

Development and Environmental Application of Microbial Bioreporters of Oxidative
Stress

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Abstract

There is a need for a sensitive, specific, rapid and cost-effective assay that can be used as an early warning signal of contamination of aquatic ecosystems. The purpose of this work was to develop a sensitive stress-specific microbial bioreporter responsive to pro-oxidants. Furthermore, the bioreporter was designed to be applicable in environments possibly affected by metal processing activities.

An *E.coli* bioreporter was developed containing a plasmid with the *katG* promoter sequence as the sensing sequence and with mCherry as the reporter protein. The bioreporter responded to metal pro-oxidants (Cd, As, Zn, Pb, Ag and Ag nanoparticles). A new assay growth-medium was developed and contributed to improve the sensitivity of our assay that has the best detection limit to inorganic pro-oxidants compared to other oxidative-stress sensitive bioreporters in the literature. The bioreporter detected pro-oxidants in environmental samples. The assay has a reasonable sensitivity, however, it still lacks sensitivity to detect pro-oxidants at concentrations lower than those shown to be toxic to many aquatic species. Within-lab reproducibility and robustness were determined to be acceptable.

For stress-specific bioreporters to be incorporated in regulative legislations and industrial monitoring programs there is a need to improve the sensitivity of these assays, they need to be calibrated with other relevant pro-oxidants, inter-lab reproducibility needs to be established and robustness to environmental samples needs to be further tested. To further validate the sensitivity and ecotoxicological relevance of the bioreporter as a relevant predictive tool, stress-specific bioreporter assays need to be performed in parallel with traditional ecotoxicological assays using contaminated environmental samples.

Résumé

Nous voulons développer un essai sensible et spécifique qui servirait à signaler précocement la contamination d'écosystème aquatique. L'objectif de ce travail de thèse était de développer un bioreporter microbien qui répond aux pro-oxydants. De plus, le bioreporteur fut développé afin de pouvoir être appliqué dans des environnements potentiellement affectés par des activités de traitements de métaux.

Un bioreporter microbien a été développé utilisant *E. coli* contenant un plasmide avec la séquence du promoteur *katG* comme la séquence sensible au pro-oxydant et avec mCherry comme protéine signal. Le bioreporteur a répondu avec succès à des métaux pro-oxydants (Cd, As, Zn, Pb, Ag et des nanoparticules d'Ag). Un nouveau médium de croissance fut développé et contribue à l'amélioration de la sensibilité de notre essai biologique qui possède la meilleure limite de détection pour des pro-oxydants inorganiques par rapport aux bioreporteurs reportés dans la littérature. Le bioreporteur a été en mesure de détecter la présence de pro-oxydants dans des échantillons environnementaux. La sensibilité du bioreporteur est jugée acceptable, cependant, la détection de plusieurs pro-oxydants se fait à des concentrations plus hautes que ce qui a été démontré être toxique pour plusieurs espèces aquatiques selon la littérature. La reproductibilité dans notre laboratoire ainsi que la robustesse du bioreporteur ont été jugée acceptable.

Afin de pouvoir éventuellement incorporer des bioreporteurs microbiens sensibles au stress oxydatif dans des programmes de suivi environnementaux de systèmes potentiellement contaminés; la sensibilité de ces essais doit être améliorée, d'autres pro-oxydants pertinents doivent être calibrés, la reproductibilité inter-laboratoire doit être établie, puis la robustesse du bioreporteur face aux échantillons environnementaux doit être testée davantage. Afin de valider la sensibilité ainsi que la pertinence écotoxicologique de tels bioreporteur en tant qu'outils prédictifs, les bioessais microbiens sensibles au stress oxydatif devraient être performés sur des échantillons environnementaux contaminés en parallèle avec des essais traditionnellement acceptés en écotoxicologie (i.e. essai sur des ménéés).

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List of Abbreviations

β-GMM	β Glucose-Minimal Media
AgNPs	Silver nanoparticles
ATP	Adenosine Triphosphate
BLM	Biotic Ligand Model
CWQG	Canadian Water Quality Guideline
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
DNA	Deoxyribonucleic Acid
EC	Effective Concentration
FIAM	Free Ion Activity Model
GlpF	Glycerol Facilitator Channel
GMM	Glucose-Minimal Media
GSH	Glutathione
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
LB	Lysogeny Broth
LC	Lethal Concentration
MATC	Maximum Allowable Toxicant Concentration
NP	Nanoparticles
OD	Optical Density
OECD	Organization for Economic Co-operation and Development
OSR	Oxidative Stress Response
PAH	Polycyclic Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction
RFU	Relative Fluorescence Units
ROS	Reactive Oxygen Species
RTA	Rio Tinto Alcan
WQG	Water Quality Guidelines

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Chapter 1. Introduction

This thesis comprises a description of the development (Chapter 2), validation (Chapter 3) and application (Chapter 4) of a microbial bioreporter sensitive to oxidative stress in the context of the development of an early warning sentinel tool for the receiving environments of our aluminum smelter industrial partner. This introduction will explain the requirements that industries need to perform to minimize impacts on the environment, the need to assist industries in making decisions as to these impacts, and the tools available to do so all leading to this particular research.

1.1 Industry and the Environment

The mining, petrochemical and the pulp and paper industries are crucial to the Canadian economy (contributing 10% to Canada's Gross Domestic Product), however industries have the potential to have a significant impact on the environment (Culp et al. 2008).

Under the Fisheries Act of 1999 (federal level), industries (e.g. mine, and pulp and paper industries) are permitted to release industrial discharges into natural water systems on the condition that they follow the Environmental Effects Monitoring Program (briefly summarized in Table 1). First, industry must comply with water quality guidelines (WQG) which set physico-chemical properties of effluents and receiving waters. In this regard, water quality must be tested four times a year to ensure they meet these guidelines (Table 1). Second, the testing program involves performing sub-lethal toxicity testing on invertebrates, plants, algae and fish (growth, survival and reproductive assays) with industrial effluent discharge samples. Third, if receiving environments have concentrations 1% higher than water quality guidelines, in-situ monitoring of wild fish (fish tissue toxicant content, fish

condition, survival, growth and reproduction assays) and benthic macro-invertebrates (taxonomic richness, population density and community structure) in receiving environments must be performed. The schedule of compliance is provided in Table 1.1.

Aluminum smelters (like our industrial partner) do not benefit from a federal regulatory framework for its discharges because they do not fall under the mining sector. As such, these industries follow the Waste Reduction Program established in 1988 under the Environmental Quality Act (provincial level; Quebec) (section IV.2; sub-section 1; articles 31.1 to 31.31). Under this legal regulation, aluminum smelter industries must comply with certain water quality guidelines and must perform toxicity tests with their effluents. Contaminants such as aluminum, fluorides, PAHs, petroleum hydrocarbons, Cd, Cu, Pb, Zn, Ni and cyanide are measured on a frequency that is site-specific and determined every 5 years when permits are re-negotiated. Acute toxicity tests must be performed (e.g. on Trout or Daphnia with a frequency that varies). Chronic toxicity tests on alga and minnows must also be performed. For a more detailed view of the requirements, see the technical guide dedicated for this program (Ministère du Développement durable de l'Environnement et des Parcs, 2009).

Table 1.1. Brief summary of requirements of Environmental Effects Monitoring Studies (annex 5 of Metal Mining Effluent Regulations Fisheries Act 1999; not applicable for smelters, only for aluminum refineries).

	Measurements	Sampling/Test Location	Frequency
Effluent Characterization	Physico-chemical properties ^a	Final emission point of effluent	4 times per year
Sublethal Toxicity Assay	Sublethal toxicity test on 1 species of fish, invertebrate, plant and algae	Final emission point of effluent	2 times per year for 3 years, once per year after first 3 years
Water Quality Evaluation	Physico-chemical properties ^a	In receiving environments; close to where effluents mix with site water and also at same location where Biological Effect Evaluations samples are taken (exposed zone)	4 times per year, with at least 1 month interval between analyses
Biological Effect Evaluation^b	-Monitoring of effects on fish population and benthic community population. -Fish mercury tissue concentration if water mercury level is higher than 0.10 µg/L -If an effect is determined, a study on the causes of toxicity must be undertaken	In exposed zone and reference zone (unexposed to effluent)	Within 30 to 42 months the first time. If no effect, once every 3 years. If no effect 2 reports in a row, every 6 years

^a Aluminum, cadmium, iron, mercury, molybdenum, arsenic, copper, lead, nickel, zinc, radium 226, selenium, cyanide, ammoniac, nitrates, hardness, alkalinity, conductivity, total solids in suspension, dissolved oxygen, temperature, pH, salinity

^b Performed only if concentrations under Water Quality Evaluation are 1% over WQG.

1.2. Impact Assessment in Ecotoxicology

1.2.1 Water Quality Guidelines (WQG)

Industries are often legally obliged to comply with WQGs. These are scientifically determined, maximum allowable concentrations of pollutants to which an aquatic community can be exposed to with what is deemed an acceptable risk (Canadian Council of Ministers of the Environment, 1999). WQG can be expressed either as total concentrations of a chemical or expressed as selected chemical species.

Although indicative of toxicity, total pollutant concentration alone is a poor predictor of toxicity (Robbens 2010). This is because the different physico-chemical properties of water affect metal pollutant speciation, which in turn greatly influences the behavior and bioavailability of these compounds (Canadian Council of Ministers of the Environment, 2007; Robbens et al. 2010). For this reason, Canadian WQGs also take into account physical-chemical properties of water. For example, water hardness (e.g. Ca^{2+} and Mg^{2+}) has been shown to have a protective effect on organisms by limiting cadmium uptake (Canadian Council of Ministers of the Environment, 2014a). That being said, total contaminant concentration is often chosen as the way to express water quality objectives because it is an easier and more conservative approach (Canadian Council of Ministers of the Environment, 2007). Over the last 30 years, advances in knowledge concerning water chemistry, ecology, aquatic toxicology and physiology have provided a better understanding of metal chemistry and its relation to a physiological effect (Wang 2013). This collaborative effort between disciplines allowed the development of metal-equilibrium predictive models of toxicity like the first ever biotic ligand model (BLM) presented by Di Toro et al. (2001). The BLM is a tool that predicts toxicity of individual metals in a sample, based on the environmental

conditions (Paquin et al. 2002). The BLM is a sophisticated way of expressing site-specific WQGs and while it is being used in some instances for regulatory purposes (U.S. EPA, 2009), the BLM is only currently applicable to a few metals (e.g. Cu, Ag, Zn, Ni) and to a few organisms (rainbow trout, phytoplankton, daphnids). BLMs remain under continuous development (Erickson et al. 2013; Wang 2013). Although the BLM derived from the Free Ion Activity (FIAM) Model, which postulated that toxicity was directly proportional to the free metal ion concentrations, many subsequent studies demonstrated many exceptions to the FIAM, hence the development of the BLM, which includes water physical and chemical properties as well as metal speciation (Wang 2013). Although offering great improvements in our ability to predict metal toxicity, the BLM still does not allow predicting the effect of mixtures (Wang 2013).

1.2.2 Bioassays

Bioassays on environmental samples do take into account bioavailability because, fundamentally, bioassays provide a biologically integrated response. For this reason, bioassays offer direct information of the effects of both known and characterized toxicants but also offer insights into the response of unknown compounds or compounds not suspected to be present. The biological integrated response renders bioassays convenient to help predict synergistic effects of mixtures on aquatic ecosystems (Freitas et al. 2011).

Ecotoxicology works by collecting information with toxicity tests on surrogate species and extrapolating the results to other species, communities and even ecosystems (Robbens et al. 2010). The use of model organisms in ecotoxicology provides important characteristics including those that are ecologically relevant or closely related to an indigenous species of the studied site, economic importance, ease of use and sensitivity (Cheung 1996). Classic model

organisms used in the field of freshwater aquatic ecotoxicology include the common crustacean *Daphnia*, fish such as fathead minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*) and several species of algae (Well et al. 1998). Although bioassays performed with these unicellular and multicellular eukaryote species are sensitive and ecologically relevant, they often are very costly, preventing their use for frequent monitoring (as seen with requirements of the Fisheries Act that imposes monitoring only every few years). This prevents industries to be pro-active because environmental problems may be detected too late (Dardenne et al. 2007).

As such, there is a need for sensitive and cost-effective bioassays that allow frequent monitoring and early detection of adverse impacts on aquatic ecosystems and thus pollution prevention measures can be performed before irreversible damage occurs. Microbial bioreporters fit this description.

1.2.2.1 Microbial Bioreporters

Three types of microbial bioreporters can be distinguished: non-specific/general bioreporters, semi- or stress-specific bioreporters and compound specific bioreporters (which will not be discussed in this thesis).

a) General Bioreporters:

In 1979, scientists at Azur Environmental (formally Microbics Corp) developed an innovative and simple test. *Vibrio fischeri* is a marine bacterium that consistently emits light (via a sequence of genes encoded by the *lux* operon) as a by-product of cell respiration and metabolism. The test relies on the ability of toxic substances to impair *V. fischerii* physiology and consequently lead to a decrease in luminescence, which can be easily quantified (Bulich et al. 1981). Different versions of the test were subsequently developed and standardized for

use in different applications (e.g. water and sediment toxicity testing) and these tests are currently known as Microtox®, with a sensitivity to contaminants comparable to other bioassays (Well et al. 1998). Microtox® has been used extensively for environmental applications for evaluating the toxicity of industrial effluents and has been incorporated into many governmental regulation programs (Well et al. 1998). While scientists and regulators commonly use it, Microtox® assays lack specificity (Sorenson et al. 2006).

b) Semi-specific Bioreporters:

Semi-specific bioreporters also known as stress-specific bioreporters, consist of genetically modified bacterium containing engineered sensing and reporting components, often on a plasmid and transformed into a bacterial host (Xu et al. 2013). The sensing component, a promoter sequence, is chosen for its ability to respond to specific groups of chemicals (e.g., pro-oxidants, genotoxic compounds, protein damaging compounds) and to control the expression of the reporting component, which is a gene coding for a protein emitting a measurable signal such as light or fluorescence. Promoter sequences are part of a complex regulatory circuitry within the cell that allows the bacteria to naturally detect and rapidly respond to environmental stressors. I used this sensing ability to engineer semi-specific bioreporters used in my study (Fig. 1.1).

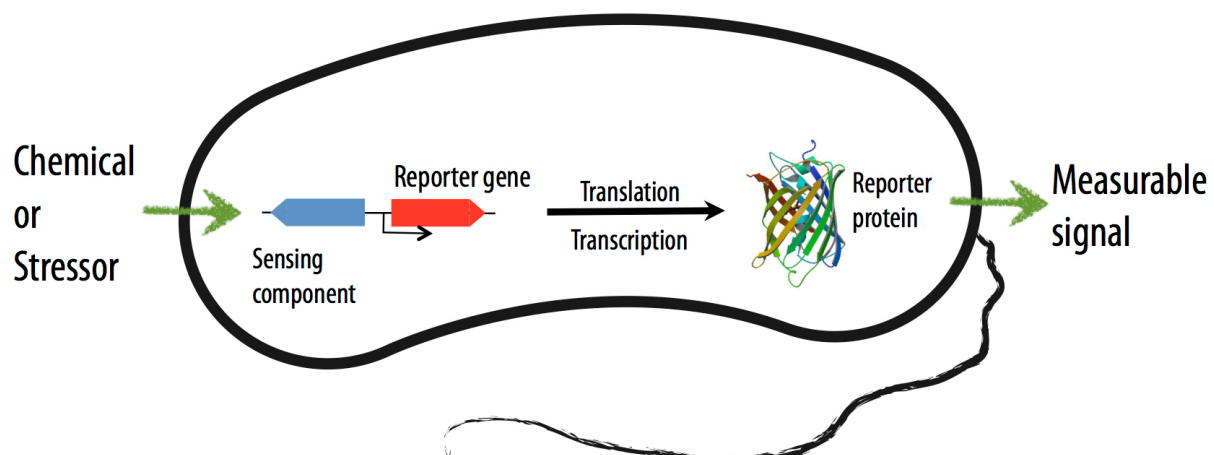


Figure 1.1. The essential components of a stress-specific or compound-specific bioreporter.

These assays offer improved specificity when compared to general bioreporters like Microtox®. One of Environment Canada's monitoring needs is to "improve the ability to detect industry specific contributions within confounded receiving environments exposed to multiple effluents" (Culp et al. 2008). Assessment of mixtures is important in a world where aquatic ecosystems are exposed to multiple pollutants and/or multiple effluents. Stress-specific bioreporters are seen as tools that could be used to better understand these mixtures and thus provide more informative risk assessments. Multi-endpoint assays, consisting of using several stress-specific bioreporters to assess water quality could offer insights into the toxicity of an environmental mixture (Culp et al. 2008).

Fundamentally, stress-specific bioreporters offer the opportunity to be more sensitive than general bioreporters because they are induced under sub-toxic conditions; stress-specific promoters/sensing systems are activated well before the metabolism of the cell is affected. With increasing knowledge of transcriptional regulation, molecular signaling and microbial physiology, the past 15 years have seen a significant number of researchers attempting to improve the sensitivity of stress-specific microbial bioreporters by carefully choosing promoters for their constructs (Robbens et al. 2010; Belkin et al. 1996; Mitchell et al. 2004). This approach of manipulating the genome of bacteria and designing novel biosensors fits with another Environment Canada program dedicated to "improving assessment capabilities for sensitive ecosystems" (Culp et al. 2008).

1.3 Project Rationale

Rio Tinto Alcan (RTA) is one of the world's leading producers of bauxite, alumina and aluminum and strives towards being an industry leader in waste management (Rio Tinto Alcan, 2015). RTA wants to better understand and mitigate the impacts of its activities and

effluents released into receiving natural waters and so developed a collaborative effort with our lab to develop novel biomonitoring strategies.

As mentioned above, many bioassays including those required within the Environmental Effects Monitoring Program (Table 1.1) and the Waste Reduction Program require complicated logistics, can be quite expensive, and are time consuming. For example, many fish bioassays require procuring the fish embryos or larvae (waiting period for hatching under proper conditions can exceed 7 days), exposing the fish to the effluent for 7 days during which time the fish are fed 2 to 3 times per day and the water in which they live is changed daily. These assays require permits and animal ethical committee approval for the procedures. Also, these assays can require large volumes of effluent water, transported from the sampling site and stored in the testing facility. An alternative is for a company to spend significant amounts of money on designing and constructing an on-site animal testing facility that would be infrequently used. Because of these issues, animal bioassays are not performed on a regular basis and so there is a lack of constant awareness of water quality. A good example of this lack of awareness is that seasonal changes can often put some of the aquatic life more at risk to some contaminants. Specifically, cyanide degrades slowly under cold temperatures and could pose a greater risk during winter (Government of Canada, Environment Canada, 2012). Also, drought periods can minimize water flow and thus reduce dilution of contaminants, rendering the receiving environments more at risk to effluents with an increased contaminant concentration (Government of Canada, Environment Canada, 2012). Other instances where a lack of constant awareness of water quality can be risky is that of the lingering possibility of unknown diffuse contamination, unknown faulty water-treatment of the effluent, and accidental spills exemplified by the recent spill from a alumina

refinery in Hungary in 2010 and of a mine tailings pond in British Columbia, Canada in 2014 (Woo and Posadzki, The Globe and Mail, 2014; Knight, CNN, 2010). With the current system in place, irregular and unpredictable increased contamination and their possible adverse effects on ecosystems could be noticed in a relatively late phase of impact and possibly too late (Dardenne et al. 2007). Microbial biosensors may circumvent these issues.

Oxidative stress is caused by the imbalance between the presence of reactive oxygen species (ROS) and the ability of the cellular antioxidant system to detoxify them (Regoli 2000). ROS are chemically reactive compounds that contain oxygen radicals; these are normally produced in the cell as a byproduct of normal cellular respiration within the mitochondria. However, pro-oxidants including a wide variety of chemicals may, through one mechanism or another, generate ROS in the cell to levels causing adverse effect via the destructive oxidation of biological molecules (lipids, DNA, proteins) (Regoli 2000). In order to overcome oxidative stress from the generation of ROS, all aerobic organisms on earth have developed relatively conserved ways to mitigate ROS and this response is known as the oxidative stress response (OSR). We have chosen to focus on bioreporters based on the oxidative stress response because:

1. The majority of chemicals released by industrial and municipal effluents consist of pro-oxidants (e.g. soft and hard metals, polycyclic aromatic hydrocarbons (PAH), pesticides like paraquat). A bioassay capable of detecting biologically active pro-oxidants can be considered a good general evaluation tool of water quality. Such an assay could serve as an integrative assessment of the joint effects of environmental insults in a receiving environment.

2. Every aerobic aquatic organism can be a victim of ROS damage, and so, looking for oxidative stress biomarkers is relevant on the ecological level. Oxidative stress is also associated with many diseases (e.g. Parkinson disease) of organisms including humans (Ahamed et al. 2007).

1.4 Project Objectives

The overarching goal of this thesis was to develop and test a sensitive microbial bioreporter of oxidative stress that could be applied in the context of metal smelting by RTA industrial process. The specific objective of Chapter 2 was the creation of a microbial bioreporter of oxidative stress. For Chapter 3, the objective was to improve the general sensitivity of the bioreporter assay and its calibration to a wide range of aluminum smelter and metal-mining related inorganic pro-oxidants. Chapter 4 had the objective of applying the bioreporter to assay environmental samples. Chapter 5 is a discussion of the current situation of stress-specific bioreporters and future steps for incorporating these tools in environmental regulations.

Chapter 2. The Development of an Oxidative-Stress Sensitive Microbial Bioreporter

2.1 Introduction

Bioreporters consist of the sensing element, the reporting element, the vector plasmid and the bacterial host. When building a bioreporter, each of these components must be carefully chosen as they define and determine how the bioreporter can be used. Changing these four components will significantly change the bioreporter. In this chapter, a description of these components is given as well as a justification of the use of some of them for our bioreporters. Finally, the construction of the bioreporter is explained.

2.1.1 Bacterial Host

Bacteria satisfy the criterion to be used as a surrogate model organism in a bioassay, as they are a life form in which a measurable signal can be quantified and act as a proxy of a toxicological effect (Bulich 1981). Although many differences exist between species and even more between different levels of organization, one of the difficult tasks in ecotoxicology consists of obtaining results with one species and extrapolating these from individuals within a population to communities and ecosystems (Robbens et al. 2010). Major differences exist between bacterial, algal and fish populations, but using bacteria as a detection system is reasonable given that Microtox® technology is used by regulatory agencies in many places across the world. The type of metal transporters found at the cell-external environment interface is often what defines the bioavailability of cations. Although countless anatomical and physiological differences exist between phylogenetic groups, metal transporter composition is a highly conserved trait (Grass et al. 2005; Guerinot 1999), explaining the similar sensitivity of daphnids and fish to toxic metals (Paquin et al. 2002) and partly justifying *E.coli* as a surrogate. For example, the ZRT- and Irt-like protein (ZIP) family of

metal transporters (for zinc and cadmium) present in all eukaryotic organisms is also present in *E.coli* (Grass et al. 2005; Guerinot 1999).

E.coli is an ideal genetic model and bioreporter host because of the in-depth knowledge concerning its genetics, transcriptional regulation, molecular signaling, biochemistry and physiology. For example, with the recent advances concerning knowledge of the structural properties of transcriptional regulators and their interaction with promoters, novel promoter sequences can be designed (Robbens 2010).

I have employed *E.coli* K-12 substr. MG1655 (the genome of which has been completely sequenced) in my study. For the purpose of increasing the sensitivity of our assay, I also used a *rfaC*-mutant *E.coli* strain (*E.coli* JW3596-1 derived from wild type *E.coli* BW25113). This mutant strain has a lipopolysaccharide (LPS) core-structure defect, having no polysaccharide chain, leading to an increase in permeability to hydrophobic compounds and plasmids (Fralick et al. 1994; Chen et al. 2010). I predicted that this mutant may also have an increased sensitivity to inorganic cations. In wild type *E.coli*, the LPS layer has a negative charge and through electrostatic interactions should result in the accumulation of metal cations by adsorption at the cell surface. In *rfaC*-mutant *E.coli*, metal cations should be less adsorbed on the cell wall and reach metal transporters on the outer-membrane more easily.

2.1.2 Vector plasmid

I chose the pUCp19 plasmid as the vector plasmid. This plasmid has several advantages: i) it has a versatile multiple cloning site, facilitating cloning (Schweizer 1991); ii) it is a high copy number plasmid (Schweizer 1991), which could influence sensitivity to chemicals being sensed by the bioreporter, as increased plasmid numbers inside the cell

equates to a higher amount of sensing component, thus being able to more efficiently bind to its respective analyte; and, iii) it is a broad-host vector and can be transformed and maintained in various gram negative bacteria such as *E.coli* (Schweizer 1991) or *Pseudomonas sp.*. This allows for the possibility to transform our construct into different hosts for eventual applications tailored to different environmental sites. For example, to assay the receiving environment of a waste-water treatment plant, isolating an indigenous bacteria from the treatment plant and using it as a host could provide a higher sensitivity and superior operational range compared to standard hosts, as illustrated in a study by Wiles et al. (2003).

2.1.3 Promoters

All domains of life, including bacteria, have evolved mechanisms to detect ROS. Two main transcription factors that control the oxidative stress response in *E.coli* are OxyR and SoxRS (Lee et al. 2003). OxyR is specifically activated by hydrogen peroxide (H_2O_2) through oxidation of a cysteine residue and a subsequent protein conformational change (Schellhorn 1995). Once activated, OxyR regulates many different genes responsible for dealing with H_2O_2 stress (e.g. *katG* that codes for catalase) by binding to their promoter region (Lee et al. 2003). SoxRS works in a similar manner, but is activated primarily by superoxide anion ($O_2^{\bullet-}$) through one-electron oxidation of its iron-sulfur center (Pomposiello et al. 2001); it then binds to the promoter of genes responsible for dealing with $O_2^{\bullet-}$ stress (e.g. *sodA* that codes for superoxide dismutase). I chose to benefit from this regulatory machinery in *E.coli* by using the promoter of the *katG* gene as the sensing component. Choosing the *katG* promoter allows for the detection of all pro-oxidants that generate H_2O_2 , but also that generate $O_2^{\bullet-}$ because it is detoxified in *E.coli* to H_2O_2 (Fig. 2.1).

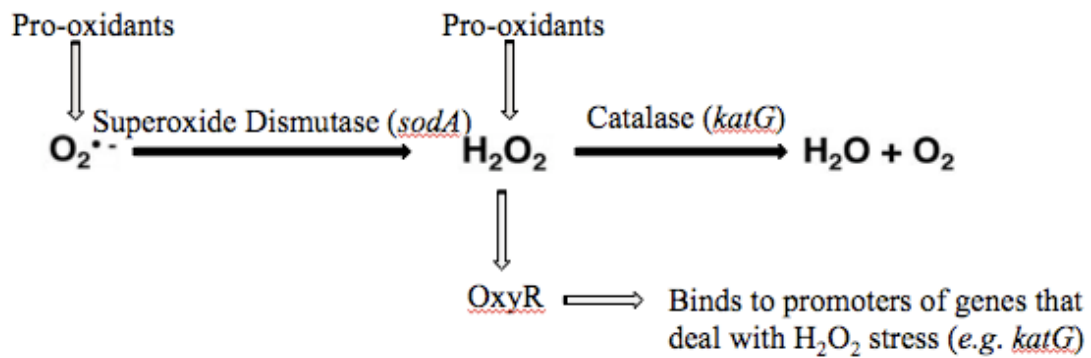


Figure 2.1. Schematic representation of some pathways of ROS generation by pro-oxidants and an OxyR-based pathway of ROS detoxification.

2.1.4 Reporters

A reporter protein is the gene product of the gene downstream of the promoter region in a bioreporter construct. This protein emits a quantifiable signal (e.g. fluorescence).

Many reporter genes on plasmids are commercially available and all offer different fluorescent/luminescent properties. Many of these are improved variants of wild type fluorescent or luminescent proteins, obtained through directed evolution of bacterial populations possessing a cloned copy of the wild type fluorescent/luminescent genes (Shaner et al. 2004). I choose the mCherry fluorescent reporter protein for my construct. It has been praised in the literature because of its very practical properties for environmental application (Shaner et al. 2005). mCherry (excitation 587 nm, emission 610 nm), is a small monomeric protein. It does not require the addition of a substrate for it to be functional, eliminating the need to add compounds that may alter the natural properties of an environmental sample (Belkin and Gu 2010). It has one of the fastest maturation times to achieve its proper protein conformation (15 min), allowing time-dependent detection. Although mCherry does not have the highest brightness, which may cause issues for samples with high autofluorescent properties, it possesses one of the best photostabilities and is thus suitable for longer assays. Finally, mCherry has a high tolerance for pH variation, again making it particularly useful for wide variety of environmental samples (Shaner et al. 2004, 2005). Tecon et al. (2009) successfully utilized mCherry in a microbial whole-cell bioreporter to detect PAH in PAH-contaminated soil. Autofluorescence of the environmental sample did not seem to affect fluorescence measurements of the mCherry chromophore.

2.1.5 Rationale and Specific Objectives

The following sections explain the construction of the bioreporters (see Table 2.1). Table 2.1 presents a summary of the new strains cloned for this thesis. The predictions are that *E.coli* K-12 MG1655 (pFR02) and *E.coli* JW3596 (pFR02) emit fluorescence in a ROS dose-dependent manner, while the negative control *E.coli* K-12 MG1655 (pFR01) would be unresponsive to varying H₂O₂ concentrations.

Figure 2.2 offers a conceptual schematic summarizing the fate of metal-mining related toxic metals in a bioreporter *E.coli* K-12 MG1655 (pFR02) cell and the processes that lead to the activation of the bioreporter.

Table 2.1. List of constructs inserted into *E.coli* hosts.

Construct	Host
mCherry in pUCP19 (pFR01)	<i>E.coli</i> K-12 substrMG1655 ^a
katGp::mCherry in pUCP19 (pFR02)	<i>E.coli</i> K-12 substrMG1655 ^b
katGp::mCherry in pUCP19 (pFR02)	<i>E.coli</i> str. JW3596 ^c

^aActs as a negative control

^bMain focus of this thesis

^cAlternate bioreporter (rfaC-mutant) designed for improved sensitivity

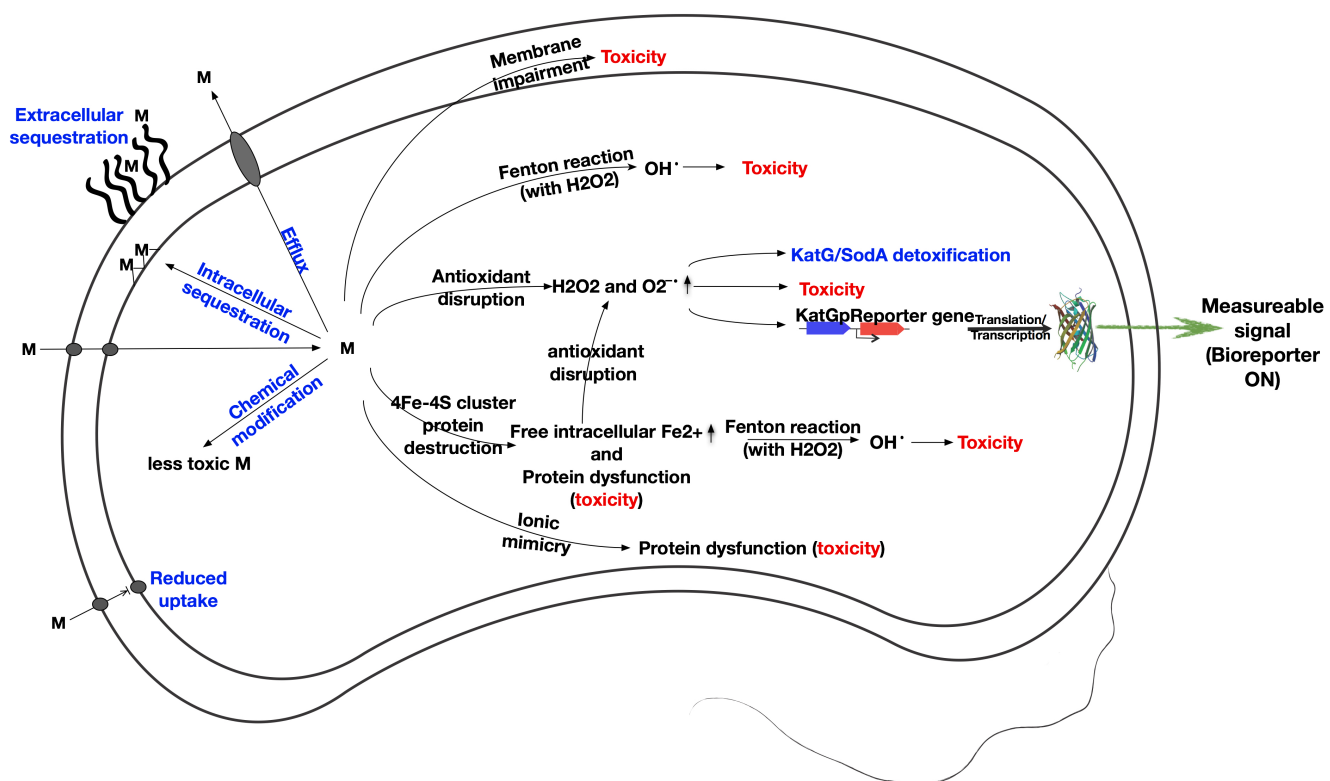


Figure 2.2. Conceptual schematic of the fate of metal-mining related metals (M) in a bioreporter *E.coli* K-12 MG1655 (pFR02) cell and bioreporter activation. Blue captions represent detoxification pathways and red captions represent toxic pathways.

2.2 Material and Methods

2.2.1 Molecular Cloning Technique and Transformation

The pFR02 plasmid (Fig. 2.3B) was assembled using a double cloning approach. First, a mCherry biomarker gene sequence was inserted into the multiple cloning site region of a pUCp19 plasmid. Then a *katG* promoter region sequence was inserted upstream of the mCherry gene. A vector containing the mCherry sequence (pmCherry) was obtained from Clontech (Mountain View, CA USA) and transformed into *E. coli* DH5 α competent cells (Agilent Technologies Canada Inc. Mississauga, ON Canada). The mCherry sequence was extracted with restriction enzymes XbaI and HindIII creating a DNA segment of 1071 base pairs (bp). mCherry is a 750 bp long gene, the 45 bp added to the 5' end of the gene creates a 15 amino acid head; the 3' end tail consists mostly of stop codons. The fragment was gel purified and ligated into pUp19, which was digested beforehand with the same restriction enzymes as mCherry (XbaI and HindIII). This created plasmid pFR01 (Figure 2.3A). The plasmid was then heat-shock transformed into *E.coli* strain K-12, substrain MG1655 (Table 2.1) and the strain preserved at -80°C.

A second round of digestion/ligation/transformation was required for the construction of pFR02. pFR01 was extracted and gel purified (Promega) to be finally digested with the restriction enzymes SacI and XbaI. This time, the insert is the promoter region of the *katG* gene found in *E.coli*. The sequence was obtained by polymerase chain reaction (PCR), amplifying a fragment of 258 bp with primers possessing the proper restriction sites corresponding to SacI and XbaI.

KatGp reverse (5'-ATATTATTCTAGATTGTGCTCCCCTCTACAGTGT-3')

KatGp forward (5'-ATATTAGGAGCTCTTGAGGGCGGGAAAATAAGGT-3')

The PCR product was then digested with the same restriction enzyme as the plasmid (SacI and XbaI) and then ligated into pFR01 to become pFR02 (Fig. 2.3). The plasmid was transformed on *E.coli* K-12 MG1655 cells by heat-shock transformation and on *E.coli* JW3596-1 (*E.coli rfaC* mutant) by electroporation transformation. All enzymes used purchased from New England Biolabs (Whitby, ON Canada).

2.2.2 Confirmation by PCR

The presence and length of the inserts in pFR02 were verified by extracting the plasmid from cultured transformed strains, and by performing a PCR using M13 primers. The expected band size was 1350 bp.

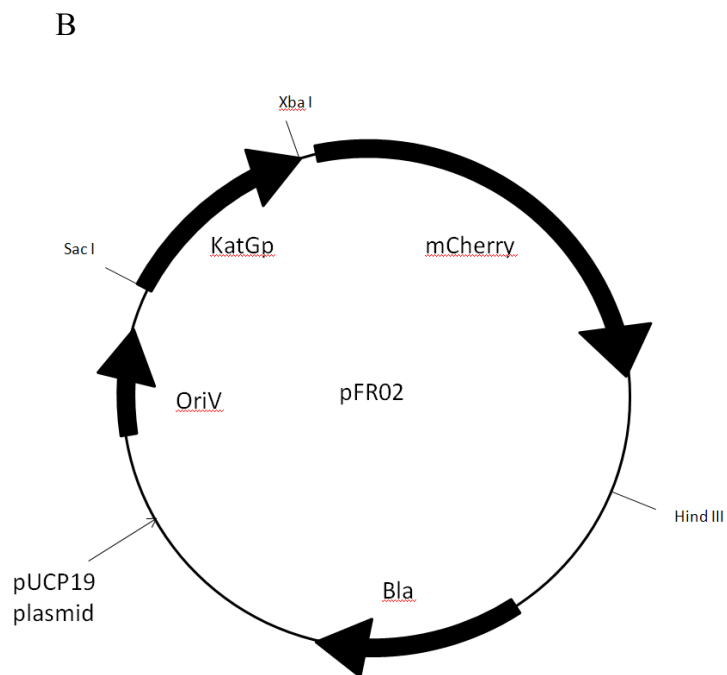
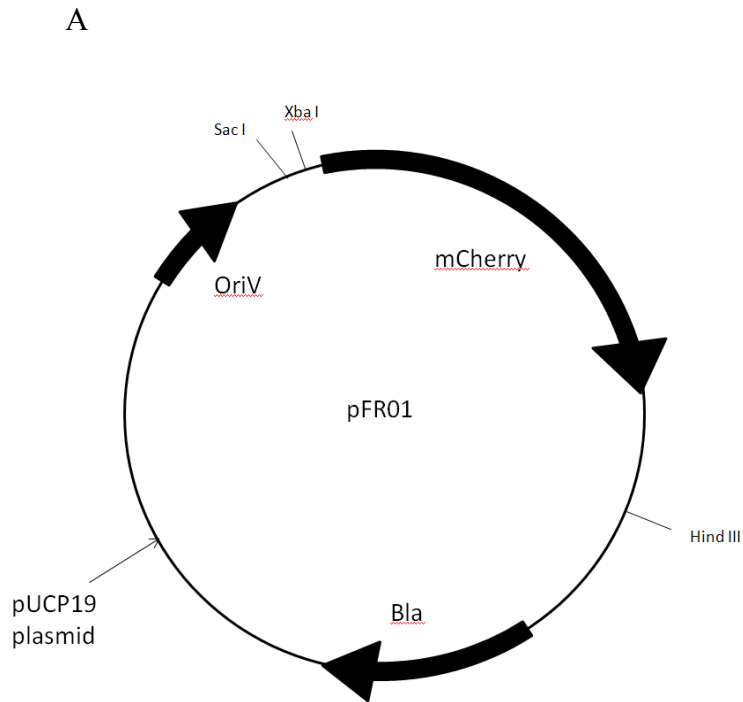


Figure 2.3. Plasmid map of pFR01 (A) and pFR02 (B). Bla, β -Lactamase; OriV, Origin of replication; SacI-HindIII-XbaI, restriction enzymes.

2.2.3 Functional Confirmation

A functional confirmation of the cloning process was also performed. From glycerol stocks, all transformed strains were plated onto lysogeny broth (LB) plates with ampicillin (100 µg/mL) and grown at 37°C overnight until the appearance of single colony units. A 1-colony inoculation in LB supplemented with ampicillin (100 µg/mL) was performed and grown until an optical density (OD₆₀₀) of 1.4, corresponding to late exponential phase (Fig. 2.4). It was important to collect the cells during exponential phase because OxyR in *E.coli* is mainly expressed during exponential phase (Pomposiello et al. 2001). I then performed a 1% inoculation of the bacterial culture into 2.5 mL LB, spiked with H₂O₂ to obtain 3 treatments (294, 1740 and 2941 µM H₂O₂) and 1 control with no H₂O₂. Two-hundred µL of these mixtures were then pipetted into the wells of a 96-well plate (transparent, flat bottom; Corning plates, Fisher Scientific Co., Ottawa, ON Canada) and placed into a TECAN Infinite 200 pro plate reader (Godrig, Austria). Measurements of absorbance at 595 nm and fluorescence (excitation 560 nm, emission 620 nm) using gain regulation and a flash set at 25 were performed every 10 min with shaking (15 s, amplitude 4) for 14 h.

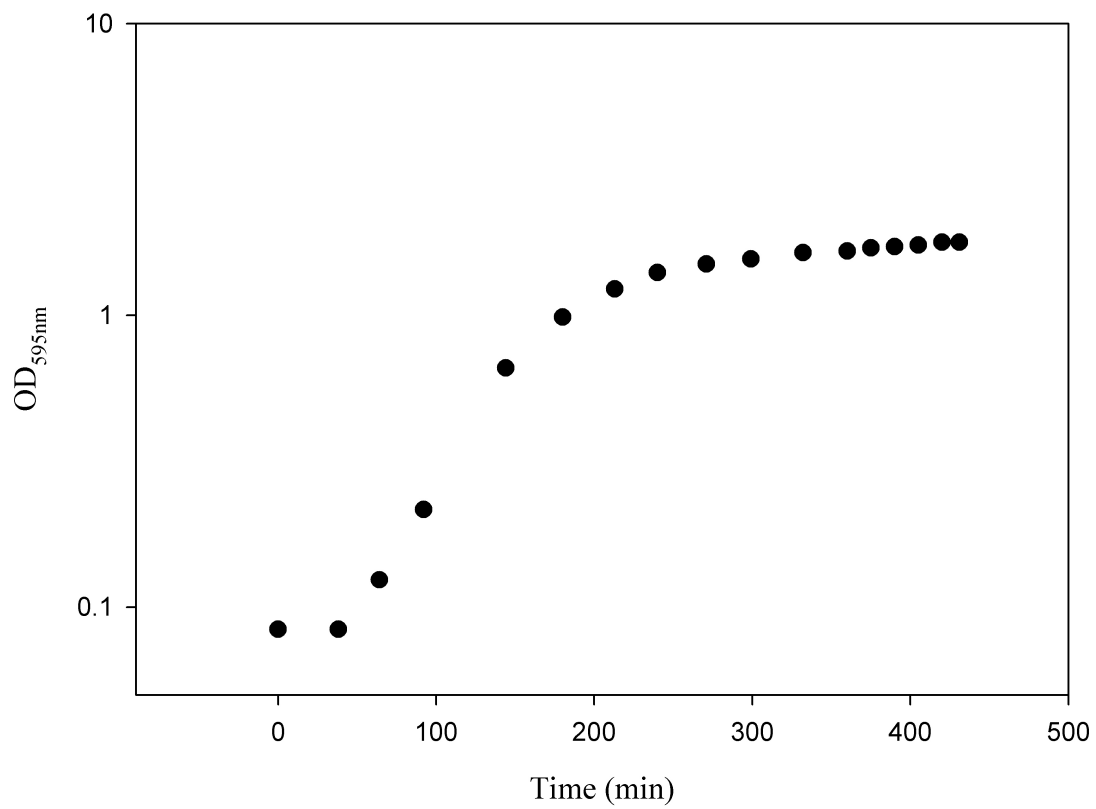


Figure 2.4. Growth curve of bioreporter *E.coli* K-12 MG1655 (pFR02) in LB media (with 100 µg/mL ampicillin). Collection of the cells occurred at an OD_{600nm} of 1.4, corresponding to late exponential phase.

2.3 Results and Discussion

2.3.1 PCR Confirmation

The length of the amplicon included mCherry, KatGp, part of the mCherry plasmid and M13 sequences. The total length was the expected 1350 bp.

2.3.2 Functional Confirmation

Figure 2.5 presents the growth and fluorescence data of *E.coli* K-12 MG1655 (pFR02) and *E.coli* K-12 MG1655 (pFR01) when exposed to hydrogen peroxide (H_2O_2). The growth curves (Fig. 2.5A and B) illustrate the toxicity for both strains when exposed to the H_2O_2 treatments. To estimate oxidative stress, fluorescence was normalized for OD_{595nm} (OD_{595nm} being a proxy of cell count) to take into account the reduced amount/number of cells resulting from oxidative stress toxicity. This provided better resolution between treatments (Fig. 2.5C and E). The comparison of fluorescence/ OD_{595nm} between *E.coli* K-12 MG1655 (pFR02) and the negative control *E.coli* K-12 MG1655 (pFR01) are perfectly in-line with our prediction (Fig. 2.5E and F). The negative control emits less basal fluorescence/ OD_{595nm} , and is not dose-sensitive, while the bioreporter *E.coli* K-12 MG1655 (pFR02) is dose-sensitive. This is also shown with Figure 2.6, with the maximum fluorescence/ OD_{595nm} (before an OD_{595nm} corresponding to 0.1; a value arbitrarily chosen), which is a proxy I used to quantify the relative amount of oxidative stress in the cell. Fluorescence data past the time corresponding to an OD_{595nm} of 0.1 is ignored because the large amount of cells in the media reduces the sensitivity of the assay. Additionally, this was done to avoid any effects that the approaching stationary phase (and medium saturation) could have on oxidative stress generation in the cell. The bioreporter *E.coli* JW3596 (pFR02) also responded in a dose-dependent manner, and thus it too qualifies as a bioreporter of oxidative stress (Fig. 2.7).

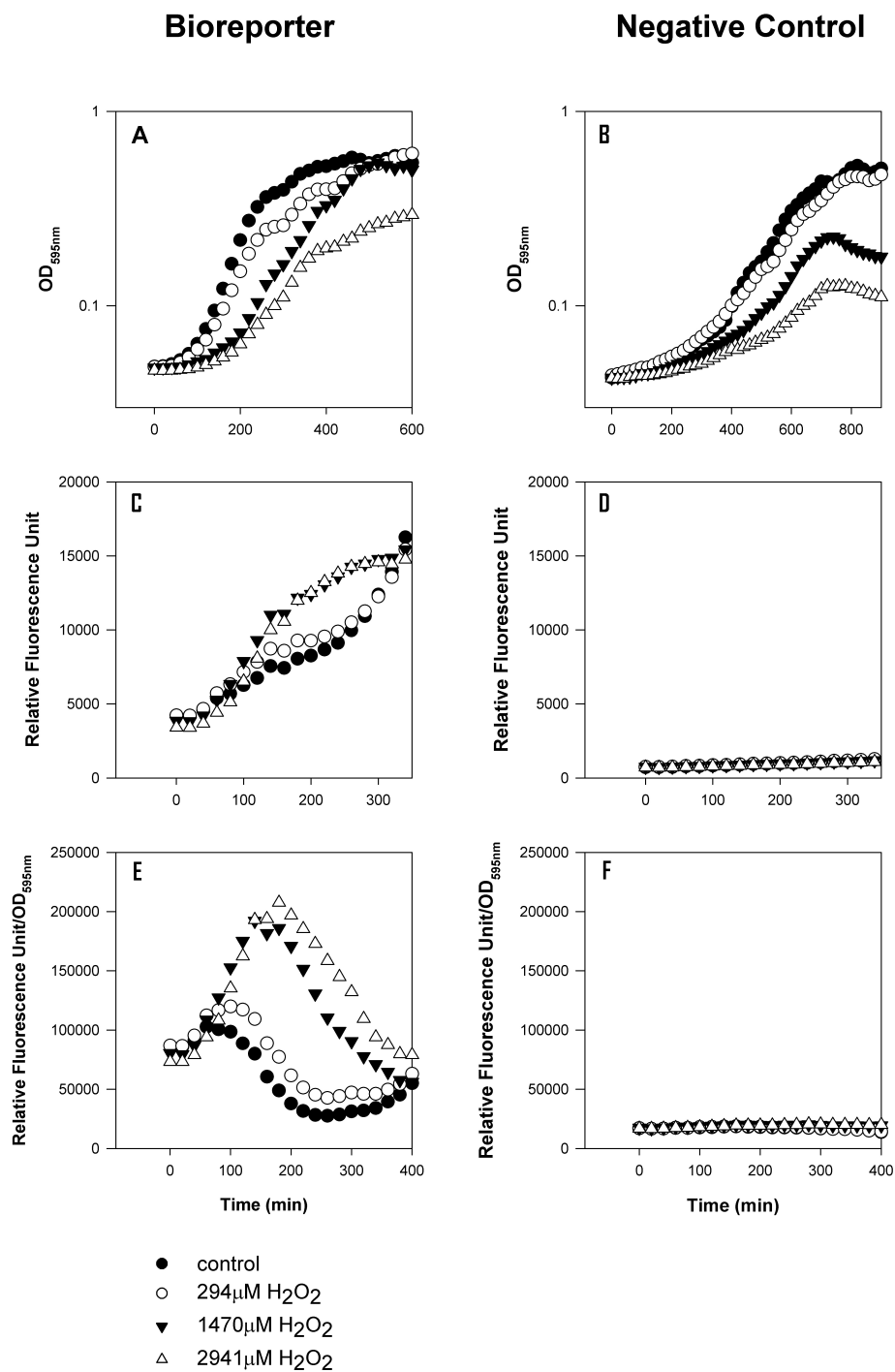


Figure 2.5. Growth and fluorescence data of the bioreporter (pFR02 in *E. coli*) (A,C,E) and the negative control (pFR01 in *E. coli*) (B,D,F) when exposed to hydrogen peroxide (H_2O_2) (0, 294, 1740, 2941 μM).

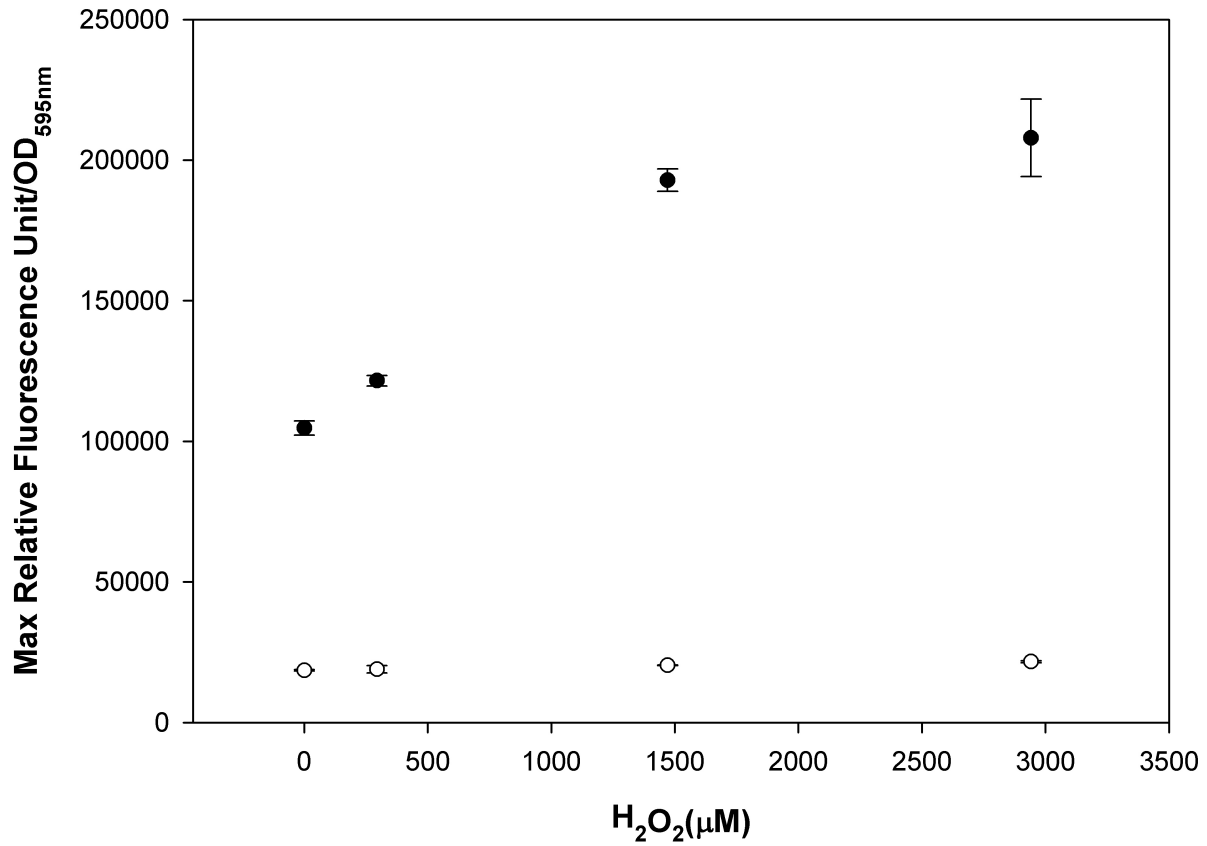


Figure 2.6. Maximum relative fluorescence unit/OD_{595nm} of the bioreporter *E.coli* K-12 MG1655 (pFR02) (black circles) and the negative control *E.coli* K-12 MG1655 (pFR01) (white circles) when exposed to varying hydrogen peroxide (H₂O₂) concentrations. Analytical replicates are presented (n=3) (replicates taken from the same bacterial culture tube).

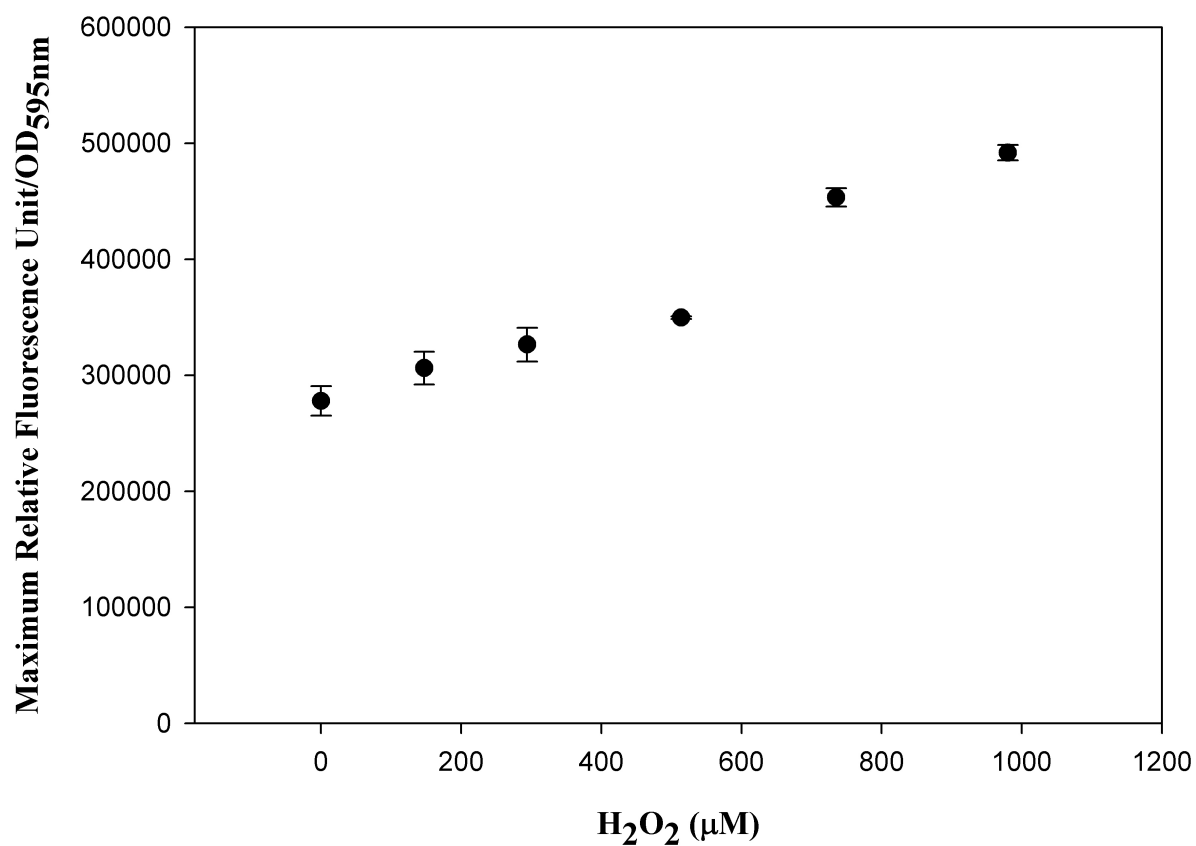


Figure 2.7. Maximum relative fluorescence unit/OD_{595nm} of the bioreporter *E.coli* JW3596 (pFR02) when exposed to varying hydrogen peroxide (H₂O₂) concentrations. Analytical standard deviations are presented (n=3).

2.4 Conclusions

I have developed 2 microbial bioreporters sensitive to varying concentrations of the oxidant H_2O_2 using standard cloning and transformation techniques. The next chapter focuses on the exploration of the sensitivity and specificity of this *E.coli* K-12 MG1655 (pFR02) bioreporter.

Chapter 3. Minimal Assay Development and Assay Calibration

3.1 Introduction

Chapter 3 has two objectives: i) to develop a minimal growth assay medium for the bioreporter assay [*E.coli* K-12 MG1655 (pFR02)]; and, ii) to calibrate the bioreporter to aluminum smelter and mining-effluent related inorganic pro-oxidants.

When environmental samples are supplemented with rich growth medium components, changes in the speciation of the various pro-oxidants of interest may occur (both in terms of their complexation and solubility). These interactions can reduce the bioavailability and thus sensitivity of the bioreporter, and also change the natural speciation of toxicants/metals. Regardless of these considerations, most microbial bioassays performed to evaluate oxidative stress are performed in a rich medium such as LB or Lysogeny Broth (Elad 2011; Dardennes 2007b; Belkin 1996; Lee 2003; Mitchel 2004).

To prevent speciation issues associated with rich media, the response of our bioreporter was compared in three different media: i) LB, which is a rich and undefined medium; ii) glucose minimal medium (GMM); and, iii) β -GMM (which is GMM free of inorganic-phosphate) (see Table 3.1 for media composition). Both minimal media (GMM and β -GMM) possess a minimum amount of nutrients, essential to sustain *E. coli* growth. They also offer the advantage of having a defined composition (see Table 3.1) allowing for the speciation of compounds with chemical equilibrium speciation software such as Visual MINTEQ (ver. 3.0) to be performed.

Additionally, the microbial bioreporter *E.coli* K-12 MG1655 (pFR02) was calibrated using typical aluminum smelter inorganic contaminants (aluminum, cadmium, lead, copper and zinc) and also mining industry related inorganic contaminants including soft and hard

metals and oxyanions [mercury, silver, silver-nanoparticles (AgNP), selenite, selenate, arsenite and arsenate]. The detection limit, dose-response range and maximum florescence induced were determined for each pro-oxidant. This calibration step was performed in β -GMM; the following section (Results and Discussion) will highlight why it is the best-suited medium for eventual environmental application. The following section discusses individual pro-oxidants, some indication as to why they were used and why chemical mixtures were used in my experiments.

3.1.1 Pro-oxidants of Interest

a) Paraquat:

Paraquat is a quaternary nitrogen herbicide, mainly used to control weeds and grasses in agricultural (applied pre-emergence) and non-agricultural contexts (U.S. EPA 1997).

Although it is relatively immobile in the soil due to its very strong binding to organic matter, paraquat is persistent, and it is possible it makes its way to surface waters (U.S. EPA 1997).

Paraquat readily shunts electrons from the respiratory chain and transfers these acquired electrons to molecular oxygen to produce the superoxide anion ($O_2^{\cdot-}$) (Suntres 2002; Bus et al. 1984). In addition to being environmentally relevant due to its presence in wastewater, paraquat is also a model compound typically used as an inducer of oxidative stress (Elad et al. 2011; Belkin et al. 1996).

b) Metals:

A wide range of environmentally relevant toxic inorganic metals were tested: soft metals (cadmium, lead, mercury, zinc, copper, silver) (Smith et al. 2002), arsenic and selenium oxyanions (arsenite, arsenate, selenite, selenate), aluminum and silver nanoparticles (AgNP). The soft metals mercury, cadmium, silver, lead, copper and zinc are all naturally

occurring in freshwaters; silver, however is extremely rare due to its low crustal abundance (U.S. EPA 1981, 1984a, 1984b, 1987, 1988, 2001, 2009). Anthropogenic sources of these metals from mining and metal-processing industries can be very important (Canadian Council of Ministers of the Environment 2007). The majority of cadmium, lead, copper, zinc, mercury and silver in most oxygenated freshwater environments is partitioned into the sediments because of their affinity with organic matter and/or their precipitation with hydrous ions and ferric and manganese oxides (U.S. EPA 1981, 1984a, 1984b, 1987, 1988, 2001, 2009). The dissolved portion of these metals in surface waters is bound to varying degrees to dissolved organic carbon, carbonates, sulphates, nitrates, and chlorides, depending on environmental conditions (Florence 1986; U.S. EPA 1981, 1984a, 1984b, 1987, 1988, 2001, 2009). Dissolved cadmium and zinc are predominantly found as free ions, contrary to copper, lead and silver which are bound to various inorganic and organic ligands (Florence 1986). Mercury occurs as inorganic (bound to chlorides, hydroxides and sulphides) and as organic mercury complexed with natural organic matter (Boszke et al. 2002; Hsu-Kim et al. 2013).

Although the toxicity of these soft metals has been known for some time, the mechanisms of action by which they exert toxicity remain under investigation. Two models describing soft metal toxicity are currently debated in terms of their relative importance in the toxicity to organisms. First, these metals interfere with the metabolism of essential cations used in biological systems; for example, Cd^{2+} and Pb^{2+} limit the biological uptake of Ca^{2+} (Xu et al. 2012; Lemire et al. 2011). Ag^+ can disrupt Na^+/K^+ transport through ionic mimicry (Galvez 1997). Second, soft metals are thiophilic, meaning that according to the hard-soft acid base theory, they have a strong affinity for soft bases like thiols (Lemire et al. 2011; Simkiss et al. 2001). Toxicity occurs through their binding to thiols and consequent

disruption of thiol containing proteins. The two main types of proteins in the cell containing abundant thiols are antioxidants and dehydratases that contain iron-sulphur clusters (Xu et al. 2012; Lemire et al. 2011). Inhibition of antioxidants like glutathione prevents the cells from maintaining a reduced environment and lowering ROS levels, resulting in oxidative damage. Binding to the sulphur of a dehydratase leads to a step-wise destruction of the iron-sulphur clusters and to the release of iron into the cytoplasm. The release of unincorporated iron into the cytoplasm increases the production of hydroxyl radical production through the Fenton reaction (equation 1), another source of oxidative damage. The dehydratase enzyme family is implicated in a multitude of very important and diverse metabolic pathways in the cell and so their destruction may also lead to cytotoxicity (Xu et al. 2012).



Although aluminum is abundant in the environment, from natural and anthropogenic sources (e.g. mining), its low solubility at pH 7 limits its bioavailability. At more acidic pHs, aluminum is solubilized. With recent anthropogenic acidification of some environments, concern over its known toxicity has arisen (Han et al. 2013). Aluminum causes toxicity mostly through ionic mimicry of iron. Displacement of iron from proteins increases the free iron in the cell that can subsequently raise ROS levels through the Fenton reaction as noted above in equation 1 (Han et al. 2013). Aluminum is also able to bind cell membrane phospholipids and disrupt cell membrane functioning (Pina et al. 1996; Han et al. 2013).

c) Oxyanions:

Arsenic water contamination constitutes a world-wide problem of high importance (Jain et al. 2000; Belkin et al. 2010). Input into freshwater is largely controlled by regional geology as well as by anthropogenic sources like mining and coal burning. Arsenic goes

through complex geological and biological cycles but is mainly present in aerobic freshwaters of neutral pH as the oxyanion arsenate [As(V)]; arsenite [As(III)], the reduced form although observed, is not stable under oxic conditions (U.S. EPA 1984; Mukhopadhyay et al. 2002). Because of its widespread occurrence, many organisms have developed dedicated detoxification strategies to arsenic (Rosen 2002). Arsenite is commonly thought to be the most toxic species (Jain et al. 2000; Jonnalagadda et al. 1993), causing oxidative stress because of its high thiophilicity as it binds and disrupts thiol containing antioxidants such as glutathione (GSH) and thioredoxins (Rosen 2002). Because of its similar structure to phosphate, arsenate causes toxicity through its disruption of phosphate metabolism (Nies et al. 1999).

Geological occurrence of selenium is very uneven across the globe. Selenium levels in freshwater can be important in regions where selenium occurrence in the soil is high and where oil refining activity, coal and oil burning, metal mining activity or agricultural irrigation of soil results in the mobilization and release of selenium into surface waters (Lenz et al. 2009). The two main species of selenium in freshwater are the oxyanions selenite (Se(IV)O_3^{2-}) and selenate (Se(VI)O_4^{2-}), the former being the most prevalent in oxic waters (U.S. EPA 2014). Selenium oxyanions are highly bioavailable to microorganisms; selenium can bioconcentrate in cells including microorganisms to factors of 1 million (U.S. EPA 2014). Thus, biological processes play an important part in the speciation of selenium in the water column (Lenz et al. 2009). Selenium is an essential element and microorganisms reduce selenium oxyanions to selenide (Se^{2-}) before incorporating Se into amino acids. When these organisms die, they release organic selenium (as selenide) associated with particulate matter (U.S. EPA 2014).

Since selenium uptake is slow and selenium bioconcentrates in microorganisms, the major exposure pathway of selenium to freshwater organisms (other than microorganisms) is through the diet (U.S. EPA 2014). As such, the U.S. EPA chronic WQG consists of a selenium water concentration criterion and a selenium concentration level in organism tissues.

Selenium is an essential element at low levels, however at high levels it becomes very toxic (Janz et al. 2010). It was thought earlier that because selenium possesses a chemistry similar to sulphur, ionic mimicry was an important mechanism of toxicity; however recent evidence casts doubt on the importance of this mode of toxicity (Janz et al. 2010). The main cause of toxicity of selenium oxyanions is now thought to be via oxidative stress generation, by interacting with GSH through a variety of mechanisms (Janz et al. 2010). Selenite and selenate react with GSH to form selenopersulfide and sulfur or thiyl radicals.

Selenopersulfide spontaneously react with molecular oxygen to produce ROS, or reacts with GSH again to form hydrogen selenide, which also reacts with oxygen to form elemental selenium and ROS. Thiyl radicals inhibit GSH (Janz et al. 2010). Selenite is more reactive than selenate because of its more polar character; it is also generally more toxic to aquatic life (U.S. EPA 2014). For example, three studies done on *Daphnia magna* by the U.S. EPA (Johnston et al. 1987; Ingersoll et al. 1990; Maier et al. 1993) as part of the water quality criterion derivation process for selenium, reported the average toxic acute value to this cladoceran was 7.8 μM for selenite and 20.9 μM for selenate.

d) Nanoparticles:

Engineered nanoparticles (NPs) are metallic particles with at least one dimension smaller than 100 nm (Massarsky et al. 2013). Their small size provides these particles with a high surface to volume ratio, a high surface curvature, a high chemical potential and a high

reactivity. Thus they have behaviours that differ markedly from their respective metal dissolved species and colloidal forms (Reidy et al. 2013; Massarsky et al. 2014). These properties of NPs have allowed novel applications and have driven engineering of various new NPs. AgNPs are presently the most abundant NPs and are mostly used for their antimicrobial properties because of their ability to be impregnated into a variety of matrices (e.g. socks) and because of their ability to release Ag^+ through dissolution in a controlled and sustained manner (Massarsky et al. 2014). The possibility of a particle-specific effect to eukaryotes or prokaryotes has raised ecotoxicological concerns (Reidy et al. 2013). AgNPs find their way to the environment (mostly from waste water treatment plants) (Massarsky et al. 2014) but their environmental fate and bioactivity is still unclear (Fabrega et al. 2011). The understanding of NP environmental fate has been limited over the last 20 years due to their multifarious nature; they differ in terms of composition (silver, gold, zinc, aluminum etc.), size, shape and coating. These factors have been shown to significantly affect the behaviour of a NP (e.g. AgNPs stability, rate of dissolution or aggregation) (Reidy et al. 2013). The water quality guidelines for AgNP are currently the same as for silver, even though AgNPs mechanisms of toxicity maybe unique.

Five proposed mechanisms of NPs toxicity to bacterial cells exist and their relative importance is under active debate (Reidy 2013). (1) Dissolution of elemental AgNP into Ag^+ and subsequent Ag^+ toxicity (discussed above). The dissolution rate of AgNPs is greatly influenced by environmental factors like ionic strength, pH, dissolved organic matter (DOM), dissolved oxygen and temperature, and the composition (core and coating) of the NP. (2) The NP can adhere to membranes and as such induce detrimental structural changes to membranes such as increased permeability. (3) Oxidative dissolution of AgNP into Ag^+ inside or outside

the cell generates ROS intermediates. (4) The oxidative dissolution also consumes H^+ and as such can disrupt the transmembrane proton gradient required for adenosine triphosphate (ATP) generation in the cell (equation 2 and 3) (Cao et al. 2011; Reidy et al. 2013).



(5) Finally, some AgNP have a neutral charge and so can more easily cross membranes and accumulate in the cell. This causes a differential localization of AgNP and Ag^+ , which causes them to affect biological targets differently. For example, the microenvironment near the membrane has a pH of 3 because of the proton gradient; this greatly increases the massive dissolution of AgNP into Ag^+ at an important site of Ag^+ toxicity (Reidy et al. 2013).

3.1.2 Mixtures

Toxicants rarely occur alone in the environment (Ince et al. 1999). The co-exposure of contaminants in mixtures is an emerging problem that needs further investigation. Metals are often found with organic pro-oxidants (e.g. PAHs, polychlorinated biphenyls) (Benedetti et al. 2009). Bioassays (e.g. bioreporters) inform on the integrative effects of all toxicants in these mixtures. To provide more environmental relevance, the bioreporter response to paraquat and its response to a mixture of paraquat and either cadmium or mercury present at sub-toxic levels is compared. I hypothesized that paraquat and soft metals tested would act synergistically in a mixture because paraquat generates ROS directly, while the soft metals tested would hamper the ability of the cell to detoxify ROS by disrupting cellular antioxidants. I predicted that the slope of the linear portion of the dose/fluorescence-response curve of a mixture composed of increasing paraquat dichloride concentrations and a constant

sub-toxic level of a soft metal would be higher than the slope of the linear portion of a curve corresponding to increasing concentrations of paraquat dichloride.

3.2 Material and Methods

3.2.1. Medium Components

The assay protocols described in Chapter 2 were used for all experiments in this section. For the development of the minimal growth assay medium, for practical purposes, only three pro-oxidants were tested; paraquat dichloride ($C_{12}H_{14}N_2$) (from Sigma-Aldrich Co. Oakville, ON Canada), $CdCl_2$ (from Sigma-Aldrich Co.), and $Pb(NO_3)_2$, which were tested in the 3 different growth media LB, GMM and β -GMM (see Table 3.1 for media composition). All pro-oxidants were dissolved into deionized and autoclaved water before being kept as stock solutions in the dark at 4°C.

Table 3.1. Molarity of constituents (M) of the different media (see Appendix A for media instructions).

Constituent	β -GMM	GMM	LB ^a
H ⁺	1x10 ⁻⁷	1x10 ⁻⁷	-
SO ₄ ⁻²	0.008615	0.0008302	-
Cl ⁻¹	0.022	0.02269	0.141
Na ⁺	0.006	0.0866	0.218
K ⁺	0.01837	0.01837	-
NH ₄ ⁺	0.01557	0.01557	-
Mg ⁺²	0.00083	0.00083	-
Zn ⁺²	2.84x10 ⁻⁷	2.84x10 ⁻⁷	-
Mn ⁺²	1.26x10 ⁻⁷	1.26 x10 ⁻⁷	-
H ₃ BO ₃	4.043 x10 ⁻⁶	4.043 x10 ⁻⁶	-
Co ⁺²	3.5 x10 ⁻⁷	3.5 x10 ⁻⁷	-
Ni ⁺²	7 x10 ⁻⁸	7 x10 ⁻⁸	-
MoO ₄ ⁻²	1.03x10 ⁻⁷	1.03x10 ⁻⁷	-
NO ₃ ⁻¹	5 x10 ⁻¹⁰	5x10 ⁻¹⁰	-
PO ₄ ⁻³	0	0.05815	-
β -Glycerophosphate	0.001	0	-
MOPS	0.04	0	-
Tryptone	0	0	10 g/L
Yeast Extract	0	0	5 g/L

^aLB is an undefined media, molarity of constituents is not known.

After establishing β -GMM as the media of choice for the assay, the bioreporter *E.coli* K-12 MG1655 (pFR02) was calibrated in the presence of Cd^{2+} , Pb^{2+} , Hg^{2+} (as $\text{Hg}(\text{NO}_3)_2$ from Wako Pure Chemical Industries, Osaka, Japan), Zn^{2+} (as ZnCl_2 from Fisher Scientific), Cu^{2+} (as CuCl_2 from J.T Baker, Center Valley, PA U.S.A.), Ag^+ , silver-nanoparticles (AgNP), $\text{Se}(\text{IV})\text{O}_3^{2-}$ (as Na_2SeO_3 from Sigma-Aldrich), $\text{Se}(\text{VI})\text{O}_4^{2-}$ (as Na_2SeO_4 from Sigma-Aldrich), $\text{As}(\text{III})\text{O}_3^{3-}$ (as NaAsO_2 from Sigma-Aldrich), $\text{As}(\text{V})\text{O}_4^{3-}$ (as Na_3AsO_4 from Sigma-Aldrich), and Al^{3+} . The AgNP used in our assay is carboxy-functionalized AgNP, stabilized by sodium polyacrylate, with an average size of 8.39 nm (see Massarsky et al. 2013). Assays were performed in triplicate with 3 different batches of *E.coli* for each pro-oxidant except for paraquat for which experiments were performed 5 times.

In the mixture experiments, the mixture was composed of sub-toxic levels of either CdCl_2 or HgCl_2 (0.1 μM) and paraquat dichloride (1.4 μM to 388 μM).

3.2.3. Data Analysis

Growth data were first analyzed by fitting a 4-parameter Gompertz model using SigmaPlot (version 9.01) to each growth curve. A Gompertz model was chosen because it has biologically relevant parameters including lag phase time, maximum specific growth rate and yield (Dardenne et al. 2007). Lag phase time and maximum specific growth rate were extracted from each treatments (see Appendix B for calculations). Pro-oxidants that increased lag phase time but did not change the maximum specific growth rate satisfied the conditions of application of the assay (see Fig. 3.1A). Pro-oxidants that altered the maximum specific growth rate did not adhere to the conditions of the assay because we could not reliably interpret their fluorescence (Fig. 3.1B).

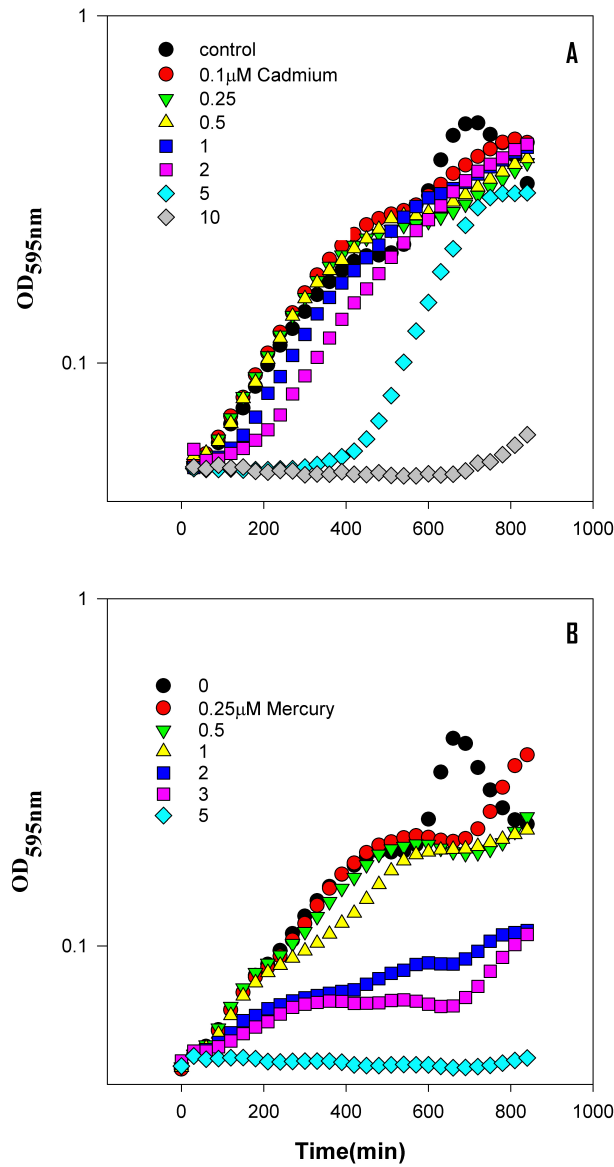


Figure 3.1. Growth of *E. coli* K-12 MG1655 (pFR02) in the presence of pro-oxidants. A) typical growth curves for pro-oxidants that increased lag phase time but did not change the maximum specific growth rate; B) typical growth curves for pro-oxidants that altered the maximum specific growth rate.

As described in Chapter 2, the maximum value of fluorescence/OD_{595nm} for each treatment was used as a proxy to quantify the relative amount of oxidative stress in the cell. Four-parameter logistic dose-response curves were fitted to the maximum fluorescence/OD_{595nm} data using SigmaPlot (version 9.01). The detection limits of the assay for each pro-oxidant was determined by plotting the fitted logistic curve and determining the concentration that corresponded to the highest value of the 2nd derivative of the curve (Fig. 3.2). Although detection limit is often determined by the concentration that emits fluorescence at a value equal to the basal fluorescence level plus 3 times the value of the standard deviation (Reeuwijk and Houba 1998), we opted for our approach because it provided a more sensitive method (yet less conservative) while being consistent and utilisable for all pro-oxidants. The upper detection limit of the assay was set as the concentration at which the logistic curve reached a plateau; further increases in pro-oxidant concentration led to toxicity. One-way ANOVA models and post-hoc multiple comparison tests (Tukey) (R version 2.14, The R Foundation for Statistical Computing) were performed to compare maximum fluorescence values, fluorescence detection limit and growth detection limit for each pro-oxidants. For the mixture experiments, the maximum slope of the linear portion of the dose-response curve was utilized as the proxy to compare the response of the bioreporter between mixtures.

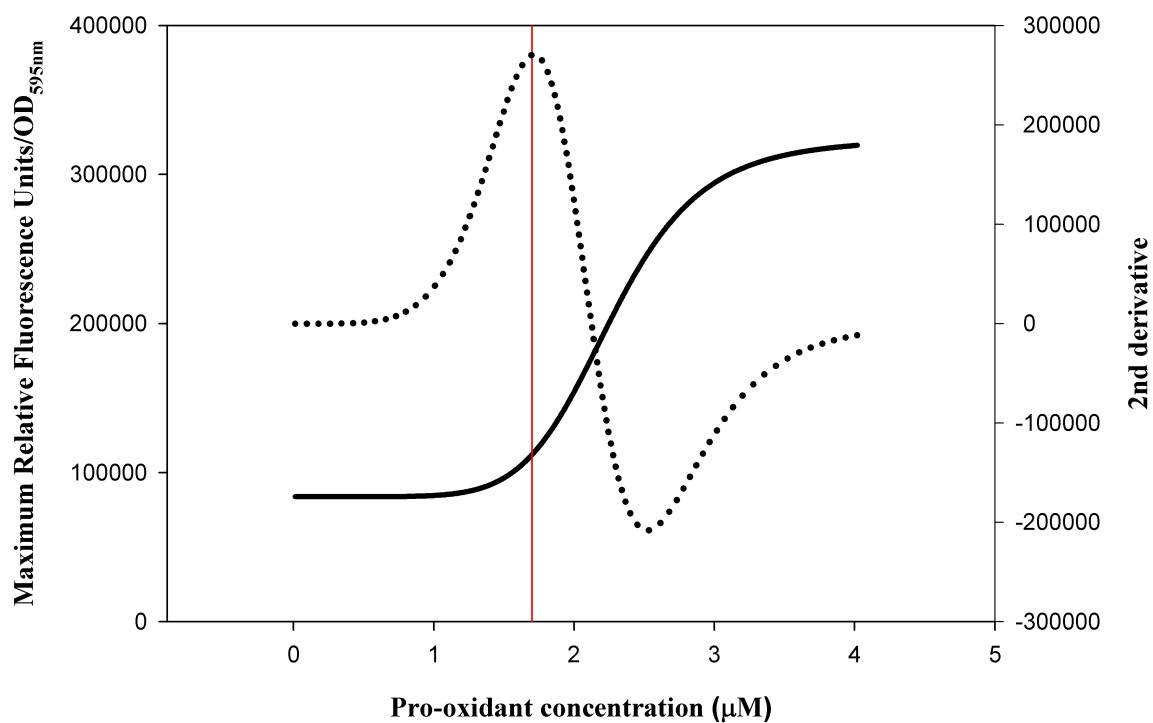


Figure 3.2. Estimation of the detection limit for a typical pro-oxidant dose-response curve (full line). The dotted line represents the 2nd derivative of the dose-response curve and the vertical red marker indicates the peak of the curve of the 2nd derivative and simultaneously the detection limit for this pro-oxidant (see text for explanation).

3.3 Results and Discussion

3.3.1 Minimal Media Development

Paraquat (PQ) was used as a positive control, as paraquat is a well-known pro-oxidant (Suntres 2002). It is a small polar organic pro-oxidant of environmental relevance (U.S. EPA 1997). We focused our medium development on Cd^{2+} , Pb^{2+} and PQ. We focused on cadmium and lead because they exhibit different solubilities with respect to the presence of phosphates. Minimal media are usually buffered using phosphates to avoid potential interaction of metals with organic buffer components (e.g. MOPS).

The results indicate an improved detection limit for all tested pro-oxidants in β -GMM (Fig. 3.3). As described in the following paragraphs, these results are further supported in light of the chemical equilibrium speciation data (Visual MINTEQ) (see Table 3.2).

The Visual MINTEQ model predicted that, in GMM, only 6% of the total cadmium was in the form of free Cd^{2+} ion, while most of the cadmium (89%) was bound to phosphates (Table 3.2). In β -GMM, where the inorganic phosphate was replaced with β -glycerophosphate, the free ion corresponded to 40.8% of the total cadmium, increasing free Cd^{2+} , the form of cadmium that is thought to be bioavailable (Hare and Tessier 1996).

Twenty percent of the total lead in GMM precipitated with phosphates as indicated using Visual MINTEQ and consequently raised $\text{OD}_{595\text{nm}}$ values and prevented the reliable interpretation of the data for lead in GMM (Table 3.2). In β -GMM, the free

Pb²⁺ proportion was 45%, allowing detection to a concentration of 0.21 µM, two orders of magnitude better than the detection in LB (53.1 µM; Table 3.3).

Altogether, the data support the use of β-GMM and GMM to improve assay sensitivity compared to LB. Furthermore, these results are supported by the literature that reports rich media typically contain a high concentration in organic carbon, typically limiting the availability of metallic cations (Barkay et al. 1997). It is surprising that a similar trend was observed for paraquat in LB, which may relate to the fact that paraquat is positively charged and thus may bind negatively charged dissolved organic carbon (DOC) and become less bioavailable as suggested by Wiegand et al. (2007). Since β-GMM allowed bacterial growth and also provided the best sensitivity to paraquat, cadmium and lead, this bioreporter medium was chosen for all further bioassays presented in this thesis.

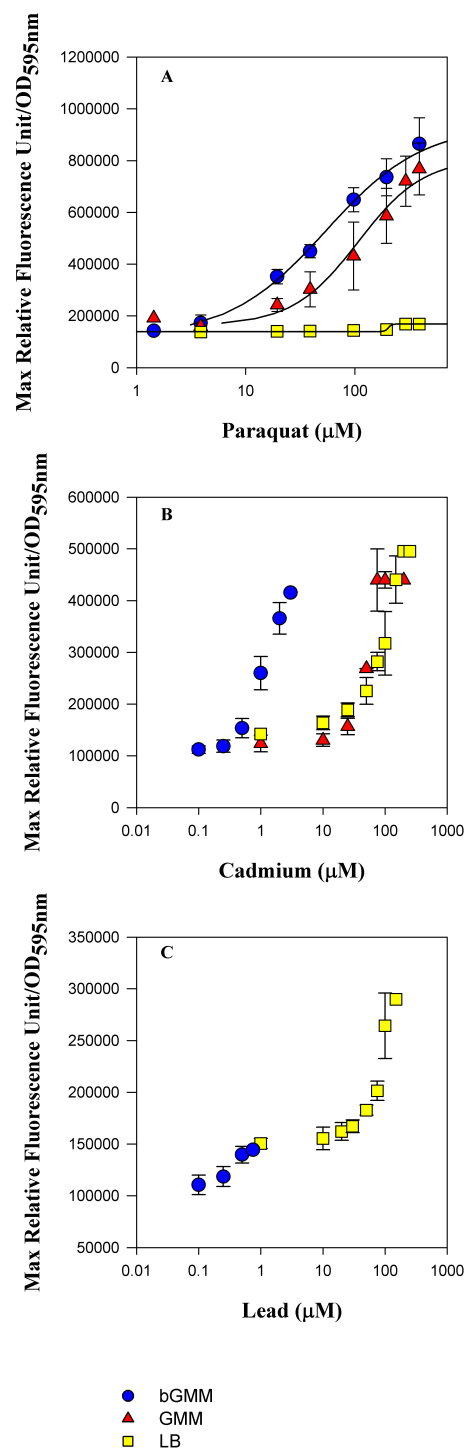


Figure 3.3. Dose-response curves for A) paraquat (β -GMM, $n = 5$; GMM, $n = 3$; LB, $n=2$), B) cadmium ($n=3$), and C) lead ($n=3$). Logistic curves were excluded from graph B and C to minimize cluttering of the data.

Table 3.2. Cadmium and lead speciation in β -GMM and GMM as predicted using Visual MINTEQ (ver.3.0).

Cationic Metal Species (%)			
β -GMM		GMM	
Cd^{2+}	40.8%	CdHPO_4	89%
CdCl^+	39.3%	Cd^2	6%
CdSO_4	14.9%		
CdCl_2	2.4%		
$\text{Cd}(\text{SO}_4)_2^{2-}$	1.5%		
Pb^{2+}	43.5%	PbHPO_4	54%
PbSO_4	33.2%	$\text{Pb}_5(\text{PO}_4)_3\text{Cl}_{(s)}^a$	20%
PbCl	15%	Pb^{2+}	15%
<u>PbOH</u>	5.2%		

^aChloropyromorphite precipitation

Table 3.3. Medium effect on the detection limit. Comparison for paraquat, cadmium and lead in LB, GMM and β -GMM. Method used is indicated in Figure 3.2.

Pro-oxidants	Medium	Detection Limit (μM) (n-value)
Paraquat	LB	187 (2)
	GMM	13.7 (3)
	β -GMM	1.4 (3)
Cadmium	LB	30.1 (3)
	GMM	44.5 (3)
	β -GMM	0.2 (3)
Lead	LB	53.1 (3)
	GMM	Not applicable ^a
	β -GMM	0.2 (3)

^a Major precipitation of lead in GMM

3.3.2 Calibration of Responses to Pro-oxidants

This section presents the toxicity response-range (growth used as proxy), the oxidative stress response-range and the maximum induced oxidative stress for all pro-oxidants tested along with their respective Canadian WQG as reported by Canadian Water Quality Guidelines for the Protection of Aquatic Life (Canadian Council of Ministers of the Environment 2014b). The data are summarized in Tables 3.4 and 3.5 as assessed from supplementary figures presented in Appendix C using the procedure described in section 3.2.3 (Data Analysis) and the calculations presented in Appendix B.

Paraquat, silver ion, AgNP, cadmium, lead, zinc, arsenite and arsenate increased lag phase duration (λ) but did not change maximum specific growth rate (μ); as such, the tested concentrations respected the conditions of application of the assay (Fig. 3.1A). Treatments with mercury, copper, selenite and selenate altered the maximum specific growth rate and therefore did not qualify to the conditions of application of the assay. The assay fluorescence could not be reliably assessed (Fig. 3.1B).

Table 3.4. Range of detection (based on growth parameters) of varying pro-oxidants. Standard deviations are presented in parenthesis (n = 3 for each except for paraquat where n = 5). Copper, mercury, selenite and selenate oxyanions were all toxic at the concentrations tested and therefore could not be fitted to a growth model. See section 3.2.3. (Data Analysis), Supplementary Figures (Appendix C) and Parameters Calculations (Appendix B) for calculation of these values.

Dose-Response Detection Range		
	μ (growth rate)	λ (lag phase duration)
Pro-oxidants	Range (μM)	Range (μM)
Paraquat		1.4-n/a ^a
Silver	no fit	9.0x10 ⁻⁴ (5x10 ⁻⁵) – 2.1x10 ⁻³ (7x10 ⁻⁴)
Cadmium	no fit	0.709(0.660)-10
Lead	no fit	0.9-5.6
Silver-NP	no fit	1.37(0.33) – 1.75(0.25)
Arsenate	no fit	3.5(5.3) - 100
Zinc	7.4(2.9)-33.3(15.2)	14.6(0.4)-33.3(15.2)
Arsenite	no fit	1,472(421)-2,333(288)
<u>Alumium</u>	no fit	100-750

^an/a, not applicable. The upper toxicity range, determined by the concentrations causing no growth, was not observed even at a paraquat treatment concentration of 1000 μM.

Table 3.5. Maximum oxidative stress [assessed as Max relative fluorescence units/OD_{595nm} (Max RFU/OD_{595nm})] and range of detection (fluorescence) of the pro-oxidants tested in decreasing order of most induced oxidative stress. Standard deviation is in parenthesis; n=3 except for paraquat, n = 5.

Assessed Oxidative Stress		
Pro-oxidants	Sublethal range tested (μM)	Max RFU/OD_{595nm}
Paraquat	1.4 - 427(87)	983,715 (366,646)
Cadmium	0.2(0.1) - 3(1.7)	417,133 (60,485)
Arsenite	972 (275) – 2,333 (288)	286,324 (71,017)
Silver-NP	1.2 (0.2) – 1.7 (0.2)	203,003 (32,502)
Zinc	9.5 (1) – 23.3 (5.7)	201,314 (32,680)
Silver	6x10 ⁻⁴ (1x10 ⁻⁴) - 1.1x10 ⁻³ (2x10 ⁻⁴)	157,060 (26,510)
Arsenate	5.1 (2.53) - 41.6 (14.43)	150,309 (10,940)
Lead	0.2 (0.01) - 0.5 (0.14)	144,233 (1,929)
Background ^a		112,000 (14,000)

^aMax RFU/OD_{595nm} when no pro-oxidant is added to the growth medium

3.3.2.1. Soft Metals

Table 3.6 illustrates the speciation of tested metals in β -GMM as calculated using Visual MINTEQ. Because speciation is an important determinant of toxicity and because medium components alter the speciation of each metal differently, interpretation of the relative toxicity and oxidative stress generation of metals must be done with caution. The free ion is generally thought to be the most bioavailable and therefore the most toxic species; however many exceptions to the free ion model are reported (Kungolos et al. 2006; Campbell 1995). For example, neutral metal-chloride complexes are lipophilic and can cross biological membranes and exert toxicity inside the cell (Campbell 1995). In our assay medium, cadmium, lead and copper have similar proportions of free ions at 40.8, 43.5 and 44%, respectively (see Table 3.6). Zinc free ion proportion is relatively high in the media, at 71%. As such, we expected a greater zinc toxicity in our assay medium than what is predicted by the literature because for a fixed total metal concentration, the free ion is present at higher levels. Chloride complexes dominated the speciation for silver and mercury.

Table 3.6. Metal speciation in β -GMM using Visual MINTEQ (ver.3.0).

Cationic Metal Species	%	Oxyanions Species	%
Cd^{2+}	40.8	$\text{H}_3\text{As(III)O}_3$	99 ^a
CdCl^+	39.3		
CdSO_4	14.9	HAs(V)O_4^{2-}	61 ^b
CdCl_2	2.4	$\text{H}_2\text{As(V)O}_4^{1-}$	39 ^b
$\text{Cd(SO}_4)_2^{2-}$	1.5		
		HSe(IV)O_3^-	94.3 ^c
Pb^{2+}	43.5	Se(IV)O_3^{2-}	5.6 ^c
PbSO_4	33.2		
PbCl^+	15	Se(VI)O_4^{2-}	99 ^d
PbOH^+	5.2		
AgCl_2^-	63		
AgCl	33		
AgCl_3^{2-}	1.8		
Ag^+	1.1		
Cu^{2+}	44		
CuNH_3^{2+}	28		
CuSO_4	15		
CuOH^+	6		
$\text{Cu(NH}_3)_2^{2+}$	3.9		
Zn^{2+}	71		
ZnSO_4	27.21		
HgCl_2	67.7		
HgCl_3^-	14.9		
$\text{Hg(NH}_3)_2^{2+}$	9.6		
HgClOH	5.7		
HgCl_4^{2-}	1.9		
$\text{AlO(OH)}_{(s)}$	99.9		

^aarsenic speciation when arsenite is added to the medium^barsenic speciation when arsenate is added to the medium^cselenium speciation when selenite is added to the medium^dselenium speciation when selenate is added to the medium

Silver, lead and zinc all induced equivalent amount of fluorescence, all considerably less than cadmium (Table 3.5, Fig. 3.4). Silver was the most toxic soft metal (although this was not statistically significant) and exhibited toxicity in the low nM range. Cadmium and lead exhibited toxicity starting at 0.7 and 0.9 μM , respectively while zinc was the least toxic and exhibited toxicity at 14.6 μM . Silver, cadmium, lead and zinc can lead to iron-sulfur cluster destruction (Xu et al. 2012), releasing free-iron to the cytoplasm. Iron present in the cytoplasm can increase hydroxyl radical production through the Fenton reaction and therefore result in enhanced ROS production. The hydroxyl radical is highly reactive and principally damage DNA (Linley et al. 2012) but hydroxyl radicals are not detected by a OxyR-based bioreporter, which senses hydrogen peroxide (H_2O_2) directly and superoxide anion ($\text{O}_2^{\bullet-}$) indirectly (see Fig. 1.2). This could explain the low fluorescence produced by our bioreporter when exposed to silver. Alternatively, cadmium and zinc toxicity could be related to deactivation of GSH (and other thiol-disulfide-containing antioxidants including thioredoxins) and consequently elevate ROS levels such as H_2O_2 and $\text{O}_2^{\bullet-}$. Relative to all soft metals, zinc has a weak affinity for thiols, which likely explains the low generation of oxidative stress when compared to cadmium. Zinc is also an essential metal and as such its intracellular concentration is highly regulated (Nies 1999). Another possible explanation for the high toxicity of silver but weak generation of oxidative stress could be its disruption of the bacterial membrane and consequently disruption of the proton motive force through an unknown mechanism (Dibrov et al. 2002). Silver is effectively used as an antimicrobial precisely because the inner membranes of bacteria are sites of energy production while eukaryote ATP production occurs in mitochondria located within the cell.

Table 3.5. Maximum oxidative stress [assessed as Max relative fluorescence units/OD_{595nm} (Max RFU/OD_{595nm})] and range of detection (fluorescence) of the pro-oxidants tested in decreasing order of most induced oxidative stress. Standard deviation is in parenthesis; n=3 except for paraquat, n = 5.

Assessed Oxidative Stress		
Pro-oxidants	Sublethal range tested (μM)	Max RFU/OD_{595nm}
Paraquat	1.4 - 427(87)	983,715 (366,646)
Cadmium	0.2(0.1) - 3(1.7)	417,133 (60,485)
Arsenite	972 (275) – 2,333 (288)	286,324 (71,017)
Silver-NP	1.2 (0.2) – 1.7 (0.2)	203,003 (32,502)
Zinc	9.5 (1) – 23.3 (5.7)	201,314 (32,680)
Silver	6x10 ⁻⁴ (1x10 ⁻⁴) - 1.1x10 ⁻³ (2x10 ⁻⁴)	157,060 (26,510)
Arsenate	5.1 (2.53) - 41.6 (14.43)	150,309 (10,940)
Lead	0.2 (0.01) - 0.5 (0.14)	144,233 (1,929)
Background ^a		112,000 (14,000)

^aMax RFU/OD_{595nm} when no pro-oxidant is added to the growth medium

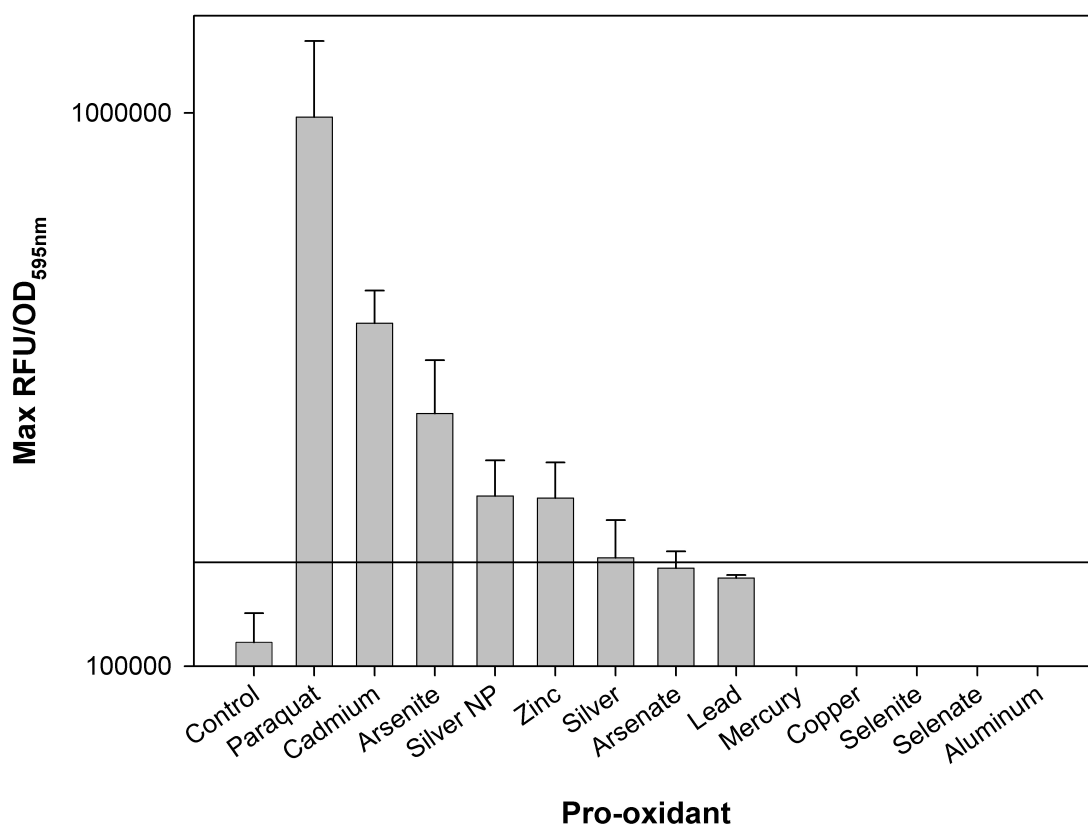


Figure 3.4. Maximum relative fluorescence units (max RFU)/OD_{595nm} comparison between all tested pro-oxidants (data from Table. 3.5). $n=3$ for all pro-oxidants with the exception of paraquat ($n = 5$). Selenite, selenate, mercury and copper could not be interpreted for their Max RFU/OD_{595nm} because of the unusual growth they caused (as explained in Material and Methods; see Table 3.5). For pro-oxidant activity to be detected in an environmental sample (i.e. detection limit), the sample must induce a fluorescence/OD_{595nm} value of 154,000 or more, represented by a horizontal reference line on this graph (see discussion of detection limits on page 70).

As the intent of these experiments is to develop a bioreporter as an early warning tool, it is important that it acts as a relevant surrogate for other organisms, and so responds to pollutants in a similar fashion and at similar concentrations to which other organisms in aquatic ecosystems respond (fish, invertebrates, amphibians, plants). Figures 3.5-3.8 (data taken from Table 3.5) present the overlap between the detection limit of each soft metal for the bioreporter *E.coli* K-12 MG1655 (pFR02) and the range of their long-term toxicity endpoints from the most sensitive species to the least sensitive species of all taxonomic groups taken from the Canadian Water Quality Guidelines for the Protection of Aquatic Life document when available; i.e. cadmium (Canadian Council of Ministers of the Environment 2014a) or the U.S. EPA Ambient Water Quality Criteria documents for silver, zinc and lead (U.S. EPA 1980, 1984a, 1987).

The oxidative stress bioreporter was sensitive to cadmium starting at 0.2 μM and responding up to 3 μM (Fig. 3.5). The WQG for cadmium (when water hardness is 50 $\text{mg CaCO}_3 \text{ L}^{-1}$) is 0.0008 μM , more than 3 orders of magnitude lower than the detection limit of our assay. The most sensitive fish species used to determine cadmium WQG (Canadian Council of Ministers of the Environment 2014a), was the rainbow trout with a 62-d EC_{10} of 0.002 μM while the least sensitive species was the northern pike with a 35-d maximum allowable toxicant concentration (MATC) of 0.071 μM . *Daphnia magna* was the most sensitive invertebrate species with a 7-d EC_{10} of 0.0004 μM while the least sensitive species was a dragon fly with a 7-d MATC of 680 μM . The most sensitive amphibian was the Northwestern salamander with a 24-d MATC of 0.942 μM . Finally, the most sensitive algae/aquatic plant species was a green algae with a 96-h NOEC of 0.043 μM while the least sensitive was duckweed with a 7-d EC_{50} of 0.702 μM . Thus,

the detection limit of the bioreporter developed here is higher than the concentrations causing adverse effects to the most sensitive species. However, it is also able to detect cadmium levels before they would be considered toxic for many other species of interest in aquatic ecosystems. This is also the case for lead and zinc. The Canadian WQG for lead is 0.0048 μM (Canadian Council of Ministers of the Environment 2014b) and the oxidative stress detection limit reported here is 0.212 μM (Table 3.5; Fig. 3.6). The CWQG for zinc is 0.450 μM and the detection limit reported for the bioreporter is 9.53 μM (Fig. 3.7). Silver is the only metal to which the bioreporter is sensitive enough to detect at its WQG level. The detection limit for oxidative stress using silver in the bioreporter is 0.0006 μM while the CWQG is 0.0009 μM (Fig. 3.8). It is not surprising that the bioreporter would be sensitive to silver at the WQG level because silver is an antimicrobial (Maillard et al. 2013) and so it is expected that bacteria will be the most sensitive species compared to all the other taxonomic groups.

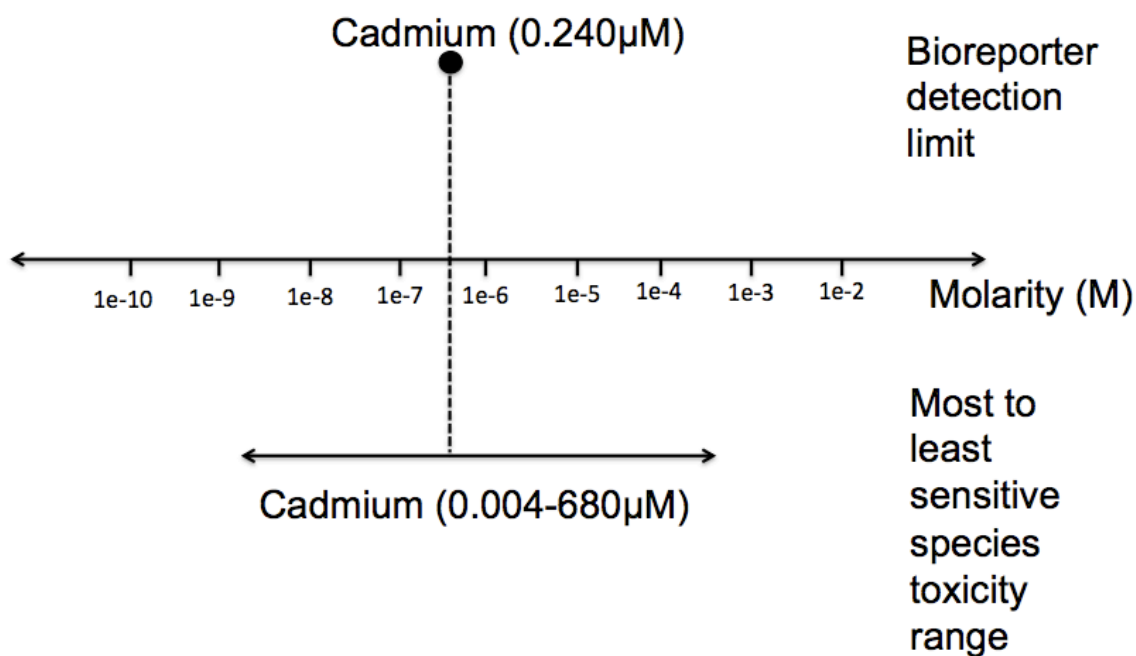


Figure 3.5. Bioreporter *E.coli* K-12 MG1655 (pFR02) detection limit (top) with the range of cadmium long-term toxicity endpoints from the most sensitive species to the least sensitive species (bottom). Long-term endpoints taken from the Canadian Water Quality Guidelines for the Protection of Aquatic Life, Cadmium (2014) (Canadian Council of Ministers of the Environment 2014a). Detection limits taken from Table 3.5.

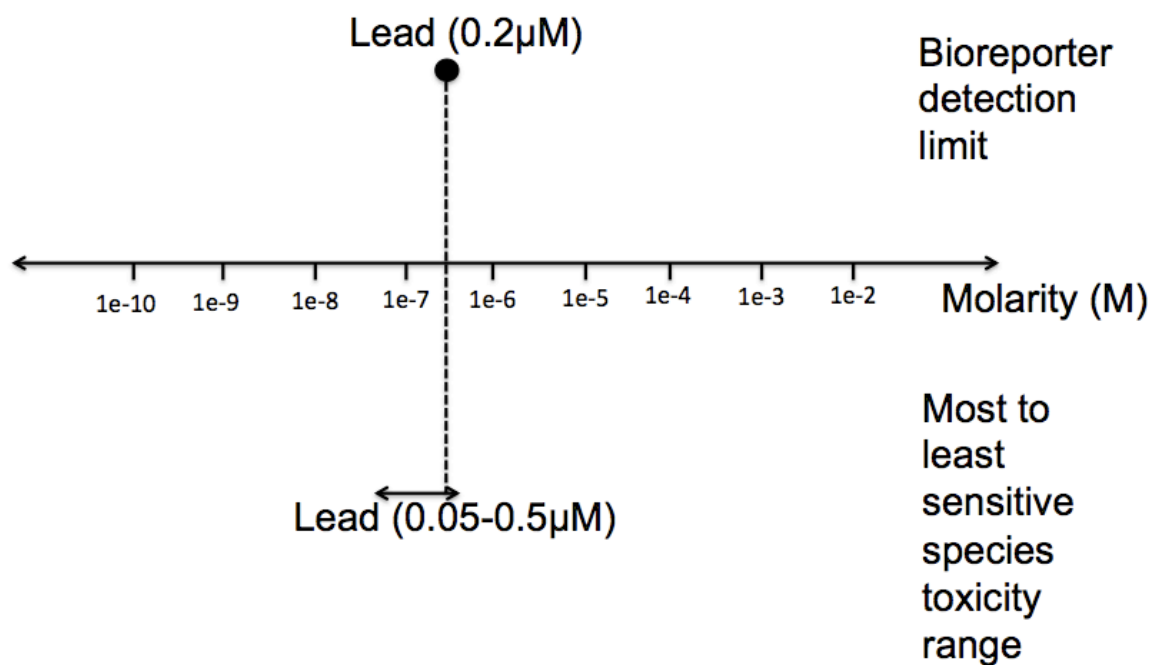


Figure 3.6. Bioreporter *E.coli* K-12 MG1655 (pFR02) detection limit (top) with the range of lead chronic minimum lethal concentration from the most sensitive species to the least sensitive species (bottom). Minimum lethal concentrations taken from U.S. EPA ambient water quality criteria for lead document (U.S. EPA1984a). Detection limits taken from Table 3.5.

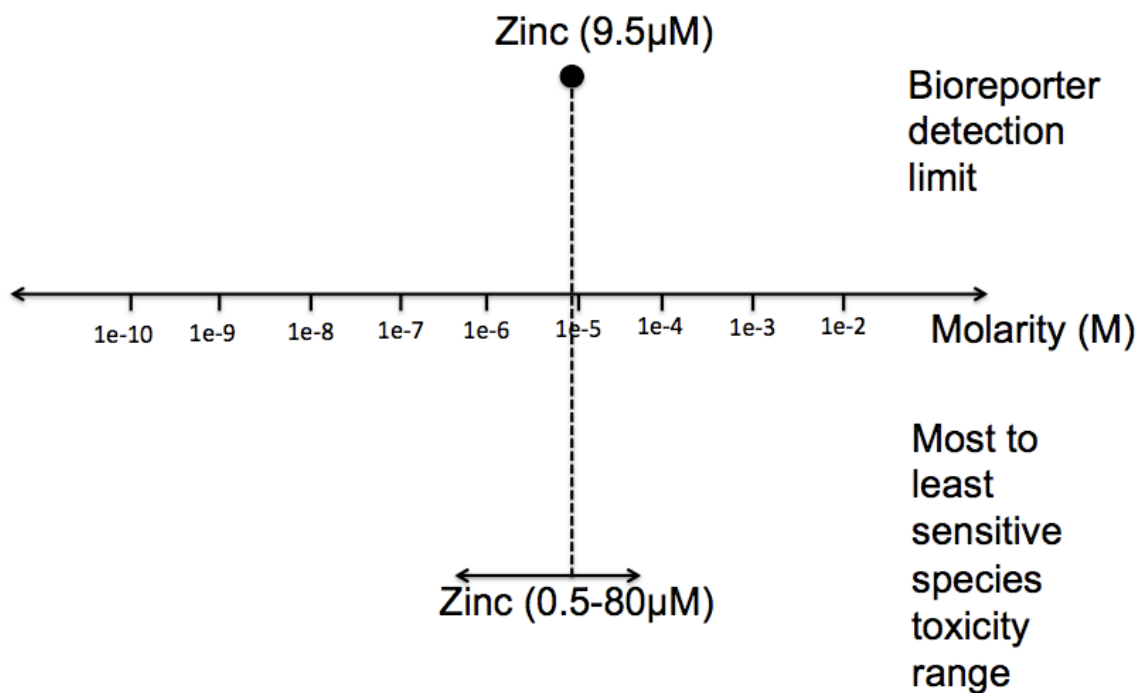


Figure 3.7. Bioreporter *E.coli* K-12 MG1655 (pFR02) detection limit (top) with the range of zinc chronic minimum lethal concentration from the most sensitive species to the least sensitive species (bottom). Minimum lethal concentrations taken from U.S. EPA ambient water quality criteria for zinc document (U.S. EPA 1987). Detection limits taken from Table 3.5.

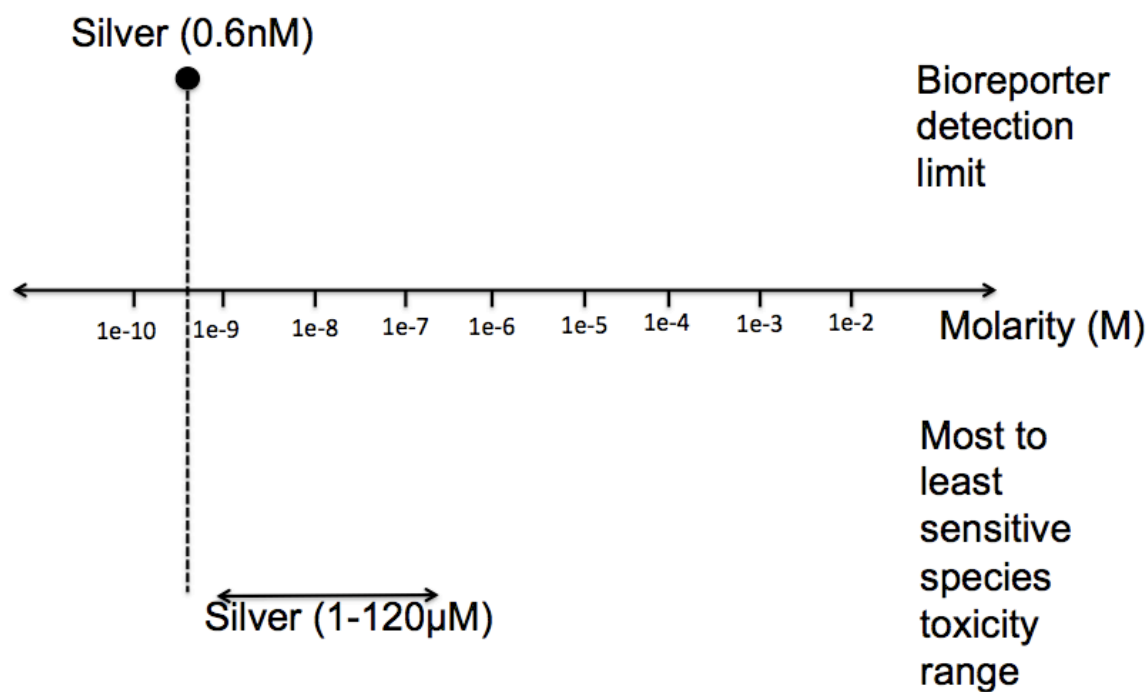


Figure 3.8. Bioreporter *E.coli* K-12 MG1655 (pFR02) detection limit (top) with the range of silver chronic minimum lethal concentration from the most sensitive species to the least sensitive species (bottom). Minimum lethal concentrations taken from U.S. EPA ambient water quality criteria for silver document (U.S. EPA 1980). Detection limits taken from Table 3.5.

3.3.2.2 Silver nanoparticles (AgNP)

The toxicity of Ag^+ was higher compared to AgNP (Table 3.4), although AgNP appeared to induce more oxidative stress than Ag^+ , although not significantly (Fig. 3.4). This difference between the toxicity of Ag^+ and AgNP is consistently seen in the literature (see Massarsky et al. 2013, 2014). The extremely small window of toxicity of AgNP (less than 500 nM ; from 1.37 μM to 1.75 μM) suggests the important contribution of ionic silver in AgNP toxicity and prevents the use of a microbial bioreporter to detect silver species toxicity.

3.3.2.3 Arsenic

The bioreporter showed a relevant response for the two prevalent species of arsenic: arsenite and arsenate. Arsenite was the element that caused the 2nd highest oxidative stress response after cadmium (Table 3.5, Fig 3.4). This was expected because arsenite or As(III) is a well-known pro-oxidant (Rosen 2002). It was also expected that arsenite induced significantly more oxidative stress than arsenate [AsO_4^{3-} or As(V)], a toxicant that causes toxicity mainly through phosphate metabolism disruption (Rosen et al. 2002). Arsenate induced oxidative stress (as much as silver and lead), and this can be explained by how arsenite is metabolized in *E. coli*. Before earth's oxic atmosphere, reduced arsenic (arsenite) was the prevalent species and so bacteria evolved detoxifying machinery involving arsenite efflux pumps. However, with the oxygenation of Earth's atmosphere, arsenate became the prevalent species. Instead of developing arsenate efflux pumps (which would be problematic because of its affinity for phosphate), evolution selected for an enzyme involved in As(V) reduction to As(III) as the first step, followed

by efflux of newly produced arsenite (Nies et al. 1999). The oxidative stress observed here may have resulted from arsenite during the detoxification process prior to its efflux.

Arsenite is generally considered more toxic than arsenate (Jain et al. 2000; Jonnalagadda et al. 1993). Surprisingly, the opposite trend was observed in this study; arsenate was toxic from 3 to 100 μM while arsenite was toxic from 1400 to 2300 μM (Table 3.4) while arsenite-induced oxidative stress was detected at 972 μM (Table 3.5). Arsenic water quality guideline in the U.S. is based on arsenite toxicity, as an overprotective measure and the CWQG was set at 0.065 μM and the most sensitive plant species had a 14-d EC_{50} of 0.65 μM while the most sensitive fish species had a 28-d LC_{50} of 7.3 μM (Canadian Council of Ministers of the Environment 2001). Clearly, our detection of arsenite is not adequate. This can be explained by the β -GMM media composition and its role in the bioavailability of arsenic oxyanions (Fig. 3.9). Arsenate is taken up by *E. coli* and by most organisms by the inorganic phosphate transport system (PitA and PitB) while arsenite is taken up by an inner membrane glycerol facilitator channel (GlpF) (Meng et al. 2004), because at neutral pH, arsenite will be present as H_3AsO_3 , an inorganic equivalent of polyols (Meng et al. 2004). β -GMM is devoid of inorganic phosphate but rich in glycerol 3-phosphate. Thus, in β -GMM, arsenite may compete with glycerol 3-phosphate (a polyol) at the GlpF uptake sites, possibly explaining the high tolerance of *E. coli* for arsenite. On the other hand, in GMM, arsenate competes with inorganic phosphate present (58 mM), possibly explaining the high tolerance to arsenate. By using β -GMM (that improved sensitivity to soft cationic metals), the relevant toxic specificity of the arsenic oxyanions may have been changed. In terms of application of the bioreporter to environmental samples, the increased toxicity

to arsenate is not problematic because the phosphate naturally present in the sample will act as a relevant inhibitor of arsenate toxicity. Also, arsenate is not a powerful pro-oxidant. While our medium poses a problem for the detection of oxidative stress induced by arsenite in the environment, it offers a novel way to investigate the route of As(III) uptake, which so far remains poorly characterized.

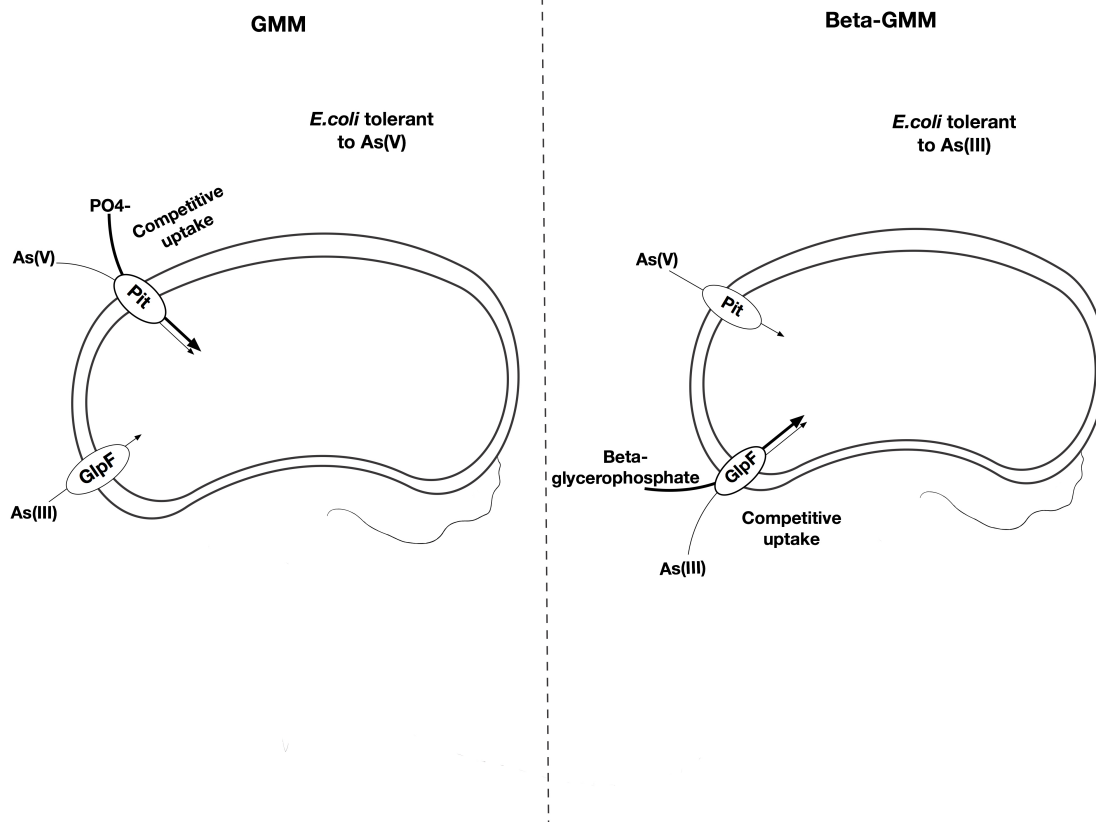


Figure 3.9. Conceptual representation of the effect of media (GMM and β -GMM) on the bioavailability of arsenic oxyanions. Pit, phosphate inorganic transport system; GlpF, glycerol facilitator channel.

3.3.2.4 Selenium

Neither selenium oxyanions satisfied the conditions of application of the assay, because both changed maximum specific growth rates, and so oxidative stress could not be interpreted reliably. Selenite was more toxic ($\sim 1\text{-}10\ \mu\text{M}$) than selenate ($\sim 100\text{-}300\ \mu\text{M}$) (Table 3.4). The chronic Canadian WQG (Canadian Council of Ministers of the Environment 2014b) for selenium is $0.013\ \mu\text{M}$ ($0.067\ \mu\text{M}$ in the U.S) and as such well below the detection limit of our assay. No reliable oxidative stress data could be generated from investigating selenium oxyanions.

3.3.2.5 Aluminum

The toxicity range of the bioreporter to aluminum was between $100\ \mu\text{M}$ and $750\ \mu\text{M}$ (Table 3.4). Considering that at pH7, the Canadian WQG for total aluminum is $3.7\ \mu\text{M}$ (Canadian Council of Ministers of the Environment 2014b), the bioreporter is not adequate to detect effects associated with environmentally relevant concentrations of aluminum. Aluminum speciation is a function of pH, anion concentrations (e.g. F^- , OH^-) and organic matter levels (Smith 1996). In our media, Visual MINTEQ predicted that 99.9% of aluminum would precipitate as the mineral diaspore [$\text{AlO}(\text{OH})$], which might explain the weak toxicity. Polymerization of aluminum hydroxide into a solid phase is a slow process that can take up to several days (Snodgrass et al. 1984).

Aluminum did not induce fluorescence at the sub-toxic or toxic levels tested. The main mode of aluminum toxicity is via the liberation of free iron into the cell that can react with H_2O_2 to produce the most reactive ROS, the hydroxyl radical ($\text{OH}^\bullet$) (Han et al. 2013). The hydroxyl radical reacts extremely fast with biomolecules to cause oxidative

damage (Reiter et al. 1995). As mentioned earlier, activated OxyR construct is designed to detect H_2O_2 , not OH^- .

3.3.3 Mixtures

Figure 3.10 illustrates the response of the bioreporter to paraquat or paraquat and a soft metal at a sub-lethal concentration. The slope of the linear portion of the dose-response curve was not higher for the paraquat-soft metal mixture although the prediction made was that it would be higher because of a possible synergistic effect. The paraquat-mercury mixture assay was only performed once, while the paraquat-cadmium mixture assay was performed twice and so more replicates are needed before conclusions can be drawn.

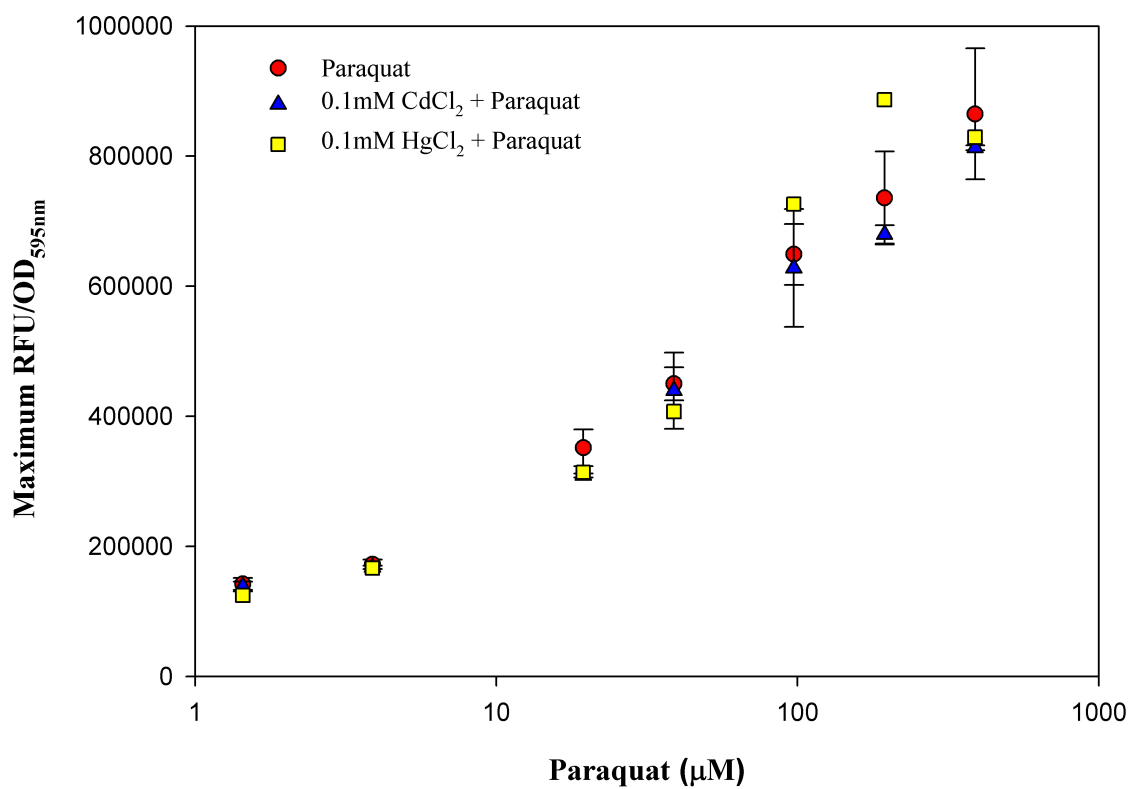


Figure 3.10. Maximum relative fluorescence units normalized to optical density at 595 nm for the *E.coli* bioreporter K-12 MG1655 (pFR02) when exposed to paraquat or to a mixture of paraquat and a soft metal (cadmium or mercury). Paraquat alone is in red (n=3), paraquat with CdCl₂ is in blue (n=2) and paraquat with HgCl₂ is in black (n=1).

3.4 Conclusions

With the use of β -GMM, the *E.coli* K-12 MG1655 (pFR02) bioreporter assay has brought some much-needed improvements with respect to the detection limit of inorganic contaminants (i.e cadmium) (Dardennes 2007b; Lee 2003; Mitchel 2004). However, when the results of the bioreporter assay are compared with long-term WQG values, further improvements are needed. The objective of this chapter was to estimate quantitatively the activity of pro-oxidants and to assess the crucial aspects of the performance of the bioreporter assay; detection limits, reproducibility and robustness from site-to-site. The average baseline level of fluorescence/OD_{595nm} obtained over the 38 experiments reported in this thesis using β -GMM was 112,000, with a relatively low noise or coefficient of variation (%) (standard deviation/average) of 12% (see Dardenne et al. 2007b). Based on this value, for pro-oxidant activity to be detected in an environmental sample (i.e. detection limit), the sample must induce a fluorescence/OD_{595nm} value of 154,000 (see equation 3; from Reeuwijk and Houba 1998 and Figure 3.4).

$$\begin{aligned}\text{Detection limit} &= \text{basal level} + \text{standard deviation} \times 3 \\ &= 112,000 + 14,000 \times 3 = 154,000\end{aligned}\tag{3}$$

The coefficient of variation for the highest pro-oxidant dose varied from 37% for paraquat to 1% for lead (see Table 3.5). The coefficient of variation for paraquat was high because of its extremely high fluorescence induction and the usually accompanying high coefficient of variation for higher inductions. The average coefficient of variation for available inorganic pro-oxidants was 14.8%, which according to Dardenne et al. (2007b), ranks well in terms of within-lab reproducibility compared to similar micro-

based assays and OECD (Organization for Economic Co-operation and Development) approved whole organism tests. Inter-lab reproducibility is an important parameter that needs to be evaluated as part of the standardization of the assay. Automation of the technical steps like pipetting has been shown in the past to greatly decrease the coefficient of variation (Dardenne et al. 2007b). As such, automation is something worth considering.

Another critical parameter is the robustness of the assay to the very wide range of environmental conditions that might affect the status of the cell, plasmid copy number, gene expression products and accompanied interferences like autofluorescence, optical density etc. All of these can affect the bioreporter output (without being related to oxidative stress) and thus affect the accuracy of the assay. Robustness will be assessed in Chapter 4 by comparing the basal normal bioreporter output (in β -GMM) and the basal output in non-polluted environmental samples. When testing in new conditions, extrinsic factors should always be considered for the interpretation of the fluorescence. For example, the presence of antibiotics can lower plasmid copy numbers and as such vary the copy number of bioreporter plasmid constructs. However, this approach of appropriately considering all extrinsic factors is not realistic because they are still poorly understood (Swain et al. 2002; Elowitz et al. 2002) and as such, calibration of the bioreporter assay with an environmental reference sample of similar composition to the sample of interest should be performed.

The general, narrow working range of the bioreporter for some contaminants (e.g. silver), defined by the detection limit to the concentration causing cell death, is an aspect of the bioreporter that is of concern for the application to environmental samples.

Elevated metal levels in the water sample can result in bacteria death and prevent them from emitting fluorescence and warn of oxidative stress. Others have observed the same narrow response before cell death in *E.coli* (Xu et al. 2012). Xu et al. suggested that the cell possesses a buffering capacity for metals through adsorption to the cell surface, but when the threshold value is exceeded, the cell is no longer able to tolerate the metal and sudden entry of metals into the cytoplasm triggers cell death. This buffering capacity could also be due to the cell's ability to perform extra- and intracellular sequestration of metals, the antioxidant repair system, the cell's ability to chemically modify the metal to a less bioactive state and the efflux system. These detoxification mechanisms may eventually become saturated, the metal exerts its toxicity and the cell dies abruptly. The narrow working range (less than an order of magnitude) of the *E.coli* K-12 MG1655 (pFR02) bioreporter was only observed for a few pro-oxidants, specifically the ones that did not induce high levels of fluorescence, such as AgNP, silver, zinc and lead.

Physicochemical changes to the bioassay can lead to improvements in sensitivity and specificity, as seen with the use of the β -GMM over GMM and LB. Additionally, one of the unique advantages of microbial bioreporters is that they can be genetically modified to create more ideal surrogate organisms. Bacteria resort to a multitude of strategies to deal with high levels of toxic metals. The creation of mutant bacteria with specific detoxification or resistance strategies can lead to bacteria that are more sensitive to specific targeted metals. For example, the ZntA P-type ATPase efflux pump in *E.coli* controls divalent soft metal homeostasis by responding to rising Cd^{2+} , Pb^{2+} , Zn^{2+} and Hg^{2+} intracellular levels (Rensing et al. 1997; Sharma et al. 2000; Binet et al. 2000). Rensing et al. (1997) illustrated that a *zntA* mutant *E.coli* showed increased sensitivity to

Cd^{2+} and Zn^{2+} . Such a mutant could be useful to us as a bioreporter host and could provide a better sensitivity to divalent soft metals.

This chapter has made a case to illustrate that the bioreporter *E.coli* K-12 MG1655 (pFR02) responds to inorganic pro-oxidants relevant to aluminum smelter and mining effluents at concentrations that are relevant to freshwater aquatic life. The next step was to validate the bioreporter by testing ICP-MS-characterized environmental samples and compare responses with a general toxicity bioreporter; this is the object of Chapter 4.

Chapter 4. Bioreporter Validation with Environmental Samples

4.1 Introduction

Chapter 3 presented evidence that the bioreporter *E.coli* K-12 MG1655 (pFR02) responds to inorganic pro-oxidants relevant to aluminum smelter and mining effluents at concentrations that in some cases are relevant to freshwater aquatic life; the aim of this chapter is to further validate the bioreporter using field samples. The validation will be done by assaying real-environmental samples with the oxidative stress bioreporter and comparing our response with a general toxicity bioreporter assay (similar to Microtox) and with water quality characteristics and the Canadian Water Quality Index (WQI) (Canadian Council of Ministers of the Environment 2014c). The WQI summarizes complex water quality data into an index that can be used to assess water quality and to provide insights concerning the extent to which the state of the water is suitable for aquatic life. The index considers frequency of instances for which water quality objectives are not met and the extent to which they are not met. The general toxicity bioreporter assay will use the *E.coli* strain, HMS174, containing plasmid pRB27 that allows for constitutive expression of bioluminescence (Golding et al. 2002). It is used as a general indicator of overall bacterial cell health. The environmental samples are taken from the receiving environment of our industrial partner the Rio Tinto Alcan (RTA) Grande-Baie (Saguenay, QC, Canada) plant of aluminum production (see section 1.3).

4.2 Material and Methods

4.2.1 Site Description

The Grande-Baie aluminum smelter produces pure aluminum from alumina (Al_2O_3) through reductive electrolysis with the Hall-Héroult process (Habashi 1998). It also produces its own carbon anodes that are used for the Hall-Héroult process. The Hall-Héroult process produces between 20 and 40 kg of waste rich in sodium fluoride and cyanide for each 1000 kg of pure aluminum produced (Jones and Haynes 2011). Two effluent streams composed of the cooling water from aluminum casting to produce aluminum lingots and from cooling towers are released into two small ditches at location 1201 and 1202 (see Fig. 4.1). Approximately 1 km downstream, the two ditches dump into a small lake (less than 500 m long) that empties into yet another small lake, which empties into a small stream that eventually (~5 km) empties into the Saguenay River. Alongside the two lakes to the north, is a golf course, while south of the lakes, agricultural fields are present.

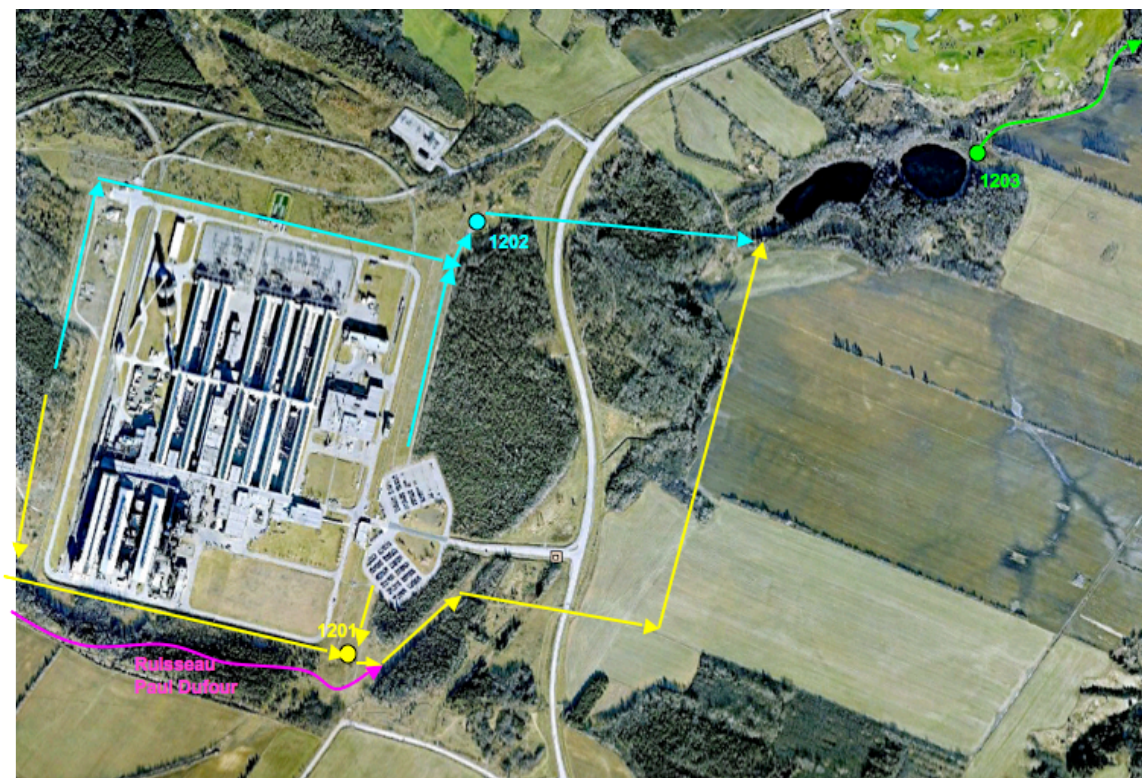


Figure 4.1. Grande-Baie refinery and receiving environments. Sampling was performed seasonally at site 1201, 1202, at the entry of the first lake and at the exit of 2nd lake (1203).

4.2.2 Sampling

Water sampling (see Fig. 4.1) was performed by the RTA personnel in the receiving environments near the effluent discharge at locations 1201 and 1202 where the estimated toxicity risk was thought to be the greatest. Samples were also taken at the entry of the first lake, to potentially observe a synergistic effect of the two effluent discharges, and so another area presenting a risk. Lastly, samples were taken at the location 1203 after both lakes, to potentially observe a pollution-sink effect of the lakes and to better understand downstream risks. Sampling was performed bi-monthly from March 2013 to September 2013.

Water samples arrived frozen at the University of Ottawa. The samples were left to thaw completely at room temperature over a period of 24 h. Each bottle was subsampled into 3 x 50 mL falcon tubes. One falcon tube was acidified to a final concentration of 1% (v/v) with trace metal nitric acid (HNO_3 ; 1 M) and bromine chloride (BrCl ; 1M) was added to the tube, then stored at 4°C for subsequent total mercury analysis. Another tube was simply acidified as above with trace metal nitric acid (HNO_3) and stored at 4°C for subsequent total metal analysis (performed within 4 months). The remaining falcon tube was stored at -20°C for subsequent bioreporter assays.

4.2.3 Chemical Analysis of Samples

Total metal concentration (Al, Cr, Mn, Co, Ni, Cu, Zn, As, Mo, Ag, Cd, Sb, Ba, Se, Ti, Pb, U, Be, Th) of each sample was determined using an Agilent Tech 7700 Series ICP-MS (Agilent Technologies, Mississauga, ON Canada) using previously published protocols (Lewen et al. 2004). Calibration standards (Environmental Calibration Standard part 5183-4688) and internal standard mixes (Agilent Internal Standard Mix; Al,

Ba, Se, Ti) were purchased from Agilent Technologies (Mississauga, ON, Canada).

Internal standard elements (Se, Ge, Rh, Ln, Tb, Bi, Er) were analysed for matrix recovery rates. Calibration standard and blank samples were run intermittently throughout the duration of the procedure.

Total mercury (THg) concentration in the samples was measured using a Tekran 2600 following method 1631 described by the U.S. EPA (EPA, 2002)

4.2.4 Bioreporter Assays

4.2.4.1 Oxidative Stress Bioreporter Assay

Bioreporter assays were performed similarly to those described in Chapter 2 using the *E.coli* bioreporter K-12 MG1655 (pFR02). From glycerol stocks, all transformed strains were plated onto LB plates with ampicillin (100 µg/mL) and grown at 37°C overnight until the appearance of single colonies. A one-colony inoculation in LB supplemented with ampicillin (100 µg/mL) was performed and grown until an optical density (OD_{600nm}) of 1.4, which corresponds to late exponential phase. We then performed a 2% inoculation of the bacterial culture into β-GMM (2X). One-hundred µL of this inoculum was then pipetted onto the wells of a 96-well plate (transparent, flat bottom, Corning). One-hundred µL of unfiltered or filtered (0.2 µm EMD Millipore Millex Sterile Syringe Filters, PES Membrane) environmental samples thawed at 4°C prior to the assay were transferred into the wells containing 100 µL 2X β-GMM. After this dilution, wells contained a mixture equivalent to a 1% inoculation from the 1.4 OD_{600nm} culture, β-GMM (1X) and the environmental sample (0.5X). As previously described (see 2.2.3), the plate was placed into a TECAN Infinite 200 Pro plate reader. Measurements of absorbance at 595 nm and fluorescence (excitation 560 nm, emission

620 nm) using gain regulation and a flash set at 25 where performed every 10 min with shaking (15 s, amplitude 4) for 14 h.

4.2.4.2 General Toxicity Bioreporter Assay

This assay is a cell suspension assay, where the bacteria are physiologically active but do not multiply or grow. Five mL of LB (with 100 µg/L kanamycin) was inoculated with a single colony of an *E.coli* strain, HMS174, containing plasmid pRB27 and constitutively expressing bioluminescence (Chiasson-Gould et al. 2014). Incubation at 37°C with shaking at 200 rotations per min was performed until late exponential phase (7 h). Five mL of GMM was inoculated with 50 µL of the LB growing culture and left to grow overnight (17 h). GMM was utilized instead of β-GMM because we wanted to use an already well-established and published microbe-based toxicity assay. Subsequently, 20 mL of GMM (100 µg/L kanamycin) was added to the culture and the culture was left to grow for an additional 3 h. Two x 2 mL Falcon polyethylene (Fisher Scientific) tubes were each filled with 1 mL of the GMM culture and centrifuged at max speed (13,000 rpm) for 30 s. Supernatant was removed and another 1 mL of the GMM culture was added to the remaining pellet that was again centrifuged. The supernatant was removed from both tubes. In each tube, 500 µL of inorganic phosphate buffer (see Appendix A) solution was added and the pellet re-suspended and transferred into the second Falcon tube. The entire content of this tube was added to a glass tube containing 5 mL inorganic phosphate buffer solution and small increments (100 µL) of the cell suspension were added until the optical density (at 600 nm) reached 0.4. This mix was then diluted 20 times into assay medium (2X) (see Appendix A). Finally, 100 µL of this cell suspension was added to the wells of a 96-well plate already containing 100 µL of environmental

sample. The plate was placed into the TECAN infinite 200 pro plate reader.

Measurements of absorbance at 595 nm and bioluminescence (integration time of 5000 ms) were performed every 10 min with shaking (15 s, amplitude 4) for 14 h.

4.2.5 Data analysis

Metal water concentrations for all 1201 and 1202 sample were arbitrarily chosen to be processed through the WQI 1.2 excel worksheet (CCME 2014). All samples (for each sampling site and each sampling date) were assigned an index numerical value (WQI) ranging from 0-100, 0 indicating very poor water quality, 100 indicating pristine water quality conditions as explained in the beginning of this chapter. Correlation between WQI and *E.coli* K-12 MG1655 (pFR02) bioreporter fluorescence was performed using R statistical software (R version 2.14, The R Foundation for Statistical Computing).

4.3 Results and Discussion

4.3.1 General Toxicity Bioreporter

Results from the general toxicity bioreporter [*E.coli* strain, HMS174 (pRB27)] demonstrated the total absence of toxicity of all samples except for one site on one sampling occasion; the filtered aliquot of site 1201 on September 5, 2013 (Fig. 4.2A). For all but this one sample, the bioreporter emitted more luminescence than the control, indicating the non-toxic nature of the environmental samples and the presence of nutrients or organic matter present benefitting the cell (Chiasson-Gould et al. 2014).

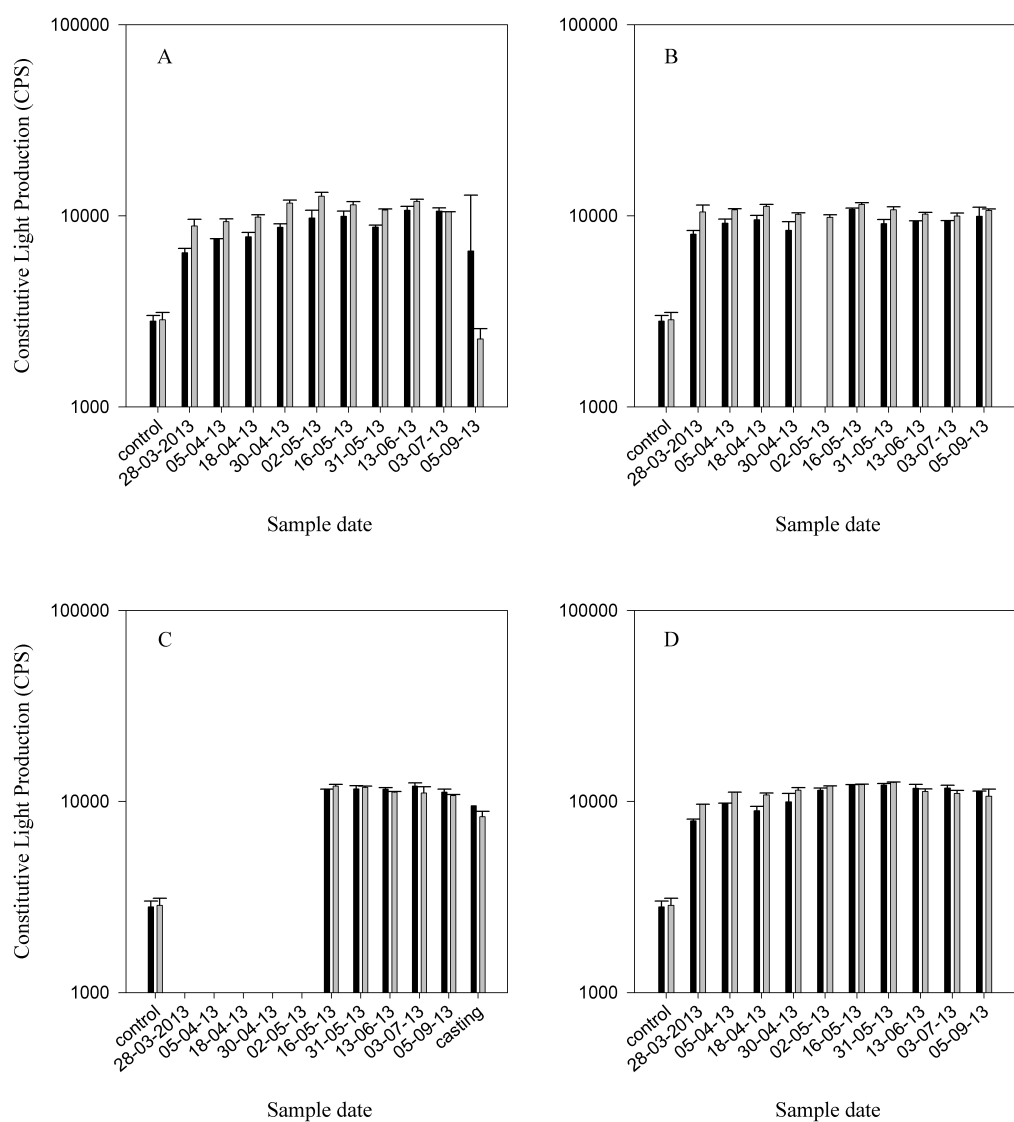


Figure 4.2. Constitutive light production of *E. coli* HMS174 (pRB27) to environmental samples over different sampling sites and dates. A) site 1201; B) site 1202; C) entry of the lake site; and, D) site 1203. Dark bars are for the unfiltered samples and light bars represent the filtered samples (0.2 μ m). Analytical replicates standard deviation for n=3 are graphed.

4.3.2 Oxidative Stress Bioreporter

The oxidative stress bioreporter [*E.coli* bioreporter K-12 MG1655 (pFR02)] confirmed the general absence of toxicity observed using the general toxicity bioreporter; it did detect a pro-oxidant signal for a few sampling dates of the sample 1201 (Fig. 4.3A). In Chapter 3, the analytical detection limit of the bioassay was determined at 154,000 RFU/OD_{595nm} (three times the standard deviation of the control + the average control fluorescence/OD_{595nm}). The five sampling dates (05-04-13; 18-04-13; 30-04-13; 02-05) for the 1201 site that reported the highest fluorescent signals did not emit at levels higher than 154,000 RFU/ OD_{595nm}. They reported signals just above or very near the standard deviation range of the control and so, according to conventional analytical chemistry, below detection limit (Reeuwijk and Houba 1998). However, these sites did report a signal outside the standard deviation range of the control, indicative of the possible presence of pro-oxidants. These data suggest the sensitivity of stress-specific bioreporters that detect sub-toxic ROS caused by pro-oxidants, before a general toxic response could be detected. These 1201 site samples (other than the toxic September 5th 1201 samples) have between 3 and 5 metals with concentrations higher than their respective CWQG (Al, Cu, Mo, Zn, Cd) (see Table 4.1). Aluminum concentration was 3 to 5 orders of magnitude higher than the pH7-CWQC for all 1201 samples, by far the highest concentration recorded with respect to its CWQC, as the other pollutants ranged from 1 to 2 orders of magnitude higher than their CWQC. These data illustrate the importance of determining speciation and/or performing bioassays (like in our case) and not only relying on total concentration in order to determine effect.

As discussed in Chapter 3, the OxyR-based bioreporter is not sensitive to the

oxidative stress generated by aluminum via Fenton reaction type oxidative stress generation. This however does not explain the lack of observed toxicity using the general response bioreporter. The aluminum present, just like the other toxicants, could be present in forms that are not bioavailable and not toxic. The site water was shown to have a protective effect on cadmium toxicity compared to cadmium in β -GMM (discussed below and Fig. 4.5).

Another explanation for the lack of toxicity or oxidative stress seen is the possible antagonistic effect of a mixture. For example, at neutral pH, toxic aluminum and toxic fluoride react together to form relatively insoluble AlF_x compounds (Barbier et al. 2010). Although AlF_x toxicity affects several cellular processes (e.g. phosphate metabolism disruption) and is a pro-oxidant, it has been suggested that the presence of aluminum and fluoride outside the cell might have an antagonistic effect and therefore decrease overall toxicity. Fluoride is expected to be produced by the refinery and thus could easily be present in the receiving environment, although we did not analyze its concentration.

The sample from Site 1201 on September 5, 2013 (that caused toxicity, according to the general toxicity bioreporter) induced the most oxidative stress (see Fig. 4.3A). Also, Site 1201 for September 5, 2013 contained 10 priority contaminants (Se, Zn, Al, As, Cr, Mo, Ni, Cd, Cu, Pb) that are higher than recommended values (CWQC) (see Table 4.1). The general toxicity bioreporter indicated that this sample affected cell physiology (lower light emission) (Fig. 4.2) and we also observed a long lag phase for the oxidative stress bioreporter. It is possible that the fluorescent capacity of the cells was lowered to the sample's toxicity. This example illustrates the way general toxicity bioreporters and stress-specific bioreporters can be used together (Mitchell et al. 2004) to

assay environmental samples.

Whether oxidative stress was a main factor in the toxicity of the 1201 September 5th remains to be determined following a dilution of the sample and subsequent bioassay. The lack of toxicity for the entry of the lake site and site 1203 illustrates that toxicity of site 1201 did not seem to extend to other sites.

What is surprising is the nature of the inorganic elements that are present in the receiving environments of the Grande-Baie refinery. The prevalence of the wide variety of inorganic compounds detected by the ICP-MS could be explained either by naturally high background levels and/or by the runoff of inorganic-based pesticide (e.g. copper) from the nearby agricultural fields (Ware and Whitacre 2004).

The robustness of the *E.coli* K-12 MG1655 (pFR02) bioreporter assay was investigated by comparing the basal normal (in β -GMM) bioreporter output and the basal output of non-polluted environmental samples that we chose here arbitrarily to be samples from site 1203. The average bioreporter fluorescence for all 1203 sites (n=10) was 100,957 with a standard deviation of 4,250. This falls within the noise (basal level $\pm$ standard deviation range) of the basal normal bioreporter output which was 1120,000 $\pm$ 14,000. This attests to the high robustness of the bioreporter output that is not sensitive to the many other environmental constituents of the sample. A high robustness is important for standardization of environmental assays.

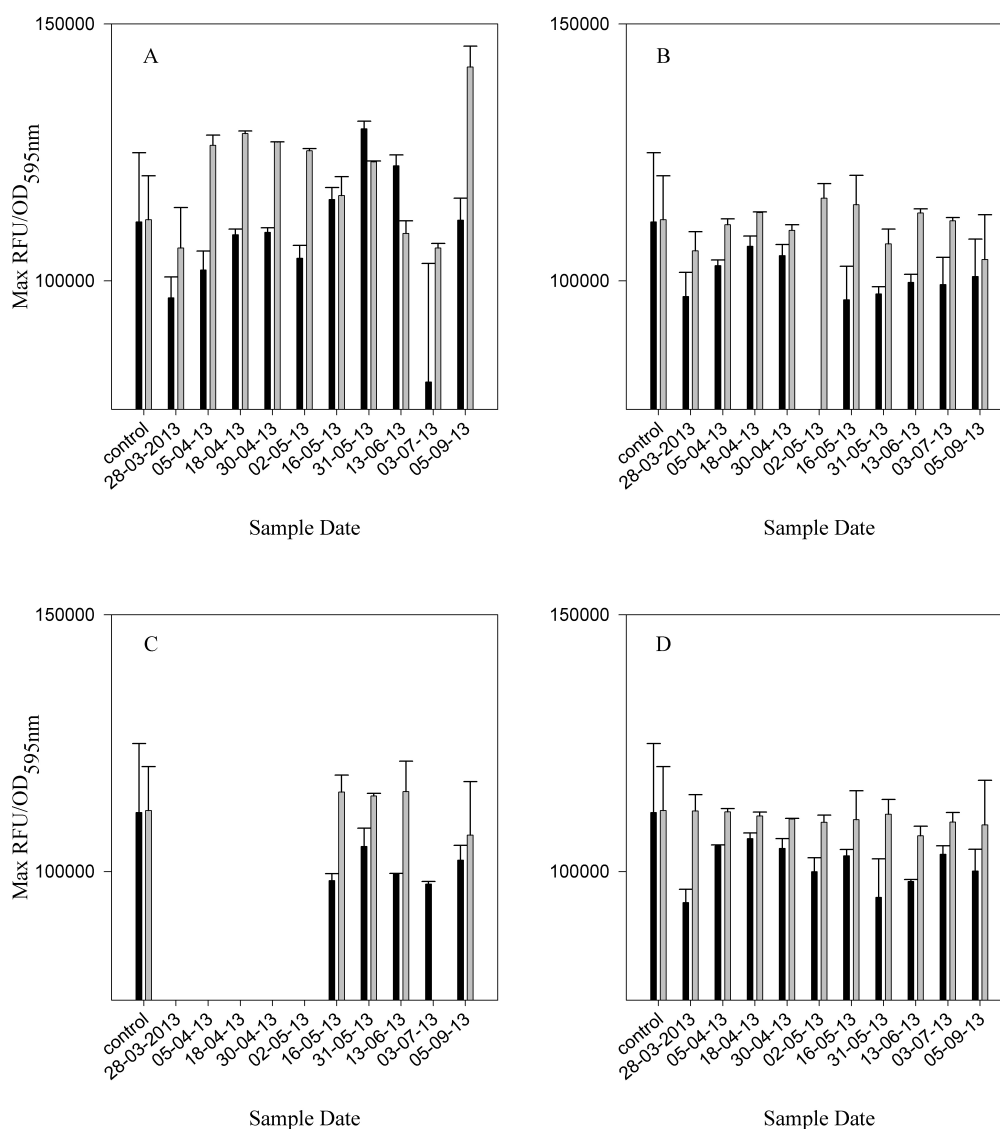


Figure 4.3. Maximum relative fluorescence unit (RFU)/OD_{595nm} of *E. coli* K-12 MG1655 (pFR02) to environmental samples over different sampling sites and dates. A) site 1201; B) site 1202; C) entry of the lake site; and, D) site 1203. Dark bars are for the unfiltered samples and light bars represent the filtered samples (0.2 μ m). Analytical replicates standard deviation for n=3 are graphed.

Table 4.1. Site 1201 water quality data for total metals in nM. Values in bold are higher than their respective Canadian water quality criteria (WQC) (Canadian Council of Ministers of the Environment 2014b). The pH of all samples was 6.8. (See Appendix D for water quality data of the other sites).

Element(WQG)	Sampling Dates									
	28/03	05/04	18/04	30/04	02/05	16/05	31/05	13/06	03/07	05/09
Se(12.6)	n/a	n/a	3.8	n/a	n/a	7.6	11.4	6	7.1	17.6
Al(3.7)	11,882	24,114	10,446	31,543	40,541	46,534	12,146	828,465	42,115	70,506
Cr(19.2)	1.2	2.2	2.2	2.2	1.9	4.6	5.3	1.9	6.1	38.9
Mn	150.9	294.1	120.1	141.2	94.9	660	688	171.5	163.9	2,498
Co	0.8	3	1	0.3	0.3	5.5	6.6	0.4	0.6	48.5
Ni(2,555)	34.5	293.3	49.3	23	21.1	819	1,304	29.8	61.1	7,348
Cu(62.9)	6,516	7,768	2,884	97.3	11.7	230	344.2	232.3	135.8	954.8
Zn(458.8)	200.6	577.5	146.6	112.8	110.6	800	1,455	42.9	95.9	9,352
As(66.7)	3.7	2.6	2.1	4.7	6.3	13.7	24.3	4.6	5.7	193.1
Mo(760.8)	1,886	1,359	1,032	9,921	9,922	3,969	3,225	12,102	6,859	1,002
Ag(0.9)	0.1	0.05	0.02	0.3	0.1	0.1	0.01	0.2	0.04	0.1
Cd(0.8)	0.8	2.6	0.3	0.3	1.1	9	16.1	1.4	1.7	156.9
Sb	0.5	1.5	0.09	1	1.6	14.9	19.6	1.7	2.5	63.2
Ba	566.3	566.6	593.7	484.7	453.7	338.4	315.6	372.2	349	172.6
Ti	0.08	0.05	0.09	0.1	0.1	0.2	0.2	0.1	0.1	0.8
Pb(4.8)	2.7	1.4	2.5	0.3	0.2	3.2	5.2	0.6	0.4	48.5
U(63)	3.3	3.5	3.3	4.3	4.3	2.3	1.8	4.5	3.1	0.5
Be	1.6	7.2	1.3	0.6	0.3	21.6	63.5	n/a	n/a	n/a
Th(3.4)	0.2	n/a	0.5	0.1	0.1	1.7	4	0.3	0.2	0.8
Hg(0.1)	3e ⁻⁴	4e ⁻⁴	5e ⁻⁴	3e ⁻⁴	3e ⁻⁴	1e ⁻³	1e ⁻³	n/a	1e ⁻³	8e ⁻³

4.3.3 Additional Bioreporter Validation

A correlation between the bioreporter oxidative stress response for each site and WQI values for each site was performed to test whether the bioreporter is able to detect changes in water quality. The correlation yielded an r-value of -0.47 (p-value of 0.03) (Fig. 4.4). However, after removing one sample (at the top left of the graph), the trend was no longer significant. This low r-value can be explained by many potential factors. First, the toxicity predicted by the index could be below the detection limit of the bioreporter. Compounds in the receiving environment could have antagonistic effects, something not considered in WQI calculations. The compounds present and calculated in the index could be toxicants that act principally through mechanism of toxicity other than hydrogen peroxide and superoxide anion generation. Also, total pollutant concentration with limited awareness of other environmental factors (such as hardness) is a poor predictor of bioactivity (Paquin et al. 2002).

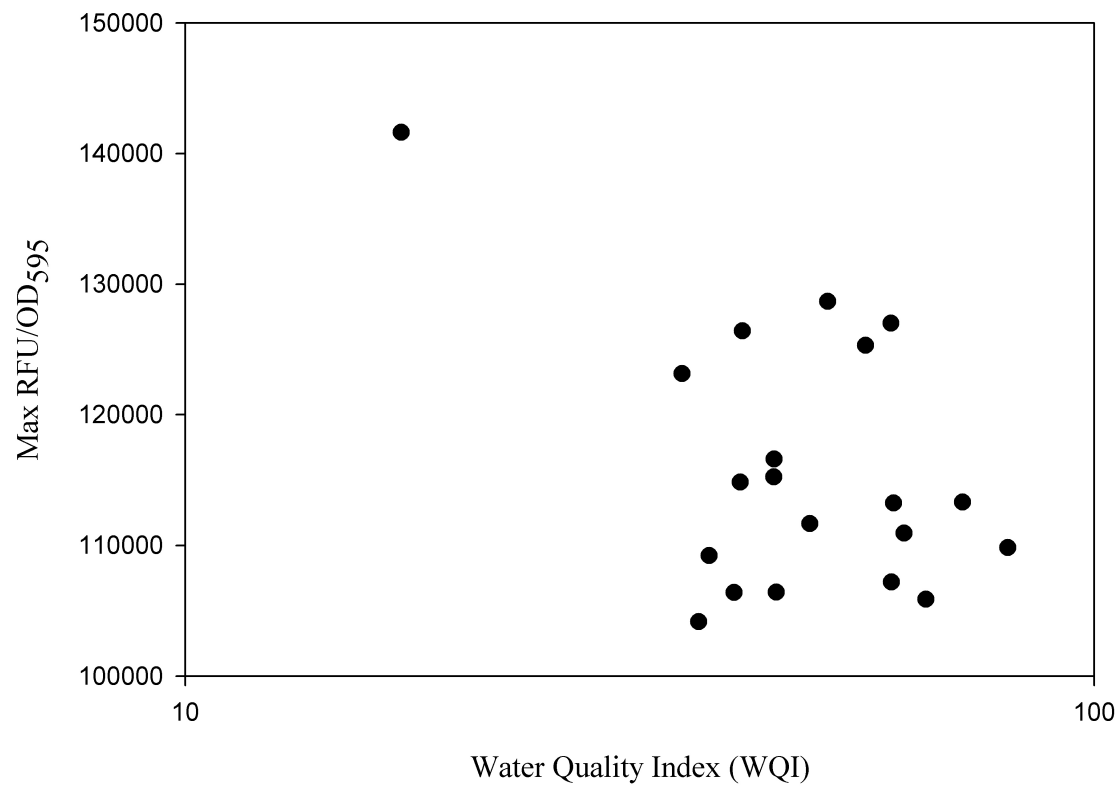


Figure 4.4. Maximum relative fluorescence unit (RFU)/OD_{595nm} of *E.coli* K-12 MG1655 (pFR02) and correlation with water quality index values for all sampling dates of site 1201 and 1202. The r-value is -0.47 (p-value =0.03).

To test whether the bioreporter could be used to detect dangerous levels of a known pro-oxidant in an environmental sample, increasing levels of cadmium were added to site 1202 (September 5, 2013) samples. Site 1202 (September 5th) samples showed no signs of toxicity or oxidative stress in the absence of this added cadmium. Figure 4.5 illustrates how the bioreporter was able to detect oxidative stress generated by cadmium in the sample from 1.7 μM to 5 μM compared to detection at 0.2 μM to 3 μM in β -GMM. Thus, the environmental sample seems to be modifying the bioreporter response. However, the bioreporter was able to detect the presence of cadmium in this complex environmental sample. The range of toxicity of cadmium to fish used by Environment Canada for the derivation of long-term WQC ranged from 0.002 μM to 0.71 μM while for invertebrates, it ranged from 0.004 μM to 680 μM (Canadian Council of Ministers of the Environment 2014b).

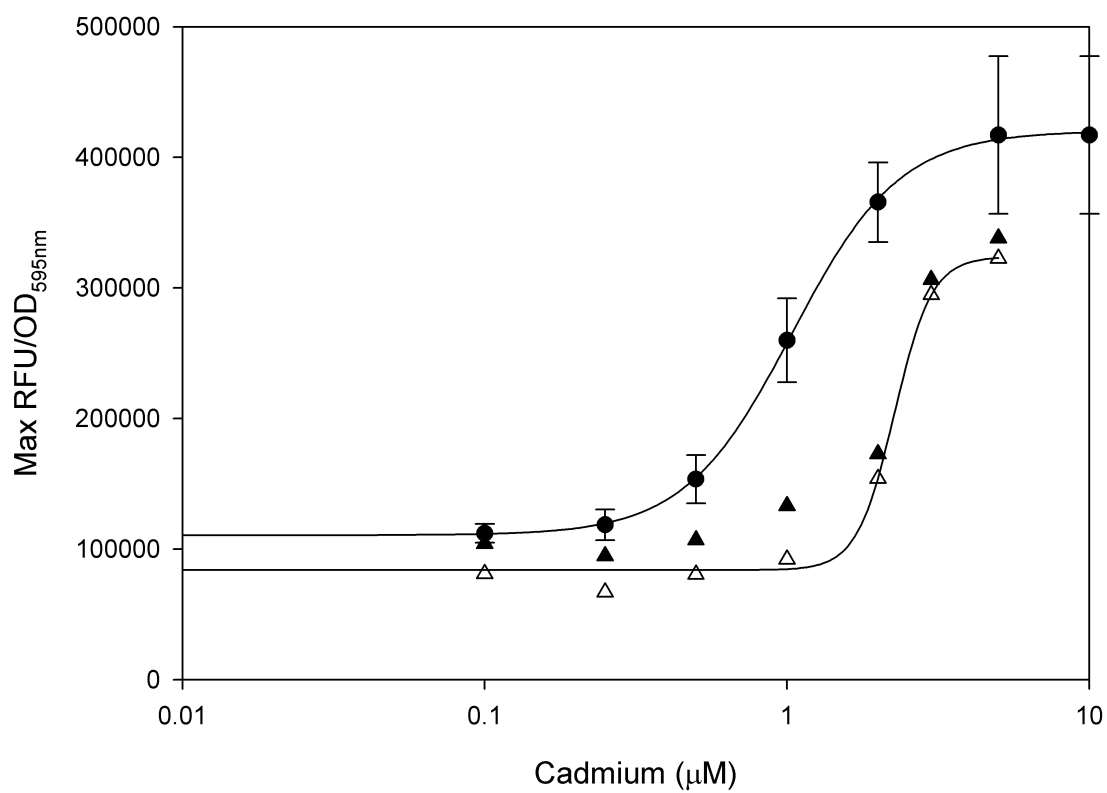


Figure 4.5. Maximum Relative Fluorescence Units/OD_{595nm} of CdCl₂ in β-GMM (closed circles) (n=3) and of site 1202 (September 5, 2013) samples spiked with varying levels of CdCl₂ for filtered sample (black triangles, n=1) and non-filtered samples (white triangles, n=1).

4.4 Conclusions

The bioreporter assay was applied in combination with a general toxicity bioreporter to receiving water samples associated with an aluminum smelter to validate its use as an early warning signal of toxicity. Two underlying questions of this chapter were:

- Can the bioreporter detect the presence of pro-oxidants in environmental matrices?
- Is the assay robust to environmental conditions?

Some samples that did not cause toxicity (according to the general bioreporter assay) but had high levels of inorganic pro-oxidants (according to the ICP-MS analysis and Canadian WQG's), elicited a very weak fluorescent activity (barely qualifying as different from basal levels), slightly indicative on the presence of pro-oxidants. Sub-toxic and toxic, artificially cadmium-polluted samples successfully elicited a detectable response from the oxidative stress bioreporter. Although cadmium detection limits in pure β -GMM (as illustrated in Fig. 3.5) would be protective of some aquatic species (Canadian Council of Ministers of the Environment 2014a), the detection in the environmental matrix raised the detection limit by an order of magnitude (0.2 to 1.7 μ M), most likely due to the protective effect of the site-water. In order to further validate the applicability of the oxidative stress bioreporter as a sensitive tool that could be used as a cost effective early warning sentinel system, parallel bioassays should be performed with the bioreporter and other species (fish, invertebrates, amphibians) using site water (Dardenne et al. 2007; Robbens et al. 2010). This would allow sensitivity and responses to be compared.

A weak oxidative stress bioreporter fluorescent signal (again barely qualifying as

different than basal levels) was detected for the one sample (1201 – September 5, 2013) that had also elicited a response by the general bioreporter assay. It is most likely that the fluorescing capacity of the oxidative stress bioreporter was hampered by the toxicity of the sample. This illustrates how general bioreporters and stress-specific bioreporters should be used together when assaying environmental samples. Furthermore, although the general bioreporter is already alerting about the poor state of the water quality, a dilution system should be applied to the sample and subsequent assaying with the oxidative stress bioreporter. This would enable one to recognize the potential pro-oxidant activity of the sample to better understand the effect of the sample and possibly link the source of pollution (especially in effluents containing more than one pollution source).

The robustness of the *E.coli* K-12 MG1655 (pFR02) bioreporter assay was investigated. The basal fluorescence in the environmental matrix was within the β -GMM basal fluorescent range and so the bioreporter assay exhibited a high robustness, illustrating that the bioreporter output was not sensitive to the many environmental constituents of the sample. A high robustness is important for standardization of environmental assays and so more testing of the bioreporter basal output on different environmental samples is recommended

Chapter 5. General Conclusions

The intent of this thesis was to develop an oxidative stress sensitive bioreporter assay applicable as an early warning signal for receiving environments of a aluminum smelter or metal mining industries. An oxidative stress sensitive bioreporter assay was developed and responded to a number of metal pro-oxidants (Cd, As, Zn, Pb, Ag, AgNP) that are known to induce elevated levels of hydrogen peroxide and potentially superoxide anion. The sensitivity of this bioreporter to inorganic pro-oxidants is the best in its category of oxidative stress bioreporters based upon its low detection limit compared to others bioreporter systems in the literature (Dardennes 2007b; Belkin 1996; Lee 2003; Mitchell 2004). However, as shown with Figures 3.5 through 3.8, the bioreporter still lacks sensitivity as it detects most inorganic pro-oxidants at levels that are already toxic to many aquatic species. Detection of contaminants at low levels is necessary to ensure detection occurs at an early stage and when impacts are limited, and so at a time not too late for remediation or preventive measures to be taken. The next steps needed are to improve the bioreporter assay, for example by crossing the species barrier and designing novel bioreporter strains and constructs with improved sensitivity to a wider range of pro-oxidants (Robbens et al. 2010). Recent advances in analytical science and genetic engineering allows for such improvements (Robbens et al. 2010).

The bioreporter needs to be calibrated with other relevant pro-oxidants associated with aluminum smelter and industrial mining receiving environments (e.g. F, PAHs, Cr, NPs, Fe). Again, the species barrier can be crossed and novel bioreporter strains can be tailored to be sensitive to contaminants that are specific to certain sites. Ideally, it might be relevant to utilize a panel of stress-responsive bioreporters (e.g. oxidative stress,

genotoxicity, DNA damage, membrane damage) as recommended and demonstrated by Dardenne et al. (2007) that utilized 14 different cell-based assays to assay environmental samples containing multiple pollutants from multiple effluents. Understanding the causative classes of compounds present in an environmental mixture allows attributing the toxicity to a specific pollution source ('signature') and helps in designing potential remediation strategies (Dardenne et al. 2007).

A good reproducibility and reasonable sensitivity to some relevant inorganic contaminant motivated applying the bioreporter in an environmental setting. The bioreporter showed signs of being able to detect pro-oxidants in an environmental matrix. The importance of a general bioreporter used in combination with the oxidative stress bioreporter was illustrated. For future steps, it is highly recommended that bioreporter assays be performed in parallel with more traditional ecotoxicological bioassays using site-water to better validate the sensitivity and ecotoxicological relevance of cell-based assays (Dardenne et al. 2007; Robbens et al. 2010). Doing so might also allow better interpretation of fluorescence values by associating them with toxicological biomarker endpoints.

For a more universal, consistent use and eventual application in a legal regulatory framework, these assays need to be standardized. Although the robustness was good and a stable basal fluorescence was observed, it should be further tested with a wider range of environmental samples. The within-lab reproducibility of the assay was evaluated to be reasonable (Dardenne et al. 2007b). Inter-lab reproducibility should now be established.

Stress-sensitive bioreporters have existed for over 2 decades with continuous focus on improvements in sensitivity, specificity and environmental application (those were

also the goals of this thesis project) (Harms et al. 2006). When will stress-sensitive bioreporters be standardized, commercialized and/or incorporated into regulations? 1979 marked the appearance of the first commercially available microbe-based bioassay; the general toxicity assay or Microtox® (Bulich et al. 1979). By virtue of their simplicity, fast results, cost-effectiveness, precision and flexibility a multitude of microbe-based bioassays have emerged since (e.g. the Ames genotoxicity test) for varied applications (e.g. sediment toxicity testing, chronic toxicity testing) (Blaise et al. 1997). A citation by Maron and Ames concerning the importance of inclusion of such tests (i.e. Ames test) also applies to the current reflections concerning the application of stress-sensitive microbial bioreporters in a more regulatory role: "It is important to recognize the limitations of the assay as well as the advantages, and, ultimately, a battery of tests is needed so that the strengths of one test can compensate for the inadequacies of another (Maron and Ames 1983)". A major advantage of stress-sensitive bioreporter assays is that they offer high throughput of samples and so offer a more continuous awareness of water quality. However, using bacteria to predict specific effects on higher organisms is daunting (Robbens et al. 2010). If focus can be put on bridging the gap between bacterial bioreporters and traditional toxicity test, combined with recent advances in analytical chemistry, nanotechnology and bioengineering to continue improving the sensitivity and specificity of bioreporters, we can be optimistic that these (stress-specific) tools could be incorporated into risk assessment plans of industries and eventually can be incorporated into regulatory legislation just like the Microtox® and Ames tests have been over the past decade.

6. References

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7. Appendices

Appendix A. Medium Instructions

GMM

For 100 mL of deionized and autoclaved H₂O; in 250 mL Erlenmeyer:

- 5.64 g DIFCO M9 minimal salts (Fisher Scientific)

Autoclave for 60 min @ 121°C. Under the flow hood, mix 20 mL into of 100 mL of deionized and autoclaved H₂O.

For the 120 mL of solution; in 250 mL bottle:

- 1 mL D-glucose (30 % stock solution)
- 100 µL MgSO₄ (1 M stock solution) (Fisher Scientific)
- 100 µL VitB1 (1 mg/mL stock solution)
- 100 µL trace elements (see Barkay et al. 1998)

β-GMM

For 100 mL of deionized and autoclaved H₂O; in 250 mL Erlenmeyer:

- 3.13 g MOPS (Sigma Aldrich chem. Co.)
- 0.16 g of β-glycerol phosphate (from Sigma-Aldrich Co.)
- 0.77 g of (NH₄)₂SO₂ (Fisher Scientific)
- 1.025g of KCl (Fisher Scientific)
- 0.175g of NaCl (Fisher Scientific)

Filter sterilize and mix 20 mL into 100 mL of deionized and autoclaved H₂O.

For the 120 mL of solution; in 250 mL bottle:

- 1 mL D-glucose (30% stock solution)
- 100 µL MgSO₄ (1 M stock solution)
- 100 µL VitB1 (1 mg/mL stock solution)
- 100 µL trace elements (see Barkay et al. 1998)

Assay medium (1X):

For 186 mL of deionized and autoclaved H₂O in 250 mL bottle:

- 600 µL D-glucose (30 % stock solution) for a final concentration of 5 mM
- 200 µL (NH₄)₂SO₄ (12% stock solution) for a final concentration of 0.9 mM
- 13.4 mL Pi buffer (1M stock solution) for a final concentration of 67 mM

Phosphate Buffer (1M):

For 250 mL of deionized and autoclaved H₂O; in 500 mL bottle:

- 26.5 g K₂HPO₄ (potassium phosphate, dibasic, anhydrous)
- 20 g NaH₂PO₄ (sodium phosphate, monobasic, monohydrate)

Add 250 mL deionized and autoclaved H₂O. Autoclave for 60 min at 121°C. Check the pH and adjust to pH 6.8 with HCl as necessary.

Appendix B. Growth Parameters Calculations

Gompertz 4-parameter equation:

$$f = y_0 + ae^{-e^{-\left(\frac{x-x_0}{b}\right)}}$$

Maximum Specific Growth Rate (μ) :

$$\mu = a * (2.728 * b)$$

Lag Phase Time (λ) :

$$\lambda = x_0 - b$$

Appendix C. Pro-oxidants Dose-Response

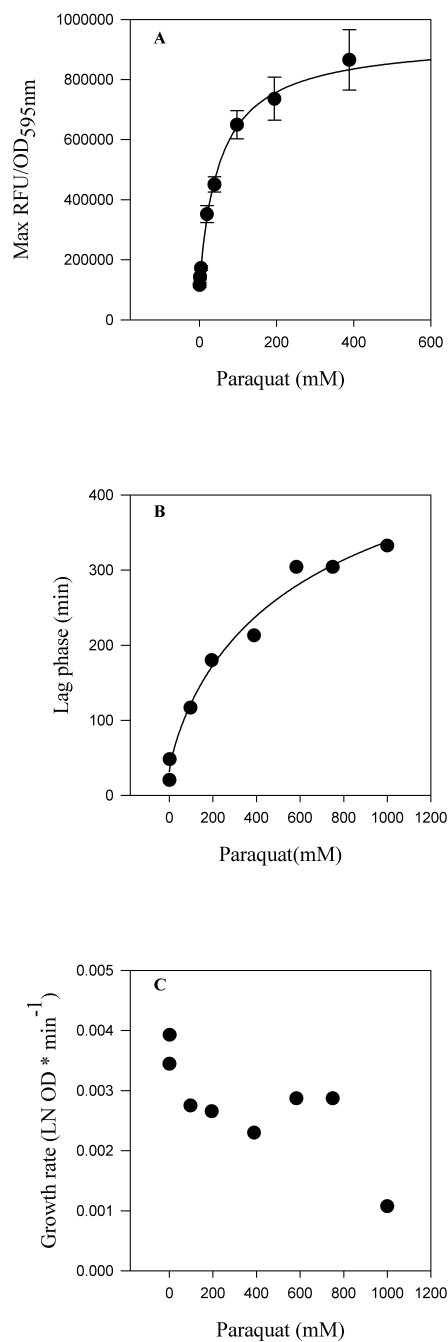


Figure S.1. Dose-response to paraquat for A) max relative fluorescence/OD_{595nm}, n=5, B) lag phase time, n=1, and C) growth rate. n=1 for each.

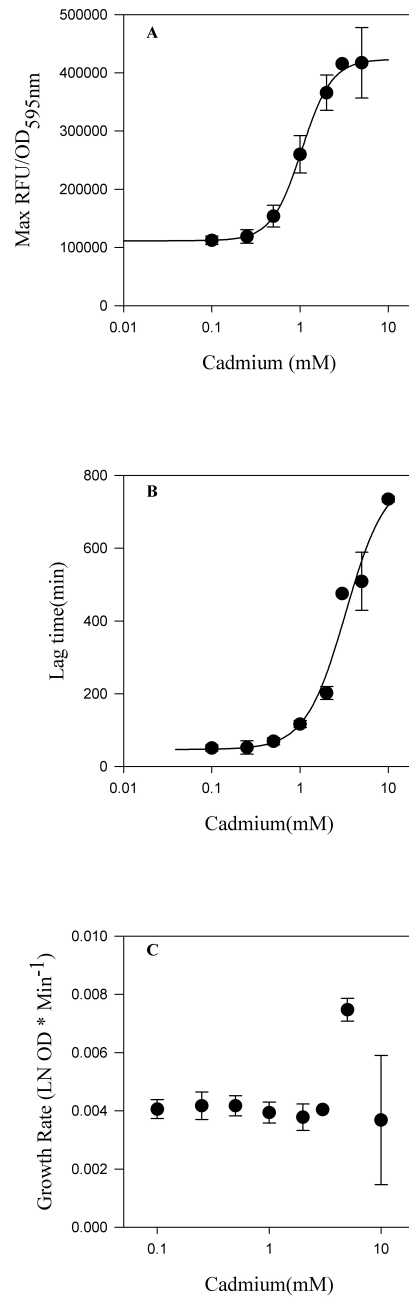


Figure S.2. Dose-response to cadmium chloride for A) max relative fluorescence/OD_{595nm}, B) lag phase time, and C) growth rate. N=3 for each.

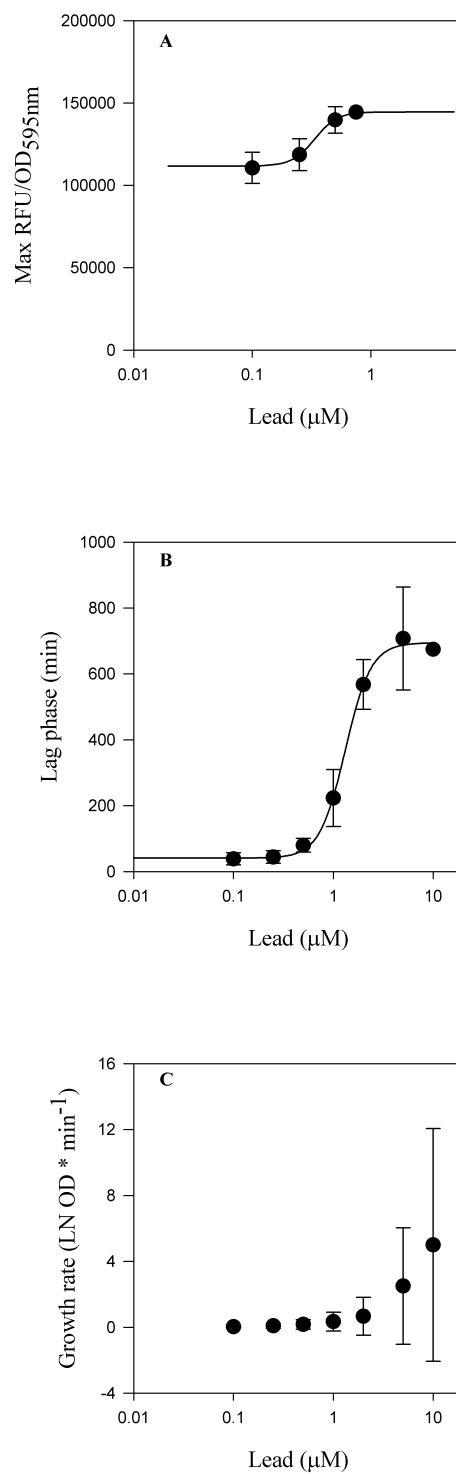


Figure S.3. Dose-response to lead nitrate for A) max relative fluorescence/OD_{595nm}, B) lag phase time, and C) growth rate. N=3 for each.

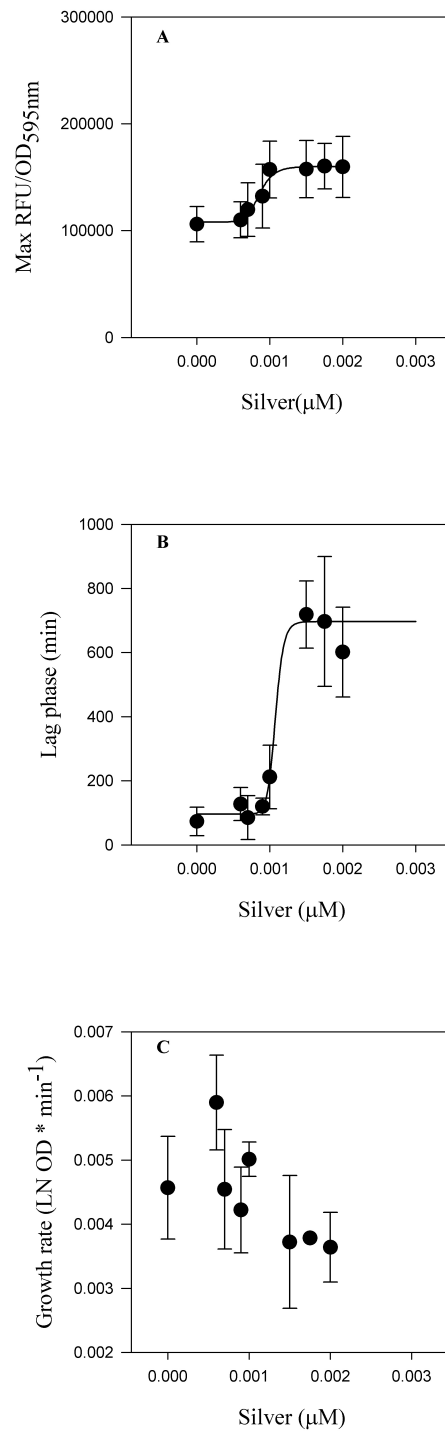


Figure S.4. Dose-response to silver nitrate for A) max relative fluorescence/OD_{595nm}, B) lag phase time, and C) growth rate. N=3 for each.

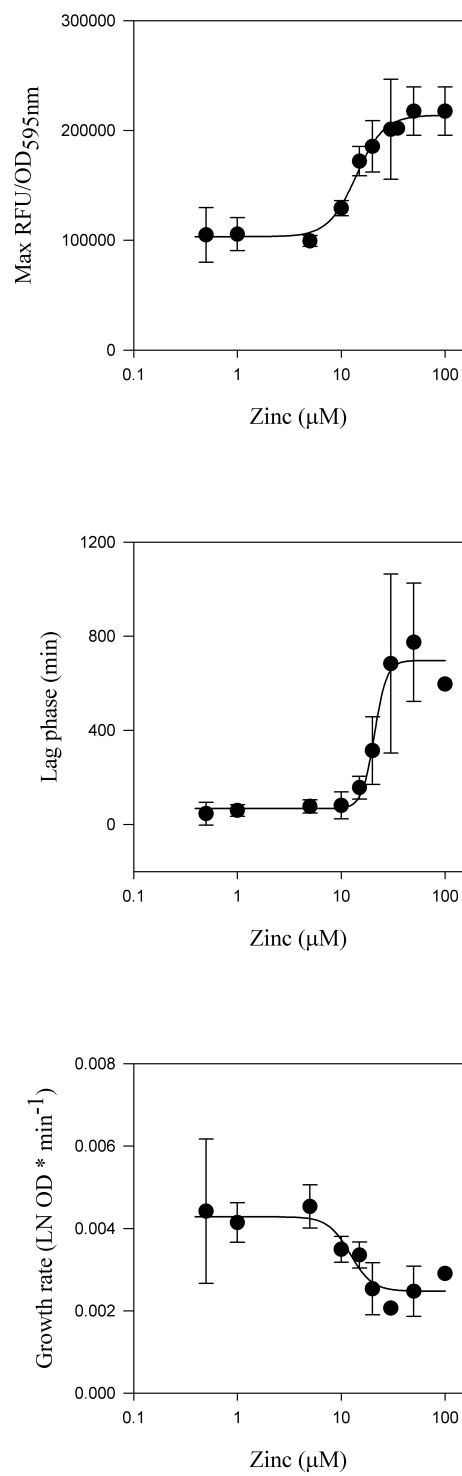


Figure S.5. Dose-response to zinc chloride for A) max relative fluorescence/OD_{595nm}, B) lag phase time, and C) growth rate. N=3 for each.

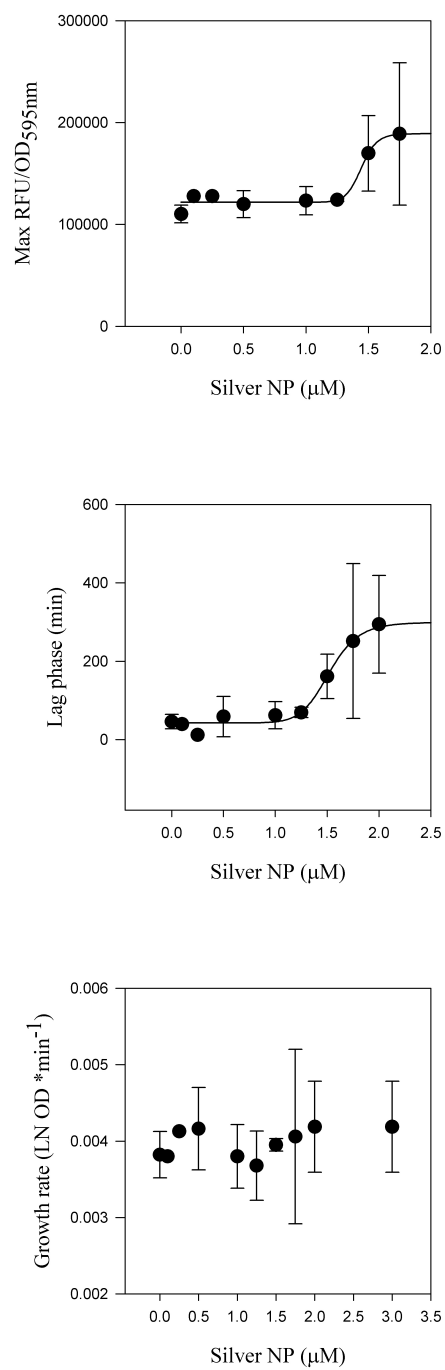


Figure S.6. Dose-response to silver nanoparticles (NP) for A) max relative fluorescence/OD_{595nm}, B) lag phase time and C) growth rate. N=3 for each.

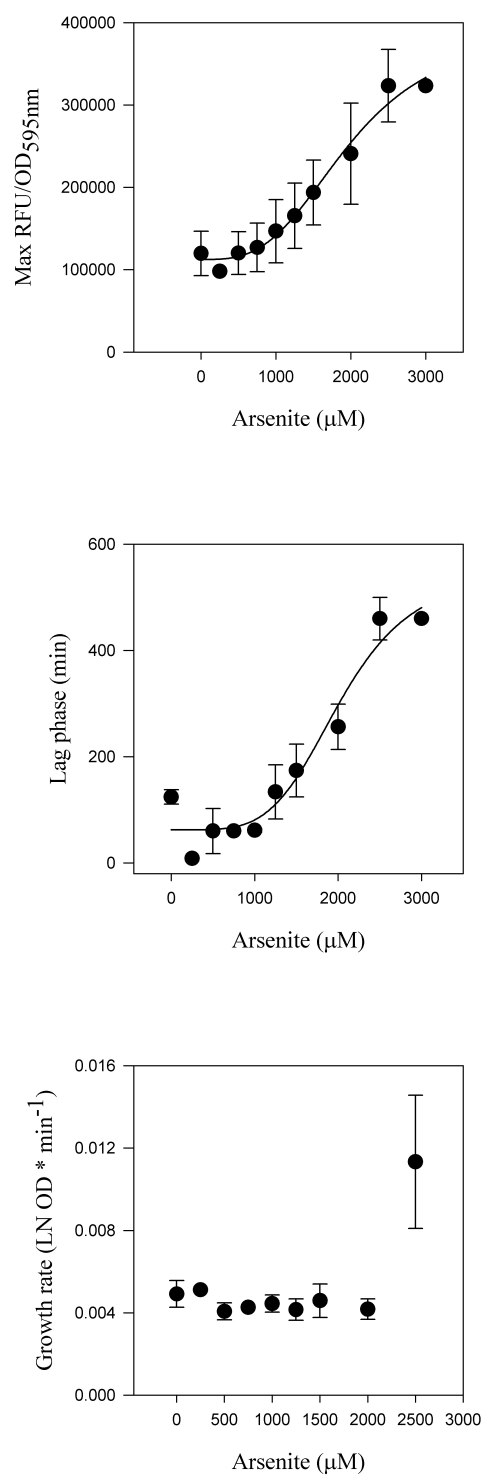


Figure S.7. Dose-response to arsenite for A) max relative fluorescence/OD_{595nm}, B) lag phase time, and C) growth rate. N=3 for each.

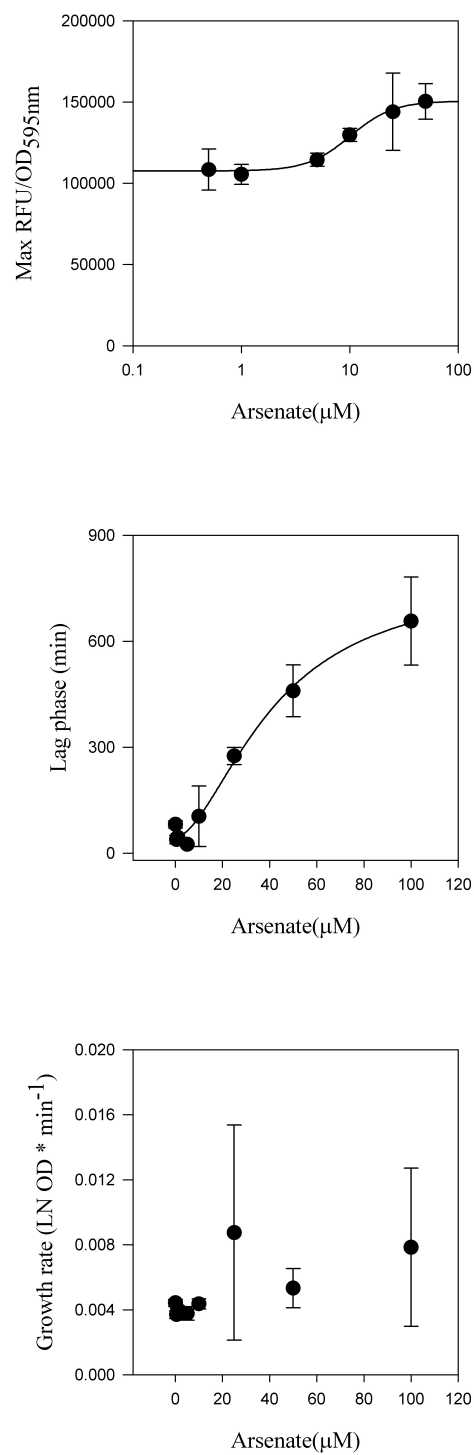


Figure S.8. Dose-response to arsenate for A) max relative fluorescence/OD_{595nm}, B) lag phase time, and C) growth rate. N=3 for each.

Appendix D. Water Quality Data for Environmental Samples

Table S.1. Site 1202 water quality data for total metals in nM. Values in bold are higher than their respective Canadian water quality criteria (WQC) (Canadian Council of Ministers of the Environment). The pH of all samples was 6.8.

Element(WQC	Sampling Dates									
	28/03	05/04	18/04	30/04	02/05	16/05	31/05	13/06	03/07	05/09
Se(12.6)	n/a	3.8	n/a	11.4	n/a	n/a	n/a	3.5	5.3	4.5
Al(3.7)	5,495	9,407	1,118	2,112	n/a	152,366	16,454	1,070	3,963	32,111
Cr(19.2)	3.7	5.7	0.9	1.4	n/a	33.6	8.1	0.6	1.5	13.1
Mn	260.7	488	184.6	83.7	n/a	668.7	302	42.2	841.3	1,119
Co	2.8	4.2	1.6	1	n/a	16.7	4.9	0.3	3.2	8.9
Ni(2555)	33.6	97.4	107	26.7	n/a	123.4	102.5	19.7	432.6	440.6
Cu(62.9)	614.9	550.2	499	295.8	n/a	430.1	531.8	808.2	1,881	797.4
Zn(458.8)	163.4	418.4	180.7	155.9	n/a	1,062	645	163.3	604.3	1,883
As(66.7)	1	4.2	1.5	3.1	n/a	8.4	6.8	1.3	2.7	5.6
Mo(760.8)	15.8	18	20.9	22.8	n/a	17.1	20.3	20.3	24.1	18.9
Ag(0.9)	0.07	0.05	0.07	0	n/a	0.08	0.1	0.06	0.01	0.02
Cd(0.8)	1.3	2.1	0.9	0.4	n/a	5.4	1.6	0.2	0.9	10
Sb	n/a	0.4	0.4	0	n/a	2.6	2.6	0.2	1.3	1.9
Ba	568.6	583.3	587.1	455.8	n/a	509	468.7	590.6	526.6	454.1
Ti	0.09	0.1	0.04	0.06	n/a	0.5	0.18	0.04	0.1	0.2
Pb(4.8)	1.7	3.4	4.8	1.3	n/a	33.4	3.8	0.4	2.7	9.2
U(63)	2.6	3	2.6	2.2	n/a	1.9	2	2.6	2.1	1.5
Be	0.7	1.7	0.1	0.99	n/a	7.9	3.1	n/a	n/a	n/a
Th(3.4)	0.2	0.3	n/a	0.6	n/a	0.3	0.7	0.1	0.1	0.1
Hg(0.1)	0.000	0.0006	0.0004	0.0005	n/a	0.005	0.001	n/a	0.001	0.001

Table S.2. Site 1203 water quality data for total metals in nM. Values in bold are higher than their respective Canadian water quality criteria (WQC) (Canadian Council of Ministers of the Environment). The pH of all samples was 6.8.

Element(WQG)	Sampling Dates									
	28/03	05/04	18/04	30/04	02/05	16/05	31/05	13/06	03/07	05/09
Se(12.6)	7.6	n/a	7.6	0	0	0	0	0.9	2.9	2.3
Al(3.7)	35,142	16,438	16,190	13,846	10,430	39,302	20,151	14,455	409,14	6,121
Cr(19.2)	18.8	10.1	9.6	7.3	7.5	26.8	10.6	8.5	302.3	3.4
Mn	879.5	541.5	503.1	412.6	390.8	2,739	540.8	388.2	6,745	374.1
Co	8.9	4.3	3.9	3.1	2.8	11.5	3.1	2.9	170.4	1
Ni(2555)	160.8	113.7	121.4	80.3	83.8	152.5	125.3	99.2	674.4	117.3
Cu(62.9)	321.9	333.9	94.7	104.1	216.9	126.9	126.4	70.6	303.8	76
Zn(458.8)	191.6	147.7	171.3	108.3	71.6	355.8	248.6	89.8	1,431	51.6
As(66.7)	4.7	3.1	2.6	3.7	2.1	9.5	3.7	4	24.6	5.5
Mo(760.8)	533.6	338.6	353.4	339.5	350.3	235.2	233.9	198.8	466.1	291.2
Ag(0.9)	0.1	0.01	0.04	0.05	0.1	0.1	0.01	0.5	0.2	0.04
Cd(0.8)	3.2	2.1	0.4	0.4	1.1	1.4	1.1	0.9	1	0.4
Sb	2.9	1.5	0.8	1.4	2.4	2.1	1.8	1.6	3.3	3
Ba	362.5	260.8	251.7	215.9	214.7	209.6	148.5	144.9	870.4	159.8
Ti	0.08	0.07	0.07	0.05	0.06	0.03	0.08	0.04	0.9	0.03
Pb(4.8)	1.3	1	1	1.1	1.1	2.6	1.6	0.7	35.9	0.3
U(63)	1.9	1.6	1.5	1	1.2	0.9	0.6	0.5	2.4	0.4
Be	4.1	1.6	3.3	1.7	1.2	5.3	5.5	n/a	n/a	n/a
Th(3.4)	0.4	0.1	0.6	n/a	1	0.5	0.5	0.5	8.6	0.1
Hg(0.1)	0.001	0.002	0.001	0.001	0.001	0.002	0.002	n/a	0.003	0.001

Table S.3. Site 1203 water quality data for total metals in nM. Values in bold are higher than their respective Canadian water quality criteria (WQC) (Canadian Council of Ministers of the Environment). The pH of all samples was 6.8.

Element(WQG)	Sampling Dates					
	02/05	16/05	31/05	13/06	03/07	05/09
Se(12.6)	0	0	7.6	0.9	2.7	3.8
Al(3.7)	14,771	48,384	54,064	6,902	12,347	19,853
Cr(19.2)	10	20.1	14.9	5.5	2.9	7.1
Mn	396.5	556.9	559.6	251	634.6	573.4
Co	4.2	11	8.2	1.8	2.1	4.6
Ni(2555)	81.9	273.9	493.4	64.2	282.2	365.3
Cu(62.9)	183.3	106.6	186.7	100.6	86.9	421.4
Zn(458.8)	178.1	604.5	813	66.4	240.4	481.7
As(66.7)	1	8.9	12.7	3.2	8.9	7.7
Mo(760.8)	656.2	200.1	290.8	545.9	2,372	1,820
Ag(0.9)	0.1	0.01	0.02	0.1	0.05	0.04
Cd(0.8)	1.6	2.6	4.2	1.1	3.1	4.2
Sb	0.09	3.4	5.7	0.7	1.7	2.2
Ba	356.6	185.7	202.2	171.3	320	395.3
Ti	0.05	0.1	0.2	0.05	0.08	0.07
Pb(4.8)	1.5	7.5	8.4	0.3	0.2	1.8
U(63)	1.5	0.5	0.5	0.6	1.7	1.9
Be	2.1	12.4	24.7	n/a	n/a	n/a
Th(3.4)	0.6	0.4	0.7	1	1.3	0.2
Hg(0.1)	0.001	0.003	0.003	n/a	0.001	0.001