. coli* may not be the best-suited host strain for testing environmental samples as well as laboratory samples, since it is not native to soil. *P.fluorescens*, a soil bacterium, may be better suited for testing soil and sediments (Petänen, and Romantschuk, 2003). *P.fluorescens* also have advantages over *E. coli* due to the low amount of substrate needed to reach the maximum luminescence (Petänen et al., 2002).

Two arsenic sensing strains were developed using the *lucGR* gene encoded for luciferin from the click beetle. The promoter and *arsR* were used from plasmid p1258 and plasmid R773 as the arsenic sensing components for plasmid pTPT21 and pTPT31, respectively. While their detection limits were similar when used in either *E. coli* or *P. fluorescens*, pTPT31 performed better than

Whole-Cell Bacterial Biosensors and the Detection of Bioavailable Arsenic

pTPT21. For *P. fluorescens* with plasmid pTPT31, induction was shown to increase steadily between 10 nm and 10 μ m, with toxic inhibition seen at 100 μ m. While *E. coli* with plasmid pTPT31 strains have detected levels of 100 nm, induction increased steadily between 10 nm and 1 μ m, with a partial drop at 10 μ m. The maximal induction for both strains was seen from 100 nm to 10 μ m. When plasmid pTPT31 was compared to plasmid pTOO31 (Tauriainen et al., 1999), no significant difference was found (Petänen et al., 2001).

Roberto et al (2002) developed an arsenic biosensor by coupling *arsR*, *arsD*, and their promoter with the gene encoding for GFP from the marine jellyfish, *A. victoria*. The recombinant plasmid was inserted in *E. coli*, which created a biosensor that produced GFP in the presence of arsenic. This biosensor is capable of detecting both As(III) and As(V) with a range of 1 to 10,000 ppb (approximately 0.01 to 100 μ m). A cell density of 10^9 cells per milliliter was necessary to exceed the background fluorescence. An incubation period of 10 to 12 hours was needed to reach a steady state of fluorescence, although growing cells to log phase before exposing them to arsenic reduced the incubation period to 2 hours.

6 Methods For Using Biosensors

The method for testing samples using bacteria as biosensors is generally the same for all strains of bacteria, with slight variations in induction time and substrate addition. Once the desired biosensor has been selected, the cultures are grown overnight, usually in a Luria-Bertani medium, which is sometimes supplemented with kanamycin at 37 °C. The next day a new dish with 50 ml of new medium is inoculated with 0.5 ml of the overnight culture. The new culture is grown at 37 °C to an optical density of 0.8 to 1.2 at 600 nm. After the cells are washed, they are suspended and then diluted in M9 medium at a concentration of 1×10^6 cells per measurement (Turpeinen et al., 2003; Tauriainen et al., 1997; Corbisiera et al., 1993; Petänen et al., 2001).

The biosensor culture is then exposed to the contaminated sample as water samples, soil water extractions, or soil suspensions. The procedures for properly exposing and reading the biosensors need to be refined for all types of medium including water, soil, and food. Arsenic sensing biosensors have not been tested in the field and therefore have not been thoroughly tested with environmental media of soil, sediment, and water. For a better understanding of arsenic bioavailability from heterogeneous medium, it is important that the biosensor be exposed to the arsenic without any alteration of its form in the medium, in order to ensure accurate information about its bioavailability.

The exposed final culture is then incubated for 60 to 90 minutes (depending on the host strain) as a mixture of one-part cells to one-part metal solution (Turpeinen et al., 2003; Tauriainen et al., 1997; Corbisiera et al., 1993; Petänen et al., 2001). Once the culture has been incubated, luminescence or fluorescence can be measured with a variety of instruments. The quantification of light emissions is one of the most sensitive means of detection. This can be performed using electrochemical transducers, where cells are immobilized onto an electrode and amperometric or potentiometer techniques measure the cellular response. Optical transducers can also be used to measure absorbance, luminescence, or fluorescence by immobilizing cells onto optical fibers (Biran et al., 2003).

6.1 Measuring Luminescence/Fluorescence

6.1.1 Luminometer/Fluorimeter

A luminometer or fluorimeter can be used to measure the intensity of the luminescence or fluorescence by counting the number of photons a cell produces. An individual cell's response is not simply on or off, but rather a sliding scale with greater intensity correlated to higher concentration of arsenic within the cell. The mixture of arsenic solution and cells is placed in a well plate, which is inserted into the luminometer. The background luminescence is measured for the first 5 seconds. A substrate is added to induce luminescence that is associated with arsenic. Induction can take 2 to 12 hours, depending upon the host strain used. Luminescence can be induced in *P. fluorescens* in 2 hours when incubated at 22 °C, while *E. coli* takes 4 hours. Induction time improves for *E. coli* when it is incubated at 37 °C (Petänen et al., 2001). GFP sensors not grown to log phase actually have the greatest induction time at 10 to 12 hours before peak luminescence is reached.

Measurements are taken periodically until luminescence peaks without adding more substrate. The time needed to reach this peak varies with the host strain and reporter gene used in the sensor. A plot can be generated of the biosensor's response to the sample as fluorescence over time. For example, Figure 3 shows the results of one biosensor using GFP as the reporter gene when exposed to a sample contaminated with arsenic. An induction coefficient is calculated by dividing the peak luminescence value of the sample by the luminescence value of a culture without arsenic (Roberto et al., 2002).

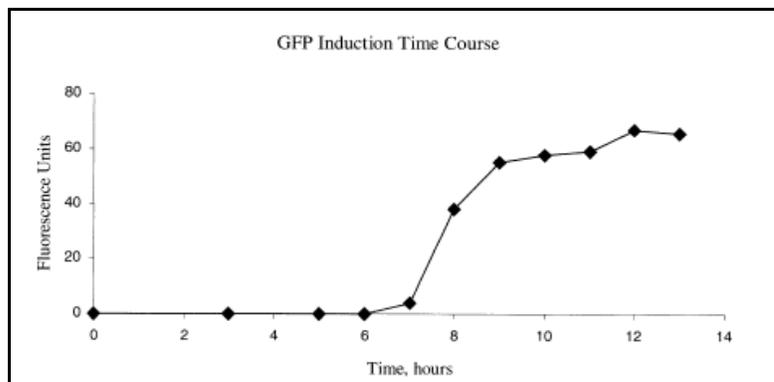


Figure 3. Plot of Induction (Roberto et al., 2002)

6.1.2 Optical Fibers

Luminescence or fluorescence can also be measured using optical fibers, which are bundles of thousands of identical, individual fibers that transmit light signals. Each fiber has a well etched into its core where single cells can be immobilized. Optical fibers allow for each cell to act as an independent sensor, while taking measurements from multiple cells (Biran et al., 2003). Unlike the luminometer, any size vial can be used, and measurements can be performed outside the sample compartment and through the walls of the container, so many vials can be analyzed at once (Leth et al., 2002).

The methods differ slightly when using optical fibers after the culture has grown to the desired optical density. The fibers are treated with a reagent that will immobilize the cells onto the fibers, which lead to an easier, more accurate detection. Reagents must not bind with the metal ions or

Whole-Cell Bacterial Biosensors and the Detection of Bioavailable Arsenic

interfere with the cell's viability and sensing activity. Currently, suitable reagents include alignate, agarose, and polyethylenimine, although their effect on cells is not completely understood. However, the cells typically retain their sensitivity and the procedure is simple (Biran et al., 2003; Leth et al., 2002).

The treated fibers are placed into tubes containing the culture and centrifuged horizontally, allowing cells to cover each fiber. The fibers covered with cells are then placed into a medium containing the contaminant. The finished fibers are placed onto an imaging system, which takes images before and after the addition of a substrate. The fibers are able to transmit the light produced by each individual cell to the imaging system, which averages the luminescence of all the cells. From this average and the measurement taken before the addition of the substrate, an induction coefficient can be calculated by dividing the peak luminescence value of the sample by the luminescence value of a culture without arsenic. Software is available to analyze the images and produce graphs showing the pattern of luminescence over time (Biran et al., 2003).

6.2 Optimizing Results

Research involving biosensors has revealed procedures that can improve the functioning of the biosensor. While the optimal induction time or the addition of a substrate may vary between strains, the results from most strains can be optimized using the same procedures. When choosing the host strain, it is important to consider the natural environment of the bacteria. For example, those strains native to soil are best for testing soil samples (Petänen and Romantschuk, 2003). Temperature, which influences induction time, should correspond to the temperature of the natural environment in which the host bacteria is found (Petänen et al., 2002).

Research has also found that the growth phase of the sensors and incubation time have the greatest affect on induction (Tauriainen et al., 1997), while luminescence has been found to correlate with the optical density of the culture (Tauriainen et al., 1999). Growing cells to log phase and incubating exposed cells for 90 minutes typically produces the best results. The sensitivity and induction coefficient are optimized with an optical density between 1×10^5 and 8×10^6 cells per assay and a pH between 5.5 and 8, depending on the host bacteria (Tauriainen et al., 1999). Biosensors using *E. coli* and *P. fluorescens* illuminated best at 1.6×10^7 and 3.8×10^7 cells per assay, respectively. If a substrate is needed, the induction coefficient is optimized and more stable when luciferin is used as the substrate at concentrations of 25 to 50 μM . Luciferin is better able to pass through the cell membrane, which better ensures that the lack of luminescence in a cell is due to the lack of arsenic and not substrate (Petänen et al., 2001).

6.3 Interpreting Results

In order to correlate the induction coefficient with a concentration of arsenic, a dose-response curve must be established. A comparison can then be made between the dose-response curve and a plot of the results from the samples. This comparison reveals the bioavailable concentration of arsenic in the unknown sample. A dose-response curve can be produced by running the experiment with known concentrations of arsenic. The graphed dose-response curve represents the luminescence measured or induction coefficient calculated against the known concentration

Whole-Cell Bacterial Biosensors and the Detection of Bioavailable Arsenic

of arsenic (Figure 4). The induction coefficient calculated for an unknown concentration can be compared to the dose-response curve to determine the concentration of arsenic associated with that induction coefficient. Current studies have generated their own dose-response curve from which to draw conclusions, but a standardized curve could be formulated.

Typically, the response of the biosensors is nonlinear until a threshold level of concentration is reached, after which the response is linear. Once the response peaks, it rapidly decreases due to concentrations so high the cell cannot expel the arsenic and begins to die. Usually the response to As(III) is higher than the response to As(V), which must be reduced to As(III) before activating the system (Roberto et al., 2002) (Figure 5).

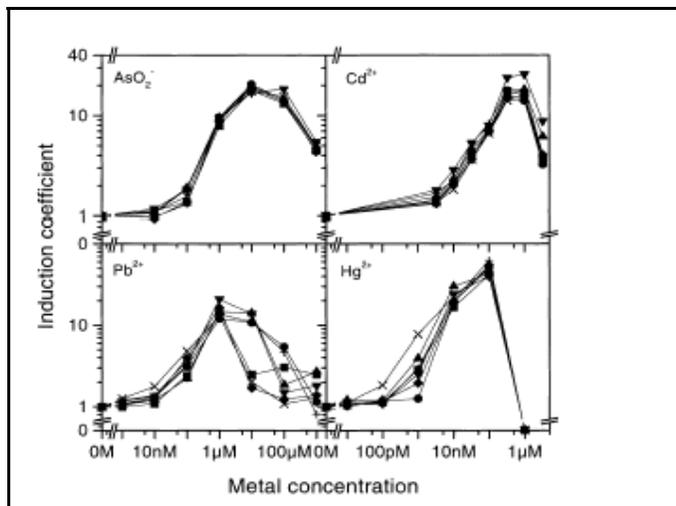


Figure 4: Example of Dose-Response Curve (Tauriainen et al., 2000)

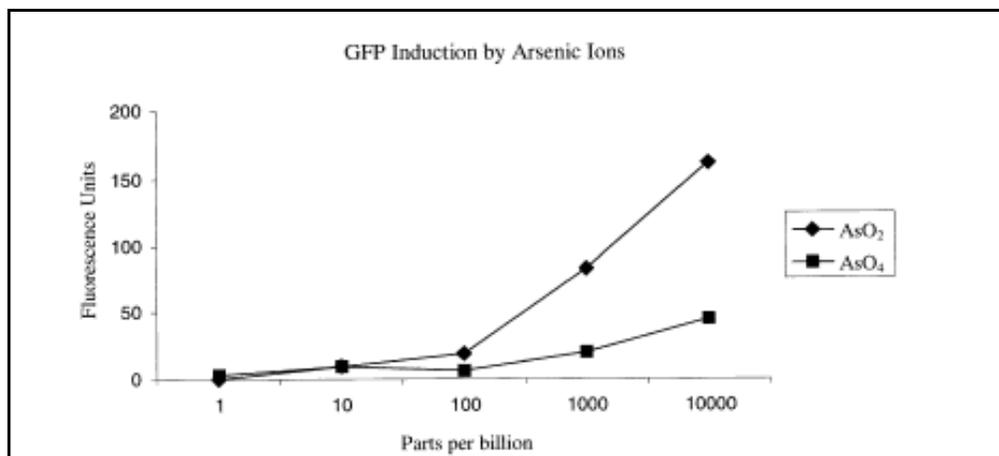


Figure 5: Example of Dose-Response Curve (Tauriainen et al., 2000)

6.4 Applying Results

The mechanism of the bacterial biosensor must be a surrogate for the mechanism within higher organisms in order for the information gathered from the biosensor to be directly relevant to ecological or human health risk assessments. A clear understanding of the mechanisms for arsenic resistance in higher organisms will improve the quantification of bioavailability and the applicability of bacterial biosensors to risk assessments. Such an understanding will improve the interpretation of the results as an indicator of the risk posed to ecological systems and human health. The relevance of bacterial biosensors to other ecological receptors is demonstrated through pathway similarities.

Whole-Cell Bacterial Biosensors and the Detection of Bioavailable Arsenic

All organisms examined by the scientific community have a pathway to respond to arsenic and share a few common themes within their pathways (Mukhopadhyay and Rosen, 2002). Similarities in the uptake of arsenic would suggest that the fraction of arsenic bioavailable to the biosensor would be equivalent to the fraction bioavailable to higher organisms. These similarities include the uptake of As(V) by phosphate transporters and the uptake of As(III) by aquaglyceroporins. The metabolism of arsenic once it has entered the cell varies greatly between organisms, but similarities exist in a few steps of the metabolism: the reduction of As(V) to As(III) and the extrusion or sequestration of As(III) (Rosen, 2002).

The uptake of arsenic depends upon its valence state and occurs through comparable transporters identified in organisms ranging from prokaryotes to eukaryotes. As(V) is structurally similar to phosphate and enters the cell via phosphate transporters. Such transporters have been identified in various strains of bacteria such as *E. coli* and in eukaryotes such as the yeast, *S. cerevisiae*. Current evidence indicates the probable presence of similar phosphate transporters in mammals. Uptake of As(III) occurs through aquaglyceroporins, which are multifunctional channels that transport neutral organic solutes such as glycerol and urea. Aquaglyceroporins have been found in prokaryotes such as *E. coli* and eukaryotes including *S. cerevisiae*, rats, mice, and humans (Rosen, 2002).

Comparing the metabolism of arsenic by microorganisms to higher organisms reveals a few similarities. Most organisms studied reduce As(V) to As(III) but not always with an arsenate reductase similar to the bacterial *arsC*. In humans a substantial fraction of As(V) is rapidly reduced to As(III) and can be reduced in the blood in addition to inside the cell. Arsenate reductase activity has been observed in vitro in human liver cells (Radabaugh and Aposhian, 2000), but the function is not completely understood (Rosen, 2002).

For humans, arsenic is further metabolized in a manner very different from microorganisms, which complicates the applicability of biosensors. A large portion of As(III) is methylated to methylarsonic acid and dimethylarsonic in the liver and can enter the cell either bound to a protein or methylated. The methylated arsenic is less toxic, less reactive with tissue, less cytotoxic, and more readily excreted in urine. The quantity of methylated arsenic found in the urine increases as the dose of arsenic increases, however the mechanism for methylation can be saturated, which would leave arsenic in its more toxic form (Klaassen, 2001).

7 Advantages and Disadvantages

Whole-cell bacterial biosensors have several advantages over conventional methods of detecting contaminants at a site. The greatest advantage is the ability of biosensors to detect the bioavailable fraction of the contaminant, as opposed to the total concentration. Knowing the bioavailable fraction allows for a more accurate assessment of the site and the potential risks involved. Biosensors create a clearer picture by providing physiologically relevant data in response to a contaminant. This response, usually luminescence, is quick and easy to measure, resulting in real-time data.

Whole-Cell Bacterial Biosensors and the Detection of Bioavailable Arsenic

Biosensors are also fast, less expensive, and less labor intensive than other traditional methods such as atomic absorption spectrometry, inductively coupled atomic electron spectrometry, and sequential extraction procedure. The results obtained from biosensors are compatible with and comparable to chemical analysis, while being free of chemical extractions and analytical procedures (Turpeinen et al., 2003; Flynn et al., 2002; Petänen and Romantschuk, 2003). Biosensors can also be more sensitive than chemical methods (Petänen and Romantschuk, 2002). The detection limit of biosensors depends on its design, but it has been demonstrated as low as 7 ppb for As(III) and 35 ppb for As(V) (Turpeinen et al., 2003). They are particularly appealing for field work or *in situ* analysis, since the procedure for using biosensors does not involve the bulky, fragile equipment or specialized training that most analytical methods require.

Advantages:

- Measures bioavailable fraction
- Inexpensive
- Produces real-time data
- Less labor intensive
- More sensitive
- Suitable for field work

Disadvantages:

- Short lifetime
- Lack of genetic stability
- Unknown rate of Type I and II errors
- Limited understanding of applicability to higher organisms
- Performance dependent on environment of procedure

Disadvantages also exist when using biosensors instead of traditional methods. A major disadvantage is the limited understanding of the biochemistry involved. It can only be assumed that the fraction of arsenic bioavailable to bacteria is equivalent to the fraction bioavailable to higher organisms. The response made by the biosensor is specific to its membrane and uptake procedures. Fortunately, the uptake of arsenic through phosphate pumps and aquaglyceroporins has been documented in a variety of organisms from bacteria to humans, indicating the relevance of the biosensors to higher organisms (Rosen, 2002). The details of bacterial response to most contaminants, including arsenic, is understood, but the response of higher organisms is still unclear. It is therefore difficult to transpose information gathered through bacterial biosensors and apply it directly to higher organisms. Discoveries are being made surrounding the responses to arsenic for higher organisms including humans. Further advances will clarify the meaning and applicability of the results for use in ecological and human health risk assessment.

Bacterial biosensors perform best under conditions similar to their natural environment (Petänen and Romantschuk, 2003). Unfortunately, little is understood about the relationship between the microorganisms and their natural environment. It is difficult to determine the effects that certain procedural conditions have on the performance of biosensors. The temperature, pH, incubation time, medium, and reagents all can have effects on their performance that can only be understood with further research and testing. Problems with performance include luminescence without the presence of the analyte or no luminescence in the presence of the analyte. Such Type I and Type II errors are not identifiable, and the rate of committing such errors is unknown. Without fully understanding the biochemistry and specificity of each sensor, such errors cannot be identified or predicted.

According to the National Research Council, the application of bacterial biosensors is limited due to their short lifetime and their lack of genetic stability, which leads to variability in the

Whole-Cell Bacterial Biosensors and the Detection of Bioavailable Arsenic

response of different cells. Such variability complicates the application of information gained from biosensors, which relies on the comparison of results to standardized dose-response curves. The results of biosensors are further complicated by the strong background response of some sensors and the strong matrix effects on the signal. The non-specificity of biosensors also serves as a hindrance. Some biosensors for arsenic can also detect antimony or cadmium (NRC, 2003), but progress has resulted in biosensors with improved sensitivity (Taurianinen et al., 1999).

8 Potential

Despite their disadvantages, whole cell bacteria biosensors do have the potential to become a common tool for site characterization. Currently, this technique is only in the research stage and needs further testing in the field. A few challenges, such as longevity, reproducibility, and linearity, must be overcome in order to fully implement the use of biosensors in the field. As these challenges are met, the applicability of biosensors may increase.

Biosensors can be freeze-dried so they are easier to handle and store. Freeze dried cells are more reagent-like, and they are suitable for field work because they do not need to be carefully stored and cultivated daily. Freeze-dried cells maintain their usability, but do have lower induction coefficients and sensitivities. Cells can be reconstituted by incubating them in water, and then placing them in a medium in order to regain sensitivity. Further research utilizing freeze-dried cells should unveil procedures that will optimize the results (Tauriainen et al., 1997).

Lysis of whole-cell bacterial biosensors has also been done in an attempt to make them easier to use. By separating the recombinant plasmid that contains the reporter gene and the arsenic sensing gene from the rest of the cell, the biosensor becomes more like a reagent without the need for cell cultivations. This reduces the day-to-day variation in the genetic make-up of the biosensor, as well as the time needed to perform the test. Using cell lysate, the plasmid without the remaining cellular components is more convenient and easier to use in the field as an *in vitro* method for determining arsenic bioavailability. Despite the increased ease of use for cell lysate, there are disadvantages. While results from tests using whole cell and cell lysate biosensors correlated well, the results varied more between replicates and samples for the cell lysate biosensor. The whole-cell biosensor created more reproducible results for all environmental samples tested (Tauriainen et al., 2000; Tauriainen et al., 1999). This may be due to the fact that the natural environment and ingredients for the mechanism provided by the whole cell. The reaction is simplified and efficiency is improved when the whole cell is used (Leth et al., 2002).

Further improvements on the applicability of biosensors include the development of a multi-analytical biosensor. By genetically labeling the bacteria used in the biosensor, a multi-analytical biosensor can be developed to test more than one contaminant at one time. Multiple strains of bacteria, which test for different specific contaminants, could be combined into one biosensor. Each strain can have another gene incorporated into its plasmid. The gene encodes for a protein, such as the cyan fluorescent protein, which emits a particular color. Measurements can be taken of the luminescence of each strain, which is identified by its individual color (Biran et al., 2003). A similar effect can be produced using mutated reporter genes that produce distinct colors

Whole-Cell Bacterial Biosensors and the Detection of Bioavailable Arsenic

ranging from green to red. Each mutant can be coupled with a specific contaminant-sensing gene to develop a multi-analytical biosensor (Daunert et al., 2000).

9 Conclusions

Continuing research will reveal the best procedures and full applicability of whole-cell bacterial biosensors. These biosensors do provide a real-time measurement of the bioavailability of arsenic, as well as other heavy metals and contaminants they can be designed to detect. Knowing the bioavailable fraction is important when assessing the true risk imposed by a contaminant. False estimates of risk can lead to a false ranking of risks, which can result in poor appropriation of time and money. Such mismanagement of time and money could lead to unnecessary clean up at one site and leave a population unprotected and unaware of danger at another. With more testing of biosensors in the field and a standardization of biosensors and their procedures, bioavailability can be incorporated into ecological and human health risk assessments.

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Appendix A: Developed Biosensors

Study	New Plasmid	As Sensing Gene	Reporter Gene	Host Strain	Detectable Metals	Detection Limits
Ji and Silver, 1992	—	<i>arsR</i> , <i>arsB</i> , <i>ArsC</i> of <i>Staphylococcus aureus</i> plasmid p1258	NA*	<i>S.aureus</i> , <i>Bacillus subtilis</i> <i>Escherichia coli</i>	Arsenate Arsenite Antimonite Arsenate Arsenite Antimonite	About 1 µm higher than Taurianinen et al., 1997 For Arsenite**
Corbisier et al., 1993	pC200	<i>arsB</i> of <i>S.aureus</i> plasmid p1258	<i>luxab</i> genes from <i>Vibrio harveyi</i>	<i>E. coli</i> (HB101) <i>S.aureus</i> (RN4220)	Arsenite Arsenate Bismuth Arsenite	About 1 µm higher than Taurianinen et al., 1997 For Arsenite**
Taurianinen et al., 1997	pTOO21	<i>arsR</i> and Promoter of <i>S.aureus</i> plasmid p1258	<i>lucFF</i> from <i>Photinus pyralis</i>	<i>S.aureus</i> (RN4220) <i>B.subtilis</i> (BR151) <i>E. coli</i> (MC1061)	Arsenite Antimonite Cadmium Arsenite Arsenate Antimonite Cadmium Arsenite Arsenate Antimonite Cadmium	100 nm 33 nm 330 nm 3.3 µm 330 µm 330 nm 330 nm 3.3 µm 33 µm 3.3 µm 33 µm
Taurianinen et al., 1999	pTOO31	Promoter and <i>arsR</i> of <i>E. coli</i> plasmid R773	<i>lucFF</i> from <i>P.pyralis</i>	<i>E. coli</i> (MC1061) <i>E. coli</i> (AW3110)	Arsenite Arsenate Arsenite Arsenate	33 nm 33 µm Similar to MC1061
Petänen et al., 2001	pTPT21 pTPT31	Promoter and <i>arsR</i> of p1258 Promoter and <i>arsR</i> of R773	<i>lucGR</i> of <i>Pyrophorus plagiophthalmus</i> <i>lucGR</i>	<i>E. coli</i> DH5, <i>Pseudomonas fluorescens</i> OS8 <i>E. coli</i> DH5, <i>P. fluorescens</i> OS8	Arsenite Arsenate Cadmium Arsenite Arsenate Cadmium	—
Roberto et al., 2002	pIRC140	Promoter, <i>arsR</i> , & <i>arsD</i>	GFP of <i>Aequorea victoria</i>	<i>E. coli</i>	Arsenite Arsenate	µm to nm, 1 ppb

* Tested ars operon in various hosts strain

** Source: Taurianinen et al., 1997

Appendix B: Commercially Available Kits

Several bacterial biosensors are available commercially as kits to test the general toxicity of environmental samples, and a few kits are available that test for specific contaminants. Microtox™, a metabolic inhibition test, can determine acute toxicity by exposing freeze-dried *V.fischeria* to environmental samples for 15 minutes. ToxAlert™ is a similar biosensor from Germany, which uses lyophilized *V.fischeria* to test general toxicity (Farré et al., 2002) BIOMET™, kit of specific biosensors, is available from the Netherlands to test the bioavailability of zinc, cadmium, copper, nickel, lead, chromium, or mercury. Cellsense™ is an amperometric biosensor that investigates specific groups of toxins such as heavy metals. This tests measures the electrical current of the biosensor's electron transport chain as a surrogate to its metabolic activity (Farré et al., 2002) An arsenic specific test is available through Aboatox as an inexpensive kit, quick enough to test up to 100 samples daily. The methods of the kit involve incubating the sample and bacteria for two hours at 37 °C. D-luciferin is added as the substrated, incubation continues for 30 minutes, and the luminescence is measured with a luminometer.

Kit	Description	Detects	Manufacturer	Website/reference
Microtox™	Metabolic inhibition test using freeze-dried <i>V.fischeria</i>	General Toxicity	Azur Environmental	http://www.azurenv.com/
ToxAlert™	Metabolic inhibition test using lyophilized <i>V.fischeria</i>	General Toxicity		(Farré et al., 2002)
BIOMET™	Uses <i>Ralstonia metallidurans</i> which naturally responds to metals and glows	Specific for Zn, Cd,Cu, Ni, Pb, Cr, or Hg	Vito	http://www.vito.be/english/environment/environmental/tech2d.htm
Cellsense™	Amperometric biosensor measures electrical current of biosensor as surrogate to metabolic activity	Specific Groups such as heavy metals		(Farré et al., 2002)
Biologically Heavy Metal Assay Kit	Whole cell bacterial biosensors, luciferase synthesis activated by presence of As	As	Aboatox	http://www.aboatox.com/environmental_analysis.html