A review on advantages of implementing luminescence inhibition test 
(*Vibrio fischeri*) for acute toxicity prediction of chemicals☆

Shahid Parvez a, Chandra Venkataraman b, Suparna Mukherji a,☆

a Centre for Environmental Science and Engineering, Indian Institute of Technology, Bombay, Powai, Mumbai 400076, India
b Department of Chemical Engineering, Indian Institute of Technology, Bombay, Powai, Mumbai 400076, India

Abstract

Evaluation of biological effects using a rapid, sensitive and cost effective method can indicate specific information on toxicity/ecotoxicity. Since assays based on animals, plants and algae are expensive, time consuming and require large sample volume, recent studies have emphasized the benefits of rapid, reproducible and cost effective bacterial assays for toxicity screening and assessment. This review focuses on a bacterial assay, i.e., *Vibrio fischeri* bioluminescence inhibition assay, which is often chosen as the first test in a test battery based on speed and cost consideration. The test protocol is simple and was originally applied for aqueous phase samples or extracts. The versatility of the assay has increased with subsequent modification, i.e., the kinetic assay for turbid and colored samples and the solid phase test for analyzing sediment toxicity. Researchers have reported the *Vibrio fischeri* bioluminescence assay as the most sensitive across a wide range of chemicals compared to other bacterial assays such as nitrification inhibition, respirometry, ATP luminescence and enzyme inhibition. This assay shows good correlations with other standard acute toxicity assays and is reported to detect toxicity across a wide spectrum of toxicants.

Keywords: Bioluminescence; *Vibrio fischeri*; Luciferase; Solid phase test; Kinetic assay

1. Introduction

Toxicity measurement of wastewater, sediments, and contaminated water bodies is a very important part of environmental pollution monitoring. Commonly, lumped parameters, e.g., dissolved oxygen (DO) level and chemical oxygen demand (COD) are used for pollution monitoring. These analyses are limited and only indicate the nature of the pollutants but do not yield any information about the biological effects. Evaluation of biological effects using a rapid, simple, sensitive and cost effective method can indicate specific information on toxicity and ecotoxicity and allow incorporation of toxicity parameters in the regulatory framework. Many researchers have studied the development of rapid and sensitive bioassays to monitor and assess the discharges of toxic materials. Traditionally, crustaceans, fish and algae are used for aquatic toxicity measurement. The tests based on these organisms require large exposure time and sample volume. Therefore, toxicity measurements based on microorganisms which are rapid, cost effective and reproducible are gaining popularity.

Farre and Barcelo (2003) provide a review on various bioassays. Fish, rat, mice and Daphnia are used as test organisms in the animal assays. Generally, fish bioassays show good sensitivity and permit real-time analysis, but they suffer from standardization problems, need specialized equipment and skilled operators and are time consuming. In plant bioassays the species typically used are *Avena sativa*, *Brassica campestris* and *Lactuca sativa*. Algal bioassays are based on microalgal species such as *Selenastrum capricornutum*, *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*. The main disadvantages associated with animal and plant bioassays are: problems with standardization of the organisms, requirements for special equipment and skilled operators, long duration of the assay and lack of reproducibility. Bacterial bioassays tend to fall into one of the five categories: population growth, substrate consumption, respiration, ATP luminescence and
bioluminescence inhibition assays. The test species used for bioluminescence inhibition assay includes *Vibrio fischeri*, *Photobacterium phosphoreum*, *Vibrio harveyi* and *Pseudomonas fluorescens*; while those used for metabolic inhibition includes *Escherichia coli* and *Pseudomonas putida*. Mixed culture from activated sludge is often used for growth inhibition studies. The popularity of bacterial assays is based on the fact that bacteria are an integral part of the ecosystem and the bacterial assays are relatively quick and simple.

This review focus on a bacterial bioassay, i.e., the bioluminescence inhibition test which is often chosen as the first test in a test battery based on speed and cost consideration (Kahru et al., 1996b). The growing interest in these tests is due to the fact that, despite the existence of different toxicity mechanisms for various organisms of different species, a substance that is toxic for an organism often demonstrates similar toxic effects on other organisms (Kaiser, 1998). Thus, luminescence inhibition in a bacterium can effectively indicate toxic effects on higher organisms.

2. Bioluminescence inhibition assay: an introduction

The bioluminescence inhibition assay is based on a marine gram negative bacterium, *Vibrio fischeri* (earlier referred as *Photobacterium phosphoreum*). The specific strain, NRRL B-11177, has been widely used for acute toxicity estimation and several commercial test kits, i.e., Microtox, LUMIStox and ToxAlert are based on this strain (Farre and Barcelo, 2003). Light production is directly proportional to the metabolic activity of the bacterial population and any inhibition of enzymatic activity causes a corresponding decrease in bioluminescence. The assay provides a measure of sub-lethal response.

3. Biochemical mechanism of luminescence in *Vibrio fischeri*

Reduced flavin mononucleotide (FMNH2) plays a key role in the bioluminescence reaction. FMN reduces to FMNH2 upon reaction with the reduced form of nicotinamide adenine dinucleotide phosphate (NAD(P)H) in presence of flavin reductase enzyme.

\[
\text{NAD(P)H} + H^+ + \text{FMN} \xrightarrow{\text{flavin reductase}} \text{NAD(P)} + \text{FMNH}_2.
\]

Reduce FMNH2 gets oxidized into FMN and H2O upon reaction with molecular oxygen in the presence of aldehyde and luciferase enzyme. In this reaction blue-green light of wavelength 490 nm is emitted (Inouye, 1994).

\[
\text{FMNH}_2 + O_2 + R - \text{CHO} \xrightarrow{\text{luciferase}} \text{FMN} + H_2O + R - \text{COOH} + \text{hv}(490\text{ nm}).
\]

Accessory proteins found in some luminous bacteria often cause a shift in the wavelength of emitted light. Both blue shift (towards lower wavelength of 475 nm) and red shift (towards higher wavelength of 540 nm) have been reported. These accessory proteins attach to luciferase to alter the emission spectrum (Karatani and Hastings, 1993).

4. Test procedure and its variations

The toxicity measurement protocol has been outlined by Kahru et al. (1996a). A 1.5 ml volume of culture suspension is contacted with 1.5 ml volume of test chemical, prepared in 2% NaCl. A control sample consisting of bacterial suspension in 2% NaCl devoid of the test chemical is included along with the test sample. Chemicals which are poorly soluble in water are first dissolved in an appropriate carrier solvent, usually methanol or ethanol and subsequently diluted with 2% NaCl such that the concentration of the carrier solvent is less than 1.5%. However, even carrier solvents with statistically insignificant toxicity may impact toxicity measurement. Mariscal et al. (2003) demonstrated the organic solvent (dimethyl sulphoxide (DMSO), ethanol, methanol, isopropanol and acetonitrile) impacts upon toxicity of heavy metals measured in a bioluminescence assay with *Vibrio harveyi*. Typically the test has been performed at 15 °C. The decrease in bacterial luminescence (INH %) due to addition of toxic chemicals can be determined as follows:

\[
\text{INH} \% = 100 - \left( \frac{I_{TF}}{I_{T0} \times KF} \right) \times 100
\]

\[
KF = \frac{IC_F}{IC_0}.
\]

Where, IC0 and IT0 are the luminescence of control and test sample at t=0. ICF and ITF are luminescence values for control and test samples measured after ‘T’ minutes exposure time and KF is the correction factor based on the control/blank. Various researchers have used variable exposure time, e.g., 5 min (DeZwart and Sloof, 1983), 15 min (Rigol et al., 2004), and 30 min (Loibner et al., 2004). The concentration of the toxicant (mg/L) which caused a 50% reduction in light after exposure for ‘T’ minutes is designated as the EC50. The luminescence emitted can be measured by luminometers that are typically provided with commercial test kits, such as Microtox (DeZwart and Sloof, 1983), LUMIstox (Wang et al., 2002), Biotox (Lappalainen et al., 2001), and Toxalert 500 (Guerra, 2001) or by fluorescence spectrophotometer (Mariscal et al., 2003), programmed to obtain an integrated response over the wavelength range 400–700 nm with excitation source switched off.

The basic assay is subject to variations in the presence of color and turbidity which interferes with luminous intensity measurements. Turbid samples require centrifugation or filtration before contacting with the test culture suspension. Recently Lappalainen et al. (1999) presented a variation of the bioluminescence inhibition assay, i.e., a kinetic mode bioassay instead of the typical static mode assay which can overcome problems due to turbidity and color. This measurement requires a luminometer or fluorescence spectrophotometer equipped with dispenser, controller and mixer. As the bacterial suspension is injected into the sample containing the test chemical, the luminous intensity increases, achieves a maximum within 0–5 s and subsequently decreases continuously. The results are
expressed as the ratio of luminescence at 30 s normalized to the peak value.

Although originally bioluminescence inhibition assay was applied for analysis of the aqueous or extracted samples, a modified solid phase assay was also developed subsequently for analysis of soil/sediments toxicity. The solid phase test protocol involves mixing the soil/sediment sample with aqueous saline diluent (2% NaCl) in the ratio 1:5 (w/v). Subsequently, several serial dilutions are prepared and bacteria are exposed to these dilutions for a fixed exposure time. The samples are then passed through a filter column for separation of soil/sediments from the aqueous suspension of bacteria and the luminescence of bacterial suspension is determined. To obtain EC$_{50}$ values based on dry weight of soil/sediment (mg) per milliliter of extracted diluent, the experimentally determined EC$_{50}$ values are corrected with the moisture content of soil/sediments. It has been suggested that uncontaminated reference sediments should be used as control. A toxicity soil/sediments. It has been suggested that uncontaminated biphenyls (PCBs) were present, they were not bioavailable to the bacteria, unless extracted from the sediments. Their results indicate that the availability of PAHs in soil/sediments from the aqueous suspension of bacteria and the luminescence of bacterial suspension is determined. To obtain EC$_{50}$ values based on dry weight of soil/sediment (mg) per milliliter of extracted diluent, the experimentally determined EC$_{50}$ values are corrected with the moisture content of soil/sediments. It has been suggested that uncontaminated reference sediments should be used as control. A toxicity reference index (TRI) may be developed by spiking an uncontaminated soil/sediment with a reference compound, e.g., pentachlorophenol (1 mg/g). Samples with EC$_{50}$ value half that of the reference would be twice as toxic and would have a TRI of two (Johnson and Long, 1998). Sediment composition was found to affect the estimated toxicity values. High proportion of silt or clay in the sediment samples is found to reduce the EC$_{50}$ values, thereby indicating higher toxicity than expected. This has been attributed to the attachment of Vibrio fischeri cells on the clay particles that settle out at the bottom, thus, reducing the intensity of the signals recorded by a luminometer (Ringwood et al., 1997).

5. Applications of bioluminescence inhibition assay

Bioluminescence inhibition assay can be used for toxicity measurement of single compounds and mixtures of organic and inorganic compounds. This bioassay is applicable for almost all kinds of samples such as surface and groundwater samples (Boyd et al., 1967), complex effluents (Reemtsma et al., 1999), municipal waste effluent and sediments (Cotou et al., 2002; Salizzato et al., 1998). It has been used for measuring short and long-term photoinduced toxicity of polynuclear aromatic hydrocarbons (PAHs) (El-Alawi et al., 2002). Bioluminescence inhibition assay has been applied as a sensitive and rapid screening tool for determining the whole effluent toxicity for various industrial effluents, i.e., dye wastewater from textile industry (Davere and Bahadur, 1994); and whitewater and effluent from paper mill (Rigol et al., 2004).

Salizzato et al. (1998) successfully applied the solid phase assay to lagoon sediments (<63 µm) using a modified protocol for correlating the toxic units (TU=100/EC$_{50}$) for sediments obtained from a lagoon to the composition of organic and inorganic toxicants. The toxic effect was primarily attributed to inorganics. Although significant level of PAHs and polychlorinated biphenyls (PCBs) were present, they were not bioavailable to the bacteria, unless extracted from the sediments. Similar results have been reported by Harkey and Young (2000) for PAH contaminated soil from manufactured gas plant sites. The extraction method, aqueous saline extraction versus supercritical fluid extraction (SFE) yielded different measures of toxicity, with SFE yielding lower EC$_{50}$ values. Toxicity of soil measured by solid phase test before and after extraction by SFE was found to be unaffected for some of the soils whereas a marked decrease was observed in other soils. No correlation was observed between total PAH content based on Soxhlet extraction and Microtox toxicity based on solid phase test. Their results indicate that the availability of PAHs in contaminated soil rather than their total concentration controls toxicity.

6. Comparison of bioluminescence inhibition test with other acute toxicity tests

Kaiser (1998) illustrated a very good correlation for EC$_{50}$ based on Vibrio fischeri with LC$_{50}$ based on other aquatic species, i.e., fathead minnow, bluegill, catfish, goldfish, golder, guppy, killifish, rainbow trout, sheepshead minnow, and zebrafish; the water flea Daphnia species; the ciliate Tetrahymena pyriformis; and algae. For mammals, i.e., rat and mouse, the EC$_{50}$ was dependent strongly on the route of exposure, i.e., oral, intravenous and intraperitoneal, the values of LD$_{50}$ being least for the intravenous route. The Vibrio fischeri EC$_{50}$ values yielded the best correlation with intravenous LD$_{50}$ data.

Abbondanzi et al. (2003) conducted the microtox test (Vibrio fischeri) and a dehydrogenase (DHase) inhibition assay (Pseudomonas fluorescens) on metal ions and organic chemicals. Both the organism yielded good sensitivity with metal ions, however, Pseudomonas fluorescens showed poor sensitivity to organic chemicals. Padrova et al. (1998) conducted toxicity experiments with various species of algae, crustaceans, rotifers, bacteria and protozoan to determine the sensitivity of different bioassays for acute toxicity measurement. Algae and the bacterial bioluminescence assays showed the highest sensitivity for most of the samples tested. They reported several limitations for the algal test, i.e., high species maintenance cost and long exposure time compared to luminescence inhibition assay, which is comparatively rapid and economical. DeZwart and Sloof (1983) also conducted similar studies with Microtox assay and different standard aquatic toxicity assays on 15 organic and inorganic chemicals to investigate the sensitivity of Vibrio fischeri with other standard tests. Microtox assay produced comparable results with other standard tests.

Dalzell (2002) conducted five rapid direct toxicity assessments to compare the relative sensitivity. Nitrification inhibition, respirometry, adenosine triphosphate luminescence, enzyme inhibition and Vibrio fischeri bioluminescence inhibition test were conducted on toxicants and industrial waste effluents. Comparisons based on sensitivity, cost of implementation, cost per test, relevance, and ease of use were made. The most sensitive bioassay was found to be Vibrio fischeri bioluminescence inhibition for all kinds of toxicants/effluents tested. It was not insensitive even for a single toxicant and demonstrated the highest sensitivity for 56% of the toxicants. A cost comparison across the five tests showed
that the ATP luminescence assay required the greatest expenditure both in initial investment and operating cost per test. The nitrification inhibition assay required the least investment, however, the operating cost per test was greatest. The *Vibrio fischeri* test involved moderate operating cost per test, and moderate investment cost while it required the least time for implementation.

7. Conclusion

Toxicity testing has grown steadily in recent years and is a useful tool in environmental risk assessment. Most of the chemical tests available for determining the concentration of toxic chemicals do not give the biological impacts of toxicants, i.e., the impacts of toxic chemicals on living beings. Different bioassays have been developed but these tests are often expensive, require large sample volume and are time consuming. Due to all these disadvantageslimitations recent studies deal with the use of rapid, reproducible and cost effective bacterial assays for screening and assessment. Among bacterial bioassays, *Vibrio fischeri* luminescence inhibition test is the most common. Based on the literature *Vibrio fischeri* inhibition test is the most sensitive test, cost effective, easy to operate and requires only 5–30 min for toxicity prediction. Although mostly applied for aqueous phase samples and organic extracts, the test can also be conducted on soil and sediment samples directly so as to reflect the true toxicity due to the bioavailability fraction. The test procedure has been modified to eliminate interferences due to turbidity and colour. The test results show good correlation with other tests and can be used for almost all kind of toxicants. Based on this literature survey we can conclude that out of the various available bioassays, *Vibrio fischeri* based luminescence inhibition test is more sensitive, rapid, cost effective, reproducible and without ethical problems ensuing from the use of higher organisms such as fish and rat.

Acknowledgement

We gratefully acknowledge the CSIR-JRF/NET (award no. 9/87(332)/2003-EMR-1) fellowship awarded by Council for Scientific and Industrial Research (CSIR), New Delhi, which provided funds for student support.

References


Guerra R. Ecotoxicological and chemical evaluation of phenolic compounds in industrial effluents. Chemosphere 2001;44:1737–47.


