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Effects of Three Organic Wastewater Contaminants on American Toad, *Bufo americanus*, Tadpoles

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Abstract. Recent surveys of aquatic habitats suggest that organic wastewater contaminants (OWCs) may be common in aquatic ecosystems. However, relatively little is known about the impacts of OWCs on amphibians. We studied the lethal and sublethal effects of three OWCs (acetaminophen, caffeine, and triclosan) on American toad (*Bufo americanus)* tadpoles. High concentrations of triclosan increased activity, whereas acetaminophen had a significant effect on activity but there was no discernable pattern or trend with concentration. Caffeine did not affect activity in *B. americanus* tadpoles. None of the OWCs we studied had a significant effect on growth. Caffeine had no effect on survivorship. Higher concentrations of acetaminophen increased mortality. Intermediate concentrations of triclosan had a negative effect on survivorship, but the highest concentration apparently had a positive effect on survivorship. Our results suggest that there is variation in the toxicity of the three OWCs we studied.

Keywords: acetaminophen; *Bufo americanus*; caffeine; lethal effects; sublethal effects; triclosan

Introduction

Amphibian larvae are potentially exposed to several pollutants in their aquatic habitats. Most of the focus has been on pesticides, herbicides, and agricultural fertilizers (e.g., Rouse et al., 1999; Cowman and Mazanti, 2000; Boone and Bridges, 2003). Recent surveys of aquatic habitats suggest another category of pollutants, organic wastewater contaminants (OWCs), may be common in aquatic ecosystems (Stuer-Lauridsen et al., 2000; Jones et al., 2001, 2002; Campagnolo et al., 2002; Kolpin et al., 2002; Boyd et al., 2003; Lalumera et al., 2004), and thus may influence amphibian performance. However, relatively little is known about the impacts of OWCs, or other organic contaminants, on amphibians (see Sparling, 2000).

We studied the lethal and sublethal effects of three OWCs, acetaminophen, caffeine, and triclosan, on American toad *(Bufo americanus)* tadpoles. Acetaminophen is an over-the-counter analgesic. Caffeine is a stimulant found in many beverages and medications. Triclosan (5-chloro-2-(2,4-dichlorophenoxyl)phenol) is an antimicrobial agent that is used widely in consumer products such as antibacterial soaps. These three OWCs are among the most widely found in a survey of freshwater habitats in the USA by Kolpin et al. (2002). Little beyond the survey done by Kolpin et al. (2002) appears to be known about the presence of acetaminophen in the environment. Caffeine has been found in the influent and effluent of wastewater treatment plants (Ternes et al., 2001; Buerge et al., 2003), as well as rivers and bays

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(Gardinali and Zhao, 2002; Siegener and Chen, 2002). Triclosan has been found in fish tissue and human milk samples (Adolfsson-Erici et al., 2002), rivers and lakes (Lindström et al., 2002), and wastewater effluent (Lindström et al., 2002; Singer et al., 2002; Sabaliunas et al., 2003; Morrall et al., 2004).

Materials and methods

We collected *B. americanus* eggs from a local pond, and incubated them in the lab until hatching. Tadpoles were introduced into the experimental conditions once they had become free-swimming (i.e., Gosner Stage 26). Tadpoles were housed in 1.2-l clear plastic containers, with 1 l of test solution (made with aged tapwater: Salinity = 0.3 ppt; pH = 8.5; dissolved oxygen = 9.40 mg l^{-1}; Nitrate-N ≤ 1 ppm; Ammonia-N ≤ 0.l ppm; Phosphate-P ≤ 1 ppm; Hardness = 176 ppm) in each container, at a density of 8 tadpoles per container. Tadpoles were fed a constant ration of food (0.040 g of ground Purina Rabbit Chow) every 3 days of the experiment. Water in the containers was changed after 7 days, refreshing the treatment at the same time. Dead individuals were removed the day they were discovered. The experiment was run for 14 days to allow for the assessment of lethal and sublethal effects of the chemicals.

Acetaminophen was obtained from ICN Biomedicals, Inc. (Aurora, Ohio, USA). Caffeine (98.5%) was obtained from Acros Organics (Geel, Belgium). Triclosan was supplied by Ciba Specialty Chemicals Corporation (High Point, North Carolina, USA). Solutions of each concentration were made by first mixing a stock solution 2000 times the maximum natural concentration found in Kolpin et al. (2002), and then aliquoting the necessary amount into each replicate container (5 replicates for each concentration) to obtain the correct overall concentration (see Table 1). Analytical determination of test concentrations was not made, thus we report nominal concentrations in our analyses. When water in the containers was changed, the replacement water was mixed in the same fashion.

To estimate activity level, we recorded the number of tadpoles in each container that were active or inactive once a day. Active was defined as movement through the water by tail motion or feeding behavior, while inactive included lying stationary at the bottom of the container or floating still in the water column with no body or tail movement. At the end of the experiment, surviving tadpoles were weighed with an electronic balance (to nearest 0.001 g) after being blotted dry.

Separate one-way ANOVAs for each chemical were performed to determine the effects of chemical concentration on the mean proportion of tadpoles active in a container (arcsin square-root transformed), and on final mass. We used separate repeated measures ANOVAs on the number of tadpoles alive in each container on each day of the experiment to determine the effects of each chemical on survivorship.

Results

Acetaminophen

Activity of *B. americanus* tadpoles was significantly affected by acetaminophen concentration; however, a pattern was not apparent (Table 2; $F_{4,20} = 3.96$, $p = 0.016$). Acetaminophen con-

Table 1. Nominal anal relative concentrations of acetaminophen, caffeine, and triclosan used in the experiment

	Nominal concentration (micrograms l^{-1})		
Relative concentration	Acetaminophen	Triclosan	Caffeine
0	0	0	0
0.l×	1.0	0.23	0.6
1×	10.0	2.3	6.0
10×	100.0	23.0	60.0
100×	1000.0	230.0	600.0

1×, maximum natural concentration found by Kolpin et al. (2002).

centration had no effect on final mass (Table 2; $F_{4,20} = 1.35$; $p = 0.29$). Tadpoles exposed to higher concentrations of acetaminophen had higher and earlier mortality (i.e., time × concentration effect in rmANOVA; Fig. 1a; $F_{52,260} = 1.63$, $p = 0.007$).

Caffeine

Activity of *B. americanus* tadpoles was unaffected by caffeine concentration (Table 3; $F_{4,20} = 1.30$, $p = 0.30$). Caffeine concentration had no significant effect on final mass (Table 3; $F_{4,20} = 1.25$, $p = 0.32$). The survivorship of *B. americanus* tadpoles was unaffected by caffeine (Fig. 1b; concentration effect in rmANOVA: $F_{4,20} = 0.69$, 0.61; time × concentration effect in rmANOVA: $F_{52,260} = 0.87$, $p = 0.73$).

Triclosan

Triclosan concentration affected activity level, with the 100× treatment being more active than the other treatments (Table 4; $F_{4,20} = 3.37$, $p = 0.029$). Triclosan concentration did not affect final mass (Table 4; $F_{4,20} = 1.71$, $p = 0.19$). Tadpoles exposed to the 1.0× concentration of triclosan had earlier and higher mortality; whereas those at 100× had lower mortality (i.e., time × concentration effect in rmANOVA; Fig 1c; $F_{52,260} = 1.39$, $p = 0.053$).

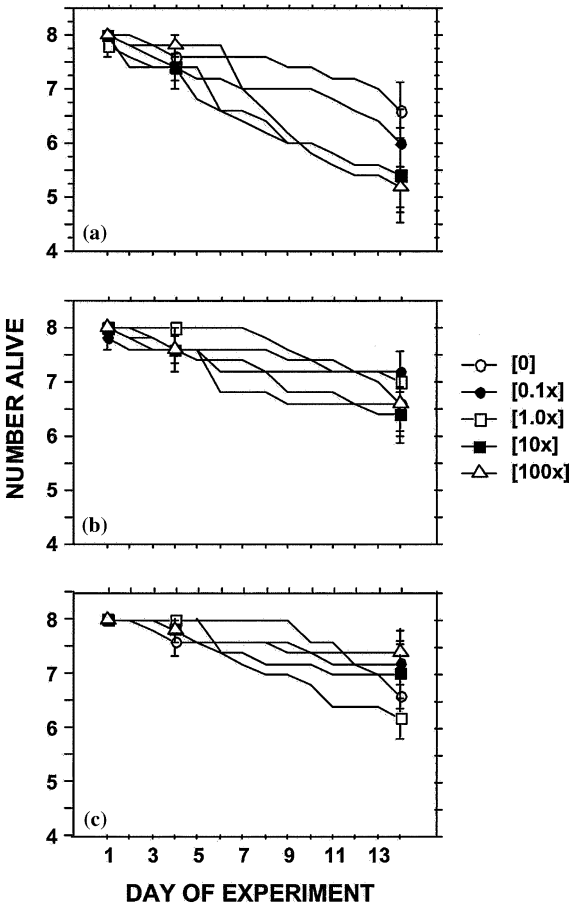

Figure 1. Effect of the relative concentration (see Table 1 for details) of (a) acetminophen, (b) caffeine, and (c) triclosan on the survivorship of American toad *(Bufo americanus)* tadpoles. For clarity, only means and 1 SE are given at 1, 4, and 14 days.

Discussion

Acetaminophen and triclosan both had significant effects on activity in *B. americanus* tadpoles; high concentrations of triclosan increased activity, whereas the effect of acetaminophen had no discernable pattern or trend. Caffeine did not affect activity in *B. americanus* tadpoles. In contrast, *Xenapus laevis* tadpoles showed decreased activity levels with exposure to higher concentrations of

Table 2. Effects of relative acetaminophen concentrations (see Table 1 for details) on activity and final mass (mean and least squares mean) of American toad *(Bufo americanus)* tadpoles

Relative concentration	Activity	Final mass (g)
0	0.259 ± 0.024	0.023 ± 0.001
0.1×	0.340 ± 0.032	0.024 ± 0.002
1×	0.261 ± 0.034	0.025 ± 0.001
10×	0.364 ± 0.024	0.028 ± 0.002
100×	0.243 ± 0.018	0.025 ± 0.002

Means are given ±1 SE; N = 5 in all cases.

Table 3. Effects of relative caffeine concentrations (see Table 1 for details) on activity and final mass (mean and least squares mean) of American toad *(Bufo americanus)* tadpoles

Relative concentration	Activity	Final mass (g)
0	0.259 ± 0.024	0.023 ± 0.001
0.l×	0.338 ± 0.024	0.024 ± 0.001
1×	0.351 ± 0.054	0.027 ± 0.001
10×	0.333 ± 0.029	0.026 ± 0.001
100×	0.360 ± 0.028	0.026 ± 0.002

Means are given ±1 SE; N = 5 in all cases.

both acetaminophen and triclosan, and for triclosan, the effects were found at concentrations observed in nature by Kolpin et al. (2002) (Fraker and Smith, in press). Activity of *Rana pipiens* tadpoles was also reduced by exposure to similar concentrations of triclosan, but not acetaminophen (Fraker and Smith, 2004). Using similar methods and concentrations to ours, Fraker and Smith (2004) found that caffeine affected the activity level of *R. pipiens* tadpoles, with tadpoles in the 0.1× treatment being less active than the control and the 100× treatment, while the 100× treatment was more active than the .0.1× and 1× treatments. Our results confirm that exposure to contaminants can have sublethal behavioral effects on amphibians, which can ultimately affect predation risk, feeding efficiency or reproductive success, and in turn the persistence of amphibian populations in contaminated areas (Semlitsch et al. 1995; Bridges, 2000; Hatch and Blaustein, 2000; Glennemeier and Denver, 2001).

None of the three OWCs we studied had a significant effect on growth (e.g., final mass). In other studies, triclosan has had significant effects on growth, with intermediate (1× and 10×) concentrations having a slightly positive effect in *R. pipiens* and *X. laevis* tadpoles, and 100× concentrations having a negative effect in *X. laevis* (Fraker and Smith, in press). Caffeine had no effect on final mass of *R. pipiens* and *X. laevis* (Fraker and Smith, 2004.). Bantle et al. (1994) found minimum concentrations of caffeine to inhibit growth ranged from 0.05 to 0.09 mg ml^{-1} (see also Fort et al., 1998). Acetaminophen had no effect on growth in *R. pipiens* tadpoles (Fraker and Smith, 2004).

The effects on the survivorship of *B. americanus* tadpoles differed among the three OWCs we studied. Caffeine had no effect on survivorship. Higher concentrations of acetaminophen increased mortality. Intermediate concentrations of triclosan had a negative effect on survivorship, but the highest concentration apparently had a positive effect on survivorship. Caffeine had no effect on survivorship of *R. pipiens* tadpoles (Fraker and Smith, 2004). Caffeine concentrations (100 mg l^{-1} led to developmental anomalies and difficulties for *X. laevis* tadpoles (Sakamoto et al., 1993). The same levels of acetaminophen used in our study had no effect on survivorship of *R. pipiens* and *X. laevis* tadpoles (Fraker and Smith, 2004, in press). Fort et al. (1992) found teratological effects of acetaminophen on *X. laevis* embryos at 100 mg l^{-1} (100 × our maximum concentration). High concentrations of triclosan, equivalent to the 100× treatment in this study, caused high mortality

Table 4. Effects of relative triclosan concentrations (see Table 1 for details) on activity and final mass (mean and least squares mean) of American toad (*Bufo americanus*) tadpoles

Relative concentration	Activity	Final mass (g)
0	0.259 ± 0.024	0.023 ± 0.001
0.l×	0.321 ± 0.020	0.025 ± 0.001
1×	0.298 ± 0.019	0.027 ± 0.001
10×	0.280 ± 0.033	0.024 ± 0.002
100×	0.380 ± 0.029	0.022 ± 0.001

Means are given ±1 SE; N = 5 in all cases.

in *R. pipiens* tadpoles, but lower concentrations had no effect relative to controls (Fraker and Smith, 2004). Triclosan had no effect on survivorship in *X. laevis* tadpoles (Fraker and Smith, in press).

For many pollutants, the effects on amphibian larvae may vary from species to species, and even from one population to another for a single species (e.g., Bridges and Semlitsch, 2000; Harris et al., 2000; Johansson et al., 2001). Our results, along with those of Fraker and Smith (2004, in press), suggest that there is variation in the toxicity of the three OWCs we studied among at least three species of amphibian larvae (*B. americanus, R. pipiens*, and *X. laevis*). For the most part, toxic effects in these three species, particularly those related to growth and survivorship, were manifested only at concentrations higher than those observed in nature by Kolpin et al. (2002), although behavioral effects appear at ecologically relevant concentrations in some cases (see also Fraker and Smith, 2004). This suggests that current environmental levels of these OWCs may have minimal impacts on amphibian populations. However, it also suggests that environmental levels of these OWCs be monitored and additional research conducted to determine the susceptibility of amphibians, and other aquatic organisms, to these and other OWCs.

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