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Effect of lead pollution on testate amoebae communities living in *Sphagnum fallax*: An experimental study

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Abstract

We studied the effects of lead pollution on testate amoebae communities living on *Sphagnum fallax* by growing this moss under controlled conditions. A progressive series of lead (Pb) concentration was used in the growing solution of the mosses: 0 (control), 625 and 2500 $\mu g\,L^{-1}$. The mosses were sampled and analysed for accumulated Pb and testate amoeba communities after 0, 6, 12, and 20 weeks. Species richness, total density and total biomass of testate amoebae declined in response to the Pb treatment and changed over time. The Pb × Time cross-effect was significant for species richness, and total density but not for the total biomass and Shannon diversity. Furthermore, the testate amoebae species richness and the total density were negatively correlated to the Pb concentrations actually accumulated in the moss at the end of the experiment. Species-specific responses of testate amoebae to Pb pollution were identified. Our results thus confirm the sensitivity of testate amoebae to lead pollution.

Keywords: Lead; Microcosm study; *Sphagnum fallax*; Testacean; Diversity; Biomonitoring

1. Introduction

Testate amoebae are a group of free-living protists that build a rigid test (shell) from siliceous, calcareous or organic materials. These microorganisms are especially numerous in peatlands, mosses and others moist substrates (Bonnet, 1973). Testate amoebae have been shown to be good bioindicators for environmental condition (e.g. water availability, water chemistry) (Foissner, 1987; Gilbert et al., 1998; Tolonen et al., 1992). In addition, thanks to their rigid test that remains identifiable even after the death of the organism, testate amoebae have been used as tools, together with pollen analysis, to infer past environmental changes (Mitchell et al., in press).

The use of living organisms for monitoring environmental quality, the so called biomonitoring approach, is biologically and economically relevant as compared to the physico-chemical approach (Markert et al., 2003). Indeed, some living organisms (referred to as biomonitors), as they are continuously exposed to pollution, accordingly can integrate the pollution over a long period of time. They 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 not be detected by the physico-chemical approach if measurements are not continuous. Biomonitoring is usually lower-cost and more accessible than the physico-chemical measurements of pollution. For these reasons, it represents an interesting alternative approach to the physico-chemical methods that are often expensive and require expensive equipments and often the access to reliable electrical power (Markert et al., 2003). However, the first step towards using a group of organisms as bioindicators is to test their sensitivity to a given stress. For testate amoebae, several studies have now dealt with the correlation between pollution and testate amoeba. For instance, the diversity, density and community structure of

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testate amoebae living in mosses were showed to be negatively correlated to atmospheric pollution by heavy metal, in particular by lead (Nguyen-Viet et al., 2007) and by nitrogen dioxide (Nguyen-Viet et al., 2004). An earlier study also showed the negative effect of road traffic pollution on testate amoebae community but the pollution level was not measured but qualitatively estimated (Balik, 1991). Furthermore, experimental acidification also affected testate amoebae (Costan and Planas, 1986).

Heavy metals are one of the most intensively monitored pollutants due to their high toxicity to organisms and environments. Several organism groups have been used as biomonitors of metal deposition. For instance, mosses have been extensively used for monitoring heavy metal atmospheric pollution (Onianwa, 2001). Lead (Pb) is one of the heavy metals that have received the most intensive attentions from research because of their particularly toxic impact on environment. Pb concentrations accumulated in the mosses are usually used to reflect the Pb pollution in environment. For example, Pb concentration in moss ranges from 2.5 to 107 $\mu g\,g^{-1}$ in France (Gombert et al., 2002), from 0.1 to 60.4 $\mu g\,g^{-1}$ in Spain (Fernández and Carballeira, 2001) and from 2.1 to 60 $\mu g\,g^{-1}$ in *Pleurozium schrebe* moss around a smelter in Poland (Samecka-Cymerman et al., 2006). Few studies have focused on the use of microorganisms for this goal. Thus, our research team has been interested in the potential use of testate amoebae living in mosses as biomonitors of general environmental pollution (Gilbert et al., 1998; Mitchell et al., 2003, 2004; Nguyen-Viet et al., 2004) and of Pb pollution in particular (Nguyen-Viet et al., 2007). Indeed, different bio-ecological characteristics of testate amoebae suggest that they may be interesting candidates for monitoring environmental pollution: (1) They live in diverse (water, soil, sub-aerial) environments as presented above, where they are directly exposed to pollutants; (2) they are very abundant, diverse (about 100 potential species in mosses alone), and most of these species appear to be cosmopolitan (Bonnet, 1973); (3) their identification is relatively easy to the species level based on the morphology of their shell and (4) they are good integrators of disturbances because of their trophic position at the end of the microbial food webs (Gilbert et al., 2000).

To our knowledge, only three other studies focused on the relationships between testate amoebae and metal pollution. Lüftenegger and Foissner (1989) studied the effect of road traffic pollution on soil testate amoebae and concluded that the density, biomass and species richness of testate amoebae were affected by Pb, total carbon and polycyclic aromatic hydrocarbon. In aquatic environments, two studies were conducted on the effects of these pollutants on testate amoebae in lake sediments. Thus, Patterson et al. (1996) and Reinhardt et al. (1998) showed that *Centropyxis aculeata*, *Centropyxis constricta* and *Arcella vulgaris* are good indicators of arsenic and mercury contamination in some lakes heavily polluted by mine tailings in Canada. In a more recent *in situ* study on the relationships between testate amoebae communities and heavy metals, we showed that species richness and density of testate amoebae were significantly correlated, negatively to Pb concentration (Nguyen-Viet et al., 2007). Pb also significantly explained the variation of testate amoebae communities. We concluded that testate amoebae are sensitive to Pb and may be good indicators for heavy metal pollution, especially Pb.

However, none of these studies were experimental. Because correlation does not necessarily imply causality, the existence of relationships between metals (or other pollutants) and testate amoebae need to be tested with investigations conducted under controlled conditions. By restricting the influences of non-desired factors and by emphasizing the effects of the pollutant of interest, this approach helps better understand how and why testate amoebae respond to pollutants. In this context, the present study aimed at investigating the effect of Pb pollution on natural testate amoebae communities living on the peat moss *Sphagnum fallax* by growing this moss under controlled conditions and by contaminating it with a Pb solution. Growing testate amoebae in a totally pure medium represents a difficult and delicate method and it would be especially difficult to be certain that the interaction among species is not affected by the artificial growing environment [(Couteaux, 1992; Couteaux and Odgen, 1988; Cowling, 1986), pers. com. with M.M. Couteaux]. The present experiment overcomes the technical constraints relative to testate amoebae culture. We hypothesized that: (1) *S. fallax* would take up the added Pb in proportion to the concentration used in the experiment, (2) the density, biomass and diversity of testate amoebae would decrease with increasing Pb pollution, and (3) species-specific responses of testate amoebae to Pb pollution would be observed resulting in changes in their community structure.

2. Methods

2.1. Field moss sampling and culture setup in the laboratory

The moss *S. fallax* was chosen as a model because this moss is common and abundant in bogs. Furthermore, numerous previous studies have shown that this moss constitutes a good habitat for testate amoebae (Mitchell et al., in press). *S. 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 13th 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) (Lacroix and Moncorge, 1999). The vascular vegetation of the sampling site is dominated by typical bog plants such as *Eriophorum vaginatum, Calluna vulgaris, Andromeda polifolia,* and *Vaccinium oxycoccos* (Lacroix and Moncorge, 1999). We selected a *S. fallax* surface of 5 × 5 m as homogenous and pure as possible. Fifteen points within this surface were randomly chosen 1–4 m apart, from which 15 moss rectangles (22.0 × 8.5 cm, 15.0 cm of 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 non-moss plant remains (e.g. vascular plant leaves and twigs) 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 (Rudolph et al., 1988). The water depth in the trays was kept at 7 cm below the top of the mosses by adding a 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); 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.

2.2. 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, Pb in the form of Pb^{2+} ions (from $PbSO_4$) was diluted in the standard nutrient solution in which the mosses rested. A progressive series of Pb concentration was used: 0, 625 and 2500 μg L^{-1}, and these treatments were respectively coded control, E625 and E2500. 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, 10 mosses (the top 3 cm) 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 hydrogen peroxide (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 graphite furnace atomic absorption spectrometer. Concentration measurement of Pb in the reference material ray grass (CRM 281, No. 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.

2.3. Testate amoebae community analyses

2.3.1. Sampling

Mosses were sampled at the onset of the experiment (coded T0), after 6 (T6), 12 (T12) and 20 (T20) weeks of contaminations for testate amoebae community analyses. At each sampling date, approximately 10 mosses (the top 3 cm) were randomly sampled from each tray and fixed in 15 ml glutaraldehyde (2% final concentration) for testate amoebae analyses.

2.3.2. Extraction

Testate amoebae were extracted from mosses using the following optimized extraction method: 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 of mosses.

2.3.3. Microscopic analyses

A 10 ml sub-sample was allowed to settle for 24 h in a plankton chamber. Living, encysted and dead amoeba tests were counted separately over the entire surface of the chamber. However, to focus on the effects of Pb on testate amoebae and avoid a possible bias due to the presence of empty shells, only the data on active testate amoebae was used.

2.3.4. Diversity index

To evaluate the diversity of testate amoebae communities, the Shannon index was calculated using the following formula:

Shannon index $H' = -\Sigma n_i/N \times \log_2(n_i/N)$, where n_i is the number of individuals of testate amoebae species i in the community and N is the total number of individuals in the community.

2.3.5. Estimation of biovolumes and biomass

The biovolume of each testate amoebae species was first estimated by assuming geometrical shapes using the image treatment software LUCIA 4.80 and then converted to carbon using the conversion factor: $1\,\mu m^3 = 1.1 \times 10^{-7}$ μg C (Weisse et al., 1990). The data were expressed as μgC g^{-1} of *S. fallax* dry mass.

2.4. Numerical analyses

2.4.1. Pb accumulation in the mosses

We used the Pb concentration measured in the mosses as support for contamination data. We plotted the Pb concentration over time in the three treatments to establish a temporal trend of Pb accumulation in mosses. As the samples were pooled for T0, T6 and T12, a measure of the variability among replicates is only available for the last sampling date (T20).

2.4.2. Effect of Pb on testate amoebae communities

We used two numerical approaches to study the effect of Pb pollution and time on testate amoebae. (1) To analyse the effect of Pb on testate amoebae communities we performed a MANOVA, with Pb treatment (control, E625, E2500), time (T0, T6, T12, T20), and treatment × time, as factors (nominal variables) using the program Statgraphics Plus 5.0. The data of species richness, Shannon index, total density and total biomass of testate amoebae were transformed using $\ln(x+1)$ to homogenize variances. (2) However, as the analysed testate amoebae communities did not live within the contaminated solution, they were more likely to respond to the Pb 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 analysed for microbial communities ("real Pb") was assessed using linear regression analysis. Only the last sampling dates (T20) were used in this analysis because their moss Pb concentrations were available for replicates as mentioned above.

3. Results

3.1. Pb accumulation in S. fallax

Pb concentrations in mosses varied between 1.1 and 15.2 μg g^{-1}, depending on treatments (Fig. 1). Pb concentrations decreased during the first 6 weeks (T6) for C and E625 and then increased until the end of the experiment (T20). 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 to the Pb loading (control < E625 < E2500) ($P < 0.05$, Mann–Whitney test). Pb 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

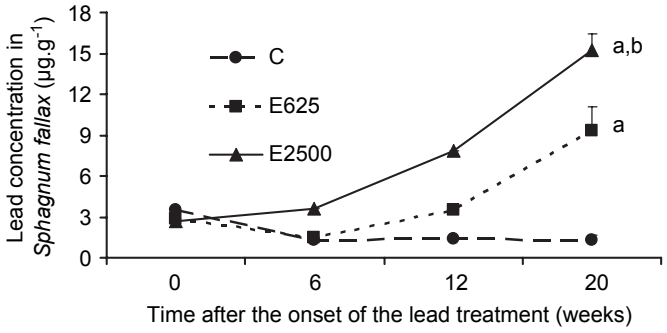

Fig. 1. Accumulation of Pb in *Sphagnum fallax* ($\mu g\,g^{-1}$) grown in the control and two different Pb concentration solutions 625 and 2500 $\mu g\,L^{-1}$. The lines show the time course trend of Pb accumulation. a: Different compared with the control; b: different compared with the E625; $P<0.05$, Mann–Whitney test.

found between moss Pb concentration and exposure time for the control mosses ($P>0.05$).

3.2. Density and diversity of testate amoebae

A total of 20 testate amoebae taxa were identified in the 36 samples analysed. However, *Heleopera rosea* and *Assulina seminulum* occurred in respectively only one and three samples (of the total of 36 samples) and in low densities compared to the average of the communities (respectively 4.6 ± 27.6 and 10.5 ± 39.3 individuals g^{-1} d.w.). Therefore, these two species were removed for further numerical analyses.

The total density of active amoebae varied from 1463 to 37 059 individuals g^{-1} d.w. in the moss samples. The average density was 13013 ± 9944 individuals g^{-1} d.w. Species richness varied between 2 and 15 species depending on the sample, treatment and sampling date (mean = 7.5 ± 3.4 species per sample). Among the recorded species, two species *Hyalosphenia papilio* and *Assulina muscorum* were present in all 36 samples. *Nebela tincta*-type, which could include *Nebela tincta, N. tincta-major, N. bohemica,* and *N. colaris*, was found in 30 samples. These three species dominated the testate amoebae community (average relative abundance of $50.2\%\pm26.5\%$, $18.9\%\pm13.8\%$ and $11.1\%\pm12.7\%$ respectively). In contrast, four other species *Heleopera sphagni, Arcella catinus, Arcella discoides* and *C. aculeata* occurred only in 4, 5, 6 and 7 samples, respectively and contributed to less than 0.4% to the overall community count. Shannon index H' ranged from 0.56 to 2.99 (mean = 1.73 ± 0.72).

3.3. Impact of Pb on the testate amoebae communities in S. fallax

The MANOVA showed that the species richness, total density and total biomass of testate amoebae responded to the Pb treatment ($P<0.05$ for all) and changed over time ($P<0.0001$ for all). The Pb × Time cross-effect was significant for the species richness, and total density ($P<0.05$ for both) but not for the total biomass and Shannon index ($P=0.08$ and 0.718, respectively). The Shannon index was not affected by the Pb treatment ($P=0.142$). To evaluate more accurately at which sampling date, testate amoebae were affected by Pb pollution, we compared the different variables for each sampling date using Mann–Whitney tests. Thus, there was no significant difference in testate amoebae species richness, total biomass and Shannon index at T0 (Fig. 2A, C and D). Species richness was significantly decreased at T20 for E625 and E2500, as compared to the control ($P<0.05$) (Fig. 2A). The biomass in E2500 was lower than in the control at T12 and T20. It was also lower in E2500 than in E625 at T6 and T20 ($P<0.05$, Fig. 2). The total density in E625 was significantly greater than in the control at T0, T6 and T12. The total density in E2500 was significantly lower than in the control at T20, and than in E625 at T12. Furthermore, the testate amoebae species richness and the total density were negatively correlated to the Pb concentrations actually accumulated in the moss (Fig. 3).

4. Discussion

4.1. Density and diversity of testate amoebae

Comparison of the testate amoebae diversity and density in the moss between studies remains always difficult as methods used have not been standardized: for example, the height of moss to be analysed, the resolution of identification to species level but also other possible reasons. Nevertheless, since *Sphagnum* are the dominant plants in peatland and represent a habitat favourable for microorganisms, testate amoebae living on these mosses species have been extensively investigated on both systematic and ecological aspects (Mitchell et al., in press). The 20 species identified in *S. fallax* in our study are also commonly described in *Sphagnum* mosses (Gilbert and Mitchell, 2006) and the total diversity is comparable to other studies focusing on *Sphagnum* sp. For instance, Kishaba and Mitchell (2005) found 33 species in 14 different *Sphagnum* species in a small raised bog in Switzerland by also analysing the first 3 cm of mosses. Mitchell et al. (2003) identified 58 testate amoebae species in the first 5 cm of different *Sphagnum* species in a study on the effect of atmospheric carbon dioxide enrichment on the structure of the microbial community in Northern peatlands (Finland, Sweden, England, Switzerland and Netherlands). However, more recently, Robson et al. (2005) recorded only 12 species in the first cm of *S. magellanicum* in a Southern peatland (South America). Finally, Mitchell et al. (2000) analysed the testate amoebae communities in the top 2 cm of *S. magellanicum*, and found a species number comparable to our finding (21 species). This latter study is perhaps the most comparable to our study because a single, relatively small area was studied instead of many sampling spots covering broader environmental gradients as in most other studies.

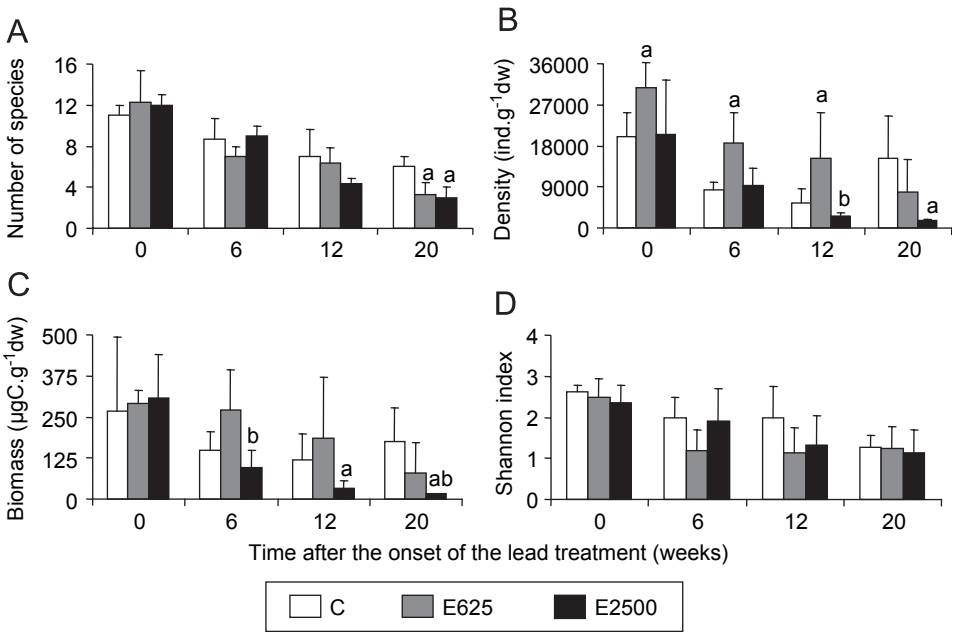

Fig. 2. Mean ± S.D. of species richness (A), total density (B), total biomass (C) and Shannon index (D) of testate amoebae in *S. fallax*. a: different compared with the control; b: different compared with the E625; $P < 0.05$, Mann–Whitney test.

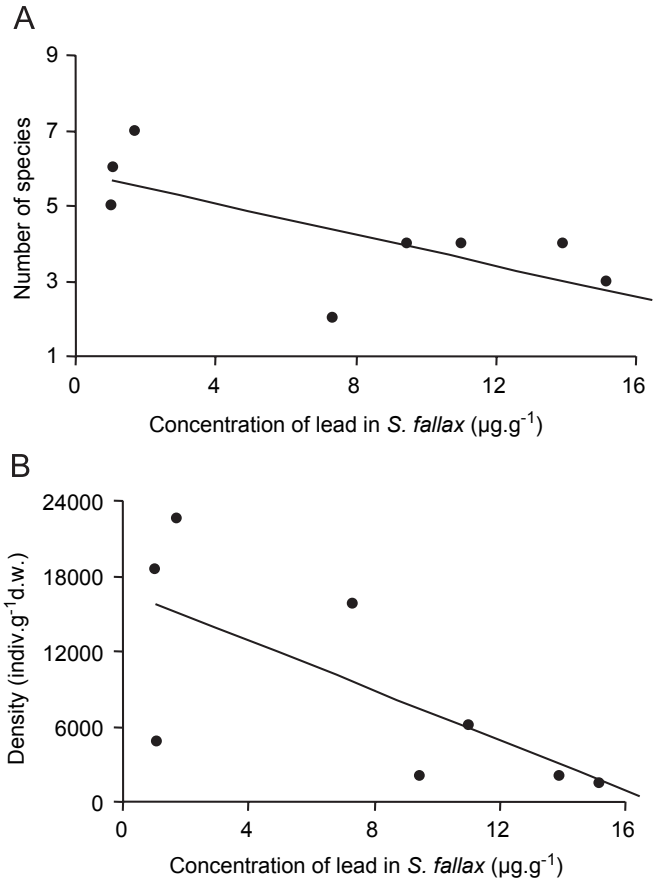

Fig. 3. Correlation between Pb concentrations actually accumulated in *S. fallax* and species richness (A) and total density (B) of testate amoebae.

The diversity and density of testate amoebae also depend strongly to environmental conditions such as humidity and pH (Mitchell et al., 1999; Tolonen et al., 1992). Furthermore, the vertical micro-distribution of testate amoebae has been demonstrated, with an increase of density and diversity of testate amoebae with the moss sample depth (compared to the surface of the moss) (Heal, 1962; Meisterfeld, 1977; Mitchell and Gilbert, 2004). Therefore, the fact that we analysed the top 3 cm of *S. fallax* certainly underestimated the real diversity of testate amoebae in the moss. Thus, the highest density recorded for the four species *A. muscorum*, *Hyalosphenia papilio*, *Nebela tincta*-type and *Amphitrema flavum* seems to be partly explained by the ecological behaviours of these species: *Hyalosphenia papilio* and *A. flavum* are mixotrophic species, which live in symbiosis with some microalgae inside their cytoplasm. So, these testate amoebae species have been mostly observed in the top part of the mosses, where their endo-symbionts can photosynthesize (Gilbert and Mitchell, 2006; Gilbert et al., 2003). *A. muscorum* is a very ubiquitous species not only in *Sphagnum* mosses but also in terrestrial mosses (Beyens and Chardez, 1984; Van Kerckvoorde et al., 2000). *A. muscorum* was the most abundant in the top segment of *Sphagnum* moss (Mitchell and Gilbert, 2004) and is an indicator of the dryness (Bonnet, 1973), associating to the top part of moss in our case. For *Nebela tincta*-type, Gilbert et al. (2003) showed that this *Nebela* group feeds on a wide range of living, senescent, or dead microorganisms (fungi, microalgae, ciliates, other testate amoebae, rotifers) and organic remains, allowing *Nebela tincta*-type to be distributed along the mosses.

Finally, it is necessary to mention the decrease in species number of testate amoebae in all the samples including the control mosses along the exposure. This reflects the effects of growth-chamber conditions, apart from the contamination effect. Indeed, this phenomenon is common in ecotoxicological studies carried out in microcosms. Controlled conditions

were surely not optimal for testate amoeba development: in our system, recolonization of our samples from the surrounding may be impossible, which would limit the possibilities of communities to exchange and refresh, like in *in situ* conditions and accordingly reducing testate amoebae diversity.

4.2. Effect of Pb on testate amoebae

Pb addition significantly affected testate amoebae communities through a diminution of species richness, density and biomass. Furthermore, the number of species and density were negatively correlated to the concentrations of Pb accumulated in the mosses at the end of the experiment. These results support our hypothesis as well as our observations from an *in situ* study of testate amoebae in the moss *Barbula indica* in Hanoi region, Vietnam (Nguyen-Viet et al., 2007). Our results also agree with other studies, showing a sensitivity of testate amoebae to pollution. Indeed, in aquatic environments *C. aculeata*, *C. constricta* and *A. vulgaris* were shown to be good indicators of arsenic and mercury contamination in Canadian lakes polluted by mine tailings (Patterson et al., 1996; Reinhardt et al., 1998). In addition, a decrease in the abundance of *Arcella* sp., *Difflugia* sp. and *Euglypha* sp. in activated sludge was observed in response to copper pollution at $20\,mg\,L^{-1}$ (Nicolau et al., 2005). Thus, our results add to the existing observations by showing the early sensitivity of testate amoebae to increasing Pb concentrations in *Sphagnum* moss under controlled conditions.

We had assumed that the Pb concentration in solution might not affect testate amoebae living in the top part of *S. fallax* but that these organisms would rather respond to the Pb actually accumulated in the precise microhabitat where they live. Indeed, the capillarity allowed *S. fallax* to remain constantly wet up to the top of the mosses, which were 7 cm above the water level. This capillarity also allowed Pb to reach the upper part of the mosses and accumulate there. In the present study, it is rather surprising to observe the effects of Pb on testate amoebae at concentrations which were much lower than those recorded in mosses *in situ* (Nguyen-Viet et al., 2007). Indeed, in the previous study, we recorded a range of Pb concentrations in mosses from 15 to $56\,\mu g\,g^{-1}$ (mean $= 26\,\mu g\,g^{-1}$) while in this study we observed a maximum concentration of $15.2\,\mu g\,g^{-1}$. However, the low concentrations of Pb observed in *S. fallax* may be due to the fact that we analysed only first 3 cm and to the short exposure period (20 weeks as compared with the long accumulation of *in situ* moss estimated to be of about 2–3 years (Nguyen-Viet et al., 2007).

The effect of Pb on testate amoebae could be also due to the indirect impact through microbial interactions within the microbial community, in particular trophic relationships between testate amoebae and their prey or predators. For example, when feeding on their preys (bacteria or other microorganisms), testate amoebae may be affected if their preys have been also affected by Pb contamination. Testate amoebae may be affected by the modification of their prey density and community structure. The favoured prey might have decreased or the competition for prey with other predators, such as ciliates, rotifers and nematodes, might have increased as a result in prey abundance or community structure. However, the limited knowledge on the food preference of testate amoebae and the lack of detailed data on the possible changes in prey community structure currently limits the interpretation of these changes.

4.3. Pb effect on the relationship between species richness and density of testate amoebae

Diversity-production dependence under disturbed conditions has been much discussed recently. This subject has mostly been addressed to plants in terrestrial ecosystems (Pfisterer and Schmid, 2002; Tilman et al., 1996). It would seem important to test these ideas also in the microbial world in order to determine if these relationships are also true for microbial ecosystems. Our results showed that there was no significant correlation between species richness and density of testate amoebae in the control mosses ($P = 0.46$, Fig. 4A). This correlation became almost significant for E625 mosses ($P = 0.0504$, Fig. 4B) and strongly significant for the highest treatment ($P < 0.001$, Fig. 4C). The changes in correlation between species richness and density show that Pb contamination reduced the number of testate amoebae species and affected the density of these species at the same time. This effect was in proportion to the Pb concentration accumulated in the mosses ($C < E625 < E2500$). Studies on the relationship between biodiversity and ecosystem function have shown that in species rich communities, redundancy exists. Thus species can be lost with no or little effect on ecosystem services or function (Naeem and Li, 1997). Beyond a certain threshold however, this redundancy is lost and the community then becomes vulnerable to any additional stress. Thus it would seem that the highest Pb concentration sufficiently affected the community to eliminate some functional groups. In case of a moderate pollution level, although species richness declined, a sufficiently broad spectrum of functional types remained for the total density to remain unchanged.

4.4. Specific response of testate amoebae to Pb contamination

At the end of the experiment (T20), two species *Halosphenia papilio* and *A. muscorum* were observed in all the samples. *A. flavum* and *Euglypha compressa* occurred in two samples of the most contaminated samples. Does this mean that these species are resistant to Pb pollution? To answer this question and to identify which species are sensitive to Pb contamination, we performed a redundancy analysis (RDA) using Canoco (Ter Braak and Smilauer, 1998). In this RDA, the explanatory variables were the sampling dates (0, 6, 12 and 20 weeks) and the nominal

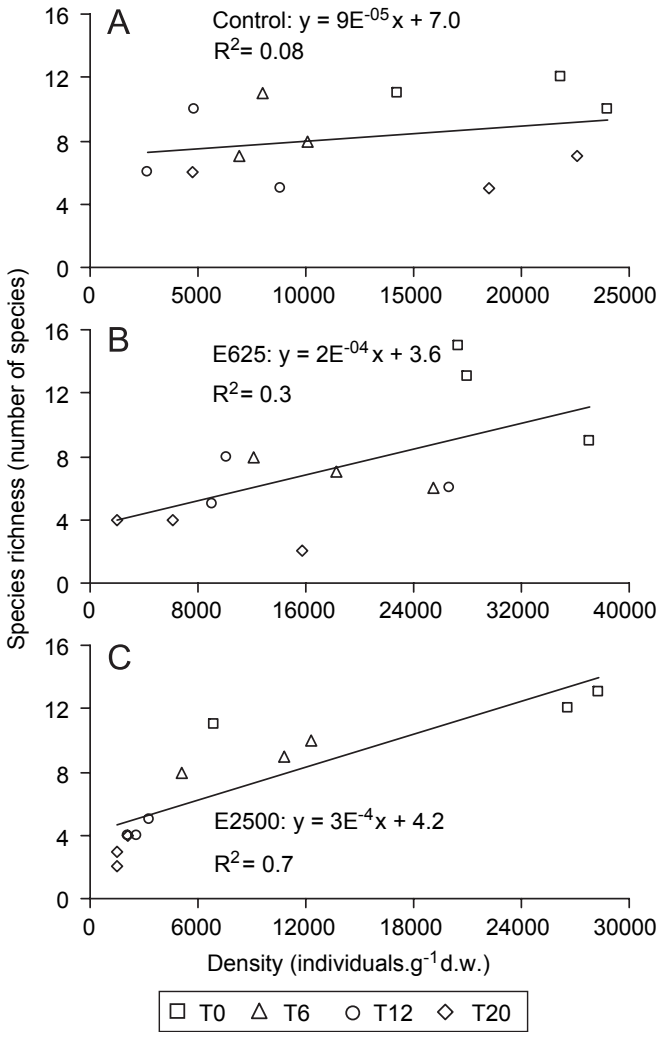

Fig. 4. Relationship between density and number of species in separate treatments: A, B and C for control, E625 and E2500, respectively.

variable for treatment (C, E625 and E2500 coded for 1, 2 and 3, respectively). The fact that we used the nominal variables helps us overcome the lack of replicate values of moss Pb concentration in T0, T6 and T12 samples. The explained variables were the density of testate amoebae for the 36 samples (transformed data using $\ln(x+1)$ to reduce the weight of dominant species). The RDA results showed that sampling date and Pb treatment together explained significantly 33% of the variation in the testate amoebae data ($P = 0.001$, Monte–Carlo permutation test, 999 permutations). Axis 1 is mainly explained by the sampling date while axis 2 is best explained by the Pb treatment. Taxa contributions on the axis are considered to be significant when their contribution is equal or higher than a threshold S (‰) calculated by the following formula: $S_n(‰) = 1/n \times 1000 \times 2$, where S_n is the contribution to axis 1 or axis 2 of the RDA; n the total number of species in the community (Renaud, 2003), accordingly $S_n(‰) = 0.11$ for the present study. Thus, although *H. papilio* was found in all 36 samples, the RDA identified this species as being sensitive and not resistant to Pb (its score on the Pb axis is 0.17, higher than threshold of 0.11). The three most sensitive species to Pb contamination were *Nebela carinata, Euglypha strigosa,* and *H. sphagni*, while the resistant species identified (in increasing order of resistance) were: *A. catinus, Nebela militaris, Corythion dubium, Trinema lineare, A. discoides,* and *C. aculeate*.

Furthermore, testate amoebae responded to Pb pollution depending on their functional characteristics. When addressing this aspect with regards to phylogenetic affiliation, the predatory or omnivorous Arcellinida are more affected by the highest Pb treatment than the mostly bacterivoreous Euglyphida. In the control the Euglyphida decline faster with time at first but at T20 the diversity of both groups was reduced by about 50%. In the intermediate Pb treatment the same trend is visible, but more marked with a final diversity reduction of 75%. In the highest Pb concentration the same trend is visible at T6, but then at T12 and T20 the Euglyphida resist better than the Arcellinida, and also better than in the intermediate treatment (in relative terms, in absolute terms the response is identical at T20). When considering the size of tests, we divided the species in four classes according to their biovolume. For the largest group (class 1), we observed no decline for the control samples and an increasingly shape decline with increasing Pb addition level. For size class 2, the diversity in control samples declined faster than for size class 1 but there was still a stronger decline for increasing Pb levels. For size class 3 the diversity drop was even stronger and the differences among treatments were less clear. For size class 4, the diversity drop was strongest and no differences remained among treatments. These results show that (1) small species are more affected by the artificial conditions of the experimental setup and (2) the species-specific (or size-class-specific) responses are marked with larger species being more affected by Pb than the smaller ones.

To our knowledge, this work is the first experimental study on the effect of the Pb pollution on testate amoebae. Our study was also the first attempt of maintaining testate amoebae communities under controlled conditions in their natural habitat. The results show that testate amoebae respond early to increasing Pb concentrations in *Sphagnum* mosses under controlled conditions. This study, together with our previous work in field conditions (Nguyen-Viet et al., 2004; Nguyen-Viet et al., 2007) suggests that, although further work remains to be done, testate amoebae could be of interest in the biological monitoring of Pb pollution.

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