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Mechanisms of Acute and Chronic Waterborne Nickel Toxicity in the Freshwater Cladoceran, *Daphnia magna*

ERIC F. PANE,*,† CLINT SMITH,‡ JAMES C. MCGEER,‡ AND CHRIS M. WOOD†

Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON L8S 4K1, Canada, and Environment Group, Mining and Mineral Sciences Laboratories, Natural Resources Canada, 555 Booth Street, Ottawa, ON K1A 0G1, Canada

We present evidence that Mg^{2+} antagonism is one mechanism for acute toxicity of waterborne Ni to *Daphnia magna*. Acutely, adult *D. magna* were exposed to either control or 694 μg Ni L^{-1} as $NiSO_4$ in moderately soft water (45 mg L^{-1} as $CaCO_3$; background Ni ≅ 1 μg Ni L^{-1}) for 48 h without feeding. Chronically, adults were exposed to either control or 131 μg Ni L^{-1} for 14 days (fed exposure). These concentrations were approximately 65% and 12%, respectively, of the measured 48-h LC_{50} (1068 μg Ni L^{-1}) for daphnid neonates in this water quality. The clearest effect of Ni exposure was on Mg^{2+} homeostasis, as whole-body [Mg^{2+}] was significantly decreased both acutely and chronically by 18%. Additionally, unidirectional Mg^{2+} uptake rate (measured with the stable isotope ^{26}Mg) was significantly decreased both acutely and chronically by 49 and 47%, respectively, strongly suggesting that Ni is toxic to *D. magna* due at least in part to Mg^{2+} antagonism. No impact was observed on the whole-body concentrations or unidirectional uptake rates of Ca^{2+} during either acute or chronic Ni exposure, while only minor effects were seen on Na^+ and Cl^- balance. No acute toxic effect was seen on respiratory parameters, as both oxygen consumption rate ($\dot{M}O_2$) and whole-body hemoglobin concentration ([Hb]) were conserved. Chronically, however, Ni impaired respiratory function, as both $\dot{M}O_2$ and [Hb] were significantly reduced by 31 and 68%, respectively. Acutely, Ni accumulation was substantial, rising to a plateau between 24 and 48 h of approximately 15 μg g^{-1} wet weight—an increase of approximately 25-fold over control concentrations. Mechanisms of acute toxicity of Ni in *D. magna* differ from those in fish; it is likely that such mechanistic differences also exist for other metals.

Introduction

In recent years, interest in the effects of waterborne and dietary nickel (Ni) on aquatic fauna has increased (*1*−*7*). Nickel is an important aquatic contaminant present at elevated concentrations in many areas that are currently, or have previously been, engaged in mining-related activities (*8*). Ni concentrations, which usually range from 1 to 10 μg L^{-1} in uncontaminated freshwaters, may reach as high as several hundred to 1000 μg L^{-1} in highly contaminated waters (*8*).

Recent work aimed at the effective regulation of certain metals (Cu, Ag, Cd, Zn, Ni, Pb) in aquatic systems has focused on developing models that will allow for accurate prediction of toxicity of a given metal across a wide range of water chemistries (e.g. the Biotic Ligand Model (BLM) (*9*). As the focus of such models is the amount of metal accumulated at a specific site within an aquatic organism (e.g. the gill), detailed knowledge of species-specific toxic mechanisms of a metal will enhance the predictive power of such models by providing physiological explanations for the toxicity associated with particular metal burdens. Although recently the acute toxic mechanism (blockade of respiratory gas exchange) of waterborne Ni to rainbow trout (*Oncorhynchus mykiss*) has been elucidated (*3*), neither the acute nor chronic toxic mechanism in *Daphnia* has been previously investigated.

The objective of this study, therefore, was to elucidate the acute and/or chronic toxic mechanism of waterborne Ni to *D. magna*. Based on our recent findings with the rainbow trout (*3*), one of our hypotheses was that Ni would elicit respiratory toxicity in *D. magna* by impairing gas exchange between the animal and the environment. Additionally, we tested for the possibility that Ni is an ionoregulatory toxicant via antagonism of homeostasis of one or more of the physiologically important ions: Na^+, Cl^-, Ca^{2+}, or Mg^{2+}. With high ion turnover rates and minute whole-body reservoirs of physiological ions (*10*), daphnids are particularly susceptible to ionoregulatory toxicants such as Ag or Cu (*11*, *12*). Accordingly, regulatory policy is often driven by these species (*13*), and the BLM, originally derived for fish, is now being applied to daphnid species for several metals (*14*−*18*).

Experimental Section

***Daphnia magna* Culture and Exposure.** Colonies of adult gravid *Daphnia magna* were obtained from Aquatic Research Organisms (Hampton, NH). Once in the laboratory, daphnids were cultured in either dechlorinated Ottawa city tap water (OCW) or synthetic water reconstituted to simulate Ottawa city tap water (ROCW). The composition of Ottawa city tap water (OCW) was (in μM) Na ≅ 400, Cl ≅ 300, Ca ≅ 250, Mg ≅ 85, SO_4 ≅ 270, Ni ≅ 1 μg L^{-1}, total organic carbon (TOC) ≅ 3.6 mg C L^{-1}, total hardness ≅ 45 mg L^{-1} as $CaCO_3$, pH 7.3−7.6. With the exception of the Mg^{2+} influx experiments with the stable isotope ^{26}Mg described below, all experiments were conducted in the media described above, and in all cases the experimental medium was that in which the daphnids were cultured. All culturing and experimentation was conducted in either an incubator or a room with temperature controlled at 20.5 ± 1.5 °C. Photoperiod was fixed at 16:00 L; 8:00 D.

Daphnids were cultured in glass beakers using a static renewal system with 25 mL of water per animal with feeding and replacement of solutions every second or third day. *D. magna* were fed 4 mL of commercially purchased YCT (Aquatic Research Organisms; a slurry of yeast, cerophyll, and trout chow) and 12 mL of algae per 800 mL of solution. The algae were either a 3:1 ratio of *Selenastrum capricornutum* to *Chlorella* (3.5×10^6 cells mL^{-1}) or *Ankistrodesmus convolutus* (3.2×10^6 cells mL^{-1}). Solutions were not aerated, as aeration drives *Daphnia* to the water surface (*19*). Rather, beakers were left uncovered to allow for atmospheric

* Corresponding author phone: (905)525-9140 ext 23237; fax: (905)522-6066; e-mail: michanderic@yahoo.com.
† McMaster University.
‡ Natural Resources Canada.

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equilibration of oxygen. Only frequently reproducing colonies (>15 neonates per female per 3 days) were used (*20*), and at no time were ephipia present in a colony.

For acute exposures, adult *D. magna* (1.5−4.0 mg wet weight) were exposed to either control or 694 μg Ni L^{-1} (about 65% of the measured 48-h LC_{50}; see Results) for 48 h, and food was withheld throughout the exposure, with two exceptions. The first exception was the Mg^{2+} influx experiment (see below), where, for methodological reasons, the exposure was only 24 h, and the second exception was a Ca^{2+} influx experiment on juvenile (4 day-old) daphnids in which animals were fed during the exposure (see below). In all other cases, animals were transferred to beakers (with no food added) the night before the exposure, and exposure solutions were not changed over the 48-h time period. Control animals were sampled at 0 (initial) and 50 (final) h, while experimental animals were sampled at 1, 8, 24, and 48 h of exposure. Water samples were taken at random periods throughout the 48-h exposure, 0.45-μm filtered, acidified with trace metal grade HNO_3, and analyzed for dissolved Ni by graphite furnace atomic absorption spectrophotometry (GFAAS; 220 SpectrAA; Varian, Australia) against certified atomic absorption standards (Fisher Scientific). All Ni was added from a concentrated stock of $NiSO_4 \cdot 6H_2O$ at the beginning of each exposure.

For chronic exposures, adult daphnids were transferred to beakers containing the appropriate exposure solution, and feeding and solution renewal were identical to the culturing regime described above, with replacement of the Ni solution from one large stock of 131 μg Ni L^{-1} prepared from a concentrated stock of $NiSO_4 \cdot 6H_2O$. Control animals were sampled at 0 (initial) and 15 (final) d, while experimental animals were sampled at 2, 4, 6, 8, 10, 12, and 14 d of exposure. Water samples were taken every third day just prior to solution renewal and analyzed as described above.

48-h Static, Nonrenewal LC_{50} Determination. Neonates (<24 h old) were collected from adults cultured in OCW and transferred randomly to 250 mL beakers at nominal Ni concentrations of 0, 200, 400, 800, 1600, 3200, and 6400 μg Ni L^{-1}. Each concentration had 2 replicates and 10 animals were loaded per 250 mL beaker. Water samples were 0.45-μm filtered, acidified with trace metal grade HNO_3, and analyzed for dissolved Ni by inductively coupled plasma mass spectrometry (ICP-MS; Sciex Elan 6100 DRC, Perkin-Elmer) using certified standards (PlasmaCal). Dead or completely immobilized animals were removed daily from beakers. The 48-h LC_{50} and 95% confidence limits were calculated using the probit method (*21*), employing mean measured dissolved Ni concentrations in the exposure beakers.

Whole-Body Ion Measurements. For acute and chronic measurements of whole-body Na^+, Ca^{2+}, Mg^{2+}, and Ni concentrations, 10 adult daphnids were removed at each sampling time, transferred by plastic pipet to #1 Whatman filter paper, blotted dry, placed on pieces of foil, weighed to the nearest 0.01 mg, and placed individually in 1.5 mL plastic assay tubes. Fifty microliters of concentrated trace metal grade HNO_3 was added to each tube, and tubes were placed in a 60 °C oven overnight. For analysis of [Na^+] and [Ni], double distilled water (1500 μL for Na^+; 250 μL for Ni) was added to the assay tubes, and the aforementioned ions were analyzed by flame atomic absorption spectrophotometry (FAAS; 220FS SpectrAA; Varian, Australia) or GFAAS, respectively. For analysis of [Ca^{2+}] and [Mg^{2+}], 1500 μL of a 0.5% La^{3+} solution (as $LaCl_3$) was added to the assay tubes, and the ions were analyzed by FAAS.

Acute and Chronic Unidirectional Influx Measurements. Unidirectional influx rates of Na^+, Cl^-, and Ca^{2+} (J_{in}^{ion}) were measured during acute Ni exposure using the radioisotopes ^{22}Na, ^{36}Cl, and ^{45}Ca. At the appropriate sampling times, 10 adult daphnids were transferred from their exposure beakers to 100 mL beakers containing 50 mL of new exposure solution with or without Ni—as appropriate. Isotopes were added to yield final concentrations of 555 kBq L^{-1} (15 μCi L^{-1}) (^{22}Na and ^{36}Cl) or 11.1 MBq L^{-1} (300 μCi L^{-1}) (^{45}Ca). Initial and final water samples were taken for ion analyses and radioisotope counting either 1 h (^{22}Na and ^{36}Cl) or 2 h (^{45}Ca) apart. After the final water samples were taken, animals were removed individually with plastic pipets, rinsed in either 600 mM NaCl (^{22}Na and ^{36}Cl experiments) or 1 mM EDTA (ethylenediaminetetraacetic acid) (^{45}Ca experiment) for 20 s to remove radioactivity loosely surface bound to the surface (*22*), followed by two successive 20 s rinses in double-distilled water. Animals were then weighed as described above.

As indicated above, a separate ^{45}Ca influx experiment was run with fed, juvenile (4 day old) daphnids. Unidirectional Ca influx rates measured on starved, adult *Daphnia* (both control and Ni-exposed) varied by as much as 20-fold between animals in the same treatment sampled at the same time point (see Results), presumably due to the effects of starvation on the adult molting cycle and subsequent whole-body Ca^{2+} dynamics (see below). We therefore concluded that in order to measure $J_{in}^{Ca^{2+}}$ more precisely and to accurately assess whether Ni exposure affects Ca^{2+} influx, it was necessary to measure the influx of juvenile (4 day old) daphnids during a fed exposure. The protocol for this experiment was identical to that described above with the exception that juvenile daphnids were loaded the previous evening into beakers containing food.

For determination of Na^+ influx rate, animals were placed individually in plastic test tubes immediately following weighing. Individual animals and water samples were then counted for ^{22}Na radioactivity in a γ counter (Cobra Auto-Gamma Counter, Packard Instrument Company, Meridian, CT). Water samples for [Na^+] determination were diluted with 1% HNO_3 and analyzed by FAAS. For determination of Cl^- and Ca^{2+} influx rates, daphnids were placed individually in 1.5 mL plastic assay tubes immediately following weighing. Fifty microliters of concentrated trace metal grade HNO_3 was added, and tubes were placed in a 60 °C oven overnight. The daphnid digest was then transferred to a scintillation vial, 10 mL of Ultima Gold fluor (Packard BioSciences) was added to the digest, and ^{36}Cl and ^{45}Ca radioactivity was measured by liquid scintillation counting (1217 Rackbeta; LKB Wallac, Turka, Finland). Water samples for ^{36}Cl and ^{45}Ca activity were diluted in ACS (Amersham) and counted similarly for β activity. No quenching was detected for either ^{36}Cl or ^{45}Ca, so quench correction was unnecessary. Water samples for [Cl^-] and [Ca^{2+}] determination were diluted in 1% HNO_3 or 0.5% La^{3+}, respectively, and analyzed by the mercuric thiocyanate spectrophotometric method (*23*) or FAAS, respectively.

Unidirectional influx rates (J_{in}^{ion}; μmol g^{-1} h^{-1}) were calculated for individual daphnids based on the incorporation of radiolabel in the whole body during the flux period (*20*, *22*).

Unidirectional Mg^{2+} influx ($J_{in}^{Mg^{2+}}$) rates were measured during both acute and chronic Ni exposure using the stable isotope ^{26}Mg and the isotopic dilution method (*24*). A Mg^{2+}-free solution was made by adding $CaCl_2$ (250 μM) and $NaHCO_3$ (500 μM) to double distilled water. ^{26}Mg O (99.4% purity − Cambridge Isotope Laboratories) was dissolved in concentrated high-purity HNO_3 (Ultrex − Fisher Scientific) and added to Mg^{2+}-free water to yield a ^{26}Mg spike solution that was 85 μM in total Mg^{2+}.

In the acute experiment, adult daphnids were then exposed (unfed) to either control or 694 μg Ni L^{-1} in OCW for 24 h. Ten animals were removed at 0 (initial control), 8, 24, and 25 (final control) h and placed in 50 mL of the ^{26}Mg spike solution described above containing the appropriate concentration of Ni. In the chronic experiment, 10 animals

from each treatment (control and 131 μg Ni L^{-1}) were removed at the end of the 14 d exposure period. The protocol described below applies to both experiments. After 2 h of exposure to the ^{26}Mg spike solution containing the appropriate concentration of Ni, individuals were rinsed for 20 s in 1 mM EDTA followed by two successive 20 s rinses in double-distilled water. Animals were blotted and weighed as described above and then placed individually at the bottom of plastic test tubes, 150 μL of Ultrex concentrated HNO_3 was added to each assay tube, and tubes were placed in a 60 °C oven overnight. After acid digestion, 4850 μL of double-distilled water was added to each tube, and the solution was analyzed by ICP-MS. Following analysis of ^{24}Mg and ^{26}Mg content of individual daphnid digests, Mg^{2+} influx rates for individuals were calculated knowing the $^{26}Mg/^{24}Mg$ ratio in the animal after the flux period, the measured $^{26}Mg/^{24}Mg$ ratio of the ^{26}Mg spike solution described above, and the natural $^{26}Mg/^{24}Mg$ ratio of the daphnids (i.e. the natural atomic abundances of stable Mg isotopes). Accordingly, the final Mg burden of each animal (after the flux period) was calculated as that which was derived from the ^{26}Mg spike solution and that which was present in the daphnid before exposure to the ^{26}Mg spike solution (i.e. the natural Mg burden). The amount of Mg transferred to the animal from the ^{26}Mg spike solution was then divided by the wet weight of the animal and the flux period to yield unidirectional Mg^{2+} influx rates (μmol g^{-1} h^{-1}).

Calculation of internal specific activities of ^{22}Na, ^{36}Cl, ^{45}Ca, and ^{26}Mg at the end of each flux period yielded values less than 10% of water specific activity, and therefore no correction for potential isotope efflux was necessary (*25*).

Oxygen Consumption and [Hb]. Given our hypothesis that Ni elicits respiratory toxicity in *D. magna* by impairing gas exchange, oxygen consumption rates were measured in control and treated daphnids after 48 h (acute exposure) or 14 d (chronic exposure) using a variation of a technique by Wiggins and Frappell (*26*). In multiple repetitions, two daphnids were sealed in a 5 mL gastight Hamilton syringe filled with the appropriate exposure water. Immediately following each respirometry trial, each pair of daphnids (n = 10) was then prepared for analysis of whole-body [Hb] (see below). A micro stirring flea was sealed in the syringe, and periodically the water was "stirred" by moving a magnet gently up and down the barrel of the syringe. At intervals, water samples of a known volume were injected into a Radiometer oxygen cell, and partial pressures were recorded using Radiometer PO_2 electrodes and an oxygen meter (Cameron Instruments). Oxygen consumption rates ($\dot{M}O_2$) in μL mg^{-1} h^{-1} were calculated based on the decrease in PO_2 with time using the formula

$$\dot{M}O_2 = \frac{(\alpha O_2)\,(\Delta\text{Torr})\,(V)}{(W)(t)} \qquad (1)$$

where αO_2 is the solubility coefficient of oxygen in water at the experimental temperature (*27*), ΔTorr is the difference in partial pressures between successive samplings, V is the volume of water corrected for sampling deficits, W is the combined wet weight of the two *D. magna*, measured as described above, and t is the time interval in hours. At no point in either experiment (acute or chronic) did the partial pressure of oxygen in the exposure water drop below 100 Torr. Oxygen consumption rates were corrected for "blank" oxygen consumption by the experimental apparatus in the absence of *D. magna*.

Post-weighing, pairs of *D. magna* used in the respirometry experiment were placed in 1.5 mL plastic assay tubes, immediately frozen in liquid nitrogen, and stored at −80 °C for 24 h before analysis of whole-body hemoglobin content using a variation of a technique by Wiggins and Frappell (*26*). A homogenate was made by adding 250 μL of Drabkin's reagent (Sigma-Aldrich) to each tube and grinding manually with a tight-fitting Teflon rod. After an additional 250 μL of reagent was added, the homogenate was centrifuged, and the supernatant was analyzed by measuring absorbance at 420 nm (the Soret region) against hemoglobin standards (Sigma-Aldrich).

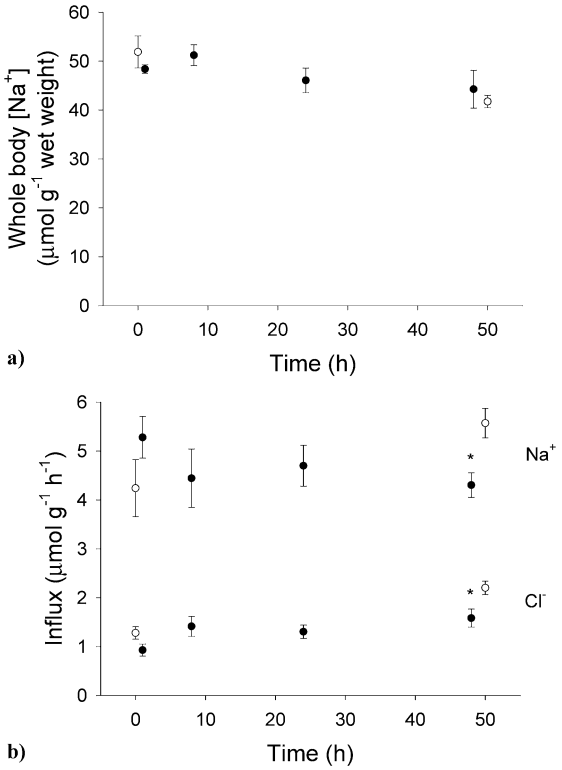

FIGURE 1. Na^+ and Cl^- homeostasis in starved, adult *D. magna* exposed for 48 h to 694 μg Ni L^{-1}. Open circles at 0 and 50 h represent initial and final control means, respectively. Dark circles represent experimental means sampled at 1, 8, 24, and 48 h of exposure. n = 8−10 in both groups. (a) Whole-body [Na^+]. (b) Unidirectional Na^+ and Cl^- influx rates. Asterisk "*" on the final experimental time points (48 h) indicate significantly different (P < 0.05; two-tailed Student's t-test) from respective final (50 h) control mean.

All concentrations and rates are expressed on a whole-body wet weight basis. All analytical values expressed per unit wet mass of daphnid were multiplied by 1.25 to correct for fluid present in the carapace, as recommended by Stobbart et al (*28*).

Statistical Analyses. All values are presented as mean ± 1 SEM (n). In all time course experiments where both initial and final control means were taken so as to temporally bracket experimental means (i.e. Figures 1−4), the initial treatment observation was compared to the time 0 control, and the final treatment observation was compared to the end-of-experiment control (50 h) by an unpaired two-tailed Student's t-test. Additionally, if the two control means (0 and 50 h) were not significantly different from one another (unpaired two-tailed Student's t-test), then time-dependent responses of experimental animals were tested against the mean of all control values by a one-way ANOVA with a two-sided Dunnett's post hoc multiple comparison. In time course experiments with only an initial (0 h) control (Figure 5), time-dependent experimental responses were tested against the initial control by a one-way ANOVA with a two-sided Dunnett's post hoc multiple comparison.

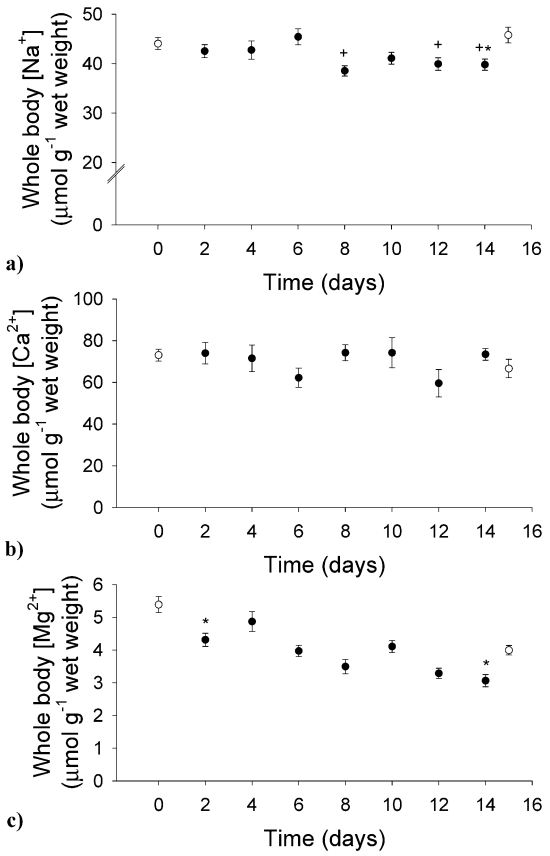

FIGURE 2. Whole-body ion concentrations in fed, adult *D. magna* exposed for 14 d to 131 μg Ni L^{-1}. Open circles at 0 and 15 d represent initial and final control means, respectively. Dark circles represent experimental means sampled at 2, 4, 6, 8, 10, 12, and 14 d of exposure. $n = 8-10$ in both groups. (a) [Na^+]. "+" indicates significant difference ($P < 0.05$; one-way ANOVA plus two-sided Dunnett's post hoc test) from the mean of all control values. Asterisk "*" on the final experimental time point (14 d) indicates significantly different ($P < 0.05$; two-tailed Student's *t*-test) from final (15 d) control mean. (b) [Ca^{2+}]. (c) [Mg^{2+}]. Asterisk "*" on the initial (2 d) and final (14 d) experimental time points indicate significantly different ($P < 0.05$; two-tailed Student's *t*-test) from initial (0 d) and final (15 d) control means, respectively ($P < 0.05$; two-tailed Student's *t*-test).

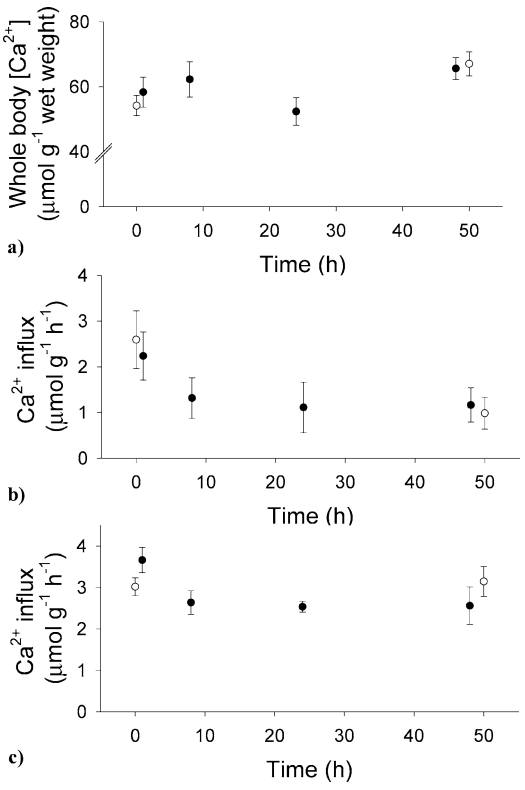

FIGURE 3. Ca^{2+} homeostasis in *D. magna* exposed for 48 h to 694 μg Ni L^{-1}. Symbols are as described in Figure 1. $n = 8-10$ in both groups. (a) Whole-body [Ca^{2+}] of starved, adult *D. magna*. (b) Unidirectional Ca^{2+} influx rates of starved, adult *D. magna*. (c) Unidirectional Ca^{2+} influx rate of fed, juvenile *D. magna*.

Results and Discussion

The measured 48-h static renewal LC_{50} for neonate *D. magna* in dechlorinated Ottawa city tap water (OCW) (hardness ≅ 45 mg L^{-1} $CaCO_3$) under normoxic conditions was 1068 μg Ni L^{-1} (dissolved) with lower and upper 95% confidence limits of 818 and 1486 μg Ni L^{-1}, respectively. Although increased water hardness protects against acute Ni toxicity (*13*), a 48-h *D. magna* LC_{50} value (510 μg Ni L^{-1}) reported by Biesinger and Christensen (*29*) is lower than the LC_{50} (1068 μg Ni L^{-1}) reported here at the same water hardness (45 mg L^{-1} as $CaCO_3$). Lake Superior water used in the Biesinger and Christensen study, however, was very low in DOC and contained nominally only 1 mg C L^{-1} (Russ Erickson; personal communication), as compared to that of the present study (3.6 mg C L^{-1}). Lower DOC concentration may explain the increased sensitivity observed in that study. Additionally, the Ni concentrations given by Biesinger and Christensen (*29*) appear to be nominal.

Acute waterborne Ni exposure at approximately 65% of the 48-h LC_{50} had only minor effects on Na^+ and Cl^- balance in *D. magna* (Figure 1). Whole-body [Na^+] in exposed animals was not significantly depressed over 48-h of exposure, despite a general trend toward loss of whole-body [Na^+] in both control and treated animals over the exposure period (Figure 1a). Influx rates of the two ions followed a similar pattern (with the exception of the 1 h time point) over the 48-h time course, though J_{in}^{Cl-}, on average, was only about 20% of J_{in}^{Na+} (Figure 1b). The influx of both ions was significantly higher in control animals than experimental animals at 48 h, due to an elevation of end-of-experiment control influx rates.

Time-dependent decreases in whole-body [Na^+] and increases in $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ in starved control daphnids are consistent with the findings of Stobbart et al (*28*). Because their filtration rate is inversely proportional to available food concentration, and because they do not show a feeding threshold at low food concentrations, *Daphnia* continue to filter at increasing rates as food concentration approaches zero (*30*), approaching maximal rates of approximately 4 mL of water per individual per hour (*31*), or approximately 1600 times their body volume per hour. As the rate of water passing over the integument of *Daphnia* increases, uptake of salts such as Na^+ and Cl^- from the water should increase for two reasons: (1) to offset increased diffusive loss of salts from the concentrated internal milieu of *D. magna* to the very dilute water, and (2) because starved animals are unable to use dietary salt to compensate for salt imbalance. It would appear, however, that in this experimental setup the rate of loss was slightly greater than the rate of uptake over time (Figure 1a). Although at 48 h, $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ were lower in exposed animals than controls (Figure 1b), it should be noted that influx rates of Ni-exposed animals were well conserved over the 48-h time course. Such marginal effects of Ni exposure on $J_{in}^{Na^+}$ in *D. magna* can be contrasted with 40% inhibition of $J_{in}^{Na^+}$ during acute Ag exposure (*22*).

Chronically, 14 days of exposure to 131 μg Ni L^{-1} had only a minor impact on whole-body [Na^+] (Figure 2a). From 8 d onward (excluding day 10), whole-body [Na^+] was signifi-

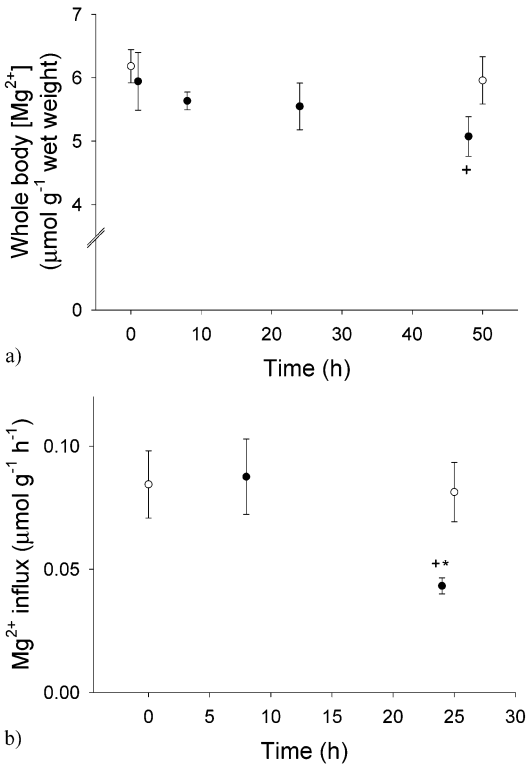

FIGURE 4. Mg^{2+} homeostasis in starved, adult *D. magna* exposed to 694 μg Ni L^{-1}. Symbols are as described in Figure 1. $n = 8-10$ in both groups. (a) Whole-body [Mg^{2+}]. "+" indicates significant difference ($P < 0.05$; one-way ANOVA followed by two-sided Dunnett's post hoc test) from the mean of all control values. (b) Unidirectional Mg^{2+} influx rates. "+" indicates significant difference ($P < 0.05$; one-way ANOVA followed by two-sided Dunnett's post hoc test) from the mean of all control values. Asterisk "*" on the final experimental time point (24 h) indicates significantly different ($P < 0.05$; two-tailed Student's *t*-test) from final (25 h) control mean. Note the 24 h time scale on the *x*-axis.

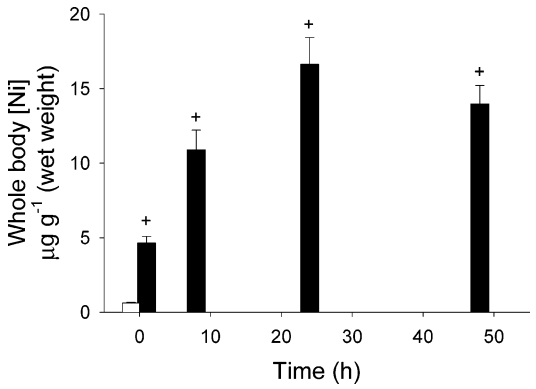

FIGURE 5. Whole-body Ni accumulation in starved, adult *D. magna* exposed for 48 h to 694 μg Ni L^{-1}. The open bar represents the initial control means, while the black bars represents experimental means at 1, 8, 24, and 48 h of exposure. $n = 8-10$ in both groups. "+" indicates significant difference ($P < 0.05$; one-way ANOVA followed by two-sided Dunnett's post hoc test) from time 0 h mean.

cantly reduced, though the decrease never exceeded 10%, and rather than worsen with time, the deficit appeared to be stabilized by the end of the exposure period. Again, in comparison to a potent Na^+ antagonist like Ag, these whole-body losses fall far short of the 65% whole-body Na^+ loss in *D. magna* reported by Bianchini and Wood (*20*) following chronic Ag exposure. Accordingly, the Na^+ loss observed in the present study is likely a secondary effect coupled to chronic Ni-induced impact on other aspects of *Daphnia*

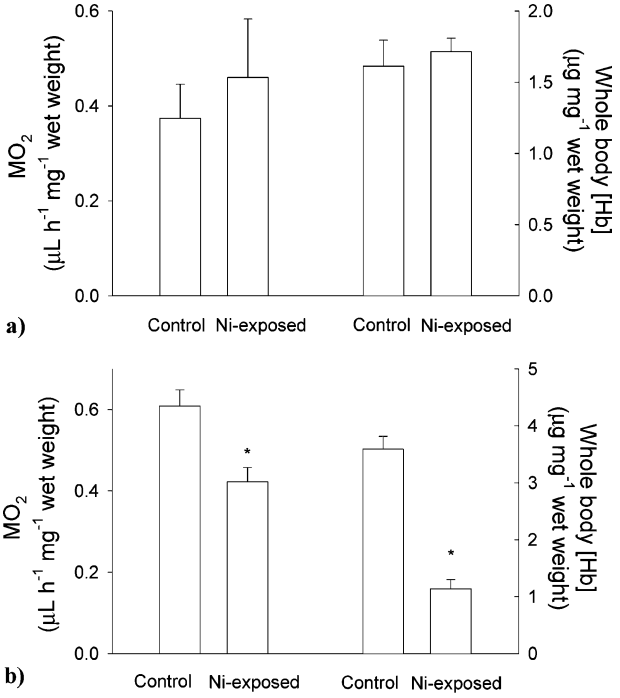

FIGURE 6. Oxygen consumption rates ($\dot{M}O_2$) and whole-body [Hb] content of adult *D. magna*. $n = 8-10$ in both groups. (a) Starved, adult *D. magna* exposed for 48 h to 694 μg Ni L^{-1}. (b) Fed, adult *D. magna* exposed for 14 d to 131 μg Ni L^{-1}. Asterisk "*" indicates significant difference ($P < 0.05$; two-tailed Student's *t*-test) from control mean.

physiology, such as potential metabolic impairment (see Figure 6b).

No evidence of specific antagonism of Ca^{2+} homeostasis was observed following either acute or chronic exposure to waterborne Ni. Whole-body [Ca^{2+}] and unidirectional Ca^{2+} influx ($J_{in}^{Ca^{2+}}$) of acutely exposed, unfed adult daphnids are presented in Figure 3, parts a and b, respectively. There were no significant differences between Ni-exposed and control *Daphnia*, as any changes in the responses of experimental daphnids over 48 h of Ni exposure were tracked by control animals, suggesting that experimental conditions had a greater effect on Ca^{2+} homeostasis in starved, adult daphnids than Ni exposure.

Additionally, Ni exposure had no significant effect on calcium influx rates in fed, juvenile *Daphnia* acutely exposed to 694 μg Ni L^{-1} (Figure 3c). Note both the decrease in variability around the mean and the lack of a time-dependent decrease when compared to $J_{in}^{Ca^{2+}}$ measured in starved, adult *Daphnia* (Figure 3b). As was the case with adults (Figure 3a), whole-body [Ca^{2+}] in juveniles after 48 h was not affected by Ni exposure. Juvenile whole-body [Ca^{2+}] in the two treatments was almost identical after 48 h (100.5 $\pm$ 16.0 μmol g^{-1} wet weight in control animals vs 102.3 $\pm$ 10.8 in Ni-exposed). Additionally, in contrast to adults, juvenile daphnid control influx rates at 0 and 48 h were very similar (Figure 3c), suggesting that juveniles are a better model for investigating the effects of known, or suspected, Ca^{2+} antagonists

Indeed, the processes of molting and calcification of the carapace so greatly dominate Ca^{2+} homeostasis in adult daphnids as to make interpretation of toxic effects difficult. Immediately following the release of a brood, *Daphnia* molt by ecdysis, shedding the exoskeleton, thereby losing a massive fraction of their whole-body [Ca^{2+}]. This Ca^{2+} loss during a molt is typical of crustaceans whose carapace contains the majority of the body Ca^{2+} load (*32*, *33*). Predictably, an extremely rapid calcification process to form a new exoskeleton necessitates that crustaceans markedly increase Ca^{2+}

uptake rate from solution (*32*, *33*). Whole-body [Ca^{2+}] and $J_{in}^{Ca^{2+}}$, therefore, are dictated almost exclusively by the stage of molt cycle and the physiological state of *Daphnia* (*32*, *34*), making it very difficult to discern any direct effect on Ca^{2+} balance. Simply put, there is no steady-state rate of Ca^{2+} influx on which to evaluate the effect of a toxicant.

The picture is complicated even further when one considers the effects of starvation on Ca^{2+} handling. Porcella and coauthors reported an approximate 11-fold range of Ca^{2+} uptake values, depending on the stage of molt cycle in starved *Daphnia* (*32*). Similarly, we observed a 20-fold range in $J_{in}^{Ca^{2+}}$ measured in adult, starved daphnids. Although not quantified, the rate of molting (and reproduction) clearly decreased with starvation in the current study (data not shown), presumably due to approximately 60 h of starvation (12 h prior to the start of the exposure plus 48 h of the exposure period). If starvation extends the time period between broods (and therefore molts), a greater percentage of animals will be in intermolt as time of starvation progresses. Intermolt crustaceans are characterized by high [Ca^{2+}] (*33*) and presumably a low $J_{in}^{Ca^{2+}}$. It follows, then, that as starvation persists, whole-body [Ca^{2+}] will increase and $J_{in}^{Ca^{2+}}$ will decrease. Both of these phenomena were observed (Figure 3, parts a and b, respectively).

Despite the aforementioned complexity, waterborne Ni did not disrupt Ca^{2+} balance either acutely or chronically, as 14 days of exposure to 131 μg Ni L^{-1} had no significant impact on whole-body [Ca^{2+}] (Figure 2b). In both treatments, whole-body [Ca^{2+}] was well conserved throughout the 14 d exposure period.

Acute waterborne Ni exposure antagonized Mg^{2+} homeostasis in *D. magna*, as both whole-body [Mg^{2+}] (Figure 4a) and $J_{in}^{Mg^{2+}}$ (Figure 4b) were significantly reduced in exposed animals. Whole-body [Mg^{2+}] dropped linearly with time in Ni-exposed daphnids, becoming significantly reduced by 18% at 48 h (Figure 4a). $J_{in}^{Mg^{2+}}$ was significantly inhibited by 49% (Figure 4b) after only 24 h of Ni exposure. For both the whole-body and influx experiments, initial and final controls gave similar results again indicating that, acutely, there were no problems associated with the exposure conditions.

By 24 h of exposure, whole-body Mg^{2+} loss was approximately 0.6 μmol g^{-1} (Figure 4a). If the degree of inhibition of $J_{in}^{Mg^{2+}}$ stayed at 49% from 24 to 48 h, the calculated loss of Mg^{2+} over the second 24 h period would be 0.8 μmol g^{-1}, yielding a calculated total loss of approximately 1.4 μmol g^{-1} over 48 h. The measured whole-body Mg^{2+} loss over 48 h of exposure amounted to 1.1 μmol g^{-1} (Figure 4a), strongly suggesting that inhibition of Mg^{2+} uptake was not reversed to any great extent from 24 h onward.

It has similarly been observed that whole-body Mg^{2+} stores of a teleost fish (*Oreochromis mossambicus*) are depleted when access to waterborne Mg^{2+} is restricted (*35*), and presumably, the problem is exacerbated in small crustaceans such as *D. magna*, given very high surface area-to-volume ratios, lower whole-body Mg^{2+} stores (typically only 25% of whole-body fish Mg^{2+} concentrations), and the lack of a mobilizable Mg^{2+} reservoir in hard tissue such as scale or bone.

Additionally, 14 days of exposure to 131 μg Ni L^{-1} caused a significant linear decrease in whole-body [Mg^{2+}] (Figure 2c), and a significant 47% inhibition of $J_{in}^{Mg^{2+}}$ when measured at the end of the exposure period (0.207 $\pm$ 0.016 μmol g^{-1} h^{-1}; control vs 0.109 $\pm$ 0.010; Ni-exposed). At both the first (2 d) and last (14 d) experimental time points, whole-body [Mg^{2+}] was significantly reduced, reaching a concentration at 14 d that was 43% lower than the initial control value (Figure 2c), although a considerable portion of this whole-body [Mg^{2+}] loss was attributable to the holding conditions, as control animals lost 25% of their whole-body [Mg^{2+}] over the same time period.

From the acute experiments, the observed net rate of loss of Mg^{2+} of 0.55 μmol g^{-1} per day during a starved exposure would deplete whole-body [Mg^{2+}] by 50% in only 6 days. Although the unidirectional rate of uptake of Mg^{2+} from the water was strongly inhibited at 14 d by Ni exposure, the decrease in whole-body [Mg^{2+}] (18%; above the whole-body Mg^{2+} loss experienced by control animals) with chronic exposure to 131 μg Ni L^{-1} was not commensurate with such a prolonged inhibition of $J_{in}^{Mg^{2+}}$, suggesting that either there is a compensatory reduction in the efflux component or that dietborne Mg^{2+} can compensate for Mg^{2+} lost from the water. Seemingly, the latter response is more likely given that freshwater fish are capable of offsetting Mg^{2+} losses from the water by dietary Mg^{2+} uptake, and epithelia are typically sparingly permeable to Mg^{2+} (*36*). It appears, however, that dietary compensation or efflux limitation were inadequate, based on the linear net loss of whole-body [Mg^{2+}].

Although historically, Mg^{2+} has been lumped together with Ca^{2+} under the label "hardness cations," the separate contribution of the two cations to mitigating metal toxicity in aquatic systems has recently been more closely examined (*37*, *38*). Welsh and coauthors (*37*) found that at a constant total hardness, adjusting Ca:Mg ratios affected Cu toxicity to two salmonid species. Furthermore, Naddy and co-workers found that while higher Ca:Mg ratios protected against Cu toxicity in the rainbow trout, lower Ca:Mg ratios protected *D. magna* (*38*).

Certainly, Ni−Mg interaction is well-known from the mammalian literature. Nickel is very similar to magnesium, chemically, sharing very similar dehydrated ionic radii (0.066 nm Ni^{2+}; 0.069 nm Mg^{2+}) (*39*), and magnesium is capable of antagonizing many of the carcinogenic processes induced by certain Ni substances, including genotoxicity, cell transformation, and tumor induction (*40*−*42*). As an example of the specificity of Mg−Ni interaction, Mg^{2+} ranks above Mn^{2+}, Ca^{2+}, Cu^{2+}, and Zn^{2+}, among essential divalent metals in its ability to compete with Ni^{2+} for DNA binding sites in vitro (*42*). Additionally, Ni inhibits the function of the Mg^{2+}-requiring enzyme, DNA polymerase (*40*).

While the specific mechanism of inhibition of Mg^{2+} uptake by Ni is not known, it presents a rich subject for future investigation in *D. magna*. It would be of great interest to examine whether Ni inhibits Mg^{2+} uptake directly via a potential Mg^{2+} channel blockade, or indirectly, perhaps by inhibition of an active Mg^{2+} transporter.

Whole-body Ni accumulation over 48 h showed a typical asymptotic increase (Figure 5) (*34*, *43*), increasing sharply over the first 8 h of exposure, only to level off from 24 h onward. Maximum Ni burden was 15.2 μg g^{-1} wet weight—an increase of approximately 25-fold over control concentrations. As accumulation was assessed at only one Ni concentration, it is difficult to conclude anything directly from these data about the mechanisms of homeostatic regulation of whole-body Ni in *D. magna*. Uptake and elimination constants were calculated at this exposure concentration, however, using a one compartment exponential model with an equation of the form

$$C = C_w * \left(\frac{k_1}{k_2}\right) * (1 - \exp(k_2(-t))) \qquad (2)$$

where C is the Ni concentration in the animal, C_w is the water [Ni], k_1 and k_2 are uptake and elimination constants, respectively, and t is the exposure time in hours. Calculated values for k_1 and k_2 were 4.17 $\pm$ 1.12 h^{-1} and 0.19 $\pm$ 0.06 h^{-1}, respectively, with the former in very good agreement with that calculated by Hall (5.16 h^{-1}) using *D. magna* exposed to nominal Ni concentrations in the range of 250−750 μg Ni

L^{-1} (*34*). The maximal burden of 15.2 μg g^{-1} wet weight, however, is over an order of magnitude lower than that predicted by Watras and co-workers (*43*) using an equation derived from acute Ni accumulation in *D. magna* exposed to several far lower Ni concentrations.

No evidence of acute respiratory toxicity was observed in *D. magna* following acute Ni exposure (Figure 6a). Neither the rate of oxygen consumption ($\dot{M}O_2$), nor whole-body hemoglobin (Hb) content were significantly affected by Ni exposure (Figure 6a), suggesting that acute waterborne Ni exposure in no way impairs the ability of *D. magna* to respire. Additionally, parallel experiments had shown that Ni is no more toxic to *D. magna* in hypoxic water (~8% O_2) than normoxic (~20% O_2) water, as 48 h LC_{50} values from both these media were very similar (data not shown).

These findings contrast with parallel work from our laboratory with the rainbow trout showing that waterborne Ni elicits acute respiratory toxicity as the mechanism of lethality (*3*). *Daphnia* have no specialized respiratory epithelium, however, rendering them potentially less vulnerable to damage by an acute respiratory toxicant targeting a gas exchange surface. Instead they exchange gases across the entire integument, with oxygen uptake facilitated by hemoglobin in the open hemocoele (*44*). In response to environmental hypoxia, *Daphnia* produce very large quantities of hemoglobin to cope with low oxygen concentrations (*44*). The lack of such a response (Figure 6a) further suggests that Ni does not limit oxygen availability acutely.

Chronic Ni exposure, however, had a significant impact on both ($\dot{M}O_2$) and whole-body [Hb] (Figure 6b). These two parameters were decreased by 31 and 68%, respectively. As the respirometry experiments of the present study did not normalize for activity, any direct Ni-induced inhibition of oxygen consumption may be confounded by an inhibitory effect of chronic exposure on daphnid swimming activity and therefore respiration. Indeed, chronically exposed daphnids appeared more sluggish and less mobile than their control counterparts. Gas diffusion limitation is unlikely in a chronic context, both for the reason of integument-based respiration given above and, more noticeably, because whole-body [Hb] after 14 d of Ni exposure was greatly reduced (Figure 6b), not increased as one would expect if oxygen were limiting. It is possible then that chronic Ni exposure impairs respiration not by limiting O_2 uptake directly but by metabolic impairment negatively affecting oxygen delivery to and/or utilization by internal tissues.

Nickel is the second metal, to our knowledge, for which an acute toxic mechanism has been elucidated in *D. magna*—the other being Ag (*22*). Based on these two studies, it is safe to say that we cannot necessarily assume that the acute toxic mechanism of a metal will be the same for *Daphnia* as for fish. While in the case of Ag, the difference between the two toxic mechanisms is subtle, the difference is marked for Ni (i.e. ionoregulatory in *Daphnia* vs respiratory in the rainbow trout).

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