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A MICROCOSM STUDY TO SUPPORT AQUATIC RISK ASSESSMENT OF NICKEL: COMMUNITY-LEVEL EFFECTS AND COMPARISON WITH BIOAVAILABILITY-NORMALIZED SPECIES SENSITIVITY DISTRIBUTIONS

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Abstract: The aquatic risk assessment for nickel (Ni) in the European Union is based on chronic species sensitivity distributions and the use of bioavailability models. To test whether a bioavailability-based safe threshold of Ni (the hazardous concentration for 5% of species [HC5]) is protective for aquatic communities, microcosms were exposed to 5 stable Ni treatments (6–96 μg/L) and a control for 4 mo to assess bioaccumulation and effects on phytoplankton, periphyton, zooplankton, and snails. Concentrations of Ni in the periphyton, macrophytes, and snails measured at the end of the exposure period increased in a dose-dependent manner but did not indicate biomagnification. Abundance of phytoplankton and snails decreased in 48 μg Ni/L and 96 μg Ni/L treatments, which may have indirectly affected the abundance of zooplankton and periphyton. Exposure up to 24 μg Ni/L had no adverse effects on algae and zooplankton, whereas the rate of population decline of the snails at 24 μg Ni/L was significantly higher than in the controls. Therefore, the study-specific overall no-observed-adverse-effect concentration (NOAEC) is 12 μg Ni/L. This NOAEC is approximately twice the HC5 derived from a chronic species sensitivity distribution considering the specific water chemistry of the microcosm by means of bioavailability models. Thus, the present study provides support to the protectiveness of the bioavailability-normalized HC5 for freshwater communities. *Environ Toxicol Chem* 2016;35:1172–1182. © 2015 The Authors. *Environmental Toxicology and Chemistry* Published by Wiley Periodicals, Inc. on behalf of SETAC.

Keywords: Metal Chronic exposure Biotic ligand model Higher tier test Community-level effect

INTRODUCTION

The potential effects of nickel (Ni) on aquatic organisms have been or are being assessed in the European Union under the Existing Substances Regulation [1]; Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) [2]; and the Water Framework Directive [3]. In the existing substances risk assessment of Ni and Ni compounds [4], a species sensitivity distribution based on chronic toxicity tests for 31 freshwater species was used to derive the hazardous concentration for 5% of the species (HC5), corresponding to the 5th percentile of the species sensitivity distribution, with the most sensitive taxa being snails (Gastropoda; i.e., *Lymnaea stagnalis*) and crustacea (Cladocera; i.e., *Ceriodaphnia dubia*). Because validated chronic bioavailability models, such as biotic ligand models, are available for fish [5], 2 invertebrate species (the Cladocera *Daphnia magna* and *C. dubia* [6,7]), and algae [8,9], site-specific HC5 values can be calculated by normalizing the species sensitivity distribution for bioavailability using site-specific conditions of pH, dissolved organic carbon (DOC) concentration, and water hardness (as explained by Schlekat et al. [10]). This approach has been applied in the European Union risk assessment of Ni and Ni compounds, which was conducted in the context of the Existing Substances Regulation [4] and, more recently, in the context of the European Union Water Framework Directive, to calculate bioavailability-based environmental quality standard values for Ni [11]. For the assessment of the chemical status of water bodies, a bioavailable environmental quality standard of 4 μg Ni/L was adopted in 2013 [12], which corresponds to water bodies with conditions of high Ni bioavailability (pH 8.2, DOC = 2 mg/L, and water hardness = 40 mg $CaCO_3$/L). Bioavailability models, such as biotic ligand models, can then be used to account for local water quality conditions (pH, DOC, and hardness) and to calculate site-specific environmental quality standard values.

As is the case with the risk assessment of all chemical substances, a critical step in deriving predicted-no-effect concentration or environmental quality standard values is to address possible uncertainties in the extrapolation from simple laboratory ecotoxicity test results to the more complex situation in the field [11]. Issues that need to be considered include the representativeness of the species tested, the relevance of the effects found in (standard) laboratory single-species toxicity tests for the population and community levels, bioavailability of Ni under field conditions (covered by the bioavailability models), and the potential of indirect effects in the food web related to ecological interactions.

Microcosm and mesocosm studies are an established higher-tier tool to address such questions because they allow investigation of effects of chemicals at the population and community levels (including indirect effects) under more

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realistic conditions than in standardized single-species tests [13,14]. This is a well-recognized approach for the risk assessment of plant protection products [15], but microcosm and mesocosm studies are also recommended tools in the assessment for industrial chemicals [16] and in deriving environmental quality standards [11]. For example, in the voluntary European risk assessment of copper [17] microcosm and mesocosm studies confirmed the low uncertainty on the protectiveness of HC5 based on bioavailability-normalized chronic toxicity values.

The aim of the present study was to determine whether the bioavailability-normalized HC5 for Ni was protective of population-level and community-level effects in a microcosm study. The microcosms were comprised of a freshwater community of algae, zooplankton, and snails that were exposed to constant Ni concentrations over 4 mo under semirealistic environmental conditions in a greenhouse. No-observed-effect concentrations (NOECs) were calculated for all populations, as well as the community structure for each sampling date, and further evaluated to derive a study-specific overall no-observed-adverse-effect-concentration (NOAEC) for chronic exposure. This microcosm NOAEC was compared with bioavailability-normalized HC5 values, which were calculated by normalizing the species sensitivity distribution to the water chemistry parameters observed during the microcosm experiment.

MATERIALS AND METHODS

The study design and performance were based on the recommendations of 2 expert workshop discussions [18,19] and the Organisation for Economic Co-operation and Development guidance on simulated freshwater lentic field tests [20]. More details can be found in the study report and its appendices, provided as Supplemental Data.

Setup and management of the test systems

Sixteen cubic microcosms of 1 m^3 installed in a temperature-controlled greenhouse at Fraunhofer IME in Schmallenberg, Germany, were set up in May 2009, with a 20-cm layer of sediment and commercial sand from the river Rhine (1:1) and approximately 750 L tap water (depth approximately 70 cm). The sediment originated from a local, extensively used fish pond stocked with rainbow trout and was not polluted with respect to pesticides, polychlorinated biphenyls, and metals (27 pesticides, <0.01 mg/kg dry wt; polychlorinated biphenyls, <0.005 mg/kg dry wt; metals, 0.2 mg/kg, 10 mg/kg, 15 000 mg/kg, 200 mg/kg, 22 mg/kg, and 77 mg/kg dry wt for Cd, Cu, Fe, Mn, Ni, and Zn, respectively). Additional illumination was provided by metal halide lamps (HQI TS 1000 W/S, Osram; wavelength range 280–780 nm) installed above the center of each microcosm. Water temperature was regulated by cooling and heating the greenhouse, with a target water temperature of 20 °C. Water temperature, pH, dissolved oxygen concentration, and conductivity were measured twice per week; DOC, hardness, and nutrient concentrations were measured at least once per month. Colonization of the microcosms with organisms occurred via the introduced sediment, overlying water from the sediment, and plankton samples taken from outdoor artificial ponds of the mesocosm (Homberg/Ohm, Germany). In addition, 4 large adult individuals of the great pond snail *L. stagnalis* were introduced into each microcosm. *Lymnaea stagnalis* was included in the study because single-species toxicity tests show high sensitivity to Ni [10]. Thirty shoots of the macrophyte *Egeria densa* were planted in one-quarter of the area per microcosm to provide additional habitat for invertebrates. However, photosynthesis by *Egeria* and algae increased the pH to values slightly above the upper limit of the pH range within which the chronic Ni bioavailability models have been developed and validated (i.e., pH 8.2) during the pre-exposure period (May–October 2009). To reduce the pH to values closer to 8.2, macrophytes were cut down and finally removed before the start of the exposure period (26 October 2009 to 15 February 2010). To further control pH, artificial aeration was installed in each microcosm and the artificial lighting was adjusted to 12:12-h light: dark intervals. During pre-exposure, the microcosms were connected to one another by a tube system allowing water (and plankton) to exchange between the systems.

During the exposure period, artificial aeration was continued and new shoots of macrophytes were removed once per month. Evaporation losses during the exposure period were balanced by adding deionized water to the microcosms (usually 20–25 L/microcosm/wk).

Calculations of bioavailability-normalized HC5 values

Chronic bioavailability models for Ni [5,7–9] were used to normalize chronic single-species toxicity data (i.e., 10% effect concentrations [EC10s] or NOECs) to the water chemistry conditions of the microcosms. A species sensitivity distribution was then fitted to these normalized toxicity data, and HC5 values were calculated for the water conditions in the study as has been done for European surface water scenarios in the European Union Ni risk-assessment report [4] and described in Schlekat et al. [10]. This was done based on pretreatment data to select appropriate Ni treatment levels and for the treatment period to be able to compare the NOAEC with the respective HC5.

Dosing

Four microcosms were used as controls, and 5 nominal Ni concentrations (6 μg/L, 12 μg/L, 24 μg/L, 48 μg/L, and 96 μg/L total Ni) were tested in 2 replicates each, which represents an optimized design for multiple testing under constraints of the total number of available test system units. Treatments were assigned randomly to the microcosms after exclusion of 2 of the 16 microcosms showing the largest deviation from the others based on physicochemical, chlorophyll a, and zooplankton data during the pre-exposure period. After the first application of nickel(II) chloride hexahydrate (solution $NiCl_2 \cdot 6\ H_2O$; Chemical Abstracts Service no. 7791-20-0; purity ≥98.0%; Merck) on 26 October 2009, more of the Ni solution had to be added to the microcosm water almost daily to achieve stable Ni concentrations over the exposure period of 4 mo. The Ni concentrations in the microcosms were sampled frequently (at least twice per week). If possible, the amount of Ni added per day was calculated based on the measurements from the previous day. On the other days, a fixed amount of the nominal loading (10–15%, depending on the previous measurements) was added. Sampling was always done before a dosing event. Thus, the measured concentrations correspond to minimum concentrations in the water, whereas the maximum (peak) concentrations could be calculated by consideration of the dosing following the sampling. Because even the mean measured concentrations (before the dosings) were close to nominal values (91%, see the *Results* section), nominal concentrations were used throughout the remaining sections of the present study.

Ni analysis in water, sediment, and biota

For the determination of dissolved Ni in water, 20-mL aliquots of the microcosm water were passed through disposable 0.45-μm membrane filters (Minisart NML, 26-mm diameter; Sartorius). Another subsample of 20 mL was used for determinations of total Ni concentrations. Both the filtered and the unfiltered samples were stabilized immediately after sampling by the addition of 1% (v/v) concentrated nitric acid to a pH of approximately 2 and stored at 4 °C following International Organization for Standardization (ISO) 11885 [21]. In water Ni was measured by inductively coupled plasma-optical emission spectrometry (ICP-OES) following standard ISO 11885 [21].

Sediment samples were taken 4 wk, 8 wk, 12 wk, and 16 wk after the start of the exposure period using a hand auger (diameter, 2 cm; length, 100 cm). At least 3 sample cores per microcosm were separated into 2 horizons (upper horizon, approximately 0–2 cm; lower horizon, approximately 2–20 cm). Total sediment Ni was measured by aqua regia extraction of sediments (according to DIN EN 13346 [22]) by ICP-OES. In addition, acid-volatile sulfides (AVS) and simultaneously extractable metals (SEM) (as operationally defined parameters for the assessment of sulfides and associated metals in aquatic sediments) were measured following the protocol of Allen et al. [23].

Periphyton, macrophytes (remaining *E. densa*), and snails were collected at the end of the study for Ni analysis by ICP-OES. Periphyton was scraped from microcosm walls. The suspended material was concentrated on a filter (0.45-μm membrane filter; Minisart NML). After drying and weighing, masses of periphyton were transferred into quartz vials for measurement of Ni. Five milliliters of 65% nitric acid were added to every sample and digested by microwave procedure (Ultraclave II microwave system, MLS; settings: 220 °C, 30 min, 90 bar). After cooling to room temperature, purified water was added to the digest to a volume of 20 mL and analyzed for Ni. Snails (mainly *L. stagnalis*) from each microcosm were sampled, weighed, homogenized, and freeze-dried. Then, samples were digested by a microwave procedure (as described above). Plant material (*E. densa*) present at the end of the study was harvested, dried, and homogenized. Aliquots were digested by a microwave procedure (described above). Bioconcentration factors (BCFs) for periphyton and macrophytes and bioaccumulation factors (BAFs) for snails were calculated by dividing the Ni concentrations in the biota (expressed on a fresh weight basis) by the nominal Ni concentration in the water. Biomagnification factors for snails were calculated as Ni concentration in snails divided by Ni concentration in periphyton or macrophytes on a dry weight basis.

Effect measurements

Water for plankton collection was taken by moving an inverted funnel connected with a vacuum pump through the microcosm. The sampling with the funnel allowed for the collection of species distributed vertically in the water column. Sampling of zooplankton was performed by filtering approximately 5 L to 10 L of water (depending on population densities) using 15-μm stainless steel sieves. The exact volumes were recorded, and the filtered water was returned to the respective microcosm except for 1 aliquot of 0.5 L used for phytoplankton-related analyses. Filtered zooplankton organisms were fixed immediately with Lugol's iodine solution. Zooplankton was differentiated into the main taxonomic groups Rotifera, Ostracoda, Copepoda (adult and nauplia), and Phyllopoda. Highly abundant groups such as Phyllopoda and Rotatoria were identified to the species level when possible. Two types of analyses were performed weekly to assess effects on the phytoplankton: 1) photosynthetic pigment concentrations (i.e., chlorophyll a and b, carotenoids, and pheophytin) were measured according to DIN 38412, part 16 [24]; and 2) water samples (200 mL) were sieved (mesh size 2 mm) and fixed with 5 mL Lugol's solution for taxonomic identification and enumeration using an inverted microscope. Periphyton was analyzed by measuring the pigments from the biofilm scraped from glass slides introduced into the microcosms just before the start of the Ni exposure period using the same methods as for the phytoplankton. Snail abundance was determined on 1 specific glass wall per microcosm by counting *L. stagnalis, Planorbarius* sp., small snails (<5 mm), and egg clutches. Zooplankton, phytoplankton, and snails were sampled and counted weekly until day 28 after the start of the exposure period and then every second week, whereas periphyton was sampled every fourth week during the exposure period.

Statistical analysis and effect evaluation

Concentrations of Ni in sediment and biota were tested for a positive correlation with the nominal Ni concentrations in the water (Pearson's correlation, $\alpha = 0.05$, one-sided). The zooplankton and phytoplankton community level was analyzed by principal response curves, follow-up redundancy analysis, and principal component analysis per sampling date according to Van den Brink and Ter Braak [25,26]. The Williams multiple *t* test [27] ($\alpha = 0.05$, one-sided) was used to determine NOECs, which are defined as the highest Ni concentration that is not significantly different from controls. The NOEC values were calculated for community structure and abundance of phytoplankton, zooplankton, and snail taxa and for pigment concentration of phytoplankton and periphyton communities. Abundance data and pigment concentrations were log-transformed, $\ln(a \times +1)$, for the analysis to achieve normal distribution and variance homogeneity. In addition, the trend of the dynamics of the snails during the exposure period was analyzed by log-linear regression.

The minimum detectable difference (MDD) was calculated for each abundance NOEC as the minimum difference of the abundance in the treatment relative to that in the control that would have been found significant

$$MDD = (\bar{x}_0 - \bar{x})^* = t_{1-\alpha,df,k} s \sqrt{\frac{1}{n_0} + \frac{1}{n}}$$

where $t_{1-\alpha,\ df,\ k}$ is the quantile of the *t* distribution with degrees of freedom (*df*) and *k* as the number of comparisons, $(\bar{x}_0\text{-}\bar{x})^*$ is the corresponding difference between the arithmetic mean of the control ($\bar{x}_0$) and the treatment ($\bar{x}$), *s* is the residual standard error (square root of the residual variance from a one-way analysis of variance), and n_0, *n* is the sample size of control (n_0) and treatment (*n*).

The minimum detectable difference is provided as a percentage of the control mean. As abundance data were log-transformed for the statistical testing, the minimum detectable differences were also calculated for the log-transformed data. Because percent effects on a log scale are difficult to interpret, these minimum detectable differences were transformed back to the abundance scale (see Brock et al. [28]).

Following the suggestion of Brock et al. [28], we considered taxa reliable for an analysis if the minimum detectable difference met at least 1 of the following conditions during

the exposure period: minimum detectable difference <100% for at least 5 sampling periods, <90% for at least 4 sampling dates, <70% for at least 3 sampling dates, or <50% for at least 2 sampling dates. Considering the frequent sampling over 4 mo of constant exposure, an NOEC was considered as reliable only if effects at the higher concentration were found to be significant over at least 2 consecutive samplings ("consistent NOEC") and as indicating a long-term effect at higher concentrations if significant deviations were found over at least 8 wk ("long-term NOEC").

RESULTS

Physicochemical water parameters

Water temperature varied between 18 °C and 23 °C. Because of permanent slight aeration of the microcosms, oxygen saturation was always above 90% and above 100% per average. Absolute oxygen concentrations varied between 7.5 mg/L and 10 mg/L. The test conditions in the present study were designed to represent conditions of high Ni bioavailability (i.e., low DOC, high pH). The median pH was 8.6, and the median DOC concentration was 3.9 mg/L. During the entire exposure period, the calculated bioavailability-normalized HC5 values varied between 4.2 μg Ni/L (day 112) and 6.8 μg Ni/L (day 0) with a median of 5.5 μg Ni/L. This is close to the bioavailable environmental quality standard of 4 μg/L set in the context of the European Union Water Framework Directive to protect waters with high Ni bioavailability [12], thus confirming the conditions of high Ni bioavailability in the microcosms. The variation in the calculated HC5 values is related to the variation in DOC (positive correlation, $R^2 = 0.83$) and pH values (negative correlation, $R^2 = 0.67$). The exact physicochemical water parameters and the HC5 values calculated over the entire exposure period are given in the Supplemental Data 3, Tables S1 and S2.

Exposure

Concentrations of Ni in the unfiltered water from control microcosms were below the limit of detection (0.4–1.7 μg/L, variable over sampling days) in 82% of the cases and always below the limit of quantification (1.5–5.5 μg/L). During the first week of the exposure period, total Ni concentrations decreased up to 60% of nominal concentrations because no Ni solution was added over the weekend. After the first week, daily Ni dosing resulted in recoveries usually between 80% and 120% of target nominal concentration (Figure 1; for measured values, see Supplemental Data 3, Table S3). The mean total Ni recovery for all samples was 91%. In fact, the real mean total Ni concentrations in the water were higher than the measured ones because the sampling was performed just before dosing events. Almost the entire total Ni was in the dissolved phase, as the concentrations measured in the 0.45-μm membrane–filtered samples were on average 97% of the total Ni concentrations (standard deviation 1.5%). We chose to report nominal concentrations throughout the text, figures, and tables because of the high average total recovery (91%) and the high average fraction of dissolved Ni (97%) in the samples and because samples were always taken before dosing events.

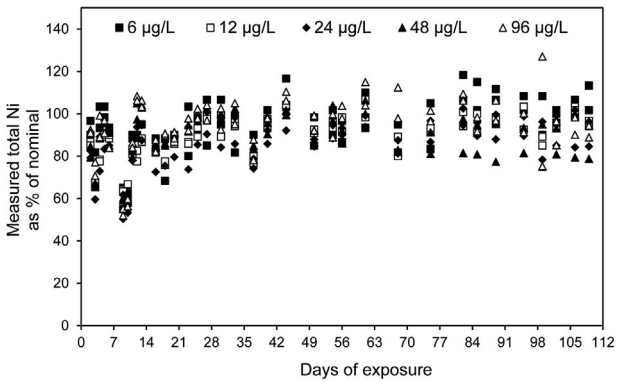

Figure 1. Total Ni concentrations measured before adding Ni solutions, expressed as a percentage of the nominal concentrations.

Fate and bioaccumulation

Analysis of Ni in the upper (0–2 cm) and lower (2–20 cm) sediment layers at the end of the study showed that the Ni concentration in the sediment was not related to the dosing regime (Figure 2A; Supplemental Data 3, Table S4). Pearson's correlation coefficient between Ni concentrations in water and sediment phases was not significant ($r = -0.241$ and 0.299, respectively, controls excluded, $n = 10$). In the lower sediment layer, the concentrations at the end of the study were higher (mean over all 14 microcosms, 28.9 mg/kg dry wt) than in the upper layer (mean, 9.9 mg/kg dry wt). In all microcosms, the SEM/AVS ratio ranged from 0.05 to 0.72 at the end of the study (Supplemental Data 3, Table S5), which indicates that there was still free capacity for metal binding in the sediment after 4 mo of Ni additions to the microcosms.

The Ni concentrations measured in periphyton, macrophytes, and snails at the end of the exposure period significantly

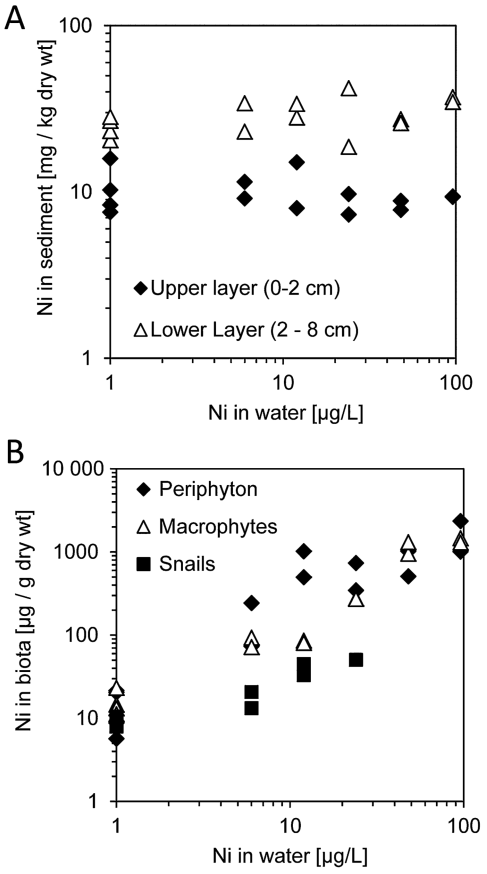

Figure 2. Concentrations of Ni in the sediment (**A**) and in biota (**B**) sampled at the end of the study. Note that Ni concentrations in the controls were plotted at 1 μg/L and that no snails were found in the 48 μg Ni/L and 96 μg Ni/L microcosms at the end of the study.

increased as a function of dissolved Ni concentration (Figure 2B; Supplemental Data 3, Table S6; r values of 0.755, 0.928, and 0.871, respectively). At the highest treatment level, Ni concentrations reached a mean of 1680 μg/g dry weight in the periphyton and 1381 μg/g dry weight in the macrophytes. In the 6 μg/L to 24 μg/L microcosms, the Ni content in the snails increased from 17 μg/g to 51 μg/g dry weight (insufficient numbers of snails were found at 48 μg/L and 96 μg/L to conduct an analysis). Among all Ni treatments (6–96 μg/L), the log BCF (L/kg dry wt) varied between 4.2 and 4.8 (mean, 4.4) for periphyton and between 3.8 and 4.4 for macrophytes (mean, 4.1). The log BAF (L/kg dry wt) for snails varied between 3.3 and 3.5 (mean, 3.4). None of the BCF or BAF values correlated significantly with the Ni concentration in the water ($r = -0.428$, 0.366, and –0.405 for periphyton, macrophytes, and snails, respectively, controls excluded). The calculated biomagnification factor for snails in the Ni treatments, assuming their main food was periphyton on the glass walls, was always below 1 and varied between 0.06 and 0.17.

Effects

In total, 141 algae, 13 zooplankton, and 2 snail taxa were identified during the study. From these, 20 taxa (9 algae, 10 zooplankton taxa, and the snails) fulfilled the minimum detectable difference criterion [28] for a reliable statistical analysis (Supplemental Data 3, Table S7). The NOEC and related minimum detectable difference values for the abundances of these taxa and higher taxonomic levels (e.g., algae classes and zooplankton groups) are given in Tables 1 and 2.

Approximately 60% of all phytoplankton cells counted in 196 samples belonged to Chlorophyceae (34%) or Cyanophyceae (27%), and Chrysophyceae, Cryptophyceae, Zygnematophyceae, and Bacilariophyceae each made up 7% to 12% of all cells. Other classes summed up to less than 1% of the cells. The total phytoplankton abundance varied between 1000 cells/mL and 10 000 cells/mL without consistent deviations from controls (Figure 3). Cryptophyceae (i.e., *Chroomonas/Rhodomonas*) were significantly less abundant in the microcosms of the 48 μg/L and 96 μg/L treatments than in the controls until the end of the study. Over the first 8 wk of exposure, Chrysophyceae were less abundant than in the controls at the 96 μg/L treatment. In contrast to this, Chlorophyceae showed temporarily increased abundances at the highest treatment level. No consistent significant deviations from controls were found for the other algae classes. On the phytoplankton species level, no significant deviations from controls were found at 6 μg/L and significant deviations at 12 μg/L (NOEC = 6 μg/L) were only found on single sampling dates for *Chlorella vulgaris* and *Coelastrum microporum*. Only for *C. vulgaris* was abundance significantly reduced at 24 μg/L over 2 consecutive samplings (day 14 and day 21), but no significant effects could be found for this species later on. For other taxa, significant effects at 24 μg/L (NOECs of 12 μg/L) were only detected on single dates, for example, for reduced abundance of *Monosiga ovata* on day 21. The green algae *Ankyra judayi* showed temporarily significantly higher abundances directly after the start of exposure at the 3 highest treatment levels. The only treatment in which effects persisted over the exposure period was the highest Ni concentration, 96 μg Ni/L. For the other phytoplankton taxa, those with higher minimum detectable differences, the statistical analysis did not indicate any greater sensitivity than for the taxa discussed in the present study (see Supplemental Data 3, Table S7). In summary, the lowest consistent NOEC was 12 μg/L for effects on algae,

Table 1. NOEC values (as nominal μg Ni/L) and percent minimum detectable difference (in parentheses) for the abundances of phytoplankton taxa with sufficiently low minimum detectable difference (see text) or significant differences from controls found over at least 2 consecutive samplings[a]

Taxon	Days after start of exposure											Consistent NOEC[b]	Long-term NOEC[c]
	0	2	7	14	21	28	42	56	70	84	98	112		
Total phytoplankton	≥96 (32)	≥96 (62)	≥96 (65)	≥96 (50)	≥96 (76)	24– (63)	≥96 (89)	≥96 (93)	≥96 (73)	≥96 (81)	≥96 (76)	48– (85)	≥96	48–
Bacillariophyceae	≥96 (29)	≥96 (74)	≥96 (95)	≥96 (75)	≥96 (85)	≥96 (92)	≥96 (100)	≥96 (100)	≥96 (85)	48+ (88)	≥96 (111)	≥96 (92)	≥96	≥96
Chlorophyceae	≥96 (24)	≥96 (64)	48+ (74)	48+ (56)	48+ (70)	≥96 (68)	≥96 (93)	≥96 (87)	≥96 (93)	≥96 (96)	≥96 (96)	≥96 (98)	48+	≥96
Chrysophyceae	≥96 (35)	≥96 (63)	≥96 (69)	≥96 (68)	48– (69)	24– (63)	≥96 (89)	48– (75)	≥96 (86)	≥96 (65)	≥96 (91)	≥96 (88)	48–	≥96
Cryptophyceae	≥96 (76)	48– (61)	24– (70)	48– (87)	24– (92)	24– (85)	48– (78)	≥96 (97)	24– (82)	24– (83)	24– (92)	24– (95)	24–	24–
Cyanophyceae	≥96 (66)	≥96 (73)	48– (85)	≥96 (85)	≥96 (92)	48– (81)	≥96 (98)	≥96 (100)	≥96 (102)	≥96 (96)	≥96 (99)	≥96 (100)	≥96	≥96
Ankyra judayi	≥96 (36)	≥96 (82)	12+ (77)	24+ (78)	48+ (98)	48+ (92)[d]	≥96 (94)	48+ (91)	48+ (94)	≥96 (107)	≥96 (108)	48+ (127)	24+	48+
Chlorella vulgaris	≥96 (66)	≥96 (71)	≥96 (90)	6– (71)	12– (88)	≥96 (95)	≥96 (91)	≥96 (94)	≥96 (90)	≥96 (92)	≥96 (107)	≥96 (125)	12–	≥96
Chromulina minima c.f.	≥96 (38)	≥96 (66)	≥96 (78)	≥96 (94)	≥96 (96)	≥96 (92)	≥96 (100)	≥96 (98)	≥96 (98)	48– (77)	≥96 (102)	≥96 (119)	≥96	48–
Chroomonas/Rhodomonas sp.	≥96 (54)	48– (70)	24– (74)	48– (83)	24– (94)	24– (88)	48– (79)	≥96 (97)	24– (82)	24– (83)	24– (93)	≥96 (100)	24–	24–
Chrysophyceae (15 μm)	≥96 (40)	≥96 (53)	≥96 (65)	≥96 (73)	≥96 (80)	≥96 (86)	≥96 (87)	≥96 (92)	≥96 (71)	≥96 (81)	≥96 (92)	≥96 (101)	≥96	≥96
Coelastrum microporum	≥96 (81)	≥96 (95)	≥96 (92)	≥96 (101)	≥96 (91)	6– (90)	≥96 (99)	≥96 (98)	≥96 (101)	≥96 (104)	≥96 (104)	≥96 (101)	≥96	≥96
Lyngbia sp.	≥96 (81)	≥96 (78)	48– (89)	≥96 (94)	48– (94)	≥96 (97)	≥96 (100)	≥96 (n.c.)	≥96 (103)	≥96 (102)	≥96 (109)	≥96 (114)	≥96	≥96
Monosiga ovata c.f.	≥96 (44)	≥96 (77)	≥96 (85)	≥96 (158)	12– (84)	24– (83)	≥96 (132)		≥96 (164)	≥96 (214)	≥96 (n.c.)	≥96 (n.c.)	24–	24–
Nephrochlamys sp. c.f.	≥96 (37)	≥96 (61)	≥96 (90)	≥96 (94)	≥96 (98)	≥96 (93)	≥96 (103)	≥96 (101)	≥96 (123)	≥96 (124)	≥96 (122)	≥96 (102)	≥96	≥96

[a]If the mean abundance in the controls is 0, a percent minimum detectable difference cannot be calculated. Signs indicate the direction of the significant deviation from controls.
[b]Lowest consistent NOEC = significant effects found over at least 2 consecutive samplings.
[c]Long-term NOEC = effects found over at least 8 wk or until or at the end of the study.
n.c. = not calculated; NOEC = no-observed-effect concentration.

Table 2. NOEC values (as nominal μg Ni/L) and minimum detectable difference (as percentage in parentheses) for the abundances of zooplankton and snails with sufficiently low minimum detectable difference (see text) or significant differences from controls found over at least 2 consecutive samplings[a]

Taxon	Days after start of exposure												Consistent NOEC[b]	Long-term NOEC[c]
	0	2	7	14	21	28	42	56	70	84	98	112		
Total zooplankton	≥96 (23)	≥96 (38)	≥96 (31)	48+ (52)	48+ (51)	≥96 (77)	≥96 (65)	≥96 (62)	48+ (63)	48+ (66)	≥96 (64)	48+ (55)	48+	48+
Rotifera	≥96 (37)	≥96 (63)	≥96 (58)	48+ (57)	48+ (50)	≥96 (85)	≥96 (70)	≥96 (65)	48+ (67)	48+ (83)	48+ (73)	48+ (77)	48+	48+
Copepoda	≥96 (43)	≥96 (44)	≥96 (62)	≥96 (65)	≥96 (80)	≥96 (80)	≥96 (80)	≥96 (62)	48–(58)	≥96 (75)	≥96 (73)	≥96 (82)	≥96	≥96
Phyllopoda	≥96 (57)	≥96 (44)	≥96 (29)	≥96 (64)	≥96 (70)	≥96 (91)	≥96 (85)	≥96 (71)	≥96 (72)	≥96 (44)	≥96 (60)	≥96 (44)	≥96	≥96
Nauplia	≥96 (44)	≥96 (49)	≥96 (62)	≥96 (68)	≥96 (81)	≥96 (88)	≥96 (83)	≥96 (59)	≥96 (71)	≥96 (94)	≥96 (82)	≥96 (86)	≥96	≥96
Daphnia magna	≥96 (50)	≥96 (60)	≥96 (37)	≥96 (60)	48+ (64)	≥96 (95)	≥96 (95)	≥96 (69)	≥96 (76)	≥96 (47)	≥96 (58)	≥96 (49)	≥96	≥96
Daphnia longispina	≥96 (54)	≥96 (46)	≥96 (41)	≥96 (65)	≥96 (64)	≥96 (89)	≥96 (89)	≥96 (65)	≥96 (66)	≥96 (53)	≥96 (60)	≥96 (50)	≥96	≥96
Acroperus harpae	≥96 (65)	≥96 (53)	≥96 (50)	≥96 (73)	≥96 (80)	≥96 (93)	≥96 (79)	≥96 (83)	≥96 (84)	48– (57)	≥96 (72)	≥96 (74)	≥96	≥96
Simocephalus vetulus	≥96 (244)	≥96 (87)	≥96 (80)	48+ (64)	≥96 (99)	≥96 (114)	≥96 (94)	≥96 (94)	≥96 (83)	≥96 (65)	≥96 (62)	≥96 (62)	≥96	≥96
Keratella quadrata	≥96 (54)	≥96 (66)	≥96 (73)	≥96 (80)	48+ (79)[d]	≥96 (95)	≥96 (86)	≥96 (71)	48+ (77)	48+ (89)	48+ (83)	≥96 (87)	48+	≥96
Keratella cochlearis	≥96 (33)	≥96 (73)	≥96 (83)	≥96 (87)	≥96 (91)	24+ (68)	≥96 (69)	≥96 (51)	≥96 (67)	24– (66)	≥96 (85)	≥96 (104)	≥96	≥96
Brachionus sp.	≥96 (75)	≥96 (76)	≥96 (80)	≥96 (50)	≥96 (23)	≥96 (46)	≥96 (49)	≥96 (58)	≥96 (51)	≥96 (54)	≥96 (43)	≥96 (62)	≥96	≥96
Hexarthra sp.	≥96 (69)	≥96 (60)	48+ (40)	12+ (72)	≥96 (46)	≥96 (114)	≥96 (96)	≥96 (67)	≥96 (55)	≥96 (73)	≥96 (84)	≥96 (77)	48+	≥96
Chephalodella sp.	≥96 (109)	24+ (201)	≥96 (96)	≥96 (112)	≥96 (99)	≥96 (126)	≥96 (123)	24+ (167)	≥96 (64)	≥96 (75)	≥96 (108)	48– (58)	≥96	48–
Total snails	≥96 (62)		≥96 (74)	≥96 (74)	≥96 (66)	≥96 (72)	≥96 (86)	24– (83)	24– (81)	24– (87)	24– (90)	24– (84)	24–	24–
Lymnaea sp.	≥96 (58)		≥96 (38)	≥96 (60)	≥96 (42)	≥96 (48)	≥96 (62)	24– (78)	24– (49)	24– (44)	24– (49)	24– (55)	24–	24–
Small snails (<5 mm)	≥96 (83)		≥96 (87)	≥96 (92)	≥96 (86)	≥96 (98)	≥96 (93)	≥96 (98)	24– (93)	24– (94)	≥96 (107)	24– (95)	24–	24–

[a]If the mean abundance in the controls is 0, a percent minimum detectable difference cannot be calculated. Signs indicate the direction of the significant deviation from controls.
[b]Lowest consistent NOEC = significant effects found over at least 2 consecutive samplings.
[c]Long-term NOEC = effects found over at least 8 wk or until or at the end of the study.
n.c. = not calculated; NOEC = no-observed-effect concentration.

whereas effects until the end of the exposure were only found at 48 μg/L and 96 μg/L (long-term NOEC = 24 μg/L).

The principal response curves explained a significant part of the total variance in the phytoplankton data set and indicated long-term deviations of the phytoplankton community structure from controls at 96 μg/L and less pronounced and temporarily restricted deviations at 48 μg/L (Figure 4). The highest positive species weight (indicating the degree of correspondence of the population dynamics of a species with the global pattern of the principal response curves) was calculated for *Chroomonas/Rhodomonas* sp. Several other taxa showed also high positive weights, while the diatom *Nitzschia palea* and the green algae *A. judayi* had high negative weights, indicating that abundances of these taxa were promoted by the treatment. Redundancy analysis per sampling date revealed a significant effect of the Ni treatment from day 21 to day 56, whereas the Williams test applied to the sample scores of principal component analysis only found significant differences for the 48 μg/L and 96 μg/L levels on day 21 and day 28. The community-level phytoplankton NOEC can therefore be concluded to be 24 μg/L.

No consistent deviations from the controls were indicated for the chlorophyll and pheophytin measurements of the phytoplankton and the periphyton. The dry weight of periphyton scraped from a larger defined area of the microcosms' walls at the end of the study to analyze Ni showed considerable variability within the controls, but periphyton biomass was significantly higher at the 2 highest treatment levels (Williams test, $\alpha = 0.05$, one-sided; see Supplemental Data 3, Table S8).

The zooplankton community was dominated by rotifers (55% of all individuals counted in the 196 samples), followed by Phyllopoda (35%) and Copepoda (10%). The principal response curves for the zooplankton did not explain a significant part or the total variance in the data set ($p = 0.258$), and thus no further ordination analysis was conducted. The rotifer *Keratella quadrata*, the most dominant zooplankton species in the present study, showed lower abundances over several weeks until the end of the study at 48 μg/L, whereas at 96 μg/L the abundances were significantly above the mean of the controls (Table 2 and Figure 5A for total rotifer abundance). No consistent significant deviations from controls over at least 2 consecutive samplings were found for other taxa (Figure 5B,C; Supplemental Data 3, Table S7; for more details, see Supplemental Data 1 and 2). Thus, based on the data for *K. quadrata*, the long-term NOEC for the zooplankton is 24 μg/L.

Lymnaea stagnalis was intentionally introduced as described in *Materials and Methods*, but other species were probably introduced via the sediment. The majority of the snails counted during the study (86.6%) were not further identified and were operationally defined as "small snails <5 mm"; 12% were identified as *Lymnaea* sp., and 1.5% were identified as *Planorbarius* sp. A clear effect on abundance of total snails, *Lymnaea* sp., and small snails was observed at 48 μg/L and 96 μg/L, where numbers decreased after 4 wk of exposure and no snails were found after 12 wk of exposure (Figure 6A). At 6 μg/L, 12 μg/L, and 24 μg/L, the mean abundances were always within or above the range of the controls and close to their mean in the last 8 wk of the study (NOEC = 24 μg/L; Table 1). However, the snail abundance also differed between the treatment levels at the start of the exposure, with mean abundance at 12 μg/L and 24 μg/L, which was clearly above the range of controls until day 28 after the start of exposure. To consider the variable snail abundance before the start of exposure and to analyze the trend of the population dynamics, the slopes of log-linear regressions of abundance over time for

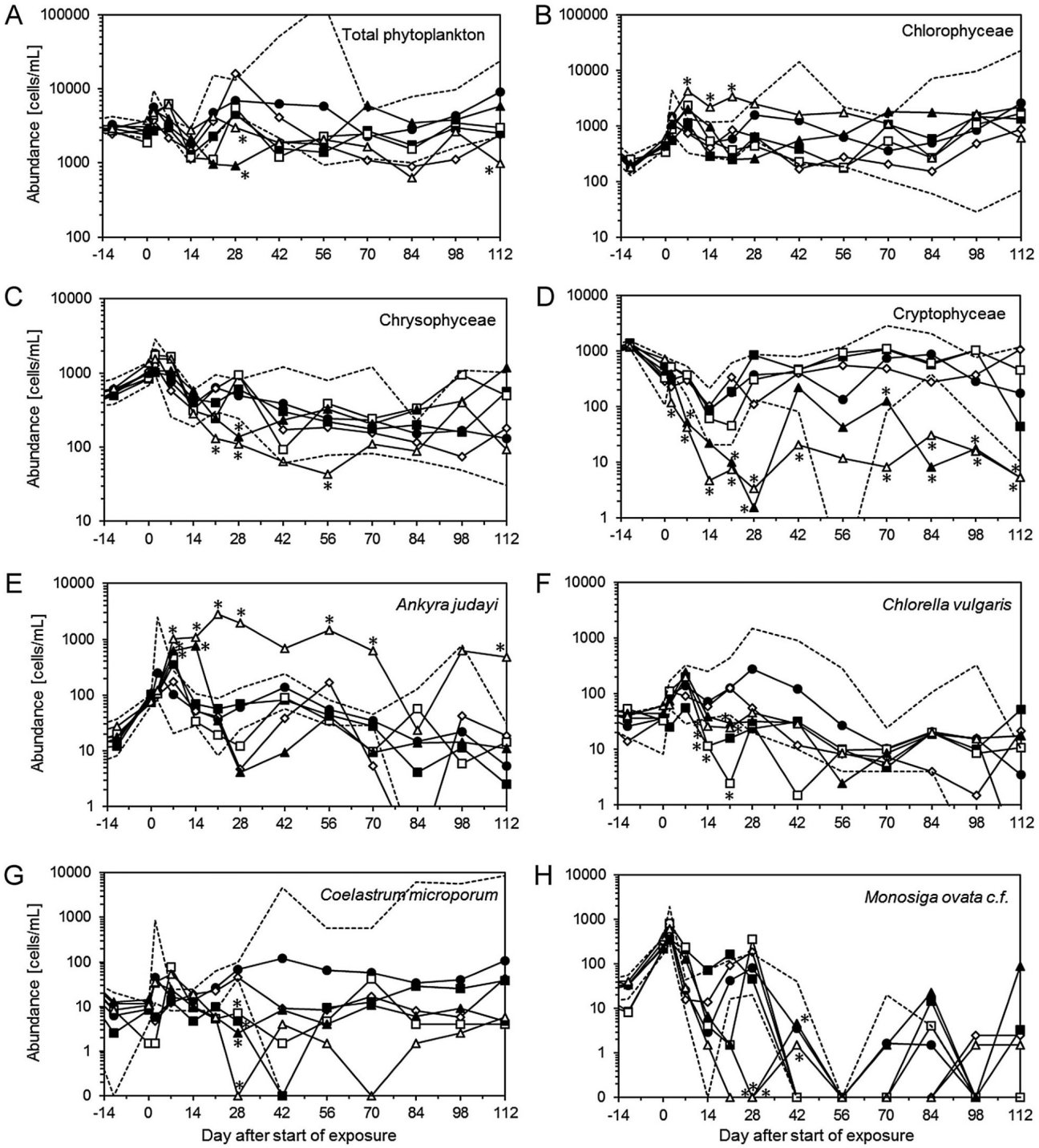

Figure 3. Population dynamics of total phytoplankton (**A**) and sensitive taxa (**B–H**). Geometric mean per treatment level and range of controls. *Significant effect according to the Williams test (see Table 1).

each microcosm were calculated (Figure 6B). Assuming that the rate of increases and decreases in snail abundance were exponential, these slopes represent the average rate of population change (increase or decrease) over the exposure period. This analysis shows that the snail population increased significantly over the 4 mo in only 1 of the 4 control replicates (microcosm 2). In the other controls and in the microcosms treated with 6 μg/L, the abundance was relatively stable, with slopes close to or slightly below 0. Up to 12 μg/L, the mean slope per treatment level was not significantly different from the slope of the controls, regardless of whether control microcosm 2 was included in the analysis or considered as an outlier (NOEC = 12 μg/L, Williams test, one-sided, $\alpha = 0.05$).

Egg clutches were rarely found during the study. In 3 microcosms, 1 clutch was found once during the exposure period. In 1 of the controls, 6 clutches were found in total (1 clutch on day 28, 1 on day 42, 3 on day 56, and 1 again on day 112). As a result, the high total number of snails in microcosm 2 reflects the hatchlings from the few clutches, whereas the number of larger *Lymnaea* was comparable to that in the other microcosms. Approximately 98% of the snails found in microcosm 2 at the end of the study were small snails.

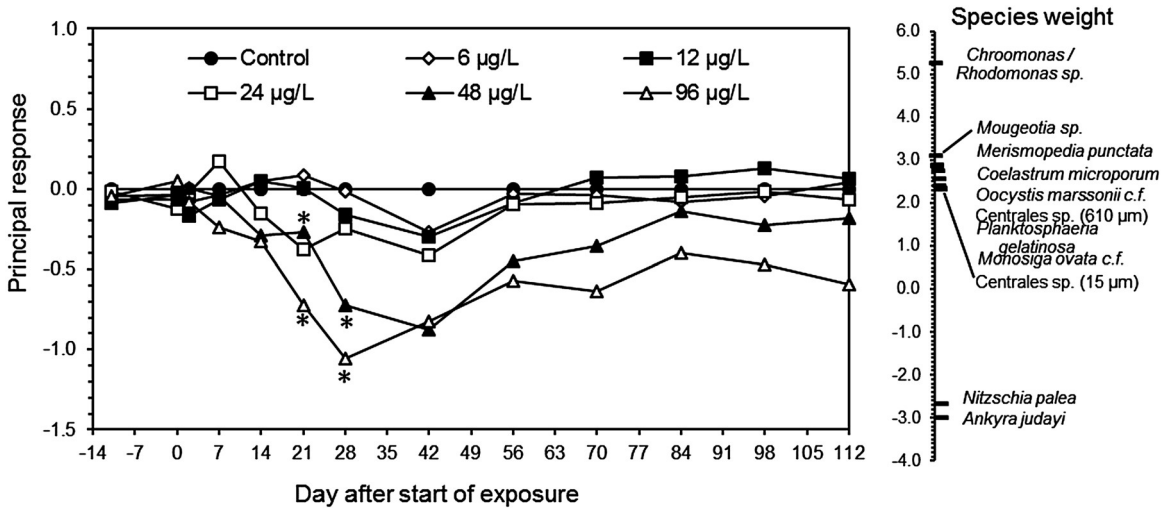

Figure 4. Principal response curves for the phytoplankton (33% of total variance explained by time, 28% explained by treatment, 20% explained by treatment is captured by the principal response curve, p value of principal response curve = 0.024). Only species with weights >2 or <2 are shown. *Significant deviations from sample scores of the principal component analysis per sampling date according to the one-sided Williams test.

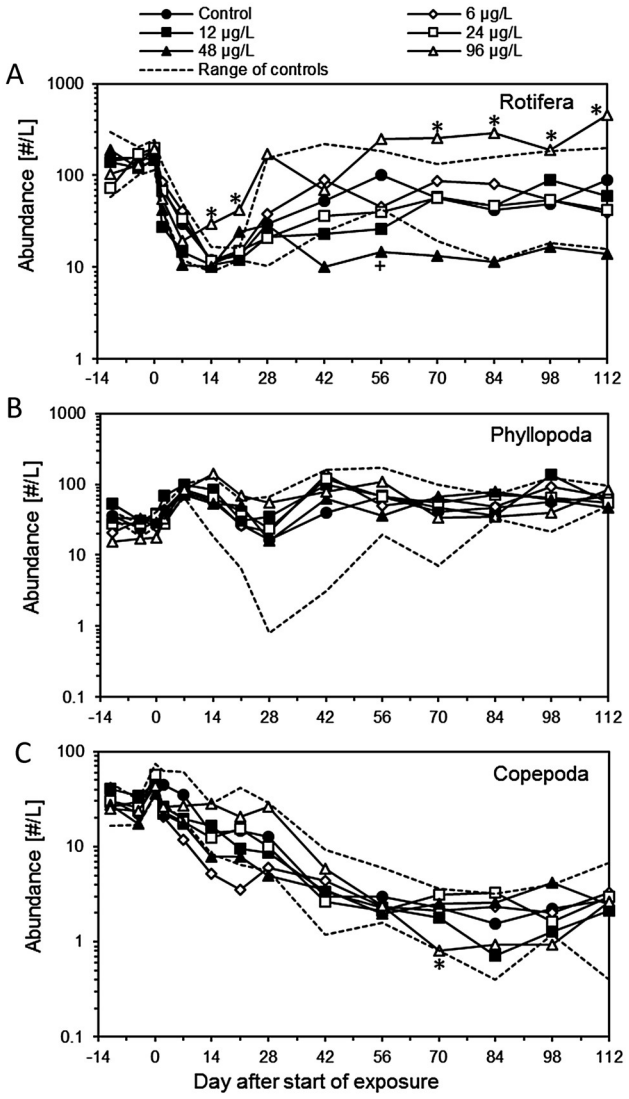

Figure 5. Geometric means per treatment level and range of controls over time for sums of Rotifera (**A**), Phyllopoda (**B**, only Cladocera), and Copepoda (**C**). *Significant deviation from controls according to the one-sided Williams test (see Table 2); +significant deviation based on the 2-sided Dunnett test (conducted for rotifer data).

DISCUSSION

Fate and bioaccumulation

The measurements of Ni in the water confirmed the target chronic exposure of the organisms over the 112-d exposure period. Nickel dissipated quickly from the overlying water, which necessitated adding 14 to 19 times the initial loading over the whole exposure period of 4 mo. From a mass balance perspective, the most likely sink for the added Ni was the sediment phase. On the other hand, the background Ni concentration (approximately 20 mg/kg dry wt) and high variability among replicate samples make it difficult to draw this conclusion with confidence. Nevertheless, the observation that excess amorphous sulfides were present at the end of the exposure indicates the continued potential for net accumulation of Ni in the sediment phase.

The Ni concentrations in periphyton, macrophytes, and snails showed a clear dose-related increase. However, the Ni concentration in the snails was always below that in periphyton and macrophytes, the snail BAFs were lower than the periphyton and macrophyte BCF, and the snail biomagnification factor was always below 1, all of which indicates that biomagnification did not occur in this part of the food chain (periphyton to snails). This conclusion is consistent with an analysis of Ni biomagnification potential for terrestrial food webs [29]. McGeer et al. [30] report in their meta-analysis of metal bioconcentration data a trend of increased Ni body concentrations with increased exposure concentration for aquatic organisms. They also reported that metal BCFs and BAFs (including for Ni) were most commonly inversely correlated with exposure, which they explained as being a consequence of active regulation of metal uptake and elimination mechanisms. In the present study, we did not find any significant relation between metal BCF or BAF and the exposure concentration, suggesting that no significant active regulation occurred at the concentrations investigated.

Effects

The established chronic exposure to 48 µg Ni/L and 96 µg Ni/L resulted in long-term adverse effects on some algae (i.e., *Chroomonas acuta/Rhodomonas* sp.) and the snails. This probably had indirect promoting effects on other algae (e.g.,

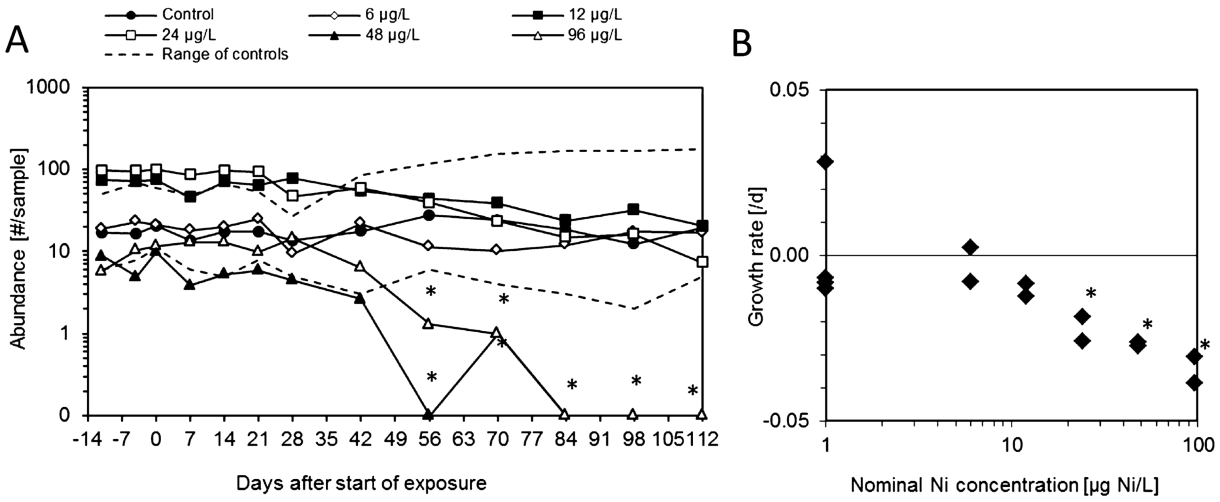

Figure 6. Total abundance of snails. (**A**) Geometric means per treatment level and range of controls over time. (**B**) Slope of linear regressions of log-transformed abundance versus time in individual microcosms. *Significant effect ($p < 0.05$) according to the one-sided Williams test.

some green algae) because of reduced competition and periphyton biomass as a result of reduced grazing. Such indirect effects are often observed in microcosm and mesocosm studies [31]. Rotifers, such as the dominating *K. quadrata*, showed a nonlinear response with abundances below the range of controls at 48 µg/L and above the range of controls at 96 µg/L. The only rotifer species included in the Ni species sensitivity distribution [4], *Brachionus calicyflorus* (mean normalized NOEC or EC10 = 460 µg/L; see Supplemental Data 3, Table S2) is 1 to 2 orders of magnitude less sensitive than the algae, cladocerans, and snails. Thus, a direct effect of the Ni exposure in the microcosms on rotifers seems to be unlikely, and the findings can be explained either as an indirect effect—for example, as a result of shifts in the phytoplankton community structure—or as stochasticity (variability between replicates).

During the exposure period, the total snail abundance at 12 µg/L and 24 µg/L declined to the level of the controls, and the rate of this population decline was significantly different from that in the controls at 24 µg/L (NOEC = 12 µg/L). However, snail abundances were close to that in the controls over the last 10 wk of the exposure period up to 24 µg/L, and the exposure to Ni is only 1 factor that might have affected the decline of the snail population at 24 µg/L. In general, the physical and chemical conditions of the microcosms may not have been favorable for snail reproduction. For example, the reduced light intensity, used to avoid a further increase of pH because of primary production, may have indirectly caused a reduction in nutritional sources of the snail community. The 12 µg Ni/L and 24 µg Ni/L microcosms showed mean abundance above the range of the controls at the start of the exposure, suggesting that the initially higher snail density could have contributed to the population decline observed in these cases by density-dependent mechanisms, that is, a decline as a result of abundances that were initially above carrying capacity. It is also possible that the snails might have experienced physiological dormancy typical of winter months that was triggered by the photoperiod and light intensity, despite the fact that the water temperature was controlled around 20 °C. These factors may have contributed to the generally small or even negative population growth and the small number of observed egg clutches. Because of the low number of egg clutches found in total and the missing egg clutch findings in 3 of the 4 controls, it is difficult to assess potential effects of Ni on the reproduction of snails in the present study. One egg clutch was found on day 98 in a 24 µg Ni/L microcosm, which indicates that at least clutch deposition was possible at this exposure level.

The study-specific NOEC is considered to be the highest concentration at which no long-term effects of the exposure over 4 mo on the populations and community structure were found. Because of these constant exposure conditions, short-term deviations from controls with demonstrated recovery within the study period were not considered to be adverse effects (e.g., the significantly lower abundance of *C. vulgaris* only on day 14 and at 24 µg/L on day 14 and day 28). No long-term effects were found on algae and zooplankton at concentrations up to 24 µg/L. Also at this concentration, no differences in the numbers of snails could be found. The NOEC of 12 µg Ni/L for the time trend of the snail population density was therefore the lowest long-term NOEC found in the present study. It is consistent with recent studies that highlight the sensitivity of the snail *L. stagnalis* to Ni [32,33]. Thus, 12 µg/L was considered to be the study-specific NOEC.

Relevance for Ni risk assessment

The community in the microcosms included several species closely related to the most sensitive species in the chronic data ecotoxicity database [4], that is, several cladoceran species and the snail *L. stagnalis* (see Supplemental Data 3, Table S2). Several phytoplankton species of classes other than Chlorophyceae were present; among these, Cryptophyceae has not been tested in the laboratory but was found to be the most sensitive algae group in the microcosms (long-term NOEC = 24 µg/L). Macroinvertebrates other than snails, such as insect larvae and benthic crustaceans, as well as fish and macrophytes were not investigated in the microcosms. However, results of previous studies suggest that these groups are less sensitive than *Lymnaea*, cladocerans, and algae [4,10,32,33].

The power of the statistical analysis conducted was sufficient for statistical analysis according to the recommendations by Brock et al. [28] for at least 20 different taxa, including representatives of sensitive taxa, such as *Lymnaea* sp., cladocerans, and green algae. In addition, multivariate analysis (principal response curve) was applied to the phytoplankton and zooplankton data set to evaluate potential effects on the community structure, also taking into account those taxa which were too rare for reliable univariate analysis.

The microcosm study should represent realistic worst-case conditions of high bioavailability of Ni. This has been shown already by application of the chronic Ni bioavailability models. The bioavailability-normalized HC5 values varied between 4.2 μg Ni/L and 6.8 μg Ni/L (see Supplemental Data 1), which is close to the bioavailable environmental quality standard of 4 μg/L proposed by the European Union and referring to a safe Ni concentration in waters with high Ni bioavailability [12]. It is also below all HC5 values for the 7 scenarios (assumed to be relevant for a range of common water types across the European Union) that were evaluated in the European Union risk-assessment report (HC5 = 7.3–43.6 μg/L [4]). By aeration, reduction of illumination, and removal of macrophytes, pH values were approximately 8.5 during the exposure period, which is considered a reasonable compromise between water conditions for diverse and abundant populations and applicability of the Ni biotic ligand model. However, the bioavailability had to be applied to pH values slightly above their upper development and validation boundary of 8.2. However, pH values above 8.2 are not unusual for European Union water bodies; thus, in the meantime, a refined bioavailability calculation tool [34] has become available to cover a broader range of water conditions.

The proposed microcosm-specific NOEC of 12 μg/L is approximately a factor of 2 higher than the microcosm-specific HC5 and above the normalized NOEC or EC10 of *L. stagnalis* (2.3–4 μg/L; see Supplemental Data 3, Table S2) as the most sensitive species in the chronic toxicity database in the water chemistry of the present microcosm study. These results are supported by those of Peters et al. [32], who evaluated monitoring data for benthic macroinvertebrates and water chemistry parameters for streams in England and Wales with respect to biological effects of potential Ni exposures. Through bioavailability normalization (with a slightly simplified version of the method used in the present study) of all monitored Ni concentrations to the same conditions (i.e., water chemistry of high bioavailability as those conditions to which the bioavailable environmental quality standard refers, 4 μg/L [12]), they concluded that in order to induce changes in snail abundance in the field, bioavailable Ni concentrations likely need to exceed 3.9 μg/L. Thus, the microcosm study confirms the protectiveness of the laboratory toxicity data-based and bioavailability-normalized HC5 values (determined with the species sensitivity distribution) and of the field analysis of Peters et al. [32].

A final interesting observation follows from the comparison of the normalized NOEC values for green algae NOECs with the NOEC for phytoplankton community dynamics observed in the microcosm, that is, 24 μg Ni/L. The latter value is clearly lower than all algae NOECs in the toxicity database, that is, between 38.5 μg Ni/L and 50.5 μg Ni/L for the most sensitive alga (*Scenedesmus accuminatus*) and between 120 μg/L and 150 μg/L for the least sensitive (*Chlorella* sp.). Thus, when normalized with the algae biotic ligand model, the single-species toxicity data with green algae species appear not to be protective of overall phytoplankton community dynamics. This suggests that some species groups (in the present study, phytoplankton) may be more sensitive in a realistic community context than what is inferred from laboratory single-species toxicity data (in the present study, only green algae toxicity data were available). However, because phytoplankton is not the most sensitive component of the freshwater community (both in the chronic toxicity database and in the present microcosm study), it does not affect the overall conclusion that the bioavailability-normalized HC5 is protective relative to the whole microcosm (i.e., study-specific NOEC > HC5).

CONCLUSIONS

The present study allowed analysis of direct and indirect effects of chronic Ni exposure over 4 mo on a diverse community of algae, zooplankton, and snails. No indication of biomagnification in snails was found. The study-specific NOEC based on the most sensitive end points studied, the population growth rate of the snails, is 12 μg total Ni/L (with 98% as dissolved Ni per average). Direct effects on snails and algae at higher concentrations probably resulted in indirect effects on periphyton and zooplankton. The microcosm study supports the protectiveness of site-specific, bioavailability-normalized HC5 values from species sensitivity distributions.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3255.

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Data availability—Part of the data are available in the Supplemental Data. Additional data can be accessed from the corresponding author on request: udo.hommen@ime.fraunhofer.de.

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