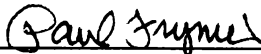


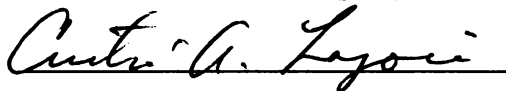
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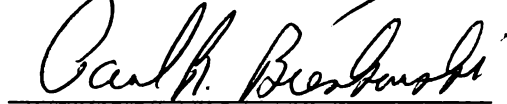
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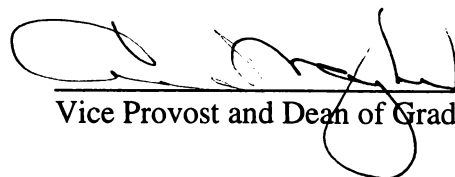


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Development of a Continuous Bioluminescent Bacteria-Based System for POTW Influent Wastewater Toxicity Monitoring

A Dissertation
Presented for the
Doctor of Philosophy
Degree

The University of Tennessee, Knoxville

Shijin Ren
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ABSTRACT

Influent wastewater toxicity causes upsets in the operations of biological wastewater treatment systems. Toxic compounds cause the loss of higher life forms in activated sludge, the formation of pin flocs that settle poorly in downstream clarifiers and increase the addition of costly polymers. Ultimately, these effects lead to the degradation of effluent quality. In the most severe circumstances, influent toxicity can lead to a plant shut-down and the discharge of untreated wastewater into the environment.

These effects can be avoided if influent wastewater is screened for toxicity and protective actions are taken to prevent damage to the activated sludge. Possible methods for the assessment of influent wastewater toxicity are chemical analysis, microscopic examination, respirometric methods, and bioluminescence-based microbiological methods. Chemical analysis is slow and requires *a priori* knowledge of wastewater components. The complexity of domestic wastewater constituents and the limited amount of time available for protective actions to be taken make chemical analysis unsuitable for the purpose of influent toxicity screening. Microscopic examination of activated sludge for population damage is also slow and cannot be used as a preventative screening technique since the damage has already occurred. Respirometry is probably the most widely used method for wastewater toxicity screening. This technique involves measuring the respiration rate of activated sludge mixed with a wastewater sample and comparing the respiration rate with a control; a decrease in respiration rate indicates toxicity. However, these methods are time-consuming and labor-intensive. Bioluminescent bacteria can be used for wastewater toxicity screening and

bioluminescence tests are quick and easy to perform. The best known bioluminescence-based test is the Microtox[®] bioassay. This assay involves rehydrating freeze-dried marine bacteria, *Photobacterium phosphoreum*, in reconstitution medium and measuring the decrease in bioluminescence when the reconstituted marine bacteria are exposed to toxicants as compared to a control. Due to its being a marine bacterial strain, *Photobacterium phosphoreum* has been shown to be excessively sensitive to many toxicants typically found in influent wastewater from industrial sources. As a consequence, the response of *Photobacterium phosphoreum* to toxicants is different from the response of the activated sludge microbial community and false positive signals are given by *Photobacterium phosphoreum* for many compounds.

An ideal method for influent wastewater toxicity should be quick, preventative, simple to perform, inexpensive, on-line, and relevant to the activated sludge organisms. In an effort to develop such a method, a strain of bioluminescent bacteria was created in the Center for Environmental Biotechnology at the University of Tennessee and designated Shk1. Shk1 was created by mating *E. coli* cells containing the plasmid pUTK2 which confers bioluminescence with activated sludge bacteria to produce a hybrid cell that grows in full strength wastewater and emits light.

In this research, the dependence of Shk1 specific bioluminescence (the ratio of bioluminescence to optical density) on dilution rate, pH, and temperature in a CSTR was quantified. The combination of dilution rate, pH, and temperature that gave the highest specific bioluminescence was identified and was chosen as the standard condition for the production of Shk1 in a toxicity monitoring application.

A continuous influent wastewater toxicity monitoring system was developed based on a CSTR. Toxicity tests were performed using a laboratory-scale system. Toxic compounds that were tested included individual heavy metals, binary mixtures of heavy metals, and organic chemicals that represented a wide range of chemical structures, such as simple and halogen substituted alkanes and aromatics, alcohols, ketones, amines, and phenol. Shk1 bioluminescence EC_{50} values, or 50% effective concentration values for the toxicants were obtained and compared to toxicant concentrations found in literature that are known to cause activated sludge upsets.

Activated sludge respiration inhibition EC_{50} values, or concentrations of toxicants that cause a 50% reduction in the respiration rate of activated sludge, are commonly used to describe damage to activated sludge caused by toxicants. Previous attempts to correlate the results of other test methods with EC_{50} values focused only on the coefficient of determination (r^2). However, for the purpose of wastewater toxicity screening, a good correlation not only needs to be statistically sound, but also needs to have some physical significance, i.e., it should be possible to predict damage to activated sludge directly from the correlation. Shk1 EC_{50} values were correlated with activated sludge EC_{50} values and a regression resulted in $EC_{50, \text{Shk1}} = 0.91 EC_{50, \text{activated sludge}}$ ($EC_{50, \text{Shk1}}$ here reflects the toxicant concentration before mixing with Shk1 cells). In other words, the possible damage to activated sludge due to influent toxicity can be predicted by exposing Shk1 to the influent and monitoring the bioluminescence: when Shk1 bioluminescence is repressed by 50%, activated sludge respiration can also be expected to be repressed by 50% if the influent is allowed to enter the plant.

A small field-scale monitoring system based on the laboratory system was constructed. This system was employed at the Kuwahee Wastewater Treatment Plant of the Knoxville Utilities Board (KUB) located in Knoxville, Tennessee. The field system continuously monitored the toxicity of the influent to the wastewater plant as indicated by Shk1 bioluminescence repression. Adjusted daily average bioluminescence repression (DABR_{adj}) obtained from the Shk1-based system was compared to plant performance data to determine the efficacy of our system as a prototype for a possible commercially produced unit. The signals generated by the Shk1-based system were found to be a meaningful piece of information. However, because the treatment efficiency could be affected by factors other than influent toxicity, such as influent BOD, temperature, etc., there was no easily observable correlation between the signals generated by the Shk1-based system and the treatment efficiency. Nonetheless, a rough alarm level could be set at 70% DABR_{adj} to warn possible decreases in treatment efficiency. Logistic regression was shown to be useful and promising for predicting the performance of the treatment plant based on relevant variables including DABR_{adj} .

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CHAPTER 1

INTRODUCTION AND BACKGROUND

Introduction

Activated sludge process has been used for treatment of both domestic and industrial wastewaters for more than 50 years, and is still one of the most widely used biological wastewater treatment systems in the world (Wong et al., 1997). However, toxicants are sometimes encountered in influent wastewater of publicly owned treatment works (POTW) and toxic loadings cause upsets in the operations of the activated sludge process and the performance of POTWs. Such upsets include formation of pin-flocs and reduction of solids separation efficiency, inhibition of waste organics removal, modification of sludge compacting properties (Evans et al., 1998, Koopman and Bitton, 1986, Neufeld, 1976, Schwartz-Mittelmann and Galil, 2000), and loss of higher life forms. These adverse effects ultimately lead to the degradation of the effluent quality. In the most severe circumstances, the activated sludge microorganisms may be completely inhibited and the treatment plant has to be shut down. This results in wastewaters being discharged to receiving water bodies without being properly treated.

Toxicities associated with influent wastewater and their inhibitory effects on treatment plants have been reported in the literature. For example, Jonsson et al. (2000) reported that 45-60% of the Swedish municipal wastewater treatment plants investigated received wastewater containing inhibitory substances. In Sao Paulo, Brazil, treatment efficiency of a biological wastewater treatment plant was significantly hindered for six

months after toxic shocks occurred (Grau and Da-Rin, 1997). In one case, because of the unavailability of a system to assess the toxic effect of wastewater on activated sludge and concerns about poisoning the activated sludge in local treatment plant, wastewater with a high concentration of phenolic compounds flowed directly to the Gulf of Finland, causing a daily discharge of 3 to 4 tons of phenolic compounds to the environment (Trapido et al., 1994).

Marine luminescent bacteria have been used in wastewater toxicity assessment. The advantages of bioluminescent bacteria-based assays over other assays such as respirometry-based assays is that bioluminescent bacteria-based assays have a quick response (usually on the order of minutes) and an easily measured signal. However, the sensitivity to toxicants of the luminescent organisms employed in existing toxicity assays is overly high compared to activated sludge microorganisms and thus the existing methods are not suitable for predicting the potential damage to activated sludge caused by toxicants in influent wastewater.

A new strain of luminescent bacteria was created at the Center for Environmental Biotechnology at the University of Tennessee, Knoxville and designated Shk1. The host strain of Shk1 was a *Pseudomonad* isolated from the activated sludge in an industrial wastewater treatment plant (Kelly et al., 1999). The genes that encode for the enzymes involved in light producing reactions are contained in the *lux* operon. The *lux* operon in Shk1 was isolated from *Photobacterium phosphoreum* (a marine luminous bacterial strain), and is under the control of a constitutive promoter. Shk1 does not require an inducer for light emission and thus emits light under normal conditions.

This research hypothesizes that Shk1, being bioluminescent bacterium whose host

strain was sludge bacteria, has a quick response to toxicants and the response is similar to that of the activated sludge microbial community.

The goals of the research were (1) to valid the use of Shk1 as a surrogate organism in wastewater toxicity testing, (2) to develop a Shk1-based system for the continuous monitoring of influent wastewater toxicity, and (3) to demonstrate the applicability of the Shk1-based system in a full-scale wastewater treatment plant.

This research is one of the pioneering efforts to apply bioluminescent bacteria-based toxicity assays to actual influent wastewater toxicity monitoring. The results of this research can serve as the basis for the future developments and perfection of Shk1-based systems that could eventually be commercialized.

Background

The following topics are discussed in this section: an overview of bioluminescence, the activated sludge wastewater treatment system, basic aspects of environmental toxicology, the requirements of an on-line wastewater toxicity monitoring system, methods for wastewater toxicity assessment, and the construction of Shk1.

Overview of bioluminescence

Bioluminescence refers to the light emitted by some living organisms. A wide range of fauna has been reported to emit bioluminescence, and includes bacteria, fungi, and higher life forms such as fireflies and fish. Nonetheless, it is bacteria that contain most of the bioluminescent species (Meighen, 1991). A large number of bioluminescent bacterial species are found in marine environments. The genera *Vibrio*, *Photobacterium*

and *Xenorhabdus* contain most luminescent bacteria (Meighen, 1991). The functional importance of light emission of higher organisms has been postulated to be useful in intraspecific communication and attracting the sexes, as a lure for food or as a warning signal (Harvey, 1952, Hastings and Nealson, 1977, Munk, 1999). For bacteria, the function of bioluminescence is less straightforward since the behavior of bacteria cannot be considered in the above-mentioned senses, and the role of bacterial luminescence is not clear (Czyz et al., 2000). However, Czyz et al. (2000) showed that bacterial luminescence stimulates DNA repair in *Vibrio harveyi*. Hastings and Nealson (1977) also imply that bioluminescence propagates the bacteria. Bacterial luminescence is not essential for the life of bacterial cells, and bacteria of the same species have both luminous and non-luminous forms (Hastings and Nealson, 1977).

The detailed reaction pathway and intermediates involved with light production have been described by Hastings and Nealson (1977). The pathway for light production branches off the electron transport chain that transports electrons to the final electron acceptor, as shown in Figure 1-1. Bioluminescence emission is associated with the oxidation of FMNH₂ and a long-chain aliphatic aldehyde by molecular oxygen. The oxidation reaction is catalyzed by bacterial luciferase which is the product of the *lux* gene cassette (Figure 1-2). FMNH₂ binds to luciferase to produce complex I, which reacts with molecular oxygen to produce complex II. Complex II decays, reducing luciferase without producing light. When complex II binds to a long-chain aldehyde, complex III is produced. Complex III is an excited intermediate, and when it decays, the aldehyde moiety is oxidized to a fatty acid and light is emitted at 490 nm. The overall stoichiometry is described by the following equation:

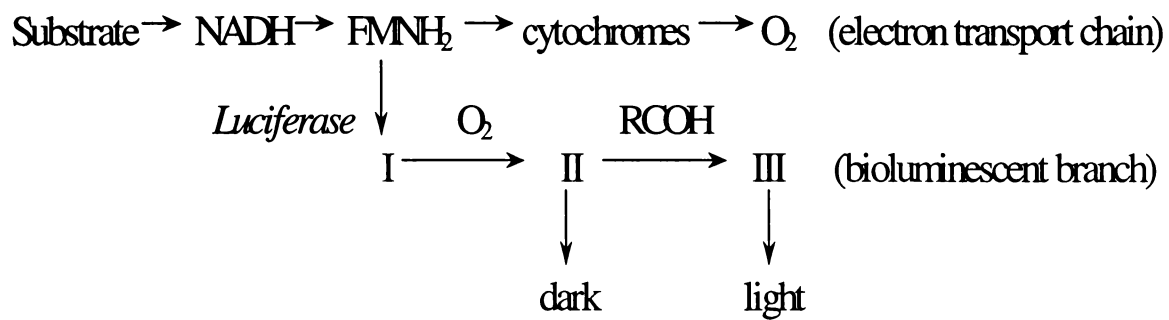


Figure 1-1. Bioluminescent system coupled to the electron transport chain. Adapted from Hastings and Nealson (1977).

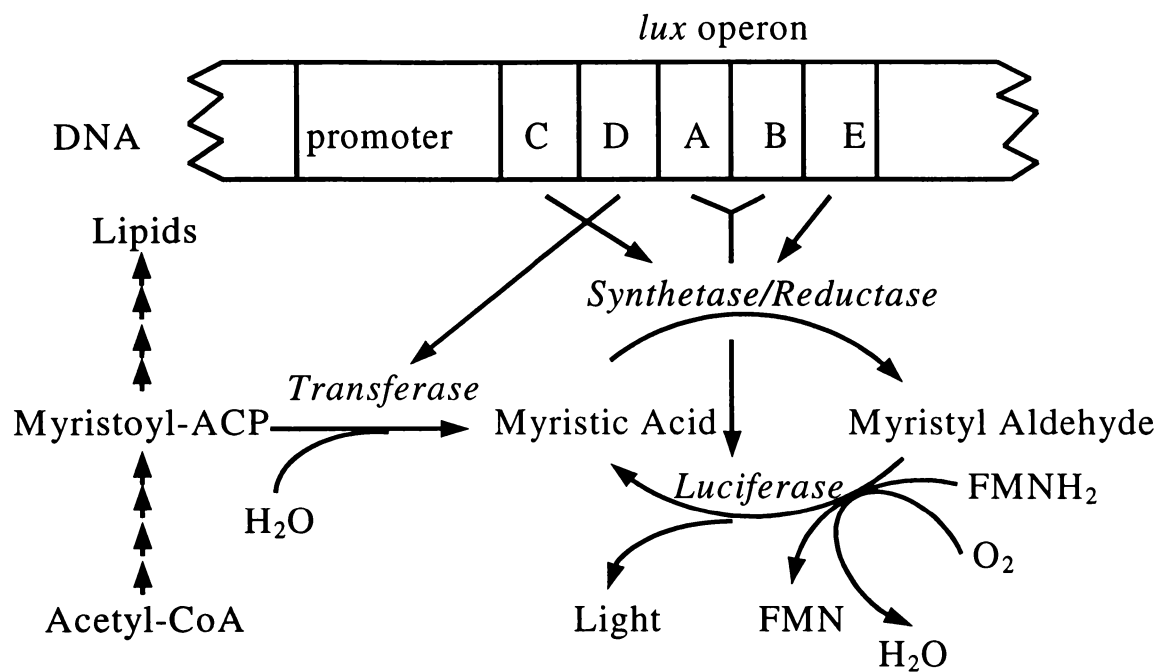
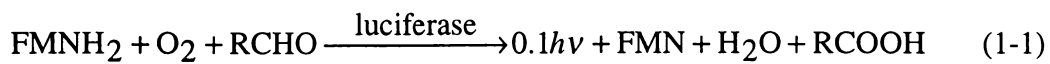


Figure 1-2. *lux* gene, associated gene products, and the reactions involved in bioluminescence. Adapted from Sayler et al. (1995).



The *lux* operon consists of five genes (*luxCDABE*) that encode for the enzymes that are involved in the light-emitting reactions (Figure 1-2). Reductase and synthetase, the gene products of *luxC* and *luxE*, respectively, catalyze the reaction to produce aldehyde from fatty acid. Transferase, the gene product of *luxD*, transfers biosynthesis of fatty acids in the cell membrane to the light-emitting reactions. The *luxAB* genes encode for bacterial luciferase which catalyzes the actual light producing reaction (Hastings and Nealson, 1977, Hastings et al., 1985, Meighen, 1991).

The primary substances involved in the light-producing reactions are luciferase, aldehyde, FMNH₂ and oxygen. The concentration of luciferase is dependent on induction, transcription and translation of the *lux* genes. Aldehyde is produced in cells by the reduction of fatty acids. FMNH₂ is a flavoprotein electron carrier that participates in the electron transport chain and is produced by biosynthesis. Oxygen is provided by mass transfer of the dissolved oxygen in the growth medium.

In addition to the naturally-occurring bioluminescent bacteria, it is possible to genetically modify non-luminous bacteria so that they are luminous (Schauer et al., 1988, Wolk et al., 1991, Eaton et al., 1993, Phillips-Jones, 1993, Mackey et al., 1994). Bioluminescence has been used in clinical assays such as immunoassays, nucleic acid assays and NAD(P)H assays (Stanley, 1981, Kricka, 1993, 1999), and in the monitoring of gene expression (Stewart and Williams, 1992). Engineering applications of bioluminescence have been studied (Sheintuch et al., 1992) but to a lesser extent. Although the idea of biological "lamp" based on bioluminescent bacteria was suggested by Dubois

(1901) at the beginning of the twentieth century, the primary engineering application for light-producing bacteria is chemical sensing. Bioluminescent bacteria can form the basis of highly selective and sensitive chemical detectors. For example, Ripp et al. (2000) reported detection of detrimental environmental contaminants in concentrations approaching 10 ppb using bioluminescent bacteria.

Activated sludge wastewater treatment systems

Wastewater treatment can be divided into four stages: primary, secondary, tertiary, and advanced (Zakrzewski, 1997); the activated sludge process is classified as secondary treatment. The activated sludge process has been utilized for treatment of both domestic and industrial wastewaters for more than 50 years and is still widely used in the world (Wong et al., 1997). For economic reasons, not every treatment plant includes all four stages. However, in the United States, primary and secondary treatments are required by law for municipal wastewater treatment in all communities (Zakrzewski, 1997).

Figure 1-3 shows a flow diagram of a biological wastewater treatment plant. Before entering the primary stage, wastewater is usually passed through a grit chamber where large solid such as stones are removed by sedimentation. In the primary treatment, wastewater is held for a few hours in the clarifier where more solid particles are removed by sedimentation. After leaving the primary treatment, wastewater enters the secondary treatment, the activated sludge process. The sludge is a mixture of various aerobic microorganisms and is aerated with air. The sludge is called activated sludge because the microorganisms are biologically active. Most pollutants in wastewater are biodegraded by these microorganisms and 85-99% of the biochemical oxygen demand (BOD) is

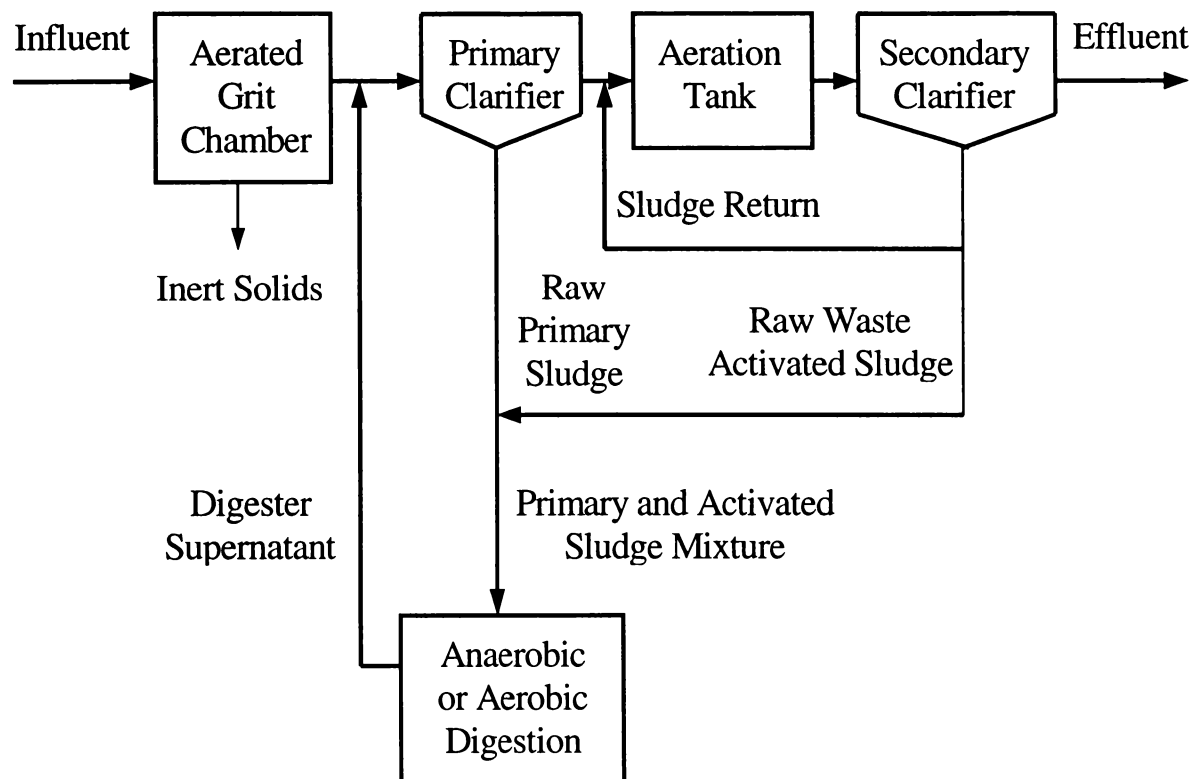


Figure 1-3. Flow diagram of a biological wastewater treatment plant.

removed by the activated sludge process. Further removal of unwanted materials in wastewater can be achieved in tertiary and advanced treatments.

Bacteria are the primary microbial constituents of activated sludge wastewater treatment systems. Protozoans and metazoans play an important role by grazing on planktonic bacteria and thus reducing the effluent suspended solids of a wastewater treatment plant. However, protozoans and metazoans occur to a lesser extent and most significantly in low-loaded activated sludge plants (Henze et al., 1995). Other microbial populations such as fungi, algae and rotifers, are found to a lesser extent in activated sludge. The major classes of bacteria in activated sludge can be divided into two groups based on function: heterotrophic bacteria and nitrifying bacteria. Heterotrophic bacteria carry out the degradation and mineralization of organic and inorganic materials in wastewaters. The major populations of nitrifying bacteria belong to two genera: *Nitrosomonas* and *Nitrobacter* (Benefield and Randall, 1980). *Nitrosomonas* bacteria convert ammonium-nitrogen to nitrite-nitrogen, and *Nitrobacter* bacteria convert nitrite-nitrogen to nitrate-nitrogen.

Basic aspects of environmental toxicology

(1) Dose, concentration, effect and response

Central to the study of environmental toxicology are the terms "concentration", "response", "concentration-response curve" and "EC₅₀". "Concentration" refers to the amount of a chemical in a unit volume solution and usually has the units of mg/L. "Concentration" can be distinguished from "dose" which refers to the amount of chemical per unit mass of an animal or human being that accepts the chemical. "Dose" has the units of mg/g, mg/kg, etc. The term "response" and "effect" are sometimes used as

synonyms, but a distinction is needed for a clearer and more precise description of changes resulting from a concentration of a chemical. When a population of individuals is exposed to a chemical at a certain concentration, both effect and response will take place simultaneously. Some individuals respond to the chemical with an effect of certain magnitude, while other individuals respond with an effect of greater or lesser magnitude. "Response" is the effect of a specified magnitude that a certain percentage of a population exhibits.

(2) Concentration-response curves

The concentration-response relationship is quantal in character and is the basis for ecotoxicological testing. Concentration-response curves are generated by plotting the responses of an organism against the concentrations of the test materials. Several other relationship curves can be plotted, e.g., concentration-effect, dose-response, dose-effect. In this research, only concentration-response curves are used. Concentration-response curves usually have a sigmoidal shape. A normally distributed sigmoidal curve approaches a response of 0% as the concentration decreases and approaches 100% as the concentration increases. The sigmoidal curves have a relatively linear portion between 16% and 84% (these values represent the limits of 1 standard deviation of the mean in a normally distributed population).

(3) End-points

To evaluate the toxic effects associated with chemical substances, it is necessary to have a quantitative method to measure their toxicity and a precise means to express the toxicity. An end-point can be thought of as a measure of toxicity and should be unequivocal, clearly relevant to the toxic effect, readily observable, describable and

measurable, and reproducible. The most frequently used end-point is the EC_{50} , or 50% effective concentration, which refers to the concentration at which 50% of the maximum response is observed. EC_{50} values can be interpolated from a concentration-response curve by drawing a horizontal line from the 50% point on the ordinate (Y-axis) to the concentration-response curve and then drawing a vertical line from the point of intersection with the curve to the abscissa (X-axis). Alternatively, EC_{50} values can be obtained from a least-square regression of the relatively linear portion of the concentration-response curve. A regression equation should have the form of $Y=aX+b$. To obtain the EC_{50} value, one substitutes 50% for Y; the corresponding value of X is the EC_{50} value. Other end-points may also be obtained from a concentration-response curve, e.g., no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) or minimum threshold concentration (MTC).

(4) Toxicity test protocols

A variety of toxicity test protocols has been developed. However, the most commonly used is aqueous-based testing. Aquatic toxicity testing is based on the principle that the response of living organisms upon exposure to a toxicant depends on the concentration of the toxicant in the aqueous phase and is modulated by toxicokinetics and toxicodynamics.

Aquatic toxicity test systems may be static, renewal, or flow-through. In a static test, the test organisms are exposed to test materials in non-moving water and there is no change of water for the duration of the test. In a renewal test, the test is also conducted in non-moving water. However, the test solutions and control water or control media are renewed periodically by transferring the test organisms to containers with freshly

prepared material, or by removing and replacing the material in the original containers. In a flow-through test, the test solution and control water or control media flow into and out of the container in which the test organisms are kept.

(5) *Mechanisms of toxic action*

Heavy metals and organic chemicals, two common groups of toxicants, differ in their mechanisms of toxic effects. Metals play an important role in cellular physiology, primarily through the interactions with proteins including enzymes. Some metals are an integral part of enzymes and the activities of enzymes depend on the presence of the metals. Metals can alter the secondary and tertiary or the quaternary structure of proteins which leads to the stabilization of protein structure (Ulmer, 1970). Metals also interact with other biological molecules such as nucleic acids and lipids (Eichhorn et al., 1970, Shah, 1970). However, only trace amounts of metals are necessary for optimal cellular function and an over-dose results in toxicity. The toxic effect of heavy metals in a microorganism depends on reactions with ligands that are essential for the normal physiological functions (Oehme, 1978). Toxicity is caused by the bonding of metals to sulfhydryl groups, amino, carboxylate, imidazole, and hydroxyl radicals of enzymes and other proteins. The simplest way to model heavy metal inhibition to microorganisms is to assume a mechanism that is similar to non-competitive inhibition to enzymes. Lewandowski et al. (1985) used this method to study the kinetics of the toxicity of chromium (VI) to activated sludge.

The mechanisms of action for organics are generally more complicated. Acute toxicity is divided into two cause-and-effect categories: reversible narcoses and irreversible reactivity. Narcosis is a general term that describes the interaction between a

xenobiotic and the cellular membrane which is the theoretical site of action. While no chemical reaction is involved in the narcotic effect, reactive toxicity is exhibited due to a chemical reaction. Most industrial organic chemicals are thought to exhibit a narcosis mode of action (Blum and Speece, 1992, Bradbury and Lipnick, 1990). Narcotic effects are thought to result in non-covalent interactions such as the disruption of van der Waals interactions between lipid and/or protein components in the cellular membrane, which leads to alterations in membrane fatty acids. Consequently, chemicals exhibiting a narcotic effect are thought to be nonbonding to macromolecules and cause only reversible physiological alterations (Schultz et al., 1998).

Requirements of an on-line wastewater toxicity monitoring system

Despite the fact that the toxic effects of heavy metals and organic chemicals to pure cultures of microorganisms and activated sludge have been studied, the toxicity of wastewater to activated sludge is not easily determined. First of all, toxicity data are not available for most of the chemicals in commercial use today and only 10% of the new chemicals submitted for approval to the Environmental Effects Branch (EEB) of the Office of Toxic Substances are provided with toxicity data (Sun et al., 1994). Secondly, the composition of wastewaters is usually complex and unknown. Thirdly, there may be interactions among the chemical constituents in wastewaters resulting in enhanced or reduced toxicity.

An influent wastewater toxicity monitoring system should be able to detect toxicity without requiring an *a priori* knowledge of the composition of the wastewater. To serve a preventative purpose, the toxicity monitoring system has to be employed upstream of the activated sludge process. Figure 1-4 shows a schematic of a biological

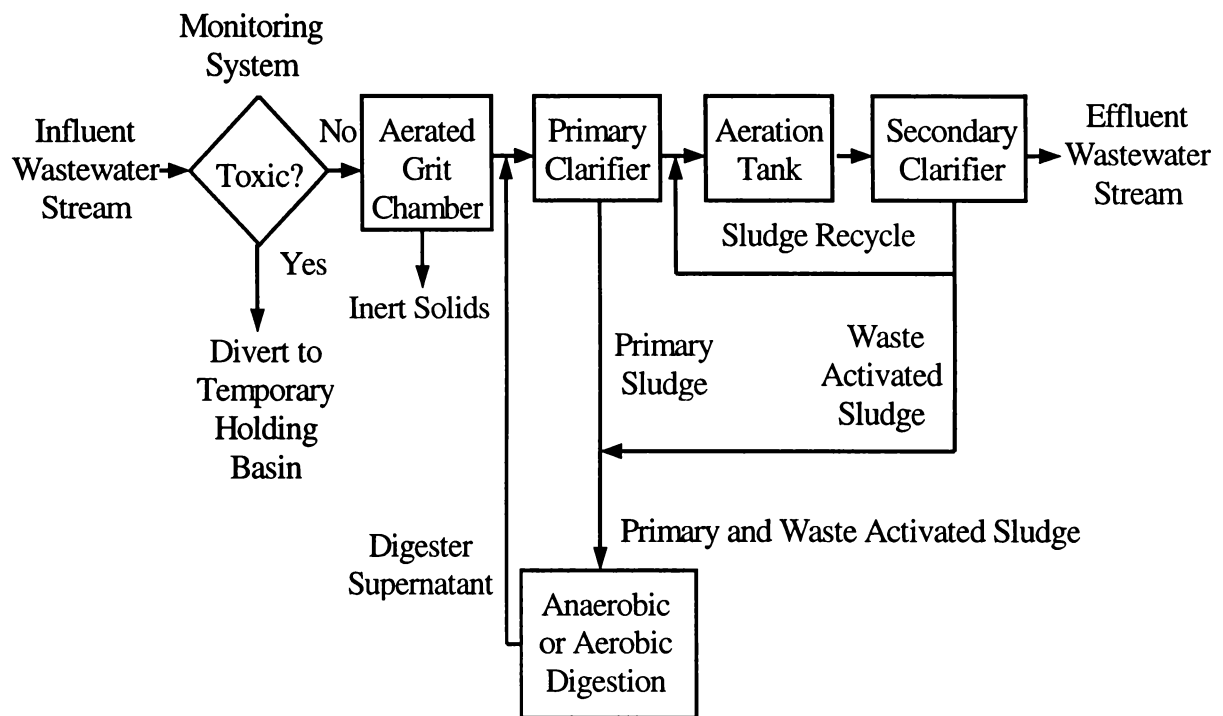


Figure 1-4. Position of the influent wastewater toxicity monitoring system in a biological wastewater treatment plant.

wastewater treatment plant with a toxicity monitoring system. Wooten (1997) pointed out the requirements for industrial effluent wastewater toxicity monitoring systems. Similar requirements can be applied to POTW influent wastewater toxicity monitoring. Accordingly, an ideal system should (1) show the effect of influent wastewater on the biomass in the treatment plant, (2) be supplied with a continuous sample that is representative of the influent wastewater, (3) not require *a priori* knowledge of wastewater composition, (4) have a quick response time, (5) be able to provide alarms should toxicity occur, (6) require minimum operation attention and (7) be inexpensive.

Methods for wastewater toxicity assessment

Currently, influent wastewater is not routinely screened for toxicity in treatment plants. However, there are a few methods that could be used for wastewater toxicity assessment. These methods could be direct or indirect and chemical or biological, and are introduced below.

(1) Chemical analysis

Chemical analysis refers to analyzing the wastewater for its constituents and concentrations. Such analysis methods include, among others, colorimetry and atomic absorption for metals (Alarcon et al., 1989, Padilla and Tavani, 1999, Lopez et al., 2000, Stehouwer et al., 2000) and chromatography for organic compounds. Chemical analysis is time-consuming, labor-intensive (usually requiring several steps to perform), and expensive (Paton et al., 1995). More importantly, *a priori* knowledge of the wastewater constituents is necessary in order to carry out chemical analysis.

(2) *Microscopic analysis*

Microscopic analysis refers to the observation of the samples of the activated sludge microorganisms taken from the aeration tank using a microscope (Fernandez Leborans and Moro, 1991, Cadavid et al., 1999, Rand, 1999, Belanche et al., 2000, Keech Gregory et al., 2000, Lishman et al., 2000). A change in the shape or other observable characteristics of the microorganisms may be related to wastewater toxicity. Microscopic analysis is slow and requires the expertise of trained personnel. It is useful in documenting a toxic shock in history, but it does not serve as an early warning method for wastewater toxicity monitoring.

(3) *Fish tests*

Fish have long been used in aquatic toxicity testing for the obvious reason that fish are living creatures in water and the lethality of fish is directly related to water toxicity. A number of fish species can be used in toxicity tests, e.g., rainbow trout and fathead minnows (Clesceri et al., 1998). The disadvantages of fish tests are that they are time-consuming, requiring 24-96 hours, and the results depend on the size (or weight) of the fish. More importantly, since fish are not indigenous species found in activated sludge, the results of fish tests may not be useful for evaluating wastewater toxicity to activated sludge.

(4) *Microorganism-based non-microscopic methods*

Microbial systems possess several advantages in toxicity testing over tests involving higher life forms (e.g., fish): small test organism size, large number of organisms, and convenient growing conditions (Kustin and McLoed, 1977). A variety of microorganisms can be employed in toxicity tests, for example, bacteria, algae

(Wojtanowicz et al., 1992), phytoplankton (Lapota et al., 1993), and yeast (Hollis et al., 2000). Toxicity tests using microbial systems are based on the effect of toxicants on some observable characteristics of the microorganism(s) employed. For example, cellular growth (Anderson et al., 1984), motility and chemoattraction (Goatcher et al., 1984, Roberts and Berk, 1990), ATP level (Arretxe et al., 1997), intracellular protein formation (Kilemade and Mothersill, 2001), respiration rate (Tyagi and Couillard, 1988, Havash and Oster, 1998) and bioluminescence (Qureshi et al., 1984, Vasseur et al., 1984, Indorato et al., 1984, Bulich, 1984) can be measured in the presence and absence of toxicants. The difference in the results can be related to toxicity. For wastewater toxicity assessment however, the most frequently used test methods are respiration inhibition and bioluminescence tests. These two methods are detailed in the following sections.

(4a) Respiration inhibition tests

Respiration inhibition tests involve mixing activated sludge or surrogate organism(s) with wastewater samples and measuring the respiration rate of the activated sludge (measured as the oxygen consumption rate). A decrease in the respiration rate compared to a control indicates toxicity. Respirometry-based tests are widely used in wastewater toxicity testing because oxygen uptake rate is the most widely accepted parameter for assessing sludge activity (Wong et al., 1997). While batchwise respiration inhibition tests have been commonly used, efforts to develop continuous respirometry methods (Clarke et al., 1978, Howell et al., 1984, Kim et al., 1994) have led to the Rodtox test (Kong et al., 1993, Temmink et al., 1993, Vanrolleghem et al., 1994). The Rodtox test uses a stirred and aerated reactor vessel containing activated sludge. The dissolved oxygen (DO) concentration is measured by a DO probe and recorded as a

respirogram. The steady-state respirogram is disturbed by the addition of a test sample or control substrate. The assessment of toxicity using the Rodtox method is based on a comparison of peak slope, peak height, and peak area of the respirogram after the addition of a test sample and a control substrate.

Another respirometry-based test is the Polytox[®] test (Polybac Corporation, Bethlehem, PA). A freeze-dried blend of 12 strains of organisms isolated from sludge cultures is reconstituted in aqueous phase according to standard methods and is used to inoculate a reactor. The oxygen uptake rate of the microorganisms in the reactor in the presence of toxicants is then measured and compared with that in a control reactor without toxicants.

Generally, the disadvantages of the respiration inhibition tests are that they are relatively slow, expensive (Slabbert, 1988), difficult to operate (Williamson and Johnson, 1981), and the results depend on the source of the sludge (Kong et al., 1993). Additionally, for the Rodtox method, since response time is dependent on calibration (using control substrate) frequency (Vanrolleghem et al., 1994), calibration has to be carried out frequently to ensure a quick response.

(4b) Bioluminescence-based tests

In recent years considerable effort has been dedicated to investigating the usefulness of bioluminescent bacteria in toxicity assessment and monitoring. In addition to the advantages mentioned previously for all microorganism-based tests, methods based on bioluminescent organisms have the additional advantages of having an easily measured signal and very quick responses (usually on the order of a minute or so). The most thoroughly studied bioluminescence-based system is the Microtox[®] assay marketed

by Azur Environmental (Carlsbad, California) and used for aquatic toxicity testing. The microorganism used in the Microtox[®] assay is *Photobacterium phosphoreum*, a marine bacterium strain. Pure cultures of the bacteria are purchased freeze-dried for storage and are resuspended as needed in an aqueous medium using a reconstitution solution, a diluent solution, and an osmotic adjustment solution provided by the manufacturer. The toxicity assay is carried out by mixing the reconstituted cells with aqueous test material for a specified contact time and measuring the luminescence at 15 °C in an analyzer.

One of the problems with *P. phosphoreum* is that, due to its being a marine bacterium, it is excessively sensitive to many toxicants compared to other methods including synthetic activated sludge respirometry tests (Dutka and Kwan, 1984, Elnabarawy et al., 1988), and as a consequence, the response of *P. phosphoreum* to toxicants is different from the response of the activated sludge microbial community (Kelly et al., 1999). The degree to which *P. phosphoreum* represents an activated sludge community is low (Reteuna et al., 1986) and care should be exercised in interpreting the Microtox[®] toxicity data (Neilson, 1994). The Microtox[®] test has been primarily used to monitor industrial effluent wastewater toxicity (Sanchez et al., 1988, Logue et al., 1989, Lin et al., 1994, Hao et al., 1996, Ince et al., 1999). Occasionally, the Microtox[®] test has also been used to monitor municipal wastewater treatment plant influent (Paxéus and Schröder, 1996, Grau and Da-Rin, 1997). However, due to the above-mentioned reasons, the application of the Microtox[®] test as a preventative influent toxicity monitoring method for biological treatment systems is limited; Arretxe (1997), Evans (1998), Reteuna et al. (1989) and Ribo (1997) pointed out that the Microtox[®] assay is not well

suited for predicting the possible toxic effects of waste waters on the activated sludge. Another problem is that the procedure for the batchwise preparation of the cells has to be repeated each time a test is to be performed, making it difficult to adapt to continuous, routine toxicity screening.

Construction of Shk1

A new strain of bacterium was created by researchers at the Center for Environmental Biotechnology at the University of Tennessee. The bacterium was designated as Shk1 and it was created for the purpose of assessing wastewater toxicity to activated sludge. Shk1 was derived from a *Pseudomonas* strain isolated from the activated sludge in an industrial wastewater treatment plant (Kelly et al., 1999). Shk1 was constructed by mating activated sludge microorganisms with *E. coli* carrying the pUTK2 plasmid (Burlage et al., 1990) with the Tn4431 *lux* transposon downstream of a putative promoter. The *lux* transposon carries the five genes coding for the enzymes that catalyze the light-producing reactions. As a result, Shk1 emits bioluminescence under normal growing conditions and does not need an inducer for light production. The plasmid also confers resistance to tetracycline to facilitate the ability to maintain the cells and the plasmid in pure culture. Screening of the 16S rDNA sequence of Shk1 against the Illinois 16S Ribosomal Database Project showed that Shk1 belongs to the genus *Pseudomonas* with a similarity of 0.817 to *Pseudomonas flavescens* strain B62 (Hildebrand et al., 1994).

CHAPTER 2

PRELIMINARY STUDIES

Preliminary experiments were performed to study the basic characteristics of Shk1. This chapter discusses the methodology and results of the preliminary studies. The topics described in this chapter are the Shk1 growth medium, Shk1 biomass quantification, Shk1 growth and light emission in batch and continuous cultures, and the bioluminescence response of Shk1 to selected toxicants.

Methodology

Organisms and growth medium

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 for Shk1 used throughout this research was YEPG medium which had the composition shown in Table 2-1. All chemicals used in the growth medium were obtained from Fisher Scientific, except for Antifoam A, which was obtained from Sigma-Aldrich.

Table 2-1. Composition of the YEPG medium (constituents dissolved in 1 L deionized water).

Medium Constituent	Quantity
Glucose	1 g
Polypeptone	2 g
Yeast extrace	0.2 g
NH ₄ NO ₃	0.2 g
Antifoam A ¹	0.1 ml

¹: Added only for cultures maintained in a bioreactor.

The medium was heat sterilized at 121°C. After cooling down, the medium was amended with 25 ppm tetracycline (final concentration). For simplicity, in this dissertation, the term “YEPG medium” refers to YEPG medium amended with 25 ppm tetracycline unless otherwise specified.

Bioreactor

The bioreactor used in the laboratory was a Bioflo III (New Brunswick Scientific) with a glass vessel having a maximum working volume of 3 L. The bioreactor was equipped with microprocessor temperature, pH, and dissolved oxygen monitoring and control. An 8 liter refrigerated water bath was used to supply cold water for temperature control. The pH probe was calibrated before autoclaving and the calibration verified after autoclaving. The dissolved oxygen probe was calibrated after autoclaving and before inoculation. The dissolved oxygen probe was calibrated to read full scale (100%) at the steady state oxygen concentration when the agitation rate was set to 500 rpm and air fed at a rate of 3 L/min.

In both batch and continuous cultures, the reaction volume in the bioreactor was 1.5 L. In continuous cultures, this was controlled by the position of the overflow port in the glass vessel. When in operation, the agitation rate was set to 400 rpm and air was fed at 1.5 L/min. 1 M NaOH (Fisher Scientific) and 10% (v/v) H₃PO₄ (Fisher Scientific) were used to control pH at desired values.

Light Detection Equipment

Two sets of light detection systems were built, one for batch cultures and the other for continuous cultures. The light detection system that was used in batch cultures is shown schematically in Figure 2-1. The light probe (tip of the light guide) was inserted

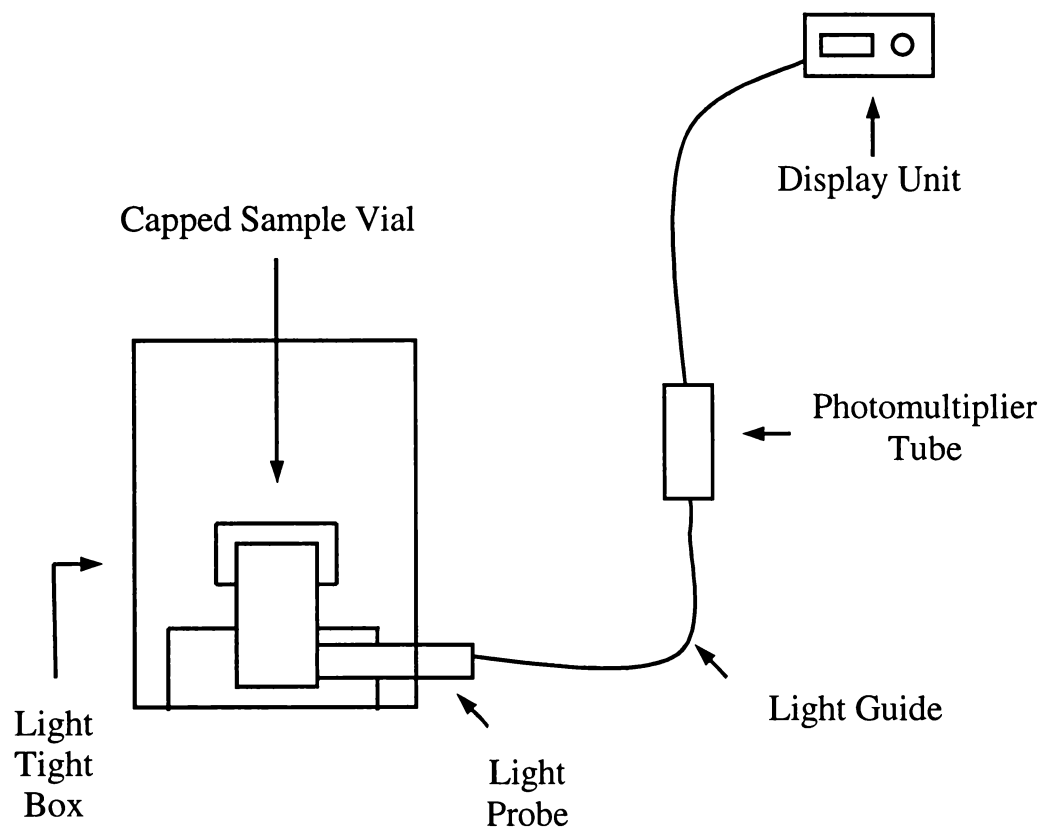


Figure 2-1. Light detection system used for batchwise sampling in batch culture studies.

horizontally into a light-tight box. The vial holder was built so that when the sample vial sat in this holder, the light probe was positioned very close to the vial (approximately 1 mm).

Bioluminescence was measured with a photomultiplier tube (Oriel Corporation, Model 70680). A radiometer (Oriel Corporation, Model 7070) was used to amplify and display the current signal resulting from light emitted by bacteria. In the light detection system that was used in continuous cultures, the plastic light sensing chamber (shown schematically in Figure 2-2) was used. A piece of ordinary glass tubing (5 cm long, with an inner diameter of 1 mm) spanned the plastic light-tight chamber and functioned as the flow cell. The tip of the light guide was placed perpendicular to the glass tubing at a distance of about 1 mm. The light guide transmitted light to the photomultiplier tube that converted the light signals to electric signals in the form of current.

Shk1 growth and light emission in batch culture

As a preliminary experiment, Shk1 was grown in batch culture. Frozen stock solutions of Shk1 were used to inoculate four 250 ml flasks containing 25 ml YEPG medium. The four 250 ml flasks were incubated in an oscillating incubator overnight at 200 rpm and 30°C until the optical density reached an OD₆₀₀ of 0.5-0.6 (absorbance at 600 nm) using a spectrophotometer (Beckman Coulter, model RU[®] 640B). The bioreactor was inoculated with 12-15 ml from each of the four over-night culture flasks described above. In this preliminary experiment, temperature and pH in the bioreactor were arbitrarily chosen to be 30°C and 5, respectively. Dissolved oxygen concentration was monitored but not controlled. Samples were taken from the bioreactor and the

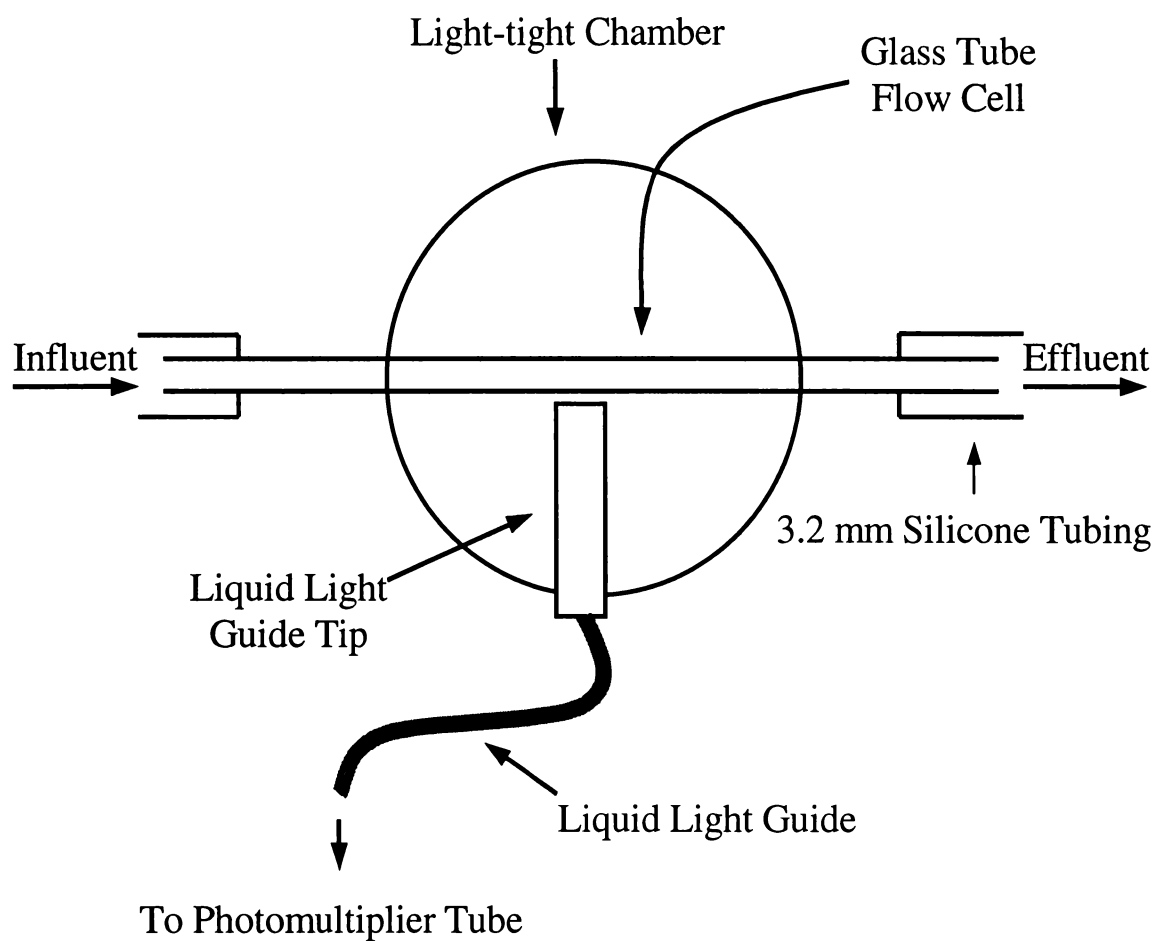


Figure 2-2. Light detection equipment used in continuous cultures. The light detection equipment was incorporated in-line in the experimental set-up for continuous cultures.

optical density was measured using the spectrophotometer while the bioluminescence was measured using the equipment shown in Figure 2-1.

Shk1 growth and light emission in continuous cultures

Modifications to the batch system were made in the continuous system. The experimental set-up, shown in Figure 2-3, differed from the one used in the batch studies in that a medium tank and a waste disposal tank were added to the system. The overflow port in the bioreactor vessel was used to keep the medium level in the bioreactor constant at 1.5 L. A peristaltic pump was used to feed fresh medium to the bioreactor. A second peristaltic pump was used to supply cell solution to the light detection chamber where bioluminescence was measured continuously by a photomultiplier tube (see Figure 2-3). The flow rate of the cell solution was approximately 200 ml/hr, resulting in a residence time in the tubing before the light detection chamber of approximately 30 seconds. The cell solution was directed to the waste disposal tank after flowing through the light detection chamber. Overnight cultures for inoculating the reactor were prepared as described previously in the batch experiment. The bioreactor was inoculated with 12-15 ml from each of the four flasks containing the overnight cultures and was operated in batch mode until the optical density reached an OD_{600} of 0.5-0.6. At that point, fresh medium was pumped into the reactor and CSTR operation began. The flow rate of fresh medium was chosen to give the desired dilution rate. In the preliminary experiment, the dilution rates were arbitrarily chosen to be 0.1 hr^{-1} , 0.2 hr^{-1} , and 0.5 hr^{-1} . Temperature was controlled at 30 °C and pH was controlled at 7. Dissolved oxygen concentration was not controlled but was monitored. Bioluminescence was measured by the light detection system shown in Figures 2-2 and 2-3.

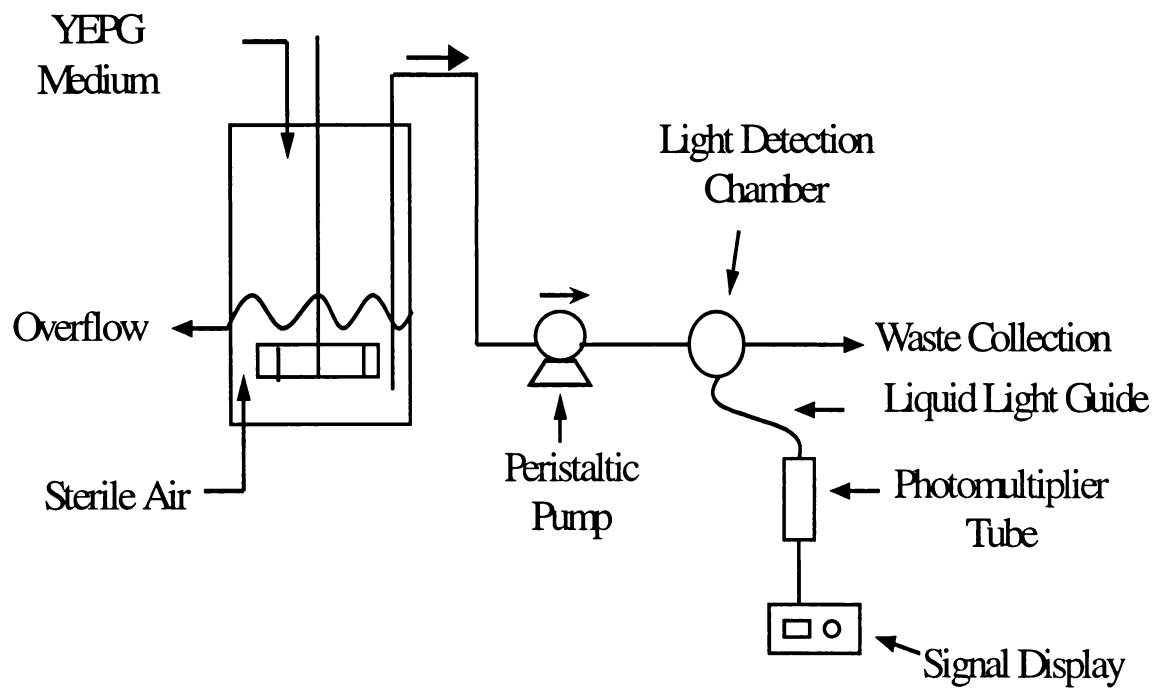


Figure 2-3. Experimental set-up for continuous culture studies.

Shk1 cell quantification

A large volume (3 L) of Shk1 cells was grown in the bioreactor using YEPG medium. A small amount of sample (5 ml) was taken to measure absorption with the spectrophotometer at 600 nm. In the meanwhile, 200 ml of the broth was taken out of the reactor. The broth was filtered using pre-dried and pre-weighed membrane filter which had a pore size of 0.22 μm . A small amount of deionized water was used to rinse the retentate and the retentate was filtered. The membrane filter was collected and kept in an oven at 105°C in a pre-weighed aluminum tray until the weight became constant. The cell dry mass was determined as the weight difference of the membrane filter before and after the filtration and drying. The above practice was repeated at regular time intervals during cell growth. Cell mass concentration was calculated and plotted versus the corresponding optical density. A least-square linear regression was performed.

Development of the prototype of the system and toxicity test using ZnCl_2

The prototype of the rapid toxicity monitoring system was developed based on a CSTR and is shown schematically in Figure 2-4, with two sets of light sensors with flow-cells (shown in Figure 2-2) used in this system. Experiments were performed with Shk1 cells grown in 1.5 L YEPG medium in the bioreactor with the following conditions: dilution rate = 0.4 hr^{-1} , pH =6, and temperature = 30°C. These initial conditions were chosen arbitrarily as in preliminary experiments. The agitation rate was set to 400 rpm and air was fed at a rate of 1.5 L/min. Shk1 cells were supplied continuously to the two light sensors. The first (upstream) sensor was used to determine the basal light level of the cells before exposure to the toxicant. After leaving the first sensor, the cells entered

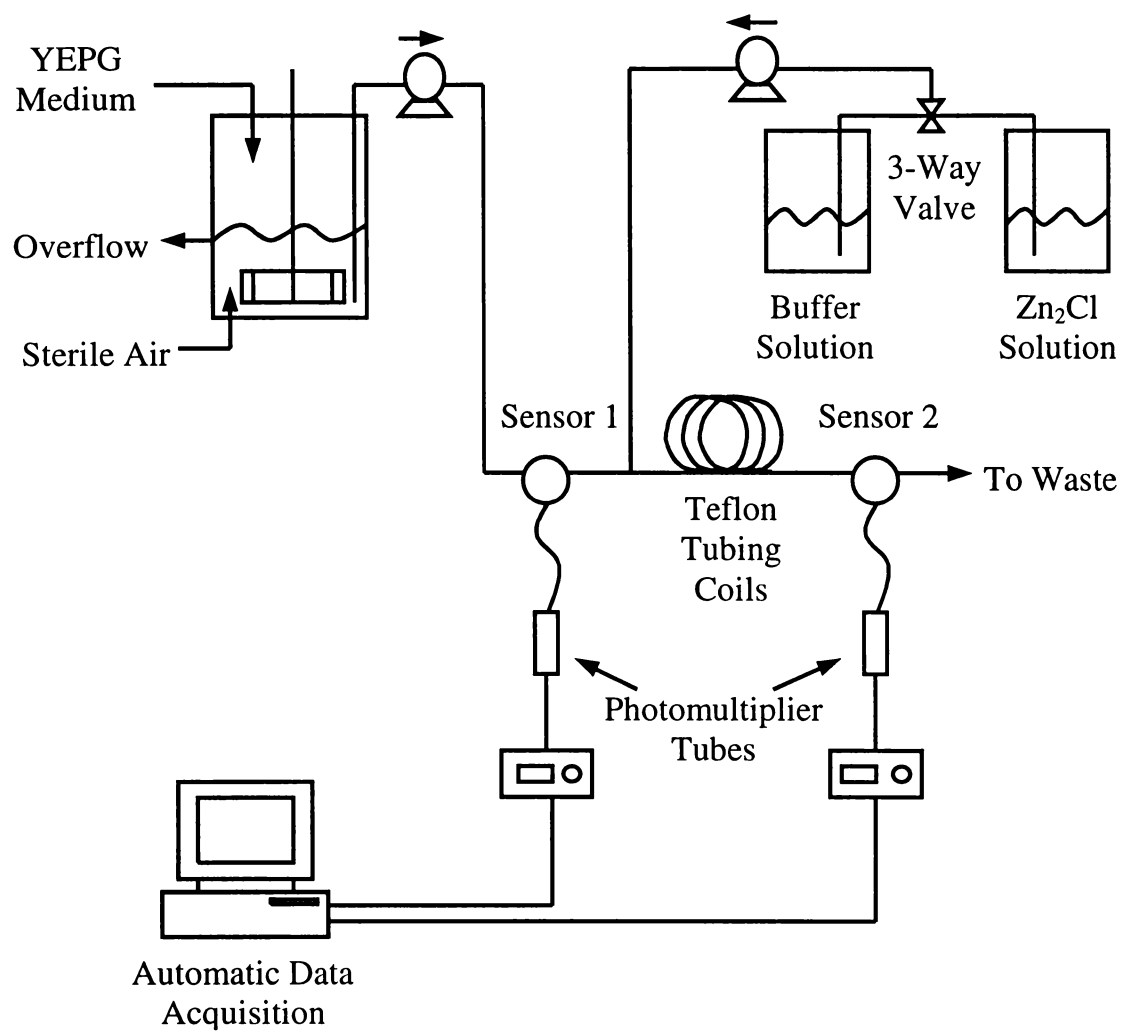


Figure 2-4. Schematic of the toxicity testing system used in the preliminary studies.

one leg of a "tee" connector where they were mixed at a 1:1 ratio alternately with either ZnCl_2 solution or a buffer solution. The mixture of Shk1 cells and ZnCl_2 solution then proceeded through several coils of tubing to achieve an appropriate residence time before entering the second light sensor. The residence time after the mixing tee and before the second light sensor was 2 minutes in this part of the preliminary study. Since there is a decrease in bioluminescence caused by the dilution effect as the Shk1 cell solution was mixed with ZnCl_2 solution, a buffer solution (6.05 g/L K_2HPO_4 and 0.97 g/L KH_2PO_4) was used to normalize the effect of dilution on the light level in the second sensor. This buffer composition gave a pH of 6, which equaled to the pH in the bioreactor when these experiments were performed. ZnCl_2 concentrations of 10, 100, and 1000 mg/L were used in the preliminary toxicity tests. The toxicity tests were performed by first mixing Shk1 cells with buffer solution for 1 hour. By manually switching the 3-way valve, Shk1 cells were then mixed with 10 mg/L ZnCl_2 solution and buffer solution alternately, each for 15 minutes, for a total of 2 hours. Similar experiments were performed using 100 and 1000 mg/L ZnCl_2 solutions.

Results

Shk1 growth and light emission in batch culture

Data for growth and light emission of Shk1 grown in batch culture at 30°C and a pH value of 7 are shown in Figure 2-5. The specific bioluminescence data shown in Figure 2-5 was calculated by dividing total measured bioluminescence at each time point by the highest calculated specific bioluminescence value in the batch experiment.

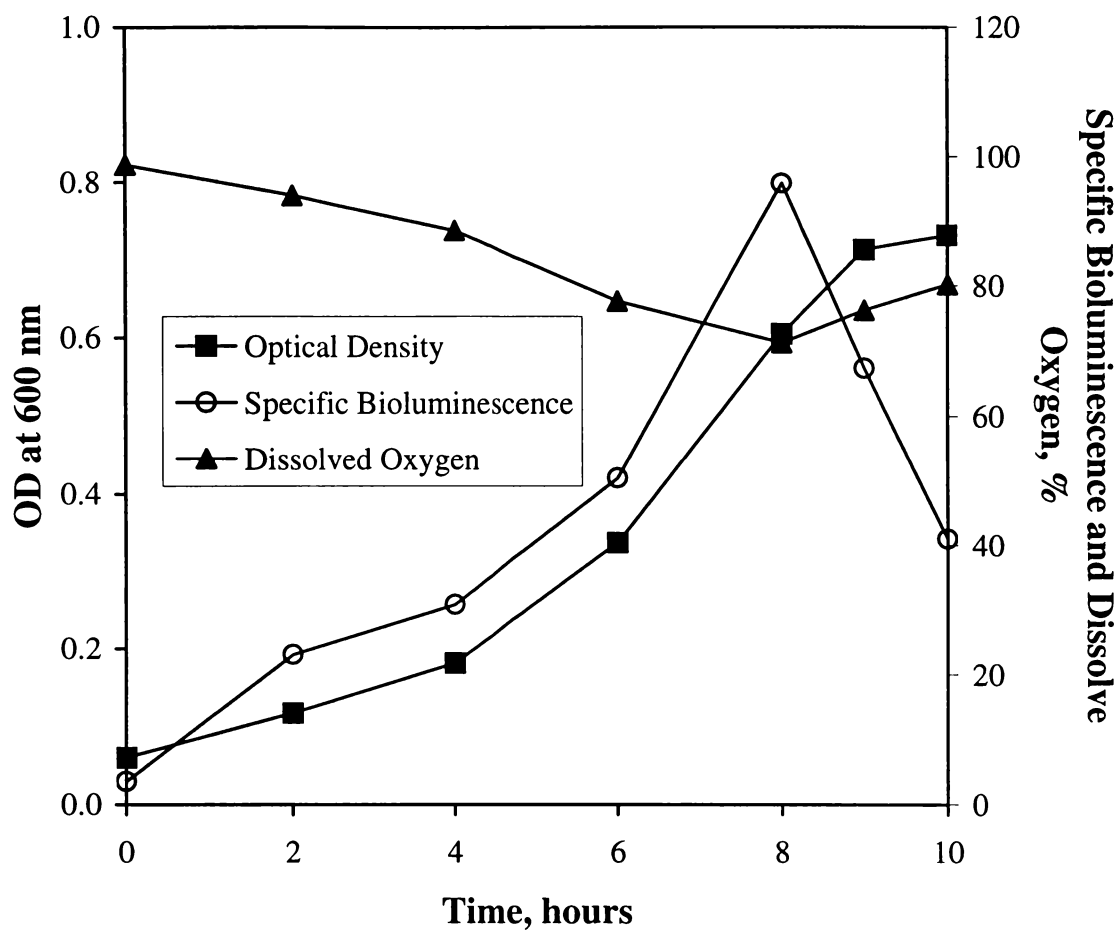


Figure 2-5. Shk1 growth and light emission in batch culture at 30°C and a pH value of 7.

The cell growth of Shk1 in batch culture exhibited the different growth phases (lag, exponential and stationary phases) common to most microorganisms. The light production of Shk1 in batch cultures was characterized by a peak in specific bioluminescence in the exponential growth phase. In the experiments just after inoculation (at $t=0$), specific bioluminescence was very low, then increased quickly as Shk1 cells grew, and finally reached a maximum. After the peak in specific bioluminescence was achieved, specific bioluminescence usually dropped by 50% within 2 hours, at which point the experiment was terminated.

Shk1 growth and light emission in continuous culture

Data for the optical density (OD_{600}) and specific bioluminescence (bioluminescence/ OD_{600}) in continuous culture are shown in Figure 2-6. In Figure 2-6, the specific bioluminescence is shown as the percentage of the highest specific bioluminescence value obtained in this continuous experiment. The dissolved oxygen concentration (data not shown) did not drop below the 50% of the air saturation level throughout the experiment. As Figure 2-6 shows, steady state was reached under the conditions (temperature, pH and dilution rate) adopted in this experiment. However, there were slight fluctuations in both optical density and specific bioluminescence even at steady state. As dilution rate changed, optical density did not change significantly. By contrast, specific bioluminescence changed significantly as dilution rate changed (dilution rate = specific growth rate). As Figure 2-6 shows, specific bioluminescence increased as dilution rate increased, and decreased as dilution rate decreased.

Shk1 cell quantification

The correlation between OD_{600} and cell dry mass is shown in Figure 2-7. A least

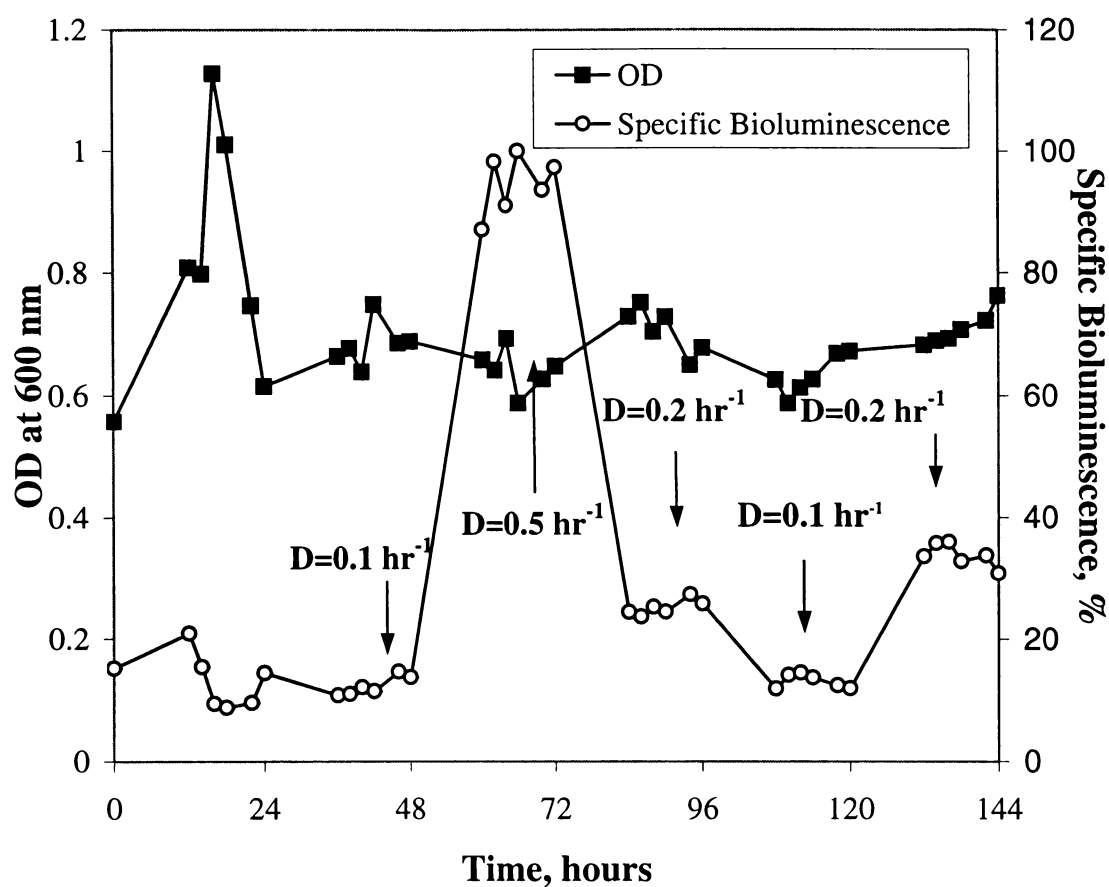


Figure 2-6. Shk1 growth and light emission in continuous culture at various dilution rates, 30°C and a pH of 7. Specific bioluminescence is shown as the percentage of the highest value.

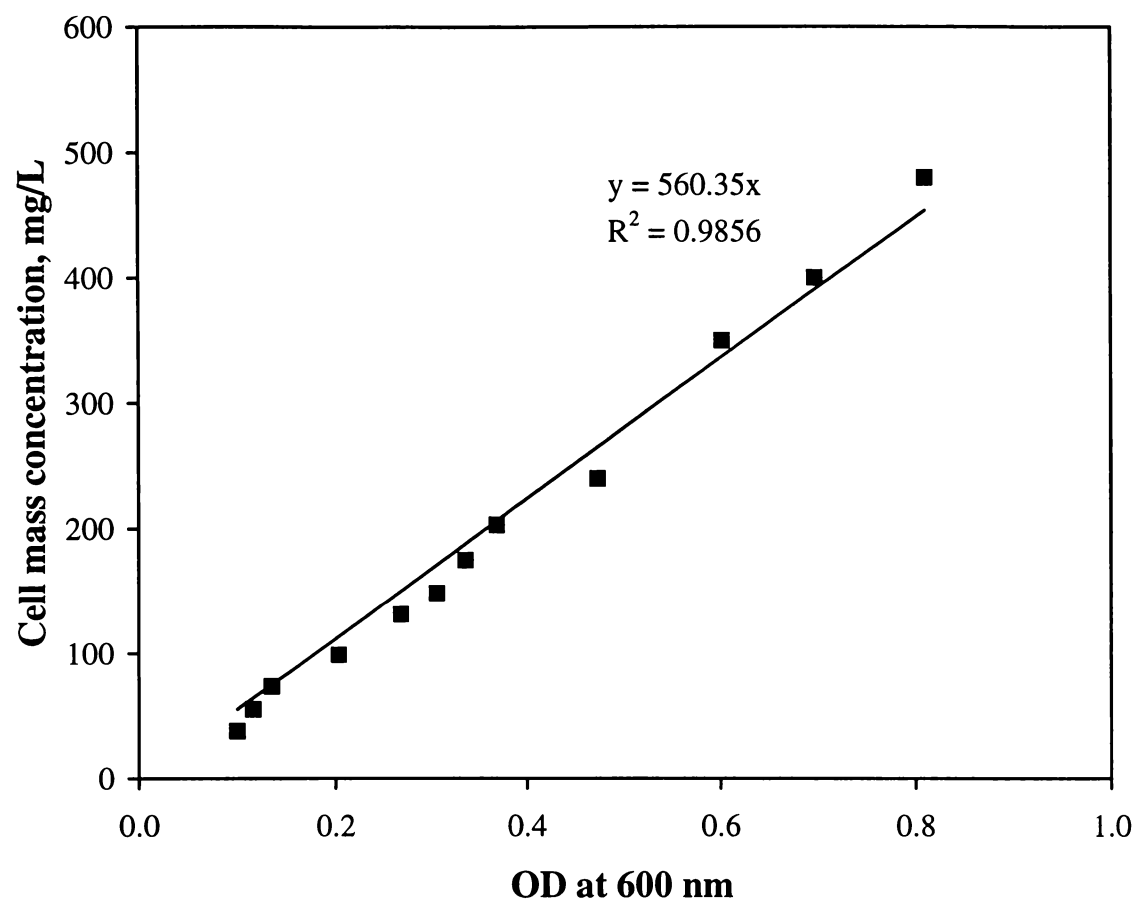


Figure 2-7. Correlation between Shk1 cell dry mass concentration and optical density at 600 nm.

square linear regression shows that cell mass concentration (mg/L) is related to optical density measured as OD₆₀₀ by Equation 2-1 up to an OD₆₀₀ of 0.8.

$$\text{Cell mass concentration (mg/L)} = 560 \times \text{OD}_{600} \quad (2-1)$$

Development of the prototype of the system and toxicity test using ZnCl₂

The results of the toxicity test using ZnCl₂ as toxicant are shown in Figure 2-8. Compared to Shk1 cells mixed with buffer solution, Shk1 cells exhibited a decrease in bioluminescence when mixed with ZnCl₂ solutions, and the decrease in bioluminescence increased as the concentrations of the zinc chloride in the test solution increased from 10 mg/L to 1000 mg/L. When buffer was fed again instead of ZnCl₂ solutions, Shk1 bioluminescence recovered and returned to its original level. Repeated experiments using ZnCl₂ at the same concentration demonstrated that the results were highly reproducible. The difference in the decrease of bioluminescence was relatively small as the concentration of ZnCl₂ increased from 10 mg/L to 100 mg/L, but quite significant as the concentration ZnCl₂ increased from 100 mg/L to 1000 mg/L. Although the response of Shk1 to toxicants as indicated by a decrease in bioluminescence was observed as expected, the sensitivity of Shk1 to toxicant (ZnCl₂) was not sufficient. The ZnCl₂ concentrations of 1000 mg/L in this preliminary experiment was significantly higher than those expected in influent wastewater, yet 100% inhibition of bioluminescence was not observed. This indicates that the 2 minute contact time may not be long enough for the toxic effect to be completely exhibited and a longer contact time should be considered.

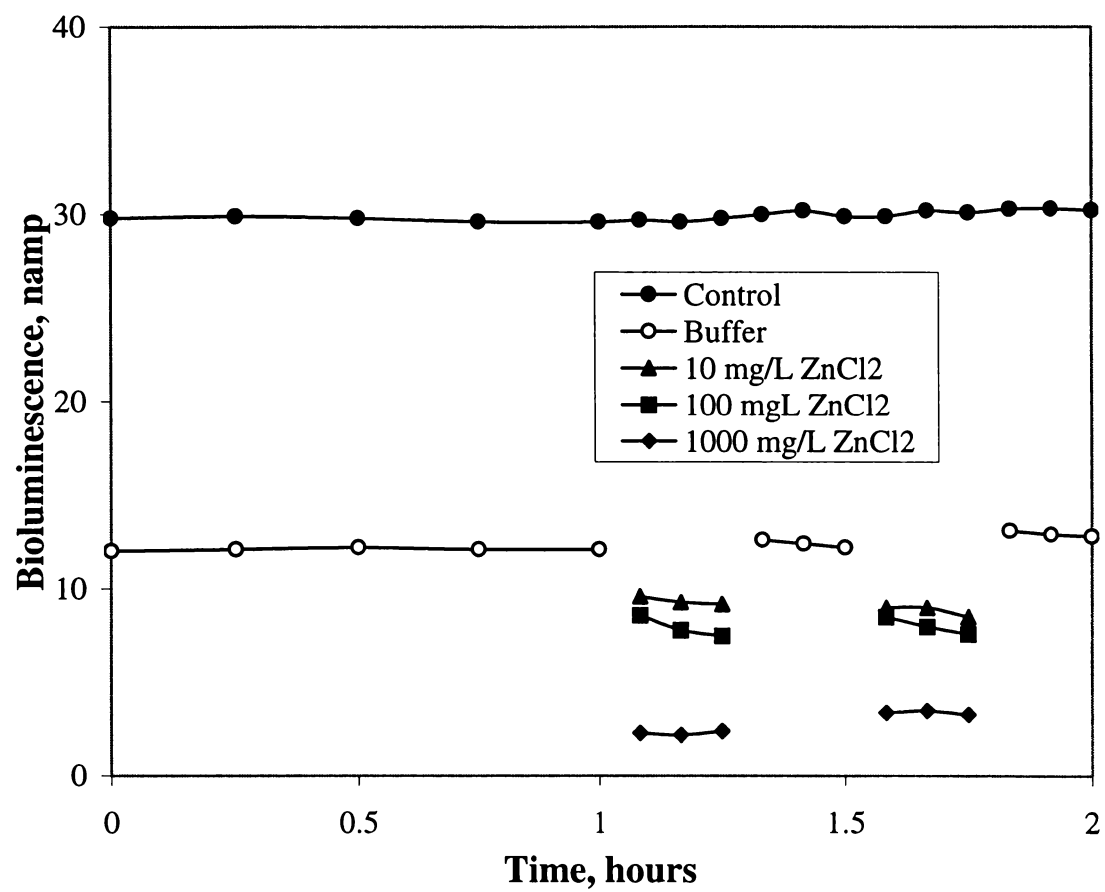


Figure 2-8. Bioluminescence response of Shk1 to ZnCl₂ at various concentrations.

CHAPTER 3

CHARACTERIZATION OF Shk1 GROWTH AND LIGHT PRODUCTION IN BATCH AND CONTINUOUS CULTURES

Shk1, which contains a constitutive-type operon for the *lux* gene cassette, does not need an inducer and therefore emits light under normal conditions. The light-producing reactions are closely related to cellular respiration and thus cell physiology. Environmental conditions such as temperature and pH determine cell physiology and also determine cell growth and light emission. This chapter discusses (1) effect of tetracycline on Shk1 growth and light production; (2) Shk1 growth and light production in batch cultures; (3) Shk1 growth and light production in continuous cultures; and (4) the effect of phosphate buffer on light production in continuous cultures. The experimental results in this chapter can provide basic information about growth and light emission of Shk1 under different environmental conditions and in different culture modes.

Methodology

Bioreactor and Light Detection Equipment

The bioreactor, light detection equipment, as well as the experimental equipment were the same as those used in the preliminary experiments. The light detection equipment and the experimental equipment for continuous cultures are shown in Figures 2-1, 2-2, and 2-3.

Effect of Tetracycline

The purpose of adding tetracycline to the medium is, in addition to preventing possible contamination by cells other than Shk1, to maintain the plasmid that contains the *lux* gene cassette. To make sure that a tetracycline concentration of 25 ppm does not inhibit cell growth and light production of Shk1, experiments were performed by amending YEPG medium with 0, 5, 10, and 25 ppm tetracycline. A frozen stock solution of Shk1 was used to inoculate a 250 ml flask containing 25 ml YEPG medium without tetracycline. The inoculated flask was incubated in an oscillating incubator overnight at 200 rpm and 30°C until optical density reached an OD₆₀₀ of 0.5-0.6. A volume of 1 ml of the overnight culture was transferred to flasks containing 24 ml fresh YEPG medium and then amended with 0, 5, 10, and 25 ppm tetracycline (final concentration). The flasks were then incubated in the oscillating incubator at 200 rpm and 30°C. Samples were taken from the flasks to monitor cell growth by optical density measurement at 600 nm and light production was monitored by measuring bioluminescence using the equipment shown in Figure 2-1.

Shk1 Cell Growth and Light Production in Batch Cultures at 30 °C and Various pH

The bioreactor was filled with 1.5 L YEPG medium amended with 0.1% (v/v) antifoam and was autoclaved at 121 °C. After cooling down to room temperature, the medium was amended with 25 ppm (final concentration) tetracycline. Frozen stock solutions of Shk1 were used to inoculate four 250 ml flasks containing 25 ml YEPG medium amended with 25 ppm tetracycline. The four 250 ml flasks were incubated in an oscillating incubator overnight at 200 rpm and 30°C until the optical density reached an

OD₆₀₀ of 0.5-0.6. The bioreactor was inoculated with 12-15 ml from each of the four over-night culture flasks described above. The agitation rate was set at 400 rpm and air was fed at 1.5 L/min. Temperature was kept constant at 30°C, and pH was set to the desired values (4, 5, 6, 7 or 8 pH units). Samples were taken and the optical density was measured. Bioluminescence was measured using the batchwise light detection system described previously and shown in Figure 2-1. The dissolved oxygen concentration was monitored to ensure that it remained high enough so that light production was not limited by the dissolved oxygen concentration (see discussion).

Shk1 Cell Growth and Light Production in Batch Cultures at a pH of 7 and Various Temperatures

Similar to the previously described experiments in which pH was varied at constant temperature, experiments were performed at a constant pH of 7 and temperatures of 14°C, 18°C, 22°C, 26°C and 34°C. All experimental procedures were the same as described above for the experiments at 30°C and various pH values.

Shk1 Cell Growth and Light Production in Continuous Cultures

To study the cell growth and light production of Shk1 in continuous culture, modifications to the experimental set-up were made as in the preliminary continuous cultures experiments. This experimental set-up was described in Chapter 2 and is shown in Figure 2-3. Overnight cultures for inoculating the bioreactor were prepared as described previously. The bioreactor was inoculated with 12-15 ml from each of the four flasks containing the overnight cultures and was operated in batch mode until the optical density reached an OD₆₀₀ of 0.5-0.6. At that point, fresh medium was pumped into the reactor and CSTR operation began. The flow rate of fresh medium was chosen to give

the desired dilution rate. In this study, the fresh medium feeding rates adopted were 390 ml/hr, 585 ml/hr, and 780 ml/hr, corresponding to dilution rates of 0.26 hr^{-1} , 0.39 hr^{-1} , and 0.52 hr^{-1} ; the temperatures studied were 18°C , 22°C , 26°C , and 30°C ; and pH values were 5, 6, and 7. Experiments were designed to incorporate all 36 possible combinations of 3 dilution rates, 4 temperatures, and 3 pH values. In each combination of these variables, the steady state optical density was measured with a spectrophotometer as described above. Steady state bioluminescence was measured by the light detection system shown in Figures 2-2 and 2-3.

Effect of Phosphate Salts

The effect of phosphate salts was studied in continuous cultures. The experimental set-up and procedure are the same as described above, the only difference being that $3.03 \text{ g/L KH}_2\text{PO}_4$ and $5 \text{ g/L K}_2\text{HPO}_4$ were added to the YEPG medium. Due to this change, glucose in the aqueous medium was autoclaved separately. The dilution rates were 0.26 hr^{-1} and 0.39 hr^{-1} , and pH values were 6 and 7. Temperature was fixed at 30°C . Experiments were performed at the 4 combinations of dilution rate and pH.

Results

Effect of Tetracycline

Data from experiments in which the concentrations of tetracycline was varied are shown in Figure 3-1. Specific bioluminescence was obtained by dividing total bioluminescence by optical density. The radiometer used for generating a current signal

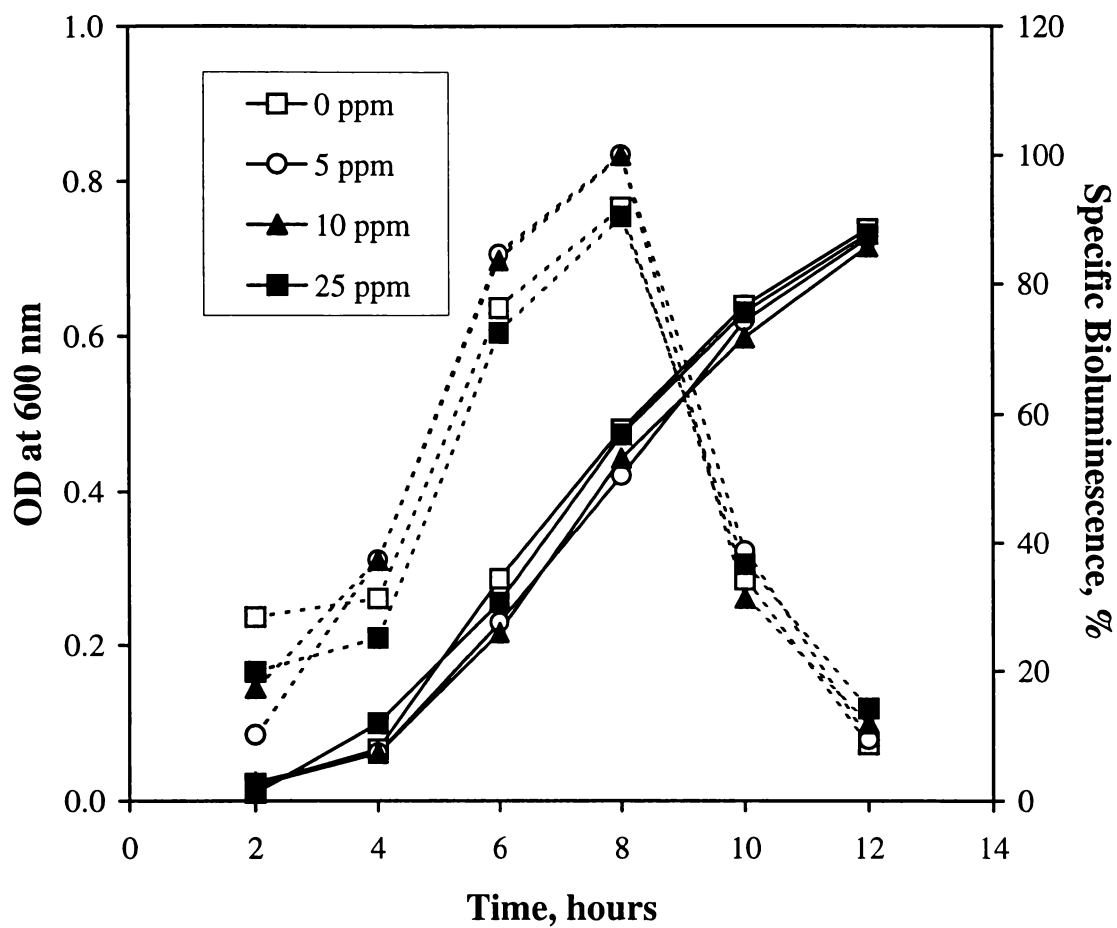


Figure 3-1. Effect of tetracycline on Shk1 cell growth and light production.

—: optical density

-----: specific bioluminescence shown as the percentage of the highest value

gave bioluminescence in units of amps. The scaling of the current signal is arbitrary since the absolute value of the current signal depends on the configuration of the light detection system. Therefore specific bioluminescence values were normalized to a common maximum value. In Figure 3-1, specific bioluminescence is shown as the percentage of the highest calculated specific bioluminescence value. It can be seen that a tetracycline concentration up to 25 ppm in the growth medium did not negatively affect the growth and light production of Shk1.

Shk1 Cell Growth and Light Production in Batch Cultures at 30°C and Various pH

Data for Shk1 cell growth and specific bioluminescence (bioluminescence/OD₆₀₀) obtained at 30°C and different pH values (4, 5, 6, 7 and 8) are shown in Figures 3-2 and 3-3. The specific bioluminescence data shown in Figure 3-3 is expressed as the percentage of the highest calculated specific bioluminescence value in all the experiments performed in batch culture studies that included experiments performed at 30°C and various pH values and experiments performed at a pH of 7 and various temperatures. The highest specific bioluminescence value was obtained at a pH of 7 and a temperature of 18°C (shown later in Figure 3-6).

The cell growth of Shk1 in batch culture underwent the different growth phases (lag, exponential and stationary phases) as in the preliminary experiments (the experiment performed at a pH of 8 and a temperature of 30°C was not performed long enough to see all the growth phases). The light production of Shk1 in batch cultures was characterized by a peak in specific bioluminescence usually in the mid- to late exponential growth phase as before. To better illustrate this, the optical density and

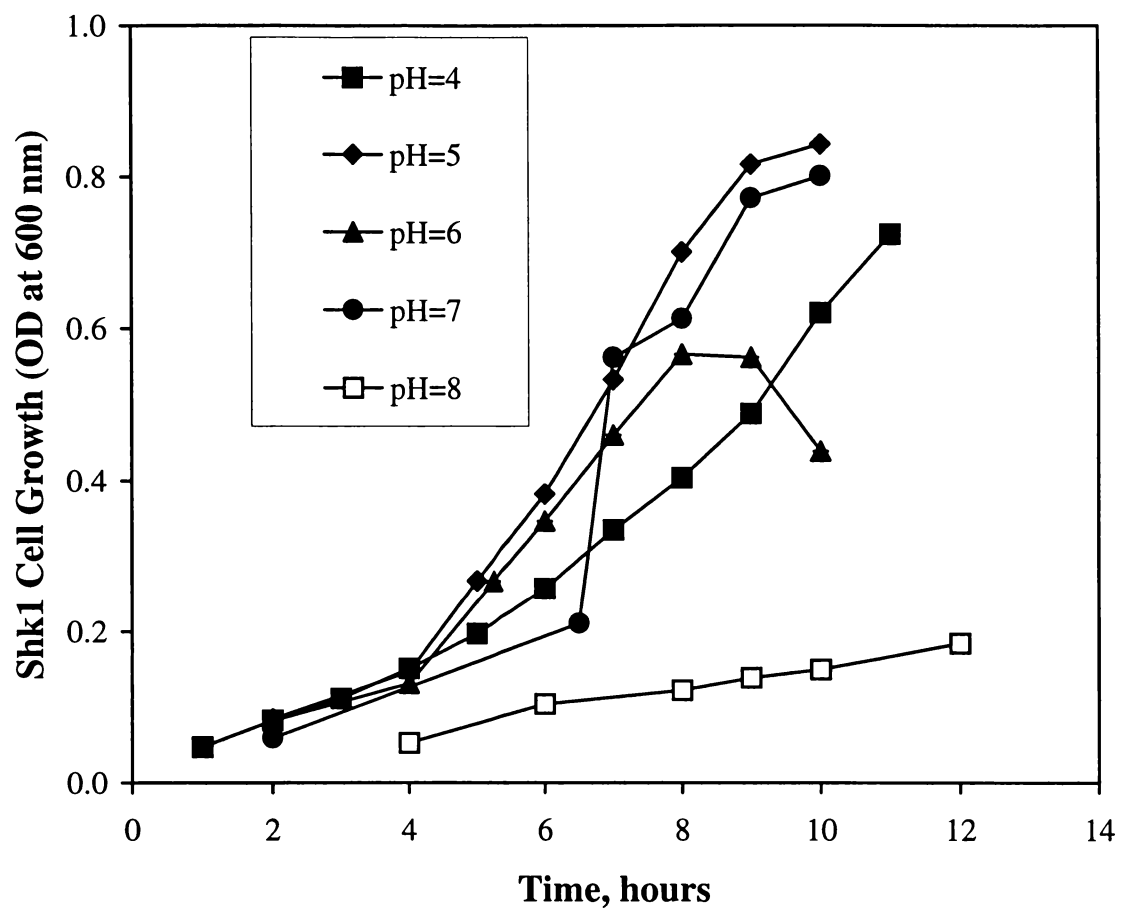


Figure 3-2. Shk1 cell growth in batch cultures at 30°C and various pH values.

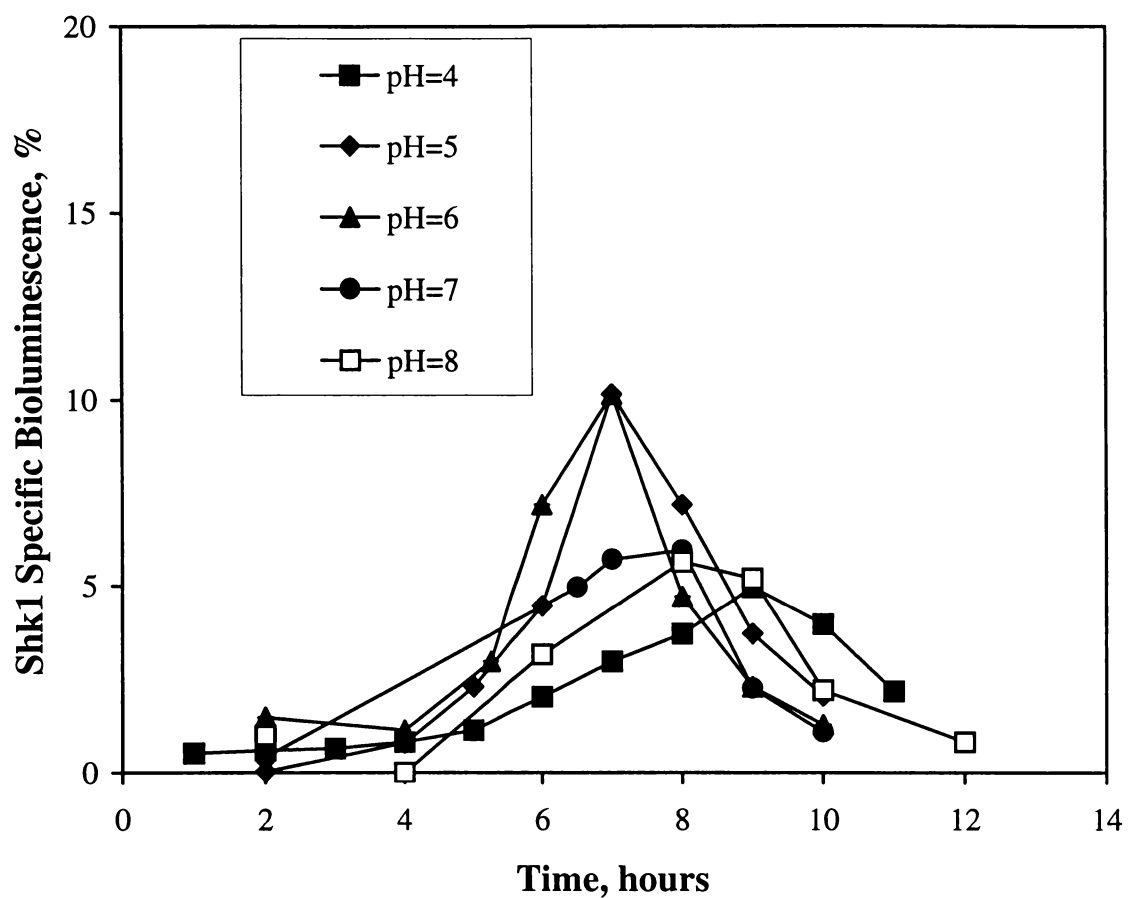


Figure 3-3. Shk1 specific bioluminescence in batch cultures at 30°C and various pH values. Specific bioluminescence is shown as the percentage of the highest value obtained in all batch experiments.

specific bioluminescence values at pH units of 5 and 7 shown in Figures 3-2 and 3-3 were plotted against time in the same graph and shown in Figure 3-4. In the experiments just after inoculation (at time $t=0$), specific bioluminescence was very low, then increased quickly as the Shk1 cells grew, and finally reached a maximum. After the peak in specific bioluminescence was achieved, specific bioluminescence usually dropped by 50% within 2-3 hours, and the experiments were terminated, as in the preliminary experiments. The maximum specific growth rates of Shk1 at various pH values were calculated by finding the maximum slope in a plot of $\ln(X)$ versus t (figure not shown), where t is time, and X is the biomass concentration at time t measured by optical density at 600 nm. The maximum specific growth rates of Shk1 at a temperature of 30°C and various pH values are listed in Table 3-1. Due to very slow growth, the experiment at a pH of 8 was not performed long enough to obtain a maximum growth rate, but the peak in specific bioluminescence was observed and the maximum specific growth rate at a pH of 8 would clearly be lower than all the calculated values. In Table 3-1 and Figure 3-2, the maximum specific growth rate of Shk1 increased as pH increased from 4 to 7, but dropped as pH increased to 8. There was also an optimum pH for specific bioluminescence. However, the optimum pH for specific bioluminescence differed from the optimum pH for specific growth rate. The peak or maximum specific bioluminescence became higher as pH decreased from 8 to 5, and decreased as pH was dropped further from 5 to 4. At pH values of 4 and 8, not only the peak specific bioluminescence, but also specific bioluminescence in general was very low as compared to other pH values.

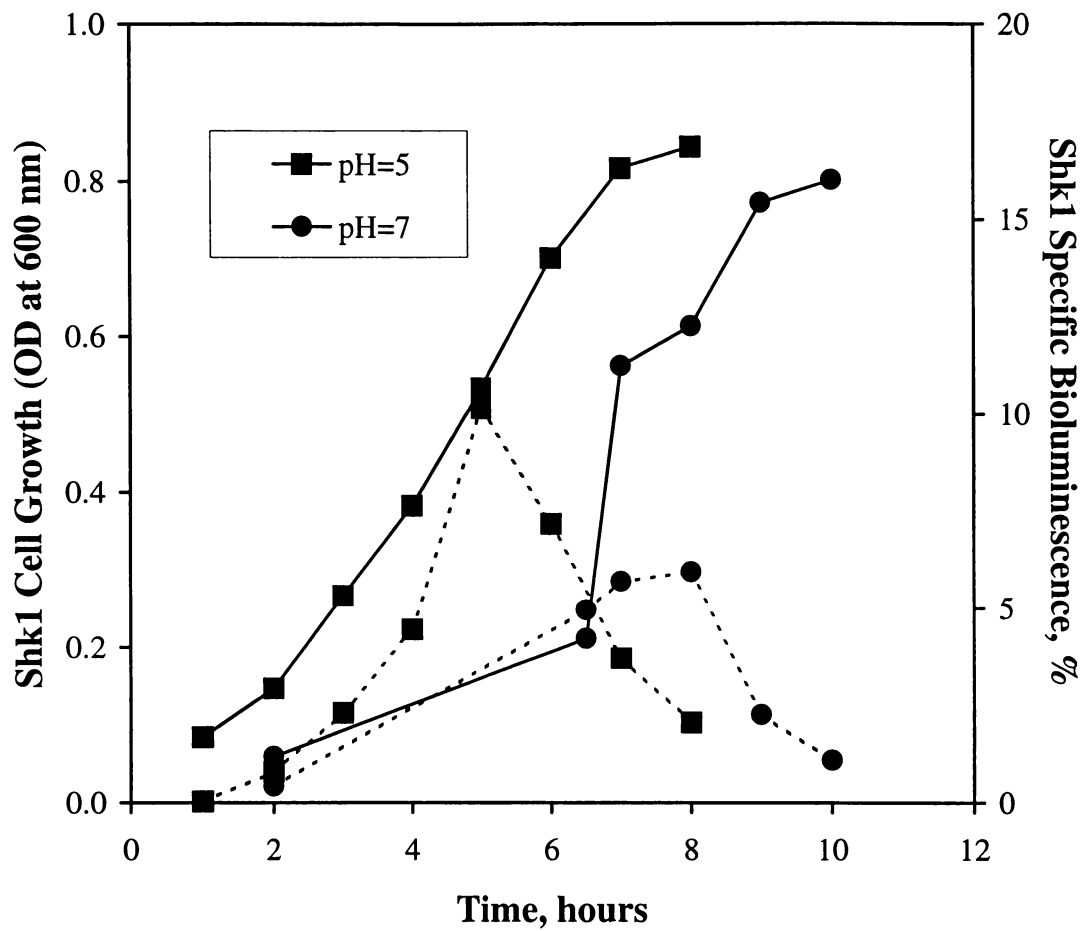


Figure 3-4. Shk1 cell growth and specific bioluminescence in batch cultures at pH values of 5 and 7 (data taken from Figures 7 and 8).

—: optical density

-----: specific bioluminescence shown as the percentage of the highest value obtained in all batch experiments.

Table 3-1. Maximum specific growth rate ($\mu_{\max}$) and normalized peak specific bioluminescence ($I_{\max}$) of Shk1 grown at 30°C and various pH.

	pH=4	pH=5	pH=6	pH=7	pH=8
$\mu_{\max}$, hr ⁻¹	0.30	0.31	0.55	0.61	-
$I_{\max}$, %	4.9	10.1	10.1	5.9	5.6

As mentioned in Methodology, the dissolved oxygen concentration was not controlled but was monitored. In all experiments, the dissolved oxygen concentration did not drop below 50% of the air saturation level.

Shk1 Cell Growth and Light Production in Batch Cultures at a pH of 7 and Various Temperatures

Data for Shk1 cell growth and specific bioluminescence obtained at a constant pH of 7 and various temperatures (14°C, 18°C, 22°C, 26°C and 34°C) are shown in Figures 3-5 and 3-6. The specific bioluminescence shown in Figure 3-6 is expressed as the percentage of the highest calculated specific bioluminescence value in all the experiments performed in batch culture studies as before. For the purposes of comparison, the data obtained at a pH of 7 and 30°C from the previous experiment are also included in the figures. Similar to the results obtained in the previous part of the study, the light production of Shk1 was characterized by a peak in specific bioluminescence in the mid- to late exponential growth phase. The peak specific bioluminescence increased as temperature decreased from 34°C to 18°C, and decreased as temperature decreased further to 14°C. The maximum growth rates at various temperatures were calculated and are shown in Table 3-2. As can be seen from the table, the maximum specific growth rate was low at a temperature of 34°C, but increased as temperature decreased to 30°C. As temperature decreased further, the maximum growth rate of Shk1 also decreased

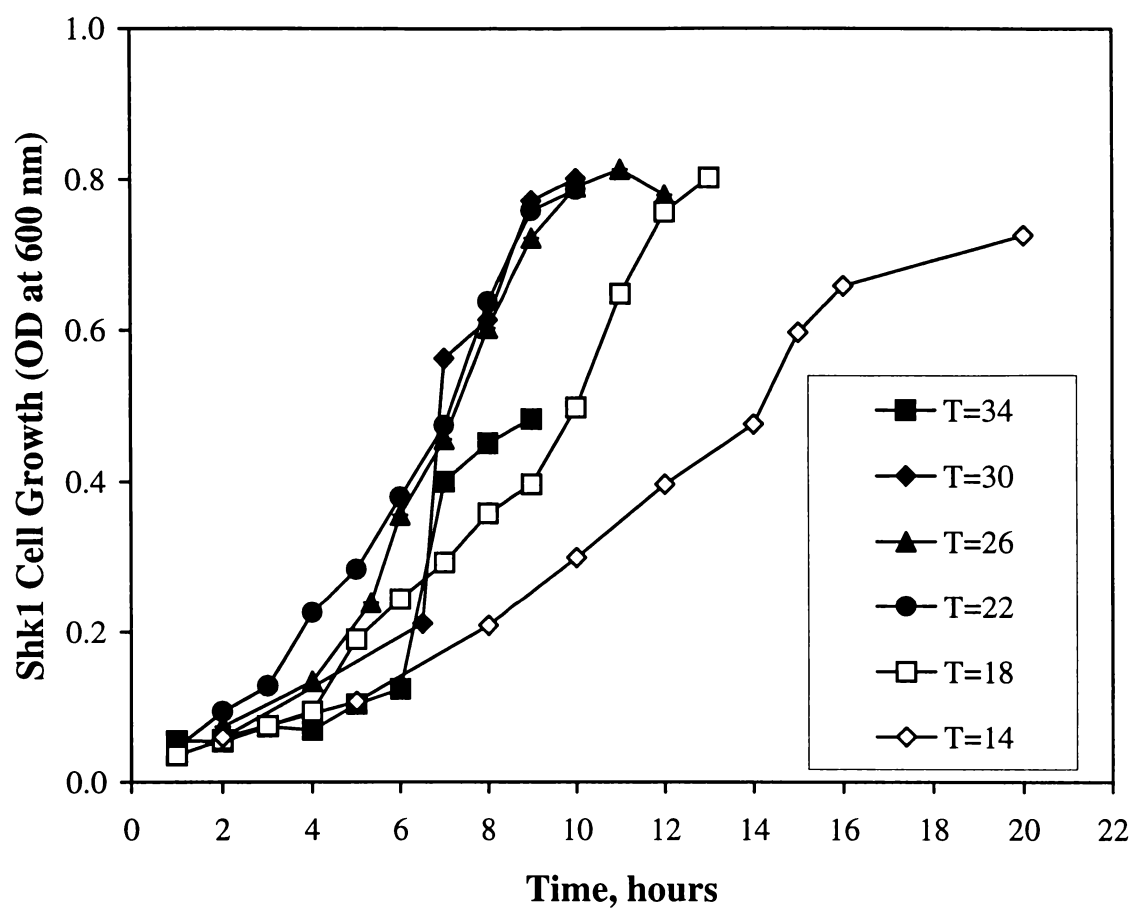


Figure 3-5. Shk1 cell growth in batch cultures at a pH of 7 and various temperatures.

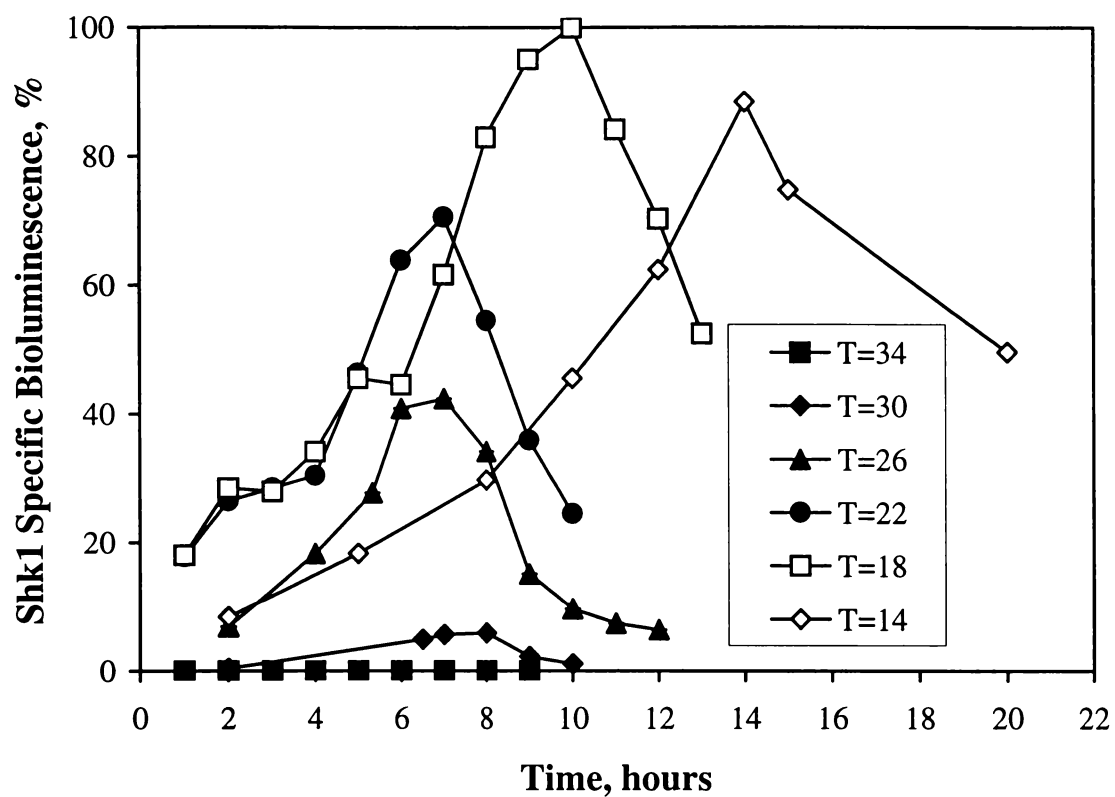


Figure 3-6. Shk1 specific bioluminescence in batch cultures at a pH of 7 and various temperatures. Specific bioluminescence is shown as the percentage of the highest value obtained in all batch experiments.

accordingly. Like optimum pH values for specific growth rate and specific bioluminescence, there was also an optimum temperature for specific bioluminescence that differed from the optimum temperature for specific growth rate. Specific bioluminescence was essentially 0 at a temperature of 34°C, but increased as temperature decreased to 18°C. As temperature decreased further to 14°C, the specific bioluminescence reversed its trend above 18°C and decreased. As in the previous experiments, the dissolved oxygen concentration did not drop below 50% of the air saturation level in all the experiments performed in this part of the study.

Shk1 Cell Growth and Light Production in Continuous Cultures

Due to decreased maximum specific growth rate at low pH and temperature, several parameter combinations were not achievable because wash-out occurred. Table 3-3 illustrates the combinations of environmental parameters (pH, temperature and dilution rate) that resulted in steady-state cell concentration and bioluminescence or wash-out.

Table 3-2. Maximum specific growth rate ($\mu_{\max}$) and normalized peak specific bioluminescence ($I_{\max}$) of Shk1 grown at a pH of 7 and various temperatures.

	34°C	30°C	26°C	22°C	18°C	14°C
$\mu_{\max}$, hr ⁻¹	0.16	0.61	0.59	0.55	0.27	0.21
$I_{\max}$, %	0	5.9	42.3	70.6	100	88.4

Table 3-3. Combinations of dilution rate and pH at 18°C, 22°C, 26°C and 30°C that resulted in steady-state (√) and wash-out (×).

pH	Dilution rate, hr ⁻¹		
	0.26	0.39	0.52
5	√	×	×
6	√	√	×
7	√	√	×

× at 18°C, √ at 22°C, 26°C and 30°C

For cases in which a steady-state was achieved, the optical density and total bioluminescence were recorded, and specific bioluminescence calculated. The dependence of specific bioluminescence on dilution rate, pH, and temperature is shown by bar graphs in Figures 3-7 to 3-10. These figures comprise the results of 74 experiments performed at different combinations of the controlled variables (pH, temperature, and dilution rate). Each bar represents the average of at least three replicates and is shown as a percentage of the highest average specific bioluminescence obtained in all the experiments using continuous cultures. The highest specific bioluminescence was obtained at a dilution rate of 0.52 hr^{-1} , a pH of 7 and a temperature of 22°C ; the lowest specific bioluminescence was obtained at a dilution rate of 0.26 hr^{-1} , a pH of 7, and a temperature of 30°C . The figures show clearly that specific bioluminescence decreased as dilution rate decreased and as temperature increased for the range of these variables studied. However, the effect of pH on specific bioluminescence in continuous cultures is ambiguous. It is clear that in batch cultures the maximum specific bioluminescence increased as pH decreased from 7 to 5. However, in continuous cultures at a dilution rate of 0.39 hr^{-1} and temperatures of 18°C and 22°C , specific bioluminescence *decreased* as pH decreased from 7 to 6. This was not expected but repeated experiments proved this result to be reproducible. A correlation between specific bioluminescence and environmental parameters was established based on the continuous culture data, and is given by Equation 3-1.

$$I = k \cdot D^{1.4} \cdot (8 - \text{pH})^{0.5} \cdot (34 - T)^{1.4} \quad (3-1)$$

where I = specific bioluminescence, amps
 k = constant which depends on the set-up of the light detection equipment

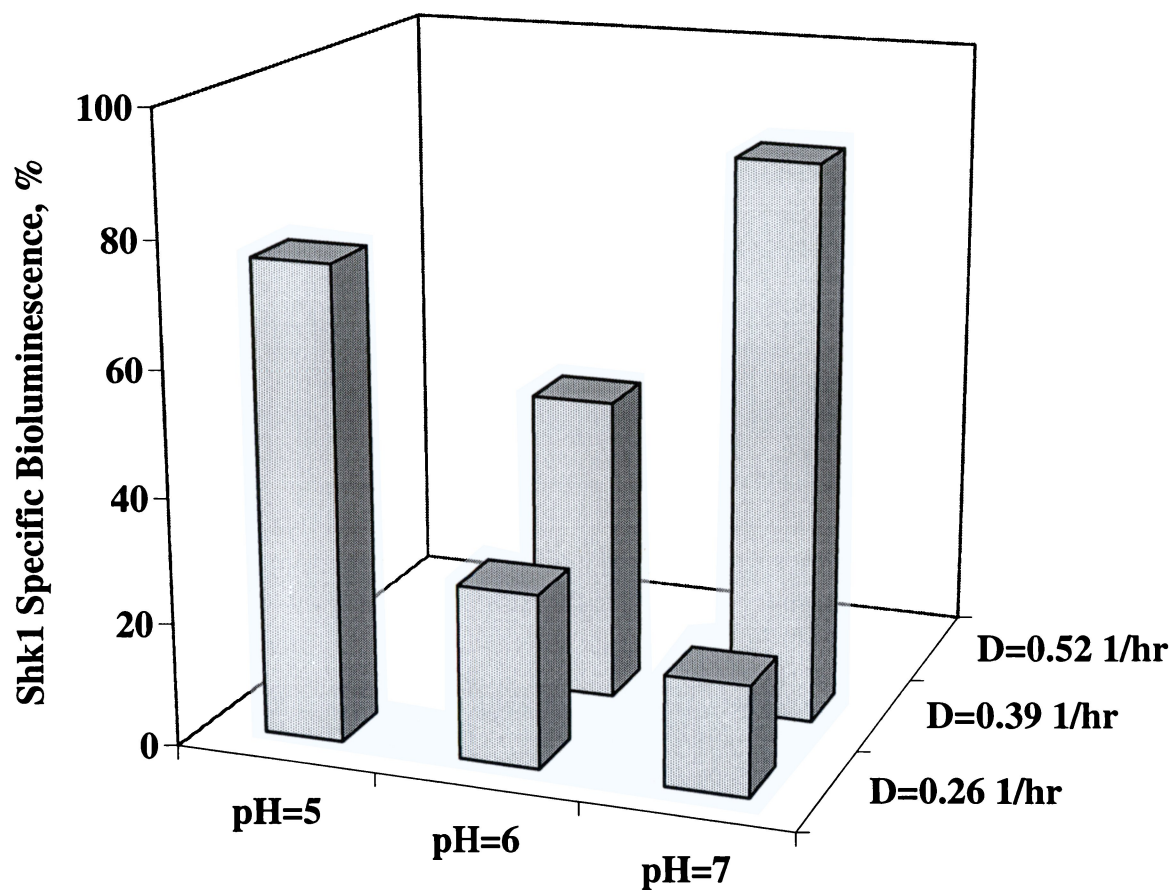


Figure 3-7. Dependence of specific bioluminescence on dilution rate and pH at 18°C. Each bar shows the average of at least 3 replicates. Specific bioluminescence is shown as the percentage of the highest average value obtained in all experiments of continuous cultures.

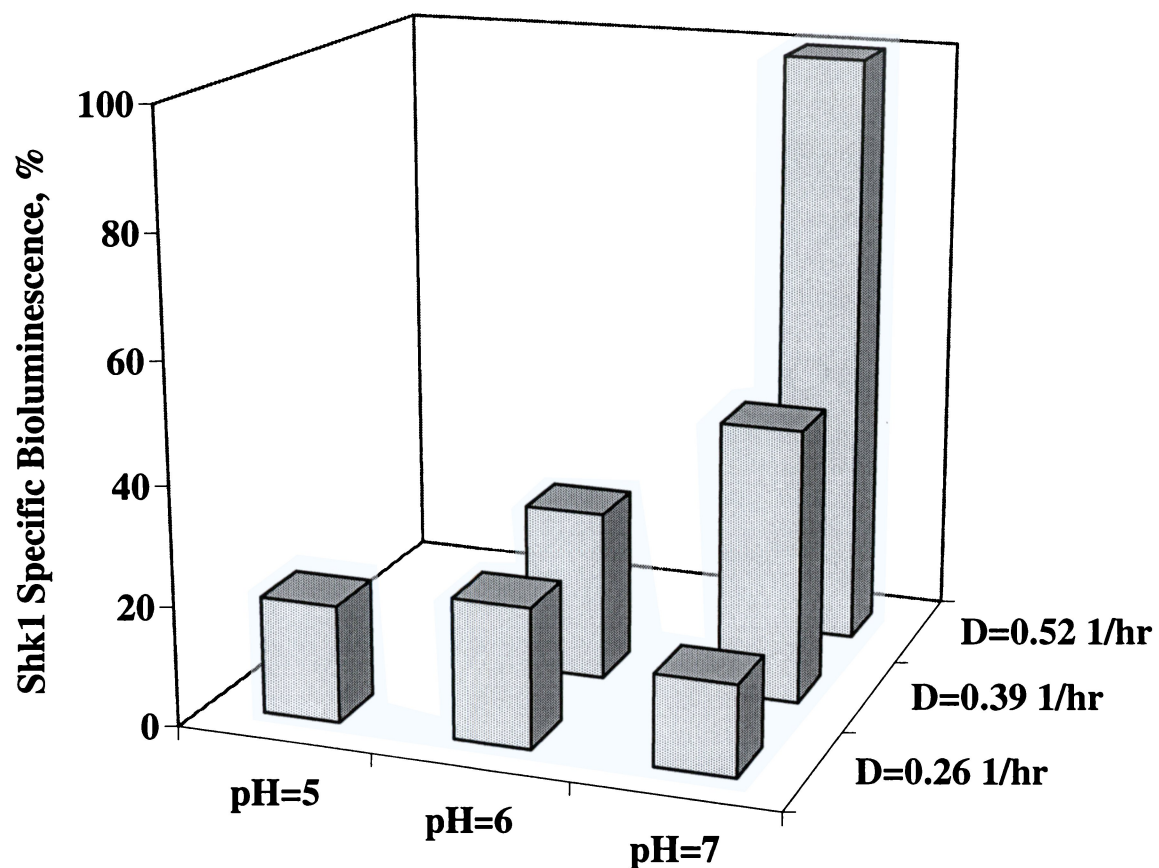


Figure 3-8. Dependence of specific bioluminescence on dilution rate and pH at 22°C. Each bar shows the average of at least 3 replicates. Specific bioluminescence is shown as the percentage of the highest average value obtained in all experiments of continuous cultures.

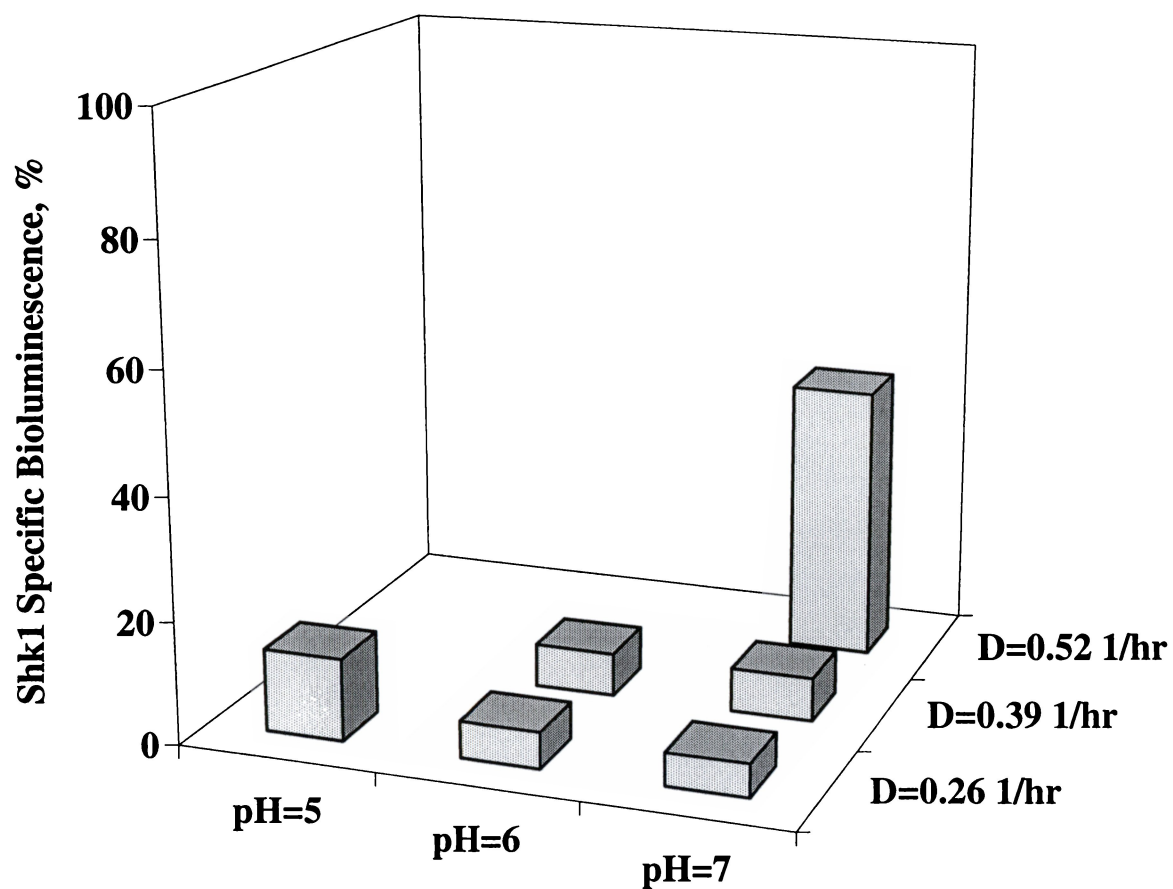


Figure 3-9. Dependence of specific bioluminescence on dilution rate and pH at 26°C. Each bar shows the average of at least 3 replicates. Specific bioluminescence is shown as the percentage of the highest average value obtained in all experiments of continuous cultures.

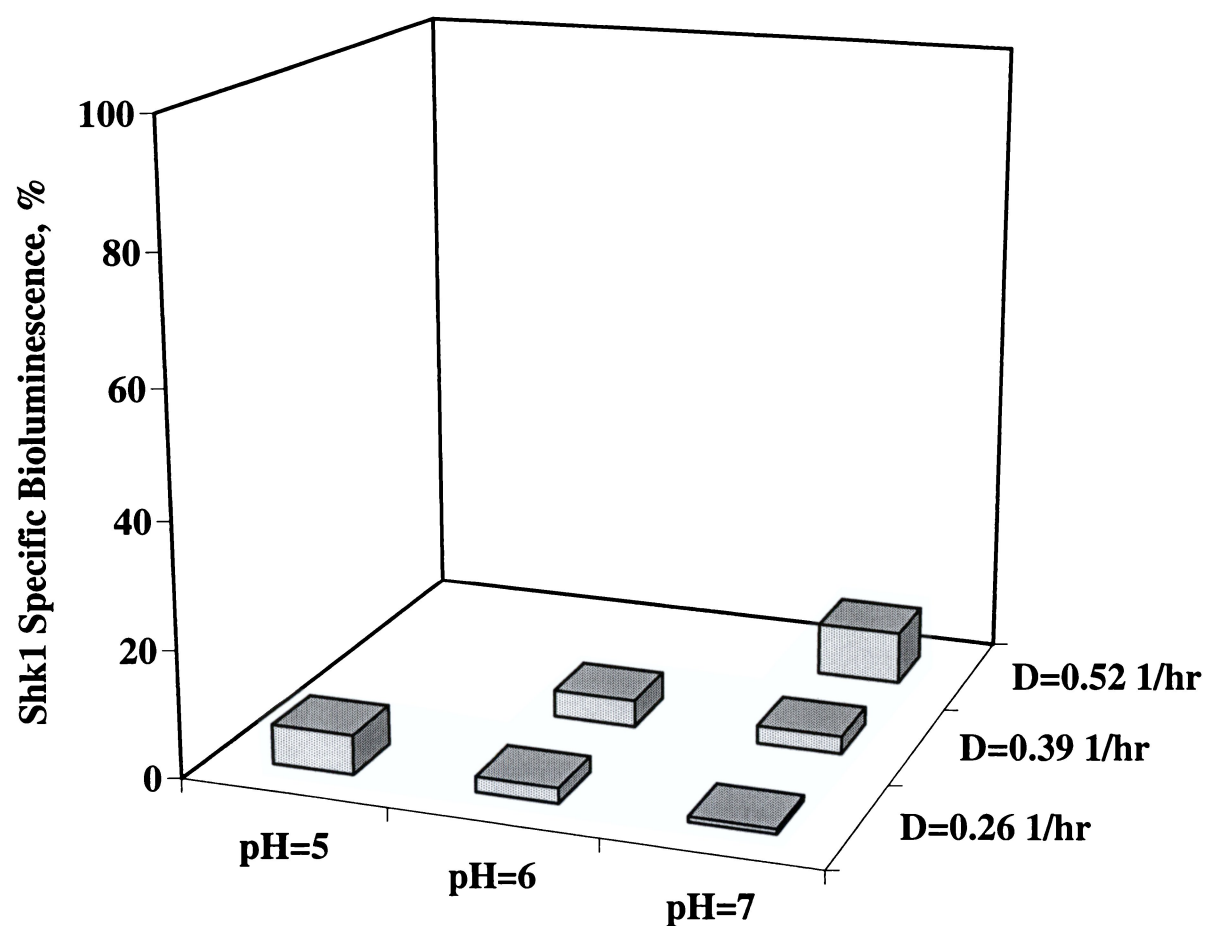


Figure 3-10. Dependence of specific bioluminescence on dilution rate and pH at 30°C. Each bar shows the average of at least 3 replicates. Specific bioluminescence is shown as the percentage of the highest average value obtained in all experiments of continuous cultures.

D = dilution rate, hr^{-1} , in the range of 0.26 hr^{-1} - 0.52 hr^{-1}
T = temperature, $^{\circ}\text{C}$, in the range of 18°C - 34°C
pH in the range of 5-8.

Based on this correlation, a plot showing the normalized predicted specific bioluminescence and normalized experimental specific bioluminescence is shown in Figure 3-11. Since the scaling of I by the constant k is arbitrary, we normalized the experimental specific bioluminescence with the highest value obtained ($D=0.52 \text{ hr}^{-1}$, $\text{pH}=7$, and $T=22^{\circ}\text{C}$, see Figure 10) and plotted I/I_{max} versus the normalized experimental specific bioluminescence in Figure 3-11. As Figure 3-11 shows, the correlation given by Equation 1 is useful for obtaining a rough prediction of the specific bioluminescence. A linear regression of the data in Figure 3-11 yields

$$-\log(I_{\text{predicted}}/I_{\text{max, predicted}}) = -0.5638 * \log(I_{\text{experimental}}/I_{\text{max, experimental}}) + 0.0576 \quad (3-2)$$

with $r^2 = 0.80$.

Effect of Phosphate Salts

The effect of phosphate salts is shown in Figure 3-12. Each bar represents the average of the results of two replicate experiments. Specific bioluminescence is shown as the percentage of the highest value obtained in the study of the effect of phosphate salts. Our data show that the presence of phosphate salts does not negatively affect Shk1 bioluminescence, and in some cases, phosphate salts increase specific bioluminescence.

Discussion

The key elements involved in bioluminescent reactions are luciferase, aldehyde, and molecular oxygen. Luciferase is synthesized by transcription and translation of the

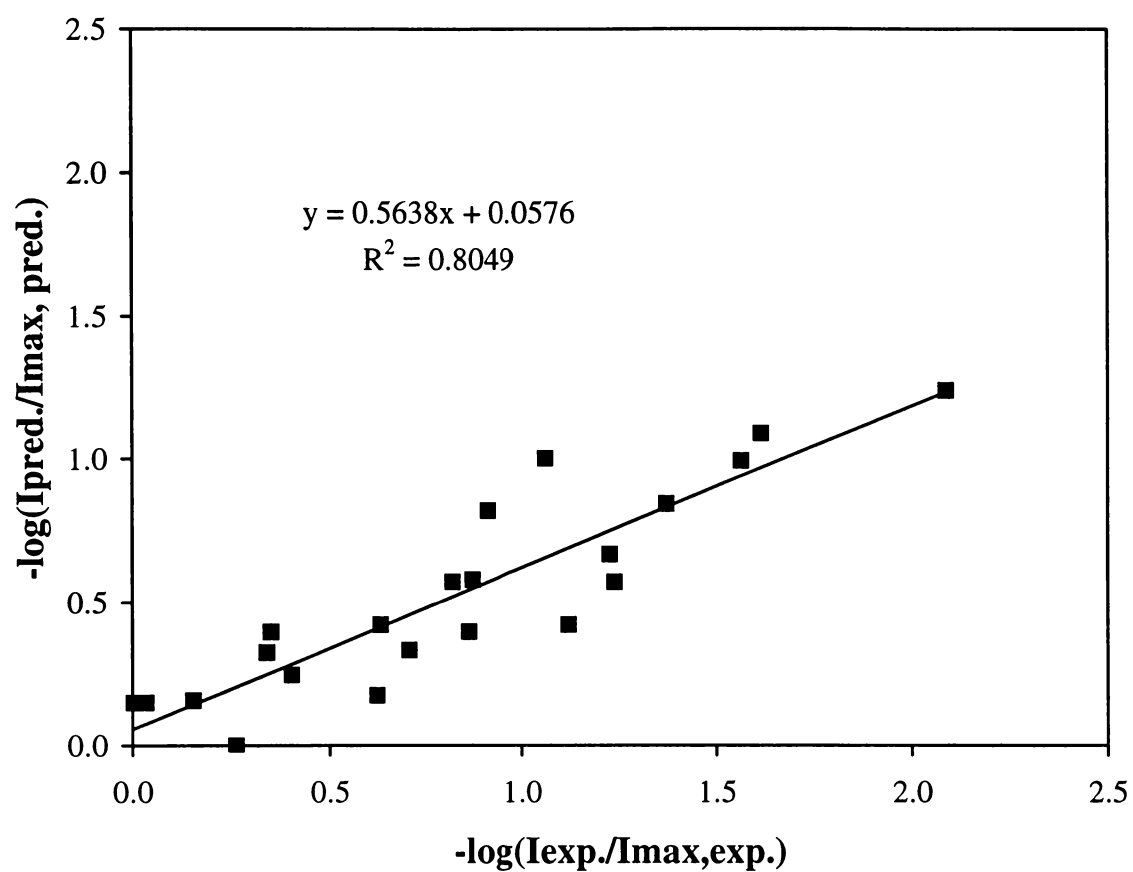


Figure 3-11. Correlation between the normalized predicted specific bioluminescence and the normalized experimental specific bioluminescence of Shk1.

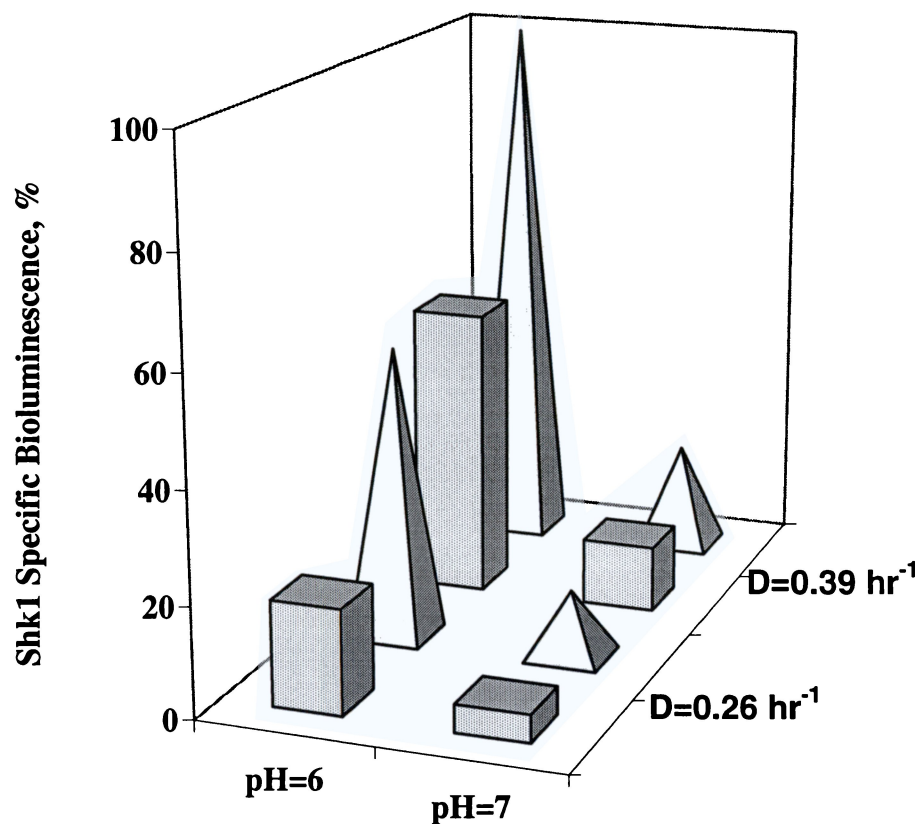


Figure 3-12. Effect of phosphate salts on specific bioluminescence. Square columns show the specific bioluminescence of Shk1 grown in medium without phosphate salt. Pyramids show the specific bioluminescence of Shk1 grown in medium with phosphate salts. Each square or pyramid shows the average of 2 replicates. Specific bioluminescence is shown as the percentage of the highest average value obtained in all experiments.

lux gene. Aldehyde is provided by biochemical reactions of cellular synthesis. Molecular oxygen is provided by dissolved oxygen in the culture medium and its concentration in the cells depends on the transport of oxygen across the cell membrane. The dissolved oxygen concentration need not be high to maintain a strong light emission. It has been shown that bacterial luminescence will be at its maximum intensity if the respiratory oxygen uptake rate is as little as 10% of normal (Hastings et al., 1985). For Shk1, Kelly (1997) showed that the dissolved oxygen concentration does not affect light production of Shk1 unless it is lower than 0.2 mg/L, or roughly 3% of its saturation value at 30°C. As stated previously, the dissolved oxygen concentration in the experiments did not drop below 50% of its saturation value at 30°C. As a result, bioluminescence was not limited by the dissolved oxygen concentration in our study.

Either due to depletion of some substrates or the accumulation of potential inhibitory substances, specific bioluminescence of Shk1 reaches a peak and then decreases in batch cultures. Comparing the growth curves and specific bioluminescence curves shown in Figures 3-2 to 3-6, the peak specific bioluminescence occurred in the mid- to late exponential growth phase. This phenomenon conforms to the findings of Farghaly (1950), Frackman et al.(1990) and Eaton et al. (1993) who used *Achromobacter fischeri*, *Xenorhabdus luminescens* and strains of *Lactococcus lactis*, respectively. The results of this study indicate that light production might be at least qualitatively correlated to cell growth of Shk1 as measured by optical density up to the point where the specific bioluminescence peaked. After that point, cells entered into mid- to late exponential or stationary phase and optical density was relatively stable; however bioluminescence dropped quickly and no correlation exists. Marincs (2000), using genetically engineered

Escherichia coli, reported that bioluminescence and cell number expressed as colony forming units were correlated during every growth phase (lag, exponential, deceleration, stationary, and death phases). Although specific bioluminescence was not calculated in his study, one can infer that specific bioluminescence must have been constant during each growth phase if total bioluminescence was correlated to cell growth. As discussed earlier, bioluminescence is closely related to cellular respiration and is dependent on cellular physiology. During the different growth phases, cells are in different physiological states. It is not clear why the specific bioluminescence of the *E.coli* strain remained constant as cells grew. However, since the lag and exponential growth phase of the *E.coli* strain were short as compared to the stationary phase, and bioluminescence reached either the maximum or a constant level fairly quickly during the exponential phase, only sparse data of cell growth and luminescence were obtained and shown in these growth phases and it is difficult to ascertain whether specific bioluminescence indeed remained constant.

Compared to batch cultures, continuous cultures have several advantages for use in a monitoring system. First, due to the continuous replenishment of fresh medium and removal of any potentially inhibitory substances, light is produced continuously in continuous cultures. Secondly, the dependence of Shk1 specific bioluminescence on dilution rate, pH, and temperature has been studied, and we have shown that specific bioluminescence can be closely controlled in continuous cultures. A third advantage of continuous cultures is that the light level is consistent, which makes possible the use of bioluminescence in process monitoring systems. Bioluminescence in continuous cultures is continuous and constant, and if the changes in bioluminescence as cells are exposed to

changing process conditions could be correlated to changes in other process parameters that are time-consuming to measure, bioluminescence alone could be used in place of those process parameters. The use of batch cultures in continuous monitoring is more complicated since they are, by their nature, dynamic.

The optimum temperature and pH for cell growth differ from those for specific bioluminescence. As Table 3-1 in **Results** show, at a fixed temperature of 30°C, the pH optimum for maximum specific growth rate is 7, but that for specific bioluminescence is 5. Similarly, at a fixed pH of 7, the temperature optimum for maximum specific growth rate is 30°C, but that for specific bioluminescence is 18°C. The general effects of temperature and pH on the growth of microorganisms have been well studied and are not discussed here. As for bioluminescence, there is an optimum temperature and an optimum pH for bioluminescence. Harvey (1952) showed the dependence of the bioluminescence of *Photobacterium phosphoreum* on temperature and pH. The curves had a characteristic bell-like shape, with bioluminescence being the highest at the optimum temperature or pH and lower at other temperatures or pH values. However, the mechanisms by which temperature and pH affect bioluminescence are not the same. Below the optimum temperature, increasing the temperature increases the rate of a reaction involved in bioluminescence, and bioluminescence increases with temperature. Above the optimum temperature, increasing the temperature increases the rate of deactivation of luciferase. As a result, bioluminescence decreases as temperature increases above the optimum. The effect of pH is not as clear. The H⁺ ions and the OH⁻ ions may combine with the luciferase above and below the pH optimum and reduce its catalytic activity (Harvey, 1952).

The specific growth rate of cells grown at steady-state in a CSTR equals the dilution rate. Using a CSTR, we found that Shk1 specific bioluminescence increases as dilution rate (or specific growth rate) increases at fixed temperatures and pH (see Figures 3-7 to 3-10). The correlation based on CSTR data to describe the dependence of specific bioluminescence on specific growth rate, pH, and temperature was given by Equation 3-2 in **Results**. The dilution rate is raised to a power other than 1 based on the observation that specific bioluminescence does not increase proportionally as dilution rate increases (see Figures 3-7 to 3-10). The terms containing (8-pH) and (34-T) are chosen to describe the fact that the specific bioluminescence is low and is regarded as 0 at a pH of 8 or a temperature of 34°C and that specific bioluminescence generally increases as pH and temperature deviate from 8 and 34°C respectively toward the lower values. They are also exponentiated to describe the hyperbolic shape of the bell-shaped curves (above optimum regions) that show the dependence of bioluminescence on pH and temperature (discussed earlier). The correlation is simple and does not imply any mechanisms. It does not take into consideration enzyme activity, intracellular substrate concentrations, or other cellular level factors. The correlation is not valid for pH values below 4 or above 8 and temperatures below 14°C or above 34°C. It cannot account for the decrease in specific bioluminescence at pH or temperature below the optima. As mentioned previously, there is some ambiguity in the effect of pH in continuous cultures. Since the term (8-pH) was exponentiated to a power of 0.5 (<1), the unaccountability of the effect of pH was reduced. Figure 3-11 shows that the correlation can be used to roughly predict specific bioluminescence in the variable space addressed in this study.

In addition to specific growth rate, temperature and pH, bioluminescence is also affected by the ionic strength. The presence of phosphate salts in the growth medium increases the ionic strength as well as the buffering capacity of the medium. The effect of salts on bacterial luminescence has been study by different researchers and the findings are contradictory (Harvey, 1952). In our study, phosphate salts were selected, which we postulated would not inhibit Shk1 bioluminescence. Johnson et al. (1945) found that phosphate buffers allowed the highest light emission of *Photobacterium phosphoreum*. Although Shk1 is quite different from *P. phosphoreum*, phosphate salts did not negatively affect its specific bioluminescence but in some cases increased specific bioluminescence as it did in the study of Johnson et al. (1945).

CHAPTER 4

TOXICITY SCREENING OF HEAVY METALS

Heavy metal ions are known to be toxic to activated sludge microorganisms. Unfortunately, heavy metals are most often found in municipal wastewater at concentrations that can adversely affect the activated sludge process (Russel et al., 1982). The toxic effects of heavy metal ions are often indicated either by inhibition to activated sludge specific oxygen uptake rate (SOUR), ammomium uptake rate (AUR), or by inhibition to microbial growth. A number of researchers have studied the toxic effect of individual heavy metals to activated sludge processes. For example, Madoni et al. (1999) showed the toxic effects of five heavy metals (Cd^{2+} , Cr^{6+} , Pb^{2+} , Zn^{2+} , and Cu^{2+}) to heterotrophic and nitrifying bacteria by SOUR and AUR tests. In another study, the same group (Madoni et al., 1996) showed the toxic effects of the same five heavy metals to activated sludge protozoan communities. Benmoussa et al. (1986), Tomlinson et al. (1966) and Loveless et al. (1968) studied the inhibitory effects of heavy metals to activated sludge nitrification and the growth of a *Nitrosomonas* strain. Mazierski et al. (1995) found that the addition of Cr^{6+} decreased the maximum growth rate of activated sludge bacteria and Yetis and Gokcay (1989) found that Ni^{2+} at a concentration of 25 mg/L caused unpredictable oscillations in the growth rate of activated sludge microorganisms.

The rapid Shk1-based toxicity screening system was developed based on a bioreactor operated as CSTR (see **Methodology**). Using this system, the EC_{50} values of

eight heavy metals were obtained and compared to the data from the open literature. Realizing that activated sludge microorganisms are sometimes exposed to a mixture of heavy metals, and that mixtures of heavy metals may significantly increase or decrease the effects of single heavy metals, the toxicity of binary heavy metal mixtures was studied using the same system.

Methodology

Chemicals

All chemicals used in the toxicity tests were obtained from Sigma-Aldrich. Stock solutions of the compounds to be tested were prepared by dissolving salts of the metals to be tested in distilled water. Assay solutions were prepared by making serial dilutions of the stock solutions to obtain the desired concentrations of the test compounds.

Rapid toxicity screening system

The rapid toxicity screening system was similar to that described in the preliminary studies and is shown in Figure 4-1. The bioreactor and light detection equipment were also described in Chapter 2: Preliminary Studies. The light detection equipment (light sensors) used in the rapid toxicity screening system was shown in Figure 2-2.

Optimization of Mixing Ratio

The mixing ratio is defined as the ratio of the volumetric flow rate of the test material solution to the flow rate of the Shk1 cell solution from the bioreactor. The different mixing ratios were achieved by fixing the flow rate of the Shk1 cell solution at 200 ml/hr while varying the flow rate of the test material solution.

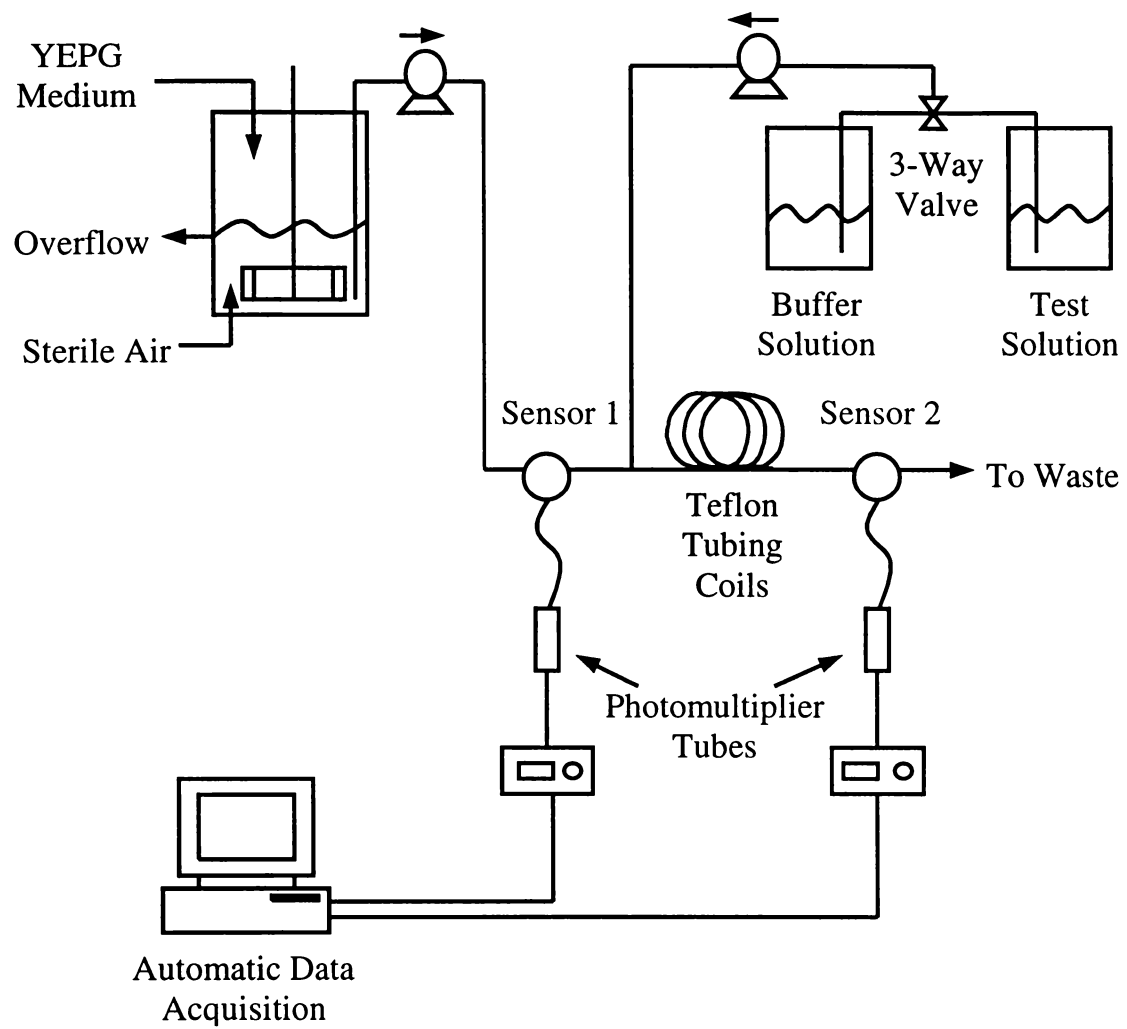


Figure 4-1. Schematic of the laboratory-scale rapid toxicity screening system.

Experiments were performed with Shk1 cells grown in the CSTR with the following conditions: dilution rate = 0.52 hr^{-1} , pH = 7, and temperature = 22°C . These conditions were chosen as optimal conditions for light emission based on previous studies (Chapter 3). Shk1 cells were supplied continuously to the two light sensors (see Figure 4-1). The first (upstream) sensor was used to determine the basal light level of the cells before exposure to the toxicant. After leaving the first sensor, the cells entered one leg of a "tee" connector where they were mixed with either the test material solution or a buffer solution. The concentrations of the test materials in the mixed stream will be termed the post-contact concentrations in this study. The mixture of Shk1 cells and test solution then proceeded through several coils of tubing to achieve an appropriate residence time (5 minutes) before entering a second light sensor. The buffer solution ($3.03 \text{ g/L K}_2\text{HPO}_4$ and $5 \text{ g/L KH}_2\text{PO}_4$) was used to normalize the effect of dilution on the light level in the second sensor (see Equation 4-1). The buffer gave a pH of 7, which equaled the pH in the bioreactor when the experiments to optimize the mixing ratio were performed (pH was varied in some experiments as discussed later).

All toxicity experiments, including those performed to determine an appropriate mixing ratio, were performed in the following way. The Shk1 cell solution (pumped continuously from the CSTR) was alternately mixed with buffer solution and test material solution using a manually operated 3-way valve (see Figure 4-1). The bioluminescence of Shk1 exposed to heavy metals was measured 5 minutes after the 3-way valve was turned to feed the test toxicant solution to the mixing tee.

The toxicity of the test material (ZnCl_2) was reflected by the repression of bioluminescence as defined by Equation 4-1.

$$\text{Bioluminescence repression} = \frac{B_b - B_t}{B_b} \times 100\% \quad (4-1)$$

where B_b = bioluminescence of Shk1 mixed with buffer

B_t = bioluminescence of Shk1 mixed with test solution, measured 5 minutes after Shk1 cell solution was mixed with the test material solution.

To determine an appropriate mixing ratio, the aqueous test solution used when optimizing the mixing ratio contained 10 mg/L ZnCl_2 and the mixing ratios (as defined previously) were 1, 2, and 3. Bioluminescence repression using the three mixing ratios were calculated and compared.

Optimization of Cell Growing Conditions

Shk1 cells can be grown under a wide variety of values of dilution rate, pH, and temperature in the CSTR. In Chapter 3, we determined that the highest and lowest specific bioluminescence (bioluminescence/optical density) were obtained under the conditions shown in Table 4-1.

Since it was not known *a priori* if low or high specific bioluminescence would result in the most effective test conditions, toxicity test experiments were performed with Shk1 cells grown in the CSTR with each of the conditions shown in Table 4-1. The residence time in the tubing after the mixing tee and before the second light sensor was 5

Table 4-1. Conditions under which high and low specific bioluminescence were obtained.

Condition	Dilution rate, hr^{-1}	pH	Temperature, $^{\circ}\text{C}$	Specific Bioluminescence
1	0.52	7	22	High
2	0.52	7	30	Low
3	0.26	5	18	High

Table 4-2. Buffer compositions.

Desired pH	K ₂ HPO ₄ , g/L	KH ₂ PO ₄ , g/L
5	3.08	0
7	3.03	5

minutes and the mixing ratio was set at 3 (3 parts test solution to 1 part cell solution). Since the pH in the bioreactor was maintained at two different values (7 for conditions 1 and 2, and 5 for condition 3), two buffer solutions containing K₂HPO₄ and KH₂PO₄ were used. The concentrations of K₂HPO₄ and KH₂PO₄ that gave the desired pH values are given in Table 4-2.

The test toxicants were aqueous solutions of CuCl₂, PbCl₂, and CdSO₄. Toxicity tests using these three heavy metal solutions were performed according to the method described earlier at a range of heavy metal concentrations. The experiments at each toxicant concentration were performed with 3 replicates. Bioluminescence repression of Shk1 (as defined by Equation 4-1) due to exposure to these heavy metal solutions was calculated and plotted against post-contact heavy metal ion concentrations. The 50% effective concentration (EC₅₀) values for heavy metals were obtained from a least-square regression of the relatively linear portions of the concentration-response curves. EC₅₀ values for the heavy metal ions represent the post-contact concentrations of the ions that caused the light level detected by sensor 2 to drop by 50% of the light level recorded when buffer alone was being fed to the mixing tee.

Assays of Single Compounds

Seven heavy metals were represented in the assays of single compounds. Aqueous solutions of salts of the metals were used and included CdSO₄, CuCl₂, PbCl₂, NiCl₂, HgCl₂, Cr(H₂O)₆Cl₃, CrO₃ and ZnCl₂. In the case of individual heavy metal

compounds, the toxicants were considered to be the cations and all concentrations are expressed on the basis of the cation concentration. No pH adjustments of the test material solutions were made since this can lead to precipitation of some of the heavy metals. In our experiments, no precipitation was observed. Assays of single compounds were performed in triplicates using the test method discussed earlier. Bioluminescence repression was calculated according to Equation 4-1 and was plotted against the post-contact concentrations of the heavy metals. EC_{50} values for single heavy metal ions were obtained according to the method described above.

Assays of Binary Mixtures

The tests of binary mixtures were carried out by maintaining the concentration of one heavy metal ion in the mixed stream (after the mixing tee, see Figure 4-1) at its EC_{50} concentration and varying the concentration of the other heavy metal ion. The metal ion pairs used in this part of the study can be found in Table 4-5. Assays of binary mixtures were performed in triplicates using the procedure described above. Bioluminescence repression was calculated according to Equation 1 and was plotted against post-contact concentrations of the heavy metals whose concentration was varied. The EC_{50} values of the heavy metals whose concentrations were varied in the binary mixtures were obtained by a least-square regression of the relatively linear portion of the concentration-response curves. Since the concentration of one component was held constant at its individual EC_{50} , the EC_{50} of the other component in the mixture was the concentration at which the bioluminescence was repressed by a total of 75% below its level in the absence of both contaminants.

The interaction of heavy metals in each test mixture can be assessed as additive, antagonistic, or synergistic by statistical testing of the difference between the observed EC_{50} of a heavy metal in binary mixture and its individual EC_{50} . The testing is based on basic concepts of statistics and is discussed by Johnson (1976). The null hypothesis for this test is defined as

$$H_0: \beta_1 = m \quad \text{where } m = EC_{50} \text{ of single heavy metals}$$

and the alternative hypothesis is written as

$$H_a: \beta_1 > m \text{ or } H_a: \beta_1 < m$$

depending on the expected behavior of β_1 if the null hypothesis is not true. The statistical testing is performed by calculating t , which is defined by

$$t = \frac{\hat{\beta}_1 - m}{s(\hat{\beta}_1)} \quad (4-2)$$

where $\hat{\beta}_1 = EC_{50}$ of a heavy metal in binary mixture

$s(\hat{\beta}_1)$ = standard error of $\hat{\beta}_1$

and by comparing the calculated t with tabulated t value, $t_{\text{tabulated}}$. If $t > t_{\text{tabulated}}$, the interaction is antagonistic, implying that the toxicity of the binary mixture is lower than additive toxicity; if $t < -t_{\text{tabulated}}$, the interaction is synergistic, implying that the toxicity of the binary mixture is higher than additive toxicity; and if $-t_{\text{tabulated}} < t < t_{\text{tabulated}}$, the interaction is simple additive.

Results

Optimization of Mixing Ratio

After being exposed to a 10 mg/L ZnCl_2 solution with a residence time after the mixing tee of 5 minutes at mixing ratios of 1 and 2, almost no bioluminescence repression was observed. However, a significant reduction in bioluminescence (a repression of 75%) was observed when the mixing ratio was increased to 3.

Optimization of Cell Growing Conditions

The response of Shk1 cells grown under three different conditions to three of the heavy metals (Pb^{2+} , Cd^{2+} , and Cu^{2+}) is shown in Figures 4-2 to 4-4 in the form of concentration-response curves. As can be seen in the figures, the toxic effects of Cd^{2+} and Pb^{2+} were greatly reduced for condition 3 (low dilution rate, high bioluminescence) when compared to conditions 1 and 2 (high dilution rate, high and low bioluminescence, respectively, see Table 4-1 for a complete description of the conditions). The toxic effect of Cu^{2+} under condition 3 was also reduced when the Cu^{2+} concentration was below 50 mg/L leading to a higher EC_{50} value. When the Cu^{2+} concentration was higher than 50 mg/L, this reduced toxic effect for condition 3 was not observed. Comparing results obtained under conditions 1 and 2 for all three metals, we found that under condition 1 and at low heavy metal concentrations, bioluminescence was higher (bioluminescence repression was lower). Under condition 2, bioluminescence repression increased more rapidly as the heavy metal concentration increased, resulting in a steeper slope of the concentration-response curve. We also observed that under condition 2, near 100% bioluminescence repression was achieved at high concentrations, while under condition 1, only about 80 - 90% bioluminescence repression was achieved even at the highest

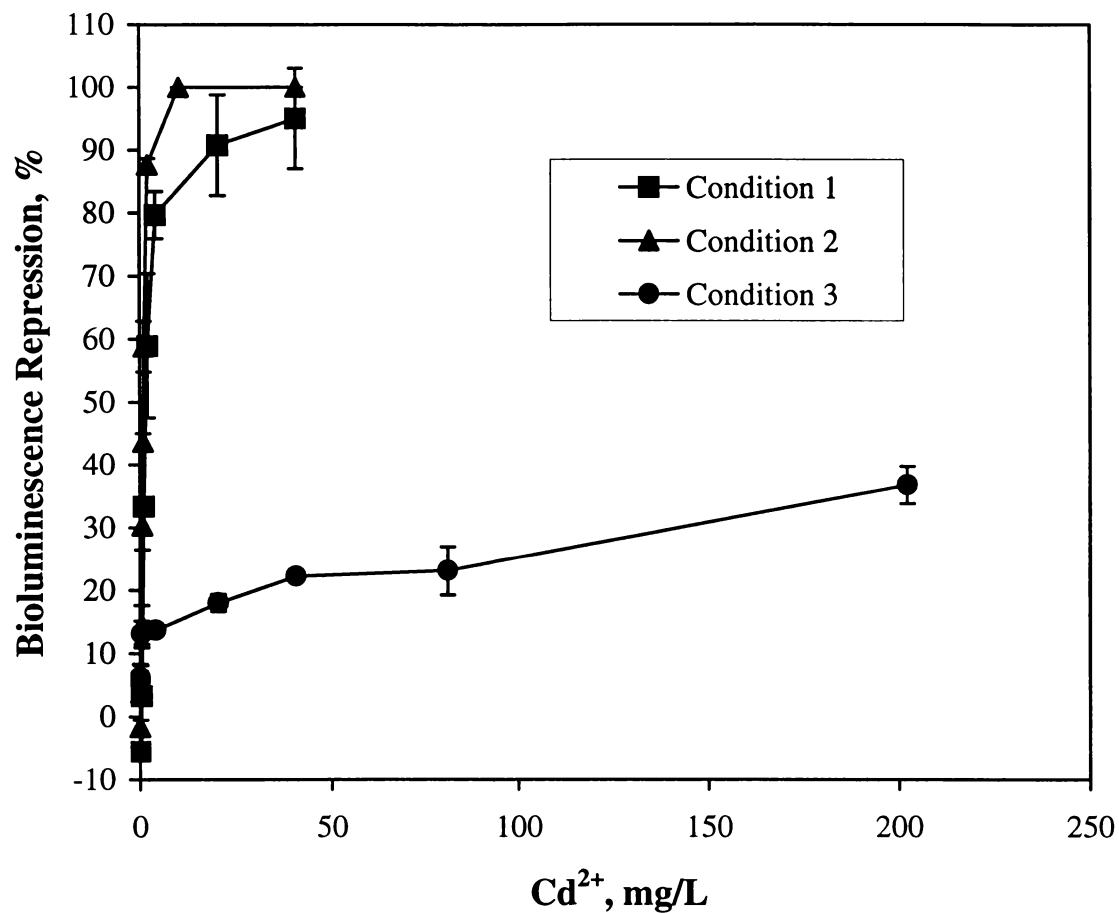


Figure 4 –2. Bioluminescence response to Cd²⁺ of Shk1 grown under 3 sets of different conditions.

Condition 1: Dilution rate = 0.52 hr⁻¹, pH = 7, temperature = 22 °C

Condition 2: Dilution rate = 0.26 hr⁻¹, pH = 7, temperature = 30 °C

Condition 3: Dilution rate = 0.26 hr⁻¹, pH = 5, temperature = 18 °C

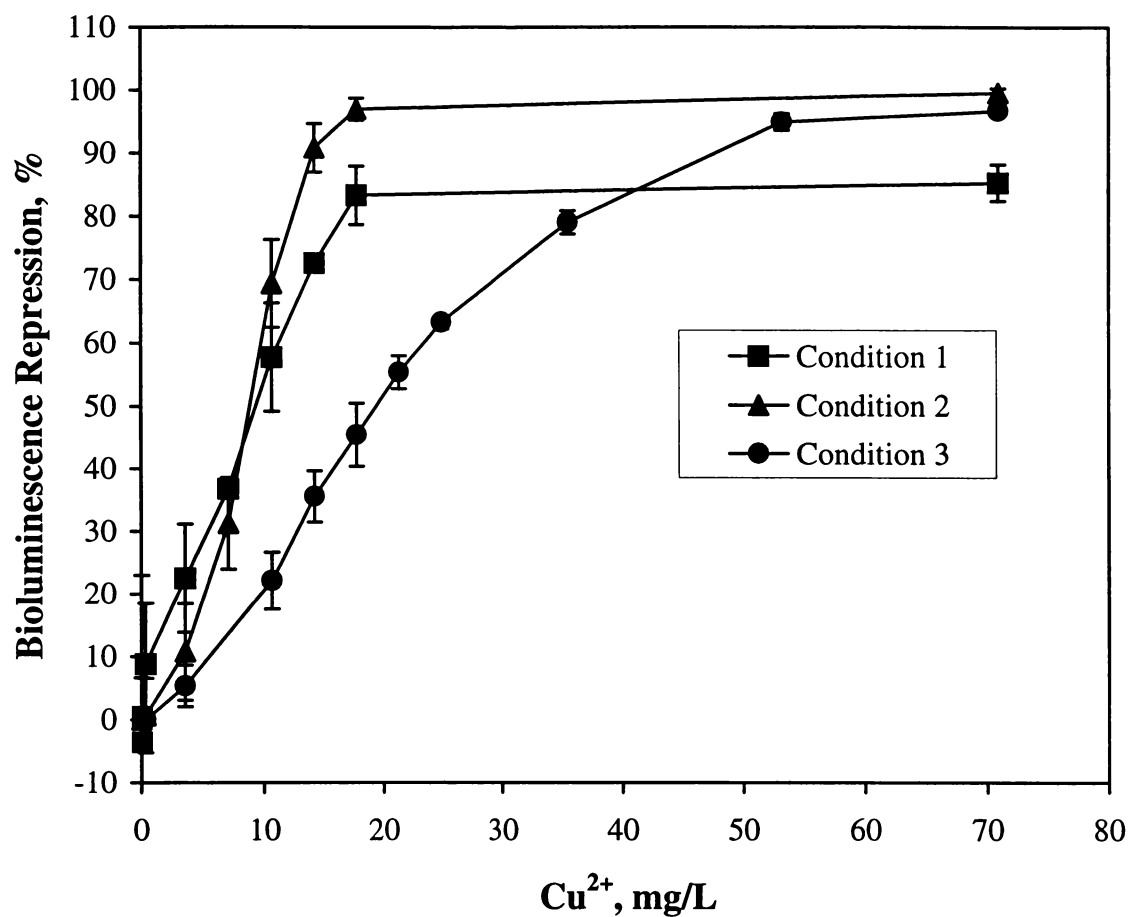


Figure 4-3. Bioluminescence response to Cu^{2+} of Shk1 grown under 3 sets of different conditions.

Condition 1: Dilution rate = 0.52 hr^{-1} , pH = 7, temperature = 22°C

Condition 2: Dilution rate = 0.26 hr^{-1} , pH = 7, temperature = 30°C

Condition 3: Dilution rate = 0.26 hr^{-1} , pH = 5, temperature = 18°C

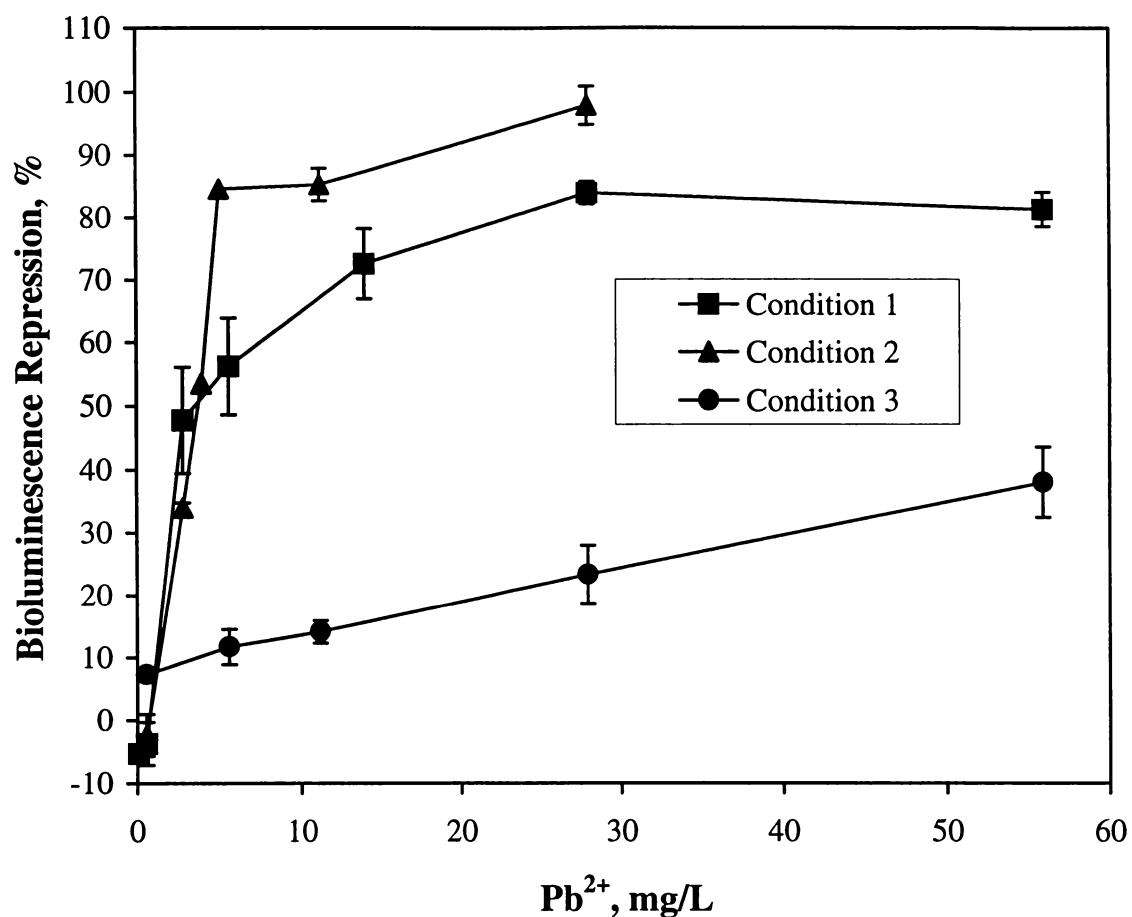


Figure 4-4. Bioluminescence response to Pb²⁺ of Shk1 grown under 3 sets of different conditions.

Condition 1: Dilution rate = 0.52 hr⁻¹, pH = 7, temperature = 22 °C

Condition 2: Dilution rate = 0.26 hr⁻¹, pH = 7, temperature = 30 °C

Condition 3: Dilution rate = 0.26 hr⁻¹, pH = 5, temperature = 18 °C

concentrations of the heavy metals. The post-contact EC₅₀ values for Cd²⁺, Cu²⁺, and Pb²⁺ under conditions 1, 2, and 3 were obtained from a linear regression of the relatively linear portion of the curves and are listed in Table 4-3.

Assays of Single Compounds

The results of the triplicate bioassays of seven binary mixtures of heavy metals were highly reproducible between replicates. At very low concentrations, it was observed that the exposure of Shk1 to five of the heavy metals (Cr³⁺, Cu²⁺, Ni²⁺, Hg²⁺, and Zn²⁺) led to negative bioluminescence repression (i.e., bioluminescence increased). The concentration-response curves (see Figures 4-2 to 4-4 for examples) exhibit a saturating behavior; initially bioluminescence repression increases quickly as the

Table 4-3. EC₅₀ values of Cd²⁺, Cu²⁺, and Pb²⁺ under the three sets of conditions.

	EC ₅₀ , mg/L (Condition 1)	EC ₅₀ , mg/L (Condition 2)	EC ₅₀ , mg/L (Condition 3)
Cd ²⁺	1.9	0.9	> 188
Cu ²⁺	9.4	8.8	19.7
Pb ²⁺	3.8	4.8	> 53

Table 4-4. Comparison of Shk1 EC₅₀ and Microtox[®] EC₅₀ and effect to activated sludge (AS) of heavy metals.

Heavy metal ion	Compound used	5 min EC ₅₀ , mg/L		Concentration s of heavy metals that affect AS process, mg/L	1 hr LC ₅₀ of ciliate species found in activated sludge ⁷ , mg/L
		This study (mean and 95% confidence intervals)	Microtox [®] ¹		
Cd ²⁺	CdSO ₄	1.9 (1.2-2.6)	98	≈ 12.5 ⁵	<0.13-3.81
Cu ²⁺	CuCl ₂	9.4 (8.6-10.1)	0.72-2.46	29 ^{2a} , 10 ³	0.31-2.05
Pb ²⁺	PbCl ₂	3.8 (3.2-4.4)	2.56	350 ^{2b} ,	<1.50-5.00
Ni ²⁺	NiCl ₂	96 (88-103)	917-1056	4.3 ^{2c} , 10 ³	-
Hg ²⁺	HgCl ₂	0.9 (0.8-1.1)	0.064	4.5 ²	-
Cr ³⁺	Cr(H ₂ O) ₆ Cl ₃	303 (299-308)	10.7	-	-
Cr ⁶⁺	CrO ₃	38.9 (33.8-43.4)	-	25 ⁴ , 0-11 ⁶	10.6-211
Zn ²⁺	ZnCl ₂	1.9 (1.5-2.2)	13.8-55.5	1.2 ² , 10 ³	0.57-84.7

- 1: Kaiser and Devillers, 1994.
- 2a: Dutka and Kwan, 1984.
- 2b: Dutka and Kwan, 1984. Most resistant data.
- 2c: Dutka and Kwan, 1984. Most sensitive data.
- 3: Yetis and Gokcay, 1989.
- 4: Gokcay and Yetis, 1991.
- 5: Estimated from data by Madoni et al., 1999.
- 6: Mazierski, 1995.
- 7: Madoni, 1996.

concentration of heavy metals increases with the response typically saturating at higher concentrations. We determined the EC₅₀ values of eight heavy metals and these are shown in Table 4-4. Also listed are EC₅₀ values from the literature for the Microtox[®] assay and concentrations of heavy metals that have been reported to cause significant adverse affects in activated sludge processes.

Assays of Binary Mixtures

The results of the triplicate bioassays of seven binary mixtures of heavy metals were highly reproducible between replicates. The individual EC₅₀ and EC₅₀ values in binary mixtures that we determined are listed in Table 4-5.

The type of interaction (synergistic or antagonistic) was determined based on the

Table 4-5. Observed EC₅₀ values for varying components in binary mixtures and the interactions of heavy metal ion pairs.

Fixed component	Varying component	Individual EC ₅₀ , mg/L	EC ₅₀ in mixture, mg/L (mean and 95% confidence intervals)	Interaction
Cd ²⁺	Cu ²⁺	9.4 (Cu ²⁺)	5.6 (4.0-7.1)	+
Cu ²⁺	Cd ²⁺	1.9 (Cd ²⁺)	3.4 (1.8-4.9)	-
Cd ²⁺	Pb ²⁺	3.8 (Pb ²⁺)	4.6 (1.5-7.5)	0
Pb ²⁺	Cd ²⁺	1.9 (Cd ²⁺)	1.7 (1.4-2.0)	0
Cu ²⁺	Pb ²⁺	3.8 (Pb ²⁺)	9.5 (8.6-10.5)	-
Pb ²⁺	Cu ²⁺	9.4 (Cu ²⁺)	16.3 (10.8-21.8)	-
Cu ²⁺	Zn ²⁺	1.9 (Zn ²⁺)	2.9 (2.0-3.8)	0

+: synergistic, -: antagonistic, 0: additive

statistical procedure outlined earlier in **Methodology**.

Discussion

The 5 minute residence time after the mixing tee was not varied in this study, though longer residence times would be expected to increase the toxic effect of most heavy metals in toxicity tests (Kaiser and Devillers, 1994, Qureshi et al., 1984). However, for the purpose of detecting toxicants in wastewater in time to serve a preventative function, a quick response is needed. The residence time of 5 minutes was chosen as a compromise between a quick response and an adequately measurable effect.

The mixing ratio determines the post-contact concentrations of toxicants and bacterial cells in the mixture; the higher the mixing ratio, the higher the post-contact concentrations of the toxicants. It is not surprising therefore that significant bioluminescence repression was observed when the mixing ratio was 3 but not when it was 1 or 2. We did not increase the mixing ratio further because the mixing ratios beyond 3 led to problems with our system. High mixing ratios (corresponding to high pumping rates) increased the pressure in the tubing and caused problems with the tubing connections. Also, high mixing ratios lowered the light level by dilution, which decreased the effective signal-to-noise ratio.

The response of Shk1 to toxicants depends on the actual concentrations (in our study, the post-contact concentrations, i.e., the concentrations of the test compounds in the mixed stream after the mixing tee) of toxicants to which Shk1 cells are exposed. However, as shown in Figure 1-4, the toxicity monitoring system must be designed to detect toxicity in the influent wastewater stream. In this case, it is the toxicity in the

influent wastewater, or the concentration of toxicants in it, that is important. While the absolute sensitivity of Shk1 to toxicants remains unchanged, the apparent sensitivity of Shk1 to the toxicity of influent wastewater is increased by having a high mixing ratio. The field application of our system to influent wastewater toxicity monitoring will be addressed in future research, and the following discussions are based on post-contact concentrations of toxicants.

By growing Shk1 under different conditions of growth rate, pH, and temperature, we sought to determine the set of conditions that gave the most appropriate toxic response. Specifically, we were interested in determining the answers to two questions. First, was Shk1 more sensitive to toxicants under growth conditions that favor high or low light production? Second, what conditions yield toxic responses in Shk1 that are comparable to respirometry repression data in the literature? For the first question, there are two plausible hypotheses based on the fact that bioluminescence is directly related to the cellular function of respiration (Harvey, 1952). One could reason that for cells emitting high light levels (implying that their metabolism rates are high), a sudden exposure to toxicant molecules may result in the most obvious repression of bioluminescence. Conversely, one could reason that when cells are not emitting high light levels (implying they are not operating near their maximum metabolism rate) a sudden exposure to toxicant molecules may have a catastrophic effect that leads to a small absolute decrease in bioluminescence but a large percent change over the basal level. Based on the fact that the experimental results shown in Figures 4-6 obtained under conditions 1 and 2 were similar, it is not possible to offer a definitive answer to the question of whether low or high light levels might indicate more sensitivity by comparing

these two cases. However, there were still some differences that warrant discussion. When the concentrations of heavy metals were relatively low, bioluminescence repression was more significant under condition 1, showing a higher sensitivity of Shk1 cells to a sudden exposure to toxicants at low concentrations. When the concentration of heavy metals was at the upper end of the concentration ranges tested, bioluminescence repression was more significant under condition 2. Since the total bioluminescence (which depends on both specific bioluminescence and cell optical density) was used to calculate bioluminescence repression, we should point out that the difference in the density of cells grown under conditions 1 and 2 was negligible. The steady state optical densities (OD₆₀₀) of the cell solutions were around 0.5 for both conditions 1 and 2 and the amount of toxicant available to each cell was almost the same in both cases at all heavy metal concentrations.

Condition 3 (which also favored light production, but at a lower growth rate than condition 1) consistently indicated much lower sensitivity of Shk1 to the metals tested (therefore higher EC₅₀ values) than the other two sets of conditions (see Figures 4-2 to 4-4). As a result, the EC₅₀ value for Cd²⁺ under condition 3 would be higher than 202 mg/L, the highest concentration of Cd²⁺ used in the experiments (see Figure 4-2). This concentration (202 mg/L) is an order of magnitude higher than the concentration of Cd²⁺ that affects activated sludge processes (12.5 mg/L, shown in Table 4-5). For this reason, condition 3 should not be considered for an influent wastewater toxicity monitoring system.

Compared to the *Photobacterium phosphoreum* (Microtox[®]) assay, our experimental results (see again Table 4-4) show that, of the eight heavy metals tested,

three (Cd^{2+} , Ni^{2+} and Zn^{2+}) showed a Shk1 EC_{50} smaller than the *P. phosphoreum* EC_{50} values (both assays use a 5 minute contact time for the cells and the toxicants), and four (Cu^{2+} , Pb^{2+} , Hg^{2+} , and Cr^{3+}) showed a Shk1 EC_{50} larger than the *P. phosphoreum* EC_{50} . The *P. phosphoreum* EC_{50} value for Cr^{6+} is not available, so no comparison can be made. Most importantly, our data shows (see again Table 4-4) that Shk1 EC_{50} values for the metals tested are closer than *P. phosphoreum* EC_{50} values to the heavy metal concentrations that negatively affect activated sludge processes as reported by other researchers. These data suggest that Shk1 would make a more appropriate reporter for influent toxicity to activated sludge wastewater treatment systems.

Our data also suggests, however, that influent monitoring using Shk1 bioluminescence alone might not be a good predictor of plant performance for all metals. For example, the differences between the Shk1 EC_{50} values and concentrations that affect activated sludge processes are large for Ni^{2+} and Pb^{2+} (see Table 4-4). Ni^{2+} affects activated sludge processes at a relatively low concentration (below 10 mg/L, see Table 4), but the Shk1 EC_{50} value for Ni^{2+} is an order of magnitude higher (96 mg/L). In the case of Pb^{2+} , we should point out that the most resistant concentration of 350 mg/L was reported and listed in Table 4-4. Therefore it is reasonable to assume that significant inhibition to activated sludge could occur at lower Pb^{2+} concentrations. This would make the response of Shk1 a better predictor of plant performance for Pb^{2+} toxicity than might be inferred from Table 4-4. In summary, our data suggest that a Shk1-based monitoring system would be more appropriate than *P. phosphoreum* based systems, which uses an organism not native to wastewater treatment plants. This is because the response of Shk1, being originally isolated from an activated sludge environment, is more similar to

the respirometric response of activated sludge microorganisms. However, there are some metals for which Shk1 toxicity is significantly different from literature values for the inhibition of respiration of activated sludge microorganisms.

The literature on the relative toxicity of Cr^{3+} and Cr^{6+} is inconsistent. Some researchers have found that Cr^{3+} is far less toxic to activated sludge microorganisms than Cr^{6+} (Mertz, 1979, Imai and Gloyna, 1990), while the contrary was also found (Mowat, 1976). In our study, Cr^{3+} has a larger Shk1 EC_{50} (303 mg/L) than Cr^{6+} (38.9 mg/L), implying that Cr^{3+} is significantly less toxic than Cr^{6+} to Shk1.

The early research literature indicated that nitrifying bacteria are more sensitive to heavy metal toxicity when compared to heterotrophic bacteria (Stensel et al., 1976, Stoveland et al., 1979). However, more recent research in this area has indicated a more complex reality. For example, in one study, Blum and Speece (1992) showed that while *Nitrobacter* had the same tolerance to heavy metals, *Nitrosomonas* was ten times more vulnerable to heavy metals, while some researchers concluded that in the activated sludge, nitrifiers are not more sensitive than aerobic heterotrophs when exposed to heavy metals (Henze et al., 1995, Madoni et al., 1999). For this reason, no special attention was given in this study to the inhibition of nitrifying bacteria in activated sludge by heavy metals. However, we will address this issue in future studies.

In Table 4-4, the 1 hour LC_{50} values of ciliate species in activated sludge are shown. LC_{50} , or 50% lethal concentration, refers to the concentration of toxicants that causes 50% lethality. The toxicity of a particular metal differs significantly for different species. For example, *Opercularia minima* has an LC_{50} of 84.7 mg/L for Zn^{2+} , while *Vorticella octava* has an LC_{50} of 0.57 for the same metal (Madoni, 1996). Since ciliated

protozoa contribute over 9% to the activated sludge biomass (Madoni, 1994) and since some species exhibit an LC_{50} lower than 5 min Shk1 EC_{50} found in this study, caution should be exercised if Shk1 is to be used as a toxicity indicator to protect the protozoan community in activated sludge.

Qureshi et al. (1984) studied the effect of the interaction of heavy metals in binary mixtures. The authors drew an "idealized line of addition" in the concentration-percent decrease in light output diagram and called the region above the line the synergistic region and the region below the line antagonistic. Ince et al. (1999) used statistical methods to study the interaction of 4 heavy metals in binary mixtures of each of 34 combinations of concentrations. The authors adopted the concept of a "toxicity unit" and tested whether the difference of the toxicity unit of a binary mixture and the sum of the individual toxicity units were statistically significant or not. While this method is valuable in ecotoxicology, it is not very useful for the purpose of monitoring wastewater toxicity using Shk1 since the concentrations of heavy metals (and often the identities of the metals themselves) in wastewater are usually not known. Since the measured parameter of the Shk1-based method in this study is the repression of bioluminescence, we tested whether the EC_{50} of a heavy metal in a binary mixture is statistically larger or smaller than its EC_{50} when it exists alone.

Our data does not suggest a general rule for the effect of interaction of heavy metals in binary mixtures (see Table 4-5). This is not unexpected since it is known that different metal pairs exhibit different interaction patterns. Even for the same metal pair, the interaction is sometimes observed to change from synergism to antagonism and vice versa as the concentrations change (Qureshi et al., 1984, Ince, 1999), though strictly

synergistic or antagonistic patterns do exist (Qureshi et al., 1984). As Table 4-5 shows, in our study, the Cd^{2+} - Cu^{2+} pair shows both a synergistic and an antagonistic interaction, depending on the concentrations of the two metals, and the Cu^{2+} - Pb^{2+} pair has an antagonistic interaction. The other metals pairs have an additive interaction.

As discussed earlier, monitoring influent wastewater using Shk1 bioluminescence alone might not be a good predictor of plant performance for all metals. Studies have shown that each bacterial species and test procedure has its own sensitivity pattern to toxicants (Dutka and Kwan, 1981, 1982, Dutka, Nyholm and Peterson, 1983). The concept of a "battery" has therefore been proposed (Dutka and Kwan, 1984, Munawar et al., 1986, Ince et al., 1996). The battery concept involves using at least two bioassays with microorganisms of different origins for toxicity assessment in aquatic environment. A more detailed discussion of the use of a test battery will be made in Chapter 5. Nonetheless, in our study, the Shk1-based method has showed its advantage in influent wastewater toxicity monitoring in that its responses are quicker than respirometry-based or growth rate-based methods and its responses are more similar to those of the activated sludge microbial community than the responses of other bioluminescence-based methods.

CHAPTER 5

TOXICITY SCREENING OF ORGANIC CHEMICALS

Organic chemicals such as industrial solvents and polymer chemistry intermediates account for a large portion of wastewater toxicity. A number of synthetic organic chemicals have been identified in industrial effluents and in municipal sewage lines that ultimately enter publicly owned treatment works (POTW) activated sludge systems (Volskay and Grady, 1988). However, the toxicity of wastewater to activated sludge is not easily determined. First of all, the composition of wastewaters is usually diversified and complex, and usually unknown. Over 100,000 chemicals are produced and used in various activities such as industries (Forget et al., 2000, Hansen et al., 1999). Secondly, interactions of chemicals in wastewater (synergistic, antagonistic, additive) also exist (Gómez et al., 2001). Unfortunately, relevant toxicological data for all individuals or combination of chemicals are not available (Arretxe et al., 1997). As mentioned previously, only 10% of the new chemicals submitted for approval to the Environmental Effects Branch (EEB) of the Office of Toxic Substances are provided with toxicity data (Sun et al., 1994).

The Shk1-based toxicity testing procedure can serve as a practical and cost-effective method for toxicity assessment. The rapid Shk1-based toxicity screening system was developed and was shown previously in Figure 4-1. Using this system, the toxicities of 102 organic chemicals were tested and compared to the results of literature data, especially the Microtox[®] data and the activated sludge respiration inhibition tests data.

Methodology

Chemicals

All organic chemicals used in toxicity tests were obtained from Sigma-Aldrich. The test toxicants were aqueous solutions of 102 organic chemicals which included simple and halogen-substituted alkanes, alkenes and aromatics, alcohols, ketones, and amines. The complete listing of the organic chemicals tested can be found in Table 6-1. All stock solutions were made by dissolving the appropriate amount of the organic chemicals in 1 L of deionized water. Among these 79 organic chemicals, 1,2-, 1,3-, 1,4-dichlorobenzene, cyclohexane, and p-xylene were dissolved in 5 ml of methanol first, and all phenols were dissolve in 5 ml of acetone first to increase aqueous solubility. These test compounds were then dissolved in 1 L of deionized water. The small amounts of methanol or acetone transferred into 1 L of deionized water did not affect Shk1 bioluminescence to an observable degree (data not shown). Serial dilutions of the stock solutions were made to achieve the desired concentrations of the organic chemicals.

Rapid toxicity screening system

The rapid toxicity screening system was the same as that described in Chapter 4 and was shown in Figure 4-1. The bioreactor and light detection equipment were also described in the preliminary studies. In the toxicity tests involving organic chemicals, the bioreactor was operated under the following conditions: dilution rate = 0.52 hr^{-1} , pH = 7, and temperature = 22°C . The light detection equipment (light sensors) used in the rapid toxicity screening system was shown in Figure 2-2.

Determination of Mixing Ratio

The mixing ratio is defined as before as the ratio of the volumetric flow rate of the test material solution to the volumetric flow rate of the Shk1 cell solution from the bioreactor. The different mixing ratios were achieved by fixing the flow rate of the Shk1 cell solution at 200 ml/hr while varying the flow rate of the test material solution. Based on previous studies (Chapter 4), a mixing ratio of 3 was selected and was used in the toxicity tests for organic chemicals.

Assays of Organic Chemicals

The toxicity of the test materials was reflected by the repression of bioluminescence as previously defined by Equation 4-1 in Chapter 4 which has the following form

$$\text{Bioluminescence repression} = \frac{B_b - B_t}{B_b} \times 100\% \quad (4-1)$$

where B_b = bioluminescence of Shk1 mixed with buffer solution

B_t = bioluminescence of Shk1 mixed with test solution, measured 5 minutes after

Shk1 cell solution was mixed with the test material solution.

All toxicity experiments were performed in the following way. Shk1 cells were supplied continuously to the two light sensors (see Figures 4-1 and 2-2). The first (upstream) sensor was used to determine the basal light level of the cells before exposure to the toxicant. After leaving the first sensor, the cells entered one leg of a "tee" connector where they were mixed alternately with either the test material solution or a buffer solution. The concentrations of the organic chemicals in the mixed solution are

referred to as post-contact concentrations as before. The mixture of Shk1 cells and test solution then proceeded through several coils of Teflon[®] tubing to achieve an appropriate residence time before entering the second light sensor. The residence time after the mixing tee and before the second light sensor was 5 minutes. The buffer solution (3.03 g/L K₂HPO₄ and 5 g/L KH₂PO₄) was used to normalize the effect of dilution on the light level in the second sensor (see Equation 4-1). This buffer composition gave a pH of 7, which equaled the pH in the bioreactor when these experiments were performed.

The Shk1 cell solution pumped continuously from the CSTR was alternately mixed with buffer solution and test material solution using a manually operated 3-way valve (see Figure 4-1). The bioluminescence of Shk1 exposed to solutions of organic chemicals was measured 5 minutes after the 3-way valve was turned to feed the test toxicant solution to the mixing tee.

Toxicity tests using these 102 toxicant solutions were performed according to the method described above at a range of concentrations of the organic chemicals. Tests with each concentration had 3 replicates. Bioluminescence repression of Shk1 (as defined by Equation 4-1) due to exposure to these solutions of organic chemicals was calculated and plotted against the post-contact concentrations of the organic chemicals to generate concentration-response curves. The 50% effective concentration, or EC₅₀, represents the concentration of an organic chemical that caused the light level detected by sensor 2 to drop by 50% of the light level recorded when buffer alone was being fed to the mixing tee. EC₅₀ values for the 102 organic chemicals were obtained from a least-square regression of the relatively linear portions of the concentration-response curves in the region between 16% and 84% bioluminescence repression.

Results

Assays of Organic Chemicals

The results of the triplicate bioassays of the 102 organic chemicals were reproducible between replicates. The Shk1 EC₅₀ values of the 102 organic chemicals are shown in Table 5-1. Since the toxicant solutions were mixed with the Shk1 cell solution at a ratio of 3 to 1, the toxicants were effectively diluted to 75% of their original concentration. The concentrations of toxicants before mixing with the Shk1 cell solution, or pre-contact concentrations, were therefore 1.33 times that after the mixing, or post-contact concentrations as defined earlier. In Table 5-1, the heading "Shk1 EC_{50, 0}" refers to the original pre-contact toxicant concentration that caused 50% bioluminescence repression. The Shk1 EC₅₀ and EC_{50,0} values marked with an asterisk are projected values, i.e., 50% bioluminescence repression was not achieved at the highest concentration (solubility limit) tested, and EC₅₀ values were obtained from the regression equation. In Table 5-1, also listed are EC₅₀ values for the same organic chemicals obtained using the Microtox[®] test, the Polytox[®] test, and activated sludge respiration inhibition test. The 5 min Microtox[®], and especially the Polytox[®] and activated sludge respiration inhibition data (from Sun et al., 1994) are not available for some organic chemicals in Table 5-1. Efforts were made to find literature data to replace the missing values and some activated sludge EC₅₀ values were found for phenolic compounds (also shown in Table 5-1). Depending on the source of the data, activated sludge EC₅₀ values are concentrations of phenolic compounds that cause a 50% inhibition in either the growth rate or the respiration rate of the activated sludge microorganisms.

Table 5-1. EC₅₀ and EC_{50,0} values of organic chemicals.

Chemical	CAS No.	MW, g/mol	EC ₅₀ , mg/L (5 min data)	EC _{50,0} mg/L (5 min data)	Microtox [®] EC ₅₀ ¹ , mg/L (5 min data unless noted)	Polytox [®] EC ₅₀ ⁵ , mg/L (6 hr data)	Activated Sludge EC ₅₀ ⁵ , mg/L (12 hr data)
Benzene	71-43-2	78.11	202	269	2.0	685	993
Toluene	108-88-3	92.14	93	124	17	207	292
2,4-Dinitrotoluene	121-14-2	182.14	242 *	323	33	-	-
Nitrobenzene	98-95-3	123.11	203	271	17.8 (15 min)	-	-
p-Xylene	106-42-3	106.17	57	76	5.7 (30 min)	140	166
Ethylbenzene	100-41-4	106.17	57	76	5.5	220	222
Chlorobenzene	108-90-7	112.56	105	140	9.36	350	155
1,2-Dichlorobenzene	95-50-1	147.00	81	108	2.74	135	49
1,3-Dichlorobenzene	541-73-1	147.00	64	85	3.07	40	63
1,4-Dichlorobenzene	106-46-7	147.00	103 *	137	4.34	6	14
Benzyl Alcohol	100-51-6	108.14	635	847	63.7	-	-
2-Chlorobenzaldehyde	89-98-5	140.57	67	89	-	-	-
4-Chlorobenzaldehyde	104-88-1	140.57	47	63	10.2	-	-
Salicylaldehyde	90-02-8	122.12	205	273	16.3	-	-
3-Chlorobenzoic Acid	535-80-8	156.57	215	287	-	-	-
2,5-Dichlorobenzoic Acid	50-79-3	191.01	202	269	-	-	-
4-Bromobenzoic Acid	586-76-5	201.02	35	47	-	-	-
2,3-Dihydroxybenzoic Acid	303-38-8	154.12	153	204	-	-	-
2,5-Dihydroxybenzoic Acid	490-79-9	154.12	249	332	-	-	-
Benzaldehyde	100-52-7	106.13	237	316	6.11	-	-
Protocatechuic Acid	99-50-3	154.12	275	367	-	-	-
Orcinol	504-15-4	124.14	1155	1540	-	-	-
Phthalic Acid (monopotassium salt)	88-99-3	166.13	1280	1707	-	-	-

Chloroform	67-66-3	119.38	701	935	441		
Methylene Chloride	75-09-2	84.93	2935	3913	1878 (15 min)	1750	1994
Iodomethane	74-88-4	141.94	621	828	-	-	-
Dibromomethane	74-95-3	173.83	1061	1415	735	1110	1572
Carbon Tetrachloride	56-23-5	153.82	93	124	5.6	325	432
1,2-Dichloroethane	107-06-2	98.96	1409	1879	157 ²	685	1385
1,1,1-Trichloroethane	71-55-6	133.40	49	65	8.04 (15 min)	415	659
1,2-Dichloropropane	78-87-5	112.99	277	369	58.9	500	861
1,3-Dichloropropane	142-28-9	112.99	444	592	71.3	-	-
1,2,3-Trichloropropane	96-18-4	147.43	211	281	18.7	-	-
1,1,3-Trichloropropene	2567-14-8	145.42	121	161	-	-	-
1,1,1,2-Tetrachlorethane	630-20-6	167.85	23	31	2.03	-	-
1,1,2,2-Tetrachloroethane	79-34-5	167.85	40	53	5.4 ³	180	197
Bromoform	75-25-2	252.73	165	220	-	-	-
Bromoethane	74-96-4	108.97	2186	2915	-	-	-
Bromochloromethane	74-97-5	129.38	3228	4304	-	1800	2672
1,2-Dibromoethane	106-93-4	187.86	921	1228	735	520	1271
1-Chlorobutane	109-69-3	92.57	278	371	485	-	-
1,4-Dichlorobutane	110-56-5	127.01	270	360	61.5	-	-
1,5-Dichloropentane	628-76-2	141.04	59	79	15.79	-	-
Trichloroethylene	79-01-6	131.39	190	253	96.84	500	770
Tetrachloroethylene	127-18-4	165.83	216 *	288	17.19	175	299
Cyclohexane	110-82-7	84.16	94	125	85.5	74	133
Hexane	110-54-3	86.18	60 *	80	104	38	47
Octane	111-65-9	114.23	163	217	730 ³	8	3025
Methanol	67-56-1	32.04	39899	53199	42237	-	-
Ethanol	64-17-5	46.07	31399	41865	31000	40000	28541
1-Propanol	71-23-8	60.10	8303	11071	9862	7200	10875
2-Propanol	67-63-0	60.10	21431	28575	35000	-	-

1-Butanol	71-36-3	74.12	2745	3660	2056	-	-
1-Hexanol	111-27-3	102.18	202	269	21.5	-	-
2-Ethyl-1-Hexanol	104-76-7	130.23	158	211	4.95	-	-
1-Pentanol	71-41-0	88.15	737	983	298	2325	3528
Isopentyl Alcohol	123-51-3	55.15	768	1024	-	-	-
1-Octanol	111-87-5	130.23	70	93	3.4	126	194
Ether	60-29-7	74.12	3536	4715	-	-	-
tert-Butyl Methyl Ether	1634-04-4	88.15	141	188	8.23	-	-
Isobutyl Acetate	110-19-0	116.16	294	392	-	1600	2156
n-Amyl Acetate	628-63-7	130.19	183	244	-	440	1031
Ethyl Acetate	141-78-6	88.11	1711	2281	5160	5400	5427
Formaldehyde	50-00-0	30.03	36	48	3.0	-	-
Glutaraldehyde	111-30-8	100.12	167	223	75.7	-	-
Acetone	67-64-1	58.08	22611	30148	8600	48000	48619
Methyl Ethyl Ketone	78-93-3	72.11	629	839	14	1900	1873
Methyl Isobutyl Ketone	108-10-1	100.16	376	501	80	2600	2811
Cyclohexanone	108-94-1	98.14	117	156	18.5	3750	5452
Formamide	75-12-7	45.04	6265	8353	-	-	-
n-Butylamine	109-73-9	73.14	318	424	41.1	90	111
Diethylamine	109-89-7	73.14	327	436	23.4	104	100
Triethylamine	121-44-8	101.19	311	415	-	-	-
Acetic Acid	64-19-7	60.05	134	179	9.24	287	299
Azelaic Acid	123-99-9	188.22	209	279	-	-	-
Cyclohexylamine	108-91-8	99.18	407	543	120 (30 min)	60	103
Ethanolamine	141-43-5	61.08	560	747	14.3	160	115
Triethanolamine	102-71-6	149.19	9470	12627	184	900	741
Aniline	62-53-3	93.13	1255	1673	139	-	-
Pyrocatechol	120-80-9	110.11	804	1072	32 ³	-	-
4-Bromophenol	106-41-2	173.01	28	37	0.41	-	120 ³

2-Chlorophenol	95-57-8	128.56	151	201	22.1	-	162 ⁶
4-Chloro-3-methylphenol	59-50-7	142.59	18	24	0.27	-	-
4-Chlorophenol	106-48-9	128.56	20	27	0.96	-	105 ⁶
m-Cresol	108-39-4	108.14	189	252	6.82	-	440 ³
2,4-Dichlorophenol	120-83-2	163.00	30	40	2.32	-	27 ⁶
2,5-Dichlorophenol	583-78-8	163.00	158	211	8.36	-	32 ⁷
2,6-Dichlorophenol	87-65-0	163.00	120	160	9.6	-	66 ⁷
3,5-Dichlorophenol	591-35-5	163.00	41	55	2.8	-	11 ⁷
2,6-Dimethoxyphenol	91-10-1	154.17	16	21	-	-	-
2,4-Dinitrophenol	51-28-5	184.11	105	140	15.8	-	110 ⁸
2-Methoxyphenol	90-05-1	124.14	583	777	-	-	-
1-Naphthol	90-15-3	144.17	53	71	3.7	-	-
2-Naphthol	135-19-3	144.17	12	16	0.22	-	-
4-Nitrophenol	100-02-7	139.11	43	57	10.9	-	59 ⁶
Phenol	108-95-2	94.11	482	643	25	-	340 ⁶
3-Phenylphenol	580-51-8	170.21	24	32	0.10 ⁴	-	-
8-Quinolinol	148-24-3	145.16	93	124	12.1 ⁴	-	-
2,3,4,5-Tetrachlorophenol	4901-51-3	231.89	7.3	9.7	0.34	-	-
2,3,6-Trichlorophenol	933-75-5	197.45	61	81	14.0	-	-
2,4,5-Trichlorophenol	95-95-4	197.45	13	17	1.18	-	23 ³
4'-Chloroacetophenone	99-02-5	154.60	67	89	6.75	-	-

¹: Kaiser and Devillers (1994)

²: Kaiser (1988)

³: Blum and speece (1991)

⁴: Calculated from data based on mmol/L in Schultz et al. (1998)

⁵: Sun et al. (1994)

⁶: Walker (1989)

⁷: Hall et al. (1992)

⁸: Volskay and Leslie Grady (1988)

Discussion

For the purpose of detecting toxicants in wastewater in time to serve a preventative function, a quick response is needed. An exposure time of 5 minutes has been shown in several studies to be sufficient for organic chemicals (Robra, 1979, Danoux, 1982, Reteuna, 1986). Additionally, Schultz et al. (1998) obtained the relationships between the 5, 15 and 30 minute Microtox[®] EC₅₀ values for 771 organic chemicals as shown by Equations 5-1 and 5-2, in which the EC₅₀ values have the units of mmol/L.

$$\log(\text{EC}_{50, 15 \text{ min}}) = 0.98 \log(\text{EC}_{50, 5 \text{ min}}) \quad (5-1)$$

$$\log(\text{EC}_{50, 30 \text{ min}}) = 0.97 \log(\text{EC}_{50, 5 \text{ min}}) + 0.02 \quad (5-2)$$

with r^2 values equal to 0.99 and 0.97 respectively. It can be seen that the EC₅₀ values for the organic chemicals do not vary significantly as the contact time increases from 5 minutes to 15 or 30 minutes. As a result, 5 minutes was chosen to be the contact time of Shk1 cells and toxicants in this study. This contact time appeared to be adequate for our study and other contact times were not considered.

As our data in Table 5-1 show, only 1-chlorobutane, hexane, octane, ethyl acetate and 2-propanol have Shk1 EC₅₀ values smaller than their corresponding Microtox[®] EC₅₀ values. This can easily be seen in Figure 5-1 which shows the relationship of 79 Shk1 and the Microtox[®] EC₅₀ values with each compound shown as a data point. Nearly all of the organic chemicals lie above the 45° line with the exceptions of the five aforementioned chemical compounds. While it is not obvious in the figure, the Shk1 EC₅₀ values are also smaller than their Microtox[®] counterparts for methanol and 1-propanol

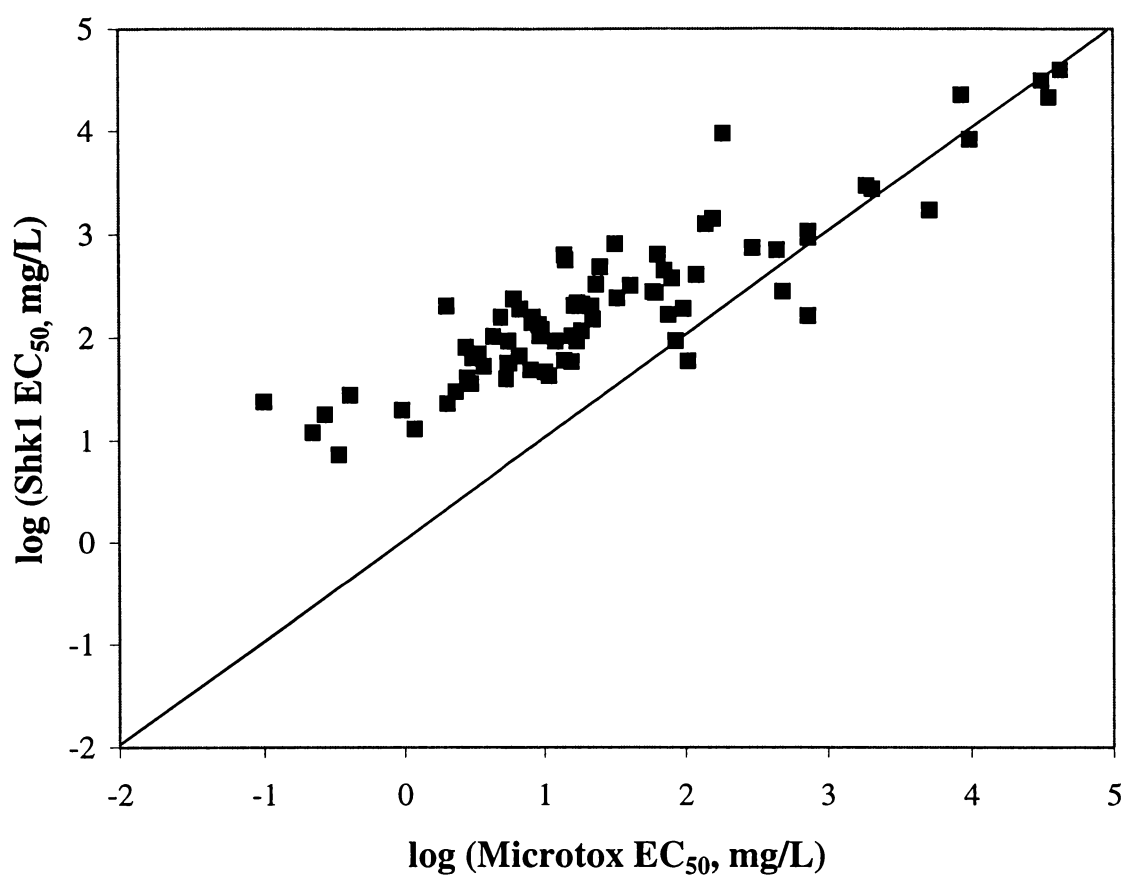


Figure 5-1. Correlation between the Shk1 EC₅₀ values and the Microtox[®] EC₅₀ values. The Shk1 assay is less sensitive to toxicants than the Microtox[®] assay.

(see Table 5-1). However, the differences are not significant so that those two compounds lie almost on the 45° line in Figure 5-1. The Microtox® EC₅₀ data for some organic chemicals are not available and so those compounds are not represented in Figure 5-1. In Figure 5-1, the horizontal axis represents the log of the Microtox® EC₅₀ values, and the vertical axis represents the log of the Shk1 EC₅₀ values. The Shk1 and the Microtox® EC₅₀ values vary from a few milligrams per liter to tens of thousands of milligrams per liter and it is more useful to present the data in a graph with logarithmic axes. The 45° diagonal line which represents equal EC₅₀ values is also shown in Figure 5-1. In Figure 5-1, the relative sensitivity for organic chemicals of the Shk1 test and the Microtox® test can be seen by the region in which the organic chemicals fall. For points in the region below the 45° line, the Shk1 assay is more sensitive than the Microtox® assay. Conversely, in the region above the 45° line, the Microtox® assay is more sensitive than the Shk1 assay. Nearly all of the organic chemicals lie above the 45° line with the five previously mentioned exceptions, and so the Shk1 assay is less sensitive than the Microtox® assay for most organic compounds tested. A comparison of the available Shk1, Microtox®, and activated sludge EC₅₀ values for phenolic compounds (shown in Figure 5-2) also shows that Shk1 is less sensitive and that the response of Shk1 is more similar to that of the activated sludge microorganisms.

Comparing Shk1 EC₅₀ values for post-contact concentrations with activated sludge EC₅₀ values in Table 5-1, most Shk1 EC₅₀ values are smaller than the corresponding activated sludge EC₅₀ values. The exceptions are the compounds 1,2-, 1,3-, 1,4-dichlorobenzene, methylene chloride, 1,2-dichloroethane, hexane, ethanol, and all

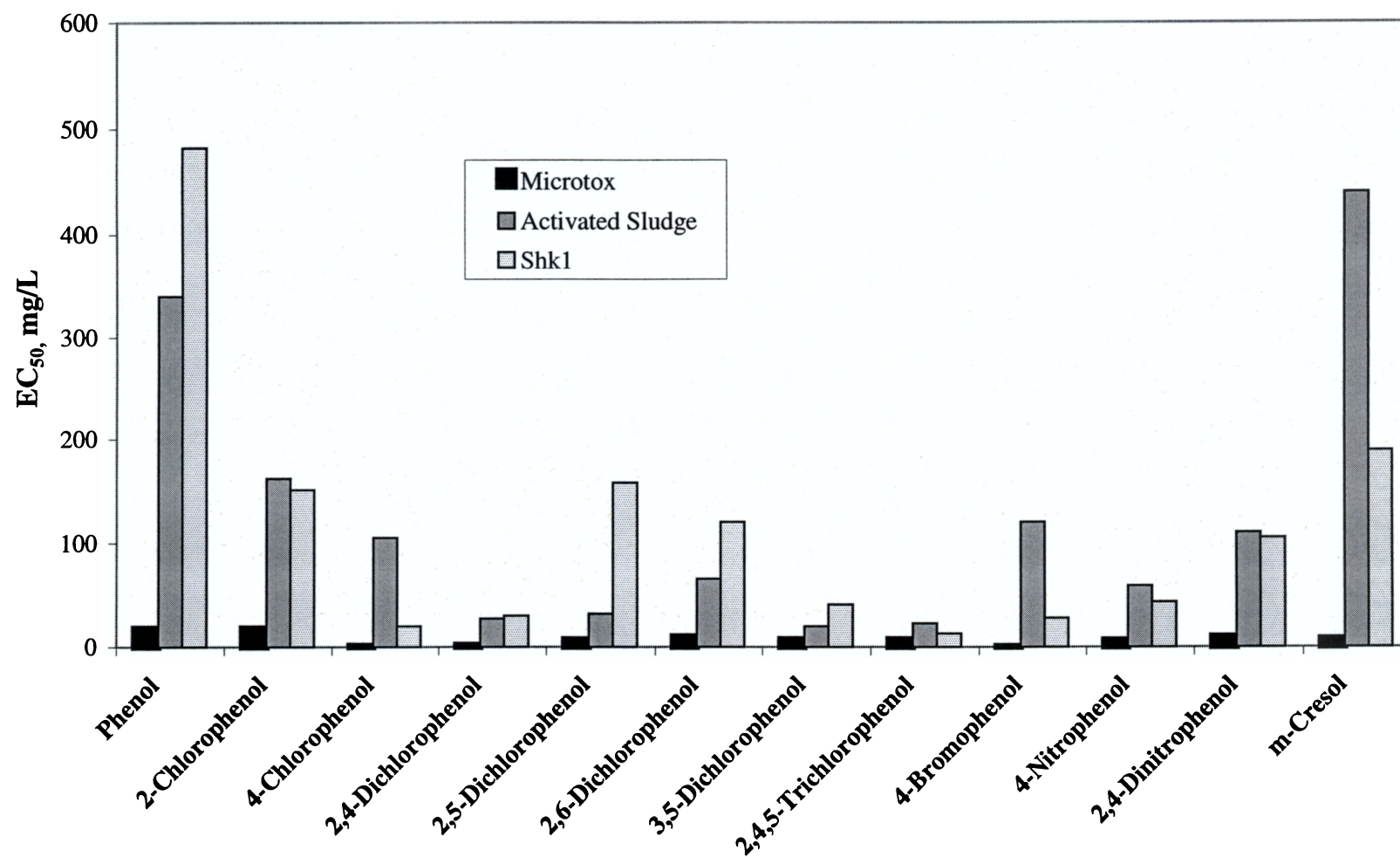


Figure 5-2. Comparison of the Microtox®, activated sludge, and Shk1 EC_{50} values for phenolic compounds.

amines, for which Shk1 EC_{50} values are larger than the corresponding activated sludge EC_{50} values. The comparison between the results obtained from the Shk1 test and the Polytox[®] test was not made due to the similarity between the Polytox[®] test and the activated sludge respiration inhibition test as discussed later.

The above comparisons are made considering the post-contact toxicant concentrations. However, as seen in Figure 1-4, the purpose of the toxicity monitoring system is to estimate the toxicity of the influent wastewater stream which depends on the pre-contact concentrations of toxicants in the wastewater. The toxicity of the test solution before mixing with Shk1 cells is reflected by the $EC_{50,0}$ values shown in Table 5-1. We correlated Shk1 $EC_{50,0}$ values instead of Shk1 EC_{50} values with activated sludge respiration inhibition EC_{50} values and the results are discussed below.

The correlation of the original 39 Shk1 $EC_{50,0}$ values and the corresponding activated sludge EC_{50} respiration inhibition values (obtained from Sun et al., 1994) is shown in Figure 5-3. For the purpose of comparison, the correlation of the 34 Microtox[®] EC_{50} values and activated sludge EC_{50} values is also shown. Similar to Figure 5-1, the x-axis in Figure 5-3 represents the log of the activated sludge EC_{50} values, and the y-axis represents the log of the Shk1 $EC_{50,0}$ values or the log of the Microtox[®] EC_{50} values. The points shown by solid squares in Figure 5-3 represent the organic chemicals tested in our study and points shown by circles represent literature values for the organic chemicals tested by the Microtox[®] assay. In the region below the 45° line, a point corresponds to activated sludge EC_{50} larger than the Shk1 $EC_{50,0}$. In this case, when a toxicant causes a 50% decrease in bioluminescence, it causes less than a 50% reduction

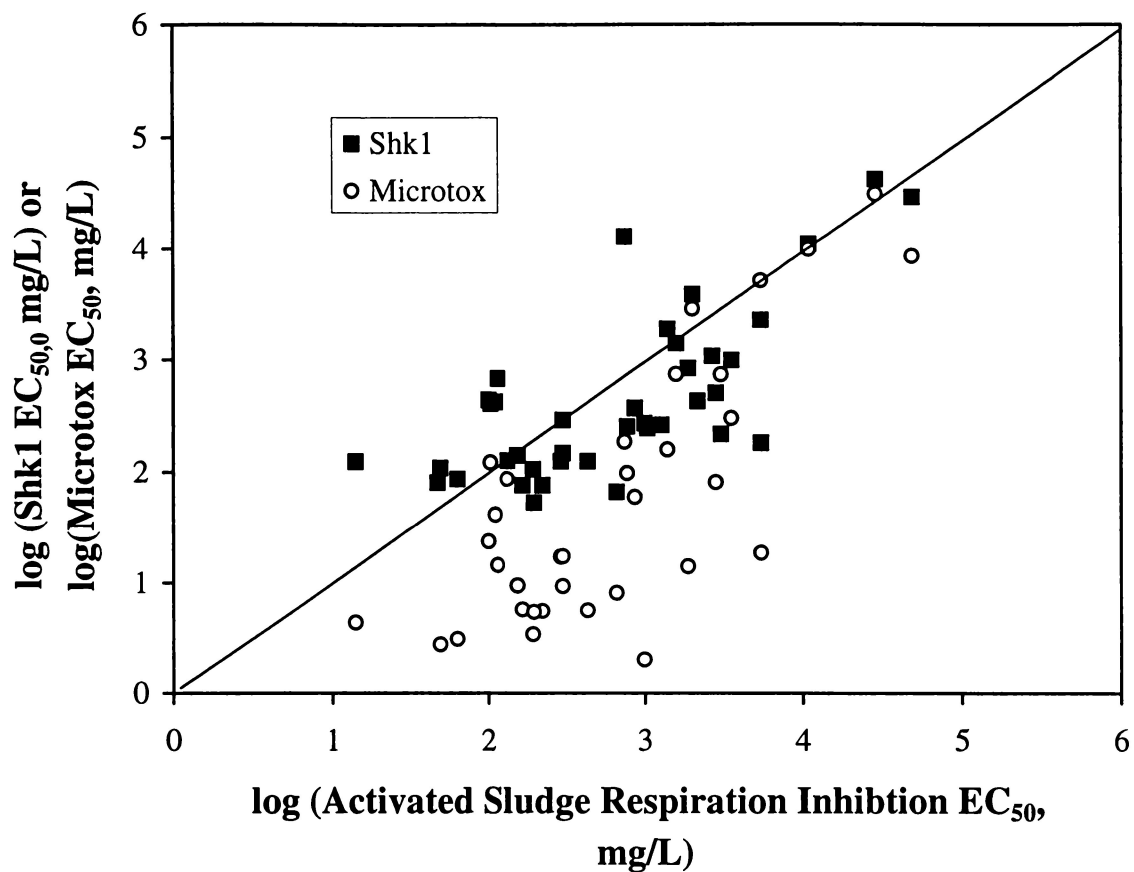


Figure 5-3. Correlation between Shk1 EC_{50,0} values, Microtox® EC₅₀ values and activated sludge respiration inhibition EC₅₀ values. The Shk1 EC_{50,0} values are closer to the 45° line than the Microtox® EC₅₀ values.

in activated sludge respiration if the activated sludge is exposed to toxicants of the same concentration (see discussion below). As a result, toxicity predicted by Shk1 for points below the 45° line is over-estimated. In the region above the 45° line, a point corresponds to activated sludge EC₅₀ values smaller than the corresponding Shk1 EC_{50, 0} values and toxicity to activated sludge as predicted by Shk1 is under-estimated. Based on the above discussion, it can be seen that the farther away from the 45° line a compound lies, the more potential there exists for over- or under-estimating toxicity.

In the above discussion, it was assumed that the activated sludge will be exposed to toxicants of the same concentrations as are in the influent wastewater, which is not the case for backmixed treatment systems. As a pulse of toxicant in a wastewater stream enters a backmixed treatment system, it becomes diluted. As a result, the toxicity in the aeration basin is lower than that in the incoming wastewater and is dependent on the volume of the aeration basin and other variables such as the flow rate of the influent wastewater stream and the intensity and duration of the toxicity in the incoming wastewater. For any test method to serve a preventative purpose, the test has to be carried out upstream of the activated sludge system, not in the activated sludge basin itself. Therefore, any test methods that correlate well with respirometry data upstream of the plant will always overestimate the effect of toxicity when diluted by mixing in the aeration basin. However, the dilution effect could serve as a safety factor in toxicity monitoring. Because the Shk1 test is less sensitive than tests based on *P. phosphoreum*, the frequency that an incoming wastewater is unnecessarily diverted would be reduced.

Unlike applications in effluent monitoring, sensitivity is important but is not the primary goal for influent wastewater toxicity monitoring, which is to predict toxic effects

of incoming wastewater on activated sludge. The three tests, Shk1, Microtox[®], and Polytox[®], are surrogate tests in that one or a mixture of surrogate organisms are tested instead of directly testing the activated sludge in the treatment system. The results of these surrogate tests can be compared to the results of the activated sludge respiration inhibition tests and a correlation can be established. The toxicity of a chemical or wastewater to activated sludge can then be estimated from the surrogate tests and the correlation. As Sun et al. (1994) pointed out, the validity of such an estimation depends on the similarity between the organism(s) used in the surrogate test and those in the activated sludge and the quality of the correlation between the measured toxicity. In one case, the so-called BASF toximeter was built to prevent toxic shocks in the activated sludge of the BASF wastewater treatment plant located in Ludwigshafen, Germany (Pagga and Günthner, 1981). The toximeter was a pilot scale activated sludge treatment unit, consisting of primary clarifier, aeration basin, secondary clarifier, etc., situated in the upstream of the treatment plant to be protected. Due to the similarity of the toximeter and the actual treatment plant in terms of the sludge quality, sludge age, etc., very good results were obtained. However, as the authors concluded, such a toxicity monitoring system is complicated and expensive, and is not suitable for practical toxicity monitoring. The Polytox[®] test is similar to the BASF toximeter in the sense that the Polytox[®] test (unlike the Shk1 or the Microtox[®] tests that use pure cultures) uses a mixture of 12 microbial strains isolated from activated sludge, thus increasing significantly the similarity between the consortia in the test and that in the activated sludge system. In addition to this similarity, respiration rate is measured in both the Polytox[®] test and the

activated sludge respiration inhibition test. Because of these similarities, it is not surprising that the Polytox[®] and the activated sludge respiration inhibition EC₅₀ values are in good correlation. Sun et al. (1994) obtained the correlation equation

$$\log EC_{50, AS} = 0.432 + 0.879 \log EC_{50, Polytox} \quad (5-3)$$

with $r^2 = 0.928$ and SE (standard error) = 0.225.

The Polytox[®] test is so similar to activated sludge respiration inhibition tests that, while it eliminates the necessity to take sludge samples in batchwise respiration inhibition tests, it is basically equivalent to an activated sludge respiration inhibition test and has the disadvantages of the activated sludge respiration inhibition tests as discussed earlier; e.g., it is time-consuming.

Previous attempts to correlate the results of other test methods with activated sludge EC₅₀ values focused only on the coefficient of determination (r^2) and standard error (Blum, 1989, Sun et al., 1994). However, for the purpose of wastewater toxicity screening, a good correlation not only needs to be statistically sound (as reflected by a high r^2 value), but also needs to be physically significant so that it can be used to predict damage to activated sludge directly from the correlation. Since the concentrations of toxicants in wastewater are usually not known, a correlation equation showing the results of a surrogate test method and activated sludge respiration inhibition tests (like Equation 4) may be useful in estimating EC₅₀ values, but is not useful in predicting the toxicity of wastewater to activated sludge using the surrogate test.

By correlating the Shk1 EC_{50,0} values with activated sludge respiration inhibition EC₅₀ values, the following regression through the origin was obtained.

$$\log EC_{50, 0 Shk1} = 0.92 \log EC_{50, AS} \quad (5-4)$$

with $n=39$, $r^2 = 0.64$ and $SE = 0.55$.

The advantage of having a regression with a 0 intercept and a slope of approximately 1 (0.92 in this case) is that the possible damage to activated sludge due to influent wastewater toxicity can be directly predicted: Expose Shk1 to the influent and monitor the bioluminescence; when Shk1 bioluminescence is repressed by 50%, activated sludge respiration can be expected to be inhibited by approximately 50% if the influent is allowed to enter the plant and toxicant concentrations remain the same (see earlier discussion). By contrast, a regression through the origin of *Photobacterium phosphoreum* EC₅₀ data resulted in a line having a slope of 0.66, or

$$\log EC_{50, P. phosphoreum} = 0.66 \log EC_{50, AS} \quad (5-5)$$

with $n=34$, $r^2 = 0.30$ and $SE = 0.82$.

A slope of less than 1 indicates that when bioluminescence of *Photobacterium phosphoreum* is repressed by 50%, the activated sludge respiration rate will be inhibited to an unknown degree of less than 50%.

Instead of correlating the actual Microtox[®] EC₅₀ values with activated sludge respiration inhibition EC₅₀ values, it is possible to correlate the Microtox[®] EC_{50,0} (pre-contact) with activated sludge respiration inhibition EC₅₀ values as was done with Shk1. However, in order to achieve a slope approximately equal to 1 and force the intercept to be 0, the test solution should be mixed with the *P. phosphoreum* solution at an approximately 1:9 (v/v) ratio. If this were done, the test solution would be diluted 10 times, whereas in the Shk1 test, the test solution is diluted by only 25%. Excess dilution of wastewater samples could change the pH and the chemistry of the wastewater. It has been shown that the toxicities of some chemical compounds, e.g., metals and organic

acids such as substituted phenols, are related to pH (Ho et al., 1999, Franklin et al., 2000, Kishino and Kobayashi, 1995, Miller et al., 1990, Larsson et al., 1993, Saarikoski and Viluksela, 1981, 1982, Schubauer-Berigan et al., 1995, Spehar et al., 1984).

Studies have shown that each bacterial species and test procedure has its own sensitivity pattern to toxicants (Dutka and Kwan, 1981, 1982, Dutka et al., 1983) and no single species is sensitive to all chemicals (Castillo et al., 2000, Toussaint et al., 1995). Sensitivity is dependent on the species used, the chemicals tested, the time of exposure, and the end-point (Kharu et al., 2000, Persoone et al., 1994). Accordingly, there has been a tendency toward the use of a "battery" of tests (Castillo et al., 2000, Codina et al., 1993, Dutka and Kwan, 1984, Fochtman et al., 2000, Kahru et al., 2000, Marwood et al., 1998, Munawar et al., 1986, Ince et al., 1996). The test battery concept is a compromise between single species toxicity tests and community-level toxicity tests (Fochtman et al., 2000). It has been pointed out that single species toxicity tests cannot explain the chemical, physical, and biological interactions that occur in the natural environment (Janssen and Persoone, 1992), however, community-level tests are too complicated to be performed on a routine basis (Fochtman et al., 2000). A test battery usually includes organisms of different trophic levels (Blaise, 1991, Burton-Allen 1991, Castillo et al., 2000, Dutka et al., 1993, Ribo, 1997). For example, Blaise et al. (1985) suggested the use of four test organisms of increasing levels of biological organization (luminescent bacteria, crustacean, alga, and fish) for assessing the toxic effect of wastewater on the aquatic environment. The choice of test organisms in a test battery to assess the wastewater toxicity to activated sludge has not been well studied. However, as a general rule, the organisms in a test battery should be representative of the whole (eco)system

(Fochtman et al., 2000). Accordingly, to represent the activated sludge microbial community, organisms representing activated sludge organisms other than bacteria (e.g., protozoans) can be considered in a test battery. We should note that in activated sludge, an important class of bacteria is the nitrifying bacteria. The nitrifying bacteria differ from other bacteria in that they use ammonia as an electron acceptor instead of oxygen, and they are responsible for the nitrification process in a biological wastewater treatment plant. Because of the distinctive characteristic of the nitrifying bacteria, a test organism should probably be included in the test battery to represent the nitrifying bacteria. Tests involving other organisms found in activated sludge, e.g., rotifers, also exist (Snell and Persoone, 1989a, 1989b, Toussaint et al., 1995). However, organisms such as rotifers represent only a small population in activated sludge and could probably be excluded from a test battery without losing much toxicity information.

In addition to the representability of the test organisms, some other requirements should be satisfied when choosing the test organisms in a test battery. Such requirements include those for a single-species bioassay: have a quick response, have appropriate sensitivity, easy to operate, be inexpensive, reproducible and reliable, etc. Additionally, in a test battery, the test organisms should be chosen in such a way that the toxicity information is as complete as possible. The results of a test battery, if they represent the complete toxicity information, can form the basis for “fingerprints” of toxicants. Also in a test battery, the toxicity information obtained from each individual test in the battery should be complimentary to the information obtained from other tests and should not be redundant.

The complementarity of a test relative to other tests in a test battery is very important (Kahru et al., 2000). Tests that lack complementarity lead to redundancy in toxicity information. In determining the number of test organisms and what organisms to use in a test battery, statistical approaches (especially multivariate techniques) have proven to be helpful. For example, Fochtman et al. (2000) used principal components analysis (PCA) to select the test battery components for pesticides toxicity assessment.

Currently, it is believed that bioassays should be considered as a complement to more traditional analytical methods such as chemical analyses and not as a replacement of them (Ince and Erdoğan, 1998, Persoone et al., 1993). The results of bioassays are not conclusive, however, they can serve as strong signals that potential toxicants are present (Williams and Johnson, 1981).

CHAPTER 6

EFFECT OF SUSPENDED SOLIDS IN WASTEWATER ON THE Shk1-BASED INFLUENT TOXICITY MONITORING SYSTEM

In the field of chemical sensing, bioluminescent microorganisms have been widely used in toxicity testing of aqueous solutions (Iizumi et al., 1998; McCloskey et al., 1996; Wojtanowicz et al., 1992). Methods based on bioluminescent bacteria not only have the advantages common to all microbiological systems (small test organism size, large number of organisms, and convenient growing conditions, Kustin and Mcleod, 1977), but also have the additional advantages of having an easily measured signal and very quick responses (usually on the order of a minute or so). As mentioned in previous chapters, the Microtox[®] assay, based on bioluminescent bacterium *Photobacterium phosphoreum*, and has been widely used in aquatic toxicology and studied extensively (Sanchez et al., 1988, Logue et al., 1989, Lin et al., 1994, Hao et al., 1996, Ince and Erdoğdu, 1998). The continuous Shk1 toxicity screening procedure, based on bioluminescent bacterium Shk1, was developed by us at the Center for Environmental Biotechnology at the University of Tennessee (Chapters 4 and 5). Both assays use bacteria containing constitutive-type promoters for expression of the *lux* genes and indicate toxicity by bioluminescence repression (BR) when the bacteria are exposed to toxicants in aqueous solution.

Special caution should be taken when applying bioluminescent bacteria-based assays developed for effluent monitoring to influent toxicity screening. One distinctive

difference between influent and effluent wastewater is that influent wastewater contains a relatively high concentration of suspended solids. These suspended solids are removed during the wastewater treatment process and effluent wastewater usually has a much lower suspended solids concentration. As a result, the effect of suspended solids needs to be taken into account when bioluminescent bacteria-based toxicity screening methods are used for influent wastewater. Suspended solids interfere with light measurement by absorbing and/or scattering light emitted by bacteria. One way to eliminate the interference of the suspended solids is to remove the solids through filtration. Using Shk1, we studied the interference of suspended solids in wastewater on light measurement and determined the pore size of filters that should be used for bioluminescent bacteria-based influent toxicity monitoring systems.

Methodology

In bioluminescence-based assays, a wastewater sample is typically diluted before being mixed with a solution of bioluminescent microorganisms. In applications involving influent wastewater toxicity screening, filtration may also be required. Therefore, two experiments were performed in our study. In the first experiment, the effect of the volume fraction of wastewater in a test solution was studied. In this experiment, a grab sample of wastewater was taken from a location between the primary clarifier and the aeration basin in the Kuwahee Wastewater Treatment Plant of the Knoxville Utilities Board located in Knoxville, Tennessee. In this initial experiment, two sets of dilutions were obtained with test solutions made up of unfiltered wastewater in

one set of dilutions and filtered wastewater (using a 0.22 μm filter) in the other. In preparing the test solutions, the wastewater (filtered and unfiltered) was mixed with de-ionized water to make three solutions with 50%, 75% and 100% (on a volume basis) wastewater. The experimental set-up included a continuous-stirred tank reactor (CSTR) operated at steady-state to produce a continuous stream of Shk1 cells in aqueous solution and light detection equipment to determine the level of light emitted by the continuous stream of Shk1 cells. The operating conditions of the CSTR in this study were a dilution rate of 0.52 hr^{-1} , a pH of 7 and a temperature of 22°C . The Shk1 cells were mixed with either buffer (pH = 7) or test solutions at a 1:3 volumetric ratio (1 part cell solution to 3 parts buffer or test solution). The bioluminescence of Shk1 was measured after alternately exposing Shk1 cells to buffer or test solutions for 5 minutes as the mixture flowed continuously from the mixing point to the light detection equipment. The experimental set-up and toxicity testing procedures used here are thoroughly described in our previous study (Chapters 4 and 5). The bioluminescence repression (BR) indicates the degree of toxicity relative to the control made up of a buffer solution. BR is calculated according to its definition in Equation 4-1. All experiments were performed in triplicates. Bioluminescence was plotted against the volume fraction of wastewater in the test solutions.

$$\text{Bioluminescence Repression} = \frac{B_b - B_t}{B_b} \times 100\% \quad (4-1)$$

where:

B_b = bioluminescence of Shk1 mixed with buffer

B_t = bioluminescence of Shk1 mixed with test solution, measured 5 minutes after Shk1 cell solution was mixed with the test material solution.

The second experiment was performed to determine the appropriate filter pore size that should be used to filter influent wastewater before applying bioluminescence-based toxicity screening assays. The suspended solids concentration in municipal wastewater varies from day to day and also during a single day (Benefield and Randall, 1980). To account for the time variability of suspended solids concentrations in wastewater, wastewater samples were taken at 6 am, 11 am, 9 pm and 12 am (midnight) on different days. According to Benefield and Randall (1980), the suspended solids concentration profile in municipal wastewater is typically at the lowest point at 6 am, and reaches its two peak values at 11 am and 9 pm. The suspended solids concentration at 12 am (midnight) is at an intermediate level. For each wastewater sample, total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined on one portion (150 ml) of the wastewater samples. A pre-dried and pre-weighed 0.2 μm filter (MPS[®] membrane filter) was used to filter the wastewater sample. TSS were measured after dessication at 105°C for 24 hours, and VSS were measured after calcinations at 500°C for 2 hours. The remaining wastewater samples were sequentially filtered using ordinary filter paper (Whatman[®] glass fiber circles) and filters (MPS[®] membrane filters) with pore sizes of 1.2 μm , 0.8 μm , 0.2 μm and 0.1 μm . Unlike the preliminary experiments, the test solutions in this part of the study were made up solely of unfiltered or filtered wastewater. Bioluminescence using the test solutions was measured and BR

calculated according to Equation 4-1. All experiments were performed in triplicates in triplicates within 3 hours of taking the wastewater samples.

Results

The result of the initial experiment is shown in Figure 6-1. Clearly, for test solutions made up of both filtered and unfiltered wastewater, BR increased as the volume fraction of wastewater (filtered or unfiltered) in the test solution increases. At all volume fractions of wastewater, BR was higher if the wastewater was not filtered.

The total suspended solids and volatile suspended solids concentrations are shown in Table 6-1. The total suspended solids concentration varied between 42.7 and 52.7 mg/L while the volatile suspended solids concentration varied between 41.3 and 50.7 mg/L. The results showed that volatile matter constituted a major fraction of the suspended solids in the wastewater samples. The results of the experiments using unfiltered and sequentially filtered wastewater are shown in Figures 6-2 to 6-5. In all cases regardless of the time the wastewater samples were taken, BR decreased as the filter pore size went down. The highest BR was obtained using unfiltered wastewater and the lowest BR was achieved using 0.1 μm and 0.2 μm filters. Using unfiltered wastewater

Table 6-1. Total suspended solids and volatile suspended solids concentrations in wastewater samples.

Sampling Time	6 am	11 am	8 pm	12 am (midnight)
Total suspended solids concentration, mg/L	44.7	42.7	52.7	52.0
Volatile suspended solids concentration, mg/L	41.3	44.0	50.0	50.7

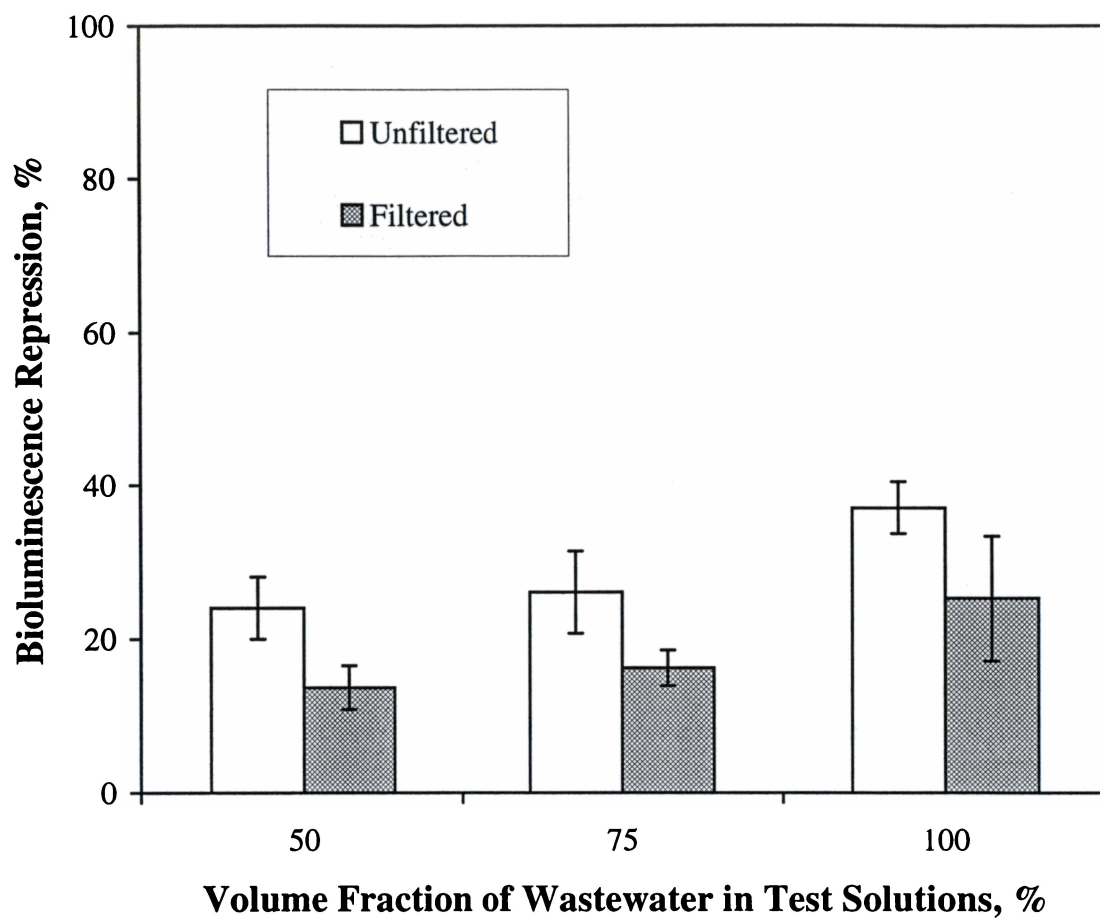


Figure 6-1. Bioluminescence repression of Shk1 exposed to test solutions made up of different percentages of unfiltered and filtered wastewater.

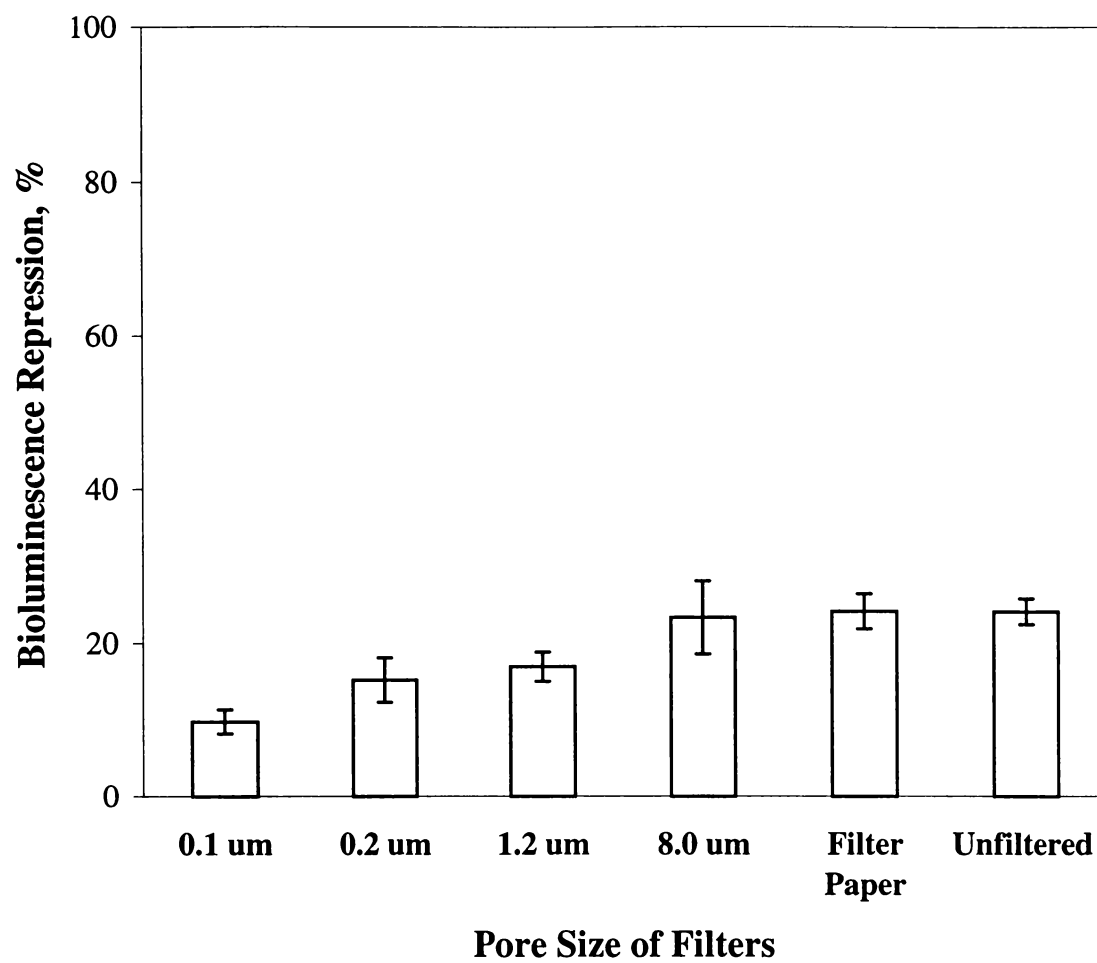


Figure 6-2. Effect of suspended solids on bioluminescence repression. The wastewater sample was taken at 6 am.

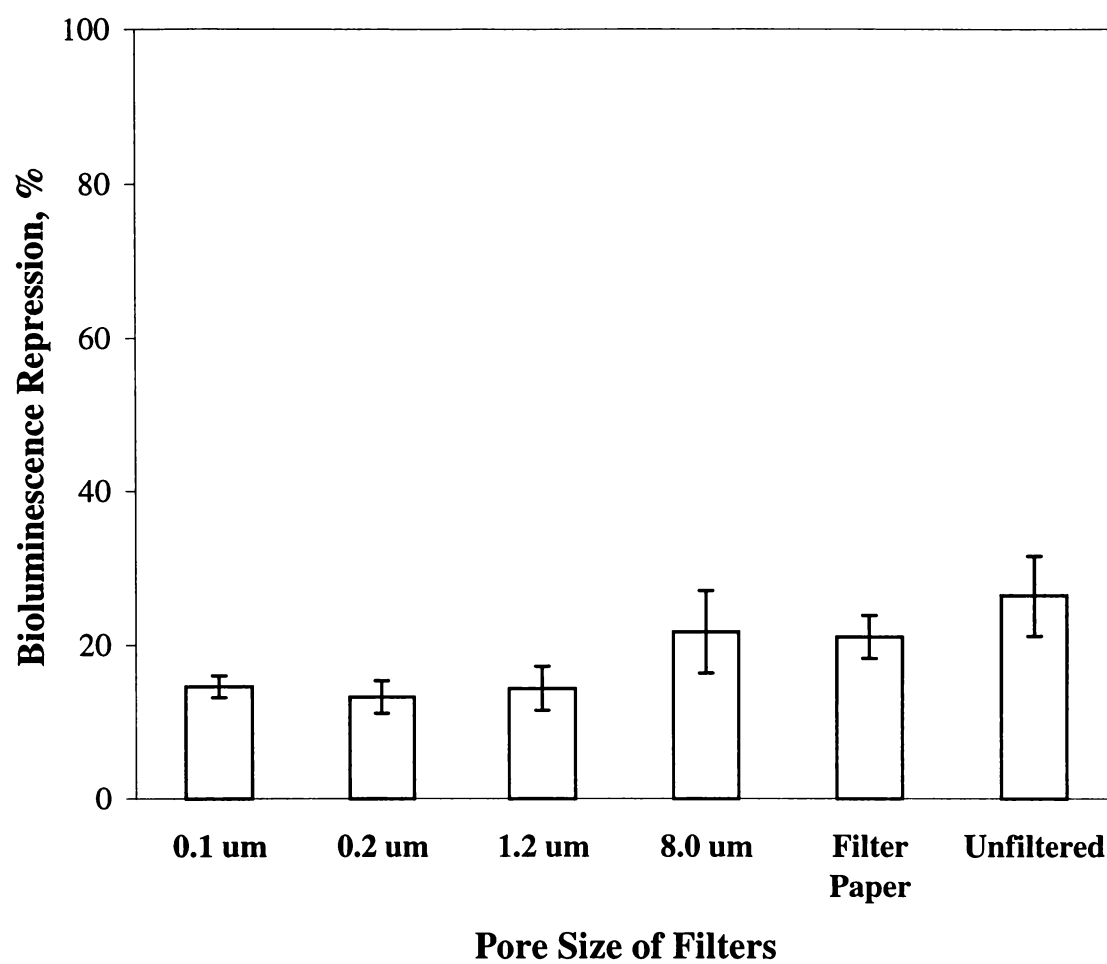


Figure 6-3. Effect of suspended solids on bioluminescence repression. The wastewater sample was taken at 11 am.

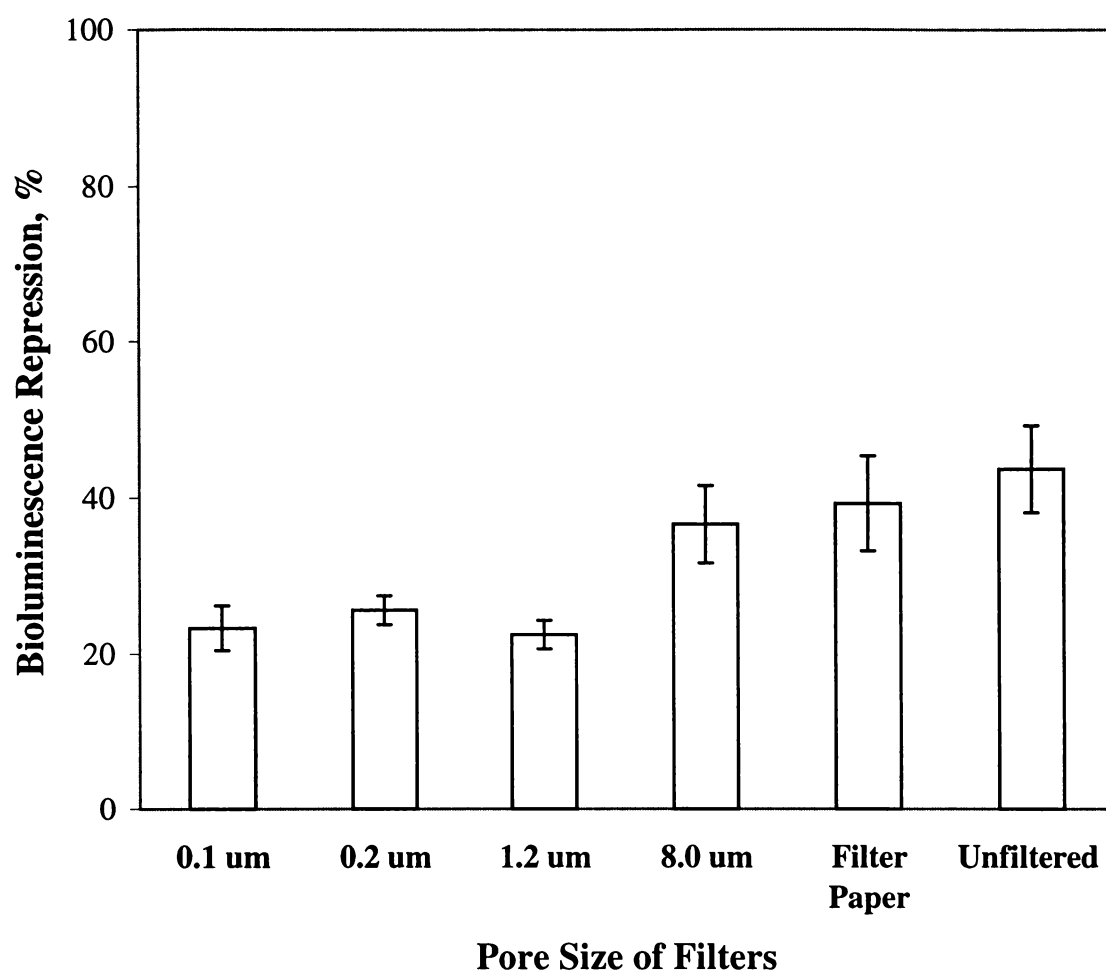


Figure 6-4. Effect of suspended solids on bioluminescence repression. The wastewater sample was taken at 8 pm.

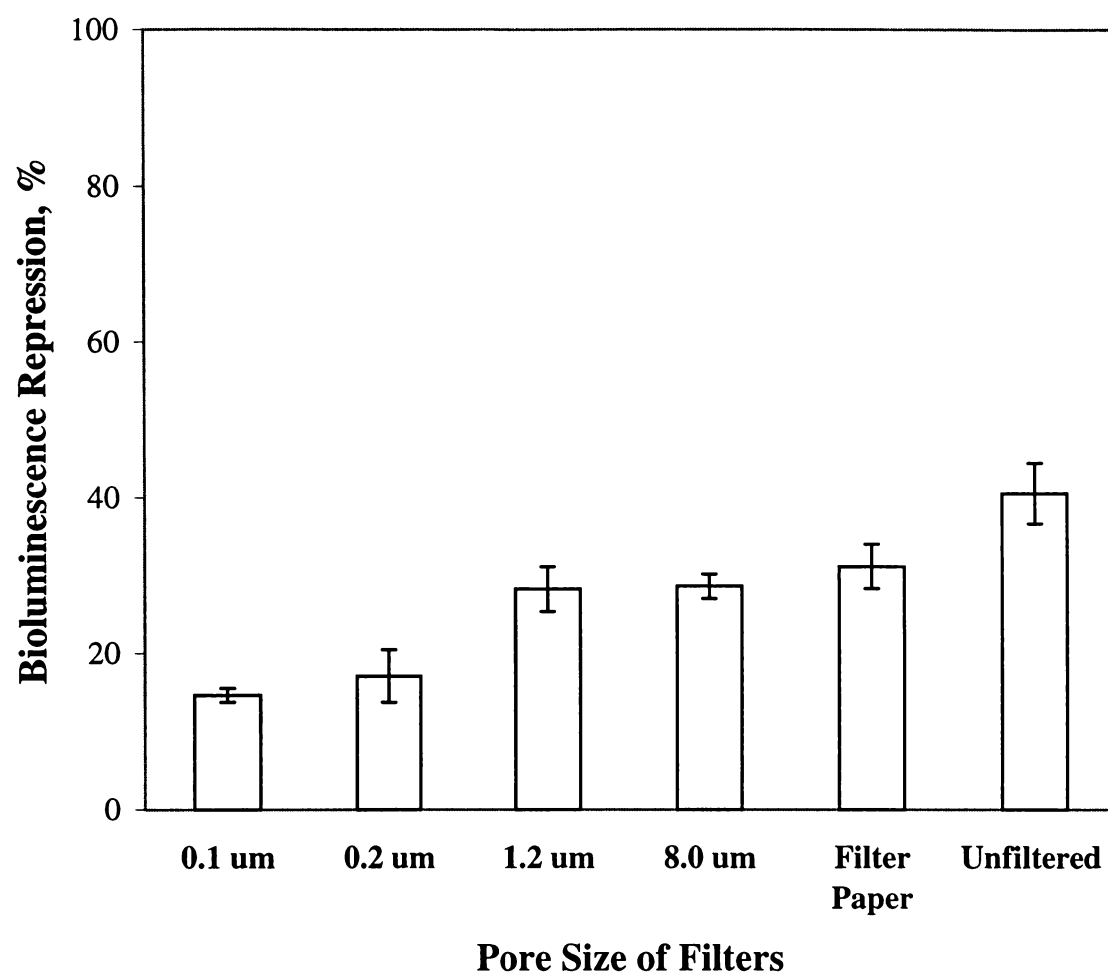


Figure 6-5. Effect of suspended solids on bioluminescence repression. The wastewater sample was taken at 12 am.

samples resulted in a BR 10-20% BR higher than that obtained using wastewater after filtration by 0.1 μm or 0.2 μm filters.

Discussion

Although we used the Shk1 assay as a specific testing method in our study, the following discussion is not limited to any particular bioluminescent bacteria-based assay. The effect of suspended solids in wastewater is common in all aquatic toxicity testing systems employing bioluminescent organisms.

Our results indicate that suspended solids in wastewater have a significant effect on light measurement and consequently affect the signal for toxicity in bioluminescent bacteria-based wastewater toxicity monitoring systems. The bioluminescence repression measured using unfiltered wastewater is higher than that measured using filtered wastewater, and the difference in the values of reduction of bioluminescence can be attributed to the existence of suspended solids. Figures 6-2 to 6-5 show that the smaller the pore size of the filter, the more significantly the bioluminescence differed from that measured when unfiltered wastewater was used.

Henze et al. (1995) discussed the distribution of particle sizes in typical raw wastewater. The number of particles of a particular diameter increases as that particle diameter increases up to its peak value at about 1 μm . The number of particles of a particular diameter is very low for particle diameters above 8 μm . Consequently, virtually all particles have diameters between 0.1 μm and 8 μm . In Figures 6-3 to 6-6, the differences in bioluminescence repression were not statistically significant between

unfiltered wastewater and filtered wastewater using coarse filter paper and an 8 μm filter, which is consistent with the typical distribution of particle sizes given by Henze et al. (1995). On the other hand, our data show that a filter with a 0.2 μm pore size filtered out almost all suspended solids (which would include microorganisms) and that reducing the pore size to 0.1 μm does not have much effect on bioluminescence measurement.

The bioluminescence repression caused by suspended solids in unfiltered wastewater can be regarded as the sum of two sources, one of which is the dissolved constituents in wastewater that inhibit bioluminescence. Bioluminescence repression due to the toxicity of the dissolved constituents is a chemical/biological effect and corresponds to the bioluminescence repression using wastewater filtered by 0.1 μm or 0.2 μm filters. The other source is the suspended solids in wastewater. This source represents a physical effect (scattering and absorption of light) and can be eliminated by filtration of the wastewater. This division of the causes of bioluminescence repression into effects associated with toxicity and those associated with light scattering/absorption caused by particles assumes that there is no toxicity associated with the particles themselves. This is not always true since toxic compounds can adsorb to particles and later desorb from them. The redistribution of toxicants between the aqueous phase and the suspended solids through adsorption/desorption can occur in the aeration tank in a biological treatment plant. However, it is difficult to predict whether the toxicity in the aqueous phase is increased or decreased because this depends on the fate of the toxicants in the aeration basin which is discussed below.

As a wastewater stream enters a biological treatment plant, it usually first flows through a primary clarifier in which 40-70% of the suspended solids will be removed by sedimentation (Benefield and Randall, 1980). Still a significant fraction of the suspended solids will enter the aeration basin since this leaves 30-60% of the BOD originally associated with suspended solids in the wastewater, which then enters the aeration basin with the remaining suspended solids. In addition to organic chemicals (which could be a part of the BOD), metals can also adsorb to suspended solids and be carried over to the aeration basin. Suspended solids can therefore be a source of toxicants downstream of a monitoring system and the rates of sorption/desorption are important in determining the bioavailability of these compounds (Koelmans et al., 1993). The degree to which the chemical compounds associated with suspended solids as well as those in the aqueous phase in influent wastewater affect the activated sludge microorganisms depends on the fate of these substances in the aeration tank. Generally, three removal mechanisms are involved in the removal of organic and some inorganic compounds in the aeration basin: volatilization through subsurface stripping and surface aeration, adsorption to suspended solids, and biodegradation (Bhattacharya et al., 1996, Haghghi-Podeh and Siyahati-Ardakani, 2000, Lee et al., 1998). Adsorption on extracellular polymers and bacterial cells of activated sludge is considered to be the removal mechanism of metals in the aeration tank (Battistoni et al., 1993). Most current research focuses on studying the fate of the chemical compounds that are in the aqueous phase of influent wastewater, and little is known about the fate of the chemical compounds associated with suspended solids in influent wastewater. Nonetheless, it can be inferred that as the suspended solids in influent wastewater enter the aeration basin, the chemical compounds released from

them also undergo one of the removal mechanisms discussed above: some compounds will be air-stripped, some will absorb to other suspended solids in the aeration basin, and the rest will be biodegraded by the activated sludge microorganisms. Those compounds that are air-stripped or absorbed by suspended solids may not be available to the activated sludge microorganisms (see discussion below) and therefore would not be toxic to them. While air-stripping definitely reduces the availability of toxicants to activated sludge microorganisms, the effect of absorption is an open issue since bacteria in activated sludge also contribute a large portion of the available surface area for adsorption (Lee et al., 1998). The fraction of chemical compounds that are either air-stripped or absorbed by suspended solids depends on the properties of both the chemical compounds and the suspended solids, e.g., volatility, hydrophobicity (Chen et al., 2000), surface acidity of suspended solids (Wang et al., 2000), etc. A quantitative relationship between the suspended solids, or the toxicities associated with the suspended solids, and their effect on activated sludge microorganisms is difficult to obtain.

As we noted previously (Chapter 5), the concentration of a toxicant in an influent wastewater stream is diluted as it enters a backmixed treatment system. Taking into account this dilution effect and the unpredictable fate of toxicants in the aeration basin, the signals produced by an influent wastewater toxicity monitoring system need to be calibrated with the overall performance of the treatment system as opposed to relating the signals to a single objective assessment of the wastewater toxicity. In this case, filtered wastewater can be used even though some toxic materials might be eliminated by removing the suspended solids in influent wastewater. For the continuous Shk1 toxicity screening procedure, filtered wastewater is preferred over unfiltered wastewater because

of problems caused by suspended solids in influent wastewater, e.g., clogging of the system. Although Ribo (1997) recommended centrifugation rather than filtration for turbidity removal, filtration can be most easily incorporated into a continuous system. Chapter 7 discusses a field-scale demonstration and evaluation of the Shk1-based influent toxicity monitoring system in which wastewater is filtered by a tangential membrane filter.

CHAPTER 7

THE FIELD-SCALE DEMONSTRATION OF THE Shk1-BASED CONTINUOUS INFLUENT WASTEWATER TOXICITY MONITORING SYSTEM

Our results in Chapters 4 and 5 indicated that the Shk1 assay is suitable for the purpose of influent wastewater toxicity monitoring. A field-scale Shk1-based influent toxicity monitoring system was developed based on the laboratory-scale rapid toxicity screening system. To assess the performance of this monitoring system and to determine the degree to which the response of Shk1 can be used to predict overall measures of plant performance (effluent BOD and ammonia, BOD removal, etc.), the field-scale monitoring system was put in field operation in the Kuwahee Wastewater Treatment Plant of the Knoxville Utilities Board (KUB) in Knoxville, Tennessee (see Figure 7-1) during the months of May and June of 2001. This chapter describes the development of the field-scale monitoring system, reports the field data, and discusses the performance of the field-scale system.

Methodology

Growth Medium

All chemicals used in the growth medium were obtained from Fisher Scientific unless otherwise specified. The growth medium for Shk1 was YEPG medium: 2g/L polypeptone, 0.2 g/L yeast extract, 1 g/L glucose, and 0.2 g/L NH_4NO_3 dissolved in de-ionized water. The medium was amended with 0.1% (v/v) antifoam A (Sigma-Aldrich)

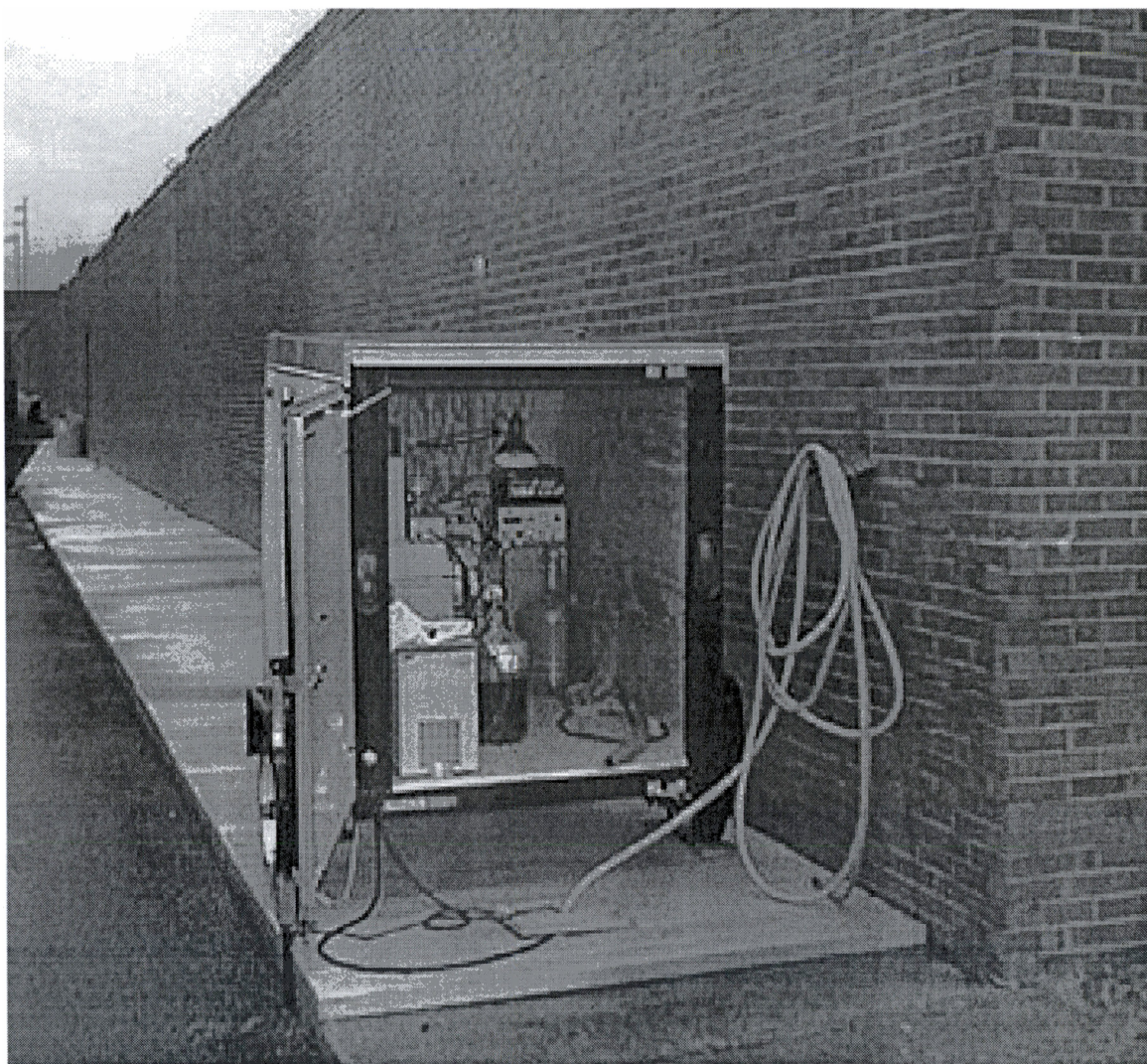


Figure 7-1. Field-scale demonstration of the influent wastewater toxicity monitoring system in a municipal wastewater treatment plant.

to prevent foaming and 3.03 g/L K_2HPO_4 and 5 g/L KH_2PO_4 to control pH at 7. The medium was first sterilized by autoclaving at 121°C (glucose in aqueous solution was autoclaved separately) and then was further amended with 25 ppm tetracycline.

Bioreactor and Accessories

A low volume bioreactor was constructed for the field-scale system (see Figure 7-2). The bioreactor consisted of a glass vessel and a Teflon[®] headplate. Sterile growth medium was fed to the nutrient port, and Shk1 cells were withdrawn from a port at the bottom of the glass vessel. An overflow port in the glass vessel controlled the volume in the bioreactor at about 150 ml. An air sparger and a thermowell for a temperature sensor were inserted into the glass vessel through the headplate. Agitation in the bioreactor was provided by a magnetic stirrer on the bottom of the bioreactor. A waterbath was made specifically for the bioreactor for temperature control (see Figures 7-2 and 7-3). The waterbath integrates metal cooling/heating coils and an acrylic lid to minimize water evaporation, and was filled with water. The heat transfer fluid inside the coils was provided by circulating heating/cooling water between the metal coils and a commercially available circulating cooling/heating waterbath.

Light Detection Equipment

Two plastic light sensing chambers as described in Chapter 2 and shown in Figure 2-2 were built and used in the field-scale system. The light emitted by the Shk1 cells was transmitted by light guides to the photomultiplier tubes (Oriel Corporation, Model 70680) that converted the light signals to electric signals in the form of current. A radiometer (Oriel Corporation, Model 7070) was used to amplify and display the current signal resulting from light emitted by bacteria flowing through the flow cell.

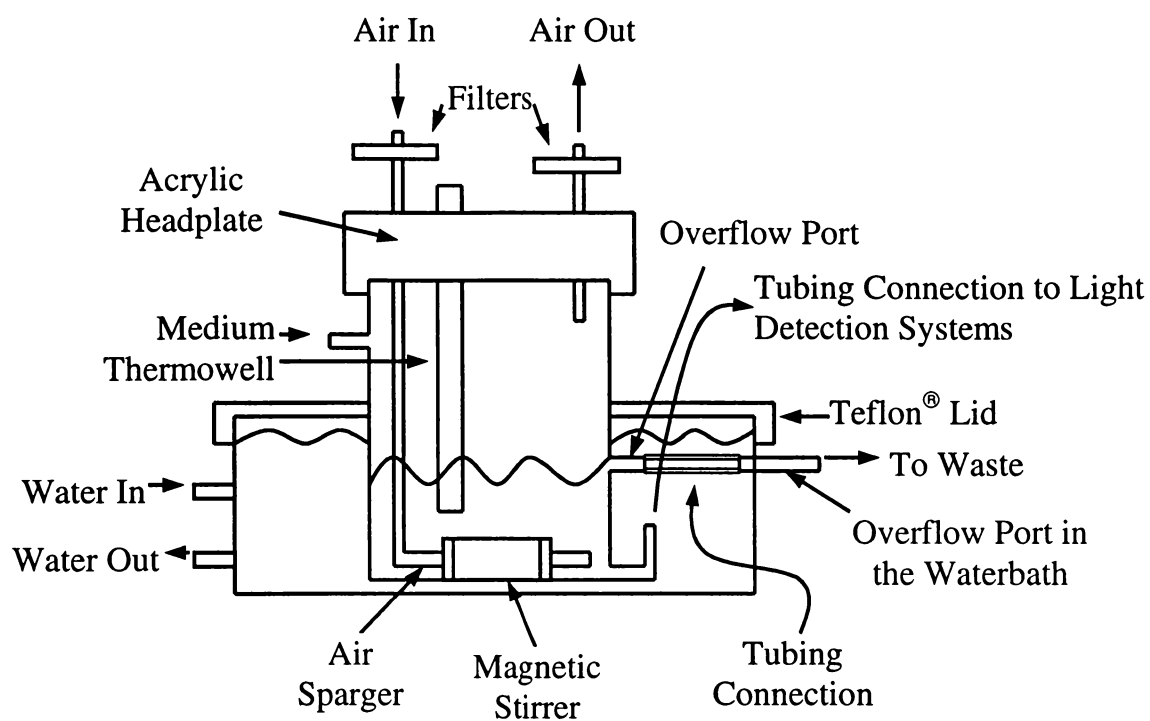


Figure 7-2. Schematic of the field-scale bioreactor and the waterbath for temperature control.

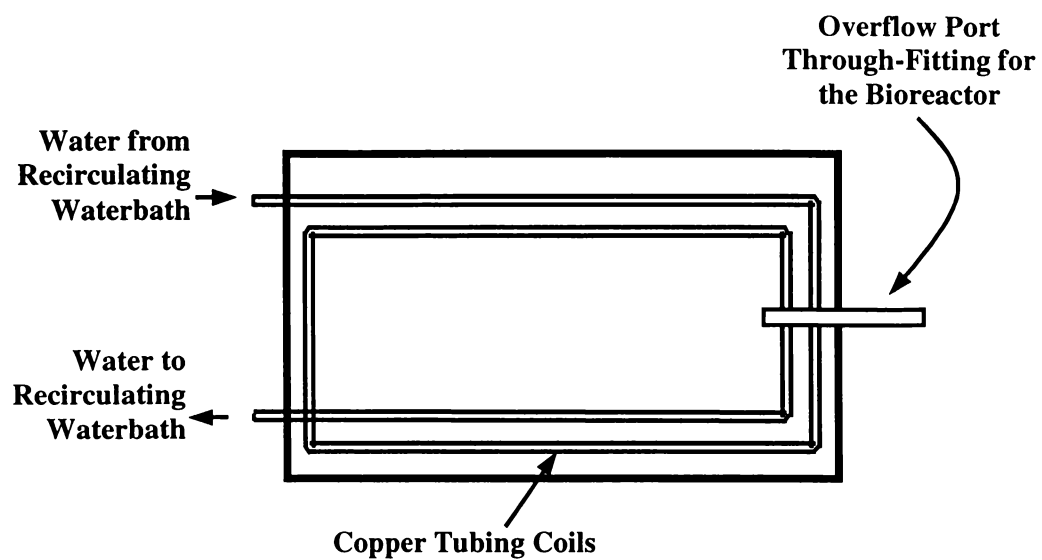


Figure 7-3. Top-view of the waterbath shown in Figure 7-2.

Field-scale System

The field-scale Shk1-based influent wastewater toxicity monitoring system was placed in a small utility trailer and was located between the primary clarifier and the aeration basin in the treatment plant (see Figure 7-1). A schematic of the field-scale system is shown in Figure 7-4. The bioreactor was operated at steady state under the following conditions: dilution rate = 0.52 hr^{-1} , pH = 7, and temperature = 22°C . These conditions were chosen as optimal for light emission based on studies discussed in Chapter 3. The pH was maintained by the buffer system described previously, and it was not necessary to actively control the pH by acid and base addition. The agitation rate was set to 200 rpm and air was fed at a rate of 1 L/min. Effluent wastewater from the primary clarifier in the wastewater treatment plant was continuously filtered using a tangential flow membrane filter (Gelman Sciences Versacap* Capsule) with a pore size of $0.2 \mu\text{m}$. The filtered wastewater was used as the influent test solution to the monitoring system.

Shk1 cells were supplied continuously to the two light sensors (see Figure 7-4). The first (upstream) sensor was used to determine the basal light level of the cells before exposure to the toxicant. After leaving the first sensor, the cells entered one leg of a "tee" connector where they were mixed with filtered wastewater. The volumetric flow rate of the wastewater into the tee was 3 times that of the Shk1 cell solution. The mixture of Shk1 cells and test solution then proceeded through several coils of Teflon[®] tubing to achieve an appropriate residence time (5 minutes) before entering the second light sensor. The Teflon[®] tubing coils were immersed in an additional waterbath (not shown) which was kept at 22°C . To help control the temperature in the bioreactor, the feed air was first

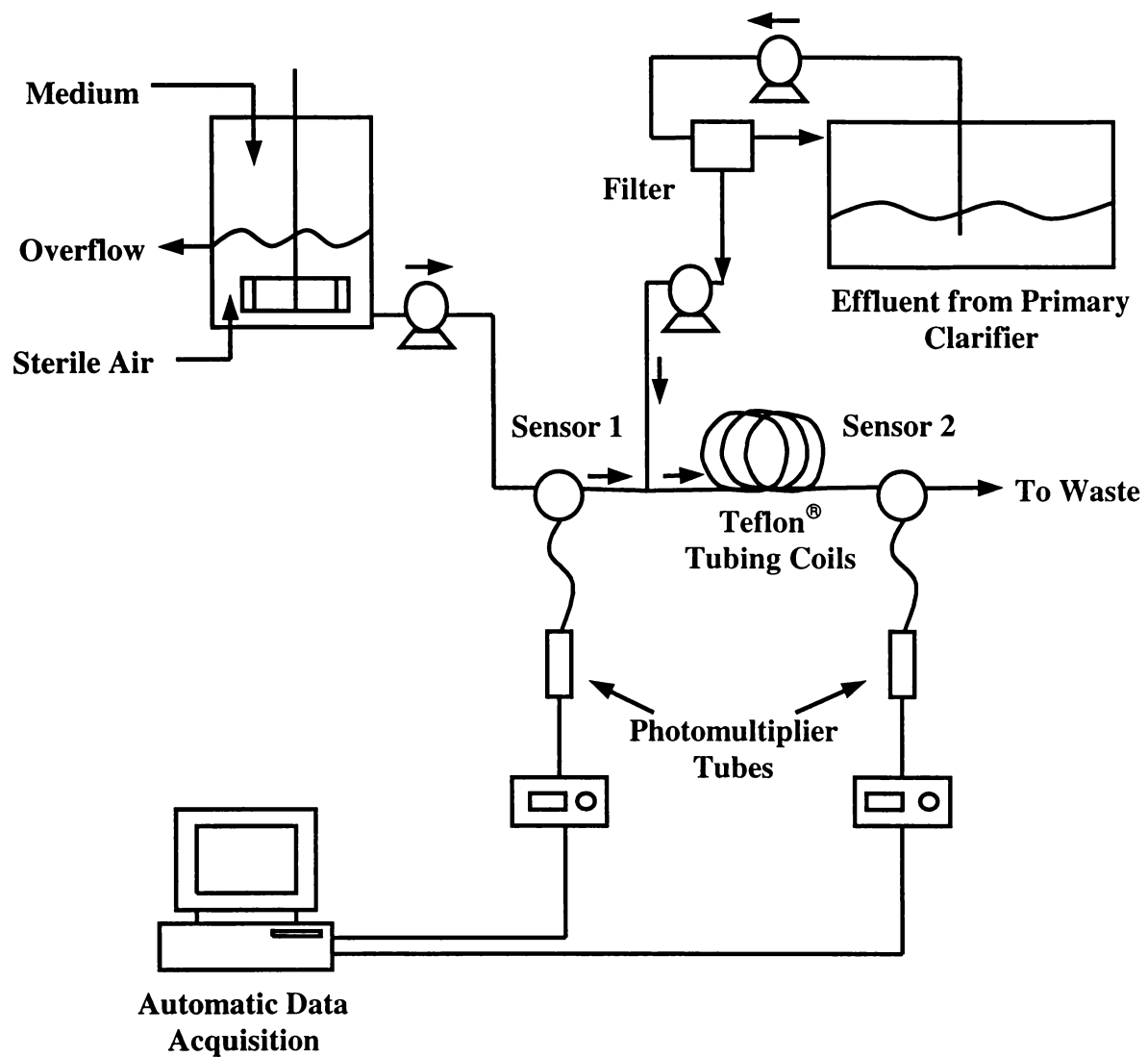


Figure 7-4. Schematic of the field-scale Shk1-based influent wastewater toxicity monitoring system.

passed through several coils of silicon tubing which was also immersed in this waterbath before passing through the filter to enter the bioreactor.

Bioluminescence of Shk1 before and after exposure to wastewater as measured by sensors 1 and 2 was recorded automatically by data loggers (Pace XR440 Pocket Loggers with millivolt modules, Pace Scientific Inc., Charlotte, North Carolina). The data loggers recorded data at a rate of once a minute. The toxicity of the filtered influent wastewater was indicated by bioluminescence repression (BR). BR was defined by

$$BR = \frac{(B_c / 4) - B_t}{(B_c / 4)} \times 100\% \quad (7-1)$$

where B_c = control bioluminescence of Shk1, measured by sensor 1

B_t = bioluminescence of Shk1 mixed with wastewater, measured by sensor 2

In Equation 7-1, the control bioluminescence B_c is divided by 4 to account for the fact that Shk1 cells were diluted by a factor of 4 by mixing with wastewater in the previously discussed 1:3 ratio.

Results

As mentioned in **Methodology**, the data loggers recorded data at a frequency of once per minute. A significant amount of data was thus obtained during the two months when the Shk1-based system was in operation. For simplicity, the average bioluminescence repression for every two hours was calculated and is shown in Figure 7-5. The field-scale system was occasionally taken back to our laboratory for maintenance and as a result data were not available on those occasions.

As seen in Figure 7-5, negative bioluminescence repression was occasionally observed. Negative bioluminescence repression indicates that the light output of Shk1 cells actually increased when they were mixed with wastewater. In Figure 7-5, negative bioluminescence repression values were truncated at -50%, though some of the negative bioluminescence repression values could be higher than -50% (see later discussion). Negative bioluminescence repression frequently occurred as a “flash”, but a few prolonged periods of negative bioluminescence repression were also observed.

In addition to 2-hour average bioluminescence repression, the average bioluminescence repression during night and day was also calculated. In this dissertation, “night” refers to the period between midnight to 8 am, and “day” refers to the remaining 16 hours in a day. Figure 7-6 shows the average bioluminescence repression by night and day during the two months. As in Figure 7-5, in Figure 7-6 negative bioluminescence repression values were truncated at -50%, though the actual values could be lower. Figure 7-6 shows that no clear day-night or weekday-weekend patterns could be seen.

Another average value calculated was daily average bioluminescence repression. Daily average bioluminescence repression was calculated for those days when bioluminescence data were available. In calculating daily average bioluminescence repression, the negative values of bioluminescence repression were neglected (see **Discussion**), and this average value was denoted as $DABR_{adj}$. $DABR_{adj}$ was plotted and is shown in Figure 7-7. Daily data for the treatment plant performance of the Kuwahee Wastewater Treatment Plant were provided by KUB in their normal monthly report. For

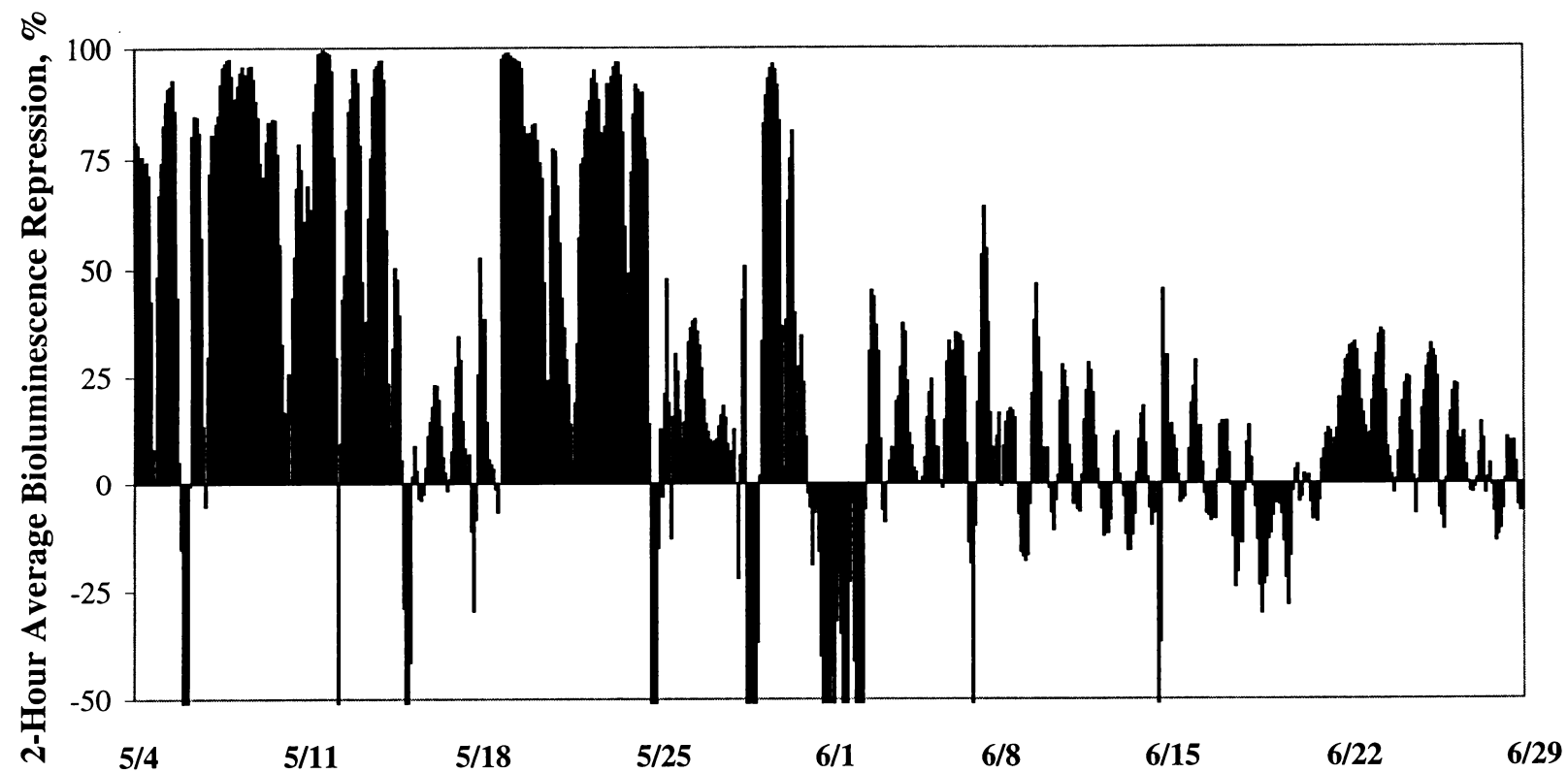


Figure 7-5. 2-Hour average bioluminescence repression profile obtained in May and June of 2001.

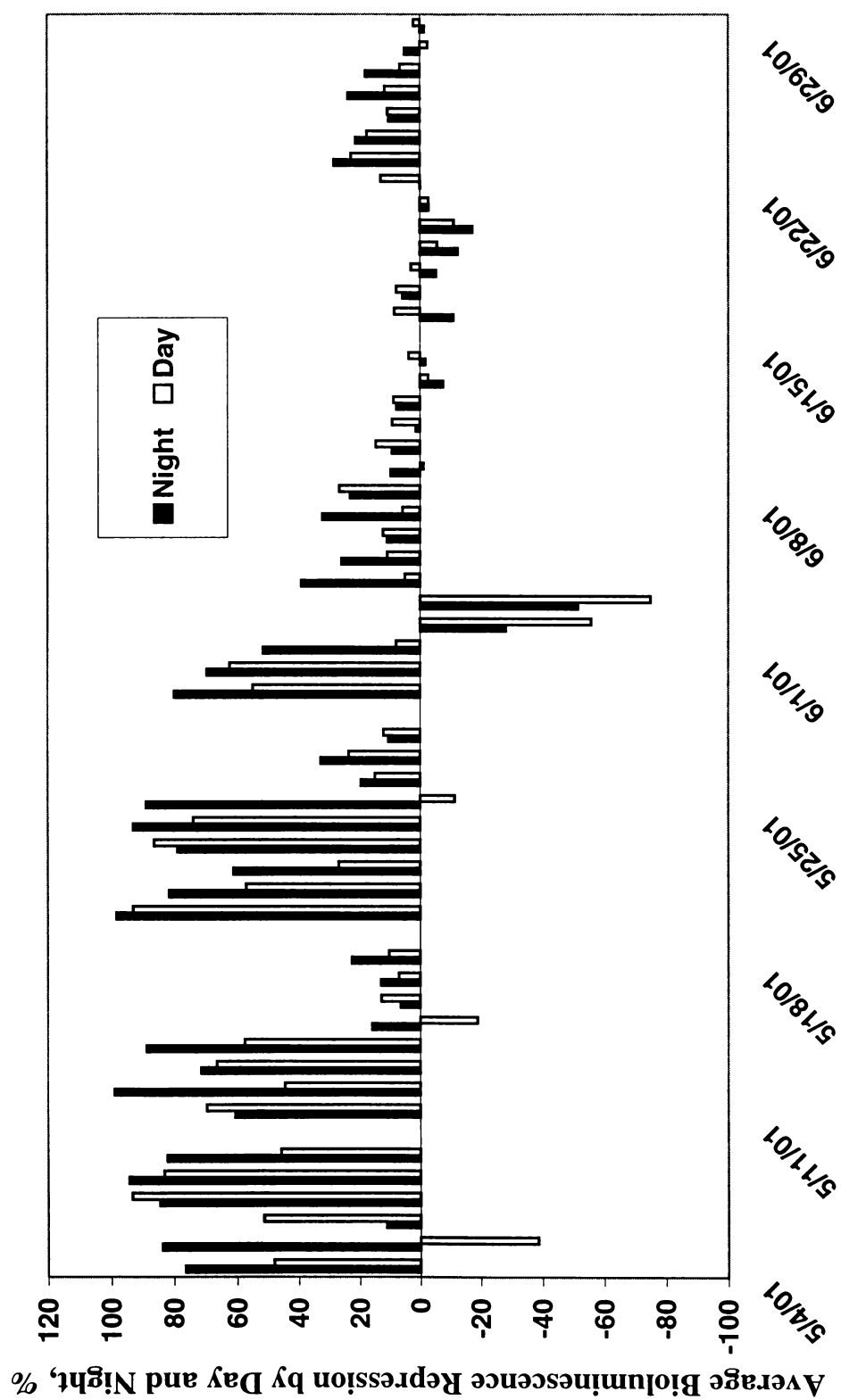


Figure 7-6. Average bioluminescence repression by night and day.

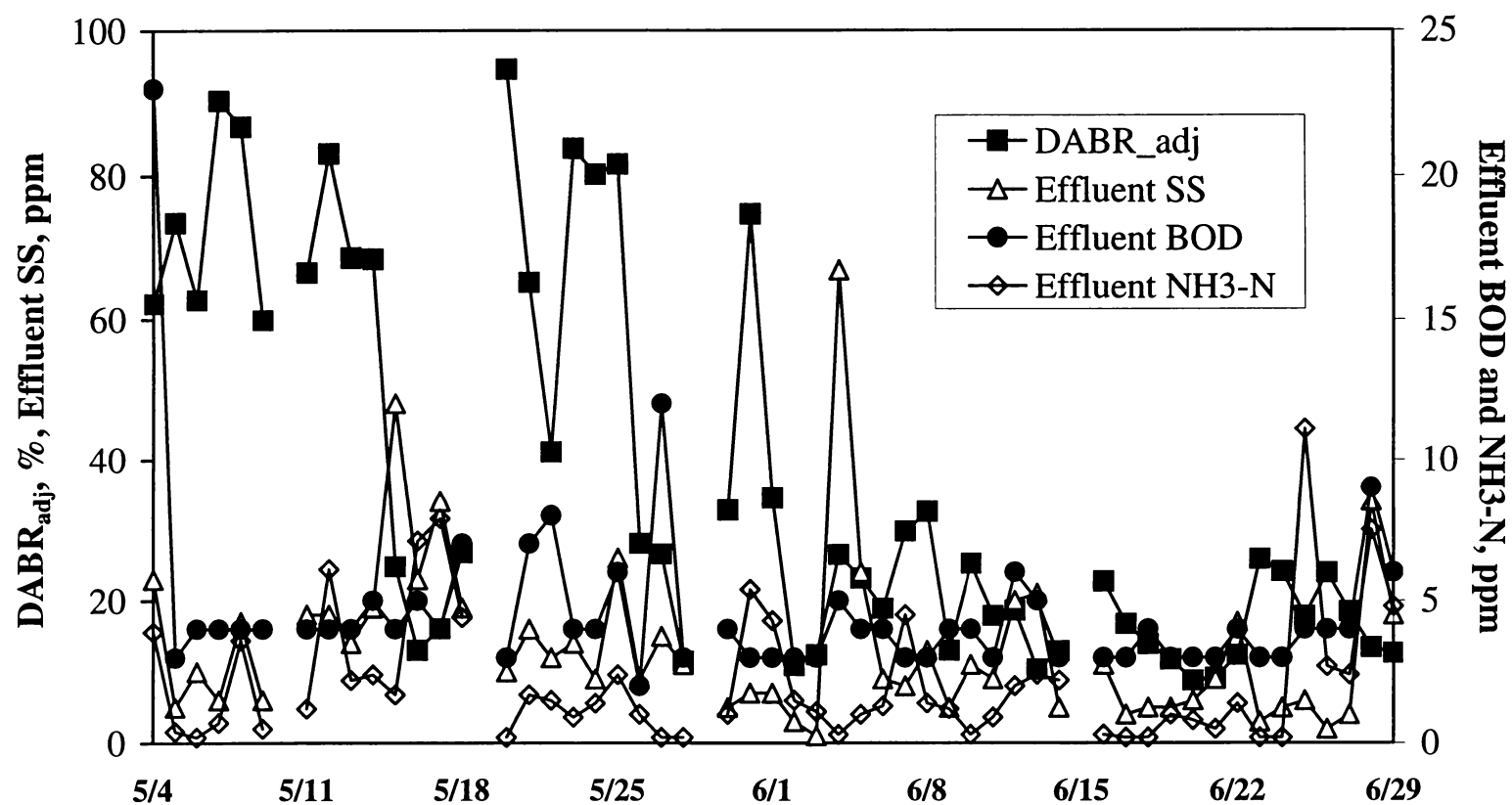


Figure 7-7. Adjusted daily average bioluminescence repression (DABR_{adj}) and effluent concentrations of BOD, suspended solids (SS) and NH₃ nitrogen (NH₃-N).

the purpose of comparison, the effluent concentrations of BOD, suspended solids, and NH_3 nitrogen were also plotted in Figure 7-7 (see **Discussion**). During the period of operation, the Shk1-based system detected 3 rough “peak” areas in DABR_{adj} . As seen from Figure 7-7, the peak areas occurred during the periods of May 4-13, 20-25 and 31, respectively. DABR_{adj} in June generally was lower than it was in May and no significant peaks could be clearly identified. Our results showed that the adjusted daily average bioluminescence repression values during the May-June period varied between 8.8% to 94.7%, with a mean value of 36.6% and a standard deviation of 27.1%.

As seen in Figure 7-7, variations in effluent concentrations of BOD, suspended solids, and NH_3 nitrogen concentration were observed during the period when the Shk1-based system was in operation. The effluent BOD concentration was significantly higher than 4 ppm (the median) on May 4 and 27 and on June 28. There were also smaller increases in effluent BOD concentration during the periods of May 18-27 and June 12. High values of the effluent suspended solids concentration could be seen on May 15 and 17, and on June 28. Significant increases in effluent NH_3 nitrogen concentration from 1.4 ppm (the median) were seen on May 12, 16-17, and 31, and on June 7, 25, and 28.

In addition to the absolute values of effluent concentrations of BOD, suspended solids and NH_3 nitrogen, the reductions of BOD and suspended solids were also provided by KUB. DABR_{adj} and BOD, suspended solids, and NH_3 nitrogen removal were plotted and are shown in Figure 7-8. As seen in Figure 7-8, BOD removal and suspended solids removal remained almost constantly near 100%. However, significant decreases in NH_3 nitrogen removal could be seen on May 8, 12, 16-17, and on June 25 and 28.

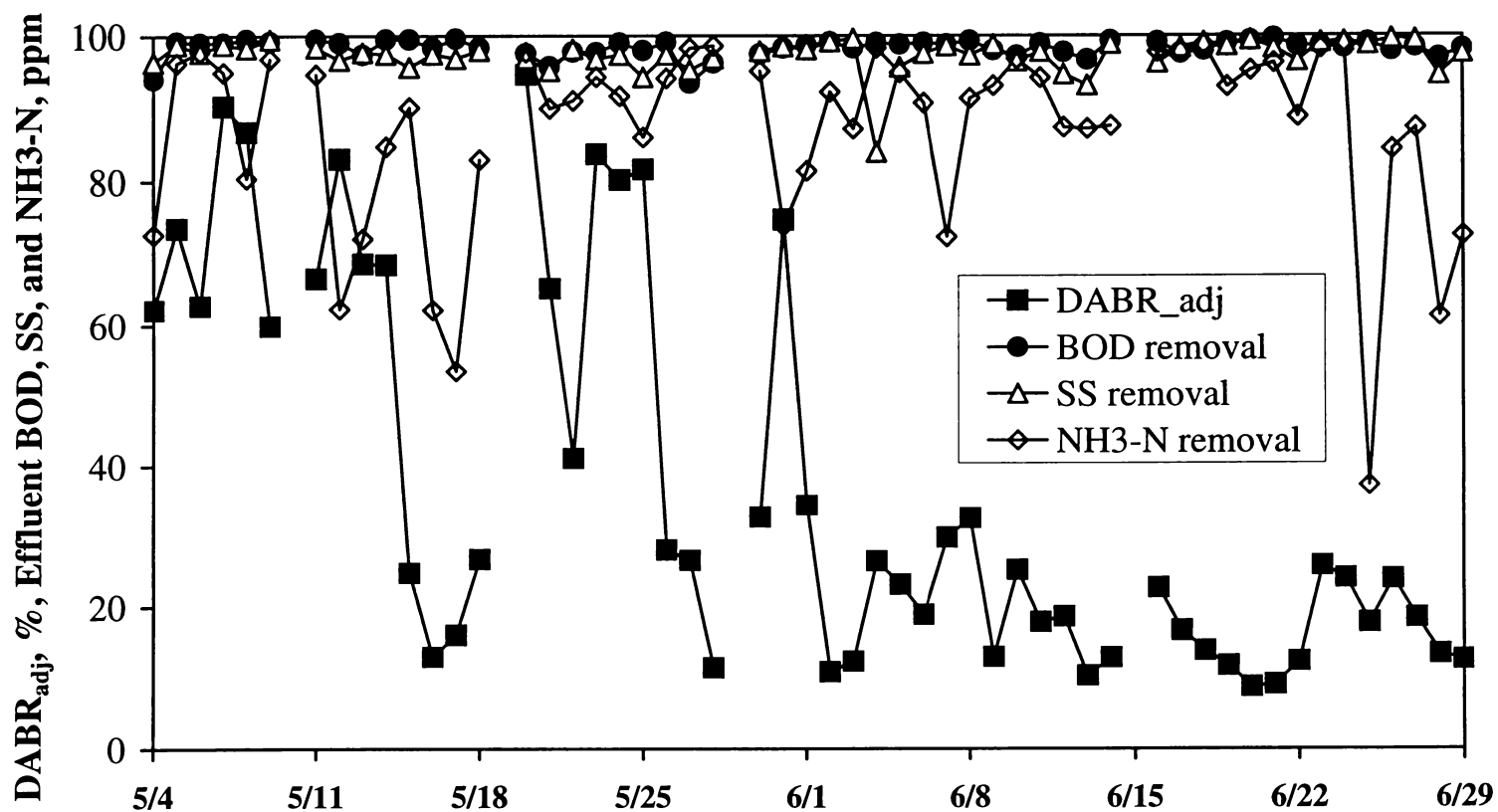


Figure 7-8. Adjusted daily average bioluminescence repression (DABR_{adj}) and BOD, suspended solids (SS), NH₃ nitrogen (NH₃-N) removals.

Fluctuations of NH_3 nitrogen removal can also be seen in the period of May 21-26 and May 31- June 7.

Discussion

As mentioned in **Introduction**, some field-scale on-line respirometers have been used for wastewater toxicity monitoring. Geenens and Thoeye (1998) reported that, on average, one toxicity measurement could be made every hour with the on-line respirometer they used. The response of the respirometer was relatively slow because the respirometer had to be frequently calibrated, and the toxicity measurement frequency depended on the calibration frequency. By contrast, our system recorded toxicity measurements in the form of bioluminescence once every minute. The measurement frequency of our system is essentially instantaneous and the frequency at which data was recorded was only limited by the memory available in the data loggers. Frequent measurements are desired because the possibility of not detecting toxic shocks that occur in short periods of time would be reduced. However, in this dissertation, average bioluminescence repression over a certain period of time is used. This is because the toxic effect of influent wastewater on the activated sludge depends not only on the toxicity of the wastewater which can be represented by bioluminescence repression, but also on the duration of the toxicity. An incoming wastewater that shows a certain level of toxicity during a short period of time may have the same toxic effect as an incoming wastewater that shows a lower toxicity for a relatively long period of time. Therefore, it is the integrate toxicity rather than instantaneous toxicity that should be taken into account when assessing the toxic effect of influent wastewater. In this dissertation, when

comparing bioluminescence repression with effluent BOD, suspended solids, and NH_3 nitrogen concentrations and their removals, the (adjusted) daily average bioluminescence repression values were used for the obvious reason that values of other parameters such as effluent BOD were provided only on a daily basis. Determining the most appropriate period of time over which bioluminescence repression should be averaged is not part of this dissertation and future research should be conducted on this aspect.

Other wastewater toxicity assessment tests have used wastewater filtered by membrane filters (Rojičková, 1998, Slabbert, 1988) as did ours. Influent domestic wastewater usually contains significant amounts of suspended solids, which interfere with light measurement by scattering or absorbing light (see Chapter 6). In a continuous system, suspended solids also could cause system clogging. We observed a significant amount of solids on the retentate side of the tangential flow filter and feel strongly that unfiltered wastewater would quickly clog the small diameter tubing in the monitoring system. In Chapter 6, we determined that a pore size of $0.2\ \mu\text{m}$ was appropriate for removing the suspended solids from influent wastewater. Therefore, in the field-scale Shk1-based system, influent wastewater was filtered using a tangential flow membrane filter with a pore size of $0.2\ \mu\text{m}$.

As mentioned in **Methodology**, negative bioluminescence repression was neglected when calculating DABR_{adj} . The reason why negative bioluminescence repression occurred was not clear. It is believed that sudden occurrence of negative bioluminescence repression with a high magnitude and a short occurrence time was caused primarily by air bubbles that traveled with the Shk1 cell solution stream. As shown in Figure 7-2, the Shk1 cell solution enters the tubing leading to the light detection

chamber through a port (harvest port) near the bottom of the bioreactor. Air coming from the air sparger presumably ascends and becomes mixed with the liquid in the bioreactor. However, it was observed that occasionally some air bubbles entered the harvest port instead of rising to the upper part of the bioreactor. When the air bubble reached the light detection system, abrupt increases in the light level readings could be observed. If the negative bioluminescence repression was indeed caused by the air bubbles, negative bioluminescence repression can be regarded as system error and can safely be neglected. For negative bioluminescence repression with relatively small magnitude that occurred during a prolonged period, however, it is possible that it occurred due to bioluminescence stimulation by certain compounds in wastewater. While these compounds are beneficial to Shk1, they may be toxic to activated sludge microorganism. If the compounds that stimulate Shk1 bioluminescence are also beneficial to activated sludge, then neglecting negative bioluminescence repression simply neglects the potential remedial effect of these compounds and therefore over-predicts influent wastewater toxicity. Neglecting negative bioluminescence can thus be regarded as adding a safety factor. On the other hand, if those compounds are toxic to the activated sludge microorganism, including negative bioluminescence repression in the analysis would lower the average bioluminescence repression and thus mistakenly create a mitigating effect. The ultimate result is under-estimating the toxicity. For these reasons, negative bioluminescence repression was completely neglected when analyzing the field data.

In order to assess the effect of influent wastewater toxicity on treatment efficiency, we chose effluent concentrations of BOD, suspended solids, and NH_3 nitrogen, and BOD, suspended solids, and ammonium reduction as indicators of

treatment efficiency. While the absolute values of the effluent concentrations of BOD, suspended solids and NH_3 nitrogen are important parameters in evaluating the effluent water quality, the BOD, suspended solids, and ammonium removal rates are, in a sense, more indicative of the efficiency of the treatment facility. As mentioned in **Results**, as influent toxicity (indicated by DABR_{adj}) varied significantly during the period when the Shk1-based system was in operation, BOD (and suspended solids) removal remained almost constant. However, significant changes in NH_3 nitrogen removal were observed. NH_3 nitrogen removal indicates the efficiency of the nitrification process in the treatment plant which is dependent on the activities of the nitrifying bacteria. The fact that NH_3 nitrogen removal varied significantly while the BOD removal did not can be explained by the relative sensitivities of the nitrifying bacteria and bacteria that are responsible for BOD removal (i.e., the heterotrophs).

Although (positive) bioluminescence repression was observed most of the time during the operation of the field-scale monitoring system, the treatment efficiency of the treatment plant as indicated by BOD and suspended solids removal was very high during the sampling period. As seen from Figures 7-7 and 7-8, there is no easily observable correlation between adjusted daily average bioluminescence repression and the treatment efficiency indicators. Several reasons could have caused this and they are discussed below.

As we pointed out in Chapter 5, there is a dilution effect as a wastewater stream enters a backmixed aeration basin. Potential toxic effects associated with the influent wastewater could be mitigated or delayed by this dilution effect. However, the Shk1-

based system must be employed upstream of the aeration basin and therefore cannot properly take into account the dilution effect.

The toxic effect of the influent wastewater also depends on the fate of the toxicants in the aeration basin, which is insufficiently understood (Vanrolleghem et al., 1996). Chapter 6 discussed the fate of toxicants associated with suspended solids in influent wastewater in the aeration basin. The fate of dissolved constituents found in influent wastewater in the aeration basin is similar. First, since the aeration basin is aerated, some toxicants could be air-stripped. Second, some toxicants including heavy metals could absorb to the surfaces of suspended solids or extracellular polymers in the aeration basin (Battistoni et al., 1993). Third, some toxicants may be biodegraded or biotransformed to less or non-toxic compounds. As we pointed out in Chapter 6, while air-stripping and bio-detoxification definitely reduce the availability of toxicants to activated sludge microorganisms, the effect of absorption is an open issue since bacteria in activated sludge also contribute a large portion of the available surface area for adsorption (Lee et al., 1998). We also pointed out in Chapter 6 that the fraction of chemical compounds that are either air-stripped or absorbed by suspended solids depends on the properties of both the chemical compounds and the suspended solids, e.g., volatility, hydrophobicity (Chen et al., 2000), surface acidity of suspended solids (Wang et al., 2000), etc. For these reasons, toxicants in influent wastewater may not be available to activated sludge microorganisms in the aeration basin which is downstream of the Shk1-based system. A quantitative relationship between the influent toxicity and the toxicity in the aeration basin is difficult to obtain.

Our system employs a pure culture of Shk1, while the microbial community in the aeration basin is a consortium of a large number of organisms of various species, the interactions among which perhaps cannot be neglected. It is possible that a toxicant in influent wastewater that is known to be toxic to one strain of microorganism in the aeration basin may be quickly biodegraded or biotransformed to another compound (less or non-toxic to this strain of microorganism) by some other sludge microorganisms, and therefore a toxic result to that particular strain of microorganism will not be seen. The sensitivities to toxicants are not the same for different microorganisms. Thus, it is also possible that when toxicants enter the aeration basin, some microorganisms are inhibited and at the same time, the growth and functioning of other microorganisms that carry out a similar function in the aeration basin are favored (due to the increased availability of nutrients). In this case, no degradation in overall treatment efficiency will be seen.

It must be realized that the treatment efficiency is not a function of influent toxicity alone. It is known that the sludge microorganisms, including the nitrifying bacteria, are also sensitive to many process variables such as temperature, pH, dissolved oxygen (Svenson et al., 2000), and hydraulic retention time, organic loading (Kim et al., 1996), rainfall, etc., that are not picked up by our system, and there are interactions among these variables. For example, on June 25, $DABR_{adj}$ was not significantly high, however, NH_3 nitrogen removal dropped significantly on that day (see Figure 9-8). Studying the plant operation parameters showed that the maximum influent flow rate and the average effluent flow rate on that day were significantly higher than a typical day (data not shown). It is known that the nitrifiers have a smaller maximum specific growth rate compared to the heterotrophs (Benefield and Randall, 1980) and therefore their

existence in the aeration basin is highly dependent on an appropriate hydraulic retention time. Thus, it was possible that the high flow rates on June 25 greatly reduced the hydraulic retention time at some time during that day and the nitrifying bacteria were removed from the aeration basin at a rate faster than they grew. This may have led to a decrease in the nitrifying bacteria population, which further resulted in a decrease in NH_3 nitrogen removal.

Despite the uncertainties of the fate of toxicants in the aeration basin and the generally lack of strict correlation between bioluminescence repression signals and plant performance, for the most convenient and most direct use of the signals generated by the Shk1-based system, an alarm level could be set at 70% DABR_{adj} , as Figure 9-8 suggests. DABR_{adj} values higher than this value generally and most obviously correspond to decreases in or perturbations of NH_3 nitrogen reduction, rather than parameters such as effluent BOD, of the treatment plant. However, note that DABR_{adj} is a quantity averaged over 24 hours. As we pointed out in Chapter 5, the toxic effect of influent toxicity not only depends on the intensity of the toxicity, but also on the duration of the toxicity. A stream of influent wastewater that causes a 99% Shk1 bioluminescence repression for 10 minutes would probably not cause any upsets in the plant performance. An algorithm that takes into account both intensity and duration should be developed. However, this is not the goal of this research.

A more rigorous analysis of the toxic effect of influent wastewater on plant performance could be achieved by considering all relevant variables together and thus requires multivariate statistical techniques. Principal component analysis (PCA) and factor analysis (FA) are used in this study. PCA is a mathematical procedure that

transforms an original set of correlated variables into a smaller set of uncorrelated variables called principal components (PCs). PCA is particularly useful for reducing data dimensionality. FA creates factors in such a way that correlation within a factor is high and correlation between factors is low. In this research, PCA and FA were performed using the software SAS 8.2 (SAS Institute, Cary, North Carolina).

In both PCA and FA, 21 variables were considered for each day and these variables were $DABR_{adj}$, rainfall amount (Rainfall), influent flow rate (Inf_flow), influent temperature (Inf_T), effluent flow rate (Eff_flow), influent BOD concentration (Inf_BOD), effluent BOD concentration (Eff_BOD), BOD removal (BOD_removal), influent suspended solids concentration (Inf_SS), effluent suspended solids concentration (Eff_SS), suspended solids removal (SS_removal), influent pH (Inf_pH), effluent pH (Eff_pH), influent NH_3 nitrogen concentration (Inf_ammonium), effluent NH_3 nitrogen concentration (Eff_ammonium), NH_3 nitrogen removal (Ammonium_removal), effluent dissolved oxygen (Eff_DO), mixed liquor suspended solids (MLSS), return suspended solids concentration (Return_SS), sludge volume index (SVI), and the volume of wet sludge to digester (Digester). The values of the variables measured on each day are shown in the Appendix.

PCA was first performed on the correlation matrix of the data set. Since the correlation matrix is a large matrix (21×21), only the correlations between $DABR_{adj}$ and other variables are shown in Table 7-1. Statistical test indicates that only the correlations between $DABR_{adj}$ and Inf_T, Eff_DO, MLSS, Return_SS and Digester are significant (test results not shown). Unfortunately, the total variability cannot be adequately described by just a few principal components (PCs). In order for approximately 85% of

Table 7-1. Correlation coefficients between daily average bioluminescence repression and other variables (numbers were rounded off to the second significant digit).

	DABR
DABR	1.00
Rainfall	0.032
Inf_flow	0.049
Inf_T	-0.50
Eff_flow	0.093
Inf_BOD	0.12
Eff_BOD	0.11
BOD_removal	-0.0076
Inf_SS	0.077
Eff_SS	-0.0017
SS_removal	0.0077
Inf_pH	-0.032
Eff_pH	-0.26
Eff_DO	0.39
Eff_ammonium	-0.053
Inf_ammonium	-0.11
Ammonium_removal	0.019
MLSS	-0.49
Return_SS	-0.47
SVI	-0.26
Digester	-0.39

the total variability to be accounted for, nine PCs are needed (see Table 7-2). Although PCA reduced the dimensionality from a 21-dimensional space to a 9-dimensional space, it is still impossible to visualize the distribution of the data in the reduced space.

The number of factors in FA was determined using the maximum likelihood method and was determined to be nine (see Table 7-3). The factor pattern is shown in Table 7-4 with the main loading components of each factor shown in blocks. The first few factors in Table 7-4 are well separated from the others and are easily interpreted. For example, the first four factors represent BOD, NH₃ nitrogen, suspended solids, and

Table 7-2. Eigenvalues and the cumulative proportion of total variability accounted for by each principal component (PC) and all previous ones (numbers were rounded off to the second significant digit).

	Eigenvalues	Cumulative proportion of total variability accounted for by each PC and all previous ones
1	3.98	0.19
2	3.39	0.35
3	2.39	0.46
4	2.37	0.58
5	1.64	0.66
6	1.54	0.73
7	1.09	0.78
8	0.90	0.82
9	0.80	0.86
10	0.65	0.89
11	0.53	0.92
12	0.41	0.94
13	0.31	0.95
14	0.28	0.97
15	0.24	0.98
16	0.21	0.99
17	0.18	1.00
18	0.042	1.00
19	0.031	1.00
20	0.013	1.00
21	0.0079	1.00

Table 7-3. Determination of the number of factors.

Test	Degrees of Freedom	χ^2	$\text{Pr} > \chi^2$
H ₀ : No common factors H ₁ : At least one common factor	210	870.1213	< 0.0001
H ₀ : 9 factors are sufficient H ₁ : More factors are needed	57	72.0590	0.0863

wastewater flow rate, respectively. However, the loading components of the remaining five factors are not as clear and the determination of the loading components was more or less at the researcher's discretion. Also note that several trivial factors, i.e., factors with

Table 7-4. Factor pattern (numbers were rounded off to the second significant digit).

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9
BOD_reduction	1.00	0	0	0	0	0	0	0	0
Inf_BOD	0.52	0.14	0.42	-	0.42	-0.16	-0.050	0.20	-
Eff_BOD	-0.71	0.19	0.25	0.038	0.27	-0.11	0.0058	0.41	-
Eff_N	0.049	0.99	0.12	0	0	0	0	0	0
N_reduction	-0.018	-0.97	-0.11	0.021	0.011	0.11	0.13	-0.024	-
Eff_SS	-0.035	0.084	1.00	0	0	0	0	0	0
SS_reduction	0.22	0.13	-0.84	-	0.37	-0.026	-0.072	0.022	-
Inf_flow	0.082	0.20	-0.036	0.064	0.038	-	-0.019	-	-
Eff_flow	0.10	0.20	-0.093	0.97	-	0.00045	0.0022	0.0088	0.043
Rainfall	-0.17	0.12	-0.043	0.96	0.030	-0.013	0.0022	0.0083	0.050
Inf_SS	0.32	0.29	0.31	0.71	-	-0.026	0.027	-0.11	-0.13
Return_SS	0.13	-	-0.33	-	0.83	0.066	-0.059	-0.042	0.036
MLSS	-0.28	0.047	-0.30	0.034	-0.21	0.67	-0.27	0.26	0.26
Inf_T	-0.012	0.066	0.050	-	-0.24	0.55	-0.36	-0.071	0.14
Eff_pH	0.056	-	-0.042	0.047	0.17	0.49	-0.15	0.13	-0.16
SVI	0.13	0.041	0.12	0.11	0.083	0.48	0.029	0.40	0.027
Digester	0.0026	-0.14	0.14	-0.12	0.32	-0.37	0.17	0.34	-0.16
DABR _{adj}	-	-	0.0026	-0.13	0.21	-0.65	0.046	0.29	-0.24
Inf_N	0.0076	0.054	0.082	0.18	0.18	-0.67	0.36	0.0060	0.43
Inf_pH	-0.15	0.073	-0.076	0.23	0.22	0.49	0.59	0.028	-
Eff_DO	0.10	-0.24	-0.18	0.13	-	0.32	0.38	0.16	0.046
					0.076	-0.16	0.21	0.12	0.028
									0.49

only one significant loading, were generated. This provides additional evidence of the complicated nature of a wastewater treatment plant.

The factor analysis indicates that the $DABR_{adj}$ is correlated with SVI more than the indicators of the treatment efficiency such as BOD removal. Considering the residence time in the aeration basin and the second clarifier, there will be a lag in time between the signals generated by the Shk1 system and effluent parameters (effluent BOD, suspended solids, and NH_3 nitrogen concentrations and their reductions). Attempts to find factor patterns relating $DABR_{adj}$ to the effluent parameters on the subsequent few days were made. In doing this, the values of $DABR_{adj}$ and all non-effluent parameters from the same day were used together with the values of effluent parameters from the 2nd, 3rd, up to the 7th day, thus creating six new data sets. Factor analysis was performed for each of these new data sets.

The number of factors was determined to be between 7 to 10 depending on the data set using the maximum likelihood method, and factor patterns were obtained. The results show that no factors can be identified containing $DABR_{adj}$ and one or more effluent parameters, i.e., effluent BOD, suspended solids, and NH_3 nitrogen (results not shown).

In addition to the effect of the variables other than influent toxicity and the effect of dilution as an influent stream enters the aeration basin (discussed in Chapter 5 and above), several other factors must be considered in the attempt to relate bioluminescence signals to plant performance.

Chapters 4 and 5 showed that the response of Shk1 to toxicants is generally similar to that of the activated sludge microorganisms; however, this will not ensure that

the responses of Shk1 and activated sludge microorganisms are the same in all circumstances. We have shown that the signals produced by the Shk1-based system is a meaningful piece of information, however, due to the reasons discussed above, we found it difficult to relate treatment efficiency to the signals of the Shk1-based system alone. While the performance of the Shk1-based system in predicting treatment efficiency is not perfectly satisfactory, it has been established that Shk1 responds to a wide variety of toxicants (Chapters 4 and 5). Consequently, the information obtained from the Shk1-based system could be used as an additional evidence of possible toxic discharges (rather than significant changes in operating conditions) should degradation in treatment efficiency and high bioluminescence repression are observed simultaneously and help identify the source of the discharges.

The bioluminescence repression signals generated by the Shk1-based system can be used in other statistical methods in addition to PCA and FA. Such methods include logistic regression. Logistic regression is one of the statistical methods that can be used for membership discrimination and classification. As an example, consider the data for $DABR_{adj}$ and plant operating variables shown in Appendix. First of all, the 95% quartiles of effluent BOD, suspended solids, and NH_3 nitrogen were found to be 9, 34, and 7.5 ppm. Then the data were artificially separated into two groups. Those days when effluent BOD is greater than 9 ppm, or when effluent suspended solids is greater than 34 ppm, or when effluent NH_3 nitrogen is greater than 7.5 ppm were put in Group 1 which represents days that degradation in effluent quality is seen. All other days were put in Group 2 which represents days with no degradation in effluent quality. Using all non-effluent parameters as variables, logistic regression was then performed to classify the

observations (i.e., days) into one the two groups. The classification results of the logistic regression and the *a priori* classification can be compared and are shown in Table 9-5. As seen from Table 9-5, only 1/3 of the days when decrease in treatment efficiency was observed was correctly classified by logistic regression. Given the complicated nature of the operations of a wastewater treatment plant, it is unrealistic to expect any classification algorithm to make 100% correct classifications.

The method of logistic regression shown above can also be used to predict whether degradation in effluent quality will occur in the subsequent days, provided appropriate data are used in the logistic regression. In logistic regression, all variables including those such as influent BOD and influent suspended solids concentration were used. Unlike parameters like pH and temperature, influent BOD and suspended solids cannot be quickly measured. When logistic regression is used with the continuous influent toxicity monitoring system, prompt measurements of the variables are necessary for decision-making purposes. It is anticipated that influent BOD and suspended solids concentration and other parameters alike can either be accurately estimated or obtained from a correlation with other parameters that can be promptly measured.

Table 7-5. Classification results of the logistic regression.

	Number of days when decrease in treatment efficiency was observed	Number of days when decrease in treatment efficiency was <i>not</i> observed
Number of days when decrease in treatment efficiency was observed that are classified as:	2	4
Number of days when decrease in treatment efficiency was <i>not</i> observed that are classified as:	0	46

CHAPTER 8

CONCLUSIONS AND RECOMMENDED WORK

Conclusions

Light production of Shk1 in batch cultures is characterized by a peak in specific bioluminescence in the mid- to late exponential phase. In batch cultures, there are optimum pH values and temperatures for maximum specific growth rate and specific bioluminescence of Shk1, above or below which the maximum specific growth rate and specific bioluminescence become lower. The optimum pH values at fixed temperatures and the optimum temperatures at fixed pH values for maximum specific growth rate differ from those for specific bioluminescence. In continuous cultures in a CSTR, the intensity of light produced by Shk1 is constant. The specific bioluminescence increases as dilution rate increases until wash-out occurs, but there are pH and temperature optima for specific bioluminescence. A correlation of specific bioluminescence with dilution rate, pH and temperature was generated and can be used to roughly predict Shk1 specific bioluminescence. The existence of phosphate salts in the growth medium does not negatively the specific bioluminescence of Shk1.

The Shk1 bioluminescence assay we describe in this study is based on the bioluminescence repression, upon exposure to toxic aqueous solutions, of a continuously supplied stream of a solution of genetically engineered *Pseudomonas* bacteria. The test is simple and its response is quick. Three mixing ratios of toxicant solution-to-cell solution as well as cell growth rates were varied to obtain appropriate conditions for toxicity screening of aqueous concentrations of heavy metal ions; these conditions are a mixing

ratio of 3 (3 parts test solution to 1 part cell solution), a dilution rate of 0.52 hr^{-1} , a pH of 7, and a temperature of 22°C .

The toxicities to Shk1 of individual and binary mixtures of heavy metals were studied. Compared to Microtox[®] toxicity data in the literature, Shk1 EC_{50} values for industrial heavy metals are closer to the concentrations of heavy metals that are known to affect the activated sludge process. EC_{50} values of the heavy metals in binary mixtures were obtained. The interaction of the two components in each binary mixture were studied and analyzed statistically for significance. The interaction was shown to exhibit both synergistic and antagonistic effects depending on the ion pair and the concentrations of its components being tested.

The toxicities to Shk1 of 102 organic chemicals that included simple and halogen substituted alkanes, alkenes and aromatics, alcohols, ketones, amines and phenols were studied. Compared to the Microtox[®] toxicity data, Shk1 is less sensitive to the organic chemicals. A comparison of the correlation between Shk1, Microtox[®] and activated sludge respiration inhibition EC_{50} values shows that the Shk1 bioluminescence assay is more suitable for predicting activated sludge respiration inhibition.

The experimental results indicate that the reduction of Shk1 bioluminescence caused by dissolved constituents in wastewater increases as the concentrations of those constituents increase in a test sample. Our results also indicate that suspended solids in influent wastewater cause interference with the light measurement in bioluminescent bacteria-based toxicity monitoring systems. The effect of suspended solids is an increased reduction of bioluminescence. For the Shk1 assay using wastewater samples,

the increased reduction of bioluminescence is approximately 10-20% higher for unfiltered wastewater samples when compared to those filtered by a 0.2 μm filter. It is difficult to evaluate the toxic effect of the suspended solids in influent wastewater to the activated sludge microorganisms since their fate is dependent on many factors. However, the interference of suspended solids can be eliminated by filtration of the wastewater using filters having a pore size equal to or less than 0.2 μm .

No correlations were obtained between the signals of the field-scale system and effluent parameters (effluent BOD, suspended solids, and NH_3 nitrogen concentration) or treatment efficiency (BOD, suspended solids, and NH_3 nitrogen removal) of the wastewater treatment plant. The signals of the continuous Shk1 assay by themselves appeared to be irrelevant to plant performance. Consequently, it is not possible to set an alarm level based on the signals of the Shk1-based system.

Recommended Work

Multi-sensor Toxicity Screening

As discussed in Chapters 4, and 5, each species of microorganism has its own sensitivity pattern to toxicants and no one is able to respond with appropriate sensitivity to all toxicants. As a result, there is an increasing tendency to use a battery of bioassays instead of a single bioassay in toxicity tests. Research has been proposed to create additional bioluminescent organisms that have origins in the activated sludge to represent other classes of sludge microorganisms. A proposed multi-organism influent wastewater toxicity monitoring system would consist of bioreactors operated in parallel to

continuously supply these microorganisms. Influent wastewater is screened for toxicity using microorganism 1, microorganism 2, and so on and so forth. The toxicity information obtained by a multi-sensor toxicity screening system will be more complete compared to a single-sensor system.

Miniaturized Bioassays

A significant amount of efforts have been made to miniaturize bioassays, also known as micro-bioassays (Blaise et al., 1986, Fishman et al., 1999, Formusoh et al., 1997, Rojíčková et al., 1997, Vidakovic et al., 1999). The advantages of miniaturized bioassays are obvious. Compared to traditional bioassays, miniaturized bioassays can handle a larger number of replicate test samples and therefore the results are more statistically convincing. Miniaturized bioassays in the form of bioassay kits are easily carried over to places where such assays are needed. The sample and reagent volumes of a miniaturized bioassay are reduced by several fold compared to traditional bioassays and are usually on the order of microliters.

Several methods could be employed when miniaturizing a bioassay, e.g., immobilized cells and microfluidic technology. However, currently most of the miniaturized bioassays are performed batchwise and are not suitable for routine influent wastewater toxicity monitoring.

For the Shk1-based assay, a continuous miniaturized system is desired. One possible approach is to develop a continuous microbioreactor which will be used to supply Shk1 cells as did the bioreactors described in this research. Kostov et al. (2001) described the development of a microbioreactor used for batch fermentation. The microbioreactor was built from a normal polystyrene cuvette normally used for

spectrophotometry and had a total volume of 4 ml. The microbioreactor was equipped with air inlet and outlet, a stirrer, and dissolved oxygen, pH and optical density measuring capacity. A continuous microbioreactor for the Shk1 assay could be developed based on the batchwise microbioreactor Kostov et al. (2001) described by adding a nutrient port and an overflow port. The Shk1-based system will have an additional advantage in that pH could be controlled by adding buffer to the growth medium and that dissolved oxygen concentration need not be strictly controlled. Therefore, while pH and dissolved oxygen measuring capacities are desirable, they are not indispensable and the configuration of the continuous microbioreactor could be no more complicated, if not less, than the batchwise microbioreactor. In operating the continuous microbioreactor, the flow rates of the medium and other streams could be controlled by high-precision pumps.

An alternative for miniaturizing the Shk1 assay is the so-called lab-on-a-chip, which conceptually means downscaling a lab bench to the size of a chip. Ramsey (1999) presented examples of lab-on-a-chip devices, some of which were continuous systems with flow rates on the order of a few milliliters per year. Obviously, the scale of lab-on-a-chip devices is several orders of magnitude smaller than the microbioreactors described and discussed above, but currently such devices are more difficult to make and naturally, more costly.

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APPENDICES

Daily average bioluminescence repression (DABR) and other plant operation variables.

Date	DABR _{adj} , %	Rainfall, inch	Total influent flow, MGD	Influent temperature, °C	Daily average effluent flow, MGD	Influent BOD, ppm	Effluent BOD, ppm
05/01/01	-	0.32	29.03	24	24.68	989	3
05/02/01	-	0	27.14	24	22.63	1004	3
05/03/01	-	0	26.23	22	21.63	730	5
05/04/01	62.23	0	25.52	24	20.84	389	23
05/05/01	73.59	0	24.5	17	20.02	360	3
05/06/01	62.79	0.31	25.89	24	21.26	394	4
05/07/01	90.36	0.08	28.56	25	24.59	408	4
05/08/01	86.8	0.11	27.35	24	23.08	757	4
05/09/01	59.92	0	26.61	24	22.53	476	4
05/10/01	-	0	25.85	25	21.43	1001	6
05/11/01	66.66	0.02	25.42	22	20.77	759	4
05/12/01	83.1	0.02	24.54	25	20.2	401	4
05/13/01	68.71	0	23.85	22	19.36	153	4
05/14/01	68.56	0	25.74	23	21.16	1038	5
05/15/01	24.81	0	25.47	25	20.48	833	4
05/16/01	13.02	0	25.4	25	20.29	311	5
05/17/01	16.1	0	25	25	20.32	1011	4
05/18/01	26.75	0	24.72	23	19.75	434	7
05/19/01	-	0.02	24.53	25	19.95	355	22
05/20/01	94.71	0	23.69	20	19.14	127	3
05/21/01	65.28	0.98	27.79	26	23.69	165	7
05/22/01	41.14	0.8	37.16	26	32.54	367	8
05/23/01	83.79	0.03	27.66	23	25.39	173	4
05/24/01	80.22	0.38	29.45	22	26.75	411	4
05/25/01	81.65	0	27.69	26	24.1	298	6
05/26/01	28.07	0	25.32	23	21.2	229	2
05/27/01	26.57	0	23.96	23	19.33	186	12
05/28/01	11.48	0.05	26.04	25	21.67	82	3
05/29/01	-	0	26.21	25	21.64	178	4
05/30/01	32.72	0	26.2	25	21.47	158	4
05/31/01	74.77	0.19	26.64	24	22.15	177	3
06/01/01	34.43	0.4	29.37	25	25.57	231	3
06/02/01	10.88	0.1	26.39	26	22.39	345	3
06/03/01	12.37	0.1	25.17	22	20.94	179	3
06/04/01	26.47	0	26	25	21.41	489	5
06/05/01	23.19	0	25.81	25	21.01	329	4
06/06/01	18.92	0	27.4	25	23.18	394	4
06/07/01	29.79	0.1	29.11	25	24.8	248	3

06/08/01	32.53	0	27.19	26	23.15	447	3
06/09/01	13	0	24.82	26	20.34	203	4
06/10/01	25.24	0	23.91	26	19.27	144	4
06/11/01	17.89	0	26.7	26	21.83	262	3
06/12/01	18.65	0	25.46	27	21.05	258	6
06/13/01	10.34	0.6	27.27	25	23.18	153	5
06/14/01	12.91	0	25.87	26	22.04	424	3
06/15/01	-	0	23.63	25	21.71	346	3
06/16/01	22.74	0	23.24	27	19.17	343	3
06/17/01	16.72	0	22.63	27	18.3	126	3
06/18/01	13.91	0	24.85	27	20.14	205	4
06/19/01	11.77	0	24.33	25	19.99	302	3
06/20/01	8.8	0.2	27.14	23	22.52	424	3
06/21/01	9.15	0	25.4	26	21.89	728	3
06/22/01	12.4	0.7	32.76	26	28.23	289	4
06/23/01	25.85	0	24.97	27	21.14	218	3
06/24/01	24.12	0	24.02	25	19.81	179	3
06/25/01	17.86	0.3	29.87	25	27	411	4
06/26/01	23.97	0	26.83	25	22.49	187	4
06/27/01	18.49	0	26.71	26	22.43	249	4
06/28/01	13.44	0.1	26.57	25	21.9	289	9
06/29/01	12.65	0.4	28.13	26	23.91	314	6
06/30/01	-	0	25.41	25	21.03	409	16

Daily average bioluminescence repression (DABR) and other plant operation variables (continued).

Date	Influent suspended solids, ppm	Effluent suspended solids, ppm	Suspended solids removal, %	Influent pH	Effluent pH	Effluent DO, mg/L	Inffluent NH ₃ -N, ppm
05/01/01	900	7	99.2	7.3	7.6	8.1	19
05/02/01	1010	7	99.3	7.2	7.9	7.6	13
05/03/01	1170	19	98.4	7	7.8	8.1	15.6
05/04/01	640	23	96.4	7.5	7.8	7.2	14.3
05/05/01	420	5	98.8	7	7.8	8	10.7
05/06/01	440	10	97.7	7.4	7.5	8.2	9.1
05/07/01	490	6	98.8	7.2	7.4	8.1	14
05/08/01	1000	17	98.3	7.3	7.4	7.6	18.4
05/09/01	1200	6	99.5	7.3	7.6	8.2	15.8
05/10/01	1200	6	99.5	7.5	7.9	7.2	18.4
05/11/01	1130	18	98.4	7.5	7.8	7.3	22.6
05/12/01	540	18	96.7	7.1	7.3	7.9	16.2
05/13/01	600	14	97.7	7.3	7.5	7.8	7.9
05/14/01	760	19	97.5	7.2	7.5	8.3	15.8
05/15/01	1110	48	95.7	7.2	7.7	7.7	17.3
05/16/01	930	23	97.5	7.2	7.6	6.9	18.8
05/17/01	1110	34	96.9	7.1	7.4	7.2	17
05/18/01	930	19	98	7.4	7.6	7	25.9
05/19/01	680	38	94.4	7.1	8	7.5	22.4
05/20/01	320	10	96.9	7.1	7	7.5	9
05/21/01	330	16	95.2	7.6	7.9	7.4	17.1
05/22/01	670	12	98.2	7.5	7.8	7.7	16.9
05/23/01	440	14	96.8	7.5	7.8	8.5	15.9
05/24/01	350	9	97.4	7.4	7.5	8.9	17
05/25/01	460	26	94.3	7.5	7.7	7.8	17.2
05/26/01	350	9	97.4	7.1	7.4	8.1	17.1
05/27/01	320	15	95.3	7	7.2	7.8	11.8
05/28/01	360	11	96.9	7.4	7.4	7.7	14.2
05/29/01	440	16	96.4	7.4	7.3	8.9	19.2
05/30/01	240	5	97.9	7.4	7.6	8.9	20.6
05/31/01	480	7	98.5	7.3	7.7	7.6	20.7
06/01/01	370	7	98.1	7.7	7.8	7.3	23.2
06/02/01	360	3	99.2	7.4	7.6	7.2	19.5
06/03/01	230	1	99.6	7.2	7.7	6.8	8.6
06/04/01	420	67	84	7.3	7.7	7.5	17.5
06/05/01	560	24	95.7	7.6	7.7	7.7	19.7
06/06/01	380	9	97.6	7.4	7.4	7.5	14.1
06/07/01	590	8	98.6	7.1	7.2	7.8	16.3
06/08/01	480	13	97.3	7.3	7.7	7.8	16.3

06/09/01	410	5	98.8	6.9	7.6	-	17.5
06/10/01	310	11	96.5	7.2	7.6	7.5	9.3
06/11/01	400	9	97.8	7.5	7.6	8	15.6
06/12/01	380	20	94.7	7.3	7.7	7.7	15.9
06/13/01	310	21	93.2	7.6	7.6	7.3	18.8
06/14/01	470	5	98.9	7.4	7.6	7.1	17.8
06/15/01	500	7	98.6	7.6	7.7	7.6	18.8
06/16/01	300	11	96.3	7	7.8	7.9	13.3
06/17/01	250	4	98.4	7.6	7.7	7.5	9
06/18/01	530	5	99.1	7.3	7.7	7.6	16.5
06/19/01	350	5	98.6	7.4	7.8	7.7	14.3
06/20/01	990	6	99.4	7.4	7.9	7.2	16.9
06/21/01	520	9	98.3	7.1	7.8	7.5	13.4
06/22/01	480	17	96.5	6.9	7.5	7.3	12.6
06/23/01	330	3	99.1	7.1	7.7	7.6	11.4
06/24/01	630	5	99.2	7.5	7.8	8.6	19.9
06/25/01	520	6	98.8	7.5	7.7	8	17.7
06/26/01	560	2	99.6	7.4	8	7.8	17.3
06/27/01	620	4	99.4	7.5	8	7.8	19
06/28/01	630	34	94.6	7.3	7.7	7.2	19.4
06/29/01	750	18	97.6	7.4	7.7	7.6	17.5
06/30/01	400	31	92.2	6.8	7.5	7.6	16.9

Daily average bioluminescence repression (DABR) and other plant operations variables (continued).

Date	Effluent NH ₃ -N	NH ₃ -N removal, %	MLSS, ppm	Return suspended solids, ppm	SVI, 100 ml/g	Wet sludge to digester, 1000 gallons,	BOD removal, %
05/01/01	0.5	97.37	2640	8850	208	130	99.7
05/02/01	2.1	83.85	2540	8375	256	162	99.7
05/03/01	1.9	87.82	2140	8675	150	176	99.3
05/04/01	3.9	72.73	2710	7700	247	197	94.1
05/05/01	0.4	96.26	2880	7825	229	195	99.2
05/06/01	0.2	97.80	2310	6425	247	142	99
05/07/01	0.7	95.00	2320	7125	168	163	99
05/08/01	3.6	80.43	2480	8075	161	115	99.5
05/09/01	0.5	96.84	2710	9300	133	131	99.2
05/10/01	1.8	90.22	2690	7725	123	163	99.4
05/11/01	1.2	94.69	2692	7725	89	165	99.5
05/12/01	6.1	62.35	2540	8000	189	167	99
05/13/01	2.2	72.15	2800	7125	104	188	97.4
05/14/01	2.4	84.81	2310	8850	182	188	99.5
05/15/01	1.7	90.17	2220	6075	189	165	99.5
05/16/01	7.1	62.23	2520	6425	190	145	98.4
05/17/01	7.9	53.53	2340	6925	188	128	99.6
05/18/01	4.4	83.01	2670	7175	172	128	98.4
05/19/01	7.8	65.18	2860	6450	143	128	93.8
05/20/01	0.2	97.78	3490	7150	43	127	97.6
05/21/01	1.7	90.06	3030	7200	132	128	95.8
05/22/01	1.5	91.12	2620	7900	141	135	97.8
05/23/01	0.9	94.34	3070	8600	130	127	97.7
05/24/01	1.4	91.76	2840	8800	109	124	99
05/25/01	2.4	86.05	3680	9800	122	123	98
05/26/01	1	94.15	3140	8900	210	125	99.1
05/27/01	0.2	98.31	4730	7475	53	125	93.5
05/28/01	0.2	98.59	4290	8300	84	124	96.3
05/29/01	0.3	98.44	3030	9475	142	124	97.8
05/30/01	1	95.15	3380	9925	148	134	97.5
05/31/01	5.4	73.91	2360	9825	102	135	98.3
06/01/01	4.3	81.47	3190	8775	122	127	98.7
06/02/01	1.5	92.31	3330	9900	99	124	99.1
06/03/01	1.1	87.21	4110	7825	73	127	98.3
06/04/01	0.3	98.29	3370	8875	92	129	99
06/05/01	1	94.92	2940	9400	95	134	98.8
06/06/01	1.3	90.78	3300	9625	88	189	99

06/07/01	4.5	72.39	3420	8625	117	126	98.8
06/08/01	1.4	91.41	2540	11650	79	126	99.3
06/09/01	1.2	93.14	2340	10575	90	126	98
06/10/01	0.3	96.77	4410	10950	68	127	97.2
06/11/01	0.9	94.23	3230	12275	84	127	98.9
06/12/01	2	87.42	3150	9875	159	129	97.7
06/13/01	2.4	87.23	3290	10200	91	144	96.7
06/14/01	2.2	87.64	3530	9900	71	130	99.3
06/15/01	3.4	81.91	4000	9025	130	129	99.1
06/16/01	0.3	97.74	3830	10375	128	127	99.1
06/17/01	0.2	97.78	3370	10800	166	128	97.6
06/18/01	0.2	98.79	3730	10250	121	133	98
06/19/01	1	93.01	4200	15175	100	127	99
06/20/01	0.8	95.27	3770	12250	154	128	99.3
06/21/01	0.5	96.27	4130	13000	111	130	99.6
06/22/01	1.4	88.89	3790	12425	103	130	98.6
06/23/01	0.2	98.25	3500	13325	86	128	98.6
06/24/01	0.2	98.99	3610	16400	139	96	98.3
06/25/01	11.1	37.29	3510	12200	97	103	99
06/26/01	2.7	84.39	5440	15025	79	93	97.9
06/27/01	2.4	87.37	5320	12675	137	110	98.4
06/28/01	7.5	61.34	3170	11150	85	95	96.9
06/29/01	4.8	72.57	4490	12700	100	107	98.1
06/30/01	0.9	94.67	4540	14125	77	94	96.1

SAS Program

```
proc import out=work.toxicity
            datafile="c:\Shijin\Paper15\Tox_all(1).xls"
            dbms=excel2000 replace;
            getnames=yes;
run;
Data tox;
set toxicity;
if DABR+Effluent_DO+Effluent_BOD='.' then delete;
run;
proc print; run;
proc corr data=tox;
var DABR_adj Rainfall--Digester;
run;
proc princomp data=tox;
var DABR_adj Rainfall--Digester;
run;
proc factor data=tox method=prin scree;
var DABR_adj Rainfall--Digester;
run;
proc factor data=tox method=ml priors=smc nfact=9 rotate=varimax reorder heywood;
var DABR_adj Rainfall--Digester;
run;

proc univariate data=tox;
var Effluent_BOD Effluent_N Effluent_SS SS_reduction BOD_reduction N_reduction;
run;

data tox1; set tox;
if Effluent_N>7.5 or Effluent_BOD>9 or Effluent_SS>34 then Effluent_N=1;
else Effluent_N=2;
run;
proc stepdisc data=tox1 stepwise SLE=0.5 SLS=0.30;
class Effluent_N;
var DABR_adj Rainfall--Influent_BOD Influent_SS Influent_pH_--Influent_N
Effluent_DO--Digester;
run;
proc discrim data=tox1 pool=yes crossvalidate;
class Effluent_N;
var DABR_adj Rainfall--Influent_BOD Influent_SS Influent_pH_--Influent_N
Effluent_DO--Digester;
run;
proc logistic data=tox1;
```

```

model Effluent_N=DABR_adj Rainfall--Influent_BOD Influent_SS Influent_pH--
Influent_N Effluent_DO--Digester;
output out=pdicts predicted=phat;
run;
data pdicts;
set pdicts;
if phat>0.5 then predicted='1';
else predicted='2';
run;
proc freq;
tables Effluent_N*predicted;

data tox2; set tox;
if N_reduction<61.3 or BOD_reduction<95.8 or SS_reduction<94.3 then Effluent_N=1;
else Effluent_N=2;
proc stepdisc data=tox2 stepwise SLE=0.5 SLS=0.30;
class Effluent_N;
var DABR_adj Rainfall--Influent_BOD Influent_SS Influent_pH--Influent_N
Effluent_DO--Digester;
run;
proc discrim data=tox2 pool=yes crossvalidate;
class Effluent_N;
var DABR_adj Rainfall--Influent_BOD Influent_SS Influent_pH--Influent_N
Effluent_DO--Digester;
run;
proc logistic data=tox1;
model Effluent_N=DABR_adj Rainfall--Influent_BOD Influent_SS Influent_pH--
Influent_N Effluent_DO--Digester;
output out=pdicts predicted=phat;
run;
data pdicts;
set pdicts;
if phat>0.5 then predicted='1';
else predicted='2';
run;
proc freq;
tables Effluent_N*predicted;
run;

```

VITA

Shijin Ren was born in Beijing, China in January 1974. He earned his Bachelor of Science degree in Chemical Engineering from Beijing University of Chemical Technology in July 1995. In 1997, he came to the University of Tennessee, Knoxville and entered the Ph.D program in the Department of Chemical Engineering. He earned his Doctor of Philosophy degree in Chemical Engineering in December 2001.