Toxic Effects of Copper, Cadmium, and Methoxychlor Shown by Neutral Red Retention Assay in Two Species of Freshwater Molluscs

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Abstract: We used neutral red retention assay in lysosomes of digestive gland cells as an indicator for stress effects by the environmental contaminants Cu, Cd, and the pesticide methoxychlor in two freshwater molluscs, the unionid mussel, *Elliptio complanata* (Lightfoot) and the ramshorn snail *Helisoma trivolvis* (Say). Mussels and snails were exposed for 7 and 14 days to Cu and Cd each at nominal concentrations of 2.5 µg/L, 5.0 µg/L, and 10.0 µg/L, and to methoxychlor concentrations of 1.0 µg/L, 10.0µg/L, and 100.0 µg/L. Both mussels and snails exposed to Cu showed a significant increase in the percent of destabilized lysosomes compared with lab control and freshly-collected (field control) mussels at both 7 and 14 days exposure for all concentrations. Cd-exposed mussels did not show a significant difference with either of the controls at 7 days, but at 14 days exposure, Cd significantly increased the percent of destabilized lysosomes at all concentrations compared to field control mussels. Compared to laboratory controls, Cd increased lysosomal destabilization at 5.0 µg/L and 10.0 µg/L. Snails exposed to Cd for 7 days had a significantly higher percentage of lysosomal destabilization than both lab and field controls but at 14 days, significant differences were only seen at the two highest Cd concentrations. Methoxychlor-exposed mussels showed no significant lysosomal destabilization at 7 days compared to controls. But at 14 days exposure, the pesticide increased the percentage of lysosomal destabilization at 10.0µg/L compared to lab controls, and increased at both 10.0µg/L and 100.0µg/L compared to field control mussels. Methoxychlor-exposed snails had a higher percentage of lysosomal destabilization at 7 and 14 days at all concentrations compared to both controls with the exception of the 1.0 µg/L -7 day exposure group. Snails were more sensitive to Cd and to methoxychlor than were mussels possibly because they lack an operculum and are thus completely exposed to the environment. The lowest observed effect concentration (LOEC) for Cd was 2.5 µg/L (14 days) for *Elliptio* and 2.5 µg/L (7 and 14 days) for *Helisoma*. For methoxychlor, the LOEC was 10.0 µg/L (14 days) for *Elliptio* and 10.0 and 1.0 µg/L (7 and 14 days, respectively) for *Helisoma*. The LOEC for Cu was 2.5 µg/L (7 and 14 day exposure) for both *Elliptio* and *Helisoma*. These results show that lysosomal destabilization as indicated by neutral red retention is a reliable indicator of stress from heavy metals and a pesticide in freshwater molluscs, including a taxon that is endangered or threatened in North America.

Keywords: Cadmium, copper, *Elliptio*, *Helisoma*, methoxychlor, lysosome, neutral red, toxicity.

INTRODUCTION

Heavy metals continue to be common pollutants in aquatic ecosystems and are toxic to aquatic organisms [1-4]. In particular, the detrimental effects of Cu and Cd have been documented in numerous studies [5-8]. Bivalve and gastropod mollusks are excellent sentinel organisms for the study of toxic effects of such metals in aquatic ecosystems [5, 9-12]. Because of their economic importance, marine bivalves have been particularly well studied and exposure to Cu and Cd has been shown to have detrimental effects on oysters [13, 14], mussels [15], and clams [16]. By contrast, relatively few studies have addressed the effects of the pesticide methoxychlor (2,2-bis(p-methoxyphenyl)-1,1,1-trichloroethane) on molluscs (but see [17]), even though the pesticide has been used for decades and was detected in freshwater mussels (*Lampsilis siliquoidea*) at concentrations as high as 222 µg/L in the Red Cedar River, Michigan

[18]. Use of methoxychlor was banned in the United States in 2003, but continues to be used worldwide, particularly in developing countries [19, 20], with recent studies showing the potential of exposure to methoxychlor and other organochlorine pesticides as risk factors for Parkinson's Disease [21]. In the aquatic environment, pesticides have been shown to reduce species richness indices by reducing the abundance of zooplankton and predatory insects [22]. Some authors [23] argue that the highest priority for research in freshwater pollutant regulation and treatment be given to pesticides since they are common pollutants which pose human health hazards at environmental concentrations.

Neutral red retention assays in lysosomes have been used as a measure of stress in various organisms under different environmental conditions [13-16, 25-33]. Since lysosomes accumulate a diverse range of toxic chemicals that can eventually lead to cell injury through lysosomal damage [34, 35], destabilization of lysosomal membranes indicated by leakage of neutral red into the cytoplasm is a good indicator of stress. Lysosomal destabilization has been shown in oysters exposed to toxic algae [36] and to Fullerene C60 nanoparticles [24], in earthworms exposed to organophosphate pesticides [37], in clams carrying trematode parasites [38], in mussels exposed to alkylbenzene sulphonate [39],

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and to human pharmaceuticals [29, 30, 32, 33]. The vast majority of these and other studies have utilized marine invertebrates even though freshwater molluscs are excellent sentinel organisms under constant pollution pressure.

We used neutral red retention assay to assess the effects of Cu, Cd, and methoxychlor exposure on two common freshwater invertebrates with a broad geographic distribution in North America, the ramshorn snail *Helisoma trivolvis* and the unionid mussel *Elliptio complanata*. We chose to test *H. trivolvis* because it lives in slow moving or even stagnant streams where toxicant-bound sediments and organic materials would tend to settle, accumulate, and become food for snails. *E. complanata* was chosen because it lives within toxicant-containing sediments and filters large volumes of water containing potential toxicants. Another reason is because unionids are endangered and threatened in North America, thus knowledge of their sensitivity to aquatic toxicants will aid conservation efforts. Finally, since both *H. trivolvis* and *E. complanata* are commonly used freshwater molluscs in aquatic toxicology experiments [40, 41], comparisons with different pollutants can be made.

MATERIALS AND METHODS

Animal Collection and Exposure to Toxicants

Mussels, *Elliptio complanata*, and snails, *Helisoma trivolvis* were collected by hand from Marsh Creek, Adams County, Pennsylvania. Mussels (10 cm shell length) were collected in September and October, 2006. Snails (1.5-2.0 cm shell length) were collected in June and July, 2007. After collection, all animals were immediately transported (10 minutes) to the laboratory at Gettysburg College where they were allowed to acclimate in the test aquaria in the water from which they were collected. All animals were used within 24 hours of collection.

Animals were placed in 3-liter plastic aquaria (1 mussel or 1 snail per aquarium) supplied with air stones for aeration. The final aquarium volume was 3000 ml for mussels and 1500 ml for snails (since snails are considerably smaller). All experiments were carried out at room temperature (20-22 °C). For mussel experiments, the control solution was de-chlorinated tap water. For snail experiments, we found de-chlorinated tap water to be unhealthy, so the control solution was creek water from the collecting site. Due to heavy parasitism by larval digenetic trematodes that was undetectable prior to toxicant exposure, some of the snails tested were unusable. We re-tested three groups of snails in July 2010 (14-day exposure to $CuSO_4$ 5.0 µg/L and $CdCl_2$ 2.5 µg/L, and the 7-day control for methoxychlor). The 2007 and 2010 groups were then pooled since there was no statistically significant difference between groups.

Following acclimation, each aquarium received nominal concentrations of toxicants in order to establish the final concentrations of 0, 2.5, 5.0, and 10.0 µg/L for both Cu (as $CuSO_4$) and Cd ($CdCl_2$), and 1.0, 10.0, and 100.0 µg/L for methoxychlor. These concentrations were selected because they are similar to environmental concentrations and have been used in previous toxicity studies on marine animals [13, 42]. All chemicals were purchased from Sigma Chemical Company. Both $CuSO_4$ and $CdCl_2$ were dissolved in either de-chlorinated tap water (for mussels) or creek water (for snails). Methoxychlor was dissolved in 100% ETOH and diluted to a final concentration of no more than 0.1% ETOH. Both mussels and snails were exposed to toxicants for 7 and 14 days. All 14-day exposure groups received fresh solutions on day 8. Mussels were fed every two days with powdered *Chlorella* algae; snails received Romaine lettuce *ad libidum*. After the 7 or 14 days exposure, animals were dissected, their digestive glands removed, and the cells processed for neutral red retention assays. We also collected animals fresh from the field and immediately dissected their digestive glands for neutral red retention assays as field controls.

Neutral Red Retention-Lysosomal Destabilization Assay

We slightly modified the methods from those of [13]. Shells of mussels and snails were crushed with a C-clamp and their digestive glands removed. A portion of the digestive gland (< 2 mm^2) was placed in a 1.5 ml microcentrifuge tube containing 1.0 ml Calcium/Magnesium Free Saline (CMFS: 1.19 g/L HEPES, 6.43 g/L NaCl, 0.37 g/L KCl, 1000 ml DI H_2O). Digestive glands were homogenized with a small Teflon tissue homogenizer to break the tissue into individual cells. Trypsin, 0.4 mg, was added to the microcentrifuge tube and shaken by hand for 5 minutes. Neutral red dye solution was prepared fresh for each assay. The primary stock solution was prepared first by the addition of 4.0 mg of neutral red to 1.0 ml of DMSO. A total of 60 µL of the primary stock solution was then added to 5.94 ml of CMFS in a 50 ml foil-covered beaker at room temperature. Three microscope slides were set up for each individual animal in each group. A total of 40 µL of cell suspension was placed on each microscope slide and 40 µL of neutral red was added to each cell suspension. Slides were covered with a cover slip and incubated in a humidified chamber at 23.5 °C for 60 minutes.

After incubation, the slides were removed from the chamber and examined under a compound microscope at 400x. For each slide, the first fifty cells encountered were evaluated for destabilized lysosomes. Lysosomes that retained neutral red were scored as stable. Cells with neutral red that had leaked into the cytoplasm were scored as having destabilized lysosomal membranes. Ambiguous cells, ones with neutral red leakage into the cytoplasm along with some retained within lysosomes were not scored. The mean percent of cells with destabilized lysosomes was calculated for each animal and thereafter for each toxicant group. Mean differences between groups were analyzed using one-way ANOVA.

RESULTS

While control animals showed some destabilized lysosomes, there was no significant difference in mean percent destabilization between any control (lab controls *vs.* field controls) in either species (for *Elliptio*: one-way ANOVA, F = 1.25, p = 0.32; for *Helisoma*: one-way ANOVA, F = 1.43, p = 0.25).

For the mussel *Elliptio complanata*, exposure to Cu had a significant effect on the mean percentage of destabilized lysosomes for both the 7-day exposure (one-way ANOVA, F = 10.01, p = 0.0006) and the 14-day exposure (F = 20.11, p<

0.0001, Fig. **1**). There were significant differences between all three Cu concentrations and both field control and lab controls at both 7 and 14 days (Fisher's PLSD, 0.0006 – 0.0001).
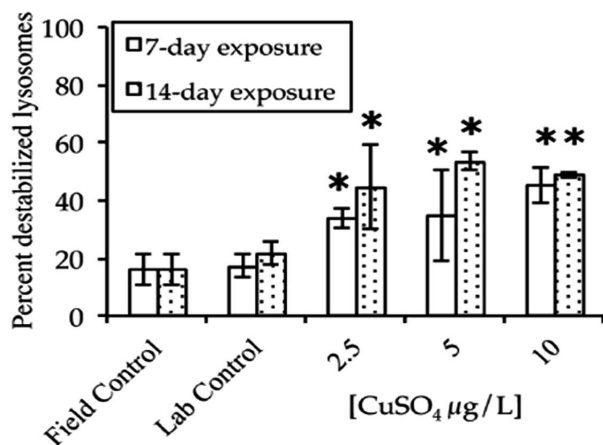

Fig. (1). Percent of destabilized lysosomes of digestive gland cells in *Elliptio complanata* exposed to three concentrations of CuSO₄ for 7 (open bars) and 14 (stippled bars) days. Field control clams were tested immediately after collection. Sample sizes were n = 3 per group. *: P < 0.0003-0.0001 *vs*. both field control and laboratory control.

Exposure to Cd also had a significant effect on the mean percentage of destabilized lysosomes, but only for the 14-day exposure (one-way ANOVA, F = 7.18, p = 0.0005, Fig. **2**). There were significant differences between the two highest Cd concentrations and both controls (Fisher's PLSD, 0.01 – 0.004). But, the 2.5 μg/L concentration was only significant when compared with the field control (p = 0.03).
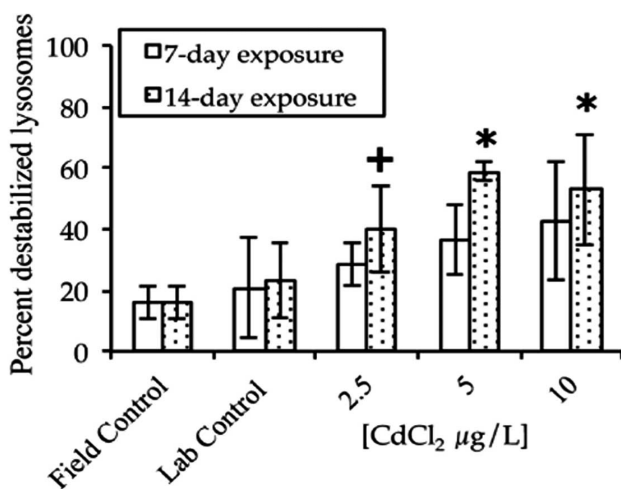

Fig. (2). Percent of destabilized lysosomes of digestive gland cells in *Elliptio complanata* exposed to three concentrations of CdCl₂ for 7 and 14 days. Field control clams as in Fig. (**1**). Sample sizes were n = 3 per group. *: P < 0.004-0.001 *vs*. both field and laboratory controls; +: P < 0.03 *vs*. field control.

Exposure of mussels to methoxychlor had a significant effect on the mean percentage of destabilized lysosomes, and similar to the results for Cd, the differences occurred only at the 14-day exposure time (one-way ANOVA, F = 4.34, p = 0.02, Fig. **3**). There were significant differences between the 10 μg/L group and both controls (Fisher's PLSD, 0.009 –

0.004). The 100 μg/L group was significantly different only from the field control (p = 0.004). Overall, for *E. complanata*, there was no dose response of toxicity for either exposure period for any of the three toxicants. In the case of the 14-day exposure, the intermediate concentrations of all three toxicants were as toxic as the high concentrations.
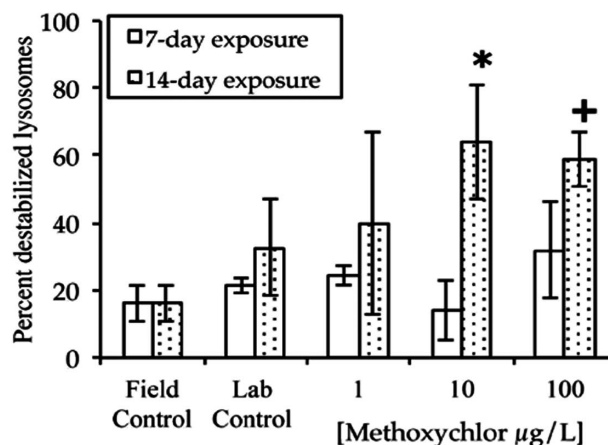

Fig. (3). Percent of destabilized lysosomes of digestive gland cells in *Elliptio complanata* exposed to three concentrations of methoxychlor for 7 and 14 days. Field control clams as in Fig. (**1**). Sample sizes were n = 3 per group. *: P < 0.004-0.009 *vs*. both field and laboratory controls; +: P < 0.004 *vs*. field control.

For the snail *Helisoma trivolvis*, exposure to Cu had a significant effect on the mean percentage of destabilized lysosomes for both a 7-day exposure (one-way ANOVA, F = 45.39, p < 0.0001) and 14-day exposure (F =23.85, p < 0.0001, Fig. **4**). There were significant differences between all three Cu concentrations and both field control and lab control snails (Fisher's PLSD, 0.003 – < 0.0001).
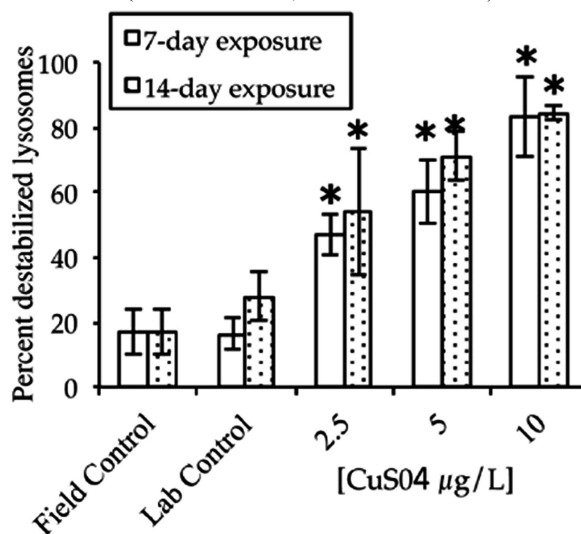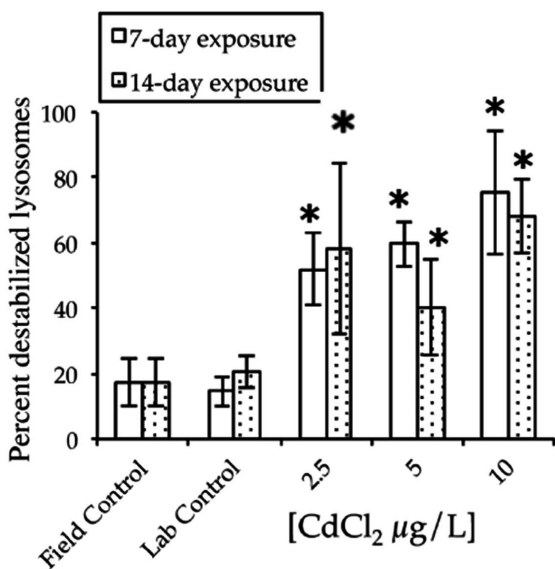

Fig. (4). Percent of destabilized lysosomes of digestive gland cells in *Helisoma trivolvis* exposed to three concentrations of CuSO₄ for 7 and 14 days. Field control snails were tested immediately after collection. Sample sizes were n = 4-5 per group. *: P < 0.003-0.0001 *vs*. both field and laboratory controls.

Similarly, exposure to Cd had a significant effect on the mean percentage of destabilized lysosomes for both a 7-day exposure (F = 20.63, p < 0.0001) and 14-day exposure (F = 7.72, p = 0.003, Fig. **5**). There were significant differences

between all three Cd concentrations and both field control and lab control snails (Fisher's PLSD, 0.03 – 0.0006).

Fig. (5). Percent of destabilized lysosomes of digestive gland cells in *Helisoma trivolvis* exposed to three concentrations of CdCl$_2$ for 7 and 14 days. Field control snails as in Fig. (**4**). Sample sizes were n = 4-5 per group. *: P < 0.03-0.0001 *vs.* both field and laboratory controls.

Exposure of snails to methoxychlor had a significant effect on the mean percentage of destabilized lysosomes for both the 7-day exposure (F = 19.57, p=0.0003) and the 14-day exposure (F =108.66, p < 0.0001, Fig. **6**). Except for the 1 µg/L-7-day exposure group (p = 0.07), there were significant differences between the three methoxychlor concentrations and both field control and lab control snails (Fisher's PLSD, 0.002 – 0.0001). Overall, for *H. trivolvis*, there was a trend of increasing lysosomal destabilization with increasing Cu and methoxychlor concentrations, but this trend was not clear with Cd.
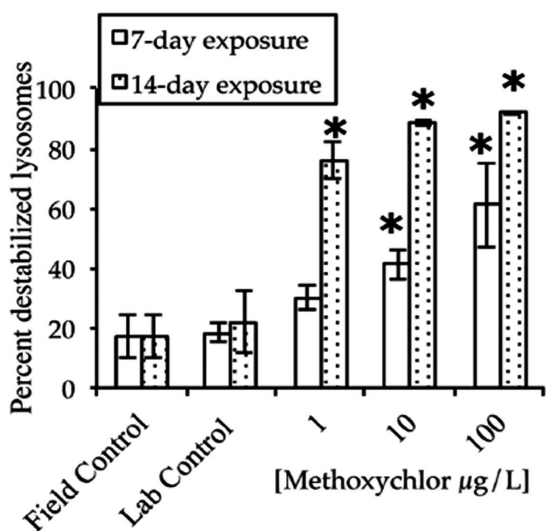
Fig. (6). Percent of destabilized lysosomes of digestive gland cells in *Helisoma trivolvis* exposed to three concentrations of methoxychlor for 7 and 14 days. Field control snails as in Fig. (**4**). Sample sizes were n = 3-4 per group. *: P < 0.02-0.0001 *vs.* field and laboratory controls.

DISCUSSION AND CONCLUSION

We found that the toxicants Cu, Cd, and methoxychlor cause stress effects in freshwater mussels and snails measurable by observing destabilized lysosomes of digestive gland cell stained with neutral red. Only a few species of freshwater invertebrates have been studied using neutral red retention as a gauge of toxicity, the snails *Vivparus contectus* [43] and *Lymnaea stagnalis* [44], and the bivalves, *Dreissena polymorpha* [29, 30], and *Lamellidens marginalis* [45]. Our use of a unionid bivalve, *Elliptio complanata*, and a planorbid snail, *Helisoma trivolvis* in the present study increases the number of freshwater invertebrate species tested, and both have a broad North American geographical distribution. Furthermore, *H. trivolvis* is more sensitive to Cd and methoxychlor than is *E. complanata*. For Cu, our lowest observed effect concentration (LOEC) was 2.5 µg/L for both *E. complanata* and *H. trivolvis* over 7 and 14 days of exposure. But for *H. trivolvis*, the Cd and methoxychlor LOECs were 2.5 and 10 µg/L, respectively over 7 days, whereas neither Cd nor methoxychlor significantly affected the percent of lysosomal destabilization in *E. complanata*. After 14 days exposure, the LOECs for Cu and Cd were the same for both species (2.5 µg/L), but the methoxychlor LOEC was 10 times lower for *H. trivolvis* (1.0 µg/L) than for *E. complanata*. It is unclear why *H. trivolvis* was more sensitive to Cd and methoxychlor than was *E. complanata*. Brown *et al.* [46], found a Cu toxicity difference over 7 days between marine snails and mussels. Their Cu LOEC for lysosomal destabilization in the limpet *Patella* was 6.1 µg/L but in the mussel *Mytilus* it was 68µg/L. They hypothesized that the difference in Cu sensitivity between species was possibly due to differences in Cu uptake via food. In our study, the food sources for *H. trivolvis* and *E. complanata* would likely be very similar. Both species occur in the same creek habitat often living within a few centimeters of each other. While *E. complanata* is a filter feeder and *H. trivolvis* consumes benthic organic material, this material is mainly settled particles. These particles could easily be re-suspended and consumed by *E. complanata*. A alternative explanation for the differences in toxicant sensitivity could be that *H. trivolvis* is a pulmonate snail, has no operculum, and thus cannot prevent exposure to the environment, whereas *E. complanata* is capable of completely closing its valves for varying periods of time, thus effectively reducing toxicant uptake.

In previous studies on Cu toxicity [43], workers exposed the snail *Viviparus contectus* to Cu concentrations from 31-100 µg/L. Snails at all concentrations had significantly lower neutral red retention time compared to controls. Russo and co-workers [44, 47] reported a significant decrease in neutral red retention time in the pond snail *Lymnaea stagnalis* exposed to the herbicides fomesafen and atrazine, respectively. Marine mollucs have been the subject of many Cu toxicity studies using neutral red retention assay in destabilized lysosomes. Shepard and Bradley [48] showed a dose-dependent, Cu-induced (20-80 µg/L) increase in lysosomal destabilization in the mussel *Mytilus edulis* after only 24 hours. Ringwood *et al.* [13] exposed oysters to Cu concentrations from 2.5 to 20 µg/L over periods of 18 hrs, and 4, 7, and 14 days. They found lysosomal destabilization significantly increased at 7 and 14 days, but with a decrease in

destabilized lysosomes at day 14 at 2.5 µg/L and 10.0 µg/L. Our study showed an increase instead of decrease in lysosomal destabilization from 7 to 14 days at all concentrations. Nicholson [49] found that elevated Cu decreased neutral red retention time in the mussel *Perna viridis*. Matozzo *et al.* [16] found significant lysosomal destabilization at concentrations from 10-110 µg/L in the bivalve *Tapes philippinarum*.

There have been even fewer studies on the toxic effects of Cd using lysosomal destabilization. Matozzo *et al.* [16] showed that Cu had a stronger effect on destabilized lysosomes than Cd did even when the concentrations of Cd were two to three times higher than that of Cu in the marine clam *T*apes *philippinarum*. In their study, they found high percentages of destabilized lysosomes at high Cd concentrations, but these were not significantly different from controls due to variance in control lysosomal destabilization. We used the same range of concentrations of Cu and Cd in our study, but the effect of Cu on lysosomal destabilization was evident after 7 days, whereas the effect of Cd became significant only after 14 days.

While no study to date has employed a neutral red lysosomal destabilization assay for effects of methoxychlor on an aquatic invertebrate, other studies on organochlorines or organophosphate pesticides have. Oysters exposed to chlordane show a negative correlation between neutral red retention time and increasing exposure [50]. Similarly, Canty *et al.* [51] showed that sea mussels exposed to the organophosphate Azamethiphos decreased neutral red retention time. Furthermore, while not an aquatic invertebrate, earthworms exposed to organophosphates (chlorpyrifos and diazinon) had significantly reduced neutral red retention time [37].

The cellular mechanism of lysosomal membrane destabilization has been shown to involve activation of Ca^{+2}-dependent phospholipase A_2 (PLA_2) in marine mussel hemocytes exposed to estradiol [52] and to Hg^{+2} and Cu^{+2} [53]. Hu *et al.* [54] have shown that PLA_2 induces the production of the lipid metabolite lysophosphatidylcholine (lysoPC) in rat liver cells. LysoPC changes the permeability of lysosomal membranes to K^+ and H^+ causing the entry of K^+ into the lysosome via a K^+/H^+ enchanger. Ultimately, the lysosome disintegrates from osmotic shock. How PLA_2 regulates lysosomal membrane destabilization in mussel hemocytes or other molluscan cells remains unknown.

Many recent toxicity studies using neutral red retention assays in lysosomes of molluscs have utilized hemocytes [29, 30, 33, 44, 47]. We measured lysosomal destabilization in molluscan digestive gland cells. Such cells have been used in neutral red retention assays previously in marine molluscs [13, 34]. Moreover, Lowe *et al.* [55] found no difference in neutral red retention time between hemocytes and digestive gland cells marine mussels exposed to fluorathene.

Neutral red retention assay has been used as a reliable indicator of stress in aquatic organisms. Our study is one of the few that addresses stress induced by heavy metals and pesticides as measured by this assay in freshwater invertebrates. It should be noted that environmental conditions such as aerial exposure [56], or high temperature and high salinity

[49] can induce lysosomal destabilization. The two species examined in our study undergo periods of seasonal environmental fluctuation. *Elliptio complanata* forms dense beds in shallow sediments near the air-water interface in small streams. During summer, water levels typically drop by several feet, leaving mussels exposed to air where they are subject to desiccation and predation. During summer, mussels are often seen migrating slowly towards the receding water line. *Helisoma trivolvis* lives in a similar habitat to *E. complanata*, except that they are found in large numbers on rocks that are frequently exposed to air during the summer. Thus, both species undergo seasonal environmental stress. Despite this, our field control animals had low percentages of lysosomal destabilization (< 20%).

Freshwater molluscs are excellent sentinel species and the two test organisms are common and widespread throughout much of North America. Our study supports the use of neutral red retention assay as a reliable indicator of environmental stress in freshwater invertebrates.

CONFLICT OF INTEREST

None declared.

ACKNOWLEDGEMENTS

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