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C.M.S. Pereira et al.

Multigenerational effects of Ni on *D. magna*

**Multigenerational Effects of Nickel on *Daphnia magna* Depend on Temperature and the Magnitude of the Effect in the First Generation**

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**Abstract:** Ecological risk assessment (ERA) is commonly based on single generation ecotoxicological tests that are usually performed at one standard temperature. We investigate the effects of nickel (Ni) on *Daphnia magna* reproduction at 15, 20 and 25°C along four generations. Multigenerational Ni effects on *D. magna* reproduction depended on the magnitude of the effect in the first generation (F0) and showed very different patterns at different temperatures. At low effect level concentrations (<EC10 of F0), chronic Ni toxicity at 15 and 20°C did not increase along four generations, and the increase of Ni toxicity at 25°C observed in F1 and F2 in some Ni treatments did not persist into F3, where complete recovery of reproduction was observed. At higher effect level concentrations, the multigenerational Ni effects depended on the test temperature.

In F0, Ni toxicity was 6.5-fold lower at 25°C than at 15°C (based on EC50), but the temperature effect on Ni toxicity was not explained by differences in Ni accumulation. At lower temperature lower internal Ni concentrations in *D. magna* were necessary to induce the same Ni toxicity than at higher temperature.

Overall, our results indicate that low single-generation chronic effect concentrations of Ni to *D. magna* (here EC10) are also protective in a long-term, multigenerational context and that temperature should be taken into account in ERA of Ni. This article is protected by copyright.

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**Keywords:** Multigenerational effects, Nickel, Temperature, Ecological risk assessment, Aquatic toxicology, Metal accumulation

## INTRODUCTION

Ecological risk assessment (ERA) is often based on single generation ecotoxicological laboratory experiments that are performed at a standard temperature. By doing so, the potential influence of temperature on chemical toxicity and effects from multigenerational exposures in the field are not taken into account. Temperature is an important abiotic factor for ectothermic organisms, including the ecotoxicological model organism *Daphnia magna*, which can be found in ponds and lakes at water temperatures between 5 and 26 °C (Carvalho 1987; Lopez et al. 1991). The increase of surface water temperature increases the ventilation rates and the respiration rates and it reduces the generation time (i.e. time to first brood) of *D. magna* (Bae et al. 2016; Paul et al. 2004a; Walsh et al. 2014). A recent study of Bae et al. 2016 (2016) exposed *D. magna* to 20 and 25°C during three generations and it was found that the levels of reactive oxygen species increased at 25°C in comparison to 20°C. Moreover, the temperature dependency of the reactive oxygen species production increased along generations. This study also showed that the length of offspring (5 days old) was significantly reduced at 25°C. At 25°C, the shorter time to reach maturity and time to first brood could reduce offspring quality. Campos et al. (2016) found that negative effects on offspring quality (e.g. assessed based on offspring length) could enhance the adverse effects of chemicals in later generations. A previous in-house study indicated that the exposure temperature had a significant effect on chronic metal toxicity to *D. magna* (Pereira et al. 2017). This study showed that in comparison with 20°C, the standard temperature recommended by the OECD guideline for the *D. magna* reproduction test (OECD 2012), chronic nickel (Ni) toxicity to *D. magna* increased at 15°C and decreased at 25°C.

Chemical toxicity can change along generations of *Daphnia* (Guan and Wang 2006; Massarin et al. 2010; Münzinger 1990; Pane et al. 2004; Vandegheuchte et al. 2009). However,

the information about the multigenerational effects of metal exposure and of temperature on metal sensitivity is relatively limited. In two multigenerational studies performed at a single temperature (20°C) Ni toxicity to *D. magna* changed along generations and demonstrated differential effects on body length of offspring, mean life span, number of broods, brood size, and the intrinsic rate of population growth (Münzinger 1990; Pane et al. 2004). Two acute studies have reported higher internal metal concentrations at higher temperatures in *Daphnia*, suggesting temperature effects on metabolic rates may influence metal uptake (Heugens et al. 2003; Sokolova and Lannig 2008; Vandenbrouck et al. 2011). However, the physiological state of the organism can influence metal uptake and also the detoxification, the sequestration and the elimination processes that determine metal toxicity (Vijver et al. 2004). It is therefore not known if differences in internal body concentrations of Ni could explain effects of temperature on chronic Ni toxicity.

Given the importance of temperature variation and multigenerational exposure in field conditions and the data gaps mentioned above, we investigated the temperature dependence on multigenerational effects of Ni. Therefore, the effects of Ni on *D. magna* reproduction and offspring length were tested at 15, 20 and 25°C along four generations. In addition, to explore possible differences in the mechanisms of Ni toxicity at different temperature and different generations, internal Ni concentrations ( $[\text{Ni}]_{\text{daphnia}}$ ) were measured. Furthermore, little information is available concerning the mechanisms that are related to the effect of temperature on Ni toxicity to *Daphnia*. Brix et al. (2017) identified four potential mechanism of Ni toxicity in aquatic organisms, that are the disruption of calcium (Ca), magnesium (Mg) and iron (Fe) homeostasis and the generation of reactive oxygen species. Also, previous studies have shown interactive effects between Ni, copper (Cu) and zinc (Zn) on daphnid reproduction (Nys et al.

2015; Nys et al. 2017), which might be related to these metals influencing each other's toxicokinetics. It is however, unknown if these potential Ni toxicity mechanisms apply across a wide temperature range. Therefore, ion homeostasis of Cu, Zn, Fe, Mg, Ca, sodium (Na) and potassium (K) (only in F0) were also measured.

## **MATERIAL AND METHODS**

### *Organism cultures and test media*

The *D. magna* clone (K6) was cultured in aquaria in a modified M4 medium that was also used for exposure (Pereira et al. 2017). Prior to actual multigenerational Ni exposure, organisms were acclimated for two generations to the temperature treatments (Mitchell and Lampert 2000). Neonates (<24h) collected from 3<sup>rd</sup> to the 4<sup>th</sup> brood of the acclimated mothers were used to start the multigenerational Ni exposure. More detailed information about the acclimation process and test medium can be found in Pereira et al. (Pereira et al. 2017).

### *Experimental design*

A multigenerational toxicity test was performed with *D. magna* exposed to Ni at 15, 20 and 25°C. The test medium was spiked with Ni three days before the start of the experiment and these test solutions were then kept in the dark at 20°C. One day before the start of the experiment or one day before the change of the medium the test solutions were placed in the different climate rooms at 15, 20 and 25°C. Every 4 weeks new medium was prepared and spiked. A scheme of the experimental design can be found in Figure 1. Seven Ni concentrations (0, 12, 25, 50, 100, 200 and 300  $\mu\text{g}\cdot\text{L}^{-1}$ ) were tested per temperature along four generations. Organisms were continuously exposed along four generations to the same Ni concentration (further defined as: Ni treatment). The 7 Ni treatments and the 3 temperature treatments of the multigenerational experiment were investigated simultaneously. Fifteen individual replicates per Ni treatment were

performed to guarantee a sufficient number of organisms to start the subsequent generation. Exposure of the first generation started with neonates (<24h) collected from 3<sup>rd</sup> to the 4<sup>th</sup> brood of pre-acclimated mothers in aquarium to the temperature treatments. Neonates (<24h) were exposed individually in 50 mL of medium in polyethylene cups. Once first generation females (F0) released their third brood of offspring, the second generation was initiated (F1). The same procedure was followed for the following generations. To start a next generation 10 neonates were collected from each of 5 randomly selected mothers and pooled together. When reproduction occurred in less than 5 mothers, neonates were collected from at least 3 mothers (10 neonates per mother). If less than 10 neonates were released per mother, 5 neonates were collected per mother from 5 to 10 mothers. Otherwise, the next generation of that Ni treatment was not started.

As *D. magna* is an ectothermic organism, time to reach maturity depends on temperature. Therefore, to ensure that *D. magna* reproduction could be assessed within each temperature treatment, the exposure of each generation continued until the daphnids had released the 5<sup>th</sup> brood in more than 50% of the replicates of the control treatment. In the present research a generation was defined as the time between the start of the Ni exposure of the neonates (<24h) and the release of the 5<sup>th</sup> brood by the adult females in control treatments. All experiments were performed under a controlled light cycle (16 h of light: 8 h of dark) and the test medium was renewed three times a week as recommended by OECD guideline No. 211 (OECD 2012). Daphnids were fed daily with *Pseudokirchneriella subcapitata* with a food density of 2.5 mg C·L<sup>-1</sup> in the first week of each generation and 5 mg C·L<sup>-1</sup> on the following weeks.

Survival of each mother and number of offspring were recorded daily. To assess the quality of the offspring, the length of neonates (<24h) released at the end of each generation was



measured in 3 replicates, 5 neonates per replicate. Different broods were used for different purposes, that is 3<sup>rd</sup> brood organisms were used to start the next generation and 5<sup>th</sup> brood organisms were used to measure length. This was logistically necessary because not enough organisms from the same brood were available for both purposes. To determine metal body concentrations in adult females, samples were taken at the end of each generation. The surviving adult females were randomly sampled and equally divided in 3 replicates to ensure sufficient body mass for the metal analysis. The internal Ni concentrations in *D. magna* ( $[Ni]_{daphnia}$ ) were measured in all generations and the internal Cu, Zn, Fe, Mg, Ca, Na and K concentrations in *D. magna* were only measured in F0.

#### *Daphnia magna sampling for metal analysis*

Daphnids were transferred to a control medium for 10 minutes, after which they were transferred to a Na<sub>2</sub>EDTA (5mM) (Sigma-Aldrich) solution where they remained during 1 minute to remove Ni adsorbed to the carapace (Adam et al. 2015). The organisms were transferred to a sieve where they were quickly rinsed with deionized water. Daphnids were transferred to a parafilm paper and all samples were placed in an oven at 60°C for at least 48 h until a constant dry weight. The daphnids were weighed on a Sartorius Digital Micro balance (type 2405), and transferred to an Eppendorf tube (2 mL).

To each vial, containing dried daphnids, 200  $\mu$ L HNO<sub>3</sub> (Normaton Ultrapure 69%, Prolabo) was added and the vial was left to stand overnight. The daphnids were microwave digested in five steps of 2 min at 90 and 160 W, and five steps of 1 min at 360 W. The different treatment steps were separated by a 2 min interval. Then 25  $\mu$ L H<sub>2</sub>O<sub>2</sub> was added to each sample (AnalaR NORMAPUR 30%) and 30 min later daphnids were microwave digested in 1 step of 2 min at 90, 160 W and 1 min at 360 W. The samples were diluted with water (ultra-pure, Chem-

lab NV) to 1 to 2% HNO<sub>3</sub>. As quality control, two reference samples (mussel tissue 2977, NIST) and two procedure blanks were included in each 40-sample rack (Mubiana and Blust 2007).

Recoveries of all metals are reported in Supportive Info (Table S1). The recovery of Mg in the reference samples was 8% of the certified values although very good recoveries (90%) had been found in earlier experiments, using the same procedure. Also, the [Mg]<sub>daphnia</sub> values found in the present study are in the same range as those reported in a previous study with *D. magna* (Pane et al. 2003). Therefore, despite the low recovery, we did report measured [Mg]<sub>daphnia</sub> values.

### *Chemical analysis*

The reference material used in all chemical analyses and the quantification limits of water chemical analysis and *D. magna* metal body analysis can be consulted in SI (Table S2). Water samples for metal analysis were taken from test solutions for each temperature and for each generation. During the four generations, samples of fresh and old medium of all treatments were collected weekly for analysis of total (only of fresh medium) and dissolved concentrations of Ni and also Cu, Zn, Fe and major cations (Na, K, Ca and Mg). Dissolved concentrations refer to 0.45 µm membrane filtered concentrations (Acrodisc, PALL Life Sciences). Samples for metal analysis were acidified to a final concentration of 0.14 mol·L<sup>-1</sup> of HNO<sub>3</sub> prior to analysis. Water metal analysis was performed using iCAP 7000 Series ICP-OES (Thermo Scientific).

Weekly samples for total (only for fresh medium) and dissolved organic carbon analysis were taken for the 0, 12, 50, 200 µg Ni·L<sup>-1</sup> treatments in F0 and samples were taken only in the control treatment and in the highest Ni concentration tested in following generations. The samples for DOC analysis were measured with TOC-L CPH (Shimadzu).

All data analyses were performed based on the mean dissolved concentrations of new and old medium measured for F0. pH was measured with a pH glass electrode (P407; Consort) and

dissolved oxygen was measured with an Oximeter WTW (probe WTW, cell Ox 325) were measured weakly. Temperature was recorded daily.

Daphnids digested samples were measured with ICP-OES to determine Cu, Zn, Fe, Na, K, Ca, Mg body concentrations and with graphite furnace atomic absorption spectrophotometry (GFAAS Furnace Autosampler, Thermo Fisher Scientific Inc., Waltham, MA, USA ) to determine Ni body concentrations.

#### *Data analysis*

All statistical analysis were performed in R software (R Team) according to the protocols of Zuur (2009).

*Daphnia magna reproduction – Analysis of variance.* To investigate whether the effect of temperature on Ni toxicity to *D. magna* reproduction changes across generations and to investigate whether the multigenerational effect on Ni toxicity to *D. magna* reproduction changes at different temperatures we tested for possible interactive effects between temperature, generation and Ni. The data that were generated were analysed by applying a general linear model. However, this analysis violates the independence assumption. We observed that organisms ( $F_{x+1}$ ) born from more healthy mothers ( $F_x$ ) (expressed as a higher offspring production) also had more offspring regardless of temperature and Ni. We tested for possible correlations (Spearman correlation) and we observed significant positive correlations of reproduction between F0 vs. F1 ( $r^2 = 0.511$ ,  $p < 0.05$ ), F1 vs. F2 ( $r^2 = 0.813$ ,  $p < 0.001$ ) and F2 vs. F3 ( $r^2 = 0.593$ ,  $p < 0.05$ ) (SI, Figure S1).

As a tool to include a dependence structure in the analysis, we therefore applied to our data set a generalized estimation equation (GEE) with a three way-interaction between temperature, Ni and generation with an auto-regressive correlation structure between generation

using the *geepack* package in R software (version 2.3-96) (RC 2017). The GEE predicted the Ni (continuous variable) effect on reproduction, expressed as the number of offspring per individual female (*Rep*) produced until more than 50% of the organisms in control treatment released the fifth brood (*Rep5*), as a function of temperature (factorial variable) and generation (factorial variable).

Prior to analysis, *Rep5* was log<sub>10</sub> transformed as log<sub>10</sub> (*Rep5* + 1). Normality was evaluated using a quantile-quantile (QQ) plot of the model residuals and by plotting the residuals against the fitted values. The homogeneity of variances of residuals across all temperatures, generations, and Ni was evaluated using a boxplot (Zuur et al. 2009).

*Daphnia magna reproduction - ECx and NOEC.* Pairwise Wilcoxon rank sum tests were used to test for statistical differences on the endpoints *Rep5* and offspring size (length) between control and Ni treatment at each generation and at each temperature. The Benjamini & Hochberg (1995) correction method was used to adjust the *p* values for multiple comparisons within each temperature at each generation. The determination of the effect concentrations (ECx) tests were performed with reproduction expressed as *Rep5* (% of control) using the *drc* package in R software (version 2.3-96). Effect concentrations were determined using the best fitted model to the data (Table S3). Pairwise Wilcoxon rank sum tests were performed *using the stats* package.

*Daphnia magna internal Ni concentrations.* A GEE analysis was also applied to the endpoint [Ni]<sub>daphnia</sub> with a three way-interaction between temperature (factorial variable), Ni (continuous variable) and generation (factorial variable) with an auto-regressive correlation structure between generation using the *geepack* package in R software (version 2.3-96) (RC 2017; Zuur et al. 2009). Prior to analysis, [Ni]<sub>daphnia</sub> and water Ni concentration were log<sub>10</sub>

( $x+0.1$ ) transformed. Normality and homogeneity of variances were evaluated as described in *Daphnia magna* reproduction – Analysis of variance.

In addition, to investigate whether the effect of temperature on Ni toxicity to *D. magna* reproduction is explained by  $[Ni]_{daphnia}$  we fitted the Michaelis-Menten equation to the data at each generation and each temperature. The Michaelis-Menten curve was fitted to the  $[Ni]_{daphnia}$  using the *nlstool* package

$$[Ni]_{daphnia} = \beta_{max} \left( \frac{[Ni]_{water}}{[Ni]_{water} + K_S} \right)$$

where  $\beta_{max}$  is the maximum  $[Ni]_{daphnia}$  ( $\mu g \cdot g^{-1}$ ),  $[Ni]_{water}$  is the water Ni concentration ( $\mu g \cdot L^{-1}$ ) and  $K_S$  is the half saturation constant ( $\mu g \cdot L^{-1}$ ) (Buchwalter and Luoma 2005).

*Daphnia magna internal cations concentrations.* A linear model was applied to each individual metal measured in *D. magna* and expressed as internal body concentrations ( $[Metal]_{daphnia}$ ), i.e. Ca, K, Na, Mg, Cu, Zn and Fe with a two way-interaction between temperature (factorial variable) and Ni (continuous variable). Prior to the linear analysis,  $[Metal]_{daphnia}$  and  $[Ni]_{water}$  were  $\log(x+0.1)$  transformed to normalize the data. To test for statistical differences on the  $[Metal]_{daphnia}$  (i.e. Ca, K, Na, Mg, Cu, Zn and Fe) in F0 between temperatures a pairwise Wilcoxon rank sum tests were performed. The Benjamini & Hochberg (Benjamini and Hochberg 1995) correction method was used to adjust the  $p$  values for multiple comparisons.

## RESULTS

### *Test water characteristics*

Temperature remained stable in the three treatments during the four generations (14.7 ( $\pm 0.8$ ), 19.9 ( $\pm 0.5$ ) and 24.8°C ( $\pm 0.6$ ) (mean  $\pm$  standard deviation) (SI, Table S4). The dissolved oxygen concentration (8.9 $\pm$ 0.7 mg·L<sup>-1</sup>), the dissolved organic carbon (3.5 $\pm$ 0.2 mg·L<sup>-1</sup>), the pH (8.0 $\pm$ 0.1), and the major ion concentrations (21.2 $\pm$ 1.3 mg Na·L<sup>-1</sup>, 9.2 $\pm$ 0.7 mg Mg·L<sup>-1</sup>, 3.3 $\pm$ 0.2 mg K·L<sup>-1</sup>, 62.1 $\pm$ 3.7 mg Ca·L<sup>-1</sup>) (overall mean  $\pm$  standard deviation) also remained stable during the multigenerational experiment (SI, Table S4, S5 and S6).

#### *Daphnia magna reproduction, offspring length*

In the control treatments, at the end of each generation a mean of  $\geq 60$  living offspring per parent survival female was reached for all generations at 15 and 20°C but not at 25°C with a mean of  $\geq 40$  offspring per female (SI, Table S8).

For F0 the length of the offspring was not reduced by Ni in any of the temperature treatments ( $p > 0.05$ ) with the exception of the 6  $\mu\text{g Ni}\cdot\text{L}^{-1}$  treatment (nominal concentration: 12  $\mu\text{g Ni}\cdot\text{L}^{-1}$ ) at 25°C ( $p < 0.05$ ) (SI, Table S9).

*Daphnia magna reproduction – Analysis of variance.* The GEE analysis revealed a significant interaction between temperature and Ni to *D. magna* reproduction, between generation and Ni, and a significant three-way interaction between generation, temperature and Ni (Table 1). The correlation coefficient between two sequential generations is 0.191 $\pm$  0.056 ( $\pm$  standard error). The log<sub>10</sub> (*Rep*5+1) transformed data met the assumption of normality based on the QQ plot of the model residuals and on the plot of the residuals vs. fitted values (SI, Figure S2). The residuals of the optimal model were homogeneously distributed around 0 when plotted against the variables temperature, generation and Ni indicating homogeneity of variance. The plot of predicted vs. observed values indicates a good model fit (SI, Figure S2) (Zuur et al. 2009).

*Daphnia magna* reproduction - ECx and NOEC. To better understand the three-way interaction between generation, temperature and Ni toxicity to *D. magna* reproduction ( $p < 0.001$ ), we performed a more detailed analysis of our data, by calculating ECx values and by testing for statistical differences on *Rep5* between control and Ni treatment in each generation and at each temperature.

Corroborating our previous study (Pereira et al. 2017), temperature had a significant effect on chronic Ni toxicity to *D. magna* in F0. The EC50 for reproduction of Ni for F0 (EC50<sub>F0</sub>) increased 6.5-fold between 15 and 25°C (Figure 2 and SI, Table S3). In some temperature treatments in later generations a dose response curve could not be fitted because some of the Ni treatments that continue to the next generation did not present adverse effects. Therefore the effects of temperature on Ni toxicity in later generations could not be estimated on the basis of ECx values.

Multigenerational Ni effects on *D. magna* reproduction showed different patterns at different temperatures (Figure 2; Table 1 and 2). At 20°C (standard temperature) when organisms were exposed to concentrations below the EC10<sub>F0</sub> ( $13.5 \mu\text{g}\cdot\text{L}^{-1} (\pm 6.1 \text{ (standard error)})$ ) chronic Ni toxicity did not increase along generations (Figure 2). However, chronic Ni toxicity significantly increased along generations when organisms were exposed to Ni concentrations higher than EC10<sub>F0</sub>. Indeed, at 23 and 54  $\mu\text{g Ni}\cdot\text{L}^{-1}$  reproduction was reduced by 23 to 36% in F0 but by 97 to 98% in F1 (Table 2). Therefore, it was also not possible to start exposures of F2 and F3 with these Ni treatments (due to a lack of neonates). At 15°C, chronic Ni toxicity was variable along generations, but was in all Ni treatments highest in F0, even though some increase of the measured total and dissolved Ni concentrations occurred along generations (SI, Table S7). At 25°C, because we observed a relatively high within-Ni-treatment variation, an unexpected

non-monotonic response in F1 and F2 and a strong effect in F1 at some concentrations below the EC10<sub>F0</sub>, this temperature treatment was entirely repeated in an additional experiment. In both experiments, the same pattern was observed (e.g., non-monotonic response in F1 and F2 and a strong effect in F1 at some concentrations below the EC10<sub>F0</sub>) but less variation within-Ni-treatment was observed in the 2<sup>nd</sup> experiment (SI, Figure S4). Therefore, we present in the main manuscript the results of the 2<sup>nd</sup> experiment. At 25°C, when organisms were exposed to Ni concentrations below the EC10<sub>F0</sub> (88.1 µg·L<sup>-1</sup> (±13.3)), chronic Ni toxicity was not significantly affected in later generations at 17 and 27 µg·L<sup>-1</sup> (Table 2). However, at 6 and 56 µg·L<sup>-1</sup> chronic Ni toxicity increased from no effect in F0 to 49% and 77% of effect in F1, respectively. This increase of Ni toxicity became less pronounced in F2 and organisms completely recovered in F3 (Table 2). At 25°C when organisms were exposed to a Ni treatment higher than EC10<sub>F0</sub> (i.e. 120 µg·L<sup>-1</sup>) the Ni effect fluctuated along generations, but a consistent trend was not observed.

#### *Daphnia magna internal Ni concentrations*

The GEE analysis applied to the endpoint [Ni]<sub>daphnia</sub> indicates that Ni, temperature and generation are significant factors (Table 3). Significant interaction effects between Ni and generation were observed but not between Ni and temperature. A significant three-way interaction was observed between Ni, temperature and generation. The correlation coefficient between two sequential generations is 0.005±0.065 (± standard error). The residuals of the model were homogeneously distributed around 0 when plotted against the variables temperature, generation and Ni indicating homogeneity of variance. The plot of predicted vs. observed values indicates a good model fit (SI, Figure S3).

To test if the effect of temperature on Ni toxicity to *D. magna* reproduction is explained by [Ni]<sub>daphnia</sub> we fitted the Michaelis-Menten equation to the data at each generation and each



temperature. In F0, we observed that at lower temperatures lower  $[\text{Ni}]_{\text{daphnia}}$  was necessary to induce the same Ni toxicity (i.e. 50% reduction of reproduction) than at higher temperatures (Figure 3, SI, Table S10). That is, when estimating the  $[\text{Ni}]_{\text{daphnia}}$  based on the  $[\text{Ni}]_{\text{water}} = \text{EC50}_{\text{F0}}$  and on the estimated values for  $\beta_{\text{max}}$  and  $K_{\text{s}}$  (SI, Table S11) the  $[\text{Ni}]_{\text{daphnia}}$  was 2.5, 6.0 and 23.8  $\mu\text{g}\cdot\text{g}^{-1}$  for 15, 20 and 25°C, respectively.

The  $[\text{Ni}]_{\text{daphnia}}$  presented a non-monotonous trend with generation-number that was different for every temperature, that is the  $[\text{Ni}]_{\text{daphnia}}$  followed the order  $\text{F1} > \text{F0} \approx \text{F2} > \text{F3}$  at 15°C,  $\text{F3} \approx \text{F2} > \text{F0} > \text{F1}$  at 20°C and  $\text{F0} \approx \text{F3} > \text{F1} > \text{F2}$  at 25°C (Figure 4).

#### *Daphnia magna internal cations concentrations*

In F0, Ni affected the  $[\text{Metal}]_{\text{daphnia}}$  for Fe and Mg ( $p < 0.05$ ) (Table 4). Also, significant interactive effects were observed between Ni and temperature on the  $[\text{Metal}]_{\text{daphnia}}$  for Ca, Na, Mg, Fe, Cu and Zn ( $p < 0.05$ ). Yet, no trends of  $[\text{Metal}]_{\text{daphnia}}$  were observed as a function of Ni that were consistent across all temperatures (Figure 5 and 6). Temperature significantly affected the internal Ca, Mg, Fe, and K concentrations in daphnids but it did not affected internal Zn, Cu and Na concentration in daphnids (Table 4, Figure 5 and 6). The  $[\text{Ca}]_{\text{daphnia}}$  significantly increased from 15°C ( $25.2 \pm 3.3 \text{ mg}\cdot\text{g}^{-1}$ ) to 20°C ( $30.7 \pm 3.0 \text{ mg}\cdot\text{g}^{-1}$ ) and to 25°C ( $35.2 \pm 4.1 \text{ mg}\cdot\text{g}^{-1}$ ). Also, the  $[\text{Mg}]_{\text{daphnia}}$  significantly increased from 15°C ( $850.2 \pm 87.4 \mu\text{g}\cdot\text{g}^{-1}$ ) to 20°C ( $936.3 \pm 111.6 \mu\text{g}\cdot\text{g}^{-1}$ ) and to 25°C ( $998.1 \pm 103.5 \mu\text{g}\cdot\text{g}^{-1}$ ). Indeed, the  $[\text{Fe}]_{\text{daphnia}}$  was significantly higher at 25°C ( $240.0 \pm 34.4 \mu\text{g}\cdot\text{g}^{-1}$ ) compared to 20°C ( $178.4 \pm 27.9 \mu\text{g}\cdot\text{g}^{-1}$ ) and 15°C ( $207.1 \pm 29.9 \mu\text{g}\cdot\text{g}^{-1}$ ). The  $[\text{K}]_{\text{daphnia}}$  was significantly lower at 25°C ( $5.5 \pm 0.5 \text{ mg}\cdot\text{g}^{-1}$ ) than at 20°C ( $6.0 \pm 0.4 \text{ mg}\cdot\text{g}^{-1}$ ) and 15°C ( $6.0 \pm 0.4 \text{ mg}\cdot\text{g}^{-1}$ ) and it was similar between 20°C and 15°C. All values were given as mean ( $\pm$  standard deviation).

## **DISCUSSION**

Our multigenerational study with *D. magna* exposed to seven concentrations of Ni at 15, 20 and 25°C provided following main insights:

- (i) We found a three-way interaction between Ni, temperature and generation on *D. magna* reproduction (Table 1), and the multigenerational Ni effects on *D. magna* reproduction showed different patterns at different temperatures (Figure 2);
- (ii) The  $[\text{Ni}]_{\text{daphnia}}$  also showed a significant three-way interaction between Ni, temperature and generation (Table 3), but the  $[\text{Ni}]_{\text{daphnia}}$  does not explain the different patterns of multigenerational Ni effects at different temperatures and neither the temperature effects on Ni toxicity in F0;
- (iii) The internal ion concentrations in *D. magna* did not explain the effects of temperature on Ni toxicity.

Below, we will discuss each of these findings in more detail, including their implications for risk assessment.

#### *Different multigenerational Ni effects on D. magna reproduction*

The multigenerational Ni effects on *D. magna* reproduction showed different patterns at different temperatures (Table 1, Figure 2). At 20°C, the magnitude of the effect in F0 determined if Ni effects weakened (at low effect levels:  $<EC_{10F0}$ ) or strengthened (at high effect levels:  $>EC_{10F0}$ ) in the following generations. At 15°C, chronic Ni toxicity at low and at high effect levels did not increase along generations. In addition, at low effect levels ( $<EC_{50F0}$ ), a recovery was observed along generations (Table 2). At 25°C, at low effect level concentrations ( $<EC_{10F0}$ ), an increase on Ni toxicity was observed in some Ni treatments in F1 and F2. This increase of Ni toxicity did, however, not persist and even a full recovery was observed in F3 (Figure 2).

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It is important to acknowledge that the three studies about the multigenerational effects of Ni to *D. magna* available, that are the present study, Pane et al. (2004) and Munzinger (1990), have similarities but also differences. These differences can be due to different endpoints but also to the fact that different *D. magna* clones were used. Indeed, it is well-known that different clones can have different tolerance to metals (Barata et al. 1998).

The study of Pane et al. (2004), performed at 20°C, indicated an increase of Ni sensitivity in F1. In their study, F1 organisms continuously exposed to 42 µg Ni·L<sup>-1</sup> (reported as no-observable-effect concentration for survival, reproduction and growth in F0) weighed significantly less than controls. This is in contrast with our results at 20°C in which Ni toxicity did not increase at low effect level concentrations.

Munzinger (1990) stated that *D. magna* acclimated to Ni during seven generations of exposure to 160 µg Ni·L<sup>-1</sup> (~EC50 for brood size) (i.e. lower Ni toxicity along generations), because the brood size increased along generations and with that the intrinsic rate of population increased. However, the mean life span and body length were significantly reduced along generations indicating a stronger Ni effect that is similar to our results at high effect level concentrations at 20°C.

*[Ni]<sub>daphnia</sub> did not explain temperature and generation effects*

The change of [Ni]<sub>daphnia</sub> along generations presented different patterns at different temperatures (Table 3 and Figure 4). These results suggest that the rates of metal uptake, detoxification and/or elimination change with both temperature and generation. The [Ni]<sub>daphnia</sub> did not explain the temperature effects on Ni toxicity (Figures 3 and 4; SI, Table S10). In F0, at lower temperature lower [Ni]<sub>daphnia</sub> was necessary to induce the same Ni toxicity than at higher temperature (Figure 3). This could be explained by the fact that metal toxicity is determined by

the metabolically available concentration and not by the total accumulation in the organism (Rainbow 2002; Vijver et al. 2004). From the few studies available about the effect of temperature on metal toxicity, only two also report internal metal concentrations. These two acute studies, one with cadmium (Cd) and the other with Ni, indicated that metal toxicity to *D. magna* is enhanced by the increase of temperature and that there was a trend of increasing internal metal concentrations in *D. magna* with increasing temperature (Heugens et al. 2003; Vandebrouck et al. 2011). This is in agreement with the hypothesis that, with metabolic rates being raised by temperature increase, metal uptake rates would also increase (Sokolova and Lannig 2008). The results of these studies are in contrast with the present study, which also showed an increase in the  $[Ni]_{daphnia}$  with increasing temperature, but which was not accompanied by increased chronic Ni toxicity with increased temperature. The contrast to the acute studies may be explained by the lack of an acclimation period in the acute studies necessary to reach a homeostatic balance to the new temperature environment (Williams et al. 2012) or to reach full sequestration capacity.

The exact mechanisms of uptake, elimination, sequestration and detoxification of Ni in *Daphnia* remain unclear (Brix et al. 2017; Pyle and Couture 2011). For instance, the metallothionein proteins are known to be involved in the regulation of some intracellular metal concentration such as Cu and Zn (Asselman et al. 2013; Fan et al. 2009) but unlikely in the case of Ni (Asselman et al. 2012; Denkhaus and Salnikow 2002; Pyle and Couture 2011). Some studies indicate that the levels of this protein can increase with the increase of temperature (Baykan et al. 2007; Serafim et al. 2002). This could be a possible explanation for the fact that lower toxicity at higher temperatures is observed. Yet, a study with *Daphnia pulex* showed no significant influence of Ni on metallothionein gene expression indicating that metallothionein is

likely not involved in Ni detoxification processes in *Daphnia* (Asselman et al. 2012). Other proteins such as serum albumin, L-histidine and 2-macroglobulin in blood serum are known to bind to Ni (Denkhaus and Salnikow 2002). Besides, other types of proteins as the heat shock proteins, which are triggered by temperature and other stressors and which work as molecular chaperons (Feder and Hofmann 1999), have also been associated with acute metal tolerance (Pestana et al. 2016). The study of Hall (1982) revealed that Ni was deposited in newly formed *D. magna* carapace but it was not deposited in the newly released eggs. It also revealed that 25 to 33% of Ni in *D. magna* is lost at molting. It is therefore possible that at higher temperatures, more Ni is eliminated by the carapace. Future toxicokinetic and toxicodynamic studies performed at different temperatures would be useful to understand the exact mechanisms involved in the effect of temperature on Ni toxicity to *Daphnia*.

#### *Effects on cations homeostasis in D. magna*

The [Metal]<sub>daphnia</sub> for Cu, Zn, Fe, Mg, Ca, Na, K and Mg did not explain the effect of temperature on chronic Ni toxicity. For none of the [Metal]<sub>daphnia</sub> was a consistent trend observed in relation to Ni across all temperatures, although some statistically significant interactions between temperature and Ni were found (Table 4, Figure 5 and 6). The study of Pane et al. (2003) showed that at 20°C Ni did not disrupt Ca and Na homeostasis in *D. magna* (which is confirmed by our results) but Mg was significantly reduced by Ni. In contrast, the results of present study showed that the [Mg]<sub>daphnia</sub> did not decrease with the increase of Ni. The contrasting results among both studies could be due to the experimental conditions, as in Pane et al. (2003) adults were exposed to Ni during 14 d and in the present study neonates (<24h) were exposed to Ni until the control treatment released the 5<sup>th</sup> brood (~ 40, 22 and 17 d at 15, 20 and 25°C respectively). Our results, however, do show that temperature by itself has an important

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effect on metal ion concentrations in *D. magna*. Temperature significantly influenced the internal levels of Ca, Mg, Fe and K in F0 (Table 4). The  $[Ca]_{daphnia}$  and the  $[Mg]_{daphnia}$  were significantly higher at the highest temperature 25°C which is also the temperature at which lower Ni toxicity was observed. Calcium is important for several biological processes such as gene expression, cell proliferation, muscle coordination, etc (Bootman 2012). Also, Mg is important for several biological functions including structural stabilization of nucleic acid, cell membranes and to promote specific structural organization of enzymes and ribosomes (Wolf and Cittadini 2003). Internal Fe concentrations are intrinsically associated to haemoglobin concentrations in *Daphnia* (Paul et al. 2004b; Smaridge 1956). Therefore, in case of Ni impairment of one of these biological processes, the highest internal concentration of Ca, Mg and Fe in *D. magna* observed at 25°C could have been an advantage and could be involved in the lower Ni toxicity at this temperature.

#### *Risk assessment implications*

Our study showed that at low effect level concentrations (estimated in the first generation) which are most relevant for ERA, Ni toxicity at 15 and 20°C did not increase along four generations, and the increase of Ni toxicity at 25°C observed in F1 and F2 in some Ni treatments did not persist into F3, in which effects were not stronger than in F0 (Figure 2). These results suggest that the low effect levels of Ni (<EC10) observed in the first generation are also protecting *D. magna* populations against multi-generational exposure. Future multigenerational studies with other substances would be useful to understand whether EC10 values, which are often considered as basis for chronic ERA, are generally protective in a multigenerational context. This is important, since risk assessment still largely ignores the possibility of multigenerational effects.

Ecological risk assessment also largely ignores the effect of temperature on metal toxicity. In the present study, for F0 the EC50 increased 6.5-fold with the increase of temperature from 15 to 25°C. This finding is in line with and even stronger than that in the study of Pereira et al. (2017) who reported a 2-fold increase of the EC10 and of the EC50 with the increase of temperature from 15 to 25°C. Together, the present study and the study of Pereira et al. (2017) provide strong evidence that temperature should be integrated as a factor in metal risk assessment.

## CONCLUSION

In our study the multigenerational Ni effects on *D. magna* reproduction showed very different patterns at different temperatures. Also, patterns of multigenerational effects on [Ni]<sub>daphnia</sub> were also very different at different temperatures. However, Ni accumulation did not explain the influence of temperature on chronic Ni toxicity to *D. magna* reproduction: at lower temperature a lower [Ni]<sub>daphnia</sub> was necessary in the first generation to induce the same Ni toxicity than at higher temperatures. In F0, the [Metal]<sub>daphnia</sub> of Ca, K, Na, Mg, Fe, Cu and Zn did not explain the effect of temperature on chronic Ni toxicity.

At relevant concentrations for ERA, that is at low effect level concentrations (EC10 or lower), chronic Ni toxicity at 15 and 20°C did not increase along four generations, and the increase of Ni toxicity at 25°C observed in F1 and F2 in some Ni treatments did not persist into F3. At higher effect level concentrations, the multigenerational Ni effects depended on the tested temperature and effects were in some cases stronger than in the first generation.

Finally, the present study and the study of Pereira et al. (2017) showed that temperature affected chronic Ni toxicity to *D. magna*, that is the EC50 increased 2 to 6.5-fold with an

increase of temperature from 15 to 25°C. Together both studies provide strong evidence that temperature should be integrated as a factor in metal risk assessment.

*Supplemental Data*—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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*Data Availability*—Data pertaining to this manuscript are deposited in figshare at DOI:xxxx.

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Figure 1. Experimental design used for the multigenerational exposure of *Daphnia magna* to nickel (Ni) at 15, 20 and 25°C. The generations (F<sub>x</sub>), the brood that was used to start the next generation (3<sup>rd</sup> brood) and the endpoints are indicated. Survival and reproduction were recorded daily. The length of neonates and metal body concentration in adult females were assessed at the end of each generation when 50 % of the organisms (adult females) in control treatments released the 5<sup>th</sup> brood.

Figure 2. Reproduction of *Daphnia magna* exposed to nickel (Ni) at 15, 20 and 25°C during four generations (F<sub>0</sub>, F<sub>1</sub>, F<sub>2</sub>, and F<sub>3</sub>). Reproduction is expressed as the number of offspring per individual female produced until the organisms in control treatment released the 5<sup>th</sup> brood as percentage of control treatment (total reproduction (% of control)). Marker points represent observations (average data) and lines are the fitted concentration response curves for F<sub>0</sub>. Vertical lines indicate the effect concentrations (EC<sub>x</sub>) estimated for F<sub>0</sub>, the EC<sub>10</sub> values are represented by dotted lines, the EC<sub>20</sub> values by dashed line and EC<sub>50</sub> values by straight lines.

Figure 3. Plot of the internal nickel (Ni) concentration in *Daphnia magna* exposed to Ni in the first generation of exposure (F0) at 15, 20 and 25°C. Internal Ni concentrations ( $[\text{Ni}]_{\text{daphnia}}$ ) expressed as  $\mu\text{g per g dry weight}$  and Ni water concentrations expressed as  $\mu\text{g per L}$ . Dots represent data points and lines are the fitted Michaelis-Menten curves. Vertical lines represent the effective Ni concentrations in the exposure medium that reduce *D. magna* reproduction by 50% at 15, 20 and 25°C.

Figure 4. Plot of the internal nickel (Ni) body concentration on *Daphnia magna* exposed to nickel during four generations (F0, F1, F2, and F3) at 15, 20 and 25°C. Internal Ni concentrations ( $[\text{Ni}]_{\text{daphnia}}$ ) expressed as  $\mu\text{g per g dry weight}$ , Ni water concentrations expressed as  $\mu\text{g per L}$ . Dots represent data points, and lines are the fitted Michaelis-Menten curves. Vertical lines indicate the effect concentrations (ECx) estimated for F0, the EC10 values are represented by dotted lines, the EC20 values by dashed line and EC50 values by straight lines.

Figure 5. Plots of the internal sodium (Na), potassium (K), calcium (Ca) and magnesium (Mg) concentrations in *Daphnia magna* on the first generation (F0) of exposure to Ni at 15, 20 and 25°C.

Figure 6. Plots of the internal iron (Fe), copper (Cu) and zinc (Zn) concentrations in *Daphnia magna* during the first generation (F0) of exposure to Ni at 15, 20 and 25°C.

Table 1. Summary of the analysis of variance performed with the generalized estimation equation that predict the nickel (Ni) (continuous variable) effect on reproduction as a function of temperature (factorial variable) and generations (factorial variable) (Ni  $\times$  Temperature  $\times$  Generation) with an auto-regressive correlation structure between generations. Reproduction expressed as  $\log_{10}$ -transformed number of offspring per individual female produced until the organisms in control treatment released the 5<sup>th</sup> brood plus 1 ( $\log_{10}(Rep5 + 1)$ ). Degrees of freedom (Df), Wald statistics ( $X^2$ ), and significance ( $p$  value) are shown. The estimated correlation coefficient between  $F_x$  and  $F_{x+1}$  was  $0.191 \pm 0.056$  ( $\pm$  standard error).

	Df	$X^2$	$p$
Ni	1	49.82	<0.001
Temperature	2	1.58	>0.05
Generation	3	11.09	<0.05
Ni $\times$ Temperature	2	53.84	<0.001
Ni $\times$ Generation	3	23.07	<0.001
Temperature $\times$ Generation	6	22.77	<0.001
Ni $\times$ Temperature $\times$ Generation	6	79.10	<0.001





Table 3. Summary of the analysis of variance with the generalized estimation equation that predict the nickel (Ni) (continuous variable) effect on internal Ni concentration ( $[\text{Ni}]_{\text{daphnia}}$ ) as a function of temperature (factorial variable) and generation (factorial variable) ( $\text{Ni} \times \text{Temperature} \times \text{Generation}$ ) with an auto-regressive correlation structure between generations. Internal Ni concentration expressed as  $\log_{10}$ -transformed ( $\log_{10}([\text{Ni}]_{\text{daphnia}} + 0.1)$ ). Degrees of freedom (Df), Wald statistics ( $X^2$ ) and significance ( $p$  value) are shown. The estimated correlation coefficient between  $F_x$  and  $F_{x+1}$  was  $0.005 \pm 0.065$  (standard error).

Predictor	Df	$X^2$	$p$
Ni	1	401	<0.001
Temperature	2	22	<0.001
Generation	3	19	<0.001
Ni $\times$ Temperature	2	4	>0.05
Ni $\times$ Generation	3	14	<0.05
Temperature $\times$ Generation	6	111	<0.001
Ni $\times$ Temperature $\times$ Generation	6	185	<0.001

Table 4. Summary of the linear model applied to each metal measured as internal body concentrations in *Daphnia magna*, i.e. calcium (Ca), potassium (K), sodium (Na), copper (Cu), and zinc (Zn) during the first generation of nickel (Ni) exposure with a two way-interaction between temperature (factorial variable) and Ni (continuous variable).

Cations	Ni	Temperature	Ni $\times$ Temperature	r <sup>2</sup>
Ca	>0.05	<0.001	<0.01	0.66
Mg	<.05	<0.01	<0.05	0.39
Fe	<0.05	<0.001	<0.05	0.54
K	>0.05	<0.01	>0.05	0.20
Cu	>0.05	>0.05	<0.01	0.23
Na	>0.05	>0.05	<0.05	0.19
Zn	>0.05	>0.05	<0.05	0.14

Experimental design

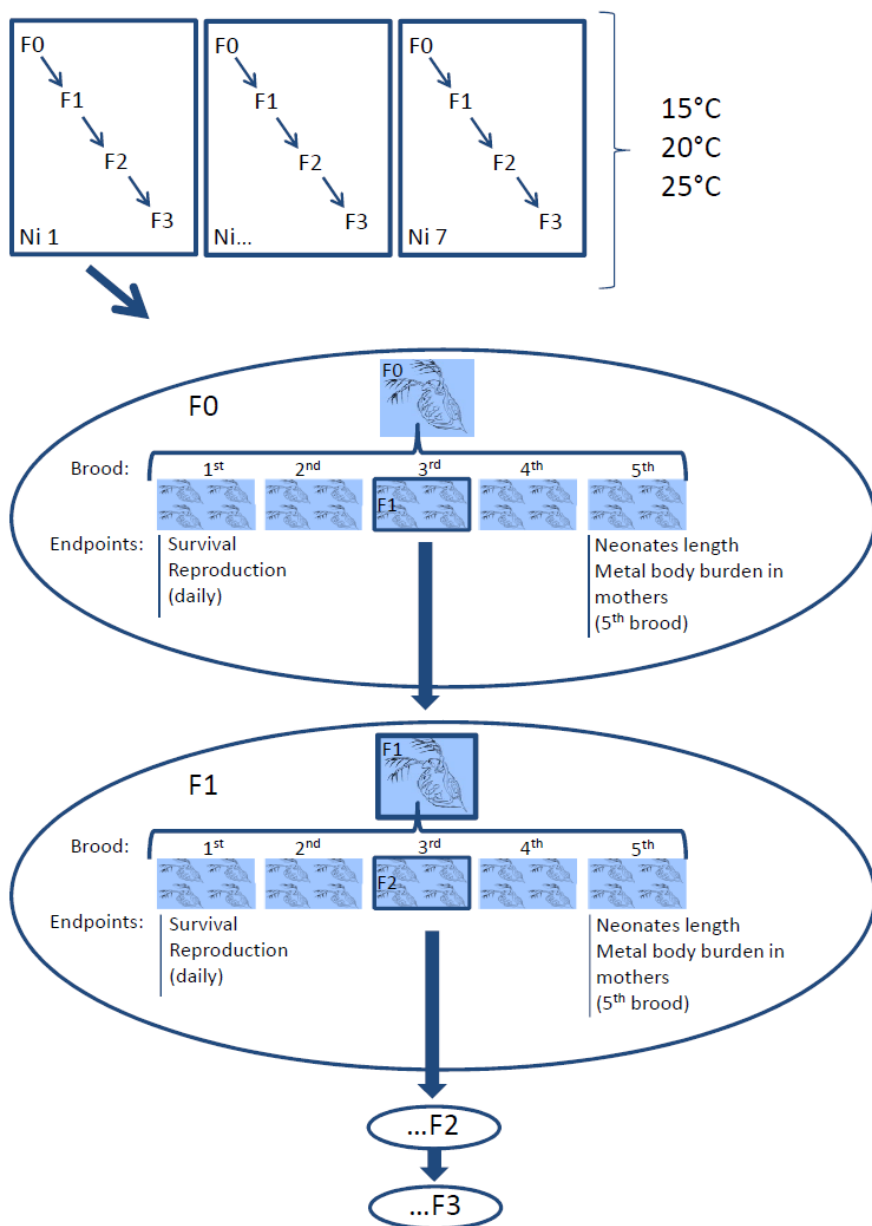


Figure 1

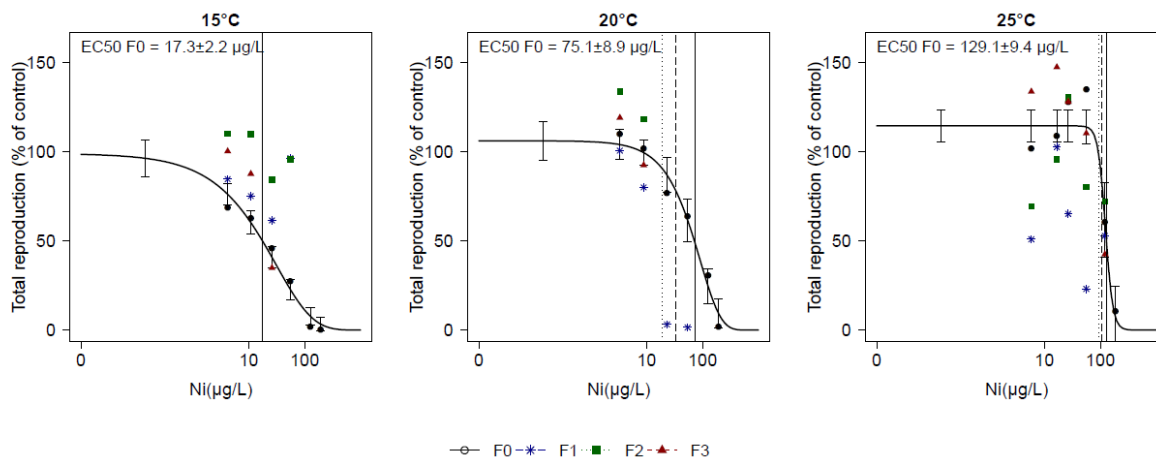


Figure 2

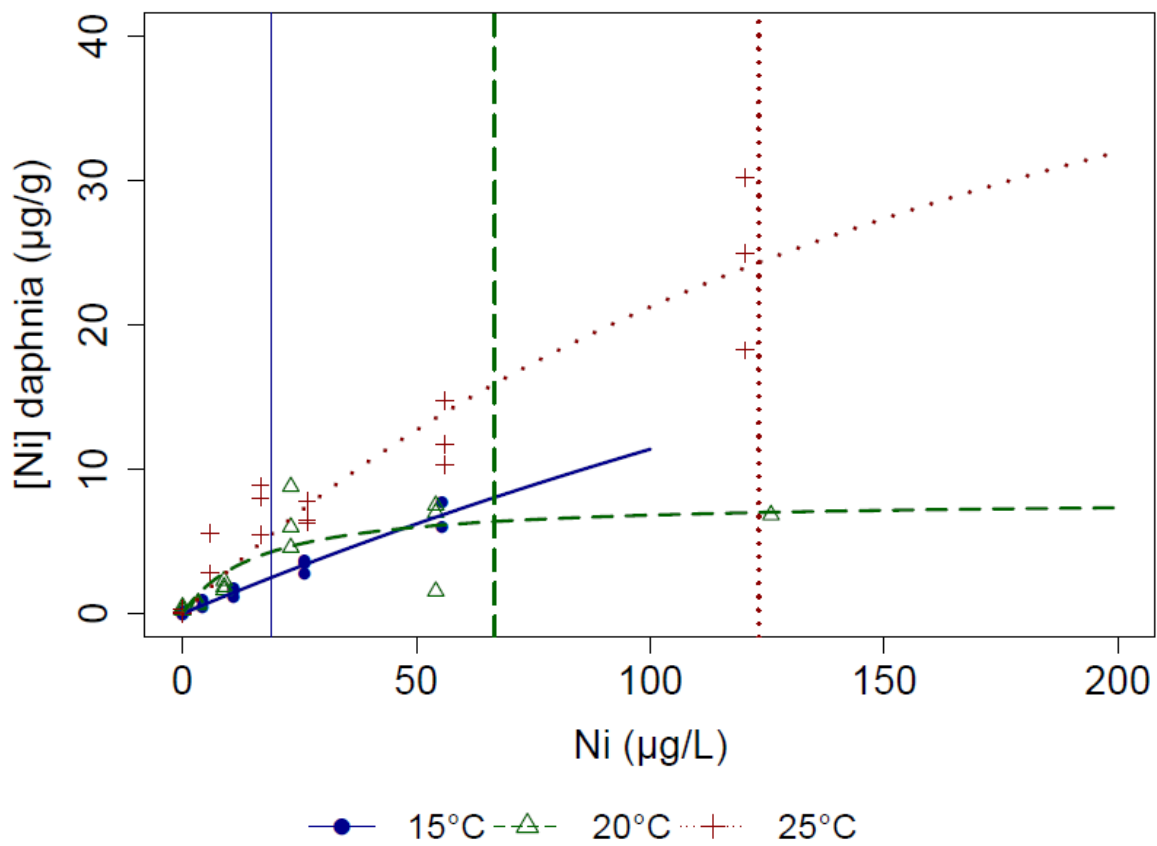


Figure 3

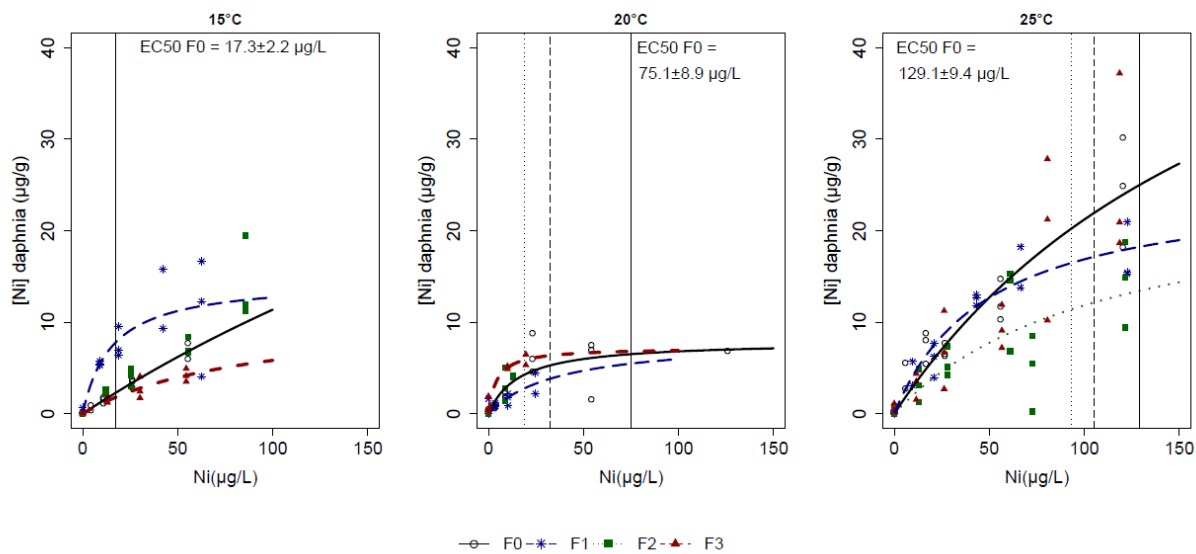


Figure 4

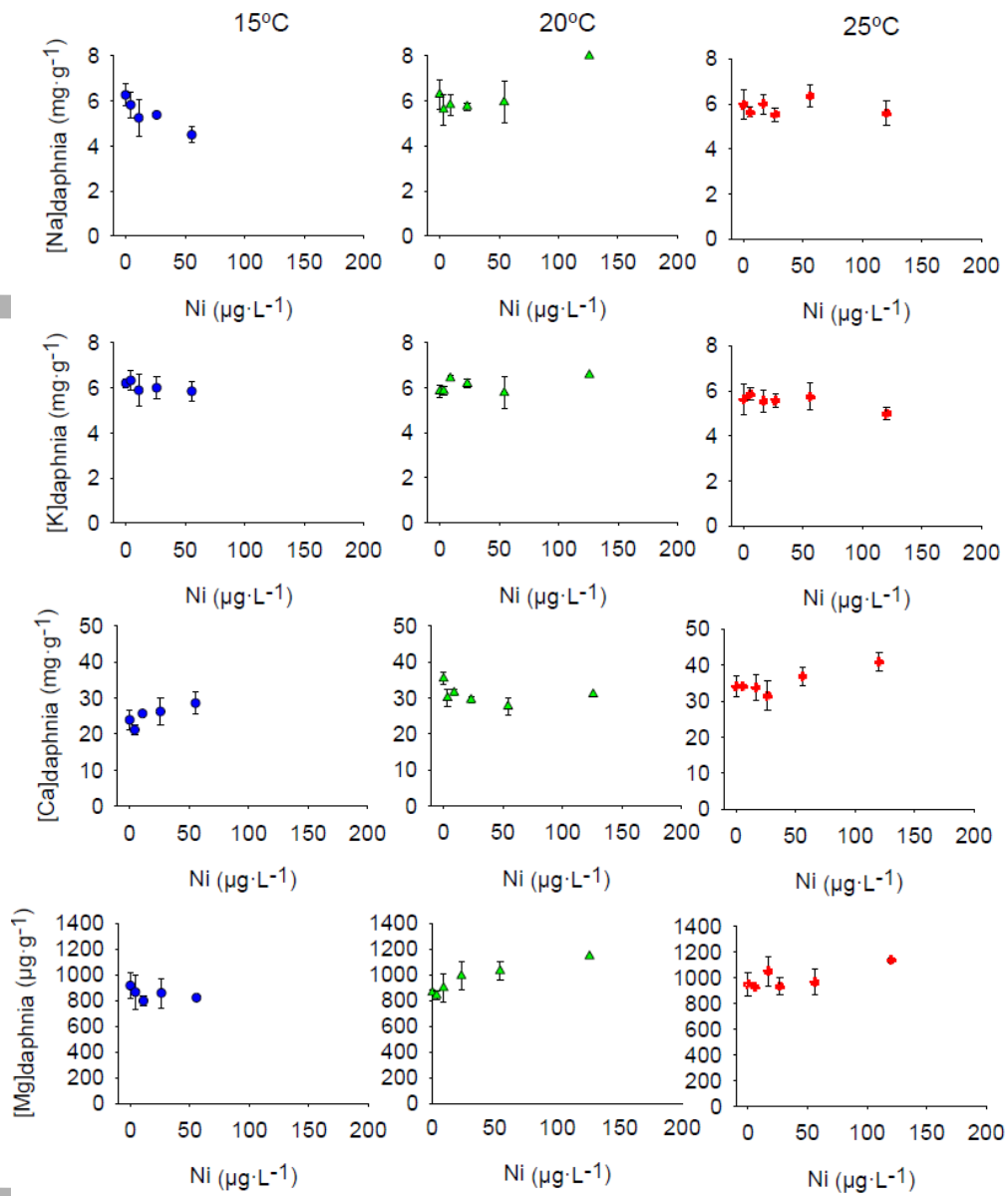


Figure 5

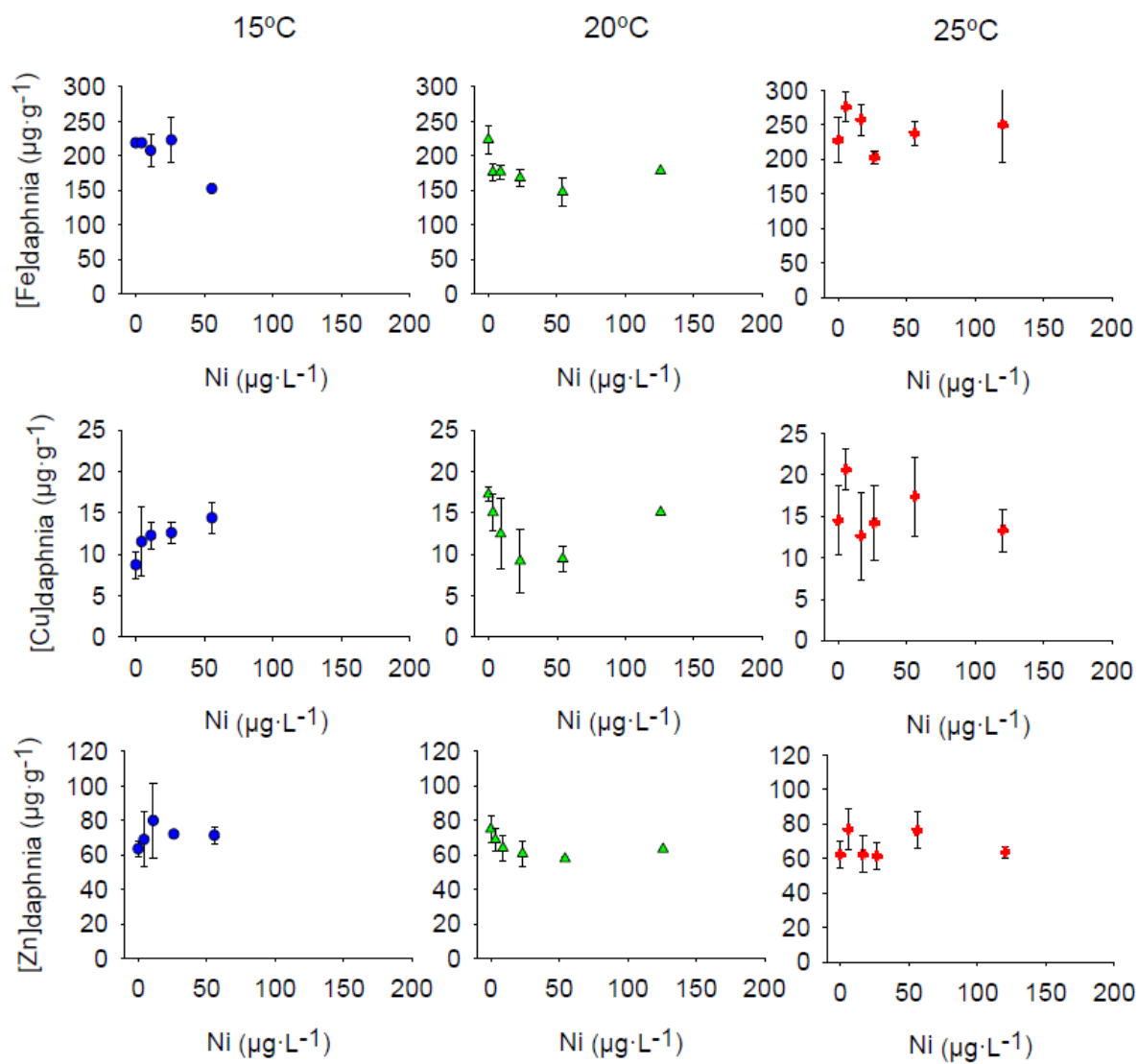


Figure 6