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Metal−Polycyclic Aromatic Hydrocarbon Mixture Toxicity in *Hyalella azteca*. 2. Metal Accumulation and Oxidative Stress as Interactive Co-toxic Mechanisms

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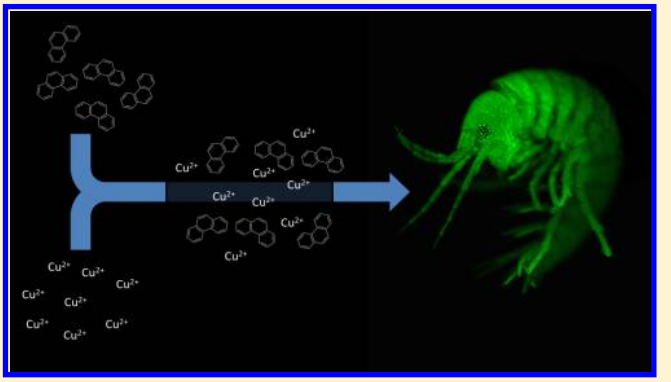
Supporting Information

ABSTRACT: Mixtures of metals and polycyclic aromatic hydrocarbons (PAHs) are commonly found in aquatic environments. Emerging reports have identified that more-than-additive mortality is common in metal−PAH mixtures. Individual aspects of PAH toxicity suggest they may alter the accumulation of metals and enhance metal-derived reactive oxygen species (ROS). Redox-active metals (e.g., Cu and Ni) are also capable of enhancing the redox cycling of PAHs. Accordingly, we explored the mutual effects redox-active metals and PAHs have on oxidative stress, and the potential for PAHs to alter the accumulation and/or homeostasis of metals in juvenile *Hyalella azteca*. Amphipods were exposed to binary mixtures of Cu, Cd, Ni, or V, with either phenanthrene (PHE) or phenanthrenequinone (PHQ). Mixture of Cu with either PAH produced striking more-than-additive mortality, whereas all other mixtures amounted to strictly additive mortality following 18-h exposures. We found no evidence to suggest that interactive effects on ROS production were involved in the more-than-additive mortality of Cu-PHE and Cu-PHQ mixtures. However, PHQ increased the tissue concentration of Cu in juvenile *H. azteca*, providing a potential mechanism for the observed more-than-additive mortality.



INTRODUCTION

The ecological risks of metal and polycyclic aromatic hydrocarbon (PAH) contamination in aquatic systems have been studied extensively considering the toxic effects of these stressors individually; however, it is difficult to estimate their co-toxicity through the summation of individual toxic effects.[1] This problem is becoming increasingly evident as more aquatic studies emerge, and is most likely a result of direct and/or indirect interactions among these contaminants at various stages of intoxication.[2]

Phenanthrene (PHE) and its oxidation/degradation products are common co-contaminants with Cu, Ni, Cd and V in a variety of contaminated marine and freshwater systems.[3−8] There are a variety of sensitive invertebrate fauna found within aquatic systems. Disturbance to their communities can produce strong bottom-up cascading effects, restructuring the biological diversity and function of an entire system.[9] *Hyalella azteca*, an abundant and widespread amphipod species in North America, is commonly used as a model organism in studies of aquatic toxicity. The toxic modes of action of PAHs and metals have not been sufficiently studied in *H. azteca* to conclusively identify the most important acute lethal mechanisms of toxicity. Nonetheless, metals and PAHs share similarities in terms of their effects on reactive oxygen species (ROS) homeostasis and ionoregulation in other aquatic biota. Low-molecular-weight PAHs such as PHE are acutely toxic in part due to their deleterious effects toward cell membranes[10,11] resulting in ionoregulatory dysfunction or membrane destabilization in severe cases (as reviewed by Gauthier et al. in 2014).[2] Likewise, Cu,[12] Cd,[13] and Ni[14] acutely disrupt osmoregulation of Mg or Na in crustaceans. Thus, ionoregulatory disruption is a potential acute lethal mechanism of action in *H. azteca* shared by Cu, Cd, Ni, and PHE.

In addition to ionoregulatory disruption, various metals and PAHs also disrupt ROS balance in aquatic organisms. Cu, Ni, and V engage in Fenton-like reactions in the presence of H_2O_2 to generate ROS (i.e., $^{\bullet}OH$).[15] Phenanthrenequinone (PHQ), the major photoderivative of PHE, generates ROS (i.e., $O_2^{\bullet-}$, $^{\bullet}OH$) through autoxidation.[16−19] Thus, oxidative stress is a second potential acute lethal mechanism of action of Cu, Ni, V, and PHQ in *H. azteca*.

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Understanding the individual mechanisms of action provides some insight into the additivity of joint exposure. The mixing of a non-ROS-producing PAH and ROS-producing metal would intuitively have a strictly additive effect on ROS production (i.e., metal-induced ROS production would account for total ROS production because the PAH would produce no ROS), as demonstrated in *Daphnia magna* exposed to Cu-PHE mixtures.[20] However, the additivity of mixtures is not always intuitive, even if the two toxicants share similar mechanisms of action. A clear example of intuition failing to predict additivity was presented for ROS production in *D. magna* exposed to Cu-PHQ and Ni-PHQ mixtures (i.e., all ROS-producing toxicants), where increases in ROS were more-than-additive.[20,21] It was proposed that PHQ-derived H_2O_2 feeds the Fenton-like reactions Cu and Ni undergo to produce ROS, and that Cu aids in the redox cycling of PHQ.[20−22]

Joint effects on ion homeostasis may be an additional co-toxic mechanism in mixtures of Cu, Cd, and Ni, with PHE and PHQ. For example, PAH-induced altered metal accumulation would produce non-additive co-toxicity in metal−PAH mixtures.[2] Waterborne co-exposures of Zn and PHE resulted in an overall less-than-additive mortality due to an unexplained reduction in Zn uptake in sheepshead minnows (*Cyprinodon variegatus*), although a more-than-additive mortality of Zn-PHE mixtures was found at low concentrations of both Zn and PHE.[23] Sediment Cd-PHE mixtures also produced a less-than-additive mortality in the oligochaete, *Hyodrilus templetoni*, which was attributed to attenuated feeding and the subsequent reduction in dietary accumulation of Cd.[24] However, bioaccumulation of Cd increased in *H. azteca* exposed to sediments containing a sublethal mixture of Cd and PHE.[25] This increase in the accumulation of Cd could explain the more-than-additive mortality observed in *H. azteca*[1,25] and the copepod, *Schizopera knabeni*,[26] exposed to Cd-PHE loaded sediments. Waterborne sublethal Cd-PHE co-exposures to *H. azteca* had no effect on Cd accumulation, and thus, led to strictly additive co-toxicity, whereas waterborne Cd-PHE mixtures were found to have a more-than-additive lethality in the copepod, *Amphiascoides atopus*.[26] Clearly, findings to date are largely equivocal and illustrate inconsistencies among test organisms and exposure scenarios, in part due to varying test methodologies.

An alteration of metal bioaccumulation or ROS homeostasis as a result of co-exposure of metals and PAHs could enhance or attenuate the ecological risk these contaminants pose to aquatic systems. Accordingly, we investigated the acute lethal additivity, bioaccumulation of metals, and ROS production associated with binary mixtures of Cu, Cd, Ni, and V with PHE and PHQ in juvenile *H. azteca*.

METHODS

Amphipod culturing and test water preparations followed those in our preceding paper.[27] Briefly, 2-L polypropylene culture containers were used to hold ca. 20 adult Burlington clade *H. azteca* in 1 L of standard artificial media (SAM)[28] at 25 °C. Animals were fed 5 mg of ground Tetramin fish flake three times per week. At the end of each week, adults and juveniles were sorted with 700 and 200 μm nylon mesh sieves. Juveniles were then transferred to a 2 L polypropylene container filled with 1 L of SAM and acclimated to 20 °C. All test containers had a 5 cm × 10 cm strip of cotton gauze as substrate.

Metal Accumulation. Tissue samples were collected from surviving amphipods obtained from the 48-h response surface experiments described in our preceding paper[27] measuring non-additive mortality. The 48-h exposure duration allowed for comparison with the Cu accumulation modeling data provided by Borgmann and Norwood.[31] Briefly, 2−10-day-old *H. azteca* were exposed to mixtures of metals and PAHs held at fixed mixture proportions tested along a standard concentration series with five concentrations (Table S1). Each treatment was replicated three times. Following 48-h exposures, surviving animals were collected for tissue analysis. Only Cu- and Cd-PAH experiments were used for the analysis metal accumulation, as Ni-PAH and V-PAH mixtures did not produced clear more-than-additive lethality (i.e., exploring altered metal accumulation as a mechanisms of more-than-additive lethality would have been pointless).

Whole-body metal concentrations were measured based on the methods described by Norwood et al.[29] However, no gut clearance was required as animals were not fed throughout the exposure period. At the end of each test, a minimum of four live amphipods were taken from all replicates of control, metal-only, and metal−PAH mixtures, and were kept separated so that each final sample only contained amphipods from a single replicate. These amphipods were bathed for 1 min in a 50 μM solution of ethylenediaminetetraacetic acid (EDTA) in culture water to remove adsorbed metals on the surface of the animals (i.e., not bioaccumulated).[29] Animals were then transferred to 1.5 mL cryovials and any transferred EDTA solution was removed with a pipet and a Kimwipe delicate task wiper. Animals were then dried for 72 h at 80 °C with the cryovial lids loosely capped to allow moisture to escape. Amphipods were measured for dry weight (dw) by transferring all animals from each cryovial into pre-tared silver weigh boats. Weighed samples were then transferred back to a clean cryovial, and weigh boats were measured again to account for any remaining tissue. Samples were then digested in 160 μL of 70% HNO_3 at room temperature for 6 days. Samples were further digested in 120 μL of 30% H_2O_2 for 24 h. Sufficient deionized water was then added to each sample to make the total volume 6 mL before analysis by ICP-MS.

Metal bioaccumulation was modeled with the saturation model described in Norwood et al.:[30]

$$c_{tb} = max[c_w(k_w + c_w)^{-1}] + c_{bk} \quad (1)$$

where c_{tb} is the total concentration ($\mu g\ g^{-1}$) of the metal within the body, c_w is the concentration ($\mu g\ L^{-1}$) of the metal in water, *max* is the maximum above-background concentration ($\mu g\ g^{-1}$) of metal that can be accumulated for the given exposure duration, k_w is the half-saturation constant ($\mu g\ L^{-1}$) for the given exposure duration, and c_{bk} is the background body metal concentration ($\mu g\ g^{-1}$) measured in control animals. For Cu accumulation modeling, a *max* of 228.7 $\mu g\ g^{-1}$, as reported by Borgmann and Norwood for 48-h accumulation trials with *H. azteca*,[31] was input into eq 1 as the c_w we tested were too low to obtain a good estimate of *max* over the short 48-h exposure period. This was an unexpected consequence of exposing amphipods to Cu c_w based on lethal effects in Cu-PAH mixtures, where Cu concentrations exceeding 15 $\mu g\ L^{-1}$ in mixture with PAHs produced high mortality, leaving too few remaining live amphipods for analysis of tissue Cu concentrations. In contrast, our c_w for Cd were in an appropriate range to estimate *max*,[32] and thus, both *max* and k_w were estimated for Cd accumulation. Parameters were estimated with the "nls" function in R.[33] Finally, lethality as a function of tissue metal

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concentration was modeled with the saturation model adapted from Norwood et al.:[29]

$$p = \ln(2)[c_{tb}(\mathrm{LBC50}^{-1} + k_{tb}{}^{-1})(1 + c_{tb}k_{tb}{}^{-1})^{-1}]^{nb} \quad (2)$$

where p is the proportional mortality attributed to background corrected whole-body metal concentrations (c_{tb}), LBC50 is the above-background lethal body concentration killing 50% of test animals (i.e., at $p = 0.5$), and k_{tb} and nb are constants. Control p was collected from all experiments, but was not considered in eq 2 as it was negligible ($\overline{p} = 0.03$). As a result of the above-mentioned restrictions for Cu accumulation analyses, it was not possible to derive an LBC50 for Cu. Differences in Cu or Cd accumulation when co-exposed with PHE or PHQ were assessed based on standard error overlap of parameter estimates.

Paired Mortality and ROS Production Assays. For paired mortality and ROS production experiments, 2−10-day-old amphipods were acclimated at 21 °C for 24 h prior to being exposed. Following acclimation, 10−20 amphipods were added to 300 mL of test water in 400 mL glass beakers containing one 2.5 cm × 5 cm strip of cotton gauze as substrate and exposed for 18 h. An 18-h exposure duration was chosen as preliminary trials with Cu indicated maximum values of ROS did not increase as exposure duration increased beyond 18 h (data not shown). All treatments were replicated three times. Exposures were carried out at 21 °C with a 16-h light:8-h dark photoperiod. Tests consisted of exposure to control conditions, each metal singly, each PAH singly, as well as metal:PAH mixtures at the same concentrations as singular exposures. The PAHs were introduced as enriched polydimethylsiloxane films.[27] Nominal concentrations of metals and PAHs were based on 48-h mixture LC50s at a 1:1 mixing proportion, and were measured with ICP-MS and HPLC respectively.[27] Cu, Cd, Ni, and V were tested at 15.3 and 17.7, 19.8, 12 480 and 15 160, and 3650 and 1217 μg L^{-1}, respectively, and were mixed with either 170, 68.8, 184.9, and 158.9 μg L^{-1} PHE, respectively, or 225, 269.7, 252.6, and 199.4 μg L^{-1} PHQ, respectively.

As tissue masses of juvenile amphipods did not allow for the analyses of both tissue metal and PAH concentrations, tissue concentrations of metals and PAHs were not measured for paired mortality and ROS production assays. Thus, the assessment of additivity was based on measured water concentrations.

Following 18-h exposures, death was assessed as immobility with no pleopod movement. Surviving animals were transferred into 1.5 mL cryovials along with 600 μL of culture water and stained with dichlorofluorescein diacetate (H_2DCFDA) which is converted to the highly fluorescent dichlorofluorescein (DCF) when oxidized (i.e., by ROS).[34] Dyeing procedures were based on those described in Xie et al.[21] An aliquot of 400 μL of 25 μM stock solution of H_2DCFDA in methanol was added to each cryovial to achieve the final staining solution concentration of 10 μM. Samples were then incubated for 4 h.

Fluorescence was measured with a confocal microscope with a fluorescence detector (FV1200, Olympus America Inc., PA, USA) set to an excitation wavelength of 485 nm and an emission wavelength of 530 nm, both with a bandwidth of 20 nm. Sample fluorescence was quantified with ImageJ 1.47,[35] and was represented as the corrected total sample fluorescence (f_{ct}), calculated as follows:

$$f_{ct} = af - af_b \quad (3)$$

where a is the area of sample containing stained tissue, f is the mean fluorescence observed in a, and f_b is the mean background fluorescence observed from all areas of the sample not containing stained tissue.

Mortality and fluorescence data were analyzed separately with a generalized and general linear model, respectively, to test for significant ($\alpha = 0.05$) differences in mortality and mean f_{ct} as a function of metal, PAH, and metal−PAH mixture concentrations, and allowed for a formal test of the interactive effect (i.e., additivity) of the metal−PAH mixture.[36] Thus, for mortality and fluorescence data, a positive or negative and significant estimate of the interactive effect indicated more- or less-than-additive toxicity, respectively. A Bayesian GLM was used for mortality data to overcome issues in applying a binomial distribution when excessive separation of binomial count data is present (i.e., excess counts of zeros or ones in mortality data). The Bayesian approach, with a Cauchy distribution having a center and scale of 0 and 2.5 respectively, incorporates a prior distribution of parameter estimates that outperforms classical GLM's in estimating parameters for data sets with excessive separation.[37] All statistical analyses were carried out with the "glm" and "bayesglm" functions contained within the statistical package, R 3.2.0.[33,38]

RESULTS AND DISCUSSION

Accumulation of Cu and Cd. Mean background tissue Cu and Cd concentrations were 34.6 and 2.7 μg g^{-1} dw, respectively. Waterborne exposures of Cu and Cd resulted in a concentration-dependent increase in juvenile *H. azteca* tissue Cu and Cd concentrations (Figure 1, Table 1). Estimated $\overline{max}$

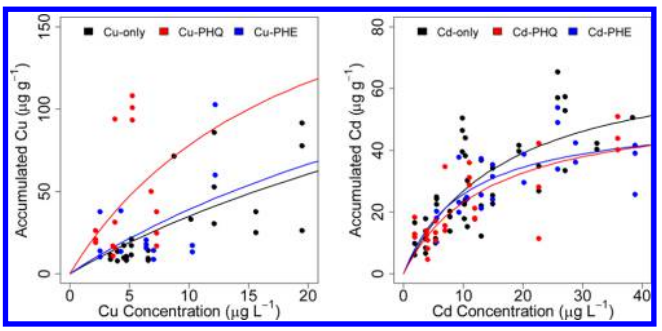

Figure 1. Tissue Cu (left panel) and Cd (right panel) concentrations in juvenile *H. azteca* following 48-h exposures to Cu-only, Cu-PHE, Cu-PHQ, Cd-only, Cd-PHE, and Cd-PHQ treatments. Curves represent model predictions from eq 1.

= 59.9 μg g^{-1} parameters for Cd accumulation (Table 1) were within the confidence limits of those reported in Borgmann et al.[32] also involving *H. azteca*; however, our estimated k_w = 13.7

Table 1. Summary of Saturation Modeling (Eq 1) Results for Mixtures of Cu or Cd with Phenanthrene (PHE) or Phenanthrenequinone (PHQ)

treatment	k_w ± se	max ± se	p-value
Cu-only	55.4 ± 7.6		<0.0001
Cu-PHE	48.5 ± 11.7	228.7	0.0012
Cu-PHQ	19.2 ± 4.9		0.0016
Cd-only	16.9 ± 5.8	72.1 ± 12.5	0.0053; <0.0001
Cd-PHE	10.2 ± 4.1	52.0 ± 7.6	0.02; <0.0001
Cd-PHQ	14.1 ± 5.8	55.7 ± 11.1	0.022; <0.0001

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Table 2. Summary of GLM Results for Lethality and Fluorescence Data from Exposure to Metals with Either Phenanthrene (PHE) or Phenanthrenequinone (PHQ)[a]

		odds ratio		f_{ct} (% above control)	
mixture	coefficient	estimate ± se	*p*-value	estimate ± se	*p*-value
Cu-PHE	Cu	0.0023 ± 6.16	0.57	1.76 ± 0.52	0.0092
	PHE	0.023 ± 2.09	0.49	0.022 ± 0.046	0.64
	Cu-PHE	0.37 ± 1.32	0.048	−0.0015 ± 0.0043	0.73
Cu-PHQ	Cu	0.078 ± 2.15	0.32	3.086 ± 0.84	0.0078
	PHQ	0.0069 ± 6.52	0.66	0.17 ± 0.066	0.039
	Cu-PHQ	2.75 ± 1.40	0.025	0.0024 ± 0.0056	0.69
Cd-PHE	Cd	0.58 ± 1.53	0.025	0.45 ± 0.54	0.43
	PHE	0.0061 ± 6.88	0.69	0.033 ± 0.16	0.84
	Cd-PHE	0.77 ± 1.45	0.59	0.0027 ± 0.011	0.81
Cd-PHQ	Cd	0.18 ± 1.64	0.028	0.65 ± 0.52	0.25
	PHQ	0.23 ± 1.59	0.015	0.054 ± 0.039	0.19
	Cd-PHQ	0.36 ± 1.42	0.11	−0.0016 ± 0.0028	0.57
V-PHE	V	0.0066 ± 6.61	0.69	0.00042 ± 0.002	0.84
	PHE	0.077 ± 2.15	0.28	0.0077 ± 0.047	0.87
	V-PHE	0.25 ± 1.58	0.33	0.0000 ± 0.0000	0.97
V-PHQ	V	0.11 ± 1.72	0.023	0.00038 ± 0.0077	0.96
	PHQ	0.022 ± 2.79	0.45	0.068 ± 0.037	0.11
	V-PHQ	0.091 ± 1.68	0.42	−0.0000 ± 0.0000	0.55
Ni-PHE	Ni	0.18 ± 1.64	0.052	0.0024 ± 0.0005	0.0012
	PHE	0.099 ± 1.85	0.18	0.025 ± 0.033	0.47
	Ni-PHE	0.36 ± 1.42	0.51	−0.0000 ± 0.0000	0.36
Ni-PHQ	Ni	0.34 ± 1.51	0.016	0.0029 ± 0.0006	0.0015
	PHQ	0.28 ± 1.54	0.026	0.12 ± 0.047	0.029
	Ni-PHQ	0.62 ± 1.37	0.13	−0.0000 ± 0.0000	0.022

[a]Mortality estimates are presented as the predicted odds ratio ± se errors at the tested concentrations. Estimates of fluorescence (f_{ct}) are presented as the % change in f_{ct} above control per unit increase in concentration.

$\mu g\ g^{-1}$ for Cd was substantially higher. We also found k_w for Cu accumulation in Cu-only (55.4 ± 7.6 $\mu g\ L^{-1}$) and Cu-PHE (48.5 ± 11.7 $\mu g\ L^{-1}$) treatments to deviate from what is reported in Borgmann and Norwood (18.5 $\mu g\ L^{-1}$).[31] Discrepancies in k_w for Cd data could be associated with differences in exposure duration (i.e., 48 h in the present study compared to 4−6 wk in Borgmann et al.[39]). However, estimated k_w from Cu data in the work of Borgmann[32] used data from 48-h exposures.[31] It is unlikely that difference in water quality would account for differences in k_w, as water hardness, pH, and dissolved organic carbon were similar. The most notable differences between the present work and the work of Borgmann and Norwood[31] in deriving k_w for Cu, is that far lower c_w were used, and animals were not fed during exposures. As k_w is influenced by c_w (eq 1), it is possible that at a lower c_w, k_w is larger (i.e., reflecting less Cu accumulation over 48 h). Moreover, the lack of accumulation of Cu from dietary sources most likely influenced k_w. Interestingly, the k_w from Cu-PHQ co-exposures (19.2 ± 4.9 $\mu g\ L^{-1}$) was similar to the k_w for Cu reported by Borgmann et al.,[32] suggesting that low c_w may not be a limiting factor in Cu accumulation when PHQ is present (see below for further discussion).

Two 48-h Cd LBC50s (47.1 ± 22.1 and 51.1 ± 10.5 $\mu g\ g^{-1}$; Figure S1) for juvenile *H. azteca* were obtained from a series of two independent reference toxicity tests from the isobole experiments described in our preceding paper.[27] The 48-h Cd LBC50s were in the same range as 6-week Cd LBC50s in a variety of test media,[39] suggesting that tissue concentration is a good indicator of Cd toxicity in *H. azteca*. Coincidentally, the threshold for lethal tissue Cd concentrations in juvenile *H. azteca* (ca. 7−10 $\mu g\ g^{-1}$; Figure S1) is similar to the threshold liver Cd concentrations indicative of in situ Cd exposure in *Perca flavecsens*, suggesting that this value may be useful for developing an aquatic regulatory guideline based on tissue Cd concentrations.[40]

Influence of PHE and PHQ on Cu and Cd Accumulation. Amphipods co-exposed to Cu and PHQ had a lower k_w (i.e., higher Cu accumulation) compared to amphipods exposed only to Cu (Table 1, Figure 1). However, PHE had no effect on Cu accumulation in juvenile *H. azteca*, and co-exposure to PHE or PHQ had no effect on Cd accumulation in juvenile *H. azteca* following 48-h (Figure 1) and 192-h[25] waterborne exposures. As PHE did not alter tissue Cu concentrations in *H. azteca* following 48-h aqueous exposures, the generalized effects of PAHs on membranes were likely not the mechanisms responsible for increasing the accumulation of Cu in juvenile *H. azteca*. Instead, the presence of the quinone group in PHQ may be important in explaining Cu accumulation.

Copper is a highly regulated essential metal in *H. azteca*,[31] likely through the activity of several proteins at the sites of uptake (i.e., gill and gut).[41,42] However, Cd is a non-regulated non-essential metal, and is likely accumulated due to its capacity to mimic Ca^{2+} and co-opt Ca^{2+} transport channels.[43,44] The regulation of Cu suggests that PHQ may affect the function of Cu transport proteins.

The arylative and oxidative properties of PAH-quinones (PAHQs) and PAHQ-derived ROS,[45,46] respectively, present mechanisms by which PAHQs may disrupt metal transport proteins and metal homeostasis. Generally, Cu^+ can pass the apical membrane by mimicking Na^+ and co-opting the Na^+ channel, but more specifically, metal ion transporters, such as divalent metal transporter 1 (DMT1) and the Cu-transporter 1

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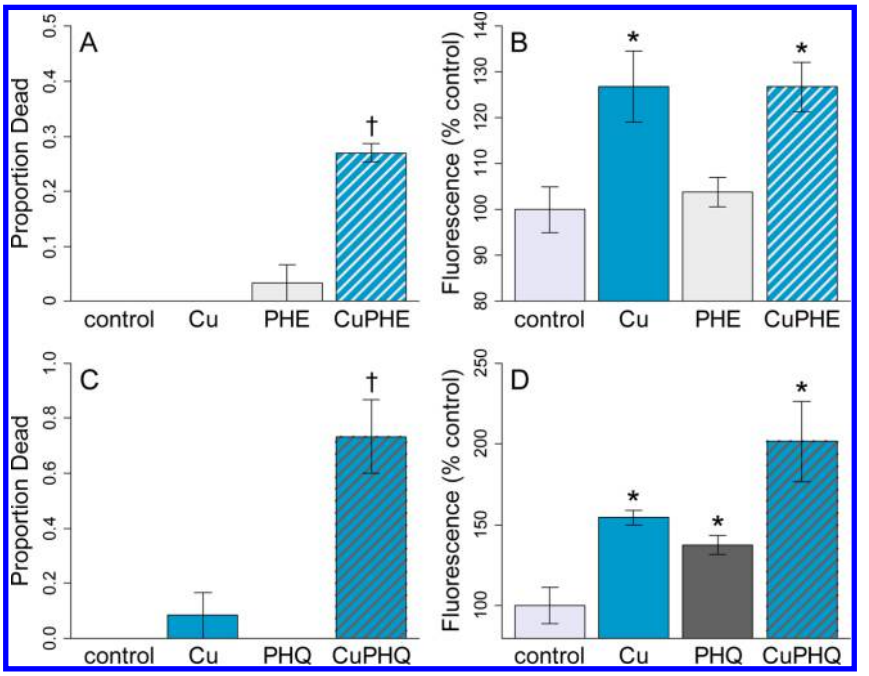

Figure 2. Mean observed mortality and ROS fluorescence ± se from Cu-PHE (panels A,B) and Cu-PHQ (panels C,D) exposures. Asterisks (*) indicate a significant difference from control. Daggers (†) indicate a significant interaction (i.e., a less- or more-than-additive effect).

(ctr1), as well as the Cu specific adenosine triphosphate ion pump (Cu-ATPase), work together to regulate Cu.[41,47−49] The uptake of Cu is assisted by ctr1[47,50] and DMT1,[41,42] whereas Cu-ATPases generally function to control the efflux of Cu from cells and tissues.[51] The critical site for ctr1[50] and DMT1[52] metal binding is at the methionine residue, which is highly susceptible to oxidation by H_2O_2 and $O_2^{\bullet-}$.[53] Oxidation of this site by PAHQ-derived ROS likely inhibits the accumulation of Cu in *H. azteca*. However, regardless of the observed increase of ROS in PHQ co-treatments, accumulation of Cu increased. An alternative explanation may be related to the effects of PHQ on Cu efflux. The putative Cu efflux enzyme in fish is Cu-ATPase.[49] Critical to the function of Cu-ATPase is the binding of Cu at the transmembrane metal binding sites (i.e., thiol groups of cysteine residues).[54] Thus, the PHQ-induced arylation[55] or PHQ-derived ROS oxidation of Cu-ATPase metal binding sites would disrupt the function of the enzyme, prevent Cu efflux, alter Cu homeostasis, and possibly explain the higher Cu tissue concentrations we observed in *H. azteca* co-exposed to Cu and PHQ.

It is likely that Cu accumulation in *H. azteca* is limited by the number of binding sites present on the animal.[31] If so, the rate of Cu accumulation would decrease when Cu is present in concentrations high enough to saturate these binding sites. However, in Cu-only and Cu-PHE treatments, we observed a slower rate of Cu accumulation, which was likely the result of efficient Cu efflux overcoming Cu influx at low water concentrations of Cu. The fact that co-exposure to PHQ increased the rate of Cu accumulation at low water Cu concentrations provides support for a PHQ-mediated decrease in Cu efflux. Nonetheless, further effort is required to validate this hypothesis, along with other competing hypotheses for PHQ-induced alteration of Cu accumulation. For example, it is also possible that PAHQs (e.g., PHQ) have the potential to form hydrogen bonds and complex with metals (e.g., Cu).[56] Similarly to other metal−hydrocarbon complexes,[57−60] metal−PAHQ complexes would likely retain their lipophilic properties and serve to facilitate the accumulation of metals, overcoming the limitations of saturable Cu binding sites that otherwise mediate the influx of Cu. The potential for PAHs to alter the bioavailability of metals has been discussed in detail elsewhere;[2,61,62] however, to date, there have been no studies that have linked metal−PAHQ complexation to increased metal bioavailability in aquatic biota.

Lethality. Control mortality was 0% in all 18-h experiments. Exposures to metals, PAHs, and metal−PAH mixtures induced varying degrees of mortality (Table 2). There was a positive interactive effect of Cu-PHE and Cu-PHQ mixtures on mortality, indicating that mortality in Cu-PHE and Cu-PHQ mixtures was more-than-additive, inducing 26.9 ± 1.7% and 73.3 ± 13.3% more mortality, respectively, than could be attributed to the observed mortality resulting from exposure to the individual toxicants (Figure 2A,C). All other mixtures produced strictly additive mortality (Table 2).

A review of the literature on non-additive co-toxicity of metal−PAH mixtures indicates that more-than-additive mortality is common in aquatic biota.[2] Simple mixtures of Cu with either PHE or PHQ produced more-than-additive mortality in *H. azteca* following 18 h of co-exposure. A more comprehensive analysis of Cu-PHE and Cu-PHQ co-toxicity in *H. azteca* similarly revealed that more-than-additive mortality was characteristic of mixtures of Cu with PHE or PHQ at a variety of different mixture ratios.[27] Experiments carried out with *D. magna* and *V. fischeri* support that Cu-PHQ mixtures produce more-than-additive lethality.[20,22] However, contrary to our work, *D. magna* exposed to Cu-PHE mixtures for 48 h exhibited strictly additive lethality.[20] The agreement between Cu-PHE mixture studies (i.e., 80%) is at par with the acceptable reproducibility criterion (i.e., 80%) proposed in Sørensen et al.[63] for isobole-based analyses. Nonetheless, inferring that Cu-PHE mixtures, or in fact any mixture, will produce the same degree of additivity in other biota or exposure scenarios must be cautioned.[64] There are many cases of conflicting mixture outcomes within the limited published data pertaining to the

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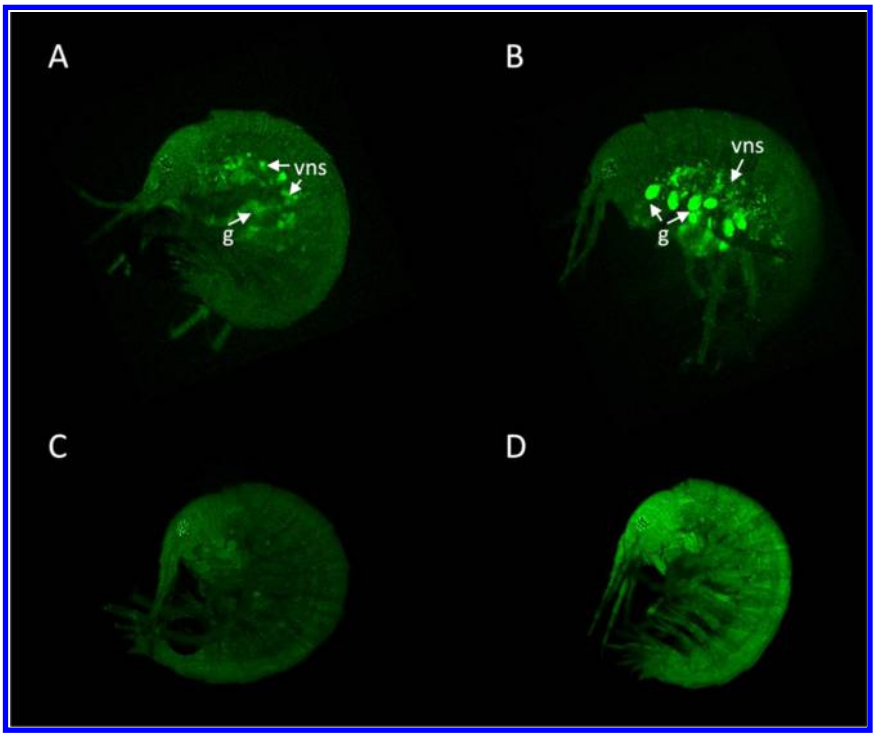

Figure 3. Dichlorofluorescein fluoresecence in juvenile *H. azteca*. Panels A and C are representative of unexposed animals. Panels B and D are representative of Cu-exposed animals. Gills and nervious tissue fluoresced in certain specimens (panels A,B), whereas some specimens exhibited a general whole-body fluorescence (panels C,D). "g" denotes gill tissue; "vns" denotes ventral nervous system tissue.

additivity of metal−PAH-induced lethality in aquatic biota.[27] One point of particular importance is that exposure duration influences the additivity of the mixture. For all practical purposes, the experimental protocol and *H. azteca* culture described in our previous paper[27] were identical to those in the present manuscript, yet at 18 h, Cd-PHE and Cd-PHQ mixtures elicited strictly additive mortality, whereas at 48 h more-than-additive and strictly additive co-toxicity was observed depending on the mixture proportion tested. This observation suggests a time-sensitive interaction between Cd-PHE and Cd-PHQ mixtures. General explanations for non-additive lethality can be found in Gauthier et al.[2] However, until the specific mechanism responsible for Cd-PHQ and Cd-PHE mixture toxicity is revealed, it is premature to speculate on time-sensitivity.

ROS produc®tion. Whole animal H_2DCFDA staining of juvenile *H. azteca* identified both tissue specific (Figure 3A,B) and general whole-body fluorescence (Figure 3C,D). Fluorescence was increased above control following 18-h exposures to Cu (40.8 ± 11.4%) and Ni (37.1 ± 7.7%; Table 2). However, there was no effect of Cd, V, and PHE on DCF fluorescence (Table 2). In Cu and Ni experiments, exposure to PHQ elicited an increase in DCF fluorescence (31.1 ± 12.1%). However, in Cd and V experiments, there was no increase in DCF fluorescence from PHQ exposure (Table 2). There were no interactive effects on DCF fluorescence following mixture exposures (Table 2). Thus, metal−PAH mixtures produced strictly additive fluorescence (i.e., ROS).

Two recent studies concluded that PHQ and redox-active metals (e.g., Cu and Ni) serve to potentiate PHQ- or metal-induced ROS production when mixed.[20,22] However, we found no evidence to suggest co-toxicity was related to interactive effects on ROS production, even though Cu-PHQ mixtures elicited more-than-additive mortality in *H. azteca*. Moreover, Ni-PHQ mixtures produced strictly additive mortality and ROS production in *H. azteca* following 18-h co-exposures, and in *H. azteca* following 48-h co-exposures, produced strictly additive and slightly less-than-additive mortality depending on the mixture proportion tested.[27]

It should be noted that the same ROS biomarker (i.e., H_2DCFDA) was used among the three studies exploring non-additive ROS production, the present study included. H_2DCFDA can be oxidized by H_2O_2 and several other ROS (e.g., other peroxides) and reactive nitrogen species (RNS).[34,65] Thus, it is expected that H_2DCFDA-stained tissue fluorescence should increase following exposure to H_2O_2-producing PHQ. This was observed in *H. azteca* as well as in *V. fischeri*,[22] but not in *D. magna* following 4-h exposures to a series of PHQ treatments from 124 to 1000 μg L^{-1}.[20] The discrepancy between *D. magna* and *V. fischeri* could stem from dissimilar experimental systems (i.e., bacteria compared to whole cladoceran crustaceans).[20] However, in whole *H. azteca*, a fellow crustacean, we found an increase in DCF fluorescence following exposure to PHQ at similar concentrations to those tested for *D. magna*. It is possible that 4 h of exposure is insufficient time to allow for increases in PHQ-induced ROS, whereas after 18 h (i.e., exposure duration in the present study) sufficient ROS have accumulated accounting for differences in H_2DCFDA oxidation and DCF fluorescence.

Nonetheless, more-than-additive DCF fluorescence was observed following 4-h Cu-PHQ exposures in *D. magna*. A more detailed discussion of PHQ redox-cycling may offer some explanation for this discrepancy. There are reactants and products in the redox-cycling of PHQ, semi-PHQ, and dihydroxyphenanthrene (dhPHQ) that lead to the formation of ROS (Figure S2). PHQ is converted to semi-PHQ with the aid of several putative NAD(P)H oxidoreductases and/or UV-A radiation.[16,18,66] Semi-PHQ can be oxidized by O_2, restoring

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PHQ while producing $O_2^{\bullet-}$. PHQ can also be directly reduced by the NAD(P)H:quinone oxidoreductase, DT-diaphorase (DTD), to produce dhPHQ without the semi-quinone intermediate.[16] Regardless, dhPHQ can still be oxidized by $O_2^{\bullet-}$ to form semi-quinone and H_2O_2 that in turn oxidizes DCF to its fluorescent form. Incidentally, Cu^+ can reduce semi-PHQ to dhPHQ and facilitate the production of H_2O_2. Moreover, Cu^+ can reduce $O_2^{\bullet-}$ to produce H_2O_2. Thus, the production of H_2O_2 is likely potentiated in animals co-exposed to Cu and PHQ.[20−22]

The degradation of PHQ to dhPHQ by DTD may represent a rate-limiting step that Cu relieves, thus explaining why there was no increase in DCF fluorescence following 4-h exposures of *D. magna* to PHQ alone, but DCF fluorescence did increase following exposure to Cu-PHQ mixtures. Unfortunately, a search of the literature revealed only one *in vivo* study investigating the time dependent activity of DTD in aquatic crustaceans, where the activity of DTD following exposure to Pb in *Gammarus pulex* did not increase until after 10 h of exposure.[67] There have been *in vitro* fish studies indicating a rapid increase in DTD activity following exposure to a variety of PAH-quinones (PAHQs),[68,69] which supports a rapid DTD-dependent PHQ-induced increase in DCF fluorescence in *V. fischeri*. It is assumed that DTD activity in aquatic invertebrates (e.g., *D. magna* and *H. azteca*) will similarly increase in response to PHQ exposure. However, given the differences in *in vivo* and *in vitro* bioaccumulation and distribution kinetics of PHQ, it is expected that DTD activity would take longer to increase following exposure. To account for the difference between the present findings and the findings in Xie et al.,[20] it is possible the cumulative activity of DTD over increasing exposure durations would diminish the effect of Cu in this scenario. A thorough time-series analysis of PHQ-induced H_2O_2 production and DTD activity is necessary to confirm this hypothesis.

One final point is that differential expression and activity of other oxidoreductases (e.g., NADH:ubiquinone oxidoreductase) and antioxidant enzymes (e.g., superoxide dismutase) involved in the cycling of PHQ may differ among species and influence rate of PHQ-induced ROS production. Although redox-cycling and PAH-induced ROS production seems largely a chemical process not to differ among species, there are a few biological considerations to made. Along these lines, it is possible that the H_2DCFDA-oxidizing ROS differ in terms of their Cu- and/or PAH-induced production among species as a function of the induction and activity of various antioxidant enzymes, producing dissimilar fluorescence responses.Interactive effects of ROS production did not account for more-than-additive co-toxicity in *H. azteca* exposed to a Cu-PHQ mixture. Moreover, the Ni-PHQ mixture produced strictly additive mortality and ROS production. Because Ni is a redox-active metal like Cu, and ROS production in Cu-PHQ mixtures was strictly additive in the present study, we suggest that a non-ROS related mechanism is responsible for the more-than-additive mortality of juvenile *H. azteca* co-exposed to Cu and PHQ.

In summary, PHQ increased the accumulation of Cu in juvenile *H. azteca*, in part explaining the more-than-additive mortality observed in *H. azteca* co-exposed to Cu and PHQ. However, PHE did not alter Cu accumulation, identifying that neither ROS-dependent mechanisms nor metal accumulation were involved in the more-than-additive mortality observed in *H. azteca* exposed to Cu and PHE for 18 and 48 h, respectively. Similarly, Cd accumulation in *H. azteca* was not influenced by PHE or PHQ, and thus cannot account for the more-than-additive mortality observed in 48-h co-exposures. Further work is required to identify the co-toxic mechanisms responsible for more-than-additive mortality in *H. azteca* exposed to Cd- or Cu-PAH mixtures.

This work contributes to an emerging area of research addressing the ecological risk associated with mixtures of metals and PAHs in aquatic environments. A better understanding of toxic and co-toxic mechanisms will aid in predictions of ecological risk associated with mixtures of metals and PAHs. Based on the findings outlined in this manuscript, and the proposed co-toxic mechanisms we have presented elsewhere,[2] we recommend further investigation into the effects of PAHs (i.e., PHQ) on metal (i.e., Cu) accumulation, and the interactive metal- and PAH-induced effects on the detoxification of metals and PAHs when present together.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03233.

Figure S1, Cd LBC50 curves; Figure S2, a schematic of metal−PAH redox cycling; and Table S1, a list of water concentrations of metals and PAHs for metal accumulation assays (PDF)

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Notes

The authors declare no competing financial interest.

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