8-2004

Development of an Accurate Toxicity Assay Using Genetically Engineered Bioluminescent Bacteria

Stacey M. Allsteadt-Barnlund
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Stacey M. Allsteadt-Barnlund entitled "Development of an Accurate Toxicity Assay Using Genetically Engineered Bioluminescent Bacteria." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemical Engineering.

Paul D. Frymier, Major Professor

We have read this thesis and recommend its acceptance:

Paul Bienkowski, Steven Ripp

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis written by Stacey M. Allsteadt-Barnlund entitled "Development of an Accurate Toxicity Assay Using Genetically Engineered Bioluminescent Bacteria." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemical Engineering.

Paul Frymier

Paul D. Frymier, Major Professor

I have read this thesis and
Recommend its acceptance:

Paul R. Bienkowski

Dr. Paul Bienkowski

Steven Ripp

Dr. Steven Ripp

Accepted for the Council:

Vice Chancellor and Dean of
Graduate Studies

DEVELOPMENT OF AN ACCURATE TOXICITY ASSAY USING GENETICALLY ENGINEERED BIOLUMINESCENT BACTERIA

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Stacey M. Allsteadt-Barnlund

August 2004

DEDICATION

This thesis is dedicated to my family especially my mom and dad who have encouraged me, helped guide me, showed me endless love, and most importantly, given me a shoulder to lean on for support every day of my life.

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Paul Frymier, for his overwhelming patience, guidance, and always knowing what is most beneficial to me not only in the research field, but also in life. I would also like to thank Drs. Paul Bienkowski and Steve Ripp for agreeing to be on my thesis committee and for their assistance and knowledge in the field of bioluminescent bacteria. An overwhelming thank you goes to my former professors and friends, Dr. Stanley Latesky and Dr. Noble Jobe, whose guidance, support, and encouragement have helped me throughout my entire college experience. A special note of thanks to one of my best friends, Barbara Cortner, whose endless support, understanding, and encouragement were always there for me. I would also like to thank another of my best friends, Jennifer Brashears, who has always given me a shoulder to lean on, support, encouragement, and a reason to smile. In addition, I thank my friend and roommate Natalie Bullock who has also given me a shoulder to lean on, support, and a place to live. Finally, I want to again thank my family, especially my mom and dad, who have never given up on me and have always been there to provide support, guidance, lessons in life and endless love.

ABSTRACT

Methods of developing and improving toxicity assays using genetically engineered bioluminescent bacteria PM6 and Shk1 were investigated. The EC_{50} values for three metals (zinc, copper, and lead) were determined using these two strains and were compared with the EC_{50} values calculated from the Microtox® assay, published inhibition to activated sludge specific oxygen uptake rate (SOUR) data (Madoni et al., 1999), and published EC_{50} values from Microtox® for the same compounds (Kaiser and Devillers, 1994). This was done to evaluate the effectiveness of using strains PM6 and Shk1 to predict toxic effects to activated sludge as indicated by respiration inhibition. A number of factors affecting toxicity assays were examined including temperature control, cell storage method (lyophilization and storage temperature), activation time, and mixing prior to luminescence measurement.

A toxicity testing protocol developed by Lajoie et al. (2002) was used and adapted to this study. Based on the results of these experiments, it was concluded that toxicity assays using PM6 and Shk1 provide EC_{50} values closer to respiration inhibition EC_{50} values than Microtox®.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1 Introduction

One of the most widely used biological wastewater treatment methods in the world is the activated sludge process. Other biological wastewater treatment methods include biological nitrogen removal, bioaugmentation, extended aeration, anaerobic processes, rotating biological contactors, sequencing batch reactors and trickling filters. Often there are toxicants encountered in the influent wastewater to treatment facilities that interfere with the normal operation of the processes. Examples of interferences are the inhibition of waste organics removal, modification of sludge compacting properties, formation of pin-flocs, the reduction of solids separation efficiency, and the loss of higher life forms; these interferences ultimately lead to the degradation of the effluent quality.

In the most severe instances, the activated sludge microorganisms may be completely inhibited, causing wastewater to be discharged that has not been properly treated. Jonsson et al. (2000) reported that 45-60% of the Swedish municipal wastewater treatment plants investigated received wastewater containing inhibitory substances. Grau and Da-Rin (1997) reported that in Sao Paulo, Brazil, treatment efficiency of a biological wastewater treatment plant was significantly hindered for six months after toxic shocks occurred. Trapido et al.,

(1994) reported that a daily discharge of 3 to 4 tons of phenolic compounds was released into the Gulf of Finland because of the inability to assess toxic effects.

Effluent violations can be avoided if the influent is screened for toxicity and protective action is taken (Love and Bott, 2004). One protective action that can be taken is the toxic stream can be diverted to a temporary holding basin and returned to the waste treatment system at a slower rate. This is to avoid high toxicant influx that may otherwise seriously affect wastewater treatment performance.

Bioluminescent bacteria are currently being used for monitoring municipal wastewater treatment plant influent (Paxéus and Schröder, 1996). However, research conducted by Love and Bott (2000) established that no one device satisfies all of the criteria required for an effective upset early warning device (UEWD). The research states that it is unlikely that any one technology will provide the perfect solution to UEW detection; however, bioluminescent bacteria show promise as a potential technology for influent wastewater toxicity testing.

The primary engineering application for bioluminescent bacteria is chemical sensing, because bioluminescent bacteria can form the basis of highly selective and sensitive chemical detectors with rapid response times. Therefore, the hypothesis of this research is that genetically engineered *Pseudomonas* spp. PM6 (WERF project no. 01-WSM-2A, 2004) and Shk1 (Kelly et al., 1999), being bioluminescent bacteria derived from sludge bacteria, have quick responses to

toxicants that are similar to those of activated sludge microbial organisms. These properties make the development of toxicity assays to predict the effect of toxicity to activated sludge a promising alternative to current, ineffective methods. The present assays used to determine wastewater toxicity are not practical, robust, or cost-effective technologies to detect and mitigate upset events (Love and Bott, 2000). Therefore, to examine the effectiveness of using strains PM6 and Shk1 to predict toxic effects to activated sludge, different assay protocols will be explored and EC_{50} values of strains PM6 and Shk1 for different chemical compounds will be compared to EC_{50} values calculated from the Microtox® assay, published inhibition to activated sludge specific oxygen uptake rate (SOUR) data (Madoni et al., 1999), and published EC_{50} values from Microtox® for the same compounds (Kaiser and Devillers, 1994).

Chapter 2 Background

2.1 Bioluminescence

The ability of living organisms to emit light has manifested itself many times during the course of evolution. There are many different species that have the ability to emit light. They range from bacteria and fungi to fireflies and fish. Bacteria, however, are the most abundant of organisms and are found in freshwater, marine, and terrestrial environments (Hastings, 1983, 1996). The function of bioluminescence in bacteria is less straightforward than the functional importance of light emission of higher organisms, but Czyz et al. (2000) showed that bacterial bioluminescence stimulates DNA repair in *Vibrio harveyi.* Hastings and Nealson (1977) also imply that bioluminescence helps to propagate bacteria. Hastings and Nealson also demonstrated that bacteria of the same species have both luminous and non-luminous forms, indicating that bioluminescence is not essential for their existence.

All known bioluminescent bacteria are Gram negative, in which the distinctive feature is the presence of a double membrane surrounding each bacterial cell. All bacteria have inner cell membranes, but Gram negative bacteria have a unique outer membrane which can block certain compounds (e.g. some antibiotics) from entering the cell, and make Gram negative cells more resistant to

these compounds than Gram positive bacteria. Most information on bioluminescent bacteria concerns only three genera: *Photobacterium*, *Photorhabdus*, and *Vibrio*. Their primary habitat is marine in free-living mode, and in symbiotic, saprophytic and parasitic relationships (Hastings et al., 1977, 1983, 1996).

Genes that code for the enzyme that catalyzes the light-emitting reaction (luciferase) and for the enzymes that convert the standard physiological metabolites into high-energy substrates (luciferins) are both required for the luminescent reaction. The enzyme-catalyzed redox reaction is shown below (Figure 2.1), where RCHO represents a generic long chain aldehyde.

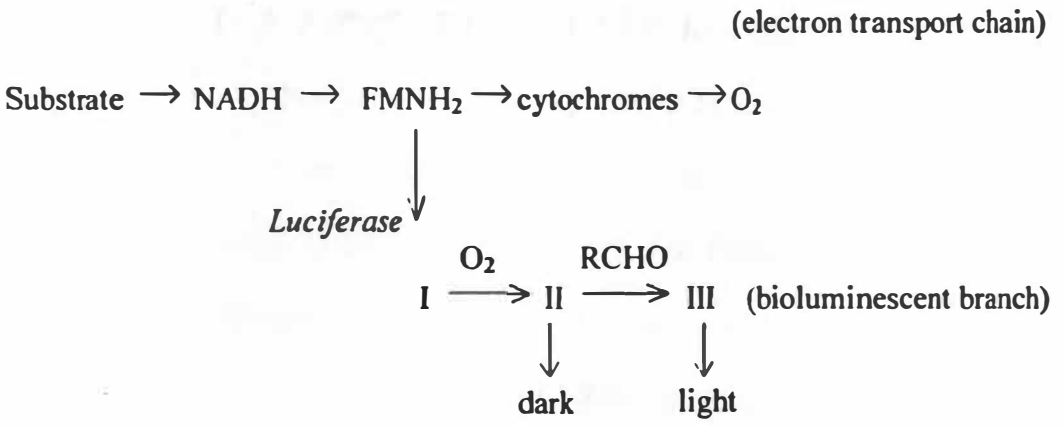

Figure 2.1: Electron transport chain joined with bioluminescent system (Hastings and Nealson, 1977).

As depicted, the reaction involves the oxidation of $FMNH_2$ and a long chain aliphatic aldehyde by molecular oxygen. $FMNH_2$ binds to luciferase, the enzyme that catalyzes this reaction and produces complex I, which reacts with molecular oxygen to produce complex II. When complex II decays, it reduces luciferase, but doesn't produce light. When complex II binds to a long-chain aldehyde (RCHO), complex III is produced, which is an excited intermediate. When it decays, the aldehyde is oxidized to a fatty acid and light is emitted at 490 nm.

The enzyme catalyzing this reaction is bacterial luciferase, a heterodimeric enzyme ($\alpha\beta$) of 78 kDa containing two non-identical subunits, α and β, with ~30% sequence identity and coded by the *luxA* and *luxB* genes, respectively. Four other genes are associated with the *lux* operon, *luxC*, *D*, and *E* (Baldwin et al., 1995, Hastings et al., 1977, 1983, 1996, Meighen, 1994).

A schematic of the *lux* operon used in the construction of Shk1 and PM6 is shown in Figure 2.2, where there is a constitutive promoter upstream of the 5 *lux* genes, *lux*A and *lux*B code for a heterodimeric luciferase (mono-oxygenase), *lux*C codes for a reductase, *lux*D codes for a transferase, and *lux*E codes for a synthetase (Nivens et al., 2004). *lux*C and *lux*E code for polypeptides that are required for the conversion of fatty acids into the long-chain aldehyde required for the luminescent reaction. *lux*D brings carbon substrates from other metabolic pathways to the light-producing reaction cycle. For chromosomal insertion, light levels are dependent on the insertion location of the plasmid and the intracellular

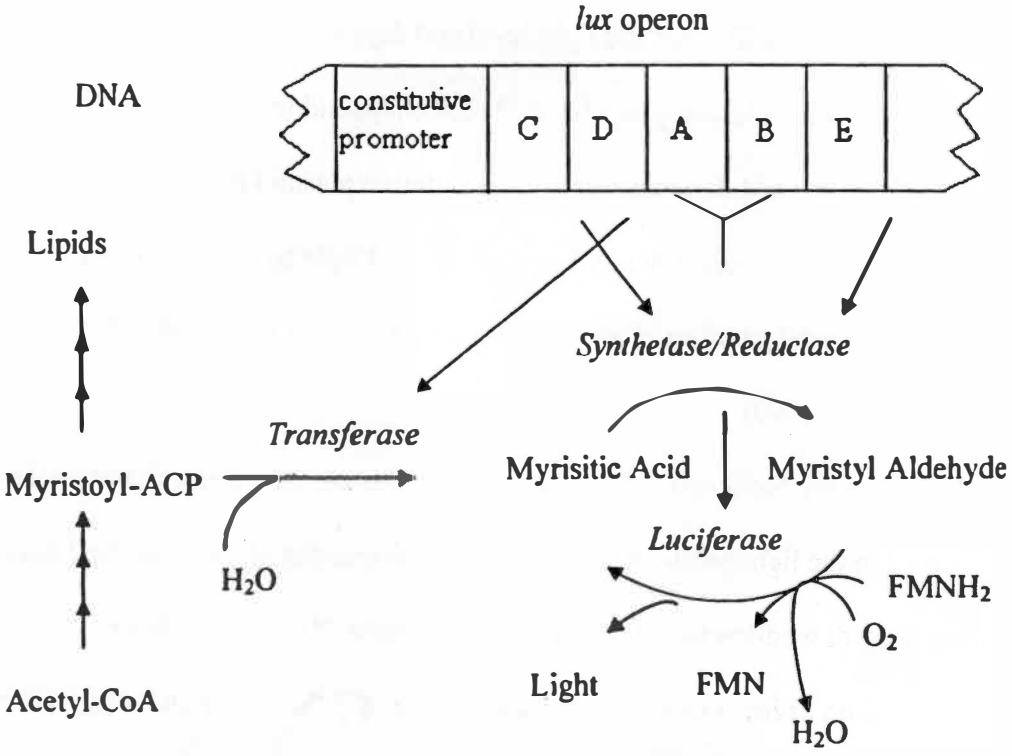

Figure 2.2: The *lux* gene cassette and its associated products (adapted to this research from WERF project no. 01-WSM-2A, 2004).

$FMNH_2$ and O_2 levels. For plasmid insertions, the nature of the promoter can be controlled more readily, but the light level still depends on the O_2 and $FMNH_2$ levels in the cell. The *lux* genes located on the plasmid used to construction PM6 and Shk1 are located downstream of a constitutive plasmid maintenance promoter as shown in the figure, allowing the production of light by expression of the *lux* genes without the addition of an inducer (WERF project no. 01-WSM-2A, 2004, Burlage et al., 1990).

Oxygen, luciferase, aldehyde, and $FMNH_2$ are the primary substances involved in the light-producing reactions. Mass transfer of the dissolved oxygen in the growth medium to the cells provides oxygen. The intracellular concentration of luciferase is dependent upon translation and transcription of the *lux* genes. Aldehyde is produced by the reduction of fatty acids, and $FMNH_2$ is produced by biosynthesis and participates in the electron transport chain (Hastings et al., 1977, 1983, 1996).

2.1.1 Construction of Shk1 and PM6

Both Shk1 and PM6 are bioluminescent Pseudomonads created by conjugal mating of naturally occurring wastewater treatment plant microorganisms with genetically engineered bioluminescent *E. coli* strains used to maintain a *lux*-containing plasmid, pUTK2. The source of the activated sludge used as a host strain for the creation of Shk1 was the Eastman Chemical Company

(Kingsport, Tennessee) wastewater treatment plant (collected in April, 1996); the bioluminescent transformant Shk1 was obtained through generous donation from the Center for Environmental Biotechnology at the University of Tennessee. The source of the activated sludge for the creation of PM6 was the aeration basin of the Syracuse Metropolitan Area Wastewater Treatment Plant (METRO) in Syracuse, New York (WERF project no. 01-WSM-2A, 2004). As mentioned above, the activated sludge strains were mated with an engineered *E. coli* strain used to maintain the plasmid; the *E. coli* strain DH5 carries the bioluminescent genes on the plasmid pUTK2.

Plasmids are self-replicating pieces of DNA not incorporated in the chromosomal DNA; the pUTK2 plasmid was created by conjugal mating of *Alcaligenes* strain A5 (Shields et al., 1985) with *E. coli* HB101 (pUCD623), and subsequent mating with *E. coli* DH5 (Burlage et al., 1990). Thus, the strain of *E. coli* DH5 is the carrier for the plasmid pUTK2, which carries the entire 5 gene *lux* operon under the control of a constitutive promoter as well as a tetracycline-resistance gene.

By mating activated sludge microorganisms with *E. coli* DH5, the plasmid was taken up by some of the naturally-occurring activated sludge microorganisms. The bioluminescent transconjugant strains PM6 and Shk1 were isolated on *Pseudomonas* isolation agar (to select against *E. coli* DH5) containing tetracycline (to select against natural strains that did not take up the plasmid). As

a result, the strains Shk1 and PM6 are Pseudomonads that contain the *lux* genes on the pUTK2 plasmid and therefore do not require an inducer to produce light. Strains created in this way produce light continuously during exponential growth and respond to toxicants in a "lights off" manner; when a toxic compound is present in the environment of Shk1 or PM6, light production is repressed. The exact mechanism of the repression is unknown but is probably related to a decrease in the intracellular level of $FMNH_2$ as a general toxic response (Hastings et al., 1985, Meighen, 1991).

2.2 Wastewater Treatment Systems

There are four stages to wastewater treatment: primary, secondary, tertiary, and advanced (Metcalf et al., 1916). Not all treatment plants incorporate process units to address each stage; some include only primary and secondary treatment for example. The activated sludge process is classified as a secondary treatment and has been used as treatment of both domestic and industrial wastewaters. Before entering the primary stage, the wastewater passes through a grit chamber where large solids are removed by sedimentation. During the primary stage, the wastewater is held for a few hours in the clarifier where solid particles are removed by sedimentation. After leaving this treatment tank, the wastewater enters the secondary treatment tank, where the activated sludge process takes place. Here the wastewater is combined with a mixture of mostly

aerobic bacteria and is aerated to promote heterotrophic aerobic metabolism. Most organic pollutants in the wastewater are biodegraded by the microorganisms and between 85 and 99% of the biochemical oxygen demand (BOD) is removed. Additional removal of unwanted materials in wastewater can be achieved in tertiary and advanced treatment.

2.3 Toxicity Test Protocols

There are a variety of protocols that have been developed for testing toxicity. The most common protocols involve aquatic toxicity testing and are based on the fact that the response of living organisms that have been exposed to toxicants depends on the concentration of the toxicant in the aqueous phase.

Aquatic toxicity testing systems may be static, renewal, or flow-through. Static tests are composed of living organisms that are exposed to test materials in non-moving water, and there is no change of water for the duration of the test (Redmond et al., 1989). The renewal test is also conducted in non-moving water, but the test solutions and control water or media are renewed periodically by transferring the organisms to freshly prepared material (Benoit et al., 1993). In the flow-through toxicity test, the test solution and control water or media are constantly flowing into and out of the container where the test organisms are kept (Bulich, 1979).

2.4 Methods for Wastewater Toxicity Assessment

There are many existing methods that have been developed for wastewater toxicity assessment. These existing methods include chemical analysis, microscopic analysis, fish tests, microorganism-based non-microscopic methods including bioluminescence-based tests and respirometric inhibition methods. Online monitoring of influent toxicity for the purpose of avoiding biomass shock is seldom used, however, because existing toxicity methods (Ince et al., 1998, Kelly et al., 1999) are difficult to adapt to continuous testing.

Chemical analysis may include colorimetry and atomic adsorption for metals, and liquid or gas chromatography for organic compounds. Problems associated with chemical analysis by these methods include the complexity and uncertain identity of potential toxicants, high equipment and operating costs, and analysis times too slow for effective process control responses; a slow monitoring process does not leave sufficient time for flow diversion to temporary basins.

Microscopic analysis is the visual inspection of the samples of activated sludge microorganisms taken from the aeration tank. Wastewater toxicity can sometimes be determined by the change in shape or observable characteristics of the microorganisms. The limitations for microscopic analysis are the same as chemical analysis, in that the process is expensive, slow, and requires the expertise of trained personnel (Ince et al., 1998).

Fish have been used as test subjects because by definition, the mortality rate of fish is directly related to water toxicity. Clesceri et al. (1998) showed that there are a number of fish that can be used in toxicity tests, such as trout and minnows. The restrictions on this test are that the tests are again time-consuming and the results are dependent upon the size of the fish. Also, since fish are not native to activated sludge, the results of fish tests may not be useful for evaluating wastewater toxicity to activated sludge.

Microorganism-based non-microscopic methods possess several advantages in wastewater toxicity testing over tests involving higher life forms, like the fish test. These advantages are convenient growing conditions, a large number of test organisms, and small test organism size (Kustin and McLeod, 1977). A variety of microorganisms can be employed in these tests, such as bacteria, algae, phytoplankton, and yeast. These tests are based on the effect of toxicants on some observable characteristics of the microorganisms, including cellular growth, motility and chemoattraction, ATP level, intracellular protein formation, respiration rate, and bioluminescence.

Respirometric inhibition is the most common method for testing wastewater toxicity to activated sludge (Love and Bott, 2004, Wong et al., 1997) and is generally accepted as the standard to which other assay methods are compared. The oxygen consumption rate of activated sludge exposed to a wastewater sample is compared to a nontoxic control and EC_{50} values (50%

oxygen consumption reduction) evaluated. Toxicity is indicated by a decrease in respiration rate.

Another respirometry-based batchwise test used is the Polytox® test (Polybac Corporation, Bethlehem, PA). This test uses a freeze-dried blend of 12 strains of organisms isolated from sludge cultures that are reconstituted in aqueous phase according to standard methods. These organisms are then used to inoculate a reactor, where the oxygen uptake rate is measured and compared with results taken from a toxicant-free reactor. Other than the fact that the distribution of organisms used in the assay may be more consistent than those from a local wastewater treatment facility, the Polytox® test is essentially an activated sludge respiration inhibition assay.

There are many disadvantages to respiration inhibition methodology. The first is that the warning of an influent disturbance does not directly identify the root of the problem. Respirometry can also take several minutes to provide sufficient information to confirm the influx of toxicity to a treatment system, possibly allowing a significant amount of toxicity to enter a plant before it is confirmed. Also, some toxins can cause process performance problems at sublethal concentrations that do not significantly impede respiration rates but may lead to false negatives (Love and Bott, 2000). In addition, significant expense in time and money and operation difficulty are incurred with respiration inhibition assays (Slabbert,1988, Williamson and Johnson, 1981). These problems have

provided an impetus to the investigation into an inexpensive, simple and sensitive wastewater biomass activity monitoring system that would allow accurate predictions of the effect of influent toxicity on activated sludge wastewater treatment systems.

Unlike respiration inhibition assays, the use of bioluminescent bacteria for toxicity measurements is relatively simple and rapid, it does not require the knowledge of the identity of the potential toxicants, and it can be used for online measurements (Girotti et al., 2002, Gu, et al., 2001). Bioluminescent toxicity assessment methods include the use of naturally luminescent marine bacteria, from genera such as *Photobacterium, Photorhabdus,* and *Vibrio*, and bacteria that have been genetically engineered to produce bioluminescence, such as the two *Pseudomonas* strains (denoted PM6 and Shk1) generated by teams of researchers at the University of Tennessee and Syracuse University (WERF project no. 01-WSM-2A, 2004, Kelly, 1999).

The most thoroughly studied bioluminescence-based system is the Microtox® assay, which is marketed by Strategic Diagnostics, Inc. (Newark, DE) and used for aquatic toxicity testing (Gu, 2000). The microorganism used in this assay is *Vibrio fischeri.* Freeze-dried cultures are purchased and resuspended, as needed, using the reconstitution solution, diluent solution, and an osmotic adjustment solution, all provided by the manufacturer. The toxicity assay is performed by mixing the reconstituted cells with test material, and measuring the

luminescence in an analyzer. The MicrotoxOmni software sold for use with this analyzer can be used to calculate EC_{50} values using the reduction in light (Azur Environmental, 1999).

The main problem with the Microtox® assay is that *V. fischeri* is extremely sensitive to many toxicants compared to other methods and as a result, the response to toxicants is different from the response of the activated sludge microbial community. Also, they are less sensitive to some compounds to which activated sludge is very sensitive. More disadvantages include the inability of analysis of anaerobic samples without first aerating the sample, the inability of turbid samples to be processed without removing particles larger than 50 μm, a time consuming correction procedure required for colored samples, and the necessity of performing the analysis at neutral pH (Love and Bott, 2000). Care should be exercised in interpreting Microtox® toxicity data because the degree to which *V. fischeri* represents an activated sludge community may be low (Reteuna et al., 1986). Therefore, application of the Microtox® toxicity test as a preventative influent toxicity monitoring method for wastewater treatment plants is limited and thus suitability is in question for predicting the possible toxic effects of wastewater on activated sludge. In addition, each time a toxicity test is performed, the procedure for batchwise preparation of the cells has to be repeated, making it difficult to adapt it to continuous toxicity screen.

The disadvantages associated with present commercially available toxicity assays and the quick responses by bioluminescent bacteria to toxicants similar to that of the activated sludge microbial organisms lead researchers to believe that toxicity of wastewater can be accurately represented by assays performed using the genetically engineered bioluminescent bacteria described above. In the following sections, the ability of two engineered strains, PM6 and Shk1, to predict toxic effects to activated sludge as indicated by respiration inhibition is investigated. Also, various methods of implementing PM6 and Shk1 in toxicity assays are studied to determine the most simple, effective, and accurate assay procedure.

Chapter 3 Materials and Methods

The parameters used to evaluate the accuracy and precision of the different protocols were the repression of light at a fixed concentration of a specific toxicant (zinc) and the EC_{50} values for three toxicants (zinc, copper, and lead) in aqueous solutions. The EC_{50} value is the sample concentration which inhibits the test organism light output by 50%. Different bioreporter storage methods were studied as well as different mixing strategies and temperature control at various periods during the performance of an assay. The EC_{50} values produced from the assays were compared to EC_{50} values determined using the Microtox® assay, published inhibition to activated sludge specific oxygen uptake rate (SOUR) data (Madoni et al., 1999), and published EC_{50} values for the Microtox® assay (Kaiser and Devillers, 1994).

Figure 3.1 shows a flow chart describing the steps taken in the course of this thesis work. The steps start with the growth of the PM6 cells obtained from Drs. Kelly and Lajoie at Syracuse University (WERF project no. 01-WSM-2A, 2004) and the Shk1 cells obtained from the Center for Environmental Biotechnology at the University of Tennessee. This is followed by the storage of the cells grown, followed by the activation of the cells. This is then followed by measurement of the bioluminescence repression in the cells, measurement of time stability in each

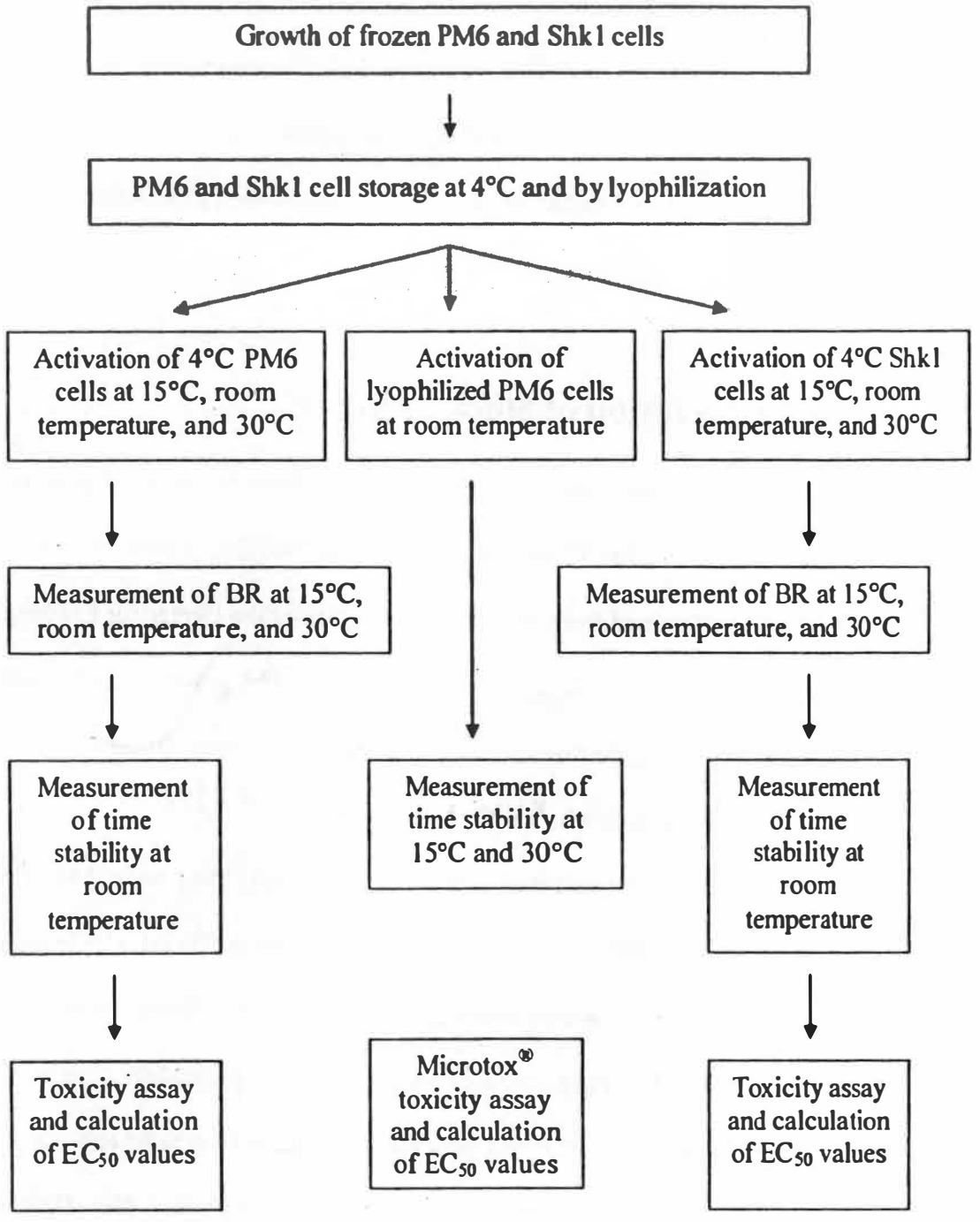

Figure 3.1: Flow chart showing steps taken in this course of study.

strain, and finally the toxicity assays performed. The usefulness of the EC_{50} values calculated is evaluated by comparing EC_{50} values determined using the Microtox® assay, published inhibition to activated sludge specific oxygen uptake rate (SOUR) data (Madoni et al., 1999), and published EC_{50} values for the Microtox® assay (Kaiser and Devillers, 1994).

3.1 Generation of Stock Cultures

A starter culture of PM6 was obtained from Drs. Kelly and Lajoie at Syracuse University (WERF project no. 01-WSM-2A, 2004). A starter culture of Shk1 was obtained from the culture collection of the Center for Environmental Biotechnology at the University of Tennessee. The growth medium used for both PM6 and Shk1 used throughout this research was Difco nutrient broth (NB) obtained from Fisher Scientific (Atlanta, GA).

Frozen cell suspension cultures of PM6 and Shk1 were thawed and 20 µL of cell suspension of each strain was inoculated into 100 mL sterile room temperature NB amended with 10 mg tetracycline/L. The tetracycline was added between 1 and 2 hours after inoculation to avoid a potentially long lag phase before the antibiotic resistant genes have adequate time to be expressed. Cells were grown in an incubator (Classic C24 Incubator Shaker, New Brunswick Scientific, Edison, NJ) at 200 rpm and 30°C. Every hour the optical density (OD) in each culture was measured using a Beckman DU 520 general purpose

laboratory UV/vis spectrophotometer at a wavelength of 600nm (Beckman Coulter Inc., Fullerton, CA), and the bioluminescence was measured using a luminometer (Sirius FB15 2C Single Tube Luminometer, Zylux Corporation, Oak Ridge, TN). When the OD_{600} in each cell culture reached 1.0, 15% (w/v) glycerol was added for freezing at -80°C. These tubes formed the base stock for all subsequent experiments.

3.2 Cell Storage

Each subsequent culture for use in toxicity assays was started by thawing the frozen cultures from the base stock and inoculating 20 µL of the thawed cell suspension into 100 mL sterile NB amended with 10 mg tetracycline/L as noted above. Cells were grown in an incubator at 200 rpm and 30 °C until the OD_{600} reached 1.0. Cells were then stored either by placing the growth flask in a refrigerator at 4°C or by lyophilizing the cells, as described below.

3.2.1 Cells stored at 4°C

Cell cultures were grown using the procedure given in section 3.2. When the OD_{600} reached 1.0, each flask containing Shk1 and PM6 cells was removed from the incubator and stored in a refrigerator at 4°C for approximately ten hours as was done in Lajoie et al. (2002).

3.2.2 Lyophilized cells

Cell cultures were grown from frozen base stock using the procedure given in section 3.2. When the OD_{600} reached 1.0, a 1000 µL aliquot was transferred into each of 18 1.5 mL microcentrifuge tubes and centrifuged at 14,000 x *g* for 8 minutes (Eppendorf Centrifuge 5415C, Brinkman Instruments, Inc., Westbury, NY). The supernatant was then carefully removed from the pellet and discarded. The tubes were placed into a 600 mL Labconco flask and attached to a Labconco Freezone® 6 Liter Freezer Dry System (Labconco Corp., Kansas, MO) for approximately 8 hours to remove any remaining liquid from the pellets. After the 8 hours, the microcentrifuge tubes were capped and placed in a freezer at -20°C. Each 1.5 mL tube contained a small pellet of dried cells (app. 3 mg) at the bottom of the tube at the end of the lyophilization process.

3.3 Cell Activation

Cells stored at 4°C and lyophilized cells were activated for 20 minutes before the toxicity assay was performed. Activation is the period of time after cells are stored when they are combined with fresh nutrient broth and brought to a specified temperature (see also Figure 3.1).

3.3.1 Activation of cells stored at 4°C

During the activation period, the temperature of both PM6 and Shk1 cells that were stored at 4°C was either controlled at 15°C or 30°C or left uncontrolled at room temperature (approximately 27°C). The activation procedure was based on a protocol published by Lajoie et al. (2002) and adapted to this work. The two controlled activation temperatures (15°C or 30°C) and the uncontrolled (room) temperature were paired with the same temperatures at which a model toxicity test was performed to form a total of 7 experiments. In each of these seven, 10 µL of PM6 or Shk1 cells was added to 1000 µL of sterile NB in a 1.5 mL microcentrifuge tube and the solution was allowed to rest for 20 minutes (the period referred to as activation) after which the model toxicant (zinc) was added. The only difference between the experiments was the activation and toxicity testing temperatures (15°C and 30°C, and room temperature). The NB solution to which the cells were added for activation was maintained at either 15°C or 30°C or left uncontrolled at room temperature both before the 10 µL addition of the cell inoculum and for the 20 minute rest period. After the activation period, the toxicant was added and the temperature was maintained at either 15°C or 30°C or left uncontrolled at room temperature. The activation (TC_{act}) and toxicity testing (TC_{tox}) temperatures were combined to form the following set of seven experimental conditions:

1. TC_{act} controlled at 30°C, TC_{tox} uncontrolled (room temp).

2. TC_{act} controlled at 15°C, TC_{tox} uncontrolled (room temp.).
3. TC_{act} uncontrolled (room temp.), TC_{tox} controlled at 30°C.
4. TC_{act} uncontrolled (room temp.), TC_{tox} controlled at 15°C.
5. TC_{act} and TC_{tox} both controlled at 30°C.
6. TC_{act} and TC_{tox} both controlled at 15°C.
7. TC_{act} and TC_{tox} both uncontrolled (room temp.).

3.3.2 Activation of lyophilized cells

Lyophilized cells were removed from the -20°C freezer, and activated by adding 1000 µL of either sterile 15°C NB or sterile room temperature NB to the 1.5 mL microcentrifuge tubes. The tubes were then incubated for 20 minutes at room temperature or 15°C and the light level monitored to study the variability in the light level during the incubation period.

In addition to the procedure described above, cells were mixed using a Vortex Genie vortex mixer (Model K-550-G, Scientific Industries, Inc., Bohemia, NY) for 2 seconds prior to each luminescence measurement. Toxicity assays were not performed using lyophilized cells.

3.4 Effect of Temperature Control on the Variability of Bioluminescence Repression and Γ

A 6 ppm solution of the model toxicant zinc (as the ion Zn^{2+}) was prepared by diluting a 50 ppm $ZnCl_2$ stock solution. 100 µL of the 6 ppm Zn^{2+} solution and 170 µL of DI water were added to 12x75 mm Fisherbrand disposable culture tubes, followed by 30 µL of cell culture (after activation, see Section 3.3.1). This gave samples with a final Zn^{2+} concentration of 2 ppm. Bioluminescence was measured at 0 minutes (immediately after the toxicant was added) and at 7 minutes; bioluminescence repression (BR) and Γ were calculated by using the equations (3.1) and (3.3) respectively. The light intensity was corrected through the use of a control (0 ppm toxicant) sample. This correction factor (R_t) was used to compensate for the tendency of the light level of PM6 and Shk1 to increase or decrease even in the absence of a toxicant. The correction factor R_t as well as BR and Γ are defined in equation 3.1-3.3:

$$BR = \frac{I_{ts}}{I_{os}} \tag{3.1}$$

$$R_t = \frac{I_{tc}}{I_{oc}} \tag{3.2}$$

$$\Gamma_t = \frac{(R_t)}{BR} - 1 \tag{3.3}$$

where:

I_{ts} = light intensity of sample at 7 minutes

I_{os} = initial (t=0) light intensity of sample

I_{tc} = light intensity of control at 7 minutes

I_{oc} = initial (t=0) light intensity of control

BR and Γ_t were determined from experiments where the temperature of the sterile NB and cell mixture was controlled at either 15°C or 30°C or left uncontrolled at room temperature, to determine if temperature should be a controlled variable. 15°C was chosen because this is the temperature at which the Microtox® Acute Toxicity Assay is performed, and the means of controlling the samples at this temperature was therefore available in the laboratory. 30°C was chosen because it is slightly above room temperature and the vials of NB could be reproducibly maintained at this temperature using a heated water bath. The decision of whether temperature should be a controlled variable was determined by measuring Γ in each experiment and comparing its variance and coefficient of variation.

The light level produced by each strain of cells needs to be fairly constant to perform a toxicity assay; if the baseline (control) is subject to a large standard deviation and if it varies significantly during the assay time, it will be a poor

control and the toxicity assay will be imprecise. In order to determine when the light level in each strain of cells was sufficiently constant, the light intensity of each sample was measured for 30 minutes at 2 minute intervals. Standard deviations of the appropriate variables (BR and Γ) were determined from the multiple measurements, and are indicated by vertical error bars in the figures. The luminometer was set to report light intensity in relative light units, or RLUs where 1 RLU = 1 photons/s measured by the detector.

3.5 Time Stability of PM6 and Shk1 Luminescence

3.5.1 Non-mixed cells

PM6 and Shk1 cells stored at 4°C were activated using room temperature sterile NB and the light level was measured for 30 minutes at 2 minute intervals, without mixing the cells. Lyophilized cells were activated using both room temperature sterile NB and by controlling the temperature of the sterile NB at 15°C to study the effect of activation temperature. After 20 minutes activation, the light level was measured again without mixing the cells.

3.5.2 Mixed cells

In addition to the time stability of the light level of PM6 and Shk1 non-mixed cells, time stability of PM6 and Shk1 luminescence was measured for

mixed cells. Cells were activated using room temperature NB and the lyophilized cells using both room temperature sterile NB and 15°C sterile NB were mixed using a vortex mixer for 2 seconds prior to each luminescence measurement.

3.6 Determination of EC_{50} Values

3.6.1 Toxicity assays for cells stored at 4°C

Stock solutions of the salts of the three metals (zinc chloride ($ZnCl_2$), copper chloride ($CuCl_2$), and lead chloride ($PbCl_2$)) were prepared and a series of dilutions performed to achieve desired concentrations. 100 µL of each dilution and 170 µL of DI water were added to 12x75 mm Fisherbrand disposable culture tubes, followed by 30 µL of cell culture (after activation). The final concentrations (the concentrations in the tubes after the final dilution) in the six samples for the toxicity assay for each heavy metal were as follows: 0, 0.4, 0.8, 1.2, 1.6, and 2 ppm for Zn^{2+} (using a 50 ppm stock solution of $ZnCl_2$), 0, 5.5, 7.0, 8.7, 10.2, and 11.8 ppm for Cu^{2+} (using a 50 ppm stock solution of $CuCl_2$), and 0, 31.03, 43.44, 55.85, 68.26, and 80.68 ppm for Pb^{2+} (using a 500 ppm stock solution of $PbCl_2$). The temperature of all solutions before inoculation, during activation, and during exposure to the toxicants was the ambient room temperature (approximately 27°C) and was not controlled. BR and Γ were

calculated by equations (3.1) and (3.3), respectively. EC_{50} values were calculated by generating a plot of log Γ values vs. log concentrations.

Bay et al. (1989) predict a power curve relationship between concentration and response when life forms are exposed to toxic chemicals, where response is measured as a ratio of activity lost to activity remaining. A log-log transformation would therefore be linear, making linear regression useful for characterizing toxic effects. The resulting linear equation, described in equations 3.4-3.6, was used to predict the EC_{50} values of each of the toxicants. This was done by setting $\ln \hat{\Gamma}_i$ equal to zero in equation 3.6 and solving for ln(c). The exponential of c is therefore the EC_{50} value for the particular toxicant.

$$m = \frac{n\sum((\ln(c_i))\ln(\Gamma_i)) - \sum\ln(c_i)\sum\ln(\Gamma_i)}{n\sum(\ln(c_i)^2) - (\sum\ln(c_i))^2} \tag{3.4}$$

$$b = \frac{\sum\ln(\Gamma_i) - m\sum\ln(c_i)}{n} \tag{3.5}$$

$$\ln\hat{\Gamma}_i = m(\ln(c)) + b \tag{3.6}$$

where:

(c_i, Γ_i) = set of each concentration and paired Γ value

$(\ln(c_i), \ln(\Gamma_i))$ = the natural log of the set of each concentration and paired Γ values

$\hat{\Gamma}_i$ = fitted Γ values

m = slope defined in equation 3.6

b = intercept defined in equation 3.6

n = number of data points

c = concentration values

The quality of the equation fit to the data is indicated by the R^2 value. This is used to measure the strength of the association between the two variables. This value is the fraction of the total squared error that is explained by the model. The formula used to calculate this value is:

$$R^2 = 1 - \frac{\sum(\ln \Gamma_i - \ln \hat{\Gamma}_i)^2}{\sum(\ln \Gamma_i - \ln \bar{\Gamma})_2} \qquad (3.7)$$

where:

$\bar{\Gamma}$ = average of all calculated Γ values

The closer the R^2 value is to unity, the better the fit of the equation to the data, meaning the larger proportion of variance in one variable that can be explained from knowledge of the second variable.

The 95% confidence intervals for the EC_{50} value from each regression were calculated using equations adapted to the Microtox® assay (Draper and Smith, 1981). These equations are:

$$CF = \sqrt{\left(S^2 \left(\frac{1}{N} + \frac{(\ln \Gamma_0 - \ln \Gamma_a)^2}{\sum \gamma^2} \right) \right)} \tag{3.8}$$

$$S^2 = \frac{\sum (\ln c)^2 - \left(\sum ((\ln c)\gamma) \right)^2 / \sum \gamma^2}{N-2} \tag{3.9}$$

where:

Γ_0 = the Γ corresponding to the EC_{50}

$\ln \Gamma_a$= the mean of ln (Γ) values

N = the number o f data pairs used for regression

S = residual variance

CF = confidence factor

$\gamma = \ln (\Gamma_i) - \ln \Gamma_a$

The product of CF and $t_{.05}$ (from t table, Walpole et al., 1998) is added to and subtracted from ln C_o to give the natural logs of the higher and lower 95% confidence limits on C_o. The exponential is then taken of ln C_o and its confidence limits. This interval is the expected deviation around each EC_{50} value calculated.

Another confidence interval was calculated by collecting all EC_{50} values for all replicates on all days. This interval represents the expected deviation around the reported EC_{50} value accounting for daily variations in temperature, technique, etc. and is given in equation 3.11.

$$s = \sqrt{\sum_{i=1}^{n} \frac{(x_i - \bar{x})^2}{n-1}} \tag{3.10}$$

$$CI = \bar{X} \pm (t_{.05})(s/\sqrt{n}) \tag{3.11}$$

where:

s = standard deviation

x_i = each EC_{50} value calculated

$\bar{x}$ = mean of EC_{50} values

n = number of data points used

CI = confidence interval

$t_{.05}$ = value from t table (Walpole et al., 1998)

Toxicity assays were randomized by performing the assay in triplicate on the same day over a span of several hours and repeating this process over a span of five days. Assays were performed on day 1, 3 and 5 with day 1 representing the day after the cells were stored at 4°C. The cells were stored in the evening of one day and assays performed the following morning so day 1 represents the elapsed time after storage of about 10 hours.

3.6.2 Microtox toxicity assays

Experiments were also run using the Microtox® Acute Test on a Microbics M500 Analyzer (Microtox®, Strategic Diagnostics, Inc., Newark, DE) to determine EC_{50} values for Zn^{2+}, Cu^{2+} and Pb^{2+}. 50 ppm stock solutions of the salts $ZnCl_2$ and $CuCl_2$ and a 500 ppm stock solution of the salt $PbCl_2$ were made and used in these experiments. First, ten Microtox® cuvettes were placed in wells A1 through B5 in the analyzer (see Figure 3.2). Then, 1.5 mL aliquots of Microtox® diluent were dispensed into two 12x75mm Fisherbrand disposable culture tubes and placed in arbitrarily chosen wells E3 and E4 (see Figure 3.2) for five minutes to bring the temperature of the diluent down to 15°C, which is controlled by the analyzer. Then, two Microtox® SOLO reagent vials were taken out of the -20°C freezer and each vial reconstituted using the 1.5 mL Microtox®

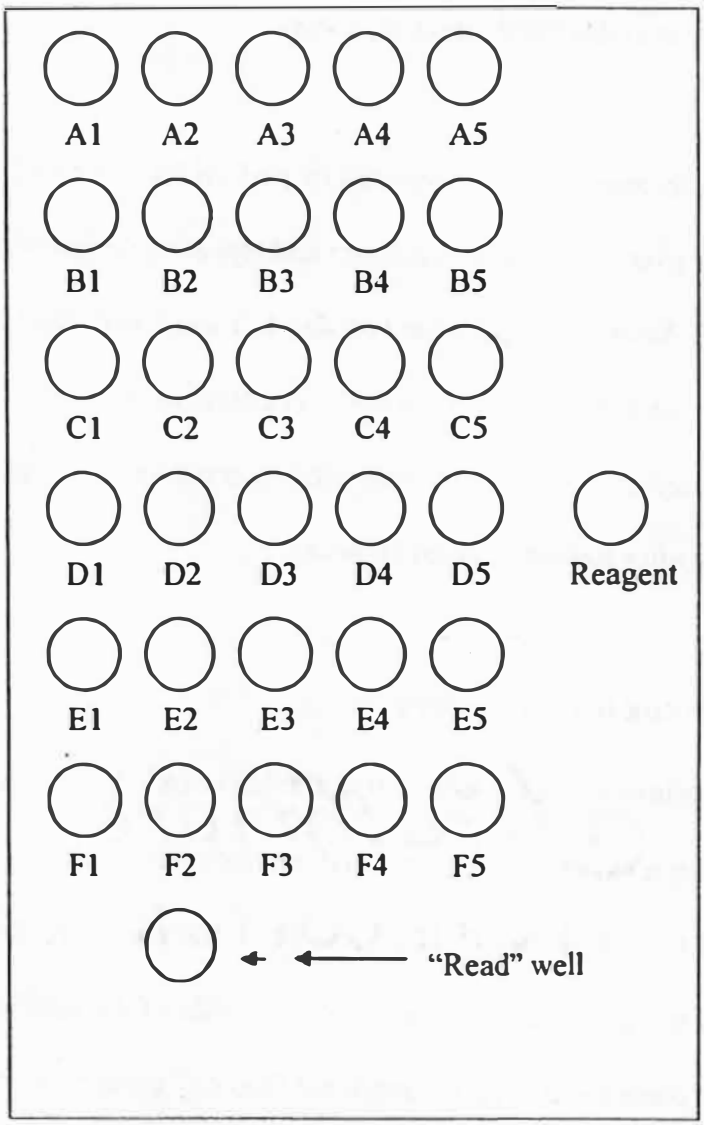

Figure 3.2: The setup of the Microbics M500 Analyzer.

diluent in wells E3 and E4 according to the manufacturer's specifications. The two tubes (E3 and E4) were then combined into one, and 500 µL of the combined reagent was added to each of five Microtox® cuvettes, in wells B1 through B5 (see Figure 3.2) and let sit for 15 minutes. 1000 µL of Microtox® diluent was then added to each of four Microtox® cuvettes, in wells A1 through A4. 2500 µL of 50 ppm $ZnCl_2$ was added to cuvette A5 along with 250 µL Microtox® osmotic adjusting solution (MOAS) using a 1000 µL pipettor and was mixed by filling and dispensing the pipettor 2-3 times. 1000 µL of A5 was then pipetted into A4 and mixed using the pipettor. 1000 µL of A4 was pipetted into A3 and mixed using the pipettor, and 1000 µL of A3 was pipetted into A2 and mixed using the pipettor. 1000 µL was discarded from A2 and 750 µL discarded from A5. The toxicant was allowed to sit for 5 minutes for temperature equilibration. After the fifteen minutes required for reagent light level stabilization, vial B1 was placed in the "read" well (Figure 3.2) and the "set" button pushed to set the analyzer so the light level in each cuvette read is expressed relative to that in B1. A reading of 100 on subsequent samples indicates the light level is equal to the original light level in B1. Each of the five cuvettes, B1 through B5 should therefore read approximately 100 before the toxicant is added. The Microtox® Basic Test timing program on the computer connected to the analyzer was started and cuvettes B1 through B5 placed in the read well and the light level measured when prompted. Then, 500 µL of A1 was pipetted into B1, A2 into B2, on through to A5, B5.

After 5 minutes, cuvettes B1 through B5 were read again when prompted by the timing program. The EC_{50} values were then calculated. This experiment was then performed again using a 50 ppm solution of $CuCl_2$ and a 500 ppm solution of $PbCl_2$. The results were then compared with the EC_{50} values determined from the Shk1 assays, PM6 assays, SOUR (Madoni et al., 1999), and the literature data for Microtox® (Kaiser and Devillers, 1994).

Chapter 4 Results and Discussion

The commercially available assays for testing wastewater toxicity and determining measured EC_{50} values for specific toxicants are expensive, time consuming, and in some cases require knowing the identity of the toxicants. This research has been dedicated to finding an alternative to the present commercially available assays while keeping the cost low, requiring no previous knowledge of the toxicants, and not requiring a lot of time to perform the assay.

Several experiments were conducted to determine the efficacy of measuring EC_{50} values using Shk1 and PM6. Results were compared to EC_{50} values determined from the Microtox® assay, published inhibition to activated sludge specific oxygen uptake rate (SOUR) data (Madoni et al., 1999), and published EC_{50} values for Microtox® (Kaiser and Devillers, 1994). Experiments were performed using aqueous solutions of zinc chloride, copper(II) chloride, and lead(II) chloride. EC_{50} values for Zn^{2+}, Cu^{2+}, and Pb^{2+} were calculated based on the experimental data. These three metals were chosen because they represent some of the materials for which the EPA requires testing in wastewater.

There were some experiments where the concentration of the toxicant was either too low or too high to yield meaningful data; these experiments did not produce an EC_{50} value because the bioluminescence repression was either greater

than 1.0 (bioluminescence increased leading to a negative Γ value) or so high the log of the Γ values did not span zero. EC_{50} values cannot be calculated with negative Γ values because the log of Γ is needed to calculate the EC_{50} values and the log of a negative number is undefined. Therefore, for the purpose of this thesis, the data from these experiments was discarded and is not shown.

4.1 Effects of Temperature Control and PM6 and Shk1 Bioluminescence Repression

Bioluminescence repression (BR) and Γ (equations 3.1 and 3.2) were the dependent variables chosen for PM6 and Shk1 cells to determine when and if temperature should be controlled during toxicity testing with these cells. BR was calculated because it is used to calculate Γ, and Γ is used to determine the EC_{50} value. There were two opportunities for temperature control in the assays being developed in this study, during "activation" of the stored (refrigerated) cells and during toxicity testing (exposure to toxicant). The purpose of the set of experiments described in this section was to determine if temperature control at various assay points would lead to better assay accuracy as indicated by the standard deviation and coefficient of variance in Γ and BR.

Cells were activated and the toxicity testing performed according to the method of Lajoie et al. (2002). This method specifies that the cells stored at 4°C

be activated by inoculating 10 μL into 1000 μL of sterile NB and letting the solution rest for 20 minutes; this stage is referred to as the activation stage. After 20 minutes, 30 μL of activated cells were injected into 270 μL of the test media for a contact time of seven minutes. This stage is referred to as the toxicity testing stage. Temperature control at either 15°C, room temperature, or 30°C was investigated during activation (TC_{act}), during toxicity testing (TC_{tox}), during both (TC_{both}), or not at all (TC_{none}). 15°C was chosen because that is the temperature at which the Microtox toxicity assay is conducted and 30°C was chosen because it is slightly above room temperature (27°C) and can be controlled by a heated water bath. Figure 4.1 shows the average BR and standard deviation for the Γ values determined during the temperature control at different steps of the assay for PM6. BR was measured after seven minutes exposure time and the average and standard deviation of the 36 replicates was calculated for each experiment. There is a significant difference in the averages of the means, as they range from 0.942 to 10.02 for the PM6 experiments performed at 15°C (see Tables 4.1 and 4.2) and range from 4.05 to 10.02 for the PM6 experiments performed at 30°C (see Tables 4.3 and 4.4). There is also a difference in the standard deviations (based on 36 replicates performed as 4 replicates each day on 3 different days from 3 different frozen culture tubes) as they range from 1.17 to 6.31 for the PM6 experiments performed at 15°C (see Tables 4.1 and 4.2) and range from 4.04 to 11.48 for the PM6 experiments performed at 30°C (see Tables 4.3 and 4.4). However, when

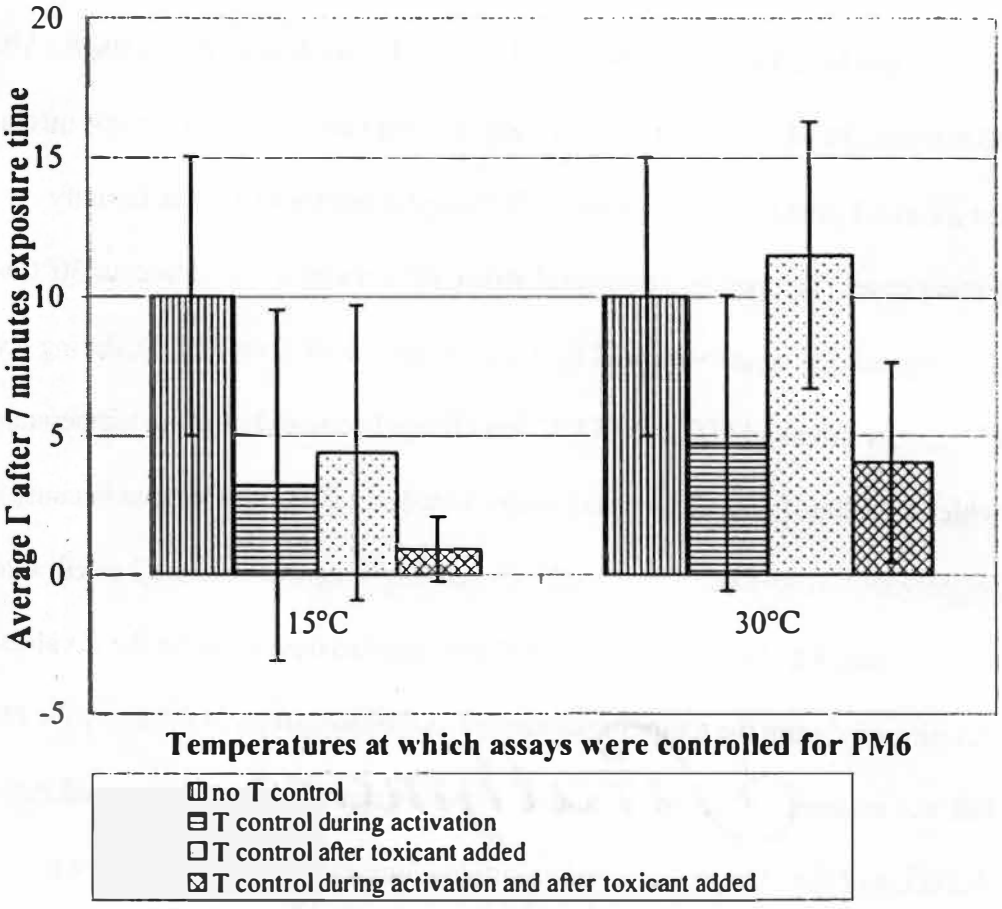

Figure 4.1: Average BR and standard deviation for Γ at the temperatures at which assays were controlled for PM6.

Table 4.1: Calculated BR values with temperature control at different steps during the assay for PM6 at 15°C.

PM6	BR-15 degrees Celcius			
	$BR\text{-}TC_{none}$	$BR\text{-}TC_{act}$ [1]	$BR\text{-}TC_{tox}$ [2]	$BR\text{-}TC_{both}$ [3]
Var	0.012	0.086	0.173	0.139
Stdev	0.111	0.293	0.415	0.373
Mean	0.182	0.507	0.680	0.836
COV	0.607	0.577	0.611	0.446

Where: (1) Temperature controlled during activation step alone.
(2) Temperature controlled during toxicity testing alone.
(3) Temperature controlled during both activation step and during toxicity testing.

Table 4.2: Calculated Γ values with temperature control at different steps during the assay for PM6 at 15°C.

PM6	Γ-15 degrees Celcius			
	$\Gamma\text{-}TC_{none}$	$\Gamma\text{-}TC_{act}$	$\Gamma\text{-}TC_{tox}$	$\Gamma\text{-}TC_{both}$
Var	25.2	39.8	28.1	1.36
Stdev	5.02	6.31	5.30	1.17
Mean	10.0	3.22	4.39	0.942
COV	0.501	1.96	1.21	1.24

Table 4.3: Calculated BR values with temperature control at different steps during the assay for PM6 at 30°C.

PM6	BR-30 degrees Celcius			
	$BR\text{-}TC_{none}$	$BR\text{-}TC_{act}$	$BR\text{-}TC_{tox}$	$BR\text{-}TC_{both}$
Var	0.012	0.142	0.004	0.115
Stdev	0.111	0.377	0.064	0.339
Mean	0.182	0.800	0.130	0.480
COV	0.607	0.473	0.492	0.706

Table 4.4: Calculated Γ values with temperature control at different steps during the assay for PM6 at 30°C.

PM6	Γ -30 degrees Celcius			
	$\Gamma\text{-TC}_{none}$	$\Gamma\text{-TC}_{act}$	$\Gamma\text{-TC}_{tox}$	$\Gamma\text{-TC}_{both}$
Var	25.2	28.3	22.9	13.0
Stdev	5.02	5.32	4.78	3.60
Mean	10.0	4.74	11.5	4.05
COV	0.501	1.12	0.417	0.890

the coefficient of variation (COV) is calculated (which is the standard deviation normalized by the mean), the differences between the assays (as indicated by the range of COV values) is much smaller. The COV values range from 0.501 to 1.96 for the PM6 experiments performed at 15°C (see Tables 4.1 and 4.2) and range from 0.501 to 1.12 for PM6 experiments performed at 30°C (see Tables 4.3 and 4.4), with the smallest COV recorded for Γ when no temperature control at all was used. The degree to which the errors in the assays are indistinguishable can also be judged by examining the error bars (which are drawn two standard deviations long, centered on the mean) in Figure 4.1; the error bars overlap for all four permutations of the assay at each temperature. For these reasons, it was judged that use of temperature control for PM6 during activation and/or toxicity testing does not improve the accuracy of the assay and there is no benefit in controlling the temperature for PM6 toxicity assays.

Figure 4.2 shows the average BR and standard deviation for the Γ values determined during the temperature control at different steps of the assay for Shk1.

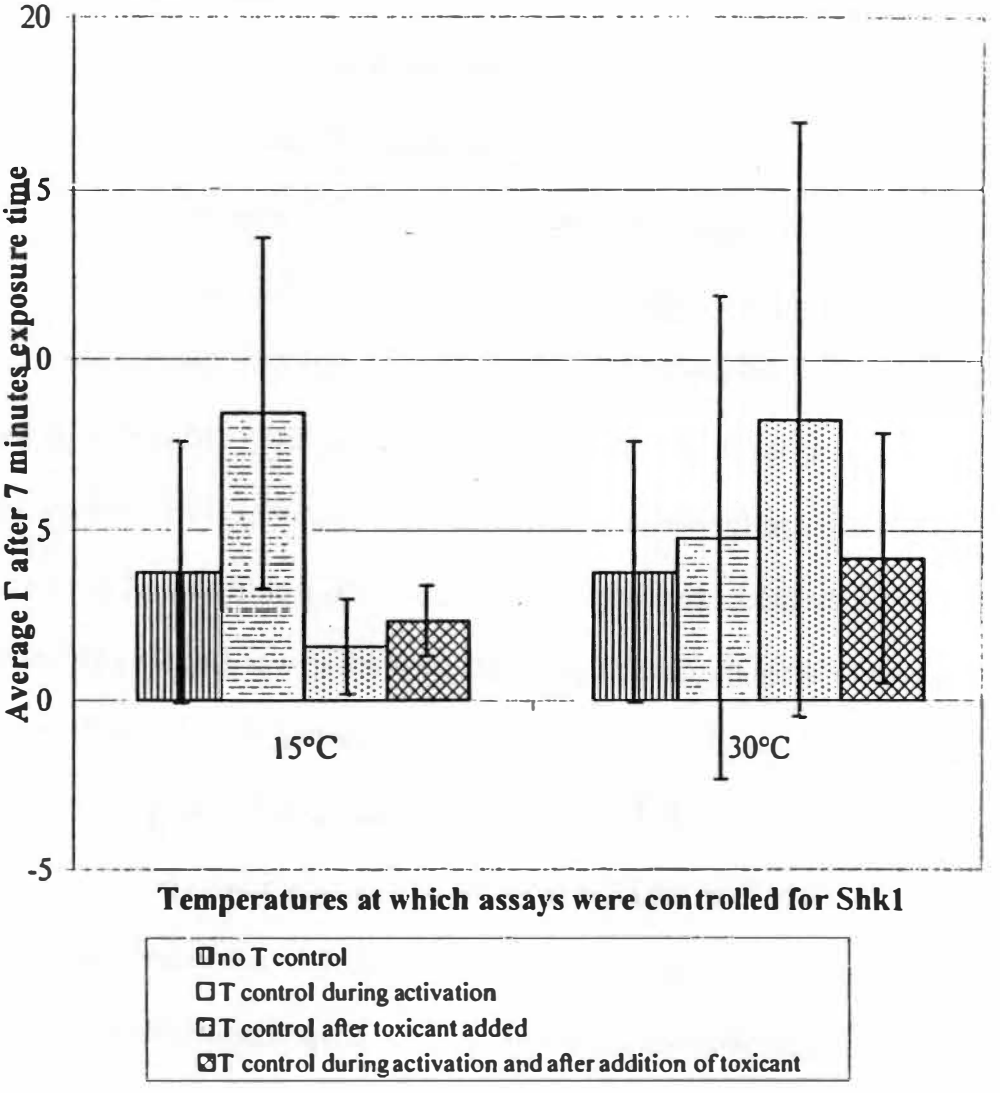

Figure 4.2: Average BR and standard deviation for Γ at the temperatures at which assays were controlled for Shk1.

BR was measured after seven minutes exposure time and the average and standard deviation of 36 replicates (chosen as discussed above) was calculated for each experiment. As for PM6, there is a significant difference in the averages of the means, as they range from 1.59 to 8.44 for the Shk1 experiments performed at 15°C (see Tables 4.5 and 4.6) and range from 3.76 to 8.24 for the Shk1 experiments performed at 30°C (see Tables 4.7 and 4.8). There is also a difference in the standard deviations as they range from 1.05 to 5.14 for the Shk1 experiments performed at 15°C (see Tables 4.5 and 4.6) and range from 3.67 to 8.71 for the Shk1 experiments performed at 30°C (see Tables 4.7 and 4.8). However, when the COV is calculated for Shk1 as was done for PM6 above, the difference between the assays is again much smaller; the COV values range from 0.450 to 1.02 for the Shk1 experiments performed at 15°C (see Tables 4.5 and 4.6) and range from 0.877 to 1.49 for the Shk1 experiments performed at 30°C see Tables 4.7 and 4.8). Examining the standard deviations and means in Figure 4.2, it is again observed that the error bars overlap in nearly all cases, the exception being the two cases where temperature was controlled either during activation alone or during toxicity testing alone for Shk1 at 15°C. However, in this case, the error bars are very close to overlapping each other. Based on the above observations, it was judged that temperature control does not improve the assay accuracy when Shk1 is used, as was the case for PM6.

Table 4.5: Calculated BR values with temperature control at different steps during the assay for Shk1 at 15°C.

Shk1	BR-15 degrees Celcius			
	$BR\text{-}TC_{none}$	$BR\text{-}TC_{act}$	$BR\text{-}TC_{tox}$	$BR\text{-}TC_{both}$
Var	0.044	0.008	0.028	0.014
Stdev	0.209	0.088	0.166	0.119
Mean	0.390	0.146	0.559	0.321
COV	0.534	0.606	0.297	0.370

Table 4.6: Calculated Γ values with temperature control at different steps during the assay for Shk1 at 15°C.

Shk1	Γ -15 degrees Celcius			
	$\Gamma\text{-}TC_{none}$	$\Gamma\text{-}TC_{act}$	$\Gamma\text{-}TC_{tox}$	$\Gamma\text{-}TC_{both}$
Var	14.8	26.4	1.94	1.10
Stdev	3.85	5.14	1.39	1.05
Mean	3.76	8.44	1.59	2.33
COV	1.02	0.609	0.876	0.450

Table 4.7: Calculated BR values with temperature control at different steps during the assay for Shk1 at 30°C.

Shk1	BR-30 degrees Celcius			
	$BR\text{-}TC_{none}$	$BR\text{-}TC_{act}$	$BR\text{-}TC_{tox}$	$BR\text{-}TC_{both}$
Var	0.044	0.347	0.023	0.093
Stdev	0.209	0.589	0.151	0.305
Mean	0.390	0.872	0.176	0.392
COV	0.534	0.675	0.861	0.779

Table 4.8: Calculated Γ values with temperature control at different steps during the assay for Shk1 at 30°C.

Shk1	Γ -30 degrees Celcius			
	Γ-TC_{none}	Γ-TC_{act}	Γ-TC_{tox}	Γ-TC_{both}
Var	14.8	50.6	75.9	13.5
Stdev	3.85	7.11	8.71	3.67
Mean	3.76	4.77	8.24	4.18
COV	1.02	1.49	1.06	0.877

4.2 Time Stability of PM6 and Shk1 Luminescence

The time stability of the light output of both strains was tested to determine if and when the light level after activation of each strain of cells was fairly constant because if the baseline (control) is subject to a large standard deviation, it will be a very poor control and the toxicity assay will be imprecise. Also, the effect of mixing was studied to determine if this would lead to a more stable light level. As noted earlier, oxygen dissolved in the media is needed by the light-producing reaction and mixing would affect the dissolved oxygen level in the samples.

As shown in section 4.1, there was no reason to control temperature during toxicity testing for cells stored at 4°C at room temperatures around 27°C because temperature control does not lead to more accurate BR or Γ values for PM6 or Shk1. Therefore, temperature was not controlled in further experiments for the cells stored at 4°C; experiments were simply conducted at room temperature.

Luminescence for PM6 and Shk1 cells that were not mixed prior to luminescence measurement was measured after activating 10 µL of the cells stored at 4°C with 1000 µL of room temperature sterile NB. Lyophilized cells were activated using 1000 µL of both room temperature sterile NB and 15°C sterile NB because it was unknown how the lyophilized cells would react to the different temperatures. PM6 and Shk1 cells that were mixed were done so by using a Vortex Genie vortex mixer (Model K-550-G, Scientific Industries, Inc., Bohemia, NY) for two seconds immediately prior to luminescence measurement. The light level was measured for both the cells stored at 4°C and the lyophilized cells, with and without mixing, for thirty minutes at two minute intervals as described in section 3.4. Activation for longer than 30 minutes was not considered because longer activation times make the assay less convenient; the Microtox® Acute Toxicity Assay uses an activation time of 15 minutes. Figures 4.3 – 4.6 display the luminescence of each strain Shk1, PM6 and lyophilized PM6 as a function of time. The optimal time to conduct the toxicity assay would be when the light level of the cells is fairly constant for at least 7 minutes; a standard exposure time of 7 minutes was set by Lajoie et al. (2002) to obtain reproducible results from toxicity assays. Error bars are shown in all non-mixed and mixed cells, PM6 cells stored at 4°C and activated at room temperature, Shk1 cells stored at 4°C and activated at room temperature, lyophilized PM6 cells activated at 15°C, and

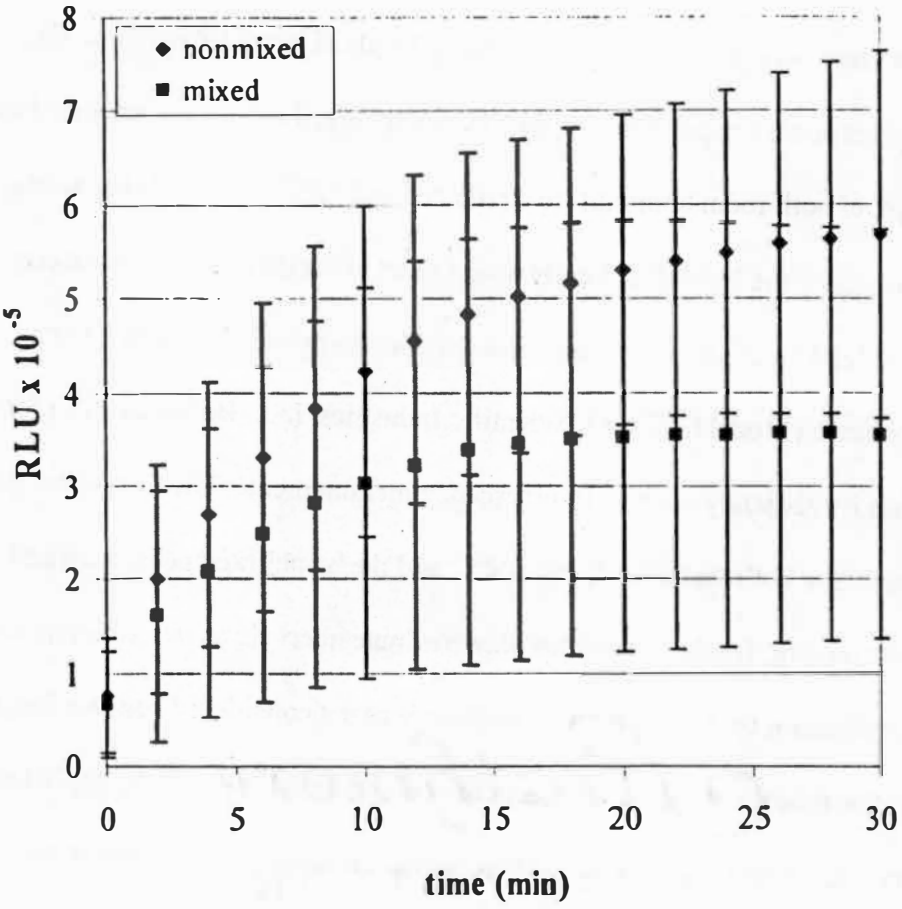

Figure 4.3: Bioluminescence of activated PM6 cells. Mixed cells were placed on the vortex mixer for two seconds prior to measurement.

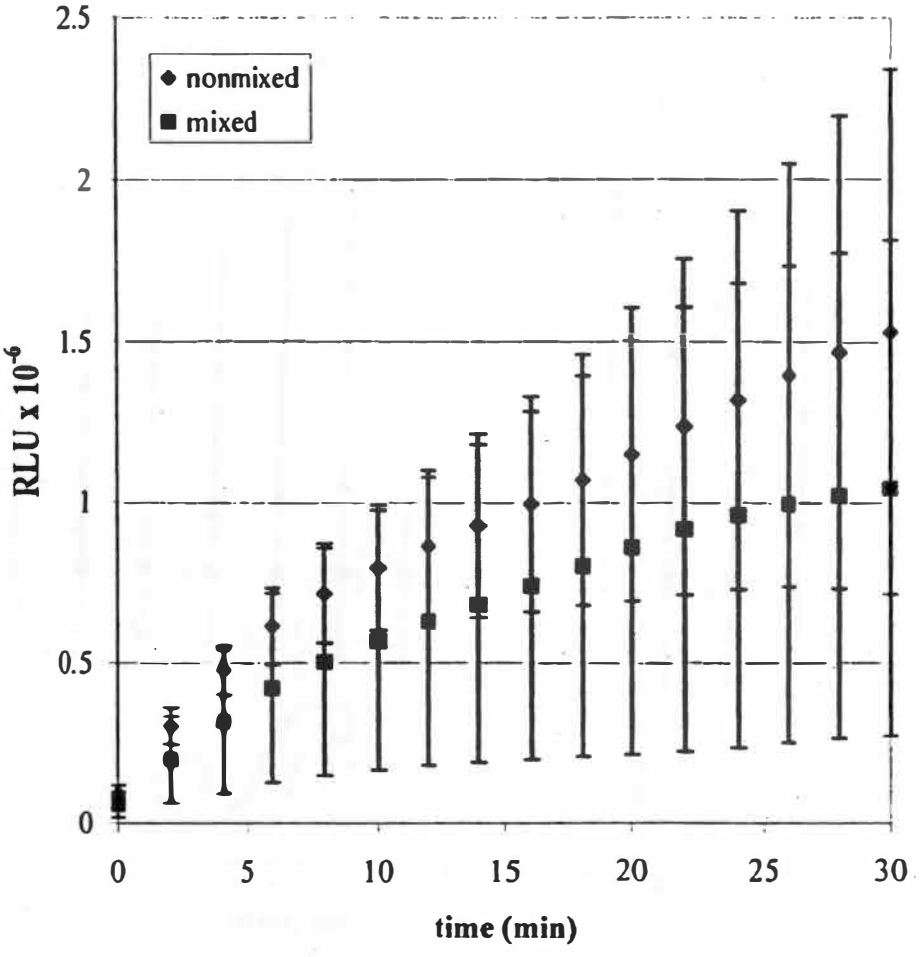

Figure 4.4: Bioluminescence of activated Shk1 cells. Mixed cells were placed on the vortex mixer for two seconds prior to measurement.

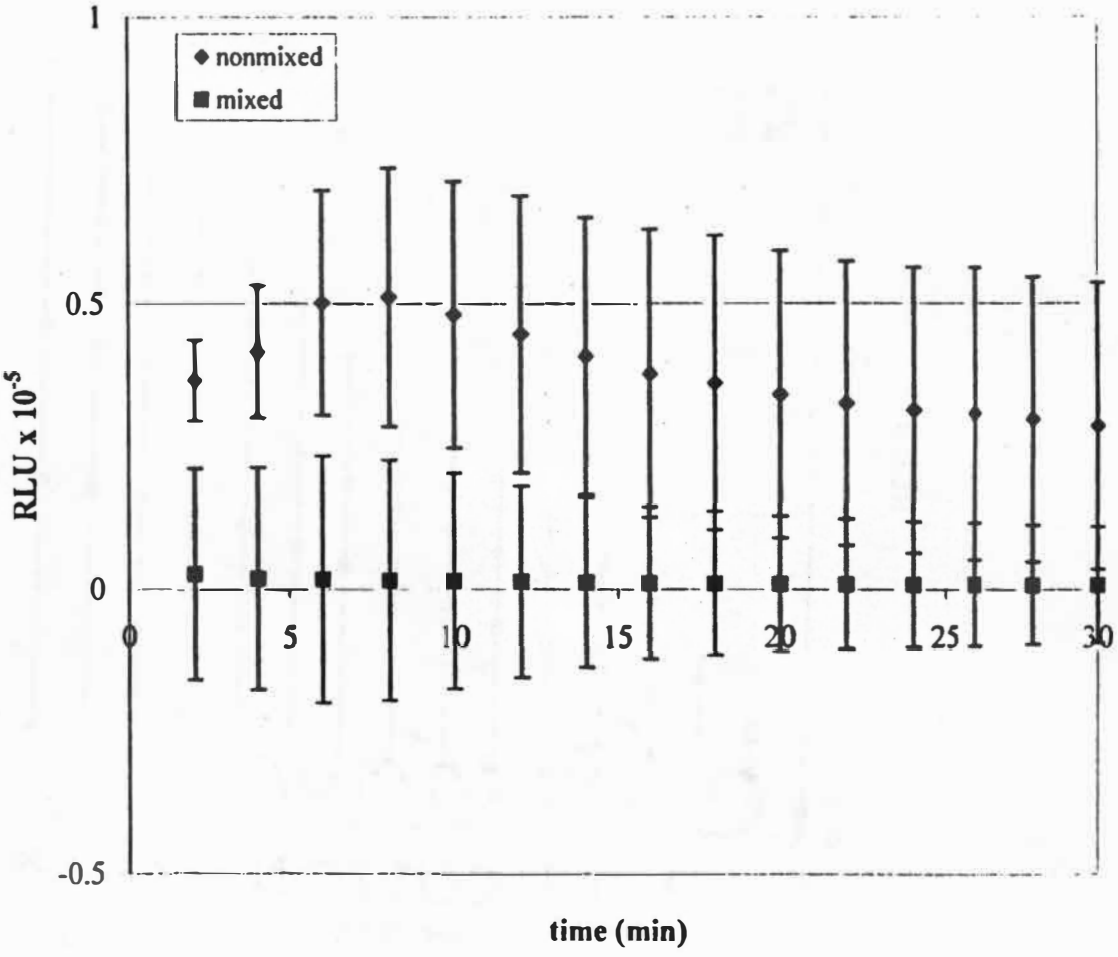

Figure 4.5: Bioluminescence of lyophilized PM6 cells activated at 15°C. Mixed cells were placed on the vortex mixer for two seconds prior to measurement.

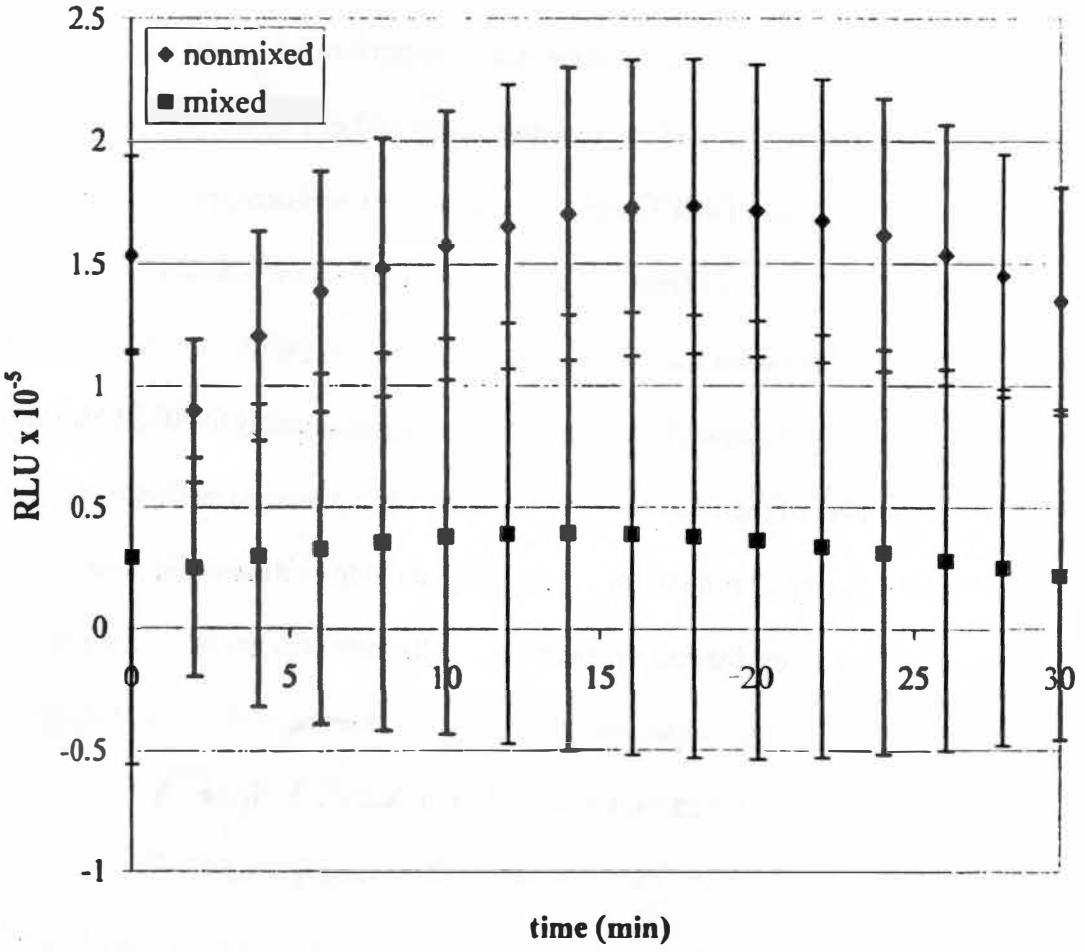

Figure 4.6: Bioluminescence of lyophilized PM6 cells activated at 27°C (room temperature). Mixed cells were placed on the vortex mixer for two seconds prior to measurement.

lyophilized PM6 cells activated at room temperature. The error bars indicate the standard deviations of six replicates that were performed on one day.

Figure 4.3 displays the bioluminescence of mixed and non-mixed PM6 cells that were stored at 4°C and activated at room temperature. The results show that between 0 minutes (immediately after the cells were activated) and 30 minutes the luminescence of non-mixed PM6 cells steadily increases between 0 minutes and 30 minutes from $1x10^5$ RLU to approximately $6x10^5$ RLU. Although the light level of the PM6 cells does not approach a plateau, assays were conducted after 20 minutes activation so that the toxicity assay is performed within 30 minutes for convenience. The cells that were mixed by the vortex mixer two seconds immediately prior to luminescence measurement display a steady increase in luminescence from 0 minutes to 20 minutes, followed by the leveling out of the light level at $4.0x10^5$ RLU (see Figure 4.3). The standard deviations of the light level of both the cells that were not mixed and those that were mixed are approximately the same. Although the mixed cells display a lower luminescence than the non-mixed cells, there is only a slight difference. The mixed cells also follow data reported by Lajoie et al. (2002) in which luminescence plateaus after 20 minutes. The standard deviations of mixed cells and non-mixed cells are also comparable; therefore mixed PM6 cells would be a plausible option to perform toxicity assays. While mixing seemed to have a

beneficial effect in this particular experiment, other assay results to follow do not indicate mixing led to a more stable light level. Therefore, in the interest of finding a uniform assay procedure for both strains, mixing was not used with the PM6 toxicity tests discussed below (section 4.3).

Figure 4.4 displays the bioluminescence of mixed and non-mixed Shk1 cells that were stored at 4°C and activated at room temperature. The results show that between 0 minutes (immediately after cells were activated) and 30 minutes the luminescence of Shk1 cells steadily increases from $1x10^5$ RLU to $1x10^6$ RLU. Although the luminescence of Shk1 cells does not level off, but increases steadily, assays were still performed between 20 minutes and 27 minutes after activation according to the protocol published by Lajoie et al. (2002). The cells that were mixed by the vortex mixer two seconds immediately prior to luminescence measurement generally display the same characteristics as those that were not mixed, a steady increase in luminescence from 0 minutes to 30 minutes, and the standard deviations of both the mixed cells and the non-mixed cells were similar. There is a small difference in the luminescence of mixed cells and non-mixed cells, with the non-mixed cells displaying lower light levels. Mixed Shk1 cells would therefore be an option to perform toxicity assays; however since both the mixed cells and the non-mixed cells steadily increase in luminescence from 0 minutes to 30 minutes and the standard deviations of both are approximately the same, mixed cells were not used for toxicity assays.

The data reported here does not appear to agree with that reported in the study performed by Lajoie et al. (2002). Lajoie et al. state that the "bioluminescence increased rapidly (minutes) after inoculation" and "continued to increase for the first 20 min, and then remained at a fairly steady value for up to 2 h before beginning to increase again". The data reported here indicates that the bioluminescence increases steadily from 0 minutes to 30 minutes and does not reach the plateau indicated by Lajoie et al. at 20 minutes. However, Lajoie et al. do not actually show the light levels and only describe this phenomenon so it is not possible to judge to what extent a plateau was reached in their work. However, if a pronounced plateau was present as was implied in the work of Lajoie et al., this may be because the Shk1 cells used in the study performed by Lajoie et al. were stored at 4°C for longer than the Shk1 cells used in this study. The Shk1 cells used in the study performed by Lajoie et al. were stored at 4°C anywhere from 1 day to 40 days, and the Shk1 cells used in this time stability study were stored at 4°C overnight (approximately 10 hours). There may be other differences between the assays (e.g., a variation in room temperature that leads to an unexpected difference between the two studies), but other than these possible explanations, it is impossible to say definitively what is the cause of the difference between the two studies based solely on this data.

Figure 4.5 displays the bioluminescence of lyophilized mixed and non-mixed PM6 cells that were activated at 15°C. The results show that between 0

minutes (immediately after cells were activated) and 30 minutes the luminescence of lyophilized cells that were not mixed first increases between 0 minutes and 10 minutes from $3x10^4$ RLU and $6.5x10^4$ RLU. In contrast to the cells stored at 4°C (see Figure 4.3), the luminescence then decreases from 10 minutes to 30 minutes. Also, as shown in Figure 4.5, each measurement of the luminescence of lyophilized PM6 cells between 0 minutes and 30 minutes that were activated at 15°C is at least an order of magnitude smaller than each luminescence measurement of the cells stored at 4°C. Although there is a significant difference in the magnitude of the light level emitted by the cells, light levels on the order of 10^4 are probably feasible for a successful assay (although more light is always preferred); the instrument background (no light) reading is usually around 10^2. Comparing the PM6 4°C and lyophilized cells, the COV for lyophilized PM6 cells activated at 15°C at 20 minutes is approximately 0.74 while that for those stored at 4°C is approximately 0.31. While this illustrates that the lyophilization-based assay leads to higher errors about the mean that the cold-storage based method, the difference in COV is no greater than that previously judged as insignificant in the studies of temperature control above. While the use of lyophilized cells may prove in the future to be a more effective method of storing PM6 cells, this was not pursued further in this study.

The cells that were mixed two seconds immediately prior to luminescence measurement display a luminescence of $1.0x10^2$ RLU. The luminescence of

lyophilized cells is expected to be lower than the cells that were not lyophilized. This is likely because of the "death" of some of the bacterial cells during the lyophilization process making the cell density lower, thus making bioluminescence lower. In the case of the mixed and lyophilized cells, the light level was even lower than for the PM6 cells stored by refrigeration only. This is interesting, because it was originally thought that mixing would cause the light level to increase due to the increase in oxygen mass transfer to the liquid phase. This was observed in each case where cells were mixed; the light level decreased significantly when compared to the case in which no mixing occurred. It is not known why this occurred. The standard deviations of the lyophilized PM6 cells that were mixed are lower than the standard deviations of the cells that were not mixed, however the bioluminescence of the cells that were mixed is so small it is hard to detect a change in light level over time. This difficulty arises at light levels under 200 RLU because the EC_{50} value is 50% of the luminescence at 0 minutes, and luminescence under 100 RLU is close to the same order as that produced by the dark current of the instrument (around 10^2). The low light level was observed from 0 minutes to 30 minutes and still did not increase after 6 hours (data not shown). These reasons (a lower luminescence of PM6 cells that were lyophilized and activated at 15°C compared to PM6 cells that were stored at 4°C, a steady decrease in luminescence from 10 to 30 minutes, and the lower magnitude of luminescence) would probably make PM6 cells lyophilized in the

manner reported here and activated at 15°C a poor choice for a control because the cells that were not mixed have a large standard deviation and the basal light level of the cells that were mixed was so low that BR cannot be accurately detected.

Figure 4.6 displays the bioluminescence of lyophilized mixed and non-mixed PM6 cells that were activated at room temperature. The results show that between 0 minutes (immediately after cells were activated) and 30 minutes the luminescence of lyophilized PM6 cells first increases between 0 minutes and 15 minutes from $1x10^5$ RLU to $2x10^5$ RLU similar to the case at 15°C then decreases between 15 minutes and 30 minutes. Although the basal light level varies significantly, it can be compared with Shk1 cells activated at room temperature where the basal light level also varies and does not plateau. The COV for PM6 cells stored at 4°C and the COV for lyophilized PM6 cells activated at room temperature are comparable. For example, the COV for PM6 cells stored at 4°C at 20 minutes of activation time is 0.31, and the COV for lyophilized PM6 cells activated at room temperature at 20 minutes is 0.35. Therefore, since there is a correction factor that partially accounts for the varying basal light level and the COV for PM6 cells stored at 4°C and lyophilized PM6 cells activated at room temperature, the results are promising and toxicity assays using lyophilized PM6 cells activated at room temperature should be explored. However, further work with the lyophilized, unmixed cells was not performed in this study. The

lyophilized PM6 cells that were mixed using a vortex mixer for two seconds immediately prior to luminescence measurement exhibited less variation in the mean light level during the activation period (see Figure 4.6). However, the mixed cells have a larger standard deviation than the non-mixed cells do and the error bars for the mixed cells bracket an RLU value of 0. Therefore, as was the case for lyophilized cells activated at 15°C, mixing is not recommended for lyophilized cells activated at room temperature.

4.3 Toxicity Assays

4.3.1 Toxicity assays for lyophilized cells

Although the light level of the non-mixed lyophilized cells is at least an order of magnitude smaller than the cells stored at 4°C anywhere within the 30 minute time interval during which light stability was studied, the varying basal light level is comparable to the Shk1 cells stored at 4°C and activated at room temperature and can be accounted for in the calculation of the correction factor as easily as that of Shk1. Therefore toxicity assays for (non-mixed) lyophilized cells should be explored in further studies, but are not examined further in this study. Mixing leads to a very low light level in one case and high standard deviation in another and should not be used when using lyophilized cells.

4.3.2 Toxicity assays for PM6 and Shk1 cells

Toxicity assays were performed using PM6 and Shk1 cells tested against toxicants Zn^{2+}, Cu^{2+}, and Pb^{2+}. Bay et al. (1989) state that a power curve relationship between concentration and response is expected when life forms are exposed to toxic chemicals, where response is measured as a ratio of activity lost to activity remaining. A log-log transform is therefore predicted to be a linear function, making linear regression useful for characterizing toxic effects as described in section 3.6.1. The exponential relationship between concentration and response is shown in Figure 4.7 for PM6 exposed to Zn^{2+}. The log-log transformation of Figure 4.7 is shown in Figure 4.8 showing the transformation to give a roughly linear relationship, as predicted. The EC_{50} values of the three heavy metals were calculated using equations 3.4-3.6 and the data is tabulated in Table 4.9. The results of the bioassays of heavy metals were highly reproducible between replicates. This reproducibility is shown in the small standard deviations of the calculated EC_{50} values. At very low concentrations, it was observed that the exposure of PM6 and Shk1 to two of the metal ions (Cu^{2+} and Pb^{2+}) led to BR greater than 1.0 (bioluminescence increased leading to a negative Γ value). An example of this is shown in Table 4.10 for PM6 cells exposed to Cu^{2+}. As shown, a concentration of 4.25 ppm Cu^{2+} leads to a bioluminescence increase above the control value instead of a decrease.

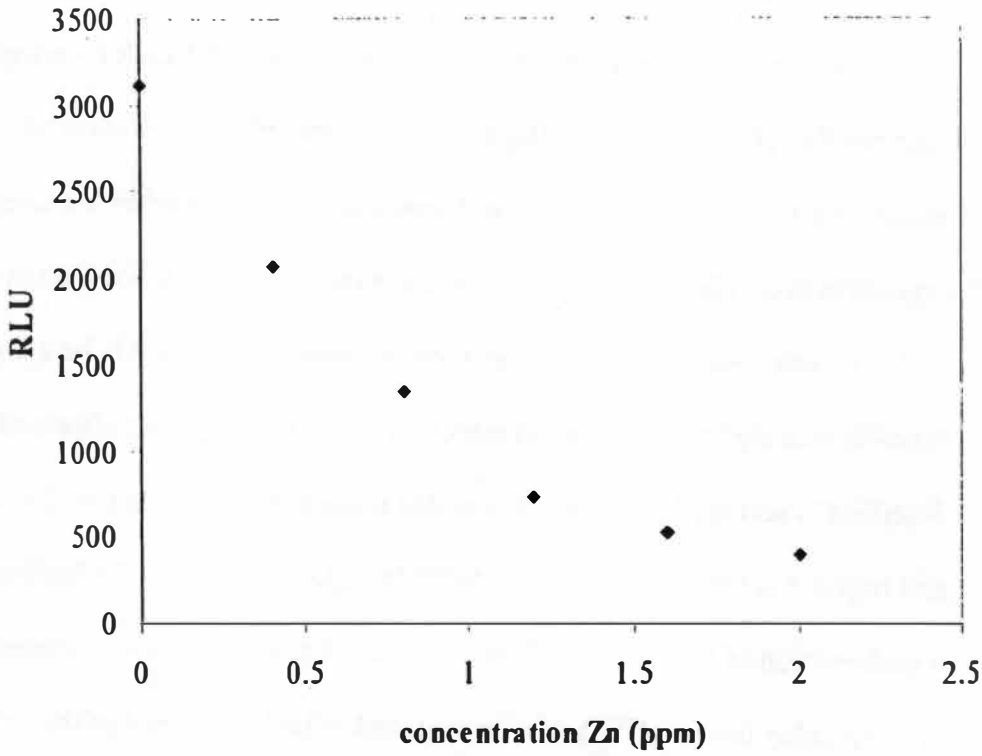

Figure 4.7: Relative light units (RLU) vs. concentration Zn (ppm) for PM6 exposed to Zn^{2+} showing a power curve relationship between concentration and response.

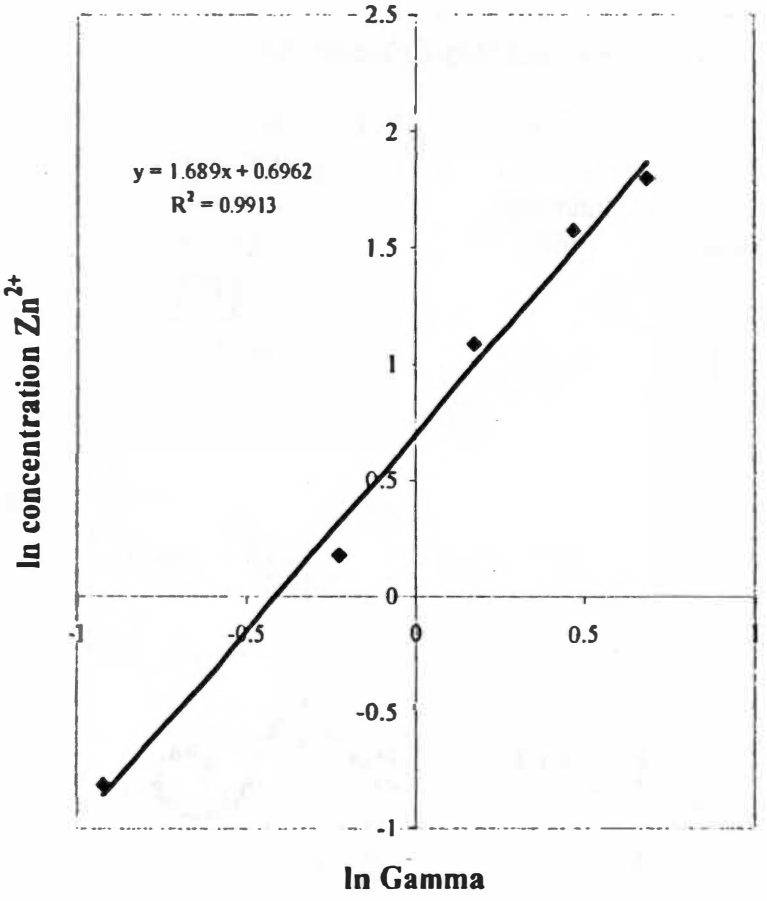

Figure 4.8: A ln-ln transformation of power curve shown in Figure 4.7 showing the transformation to be a linear function, as predicted. The EC_{50} value calculated by this data is 0.66 ppm and the 95% confidence interval is 0.23 ppm – 1.60 ppm.

Table 4.9. Comparison of Shk1 and PM6 EC_{50} and Microtox® EC_{50} values.

Heavy Metal Ion	Shk1 EC_{50} (95% conf. interval) (ppm)	PM6 EC_{50} (95% conf. interval) (ppm)	SOUR[1] EC_{50} (ppm)	Microtox® EC_{50} (95% conf. interval)[2] (ppm)	Microtox® (5 min. lit. data[3]) (ppm)
Zn^{2+}	1.10 (1.05-1.15)•	1.34 (0.45-2.23)	0.0-1.0	28.01 (26.75-29.26)	13.8-55.5
	(0.37-1.85)·	(0.73-2.28)			
COV	0.121	1.95			
Cu^{2+}	2.65 (2.48-2.82)	3.37 (1.71-5.03)	0.0-1.0	4.07 (2.47-5.68)	0.72-2.46
	(0.94-4.60)	(1.61-5.40)			
COV	0.186	1.44			
Pb^{2+}	4.96 (4.53-5.40)	4.67 (2.94-6.40)	10.0-15.0	1.24 (0.18-2.29)	2.56
	(0.93-9.50)	(0.73-8.73)			
COV	0.209	0.182			

Where:

• = Confidence interval of the average of EC_{50} values.

· = Average of the confidence intervals calculated for each EC_{50} value.

1: Estimated from Madoni et al., 1999.
2: This study.
3: Kaiser and Devillers, 1994.

Table 4.10: Calculated BR and Γ values for PM6 exposed to low [Cu^{2+}] leading to negative Γ values.

concentration of Cu^{2+} (ppm)	ln [Cu^{2+}]	RLU (initial)	RLU (final)	BR	Γ	ln Γ
0		9752	14894			
2.13	0.755	8756	16555	1.89	-0.192	UNDEF.
4.25	1.45	8724	16326	1.87	-0.184	UNDEF.
6.14	1.82	9274	12456	1.34	0.137	-1.99
8.03	2.08	8967	5269	0.588	1.60	0.469
10.4	2.34	8086	1605	0.198	6.69	1.90

Where:

UNDEF = UNDEFINED

4.3.3 Microtox® toxicity assays

Microtox toxicity assays were performed as described in section 3.6.3. The EC_{50} values calculated from this assay are listed in Table 4.9. Also listed in Table 4.9 are the EC_{50} values from the published inhibition to activated sludge specific oxygen uptake rate (SOUR) data (Madoni et al., 1999), and published EC_{50} values for the Microtox® assay from the literature (Kaiser and Devillers, 1994). The Microtox® assay uses a 5 minute contact time with the toxicant, while for the PM6 and Shk1 assays, a 7 minute contact time was established by Lajoie et al. (2002). The 7 minute contact time after adding the toxicant to the Shk1 and PM6 assays was not varied in this study, because for the purpose of detecting toxicants in wastewater in time to serve a preventative function, a rapid and reproducible response is desirable.

Compared to the specific oxygen uptake rate (SOUR) data estimated from Madoni et al. (1999), the experimental results (Table 4.9) show that of the three heavy metals tested, two (Zn^{2+} and Cu^{2+}) showed a PM6 and Shk1 EC_{50} larger than the SOUR EC_{50} data, meaning that Zn^{2+} and Cu^{2+} are less sensitive to PM6 and Shk1 than they are to inhibition to respiration, and one (Pb^{2+}) showed PM6 and Shk1 EC_{50} values smaller than the SOUR EC_{50} values, meaning that Pb^{2+} is more sensitive to Microtox® than it is to PM6 and Shk1. The EC_{50} values for Zn^{2+} and Cu^{2+} calculated from the Microtox® assay and the Microtox® literature values are both larger than the SOUR EC_{50} data, meaning that Zn^{2+} and Cu^{2+} are

less sensitive to Microtox® than they are to inhibition to respiration also. However, there is an order of magnitude difference between the PM6 and Shk1 EC_{50} values calculated for Zn^{2+} and those from Microtox®. For example, the SOUR EC_{50} values for Zn^{2+} estimated from Madoni et al. (1999) are between 0.0 and 1.0 ppm, the PM6 and Shk1 EC_{50} values calculated are 1.10 ppm and 1.34 ppm respectively. As shown in Table 4.9, the calculated Microtox® EC_{50} value is 28.01 ppm and the literature value for Microtox® is between 13.8 and 55.5 ppm. Therefore, even though neither Shk1 and PM6 nor Microtox® are as sensitive to SOUR data for Zn^{2+}, Shk1 and PM6 are more sensitive than Microtox®. The SOUR EC_{50} value data for Cu^{2+} is between 0.0 and 1.0 ppm and PM6, Shk1, and the EC_{50} value calculated from the Microtox® assay are larger than that interval. The EC_{50} value for Cu^{2+} for Shk1 is 2.65 ppm, for PM6 is 3.37 ppm, and for the Microtox® assays is 4.07 ppm, meaning PM6, Shk1 and Microtox® are all less sensitive to Cu^{2+} than inhibition to respiration is. However, unlike Zn^{2+} and Cu^{2+}, PM6, Shk1, and Microtox® are all more sensitive to Pb^{2+} than inhibition to respiration. As shown in Table 4.9, the SOUR EC_{50} values for Pb^{2+} estimated from Madoni et al. (1999) is between 10.0 and 15.0 ppm, Shk1 is 4.96 ppm, PM6 is 4.67 ppm, and Microtox® is 1.24 ppm. The EC_{50} values for Shk1, PM6, and Microtox® for Pb^{2+} being less than the EC_{50} value for SOUR data means that Shk1, PM6 and Microtox® are more sensitive to Pb^{2+} than inhibition to respiration.

Therefore, for the three metals examined in this research (Zn^{2+}, Cu^{2+}, and Pb^{2+}), the assays performed with PM6 and Shk1 are more appropriate for predicting respiration inhibition EC_{50} values than the Microtox® assay; due to the sensitivity of the PM6 and Shk1 assays to the toxicants chosen, the EC_{50} values calculated for these strains are closer to values for inhibition to respiration than the Microtox® assays.

Chapter 5 Conclusion

The objective of this research was to develop an accurate toxicity assay using genetically engineered bioluminescent bacteria PM6 and Shk1. Different assay protocols were explored and EC_{50} values of strains PM6 and Shk1 for three chemical compounds ($ZnCl_2$, $CuCl_2$, $PbCl_2$) were compared to EC_{50} values from the Microtox® assay, published inhibition to activated sludge specific oxygen uptake rate (SOUR) data (Madoni et al., 1999), and published EC_{50} values from Microtox® for the same compounds (Kaiser and Devillers, 1994).

The toxicity assay protocol used in this study was based on one developed by Lajoie et al. (2002) and was adapted to this study. Temperature control during activation and exposure to the toxicant was studied to determine if the accuracy of the original protocol of Lajoie et al. could be improved. However, it was determined that temperature control did not lead to an improvement in the accuracy of the assay. Experiments to determine the appropriate time interval for cell activation indicated that the activation time originally selected by Lajoie et al. (20 minute) was appropriate. The time stability experiments also showed that mixing the cells for 2 seconds immediately prior to measurement of luminescence does not significantly change the results in comparison to non-mixed cells for the PM6 and Shk1 cells stored at 4°C, with the exception that the light level was slightly more constant for PM6 cells after mixing. The lyophilized cells that were

activated at 15°C and mixed display a luminescence close to the dark current of the luminometer, and the lyophilized cells that were activated at room temperature and mixed display a higher standard deviation than the lyophilized cells that were not mixed. Therefore the final protocol specified in this work used no temperature control or mixing, and a 20 minute activation period. The protocol proved adequate in determining EC_{50} values comparable to those determined from inhibition to respiration assays. Toxicity assays were also performed using the Microtox® analyzer and EC_{50} values calculated. When compared with Microtox®, the Shk1 and PM6 assays display EC_{50} values closer to respiration inhibition EC_{50} values.

Future work should include lyophilizing Shk1 cells and performing time stability studies and toxicity assays, and performing toxicity assays using lyophilized PM6 cells. There are various procedures for lyophilization that could be tried to potentially stabilize and increase the light level of reconstituted cells. Various lyoprotectants have been studied in the research literature; in the original study on which the Microtox® assay is based, skim milk was used (Bulich, 1979). If an assay based on lyophilized PM6 or Shk1 is desired in future work, the effects of lyoprotectant addition should be studied.

LIST OF REFERENCES

REFERENCES

Baldwin T.O., Christopher J. A., Raushel F.M., Sinclair J.F., Ziegler M.M., Fisher A.J., Rayment I. (1995). "Structure of bacterial luciferase." *Current Opinion in Structural Biology* 5: 798-809.

Bay S., Greenstein D., Raco V., Anderson J.W. (1989). "Responses of marine organisms to wastewater: comparisons between species and effluent types." Contribution No. C-320. Southern California Coastal Water Research Project, Long Beach, CA. ARL #196-89.

Benoit D.A., Phipps G.L., Ankley G.T. (1993). "A simple inexpensive miniflow system for the automated renewal of overlying water in toxicity tests with contaminated sediments." *Water Research* 27: 1403-1412.

Bulich, A.A., (1979) "Use of luminescent bacteria for determining toxicity in aquatic environments," *Aquatic Toxicoloy.* ASTM Special Technical Publication 667 (STP 667), Symposium on Aquatic Toxicology, L.L. Marking and R.A. Kimerle, Eds., American Society for Testing and Materials, 1979, pp. 98-106. Out of print.

Burlage R.S., Bemis L.A., Layton A.C., Sayler G.S., Larimer F. (1990). "Comparative Genetic Organization of incompatibility Group P Degradative Plasmids." *Journal of Bacteriology* 172(12): 6818-6825.

Choi S.H., Gu M. B. (2002). "A portable toxicity biosensor using freeze-dried recombinant bioluminescent bacteria." *Biosensors and Bioelectronics* 17: 433-440.

Clesceri L.S., Greenberg A.E., Eaton A.D. (1998). *Standard Methods for the examination of water and wasterwater.* 20th edition. American Public Health Association, American Water Works Association, Water Environment Federation, Washington D.C.

Czyz A., W.B., Webgrzyn G. (2000). "*Vibrio harveyi* bioluminescence plays a role in stimulation of DNA repair." *Microbiology Reading* 146: 283-288.

Draper N.R., Smith H. (1981). *Applied Regression Analysis.* 2nd ed. John Wiley & Sons, Inc., NY.

Frymier P., Ren S., Lajoie C., Kelly C. (2004). Sensor Technology for Water Quality Monitoring: Bioluminescent Microorganisms. Water Environment Research Foundation Project 01-WSM-2A, in publication.

Girotti S., Bolelli L., Roda A., Gentilomi G., Musiani M. (2002). "Improved detection of toxic chemicals using bioluminescent bacteria." *Analytica Chimica Acta* 471(1): 113-120.

Grau P., Da-Rin B.P. (1997). "Management of toxicity effects in a large wastewater treatment plant." *Water Science and Technology* 36: 1-8.

Gu M.B., Dhurjati P. S. (1996). "A miniature bioreactor for sensing toxicity using recominbant bioluminescent *Escherichia coli* cells." *Biotechnology Progress* 12: 393-397.

Gu M.B., Mitchell R. J., Kim J.H. (2000). "Continuous monitoring of protein damaging toxicity using a recombinant bioluminescent *Escherichia coli*." *Journal of American Chemical Society*: 185-196.

Gu M.B., Gil G. C. (2001). "A multi-channel continuous toxicity monitoring system using recombinant bioluminescent bacteria for classification of toxicity." *Biosensors and Bioelectronics* 16: 661-666.

Hastings J.W., Nealson K.H. (1977). "Bacterial bioluminescence." *Annual Review of Microbiology* 31: 549-595.

Hastings, J. W. (1983). "Biological diversity, chemical mechanisms, and the evolutionary origins of bioluminescent systems." *Journal of Molecular Evolution* 19: 309-321.

Hastings, J. W. (1996). "Chemistries and colors of bioluminescent reactions: a review." *Gene* 173: 5-11.

Horsburgh A.M., Mardlin D. P., Turner N.L., Henkler R., Strachan N., Glover L.A., Paton G.I., Killham K. (2002). "On-line microbial biosensing and fingerprinting of water pollutants." *Biosensors and Bioelectronics* 17(6-7): 495-501.

Ince N.H., Erdoğdu G. (1998). "Toxicity screening, assessment, and reduction in an industrial wastewater treatment plant." *Water Environment Research* 70(6): 1170-1177.

Jonsson K., Grunditz C., Dalhammar J. J. La Cour. (2000). "Occurrence of nitrification inhibition in Swedish municipal wastewaters." *Water Research* 34: 2455-2462.

Kaiser K.L.E., Devillers J. (1994). "Ecotoxicity of chemicals to *Photobacterium phosphoreum*." *Handbooks of Ecotoxicology Data.* Volume 2. Gordon and Breach Science, Langhorne, Pennsylvania.

Kelly C.J., Lajoie C.A., Layton A.C., Sayler G.S. (1999). "Bioluminescent reporter bacterium for toxicity monitoring in biological wastewater treatment systems." *Water Environment Research* 71: 31-35.

Kustin K., Mcleod G.C. (1977). "Interactions between metal ions and living organisms in sea water." *Topics in Current Chemistry* 69: 1-37.

Lajoie C.A., Lin S.-C., Nguyen H., Kelly C.J. (2002). "A toxicity testing protocol using bioluminescent reporter bacterium from activated sludge." *Journal of Microbiological Methods* 50(3): 273-282.

Love N.G., Bott C.B. (2000). A review and needs survey of upset early warning devices. Project Report 99-WWF-2 Water Environment Research Foundation, Alexandria, Virginia.

Madoni P., Davoli D., Guglielmi L. (1999). "Response of SOUR and AUR to heavy metal contamination in activated sludge." *Water Research* 33(10): 2459-2464.

Meighen, E. A. (1994). "Genetics of bacterial bioluminescence." *Annual Review of Genetics* 28: 117-139.

Metcalf L., Eddy H.P. (1916). *American Sewerage Practice, Vol. III – Disposal of Sewage*. McGraw-Hill Book Company, Inc., New York.

Nivens D.E., McKnight T.E., Moser S.A., Osbourn S.J., Simpson M.L., Sayler G.S. (2004). "Bioluminescent bioreporter integrated circuits: potentially small, rugged and inexpensive whole-cell biosensors for remote environmental monitoring." *Journal of Applied Microbiology* 96: 33-46.

Paxéus N., Schröder H.F. (1996). "Screening for non-regulated organic compounds in municipal wastewater in Goteborg, Sweden." *Water Science and Technology* 33: 9-15.

Redmond M.S., Scott K.J., McKenna K.M., Robson D. (1989). Evaluation of the relative acute toxicity of nearshore sediments in Narragansett Bay. Report prepared by Science Applications International Corporation for U.S. EPA, Narragansett Bay Program Office.

Ren S. (2001) "Development of a continuous bioluminescent bacteria-based system for POTW influent wastewater toxicity monitoring." Dissertation for PhD at the University of Tennessee, Knoxville.

Reteuna C., Vassuer P., Cadridenc R., Lepailleur H., (1986). "Comparison of respiration and luminescent tests in bacterial toxicity assessment." *Toxicity Assessment* 1: 159-168.

Shields M.S., Hooper S.W., Sayler G.S. (1985). "Plasmid-mediated mineralization of 4-chlorobiphenyl." *Journal of Bacteriology* 163: 882-889.

Slabbert J.L. (1988). "Microbial toxicity assays used for water quality evaluation in South Africa." *Toxicity Assessment* 3: 101-105.

Trapido M., Munter R., Kallas J. (1994). "Oil shale ash dump waste water as polycyclic aromatic hydrocarbons and phenols pollution source." Proceedings of Estonian Conference on Ecology.

Vanrolleghem P. A., Kong Z., Rombouts G., Verstraete W. (1994). "An on-line respirographic biosensor for the characterization of load and toxicity of wastewaters." *Journal of Chemical Technology and Biotechnology* 59: 321-333.

Walpole R.E., Myers R.H., Myers S.L., Ye K. (1998). *Probability & Statistics for Engineers & Scientists.* Prentice Hall, Inc., Upper Saddle River, NJ.

Williamson K.J., Johnson D.G. (1981). "A bacterial bioassay for assessment of wastewater toxicity." *Water Environment Research* 15: 383-390.

Wong K.-Y., Zhang M.-Q., Li X.-M., Lo W. (1997). "A luminescence-based scanning respirometer for heavy metal toxicity monitoring." *Biosensors and Bioelectronics* 12(2): 125-133.

VITA

Stacey M. Allsteadt-Barnlund was born in Watseka, Illinois on September 4, 1980. She graduated from Bradley Bourbonnais Community High School in Bradley, Illinois in May of 1998. The following august she entered Lincoln Memorial University and in May, 2001 received a degree of Bachelor of Science in Chemistry. In August of 2001, she entered The University of Tennessee, Knoxville, and in August, 2004 received a Master of Science in Chemical Engineering.