Realistic low-doses of two emerging contaminants change size distribution of an annual flowering plant population

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Abstract

HHCB [1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran] and 4-*tert*-octylphenol [4-(1,1,3,3-tetramethylbutyl)phenol] are widely used emerging contaminants that have the potential to cause adverse effects in the environment. The purpose of this study was to observe if and how environmentally realistic concentrations of these contaminants alter growth in plant populations. It was hypothesized that within an exposed *Gypsophila elegans* Bieb (annual baby's breath) population especially fast-growing seedlings are impaired even when the population mean is unaffected, and small doses can cause hormesis and, thus, an increase in shoot or root length. In a dose-response experiment, an experimental population of *G. elegans* was established (total 15.600 seeds, 50 seeds per replicate, 24 replicates per concentration, 5.2 seedlings/cm^2) and exposed to 12 doses of HHCB or 4-*tert*-octylphenol. After five days, shoot and root length values were measured and population averages, as well as slow- and fast-growing subpopulations, were compared with unexposed controls. Growth responses were predominantly monophasic. HHCB seemed to

selectively inhibit both root and shoot elongation among slow- and fast-growing individuals, while 4-*tert*-octylphenol selectively inhibited both root and shoot elongation of mainly fast-growing seedlings. The ED_{50} values (dose causing 50 % inhibition) revealed that the slow-growing seedlings were more sensitive and fast-growing seedlings less sensitive than the average of all individuals. Although there was toxicant specific variation between the effects, selective toxicity was consistently found among both slow- and fast-growing plants starting already at concentrations of 0.0067 µ*M*, that are usually considered to be harmless. This study indicates that these contaminants can change size distribution of a plant population at low concentrations in the n*M*/µ*M* range.

Keywords – Dose-response, Growth stimulation, Hormesis, Low toxin doses, Selective toxicity.

Introduction

Some specific classes of substances called ‘emerging contaminants’ have been defined to be chemicals or materials which cause or have the potential to cause adverse effects on humans and/or the environment and, thus, require our special attention (Sauvé and Desrosiers 2014). Usually, these compounds are present in many widely used everyday products such as plastics, flame retardants and cosmetics. They have become a serious environmental issue after being detected in trace concentrations around the globe thanks to the rapid development of analytical techniques enabling identification and quantification of these contaminants (Klaschka et al. 2012; Tao et al. 2011).

Once being released into the environment, many of these chemicals have been observed to cause adverse effects on wildlife (Pablos et al. 2015). In contrast, effects on plant populations, especially in environmentally realistic trace amounts, are seldom addressed and do not seem to cause significant inhibition (An et al. 2009). However, low-doses of plant toxins are well-known to have an impact on plant populations. Low-doses of plant toxins can induce stimulatory responses in many plant traits and species (Calabrese and Howe 1976; Duke et al. 2006; Cedergreen et al. 2007; Calabrese and Blain 2009). This enhancement in plant performance due to low chemical exposure is believed to be a widespread phenomenon, generally known as hormesis (Calabrese 2008). In order to detect this growth enhancement, one should concentrate on very low concentrations that are below the concentrations causing significant toxic effects.

Even though such stimulatory responses can be present at the mean population level, the phenomenon does not always seem to occur homogeneously throughout a population in dense plant stands (Belz and Sinkkonen 2016a, b). Moreover, hormesis may remain hidden at the mean population level even though slow-growing individuals with short root/shoot elongation show strong hormetic responses. The associated lack of hormesis among the fast-growing individuals with long root/shoot elongation may be due to a more limited capacity for enhanced growth since these vigorously growing individuals may already have allocated all possible resources to growth (Belz and Sinkkonen 2016a).

However, if hormesis is observed at the mean population level, it usually involves a stimulation of slow- and fast-growing individuals (Belz and Sinkkonen 2016a).

Besides hormesis leading to significantly enhanced responses in population mean, another low-dose phenomenon may occur for plant toxins leading to significant effects on individual plants within a population without changing the overall response. This phenomenon is called ‘selective low-dose toxicity’ in the case of toxic effects and ‘selective low-dose stimulation’ in the case of stimulatory effects. These selective low-dose effects have been observed to appear differently among individuals having a different growth rate and, thus, whether they are fast- or slow-growing (Sinkkonen et al. 2008, 2011). Exposing high-density plant populations to low toxicant concentrations has caused a significant decrease in growth especially in the fast-growing part of a population (Sinkkonen et al. 2011; Belz and Sinkkonen 2016a, b). It has been proposed that the higher growth rate of the fast-growing individuals leads to a faster toxicant uptake affecting growth in an adverse manner (Aina et al. 2006).

Due to the possibility that environmental pollutants cause such low-dose phenomena in toxin-exposed natural plant populations, there is a risk for long-term environmental consequences. Since low toxicant exposures have been confirmed to inhibit mainly the growth of fast-growing seedlings, Sinkkonen et al. (2011) hypothesized that if natural conditions favor the survival of the fastest growing individuals, a low chemical exposure can drastically affect the overall survival of a plant population because root growth is directly linked to the efficiency of water uptake. The authors confirmed that two ‘emerging contaminants’, namely HHCB [1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran] and 4-*tert*-octylphenol [4-(1,1,3,3-tetramethylbutyl)phenol] can cause selective low-dose inhibition on root growth of the most fast-growing individuals of *Gypsophila elegans* Bieb. (annual baby’s breath). As Sinkkonen et al. (2011) did not observe hormesis possibly because of limited replications, and as natural plant populations are commonly exposed to trace amounts of these two toxicants, HHCB and 4-*tert*-octylphenol were

chosen to investigate potential selective low-dose effects in more detail and the interplay with a possible induction of hormesis.

HHCB, also known as galaxolide, is a synthetic musk compound widely used as an ingredient in consumer products such as cosmetics and fragrances (HERA 2004). It has been detected in both effluent waters from wastewater treatment plants (Klaschka et al. 2012) and sewage sludge (Kupper et al. 2004), which often have applications in agricultural use. Due to its high sorption in soil, low leaching and low soil degradation, HHCB is likely to remain in the upper soil layers after being applied to soils exposing also a putative risk for terrestrial plants (Litz et al. 2007). In addition, HHCB has been observed to affect also root elongation of wheat seedlings and of *Lactuca sativa* L. by inhibition at higher and stimulation at lower doses (An et al. 2009; Agathokleous et al. 2018; Belz et al. 2018). This biphasic action was said to be caused by the hormone-like characteristics of HHCB (An et al. 2009).

4-*tert*-Octylphenol is a high-production volume alkylphenol substance with applications in industrial processes, for example in rubber processing or production of ethoxylates which are further used in emulsion polymerization or water-based paints (Brooke et al. 2005). The compound is especially found in aquatic environments including groundwater (Hernando et al. 2004, Tao et al. 2011), and can reach terrestrial environments when soils are irrigated with reclaimed wastewater (Chen et al. 2013). Furthermore, 4-*tert*-octylphenol seems to accumulate in soils (Chen et al. 2013). Ecotoxicological data about the phytotoxicity of 4-*tert*-octylphenol seems to be lacking, yet Sinkkonen et al. (2011) observed a significant reduction of root length by low doses of the compound among the fast-growing seedlings of a *G. elegans* population. Moreover, 4-*tert*-octylphenol has been observed to cause o biphasic response on root elongation of *L. sativa* (Agathokleous et al. 2018; Belz et al. 2018). Therefore, when studying low-dose effects of the two emerging contaminants, HHCB and 4-*tert*-octylphenol, it is important to manifest possible ecological risks of such pollutant-driven low-dose effects on natural vegetation.

Previous studies about the selective low-dose effects explored the topic from an agricultural point of view. Commercially cultivated plant species or weed species were exposed to herbicides that are common in agriculture (Belz and Sinkkonen 2016a, b). Other studies focused on selective low-dose toxicity only (Sinkkonen *et al.* 2009, 2011). Therefore, the two main objectives of this study were to study the effects of emerging contaminants on a wild plant species, namely the wildflower *G. elegans,* and to explore low-dose stimulatory effects in order to better assess possible environmental consequences. We focused on the following hypotheses: a) the emerging contaminants HHCB and 4-*tert*-octylphenol induce hormesis in a *G. elegans* population; and b) selective low-dose effects and/or hormesis appear and vary among the fast- and slow-growing individuals of *G. elegans* even though the population mean remains unchanged. Based on previous findings, selective low-dose toxicity among the fast-growing seedlings and more pronounced hormesis was expected to occur among the slow-growing seedlings, so that these low-dose phenomena would occur heterogeneously within the plant population and consequently alter the plant size distribution within the population.

Materials and methods

Bioassay

An experimental high-density population of *G. elegans* (cv. Covent Garden; Saatgut-Vielfalt, Germany) (total 15,600 seeds; 5.2 seedlings/cm^2) was used as the test population and exposed in complete dose-response germination experiments to HHCB and 4-*tert*-octylphenol (**Table 1**). Since plants are exposed to HHCB and 4-*tert*-Octylphenol mainly in the upper soil layers, where most plant seeds usually germinate (Litz et al. 2007, Chen et al. 2013), we chose to expose seeds in a germination bioassay. The test method has been used and published previously (Belz and Sinkkonen 2016a, b). Briefly, the assays were done in 6-well cell culture plates (Cellstar, Greiner bio-one) for 5-d prior to measuring shoot and root elongation as endpoints. One layer of filter paper (MN 615, Macherey-Nagel) was placed inside each well before applying the chemicals. The applied concentrations of the

test chemicals were chosen based on preliminary tests and comprised besides an untreated control in total 12 concentrations ranging from 0.000067 to 0.67 m*M* for 4-*tert*-octylphenol (Sigma-Aldrich, Germany; purity 97 %) and from 0.0000067 to 2.00 m*M* for HHCB (Sigma-Aldrich, Germany; purity 50 %). The number of replicates (one replicate equals one well) per treatment was 24 arranged in blocks of six replicates (one 6-well-plate) that were randomly placed in a climate chamber. Due to the low water solubility of the test chemicals, the various concentrations were prepared from ethanol stock solutions by adding increasing amounts to wells. All plates were left open for 1 d in order to let the ethanol evaporate. Then, 60 seeds of *G. elegans* and 1.5 mL of demineralized water were added to each well/replicate. With HHCB, 65 seeds/replicate were initially added because of a low germination rate in the first experiment with 4-*tert*-octylphenol. For the control treatment, only 1.5 mL of demineralized water was added. The plates were sealed with parafilm before placing them in a completely randomized design into a climate chamber. The climate conditions were set to a day/night cycle of 12/12 h staring at 8 am with 24/18 °C and a 12 h light period of 50-70 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR). After 2-3 days, the number of seeds was harmonized to 50 seeds/replicate. After 5 d of exposure, plates were frozen at -20 °C until the shoot and root growth of the seedlings was evaluated. The evaluation was done using Fitomed (Castellano et al. 2001). If the shoot/root length was <1 mm, it was counted as zero.

Statistical analysis

The statistical analysis applied has been largely used and published previously (for example, Sinkkonen *et al.* 2009, 2011; Belz and Sinkkonen 2016a, b), but it was now optimized to consider block effects and data normalization. All analyses were done with SAS® 9.4. At first, the mean values per replicate were calculated for absolute shoot and root length values (mean of the 50 seeds per replicate) as well as the percentile (%ile) values per replicate. At the left tail of the size distribution (the most short-grown individuals referred to as the slow-growing subpopulations), the 5, 8, 10, 15 and 20 %iles were calculated for HHCB and the 20, 22, 23 and 25 %iles for 4-*tert*-octylphenol due

to a high number of ungerminated seeds in this experiment. At the right tail of the size distribution (the most long-grown individuals referred to as the fast-growing subpopulations), the 90, 95, 97 and 99 %iles were calculated for both toxicants. Because at each treatment six replicates were blocked on one 6-well-plate, we first analyzed for significant differences between these four blocks within a treatment by a univariate analysis of variance (Anova; α=0.05). Since absolute and %ile values for root and shoot data of both contaminants showed partly significant differences between blocks and in parts a non-normal data distribution (*Shapiro-Wilk's* test, p>0.05), we decided to consider block effects in the further statistical analysis and to transform any non-normal, blocked data via Box-Cox power transformation.

Block effects were considered in the form of calculating a mean value per block based on all six replicates blocked on one plate, so that a treatment was characterized by four block values. The Box-Cox transformation for datasets violating the assumption of normality was done after estimating the optimal value of the transformation parameter λ from the data by the maximum likelihood method (-3 < λ < 3) using the TRANSREG procedure (Piepho 2009, Osborne 2010, Perla 2016, Damesa et al. 2018). Transformation of data was necessary for seven datasets out of entirely 38. For all datasets transformed, the application of the Box-Cox transformation fixed the problem of violating normality. The mean values per block formed the basis for the evaluations at the subpopulation level. After that, these data were used to calculate the mean response per treatment (mean of the four block values per treatment). This formed the basis for the evaluations at the population level.

Selective low-dose effects

An ANOVA together with a *Tukey* test (α=0.05) was done to exclude the treatments with significantly different absolute mean shoot/root length values per block compared to the control treatment. For those treatments that did not show significant differences in absolute mean values at the population level, %ile values per block were compared by a *Mann-Whitney U* test (α=0.05) for significant differences between treatments and the control.

Dose-response modelling

Dose-response relationships were modelled at the population level based on the mean response per treatment and at the 'percentile-dependent' subpopulation level based on percentile values, namely the mean response per treatment for the 95 %ile and, thus, the fast-growing subpopulation represented by the most long-grown plants, and for the ≤25 %ile and, thus, the slow-growing subpopulation represented by the most short-grown plants. Reduced forms of either a monophasic function (Streibig 1988) (**Eq. 1**) or a hormetic model (Brain and Cousens 1989) was modelled (**Eq. 2**):

Eq. (1) $$y = d/(1 + \exp(b * \log(x/ED_{50})))$$

Eq. (2) $$y = (d + f\ x)/(1 + \exp(b * \log(x/e)))$$

where *d* corresponds to the mean response of the untreated control, *f* reveals the degree of hormetic increase, *b* equals the slope of the decreasing curve part, and ED_{50} the dose causing 50 % inhibition while parameter *e* does not correspond to any actual biological factor (Brain and Cousens 1989). In case of a biphasic modelling, the following quantitative features were further deduced using reparameterizations of Eq. 2 (Schabenberger et al. 1999; Belz and Piepho, 2012, 2013): the ED_{50}, the maximum stimulatory response y_{max} and the dose *M* where stimulation is maximal.

Based on the nature of the data, either the mono- or the biphasic dose-response model was used. The choice of the specific model fitted was primarily based on the significance of parameter *f* as indicated by an estimate of f with a 95 % confidence interval that did not cover the value zero ($f > 0$) (Schabenberger et al. 1999). If $f > 0$ was not fulfilled, but the graph of the data for response *y* versus dose *x* indicated hormesis, a pairwise likelihood ratio test with the monophasic model (Eq. (1)) as the reduced model was performed as goodness-of-fit test with the *p*-value of the test statistic being approximated by the chi-square distribution χ^2 (Seber and Wild 1989; Belz and Piepho 2017). Additionally, the ED_{10} dose level was calculated to distinguish the low-dose range from high-dose inhibitory effects (Streibig and Jensen 2000) (**Eq. 3**):

Eq. (3) $$ED_{10} = ED_{50} * (10/100 - 10)^{1/b}$$

Starting values for the regression parameters were selected based on the graph of the data for response *y* versus dose *x*. Response variance heterogeneity was accounted for by using the inverse variance of replicates at each dose as weight. Significant differences between dose-response curves were evaluated by comparing regression parameters using the CONTRAST statement within the NLMIXED procedure (α=0.05).

Results

HHCB

Selective low-dose effects

After an HHCB exposure, seven out of 12 treatments showed no significant difference in terms of mean shoot elongation when compared to the control (**Table 2**). Shoot elongation of the slow-growing seedlings (≤20 %iles) was significantly inhibited at six doses (excluding 0.002 m*M*) at all percentiles tested. The last treatment, however, may already account for beginning of high-dose inhibition. The fast-growing seedlings (≥90 %iles) were negatively affected only by a dose of 0.013 m*M* at two percentiles tested. This indicates that low-dose toxicity affected shoot elongation by HHCB among both slow- and fast-growing seedlings, while selective low-dose stimulation was absent.

Regarding mean root elongation, nine out of 12 treatments were not significantly different from the control. Among the slow-growing seedlings, two doses (0.0000067 and 0.00013 m*M*) negatively affected root elongation at two percentiles tested. Root elongation of the fast-growing seedlings was selectively inhibited at three doses (0.0000067, 0.013 and 0.067 m*M*) at all percentiles tested. The highest treatment, however, may already account for beginning high-dose inhibition. Hence, results indicate that low-dose toxicity by HHCB was less pronounced on root elongation compared to shoot elongation.

Dose-response modelling

HHCB exposure lead to monophasic responses at the population level for both endpoints measured (**Fig. 1; Supplement Table A.1 and A.2**). Regarding shoot growth, the ED_{50} value was 0.316±0.031 m*M*. Modelling dose-response curves for the 20 and 95 %ile revealed as well only monophasic relationships (**Fig. 2; Supplement Table A.1**). A high variability was observed among the slow-growing individuals, so that the biphasic model could not provide a significant fit to the data despite a triphasic low-dose trend in the data in the form of a horizontal s-shaped curve in the low-dose range with a slight inhibition before the stimulatory peak. However, this kind of dose-response pattern cannot be captured well by the current biphasic model (Brain and Cousens 1989), but for example by the hormetic model by Cedergreen et al. (2005) which allows the curve to go down before the hormetic increase. Nevertheless, due to the high variability the Cedergreen et al. (2005) model could not be fitted to the data.

The ED_{50} for the 20 %ile was 0.076±0.026 m*M* and, thus, significantly lower (4.2-fold) as compared to the ED_{50} of the entire population indicating a higher sensitivity of the slow-growing seedlings. The fast-growing part seemed to be less prone to HHCB and showed an ED_{50} of 0.876±0.074 m*M* corresponding to a 2.8-fold higher value compared to the mean population and 11.5-fold compared to the slow-growing seedlings. Hence, the more vigorously growing seedlings were significantly less sensitive to HHCB than most of the population and needed considerably higher doses to be inhibited. Therefore, shoot elongation of the slow- and fast-growing seedlings clearly showed selective dose inhibition after an HHCB exposure and indicated an impact on the size distribution.

Regarding root growth, the ED_{50} value at the population level was 0.216±0.025 m*M*. Modelling dose-response curves for the 10 and 95 %ile revealed only monophasic relationships that did however not significantly differ in ED_{50} from the population mean (**Fig. 2**). A high variability occurred again among the slow-growing individuals so that any visible bi- or triphasic trend in the data could not be significantly modelled. The ED_{50} for the 10 %ile was 0.267±0.195 m*M*. Root growth of the fast-

growing individuals was more homogenous and showed an ED_{50} of 0.200±0.013 m*M*. For that reason, HHCB exposure did not cause selective effects on root elongation of the slow- and fast-growing seedlings.

4*-tert-*Octylphenol

Selective low-dose effects

According to *Tukey* test, the mean shoot growth of four treatments was not statistically different from the control (**Table 3**). Regarding the slow-growing seedlings (≤25 %iles), no significant selective low-dose effects occurred. Shoot growth of the most fast-growing seedlings (≥90 %iles) was also not selectively stimulated, but inhibited at two doses (0.002 and 0.02 m*M*) at all percentiles tested. This indicated the presence of some low-dose toxicity on shoot growth in the fast-growing subpopulations, although not very prevalent.

Regarding mean root elongation, seven out of 12 treatments were not significantly different from the control (**Table 3**). Root growth of the slow-growing seedlings showed no significant selective low-dose effects. Among the fast-growing seedlings, five treatments caused significant inhibition at between one and all percentiles tested (0.000067, 0.00067, 0.002, 0.0067 and 0.02 m*M*). This widespread selective inhibition of root elongation among mainly the fast-growing part of the population clearly indicated the presence of low-dose toxicity, while selective low-dose stimulation was again absent.

Dose-response modelling

4-*tert*-Octylphenol exposure led to monophasic responses at the population level for both endpoints measured (**Fig. 3; Supplement Table A.1 and A.2**). The ED_{50} value for shoot elongation was 0.099±0.008 m*M*. Modelling dose-response curves for shoot growth at the 25 and 95 %ile revealed as well only monophasic relationships (**Fig. 4; Supplement Table A.1**). A high variability was again observed among the slow-growing 25 %ile showing an ED_{50} of 0.062±0.018 m*M*. This value was significantly lower (1.6-fold) as compared to the ED_{50} of the entire population indicating a higher

sensitivity of the slow-growing seedlings. The response of the fast-growing part (95 %ile) seemed to be more stable with an ED_{50} for shoot elongation of 0.117±0.008 m*M*. This value was not significantly higher than that of the mean population, but corresponded to a 1.9-fold higher value compared to the slow-growing seedlings. Therefore, the most fast-growing seedlings were less sensitive to 4-*tert*-octylphenol than the slow-growing seedlings and needed considerably higher doses in order to be inhibited.

Regarding root growth, the ED_{50} value for 4-*tert*-octylphenol at the population level was 0.102±0.008 m*M* (**Fig. 3**). As to the subpopulation levels (23 and 95 %ile), modelling dose-response curves for root length revealed that a biphasic dose-response curve provided the best fit for the slow-growing seedlings despite an insignificant *f* value, while responses of the fast-growing seedlings were monophasic (**Fig. 4; Supplement Table A.2**). The maximum stimulation (y_{max}) was 185±51 % at a dose *M* of 5.8 ± 2.7 µ*M*. The ED_{50} value for the slow-growing seedlings was 0.074±0.037 m*M*, which was not significantly different compared to the ED_{50} of the mean population. Considering the fast-growing 95 %ile, the ED_{50} for root growth was 0.124±0.008 m*M*. This value was significantly higher as compared to the mean population (1.2-fold) and the slow-growing subpopulation (1.7-fold). Consequently, there was some selective hormesis in the population albeit restricted to the slow-growing seedlings and masked at the population level. The most fast-growing seedlings were also significantly less sensitive to 4-*tert*-octylphenol in root growth as most of the population.

Discussion

This study aimed at investigating selective low-dose toxin effects and dose-dependent selectivity from an environmental perspective by exposing a wild plant population to two emerging contaminants at environmentally relevant concentrations. The two chosen toxicants did not cause selective low-dose stimulation without changing the mean plant size and did not induce significant hormesis in root or shoot growth despite one exception observed with 4-*tert*-octylphenol at the slow-growing

subpopulation. A wide absence of hormesis does not necessarily mean that a compound is generally not hormetic since there are several factors that influence the occurrence and expression of a hormetic response in plants (Belz and Piepho 2014). Changing the experimental setup in terms of a prolonged timeframe, different test parameters, or several lower concentrations can sometimes reveal hormesis. Microbial interactions, resource competition and pests can be the reason for the lack of hormesis in many plant stands (Hansi et al. 2014, Płociniczak et al. 2013, 2016, Yu et al. 2015). Additionally, the expression of stimulatory responses seems to be linked to the species or biotype used, so that some plants are simply prone to lack hormesis. For example, Rodriguez et al. (2012) identified one quantitative trait locus (QTL) on a chromosome that caused the natural variation and the lack of hormesis in the heat-stress response of different biotypes of the nematode *Caenorhabditis elegans* Maupas. This finding suggests that natural variation in hormesis and its absence may have a genetic background. Furthermore, the lack of hormesis can also be due to growth conditions since both poor and exceptionally optimized conditions have been shown to cause the absence of hormesis even though a compound would otherwise induce hormesis (Belz and Cedergreen 2010). For instance, an increase in leaf area of *Sinapis arvensis* L. (wild mustard) under a parthenin exposure was lacking during a non-optimal warm period, yet the enhancement occurred under cooler conditions (Belz 2008). The same phenomenon was observed with glyphosate-exposed *Hordeum vulgare* L. (barley) by altering the CO_2 supply. The amount of biomass of *H. vulgare* in response to glyphosate did not increase at below ambient concentrations even though the hormetic response was observed at ambient and even higher CO_2 levels (Cedergreen and Olesen 2010). Additionally, a study conducted with parthenin-exposed *Lactuca sativa* L. (lettuce) revealed that hormesis was absent under exceptionally good growing conditions (Belz and Cedergreen 2010). It has been surmised that the cell growth rate under optimal conditions is already at maximum and cannot be further enhanced (Vichi and Tritton 1989). Hence, a toxicant-induced hormesis seems to be most pronounced at below maximal, but still at favorable environmental conditions.

Selective low-dose toxicity of mainly fast-growing seedlings seemed to be characteristic for low-dose effects of HHCB and 4-*tert*-octylphenol on *G. elegans*, although this phenomenon was not very prevalent. Previous studies by Sinkkonen et al. (2008, 2011) with the same contaminants were ten times smaller in scale than the current well replicated study and used separate individuals as replicates, while the current study uses percentile and mean values per dish as replicates. Furthermore, previous studies used commercially cultivated plant species or weed species rather than a more heterogeneous, wild plant population. Therefore, this study clearly indicates that the phenomenon of selective low-dose effects within a population also holds true under ecologically more relevant conditions and represents a further step towards the elucidation of the ecological significance of this low-dose phenomenon.

Low-dose toxicity may be linked to density-dependent phytotoxicity, so that when plants share the same toxicant pool in dense plant stands (Weidenhamer et al. 1989, Sinkkonen 2001, 2003), the fast-growing individuals can take up higher amounts of toxicants due to their higher activity (Sinkkonen et al. 2009). Regarding high-dose selective toxicity, the slow-growing seedlings showed the highest sensitivity to the chosen compounds, followed by the mean population, while the fast-growing subpopulation seemed to be the most inert part of the population. This pattern of sensitivity was rather consistent throughout our findings irrespective of the compound or endpoint investigated. A decrease in sensitivity among the fast-growing subpopulation and a respective increased sensitivity among the slow-growing seedlings has also been observed in previous studies using other test species and chemicals (Belz and Sinkkonen 2016a). Nonetheless, compared to our previous studies with other toxins and plant species, both compounds showed a rather low capacity to differentiate populations of *G. elegans*, especially at low doses.

Selective effects of HHCB exposure

Under an HHCB exposure, the shoot growth of the slow-growing subpopulation was adversely affected by several different doses, yet the fast-growing subpopulation remained widely unaffected

whether shoot or root growth was considered. Based on this, it seems that there is some low-dose toxicity with HHCB, but it did not seem to be as prevalent and characteristic mainly for the fast-growing subpopulation as expected from earlier findings with other plant species. This indicated that the pattern and expression of selective low-dose effects may depend on the effective compound and/or the exposed plant species. The finding that *G. elegans* is rather inert to low-dose selective effects of HHCB corresponds to previous reports where low-dose toxicity of HHCB was also not very pronounced (Sinkkonen et al. 2011).

The detected environmental concentration of 0.00631 µ*M* (**Table 1**) (Litz et al. 2007) closely corresponds to the lowest dose actually tested and causing significant, selective inhibition of shoot growth in both fast- and slow-growing seedlings (0.0067 µ*M*). Although the observed ED_{50} values are several magnitudes higher, this observation clearly substantiates the environmental significance of low-dose exposures of HHCB in terms of alterations in size distribution of exposed plant populations by selective low-dose toxicity.

Selective effects of 4-*tert*-octylphenol exposure

Regarding 4-*tert*-octylphenol, selective low-dose toxicity occurred more profoundly among the fast-growing subpopulation since both shoot and root elongation were selectively inhibited at certain low-doses, while the slow-growing part of the population remained unaffected. Especially root growth of the fast-growing seedlings showed selective low-dose toxicity by 4-*tert*-octylphenol, which is in line with a previous study showing significant reduction in root length of *G. elegans* among the fast-growing subpopulation after 4-*tert*-octylphenol exposure (Sinkkonen et al. 2011). This is an important revelation as the previous study was produced in a different laboratory using another root measurement method. Therefore, the current study finds the first proof that selective low-dose toxicity is a persistent phenomenon.

Regarding selective hormetic effects, 4-*tert*-octylphenol was only stimulatory towards root growth of slow-growing *G. elegans* plants with a maximum of 85 % stimulation above control. Because this

selective enhancement of growth was masked at the population level, the results confirm the assumption that the stimulation of fast-growing seedlings is the decisive factor for the formation of hormesis at the population level (Belz and Sinkkonen 2016a, b). Additionally, the ED_{50} values revealed significant differences in sensitivity of the different subpopulations compared to the mean population with the slow-growing seedlings being the most sensitive group and then the fast-growing seedlings being the least sensitive. Hence, 4-*tert*-octylphenol clearly has a potential to dissect a plant population at low-doses. However, compared to the reported environmental concentration of 0.00044 µ*M* in wastewater treatment plant effluent (**Table 1**) (Höhne et al. 2008), the lowest dose actually tested and causing significant, selective inhibition of root growth was several fold higher (0.067 µ*M*) so that 4-*tert*-octylphenol would not seem to cause either adverse or stimulatory selective effects on *G. elegans* at such environmental levels.

Dose-response modelling

For several curves generated for HHCB, there was clearly a trend that a slight decrease in response was followed by an enhancement in both shoot and root lengths not only among the slow-growing seedlings, but also fast-growing ones and even at the mean population level. This so called triphasic dose-response cannot be captured by a monophasic model (Streibig 1988), which widely ignores this low-dose depression in responses. However, to a certain extent the biphasic model of Cedergreen et al. (2005) is flexible enough to capture a triphasic pattern (Belz and Piepho 2012). In this study, a triphasic pattern could however not be significantly fitted whether the dose-response modelling was based on the individual values from each replicate per treatment or the mean values per treatment in order to dampen the variability observed in the data especially at the low percentiles. This apparent lack of a better fit than the monophasic model may account for the partly high variability of responses, but also an insufficient number of treatments covering the observed low-dose depression. In the latter case, a triphasic curve is not easy to model which is probably why triphasic curve shapes are seldom

reported. However, there are some previous studies showing this phenomenon (Belz and Piepho 2012).

Practical implications

Previous studies performed with commercially cultivated *L. sativa* have established the parallel occurrence of hormesis, selective low-dose toxicity and selective low-dose stimulation within a dense plant population (Belz and Sinkkonen 2016a). Based on the genetic homogeneity of a cultured species such as *L. sativa*, these findings should be applied carefully to natural plant populations since the genetic variation of wild plants is expected to be more pronounced (Belz and Sinkkonen 2016a). Nevertheless, the hypothesis that low toxicant levels can segregate certain plant populations has already been confirmed with an agricultural weed population (Belz and Sinkkonen 2016b). Compared to this, our results showed rather weak low-dose effects of the chosen compounds on populations of *G. elegans.* This provokes the question of whether this is due to the compounds tested or the plant species used. Hormetic effects on plants have not been studied before using HHCB and 4-*tert*-octylphenol, so that it is unknown if the observed lack of hormesis is a common situation. However, *G. elegans*, as a wild plant, expressed a rather pronounced variation response-wise, which made it difficult to observe significant low-dose effects. The high variability of responses among the slow-growing seedlings seemed to disturb the detection of significant low-dose effects and thus acted as an argument for focusing on higher %iles for dose-response modelling compared to our previous studies. Additionally, due to the relative slow development of *G. elegans* compared to the previously studied fast-developing *L. sativa* and the more pronounced occurrence of selective low-dose toxicity in *L. sativa* (Belz and Sinkkonen 2016a), it can be hypothesized that species with a low overall growth rate are less prone to develop selective low-dose effects.

Since there is a vast amount of harmful chemicals at low doses in the environment (for example, Klaschka et al. 2012), the likelihood that natural vegetation is exposed to compounds causing selective effects at low-doses seems to be very realistic. The importance of studying the topic of low-

dose effects has been emphasized before (Belz and Sinkkonen 2016a), especially in relation to extreme weather conditions such as drought, since a change in root size distribution is of utmost importance for the efficiency of water uptake and, thus, the survival of plants (Sinkkonen et al. 2009). If a population is simultaneously exposed to both drought and low toxicant doses, the fast-growing seedlings seem to be more prone to an inhibition of root growth, as observed in this study. This can decrease the overall survival of that plant population, such that the plants that would be the most likely to survive from drought are now inhibited and may not be able to contribute to the survival of the whole plant population. Additionally, in natural conditions, chemical exposure is likely to be rather continuous, especially since both of the studied compounds have shown a tendency to persist in soil (Litz et al. 2007, Chen et al. 2013). This kind of prolonged low-dose exposure could cause further effects even on more slow-growing species such as *G. elegans.* Overall, it has been surmised that such low-dose driven changes in the structure of a population are directly related to plant performance and survival under extreme environmental conditions (Chu et al., 2008, 2009) and, hence, ultimately to reproduction. This may change population dynamics and lead to genotypic adaptations and/or ecotype formation in the longer term with ecologically significant consequences for the ecosystem or biodiversity (Sinkkonen et al. 2009).

Despite the fact that toxicant levels detected from the environment tend to be rather low, usually in the ng/L to µg/L range (Klaschka et al. 2012, Tao et al. 2011), it indeed seems that such negligible concentrations have the potential to lead to negative growth effects within a plant population even though such concentrations would have been considered safe in ecotoxicological bioassays. A similar trend was observed by Sinkkonen et al. (2009) who further suggested that current laboratory standards in risk evaluation should include the possibility of low-dose effects and that re-evaluation of the threshold levels for environmental contaminants would be needed. One of the lowest predicted no effect concentrations (PNEC) in aquatic environments regarding HHCB is 0.23 µ*M* for the fish *Pimephales promelas* Rafinesque (fathead minnow) (HERA 2004). As we observed adverse selective

effects at levels that were tens of times lower, the current study implicates a need to re-evaluate the test protocol of PNECs.

Supplementary data

Table A.1 Regression parameters from the monophasic modeling (Streibig 1988) of toxin effects on shoot growth of *Gypsophila elegans*. Data given as mean ± standard error.

Table A.2 Regression parameters and estimated quantitative features from the monophasic (Streibig 1988) or biphasic (Brain and Cousens 1989) modeling of toxin effects on root growth of *Gypsophila elegans*. Data given as mean ± standard error.

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Compliance with Ethical Standards

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Conflict of Interest: All authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

Table 1 Some chemical properties and environmental concentrations of HHCB and 4-*tert*-octylphenol.

Chemical name	Formula	Molecular weight [g/mol]	CAS no.	Effluent [µ*M*]	Sludge [mg/kg d.m.]
HHCB 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[g]-2-benzopyran	$C_{18}H_{26}O$	258.41	1222-05-5	0.00631 [1]	20.3 [2]
4-*tert*-octylphenol 4-(1,1,3,3-tetramethylbutyl)phenol	$C_{14}H_{22}O$	206.32	140-66-9	0.00044 [3]	0.08-0.20 [4]

[1] Klascka *et al.* 2012, [2] Kupper *et al.* 2004, [3] Höhne *et al.* 2008, [4] Bolz *et al.* 2001; d.m.=dry mass

Table 2 Statistical significance of low-dose effects of HHCB on shoot and root length of *Gypsophila elegans* at different percentiles. Displayed are only concentrations for which mean root/shoot length at the population level was not significantly different from control (*Tukey*-test at α=0.05 for four blocks with six replicates per concentration (mm)).

End-point	Dose [mM]	Mean	Percentile 5 %	8 %	10 %	15 %	20 %	90 %	95 %	97 %	99 %
shoot length [mm]	**control**	**6.6**	**0.8**	**1.5**	**2.0**	**3.0**	**3.8**	**10.6**	**11.6**	**12.0**	**12.9**
	0.0000067	5.8	0.1*	0.3*	0.6*	2.0*	3.1	9.7	10.6	11.2	12.1
	0.000067	6.2	0.3	0.5	1.1*	2.3	2.9	10.5	11.7	12.2	13.1
	0.00067	6.3	0.2	0.6*	1.1*	2.1	3.1	10.5	11.4	11.9	12.6
	0.002	6.8	0.7	1.3	1.8	3.0	4.0	11.0	11.7	12.4	13.3
	0.0067	6.1	0.0*	0.2*	0.3*	1.1*	2.0*	10.8	11.9	12.5	13.6
	0.013	5.4	0.3	0.5	0.8*	1.4*	2.3	9.3*	10.3*	10.8	11.7
	0.02	5.6	0.3	0.7*	0.9*	1.5*	2.3	9.8	10.7	11.2	12.1
root length [mm]	**control**	**13.3**	**0.8**	**1.5**	**1.8**	**3.6**	**5.4**	**23.5**	**25.6**	**27.2**	**30.4**
	0.0000067	11.5	0.1*	0.4	1.3	2.7*	4.2	20.7*	23.5*	25.0*	27.7*
	0.000067	12.2	0.8	1.4	1.6	3.0	4.4	22.8	25.6	27.2	29.6
	0.00013	11.7	0.3	0.7	1.2	2.5*	4.1	22.1	24.7	26.4	28.9
	0.00067	12.8	0.5	1.1	2.0	3.7	5.4	22.9	25.4	26.4*	28.7
	0.002	14.0	1.1	2.0	2.8	4.8	6.4	23.9	26.3	28.2*	30.9
	0.0067	13.1	0.2	0.9	1.4	2.9	4.8	23.9	27.0	28.2	30.4
	0.013	12.0	0.5	1.0	1.9	3.3	4.6	21.6	23.8	25.1	27.0*
	0.02	12.5	0.9	1.7	2.3	3.4	4.7	22.5	25.1	26.4	29.1
	0.067	11.3	0.9	1.6	1.9	3.2	4.4	20.8*	22.8*	24.0*	25.9*

'*' significantly different from control according to *Mann-Whitney U* test at α=0.05.

Table 3 Statistical significance of low-dose effects of 4-*tert*-octylphenol on shoot and root length of *Gypsophila elegans* at different percentiles. Displayed are only concentrations for which mean root/shoot length at the population level was not significantly different (ns) from control (*Tukey*-test at α=0.05 for four blocks with six replicates per concentration (mm)).

End-point	Dose [mM]	Mean	Percentile 20 %	22 %	23 %	25 %	90 %	95 %	97 %	99 %
shoot length [mm]	**control**	**7.0**	**1.4**	**1.6**	**1.9**	**2.6**	**12.2**	**13.0**	**13.5**	**14.8**
	0.000067	6.3	0.2	0.5	0.9	2.0	11.6	12.6	13.3	14.5
	0.00067	6.8	1.3	2.0	2.2	2.4	12.4	13.7	14.6	15.7
	0.002	6.2	1.5	2.0	2.2	2.4	10.9*	11.7	12.3	13.3
	0.02	5.9	1.5	1.7	1.9	2.5	10.6*	11.5*	12.1*	13.1*
root length [mm]	**control**	**13.3**	**1.4**	**1.7**	**2.0**	**3.0**	**27.6**	**30.7**	**32.4**	**34.7**
	0.000067	12.0	0.3	0.6	1.0	2.3	24.7*	28.1*	29.3*	31.0*
	0.00067	12.5	1.3	2.0	2.2	2.7	26.1*	28.8*	30.6	33.7
	0.002	13.1	1.7	2.4	2.7	3.3	26.0*	28.9*	31.1	34.8
	0.0033	12.7	0.8	1.5	1.9	2.4	26.9	29.7	32.0	35.0
	0.0067	12.2	1.5	2.2	2.5	3.0	25.3*	28.5*	30.3	33.0
	0.013	12.0	1.0	1.2	1.4	1.8	26.4	29.6	31.3	34.2
	0.02	12.3	1.5	1.9	2.3	3.3	25.7*	30.2	32.2	35.5

'*'significantly different from control according to *Mann-Whitney U* test at α=0.05.

Figure Captions

Fig. 1 Dose-response curves for the effects of HHCB on shoot length (a) and on root length (b) of *Gypsophila elegans* at the population level. The dotted line shows the ED_{10} value which distinguishes the low-dose range from high-dose inhibitory effects. Doses that are significantly different from the control are marked with an asterisk '*' (*Tukey* test, $\alpha = 0.05$).

Fig. 2 Dose-response curves for the effects of HHCB on shoot length of *Gypsophila elegans* at the 20 and 95 % percentiles (%iles) (a) and on root length at the 10 and 95%ile (b) of the tested population. The dotted line shows the ED_{10} value which distinguishes the low-dose range from high-dose inhibitory effects. Doses that are significantly different from the control are marked with an asterisk '*' (*Mann-Whitney U* test, $\alpha = 0.05$).

Fig. 3 Dose-response curves for the effects of 4-*tert*-octylphenol on shoot length (a) and on root length (b) of *Gypsophila elegans* at the mean population level. The dotted line shows the ED_{10} value which distinguishes the low-dose range from high-dose inhibitory effects. Doses that are significantly different from the control are marked with an asterisk '*' (*Tukey* test, $\alpha = 0.05$).

Fig. 4 Dose-response curves for the effects of 4-*tert*-octylphenol on shoot length of *Gypsophila elegans* at the 25 and 95 %iles (a) and on root length at the 23 and 95%ile (b) of the tested population**.** The dotted line shows the ED_{10} value which distinguishes the low-dose range from high-dose inhibitory effects. Doses that are significantly different from the control are marked with an asterisk '*' (*Mann-Whitney U* test, $\alpha = 0.05$).

Figure 1

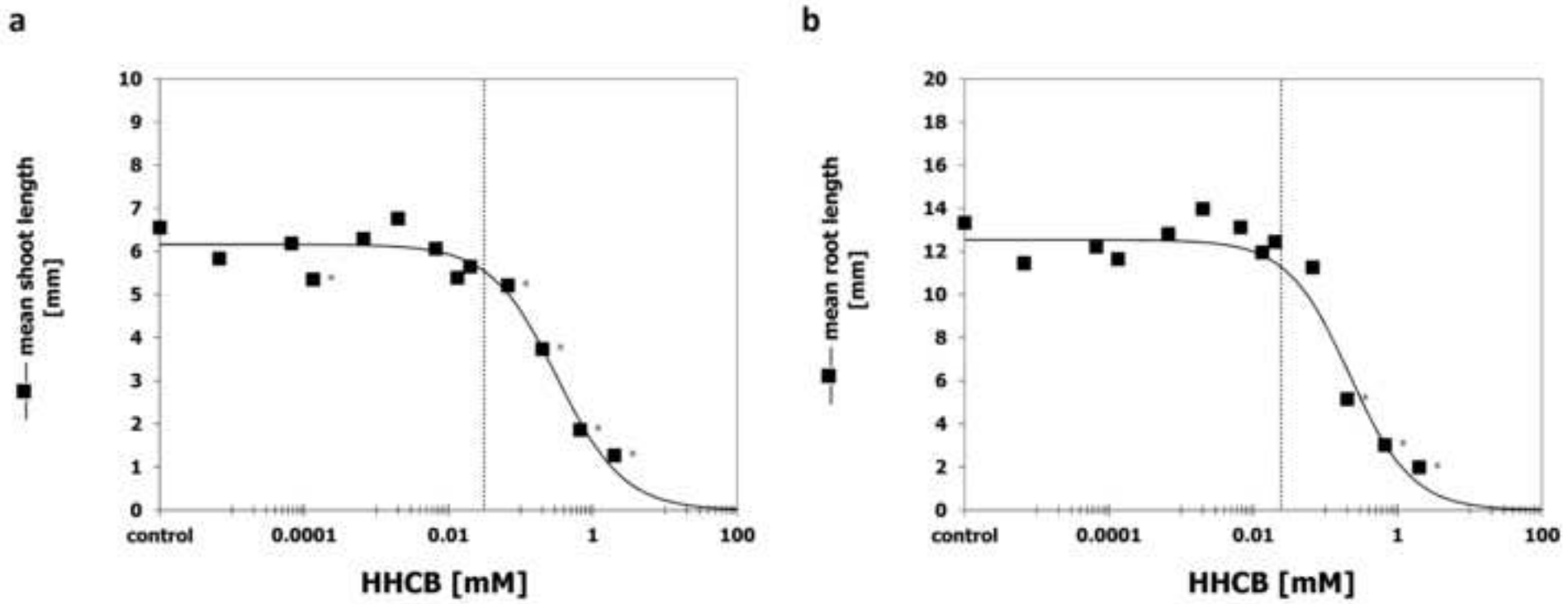


Figure 2

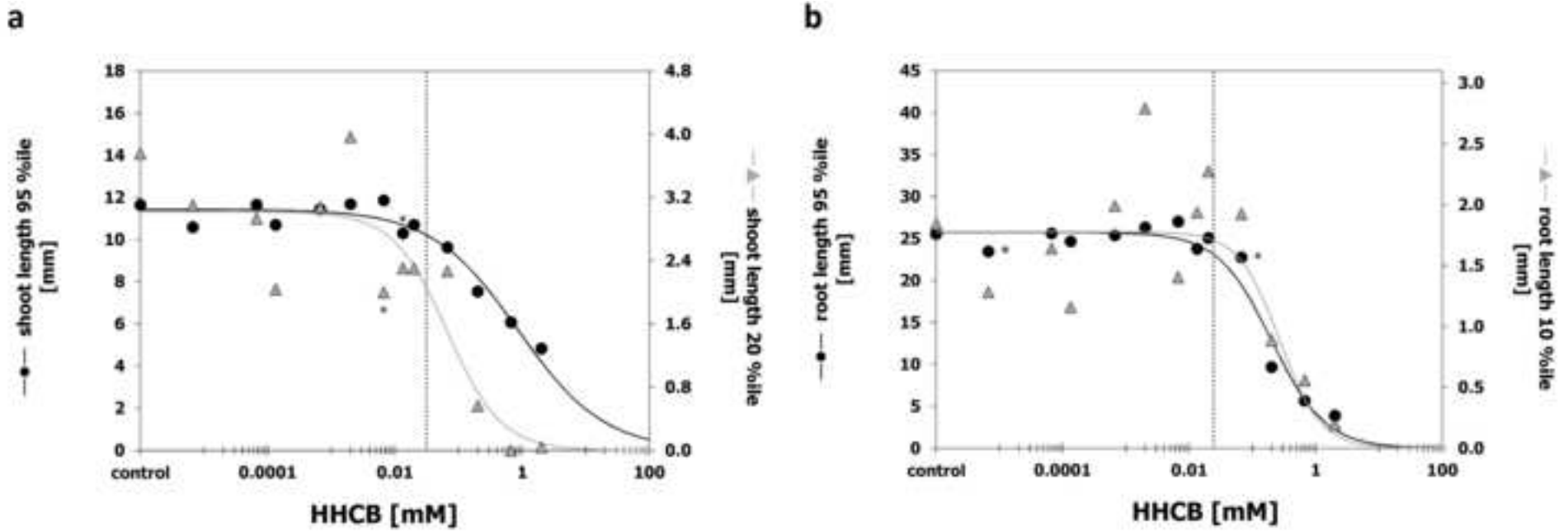


Figure 3

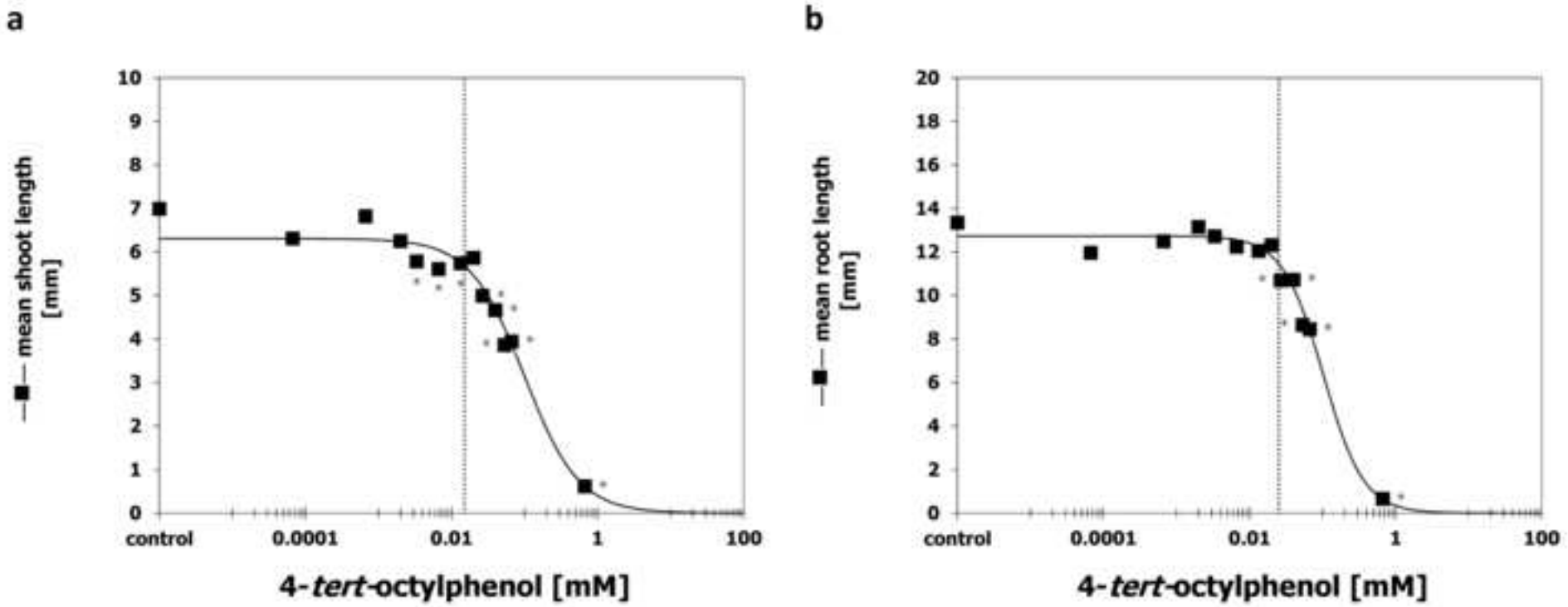
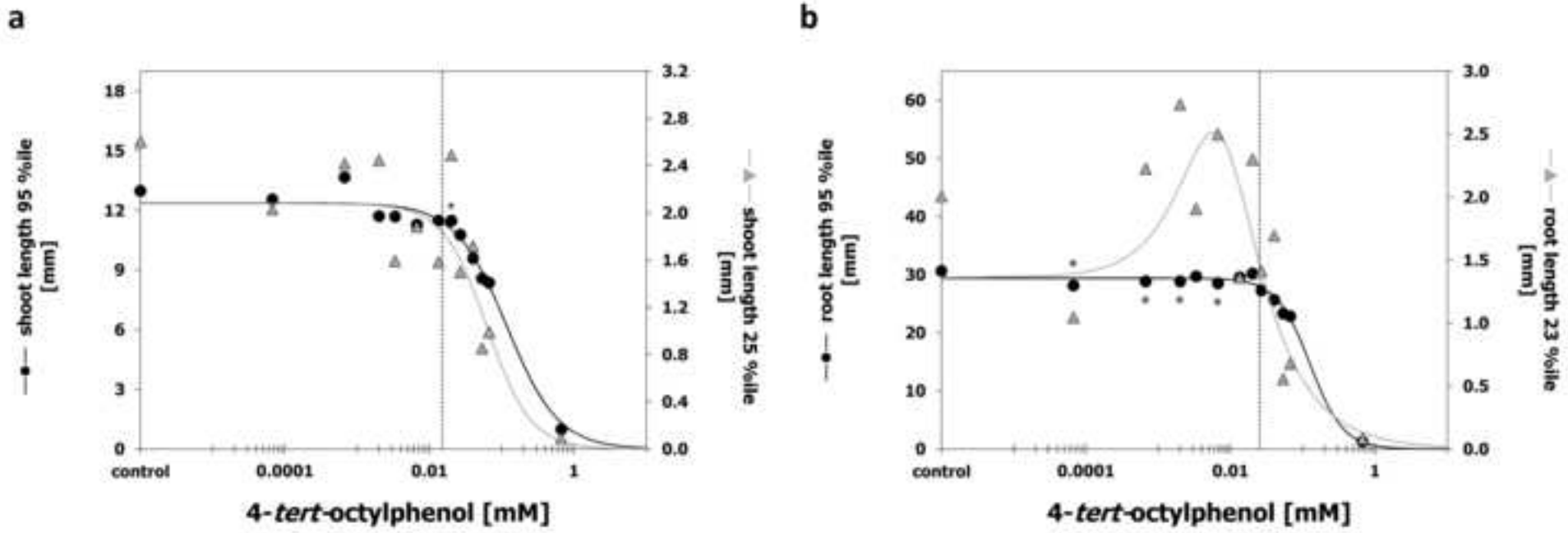


Figure 4



Supplement Table A.1 Regression parameters from the monophasic modeling (Streibig 1988) of toxin effects on shoot growth of *Gypsophila elegans* . Data given as mean ± standard error.

toxin	percentile	model providing best fit	*d* [mm]	*b*	ED_{50} [m*M*]	ED_{50} 95% CI [m*M*]
HHCB	**20%**	monophasic	3.01 ± 0.16	1.17 ± 0.32	0.076 ± 0.026 c	0.024-0.127
	95%	monophasic	11.46 ± 0.10	0.60 ± 0.03	0.876 ± 0.074 b	0.732-1.021
	population mean	monophasic	6.17 ± 0.09	0.91 ± 0.08	0.316 ± 0.031 a	0.256-0.376
4-*tert*-octylphenol	**25%**	monophasic	2.08 ± 0.19	1.43 ± 0.68	0.062 ± 0.018 b	0.026-0.098
	95%	monophasic	12.37 ± 0.15	1.22 ± 0.11	0.117 ± 0.008 a	0.101-0.133
	population mean	monophasic	6.31 ± 0.11	1.16 ± 0.07	0.099 ± 0.008 a	0.084-0.115

CI = confidence interval; small letters indicate significant differences between ED_{50} values at α=0.05

Supplement Table A.2 Regression parameters and estimated quantitative features from the monophasic (Streibig 1988) or biphasic (Brain and Cousens 1989) modeling of toxin effects on root growth of *Gypsophila elegans* . Data given as mean ± standard error.

toxin	percentile	model providing best fit	*d* [mm]	*b*	*f*	*M* [µ*M*]	ED_{50} [m*M*]	ED_{50} 95% CI [m*M*]	y_{max} [%]
HHCB	**10%**	monophasic	1.78 ± 0.24	1.34 ± 1.00	n.s.	-	0.267 ± 0.195 a	-0.118-0.651	-
	95%	monophasic	25.74 ± 0.29	1.03 ± 0.06	n.s.	-	0.200 ± 0.013 a	0.173-0.226	-
	population mean	monophasic	12.54 ± 0.23	1.00 ± 0.09	n.s.	-	0.216 ± 0.025 a	0.167-0.265	-
4-*tert*-octylphenol	**23%**	biphasic	1.36 ± 0.30	1.74 ± 0.26	470 ± 440	5.789 ± 2.669	0.074 ± 0.037 a	0.002-0.147	185 ± 51
	95%	monophasic	29.39 ± 0.22	1.88± 0.13	n.s.	-	0.124 ± 0.008 b	0.108-0.141	-
	population mean	monophasic	12.74 ± 0.18	1.57 ± 0.11	n.s.	-	0.102 ± 0.008 a	0.086-0.118	-

n.s. = no significant hormesis; CI = confidence interval; small letters indicate significant differences between ED50 values at α=0.05