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Critical Reviews

THE MECHANISMS OF NICKEL TOXICITY IN AQUATIC ENVIRONMENTS: AN ADVERSE OUTCOME PATHWAY ANALYSIS

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Abstract: Current ecological risk assessment and water quality regulations for nickel (Ni) use mechanistically based, predictive tools such as biotic ligand models (BLMs). However, despite many detailed studies, the precise mechanism(s) of Ni toxicity to aquatic organisms remains elusive. This uncertainty in the mechanism(s) of action for Ni has led to concern over the use of tools like the BLM in some regulatory settings. To address this knowledge gap, the authors used an adverse outcome pathway (AOP) analysis, the first AOP for a metal, to identify multiple potential mechanisms of Ni toxicity and their interactions with freshwater aquatic organisms. The analysis considered potential mechanisms of action based on data from a wide range of organisms in aquatic and terrestrial environments on the premise that molecular initiating events for an essential metal would potentially be conserved across taxa. Through this analysis the authors identified 5 potential molecular initiating events by which Ni may exert toxicity on aquatic organisms: disruption of Ca^{2+} homeostasis, disruption of Mg^{2+} homeostasis, disruption of $Fe^{2+/3+}$ homeostasis, reactive oxygen species–induced oxidative damage, and an allergic-type response of respiratory epithelia. At the organ level of biological organization, these 5 potential molecular initiating events collapse into 3 potential pathways: reduced Ca^{2+} availability to support formation of exoskeleton, shell, and bone for growth; impaired respiration; and cytotoxicity and tumor formation. At the level of the whole organism, the organ-level responses contribute to potential reductions in growth and reproduction and/or alterations in energy metabolism, with several potential feedback loops between each of the pathways. Overall, the present AOP analysis provides a robust framework for future directed studies on the mechanisms of Ni toxicity and for developing AOPs for other metals. *Environ Toxicol Chem* 2017;36:1128–1137. © 2016 SETAC

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INTRODUCTION

Over the past 15 yr there has been extensive development of biotic ligand models (BLMs) for various divalent metals, including nickel (Ni), that allow for the prediction of acute and chronic metal toxicity to aquatic organisms as a function of chemical parameters typical of freshwater systems. The foundation of the BLM is an understanding of the physiological mechanisms by which metals exert toxicity on organisms. For several metals (e.g., silver [Ag], copper [Cu], lead [Pb], zinc [Zn]) there is a relatively robust understanding of these physiological mechanisms, at least for acute toxicity. This mechanistic understanding has contributed greatly to the wide acceptance of using BLMs for setting water quality guidelines for the protection of aquatic life by regulatory agencies around the world. In contrast, our understanding of the mechanisms of Ni toxicity to aquatic organisms is more limited. This limitation exists despite numerous studies that have been conducted in the past 15 yr exploring potential mechanisms of action for Ni. These studies suggest several potential mechanisms, but a firm understanding of the underlying mechanisms and their links to higher levels of biological organization has remained elusive.

Nickel is used in a range of industrial practices, the most important of which is the production of stainless steel. In addition to point source releases from industrial practices, there are a number of diffuse sources (natural weathering, atmospheric deposition, surface runoff) that contribute to environmental Ni exposure [1]. Typical background waterborne Ni concentrations range from 0.002 μM to 0.010 μM, although impacted systems can have as much as 3 μM Ni in some cases [1,2]. In the European Union, Ni is a priority substance, and a BLM–based threshold environmental quality standard of 0.068 μM has been established. There has been extensive validation of Ni BLMs across ranges of water chemistry parameters, species, and geographical regions [3,4]. However, because of the uncertainty in mechanisms of action, adaptation of existing Ni BLMs developed for North America and Europe to other environments (e.g., Australasia) has been met with some resistance by local regulatory agencies, requiring the development of new BLMs with local species and extensive testing with local waters to validate applicability.

To address this issue, an expert workshop was convened (Vancouver, BC; November 9, 2014; see *Acknowledgment* for workshop participants) to review the mechanisms of Ni toxicity to aquatic organisms and discuss future research objectives that would improve our understanding of the mechanisms of Ni toxicity across a broad range of taxonomic groups. Although the focus of the workshop was on the freshwater aquatic environment, knowledge on mechanisms of Ni toxicity from other environmental compartments (marine, sediments, soil) was also discussed.

The workshop participants concluded that evidence exists in the literature to support a number of possible mechanisms, including disruption of trace element and ion homeostasis (e.g., calcium [Ca], magnesium [Mg], and iron [Fe]), allergic reactions at respiratory epithelia, disruption of energy

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metabolism, and oxidative stress. Participants recommended using an adverse outcome pathway (AOP) analysis as an organizing framework to link mechanisms of action at the protein level to responses that are relevant at the population level for sensitive freshwater taxa (*Lymnaea stagnalis*, *Lemna* sp., and daphnids), recognizing that a specific common mechanism of action may not exist across different aquatic taxa. Participants also recommended using the AOP framework to evaluate a broader range of possible mechanisms of action, taking advantage of genomic/transcriptomic techniques and extant mechanistic knowledge from other environmental compartments (e.g., terrestrial plants, mammals) to identify potential mechanisms not previously considered in aquatic environments. The goal of the present AOP–based evaluation is to guide future research that will develop a sufficiently robust mechanistic understanding of Ni toxicity to allow the application of predictive tools like the BLM to new species and a broader range of geochemical conditions.

The present study provides a more thorough documentation of the discussions and outcomes from the workshop along with a summary review of the extant literature on mechanisms of Ni toxicity. The present study is organized within an AOP framework, drawing information from multiple compartments (aquatic, terrestrial plants, mammalian) to provide a broad perspective. We review interactions of Ni with known and candidate mechanisms of action, beginning at the interface between Ni and proteins and then moving to higher levels of biological organization. The objective of the present review is to provide a synthesis overview rather than a comprehensive detailed discussion of every study in the literature. The conclusion highlights key research needs that will most effectively reduce uncertainty in the Ni AOP model.

DEVELOPMENT OF AN AOP ANALYSIS FOR Ni

An AOP analysis [5] was used to place the available information on Ni toxicity into a conceptual framework describing plausible mechanisms of Ni toxicity. Briefly, AOP analysis provides a framework for evaluating pathways by which a toxicant may interact with an organism to cause an adverse outcome. The analysis begins at the molecular level with molecular initiating events (MIEs) and provides a plausible set of linkages to increasingly higher levels of biological organization, from macromolecular interactions to cellular responses to organ and then whole-animal responses and ultimately population-level and community-level responses that are the focus of environmental risk assessment and water quality management.

To develop an AOP for Ni in aquatic systems, published studies were reviewed on both aquatic and terrestrial organisms, considering both prokaryotes and eukaryotes. Potentially relevant studies were identified using the Web of Knowledge and Google Scholar search engines, as well as the literature cited in identified studies. Identified studies were evaluated for reliability by considering whether appropriate controls and statistics were used and whether exposure concentrations were analytically verified. For many of the in vitro studies evaluating mechanisms of action on isolated tissues or cells, exposure concentrations were not analytically verified, and it is unclear whether the exposure concentrations used in these experiments correspond to environmentally realistic in vivo exposures in whole organisms. However, because the objective of the present study was to evaluate potential pathways for mechanisms of action rather than to quantify specific concentrations in the environment that cause effects, these studies were still considered useful in the present assessment.

Several studies have addressed plausible mechanisms of Ni toxicity in aquatic eukaryotes (e.g., fish and invertebrates) that are typically the focus of aquatic environmental risk assessment. However, these studies have mostly focused on a limited number of mechanisms of action that are specifically relevant to these organisms. Additional potential mechanisms of action have been identified in terrestrial systems (plants and mammals) and prokaryotes that have not been previously considered in aquatic eukaryotes. We hypothesize that some of these mechanisms may be evolutionarily conserved and may also occur in aquatic eukaryotes, so they were included in the AOP analysis for consideration.

Ultimately, 5 potential pathways by which Ni may exert toxicity on aquatic organisms were identified: 1) disruption of Ca^{2+} homeostasis, 2) disruption of Mg^{2+} homeostasis, 3) disruption of $Fe^{2+/3+}$ homeostasis, 4) an allergic reaction at respiratory epithelia, and 5) generation of reactive oxygen species (ROS). We did not distinguish between waterborne and dietary exposure in the following analysis. All of the data in the analysis are based on waterborne exposures because, to the best of our knowledge, no information is available on the mechanisms of dietary Ni toxicity. However, the diet is a significant Ni exposure pathway for some organisms [6,7], and there is evidence that some species are more sensitive to dietary Ni exposure than waterborne Ni exposure [8,9] and that subcellular partitioning of Ni is similar regardless of exposure pathway [10]. We hypothesize that all of the potential MIEs identified except the allergic reaction pathway are also potentially viable pathways for dietary Ni exposure, but no data are available to test this hypothesis.

In the remainder of the present review the scientific support for each of the identified pathways at the level of MIEs is evaluated, followed by consideration of how these effects would translate and interact at higher levels of biological organization. Based on this analysis, we conclude with recommendations for future research priorities regarding the effects of Ni in aquatic systems.

DISRUPTION OF Ca^{2+} HOMEOSTASIS

Calcium is involved in a variety of biological processes including acting as a component of structural systems (e.g., skeleton, shell), as a carrier of action potentials in smooth and cardiac muscle, and as an intracellular secondary messenger in all cells. As a result of this functional diversity, there are also a variety of proteins involved in Ca^{2+} transport in different tissues. These can be divided into 5 groups: voltage-independent Ca^{2+} channels, voltage-dependent Ca^{2+} channels, Na^{+}/Ca^{2+} exchangers, Ca^{2+}/H^{+} exchangers, and Ca^{2+}-adenosine triphosphatases (ATPases). Each of these transporter groups has multiple protein and/or protein isoforms, some of which have not been tested for Ni sensitivity. With respect to Ca^{2+} uptake in freshwater environments, which is a hypothesized mechanism of action for Ni, there are distinct differences in the use of these transporters for Ca^{2+} uptake in fish versus invertebrates (Figure 1) [11–13].

There is no evidence that Ni blocks voltage-independent Ca^{2+} channels like the epithelial Ca^{2+} channel in the fish gill or Ca^{2+}-ATPases (plasma membrane) involved in Ca^{2+} uptake at the respiratory surface of aquatic organisms [13]. Studies in mammalian systems have demonstrated that Ni inhibits Ca^{2+} transport in both voltage-dependent Ca^{2+} channels and

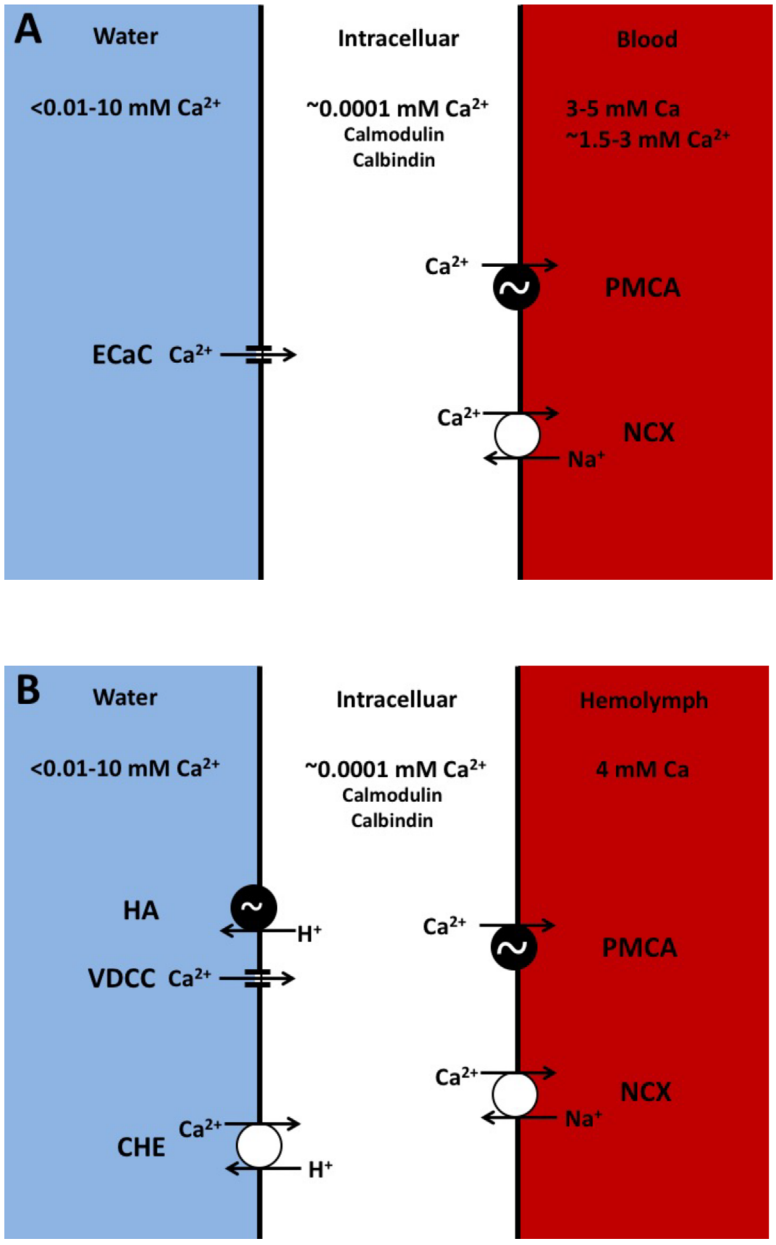

Figure 1. Comparison of mechanisms for Ca^{2+} uptake in (**A**) fish and (**B**) a nickel-sensitive invertebrate, *Lymnaea stagnalis* [11–13]. Uptake of Ca^{2+} across the basolateral membrane is accomplished by the same proteins, although there are significant differences at the apical membrane. Intracellular Ca^{2+} is extremely low because most Ca is bound to proteins (calmodulin, calbindin). Some isoforms of voltage-dependent calcium channels and the Na^+/Ca^{2+} exchanger are known to be Ni-sensitive, but no studies have been performed on these proteins in aquatic organisms. CHE = Ca^{2+}/H^+ exchanger; ECaC = epithelial calcium channel; HA = H^+-ATPase; NCX = Na^+/Ca^{2+} exchanger; PMCA = plasma membrane Ca^{2+}-ATPase; VDCC = voltage-dependent calcium channel.

Na^+/Ca^{2+} exchangers [14–17]. However, in these studies, Ni was being used as a pharmacological blocker at high (e.g., 5000 μM) concentrations. Consequently, it is unknown whether these proteins are sensitive to Ni at environmentally or physiologically relevant concentrations. No studies were identified that assessed the sensitivity of the invertebrate Ca^{2+}/H^+ exchanger to Ni.

At the whole-animal level, a negative relationship between increasing water hardness (Ca^{2+} and Mg^{2+}) and metal toxicity to aquatic organisms has been well documented for over 30 yr for a number of divalent metals including Ni [18]. In some cases, these relationships may have been confounded by other covarying water quality parameters such as alkalinity and pH because of the specific ionic composition of natural and synthetic waters, but subsequent ion-specific studies have confirmed that increasing ambient Ca^{2+} reduces both acute and chronic Ni toxicity to a variety of aquatic and terrestrial animals [3,19–24]. In contrast, most studies indicate that Ca^{2+} has no effect on Ni toxicity to aquatic or terrestrial plants, suggesting that Ni uptake occurs via different mechanisms in plants [25–29]. This may be related to Ni being an essential cofactor in plant ureases and hydrogenases [30].

Additional studies have directly measured the effect of Ca^{2+} on Ni uptake on aquatic vertebrates and invertebrates. In several aquatic species, including daphnids [31], amphipods [32], and the fathead minnow (*Pimephales promelas*) [33], Ca^{2+} has been shown to inhibit Ni uptake. However, Ca^{2+} appears to have no effect on Ni uptake in other species such as the zebrafish (*Danio*

rerio) [34]. Differences in response between the 2 fish species may reflect differences in Ni exposure concentration between the 2 studies, with a relatively low Ni concentration in the *D. rerio* study (0.1 μM) and relatively high concentrations in the fathead minnow study (range of concentrations 0.1–2377 μM). If this hypothesis is correct, these data suggest that Ca-sensitive and Ca-insensitive Ni uptake pathways may exist in fish.

Nickel does not appear to affect Ca^{2+} homeostasis as measured by Ca^{2+} influx, extracellular fluid Ca concentration, or whole-body Ca concentration in rainbow trout (*Oncorhynchus mykiss*) or daphnids [35,36] but does inhibit Ca^{2+} uptake and reduce hemolymph and soft tissue Ca concentrations in the snail *L. stagnalis* [37]. Despite this result for *L. stagnalis*, pharmacological inhibitors that block various proteins involved in Ca^{2+} uptake in *L. stagnalis* have no effect on Ni uptake, suggesting that this is a secondary effect of some other mechanism of action rather than a direct effect of Ni on Ca^{2+} uptake [37].

Although the above data suggest that, in at least some aquatic organisms, Ni may be taken up via Ca^{2+} uptake pathways as ambient Ca^{2+} can affect Ni bioavailability, it is not clear whether Ni can directly affect Ca^{2+} homeostasis. Nickel has only been shown to affect Ca^{2+} homeostasis in a single species, *L. stagnalis*; and it has been hypothesized that this was a secondary effect of some other mechanism of action rather than a direct effect of Ni [37]. An alternative hypothesis has recently been developed that Ni may be taken up by at least some aquatic organisms via paracellular pathways [38]. Under this hypothesis, the effect of Ca^{2+} on Ni bioavailability could be the result of Ca^{2+} reducing ion permeability at tight junctions between cells [39]. Regardless, given the potential sensitivity of some Ca^{2+} transport proteins to Ni, there is still a possibility that this is an important pathway for Ni toxicity in some aquatic organisms.

Table 1. Summary of known Mg^{2+} transporters and their Ni transporting characteristics[a]

Protein	Organism	Transports Ni?	Location
CorA			
CorA	Bacteria	Yes	Surface membrane
lr1	Fungi	Yes	Surface membrane
Mrs2, Mrs2p, MRS2	Yeast, invertebrates, vertebrates	Yes	Mitochondrial membrane
MgtA/B	Bacteria	Yes	Surface membrane
MgtE	Bacteria	No	Surface membrane
TRPM			
TRPM 6	Vertebrates	Yes	Intestine, kidney
TRPM 7	Vertebrates	No	Plasma membrane
SLC41			
SLC41a1	Vertebrates	No	Kidney
SLC41a2	Vertebrates	Yes	Plasma membrane
SLC41a3	Vertebrates	Yes	Muscle, heart
MagT			
MagT1	Vertebrates	No	Liver
TUSC3	Vertebrates	No	Lung, liver
APDC			
APDC1	Vertebrates	?	Brain
APDC2	Vertebrates	Yes	Brain
APDC3	Vertebrates	No	Ubiquitous
APDC4	Vertebrates	?	Ubiquitous
NIPA			
NIPA1	Vertebrates	No	Brain
NIPA2	Vertebrates	No	Brain, kidney
NIPA3	Vertebrates	No	?
NIPA4	Vertebrates	No	?
MMgT			
MMgT1	Vertebrates	No	Golgi complex
MMgT2	Vertebrates	Yes	Golgi complex
HIP			
HIP14	Vertebrates	Yes	Golgi complex
HIP14L	Vertebrates	Yes	Golgi complex

[a]Adapted from Quamme [48].

DISRUPTION OF Mg^{2+} HOMEOSTASIS

Magnesium has many roles in biological systems including stabilizing nucleic acids and protein function, having a catalytic role in >200 Mg^{2+}-dependent enzymatic reactions, including those related to ATP and photosynthesis [40,41]. Interactions of Ni with a variety of Mg^{2+} transporters have been widely documented in bacteria. There are 3 main prokaryotic families of Mg^{2+} transporters: CorA, MgtA/B, and MgtE. Of these, members of the CorA and MgtA/B families have been shown to transport Ni (though with relatively low affinity; Michaelis constant K_m 200–300 μM) and inhibit Mg^{2+} uptake [42,43]. The CorA family homologs in fungi, yeast, and plants also interact competitively with Ni [44–46]. The MgtE family, which occurs only in bacteria, does not interact with Ni [43,47]. There is a much greater diversity of Mg^{2+} transporters in invertebrates and vertebrates, most of which do not appear to have a prokaryotic homolog. The Mg^{2+} transporter Mrs2p located in the mitochondrial membrane of vertebrates is the only clear homolog with the prokaryotic CorA. It is possible that the SLC41 family in vertebrates evolved from MgtE in prokaryotes, although amino acid sequence similarity is low (<15%). Overall, the distribution and Ni sensitivity of Mg^{2+} transporters unique to higher eukaryotes are not well characterized, with the majority of studies focused on transporters isolated from mice and humans [48] (Table 1).

Similar to Ca^{2+}, at the whole-animal level there is an abundance of data showing that Mg^{2+} reduces Ni accumulation and toxicity in a variety of organisms. Increasing waterborne Mg^{2+} concentrations reduce Ni toxicity to daphnids [21,23], oligochaetes [24], rainbow trout [19], and frog embryos [49]. Unlike Ca^{2+}, ambient Mg^{2+} also reduces Ni toxicity in terrestrial [25,28,29], but not aquatic [27], plants. Conversely, Ni has been shown to disrupt Mg^{2+} homeostasis at the whole-animal level in daphnids and snails [36,37] as well as to inhibit Mg^{2+} uptake at the organ and cellular levels of the rainbow trout kidney [50,51]. In aquatic plants, Ni can displace Mg^{2+} from the chlorophyll molecule, leading to inhibition of photosynthesis [52]. The specific physiological consequences of disrupting Mg^{2+} homeostasis in aquatic eukaryotes are not known. However, given the variety of biochemical and physiological functions involving Mg^{2+}, disruption of Mg^{2+} homeostasis by Ni in one or more of these biological systems is a probable mode of action.

DISRUPTION OF Fe HOMEOSTASIS

Iron is essential for life because of its role as an electron donor/receptor for a large number of proteins including those involved in ATP synthesis (cytochrome *c* oxidase), deoxyribonucleic acid (DNA) synthesis (DNA polymerases and helicases), the immune response (Nramp), and respiration (in organisms using hemoglobin). Competitive interactions between Ni and Fe have been observed in several biological systems in both aquatic and terrestrial environments. In freshwater fish, the intestine is the primary site for Fe acquisition, although the gill also likely contributes, especially when dietary sources are Fe-deficient [53–55]. In both tissues,

the divalent metal transporter DMT-1 (SLC11a2) is believed to be the primary transport protein involved in Fe uptake [53,54,56]. Mammalian DMT-1 has been demonstrated to transport Ni, although with relatively low affinity compared with other metals [57]. There is conflicting evidence regarding the Ni sensitivity of DMT-1 in fish. In zebrafish, Fe uptake across the gill was not sensitive to 0.2 μM of Ni [58], whereas Fe uptake across the rainbow trout intestine was sensitive to 2 μM Ni [59]. These disparate observations may be the result of differences in Ni exposure concentration or species-specific or tissue-specific differences in DMT-1 isoforms. For example, 2 different SLC11 isoforms have been isolated from rainbow trout gills for which Fe transport is differentially inhibited by various divalent metals (Ni was not studied) [60].

In terrestrial plants, Fe transport from the root to the shoot is accomplished by the divalent cation transporter IRT-1, and Ni competitively inhibits this process, leading to reduced shoot chlorophyll production and oxidative damage in roots as a result of excess Fe [61,62]; IRT-1 is a member of the ZIP zinc transporter family (SLC39a), with ZIP-14 being the homologous gene in vertebrates. Like IRT-1, the primary role of ZIP-14 is Fe transport [63], which can be competitively inhibited by Ni [64]. Based on studies in mammals, ZIP-14 is most prevalent in the liver, where it is involved in the uptake of non-transferrin-bound Fe at the cell membrane and export of transferrin-bound Fe from endosomes [65,66]. The role of ZIP-14 in fish and other aquatic organisms has not been studied.

In addition to its roles in respiration, DNA replication, and the immune response, Fe-dependent enzymes are involved in the destabilization of hypoxia inducible factor 1α (HIF-1α), the master regulator of the cellular hypoxia response. In the absence of these enzymes, stable HIF-1α induces transcription of over 60 genes involved in red blood cell production, glucose metabolism, and Fe metabolism. In mammalian cell systems, Ni exposure has been shown to induce a physiological cascade resulting in a hypoxia response at the cellular level even under oxic conditions [67–69]. Inappropriate induction of the hypoxia response when cells are normoxic is thought to lead to increased generation of ROS and subsequent oxidative stress (see section, *Formation of ROS*).

Collectively, the available data suggest that disruption of Fe homeostasis by Ni is a potentially important mechanism of action. The above observations of transporter inhibition are supported by gene expression studies in the yellow perch (*Perca flavescens*), where chronic Ni exposure leads to changes in expression of multiple genes involved in Fe homeostasis (transferrin receptors, transferrin, ferritin) indicative of a compensatory response to Ni-induced Fe deficiency [70]. Consequently, Ni-induced Fe deficiency resulting from inhibition of DMT-1 and/or ZIP-14 could impair both the transfer of oxygen through the circulatory system and the electron transport chain that facilitates generation of ATP. This potential mechanism of action is supported by observations that chronic Ni exposure leads to a 68% reduction in hemoglobin content and a corresponding 31% reduction in oxygen consumption rate in *Daphnia magna* [36]. These effects could be further enhanced by the inappropriate induction of the hypoxia response under oxic conditions leading to a biologically significant impairment of organism respiration along multiple pathways.

ALLERGIC RESPONSE AT RESPIRATORY EPITHELIA

Hughes et al. [71] demonstrated a potential allergic response in the gills of Ni-exposed (34–51 μM) rainbow trout. Exposed gills were characterized by edema in the secondary lamellae and increased oxygen diffusion distance. Subsequent studies demonstrated similar morphological responses in rainbow trout gills with corresponding reductions in oxygen extraction efficiency and arterial oxygen content, increased arterial CO_2, and reduced blood pH. Compensatory responses included an increase in ventilation rate, ventilation volume, and hematocrit. Direct infusion of Ni into fish did not elicit a comparable effect, indicating that observed effects at the gill were potentially a surface response to external Ni exposure [35,72]. Gill edema has been observed in rainbow trout in both acute and chronic Ni exposures at concentrations $\geq$5.1 μM, but lower Ni exposure concentrations have not been tested [72,73].

Nickel is a well-known contact allergen in humans, with Ni-induced allergic contact dermatitis affecting 10% to 12% of the general population [74]. The prevalence of this response has led to the hypothesis that observed effects on rainbow trout gills may be an allergic reaction. Allergic reactions are a response of the immune system, which has innate and adaptive components. The innate immune system is present in invertebrates and vertebrates, whereas the adaptive immune system is present only in vertebrates [75]. In humans, Ni-induced allergic contact dermatitis can be elicited via both the innate and adaptive immune systems. The primary pathway within the innate immune system is a proinflammatory signal initiated by Ni interaction with the toll-like receptor 4 (TLR4)/MD-2 complex. This complex, which normally responds to lipopolysaccharide in the outer membrane of gram-negative bacteria, activates a signaling cascade, leading to the expression of cytokines, adhesion molecules, and type 1 interferons that elicit the inflammatory response. The binding site for Ni on TLR4 is comprised of 2 histidine residues that are not well conserved across taxa. For example, these residues are not present in mice, which consequently are not susceptible to Ni-induced allergic contact dermatitis without the presence of an adjuvant to induce sensitivity [74,76,77]. There is a low likelihood that this mechanism of action occurs in fish. First, a TLR4 ortholog has not been found in any fish genome, although a paralog appears to be present in some groups (salmonids, cyprinids) [78,79]. Further, the MD-2 molecule necessary to form the TLR4/MD-2 complex is also lacking in all fish genomes, and the immune response of fish to gram-negative bacteria is via an entirely different pathway [79]. Toll-like receptor proteins are also present in most invertebrates and are part of the invertebrate innate immune response, but the protein structures of invertebrate TLRs are quite different from vertebrate TLRs in general, and TLR4 in particular, again suggesting that this is an unlikely pathway for an Ni allergic response in invertebrates [80].

A second pathway for Ni-induced allergic contact dermatitis, limited to the adaptive immune system, involves T cells. There appear to be a variety of different potential mechanisms for T-cell activation by Ni including Ni–major histocompatibility complex–peptide complexes [81] and direct interaction with T-cell receptors in both $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells [82–84]. Both $CD4^+$ and $CD8^+$ T cells are found in fish and have the same functional roles as in mammals [85,86], with $CD8^+$ T cells in particular present at high densities in the fish gill [87]. However, this similarity in functional roles has only been demonstrated in responses to allografts and other cells, not in response to xenobiotic substances [88,89]. Consequently, although T-cell activation by Ni in fish gills is a potentially viable mechanism for an allergic response, it is not considered a likely mechanism of action for Ni toxicity in fish. Given that invertebrates lack an adaptive immune system and

generally have respiratory structures different from the fish gill, this is considered an even less likely mechanism of action for Ni toxicity in invertebrates.

FORMATION OF ROS

Formation of ROS is another potential mechanism for Ni toxicity in all organisms. Reactive oxygen species can be generated by both endogenous and exogenous mechanisms and at low levels are an important component of several intracellular signaling pathways. Excessive ROS generation is regulated by a variety of nonenzymatic (e.g., glutathione) and enzymatic (e.g., superoxide dismutase, catalase, glutathione peroxidase) antioxidants, but these regulatory processes can be overwhelmed. Depletion of these antioxidants is an important biomarker of excessive ROS generation.

One of the most important reactions for generation of ROS is the Fenton reaction, which leads to the formation of the hydroxyl radical (·OH):

$$\text{metal}^{n+} + H_2O_2 \rightarrow \text{metal}^{n+1} + \cdot OH + OH^-$$

Compared with metals like Fe^{2+}, the Ni^{2+} ion has relatively low efficiency with respect to the Fenton reaction. However, Ni reactivity is substantially enhanced when chelated by oligopeptides or histidine [90,91]. Nickel can also induce oxidation by interfering with Fe homeostasis. For example, in terrestrial plants, Ni inhibits root to shoot Fe transport, leading to accumulation of excessive Fe in the roots and generation of ROS [61].

Although few studies have investigated the effects of Ni on excessive ROS generation in aquatic animals, there is extensive literature on mammalian systems because this is considered a potential mechanism for Ni-induced carcinogenesis. At elevated levels, ROS will damage various cell structures including lipids, membranes, proteins, and nucleic acids, all of which collectively are called "oxidative stress." Nickel-induced ROS have been observed to elicit these effects in mammalian systems and in terrestrial plants [69,92,93].

Reactive oxygen species can also elicit changes in a variety of interacting signal transduction pathways that are related to the cell cycle and apoptosis. Disruption of these pathways (either stimulation or inhibition) leads to abnormal cell cycles and, potentially, to the formation of tumors. Many of these pathways are also directly affected by Ni^{2+}, leading to the potential for additive or reinforcing effects (Figure 2) [94,95]. At the cell membrane, Ni^{2+} and Ni-induced ROS can activate kinases, guanosine triphosphatases (GTPases) (*ras*), and growth factors (epidermal growth factor, vascular endothelial growth factor, platelet-derived growth factor). Both *ras* and growth factors induce intracellular mitogen-activated protein kinases (MAPKs), which in turn activate the inactive forms of transcription factors (HIF 1α, nuclear factor κB [NF-κB], nuclear factor of activated T cells [NFAT]), leading to stimulated transcription of genes controlling the cell cycle, apoptosis, and the hypoxia response. Intracellular Ni^{2+} can generate additional ROS as well as directly affect proteins (e.g., HIF 1α). Intracellular ROS activate MAPKs and can cross into the nucleus, where they can cause direct DNA damage (single or double strand breaks; purine, pyrimidine, or deoxyribose modification; and DNA crosslinks) and activate nuclear transcription factors (p53) that regulate cell apoptosis [95].

Nickel-induced ROS have also been shown to elicit effects in a variety of terrestrial plants similar to those observed in animals

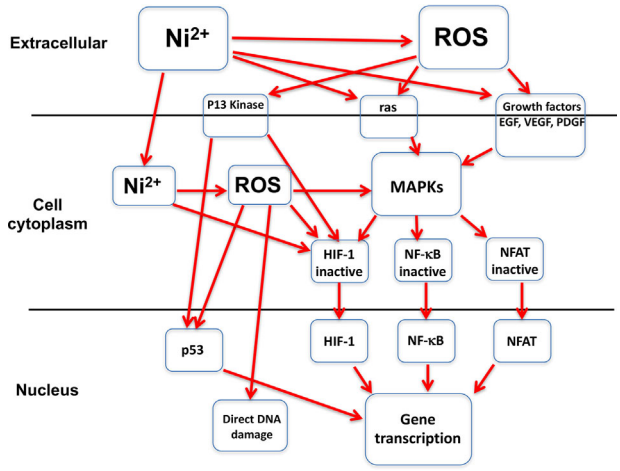

Figure 2. Interactions between nickel and reactive oxygen species (ROS) based on studies in mammalian systems. Both Ni^{2+} and ROS can affect signal transduction pathways related to cell cycling and apoptosis. Adapted from Leonard et al. [94] and Valko et al. [95]. EGF = epidermal growth factor; MAPK = mitogen-activated protein kinase; HIF-1 = hypoxia inducible factor 1; NFAT = nuclear factor of activated T cells; NF-κB = nuclear factor kappa B; PDGF = platelet-derived growth factor; PI3 kinase = phosphoinositide 3-kinase; ROS = reactive oxygen species; VEGF = vascular endothelial growth factor.

with increased lipid and protein peroxidation, as well as depletion of antioxidants [96–99]. In the sole example of Ni causing oxidative damage in aquatic organisms at high concentrations (10 μM), Randhawa et al. [100] demonstrated differences in Ni sensitivity between strains of the green alga *Scenedesmus acutus*, correlated with the level of oxidative damage (lipid peroxidation) observed after Ni exposure. Nickel tolerance and reduced oxidative stress were attributed to the approximately 10 times higher levels of glutathione reductase and catalase activities (antioxidants) naturally occurring in the tolerant strain.

Overall, it appears that Ni-induced oxidative stress may be a viable mechanism of action in aquatic systems. What is not clear is whether oxidative stress occurs at environmentally relevant concentrations (e.g., 2.5 μM and below) and, if so, whether this stress at the cellular level translates to significant effects at higher levels of biological organization for multicellular organisms.

AOP FOR Ni AND FUTURE RESEARCH NEEDS

Ultimately, 5 potential pathways by which Ni may exert toxicity on aquatic organisms were identified (Figure 3). The MIE for 3 of these pathways involved disruption of ion homeostasis for Ca^{2+}, Mg^{2+}, and $Fe^{2+/3+}$. A fourth potential pathway involves an allergic-type response at the respiratory epithelia of aquatic organisms that is potentially initiated by activation of T cells. The fifth potential pathway is initiated by the generation of reactive oxygen species in response to Ni exposure. At the organ level of biological organization, these 5 potential MIEs collapse into 3 potential pathways—reduced Ca^{2+} availability to support formation of exoskeleton, shell, and bone for growth; impaired respiration; and cytotoxicity and tumor formation. At the level of the whole organism, the organ-level responses contribute to potential reductions in growth and reproduction and/or alterations in energy metabolism.

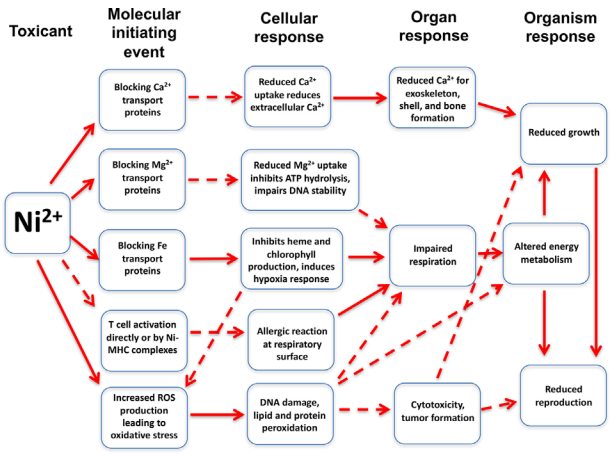

Figure 3. Adverse outcome pathway analysis for Ni in aquatic systems. Solid arrows represent pathways with supporting data; dashed arrows are hypothesized pathways. Note that development and evaluation of pathways consider multiple species. To date, a complete pathway has not been demonstrated for any individual species. ATP = adenosine triphosphate; MHC = major histocompatibility complex; DNA = deoxyribonucleic acid; ROS = reactive oxygen species.

There are potential feedback loops between each of the whole-organism responses. For example, altered energy metabolism could result in reductions in organism growth and/or reproduction, although reductions in growth can also lead to reductions in reproductive output. In addition, at least 1 of the cellular effects, induction of the hypoxia response, has the potential to contribute to the MIE for generation of ROS (Figure 3).

Development of this AOP analysis allowed for the identification of several major uncertainties in our understanding of the mechanisms of Ni toxicity to aquatic organisms. There are data supporting the effects of Ni on all of the MIEs identified. However, for the Ca^{2+}, Mg^{2+}, and allergic response pathways, all available data are in prokaryotic or mammalian systems. Similarly, for these same 3 pathways, no studies supporting (or refuting) the hypothesized link between the MIE and the response at the cellular level were identified, while the linkage to the cellular level for the Fe pathway is limited to mammalian systems. Moving from the hypothesized effects at the cellular level, there were also no studies identified supporting the subsequent hypothesized effects at the organ level and above for the Mg^{2+} and ROS pathways, although inferences can be made based on the disruption of whole-animal or extracellular fluid Mg^{2+} homeostasis in some aquatic organisms. Data on MIEs for aquatic plants are largely absent, although some pathways can be hypothesized based on observations in terrestrial plants. Importantly, the analysis presented in Figure 3 is based on data from multiple species. To date, a complete pathway (i.e., from MIE to whole-organism response) has not been demonstrated for any single species. Nickel is not unusual in this sense as, to the best of our knowledge, a complete pathway for chronic toxicity is not available for any of the other divalent metals.

Considering the uncertainties in this AOP analysis, a series of research priorities, listed in order of priority based on our qualitative assessment of the potential importance of a given pathway and level of uncertainty in the pathway, were identified for improving our understanding of Ni toxicity to aquatic organisms. As previously noted in *Development of an AOP analysis for Ni*, research to date has focused on the waterborne exposure pathway, and dietary exposure pathways should also be considered in the research priorities described below.

Determine whether MIEs for Ca^{2+} and Mg^{2+} response pathways exist in aquatic organisms

Given the diversity of proteins potentially involved in each of these pathways, transcriptomic/proteomic screening may be the most efficient approach for assessing the potential effects of Ni on Ca^{2+} and Mg^{2+} homeostasis [101–103]. Use of a model fish, invertebrate, and algae is recommended because these taxonomic groups use different proteins in each of these pathways. In these experiments, model organisms would be exposed to environmentally relevant Ni concentrations and changes in expression across the transcriptome/proteome would be used to identify potential MIEs. Confirmation of MIEs for Ca^{2+} and Mg^{2+} could be accomplished by evaluating Ni effects on specific proteins in expression systems (e.g., *Xenopus*), similar to the approach used by Cooper et al. [60].

Evaluate mechanistic links between MIEs and cellular effects

Except for the ROS pathway, data supporting mechanistic links between MIEs and cellular effects either are not available or are limited to mammalian systems. For the Ca^{2+} pathway, candidate MIEs have been identified in mammalian systems and cellular effects (reduced Ca^{2+} uptake) have been observed in freshwater snails. Consequently, identifying a MIE in snails would complete this pathway. For the Mg^{2+} pathway, there are numerous potential mechanisms at the cellular level. It is possible that specific pathways might be identified based on results of the transcriptomic/proteomic screening previously described. The exposure concentrations leading to Mg^{2+} inhibition of photosynthesis should be studied in aquatic plants. The cell and tissue culture techniques previously described would also be useful for linking MIEs to cellular effects for the allergic response pathway.

Evaluate interactions of multiple AOPs on organism respiration and metabolism

Currently, it is hypothesized that multiple pathways (Mg^{2+}, Fe, ROS) have the potential to negatively impact organism respiration, photosynthesis, ATP production, and subsequently metabolism. One of the few consistencies across aquatic invertebrate and vertebrate taxa in response to chronic Ni exposure is impaired respiration and metabolic rate [36,73]. It is possible that very modest (e.g., 5–10%) effects manifested along multiple adverse outcome pathways interact to induce significant impacts on organism metabolism at the whole-animal level. It will be important to consider data from the above research priorities in this context. It will also be important to develop an understanding of how already observed effects on organism metabolic rates do or do not relate to more traditional toxicity endpoints like survival, growth, and reproduction at the individual and population levels. For example, does the approximately 30% reduction in MO_2 (routine metabolic rate) for Ni-exposed *D. magna* explain corresponding reductions in reproductive output [36,104]? A dynamic energy budget modeling approach is recommended to address these questions [105]. Dynamic energy budget models are now available for aquatic taxa

sensitive to Ni (*Daphnia*, *Lymnaea*) and could be modified and parameterized to account for multiple mechanisms of action that might be involved in the chronic toxicity of Ni to aquatic organisms [106,107].

Evaluate effects of ROS at the organ and whole-animal levels

Although the MIE and links to cellular effects of the ROS pathway are well established, it is unclear whether cellular effects translate to effects at higher levels of biological organization when organisms are exposed to environmentally relevant Ni concentrations. This uncertainty applies to all metals that have been observed to induce ROS. To evaluate this uncertainty, it is recommended that chronic toxicity studies be performed on model taxa (plant, invertebrate, and fish) monitoring ROS production and various biomarkers of ROS effects at the cellular level along with traditional toxicity testing endpoints (e.g., survival, growth, reproduction). Experiments should be performed using multiple ROS–inducing stressors (e.g., Ni, Cu, H_2O_2, hyperoxia). If ROS are the principal mechanism of action, relationships between ROS, ROS biomarkers, and traditional toxicity endpoints should be similar across stressors.

Evaluate the allergic response pathway in fish

Given the analysis above, we conclude that the allergic response pathway is the least likely mechanism of action for Ni and that if it occurs at all, it is limited to fish. Consequently, research on this pathway is considered a low priority. There are several potential approaches that could be used to evaluate the MIE(s) for the allergic response. These include characterizing $CD4^+$ and $CD8^+$ T-cell density in Ni-exposed fish gills, the use of isolated fish T-cell cultures [108], cultured gill epithelia [109], and targeted gene expression studies to characterize the mechanisms underlying the whole-tissue inflammatory response.

CONCLUSIONS

The use of models like the BLM for regulating metals in the environment is predicated on a strong understanding of the mechanisms underlying toxicological effects. Based on the recommendations of an expert workshop, 5 potential mechanisms for chronic Ni toxicity to aquatic organisms were evaluated within an AOP framework. Each of the potential pathways was found to have some support in the literature, although the allergic response pathway is not considered likely based on our analysis. Further, data supporting one or more links between MIEs and whole animal–level or population-level effects were unavailable for each pathway. Overall, the AOP analysis was a useful tool for conceptualizing interactions between potential mechanisms of action and identifying knowledge gaps that should be the focus of future research efforts on the aquatic toxicity of Ni.

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Data Availability—Readers may contact the first author (kevinbrix@icloud.com) for any information or details regarding calculations in this review.

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