



Plant growth responses to inorganic environmental contaminants are density-dependent: Experiments with copper sulfate, barley and lettuce



Mari Hansi^a, Jeffrey D. Weidenhamer^b, Aki Sinkkonen^{a,c,*}

^a University of Helsinki, Department of Environmental Sciences, Section of Environmental Ecology, Niemenkatu 73, 15140 Lahti, Finland

^b Ashland University, Department of Chemistry, Ashland, OH 44805, USA

^c Lawrence Berkeley National Laboratory, Earth Sciences Division, 1 Cyclotron Road, MS 70A, Berkeley, CA 94720, USA

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ABSTRACT

The density-dependence of terrestrial plant–plant interactions in the presence of toxins has previously been explored using biodegradable compounds. We exposed barley and lettuce to four copper concentrations at four stand densities. We hypothesized that toxin effects would decrease and Cu uptake would increase at increasing plant densities. We analyzed toxin effects by (a) comparing plant biomasses and (b) using a recent regression model that has a separate parameter for the interaction of resource competition and toxin interference. Plant response to Cu was density-dependent in both experiments. Total Cu uptake by barley increased and the dose per plant decreased as plant density increased. This study is the first to demonstrate that plant density mediates plant response to metals in soil in a predictable way. This highlights the need to explore the mechanisms for and consequences of these effects, and to integrate the use of several plant densities into standard ecotoxicological testing.

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1. Introduction

Environmental pollutants are a serious worldwide problem, and may affect all ecological functions ranging from microbial activity to primary production by exposed plants (Mallick and Mohn, 2003; Sinkkonen et al., 2010; Kauppi et al., 2012). Although standardized ecotoxicological assays have been developed to measure plant response to pollutants *in vitro*, plant response to toxins in field conditions varies so much that reliable *in situ* estimation of the response is currently lacking (Navarro et al., 2008). One largely unappreciated reason for variability in plant response to pollutants is the phenomenon of toxin dilution by plants in dense plant stands (Yoda et al., 1963; Weidenhamer et al., 1989). Toxin dilution is thought to occur because plants share and “compete” not only for resources but also for toxins (Hoffman and Lavy, 1978). Thus, the dose of a pollutant received by a plant is inversely related to plant density.

Toxin dilution can be studied using density–response experiments. The basic idea of density–response experiments is simple.

Plants are grown at several plant densities, and the dose of toxin added per unit soil volume is kept constant (Weidenhamer et al., 1989; Sinkkonen, 2001, 2003). As a result, the toxin dose received by a plant is inversely related to plant density. In other words, the dose per plant becomes diluted as plant number increases. The higher the plant density, the less plant response is altered by the toxin, as compared to control plants that grow in toxin-free but otherwise identical conditions. In the 1980’s, Weidenhamer et al. (1987, 1989) formulated a graphical model where toxin dose per plant determined the outcome of a density–response experiment (see also Thijs et al., 1994). They predicted that depending on concentration, maximum plant size may occur at intermediate plant densities if homogeneously spread phytotoxins affect plant growth, with plant size greatly reduced at low plant densities (due to effects of high toxin doses per plant), and also at high plant densities (due to intense resource competition). Such a result is contrary to the predicted effects of resource competition, which predicts maximum plant size at low plant density, and thus this phenomenon is a potential tool for exploring the effects of toxins on plant growth.

Based on enzyme kinetics, Sinkkonen (2001, 2003, 2006, 2007) modeled the outcome of density–response experiments. He also added the possibility of density-dependent hormesis to the dilution

* Corresponding author.

E-mail addresses: ATSinkkonen@lbl.gov, aki.sinkkonen@helsinki.fi (A. Sinkkonen).

model by Weidenhamer et al. (1989). San Emeterio et al. (2007) used the work of Sinkkonen, Weidenhamer and colleagues to develop a model which takes into account the effects of both chemical interference and resource competition on the growth of individual plants in density–response experiments. The model includes separate parameters for resource competition and chemical interference, making it possible to estimate whether – under certain conditions – resource competition is significant but chemical interference or the interaction of these two is not (details in Materials and Methods). If the interaction is not significant, toxin interference is constant regardless of the intensity of resource competition, and toxin effects are not diluted as plant density increases.

The phenomenon of density-dependence of toxin interactions has thus been firmly established but it has been tested only with natural phytotoxins and herbicides (Weidenhamer, 2006). The aim of this study was to test the phenomenon by exposing two annual plant species to copper sulfate, an inorganic compound known to cause toxicity symptoms in plants at high doses (Wong and Bradshaw, 1982; Michaud et al., 2008). We hypothesized that toxicity will vary with plant density as predicted by San Emeterio et al. (2007)'s model, and that toxin dilution can also be distinguished by characteristic density-dependent growth responses as suggested by Weidenhamer et al. (1989). Copper concentrations in plants were analyzed to estimate whether plant density changes the total amount of copper taken up from soil. As far as we know, this is the first time that the density-dependence of plant growth responses has been evaluated in plants with an elemental environmental contaminant.

2. Materials and methods

2.1. Experimental setup

Barley (*Hordeum vulgare* L. var. Scarlett) and lettuce (*Lactuca sativa* var. crispata) were used as their response to Cu has been studied: barley is known to tolerate higher Cu concentrations than lettuce (Ait Ali et al., 2004; Inaba and Takenaka, 2005). Seeds of barley and lettuce were germinated in Petri dishes on moist Whatman filter paper. Seedlings (root length at least 1 mm) were transferred to pots on day 0. Plants were grown in 300 ml polyethylene pots that contained 350 g soil (surface area 0.5 dm²). The pots were randomized once a week in the growth room. Soil was a 2:1 mixture of glacial sand (grain size 1–2 mm; Loimaan hiekka, Loimaa, Finland) and quartz (grain size 0.1–0.6; Nilsian kvartsi, Nilsia, Finland) in both experiments. Four copper sulfate (Cu(II)SO₄·5H₂O; J.T. Baker, Baker analyzed reagent) concentrations and plant densities (1, 5, 15 and 30 plants per pot) were used, and there were always six replicates per treatment. Toxic Cu concentrations were estimated in pre-experiments and they were 0, 15, 50 and 100 µg Cu g⁻¹ dw soil for barley and 0, 7, 12.5 and 15 µg Cu g⁻¹ dw soil for lettuce. Diurnal rhythm was 16 h light (at least 250 µE m⁻² s⁻¹); 8 h darkness. Plants were watered at least every second day by adding 5–20 ml deionized water, depending on evaporation. This ensured that plants did not experience water deficiency or flooding. Aqueous fertilizer solution (deionized water + Substral® Vital-Aktiv, Henkel-Norden Oy, Germany; 500 mg N (55% nitrate, 45% ammonium), 108 mg P, 416 mg K, 2.5 mg Fe, 0.83 mg B and Mn, 0.42 mg Cu, 0.17 mg Zn, and 0.083 Mo mg l⁻¹ Milli-Q grade water) was added on day 0 (60 ml for barley and 70 ml for lettuce), on days 10 (15 ml) and 16 (25 ml) in the barley experiment and on days 12 (20 ml) and 19 (40 ml) in the lettuce experiment. Instead of receiving 25 ml of normal fertilizer solution, the two lowest densities of barley received 12.5 ml of double dose fertilizer solution on day 16 as evaporation had been significantly smaller compared to higher densities. Plants were harvested on days 20 (barley) and 25 (lettuce), shoots and roots were dried separately at 70 °C for 24 h and plant parts were weighed.

2.2. Chemical analyses

Copper content was determined from barley shoot and roots grown at 100 µg Cu g⁻¹ dw soil treatment following the method by Xiong and Wang (2005). To increase sample size, the dry weights of two randomly selected pots were combined which resulted in three replicate shoot and three replicate root samples per treatment. Each of these samples consisted of all root or shoot biomass from two pots. The samples were ashed in a muffle furnace at 700 °C for 15 h. The ash was dissolved in concentrated HNO₃/HClO₄ [2:1 (v/v)], heated until ash had dissolved, cooled and the extract was diluted to 10 ml with 1 M HNO₃ (Xiong and Wang, 2005).

Table 1

San Emeterio et al. (2007) model parameters for barley and lettuce experiments. Both plant species were grown at four densities and copper sulfate concentrations. α and β describe resource competition, a describes toxin interference, and b describes the interaction term of resource competition and toxin interference.

Parameter	Mean	95% Confidence interval
Barley		
α	0.466	0.456–0.475
β	0.00342	0.00275–0.00410
a	–0.00282	–0.0322 to –0.0242
b	–0.00008	–0.00010 to –0.00005
Lettuce		
α	0.460	0.421–0.499
β	0.00814	0.00463–0.0117
a	–0.0594	–0.0702 to –0.0486
b	–0.00048	–0.0012 to 0.00026

The dilution was stored in glass tubes until rebalanced to 10 ml and analyzed with a flame atomic absorption spectrophotometer (Thermo M-Series; Thermo Electron, Cambridge, UK) according to standards SFS 3044 and SFS 3047.

2.3. Statistical analyses

Plant dry weights (plants/pot) were analyzed using two statistical approaches. The first one is a model adapted from San Emeterio et al. (2007). The simplest form of the model consists of three terms:

$$f(x_d, x_a) = \frac{1}{\alpha + \beta x_d} \exp(a x_a) \exp(-b x_a x_d) \quad (1)$$

In Equation (1), biomass per plant (f) is presented as a function of plant density (x_d) and chemical concentration (x_a) (San Emeterio et al., 2007). The first term is a hyperbolic size-density response function that measures the effect of density. Parameters α and β measure the effect of competition.

The second term measures the effect of the chemical concentration on mean biomass per plant independently of density. If parameter $a > 0$, the effect on biomass is stimulatory and if $a < 0$ toxin effect is inhibitory. The final term measures the interaction between plant density and chemical concentration. Parameter b measures the interaction effect which should be affected by plant density (Weidenhamer et al., 1989; Sinkkonen, 2001; San Emeterio et al., 2007). Parameters were calculated with NLIN method in SAS 9.1. (SAS Institute Inc., Cary, North Carolina). Plant dry weights were log transformed before the analysis.

San Emeterio et al. (2007) also introduced a piecewise version of the model; the authors supposed that if plant density is high enough, toxin dilution disappears as toxin effects are negligible. We tested the piecewise version of the model but do not present the results as the piecewise model decreased F value by 48% in case of barley and the confidence interval of the cutting point included 0 in case of lettuce.

In addition to San Emeterio et al. (2007)'s model, plant dry weights were compared using the method of Weidenhamer et al. (1989): the mean dry weight of control (= no Cu addition) plants at density i was set to 100%, and the mean dry weights of each Cu level at density i were compared with the mean dry weight of the corresponding control. The null hypothesis was that the response of plants exposed to toxins was unaffected by density. According to the toxin dilution hypothesis of Weidenhamer et al. (1989), the toxicity of copper, as measured by a decrease in mean plant weight (% of the corresponding control at that density) was predicted to decrease as plant density increased at one or more Cu treatments. Significant differences between control and exposed plants at each plant density were evaluated using univariate analysis of variance with Tukey's test, and Pearson correlation analysis was used to compare Cu uptake with dry weights and the shoot: root ratio of barley. % dry weights were log transformed before the analyses. Treatments were normally distributed in Kolmogorov–Smirnov test, except in the density 30 – concentration 15 treatment ($p = 0.004$) in the lettuce experiment. Variances were equal in Levene's tests, except at densities 15 ($p = 0.042$) and 30 ($p = 0.005$) in the lettuce experiment and at the density 1 ($p = 0.001$) in the barley experiment. We consider the violations to not change the conclusions drawn in this paper as statistically significant differences existed between the same treatments using parametric (shown in results) or non-parametric Mann–Whitney U and χ^2 tests (not shown). Cu uptake by barley and shoot Cu concentration in barley shoots were analyzed using polynomial regression analysis (100 µg g⁻¹ dw soil treatment).

3. Results

The San Emeterio et al. (2007) model explained plant growth at different plant densities and copper sulfate concentrations (Table 1; $F = 423.6$, $MS = 163,593$, $df = 4, 92$, $p < 0.0001$ for barley; $F = 520.8$,

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