MIXTURES OF METALS AND POLYCYCLIC AROMATIC HYDROCARBONS LEAD TO COMPLEX TOXIC OUTCOMES IN THE FRESHWATER AMPHIPOD, *HYALELLA AZTECA*

Patrick T. Gauthier

Faculty of Natural Resources Management

Lakehead University

Thunder Bay, Ontario

A dissertation submitted in partial fulfilment of the requirements of the degree of

Doctor of Philosophy in Forest Sciences

Supervisory Committee:

Dr. G. Pyle (supervisor), Dr. E. Prepas (supervisor), Dr. W. Norwood,

and Dr. B. McLaren

Submitted August 26, 2015

Abstract

Gauthier, P.T. 2015. Mixtures of metals and polycyclic aromatic hydrocarbons lead to complex toxic outcomes in the freshwater amphipod, *Hyalella azteca*.

The study of ecotoxicology inevitably must address the fact that toxicants always occur in mixture. Unfortunately, our present state of knowledge in terms of predicting the effects of contaminant mixtures and understanding the mechanisms by which a mixture can produce non-additive (e.g., antagonistic or synergistic) toxicity is insufficient to advise regulatory authorities on appropriate water quality objectives for the protection of aquatic life. Metals and polycyclic aromatic hydrocarbons (PAHs) are two ubiquitous contaminants that are often associated with similar effluent sources, such as bitumen and municipal waste. The adverse toxicological effects that metals and PAHs produce have given these contaminants the status of "priority pollutants" in many countries, including the USA and Canada. Thus, understanding their toxicological effects when in mixture should also be a priority. However, to date, there have been only 11 studies that have investigated the potential for metals and PAHs to produce non-additive toxicity. Of these 11 studies, reports of more-than-additive lethality have been equally common as strictly-additive lethality, raising concern over the largely ignored ecological risk these contaminants types produce when in mixture.

The research outlined in this dissertation expands our understanding by providing the first comprehensive review of mechanistic aspects of metal-PAH mixture toxicity that likely amount to more-than-additive co-toxicity. This dissertation outlines experimental work investigating the additivity of binary mixtures of Cu, Cd, Ni, and V, with either phenanthrene (PHE) or phenanthrenequinone (PHQ), two common PAHs. For cases where more-than-additive mortality was found, additional experimentation was carried out to explore interactive toxic mechanisms in attempt to explain why certain mixtures of metals and PAHs produce more-than-additive lethality. Finally, the effects of Cu, PHE, and Cu-PHE mixtures were studied in terms of their effects on behaviour, a sublethal endpoint that mediates ecological effects which can also be used to predict toxic mechanisms. All experimental work outlined in this dissertation involved the aquatic crustacean amphipod, *Hyalella azteca*, which was selected due to its tractability in a laboratory setting, its ecological importance as a food source for fish, amphibians, and waterfowl, and its widespread distribution throughout North and Central America.

The literature review provided within this dissertation outlines several candidate interactive toxic mechanisms by which metal-PAH mixtures may produce more-than-additive toxicity. Firstly, metal-induced changes in gene expression and protein activity of the highly conserved xenobiotic detoxification system, cytochrome P450, most likely decreases the metabolism of PAHs *in vivo*, potentially prolonging and exacerbating the effects of PAH parent compounds. Secondly, the PAH-induced inhibition of metal sequestering/detoxifying metallothionein proteins would increase the capacity of metals to interact with other biomolecules in vivo and potentially exacerbate metal toxicity. Thirdly, the deleterious nature of PAHs toward biomembranes may alter their permeability to metals, potentially increasing their uptake and distribution among various tissues. Fourthly, the capacity for PAHs and metals to catalyse each other's production of reactive oxygen species (ROS) suggests potentiated oxidative stress may be an important mechanism mediating more-than-additive metal-PAH mixture toxicity. Finally, the mutual inhibition of detoxification could lead to positive feedback among these outlined interactive mechanisms.

Mixtures of Cu and Cd with either PHE or PHQ produced more-than-additive lethality in *H. azteca* following acute 48-h waterborne exposures. Mixtures of Cu with either PHE or PHQ also produced more-than-additive lethality in *H. azteca* following 18 h-exposures. However, mixtures of Cd with PHE or PHE produced strictly additive lethality following 18-h exposures. Additionally, mixtures of Ni with either PHE or PHQ produced mostly strictly-additive lethality in both 48- and 18-h exposures, and mixtures of V with either PHE or PHQ produced mostly strictly- and less-than-additive lethality in 48- and 18-h exposures. The degree of more-than-additive toxicity induced by Cu-PHE, Cu-PHQ, Cd-PHE, and Cd-PHQ mixtures over 48 h is presented in terms of the protectiveness of Canada's water quality guidelines for the protection of aquatic life, where Cu-PHE, Cu-PHQ, Cd-PHE, and Cd-PHQ mixtures at their guideline values are predicted to induce 7.5%, 3.7%, 4.4%, and 1.4% mortality over 48 h.

Investigation into interactive aspects of oxidative stress and metal accumulation revealed that ROS production was not potentiated in metal-PAH mixtures, and therefore does not account for any more-than-additive toxicity in *H. azteca*. Moreover, the accumulation of Cd was not affected by co-exposure to PHE or PHQ, ruling out the possibility that altered Cd accumulation may in part explain the more-than-additive lethality observed in 48-h Cd-PHE and Cd-PHQ exposures. The accumulation of Cu was also not influenced by co-exposure to PHE. However, Cu accumulation was increased by co-exposure with PHQ, which in part explains the more-than-additive lethality observed in Cu-PHQ mixtures.

Exposure to sublethal concentrations of PHE led to drastic behavioural impairment in *H. azteca* associated with an increase in respiratory rate and a decrease in the activity of the acetylcholine neurotransmitter hydrolase, acetylcholinesterase (AChE; i.e., neurotoxicity). The behavioural, respiratory and neurotoxic effects of PHE mirrored those induced by organophosphate pesticides (e.g., Malathion) in invertebrates, suggesting that PHE is a potent neurotoxicant in *H. azteca*, and that neurotoxicity is an important mechanism in the acute lethality of PHE in *H. azteca*. Sublethal concentrations of Cu had no effect on amphipod behaviour, respiration, or AChE activity. However, Cu antagonized the PHE-induced reduction and increase in ventilatory behaviour and respiration, respectively, and most likely antagonized the PHE-induced decrease in AChE activity. Thus, interactive aspects of neurotoxicity do not account for the more-than-additive lethality observed in Cu-PHE mixtures in *H. azteca*.

This study concludes that certain metal-PAH mixtures induced more-than-additive lethality in *H. azteca*, and that an assortment of interactive aspects of metal and PAH toxicity are likely involved in manifesting more-than-additive toxicity.

Keywords: amphipods; aquatic toxicology; behaviour; bioavailability; detoxification; mixture toxicology; metals; more-than-additive toxicity; neurotoxicity; oxidative stress; PAHs; reactive oxygen species; respiration; regulatory guidelines

Contents

Figures

Tables

Acknowledgements

It goes without saying that my supervisors, Drs. Greg Pyle and Ellie Prepas, and committee members, Drs. Warren Norwood and Brian McLaren, have played an instrumental role in the development and completion of this research. Each with a diverse background, they have provided a wide spectrum of perspectives, always pushing me to think outside of the narrow scope of my individual projects and produce a dissertation that is cohesive, interdisciplinary, and touches on many aspects of aquatic toxicology and ecological risk assessment. I am also very grateful to Dr. Chris Wood for his comments on this disseration and participation as external examiner.

The response-surface analysis outlined in Chapter 3 was suggested as a tool for mixtures analysis by Dr. Dominique Turcotte. She likely has no idea what amounted from a casual 5 minute conversation. Further support for this analysis came from Dr. Christian Ritz, who not only authored the 'drc' package in R, which was instrumental for isobole modelling, but provided thoughtful and encouraging feedback and support for my specific statistical requirements. Again, Dr. Ritz probably has no idea how useful the few e-mails we've shared were for my work.

There are many people who have helped me with general laboratory and analytical procedures, and administrative aspects of a PhD. I thank Mitra Brown from Environment Canada for her assistance in supplying us with amphipods, Maarit Wolfe for her help with amphipod culturing and sample analyses, and Debbie Puumala for her assistance with HPLC methods and instrumentation, Greg Kepka and Ain Raitsakas from LUIL for all the training and advise for various analyses, and Anne Willis for her assistance in the lab(s). Thanks to Trish Sokoloski for her help over the years organizing my personal funding. Most importantly, thank you Virginia Antoniak. I am forever grateful for your help organizing pretty much every aspect of my work, from expense claims, purchase orders, funding, travel plans, committee meetings, office and lab space, and so much more.

Financial support for this research came from the Ontario Ministry of Training, Colleges, and Universities Ontario Graduate Scholarship (OGS) award program, and the Forest Watershed & Riparian Disturbance Project (FORWARD II & III) funded by the Natural Sciences and Engineering Research Council of Canada Collaborative Research and Development (CRD) Program with Suncor Energy Inc., Total E&P Canada Ltd., Canadian Natural Resources Limited, Tervita Corporation, Syncrude Canada Ltd., Alberta Newsprint Company, Alberta-Pacific Forest Industries, Hinton Pulp, Millar Western Forest Products Ltd., Slave Lake Pulp, Oil Sands Research and Information Network and Environment Canada.

Finally, sometimes the most important aspect of being productive is the quality of time spent not doing work. Thanks to all my old friends and family, and my new friends and colleagues I've met over the years at the various conferences I've been fortunate enough to attend. Most importantly, thanks Anne for putting up with my PhD-induced irritability and impatience, you're amazing. Will you marry me?

Chapter 1: Introduction

Much has been done to understand the environmental consequences associated with the contamination of aquatic systems. Contaminant-induced effects span a wide canvas of ecological, behavioural, physiological, cellular, and molecular endpoints. On the largest scale, increased presence of contaminants can lead to the extirpation of less contaminant-tolerant aquatic species and a general decrease in species diversity (Bickman et al., 2000). Observing individual animals reveals that exposure to contaminants can have widespread physiological consequences in terms of reproductive health, which are often a result of tissue damage to the ovaries or gonads of reproductive adults (Vos et al., 2000), or the developmental toxicity incurred by early-life stages (Finn 2007). These physiological effects are triggered by changes at the level of the cell, where differential gene expression or protein activity can trigger a cascade of cellular effects that alter cell function or lead to cell death. Moreover, contaminants can damage and/or alter the structure of deoxyribonucleic acid (DNA), amounting to genetic defects that can manifest adverse toxicological effect at all levels of biological organisation.

Two ubiquitous groups of contaminants are metals and polycyclic aromatic hydrocarbons (PAHs). The contamination of aquatic systems with metals and PAHs has been ongoing since the dawn of the industrial revolution. Metals are mined and refined for their desirable physical and chemical properties for a variety of products (e.g., construction materials, electrical components, chemical catalysts, jewellery, etc.). Atmospheric and water-based effluents (i.e., mine tailings) from smelting activities are primary sources of metals contamination. Similarly, as PAHs are a major constituent of oil, the extraction and refinement of oil for the production of petroleum products is the major route by which PAHs are released into aquatic environments. Based on the extent of these industrial activities on a global scale, it can be expected that low concentrations of metals and PAHs can be detected in virtually every aquatic system, and in the past two decades there have been

emerging reports of substantial co-contamination of metals and PAHs in a variety of freshwater and marine environments (Ho et al., 1997, Valette-Silver et al., 1999, Curran et al., 2000, Mielke et al., 2001, Muniz et al., 2004, Donahue et al., 2006 and Sprovieri et al., 2007). Moreover, accidental oil/petroleum spills and failure of wastewater (e.g., mine tailings) containment are of public concern, and would present aquatic life within receiving waters an acute, potentially lethal, toxicological challenge.

As aquatic organisms are most likely subjected to mixtures of metals and PAHs, among other toxicants, it is essential to understand and predict how the mixing of contaminants influences the additivity of the toxic response. Additivity can be described in terms of three potential outcomes: 1) less-than-additive toxicity (e.g., antagonism), where the net toxicity of the mixture is lesser than that would be expected based on the addition of the toxicities of the individual contaminants when present on their own, 2) strictly-additive toxicity, where the net toxicity is a simple addition of the individual toxic effects, and 3) more-than-additive toxicity (e.g., potentiation and synergy), where the net toxicity is greater than can be attributed to the addition of the toxic effects of the individual contaminants when present on their own. A review of aquatic studies that have measured the additivity of metal-PAH mixture toxicity reveals that more-than-additive co-toxicity is equally common as strictly-additive toxicity (Chapter 2; Gauthier et al., 2014). More-than-additive co-toxicity is particularly troubling as environmental regulatory authorities (e.g., in Canada, the USA, Australia, and New Zealand), either do not consider mixture toxicity or assume strictly-additive toxicity when developing environmental protection policy (Warne, 2003).

A great deal of effort is required to provide regulatory authorities with recommendations for the environmental protection from mixtures of metals and PAHs. As our understanding of metal-PAH mixture toxicity is in its infancy, sensible actions would be to: 1) measure and detect the most commonly found metals and PAHs in co-contaminated environments, 2) conduct toxicity bioassays

to map and predict additivity on a variety of whole-organism- (e.g., survival and behaviour), cellular- (i.e., survival and function), and molecular-endpoints with commonly co-occurring metals and PAHs, 3) compare toxicological results to regulatory guideline values to assess their protectiveness in a mixture scenario, and 4) use mixtures that produce more-than-additive lethality as model mixtures in further toxicity bioassay experiments to discover interactive cellular/molecular mechanisms by which more-than-additive toxicity occurs.

Among the various reports describing metal-PAH co-contamination, there are several metals and PAHs that are reported with relatively high frequency, such as copper (Cu), cadmium (Cd), nickel (Ni), vanadium (V), and phenanthrene (PHE; Valette-Silver et al., 1999, Curran et al., 2000, Mielke et al., 2001, Muniz et al., 2004, Donahue et al., 2006 and Sprovieri et al., 2007). Phenanthrene is a three-ringed PAH and is one of the most abundant PAH parent compounds found in petroleum products. Phenanthrene undergoes photodegradation to produce phenanthrenequinone (PHQ) when sufficient UV and visible light is present (Yu et al., 2006). Thus, it is likely that PHE and PHQ occur together. Although similar in structure, PHE and PHQ have unique physical properties that presumably amount to dissimilar mechanisms of toxicity. Unfortunately, the exact mechanisms by which PHE and PHQ exert their toxicity in aquatic biota, particularly from short-term exposures, have not been well described. Nonetheless, PHE, among other low-molecular weight PAH-parent compounds, are highly lipophilic and can infiltrate and disrupt biomembrane structure and function (e.g., ionoregulation; Sikkema et al., 1995; Schirmer et al., 1998), whereas PHQ engages in futile redox cycling producing reactive oxygen species (ROS) that can disrupt cellular ROS balance leading to oxidative stress (Penning et al., 1996; Fu et al., 2012).

Slightly more is known regarding the mechanisms of metal toxicity, where exposure to Cu, Cd, and Ni have been shown to disrupt ionoregulation (Brooks and Mills, 2003; Pane et al., 2003; Issartel et al., 2010). Metals are also known to disrupt ROS balance, either directly as ROS

producers (i.e., Cu, Ni, V) or indirectly though the inhibition of ROS scavenging enzymes (i.e., Cd; Stohs and Bagchi, 1995). Thus, metals and PAHs share some mechanisms of toxicity, such as ionoregulatory dysfunction and oxidative stress, which likely play an important role in acute whole-organism endpoints such as mortality. Nonetheless, it is near impossible to predict the additivity of co-toxicity based on these mechanisms without understanding how co-exposure to metals and PAHs may also produce cellular/molecular effects that alter toxicity pathways in terms of detoxification and toxicokinetics (as review in Chapter 2; Gauthier et al., 2014). Thus, a better understanding of interactive mechanistic aspects of contaminants co-exposure is essential to understand and predict metal-PAH toxicity on whole-organism endpoints.

Given the diversity of aquatic life, toxicological effects cannot be investigated for every aquatic species. Many species have been chosen as model organisms for the investigation of adverse toxicological effects, where effects on these model species are suspected to be representative of effects on other organisms of similar taxa. These model organisms are chosen based on their ecological importance (i.e., keystone predator or prey species), sensitivity to contaminants, distribution (i.e., relevance on a spatial scale), and tractability in a laboratory setting (i.e., ability to culture organisms/cells; Nikinmaa, 2014). The crustacean amphipod, *Hyalella azteca*, is an exceptional model organism as it is an important prey species for a variety of other macroinvertebrates, fish, amphibians, and waterfowl. In fact, *H. azteca* may represent the majority of food for white-winged scoters (*Melanitta fusca deglandi*; Brown and Fredrickson, 1986), and a decline in amphipod abundance likely contributed to the decline in lesser scaup (*Aythya affinis*; Lindeman and Clark, 1999) in Saskatchewan, Canada. Contaminant-induced changes in *H. azteca* distribution and abundance can lead to indirect effects on organisms at higher trophic scales. Combined with its widespread distribution spanning most of North and Central America, and that it

can be cultured in a lab setting to produce 1,000s of juveniles per week, *H. azteca* represents an ideal model organism for toxicity bioassays.

The goal of the research outlined in this dissertation is to expand our knowledge on the toxicological effects of metal-PAH mixtures in aquatic biota. Specifically, the first objective was to produce the first comprehensive literature review synthesizing a variety of disparate toxicological literature in attempt to stimulate and direct further research into the exploration of co-toxic mechanisms that may account for the common more-than-additive lethality observed in metal-PAH mixtures. The second objective was to carry out acute toxicity experiments with *H. azteca* to map the additivity of acute lethality induced by Cu, Cd, Ni, and V mixed with either PHE or PHQ. In the process of mapping the additivity of these metal-PAH mixtures, the third objective was to incorporate an experimental design that allowed mixture toxicity estimates to be compared to aquatic regulatory guidelines to assess their protectiveness in a mixture scenario. Following mortality experiments, in cases where more-than-additive lethality was observed, the fourth objective was to explore oxidative stress and metal accumulation in attempt to find an explanation for any observed more-than-additive lethality. Finally, the fifth objective was to address metal-PAH mixture toxicity in terms of behavioural effects and the mechanisms (i.e., respiration, oxidative stress, and neurotoxicity) responsible for behavioural effects in *H. azteca*.

1.1 References

Bickman, J.W., Sandhu, S., Hebert, P.D.N., Chickhi, L., Athwal, R., 2000. Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. Mutation Research 463, 33-51.

Brooks, S.J., Mills, C.L., 2003. The effect of copper on osmoregulation in the freshwater amphipod *Gammarus pulex*. Comparative Biochemistry and Physiology Part A 135, 527-537.

Brown, P.W., Fredrickson, L.H., 1986. Food habits of breeding white-winged scoters. Canadian Journal of Zoology 64, 1652-1654.

Curran, K.J., Irvine, K.N., Droppo, I.G., Murphy, T.P., 2000. Suspended solids, trace metal and PAH concentrations and loadings from coal pile runoff to Hamilton Harbour, Ontario. Journal of Great Lakes Research 281, 217-227.

Donahue, W.F., Allen, E.W., Schindler, D.W., 2006. Impacts of coal-fired power plants on trace metals and polycyclic aromatic hydrocarbons (PAHs) in lake sediments in central Alberta, Canada. Journal of Paleolimnology 35, 111-128.

Finn, R.N., 2007. The physiology and toxicology of salmonid eggs and larvae in relation to water quality criteria. Aquatic Toxicology 81, 337-354.

Fu, P., Xia, Q., Sun, X., Yu, H., 2012. Phototoxicity and environmental transformation of polycyclic aromatic hydrocarbons (PAHs)-light-induced reactive oxygen species, lipid peroxidation, and DNA damage. Journal of Environmental Science and Health, Part C. 30, 1-41.

Gauthier, P.T., Norwood, W.P., Prepas, E.E., Pyle, G.G, 2014. Metal-PAH mixtures in the aquatic environment: A review of co-toxic mechanisms leading to more-than-additive outcomes. Aquatic Toxicology 154, 253-269.

Ho, K.T., McKinney, R.A., Kuhn, A., Pelletier, M.C., Burgess, R.M., 1997. Identification of acute toxicants in New Bedford Harbour sediments. Environmental Toxicology and Chemistry 16, 551-558.

Issartel, J., Boulo, V., Wallon, S., Geffard, O., Charmantier, G., 2010. Cellular and molecular osmoregulatory responses to cadmium exposure in *Gammarus fossarum* (Crustacea, Amphipod). Chemosphere 81, 701-710.

Lindeman, D.H., Clark, R.G., 1999. Amphipods, land-use impacts, and lesser scaup (*Aythya affinis*) distribution in Saskatchewan wetlands. Wetlands 19, 627-638.

Mielke, H.W., Wang, G., Gonzales, C.R., Le, B., Quach, V.N., Mielke, P.W., 2001. PAH and metal mixtures in New Orleans soils and sediments. Science of the Total Environnent 281, 217-227.

Muniz, P., Danulat, E., Yannicelli, B., García-Alonso, J., Medina, G., Bícego, M.C., 2004. Assessment of contamination by heavy metals and petroleum hydrocarbons in sediments of Montevideo Harbour (Uruguay). Environment International 29, 1019-1028.

Nikinmaa, M., 2014. The most important experimental designs and organisms in aquatic toxicology. In: Nikinmaa, M. (Ed.), An Introduction to Aquatic Toxicology. Academic Press Elsevier Inc., Waltham, MA, USA, pp. 54- 66.

Pane, E.F., Smith, C., McGeer, J.C., Wood, C.M., 2003. Mechanisms of acute and chronic waterborne nickel toxicity in the freshwater cladoceran, *Daphnia magna*. Environmental Science & Technology 37, 4382-4389.

Penning, T. Ohnishi, S. Ohnishi, T. Harvey, R., 1996. Generation of reactive oxygen species during the enzymatic oxidation of polycyclic aromatic hydrocarbon trans-dihydrodiols catalyzed by dihydrodiol dehydrogenase. Chemical Research in Toxicology. 9, 84-92.

Schirmer, K., Dixon, D. G., Greenberg, B. M., Bols, N. C., 1998. Ability of 16 priority PAHs to be directly cytotoxic to a cell line from the rainbow trout gill. Toxicology 127, 129-141.

Sikkema, J., de Bont, J.A., Poolman, B., 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiology and Molecular Biology Reviews 59, 201-222.

Sprovieri, M., Feo, M.L., Prevedello, L., Manta, D.S., Sammartino, S., Tamburrino, S., Marsella, E., 2007. Heavy metals, polycyclic aromatic hydrocarbons and polychlorinated biphenyls in surface sediments of the Naples harbour (southern Italy). Chemosphere 67, 998-1009.

Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radical Biology & Medicine 18, 321-336.

Valette-Silver, N., Hameedi, M.J., Erfurd, D.W., Robertson, A., 1999. Status of the contamination in sediments and biota from the western Beaufort Sea (Alaska). Marine Pollution Bulletin 38, 702-722.

Vos, J.G., Dybing, E., Greim, H.A., Ladefoged, O., Lambre, C., Tarazona, J.V., Brandt, I., Vethaak, A.D., 2000. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Critical Reviews in Toxicology 30, 71-133.

Warne, M. St J., 2003. A review of the ecotoxicity of mixtures, approaches to, and recommendations for, their management. Proceedings of the Fifth National Workshop on the Assessment of Site Contamination, 253-276.

Yu, H., Xia, Q., Yan, J., Herreno Saenz, D., Wu, Y., Fu, P., 2006. Photoirradiation of polycyclic aromatic hydrocarbons with UVA light - a pathway leading to the generation of reactive oxygen species, lipid peroxidation, and DNA damage. International Journal of Environmental Research and Public Health 3, 348-354.

Chapter 2: Metal-PAH mixtures in the aquatic environment: a review of co-toxic mechanisms leading to more-than-additive outcomes[1]

2.1 Abstract

Mixtures of metals and polycyclic aromatic hydrocarbons (PAHs) occur ubiquitously in aquatic environments, yet relatively little is known regarding their combined toxicities. Emerging reports investigating the additive mortality in metal–PAH mixtures have indicated that more-than-additive effects are equally as common as strictly-additive effects, raising concern for ecological risk assessment typically based on the summation of individual toxicities. Moreover, the current separation of focus between in vivo and in vitro studies, and fine- and coarse-scale endpoints, creates uncertainty regarding the mechanisms of co-toxicity involved in more-than-additive effects on whole organisms. Drawing from literature on metal and PAH toxicity in bacteria, protozoa, invertebrates, fish, and mammalian models, this review outlines several key mechanistic interactions likely to promote more-than-additive toxicity in metal–PAH mixtures. Namely, the deleterious effects of PAHs on membrane integrity and permeability to metals, the potential for metal–PAH complexation, the inhibitory nature of metals to the detoxification of PAHs via the cytochrome P450 pathway, the inhibitory nature of PAHs towards the detoxification of metals via metallothionein, and the potentiated production of reactive oxygenated species (ROS) in certain metal (e.g. Cu) and PAH (e.g., phenanthrenequinone) mixtures. Moreover, the mutual inhibition of detoxification suggests the possibility of positive feedback among these mechanisms. The individual toxicities and interactive aspects of contaminant transport, detoxification, and the production of ROS are herein discussed.

[1] This chapter is published. Gauthier, P.G., Norwood, W.P., Prepas, E.E., Pyle, G.G., 2014. Metal-PAH mixtures in the aquatic environment: a review of co-toxic mechanisms leading to more-than-additive outcomes. Aquatic Toxicology 154, 253-269.

2.2 Introduction

Environmental contamination comes in the form of a mixture of toxicants, some of which have drastically different chemical properties, modes of toxicity, and potential to interact ex- and in-vivo. Exploration into co-toxic effects of metal mixtures has revealed that most co-toxic outcomes are not simple additions of individual toxicities (Norwood et al., 2003). Non-additive co-toxic outcomes, either less- or more-than-additive, are common and complicate attempts to address the ecological risk posed by environmental contamination by metal mixtures. It is even less clear when trying to assess the risks associated with mixtures of metals and organic contaminants, such as polycyclic aromatic hydrocarbons (PAHs).

The contamination of aquatic environments with metals has been on-going since the onset of industrialization in the eighteenth century (Pyle and Couture, 2012). An iconic example of the extent of 20th century metal contamination can be found in the smelting activities that have led to the contamination of over 7,000 lakes in the Sudbury region of Ontario, Canada (Keller et al., 1999). Coincident with extraction and redistribution of metals from the Earth's crust, industrialization also introduced the extraction, refining, and combustion of petroleum products rich in PAHs to fuel the increasing energy demands associated with industrialization. As a result, PAHs can be found ubiquitously in aquatic and terrestrial environments (as reviewed by Lima et al., 2005). Environmental contamination of metals and PAHs is a global concern, as there have been reports of substantial co-contamination in a variety of coastal (Ho et al., 1997; Valette-Silver et al., 1999; Mielke et al., 2001; Muniz et al., 2004; Sprovieri et al., 2007) and freshwater (Curran et al., 2000; Donahue et al., 2006) systems.

Numerous aquatic studies of metal–PAH co-toxicity have emerged in the past two decades. However, the question of whether joint toxicity is additive or non-additive has rarely been

addressed. Availing to the literature catalogued in ISI Web of Knowledge and Google Scholar while using various combinations of the keywords: metal, PAH, polycyclic aromatic hydrocarbons, mixtures, toxicity, concentration addition, effects addition, independent action, aquatic, invertebrates, and fish, only ten studies were found in which non-additive toxicity was measured (Table 2.1). Perhaps the simplest explanation for this shortcoming is the difficulty involved in conducting experiments catered to data analyses that can extract such information. Accordingly, there have been several reviews describing mixtures theory, modelling, and experimental designs capable of discerning between non-additive and additive toxicity (Vouk et al., 1983; Berenbaum, 1989; Teuschler et al., 2002; Norwood et al., 2003; Sørensen et al., 2007; Abendroth et al., 2011).

The capacity for metal–PAH mixtures to elicit non-additive toxicity most likely relies on the contaminants' individual chemical properties and mechanisms of toxicity. From a mechanistic point of view, perhaps the most straightforward explanation is that the co-toxicity is co-dependent. Thus, the toxic agents interact, whereby the presence of one agent directly influences the toxicity of the other (e.g., competition for receptor sites, or the speciation of metal–PAH complexes). However, a deeper understanding of the cell supports that non-additive effects can arise even if the two agents do not directly interact. Non-additive co-toxicity may not depend on direct metal–PAH interactions. Instead, the toxic actions of the co-occurring toxicants may influence the transport, metabolism, and detoxification upstream or downstream of the sites of action.

Reports describing the mechanisms of metal–PAH co-toxicity are scarce and the nature of co-toxic interactions remain virtually unknown. As such, the limited number of studies investigating the additivity of metal–PAH mixtures have had to avail to coarse endpoints (e.g., mortality) and ultimately have produced ambiguous results with limited mechanistic explanation. The goal of this review is to stimulate further discussion and exploration into metal–PAH co-toxicity with an

Table 2.1 The co-toxicity of metals and polycyclic aromatic hydrocarbons (PAHs) in aquatic organisms. Less-than-, more-than-, and strictly-additive outcomes are indicated by '−', '+', and '=', respectively. Tallied outcomes are not replicated by concentration (e.g., three more-than-additive and two strictly-additive mixture outcomes within a single concentration response curve are tallied as '+ =='). However, for publications including multiple mixture 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 '+ +'). Therefore every symbol is counted as one case with a total of 63 cases. For cases where multiple exposure durations were reported in the same publication, outcomes were separated as per the duration column. Additivity varied by metal, PAH, species, exposure regime, and endpoint observed. In terms of mortality, 44.7%, 44.7%, and 10.5% of mixtures were more-than-additive, additive, and less-than-additive respectively. In terms of accumulation, 46.6%, 26.7%, and 26.7% of mixtures were more-than-additive, additive, and less-than-additive respectively. In terms of reactive oxygenated species (ROS) production, 37.5%, 50%, and 12.5% of mixtures produced more-than-additive, additive, and less-than-additive effects respectively. 'mort' denotes mortality, 'accum' denotes metal accumulation.

Metal	PAH	Species	Exposures	Duration (h)	Endpoint	+/=/-	Source
Cd	phenanthrene	*S. knabeni*	sediment	96	mort	+	Fleeger et al. (2007)
Cd	phenanthrene	*S. knabeni*	aqueous	96	mort	+	Fleeger et al. (2007)
Cd	phenanthrene	*A. atopus*	aqueous	96	mort	+	Fleeger et al. (2007)
Cd	phenanthrene	*H. azteca*	sediment	240	mort	+	Gust (2006)
Cd	phenanthrene	*H. azteca*	sediment	240	growth	=	Gust (2006)
Cd	phenanthrene	*H. azteca*	aqueous	24; 48; 72	mort	=; -; =	Gust (2006)
Cd	phenanthrene	*H. azteca*	sediment	240	accum	+	Gust and Fleeger (2005)
Cd	phenanthrene	*H. azteca*	sediment	240	growth	=	Gust and Fleeger (2005)
Cd	phenanthrene	*H. azteca*	aqueous	192	mort	=	Gust and Fleeger (2005)
Cd	phenanthrene	*H. azteca*	aqueous	192	accum	=	Gust and Fleeger (2005)
Cd	fluoranthene	*A. atopus*	aqueous	96	mort	+	Fleeger et al. (2007)
Cd	benzo[α]pyrene	*R. phillipinarum*	aqueous	24; 72; 144; 288; 504	accum	- =; + -; + =; + +; + +	Wang et al. (2011)
Cd	phenanthrenequinone	*V. fischeri*	aqueous	0.5; 0.75; 1	mort	+ + =; + = =; = = =	Wang et al. (2009)
Cd	phenanthrenequinone	*V. fischeri*	aqueous	1	ROS	+	Wang et al. (2009)
Cd	phenanthrenequinone	*D. magna*	aqueous	48	mort	=	Xie et al. (2007)
Cd	phenanthrenequinone	*D. magna*	aqueous	4	ROS	=	Xie et al. (2007)
Cu	phenanthrene	*D. magna*	aqueous	48	mort	=	Xie et al. (2006)
Cu	phenanthrenequinone	*D. magna*	aqueous	48	mort	+ +	Xie et al. (2006)
Cu	phenanthrenequinone	*D. magna*	aqueous	48	accum	= -	Xie et al. (2006)
Cu	phenanthrenequinone	*D. magna*	aqueous	4	ROS	+ = -	Xie et al. (2006)
Cu	phenanthrenequinone	*V. fischeri*	aqueous	0.5; 0.75; 1	mort	+ + = =; + + = -; = = = -	Wang et al. (2009)
Cu	phenanthrenequinone	*V. fischeri*	aqueous	1	ROS	+	Wang et al. (2009)
Ni	phenanthrenequinone	*D. magna*	aqueous	48	mort	+	Xie et al. (2007)
Ni	phenanthrenequinone	*D. magna*	aqueous	4	ROS	+ =	Xie et al. (2007)
Zn	phenanthrene	*C. variegatus*	aqueous	96	mort	-	Moreau et al. (1999)
Zn	phenanthrene	*C. variegatus*	aqueous	24	accum	-	Moreau et al. (1999)
nano-Zn	phenanthrene	*D. magna*	aqueous	24; 48	mort	+; +	Naddafi et al. (2011)

emphasis on linking coarse and fine scale (e.g., gene expression) endpoints. Drawing from literature on metal and PAH toxicity in bacteria, protozoa, invertebrates, fish, and mammalian models, this review briefly outlines the toxic mechanisms of metals and PAH and discusses how the individual toxicities may lead to non-additive effects under co-exposure.

2.3 Factors influencing metal-PAH co-toxicity

It is necessary to explore the toxic mechanisms of each contaminant to understand how co-exposure might influence co-toxicity. The goal of this section is to elucidate the mechanisms by which metals and PAHs exert toxic effects, in order to outline particular stages at which co-acting metals and PAHs may effectuate a non-additive toxic response.

2.3.1 Toxicity of PAHs

The uptake of PAHs is a result of passive diffusion across membranes, a feature attributed to the non-polar nature of PAHs and the shared lipophilicity between PAHs and lipid membranes. Yet, regardless of the non-specific nature by which PAHs are accumulated, the distribution of PAHs among tissues once taken up does appear to be PAH-specific. For example, identical waterborne exposures of naphthalene (NAP) and anthracene (ANT) involving the fish *Rasbora daniconius* revealed that NAP was accumulated almost exclusively in the intestine, whereas ANT was mostly accumulated in the liver and kidneys (Advaiti et al., 2013). Based on these general differences in tissue distribution, it can be expected that the toxicity of PAHs will vary based on the capacity of these tissues to metabolize and detoxify PAHs.

The toxicity of PAHs is most widely attributed to the metabolism of parent compounds to genotoxic, carcinogenic, and reactive oxygenated metabolites, which is universally mediated by the heme-thiolate monooxygenase enzyme super family, cytochromeP450 (CYP). As phase 1 enzymes, CYP “functionalizes” PAHs (e.g., through hydroxylation), allowing for further modification by

other phase 1 and 2 enzymes into more soluble and readily excretable derivatives (see reviews by Conney, 1982; Nebert and Dalton, 2006).

The activity of CYP in PAH metabolism has received consider-able attention because of the capacity of PAHs to alter the CYP1metabolic pathway upstream of CYP1 induction. The induction of CYP1 is mediated by aryl hydrocarbon receptor (AHR) transcription factors (Hahn, 2002) that can be activated by binding with coplanar PAHs. Coplanar PAHs tend to be AHR-agonists and therefore stimulate PAH metabolism by inducing the transcription of CYP1A1 (Conney, 1982). However, there are exceptions, such as fluoranthene (FLA; Van Tiem and Di Giulio, 2011), carbazole, and dibenzothiophene (Wassenberg et al., 2005), where coplanar PAHs have no, or almost no, affinity for the AHR. Unfortunately, the metabolism of many PAHs, particularly benzopyrene congeners (Schober et al., 2006; Wills et al., 2009; Incardona et al., 2011; Mu et al., 2012), produces reactive oxygenated intermediates (e.g., epoxides and dihydrodiols) often with enhanced toxicity compared to the parent compound.

In general, carcinogenesis, effectuated by CYP-metabolites, is the most recognized mechanism by which PAHs exert toxic effects (see Baird et al., 2005 for an in depth review). However, the ramifications of carcinogenesis may be inconsequential compared to more severe acute and/or other chronic effects related to PAH exposure (Fig. 2.1). For example, recent investigation into the toxicity of benzo[α]pyrene (B[α]P), a strong AHR-agonist, illustrated that CYP1A-metabolism is protective in the early lifecycle stage (ELS) of fish (Willett et al., 2001;Wassenberg and Di Giulio, 2004; Billiard et al., 2006; Scott and Hodson, 2008; Wills et al., 2009; Van Tiem and DiGiulio, 2011), and that the inhibition of CYP1A by various inhibitors leads to increased toxicity in the form of cardiovascular dysfunction and craniofacial deformities. However, the specific mechanisms involved may not be directly associated with CYP1A inhibition, as altered CYP1A activity may simply be a side effect of other mechanisms (e.g., AHR inhibition)

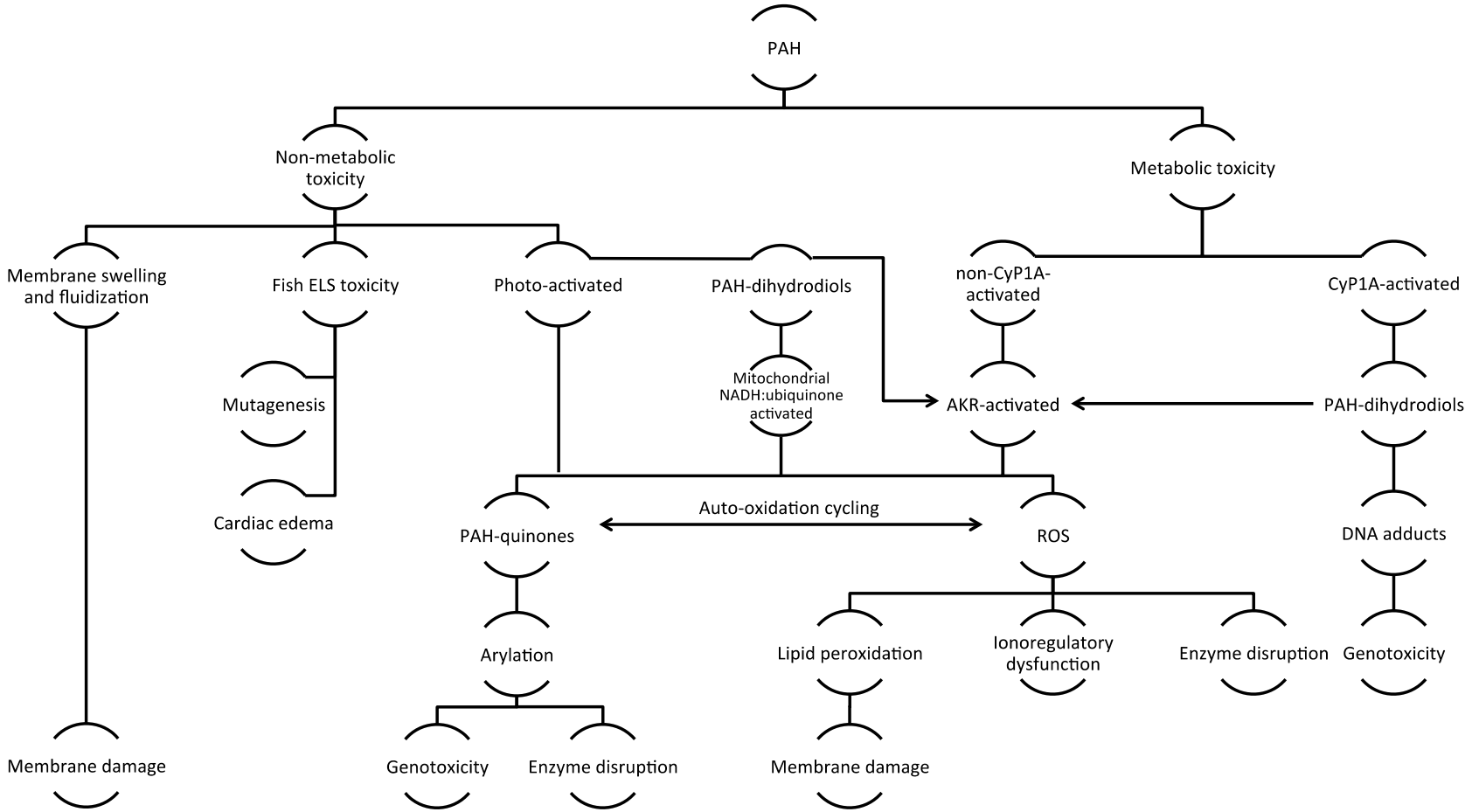

Figure 2.1 A generalized pathway of polycyclic aromatic hydrocarbon (PAH) toxicity. Although PAH toxicity can be divided into metabolic and non-metabolic pathways, the photodegradation of PAHs allows for an interaction among these pathways. Moreover, there are many shared endpoints of metabolic and non-metabolic PAH toxicity, such as membrane damage, ionoregulatory dysfunction, and deoxyribonucleic acid (DNA) damage.

leading to ELS toxicity (see Billiard et al., 2008 for a review of this topic). Nonetheless, it is evident that carcinogenesis is not the most important form of toxicity during fish embryogenesis when CYP activity is uninhibited (Billiard et al., 2006), regardless of the presence of known carcinogens, such as B[α]P 9,10-dihydrodiol (Wills et al., 2009). These findings are consistent with ELS studies of fish using PAHs of dissimilar structure and AHR binding affinities, such as phenanthrene (PHE), a three-ring PAH and weak AHR-agonist, and pyrene (PYR), a four-ring PAH and average AHR-agonist (Mu et al., 2012), and suggest that CYP1A is protective of B[α]P, PHE, and PYR ELS teratogenicity in fish (Billiard et al., 2008).

One explanation for these findings, and analogous to endpoints other than ELS teratogenicity, is that a reduction in PAH metabolism increases the half-lives of parent compounds and prolongs their toxic effects (Mu et al., 2012). Although PAH parent compounds are often regarded as biologically inactive (Fu et al., 2012), direct toxicity can occur (Schirmer et al., 1998), particularly when co-occurring xenobiotics have potential to inhibit PAH metabolism. There is good evidence that at least one key mechanism in PAH-induced fish teratogenicity is the inhibition of $Ca^{2+}$and Na^{+}/K^{+}adenosinetriphosphate ion pumps (e.g., P-type ATPases; Englehardt et al.,1981; McCloskey and Oris,1993; Kennedy and Farrell, 2005; Li et al., 2011), yet the mechanisms of such inhibition in fish require further clarification (see Section 3.2). As these P-ATPases are located within membranes, one explanation for their inhibition is the membrane damage associated with PAH parent compounds (Sikkema et al., 1995). Direct membrane damage has been ascribed to the partitioning of PAHs into lipid membranes, a phenomenon attributed to their shared lipophilicity. The resulting change in membrane structure, caused by expansion and increased fluidity (Sikkema et al., 1994), compromises the capacity of the membrane to regulate ions (see Section 2.3.1.1), most likely in part through altered P-ATPases activity. The resulting effects on the function of cell and

organelle membranes will alter homeostasis (e.g., pH balance, ion permeability, and respiration), impairing cellular function and inevitably causing cell death if intoxication persists.

A comprehensive study of the direct cytotoxicity of 16 PAHs in rainbow trout (*Oncorhynchus mykiss*) gill cell lines revealed that water solubility, lipophilicity, and ring structure are key factors in determining membrane toxicity (Schirmer et al., 1998). A common negative relationship between solubility and toxicity was observed, and as solubility among PAHs typically decreases with increasing numbers of rings (Mackay et al., 2006), only PAHs with ≤ 3 rings (e.g., NAP to PHE) were found to directly damage membranes. Schirmer et al. (1998) concluded that only NAP was of environmental relevance, as EC50 values for all other tested PAHs were greater than their solubility limits. However, these findings were restricted to acute 2-h exposures in an effort to eliminate any toxicity associated with metabolic derivatives, yet parent compounds would still be bioavailable in the exposure media of assays of longer duration, and likely still present in vivo, resulting in lower EC50 values possibly within their solubility range. In addition, the bioavailable fraction of PAHs tends to be lower in in vitro studies (i.e., cell line assays) as a result of PAHs partitioning into non-relevant materials present in the assay (e.g., well plate plastic; Kramer et al., 2012). Membrane damage will vary for each specific membrane as partition coefficients are affected by their unique lipid composition (Sikkema et al., 1995), limiting the inference of these data to salmonid gill cells. Taken together, these points identify the need for a better understanding of PAH membrane toxicity in other model systems, and how it may be aggravated by CYP1A inhibition.

An alternative explanation for the increased toxicity coincident with CYP1A inhibition is that PAH metabolism is shifted to less efficient pathways that produce derivatives with greater toxicity compared to the parent compounds or CYP-metabolites (Matson et al., 2008). For example, the aldo-keto reductase (AKR) super family competes with CYP1A (Jiang et al., 2006) to further metabolize dihydrodiol intermediates. When AKR (e.g., dihydro-diol dehydrogenase) prevails,

dihydrodiols are metabolized into catechols, at which point $O_2^{\bullet-}$ anions initiate autoxidation cycling of o-semiquinones, o-quinones, H_2O_2, and their free radicals (e.g., $O_2^{\bullet-}$, and ●OH; Penning et al., 1996). This explanation becomes increasingly plausible considering the metabolic role of CYP1A can also be achieved by photoirradiation, as PAHs absorb UVA and visible light exciting them to a singlet state (as reviewed by Fu et al., 2012) and promoting their degradation to dihydrodiol and PAH quinone (PAHQ) species (Yu et al., 2006). Furthermore, the autoxidation cycling of photo-derived PAHQs and ROS can be initiated by mitochondrial nicotinamide adenine dinucleotide(NADH):ubiquinone (Flowers-Geary et al., 1993) in the absence of both AKR and CYP activity.

The cytotoxicity of ROS can be attributed largely to lipid peroxidation, but also enzymatic disruption (discussed in more detail in Section 2.3). Following exposure to ROS-active toxicants, associated free radical species (e.g., $O_2^{\bullet-}$, and ●OH) oxidize lipid membranes producing toxic unsaturated aldehydes that are deleterious to proteins and DNA (as reviewed by Schlenk et al., 2008). More directly, oxidized membranes have decreased membrane fluidity (Nagasaka et al., 2004; Tai et al., 2010) and conductivity (Richter, 1987), altering permeability and resulting in the disruption of ion homeostasis (see Section 2.3.1.1). Lipid peroxidation is particularly damaging due to the potential propagation of oxidation along lipid chains (e.g., cell membranes; see review by Di Giulio and Meyer, 2008). In response, several detoxification enzymes, such as catalase (CAT), superoxide dismutase (SOD), and the glutathione system are available to scavenge ROS or their toxic by-products. For example, glutathione S-transferases (GSTs) catalyze the conjugation of unsaturated aldehydes with glutathione (GSH). The end product is glutathione disulfide (GSSG)-conjugated aldehydes, which are transported out of the cell by various GSH-ATPases (Hayes and Pulford, 1995) and passed on for excretion. In fact, GST activity has been frequently presented as a

useful biomarker of ROS in fish (Teles et al., 2003; Luet al., 2009; Pathiratne and Hemachandra, 2010; Palanikumar et al., 2012) and aquatic invertebrates (Le Pennec and Le Pennec, 2003; Solé et al., 2009; Sureda et al., 2011).

Aside from oxidative stress, there is evidence that PAHs can act as immunosuppressors (Wootton et al., 2003), a mechanism possibly linked to the accumulation and direct toxicity of PAHs in lysosomes (Grundy et al., 1996). PAHQs also have the potential to manifest direct toxicity through the arylation of protein sulfhydryl (PrSH) groups (see review by O'Brien, 1991). Arylation has been implicated (Tapper et al., 2000) as the primary mechanism involved in quinone-induced PrSH elimination and cell death in *O. mykiss* (Schmieder et al., 2003). Of great consequence, concerning the dual-role of quinones in redox cycling, is that GSH is the first line of defence protecting other PrSH from arylation and once depleted, cellular impairment and/or death is imminent. The link between arylation and ROS toxicity raises an important point regarding the toxicity of PAHs in mixture with other redox-active toxicants, such as metals.

2.3.2 Toxicity of metals

Organisms have evolved under exposure to various metals and utilize a number of metals in essential biological processes. Nonetheless, certain trace metals provide no biological benefit and serve only to induce toxicity (e.g., Cd and Pb). Moreover, most essential-metals become toxic when present in excessive concentrations. Due to the chemical and physical similarities among metals, non-essential metals have the potential to mimic essential-metals and bind with various metal-binding ligands in the gill (i.e., waterborne exposure) and gastrointestinal (i.e., dietary exposure) epithelia. The similarities among metals allows non-essential metals, that are otherwise not strictly regulated, to gain entry to the body and alter a variety of biologically crucial metal-mediated processes at various receptor sites inside and outside of cells. Metals can cause toxicity through

several key mechanisms, namely the disruption of vital enzymatic functions, reacting as redox catalysts in the production of ROS, disruption of ion regulation, and the formation of DNA and protein adducts (Liu et al., 2008).

Many metals disrupt enzymatic function through competitive interactions with substrates over binding sites, non-competitive binding causing conformational changes in enzymes, alterations of enzyme gene expression, and the formation of ROS leading to enzyme oxidation forming carbonyl adducts (Dalle Donne et al., 2003). A good example of non-competitive metal-induced enzyme inhibition has been extensively studied using carbonic anhydrase (CAH), a ubiquitous group of enzymes mainly responsible for balancing pH, and controlling respiration and gas balance through the hydrolysis of CO_2 to HCO_3^- and H^+ (Ceyhun et al., 2011a; DeSimone and Supuran, 2012). There has been considerable research into the inhibitory effects of various metals (e.g., Cu and Cd) on the activity of CAH isoforms extracted from a variety of aquatic organisms (Henry et al., 1995; Lionetto et al., 2000; Skaggs and Henry, 2002; Ceyhun et al., 2011a; Demirdag et al., 2013). These findings strongly suggest a metal-, species-, and isoform-specific response, with most cases indicating a post-transcriptional metal-induced inhibition of CAH activity. Although direct inhibition is most likely due to metals binding with the functional groups of amino acid sidechains (e.g., imidazole group of histidine) of CAH, it is likely that metals do not compete with CO_2 over the active site of CAH (Tu et al., 1981). Instead, metals bind with surrounding functional groups, thereby inducing a conformational change and inhibiting either the ability of CAH to bind with or hydrolyze CO_2. The inhibition of CAH in part explains the specificity of the observed metal-induced CAH inhibition, as structural differences in CAH amino acid profiles have been identified within and among species (Skaggs and Henry, 2002). The resulting outcome is a loss of CAH activity,

impairing the ability of cells to regulate CO_2 and pH resulting in the impairment of cellular function or even cell death.

Carbonic anhydrase gene expression was 9-fold greater in zebrafish (*Danio rerio*) exposed to Cd compared to unexposed fish (Luet al., 2012), regardless of inhibited CAH activity. Metal-induced differential (i.e., up- or down-regulated) gene expression has been studied considerably in aquatic biota, particularly regarding antioxidant systems. Through Fenton-like reactions, various metals (e.g., Cu and Ni) catalyze the production of ROS (for a review of metal-induced ROS formation in fish see Di Giulio and Meyer, 2008). These species (e.g., peroxyl ($RO_2^{\bullet}$), $O_2^{\bullet-}$, and ●OH) have the potential to oxidize proteins (e.g., enzymes) to form carbonyl adducts (Dalle Donne et al., 2003), inducing conformational changes and inhibiting enzyme function. Moreover, as described above, ROS imbalance can lead to lipid peroxidation (see reviews by Stohs and Bagchi, 1995; Sevanian and Ursini, 2000). As oxidative stress is a major mechanism of metal toxicity, an effective cellular response is the up-regulation of GSH production and related antioxidant proteins such as GST. Accordingly, considerable research has been carried out on the usefulness of the response of the antioxidant system as a biomarker of environmental metal exposure (Tables 2.2 and 2.3). However, the transcriptional response is complex, and many of these reports have conflicting results. Evidently, the transcriptional response is a function of the specific metal, concentration, expo-sure duration, tissue, and species tested (Tables 2.2 and 2.3), making generalizations vague in terms of ecological risk assessment.

Clearly, absorbed metals can be detrimental to aquatic organisms, and thus, many sequestering and detoxification systems are present to counteract potential metal toxicity. A basic detoxification mechanism is to facilitate the efflux of toxic metals, reducing their presence within cells. For example, a variety of metal-specific P-type ATPases may be responsible for maintaining

Table 2.2 Differential gene expression of aquatic invertebrate glutathione S-transferases (GST) and glutathione peroxidases (GP) enzymes in response to various metals. Up- and down-regulated outcomes are indicated by '+' and '-' respectively. Gene regulation varied by metal, species, sex, tissue, enzyme, and isozyme, with 71.2-,18.6-, and 10.2-% of cases reporting an up-, down-, and mixed-regulated expression, respectively, in response to metal exposure. All GSTα, GSTΔ, GSTε, GSTκ, and GSTω, 93% of GSTσ, 80% of GSTμ and GSTπ, and 50% of GSTζ were up-regulated. By contrast, 100% of GSTρ and 71.4% of GSTm enzymes were down-regulated. 'wb' denotes whole body, 'gil' denotes gill, 'dig' denotes digestive tract, and '-' and '+' denote down-and up-regulated responses.

Metal	Species	Tissue	Enzyme	Isozyme	+/-	Source
As	*T. japonicus*	wb	GST	ΔE; μ5; σ	+	Lee et al. (2008)
Cd	*T. japonicus*	wb	GST	ω; σ; m1	+	Lee et al. (2008)
Cd	*T. japonicus*	wb	GST	ζ; m3	-	Lee et al. (2008)
Cd	*C. riparius*	wb	GST	Δ3; σ1; σ2; σ3; σ4; ε1; ω1	+	Nair and Choi (2011)
Cd	*C. riparius*	wb	GST	Δ1; Δ2; ζ1; θ1	+ -	Nair and Choi (2011)
Cd	*C. riparius*	wb	GPx	nr	+ -	Nair et al. (2012)
Cd	*P. nuntia*	wb	GST	κ; ω; ζ; σ; α; π	+	Won et al. (2011)
Cd	*P. nuntia*	wb	GST	μ	+ -	Won et al. (2011)
Cd	*V. philippinarum*	wb	GST	ρ	-	Zhang et al. (2012)
Cd	*V. philippinarum*	wb	GST	σ2	-	Zhang et al. (2012)
Cd	*V. philippinarum*	wb	GST	σ3	+	Zhang et al. (2012)
Cd	*R. philippinarum*	gil	GST	π	+	Wang et al. (2011)
Cd	*R. philippinarum*	dig	GST	π	+	Wang et al. (2011)
Cr	*M. galloprovincialis*	wb	GST	♂-π	-	Ciacci et al. (2012)
Cr	*M. galloprovincialis*	wb	GST	♀-π	+	Ciacci et al. (2012)
Cu	*V. philippinarum*	wb	GST	ΔE; ω; σ; m1	+	Lee et al. (2008)
Cu	*V. philippinarum*	wb	GST	ζ; m3	-	Lee et al. (2008)
Cu	*N. succinea*	wb	GST	θ	+	Rhee et al. (2007)
Cu	*P. nuntia*	wb	GST	α; μ; ω; π; σ; ζ; κ	+	Won et al. (2012)
Cu	*V. philippinarum*	wb	GST	ω, m	-	Zhang et al. (2012)
Cu	*V. philippinarum*	wb	GST	σ1; σ2; σ3; ω; μ	+	Zhang et al. (2012)
Ag	*T. japonicus*	wb	GST	ΔE; σ	+	Lee et al. (2008)
Ag	*T. japonicus*	wb	GST	m1; m2	-	Lee et al. (2008)

Table 2.3 Differential gene expression of fish glutathione S-transferases (GST) and glutathione peroxidases (GP) enzymes in response to various metals. Up- and down-regulated outcomes are indicated by '+' and '-' respectively. Gene regulation varied by metal, species, tissue, enzyme, and isozyme, with 61.1-, 47.2-, and 8.3-% of cases reporting an up-, down-, and mixed-regulated expression of GST, respectively, in response to metal exposure. All GSTm, GST-MAPEG, GSTμ, GSTω, GSTπ, and GSTρ, and 67% of GST ζ were up-regulated. By contrast, all GSTα and GSTκ, and 75% of GSTθ enzymes were down-regulated. In cases where specific enzymes were not differentiated, 67% of cases reported a down-regulation. Class 1 and class 2 GPx were up-and down-regulated respectively, and class GPxλ exhibited a mixed-regulation. 'liv' denotes liver, 'olf' denotes olfactory, 'gil' denotes gill, 'blo' denotes blood, 'kid' denotes kidney, 'int' denotes intestine, 'nr' denotes isozyme not reported, and '-' and '+' denote down-and up-regulated responses.

Metal	Species	Tissue	Enzyme	Isozyme	+/-	Source
Cd	*T. obscurus*	liv	GST	μ; MAPEG; ω; θ; ζ	+	Kim et al. (2010a; 2009)
Cd	*T. obscurus*	liv	GST	α	-	Kim et al. (2010a; 2009)
Cd	*T. obscurus*	liv	GST	m3	+	Kim et al. (2009)
Cd	*T. obscurus*	liv	GR	nr	+	Kim et al. (2010b)
Cd	*T. obscurus*	liv	GPx	1α; 1β	+	Kim et al. (2010b)
Cd	*O. kisutch*	olf	GPx	4α	-	Wang et al. (2012)
Cd	*O. kisutch*	olf	GST	α; κ; θ	-	Espinoza et al. (2012)
Cd	*O. kisutch*	olf	GST	ζ; ρ	+	Espinoza et al. (2012)
Cd	*O. kisutch*	liv	GPx	4α; 4β	-	Wang et al. (2012)
Cd	*O. kisutch*	liv	GST	θ	-	Espinoza et al. (2012)
Cd	*O. kisutch*	liv	GST	ρ	+	Espinoza et al. (2012)
Cd	*O. kisutch*	gil	GST	θ; ζ	-	Espinoza et al. (2012)
Cd	*O. kisutch*	gil	GST	m, π, ρ	+	Espinoza et al. (2012)
Cd	*D. rerio*	olf	GST	π	+	Wang et al. (2013)
Cd	*P. flesus*	liv	GST	nr	+	Sheader et al. (2006)
Cu	*S. aurata*	blo	GST	nr	-	Isani et al. (2011)
Cu	*S. aurata*	liv	GR	nr	+ -	Minghetti et al. (2008)
Cu	*S. aurata*	gil	GR	nr	+ -	Minghetti et al. (2008)
Cu	*S. aurata*	kid	GR	nr	+	Minghetti et al. (2008)
Cu	*S. aurata*	int	GR	nr	-	Minghetti et al. (2008)
Cr	*C. auratus*	liv	GPx	λ	+ -	Li et al. (2013)
Cr	*C. auratus*	gil	GPx	λ	+	Li et al. (2013)
Cr	*C. auratus*	int	GPx	λ	-	Li et al. (2013)
Hg	*O. melastigma*	liv	GST	nr	-	Wang et al. (2011)

metal homeostasis (Rosen, 2002). The transcription of metal-ATPases is responsive to metal exposure, as has been clearly demonstrated in fish cell lines, which effectively up-regulate the transcription of Cu-ATPase following exposure to Cu (Minghetti et al., 2008, 2010, 2011). If metal accumulation is persistent enough to overwhelm ATPase metal-efflux capacity, there are numerous biotic systems that serve to transport and reduce the presence of free metal ions within the cell. For example, metallothionein (MT), a cysteine-rich protein considered to be highly conserved due to its service in regulating the presence of the essential metals Zn and Cu within the cell (see reviews by Hamilton and Merhle, 1986 for fish, and Amiard et al., 2006 for aquatic invertebrates), serves principally as metal-binding protein, rendering the metal unavailable to exert toxicity. Metallothionein also has an affinity for other transition metals occupying periodic groups IB and IIB (Olsson, 1996), namely Cd, Hg, and less well documented Ag (Hogstrand et al., 1996). There have also been several reports of Co (Ceyhun et al., 2011b), Ni, and Pb (Cheung et al., 2004) inducing MT in fish. In general, MT production is induced following exposure to these metals, and studies using various aquatic species have reported an up-regulated MT gene expression post-exposure to Cu (Ghedira et al., 2010; Ivankovic et al., 2010; Nugroho and Frank, 2012), Cd (Espinoza et al., 2012; Tiwari et al., 2012; Ragusa et al., 2013), Hg (Sinaie et al., 2010; Yamuna et al., 2012; Simpkins et al., 2013), Ni, and Co (Cheung et al.,2004).

In addition to being bound by MT, metals can be bound/sequestered in a variety of other ways, such as being bound to lysosomes as intracellular metal rich granules (Vijver et al., 2004). When metal accumulation is persistent enough to overwhelm the sequestering systems, there are other systems (e.g., the GSH system) present that can mediate metal-induced toxicity such as that caused by a ROS imbalance. Following exposure to metals, a drop in GSH content has been commonly observed (Schlenk and Rice, 1998; Ahmad et al., 2005; Wang and Wang, 2010; Eyckmans et al., 2011; Cirillo et al., 2012), but not without exception (Wang and Wang, 2010;

Eyckmans et al., 2011; Yamuna et al., 2012; Jorge et al., 2013). This discrepancy is possibly related to exposure concentrations and durations, as has been clearly illustrated by Wang et al. (2011). A drop in GSH is likely due to its consumption while conjugating ROS by-products, which is evidenced by an increase in GST activity (Ahmad et al., 2005; Wang and Wang, 2010; Yamuna et al., 2012b; Won et al., 2011, 2012) and decrease in the GSH/GSSG ratio (Spokas et al., 2006; Wang and Wang, 2010). While GSH is gradually depleted, glutathionereductase (GR) activity increases (Barmo et al., 2011) and GR levels drop (Cirillo et al., 2012), which is indicative of a cellular effort to recycle ROS-oxidized GSH. However, direct interactions between metals and GSH (as reviewed by Christie and Costa, 1984) as well as transcriptional modulation (Tables 2.2 and 2.3) may also inhibit ROS detoxification (see Section 2.3.3). Nonetheless, whatever the transcriptional and post-transcription outcomes might be, metal-induced cytotoxicity increases in GSH depleted scenarios (Maracine and Segner, 1998).

2.4 Co-toxicity of metals and PAHs

Co-exposure to metals and PAHs will lead to either additive or non-additive co-toxicity. In terms of ecological risk assessment, it is the non-additive effects that are most concerning, particular more-than-additive effects, where the contaminant mixture toxicity is greater than the summed toxicity of each of its constituents. More-than-additive outcomes can arise from a variety of interactions, either directly among the co-occurring toxicants, or indirectly through the effect of one toxicant on the various pro-cesses involved in the transport, metabolism, and detoxification of the co-occurring toxicant (Fig. 2.2). The complexity of potential interactions is immense. Thus, it is helpful to group them in terms of their position along the toxic pathway. The proceeding sections will outline several examples of toxic interactions among metals and PAHs that can result in more-than-additive co-toxicity in terms of altered cellular transport, detoxification, and redox imbalance.

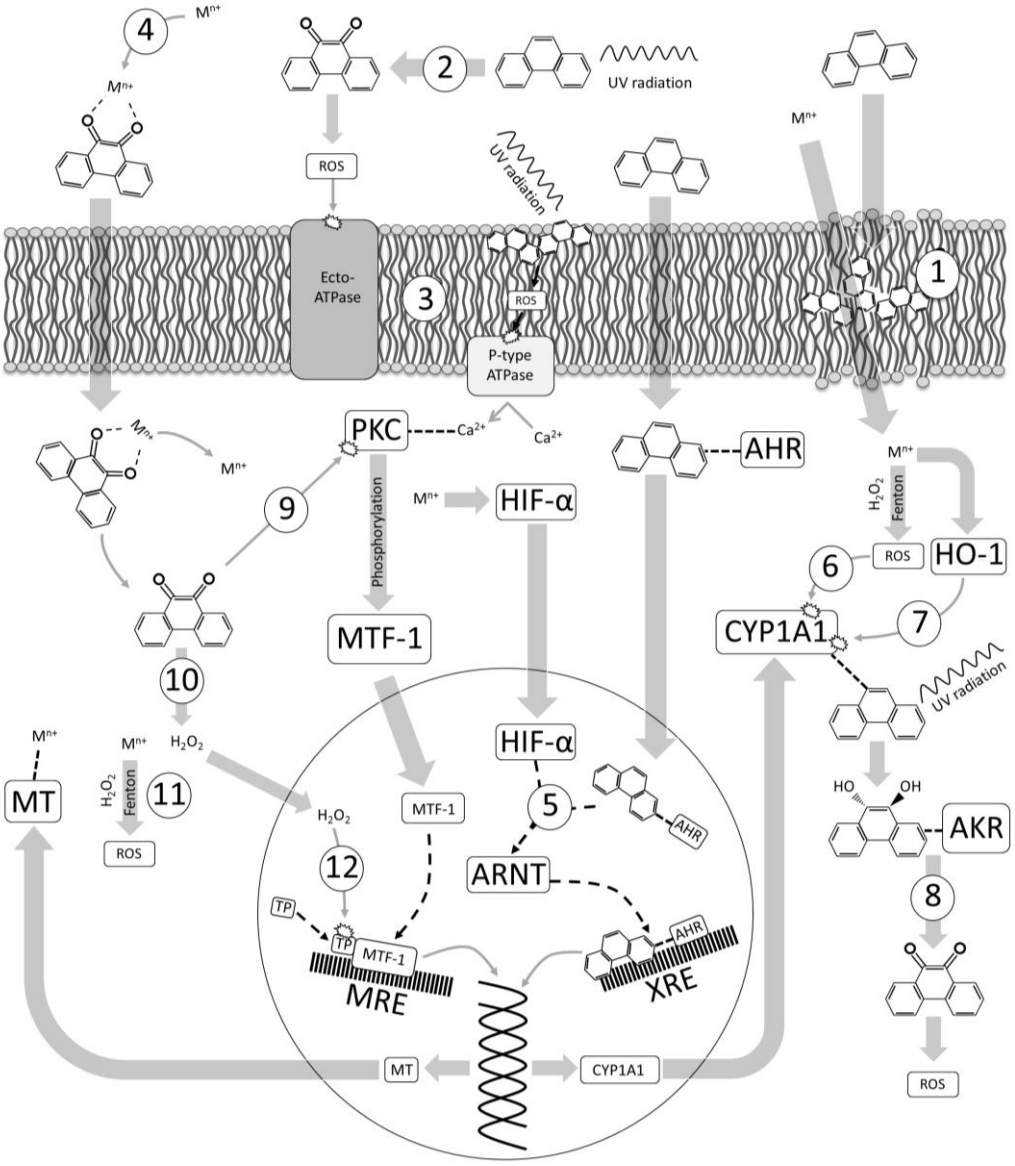

Figure 2.2 A schematic of metal–PAH interactions that may contribute to more-than-additive toxicity. Phenanthrene and phenanthrenequinone are used as example polycyclic aromatic hydrocarbons (PAHs) and PAH-quinones (PAHQs). (1) The accumulation of PAHs in lipid membranes alters membrane permeability to metals. (2) Photoirradiation of PAHs produces PAHQs leading to the production of reactive oxygenated species (ROS). (3) Photoderived ROS inhibit ATPase (e.g., Cu-ATPase and Ca^{2+}-ATPase) function deactivating ion efflux and increasing cellular trace metal and Ca^{2+} content. (4) Metal–PAH complexation may facilitate the transport of metals into cells. (5) Metal-derived hypoxia-inducible factor-1 (HIF-1) competitively inhibits the binding of aryl hydrocarbon receptor (AHR)-bound PAHs to the aryl hydrocarbon nuclear translocator (ARNT) and the expression of cytochrome P450 (CYP) enzymes (e.g., CYP1A1). (6) Metal-catalyzed ROS can bind with CYP1A1 to deactivate the enzyme. (7) The production of heme oxygenase (HO-1) as an anti-oxidant response to metals likely degrades heme rich CYP proteins, inhibiting their function in detoxifying PAHs. (8) In a CYP inhibited scenario, PAH metabolism by aldo-keto reductase (AKR) enzymes becomes increasingly likely, inevitably leading to the production of ROS. (9) The phosphorylation of metal transcription factor 1 (MTF-1) is inhibited by the PAHQ-induced arylation of protein kinase C (PKC). (10) The autooxidation of PAHQs produces H_2O_2. (11) The PAHQ-derived H_2O_2 increases the capacity of redox-active metals to produce ROS through Fenton-like reactions. (12) Dephosphorylation of MRE-bound MTF-1 is inhibited by the deactivation of tyrosine phosphatase (TP) by H_2O_2, reducing transcription of metallothionein (MT) in response to metals. 'XRE' and 'MRE' denote the xenobiotic- and metal-responsive elements, respectively.

2.4.1 Cellular transport

2.4.1.1 PAH-induced membrane damage

Perhaps the most obvious interaction that could lead to an altered transport of metals is the deleterious effects of PAHs on membranes. The transport of metal ions is inhibited by their low solubility with biomembrane lipids and regulated by a trans membrane electrical potential that serves to inhibit the adsorption of cations and control the function of a variety of membrane metal ion receptors (e.g., P-ATPase) and channel proteins (Bhattacharya, 2005). Thus, alterations in membrane structure may have several consequences in terms of the bioavailability of metals. Fundamentally, ion permeability is a function of the ordering of lipids (i.e., fluidity) within the membrane (Rossignol et al., 1985). PAH-induced increases in membrane fluidity (Sikkema et al., 1994) have been shown to alter the cell's ability to regulate H^+, disrupting pH gradients and electrical potential across the membrane (Sikkema et al., 1992). As membrane potential is considered critical to the bioavailability of metals (Kinraide, 2006), changes in membrane fluidity may indirectly alter metal uptake. It should be noted that metal uptake as a function of membrane permeability can be influenced by a variety of physiological factors, such as the osmotic status of the organism. For example, freshwater (i.e., hyperosmotic) fish require various channels and exchangers to absorb electrolytes (e.g., Na^+) against the osmotic gradient. A PAH-induced increase in membrane Na^+ permeability would result in decreased Na^+ plasma concentrations as a result of increased passive Na^+ efflux. Decreased Na^+ plasma concentrations would serve to increase the activity of Na^+ uptake channels to modulate the homeostasis of Na^+. This Na^+-channel up-regulation would also serve to increase the uptake of any other metals that co-opt the Na^+ channel (e.g., Cu; Pyle et al., 2003). Oppositely, saltwater (i.e., hyposmotic) fish faced with increased membrane Na^+ permeability would experience increased Na^+ plasma concentration. Consequently, increased Na^+

plasma concentrations would serve to reduce the activity of Na^+ channels, and potentially reduce the uptake of metals that co-opt the Na^+ channel.

In addition, the effects of PAHs on membrane enzymes (Sikkema et al., 1995) have been linked to a disruption of ionoregulation, as several studies have reported both increases (Lemaire-Gony et al., 1995) and decreases (Englehardt et al., 1981; McCloskey and Oris,1993) in Ca_2^+- and Na^+/K^+-ATPase activity following exposure of fish to various PAHs. The mechanisms involved in P-type ATPase alteration remain unclear and have not been investigated using PAHs with aquatic organisms. Nonetheless, there is convincing evidence from mammalian models that suggests ROS are chiefly responsible for P-ATPase modulation (Rodrigo et al., 2002), either by disrupting the enzyme microenvironment within the membrane through lipid peroxidation or by oxidizing thiol groups of the enzymes themselves (Gamaley and Klyubin, 1999). The inhibition of P-type ATPases has clear consequences in terms of ion homeostasis and is also likely to inhibit the efflux of metals, such as Cu, through the deactivation of Cu-ATPase (Minghetti et al., 2008).

2.4.1.2 Metal-PAH complexation

There are emerging reports of metals and PAHs engaging in cation–π interactions, illustrating the potential for metal–PAH complexation. The symmetry of aromatic rings allows the p-orbital electrons of hydrogen atoms to be shared equally by all six hydrogen atoms in the ring. This results in a circular, or π-symmetry, distribution of electrons above and below the plane of the ring, and enables aromatic rings (i.e., PAHs), which are otherwise non-polar, the capacity to interact with cations. The majority of studies which have observed metal–PAH cation–π interactions were carried out in organic solvents or the gas phase (Crowley and Haendler, 1962; Lee et al., 2011), or have shown cation–π bonds using theoretical computations (Stöckigt, 1997; Lee et al., 2011; Dinadayalane and Hassan, 2012). Nonetheless, it seems likely that metals and PAHs have the

potential to form metal–PAH complexes in aqueous environments (Zhu et al., 2004a; Zhu et al., 2004b). It has been demonstrated that gas phase data are appropriate for modelling cation–π interactions among PAHs and several species of Al in water, as metal solvation has little influence (Kubicki et al., 1999). Moreover, it has been suggested that metal–phospholipid interactions can promote cation–π bonding in the vicinity of lipid membranes by negating the metal solvation penalty (Qu et al., 2007). The latter phenomenon is supported by the importance of cation–π bonding in a variety of biological molecules and structures (e.g., membranes), including ion channels, suggesting that metal–PAH cation–π interactions in aqueous media are common (Ma and Dougherty, 1997). In addition, PAHQs (e.g., hydroxylnapthoquinones) can also form hydrogen bonds with transition metals (e.g., Cu) via their quinone groups (Salunke-Gawali et al., 2005).

It is generally accepted that the free metal ion is the bioavailable metal species, and that metal complexation with ligands not specifically involved in the trans membrane transport of metals serves to reduce metal bioavailability (Morel, 1983; Niyogi and Wood, 2004). However, there has been considerable work demonstrating that lipophilic organometallic complexes can facilitate the bioaccumulation of metals (Poldoski, 1979; Phinney and Bruland, 1994; Tjalve and Borgneczak, 1994; Parthasarathy et al., 2008, 2010; Boullemant et al., 2009). In almost all reported cases, the complexation of metals with 8-hydroxyquinoline (HQ) and diethyldithiocarbamate (DDC) potentiated metal accumulation. Once taken up, the organometallic complexes allegedly dissociate, leaving each component to exert its individual toxicity. Thus, the increased toxicity observed in these studies has been attributed solely to increased metal body concentrations. It remains uncertain that metal–PAH complexation would have the same effect as metal–HQ and metal–DDC complexation on metal accumulation. However, the favourable conditions for metal–PAH complexation in the vicinity of lipid membranes would promote the accumulation of metal–PAH complexes within membranes, and potentially the trans membrane transport of metals.

Research on the potential toxicity and environmental risk associated with metal–PAH complexes, whether it be by increasing metal accumulation or by novel mechanisms associated with these unique complexes, remains scarce. Investigation into altered metal (e.g., Cu, Cd, and Ni) accumulation in the presence of PAHs has been limited to a few studies. Combined with the findings that PAHs can increase (Fair and Fortner, 1987; Gust and Fleeger, 2005; Wang et al., 2011b), decrease (Moreau et al., 1999; Xie et al., 2006), and have no effect on (Moore et al., 1984; Viarengo et al., 1987; Xie et al., 2006) metal accumulation in aquatic biota, it is difficult to make generalizations regarding the possibility of altered transport. Nonetheless, in none of these cases has Cu accumulation been enhanced, nor have PAHQs been shown to increase metal accumulation. The only cases where accumulation was seen to increase involved mixtures of Cd and PHE.

Metal–PAH complexes have yet to be identified in natural waters. However, the manufacturing of metal–PAH complexes for their desirable properties as nanomaterials (Sovoca et al., 2001; Murahashi et al., 2003; Baker and Head-Gordon, 2010) may represent a growing source of metal–PAH complexes. Furthermore, increasing exploration into the utility of engineered metal nanoparticles (NPs) presents a new avenue of potential metal–PAH complexation, as metal-NPs possess a high amount of unsatisfied bonds on their surface that have an enhanced affinity for neighbouring particles (as reviewed by Li et al., 2006), possibly even PAHs. For example, citrate-coated Au-NPs ($AuNP_{CIT}$) can bond with PHE. Similar to the increased bioavailability of metals when present as metal–HQ and metal–DDC complexes, PHE-bound $AuNP_{CIT}$ are more bioavailable than $AuNP_{CIT}$ (Farkas et al., 2012). As $AuNP_{CIT}$ are manufactured for their capacity to sequester Hg^{2+} from polluted waters (Ojea-Jimenez et al., 2012), there is also the potential for PHE-bound $AuNP_{CIT}$ to increase the bioavailability of any bound Hg^{2+}.

2.4.1.3 PAH-mucus complexation

PAHs are taken up by passive diffusion, substantially reducing the potential for metals to influence PAH transport (Bridges et al., 1987). Thus, reports of such outcomes are few and provide conflicting results (Fair and Sick, 1983; Benedetti et al., 2007; Ke et al., 2010; Wang et al., 2011b). Moreover, the endpoints used do not distinguish between altered accumulation and decreased PAH metabolism that is likely concurrent with metal exposure (see Section 2.3.2.1). Nonetheless, the favourable partitioning of waterborne PAHs to the mucus of epithelial tissue may be relevant in regards to co-exposure with metals. For example, exposures of Zn, Cu, Be, and Pb have been shown to increase the secretion of mucus in various tissues of aquatic invertebrates (Bouché et al., 2000; Rathore and Khangarot, 2003; Main et al., 2010) and fish (Jagoe et al., 1993; Sola et al., 1995; Khan and McGeer, 2013).

In the context of dietary exposure, the binding of metals to the gastrointestinal mucus is an essential process involved in the absorption of essential metals from the diet (Ojo and Wood, 2007). However, increased mucus production in response to elevated metal exposure could be an adaptive response to temporarily sequester excess metals prior to their excretion along with the sloughing of the mucus (Khan and McGeer, 2013). This process is supported by the observation that when gut mucus secretions were increased in response to elevated Zn, the bioavailability and subsequent lipid peroxidation of gut epithelia induced by dietary Cd exposure was decreased (Khan and McGeer, 2013). However, contrary to being an effective means to reduce the uptake and subsequent toxicity of excess consumed metals, the role of mucus on PAH uptake is not well described, and a recent report has indicated that PAH–mucus interactions, specifically the complexation of PAHs with mucin, an abundant glycoprotein in mucus, facilitates the accumulation of PAHs in protozoans (Drug et al., 2011). An increased production of mucus coincident with metal exposure potentially increases the capacity for PAHs to complex with mucin by virtue of more mucin being present,

suggesting that increased mucus production in epithelial tissues (e.g., gill and gastrointestinal) may lead to an increase in the bioavailability of waterborne and dietary PAHs. Nonetheless, due to the scarcity of evidence to support this mechanism of altered PAH bioavailability, further study is required to illustrate the importance of mucus in regards to PAH uptake.

2.4.2 Detoxification

Enzyme inhibition is an important mechanism to consider in terms of co-toxicity, particularly when the enzymes are involved in the transport, sequestration, or detoxification of a co-occurring contaminant. Whether by direct effects on the enzymes themselves, or by disrupting enzyme production, enzyme inhibition most likely plays a role in developing non-additive co-toxicity of metal–PAH mixtures. This sub-section will focus on modulatory effects of metals and PAHs on CYP1A1 and MT detoxification pathways involved with the co-occurring toxicant.

2.4.2.1 Metal-induced inhibition of CYP1A1

Cytochrome P450 1A1 enzymes are chiefly responsible for the phase 1 metabolism of PAHs, among other xenobiotics. As such, an inhibition of CYP1A1 production and/or activity has the potential to alter the toxic pathway of PAHs, as has been observed in fish co-exposed to PAHs and CYP1A1-inhibitors during their ELS. Although these types of studies typically use α-naphthoflavone and AHR-morpholinos as CYP and AHR inhibitors, there have been many reports of various metals inhibiting CYP1A1 activity as well. For example, the activity of CYP1A1 in several aquatic species was inhibited following exposure to As, Cd, Cu, Cr, Fe, Hg, Ni, and Zn (Bruschweiler et al., 1996; Sandvik et al., 1997; Faverney et al., 1999; Risso-De Faverney et al., 2000; Oliveira et al., 2003; Oliveira et al., 2004; Thompson et al., 2010; Wang et al., 2011a).

One explanation for metal-induced CYP1A1 enzyme inhibition arises from numerous reports of metals unanimously down-regulating the expression of PAH-induced CYP1A1 mRNA in fish

(Table 2.4). It may be that metals and metal-catalyzed ROS disrupt the activity of AHR transcription factors themselves. Alternatively, the transcription of CYP1A1 may be specifically inhibited downstream from PAH-induced AHR activation. Following the binding of a PAH with the AHR, the AHR is translocated to the nucleus where the aryl hydrocarbon nuclear translocator (ARNT) further directs the bonding of the PAH-bound AHR with the xenobiotic responsive element (XRE), which triggers the transcription of CYP1A1 mRNA (see Denison and Nagy, 2003 for a review of the AHR system). Although no aquatic models have been used to describe the role of metals in this process, the study of human hepatocytes has provided considerable mechanistic insight. For example, As (Anwar-Mohamed and El-Kadi, 2010), Hg (Amara and Anwar Mohamed, 2010), Pb (Korashy and El-Kadi, 2012), and V (Anwar-Mohamed and El-Kadi, 2008) decreased the induction of XRE-luciferase genes in HepG2 cells following exposure to various AHR agonists. Moreover, Pb, V, and most likely As and Hg as well due to the ligand binding properties of the AHR (Denison and Nagy, 2003), had no influence on total AHR protein content. Taken together, it is evident that these metals disrupt the transcription of CYP1A1 without being deleterious to AHRs.

Nonetheless, mechanistic details outlining why metal exposure decreases CYP1A1 gene expression is scarce. One partial explanation is the increased presence of other transcription factors concurrent with metal exposure that bind competitively with the ARNT. It has been postulated that certain metals, such as Co (Yuan et al., 2003), Cu (van-Heerden et al., 2004), V (Gao et al., 2002), and Ni (Salnikow et al., 2002) induce the transcription of hypoxia-inducible factor-α (HIF-α) which competes with the AHR to bind with ARNT (Kim and Sheen, 2000). Since this postulation was presented, several fish studies have supported an AHR-dependent down-regulation of CYP1A1 that is mediated by interactions with HIF-α (Fleming et al., 2009, Fleming and Di Giulio, 2011 and Rahman et al., 2012). Thus, it seems plausible that metals may be down-regulating CYP1A1 in this fashion. Alternatively, V-induced XRE gene modulation has been linked to an ATP-dependent

Table 2.4 Differential gene expression of fish cytochrome P450 1A (CYP1A) enzymes in response to various metals. Up- and down-regulated outcomes are indicated by '+' and '-' respectively. In cases where CYP1A was not pre-induced (i.e., no co-exposure to CYP1A inducers), differential gene expression was species and/or metal dependent. In 66.6% of cases where CYP1A was not pre-induced, CYP1A was up-regulated in response to metals. However, for all cases where CYP1A was pre-induced (i.e., in response to PAH exposure), CYP1A expression was down-regulated. 'liv' denotes liver, 'ova' denotes ovaries, 'mus' denotes muscle, 'B[α]P' denotes benzo[α]pyrene, '3MC' denotes 3-methylcholanthrene, 'LH' denotes luteinizing hormone, 'TCDD' denotes tetrachlorodibenzo-p-dioxin, and 'PCB-77' denotes polychlorinated biphenyl 77, and '-' and '+' denote down-and up-regulated responses.

Metal	Species	Assay	Tissue	CYP inducer	+/-	Source
As	*M. tomcod*	*in vivo*	liv	B[α]P	-	Sorrentino et al. (2005)
As	*D. rerio*	*in vivo*	liv	B[α]P	-	Thompson et al. (2010)
Cd	*S. cantharus*	*in vitro*	liv	3MC	-	Risso-De Faverney et al. (1999)
Cd	*O. mykiss*	*in vitro*	liv	3MC	-	Risso-De Faverney et al. (2000)
Cd	*M. tomcod*	*in vivo*	liv	B[α]P	-	Sorrentino et al. (2005)
Cd	*P. flesus*	*in vivo*	liv	B[α]P	-	Sheader et al. (2006)
Cd	*P. flesus*	*in vitro*	liv	3MC	-	Lewis et al. (2006)
Cd	*C. carpio*	*in vivo; in vitro*	ova	LH	-	Das and Mukherjee (2013)
Cd	*G. morhua*	*in vitro*	liv	baseline	-	Søfteland et al. (2010)
Cd	*T. bernacchii*	*in vivo*	liv	B[α]P	-	Benedetti et al. (2007)
Co	*O. mykiss*	*in vivo*	mus	baseline	+	Ceyhun et al. (2011b)
Cu	*T. bernacchii*	*in vivo*	liv	TCDD	-	Benedetti et al. (2009)
Cr	*M. tomcod*	*in vivo*	liv	B[α]P; PCB-77	-	Sorrentino et al. (2005)
Ni	*M. tomcod*	*in vivo*	liv	B[α]P	-	Sorrentino et al. (2005)
Zn	*O. mykiss*	*in vivo*	mus	baseline	+	Das and Mukherjee (2013)

mechanism through the inhibition of ecto-ATPases (Anwar-Mohamedand El-Kadi, 2008). As various other metals (e.g., Cd, Cu, and Hg) inhibit ecto-ATPase in mammalian models (Milosevic et al., 2005; Milosevic et al., 2009), it is possible that ATP-dependent mechanisms are also involved in the transcriptional inhibition of CYP1A1in aquatic biota.

Metal-induced transcriptional effects do not solely account for the level of CYP1A1 inhibition observed, suggesting that there must also be a post-transcriptional mechanism. One fundamental explanation for fish (Oliveira et al., 2003; Oliveira et al., 2004) and mammalian (Elbekai and El Kadi, 2004, 2005) models, is that the thiol group of CYP1A1 could be bound by metals, or oxidized by metal-catalyzed ROS, deactivating the enzyme. However, a more detailed explanation of ROS-related post-transcriptional inhibition is found in the activity of the antioxidant producer hemeoxygenase-1 (HO-1). Redox-active metals induce the transcription of HO-1 in fish (Søfteland et al., 2010; Wang and Gallagher, 2013), which serves to convert heme into the antioxidants biliverdin and bilirubin (Ariyoshi et al., 1990; Jorgensen et al., 1998), leading to a reduction in cellular heme content. As all CYP enzymes are heme proteins, the increased activity of HO-1 serves to render CYPs inactive by degrading their catalytic heme domains (Anwar Mohamed et al., 2012). Remarkably, up-regulation of HO-1 is more-than-additive in metal–PAH co-exposures (Kann et al., 2005), which illustrates the potential of metals to inhibit CYP1A1activity when co-exposed with PAHs. Furthermore, the combined effect of heme degradation and ROS-thiol interactions may serve to further potentiate CYP1A1 inhibition.

2.4.2.2 PAH-induced MT inhibition

As MT plays a key role in the detoxification of a variety of metals, MT modulation concomitant with metal exposure has potential to exacerbate metal toxicity. Although reports of PAH-induced MT modulation in aquatic biota are rare, virtually all have indicated that PAHs have

an inhibitory effect (George and Young, 1986; Sandvik et al., 1997; Risso-De Faverney et al., 2000; van den Hurk et al., 2000; Costa et al., 2010; Maria and Bebianno, 2011; Wang et al., 2011). The one reported exception used dietary exposures which effectively limited the MT response to the digestive tract in fish (Roesijadi et al., 2009) suggesting that MT induction is organ/tissue specific, and that altered MT activity in the gastrointestinal system of fish may not be involved in the potential more-than-additive toxicity of metal–PAH mixtures. Moreover, the nature of the co-toxic outcome in regards to MT modulation may be specific to waterborne (i.e., gill based) and dietary (i.e., gastrointestinal) exposure scenarios. Nonetheless, MT content in fish hepatocytes and mussel gill and hepatopancreas following waterborne co-exposure to metals and PAHs was significantly reduced in comparison to metal exposures alone, suggesting an increase in metal bioavailability and ensuing toxicity. Unfortunately, there is little evidence regarding the mechanisms by which PAHs inhibit MT. However, a comprehensive investigation into the processes involved in MT transcription, in combination with known PAH-induced toxicological outcomes, allows for speculation.

The metal-induced transcription of MT is ubiquitously controlled via the activation of metal–responsive elements (MREs) by the metal transcription factor-1 (MTF-1). MTF-1 is characterized by having six Zn fingers which upon binding with Zn induce a conformational change that triggers the translocation MTF-1 from the cytosol to the nucleus where it interacts with MREs (Chen et al., 2002). Zn clearly plays a role in MT expression, but it remains less clear as to how various other metals (e.g., Cd) up-regulate MT, as Cd produces an inhibitory effect on MTF-1/MRE binding when bound within the Zn fingers (Bittel et al., 1998). Zhang et al. (2003) proposed that exposure to non-Zn metals displaces Zn from storage proteins, allowing for the activation of MTF-1, and circumventing the requirement of direct non-Zn metal–MTF-1 interactions. Although this postulation provides a convenient explanation that has been supported in subsequent studies

(Cortese Krott et al., 2009; Nemec et al., 2009), it provides little explanation for PAH-induced MT-inhibition. Moreover, the only reported study of MT-related Zn–PAH co-toxicity found that 3-methylcholanthrene had no effect on Zn-induced MT induction (Risso-De Faverney et al., 2000), suggesting that Zn-dependent mechanisms may not be directly involved in PAH-induced MT inhibition in fish.

An alternative explanation resides in MT transcription increasing concomitantly with a rise in MTF-1 phosphorylation, which facilitates the translocation of MTF-1 to the nucleus. Multiple kinases are believed to be responsible for MTF-1 phosphorylation, including the Ca^{2+}-activated protein kinase C (PKC; Saydam et al., 2002). This is evidenced by the down-regulation of MT gene expression coincident with PKC-inhibition (Yuet al., 1997; Saydam et al., 2002). As PKC is Ca^{2+}-activated, the inhibition of Ca^{2+}-ATPase and subsequent decrease in cellular Ca^{2+} efflux associated with the exposure of aquatic organisms to a variety of metals (Shephard and Simkiss, 1978; Viarengo et al., 1996; Pattnaik and Jena, 2007; Vergani et al., 2007) in part explains why MT is up-regulated by non-Zn metals. However, although MTF-1 phosphorylation has been linked with translocation and MRT-1/MRE binding, a variety of MT-inhibitors also promote the phosphorylation of MTF-1 (Saydam et al., 2002). To reconcile this issue, Saydam et al. (2002) proposed that dephosphorylation after MTF-1/MRE binding must also take place for transcription to occur.

Similar to metals, exposure to PAHs elevates intracellular Ca^{2+} concentrations in fish (as reviewed by Reynaud et al., 2001; Reynaud and Deschaux, 2006), which is at least in part a result of Ca^{2+}-ATPase inhibition in the endoplasmic reticulum (Reynaud et al., 2001). Again, elevated Ca^{2+} provides one explanation for the increases in protein phosphorylation seen in aquatic organisms exposed to PAHs (Burlando et al., 2006; Châtel et al., 2010; Connelly and Means, 2010). However, the expected increase in Ca^{2+}-activated kinase activity and MTF-1 phosphorylation would facilitate

MFT-1 translocation and presumably MT transcription. Elevated intracellular Ca^{2+} following PAH exposure suggests that PAH-induced MT-inhibition may be independent of phosphorylation, as metal-induced phosphorylation typically serves to up-regulate MT transcription, and that inhibition is dependent on other mechanisms, such as phosphatase inhibition which would reduce MRE/MTF-1 dephosphorylation. Evidence for the latter explanation comes from the finding that H_2O_2, a product of PAHQ autoxidation, inactivates tyrosine phosphatase by oxidizing its sulfhydryl groups (Lee et al., 2002). This point is of particular relevance regarding MTF-1, as the phosphorylation of its tyrosine residues is required for its translocation (Saydam et al., 2002), and thus, according to the MTF-1 dephosphorylation hypothesis, dephosphorylation of its tyrosine residues is presumably required for MT transcription. Finally, PAHQs also have potential to irreversibly inactivate PKC, likely by arylation of cysteine sites in its catalytic domain (Yu et al., 2002). This point is also of particular importance regarding MT transcription, as PKC is the most important kinase involved in the phosphorylation of MTF-1 (Saydam et al., 2002).

2.4.3 Redox imbalance

The mutual induction of ROS by metals and PAHs, together with their effects on each other's transport and detoxification, suggest that the interactive contribution to ROS imbalance may be an important factor governing co-toxicity. Fundamentally, either metal–PAH mixtures facilitate the production of ROS or disrupt the homeostasis of ROS by various antioxidants (e.g., GST). Investigation into the transcriptional effects of metals on the GSH enzymes has revealed that GST transcription is in general up-regulated, as would be expected and most effective in response to oxidative stress. Nonetheless, there is considerable variation among species and isozymes (Tables 2.2 and 2.3). Furthermore, the effects of PAHs on GST transcription are equivocal, as there are reports describing up- (Nahrgang et al., 2009; Bilbao et al., 2010; Garner and Di Giulio, 2012; Yang

et al., 2012; Zhang et al., 2012) and down-regulation (Roh et al., 2012; Yang et al., 2012; Zhang et al., 2012).

One possible explanation for this inconsistency is that GST isozymes are distributed differently among (Srikanth et al., 2013) and within various tissues (Awasthi, 2007), which would influence their response based on a contaminant's toxicokinetics. For example, microsomal GSTs (membrane-associated proteins in eicosanoid and glutathione metabolism; MAPEG) are associated with membranes and thus would respond more effectively to lipid peroxidation compared to cytosolic GSTs (Hayes and Pulford, 1995). Additionally, the biological function of isozymes is believed to vary substantially between aquatic and terrestrial organisms (Konishi et al., 2005) and most likely between fish and invertebrates as well. For example, a closer look at specific GST isozymes reveals a trend in transcriptional effects among, but not between, fish (Table 3) and invertebrates (Table 2). Metals down-regulate the transcription of GSTρ and GSTm, and up-regulated the transcription GSTα and GSTκ in invertebrates, but the exact opposite is found for fish.

Additional problems with using GSTs to address co-toxicity include GST activity not always being intuitive given the differential gene expression (Bilbao et al., 2010) and the specificity of GST isozymes for the diverse assortment of ROS and ROS by-products associated with metal and PAH exposure. Taken together, regardless of the numerous studies observing contaminant effects on GST, there is still too much confusion to support whether transcriptional modulations of GST is a factor involved in metal–PAH co-toxicity. Nonetheless, post-transcriptional inhibition is still possible, as the arylation and elimination of PrSH by PAHQs suggests PAHQs may also arylate the cysteine sulfhydryl group of GSH. Together with the affinity of metals to bind with thiol groups, metal and PAH interactions with the GSH cysteine may deactivate its ROS scavenging and conjugative capabilities.

Additionally, there is evidence to support the facilitative role of PAHQs in metal–catalyzed ROS production. Recent works using the marine bacterium *Vibrio fischeri* (Wang et al., 2009) and the cladoceran *Daphnia magna* (Xie et al., 2006; Xie et al., 2007) have provided insight into a ROS-dependent mechanism involved in the more-than-additive lethality observed in metal–PAHQ mixtures. The reduction of PAHQs by CYP reductase or mitochondrial NADH:ubiquinone produces o-semiquinones that engage in futile redox cycling with o-quinone. This cycling provides an ideal reducing environment for the conversion of O_2 to $O_2^{\bullet-}$ (Flowers-Geary et al., 1993) which can then be converted to H_2O_2 by SOD or metal redox reactions. In the presence of H_2O_2, redox-active metals can engage in Fenton-like reactions to produce ●OH, and thus oxidative damage (e.g., lipid peroxidation). This phenomenon is supported by the finding that in the presence of Cu (i.e., a redox-active metal), phenanthrenequinone (PHEQ)-derived H_2O_2 content was significantly lower compared to mixtures of PHEQ with Cd (i.e., not redox-active), suggesting that Cu engaged in Fenton-like reactions (Wang et al., 2009).

In the absence of PAHQs, $O_2^{\bullet-}$ and H_2O_2 would be limited to endogenous sources, thus reducing the capacity of metals to produce ●OH. However, in the presence of PAHQs, increased H_2O_2 would potentiate the production of ●OH by redox-active metals, which would result in more-than-additive oxidative damage. Moreover, the transcription of SOD in a variety of aquatic organisms is mostly up-regulated in the presence of metals (Sheader et al., 2006; Kim et al., 2010a,b, 2011; Jiang et al., 2013), suggesting that the presence of metals may facilitate the production of H_2O_2, in turn enhancing the potential for redox-active metals to convert H_2O_2 to ●OH. It should be noted that the same experiments found no indication of non-additivity when PHE was mixed with Cu (Xie et al., 2006), which the authors attributed to the fact that PHE is not redox-active (i.e., not capable producing $O_2^{\bullet-}$). The conclusion that PHE exposure did not produce $O_2^{\bullet-}$

assumes that the degradation of PHE to PHEQ was minimal throughout the 48-h exposure period. However, in tests of longer duration the in vivo production of PAHQs may be an important consideration regarding the potentiation of oxidative stress, among other effects.

2.5 Summary

There are many similarities in the individual toxicities of metals and PAHs, as the major mechanisms involve ionoregulatory dysfunction and ROS imbalance. For this reason, it seems evident that metal–PAH co-toxicity could be described by the simple addition of individual contributions to these endpoints. However, 44.7% of the reported cases investigating metal–PAH co-toxicity in aquatic systems have found more-than-additive mortality (Table 2.1). Thus, co-exposure to metals and PAHs may produce unexpected effects that exacerbate the combined toxicities.

Most of the reported studies have applied exposure scenarios in which one of the contaminants is held below the threshold where it induces an effect towards the endpoint being observed. It is apparent from using no effect concentrations (NOECs) that even in the absence of substantive toxicity from one toxicant (i.e., the NOEC-toxicant), the toxicity of the mixture is still altered. The contribution of ionoregulatory dysfunction, enzyme disruption, or ROS imbalance provided by the NOEC-toxicant is not expected to be responsible for the observed more-than-additive co-toxicity. Thus, there must be an interactive effect of co-exposure whereby the >NOEC-toxicant by some means elevates the effect of the NOEC-toxicant beyond its no effect threshold, or the NOEC-toxicant serves to exacerbate the toxicity of the >NOEC-toxicant. As reviewed herein, there are several proposed mechanisms that could be responsible for either phenomenon.

Firstly, co-exposure of PAHs with metals has clear potential to elicit non-additive co-toxicity through CYP inhibition. In the context of sub-chronic carcinogenesis, metal co-exposures may be

beneficial to attenuate the development of cancer, as CYP metabolism contributes to the formation of carcinogenic PAH derivatives. However, the benefits of CYP inhibition by metal expo-sure have to be weighed against the costs of the toxicity incurred, which inevitably depends on the specific exposure scenario. Metal-induced CYP-inhibition most likely triggers a shift in PAH toxicity away from carcinogenicity to other chronic and/or acute effects. Reducing PAH metabolism increases the threat of acute membrane damage incurred from PAH parent compounds, as has been demonstrated for salmonid gill cell membranes. As the gills represent a major waterborne uptake pathway for many metals, PAH-induced gill membrane damage has the potential to alter metal bioavailability and disrupt ion homeostasis in waterborne exposure scenarios. It is also likely that an increased AKR metabolism of photo-derived dihydrodiols to o-quinones in a CYP-inhibited scenario increases the potential for oxidative damage and protein arylation.

Secondly, the role of PAHQs and PAHQ-derived ROS in MT inhibition becomes increasingly plausible with co-exposure to metals due to metal-induced CYP1A1 inhibition. This would shift the metabolism of PAHs to the AKR pathway promoting the formation of PAHQ metabolites. It is feasible that PAHQs are inhibiting the phosphorylation and dephosphorylation of MTF-1 by arylating PKC and oxidizing MTF-1 phosphatases, respectively, thereby down-regulating the transcription of MT. Further efforts are required to validate these mechanisms using aquatic species. Nonetheless, the observed PAH-induced MT-inhibition coinciding with metal exposure, regardless of its specific mechanism, most likely results in a more-than-additive toxicity due to an increase in the concentration of metals capable of interacting with other non-MT proteins in vivo.

Thirdly, the capacity for PAHs to increase metal bioavailability, either through membrane damage, or complexation, again suggests PAHs have the potential to exacerbate metal toxicity. Although studies regarding this phenomenon have produced ambiguous results, Cd influx appears to be increased in the presence of PHE. Still, the limited scope of investigations to date makes such

generalization premature. The potential for metal–PAH complexes warrants further investigation in terms of their occurrence in aquatic environments as well as their toxicity.

Fourthly, there is potential for interactive effects among these mechanisms leading to the enhanced toxicity of both contaminants. The likelihood that metals and PAHs are mutually disruptive to each other's detoxification suggests the possibility of positive feedback among these mechanisms. For example, the metal-induced CYP1A1inhibition leading to the formation of PAHQs would also serve to reduce MT transcription, elevating free metal concentrations, which in turn would further promote the production of PAHQs, and so on. Moreover, the heightened membrane damage due to the increased half-lives of parent PAHs associated with CYP1A1 inhibition could be yet another means to exacerbate exposure to metals, feeding into this cycle of co-toxicity.

Lastly, this review has focussed on the mechanisms which were best supported by evidence within the literature. This is not to say that other potential mechanisms are inconsequential in terms of more-than-additive metal–PAH toxicity. For example, the potential for increased bioavailability of PAHs in the form of PAH–mucin complexes as a result of metal-induced mucus production, or the possible role of PAH-induced lysosomal damage in altering the sequestration of metals in metal rich granules, are mechanisms requiring future investigation. However, there was simply insufficient evidence in the literature to warrant an in-depth discussion of the potential non-additive co-toxicity resulting from PAH–mucin complexation or PAH-induced lysosomal damage.

The individual mechanisms of metal and PAH toxicity vary by the specific metal and PAH involved, as well as the organism being exposed. Thus, it is reasonable to assume there will also be a certain degree of specificity associated with co-toxicity of their mixing. Moreover, the additivity of metal–PAH co-toxic interactions is likely to change based on mixture ratios (Wang et al., 2009). For example, if PAH-induced membrane damage were to substantially influence toxicity based on increased metal accumulation, a threshold concentration of the PAH would likely be required. Thus,

this mechanism may not be particularly important in mixtures where PAHs are at very low concentrations. Similarly, a particular range of mixture ratios would likely be necessary for metal–PAH complexation to influence toxicity. For example, in exposures where metals are present in very low concentrations, metal–PAH complexation may not occur due to the abundance of ligands with higher affinity than PAHs. Moreover, in exposure scenarios where one toxicant is present in very low concentration, complexation and increased influx may be inconsequential in light of the toxicity incurred by the second toxicant already occurring in lethal concentrations. These principles likely apply to the effects of metals and PAHs on each other's detoxification as well. However, as NOECs are typically based on cell viability in vitro or mortality in vivo, they are likely to still have effects on finer-scale endpoints, such as enzymatic activity, as has been indicated by the finding that sub-lethal concentrations of PHEQ potentiate Cu-induced ROS (Xie et al., 2006; Wang et al., 2009).

To date, all but one metal–PAH mixture study exploring additivity have used the NOECs approach, and thus, provide narrow insight into the infinite possibilities of mixture ratio scenarios in aquatic environments. Thus, the use of more comprehensive experimental designs (e.g., isoboles, as used by Wang et al., 2009) will aid in attaining a panoramic view of the possible ecological risks associated with metal–PAH mixtures. Finally, experiments that have addressed metal–PAH additivity have only incorporated binary mixtures, whereas environmental contamination is likely to include multiple contaminants, emphasizing that additivity must be addressed in terms of multiple contaminants in order to responsibly address the environmental threat metal–PAH mixtures pose.

2.6 References

Abendroth, J.A., Blankenship, E.E., Martin, A.R., Roeth, F.W., 2011. Joint action analysis utilizing concentration addition and independent action models. Weed Technology 25, 436-446.

Advaiti, B., Shanta, S., Abhijit, D., 2013. Bioaccumulation kinetics and bioconcentration factors for polycyclic aromatic hydrocarbons in tissues of *Rasbora daniconius*. International Journal of Chemical and Physical Sciences 2, 82-94.

Ahmad, I., Oliveira, M., Pacheco, M., Santos, M., 2005. *Anguilla anguilla* L. oxidative stress biomarkers responses to copper exposure with or without beta-naphthoflavone pre-exposure. Chemosphere 61, 267-275.

Amara, I.E.A., Anwar Mohamed, A., 2010. Mercury modulates the CYP1A1 at transcriptional and posttranslational levels in human hepatoma HepG2 cells. T 199, 225-233.

Amiard, J.C., Amiard Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquatic Toxicology 76, 160-202.

Anwar Mohamed, A., Klotz, L., El-Kadi, A.O.S., 2012. Inhibition of heme oxygenase-1 partially reverses the arsenite-mediated decrease of CYP1A1, CYP1A2, CYP3A23, and CYP3A2 catalytic activity in isolated rat hepatocytes. Drug Metabolism and Disposition 40, 504-514.

Anwar-Mohamed, A., El-Kadi, A.O.S., 2010. Arsenite down-regulates cytochrome P450 1A1 at the transcriptional and posttranscriptional levels in human HepG2 cells. Free Radical Biology & Medicine. 48, 1399-1409.

Anwar-Mohamed, A., El-Kadi, A.O.S., 2008. Down-regulation of the carcinogen-metabolizing enzyme cytochrome P450 1a1 by vanadium. Drug Metabolism and Disposition 36, 1819-1827.

Ariyoshi, T., Shiiba, S., Hasegawa, H., Arizono, K., 1990. Effects of the environmental pollutants on heme oxygenase activity and cytochrome P-450 content in fish. Bull. Environmental Contamination and Toxicology 44, 189-196.

Awasthi, Y.C., 2007. Toxicology of Glutathione Transferases. Taylor & Francis Group, LLC, Boca Raton, Fl.

Baird, W., Hooven, L., Mahadevan, B., 2005. Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environmental and Molecular Mutagenesis 45, 106-114.

Baker, T.A., Head-Gordon, M., 2010. Modeling the charge transfer between alkali metals and polycyclic aromatic hydrocarbons using electronic structure models. Journal of Physical Chemistry A 114, 10326-10333.

Barmo, C., Ciacci, C., Fabbri, R., Olivieri, S., Bianchi, N., Gallo, G., Canesi, L., 2011. Pleiotropic effects of hexavalent chromium (CrVI) in *Mytilus galloprovincialis* digestive gland. Chemosphere 83, 1087-1095.

Benedetti, M., Martuccio, G., Fattorini, D., Canapa, A., Barucca, M., Nigro, M., Regoli, F., 2007. Oxidative and modulatory effects of trace metals on metabolism of polycyclic aromatic hydrocarbons in the Antarctic fish *Trematomus bernacchii*. Aquatic Toxicology 85, 167-175.

Berenbaum, M.C., 1989. What is synergy? Pharmacological Reviews 41, 93-141.

Bhattacharya, P.K., 2005. 3. Alkali and alkaline earth metals ions in biochemical systems, in Metal Ions in Biochemistry. Alpha Science International Ltd. Harrow, UK, pp 66-85.

Bilbao, E., Raingeard, D., de Cerio, O.D., Ortiz Zarragoitia, M., Ruiz, P., Izagirre, U., Orbea, A., Marigómez, I., Cajaraville, M.P., Cancio, I., 2010. Effects of exposure to Prestige-like heavy fuel oil and to perfluorooctane sulfonate on conventional biomarkers and target gene transcription in the thicklip grey mullet *Chelon labrosus*. Aquatic Toxicology 98, 282-296.

Billiard, S., Meyer, J., Wassenberg, D., Hodson, P., Di Giulio, R., 2008. Nonadditive effects of PAHs on early vertebrate development: Mechanisms and implications for risk assessment. Toxicological Sciences 105, 5-23.

Billiard, S., Timme-Laragy, A., Wassenberg, D., Cockman, C., Di Giulio, R., 2006. The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycyclic aromatic hydrocarbons to zebrafish. Toxicological Sciences 92, 526-536.

Bittel, D., Dalton, T., Samson, S., Gedamu, L., Andrews, G.K., 1998. The DNA binding activity of metal response element-binding transcription factor-1 is activated *in vivo* and *in vitro* by zinc, but not by other transition metals. Journal of Biological Chemistry 273, 7127-7133.

Bouché, M.-L., Habets, F., Biagianti-Risbourg, S., Vernet, G., 2000. Toxic effects and bioaccumulation of cadmium in the aquatic oligochaete *Tubifex tubifex*. Ecotoxicology and Environmental Safety 46, 246-251.

Boullemant, A., Lavoie, M., Fortin, C., Campbell, P., 2009. Uptake of Hydrophobic Metal Complexes by Three Freshwater Algae: Unexpected Influence of pH. Environmental Sciences & Technology 43, 3308-3314.

Bridges, J.W., Ketterer, B., King, L.J., Matsumura, F., McGregor, D.B., Newberne, P.N., Parizek, J., Peakall, D.B., Wellburn, A.R., 1987. Metabolism and biochemical mechanisms of combined action, in Vouk, V.B., Butler, G.C., Upton, A.C., Parke, D.V., Asher, S.C. (Eds.), Methods for Assessing the Effects of Mixtures of Chemicals. John Wiley & Sons, Chichester, UK, pp. 73-98.

Bruschweiler, B.J., Wurgler, F.E., Fent, K., 1996. Inhibitory effects of heavy metals on cytochrome P4501A induction in permanent fish hepatoma cells. Archives of Environmental Contamination and Toxicology 31, 475-482.

Burlando, B., Berti, E., Viarengo, A., 2006. Effects of seawater pollutants on protein tyrosine phosphorylation in mussel tissues. Aquatic Toxicology 78 Suppl 1, S79-85.

Ceyhun, S.B., Şentürk, M., Yerlikaya, E., Erdoğan, O., Küfrevioğlu, Ö.İ., Ekinci, D., 2011a. Purification and characterization of carbonic anhydrase from the teleost fish *Dicentrarchus labrax* (European seabass) liver and toxicological effects of metals on enzyme activity. Environmental Toxicology and Pharmacology 32, 69-74.

Ceyhun, S.B., Aksakal, E., Ekinci, D., Erdogan, O., Beydemir, S., 2011b. Influence of cobalt and zinc exposure on mRNA expression profiles of metallothionein and cytocrome P450 in rainbow trout. Biological Trace Element Research 144, 781-789.

Châtel, A., Hamer, B., Talarmin, H., Dorange, G., Schroeder, H.C., Schröder, H.C., 2010. Activation of MAP kinase signaling pathway in the mussel *Mytilus galloprovincialis* as biomarker of environmental pollution. Aquatic Toxicology 96, 247-255.

Chen, W., John, J.A.C., Lin, C., Chang, C., 2002. Molecular cloning and developmental expression of zinc finger transcription factor MTF-1 gene in zebrafish, *Danio rerio*. Biochemical and Biophysical Research Communications 291, 798-805.

Cheung, A.P.L., Lam, T.H.J., Chan, K., 2004. Regulation of Tilapia metallothionein gene expression by heavy metal ions. Marine Environmental Research 58, 389-394.

Christie, N., Costa, M., 1984. In vitro assessment of the toxicity of metal compounds. IV. disposition of metals in cells: interactions with membranes, glutathione, metallothionein, and DNA. Biological Trace Element Research 6, 139-158.

Ciacci, C., Barmo, C., Gallo, G., Maisano, M., Cappello, T., D'Agata, A., Leonzio, C., Mauceri, A., Fasulo, S., Canesi, L., 2012. Effects of sublethal, environmentally relevant concentrations of hexavalent chromium in the gills of *Mytilus galloprovincialis*. Aquatic Toxicology 120-121, 109-118.

Cirillo, T., Cocchieri, R.A., Fasano, E., Lucisano, A., Tafuri, S., Amodio Cocchieri, R., Ferrante, M.C., Carpenè, E., Andreani, G., Isani, G., 2012. Cadmium accumulation and antioxidant responses in *Sparus aurata* exposed to waterborne cadmium. Archives of Environmental Contamination and Toxicology 62, 118-126.

Connelly, H., Means, J., 2010. Immunomodulatory effects of dietary exposure to selected polycyclic aromatic hydrocarbons in the bluegill (*Lepomis macrochirus*). International Journal of Toxicology 29, 532-545.

Conney, A.H., 1982. Induction of microsomal-enzymes by foreign chemicals and carcinogens by polycyclic aromatic-hydrocarbons. Cancer Research 42, 4875-4917.

Cortese Krott, M., Muenchow, M., Pirev, E., Hessner, F., Bozkurt, A., Münchow, M., Uciechowski, P., Pallua, N., Suschek, C., 2009. Silver ions induce oxidative stress and intracellular zinc release in human skin fibroblasts. Free Radical Biology & Medicine 47, 1570-1577.

Costa, P.M., Chicano-Galvez, E., Lopez Barea, J., DelValls, T.A., Costa, M.H., Chicano Gálvez, E., López Barea, J., 2010. Alterations to proteome and tissue recovery responses in fish liver caused by a short-term combination treatment with cadmium and benzo[a]pyrene. Environmental Pollution 158, 3338-3346.

Crowley, P.J., Haendler, H.M., 1962. Metal halide complexes of *o*-quinones. I. Preparation and preliminary characterization. Inorganic Chemistry 1, 904-909.

Curran, K.J., Irvine, K.N., Droppo, I.G., Murphy, T.P., 2000. Suspended solids, trace metal and PAH concentrations and loadings from coal pile runoff to Hamilton Harbour, Ontario. Journal of Great Lakes Research 281, 217-227.

Dalle Donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clinica Chemica Acta 329, 23-38.

Das, S., Mukherjee, D., 2013. Effect of cadmium chloride on secretion of 17β-estradiol by the ovarian follicles of common carp, *Cyprinus carpio*. General and Comparative Endocrinology 181, 107-114.

De Simone, G., Supuran, C.T., 2012. (In)organic anions as carbonic anhydrase inhibitors. Journal of Inorganic Biochemistry 111, 117-129.

Demirdag, R., Yerlikaya, E., Senturk, M., Kufrevioglu, O.I., Supuran, C., 2013. Heavy metal ion inhibition studies of human, sheep and fish alpha-carbonic anhydrases. Journal of Enzyme Inhibition and Medicinal Chemistry 28, 278-282.

Denison, M., Nagy, S., 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annual Reviews of Pharmacology and Toxicology 43, 309-334.

Di Giulio, R.T., Meyer, J.N., 2008. Reactive oxygen species and oxidative stress, in Di Guilio, R.T., Hinton, D.E. (Eds.), The Toxicology of Fishes. CRC Press, New York, NY, USA, pp. 273-324.

Dinadayalane, T.C., Hassan, A., 2012. A theoretical study of cation-p interactions: Li^{+}, Na^{+}, K^{+}, Be^{2+}, Mg^{2+} and Ca^{2+} complexation with mono- and bicyclic ring-fused benzene derivatives. Theoretical Chemistry Accounts 1, 131:1131.

Donahue, W.F., Allen, E.W., Schindler, D.W., 2006. Impacts of coal-fired power plants on trace metals and polycyclic aromatic hydrocarbons (PAHs) in lake sediments in central Alberta, Canada. Journal of Paleolimnology 35, 111-128.

Drug, E., Landesman-Milo, D., Belgorodsky, B., Ermakov, N., Frenkel-Pinter, M., Fadeev, L., Peer, D., Gozin, M., 2011. Enhanced bioavailability of polyaromatic hydrocarbons in the form of mucin complexes. Chemical Research in Toxicology 24, 314-320.

Elbekai, R., El Kadi, A.O.S., 2005. The role of oxidative stress in the modulation of aryl hydrocarbon receptor-regulated genes by As^{3+}, Cd^{2+}, and Cr^{6+}. Free Radical Biology & Medicine 39, 1499-1511.

Elbekai, R., El Kadi, A.O.S., 2004. Modulation of aryl hydrocarbon receptor-regulated gene expression by arsenite, cadmium, and chromium. Toxicology 202, 249-269.

Englehardt, F.R., Wong, M.P., Duey, M.E., 1981. Hydromineral balance and gill morphology in rainbow trout, *Salmo gairdneri*, acclimated to fresh and seawater, as affected by petroleum exposure. Aquatic Toxicology 1, 175-186.

Espinoza, H., Williams, C., Gallagher, E., 2012. Effect of cadmium on glutathione S-transferase and metallothionein gene expression in coho salmon liver, gill and olfactory tissues. Aquatic Toxicology 110-111, 37-44.

Eyckmans, M., Celis, N., Horemans, N., Blust, R., De Boeck, G., 2011. Exposure to waterborne copper reveals differences in oxidative stress response in three freshwater fish species. Aquatic Toxicology 103, 112-120.

Fair, P.A., Sick, L.V., 1983. Accumulations of naphthalene and cadmium after simultaneous ingestion by the black sea bass, *Centropristis striata*. Archives of Environmental Contamination and Toxicology 12, 551-557.

Fair, P.H., Fortner, A.R., 1987. Effect of ingested benzo[α]pyrene and cadmium on tissue accumulation, hydroxylase activity, and intestinal morphology of the black sea bass, *Centropristis striata*. Environ. Res. 42, 185-195.

Farkas, J., Nizzetto, L., Thomas, K.V., 2012. The binding of phenanthrene to engineered silver and gold nanoparticles. Science of the Total Environment 425, 283-288.

Fleeger, J.W., Gust, K.A., Marlborough, S.J., Tita, G., 2007. Mixtures of metals and polynuclear aromatic hydrocarbons elicit complex, nonadditive toxicological interactions in meiobenthic copepods. Environmental Toxicology and Chemistry 26, 1677-1685.

Fleming, C., Billiard, S., Di Giulio, R., 2009. Hypoxia inhibits induction of aryl hydrocarbon receptor activity in topminnow hepatocarcinoma cells in an ARNT-dependent manner. Comparative Biochemistry and Physiology C 150, 383-389.

Fleming, C., Di Giulio, R., 2011. The role of CYP1A inhibition in the embryotoxic interactions between hypoxia and polycyclic aromatic hydrocarbons (PAHs) and PAH mixtures in zebrafish (*Danio rerio*). Ecotoxicology 20, 1300-1314.

Flowers-Geary, L., Harvey, R., Penning, T., 1993. Cytotoxicity of polycyclic aromatic hydrocarbon o-quinones in rat and human hepatoma cells. Chemical Research in Toxicology 6, 252-260.

Fu, P., Xia, Q., Sun, X., Yu, H., 2012. Phototoxicity and Environmental Transformation of Polycyclic Aromatic Hydrocarbons (PAHs)-Light-Induced Reactive Oxygen Species, Lipid Peroxidation, and DNA Damage. J Environ. Sci. Heal. C 30, 1-41.

Gamaley, I.A., Klyubin, I.V., 1999. Roles of reactive oxygen species: signalling and regulation of cellular functions. Int. Rev. Cytol. 188, 203-255.

Gao, N., Ding, M., Zheng, J., Zhang, Z., Leonard, S., Liu, K., Shi, X., Jiang, B., 2002. Vanadate-induced expression of hypoxia-inducible factor 1 alpha and vascular endothelial growth factor through phosphatidylinositol 3-kinase/Akt pathway and reactive oxygen species. Journal of Biological Chemistry 277, 31963-31971.

Garner, L.V.T., Di Giulio, R., 2012. Glutathione transferase pi class 2 (GSTp2) protects against the cardiac deformities caused by exposure to PAHs but not PCB-126 in zebrafish embryos. Comparative Biochemistry and Physiology C 155, 573-579.

George, S.G., Young, P., 1986. The time course of effects of cadmium and 3-methylcholanthrene on activities of enzymes of xenobiotic metabolism and metallothionein levels in the plaice, *Pleuronectes platessa*. Comparative Biochemistry and Physiology C 83, 37-44.

Ghedira, J., Jebali, J., Bouraoui, Z., Banni, M., Guerbej, H., Boussetta, H., 2010. Metallothionein and metal levels in liver, gills and kidney of *Sparus aurata* exposed to sublethal doses of cadmium and copper. Fish Physiology and Biochemistry 36, 101-107.

Grintzalis, K., Georgiou, C., Dailianis, S., 2012. Total thiol redox status as a potent biomarker of PAH-mediated effects on mussels. Marine Environmental Research 81, 26-34.

Grundy, M.M., Ratcliffe, N.A., Moore, M.N., 1996. Immune inhibition in marine mussels by polycyclic aromatic hydrocarbons. Marine Environmental Research 42, 187-190.

Guschina, I.A., Harwood, J.L., 2006. Mechanisms of temperature adaptation in poikilotherms. FEBS Letters 580, 5477-5483.

Gust, K.A., 2006. Joint toxicity of cadmium and phenanthrene in the freshwater amphipod *Hyalella azteca*. Archives of Environmental Contamination and Toxicology 50, 7-13.

Gust, K.A., Fleeger, J.W., 2005. Exposure-related effects on Cd bioaccumulation explain toxicity of Cd-phenanthrene mixtures in *Hyalella azteca*. Environmental Toxicology and Chemistry 24, 2918-2926.

Hahn, M.E., 2002. Aryl hydrocarbon receptors: diversity and evolution. Chemico-Biological Interactions 141, 131-160.

Hamilton, S.J., Merhle, P.M., 1986. Metallothionein in fish: review of its importance in assessing stress from metal contaminants. Transactions of the American Fisheries Society 115, 596-609.

Hayes, J.D., Pulford, D.J., 1995. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Critical Reviews in Biochemistry and Molecular Biology 30, 445-600.

Henry, R.P., Boutilier, R.G., Tufts, B.L., 1995. Effects of carbonic anhydrase inhibition on the acid base status in lamprey and trout. Respiration Physiology 99, 241-248.

Ho, K.T., McKinney, R.A., Kuhn, A., Pelletier, M.C., Burgess, R.M., 1997. Identification of acute toxicants in New Bedford Harbour sediments. Environmental Toxicology and Chemistry 16, 551-558.

Hogstrand, C., Galvez, F., Wood, C.M., 1996. Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. Environmental Toxicology and Chemistry 15, 1102-1108.

Incardona, J.P., Linbo, T.L., Scholz, N.L., 2011. Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development. Toxicology and Applied Pharmacology 257, 242-249.

Isani, G., Andreani, G., Carpenè, E., Di Molfetta, S., Eletto, D., Spisni, E., 2011. Effects of waterborne Cu exposure in gilthead sea bream (*Sparus aurata*): a proteomic approach. Fish and Shellfish Immunology 31, 1051-1058.

Ivankovic, D., Pavicic, J., Beatovic, V., Klobucar, R., Klobucar, G.I.V., 2010. Inducibility of metallothionein biosynthesis in the whole soft tissue of zebra mussels *Dreissena polymorpha* exposed to cadmium, copper, and pentachlorophenol. Environmental Toxicology 25, 198-211.

Jagoe, C.H., Matey, V.E., Haines, T.A., Komov, V.T., 1993. Effect of beryllium on fish in acid water is analogous to aluminum toxicity. Aquatic Toxicology 24, 241-256.

Jiang, H., Vudathala, D., Blair, I., Penning, T., 2006. Competing roles of aldo-keto reductase 1A1 and cytochrome P4501B1 in benzo[a]pyrene-7,8-diol activation in human bronchoalveolar H358 cells: role of AKRs in P4501B1 induction. Chemical Research in Toxicology 19, 68-78.

Jiang, J., Wang, G., Mao, M., Wang, K., Li, S., Zeng, C., 2013. Differential gene expression profile of the calanoid copepod, *Pseudodiaptomus annandalei*, in response to nickel exposure. Comparative Biochemistry and Physiology C 157, 203-211.

Jorge, M., Loro, V., Bianchini, A., Wood, C., Gillis, P., 2013. Mortality, bioaccumulation and physiological responses in juvenile freshwater mussels (*Lampsilis siliquoidea*) chronically exposed to copper. Aquatic Toxicology 126, 137-147.

Jorgensen, M., Grosvik, B., Hamre, K., Goksoyr, A., Jørgensen, M., Grøsvik, B.E., Goksøyr, A., 1998. Induction of heme oxygenase in fish by heavy metals, phenylhydrazine and high lipid diets. Marine Environmental Research 46, 559-561.

Kann, S., Huang, M., Estes, C., Reichard, J., Sartor, M., Xia, Y., Puga, A., 2005. Arsenite-induced aryl hydrocarbon receptor nuclear translocation results in additive induction of phase I genes and synergistic induction of phase II genes. Molecular Pharmacology 68, 336-346.

Ke, L., Luo, L., Wang, P., Luan, T., Tam, N., 2010. Effects of metals on biosorption and biodegradation of mixed polycyclic aromatic hydrocarbons by a freshwater green alga *Selenastrum capricornutum*. Bioresource Technology 101, 6950-6961.

Keller, W., Heneberry, J.H., Gunn, J.M., 1999. Effects of emission reductions from the Sudbury smelters on the recovery of acid- and metal-damaged lakes. Journal of Aquatic Ecosystem Stress and Recovery 6, 189-198.

Kennedy, C., Farrell, A., 2005. Ion homeostasis and interrenal stress responses in juvenile Pacific herring, *Clupea pallasi*, exposed to the water-soluble fraction of crude oil. Journal of Experimental Marine Biology and Ecology 323, 43-56.

Khan, F.R., McGeer, J.C., 2013. Zn-stimulated mucus secretion in the rainbow trout (*Oncorhynchus mykiss*) intestine inhibits Cd accumulation and Cd-induced lipid peroxidation. Aquatic Toxicology 142-143, 17-25.

Kim, B., Rhee, J., Park, G., Lee, Y., Lee, J., 2011. Cu/Zn- and Mn-superoxide dismutase (SOD) from the copepod *Tigriopus japonicus*: molecular cloning and expression in response to environmental pollutants. Chemosphere 84, 1467-1475.

Kim, J.-H., Raisuddin, S., Rhee, J.-S., Lee, Y.-M., Han, K.-N., Lee, J.-S., 2009. Molecular cloning, phylogenetic analysis and expression of a MAPEG superfamily gene from the pufferfish *Takifugu obscura*. Comparative Biochemistry and Physiology C 149, 358-362.

Kim, J., Rhee, J., Dahms, H., Han, K., Lee, J., 2010a. Effect of cadmium exposure on expression of antioxidant gene transcripts in the river pufferfish, *Takifugu obscurus* (Tetraodontiformes). Comparative Biochemistry and Physiology C 152, 473-479.

Kim, J.-H., Dahms, H.-U., Rhee, J.-S., Lee, Y.-M., Lee, J., Han, K.-H., Lee, J.-S., 2010b. Expression profiles of seven glutathione S-transferase (GST) genes in cadmium-exposed river pufferfish (*Takifugu obscurus*). Comparative Biochemistry and Physiology C 151, 99-106.

Kim, J.E., Sheen, Y.Y., 2000. Inhibition of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-stimulated Cyp1a1 promoter activity by hypoxic agents. Biochemical Pharmacology 59, 1549-1556.

Kinraide, T.B., 2006. Plasma membrane surface potential (ψ_{pm}) as a determinant of ion bioavailability: a critical analysis of new and published toxicological studies and a simplified method for the computation of plant ψ_{pm}. Environmental Toxicology Chem 25, 3188-3198.

Konishi, T., Kato, K., Araki, T., Shiraki, K., Takagi, M., Tamaru, Y., 2005. A new class of glutathione S-transferase from the hepatopancreas of the red sea bream *Pagrus major*. Biochemical Journal 388, 299-307.

Korashy, H., El-Kadi, A.O.S., 2012. Transcriptional and posttranslational mechanisms modulating the expression of the cytochrome P450 1A1 gene by lead in HepG2 cells: A role of heme oxygenase. Toxicology 291, 113-121.

Kramer, N., Krismartina, M., Rico Rico, A., Blaauboer, B., 2012. Quantifying Processes Determining the Free Concentration of Phenanthrene in Basal Cytotoxicity Assays. Chemical Research in Toxicology 25, 436-445.

Kubicki, J.D., Blake, G.A., Apitz, S.E., 1999. Molecular models of benzene and selected polycyclic aromatic hydrocarbons in the aqueous and absorbed states. Environmental Toxicology and Chemistry 18, 1656-1662.

Lee, J.S., Krasnokutski, S.A., Yang, D., 2011. High-resolution electron spectroscopy, preferential metal-binding sites, and thermochemistry of lithium complexes of polycyclic aromatic hydrocarbons. Journal of Chemical Physics 134, 024301-1-024301-9.

Lee, K.-W., Raisuddin, S., Rhee, J.-S., Hwang, D.-S., Yu, I.T., Lee, Y.-M., Park, H.G., Lee, J.-S., 2008. Expression of glutathione *S*-transferase (GST) genes in the marine copepod *Tigriopus japonicas* exposed to trace metals. Aquatic Toxicology 89, 158-166.

Lee, S., Yang, K., Kwon, J., Lee, C., Jeong, W., Rhee, S., 2002. Reversible inactivation of the tumor suppressor PTEN by H_2O_2. Journal of Biological Chemistry 277, 20336-20342.

Lemaire-Gony, S., Lemaire, P., Pulsford, A.L., 1995. Effects of cadmium and benzo(a)pyrene on the immune system, gill ATPase and EROD activity of European sea bass *Dicentrarchus labrax*. Aquatic Toxicology 31, 297-313.

Le Pennec, G., Le Pennec, M., 2003. Induction of glutathione-S-transferases in primary cultured digestive gland acini from the mollusk bivalve *Pecten maximus* (L.): application of a new cellular model in biomonitoring studies. Aquatic Toxicology 64, 131-142.

Lewis, N.A., Williams, T.D., Chipman, J.K., 2006. Functional analysis of a metal response element in the regulatory region of flounder cytochrome P450 1A and implications for environmental monitoring of pollutants. Toxicological Sciences 92, 387-393.

Li, M., Zheng, Y., Liang, H., Zou, L., Sun, J., Zhang, Y., Qin, F., Liu, S., Wang, Z., 2013. Molecular cloning and characterization of *cat*, *gpx1* and Cu/Zn-*sod* genes in pengze crucian carp (*Carassius auratus* var. Pengze) and antioxidant enzyme modulation induced by hexavalent chromium in juveniles. Comparative Biochemistry and Physiology C 157, 310-321.

Li, R., Zuo, Z., Chen, D., He, C., Chen, R., 2011. Inhibition by polycyclic aromatic hydrocarbons of ATPase activities in *Sebastiscus marmoratus* larvae: Relationship with the development of early life stages. Marine Environmental Research 71, 86-90.

Li, X., Elliott, D., Zhang, W., 2006. Zero-valent iron nanoparticles for abatement of environmental pollutants: materials and engineering aspects. Critical Reviews in Solid State and Materials Sciences 31, 111-122.

Lima, A.L.C., Farrington, J.W., Reddy, C.M., 2005. Combustion-derived polycyclic aromatic hydrocarbons in the environment – a review. Environmental Forensics 6, 109-131.

Lionetto, M.G., Giordano, M.E., Vilella, S., Schettino, T., 2000. Inhibition of eel enzymatic activities by cadmium. Aquatic Toxicology 48, 561-571.

Liu, J., Goyer, R.A., Waalkes, M.P., 2008. Toxic effects of metals, in Klassen, C.D. (Ed.), Casarett & Doull's Toxicology: The Basic Science of Poisons, 7 ed. McGraw-Hill, Toronto, pp. 931-980.

Lu, G.H., Wang, C., Zhu, Z., 2009. The dose-response relationships for EROD and GST induced by polyaromatic hydrocarbons in *Carassius auratus.* Bulletin of Environmental Contamination and Toxicolgy 82, 194-199.

Lu, X., Chen, J., Huang, Z., Zhuang, L., Peng, L., Shi, Y., 2012. Influence of acute cadmium exposure on the liver proteome of a teleost fish, ayu (*Plecoglossus altivelis*). Molecular Biology Reports 39, 2851-2859.

Ma, J., Dougherty, D., 1997. The cation-π interaction. Chemical Reviews 97, 1303-1324.

Mackay, D., Shiu, W.Y., Ma, K.C., 2006. Illustrated Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals, 2 ed. Taylor & Francis Group, Boca Raton, FL.

Main, W.P.L., Ross, C., Bielmyer, G.K., 2010. Copper accumulation and oxidative stress in the sea anemone, *Aiptasia pallida*, after waterborne copper exposure. Comparative Biochemistry and Physiology C 151, 216-221.

Maracine, M., Segner, H., 1998. Cytotoxicity of metals in isolated fish cells: Importance of the cellular glutathione status. Comparative Biochemistry and Physiology A 120, 83-88.

Maria, V.L., Bebianno, M.J., 2011. Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper. Comparative Biochemistry and Physiology C 154, 56-63.

Matson, C.W., Timme-Laragy, A.R., Di Giulio, R.T., 2008. Fluoranthene, but not benzo[a]pyrene, interacts with hypoxia resulting in pericardial effusion and lordosis in developing zebrafish. Chemosphere 74, 149-154.

McCloskey, J., Oris, J., 1993. Effect of anthracene and solar ultraviolet radiation exposure on gill ATPase and selected hematologic measurements m the bluegill sunfish (*Lepomis macrochirus*). Aquatic Toxicology 24, 207-217.

Mielke, H.W., Wang, G., Gonzales, C.R., Le, B., Quach, V.N., Mielke, P.W., 2001. PAH and metal mixtures in New Orleans soils and sediments. Science of the Total Environment 281, 217-227.

Milosevic, M., Petrovic, S., Demajo, M., Horvat, A., 2005. Effects of metal ions on plasma membrane Mg^{2+}-ATPase in rat uterus and ovaries. Annals of the New York Academy of Sciences 1048, 445-448.

Milosevic, M., Petrovic, S., Stanojevic, I., Drakulic, D., Velickovic, N., Horvat, A., 2009. Effect of EDTA on copper-induced inhibition of rat myometrial ecto-ATPase activity. Russian Journal of Physical Chemistry A 83, 1592-1595.

Minghetti, M., Leaver, M.J., Carpenè, E., George, S.G., 2008. Copper transporter 1, metallothionein and glutathione reductase genes are differentially expressed in tissues of sea bream (*Sparus aurata*) after exposure to dietary or waterborne copper. Comparative Biochemistry and Physiology C 147, 450-459.

Minghetti, M., Leaver, M.J., George, S.G., 2010. Multiple Cu-ATPase genes are differentially expressed and transcriptionally regulated by Cu exposure in sea bream, *Sparus aurata*. Aquatic Toxicology 97, 23-33.

Minghetti, M., Leaver, M., Taggart, J., Casadei, E., Auslander, M., Tom, M., George, S., 2011. Copper induces Cu-ATPase ATP7A mRNA in a fish cell line, SAF1. Comparative Biochemistry and Physiology C 154, 93-99.

Moore, M.N., Widdows, J., Cleary, J.J., Pipe, R.K., Salkeld, P.N., Donkin, P., Farrar, S.V., Evans, S.V., Thomson, P.E., 1984. Responses of the mussel *Mytilus edulis* to copper and phenanthrene: Interactive effects. Marine Environmental Research 14, 167-183.

Moreau, C.J., Klerks, P.L., Haas, C.N., 1999. Interaction between phenanthrene and zinc in their toxicity to the sheepshead minnow (*Cyprinodon variegatus*). Archives of Environmental Contamination and Toxicology 37, 251-257.

Morel, F.M.M., 1983. Principles of Aquatic Chemistry. John Wiley & Sons. New York, NY.

Mu, J., Wang, X., Jin, F., Wang, J., Hong, H., 2012. The role of cytochrome P4501A activity inhibition in three- to five-ringed polycyclic aromatic hydrocarbons embryotoxicity of marine medaka (*Oryzias melastigma*). Marine Pollution Bulletin 64, 1445-1451.

Muniz, P., Danulat, E., Yannicelli, B., García-Alonso, J., Medina, G., Bícego, M.C., 2004. Assessment of contamination by heavy metals and petroleum hydrocarbons in sediments of Montevideo Harbour (Uruguay). Environment International 29, 1019-1028.

Murahashi, T., Uemura, T., Kurosawa, H., 2003. Perylene-tetrapalladium sandwhich complexes. Journal of the American Chemical Society 125, 8436-8437.

Naddafi, K., Zare, M.R., Nazmara, Sh., 2011. Investigating the potential toxicity of phenanthrene adsorbed to nano-ZnO using *Daphnia magna*. Toxicology & Environmental Chemistry 93, 729-737.

Nagasaka, R., Okamoto, N., Ushio, H., 2004. Partial oxidative-stress perturbs membrane permeability and fluidity of fish nucleated red blood cells. Comparative Biochemistry and Physiology C 139, 259-266.

Nahrgang, J., Camus, L., Gonzalez, P., Goksoyr, A., Christiansen, J., Goksøyr, A., Hop, H., 2009. PAH biomarker responses in polar cod (*Boreogadus saida*) exposed to benzo(a)pyrene. Aquatic Toxicology 94, 309-319.

Nair, P.M.G., Choi, J., 2011. Identification, characterization and expression profiles of *Chironomus riparius* glutathione S-transferase (GST) genes in response to cadmium and silver nanoparticles exposure. Aquatic Toxicology 101, 550-560.

Nair, P.M.G., Park, S.Y., Choi, J., 2012. Characterization and expression analysis of phospholipid hydroperoxide glutathione peroxidase cDNA from *Chironomus riparius* on exposure to cadmium. Comparative Biochemistry and Physiology B 163, 37-42.

Nebert, D.W., Dalton, T.P., 2006. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. Nature Reviews Cancer 6, 947-960.

Nemec, A., Leikauf, G., Pitt, B., Wasserloos, K., Barchowsky, A., 2009. Nickel mobilizes intracellular zinc to induce metallothionein in human airway epithelial cells. American Journal of Respiratory Cell and Molecular Biology 41, 69-75.

Niyogi, S., Wood, C., 2004. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. Environmental Sciences & Technology 38, 6177-6192.

Norwood, W.P., Borgmann, U., Dixon, D.G., Wallace, A., 2003. Effects of metal mixtures on aquatic biota: A review of observations and methods. Hum. Ecol. Risk Assess. 9, 795-811.

Nugroho, A., Frank, H., 2012. Effects of copper on lipid peroxidation, glutathione, metallothionein, and antioxidative enzymes in the freshwater mussel *Anodonta anatina*. Toxicology & Environmental Chemistry 94, 918-929.

O'Brien, P., 1991. Molecular Mechanisms of Quinone Cytotoxicity. Chemico-Biological Interactions 80, 1-41.

Ojea-Jimenez, I., Lopez, X., Arbiol, J., Puntes, V., 2012. Citrate-coated gold nanoparticles as smart scavengers for mercury(II) removal from polluted waters. ACS Nano 6, 2253-2260.

Ojo, A.A., Wood, C.M., 2007. *In vitro* analysis of the bioavailability of six metals via the gastro-intestinal tract of the rainbow trout (*Oncorhyncus mykiss*). Aquatic Toxicology 83, 10-23.

Oliveira, M., Santos, M.S., Pacheco, M., 2004. Glutathione protects heavy metal-induced inhibition of hepatic microsomal ethoxyresorufin *O*-deethylase activity in *Dicentrarchus labrax* L. Ecotoxicology and Environmental Safety 58, 379-385.

Oliveira, M., Santos, M.A., Gravato, C., Pacheco, M., 2003. Chromium effects on *Anguilla anguilla* liver organ culture. Fresenius Environmental Bulletin 12, 349-352.

Olsson, P.-E., 1996. Metallothioneins in fish: induction and use in environmental monitoring, in Taylor, E.W. (Ed.), Toxicology of Aquatic Pollution. Cambridge University Press, Cambridge, pp. 187-204.

Palanikumar, L., Kumaraguru, A.K., Ramakritinan, C.M., Anand, M., 2012. Biochemical response of anthracene and benzo[a]pyrene in milkfish *Chanos chanos*. Ecotoxicology and Environmental Safety 75, 187-197.

Parthasarathy, N., Pelletier, M., Buffle, J., 2010. Transport of lipophilic metal complexes through permeation liquid membrane, in relation to natural water analysis: Cu(II)-8-hydroxyquinoline complex as a model compound. Journal of Membrane Science 355, 78-84.

Parthasarathy, N., Pelletier, M., Buffle, J., 2008. Transport of lipophilic ligands through permeation liquid membrane in relation to natural water analysis. Journal of Membrane Science 309, 182-188.

Pathiratne, A., Hemachandra, C.K., 2010. Modulation of ethoxyresorufin O-deethylase and glutathione S-transferase activities in Nile tilapia (*Oreochromis niloticus*) by polycyclic aromatic

hydrocarbons containing two to four rings: implications in biomonitoring aquatic pollution. Ecotoxicology 19, 1012-1018.

Pattnaik, S., Jena, J.K., 2007. Characterization of Ca^{2+}-ATPase activity in gill microsomes of freshwater mussel, *Lamellidens marginalis* (Lamarck) and heavy metal modulations. Aquaculture 270, 443-450.

Penning, T., Ohnishi, S., Ohnishi, T., Harvey, R., 1996. Generation of reactive oxygen species during the enzymatic oxidation of polycyclic aromatic hydrocarbon trans-dihydrodiols catalyzed by dihydrodiol dehydrogenase. Chemical Research in Toxicology 9, 84-92.

Phinney, J.T., Bruland, K.W., 1994. Uptake of lipophilic organic Cu, Cd, and Pb complexes in the coastal diatom *Thalassiosira-weissflogii.* Environmental Sciences & Technology 28, 1781-1790.

Poldoski, J., 1979. Cadmium accumulation assays - their relationship to various ionic equilibria in Lake-Superior water. Environmental Sciences & Technology 13, 701-706.

Pyle, G.G., Couture, P., 2012. Field studies on metal accumulation and effects in fish in Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), Homeostasis and Toxicology of Essential Metals. Academic Press, Elsevier Inc., New York, NY, USA, pp 417-473.

Pyle, G.G., Kamunde, C.N., McDonald, D.G., Wood, C.M., 2003. Dietary sodium inhibits aqueous copper uptake in rainbow trout (*Oncorhynchus mykiss*). Journal of Experimental Biology 206, 609-618.

Qu, X., Wang, X., Zhu, D., 2007. The partitioning of PAHs to egg phospholipids facilitated by copper and proton binding via cation-π interactions. Environmental Sciences & Technology 41, 8321-8327.

Ragusa, M., Costa, S., Gianguzza, M., Roccheri, M., Gianguzza, F., 2013. Effects of cadmium exposure on sea urchin development assessed by SSH and RT-qPCR: metallothionein genes and their differential induction. Molecular Biology Reports 40, 2157-2167.

Rahman, M., Thomas, P., Karbstein, K., 2012. Effects of Hypoxia Exposure on Hepatic Cytochrome P450 1A (CYP1A) Expression in Atlantic Croaker: Molecular Mechanisms of CYP1A Down-Regulation. PLoS ONE 7, e40825.

Reynaud, S., Deschaux, P., 2006. The effects of polycyclic aromatic hydrocarbons on the immune system of fish: a review. Aquatic Toxicology 77, 229-238.

Reynaud, S., Duchiron, C., Deschaux, P., 2001. 3-Methylcholanthrene increases phorbol 12-myristate 13-acetate-induced respiratory burst activity and intracellular calcium levels in common carp (*Cyprinus carpio* L) macrophages. Toxicology and Applied Pharmacology 175, 1-9.

Rhee, J.-S., Lee, Y.-M., Hwang, D.-S., Won, E.-J., Raisuddin, S., Shin, K.-H., Lee, J.-S., 2007. Molecular cloning, expression, biochemical characteristics, and biomarker potential of theta class glutathione *S*-transferase (GST-T) from the polychaete *Neanthes succinea*. Aquatic Toxicology 83: 104-115.

Richter, C., 1987. Biophysical consequences of lipid peroxidation in membranes. Chemistry and Physics of Lipids 44, 175-189.

Risso-De Faverney, C., De Sousa, G., LaFaurie, M., Rahmani, R., 1999. Expression and induction of CYP1A1 in black seabream (*Spondyliosoma cantharus*) hepatoctye culture: effects of heavy metals. Polycyclic Aromatic Compounds 13, 285-299.

Risso-De Faverney, C., LaFaurie, M., Girard, J.P., Rahmani, R., 2000. Effects of heavy metals and 3-methylcholanthrene on expression and induction of CYP1A1 and metallothionein levels in trout (*Oncorhynchus mykiss*) hepatocyte cultures. Environmental Toxicology and Chemistry 19, 2239-2248.

Rodrigo, R., Trujillo, S., Bosco, C., Orellana, M., Thielemann, L., Araya, J., 2002. Changes in (Na+ K)-adenosine triphosphatase activity and ultrastructure of lung and kidney associated with oxidative stress induced by acute ethanol intoxication. Chest 121, 589-596.

Roesijadi, G., Rezvankhah, S., Perez Matus, A., Mitelberg, A., Torruellas, K., Van Veld, P.A., 2009. Dietary cadmium and benzo(a)pyrene increased intestinal metallothionein expression in the fish *Fundulus heteroclitus*. Marine Environmental Research 67, 25-30.

Roh, T., Kwak, M., Kwak, E., Kim, D., Han, E., Bae, J., Bang, D., Lim, D., Ahn, I., Jang, D., Lim, S., Yoo, S., Kwack, S., Park, K., Lee, Y., Kim, K., Lee, J., Kim, H., Lee, B., 2012. Chemopreventive mechanisms of methionine on inhibition of benzo(a)pyrene-DNA adducts formation in human hepatocellular carcinoma HepG2 cells. Toxicology Letters 208, 232-238.

Romero, D.L., Mounho, B.J., Lauer, F.T., Born, J.L., Burchiel, S.W., 1997. Depletion of glutathione by benzo(a)pyrene metabolites, ionomycin, thapsigargin, and phorbol myristate in human peripheral blood mononuclear cells. Toxicology and Applied Pharmacology 144, 62-69.

Rosen, B., 2002. Transport and detoxification systems for transition metals, heavy metals and metalloids in eukaryotic and prokaryotic microbes. Comparative Biochemistry and Physiology A 133, 689-693.

Rossignol, M., Uso, T., Thomas, P., 1985. Relationship between fluidity and ionic permeability of bilayers from natural mixtures of phospholipid. Journal of Membrane Biology 87, 269-275.

Salnikow, K., Davidson, T., Costa, M., 2002. The role of hypoxia-inducible signaling pathway in nickel carcinogenesis. Environmental Health Perspectives 110 Suppl 5, 831-834.

Salunke-Gawali, S., Rane, S., Boukheddaden, K., Codjovi, E., Linares, J., 2005. Thermal, magnetic and spectral studies of metal-quinone complexes Part III. Radical coordination and hydrogen bonding mediated exchange interaction in copper-hydroxyquinone complex. Journal of Thermal Analysis and Calorimetry 79, 669-675.

Sandvik, M., Beyer, J., Goksøyr, A., Hylland, K., Egaas, E., Skaare, J.U., 1997. Interaction benzo[*a*]pyrene, 2,3,3',4,4',5-hexachlorobyphenyl (PCB-156) and cadmium on biomarker responses in flounder (*Platichthys flesus* L.). Biomarkers 2, 153-160.

Saydam, N., Adams, T., Steiner, F., Schaffner, W., Freedman, J., 2002. Regulation of metallothionein transcription by the metal-responsive transcription factor MTF-1: identification of signal transduction cascades that control metal-inducible transcription. Journal of Biological Chemistry 277, 20438-20445.

Schirmer, K., Dixon, D.G., Greenberg, B.M., Bols, N.C., 1998. Ability of 16 priority PAHs to be directly cytotoxic to a cell line from the rainbow trout gill. Toxicology 127, 129-141.

Schlenk, D., Celander, M., Gallagher, E.P., George, S., James, M., Kullman, S.W., van der Hurk, P., Willet, K., 2008. Biotransformation in fishes, in Di Guilio, R.T., Hinton, D.E. (Eds.), The Toxicology of Fishes. CRC Press, New York, NY, USA, pp. 153-235.

Schlenk, D., Rice, C.D., 1998. Effect of zinc and cadmium treatment on hydrogen peroxide-induced mortality and expression of glutathione and metallothionein in a teleost hepatoma cell line. Aquatic Toxicology 43, 121-129.

Schmieder, P., Tapper, M., Kolanczyk, R., Hammermeister, D., Sheedy, B., 2003. Discriminating redox cycling and arylation pathways of reactive chemical toxicity in trout hepatocytes. Toxicological Sciences 72, 66-76.

Schober, W., Luch, A., Soballa, V.J., Raab, G., Stegeman, J.J., Doehmer, J., Jacob, J., Seidel, A., 2006. On the species-specific biotransformation of dibenzo[a,l]pyrene. Chemico-Biological Interactions 161, 37-48.

Scott, J.A., Hodson, P.V., 2008. Evidence for multiple mechanisms of toxicity in larval rainbow trout (*Oncorhynchus mykiss*) co-treated with retene and α-naphthoflavone. Aquatic Toxicology 88, 200-206.

Sevanian, A., Ursini, F., 2000. Lipid peroxidation in membranes and low-density lipoproteins: similarities and differences. Free Radical Biology & Medicine 29, 306-311.

Sheader, D.L., Williams, T.D., Lyons, B.P., Chipman, J.K., 2006. Oxidative stress response of European flounder (*Platichthys flesus*) to cadmium determined by a custom cDNA microarray. Marine Environmental Research 62, 33-44.

Shephard, K., Simkiss, K., 1978. The effects of heavy metal ions on Ca^{2+}ATPase extracted from fish gills. Comparative Biochemistry and Physiology B 61, 69-72.

Sikkema, J., de Bont, J.A., Poolman, B., 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiology and Molecular Biology Reviews 59, 201-222.

Sikkema, J., de Bont, J.A.M., Poolman, B., 1994. Interactions of cyclic hydrocarbons with biological membranes. Biological Chemistry 269, 8022-8028.

Sikkema, J., Poolman, B., Konings, W.N., de Bont, J.A.M., 1992. Effects of the membrane action of tetralin on the functional and structural properties of artificial and bacterial membranes. Journal of Bacteriology 174, 2986-2992.

Simpkins, A., Tatum, T., Cardin, D., Wolf, W., 2013. Metallothionein and heat-shock protein 70 induction in caged and wild fathead minnows (*Pimephales promelas*) exposed to the Ouachita River, Louisiana. Journal of Toxicology and Environmental Health A 76, 98-106.

Sinaie, M., Bastami, K., Ghorbanpour, M., Najafzadeh, H., Shekari, M., Haghparast, S., 2010. Metallothionein biosynthesis as a detoxification mechanism in mercury exposure in fish, spotted scat (*Scatophagus argus*). Fish Physiology and Biochemistry 36, 1235-1242.

Skaggs, H.S., Henry, R.P., 2002. Inhibition of carbonic anhydrase in the gills of two euryhaline crabs, *Callinectes sapidus* and *Carcinus maenas*, by heavy metals. Comparative Biochemistry and Physiology C 133, 605-612.

Søfteland, L., Holen, E., Olsvik, P., 2010. Toxicological application of primary hepatocyte cell cultures of Atlantic cod (*Gadus morhua*)--effects of BNF, PCDD and Cd. Comparative Biochemistry and Physiology C 151, 401-411.

Sola, F., Isaia, J., Masoni, A., 1995. Effects of copper on gill structure and transport function in the rainbow trout, *Oncorhynchus mykiss*. Journal of Applied Toxicology 15, 391-398.

Solé, M., Kopecka Pilarczyk, J., Blasco, J., 2009. Pollution biomarkers in two estuarine invertebrates, *Nereis diversicolor* and *Scrobicularia plana*, from a Marsh ecosystem in SW Spain. Environment International 35, 523-531.

Sørensen, H., Cedergreen, N., Skovgaard, Ib M., Streibig, J.C., 2007. An isobole-based statistical model and test for synergism/antagonism in binary mixture toxicity experiments. Environmental and Ecological Statistics 14, 383-397.

Sorrentino, C., Roy, N.K., Courtenay, S.C., Wirgin, I., 2005. Co-exposure to metals modulates CYP1A mRNA inducibility in Atlantic tomcod *Microgadus tomcod* from two populations. Aquatic Toxicology 75, 238-252.

Sovoca, M., Wende, T., Jiang, L., Langer, J., Meijer, G., Dopfer, O., Asmis, K.R., 2001. Infrared spectra and strcutres of silver-PAH cation complexes. Journal of Physical and Chemical Letters 2, 2052-2056.

Spokas, E., Spur, B., Smith, H., Kemp, F., Bogden, J., 2006. Tissue lead concentration during chronic exposure of *Pimephales promelas* (fathead minnow) to lead nitrate in aquarium water. Environmental Sciences & Technology 40, 6852-6858.

Sprovieri, M., Feo, M.L., Prevedello, L., Manta, D.S., Sammartino, S., Tamburrino, S., Marsella, E., 2007. Heavy metals, polycyclic aromatic hydrocarbons and polychlorinated biphenyls in surface sediments of the Naples harbour (southern Italy). Chemosphere 67, 998-1009.

Srikanth, K., Pereira, E., Duarte, A.C., Ahmad, I., 2013. Glutathione and its dependent enzymes' modulatory responses to toxic metals and metalloids in fish--a review. Environmental Science and Pollution Research 20, 2133-2149.

Stöckigt, D., 1997. Cation-π interaction in $Al(L)^+$ complexes (L = C_6H_6, C_5H_5N, C_5H_6, C_4H_4NH, C_4H_4O). Journal of Physical Chemistry A 101, 3800-3807.

Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radical Biology & Medicine 18, 321-336.

Sureda, A., Box, A., Tejada, S., Blanco, A., Caixach, J., Deudero, S., 2011. Biochemical responses of *Mytilus galloprovincialis* as biomarkers of acute environmental pollution caused by the Don Pedro oil spill (Eivissa Island, Spain). Aquatic Toxicology 101, 540-549.

Tai, W., Yang, Y., Lin, H., Huang, C., Cheng, Y., Chen, M., Yen, H., Liau, I., 2010. Interplay between structure and fluidity of model lipid membranes under oxidative attack. Journal of Physical Chemistry B 114, 15642-15649.

Tapper, M., Sheedy, B., Hammermeister, D., Schmieder, P., 2000. Depletion of cellular protein thiols as an indicator of arylation in isolated trout hepatocytes exposed to 1,4-benzoquinone. Toxicological Sciences 55, 327-334.

Teles, M., Pacheco, M., Santos, M.A., 2003. *Anguilla anguilla* L. liver ethoxyresorufin O-deethylation, glutathione S-transferase, erythrocytic nuclear abnormalities, and endocrine responses to naphthalene and beta-naphthoflavone. Ecotoxicology and Environmental Safety 55, 98-107.

Teuschler, L., Klaunig, J., Carney, E., Chambers, J., Conolly, R., Gennings, C., Giesy, J., Hertzberg, R., Klaassen, C., Kodell, R., Paustenbach, D., Yang, R., 2002. Support of science-based decisions concerning the evaluation of the toxicology of mixtures: a new beginning. Regulatory Toxicology and Pharmacology 36, 34-39.

Thompson, E.D., Burwinkel, K., Chava, A., Notch, E., Mayer, G., 2010. Activity of Phase I and Phase II enzymes of the benzo[a]pyrene transformation pathway in zebrafish (*Danio rerio*) following waterborne exposure to arsenite. Comparative Biochemistry and Physiology C 152, 371-378.

Tiwari, M., Nagpure, N.S., Saksena, D.N., Paul, B.N., 2012. Sequencing and expression of metallothionein mRNA in freshwater murrel after cadmium exposure. National Academy Science Letters 35, 323-330.

Tjalve, H., Borgneczak, K., 1994. Effects of lipophilic complex-formation on the disposition of nickel in experimental-animals. Science of the Total Environment 148, 217-242.

Tu, C.K., Wynns, G.C., Silverman, D.N., 1981. Inhibition by cupric Ions of ^{18}O exchange catalyzed by human carbonic anhydrase II - relation to the interaction between carbonic anhydrase and hemoglobin. Journal of Biological Chemistry 256, 9466-9470.

Vakharia, D.D., Liu, N., Pause, R., Fasco, M., Bessette, E., 2001. Effect of metals on polycyclic aromatic hydrocarbon induction of CYP1A1 and CYP1A2 in human hepatocyte cultures. Toxicology and Applied Pharmacology 170, 93-103.

Valette-Silver, N., Hameedi, M.J., Erfurd, D.W., Robertson, A., 1999. Status of the contamination in sediments and biota from the western Beaufort Sea (Alaska). Marine Pollution Bulletin 38, 702-722.

van den Hurk, P., Faisal, M., Roberts, M.H., 2000. Interactive effects of cadmium and benzo[a]pyrene on metallothionein induction in mummichog (*Fundulus heteroclitus*). Marine Environmental Research 50, 83-87.

Van Tiem, L., Di Giulio, R., 2011. AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE- and ARE-associated gene induction in zebrafish (*Danio rerio*). Toxicology and Applied Pharmacology 254, 280-287.

van-Heerden, D., Vosloo, A., Nikinmaa, M., 2004. Effects of short-term copper exposure on gill structure, metallothionein and hypoxia-inducible factor-1alpha (HIF-1α) levels in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 69, 271-280.

Vergani, L., Lanza, C., Borghi, C., Scarabelli, L., Panfoli, I., Burlando, B., Dondero, F., Viarengo, A., Gallo, G., 2007. Effects of growth hormone and cadmium on the transcription regulation of two metallothionein isoforms. Molecular and Cellular Endocrinology 263, 29-37.

Viarengo, A., Mancinelli, G., Mazzucotelli, A., Moore, M.N., Pipe, R.K., Farrar, S.V., 1987. Metallothioneins and lysosomes in metal toxicity and accumulation in marine mussels: the effect of cadmium in the presence and absence of phenanthrene. Marine Biology 94, 251-257.

Viarengo, A., Pertica, M., Mancinelli, G., Burlando, B., Canesi, L., Orunesu, M., 1996. In vivo effects of copper on the calcium homeostasis mechanisms of mussel gill cell plasma membranes. Comparative Biochemistry and Physiology C 113, 421-425.

Vijver, M., Van Gestel, C., Peijnenburg, W., Lanno, R., Van Straalen, N., 2004. Internal metal sequestration and its ecotoxicological relevance: a review. Environmental Sciences & Technology 38, 4705-4712.

Vouk, V.B., Butler, G.C., Upton, A.C., Parke, D.V., Asher, S.C., 1983. Methods For Assessing the Effects of Mixtures of Chemicals. John Wiley & Sons, Chichester, UK.

Wang, M., Wang, G., 2010. Oxidative damage effects in the copepod *Tigriopus japonicus* Mori experimentally exposed to nickel. Ecotoxicology 19, 273-284.

Wang, L., Pan, L., Liu, N., Liu, D., Xu, C., Miao, J., 2011. Biomarkers of bioaccumulation of clam *Ruditapes philippinarum* in response to combined cadmium and benzo[*a*]pyrene exposure. Food and Chemical Toxicology 49, 3407-3417.

Wang, L., Gallagher, E., 2013. Role of Nrf2 antioxidant defence in mitigating cadmium-induced oxidative stress in the olfactory system of zebrafish. Toxicology and Applied Pharmacology 266, 177-186.

Wang, W., Lampi, M., Huang, X., Gerhardt, K., Dixon, D.G., Greenberg, B., 2009. Assessment of mixture toxicity of copper, cadmium, and phenanthrenequinone to the marine bacterium *Vibrio fischeri*. Environmental Toxicology 24, 166-177.

Wassenberg, D.M., Di Giulio, R.T., 2004. Synergistic Embryotoxicity of Polycyclic Aromatic Hydrocarbon Aryl Hydrocarbon Receptor Agonists with Cytochrome P4501A Inhibitors in Fundulus heteroclitus. Environmental Health Perspectives 112, 1658-1664.

Wassenberg, D., Nerlinger, A., Battle, L., Di Giulio, R., 2005. Effects of the polycyclic aromatic hydrocarbon heterocycles, carbazole and dibenzothiophene, on in vivo and in vitro CYP1A activity and polycyclic aromatic hydrocarbon-derived embryonic deformities. Environmental Toxicology and Chemistry 24, 2526-2532.

Willett, K.L., Wassenberg, D., Lienesch, L., Reichert, W., Di Giulio, R.T., 2001. In Vivo and in Vitro Inhibition of CYP1A-Dependent Activity in *Fundulus heteroclitus* by the Polynuclear Aromatic Hydrocarbon Fluoranthene. Toxicology and Applied Pharmacology 177, 264-271.

Wills, L.P., Zhu, S., Willett, K.L., Di Giulio, R.T., 2009. Effect of CYP1A inhibition on the biotransformation of benzo[a]pyrene in two populations of *Fundulus heteroclitus* with different exposure histories. Aquatic Toxicology 92, 195-201.

Won, E., Kim, R., Rhee, J., Park, G., Shin, K., Lee, Y., Lee, J., 2011. Response of glutathione S-transferase (GST) genes to cadmium exposure in the marine pollution indicator worm, *Perinereis nuntia*. Comparative Biochemistry and Physiology C 154, 82-92.

Won, E., Rhee, J., Kim, R., Ra, K., Kim, K., Shin, K., Lee, J., 2012. Susceptibility to oxidative stress and modulated expression of antioxidant genes in the copper-exposed polychaete *Perinereis nuntia*. Comparative Biochemistry and Physiology C 155, 344-351.

Wootton, E.C., Dyrynda, E.A., Pipe, R.K., Ratcliffe, N.A., 2003. Comparisons of PAH-induced immunomodulation in three bivalve molluscs. Aquatic Toxicology 65, 13-25.

Xie, F.L., Koziar, S.A., Lampi, M.A., Dixon, D.G., Norwood, W.P., Borgmann, U., Huang, X.D., Greenberg, B.M., 2006. Assessment of the toxicity of mixtures of copper, 9,10-phenanthrenequinone, and phenanthrene to *Daphnia magna*: Evidence for a reactive oxygen mechanism. Environmental Toxicology and Chemistry 25, 613-622.

Xie, F., Lampi, M., Dixon, D.G., Greenberg, B., 2007. Assessment of the toxicity of mixtures of nickel or cadmium with 9,10-phenanthrenequinone to *Daphnia magna*: impact of a reactive oxygen-mediated mechanism with different redox-active metals. Environmental Toxicology and Chemistry 26, 1425-1432.

Yamuna, A., Bhavan, P.S., Geraldine, P., 2012. Glutathione S-transferase and metallothionein levels in the freshwater prawn *Macrobrachium malcolmsonhi* exposed to mercury. Journal of Environmental Biology 33, 133-137.

Yang, J., Wei, X., Xu, J., Yang, D., Liu, X., Fang, J., Hu, X., 2012. A sigma-class glutathione S-transferase from *Solen grandis* that responded to microorganism glycan and organic contaminants. Fish and Shellfish Immunology 32, 1198-1204.

Yu, C.W., Chen, J.H., Lin, L.Y., 1997. Metal-induced metallothionein gene expression can be inactivated by protein kinase C inhibitor. FEBS Letters 420, 69-73.

Yu, D., Kazanietz, M., Harvey, R., Penning, T., 2002. Polycyclic aromatic hydrocarbon *o*-quinones inhibit the activity of the catalytic fragment of protein kinase C. Biochemistry 41, 11888-11894.

Yu, H., Xia, Q., Yan, J., Herreno Saenz, D., Wu, Y., Fu, P., 2006. Photoirradiation of polycyclic aromatic hydrocarbons with UVA light - a pathway leading to the generation of reactive oxygen species, lipid peroxidation, and dna damage. International Journal of Environmental Research and Public Health 3, 348-354.

Yuan, Y., Hilliard, G., Ferguson, T., Millhorn, D., 2003. Cobalt inhibits the interaction between hypoxia-inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-alpha. Journal of Biological Chemistry 278, 15911-15916.

Zhang, B., Gunes, C., Georgiev, O., Hagmann, M., Günes, C., Cramer, M., Faller, P., Vasák, M., Schaffner, W., 2003. Activity of metal-responsive transcription factor 1 by toxic heavy metals and H_2O_2 in vitro is modulated by metallothionein. Molecular and Cellular Biology 23, 8471-8485.

Zhang, L., Qiu, L., Wu, H., Liu, X., You, L., Pei, D., Chen, L., Wang, Q., Zhao, J., 2012. Expression profiles of seven glutathione S-transferase (GST) genes from *Venerupis philippinarum* exposed to heavy metals and benzo[a]pyrene. Comparative Biochemistry and Physiology C 155, 517-527.

Zhu, D., Herbert, B.E., Schlautman, M.A., Carraway, E.R., 2004a. Characterization of cation-p interactions in aqueous solution using deuterium nuclear magnetic resonance spectroscopy. Journal of Environmental Quality 33, 276-284.

Zhu, D., Herbert, B., Schlautman, M., Carraway, E., Hur, J., 2004b. Cation-pi bonding: a new perspective on the sorption of polycyclic aromatic hydrocarbons to mineral surfaces. Journal of Environmental Quality 33, 1322-1330.

Chapter 3: Metal-polycyclic aromatic hydrocarbon mixture toxicity in *Hyalella azteca*. I. Response-surfaces and isoboles to measure non-additive mixture toxicity and ecological risk[1]

3.1 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-h 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, Canada's environmental water quality guidelines were analyzed to see if they would be protective. A 3-dimensional response-surface isobole model-based approach was used 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. The 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.

3.2 Introduction

The co-contamination of aquatic environments by polycyclic aromatic hydrocarbons (PAHs) and metals is an underexplored area of ecological risk assessment (Gauthier et al. 2014). Severe co-

[1] A modified version of this chapter has been published. Gauthier, P.G., Norwood, W.P., Prepas, E.E., Pyle, G.G., 2015. Metal-polycyclic aromatic hydrocarbon mixture toxicity in *Hyalella azteca*. I. Response-surfaces and isoboles to measure non-additive mixture toxicity and ecological risk. Environmental Science & Technology "Just Accepted", DOI: 10.1021/acs.est.5b03231

contamination of metals and PAHs occurs in a variety of coastal and freshwater environments around the globe (Ho et al., 1997, Valette-Silver et al., 1999, Curran et al., 2000, Mielke et al., 2001, Muniz et al., 2004, Donahue et al., 2006 and Sprovieri et al., 2007). 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 (Gauthier et al. 2014). However, regulatory authorities typically avoid considering the additivity of contaminants altogether, opting for whole effluent testing (WET) on a case-by-case basis (e.g., Canada, USA, Australia, and New Zealand) or when additivity is considered operate under the assumption of strictly-additive toxicity (e.g., Australia and New Zealand; Warne, 2003).

Unfortunately, the present state of knowledge regarding whole-organism (e.g., mortality and growth) and cellular (e.g., protein activity and gene transcription) endpoints 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. Firstly, researchers must employ experimental and statistical designs capable of discriminating non-additive toxicity from strictly-additive toxicity. Secondly, the application of these designs must be able to provide environmentally relevant information (i.e., relevant exposure concentrations or effect levels). Thirdly, experimental designs must encompass an array of mixture concentration scenarios to represent a variety of 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-surfaces (i.e., isobole analysis), providing predicted toxicity across all possible mixture scenarios (Sørensen et al. 2007). This approach can also be used to assess ecological risk by conducting the analyses at lower effect levels occurring at ecologically relevant concentrations. Accordingly, an isobole-based ecological risk assessment is presented for binary mixtures of metals (i.e., Cd, Cu, Ni, and V) and two PAHs, phenanthrene (PHE) and phenanthrenequinone (PHQ).

3.3 Theory

3.3.1 LC50 estimation

Concentration-response relationships for lethality experiments can be modelled according to proportional responses using the two-parameter logistic model:

$$p_i = 1/[1 + (c_i/LC50_i)^{ri}], \quad (3.1)$$

where p_i is the proportion of animals dying in response to the i^{th} toxicant, c_i is the concentration of the i^{th} toxicant, and r_i is the rate of increase in toxicity at the LC50 of the i^{th} 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 (Haanstra et al., 1985).

3.3.2 Response-surface construction and isobole modelling

An isobologram is produced from a 3-dimensional concentration-response surface with the concentration of each contaminant on the x-and y-axes, and proportional response on the z-axis (Fig. 3.1), where the isobologram is a cross section of the response-surface at the desired effect level. The

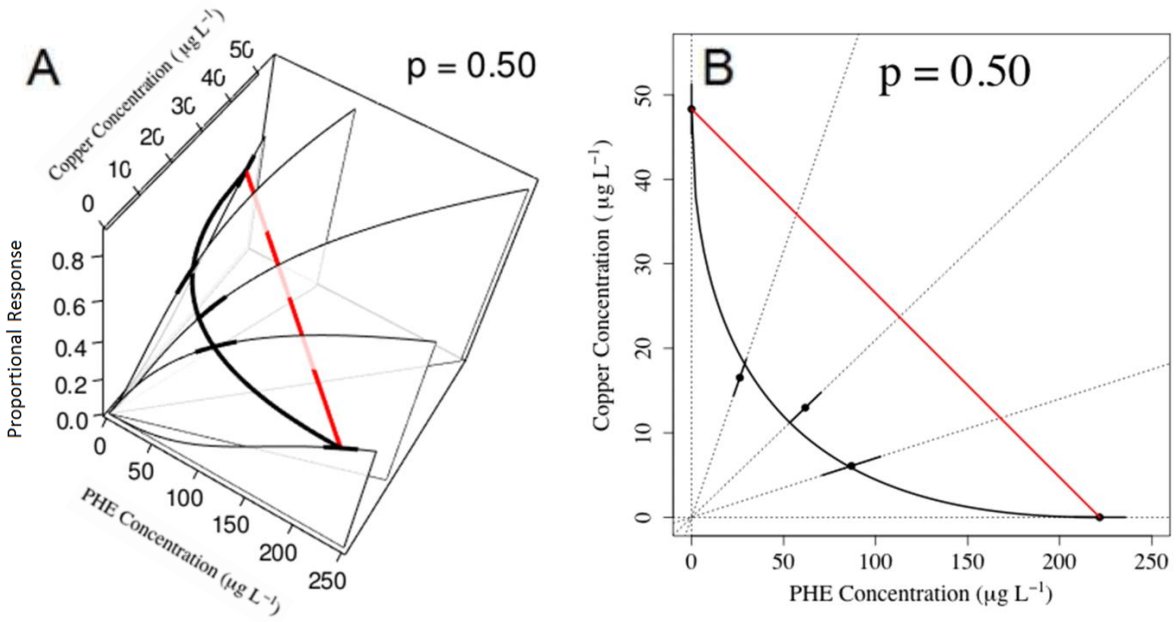

Figure 3.1 Three-dimensional response-surface (panel A) and two-dimensional isobologram containing 5 mixture proportions (vertical black curves in panel A and dotted lines in panel B). The response-surface x-, y-, and z-axes represent the concentration of phenanthrene (PHE) and Cu, and the proportion (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 curve represents the predicted isobole assuming strictly-additive co-toxicity.

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 (Gessner, 1988). The mixture concentrations are established with fixed proportions based on individual contaminant concentrations or toxic units (see sub-section 3.4.4. for details). The EC50s for the individual contaminants and fixed-mixture proportions of the contaminants can be estimated using Eq. 3.1, or by other LC50 estimation techniques. These EC50 data are then modelled to produce an isobole (i.e., a best-fit curve through the EC50s at $p = 0.5$ on the z-axis of the response-surface). An isobole

is literally a curve of equieffective response modelled to mixture EC50 data and predicts the mixture concentration required to elicit a 50% response across the entire isobologram. Isoboles can be plotted on the isobologram and are particularly useful in terms of visualizing mixture toxicity across a wide range of mixture proportions (Fig. 3.1). 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 (Hewlett and Plackett, 1959; Ritz and Striebig, 2005; Ritz and Striebig, 2014; R Core Team, 2015). For a detailed description of the theory and practice of Hewlett and Vølund models, see Ritz and Streibig (2014).

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 (Norwood et al., 2003):

$c_m = c_1 + (e \times c_2)$, (3.2)

where e is the proportional exchange rate between toxicants 1 and 2 calculated as $e = EC50_1/EC50_2$, 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. Predictions based on EA can be obtained using the following equation modified from Gessner (1988):

$p_m = p_1 + [p_2 \times (1 - p_1)]$, (3.3)

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. 3.2 and 3.3 for the desired response level (i.e., 50%) according to the specific fixed-mixture proportions. An iterative approach using Eq 3.1. 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 (Ritz and Striebig, 2005; R Core Team, 2015):

$$c_2 = [EC50_2^{1/\lambda} - (c_1 \times EC50_2/EC50_1)^{1/\lambda}]^{\lambda}, \quad (3.4)$$

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 modelling 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 λ ± se produces uncertainty intervals along the entire isoboles for comparisons at various mixture proportions.

3.3.3 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.

3.1 to 3.4 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. 3.1 to 3.4. 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.

3.4 Methods

3.4.1 Amphipod culturing

Hyalella azteca were obtained from the Aquatic Contaminants Research Division, Environment Canada (Burlington, ON) and cultured according to Borgmann (1996). Briefly, cultures were incubated in 2 L polyethylene containers held in water baths heated to 25°C and illuminated by two 40 watt 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 to 30 amphipods was fed 5 mg of finely ground Tetramin® fish flake three times per week.

3.4.2 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 counter ion 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 7 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 (Fisher Scientific).

3.4.3 Polydimethylsiloxane film preparation

Test concentrations of PAHs were controlled using a partition controlled delivery (PCD) system using PAH-enriched PDMS films. Enriched PDMS films were prepared by modifying the procedures described by Brown et al. (2001) and Kiparissis et al. (2003) to incorporate larger test volumes. Calibration values of PAH enriched films were based on Turcotte et al. (2011). A series of films were produced by mixing aliquots of PAH stock solutions into a 6 mg mL^{-1} PDMS:hexanes solution. Nine mL 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).

3.4.4 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 8 response-surfaces. Standard concentration series were applied for the estimation of LC50s with Eq. 3.1 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. The p_{ri} can be expressed as the proportion of PAH in mixture. Thus, each isobole contained 0, 0.25, 0.5, 0.75, and 1 proportional units of PAH, with $p_{ri} = 0$ and 1 representing the unary exposures for each metal and PAH respectively. Metal (C_{Mi}) and PAH (C_{PAHi})

mixture concentration series' for the i^{th} mixture proportion were based on the unary PAH concentration series' (U_{PAH}) and e, and were calculated as follows:

$C_{PAHi} = U_{PAH} \times p_{ri,}$ (3.5)

$C_{Mi} = U_{PAH} \times (1 - p_{ri}) \times e$ (3.6)

Following measurements for metal and PAH concentrations, p_{ri} 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. Ten to twenty 2 to 10 day old amphipods 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 3 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 endpoint and was determined at the end of each test as immobility with no pleopod movement.

For every p_{ri} tested (i.e., 5 per response-surface), identical metal and PAH concentration series (i.e., C_{PAHi} and C_{Mi}) 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_{ri} experiment was either repeated, or unary reference test data were used to derive a multiplication factor (Eqs. S3.1 and S3.3 in Supporting Information) to correct the mixture LC50 (see Supporting Information). A two-parameter log-logistic model (Eq. 3.1) was used for all LC50 estimations. The Hewlett model (Eq. 3.4) was used for all isoboles except the V-PHQ isobole as it exhibited substantial antagonism at one p_{ri}. 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 (Ritz and Striebig, 2005), was used to produce isoboles from CA and EA predictions. All statistical analyses were carried out in R 3.03 (R Core Team, 2015). An $\alpha = 0.05$ was applied for all tests of significance.

3.4.5 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_{ri} tested (i.e., 5 p_{ri} 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 simply 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 dissolved Ca^{2+} and Mg^{2+} and titration with 0.1N

H_2SO_4 to a pH of 4.5, respectively, and reported as mg $CaCO_3$ equivalents according to Clesceri et al. (1998a,b).

All PAHs were measured by HPLC (1200 Series, Agilent Technologies, ON, Canada) with a SDB-C18 5 μm 4.6x150 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 hours of sampling. Concentrations were determined with a calibration curve obtained from 10- to 1000-μg L^{-1} standard solutions (60:40 acetonitrile:test water) for PHE and PHQ.

3.5 Results

All log-logistic concentration-response model parameter estimates were satisfactory with appropriate uncertainty for the purpose of this analysis (Table S3.3). All Hewlett isoboles were well fit (Table S3.4). Estimation of η_1 for the V-PHQ Vølund model was not satisfactory (Table S3.4), and thus comparisons of observed versus predicted toxicity for V-PHQ mixtures was carried out based on LC50s from each p_{ri}. For each isobole and p_{ri} tested, Cu-PHE ($\lambda = 2.12 \pm 0.11$) and Cu-PHQ ($\lambda = 3.16 \pm .025$) mixture toxicity 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 3.2A,B). Similarly, Cd-PHE ($\lambda = 1.57 \pm 0.13$) and Cd-PHQ ($\lambda = 1.51 \pm 0.32$) isoboles were more-than-additive toxicity 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 3.2C,D). However, unlike the Cu-PAH mixtures, $p_{r0.36}$ and $p_{r0.59}$ for Cd-PHE and Cd-PHQ, respectively, produced strictly-additive

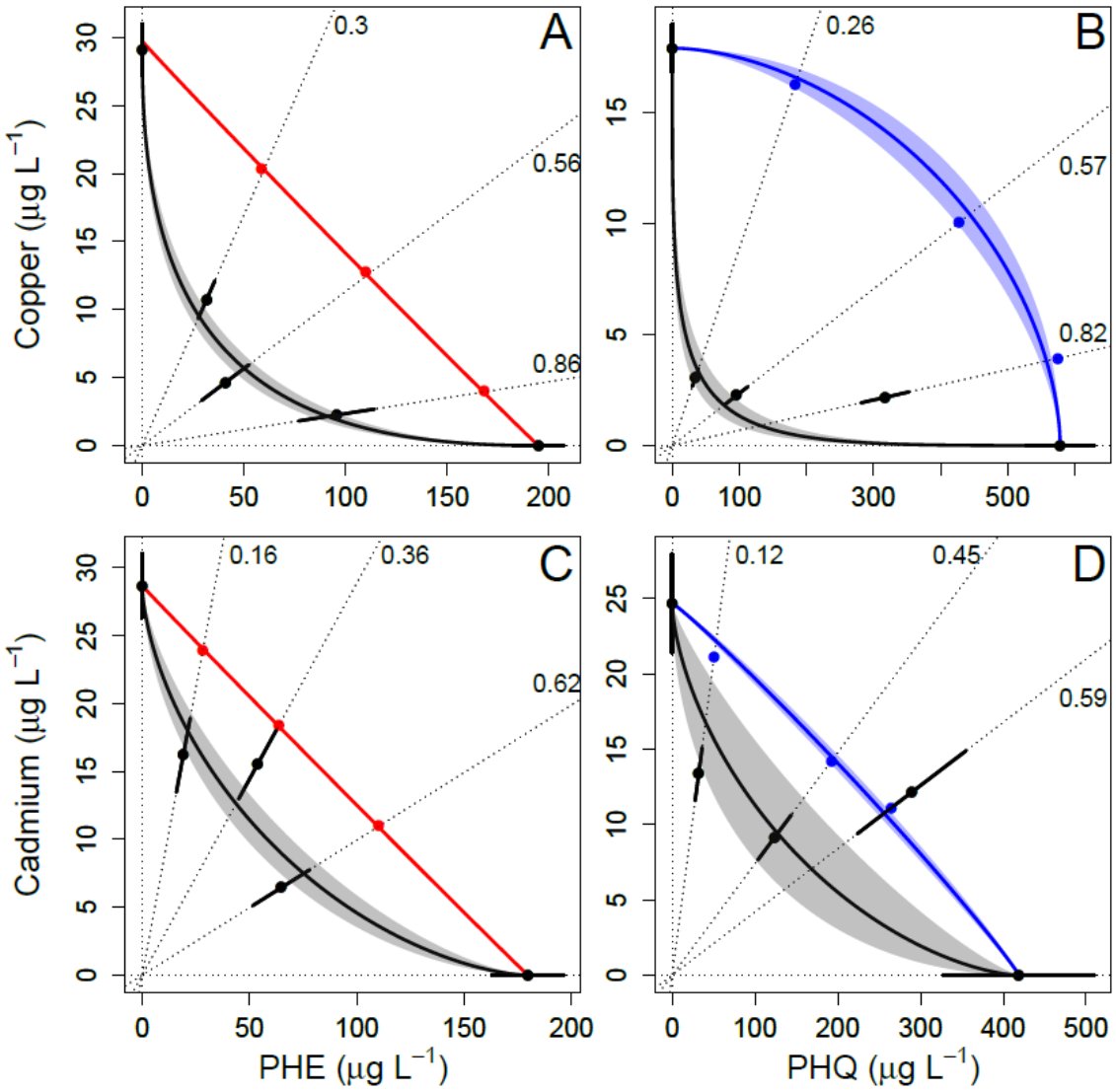

Figure 3.2 Isobolograms for mixtures of Cu (panels A and B) and Cd (panels C and D) with phenanthrene (PHE) or phenanthrenequinone (PHQ). Closed black circles represent LC50 ± se estimates, where red and blue closed circles represent LC50 predictions by concentration addition (CA) and effects addition (EA) respectively. Black curves and grey 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_{ri}), which are indicated numerically next to each curve.

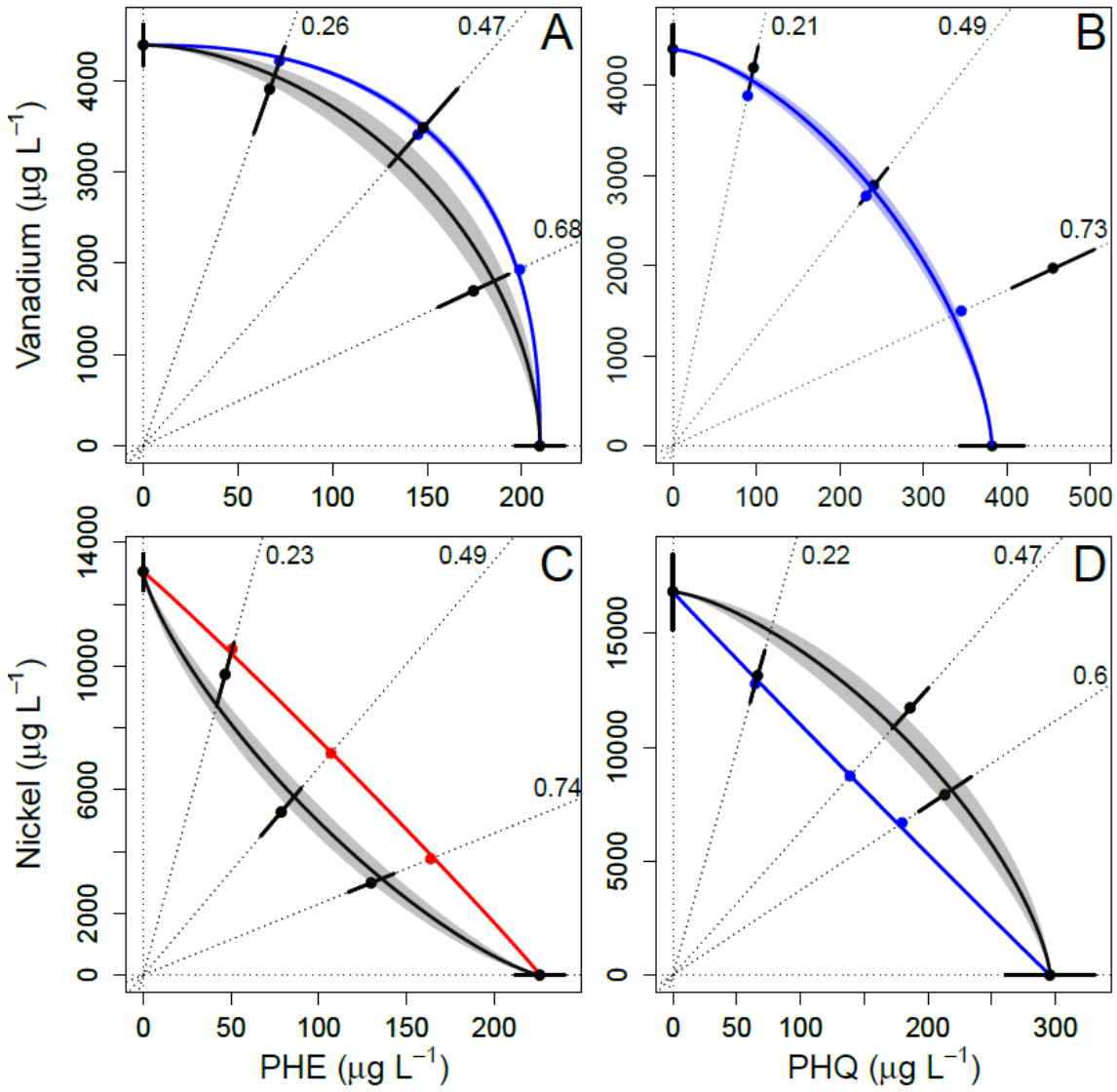

Figure 3.3 Isobolograms for mixtures of V and Ni with phenanthrene (PHE) or phenanthrenequinone (PHQ). Closed black, red, and blue circles are as described in Figure 3,2. Black curves and grey shaded regions, red curves and red shaded regions, and blue curves and blue shaded regions are as described in Figure 3.2. Dotted lines are as described in Figure 3.2. Neither Hewlett nor Vølund isobole models could be fit to experimental VPHQ data. Thus additivity for VPHQ mixtures can only be assessed based on the p_{ri} tested.

mortality. The effect of p_{ri} was also evident in mixtures involving Ni, where a $p_{r0.23}$ produced strictly-additive mortality, while $p_{r0.49}$ and $p_{r0.74}$ produced more-than-additive mortality (Figure 3.3C). Nonetheless, the Ni-PHE ($\lambda = 1.28 \pm 0.07$) isobole predicted slightly more-than-additive co-toxicity across all p_{ri} values compared to the CA isobole ($\lambda = 0.96 \pm 0.01$). The Ni-PHQ ($\lambda = 0.66 \pm 0.07$) isobole predicted less-than-additive co-toxicity compared to the EA isobole ($\lambda = 1.01 \pm 0.01$), although $p_{r0.22}$ produced strictly-additive mortality (Figure 3.3D). Although observed ($\lambda = 0.57 \pm 0.06$) and EA ($\lambda = 0.41 \pm 0.02$) VPHE isoboles indicated subtle more-than-additive toxicity, strictly-additive mortality was observed at $p_{r0.26}$ and $p_{r0.47}$ (Figure 3.3A). In V-PHQ mixtures, $p_{ri0.73}$ produced less-than-additive mortality, whereas strictly-additive mortality was observed at $p_{r0.21}$ and $p_{r0.49}$ (Figure 3.3B).

3.6 Discussion

3.6.1 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 3.1). Nonetheless, more-than-additive mixture mortality is common from exposure to binary mixtures of metal and PAHs. As these contaminants occur together in a variety of aquatic systems (Ho et al., 1997, Valette-Silver et al., 1999, Curran et al., 2000, Mielke et al., 2001, Muniz et al., 2004, Donahue et al., 2006 and Sprovieri et al., 2007), the potential for enhanced toxicity should be considered in terms of assessing regulatory guidelines for the protection of aquatic ecosystem health.

Table 3.1 Summary of cases identifying non-additive mortality in aquatic biota.[a]

Metal	PAH	Species	Exposures	Duration (h)	+/=/-	Source
Cd	phenanthrene	*S. knabeni*	sediment	96	+	[23]
Cd	phenanthrene	*S. knabeni*	aqueous	96	+	[23]
Cd	phenanthrene	*A. atopus*	aqueous	96	+	[23]
Cd	Phenanthrene	*H. templetoni*	sediment	240	- - -	[24]
Cd	phenanthrene	*H. azteca*	sediment	240	+	[25]
Cd	phenanthrene	*H. azteca*	aqueous	24; 48; 72	=; -; =	[25]
Cd	phenanthrene	*H. azteca*	aqueous	192	=	[26]
Cd	phenanthrene	*H. azteca*	aqueous	18	=	[27]
Cd	phenanthrene	*H. azteca*	aqueous	48	+ + =	
Cd	fluoranthene	*A. atopus*	aqueous	96	+	[23]
Cd	phenanthrenequinone	*V. fischeri*	aqueous	0.5; 0.75; 1	+ + =; + = =; = = =	[28]
Cd	phenanthrenequinone	*D. magna*	aqueous	48	=	[29]
Cd	phenanthrenequinone	*H. azteca*	aqueous	18	=	[27]
Cd	phenanthrenequinone	*H. azteca*	aqueous	48	+ + =	
Cu	phenanthrene	*D. magna*	aqueous	48	=	[30]
Cu	phenanthrene	*H. azteca*	aqueous	18	+	[27]
Cu	phenanthrene	*H. azteca*	aqueous	48	+ + +	
Cu	phenanthrenequinone	*D. magna*	aqueous	48	+ +	[30]
Cu	phenanthrenequinone	*V. fischeri*	aqueous	0.5; 0.75; 1	+ + = =; + + = -; = = = -	[28]
Cu	phenanthrenequinone	*H. azteca*	aqueous	18	+	[27]
Cu	phenanthrenequinone	*H. azteca*	aqueous	48	+ + +	
Ni	phenanthrene	*H. azteca*	aqueous	18	=	[27]
Ni	phenanthrene	*H. azteca*	aqueous	48	+ + =	
Ni	phenanthrenequinone	*D. magna*	aqueous	48	+	[29]
Ni	phenanthrenequinone	*H. azteca*	aqueous	18	=	[27]
Ni	phenanthrenequinone	*H. azteca*	aqueous	48	= - -	
V	phenanthrene	*H. azteca*	aqueous	18	=	[26]
V	phenanthrene	*H. azteca*	aqueous	48	= = =	
V	phenanthrenequinone	*H. azteca*	aqueous	18	=	[27]
V	phenanthrenequinone	*H. azteca*	aqueous	48	= = -	
Zn	phenanthrene	*C. variegatus*	aqueous	96	-	[31]
nano-Zn	phenanthrene	*D. magna*	aqueous	24; 48	+; +	[32]

[a]Adapted from Gauthier et al. (2014). More-than-, less-than-, 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.

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. (2006, 2007) 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. (2009) 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. 3.2 and 3.3. 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 (Gauthier et al, 2014). 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_{ri}. For example, in the analysis of Cu-PHQ mixtures, a small addition of either contaminant increased the toxicity of the mixture substantially, but additional relative increases of either contaminant by manipulating p_{ri} did not noticeably alter the LC50 values (Figure 3.2), 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 modelled using the Hewlett or Vølund models, or experimentally derived mechanistic models (Cheng and Bois, 2011), can provide predictions across a wide range of p_{ri}.

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]

3.6.2 Environmental risk of more-than-additive mixtures

More-than-additive metal-PAH mixture toxicity is a common and increasingly well-described phenomenon (Gauthier et al. 2014), and must be addressed in terms of ecological risk assessment and regulatory water quality guidelines (McCarty and Borgert, 2006). Based on the water quality criteria for SAM, with a water temperature of 20-to 22$^{-\circ}$C, a pH of 7.8 to 8.1, hardness of 121-to 126-mg L^{-1} as $CaCO_3$ eq., alkalinity of 49-to 52-mEq L^{-1}, and DOC <1 mg L^{-1}, the short-term Canadian Council of Ministers of the Environment (CCME) guideline 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 (CCME, nda-c; CCME, 1999; CCME, 2007a,b). There is no short-term CCME guideline for PHQ. For the purpose of this analysis, the PHE CCME guidelines will also be applied to PHQ. The guidelines for Cu, Cd, V, and Ni were derived using acute LC50 values at the 5th percentile of a species sensitivity distribution curve (CCME, 2007b). The guidelines value for PHE was determined using the lowest observable effect level (LOEL) multiplied by a protection factor of 0.1 (CCME, 1999). These guideline values are intended to represent safe concentrations for aquatic biota, and thus, should exert no adverse toxic effects (CCME, 2007b).

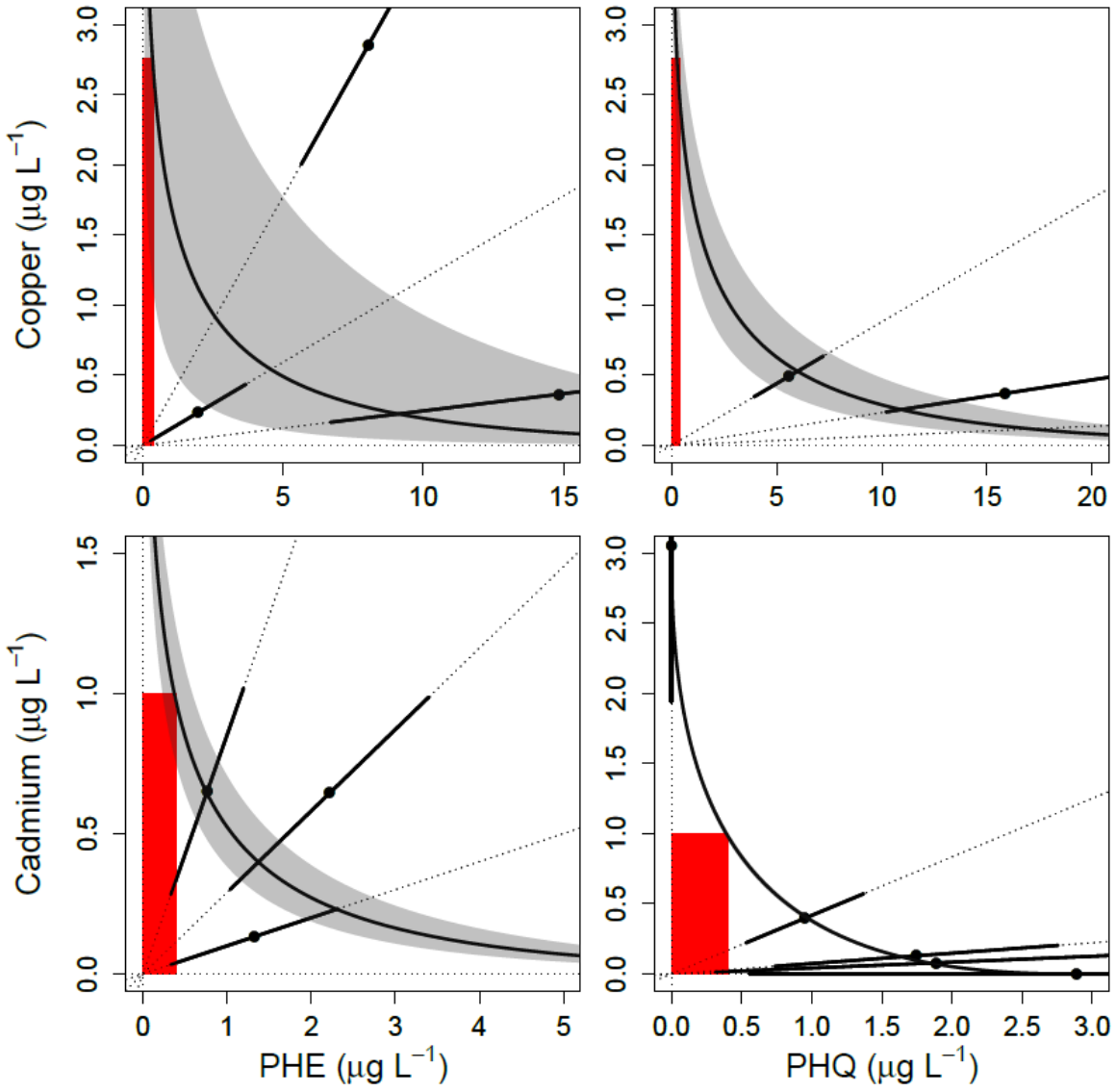

Figure 3.4 Isobole-based analysis of the protectiveness of the Canadian Council of Ministers of the Environment (CCME) short-term water quality guidelines of the protection of aquatic life from mixtures of Cu or Cd with phenanthrene (PHE) or phenanthrenequinone (PHQ). Closed black circles represent LC07.5, LC03.7, LC04.4, and LC01.4 ± se estimates for CuPHE, CuPHQ, CdPHE, and CdPHQ 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 CuPHE, CuPHQ, and CdPHE 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 grey shaded regions) and the guideline concentrations (red polygon).

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 3.4; Table S3.4). Mixtures of the 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.[40] However, these predictions must be validated experimentally, and must also be considered carefully in light of the following experimental details.

This 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. Also, only binary mixtures were considered, 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, only acute co-toxicity was considered, 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 the water quality guideline analysis must be sensitive to thr specific experimental conditions outlined in this work. 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.

3.7 References

Borgmann, U., 1996. Systematic analysis of aqueous ion requirements of *Hyalella azteca*: a standard artificial medium including the essential bromide ion. Archives of Environmental Contamination and Toxicology 30, 356-363.

Brown, R.S., Akhtar, P., Åkerman, J., Hampel, L., Kozin, I.S., Villerius, L.A., Klamer, H.J.C., 2001. Partition controlled delivery of hydrophobic substances in toxicity tests using poly(dimethylsiloxane) PDMS films. Environmental Science & Technology 35, 4097-4102.

CCME. nda. Canadian Council of Ministers of the Environment copper factsheet Website: http://st-ts.ccme.ca/en/index.html?lang=en&factsheet=71#aql_fresh_concentration.

CCME. ndb. Canadian Council of Ministers of the Environment nickel factsheet Website: http://st-ts.ccme.ca/en/index.html?lang=en&factsheet=139#aql_fresh_concentration.

CCME. ndc. Canadian Council of Ministers of the Environment vanadium factsheet Website: http://st-ts.ccme.ca/en/index.html?lang=fr&factsheet=226.

CCME. 1999. Canadian water quality guidelines for the protection of aquatic life: polycyclic aromatic hydrocarbons (PAHs): Canadian environmental quality guidelines, 1999, Canadian Council of Ministers of the Environment, 1999. http://ceqg-rcqe.ccme.ca/download/en/148.

CCME. 2007a. Canadian water quality guidelines for the protection of aquatic life: cadmium: Canadian environmental quality guidelines, 1999, Canadian Council of Ministers of the Environment, 2007. http://ceqg-rcqe.ccme.ca/download/en/148.

CCME. 2007b. A protocol for the derivation of water quality guidelines for the protection of aquatic life 2007: Canadian environmental quality guidelines, 1999, Canadian Council of Ministers of the Environment, 2007, http://www.ccme.ca/files/Resources/supporting_scientific_documents/protocol_aql_2007e.pdf

Cheng, S., Bois, F.Y., 2011. A mechanistic modeling framework for predicting metabolic interactions in complex mixtures. Environmental Health Perspectives 119, 1712-1718.

Clesceri, L.S., Greenberg, A.E., Eaton, A.D., 1998a. 2340 B. Hardness by calculation. In Standard Methods for the Examination of Water and Wastewater, 20th edition. American Public Health Association, Washington, DC, pp 2−37.

Clesceri, L.S., Greenberg, A.E., Eaton, A.D., 1998b. 2320 Alkalinity B. Titration Method. In Standard Methods for the Examination of Water and Wastewater, 20th edition. American Public Health Association, Washington, DC, pp 2−27-2−29.

Curran, K. J. Irvine, K.N. Droppo, I.G. Murphy, T.P., 2000. Suspended solids, trace metal and PAH concentrations and loadings from coal pule runoff to Hamilton Harbour, Ontario. Journal of Great Lakes Research 281, 217-227.

Donahue, W.F. Allen, E.W. Schindler, D.W., 2006. Impacts of coal-fired power plants on trace metals and polycyclic aromatic hydrocarbons (PAHs) in lake sediments in central Alberta, Canada. Journal of Paleolimnology 35, 111-128.

Fleeger, J.W., Gust, K.A., Marlborough, S.J., Tita, G., Mixtures of metals and polycyclic aromatic hydrocarbons elicit complex, nonadditive toxicological interactions in meiobenthic copepods. Environmental Toxicology and Chemistry 26, 1677-1685.

Gauthier, P.T. Norwood, W.P. Prepas, E.E. Pyle, G.G., 2014. Metal-PAH mixtures in the aquatic environment: A review of co-toxic mechanisms leading to more-than-additive outcomes. Aquatic Toxicology 154, 253-269.

Gessner, P.K., 1988. A straightforward method for the study of drug interactions: an isobolographic analysis primer. Journal of the American College of Toxicology 7, 987-1011.

Gust, K.A., Fleeger, J.W., 2006. Exposure to cadmium-phenanthrene mixtures elicits complex toxic responses in the freshwater tubificid oligochaete, *Hyodrilus templetoni*. Archives of Environmental Contamination and Toxicology 51, 54-60.

Gust, K.A., 2006. Joint toxicity of cadmium and phenanthrene to the freshwater amphipod *Hyalella azteca*. Archives of Environmental Contamination and Toxicology 50, 7-13.

Gust, K.A., Fleeger, J.W., 2005. Exposure-related effects on Cd bioaccumulation explain toxicity of Cd-phenanthrene mixtures in *Hyalella azteca*. Environmental Toxicology and Chemistry 24, 2918-2926.

Haanstra, L. Doelman, P. Oude Voshaar, J.H., 1985. The use of sigmoidal dose response curves in soil ecotoxicological research. Plant and Soil 84, 293-287.

Hewlett, P.S. Plackett, R.L., 1959. A unified theory for quantal responses to mixtures of drugs: non-interactive action. Biometrics 15, 591-610.

Ho, K.T. McKinney, R.A. Kuhn, A. Pelletier, M.C., Burgess, R.M., 1997. Identification of acute toxicants in New Bedford Harbour sediments. Environmental Toxicology and Chemistry 16, 551-558.

Kiparissis, Y., Akhtar, P., Hodson, P., Brown, R.S., 2003. Partition-controlled delivery of toxicants: a novel in vivo approach for embryo toxicity testing. Environmental Science & Technology 37, 2262-2266.

McCarty, L.S., Borgert, C.J., 2006. Review of the toxicity of chemical mixtures: theory, policy, and regulatory practice. Regulatory Toxicology and Pharmacology 45, 119-143.

Mielke, H.W. Wang, G. Gonzales, C.R. Le, B. Quach, V.N. Mielke, P.W., 2001. PAH and metal mixtures in New Orleans soils and sediments. Science of the Total Environment 281, 217-227.

Moreau, C.J., Klerks, P.L., Haas, C.N., 1999. Interaction between phenanthrene and zinc in their toxicity to the sheepshead minnow (*Cyprinodon variegatus*). Archives of Environmental Contamination and Toxicology 37, 251-257.

Muniz, P. Danulat, E. Yannicelli, B. García-Alonso, J. Medina, G. Bícego, M.C., 2004. Assessment of contamination by heavy metals and petroleum hydrocarbons in sediments of Montevideo Harbour (Uruguay). Environment International 29, 1019-1028.

Naddafi, K., Zare, M. R., Nazmara, Sh., 2011. Investigating the potential toxicity of phenanthrene adsorbed to nano-ZnO using *Daphnia magna*. Toxicology & Environmental Chemistry 93, 729-737.

Norwood, W.P. Borgmann, U., Dixon, D.G. Wallace, A., 2003. Effects of metal mixtures on aquatic biota: a review of observations and methods. Human and Ecological Risk Assessment 9, 795-811.

Ritz, C. Streibig, J.C., 2005. Bioassay analysis using R. Journal of Statistical Software 12, 1-22.

Ritz, C. Striebig, J.C., 2014. From additivity to synergism – A modelling perspective. Synergy 1, 22-29.

R Core Team, 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Sørensen, H. Cedergreen, N. Skovgaard, I.M. Streibig, J.C., 2007. An isobole-based statistical model and test for synergism/antagonism in binary mixture toxicity experiments. Environmental and Ecological Statistics 14, 383-397.

Sprovieri, M. Feo, M.L., Prevedello, L. Manta, D.S. Sammartino, S. Tamburrino, S. Marsella, E., 2007. Heavy metals, polycyclic aromatic hydrocarbons and polychlorinated biphenyls in surface sediments of the Naples harbor (southern Italy). Chemosphere 67, 998-1009.

Turcotte, D., Akhtar, P., Bowerman, M., Kiparissis, Y., Brown, R.S., Hodson, P.V., 2011. Measuring the toxicity of alkyl-phenanthrenes to early life stages of medaka (*Oryzias latipes*) using partition-controlled delivery. Environmental Toxicology and Chemistry 30, 487-495.

Valette-Silver, N. Hameedi, M.J., Erfurd, D.W. Robertson, A., 1999. Status of the contamination in sediments and biota from the western Beaufort Sea (Alaska). Marine Pollution Bulletin 38, 702-722.

Wang, W., Nykamp, J., Huang, X., Gerhardt, K., Dixon, D.G., Greenberg, B.M., 2009. Examination of the mechanism of phenanthrenequinone toxicity to *Vibrio fischeri*: evidence for a reactive oxygen species-mediated toxicity mechanism. Environmental Toxicology and Chemistry 28, 1655-1662.

Warne, M. St J., 2003. A review of the ecotoxicity of mixtures, approaches to, and recommendations for, their management. Proceedings of the Fifth National Workshop on the Assessment of Site Contamination 253-276.

Xie, F., Lampi, M.A., Dixon, D.G., Greenberg, B.M., 2007. Assessment of the toxicity of mixtures of nickel or cadmium with 9,10-Phenanthrenequinone to *Daphnia magna*: impact of reactive oxygen-mediated mechanisms with different redox-active metals. Environmental Toxicology and Chemistry 26, 1425-1432.

Xie, F., Koziar, S.A., Lampi, M.A., Dixon, D.G., Norwood, W.P., Borgmann, U., Huang, X., Greenberg, B.G., 2006. Assessment of the toxicity of mixtures of copper and 9,10-phenanthrenequinone, and phenanthrene to *Daphnia magna*: evidence for a reactive oxygen mechanism. Environmental Toxicology and Chemistry 25, 613-622.

3.8 Supporting information

3.8.1 PAH enriched PDMS film calibration

The film:water phenanthrene (PHE) and phenanthrenequinone (PHQ) concentration ratios were fitted by linear segmented regression (Muggeo, 2003; Figure S3.1). In order to assure polycyclic aromatic hydrocarbon (PAH) concentrations were static throughout the 48-h exposure period, samples were taken at the beginning and end of a range finder test (i.e., identical to unary toxicity test procedures). A two-way ANOVA of PAH concentrations in response to sampling date ($p > 0.05$) as well as the interaction between sampling date and treatment concentration ($p > 0.05$), followed by a Tukey's post-hoc test on each treatment concentration revealed there were no significant differences in PAH concentration throughout the 48-h exposure period (data not shown).

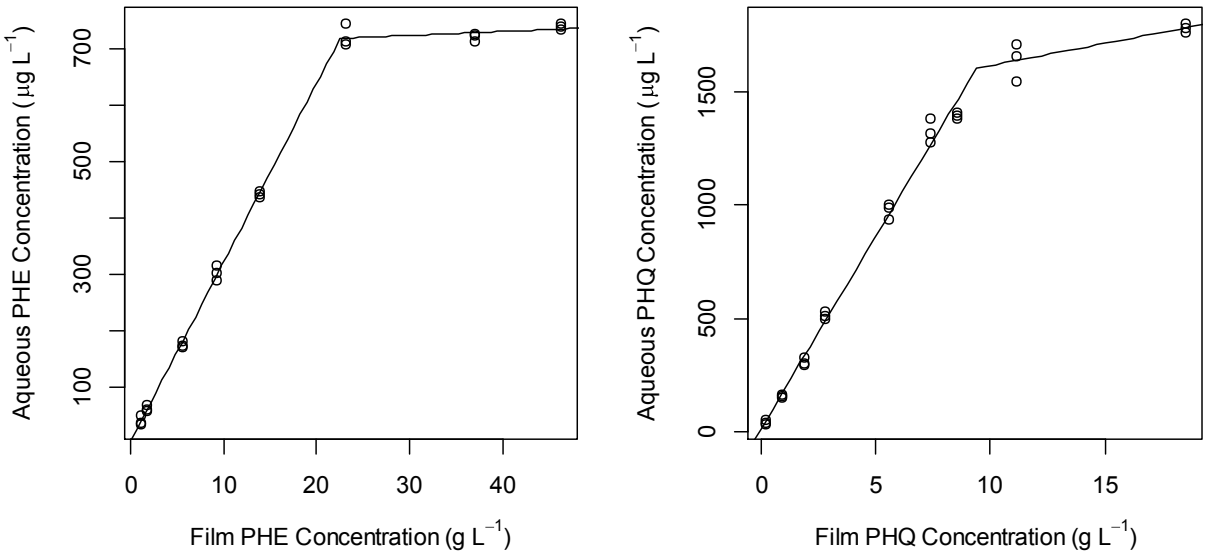

Figure S3.1 Phenanthrene (PHE) and phenanthrenequinone (PHQ) enriched polydimethylsiloxane (PDMS) films and their corresponding water concentrations at equilibrium.

3.8.2 Reference model selection

There has been much debate over the appropriate reference model for testing for non-additive mixture toxicity (Cedergreen et al. 2008). There are many who advocate the use of the

concentration addition (CA) model (Berenbaum, 1989; Greco, 1995). This is typically because the CA model is believed to be less fallible and more conservative than alternative methods regardless of what mechanistic processes are responsible for the toxicity of either contaminant, where the fallibility of the model is typically based on the ability of the model to accurately predict the mixture toxicity. However, the lack of fit of a given reference model to experimental data is just as likely to result from a non- additive co-toxic outcome as it is to result from the improper choice of the reference model (Abendroth et al., 2011). Ultimately, sound mechanistic rationale must be applied in selecting the appropriate reference model. Unfortunately, mechanistic data are generally incomplete. In fact, no published studies were found revealing toxic mechanisms for *H. azteca* for the contaminants studied herein. Thus, the criterion for model selection was to identify the most probable most lethal (MPML) mechanisms of action of Cu, Cd, Ni, V, PHE, and PHQ using literature involving crustaceans where possible. If the contaminants shared a MPML mechanism, the CA reference model was applied. If the contaminants did not share the MPML mechanism, the effects addition (EA) reference model was applied.

It was found that Cu, Cd, Ni, and PHE shared the MPML mechanism of ionoregulatory dysfunction (Brooks and Mills, 2003; Pane et al., 2003; Rainbow and Black, 2005; Felton et al., 2008; Hannam et al., 2010; Vellinger et al., 2012; Leonard and Wood, 2013; Zhang et al. 2013) and applied the CA reference model for mixtures involving Cu, Cd, Ni, and PHE. Quinone (O'Brien, 1991) and V (Goc, 2006) species in general are known to be diverse toxicants with potential to cause oxidative stress and enzyme dysfunction. However, no conclusive mechanisms for acute PHQ and V toxicity in aquatic invertebrates were found, and thus, applied the EA reference model for all mixtures involving V and/or PHQ.

3.8.3 Accounting for biotemporal variability in isobole experiments

Depending on the test organism being used and the number of mixture proportions being tested, it may not be possible to conduct all mixing proportion experiments simultaneously. Not being able to conduct all mixture proportion experiments simultaneously presents a challenge to precisely measuring the additivity of the mixture, as weekly variability in contaminant sensitivity jeopardizes the assumption that individual toxicities remain unchanged. For example, if a weekly cohorts' sensitivity to one or both of the contaminants decreased in comparison to the initial sensitivity for unary exposures, the interpretation of the additive nature of the mixture would be biased to reporting a lesser degree of potentiated co-toxicity (e.g., a true more-than-additive outcome could be observed as a strictly-additive or less-than-additive outcome). In the present study, changes in weekly tolerances were identified by non-overlapping standard errors of the estimated weekly LC50s. We chose to use standard errors instead of 95% confidence intervals (CI) as standard errors produced more conservative comparisons (i.e., the use of standard errors were more likely to detect changes in weekly tolerances), whereas the use of CI's failed to detect differences that were likely to be biologically/toxicologically important (Table S3.1 and S3.2).

Repeating experiments where references tests have failed is not ideal due to the time constraints, cost of materials, unnecessary sacrifice of additional test animals, and the effects on the mental wellbeing of the researcher. As such, an alternative method to account for weekly shifts in individual contaminant toxicities is desirable. Here we propose a simple algorithm for binomial response data based on weekly reference tests and CA and EA models that applies a multiplication factor (*MF*) to transform LC50 estimates for any given mixture proportion (p_{ri}) experiment. As mixture concentrations for isobole experiments are typically derived from the concentrations tested for $p_{ri} = 0$ or 1 (see Section 3.4.4), the following approach transforms mixture LC50 estimates for $p_{ri} \neq 0$ or 1 having a shift in tolerance for one or both contaminants in the mixture to allow for the

assumption that individual toxicant tolerances were unchanged compared $p_{ri} = 0$ or 1. For simplicities sake, the following explanation shall be in the context of the LC50. However, the algorithm can be applied to any effect level.

For CA scenarios, a multiplication factor (*MF*) was obtained using the following equation:

$$MF_i = TU_i/(uc_{mi}/LC50_1), \text{ (S3.1)}$$

where c_{mi} represents the uncorrected LC50s (i.e., calculated as if there were no change in weekly tolerance or exchange rate of toxicant 1) for the i^{th} mixture proportion calculated using Eq. 3.1, TU_i represents the toxic units of uc_{mi} according to the original LC50 for toxicant 1 (i.e., obtained from $p_{ri} = 1$; $LC50_1$), and $LC50_1$ is as previously described in the main article. The toxic units of the i^{th} mixture proportion can be calculated using the following equation:

$$TU = c_{mi}/LC50_i, \text{ (S3.2)}$$

where c_{mi} represents the CA prediction based on the i^{th} weekly reference test (Eq. 3.2), and $LC50_i$ represents the reference test LC50s for the i^{th} mixture proportion of toxicant 1. The exchange rates (e_i) used to calculate c_{mi} should be obtained using the weekly reference test LC50s.

For EA scenarios, the *MF* was obtained using the following equation:

$$MF = [(LC50_1/LC50_i) + (LC50_2/LC50_j)]/2, \text{ (S3.3)}$$

where $LC50_2$ represents the original LC50 for toxicant 2 (i.e., obtained from $p_{ri} = 1$), and $LC50_j$ represents the weekly reference LC50 for toxicant 2 for the j^{th} mixture proportion. After the *MF* was applied to the original isobole concentrations, the corrected data were re-analyzed to obtain the corrected estimated mixture LC50s.

Table S3.1 Reference toxicity test data for mixtures of Cu and Cd with phenanthrene (PHE) or phenanthrenequinone (PHQ). Non-overlapping standard errors (se) for binary and unary mixing proportions (p_{ri}) were used to test for weekly variability in LC50s.

Mixture	p_{ri}	Toxicant	Reference LC50 (μg L^{-1})	se	- se	+ se	overlap (y/n)
Cu-PHE	0.00	Cu	29.1	2.7	26.4	31.8	
	0.86	Cu	38.5	22.5	15.9	61.0	y
	0.56	Cu	27.9	1.6	26.3	29.5	y
	0.30	Cu	25.0	2.4	22.6	27.3	y
	0.86	PHE	174.6	24.2	150.4	198.8	y
	0.56	PHE	166.9	31.9	135.1	198.8	y
	0.30	PHE	175.5	10.7	164.8	186.3	y
	1.00	PHE	194.9	12.6	182.2	207.5	
Cu-PHQ	0.00	Cu	17.9	1.0	16.9	18.9	
	0.82	Cu	13.3	3.4	9.9	16.7	n
	0.57	Cu	12.0	0.9	11.0	12.9	n
	0.26	Cu	15.8	1.0	14.9	16.8	y
	0.82	PHQ	532.3	50.2	482.1	582.6	y
	0.57	PHQ	455.3	47.8	407.5	503.1	n
	0.26	PHQ	426.2	46.8	379.4	473.0	n
	1.00	PHQ	578.2	50.3	527.9	628.5	
Cd-PHE	0.00	Cd	28.7	2.8	25.8	31.5	
	0.62	Cd	30.1	8.6	21.5	38.7	y
	0.36	Cd	23.1	2.0	21.1	25.2	n
	0.16	Cd	23.5	1.8	21.7	25.3	n
	0.62	PHE	151.3	13.7	137.6	164.9	y
	0.36	PHE	233.3	47.7	185.6	281.0	y
	0.16	PHE	287.0	82.5	204.5	369.5	n
	1.00	PHE	179.7	16.8	162.9	196.5	
Cd-PHQ	0.00	Cd	24.8	3.4	21.4	28.2	
	0.59	Cd	24.6	7.2	17.4	31.9	y
	0.45	Cd	28.5	4.6	23.9	33.0	y
	0.12	Cd	23.6	2.4	21.2	26.0	y
	0.59	PHQ	444.8	102.5	342.4	547.3	y
	0.45	PHQ	408.3	102.1	306.1	510.4	y
	0.12	PHQ	434.9	73.3	361.6	508.2	y
	1.00	PHQ	419.1	91.0	328.0	510.1	

Table S3.2. Reference toxicity test data for mixtures of V and Ni with phenanthrene (PHE) or phenanthrenequinone (PHQ). Non-overlapping standard errors (se) for binary and unary mixing proportions (p_{ri}) were used to test for weekly variability in LC50s.

Mixture	p_{ri}	Toxicant	Reference LC50 (μg L^{-1})	se	- se	+ se	overlap (y/n)
V-PHE	0.00	V	4402.0	210.7	4191.4	4612.7	
	0.68	V	4733.6	641.3	4092.3	5375.0	y
	0.47	V	4028.0	565.6	3462.4	4593.6	y
	0.26	V	3482.4	211.0	3271.4	3693.4	n
	0.68	PHE	202.0	27.3	174.7	229.3	y
	0.47	PHE	273.8	62.4	211.4	336.2	y
	0.26	PHE	209.7	13.1	196.5	222.8	y
	1.00	PHE	209.7	13.1	196.5	222.8	
V-PHQ	0.00	V	4399.5	261.9	4137.6	4661.5	
	0.73	V	4185.0	205.3	3979.6	4390.3	y
	0.49	V	4283.7	321.3	3962.4	4605.0	y
	0.21	V	4284.3	244.7	4039.5	4529.0	y
	0.73	PHQ	378.3	64.6	313.8	442.9	y
	0.49	PHQ	419.3	76.3	343.0	495.5	y
	0.21	PHQ	402.2	44.2	358.0	446.4	y
	1.00	PHQ	381.9	38.5	343.3	420.4	
Ni-PHE	0.00	Ni	13675.6	1060.7	12614.9	14736.4	
	0.74	Ni	16376.9	6327.9	10049.0	22704.7	y
	0.49	Ni	11319.3	880.0	10439.3	12199.4	n
	0.23	Ni	15494.7	1538.0	13956.8	17032.7	y
	0.74	PHE	275.2	27.2	248.0	302.5	n
	0.49	PHE	142.6	13.3	129.3	155.9	n
	0.23	PHE	162.2	54.9	107.3	217.1	y
	1.00	PHE	226.2	14.2	212.0	240.3	
Ni-PHQ	0.00	Ni	16494.5	1647.7	14846.8	18142.2	
	0.60	Ni	21290.1	2949.8	18340.3	24240.0	n
	0.47	Ni	19913.7	1909.7	18003.9	21823.4	y
	0.22	Ni	20363.3	1358.7	19004.6	21722.1	n
	0.60	PHQ	292.8	42.8	250.1	335.6	y
	0.47	PHQ	335.4	22.7	312.7	358.0	y
	0.22	PHQ	327.8	18.9	308.9	346.8	y
	1.00	PHQ	295.8	34.9	260.9	330.7	

Table S3.3. Log-logistic *LC50* estimates and their derivatives (r_i) at various mixing proportions (p_{ri}) of metals with either phenanthrene (PHE) or phenanthrenequinone (PHQ).

Mixture	p_{ri}	$r_i \pm$ se	*LC50* ± se	p-value
Cu-PHE	1	-2.34 ± 0.31	194.8 ± 12.6	<0.0001; <0.0001
	0.86	-1.01 ± 0.18	110.9 ± 21.5	<0.0001; <0.0001
	0.56	-0.68 ± 0.13	72.5 ± 20.7	<0.0001; <0.0001
	0.3	-1.66 ± 0.25	104.9 ± 13.6	<0.0001; <0.0001
	0	-2.29 ± 0.30	201.4 ± 13.2	<0.0001; <0.0001
Cu-PHQ	1	-1.54 ± 0.22	578.0 ± 50.3	<0.0001; <0.0001
	0.82	-1.91 ± 0.25	358.9 ± 40.3	<0.0001; <0.0001
	0.57	-1.69 ± 0.21	164.3 ± 28.5	<0.0001; <0.0001
	0.26	-2.02 ± 0.29	125.0 ± 16.7	<0.0001; <0.0001
	0	-3.49 ± 0.51	592.9 ± 34.6	<0.0001; <0.0001
Cd-PHE	1	-1.59 ± 0.19	179.7 ± 16.8	<0.0001; <0.0001
	0.62	-0.89 ± 0.17	105.5 ± 21.5	<0.0001; <0.0001
	0.36	-1.04 ± 0.16	153.6 ± 25.1	<0.0001; <0.0001
	0.16	-0.98 ± 0.15	123.3 ± 20.7	<0.0001; <0.0001
	0	-1.97 ± 0.21	177.5 ± 14.6	<0.0001; <0.0001
Cd-PHQ	1	-0.85 ± 0.15	419.2 ± 91.1	<0.0001; <0.0001
	0.59	-0.73 ± 0.11	493.7 ± 111.8	<0.0001; <0.0001
	0.45	-0.99 ± 0.14	278.6 ± 45.4	<0.0001; <0.0001
	0.12	-1.21 ± 0.15	257.3 ± 33.9	<0.0001; <0.0001
	0	-2.06 ± 0.34	410.6 ± 51.7	<0.0001; <0.0001
V-PHE	1	-2.47 ± 0.32	209.7 ± 13.1	<0.0001; <0.0001
	0.68	-1.45 ± 0.22	255.4 ± 27.5	<0.0001; <0.0001
	0.47	-1.46 ± 0.29	314.2 ± 38.6	<0.0001; <0.0001
	0.26	-1.47 ± 0.25	252.2 ± 30.8	<0.0001; <0.0001
	0	-3.25 ± 0.42	209.4 ± 10.7	<0.0001; <0.0001
V-PHQ	1	-1.29 ± 0.19	381.8 ± 38.3	<0.0001; <0.0001
	0.73	-1.56 ± 0.29	625.3 ± 68.3	<0.0001; <0.0001
	0.49	-1.97 ± 0.27	490.4 ± 34.3	<0.0001; <0.0001
	0.21	-2.49 ± 0.31	459.8 ± 26.8	<0.0001; <0.0001
	0	-2.58 ± 0.32	383.5 ± 23.5	<0.0001; <0.0001
Ni-PHE	1	-2.47 ± 0.32	226.2 ± 14.2	<0.0001; <0.0001
	0.74	-1.91 ± 0.31	179.3 ± 17.9	<0.0001; <0.0001
	0.49	-1.01 ± 0.15	165.9 ± 24.5	<0.0001; <0.0001
	0.23	-1.45 ± 0.22	207.6 ± 22.0	<0.0001; <0.0001
	0	-3.92 ± 0.50	215.6 ± 9.79	<0.0001; <0.0001
Ni-PHQ	1	-1.14 ± 0.17	295.9 ± 34.9	<0.0001; <0.0001
	0.6	-1.49 ± 0.22	355.3 ± 34.4	<0.0001; <0.0001
	0.47	-1.76 ± 0.26	396.1 ± 30.6	<0.0001; <0.0001
	0.22	-1.63 ± 0.22	301.4 ± 26.4	<0.0001; <0.0001
	0	-1.42 ± 0.18	300.6 ± 30.2	<0.0001; <0.0001

Table S3.4. Isobole estimates for Hewlett (λ) and Vølund (η_1 and η_2) interactions terms and their levels of significance for mixtures of metals with either phenanthrene (PHE) or phenanthrenequinone (PHQ) from observed (Obs) and predicted concentration addition (CA) or effects addition (EA) estimates as various levels of proportional response (*p*).

Mixture	Isobole	*p*	λ ± se	η_1 ± se	η_2 ± se	p-value
Cu-PHE	Obs	0.500	2.15 ± 0.11	NA	NA	<0.0001
	CA	0.500	1.03 ± 0.01	NA	NA	<0.0001
	Obs	0.075	4.13 ± 1.03	NA	NA	0.02
Cu-PHQ	Obs	0.500	3.16 ± 0.25	NA	NA	0.0002
	EA	0.500	0.58 ± 0.07	NA	NA	0.0001
	Obs	0.037	3.65 ± 0.31	NA	NA	0.0003
Cd-PHE	Obs	0.500	1.57 ± 0.13	NA	NA	0.0002
	CA	0.500	0.99 ± 0.01	NA	NA	<0.0001
	Obs	0.044	4.18 ± 0.24	NA	NA	<0.0001
Cd-PHQ	Obs	0.500	1.51 ± 0.32	NA	NA	0.009
	EA	0.500	0.94 ± 0.03	NA	NA	<0.0001
	Obs	0.014	2.09 ± 0.04	NA	NA	<0.0001
V-PHE	Obs	0.500	0.57 ± 0.06	NA	NA	0.0008
	EA	0.500	0.41 ± 0.02	NA	NA	<0.0001
V-PHQ	Obs	0.500	NA	10.9 ± 9.26	0.39 ± 0.12	0.2; 0.0001
	EA	0.500	0.66 ± 0.03	NA	NA	<0.0001
Ni-PHE	Obs	0.500	1.28 ± 0.07	NA	NA	<0.0001
	CA	0.500	0.96 ± 0.01	NA	NA	<0.0001
Ni-PHQ	Obs	0.500	0.69 ± 0.07	NA	NA	0.0004
	EA	0.500	1.01 ± 0.01	NA	NA	<0.0001

3.8.4 Supporting information references

Abendroth, J.A. Blankenship, E.E., Martin, A.R., Roeth, F.W., 2011. Joint action analysis utilizing concentration addition and independent action models. Weed Technology, 25, 436-446.

Berenbaum, M.C., 1989. What is synergy? Pharmacological Reviews 41, 93-141.

Brooks, S.J., Mills, C.L., 2003. The effect of copper on osmoregulation in the freshwater amphipod *Gammarus pulex*. Comparative Biochemistry and Physiology Part A 135, 527-537.

Cedergreen, N., Christensen, A.M., Kamper, A., Kudsk, P., Mathiassen, S.K., Streibig, J.C., Sorensen, H., 2008. A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. Environmental Toxicology and Chemistry 27, 1621-1632.

Felton, V., Charmantier, G., Mons, R., Geffard, A., Rousselle, P., Coquery, M., Garric, J., Geffard, O., 2008. Physiological and behavioural responses of *Gammarus pulex* (Crustacea: Amphipoda) exposed to cadmium. Aquatic Toxicology 86, 413-425.

Goc, A., 2006. Biological activity of vanadium compounds. Central European Journal of Biology 1, 314-332.

Greco, W.R., Bravo, G., Parsons, J.C., 1995. The search for synergy: A critical review from a response surface perspective. Pharmacological Reviews 47, 331-385.

Hannam, M.L., Bamber, S.D., Galloway, T.S., Moody, A.J., Jones, M.B., 2010. Effects of the model PAH phenanthrene on immune function and oxidative stress in the haemolymph of the temperate scallop *Pecten maximus*. Chemosphere 78, 779-784.

Leonard, E.M., Wood, C.M., 2013. Acute toxicity, critical body residues, Michaelis-Menten analysis of bioaccumulation, and ionoregulatory disturbance in response to waterborne nickel in four invertebrates: *Chironomus riparius*, *Lymnaea stagnalis*, *Lumbriculus variegatus* and *Daphnia pulex*. Comparative Biochemistry and Physiology, Part C 158, 10-21.

Muggeo, V.M.R., 2003. Estimating regression models with unknown break-points. Statistics in Medicine 22, 3055-3071.

O'Brien, P. Molecular mechanisms of quinone cytotoxicity. Chemico-Biological Interactions. 1991, 80, 1-41.

Pane, E.F., Smith, C., McGeer, J.C., Wood, C.M., 2003. Mechanisms of acute and chronic waterborne nickel toxicity in the freshwater cladoceran, *Daphnia magna*. Environmental Science & Technology 37, 4382-4389.

Rainbow, P.S., Black, W.H., 2005. Cadmium, zinc and the uptake of calcium by two crabs, *Carcinus maenas* and *Eriocheir sinensis*. Aquatic Toxicology 72, 45-65.

Vellinger, C., Felton, V., Sornom, P., Rouselle, P., Beisel, J.-N., Usseglio-Polatera, P., 2012. Behavioural and physiological responses of *Gammarus pulex* exposed to cadmium and arsenate at three temperatures: Individual and combined effects. PLoS ONE 7, e39153.

Zhang, Y., Huang, L., Zuo, Z., Chen, Y., Wang, C., 2013. Phenanthrene exposure causes cardiac arrhythmia in embryonic zebrafish via perturbing calcium handling. Aquatic Toxicology142-143, 26-32.

Chapter 4: Metal-polycyclic aromatic hydrocarbon mixture toxicity in *Hyalella azteca*. II. Metal accumulation and oxidative stress as interactive co-toxic mechanisms[1]

4.1 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. Redox-active metals (e.g., Cu and Ni) are also capable of enhancing the redox cycling of PAHs. Accordingly, 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 was explored 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. No evidence was found 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.

4.2 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 (Gust, 2006). 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

[1] This chapter has been published. Gauthier, P.G., Norwood, W.P., Prepas, E.E., Pyle, G.G., 2015. Metal-polycyclic aromatic hydrocarbon mixture toxicity in *Hyalella azteca*. II. Metal accumulation and oxidative stress as interactive co-toxic mechanisms. Environmental Science & Technology "Just Accepted" DOI: 10.1021/acs.est.5b03233.

contaminants at various stages of intoxication (Chapter 2; Gauthier et al., 2014). 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 (Valette-Silver et al., 1999, Curran et al., 2000, Mielke et al., 2001, Muniz et al., 2004, Donahue et al., 2006 and Sprovieri et al., 2007). 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 (Borer et al. 2005). *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 towards cell membranes (Sikkema et al., 1995; Schirmer et al., 1998) resulting in ionoregulatory dysfunction or membrane destabilization in severe cases (as reviewed by Gauthier et al. 2014). Likewise, Cu (Brooks and Mills, 2003), Cd (Issartel et al., 2010), and Ni (Pane et al., 2003) 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. Copper, Ni, and V engage in Fenton-like reactions in the presence of H_2O_2 to generate ROS (i.e., ●OH; Stohs and Bagchi, 1995). Phenanthrenequione (PHQ), the major photoderivative of PHE, generates ROS (i.e., $O_2^{\bullet-}$, ●OH) through autoxidation (Flowers-Geary et al., 1993; Penning et al., 1996; Yu et al., 2006; Fu et al., 2012). Thus, oxidative stress is a second potential acute lethal mechanism of action of Cu, Ni, V, and PHQ in *H. azteca*.

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 (Xie et al., 2006). 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 (Xie et al., 2006, 2007). 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 (Xie et al., 2006, 2007; Wang et al., 2009).

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 (Chapter 2; Gauthier et al., 2014). 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 (Moreau et al., 1999). 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 (Gust and Fleeger, 2006). However, bioaccumulation of Cd increased in *Hyalella azteca* exposed to sediments containing a sublethal mixture of Cd and PHE (Gust and Fleeger, 2005). This increase in the accumulation of Cd could explain the more-than-additive mortality observed in *H. azteca* (Gust and Fleeger, 2005; Gust, 2006; Chapter 3) and the copepod, *Schizopera knabeni* (Fleeger et al., 2009), 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* (Fleeger et al., 2009). 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, 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* was investigated.

4.3 Methods

Amphipod culturing and test water preparations followed those described in Chapter 3. 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; Borgmann, 1996) 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 by 10 cm strip of cotton gauze as substrate.

4.3.1 Metal accumulation

Tissue samples were collected from surviving amphipods obtained from the 48-h isobole experiments described Chapter 3 measuring non-additive mortality. The 48-h exposure duration allowed for comparison with the Cu accumulation modelling data provided by Borgmann and Norwood (1995). Briefly, 2- to 10-d old *H. azteca* were exposed to mixtures of metals and PAHs

held at fixed mixture proportions tested along a standard concentration series with 5 concentrations (Table S4.1). Each treatment was replicated 3 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. (2007). However, no gut clearance was required as animals were not fed throughout the exposure period. At the end of each test, a minimum of 4 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 ethylene-diamine-tetra-acetic acid (EDTA) in culture water to remove adsorbed metals on the surface of the animals (i.e., not bioaccumulated; Norwood et al., 2007). Animals were then transferred to 1.5 mL cryovials and any transferred EDTA solution was removed with a pipette and Kimwipe®. 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 modelled with the saturation model described in Norwood et al. (2006):

$$c_{tb} = max \times [c_w \times (k_w + c_w)^{-1}] + c_{bk}, \quad (4.1)$$

where c_{tb} is the total concentration (μg g^{-1}) of the metal within the body, c_w is the concentration (μg L^{-1}) of the metal in water, *max* is the maximum above-background concentration (μg g^{-1}) of metal that can be accumulated for the given exposure duration, k_w is the half saturation constant (μg L^{-1}) for the given exposure duration, and c_{bk} is the background body metal concentration (μg g^{-1}) measured in control animals. For Cu accumulation modelling, a *max* of 228.7 μg g^{-1}, as reported by Borgmann and Norwood (1995) for 48-h accumulation trials with *H. azteca*, was inputted into Eq. 4.1 as the c_w 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 μg L^{-1} in mixture with PAHs produced high mortality leaving too few remaining live amphipods for analysis of tissue Cu concentrations. In contrast, the c_w for Cd were in an appropriate range to estimate *max* (Borgmann et al., 2004), and thus, both *max* and k_w were estimated for Cd accumulation. Parameters were estimated with the 'nls' function in R (R Core Team, 2015). Finally, lethality as a function of metal body concentration was modelled with the saturation model adapted from Norwood et al. (2007):

$$p = p' + \ln(2) \times [c_{tb} \times (LBC50^{-1} + k_{tb}^{-1}) \times (1 + c_{tb} \times k_{tb}^{-1})^{-1}]^{nb}, \quad (4.2)$$

where p is the proportional response attributed to background corrected whole-body metal concentrations (c_{tb}), p' is the control response, *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. 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.

4.3.2 Paired mortality and ROS production assays

For paired mortality and ROS production experiments, 2- to 10-d old amphipods were acclimated at 21°C for 24 h prior to being exposed. Following acclimation, 10 to 20 amphipods were added to 300 mL of test water in 400 mL glass beakers containing one 2.5 by 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 3 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 (Chapter 3). 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 (Chapter 3). Copper, Cd, Ni, and V were tested at 15.3-and 17.7-, 19.8-, 12,480-and 15,160-, and 3,650-and 1,217-μg L^{-1}, respectively, and were mixed with either 170-, 68.8-, 184.9-, and158.9-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; Cathcart et al., 1983). Dyeing procedures were based on those described in Xie et al. (2006) 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 hours.

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 (Rasband, 2014), and was represented as the corrected total sample fluorescence (f_{ct}) calculated as follows:

$$f_{ct} = a \times f - a \times f_b, \quad (4.3)$$

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} among control, metal-only, and metal-PAH mixture treatments, and allowed for a formal test of the interactive effect (i.e., additivity) of the metal-PAH mixture (Iwasaki and Brinkman, 2014). Thus, for mortality and fluorescence data, a positive or negative and significant estimate of the interactive effect indicated more-and less-than-additive toxicity respectively. The assessment of additivity was based on measured water concentrations. 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 centre and scale of 0 and 2.5 respectively, incorporates a prior distribution of parameter estimates that outperforms classical GLM's in estimating parameters for datasets with excessive separation (Gelman et al., 2008). All statistical analyses were carried out

with the 'glm' and 'bayesglm' functions contained within the statistical package, R 3.2.0 (Gelman and Su, 2015; R Core Team, 2015).

4.4 Results and Discussion

4.4.1 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 (Fig. 4.1; Table 4.1). Estimated *max* (μ = 59.9 μg g^{-1}) parameters for Cd accumulation (Table 4.1) were within the confidence limits of those reported in Borgmann et al. (2004) also involving *H. azteca*; however, estimated k_w (μ = 13.7 μg g^{-1}) for Cd was substantially higher. It was also found that k_w for Cu accumulation in Cu-only (55.4 ± 7.6 μg L^{-1}) and Cu-PHE (48.5 ± 11.7 μg L^{-1}) treatments to deviate from what is reported in Borgmann and Norwood (1995; 18.5 μg L^{-1}). 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-to 6-wk in Borgmann et al., 1991). However, estimated k_w from Cu data in the work of Borgmann et al. (2004) used data from 48-h exposures (Borgmann and Norwood, 1995). 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 (1995) 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. 4.1), 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 μg L^{-1}) was similar to the k_w for Cu reported by Borgmann et al. (2004), suggesting that low c_w may not be a limiting factor in Cu accumulation when PHQ is present (see below for further discussion).

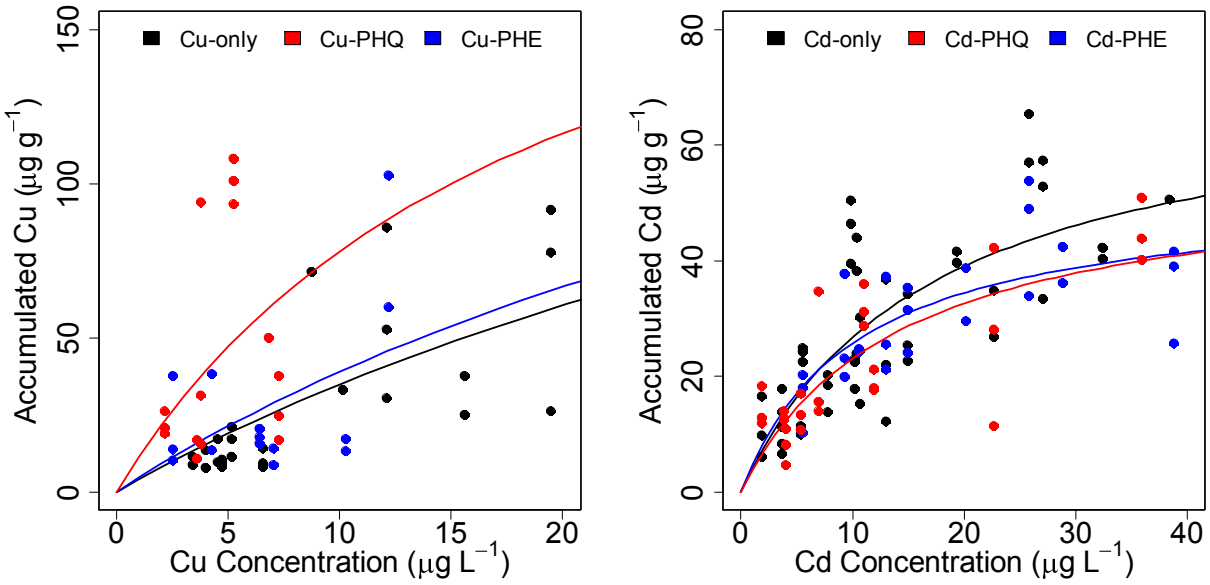

Figure 4.1 Tissue Cu (left panel) and Cd (right panel) concentrations in juvenile *H. azteca* following 48-h exposures to Cu-only, Cu-PHE, Cu-PHQ, Cd-only, Cd-PHE, and Cd-PHQ treatments. Curves represent model predictions from Eq. 4.1.

Table 4.1 Summary of saturation modelling (Eq. 4.1) 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

Two 48-h Cd LBC50s (47.1 ± 22.1 and 51.1 ± 10.5 µg g^{-1}; Fig. S4.1) for juvenile *H. azteca* were obtained from a series of two independent reference toxicity tests from the isobole experiments described in Chapter 3. The 48-h Cd LBC50s were in the same range as 6-week Cd LBC50s in a

variety of test media (Borgmann et al., 1991), 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- to10-µg g^{-1}; Fig. S4.1), 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 (Couture and Pyle, 2008).

4.4.2 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 (Fig. 4.1; Table 4.1). 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 (Fig. 4.1) and 192-h (Gust and Fleeger, 2005) 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* (Borgmann and Norwood, 1995), likely through the activity of several proteins at the sites of uptake (i.e., gill and gut; Grosell, 2012; Grosell and Wood, 2002). 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 (Wood, 2012; Chmielowska-Bąk et al., 2013). 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 (O'Brien, 1991; Schmieder et al., 2003), 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 (ctr1), as well as the Cu specific adenosine triphosphate ion pump (Cu-ATPase), work together to regulate Cu (Minghetti et al., 2008, 2010; 2011; Grosell, 2012). The uptake of Cu is assisted by ctr1 and DMT1 (Grosell, 2012; Grosell and Wood, 2002), whereas Cu-ATPases generally function to control the efflux of Cu from cells and tissues (Lutsenko et al. 2008). The critical site for ctr1 (Mackenzie et al., 2004) and DMT1 (Ehrnstorfer et al., 2014) metal binding is at the methionine residue, which is highly susceptible to oxidation by H_2O_2 and $O_2^{\bullet-}$ (Levine et al., 1996). 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 (Minghetti et al., 2011). 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; Gonzáles-Guerrero and Argüello, 2008). Thus, the PHQ-induced arylation (Tapper et al., 2000) 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 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 (Borgmann and Norwood, 1995). 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, a slower rate of Cu accumulation was observed, 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; Salunke-Gawali et al., 2005). Similarly to other metal-hydrocarbon complexes (Phinney and Bruland, 1994; Tjalve and Borg-Neczak, 1994; Parathasarathy et al., 2008, 2010), 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 (Zhu et al., 2004a, 2004b; Chapter 2; Gauthier et al., 2014); however, to date, there have been no studies that have linked metal-PAHQ complexation to increased metal bioavailability in aquatic biota.

4.4.3 Lethality

Exposures induced varying degrees of mortality (Table 4.2), and 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 (Fig. 4.2A and C). All other mixtures produced strictly-additive mortality (Table 4.2).

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 (Gauthier et al. 2014). 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

Table 4.2 Summary of GLM results for lethality and fluorescence data from exposure to metals with either phenanthrene (PHE) or phenanthrenequinone (PHQ). 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.

		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	0.82
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

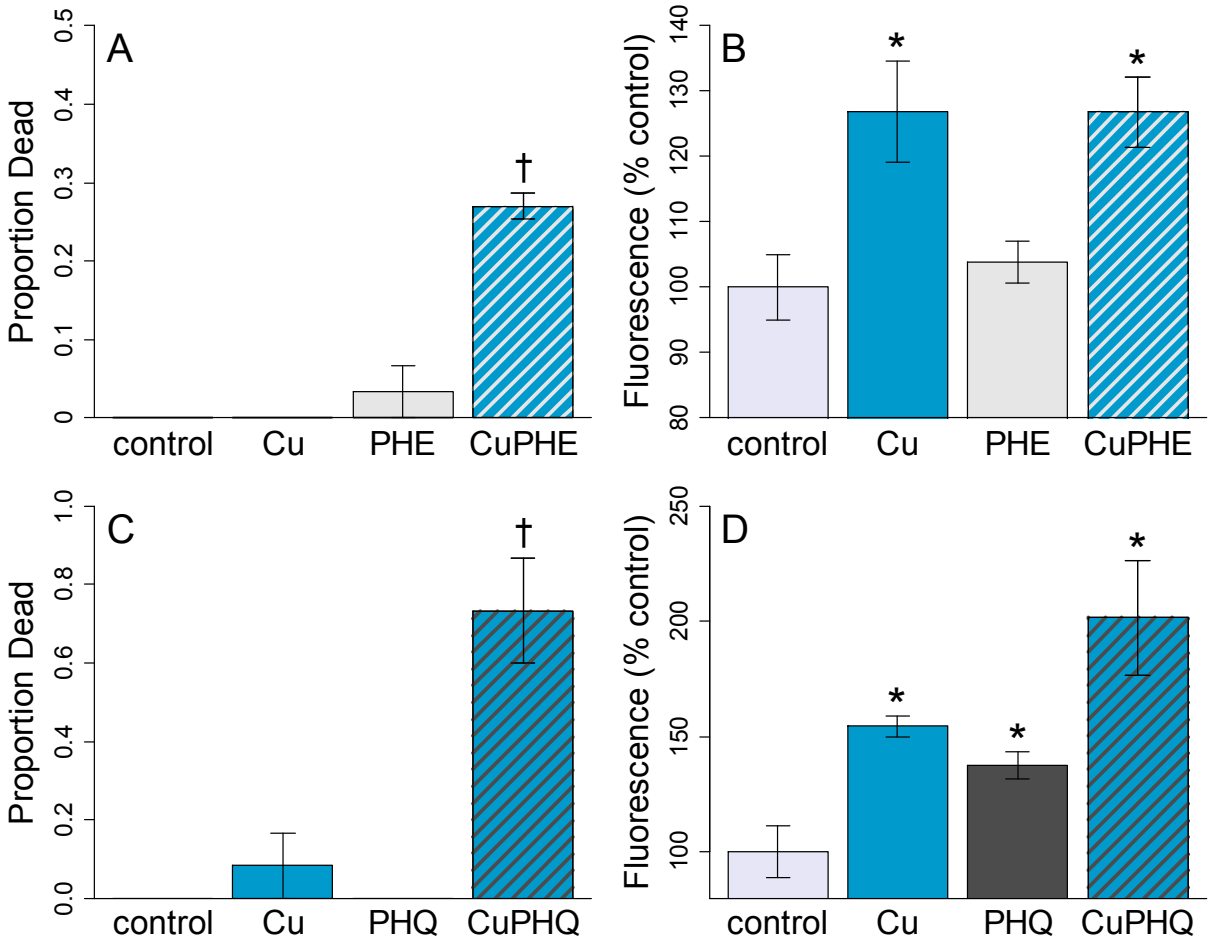

Figure 4.2 Mean observed mortality and ROS fluorescence ± se from Cu-PHE and Cu-PHQ exposures. Asterisks indicate a significant difference from control. Daggers (†) indicate a significant interaction (i.e., a less- or more-than-additive effect).

similarly revealed that more-than-additive mortality was characteristic of mixtures of Cu with PHE or PHQ at a variety of different mixture ratios (Chapter 3). Experiments carried out with *D. magna* and *V. fischeri* support that Cu-PHQ mixtures produce more-than-additive lethality (Xie et al., 2006; Wang et al., 2009). However, contrary to the present work, *D. magna* exposed to Cu-PHE mixtures for 48 h exhibited strictly additive lethality (Xie et al., 2006). 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. (2007) 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 (Cedergreen et al., 2007). There are many cases of conflicting mixture outcomes within the limited published data pertaining to the additivity of metal-PAH-induced lethality in aquatic biota (Chapter 3). 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 Chapter 3 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. (2014). However, until the specific mechanism responsible for Cd-PHQ and Cd-PHE mixture toxicity is revealed, it is premature to speculate on time-sensitivity.

4.4.4 ROS production

Whole animal H_2DCFDA staining of juvenile *H. azteca* identified both tissue specific (Fig. 4.3A and 4.3B) and general whole-body fluorescence (Fig. 4.3C and 4.3D). Fluorescence was increased above control following 18-h exposures to Cu ($\mu = 40.8 \pm 11.4\%$) and Ni ($\mu = 37.1 \pm 7.7\%$; Table 4.2). However, there was no effect of Cd, V, and PHE on DCF fluorescence (Table 4.2). In Cu and Ni experiments, exposure to PHQ elicited an increase in DCF fluorescence ($\mu = 31.1 \pm 12.1\%$). However, in Cd and V experiments, there was no increase in DCF fluorescence from PHQ exposure (Table 4.2). There were no interactive effects on DCF fluorescence following mixture exposures (Table 4.2). Thus, metal-PAH mixtures produced strictly-additive fluorescence (i.e., ROS).

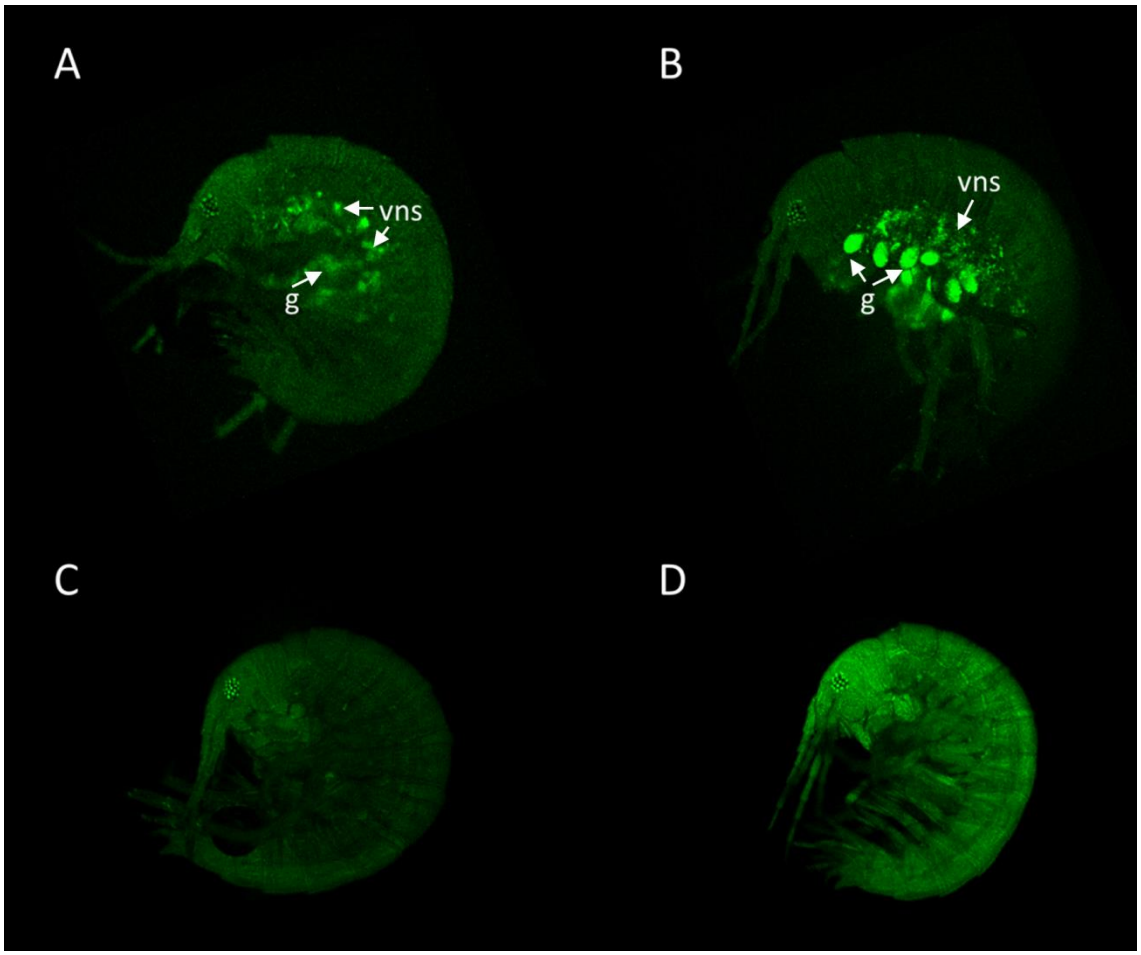

Figure 4.3 Dichlorofluorescein fluoresecence in juvenile *H. azteca*. Panels A and C are representative of unexposed animals. Panels B and D are representative of Cu exposed animals. Gills and nervious tissue fluoresced in certain specimens (panels A and B), whereas some specimens exhibited a general whole-body fluorescence (panels C and D). 'g' denotes gill tissue. 'vns' denotes ventral nervous system tissue.

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 (Xie et al., 2006; Wang et al., 2009). However, no evidence was found 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-hr co-exposures, and 48-h co-exposures in *H. azteca* produced strictly-additive and slightly less-than-additive mortality depending on the mixture proportion tested (Chapter 3).

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. Dichlorofluorescein-diacetate can be oxidized by H_2O_2 and several other ROS (e.g., other peroxides) and reactive nitrogen species (RNS; Cathcart et al., 1983; Possel et al., 1997). 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* (Wang et al., 2009), but not in *D. magna* following 4-h exposures to a series of PHQ treatments from 124- to 1,000-μg L^{-1} (Xie et al., 2006). The discrepancy between *D. magna* and *V. fischeri* could stem from dissimilar experimental systems (i.e., bacteria compared to whole cladoceran crustaceans; Xie et al., 2006). However, in whole *H. azteca*, a fellow crustacean, an increase in DCF fluorescence was observed 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 S4.2). Phenanthrenequinone is converted to semi-PHQ with the aid of several putative NAD(P)H oxidoreductases and/or UV-A radiation (Flowers-Geary et al., 1993; Penning et al., 1996; Zhao et al., 2011). Semi-PHQ can be oxidized by O_2, restoring PHQ while producing $O_2^{\bullet-}$. Phenanthrenequinone can also be directly reduced by the NAD(P)H:quinone oxidoreductase, DT-

diaphorase (DTD), to produce dhPHQ without the semi-quinone intermediate (Flowers-Geary et al., 1993). 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 (Xie et al., 2006, 2007; Wang et al., 2009).

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 (Kutlu et al., 2003). There have been *in vitro* fish studies indicating a rapid increase in DTD activity following exposure to a variety of PAH-quinones (PAHQs; Hasspieler et al., 1992; Hasspieler and Di Giulio, 1994), 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. (2006), 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, it is predicted that a non-ROS related mechanism is responsible for the more-than-additive mortality of juvenile *H. azteca* co-exposed to Cu and PHQ.

4.5 Conclusions

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 presented elsewhere (Chapter 2; Gauthier et al. 2014), further investigation should focus on 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.

4.6 References

Borer, E.T., Seabloom, E.W., Shurin, J.B., Anderson, K.E., Blanchette, C.A., Broitman, B., Cooper, S.D., Halpern, B.S., 2005. What determines the strength of a trophic cascade? Ecology 86, 528-537.

Borgmann, U., Norwood, W.P., Babirad, I.M., 1991. Relationship between chronic toxicity and bioaccumulation of cadmium in *Hyalella azteca*. Canadian Journal of Fisheries and Aquatic Sciences 48, 1055-1060.

Borgmann, U., Norwood, W.P., 1995. Kinetics of excess (above background) copper and zinc in *Hyalella azteca* and their relationship to chronic toxicity. Canadian Journal of Fisheries and Aquatic Sciences 52, 864-874.

Borgmann, U., 1996. Systematic analysis of aqueous ion requirements of *Hyalella azteca*: a standard artificial medium including the essential bromide ion. Archives of Environmental Contamination and Toxicology 30, 356-363.

Borgmann, U., Norwood, W.P., Dixon, D.G., 2004. Re-evaluation of metal bioaccumulation and chronic toxicity in *Hyalella azteca* using saturation curves and the biotic ligand model. Environmental Pollution 131, 469-484.

Brooks, S.J., Mills, C.L., 2003. The effect of copper on osmoregulation in the freshwater amphipod *Gammarus pulex*. Comparative Biochemistry and Physiology, Part A 135, 527-537.

Cathcart, R., Schwiers, E., Ames, B.N., 1983. Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. Analytical Biochemistry 134, 111-116.

Cedergreen, N., Kudsk, P., Mathiassen, S.K., Sørensen, H., Streibig, J.C., 2007. Reproducibility of binary-mixture toxicity studies. Environmental Toxicology and Chemistry 26, 149-156.

Chmielowska-Bąk, J., Izbiańska, K., Deckert, J., 2013. The toxic doppelganger: on the ionic and molecular mimicry of cadmium. Acta Biochimica Polonica 60, 369-374.

Couture, P., Pyle, G., 2008. Live fast and die young: metal effects on condition and physiology of wild yellow perch from along two metal contamination gradients. Human and Ecological Risk Assessment, 14, 73-96.

Curran, K. J. Irvine, K.N. Droppo, I.G. Murphy, T.P., 2000. Suspended solids, trace metal and PAH concentrations and loadings from coal pule runoff to Hamilton Harbour, Ontario. Journal of Great Lakes Research 281, 217-227.

Donahue, W.F. Allen, E.W. Schindler, D.W., 2006. Impacts of coal-fired power plants on trace metals and polycyclic aromatic hydrocarbons (PAHs) in lake sediments in central Alberta, Canada. Journal of Paleolimnology 35, 111-128.

Ehrnstorfer, I.A., Geerstma, E.R., Pardon, E., Steyaert, J., Dutzler, R., 2014. Crystal structure of a SLC11 (NRAMP) transporter reveals the basis for transition-metal ion transport. Nature Structural & Molecular Biology 21, 990-996.

Fleeger, J.W., Gust, K.A., Marlborough, S.J., Tita, G., 2009 Mixtures of metals and polycyclic aromatic hydrocarbons elicit complex, nonadditive toxicological interactions in meiobenthic copepods. Environmental Toxicology and Chemistry 26, 1677-1685.

Flowers-Geary, L., Harvey, R., Penning, T., 1993. Cytotoxicity of polycyclic aromatic hydrocarbon *o*-quinones in rat and human hepatoma cells. Chemical Research in Toxicology 6, 252-260.

Fu, P., Xia, Q., Sun, X., Yu, H., 2012. Phototoxicity and environmental transformation of polycyclic aromatic hydrocarbons (PAHs)-light-induced reactive oxygen species, lipid peroxidation, and DNA damage. Journal of Environmental Science and Health, Part C 30, 1-41.

Gauthier, P.T. Norwood, W.P. Prepas, E.E. Pyle, G.G., 2014. Metal-PAH mixtures in the aquatic environment: A review of co-toxic mechanisms leading to more-than-additive outcomes. Aquatic Toxicology 154, 253-269.

Gelman, A., Jakulin, A., Pittau, M.G., Su, Y.-S., 2008. A weakly informative default prior distribution for logistic and other regression models. The Annals of Applied Statistics 2, 1360-1383.

Gelman, A., Su, Y-.S. 2015. arm: data analysis using regression and multilevel/hierarchical models. R package version 1.8-5. http://CRAN.R-project.org/package=arm

Gonzáles-Guerrero, M., Argüello, J.M., 2008. Mechanisms of Cu^{+}-transporting ATPases: soluble Cu^{+} chaperones directly transfer Cu+ to transmembrane transport sites. Proceedings of the National Academy of Sciences 105, 5992-5997.

Grosell, M., Wood, C.M., 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. Journal of Experimental Biology 205, 1179-1188.

Grosell, M., 2012. Copper. In Homeostasis and Toxicology of Essential Metals, Wood, C.M., Farrell, A.P., Brauner, C.J., Eds., Academic Press, Elsevier Inc. New York, USA, pp 54-135.

Gust, K.A., 2006. Joint toxicity of cadmium and Phenanthrene to the freshwater amphipod *Hyalella azteca*. Archives of Environmental Contamination and Toxicology 50, 7-13.

Gust, K.A., Fleeger, J.W., 2005. Exposure-related effects on Cd bioaccumulation explain toxicity of Cd-phenanthrene mixtures in *Hyalella azteca*. Environmental Toxicology and Chemistry 24, 2918-2926.

Gust, K.A., Fleeger, J.W., 2006. Exposure to cadmium-phenanthrene mixtures elicits complex toxic responses in the freshwater tubificid oligochaete, *Hyodrilus templetoni*. Archives of Environmental Contamination and Toxicology 51, 54-60.

Hasspieler, B.M., Di Giulio, R.T., 1992. DT diaphorase [NAD(P)H-(quinone acceptor) oxidoreductase] in channel catfish *(Ictalurus punctatus*) – kinetics and distribution. Aquatic Toxicology 24, 143-151.

Hasspieler, B.M., Di Giulio, R.T., 1994. Dicuomarol-sensitive NADPH – phenanthrenequinone oxidoreductase in channel catfish (*Ictalurus punctatus*). Toxicology and Applied Pharmacology 125, 184-191.

Issartel, J., Boulo, V., Wallon, S., Geffard, O., Charmantier, G., 2010. Cellular and molecular osmoregulatory responses to cadmium exposure in *Gammarus fossarum* (Crustacea, Amphipod). Chemosphere 81, 701-710.

Iwasaki, Y., Brinkman, S.F., 2014. Application of generalized linear mixed model to analyze mixture toxicity: survival of brown trout affected by copper and zinc. Environmental Toxicology and Chemistry 34, 816-820.

Kutlu, M., Sümer, S., Özata, A., 2003. DT-Diaphorase [NAD(P)H: (quinone acceptor) oxidoreductase] in *Gammarus pulex*: kinetics and some biochemical properties. Environmental Contamination and Toxicology 71, 520-526.

Levine, R.L., Mosoni, L., Berlett, B.S., Stadtman, E.R., 1996. Methionine residues as endogenous antioxidants in proteins. Proceedings of the National Academy of Science 93, 15036-15040.

Lutsenko, S., Gupta, A., Burkhead, J.L., Zuzel, V., 2008. Cellular multitasking: the dual role of human Cu-ATPases in cofactor delivery and intracellular copper balance. Archives of Biochemistry and Biophysics 476, 22-32.

Mackenzie, N.C., Brito, M., Reyes, A.E., Allende, M.L., 2004. Cloning, expression pattern and essentiality of the high-affinity copper transporter 1 (*ctr1*) gene in zebrafish. Gene 328, 113-120.

Mielke, H.W., Wang, G., Gonzales, C.R., Le, B., Quach, V.N., Mielke, P.W., 2001. PAH and metal mixtures in New Orleans soils and sediments. Science of the Total Environment 281, 217-227.

Minghetti, M., Leaver, M.J., Carpenè, E., George, S.G., 2008. Copper transporter 1, metallothionein and glutathione reductase genes are differentially expressed in tissues of sea bream (*Sparus aurata*) after exposure to dietary or waterborne copper. Comparative Biochemistry and Physiology, Part C 147, 450-459.

Minghetti, M., Leaver, M.J., George, S.G., 2010. Multiple Cu-ATPase genes are differentially expressed and transcriptionally regulated by Cu exposure in sea bream, *Sparus aurata*. Aquatic Toxicology 97, 23-33.

Minghetti, M., Leaver, M.J., Taggart, J.B., Casadei, E., Auslander, M., Tom, M., George, S.G., 2011. Copper induces Cu-ATPase ATP7A mRNA in a fish cell line, SAF1. Comparative Biochemistry and Physiology, Part C 154, 93-99.

Moreau, C.J., Klerks, P.L., Haas, C.N., 1999. Interaction between phenanthrene and zinc in their toxicity to the sheepshead minnow (*Cyprinodon variegatus*). Archives of Environmental Contamination and Toxicology 37, 251-257.

Muniz, P. Danulat, E. Yannicelli, B. García-Alonso, J. Medina, G. Bícego, M.C., 2004. Assessment of contamination by heavy metals and petroleum hydrocarbons in sediments of Montevideo Harbour (Uruguay). Environment International 29, 1019-1028.

Norwood, W.P., Borgmann, U., Dixon, D.G., 2006. Saturation models of arsenic, cobalt, chromium and manganese bioaccumulation by *Hyalella azteca*. Environmental Pollution 143, 519-528.

Norwood, W.P., Borgmann, U., Dixon, D.G., 2007. Chronic toxicity of arsenic, cobalt, chromium and manganese to *Hyalella azteca* in relation to exposure and bioaccumulation. Environmental Pollution 147, 262-272.

O'Brien, P. 1991. Molecular mechanisms of quinone cytotoxicity. Chemico-Biological Interactions 80, 1-41.

Pane, E.F., Smith, C., McGeer, J.C., Wood, C.M., 2003. Mechanisms of acute and chronic waterborne nickel toxicity in the freshwater cladoceran, *Daphnia magna*. Environmental Science and Technology 37, 4382-4389.

Parthasarathy, N., Pelletier, M., Buffle, J., 2008. Transport of lipophilic ligands through permeation liquid membrane in relation to natural water analysis. Journal of Membrane Science 309, 182-188.

Parthasarathy, N., Pelletier, M., Buffle, J., 2010. Transport of lipophilic metal complexes through permeation liquid membrane, in relation to natural water analysis: Cu(II)-8-hydroxyquinoline complex as a model compound. Journal of Membrane Science 355, 78-84.

Penning, T., Ohnishi, S., Ohnishi, T., Harvey, R., 1996. Generation of reactive oxygen species during the enzymatic oxidation of polycyclic aromatic hydrocarbon trans-dihydrodiols catalyzed by dihydrodiol dehydrogenase. Chemical Research in Toxicology 9, 84-92.

Phinney, J.T., Bruland, K.W., 1994. Uptake of lipophilic organic Cu, Cd, and Pb complexes in the coastal diatom *Thalassiosira weissflogii*. Environmental Science Technology 28: 1781-1790.

Possel, H., Noack, H., Augustin, W., Keilhoff, G., Wolf, G., 1997. 2,7-Dihydrodichlorofluorescein diacetate as a fluorescent marker for peroxynitrite formation. FEBS Letters 416, 175-178.

R Core Team, 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rasband, W. S. 2014. ImageJ. National Institute of Health, Maryland. http://imagej.nih.gov/ij/.

Salunke-Gawali, S., Rane, S.Y., Boukheddaden, K., Codjovi, E., Linares, J., Varret, F., Bakare, P.P., 2005. Thermal, magnetic and spectral studies of metal-quinone complexes: Part III. Radical coodination and hydrogen bonding mediated exchange interaction in copper-hydroxyquinone complex. Journal of Thermal Analysis and Calorimetry 79, 669-675.

Schirmer, K., Dixon, D.G., Greenberg, B.M., Bols, N.C., 1998. Ability of 16 priority PAHs to be directly cytotoxic to a cell line from the rainbow trout gill. Toxicology 127, 129-141.

Schmieder, P., Tapper, M., Kolanczyk, R., Hammermeister, D., Sheedy, B., 2003. Discriminating redox cycling and arylation pathways of reactive chemical toxicity in trout hepatocytes. Toxicological Sciences 72, 66-76.

Sikkema, J., de Bont, J.A., Poolman, B., 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiology and Molecular Biology Reviews 59, 201-222.

Sørensen, H., Cedergreen, N., Skovgaard, I.M., Streibig, J.C., 2007. An isobole-based statistical model and test for synergism/antagonism in binary mixture toxicity experiments. Environmental and Ecological Statistics 14, 383-397.

Sprovieri, M. Feo, M.L., Prevedello, L. Manta, D.S. Sammartino, S. Tamburrino, S. Marsella, E., 2007. Heavy metals, polycyclic aromatic hydrocarbons and polychlorinated biphenyls in surface sediments of the Naples harbor (southern Italy). Chemosphere 67, 998-1009.

Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radical Biology & Medicine 18, 321-336.

Tapper, M., Sheedy, B., Hammermeister, D., Schmieder, P., 2000. Depletion of cellular protein thiols as an indicator of arylation in isolated trout hepatocytes exposed to 1,4-benzoquinone. Toxicological Sciences 55, 327-334.

Tjalve, H., Borg-Neczak, K., 1994. Effects of lipophilic complex formation on the disposition of nickel in experimental animals. Science of the Total Environment 148, 217-242.

Valette-Silver, N. Hameedi, M.J., Erfurd, D.W. Robertson, A., 1999. Status of the contamination in sediments and biota from the western Beaufort Sea (Alaska). Marine Pollution Bulletin 38, 702-722.

Wang, W., Nykamp, J., Huang, X., Gerhardt, K., Dixon, D.G., Greenberg, B.M., 2009. Examination of the mechanism of phenanthrenequinone toxicity to *Vibrio fischeri*: evidence for a reactive oxygen species-mediated toxicity mechanism. Environmental Toxicology and Chemistry 28, 1655-1662.

Wood, C.M., 2012. An introduction to metals in fish physiology and toxicology: basic principles. In Homeostasis and Toxicology of Essential Metals, Wood, C.M., Farrell, A.P., Brauner, C.J., (Eds.), Academic Press, Elsevier Inc. New York, NY, USA, pp 1-53.

Xie, F., Koziar, S.A., Lampi, M.A., Dixon, D.G., Norwood, W.P., Borgmann, U., Huang, X., Greenberg, B.G., 2006. Assessment of the toxicity of mixtures of copper and 9,10-phenanthrenequinone, and phenanthrene to *Daphnia magna*: evidence for a reactive oxygen mechanism. Environmental Toxicology and Chemistry 25, 613-622.

Xie, F., Lampi, M.A., Dixon, D.G., Greenberg, B.M., 2007. Assessment of the toxicity of mixtures of nickel or cadmium with 9,10-phenanthrenequinone to *Daphnia magna*: impact of reactive oxygen-mediated mechanisms with different redox-active metals. Environmental Toxicology and Chemistry 26, 1425-1432.

Yu, H., Xia, Q., Yan, J., Herreno Saenz, D., Wu, Y., Fu, P., 2006. Photoirradiation of polycyclic aromatic hydrocarbons with UVA light - a pathway leading to the generation of reactive oxygen species, lipid peroxidation, and DNA damage. International Journal of Environmental Research and Public Health 3, 348-354.

Zhu, D., Herbert, B.E., Schlautman, M.A., Carraway, E.R., 2004a. Characterization of cation-p interactions in aqueous solution using deuterium nuclear magnetic resonance spectroscopy. Journal of Environmental Quality 33, 276-284.

Zhu, D., Herbert, B.E., Schlautman, M.A., Carraway, E.R., Hur, J., 2004b. Cation-pi bonding: a new perspective on the sorption of polycyclic aromatic hydrocarbons to mineral surfaces. Journal of Environmental Quality 33, 276-284.

Zhao, Y., Xia, Q., Yin, J.-J., Yu, H., Fu, P., 2011. Photoirradiation of polycyclic aromatic hydrocarbon diones by UVA light leading to lipid peroxidation. Chemosphere 85, 83-91.

4.7 Supporting information

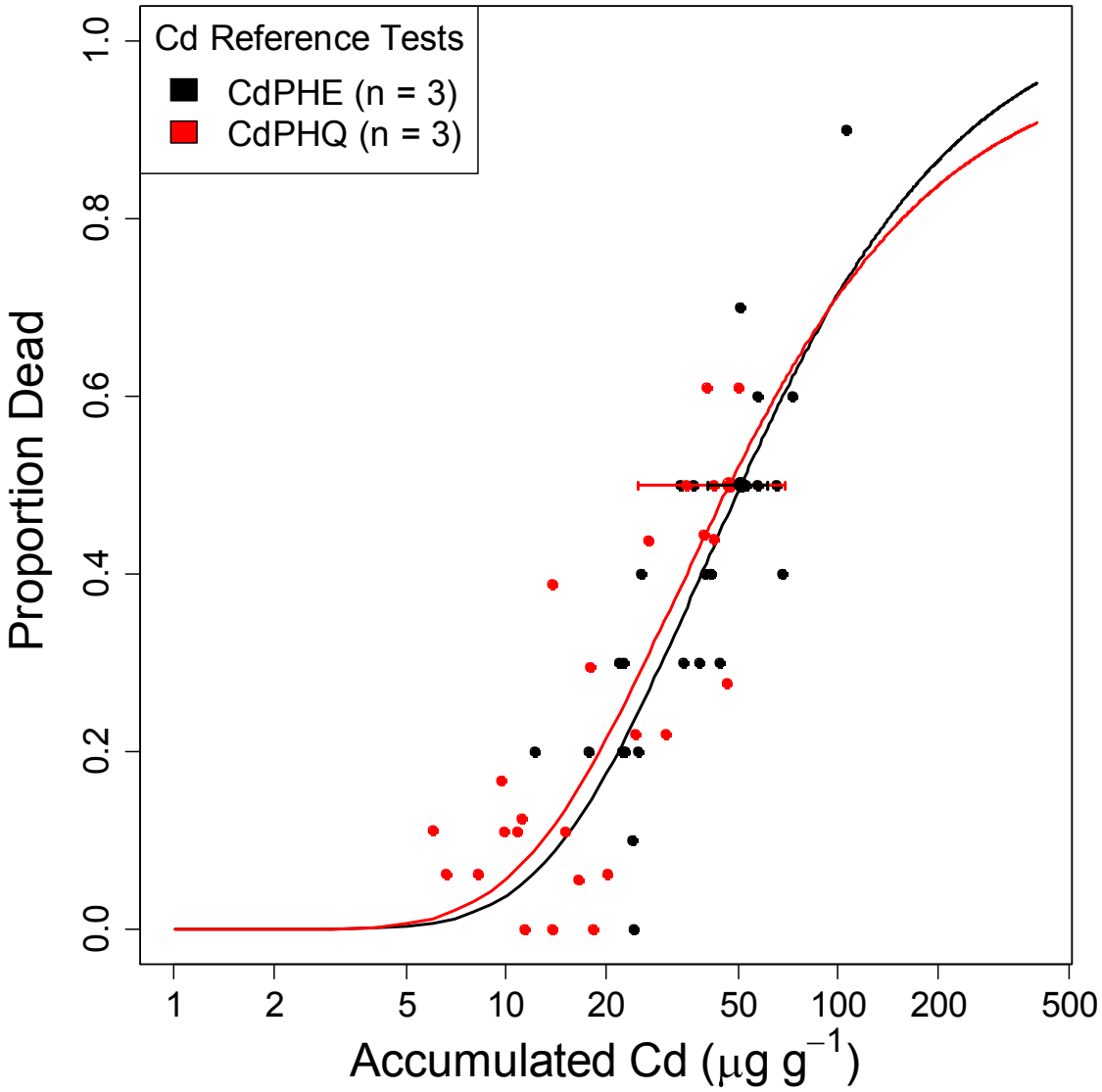

Figure S4.1 Tissue Cd concentrations and lethal response of juvenile H. azteca in Cd-only exposures (i.e., weekly references tests) from Cd-phenanthrene (PHE) and Cd-phenanthrenequinone (PHQ) isobole experiments. Closed circles with standard errors represent LBC50 estimates for each curve.

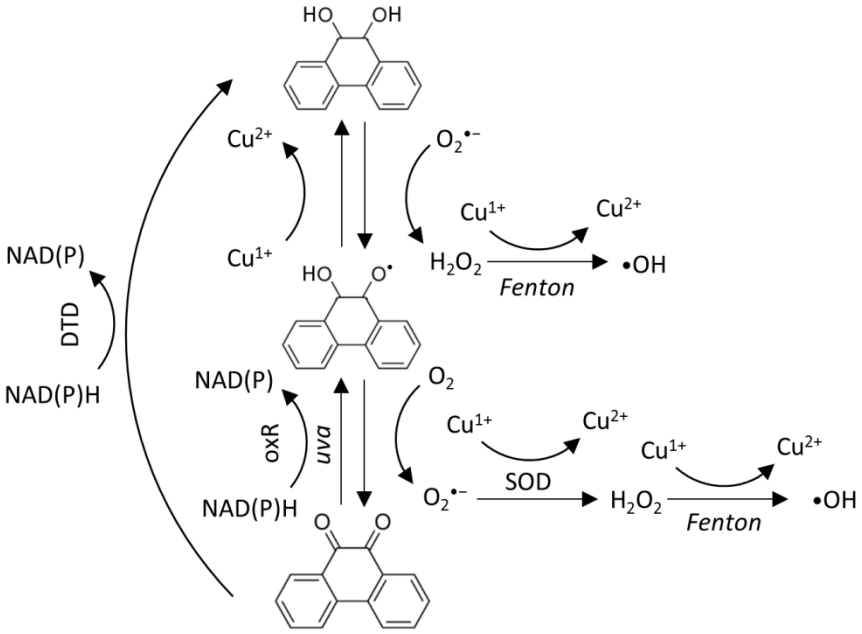

Figure S4.2 Redox-cycling of phenanthrenequinone and Cu. ‘SOD’ denotes superoxide dismutase. ‘oxR’ denotes NADH:ubiquinone oxidoreductase. ‘DTD’ denotes NAD(P)H:quinone oxidoreductase.

Table S4.1 Measured concentrations of metals and polycyclic aromatic hydrocarbons (PAHs) from metal accumulation assays. 'p_{ri}' denotes the mixture proportions based on equitoxic concentrations, and represents the proportion of PAH in the mixture. Thus, a p_{ri} = 0.5 indicates that both contaminants are present at equitoxic concentrations.

mixture	p_{ri}	metal concentration (μg L^{-1})	PAH concentration (μg L^{-1})
CuPHE	0.86	2.5	77.7
	0.86	4.3	141.0
	0.86	7.0	221.3
	0.56	6.4	61.4
	0.56	12.2	86.3
	0.30	15.6	24.3
CuPHQ	0.82	2.2	240.9
	0.82	3.6	499.4
	0.82	5.3	765.4
	0.57	3.8	144.1
	0.57	6.8	321.3
	0.27	7.3	51.2
CdPHE	0.62	5.5	87.9
	0.62	9.3	192.0
	0.62	13.0	317.1
	0.36	10.6	61.3
	0.36	20.2	146.6
	0.36	28.8	248.2
	0.16	14.9	28.9
	0.16	25.9	73.7
	0.16	38.8	145.0
CdPHQ	0.59	1.8	42.9
	0.59	3.9	95.2
	0.59	11.1	324.3
	0.45	4.1	35.4
	0.45	6.9	53.6
	0.45	22.7	169.7
	0.12	5.4	1.3
	0.12	11.9	32.9
	0.12	35.9	95.7

Chapter 5: Behavioural alterations from exposure to Cu, phenanthrene, and Cu-phenanthrene mixtures: linking behaviour to acute toxic mechanisms in the aquatic amphipod, *Hyalella azteca*

5.1 Abstract

Phenanthrene (PHE) and Cu are two contaminants commonly co-occurring in marine and freshwater environments. Mixtures of PHE and Cu have been reported to induce more-than-additive lethality in the amphipod, *Hyalella azteca*, a keystone aquatic invertebrate, yet little is understood regarding the interactive toxic mechanisms that mediate more-than-additive toxicity. Understanding the interactions among toxic mechanisms among Cu and PHE will allow for better predictive power in assessing the ecological risks of Cu-PHE mixtures in aquatic environments. Here we use behavioural impairment to help understand the toxic mechanisms of Cu, PHE, and Cu-PHE mixture toxicity in the aquatic amphipod crustacean, *Hyalella azteca*. Our principal objective was to link alterations in activity and ventilation with respiratory rates, oxidative stress, and neurotoxicity in adult *H. azteca*. Adult amphipods were used for all toxicity tests. Amphipods were tested at sublethal exposures of 91.8- and 195 μg L^{-1} Cu and PHE, respectively, and a Cu-PHE mixture at the same concentrations for 24 h. Neurotoxicity was measured as acetylcholinesterase (AChE) activity, where malathion was used as a positive control. Oxidative stress was measured as reactive oxygen species (ROS) production. Phenanthrene-exposed amphipods exhibited severe behavioural impairment, being hyperstimulated to the extent that they were incapable of coordinating muscle movements. In addition, respiration and AChE activity in PHE-exposed amphipods were increased and reduced by 51 and 23% respectively. However, ROS did not increased following exposure to phenanthrene. In contrast, Cu had no effect on amphipod behaviour, respiration or AChE activity, but did lead to an increased in ROS. However, co-exposure to Cu antagonized the PHE-induced reduction in ventilation and negated any increase in respiration. The results suggest that PHE acts like an organophosphate pesticide (e.g., Malathion) in *H. azteca* following 24-h sublethal exposures,

and that AChE inhibition is the likely mechanisms by which PHE alters *H. azteca* behaviour. However, interactive aspects of neurotoxicity do not account for the previously observed more-than-additive mortality in *H. azteca* following exposure to Cu-PHE mixtures.

5.2 Introduction

The ecological risk associated with the co-occurrence of metals and polycyclic aromatic hydrocarbons (PAHs) in aquatic environments is gaining increasing attention, as there have been emerging reports of substantial co-contamination around the world (Valette-Silver et al., 1999, Curran et al., 2000, Mielke et al., 2001, Muniz et al., 2004, Donahue et al., 2006 and Sprovieri et al., 2007), with certain mixtures, such as Cu and phenanthrene (PHE), producing enhanced (i.e., more-than-additive) toxicity toward aquatic life (Chapters 3 and 4). Unfortunately, insufficient information on metal-PAH mixture toxicity is available to advise aquatic regulators on how potential more-than-additive toxicity should be considered in terms of ecological risk assessment and environmental policy (Chapter 2).

One major gap in our understanding of metal-PAH co-toxicity, and mixture toxicology in general, is linking more-than-additive whole-organism toxicity (e.g., mortality and altered behaviour) to interactions among biomolecular mechanisms of metal and PAH toxicity responsible for manifesting whole-organism responses (e.g., ionoregulatory dysfunction, oxidative stress, enzyme inhibition, etc.; Chapter 2). The majority of literature describing non-additive metal-PAH co-toxicity have dealt with mortality, an endpoint providing limited inference regarding toxic mechanism when tested in vacuo. In contrast, changes in animal behaviours are typically triggered by specific physiological and neurological effects (Amiard-Triquet 2009; Sloman and McNeil 2012), and can be used to predict mechanisms of toxicity occurring within the animal. For example, altered swimming behaviour in fish is characteristic of exposure to cholinergic pesticides (e.g.,

malathion), and is triggered by an inhibition of acetylcholinesterase (AChE; Brewer et al. 2001). Contaminant-induced behavioural effects can be used as a powerful tool for risk assessment as they provide non-invasive insight into adverse ecotoxicological and physiological effects (Melvin and Wilson 2013). However, behaviour is rarely used as an endpoint in mixtures toxicity testing, and has never been applied to understanding the additive toxicity of metal-PAH mixtures.

The potential for PHE to increase the accumulation of Cu, and for Cu and PHE to potentiated each other's production of reactive oxygen species (ROS; Chapter 2) previously led me to explore these interactions as possible mechanisms through which Cu and PHE produce more-than-additive mortality in the aquatic crustacean amphipod, *Hyalella azteca*. Nonetheless, I found no evidence suggesting interactive aspects of oxidative stress or metal accumulation were responsible for more-than-additive mortality in Cu-PHE mixtures (Chapter 4). Other candidate interactions have been proposed (Chapter 2); however, there is emerging evidence demonstrating that PAHs are neurotoxic to aquatic invertebrates (Martínez-Tabche et al. 1997; Zhang et al. 2008; Han and Wang 2009). The neurotoxic effects of PAHs (e.g., PHE) may be underappreciated in terms of the major mechanism of action of acute PHE-induced toxicity, and may be an important mechanism involved in the more-than-additive mortality following co-exposure with Cu.

There is currently no description of the effects of Cu and PHE on *H. azteca* behaviour (i.e., a powerful biomarker of neurotoxicity). Accordingly, I explored alterations in *H. azteca* activity and ventilation following acute 24-h exposures to sublethal concentrations of Cu and PHE present singly and in mixture. Behavioural trials were accompanied with assays measuring changes in respiratory rates, oxidative stress, and neurotoxicity. My principal goal was to link changes in amphipod behaviour, respiration, oxidative stress, and neurotoxicity in attempt to explain why Cu-PHE mixtures produce more-than-additive toxicity (i.e., mortality) in *H. azteca*.

5.3 Methods

5.3.1 Materials and supplies

A 250 mg L^{-1} Cu stock solution was prepared with $CuSO_4$ salt (purity >99%; Sigma-Aldrich Co. LLC, Oakville, Canada) and 1% OmniTrace® HNO_3 (Thermo Fisher Scientific, Mississauga, Canada) in deionized water (DW; Millipore Canada Ltd., Etobicoke, Canada). A 10 g L^{-1} PHE stock was prepared with the addition of PHE (purity >98%: Sigma) to hexanes (Fisher). The PHE stock solution was kept in the dark at 2°C to prevent degradation. The degradation of the PHE stock solution was monitored by weekly measurements of a 1 mg L^{-1} stock solution of PHE in 100% HPLC grade acetonitrile (Fisher). The 1 mg L^{-1} PHE stock solution was also used as an analytical standard for high potential liquid chromatography (HPLC). For ROS determination assays, dichlorofluorescein-diacetate (H_2DCFDA; Fisher) was used as a fluorescent marker (Cathcart et al. 1983). A 25 uM stock solution was prepared by adding H_2DCFDA to methanol. PESTANAL® malathion (purity <=100%; Sigma-Aldrich) was used as a positive control for neurotoxicity, respiratory, and behavioural assays. A 50 mg L^{-1} stock solution of malathion was prepared with the addition of 10 mg of malathion to 200 mL DW, where the stock solution was titrated with HNO_3 to obtain a pH of 4 to reduce the degradation of malathion in solution (Wolfe et al. 1977). The malathion stock solution was refrigerated in the dark and replaced weekly. Thioflavin T (ThT; Fisher) was used as a fluorescent marker of AChE activity (De Ferrari et al. 2001). A 100 μM stock solution of ThT was prepared in 100 mL of DW. ThT stock solutions were refrigerated in the dark and replaced twice weekly.

5.3.2 Amphipod culturing and bioassays

Hyalella azteca were cultured in a standard artificial medium (SAM; Borgmann 1989). Animals were kept in a 120 L tank filled with 80 L of SAM at 21°C, with two 50% water changes

per week. Animals were fed ca. 200 mg of finely ground Tetramin® fish flake twice per week following each water change. Cotton gauze strips were added to the culture tank to serve as substrate. The culture was illuminated with two 48" fluorescent light bulbs emitting ca. 55 μE m^{-1} s^{-1} with a 16 h light:8 h dark photoperiod.

All toxicity tests involved adult *H. azteca* and were carried out at 21°C with a 16 h light:8 h dark photoperiod. Adults were sorted from juveniles with a mesh net with a 2 mm mesh size, and were transferred to a 2 L polypropylene container with 1.5 L of SAM and gauze mesh to acclimate for 24 h prior to toxicity testing. All tests were carried out in 400 mL glass beakers filled with 300 mL of test water in replicates of three. Each replicate contained 5 to 10 amphipods. Copper test water consisted of 2 L of Cu-spiked SAM with the addition of aliquots of Cu stock solutions. Tests involving PHE used passive dosing with PHE spiked polydimethylsiloxane (PDMS) GE silicone II (General Electric, Salisbury, USA):hexanes solutions as described in Gauthier et al. (2015a), which were added to 400 mL glass beakers (Fisher) where the hexanes was allowed to evaporate leaving an PHE-enriched PDMS film. The PDMS film was allowed to cure for 2 h before test media was added. Beakers containing PDMS films and test water (i.e., SAM for PHE-only treatments, and Cu-spiked SAM Cu-PHE treatments) were then mixed on an orbital shaker at 50 rpm for 24 h to allow PHE to come to equilibrium in solution.

Concentrations of Cu and PHE were chosen based on the results of range finder assays that determined the maximum non-lethal concentration over the 24 h exposure duration (data not shown). Amphipods were exposed to measured concentrations of 91.8- and 195.0-μg L^{-1} of Cu and PHE, and a binary mixture at the same concentrations. For positive control experiments, amphipods were exposed to nominal concentrations of 20-, 40-, or 50-μg L^{-1} malathion.

All 2 L Cu-spiked SAM solutions were sampled and analyzed for metal analyses by ICP-AES at the Lakehead University Instrumentation Laboratory (Thunder Bay, Canada). Phenanthrene

was measured within 3 h of sampling by HPLC (1200 Series, Agilent Technologies, Mississauga, Canada) with a SDB-C18 5 µm 4.6 x 150 mm column, in an initial mobile phase of 60:40 acetonitrile:test water that 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. Concentrations of PHE were determined with a calibration curve obtained from 10- to 1000-µg L^{-1} standards.

5.3.3 Amphipod behaviour

All behavioural trials were carried out during the illuminated segment of the 16 h light:8 h dark photoperiod. Video was captured at 30 frames per second with a Sony Nex-5 mirrorless interchangeable lens digital camera (Sony Electronics Inc., Toronto, Ontario) coupled to a Minolta 100 mm 1:1 macro lens (Konica Minolta Inc., Mississauga, Ontario) via a Fotodiox MD-Nex adaptor (Fotodiox Inc., Waukegan, Illinois) and mounted on a tripod. For ventilatory trials, a 2X extension tube (Konica Minolta) was paired with the lens to double magnification. Following 24-h exposures, animals and 50 mL of test water from each replicate were transferred to petri dishes, allowed to acclimate for 1 min, and then video recorded for 3 min. Following activity trials, animals from each replicate were transferred to well slides (Fisher) with sufficient treatment water to keep the animal submerged. Coverslips were used to keep the animals held in place on their sides. Slides were backlit with a single LED to illuminate the pleopods, and video was recorded for 1 min.

Activity was quantified as % of total time spent swimming using Lolitrack behavioural analysis software (Loligo Systems, Tjele, Denmark). Pleopod beats were counted manually during a randomly selected 10 s segment of video. Ventilation data were analyzed as pleopod beats per min (bpm).

5.3.4 Respiration

Oxygen consumption was measured with closed-system respirometry during the the illuminated segment of the 16 h light:8 h dark photoperiod. Amphipods and 0.7 mL of SAM were placed into glass micro-respirometry chambers made by Kris Fisher (University of Lethbridge, Lethbridge, Canada). Oxygen saturation in mg L^{-1} was then measured with a FireStingO_2 fiber-optic oxygen meter (Pyro Science, Aachen, Germany). Chambers were then sealed and oxygen saturation was measured again after 1 h. Amphipods were removed from the chambers and transferred to weigh paper, patted dry with Kimwipes®, and allowed to dry for an additional 5 min. Amphipods were then transferred to a pre-weighed weigh paper and wet weight was measured. Respiratory rates are reported as mg O_2 g^{-1} h^{-1}.

5.3.5 Oxidative stress

The production of ROS in vivo was used to describe oxidative stress following exposures. Surviving animals were collected from each treatment and transferred to 1.5 mL cryovials along with 600 µL of SAM. Amphipods were then dyed with the addition of 400 µL of H_2DCFDA stock to achieve a final H_2DCFDA concentration of 10 µM. Samples were incubated for 4 h and fluorescence was measured by confocal microscopy with a fluorescence detector (FV1200, Olympus America Inc., PA, USA). Excitation and emission wavelengths of 485 and 530 nm, respectively, both with a 20 nm bandwidth, were used for fluorescence imaging. Sample fluorescence was quantified with ImageJ 1.47 (Rasband 2014), and was reported as the corrected total sample fluorescence (f_{ct}) calculated as $f_{ct} = a \times f - a \times f_b$, 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.

5.3.6 Neurotoxicity

Neurotoxicity was measured as inhibition of acetylcholinesterase (AChE) activity. Pre-exposed amphipods from each replicate were collected, kept separate, and transferred to weigh paper. Amphipods were patted dry with Kimwipes® and left for an additional 5 min to air dry. Amphipods were then transferred to pre-weighed weigh paper and wet weight was measured. Weighed whole-animals were then transferred to a Dounce tissue grinder (Sigma-Aldrich) and homogenized. Whole animals were used for AChE determination as preliminary trials revealed that exposure to Cu, PHE, and the Cu-PHE mixture did not affect ThT fluorescence in the head (i.e., brain), and that the inclusion of head tissue in addition to body tissue in the analysis did not noticeably diminish AChE measurements in body tissue. Homogenized tissue was flushed from the Dounce mortar into 1.5 mL centrifuge vials with 3 sequential aliquots totaling 1 mL of a 0.02% Triton X-100 20 mM phosphate buffer at pH 7. Sample vials were chilled on ice until all samples from the ongoing experiment were processed. Vials were then centrifuged at 15,000 rpm for 15 min at room temperature and the supernatant (ca. 1 mL of solution) was collected and transferred to 3 mL cuvettes (Sigma-Aldrich). Cuvettes were then topped-up with phosphate buffer to achieve a total volume of 3 mL, and 200 µL of ThT stock solution was added to each cuvette to obtain a final ThT concentration of 6.25 µM. Fluorescence was measured with a Perkin Elmer LS50B luminescence spectrophotometer (PerkinElmer LAS Canada Inc., Woodbridge, Canada) at 23°C, with an excitation and emission wavelength of 450 and 480 nm respectively, both with a bandwidth of 10 nm, according to De Ferrari et al. (2001). Fluorescence intensity was then corrected by wet weight.

5.3.7 Statistical analyses

A general linear model, $Y_{ijk} = \mu + Cu_i + PHE_j + Cu\text{-}PHE_{ij} + e_{ijk}$, was used to analyze differences between control, Cu, PHE, and Cu-PHE treatments for all endpoints tested. Significance

of the interaction term, Cu-PHE$_{ij}$, indicates non-additive co-toxicity (Iwasaki et al. 2015). All statistical analyses were carried out with the 'lm' function in the 'stats' package of the open-source statistical software, R (R core team 2015). A significance level of $\alpha = 0.05$ was used for all tests. All data met the assumptions of normal distribution and homogeneity of variance according to Shapiro-Wilk's (R core team 2015) and Levene's test (Fox and Weisberg 2011) respectively.

5.4 Results

5.4.1 Behaviour

Mean control amphipod ventilation was 350 bpm (Figure 5.1B). Mean ventilation following exposure to Cu was 346 bpm and was not altered from control ($t_{(14)} = 0.16$; $p = 0.88$). Exposure to PHE reduced ventilation to 132 bpm ($t_{(14)} = 11.0$; $p = <0.0001$). There was an interactive effect of mixing Cu and PHE on ventilatory behaviour ($t_{(14)} = 3.29$; $p = 0.0053$), where co-exposure to Cu partly attenuated the PHE-induced ventilatory-inhibition down to 241 bpm Cu-PHE.

Control amphipods were active for 49% of recording period (Figure 5.1A). Mean % activity from Cu-exposed animals did not increase above control ($t_{(8)} = 1.23$; $p = 0.25$). However, mean % activity from PHE-exposed animals was lower than control, with amphipods swimming for 23% of the recording period ($t_{(8)} = 2.87$; $p = 0.021$). Mean % activity from Cu-PHE-exposed animals was lower than control, with amphipods in treated containers swimming for 20% of the recording period; however, there was no detectable interactive effect of co-exposure ($t_{(8)} = 1.15$; $p = 0.29$).

Observations of PHE-exposed animals revealed that amphipods were hyperstimulated. For example, control animals were fully capable of swimming in activity trials and had regular ventilation. In contrast, PHE-exposed animals were seemingly incapable of coordinating muscle movements, where animals were moving all appendages, antennae, and abdominal muscles movements, where animals were moving all appendages, antennae, and abdominal muscles in an

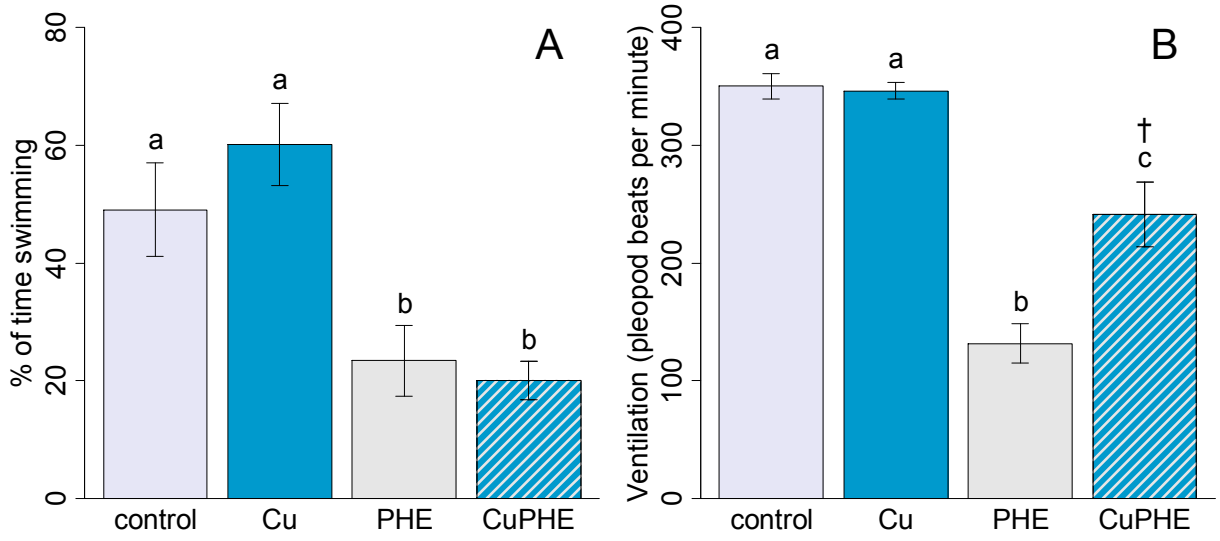

Figure 5.1 Activity (panel A) and ventilatory (panel B) inhibition following exposure to Cu, phenanthrene (PHE), and Cu-PHE mixtures. Daggers (†) denote a significant interactive effect (i.e., non-additive toxicity).

uncontrolled fashion. As a result, PHE-exposed amphipods were severely inhibited in terms of swimming performance and had irregular ventilation. Similarly debilitating hyperstimulation was also observed in Malathion-exposed amphipods.

5.4.2 Respiration, oxidative stress, and neurotoxicity

Control amphipods had a mean respiratory rate of 205 mg O_2 g^{-1} h^{-1} (Figure 5.2A). Exposure to Cu did not affect respiration ($t_{(20)}$ = 0.39; p = 0.69), while exposure to PHE substantially increased respiration to 311O_2 g^{-1} h^{-1} ($t_{(20)}$ = 4.85; p = <0.0001). Co-exposure to Cu attenuated the PHE-induced increase in respiration ($t_{(20)}$ = 2.97; p = 0.0077) and returned respiration to control levels.

Exposure to Cu increased ROS by 91% above control ($t_{(8)}$ = 3.63; p = 0.0067). However, exposure to PHE had no detectable effect on ROS production ($t_{(8)}$ = 0.58; p = 0.58). There was no interactive effect on ROS production from mixing Cu and PHE ($t_{(8)}$ = 0.08; p = 0.94).

Mean measured whole-body amphipod AChE activity was not elevated above control following exposure to Cu ($t_{(38)}$ = 0.89; p = 0.38; Figure 5.2B). Exposure to PHE significantly

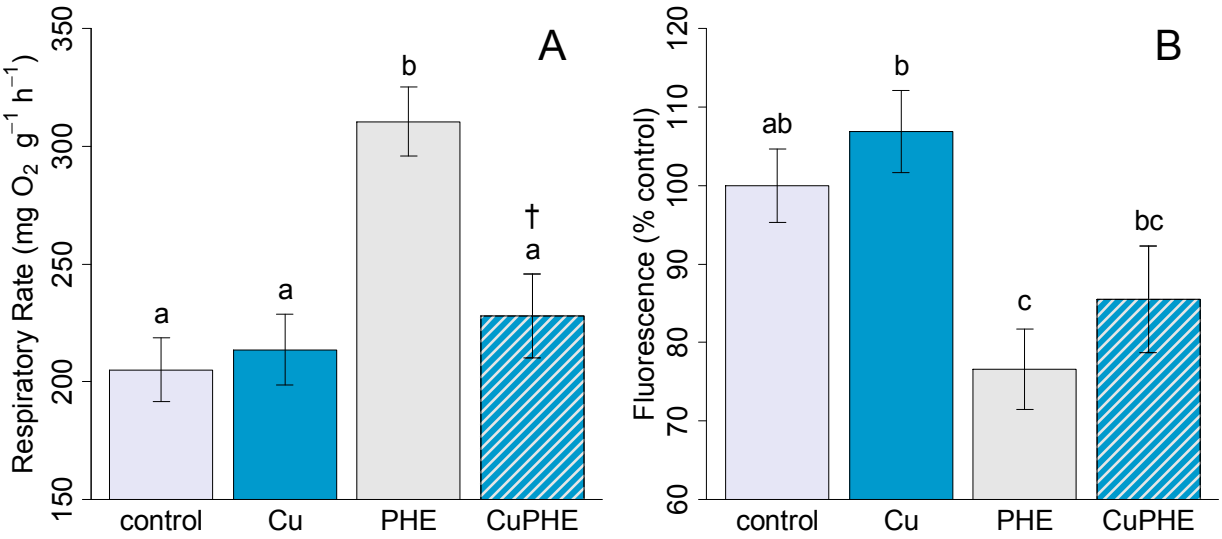

Figure 5.2 Respiratory (panel A) and neurotoxic (panel B) effects following exposure to Cu, phenanthrene (PHE), and Cu-PHE mixtures. Daggers (†) denote a significant interactive effect (i.e., non-additive toxicity)

decreased ThT-fluorescence to 77% of control ($t_{(38)} = 3.29$; p = 0.0022). There was no detectable interactive effect from Cu-PHE co-exposures ($t_{(38)} = 0.18$; p = 0.87). However, co-exposure to Cu diminished the effect of PHE so that Cu-PHE-exposed amphipod fluorescence was no longer distinguishable from control.

Similarly to PHE-exposed animals, respiratory rate increased in Malathion-exposed amphipods in a concentration-dependent manner (Figure 5.3A; $F_{(3,11)} = 4.95$; p = 0.021), and AChE activity was reduced by 33% in amphipods exposed to 40 µg L^{-1} Malathion compared to control amphipods (Figure 5.3B; $t_{(10)} = 4.28$; p = 0.0016).

5.5 Discussion

It was found that PHE acts as a potent neurotoxicant in *H. azteca*, with a similar mechanism of action as Malathion (i.e., AChE inhibition). The PHE-induced neurotoxic effects amounted to

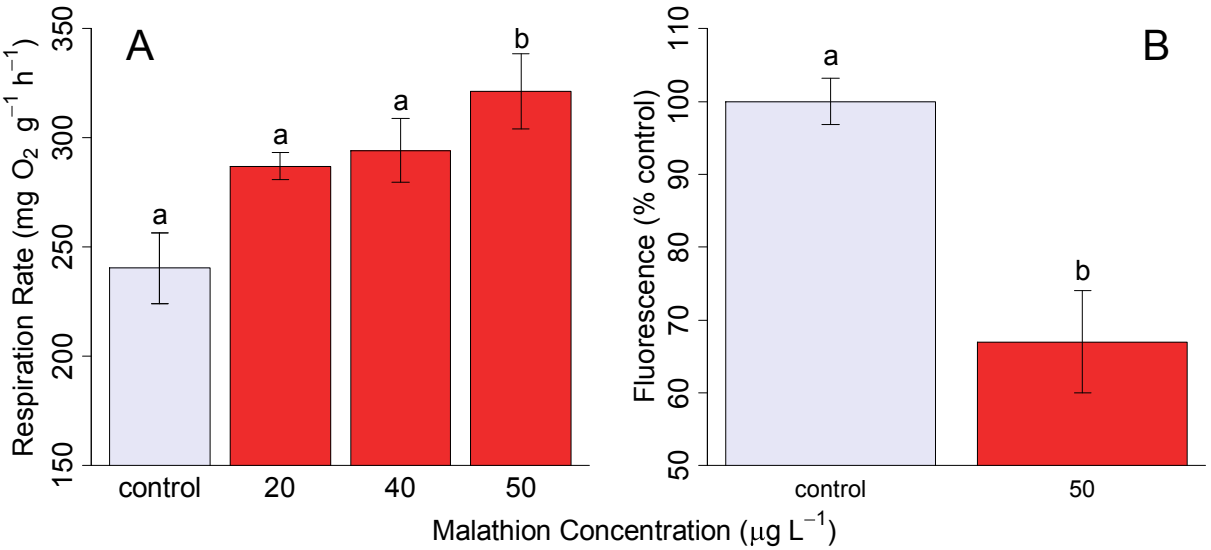

Figure 5.3 Respiratory (panel A) and neurotoxic (panel B) effects following exposure to Malathion.

severe behavioural impairment following 24-h exposure to a sublethal concentration of 195.0 μg L^{-1} PHE, where co-exposure to Cu attenuated several of the pesticide-like effects of PHE (e.g., increased respiration and hyperstimulation).

I observed a decrease in ventilation that would suggest a concomitant decrease in respiration. However, respiration actually increased by 51% in PHE-exposed amphipods, which can be most likely attributed to the fact that amphipods were hyperstimulated following exposure to PHE. Hyperstimulation would not only suggest an increase in energy demand requiring greater respiration, but would also likely increase ventilation at the gill regardless of the reduction and irregularity of pleopod beating, as the twitching movements of all other appendages and body segments likely stimulated the flow of water over the gills.

The PHE-induced reduction in AChE activity is the most likely toxic mechanism responsible for the observed hyperstimulation and behavioural impairment. Acetylcholine (ACh) is a highly conserved neurotransmitter found in neuromuscular junctions that binds with the post-synaptic cleft following its Ca^{2+}-triggered released from the synaptic vesicles of the pre-synaptic cleft (Zubay 1988). Binding of ACh to the post-synaptic cleft initiates an action potential that subsequently

propagates down the muscle to stimulate muscle fiber contraction. Acetylcholinesterase hydrolyzes ACh to remove it from the post-synaptic cleft. However, when AChE is inhibited, a buildup of ACh at the post-synaptic cleft leads to overstimulation of the neuromuscular tissue, producing rapid involuntary muscle twitching in both fish and aquatic invertebrates (as reviewed by Fulton and Key 2001). This rapid twitching is exactly what was observed in *H. azteca* following exposure to PHE and Malathion. Combined with the observed reduction in AChE activity, the observed hyperstimulation strongly suggests that PHE is a potent neurotoxicant in *H. azteca* producing toxicological effects that closely resemble those of an organophosphate pesticide.

Oxidative stress is another mechanism through which aquatic species may experience behavioural impairment (Correia et al. 2007; Vieira et al. 2009). However, while exposure to Cu increased the production of ROS, exposure to PHE did not, ruling out the possibility that ROS imbalance is largely responsible for behavioural impairments associated with exposure to PHE. Moreover, the increases in ROS associated with exposure to Cu did not coincide with any observed behavioural impairment in the present study, further supporting that behavioural alterations in *H. azteca*, at least from acute 24-h exposures, are not linked to ROS imbalance. It is most likely that the reduction in AChE induced by PHE is responsible for altered behaviour in *H. azteca*.

The exact mechanism by which PHE, among other PAHs, inhibits AChE activity is unknown. Holth and Tollefsen (2012) found that the aromatic fraction of oil production process water (i.e., the fraction containing PAHs) inhibited AChE activity in a competitive manner resembling organophosphate pesticides (e.g., malathion). Malathion is metabolized by CYP enzymes to produce the oxygenated analogue malaoxon containing a P=O moiety (Costa 2008), which phosphorylates the esteratic β-anionic site within the cavity of the active region of AChE (Pohanka 2011) inactivating the enzyme. As PHE, among other PAHs, is also readily metabolized via the cytochrome P450 (CYP) pathway (Conney 1982), it is possible that just as with malathion

and its malaoxon metabolite, PHE metabolites are responsible for AChE inhibition. However, as PAH-metabolites (e.g., PAH-epoxides, -hydrodiols, -quinones, etc.) lack a P=O moiety, it is unlikely that PHE-metabolites would act on AChE in a similar fashion as malaoxon. Alternatively, AChE can be inactivated through binding of nitro-aromatic compounds at the α-anionic site AChE (Pohanka 2011). The likely presence of nitro-PAHs in petroleum products and associated effluents (Sawicki et al. 1965) may in part account for the frequently reported AChE inhibition following exposure to these PAH sources. However, in laboratory-based studies, the present study included, animals exposed to non-nitro-PAHs experienced AChE inhibition. The endogenous transformation of PAHs to nitro-PAHs in vivo is possible and has been demonstrated in mammals (Miyanishi et al. 1996) and fish (Shailaja et al. 2006). However, further effort is required to validate that the potential production of nitro-PAHs in vivo is substantial enough to inhibit AChE to the extent reported in the literature following exposure to non-nitro-PAHs. Moreover, further effort is required to determine if PAHs inhibit AChE activity by other indirect means, such as through changes in gene expression. A final piece of evidence suggesting that PHE and malathion inhibit AChE via dissimilar pathways is that Cu has been shown to enhance AChE inhibition in marine copepods exposed to malathion (Forget et al. 1999), whereas Cu antagonized the PHE-induced AChE inhibition observed in the present study.

Although statistical analysis did not detect a significant interactive effect on AChE activity from Cu-PHE co-exposure in this experiment, co-exposure to Cu attenuated the PHE-induced changes in ventilation and respiration observed in *H. azteca*. As PHE-induced ventilatory and respiratory effects are most likely attributed to the inhibition of AChE activity in neuromuscular tissue, it is likely that Cu antagonized AChE activity, and that the sample size and/or observed effect size was merely insufficient to detect antagonism. In fact, although no significant interaction was found, AChE activity in Cu-PHE treatments was not distinguishable from the control treatment,

suggesting a shift away from the clear PHE-induced reduction in AChE activity. Others have found that Cu antagonizes PAH-induced reductions in AChE activity (Zhang et al. 2008). Han and Wang (2009) found that Cu concentrations ≤ 100 μg L^{-1}, antagonized benzo[α]pyrene-induced AChE inhibition in the Korean lugworm (*Perinereis aibuhitensis*), while Cu concentrations >200 μg L^{-1} had no antagonistic effect. Although cross study/animal comparisons should be considered with caution, it is interesting to note that the Cu concentrations in the present study would be right on the margin of inducing antagonism in *P. aibuhitensis*, and were seemingly on the margin of inducing antagonism in *H. azteca* as well. As the mechanism by which PAHs inhibit AChE activity is unknown, it is perhaps premature to speculate as to what interactive co-toxic mechanism accounts for the antagonistic effect of Cu to PAH-induced AChE inhibition. However, as discussed above, it is possible that PAH metabolites, rather than PAH parent compounds, are responsible for AChE inhibition and that the metal-induced inhibition of CYP gene expression and activity may reduce the presence of PAH-metabolites in biota co-exposed to metals (as reviewed in Chapter 2), thereby reducing AChE inhibition. Testing PAH-induced AChE inhibition alone with various CYP inhibitors (e.g., α-napthoflavone and/or aryl hydrocarbon receptor morpholinos) would help elucidate the role of PAH parent compounds and metabolites play in AChE inhibition.

In previous work, co-exposure to Cu and PHE produced more-than-additive lethality in *H. azteca* (Chapters 3 and 4). There is evidence to suggest the mechanisms responsible for more-than-additive lethality in *H. azteca* are not related to potentiated oxidative stress or altered Cu accumulation (Chapter 4). Here, further evidence is presented revealing that the more-than-additive lethality of Cu-PHE mixtures in *H. azteca* is also not related to potentiated neurotoxicity. Nonetheless, I do present evidence outlining the critical role neurotoxicity plays in PHE-induced toxicity in *H. azteca*. It seems likely that multiple pathways of Cu and PHE toxicity are involved in ultimately producing more-than-additive lethality in *H. azteca* exposed to Cu-PHE mixtures, where

the effects of Cu and PHE on the co-exposed toxicants detoxification may play a role (Chapter 2), and should be investigated further.

5.6 References

Amiard-Triquet, C., 2009. Behavioural disturbance: the missing link between sub-organismal and supra-organismal responses to stress? Prospects based on aquatic research. Human and Ecological Risk Assessment 15, 87-110.

Borgmann, U., 1996. Systematic analysis of aqueous ion requirements of *Hyalella azteca*: a standard artificial medium including the essential bromide ion. Archives of Environmental Contamination and Toxicology 30, 356-363.

Brewer, S. K., Little, E. E., DeLonay, A. J., Beauvais, S. L., Jones, S. B., Ellersieck, M. R., 2001. Behavioural dysfunctions correlate to altered physiology in rainbow trout (*Oncorynchus mykiss*) exposed to cholinesterase-inhibiting chemicals. Achrives and Environmental Contamination and Toxicology, 40, 70-76.

Cathcart, R., Schwiers, E., Ames, B.N., 1983. Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. Analytical Biochemistry 134, 111-116.

Conney, A.H., 1982. Induction of microsomal-enzymes by foreign chemicals and carcinogens by polycyclic aromatic-hydrocarbons. Cancer Research 42, 4875-4917.

Correia A.D., Goncalves R., Scholze M., Ferreira M., Henriques M.A.R., 2007. Biochemical and behavioural responses in gilthead seabream (*Sparus aurata*) to phenanthrene. Journal of Experimental Marine Biology and Ecology 347,109-122.

Costa, L.G., 2008. Toxic effects of Pesticides, in Klassen, C.D. (Ed.), Casarett & Doull's Toxicology: The Basic Science of Poisons, 7 ed. McGraw-Hill, Toronto, pp. 883-930.

Curran, K.J., Irvine, K.N., Droppo, I.G., Murphy, T.P., 2000. Suspended solids, trace metal and PAH concentrations and loadings from coal pile runoff to Hamilton Harbour, Ontario. Journal of Great Lakes Research 281, 217-227.

De Ferrari G.V., Mallender, W.D., Inestrosa, N.C., Rosenberry, T.L., 2001. Thioflavin T is a fluorescent probe of the acetylcholinesterase peripheral site that reveals conformational interactions between the peripheral and acylation sites. The Journal of Biological Chemistry 276, 23282-23287.

Donahue, W.F., Allen, E.W., Schindler, D.W., 2006. Impacts of coal-fired power plants on trace metals and polycyclic aromatic hydrocarbons (PAHs) in lake sediments in central Alberta, Canada. Journal of Paleolimnology 35, 111-128.

Forget, J., Pavillon, J.-F., Beliaeff, B., Bocquené, G., 1999. Joint action of pollutant combinations (pesticides and metals) on survival (LC50 values) and acetylcholinesterase activity of *Tigriopus brevicornis* (Copepoda, Harpacticoida). Environmental Toxicology and Chemistry, 18, 912-918.

Fox, J., Weisberg, S., 2011. An {R} Companion to Applied Regression. Sage, Thoussand Oaks. 472 pp.

Fulton, M.H., Key, P.B., 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environmental Toxicology and Chemistry, 20, 37-45.

Han, Z.-H., Wang, J.-H., 2009. Interactive effects of heavy metals and BaP on AChE activity of polycheate *Perinereis aibuhitensis*. Synthesis and Reactivity in Inorganic, Metal-Organic, and Nano-Metal Chemistry 39, 193-188.

Holth, T.F., Tollefsen, K.E., 2012. Acetylcholine esterase inhibitors in effluents from oil production platforms in the North Sea. Aqautic Toxicology 112-113, 92-98.

Iwasaki, Y., Brinkman, S.F., 2015. Application of generalized linear mixed model to analyze mixture toxicity: survival of brown trout affected by copper and zinc. Environmental Toxicology and Chemistry 34, 816-820.

Martínez-Tabche, L., Ramírez, B.M., Germán, F.C., Galar, C.I., Madrigal, C.I., Madrigal, O.M., Ulloa, G.V., Orozco, F.M., 1997. Toxic effect of sodium dodecylbenzensulfonate, lead, petroleum, and their mixtures on the activity of acetylcholinesterase of *Moina macrocopa* in vitro. Environmental Toxicology and Water Quality 12: 211–215.

Melvin, S.D., Wilson, S.P., 2013. The utility of behavioural studies for aquatic toxicology testing: a meta-analysis. Chemosphere 93, 2217-2223.

Mielke, H.W., Wang, G., Gonzales, C.R., Le, B., Quach, V.N., Mielke, P.W., 2001. PAH and metal mixtures in New Orleans soils and sediments. Science of the Total Environment 281, 217-227.

Miyanishi, K., Kinouchi, T., Kataoka, K., Kanoh, T., Ohnishi, Y., 1996. In vivo formation of mutagens by intraperitoneal administration of polycyclic aromatic hydrocarbons in animals during exposure to nitrogen dioxide. Carcinogenesis 17, 1483-1490.

Muniz, P., Danulat, E., Yannicelli, B., García-Alonso, J., Medina, G., Bícego, M.C., 2004. Assessment of contamination by heavy metals and petroleum hydrocarbons in sediments of Montevideo Harbour (Uruguay). Environment International 29, 1019-1028.

Pohanka, M., 2011. Cholinesterases, a target of pharmacology and toxicology. Biomedical Papers of the Faculty of Medicine of Palacky University, Olomouc Czech Republic 155, 219-223.

R Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rasband, W.S., 2014. ImageJ. National Institute of Health, Maryland. http://imagej.nih.gov/ij/.

Sawicki, E., Meeker, J.E., Morgan, M.J., 1965. The quantitative composition of air pollution: source of effluents in terms of aza heterocyclic compounds and polynuclear aromatic hydrocarbons. International Journal of Air and Water Pollution 9, 291-298.

Shailaja, M.S., Rajamanickam, R., Wahidulla, S., 2006. Formation of genotoxic nitro-PAH compounds in fish exposed to ambient nitrite and PAH. Toxicological Sciences 91, 440-447.

Sloman, K.A., McNeil, P.L., 2012. Using physiology and behavioura to understand the responses of fish to early life stages to toxicants. Journal of Fish Biology 81, 2175-2198.

Sprovieri, M., Feo, M.L., Prevedello, L., Manta, D.S., Sammartino, S., Tamburrino, S., Marsella, E., 2007. Heavy metals, polycyclic aromatic hydrocarbons and polychlorinated biphenyls in surface sediments of the Naples harbour (southern Italy). Chemosphere 67, 998-1009.

Valette-Silver, N., Hameedi, M.J., Erfurd, D.W., Robertson, A., 1999. Status of the contamination in sediments and biota from the western Beaufort Sea (Alaska). Marine Pollution Bulletin 38, 702-722.

Vieira L.R., Gravato C., Soares A.M.V.M., Morgado F., Guilhermino L., 2009. Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: Linking biomarks to behaviour. Chemosphere 76, 1416-1427.

Wolfe, N.L., Zepp, R.G., Gordon, J.A., Baughman, G.L., Cline, D.M., 1977. Kinetics of chemical degradation of malathion in water. Environmental Science & Technology 11, 88-93.

Xie, F.L., Koziar, S.A., Lampi, M.A., Dixon, D.G., Norwood, W.P., Borgmann, U., Huang, X.D., Greenberg, B.M., 2006. Assessment of the toxicity of mixtures of copper, 9,10-phenanthrenequinone, and phenanthrene to Daphnia magna: Evidence for a reactive oxygen mechanism. Environmental Toxicology and Chemistry 25, 613-622.

Zhang, Q., Zhou, Q., Wang, J., Sun, S., Hua, T., Ren, L., 2008. Influences of Cu or Cd on the neurotoxicity induced by petroleum hydrocarbons in ragworm *Perinereis aibuhitensis*. Journal of Environmental Sciences 20, 364-371.

Zubay, G., 1988. Biochemistry. Macmillan Publishing, Basingstoke, UK, pp 1226.

Chapter 6 Summary and conclusions

Mixtures of contaminants often produce unexpected toxicity compared to predictions based on individual toxic responses. It may be that co-occurring contaminants attenuate the predicted toxicity of the mixture. However, it may be that the toxicity of the mixture is greater than expected based on the individual toxicities of the contaminants present. The latter scenario is common for binary mixtures of metals and polycyclic aromatic hydrocarbons. Understanding which mixtures produce more-than-additive toxicity and by what means more-than-additive toxicity is manifested was the central thesis of the work described in this dissertation.

This dissertation describes the toxicological effects of metal-PAH mixtures at several levels of organization in the aquatic crustacean amphipod, *Hyalella azteca*. Whole-organism effects (i.e., mortality and behaviour) and cellular/molecular effects (i.e., metal accumulation, reactive oxygen species (ROS) production, and acetylcholinesterase (ACHE) activity) were explored in attempt to address the ecological risk and underlying interactive co-toxic mechanisms of metal-PAH mixtures in aquatic environments and organisms.

I present several major conclusions. Firstly, in terms of more-than-additive lethality: 1) the mixing of Cu with either PHE or PHQ unanimously produced more-than-additive lethality in *H. azteca*, 2) mixtures of Cd with either PHE or PHQ produced mostly more-than-additive lethality in *H. azteca*; however, they also produced strictly-additive lethality at specific mixing proportions (p_{ri}), 3) mixtures of Ni with PHE produced strictly-additive lethality when the p_{ri} of PHE was low, and more-than-additive lethality when the p_{ri} of PHE was higher; however, the degree of more-than-additive mortality observed at these p_{ri} was not substantial enough to appreciably decrease the effective concentrations of the mixture in light of concentrations of Ni and PHE present in the environment, 4) mixtures of Ni with PHQ also produced strictly-additive lethality when the p_{ri} of

PHE was low, but produced less-than-additive lethality when the p_{ri} of PHQ was higher; 5) mixtures of V with either PHE or PHQ generally produced strictly-additive lethality.

Secondly, the degree of more-than-additive lethality in Cu-PHE, Cu-PHQ, Cd-PHE, and Cd-PHQ mixtures was large enough to warrant an investigation into the protectiveness of the short-term waterborne water quality guideline concentrations for the protection of aquatic life issued by the Canadian Council of Ministers of the Environment (CCME). The response-surface approach allowed for the prediction that Cu-PHE, Cu-PHQ, Cd-PHE, and Cd-PHQ mixtures would produce 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 at the CCME guideline concentrations, emphasizing the importance of considering mixture toxicity moving forward with the determination of safe concentrations of metals and PAHs in aquatic systems. Nonetheless, further experimental effort is required to validate these predictions.

Thirdly, in attempt to explain why Cu- and Cd-PHE/PHQ mixtures produce more-than-additive lethality in *H. azteca*, no evidence was found in support of a potentiated ROS production mechanism. Moreover, Cu and Cd accumulation was not altered in Cu-PHE, Cd-PHE, and Cd-PHQ exposures. However, the observed increase in Cu accumulation when co-exposed with PHQ likely contributed to the more-than-additive lethality observed in Cu-PHQ mixtures.

Lastly, acute exposure to PHE induced pesticide-like neurotoxicity in *H. azteca*, amounting to severe behavioural impairment, potentially represents the major mechanism of acute PHE-induced mortality in *H. azteca*. However, no evidence was found to suggest interactions of PHE and Cu on neurotoxicity were involved in the more-than-additive lethality of Cu-PHE mixtures. In fact, PHE-induced neurotoxicity was attenuated by co-exposure with Cu, regardless of the observed more-than-additive lethality of Cu-PHE mixtures.

The conclusions of this work are based on acute waterborne exposures to *H. azteca* in a standard artificial media having a tight range of water quality characteristics (e.g., pH, temperature,

hardness, etc.). Just as biological and environmental variability amount to differences in the toxicity of a single contaminant, they will also likely lead to differences in the additivity of a mixture of contaminants. Throughout this dissertation, cross-study comparisons have been made carefully in light of experimental and biological differences. Thus, future investigation of metal-PAH mixture toxicity should make similar considerations in regards to inter-experimental differences, and should strive to compare observed whole-organisms effects with toxic mechanisms in vivo. Discovering interactive mechanisms will help overcome inter-study differences, as it is probable that there are certain interactive mechanisms largely responsible for more-than-additive co-toxicity. It is likely that metal- and PAH-induced inhibition of PAH- and metal-detoxification (Chapter 2), respectively, are likely to play an important role in the additivity of metal-PAH mixture toxicity. Further investigation into metal-PAH mixture toxicity should consider the inhibitory effects of these contaminants on each other's detoxification.