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A LONG-TERM COPPER EXPOSURE IN A FRESHWATER ECOSYSTEM USING LOTIC MESOCOSMS: INVERTEBRATE COMMUNITY RESPONSES

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Abstract: A lotic mesocosm study was carried out in 20-m-long channels, under continuous, environmentally realistic concentrations of copper (Cu) in low, medium, and high exposures (nominally 0, 5, 25, and 75 $\mu g\,L^{-1}$; average effective concentrations <0.5, 4, 20, and 57 $\mu g\,L^{-1}$ respectively) for 18 mo. Total abundance, taxa richness, and community structure of zooplankton, macroinvertebrates, and emerging insects were severely affected at Cu treatment levels of 25 and 75 $\mu g\,L^{-1}$. Some taxa were sensitive to Cu, including gastropods such as *Lymnaea* spp. and *Physa* sp., crustaceans such as *Chydorus sphaericus, Gammarus pulex*, and *Asellus aquaticus*, rotifers such as *Mytilina* sp. and *Trichocerca* sp., leeches such as *Erpobdella* sp., and the emergence of dipteran insects such as Chironomini. Other taxa appeared to be tolerant or favored by indirect effects, as in Chironimidae larvae, the emergence of Orthocladiinae, and the zooplankter *Vorticella* sp., which increased in the 25 and 75 $\mu g\,L^{-1}$ treatments. After approximately 8 mo of Cu exposure, the macroinvertebrate community in the high treatment was decimated to the point that few organisms could be detected, with moderate effects in the medium treatment, and very slight effects in the low-Cu treatment. Subsequently, most taxa in the high-Cu exposure began a gradual and partial recovery. By the end of the study at 18 mo, macroinvertebrate taxa richness was similar to control richness, although overall abundances remained lower than controls. After 18 mo of copper exposure, a no-observed-effect concentration at the community level for consumers was set at 5 $\mu g\,L^{-1}$ (4 $\mu g\,L^{-1}$ as average effective concentration), and a lowest-observed-effect concentration at 25 $\mu g\,L^{-1}$(20 $\mu g\,L^{-1}$ as average effective concentration). *Environ Toxicol Chem* 2017;36:2698–2714. © 2017 SETAC

Keywords: Zooplankton Macroinvertebrates Emerging insects Copper Mesocosms Tolerance Recovery

INTRODUCTION

Copper exists naturally in a variety of mineral forms (cuprite, malachite, and ores) and is associated with aqueous discharges from mining, metal plating, power generation, and the manufacturing of electrical equipment. Copper pollution may be of concern in aquatic environments because they are receptors of urban wastewater, industrial and mine effluents, agriculture runoff, and atmospheric deposition [1]. In European freshwaters that were considered to be in a mostly natural state, copper concentrations commonly range from approximately 0.4 to 16 $\mu g/L$ in unfiltered water samples [2]; yet in severely polluted industrial settings such as streams receiving unmanaged mine drainage, concentrations can be orders of magnitude higher [3].

Copper is an efficient fungicide, bactericide, plant herbicide, molluscicide, and algicide, and can thus be considered as a nonspecific toxicant [1]. Copper at trace concentrations is an essential element, but at higher concentrations, in excess of nutritional needs, copper can cause internal hydromineral regulatory functions to be overwhelmed, stressing or killing organisms [4]. The bioavailability and hence the toxicity of copper in freshwaters is strongly influenced by water chemistry, with the pH and dissolved organic carbon (DOC) being particularly influential parameters [5]. The differing physiological sensitivity of organisms comprising the exposed communities can also influence the toxicity of copper. The mechanisms behind the differing physiological sensitivities of aquatic organisms to copper are incompletely understood, but small size and differing capacities for depuration or sequestration of excess copper are likely factors [6].

Because of their abundance and position in the aquatic food chain of lotic systems, invertebrates play a critical role in the natural flow of energy and nutrients [7,8]. Owing to their ecological importance and vulnerability to elevated copper concentrations, numerous studies of the long-term effects of copper on freshwater invertebrates at the community level have been reported. Examples of prior community-level work include stream ecosystem field experiments [9,10], field surveys of copper-contaminated streams from mining [3,11,12], lentic mesocosm tests [13–15], and lotic laboratory microcosm and stream-side tests [16–18]. In the present study, we build on this previous knowledge by evaluating the effects of copper in long-term exposures in lotic mesocosm experiments. Our work differs from previous lotic experiments in both spatial and temporal scales. Where previous lotic experiments sought to represent small-stream communities, we evaluated an ecological scenario more reminiscent of large, low-gradient rivers with mixed pelagic planktonic and benthic communities along with one fish species. The 18-mo duration exposures were long enough to encompass seasonal changes and reproductive cycles.

In this context, the primary objective of the present study was to evaluate the effects of copper on freshwater invertebrate

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communities in a low-gradient river system exposed to copper using an outdoor lotic mesocosm experimental design. The effects of copper on zooplankton, macroinvertebrates, and emerging insects were studied over 18 mo using environmentally realistic concentrations. We hypothesized that copper would reduce the abundance and diversity of freshwater invertebrates, especially gastropods, in virtue of its high toxicity to molluscs [19]. We also suspected indirect effects through ecological cascades at the community level. Previous published reports from this experiment include individual- and population-level effects of copper exposures on a small-bodied fish species, the 3-spined stickleback (*Gasterosteus aculeatus*) [20], effects on primary producers [2], and on litter decomposition processes [21].

Secondarily, the results from this experiment serve to evaluate ecological risk assessment approaches for protecting aquatic environments. The approach used in the European Union risk assessment report for copper in freshwaters is to calculate predicted-no-effect concentrations (PNECs), which serve as a "safe" benchmark for copper concentrations that would generally protect aquatic communities. The PNECs are calculated by compiling and averaging the no-observed-effect concentrations (NOECs) from standardized, laboratory single-species chronic toxicity tests into mean values for each tested species, ranking the species mean values, and then calculating concentration hazardous to the 5th percentile of the ranked species sensitivity distribution (HC5). In effect, this approach assumes that a compilation of toxicity results conducted with cultured organisms under artificial laboratory conditions would reflect ecosystem responses. Furthermore, it assumes that allowing the most sensitive 5% of the taxa in aquatic ecosystems to experience some level of harm would still sufficiently protect ecosystems [22,23]. These assumptions have been controversial [24,25]. Comparison of the theoretical PNEC concentrations of European freshwaters with observed effects from our long-term mesocosm ecosystem approaches provides a field test of the PNEC extrapolation procedure.

MATERIALS AND METHODS

Mesocosm set-up and copper exposure

The experimental platform (INERIS, Verneuil-en-Halatte, France) was composed of 12 mesocosms, each 20 m long and 1 m in width. Each mesocosm had 2 sections. The upper section contained coarse grain sediments, mainly pebbles, with a water depth of 0.3 m. The lower section was filled with fine-grain sediments with a water depth of 0.7 m (Figure 1A and B). Overall, the water volume for each mesocosm was 10.9 m^3. Fine-grain sediment was made of a mixture of natural and artificial sediment at a proportion of 15% natural and 85% artificial sediment. Artificial sediment was composed of 65.4% sand and 14.3% clay. Natural sediment was taken from the Aronde Stream near Gournay-sur-Aronde in France (Supplemental Data, SI-1, Figure SI).

Mesocosms were set up with macrophytes, phytoplankton, periphyton, benthic and pelagic invertebrates, decomposer microorganism inocula, and one fish species (*G. aculeatus* L.) coming from nearby unpolluted streams (Figure 1A and C). The macrophytes that were introduced in the upper part were *Callitriche platycarpa* Kütz and *Nasturtium officinale* R. Br., and in the lower part they were *C. platycarpa*, *Iris pseudacorus* L., *Myriophyllum verticillatum* L., and *Nymphaea alba* L. (Supplemental Data, SI-1, Figure S2). The introduced invertebrates were mainly scrapers (gastropod species, e.g., *Lymnaea* sp., *Radix balthica*), shredders (*Gammarus pulex*, *Asellus aquaticus*), collector–gatherers (e.g., Limnephilidae), and invertebrate predators (e.g., *Glossiphonia*, *Libellula*). The species were selected according to their trophic level along with their structural and functional relevance in aquatic ecosystems. Equal stocking densities were introduced in each mesocosm. Other invertebrates, such as larvae of Chironomidae, naturally colonized the mesocosms mainly by aerial deposition of eggs by adults.

A tap water flow rate of 800 L h^{-1} was maintained in each mesocosm. The water velocities were respectively 2.7 and 1.1 m/h for the upper and lower sections, which resulted in an average water transit time through the mesocosms of approximately 12 to 13 h, or approximately 2 volume replacements/d. A 2-mm mesh was placed at the outlet of the mesocosms to avoid adult macroinvertebrate and fish drift. Constructed mesocosms represent low-gradient shallow water ecosystems (Figure 1; Supplemental Data, SI-1, S1).

For 18 mo and using an automated system, a continuous dose of copper from stock solutions using copper sulfate pentahydrate ($CuSO_4$, $5H_2O$; Acros Organics) was performed at 3 nominal concentrations in triplicate at 5, 25, and 75 μg L^{-1} corresponding to low, medium, and high treatments. Three controls were also established (Supplemental Data, Figure S3 and Table S1). Mesocosms were set up gradually between September and December 2001 and left to settle before the beginning of exposure, which started on the 15 April 2002 and ended 18 mo later on 15 October 2003. More details about the mesocosm set-up can be found in Roussel et al. [2].

Total copper was measured every week at the inlet of each mesocosm for the first 5 mo to check the validity of the dosing system. Water samples were taken in each mesocosm each month at 5, 10, 15, and 19 m at a depth of 0.2 m and pooled to measure the dissolved copper concentrations. The water samples were filtered with a 0.45-μm pore prior to analysis. Two mesocosms were sampled for each treatment. Spatial distribution of dissolved copper along the mesocosms was determined in the 4 other mesocosms (one for each concentration) with samples taken at 5, 10, 15, and 19 m. Total and dissolved copper were analyzed by graphite furnace atomic absorption spectrometry (SpectrAA 220 Zeeman; Varian), in accordance with the norm NF EN ISO 15586 [26]. An average effective concentration for dissolved copper was calculated for each mesocosm, following the method of Van Wijngaarden et al. [27] to integrate concentrations over space and time. The method averages the results of measurements at several depth-integrated sampling locations in the macrophyte-dominated and macrophyte-free locations within the mesocosm [27]. In addition, free copper analyses were performed once on samples collected in September 2003. This analysis was performed by the WRc-NSF Medmenham Water Research Centre (Medmenham, UK) by anodic stripping voltammetry [28].

Water quality was followed every week during the first month of dosing. Because the water quality was relatively constant, the sampling period was extended to every 2 wk for the following 7 mo and finally to once a month until the end of the experiment. Chemical analyses were Al, Cl, Fe, Si, Ca, Mg, Na, K, NH_4^+, $CaCO_3^-$, NO_3^-, PO_4^{3-}, SO_4^{2-}, suspended matter, and total and dissolved organic carbon. Routine water quality parameters, such as pH, temperature, conductivity, and dissolved oxygen were measured every week in each mesocosm.

Total copper concentrations were also measured in sediment samples of each mesocosm taken at 11, 13, 15, 17, and 19 m length after 3, 6, 12, and 18 mo of exposure. A Perspex core

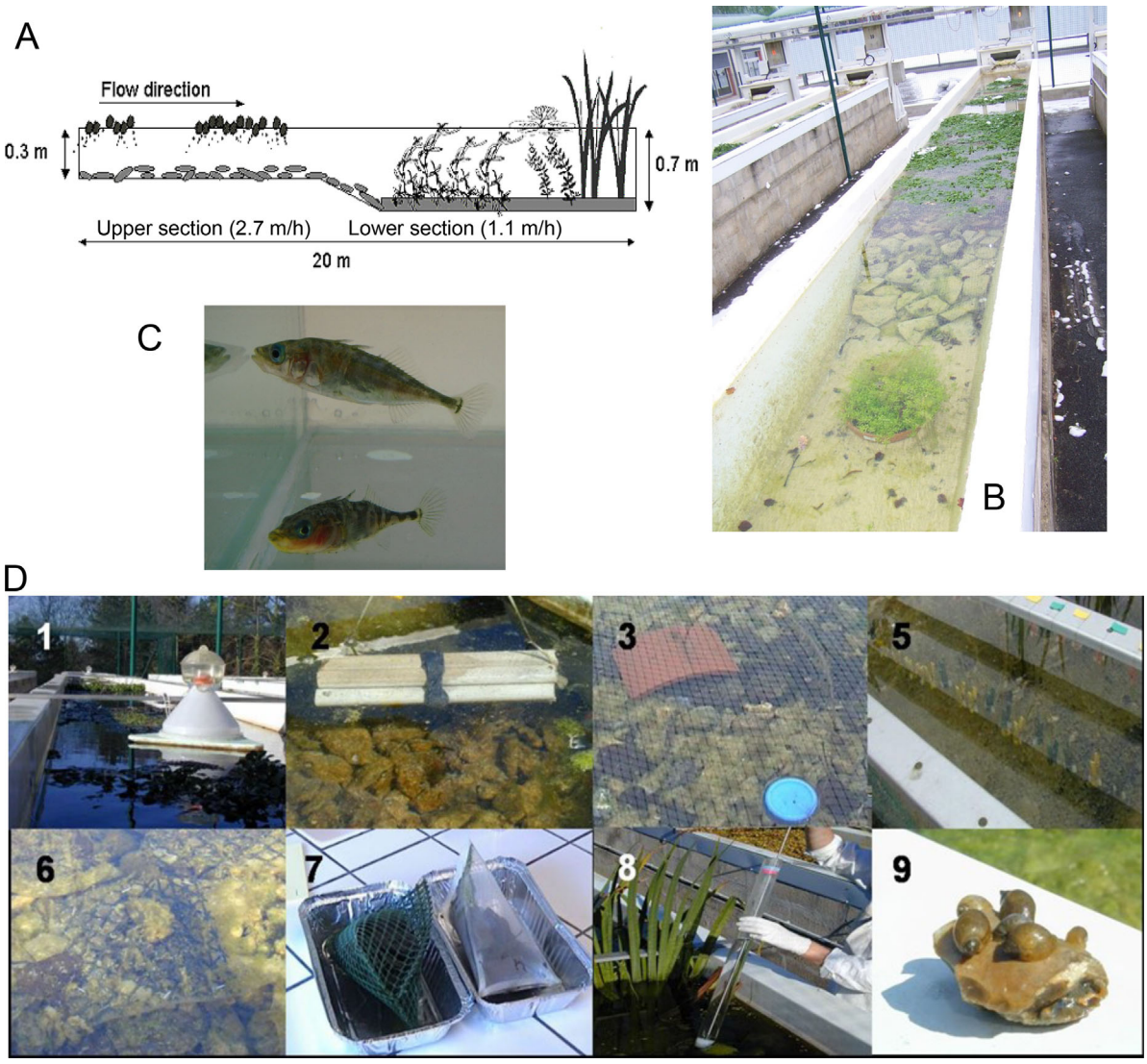

Figure 1. Overview of key mesocosm features and types of sampling traps used during the study: (**A**) sketch of the dimensions and average velocities in the upper and lower sections of a mesocosm; (**B**) photo showing transition from shallow to deeper water sections of a mesocosm at the start of the study; (**C**) the three-spined stickleback (*Gasterosteus aculeatus*), a predatory fish introduced in all mesocosms; (**D**) types of sampling traps used: 1) emergence trap, 2) tube substrate for macroinvertebrate sampling, 3) tile substrate for nondestructive macroinvertebrate sampling, 4) landing net traps (no photo available), 5) periphyton sampling device, 6 and 7) litter bags, 8) zooplankton sampler, and 9) gastropods sampled on the lining of a channel. (Photo credit: INERIS)

(5 cm in diameter) was used to obtain the samples. Samples were pooled to analyze the concentration of total copper once they were mineralized with acid digestion, in accordance with the norm NF EN ISO 11885 [29] with inductively coupled plasma–atomic emission spectrometry (Ultima; Jobin Yvon Horiba).

Zooplankton sampling

Zooplankton was sampled every 4 wk in each mesocosm with a Perspex tube, 5 cm in diameter and 0.8 m in height, closed with a positionable silicone cork (Figure 1D). This tool, adapted for shallow water lake plankton sampling, allows sampling of the entire water column [30,31]. A water sample was collected every meter (251 cm^3 in the upper section and 691 cm^3 in the lower section), until 9 L were obtained. Water was then passed through 2-mm, 1-mm, and 53-μm mesh nets. The 2 wider meshes were used to take out most of the filamentous algae from the samples. Zooplankton material on the 53-μm mesh was then fixed with 70% ethanol up to a volume of 50 mL. Before processing, samples were stained with a pink dye (Rose Bengal; Sigma-Aldrich). Subsamples of 1 mL were transferred to a Ward counting wheel, and the organisms were enumerated and identified under a stereo microscope [32–34]. All individuals were identified in the 3 first aliquots. Then, only taxa that had fewer than 30 individuals in the previous aliquots were counted. Up to 10 aliquots were identified to allow rare taxa to be identified [35]. Results were expressed as number of individuals per liter.

Macroinvertebrate sampling

Macroinvertebrates were sampled every 4 wk using different types of artificial substrates: tubes, landing nets, and tiles. Each tube substrate was composed of 7 polyvinyl chloride (PVC) tubes strapped together (2 cm wide and 20 cm long). Four tubes were placed horizontally along each mesocosm at 2, 8, 13, and 19 m at different water depths. At 2 and 13 m, the tubes were placed on the bottom of the mesocosm, representing 30 and 70 cm of water depth. At 8 and 19 m, the tubes were suspended

on stainless steel bars using fishing wire at, respectively, 10 and 40 cm in water depth (Figure 1D). On each sampling date, the artificial substrates were retrieved from each mesocosm using a landing net to prevent the loss of organisms. Each substrate was then washed in a container to remove the invertebrates trapped inside. Each trap was then replaced in each mesocosm. The rinsing water was then passed through a 50-μm sieve. Invertebrates were preserved in 70% ethanol prior to enumeration and identification.

Two landing nets (10 cm wide and mesh size of 50 μm) were filled with pebbles and placed along each mesocosm at 5 and 8.5 m in the coarse-grain sediment. During sampling, the landing nets and the corresponding pebbles were rinsed and scrubbed in a container before being replaced in each mesocosm. The rinsing water was then passed through a 50-μm sieve. Collected invertebrates were preserved in 70% ethanol until enumeration and identification.

Four tiles (11 × 16 × 1.5 cm) were placed horizontally along each mesocosm at 4, 8, 13, and 18 m at different water depths. At 4, 8, and 18 m, the tiles were placed on the bottom of the mesocosm, representing, respectively, 30, 30, and 70 cm in water depth. At 13 m, the tiles were suspended on stainless steel bars using fishing wire at 40-cm water depth (Figure 1D). As for tube substrates, a landing net was used during sampling to prevent the loss of organisms. The tiles were then placed in a dish half-filled with water. As we did not want to remove too many organisms from the mesocosms, enumeration and identification of macroinvertebrates were done immediately before replacing them in each mesocosm. The tiles were then scrubbed clean and also replaced in each mesocosm.

In addition to the use of artificial substrates, sampling of gastropods on the wall of each mesocosm was performed using a landing net. Along each mesocosm at 1, 7, 13, and 19 m (at 1 and 13 m on the left-hand side and at 7 and 19 m on the right-hand side), gastropods were scraped off the wall and placed in a dish half-filled with water. As we did not want to remove too many organisms from the mesocosms, enumeration and identification of gastropods were done immediately before replacing them in each mesocosm.

Macroinvertebrates were identified to the lowest practical taxonomic level (genus except for Chironomidae and Oligochaeta, which were identified to the family level) [8]. For crustaceans and some families of gastropods, a distinction was made between adults and juveniles. For gastropods, individuals with shell size inferior to 5 mm were considered to be juveniles, and individuals with a shell size greater than 5 mm were considered to be adults. For *G. pulex* and *A. aquaticus*, individuals with a body size less than 5 mm were considered as juveniles and as adults when they had a body size larger than 5 mm [36]. All data from the 4 trapping methods for each mesocosm were pooled, and results were expressed as number of individuals per mesocosm.

Emerging insects

Emerging insects were sampled during 2 periods, from March 2002 to October 2002 and from March 2003 to October 2003. Surface-level funnel emergence traps (Figure 1D and Supplemental Data, SI-1, Figure S4) containing formaldehyde at 4% were placed along each mesocosm at 2 and 16 m. Every 2 wk, the lid of the traps was opened, and the organisms were retrieved using a plastic pipettor. Identification of the individuals was performed to the lowest practical taxonomic level (family or subfamily taxonomic level except for chironomids, which were identified to the tribe level [37–39].

An overview of all the compartments, populations, and communities sampled throughout the experiment along with the methodologies is given in Table S2 of the Supplemental Data (SI-1).

Statistical analysis

Prior to analysis, the abundance data for zooplankton, macroinvertebrates, and emerging insects were Ln ($a \times X + 1$) transformed, where X stands for abundance. The value of a was chosen in such a way that when the lowest value of the dataset above 0 was taken for X, $a \times X$ yields 2. This was done to downweight high-abundance values and to approximate a normal distribution of the data (for rationale, see Van den Brink et al. [40]). For total abundance, taxa richness and abundance of certain species of zooplankton, macroinvertebrates, and emerging insects, a Williams test was used to determine the eventual differences between the control and treatments [41]. This test is an analysis of variance (ANOVA) that assumes that the mean response of the variable is a monotonic function of the treatment, and thus greater effects are expected with increasing dose [41]. The tests were performed using the TOXSTAT 3.0 computer program [42].

The responses of the communities to copper treatment were analyzed using the principal response curve (PRC) method [43]. As sampling of emerging insects was discontinuous, 2 PRC analyses were performed for 2002 and 2003. To test the significance of the treatment effects on communities, a Monte Carlo permutation was done following the PRC. Monte Carlo permutation was also performed for each individual sampling date, to test for the significance of the treatment effects. To know which treatment differed from the control for each sampling date, principal component analysis was used. Then a Williams test was applied to the first principal component to determine the NOEC at the community level ($NOEC_{community}$). In addition, NOECs at the population level were calculated for the taxa that had the highest species weight in the PRC. For each season and for the entire experiment (NOEC experiment), effects were considered valid if the treatment effect was observed for at least 2 consecutive sampling dates. Note that species weights between 0.5 and –0.5 were not presented, as they were likely to show a weak or unrelated response. All multivariate analysis were performed using CANOCO version 4 [44]. For further information, see Roussel et al. [2].

When results from our experiment conflicted with published ecotoxicological studies with copper, if sufficient water chemistry was reported, we used the Bio-Met tool to adjust for copper bioavailability differences between study conditions [45]. Bio-Met is a simplified bioavailability calculator that approximates the biotic ligand modeling (BLM) used in the European Union Risk Assessment Report to calculate PNECs for copper as a function pH, DOC, and calcium concentrations in different waters [23,46]. In comparisons among literature reports, we treated the PNEC values as an approximate threshold for the onset of toxicity.

RESULTS

Copper concentrations

The average effective concentration of dissolved copper found in each treatment were stable and close to 80% of the nominal concentrations for all treatments (Table 1). Therefore, we present the treatment results by the nominal control 5, 25, and 75 $\mu g\,L^{-1}$ treatments, rather than by their respective average effective concentrations of <0.5, 4, 20, and 57 $\mu g\,L^{-1}$.

Table 1. Average (± SEM) copper composition of the mesocosm treatments and biological metrics after 6 and 18 mo[a]

	Control	Low copper	Medium copper	High copper
Nominal Cu ($\mu g\,L^{-1}$)	0	5	25	75
Average effective dissolved Cu ($\mu g\,L^{-1}$)	<0.5	4 (± 0.4)	20 (± 0.7)	57 (± 1.1)
Measured free Cu^{2+} ($\mu g\,L^{-1}$) in Sept. 2003	0.026 (± 0.001)	0.022 (± 0.003)	0.033 (± 0.001)	0.050 (± 0.001)
Sediment Cu ($mg\,kg^{-1}$, dry wt, at 18 mo)	17 (± 0.7)	22 (± 3)	80 (± 20)	196 (± 9)
Biological metrics, average (± SEM), end of growing season 1 (Oct. 2002, 6-mo exposure)				
Macroinvertebrate taxa richness	11 (± 0.0)	11 (± 2)	9.3 (± 1.5)	6* (± 1)
Macroinvertebrate total abundance	394 (± 105)	336 (± 57)	133* (± 33)	40* (± 6.5)
Gastropoda abundance	315 (± 109)	258 (± 76)	98* (± 30)	6* (± 1)
Amphipoda abundance	26 (± 9)	27 (± 21)	5* (± 3)	1* (± 1.7)
Isopoda abundance	25 (± 8)	25 (± 12)	6 (± 3)	13 (± 1)
Diptera abundance	3 (± 1)	3 (± 3)	0 (± 0)	16 (± 5)
Cumulative emerging insect abundance	1154 (± 192)	1260 (± 152)	958 (± 49)	550*(± 98)
Zooplankton taxa richness	16.3 (± 0.3)	15.0 (± 0.6)	14.3 (± 0.3)	11.3* (± 1.8)
Zooplankton total abundance	1598 (± 291)	610* (± 424)	792* (± 130)	180* (± 120)
Copepoda–Cyclopoida abundance	135 (± 22)	72* (± 13)	83 (± 21)	24* (± 13)
Cladoceran abundance	33 (± 7.5)	20 (± 1.5)	7* (± 2.9)	6* (± 2.6)
Rotifer abundance	1430 (± 288)	518* (± 108)	701* (± 113)	30* (± 17)
Protozoa abundance	0	0	0	0
Biological metrics, end of growing season 2 (Oct. 2003, 18-mo exposure, test end)				
Macroinvertebrate taxa richness	14 (± 2)	13 (± 0.0)	11.3 (± 2)	12.3 (± 0.5)
Macroinvertebrate total abundance	728 (± 52)	578 (± 145)	517 (± 164)	271* (± 34)
Gastropoda abundance	20 (± 9)	14 (± 3)	347* (± 173)	52 (± 27)
Amphipoda abundance	3 (± 2)	10 (± 10)	0 (± 0)	0 (± 0)
Isopoda abundance	410 (± 75)	305 (± 129)	26* (± 13)	36* (± 21)
Diptera abundance	72 (± 17)	56 (± 12)	60 (± 16)	119 (± 38)
Cumulative emerging insect abundance	334 (± 69)	480 (± 101)	789* (± 98)	633* (± 96)
Zooplankton taxa richness	16 (± 0.6)	16 (± 0)	13.0* (± 0.6)	10.7* (± 0.3)
Zooplankton total abundance	1155 (± 309)	976 (± 85)	710 (± 50)	177* (± 29)
Copepoda–Cyclopoida abundance	464 (± 158)	443 (± 74)	155* (± 23)	55* (± 23)
Cladoceran abundance	50 (± 8.5)	42 (± 5)	24* (± 11)	4* (± 1.8)
Rotifer abundance	642 (± 155)	490 (± 28)	530 (± 80)	118* (± 7)
Protozoa abundance	109 (± 32)	337 (± 186)	118 (± 58)	46 (± 27)

[a]Chemical parameters other than copper did not differ appreciably between treatments and are shown as long-term averages. Selected average unmanipulated chemical parameters, grand means across treatments, ± SEM: dissolved oxygen, 11.2 ± 1.7 mg/L; temperature, 13.6 ± 2.3 °C; pH, 7.6 ± 0.1; alkalinity as $CaCO_3$, 250 ± 25 mg/L; dissolved organic carbon, 1.8 ± 0.7 mg/L; Ca, 119 ± 10.5 mg/L; Mg, 11.6 ± 0.6 mg/L; hardness as $CaCO_3$, 342 mg/L; Na, 11.2 ± 0.7 mg/L; K, 3.1 ± 0.3 mg/L; SO_4, 7.6 ± 4.6 mg/L; Cl, 66.2 ± 11.7 mg/L. See Roussel et al. [2] for more details.
*Significantly different from control ($p < 0.05$) by Williams test.
SEM = standard error of the mean.

Dissolved copper concentrations were relatively constant throughout time for all treatments (Supplemental Data, SI-2, Chemistry). In September 2003, free copper (Cu^{2+}) concentrations were 1.36, 0.26, and 0.08% of the total dissolved copper concentrations in the low, medium, and high treatments, respectively. After 18 mo of continuous exposure, copper concentrations in the sediment reached a level (mean ± standard error of the mean [SEM]) of 22 (± 3), 80 (± 20), and 196 (± 9) mg of copper/kg of dry sediment, respectively, for the low, medium, and high treatments (Table 1). In comparison, a threshold for safe levels for copper in freshwater sediments was estimated at 87 mg copper/kg of dry sediment [23]. As for the unmanipulated chemical parameters, no significant differences were observed between the control and the copper treatments. The mean values for the entire experiment are presented in Table 1, and more information is available in the Supplemental Data and in Roussel et al. [2].

Zooplankton

In total, 21 zooplankton taxa were identified. At the beginning of the experiment in March 2002, the zooplankton community in the control mesocosms was equally dominated by cladocerans, rotifers, and Cyclopoida copepods (Figure 2). The most abundant rotifers were *Colurella* sp., *Lecane* sp., and *Mytilina* sp., and the dominant cladocerans were *Chydorus sphaericus* and *Alona quadrangularis*. At the end of the experiment in October 2003 in the control mesocosms, rotifers were largely dominant, followed by copepods mainly composed of cyclopoids and nauplii. The protozoan ciliate *Vorticella* sp. was integrated into the analysis because of its abundance in our macrophyte-dominated system and was considered as protozooplankton.

Nearly 3 mo after the beginning of the exposure, zooplankton total abundance, in the medium and high treatments, was significantly higher than in the control on only one sampling date in July 2002 (Figure 2). Total abundance was then significantly lower compared with the control (from September and October 2002 for the medium treatment and from September to December 2002 for the high treatment). Thereafter, no significant differences were observed in the medium treatment. As for the high treatment, lower abundance compared with the control continued to be observed in 2003 on 5 sampling dates. The low treatment had a significantly lower abundance than the control on only 2 sampling dates (September and October 2002).

Taxa richness was significantly lower in the high treatment 1 mo after the beginning of the exposure up to the end of the experiment (Figure 3). As for the medium treatment, the number of species was significantly lower after 9 mo of exposure (Williams test, $p < 0.05$) up to the end of the experiment. The low treatment showed significant lower taxa richness in January and March 2003.

The PRCs followed by a Monte Carlo permutation showed that the structure of the zooplankton community treated with copper deviated significantly from the control ($p = 0.005$, Figure 4A). Time explained 40% of total variance and is shown

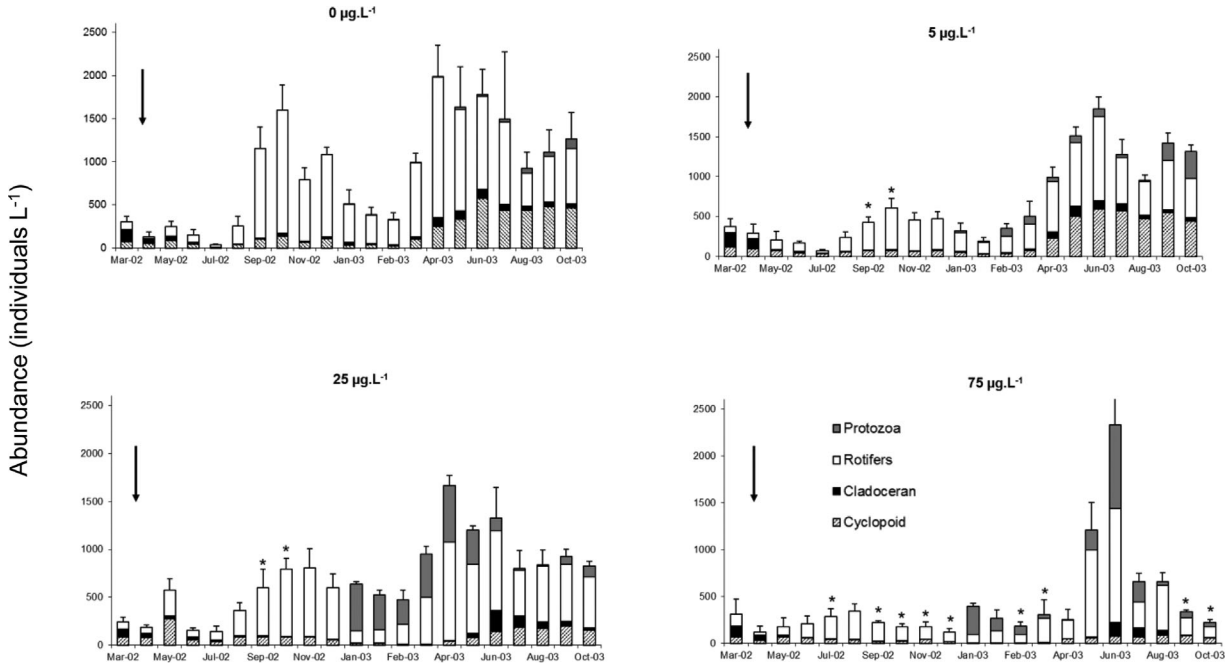

Figure 2. Zooplankton abundance for control and treated mesocosms. Asterisks indicate significant difference relative to controls (Williams test, $p < 0.05$). Arrow indicates the beginning of exposure. Shading indicates different taxa groups (protozoan, rotifers, cladocerans, and cyclopoids).

on the *x* axis, while 40% was also explained by the treatment. Difference between replicates explained 20% of the total variance. The first PRC axis displayed 51% of the variance and was explained by the treatment.

One month after the beginning of exposure, a significant impact on the zooplankton community structure was observed in the high treatment. Similarly, 5 mo after the beginning of the exposure, a significant impact on the zooplankton community structure was observed in the medium treatment. The low treatment had a sporadic impact on the zooplankton community. The $NOEC_{community}$ was thus estimated to be 5 $\mu g\ L^{-1}$ of copper for the entire experiment (Table 2).

Taxa with a positive weight decreased in abundance in the treated mesocosms. This was the case for the rotifers *Mytilina* sp. and *Trichocerca* sp. and the cladoceran *C. sphaericus* (Figure 4B). The most sensitive species was the rotifer *Mytilina* sp. (Figure 4C), which was significantly less abundant 1 mo after the beginning of the exposure in the medium and high treatments (Williams test, $p < 0.05$). However, after 1 yr of exposure in Spring 2003, abundance of this taxa increased in the medium treatment. *Chydorus sphaericus* (Figure 4E) decreased in the high treatment 2 mo after the beginning of the exposure. As for the medium treatment, a significant decrease of abundance was observed 5 mo after the beginning of the exposure. Surprisingly, *C. sphaericus* abundance was higher in the low and medium treatments compared with the control in June and July 2002. Taxa with a negative weight increased in abundance. This was the case for the protozoan *Vorticella* sp. (Figure 4F); negative weight appeared in January 2003 and developed mainly in the medium and high treatments at high abundances. The NOECs for each taxon are presented in Table 2 and vary with time.

The raw data are available in the Supplemental Data (SI-3, Zooplankton raw data).

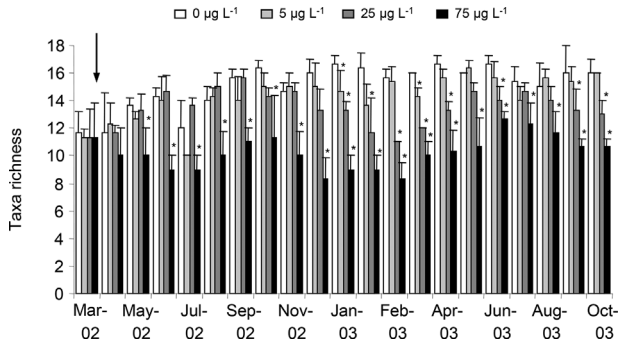

Figure 3. Mean taxa richness for zooplankton. Asterisks indicate significant difference relative to controls (Williams test, $p < 0.05$). Arrows indicate the beginning of dosing.

Macroinvertebrates

In total, 38 different taxonomic groups of macroinvertebrates were sampled throughout the study period, including 2 families of Turbellaria, 1 of Annelida, 4 of Clitellata (leeches), 6 of Gastropoda, 2 of Crustacea, and 6 of Insecta. Prior to exposure, the most abundant taxonomic group was gastropods, which represented approximately 50% of the total abundance. In this group, the pulmonate gastropod *Lymnaea* spp. was the most abundant taxa, followed by *Physa* sp. The prosobranch gastropods such as *Bithynia* sp. and Hydrobiidae were less represented. The second most abundant taxonomic group was Diptera and more particularly Chironomidae larvae, which represented 27% of the total abundance. Crustaceans represented 21% of the total abundance, with *G. pulex* as the main taxa and *A. aquaticus* being less common. Finally, in decreasing order of importance and representing approximately 2% of the total abundance, the following taxa were sampled: Oligochaeta, leeches, Coleoptera, Trichoptera, Heteroptera, and Odonata. Ephemeroptera (Baetidae) and Plecoptera (*Nemoura*) were occasionally collected but were always rare.

In the high treatment, macroinvertebrates' total abundance decreased significantly immediately after the beginning of the

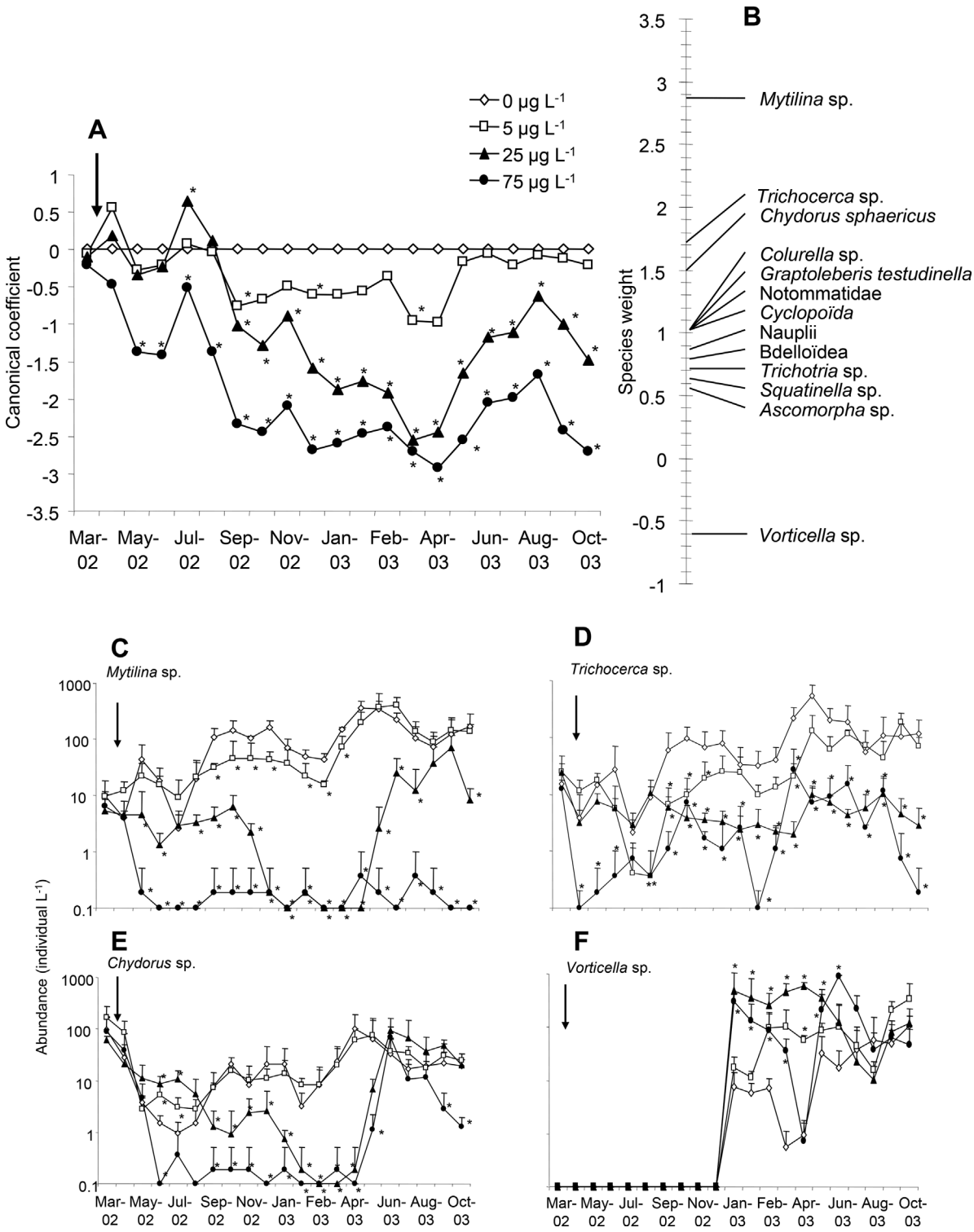

Figure 4. Principal response curves (**A**) indicating the effect of copper through time on the abundance of the zooplankton community. Curves deviating from the reference value of 0 indicate treatment effects. The species weight can be interpreted as the affinity of the taxon with the curves (**B**). Arrow indicates the beginning of exposure. Notice that species weights between 0.5 and –0.5 are not presented, as they were likely to show a weak or unrelated response. Dynamics of the 4 most important species of zooplankton are presented in a logarithmic scale: *Mytilina* sp. (**C**), *Trichocerca* sp. (**D**), *Chydorus* sp. (**E**), and *Vorticella* sp. (**F**). Absence of cells is denoted by 0.1. Asterisks indicate significant difference relative to controls (Williams test, $p < 0.05$).

exposure until the following spring (Williams test, $p < 0.05$; Figure 5). In June 2003, total abundance was significantly higher in the high treatment. Finally, from August 2003 to the end of the exposure, total abundance in the high treatment was once more significantly lower than the control. In the medium treatment, total abundance showed a significant decrease on only 5 sampling dates. In the low treatment, total abundance was not significantly different from the control.

A significant decrease in taxa richness was observed in the high treatment during 16 mo (Williams test, $p < 0.05$; Figure 6). For the medium and low treatments, taxa richness was sporadically significantly lower than in the control.

Table 2. $NOEC_{community}$ and NOEC for the taxa that had the most important species weight in the zooplankton PRC[a]

		Spring 2002	Summer 2002	Autumn 2002	Winter 2002/2003	Spring 2003	Summer 2003	Autumn 2003	NOEC experiment
Community	–	25	25	5	5	5	5	5	5
Mytilina sp.	–	5	5	<5	<5	5	25	5	<5
Trichocerca sp.	–	25	25	<5	5	5	5	5	5
Chydorus sphaericus	–	≥75	≥75	5	5	5	≥75	25	5
Vorticella sp.	+				5	<5	≥75	≥75	≥75

[a]Values are given in $\mu g\,L^{-1}$. Plus (+) and minus (−) symbols indicate significantly higher or significantly lower abundance, respectively, relative to controls. For each season, only NOECs calculated for at least 2 consecutive sampling dates were considered.
NOEC = no-observed-effect concentration; PRC = principal response curve.

Community structure showed a significant concentration-related deviation ($p = 0.005$; Figure 7A). In the PRC, time explained 42% of the total variance and is shown on the horizontal axis while 33% is explained by the treatment. The difference between replicates explained 25% of the total variance. The first PRC axis displayed 51% of the variance explained by the treatment. Principal component analysis followed by a Williams test per date showed that the community structure in the high treatment differed significantly 1 mo after the beginning of exposure until the end of the experiment. In the medium treatment, significant differences were observed from almost all sampling dates. As for the low treatment, the community structure differed significantly on one sampling date. Even though the medium and high treatments remained significantly lower in relative abundance, the trend in community structure suggests a potential recovery (Figure 7A) beginning in summer 2003. The $NOEC_{community}$ was set to 5 $\mu g\,L^{-1}$ for the entire experiment (Table 3).

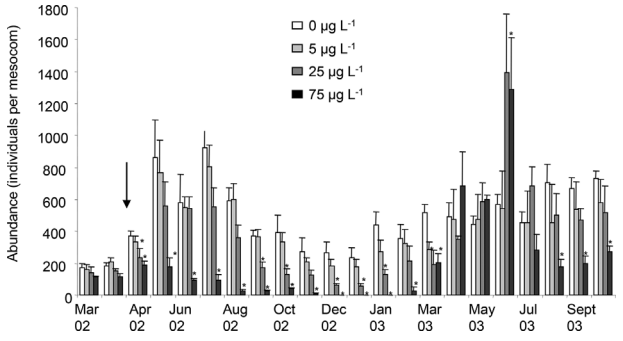

Figure 5. Macroinvertebrate abundance for control and treated mesocosms. Asterisks indicate significant difference relative to controls (Williams test, $p < 0.05$). Arrow indicates the beginning of exposure.

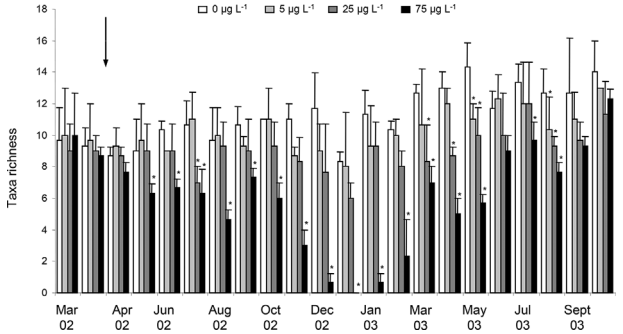

Figure 6. Mean taxa richness for macroinvertebrates. Asterisks indicate significant difference relative to controls (Williams test, $p < 0.05$). Arrow indicates the beginning of exposure.

Taxa with a positive weight decreased in abundance in the treated mesocosms (Figure 7B). In order of decreasing weight, *Lymnaea* spp. juveniles (Figure 8A), *Lymnaea* spp. adults (Figure 8B), *Physa* sp. juvenile (Figure 8E), *Erpobdella* sp. (Figure 8D), *G. pulex* adults (Figure 8C–E), *G. pulex* juveniles, *A. aquaticus* adults (Figure 8G), *A. aquaticus* juveniles, *Physa* sp. adults (Figure 7F), *Glossiphonia* sp., Hydrobiidae, Planariidae, and *Bithynia* sp. were the taxa that decreased in abundance, for a certain period of time, in the treated communities. In contrast, Chironomidae increased in the treated mesocosms in spring and summer 2003 (Figure 8H). Because the responses of adult and juvenile *G. pulex* and *A. aquaticus* were similar, only the adults were represented (Figure 7C and G). The NOECs for each taxa are presented in Table 3 and are shown to vary with time. The dynamics and potential recovery of each population will be discussed below.

The raw data can be found in the Supplemental Data (SI-3, Macroinvertebrate raw data).

Emerging insects

The only taxonomic group represented was Diptera. Seven taxa were identified (listed by order of importance in the control): Chironomini, Tanytarsini, Corynoneurina, Tanypodinae, Orthocladiinae, Ceratopogonidae, and Ephydridae.

Because the pattern of emergence was variable, biweekly values of total abundance did not give a consistent pattern in terms of copper effects. The cumulative total number of emerged Diptera gave more consistent indications of the effects of copper (Figure 9A and B). Compared with the control, total abundance was significantly lower only for the high treatment during the first sampling period (Figure 9A). During the second sampling period, total abundance in the medium and high

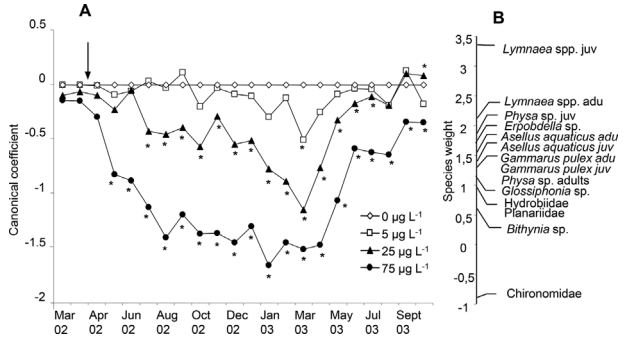

Figure 7. Principal response curves (**A**) indicating the effects of copper through time on the abundance of the macroinvertebrate community. Curves deviating from the reference value of 0 indicate treatment effects. Asterisks indicate significant difference relative to controls (Williams test, $p < 0.05$). The species weight can be interpreted as the affinity of the taxon with the curves (**B**). Species with a weight between 0.5 and –0.5 are not shown. Arrow indicates the beginning of exposure.

Table 3. $NOEC_{community}$ and NOEC for the taxa that had the most important species weight in the macroinvertebrates PRC[a]

		Spring 2002	Summer 2002	Autumn 2002	Winter 2002/2003	Spring 2003	Summer 2003	Autumn 2003	NOEC experiment
Community	–	25	5	5	5	5	5	25	5
Lymnaea spp. adults	–/+	25	25	25	25	5	≥75	5	25
Lymnaea spp. juveniles	–/+	25	25	25	5	5	≥75	≥75	5
Physa sp. juveniles	–	≥75	25	25	5	≥75	≥75	≥75	25
Erpobdella sp.	–	≥75	25	25	25	25	25	25	25
Gammarus pulex adults	–	≥75	5	25	≥75	25	≥75	≥75	≥75
G. pulex juveniles	–	25	5	25	≥75	≥75	≥75	≥75	≥75
Asellus aquaticus adults	–	25	≥75	≥75	5	5	≥75	5	5
A. aquaticus juveniles	–	≥75	5	≥75	5	≥75	≥75	5	≥75
Physa sp. adults	–	≥75	5	5	5	5	≥75	≥75	5
Chironomidae	+	≥75	≥75	5	≥75	5	5	≥75	5

[a]Values are given in $\mu g\,L^{-1}$. Plus (+) and minus (−) symbols indicate significantly higher or significantly lower abundance, respectively, relative to controls. Only NOECs calculated for at least 2 consecutive sampling dates were considered.
NOEC = no-observed-effect concentration; PRC = principal response curve.

treatment was significantly higher than the control (Williams test, $p < 0.05$; Figure 9B). As the number of taxa was low, analysis of taxa richness did not yield interpretable results.

The emerging insects in 2002 significantly deviated in the treated mesocosms compared with the control ($p = 0.005$; Figure 10A). Time explained 61% of the total variance, whereas 17% was explained by the treatment. The difference between replicates explained 22% of the total variance. The first PRC axis displayed 53% of the variance explained by the treatment. The abundance of emergent taxa in the high treatment differed significantly from the control for 8 sampling dates (mainly in summer). In the medium treatment, significant differences were observed on 3 sampling dates but were not consecutive. For the low treatment, no significant differences were observed. The $NOEC_{community}$ was set to 25 $\mu g\,L^{-1}$ of dissolved copper for 2002 (Table 4). Taxa with a negative weight (Figure 10B), such as Chironomini, decreased in the treated mesocosms (Figure 10C). Taxa with a positive weight, such as Orthocladiinae (Figure 10D), increased in the treated mesocosms.

The emerging insects in 2003 significantly deviated in the treated mesocosms compared with the control ($p = 0.005$; Figure 11A). Time explained 49% of the total variance, whereas 26% was explained by the treatment. The difference between replicates explained 25% of the total variance. The first PRC axis displayed 36% of the variance explained by the treatment. The abundance of emergent taxa in the high treatment differed significantly from the control from early spring to midsummer. In the medium treatment, significant differences were observed sporadically. For the low treatment, a significant difference of the abundance of emerging taxa was observed only for one sampling date. The $NOEC_{community}$ was set to 5 $\mu g\,L^{-1}$ of dissolved copper for 2003 (Table 4). There were no taxa with a negative weight (Figure 11B). Taxa with a positive weight, such as Orthocladiinae and Corynoneurina (Figure 11C and D) increased in the treated mesocosms. The NOECs for the taxa Chironomini, Corynoneurina, and Orthocladiinae are presented in Table 4 and are shown to vary with time. The dynamics and potential recovery of each population will be discussed below.

The raw data are available in the Supplemental data (SI-3, Emerging insects raw data).

DISCUSSION

Direct and indirect effects at the population and community levels

Zooplankton. Total abundance, taxa richness, and community structure were affected mainly in the medium and high treatments immediately after the beginning of the exposure. The most sensitive taxa were the rotifers Mytilina sp. and Trichocerca sp. and the cladoceran C. sphaericus. Moreover, the observed overall decrease in total zooplankton abundance was caused by a much lower rotifer abundance compared with the control mesocosms. Direct effects of copper on some rotifer and cladoceran taxa are suspected.

Indeed, rotifers previously have been shown to be sensitive to copper at a dissolved copper concentration of 20 $\mu g\,L^{-1}$ in lentic mesocosms [13]. No toxicity data were found for the *Mytilina* sp. However, Leland et al. [46] reported that a species of *Trichocerca* was adversely affected at 7 $\mu g\,L^{-1}$ of copper, showing a possible sensitivity of this taxon to copper.

Likewise, it has been reported that *C. sphaericus* is very sensitive to copper under the neonate stage with a 48-h median effective concentration (EC50-48 h) as low as 3.3 $\mu g\,L^{-1}$ of copper [47]. Moore and Winner [13] reported a depressed density of chydorids in enclosures maintained at 40 $\mu g\,L^{-1}$ of copper. Although the total dissolved copper concentrations of that mesocosm experiment were similar to that of our medium treatment, the Bio-Met calculations suggest that the bioavailable (toxic) fraction of copper was lower in the Moore and Winner [13] study than in the present study. Their mean pH of 8.2, hardness 92 $\mu g\,L^{-1}$ as CaCO3, and DOC ranging from 7.2 to 8.7 $\mu g\,L^{-1}$ [13,14] result in calculated copper PNECs of approximately 14 to 20 $\mu g\,L^{-1}$ compared with approximately 4 to 7 $\mu g\,L^{-1}$ for our study.

In contrast, the ciliate *Vorticella* sp. showed a large development in the medium and high treatments after 8 mo of exposure. Indirect effects are suspected. This is most likely an improved competitive position relative to the other zooplankton taxa in terms of copper tolerance, predation pressure, and food supply [40]. In fact, *Vorticella* sp. has been reported to be tolerant to various heavy metals including copper [48,49]. As an example, it has been shown that *Vorticella microstoma* mobility was not affected by copper concentrations over 220 $mg\,L^{-1}$ [50]. As a probable result of its tolerance to copper and its improved competitive position, *Vorticella* sp. had a larger development in the medium and high treatments, with no visible effect of copper on total abundance of zooplankton at some dates (Figure 2).

Top-down predation pressure from fish may have had an additional indirect effect on the zooplankton community. Juvenile stickleback were mainly planktonic feeders, feeding primary on cladocerans and copepods, with larger fish switching to benthic prey. Although there were no significant differences in stickleback populations between the copper treatments during

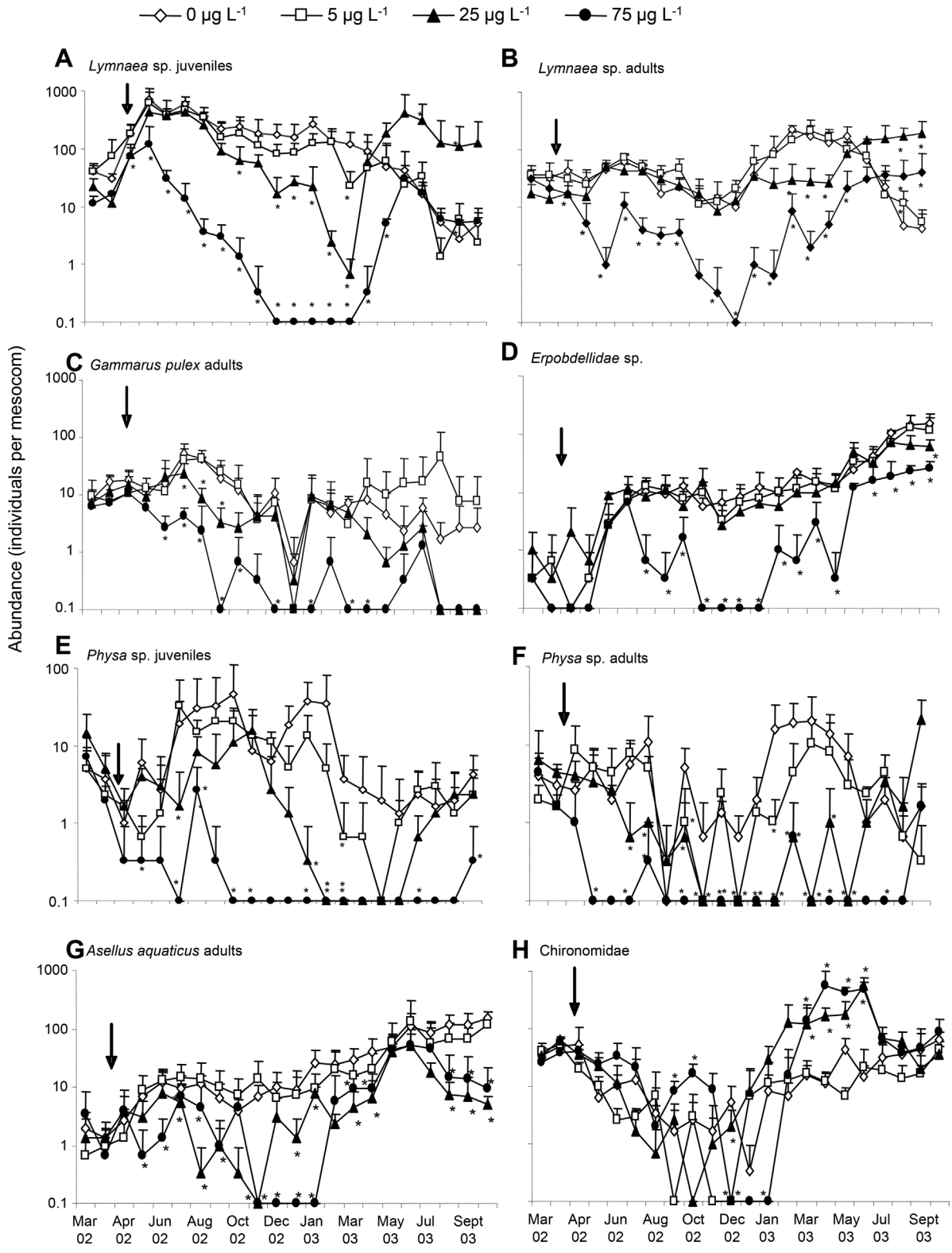

Figure 8. Dynamics of the most important species of macroinvertebrates contributing the most to the principal response curve (PRC) are presented: *Lymnaea* spp. juveniles (**A**), *Lymnaea* spp. adults (**B**), *Gammarus pulex* adults (**C**), Erpobdellidae sp. (**D**), *Physa* sp. juveniles (**E**), *Physa* sp. adults (**F**), *Asellus aquaticus* adults (**G**), and Chironomidae (**H**), presented in a logarithmic scale. Absence of cells is denoted by 0.1. Asterisks indicate significant difference relative to controls (Williams test, $p < 0.05$). Arrows indicates the beginning of exposure.

the first season of exposure, stickleback abundances were highest in the highest copper treatment by the end of the second season. The increased fish abundances in the high copper concentration were thought to be in response to declines in gastropods and leeches, which feed on stickleback eggs [20]. Abundant, metal-tolerant, planktivorous fish were elsewhere shown to constrain recovery of zooplankton communities in historically polluted lakes, following the decline of copper and other metals [51]. This finding suggests to us that the lower abundances of zooplankton in the medium and high copper

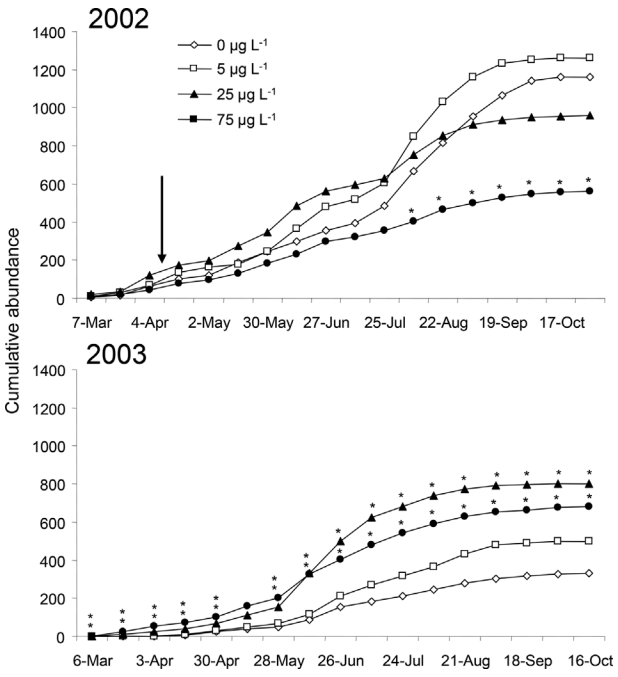

Figure 9. Cumulative total abundance of emerging insects for the control and treated mesocosms. Asterisks indicate significant difference relative to controls (Williams test, p 0.05). Arrow indicates the beginning of exposure.

treatments were likely the result of both direct and indirect effects of copper toxicity.

Macroinvertebrates. Total abundance, taxa richness, and community structure were severely affected in the medium and the high treatment throughout the exposure period. Some other studies have also reported reduced total abundance and taxa richness of macroinvertebrates, particularly in situations with very high copper concentrations [3,10,52] or in relatively short-term mesocosm experiments in which the exposure duration was one generation or less [17,52].

The sensitive taxa that contributed the most to the observed community response were gastropods such as *Lymnaea* spp. and *Physa* sp., crustaceans such as *G. pulex* and *A. aquaticus*, and leeches such as *Erpobdella* sp. Because a decrease in abundance of gastropods and crustaceans occurred shortly after the beginning of exposure, direct effects are suspected.

Our study showed that gastropods and especially juveniles of *Lymnaea* spp., and *Physa* sp. were impacted. Direct comparisons of the specific effect concentrations from our exposures with those in the previous literature may not be appropriate without modeling the relative bioavailability of copper in different waters, and some reports did not give sufficient chemical details to permit the modeling of bioavailability differences. However, freshwater molluscs have been reported to be sensitive to copper in laboratory toxicity testing [53–55], and in microcosm and mesocosm experiments [15,16,36].

Crustaceans, especially *G. pulex* and *A. aquaticus* were also affected at the medium and high mesocosm treatments. These observations are consistent with previous reports of *G. pulex* being sensitive to copper, but are in contrast with reports of *A. aquaticus*'s high tolerance of copper. In a stream mesocosm experiment with copper sulfate, Girling et al. [36] found NOEC values of $10\,\mu g\,L^{-1}$ for drift and $30\,\mu g\,L^{-1}$ for precopula separation of *G. pulex* using exposure chambers. The same authors performed a single species test on juvenile mortality, which yielded a median lethal concentration (LC50, 96 h) of $37\,\mu g\,L^{-1}$. Maund et al. [56] also reported that copper reduced the population density of *G. pulex* between an NOEC and a lowest-observed-effective concentration (LOEC) of 11 and $14.6\,\mu g\,L^{-1}$ in hard water laboratory waters. In contrast, *A. aquaticus* were able to survive and continue to grow in 10-d exposures of up to $1481\,\mu g\,L^{-1}$ of copper [57]. These latter results cannot be explained by bioavailability differences, because while unreported, the reconstituted freshwater medium-hard water exposures would be expected to produce waters with pH values in a range of 7.6 to 8.0, and DOC $< 1\,mg\,L^{-1}$ [58].

Leeches such as *Erpobdella* sp. were negatively affected in the high treatment after 4 mo of exposure. No reliable toxicity data were found for *Erpobdella* sp. However, it is thus highly probable that indirect effects such as a reduction in prey availability or predator occurrence were responsible for the observed response. Indeed, the main prey of *Erpodella* sp. are gastropods and crustaceans [59], which were directly affected in the mesocosms by copper at the medium and high treatments. Although there were still significant differences from control, the leech population increased in spring 2003 in the high and medium treatments, following the trend of *Lymnaea, Gammarus*, and *Asellus* populations.

In contrast, larvae of the family Chironomidae seemed to be indirectly favored by high copper concentrations, as they increased in abundance in the medium and high treatments mainly after 12 mo of exposure. Oligochaeta were also favored but only during a short period, thus explaining their low species weight in the PRC (Figure 3). Indeed, in June 2003, higher total abundance compared with the control was even recorded in the high treatment (Figure 5). Chironomidae and Oligochaeta represented around 80% of the total abundance at that date. On the whole, these groups have been shown to be generally tolerant to copper [3,10,15,16,60]. Indeed, Girling et al., [36], in a single-species laboratory test done on *Chironomus riparius*, reported a 96-h LC50 of $700\,\mu g\,L^{-1}$ for second-instar mortality, an NOEC of $800\,\mu g\,L^{-1}$ for third-instar growth, and an NOEC of $660\,\mu g\,L^{-1}$ for the percentage of egg hatching. For *Chironomus tentans,* the LC50 for copper ranged from 77.5 to $1690\,\mu g\,L^{-1}$ depending on the instar and whether the test included reproductive endpoints [61]. Conversely, other members of the family Chironomidae may be relatively sensitive to copper, particularly the tribe Tanytarsini chironomids [3,17,62–64]. For instance, in a long-term study of streams recovering from copper pollution, the order of copper tolerance among chironomids was Orthocladinii > Chironimini > Tanytarsini [3]. In our study, for practical reasons, the lowest taxonomic level for dipteran larvae was the family. It was thus not possible to determine which taxa were responsible for this major Chironomidae larvae development. However, based on the literature data cited above and the data on emergence (see further discussion), we suspected that Orthocladiinae and Corynoneura were the major taxa accounting for the development in the treated mesocosms. Given sufficient time for colonization and development, they numerically replaced the most sensitive taxa. Indirect effects such as a release of interspecific competition coupled with an increase in food resources could explain the development of these relative tolerant taxa in the treated mesocosms [65].

Community structure in the medium and high treatments thus shifted from a gastropod/dipteran/crustacean-dominated community to a dipteran-dominated community. Other studies

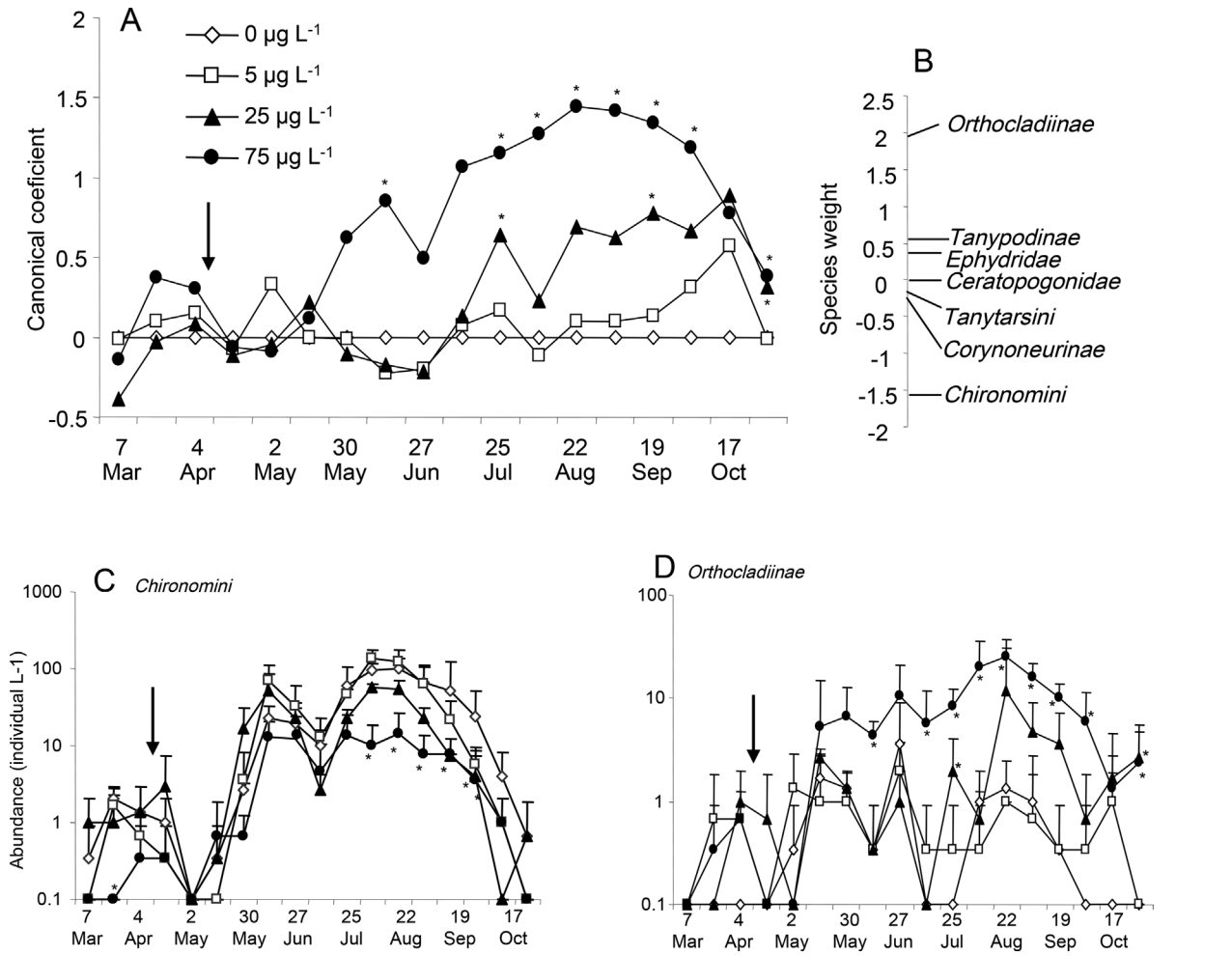

Figure 10. Principal response curves (**A**), indicating the effects of copper through time on the abundance of the emerging insect in 2002. Curves deviating from the reference value of 0 indicate treatment effects. The species weight can be interpreted as the affinity of the taxon with the curves (**B**). Arrow indicates the beginning of exposure. Note that species weights between 0.5 and –0.5 are not presented, as they were likely to show a weak or unrelated response. Dynamics of the 2 most important species are presented in a logarithmic scale: Chironomini sp. (**C**) and Orthocladiinae sp. (**D**). Asterisks indicate significant difference relative to controls (Williams test, $p < 0.05$).

have shown comparable shifts, depending on their initial conditions. For example, in Hedtke's [16] 32-wk copper microcosm exposures, the community shifted from a gastropod/oligochaete-dominated community to an oligochaete-dominated community. In Gardham et al.'s [15] 71-wk copper mesocosm exposures, the macrofauna community in controls and copper-exposed treatments was initially dominated by dipterans with gastropods also commonly occurring. Gastropods (Physidae) were largely eliminated, along with cladocerans and benthic Chironominae [15].

Table 4. $NOEC_{community}$ and NOEC for the taxa that had the most important species weight in the emerging insects PRC for 2002 and 2003[a]

		Spring	Summer	NOEC experiment
2002				
Community	–	75	25	25[b]
Chironomini	–	75	25	25[b]
Orthocladiinae	+	75	25	25[b]
2003				
Community	+	5	5	5
Corynoneurina	+	5	5	5
Orthocladiinae	+	5	25	5[b]

[a]Only NOECs calculated for at least 2 consecutive sampling dates were considered for the PRCs. Values are given in µg L^{-1}. Plus (+) and minus (–) symbols indicate significantly higher or significantly lower abundance, respectively, relative to controls.
[b]In this case, the lowest NOEC was considered valid.
NOEC = no-observed-effect concentration; PRC = principal response curve.

To what extent has this shift in community structure of macroinvertebrates affected the functioning of the ecosystem? During our experiment, the effects of copper on aquatic decomposers were also investigated, and were reported separately [21]. Two main endpoints were assessed: leaf decomposition rate and the qualitative and quantitative composition of the aquatic hyphomycete and invertebrate communities. Roussel et al. [21] showed that the functioning of the ecosystem, measured by the breakdown rate, was altered in the high treatment. This effect was mainly because of the loss of decomposer invertebrates such as *G. pulex* and *A. aquaticus*. When the results of the decomposition measurements and the results obtained on the structure of the macroinvertebrate community are considered together, we can conclude that replacement of sensitive taxa (which are mainly grazers and shedders) by the more tolerant taxa (which are mainly collectors and deposit feeders) has altered natural ecosystem functioning. Furthermore, after 6 mo of exposure, periphyton biomass was significantly higher in the medium and high treatments than in the control [2]. This increase can be explained both by the shift

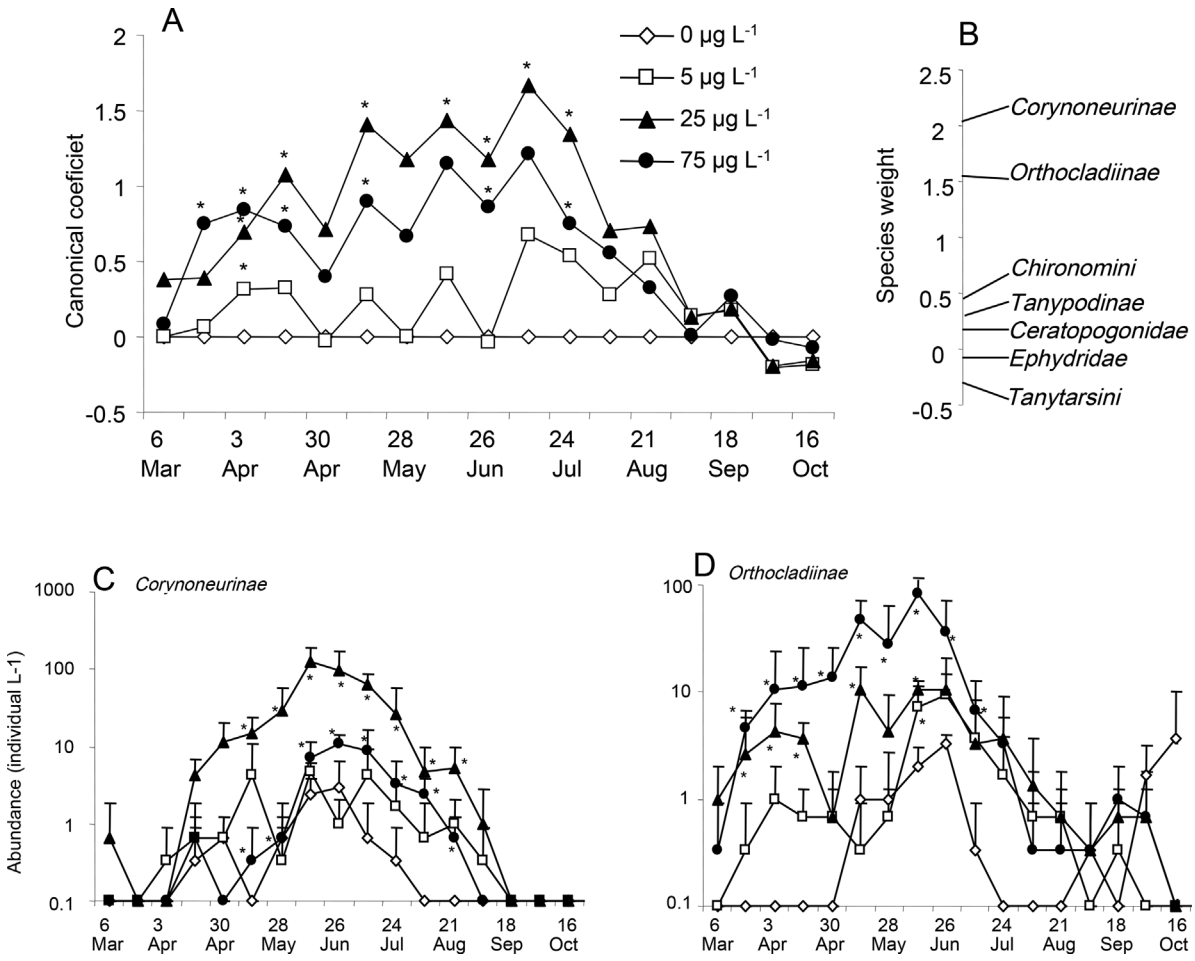

Figure 11. Principal response curves (**A**), indicating the effect of copper through time on the abundance of the emerging insects in 2003. Curves deviating from the reference value of 0 indicate treatment effects. The species weight can be interpreted as the affinity of the taxon with the curves (**B**). Arrow indicates the beginning of exposure. Note that species weights between 0.5 and –0.5 are not presented, as they were likely to show a weak or unrelated response. Dynamics of the 2 most important species are presented in a logarithmic scale: Corynoneurina sp. (**C**) and Orthocladiinae sp. (**D**). Asterisks indicate significant difference relative to controls (Williams test, $p < 0.05$).

in the algal composition of the periphyton community and by a decrease in grazing gastropods. An increase in filamentous algal morphotypes, such as *Leptolyngbya* sp., *Microspora* sp., *Mougeotia* sp., *Oscillatoria* sp., *Pseudanabaena* sp., and *Ulothrix* sp. was observed [21], which could have contributed to the increase in overall periphyton biomass. Furthermore, as grazing controls periphyton biomass and diversity [66], a decrease in its pressure can thus also have an impact on primary production.

Emerging insects. During the first sampling period (March 2002 to October 2002), emergence of Chironomini was depressed in the high treatment, as shown in the PRC. A NOEC of 25 $\mu g\,L^{-1}$ was determined and is the first to be recorded for this tribe. In contrast, emergence of Orthocladiinae seemed to be favored in the high treatment, as the number of emerging insects was greater than in the control. Overall, total abundance of emerging dipteran was lower in the high treatment compared with the control. Chironomidae larvae abundance in the high treatment gradually decreased throughout time but followed the same trend as the control (Supplemental Data, SI-3, Macroinvertebrate raw data). This finding suggests that copper may be directly impairing the pupal stages or the emergence of some taxa such as Chironomini.

During the second sampling period (March 2003 to October 2003), the number of total emerging dipterans was higher in the medium and high treatments compared with the control. During this period, total abundance in the control was very low compared with the values obtained during the first peak of emergence found in August through September 2002. The highest larval densities of Chironomidae occurred in the highest copper treatment, yet the highest numbers of emerging Chironomidae occurred in the medium copper treatment (Supplemental Data, SI-3, Emerging insects raw data). This may suggest what while chironomids increased in larvae densities, emergence did not keep pace with the increases in larvae. This pattern is consistent with the concept that metamorphosis and emergence are stressful parts of the insect life cycle, making them vulnerable to other stressors (discussed below) in this sub-section.

As shown by the PRC, emergence of both Orthocladiinae and *Corynoneura* (a genus of the subfamily of Orthocladiinae) was positively affected by the copper treatments. No taxa were significantly negatively affected during the second sampling season (Figure 10), which suggests that overall emergence of chironomids is favored in the medium and high treatments compared to the control. In the previous section on macroinvertebrates, it was reported that Chironomidae larvae were favored at the medium and high treatments and therefore increased in abundance only in 2003 (also see Supplemental Data, SI-3, Macroinvertebrates raw data). Emergence of 2 taxa

of this family, Orthocladiinae and *Corynoneura*, also increased. We can thus suppose that the larvae of these 2 taxa increased significantly in the high treatment and were responsible for the observed effects. These results highlight the fact that taxonomic identification only to the family level for larvae is insufficient to fully understand the effects of copper on chironomids. Moreover, the increased emergence of dipterans in the medium and high treatments could be influenced by several factors. Selection caused by a pollutant (copper in the present study) may act directly on some life-history or behavioral traits that reduce the impact of the pollutants on the organism. For example, rapid growth and early reproduction can reduce the concentration of a pollutant in an organism and allow it to reproduce before the pollutant damage is too great [67]. Such selection may thus shorten the life cycle of an organism. Acquired tolerance to metals could provide protection to latter generations (discussed further in the section *Implications for water quality guidelines or risk assessment*), or chironomids could have benefited from reduced competition for food, following the decline in gastropods [2,65,68]. These factors are doubtfully mutually exclusive.

Furthermore, during the first sampling period, we have shown that emergence of some species of the Chironomini (tribe of the subfamily Chironominae, which is a subfamily of Chironomidae) is sensitive to copper, suggesting again that the level of taxonomic identification should be the tribe or species levels. The difference in the sensitivity of different members of the family Chironomidae is supported by the artificial stream studies of Clements et al. [17] as well as field surveys [3]. However, our work and that of some other researchers show that differing taxa show markedly different susceptibility to impaired emergence [69]. Metamorphosis is a stressful part of the insect life cycle, and developmental endpoints are often much more sensitive than survival-based endpoints [61,63,64,70]. This was the case in our study for the taxa Chironomini. The inclusion of sublethal endpoints, such as insect emergence, thus assists in the understanding of multigenerational effects that a contaminant can have on a population and can also improve our understanding of community-level changes [71]. The study of these endpoints should thus endure in artificial stream experiments.

Partial recovery

Copper appeared to affect both zooplankton and macroinvertebrate communities more severely during the first several months of exposure than during the second season of exposure. While attempting to define the mechanisms behind this observed partial recovery was beyond the scope of our experiment, we think that indirect effects arising from interactions with the primary producers and acquired tolerance were possible explanatory factors.

Reduced copper exposures would obviously lead to recovery of invertebrate communities [3,72]. We examined whether copper exposure conditions could have changed during the experiment and contributed to the observed, partial recovery. Copper, pH, and DOC did vary in the experimental streams during the experiment, but not in a direction that would lessen copper toxic stress. Measured dissolved copper concentrations in the mesocosms were similar among replicate streams and showed no appreciable trends over time. Measurements of pH were mostly stable and consistent between streams. The pH showed a slight but noticeable decline across treatments throughout the experiment, declining from an average of 7.8 in April 2002 to 7.4 in October 2003. Dissolved organic carbon concentrations also were generally lower and more stable in the second season compared with the first season, averaging 2.3 mg/L in 2002 and 1.2 mg/L in the 2003 season. Sporadic high DOC concentrations $>$ 6 mg/L were observed in April 2002. Measured dissolved copper concentrations showed no obvious trends over time (Supplemental Data, SI-2, Chemistry).

The reasons for the patterns of declining pH and DOC over time as the mesocosm experiment matured are unexplained. Both pH and DOC can be influenced by primary producers [72], but there was no obvious correspondence between the pH and DOC patterns and macrophyte, periphyton, or phytoplankton production [2]. The implications of the lower pH and DOC in season 2 are that a given concentration of copper would be more bioavailable and toxic. For instance, the Bio-Met tool provides a PNEC estimate adjusted to the copper bioavailability conditions of specific water conditions [55] (see *Implications for water quality guidelines or risk assessment* for further explanation). The season 1 average test pH of 7.8 and DOC of 2.3 mg/L resulted in a PNEC estimate of 6.3 μg/L, compared with a PNEC estimate of 4.2 μg/L, for the season 2 average pH of 7.4 and DOC of 1.2 mg/L. Thus, changing dosing or water characteristics are inconsistent with the partial recovery observations, supporting our view that acquired tolerances and indirect effects were the most plausible factors.

Food availability can certainly influence populations of any consumer. During summer 2003, the population of the zooplankter *C. sphaericus* showed a recovery in the medium and high treatments. A large increase of periphyton biomass in these treatments was observed during this period, which may in turn have been partially related to the decline in gastropods [2]. An increase in food availability might have favored population survival and recovery. Because it has been shown that the nutritional quality and quantity of food has a marked effect on the direct or indirect effects of copper on zooplankton [72–74], we can suppose that food availability might have been partially responsible for the observed increase in abundance of this species.

A partial recovery of the macroinvertebrate community was also observed in our experiment. After approximately 8 mo of exposure, the macroinvertebrate community in the high treatment was practically decimated. Abundances of all taxa reached very low levels in December 2002. Abundances of several dominant taxa such as snails (*Lymnaea* and *Physa*), isopods (*Asellus*), amphipods (*Gammarus*), and leeches (Erpobdellidae) were depressed to the point of being undetectable in the December 2002 samples (Figure 5). Only moderate effects were observed in the medium treatment, and no detectable effects were observed in the low treatment (Table 1 and Figures 4 and 5). Subsequently, most taxa in the high treatment began a gradual and partial recovery. By the end of the study at 18 mo, macroinvertebrate taxa richness in the copper treatments had increased and had approached that of control richness, although overall abundances remained lower than controls.

Acquired tolerance was likely a factor for the partial recoveries. Populations of organisms that have been chronically exposed to chemical pollutants may develop increased tolerance, or resistance, to those toxicants. For populations exposed over many generations, tolerance may be acquired from genetic adaptation, and in shorter exposures, tolerance may be acquired through physiological acclimation to the polluted environment, such as stimulated production of metallothionein (metal-binding proteins) [75]. Chironomids, isopods, and daphnids have been shown to adapt to metal-contaminated

environments [4,76,77], and acquired tolerance mechanisms have been proposed to be generally applicable to aquatic organisms [75]. While acquired tolerance can allow organisms to survive and reproduce in metal-contaminated areas, there is an energetic cost to detoxifying metals. Energy devoted to detoxification and maintaining internal mineral balances is energy not available for growth and reproduction. For example, brood sizes may be smaller in metal-stressed and metal-acclimated populations [4,75]. Thus recoveries under continued metal stress may be partial.

Implications for water quality guidelines or risk assessment

A key attribute of mesocosm experiments is the bridging of the gap between laboratory aquatic toxicity testing and natural ecosystems. The former offers high experimental control and sometimes the ability to test a large number of conditions, but in an artificial setting that may not be obviously relevant to natural conditions. However, attributing cause and effects of contaminants in natural ecosystems is difficult, with the confounding influence of multiple uncontrolled potential stressors and the inability to experimentally dose natural environments. Mesocosms provide an intermediate condition that may help evaluate the protectiveness of PNECs that were derived from compilations of laboratory toxicity tests [25]. In communities or jurisdictions such as the European Union or the United States, for example, water quality guidelines for priority metals such as copper are typically derived by normalizing laboratory toxicity tests to specific water chemistry conditions with a bioavailability model, and then plotting a species sensitivity distribution to derive a site-specific HC5 of species or the 95% protection level [45,58]. The appropriateness of these PNECs can be evaluated in comparison with the mesocosm NOECs, but only after adjusting for the differing bioavailability of copper in the different waters. Because the EU approach of normalizing all the data making up the species sensitivity distribution for different waters and then determining a site-specific HC5 is computationally tedious, simplified approaches have been developed, including the Bio-Met tool. The Bio-Met tool approximates BLM as a function of pH, DOC, and calcium concentrations [45]. Using the Bio-Met tool, the estimated EU PNEC for the grand average mesocosm conditions from Table 1 was 5.5 $\mu g\,L^{-1}$. This estimate is slightly lower than the PNEC of 6.9 $\mu g\,L^{-1}$ calculated by Van Sprang et al. [23] for the same conditions using the EU full normalization procedure. Similarly, the US Environmental Protection Agency's [58] BLM adjusted chronic copper criterion value calculated for the average conditions given in Table 1 was 6.8 $\mu g\,L^{-1}$. In these instances, the laboratory-based predicted safe values were quite similar to our lowest average effective concentration based NOECs of 4 $\mu g\,L^{-1}$ from the low copper treatment and were well under the 20 $\mu g\,L^{-1}$ medium copper treatment, which produced pronounced adverse effects on the zooplankton and macroinvertebrate communities, in addition to altering the macrophyte community [2] and carbon cycles [21] within the streams.

CONCLUSION

Effects of copper were observed in the medium and high copper treatments at the population and community levels for zooplankton, macroinvertebrates, and emerging insects. The length of the exposure period revealed seasonal variations in copper toxicity, and in some cases the partial recovery of certain populations. Acquired tolerance along with indirect effects (e.g., increased food availability) are believed to be responsible for the observed responses. Dominance development of tolerant taxa also induced important shifts in the community structure of zooplankton and macroinvertebrates, which in turn are believed to have caused indirect effects on other communities (primary producers and decomposers). The importance of indirect effects in modulating community-level responses was thus highlighted in the present study.

When we considered all of the results, the NOEC for consumers was set to 5 $\mu g\,L^{-1}$ of copper (nominal concentration, 4 $\mu g\,L^{-1}$ as average effective concentrations), and the LOEC was 25 $\mu g\,L^{-1}$ (nominal concentration) or 20 $\mu g\,L^{-1}$ (average effective concentrations). Using the Bio-Met tool, a PNEC of 5.5 $\mu g\,L^{-1}$ was estimated using our experimental conditions. This value is slightly lower than the PNECs of 6.9 $\mu g\,L^{-1}$ and 6.8 $\mu g\,L^{-1}$ estimated with our experimental conditions using the full European Union normalization procedure and the US Environmental Protection Agency's BLM, respectively.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3822.

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Data availability—Supporting data are available through the figshare digital data repository at https://doi.org/10.6084/m9.figshare.4769635, and also as online supplemental files. These include methods details (SI-1), chemical data (SI-2), and biological data (SI-3).

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