

DETERMINATION OF THE TOXICITY OF FOUR HEAVY METAL COMPOUNDS  
AND THREE CARCINOGENS USING TWO MARINE NEMATODE SPECIES,  
MONHYSTERA MICROPHTHALMA AND DIPLOLAIMELLOIDES BRUCIEI

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**ABSTRACT**

Bioassays with two marine nematode species were used to determine the toxicity of four heavy metal compounds and three carcinogens. The test using the marine species Diplolaimelloides brucei is more sensitive than the existing nematode test using the freshwater nematode Panagrellus redivivus for mercury chloride and 2-acetamidofluorene, but less sensitive for methylmercury chloride, phenacetin, and 4-aminobiphenyl. The Monhyстера microphthalma test is less sensitive than the nematode test with P. redivivus for mercury chloride, selenium oxide, and cesium chloride.

The range between the concentration at which no toxic effects are observed and the concentration at which 100 % lethality is observed is for most compounds smaller in both nematode tests with marine species than in the test using P. redivivus. This suggests that the marine analogs of the P.

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redivivus test are less suited to detect, map, and rank the primary sites and sources of biotoxicity in an ecosystem. Further validation of the nematode tests using D. brucei and M. microphthalma is required.

### KEYWORDS

Marine ecotoxicology, Methods, Nematodes, Monhystera microphthalma, Diplolaimelloides brucei, Mercury, Selenium, Cesium, 2-Acetamidofluorene, 4-Aminobiphenyl, Phenacetin.

### INTRODUCTION

Although nematodes are the most abundant metazoans in littoral, estuarine, coastal, and oceanic sediments (Nicholas, 1975), and a very significant portion of the energy flow of marine benthic systems passes through the nematodes (Heip et al., 1982), no simple toxicity tests using marine nematodes have been reported (Samoiloff and Bogaert, 1984), and only few yardstick assays for the determination of toxicity in a marine environment are available (Samoiloff and Wells, 1984).

A quantitative cost-effective 96 h chronic toxicity test using the freshwater free-living nematode Panagrellus redivivus, has been developed and extensively applied to detect the toxicity of complex environmental samples (fish-tissue homogenates, mine tailings, crude oil, ureaformaldehyde-foam insulation), as well as chemical fractions of river sediments, and samples of indoor air. It has also been used to determine the primary sites and sources of biotoxicity in a freshwater river system by mapping out the distribution of the biotoxicity (Samoiloff, 1980 ; Samoiloff et al., 1980, 1983ab, 1984).

The nematode bioassay detects a gradient of sublethal and lethal effects over a wide range of concentrations (several orders of magnitude). This allows a precise quantification of the toxicity of a sample as well as the distinction of three distinct toxic effects : lethality, developmental inhibition, and mutagenicity. These features make the nematode bioassay with Panagrellus redivivus extremely well-suited for toxicity ranking of samples.

In the Panagrellus redivivus test, a population of 100 L2-juveniles are grown in the presence of the substance to be tested through the three remaining postembryonic juvenile stages until approximately 50 % of the control population is adult. The primary set of data from which toxicity is determined, consists of the number of surviving nematodes as well their distribution over the four postembryonic stages in both control and test populations (Samoiloff et al., 1980). The stage distribution is analyzed to provide evidence of three distinct toxicological effects :

1. Lethality, characterized by the fact that a significant proportion of the tested population dies, while the control shows no significant mortality.
2. Inhibition of physiological or developmental processes, expressed by a decrease in the proportion of growth to each stage as compared to the controls. This effect indicates that exposure to the tested materials is inhibitory or debilitating to normal physiological processes.
3. Inhibition of, or damage to genetic mechanisms, which is manifested as the specific inhibition of the moult to the adult stage, relative to the controls. Growth through the final moult requires extensive gene expression and this growth is highly sensitive to known mutagens (Samoiloff et al., 1983a).

With the objective of applying the above toxicity test for the marine environment, we have initiated the development of a marine analog of the Panagrellus bioassay using Diplolaimelloides brucei and Monhystera microphthalma, two fairly large (approximately 1 mm), abundant, and cosmopolitan Monhysterid marine nematodes with a short life cycle. Simple methods for the cultivation and handling of these two nematodes including a method for the cryogenic storage of D. brucei, and the development of the test protocol will be reported elsewhere. Here we report on the toxicity of a series of reference chemicals using the D. brucei, and M. microphthalma bioassay, in comparison to the P. redivivus bioassay.

## MATERIALS AND METHODS

### STOCK CULTURES

M. microphthalma was isolated in September 1982 from a polyhaline-marine pond in Belgium (the Sluice Dock in Ostend). Cultures were obtained

from a strain of Diplolaimelloides brucei isolated in England (Lynher Estuary), and a strain isolated from the Gulf of Mexico by Dr. Warwick. M. microphthalma and D. brucei stocks were maintained in petri dishes on a 2% agar containing 10 mg cholesterol, and 25 g artificial seasalt (Instant Ocean) per liter distilled water (CS-agar). The animals were fed 1 to 3 drops per week of Yeast-Cholesterol-Saline medium (YCS) containing 50 mg autoclaved dried baker's yeast, 10 mg cholesterol, and 25 g artificial seasalt (Instant Ocean) per liter of distilled water.

Inbred tester strains of both species were constructed by nine generations of sib-sib matings.

#### TEST PROTOCOL

Synchronized L2-juveniles were obtained by transferring gravid females to a fresh culture medium allowing them to drop eggs for 48 h at 28 °C. A needle was used to transfer the animals and no YCS was added to the new petri dish to minimize the carry over and growth of bacteria. L2-juveniles of similar size, synchronized through starvation were collected 48 h later with a micropipette after flooding the agar with YCS-solution from which the yeast was omitted. If, for an experiment, more juveniles were required than could be produced in one petri dish, juveniles from several petri dishes were pooled to eliminate differences between petri dishes.

The actual test protocol is similar to that used with P. redivivus (Samoiloff et al., 1980). Synchronized L2-juveniles are transferred ten by ten to autoanalyzer cups with 0.3 or 0.5 ml of toxicant dissolved in YCS containing 15 mg.l<sup>-1</sup> yeast. Hydrophobic toxicants are tested in YCS with up to 5 ml.l<sup>-1</sup> acetone. The sealed autoanalyzer cups are incubated until 50% of the animals in the control series have reached the adult stage which is approximately 7 days at 25 °C.

The surviving animals of each series of ten cups are counted and transferred to a small drop of liquid on a slide, heat-killed, and dried on a slidewarmer at approximately 45 °C and mounted in lactophenol cotton blue. The nematodes on the slide are photographed using a microfilm viewer equipped with a photocopier and measured using a Houston Hipad Digitizer interfaced to an Apple computer. The lengths of the nematodes of each sample are stored on floppy disks for further analysis.

The total number of L2, L3, L4, and adult animals are determined for each sample, using the length of the animals as criterion and the frequency of completion of the L2-L3 molt ( $p_1$ ), L3-L4 molt ( $p_2$ ), and L4-adult molt ( $p_3$ ) values are calculated (Samoiloff, 1980).

The development of the nematodes in the experimental sample (usually YCS to which toxicant was added with or without carrier) is compared to the development of the nematodes in the control sample (usually YCS or YCS with  $5 \text{ ml.l}^{-1}$  acetone), using the following procedure :

1. The percent survival in the control sample is compared to the percent survival in the experimental sample using a chi-square test to determine whether significant mortality occurred in the experimental sample as compared to the control sample.
2. The stage distribution of the animals grown in the experimental sample is compared to that of the animals grown in the control sample using a  $2 \times 4$  contingency analysis (chi-square test).
3. The relative ratios of  $p_1$ ,  $p_2$ , and  $p_3$  (experimental divided by control) are calculated. These  $P_1$ ,  $P_2$ , and  $P_3$  values are independent indicators for the degree of inhibition (value  $< 1$ ) or stimulation (value  $> 1$ ) of respectively the second, third, and fourth moult. (Samoiloff et al., 1980).

## TEST COMPOUNDS

The toxicity of the heavy metal compounds mercury chloride, methylmercury chloride, and the known or putative carcinogens phenacetin, 2-acetamidofluorene, and 4-aminobiphenyl was determined using D. brucei. Mercury chloride, selenium oxide, and cesium chloride were tested using M. microphthalma.

## RESULTS

### MERCURY CHLORIDE

Exposure to concentrations of  $10^{-7}$  and  $10^{-6} \text{M}$  has no effect on M. microphthalma. In the D. brucei test, mercury chloride causes significant inhibition of the L4 to adult moult at a  $10^{-7} \text{M}$  concentration and significant

mortality together with inhibition of all three moults at  $10^{-6}$ M. A  $3.2 \times 10^{-6}$ M concentration causes significant mortality and no growth in D. brucei, and 100 % mortality in M. microphthalma.

#### **METHYLMERCURY CHLORIDE**

Exposure to a concentration of  $10^{-8}$ M does not affect the development of D. brucei, while a concentration which is 3.2 times higher causes 100 % mortality.

#### **2-ACETAMIDOFLUORENE**

Concentrations of  $10^{-3}$  and  $10^{-4}$ M are 90 to 100 % lethal to D. brucei. At a concentration of  $10^{-5}$ M significant lethality and inhibition of all three moults is observed.

#### **PHENACETIN**

This compound has no effect on the development of D. brucei at a  $10^{-4}$ M concentration but is 100 % lethal at  $10^{-3}$ M.

#### **4-AMINOBIPHENYL**

All moults of D. brucei are inhibited at a concentration of  $10^{-5}$ M but no effect is observed at  $10^{-6}$ M. Exposure to  $10^{-4}$ M causes 95 % mortality in D. brucei.

#### **SELENIUM OXIDE**

The development of M. microphthalma is not affected at a concentration of  $10^{-7}$ M. The L4 to adult moult is inhibited at  $10^{-6}$ M. At concentrations of  $10^{-4}$ M mortalities of M. microphthalma of respectively 47 % and 100 % were observed.

#### **CAESIUM CHLORIDE**

This chemical does not affect the development of M. microphthalma at  $10^{-3}$ M but causes 65 % mortality and inhibition of all three moults at  $3.2 \times 10^{-3}$ M. At  $10^{-2}$ M 79 % mortality and no growth was observed and  $3.2 \times 10^{-2}$ M was 100 % lethal to this species.

## DISCUSSION

The toxicities of the chemicals tested in this study can be compared to that determined with the Panagrellus redivivus assay system (Samoiloff et al., 1980) (Table I). The assay with Diplomaimelloides brucei is more sensitive to mercury chloride and 2-acetamidofluorene, but less to methylmercury chloride, phenacetin, and 4-aminobiphenyl than the Panagrellus redivivus assay. The nematode test using P. redivivus is more sensitive to mercury chloride, selenium oxide, and cesium chloride at lower concentrations than the test with M. microphthalma.

The range between the concentration at which no toxic effects are observed and the concentration at which 100 % lethality is observed is for most compounds tested smaller in the test with the marine nematode species than in the test with P. redivivus. This can more likely be attributed to differences in sensitivity and life strategy between the species than to differences in chemical composition between the culture media used in the two different assay systems. The nematode tests using D. brucei and M. microphthalma might be less suited as a general yardstick assay than the nematode test using P. redivivus. However, no other simple rapid tests using marine nematode species have been developed so far for toxicity assessment in the marine environment.

The main goal of the nematode assay system is not to provide regulatory information on the toxicity of a sample to this specific group of organisms, but to provide rapid cost-effective yardstick information which can be used to detect, rank or map the sites and sources of biotoxicity in a given ecosystem. Since the nematode assay system Panagrellus redivivus has been extensively used to detect and rank toxic effects of samples from a variety of environments, there are good reasons to assume that this test can be applied to detect toxic compounds in samples of the marine environment. However, the Panagrellus test can not be directly applied in cases where it is required that an extract of a sample of marine origin is tested in a solution with an ionic composition which is identical to that of seawater. This is the only drawback to the use of this test as a "yardstick test" to detect and map the primary sites and sources of biotoxicity in a marine ecosystem.

Table I. Comparison of the toxicity of four heavy metal compounds and three carcinogens in the nematode test using marine species and the nematode test using *P. redivivus*. (The results of the nematode test using *Panagrellus redivivus* were obtained from Samoiloff *et al.* (1980)).

Test compound/ concentration	Test species		
	<i>D. brucei</i>	<i>M. microphthalma</i>	<i>P. redivivus</i>
<b>Mercury chloride</b>			
$10^{-7}M$	P3 reduced	no effect	no effect
$10^{-6}M$	mortality, P1, P2, P3 reduced	no effect	P3 reduced
$3.2 \times 10^{-6}M$	mortality, no growth	100 % mortality	not tested
$10^{-5}M$	100 % mortality	100 % mortality	100 % mortality
<b>Methylmercury chloride</b>			
$10^{-8}M$	no effect		P2 and P3 reduced
$3.2 \times 10^{-8}M$	100 % mortality		not tested
$10^{-7}M$	100 % mortality		P1, P2 and P3 reduced
$10^{-6}M$	100 % mortality		mortality
$10^{-5}M$	100 % mortality		100 % mortality
<b>2-acetamidofluorene</b>			
$10^{-5}M$	mortality, P1, P2 and P3 reduced		stimulatory effect
$10^{-4}M$	91 % mortality		no effect
$10^{-3}M$	100 % mortality		P2 and P3 reduced
<b>Phenacetin</b>			
$10^{-5}M$	no effect		P2 and P3 reduced
$10^{-4}M$	no effect		P2 and P3 reduced
$10^{-3}M$	100 % mortality		P2 and P3 reduced
<b>4-Aminobiphenyl</b>			
$10^{-8}M$	no effect		P2 and P3 reduced
$10^{-7}M$	no effect		P2 and P3 reduced
$10^{-6}M$	P3 reduced		P2 and P3 reduced
$10^{-5}M$	47 % mortality		P2 and P3 reduced
$10^{-4}M$	100 % mortality		significant mortality
			P1, P2 and P3, reduced
<b>Cesium chloride</b>			
$10^{-5}M$	no effect		P1 and P3 reduced
$10^{-3}M$	no effect		P3 reduced
$3.2 \times 10^{-3}M$	65 % mortality		P3 reduced
$10^{-2}M$	79 % mortality		P3 reduced
$3.2 \times 10^{-2}M$	100 % mortality		P3 reduced

Further validation is required of the D. bruciei and M. microphthalma nematode assays using known chemicals as well as a variety of complex environmental samples and extracts thereof to determine whether the specific inhibition of the L4 to adult moult can be correlated with a mutagenic effect of a toxicant as is the case in the nematode test using P. redivivus. Heip et al. (1978) reported good growth of P. redivivus in natural seawater with a salinity of 17 - 23 ‰. An alternative route towards the development of a yardstick biomonitoring assay for the marine environment would be to use or develop a strain of P. redivivus which can be grown in a culture medium with an ionic composition similar to that of seawater.

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