BIOLOGICAL SCIENCES

LIGHT INHIBITION OF *Photobacterium leiognathi* AS BIOINDICATOR OF HEAVY METAL POLLUTION

THOMAS EDISON E. DELA CRUZ*, MARIPAZ B. ANCHETA,
ROSEMARIE P. CAPAQUE, ARNEL E. MANTES AND MA. ESTELLITA PEREZ
Department of Biological Sciences
University of Sto. Tomas
España, Sampaloc
1008 Manila

ABSTRACT

Heavy metal pollution in the biosphere has become a serious health and ecological concern. Its occurrence in nature is usually monitored by chemical analyzes with the use of high-cost instruments. An alternative method is presented for determination of water pollution with heavy metals and detergents using biological system. It is a test tube assay involving the naturally luminescent ***Photobacterium leiognathi*** *as bioindicator. Three-milliliter bacterial suspension (10^6 cells/ml) is mixed with 0.5 ml of the test solution to find the minimum anti-luminescent concentration (MAC) of the pollutant. Light inhibition is determined simply by gross visual observation inside the dark room. Tests showed MAC at 714.29 ppm for PbNO3, 142.86 ppm for CuS04, 14.29 ppm for ZnSO4 and 1.43 ppm for HgCI2. A local commercial detergent inhibited bioluminescence of the bioindicator bacterium at 14 ppm MAC. Inhibition of light emission in the bioassay literally means "turning off" the light of the bioindicator bacterium. Light is "turned on" again, i. e., light emission is restored, when the bacterium is transferred to a fresh medium. Since the assay simply involves "on" and "off" of luminescence, this can serve as a preliminary test for toxicity of heavy metals in polluted waters.*

Keywords: *Bioluminescence, Photobacterium leiognathi, Bioindicator, Heavy Metals, Bioassay, Minimum Anti-luminescent Concentration (MAC), Light Inhibition*

INTRODUCTION

Metals mostly exist as mineral deposits in nature. They may be derived from the lithosphere by mining and leaching or from the more familiar industrial, agricultural, and domestic wastes. With the advances in technology, the accumulation of, and pollution with, heavy metals in the biosphere especially in rivers, lakes, and estuaries in urban areas has become a serious health and ecological concern.

Cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdemum (Mo), and zinc (Zn) are important trace metals for growth and development in most living systems. However, other metals like lead (Pb), mercuy (Hg), and cadmium (CD) do not seem to have any importance and, in large quantities, may become hazardous or toxic to various life forms in the environment (Haslam 1990). Because of the need to determine the occurrence and monitor the concentrations of these elements in nature, chemical analyzes are done with the use of high-cost instruments. On the other hand, recent studies explore the possibility of using alternate biological systems such as bacterial bioluminescence and fish bioassay in determining the concentrations of these metals (Bulich 1979).

Bioluminescence is a natural lighting phenomenon associated with fireflies (e.g. *Plotinus pyralis*) and bacteria, for example, *Photobacterium* and *Vibrio* (Sayler *et al.*, 1990). It refers to the ability of the organism to give off light. Luminescence here is a result of endogenous enzymatic process that transforms the chemical energy into light energy. The enzyme involved is luciferase which catalyzes the bioluminescent mixed-function oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long chain aldehyde (RCHO) in the presence of molecular oxygen. The balanced over-all equation for the reaction (Brock *et al.*, 1994) is:

$$FMNH_2 + O_2 + RCHO \longrightarrow FMN + RCOOH + H_2O + \text{light}$$

Bioluminescent microorganisms exist symbiotically with nocturnal and benthic marine life forms although some are free-living. They can be found within sac-like organs in fishes. The emitted light functions in communication and mating (Atlas and Bartha 1993).

The bioluminescent *Photobacterium* and *Vibrio* species are gram-negative, straight or curved rods measuring 1.8-2.4 x 0.8-1.3 μm. They are motile with 1-3 unsheated polar flagella and capable of respiratory and fermentative metabolisms. They accumulate poly-B-hydroxybutyrate but do not metabolize exogenous B-hydroxybutyrate monomers. They do not form endospore or microcyst. These bacteria inhabit the marine environment and thus, have an absolute requirement for Na^+ (Baumann and Baumann 1977).

The objectives of this research work are to: isolate, characterize, and identify bioluminescent bacterial from local marine fishes; devise a bioassay method of determining the pollution levels of heavy metals using inhibition of bioluminescence in the bioindicator bacterium; and, determine the applicability of the

bioassay to other pollutants such as detergents. However, this work does not aim to establish the mechanism involved in the inhibition of luminescence.

MATERIALS AND METHODS

Isolation of Bioluminescent Bacteria

Specimens of four marine fishes (asungos, bangus, loro, lapu-lapu) were obtained for the isolation of the organism. The eyes, scales and intestine of the samples were removed and teased apart with scalpels, and mixed thoroughly. The mixture was suspended in distilled water containing rock salt. A 10-ml aliqout was transferred to 125-ml Erlenmeyer flask containing 100 ml 2% NaCl solution. The flask was then subjected to shaking for few minutes; thereafter, 5 ml was added to a 500-ml Erlenmeyer flask containing 100 ml modified nutrient broth for enrichment for 12 hr in a shaker. Final isolation and subsequent purification were done by streaking on modified nutrient agar plates which were then incubated at 18-20°C. Bioluminescent bacterial colonies were identified in the dark room.

Characterization and Identification of Bacterial isolates

Cultural characterization of the two bioluminescent isolates was done based on colony growth on Egorova-Yarmolink agar (EYA). Gram-staining and morphometry of the bacterial isolates were also done.

For the biochemical characterization, the isolates were tested for: *gas production* from glucose using glucose-yeast extract broth; *xylose* and *maltose utilization* using basal medium agar, *acetate utilization* using acetate agar; and, *gelatinase production* using nutrient gelatin. The temperature requirement for optimal growth was also determined. NaCl (3%) was added to all media, and the pH in all cases was adjusted to 7.0.

Bioassay of Heavy Metals

For the assay of heavy metals, a 100-ml EY broth culture grown for 9 hr at room temperature was centrifuged for 5 min at 3000 rpm. The harvested cells were then washed and suspended in 2% NaCl solution. Cell density of the inoculum was quantified and adjusted to 10^6 cells/ml using Breed's method.

Four heavy metals in salt form ($ZnSO_4$, $HgCL_2$, $PbNO_3$, $CuSO_4$) were tested. One gram of each heavy metals salt was dissolved in 100 ml of water for an initial standard of 10,000 ppm. From this standard, concentrations of 7000, 5000, 3000, 2000, 1000, 100, 10, 1 and 0.1 ppm were prepared. Aliquots (0.5 ml) of these preparations were added to 3 ml bacterial suspension in test tubes to come up with final concentrations of 1000, 714429, 286, 143, 14.3, 0.14 and 0.014 ppm. The tubes were shaken for 5 min to allow aeration and then were observed in the dark room. The minimum anti-luminescent concentration (MAC) was recorded.

Bioassay of Complex Effluents

A commercial detergent (Tide powder) (0.01 g) was diluted with 10 ml distilled water to come up with a standard concentration of 1000 ppm. From this standard solution, serial dilutions were done down to a concentration of 1 ppm. The same procedure was done as in the assay of heavy metals. The final concentrations of the diluted detergent would then be 143, 14, 1.4, 0.14 and 0.014 ppm. The MAC was recorded.

Bioassay of Polluted Water

A 10-ml aliquot of a polluted water sample from San Juan River was serially diluted in 9 ml 2% NaCl solution down to a concentration of 1 ppm. Aliquots (0.5 ml) of each solution were transferred to tubes containing 3 ml of the bacterial suspension. These were shaken and observed inside the dark room. The MAC was recorded.

RESULTS

Identification of Bacterial Isolates

Two bacterial isolates were obtained from fish specimens. Both were identified as *Photobacterium* sp. belonging to the family Vibrionaceae. The cells were gram-negative rods, grew at room temperature, did not produce gas from glucose, and utilized acetate but not maltose and xylose as carbon source. Results for gelatinase production were negative.

Of the three known species of *Photobacterium*, two were able to emit light: *P. phosphoreum* and *P. leiognathi*. The biochemical characteristics of these two species and those of our two isolates (from asungos) are summarized in Table 1. Our isolates clearly are allied to *P. leiognathi* and thus, were identified to be this species.

Light Inhibition Assay for Heavy Metals

With the test tube assay done at room temperature, the minimum anti-luminescent concentration (MAC) (Fig. 1) for the four heavy metals tested was lowest at 1.43 ppm for mercury and highest at 714 ppm for lead. MAC for zinc was 14.3 ppm, and for copper, 143 ppm (Table 2; Fig. 1).

Light Inhibition Assay For Detergent and Polluted Water

The detergent concentration of 14 ppm was the minimum anti-luminescent concentration (Table 3) (Fig. 2). The water sample from San Juan River was diluted down to 10^{-4}. All dilutions in three trials as well as the control (saline solution) and undiluted water sample failed to inhibit bioluminescence of the bacterium.

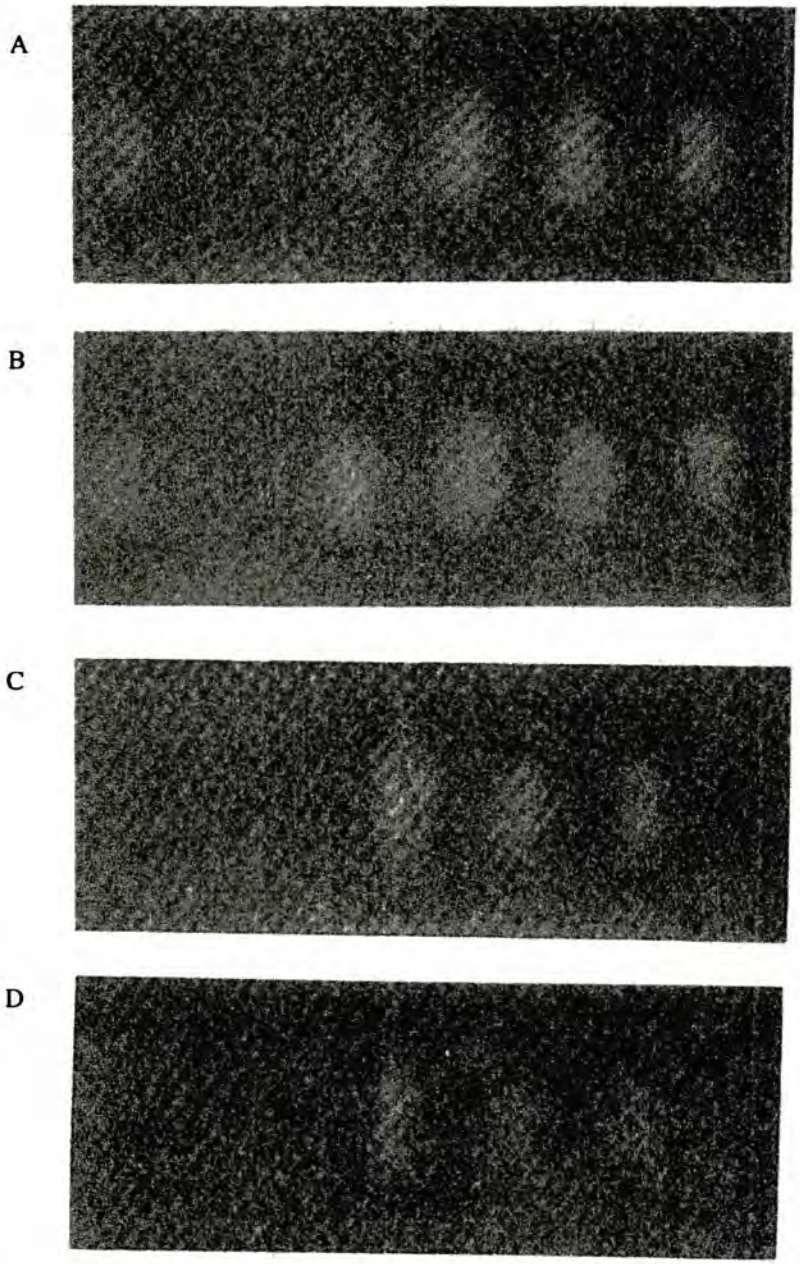

Figure 1. Minimum anti-luminescent concentration (MAC) of Pb (A), Cu (B), Zn (C), and Hg (D).

Figure 2. Minimum anti-luminescent concentration (MAC) of detergent.

Table 1. Biochemical characteristics of bioluminescent bacteria from fishes

Biochemical Test	Isolate A	Isolate B	*P. phosphoreum*	*P. leiognathi*
Gelatinase Production	-	-	-	-
Utilization of				
Xylose	-	-	-	-
Maltose	-	-	+	-
Acetate	+	+	-	+
Fermentation and Gas Production	-	-	+	-
Optimum Growth (°C)	33.35	33.35	4	35

(+) positive; (-) negative test

Table 2. Minimum anti-luminescent concentration (MAC) of heavy metals

Concentration (ppm)	Hg	Zn	Cu	Pb
1000	-	-	-	-
714	-	-	-	-
429	-	-	-	+
286	-	-	-	+
143	-	-	-	+
14.3	-	-	-	+
1.43	-	+	+	+
0.14	+	+	+	+
0.014	+	+	+	+

(+) light on; (-) light off

Table 3. Minimum anti-luminescent concentration of detergent (Tide powder)

Concentration (ppm)	Detergent
143	--
14	--
1.4	+
0.14	+
0.014	+

(+) light on; (–) light off

DISCUSSION

The bioluminescent *Photobacterium leiognathi* could be isolated from the intestines and guts of non-luminous Philippine marine fishes (asungos). Growth at 35°C or at room temperature (Hasting 1977) makes easy identification of this species. The morphological, cultural and biochemical characteristics (Table 1) confirmed that our two isolates belong to this species of *Photobacterium.*

Bacterial luminosity has been employed in determining toxicity in aquatic environments (Bulich 1979). Its sensitivity and reliability compared to the fish test has already been established. A good correlation exists between bacterial luminescence assay and the conventional fish test (Bulich *et al.* 1981).

In this experiment, the luminescence of *P. leiognathi* was used to determine the minimum anti-luminescent concentration (MAC) of four heavy metal samples. These metals were chosen based on their solubility in water and their occurrence in polluted waters. The MAC for mercury was lowest at 1.43 ppm. Mac values for copper and zinc were 143 and 14.3 ppm, respectively. Lead had the highest MAC at 714 ppm (Table 2). These concentrations inhibited light production of our bioindicator bacterium (isolate B). Light inhibition could well be due to the inhibition of the enzyme luciferace by heavy metals which are known to targets the cell's functional enzymes. However, light inhibition is not related to the death of the cells. This was proven when light-inhibited bacterial cells used in the assay were restreaked in fresh culture media. After 9 hr of incubation at room temperature, the cells grew and again exhibited luminescence.

The assay procedure was further applied to detergent, a common water pollutant, and to a polluted water sample. A local commercial detergent (Tide powder) gave a MAC of 14 ppm (Table 3). This concentration level is the same MAC for Hg (Table 2). Light inhibition here again did not mean cell death but simply turning-off the light.

Rebioluminescence or the resumption of light emission upon transfer of the bioindicator bacterium to a fresh medium from the assay tube needs some explanation. Microorganisms have specialized transport systems for different solutes and toxic inorganic ions such as heavy metals. Thus, the influx of sodium ions in

the cells acts as a transport system for inorganic ions transforming toxic heavy metals to less toxic and sometimes volatile forms (Selifonova and Barkay 1994; Mitchell, 1991). Perhaps the same mechanisms holds true in the rebioluminescence of *P. leiognathi.*

The other unexpected result was the failure of the indicator bacterium to turn off its light in the presence of the polluted water sample from a river. It is not likely that heavy metals were not present in that river. According to the National Pollution Control Center, heavy metal levels in San Juan River in 1980-1985 decreased from 2.8 to 0.4 ppm. Based on our results with Cu, Zn, Pb, Hg (Table 2), this level would not be sufficient to inhibit bioluminescent of our test bacterium.

CONCLUSION

Our experiment proved that inhibition of bacterial luminescence can be used to indicate heavy metal pollution in aquatic environment. The applicability of the bioassay using *Photobacterium leiognathi* worked for both metals and detergents. Its sensitivity to low concentrations of pollutants makes it an effective, alternative biological method for testing pollution.

ACKNOWLEDGMENT

We would like to express our sincerest gratitude to Dr. Irineo Dogma, Jr. and Mr. Edward Quinto, our mentors, who had been very accommodating in sharing with us reference materials and assisting us during our experimental work.

REFERENCES

Atlas, R. and **R. Bartha.** 1993. Microbial Ecology: Fundamentals and Applications. Benjamin Cumming Pub., USA.

Bauman, P. and **L. Bauman.** 1977. Biology of Marine Enterobacteriaceae. Ann. Rev. Microbiol. 31:39-61.

Brock, T. and **M. Madigan.** 1994. Biology of Microorganisms. Prentice-Hall International, Inc., USA.

Bulich, A.A. 1979. Use of luminescent bacteria for determining toxicity in aquatic environments. Aquatic Toxicology 667:98-106.

Bulich, A.A., M.W. Greene, and D.L. Senberg. 1981. Reliability of the Bacterial Luminescence assay for determination of the toxicity of pure compounds and complex effluents. Aquatic Toxicology 737:338-347.

Haslam, S.M. 1990. River Pollution: An Ecological Perspective. John Wiley & Sons, USA.

Hastings J. W. 1977. Bacterial Luminescence. Ann. Rev. Microbial. 31:549-591.

Mitchell, R. 1991. Environment Microbiology. John Wiley & Sons, USA.

Sayler, G.S., J.M. King, P.M. Grazia, B. Applegate, J. Severino, P. Dunban, and **F. Carmen.** Rapid sensitive bioluminescent reporter technology for napthalene exposure and biodegradation. Science 249:778-779.

Selifonova, O.V. and **T. Barkay.** 1994. Role of Na(sup$^+$) in transport of Hg(sup^{2+}) and induction of the Tn 21 over operon (dag). Appl. & Environ. Microbiol. 60:3503-3607.