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Metal–PAH mixtures in the aquatic environment: A review of co-toxic mechanisms leading to more-than-additive outcomes

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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. 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 7000 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

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Table 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	Fuoranthene	*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)

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 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 (Abendroth et al., 2011; Berenbaum, 1989; Norwood et al., 2003; Sørensen et al., 2007; Teuschler et al., 2002; Vouk et al., 1983).

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 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. 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.

2.1. 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.

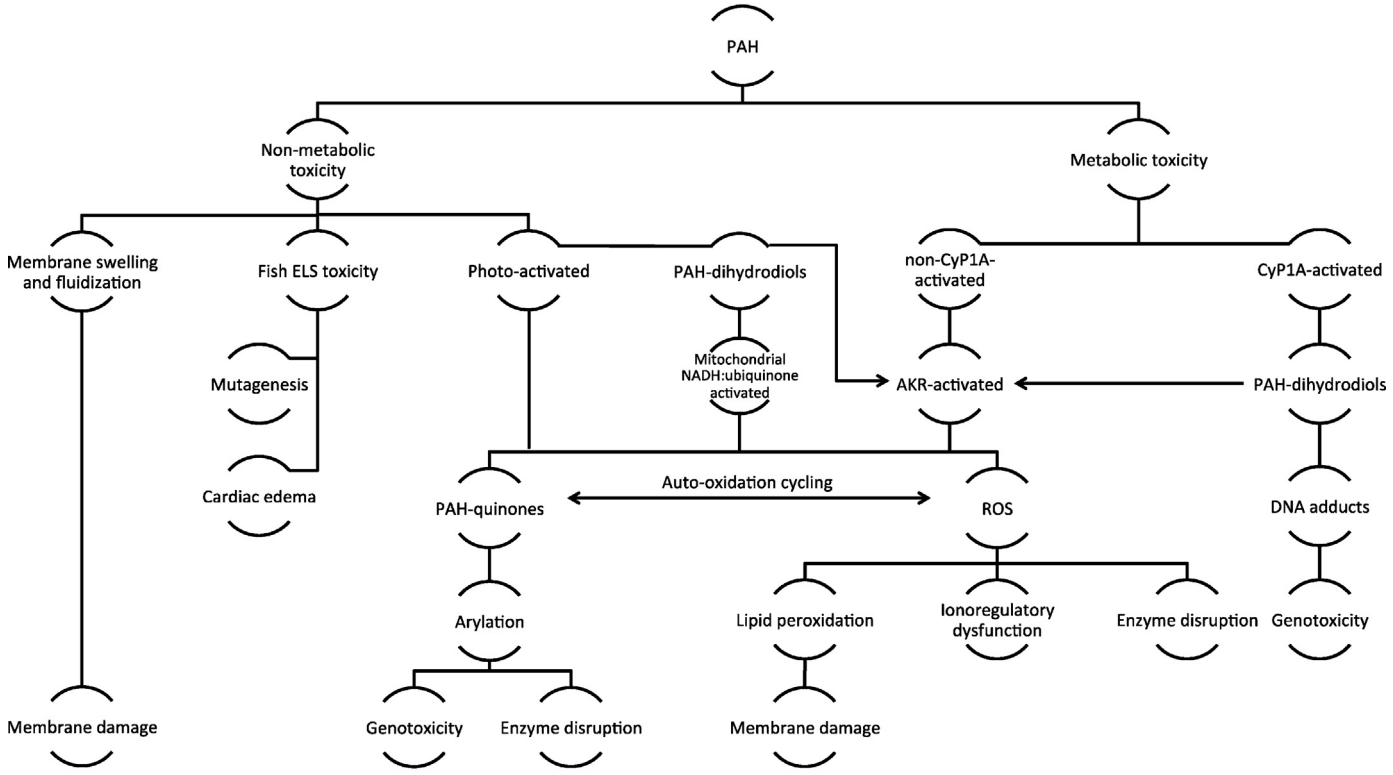

Fig. 1. A generalized pathway of polycyclic aromatic hydrocarbon (PAH) toxicity. Although PAH toxicity can be divided into metabolic and non-metabolic pathways, the photodegradation of PAHs allows for an interaction among these pathways. Moreover, there are many shared endpoints of metabolic and non-metabolic PAH toxicity, such as membrane damage, ionoregulatory dysfunction, and deoxyribonucleic acid (DNA) damage.

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, cytochrome P450 (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 considerable attention because of the capacity of PAHs to alter the CYP1 metabolic 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 (Incardona et al., 2011; Mu et al., 2012; Schober et al., 2006; Wills et al., 2009), 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. 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 (Billiard et al., 2006; Scott and Hodson, 2008; Van Tiem and Di Giulio, 2011; Wassenberg and Di Giulio, 2004; Willett et al., 2001; Wills et al., 2009), 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) 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^{+} adenosine triphosphate ion pumps (e.g., P-type ATPases; Englehardt et al., 1981; Kennedy and Farrell, 2005; Li et al., 2011; McCloskey and Oris, 1993), 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 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., dihydrodiol 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 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 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 (Lu et al., 2009; Palanikumar et al., 2012; Pathiratne and Hemachandra, 2010; Teles et al., 2003) 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.

2.2. 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; De Simone 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 (Ceyhun et al., 2011a; Demirdag et al., 2013; Henry et al., 1995; Lionetto et al., 2000; Skaggs and Henry, 2002). These findings strongly suggest a metal-, species-, and isoform-specific response,

Table 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)

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 side chains (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 zebra fish (*Danio rerio*) exposed to Cd compared to unexposed fish (Lu et 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 $^{\bullet}OH$) 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 and 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, exposure duration, tissue, and species tested (Tables 2 and 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 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; Ragusa et al., 2013; Tiwari et al., 2012), Hg (Simpkins et al., 2013; Sinaie et al., 2010; Yamuna et al., 2012), 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

Table 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 and Gallagher (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)

to metals, a drop in GSH content has been commonly observed (Ahmad et al., 2005; Cirillo et al., 2012; Eyckmans et al., 2011; Schlenk and Rice, 1998; Wang and Wang, 2010), but not without exception (Eyckmans et al., 2011; Wang and Wang, 2010; Jorge et al., 2013; Yamuna et al., 2012). 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, glutathione reductase (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 and 3) may also inhibit ROS detoxification (see Section 3.3). Nonetheless, whatever the transcriptional and post-transcription outcomes might be, metal-induced cytotoxicity increases in GSH depleted scenarios (Maracine and Segner, 1998).

3. 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 processes involved in the transport, metabolism, and detoxification of the co-occurring toxicant (Fig. 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.

3.1. Cellular transport

3.1.1. 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

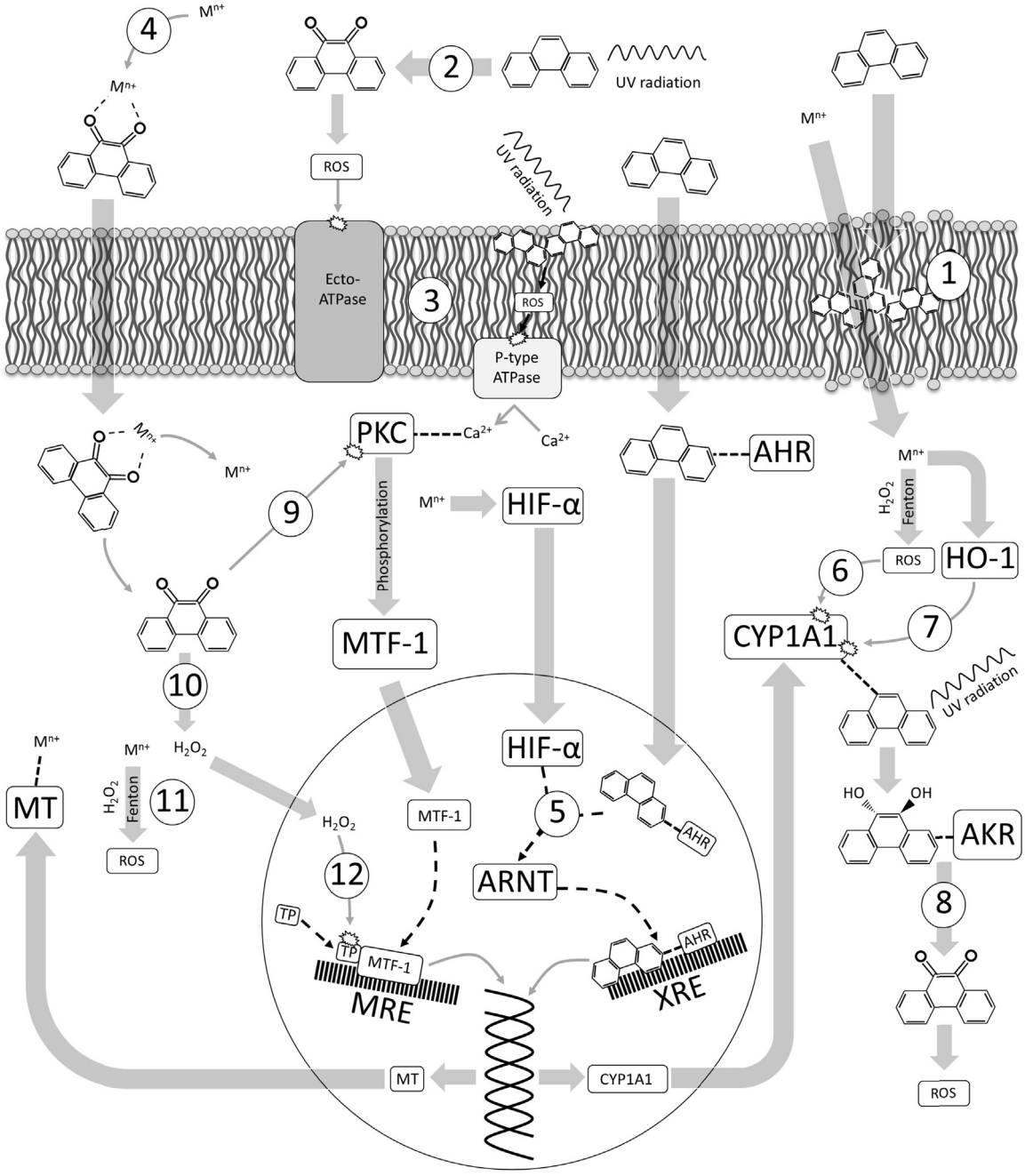

Fig. 2. A schematic of metal–PAH interactions that may contribute to more-than-additive toxicity. Phenanthrene and phenanthrenequinone are used as example polycyclic aromatic hydrocarbons (PAHs) and PAH-quinones (PAHQs). (1) The accumulation of PAHs in lipid membranes alters membrane permeability to 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.

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 to the best of our knowledge 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).

3.1.2. 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., hydroxyl napthoquinones) 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 (Boullemant et al., 2009; Parthasarathy et al., 2008, 2010; Phinney and Bruland, 1994; Poldoski, 1979; Tjalve and Borgneczak, 1994). 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 (Wang et al., 2011b; Fair and Fortner, 1987; Gust and Fleeger, 2005), decrease (Moreau et al., 1999; Xie et al., 2006), and have no effect on (Xie et al., 2006; Moore et al., 1984; Viarengo et al., 1987) 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 (Baker and Head-Gordon, 2010; Murahashi et al., 2003; Sovoca et al., 2001) 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+}.

3.1.3. 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 (Benedetti et al., 2007; Ke et al., 2010; Fair and Sick, 1983; 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 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; Main et al., 2010; and Rathore and Khangarot, 2003) and fish (Jagoe et al., 1993; Khan and McGeer, 2013; Sola et al., 1995).

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).

Table 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, '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)
Cu	*S. cantharus*	In vitro	liv	3MC	–	Risso-De Faverney et al. (1999)
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)
Pb	*S. cantharus*	In vitro	liv	3MC	–	Risso-De Faverney et al. (1999)
Zn	*O. mykiss*	In vivo	mus	baseline	+	Das and Mukherjee (2013)
Zn	*S. cantharus*	In vitro	liv	3MC	–	Risso-De Faverney et al. (1999)

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.

3.2. 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.

3.2.1. 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; Oliveira et al., 2003; Oliveira et al., 2004; Risso-De Faverney et al., 1999; Risso-De Faverney et al., 2000; Sandvik et al., 1997; 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 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 mechanism through the inhibition of ecto-ATPases (Anwar-Mohamed and El-Kadi, 2008). As various other metals (e.g., Cd, Cu, and Hg) inhibit ecto-ATPase in mammalian models (Milosevic et al., 2005 and Milosevic et al., 2009), it is possible that ATP-dependent mechanisms are also involved in the transcriptional inhibition of CYP1A1 in 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 heme oxygenase-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 CYP1A1 activity when co-exposed with PAHs. Furthermore, the combined effect of heme degradation and ROS-thiol interactions may serve to further potentiate CYP1A1 inhibition.

3.2.2. 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 (Costa et al., 2010; George and Young, 1986; Maria and Bebianno, 2011; Risso-De Faverney et al., 2000; Sandvik et al., 1997; van den Hurk et al., 2000; 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 (Saydam et al., 2002; Yu et al., 1997). 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 (Pattnaik and Jena, 2007; Shephard and Simkiss, 1978; Vergani et al., 2007; Viarengo et al., 1996) 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).

3.3. 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 and 3). Furthermore, the effects of PAHs on GST transcription are equivocal, as there are reports describing up- (Bilbao et al., 2010; Garner and Di Giulio, 2012; Nahrgang et al.,

2009; 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 (Jiang et al., 2013; Kim et al., 2010a,b, 2011; Sheader et al., 2006), 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.

4. 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 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 exposure 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 CYP1A1 inhibition 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 sublethal 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.

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