REDUCED GRAZING RATES IN DAPHNIA PULEX CAUSED BY CONTAMINANTS: IMPLICATIONS FOR TROPHIC CASCADES

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Abstract—Ecotoxicological endpoints based on behavioral traits (e.g., predator avoidance, feeding, and locomotion) may be more sensitive and give more insights into patterns of sublethal toxicity than survivorship tests. In this study, the density-dependent grazing rate of Daphnia pulex pre-exposed to p,p’-dichlorodiphenyldichloroethylene (DDE) (insecticide metabolite) and glyphosate (herbicide), via water or a vector, Scenedesmus spp., was assayed in laboratory experiments. The phytoplankton biomass was estimated from the chlorophyll content, and the pesticide uptake and turnover pattern in Daphnia and Scenedesmus were determined from parallel experiments with a radiolabeled source. Scenedesmus spp. relative net growth rate was inversely and linearly related to the density of the grazer. Daphnia pulex exhibited significant reductions in grazing rate: 30% for those pre-exposed to p,p’-DDE via water and 40% for D. pulex pre-exposed to glyphosate via Scenedesmus spp. Through the process of trophic cascading, this impaired grazing allowed Scenedesmus spp. to grow at higher rates, 70 and 60%, respectively. The reduced grazing efficiencies were associated with the treatments that gave the highest body burden of p,p’-DDE (70 µg/g dry wt) and the lowest of glyphosate (13 mg/g dry wt). The pattern of results suggests a toxic effect of p,p’-DDE on D. pulex and a growth enhancement of Scenedesmus spp. in response to nitrogen and phosphorus in glyphosate excracted by D. pulex.

Keywords—Behavioral endpoint Scenedesmus Body burden Glyphosate Dichlorodiphenyldichloroethylene

INTRODUCTION

Applied successfully to explain differences in primary productivity among lakes with contrasting food webs, the concept of trophic cascading has moved attention toward the interactions between herbivorous zooplankton and phytoplankton and away from the complementary nutrient supply processes [1,2]. It is difficult to generalize about the conditions that are conducive to trophic cascading, from the level of piscivorous fish to phytoplankton [3]. Nonetheless, numerous studies demonstrate the positive effects of planktivorous fish on phytoplankton biomass and accumulation [4,5], and others reveal the inverse relationship of the numbers of large-bodied zooplankton with the quantity and net production rates of phytoplankton [6].

Xenobiotics, such as insecticides and metals, can affect primary production in much the same way as nutrient supply. Higher trophic levels (e.g., piscivores) are more susceptible than phytoplankton to xenobiotics, and they propagate their effects down food webs via predator performance [7]. Herbivores (e.g., zooplankton) often are more susceptible to pesticides, especially insecticides, than are phytoplankton, and median effect concentration (EC50) values for these two groups may differ by orders of magnitude [8]. Pesticide effects on zooplankton may be transmitted, both up the food webs, reducing fish growth, and down the food webs increasing primary production. The resulting phytoplankton bloom is a characteristic secondary effect of xenobiotic exposure in aquatic ecosystems [9].

Reduced grazing pressure by zooplankton on phytoplankton may follow from sublethal pesticide effects on feeding behavior (e.g., filtration, ingestion, and assimilation rates) and on locomotory behavior, such as swimming performance, coordination, predator avoidance, and prey handling [10]. Feeding behavior has been recognized as an ecotoxicological endpoint in filter feeders [11] because it has a physiological implementation closely associated with growth, metabolism, and reproduction [12]. Further, feeding behavior has a higher sensitivity than mortality. For instance, the median lethal concentration (LC50) for the insecticide fenvalerate is 0.21 µg/L in Ceriodaphnia lacustris, but the filtration rate decreases significantly only at 0.01 µg/L [12].

Daphnids are key species in freshwater ecosystems (e.g., as grazers on phytoplankton) and a favorite food item for many predators [13,14]. Their ability to feed efficiently is, at the same time, their success and a major disadvantage because, in so doing, they are more exposed to contamination and thus, more susceptible, which explains their frequent use in ecotoxicological tests. Xenobiotics, inorganic as well as organic, may affect food filtering and subsequent food processing (ingestion and assimilation) in Daphnia, causing a net reduction in rates of food attainment [15]. Filtration rates in Daphnia species are affected by fenvalerate [16] and endosulfan and diazinon [17]. Ingestion rates are less sensitive to toxic compounds than filtration rates [15,17], and assimilation seems to be the least sensitive process. In order for a concentration of a compound to cause significant effects on assimilation it must be close to the LC50 [16].

The present study is an effort to quantify the impact of two xenobiotics, p,p’-dichlorodiphenyldichloroethylene (DDE) and glyphosate, on phytoplankton growth rate due to cascading through reduced grazing rate in Daphnia pulex. Herein we also report on development of a bioassay for Daphnia species with grazing efficiency as an ecotoxicological endpoint. The...
p,p'-DDE and glyphosate represent different types of substances due to their physical-chemical properties (e.g., lipophilic vs hydrophilic), their mode and site of action, and their application targets (insecticide metabolite vs herbicide). Special attention was given to the importance of the source of contaminant exposure, because the literature is equivocal as to what extent exposure through water or food makes a contribution to the body burden of contaminants in aquatic organisms.

We compared the grazing rate of *D. pulex* on *Scenedesmus* spp. in laboratory-controlled uncontaminated conditions with those in which *D. pulex* had been pre-exposed to sublethal concentrations of p,p'-DDE and glyphosate through water and algae. We assumed that the growth rate of *Scenedesmus* spp. would be negatively and linearly related to the density of *D. pulex*, reflecting a constant individual grazing rate for the given set of growth conditions. Further, that the grazing rate would be reduced for *D. pulex* pre-exposed to the contaminants, and that this reduction would be greater in *D. pulex* pre-exposed to p,p'-DDE than to glyphosate due to differences in mode of toxic action in animal cells.

**MATERIALS AND METHODS**

**Test species**

Specimens of *Daphnia pulex* De Geer were collected in cohorts between May and October 2000 from a 50-m² pond in a recreational area of Lund, southern Sweden, using a plankton net (mesh size 150 μm) towed horizontally just below the water surface. The animals were kept in plastic aquaria (6 L) with Whatman glass fiber/C (Maidstone, UK) filtered copper-free tap water (pH 8.1, electrolytic conductivity 160 μS/cm, total hardness 58.4 mg/L as CaCO₃), which was replaced twice a week. The laboratory cultures were fed with suspensions of *Scenedesmus* spp. every second or third day, as needed, depending on the density of the cultures. Following acclimation in the aquaria for 3 to 4 d, *D. pulex* individuals, 1.3- to 1.9-mm long, were chosen for the experiments. *Scenedesmus* spp. was obtained from an in-house culture that was maintained continuously in filtered copper-free tap water, and amended with 10 ml of a commercial liquid fertilizer once a week (N 1.8%, P 1.8%, K 1.6% w/v). The same room (temperature-controlled, [20 ± 1°C] and illuminated by four 58-W fluorescent lamps, with a daily 12:12-h light:dark cycle) was used for maintenance of both the animal and algal cultures and for the experiments. The algal cultures were supported by extra lights (60-W bulb, 24 h/d) and aerated by a diaphragm air pump for 12 nonconsecutive h daily.

**Chemicals**

The p,p'-DDE (International Union of Pure and Applied Chemistry: N-[phosphonomethyl]glycine) is a broad-spectrum, nonselective, postemergence, systemic herbicide, which acts by competitive inhibition of 5-enolpyruvylshikimic acid-3-phosphate synthase, an enzyme in the shikimic acid pathway of the aromatic amino acid biosynthesis pathways, which links primary and secondary metabolism in plants and bacteria [8]. Glyphosate is an amphoteric and hydrophilic compound that undergoes degradation to aminomethylphosphonic acid (AMPA) and is not expected to bioaccumulate [8]. At the present time, it is the largest-selling crop-protection chemical product [19].

**Survival assays**

*N*-phosphonomethyl-glycine (glyphosate) (95%; Sigma Chemical, St. Louis, MO, USA), and 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (p,p'-DDE) (Sigma Chemical, 99%) working solutions were prepared in water (glyphosate) and in acetone (p,p'-DDE) and diluted in filtered copper-free tap water to determine the highest water concentration at which *D. pulex* and *Scenedesmus* spp. would survive a 4-d exposure. Two concentrations of each contaminant (10 and 100 mg/L of glyphosate, and 1 and 10 μg/L of p,p'-DDE) were used after reviewing LC50 and bioconcentration factor (BCF) data of both contaminants for the test species. Five individuals of *D. pulex* in 10 ml water and a 10-ml suspension of *Scenedesmus* spp. (~100 μg/L chlorophyll) were exposed separately in a 15-ml culture tube. The test species were assayed in parallel with a control (no contaminants added). *Daphnia pulex* was assayed by recording the number of live specimens after each day of exposure. The total chlorophyll (Chl) concentration of the *Scenedesmus* spp. suspensions was quantified at 665 and 750 nm according to Marker et al. [20], using an ultraviolet-visible spectrophotometer (Beckman DU 650; Fullerton, CA, USA) equipped with a 10-mm (path length) flow cell.

**Contaminant uptake and metabolism assays**

Glyphosate-(glycine-2-14C) sodium salt (aqueous solution) (Sigma Chemical, 7.4 mCi/mmol) and p,p'-DDE-ring-UL-14C (toluene solution) (Sigma Chemical, 13 mCi/mmol) were used to obtain the uptake pattern and BCF. Afterward, this information was used to assess the length of the pre-exposure and of the grazing periods for the grazing experiment.

Five *D. pulex* in 10-ml water or 10-ml algal suspension (~20 μg Chl/L for glyphosate, 100 μg Chl/L for p,p'-DDE) were exposed in 15-ml culture tubes to a combination of glyphosate (10 mg/L) and 14C-labeled glyphosate (0.03 mg/L, 1.136 nCi) or to 14C-labeled p,p'-DDE (5 μg/L, 213 nCi). Controls, without contaminants added, were assayed in parallel with the treatments. Five replicate culture tubes were sampled daily (scintillation counted) during 4 d of exposure to the labeled compounds.

*Daphnia pulex* were fixed in the culture tube with Lugol’s solution and transferred with a glass Pasteur pipette (carrying a small water volume) to 6-ml scintillation vials and combined with 0.2 ml of Beckman BTS-450 tissue solubilizer. The samples were digested at room temperature for at least 48 h following this; 4 ml of Beckman Ready Organic scintillation cocktail was added to each sample with subsequent stirring. One milliliter of the culture tube solution was mixed with 5 ml of Beckman Ready Safe scintillation cocktail to quantify 14C activity in the water.

The algae suspension (10 ml) was filtered onto a 0.45 μm,
25 mm diameter, HAWP Millipore® (Bedford, MA, USA) filter. Each filter was digested at room temperature for at least 48 h in a 20 ml scintillation vial with one ml of Beckman BTA-450. After digestion, 0.2 ml glacial acetic acid was added to decrease the sample chemiluminescence. Fourteen ml of Beckman Ready Organic scintillation cocktail was added to each sample with subsequent stirring. Samples of the water that passed through the filter were collected, and one ml mixed with 5 ml of Beckman Ready Safe scintillation cocktail.

All samples were stored in their respective cocktails for at least 12 h before 14C activity was measured in a Beckman Model LS 6500 scintillation counter, with color quench correction and error of less than 10% disintegrations per minute (dpm). The counting efficiency was obtained from a quench curve established from a set of 14C unquenched standards.

Once the uptake pattern of the contaminants was established by this assay, the length of the pre-exposure and grazing periods of the grazing experiment (see below) was determined. In parallel with the grazing assay, a small-scale experiment was performed with the labeled compounds to determine their fate during all steps of the assay. Across all assays, both organisms survived both contaminants to between 90 and 100%.

Preparation of cultures for grazing experiments

The general strategy was to pre-expose grazers to uncontaminated conditions (control animals), contaminated water, and contaminated food, and then compare their performance in assays with uncontaminated water and food.

Preparation of Scenedesmus spp. cultures

An inoculum of Scenedesmus spp. from the stock culture was added to tap water to obtain approximately 2.5 L of approximately 100 µg Chl/L in each of three 5-L beakers used for this procedure per contaminant. One beaker of each trio was spiked with the contaminant (50 mg/L for glyphosate, 5 µg/L for p,p'-DDE) and the other two were not (Fig. 1). The trios were incubated for 7 (glyphosate) or 4 (p,p'-DDE) d, and stirred slowly by hand each day and repositioned with respect to the light sources. The chlorophyll concentration was measured at the beginning and end of the incubation. A small-scale phytoplankton exposure assay in 100-ml beakers (1 replicate per treatment and per contaminant) was made with the labeled compounds ( glyphosate [50 mg/L] + 14C-glyphosate [0.03 mg/L, 1.136 nCi] or 5 µg/L 14C-p,p'-DDE [213 nCi]) and inoculated with Scenedesmus spp. at an initial density of about 100 µg Chl/L. The cultures were incubated and treated as described above for the pre-exposure experiment. The 14C activity of the phytoplankton biomass and of the water was measured after filtration at the beginning and end of the exposure period.

Pre-exposure of D. pulex

Up to 225 D. pulex from the stock cultures were added to each of three feeding chambers that were positioned in 5-L beakers containing 2.5 L of tap water. The feeding chambers were constructed from rectangular 500-ml plastic jars, with a plankton net fixed into the bottom and two walls (mesh size 150 µm). This allowed algae to enter the chamber from the surrounding water and D. pulex to feed on them. For this procedure, three beakers were used per contaminant. One of the beakers was spiked with the contaminant, 50 mg/L glyphosate, or 5 µg/L p,p'-DDE (Fig. 1). The feeding chambers were moved to beakers with the Scenedesmus spp. suspensions twice a day for 1.5 h each time to allow the animals to feed. Uncontaminated Scenedesmus was used to feed the animals that were pre-exposed to contaminants dissolved in water, and contaminated Scenedesmus was used to feed those pre-exposed to the contaminants through the food. This feeding procedure started on the fifth (glyphosate) or third day (p,p'-DDE) of incubation and lasted for 3 d for glyphosate and 2 d for p,p'-DDE.

A small-scale D. pulex exposure assay with labeled compounds was performed to test the possibility that any changes in grazing efficiency were related to differences in body burden of contaminants in D. pulex. Twenty-two specimens were kept in a 50-ml glass beaker placed inside a 100-ml beaker containing 75 ml of filtered tap water. The inner beaker had three holes (two in the walls and one in the bottom) covered by plankton net (mesh size 150 µm, attached with glue) to allow algae to enter from the surrounding water. This small-scale assay was made as the pre-exposure assay described above, with three replicates used for each treatment and contaminant. The 14C activity of D. pulex and of the assay water was measured at the beginning and end of the exposure period.

Grazing experiments

Specimens of D. pulex from the pre-exposure assay were added to 250 ml of filtered tap water in 1-L glass jars and inoculated with uncontaminated Scenedesmus spp. at approximately 100 µg Chl/L. The chlorophyll content of the water was measured before the animals were added (N0) and at the end of the assay (Nt). The net growth rate (r) was calculated for each replicate according to $r = \ln(N_t / N_0) / \Delta t$, where $\Delta t$ was the duration of the assay in days [21]. The relative change in

Fig. 1. Schematic representation of bioassays with Scenedesmus spp. and Daphnia pulex cultures. Preparation of Scenedesmus: A set of three containers were inoculated at approximately 100 µg/L of chlorophyll (Chl). These assays lasted for 2 (p,p'-dichlorodiphenyldichlooroethylene [DDE]) or 4 (glyphosate) days. The contaminants were added to one of the containers each (contaminated water). Pre-exposure of D. pulex: 225 D. pulex were added to each of a set of three feeding chambers that were placed inside containers filled with filtered tap water. These assays lasted for 2 (p,p'-DDE) or 3 (glyphosate) d. The contaminants were added to one container each (treatment water). The feeding chambers containing D. pulex were moved twice a day (1.5 h each time) to the container prepared with Scenedesmus. Grazing experiments: D. pulex from the pre-exposure assays were moved to containers with uncontaminated Scenedesmus spp. at an initial Chl concentration of about 100 µg/L. The assay was made with five densities of D. pulex (8, 20, 40, 80, and 100 individuals/L).

25 mm diameter, HAWP Millipore® (Bedford, MA, USA) filter. Each filter was digested at room temperature for at least 48 h in a 20 ml scintillation vial with one ml of Beckman BTA-450. After digestion, 0.2 ml glacial acetic acid was added to decrease the sample chemiluminescence. Fourteen ml of Beckman Ready Organic scintillation cocktail was added to each sample with subsequent stirring. Samples of the water that passed through the filter were collected, and one ml mixed with 5 ml of Beckman Ready Safe scintillation cocktail.

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algal biomass was calculated as $\Delta r = r_2 - r_1$, where $r_1$ and $r_2$ are the net growth rates for $D. pulex$ density 1 and 2. Five densities of $D. pulex$ (8, 20, 40, 80, 100 individuals/L) were used, with three replicates per density and treatment each time the assay was made (batch). At least two batches were made per contaminant. In this way, 15 jars were used to observe grazing by $D. pulex$ pre-exposed to uncontaminated water, 15 jars were used with $D. pulex$ pre-exposed to glyphosate or $p,p'$-DDE dissolved in water, and 15 jars with $D. pulex$ pre-exposed to Scenedesmus spp. contaminated with glyphosate or $p,p'$-DDE (Fig. 1). The relative net growth rates (See Statistical analyses section) were plotted versus the densities of $D. pulex$ for each treatment. The slope of the linear regression expressed the grazing rate, and the intercept expressed the in situ algal growth rate in the absence of grazers.

The assay lasted for 4 d, and the jars were stirred daily by hand and repositioned randomly. At the end of the assay, the health condition and the number of live animals were recorded. Specimens were preserved with Lugol’s solution for subsequent length measurements.

A small-scale grazing experiment was performed in parallel with the main experiment. Seven individuals of $D. pulex$ were used from each of the nine replicates of the small-scale pre-exposure experiment (three replicates per treatment per contaminant). They were transferred to 100-ml beakers with 75 ml of filtered tap water inoculated with Scenedesmus spp. at approximately 100 $\mu$g Chl/L. The assay lasted for 4 d. The $^{14}$C activity was measured in the water, $D. pulex$, and Scenedesmus spp. at the end of the assay.

Calculations of body burden of the contaminants in both phytoplankton and zooplankton were done based on specific activity (mCi/mmol), molecular weight of the labeled compounds (mg/mmol), and average dry weight (dry wt) of the filtered algal suspension (10 ml at different Chl concentrations) and five $D. pulex$ (estimations from length). The dry weight–based bioconcentration factors (BCF$_W$) were calculated as (dpm in tissue/weight of tissue [g])/dpm in water/ml of water) and measured at steady state conditions.

Statistical analyses

The algal net growth rate of the control varied from one batch to another. In order to compare results from different batches, we calculated a dimensionless relative net growth rate in the following way: Within each batch, each net growth rate (from the various treatments) was divided by the maximum net growth rate found in that batch. A confidence interval (95% probability) was used to delimit treatment data that was amenable to a statistical comparison. Normal distribution of data and homogeneity of variances were evaluated with Kolmogorov–Smirnov test and Levene’s test, respectively. The observed differences between the control and contaminant treatments were evaluated by a one-way analysis of covariance, using density of $D. pulex$ as the covariate, and using the interaction between treatment and density to test for the homogeneity of the slopes. All possible pairs of regression lines were compared and the significance of the regression between the relative $r$ and density was assessed for each treatment and pesticide. All statistical tests were done with SPSS® for Windows 9.0.1 (Chicago, IL, USA).

RESULTS

The inverse linear relationship between the net growth rate of Scenedesmus spp. and the density of $D. pulex$, expressed by significant negative slopes of the regression lines (Fig. 2A,B; Density, Table 1), was taken as an estimator of grazing. In general, slopes for the control were steeper than for the treatments (Fig. 2A,B), suggesting that grazing became less efficient in $D. pulex$ that were pre-exposed to the contaminants. The loss of efficiency was significant for $D. pulex$ pre-exposed to $p,p'$-DDE via water (~30% compared to the control) and to glyphosate via Scenedesmus spp. (~40%) (Table 1, treatment-density). Hence, as a consequence of impaired grazing, the relative algal biomass increase ($\Delta r_\text{T}/\Delta r_\text{C}=100$, where $T =$ treatment and $C =$ control) was larger for $p,p'$-DDE (70%) than for glyphosate (60%). The $y$-intercepts, which are estimates of the net growth rate in absence of grazers, of the...
Table 1. One-way analysis of covariance of the effects of p,p′-dichlorodiphenyldichloroethylene (DDE) and glyphosate on the grazing efficiency of Daphnia pulex. The density of D. pulex was used as covariate and the relative net growth rate of Scenedesmus spp. as the dependent variable. The y-intercepts (treatment), the significance of the linearity of the response of the dependent on the independent variable (density), and the homogeneity of slopes (treatment-density) between relevant pairs of regression lines (control vs treatments) were tested at a significance level of 5% (p = 0.05).

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The data from the parallel small-scale experiment with 14C-labeled contaminants showed that D. pulex took up p,p′-DDE and glyphosate from water as well as from Scenedesmus spp. (Fig. 3A,B; Table 2) and were carrying orders of magnitude higher concentrations of contaminants than the controls at the end of the grazing period. At the end of a period of pre-exposure to the contaminants via water, D. pulex had an average of 71 µg/g (dry wt) of p,p′-DDE and 50 mg/g (dry wt) of glyphosate (Table 2). The concentrations were at least 50% less when the pre-exposure was via Scenedesmus spp. (Table 2). Scenedesmus spp. took up less glyphosate (39 mg/g dry wt) and p,p′-DDE (30 µg/g dry wt) than D. pulex.

The length of the pre-exposure and grazing intervals were based on the pattern of uptake of the labeled pesticides in the separate culture tube experiments (Fig. 4A,B). The p,p′-DDE concentration peaked in Scenedesmus spp. within 12 h of exposure (Fig. 4A), corresponding to BCFd = 19,989, and then declined within the next 36 h. The uptake of glyphosate (Fig. 4B) was slower and 3 to 4 d were required to reach a peak (BCFd = 2,000). These observations suggested that 2 d of exposure to p,p′-DDE and 4 d to glyphosate were sufficient before the cultures were used to expose D. pulex. The p,p′-DDE concentration (BCFd = 69,394) in D. pulex reached an equilibrium within 12 h and the glyphosate concentration (BCFd = 2,200) within 2 to 3 d (Fig. 4A,B). Accordingly, D. pulex were pre-exposed to p,p′-DDE for 2 d and to glyphosate for 4 d before the grazing experiment started, and the grazing period was limited to 4 d.

**DISCUSSION**

Although the genus Daphnia is recognized for its high rates of filtration of phytoplankton [6,22,23], the grazing rates realized in our assays (and in the enclosures used by Lehman and Sandgren [21]) would have only a marginal impact on the net growth rate of phytoplankton, unless the density of grazers is high (e.g., >40 individuals/L). Our results give strong support for the assumption of a linear and negative relationship between the density of D. pulex and the net growth rate of Scenedesmus spp. However, the slopes of the regression lines control and the treatments statistically were indistinguishable (Table 1, treatment). This was expected, because the algae used in the treatments had not been exposed to contaminants and were sampled from the same population as the control. The Levene’s test reported a low level of significance (p = 0.131–0.678) for all data groups, meaning that the dependent variable was measured with the same error for control as for treatments. The error terms from the significantly different pairs of treatments were distributed normally (p = 0.200 in Kolmogorov-Smirnov test).

The **Fig. 3.** 14C-p,p′-dichlorodiphenyldichloroethylene (DDE) (A) and 14C-glyphosate (B) (disintegrations per minute [dpm]) in tissue of Scenedesmus spp. after exposure via water and in tissue of Daphnia pulex after exposure via water or via Scenedesmus spp. were exposed for 2 (p,p′-DDE) or 4 (glyphosate) d. D. pulex were exposed (See Pre-exposure of D. pulex section) for another 2 (p,p′-DDE) or 3 (glyphosate) d to contaminated water or Scenedesmus. D. pulex were then moved to graze on uncontaminated Scenedesmus spp. between day 4 and day 8 (p,p′-DDE) and day 7 and 11 (glyphosate). Average values of three replicates and standard deviations (error bars) are given.
were shallow and, for the differences between the control and treatments, significance was produced by the impact of the higher densities of *D. pulex*. By converting the phytoplankton growth rates in our study to the dimension/d, and the densities to µg/L (144–1,800) and then multiplying the resulting slopes (0.0001–0.0003/µg d) by the average density, we obtained average grazing rates of −0.089 to −0.178/d; these are more or less comparable to those from the 98 enclosures in Lehman and Sandgren [21] (average −0.053, standard deviation 0.159). The grazing rates in our controls obviously were rather high, possibly reflecting maximum biomasses that were twice as high as in most of the enclosures of Lehman and Sandgren [21], but still within one standard deviation of their mean.

The comparison here suggests that the densities of *D. pulex* used in the assays were representative of the range found in lakes and were large enough to allow detection of the impact of contaminants through grazing effects. The influence of contaminants, through grazing effects, on phytoplankton growth may be possible to observe only at relatively high grazer densities.

The reduced grazing efficiency observed in *D. pulex* pre-exposed to *p,p*-DDE via water and pre-exposed to glyphosate via *Scenedesmus* spp. was associated with the treatments that gave the highest body burden of *p,p*-DDE (70 µg/g dry wt) and the lowest of glyphosate (13 µg/g dry wt). Similar body burdens of *p,p*-DDE (80 ppm dry wt) are known to cause thinning of shells in krestel and peregrine eggs [18], and sublethal levels in the diet (10–100 µg/g) can impair reproductive behavior in guppies [24]. Insecticides, at concentrations below LC50, are known to reduce the movement of filter-feeding appendages, disrupt body coordination, and even cause immobility in zooplankton, leading to reduced filtration and/or ingestion of algal cells [11,16,17,25].

Aquatic organisms usually have much lower body burdens of glyphosate than those reported in this study [26,27]. However, glyphosate toxicity did not seem to cause the reduced grazing rate observed; we came to this conclusion because *D. pulex* pre-exposed via water had a higher body burden of glyphosate (50 mg/g dry wt) than those exposed via *Scenedesmus* spp. (13 mg/g dry wt), yet the lower level caused a greater reduction in grazing effect. A more likely explanation for this pattern of results is that *Scenedesmus* spp. growth was enhanced by excretions from *D. pulex* [28] (of glyphosate or its degradation products) that had been pre-exposed to glyphosate-contaminated *Scenedesmus* spp. During the assay, the body burden of glyphosate in *D. pulex* decreased from 13 mg/g at the beginning to 3 mg/g at the end, corresponding to a final glyphosate concentration in the assay solution of approximately 20 µg/L (2 µg/L N and 4 P). This is a concentration that has been shown to stimulate growth, photosynthesis, and chlorophyll-a synthesis in *Scenedesmus quadricauda* (potential bottom-up effect), probably as a result of its nitrogen or phosphorus content [29]. Further, the resulting biomass increase may slow down the filtering rates of cladocerans [30,31]. *D. pulex* pre-exposed via water also excreted glyphosate; the body burden decreased from 50 mg/g to 10, cor-

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Table 2. Average body burdens (three replicates) in *Daphnia pulex* and in *Scenedesmus* spp. after 2 d of exposure to *14C-p,p’-dichlorodiphenyldichloroethylene (DDE)*. Calculations were made based on a specific activity of 13 mCi/mmol, molecular weight = 318 mg/mmol, the average weight of five specimens of *D. pulex* (90 µg, dry wt), and the average weight of 10 ml of *Scenedesmus* spp. suspension (950 µg, dry wt).

<table>
<thead>
<tr>
<th>Test organism</th>
<th>dpm* in tissue</th>
<th>mCi</th>
<th>mmol</th>
<th>µg</th>
<th>µg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Daphnia pulex</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment via water</td>
<td>573</td>
<td>$2.6 \times 10^{-7}$</td>
<td>$2.0 \times 10^{-8}$</td>
<td>$6.4 \times 10^{-3}$</td>
<td>70.8</td>
</tr>
<tr>
<td>Treatment via <em>Scenedesmus</em> spp.</td>
<td>256</td>
<td>$1.2 \times 10^{-7}$</td>
<td>$9.0 \times 10^{-9}$</td>
<td>$2.8 \times 10^{-3}$</td>
<td>31.6</td>
</tr>
<tr>
<td><em>Scenedesmus</em> spp.</td>
<td>2,542</td>
<td>$1.2 \times 10^{-6}$</td>
<td>$8.9 \times 10^{-8}$</td>
<td>$28.3 \times 10^{-3}$</td>
<td>29.8</td>
</tr>
</tbody>
</table>

* dpm = Disintegrations per minute.
responding to approximately 75 μg/L glyphosate (6 μg/L N and 13 P) in the assay solution. The absence of a grazing effect at this higher glyphosate concentration has support in the observations by Wong [29], who noted that 200 μg/L of glyphosate had no influence on the chlorophyll-a content of S. quadricauda.

A review on freshwater cascading made by Brett and Goldman [13] showed that zooplanktivorous fish decreased zooplankton biomass by 29%, correspondingly increasing phytoplankton biomass by 176% (range from 38–711%) of the control values. Many of the studies used fish abundances that were higher than those commonly found in similar lakes. In comparison, xenobiotics such as DDT, dibenzofuran, and esfenvalerate, used at concentrations above the LC50 for zooplankton, decreased zooplankton populations by 70% (range 50–85%) with an increase in phytoplankton biomass of 230% (range 210–260%) [9,32,33]. The trophic level ratios of biomass effects, 6 (176/29) for zooplanktivorous fish and 3 (230/70) for contaminants, suggest that contaminants have at least half the impact, compared to zooplanktivorous fish, in causing cascading effects. Variations in cascading effects, to some extent, reflect variations in biological characteristics of the dominating zooplankton (e.g., large vs small species and individuals) or phytoplankton (e.g., edible green algae vs colonial cyanobacteria) [13,14]. For instance, the genus Daphnia has a larger impact on phytoplankton biomass than many other zooplankton genera (e.g., rotifers) [13,14].

Grazing efficiency in D. pulex was more sensitive (15× for glyphosate and 3× for p,p′-DDE) as an ecotoxicological endpoint than the 48-h LC50 for glyphosate (780 μg/L, [34]), and our estimated 48-h LC50 for p,p′-DDE (13.5 μg/L) (lacking data on p,p′-DDE LC50 for Daphnia spp.) we estimated it from linear correlations for p,p′-DDT [k1 = 15] and p,p′-DDD [k2 = 7.6] between LC50s for fish and Daphnia spp. Once k1 and k2 were known, we calculated a slope k1 + k2/2 for p,p′-DDE. The average LC50 for D. pulex was extrapolated from k1, using averaged reported p,p′-DDE LC50 for fish. The calculated LC50 was compared and found to agree with the observed survival of D. pulex at different concentrations of p,p′-DDE in the assays). Median lethal concentration is a logical first, fast, and cheap endpoint for acute toxicity assessment and can be used to rank toxicity of substances whose mechanisms of action may be quite different [35,36]. Compared to LC50s, behavioral changes may be more sensitive and may anticipate reductions of survival, growth, and reproduction; furthermore, they become apparent at shorter exposure durations and at lower contaminant concentrations [16,17,37]. For example, the increased swimming activity in D. pulex at sublethal concentrations of the insecticide carbaryl (one-fifth of its 48-h LC50) seems to increase the vulnerability of specimens to predation and reduce their body size by allocating energy away from growth [37]. More generally, for filter feeders there are two consequences of a decreased grazing efficiency. First, the current produced by movement of the thoracic appendages, in addition to feeding, subserves respiration, and it is essential that filtration continues even if food is not going to be collected. Second, there is a threshold concentration of food that must be ingested for an individual to fulfill its basic metabolic needs [12].

The BCFs for p,p′-DDE in this study were similar to those reported by Verschueren [8] for different organisms, and this is most likely attributed to the high Kow of p,p′-DDE [38]. The glyphosate BCFs that we observed were lower than for p,p′-DDE, but were higher than expected from its low Kow. However, higher-than-expected BCFs are known for other substances with negative log Kow such as methanol, ethyl acetate, and urea [39]. It is possible that these substances are taken up rapidly and then metabolized. We also observed a biodilution of both contaminants, that is, a reduction in contaminant concentration per unit weight during uptake from Scenedesmus spp. (Fig. 4A). This phenomenon has been reported by some authors [40,41] and mainly is attributed to high growth rates and slow partitioning of compounds between water and phytoplankton.

The bioassays demonstrated that the insecticide metabolite, p,p′-DDE, at concentrations above ambient levels, increased the primary producer biomass through cascading by releasing the top-down control exerted by zooplankton. Similarly, the herbicide glyphosate increased phytoplankton biomass, probably by a combination of cascading and bottom-up control. The mixed effects of glyphosate on the bioassay system call for a more detailed evaluation of its mode of action on trophic interactions of varying complexity. Though grazing assays like the one described here are laborious and critically dependent on assay conditions, they help to elucidate secondary effects of contaminants on community processes (e.g., biological interactions) that would escape detection by other ecotoxicological endpoints.

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REFERENCES


