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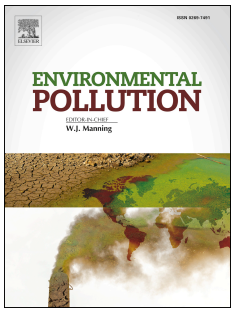

The effects of temperature on nickel bioaccumulation and toxicity in the freshwater snail *Lymnaea stagnalis*

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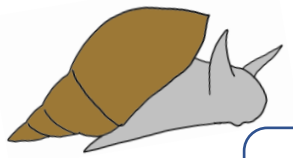
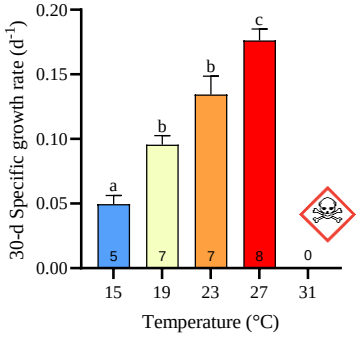


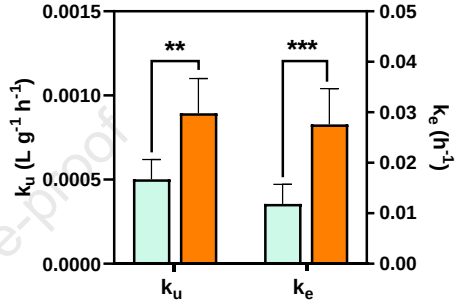
EFFECTS OF ↗ TEMPERATURE ON:

Juvenile growth (15 – 31°C)



- Growth rate ↗ up to 27°C
- Mortality ↗ at 31°C

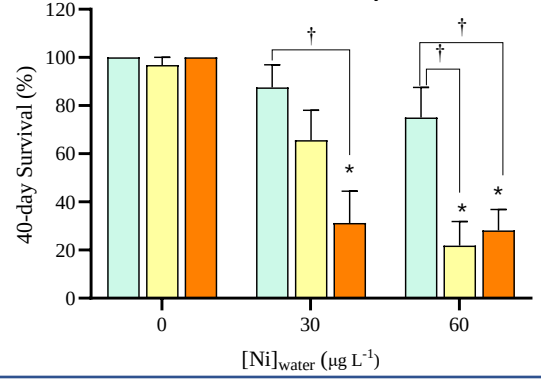
Ni uptake and elimination rates (18 – 26°C)



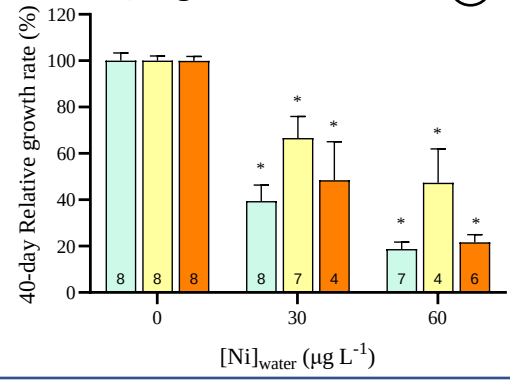
- Ni uptake rate ↗
- Ni elimination rate ↗

Ni bioaccumulation and toxicity (18 – 26°C)

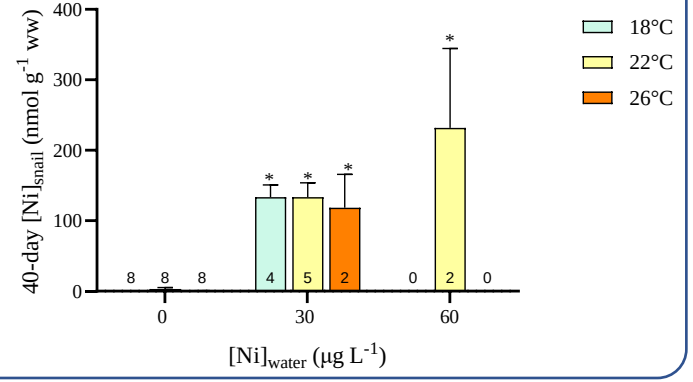
- Ni lethality ↗



- Ni growth inhibition =



- Ni bioaccumulation =



The effects of temperature on nickel bioaccumulation and toxicity in the freshwater snail *Lymnaea stagnalis*

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ABSTRACT

It is well known that temperature can have important effects on the toxicity of metals (and other contaminants) to aquatic organisms. To date, research has mostly focused on thermal effects on acute metal toxicity, and there is a data gap on thermal effects on chronic metal toxicity to sensitive organisms that are particularly relevant to environmental risk assessment. This latter research is especially needed in the context of increased global temperature and heat waves frequency associated with climate change. We investigated temperature effects on chronic nickel (Ni) bioaccumulation and toxicity to the metal-sensitive freshwater snail *Lymnaea stagnalis*. In the laboratory, we conducted a series of experiments with juvenile snails that were pre-acclimated to different temperatures since their embryonic stage. We found that temperature and nickel separately had strong effects on juvenile growth rate and survival. Rising temperature from 18 to 26°C had no noticeable effect on Ni-induced growth inhibition and Ni bioaccumulation in juvenile *L. stagnalis* exposed over 40 days to 0, 30 and 60 µg L^{-1} of dissolved Ni. These results agreed with estimates of Ni uptake and elimination rates (k_u and k_e), which were either unaffected by temperature or increased by similar factors from 18 to 26°C. On the other hand, a temperature increase from 18 to 26°C appeared to exacerbate Ni lethality to juvenile snails in the 40-day toxicity test. This exacerbation might have been due to a combination of factors, including detrimental changes in metabolically available Ni pools and/or to sensitization of the organism under sub-optimal temperatures. Overall, our study shows that thermal effects on metal chronic toxicity are complex, with effects that can be response-specific and not directly related to metal toxicokinetic.

Keywords: temperature, nickel, bioaccumulation, mortality, growth inhibition, uptake rate, elimination rate

INTRODUCTION

Temperature has profound effects on metabolic rates and physiological processes in ectotherms, with potentially strong consequences on contaminants toxicity (Heugens et al., 2001; Sokolova and Lannig, 2008). Currently, metal environmental risk is regulated based on laboratory ecotoxicological studies generally conducted around 20°C and/or at animal optimal temperatures. Yet, wild aquatic organisms experience important seasonal temperature fluctuations in temperate surface waters, along with global warming and more frequent and intense heat waves associated with climate change (Parmesan et al., 2022; Schär et al., 2004). Increased temperatures have most often been associated with increased metal toxicity in aquatic ectotherms (Heugens et al., 2001; Sokolova and Lannig, 2008). Despite a general lack of mechanistic studies, this increased toxicity has often been explained by increased metal uptake rates and/or by increased organism sensitivity under thermal stress (Sokolova and Lannig, 2008). It is recognized that more studies are needed to elucidate the complex temperature-metal interactions for model species in ecotoxicology. Notably, few studies have investigated the influence of temperature on metal-sensitive species in a chronic exposure scenario (as opposed to acute) that are particularly relevant to environmental risk assessment (Pereira et al., 2019, 2017)

One of the most metal-sensitive freshwater organism tested to date is the great pond snail (*Lymnaea stagnalis*). This pulmonate snail is an abundant species of the northern temperate zone, inhabiting shallow low-flowing environments where temperature can change drastically on daily and seasonal bases (Brown, 1979; Kuroda and Abe, 2020). Their habitats may reach near-freezing temperatures in the winter, to nearly 35°C in shallow ponds during summer heat waves (Brown, 1979). The growth rate of juvenile *L. stagnalis* has been shown to be inhibited by exposure to very low concentrations of various trace metals (in $\mu g \cdot L^{-1}$ range), including nickel

(Ni) (Brix et al., 2012, 2011; Niyogi et al., 2014; Nys et al., 2016; Schlekat et al., 2010). Despite its resulting relevance to environmental regulation, there is currently no standardized toxicity testing protocol for growth effects of contaminants with *L. stagnalis*. Notably, chronic Ni toxicity studies have been conducted at water temperatures ranging from 19 to 26°C (Crémazy et al., 2020), with no knowledge on how this temperature variation may affect the toxicity outcome.

The objective of this study was to address the knowledge gaps on temperature effects on metal bioaccumulation and toxicity in the metal-sensitive freshwater snail *L. stagnalis.* In the laboratory, we first characterized the growth of newly hatched *L. stagnalis* over 30 days at various water temperatures ranging from 15 to 31°C. This range covers the optimum temperature for the growth of juvenile *L. stagnalis* (Vaughn, 1953) and the typical to maximum temperatures encountered by this early life stage in their natural environments (Kuroda and Abe, 2020; Salo et al., 2019, 2017). Then, we measured Ni bioaccumulation and effects on snail growth and survival over a 40-day toxicity test at 18, 22 and 26°C. This narrower temperature range was selected to limit snail mortality in the control treatments, and to cover the typical temperature range of toxicity tests for this organism (Crémazy et al., 2020; Niyogi et al., 2014; Nys et al., 2016; Schlekat et al., 2010). Finally, using ^{63}Ni radiotracing, we estimated Ni uptake and elimination rates in juvenile snails at 18 and 26°C, to get better insights into the mechanisms of temperature effects on Ni bioaccumulation and toxicity. Based on previous studies that separately investigated Ni effects and temperature effects on the great pond snail, we predicted that increasing Ni exposure concentration would increase Ni bioaccumulation and decrease growth rate in *L. stagnalis*, and that increasing temperature from 15 to 31°C would increase growth rate up to a critical thermal maximum. Furthermore, based on previous studies on temperature and metal interactions, we anticipated that increasing temperature from 18 to 26°C

would increase Ni bioaccumulation (by increasing Ni uptake rate faster than elimination rate) and Ni toxicity (mainly by increasing Ni bioaccumulation) in *L. stagnalis*.

MATERIALS AND METHODS

Snail culture and temperature acclimation

Egg masses of *Lymnaea stagnalis* were obtained from an in-house culture at the University of British Columbia (Vancouver, BC) and snails were cultured at the University of New Brunswick (UNB) (Saint John, NB) for about a year prior to the experiments. They were cultured at 21 ± 1°C, under a 12D:12L light cycle, in aerated UNB dechlorinated tap water supplemented with salts. Final culture water composition was (mean ± SD (n)): pH = 7.2 ± 0.2 (264), dissolved organic carbon (DOC) = 1.4 ± 0.5 $mg \cdot L^{-1}$ (99), [Ca] = 40.08 ± 0.03 $mg \cdot L^{-1}$ (3), [Mg] = 4.37 ± 0.006 $mg \cdot L^{-1}$ (3), [Na] = 26.67 ± 0.02 $mg \cdot L^{-1}$ (3), [K] = 3.13 ± 0.006 $mg \cdot L^{-1}$ (3), [Cl] = 69.84 ± 0.26 $mg \cdot L^{-1}$ (3), and $[SO_4]$ = 72 $mg \cdot L^{-1}$ (nominal). The culture was kept under static renewal conditions and was fed with romaine lettuce (adult snails) or a mix of butterhead lettuce and sweet potato (juvenile snails).

Experimental snails were acclimated at the future test temperature prior to each experiment. Briefly, freshly laid egg masses (<2d after laying) were collected and acclimated to test temperatures in 1-L plastic containers filled with aerated culture water and immersed in different water baths brought to the targeted experimental temperatures, using submersible heaters (Marina) or chillers (DBM-250, 1/3 HP Arctica, JBJ Lighting). When juvenile snails of a few weeks old were required (in Ni toxicokinetic tests), hatched snails were fed with a butterhead lettuce diet (*ad libitum*) during temperature acclimation.

Growth test at different temperatures

The effect of temperature on juvenile snail growth was characterized with a 30-day growth experiment at five water temperatures (15, 19, 23, 27 and 31°C) with newly hatched snails. There were n = 8 replicates per temperature treatment, with a replicate consisting of an individual snail in 250 mL of aerated culture water in a 350 mL polypropylene container in a temperature-controlled water bath.

During the growth experiment, snails were fed *ad libitum* with rinsed butterhead lettuce. Lettuce and test water were renewed three times per week. Snails were weighed (blotted-dry wet weigh, ± 0.1 mg, ME235P, Sartorius) on days 0, 10, 20 and 30, and survival was monitored daily. Every 10 days, water samples were collected (fresh and 2 to 3-d old water), filtered (<0.45µm, PES membrane syringe filter, Cytiva Whatman™) and stored at 4°C until chemical analyses (DOC, Ni, Ca, K, Na, and Mg concentrations). Water temperature was measured daily (Traceable Model 4378, ITM) and pH was measured every 10 days on fresh and 2 to 3-d old water.

Nickel bioaccumulation and toxicity at different temperatures

Nickel effects on the growth and survival of newly hatched snails were assessed over 40 days at 18, 22 or 26°C. Test Ni nominal concentrations were 0 (control), 30 and 60 $\mu g \cdot L^{-1}$. The test solutions were prepared by adding $NiCl_2 \bullet 6H_2O$ (Fisher Chemical) in culture water and adjusting to test temperatures two days before use. There were n = 8 replicates per temperature/Ni treatment, with a replicate consisting of four snails in 400 mL of aerated test water in a 500 mL polypropylene container randomly positioned in a temperature-controlled water bath.

At the beginning of the experiment, newly hatched snails (<24h old) were transferred from their temperature acclimation containers into the various test containers. For the duration of the experiment, test water was renewed completely every three days and snails were fed daily *ad libitum* with rinsed butterhead lettuce. Snails were weighed (blotted-dry wet weigh, ± 0.1 mg, ME235P, Sartorius) on days 0, 10, 20, 30 and 40, and mortality was monitored daily. Filtered water samples for chemical analyses were collected and stored exactly as described in the previous experiment. Water temperature was measured daily (Traceable Model 4378, ITM) and pH was measured every 10 days on fresh and 3-d old water. At the end of the experiment, snails were euthanized at -20°C. For Ni tissue analyses, the soft tissues of the surviving snails of each test replicate were dissected, pooled together and digested at 60°C overnight in concentrated nitric acid (HNO_3, trace metal grade, Fisher Chemical), then at room temperature for 24 h in concentrated hydrogen peroxide (H_2O_2, ultrapure grade for trace metal analysis, VWR). The volumetric ratio of HNO_3 to H_2O_2 was 5:4.

Nickel uptake and elimination at different temperatures

Nickel uptake and elimination in juvenile snails were measured at 18 and 26°C over a 72-h Ni exposure phase (60 $\mu g \cdot L^{-1}$ of Ni) followed by a 96-h elimination phase (in Ni-free water). There were n = 6 replicates per temperature and sampling time (72 h of Ni exposure, then 24, 48 and 96 h of elimination). Each replicate consisted of an individual snail in a 50 mL polypropylene tube with 40 mL of aerated test water in a temperature-controlled water bath. Test waters for the loading phase were prepared by adding $NiCl_2 \cdot 6H_2O$ (Fisher Chemical) and 0.2 $mCi \cdot mL^{-1}$ of ^{63}Ni (as $NiCl_2$, Eckert & Ziegler) in the culture water and bringing it to the desired temperature 24 h prior to the experiment. This toxicokinetic experiment was conducted on

juvenile snails of a few weeks old to obtain enough biological tissues for ^{63}Ni analysis. As the 18°C and 26°C snails grew at widely different rates during the temperature acclimation period, we conducted two experiments to decipher weight-driven *vs.* age-driven effects on Ni toxicokinetics. Indeed, we conducted an experiment with snails of the same age but different weights (40 d old; 0.053 – 0.19 g whole body weight) and another experiment with snails of the weight but different ages (0.35 ± 0.01 g whole body weight; 40 – 50 d old).

At the beginning of each toxicokinetic experiment, snails were transferred from their temperature acclimation containers to their test containers. Snails were fed with rinsed butterhead lettuce (*ad libitum*) during the experiment. After 72 h of Ni exposure, snails were moved to Ni-free waters and were allowed to eliminate their Ni content over a 96-h period. Water was renewed daily during the Ni exposure phase, then twice per day during the elimination phase to limit ^{63}Ni re-absorption. Filtered water samples (< 0.45µm, PES membrane syringe filter, Cytiva Whatman) were collected daily for ^{63}Ni analyses and once at the beginning of the test for DOC, Ni and major cation concentrations. Water temperature was measured daily (Traceable Model 4378, ITM). For ^{63}Ni tissue analysis, six replicated snails were collected after 72 h of Ni exposure then after 24, 48 and 96 h of elimination. Collected snails were rinsed with dechlorinated water, exposed for 5 min to 20 mL of 1 mM EDTA solution (ACS grade, Fisher Chemical) to desorb Ni adsorbed on snail surface, rinsed again with dechlorinated water, blotted dried on paper towel and frozen at -20°C. For ^{63}Ni analyses, soft tissues were dissected, weighed (ME235P, Sartorius), and digested in 50% v/v HNO_3 (ACS grade, Fisher Chemical) at 65°C for two days, then in concentrated H_2O_2 (Ultrapure for trace metal analysis, VWR) at room temperature for 24 h.

178 **Chemical analyses**

179 Concentrations of inorganic elements in water samples were determined by inductively
180 coupled plasma – atomic emission spectrometry (ICP-AES; Thermo Agilent Dual View 5110).
181 The Ni, Ca, Mg, K, and Na detection limits were 3.0 $\mu g \cdot L^{-1}$, 3.0 $\mu g \cdot L^{-1}$, 0.1 $\mu g \cdot L^{-1}$, 2.0 $\mu g \cdot L^{-1}$
182 and 0.3 $\mu g \cdot L^{-1}$, respectively. Instrument calibration was checked with a certified reference water
183 (SCP Science, 140-025-031), and instrument signal drift was corrected by analyzing blanks and
184 standards every 12 samples. Tissue concentrations of Ni were analyzed by inductively coupled
185 plasma – mass spectrometry (ICP-MS, Thermo Scientific iCAP RQ) (detection limit of 0.003
186 $\mu g \cdot L^{-1}$), along with digestion blanks and certified reference material (TORT-2; National
187 Research Council Canada) to check the quality of the digestion and analysis. Radioactivity from
188 ^{63}Ni in water and tissue samples was determined by liquid scintillation counting (LS 6000 Multi-
189 Purpose, Beckman Coulter), after mixing with scintillation cocktail (water samples in Optiphase
190 HiSafe and acid digests in UltimaGold AB, PerkinElmer). Quenching was constant in water
191 analyses, but was corrected in tissue analysis using different volumes of acid digests and the
192 external standard method to correct for the counting efficiency difference with that in the water.
193 The DOC concentrations in filtered water samples were analyzed with a total organic carbon
194 analyzer (Shimadzu TOC-L) using the non-purgeable organic carbon method.
195
196 **Data analyses**
197 *Data calculations*
198 The specific growth rates (SGR, in d^{-1}) of newly hatched snails were calculated every ten
199 days in the growth and chronic Ni toxicity tests. Since juvenile *L. stagnalis* grow exponentially
200 with time, the SGR of each individual snail was calculated using the following equation:
201

$$SGR = \frac{\ln(w_{final}) - \ln(w_{initial})}{t} \qquad \text{Eq 1}$$

where $w_{initial}$ and w_{final} are, respectively, the mean initial (at day 0) and mean final whole body weights (at days 10, 20, 30 or 40) (in g) for a given container replicate, and *t* is the growth period (in d) (i.e., 10, 20, 30 or 40 days).

Furthermore, for the Ni toxicity test only, the SGR value of each replicate was normalized by the mean SGR in the control treatments of the same temperature ($SGR_{control}$), to obtain the relative growth rate (RGR, in %):

$$RGR = \left(\frac{SGR}{SGR_{control}}\right) \times 100 \qquad \text{Eq 2}$$

Mean percent snail survival was calculated for each treatment of the Ni toxicity test at 10, 20, 30 and 40 days of Ni exposure.

We used a one-compartment toxicokinetic model to calculate Ni uptake rate constants (k_u, in L g^{-1} h^{-1}) and Ni elimination rate constants (k_e, in h^{-1}) with the uptake and elimination data, as described in Pereira et al. (2019). The k_e parameters were first determined by non-linear regression of the mean Ni tissue concentration as a function of time, using this first-order elimination kinetics equation:

$$[Ni]_{snail,t} = [Ni]_{snail,0} \times exp^{(-k_e \times t)} \qquad \text{Eq 3}$$

Where $[Ni]_{snail,\,t}$ is the mean soft tissue Ni concentration (in nmol·g^{-1}) after a given elimination time *t* (in h, t = 0, 24, 48 and 96 h), and $[Ni]_{snail,\,0}$ is the mean soft tissue Ni concentration (in nmol·g^{-1}) at the start of the elimination phase (i.e. at t = 0 h).

The k_u parameters were then calculated for each snail replicate, with the 72-h Ni exposure data and the previously determined mean k_e estimates, using the following equation:

$$[Ni]_{snail,t} = [Ni]_{water} \times \frac{k_u}{k_e} \times (1 - exp^{(-k_e \times t)}) \qquad \text{Eq 4}$$

Where $[Ni]_{snail,\,t}$ is the soft tissue Ni concentration (in nmol·g^{-1}) after *t* = 72 h of Ni exposure, and $[Ni]_{water}$ is the water concentration of Ni (in nmol·L^{-1}) during the Ni exposure phase.

Statistical analyses

Data were analyzed in GraphPad Prism 9.3.1, with a significance threshold of 0.05. A One-Way ANOVA and Tukey's *post hoc* test was used to analyze the effects of water temperature on SGR at each exposure duration (10, 20 and 30 days) of the growth test. Two-Way ANOVAs and Tukey's *post hoc* test were used to analyze the effects of Ni exposure and temperature on precent snail survival, SGR, RGR and 40-day Ni bioaccumulation, at each exposure duration (10, 20, 30 and 40 days) of the Ni toxicity test. Estimated mean Ni uptake and elimination rate constants (k_u and k_e) were compared at each temperature (18 and 26°C) for each snail group (same-age and same-weight snails) using unpaired t-tests for k_u and extra-sum-of-square F-tests for k_e.

RESULTS

Juvenile snail growth at various temperatures

Over the 30-day growth experiment, the water physico-chemistry parameters (mean ± SD (n)) for all treatments was: pH = 7.33 ± 0.06 (105), [DOC] = 1.82 ± 0.36 mg·L^{-1} (21), [Ca] = 44.92 ± 5.85 mg·L^{-1} (119), [K] = 5.09 ± 3.15 mg·L^{-1} (119), [Mg] = 5.12 ± 0.44 mg·L^{-1} (119), and [Na] = 37.62 ± 3.47 mg·L^{-1} (119) (Table SI.1 of the supplemental information). The water temperatures remained within ± 1°C of the target temperatures throughout the experiment. The overall mean dissolved Ni concentration in the test water was < 3.0 µg·L^{-1} (analytical detection limit) (n=119). Whole-body weight of snails are given in Table SI. 2 of the supplemental information.

Temperature had a significant effect on SGR ($p < 0.001$, one-way ANOVA): at each exposure time, SGR increased with increasing temperature, up to 27°C (Fig. 1). At 31°C, snail growth and survival were dramatically decreased at all exposure times, with 100% mortality observed by day 20 (Fig. 1B). Increased mortality was also apparent at 15°C, with only 63% snail survival observed by day 30 at this temperature, compared to the 88 – 100% survival observed at 19 – 27°C (Fig. 1C).

Nickel bioaccumulation and toxicity at various temperatures

Over the 40-day Ni toxicity experiment, the water quality parameters (mean ± SD (n)) from all treatments were: pH = 7.09 ± 0.29 (126), [DOC] = 1.52 ± 0.38 mg·L^{-1} (48), [Ca] = 39.70 ± 0.77 mg·L^{-1} (144), [K] = 3.48 ± 1.16 mg·L^{-1} (144), [Mg] = 4.18 ± 0.11 mg·L^{-1} (144) and [Na] = 34.25 ± 0.48 mg·L^{-1} (144) (Table SI.3 of the supplemental information). Mean dissolved Ni concentrations and temperatures were within 90% and 99% of the 30 and 60 µg L^{-1} target values,

respectively (Table SI.3 of the supplemental information). Snail counts and weights are given in Table SI.4 of the supplemental information.

Temperature did not affect survival of snails in the Ni-free (control) water over the course of the 40-day experiment (Figure 2). Nickel became lethal to snails after 30 days of Ni exposure ($p < 0.0001$, two-way ANOVA), and lethality was exacerbated by an increase in water temperature at both 30 and 40 days of Ni exposure (two-way ANOVA interaction term: $p < 0.0001$ and < 0.01 at day 30 and 40 respectively) (Fig. 2C and 2D).

At each test duration, both nickel and temperature had significant effects on snail SGR, and a significant interaction was observed between these two factors ($p < 0.05$, two-way ANOVA) (Figure 3). In the control (Ni-free) treatments, SGR generally increased with temperature from 18 to 26°C, in agreement with the previous growth experiment (cf. Fig. 2). This positive thermal effect on SGR was virtually erased in the presence of Ni exposure, which had a negative effect on SGR (Fig. 3). Indeed, for a given Ni concentration and exposure duration, there was no difference in SGR between the three temperatures (Fig. 3).

Growth rates are given relative to the control growth rate (RGR) in Figure 4, to allow a better assessment of thermal effects on Ni toxicity. With RGR, we also observed an inhibition of snail growth with Ni exposure at each temperature ($p < 0.0001$, two-way ANOVA) (Figure 4), but there was no interaction between temperature and Ni effects ($p > 0.1$ at all days, two-way ANOVA). Snails exposed to Ni at 22°C almost always had the highest mean RGR compared to the other two temperatures, but a small temperature effect was only statistically significant at day 20 between 22 and 26°C ($p = 0.040$, Tukey test) (Fig. 4B).

At the end of this 40-day toxicity test, significant Ni bioaccumulation was detected in snail tissue as [Ni] increased in the water (Figure 5). There was no detectable temperature effect on

this Ni bioaccumulation. Note that there was limited statistical power for this analysis, as elevated test mortalities and difficulties extracting tissues from very small snails led to very small n values, and even completely missing 18 and 26°C data at the highest Ni treatment.

Nickel uptake and elimination at various temperatures

The mean dissolved Ni concentrations and water temperatures were on average within 83% and 99% of target values, respectively (Table SI.5 in the supplemental information). After 72 h of Ni exposure to 60 µg L^{-1}, 18°C snails of the same age (40-d old; 0.053 – 0.19 g) accumulated slightly more Ni (~1.5x increase) than the 26°C snails ($p = 0.0122$, t-test) (Figure 6A). On the other hand, there was no difference in the levels of Ni accumulated by snails of the same weight (0.35 ± 0.01 g; 40 – 50-d old) ($p = 0.365$, t-test) (Figure 6B). After 96-h in Ni-free water, about 95% and 80% of accumulated Ni was eliminated from same-age snails (Fig. 6A) and from same-weight snails (Fig. 6B), respectively.

The uptake and elimination rate constants derived from these data are presented in Figure 7 and in Table SI.6 (supplemental information). For snails of the same age (Fig. 7A), increasing temperature from 18 to 26°C did not lead to significant changes in k_u estimate ($p = 0.196$, t-test) and k_e estimate ($p = 0.07$, F-test). For snails of the same weight (Fig. 7B), increasing temperature from 18 to 26°C led to a 1.8-fold increase in k_u estimate ($p = 0.0017$, t-test) and a 2.3-fold increase in k_e estimate ($p < 0.0001$, F-test).

DISCUSSION

We found a profound effect of temperature (15 – 31°C) on juvenile growth rate and survival, with an optimum temperature for growth around 27°C and acute thermal stress

317 occurring at 31°C. Raising temperature from 18 to 26°C exacerbated Ni lethality to juvenile *L.*
318 *stagnalis* in a 40-day Ni toxicity test. On the other hand, this temperature change had no
319 observable effect on Ni-induced growth inhibition or Ni bioaccumulation. These latter lack of
320 temperature effects agreed with Ni uptake and elimination kinetics data, that predicted similar Ni
321 bioaccumulation in this temperature range.
322
323 **Nickel effects on juvenile *L. stagnalis***
324 In our study, Ni inhibited growth of juvenile *L. stagnalis*, with growth reduction observed
325 after only 10 days of exposure to 30 µg Ni $\cdot L^{-1}$ at each tested temperature (18, 22 and 26°C) (cf.
326 Fig. 3 and 4). This effect was relatively constant by day 20, agreeing with Niyogi et al. (2014)
327 that Ni toxicity testing with *L. stagnalis* may be conducted within 3 weeks. Effects on snail
328 survival were also observed, with Ni becoming lethal to snails after 30 days of exposure to 30 µg
329 Ni $\cdot L^{-1}$ in the 26°C snails (cf. Fig. 2). These findings agree with studies that have reported *L.*
330 *stagnalis* as a very sensitive species to chronic Ni exposure (Schlekat *et al*., 2010; Niyogi *et al*.,
331 2014; Nys *et al*., 2016). Indeed, our data suggests a EC50 on growth around 30 µg Ni $\cdot L^{-1}$,
332 similar to what Niyogi *et al*. (2014) observed at 25°C with similar water composition (after
333 converting their biomass-based EC50 to SGR-based EC50, as reported in Crémazy et al. (2020)).
334 On the other hand, our findings contrast with the high EC values reported by Crémazy *et al*.
335 (2020, 2018) at 25°C using similar water chemistry (i.e., EC50 based on SGR of 220 µg$\cdot L^{-1}$).
336 The lower snail sensitivity observed in this latter study might be due to the use of older, more
337 tolerant snails (2-3-week old), compared to the present study (24-h old snails) and the Niyogi *et*
338 *al*. (2014) study (7-8-d old snails), as suggested by Crémazy et al. (2020).
339
340 **Temperature effects on growth and survival of juvenile snails**

In uncontaminated water, temperature (15 – 31°C) strongly affected growth and survival of *L. stagnalis*, as expected for an ectotherm organism (Schulte et al., 2011). Over the 30-day growth experiment, snail survival was reduced to 60% at 15°C and to 0% at 31°C, compared to the 88-100% survival observed at 19, 23 and 27°C (cf. Fig 1). Overall, these observations agree with previous reports of reduced survival at around 12°C (Blehrádek, 1935) and above 28-30°C for juvenile *L. stagnalis* (Moore et al., 2021; Salo et al., 2019; Van der Schalie and Berry, 1973). In our study, increasing temperature from 15 to 27°C increased growth rate, with a 30-day specific growth rate at 27°C (0.18 d^{-1}) about 3.6x higher than at 15°C (0.05 d^{-1}) (cf. Figure 1). Since juvenile growth of *L. stagnalis* is exponential, such increase translates into a large ~50-fold increase of snail weight from 15 to at 27°C. Similar thermal effects on *L. stagnalis* growth have been reported (Van der Schalie and Berry, 1973; Vaughn, 1953), and have been associated with increased feeding rates (Vaughn, 1953). Growth was sharply reduced (along with survival) at 31°C (cf. Fig. 1), and this reduction has been associated with reduced feeding activity as a consequence of oxygen limitation in ectotherms above their suitable temperature (Jutfelt et al., 2021). Thus, while natural habitats of the great pond snail may occasionally exceed 30°C during summer heat waves (Brown, 1979), we can assume that long-term exposure to such temperatures will lead to significant population decline for this species. The thermal growth optimum ~ 27°C observed in our study was only slightly lower than the critical thermal maximum, conforming with the typical shape of ectotherms thermal performance curves where rates increase up to a maximum then steeply decline (as high temperatures lead to catastrophic failure of biological processes) (Schulte et al., 2011). Note that lower growth optima around 24°C have been reported for *L. stagnalis* in other studies (Van der Schalie and Berry, 1973; Vaughn, 1953). For a given species, such measurement difference in the breadth of the temperature optimum/tolerance zone

might be due to various factors, such as population, age and life history (e.g. acclimation vs. no acclimation) (Axenov-Gribanov et al., 2015; Schulte et al., 2011).

Temperature effects on Ni bioaccumulation in juvenile snails

Our data suggests that temperature has limited effects on chronic Ni tissue bioaccumulation in *L. stagnalis*, under the conditions tested in our study. When using snails of the same weight (~0.35 g wet tissue weight) that were pre-acclimated to 18°C or 26°C, both k_u and k_e estimates were about 2-fold higher at 26°C than at 18°C (cf. Fig. 7B). In this experiment, the slightly older age of the 18°C snails (50-d old vs. 40-d old at 26°C) was due to their slower growth (they required 10 additional days to reach a similar weight to the 26°C snails). It is unlikely that this relatively small age difference confounded the temperature effect observed on k_u and k_e estimates. The observation of greater uptake/elimination rates at warmer temperature conforms with the prediction of the Arrhenius law, describing the thermal dependency of chemical reaction rates (Schulte et al., 2011). This observation is also in relatively good agreement with previous studies. Indeed, a review by Sokolova and Lannig (2008) on aquatic ectotherms showed that an increase in temperature increased metal uptake (or accumulation) and elimination in 85% (n=45) and 26% (n=35) of the studies. For elimination, the most common observation was an absence of temperature effect (Sokolova and Lannig, 2008).

Interestingly, we observed an absence of thermal effect on k_u and k_e estimated with snails of the same age (40-d old) but different weights (cf. Fig. 7A). For this experiment, it is possible that the thermal effect on Ni toxicokinetic was confounded by a size effect, as 18°C snails were about 3.5-fold smaller than the 26°C snails. Indeed, smaller organisms have a larger surface-to-volume ratio, typically leading to faster exchange with the surrounding water (Grosell et al.,

2002). In any case, the data from both toxicokinetics experiments predict a similar Ni tissue accumulation at 18 and 26 °C, which is indeed what was observed at the end of the 40-d Ni toxicity test (cf. Fig. 5).

Temperature effects on Ni toxicity in juvenile snails

Thermal effects on Ni toxicity to juvenile *L. stagnalis* was response dependent. Perhaps most importantly from a risk assessment perspective, our study showed limited thermal effect (18 – 26°C) on the most sensitive response to chronic Ni exposure: growth inhibition (cf. Figures 3 and 4). In accordance with the critical body concentration hypothesis, this lack of thermal effect might be due to similar Ni tissue concentration at both temperatures (as previously discussed). Indeed, this theory states that the internal concentration of a chemical determines the toxicity to the organism (Vijver et al., 2004). A lack of thermal effect on metal toxicity has been shown in previous studies, although it is not the most common observation. In their review, Sokolova and Lannig (2008) showed that an increase in temperature increased toxicity in 80% of the studies and led to no change in 15% of the studies (n=115). However, the majority of these latter studies were acute toxicity studies where test duration might be too short for elimination/detoxification processes to play an important role in the toxicity outcome. Furthermore, most of these studies reviewed by Sokolova and Lannig (2008) did not pre-acclimate their test organisms to the various temperature treatments. In a recent chronic study with temperature pre-acclimation, Pereira et al. (2017) showed modest and counter-intuitive thermal effects on Ni chronic toxicity with *Daphnia magna*: at the standard temperature of 20°C, toxicity was 1.3 times lower than at 15°C and 1.6 times higher than at 25°C.

Contrary to Ni-induced growth inhibition, Ni lethality increased with increasing temperature in our study (cf. Fig 2). Notably, percent mortality was ~2.5-fold greater at 26°C than at 18°C for snails exposed for 40 days to 60 $\mu g \cdot L^{-1}$ of Ni (cf. Fig. 2D). The difference in thermal effects between the growth and survival data suggests different Ni modes of action for these two biological responses, which remain to be unravelled for *L. stagnalis* (Niyogi et al., 2014). While conforming with most metal toxicity studies (Heugens et al., 2001; Sokolova and Lannig, 2008), this increased Ni lethality with increasing temperature disagrees with our Ni bioaccumulation/toxicokinetics data (cf. Fig. 5 – 7) with regards to the critical body concentration theory (Vijver et al., 2004). Yet, this relationship between internal concentration and toxicity is not always observed (Heugens et al., 2003; Pereira et al., 2019; Rainbow, 2007; Vijver et al., 2004). For example, Heugens *et al*. (2003) reported a decrease in internal threshold Cd concentration estimates in daphnids from 10 to 26°C, meaning that less Cd accumulation was needed to induce lethal effects at 26°C than at 10°C. Other factors may explain elevated metal toxicity with elevated temperatures. Notably, increased temperature may detrimentally change the distribution between detoxified and metabolically-available metal pools, without changing the total tissue concentration (Rainbow, 2007). For example, this scenario could arise if changes in the production rate of metal-sequestering proteins (e.g., metallothionein) do not closely follow changes in metal uptake rates. Finally, as reviewed by Sokova and Lanning (2004), elevated temperature and metal exposure can act individually and jointly to affect aerobic metabolism in aquatic ectotherms (e.g. elevating maintenance costs, reducing mitochondrial efficiency, increasing ROS production), which can lead to i) increase metal susceptibility/sensitivity and ii) decrease thermal tolerance of the organism. It is possible that 26°C was a sub-optimal temperature and thus sensitized our snails to Ni lethality, even though this temperature did not

have direct negative effects on growth and survival of our test organims. Indeed, several studies on *L. stagnalis* have shown that 24-25°C exposures had negative impacts on reproduction, immune function and contaminants sensitivity (Leicht et al., 2013; Leicht and Seppälä, 2019; Salo et al., 2017; Van der Schalie and Berry, 1973). Notably, Salo et al. (2017) showed that micropollutants (a mixture of mainly pharmaceuticals and pesticides) and heat wave (8 days at 23.5°C) had additive effects on *L. stagnalis* fecundity. Conversely, the increased mortality in the Ni/high temperature treatments might partially be due to decreased thermal tolerance in metal-exposed organisms (Negri and Hoogenboom, 2011; Sokolova and Lannig, 2008). This reduction in the critical thermal limit can be explained by the above-mentioned effects of metals on the aerobic metabolic capacities of aquatic ectotherms (e.g. decreased aerobic scope due to energy costs for metal detoxification) (Couture and Kumar, 2003). Overall, various factors might explain the interaction between temperature and nickel on the survival rate of *L. stagnalis* in our study.

CONCLUSION

Our study is the first to investigate the effects of temperature on Ni toxicity to the metal-sensitive species *Lymnaea stagnalis*. As expected for this ectothermic animal, we observed a profound effect of temperature on juvenile *L. stagnalis* growth and survival, with increasing growth rate up to 27°C followed by a steep decline in growth and survival rates. The effects of temperature on Ni chronic toxicity were not as straightforward, as increasing temperature increased Ni lethality but did not affect Ni-induced growth inhibition to juvenile snails. The latter lack of effect could be attributed to the apparent absence of temperature effect on Ni tissue concentration, due to counter-acting effects of temperature on Ni uptake and elimination rates.

455 However, increased Ni lethality with increased temperature might be due to a combination of
456 detrimental changes in metabolically available metal pools and/or to increased sensitivity of
457 snails to nickel and/or to reduced thermal tolerance of nickel-exposed snails. Additional research
458 is needed to characterize the extent and the mechanisms by which temperature and metal stress
459 interact within metal-sensitive aquatic organisms like the great pond snail. This knowledge is
460 especially important to adequately assess the environmental risk of metals in the context of
461 climate change. Indeed, it is predicted that *L. stagnalis* will experience increasingly frequent heat
462 waves in its natural habitats, which may increase its susceptibility to metals (Schär et al., 2004).
463

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470 **COMPETING INTERESTS**

471 The authors declare no competing interests.
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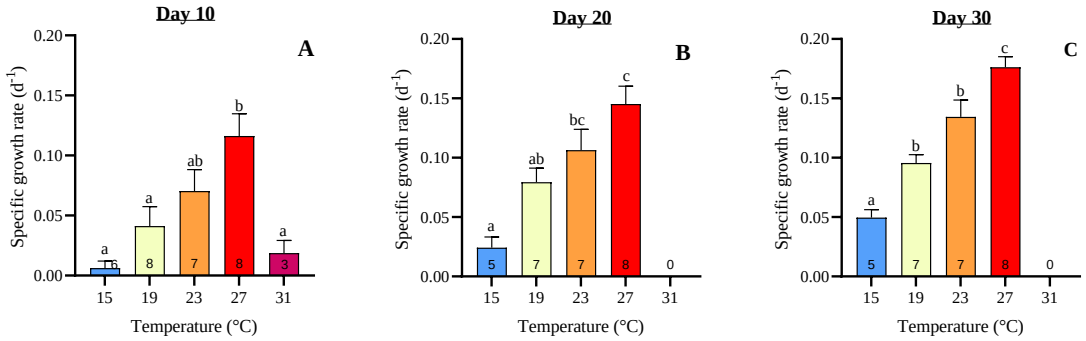

Figure 1: Specific growth rate of *L. stagnalis* after **A)** 10, **B)** 20 and **C)** 30 days in Ni-free water at 15, 19, 23, 27 or 31°C. Data are represented as mean ± SEM (*n* values given at the bottom of each bar). Different letters indicate significant differences between temperature groups ($p < 0.05$, one way ANOVA with Tukey test).

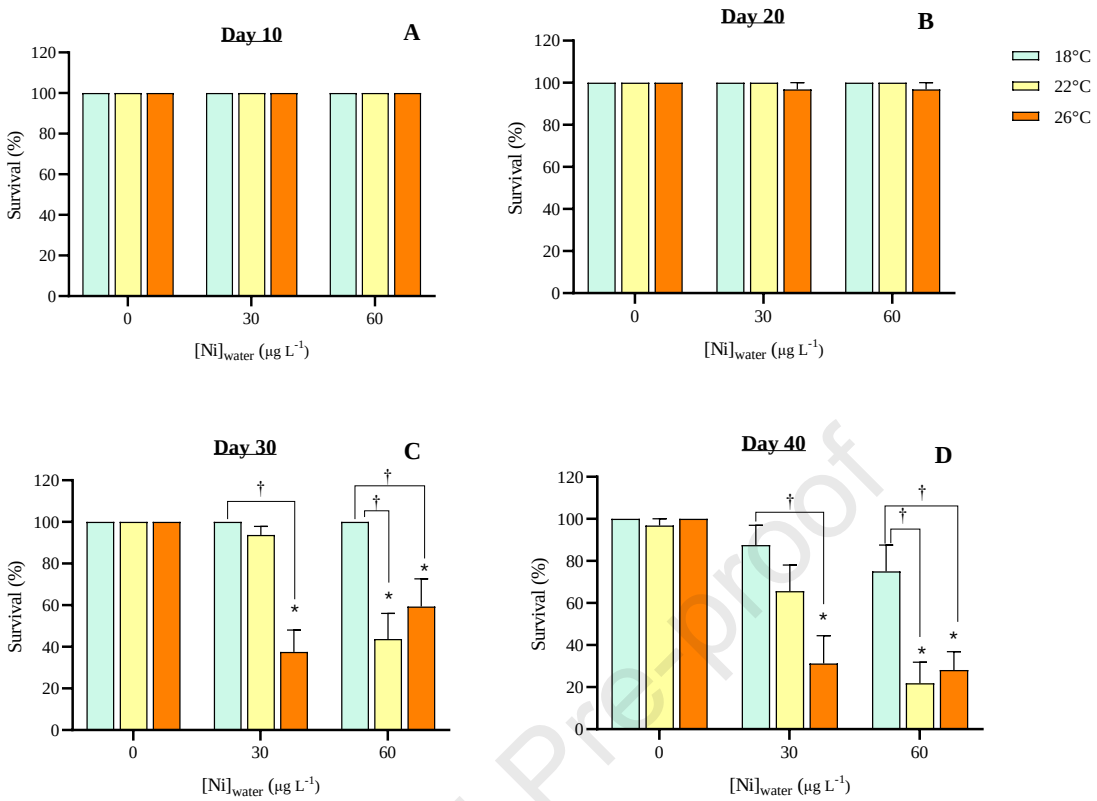

Figure 2: Percent survival of *L. stagnalis* after A) 10, B) 20, C) 30 and D) 40 days of Ni exposure at 18, 22 or 26°C, as a function of nominal Ni water concentration. Data are presented as mean ± SEM (n = 8). Asterisks indicate differences of Ni treatments compared to the control (0 µg·L^{-1}), and daggers indicate differences between temperature groups for a given Ni treatment ($p < 0.05$, two-way ANOVA with Tukey test).

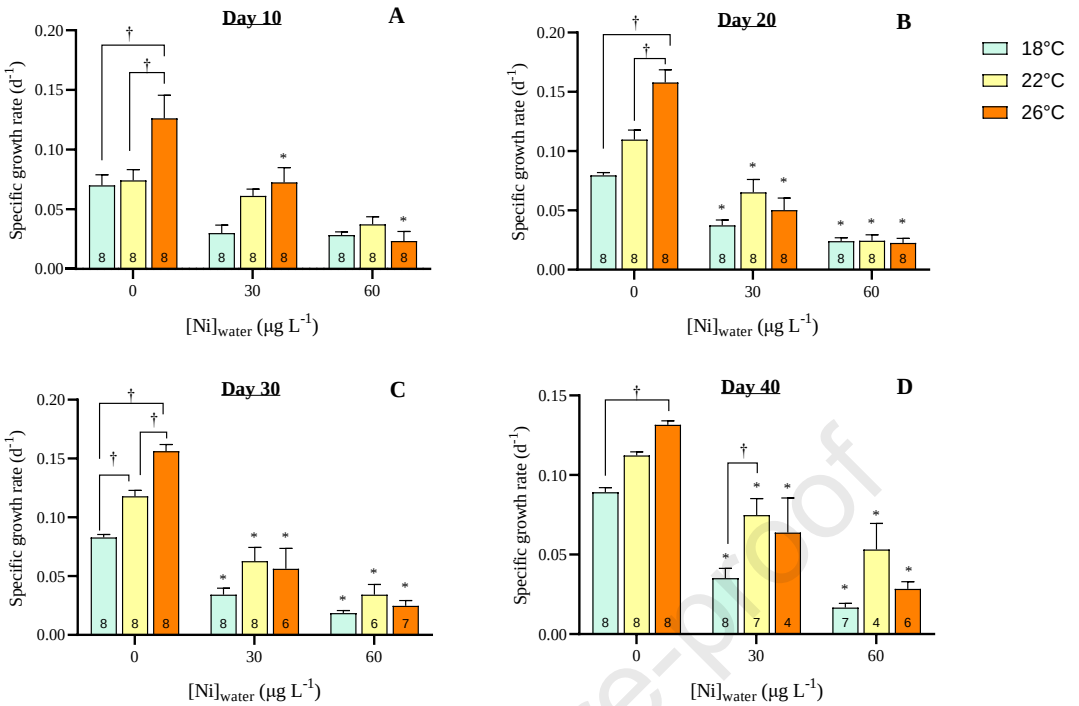

Figure 3: Specific growth rate of *L. stagnalis* after A) 10, B) 20, C) 30 and D) 40 days of Ni exposure at 18, 22 or 26°C, as a function of nominal Ni water concentration. Data are presented as mean ± SEM (*n* values given at the bottom of each bar). Asterisks indicate differences of Ni treatments compared to the control (0 µg·L^{-1}) for a given temperature, and daggers indicate significant between temperature groups for a given Ni treatment ($p < 0.05$, two-way ANOVA with Tukey test).

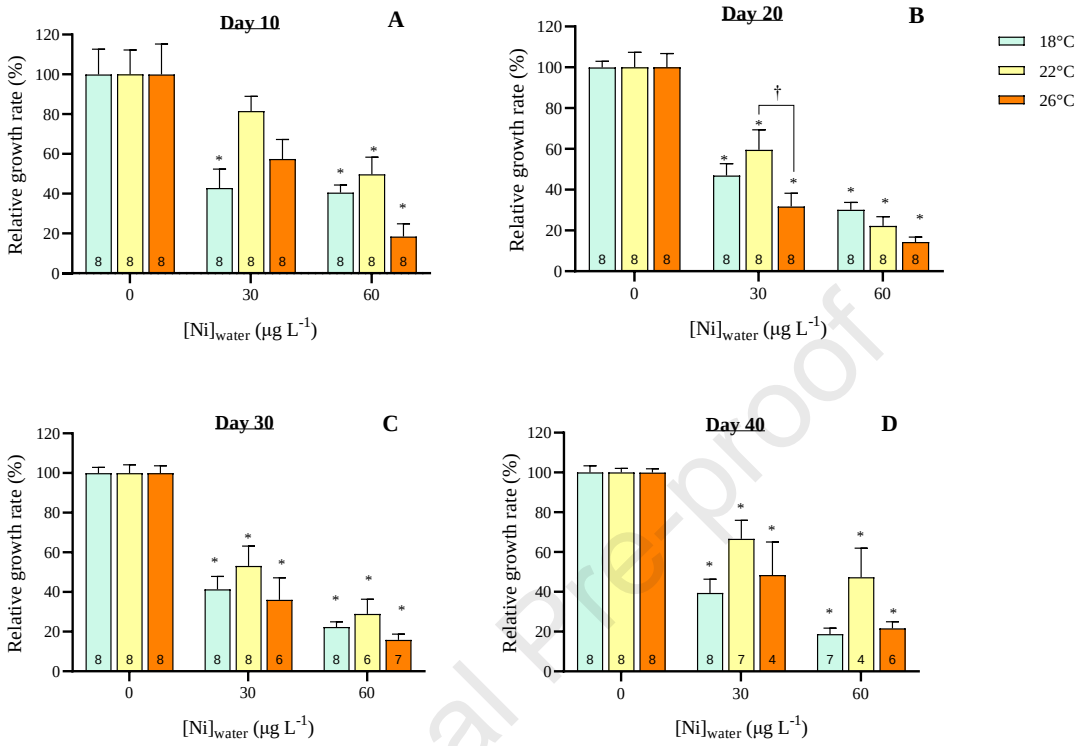

Figure 4: Relative growth rate of *L. stagnalis* after A) 10, B) 20, C) 30 and D) 40 days of Ni exposure at 18, 22 or 26°C, as a function of nominal Ni water concentration. Data are presented as mean ± SEM (*n* values given at the bottom of each bar). Asterisks indicate differences of Ni treatments compared to the control (0 µg·L^{-1}) for a given temperature, and daggers indicate significant between temperature groups for a given Ni treatment ($p < 0.05$, two-way ANOVA with Tukey test).

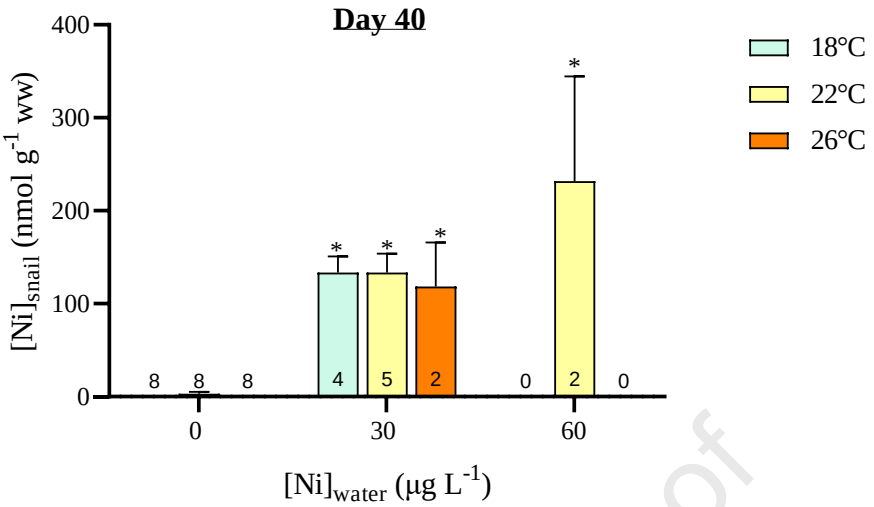

Figure 5: Nickel in snail soft tissues after 40 days of Ni exposure at 18, 22 or 26°C, as a function of waterborne Ni exposure concentration. Data are presented as mean ± SEM (*n* values given at the bottom of each bar). Asterisks indicate differences of Ni treatments compared to the control (0 µg·L^{-1}), and no differences were observed between temperature groups for a given Ni treatment ($p < 0.05$, two-way ANOVA with Tukey tests).

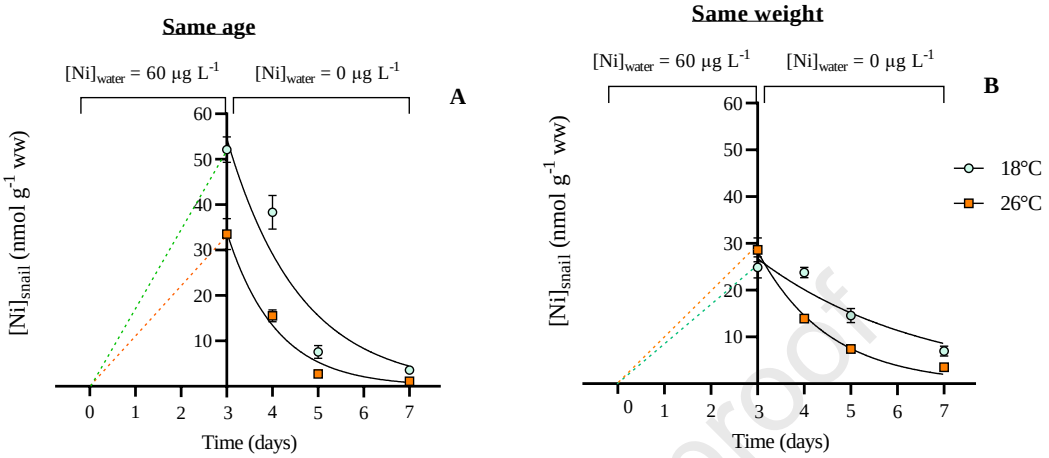

Figure 6: Nickel tissue concentration after a 72-h exposure phase to 60 µg·L^{-1} Ni, then over a 96-h elimination phase (in Ni-free water), for **A)** snails of the same age (40-d old; 0.053 – 0.19 g whole soft tissue wet weight) and **B)** snails of the same weight (0.35 ± 0.01 g whole soft tissue wet weight; 40 – 50-d old). Data points are mean ± SEM (*n* = 6). Plain lines represent the best fits with Eq 3, with A) R^2 = 0.859 and 0.951 at 18 and 26°C respectively, and B) R^2 = 0.686 and 0.956 at 18 and 26°C respectively). Dashed lines are linear extrapolation of Ni tissue concentration during the loading phase.

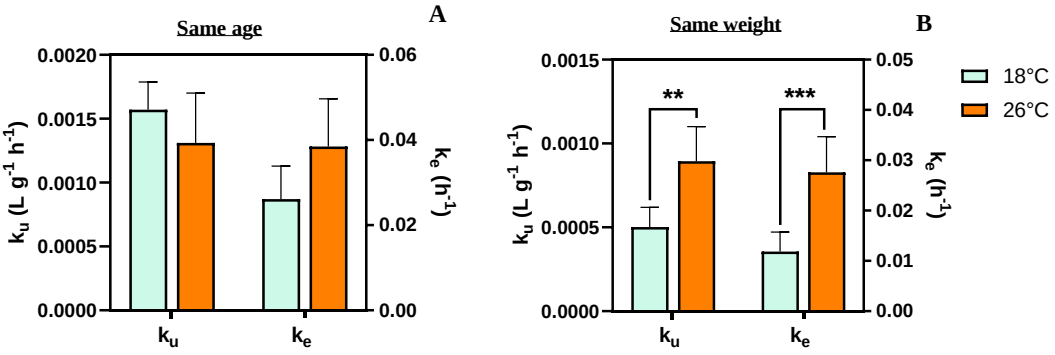

Figure 7: Temperature effects on Ni uptake and elimination rate constants in juvenile snails of **A)** the same age (40-d old; 0.053 – 0.19 g whole soft tissue wet weight) and **B)** snails of the same weight (0.35 ± 0.01 g whole soft tissue wet weight; 40 – 50-d old). The k_u and k_e values were estimated from Fig. 5 data, using eq. 4 and 3 respectively. Each bar is a mean ± SEM and asterisks indicate differences between temperature treatments (k_u: t-tests wih n = 6, k_e: F-test with df =22) (** $p < 0.01$, ***$p < 0.001$).

Highlights

- Juvenile growth and survival were affected by temperature and by nickel
- Warming (18 to 26°C) appeared to aggravate Ni lethality in 40-day chronic toxicity test
- However, temperature had no effect on Ni-induced growth inhibition and Ni bioaccumulation
- Temperature had either limited or counter-acting effects on nickel uptake and elimination rates

AUTHOR CONTRIBUTIONS

Megan Mattsson: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Data curation, Writing – Original draft.

Anne Cremazy: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – Review & editing, Supervision, Project administration, Funding acquisition.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: