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TOXIC EFFECTS OF COMBINED STRESSORS ON *DAPHNIA PULEX*: INTERACTIONS BETWEEN DIAZINON, 4-NONYLPHENOL, AND WASTEWATER EFFLUENT

Maya A. Zein,† Shawn P. McElmurry,*† Donna R. Kashian,‡ Peter T. Savolainen,† and David K. Pitts§
†Department of Civil & Environmental Engineering, Wayne State University, Detroit, Michigan, USA
‡Department of Biological Sciences, Wayne State University, Detroit, Michigan, USA
§Department of Pharmaceutical Sciences, Wayne State University, Detroit, Michigan, USA

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Abstract: Contaminant exposure in aqueous systems typically involves complex chemical mixtures. Given the large number of compounds present in the environment, it is critical to identify hazardous chemical interactions rapidly. The present study utilized a prototype for a novel high-throughput assay to quantify behavioral changes over time to identify chemical interactions that affect toxicity. The independent and combined effects of 2 chemicals, diazinon (an insecticide) and 4-nonylphenol (a detergent metabolite), on the swimming behavior of the freshwater crustacean *Daphnia pulex* were examined. Cumulative distance and change in direction were measured repeatedly via optical tracking over 90 min. Exposure to low concentrations of diazinon (0.125–2 μM) or 4-nonylphenol (0.25–4 μM) elicited significant concentration- and time-dependent effects on swimming behavior. Exposure to 0.5 μM 4-nonylphenol alone did not significantly alter mean cumulative distance but did elicit a small, significant increase in mean angle, the measure of change in direction. When 0.5 μM 4-nonylphenol was used in combination with diazinon (0.125–0.5 μM), it augmented the adverse impact of diazinon on the swimming behavior of *Daphnia*. Additionally, enhanced sensitivity to diazinon was observed in animals exposed to treated wastewater effluent for 24 h prior to a diazinon challenge. The present experiments demonstrate that exposure to 4-nonylphenol and complex chemical mixtures (e.g., treated wastewater) can enhance the toxicity of exposure to the insecticide diazinon. *Environ Toxicol Chem* 2015;34:1145–1153. © 2015 SETAC

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INTRODUCTION

Many factors may influence the toxicity of water contaminants. These include temperature, pH, dissolved oxygen, food density, and exposure to mixtures of chemicals, both natural and anthropogenic [1]. The toxicity of specific chemicals can be amplified or reduced by the presence of other chemicals in solution [2]. Understanding how these chemicals interact to affect aquatic biota is becoming increasingly important as many chemicals, with known and unknown biological targets, are entering the environment [3]. For example, Kolpin et al. [4] detected complex anthropogenic chemical mixtures in 100% of the 139 US streams investigated. The increased awareness of these mixtures in the environment has led to growing concern about the potential adverse effects such complex chemical mixtures have on aquatic ecosystems [1,3–5].

An estimated 1.1 billion pounds of pesticides were used in the United States as of 2007, with most of the use (80%) in agriculture [6]. Pesticides used in intensive agricultural systems are known to affect nontarget biota (e.g., honeybees [7]), and often these chemicals end up in surface waters. Many of the insecticides, a subcategory of pesticides, are designed to target biological mechanisms associated with the intercellular signaling molecule acetylcholine (ACh). Neurons that use ACh as a neurotransmitter are referred to as "cholinergic." Acetylcholine is found as a neurotransmitter throughout the animal kingdom [8] and is a major neurotransmitter utilized in the central nervous system of insects [9]. The neurotransmitter receptors that are stimulated by ACh have been classified as muscarinic or nicotinic subtypes according to agonist sensitivity, and these receptors have also been identified in many invertebrates [8], including the arthropod and aquatic freshwater crustacean *Daphnia* [10]. Insecticides that target cholinergic mechanisms do so by either inhibiting the enzyme acetylcholinesterase (AChE; e.g., diazinon) or directly stimulating ACh receptors (e.g., neonicotinoids). As insecticides, AChE inhibitors increase ACh to toxic levels by inhibiting the activity of this enzyme, which is responsible for the rapid hydrolysis of ACh and termination of the cholinergic signal [11]. When the enzyme is inhibited, overstimulation of ACh receptors occurs; if concentrations are sufficient, the cholinergic overstimulation is lethal.

Diazinon is an organophosphate AChE inhibitor insecticide, widely used in agriculture to control insects on crops [12]. Because of its known toxicity to aquatic organisms [13], its use has been banned in the European Union [14] and is increasingly being restricted in the United States [15]. Following restrictions on the use of diazinon in Texas, concentrations of this compound and its occurrence in surface waters have decreased significantly [15]. Because it is readily transported, is persistent, and continues to be used in commercial agriculture, however, it is still detected in surface waters in the United States and other countries [16,17]. Diazinon is known to be toxic to *Daphnia* at very low concentrations [18].

In addition to insecticides that are designed to target the cholinergic system, there is some evidence in the literature that other chemicals, such as surfactants, may unexpectedly act on biological targets by inhibiting AChE similarly [19–21]. Surfactants are found in most personal care and household products (e.g., detergents, cosmetics), industrial products, and even agricultural chemicals (pesticide adjuvants [22]). As a result, they are fairly ubiquitous in the environment and are found in wastewater effluent (WWE) along with an array of

* Address correspondence to s.mcelmurry@wayne.edu.
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breakdown products [4,23,24]. The detergent metabolite and surfactant precursor 4-nonylphenol is commonly found in wastewater and surface waters and is of particular concern because of its ability to act as an endocrine disruptor, causing toxicity in various aquatic organisms [22,24,25]. The behavioral toxicity of 4-nonylphenol to aquatic life is less well characterized. Given recent reports of surfactants affecting AChE, it is reasonable to expect that there may be significant interactions between surfactants (such as 4-nonylphenol) and insecticides (such as diazinon) that target and inhibit the important hydrolytic activity of AChE.

It has been reported that 4-nonylphenol interacts with AChE in planaria [20] and fish [21], and surfactant interactions with AChE have also been reported in *Daphnia* [19]. These findings suggest there is a potential for 4-nonylphenol also to interact with insecticide contaminants in the aquatic environment and potentially enhance toxicity, particularly the AChE inhibitors. There is also the distinct possibility that low-level wastewater contaminants, such as the surfactants, could interact in complex and unknown ways to produce antagonistic, additive, or synergistic toxic effects with chemicals from a different class, such as insecticides. These kinds of toxic interactions cannot be detected through examination of the lethality of individual chemicals (e.g., median lethal concentration [LC50]). Because many different chemicals can be found in wastewater [4], some of which are known to bioaccumulate, such as 4-nonylphenol [26], there is a potential for these mixtures to enhance the toxicity of individual contaminants on biota through complex and undefined interactions.

Most wastewater treatment plants do not remove or inactivate many low–molecular weight chemicals that are biologically active at low concentrations [23]. Methods commonly used for disinfection—chlorination, ozonation, and ultraviolet irradiation—also create new transformation products; and the biological impact of these products is not well characterized [27]. In some instances wastewater-treatment methods can create more toxic transformation products [28].

Evaluating the full ecological impacts of emerging contaminants will require assessments of toxicity that extend beyond simple lethality tests and include evaluations of sublethal behavioral effects, specific chemical interactions, and the effects of complex mixtures on biota. In addition to survival, methods have been developed to assess aggregate toxicity on the growth and reproduction of aquatic organisms (e.g., US Environmental Protection Agency whole-effluent toxicity test methods [29]). However, existing tests are limited in the amount of insight they provide into potential subtle neurotoxic effects affecting behavior and in their ability to examine a large number of samples or chemical interactions. Behavioral alterations induced by chemical exposure can have multiple impacts on the fitness of an individual by altering its ability to avoid predation and find mates, food, and shelter [24,30]. These kinds of behavioral changes may be maladaptive and have serious ecological consequences, making them ideal for assessing toxicity. In fact, changes in behavior have proven effective in identifying toxic effects [1,31] and have been used as biomarkers [24,32,33].

In a previous study, we developed an optical tracking technique capable of quantifying changes in swimming behavior of *Daphnia* when exposed to chemical toxicants [34]. Two prototypical compounds exhibiting different modes of action (physostigmine and nicotine) as well as insecticides commonly found in surface waters with similar modes of action (chlorpyrifos and imidacloprid) were investigated. The results demonstrated the ability to detect concentration- and time-dependent behavioral effects. Furthermore, we found that biologically active compounds with similar modes of action produced similar behavioral effects.

The objective of the present study was to test a prototype of a high-throughput *Daphnia pulex* assay in evaluating the interaction between 2 chemical contaminants commonly found in water, 4-nonylphenol and diazinon, and the effect of prior exposure to the complex chemical mixture associated with treated wastewater on the toxicity to a subsequent diazinon challenge. *Daphnia* was selected as the model organism for studying mixtures using the optical bioassay because it is recognized as an ideal organism for studying toxicity [1,18,19,30,35,36]. To assess the ability of this optical bioassay to detect the effect of multiple chemical stressors on toxic outcomes, the following hypotheses were evaluated: 1) different classes of contaminants, based on intended use, can interact in a manner that alters toxicity (e.g., additive, synergistic, or antagonistic effects); and 2) exposure to chemicals in treated wastewater can alter the level of toxicity resulting from subsequent chemical challenges.

MATERIALS AND METHODS

Daphnia utilized in the bioassay came from a *Daphnia* clone isolated from Lake Michigan (USA) and maintained in the laboratory in COMBO water, a freshwater medium [37], at 21 °C and exposed to a light cycle of 16 h light followed by 8 h of darkness. *Daphnia* of approximately the same size (>1.4 mm in length) were used in these experiments by sorting them with a mesh screen. Size was used as a proxy for age.

Diazinon (98.5%; Fluka), 4-nonylphenol (99.9%; Fluka), and acetone (>99.8% purity; Fisher Scientific) were obtained from Sigma-Aldrich. Acetone was used as the solvent to make 10-mM stock solutions of both diazinon and 4-nonylphenol. The 10-mM stock solutions were made by adding 13.6 μL of diazinon or 11 mg of 4-nonylphenol to 5 mL acetone, respectively. By making appropriate dilutions in COMBO water, all solutions for the assay that contained diazinon or 4-nonylphenol were made from the acetone-based stock solutions. The maximum concentration of chemical used in experiments with the optical assay was the 4 μM solution of 4-nonylphenol. Therefore, the maximum concentration of acetone used was 0.04%. All experimental controls where the concentration of diazinon or nonylphenol was 0 μM contained 0.04% acetone. Previous research has documented that these low concentrations of acetone do not have a detectable effect on *Daphnia* [35]. All solutions were prepared immediately prior to experiments.

Individual *Daphnia* were placed in separate beakers containing the desired concentrations of chemical contaminants before their transfer to 24-well plates. Within 5 min of being placed in these beakers, a single animal from each treatment solution was randomly placed into a well (surface area 256 mm^2) of a 24-well plate for optical tracking along with the treatment solution. Each well was then filled to 3 mL with the appropriate treatment solution. The 24-well plate chambers allowed for horizontal swimming behavior to be observed with limited vertical movement.

The optical tracking assay was performed in the 24-well plates on a raised Plexiglas platform backlit by fiber-optic lights. Video recordings of *Daphnia* movement were obtained using an Infinity 2-1 M monochrome camera, and the resulting video was analyzed using Image Pro Plus 7 software (Media Cybernetics). Prior to video recordings, *Daphnia* were acclimated for 10 min in the 24-well plate. After the initial 10-min exposure, 5-s videos were recorded every 10 min for 90 min. *Daphnia* were therefore exposed to each treatment solution during the recording session for a total of approximately 100 min. The ambient temperature in the lab during experiments with the optical bioassay was $21 \pm 1\,^{\circ}C$.

Two parameters were quantified: the cumulative distance *Daphnia* traveled and the change in angle of direction. The change in distance was based on the calculation of the difference in position between 2 consecutive frames collected at a rate of 29 frames/s. The change in angle was derived from the movement detected in 3 sequential frames. The change in position between the first 2 frames describes an initial vector. The change in position between the second and third frames describes a second vector. The change in angle is a measure of difference in direction between these 2 vectors. The accumulated distance traveled by individual *Daphnia* was measured in millimeters, and the change in the direction of travel was measured in degrees. See Zein et al. [34] for further details.

The behavioral response of *Daphnia* to single-chemical exposure was assessed for both diazinon and 4-nonyphenol. Six different concentrations were selected for the optical assay based on preliminary visual observations of *Daphnia* behavior when exposed to a range of 10 to 12 concentrations over a 90-min period that bracketed reported LC50 values [34,38]. Qualitative visual observations that were noted included fast swimming, slow swimming, spinning, barely moving, and immobility. Based on these initial behavioral observations, a final set of treatment concentrations was investigated: 0 μM, 0.125 μM, 0.25 μM, 0.5 μM, 1 μM, and 2 μM diazinon, and 0 μM, 0.25 μM, 0.5 μM, 1 μM, 2 μM, and 4 μM 4-nonylphenol.

To evaluate potential interactions between diazinon and 4-nonylphenol exposure, we added 4 concentrations of diazinon (0.0625 μM, 0.125 μM, 0.25 μM, 0.5 μM) to solutions in the absence or the presence of 0.5 μM 4-nonylphenol. This set of experiments included 2 controls: control 1 had 0.5 μM 4-nonylphenol with 0.04% acetone (4-nonylphenol control), and control 2 had 0.04% acetone (acetone alone; 0 μM diazinon and 0 μM 4-nonylphenol).

The WWE used during the present study was collected from the effluent of the Detroit Water and Sewage Department wastewater-treatment plant located on Jefferson Avenue in Detroit, Michigan, USA, on 18 August 2013. Wastewater at this plant is subjected to primary treatment (solids settling), secondary treatment (aerobic digestion), and advanced treatment (addition of $FeCl_3$ into primary treatment tanks to enhance phosphorus removal). The effluent was collected in a glass container, immediately transported in a dark box back to the laboratory, and stored at 4 °C for less than 24 h until experiments were conducted. Prior to conducting experiments, the concentration of dissolved oxygen was confirmed to be the same (± 0.1 mg/L) in both COMBO water and WWE.

To evaluate the effect of prior exposure to a complex chemical mixture, *Daphnia* were placed in treated WWE for 24 h. This 24-h treatment was intended to mimic real systems where WWE provides a nearly constant exposure and to avoid possible differences in uptake rates of unknown compounds. This pretreatment was used only for experiments evaluating the impact of WWE. For this set of experiments, 2 sets of media controls were evaluated: 1 in which the organisms were placed into WWE and exposed to diazinon (including the 0 μM control with 0.04% acetone) and 1 in which organisms were placed into COMBO water and exposed to diazinon (including the 0 μM control with 0.04% acetone). After the 24-h exposures, *Daphnia* were challenged with a range of diazinon concentrations (0 μM, 0.125 μM, 0.25 μM, 0.5 μM) in the optical bioassay as described above.

All statistical analyses were performed using Statistica (Statsoft, Ver 10). Mean values for the 5-s videos were calculated for the dependent variables, cumulative distance and change in angle. These mean values were used in the statistical analysis and obtained at 10-min intervals during 90 min of optical tracking. Independent variables included time (0–90 min), concentration, and treatments (e.g., media). An analysis of covariance with repeated measures over time was used for statistical analysis, and basal activity at time 0 was used as the covariate to control for between-group variations in basal motor activity [34]. The experiments examining single-chemical exposures had a 2-way design with the factors of concentration (6 levels, including 0 μM) and time (9 levels). The experiments examining the time course for responses to single-chemical exposures were also used to focus subsequent analysis of interactions between individual chemicals or WWE on the time period when maximal effects for the individual chemicals were occurring during the exposure period (50–90 min) and to optimize statistical power. When the interaction between diazinon and 4-nonylphenol or diazinon and WWE was examined, a 3-way design was used with the factors concentration (4 levels including 0 μM), time (9 levels), and treatment (2 levels: present or absent). The well positions within the 24-well plate for the independent factors were randomized. The post hoc Fisher's least significant difference test was employed to evaluate pairwise comparisons. All experiments included 6 animals per treatment condition.

RESULTS

Diazinon

Diazinon caused a significant concentration-dependent change in cumulative swimming distance (Table 1 and Figure 1A; concentration effect $F_{5,29} = 2.72$, $p < 0.05$). The maximum cumulative swimming distance was observed at the lowest concentration (0.125 μM), and it decreased at higher concentrations, resulting in mean values below that observed for the control at 1 μM and 2 μM. This concentration-dependent effect of diazinon on cumulative swimming distance was time-dependent (concentration × time effect $F_{40,232} = 1.58$, $p < 0.05$). The cumulative distance observed for animals exposed to 0.125 μM were elevated above the control at all time points (Figure 1B). The 2 μM concentration of diazinon exhibited a time-dependent decrease in cumulative distance relative to the control. At the higher concentrations (1–2 μM), the *Daphnia* were hardly moving or immobile, with all of the animals ($n = 6$) immobilized after approximately 90 min of exposure to the 2 μM concentration. The stimulatory effect of diazinon could be observed through most of the time course for the lowest concentration of diazinon (0.125 μM), whereas the stimulation of swimming behavior was observed only in the first 10 min during exposure to 2 μM diazinon. Overall, a diazinon concentration of 0.125 μM induced an effect that was significantly different from the control (Figure 1A, least significant difference $p < 0.05$).

Diazinon exposure also induced a significant concentration-dependent change in angle (Table 2 and Figure 1C; concentration effect $F_{5,29} = 6.74$, $p < 0.001$), with the lowest mean change in angle being observed at the lowest concentration (0.125 μM), where cumulative swimming

Table 1. Summary of repeated measures analysis of covariance: Cumulative distance (mm)

Test	Effect	*df*[a]	*F*	*p*
1	Diazinon concentration	5, 29	2.72	0.039
1	Time × diazinon concentration	40, 232	1.58	0.020
2	4-Nonylphenol concentration	5, 29	3.54	0.013
2	Time × 4-nonylphenol concentration	40, 232	1.29	0.126
3	Diazinon concentration × 4-nonylphenol concentration	3, 41	3.29	0.030
3	Time × diazinon concentration × 4-nonylphenol concentration	12, 164	0.91	0.533
4	Media (COMBO vs WWE)	1, 45	4.15	0.048
4	Media (COMBO vs WWE) × diazinon concentration	3, 45	1.80	0.160
4	Time × media (COMBO vs WWE)	4, 180	1.44	0.221
4	Time × media (COMBO vs WWE) × diazinon concentration	12, 180	1.03	0.420

[a]Degrees of freedom effect, degrees of freedom error.
WWE = wastewater effluent.

distance was greatest. Alternatively, the highest mean change in angle was observed at the highest concentration (2 μM), where the mean cumulative swimming distance was lowest. The concentration-dependent effect on change in angle was also time-dependent (concentration × time $F_{40,232} = 3.31$, $p < 0.001$). Figure 1D illustrates the time-dependent effect of diazinon at 0.125 μM and at 2 μM. The mean change in angle for the 2 μM concentration reached a plateau at approximately 60 min (all 6 *Daphnia* were immobile).

4-Nonylphenol

Exposure to 4-nonylphenol produced a significant concentration-dependent change in cumulative distance (Table 1 and Figure 2A; $F_{5,29} = 3.54$, $p < 0.05$). The cumulative distances for

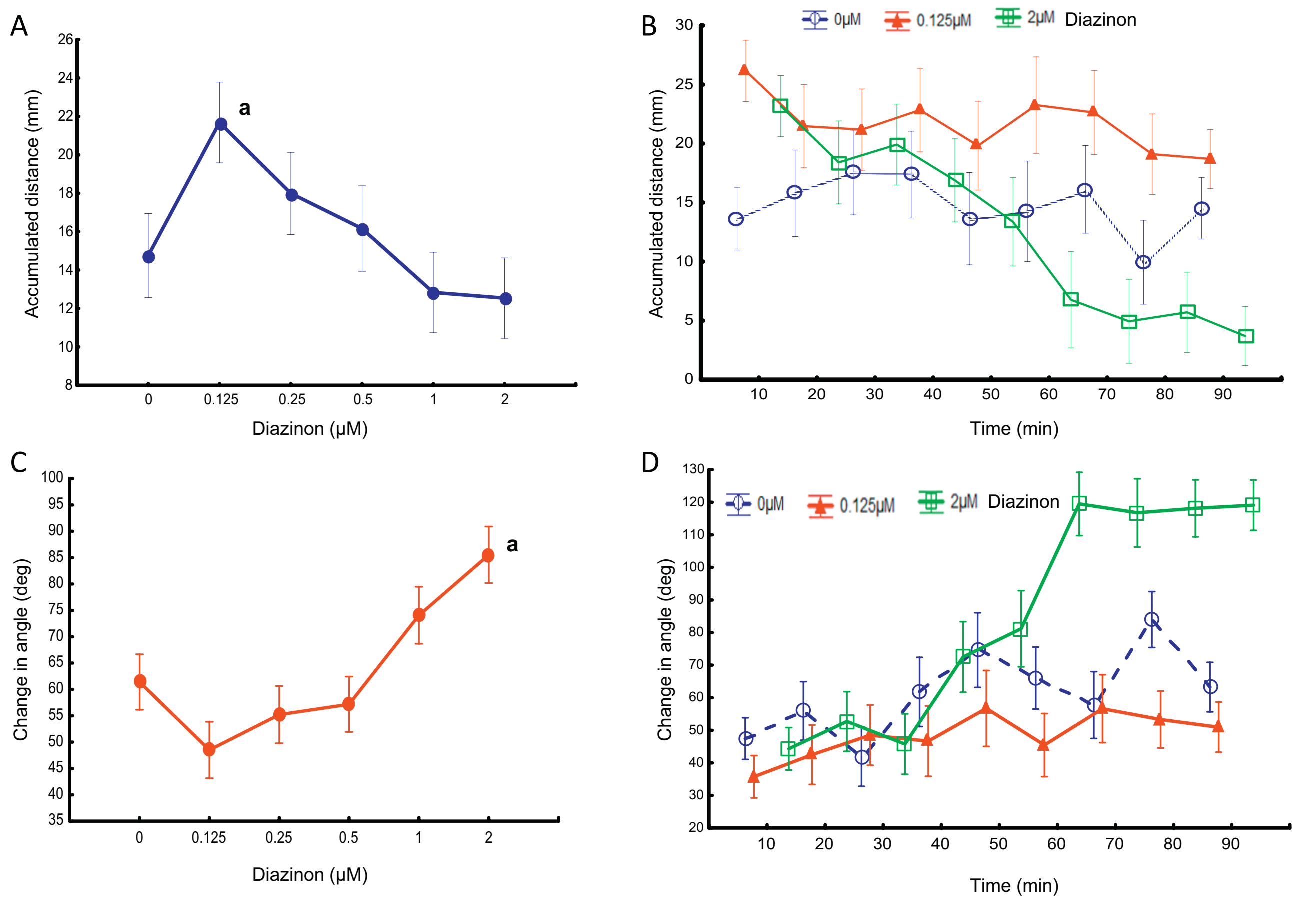

Figure 1. Concentration-dependent effect of diazinon (**A**,**B**) on the swimming behavior of *Daphnia pulex* when measured by accumulated distance (**A**) averaged over the 90-min experiment and (**B**) with the time-dependent effect, and (**C**,**D**) on the average change in angle (**C**) over the 90-min experiment and (**D**) with the time-dependent effect. Error bars are the standard error; data points identified with "a" in (**A**) and (**C**) indicate differences from control (least significant difference test, $p < 0.05$).

Table 2. Summary of repeated measures analysis of covariance: Change in angle

Test	Effect	*df* [a]	*F*	*p*
1	Diazinon concentration	5, 29	6.74	<0.001
1	Time × diazinon concentration	40, 232	3.31	<0.001
2	4-Nonylphenol concentration	5, 29	3.30	0.018
2	Time × 4-nonylphenol concentration	40, 232	1.82	0.004
3	Diazinon concentration × 4-nonylphenol concentration	3, 41	2.37	0.084
3	Time × diazinon concentration × 4-nonylphenol concentration	12, 164	0.84	0.607
4	Media (COMBO vs WWE)	1, 45	30.64	<0.001
4	Media (COMBO vs WWE) × diazinon concentration	3, 45	3.71	<0.001
4	Time × media (COMBO vs WWE)	4, 180	1.85	<0.001
4	Time × media (COMBO vs WWE) × diazinon concentration	12, 180	1.42	<0.001

[a]Degrees of freedom effect, degrees of freedom error.
WWE = wastewater effluent.

the 3 highest concentrations (1 μM, 2 μM, and 4 μM) were significantly different from control (least significant difference test, $p < 0.005$). There was a nonsignificant trend for a time-dependent effect of concentration ($F_{40,232} = 1.29$, $p \sim 0.126$). As can be observed in Figure 2B, the highest concentration of 4-nonylphenol caused a reduction in the cumulative swimming distance, which plateaued after about 40 min of exposure.

The change in angle was significantly altered with exposure to 4-nonylphenol in a concentration-dependent manner (Table 2 and Figure 2C; $F_{5,29} = 3.30$, $p < 0.05$). The highest mean value for the change in angle occurred at 4 μM, where all of the animals ($n = 6$) were found immobilized. A significant time-dependent effect of concentration was observed (time × concentration effect, $F_{40,232} = 1.82$, $p < 0.005$), and the effect of the

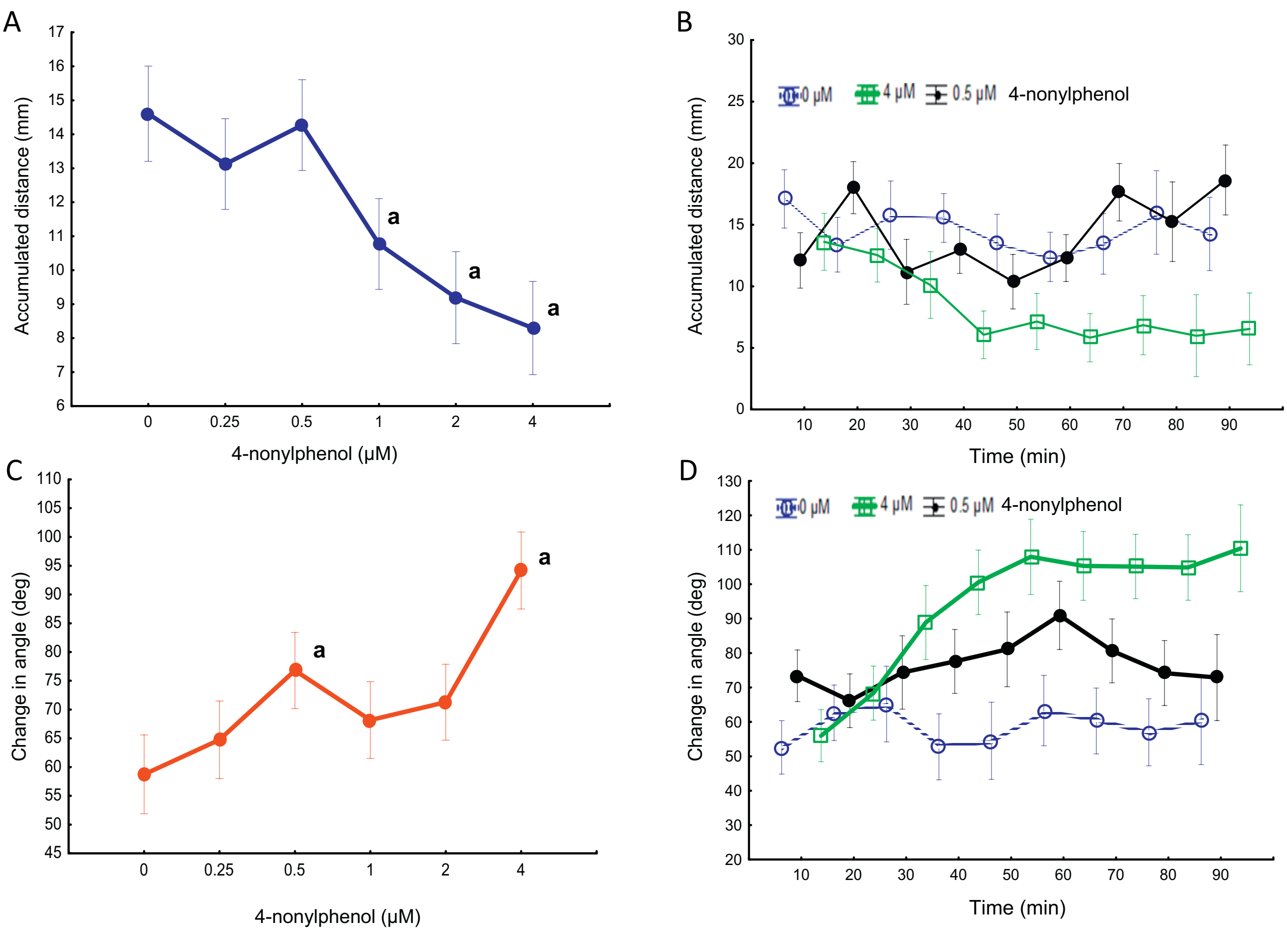

Figure 2. Concentration-dependent effect of 4-nonylphenol (**A**,**B**) on the swimming behavior of *Daphnia* when measured by accumulated distance (**A**) averaged over the 90-min experiment and (**B**) with the time-dependent effect, and (**C**,**D**) on the average change in angle (**C**) over the 90-min experiment and (**D**) with the time-dependent effect. Error bars are the standard error; data points identified with "a" in (**A**) and (**C**) indicate differences from control within the treatment group (least significant difference test, $p < 0.05$).

highest concentration (4 μM) plateaued at approximately 50 min during the exposure (Figure 2D). Based on the time course for the responses observed, subsequent analyses focused on the 50-min to 90-min exposure period.

Combination diazinon and 4-nonylphenol

Figure 3 describes the concentration–response relationship of diazinon (0 μM, 0.125 μM, 0.25 μM, 0.5 μM) in the presence and absence of 4-nonylphenol (0 μM, 0.5 μM) during the 50-min to 90-min exposure period. Overall, there was a significant 4-nonylphenol effect (Table 1; $F_{1,41} = 7.51$, $p < 0.01$) on cumulative swimming distance. There was also a significant diazinon by 4-nonylphenol interaction ($F_{3,41} = 3.29$, $p < 0.05$). A least significant difference post hoc test indicated a significant difference between groups (with and without 4-nonylphenol) at a diazinon concentration of 0.125 μM (Table 1 and Figure 3A and B). When the change in angle was examined for all concentrations (Figure 3B), there was a trend toward a significant ($F_{3,41} = 2.37$, $p \sim 0.084$) interaction between diazinon and 4-nonylphenol.

Wastewater pretreatment

The toxicity of diazinon was assessed in animals in COMBO water and animals subjected to WWE pretreatment for 24 h before being challenged by diazinon exposure. When the 50-min to 90-min period of diazinon exposure was examined, a significant increase in cumulative swimming distance was observed for animals challenged by 0.125 μM diazinon in COMBO but not for animal subjected to the WWE pretreatment (Figure 4A; least significant difference test, $p < 0.01$). Overall, a significant media effect (COMBO vs WWE) on cumulative swimming distance was observed (Table 1; $F_{1,45} = 4.15$, $p < 0.05$), with the cumulative swimming distance observed in WWE being less than that for COMBO water. The interaction between the type of medium and the concentration of diazinon was not significant (Figure 4B; $F_{3,45} = 1.8$, $p > 0.10$), suggesting a similar depression of cumulative swimming distance across all 3 diazinon concentrations. The least significant difference post hoc test identified a significant difference between groups at diazinon concentrations of 0.125 μM ($p < 0.05$) and 0.5 μM ($p < 0.05$). After 90 min of exposure to 0.5 μM of diazinon, all of the animals in COMBO were still moving, whereas all of the animals in WWE were immobilized.

The effect of WWE on the change in angle was significant (Table 2; $F_{1,45} = 30.6$, $p < 0.001$) and dependent on diazinon concentration (media × concentration effect, $F_{3,45} = 3.70$, $p < 0.05$; Figure 4C). The mean values for angle were different between groups at each concentration studied (least significant difference test, $p < 0.005$ for all 3). In contrast to what was observed for COMBO media (Figure 1C), the effect of 0.5 μM diazinon on the change in angle in the group exposed to WWE was different from the COMBO control (least significant difference test, $p < 0.05$). This effect, resulting from exposure to 0.5 μM diazinon in WWE, was comparable to the large increase in the change in angle observed at 2.0 μM diazinon in COMBO (Figure 1C).

DISCUSSION

Previously, we demonstrated the utility of this optically based behavioral assay in quantifying the effects of individual chemicals on *Daphnia* swimming behavior [34]. In the present study, we have extended the use of this behavioral assay to evaluate potential interactions between contaminants commonly found in surface waters [4]. The organophosphate diazinon and the detergent metabolite 4-nonylphenol were investigated because of their common occurrence as contaminants in surface waters and suspected ability to cause behavioral effects at sublethal concentrations via the same mode of action, AChE inhibition [39–42]. The impact of prior treatment with WWE to a subsequent diazinon challenge also was examined.

Both diazinon and 4-nonylphenol were found to have a similar response profile in their effects on mean swimming angle, causing a significant concentration- and time-dependent increase in mean angle. This significant change in mean angle is associated with an increased rate in change of direction, which culminates in the animals spinning in circles with little forward

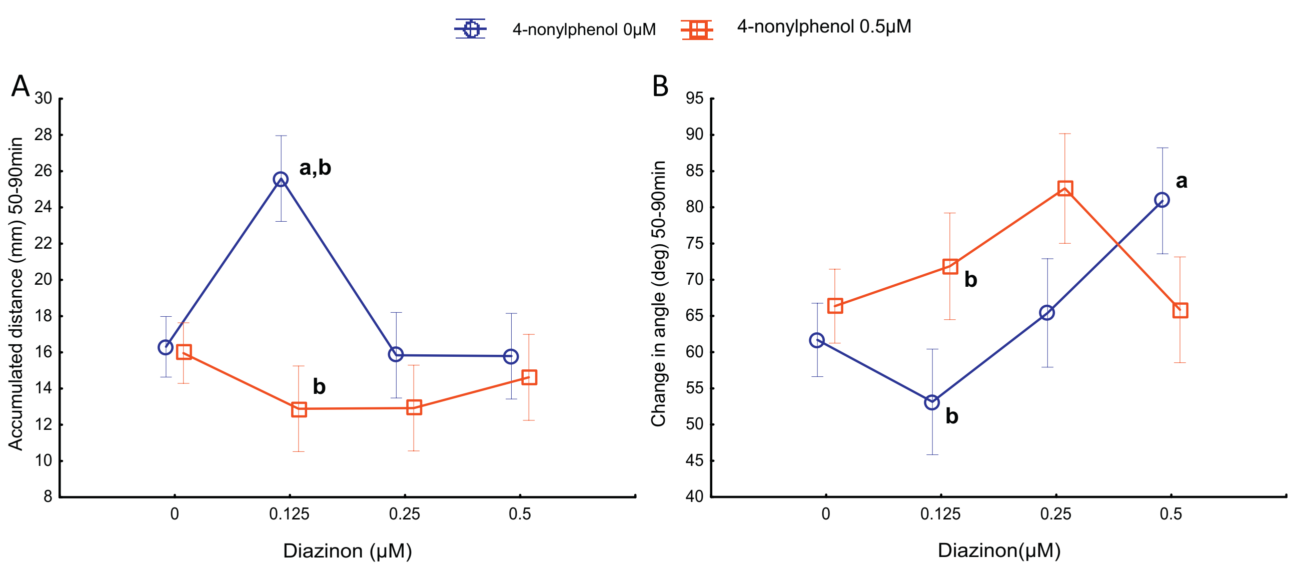

Figure 3. Behavioral response, measured as (**A**) accumulated distance and (**B**) average change in angle, of *Daphnia* exposed to diazinon with 4-nonylphenol present and absent. Error bars are the standard error. Data points identified with “a” indicate differences from the control within the treatment group, and “b” indicates differences between groups (least significant difference test, $p < 0.05$). This analysis focuses on the 50-min to 90-min period of exposure based on results depicted in Figures 1 and 2.

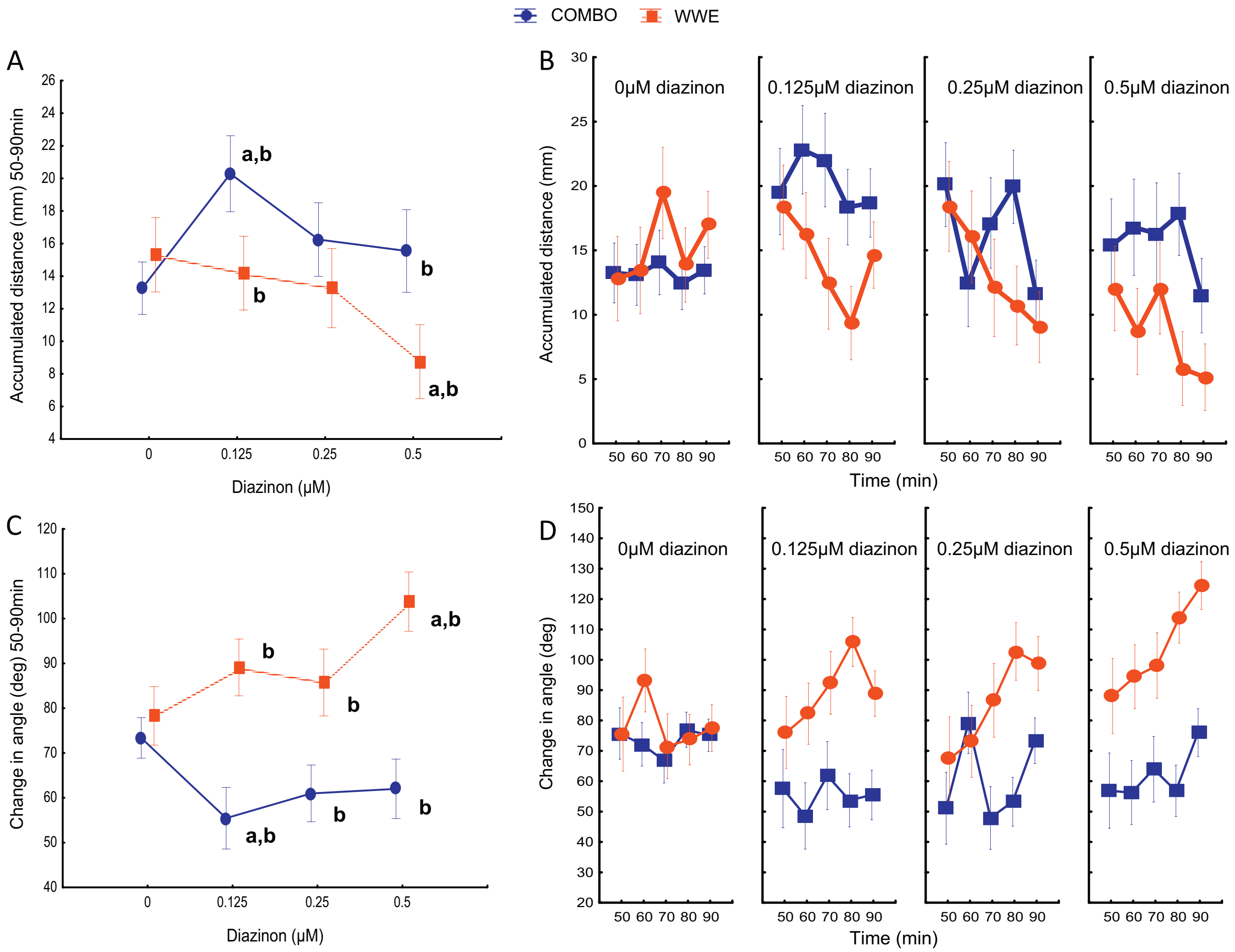

Figure 4. The behavioral response of *Daphnia* to exposure to diazinon in COMBO water and treated wastewater effluent (WWE) as measured by (**A**,**B**) accumulated distance (**A**) averaged over the 90-min experiment and (**B**) with the time-dependent effect, and by (**C**,**D**) the average change in angle (**C**) over the 90-min experiment and (**D**) with the time-dependent effect. Error bars are the standard error. For (**A**) and (**C**), data points identified with "a" indicate differences from the control within the treatment group and "b" indicates differences between groups (least significant difference test, $p < 0.05$). This analysis focuses on the 50-min to 90-min period of exposure as also depicted in Figure 3.

movement, and is eventually associated with immobility at the higher concentrations and later in the time course. Diazinon also elicited a significant concentration- and time-dependent effect on mean cumulative distance, with a stimulatory effect that was most evident at the lower concentrations (e.g., 0.125 μM) and earlier in the time course. At higher concentrations and later in the time course, the effects of diazinon on accumulated distance were inhibitory and associated with immobility. This result coincides with our findings in a previous study using another carbamoylating AChE inhibitor, physostigmine [34]. In contrast, 4-nonylphenol did not elicit this stimulatory effect on swimming behavior (increase in accumulated distance) in the concentration range studied. Thus, the response profiles for these 2 agents were similar but not identical. When the 0.5 μM concentration of 4-nonylphenol was combined with diazinon, it eliminated the stimulatory effect seen with diazinon alone (e.g., 0.125 μM) and augmented the toxic effects of diazinon (e.g., increase in mean angle and incidence of immobility). The effect of a 24-h pretreatment with WWE was similar, also eliminating the stimulatory effect seen with diazinon alone and augmenting toxicity.

As demonstrated with diazinon in the present study (Figure 1) and physostigmine during a previous study [34], an initial stimulation of swimming behavior was seen at lower concentrations, followed by immobility at higher concentrations after 100 min. Such a stimulatory effect on swimming behavior was not observed for chlorpyrifos at lower concentrations studied [34]. Because a stimulatory effect was not observed for chlorpyrifos, it is not yet clear if such behavioral stimulation at lower concentrations can be ascribed to all AChE inhibitors. The lack of a stimulatory effect on swimming behavior by chlorpyrifos could be attributable to the concentration range selected or some other unknown toxicokinetic or toxicodynamic difference from the other AChE inhibitors. Exploring this stimulatory effect of AChE inhibitors further will require a more thorough analysis of a wider range of concentrations and an examination of longer time courses. Understanding the differences in response to AChE inhibitors would be further enhanced by additional toxicokinetic studies of AChE inhibitors (e.g., Kretschmann et al. [18]). However, animals exposed to diazinon did exhibit decreased swimming activity that progressed toward immobility in a concentration- and time-dependent manner, consistent with our previous work [34].

In vertebrates, AChE inhibitors are known to cause increased cholinergic stimulation of effector organs such as skeletal muscle, autonomic ganglia, and other autonomic effector organs. Toxic doses can cause depression or paralysis of autonomic ganglia and skeletal muscle that is associated with excessive stimulation of nicotinic receptors [11]. Acetylcholine is known to be present in the central nervous system of arthropods such as insects [8,9], and both muscarinic and nicotinic ACh receptors can be found in the *Daphnia pulex* genome [10]. The immobility observed in *Daphnia* following exposure to toxic concentrations of AChE inhibitors may be the result of impaired neurotransmission from overstimulation of ACh receptors and/or altered neuromodulatory effects of the cholinergic system as a result of inhibition of ACh degradation. However, the precise anatomical site(s) for the effect(s) of AChE inhibitors in *Daphnia* remains to be determined.

The effects of diazinon on the swimming behavior of *Daphnia* were altered in the presence of 4-nonylphenol (Figure 3). Inhibitory effects of 4-nonylphenol on AChE previously have been reported in planaria and fish [20,21], and this suggests that exposure to sufficient concentrations might elicit similar inhibition of AChE in *Daphnia*. If AChE proved to be a shared target, it is possible that exposure to both an AChE inhibitor and 4-nonylphenol could lead to additive or synergistic effects. However, little is known about the nature of the interaction of 4-nonylphenol with AChE at the molecular level. The pattern of behavioral response observed was indicative of an additive or synergistic effect. The stimulatory effect observed during exposure to diazinon alone was apparently suppressed in the presence of 4-nonylphenol (i.e., mean swimming distance decreased when 4-nonylphenol and diazinon were present in combination relative to diazinon alone). Because the mean change in angle was also elevated at the 0.125 μM concentration relative to diazinon alone, this suggests that the toxic effect of the combined agents was augmented, shifting the response beyond stimulation toward immobility. Similarly, the combination of 4-nonylphenol and diazinon in solution appeared to increase the mean change in angle relative to isolated diazinon exposure. Both of these effects, decreasing distance traveled and increasing mean change in angle, are indicative of *Daphnia* approaching immobility and suggest additive or synergistic effects. The similarity of changes in behavior resulting from exposure to 4-nonylphenol to changes resulting from exposure to other types of AChE inhibitors and the enhanced toxicity of diazinon in the presence of 4-nonylphenol are consistent with an inhibitory effect of 4-nonylphenol on AChE reported by Li [21]. These results support the hypothesis that different classes of contaminants can interact with each other and influence toxicity.

The potential for complex interactions is particularly concerning because WWE typically contains myriad surfactants and detergent metabolites, along with pesticides, pharmaceuticals and personal care products, and other organic contaminants [4,5,23]. Exposure to WWE for 24 h alone did not appear to have an effect on swimming behavior (i.e., controls at 0 μM concentration). However, a significant alteration in the effects of diazinon on swimming behavior was observed when the experiment was conducted in WWE and compared with diazinon exposure in COMBO media (Table 2 and Figure 4).

It is important to note that the WWE sample was not concentrated or altered for these experiments. As seen with 4-nonylphenol, the pattern of behavioral response observed was indicative of additive or synergistic effects, likely caused by uncharacterized chemicals within the WWE. In the presence of WWE, the concentration-dependent effects of diazinon previously observed for diazinon alone (stimulatory at low concentrations and suppressive at high concentrations) were altered, resulting in elimination of the stimulatory effect and an enhanced suppressive effect. The potential for diazinon exposure to cause immobility was also enhanced in the WWE. At the end of our present experiments, except for the highest concentrations of diazinon, *Daphnia* appeared to be swimming normally in diazinon solutions containing COMBO, whereas all animals were immobilized in diazinon solutions containing WWE. These results suggest that the toxicity of diazinon was greater in the WWE and that the ability of diazinon to cause immobility was enhanced. The interaction between WWE and diazinon is consistent with our second hypothesis, that the mixture of chemicals found within treated wastewater can influence the toxicity of individual chemical agents.

The exact nature of the interactions observed for mixtures of chemicals (additive, synergistic, or antagonistic) was not fully characterized. This could be accomplished in future studies using models such as concentration addition and independent joint action [36]. Additionally, since the present study utilized a short 100-min exposure paradigm, it likely underestimated the behavioral toxicity of these chemical stressors relative to longer exposure regimens [18,43]. However, the assay we applied has a number of potential advantages over other assays using immobility as an end point (e.g., LC50 values) because it: 1) is scalable and can be used to examine sublethal behavioral effects of multiple chemical stressors simultaneously with limitations only restricted to optics, sensor size, and computing power; 2) is rapid and can produce results from an experiment in a few hours; 3) can be used to evaluate many different exposure paradigms of varying lengths (e.g., acute, chronic, or pulsed exposures in the laboratory or water samples from the field); and 4) has the power of a repeated measures design.

CONCLUSION

In the present study, we observed significant interactions between diazinon, an organophosphate AChE inhibitor insecticide, and 4-nonylphenol. We also found that prior exposure to WWE enhanced the behavioral toxicity of diazinon. The significant interactions between chemical stressors that were identified indicate that concern about the combination effects is warranted, and the precise nature of these interactions should be characterized. The interactions described emphasize the need for more studies evaluating the impact of multiple chemical stressors on biota. Ultimately, a better understanding of how complex chemical mixtures affect biota is critical to the assessment of the environmental impacts of emerging contaminants [44,45] and underscores the need for environmental regulations that can adequately address the potential impact of complex mixtures.

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