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# New Algal Enzyme Bioassay for the Rapid Assessment of Aquatic Toxicity

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Bioassays with single-celled algae have been used extensively over the past 10 years to determine the toxicity of complex effluents, leachates, natural waters and specific chemicals. The standard bioassay measures the inhibition of algal growth rate over 72 hr exposure to a toxicant. Although such growth inhibition tests are sensitive and use an ecologically relevant endpoint, cost-effective assessment of toxicity is limited. There has been an increasing demand for tests that are rapid and inexpensive. Bioassays that detect sub-acute endpoints such as inhibition of enzyme activity (Gilbert *et al.* 1992; Jung *et al.* 1995; Mariscal *et al.* 1995) show promise in meeting these requirements.

The enzyme β-D-galactosidase has recently been used in a rapid toxicity test with the invertebrate *Daphnia magna* (Janssen and Persoone 1993). Juvenile *D. magna* were exposed to a series of toxicant dilutions for one hour before the addition of a fluorogenic substrate, 4-methylumbelliferyl β-D-galactoside. Fifteen minutes later, the number of fluorescent test animals was compared to controls.

The galactosidase enzyme has also been found in other organisms such as sediment bacteria and algae (Casal *et al.* 1985; Stemmer *et al.* 1990; Davies *et al.* 1994). This paper describes the development of a rapid toxicity test based on galactosidase activity in the green alga *Dunaliella tertiolecta.* This 3-hr bioassay is compared to standard 72-hr algal growth inhibition tests.

## MATERIALS AND METHODS

The fluorogenic substrate was prepared by dissolving 0.0625 g of 4-methylumbelliferyl β-D-galactoside (MU-gal) in 10 mL of dimethylformamide with sonication and then diluting with 10 mL of Milli-Q water. To ensure a low background fluorescence, the reagent was cleaned according to the method of Apte and Batley (1994). The purified reagent was diluted to 250 mL with Milli-Q water and filter sterilized before use in the assay.

A base solution, required at the end of the assay to enhance the fluorescent signal, was prepared by dissolving 2.8 g sodium carbonate, 1.2 g sodium bicarbonate and 10 g sodium citrate in 100 mL of Milli-Q water. All reagents were AR grade.

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*Dunaliella tertiolecta* Butcher was chosen as the test species as it had a suitably high galactosidase activity (Davies *et al.* 1994) and was available in axenic culture (CSIRO Culture Collection Strain CS-175, Hobart, Tasmania, Australia). *Dunaliella* was cultured in a modified half-strength f medium (Guillard and Ryther 1962). The culture was maintained on a 12:12 hr 1ight:dark cycle (Philips TL 40W fluorescent white, 4000 lux) at 21°C.

Exponentially growing cells of *Dunaliella tertiolecta* were washed three times in seawater to remove culture medium and counted using a Coulter Multisizer II with 70 µm aperture. For the enzyme assay, algal inoculum was added to each test flask containing the toxicant and 50 mL of filter-sterilized seawater, to produce a final cell density of 2 x $10^5$cells/mL. The test flasks were incubated in the light at 21°C for 2 or 24 hr, and then 5 mL aliquots of algal solution were asceptically transferred to sterile glass culture tubes (16x100 mm) containing 4 mL of MU-gal solution and 1 mL of 0.5 M PIPES buffer. After mixing, the tubes were incubated in a water bath. Incubation temperatures of 21, 30 and 44.5°C were used and the incubation period was varied from 30 to 120 min. At the end of the incubation, the tubes were cooled under running water for 5 min and 400 µL of base solution was added to convert all the liberated MU to its anionic (and most fluorescent) form.

*Dunaliella* was exposed to five different metals ($Hg^+$, $Cd^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Cr^{6+}$), which were prepared as aqueous solutions in dilute acid, and five different organic compounds (sodium dodecyl sulfate (SDS), phenol, 4-chlorophenol, diuron, atrazine). All organic compounds were dissolved in Milli-Q water, except diuron and atrazine which were dissolved in AR grade methanol. For each toxicity assay, five replicate tubes of five different concentrations of toxicant were assayed along with five replicates of the control (no toxicant). Methanol controls were also run during the diuron and atrazine experiments. As the substrate underwent a limited amount of chemical hydrolysis during the incubation period, it was necessary to run blank solutions of MU-gal with no algae, to account for this contribution to the fluorescent signal. In addition, a blank containing algae plus toxicant (no MU-gal) was included to correct for any background algal fluorescence.

The fluorescence was measured using a Perkin-Elmer LS-5 Luminescence Spectrometer with the following settings: excitation wavelength 375 nm (slit width 10 nm); emission wavelength 465 nm (slit width 20 nm). The instrument response was calibrated daily using standard 4-methylumbelliferone (MU) solutions (20 nM, 50 nM and 80 nM). All results were reported as concentrations of MU.

Toxicity was determined by the reduction in fluorescence in the presence of toxicant compared to the controls. EC50 values (the effective concentration of toxicants to reduce enzyme activity by 50%) were calculated using trimmed Spearman Karber analysis, probit analysis or Microtox® (Microbics Corp.) software.

Growth inhibition bioassays with *Dunaliella tertiolecta* were carried out according to the method of Stauber *et al.* (1994), using the same washed inoculum as the enzyme bioassays. Triplicate seawater controls (50 mL) together

with at least 5 concentrations of toxicant (in triplicate) were prepared, To each flask, 0.5 mL of 26 mM sodium nitrate and 0.5 mL of 1.3 mM potassium dihydrogen phosphate were added. Each flask was inoculated with 2-4 x $10^4$ cells/ml of the prewashed *Dunaliella* suspension and incubated at 21°C on a 12:12 hr 1ight:dark cycle at 14000 lux.

Cell density in each flask was determined daily for three days using a Coulter counter. A regression line was fitted to a plot of log,, cell density versus time (hr) for each flask and the cell division rate determined from the slope. EC50 values were determined using the same statistics protocols as the enzyme bioassays. The bioassay was acceptable if the cell division rate in the controls was 1.3±0.3 doublings per day.

## RESULTS AND DISCUSSION

The effect of incubation temperature on fluorescence response of the controls is shown in Figure 1. The fluorescence response of the assay increased only slightly from 21°C (the usual *Dunaliella* culture temperature) to 30°C and then increased by nearly two orders of magnitude at 44.5°C. Even at 44.5°C, microscopic examination showed that the *Dunaliella* cells were intact and healthy. A separate experiment in which cells were killed with 4% formalin prior to incubation with MU-gal confirmed that the enzyme was only present in live algae.

The fluorescent signal was also found to increase with increasing incubation time from 30 to 120 min (Fig. 2), with a maximum fluorescence soon after 120 min. Background fluorescence from chemical hydrolysis of the substrate also increased with increasing incubation time, with a rapid increase after 90 min. Coefficients of variation also increased with length of incubation. Consequently, a one hour incubation time was chosen as a compromise between maximum fluorescence response and minimum background fluorescence.

The assay was performed with a number of different cell densities to ensure that fluorescence response increased linearly with the number of algal cells. Figure 3 shows that the fluorescence response was proportional to the number of cells over 2 orders of magnitude ($10^4$-$10^6$ cells/ml).

Once the optimal bioassay conditions were established, the toxicity of 5 metals and 5 organic compounds to *Dunaliella* was tested (Table 1). The alga was much more sensitive to metals than to organics. Similar insensitivity to organic compounds has been observed in other enzyme tests, including bacteria galactosidase assays (Dutton *et al.* 1988; Barnhart *et al.* 1983; Jung *et al.* 1995). Longer exposure times increased the sensitivity of the alga to organics, whereas metals were less toxic after 24 hr exposure than after 2 hr. It is possible that the metals may affect only enzyme that has already been expressed, but do not inhibit further biosynthesis of enzymes (which may be more resistant to the metals). The organics, on the other hand, may restrict enzyme expression so that the activity appears to decrease with time relative to the controls. This may explain the observations of Dutton *et al.* (1988) who found that galactosidase activity in bacteria was not affected by organic toxicants, whereas galactosidase biosynthesis was.

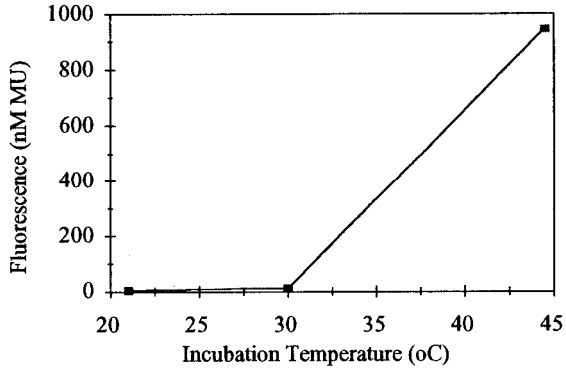


**Figure 1.** Effect of incubation temperature on utilization of substrate by *Dunaliella tertiolecta.* Incubation time: 60 min.

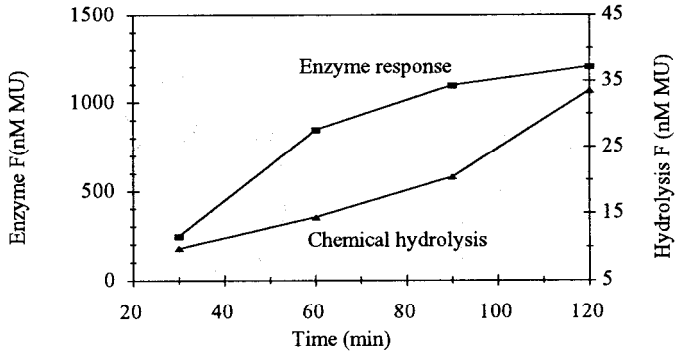


**Figure 2.** Effect of incubation time on fluorescent signal by *Dunaliella tertiolecta* and on chemical hydrolysis of substrate. (Note: Different scales on Y-axes) Incubation temperature: 44.5°C.

**Table 1.** Inhibition of β-D-galactosidase in *Dunaliella tertiolecta* after exposure to metals and organic compounds for 2 hr (Concentrations in mg/L; Coefficients of variation from 3-5 replicate experiments in parentheses).

| Toxicant | 2-hr EC50 (CV) | Toxicant | 2-hr EC50 |
|---|---|---|---|
| Copper | 0.035 (10%) | Sodium Dodecyl Sulfate | 50 |
| Mercury | 0.081 (10%) | 4-Chlorophenol | >230 |
| Zinc | 0.076 (22%) | Phenol | >470 |
| Cadmium | 0.351 (3%) | Diuron | >200 |
| Chromium (VI) | 7.0 (5%) | Atrazine | >100 |

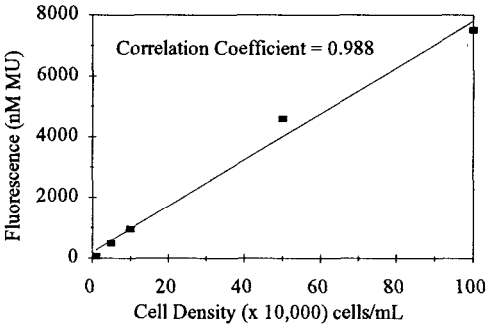


**Figure 3.** Effect of cell numbers of *Dunaliella* on fluorescent signal. Incubation temperature: 44.5°C; incubation time: 60 min.

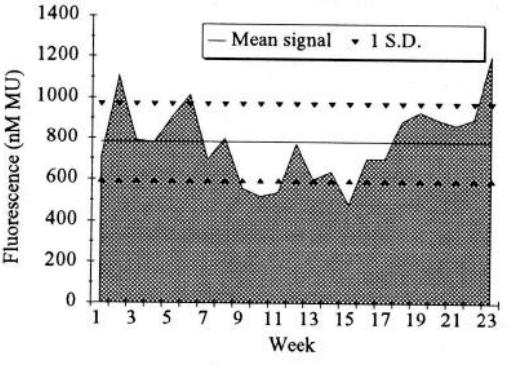


**Figure 4.** Variation of fluorescence produced by control samples from weekly cultures of *Dunaliella.* Mean signal ± one standard deviation is shown.

Weekly variations in the fluorescence response of the controls is shown in Figure 4. This variability was due to different inoculum sizes in the algal culturing procedure prior to the enzyme assay, rather than variation in the enzyme assay itself. Evidently, toxicity is a relative response compared to healthy controls rather than an absolute response to enzyme activity. Despite this, the new bioassay was highly reproducible with excellent replication within experiments (mean coefficient of variation: 4%), as well as between experiments (coefficients of variation: 3-22%).

Table 2 compares the sensitivity of the new *Dunaliella* galactosidase test with standard 72-hr growth tests with *Dunaliella* and the marine diatom *Nitzschia closterium.* The new enzyme assay was much more sensitive than 72-hr growth tests with *Dunaliella* for all compounds tested, except SDS (Table 2). Because *Dunaliella* growth is not a particularly sensitive endpoint (Abalde *et al.* 1995), the *Dunaliella* enzyme test was also compared to 72-hr growth tests using the sensitive diatom *Nitzschia closterium. Nitzschia* was more sensitive than the enzyme assay for some toxicants, while similar toxicity to zinc and cadmium was

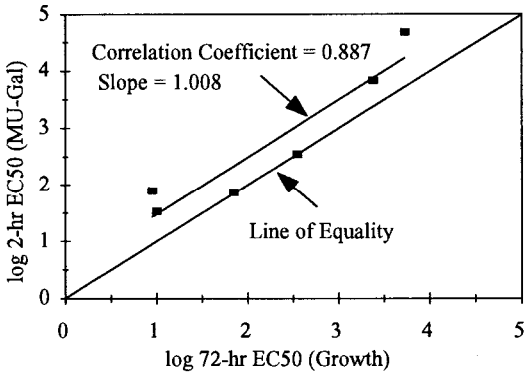


**Figure 5.** Correlation between 2-hr EC50 from β-D-galactosidase activity in *Dunaliella tertiolecta* compared to 72-hr EC50 from growth of *Nitzschia*

observed for both tests (Table 2). Figure 5 shows the correlation between 2-hr EC50 values for the new enzyme assay, compared to 72-hr EC50 values for *Nitzschia* with six toxicants. Good correlation for the 6 toxicants was obtained ($r^2$= 0.887; slope = 1.008).

The *Daphnia* test (Janssen and Persoone 1993) was more sensitive than *Dunaliella* to mercury, cadmium and chromium, whereas *Dunaliella* was more sensitive to copper, zinc and SDS (Table 2). However, the coefficients of variation for the *Daphnia* test were higher (15-32%), probably due to the qualitative determination of fluorescence in the water fleas.

**Table 2.** Comparison of EC50 values obtained from different tests (mg/L) for six different toxicants.

| Toxicant | *Dunaliella* Galactosidase test | *Dunaliella* Growth test | *Nitzschia* Growth test[a] | *Daphnia* Galactosidase test[b] |
|---|---|---|---|---|
| Mercury | 0.081 | 0.300 | 0.009 | 0.015 |
| Copper | 0.035 | 0.576 | 0.010 | 0.059 |
| Zinc | 0.076 | >6 | 0.070 | 0.978 |
| Cadmium | 0.351 | >6 | 0.350 | 0.210 |
| Chromium (VI) | 7.0 | 17.8 | 2.4 | 0.250 |
| SDS | 50 | 15 | 5.3 | 74 |

[a] From Stauber *et al.* 1994; [b] From Janssen and Persoone 1993

The new algal enzyme bioassay was both sensitive and reproducible and correlated well with other environmentally relevant parameters such as growth. The major advantage of this test is that toxicity information can be obtained

within 3 hr rather than 72 hr in a standard growth test, making the algal enzyme test a cost-effective way to monitor the toxicity of a range of chemicals.

Further work is underway to reduce the test time to 1 hr and to assess its suitability for monitoring complex effluents. In addition, an enzyme test using freshwater algae is currently being developed for monitoring pollutants in freshwater aquatic systems.

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