NEW DEVELOPMENTS IN THE BIOLUMINESCENCE ASSAY

BROERS, C.A.M[1] AND LAPPALAINEN J.[2]

[1] Skalar BV, Tinstraat 12, NL-4823 AA, Breda, The Netherlands. Email: broers.c@skalar.com

[2] AboaTox Oy, Lemminkäsenkatu 36 FIN-20520 Turku, Finland. Email: juha.lappalainen@aboatox.com

1 INTRODUCTION

The industrial development, the use of pesticides in agriculture and the urbanization threaten the natural water resources. Legislation was introduced for monitoring and preventing the release of toxic substances to protect water quality. The traditional approach to toxicant monitoring in water involves standard analytic procedures. In general these techniques are selective and very sensitive. One is able to detect very low concentrations of a single chemical. However, these benefits have their drawback. Water may contain thousands of chemicals. Because of the selectivity only a limited number of compounds can be evaluated adequately. A broad based chemical analysis is expensive or sometimes impossible. Furthermore, most of the methods are laborious and time-consuming and cannot assess toxicity.

An alternative to the specific chemical methods is the bioassay. In bioassays, whole organisms are used for testing the quality of aqueous samples. Living organisms are sensitive to a broad spectrum of bio-available substances. The conventional aquatic bioassays use fish and waterfleas. However, these tests are unpractical for routine screening, because culturing and testing is costly and laborious as well as time and space consuming. This has led to the development of the microbiotests. Because microorganisms are used in these tests, many of the drawbacks mentioned above are overcome. They are cheap, available as kits and use small test volumes. The demand was a test that was technical simply, using organisms with sufficient sensitivity to a broad spectrum of toxic compounds, an easy detection, reproducible, rapid results and minimal sample preparation. The bioluminescence assay fulfills these needs and the *Vibrio fischeri* bioluminescence assay is standardized and widely used now. Despite the benefits of this system, there is a growing demand for bacteria that are more sensitive than the previous mentioned bacteria especially to metals and pesticides (chlorinated organic compounds). In this presentation we will discuss the traditional *Vibrio fischeri* assay and introduce some novel approaches in toxicity testing using metal biosensors and a luminescent bacterial strain which is much more sensitive to metals and organic toxicants.

2 MICROBIOTESTS FOR DETECTION OF TOXICANTS IN WATER

The *Vibrio fischeri* assay is the most widely used. The test organism is *Vibrio fischeri*, a luminescent marine bacterium. The luminescence is part of their natural metabolism (Nealson and Hastings, 1979). It is an aerobic oxidation process as a sideway of their respiration pathway. The enzyme luciferase is involved in the generation of bacterial luminescence. The reaction catalyzed by this enzyme involves the oxidation of a long-chain aldehyde and a reduced coenzyme flavin mononucleotide (Hastings and Nealson 1977, Meighen, 1991):

$$FMNH_2+O_2+R\text{-}COOH \xrightarrow{\text{Luciferase}} FMN+H_2O+R\text{-}COOH+Light$$

The intensity of the light output depends on several external factors including temperature, pH, salinity, nature and concentration of the toxicant (Kaiser and Ribo 1987, Reichelt and Baumann, 1974). Toxic compounds interact with cellular structures and functions: DNA, membranes, enzymes and energy fluxes, which are fundamental to all living organisms. In *Vibrio fisheri* these interactions result directly or indirectly in the inhibition of the light production. Because the metabolic activity in the bacteria is much faster than in eukaryotic cells, the exposure time to the toxic compound is very short. Within 30 minutes, but often after 5 minutes, a notably inhibitory effect of the toxicant on the light emission is measured (Bulich, 1982).

The procedure for toxicity testing is a very simple procedure. A dilution series of the sample is prepared. Each dilution is added to a test tube with a suspension of bacteria. The bacteria are incubated for 5, 15 or 30 minutes at 15°C and the light is measured in a luminometer, containing a photomultiplyer tube. The light production of each dilution is compared to a blank. The results are plot out in an inhibition curve and the dilution factor causing 50% inhibition is calculated. This value is the EC_{50}. The method using the bacterial strain *Vibrio fisheri NRRL B-111 77* is standardized (ISO 11348-3).

Commercial kits from several companies are available now. These contain in general freeze-dried bacteria. The bacteria

are treated as a reagent. They can be stored for one year in a freezer. The advantage is that they can be used at moment when it is needed. The freeze-dried bacteria are hydrated and can be used immediately.

The freeze-dried bacteria need to be transported on dry ice. This is quite expensive and sometimes impossible by the recent restrictions in air transport. Exposure to ambient temperatures is disastrous for the quality of the bacteria. An alternative is the use of freshly prepared bacteria. This method is also standardized (ISO 11348-1). This is especially beneficial when many samples have to be analyzed. The sensitivity of the bacteria is influenced by the way the bacteria are cultivated and by the method of preparation for analysis. In table 1 the freeze-dried bacteria are compared to the freshly prepared bacteria.

Table 1 : *Comparison of EC50 values*

Chemical	ISO 11348-3 Freeze-dried bacteria	ISO-11348-1 Freshly prepared bacteria	ToxTracer methods Freshly prepared bacteria
$ZnSO_4 \cdot 7H_2O$	2.2 mg/l	25 mg/l	8 mg/l
3,5-Dichlorophenol	3.4 mg/l	6 mg/l	4 mg/l
$K_2Cr_2O_7$	18.7 mg/l	4 mg/l	5 mg/l

In the last column the results of our method of preparation of fresh bacteria are shown. This method combines the good results of the ISO standards of freeze-dried and freshly prepared bacteria (table 1).

Recently some new applications using the bioluminescence assay have been developed. A method for the determination of BOD in water using *V. fischeri* is described now. Furthermore, a strain variant of the same bacterium is used for assimilable organic compounds (AOC)

3 NEW LUMINESCENT BACTERIA STRAINS

Despite the ease and sensitivity of the *V. fischeri* test, there are some serious disadvantages. First, the freeze-dried bacterial reagent preparation needs freezing during shipment what is expensive. Secondly, it was found that for some organic compounds and metals, the bacteria are not sensitive enough. Furthermore, for work in the field the test is not suitable because of the restriction in an exposure temperature of 15°C. A thermostat is required.

Alternative organisms were tested, such as *Vibrio harveyi* (Thomulka et al, 1993). Despite the positive evaluation of this organism, the bioassay method using this organism was not commercialized (Thomulka et al, 1993). Girotti et al described an assay using the bacterium *Vibrio logei*. The bacterium showed sensitivity characteristics similar to the ISO procedure. However, stringent temperature conditions are not required. There is no need for a thermostat, making the test simpler and cheaper (Girotti et al, 2002).

The sensitivity of V. harvey and V. logei for heavy metals is good (Table 2) but they seem to be less sensitive to organic toxicants as compared to the *V. fischeri* test.

Most promising is a novel test utilizing a highly sensitive variant of the luminescent bacterium *Photobacterium leiognathi* (Ulitzur et al, 2002). It allows the detection of sub-mg/L levels of a diverse group of toxicants, including heavy metals, pesticides, PCB, PAH's and fuel traces in water (Table 2 and 3). For most of the toxic agents studied so far, the new assay is much more sensitive than the toxicity data reported for *V. fischeri.* The test can be performed at ambient temperatures, between 18°C and 27°C. This enables to perform the test on-site. At this moment the test is not conform to national or international toxicity testing standards. Moreover, the lyophilized bacteria do not need to be transported on dry ice.

Table.2 : *EC50 (ppm) values after 30 minutes exposure to heavy metals.*

Heavy metal	V. fischeri	V. harvey[2]	V. logei[3]	P. leiognathi[4*]
Cadmium	5.4[5]	ND	0.5	0.06
Cobalt	3	0.046	ND	0.05
Copper	8[6]	0.62	0.3	0.02
Lead	0.31[5]	>100	0.2	0.1
Mercury	0.065[6]	0.03	0.05	0.07
Silver	ND	0.046	ND	0.04
Zinc	2.5[6]	0.22	3.0	0.6

Table. 3 : *EC50 (mg/L) values for pesticides*

Chemical	V. fischeri[1]	P leiognathi[4]	V logei[3]
Dieldrin	15	0.77	250
DDT	7	0.12	250
Chlorpyrofos	46.2	1	300
Carbaryl	2	ND	40

EC50 values (Kaiser and Palabrica)[1], (Thomulka et al., 1993)[2], (Goretti et al 2002)[3], (Ulitzur et al 2002)[4], (Tchounwou P.B. and Reed L. 1999)[5], (Bulich and Isenberg, 1981)[6],

4 OTHER APPLICATIONS OF THE BIOLUMINESCENCE ASSAY

Except for the toxicity testing of samples, the bioluminescence assay has found some other applications. Recently a new method for the determination of BOD in water using *V. fischeri* was developed. Furthermore, a strain variant of the same bacterium is used for the detection of assimilable organic compounds (AOC), which is a measure for water quality. As compared to the standard procedures relying on the direct measurement of microbial growth, in the new method the incubation time is drastically reduced. The light production of the inoculated bacteria in the sample is measured after 2 hours. The amount of light produced is proportional to the amount of AOC present.

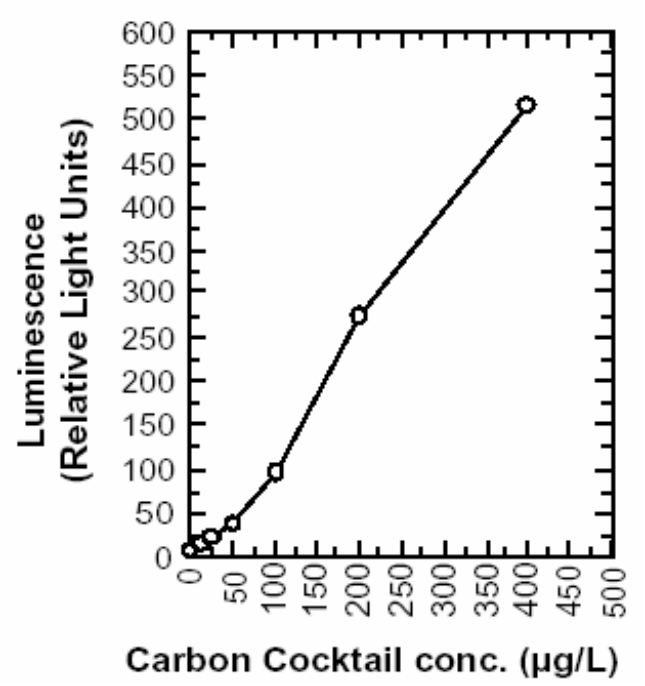

Fig. 1 : *The Vibrio fischeri bacteria start to produce light in the presence of AOC*

5 METALSENSORS

The bioavailability of heavy metals can be measured with specific biosensors. These biosensors are genetically modified *Escherischia Coli* bacteria. They produce light if the cell takes up a specific metal. The biosensors carry a plasmid with a firefly luciferase gene (Lucff). The promotor region has a metal responsive element that recognizes the metal and controls the expression of the luciferase reporter gene (Virta et al, 1994, Ivask et al, 2001). In the presence of a specific metal the gene is induced and the production of luciferase mRNA and corresponding protein begins. After addition of the substrate D-luciferin the cells starts to emit light which is measured by the ToxTracer luminometer

$$\text{ATP + D-luciferin + } O_2 \xrightarrow{\text{Luciferase}} \text{AMP + Oxyluciferin + PPi + } CO_2 \text{ + Light (560 nm)}$$

Several strains of genetically engineered *E. coli* bacteria are available now: for inorganic mercury, organomercurials, arsenics, cadmium, chromate and lead (Tauriaien et al, 1998, Ivask et al, 2001). Sub-toxic levels are measured and detection limits of 0.05 ppb for organomercurals and 4 ppb for Arsenic (V) are reported (see fig 2 and 3).

In contrast to the marine luminescent bacteria test there is no requirement for adjustment of the osmotic value of the sample. The method is extremely sensitive as compared to the traditional toxicity test (Lappalainen et al, 2000).

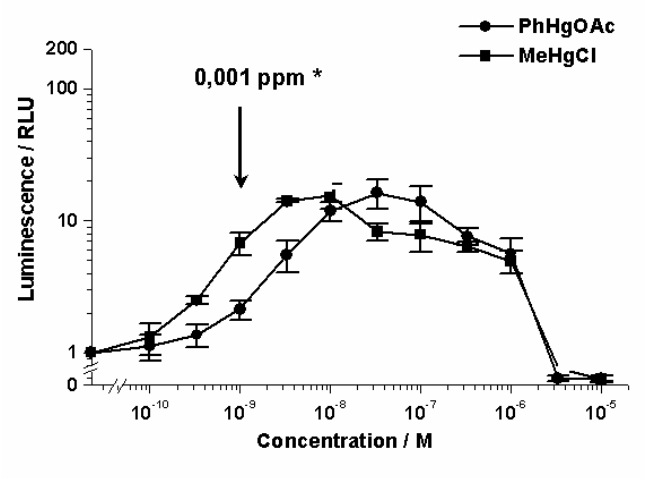

Fig. 2 : *Response curve of the mercury sensor.*

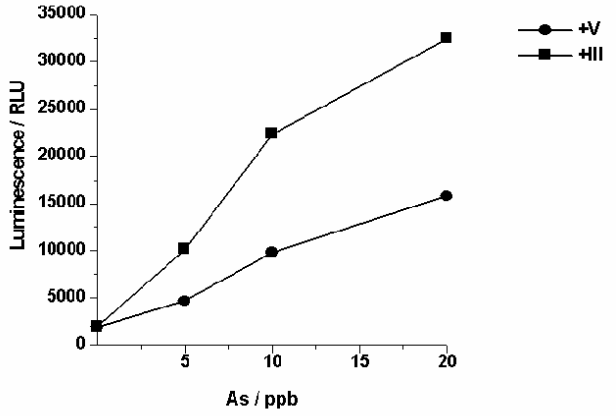

Fig. 3 : *Response curves of the Arsenic-III and Arsenic-V sensors*

6 CONCLUSION

The traditional *V. fischeri* bioluminescence assay has been proofed to be a stable test. A long list with EC50 values for pure chemical compounds is available. Results are highly reproducible and the method is standardized. The transport and storage of the lyophilized reagent at temperatures below zero and the restriction to perform the test at 15°C are serious drawbacks. A good alternative for the freeze-dried bacteria is the use of freshly prepared *V. fischeri* bacteria. Recent research has shown that other strains of light emitting bacteria are more sensitive to toxic compounds. Improvement of the freeze-drying technique has made dry ice transport unnecessary. Progress is made in the utilization of the light-producing bacteria in other application fields such as AOC testing.

Recombinant DNA-techniques have resulted in the construction of whole-cell bacterial sensors. This allows the detection of single heavy metals at ppb levels by measuring bioluminescence.

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