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Original article

Effects of heavy metals on free-living nematodes: A multifaceted approach using growth, reproduction and behavioural assays

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ABSTRACT

Toxicity tests, using single species bioassays, are a promising way to understand the biological effects of toxicants. The aims of this study are: (a) to assess sublethal effect concentrations of the heavy metals Pb, Zn and Ni for the free-living nematode *Caenorhabditis elegans* using a liquid assay focussing on growth and reproduction as endpoints; and (b) to test a behavioural assay with taxis to food in polluted vs unpolluted food spots as an endpoint. In general, reproduction and behaviour proved to be much more sensitive endpoints than growth. Different endpoints, however, did not always yield concordant results. As an example, Ni already affected growth and reproduction at lower concentrations than did Zn and Pb, but did not affect taxis to food, not even at strongly elevated Ni concentrations. Taxis towards polluted food spots was deferentially affected by different metals and metal concentrations: nematodes avoided food spots containing low concentrations (1–2 mg L^{-1}) of Zn and Pb and very high concentrations of Ni. By contrast, they preferred food spots polluted with Ni at 1 mg L^{-1} over unpolluted food. Pb at high concentration (129 mg L^{-1}) was the only metal which interfered with food finding, i.e. *C. elegans* did not exhibit a significant taxis to either polluted or unpolluted food spots. We conclude that a combination of growth, reproduction and behavioural assays provides a more complete picture and allows a better assessment of the different modes of action of pollutants and their corresponding effect concentrations.
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1. Introduction

Toxicity tests using nematodes are a promising way to identify the biological effects of toxicants in soil and aquatic environments [1,2]. Nematodes are among the most abundant, ubiquitous and diverse metazoans in soil [3,4], freshwater [5–8], and marine habitats [9,10]. They occupy important positions in benthic food webs, representing different feeding types, trophic levels and life strategies [11,12] as well as different levels of tolerance to changes in environmental conditions [3,1,13]. Some species have short generation times, varying from a few days to weeks, and can be easily cultivated [14–16]. The soil bacterivore *Caenorhabditis elegans* is the only nematode species used so far in international standardized single-species toxicity tests, such as the ones approved by the American Society for Testing and Material [17] and the one issued by the International Organization for Standardization [18]. Experimental setups with this nematode may utilize solid as well as liquid substrata [19,20].

Hitherto, the focus in standardized toxicity assays has mainly been on survival, growth and reproduction [1]. Another way of evaluating toxicant effects is through behaviour experiments [21–26]. Behavioural responses to toxicant exposure are very sensitive sublethal endpoints that help to assess the pollutant effects on exposed populations and to unravel the mechanisms that underlay these effects [22].

Metals are considered conservative pollutants, as they are not subject to microbial decomposition [27] and industrial discharges polluted with heavy metals still represent an environmental threat [27–29]. Lead is a non-essential metal with a total world production of about 43 million tonnes/year [27,28], ca. 10% of which is used

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as gasoline additives [27]. Nickel is a significant contaminant of sediments in industrialized areas, and is often heavily deposited in estuarine [27] or freshwater sediments [7]. Zinc is one of the most commonly used metals, mainly as an anti-corrosion agent. About 70% of its production is from mining activities, while 30% is from the recycling of secondary zinc [29].

The present study has two main aims: (a) to assess sublethal effect concentrations of lead, zinc and nickel for the free-living nematode *C. elegans* using a liquid assay focussing on growth and reproduction as endpoints; and (b) to compare the sensitivity of reproduction and growth with food taxis as a behavioural endpoint. To test effects on nematode taxis towards food, a bacterial (*Escherichia coli*) suspension spiked with a heavy-metal solution was used to test for any difference in the response of *C. elegans* towards contaminated vs uncontaminated food spots.

2. Materials and methods

2.1. Origin and cultivation of nematodes

C. elegans var. Bristol, strain N2 was provided by the Caenorhabditis Genetics Center (Minneapolis, MN, USA) and maintained as stocks of dauer larvae [14,30]. *E. coli* strain OP50 was used as a food source. 50 mL lysogeny-broth medium (1% tryptone/peptone; 0.5% yeast extract; 1% NaCl) in 250-mL Erlenmeyer flasks were inoculated with 30 μL of *E. coli* stock. After incubation for 17 h at 37 °C on a shaker, the bacteria were suspended in K-medium (3.1 g L^{-1} NaCl and 2.4 g L^{-1} KCl in deionized water) and the bacterial density was adjusted to 1000 FAU (Formazin Absorption Units [31]), corresponding to an approximate cell density of 2.14×10^9 cells mL^{-1}. The final density was half of this value.

To obtain a synchronous culture of *C. elegans*, dauer larvae were transferred to an agar plate with a fresh bacterial lawn (*E. coli*) and incubated for 72 h at 20 °C. Because the (minimum) generation time of *C. elegans* is approximately 72 h under non-limiting conditions at a temperature of 20 °C [32], adults together with first- (J1) and second-stage juveniles were found in the culture plate. To separate the J1 individuals, the plate was rinsed with K-medium, filtered through a cascade of filter gauze of 5 μm and 10 μm mesh size, and recovered on a petri plate [21].

2.2. Liquid assay

To assess toxicity of Pb, Zn and Ni in *C. elegans*, a bioassay based on the standard [18] was performed using 12-well plates. We have initially tested five different concentrations of Pb, Zn and Ni and a control without heavy metals, assessing growth and reproduction as sublethal endpoints. These concentrations are henceforth referred to as C1 (0.01 mg L^{-1}), C2 (0.05 mg L^{-1}), C3 (0.1 mg L^{-1}), C4 (0.5 mg L^{-1}) and C5 (1.0 mg L^{-1}). For Zn, we also tested 2 higher concentrations, 2 mg L^{-1} and 4 mg L^{-1} (referred as C6 and C7, respectively), because none of the lower concentrations had any measurable effect on the nematodes. The test consisted of the exposure of first-stage (J1) juveniles for an entire development time. Three endpoints were determined: % of gravid adults in the total population; reproduction, calculated as: $O/(I - M)$, where O is the number of juveniles/replicate, I is the number of introduced J1 juveniles, and M is the number of males (note that *C. elegans* is mostly hermaphroditic with males occurring rarely). Growth was determined as the difference of the body length of the adult nematodes after 3 days of incubation (BL) and the mean body length of the J1 at the start of the assay (BLJ1): Growth = BL − BLJ1. The latter was derived from measurements of 30 J1 individuals preserved at the start of the experiment.

Controls and metal concentrations were set up in 5 replicates each, in the wells of 12-well sterile culture plates (CELLSTAR®, Greiner bio-one). The controls consisted of 0.5 mL of K-medium and 0.5 mL of *E. coli* suspension, and the different treatments of 0.5 mL of the corresponding heavy-metal solution and 0.5 mL of the bacterial suspension. Ten J1 individuals were transferred to each replicate well using a micropipette. The multiwell plates were closed and incubated at 20 (±1) °C in the dark for 96 h (4 days). Then, ±0.1 mL of bengal rose solution was added to each well to facilitate counting of the offspring, and the plates were heated for 15–20 min at 80 °C, to kill the worms and terminate the test. This method results in outstretched, easily measurable worms.

2.3. Behaviour experiments

The behaviour experiments tested the food-finding behaviour of *C. elegans*. For this purpose, we used petri dishes bottom-covered with an agar layer in which several round holes were cut out at equal distances from the plate centre (method described in Ref. [26]). Each hole was filled up with either a bacterial suspension in K-medium or with plain K-medium (control). Pollutant was only present inside destination spots (so inside half of the bacterial spots), nematodes were inoculated at the centre of the petri dish, and the number of nematodes inside bacterial and control spots was quantified at several time intervals (see further). This assay tests whether nematodes exhibit any avoidance of contaminated food. We first performed the experiments with metal concentrations that proved lethal to *C. elegans* in 24-h liquid assays, and in a second phase also at the sublethal metal concentrations that yielded an adverse effect in our liquid assays.

Petri plates (8 cm internal diameter) were bottom-covered with 16 mL of 0.8% bacto-agar. We used bacto-agar so as to provide the nematodes with a nutrient-poor substratum, which limits the growth of any bacteria co-transferred with the nematodes. Six holes, at equal distances from the plate centre, were made in the agar using a sterile tube (1.5 cm diameter) and filled with 300 μL each of either control medium or *E. coli* suspension. Two holes received 'clean' *E. coli* suspension, two received *E. coli* mixed with a heavy-metal solution, and the remaining two received control medium. Before adding the solutions to each spot, the holes were first bottom-covered again with a very thin layer of agar (100 μL) to avoid that the aqueous solutions or suspensions spread underneath the agar. For a first test, established lethal concentrations (LC50s of 24-h test, [21]) of Pb, Zn and Ni were tested: 129 mg L^{-1} of Pb; 202 mg L^{-1} of Zn; and 2916 mg L^{-1} of Ni. Then, according to the results of the liquid assays, metal concentrations that indicated a significant sublethal effect (in the form of a pronounced inhibition of reproduction) were tested in the same way: 1 mg L^{-1} of Pb, 2 mg L^{-1} of Zn and 1 mg L^{-1} of Ni. Each treatment was run in five replicates.

Only adults and larger juveniles (J4) were used in the behaviour experiments. We hand-picked ca. 600 active nematodes into each of two staining blocks filled with sterile K-medium. We then transferred 30 specimens to each replicate in the middle of the plate. This took at most five minutes per replicate. Replicates of different metal treatments were inoculated in a random order to avoid any bias resulting from a different duration of the washing step in K-medium.

The starting point of the experiment was the moment where the last nematode was transferred to a particular plate. Observations were done at four moments in time: after 1, 4, 6 and 24 h of incubation. The number of nematodes present in each spot was quantified at each moment in time.

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