

**EFFECTS OF PREDATOR CUES ON PESTICIDE TOXICITY: TOWARD
AN UNDERSTANDING OF THE MECHANISM OF THE INTERACTION**

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Abstract—Pesticide toxicity may be modified by a number of co-occurring environmental and ecological stressors. Co-exposure to predator cues has been shown to potentiate and/or synergize toxicity of pesticides. However, the mechanisms behind these interactions are not well understood. Here we examine the effects of fish predator (bluegill, *Lepomis macrochirus*) cues on toxicity of five different pesticides to the freshwater cladoceran, *Ceriodaphnia dubia*. The purpose for examining patterns among pesticides was to test the idea that the mechanism of the interaction could be explained by a general stress response (i.e., the interaction patterns would be similar regardless of the pesticide's mechanism of action [MOA]). Acute 96-h concentration-response experiments were conducted for pesticides with and without fish cues. Predator cues influenced the toxicity of pesticides and the interaction patterns varied among pesticides. Fipronil exhibited a synergistic interaction, while predator cues interacted antagonistically for bifenthrin and thiacloprid. Other compounds previously reported to potentiate toxicity (malathion) were found to act additively. The results demonstrate that factors such as pesticide bioavailability, K_{OC} , and exposure concentration may be important for predicting the occurrence of these interactions and that patterns were not consistent among pesticides varying in MOA. Predator stress is an important component for structuring communities and ecosystem processes. Fully understanding how this process may interact with organic contaminants may best be achieved by examination at toxicokinetic and toxicodynamic scales.

Keywords: *Ceriodaphnia dubia*; Mixture effects; Multiple stressors, Non-additive interactions, Predator cues

INTRODUCTION

The toxicity of pesticides may be modified by a number of co-occurring environmental stressors, and there has been a recent shift in research focus to examine pesticide toxicity under more environmentally realistic conditions that include these toxicity modifying factors. Contaminant toxicity may be modified by environmental and ecological factors such as temperature [1-2], pH [3], resource conditions [4], competition [5], and predation [6,7]. Exposure to predatory cues is a biotic stressor that has received attention recently for its interaction with pesticides on biological responses in both freshwater invertebrate and vertebrate organisms [7-10].

As opposed to direct effects of predation (consumptive predation), predators can transmit cues that warn of imminent predation events and elicit predator-cue driven responses in prey organisms. Predator cues in freshwater systems can be chemical, visual, or auditory, although most focus has been on chemical-based signals such as kairomones or alarm cues [11]. Predator cues may influence proximate prey responses such as behavior [12-15], as well as ultimate responses such as life-history traits and population-level responses [16-17]. How predator cues are released into the environment and the exact nature of predator cues are unclear. Potential sources of cues may be prey conspecifics, predators, or bacteria associated with these organisms [18], and may be released from body surfaces, injured tissues, or feces [19]. Variation in prey responses to cues from different predators suggests that predator species are likely to excrete different cues [15]. However, chemical characteristics suggested that predator cues from different predator species may be similar [13,20]. It was reported that the active compound of predator fish cues is a low molecular weight compound that is water soluble, non-volatile, and

stable to a broad range of temperatures and pH [21], while Weber [22] suggested that predator cues are a mixture of different substances.

The interaction of predator cues and pesticides has resulted in greater or less than additive responses (synergistic or antagonistic effects). Within the same experimental system and response variable, these interaction patterns have varied among contaminants [9,10,23,24], suggesting that the process driving these interactions may be related to the mechanism of action of the chemical contaminant. Only a few studies have attempted to explore the mechanisms of the predator cue-contaminant interaction by conducting co-exposures between predator cues and multiple chemicals varying in mechanism of toxic action. Barry [23] examined nine chemicals and found synergistic interaction effects between predator cues and physostigmine, a cholinesterase (ChE) inhibitor, and picrotoxin, a γ -aminobutyric acid (GABA) receptor antagonist, on induction of neckteeth in *Daphnia pulex*. Additional studies have reported interaction effects, primarily synergistic, between predator cues and ChE-inhibiting chemicals, including both organophosphorous and carbamate pesticides [25,26]. However, there are inconsistencies among these patterns. For example, Hanazato and Dodson [26] reported a synergistic interaction of carbaryl and *Chaoborus americanus* cues on *Daphnia pulex* time to maturity, whereas Coors and De Meester [27] reported no interaction effect on *Daphnia magna* time to maturity for co-exposures of carbaryl and *Chaoborus* spp. cues. Other factors such as concentration of chemical stressor may be important for whether these interactions are observed in experimental studies and may introduce variability among studies. Understanding how multiple stressor interactions may vary among changing contaminant concentrations would have implications toward understanding risk of multiple stressors in aquatic systems.

The aim of this study was to gain insight into the patterns and possible mechanisms of this multiple stressor interaction by addressing the following objectives: examine the interaction between predator cues and pesticides across a suite of commonly used pesticides that vary in their mechanism of toxic action on the acute lethal response of the cladoceran *Ceriodaphnia dubia*, examine how the interaction behaves across multiple contaminant concentrations, and examine how predatory chemical cues may affect the stability and/or bioavailability of pesticides, potentially influencing pesticide toxicity. Ultimately, understanding pesticide toxicity within a multiple stressor context that includes biotic modifying factors will improve the understanding of risk of pesticides in aquatic ecosystems.

MATERIALS AND METHODS

Test organisms

The test organism in this study was the cladoceran *C. dubia*. This species was selected because it is a sensitive indicator of pesticide toxicity, represents a model zooplankton species, and toxicity interactions with biotic factors would likely occur in the range of environmentally relevant pesticide concentrations. In addition, an abundance of background data exists on *C. dubia* response spectrum to pesticides, facilitating use of data generated in these experiments for ecological risk assessment and comparison to previously published toxicity data. In addition, our previous study reported an interaction between predator cues and pesticides on *C. dubia* survival [28]. Organisms used in these experiments were originally obtained from the Ecotoxicology Research Facility at Arkansas State University, and reared in the Aquatic Toxicology Laboratory at Texas Tech University following standardized culturing procedures [29]. *Ceriodaphnia dubia* cultures were maintained at $24\pm 1^{\circ}\text{C}$ in synthetic moderate hard water and a 16:8 h light:dark

photoperiod. The synthetic moderate hard water was prepared following standard procedures [29]. *Ceriodaphnia dubia* were fed a solution containing green algae, *Pseudokirchneriella subcapitata*, at a concentration of 3×10^7 cells/ml, and a mixture of yeast/cereal/trout chow (YCT) [29]. Bluegill (*Lepomis macrochirus*) were obtained from Aquatic Research Organism Company (Hampton, NH, USA), reared in 284 L tanks filled with aged tap water, and fed flake fish food *ad libitum*. All bluegill culturing and experimental procedures followed approved Texas Tech University Animal Care and Use Committee protocols (Protocol 08014-05).

Test Chemicals

Five different pesticides were examined in this study with the objective of providing insight into the extent of predator-pesticide interactions among pesticides that vary in their mechanism of action (MOA) and/or metabolic-detoxification pathways. Specific pesticides, purity (%), their chemical class, and their mechanism of action included: bifenthrin (99.0%; pyrethroid; Na⁺ channel inhibitor); fipronil (98.7%; phenylpyrazole; GABA gate and Cl⁻ channel inhibitor); malathion (99.4%; organophosphate, subclass: phosphorodithioate; ChE inhibitor); thiacloprid (99.5%; neonicotinoid; nicotinic acetylcholine inhibitor); and tribufos (98.0%; organophosphate, subclass: phosphate; ChE inhibitor) [30]. All pesticides were obtained from ChemService (West Chester, PA, USA). Not only did these chemicals represent diverse classes of pesticides with varying functional groups and mechanisms of toxic action, but they represented pesticides that are commonly used in agricultural practices (National Agriculture Statistics Service, <http://www.pestmanagement.info/nass/>).

Stock solutions were prepared by dissolving chemicals in pesticide-grade acetone. All stock and working solutions were stored in the dark at 4 °C and all working solutions were used within

24 h. Exposure solutions were prepared by spiking known amounts of stock solution into exposure media. For bifenthrin, malathion, and tribufos, toxicity experiments and the stability experiments (described below) were conducted on aliquots of the same exposure solution preparation. For fipronil and thiacloprid, toxicity and stability experiments were derived from separate solution preparations from the same stock solution, following identical solution preparation methods.

Mixture toxicity tests

Freshwater acute toxicity tests were conducted to examine the concentration-response relationships and point estimates (LC50s [concentration resulting in 50% mortality of a population]) for each individual pesticide in the presence or absence of predator-conditioned water. The potential interaction between predatory stress and each pesticide was examined separately using a multiple stressor dose-response approach. Each experiment consisted of 2×6 completely randomized factorial designs with predator conditioned water either present or absent and five or six pesticide levels (not including the control). To produce predator-conditioned water, two healthy adult bluegill (total body length = 10-12 cm) were randomly selected from a culture tank that contained about 20 individuals, and placed in 8 L of synthetic moderately hard water for two hours. The culture water was then filtered through a 1 μ m glass fiber filter to remove particles and used as the fish-conditioned water in experiments [8,9]. Bluegill were then returned to the culture tank. Previous studies have demonstrated that this methodology of water conditioning resulted in a solution of chemical cues that led to significant effects on life history traits of *C. dubia* [9].

To examine total organic carbon (TOC) content, fish-conditioned water was prepared in three different 8 L tanks. Three additional tanks were prepared identically with only moderately hard water. Water samples were filtered through a 1 µm glass fiber filter, and TOC of the water samples was determined with an OI Analytical TOC analyzer (model 1020A). Standard solutions were made by dissolving known amounts of glucose in Milli-Q water to develop a four-point standard curve.

Acute toxicity tests (96-h) were conducted following standardized methodology [29]. Experimental chambers consisted of 40-ml borosilicate glass jars and each contained 30 ml of synthetic moderate hard water or predator-conditioned water for every pesticide concentration tested. Five randomly selected *C. dubia* neonates (< 24 h old) were added to each experimental chamber at the start of each experiment and there were six replicates for every treatment combination. Solvent controls contained pesticide-grade acetone at a concentration not exceeding 0.06 % of the exposure media volume. In preliminary studies, no difference in *C. dubia* mortality was observed between organisms exposed to synthetic moderately hard water with and without the % volume of acetone used in the present studies. Experiments were conducted in an incubator (Thermo Scientific Precision Refrigerated Incubator 818) at 24±1°C, with a 16:8 h light:dark photoperiod. Each experimental unit was fed 50 µl of an algae solution (*P. subcapitata*; 3×10^7 cells/ml) and 50 µl YCT. Water quality parameters including temperature, dissolved oxygen (DO), pH, and conductivity were monitored on the freshly prepared solutions, using an Accumet XL 60 bench top dual channel pH, ion, DO meter and probes and an Orion 3-Star bench top conductivity meter.

Chemical stability tests

Separate experiments were conducted to examine the stability of bifenthrin, fipronil, malathion, thiacloprid, and tribufos with and without the presence of predator fish cues. The pesticide stability tests were conducted under similar experimental conditions as the acute toxicity tests. Pesticide solutions were made in moderate hard water and predator-conditioned water. Individual experimental chambers were 40-ml borosilicate glass jars with 30 ml pesticide solution and five *C. dubia* neonates. Each experimental chamber was fed with 50 µl of *P. subcapitata* (3×10^7 cells/ml) and 50 µl YCT and experiments were conducted in an incubator at 24 ± 1 °C with a 16:8 h light:dark photoperiod. Pesticide concentrations were examined 1 h after solution preparation and 72 h of exposure. These time points were selected to be within the overall exposure period.

Chemical Analyses

Pesticides were extracted using Strata™ C18 solid phase extraction (SPE) cartridges. Cartridges were first conditioned with 6 ml of methanol, followed by 6 ml of deionized water. Samples were passed through the cartridges at a rate less than 1 ml/min until complete drying of the SPE column. For each cartridge, pesticide was eluted with two 3 ml volumes of a 20/80 (v/v) mixture of acetone/hexane. Eluates were evaporated under a gentle stream of nitrogen gas at 25 °C until near dryness. Samples were then reconstituted in 1 ml of acetone and then quantified using an Agilent 6890 series gas chromatograph.

For tribufos and malation, a Phenomenex ZB-5 column (30 m × 0.32 mm × 0.25 µm) and a flame photometric detector were used. Helium was used as the carrier gas and makeup gas, with the combined flow rate of 60.0 ml/min. The injection volume was 2 µl and inlet was set to the

splitless mode. Oven temperature was 60 °C initially, heated to 260 °C at a rate of 20 °C/min, and held for 2 min.

For bifenthrin, an Alltech EC-5 column (30 m × 0.32 mm × 0.25 µm) and a microcell electron capture detector (µECD) was used. Helium was used as the carrier gas and argon-methane (5%) as the makeup gas, with a combined flow rate at 60.0 ml/min. The injection volume was 2 µl and inlet was set to the splitless mode. Oven temperature was 60 °C initially, heated to 260 °C at a rate of 20 °C/min, and held for 2 min.

For fipronil and thiacloprid, a Phenomenex ZB-5 column (30 m × 0.32 mm × 0.25 µm) and a µECD were used. Helium was used as the carrier gas and argon-methane (5%) as the makeup gas, with a combined flow rate of 30.0 ml/min. The injection volume was 2 µl and inlet was set to splitless mode. Oven temperature was 35 °C initially, heated to 320 °C at a rate of 20 °C/min, and held for 5 min.

For each pesticide, five external standards were used for linear calibration. Standards were run before each set of blanks and samples.

Prediction of joint effects and data analyses

The joint or interaction effects between pesticide and predator cues were evaluated using several methods in a weight of evidence approach. First, a commonly examined point estimate for ecological risk assessments, the LC50, was estimated for each pesticide and compared between moderately hard water [MH] and fish predator cue [FC] treatments. Logistic regressions were used to estimate LC50s using a generalized linear model with a logit link function, binomial probability distribution, and a maximum likelihood estimation method with the software JMP version 8.0.2 (SAS Institute). From these LC50 estimates, 95% confidence

intervals (CIs) were visually inspected and if there was no overlap between the MH or FC groups then the null hypothesis ($H_0: LC50_{MH} = LC50_{FC}$) was rejected.

A second approach, also based on these LC50 estimates, was the LC50 ratio test [31]. The natural log of the ratio was defined as: $\ln(\zeta) = \ln(LC50_{MH}) - \ln(LC50_{FC})$ and used to derive a z test statistic: $z = \ln(\zeta) / SE[\ln(\zeta)]$, where $SE[\ln(\zeta)]$ was the standard error of the ratio [31]. The standard error was estimated using the formula described in Wheeler et al. [31]. The calculated z test statistic was compared to a Z score table to estimate p -values.

In a third approach, survival data were analyzed using a two-way analysis of variance (ANOVA). Each analysis consisted of two factors: pesticide at six or seven levels (i.e., control and either five or six concentrations) and predator cue at two levels (present or absent). All percentage data were arcsine square root transformed. A significant two-way interaction between the insecticide factor and the predator cue factor was considered to be a non-additive response, either synergistic or antagonistic. If the response from the interaction was greater than additive, we considered this a synergistic interaction. If the response from the interaction was less than additive, we considered this an antagonistic interaction. Pairwise t tests were used as multiple comparison tests. All tests were performed using JMP version 8.0.2. (SAS Institute).

Fourth, equivalence of concentration-response relationships with and without predator cues was examined using the methods described in Oris and Bailer [32]. Briefly, a series of hypotheses related to parameters of the relationship from the logistic regression (i.e., β_1 or β_0) were tested. Null hypothesis 1 was that β_1^{MH} and β_0^{MH} did not differ from β_1^{FC} and β_0^{FC} . Null hypothesis 2 was that $\beta_0^{MH} = \beta_0^{FC}$ and null hypothesis 3 was that $\beta_1^{MH} = \beta_1^{FC}$ (see Yu [30]). Parameters were derived from logistic regressions of mortality and pesticide concentration, and likelihood estimates were used to compare models by generating likelihood ratio test statistics.

This test statistic was then compared to a table of χ^2 values [32]. Rejection of hypothesis 2 (i.e., intercepts differ) may be interpreted as the presence of predator cues having altered *C. dubia* sensitivity to the pesticide. Rejection of hypothesis 3 (i.e., slopes differ) may be interpreted as the presence of predator cues altering the mechanism of action of the pesticide or how toxicity was occurring, and this may suggest effects on toxicokinetic or toxicodynamic processes.

And finally, an independent action (IA) model was used to evaluate interaction effects of pesticides and predator cues. We followed the methods described in Coors and De Meester [27] to estimate the predicted joint effects from responses measured from individual stressors using the following equation:

$$E_{mix} = \prod^i (1 - E_i)$$

Where E_{mix} was the effect of the predator cue and pesticide combination and E_i was the effect of each individual stressor (i.e., i = predator cue or pesticide). It is suggested that responses for individual stressors be modified to proportional effects to account for maximal responses [33] and responses observed for control treatments [27]. Thus, E_i was estimated by the approach described in Coors and De Meester [27]:

$$E_i = \frac{(e_i - e_c)}{(e_{max} - e_c)}$$

Whereas, e_i was the observed effect (i.e., proportion mortality) of either predator cue or pesticide, e_c was the response observed for the control, and e_{max} was the maximum possible response of an individual stressor. For mortality data in the present study, e_{max} was one.

For the observed effect of the mixture of predator cues and pesticides, 95% confidence intervals were calculated and the predicted mixture effect was compared to the mean observed

effect and associated 95% CIs. We followed previous interpretations of this comparison such that if the predicted effect was within the 95% CI of the observed, the interaction was additive [27]. If the predicted mixture effect was greater than the upper 95% CI then the interaction was considered antagonistic, and less than the lower 95% CI the interaction was considered synergistic. Observed and predicted values were compared for each concentration separately. This was identical to the approach of Coors and De Meester [27] except it was conducted over multiple pesticide concentrations ~~in order~~ to demonstrate how concentration may influence this interaction.

RESULTS

Ranges of water quality parameters in prepared exposure solutions were as follows: temperature, 22.0 to 22.4 °C; pH, 7.78 to 8.33; DO, 4.70 to 5.66 mg/L; and conductivity, 310 to 346 µS/cm. No mortality was observed in the solvent control treatment and results indicate that predator conditioned water alone had little influence on the mortality of *C. dubia* neonates (mean = 1.25%, standard deviation = 2.49%, range = 0 to 6.67%). Of all the chemicals tested, bifenthrin was found to be the most toxic to *C. dubia*, while thiacloprid was the least toxic pesticide (**Table 1**). Mean \pm standard deviation of TOC in freshly prepared fish-conditioned water was 14.1 ± 6.8 mg/L and 3.4 ± 3.3 mg/L in moderately hard water.

From the exposure-response analyses, LC50 95% CIs did not overlap for bifenthrin, fipronil, and thiacloprid (Table 1). Likewise, the LC50 ratio test indicated significantly different LC50s for these same compounds ($p < 0.01$) (Table 1). From these results, bifenthrin and thiacloprid were less toxic to *C. dubia* neonates in the presence of predator cues (i.e., interacted antagonistically), while fish cues interacted synergistically with fipronil on the survival response

(Table 1). Tribufos and malathion had lower LC50s in the presence of predator cues but 95% CIs overlapped and the LC50 ratio test was not significant ($p > 0.05$) (Table 1).

From the results of the 2-way ANOVA of pesticide and predator cue, bifenthrin, fipronil, thiacloprid, and tribufos exhibited a significant interaction effect ($p < 0.05$) with predator cues, suggesting that assessment of interaction effects with ANOVA may be less conservative than other methods included here in the weight of evidence approach (Table 2).

Results of the slope-intercept equivalence test indicated that intercepts (β_0) for bifenthrin, fipronil, and tribufos varied between treatment with and without predator cue, suggesting either a shift in sensitivity of *C. dubia* or a shift in potency of the pesticide (Table 3). For these chemicals, the direction of this shift was consistent with other assays in the weight of evidence approach (i.e., bifenthrin was antagonistic and the others were synergistic) (Table 4). Slopes (β_1) for fipronil and tribufos differed when the exposure-response relationship was examined in the presence or absence of predator cues (Table 3). Typically this result is interpreted as either a difference in mechanism of toxic action or genetic variation among populations of the receptor organisms [32]. Both explanations are unlikely in the present study, but variation in slopes may suggest that effects of predator cues may be operating at the toxicodynamic scale.

Observed effects of the joint exposure of predator cues and pesticides were compared to those predicted using an IA model. Deviation of the predicted value from the 95% CI of the observed data occurred for at least two concentrations for bifenthrin, fipronil, and thiacloprid (Figure 1). These patterns were consistent with other methodologies used in the weight of evidence and provided further support of an antagonistic interaction of predator cues with bifenthrin and thiacloprid and a synergistic interaction with fipronil (Table 4).

The IA model analysis reveals a pattern that suggests that the influence of predator cues on pesticide toxicity may be dependent on exposure concentrations. For example, bifenthrin antagonistically interacted with predator cue when bifenthrin concentration was between 0.12 and 1.92 $\mu\text{g/L}$, while at the high and low effect concentrations predicted joint effects were closer to the observed response and associated variability (Figure 1). A similar pattern was observed for thiacloprid for concentrations between 3.6 and 6.0 mg/L .

For pesticides exhibiting strong synergistic effects with predator cues (i.e., fipronil) and those indicating some evidence of synergism (i.e., tribufos), it appears that these effects are most prevalent at lower effective concentrations. For example, predator cue only increased the toxicity of fipronil when its concentration was lower than 150 $\mu\text{g/L}$ and less than a 30% effect on survival (Figure 1). Deviation from the 95% CI of the measured toxicity data was also observed for tribufos, but only at a single tested concentration and also at concentrations that resulted in around 30% effect or less. These patterns indicate that exposure concentration is important and at higher pesticide concentrations, the effect of contaminant stressor may mask detection of interaction effects.

For the chemical stability experiments it was found that after 72 h, concentrations of tribufos and malathion were somewhat lower in the fish-conditioned water treatment than in moderately hard water (Table 5). The concentrations of bifenthrin, fipronil, and thiacloprid in fish-conditioned water were similar with those in synthetic moderately hard water (Table 5).

DISCUSSION

Interaction between predator cues and pesticides

Interaction effects between predator cues and contaminants on aquatic invertebrates and vertebrates have been observed in laboratory and outdoor mesocosm experiments [8-10,25]. Results of the current study support the view that predator stress may interact with pesticides and influence their toxicity in aquatic environments [25]. Without the presence of pesticides, predator cues alone did not significantly influence *C. dubia* neonate survival, which was expected since there was no occurrence of consumptive predation throughout the experiments. However, predator cues were shown to modify the toxicity of pesticides. Among the pesticides examined, all possible outcomes of combined effects such as synergistic, antagonistic, and additive effects were observed.

A significant synergistic interaction on *C. dubia* survival was found between predator cues and fipronil. To our knowledge, this is the first report of an interaction between predator cues and fipronil, although the interaction between predator cues and another GABA-gated chloride channel antagonist (endosulfan) on invertebrate responses has been reported [24,34]. Synergistic interaction between predator cues and chemicals has been widely observed in the aquatic environment, and multiple stressor combinations (including predator cues and pesticides) have been suggested to be pervasive enough as to contribute to amphibian population declines [35]. Given that many other natural stressors, including temperature, resource limitation, and pathogens have been observed to interact (mostly synergistically) with chemical stressors [36], it is reasonable to conclude that traditional single stressor laboratory experiments may significantly underestimate risk of toxicants under environmentally realistic conditions.

Significant antagonistic interactions between predator cues and bifenthrin on *C. dubia* survival were observed, providing additional evidence that factors related to bioavailability may be important drivers in pesticide-predator cue interactions. Previously, several pesticides have been reported to have antagonistic interactions with predator cues and have included fenvalerate [10], endosulfan [24,34], atrazine [24], fenoxycarb [9], carbaryl [26], and glyphosate [37]. In the present study, a relatively new class of pesticide, a neonicotinoid (thiacloprid), was also found to have antagonistic interactions with predator cues on *C. dubia* survival. These examples further support the idea that pesticide-predator cue interaction may be a prevalent phenomenon in aquatic systems, but the characteristics of the interaction (i.e., additive, synergistic, antagonistic, or potentiation) may be highly variable and difficult to predict.

One hypothesis for the occurrence of antagonistic interactions is that predator cues and/or other constituents of exuded material from predators may directly interact with organic contaminants. Results of the current study showed that TOC content in fish conditioned water was about 4.7 times higher than that in moderately hard water, suggesting that the fish conditioned water contained dissolved organic matter from fish. Antagonistic effects observed in the current study may be the result of properties such as K_{OC} driving a chemical interaction between exuded organic matter, reducing the bioavailable fraction of the pesticides and resulting in lower toxicity. Bifenthrin has a high $\log K_{OC}$ relative to other pesticides ($\log K_{OC} \approx 5.0$) and has been shown to adsorb to a variety of organic matter sources common within aquatic ecosystems [38]. Unfortunately, the exact chemical nature of fish cues is not clear. Further research in this area may yield information that could be useful for predicting chemical interactions between predator cues and pesticides that may help to explain mechanisms for both synergistic and antagonistic interactions.

Microbial degradation may also play a role in antagonistic interactions as this process can affect the persistence of pesticides in the environment [39]. It has been suggested that bacteria associated with fish, rather than fish themselves, are responsible for the production of predator cues [22] and these bacteria could facilitate the degradation of pesticides.

Simultaneous exposure to malathion (a ChE inhibitor) and predator cues in the present study resulted in an additive effect on *C. dubia* survival, whereas previous work has shown that interactions between predator cues and malathion has resulted in greater than additive responses. For example, a synergistic interaction between malathion and predator (*Notophthalmus viridescens*) kairomones was observed on *Hyla versicolor* survival [40]. In addition, a greater than additive response in *C. dubia* survival occurred with simultaneous exposure to malathion and *Pimephales promelas* extracts [28]. In other joint stressor studies involving predator cues and ChE inhibitors, results of the interaction have been mixed. Carbaryl was found to have antagonistic interactions with predator (*Chaoborus* spp.) cues on *Daphnia* spp. growth and reproduction [26,27]. Alternatively, several studies reported that predator stress did not affect the acute toxicity of carbaryl (i.e., an additive response was observed) [8,24,27] and dicrotophos (a phosphate subclass OP) [28]. These patterns demonstrate that even within a group of contaminants (e.g., ChE-inhibitors) that should behave similar from a toxicodynamic perspective, their interactions with predator cues may result in widely varying outcomes. Results of the present study also showed that predator cues could affect the toxicity of pesticides from different classes, such as bifenthrin and thiacloprid, similarly. Thus, in terms of risk assessment, it may not be feasible to predict pesticide toxicity modification by predator cues at broader levels of pesticide classification, such as the mechanism of toxic action. It is likely that other variables such as the specific predator-prey combination, resource conditions, response quantified,

pesticide bioavailability and hydrophobicity, and pesticide exposure regime and concentration are important for evaluating the predator-pesticide interaction.

In the present study we used several approaches to estimate the interaction between predator cues and pesticides on *C. dubia* survival in a weight of evidence approach. These included an LC50 95% CI comparison, LC50 ratio test, observed versus predicted effects from an IA model, two-way ANOVAs, and comparisons of parameters (β_1 and β_0) from exposure-response relationships. All methods suggested that bifenthrin and thiacloprid antagonistically interacted with predator cues, fipronil synergistically interacted with predator cues, and malathion had no interaction with predator cues. Because each approach is weighted by different aspects of the exposure-response relationship, variation in outcome among approaches (within a particular pesticide) was not surprising and this was observed for one pesticide examined, tribufos. For example, significant interaction between tribufos and predator cues was observed from ANOVA results and comparison of β_0 , but no evidence of interaction was found when comparing LC50 95% CIs or from the LC50 ratio test. This presents a challenge for determining if or to what extent these compounds interact with predator cues, but provides information on the mechanism of the interaction. Therefore, the pattern for both compounds suggests that comparisons of single point estimates (LC50s) associated with the concentration-response relationship may miss important information that is revealed when considering parameters of the relationship such as the slope and intercept.

Pesticide concentration effects on interaction patterns

Toxicity is predictably a function of exposure concentrations and this fundamental concept should also be applied when examining interactions among stressors that include contaminants.

To our knowledge, only a limited number of studies have applied multiple pesticide exposure concentrations to examine the interaction between predator cues and pesticides, and for larger and more complex mesocosm study designs, this is an understandable tradeoff. The value of examining responses within the context of a concentration gradient is that information related to alteration in mechanisms and/or potency can be extracted. Here we examine multiple exposure concentrations that result in partial responses, and demonstrate that the modifying effects of predator cues on pesticide toxicity vary over the concentration gradient examined. This is evident by comparison of observed joint effect responses to those predicted from the IA model. For example, it was found that the variation between observed and predicted effects of predator cues and pesticide combination varied across the thiacloprid and bifenthrin concentration gradient: the predicted value was farthest from the 95% CI of the observed data around the LC50 value. Differences in test concentrations may explain inconsistent responses to predator cue and pesticide mixtures among different studies. In the present study we also found that predator cues interacted synergistically with fipronil when concentrations were less than 172.8 $\mu\text{g/L}$, while acting additively when concentrations were over 288.0 $\mu\text{g/L}$ (again from the comparison of observed to predicted by the IA model). This supports the idea that at least for a mortality response, exposure to concentrations at which low partial responses are observed is the window in which the strongest interaction with other stressors may occur [24]. As concentrations increase, the main effect of the pesticide may overwhelm the subtle effects of ecological stressors, and this may occur most dramatically with joint stressors that act through potentiation, such as predator cues. Essentially, it is reasonable to conclude that under some experimental designs, interactions between predator cues and pesticides or other chemicals may go undetected because of selection of inappropriate exposure concentrations. In terms of understanding risk

within aquatic ecosystems, inclusion of exposure concentrations in experiments that occur most frequently in the environment and lower will yield the most useful information on the importance of predator cue interactions with pesticides.

Effects of predator cues on chemical stability

To better understand the mechanisms of the interaction, it is important to monitor pesticide fate in the presence of predator cues. Results of the chemical stability tests in the current study indicated that stability in the presence of predator cues varied among compounds relative to absence of predator cues. After 72 h, concentrations of tribufos in the predator cue conditioned water were slightly more reduced (15-25%) than the experimental units containing only synthetic moderately hard water. Because the weight of evidence was mixed with half of the endpoints indicating similar toxicity between the two treatments and 1 h tribufos concentrations were similar, the majority of the toxic effect may have occurred during the early part of the exposure period. Fipronil was the only pesticide to have consistent synergistic effects in the present study and also exhibited little variation in water concentrations between the MH and FC treatments. This may suggest that the mechanism of the interaction is not due to bioavailability of the pesticide but rather processes within the organism. Whether the interaction was a result of repartitioning of finite energy due to an additional stress response (i.e., less energetic resources allocated to metabolism) or related to toxicokinetic or toxicodynamic processes is unknown at this time.

Thiacloprid and bifenthrin concentrations were relatively similar between the MH and FC groups and over time. The antagonistic interaction may be explained by alteration in the

bioavailable fraction when predator cues were present within the system or perhaps effects at the toxicodynamic scale.

It should be noted that the chemical stability data provides information on only the quantity of parent compound present in these systems. From this we only gain a portion of the information related to bioavailability (i.e., the parent compound may be present and detected but not available for uptake and toxicity). Furthermore, these data should be interpreted with caution because only two concentrations were examined with two samples each. Further work with a more complete design would more thoroughly answer questions related to pesticide bioavailability in the presence of predator cues.

CONCLUSIONS

The results of the current study demonstrate that predator (*L. macrochirus*) chemical cues alone did not have a significant impact on the survival of *C. dubia*. However, predator cues influenced the acute toxicity of some pesticides and varied among predator × pesticide combinations, exhibiting both synergistic and antagonistic patterns. Results of the weight of evidence approach to all assays in the current study suggest a synergistic interaction between fipronil and predator cues on *C. dubia* survival, an antagonistic interaction between bifenthrin and thiacloprid and predator cues, and generally an additive effect on survival for malathion and tribufos. It is suggested that the interaction between predator cues and pesticides may depend on factors such as the particular prey-predator model, pesticide bioavailability, partitioning to organic matter, and exposure concentrations; while a pesticides MOA may be less important. Mechanistically predator cues may influence the bioavailability or stability of pesticides and affect toxicity. Results of the current study indicate the importance of considering environmental

and ecological modifying factors, such as species interactions (predation), in pesticide risk assessments within aquatic ecosystems. More studies are needed to fully understand the mechanisms driving these interactions.

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Figure Text

Figure 1. Observed *Ceriodaphnia dubia* mortality response (●) when exposed jointly to a pesticide and the presence of predator *Lepomis macrochirus* chemical cues (mean \pm 95% confidence interval, $n = 6$), predicted mortality (○) of the joint stressors estimated from the independent action (IA) model described in Coors and De Meester [27], and logistic regression of observed mortality on concentration (solid line) and associated 95% confidence intervals (dotted line) for the joint exposure. Error bars associated with the observed response (●) are 95% confidence intervals. Interpretation of synergistic or antagonistic responses follow the approach of Coors and De Meester [27] such that when the predicted response was greater than the observed response (i.e., above the 95% CI) the interaction was considered antagonistic. When the predicted response was less than the 95% CI of the observed response, a synergistic interaction between pesticide and predator cue occurred.

Table 1. Median lethal concentrations (LC50s) (96-h) and 95% confidence intervals (CI) for *Ceriodaphnia dubia* exposed to pesticides in the presence and absence of predator *Lepomis macrochirus* chemical cues, and results of the LC50 ratio test based on methods described by Wheeler et al. [31]. The z test statistic was calculated as the LC50 ratio $[\ln(\zeta)]$ divided by the standard error of the ratio (SE $[\ln(\zeta)]$). MH = moderately hard water treatment and FC = fish cue treatment.

Pesticide	Group	LC50		LC50			
		($\mu\text{g/L}$) ^a	95% CI	Ratio [$\ln(\zeta)$]	SE [$\ln(\zeta)$]	z Test Statistic	p
Bifenthrin	MH	0.39	0.29-0.49	1.052	0.154	6.85	<0.001
	FC	1.12	0.92-1.36				
Fipronil	MH	143.43	126.40-163.43	0.398	0.157	2.53	0.011
	FC	96.36	61.30-122.18				
Malathion	MH	2.01	1.81-2.24	0.102	0.071	1.43	0.153
	FC	1.81	1.63-2.01				
Thiacloprid	MH	3.39	3.05-3.82	0.735	0.079	9.19	<0.001
	FC	7.07	6.32-8.03				
Tribufos	MH	4.62	4.14-5.28	0.122	0.076	1.60	0.110
	FC	4.09	3.71-4.61				

^a Thiacloprid concentration is mg/L.

Table 2. Results of the two-way analysis of variance (ANOVA) testing the effects of predator *Lepomis macrochirus* chemical cues (FC), pesticide (P), and their interaction (FC × P) on *Ceriodaphnia dubia* 96-h survival.

Chemical	Factor	<i>F</i>	<i>p</i> -value
Bifenthrin	FC	51.0	<0.0001
	P	71.5	<0.0001
	FC × P	2.8	0.024
Fipronil	FC	12.7	0.001
	P	186.9	<0.0001
	FC × P	6.1	<0.0001
Malathion	FC	0.5	0.467
	P	175.3	<0.0001
	FC × P	1.0	0.419
Thiacloprid	FC	70.4	<0.0001
	P	153.6	<0.0001
	FC × P	14.5	<0.0001
Tribufos	FC	9.5	0.003
	P	212.0	<0.0001
	FC × P	4.5	<0.001

Table 3. Results of slope-intercept equivalence tests for concentration-response relationships of *Ceriodaphnia dubia* 96-h survival in the presence and absence of predator *Lepomis macrochirus* chemical cues. MH = moderately hard water treatment, FC = fish cue treatment, NS = not significant, and β_0 = the intercept and β_1 = slope of the corresponding concentration-response relationship. Methods followed those described by Oris and Bailer [32]. The value of the likelihood ratio test statistic (LRT) was derived from subtracting the likelihood estimates ($-2\ln(L)$) of the full model from those of each corresponding model. The p value was derived by comparing the LRT value to the chi-square distribution with degrees of freedom equal to the number of parameters missing from the reduced model compared to the full model.

Pesticide	Model	$-2\ln(L)$	LRT	p value
Bifenthrin	Full	248.6	--	--
	H1: $\beta_0^{FC}, \beta_1^{FC} = \beta_0^{MH}, \beta_1^{MH}$	290.7	42.17	<0.001
	H2: $\beta_0^{FC} = \beta_0^{MH}$	275.1	26.52	<0.001
	H3: $\beta_1^{FC} = \beta_1^{MH}$	248.7	0.08	NS
Fipronil	Full	203.8	--	--
	H1: $\beta_0^{FC}, \beta_1^{FC} = \beta_0^{MH}, \beta_1^{MH}$	221.0	17.25	<0.001
	H2: $\beta_0^{FC} = \beta_0^{MH}$	212.0	8.29	<0.005
	H3: $\beta_1^{FC} = \beta_1^{MH}$	210.4	6.64	0.01
Malathion	Full	134.0	--	--
	H1: $\beta_0^{FC}, \beta_1^{FC} = \beta_0^{MH}, \beta_1^{MH}$	136.1	2.15	NS
	H2: $\beta_0^{FC} = \beta_0^{MH}$	134.0	0.01	NS
	H3: $\beta_1^{FC} = \beta_1^{MH}$	134.4	0.37	NS
Thiacloprid	Full	170.9	--	--

	H1: $\beta_0^{FC}, \beta_1^{FC} = \beta_0^{MH}, \beta_1^{MH}$	227.7	56.71	<0.001
	H2: $\beta_0^{FC} = \beta_0^{MH}$	174.6	3.65	NS
	H3: $\beta_1^{FC} = \beta_1^{MH}$	171.0	0.01	NS
Tribufos	Full	91.3	--	--
	H1: $\beta_0^{FC}, \beta_1^{FC} = \beta_0^{MH}, \beta_1^{MH}$	102.9	11.64	<0.005
	H2: $\beta_0^{FC} = \beta_0^{MH}$	100.5	9.17	<0.005
	H3: $\beta_1^{FC} = \beta_1^{MH}$	98.7	7.38	<0.01

Table 4. Weight of evidence summary for evaluating the interaction effects of co-exposures to predator cues and pesticides on *Ceriodaphnia dubia* acute toxicity compared to the pesticides alone. Approaches considered within the weight of evidence included an LC50 confidence interval (CI) comparison, LC50 ratio test (ratio), two-way analysis of variance (ANOVA), comparisons of intercepts (β_0) and slopes (β_1) from exposure-response relationships, and comparison of observed responses to the mixture to the predicted response from an independent action (IA) joint stressor model. Interpreted synergistic interactions (+) and antagonistic interactions (-) are indicated. Differences in slopes (β_1) are indicated with an asterisk (*) and NS = not significant.

Pesticide	CI	Ratio	ANOVA	β_0	β_1	IA
Bifenthrin	-	-	-	-	ns	-
Fipronil	+	+	+	+	*	+
Malathion	NS	NS	NS	NS	NS	NS
Thiacloprid	-	-	-	NS	NS	-
Tribufos	NS	NS	+	+	*	NS

Table 5. Mean (range) concentration of pesticides in experimental exposure water 1 h after solution preparation and 72 h of exposure from the chemical stability experiments with and without *Lepomis macrochirus* predator cues. For

malathion $n = 1$, for all other pesticides $n = 2$. MH = moderately hard water treatment, FC = fish cue treatment.

Pesticide ($\mu\text{g/L}$)	Nominal concentration	1 h		72 h		Reduction after 72 h (%)	
		MH	FC	MH	FC	MH	FC
Bifenthrin	0.10	0.04	0.05	0.002	0.005	95	90
		(0.04-0.04)	(0.04-0.05)	(0.003-0.002)	(0.005-0.005)		
		0.09	0.10	0.014	0.021	84	79
Fipronil	80.0	(0.09-0.09)	(0.10-0.10)	(0.01-0.01)	(0.02-0.02)		
		75.8	79.4	57.6	57.5	24	28
		(74.1-77.4)	(78.2-80.6)	(52.6-62.6)	(56.3-58.7)		
Malathion	160.0	150.2	177.9	114.6	118.7	24	33
		(145.1-155.3)	(172.5-183.4)	(108.4-120.8)	(116.8-120.7)		
		0.16	0.11	0.09	0.03	25	73
	0.43	0.49	0.40	0.21	17	57	

Thiacloprid ^a	4.0	3.6	3.3	2.9	2.6	19	21
	(2.9-4.4)	(2.9-3.7)	(2.3-3.7)	(2.5-2.8)			
	8.0	6.9	5.6	5.4	4.9	22	13
		(6.4-7.5)	(5.4-5.8)	(5.2-5.7)	(4.7-5.0)		
Tribufos	0.13	0.10	0.11	0.07	0.06	30	45
		(0.10-0.10)	(0.10-0.12)	(0.07-0.07)	(0.06-0.06)		
	0.36	0.27	0.28	0.22	0.15	19	46
		(0.25-0.28)	(0.24-0.31)	(0.22-0.22)	(0.15-0.15)		

^a Thiacloprid concentration units = mg/L.

Figure 1

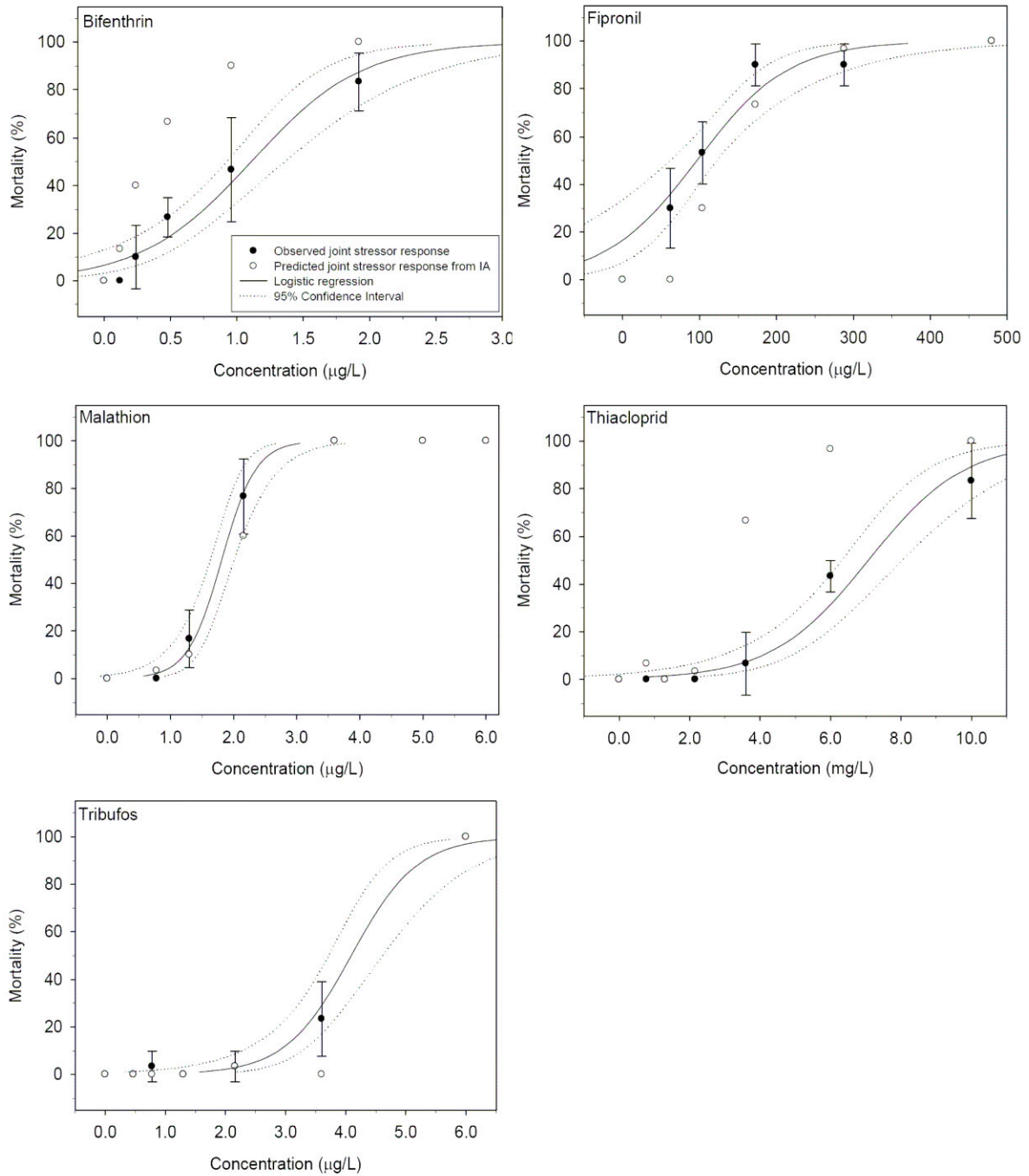


Figure 1