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# Use of Mfu-Galactoside Enzymatic Activity as Ecotoxicological Endpoint on Rainbow Trout Red Blood Cells

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Standard single-species toxicity tests are the most common tool for the toxicological assessment of chemical substances. These tests are also used for related purposes such as whole effluent toxicity assessment. In sewage samples, there are many difficulties to detect which toxic compound/s is/are the suspected responsible/s of the toxicity (Calow 1994); the potential solution, Toxicity Identification Evaluations (TIE), requires testing large number of samples. When this is the case, conventional tests have some disadvantages; they are time consuming, require large sample volume and are expensive. In human toxicology, *in vitro* tests are frequently proposed as an alternative to detect toxicity without using experimental animals. Similarly, *in vitro* methods with fish cells have been developed in aquatic toxicology (Tarazona et al., 1993) and used in TIE protocols (Vega et al. 1994). Most *in vitro* tests use proliferation endpoints on primary cultures or established cell lines. Special handling techniques are required. Fish red blood cells are a good model to detect effects of many chemicals; these cells are directly exposed to pollutants absorbed by different routes (gills, digestive tract, etc), and can be easily obtained.

Mortality is recognized as the classical endpoint for acute toxic effects. The use of more sophisticated endpoints as biochemical, physiological, or behavioural effects is growing (Walker et al. 1996). The inhibition of enzymatic activities has been used as a way to assess ecotoxicological problems. The fluorogenic substrate 4-methylumbelliferyl- β-D-galactoside (MFU-galactoside) is frequently used as enzymatic substrate (Janssen et al. 1993; Davies et al. 1994; Apte and Batley 1994. Peterson and Stauber 1996). This substrate is hydrolysed by an enzyme, producing a fluorescence product (4-methylumbelliferone); the presence of toxic compounds affects this enzymatic activity, and inhibition is used as a sublethal stress indicator. This activity has been found in the majority of the organisms (Davies et al. 1994. Peterson and Stauber 1996) and this allows its use to detect toxic effects. Enzymatic inhibitions can be monitoring at different exposure times, allowing concentration-time-response assessment. A new method developed by Kooijman and Bedaux will be use to estimate the No Effect Concentration (NEC) based on changes in the parameters of the Dynamic Energy Budget (DEB).

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## MATERIALS AND METHODS

Cortland saline (Wolf 1963) was used as maintenance cell medium. Concentrations of the chemicals were prepared using methanol (Merk, Germany) as solvent. Fluorogenic substrate MFU-galactoside was added to the medium at 75 ppm final concentration, Then, erythrocytes were incorporated at the Cortland solution by adding rainbow trout (*Oncorhynchs mykiss*) whole blood (dilution factor of 1/500). At predetermined times, supernatants were removed and the fluorescence was measured in a spectrofluorometer ($\lambda$ excitation = 365 mn; $\lambda$ emission = 450 nm). Handling was made under sterile conditions.

The method calibration included fluorescence measurements of unexposed supernatants at different times (1, 2, 3, 22, 23, 24, 46, 47, 48 hr). The method development also incorporated additional checking points to prove the absence of interferences when quantifying the fluorogenic product. The fluorescence of red blood cells, prior and after cellular lysis was measured. Similarly, possible interferences with the tested substances were investigated in presence and absence MFU-galactoside. Considering the short exposure times, and aiming a cost reduction, an attempt of maintenance without PVP and bovine albumin was also made.

In a first assay, cells were exposed to six different concentrations of 4-chloro-phenol (Aldrich, Germany); solvent and medium controls were included. Experiments were run in 96-well plastic plates (one for each measured time). Plates were maintained in darkness, at 20°C on an orbit shaker (Lab-Line, USA) to provide constant agitation. In a second assay, red blood cells were exposed to six different concentration of a pesticide (fenarimol (DowElanco Europe, UK); solvent and medium controls were also considered. In this case, cells were maintained in flasks with short periods of shaking. At predetermined times (1, 2, 3 and 4 hr of exposure), aliquots were removed from the flasks, and fluorescence was measured.

Data analysis included the estimation of $EC_{50}$ and NEC values using the log-probit method and the DEBtox model respectively.

## RESULTS AND DISCUSSION

In the calibration study, measuring supernatants from unexposed cells, initial linear responses followed by a progressive decrease in the production rate were observed. Plateau values were reached after a few hours (Figure 1). These test conditions permitted the design of a very rapid and sensitive assay, reducing the exposure time to a few hours with good cost/effect ratio.

Preliminary assays showed a lack of interference with the fluorometric determination of the 4-methyl-umbelliferone. No fluorescence was observed in the different assays with undisturbed or lysed red blood cells, with the tested chemicals

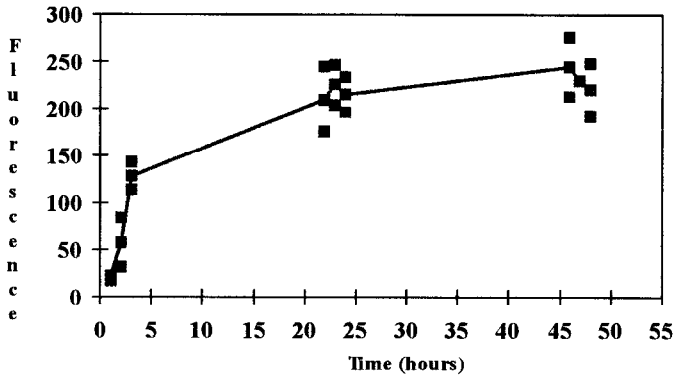


**Figure 1.** Fluorescence in control supernatants.

and so on. On the other hand, the attempt to rule out different components of Cortland saline solution, particularly PVP and bovine albumin, was fruitless, Despite the selected short exposure periods, a poor reproducibility was found in the absence of these compounds. It was concluded that under these experimental conditions, PVP and bovine albumin are essential to maintain the physiological status of red blood cells.

The exposure to 4-chlorophenol provoked an increase in the enzymatic activity. The increase was dose-related, reaching a plateau at the highest tested concentration, 170 ppm. The fluorescence enhancement was linear with time. To prove no interactions between 4-chlorophenol, Cortland medium and the fluorogenic substrate, exposures of different concentrations of 4-chlorophenol in a Cortland medium with and without MFU-galactoside were done. Results showed that the fluorescence increase could not be related to the presence of this phenolic compound. Measured fluorescence ranged between 16±15 and 36±18 units in absence of the substrate, and from 132±11 to 141±12 units in presence of the substrate. Neither significant differences for the 4-chlorophenol nor concentration-dependent responses were observed. Fluorescence was between 5 and 10 times higher when MFU-galactoside was present. This was related to the substrate fluorescence background.

Therefore, the increase in fluorescence observed due to 4-chlorophenol exposure cannot be considered an artefact, and must be explained by the induction of the enzymatic activity. It is well known that β-galactosidase can be induced by β-galactosides and other compounds. Other enzymatic activities are also induced by the exposure to certain pollutants. For toxic chemicals, enzymatic induction is only a first step in the concentration/response curve, obviously when the concentrations are high enough to affect the vital structures of the cell/organisms, the enzymatic activity is not longer supported and inhibition is observed. Thus, the induction of

an activity used as toxicity endpoint must be considered a hormesis phenomena. Hormesis has been observed for several pollutants and endpoints with and without biochemical-physiological explanations. In our experiment, the induction was observed even for the highest tested concentration, therefore the DEBtox model was not used for data analysis. An alternative dose-time-effect representation is showed in Figure 2.

The response of red blood cells exposed to fenarimol was the expected; a concentration-related inhibition of the enzymatic activity. The $LC_{50}$ values of fenarimol for rainbow trout red blood cells were estimated by log-probit analysis at different exposure times. Calculated values were 262.7 ppm, 237.1 ppm and 168.9 ppm for 1, 2 and 3 hrs respectively. The $LC_{50}$s showed a linear distribution with time (correlation coefficient of -0.968), therefore no asymptotic $CL_{50}$ value could be estimated. The DEBtox model was used to calculate the NoEC concentration of fenarimol. The population growth, model hazard DEBtox alternative was applied to the fenarimol results. NEC concentration calculated by this system was 31.04 ppm. Dose-time-effect representation is showed on Figure 3. The DEBtox model has the main advantage of considering all the available information on concentration and time related response to estimate a no-effect concentration (Kooijman and Bedaux 1996). The model requires the estimation of responses at different exposure times, requisite that can be easily fulfilled using enzymatic activities as endpoint.

This work presents, for fish red blood cells, the use of β-galactosidase, an unspecific activity that has been used as screening toxicity method in several organisms (Janssen et al. 1993; Apte and Batley 1994; Davies et al. 1994; Peterson and Stauber 1996). The activity was proposed for the detection of total and fecal coliforms in drinking water (Berg and Fiksdal 1988). Later it was incorporated on different bioassays on the aquatic invertebrate *Daphnia magna* (Barber and Cordell 1992; Janssen et al. 1993; Weltens et al. 1995; Hayes et al. 1996); algae (Peterson and Stauber 1996); or bacterial populations (Bitton et al. 1992; Davies et al. 1994). In our laboratory the system has been incorporated to *in vitro* assays, using the fish cell line RTG-2 (Pablos et al., in preparation) and red blood cells. The use of a fluorogenic substrate allows rapid, easy and sensitive measurements (Mariscal et al. 1995).

In microorganisms, β-galactosidase activity was observed to be more sensitive to metals than other enzyme groups (Obst et al. 1988). The inhibition of this activity in bacterial populations was the basis for the development of MetPAD™, an specific bioassay for metals (Bitton et al. 1992). However, the results obtained for 4-chlorophenol and fenarimol confirm that, as observed for other taxonomic groups, the assay using trout cells is sensitive to organic chemicals, and therefore can be proposed as a general cost/effective tool for toxicological screenings.

Cost/effective bioassays are continuously developed as screening alternatives to the

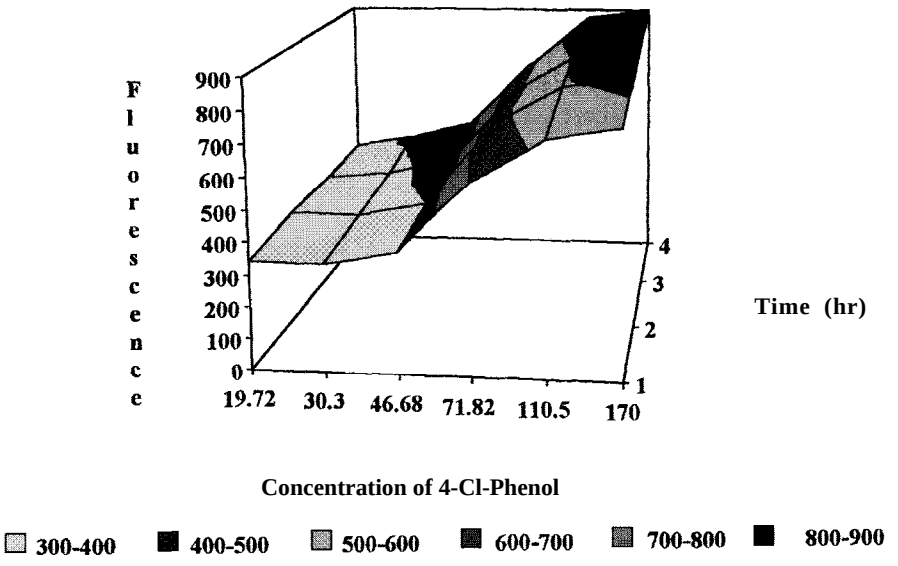


**Figure 2.** Dose-time-effect representation of red blood cells exposed to 4-cl-phenol

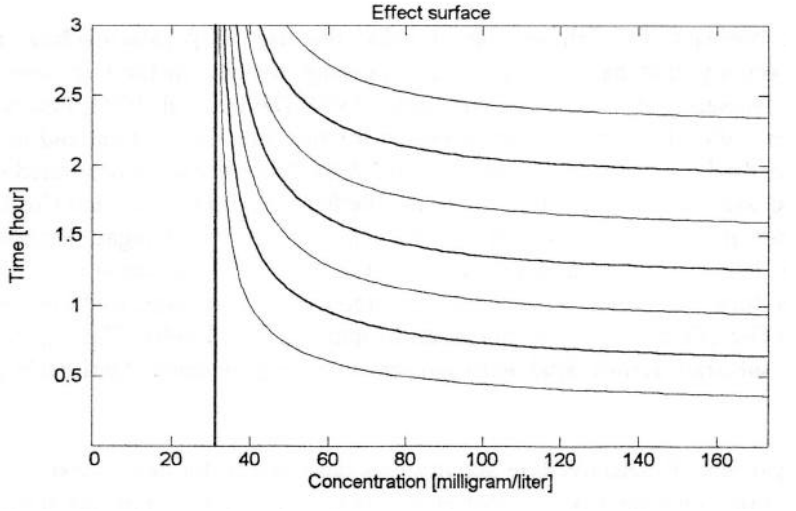


**Figure 3.** Dose-time-effect representation of red blood cells exposed to fenarimol (from population growth, model hazard. DEBtox model).

standardised toxicity tests. Most of these new methods are rapid, inexpensive and require little space as compared to fish bioassays (Bitton, et al. 1992). Small size organisms, from micro-organisms to several invertebrate taxon are normally used. In vitro methods offers the possibility of develop similar approaches using fish cells (i.e Tarazona et al., 1993). Both primary and established cell cultures can be used. Under certain circumstances, good correlations between in vitro and in vivo methods are obtained (Castaño et al. 1996). Fish erythrocytes have bee used to assess membrane alterations provoked by xenobiotics. The inhibition of antioxidant enzymatic activities is used for this assessment. It was proved that metals (Gwozdzinski et al. 1992; Roche and Bogé 1993) and phenolics compounds could affect this activity (Roche and Bogé 1993; Bogé and Roche 1996). In this paper a rapid bioassay on rainbow trout cells has been presented. The assays can produce concentration and time related responses rapidly and at a very low cost. The endpoint has been previously recognised as good possibility to reduce the bioassay duration in screening toxicity tests (Janssen et al. 1993).

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