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Automated β Galactosidase Activity Bioassay for Adult *Daphnia magna* versus Classic Immobilization Test

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Wallenfels and Weil (1972) reported that the β-galactosidase enzyme was present in numerous species of plants and in the gut of most animals. The activity of this enzyme is inhibited by many toxicants and can, therefore, be used as an ecotoxicological endpoint. For example, ß -galactosidase inhibition is the basis for many bacterial assays (Kilroy and Gray, 1995). In the need for rapid and cost-effective bioassays, the β-galactosidase activity test is now also described for rapid assessment of toxicity in a variety of organisms (Janssen and Persoone 1993). The test is much faster than tests with classic endpoints (1-4 versus 48-72 hr of exposure time, respectively) while the dose-response relations are often comparable. The test is considered to be a valuable alternative for classic ecotoxicity tests as f.i. *Daphnia* immobilisation or algal growth inhibition (Janssen and Persoone 1993; Peterson and Stauber 1996). Many toxicants are known to interfere with enzyme activities (Van Straalen 1991) or sytheses (Kilroy and Gray 1995). In *Daphnids* food uptake is also known as a sensitive ecotoxicological endpoint (Allen et al. 1995). The enzyme activity, measured in *Daphnids*, reflects both the effect of the toxicant on food uptake (indirect effect) and direct enzyme interference after the toxicant has entered the gut.

Originally the protocol was described for neonate *Daphnids (*age < 24 hr) (ASTM 1993). We planned short term toxicity tests on *adult Daphnids* for evaluating toxicity of complex environmental samples. The method was adapted: (a) adult organisms were used, (b) fluorescence was measured automatically in a cytofluormeter, instead of counting the number of fluorescent organisms (aut-ß-GAL) and (c) different exposure conditions were tested. Tests were performed on pure chemicals and on environmental samples, to evaluate the suitability of the adapted method.

MATERIALS AND METHODS

The test organisms used were *Daphnia magna,* cultured in the lab since 1994. For the acute toxicity test neonate (maximum 24 hr old) and/or adult (7-14 d) organisms were used. For the β-galactosidase test adult organisms were used which were between 7 and 14 d old.

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Daphnia medium was prepared according to OECD guideline 202. Table 1 summarises the composition of the medium. MilliRO water with a conductivity ≤ 20 µS/cm was used as diluting medium
pH = 7.9 +/– 0.3; Hardness = 250 +/– 25 mg/L $CaCO_3$; Ca/Mg ~ 4; Na/K ~ 10

Table 1. Composition of *Daphnia* medium (based upon OECD guideline 202).

Substance	**Final concentration**
$CaCl_2.2H_2O$	294.0 mg/L
$MgSO_4.7H_2O$	123.3 mg/L
KCl	5.8 mg/L
$NaHCO_3$	64.8 mg/L
K_2-EDTA.$2H_2O$	1086 µg/L
$FeCl_3.6H_2O$	1500 µg/L
$MnSO_4.7H_2O$	31 µg/L
$Na_2MoO_4.2H_2O$	12.6 µg/L
$ZnSO_4.7H_2O$	4.4 µg/L
SeO_2	1.4 µg/L
Vitamine B_{12}	1.0 µg/L

Fluorometrically tagged substrate (MUG) was prepared using 10.5 mg 4-methyl umbelliferyl ß-D- galactopyranoside in 5 mL of distilled water. The suspension is mixed with a mechanic mixer during a few min. The solution is freshly prepared each week.
All products used were pro analyse. Stocks of 10 mM were prepared of the following salts: $CdCl_2$, $HgCl_2$, $ZnCl_2$, $AlCl_3.6H_20$, $MgCl_2.6H_2O$, $CuCl_2.2H_2O$. Stocks of 200 g/L were prepared of the following products: SDS (Sodium Dodecyl sulphate) and Selenium dioxide.
Stocks were diluted in *Daphnia* medium to obtain the LC_{50} and EC_{50} concentration range as derived from preliminary range finding tests (see below). Maximum test concentration in the preliminary tests was 1 g/L. Carbaryl, lindane and PCP are only slightly soluble in water: 45, 7 and 14 mg/L respectively. Stocks were prepared in DMSO that were 10000 times more concentrated than the maximum water solubility (450 g/L carbaryl, 70 g/L and 140 g/L PCP). The maximum test concentrations were equal to the maximum water soluble concentrations and a solvent concentration of 0.1 mL/L.
The *Daphnia* immobilisation test was based on OECD guideline 202 (1984). 5 *Daphnids* (neonates – max.24 hr of age or adult) are exposed to 20 mL of fluid media in a concentration series of the test substance (4 replicates per concentration, standard room conditions, 16/8 light/dark). In (solvent)control conditions, at least 90% of the animals should survive within 48 hr. Depending upon the toxic effects of the test substance, mortality will increase with increasing concentration. Mortality is evaluated after 24 and 48 hr of

exposure. The effect is calculated as % immobile organisms relative to the initial number of animals (100%).
For the ß-galactosidase activity measurements, 20 organisms per concentration are exposed to different concentrations of the test substance during 2-4 hr. The organisms are then allowed to feed on a MUG suspension during 15 min (100 µL suspension/mL). Organisms with active ß-galactosidase and normal feeding behavior will fluoresce because of the enzymatic cleavage of the substrate. Two protocols were compared: In the 96 well method, the whole procedure is carried out directly in the wells of a 96 multiwell plate (corning). Each well contains 200 µL test solution (4 replicates) and 5 organisms. After 2 hr 20 µL of the MUG suspension is added to the well and fluorescence is measured after another 15 min. In the vial method, 10-20 organisms are exposed to 1 - 2 mL test solution during 2-4 hr. The organisms are then removed from the vial, rinsed with distilled water and transferred to a fresh vial containing 1 mL of distilled water. 100 µL of the MUG suspension is added and the animals are allowed to feed on the suspension during 15 min. Again, the animals are rinsed with distilled water and then transferred to the wells of a 96 multiwell plate (5 organisms/well) with 200 µL of either distilled water (alive organisms) or a 1:1 mixture of aceton and water (dead organisms). Fluorescence of the separate wells is automatically measured by cytofluometry ($\lambda_{Excitation}$ = 360 nm, $\lambda_{Emission}$ = 460 nm).

RESULTS AND DISCUSSION

This study was set up to evaluate the practical use of the adapted protocol for the ß-galactosidase inhibition test (aut-ß-GAL), to evaluate toxic effects of environmental samples for *adult Daphnids.* First some technical aspects were investigated.
Espiritu (1994) stated that automatisation could simplify the measurement if used in routine. We did not systematically compare visual and automated measurements, but in our experience the automated method is very rapid and more easy than the visual measurement. When visually counting, problems arise because of the limited amount of organisms that can be observed at one time, and because of gradual fading. An automated system permits a more objective and more gradual measurement.
A critical number of animals/well is necessary to measure the fluorescent yield. In a preliminary test, 1 to 10 animals/well were used. For stable measurement of fluorescence automatically in individual wells, 5 animals/well are needed. An exposure time of 2-4 hr for the adults yielded results that were comparable to the lethality data for the adult *Daphnids* for most toxicants tested (see below).
Clearly matrix effects interfered. A time dependent and matrix dependent fluorescent background signal was measured when the multiwell method was used. To overcome these matrix effects the *vial method* was tested, separating exposure - feeding - and measurement in three different vials.

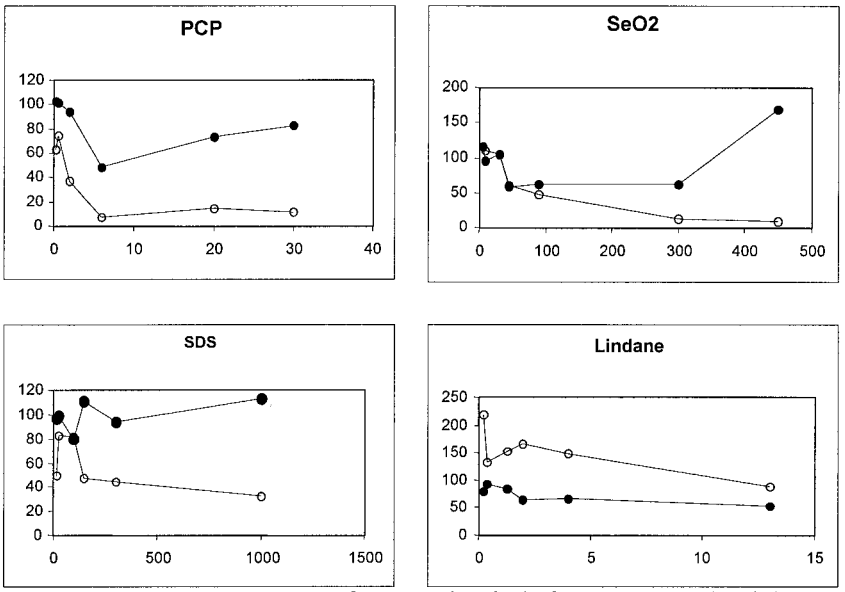

Figure 1. Dose response curves for pure chemicals for two protocols of the aut-β-GAL assay in adult Daphnids: multiwell (filled bullets) and vial metaod (open bullets). Y-axis: inhibition of enzyme activity as % of the control value, X-axis: concentration of the test substance (μM).

Results are summarised in Fig.1. The vial method improved the dose-response correlation. In the multiwell method, high standard deviations (up to 100%) and inconsistent results were seen. Although better, the standard deviations were still high with the vial method. This extreme variability can be due to natural and conditional factors. Intra individual variation can arise from differences in the amount of available enzyme, in feeding rate, in the sensitivity of the feeding and/or enzymatic responses, in the development of the gut microflora which also shows ß-GAL activity and responds to toxicants (Kilroy and Gray 1995). Conditional factors of importance are matrix effects, the time difference between measurements, homogeneity of the MUG feeding suspension or the fact that the organisms are alive and moving during the measurement, inhibiting a homogenous signal. In an attempt to reduce variability, we tried to rule out as many of these conditional factors as possible. Although we standardized the protocol and tried to avoid conditonal sources of variation, the standard deviations were still rather high with the aut-ß-GAL method. Possibly the natural variation of this parameter is high.

To test the suitability of using adult *daphnids* for toxicity evaluation, we compared the sensitivity of adults and neonates for Cd, Zn and Hg salts. As table 2 shows, no major differences for the LC_{50} values were noticed for these metals between adults and neonates. Buikema et al. (1980) discussed the use of neonate *Daphnia* for toxicity tests. The main advantages of using neonate organisms are their smaller size and larger surface area, and their higher molting frequency (3-5 times during the first 48 hr). However, the use of

neonates in tests lasting more than 24 hr is also questionable because of starvation. The starvation proces interferes with the health condition of the organisms, and therefore, toxicity. The authors conclude that 2-2.5 mm *Daphnia* may be more appropriate for 48 hr toxicity tests. The adults used in our tests were approximately 2.5-3 mm of length.

Table 2. LC_{50} (48 hr acute toxicity) and EC_{50} (ß-Galactosidase inhibition test) values for adult and neonate *Daphnia magna.*

	LC 50 Acute toxicity (48hr)		**EC 50** ß-Galactosidase
	adults	**neonates**	**adults**
CdCl2	1.5 mg/L [c]	1.1 mg/L [f]	1.0 mg/L [c]
HgCl2	0.004 mg/L [c]	0.0022 mg/L [e] 0.005 mg/L [e]	0.005 mg/L [c]
ZnCl2	5.4 mg/L [c]	7.9 mg/L [f] 2.1 mg/L [a] 8.4 mg/L [b]	1.9 mg/L [c] 4.3 mg/L [a(neonates)] 0.82 mg/L [b] 0.8 mg/L [d]
Carbaryl		0.23 mg/L [b]	0.08 mg/L [b]
Lindane		2.6 mg/L [b] 27 mg/L [a] 14.5 mg/L [e]	0.8 mg/L [b] 2.8 mg/L [a(neonates)]
PCP		1.2 mg/L [b] 0.86 mg/L [e] 0.33 mg/L [a]	0.8 mg/L [b] 1 mg/L [a(neonates)]
SDS		12.4 mg/L [b] 31 mg/L [a]	264 mg/L [b]
SeO2		4.8 mg/L [b]	9.7 mg/L [b]

[a] (Janssen and Persoone 1993), [b] present study, [c] (Goossens 1998), [d] (Verhulst 1996), [e] (Wren et al. 1995), [f] Test performed at our lab according to GLP principles.

Table 2 also shows the results obtained with the classic acute 48 hr toxicity test on neonates and the alternative ß-GAL for pure chemicals. For most test substances the results for the LC_{50} in neonates and EC_{50} in adults were comparable, but important differences were noticed for some chemicals (e.g., SDS and $ZnCl_2$.).

Despite large standard deviations in the ß-GAL response (see above), the dose response curves (fig.2) and EC_{50} values (table 2) are comparable to the acute response for most toxicants, with a slightly higher sensitivity in ß-GAL in most cases. For Carbaryl, Lindane and $ZnCl_2$ the EC_{50} ß-GAL however is significantly lower than LC_{50} value, while the opposite is true for SDS. The same results were obtained by Janssen and Persoone (1993). They compared for 28 substances the *Daphnia* IQ test (1 hr), which is also based upon ß-GAL activity (visual) measurements, with the 48 hr acute LC_{50} values for neonates. They found a good relationship between both tests (Linear regression r^2 value of 0.99 for raw data, and 0.89 for log transformed data), but also noticed marked differences for some chemicals (e.g. Lindane: LC_{50} = 27 mg/L; EC_{50} =

2.7 mg/L; Paraquat: LC_{50} = 2.2 mg/L; EC_{50} = 16.5 mg/L). This confirms that the test results of the enzymatic inhibition tests should be interpreted with care. The aut-ß-GAL method is not an accurate method to measure EC_{50} values for pure chemicals, but can be used as a rapid range finding test for pure chemicals, providing a good estimate of the dose/response range for *daphnids* for most products. LC_{50} values should always be verified in a classic immobilisation test.

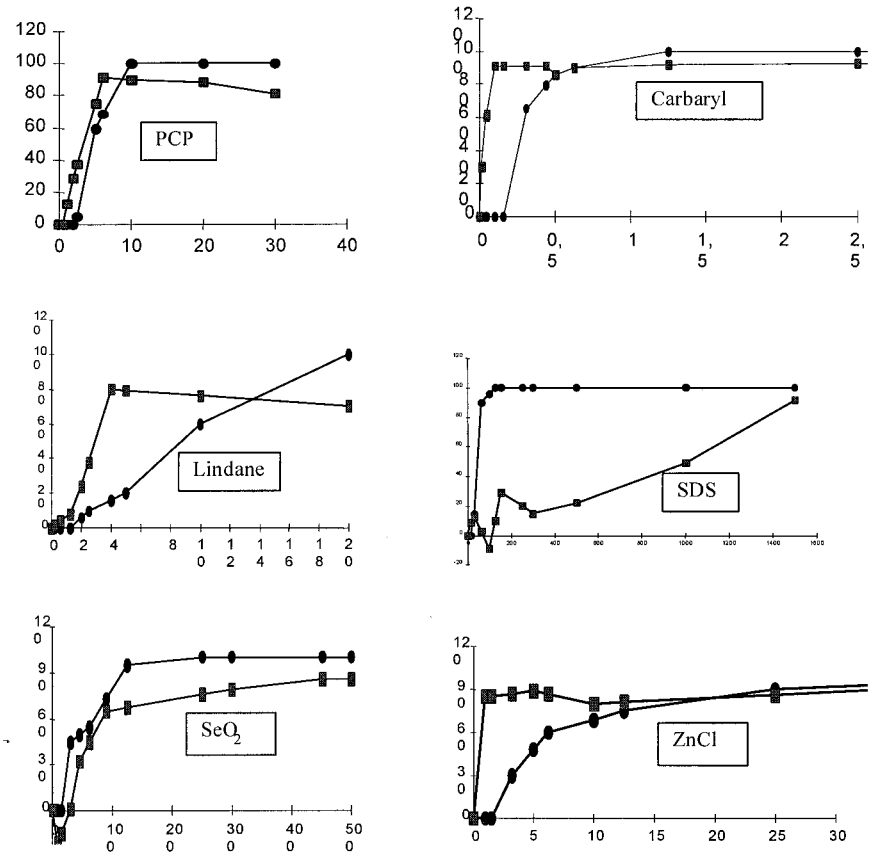

Figure 2. Dose response curves for pure chemicals for the acute 48 hr toxicity test in neonates (bullets) and b-GAL inhibition in adults (squares). Y-axis: % inhibiton, X-axis: concentration (µM).

We also evaluated the suitability of the aut-ß-GAL to measure the toxicity of complex environmental samples. Table 3 shows results obtained with elutriate fractions of natural suspended solids.

Results of the two bioassays were not at all comparable for these samples. It was previously shown in our lab that the physical presence of even uncontaminated particulate matter decreased the fluorescent signal

significantly by 40% (Verhulst 1996). In a study on complex effluents the *Daphnia* IQ test often yielded EC_{50} values, while the 48 hr exposures yielded no acute toxic response (Hayes et al. 1998). These differences and unpredictable interactions, and the impact of physical (non-toxic) agents, makes the test unsuitable for ecotoxicological evaluation of complex environmental samples. The correlation between lethality and aut-ß-GAL response is rather an emperical statement that is not yet well understood.

Table 3: Results of the acute toxicity bioassay and the aut- ß-GAL performed on elutriates of 2 g/L suspended solids collected from contaminated surface waters in Flanders (study sponsored by the Flemish Environmental Agency, reported in Weltens and Schoeters (1997)).

Sample	Acute toxicity	aut- ß-GAL assay
4	-[a]	-[a]
5	++++[b]	-[a]
6	++++[b]	-[a]
7	-[a]	-[a]
1b	-[a]	+[e]
2b	-[a]	-[a]
3b	-[a]	-[a]
4b	-[a]	-[a]
7b	-[a]	++[d]
8	++[d]	-[a]
9	-[a]	-[a]
10	-[a]	+[e]
11	-[a]	++[d]
13	+[e]	-[a]
14	-[a]	+[e]
16	+++[c]	++[d]

[b]: 100 % mortality, [c] 75-99% mortality, [d] 50-74% mortality or 50- 75% and significant inhibition of ß-GAL activity, [e] 25-49 % mortality or up to 50% and significant inhibition of ß-GAL activity, [a] < 25% mortality or no significant inhibition of ß-GAL activity.

Clearly more research is needed to fully understand all possible interactions between chemicals and enzyme, and to explore the possibilities of this bioassay or to interprete results in terms of toxicity (Kubitz et al. 1995).

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