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A Mystery Tale: Nickel Is Fickle When Snails Fail—Investigating the Variability in Ni Toxicity to the Great Pond Snail

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ABSTRACT

Dissolved Ni concentrations inhibiting the growth of juvenile great pond snails (*Lymnaea stagnalis*) have been documented to vary from about 1 to 200 μg L^{-1} Ni. This variability makes *L. stagnalis* either a moderately sensitive or the most sensitive freshwater species to chronic Ni exposure tested to date. Given the role of sensitive species in environmental risk assessment frameworks, it is particularly important to understand this variability, i.e., to characterize the factors that modulate Ni toxicity and that may confound toxicity test outcomes when uncontrolled. In the present study, we tested if this variability was due to analytical (growth calculation: biomass versus growth rate), environmental (water quality), lab-specific practices, and/or snail population differences among earlier studies. Specifically, we reanalyzed previously published Ni toxicity data and conducted additional measurements of Ni aqueous speciation, short-term Ni uptake, and chronic Ni toxicity with test waters and snail cultures used in previous studies. Corrections for Ni bioavailability and growth calculations explained a large degree of variability in the published literature. However, a residual 16-fold difference remained puzzling between 2 studies: Niyogi et al. (2014) (low ECxs) and Crémazy et al. (2018) (high ECxs). Indeed, differences in metal bioavailability due to water chemistry, lab-specific practices, and snail population sensitivity could not explain the large variation in Ni toxicity in these 2 very similar studies. Other potentially important toxicity-modifying factors were not directly evaluated in the present work: test duration, diet, snail holding conditions, and snail age at onset of testing. The present analysis highlights the need for further studies to elucidate 1) the mechanisms of growth inhibition in Ni-exposed *L. stagnalis* and 2) the important abiotic and biotic factors affecting this biological response. Until these processes are understood, substantial uncertainties will remain about inclusion of this species in Ni environmental risk assessment. *Integr Environ Assess Manag* 2020;16: 983–997. © 2020 SETAC

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INTRODUCTION

The great pond snail *Lymnaea stagnalis* is an abundant and widespread pulmonate snail present in temperate fresh and brackish waters in North America, Europe, Asia, and North Africa (Amorim et al. 2019). As part of the diet of many species (fish, crustaceans, amphibians, mammals, and birds), it is considered a key organism for ecosystem health and function (Bronmark 1994; Nyström and Pérez 1998). Since the early 2000s, *L. stagnalis* has been increasingly used as a model organism in ecotoxicology studies, mainly because the snails are easy to culture in the lab, ecologically important, and highly sensitive to pollutants (Amorim et al. 2019). Regarding this latter aspect, the extreme sensitivity of *L. stagnalis* to long-term exposures to many trace metals also makes it a key organism for the environmental regulation of these inorganic contaminants. Indeed, over the past decade, the great pond snail has been shown to be either the most sensitive, or the second most sensitive organism to Co, Cu, Ni, and Pb in chronic exposures (Grosell et al. 2006; De Schamphelaere et al. 2008; Schlekat et al. 2010; Brix et al. 2011; Niyogi et al. 2014). In these studies, effects on juvenile growth were observed at low μg L^{-1} aqueous metal concentrations, which are close to protective values for aquatic life. For example, Brix et al. (2011) measured a 30 d EC20 of 1.8 μg L^{-1} Cu for juvenile snail growth, indicating that *L. stagnalis* is under protected by the United States Environmental Protection Agency

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Table 1. Conditions of the various chronic Ni toxicity tests that measured growth inhibition in juvenile *Lymnaea stagnalis*

	Crémazy et al. 2018	Niyogi et al. 2014	Nys et al. 2016	Schlekat et al. 2010
Test snails origin	University of British Columbia (in-house culture)	University of Miami (in-house culture)	Eggs shipped from Vrije Universiteit Amsterdam	Eggs shipped from University of Miami
Light: dark cycle	16:8 light: dark	16:8 light: dark	12:12 light: dark	16:8 light: dark
Water T (°C)	25 ± 1	25 ± 1	20	24 ± 2
Culture food	Sweet potato, romaine lettuce	Sweet potato, romaine lettuce	Organic butterhead lettuce	Sweet potato, romaine lettuce
Test food	Romaine lettuce, ad libitum	Romaine lettuce, ad libitum	Organic butterhead lettuce, 25 mg L^{-1}	Carrot, romaine lettuce, sweet potato, ad libitum
Test duration	14 d	21 d	28 d	30 d
Test initial snail age	2–3 wk old	7–8 d old	2–3 d old	<24 h old
Test initial snail weight	5–11 mg	2–4 mg	Not measured	Not measured
Test snail density	2.5 snails/L	4 snails/L	2 snails/L	8.3 snails/L
Test water regime	Static renewal (every day)	Static renewal (every day)	Static renewal (3 times/week)	Static renewal (3 times/week)
Test water(s) type	Vancouver water + salts	Miami water	Artificial waters	Natural waters
Test Ni salt	$NiCl_2$	$NiCl_2$	$NiCl_2$	$NiCl_2$
Water equilibration	>12 h	24 h	24 h	>18 h
Snail measurement	Wet weight	Wet weight	Shell length	Wet weight
Reported growth based on	Growth rate	Biomass	Growth rate	Biomass

(USEPA) hardness-based Cu water quality criteria (7.8 µg L^{-1} under the test conditions). While the exact mechanisms of metal toxicity remain elusive, this high sensitivity seems to be linked to disruption of the homeostasis of Ca^{2+}, a substrate that sustains the extremely fast shell formation required for high growth rates of juvenile *L. stagnalis* (~20%–25% biomass gain per day) (Grosell and Brix 2009; Brix et al. 2012). Nickel may also disturb Na^{+}, Mg^{2+}, and Fe^{2+} homeostasis (Leonard and Wood 2013; Niyogi et al. 2014; Brix et al. 2017). These data suggest that this sensitive organism may serve an important role in revisions of current water quality guidelines. Yet, conflicting data on *L. stagnalis* sensitivity have recently emerged for Ni, creating uncertainty regarding how to incorporate data from this species in Ni risk assessments.

Lymnaea stagnalis has been reported to be among the most chronically sensitive species tested to date for Ni. Using newly hatched (<24 h old) snails, Schlekat et al. (2010) measured 30 d EC20s for growth ranging from 1.6 to 21 µg L^{-1} in 5 natural waters representing the typical range of water composition in temperate regions. This variability could mainly be explained by differences in water chemistry, i.e., by changes in Ni bioavailability using a Biotic Ligand Model (BLM). When these toxicity data were normalized to a common water chemistry, *L. stagnalis* and *Ceriodaphnia dubia* were found to be the 2 most sensitive species (with comparable sensitivities) in the Ni ecotoxicity database (European Chemicals Bureau 2008). By comparison, the species in the middle of the list of sensitivity, the green alga *Pediastrum duplex* is approximately 16 times less sensitive than *L. stagnalis* and 14 times less sensitive than *C. dubia*. Other growth inhibition tests support these findings: Niyogi et al. (2014) reported a 21 d EC20 <1.3 µg L^{-1} (lowest tested concentration) using 1-wk-old snails and Nys et al. (2016) reported 28 d EC20s of 10 and 32 µg L^{-1} using 2-d or 3-d-old snails in waters of higher pH and hardness. These lab results are further corroborated in the field by Peters et al. (2014), with effects on snail abundance occurring at lower levels (3.9 µg L^{-1} bioavailable Ni) than on other sensitive benthic macroinvertebrates (*Ephemeroptera*, *Plecoptera*, or *Tricoptera*). Additionally, Hommen et al. (2016) showed that *L. stagnalis* was the most sensitive species out of >150 species exposed to Ni in a 12 wk microcosm study. Meanwhile, a more recent study found *L. stagnalis* to be much more tolerant to Ni, with a 14 d EC20 of 150 µg L^{-1} using 2- to 3-wk-old snails (Crémazy et al. 2018). The various Ni toxicity lab studies were carried out using different test protocols and conditions (Table 1), in part due to the absence of a standardized method for testing growth inhibition in *L. stagnalis*. Yet, no obvious reason explains the high variability in Ni toxicity, which ranges approximately from 1 to 200 µg L^{-1}. Notably, test conditions (including water chemistry) used in Crémazy et al. (2018) were very similar to those used in Niyogi et al. (2014). Interestingly, Crémazy et al. (2018) measured EC20s for 5 other metals that were similar to other published data:

Ag (Brancho 2017), Cd (Pais 2012), Cu (Brix et al. 2011; Brancho 2017), Pb (Brix et al. 2012), and Zn (De Schamphelaere and Janssen 2010). Therefore, this sensitivity variability seems to be a Ni-specific issue.

In the present study, we investigated the large discrepancies reported in the literature for chronic Ni toxicity to *L. stagnalis*, with a focus on the Crémazy et al. (2018) and the Niyogi et al. (2014) studies. These 2 studies were selected mainly because their test conditions were the closest (Table 1), yet their reported Ni EC20s were the most different (Table 2). Four potential factors were tested to explain the variability: 1) differences in methods used to calculate the toxicity endpoint, 2) differences in Ni bioavailability in the test waters (water chemistry effects), 3) differences in sensitivity of the snail cultures (population effect), and 4) differences in lab-specific practices (lab effect). To test these factors, we reanalyzed the literature data (Tables 1 and 2) and performed additional physiological and toxicological measurements using the 2 snail populations and the 2 test waters used in Crémazy et al. (2018) and in Niyogi et al. (2014) (Table 3). These additional experiments were performed at the lab sites where these original studies were carried out, i.e., at the University of British Columbia (UBC, Vancouver, Canada) and at the University of Miami (UM, Miami, FL), with local and shipped (from the other lab) snails and test waters. We measured Ni^{2+} aqueous concentrations (with an ion exchange technique [IET]), short-term Ni uptake fluxes (3 h flux measurements with radiolabeled Ni), and chronic Ni toxicity (7 d and 14 d growth tests) in juvenile snails.

The present study addresses an issue of particular relevance to the regulatory community. Indeed, accurate risk management requires a clear understanding of the factors affecting toxicity variability and test reproducibility with key sensitive species. While the present study could not explain the full extent of the variability in snail sensitivity to Ni, it provides some answers, raises important considerations for snail toxicity testing, and makes recommendations for subsequent studies and the future development of standardized methods.

MATERIALS AND METHODS

Literature data analyses

To our knowledge, only 4 studies have reported the effects of Ni on the growth of juvenile *L. stagnalis* (Table 1).

Table 2. Literature observed and predicted EC20s and EC50s (dissolved Ni concentration, in $\mu g\,L^{-1}$) for growth inhibition in juvenile *L. stagnalis*

			Biomass		Specific Growth Rate	
Study	Water	Effect concentration	Observed ECx	Predicted ECx	Observed ECx	Predicted ECx
Crémazy et al. 2018	UBC water	EC20	98 [73–120]	–	150 [130–170]	41
		EC50	160 [140–190]	7.0	220 [200–240]	–
Niyogi et al. 2014	UM water	EC20	<1.3	–	9.4	36
		EC50	1.5	7.8	>17	–
Nys et al. 2016	Artificial water pH 8.1	EC20	34 [21–54]	–	32 [21–50]	35
		EC50	>39	5.4	>39	–
Nys et al. 2016	Artificial water pH 8.6	EC20	13 [8.7–20]	–	10 [6.5–16]	21
		EC50	>42	3.3	40 [26–62]	–
Schlekat et al. 2010	Calapooia River	EC20	1.6 [1.4–2.3]	–	7.3 [1.1–50]	48
		EC50	6.2 [2.7–7.2]	8.1	10 [4.7–23]	–
Schlekat et al. 2010	S. Platte River	EC20	27 [12–31]	–	48 [33–69]	120
		EC50	40 [34–44]	26	98 [80–120]	–
Schlekat et al. 2010	S. Platte River-pH amended	EC20	21 [6.9–62]	–	84 [55–130]	490
		EC50	78 [66–85]	91	160 [130–200]	–
Schlekat et al. 2010	S. Santiam River	EC20	6.3 [1.3–14]	–	21 [18–24]	49
		EC50	15 [8.2–20]	9.8	34 [31–36]	
Schlekat et al. 2010	Zollner Creek	EC20	1.9 [1.7–3.0]	–	52 [42–65]	160
		EC50	23 [12–36]	36	110 [92–120]	–

The observed ECxs are given as means with their 95% confidence intervals, based on snail biomass or specific growth rate.
ECx predictions were made with WHAM VII and a *C. dubia* chronic Ni BLM (Equation 2) with adapted sensitivity constants for *L. stagnalis* from Schlekat et al. (2010) ($Q_{50,Lymnaea,biomass}$) and Nys et al. (2016) ($Q_{20,Lymnaea,SGR}$). These data are also illustrated in Figure 1.

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Table 3. The experiment ID, Ni concentration range and measurement(s) (3 h uptake fluxes [J_{int}], 7 d and 14 d Specific Growth Rate [7 d SGR, 14 d SGR]) for each snail source and water type combination tested in the 4 experiments carried out at UBC or UM in the present study

Exp ID	[Ni] (μg L^{-1})	UBC snails		UM snails	
		UBC water	UM water	UBC water	UM water
Exp#1 (at UBC)	40, 300	3 h J_{int} [a] 3 h J_{int}		–	–
Exp#2 (at UBC)	0, 10, 250	7 d SGR [a] 7 d SGR [c]		–	–
Exp#3 (at UM)	0, 10, 250	–	7 d SGR, 14 d SGR [b]	7 d SGR, 14 d SGR [a]	7 d SGR, 14 d SGR [b]
Exp#4 (at UBC)	13 conc. [0–290]	14 d SGR [b]		14 d SGR	–

[a] Testing of water type effects.
[b] Testing of snail population effects.
[c] Testing of lab effects.

These studies did not estimate effect concentrations on growth (ECx) with the same method: Schlekat et al. (2010) and Niyogi et al. (2014) used snail biomass ($ECx_{biomass}$) while Nys et al. (2016) and Crémazy et al. (2018) used snail specific growth rate (ECx_{SGR}) (Table 2). For each study, we calculated EC20s and EC50s based on biomass (final snail weight or shell length) and specific growth rate (SGR) to allow for interstudy comparison. The measurement of both response variables is traditionally proposed in standardized methods, as there is still no consensus on which variable should be used for risk assessment (OECD 2011). SGR (in d^{-1}) is calculated using the following equation:

$$SGR = \frac{\ln(w_f) - \ln(w_i)}{t} \quad (1)$$

where w_f is the initial snail wet weight, w_i is the final snail wet weight (soft tissue + shell) (Som Niyogi, University of Saskatchewan, personal communication; Chris Schlekat, NiPERA, personal communication; Charlotte Nys, Arche Consulting, personal communication), and t is the test duration. The ECx_{SGR} in Niyogi et al. (2014) and in Schlekat et al. (2010) and the $ECx_{biomass}$ in Nys et al. (2016) and Crémazy et al. (2018) were then estimated with the methods used for the original ECx estimates. That is, ECx_{SGR} in Niyogi et al. (2014) were estimated via graphical interpolation, while ECx_{SGR} in Schlekat et al. (2010) and $ECx_{biomass}$ in Nys et al. (2016) and Crémazy et al. (2018) were estimated with the Toxicity Relationship Analyses Program (TRAP, Version 1.10, USEPA, NHERL, Duluth, MN) using logistic equations after log transformation of dissolved Ni concentrations. The logistic equation had 3 parameters in Schlekat et al. (2010) and Crémazy et al. (2018) and 2 parameters in Nys et al. (2016).

To evaluate the test water chemistry effects (i.e., metal bioavailability changes) in these studies, we predicted the ECx in each test water using BLMs parameterized by Schlekat et al. (2010) and Nys et al. (2016). These studies used an existing chronic Ni BLM for *C. dubia*, with adjusted sensitivity parameters for *L. stagnalis*, to obtain ECxs based on bioavailable Ni^{2+} concentration:

$$\text{Predicted } ECx^{Ni2+} = 10^{-(Q_{x,Lymnaea} + S_{pH} \times pH)} \times \{1 + K_{CaBL} \times [Ca^{2+}] + K_{MgBL} \times [Mg^{2+}]\} \quad (2)$$

with K_{CaBL} ($=10^{3.53}$) and K_{MgBL} ($=10^{3.57}$) being the biotic ligand binding stability constants for Ca^{2+} and Mg^{2+} respectively, S_{pH} (=0.8587) being the slope of the pH effect, and $Q_{x,Lymnaea}$ being the intrinsic sensitivity parameter for x% effect on *L. stagnalis* growth. Schlekat et al. (2010) derived a $Q_{50,Lymnaea,biomass}$ of 1.099 for the prediction of $EC50^{Ni2+}_{biomass}$. Nys et al. (2016) derived a $Q_{20,Lymnaea,SGR}$ of 0.29 for the prediction of $EC20^{Ni2+}_{SGR}$. The corresponding $EC50_{biomass}$ and $EC20_{SGR}$ based on total Ni concentrations were then obtained with the Windermere Humic Aqueous Model (WHAM, version VII [Tipping et al. 2011]), following the modeling recommendations from Van Laer et al. (2006), as in Schlekat et al. (2010).

Experimental water compositions

We conducted additional measurements of Ni speciation, uptake flux rate, and chronic toxicity in fresh test waters used in Niyogi et al. (2014) at the University of Miami (UM) and in Crémazy et al. (2018) at the University of British Columbia (UBC) (Table 3). In the remainder of this manuscript, these 2 waters will be referred to as "UM water" and "UBC water" respectively. In some cases, test water was shipped from UBC to UM and vice versa.

The UM water corresponds to dechlorinated Miami city water. Its composition is given in Niyogi et al. (2014), but the concentrations of major cations, dissolved organic carbon (DOC), and dissolved inorganic carbon (DIC) were also remeasured during the present study (Table 4). These

Table 4. Composition of the 2 exposure waters tested in the present study

	UM water	UBC water
pH	7.79 ± 0.02	7.81 ± 0.20
DOC (mg of C L^{-1})	2.16 ± 0.21	0.76 ± 0.08
DIC (mg of C L^{-1})	10.00 ± 0.10	9.56 ± 0.06
[Ca] (mM)	0.454 ± 0.006	0.94 ± 0.01
[Mg] (mM)	0.154 ± 0.005	0.22 ± 0.02
[Na] (mM)	1.50 ± 0.05	1.7 ± 0.2
[K] (mM)	0.087 ± 0.007	0.054 ± 0.015
[Cl] (mM)	1.21	1
[SO_4] (mM)	0.14	0.79

The composition of UM water (dechlorinated Miami city water) was measured in the present study and is given as mean ± SD, n = 3 (except for Cl and SO_4 concentrations which are nominal).
The composition of UBC water (dechlorinated Vancouver city water + salts) was measured in Crémazy et al. (2018) and is given as mean ± SD, n = 252 (except for Cl and SO_4 concentrations which are nominal).

chemistry parameters were not noticeably different between the 2 studies. The UBC water corresponds to dechlorinated Vancouver city water, to which salts were added ($CaSO_4 \cdot 2H_2O$, $CaCl_2 \cdot 2H_2O$, $NaHCO_3$, $KHCO_3$, NaCl, and $MgSO_4 \cdot 7H_2O$) to mimic the ionic composition of Miami water (although Ca and SO_4 levels in the UBC water were respectively 2- and 6-fold greater). Its composition was measured in Crémazy et al. (2018) and is also given in Table 3. There were 2 reasons for this salt addition to Vancouver water: First, this water is very soft and snails perform better in moderately hard to hard waters. Second, a series of metal chronic toxicity tests had already been performed at UM (Cu: Brix et al. 2011; Pb: Brix et al. 2012; Ni: Niyogi et al. 2014), and they could provide useful comparisons to the UBC tests carried out in Crémazy et al. (2018, 2019) if conducted in relatively similar water chemistry. The main differences between the 2 waters were their Ca (2-fold higher in the UBC water), DOC (3-fold higher in the UM water), and SO_4 concentrations (6-fold higher in the UBC water).

Experimental snail culture conditions

The additional Ni uptake flux rate and chronic toxicity tests were conducted with 2 snail cultures: 1) the culture used by Crémazy et al. (2018, 2019) at UBC, and 2) the culture used by Niyogi et al. (2014) at UM (Table 3). In some cases, egg masses were shipped from UBC to UM and vice versa.

The UBC snails have been continuously cultured since 2014 from a colony held at McMaster University since 2006. The McMaster culture results from the mixing of 5 cultures from the following sources: N Syed (University of Calgary, Calgary, AB, Canada), M Grosell and S Ebanks (University of Miami, Miami, FL), ZP Feng (University of Toronto, Toronto, ON, Canada), G Spencer (Brock University, St. Catharine's, ON, Canada), and D Spafford (University of Waterloo, Waterloo, ON, Canada). Therefore, the UM culture and the McMaster/UBC culture are somewhat related. At UBC, the snails were kept in UBC water, at 25 ± 1 °C, with a 16 h light: 8 h dark photoperiod. They were fed with excess peels of washed sweet potato (*Ipomoea batatas*) for newly hatched snails to approximately 1 to 2 wk old snails, then with washed romaine lettuce (*Lactuca sativa*) 3 times a week. Holding tank water was changed twice a week. At UM, the snails have been continuously cultured since 2002. The original source of this culture was Dr. N Syed at the University of Calgary—also one of the sources of the McMaster/UBC colony; therefore, the 2 cultures have been separated for about a decade. The UM culture conditions were the same as described above for UBC, except that the adult snail cultures were maintained under flow-through conditions using UM water.

Egg masses for experiments were isolated and maintained under static renewal conditions through hatch and until use in toxicity tests. Shipped egg masses were acclimated to the local culture conditions until tests were initiated. They were provided sweet potato until approximately 10 d post hatch, then a mix of sweet potato and lettuce. The snails were primarily feeding on lettuce when the Ni uptake and toxicity tests were initiated (2–4 wk post hatch). These tests were performed under the same temperature and light regime as described above.

Short-term Ni uptake tests

Short-term Ni uptake rates were measured in UBC snails exposed in parallel in UM water or in UBC water (Experiment 1 in Table 3) as a physiological indicator of Ni bioavailability in both waters. These tests were performed at UBC, as previously detailed in Crémazy et al. (2019). Exposure solutions were prepared 24 h prior to the test. They consisted of test waters spiked with $NiCl_2 \cdot 6H_2O$ (ACS grade, Fisher Scientific, Ottawa, ON, Canada) at 40 µg L^{-1} and 300 µg L^{-1} Ni, and with 0.02 µCi · mL^{-1} of radioactive ^{63}Ni (Stuart Hunt & Associates Ltd, Edmonton, AB, Canada). Snails approximately 4-wk-old (100–150 mg wet weight) were selected for these tests. They had been switched from a sweet potato diet to a lettuce diet about 1 wk before the test and were starved for 24 h prior to exposure. The snails were exposed for 3 h in 45 mL of aerated solution in individual containers (n = 6 replicates per treatment). This duration was long enough to measure influx, yet short enough to avoid noticeable backflux of the radioisotope, as demonstrated in Crémazy et al. (2019). After exposure, each snail was quickly rinsed in Ni-free test water, then for 5 min in 20 mL of 1 mM ethylenediaminetetraacetic ($EDTA \cdot 2H_2O$, ACS grade, Fisher Scientific) prepared in Ni-free test water ([Ni] <0.7 µg L^{-1}, method quantification limit), and finally rinsed quickly again in Ni-free test water. Soft tissues were dissected, weighed, then digested for 2 d at 65 °C in 50% HNO_3 v/v (ACS grade, Fisher Scientific). Filtered exposure water samples (0.45 µm polyethersulfone (PES) membrane, Membrane Solution, Dallas, TX) were also collected at the

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beginning and at the end of the uptake test for physicochemical analyses.

Chronic Ni toxicity tests

As shown in Table 3, 3 chronic toxicity experiments were conducted in the present study: Experiment 2, Experiment 3, and Experiment 4. Experiment 2 (conducted at UBC) was designed to evaluate water effects using UBC snails. In this experiment, 7 d SGR were measured with UBC snails exposed in UBC water or UM water. The test waters were spiked with 0 μg L^{-1}, 10 μg L^{-1}, and 250 μg L^{-1} Ni and there were 5 replicates per treatment. The limited test duration and Ni treatments were due to the limited amount of shipped UM water.

Experiment 3 (conducted at UM) was designed to further evaluate water effects (using UM snails) and to evaluate snail population effects in UM water. In this experiment, 7 d and 14 d SGR were measured with UM snails exposed in UBC or UM water and with UBC snails exposed in UM water. As in Experiment 2, the test waters were spiked with 0 μg L^{-1}, 10 μg L^{-1}, and 250 μg L^{-1} Ni, and there were 5 replicates per treatment. Note that the same 7 d SGR measurements with UBC snails in UM water were performed at UBC (Experiment 2) and UM (Experiment 3), thereby facilitating the assessment of test reproducibility between the 2 labs.

Finally, Experiment 4 (conducted at UBC) was designed to further evaluate snail population effects (in UBC water). In this experiment, 14 d SGR were measured with UBC snails or UM snails exposed in UBC water. Five snails per treatment were exposed in bulk in 2 L of exposure water at 13 Ni concentrations ranging from 0 to 300 μg L^{-1} Ni, as in Crémazy et al. (2018). The treatments were not replicated.

For all 3 tests, exposure solutions were prepared by spiking stock solutions of $NiCl_2 \cdot 6H_2O$, ACS grade, Fisher Scientific) into both waters 24 h prior to test initiation. Snails approximately 2-wk-old (5–10 mg) were exposed in aerated exposure solutions, either individually (in 250 mL of water, Experiment 2 and Experiment 3) or in bulk (5 snails in 2 L of waters, Experiment 4). Every day, water was renewed and snails were fed ad libitum with fresh washed romaine lettuce. Water samples were collected at days 1, 7, and 14 (when applicable), before water changes (24 h old water) and after water changes (fresh water), and filtered (0.45 μm PES membrane, Membrane Solution, Dallas, TX) for measurements of Ni concentration and other physicochemical parameters (pH, major cations, and DOC concentrations). At the end of the test, the snails were blotted dry and their wet weight was determined with an accuracy of ±0.1 mg with an analytical balance.

Physicochemical analyses and free Ni^{2+} measurements

The pH was measured with an Orion™ Green pH combination electrode (Fisher Scientific). Inorganic elements were analyzed via atomic absorption spectrometry (UBC: AA240 FS, Varian, Palo Alto, CA) in furnace mode (GFAAS) for dissolved Ni, and in flame mode (FAAS) for dissolved Ca, Mg, K, and Na. The DOC and DIC concentrations were measured with a Total Organic Carbon analyzer (V-series TOC analyzer, Shimadzu). Instrument calibrations were verified with certified reference waters TMDA-54.5 and TM-25.4 (Natural Resources Canada, Burlington, ON, Canada) (percent recovery was >95%). A blank and calibration standard was reanalyzed every 8 (TOC analysis) or 12 (AAS analysis) samples to check and correct for potential instrument drift. For the short-term uptake tests, beta radiation from ^{63}Ni in water and snail tissues was measured as cpm (counts per minute) using an automated scintillation counter (LS 6500, Beckman Coulter, Mississauga, ON, Canada), with appropriate quench correction (Crémazy et al. 2019).

To characterize potential differences in Ni bioavailability in UBC and UM waters, we measured Ni^{2+} concentrations in both waters using a solid-phase IET. This technique has been shown to selectively measure Ni^{2+} species in natural waters (Worms and Wilkinson 2008). These measurements were performed in the range of dissolved Ni concentrations eliciting growth inhibition in UBC water (10–220 μg L^{-1}). The working principle of IET has been described in detail by Worms and Wilkinson (2008). Briefly, samples and standard solutions were passed through a column with 50 to 100 mg of cation-exchange resin (Dowex 50W-X8, 50–100 mesh; Sigma-Aldrich, Oakville, ON, Canada) until equilibrium was reached (i.e., when the total dissolved [Ni] entering and exiting the resin column became equal). The resin was then eluted with nitric acid (Omni-trace grade, Sigma Aldrich) and the Ni concentration in the eluate was measured by GFAAS (Perkin Elmer PinAAcle 900T AA, Woodbridge, ON, Canada). After calibration with standard solutions of known Ni^{2+} concentrations, the Ni^{2+} concentration in the test solutions could be calculated from the Ni concentration in the eluates for a given acid volume and resin mass.

Finally, to characterize potential differences in the Ni-binding properties of the dissolved organic matter (DOM) in UBC and UM waters, Fluorescence Excitation Emission Matrices (FEEMs) were measured using a spectrofluorometer (Varian ECLIPSE Cary fluorescence spectrophotometer [Varian Inc., Old Oak, NJ]) and a 1 cm quartz cuvette (Starna Cells Inc., Atascadero, CA) (Tait et al. 2018). For scans, the excitation monochrometer was varied from 200 to 450 nm in 10 nm increments, and for each excitation wavelength the sample emission was recorded at 250 to 600 nm in 1 nm increments. For fluorescence quenching experiments, Ni was titrated at pH 7.8 (pH adjusted using dilute NaOH additions). Samples were equilibrated 24 h before measurement of the FEEM.

Experimental data analyses

If not stated otherwise, results are presented as mean ± standard error (SE) or as mean with 95% confidence intervals. Statistical analyses of the new experimental data were conducted with the software GraphPad Prism, with a significance level of 0.05.

The IET data in the 2 waters were analyzed via linear regression (IET-Ni^{2+} concentration as a function of the total Ni concentration) and compared with an extra sum-of-squares *F* test.

Nickel internalization (uptake) rates (J_{int}, in $\mu g \cdot g \cdot h^{-1}$) (Experiment 1) were calculated using the following equation, with a = cpm g^{-1} of whole snail body (wet weight), b = cpm ml^{-1} in the water, c = the measured dissolved Ni concentration in the water ($\mu g\ ml^{-1}$), and t = time (3 h):

$$J_{int} = \frac{a \times c}{b \times t} \quad (3)$$

where the J_{int} values were compared at the different Ni exposure concentrations, and for both water types, using a 2-way ANOVA and Tukey's post hoc test.

Nickel chronic toxicity (Experiments 2–4) is reported both as effects on w_f (in milligram wet weight) and effects on SGR (in d^{-1}, calculated with Equation 1). For chronic toxicity tests conducted at 3 Ni concentrations (Experiment 2 and Experiment 3), the growth measurements were compared at the various Ni exposure concentrations and for both water types, snail populations, or labs, using 2-way ANOVAs and Tukey's post hoc tests. For the toxicity tests performed according to the Crémazy et al. (2018) protocol (Experiment 4), the data were fitted against Ni exposure concentrations with a 3-parameter logistic equation after log transformation of dissolved Ni concentrations. The regressions in both waters were compared using an extra sum-of-squares *F* test.

RESULTS

Literature data

Table 2 shows the EC20s and EC50s obtained in the 4 literature studies, based on snail final biomass (including the final shell length) and SGR. The ECx estimates based on biomass were consistently lower than the estimates based on SGR, with the exception of Nys et al. (2016) where they were similar. Estimated $EC20_{biomass}$ were up to 27-fold (average 5.7-fold) lower than $EC20_{SGR}$ and estimated $EC50_{biomass}$ were up to >11-fold (average 3.3-fold) lower than $EC50_{SGR}$. Interstudy variability was higher when toxicity was expressed based on biomass when compared to SGR. Estimated $EC20_{biomass}$ and $EC50_{biomass}$ observed in the various tests varied by >126-fold and 109-fold respectively, with the greatest difference observed between the Niyogi et al. (2014) and the Crémazy et al. (2018) studies. In comparison, the interstudy variation was about 20-fold and 22-fold when considering $EC20_{SGR}$ (7.3–150 $\mu g\ L^{-1}$) and $EC50_{SGR}$ (10–220 $\mu g\ L^{-1}$) respectively, with the greatest difference observed between the Schlekat et al. (2010) (Calapooia River water) and the Crémazy et al. (2018) studies.

Table 2 also shows that $EC50_{biomass}$ and $EC20_{SGR}$ predicted with the chronic Ni BLM for *L. stagnalis* developed by Schlekat et al. (2010) and Nys et al. (2016), respectively. The performance of each BLM for the prediction of the literature ECx data is illustrated by Figure 1. The biomass BLM explained the 109-fold variation in $EC50_{biomass}$ within a 23-fold maximum deviation from the prediction. Notably, Ni toxicity was over-predicted by a factor of 23 in Crémazy et al. (2018) and under-predicted by a factor of 5 in Niyogi et al. (2014) (Table 2 and Figure 1A). The SGR BLM explained the 22-fold interstudy variability within a factor of 6.6 (Table 2 and Figure 1B). It over-predicted most measured $EC20_{SGR}$ values, with the exception of the Crémazy et al. (2018) $EC20_{SGR}$ that was underpredicted by a factor 3.6. Because of the very similar water chemistry in Niyogi et al. (2014) (UM water) and Crémazy et al. (2018) (UBC water), both BLMs predicted nearly identical chronic Ni toxicity in these 2 studies (Table 2 and Figure 1). Yet, Crémazy et al. (2018) measured $EC50_{biomass}$ and $EC20_{SGR}$ that were respectively 109 times and 16 times higher than in Niyogi et al. (2014).

In UBC and UM waters respectively, Crémazy et al. (2019) and Niyogi et al. (2014) measured short-term Ni uptake flux rates in juvenile *L. stagnalis* using ^{63}Ni. The comparison of these literature data is shown in Supplemental Data Figure S1, where J_{int} values in UM water (Niyogi et al. 2014) were about 5-fold higher than in UBC water at comparable dissolved Ni concentrations (Crémazy et al. 2019).

Ni speciation in test waters

At the observed $EC20_{SGR}$ in UBC water (150 $\mu g\ L^{-1}$) and UM water (9.4 $\mu g\ L^{-1}$), WHAM VII predicted that 71% and

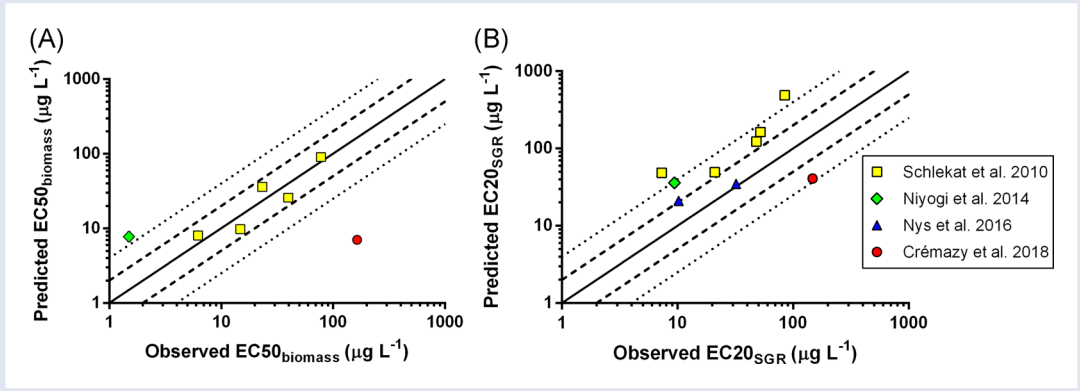

Figure 1. Predicted versus observed A) $EC20_{biomass}$ and B) $EC20_{SGR}$ (dissolved Ni concentration) for growth inhibition of *L. stagnalis* in literature studies. Predictions were made with WHAM VII and a *C. dubia* chronic Ni BLM (Equation 2) with adapted sensitivity constants for *L. stagnalis* from Schlekat et al. (2010) ($Q_{50,\ Lymnaea,\ biomass}$) and Nys et al. 2016 ($Q_{20,\ Lymnaea,\ SGR}$). Plain lines represent perfect predictions. The dashed and dotted lines represent predictions within a factor 2 and 4, respectively, for easier assessment of deviation from the 1:1 line. These data are also reported in Table 2.

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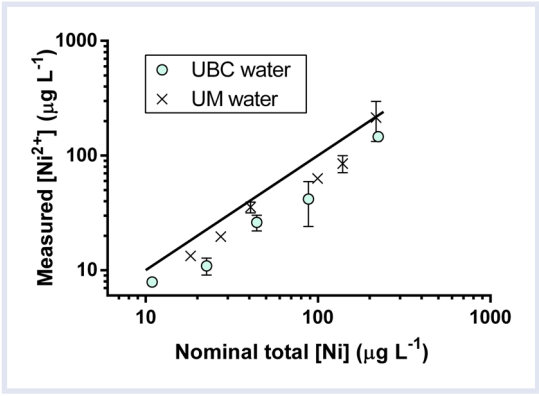

Figure 2. Free Ni^{2+} concentration (measured by an ion-exchange technique) as a function of total Ni concentration (nominal) in UBC and UM waters. Data are presented as mean ± SD (n = 2). The line corresponds to the 1:1 line.

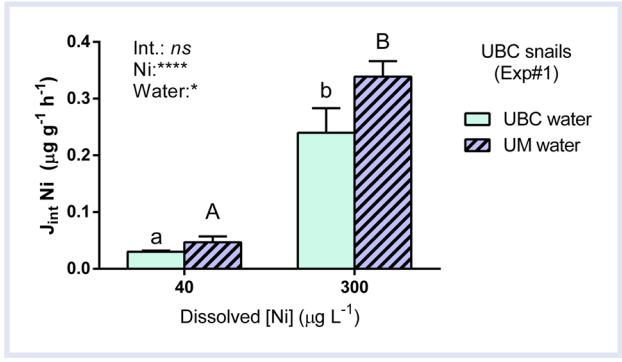

Figure 4. Effect of water type on Ni 3 h uptake rate in UBC snails, as a function of dissolved Ni concentration. Data are presented as means ± SE (n = 6). Results of the 2-way ANOVA are given in each panel (ns: not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$) and in more detail in Table S1 (Supplemental Data). The interaction effect is abbreviated Int. Furthermore, statistically significant effects of [Ni] on J_{int} are shown by different letters (Tukey's test). No significant effect of water type on J_{int} was observed at each [Ni] with the Tukey's test.

51% of Ni was present, respectively, as free bioavailable Ni^{2+}. At the observed $EC50_{biomass}$ (160 µg L^{-1} in the UBC water and 1.5 µg L^{-1} in the UM water), these predictions were respectively 65% and 35%. The 6-fold SO_4 concentration difference in the 2 waters had only a small effect on these Ni^{2+} percentages, since Ni-SO_4 complexes were not very abundant (6% and 1% of total Ni species in UBC and UM waters, respectively). The lower proportion of Ni^{2+} in the UM water was mainly attributed to its 3-fold higher DOC concentration, leading to increased Ni-DOC complexation.

Figure 2 shows the [Ni^{2+}] measured by IET as a function of the total [Ni] in each water. These measurements seemed to slightly disagree with the WHAM predictions, since the measured Ni^{2+} was 58 ± 5% in the UBC water and 76 ± 6% in the UM water. However, our analysis of the IET data concluded that there was no statistical difference in Ni speciation between the 2 waters (*F*-test, $p = 0.1389$; regression equation: log [Ni^{2+}] = 0.7998 x log [Ni] – 7.379).

Fluorescence Excitation Emission Matrices (FEEM) of the UBC and UM waters indicated that the DOCs of both waters exhibited fulvic-like components, which could be detected in the emission-excitation ranges of 250 to 390 nm to 460 to 520 nm (Figure 3). However, DOM in the UBC water also has a tyrosine-like component, which could be detected in the emission-excitation ranges of 225 to 270 nm to 300 nm. When UBC water was incubated with 40 µg L^{-1} of total Ni, fluorescence of the tyrosine-like peak increased (Figure 3C), likely indicating interactions between this fluorophore and Ni ions.

Short-term Ni uptake

A slightly significant effect of water type was observed in newly measured short-term Ni uptake flux rates in Experiment 1 (2-way ANOVA, $p = 0.039$, 4% of total variation) (Figure 4). However, the Tukey's test did not find any difference between the water types for each of the tested [Ni]. Indeed, the 3 h Ni uptake flux rates measured in UBC snails were very similar in UBC water and in UM water at the 2 tested Ni concentrations. These Ni flux rate levels were similar to the levels reported by Crémazy et al. (2019) for UBC snails in UBC water.

Chronic Ni toxicity

No mortality was observed among the control snails across all of the toxicity tests performed in the present

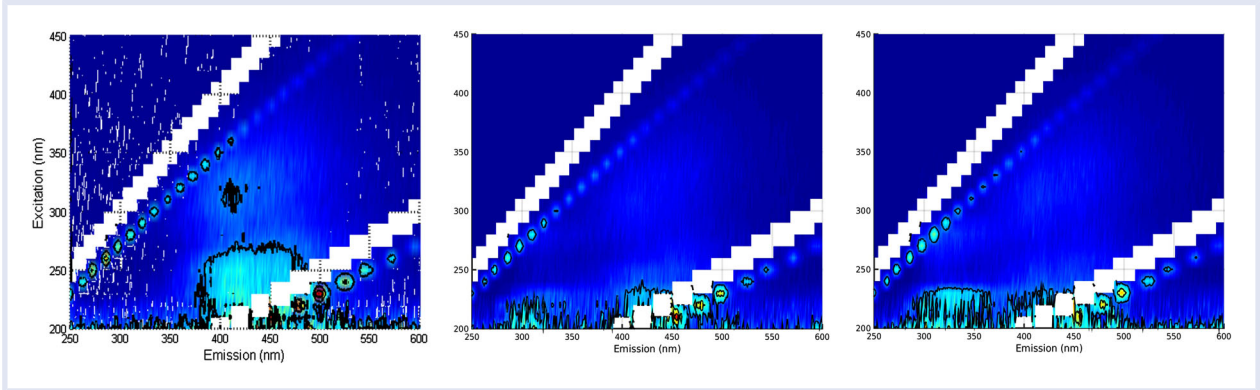

Figure 3. Excitation-emission spectrum (wavelength in nm) of (**A**) UM water, (**B**) UBC water, and **C**) UBC water incubated with 40 µg L^{-1} of total Ni.

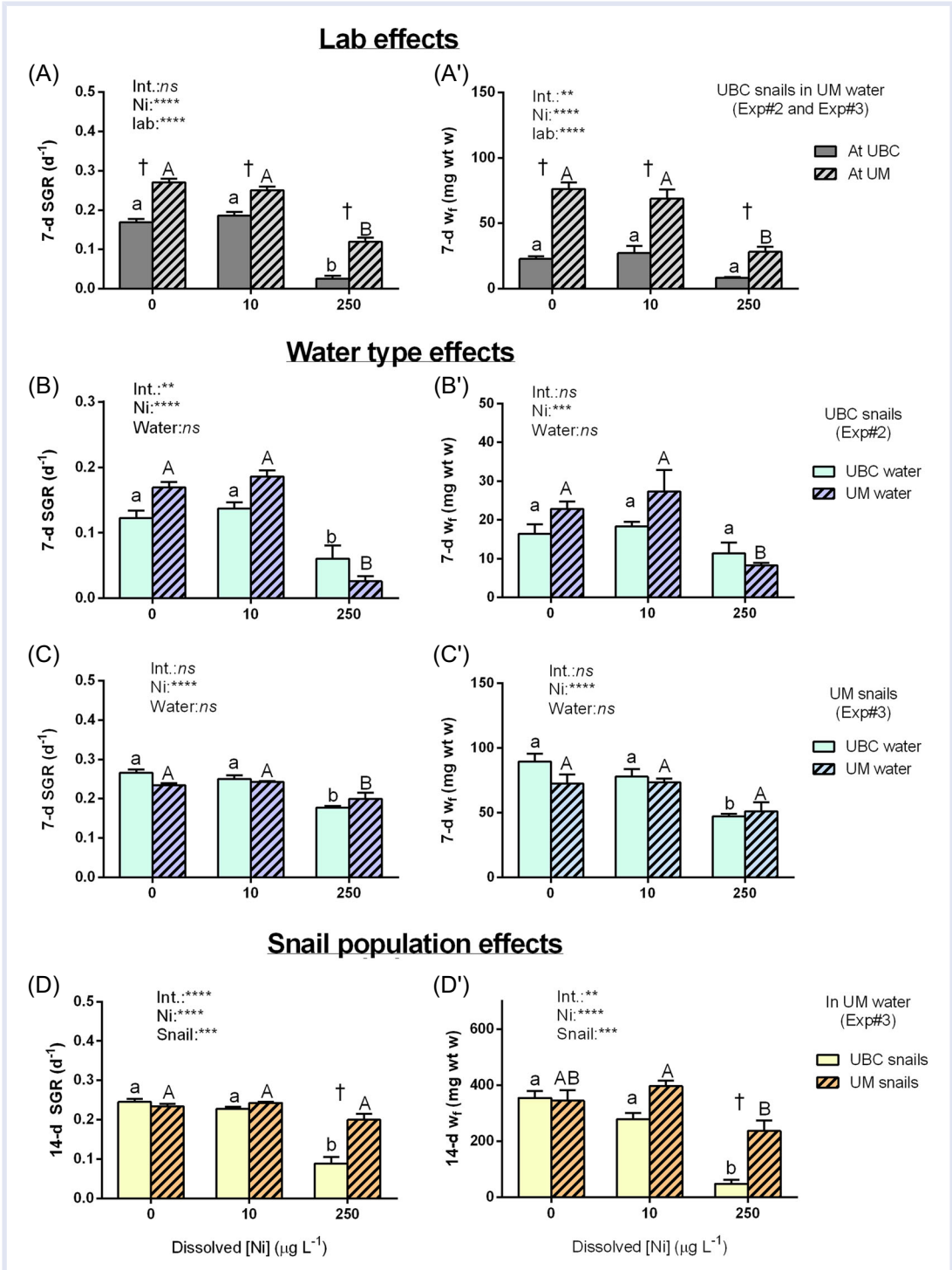

Figure 5. Effects of lab (A and A'), water type (with UBC snails: B and B'; with UM snails: C and C') and snail population (D and D', in UM water) on snail SGR (left panel) and wf (right panel), as a function of dissolved Ni concentration. Results are shown as means ± SE (n = 5). The 2-way ANOVA results are given in each panel (ns: not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$) and in more details in Table SI.1 (Supplemental Data). The interaction effect is abbreviated Int. Furthermore, statistically significant effects of lab, water, and snail on SGR/w_f are shown by daggers and statistically significant effects of [Ni] on SGR/w_f are shown by different letters (Tukey's test).

study. The average SGR in the control snails was 0.221 ± 0.008 d^{-1} (n = 42) and ranged from 0.098 to 0.314 d^{-1}. These growth rates are consistent with control SGR measured under similar test conditions by Schlekat et al. (2010), Niyogi et al. (2014), and Crémazy et al. (2018): from 0.11 to 0.24 d^{-1} with an average of 0.20 d^{-1}. Note that Nys et al. (2016) measured a much lower control SGR, from 0.022 to 0.047 d^{-1} (average of 0.032 d^{-1}), probably due to

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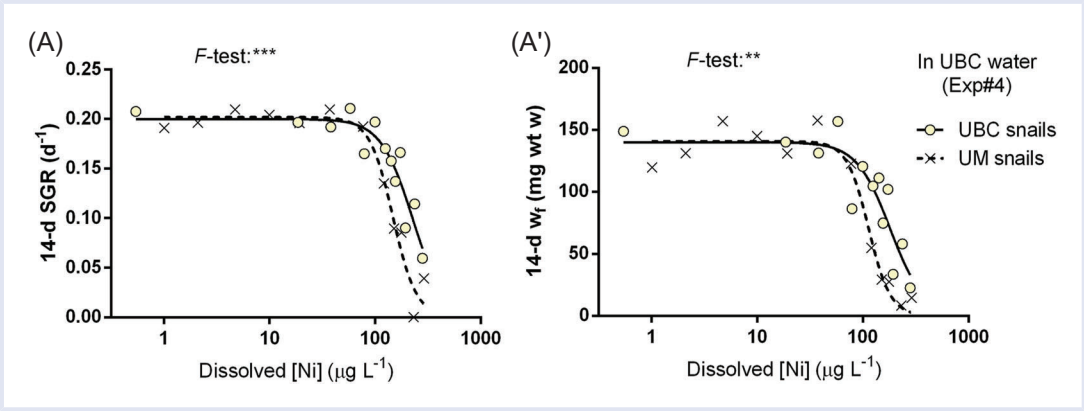

Figure 6. Effects of snail population (in UBC water) on snail A) SGR and A') wf, as a function of dissolved Ni concentration. Each data point corresponds to a treatment (n = 13 treatments). Regression and *F*-tests (**$p < 0.01$, ***$p < 0.001$) results are given in Table S2 (Supplemental Data).

differences in the test conditions (darker light cycle, lower temperature, and less nutritious food). No conclusion on test validity was made based on the growth rate of the control snails due to a lack of information on what is considered acceptable under our test conditions.

Figure 5 shows snail growth measured as either specific growth rate (SGR, left panel) or biomass (w_f, right panel) as a function of the dissolved Ni concentration, in Experiment 2 and Experiment 3, which tested the effects of test location (lab), water type, and snail population on chronic Ni toxicity. Figure 6 also shows the effects of the snail population source on chronic Ni toxicity, but in Experiment 4 which was conducted with a different experimental design.

Interlab experimental reproducibility (lab effects) was evaluated with an identical 7 d chronic toxicity test performed at UBC (Experiment 2) and at UM (Experiment 3) (Figures 5A and 5A'). Snails at UBC grew consistently less than at UM in each Ni treatment (including the controls). Significant decreases of snail growth were observed at 250 µg L^{-1} Ni in both labs. When considering SGR (Figure 5A), this growth inhibition at 250 µg L^{-1} was 3-fold higher in UBC compared to UM, but no significant interaction between the test location and Ni toxicity was observed. When considering biomass (w_f) (Figure 5A'), growth inhibition at 250 µg L^{-1} was only significant at UM, although both w_f decreased by the same 3-fold factor in both labs. Overall, these data suggest that, while the test location affected absolute snail growth, it had little impact on chronic Ni toxicity.

The effects of water type were evaluated with UBC snails (Figures 5B and 5B', Experiment 2) and with UM snails (Figures 5C and 5C', Experiment 3). As in the above test, the effects of Ni on snail growth were only observed at 250 µg L^{-1} Ni. There was no effect of water type on snail growth. A relatively small interaction effect (10% of the total variability) was observed with SGR of UBC snails (Figure 5B), such that growth tended to be higher in UM water at low Ni concentrations but lower in UM water at high Ni concentrations.

The effects of the snail population source were evaluated in UM water (Figures 5D and 5D', Experiment 3) and in UBC water (Figures 6A and 6A', Experiment 4). In UM water (Experiment 3), both of the main effects (Ni concentration and population) were significant, but there was also a significant interaction effect (Figures 5D and 5D'). The effects of Ni on snail growth only occurred at 250 µg L^{-1} Ni, with UBC snails growing significantly less than UM snails at the highest Ni concentration (Figures 5D and 5D'). However, this finding was not confirmed in UBC water (Experiment 4) (Figures 6A and 6A'). In this latter test, Ni effects also occurred in the 100 to 300 µg L^{-1} Ni range and different concentration-response curves (*F*-test, $p < 0.001$) also confirmed a snail population effect on Ni toxicity. However, this time, UM snails were more sensitive to Ni. Indeed, estimated $EC50_{SGR}$ was 230 [190–270] µg L^{-1} for UBC snails and 150 [130–170] µg L^{-1} for UM snails based on SGR (Figure 6A). Similarly, estimated $EC50_{biomass}$ was 180 [140–240] µg L^{-1} for UBC snails and 110 [96–130] µg L^{-1} for UM snails (Figure 6A') (see Supplemental Data Table S2 for detailed regression results). However, when considering EC20s, there was no significant difference between the two snail populations, mainly due to larger 95% CL on these estimates. For the UBC snails, $EC20_{SGR}$ was 140 [100–190] µg L^{-1} and $EC20_{biomass}$ was 110 [63–190] µg L^{-1}. For the UM snails, $EC20_{SGR}$ was 100 [82–130] µg L^{-1} and $EC20_{biomass}$ was 82 [61–110] µg L^{-1}.

DISCUSSION

Major findings

First, we showed that a large proportion of the variability in published Ni toxicity data could be accounted for by bioavailability correction (using the BLM) and by using SGR rather than w_f as a response variable. The latter is mainly because SGR is less dependent on test duration, which varied by 2-fold among the studies. Second, our new experiments showed that test water composition (UBC vs. UM water) or test location (at UBC vs. UM) had only small effects or no effects on the short-term uptake and chronic toxicity of Ni. We observed the snail population source (UBC vs. UM snails) effects on Ni toxicity, but these effects were

inconsistent and small relative to the differences between Niyogi et al. (2014) and Crémazy et al. (2018). Finally, the Niyogi et al. (2014) toxicity data could not be reproduced, as snail Ni sensitivity measured in the present study was systematically closer to the sensitivity reported in Crémazy et al. (2018) (i.e., with toxic effects in the low hundreds $\mu g\ L^{-1}$).

Variability in chronic Ni toxicity to L. stagnalis *in the literature*

Both literature EC20s and EC50s are reported in the present study. The low-effect estimate EC20 is typically considered a better parameter than the EC50 for regulatory purposes. However, among all of the statistical estimates, the EC50 generally has the narrowest confidence interval (i.e., the lowest measurement uncertainties), making it a more robust parameter for interstudy comparison (Christensen et al. 2009). Furthermore, we reported ECx based on 2 response variables: final biomass (used in Schlekat et al. [2010] and Niyogi et al. [2014]) and SGR (used in Nys et al. [2016] and Crémazy et al. [2018]). Although both response variables are derived from the same measurements and assess percent growth inhibition (by relating to the response in control treatments), the resulting ECx figures are markedly different, for systemic and mathematical reasons. Indeed, for organisms with exponential growth (e.g., *L. stagnalis*, most unicellular algae, and some aquatic plants), small changes in growth rate result in much larger changes in biomass. Therefore, biomass will generally give lower ECx estimates than SGR. Indeed, this was generally observed across the various literature studies (Table 2). The use of biomass or growth rate to assess growth inhibition is an old controversy that has received much attention in algae studies (Nyholm 1985; Ratte et al. 1998; Bergtold and Dohmen 2011) but remains globally unresolved in the regulatory community (Weyers and Vollmer 2000; OECD 2011). On the one hand, it is common practice for regulatory purposes to use the response variable exhibiting the lowest value. On the other hand, for the same reasons that biomass is mathematically more sensitive than SGR, it also has a larger variance (Ratte et al. 1998). Indeed, SGR averages over the growth curve, whereas the final biomass sums up all of the deviations in the growth and thus also the variability. As a result of its higher variance, biomass is not always found to be more sensitive for detecting biological effects than SGR, as observed in some tests of the present study (Figures 5B vs. 5B' and Figures 5C vs. 5C'). However, had these tests been conducted for a longer time (i.e., >7 d), it is likely that the biomass would have been found more sensitive than SGR ($ECx_{biomass}$ estimates substantially decrease with time). Indeed, relative to SGR, biomass is more dependent on test conditions, such as biomass at the start of the test, the snail intrinsic growth rate, and more importantly test duration (Nyholm 1985; Ratte et al. 1998; Bergtold and Dohmen 2011). Therefore, the use of biomass has been argued to be one of the reasons for the lack of comparability among algal inhibition studies (Nyholm 1985). The larger variance of the toxicity data derived from biomass could possibly be diminished by strict standardization of the test conditions across studies, although this may not always be practical. In part due to the absence of a standardized method for testing the growth effects with *L. stagnalis*, the 4 literature studies had many methodological differences (e.g., test duration, starting size and/or age, photoperiod, temperature, food) (Table 1). Based on the test duration differences alone, these tests are best compared based on toxicity estimates derived from SGR, which are much less time-dependent (Nyholm 1985; Ratte et al. 1998; Bergtold and Dohmen 2011). Indeed, in the present study, test duration (7 d or 14 d) had no effects or negligible effects on SGR inhibition by Ni (Supplemental Data Figure S2). Likewise, time (7 d, 14 d, and 21 d) had no effect on SGR inhibition in Niyogi et al. (2014) (Supplemental Data Figure S2). Therefore, it was not surprising that we observed a 5- to 6-fold lower interstudy variability when comparing ECx_{SGR} rather than $ECx_{biomass}$ (Figure 1 and Table 2).

Even with SGR, there was still an approximately 20-fold variation in chronic Ni toxicity among the various literature tests. Beyond differences in the test methods, intraspecies metal toxicity variability is usually largely attributed to differences in test water chemistry (e.g., pH, DOC concentration, major cation concentrations, etc.) affecting metal bioavailability to aquatic organisms (Paquin et al. 2002). According to the BLM conceptual framework, metal bioavailability (and thus toxicity) is associated with the free metal ion concentration (e.g., Ni^{2+}). Major cations (e.g., Ca^{2+} and Mg^{2+}) decrease metal bioavailability by competing with the free metal ion to bind to biologically sensitive sites (biotic ligands, e.g., membrane transporters), while abiotic ligands (e.g., DOC and Cl^-) compete with these biotic ligands for binding the free metal ions. Therefore, both major cations and abiotic ligands exert protection against metal toxicity. The 4 Ni literature studies were conducted in test waters of varying water chemistry (pH = [6.9–8.6], $[Ca^{2+}]$ = [0.085–1.80 mM], $[Mg^{2+}]$ = [0.033–0.699 mM], DOC = [0–7.1 $mg\ L^{-1}$]). The water chemistry effects can be accounted for by using computational BLMs that have been parameterized for a given metal, organism, and endpoint. Schlekat et al. (2010) and Nys et al. (2016) adjusted the species-sensitivity parameter (to very different values) of an existing *C. dubia* chronic Ni BLM (Equation 2) to fit their $EC50_{biomass}$ and $EC20_{SGR}$ measurements respectively with *L. stagnalis*. The biomass BLM explained the 109-fold variation in $EC50_{biomass}$ within a 23-fold maximum deviation from the prediction. However, as discussed in the earlier paragraph, biomass should not be used when comparing toxicity tests with different test durations. The SGR BLM explained the 22-fold variation in $EC20_{SGR}$ within a factor of 6.6-fold.

The residual interstudy variability after bioavailability-correction could reflect uncertainties of water chemistry (e.g., pH) measurements that are fed into the BLM, and/or may indicate that the BLM is not adequately parameterized. Regarding this latter point, it is important to remember that

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the Ni BLMs used here were developed for *C. dubia* and only their sensitivity parameters were adjusted to fit the very small *L. stagnalis* datasets (respectively a 5-point and 1-point dataset in Schlekat et al. [2010] and in Nys et al. [2016]). The BLM predictions could probably be improved if a new model was developed from a dedicated and larger *L. stagnalis* toxicity dataset. However, it is very unlikely that the BLM could be improved to the point of reconciling the Niyogi et al. (2014) and Crémazy et al. (2018) toxicity data within an acceptable margin of error. Indeed, the current BLM modeling exercise highlighted the surprising 16-fold difference in the $EC20_{SGR}$ measured by Niyogi et al. (2014) and Crémazy et al. (2018). For these 2 studies, the chemistry of the test waters (UM and UBC waters) was quite similar (Table 4), so that the BLMs predicted nearly identical Ni toxicity. More precisely, at the $EC20_{SGR}$, WHAM VII predicted slightly more bioavailable Ni^{2+} in UBC water because of its 3-fold lower DOC concentration. However, this lower complexation-based protection in the UBC was counteracted by a larger competition-based protection due to the 2-fold higher [Ca^{2+}] in UBC water. Yet, despite a similar Ni bioavailability predicted in both studies, the chronic Ni toxicity (Figure 1) and uptake rate (Supplemental Data Figure S1) were much higher in Niyogi et al. (2014) at UM than in Crémazy et al. (2018 and 2019) at UBC, respectively. These observations suggested that Ni bioavailability predictions were somehow erroneous in these studies, which triggered additional tests.

Effects of test waters

With new measurements, we first investigated if the reported lower Ni uptake rates and lower chronic toxicity in the UBC water could be due to lower Ni^{2+} concentrations, in contradiction with the WHAM VII predictions. This lower Ni bioavailability could be due to the presence of an uncharacterized Ni-complexing ligand, or to a DOC in the UBC water with remarkably larger Ni-binding properties. Our FEEM results suggested that despite the lower concentration of DOC in the UBC water, an unknown tyrosine-like component with unusual Ni-binding properties might be reducing the concentration of free ionic Ni^{2+} below that in the UM water at the same total Ni concentration. However, the IET analysis indicated that overall, free ionic Ni^{2+} was not statistically different in either the UBC or the UM water. The similar Ni bioavailability in the 2 waters was further supported by new measurements of Ni uptake rates and chronic toxicity, which showed no significant difference between the snails exposed to the 2 test waters. In these tests, the Ni uptake rates and chronic Ni toxicity were similar to those observed in Crémazy et al. (2019 and 2018, respectively). Altogether, these different measurements suggest that, as expected from their similar chemical compositions, the difference in water chemistry could not explain the large difference in Ni uptake rate and chronic toxicity observed between Niyogi et al. (2014) and Crémazy et al. (2018, 2019).

Snail population and lab effects

We then investigated if UBC snails were inherently more tolerant to Ni than the UM snails. Since these 2 snail cultures had been separated for over a decade, this was a distinct possibility. Over this time, the 2 isolated and small-sized cultures may have experienced significant genetic differentiation (and various levels of genetic impoverishment as compared to wild animals), which can lead to different sensitivities to contaminants (Nowak et al. 2008). Two new toxicity tests concluded that there was a relatively small difference in Ni sensitivity between the snail cultures: a 2.3-fold difference in SGR measured at 250 $\mu g\text{-}L^{-1}$ Ni in UM water (Figure 5D) and a 1.5-fold difference in $EC50_{SGR}$ measured in UBC water (Figure 6A). However, in the former experiment, UBC snails exhibited a higher Ni sensitivity than the UM snails (Figure 5D), while the opposite was found in the latter experiment (Figure 6A). While the UBC snails showed a Ni sensitivity similar to that reported in Crémazy et al. (2018), the UM snails appeared much less sensitive in these new tests than in Niyogi et al. (2014). In fact, using UM snails in either test water, the present study could not reproduce the extremely low chronic Ni toxicity levels (>one order of magnitude lower) reported by Niyogi et al. (2014). Using the same water, snail source population, and methods, we showed that between-lab reproducibility for the assessment of chronic Ni toxicity was quite reasonable (Figures 5A and 5A'). Therefore, potential explanations for these discrepancies are that 1) the UM snail culture has changed since the Niyogi et al. (2014) study, and/or that 2) there were important differences in test conditions and methods. Because there was no apparent reason that could explain the former point, we focus on the latter in the following discussion.

Other potentially important factors

Besides water composition and snail culture, there were methodological differences between the Ni studies, e.g., test animal housing, exposure duration, test food, and snail age and weight at test start (Table 1).

Snails in Schlekat et al. (2010) and Niyogi et al. (2014) (with the highest Ni sensitivity, Table 2 and Figure 1) were exposed in individual containers, while snails in Crémazy et al. (2018) and Nys et al. (2016) (with the lowest Ni sensitivity) were exposed in groups. Multiple snail studies have shown that individual interaction through waterborne substances (pheromones and metabolic chemicals) may affect the growth of conspecifics in both positive and negative manners (Levy et al. 1973; Thomas and Aram 1974; Thomas and Benjamin 1974; Thomas et al. 1975). However, in the present study, there was no remarkable difference in Ni sensitivity between snails exposed in a group (Figure 6A) or in isolation (Figures 5A, 5B, 5C, and 5D).

The test duration varied from 14 to 30 d in the literature studies. While this factor will strongly affect toxicity estimates based on biomass, ECxs derived from SGR are not as dependent on test duration (Bergtold and Dohmen 2011). Indeed, in the present study and in Niyogi et al. (2014),

the test duration had little or no effects on SGR inhibition (Supplemental Data Figure S2), indicating no important change in snail sensitivity throughout the test.

With growth being directly related to energetic resources, the food quality may greatly affect this biological response in toxicity tests. *L. stagnalis* raised on different diets have shown significant variations in their chemical sensitivity (Fidder et al. 2018). For example, Reátegui-Zirena et al. (2016) showed that *L. stagnalis* growth was more inhibited by Cd when snails were fed high-caloric pellets rather than romaine lettuce (although their control growth rate was higher). These food effects have also been shown to be transgenerational in other snails (Plautz et al. 2013), so that parental diet should also be considered. While *L. stagnalis* feeds on plants, carrion, algae, and microorganisms in their natural environment (Fidder et al. 2018), a typical laboratory diet for *L. stagnalis* is romaine lettuce. During the toxicity tests, Crémazy et al. (2018) and Niyogi et al. (2014) used romaine lettuce, Nys et al. (2016) used butterhead lettuce, and Schlekat et al. (2010) used a mix of carrot, sweet potato, and romaine lettuce (Cf., Table 1). Also, only the lettuce in Nys et al. (2016) was of organic quality. Furthermore, newly hatched snails in Crémazy et al. (2018) and Niyogi et al. (2014) were fed with sweet potato prior to the toxicity test. These variations in food quality may significantly influence the bioenergetic resources and tolerance of snails to Ni. However, because the snails in Crémazy et al. (2018) and Niyogi et al. (2014) were raised and tested using the same diet, food does not appear to be an important factor in explaining the large variation between the 2 studies. Yet, it is possible that the lettuces used in these 2 studies had different nutrient contents. Indeed, Hartz et al. (2007) showed that the nutrient composition (e.g., Ca and Mg) of romaine lettuce can vary substantially with its source. Therefore, we believe that this particular factor should be investigated further.

The age and weight of snails at the beginning of the toxicity tests were different in each literature study, ranging from <24 h old (Schlekat et al. 2010) to 3-wk-old (Crémazy et al. 2018). With a life expectancy around 2 y for *L. stagnalis* (Amorim et al. 2019), a 3-wk-age difference may appear insignificant. Yet, critical physiological development occurs at a fast rate during the early life (embryonic and juvenile) stages, and surface area-to-volume ratio decreases progressively. Therefore, newly hatched snails may be significantly more impacted by Ni than 3-wk-old snails, because of lower metabolic capacity for excretion or greater relative surface area for uptake, and/or enhanced sensitivity (e.g., due to functional immaturity of detoxification processes). The age difference between the snails used in Niyogi et al. (2014), Nys et al. (2016) and Schlekat et al. (2010), as opposed to Crémazy et al. (2018) and the present study was relatively small (2 to 3 wk), with the age ranges over the toxicity tests overlapping among studies. Yet, a sensitive early life stage, even if brief, may result in a prolonged lower growth trajectory. The most sensitive reports (Schlekat et al. 2010; Niyogi et al. 2014; Nys et al. 2016) all started with younger animals (<8 d at test onset) than the Crémazy et al. (2018) and the present study (>14 d at test onset). In the 4 published studies, we found no statistical correlation ($p = 0.1211$) between the snail age at the start of toxicity testing and chronic Ni toxicity (Supplemental Data Figure S3). For the present analysis, we compared the snail age with the BLM $EC20_{SGR}$ residuals (differences between observed and predicted $EC20_{SGR}$ in Table 2) to account for the bioavailability effects predicted by the BLM. However, the present analysis was made on a relatively small dataset, with potential confounding factors (such as the factors discussed above) that may have masked the age effect. The possible influence of snail age should be investigated further.

CONCLUSIONS

While the Crémazy et al. (2018) study appears to be an outlier among the various literature studies, its relatively high ECs were reproduced multiple times in the present study, and, as noted earlier, it was an outlier only for Ni and not for other metals. Conversely, while the Niyogi et al. (2014) data could not be reproduced, the very low ECs of that study were supported by other studies (Schlekat et al. 2010; Peters et al. 2014; Nys et al. 2016). Our present investigation does not conclude that one study has better assessed the "intrinsic sensitivity" of *L. stagnalis* to Ni. It simply emphasizes that this sensitivity varies greatly under the influence of abiotic and/or biotic factors that remain to be characterized. Differences in the metal bioavailability or the source of snails could not explain the large Ni toxicity difference between Niyogi et al. (2014) and Crémazy et al. (2018). In future studies, we recommend separately characterizing the possible influence(s) of test animal housing, test duration, diet, and snail age as possible toxicity modifying factors. Taken together, these various parameters may have a significant impact on toxicity test results. Until this variability is resolved/better understood, caution should be used before applying *L. stagnalis* data in a regulatory setting or in a guideline. Our study emphasizes the importance of developing standard methodologies for toxicity testing, especially for highly sensitivity species whose toxicity levels may affect the regulatory guidelines. We also encourage further studies aimed at unraveling the exact mechanism leading to snail growth inhibition during chronic Ni exposure. This knowledge will be valuable for the development of a standardized toxicity test for assessing the growth effects of *L. stagnalis*. Ultimately, this knowledge and these methodological guidelines will help better assess Ni environmental risk to this important freshwater species.

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Data Availability Statement—Details of the statistical analyses (Supplemental Data Tables S1 and S2), BLM calculations (Supplemental Data Table S3) and all the data illustrated in the present paper (Supplemental Data Tables S4 to S8) are available as Supplemental Data. Supplemental Data Figure S1 compares the J_{int} values reported in Niyogi et al. (2014) and Crémazy et al. (2019). Supplemental Data Figure S2 shows the effect of test duration on snail SGR in Exp#3 and Niyogi et al. (2014). Supplemental Data Figure S3 shows the effect of snail age at toxicity test start on EC_{20SGR} BLM residuals (calculated from Table 2) of the four published Ni studies.

SUPPLEMENTAL DATA

Figure S1. Literature short-term Ni uptake flux rates measured in *L. stagnalis* as a function of the dissolved Ni concentration. The Niyogi et al. (2014) data were obtained in 7 h tests in UM water. The Crémazy et al. (2019) data were obtained in 3 h tests in UBC water. Data are presented as mean ± SE (n = 3 in Crémazy et al. (2019) and n = 8 in Niyogi et al. (2014)). Lines are data-connecting lines.

Figure S2. Effect of test duration on the SGR of snails in the chronic Ni tests of Experiment 3 performed at UM (a, b, and c) and in the test performed by Niyogi et al. (2014) (d) (Som Niyogi, University of Saskatchewan, personal communication). Means ± SE (n = 5 for a, b, c, and 9 for d). Results of the 2-way ANOVA are given in each panel (ns: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$) and in more detail in Table S1. The interaction effect is abbreviated Int. Furthermore, statistically significant effects of time on SGR are shown by daggers and statistically significant effects of [Ni] on SGR are shown by different letters (2-way ANOVA with a Tukey's test).

Figure S3. $EC20_{SGR}$ BLM prediction residuals as a function of snail age at the beginning of the toxicity test, in 4 published studies. There was no statistical correlation between snail age and BLM prediction error ($p = 0.1211$).

Table S1. Results of each 2-way ANOVA analysis.

SS are the sum of the squares, DF the degrees of freedom, MS the mean squares and F (DFn, DFd) the F ratios. The % of total variation gives the percentage of the variability due to each main effect and their interaction

Table S2. Regression parameters and 95% confidence intervals of Experiment 4 (Figure 6 in the main manuscript): chronic Ni toxicity of UBC and UM snails in UBC water. The equation used was $y = \max/(1+10\text{^}((\log EC50-X)\cdot H))$, where max is the maximum SGR (in d^{-1}) or w_f (in mg wet wt), EC50 the Ni exposure concentration at 50% effect (in $\mu g\,L^{-1}$), X the dissolved Ni concentration (in $\mu g\,L^{-1}$) and H the Hill slope parameter (unitless)

Table S3. Details of BLM predictions of $EC20_{SGR}$ and $EC50_{biomass}$ values (free Ni^{2+} and total Ni concentration, in $\mu g\,L^{-1}$) for growth inhibition in juvenile *L. stagnalis*. These literature data are given in Figure 1 and Table 2 of the main manuscript. The ECx Ni^{2+} predictions were made with a *Ceriodaphnia dubia* chronic Ni BLM (Equation 2) with adapted sensitivity constants for *L. stagnalis* from Schlekat et al. 2010 ($Q_{50,Lymnaea,biomass}$) and Nys et al. 2016 ($Q_{20,Lymnaea,SGR}$). The ECx Ni predictions were made with WHAM VII (conversion from Ni^{2+} to total Ni concentrations), as detailed in the manuscript. **Note the use of molar units here**.

Table S4. Free Ni^{2+} concentrations measured with an Ion Exchange Technique (IET), in various UBC and UM waters spiked with nominal Ni concentrations. The IET data is illustrated in Figure 2 of the main manuscript. These data were collected at Wilfrid Laurier University in the lab of Dr. DS Smith

Table S5. Internalization fluxes of Ni in UBC snails exposed for 3 h to 2 Ni concentrations in UBC water or in UM water. Each row corresponds to a treatment replicate with one snail (n = 6 replicates per Ni concentration). These data are illustrated in Figure 4 in the main manuscript. These data were collected in Experiment 1, at UBC in the lab of Dr. CM Wood

Table S6. Chronic (7-d) toxicity test with UBC snails exposed to Ni in UBC water and in UM water. Each row corresponds to a treatment replicate with one snail (n = 5 replicates per Ni concentration). The initial and final snail wet weight and SGR are given. Data illustrated in Figures 5A and 5A' (gray bars only) and in Figures 5B and 5B' in the main manuscript. These data were collected in Experiment 2, at UBC in the lab of Dr. CM Wood

Table S7. Three chronic (7, 14 d) toxicity tests with UBC and UM snails exposed to Ni in UBC and/or UM water. Each row corresponds to a treatment replicate with one snail (n = 5 replicates per Ni concentration). The day 0, day 7, and day 14 final snail wet weight was measured and the 7 d and 14 d SGR were calculated. Data are illustrated in Figures 5A and 5A' (gray dashed bars only) and in Figures 5C, 5C', 5D, and 5D' and of the manuscript. These data were collected in Experiment 3, at UM in the lab of Dr. M Grosell

Table S8. Chronic (14 d) toxicity test with UBC and UM snails exposed to Ni in UBC water. Each row corresponds to a treatment with n = 5 snails exposed in bulk. The mean initial and final snail wet weight and SGR are given. Data are illustrated in Figure 5B in the main manuscript. These data were collected in Experiment 4, at UBC in the lab of Wood.

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