Dose-Response: An International Journal

Volume 9 | Issue 1 Article 10

3-2011

SELECTIVE TOXICITY AT LOW DOSES: EXPERIMENTS WITH THREE PLANT SPECIES AND TOXICANTS

Aki Sinkkonen
University of Helsinki, Finland

Mervi Myyrä
University of Helsinki, Finland

Olli-Pekka Penttinen
University of Helsinki, Finland

Anna-Lea Rantalainen
University of Helsinki, Finland

Dose-Response, 9:130–143, 2011
Formerly Nonlinearity in Biology, Toxicology, and Medicine

ISSN: 1559-3258
DOI: 10.2203/dose-response.09-045.Sinkkonen

International Dose-Response Society
www.Dose-Response.org

SELECTIVE TOXICITY AT LOW DOSES: EXPERIMENTS WITH THREE PLANT SPECIES AND TOXICANTS

Aki Sinkkonen, Mervi Myyrä, Olli-Pekka Penttinen, Anna-Lea Rantalainen
□ Department of Environmental Sciences, University of Helsinki, Finland

□ During the last decade, the paradigm that low toxicant doses often have stimulatory effects on plants has become widely accepted. At the same time, low toxicant doses of metal salts have been observed to inhibit the growth of the most vigorous seedlings of a population *in vitro*, although mean plant size has remained unaffected. We hypothesized that this kind of selective low-dose toxicity is not restricted to inorganic contaminants. We exposed annual plants (baby's breath *Gypsophila elegans*, purslane *Portulaca oleracea*, and duckweed *Lemna minor*) to 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran (HHCB) and 4-*tert*-octylphenol and lead acetate. As compared to unexposed *G. elegans* roots, 4-*tert*-octylphenol did not affect the mean root size of all seedlings, but it reduced the average length of roots longer than the 98th percentile. A comparable response was found in case of *G. elegans* roots treated with lead acetate beyond the 90th percentile. The average size of roots beyond the 90th percentile was decreased also when *L. minor* was exposed to lead acetate though the means of all roots were constant. *P. oleracea* seemed to be insensitive to selective toxicity. We conclude that selective toxicity at low doses should be considered in parallel with hormesis.

Keywords: HHCB, lead acetate, NOEC, octylphenol, seedling growth, selective toxicity

INTRODUCTION

Numerous xenobiotics are toxicants at high concentrations, but many of them have stimulatory effects on plants at low concentrations (Calabrese and Blain 2009). The phenomenon is called hormesis, and its existence has led to an intense debate on the safe threshold levels of environmental contaminants (Rietjens and Alink 2006). Especially, it has been argued that if commonly used xenobiotics have unexpected stimulatory effects on human health and wildlife at low concentrations, legislation on threshold levels should not be based on traditional toxicity assays that do not take into account the possibility of low-dose stimulation (Hoffmann and Stempsey 2008). The counterarguments have been that hormesis may not be a universal phenomenon, and that the level of hormesis may depend on environmental conditions, like plant stress (Pickrell and Oehme 2006; Hoffmann and Stempsey 2008; Chobot and Hadacek 2009; Sharma and Diez 2009). Most of this argumentation is

Address correspondence to Aki Sinkkonen, University of Helsinki, Department of Environmental Sciences, Niemenkatu 73, FIN-15140 Lahti, Finland; Phone: +358-9-1912 0344; Fax: +358-9-1912 0331, E-mail: aki.sinkkonen@helsinki.fi

based on statistical differences in mean response between treated and control individuals, probably because mean values have been thought to be a proper way to explore potential ecological and toxicological effects of any compound (references above).

Low-dose stimulatory effects are not the only biological phenomenon occurring in the low dose range. Sinkkonen et al. (2008, 2009) have recently proposed that it may be ecologically more valuable to understand the response of the most vigorous individuals in exposed stands or plant populations, instead of purely focusing on mean responses. The authors have also presented evidence that aqueous solutions of lead acetate and copper sulfate reduce the number of exceptionally long-rooted seedlings *in vitro* at toxicant concentrations that do not affect mean plant size (Sinkkonen et al. 2008, 2009). The phenomenon may be related to density-dependent phytotoxicity (San Emeterio et al. 2007; Sinkkonen 2001, 2006, 2007), or it may be caused by genotoxic or gene regulation effects of the contaminants on fast-growing seedlings of a population (Aina et al. 2006; Quaggiotti et al. 2007). Further, fast-growing seedlings may simply be exceptionally sensitive to a toxicant due to within-population genetic differences. No matter what factors are the ultimate reasons for the selective low-dose toxicity, the phenomenon may be of ecological significance in wild plant populations. If selective mortality by a natural environmental factor allows the survival of fast-growing individuals only, and if low-dose toxicity alters the number of the fast-growing i.e. surviving individuals, the total number of surviving plants is changed by the very low toxicant exposure. Light competition, drought and declining water table are known to lead to the survival of the largest or deep-rooted individuals (Mahoney and Rood 1991; Horton and Clark 2001).

In this paper, we ask whether organic xenobiotics can be associated with selective low-dose toxicity, whether the degradation of organic xenobiotics with time annuls the low-dose effects on seedlings, and how essential is the role of plant species in the context of selective low-dose toxicity on vigorous individuals. In addition, we explore if the frequency of long-rooted aqueous plants is reduced when exposed to very low lead acetate concentrations. Our ultimate goal is to investigate how regularly the frequency of long-rooted seedlings is reduced with a variety of toxicants under laboratory conditions.

MATERIALS AND METHODS

Toxicant and species selection

HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran and related isomers; CAS No: 1222-05-5) is a synthetic polycyclic musk that is used as fragrance in a variety of consumer products such as cosmetics, perfumes, air fresheners and household and laundry

cleaning products. Low levels of HHCB have been detected in aqueous ecosystems and in urban soils and high concentrations in sewage sludge. HHCB is very persistent in soil (half life 10-17 months) and has been shown to accumulate in carrot roots from soil (Litz et al 2007). Potential acute toxicity of HHCB on plants is largely unknown (Anonymous 2004).

Alkylphenol ethoxylates are a class of nonionic surfactants commonly used in the manufacture of plastics, detergents and other products (Nimrod and Benson 1996). 4-*Tert*-octylphenol (4-(1,1,3,3-tetramethylbutyl)phenol, CAS No: 140-66-9) is a degradation product of 4-*Tert*-octylphenolethoxylate. Octylphenol is found in the wastewater and sludge of sewage treatment works and river sediments (Giger et al. 1984; Hernando et al. 2004). Alkylphenols are even found in drinking water and they are generally considered as estrogenic compounds (Kuch and Ballschmiter 2001; Koh et al. 2009).

Lead acetate [$Pb(CH_3COO)_2$] is an inorganic toxicant that has been observed to create selective toxicity on long-rooted seedlings in even-aged stands at concentrations that do not affect mean plant size *in vitro* (Sinkkonen et al. 2008). Lead acetate consists of two potentially toxic components, i.e. lead and acetate ions. In this paper, lead acetate trihydrate ([$Pb(CH_3COO)_2 \cdot 3H_2O$], CAS No: 6080-56-4) was used.

Annual baby's breath (*Gypsophila elegans* M. Bieb.) grows naturally at open, arid environments in South-Eastern Europe and Western Asia. It is a naturalized, sometimes invasive species in many geographical areas ranging from North America to Australia. The plant germinates rapidly and is insensitive to soil characteristics. Sinkkonen et al. (2008) have previously demonstrated that long-rooted seedlings of *G. elegans* are sensitive to lead acetate levels that do not affect mean plant size.

Purslane [*Portulaca oleracea* L. ssp. *sativa* (Haw.) Celak] is one of the most widespread plants in the world. Typically, it plagues agricultural areas in mild and warm climates. *P. oleracea* has several ecotypes that all germinate and grow rapidly in environments characterized by warm climate and periods of drought (Singh 1973; Zimmerman 1977). Sinkkonen et al. (2009) have recently demonstrated that the growth of the most vigorous *P. oleracea* ssp. *sativa* seedlings is inhibited at copper sulfate levels that do not affect mean plant size.

Common duckweed (*Lemna minor* L.) is one of the most widespread aquatic freshwater plants at temperate and high latitudes. The species occupies and can sometimes form large colonies in a wide array of habitats, ranging from small pools and ponds to river basins and large lakes. Duckweeds, and especially *L. minor*, are model organisms for ecotoxicological studies with a standardized test protocol (ISO 2005), and are used commonly as a part of different ecotoxicological test batteries (e.g. Hagner et al. 2010). Here the growth inhibition test with *L. minor* followed ISO (2005), with slight modifications (explained below).

Plant material and growth conditions

Seeds of *G. elegans* and *P. oleracea* ssp. *sativa* were purchased from commercial horticultural companies (*G. elegans* from Nelson Garden Oy, Turku, Finland; *P. oleracea* from Oy Schetelig Ab, Vantaa, Finland,). In July 2008, leaflets of a local strain of *L. minor* were collected from a pond (EUREF-FIN N 61° 2.664' E 25° 45.322') in Päijät-Häme, Finland, next to a nature reserve area. The strain was grown at 22 °C and 24 h light for at least one month before used for testing.

Fifty *P. oleracea* seeds per dish were germinated for one day in tightly closed 53 mm diameter polyethylene Petri dishes on a filter paper (Whatman® Grade 41) in 1 ml aqueous solution that consisted of deionized Milli-Q grade water and 5.75 KCl, 123.25 $MgSO_4 \cdot 7H_2O$, 294.0 $CaCl_2 \cdot 2H_2O$, and 64.75 mg l^{-1} $NaHCO_3$ (total Ca+Mg: 0.5 mM, ratio Ca:Mg=4:1). On the second day, seedling number of *P. oleracea* was harmonized to 47 per dish by removing all potentially ungerminated seeds and if necessary also some random seedlings. The procedure harmonized the mean dose per seedling (Sinkkonen 2001, 2007). In case of *G. elegans*, the setup was the same, except that seed number was 60 per dish in the beginning of the experiment and seedling number 50 per dish after the removal process on the second day.

Thereafter, treatments as randomized replicates were established by adding 2 ml of deionized water that had been enriched with micronutrients described above and contaminated with aqueous HHCB (LGC Promochem, Germany), octylphenol (Supelco, USA) or lead acetate (J.T. Baker, The Netherlands) at the following concentrations (Table 1). *Gypsophila elegans* was treated with 0, 1.25 or 1.75 mg l^{-1} HHCB in one experiment; with 0, 0.0425, 0.425 or 4.25 mg l^{-1} octylphenol in another experiment; and with 0, 0.38, 3.79 or 37.93 mg l^{-1} lead acetate trihydrate in a third experiment. *P. oleracea* was treated either with 0, 0.01, 0.10 or 1.00 mg l^{-1} HHCB; 0, 0.042, 0.425 or 4.25 mg l^{-1} octylphenol; or 0, 3.79 or 37.92 mg l^{-1} lead acetate trihydrate, depending on the experiment. The dishes were closed airtight and the seedlings were grown at 28 °C and 16 h light:8 h darkness diurnal rhythm in a growth chamber. Root lengths of *G. elegans* seedlings were measured after three more days. *P. oleracea* seedlings were measured similarly after four more days.

For the *L. minor* experiment, all roots of plant individuals consisting of 2 mature leaves were cut off and ten individuals per 135 mm diameter dish were let to grow on 150 ml ultra pure water enriched with nutrients and 0, 0.15 or 9.5 mg l^{-1} lead acetate trihydrate. Nutrient concentrations were 42.5 $NaNO_3$, 37.5 $MgSO_4 \cdot 7H_2O$, 18.0 $CaCl_2 \cdot 2H_2O$, 0.5 H_3BO_3, 0.1 $MnCl_2 \cdot 4H_2O$, 0.025 $ZnSO_4 \cdot 7H_2O$, 0.005 $Co(NO_3)_2 \cdot 6H_2O$, 0.0025 $CuSO_4 \cdot 5H_2O$, 0.005 $Na_2MoO_4 \cdot 2H_2O$, 0.42 $FeCl_3 \cdot 6H_2O$ and 0.7 mg l^{-1} NaEDTA $\cdot 2H_2O$. Treatments as six randomized replicates were let to grow for 6 days at 28 °C and 16 h light: 8 h darkness diurnal rhythm and

TABLE 1. Replicate dishes per treatment and plants per treatment in the experiments performed.

Contaminant	Species	Replicate dishes	Plants per treatment
HHCB	*Gypsophila elegans*	6, except 5 in 1.25 mg l^{-1}	300, except 250 in 1.25 mg l^{-1}
HHCB	*Portulaca oleracea*	6	282
Octylphenol	*G. elegans*	7 (0 mg l^{-1}), 9 (0.04 mg l^{-1}), 7 (0.4 mg l^{-1}), 10 (4.0 mg l^{-1})	350 (0 mg l^{-1}), 450 (0.04 mg l^{-1}), 350 (0.4 mg l^{-1}), 500 (4.0 mg l^{-1})
Octylphenol	*P. oleracea*	12	564
Lead acetate trihydrate	*G. elegans*	6, except 5 in 0.38 mg l^{-1}	300, except 250 in 0.38 mg l^{-1}
Lead acetate trihydrate	*P. oleracea*	6	282
Lead acetate trihydrate	*Lemna minor*	6	60

root lengths and the number of leaves per dish were measured.

Chemical analyses

After removing the plants from the Petri dishes in *G. elegans* + HHCB and *P. oleracea* + octylphenol experiments, the filter papers were transferred to glass bottles with a 1 ml ethanol rinse. The samples were stored in a freezer until HHCB and octylphenol levels were analyzed. Furthermore, control treatments without plants and without chemicals, reagent blanks, and blanks with spiked water and filter paper were analyzed.

Filter papers used in octylphenol exposures were transferred to 30 ml test tubes with an ethyl acetate rinse. Ethyl acetate (20 ml) and internal standard deuterated bisphenolA (120 ng or 480 ng) were added into the tubes. The samples were sonicated for 30 minutes and the extract was transferred to an Erlenmeyer flask. The extraction was repeated and both extracts were combined. The sample was concentrated (2 ml) with a rotary evaporator and quantitatively transferred to a Kimax tube, where an aqueous layer was separated. The top layer was transferred into a new Kimax tube through a sodium sulphate column to remove remaining moisture. After that the sample was concentrated under gentle nitrogen flow until 0.5 ml was remaining. The sample was transferred to a 1.5 ml gas chromatography vial and concentrated further until 100 µl was left. Silylation reagent N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, 200 µl) was added to the vial and phenolic compounds were silylated at 85 °C for one hour. The sample was concentrated and depending on the concentration either 100 or 500 ng deuterated

anthracene was added as a recovery standard prior to analysis in gas chromatograph-mass spectrometer (GC-MS).

Filter papers used in HHCB exposures were fortified with deuterated phenanthrene as an internal standard. The amount of internal standard was either 100 or 400 ng depending on the HHCB concentration of the sample. The sample was extracted with 20 ml dichloromethane/hexane (3:7, v:v) for 30 min in an ultrasound bath and overnight in a shaker (300 rpm). The extraction was repeated with a shorter 2 h shaking. After concentrating and drying the sample in a sodium sulphate column it was transferred to a GC vial. Deuterated anthracene (100 or 200 ng) was added as a recovery standard prior to analysis on GC-MS.

All samples were analyzed using a gas chromatograph (Shimadzu GC-17A) equipped with a low resolution mass spectrometer (Shimadzu GCMS-QP5000). ZB-5MS column length was 30 m, i.d. 0.25 mm and phase thickness 0.25 µm. The helium carrier gas was set at 1 ml min^{-1}. Splitless injection mode was used for 1 min. Both injector and detector temperatures were maintained at 280 °C. The GC oven program for octylphenol samples began at 100 °C for 1 min. Thereafter, the temperature was ramped to 200 °C at 10 °C min^{-1}, then to 260 °C at 15 °C min^{-1}, then to 300 °C at 3 °C min^{-1}. The final temperature was held for 2 min. The GC oven program for HHCB samples began at 80 °C that was held for 1 min. Thereafter, the temperature was ramped to 250 °C at 10 °C min^{-1}, then to 280 °C at 7 °C min^{-1}, then to 320 °C at 20 °C min^{-1}. The final temperature was held for 10 min.

At least two ions per compound were monitored in MS (SIM mode). Target ion m/z was used to quantify the concentration of an analyte and additional ion m/z was used to confirm the qualitative result in addition to correct retention time. Quantitation was based on an internal standard and performance was evaluated with the aid of a recovery standard. The mean recovery for internal standard in HHCB analysis was 75 ± 9 % and in octylphenol analysis 122 ± 20 %.

Statistical analyses

Statistical analyses were executed with SPSS for Windows 15.0.1. The first goal was to find exposed treatments that were not significantly different from non-exposed control treatments in mean root lengths. The major goal was thereafter to investigate if there still was a significant difference between the most long-rooted seedlings in any of the treatments that were not significantly different in treatment means from control.

Univariate analysis of variance (Anova) was performed with Tukey honestly significant difference tests to sort out treatments with significantly different mean values. Whenever the assumption of normality was seriously violated, the probability of rejecting a true null hypothesis was higher than indicated by the analysis. Then, Kruskal-Wallis test was per-

formed and two-treatment differences between medians were analyzed non-parametrically with Mann–Whitney U tests.

If Anova or its non-parametric alternatives indicated no difference in treatment means, Moses test of extreme reactions was executed to find out if individuals were equally distributed in the two treatments compared (Moses 1952; Sinkkonen et al. 2009). Because the Moses extreme reactions test tests for one-tailed differences, exposed treatments were set as Group 1 in the test. Because this test also is affected by extreme values that may occur due to experimental or systematic errors, the three longest seedling roots were excluded as potential outliers at the right tails of the two distributions compared (Sinkkonen et al. 2009). As Moses test cannot be used to discover if large but not short individuals are sensitive to low toxicant levels in the substratum, we compared seedlings at the right tails of the size distribution diagrams of control and exposed treatments with Mann–Whitney U tests. We tested 99, 97, 95 and 90% percentiles adjusted for ties (Sinkkonen et al. 2009). The 98th percentiles were tested instead of the 99th percentiles whenever there were less than four replicates in any of the 99th percentiles explored. If the distributions compared were not similar, Kolmogorov-Smirnov Z tests were performed instead of the Mann-Whitney U tests.

Since roots of any single experiment were measured during 2-3 consecutive days, we also analyzed the effect of day on root length mean, standard deviation, and min and max values per dish, despite the fact that plants were stored at +5 °C during those days.

The EC50 for plant roots was also estimated, according to the logistic model by Haanstra et al. (1985). However, not a single significant inhibitory effect was found in any of the experiments for plant responses within the exposure regimes that were used in this study.

RESULTS

Extreme values in octylphenol treatments.

Root lengths of *G. elegans* (mean ± SD) were 18.0 ± 8.3, 18.6 ± 7.9, 18.2 ± 8.6 and 18.4 ± 8.0 mm in 0, 0.043, 0.425 and 4.250 mg l^{-1} treatments, respectively. There were no significant differences between treatments in mean root length of *G. elegans* ($F = 0.396$, $df = 1495, 3$, $p = 0.76$). In Moses extreme reactions tests, root lengths in 4.250 mg l^{-1} treatment were significantly different from those in 0 and 0.425 mg l^{-1} treatments ($p < 0.05$) but not from root lengths in 0.043 mg l^{-1} treatment ($p > 0.1$). In trimmed Moses tests, 4.250 mg l^{-1} treatment was significantly different from 0 mg l^{-1} ($p = 0.003$), 0.043 mg l^{-1} ($p = 0.025$) and 0.425 mg l^{-1} ($p = 0.001$) treatments. When roots that were equal to or longer than the 98 % percentiles were compared, 4.250 mg l^{-1} treatment was significantly different from 0

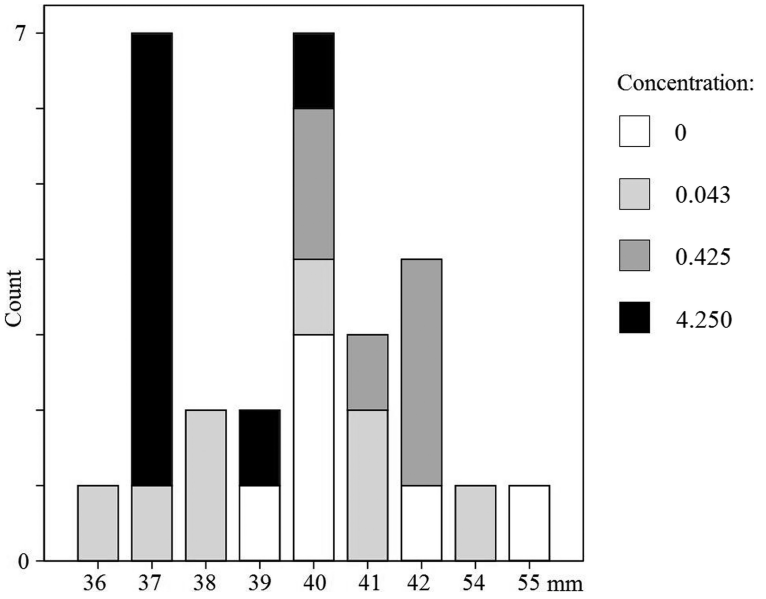

FIGURE 1. Roots of *G. elegans* beyond the 98th percentiles of treatments exposed to different concentrations of 4-*tert*-octylphenol (mg l^{-1}).

mg l^{-1} (K-S Z =1.39, p = 0.042) and 0.425 mg l^{-1} (K-S Z = 1.62, p = 0.01) treatments in Two-Sample Kolmogorov-Smirnov tests (Figure 1).

Root lengths of *P. oleracea* (mean ± SD) were 20.8±5.5, 21.2±6.0, 20.1±5.4 and 19.4±5.2 mm in 0, 0.043, 0.425 and 4.250 mg l^{-1} treatments, respectively. In comparison with 0 mg l^{-1} treatment, octylphenol affected mean root length of *P. oleracea* at 4.250 mg l^{-1} concentration but not at the three weaker concentrations (data not shown). There were no significant differences in Moses extreme reactions tests between 0 mg l^{-1} and other treatments (data not shown).

Extreme values in HHCB treatments.

Root lengths of *G. elegans* (mean ± SD) were 17.6±8.2, 17.8±7.9 and 18.9±9.1 mm in 0, 1.25 and 1.75 mg l^{-1} treatments, respectively. There were no significant between-treatment differences in mean root length of *G. elegans* (F = 1.39, df = 1041, 3, p = 0.24). In Moses extreme reactions tests, 1.75 mg l^{-1} was significantly different from 1.25 mg l^{-1} treatment (p = 0.038) but these treatments were not significantly different when mean values of 97-90 % percentiles were analyzed in Mann-Whitney U-tests (p > 0.05).

Root lengths of *P. oleracea* (mean ± SD) were 21.8±5.9, 20.0±5.9, 21.6±6.7 and 20.2±6.3 mm in 0, 0.01, 0.10 and 1.00 mg l^{-1} treatments, respectively. HHCB affected mean root length of *P. oleracea* at 0.01 and 1.00 mg l^{-1} but not at 0.10 mg l^{-1}, as compared to 0 mg l^{-1} treatment (K-W

χ^2 = 22.49, df = 3, p = 0.024). The latter two treatments were not significantly different in Moses extreme reactions test (data not shown).

Extreme values in lead acetate treatments.

Root lengths of *G. elegans* (mean ± SD) were 19.1±8.7, 18.9±8.3, 18.3±7.5 and 17.1±7.1 mm in 0, 0.38, 3.79 and 37.90 mg l^{-1} treatments, respectively. There were significant between-treatment differences in mean root length of *G. elegans* (χ^2 = 7.95, df = 3, p = 0.047). 0 mg l^{-1} treatment was not significantly different from 0.38 and 3.79 mg l^{-1} treatments (p > 0.50) but significantly different from the 37.90 mg l^{-1} treatment. In Moses extreme reactions tests, there was a significant difference between 0 mg l^{-1} and 0.38 mg l^{-1} treatments (p = 0.044). After trimming, 0 mg l^{-1} treatment was significantly different from both 0.38 and 3.79 mg l^{-1} treatments in Moses extreme reactions tests (p = 0.01 and p = 0.004, respectively). When 97-90 percentiles were compared, 3.79 mg l^{-1} differed significantly from the 0 mg l^{-1} treatment (Table 2).

Root lengths of *P. oleracea* (mean ± SD) were 22.2±5.9, 22.1±5.7 and 19.5±4.6 mm in 0, 3.79 and 37.90 mg l^{-1} treatments, respectively. As compared to the 0 mg l^{-1} treatment, lead acetate affected mean root length of *P. oleracea* at 37.90 mg l^{-1} but not at 3.79 mg l^{-1} concentration (data not shown). There were no significant differences between 3.79 mg l^{-1} and 0 mg l^{-1} treatments in Moses extreme reactions or 97-90 percentile tests.

Root lengths of *L. minor* (mean ± SD) were 16.6±18.2, 16.0±17.1 and 12.3±16.5 mm in 0.0, 0.1 and 9.5 mg l^{-1} treatments, respectively. Lead acetate reduced root lengths of *L. minor* at 9.5 mg l^{-1} but not at 0.1 mg l^{-1} treatments, as compared to the 0 mg l^{-1} treatment (χ^2 = 179.4, df = 2, p < 0.0005). Control and 0.1 mg l^{-1} treatments were significantly different in trimmed and untrimmed Moses tests (p < 0.0005). When 90th percentiles were compared with Mann-Whitney U-tests, mean root length was shorter in 0.1 mg l^{-1} treatment as compared to the 0 mg l^{-1} treatment (U = 37, p =0.032). However, there were no differences when the 95th and 97th percentiles were compared between these two treatments.

TABLE 2. Statistically significant effects of aqueous 0.38 and 3.79 mg l^{-1} lead acetate at 97th 95th and 90th percentile (adjusted for ties) on seedling roots of *G. elegans in vitro* (n = 11, 14, 34, respectively). 99th percentile was not tested due to small number of replicates.

Percentile	97	95	90
Mann-Whitney U:			
0.38 vs 0 mg l^{-1}	22.0	63.0	303.0
3.79 vs. 0 mg l^{-1}	18.0	40.0	220.0
p-values:			
0.38 vs 0 mg l^{-1}	0.301	0.302	0.572
3.79 vs. 0 mg l^{-1}	0.082	0.004	0.003

Effect of day on root length.

When the effect of harvesting day was analyzed, there was only one statistically significant difference in mean values, standard deviations, and min and max values per dish in all the experiments. When *P. oleracea* was treated with different octylphenol levels, the standard deviations of root lengths on day five were greater than standard deviations on day seven ($F = 3.47$, $df = 45, 2$, $p = 0.04$).

Chemical analyses.

At the end of the experiment, HHCB concentrations (mean ± SD) were zero or lower in Petri dishes with *G. elegans* seedlings (0.2 ± 0.1, 0 and 1.4 ± 2.4 ng sample^{-1} in 0, 1.25 and 1.75 mg l^{-1} treatments) compared to the level found in 0 mg l^{-1} + filter paper controls. HHCB levels in the filter paper controls and GC-MS blanks were 5.7 ± 0.6 ng sample^{-1} and 0.7 ± 0.5 ng sample^{-1}, respectively. Petri dishes with 1.75 mg l^{-1} HHCB but without seedlings contained 510.7 ± 8.5 and 46.6 ± 15.5 ng HHCB sample^{-1} at the beginning and at the end of the experiment, respectively. Also 4-*tert*-octylphenol concentrations decreased below the level found in 0 mg l^{-1} + filter paper controls (Table 3.). At the end of the experiment, 4-*tert*-octylphenol levels in spiked samples were higher in treatments without *P. oleracea*, as compared to treatments with *P. oleracea* (Table 3.). One sample in the 4.250 mg l^{-1} treatment contained 651.7 ng 4-*tert*-octylphenol, and was removed as a statistical outlier due to potential contamination during chemical analysis.

DISCUSSION

In the three experiments performed with *G. elegans*, significant differences were observed in Moses extreme reaction tests. Similarly, two of the three experiments ended up in significant differences in Mann-

TABLE 3. The amount of 4-*tert*-octylphenol (ng sample^{-1}) in different treatments with and without 47 seedlings of *P. oleracea*.

Treatment	Time	Amount of octylphenol	n
0.043 mg, no seedlings	Start of experiment	60.3 ± 2.2	2
4.250 mg, no seedlings	Start of experiment	7073.4 ± 177.0	2
0 mg + seedlings	End of experiment	9.9 ± 8.34	3
0.043 mg + seedlings	End of experiment	6.9 ± 5.1	3
0.425 mg + seedlings	End of experiment	4.1 ± 0.6	3
4.250 mg + seedlings	End of experiment	11.0 ± 1.4	2
0.043 mg, no seedlings	End of experiment	19.0 ± 5.7	2
4.250 mg, no seedlings	End of experiment	1640.1 ± 265.7	2
Blank samples in GC-MS		3.2 ± 0.3	2
0 mg and filter paper but no seedlings		17.8 ± 11.2	2

Whitney U tests made with ninety or higher percentiles. The differences were found when control roots were compared with roots exposed to toxicant concentrations that did not affect mean root length. This supports the hypothesis that very low toxicant concentrations may shape the size distribution diagram even if toxicant concentrations are so negligible that mean values are not affected. The effect on *G. elegans* was significant also with lead acetate, which confirms our earlier observation (Sinkkonen et al. 2008).

The fact that the frequency of long-rooted *P. oleracea* seedlings did not differ from root lengths in control treatments is in contrast with our previous observation that low concentrations of copper sulphate reduced the number of long-rooted *P. oleracea* seedlings (Sinkkonen et al. 2009). It is possible that *P. oleracea* is not especially prone to the selective toxicity studied in this paper, but an alternative explanation is that the concentrations used and the short turnover time of HHCB and octylphenol masked significant effects. Belz (2008) has suggested that the concentration range at which stimulatory hormesis can be observed may be very narrow. If the same is true with selective toxicity on long-rooted seedlings in dense plant stands, degradation of organic compounds may complicate efforts to verify the phenomenon. Moreover, since organic toxicants are not often recalcitrant, the ecological effects of selective toxicity at low doses may not be as severe as in case of low levels of inorganic or recalcitrant toxicants.

The effect of the lowest lead acetate concentration on long-rooted *L. minor* plants supports the hypothesis that selective toxicity is not restricted to terrestrial ecosystems. In the experiment performed, nutrients did not limit plant growth. If natural aquatic ecosystems are considered, slow growth of the largest individuals may affect light competition between different plant species, or it could delay sexual reproduction. Since those factors often play a major role in the determination of plant fitness, the selective toxicity at low doses deserves further attention in aquatic environments.

In this study, selective low-dose toxicity was studied by comparing all plants at one concentration to all plants at another concentration without separating replicate dishes from each other. Although mean root lengths were not significantly different between different dishes within any treatment involved in Moses extreme reactions tests (data not shown), a more optimal strategy might be to compare if 90-99 percentile values of plants at different dishes of an exposed treatment are statistically different from the respective values of a control treatment. This, however, requires many replicate dishes per treatment in order to separate variation between treatments from random variation within treatments. If every dish contains 40-60 seedlings, and if there are 30 dishes per treatment, the root lengths of all plants may be difficult to measure within an acceptable time limit. Another possible method to evaluate how commonly selective low-dose toxicity exists is to make a literature survey similar to the one made

by Calabrese and co-authors on the prevalence of hormetic growth stimulation in low-dose studies (see Calabrese and Blain 2009). Since many published studies tell treatment means and standard deviations but not high percentiles, direct contacts with authors may be needed to complete such a survey.

The species studied seem to be quite inert to low-dose effects of HHCB. Although there were no differences in mean values of roots of *G. elegans* exposed to HHCB, the two exposed treatments were significantly different in Moses extreme reactions test. This opens an interesting view that xenobiotics that have traditionally been considered relatively harmless may change the frequency of very low or very high values. Many surfactants and other estrogenic compounds that are continuously spread by human activities to numerous ecosystems in industrialized countries belong to this category.

The purpose of this study was to explore the effects of plant species and toxicant type on selective toxicity at low doses, and the relationship between stimulatory hormesis and the selective toxicity. The results lend support to the hypothesis that the selective toxicity can be found with many toxicants and species. Thus, it is possible that fairly significant effects of low doses on subpopulations are missed because the effects are masked by a lack of effect on most of the population. Alternatively, there could be a stimulation of a part of the population that would mask inhibitory effects on the normally faster growing part of the population. However, we were not successful in our efforts to verify hormesis statistically though we observed selective toxicity together with slightly increased treatment means. Thus, it is possible that hormesis and selective toxicity occur in parallel i.e. the number of the most-long rooted plant individuals may decrease even if stimulatory hormesis exists. The results also indicate that there are differences between plant species. Much more research effort will be needed to find out if the growth of fast-growing plant seedlings is decreased in nature due to very low toxicant doses, and what are the possible consequences of the potential decrease on plant fitness and population dynamics.

ACKNOWLEDGMENTS

Three anonymous reviewers gave valuable comments on earlier versions of the manuscript.

REFERENCES

Aina R, Palin L, Citterio S 2006. Molecular evidence for benzo[a]pyrene and naphthalene genotoxicity in *Trifolium repens* L. Chemosphere 65:666-673.

Anonymous 2004. HERA Risk Assessment of HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran and related isomers). HERA Human and Environmental Risk Assessment on Ingredients of Household Cleaning Products. Hera Secretariat. Brussels, Belgium.

Belz R 2008. Stimulation versus inhibition – bioactivity of parthenin, a phytochemical from *Parthenium hysterophorus* L. Dose-Response 6:80-96.

Calabrese EJ, Blain RB 2009. Hormesis and plant biology. Env Poll 157:42-48.

Chobot V, Hadacek F 2009. Milieu-Dependent Pro-and Antioxidant Activity of Juglone May Explain Linear and Nonlinear Effects on Seedling Development. J Chem Ecol 35:383-390.

Giger W, Brunner PH, Schaffiner C 1984. 4-Nonylphenol in sewage sludge: accumulation of toxic metabolites from nonionic surfactants. Science 225:623-625.

Haanstra L, Doelman P, Oude Voshaar JH 1985. The use of sigmoidal dose response curves in soil ecotoxicological research. Plant Soil 84:293-297.

Hagner, M, Penttinen, O-P, Pasanen T, Tiilikkala K, Setälä H. 2010. Acute toxicity of birch tar oil on aquatic organisms. Agric Food Sci 19:24-33.

Hernando MD, Mezcua M, Gómez MJ, Malato O, Agüera A, Fernández-Alba AR 2004. Comparative study of analytical methods involving gas chromatography-mass spectrometry after derivatization and gas chromatography-tandem mass spectrometry for the determination of selected endocrine disrupting compounds in wastewaters. J Chromatogr A 1047:129-135.

Hoffmann GR, Stempsey WE 2008. The hormesis concept and risk assessment: are there unique ethical and policy considerations? Hum Exp Toxicol 27:613-620.

Horton JL, Clark JL 2001. Water table decline alters growth and survival of *Salix gooddingii* and *Tamarix chinensis* seedlings. For Ecol Manag 140:239-247.

[ISO] International Organization for Standardization. 2005. Water quality – determination of the toxic effect of water constituents and waste water on duckweed (*Lemna minor*) – duckweed growth inhibition test. ISO guideline 20079. Geneva, Switzerland.

Koh YK, Chiu TY, Boobis AR, Scrimshaw MD, Bagnall JP, Soares A, Pollard S, Cartmell E, Lester JN 2009. Influence of operating parameters on the biodegradation of steroid estrogens and nonylphenolic compounds during biological wastewater treatment processes. Environ Sci Technol 43:6646-6654.

Kuch HM, Ballschmiter K 2001. Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC–(NCI)–MS in the Picogram per liter Range. Environ Sci Technol 35:3201-3206.

Litz NTh, Müller J, Böhmer W 2007. Occurrence of polycyclic musks in sewage sludge and their behaviour in soils and plants. Part 2: Investigation of polycyclic musks in soils and plants. J Soils Sediments 7:36-44.

Mahoney JM, Rood SB 1991. A device for studying the influence of declining water table on poplar growth and survival. Tree Physiol 8:305-314.

Moses LE 1952. A two-sample test. Psychometrika 17:239-47.

Nimrod AC, Benson WH 1996. Estrogenic responses to xenobiotics in channel catfish (*Ictalurus punctatus*). Mar. Environ. Res. 42:155-160.

Pickrell JA, Oehme FW 2006. Examining the risks and benefits of considering both the traditional dose–response and hormesis in arriving at an acceptable exposure level. Hum Exp Toxicol 25:23-27.

Quaggiotti S, Barcaccia G, Schiavon M, Nicolé S, Galla G, Rossignolo V, et al. 2007. Phytoremediation of chromiumusing Salix species: cloning ESTs and candidate genes involved in the Cr response. Gene 402:68-80.

Rietjens IMCM, Alink GM 2006. Future of toxicology — low-dose toxicology and risk–benefit analysis. Chem Res Toxicol 19:977-981.

San Emeterio L, Damgaard C, Cannals RM 2007. Modelling the combined effect of chemical interference and resource competition on the individual growth of two herbaceous populations. Plant Soil 292:95-103.

Sharma SS, Diez KJ 2009. The relationship between metal toxicity and cellular redox imbalance. Trends Plant Sci 14:43-50.

Singh KP 1973. Effect of temperature and light on seed germination of two ecotypes of *Portulaca oleracea* L. New Phytol 72:289-95.

Sinkkonen A 2001. Density-dependent chemical interference — an extension of the biological response model. J Chem Ecol 27:1513-23.

Sinkkonen A 2006. Modelling the effect of density-dependent chemical interference upon seed germination. Dose-Response 4:217-224.

Sinkkonen A 2007. Modelling the effect of autotoxicity on density-dependent phytotoxicity. J Theor Biol 244:218-27.

Sinkkonen A, Strömmer R, Penttinen OP 2008. Low toxicant concentrations decrease the frequency of fast-growing seedlings at high densities of annual baby's breath (*Gypsophila elegans*). Environ Pollut 153:523-5.

Sinkkonen A, Penttinen OP, Strömmer R 2009. Testing the homogenizing effect of low copper sulfate concentrations on the size distribution of *Portulaca oleracea* seedlings *in vitro*. Sci Tot Env 407:4461-4464.

Zimmerman CA 1977. A Comparison of breeding systems and seed physiologies in three species of *Portulaca* L. Ecology 568:860-868.