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Comparative Toxicity of Herbicide Active Ingredients, Safener Additives, and Commercial Formulations to the Nontarget Alga *Raphidocelis Subcapitata*

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Abstract: Chloroacetanilide herbicides are used worldwide to control weeds that affect crops such as corn, soybeans, and cotton. These herbicides are frequently paired with a "safener," which prevents herbicidal damage to the crop without diminishing weed control. Formulated herbicide products that include safeners and other ingredients are infrequently assessed for toxicity. Our goal was to understand the potential toxicity of safeners and herbicide + safener formulations relative to the toxicity of associated active ingredients. We quantified the concentration of safeners in commercially available formulations and tested effects on nontarget algae, *Raphidocelis subcapitata*, when exposed to individual herbicide active ingredients, safeners, and commercial formulations. The median effective concentrations (EC50s) causing 50% reduction in population growth for the herbicide active ingredients *S*-metolachlor and acetochlor were 0.046 and 0.003 ppm, respectively. The safeners benoxacor, AD-67, furilazole, and dichlormid were all substantially less toxic than the herbicides and were not toxic at environmentally relevant concentrations. The commercial formulations Dual II Magnum®, Me-Too-Lachlor II®, Harness®, and Surpass EC® all resulted in EC50 values that fell within the 95% confidence interval of the associated active ingredient herbicide. Interestingly, a significant increase in cell size was observed when algae were exposed to all the formulations, herbicides (acetochlor and *S*-metolachlor), and safener (dichlormid). The safener furilazole caused a significant decrease in cell size, whereas benoxacor and AD-67 had no observed effect on algae cell size. Significant algae cell size effects all occurred at or above the EC50 concentrations for each chemical, suggesting that other morphological effects may be occurring. Importantly, safeners in commercial formulations appeared not to impact toxicity to *R. subcapitata* compared with the active ingredient alone. *Environ Toxicol Chem* 2022;41:1466–1476. © 2022 SETAC

Keywords: Metolochlor; Acetochlor; Herbicide formulations; Algae

INTRODUCTION

Herbicides are widely used to increase crop yields by inhibiting the growth of unwanted plants. A common group of herbicides is the chloroacetanilide herbicides, with *S*-metolachlor (SMET) and acetochlor (ACE) being among the most widely used (Abigail et al., 2015). Based on 2012 herbicide usage data, in the United States, SMET was ranked third, and ACE was ranked seventh (Atwood & Paisley-Jones, 2017). Because of their heavy usage, both SMET and ACE as well as their transformation products have been found in streams across the United States (Mahler et al., 2020). Although the popularity of SMET and ACE stems from their effectiveness in controlling grasses and broadleaf weeds for more than 20 crops worldwide, they are mostly used for maize crops (corn) and soybeans (O'Connell et al., 1998). In addition, SMET and ACE are considered sustainable herbicides due to their short soil half-lives (~11–26 days) and the lack of resistance development in weeds (O'Connell et al., 1998, Sivey et al., 2015). The SMET and ACE herbicides control weeds by disrupting lipid synthesis (Maronić et al., 2018), but they can also sometimes negatively impact crops (Jablonkai, 2013). To prevent an herbicide from damaging crops, ingredients referred to as "safeners" are added to herbicide formulations. Commercial formulations containing both herbicides and safeners increase crop size and yields compared with the herbicide alone (Davies & Caseley, 1999; Jablonkai, 2013; O'Connell et al., 1998).

From a regulatory perspective, safeners are classified as "inert," meaning they do not prevent, destroy, repel, or

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mitigate a pest (U.S. Environmental Protection Agency [USEPA], 2019). Hence pesticide companies are not required to list the identity or amounts of safeners on pesticide labels (USEPA, 2019). However, some companies advertise that safeners are included in the formulations and include safeners in safety data sheets for their product (DOW, 2015; Monsanto, 2015; Syngenta, 2015). The mode of action for dichloroacetamide safeners is not entirely understood. Studies suggest that safeners promote gene expression to produce enzymes, such as glutathione S-transferases, as well as cytochrome P45 monogeneses, that help crops to detoxify herbicides (Gatz, 1997; Hatzios & Burgos, 2004; Jablonkai, 2013; Riechers et al., 2010).

Since their development in the 1940s and commercial implementation in the 1970s (Davies & Caseley, 1999), safener incorporation into commercial formulations has increased (Sivey et al., 2015). Because pesticide companies in the United States are not obligated to report precise concentrations of safeners in formulations, there is some uncertainty as to the extent of safener usage, which is estimated to be approximately 2×10^6 kg/year in the United States and approximately 8×10^6 kg/year worldwide (Sivey et al., 2015). These estimates are based on the usage of herbicide active ingredients commonly applied with safeners in formulations and the estimated amount of safeners in the formulations. Greater certainty in safener usage and applications may be important because although safeners are considered "inert" in a regulatory sense, they are biologically active (Sivey & Roberts, 2012). Safeners typically have chemical structures similar to the herbicide they are applied with (Davies & Caseley, 1999). Dichloroacetamide safeners are typically applied with SMET and ACE, making them the most prevalent safener class (Abu-Qare & Duncan, 2002; Schulze et al., 2002). An occurrence study by Woodward et al. (2018) found a strong correlation between detection of chloroacetanilide herbicides (SMET and ACE) and detection of dichloroacetamide safeners in streams in the midwestern United States. These included benoxacor (BEN), dichlormid (DICH), furilazole (FUR), and AD-67 (AD). Similarly, cyprosulfamide, a relatively new herbicide safener, has been found along with two of its degradates in surface water samples in the midwestern United States (McFadden & Hladik, 2021). Collectively, these studies provide evidence of the environmental occurrence of safeners and hence a potential exposure to ecological receptors.

The toxicity of SMET and ACE to ecological receptors has been extensively studied. Chloroacetanilide herbicides are toxic to selected terrestrial plants (Davies & Caseley, 1999; Jablonkai, 2013) and aquatic phototrophs (H. Lui et al., 2006; USEPA, 1992). For example, a primary producer such as the green alga *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum* or *Pseudokirchneriella subcapitata*) is sensitive to SMET and ACE, with effective concentrations causing 50% reduction in population growth (EC50) of 0.008 and 0.0001 ppm, respectively (H. Lui et al., 2006; USEPA, 1992). It is unclear, however, whether *R. subcapitata* would exhibit the same sensitivity to safeners and whether herbicide formulations would be more or less toxic than herbicide active ingredients. For example, SMET and the safener BEN had a synergistic effect on female *Chironomus riparius* that was greater than the impacts of either compound individually (Bolyard et al., 2017). It is important to understand the individual and combined effects of the active and other chemicals within a commercial formulation because environmental exposures may involve both simple and complex mixtures of contaminants and stressors. The combined effect can conceivably differ from individual effects and should be considered when one is assessing the environmental risk of safeners (Bolyard et al., 2017; Silva et al., 2018).

The first goal of the present study was to quantify and identify (if not labeled) each safener in four commercial formulations, Dual II Magnum® (DUAL) containing less than 5.0% BEN (based on safety data sheets) and 82.4% SMET (Lee et al., 2019; Syngenta, 2015); Surpass EC® (SUR) containing 12.2% DICH and 70.87% ACE (DOW, 2019); Harness® (HAR) containing less than 2.5% FUR and 75.9% ACE (Monsanto, 2015); and Me-Too-Lachlor II® (METOO) containing 84.4% racemic metolachlor and unidentified safener (Drexel, 2019; Lee et al., 2019). The characterization of safener concentrations in herbicide formulations will allow for improved estimates of safener use and potential prevalence in the environment. Our second goal was to assess the toxicity to *R. subcapitata* exposed to each herbicide (SMET and ACE) and safener (DICH, BEN, FUR, and AD) independently and combined through commercial formulations (DUAL, SUR, HAR, and METOO). The endpoints were inhibition of population growth and individual cell size measured as area. We hypothesized that safeners would have a protective effect on nontarget algae similar to their protective effect on crops (e.g., via induction of enzymes that facilitate detoxification of herbicides in crops), causing a decrease in toxicity of the formulations compared with herbicide active ingredients (Gatz, 1997; Hatzios & Burgos, 2004; Jablonkai, 2013; Riechers et al., 2010). Collectively, these data will help to further our understanding of safener toxicity and potential risk to nontarget aquatic phototrophs.

MATERIALS AND METHODS

Chemicals

The herbicides SMET (purity 99.1%) and ACE (purity 98.0%) were purchased from Chem Service. The safeners FUR (purity 98%) and DICH (purity 97%) were purchased from AstaTech and Tokyo Chemical Industry, respectively. The safener AD-67 (purity 97%) was donated by Nanjing Essence Fine-Chemical. Due to purity concerns associated with commercially available technical grades of BEN, we chose to synthesize BEN (purity 99.8%); see the Supporting Information for details. The formulations DUAL, HAR, SUR, and METOO were purchased from Syngenta, Monsanto, Syngenta, and Drexel, respectively. The internal standard 2-chlorobenzonitrile (CBN; purity 99.8%) was purchased from Acros Organics. The solvents acetone,

methanol, and toluene, all ACS grade, were purchased from Fisher Scientific.

Determination of safener concentrations in commercial formulations

The formulations were diluted into methanol based on reported maximum safener concentrations to achieve diluted concentrations of approximately 0.5 mM. Methanolic stock solutions were diluted further into deionized water purified to a resistivity of 18 MW/cm or more (NANOpure; Thermo Scientific) to achieve safener concentrations of approximately 30 µM. The diluted formulations were then filtered using nylon syringe filters (0.2-µm pore size) into 2-ml clear glass vials. Filtered solutions (2.0 µl) were analyzed by high-performance liquid chromatography (HPLC; Shimadzu LC-20AT) with a diode array detector (220-nm quantitation wavelength). Calibration was performed using external standards containing (safener) = 1–40 µM in 35 vol% acetonitrile/65 vol% Nanopure water. Analyte separations were achieved on a Poroshell 120 EC-C18/Bonus RP column (Agilent, length = 50 mm, diameter = 2.1 mm, pore size = 2.7 µM). An isocratic mobile phase consisting of 65 vol% water (18 MW/cm) and 35 vol% acetonitrile (0.55 ml/min, total analysis time of 8.5 min). For DUAL and HAR, the safener's identity and maximal concentration were provided in the safety data sheets. For SUR, the precise concentration of DICH (12.2 wt%) was included in the safety data sheets; therefore we did not quantify DICH in this formulation. For METOO, the identity and concentration of the safener were not included on the label or within the safety data sheet; therefore qualitative analysis (via HPLC retention time matching to authentic standards) was performed followed by quantitation of the identified safener.

Algae culture medium and algae test organisms

Algae culture medium and *R. subcapitata* algae were purchased from EBPI. Algae culture medium contains essential micronutrients and trace metals needed for algae growth following the Organisation for Economic Co-operation and Development (OECD, 2011) test guideline 201 for algal growth inhibition and the International Organization for Standardization (2012) standard 8692, Water quality—Freshwater algal growth inhibition tests with unicellular green algae.

Chemical analysis in algae toxicity experiments

All chemical concentrations were analytically verified at the initiation of algal toxicity experiments. After algae cuvettes were prepared, 2 ml of the treatment stocks for each exposure concentration were sampled. The aqueous samples underwent liquid–liquid extraction; 500 µl of toluene containing 14 µM 2-CBN and 300 µl of 2 M NaCl were added to the 2-ml aqueous samples. Toluene extracts were analyzed using gas chromatography (GC; Agilent 7890A) interfaced with a mass spectrometer (MS; Agilent 5975C). Separations were effected using an Agilent DB-35MS column (length 30 m, diameter 0.250 mm, film thickness 0.25 µm).

Toxicity test and morphometric analysis

Algal toxicity tests were performed with a range of concentrations of herbicides (SMET and ACE), safeners (BEN, AD, DICH, and FUR), and formulations (DUAL, SUR, HAR, and METOO; Supporting Information, Table S1). All chemicals were tested individually. A range-finding experiment was conducted (data not shown) to establish exposure concentrations in the series of definitive studies we reported. Modified 72-h algae toxicity tests were performed following OECD (2011) test guideline 201. Modifications included gathering measurements every 24 h to obtain additional insight, but we report toxicity values for 72 h. In addition, solvent controls containing less than 0.01 vol% of either methanol or acetone were added to each test along with experimental controls (algae in algae culture medium only). Also, a positive control containing 0.067 ppm of SMET was added to each test. The positive control was considered acceptable if it resulted in 50%–60% reduction in algal population density. The solvent control was considered acceptable if there was no significant difference in population growth compared with the experimental controls. For selected experiments, the pH was measured after 72 h. When the pH was 6.7 and did not change after 72 h, we concluded that algae did not alter the pH in the 72-h experimental duration. Algae were housed in long-cell plastic cuvettes (10-cm pathlength) randomly placed on the incubator shelf. Each cuvette contained algae culture medium prefiltered with a 0.45-µm polytetrafluoroethylene membrane filter and amended with a given treatment (or no treatment for controls). Optical density (absorbance) at 670 nm was measured for all replicates at 0, 24, 48, 72, and 96 h using a DLAB SP-V1100 spectrophotometer. Algal population density was estimated from optical density using a standard curve specific to time point and test. The algal population density of each replicate was compared with the control, and the inhibition of growth rate relative to control was calculated. The percentage of population growth inhibition was used to form a dose–response curve for each chemical, which was then used to estimate the EC50 and 10% effective concentration (EC10) toxicity thresholds using the "drc" package in R statistical software (R Core Team, 2019).

Anecdotal observations from preliminary toxicity tests for SMET revealed that the size of the algae cells appeared to increase with increasing concentrations of SMET. This observation suggested that some treatments may impact algal physiology, as indicated by cell size differences across treatments and/or treatment concentrations. Therefore, cell size analysis was incorporated as a potential endpoint of interest by capturing images of individual algal cells in each treatment and control. A 16-µl sample was taken from one randomly chosen replicate for each treatment and placed onto a hemocytometer microscope slide. The hemocytometer grid was divided into

0.02-mm^2 areas; 10 were chosen randomly and imaged with Motic Image Plus 2.0. The area (mm^2) of algal cells within the 10^2 chosen was measured in ImageJ (Schneider et al., 2012). For all chemicals, images were taken at 0-, 24-, 48-, 72-, and 96-h time points.

Statistical analysis

Algal population density was analyzed for each treatment and control by first calculating the growth rate (GR; Equation 1) and percentage of growth inhibition (Equation 2). Subsequently, the EC50 and EC10 values were generated using the "drc" package (Ritz et al., 2015) in R software (R Core Team, 2019).

$$\mathrm{GR} = \frac{\log\left(\frac{\text{algal cells}}{L}\right) - \log\left(\frac{\text{inital algal cells}}{L}\right)}{\text{time (days)}} \quad (1)$$

$$\text{Percentage of growth inhibition} = \frac{100 \times (\text{Average control GR}) - \text{GR}}{(\text{Average control GR})} \quad (2)$$

The effects of test chemicals on algal population density were assessed by comparing the 95% confidence limits of EC50 and EC10 values with the respective controls. The EC50 and EC10 values were estimated using the "LL.2()" function fitted to observed percentage of difference from control data (Ritz et al., 2015). The average cell sizes of algae at various exposure concentrations were compared using analysis of variance (ANOVA) separately for each day of exposure. If the ANOVA indicated a significant overall effect of treatment, then the average cell size at each exposure concentration was compared with the control using a Dunnett's test, with the "multcomp" package (Hothorn et al., 2008) in R software (R Core Team, 2019). For hypothesis testing, homogeneity of variance was tested with Levene's test using the "car" package (Fox & Weisberg, 2019) in R software (R Core Team, 2019). The raw data sometimes violated the homogeneity of variance assumption, but after log-transformation, variances were homogenous. All data met the assumptions of normality. For all statistical analyses, alpha was 0.05.

RESULTS

Safener concentration in commercial formulations

The results from HPLC quantification of safeners are shown in Table 1. For METOO, the identity and concentration of the safener was not included on the label or within the safety data sheets. We were able to identify DICH as being present in the formulation by matching the retention times of a DICH standard with a peak from the METOO HPLC chromatogram. Qualitative GC–MS analysis of the sample was also used to verify the identity of the safener. In general, the safener content provided on herbicide formulation labels, when available, was in good agreement with our measured concentrations.

Acute toxicity to *R. subcapitata*

In controls, the highest algal population growth occurred between 48 and 72 h (Figure 1). As the exposure concentrations approached the EC50 of each chemical, the population growth between time points decreased and became zero at concentrations above the EC50 (Figure 1). Specifically, for all chemicals, when exposure concentrations were higher than the EC50, the number of cells was equal to or less than the initial number of cells. Also, for all tests the SMET positive control resulted in 50%–60% reduction in algal population growth, indicating that the tests were acceptable. In addition, all solvent controls containing 0.01% of acetone or methanol were not significantly different compared with controls for any endpoint analyzed.

The 72-h predicted EC50, and the EC10 values of all chemicals tested with 95% confidence intervals (CIs) are shown in Table 2. The most toxic herbicide to *R. subcapitata* was ACE, with an EC50 of 0.003 ppm and an EC10 of 0.001 ppm. These EC10 and EC50 values are below the maximum concentration of ACE (0.012 ppm) measured in stream waters (Woodward et al., 2019). At 1 order of magnitude less toxicity to *R. subcapitata* than ACE, SMET had an EC50 of 0.046 ppm and an EC10 of 0.022 ppm. All the safeners were considerably less toxic than the herbicide active ingredients, with EC50s ranging from 4 to 213 ppm and EC10s ranging from 0.62 to 11.1 ppm (Table 2). The most toxic safener to *R. subcapitata* was AD, followed by BEN, DICH, and then FUR; the EC50 for FUR was 2 orders of magnitude higher (less toxic) than the other safeners (Table 2).

The formulation exposures were normalized to the concentration of active herbicide to facilitate comparisons. The most toxic of the formulations tested were HAR and SUR, with EC50s of approximately 0.002 ppm ACE (mg of ACE/L) and EC10s of approximately 0.001 ppm ACE. Toxicities of both HAR and SUR were shifted slightly left (i.e., more toxic) but still within the 95% CI of ACE's fitted dose–response model

TABLE 1: Percentage by weight (%wt) of safeners in commercial herbicide formulations

Formulation	Safener used	%wt reported by manufacturer	%wt (±95% CI) measured by HPLC
METOO	DICH[a]	Not reported	2.6 (±0.9)
DUAL	BEN	<5.0	4.7 (±0.4)
HAR	FUR	<2.5	4 (±2)
SUR	DICH	12.2[a]	Not measured

[a]From the Surpass EC Safety Data Sheet (DOW, 2015).
HPLC = high-performance liquid chromatography; METOO = Me-Too-Lachlor II; DUAL = Dual II Magnum; HAR = Harness; SUR = Surpass EC; DICH = dichlormid; BEN = benoxacor; FUR = furilazor.

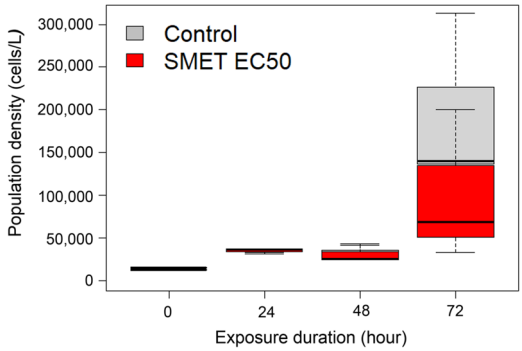

FIGURE 1: Population density (cells/L) of *Raphidocelis subcapitata* over time with controls (no chemical present) and *S*-metolachlor (SMET) present at the median effect concentration (EC50) for *R. subcapitata* at 72 h (indicated in legend top left).

(Figure 2). The safeners FUR and DICH, which are paired with ACE in formulations, did not fall within the ACE 95% CI (Figure 2). The DUAL formulation was more toxic to *R. subcapitata* than METOO (EC50 of 0.051 ppm SMET and EC10 of 0.03 ppm SMET). The EC50 and EC10 values for DUAL were both within the 95% CI of SMET (Figure 2). The safener in DUAL, BEN, was substantially less toxic than SMET (Figure 2). The dose response for METOO was shifted to the right from that of SMET (Figure 2), only overlapping CIs with SMET in the EC10 range (Table 2). The safener DICH was substantially less toxic than SMET, with no overlapping CIs in the dose responses (Figure 2).

Algae size

There was a significant effect of exposure on algal cell size measured for at least one time point for all 10 chemicals tested (Supporting Information, Table S2). The algae cell size will only be discussed for the 72-h results; the results for additional time points can be found in the Supporting Information, Table S2. Both SMET and ACE caused significant effects on algae size at 72 h. A Dunnett's multiple comparison test showed that algae exposed to the herbicide active ingredients were significantly larger compared with controls, at 0.112 ppm or more and 0.004 ppm or more for SMET and ACE, respectively (Figure 3). The ANOVA showed that the safeners BEN and AD did not cause significant changes in algae cell size at 72 h (Figure 3). A significant effect on algal cell size was caused by DICH; at 72 h, DICH treatments of 80 ppm or more resulted in cell sizes that were significantly larger than the controls. A significant effect at 72 h of exposure was seen for FUR, for which the multiple comparison analysis showed that concentrations of 0.05 ppm or more produced cell sizes that were statistically smaller than the controls.

All formulations caused significant effects on algal cell size after 72 h of exposure (Figure 3). A Dunnett's multiple comparison test for DUAL at 72 h showed significantly larger cell sizes than controls at 0.112 ppm or more. Algae in the HAR treatments were significantly larger than the controls at 0.005 ppm or more at 72 h. Multiple comparisons for SUR showed that algae were significantly larger at 0.006 ppm or more at 72 h compared with the respective controls. Finally, algae from the METOO treatments were significantly larger than the controls at all concentrations at 0.045 ppm or more at 72 h.

TABLE 2: Effective concentrations causing 50% and 10% reduction (EC50 and EC10) in *Raphidocelis subcapitata* cell density after 72 h of chemical exposure[a]

Chemical	EC50 (ppm 95% CI)	EC10 (ppm; 95% CI)
Herbicides		
Acetochlor (ACE)	0.003 (0.0027–0.0032)	0.001 (0.0009–0.0013)
S-metolachlor (SMET)	0.046 (0.035–0.057)	0.022 (0.010–0.034)
Safeners		
Benoxacor (BEN)	4.0 (2.6–5.4)	0.62 (0.05–1.19)
AD-67 (AD)	3.8 (0.93–6.7)	0.48 (−0.63–1.6)
Furilazole (FUR)	213.8 (9.9–417.63)	2.7 (−0.06–5.51)
Dichlormid (DICH)	32.1 (17.8–46.5)	11.1 (−3.2–25.4)
Formulations		
Harness (HAR)	0.0022 (0.0018–0.0026)	0.001 (0.0007–0.0014)
Surpass (SUR)	0.002 (0.0022–0.0035)	0.0014 (0.0008–0.002)
Dual II Magnum (DUAL)	0.051 (0.043–0.056)	0.03 (0.026–0.038)
Me-Too-Lachlor (METOO)	0.15 (0.13–0.17)	0.11 (0.07–0.14)

[a]Formulation concentrations were normalized to concentration of active herbicide ingredient.

DISCUSSION

Herbicide active ingredients decreased the growth rate of *R. subcapitata*; the effect of herbicide active ingredients on growth rate was greater than the effect of safeners. The toxicity of the formulations DUAL, HAR, and SUR was similar to that of the respective active herbicide. The only exception was METOO, which was less toxic than the associated active herbicide, SMET. Even though the formulations contained 2%–12% safener, the safener did not appear to impact the effect of the herbicide active ingredients. Initially we hypothesized that safeners would have a protective effect on nontarget algae similar to their protective effect on crops (e.g., via induction of enzymes that facilitate detoxification of herbicides in crops; Gatz, 1997; Hatzios & Burgos, 2004; Jablonkai, 2013; Riechers et al., 2010). Our results suggest that safeners do not provide an analogous protective effect on nontarget algae. In addition to standard toxicity endpoints, significant effects on cell size were also observed in algae exposed to the herbicide active ingredients, formulations, and safeners FUR and DICH. Collectively, these data show that the herbicide and formulations are equally toxic to *R. subcapitata* whereas safeners are not, although all safeners except BEN caused a significant increase in cell size, which may be indicative of more subtle physiological effects.

The herbicide active ingredient SMET was toxic to *R. subcapitata* at 0.046 ppm, causing a 50% reduction in algal

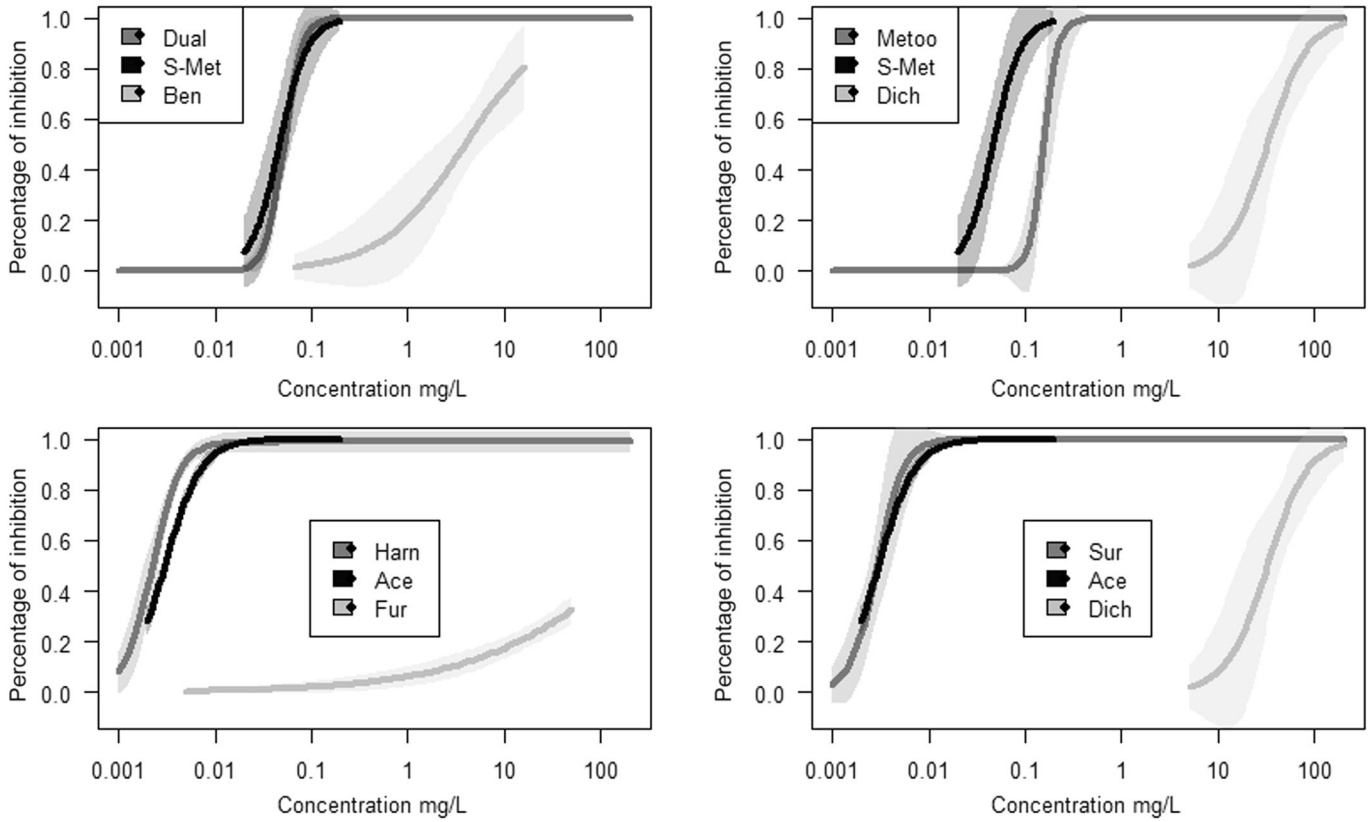

FIGURE 2: Combined dose–response curves for *Raphidocelis subcapitata* exposed for 72 h separately to Dual II Magnum® (Dual), S-metolachlor (S-Met), and benoxacor (Ben; top left), Me-Too-Lachlor II® (Metoo), S-Met, and dichlormid (Dich; top right), Harness® (Harn), acetochlor (Ace), and furilazole (Fur; bottom left), Surpass EC® (Sur), Ace, and Dich (bottom right). Model is a two-parameter log-logistic function fitted using the "drc" package, and estimates are presented with 95% confidence intervals (shaded regions). Formulation concentrations were normalized to concentration of active herbicide ingredient.

population growth (Table 2). The active ingredient ACE was more toxic, causing the same magnitude of effect at 0.003 ppm (Table 2). The EC10 values for SMET and ACE were 0.022 and 0.001 ppm, respectively. Importantly, the EC50 and EC10 values for both herbicide active ingredients occurred at environmentally relevant concentrations; for example, SMET and ACE have been detected in surface, ground, and drinking water at concentrations ranging from 0.001 to 15 ppm (Hidebrandt et al., 2008; Hladik et al., 2005, 2008; Woodward et al., 2018). The toxicity data we present and the available data on environmental concentrations in surface water suggest that effects on nontarget algal species may occur in contaminated environments.

There was a significant increase in algal cell size when algae were exposed to ACE at 72 h at concentrations of 0.004 ppm or more. At 72 h, SMET caused an increase in algal cell size at 0.112 ppm or more (Figure 3). Hence, both ACE and SMET had a significant effect on algal cell size at concentrations similar to their respective EC50 concentrations. Increased cell size has been observed for *R. subcapitata* after exposure to various metals (copper, cadmium, zinc) and SMET (Franklin et al., 2001; Machado & Soares, 2014; Yamagishi et al., 2017). The causes or implications of the observed changes in algal size are unclear. Some research suggests that cell size increases in algae may be due to uptake of water, changes in the reproductive cycle, or change in amount of cell structures like starches or vacuoles (Bišová et al., 2003; Nishikawa et al., 2003; Yamagishi et al., 2017). Yamagishi et al. (2017) found that when exposed to a chemical, *R. subcapitata* favored a reproductive mode whereby one cell produced eight autospores or daughters and the delay in release made them larger. Under less stressful conditions, algal reproduction occurs via a two-autospore mode, which is not accompanied by an increase in cell size. In the case of SMET, researchers found that the cells' ability to split and perform the final stage of mitosis was being inhibited, which resulted in an increase in algal size (Machado & Soares, 2020, 2021). Changes in the reproductive mode and physiology in response to stress were seen in the alga *Parachlorella kessleri* after exposure to SMET (Maronić et al., 2018). Increased size effects seen in ACE and SMET treatments may be indicative of additional impacts of the chemical coupled with toxicity.

The safeners were less toxic to *R. subcapitata* compared with the herbicides. The most toxic safeners were BEN and FUR, with EC50s of 4.0 and 3.8 ppm and EC10s of 0.62 and 0.48 ppm, respectively. The safeners FUR and DICH were far less toxic, with EC50s of 213 and 32.1 ppm and EC10s of 2.7 and 11.1 ppm, respectively. Both BEN and AD are more lipophilic (larger octanol–water partition coefficient) than DICH and FUR (Sivey et al., 2015), which could have caused their

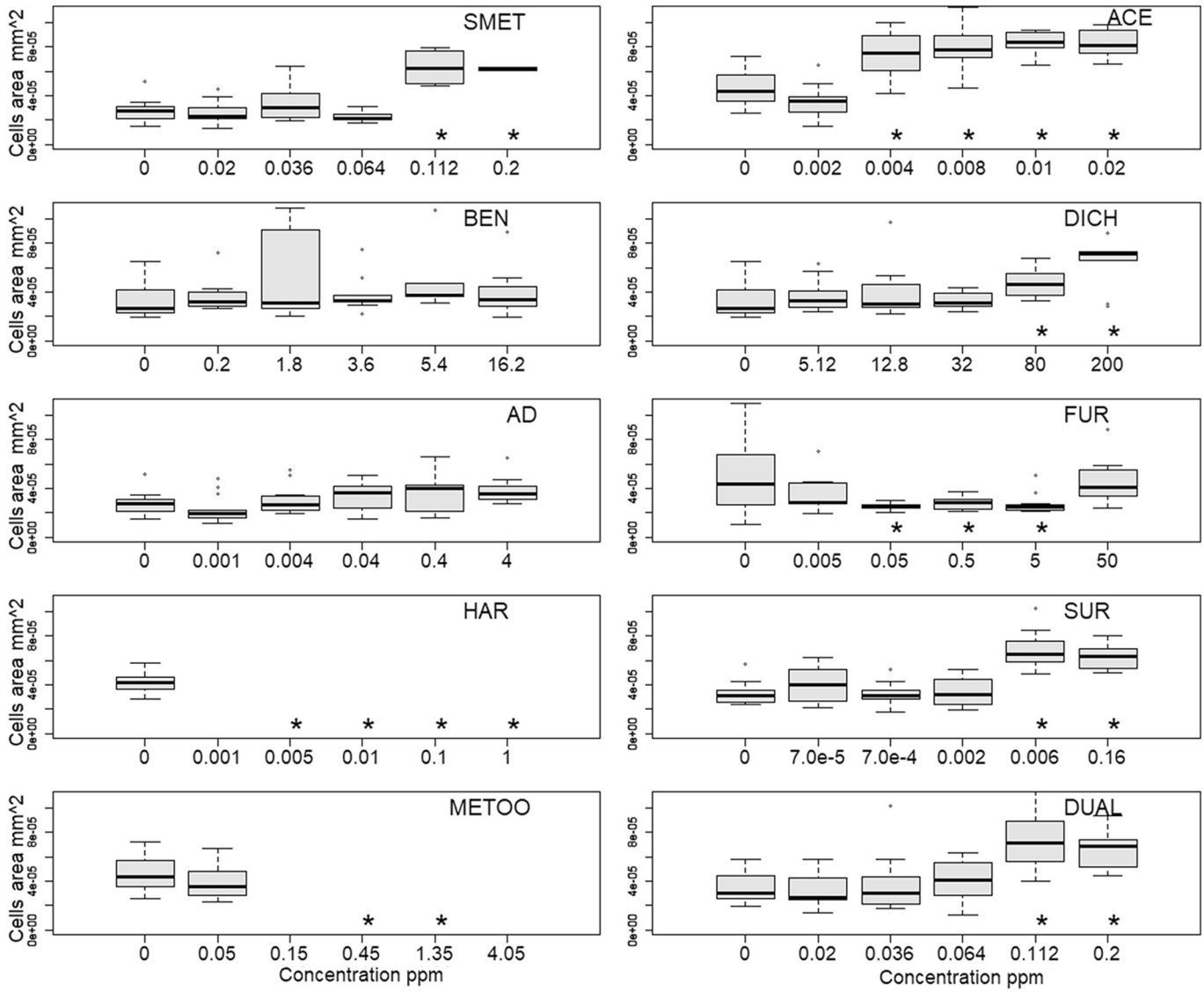

FIGURE 3: Boxplots of *Raphidocelis subcapitata* cell area (mm^2) exposed for 72 h to each chemical separately (indicated left of each plot). *Indicates significant difference compared with controls (shown as 0 for each figure). AD, AD-67. For other abbreviations, see Table 1 footnote.

increased toxicity to algae. Although our data show little toxicity to algae, safeners might be more toxic to aquatic animals. The lethal concentrations that immobilized/killed 50% (LC50) of *Daphnia magna* after exposure to BEN were 4.7 and 26 ppm after exposure to FUR (Lewis et al., 2015). When zebrafish embryos were exposed to AD, the 96-h LC50 was 2.52 ppm, and hatching rates were decreased at 0.02 ppm accompanied by signs of oxidative stress (S. Lui et al., 2021). The effects thresholds we report for a representative freshwater algal species and those reported in the literature for a few aquatic species (Lewis et al., 2015; S. Lui et al., 2021) exposed to the safeners we tested are substantially higher than what has been reported in the environment (42–190 ng/L [0.000042–0.000190 ppm]; Woodward et al., 2018). Similarly, another, newer class of safener that we did not test, cyprosulfamide, has been recently detected at higher concentrations, 22–5185 ng/L (2.2×10^{-5}–0.005 ppm), in surface waters of the midwestern United States (McFadden & Hladik, 2021). It is uncertain, however, whether the higher concentrations of safeners we tested may occur in other water bodies because there is only one environmental concentration study available in the literature to date, that of Woodward et al. (2018). That said, it is also possible that the data reported by Woodward et al. (2018) represent a relatively high-end environmental exposure scenario given the prevalence of row-crop agriculture in their study area. Additional safeners are still being developed, and their ecotoxicological effects merit research attention (Deng et al., 2021). More research is also needed to better understand the magnitude and frequency of safener occurrence in environmental media as well the toxicity of safeners to a broader range of aquatic receptors.

Importantly, dichloroacetamide safeners can undergo photolysis (Kral et al., 2019; Su et al., 2019), hydrolysis (McFadden et al., 2022), abiotic reductive dechlorination (Ricko et al., 2020; Sivey & Roberts, 2012; Xu et al., 2020, 2022), and

TABLE 3: Effective concentrations causing 50% and 10% reductions (EC50s and EC10s) associated with reductions in *Raphidocelis subcapitata* cell density after a 72-h exposure to a mixture of *S*-metolachlor (SMET) and benoxacor, in concentration of the active ingredient SMET[a]

Chemical	EC50 (ppm; 95% CI)	EC10 (ppm; 95% CI)
SMET with 15% BEN	0.058 (0.047–0.063)	0.027 (0.018–0.035)
SMET	0.046 (0.035–0.057)	0.022 (0.010–0.034)

[a]For abbreviations, see Table 1 footnote.

biotransformation (Abu-Qare & Duncan, 2002) in the environment. These processes can result in the safener changing into structures like those of herbicides, which could be more toxic than the parent (Abu-Qare & Duncan, 2002; Sivey & Roberts, 2012). For example, DICH can undergo abiotic reductive dechlorination to produce allidochlor (Sivey & Roberts, 2012). Allidochlor was removed from the market in the 1980s because it is highly irritant to human eyes and skin (Hamm, 1974; Sivey & Roberts, 2012). In rat livers BEN was metabolized by glutathione S-transferases, hepatic cytochrome P450 enzymes, and microsomal carboxylesterases, and BEN concentrations were decreased in the process, suggesting that metabolites were formed (Simonsen et al., 2020). These metabolites are inherently different from the parent compounds and could potentially have different toxicity and environmental effects. Additional studies on the transformation of safeners seem warranted to better understand their potential effects and risks to ecological receptors, including algae and other nontarget organisms.

Although safeners are not substantially toxic to *R. subcapitata* based on a lack of effects ono algal growth, some safeners did cause significant increases in algal cell size. Whereas algae exposed to BEN and AD did not increase in cell size compared with controls at 72 h, DICH caused increased algal cell size at 72 h, but these effects still occurred above environmentally relevant concentrations (Woodward et al., 2018). The only chemical or safener to cause a decrease in cell size at 72 h was FUR, but this still occurred above environmental concentrations (Woodward et al., 2018). More research is needed to better understand algal responses to chemical stressors that result in changes in algal size. As an example, it may be worth exploring whether the change in observed algal size somehow alters the energetic density of algae, which could conceivably result in indirect nutritional effects on consumers.

The formulations HAR, SUR, and DUAL all had EC50s and EC10s (concentration normalized to active herbicide) within the CI of their respective herbicide active ingredient (Table 2). The formulation METOO was less toxic by 1 order of magnitude than active SMET, likely because METOO is a racemic mixture of *R*- and *S*-metolachlor; SMET is a more efficacious herbicide than *R*-metolachlor (Blaser et al., 1999; Moser et al., 1983). We did not have the ability to differentiate *R*- and *S*-chirality with our analytical methods. Although we did not quantify the amount of SMET in METOO, it is still apparent that SMET is the driver of toxicity because the EC50 and EC10 values are very similar between the formulation and active only, and both values are far lower than the safeners. The highest amount of safener (12.2% DICH) of the formulations tested was in SUR, and this did not provide any change in toxicity. In addition, *R. subcapitata* was exposed to a formulation of 85% SMET and 15% BEN to compare the results with DUAL. When *R. subcapitata* was exposed to this formulation, the 72-h EC50 and EC10 values were within the CI for SMET only, further suggesting no modification of toxicity by the safener (Table 3). The EC50 and EC10 values for each formulation presented in the concentration of the total formulation can be found in Table 4. The ECx values have been previously presented as normalized to the active ingredient (herbicide), so they can easily be compared with the ECx values of the active ingredients (Table 2). Each CI range is slightly shifted right of the herbicide active CI range because the formulations are 70%–80% active ingredient, the driver of toxicity. Collectively, our results indicate that the tested herbicide formulations are similarly toxic compared with their respective active ingredients and that "other" ingredients in the formulations, including safeners, do not have a significant impact on algal growth in *R. subcapitata*. However, in actual habitats, organisms may be exposed to more complex mixtures of anthropogenic chemicals (see Bradley et al., 2017). Although exploring the toxicity of active ingredients versus formulations is an important step, more research is needed to better understand both exposure and effects related to complex, real-world mixtures of agrochemicals.

The formulations all had a significant effect on algal cell size, as expected based on similar effects of the respective herbicide active ingredients at 72 h. The formulations DUAL, HAR, and SUR had a significant effect on algal cell size at treatments 0.112 or more, 0.005 or more, and 0.006 ppm or more, respectively. The formulation METOO also caused significant effects on algal cell size at 72 h; exposure to METOO caused a significant effect at treatments of 0.45–1.35 ppm but not at the highest treatment. The only formulation that caused significant increases in algal cell size at concentrations higher than the

TABLE 4: Percentage of active herbicide and safener ingredients in each formulation[a]

Chemical	% Herbicide active	% Safener	EC50 (ppm; 95% CI)	EC10 (ppm; 95% CI)
HAR	75.9	4	0.0029 (0.0024–0.0034)	0.001 (0.0009–0.0019)
SUR	70.87	12.2	0.004 (0.002–0.005)	0.001 (0.0004–0.002)
DUAL	82.4	4.7	0.069 (0.053–0.070)	0.038 (0.030–0.047)
METOO	84.4	2.6	0.18 (0.16–2.20)	0.12 (0.083–0.17)

[a]EC50 and EC10 concentrations (ppm) for growth rate of *Raphidocelis subcapitata* exposed to herbicide formulations. Concentrations of total formulation concentration and not normalized to active herbicide.
DUAL = Dual II Magnum; EC = effective concentration; HAR = Harness; METOO = Me-Too-Lachlor II; SUR = Surpass EC.

TABLE 5: Results from *Raphidocelis subcapitata* exposed to 85% *S*-metolachlor and 15% benoxacor of model I one-way analysis of variance followed by a Dunnett's multicomparisons test

Chemical	Time (h)	ANOVA *p* value	*F* value	*df* among group	*df* within group	Dunnett's test comparisons (mg/L chemical)	Dunnett's *p* value
SMET with 15% BEN	72	1.23×10^{-8}[a]	12.6	5	67	0–0.017	0.4
						0–0.030	0.1
						0–0.054	0.9
						0–0.095	0.003[a]
						0–0.17	0.001[a]

[a]Indicates significant effect at $p < 0.05$.
ANOVA = analysis of variance; BEN = benoxacor; SMET = *S*-metolachlor.

active only was METOO, but the size effects of METOO occurred just above the EC50, similar to the herbicide active ingredients and other formulations. The formulation containing 85% SMET and 15% BEN also caused a significant increase in cell size at a concentration just above the EC50 of the active ingredient alone (Table 5). All formulations had a significant cell size effect at concentrations at or just above their EC50, again similar to their respective herbicide active ingredients.

CONCLUSIONS

Herbicides are added to crops as a formulation, which typically contains an herbicide active ingredient and "other" ingredients that are not always indicated on product labels. It is important to know how much is included and what effects these "other" ingredients may have because they are being introduced to the environment with the herbicides, but these chemicals are generally tested less than active ingredients. One important outcome of our study is that the quantity of safeners in the tested formulations is now more precisely known. The algal toxicity data and cell size effects allowed us to compare safeners with herbicide active ingredients. We found that the safeners had little effect on *R. subcapitata*, and we confirmed that toxicity of the active herbicide to algae can occur at environmentally relevant concentrations. In addition, our data suggest that the other ingredients, including safeners, in the formulations do not modify toxicity to *R. subcapitata*. We also showed an increase in cell size with all herbicide active ingredients, formulations, and two of the safeners tested. These data indicate that there are sublethal effects on *R. subcapitata* and potential indirect effects on algal consumers. Nevertheless, the environmental hazards posed by dichloroacetamide safeners may not be negligible, particularly because these safeners can transform into products with potentially greater bioactivity relative to the parent compounds (Kral et al., 2019; Sivey et al., 2015). Population growth and cell size of algae are common and important endpoints, but additional endpoints should still be investigated to fully understand the potential effects of exposure to herbicide active ingredients, safeners, and formulations. Alternative endpoints could include multispecies impacts, bioaccumulation, and biotransformation. How the safeners interact when under dynamic environmental conditions and if they affect other nontarget organisms is still not yet fully understood. Moreover, if we consider interactions with a broader range of chemicals within complex mixtures, even greater uncertainty exists (Bradley et al., 2017).

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