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Metal−Polycyclic Aromatic Hydrocarbon Mixture Toxicity in *Hyalella azteca*. 1. Response Surfaces and Isoboles To Measure Non-additive Mixture Toxicity and Ecological Risk

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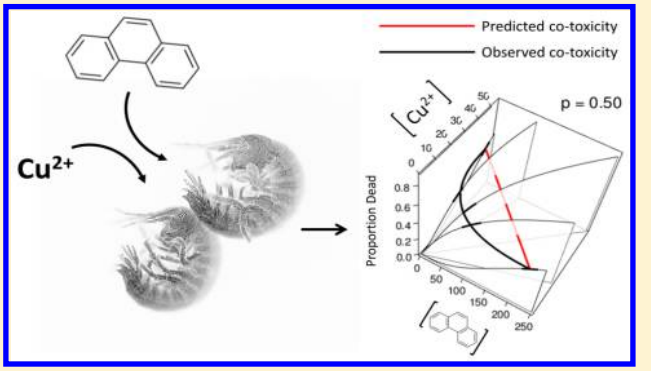
Supporting Information

ABSTRACT: Mixtures of metals and polycyclic aromatic hydrocarbons (PAHs) occur ubiquitously in aquatic environments, yet relatively little is known regarding their potential to produce non-additive toxicity (i.e., antagonism or potentiation). A review of the lethality of metal−PAH mixtures in aquatic biota revealed that more-than-additive lethality is as common as strictly additive effects. Approaches to ecological risk assessment do not consider non-additive toxicity of metal−PAH mixtures. Forty-eight-hour water-only binary mixture toxicity experiments were conducted to determine the additive toxic nature of mixtures of Cu, Cd, V, or Ni with phenanthrene (PHE) or phenanthrenequinone (PHQ) using the aquatic amphipod *Hyalella azteca*. In cases where more-than-additive toxicity was observed, we analyzed Canada's environmental water quality guidelines to see if they would be protective. We used a three-dimensional response surface isobole model-based approach to compare the observed co-toxicity in juvenile amphipods to predicted outcomes based on concentration addition or effects addition mixtures models. More-than-additive lethality was observed for all Cu-PHE, Cu-PHQ, and several Cd-PHE, Cd-PHQ, and Ni-PHE mixtures. Our analysis predicts Cu-PHE, Cu-PHQ, Cd-PHE, and Cd-PHQ mixtures at the Canadian Water Quality Guidelines for the Protection of Aquatic Life concentrations would produce 7.5%, 3.7%, 4.4%, and 1.4% mortality, respectively, suggesting these guideline values may be under-protective.



INTRODUCTION

The co-contamination of aquatic environments by polycyclic aromatic hydrocarbons (PAHs) and metals is an underexplored area of ecological risk assessment.[1] Severe co-contamination of metals and PAHs occurs in a variety of coastal and freshwater environments around the globe.[2−8] The threat these individual contaminants pose to aquatic organisms has been studied extensively in a variety of model aquatic organisms. For example, the aquatic crustacean amphipod, *Hyalella azteca*, is commonly incorporated into toxicity bioassays due to its tractability in laboratory and field settings, relatively high reproductive rate, and its widespread distribution and importance as an invertebrate herbivore prey species to a variety of other aquatic organisms (e.g., fish, amphibians, and waterfowl). However, comparatively little effort has been placed in understanding interactive effects of joint contamination. A recent review of metal−PAH mixture toxicity revealed that more-than-additive and strictly additive lethality were equally common.[1] However, regulatory authorities typically avoid considering the additivity of contaminants altogether, opting for whole effluent testing on a case-by-case basis (e.g., Canada, USA, Australia, and New Zealand) or when additivity is considered to operate under the assumption of strictly additive toxicity (e.g., Australia and New Zealand).[9]

Unfortunately, the present state of knowledge regarding whole-organism (e.g., mortality and growth) and cellular (e.g., protein activity and gene transcription) end points is incapable of informing regulatory authorities of appropriate water quality objectives for the protection of aquatic life from metal−PAH mixtures. There are several obstacles in overcoming this problem. First, researchers must employ experimental and statistical designs capable of discriminating non-additive toxicity from strictly additive toxicity. Second, the application of these designs must be able to provide environmentally relevant information (i.e., relevant exposure concentrations or effect levels). Third, experimental designs must encompass an array of mixture concentration scenarios to represent a variety of

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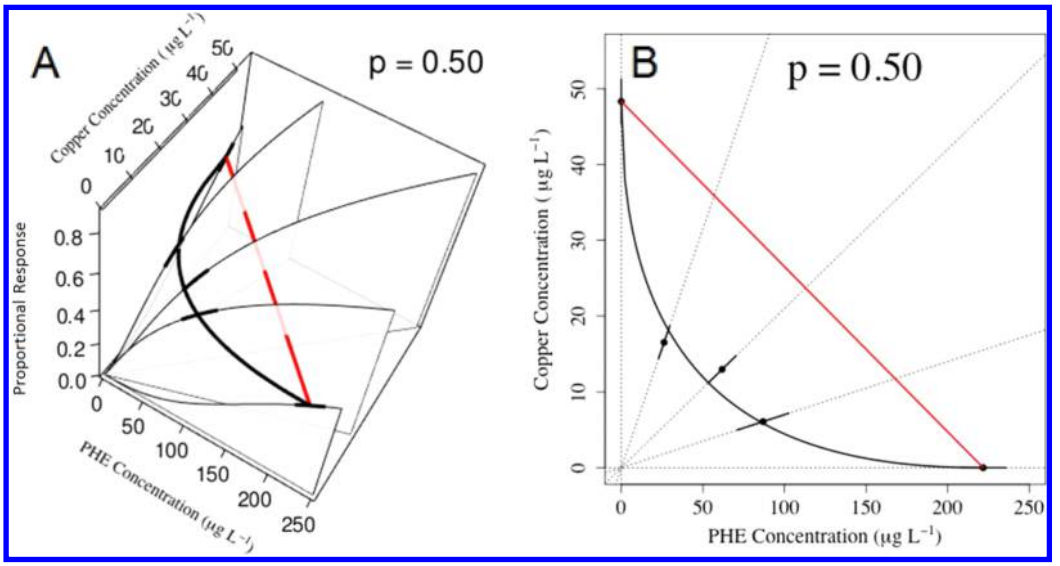

Figure 1. Three-dimensional response surface (panel A) and two-dimentional isobologram containing five mixture proportions (vertical black curves in panel A and dotted lines in panel B). The response surface *x*-, *y*-, and *z*-axes represent the concentrations of phenanthrene (PHE) and Cu and the proportion (p) of *H. azteca* succumbing to joint exposure, respectively. Panel B illustrates the same data without the *z*-axis (i.e., a cross section of panel A at $p = 0.50$). The closed black circles in panel B are the estimated LC50s with standard errors for each mixture proportion. The black curves in each plot represent the observed isobole. The red curves represent the predicted isobole assuming strictly additive co-toxicity.

environmental scenarios. Lastly, experimental designs must be robust enough to encompass biological and temporal variation. These obstacles greatly increase the complexity and difficulty to conduct experiments and to predict the ecological risk of metal−PAH mixtures.

One approach that satisfies these criteria is to model toxicity data obtained from multiple fixed-mixture-proportion concentration−response curves (i.e., isobole analysis), providing predicted toxicity across all possible mixture scenarios.[10] This approach can also be used to assess ecological risk by conducting the analyses at lower effect levels occurring at ecologically relevant concentrations. Accordingly, we present an isobole-based ecological risk assessment of binary mixtures of metals (i.e., Cd, Cu, Ni, and V) and two PAHs, phenanthrene (PHE) and phenanthrenequinone (PHQ), to *H. azteca*.

THEORY

LC50 Estimation. Concentration−response relationships for lethality experiments can be modeled according to proportional responses using the two-parameter logistic model:

$$p_i = 1/[1 + (c_i/\mathrm{LC50}_i)^{r_i}] \quad (1)$$

where p_i is the proportion of animals dying in response to the ith toxicant, c_i is the concentration of the ith toxicant, and r_i is the rate of increase in toxicity at the LC50 of the ith toxicant (i.e., the derivative of the LC50). Logistic models are aptly applied to mortality data as mortality typically follows a sigmoidal relationship with concentration.[11]

Response Surface Construction and Isobole Modeling. An isobologram is produced from a three-dimensional concentration−response surface with the concentration of each contaminant on the *x*- and *y*-axes, and proportional response on the *z*-axis (Figure 1). The isobologram is a cross section of the response surface at the desired effect level. The response surface is constructed by carrying out traditional EC50, or other effects levels, experiments for the individual contaminants and binary mixtures of the two contaminants.[12] The mixture concentrations are established with fixed proportions based on individual contaminant concentrations or toxic units. The EC50s for the individual contaminants and fixed-mixture proportions of the contaminants can be estimated using eq 1, or by other LC50 estimation techniques. These EC50 data are then modeled further to produce an isobole. An isobole is literally a curve of equieffective response modeled to mixture EC50 data and predicts the mixture concentration required to elicit a 50% response. Isoboles can be plotted on the isobologram and are particularly useful in terms of visualizing mixture toxicity across a wide range of mixture proportions (Figure 1). A Hewlett model, and less commonly, Vølund model, can be applied to produce an isobole using the isobole function of the "drc" package in R.[13−16] For a detailed description of the theory and practice of Hewlett and Vølund models, see ref 14.

Perhaps the most beneficial component of isobolographic analyses is their ability to clearly present experimental data and compare them to predicted mixture toxicity data obtained from concentration addition (CA) and/or effects addition (EA) reference models (see Supporting Information). The predictions from either reference model can be calculated for each fixed mixture proportion experiment by carrying out reference toxicity tests for both contaminants alongside mixture experiments. Reference data can be used to calculate CA and EA isoboles instead of relying on purely theoretical CA and EA isoboles (e.g., straight diagonal line on the isobologram between EC50 of the two contaminants present on their own for CA). Predictions based on CA can be obtained using the following equation:[17]

$$c_m = c_1 + ec_2 \quad (2)$$

where e is the proportional exchange rate between toxicants 1 and 2, calculated as $e = \mathrm{EC50}_1/\mathrm{EC50}_2$, c_m is the summed concentration of the mixture, represented as relative units of toxicant 1, and c_1 and c_2 are the concentrations of toxicants 1 and 2 in the mixture, respectively. Predictions based on EA can be obtained using the following equation, modified from ref 12:

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$$p_m = p_1 + p_2(1 - p_1) \quad (3)$$

where p_m is the predicted proportional response of the mixture, and p_1 and p_2 are the individual proportional responses induced by toxicants 1 and 2, respectively. It is necessary to produce estimates from eqs 2 and 3 for the desired response level (i.e., 50%) according to the specific fixed-mixture proportions. An iterative approach using eq 1 individually for each toxicant can be used to obtain the appropriate values for c_1, c_2, p_1, and p_2. Finally, the Hewlett model can be applied to CA and EA estimates to produce an isobole of the predicted mixture toxicity with the following equation modified from the isobole function from the "drc" package in R:[15,16]

$$c_2 = [EC50_2^{1/\lambda} - (c_1 EC50_2/EC50_1)^{1/\lambda}]^{\lambda} \quad (4)$$

where λ describes the curvature of the isobole which can be estimated using non-linear least-squares or maximum likelihood estimation. An observed λ (i.e., estimated from modeling experimental data) greater than the predicted CA or EA λ indicates more-than-additive toxicity, where the overlap of standard errors (se) of the λ estimates distinguishes between observed and predicted toxicity. Moreover, plotting isoboles with $\lambda \pm$ se produces uncertainty intervals along the entire isoboles for comparisons at various mixture proportions.

Applying Isoboles to Ecological Risk Assessment. Isoboles are typically used to illustrate mixture effects at $p = 0.5$. However, $p = 0.5$ may not provide ecologically relevant concentrations to compare to the environment and regulatory guidelines. This is especially true for acute mortality toxicity tests, as the concentrations applied to induce $p = 0.5$ are typically far greater than concentrations present in the environment. Fortunately, the response surface approach allows for the analysis of mixture toxicity at any p, where isoboles can be used to predict the effect associated with a mixture of two contaminants present at their guideline concentrations. This is accomplished by fitting isoboles to various values of p, using eqs 1−4 in the same iterative fashion as described above, until the maximum value of p induced from the guideline concentrations is found. If several mixture proportions are applied, the specific mixture proportion of the guideline concentrations does not matter, as the isobole model will provide LC_p estimates ± se across all mixture proportions for any given value of p. Lower and upper estimates of p induced by guideline concentrations can be calculated from the se of the LC_p estimate again using eqs 1−4. Finally, isobolograms can be used to identify the regulatory guideline values of each contaminant in comparison to the isobole predictions (e.g., by plotting a polygon based on the two guideline concentrations). If the experimentally derived isobole and/or its standard errors overlap the guideline "safe concentration" polygon, the guidelines may not be protective. Further experimental validation of isobole predictions would provide strong evidence that guideline concentrations are not protective when mixed.

MATERIALS AND METHODS

Amphipod Culturing. *Hyalella azteca* were obtained from the Aquatic Contaminants Research Division, Environment Canada (Burlington, ON) and cultured according to Borgmann.[18] Briefly, cultures were incubated in 2 L polyethylene containers held in water baths heated to 25 °C and illuminated by two 40 W full spectrum fluorescence light bulbs with a 16-h light:8-h dark photoperiod. All amphipods were cultured and tested in standard artificial media (SAM) containing 147 mg L^{-1} $CaCl_2$, 84 mg L^{-1} $NaHCO_3$, 1 mg L^{-1} NaBr, 3.7 mg L^{-1} KCL, and 62 mg L^{-1} $MgSO_4$ prepared in deionized water (DW; Millipore, ON, Canada).[18] Each 2 L culture containing 20−30 amphipods was fed 5 mg of finely ground Tetramin fish flake three times per week.

Materials and Supplies. Stock solutions of $CdSO_4$, $CuSO_4$, Na_3O_4V, and $NiSO_4$ were prepared from metal salts (purity >99%; Sigma, St. Louis, MO, USA) made with DW and preserved with 1% trace metal grade HNO_3 (Fisher Scientific, ON, Canada). The SO_4 counterion was evaluated in Na_2SO_4 reference tests. No lethal effects were observed at SO_4 concentrations representative of those found in metal exposures (data not shown). The pH in metal exposures was controlled with aliquots of 1 M NaOH solution made with DW. Non-metal labware was washed in a 3% solution of HCl (purity >36.5%, Anachemia, Mississauga, ON, Canada). Metal labware was washed in 2% solution of FLO-70 detergent (Fisher Scientific). All labware was rinsed seven times with DW and oven-dried at 40 °C.

Polydimethylsiloxane (PDMS) GE silicone II (General Electric, NC, USA) was purchased from a local hardware store. All PDMS was dissolved in hexanes (purity >98.5%, Anachemia, ON, Canada). Stock calibration and film solutions of PHE and PHQ (purity >98%: Sigma) were prepared using 100% HPLC grade acetonitrile or acetone (Fisher Scientific) respectively, and refrigerated in the dark. Glassware containing PDMS films were first cleaned with Dynasolve 230 silicone digestant (Ellsworth, ON, Canada), and then acid washed as described above. All tests were carried out in 400 mL glass beakers (Fisher Scientific) covered with Parafilm sealing film (Fisher Scientific).

Polydimethylsiloxane Film Preparation. Test concentrations of PAHs were controlled using a partition-controlled delivery system using PAH-enriched PDMS films. Enriched PDMS films were prepared by modifying the procedures described by Brown et al.[19] and Kiparissis et al.[20] to incorporate larger test volumes. Calibration values of PAH enriched films were based on Turcotte et al.[21] A series of films were produced by mixing aliquots of PAH stock solutions into a 6 mg mL^{-1} PDMS:hexanes solution. A 9 mL aliquot of the PDMS solution was deposited into each beaker followed by the respective volume of PAH stock solution. The contents of each beaker were then gently mixed using compressed air directed with vinyl tubing. The hexanes and acetonitrile from the stock solutions were allowed to evaporate, after which the enriched PDMS films were allowed to cure for 2 h. After curing, 300 mL of test water was added to each beaker. Beakers were then placed on an orbital shaker at 50 rpm for 24 h to establish equilibrium between the PAH concentrations within the PDMS film and test water.

Enriched PDMS films were calibrated according to the nominal initial concentration of PAH within each film and the measured aqueous PAH concentration at equilibrium. A linear regression was used to determine the film:water PAH concentration ratio (see Supporting Information). An ANOVA failed to detect any significant differences between aqueous PAH concentrations at the beginning and end of tests (data not shown).

Toxicity Tests. A concentration−response surface experimental design was applied to incorporate an isobole-based statistical analysis (see Theory section). All binary combinations of Cu, Cd, V, and Ni with either PHE or PHQ were tested, totalling eight response surfaces. Standard concentration

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series were applied for the estimation of LC50s with eq 1 for unary and binary exposures. Concentrations for unary exposures were based on data gathered from range finder assays, and were uniformly distributed between 0% and 100% lethal concentrations typically with two concentrations below and above the LC50s estimated in the range finder assays. No fewer than five metal or PAH concentrations per assay were used. Mixture proportions (p_{r_i}) can be expressed as the proportion of PAH in mixture. Thus, each isobole contained 0, 0.25, 0.5, 0.75, and 1 proportional unit of PAH, with $p_{r_i} = 0$ and 1 representing the unary exposures for each metal and PAH, respectively. Metal (C_{M_i}) and PAH (C_{PAH_i}) mixture concentration series for the ith mixture proportion were based on the unary PAH concentration series (U_{PAH}) and e, and were calculated as follows:

$$C_{PAH_i} = U_{PAH_i} p_{r_i} \quad (5)$$

$$C_{M_i} = U_{PAH}(1 - p_{r_i})e \quad (6)$$

Following measurements for metal and PAH concentrations, p_{r_i} were recalculated based on measured concentrations (see Supporting Information).

All toxicity assays were 48-h non-renewal tests and were conducted at 21 °C with a 16-h light:8-h dark cycle. Young amphipods were acclimated to 21 °C for 24 h prior to testing. Tests contained no food to eliminate confounding interactions with analytes. Renewal of test water was not necessary given the short exposure length and the use of enriched PDMS films. A total of 10−20 amphipods, 2−10 days old, were randomly allocated to 300 mL of test water. Test water containing PAHs was prepared as per the PDMS film preparation section above. Metal test water for metal-only and metal−PAH exposures was prepared with the addition of aliquots of metal stock solutions into 2 L of SAM, which was allowed to equilibrate for 24 h, and then divided into the metal-only metal−PAH treatment beakers (i.e., 300 mL per beaker with three replicates per treatment). The remaining 200 mL of metal-loaded water was used for quantitative metal analyses (see below). Control water was prepared in the same fashion except no metals aliquots were added and PAHs were not added to PDMS films. Each treatment concentration was replicated three times. Mortality was the end point and was determined at the end of each test as immobility with no pleopod movement.

For every p_{r_i} tested (i.e., five per response surface), identical metal and PAH concentration series (i.e., C_{PAH_i} and C_{M_i}) were used to determine amphipod mortality to the individual contaminants. These served as reference toxicity tests for each mixture experiment and were used to calculate CA and EA predictions based on toxicity data from the same animal cohort. In cases where weekly reference tests indicated a shift in the sensitivity the metal−PAH pair being tested, measured as non-overlapping se of the LC50 estimate, the p_{r_i} experiment was either repeated, or unary reference test data were used to derive a multiplication factor (eqs S1 and S3 in Supporting Information) to correct the mixture LC50 (see Supporting Information). A two-parameter log−logistic model (eq 1) was used for all LC50 estimations. The Hewlett model (eq 4) was used for all isoboles except the V-PHQ isobole, as it exhibited substantial antagonism at one p_{r_i}. Thus, the Vølund model was used to model V-PHQ mixture toxicity (see Theory section). The CA model was used to produce estimated mixture toxicity for Cu-PHE, Cd-PHE, and Ni-PHE mixtures (see Supporting Information for rationale). The EA model was used to estimate mixture toxicity for mixtures involving V and PHQ. The Hewlett model, as adapted from the isobole function in the "drc" package in R,[15] was used to produce isoboles from CA and EA predictions. All statistical analyses were carried out in R 3.03.[16] An $\alpha = 0.05$ was applied for all tests of significance.

Analytical Procedures. Water hardness, alkalinity, temperature, pH, and metal concentrations were analyzed in all control and two randomly assigned treatments at the beginning and end of each test for every p_{r_i} tested (i.e., five p_{r_i} per isobole), representing a 50% sampling effort. Differences between measured and nominal metal concentrations were consistent within and between isobole experiments, and were used to correct nominal values for unmeasured treatments by multiplying nominal concentrations by the proportion of measured metal or PAH. Temperature and pH were measured with an Accumet Basic pH meter (Fisher Scientific). Hardness and metal samples were filtered through a 0.45 μm filter and preserved in 1% HNO_3 prior to analysis by ICP-MS (Perkin Elmer). Hardness and alkalinity were calculated from measured Ca^{2+} and Mg^{2+}, and titration with 0.1 N H_2SO_4 to a pH of 4.5, respectively, and reported as mg $CaCO_3$ equivalents according to Clesceri et al.[22,23]

All PAHs were measured by HPLC (1200 Series, Agilent Technologies, ON, Canada) with a SDB-C18 5 μm 4.6 × 150 mm column and a dual channel UV detector. The mobile phase was 60:40 acetonitrile:test water initially and moved to 100% acetonitrile at 10 min with a total run time of 10 min and flow rate of 1 mL min^{-1}. Best results were obtained with a 254 nm wavelength and an injection volume of 50 μL. Temperature was held constant at 25 °C. For toxicity assays, water samples were collected from the control and three randomly assigned treatments. Just as for metal samples, a correction factor applied to correct nominal values for unmeasured treatments. All samples were analyzed within 3 h of sampling. Concentrations were determined with a calibration curve obtained from 10 to 1000 $\mu g\ L^{-1}$ standard solutions (60:40 acetonitrile:test water) for PHE and PHQ.

RESULTS

Mean control mortality from all response surface experiments was 2.7%. All log−logistic concentration−response model parameter estimates were satisfactory with appropriate uncertainty for the purpose of this analysis (Table S3). All Hewlett isoboles were well fit (Table S4). Estimation of η_1 for the V-PHQ Vølund model was not satisfactory (Table S4), and thus comparisons of observed versus predicted toxicity for V-PHQ mixtures was carried out based on LC50s from each p_{r_i}. For each isobole and p_{r_i} tested, Cu-PHE ($\lambda = 2.12 \pm 0.11$) and Cu-PHQ ($\lambda = 3.16 \pm 0.025$) mortality was more-than-additive compared to CA or EA isoboles ($\lambda_{Cu\text{-}PHE} = 1.03 \pm 0.01$; $\lambda_{Cu\text{-}PHQ} = 0.58 \pm 0.07$; Figure 2A,B). Similarly, Cd-PHE ($\lambda = 1.57 \pm 0.13$) and Cd-PHQ ($\lambda = 1.51 \pm 0.32$) isoboles predicted more-than-additive mortality compared to CA or EA isoboles ($\lambda_{Cd\text{-}PHE} = 0.99 \pm 0.01$; $\lambda_{Cd\text{-}PHQ} = 0.94 \pm 0.03$; Figure 2C,D). However, unlike the Cu-PAH mixtures, $p_{r0.36}$ and $p_{r0.59}$ for Cd-PHE and Cd-PHQ, respectively, produced strictly additive mortality. The effect of p_{r_i} was also evident in mixtures involving Ni, where $p_{r0.23}$ produced strictly additive mortality,

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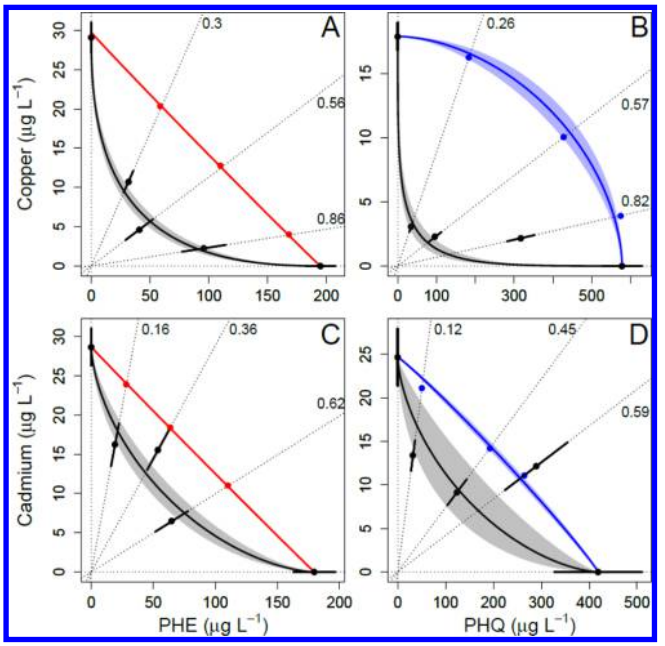

Figure 2. Isobolograms for mixtures of Cu (panels A and B) and Cd (panels C and D) with phenanthrene (PHE) or phenanthrenequinone (PHQ). Closed black circles represent LC50 ± se estimates, whereas red and blue closed circles represent LC50 predictions by concentration addition (CA) and effects addition (EA) respectively. Black curves and gray shaded regions, red curves and red shaded regions, and blue curves and blue shaded regions represent experimental, CA, and EA, respectively, isobole predictions ± standard errors of λ. Dotted lines represent concentration−response curves at each mixture proportion (p_{r_i}), which are indicated numerically next to each curve.

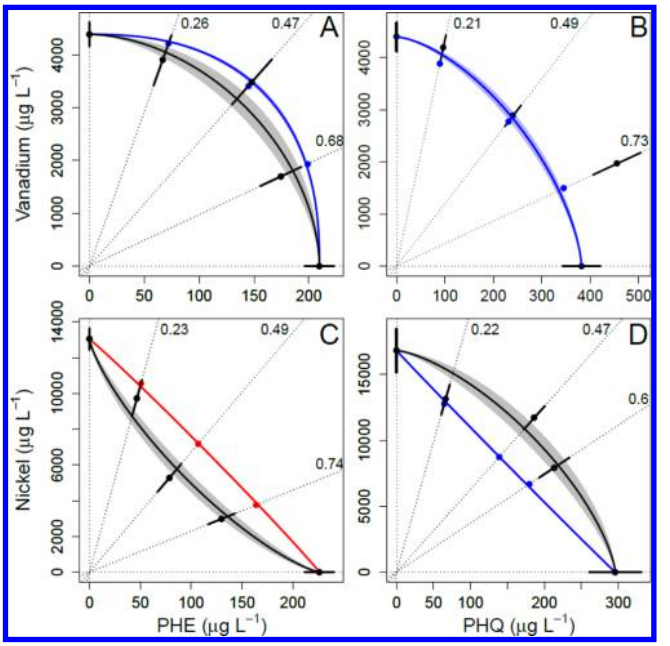

Figure 3. Isobolograms for mixtures of V (panels A,B) and Ni (panels C,D) with phenanthrene (PHE) or phenanthrenequinone (PHQ). Closed black, red, and blue circles are as described in Figure 2. Black curves and gray shaded regions, red curves and red shaded regions, and blue curves and blue shaded regions are as described in Figure 2. Dotted lines are as described in Figure 2. Neither Hewlett nor Vølund isobole models could be fit to experimental V-PHQ data. Thus, additivity for V-PHQ mixtures can only be assessed based on the p_{r_i} tested.

while $p_{r0.49}$ and $p_{r0.74}$ produced more-than-additive mortality (Figure 3C). Nonetheless, the Ni-PHE (λ = 1.28 ± 0.07) isobole predicted slightly more-than-additive co-toxicity across all p_{r_i} values compared to the CA isobole (λ = 0.96 ± 0.01). The Ni-PHQ (λ = 0.66 ± 0.07) isobole predicted less-than-additive co-toxicity compared to the EA isobole (λ = 1.01 ± 0.01), although $p_{r0.22}$ produced strictly additive mortality (Figure 3D). Although observed (λ = 0.57 ± 0.06) and EA (λ = 0.41 ± 0.02) V-PHE isoboles indicated subtle more-than-additive toxicity, strictly additive mortality was observed at $p_{r0.26}$ and $p_{r0.47}$ (Figure 3A). In V-PHQ mixtures, $p_{r0.73}$ produced less-than-additive mortality, whereas strictly additive mortality was observed at $p_{r0.21}$ and $p_{r0.49}$ (Figure 3B).

DISCUSSION

Non-additive Co-toxicity. To date, there have been 73 published cases that have measured non-additive mortality in aquatic biota exposed to metal−PAH mixtures (Table 1). Of these 73 cases, 42.5%, 43.8%, and 13.7% produced more-than-additive, strictly additive, and less-than-additive mortality, respectively. However, there are conflicts in terms of the outcomes for particular mixtures (Table 1). Nonetheless, more-than-additive mixture mortality is common from exposure to binary mixtures of metals and PAHs. As these contaminants occur together in a variety of aquatic systems,[2−8] the potential for enhanced toxicity should be considered in terms of assessing regulatory guidelines for the protection of aquatic ecosystem health.

The discrepancies in additivity of metal−PAH lethality are likely, at least in part, attributed to the use of dissimilar test animals and/or populations, test media, and exposure duration and route (i.e., waterborne, sediment, or dietary). For example, the hardness of the media used by Xie et al.[30,31] in their Cu-PHE and Ni-PHQ mixture experiments on *D. magna* was roughly twice as high as the hardness in the present study. Moreover, Wang et al.[29] reported that 0.5-, 0.75-, and 1-h exposures resulted in notable differences in the additivity of Cd-PHQ mixtures in *Vibro fischeri* when tested at the same mixture ratio. Just as the degree of toxicity of individual contaminants is expected to vary among species/cultures (e.g., through biological tolerance), test media (i.e., through toxicity modifying factors such as hardness), and exposure duration (e.g., through toxicokinetics), it is reasonable to expect the degree of additivity in mixtures to vary among species, test media and exposure duration as well.

Experimental design and statistical analyses represent additional considerations that may account for the differences in findings. The majority of relevant published cases search for non-additive effects by holding one of the contaminants at a no observable effect concentration (NOEC). The NOEC approach is typically applied to simplify the prediction of the mixture toxicity according to eqs 2 and 3. Unfortunately, the NOEC approach does not account for the likely scenario that threshold concentrations of either contaminant are required to drive mixture toxicity away from being strictly additive.[1] It is also likely that there are sites of action involved in producing more-than-additive toxicity that become saturated when either contaminant is present at a certain concentration and/or p_{r_i}. For example, in our analysis of Cu-PHQ mixtures, we found

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Table 1. Summary of Cases Identifying Non-additive Mortality in Aquatic Biota[a]

metal	PAH	species	exposure	duration (h)	+ / = / −	ref
Cd	phenanthrene	*S. knabeni*	sediment	96	+	24
Cd	phenanthrene	*S. knabeni*	aqueous	96	+	24
Cd	phenanthrene	*A. atopus*	aqueous	96	+	24
Cd	phenanthrene	*H. templetoni*	sediment	240	− − −	25
Cd	phenanthrene	*H. azteca*	sediment	240	+	26
Cd	phenanthrene	*H. azteca*	aqueous	24; 48; 72	= ; −; =	26
Cd	phenanthrene	*H. azteca*	aqueous	192	=	27
Cd	phenanthrene	*H. azteca*	aqueous	18	=	28
Cd	phenanthrene	*H. azteca*	aqueous	48	+ + =	
Cd	fluoranthene	*A. atopus*	aqueous	96	+	24
Cd	phenanthrenequinone	*V. fischeri*	aqueous	0.5; 0.75; 1	+ + =; + = =; = = =	29
Cd	phenanthrenequinone	*D. magna*	aqueous	48	=	30
Cd	phenanthrenequinone	*H. azteca*	aqueous	18	=	28
Cd	phenanthrenequinone	*H. azteca*	aqueous	48	+ + =	
Cu	phenanthrene	*D. magna*	aqueous	48	=	31
Cu	phenanthrene	*H. azteca*	aqueous	18	+	28
Cu	phenanthrene	*H. azteca*	aqueous	48	+ + +	
Cu	phenanthrenequinone	*D. magna*	aqueous	48	+ +	31
Cu	phenanthrenequinone	*V. fischeri*	aqueous	0.5; 0.75; 1	+ + = =; + + = −; = = = −	29
Cu	phenanthrenequinone	*H. azteca*	aqueous	18	+	28
Cu	phenanthrenequinone	*H. azteca*	aqueous	48	+ + +	
Ni	phenanthrene	*H. azteca*	aqueous	18	=	28
Ni	phenanthrene	*H. azteca*	aqueous	48	+ + =	
Ni	phenanthrenequinone	*D. magna*	aqueous	48	+	30
Ni	phenanthrenequinone	*H. azteca*	aqueous	18	=	28
Ni	phenanthrenequinone	*H. azteca*	aqueous	48	= − −	
V	phenanthrene	*H. azteca*	aqueous	18	=	27
V	phenanthrene	*H. azteca*	aqueous	48	= = =	
V	phenanthrenequinone	*H. azteca*	aqueous	18	=	28
V	phenanthrenequinone	*H. azteca*	aqueous	48	= = −	
Zn	phenanthrene	*C. variegatus*	aqueous	96	−	32
nano-Zn	phenanthrene	*D. magna*	aqueous	24; 48	+; +	33

[a]Adapted from Gauthier et al. 2014.[1] More-than-additive, less-than-additive, and strictly additive mortality is indicated by +, −, and =, respectively. Tallied outcomes are not replicated by concentration (e.g., three more-than-additive and two strictly additive outcomes within a single concentration response curve are tallied as + −). For publications including multiple concentration response curves, multiple outcomes from each curve are reported with multiple symbols (e.g., two more-than-additive outcomes observed from two independent mixture concentration response curves are tallied as + +). For cases where multiple exposure durations were reported in the same publication, outcomes were separated as per the duration column.

that a small addition of either contaminant increased the toxicity of the mixture substantially, but additional relative increases of either contaminant by manipulating p_{r_i} did not noticeably alter the LC50 values (Figure 2), suggesting a saturable site of action is potentially involved in the short term, more-than-additive co-lethality of Cu-PHQ mixtures in *H. azteca*. As such, findings based on a mixture where one contaminant concentration is held static can only be interpreted for that concentration, whereas response surfaces modeled using the Hewlett or Vølund models, or experimentally derived mechanistic models,[34] can provide predictions across a wide range of p_{r_i}.

Given the above-mentioned cross-experimental considerations and the scarcity of studies investigating non-additive metal−PAH mixture toxicity, it is not surprising that contradictory results have been published. Further research is required to determine the effects of water quality parameters, exposure duration, and mixing proportions (i.e., threshold and saturation concentrations) on mixture additivity while considering proposed mechanisms of non-additive metal−PAH toxicity.[1]

Environmental Risk of More-than-Additive Mixtures. More-than-additive metal−PAH mixture toxicity is a common and increasingly well-described phenomenon[1] that must be addressed in terms of ecological risk assessment and regulatory water quality guidelines.[35] Based on the water quality criteria for SAM, with a water temperature of 20−22 °C, a pH of 7.8−8.1, hardness of 121−126 mg L^{-1} as $CaCO_3$ equiv, alkalinity of 49−52 mequiv L^{-1}, and DOC < 1 mg L^{-1}, the short-term Canadian Council of Ministers of the Environment (CCME) guidelines for protection of aquatic life for Cu, Cd, V, Ni, and PHE are 2.8, 1.0, 109.8, 100, and 0.4 μg L^{-1}, respectively.[36−40] There is no short-term CCME guideline for PHQ. For the purpose of this analysis, the PHE CCME guideline provides the closes approximation of a guideline for PHQ and will also be applied to PHQ. The guidelines for Cu, Cd, V, and Ni were derived using acute LC50 values at the fifth percentile of a species sensitivity distribution curve.[41] The guidelines value for PHE was determined using the lowest observable effect level (LOEL) multiplied by a protection factor of 0.1.[40] These guideline values are intended to represent safe concentrations for aquatic biota, and thus, should exert no adverse toxic effects.[41]

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The response surface isobole-based approach (see Theory section) allowed for the prediction that mixtures of the guideline concentrations would induce 7.5% (3.5−14.6%), 3.7% (2.4−8.1%), 4.4% (4.0−7.4%), and 1.4% (1.3−1.5%) mortality above control in Cu-PHE, Cu-PHQ, Cd-PHE, and Cd-PHQ mixtures, respectively (Figure 4; Table S4). Mixtures of the

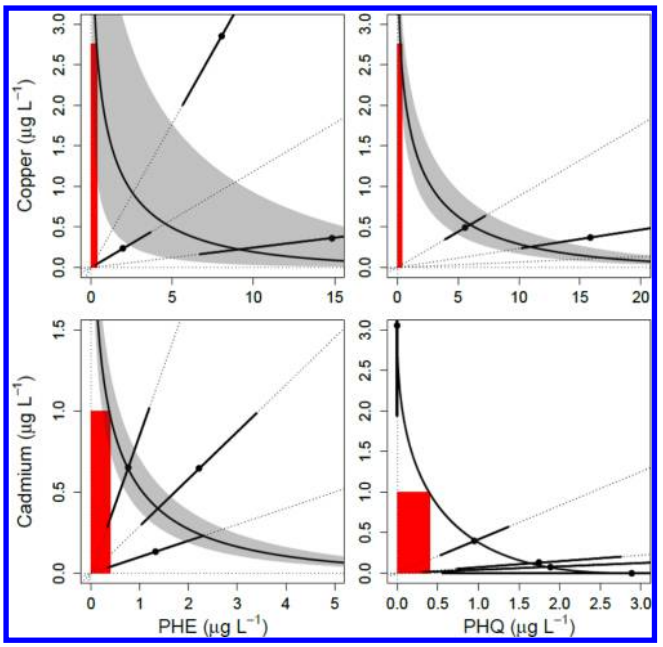

Figure 4. Isobole-based analysis of the protectiveness of the Canadian Council of Ministers of the Environment (CCME) short-term water quality guidelines of the protection of aquatic life from mixtures of Cu or Cd with phenanthrene (PHE) or phenanthrenequinone (PHQ). Closed black circles represent LC07.5, LC03.7, LC04.4, and LC01.4 ± se estimates for Cu-PHE, Cu-PHQ, Cd-PHE, and Cd-PHQ mixtures, respectively. The red polygons indicate the respective CCME guideline concentrations of Cu, Cd, PHE, or PHQ at which no adverse toxicological effects should occur. For Cu-PHE, Cu-PHQ, and Cd-PHE mixtures, it was necessary to crop and magnify an area close to the origin of the plot to clearly show the interaction of the isobole predictions ± se of λ (black curves with gray shaded regions) and the guideline concentrations (red polygon).

PAHs with Ni and V would not result in sufficiently altered co-toxicity to jeopardize the protectiveness of the CCME guidelines (data not shown). The use of isoboles at low effects levels predicted the guideline concentrations would not be fully protective for mixtures of Cu or Cd with PHE or PHQ even though the guidelines were derived from LOEL data multiplied by a 0.1 correction factor.[41] However, these predictions must be validated experimentally, and must also be considered carefully in light of the following experimental details.

Our study was carried out within a narrow range of water quality parameters, whereas water quality parameters found in the environment are subject to substantial spatiotemporal variation. We also only considered binary mixtures, whereas natural systems contain a mixture of many contaminants. It is likely that the inclusion of additional contaminants would alter the additivity of the response. Additionally, we only considered acute co-toxicity representing short-term spikes in contaminant loading, and only applied waterborne exposures, where there may be differences in mixture additivity among waterborne, sediment, and dietary exposures. As such, the interpretation of our water quality guideline analysis must be sensitive to our specific experimental conditions. Nonetheless, there is potential for a response surface isobole-based analysis to be applied in testing and/or developing water quality guidelines for mixtures of contaminants.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03231.

Details regarding PDMS film calibration, including Figure S1; mixture reference model selection; and a practical way to correct weekly isobole data for biotemporal variability in contaminant sensitivity, including Tables S1 and S2, listing the reference LC50s for weekly mixture experiments, indicating where a biotemporal multiplication factor was required, and Tables S3 and S4, describing log−logistic and isobole model parameter estimates and fits (PDF)

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Notes

The authors declare no competing financial interest.

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