Using Bioluminescent Bacteria to Detect Water Contaminants

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ABSTRACT

Conventional chemical analyses typically have a specificity that makes it difficult to apply them efficiently in the assay of a broad range of water contaminants. An economical bioassay method for determining the toxicity of aquatic contaminants in developing countries should help improve public health worldwide. This research explored the effect of six contaminants - CuSO₄, ZnSO₄, NaNO₃, HgCl₂, Atrazine, and Permetherin, on the bioluminescence of the bacterium *Vibrio fischeri*. A decrease in *V. fischeri* bioluminescence was correlated to the presence of contamination. Bacteria were cultured in both liquid flasks and on agar plates, and contaminants were added according to EPA Maximum Contaminant Level values. Bioluminescence was determined by photographing cultures at a 30-second exposure in a lightproof box with a digital camera connected to a PC. The mean light intensity of each image was determined with Image J Batch Measure Macro. The effect of all contaminants on bioluminescence could be detected within 150 min. of their introduction.

KEYWORDS: Vibrio fischeri; bioluminescence, cell biosensor, Image J, Batch Measure Macro

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INTRODUCTION

Vibrio fischeri is a Gram-negative, rod-shaped bacterium that bioluminesces through a population-dependent mechanism called quorum sensing. Colonies of *V. fischeri* collectively luminesce upon reaching a certain cell density [1, 2]. The bacterial luminescence reaction, catalyzed by luciferase, involves the oxidation of a long-chain aliphatic aldehyde and a reduced flavin mononucleotide (FMNH₂), generating luciferin (FMN), the oxidized form of the aldehyde, and water, with the liberation of excess free energy in the form of a blue-green light at 490nm [3]:

 $FMNH_2 + RCHO + O_2 ----> FMN + RCOOH + H_2O + light (490nm)$

The bioluminescence intensity reflects the overall health of the organisms and the luminescence reaction, which reflects metabolism, is sensitive to a wide variety of toxic substances [1-3]. This sensitivity has made them a popular choice for methods to detect environmental pollutants, such as heavy metals and pesticides.

In the United States approximately 14 million people drink water contaminated with major agricultural herbicides such as Atrazine [4]. Herbicides sprayed on crops such as corn and sugarcane result in the presence of more than 60 million lb/year of residual Atrazine that can percolate into streams, rivers, lakes and ultimately drinking water supplies [4, 5].

The presence of environmental contaminants such as ZnSO₄, CuSO₄, NaNO₃, and HgCl₂, pesticides, and herbicides in runoff that feeds into drinking supplies is a major current health concern. For example, Atrazine causes mammary gland tumors if ingested at toxic levels [4, 5]. Copper, zinc, and nitrate cause gastrointestinal damage, especially in fragile infants, who may die if exposed to even a relatively small amount of the toxin [5]. Thus, a proper method of determining the presence of such contaminants is crucial to improving public health. Globally, a cost effective method for detecting contamination is key to improving water quality.

Purpose

The purpose of this experiment is to explore the use of bioluminescent bacteria as a rapid, versatile and economic method of testing water contamination. Practical experience with water treatment has shown that chemical specific assays can be sensitive and precise [4, 5] but they can be expensive and time-consuming. In addition, chemical specific tests detect a narrow range of compounds and do not identify toxins for which the analysis is insensitive. This could allow unanticipated toxins to remain undetected.

Because *V. fischeri* bioluminescence intensity reflects overall health [1, 2, 6, 7] its measurement can detect and quantify the presence of unanticipated toxic chemicals. A substance toxic to *V. fischeri* will inhibit its metabolic activity and reduce or completely suppress the intensity of bioluminescence. A biosensor based on *V. fischeri* bioluminescence could offer a rapid, simple, and precise method to test a wide spectrum of chemical substances in environmental samples.

Hypothesis

The reduction in bioluminescence intensity of *V. fischeri* cultures grown in liquid flask or solid plate cultures can be correlated with the amount of toxicity from environmental contaminants such as ZnSO₄, CuSO₄, NaNO₃, Atrazine, pesticides, and HgCl₂.

MATERIALS AND METHODS

Reagents and Materials

Unless otherwise stated, all aqueous solutions were prepared with distilled, deionized water; reagent-grade or analytical-grade chemicals were obtained from Sigma Chemical Co., St. Louis, MO. Atrazine was purchased from Home-Depot in the form of the product IMAGE[®] which contained 4% Atrazine and 96% of other inert ingredients. The pesticide Permetherin was also purchased from Home-Depot in the form of the product High-Yield[®] Kill-a-Bug which contained 2.5% Permetherin and 97.5% other inert ingredients.

Bacterial Strains and Media

V. fischeri on photobacterium agar (Carolina Biological Supply Company), was used to study luminescence characteristics. The bacterium was grown at room temperature (20-22°C) in a water bath shaker and inoculated every 48-96 hours [1-3, 6, 7].

Three types of media were tested for this experiment; all media were sterilized by autoclaving at 121°C for 15 minutes. The first medium selected for culturing was a liquid photobacterium broth (PB, Carolina Biological Supply Company). The PB differed from Luria Burtani (LB) broth (most commonly used to culture bacteria such as *E. coli*) in two significant ways [6-8]. First, to simulate seawater, the broth contained several salts, most notably 0.58M of NaCl. In addition, glycerol was used in place of the glucose typically found in LB broth because glucose acts to inhibit *V. fischeri* bioluminescence [9, 10].

Because the salts present in the PB broth formed precipitates that interfered with the bioluminescence intensity measurements [11], LA medium (per L: NaCl, 10 g; Yeast Extract, 5 g; Peptone, 10 g and also 15 g agar for solid media) and LBS medium (per L: Tryptone, 10g; Yeast Extract, 5 g; NaCl, 20 g; glycerol, 0.3% (vol/vol) and Tris-HCl buffer, 50 mM to pH 7.5) were tested [8, 9]. *V. fischeri* grew more slowly in LA medium and produced less bioluminescence than in LBS medium. LBS was therefore selected for these experiments.

Culturing Conditions

For solid plates, a colony of *V. fischeri* was selected using a sterile wooden stick and streaked across the plate. The different sections of streaks were intended to dilute the cells so that by the last streak, only single colonies grew rather than the lawn of bacteria that developed from the first streak. For liquid culture, a metal loop was sterilized over a Bunsen burner, and one loopful of *V. fischeri* grown in LBS agar was inoculated to a 5 mL LBS medium in a test tube. Subsequent liquid cultures were inoculated directly from this stock (1% v/v, e.g. 0.5 mL to each 50 mL flask). Since the amount of luminescence varies to some extent with the degree of aeration, care was taken to keep the shaking conditions identical in different experiments by always using 150-mL flasks containing a culture volume of 50 mL. Cultures were aerated by continuous shaking at 150 rpm and kept at room temperature (20-22 °C) for a minimum period of 24 hours to reach peak luminescence.

Cell Density Measurement

Cell density was determined by measuring optical density at 600 nm and a 1-cm path in a spectrophotometer (Beckman Model DU-70). There are three phases in the growth of a cell culture: lag phase, exponential (or logarithmic) phase, and stationary phase. At various times after inoculation, either 1-mL (lag to mid-exponential phase) or 0.1-mL (mid-exponential to stationary phase, mixed with 0.9-mL LBS to dilute 10-fold for accurate measurement) samples were removed from batch cultures for determinations of culture density. West (2006) showed that an absorbance of 1.0 corresponded to a *V*. *fischeri* concentration of approximately 5 x 10^9 cells/mL [11].

Qualitative and Quantitative Measurement of Luminescence

A fast and qualitative luminescence measurement was made by swirling the flask in a completely dark room. When the observer's eyes have become accustomed to the dark, it is possible to judge the intensity of the blue-green fluorescence.

For quantitative measurement, a digital photographic method was designed using the open source ImageJ Macro software [12]. The fluorescence intensity (in light units) was determined in a series of photos taken over time. A digital camera was stationed inside a self-made lightproof box. Above the camera, openings were carved out in boards to hold either the flasks or petri dishes so that the distance from the bottom of them to the camera lens was fixed. Pictures were taken with a constant setting and exposure time of 30 seconds. The pictures were then transferred to the ImageJ Batch Measure Macro to obtain a mean light unit (LU) reading. The results were transferred to Microsoft Excel and analyzed and presented numerically as bioluminescence LU. Normalized data were determined by comparing the LU at a given time to the LU at the start time at which just prior to adding the contaminant. To determine whether the cells were affected by the contaminant treatment, their light output was compared with the light output of the control cells.

Factors significantly impacting bioluminescence include oxygen supply, medium type, and culture [11]. Because most cultures, even those in the phase of maximum light production, experienced a substantial reduction in luminescence within a relatively short period of time unless they were agitated to allow oxygen access, all flasks were shaken continuously at the same rate until they were removed from the water bath shaker for luminescence measurement. The time between removing the flasks from the shaker to taking the pictures was maintained constant for all liquid samples.

Addition of Pollutants

Four inorganic (CuSO₄.5H₂O, NaNO₃, HgCl₂, and ZnSO₄.7H₂O) and two organic (herbicide-Atrazine and pesticide-Permetherin) compounds were evaluated. The EPA limits [5] for these contaminants in drinking water are shown in Table 1. Stock solutions of the concentrations listed in Table 1 were prepared in distilled water, autoclaved, and then diluted as necessary before addition to the flask culture or agar plate. The final amount of contaminants added to either the liquid culture or agar plates was determined by using the EPA limit as a standard and magnifying this concentration by ten times, as specified in Table 1. Similar procedures were also applied to bacteria grown on agar plates. Contaminants

were sprayed in standardized quantities, with about 135 μ L per spray, onto the bacteria cultures on agar plates.

	FDA		Amount of Stock Solution Added (µL)		Final Concentration in 50 mL
Contaminant	MCL* (mg/L)**	Chemicals and Stock Solution Used in this Study	50 mL Liq.	Plate	liquid culture (μM)
		Image Herbicide from Home-depot containing 4% Atrazine, diluted 10 ⁴			
Herbicide	0.003	times	370	$2 \text{ spray} = 270 \ \mu \text{L}$	0.14
Nitrate	10	1M NaNO ₃ then diluted 10x to 0.1M	81	2 spray = 270 μ L	$1.6 \ge 10^3$
Copper	1.3	0.5M CuSO ₄ then dilute 5x to 0.1M	200	$2 \text{ spray} = 270 \ \mu \text{L}$	$2.0 \text{ x } 10^2$
Zinc	5	1.0M ZnSO ₄ then diluted 10x to 0.1M	380	2 spray = 270 μ L	7.6×10^2
Pesticide	0.25	High-Yield Kill-a-Bug from Home Depot containing 2.5% Permetherin, diluted 10 ³ times	32	2 spray = 270 μL	6.4
Hg	0.002	1.0M HgCl ₂ then diluted 10^3 times to 0.001M	50	$2 \text{ spray} = 270 \ \mu \text{L}$	0.10

Table 1. Contaminants Used

* Maximum Contaminant Level (MCL) - The highest level of a contaminant allowed in drinking water

RESULTS

Effect of contaminants on bioluminescence in liquid cultures

The optical density value correlates to the cell population and indicates bacterial growth; the bioluminescence is correlated to luminescent efficiency and bacterial health.

The series of photographs for each liquid flask culture at increasing times after adding contaminants is shown in Figure 1. Results from the liquid cultures are summarized in Table 2 and shown graphically in Figure 2. Cultures continued to grow after adding the contaminants, as evidenced by the increase in the OD_{600} reading over time. Bioluminescence intensity decreased within 60 min. after adding contaminants and continued to decrease to 30-58% of its original value within 150 min. after adding the contaminants. Atrazine resulted in the highest light intensity decrease and ZnSO₄ and NaNO₃ the lowest. The light intensity of the control sample was relatively constant throughout the experiment (Table 2).



Figure 1. Progressive photographs of *V. fischeri* bioluminescence grown in liquid cultures after adding contaminants



Figure 2. Effect of contaminants on bioluminescence for *V. fischeri* grown in liquid cultures

Table 2. Effect of contaminants on bioluminesco	ence of V. fischeri grown in liquid cultures
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Measured Light Units*						
Time after Adding Contaminants	0 min (Start)	30 min	60 min	90 min	120 min	150 min
Control	27.76	28.66	28.74	26.23	27.17	27.43
CuSO4	29.87	28.92	21.39	19.49	15.76	13.96
HgCl2	26.28	26.79	22.12	22.66	16.91	12.49
ZnSO4	28.88	29.87	24.98	20.25	15.44	18.41
NaNO3	30.27	24.32	22.57	18.13	15.65	18.73
Herbicide (Atrazine)	28.21	21.97	14.98	12.29	10.35	9.52
Pesticide	31.36	24.33	18.61	17.13	13.78	12.83
OD ₆₀₀						
Time after Adding Contaminants	0 min (Start)	30 min	60 min	90 min	120 min	150 min
Control	3.11	3.19	3.21	3.21	3.23	3.33
CuSO4	3.13	3.25	3.17	3.27	3.38	3.46

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HgCl2	3.30	3.40	3.36	3.45	3.50	3.66	
ZnSO4	3.22	3.29	3.30	3.38	3.45	3.55	
NaNO3	3.25	3.30	3.28	3.38	3.37	3.56	
Herbicide (Atrazine)	3.19	3.38	3.40	3.41	3.50	3.58	
Pesticide	3.15	3.23	3.24	3.26	3.36	3.42	
Normalized Light Unit/OD ₆₀₀							
Time after Adding Contaminants	0 min (Start)	30 min	60 min	90 min	120 min	150 min	
Control	1.00	1.01	1.00	0.92	0.94	0.92	
CuSO4	1.00	0.93	0.71	0.62	0.49	0.42	
HgCl2	1.00	0.99	0.83	0.83	0.61	0.43	
ZnSO4	1.00	1.01	0.84	0.67	0.50	0.58	
NaNO3	1.00	0.79	0.74	0.58	0.50	0.57	
Herbicide (Atrazine)	1.00	0.74	0.50	0.41	0.34	0.30	
Pesticide	1.00	0.76	0.58	0.53	0.41	0.38	

* Results are the average of 3 separate trials

Effect of contaminants on bioluminescence on solid plate cultures

Photos of the plate cultures are shown in Figure 3 and bioluminescence intensities are summarized in Table 3 and presented graphically in Figure 4. Bioluminescence intensity decreased within 30 minutes after addition of all metal contaminants; after 30 minutes luminescence had decreased by 72 to 84% and after 150 minutes by 64 to 87%. HgCl₂ seemed to have the most significant effect on bioluminescence intensity, reducing it by 87%. Atrazine and NaNO₃ had little effect on the bioluminescence intensity of the plate cultures- indeed, in the presence of NaNO₃, the bioluminescence intensity increased after contaminant addition.

Table 3. Effect of contaminants on bioluminescence of V. fisc	scheri grown on agar plates
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Time after Adding	0 min						0 min					
Contaminants	(Start)	30 min	60 min	90 min	120 min	150 min	(Start)	30 min	60 min	90 min	120 min	150 min
		Measured Light Units*				Normalized Light Unit						
Control	64.53	66.84	67.40	66.35	65.80	63.48	1.00	1.04	1.04	1.03	1.02	0.98
CuSO4	65.75	16.33	16.73	18.39	19.47	20.48	1.00	0.25	0.25	0.28	0.30	0.31
HgCl2	51.75	8.29	7.54	6.90	6.81	6.71	1.00	0.16	0.15	0.13	0.13	0.13
ZnSO4	63.86	17.67	18.45	20.27	21.85	22.84	1.00	0.28	0.29	0.32	0.34	0.36
NaNO3	55.97	57.27	59.59	65.26	67.74	71.07	1.00	1.02	1.06	1.17	1.21	1.27
Herbicide (Atrazine)	56.75	53.15	55.16	57.00	57.51	59.58	1.00	0.94	0.97	1.00	1.01	1.05
Pesticide	51.31	45.80	47.24	48.68	48.56	51.18	1.00	0.89	0.92	0.95	0.95	1.00
* Results are the average of 3 separate trials												

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Measured Light Units*						
Time after Adding Contaminants	0 min (Start)	30 min	60 min	90 min	120 min	150 min
Control	64.53	66.84	67.40	66.35	65.80	63.48
CuSO4	65.75	16.33	16.73	18.39	19.47	20.48
HgCl2	51.75	8.29	7.54	6.90	6.81	6.71
ZnSO4	63.86	17.67	18.45	20.27	21.85	22.84
NaNO3	55.97	57.27	59.59	65.26	67.74	71.07
Herbicide (Atrazine)	56.75	53.15	55.16	57.00	57.51	59.58
Pesticide	51.31	45.80	47.24	48.68	48.56	51.18
Normalized Light Unit						
Time after Adding Contaminants	0 min (Start)	30 min	60 min	90 min	120 min	150 min
Control	1.00	1.04	1.04	1.03	1.02	0.98
CuSO4	1.00	0.25	0.25	0.28	0.30	0.31
HgCl2	1.00	0.16	0.15	0.13	0.13	0.13
ZnSO4	1.00	0.28	0.29	0.32	0.34	0.36
NaNO3	1.00	1.02	1.06	1.17	1.21	1.27
Herbicide (Atrazine)	1.00	0.94	0.97	1.00	1.01	1.05
Pesticide	1.00	0.89	0.92	0.95	0.95	1.00

* Results are the average of 3 separate trials



Figure 3. Progressive photographs of bioluminescence of *V. fischeri* grown on agar plates after adding contaminants



Figure 4. Effect of contaminants on bioluminescence of *V. fischeri* grown on agar plates

DISCUSSION AND CONCLUSIONS

An economical method was designed to detect six environmental contaminants by measuring their effect on decreasing *V. fischeri* bioluminescence intensity. Using a series of photographs taken at 30 minute increments following exposure to contaminants, the *V. fischeri* bioluminescence intensity in both liquid and solid plate cultures decreased within 30-60 minutes of exposure to metal salts (Figures 1 and 3). In all exposed liquid cultures, bioluminescence intensity decreased 32-70% within 150 minutes, while there was very little or no light intensity reduction in the control culture (Table 2 and Figure 2). In solid plate cultures, bioluminescence intensity decreased by 72-84% of its original level within 30 minutes when the culture was exposed to metal pollutants (Table 3 and Figure 4). These results show the potential of bioluminescent bacteria as a rapid and effective method for detecting the toxicity of pesticide and metal contaminants in water.

The herbicide Atrazine, the pesticide Permetherin, and NaNO₃ had little effect on the *V. fischeri* bioluminescence intensity of plate cultures (Table 3 and Figure 4). It is possible that the method for spraying the contaminants on to the agar plates was not effective for Atrazine and Permetherin. The presence of large amounts of inert materials in the products used to evaluate these compounds and the poor aqueous solubility of these compounds may have caused uneven distribution of the diluted chemical on to the bacteria. Further studies with pure Atrazine or Permetherin should be conducted to elucidate this

discrepancy. It is not certain that nitrate in NaNO₃ is toxic to *V. fischeri;* indeed, the nitrate might actually stimulate its growth.

The *V. fischeri* bioluminescence assay is a simple, rapid, and versatile method that could be applied in practice for the detection of water contaminants. Its application would be as a qualitative tool for the rapid determination of the presence of contaminants, and it could be followed by various quantitative tests to ascertain a more precise and specific measurement. In addition, since it is non-specific, it can be used as a versatile method for detecting the presence of unanticipated pollutants. The ability to detect hazardous chemicals such as those tested in this experiment is crucial to public health worldwide. It is recommended that future experimentation on other types of contaminant be conducted. The method could also be improved by using a luminometer for measuring bioluminescence intensity.

ABBREVIATIONS AND ACRONYMS

EPA: US Environmental Protection Agency
LBS: Lactobacillus selection
LA: Luria agar
PB: Photobacterium
ppm: parts per million, the equivalent of mg/kg
OD: Optical density
RLU: Relative light unit
LU: Light unit

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Author

I am a junior at Bellaire High School in Houston, Texas with interests in the sciences, writing, and creative arts. My parents supported me in pursuing a variety of interests in addition to science as part of their ongoing aspiration to live life to the fullest. Therefore, I participated in Science Fair, Science Connection, my school newspaper, and the Glassell School of Art, among other higher-level painting classes, during my high school career. I have also pursued Speech and Public Forum Debate to help me express myself more eloquently to a wide range of audiences. Outside of academics, I volunteer at numerous events through my high school chapter of the National Honor Society. I have organized various recreational events for the elderly; including a Thanksgiving Banquet to bring together seniors whose closest "family" on the holidays are other members of the community. This event really taught me that you get the most of life not just by taking what you can for yourself, but by giving to others. Some of my accomplishments include the 1st Place Grand Award Winner at Intel ISEF 2009, the 2009 Intel International Young Scientist Award, and a Silver Award at the International Sustainable World Project Olympiad in 2009.

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