

Effects of Experimental Lead Pollution on the Microbial Communities Associated with *Sphagnum fallax* (Bryophyta)

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Abstract

Ecotoxicological studies usually focus on single microbial species under controlled conditions. As a result, little is known about the responses of different microbial functional groups or individual species to stresses. In an aim to assess the response of complex microbial communities to pollution in their natural habitat, we studied the effect of a simulated lead pollution on the microbial community (bacteria, cyanobacteria, protists, fungi, and micrometazoa) living on *Sphagnum fallax*. Mosses were grown in the laboratory with 0 (control), 625, and 2,500 $\mu\text{g L}^{-1}$ of Pb^{2+} diluted in a standard nutrient solution and were sampled after 0, 6, 12, and 20 weeks. The biomasses of bacteria, microalgae, testate amoebae, and ciliates were dramatically and significantly decreased in both Pb addition treatments after 6, 12, and 20 weeks in comparison with the control. The biomass of cyanobacteria declined after 6 and 12 weeks in the highest Pb treatment. The biomasses of fungi, rotifers, and nematodes decreased along the duration of the experiment but were not significantly affected by lead addition. Consequently, the total microbial biomass was lower for both Pb addition treatments after 12 and 20 weeks than in the controls. The community structure was strongly modified due to changes in the densities of testate amoebae and ciliates, whereas the relative contribution of bacteria to the microbial biomass was stable. Differences in responses among the microbial groups suggest changes in the trophic links among them. The correlation between the biomass of bacteria and that of

ciliates or testate amoebae increased with increasing Pb loading. We interpret this result as an effect on the grazing pathways of these predators and by the Pb effect on other potential prey (i.e., smaller protists). The community approach used here complements classical ecotoxicological studies by providing clues to the complex effect of pollutant-affecting organisms both directly and indirectly through trophic effects and could potentially find applications for pollution monitoring.

Introduction

The release of various toxic pollutants in the environment affects both the natural environment and human health and is one of the most challenging impacts of human activities on the biosphere. This pollution is therefore monitored and its effects are studied at various levels, including the study of pollutant impact on living organisms with the aim of using them as bioindicators or biomonitors [22]. This approach represents an alternative to the more classical physical-chemical measurement of pollution. Bioindicator organisms can integrate the pollution level (or other stresses) over a long period of time and therefore provide data on an average pollution level for a given place, while also allowing for the detection of short-lasting, but biologically relevant, extreme pollution events that may go unnoticed with the physicochemical approach if measurements are not continuous. Sensitive bioindicators are also useful as early warning sentinels of environmental and human health [1].

Due to the toxicity of heavy metals in general, and lead in particular, to living organisms, including humans, a number of studies have focused on the effect of lead on microorganisms [3, 11, 16, 27]. However, most ecotoxicological studies on microorganisms have dealt only with bacteria using molecular (community structure

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profile: DGGE, PLFA) or microbial activity approaches [18, 37]. Among the eukaryotic microorganisms, protozoa, especially ciliates [11, 20, 21], and microalgae, especially the genus *Chlorella* [38], have also been used as models for some investigations. Other taxonomic groups have, by contrast, been mostly ignored with respect to ecotoxicology. In addition to this unbalanced effort among taxonomic groups, almost all ecotoxicological studies have focused on a single, well-known, model organism and on the molecular and biochemical aspects of toxicity. While these approaches have clear advantages, they fail to inform on the behavior of a more complex system. Indeed, little is known about the group-specific or species-specific responses to stress and their possible causes [7]. The point is not to suggest that one approach is better than the other; molecular-, population-, and community-level studies are all needed, as they each inform on different aspects of the impact of toxic substances on the environment.

In addition to the above-mentioned issue of level of integration, a bias may also exist with respect to the ecosystem chosen. Indeed, the effects of heavy metals on microbial communities have been mostly studied in aquatic [11, 21, 34] and soil environments [33, 40, 42], whereas wetlands have been much less studied in this respect. Several studies have focused on the effect of metals and radionuclides on microorganisms in wetlands and some bacteria, and algae have been proposed to be used for removing these pollutants in wetlands [15, 17, 46]. Wetlands, both natural and man-made, play an important role as natural filters and buffer zones [26]. It therefore appears important to better understand how pollutants affect wetland microorganisms.

To fill some of these existing gaps, we have initiated a research program on the effect of pollutants on microbial communities in bryophytes [28, 29]. We present here an experimental study on the uptake of lead by a common peat moss, *Sphagnum fallax*, and the effect of this simulated lead pollution on natural microbial communities living on this moss. Our primary focus is not the effect of pollution on the mosses or the ecosystem, but rather on the microorganisms that live on the surface of the mosses and how useful they might be as pollution bioindicators. We hypothesized that: (H1) *S. fallax* would take up the added Pb in proportion to the concentration used in the experiment, (H2) the biomass and diversity of the different microbial groups analyzed (bacteria, cyanobacteria, microalgae, ciliates, testate amoebae, rotifera, and nematoda) would decrease with increasing Pb pollution, and (H3) group-specific responses to Pb would result in changes in the structure of communities (measured as the relative contribution of different groups in the overall microbial biomass). More specifically, the differences in

anatomical, physiological, ecological, and evolutionary characteristics of microorganisms studied lead us to hypothesize that testate amoeba and ciliates would be more sensitive to Pb than micrometazoa (rotifers and nematodes) and fungi because these protists have a simpler structure (single cell) and are predators of other microorganisms, and thus are likely to accumulate toxic substances through biological magnification.

Material and Methods

Field Moss Sampling and Culture Setup in the Laboratory. *Sphagnum fallax* was chosen as a model because this moss species is common and abundant in peatlands. Furthermore, previous research has shown that this moss constitutes a good habitat for numerous microorganisms [14, 25]. *Sphagnum fallax* was collected in the bog “Frambouhans-Les Ecorces” (47°18'N, 6°79'E, at an altitude of 846 m in Franche-Comté, France) on April 13, 2004. The climate of the area is characterized by cold winters (on average, -2.4°C in January) and mild summers (on average, 14.6°C in July) [19]. The vascular vegetation of the sampling site is dominated by typical bog plants such as *Eriophorum vaginatum*, *Calluna vulgaris*, *Andromeda polifolia*, and *Vaccinium oxycoccos* [19]. We selected a *S. fallax* surface of 5 × 5 m as homogenous and pure as possible. Fifteen points within this surface were randomly chosen 1 to 4 m apart, from which 15 moss rectangles (22.0 × 8.5 cm, 15.0 cm in depth) were cut with a knife, carefully removed, and placed into plastic trays of the same size (Charles River Laboratories, E1DBBAC004). The trays containing the mosses were transported to a growth chamber in the laboratory for further manipulations. In the laboratory, all nonmoss plant remains were removed. Each moss tray was placed into a larger plastic tray (26.5 × 16.0 cm, 20.0 cm of depth, Charles River Laboratories, E1DBBAC001), which contained a standard nutrient solution [36]. The water depth in the trays was kept at 7 cm below the top of the mosses by adding nutrient solution as needed every 2 days. The following growth conditions were used: temperature, 20–23°C (day), 15–20°C (night); relative humidity, 50–75% (day), >90% (night); and light cycle, 14/10 h (light/dark), and light intensity, 105 μmol s⁻¹ m⁻². The position of the trays was randomly changed every 2 days.

Experimental Setup and Pb Analysis. After 4 weeks of acclimatization in the growth chamber, the nine moss trays were randomly assigned to three treatments in triplicates. For contamination, lead in the form of Pb²⁺ ions (from PbSO₄) was diluted in the standard nutrient solution in which the mosses rested. A

progressive series of Pb concentration was used: 0, 625, and 2,500 $\mu\text{g L}^{-1}$, and these treatments were, respectively, coded as control, E625, and E2500 (E for “exposed” followed by the respective Pb concentrations above the control). The mosses were exposed continually to Pb during the experiment. The water table depth and other environmental conditions were also maintained as described above. The experiment was carried out during 20 weeks.

For Pb analysis, ten mosses (the top three centimeters) were randomly sampled from each tray. The three moss replicates were combined to make a composite sample (i.e., 30 mosses) for each treatment at all sampling dates except for the last sampling (T20), for which 30 mosses were taken from each tray and kept separately for replicated Pb analysis. This sampling strategy was dictated by the space limitations in the growth chambers that did not allow more moss material to be grown.

The mosses to be used for Pb analyses were dried at 40°C to constant weight, ground, then digested in a mixture of 3.4 ml of concentrated nitric acid (HNO_3 , 65%) and 0.6 ml of peroxide oxide (H_2O_2) at 65°C during 48 h. The resulting solution was diluted in 16 ml of deionized water and filtered at 0.2 μm . The Pb concentrations in the moss were determined using a furnace atomic absorption spectrometer. Concentration measurement of Pb in the reference material ray grass (CRM 281, number 766 of the community bureau of reference, Commission of the European Community) was also performed to check the accuracy of the moss analysis. Analytical replication was used for every analysis; the same material reference was used after every tenth analysis and during the whole period.

Microbial Community Analyses

Sampling. Mosses for microbial community analyses were sampled after 6, 12, and 20 weeks (hereafter coded T6, T12, and T20). At each sampling date, approximately ten mosses (the top 3 cm) were randomly sampled from each tray and fixed in 15 ml glutaraldehyde (2% final concentration) for microbial analyses. Mosses growing near the edge of the tray were not collected for analysis to avoid bias due to possible edge effects.

Extraction. To extract microorganisms from the mosses, each sample was first shaken for 1 min on a vortex and then filtered through a 250- μm -mesh filter. A 15-ml volume of glutaraldehyde (2% final concentration) was then added to the sample. The sample was then shaken again on the vortex for 1 min and filtered in the same way. The process was repeated six times and all filtrate fractions were combined to obtain a final composite sample of 105 ml. The remaining fraction on the filter

was dried at 80°C for 48 h and weighed to express microbial biomass by gram dry weight (DW) of mosses.

Microscopic Analyses

Primary Producers (Cyanobacteria and Microalgae). For cyanobacteria, a 5-ml subsample was allowed to settle for 24 h in a plankton chamber. Ten to 42 (22.5 ± 6.5) random fields were analyzed at $\times 400$ magnification with an inverted microscope following Uthermöhler's [43] method for different groups of cyanobacteria: *Anabaena*, *Aphanocapsa*, *Nostoc*, *Chroococcus*, etc. For microalgae, a 10-ml subsample was left to settle and the whole slide was analyzed.

Decomposers (Bacteria and Fungi). For bacteria that include in this study both bacteria (heterotrophic and autotrophic) and archaea as methanogens, a 200- μL subsample was stained with 4,6 diamino 2 phenylindol (DAPI, 0.2% of final concentration) for 15 min in the dark, filtered through 0.2 μm black membrane filters, and examined by epifluorescence microscopy at $\times 1,000$ magnification. The image was recorded using a digital camera connected to a computer. Bacteria numbers and sizes were estimated using an image analysis program (LUCIA 4.0). Ten to 24 (11.9 ± 3.4) random fields were counted for each sample. The number of cells counted and measured for each sample ranged from 407 to 1,308. This direct counting method using DAPI allowed us to assess only the biomass of bacteria and not their diversity, for which a molecular approach would be needed. For fungi, the same procedure was applied as for cyanobacteria. Hyphae and spores were counted and measured.

Predators. For predator protists (testate amoebae and ciliates *Uronema* sp.) and micrometazoans (here restricted to nematodes and rotifers), we used the same preparation as for microalgae. The whole slide was analyzed. Each nematode observed was photographed for size measurement. Rotifers belonged to three genera: *Lecane*, *Philodina*, and *Habrotrochoa*. They were divided into four groups depending on the size to estimate biomass.

Estimation of Biovolumes and Biomass. The biovolume of each community was first estimated by assuming geometrical shapes and then converted to carbon using the following conversion factors: bacteria, $1 \mu\text{m}^3 = 5.6 \times 10^{-7} \mu\text{g C}$ [5]; cyanobacteria and algae, $1 \mu\text{m}^3 = 1.2 \times 10^{-7} \mu\text{g C}$ [4]; ciliates and testate amoebae, $1 \mu\text{m}^3 = 1.1 \times 10^{-7} \mu\text{g C}$ [45]; fungi $1 \mu\text{m}^3 = 2.5 \times 10^{-7} \mu\text{g C}$; nematodes and rotifers $1 \mu\text{m}^3 = 1.25 \times 10^{-7} \mu\text{g C}$ [14]. These data were expressed as micrograms of C per gram of *S. fallax* dry mass.

Numerical Analyses

Pb Accumulation in the Mosses. We compared the Pb accumulation of *S. fallax* at T20 in the three treatments using Mann–Whitney tests. Because the samples were pooled, this was not done for other dates. We plotted the Pb concentration over time in the three treatments to establish a temporal trend of lead accumulation in mosses.

Effect of Pb on Microbial Communities. We used two numerical approaches to study the effect of lead pollution and time on microbial communities. (1) To analyze the effect of lead on microbial communities, we performed a MANOVA, with lead treatment, time, and treatment \times time as factors (nominal variables) using the software JMP 3.2.6. When needed, the carbon biomass data of individual microbial groups were transformed using $\ln(x+1)$ or $\log(x+10)$ to homogenize variances. The total microbial biomass was analyzed using an ANOVA with the same factors as in the MANOVA. The significance level was set at $P < 0.05$. (2) However, as the analyzed microbial communities did not live within the contaminated solution, they were more likely to respond to the lead concentration really accumulated in mosses than to the concentration in the solution in which the lower part of the mosses was immersed. Therefore, in a second approach, the response of microbial communities to the Pb actually accumulated in the portion of the mosses that were analyzed for microbial communities (“real Pb”) was assessed using regression analysis. Because lead concentration in the mosses was not measured in replicates at T6 and T12, only the last sampling date (T20) was used in this analysis. To assess the effects of Pb pollution on the trophic relationships among groups of organisms, we calculated either the correlation between the biomasses or the density of different groups. While this correlative approach is not as powerful as manipulations of communities, it has been shown to be useful [9].

Results

Lead Accumulation in *S. fallax*. Pb concentrations in mosses varied between 1.1 and 15.2 $\mu\text{g g}^{-1}$, depending on treatments (Fig. 1). Pb concentrations tended to decrease during the first 6 weeks (T6) for control and E625, and then increased until the end of the experiment (T20). However, as these measurements were not replicated, it is unclear if this trend is significant. By contrast, a continuous increase in Pb accumulation was recorded from the beginning to the end of the experiment for E2500 (Fig. 1). At T20, Pb concentrations differed significantly among treatments and increased according

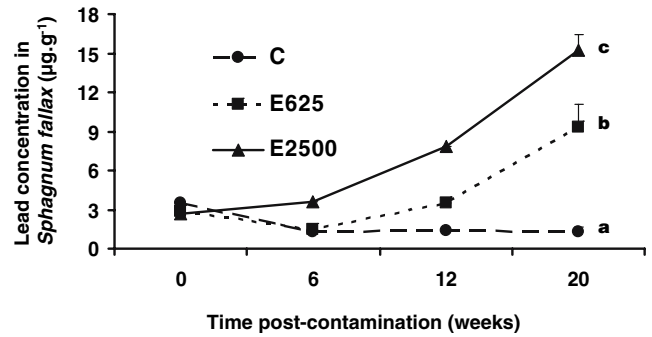


Figure 1. Accumulation of lead in *S. fallax* ($\mu\text{g g}^{-1}$) grown in the control and two different Pb concentration solutions 625 and 2,500 $\mu\text{g L}^{-1}$. The lines show the time course trend of lead accumulation. Different letters at T20 indicate significant differences between treatments. C control.

to the Pb loading (control $<$ E625 $<$ E2500) ($P < 0.05$, Mann–Whitney test). Lead concentrations increased exponentially in treatments E625 and E2500 over the course of the experiment, but this trend was significant only for E2500 ($R^2 = 0.98$, $P = 0.015$, exponential regression on the value from the pooled samples in T0, T6, and T12 and the average values for T20). No correlation was found between moss Pb concentration and exposure time for the control mosses ($P > 0.05$).

Impact of Lead on the Microbial Communities in *S. fallax*. The temporal variation of microbial biomasses for each treatment and sampling date is presented in Fig. 2. Bacteria and ciliates were the dominant microbial groups (on average, representing 45.4 and 18.2%, respectively, of the total community biomass).

The biomass of bacteria varied from 226 to 2,419 $\mu\text{g C g}^{-1}$ DW. The coccus morphotype was dominant (a cell was considered to have the coccus shape when the circularity value was above 0.8). The cyanobacterial biomass varied from 4.4 to 978.1 $\mu\text{g C g}^{-1}$ DW. Among the taxa recorded, two *Chroococcus* morphotypes were dominant, whereas three other genera, *Nostoc*, *Anabaena*, and *Aphanocapsa*, accounted for only a small percentage (1 to 8% depending on treatments and sampling dates) of the cyanobacterial biomass. The biomass of microalgae varied from 2.1 to 369.0 $\mu\text{g C g}^{-1}$ DW. *Penium* sp. was the dominant microalgal taxon (78.8%). The biomass of ciliates (*Uronema*) ranged from 18.7 to 4.1×10^3 $\mu\text{g C g}^{-1}$ DW (on average, 20.7% of the total community biomass). The total testate amoebae biomass varied between 14.9 and 524.9 $\mu\text{g C g}^{-1}$ DW. Among micrometazoa, rotifers strongly dominated over nematodes in terms of biomass. Only bdelloid rotifers were observed, and their biomass varied between 37.1 and 479.2 $\mu\text{g C g}^{-1}$ DW.

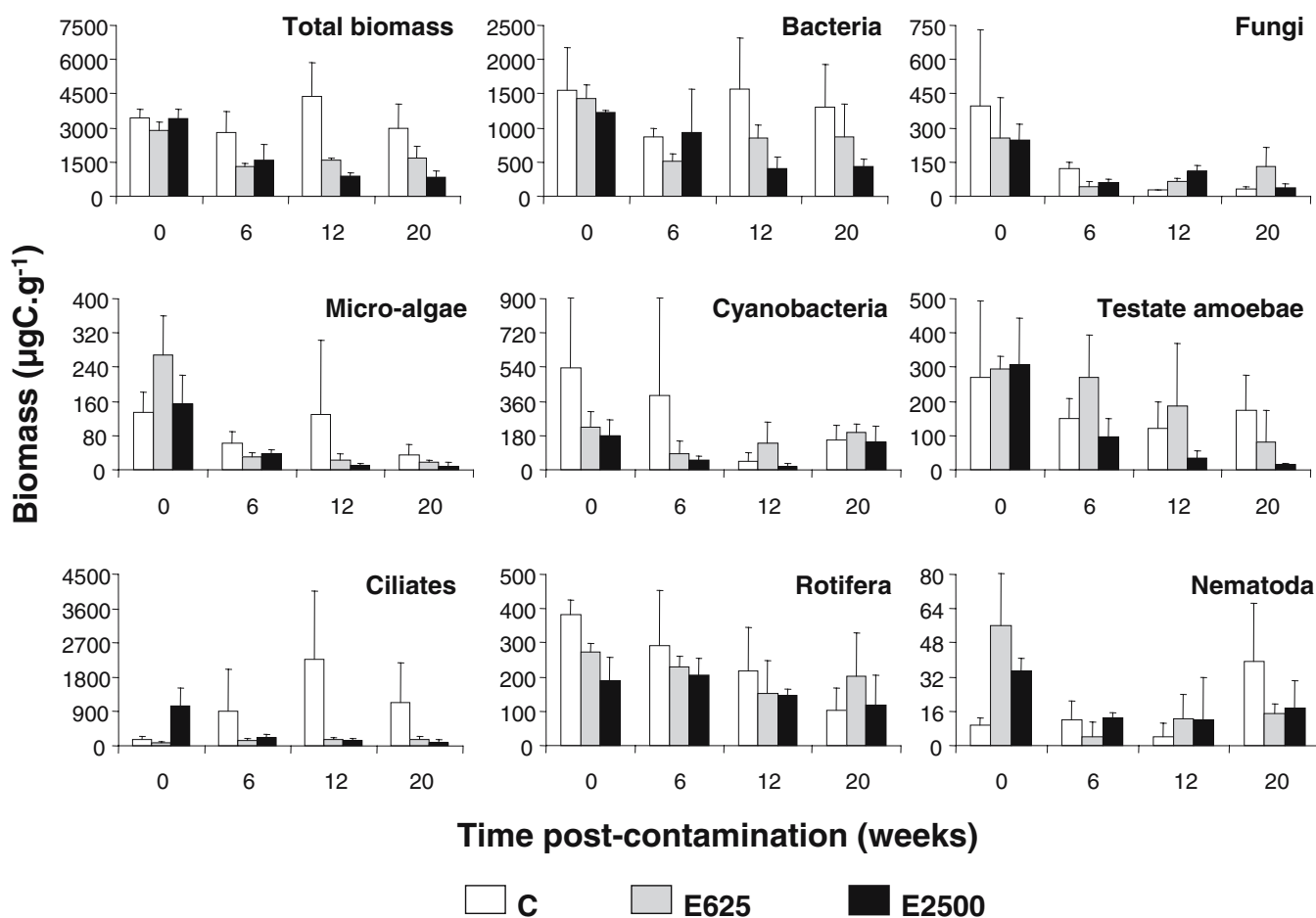


Figure 2. Carbon biomass (mean \pm SD) of microorganisms in *S. fallax*. Total biomass and detail for each microbial group at four sampling dates for the control and two lead treatments 625 and 2,500 $\mu\text{g L}^{-1}$. C control.

Both the total microbial biomass and the structure of microbial communities varied significantly over time, in response to the Pb treatment, and differently over time in function of the Pb treatment (ANOVA and MANOVA, $P < 0.005$). The biomass of all groups except ciliates decreased significantly over time ($P < 0.01$ for all). The Pb \times time cross-effect was significant for three microbial groups: (1) bacteria, which showed no clear trend in the control but declined in the treatments; (2) fungi, which declined more markedly in the control than in the treatments; and (3) ciliates, which increased in the control but decreased in the highest Pb treatment (Fig. 2). The Pb treatment effect was also significant for bacteria, ciliates, testate amoebae, and microalgae. For testate amoebae and microalgae, the cross effect was marginally significant ($P = 0.08$). This was also true for nematodes, but in this case, the Pb effect was not significant.

The correlation between bacterial (prey) and ciliate (predator) biomasses changed from negative to positive with increasing Pb levels, but this relationship was only significant for E2500 ($R = -0.72, 0.02, \text{ and } 0.61$; $P = 0.3,$

$0.84, \text{ and } <0.05$, respectively, for control, E625, and E2500; Fig. 3A–C). Similar results were recorded for the relationships between bacterial (prey) and testate amoebae (predator) biomasses ($R = -0.04, -0.03, \text{ and } 0.21$; $P = 0.53, 0.76, \text{ and } <0.01$, respectively, for control, E625, and E2500; Fig. 3D–F). No significant correlation was found for the other potential prey–predator pairs (bacteria, cyanobacteria, and microalgae prey vs rotifer and nematode predators).

Discussion

Lead Accumulation in *S. fallax*. *Sphagnum fallax* accumulated lead significantly at 625 and 2,500 $\mu\text{g L}^{-1}$ (Fig. 1), therefore partly supporting our first hypothesis that supposed that *S. fallax* takes up the added Pb in proportion to the concentration used in the experiment. The data presented in Fig. 1 suggest that lead accumulation for E625 and E2500 had not reached equilibrium by the end of the experiment. Based on results we obtained in a previous study on heavy metal concentrations in mosses

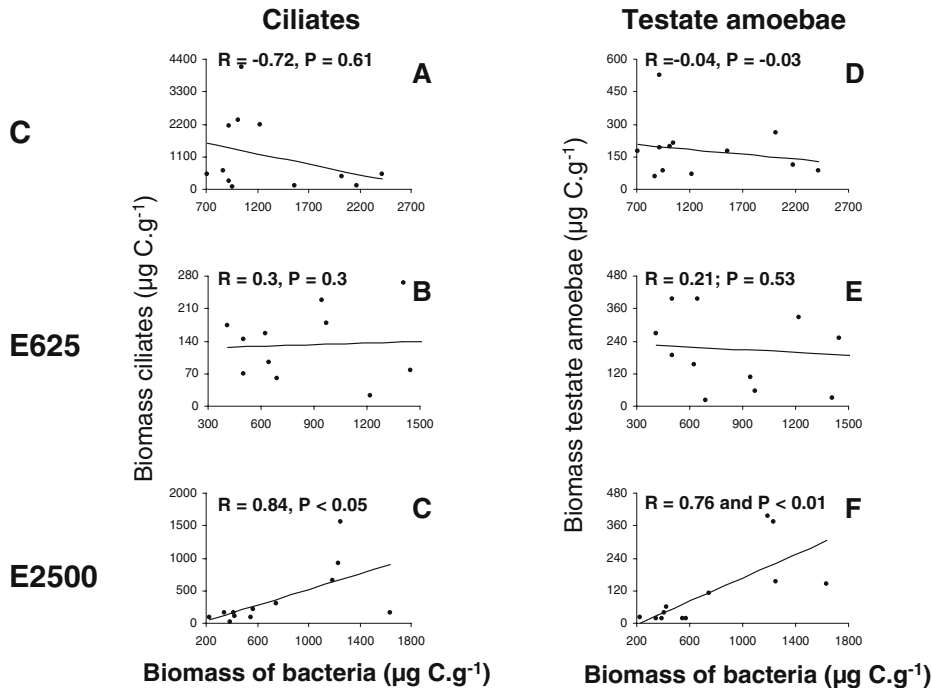


Figure 3. Slope of correlation between the biomass of bacteria and ciliates and testate amoebae in *S. fallax* of the control and two lead treatments 625 and 2,500 $\mu\text{g L}^{-1}$ ($n = 12$). A, B, and C corresponding to control, E625, and E2500, respectively, for ciliates and D, E, and F for testate amoebae. C control.

in the region of Hanoi, Vietnam [28], we expected higher concentrations of lead in the mosses. However, our results can be explained by the fact that we analyzed only the top 3 cm of the mosses, where the growth takes place, whereas the water level was kept at 7 cm under the moss surface. The top part can therefore be assumed to accumulate lead from the solution at a much lower rate than the lower part that was soaking in it. This lower accumulation in the top part is likely caused by (1) a gradual depletion of Pb through absorption by the *Sphagnum* cell walls during the capillary rise of water up to the top of the mosses and (2) the continuous growth of the mosses at their apex causing further dilution of Pb content.

Pb Effect on the Structure of the Microbial Communities. The addition of lead decreased the total microbial biomass significantly (Fig. 2). There was a significant negative linear correlation between the total microbial biomass and lead concentration in mosses at the end of the experiment ($P = 0.008$, $R^2 = 0.66$). Beyond total microbial biomass, our results show that Pb affected bacteria, protozoa, cyanobacteria, and microalgae, but not the micrometazoa and fungi, in partial agreement with our hypothesis H2 that the biomass and diversity of the different microbial groups analyzed would decrease with increasing Pb pollution (Fig. 2). Furthermore, in accordance with H3 (group-specific responses to Pb would result in changes in the structure of communities), the

structure of the whole microbial community was significantly modified by the addition of lead (MANOVA, $P < 0.0001$, Fig. 2). To put these results in the perspective of the functioning of the microbial loop, we will discuss the effect of lead on three functional groups of microorganisms: primary producers, decomposers, and predators.

Primary Producers. Cyanobacteria and microalgae are the two groups of microbial primary producers in peatlands. The biomass of cyanobacteria was marginally significantly affected by Pb treatment but the Pb \times time cross effect was not significant. The cyanobacterial biomass decreased after 6 weeks of treatment for E625 and E2500 (Fig. 2), and then recovered in the last two sampling dates. This pattern of apparent recovery brings some support to the proposed use of cyanobacteria for heavy metal removal [8] that is attributed to their capacity to either resist to or accumulate metals. Moreover, cyanobacterial biomass was dominated by two large-size species *Chroococcus* sp. (80% of relative biomass on average), whereas the contribution of nitrogen-fixing taxa such as *Anabaena* spp. and *Nostoc* spp. remained small (16%). The lack of correlation between individual cyanobacterial groups, in particular, heterocystic (nitrogen-fixing) taxa, and lead concentration in the moss ($P = 0.85$, $R^2 = 0.008$, $n = 9$, linear correlation) may tentatively be explained by their lack of sensitivity to lead. Unlike cyanobacterial biomass, microalgal biomass declined both over time and in response to Pb treatment, but the cross effect was not significant.

Decomposers. In accordance with previous reports of microbial communities in *Sphagnum* [14, 25], bacteria dominated the total microbial biomass for all treatments and sampling dates. Although the absolute biomass of bacteria decreased in treatments E625 and E2500 (Fig. 2), their relative contribution to the total microbial biomass remained relatively stable (on average $45.3 \pm 13.6\%$) regardless of treatment or sampling date. This stability may be due either to a general resistance of bacteria to lead or to a selection effect on bacterial species that had no effect on total biomass, as resistant species replaced sensitive ones in the community. The very fast turnover of bacteria allows for rapid renewal of their community structure. However, a more detailed assessment of the bacterial community structure was beyond the scope of this study. The fungi were not affected significantly by the lead treatments, in accordance with H2.

Predators. We hypothesized (H2) that protozoa would be especially affected by the added Pb due to the lack of cell wall [32]. In agreement with our hypothesis, both testate amoebae and ciliates were affected by Pb pollution.

In this study, the ciliate community was dominated by one morpho-species belonging to genus *Uronema*, which was affected by lead pollution. In addition to this strong dominance of a single taxon, significant differences existed at the onset of the experiment for ciliate biomass. To focus on the treatment-induced changes, we expressed the ciliate data as a percentage change relative to the initial biomass. Using this approach, the percentage of ciliate biomass at T6, T12, and T20 relative to T0 was lower in E2500 than in both control and E625 ($P < 0.05$, Mann–Whitney test). This would suggest that Pb affected ciliates already after 6 weeks. However, this interpretation clearly needs to be confirmed by additional studies. Nevertheless, our results are in agreement with previous studies on the effect of metals in general and lead in particular on ciliates in activated sludge and in the seawater. These studies have suggested that some ciliate species, like *Colpoda steinii*, *Cyrtolophosis elongata*, and *Drepanomonas revoluta*, suffer from alterations at the cellular and the nucleus level by cadmium and zinc [23]. In marine water, the addition of lead and other metals caused a shift in the community structure of ciliates [11]. The same phenomenon was observed for ciliates living in activated sludge [21].

Testate amoebae have repeatedly been shown to be good bioindicators for natural microenvironmental gradients, as well as pollutants including heavy metals, in aquatic and soil environments [6, 12, 13, 25]. Their response to Pb is therefore not surprising. For example, in a previous *in situ* investigation, we observed a decline in testate amoeba species richness, abundance, and

diversity index with increasing Pb concentration accumulated through atmospheric deposition on the moss *Barbula indica* in the Hanoi region, Vietnam [28]. In aquatic environments, Patterson *et al.* (1996) and Reinhardt *et al.* (1998) have shown that *Centropyxis aculeata*, *Centropyxis constricta*, and *Arcella vulgaris* resist well to arsenic and mercury contamination in Canadian lakes polluted by mine tailings [31, 35]. In addition, a decrease in the abundance of *Arcella* sp., *Diffflugia* sp., and *Euglypha* sp. in activated sludge was observed in response to copper pollution at 20 mg L^{-1} [30].

In accordance with our hypothesis, micrometazoa were more resistant to Pb pollution than unicellular eukaryotes. This lack of response for the larger organisms studied here (rotifers and nematodes) may be due to their higher structural complexity. The membranes of the latter are in direct contact with the external environment and pollutants, whereas the former are better protected by specialized tissues.

Potential Trophic Effects. In addition to direct toxicity effects, lead could also affect predatory microorganisms such as ciliates, testate amoebae, or rotifers through a modification of trophic relationships. For example, the main food source of the ciliate *Uronema* sp. is bacteria, and therefore a decline in bacteria or a shift in the composition of bacterial communities resulting in a dominance of unpalatable species would affect the population dynamics of *Uronema* sp. This could cause ciliates to shift to other prey, for example from bacteria to cyanobacteria. Furthermore, Pb pollution may affect the ability of *Uronema* sp. to catch bacteria (in terms of number of bacteria eaten/time unit), leading to a nonproportional response of *Uronema* as compared to bacteria. However, the same reasoning could be applied to rotifers.

To assess the relationships among groups of organisms, one possible approach is to calculate the correlation between the biomasses or the density of different groups [9]. When doing this separately for the different treatments, the correlation between prey (bacterial) and predator (ciliate or testate amoeba) biomasses changed from negative to positive with increasing Pb levels, but this relationship was only significant for E2500 (Fig. 3). No significant correlation was found for the other potential prey–predator pairs (bacteria, cyanobacteria, and microalgae prey vs rotifer and nematode predators). We tentatively explain these results by the pressure that Pb pollution exerts on the grazing pathways of these predators and by the effect Pb has on prey. For instance, in the unpolluted and medium-polluted conditions (control and E625), predators may have access to, and therefore may feed on, a wider range of prey (bacteria, cyanobacteria, algae, etc.). The negative, or lack of, correlation between the biomasses of bacteria and ciliates

or testate amoebae would therefore either mean that these protozoa feed preferentially on cyanobacteria and algae or that they are opportunistic feeders. In contrast, for the highly polluted condition (E2500), bacteria may represent the main food source for ciliates and testate amoebae, which would explain the strong and significant correlations between prey and predator biomasses (Fig. 3C, F). Testate amoebae prey on a large spectrum of organisms (bacteria cyanobacteria, algae, protozoa, rotifers, and nematodes) [47], and Pb contamination decreased the density of several of these. This effect could have restricted the choice of prey for testate amoebae, and decreased their density. However, such correlations are not straightforward to interpret because Pb simultaneously affects each group, and not necessarily in the same way. It is therefore impossible with our data to separate the direct effects from the indirect ones. For this, a study of each group alone and manipulative experiments with different combinations or organisms would be needed as a complement to the community approach we used. For example, specific functional bacterial groups (i.e., appropriate bacterial size for ciliates' feeding) could be studied, as suggested by Epstein *et al.* [10].

Among the microbial groups living in *Sphagnum*, testate amoebae have been suggested as a key functional group because of the multiple trophic relationships linking them to other groups [14, 25]. Our observations further suggest that phagotrophic unicellular eukaryotic microorganisms living in mosses are more sensitive to Pb pollution than prokaryotes or micrometazoa. Taken together, these results illustrate how complex the responses of communities to Pb contamination can be and how the observation of any single group or species may hide more subtle effects on the structure of communities and functioning of the ecosystems.

Advantages and Limitations of the Experimental Setup. As mentioned in the "Introduction," whole communities have not been much used as models in ecotoxicological studies and even less so for microbial communities in microcosms [7]. The model we used here is powerful and original because a small quantity of material (only ten mosses) is sufficient to extract a relatively complex community, with numerous trophic relationships among them. It is thus possible to work at the community level. Furthermore, our experiment combines the advantages of field microcosm approaches and may be an appropriate way to lean towards more realistic field conditions while maintaining a high level of control over microenvironmental conditions [7]. Our assumption was that this approach would yield results that are closer to those that can be expected in nature, as compared to studies on less complex models, such as single-species tests. The negative side is, of course, that a

more complex system is more likely to yield more "noisy" data. The challenge is to hear the music through the noise.

To our knowledge, this is the first study to assess the effect of pollution on various microbial groups in mosses under controlled conditions. In general, microcosm studies have essentially focused on aquatic invertebrate communities [44], but some microcosm studies have dealt with aquatic microbial communities [2, 39, 41]. In field conditions, among the different microbial groups, only testate amoebae have received substantial attention including experimental studies simulating environmental perturbation.

Our approach also presents some disadvantages. If the difference in community structure after contamination can be interpreted by the effect of lead concentration in mosses over time, the difference at the onset of the lead contamination can only be explained by the spatial heterogeneity of the community as has indeed been observed for testate amoebae in *Sphagnum* [24]. Although the acclimatization phase lasted 1 month and the treatments were randomly assigned to the plots, statistically significant differences remained ($P < 0.01$, Khi-2 test). Unfortunately, the microscopic analyses only revealed this once the treatments had started. Studying the effect of pollutants on communities of living organisms in complex environments and using experimental study conditions similar to *in situ* ones clearly represent important challenges for ecotoxicological studies, but these are needed.

Acknowledgments

We thank Marie-Laure Toussaint for her assistance in Pb analysis, Stéphanie Nicopoulos for determining cyanobacterial species, and Dominique Rieffel for nematode measurement. We express our acknowledgements to Michael Coeurdassier, Jérôme Cortet, and Renaud Scheifler for giving comments to improve the experimental design and helpful discussion throughout the work. Jyrki Jauhainen (University of Helsinki, Finland), Catherine Rausch, and Sandrine Gombert (Muséum National d'Histoire Naturelle de Paris, France) are acknowledged for discussion on *Sphagnum* growth and supplying references on this issue. We thank the two anonymous reviewers for helpful comments on an earlier version of the manuscript. H. Nguyen-Viet has been supported by the University of Franche-Comté through a temporary lecturer contract (2004–2006) and by the European Science Foundation through an exchange grant (program RSTCB, ESF, 2006). E. Mitchell was at the University of Alaska Anchorage at the onset of this experiment and was later supported by the Swiss contribution to EU project RECIPE (no. EVK2-2002-00269).

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