HUNGARIAN JOURNAL
OF INDUSTRIAL CHEMISTRY
VESZPRÉM
Vol. 30. pp. 271 - 274 (2002)

ASSESSMENT OF DEGRADABILITY IN WHOLE EFFLUENT TOXICITY TESTING USING BIOLUMINESCENT BACTERIA

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Received: October 10, 2002

In whole effluent toxicity (WET) testing the aggregate toxicity of an effluent is measured, using different test organisms. Although these predicted deleterious effects will occur in natural (real-world) ecosystems, the tests are performed in the laboratory, under strictly controlled conditions. However, there are many reasons why these test results cannot be directly applied for real-world ecosystems. One basic problem is that exposure changes with space and time. Several physical, chemical and biological processes can result in significant decreases in exposure concentrations of a test substance over time.

For many industrial effluents biodegradation is perhaps the most important process which affects the environmental concentration of the test substance. Biodegradation is a specific process as not only appropriate abiotic conditions are necessary but also a competent bacterial population, both anaerob and aerob should be established.

Our main goal was to assess how toxicity of a selected industrial effluent changes over time, caused by degradation. In order to measure the aggregate toxicity of the effluent the ToxAlert ®100 luminometer was used, developed by Merck. This test is in compliance with ISO/EN/DIN 11348. The use of the bioluminescent bacterium *Vibrio fischeri* (or other bacteria) has several advantages comparing to conventional toxicity testing. The test is rapid and causes no ethical problems.

Our results has shown that toxicity changes caused by degradation can be appropriately followed by bioluminescent bacteria.

Keywords: whole effluent toxicity, biodegradation, bioluminescence, Microtox

Introduction

There is an increasing awareness of the need to protect aquatic habitats, as many chemicals may ultimately find their way into recipients. The role of scientists is to provide environmental decision makers with applicable information on how to assess the impact of man's activities. In order to create the framework for assessing the toxic effects of such chemicals on aquatic organisms, national and international authorities have developed several test guidelines and protocols and are continuing to do so. These aim to produce quantitative data using standardised procedures.

By definition, whole effluent toxicity (WET) test methods measure "the aggregate acute and chronic toxicity of an effluent using standardised freshwater, marine, and estuarine plants, invertebrates and vertebrates" [1]. In WET testing our null hypothesis was that there is no difference between the control and the test treatment —in other words, the effluent is not toxic. On the contrary, if there is a statistically significant difference between the control treatment and any other test treatment the effluent is determined to be toxic and our null hypothesis is rejected.

Basically the main goal of such testing is to determine what risk the effluent might pose to the environment. Although these predicted deleterious effects will occur in natural (real-world) ecosystems, the tests are performed in the laboratory, under strictly controlled conditions. However, there are many reasons why these test results cannot be directly applied for real-world ecosystems.

First of all, quite many test organisms have been selected because they can be maintained in the

laboratory sufficiently, so they are seldom good representatives of natural ecosystems. In order to use test results for other species not tested in laboratory, specific taxonomic extrapolations are needed, for which some models have been elaborated [2]. Different responses can occur within the same species as well. Kiss et al. [3] investigated how different natural populations of *Lemna minor* behave when tested for potassium-dichromate. Significant differences were observed in growth rate expressed as doubling time and growth inhibition as well.

Another important criteria are life stage and size. Under standard test conditions these parameters cannot vary, but do so in natural populations. Young organisms for example have incompletely developed organs and metabolic capacities and therefore they are more sensitive to toxic pollutants [4].

For our study, the main factor is exposure. In standard ecotoxicological tests the exposure duration is fixed, in acute tests it mostly takes 24, 48 or 72 hours. As real-world ecosystems have to suffer from exposure duration other than these, interpolation or extrapolation is needed. However, temporal extrapolation models are intended to deal with constant exposure, but exposure concentrations are irregular and significantly change with time.

Several physical, chemical and biological processes can result in significant declines in exposure concentrations of a test substance over time [5]. The substance in question can be volatile, in this case the main difficulty during the test is to maintain exposure concentration. Photo-degradable, hydrolytically unstable, oxidizable and biodegradable substances in addition may form such breakdown products which can be even more toxic than the parent substance was. In many cases where data are absent or insufficient to identify the process responsible for the decline in exposure concentration preliminary tests are suggested to assess the stability of the test substance. The result of the preliminary stability study (which is carried out in the absence of test organisms) will then be used for selecting the exposure regime. The exposure regime can be static (the test medium is not replaced for the duration of the experiment), semi-static (the test medium is periodically replaced on a batch basis), intermittent flow-through (the test medium is replaced over set periods during the exposure) and continuous flow-through (the test medium is continually replaced).

For many industrial effluents biodegradation is perhaps the most important process which affects the environmental concentration of the test substance. Biodegradation is a specific process as not only appropriate abiotic conditions are necessary but also a competent bacterial population should be established. The ability of cultures of common bacteria such as *Pseudomonas* spp, *Flavobacterium* spp. or *Aerobacter* spp. to degrade a variety of test chemicals has been extensively examined [6]. However, it was soon demonstrated that mixed cultures of natural origin (lakes, rivers, sewage, etc.) were even more capable to degrade the test compounds.

However, present OECD tests [7] for ready biodegradability are not designed to predict in detail the fate and behaviour of a toxicant in a specific compartment of the aquatic environment. This is partly due to the fact that OECD tests are carried out at much higher concentrations than one can expect to occur in real-world ecosystems. Moreover, several factors appear to influence the biodegradation of xenobiotic pollutants.

Therefore although some methods have been described to evaluate the biodegradability of chemicals in natural freshwaters [8], no relevant OECD Test Guidelines have been proposed so far. Instead, simulation tests exist such as the stream model of Shimp et al. [9] or the die-away test of Anderson et al. [10]

In WET testing basically two types of question can be answered: 1. What is the absolute toxicity of the effluent? 2. What is the actual toxicity of the effluent in the receiving system? In the former case, standard synthetic or acceptable natural dilution water is used that matches the organism culture water. In the second case, the local receiving water is suggested for dilution water. The use of receiving water increases the environmental relevance of the test simulating actual effluent/receiving water system. However, it might occur that receiving water is also toxic, which provides inadequate conditions for WET testing.

Our basic aim was to test how degradation processes, especially biodegradation affect the toxicity of an industrial effluent, and to predict what changes in toxicity can be expected in natural freshwaters. Test series was designed in a way that it is in concordance with USEPA WET methods but simultaneously direct toxicity is measured.

Materials and methods

Bioluminescence inhibition of Vibrio fischeri

Bioluminescence is a rapid indicator of the metabolic status and of the viability of the cell. The enzyme involved in the process is bacterial luciferase. A toxic substance will cause changes in some cellular structures or functions such as the electron transport system, cytoplasmic constituents or the cell membrane, which are directly reflected in a decrease in bioluminescence.

The use of the bioluminescent bacterium *Vibrio fischeri* (or other bacteria) has several advantages comparing to conventional toxicity testing. The test is rapid and causes no ethical problems. For our tests the ToxAlert ®100 luminometer was used, developed by Merck. This test is in compliance with ISO/EN/DIN 11348. The test is highly recommended for industrial waste waters and effluents [11].

Test design

A textile industrial waste water was used as test substance. The WET method manuals [12, 13] suggest a

dilution series of 6.25 %, 12.5 %, 25 %, 50 % and 100 % effluent but preliminary toxicity tests showed that the substance was highly toxic in high concentration therefore a 25 % initial sample concentration was used throughout the tests. Selection of the proper dilution water was of crucial importance. In order to simulate the behaviour of the test substance in natural ecosystems the water used for diluting the test substance was collected in the Kis-Balaton Water Protection System. In fact this water served as inoculum. This inoculum contained a mixed (aerob-anaerob) culture, due to the natural characteristics of the Water Protection System.

The diluted sample (50 % waste water + 50 % pure water) was kept in an incubator in darkness (thus no photodegradation could take place), at 24 °C.

Microtox tests were carried out at the following intervals:

Day 0	sample collection	Day 41	assay 9
Day 1	assay 1	Day 73	assay 10
Day 4	assay 2	Day 76	assay 11
Day 6	assay 3	Day 78	assay 12
Day 8	assay 4	Day 80	assay 13
Day 15	assay 5	Day 87	assay 14
Day 28	assay 6	Day 100	assay 15
Day 32	assay 7	Day 104	assay 16
Day 36	assay 8		

Analysis

Sample preparation

In order to provide optimal conditions for the test pH of the samples was adjusted to pH 7.0.

Preparation of liquid dried bacteria reagent and test suspension

The reconstitution solution and the liquid dried bacteria are kept at –18 °C. First the reconstitution solution was thawed and shaken well to ensure sufficient dissolved oxygen. The ToxAlert ®100 luminometer has a separate block for the liquid dried reagent vials ensuring the required temperature of 15 °C. 12.5 ml of reconstitution solution was added into the Microquant vial and kept in the liquid dried reagent block for at least 15 minutes.

One vial of liquid dried bacteria was removed from the freezer (it is also kept at –18 °C) and placed in the liquid dried bacteria well for 2 minutes. Than 0.5 ml Reconstitution Solution was pipetted into the Liquid Dried Bacteria vial and mixed. After 15 minutes the bacteria suspension was transferred back to the Microquant vial was used as test suspension.

Conducting measurements

Firstly, a pre-incubation time of 15 minutes was set. During this time 500 µl of test suspension was pipetted into all cuvettes, including control cuvettes as well. Shortly before contact time begins (before timer reaches zero) cuvette A1 was placed into the turret. At contact time t = 0 RLU (relative luminescence unit) of the solution was measured. After measurement 500 µl of Sodium Chloride solution was added to cuvette A1 and gently mixed by hand. As the intercuvette time was set at 30 seconds, the same procedure followed for cuvette B1 (the other control cuvette) at t = 30 sec. At t = 60 and 90 sec. a slightly different procedure was followed for sample cuvettes. In their case after measuring RLU 500 µl of the diluted sample was added and gently mixed by hand.

Exposure time ended at t = 30 mins. Than RLU of all cuvettes was measured again, in the same sequence, also keeping an intercuvette time of 30 secs.

Calculation of results

The ToxAlert ®100 luminometer calculates all values automatically. Firstly the f_{kt} correction factor is calculated from the measured luminescence (*Eq.(1)*).

$$f_{kt} = l_{kt} / l_0 \ (t = 30 \text{ min in our test}) \qquad (1)$$

where

- f_{kt} the correction factor for the contact time
- l_{kt} luminescence intensity in the control sample measured in RLU (relative luminescence units), after the contact time
- l_0 luminescence intensity of the control test suspension.

Using the correction factor, than the corrected value of for every test sample cuvettes are calculated (*Eq.(2)*).

$$l_{ct} = l_0 \times f_{kt} \qquad (2)$$

where

- f_{kt} mean of f_{kt} of the two control samples
- l_0 luminescence intensity of the control test suspension
- l_{ct} corrected value of for test sample cuvettes immediately before the addition of the test sample

Then the inhibitory effect H_t of the test sample was calculated (*Eq.(3)*).

$$H_t = [(l_{ct} - l_{Tt} / l_{ct})] \times 100 \qquad (3)$$

where

- H_t the inhibitory effect of the test sample after the contact time, in%
- l_{ct} corrected value for test sample cuvettes immediately before the addition of the test sample
- l_{Tt} luminescence intensity of the test sample after the contact time, in RLU.

Table 1 Results of toxicity tests made using ToxAlert. Day 0 represents the day of sample collection

Days	1	4	6	8	15	28	32	36	41	73	76	78	80	87	100	104
Inhib Aver.	34,7	44,05	45,5	55,6	42,6	46,6	46,3	80,9	74,7	63,4	81,95	73,2	71,45	68,75	68,5	66,95

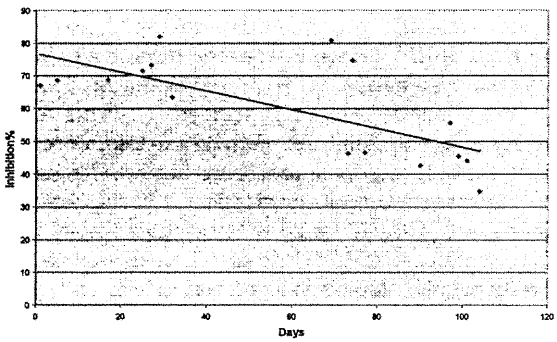

Fig.1 Degradation expressed as changes in ecotoxicity

Results and conclusions

Table 1 and *Fig.1* summarize the results of the toxicity tests. As the trend shows, there was a significant decrease in the toxicity of the samples between Day 1 and Day 104, as the average bioluminescence inhibition shows.

The results have proved our expectations, as far as the whole effluent toxicity changes can be precisely followed by Microtox test. It was possible to assess how toxicity of the selected industrial effluent changes over time caused by biodegradation.

In our case the classical WET testing was combined with a chronic test for degradability. Degradation can be resulted by various physical, chemical and biological processes. In the present examination the causes are unknown, the decline in exposure concentration can be of any origin including both inorganic and biological processes. However, they are not specifically important in this case, as the basic goal of the test was to determine what risk the effluent might pose to the receiving water.

The question arises, what components of the effluents may be responsible for the toxicity. All these constituents are known as environmental toxicants. In this respect there is an interesting result: in spite of the complex composition of the effluent tested the decrease in toxicity - evidently being in strong correlation with the degradation - can be approximated by a simple regression line. The existence of such a simple mathematical function suggests that the main and limiting component of the degradation might be determined by not more than one xenobiotic substance. This material must have a high initial toxicity and a probably slow inherent biodegradation indicated by the relatively high inhibition measured on the 101st day.

In the future the test system will be characterised by using various effluents and receiving waters. It should also be examined what microorganisms are responsible for the biodegradation.

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