AN ABSTRACT OF THE THESIS OF

Sandra L. Uesugi for the degree of Master of Science in Soil Science and
Bioresource Engineering presented on June 14, 2000.

Title: Evaluation of Bioluminescence as a Measure of Bacterial Cell Density in
Porous Media.

Redacted for Privacy

Abstract approved: _____
                                         Peter J. Bottomley

Redacted for Privacy

_____
                              John S. Selker

Salicylate-induced, *lux* gene dependent bioluminescence was measured
using a cooled charge-coupled device (CCD) camera to evaluate its potential as a
quantitative measurement of cell density of *Pseudomonas fluorescens* HK44 in
porous media. The CCD camera was able to detect bioluminescence from cell
densities between 1×10^6 and 1×10^8 cells/ml in both liquid suspensions and saturated
sand, and between 5×10^6 and 8.5×10^8 cells/ml in unsaturated sand at 7.0%
(cm^3/cm^3) volumetric water content. After *lux* gene induction by 100 mg/L
salicylate, light emission increased with the square of time and linearly with
increasing cell density. A model was developed to relate light emission with cell
density. Similar values were determined for the rate of increase in light emission,
B', [12 ($\pm$ 0.2) $\times 10^{-10}$ light units/(cell-min^2)] for suspensions of cells in aqueous
media and in saturated or unsaturated sand. Growth phase of HK44 significantly

influenced the first detectable time (FDT) of bioluminescence response with log and stationary phase cells expressing FDT values of 2.5 and 1.8 hours, respectively, after induction. The effect of growth phase was eliminated by the addition of 10 to 250 mg/L glucose and salicylate simultaneously. Oxygen availability limited the upper cell density limit (1×10^8 cells/ml) that could be measured by bioluminescence with the CCD camera. While each camera system and bioluminescent organism combination will require calibration, a CCD camera system has the potential to quantify bacterial bioluminescence as a means to study microbial growth and dynamics non-destructively in two-dimensional porous media.

Evaluation of Bioluminescence as a Measure of Bacterial
Cell Density in Porous Media

by

Sandra L. Uesugi

A THESIS

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 14, 2000
Commencement June 2001

Master of Science thesis of <u>Sandra L. Uesugi</u> presented on <u>June 14, 2000</u>.

APPROVED:

Redacted for Privacy

Co-Major Professor, representing Soil Science

Redacted for Privacy

Co-Major Professor, representing Bioresource Engineering

Redacted for Privacy

Head of Department of Crop and Soil Science

Redacted for Privacy

Head of Department of Bioresource Engineering

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Redacted for Privacy

Sandra L. Uesugi, Author

ACKNOWLEGEMENTS

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

Dedicated to my parents and grandparents
who have continued to support me
throughout my education
and
to the memory of my grandpa,
Dr. Masaru Kurashima
a wise man and a great fisherman.

Evaluation of Bioluminescence as a Measure of Bacterial Cell Density in Porous Media

INTRODUCTION

Increasing demands on groundwater supplies require intensified efforts to remediate contaminated subsurface soils and aquifers. In order to meet these requirements, new technology must be developed to expedite the removal of hazardous substances from polluted and threatened water supplies. One such favorable process, in-situ bioremediation, utilizes existing microbial communities to degrade pollutants in soil and groundwater (Hart 1996; Mohammed et al., 1996). However, a lack of understanding of microbial community growth and development within the subsurface greatly impedes these efforts. There is a critical need for the development of methods to accurately study subsurface microbial activities and growth in order to design more effective and efficient remediation techniques.

The utilization of bioluminescent bacteria provides a unique ability to visualize bacteria in some porous media. Bioluminescent bacteria have been used for a multitude of purposes including biosensors to detect chemicals in the environment (Heitzer et al., 1992; Jensen et al., 1998; King et al., 1990; Yeomans et al., 1999), cell detection in the environment (Shaw et al., 1992; Silcock et al., 1992), pathogen detection (Hibma et al., 1996; Soren et al., 1995; Sutherland et al., 1994) and as indicators of bacterial metabolic status (Duncan et al., 1994;

Ellison et al., 1991). The bacterial bioluminescence reaction involves a heterodimeric enzyme, luciferase, which catalyzes the reaction between molecular oxygen, a long-chain aldehyde and reduced flavin mononucleotide ($FMNH_2$), resulting in the emission of visible blue-green light at 490 nm (Meighen & Dunlap 1994). In the bioluminescence reaction, the aldehyde is oxidized to a fatty-acid by luciferase with the production of water and FMN. Aldehyde substrates are generated by a fatty-acid reductase complex requiring ATP, NADPH, and acyl-CoA. Figure 1 illustrates the relationship between the fatty-acid reduction system and the luminescent reaction. The *lux* CDABE gene cassette codes for luciferase and its corresponding reductase complex. The α and β subunits of the luciferase enzyme are encoded by the *lux* AB genes while the reductase, transferase and synthetase enzymes are encoded by *lux* CDE, respectively (Meighen & Dunlap 1994).

The bacterium used in this project, *Pseudomonas fluorescens* HK 44, contains the stable plasmid pUTK21. The plasmid was created by the insertion of the *lux* CDABE gene cassette from the marine bacterium, *Vibrio fischeri*, into the upper naphthalene degradative pathway located on a catabolic plasmid NAH7. The *lux* genes are induced by salicylate, a metabolite of naphthalene degradation, or by naphthalene itself (King et al., 1990). For the purpose of this project, salicylate was used for induction due to its relatively high aqueous solubility. A number of
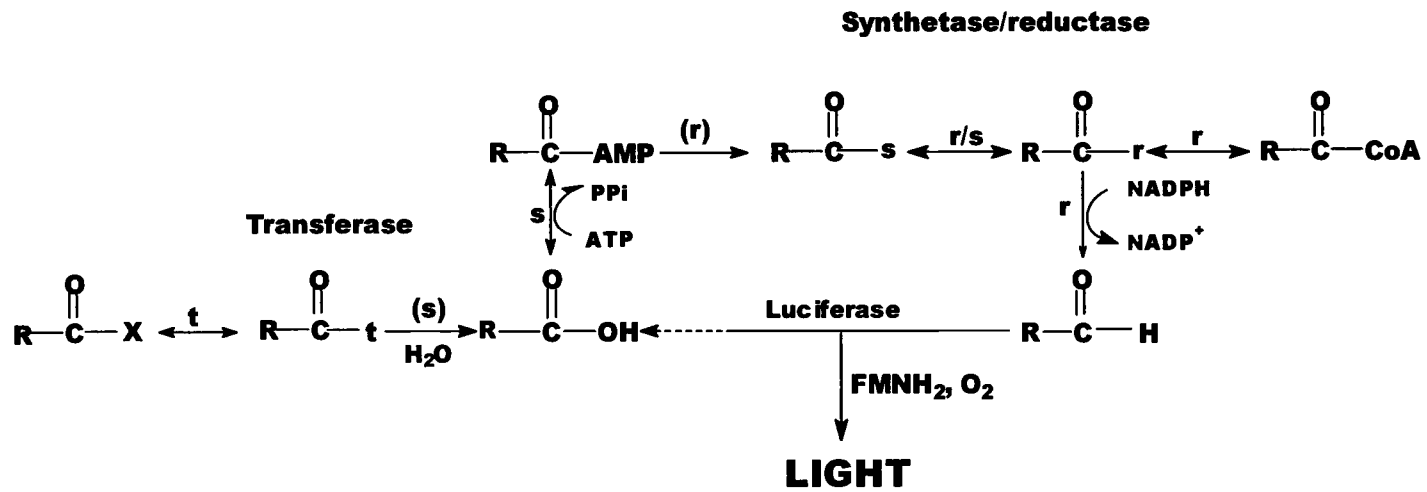
Figure 1. Interaction between the light-producing luciferase reaction and the fatty-acid reduction pathway. Transferase (t) cleaves the activated acyl group (R-CO-X). The complex is then activated by synthetase (s) and reduced by reductase (r) to form the fatty aldehyde substrate for the luminescence reaction. Fatty acids produced by the luminescence reaction can be recycled via this reduction pathway (from Meighen and Dunlap, 1994)

papers exist on the kinetics and bioluminescence response of HK44 when used as a biosensor for naphthalene, salicylate, and other hydrocarbon contaminants in soil and water (Ford et al., 1999; Heitzer et al., 1998; Heitzer et al., 1994; Heitzer et al., 1992; King et al., 1990; Matrubutham et al., 1997; Webb et al., 1997).

Instruments used to measure bacterial bioluminescence include luminometers (Duncan et al., 1994; King et al., 1990), scintillation counters (Neilson et al., 1999), x-ray autoradiography (Langridge et al., 1994), 35-mm film (Grant et al., 1991; Shaw et al., 1987) and CCD (Flemming et al., 1994; Shaw et al., 1987; Silcock et al., 1992; Waterhouse et al., 1993; Waterhouse & Glover 1994; Waterhouse et al., 1996). However, with the exception of CCD imaging, these approaches do not allow for continuous non-destructive and non-intrusive observation of microbial processes over time. Hydrologic relationships are especially sensitive to invasive methods. This project will focus on initial development and characterization of a non-invasive technique for observing microbial activity in saturated and unsaturated sand. To date, no one has determined if it is possible to use a CCD camera to quantify bacterial biomass by bioluminescence in a non-destructive, non-intrusive method in liquid cell suspensions and porous media under both saturated and unsaturated water contents. A few studies have used bioluminescence to quantify bacteria in the soil environment, but these involve the removal of a sample aliquot for the quantification of light emission (Bolenes et al., 1993; Duncan et al., 1994; Grant et al., 1992; Neilson et al., 1999; Rattray et al., 1990). This study makes

quantification of microbial biomass in porous media possible by the measurement of bioluminescence of HK44 with a CCD camera.

The use of a CCD camera provides several benefits, the most important for our study being the ability to quantify light in a completely non-destructive manner. It is also a non-intrusive technique, allowing microbial processes to occur undisturbed. Additionally, the use of a programmed time-series of image captures allows for recording of light emission throughout the length of the experiment. Variable exposure lengths allow for detection of low light levels without significant background noise in long exposures as well as detection of rapid changes in light levels during times of greater light emission. Cameras have also been used to non-destructively quantify water content in porous media via light transmission in two-dimensional chambers (Glass et al., 1989) and recently, this technique has been enhanced by the use of a sensitive CCD camera (Schroth et al., 1995; Schroth et al., 1996). The combination of a bioluminescent reporter bacterium in conjunction with the use of a CCD camera for light detection provides a unique method for monitoring changes in both microbial and hydrologic properties in two-dimensional porous media systems.

The objectives of this project were to:

1. Determine if a CCD camera can be used to characterize the relationship between bioluminescence and cell density under three conditions: liquid suspension, saturated and unsaturated sand.

2. Identify the major physiological and environmental factors influencing bioluminescence of *P. fluorescens* HK 44.

MATERIALS AND METHODS

Bacteria and Growth Media

P. fluorescens HK 44, containing the plasmid pUTK21, was generously

supplied by Gary Sayler, University of Tennessee, Knoxville, TN. The plasmid

encodes tetracycline resistance so all growth media contained 15 mg/L tetracycline

to prevent plasmid loss. Stock cultures were stored in 40% glycerol at -80° C.

From the freezer stocks, cells were grown at 27° C on Luria broth agar (LB tet15)

plates which contained 5 g NaCl, 10 g tryptone, 5 g yeast extract, 15 mg

tetracycline and 15 g Bacto Agar (Difco Laboratories, Detroit, Michigan) per 1 L

distilled water (pH 7.0). For the experiments, cells were grown in a minimal

mineral salts medium (MMS) with glucose as the carbon source. The mineral salts

component of MMS contained the following (in grams per liter): 0.05, $CaCl_2 \cdot H_2O$,

1.2 $(NH_4)_2HPO_4$; 5.6, KH_2PO_4; 0.2, $MgSO_4 \cdot 7H_2O$; 0.009, $Na_2EDTA_2 \cdot H_2O$; 0.004,

$FeCl_3$; 0.00143, H_3BO_3; 0.00102, $MnSO_4 \cdot 4H_2O$; 0.00022, $ZnSO_4 \cdot 2H_2O$; 0.00008,

$CuSO_4 \cdot 5H_2O$; 0.0001, $CoCl_2 \cdot 4H_2O$ and 0.00005, $Na_2MoO_4 \cdot 2H_2O$. The pH of the

MMS was adjusted to 7.0. The glucose and salicylate for growth and

bioluminescence experiments were obtained from Sigma Chemical Co., St. Louis,

Missouri.

Cell Preparation

For studies of cells in stationary phase, cells from the LB tet15 plates were grown in a 500 ml Erlenmeyer flask in 200 ml MMS containing 1 g/L glucose in a shaker incubator at 28° C, 200 rpm. After 18 hours (OD_{600} ~2.5), the cell suspension was supplemented with sufficient 50 mM potassium phosphate buffer solution, pH 7.0, to fill a 480-ml Nalgene centrifuge tube and centrifuged at 8000 rpm (11,325x g) for 15 minutes. The cell pellet was resuspended in approximately 100 mL of buffer, transferred into two 50 ml Nalgene centrifuge tubes and centrifuged at 8000 rpm for 15 minutes. The cell pellets were resuspended in buffer and centrifuged again for 15 minutes. The washed cells were then resuspended to selected cell densities in MMS with 15 mg/L tetracycline and selected concentrations of glucose and/or salicylate.

For preparation of log phase cells, an inoculum was taken from an LB tet15 plate and was grown for 8-12 hours in a shaker incubator at 27° C in MMS. An aliquot of approximately 1 ml of this culture was added to 1 L of fresh MMS-glucose to obtain a density of 10^6 cells/ml (OD_{600} 0.001). Cells were grown at 27° C for 8 hours to a density of approximately 4×10^8 cells/ml (OD_{600} 0.4). Cells were centrifuged as described above, washed once in buffer, and resuspended to selected densities in MMS with 15 mg/L tetracycline and selected concentrations of glucose and/or salicylate.

Sand Characteristics and Preparation

Four grades of silica sand (12/20, 20/30, 30/40 and 40/50 sieve sizes) were

obtained from Unimin Corporation (Le Sueur, MN), under the trade name

Accusand. The physical characteristics of the sand are described by Schroth, et al.,

(1996) with pertinent details summarized in Table 1. Although the sand was

presieved and prewashed with water by the manufacturer, the sand was again

rinsed with distilled water to remove fine particulates. Before use in experiments,

wet sand was autoclaved for 45 minutes, allowed to sit at room temperature for 24

hours, autoclaved again for 45 minutes, and oven dried at 45° C for 3-5 days.

Table 1. Physical properties of the four Accusand grades (adapted from Schroth, et al., 1996)

Sand	Particle Diameter d_{50} (mm)	Particle Density (Mg/m^3)	Bulk Density (total Mg/ m^3Sand)	Porosity, n (m^3 pore space/ m^3 total)
12/20	1.105 ± 0.014	2.665	1.72	0.348
20/30	0.713 ± 0.023	2.664	1.72	0.348
30/40	0.532 ± 0.003	2.665	1.72	0.348
40/50	0.359 ± 0.010	2.663	1.72	0.348

Quantification of Bioluminescence

The CCD camera system consisted of a 16-bit backlit SITe 512x512 CCD

and ST-138 CCD camera controller (Princeton Instruments, Inc., Trenton, New

Jersey), Nikon 50 mm 1:1.8 lens and Tristar 52 mm UV filter. This system was

chosen due to its very low dark noise, large pixel size (841 μm^2), high quantum

efficiency (>80%) and large dynamic range (16-bit with 300,000 e⁻ well capacity).

All images were taken with an f-stop setting of 1.8. The CCD chip was cooled to

−50° C by the 0° C liquid cooled thermoelectric system.

Bioluminescence of HK 44 was imaged in 70 ml tissue culture flasks (75

ml capacity) fitted with black phenolic caps. In order to eliminate headspace in the

liquid culture and saturated sand experiments, the flasks were completely filled

with either liquid culture or a combination of liquid culture and sand. For these

experiments, the flasks were capped to prevent additional oxygen diffusion into

the flasks. The flasks were placed vertically in an array made of black matte

posterboard located 1.5 m from the CCD camera. The entire system was contained

within a blackened dark room in order to minimize background light. The room

temperature was maintained at 70° F (21° C) and humidity was maintained below

50% with a dehumidifier to prevent the formation of water condensation within the

camera apparatus.

In experiments with liquid cell suspensions, bioluminescence was

measured with 10 minute exposures taken every 20 minutes (with 10 minutes

between each exposure). In experiments conducted with cell densities >10^8

cells/ml and saturated sand, 2 minute exposures were taken every 4 minutes. In

saturated sand experiments with cell densities greater than 1x10^8 cells/ml and all

the unsaturated sand, 5 minute exposures were taken every 10 minutes. Time zero

corresponds to the time which salicylate was added. A custom Visual Basic software program regulated camera automation.

Cell Density Experiments

The relationship between the intensity of bioluminescence and cell density was examined both in liquid suspension and saturated sand. All cell density experiments were conducted with stationary phase cells. Suspensions of HK 44 were prepared in MMS with 100 mg/L salicylate to the following densities: 1 x, 2 x, 4 x, 6 x, 8 x 10^7 and 1x 10^8 cells/ml. These densities were confirmed by dilution plating on LB tet15 plates. Another series of experiments was conducted with cell densities ranging over three orders of magnitude: 1x, 5x10^5, 1x, 5x10^6, 1, 5x10^7, 6 x10^7, 1x, and 3.5x10^8 cells/ml respectively. All experiments were performed in duplicate with 45 g 40/50 sand in each flask and the remaining volume of the flask, approximately 60 ml, filled with cell suspension.

Unsaturated Sand Experiments

The relationship between cell density and bioluminescence was examined in unsaturated sand. Stationary phase cells were prepared as described above and resuspended to 5x10^6, 1x, 3x, 5x10^7, 1x, 3x, 5x, or 8x10^8 cells/ml. All unsaturated sand experiments were conducted with 40/50 sand, and salicylate was added at 100 mg/L. These experiments were conducted in 70 ml tissue culture flasks (Falcon Products, Inc., St. Louis, MO) that had their bottom panels sawed off. The flasks were inverted and attached to a vacuum manifold that could accommodate nine flasks (Fig. 2). A small ball of glass wool was inserted into the neck of each flask

Tissue culture flask with bottom
panel removed

Glass wool

Glass tubing

Rubber tubing and tubing clamp

Vacuum Pump

Vacuum manifold
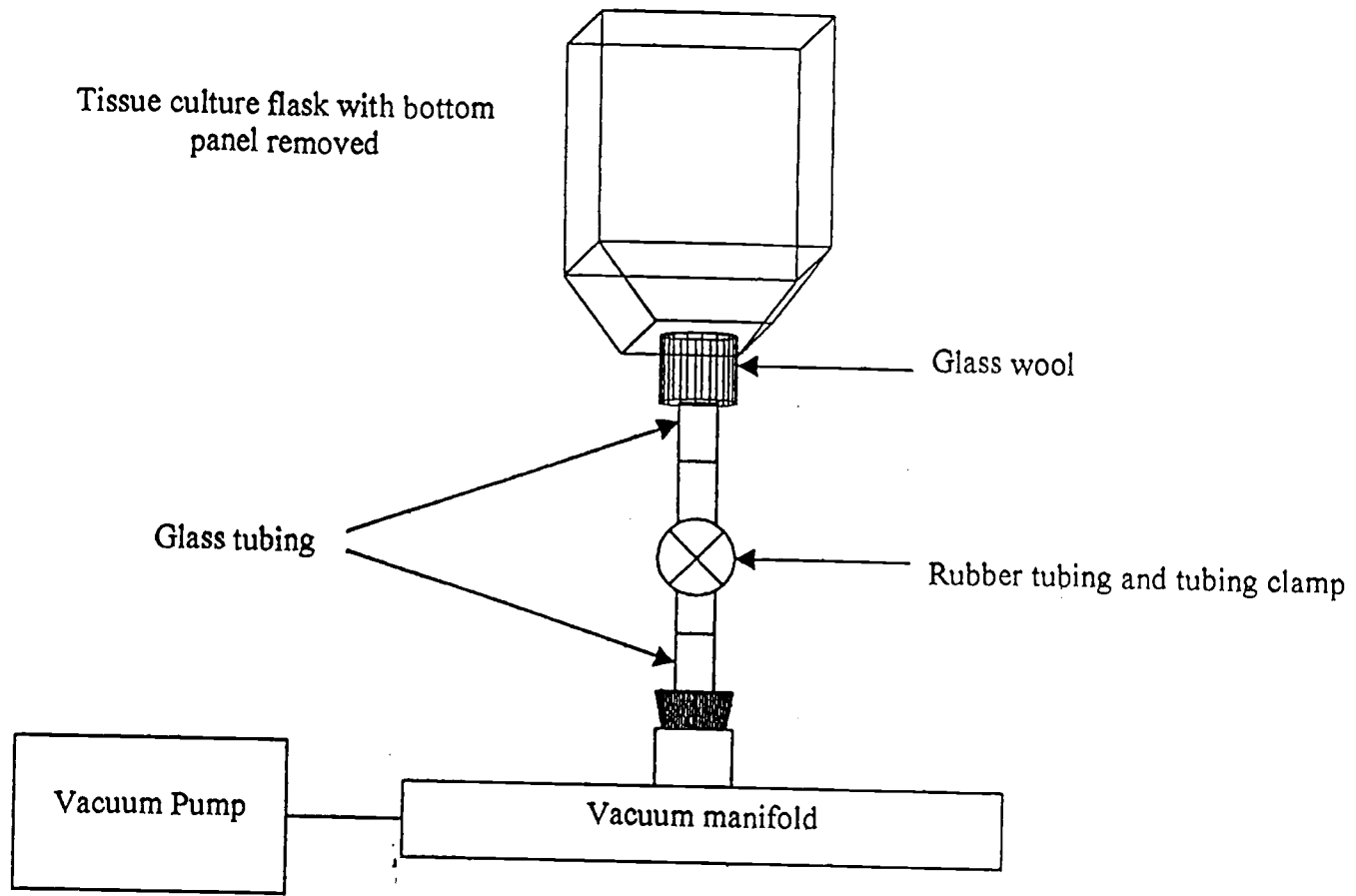
Figure 2. Schematic for unsaturated sand experiment preparation

to prevent sand from falling through the hole in the rubber stopper. With the tube clamps fastened, approximately 40 ml of cell suspension was poured into the flasks and then 90 g of sand was added while the flasks were tapped to ensure uniform sand packing. With the tube clamp removed, the liquid suspension was removed from one flask at a time using a vacuum pump. Each flask was allowed to drain under an initial pressure of -40 kPa for 30 seconds, which rose to approximately -15 kPa as the sand drained. Moistened cheesecloth was placed over the open tops of the flasks during the vacuum procedure to prevent evaporative drying. The flasks remained inverted during with screw caps fastened to prevent the sand from falling out of the flasks.

Growth Phase and Mixed Substrate Experiments

The effect of a non-inducing growth substrate both alone and in combination with salicylate on bioluminescence was examined. Treatments consisted of suspensions of 2×10^7 cells/ml in MMS containing 250 mg/L glucose alone, and in MMS containing 100 mg/L salicylate plus either 0, 50, 125 or 250 mg/L glucose. The control consisted of non-amended cells. Each treatment contained three replicates and the experiment was repeated. The experiment was conducted with both stationary phase and log phase cells and with 45 g of 40/50 sand in each flask and the remaining volume, approximately 60 mls, of the flask filled with cell suspension. Another set of experiments examined the effect of lower concentrations of glucose (0, 10, 20, 30, 40 and 50 mg/L) in combination with 100 mg/L salicylate on HK 44 bioluminescence in the absence of sand.

Sand Texture Experiment

An experiment was conducted to compare the properties of bioluminescence by HK44 suspended in three grades of Accusand (12/20, 20/30, and 30/40) with those obtained from the 40/50 material. Stationary phase cells were prepared as described above and resuspended to 2×10^7 cells/ml in MMS with 100 mg/L salicylate. Forty-five gram portions of sand were added to each flask with the remaining volume occupied by cell suspension. Each texture was measured in triplicate and the experiment was repeated.

Oxygen Consumption Rate Measurements

Oxygen concentration was measured at 25° C for 5-8 minutes on a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) mounted in a 1.8 ml water-jacketed reaction vessel. The instrument was calibrated with a saturated aqueous solution of sodium dithionite and deionized water for 0- and 8.24-ppm (ambient) values, respectively.

Cell suspensions (2×10^8 cells/ml) were prepared from both log and stationary phase cultures as described above except that the final resuspension was made in MMS minus ammonium phosphate to prevent cell growth. Four 150 ml portions of liquid culture were added to sterile 500 ml Erlenmeyer flasks. Treatments consisted of 250 mg/L glucose, 250 mg/L glucose plus 100 mg/L salicylate, 100 mg/L salicylate alone and a non-amended control. For stationary phase cells, oxygen uptake rates were measured at 0.5, 1, 1.5, 3, 4.5 and 6 hours

after addition of substrates. For log phase cells, rates were measured at 0.5, 1.5, 3, 6 and 10 hours after addition of substrates.

Salicylate Consumption Experiments

To ensure that salicylate was not limiting nor significantly consumed during the typical time course of a bioluminescence experiment, salicylate disappearance was monitored. Stationary and log phase cells were used for two separate experiments, prepared as described above, and resuspended to $2x10^7$ cells/ml. Both experiments contained an unamended control as well as the following treatments: 100 mg/L salicylate with 0, 50, 125 and 250 mg/L glucose. These experiments were conducted in triplicate in completely filled 60 ml glass serum vials sealed with gray butyl rubber stoppers.

Samples were extracted from the stationary phase cells after 0.5, 1.5, 3.5, 8, 12 and 24 hours of incubation and from the log growth cells at 0.25, 2, 4, 12 and 24 hours. Time zero corresponded to the addition of substrates. The sampling scheme consisted of a 3 ml extraction of liquid culture and a subsequent 3 ml injection of N_2 gas into the vial. This prevented the formation of a vacuum or the introduction of additional oxygen into the vials. Each 3-ml sample was divided into two 1.5 ml sterile Eppendorf tubes and spun for 5 minutes at 12,500x g. One ml of supernatant was removed from each tube, recombined into one sample and filtered through a 0.22 μm pore size membrane (Millipore, Mass.) to remove any cells and particulates. Samples were stored at -20° C until they could be analyzed by HPLC.

Salicylate was measured on a Waters (Milford, Mass.) HPLC system which was comprised of a quaternary pump (Model 600), UV detector (Model 486), Alltima C-18 column (Alltech Associates, Inc., Deerfield, IL) and an autosampler (Model 717 plus). The system was controlled by a DEC (Nashua, NH) personal computer using Waters Millennium software. The mobile phase contained 45% (v/v) HPLC-grade acetonitrile (EM Science, Gibson, NJ), 55% (v/v) distilled and deionized water from a Milli-Q ultrapure water system (Millipore, Bedford, MA) and 0.05% (v/v) sequanal-grade trifluoroacetic acid (Pierce, Rockford, IL). Salicylate concentration was determined by its absorbance at 254 nm of two separate 200 μl-volume injections of each sample. Each sample injection was separated with the isocratic mobile phase for 10 minutes and salicylate concentration was calculated from a standard curve.

Image Analysis

At the completion of each experiment, the flasks were removed from the darkroom and a background image was taken. The exposure time of the background image was the same as those used during the experiment. The light level recorded in this image was subtracted from each picture.

In images of flasks containing only liquid culture or unsaturated sand, a 32 x 32 pixel area was selected in the middle of each flask, representing a 2.6 x 2.6 cm area of the flask. In images of flasks containing both sand and liquid culture, a 16 x 32 pixel area (1.3 x 2.6 cm of the flask) was selected in the middle of both the sand and liquid portions of the flask (Fig. 3). These two areas of analysis allowed

for an ample number of sample pixels while avoiding edge effects at the sand-liquid, liquid-flask or sand-flask interfaces. Using Winview 3.0 (Princeton Instruments, Inc.) in combination with another custom Visual Basic automation program, the average pixel intensity was obtained for the selected areas. Light levels were divided by length of exposure in order to compare images from experiments with different exposure lengths. For example, light levels from experiments with ten-minute exposures were divided by ten, and light levels from experiments with five-minute exposures were divided by five. For curves displaying light emission over time, the units are reported as light units per minute (LUPM).
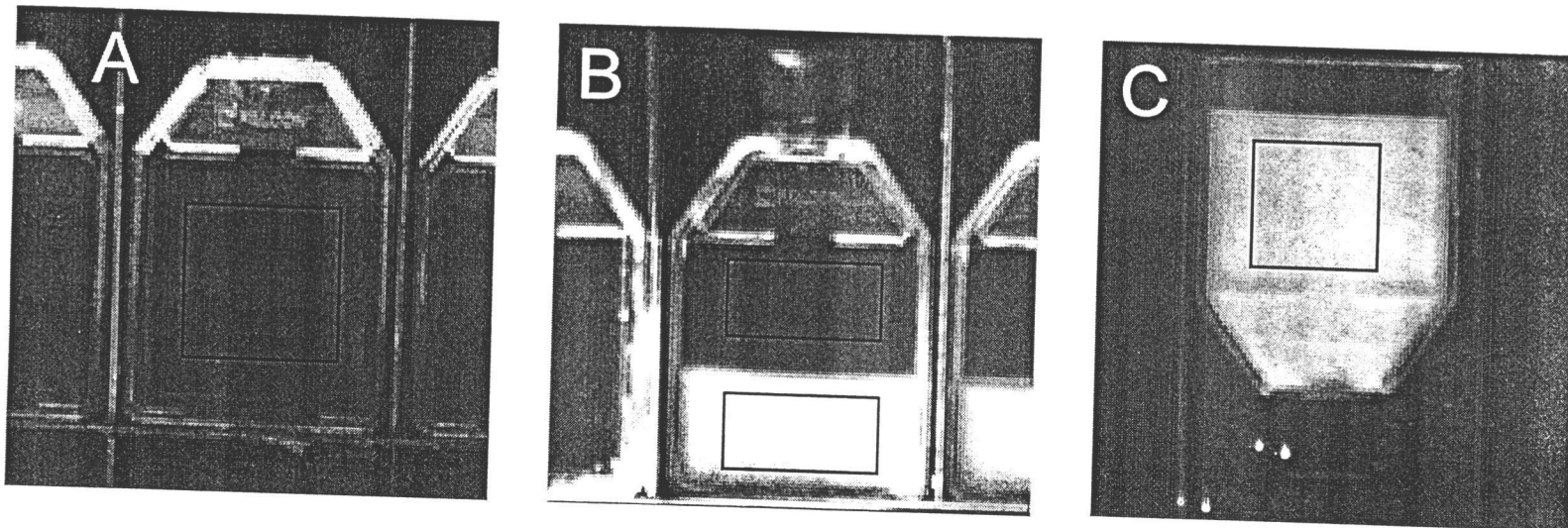
Figure 3. Selected areas for light quantification in liquid culture (A), liquid culture and saturated sand (B), and unsaturated sand (C)

RESULTS

The geometry of the experimental container was found to influence bioluminescence measurements with the CCD camera. Initially, glass scintillation vials were used to hold the cell suspensions. However, the curved surface of the vial caused the appearance of non-uniform light emission, and the small cross-sectional area proved difficult for image analysis. The vials were replaced with upright transparent ("plastic type") tissue culture flasks (75 ml capacity) because of their relatively large planar surface available for image analysis.

The typical salicylate-induced bioluminescence response of HK 44 is depicted in Figure 4. Initially after salicylate addition there is a period in which a lower level of light detection by the CCD camera. The time at which light emission exceeds 5 LUPM is referred to as the First Detectable Time, FDT. Following FDT is a period when the rate of light emission is increasing and oxygen is not limiting. The subsequent decline in the rate of light emission is most likely due to oxygen depletion in the flasks.

Cell Density Experiments

A series of experiments were conducted with stationary phase cells of HK44 to determine the relationship between cell density and bioluminescence both in liquid suspension and in saturated 40/50 sand. The cell densities ranged over three orders of magnitude, from 1×10^5 to 3.5×10^8 cells/ml. In both liquid suspension and saturated 40/50 sand, higher cell densities exhibited more rapid
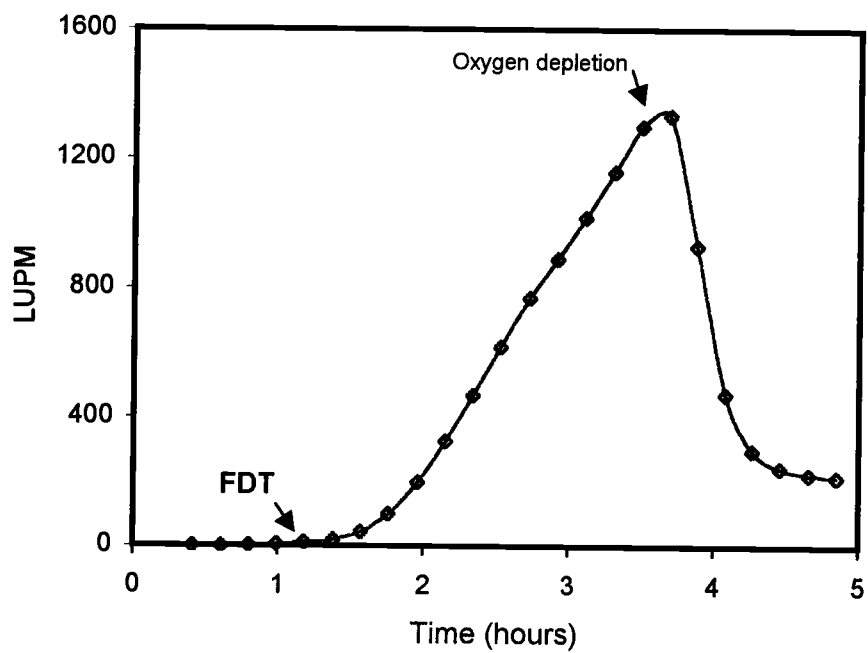
Figure 4. Typical bioluminescence response of HK44. Time zero corresponds to time after salicylate addition.

increases in the rate of light production (Figs. 5 and 6). The FDT ranged from 0.8 to 5.5 hours for the range of cell densities with higher densities producing detectable light levels earlier than lower densities.

This range of cell densities established the maximum and minimum detectable cell densities for these experimental conditions and camera settings. The highest cell density that could be successfully examined was 1×10^8 cells/ml. The oxygen demand of the highest cell density, 3.5×10^8 cells/ml, exhausted the available oxygen before bioluminescence could be measured with the CCD camera. The camera could successfully detect light emission from 1×10^6 to 1×10^8 cells/ml under minus and plus sand conditions. Although the camera was able to detect cells at a density as low as 1×10^5 cells, the FDT was 5 hours in liquid suspension and 8.25 hours in saturated sand (Fig. 5). This may not be desirable in dynamic and flowing systems where cell growth might occur during these long periods before light detection. Also, the attenuating effect of sand on light emission will exaggerate the problem.

The fraction of light emitted in saturated 40/50 sand compared to the liquid suspension ranged from 0.094 to 0.171 with an average of 0.130 (SD 0.019) (Table 2). This reduction in light emission was probably due to the combination of a reduction in the total amount of cells per unit volume caused by the presence of sand (n=0.348), and attenuation of light emission by the sand particles.
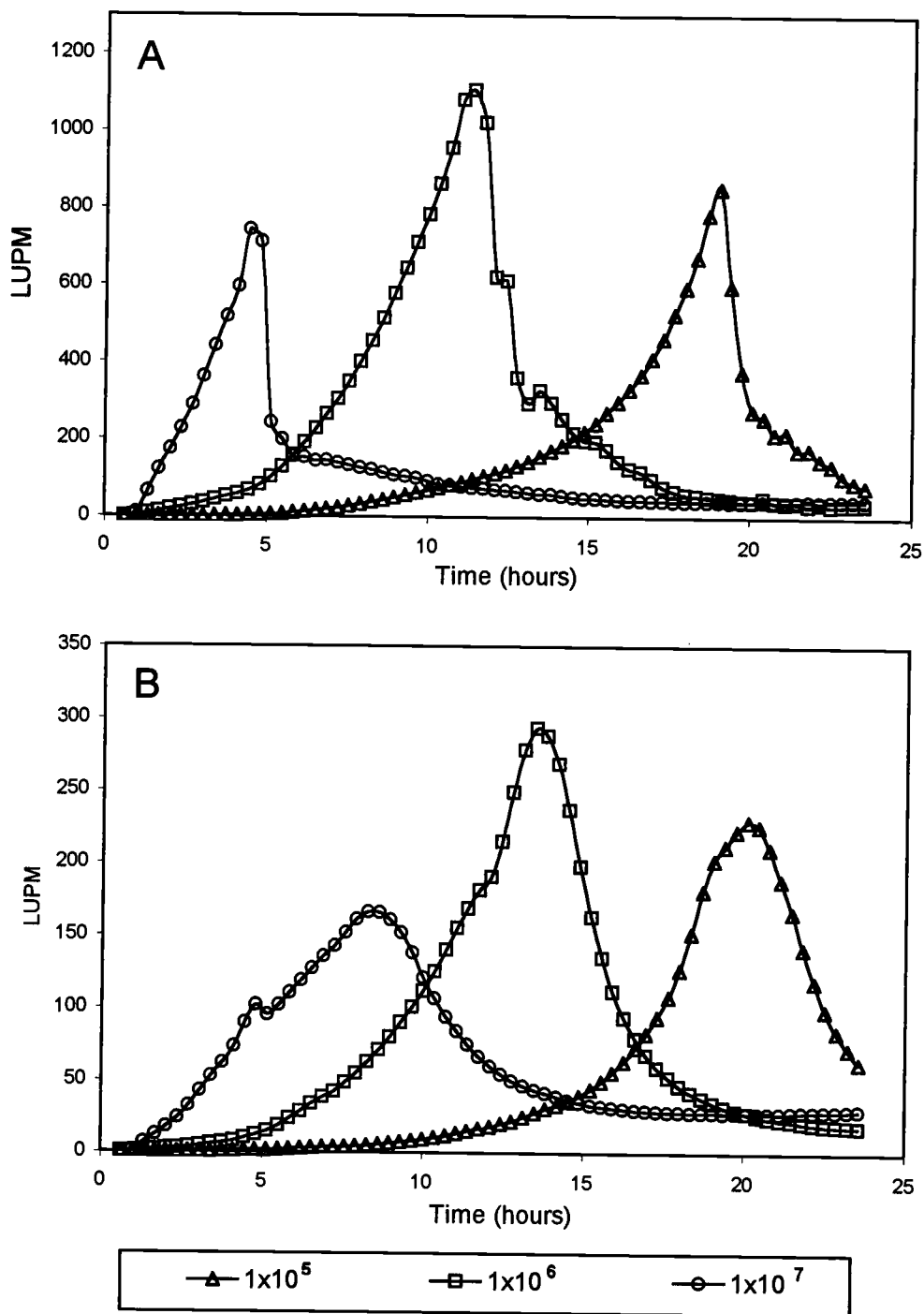
Figure 5. Bioluminescence response of stationary phase cells in liquid suspension (A) and saturated sand (B) resuspended to 1x105, 1x106, 1x107 cells/ml in 100 mg/L salicylate in MMS.
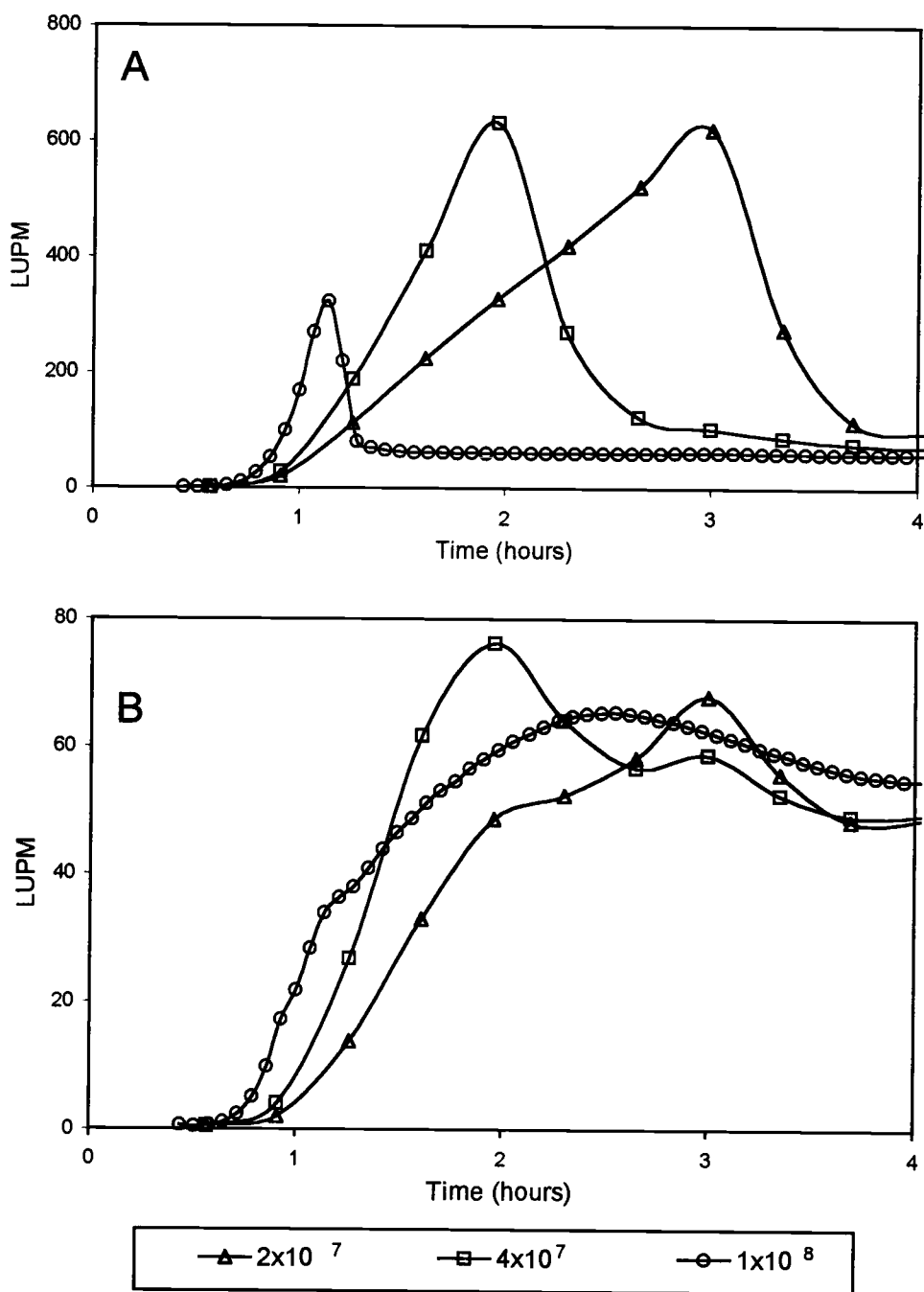
Figure 6. Bioluminescence response of stationary phase cells in liquid
Suspension (A) and saturated sand (B) resuspended to 2x107,
4x107, 1x108 cells/ml in 100 mg/L salicylate in MMS.

Table 2. Fractions of light emitted by HK44 from saturated 40/50
sand and liquid suspensions.

Density (cells/ml liquid)	Fraction (SD)
1 x 10^5	0.14 (0.003)
5 x 10^5	0.13 (0.012)
1 x 10^6	0.13 (0.019)
5 x 10^6	0.11 (0.016)
1 x 10^7	0.11 (0.015)
2 x 10^7	0.13 (0.005)
4 x 10^7	0.15 (0.022)
6 x 10^7	0.15 (0.009)
1 x 10^8	0.14 (0.017)
Average	**0.13 (0.019)**

Unsaturated Sand Experiments

Experiments were conducted to characterize bioluminescence emitted

under unsaturated sand conditions by densities of stationary phase cells ranging

from 5 x 10^6 to 8.5 x 10^8 cells/ml. In preparing the colonized unsaturated sand, it

was assumed that no preferential cell attachment to the sand occurred and that the

liquid phase within the unsaturated sand contained the same cell density as the

original inoculum. This was confirmed by dilution plating of sand samples onto

LB tet15 plates (data not shown).

Water content of the unsaturated sand, as determined by oven drying, was

4.0% (g/g) gravimetric or 7.0% volumetric (cm^3/cm^3). The moisture content was

uniform throughout the top two thirds of the sand volume, where bioluminescence

was measured. Uniform cell distribution was indicated by the uniform glow

achieved in this portion of the flask (Fig. 7A).
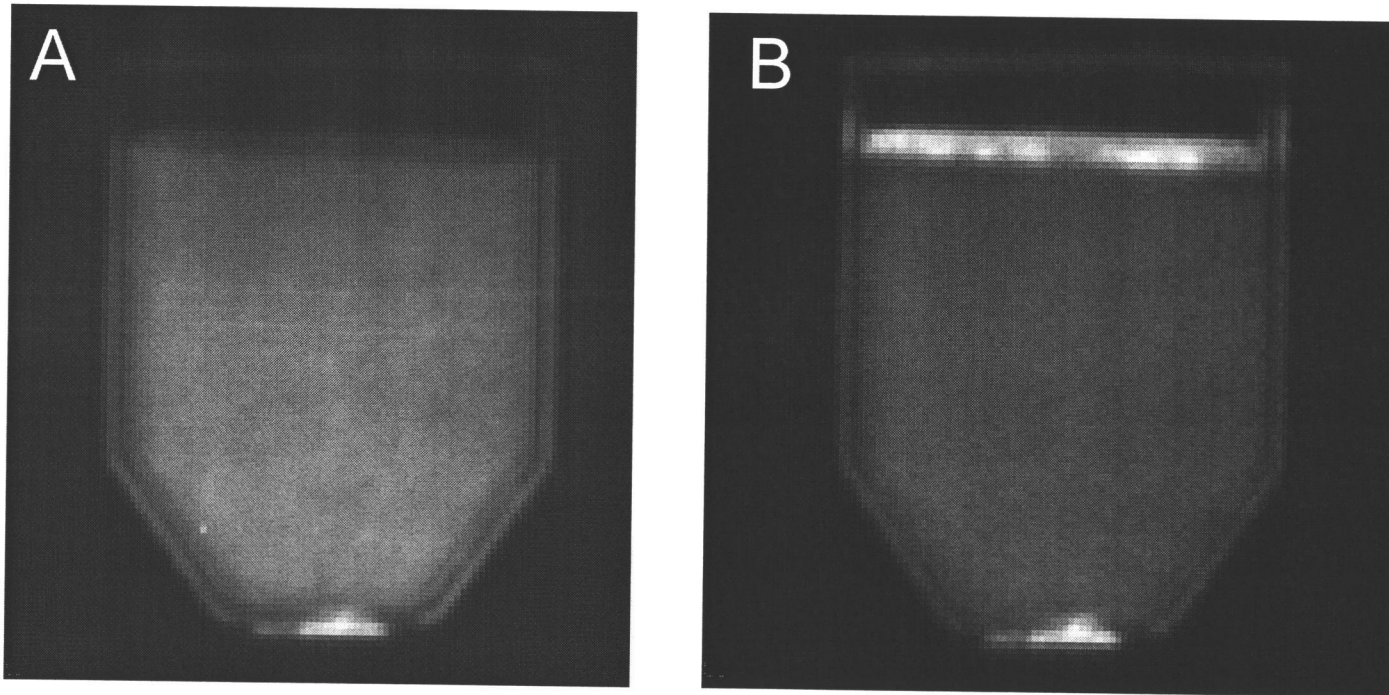
Figure 7. Images of bioluminescence in unsaturated sand 1 hour after salicylate addition (A) with uniform glow throughout the imaging area of the sand and after 6 hours (B) with glow observed most intensely at the sand surface due to oxygen depletion below surface.

As with the saturated sand experiments, FDT decreased with increasing

cell densities and the rate of light production increased with cell density. Again,

the decline in light production is most likely due to oxygen limitation. Although

more oxygen was available in the pores under unsaturated conditions, presumably,

the rate of oxygen consumption exceeded the ability of oxygen to diffuse

throughout the depth of the indicated by glow being restricted to the surface

(Figure 7B). Light production in unsaturated sand exhibited a trend to increase

non-linearly over time and showed a positive correlation with cell density similar

to that observed in liquid suspensions and saturated sand (Fig. 8).

Characterization of Density Dependent Bioluminescence

As shown in Figures 5, 6 and 8, the rate of light production increased non-

linearly with cell density over time. Although the bioluminescence did not appear

to be non-linear in experiments with densities between 1×10^7 and 4×10^7 cells/ml,

this was probably due to the length of exposure of the images. The more frequent

and shorter exposures used to observe 1×10^8 cells/ml (Fig. 6) demonstrate the

same non-linear response as the experiments with cell densities lower than 1×10^7

cells/ml (Fig.5).

The bioluminescence response can be empirically modeled as a function of

cell density and time. With the constant presence of salicylate, it is assumed that

the lux system is continually induced and producing luciferase. Furthermore, it is

assumed that luciferase accumulates within the cell and that components for the
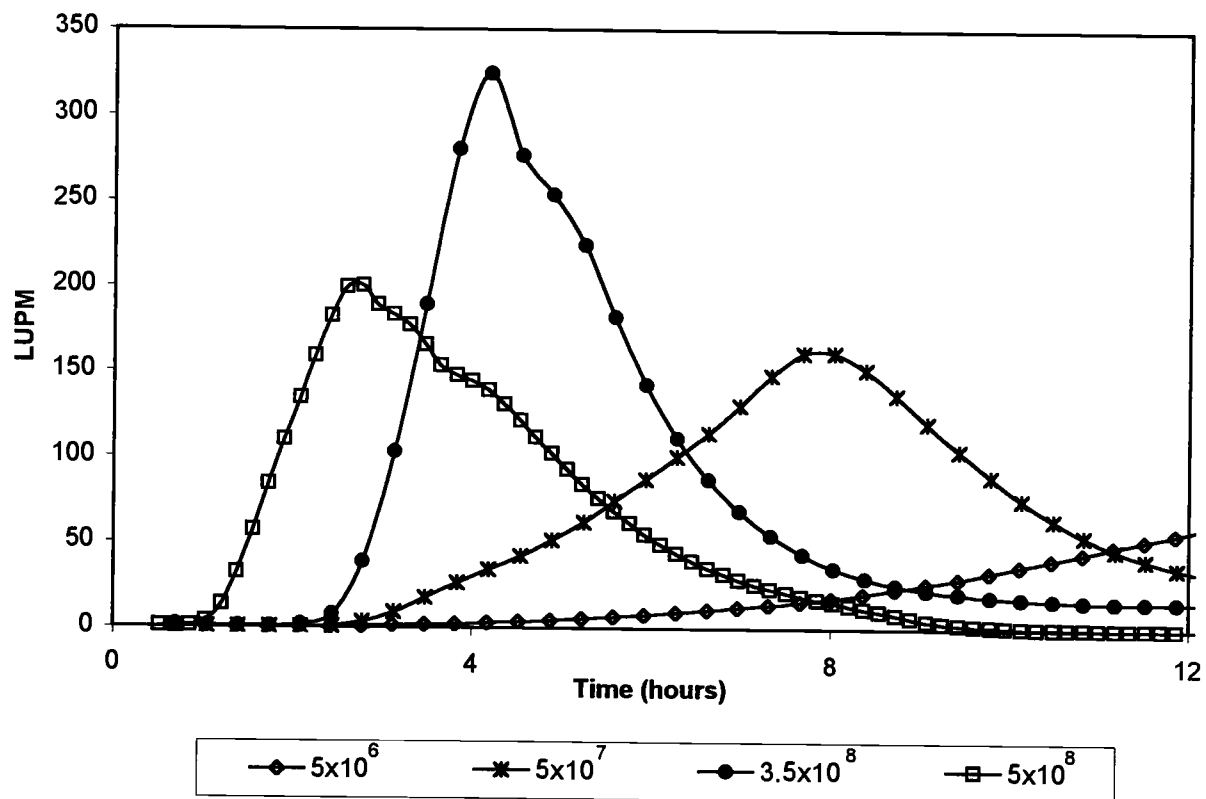
Figure 8. Bioluminescence response curves of HK 44 in unsaturated sand at 7% water content. Stationary cell concentrations in the liquid phase are 5×10^6, 5×10^7, 3.5×10^8 and 5×10^8 cells/ml with 100 mg/L salicylate in MMS.

lux reaction are not limiting until the time of oxygen depletion, accounting for the steady increase in the rate of light emission over time. Mathematically, this can be represented by the following equation:

(1) $\dfrac{dE}{dt} = tBc$

where E is the concentration of luciferase enzymes (active enzyme/ml), c is cell density (cells/ml), B is the rate of increase in cellular enzyme production rate (enzymes/[cell-min^2]) and t is time in minutes. Integrating eq. (1) and imposing the condition that E=0 at t=0 gives the total number of luciferase enzymes at time t:

(2) $E = \dfrac{1}{2}t^2 Bc$

Assuming that light emission is related to the number of luciferase enzymes by the proportionality constant k (LU/enzyme), the amount of light emitted can be described by

(3) $L = kE = \dfrac{1}{2}t^2 B'c$

where B' = kB with units of (LU/[cell-min^2]).

Taking the square root of eq. (3) gives the density dependent light equation:

(4) $L^{1/2} = t\left(\dfrac{B'c}{2}\right)^{1/2}$

After the liquid suspension bioluminescence values were transformed by the square root function, the non-linear portions of the light curves became linear

between FDT and bioluminescence decline with R^2 values ranging from 0.948 to 0.999. Eq. (4) can be used to calculate B' by dividing by cell density.

(5) $(L/c)^{1/2} = t(B'/2)^{1/2}$

When $(L/c)^{1/2}$ is plotted against t, the slope of the linear portion of each curve is $(B'/2)^{1/2}$ and B' can be calculated for the each cell density (Figure 9). The B' values for cell densities 1×10^5 to 1×10^8 cells/ml in liquid suspension are summarized in Table 3.

Table 3. B' values for liquid cell suspensions

Density (cells/ml)	B' $(x10^{-10})$ (LU/[cell-min^2])
1×10^5	16.52
5×10^5	14.68
1×10^6	13.32
5×10^6	8.76
1×10^7	4.84
2×10^7	8.54
4×10^7	11.72
1×10^8	18.74
Average	12.14
SD	4.61

Under saturated and unsaturated sand conditions, two additional parameters must be considered in order to account for light attenuation due to the sand. The sand particles occupy a fixed volume, and, therefore, automatically reduce the total amount of cells within the system for a given cell density. The sand used in these
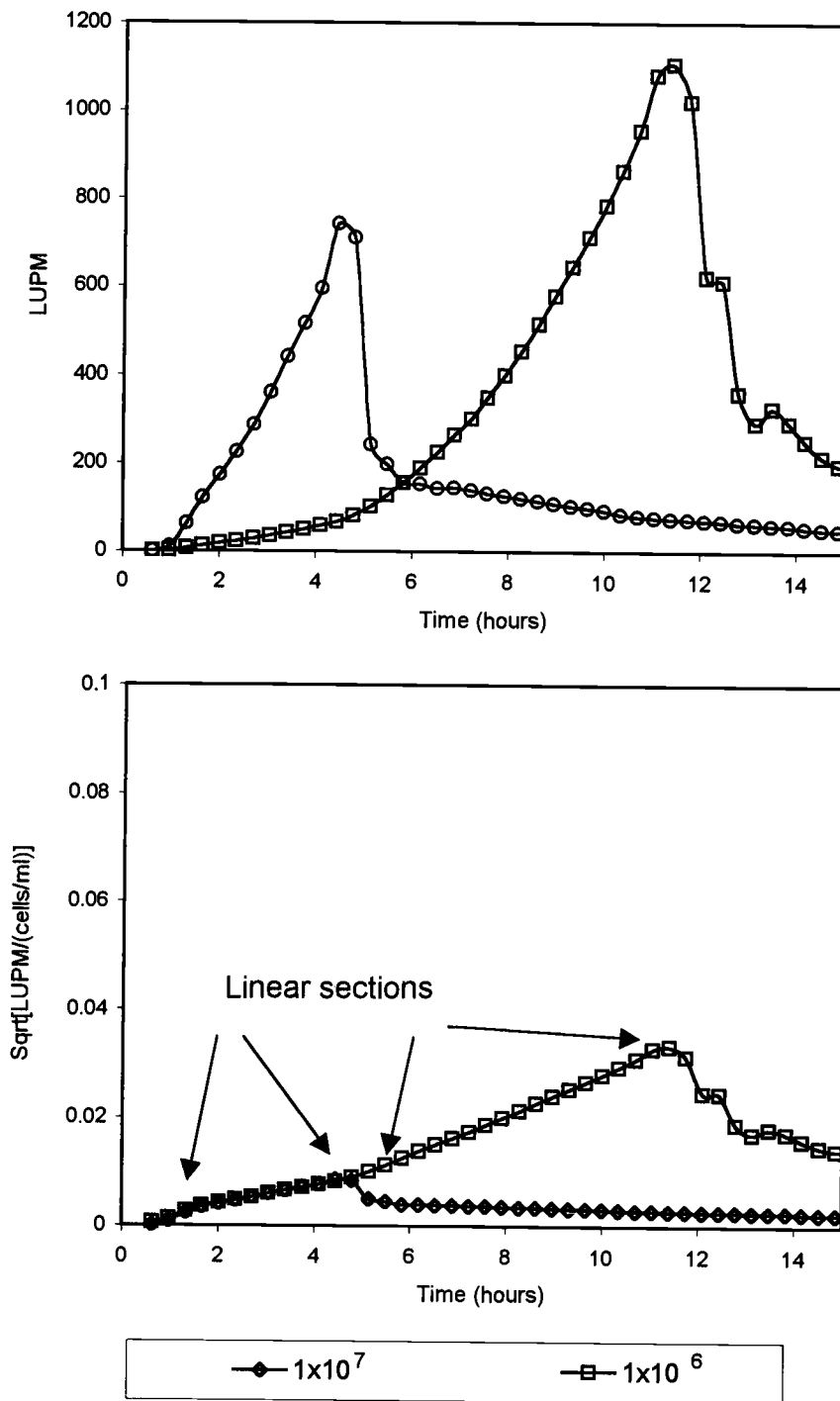
Figure 9. Bioluminescence responses of cell densities of
1x106 and 1x107 cells/ml cell densities before (A) and after
(B) transformation with the square root(LUPM/density)
function.

experiments has a porosity, n, of 0.348, thereby reducing the concentration of cells to 0.348 of that contained in the liquid suspension for saturated conditions. Additionally, the sand is not optically transparent and will contribute to the physical attenuation of light by absorption.

The extent of sand opaqueness is dependent upon its water content, θ, and the introduction of air-water interfaces under unsaturated conditions (Niemet & Selker 2000; Tidwell & Glass 1994). Light attenuation as a function of sand porosity, and physical occlusion as a function of water content, $\phi(\theta)$, can be incorporated into eq. (5) resulting in the following equation:

(6) $(L/c)^{\frac{1}{2}} = tn\phi(\theta)(B'/2)^{\frac{1}{2}}$

Further research needs to be performed to experimentally determine the relationship $\phi(\theta)$ under different unsaturated conditions. However, the values of $\phi(\theta)$ have been empirically calculated as 0.41 and 0.038 in saturated ($\theta=0.348$) and unsaturated ($\theta=0.07$) conditions, respectively. When eq. (6) was applied to the unsaturated sand data, the data exhibited a linear portion between the time of light induction and bioluminescence decline with R^2 values ranging from 0.962 to 0.999. The B' values in saturated and unsaturated sand are summarized in Table 4.

Table 4. B' values for saturated and unsaturated sand

Saturated Sand		Unsaturated Sand	
Density (cells/ml liquid)	B' ((x10^{-10}) (LU/[cell-min^2])	Density (cells/ml liquid)	B' (x10^{-10}) (LU/[cell-min^2])
1×10^5	11.67	5×10^6	8.94
5×10^5	12.46	1×10^7	7.11
1×10^6	12.55	3×10^7	7.02
5×10^6	9.10	5×10^7	8.28
1×10^7	3.92	1×10^8	9.73
2×10^7	13.98	3.5×10^8	25.36
4×10^7	19.55	5×10^8	18.95
1×10^8	13.21	8.5×10^8	9.89
Average	12.05	Average	11.91
SD	4.42	SD	6.64

Growth Phase and Mixed Substrate Experiments

Cells harvested from log phase displayed different bioluminescence

dynamics than stationary phase cells with respect to FDT. In the liquid

suspensions above 40/50 sand, stationary phase cell FDT was shorter than that of

log phase cells, 1.8 versus 2.5 hours, respectively (Figs. 10A and 11A). Within

saturated 40/50 sand, light could be detected at 1.8 hours for both log and

stationary phase cells (Figs. 10B and 11B).

Experiments were conducted to examine the properties of salicylate-

induced bioluminescence in the presence of glucose to determine if a non-inducing

growth substrate would modify the bioluminescence response. Stationary phase

cells in liquid suspension and saturated 40/50 sand with combinations of 100 mg/L
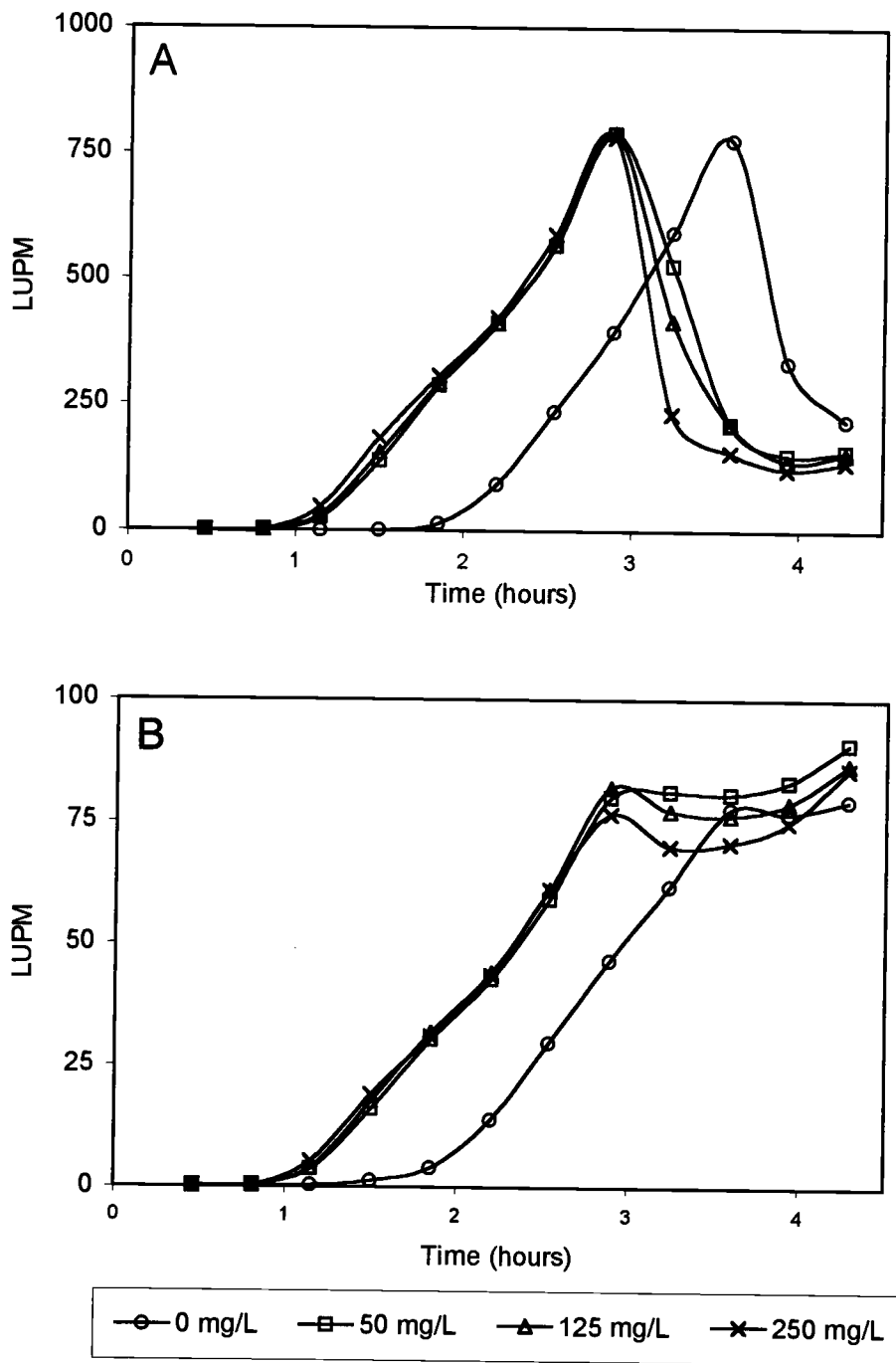
Figure 10. Bioluminescence response of 2×10^7 cells/ml stationary cells in liquid suspension (A) or saturated sand (B) with 100 mg/L salicylate and 0, 50, 125 or 250 mg/L glucose
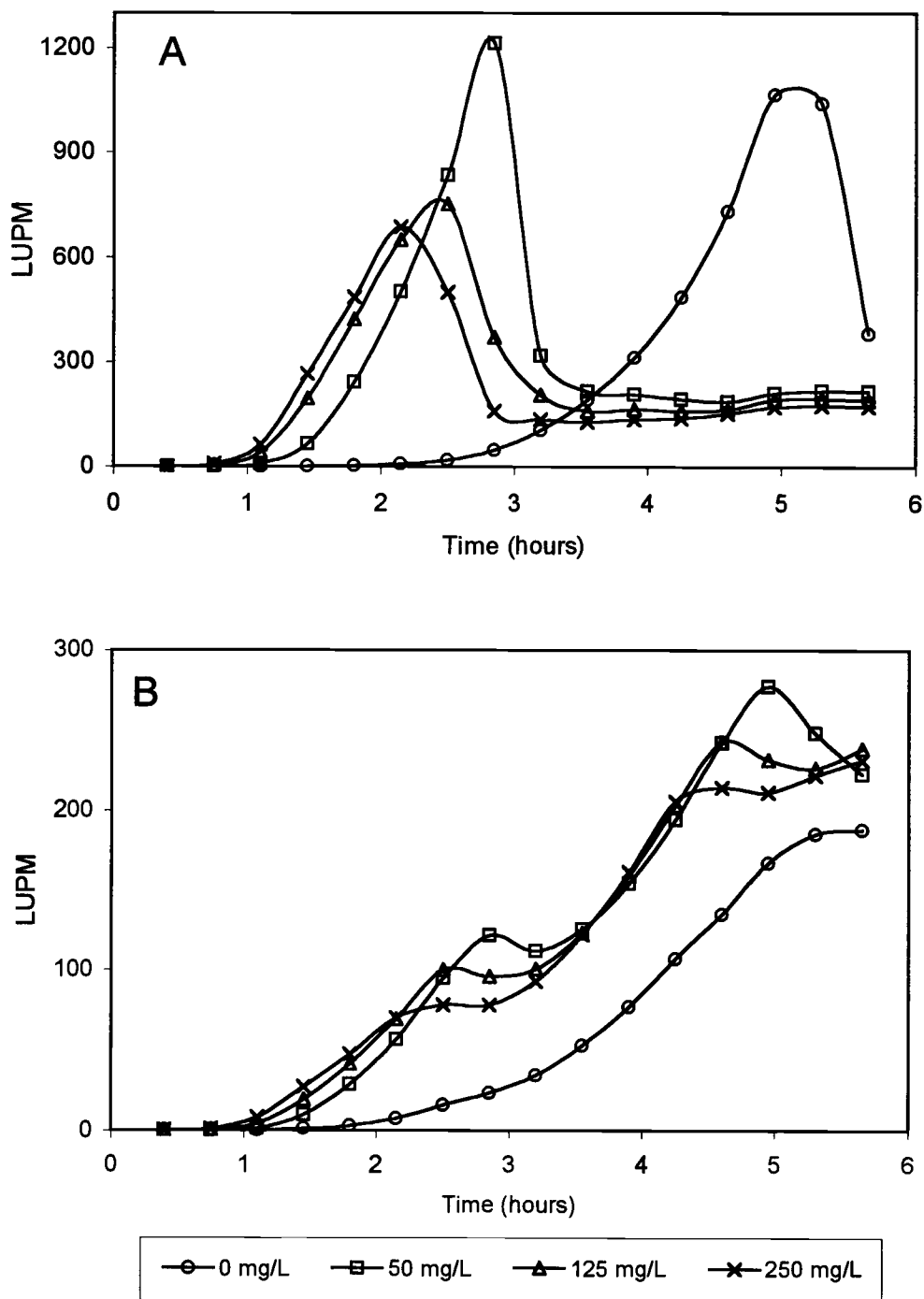
Figure 11. Bioluminescence response of 2×10^7 cells/ml log phase cells in liquid suspension (A) or saturated sand (B) with 100 mg/L salicylate and 0, 50, 125 or 250 mg/L glucose.

salicylate and 50, 125 and 250 mg/L glucose reduced FDT to 1 hour versus 1.8 in the absence of glucose (Fig. 10). Another experiment demonstrated this effect to occur at glucose concentrations as low as 10 mg/L (data not shown).

Similar experiments were conducted with log phase cells. In both liquid suspensions and cells in saturated 40/50 sand, log phase cells exhibited a bioluminescence FDT of 2.5 hours (Fig. 11). The presence of glucose at 50, 125, or 250 mg/L reduced the FDT 1 hour, which is similar to the FDT value of stationary phase cells with the same treatment (Fig. 10).

The fraction of detectable light emitted from HK 44 in saturated 40/50 sand compared to liquid suspension was slightly higher from stationary phase cells treated with both glucose and salicylate, 0.178, (SD 0.081) than from log phase cells, 0.116 (SD 0.016). The fraction emitted from the salicylate alone treatment was similar for stationary phase and growing phase cells, 0.151 (SD 0.044) and 0.163 (SD 0.015) respectively.

Determining *lux*-dependent Oxygen Consumption by HK44

Experiments were conducted to determine the rate of oxygen consumption by *lux* induced cells and the time required for oxygen depletion in the saturated closed flask system. The decline in bioluminescence was likely due to oxygen depletion in the closed flask system.

The time required to observe decline in light production decreased with increasing cell density, indicating that higher cell densities had depleted the available oxygen sooner than lower cell densities. Treatments for both stationary and log phase cells consisted of 100 mg/ L salicylate, 100 mg/L salicylate plus 250 mg/L glucose, 250 mg/L glucose, and an unamended control.

Figure 12A shows the oxygen consumption rate over time for stationary phase cells. The basal respiration rate of the control cells gradually declined from 2.9×10^{-6} to 1.9×10^{-6} moles/ml-min over 6 hours. The combination of glucose and salicylate produced similar rates of oxygen consumption to that of glucose alone, 1.13×10^{-5} (SD 5.2×10^{-7}) and 1.09×10^{-5} (SD 4.8×10^{-7}) mole/ml-min, respectively. Between 1 and 3.5 hours after salicylate addition, oxygen uptake began to increase from 4.4×10^{-6} mole/ml-min and after 6 hours reached a value similar to glucose stimulated uptake, 9.1×10^{-6} mole/ml-min. This suggests that the oxygen uptake rate parallels to *lux* system induction where stationary phase cell light can be detected at approximately 1.8 hours.

In the case of log phase cells, basal respiration was lower than that of stationary cells, with an average of 7.4×10^{-7} mole/ml-min (SD 1.5×10^{-7}) (Fig. 12B). Salicylate-induced oxygen uptake responded more slowly in log phase cells. After about 3.8 hours, the rate rapidly increased to 9.1×10^{-6} mole/(ml-min), similar to that observed in the stationary phase cells. The difference in response times of salicylate-induced oxygen uptake by log and stationary phase cells was
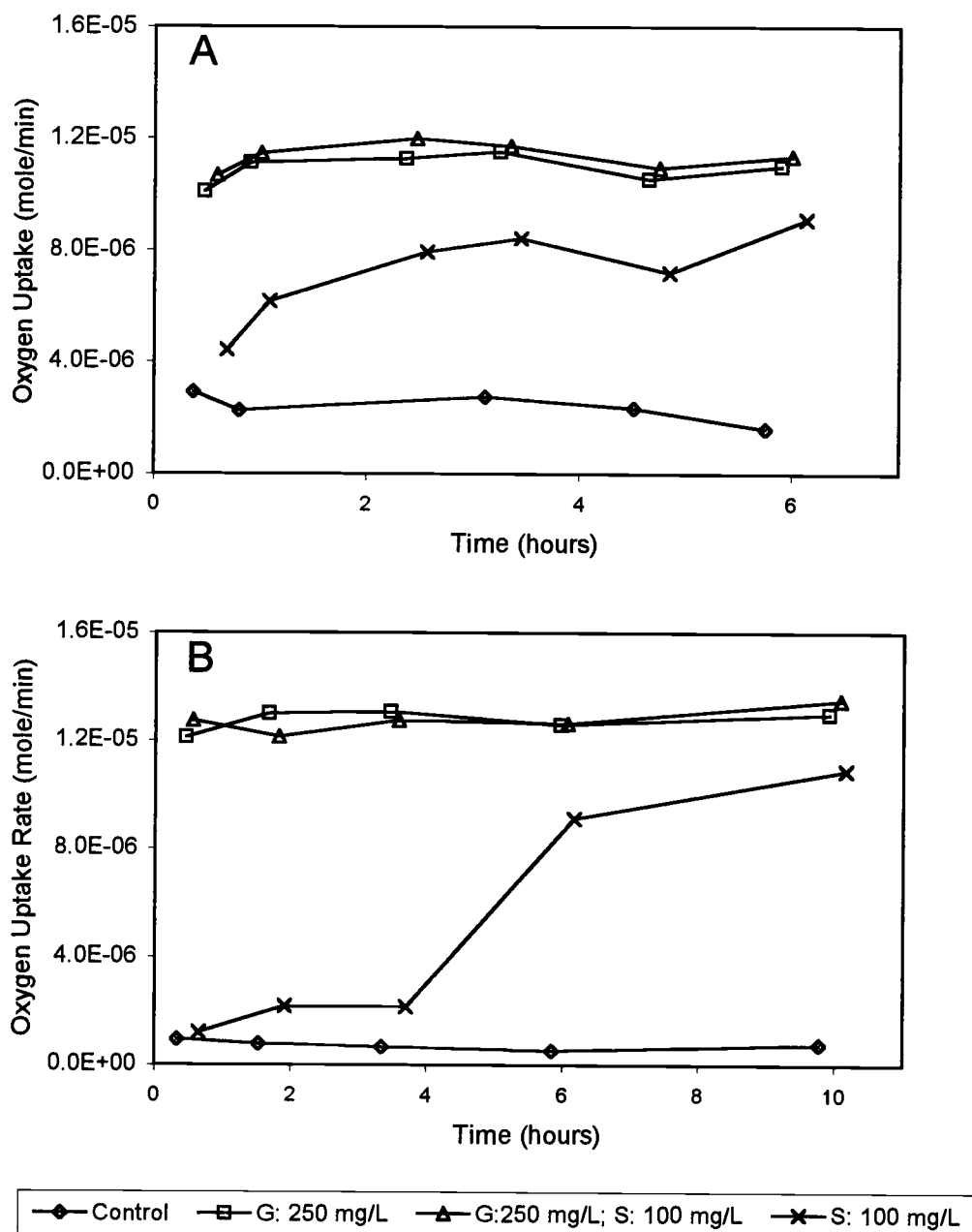
Figure 12. Response of oxygen uptake rates by in stationary (A) and log phase (B) cells with lux genes induced or not induced. $(2 \times 10^8$ cells/ml). Treatments consisted of an unamended control, 250 mg/L glucose (G), 250 mg/L glucose and 100 mg/L salicylate (S) and 100 mg/L salicylate.

consistent with light detection after 3 hours in log phase cells (Fig. 11A) and 1.75 hours in stationary phase cells (Fig 10A).

It should be noted that the cell density of the suspensions used for the oxygen uptake experiments were 2×10^8 cells/ml, ten-fold higher than that used in most of the bioluminescence experiments. This density was necessary to accurately measure oxygen consumption rates with the oxygen probe. Assuming that a 2×10^7 cell/ml suspension has a 10-fold lower oxygen consumption rate of approximately 9×10^{-7} mole/ml-min and an initial ambient oxygen concentration of 8 mg/L, oxygen depletion would occur 3.5 hours after cells were treated with 100 mg/L salicylate. With an oxygen consumption rate of 1.13×10^{-6} mole/ml-min for cells treated with 250 mg/L glucose and 100 mg/L salicylate, oxygen would be depleted after 2.8 hours. These predicted oxygen depletion times are consistent with the bioluminescence decline shown in Figure 6.

Different Sand Textures

In addition to the experiments conducted with 40/50 sand, three other sand textures, 12/20, 20/30 and 30/40, were tested to compare the amount of light emitted from saturated sand. The fractions emitted from saturated 30/40 and 12/20 sand, 0.17 (SD 0.025) and 0.16 (SD 0.026), respectively, were similar to that emitted from the saturated 40/50 sand (Table 2). However, the fraction of light emitted from the 20/30 sand was slightly higher at 0.23 (SD 0.058). Bioluminescence in the three textures of sand exhibited the same temporal patterns

as that in 40/50 sand. FDT was 1 hour and the rate of light increase was consistent with experiments conducted with 40/50 sand (data not shown).

Salicylate Consumption

To ensure that the decline in light production was not due to salicylate depletion, HPLC was used to measure salicylate in a closed system similar to the saturated sand tissue culture flasks. Nitrogen gas was injected into the serum vials to prevent to formation of a vacuum as samples were withdrawn. Salicylate levels in stationary and growth phase cell suspensions did not significantly decline over 12 hours, and salicylate availability was not a limiting factor (data not shown).

DISCUSSION

Density Dependent Bioluminescence

Although the *lux* phenotype has been widely used as a means of detecting

bacterial cells in the environment and as a biosensor for detecting specific

chemicals, we wished to determine its potential for following bacterial growth in

porous media in a non-destructive manner. There is considerable interest in

characterizing growth parameters of bacteria under conditions of saturated and

unsaturated flow. A greater understanding of bacterial growth in porous media

will provide information to microbiologists and engineers concerned with

improving the understanding of the interaction between microbial growth and

hydraulic properties in subsurface environments. Previous studies have used

bioluminescence to qualitatively determine bacterial biomass in porous media

(Bolenes et al., 1993; Duncan et al., 1994; Grant et al., 1992; Neilson et al., 1999;

Rattray et al., 1990) however this study is the first to utilize a CCD camera as a

non-destructive sampling instrument.

In this study, we established that the rate of light production until oxygen

limitation is related to cell density with a model that is based on the assumption

that luciferase accumulates non-linearly within each cell after induction by

salicylate. The model was applied to cell suspensions in aqueous medium, and in

both saturated and unsaturated sand conditions. B' was found to be stable over a

wide range of cell densities and in both saturated and unsaturated conditions,

suggesting it is a robust intrinsic property of HK44. This fact helps to support the

fundamental assumption of the model by demonstrating that the rate of increase in light production is due to biological factors (i.e. enzyme accumulation in the cells, availability of *lux*-system requirements), rather than changes in physical conditions such as the presence or absence of sand and degree of saturation.

CCD Camera System

These studies established that the CCD camera is a useful tool for making undisturbed measurements of light emission in two-dimensional systems over real time. The most favorable characteristic of the CCD camera system is its capacity for the non-destructive and non-intrusive quantification of bioluminescence. The CCD camera system used in these experiments is optimal at the bench scale and is most suitable for measuring bioluminescence in experimental systems with dimensions between 0.1 and 1 meter. Care must be taken in designing the experimental apparatus since a larger chamber must be placed further from the camera to fit into its. Additionally, light intensity decreases with distance from the light source so the distance from the chamber to the camera is an important factor.

Another important parameter is the length and timing of the pictures. Exposure lengths need to be long enough to capture low light levels, which may vary depending on the sensitivity of the camera and features of the lens, and exposures must be frequent enough to provide temporal resolution for the given experimental conditions. A rapidly changing system would not be adequately sampled with long exposure lengths which would serve to average light intensity over time rather than act as snapshots at specific moments in time. Additionally,

bioluminescence response dynamics such as a non-linear light response may not be detected with long, infrequent exposures.

Growth Phase and Mixed Substrate Effects

Other researchers have observed that light emission varies with growth phase (Neilson et al., 1999; Wilson & Hastings 1998) indicating that the luciferase reaction is influenced by the physiological status of the cells. Growing cells must channel fatty acids, reductant and ATP away from the cellular reproduction pathway to the *lux* pathway as shown by the prolonged FDT displayed by cells in log phase as compared to stationary phase.

The presence of glucose with salicylate significantly reduces the amount of time before bioluminescence is observed in log phase cells. Glucose serves as an easily catabolized source of energy for the cells, providing ATP, $FMNH_2$ and NADPH for the luciferase reaction as well as a carbon source for the production of aldehyde, another component of the light reaction. In the presence of glucose, the cells are not limited to carbon and energy reserves within the cell. Hence, growth phase cells produce light one hour after glucose and salicylate addition as opposed to 4 hours after the addition of only salicylate (Fig. 10 and 11). The fact that the growth phase effect is eliminated by the addition of glucose with salicylate to produce a consistent FDT is important for research in systems were the population will be a mixture of both growing and non-growing phase cells. However, this observation serves to illustrate that properties of bioluminescence need a thorough examine under the growth conditions employed in the study.

Oxygen Requirements

The oxygen demand of the luciferase system places a limitation on the maximum cell density that can be accurately quantified in both the saturated and unsaturated sand systems. While unsaturated sand conditions allowed for a higher maximum cell density to be observed relative to saturated sand, both systems demonstrated bioluminescence decline due to oxygen depletion. Nevertheless, the flexibility of the CCD camera system revealed itself by using short and frequent camera exposures. For saturated sand, 1×10^8 cells/ml is the maximum density as light production only lasts approximately 30 minutes before oxygen is depleted, and 3.5×10^8 cells/ml suspension proved to be too high for the system. In unsaturated sand, light production proceeded for approximately 60 minutes before oxygen was depleted by in 8.5×10^8 cells/ml. It may be possible quantify reasonably high cell densities under unsaturated flow conditions before severe oxygen limitation becomes a problem.

CONCLUSIONS

Bacterial growth phase has been shown to significantly increase the time before bioluminescence is observed, however this effect is minimized when a non-inducing substrate such as glucose is introduced concurrently with salicylate. The increased oxygen requirement of the induced luciferase system dictates the upper limit of detection of a *lux*-modified organism as an indicator of biomass in porous media.

This study has shown that a CCD camera can be used to characterize the relationship between bioluminescence and cell density in liquid suspension and saturated and unsaturated sand. The sensitivity of the CCD device as well as type of camera lens and the geometry and dimensions of the experimental setup determine the lower limit of detection of the camera. Utilization of this system for non-destructive observation of microbial growth in porous media has great potential for applications in two-dimensional flowing systems.

BIBLIOGRAPHY

Bolenes, J., Zoutman, D., Campbell, J., Verstraete, W., Paranchych, W. 1993. The use of bioluminescence as a reporter to study the adherence of the plant growth promoting rhizopseudomonads 7NSK2 and ANP15 to canola roots. *Canadian Journal of Microbiology* 39:329-34.

Duncan, S., Glover, L. A., Killham, K., Prosser, J. I. 1994. Luminescence-based detection of activity of starved and viable but nonculturable bacteria. *Applied and Environmental Microbiology* 60:1308-1316.

Ellison, A., Perry, S. F., Stewart, G. S. 1991. Bioluminescence as a real time monitor of injury and recovery in *Salmonella typhimurium. International Journal of Food Microbiology* 12:323-32.

Flemming, C. A., Leung, K. T., Lee, H., Trevors, J. T., Greer, C. W. 1994. Survival of *lux-lac*-marked biosurfactant-producing *Pseudomonas aeruginosa* UG2L in soil monitored by nonselective plating and PCR. *Applied and Environmental Microbiology* 60:1606-1613.

Ford, C. Z., Sayler, G. S., Burlage, R. S. 1999. Containment of a genetically engineered microorganism during a field bioremediation application. *Applied Microbiology and Biotechnology* 51:397-400.

Glass, R. J., Steenhuis, T. S., Parlange, J.-Y. 1989. Mechanism for finger persistence in homogeneous, unsaturated, porous media: Theory and Verification. *Soil Science* 148:60-70.

Grant, F. A., Glover, L. A., Killham, K., Prosser, J. I. 1991. Luminescence-based viable cell enumeration of *Erwinia carotovora* in the soil. *Soil Biology and Biochemistry* 23:1021-1024.

Grant, F. A., Prosser, J. I., Killman, K., Glover, L. A. 1992. Luminescence based detection of *Erwinia cartovora* subsp. *carotovora* in soil. *Soil Biology and Biochemistry* 24:961-967.

Hart, S. 1996. In situ bioremediation: Defining the limits. *Environmental Science and Technology* 30:398A-401A.

Heitzer, A., Applegate, B., Kehrmeyer, S., Pinkart, H., Webb, O. F., Phelps, T. J., White, D. C., Sayler, G. 1998. Physiological considerations of environmental applications of *lux* reporter fusions. *Journal of Microbiological Methods* 33:45-57.

Heitzer, A., Malachowsky, K., Thonnard, J. E., Bienkowski, P. R., White, D. C., Sayler, G. S. 1994. Optical biosensor for environmental on-line monitoring of naphthalene and salicylate bioavailability with an immobilized bioluminescent catabolic reporter bacterium. *Applied and Environmental Microbiology* 60:1487-1494.

Heitzer, A., Webb, O. F., Thonnard, J. E., Sayler, G. S. 1992. Specific and quantitative assessment of naphthalene and salicylate bioavailability using a bioluminescent catabolic reporter bacterium. *Applied and Environmental Microbiology* 58:1839-1846.

Hibma, A. M., Jassim, S. A. A., Griffiths, M. W. 1996. In vivo bioluminescence to detect the attachment of L-forms of *Listeria monocytogenes* to food and clinical contact surfaces. *International Journal of Food Microbiology* 33:157-167.

Jensen, L. E., Kragelund, L., Nybroe, O. 1998. Expression of a nitrogen regulated *lux* gene fusion in *Pseudomonas fluorescens* DF57 studied in pure culture and in soil. *FEMS Microbiology Ecology* 25:23-32.

King, J. M. H., DiGrazia, P. M., Applegate, B., Burlage, R., Sanseverino, J., Dunbar, P., Larimer, F, Sayler, G. S. 1990. Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. *Science* 249:778-781.

Langridge, W., Escher, A., Wang, G., Ayre, B., Fodor, I., Szalay, A. 1994. Low-light image analysis of transgenic organisms using bacterial luciferase as a marker. *Journal of Bioluminescence and Chemiluminescence* 9:185-200.

Matrubutham, U., Thonnard, J. E., Sayler, G. S. 1997. Bioluminescence induction response and survival of the bioreporter bacterium *Pseudomonas fluorescens* HK44 in nutrient-deprived conditions. *Applied Microbiology and Biotechnology* 47:604-609.

Meighen, E. A., Dunlap, P. V. 1994. Physiological, biochemical and genetic control of bacterial bioluminesence. *Advances in Microbial Physiology* 34:2-67.

Mohammed, N., Allayla, R. I., Nakhla, G. F., Farooq, S., Husain, T. 1996. State-of-the-art review of bioremediation studies. *Journal of Environmental Science and Health* A31:1547-1574.

Neilson, J. W., Pierce, S. A., Maier, R. M. 1999. Factors influencing expression of *lux*CDABE and *nah* genes in *Pseudomonas putida* RB1353(NAH7,pUTK9) in dynamic systems. *Applied and Environmental Microbiology* 65:3473-3482.

Niemet, M. R., Selker, J. S. 2000. A new method for quantification of liquid saturation in 2-d translucent porous media systems using light transmission. Submitted to *Advances in Water Resources*.

Rattray, E. A. S., Prosser, J. I., Killham, K., Glover, L. A. 1990. Luminescence-based nonextractive technique for in situ detection of *Escherichia coli* in soil. *Applied and Environmental Microbiology* 56:3368-3374.

Schroth, M., Istok, J. D., Ahern, S. J., Selker, J. S. 1995. Geometry and position of light nonaqueous-phase liquid lenses in water-wetted porous media. *Journal of Contaminant Hydrology* 19:269-287.

Schroth, M. H., Ahearn, S. J., Selker, J. S., Istok, J. D. 1996. Characterization of Miller-similar silica sands for laboratory hydrologic studies. *Soil Science Society of America Journal* 60:1331-1339.

Shaw, J. J., Dane, F., Geiger, D., Kloepper, J. W. 1992. Use of bioluminescence for detection of genetically engineered microorganisms released into the environment. *Applied and Environmental Microbiology* 58:267-273.

Shaw, J. J., Rogowsky, P., Close, T. J., Kado, C. I. 1987. Working with Bacterial Bioluminescence. *Plant Molecular Biology Reporter* 5:225-236.

Silcock, D. J., Waterhouse, R. N., L. A. Glover, Prosser, J. I., Killham, K. 1992. Detection of a single genetically modified bacterial cell in soil by using charge coupled device enhanced microscopy. *Applied and Environmental Microbiology* 58:2444-2448.

Soren, L., Nilsson, M., Nilsson, L. E. 1995. Quantitation of antibiotic effects on bacteria by bioluminescence, viable counting and quantal analysis. *Journal of Antimicrobial Chemotherapy* 35:669-674.

Sutherland, A. D., C. Bell, A. L., Deakin, J., Hunter, E. A. 1994. The Biotrace method for estimating bacerial numbers in milk by bioluminescence. *Journal of the Society of Dairy Technology* 47:117-120.

Tidwell, V. C., Glass, R. J. 1994. X-ray and visible light transmission for laboratory measurement of two-dimensional saturation fields in thin-slab systems. *Water Resources Research* 30:2873-82.

Waterhouse, R. N., Buhariwalla, H., Bourn, D., Rattray, E. J., Glover, L. A. 1996. CCD detection of *lux*-marked *Pseudomonas syringae* pv. *phaseolicola* L-forms associated with Chinese cabbage and the resulting disease protection against *Xanthomonas campestris*. *Letters in Applied Microbiology* 22:262-266.

Waterhouse, R. N., Glover, L. A. 1994. CCD-monitoring of bioluminescence during the induction of the cell-wall deficient, L-form state of a genetically modified strain of *Pseudomonas syringae* pv. *phaseolicola*. *Letters in Applied Microbiology* 19:88-91.

Waterhouse, R. N., Silcock, D. J., Glover, L. A. 1993. Use of charge-coupled device (CCD) image-enhancement for rapid screening and monitoring of prokaryotic promoter expression. *Letters in Applied Microbiology* 16:136-141.

Webb, O. F., Bienkowski, P. R., Matrubutham, U., Evans, F. A., Heitzer, A., Sayler, G. S. 1997. Kinetics and response of a *Pseudomonas fluorescens* HK44 biosensor. *Biotechnology and Bioengineering* 54:491-502.

Wilson, T., Hastings, J. W. 1998. Bioluminescence. *Annual Review of Cell and Developmental Biology* 14:197-230.

Yeomans, C. V., Porteous, F., Paterson, E., Meharg, A. A., Killham, K. 1999. Assessment of lux-marked *Pseudomonas fluorescens* for reporting on organic compounds. *FEMS Microbiology Letters* 176:790-83.