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## Relationship between Atmospheric Pollution Characterized by NO<sub>2</sub> Concentrations and Testate Amoebae Density and Diversity

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**Summary.** To assess the potential use of testate amoebae as biomonitors of atmospheric pollution we studied the relationship between atmospheric nitrogen dioxide (NO<sub>2</sub>) pollution and testate amoebae density, diversity, and community structure (Protista: Rhizopoda) in (zone 1) and around (zone 2) the city of Besançon, France. NO<sub>2</sub> concentrations were on average significantly lower in the city zone (mean: 34.8 ± 9.5 µg/m<sup>3</sup>) than in the periphery zone (mean: 14.6 ± 4.7 µg/m<sup>3</sup>). The density of living amoebae was correlated with that of empty tests (0.001 < p < 0.043 depending on the species), therefore we used the total of dead and living amoebae in all analyses. Testate amoebae species richness was significantly lower in the city (4.7 species) than in the less polluted surrounding areas (6.0 species) but the total density did not vary between the two zones. Of the nine recorded taxa, the density of only one, *Paraquadrula irregularis* differed significantly between the two zones (p = 0.017), being present in all periphery samples and absent from all city samples. These results are interesting because the pollution level recorded was very low. Although further work is needed before testate amoebae can be used as a monitoring tool for atmospheric pollution, these results suggest they may have a potential for such a use. Further work should focus on potential effects of other pollutants and studies under controlled conditions.

**Key words:** air pollution, bioindication, bryophyte, nitrogen dioxide, protist, testate amoebae.

### INTRODUCTION

The degradation of air quality in urban areas is a major problem and many countries have developed programs to monitor and control its intensity. Atmospheric pollution is a complex notion that is mainly

defined by its negative consequences. The perception of this threat changes as progresses are made in atmospheric physics and chemistry, toxicology (Sandström 1995, Sega 1995) and epidemiology (Last *et al.* 1994, Nakai *et al.* 1995, Société Française de Santé Publique 1996, Bernard *et al.* 1998). In addition, atmospheric pollution varies temporally in relation to climate, especially wind and temperature. For this reason, measurement must be done almost on a continuous basis. A complementary option is to use the sensitivity of organisms to pollution level. This biomonitoring approach

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integrates the pollution level over a long period of time and therefore provides data on an average pollution level for a given place. It may also allow the detection of extreme or catastrophic events than may not be recorded by non-continuous pollution monitoring. The sensitivity of an organism to environmental stress depends on its ability to recover from stress, its mobility and its live cycle pattern.

Several groups or organisms have been used as bioindicators of atmospheric pollution. The most commonly used are mosses (Palmieri *et al.* 1997, Pearson *et al.* 2000), and lichens (Hamada and Miyawaki 1998, Conti and Cechetti 2001). Farmer *et al.* (1992) performed fumigation experiments with bryophytes and lichens and criticized the utilization of unrealistically high concentration of NO<sub>2</sub> used in many experiments. The effects of a realistic NO<sub>2</sub> concentration (122.2 µg/m<sup>3</sup> = 64 ppb - using for NO<sub>2</sub> 1 ppb = 1.91 µg/m<sup>3</sup> at 21.1°C) on the endohydric moss *Polytrichum formosum* were varied: the growth of existing shoots was a first stimulated but later these shoots suffered a 46% reduction in number, and there was a 36% reduction of new shoot production (Bell *et al.* 1992). In a fumigation experiment, Morgan *et al.* (1992) showed that, at about half that concentration (35 ppb = 66.9 µg/m<sup>3</sup>), NO<sub>2</sub> stimulated nitrate reductase activity in four mosses. In another laboratory experiment nitrite was showed to affect the shoot respiration of two terricolous (i.e. growing on the soil) mosses *Pleurozium schreberi* and *Rhytidiadelphus triquetrus* (Bharali 1999).

By comparison to bryophytes and lichen, only few studies have focused on microorganisms. The rare exceptions concern fungi and algae. For example, Garcia *et al.* (1998) showed that the lead concentration of *Coprinus comatus* (Fungi) was correlated to pollution levels. Green algae have also been shown to be good indicators of nitrogen, sulphur and metal pollution (Poikolainen *et al.* 1998, Shubert *et al.* 2001). To our knowledge, although testate amoebae were shown to be valuable indicators for soil conditions (Foissner 1987, 1999; Tolonen *et al.* 1992, 1994; Gilbert *et al.* 1998; Muqi and Wood 1999), only two studies have focused on using these protists as bioindicators of atmospheric pollution (Lüftenegger and Foissner 1989, Balik 1991).

In a study on the effect of road pollution on testate amoebae in Warsaw (Poland), Balik reported a decrease in abundance, species richness, species diversity (Shannon Weaver index), and the index of equitability (Balik 1991). Furthermore a shift in community composition was also observed. Of a total of 42 species and subspe-

cies recorded, 16 were found only in the less polluted sites, while three were found only in the more polluted sites, but two of these each in a single sample and in relatively low abundance. Unfortunately, no data on the atmospheric or soil pollution were given in this study and therefore the information is qualitative.

Lüftenegger and Foissner (1989) also studied the effect of road traffic pollution on soil testate amoebae by analysing two 100 m transects on both sides of a high-traffic road. In this study several pollutants were measured: lead, cadmium, chloride, and polycyclic aromatic hydrocarbons. The lowest density, biomass and species richness of testate amoebae occurred near the road, but were not correlated with the highest concentrations of the measured pollutants, most of which peaked at 50 m from the road. Testate amoebae were thus correlated with higher lead, total organic carbon, and polycyclic aromatic hydrocarbon concentrations but the reason for their lower abundance near the road could not be established.

Testate amoeba may be interesting candidates for the monitoring of air pollution for several reasons: (1) they can live in a sub-aerial environment where they are directly exposed to atmospheric pollutants, (2) they are very abundant, diverse (about 100 potential species in mosses alone although the number in any given samples is much lower), and most species are cosmopolitan (although exceptions exist) (Bonnet 1973), (3) their identification is relatively easy based on the morphology of their test (shell) that remains even after the death of the organism and (4) they are good integrators of perturbations because of their trophic position at the end of the microbial food webs (Gilbert *et al.* 1998, 2000). Mosses growing on vertical surfaces, such as trees and walls, where water drains fast, represent an extreme environment for aquatic microorganisms including amoebae. Water availability appears to be the main limiting factor for the amoebae, and accordingly, the species found in these mosses often have adaptations such as small size or a flattened test (shell) with a ventral aperture (Bonnet 1973).

The aim of this study was to evaluate the relationship between anthropogenic atmospheric NO<sub>2</sub> pollution and the density, diversity and community structure of testate amoebae living in *Tortula ruralis* (Hedw), a common moss species in urban and suburban areas. Testate amoebae are likely to be indirectly affected by NO<sub>2</sub> pollution through the bacteria and other microorganisms on which they feed if these were themselves feeding on contaminated remains of mosses. In addition, gases may

directly affect amoebae as they diffuse in the water film and change the water chemistry. Our working hypotheses were: (1) NO<sub>2</sub> pollution levels would be higher in the city than in the surrounding areas. (2) The density and diversity of testate amoebae and the structure of communities would differ between the city and the surrounding area, and along the NO<sub>2</sub> pollution gradient.

## METHODS

**Study sites.** This study took place between April 20<sup>th</sup> and May 3<sup>rd</sup> 2001 in 15 sites located in the town of Besançon (Franche-Comté, France) and surrounding villages (Fig. 1). The sites were selected along an East-West axis in two zones: (1) city: seven sites located within the limits of Besançon and (2) periphery: eight sites located in the surrounding outskirts and rural area. The periphery zone included the city outskirts where shopping areas are located but with no industrial source of pollution, and rural areas. As the dominant winds blow from the Southwest, the peripheral sites were presumably not contaminated by the “plume” of higher pollution levels produced in the city, and therefore the pollution gradient was maximized.

**Meteorological data.** Data from MeteoFrance stations located in Besançon (temperature, rain, humidity and wind speed) and in Marnay (rain) were used. In Besançon, the minimal temperature varied from -0.9 to 11.4°C and the maximal from 7.2 to 20.4°C. The sum of rain during this period was 38.4 and 36.1 mm of water respectively in Besançon and Marnay. The humidity varied from 67.4 to 90.5% (mean = 78.7 ± 7.3%). The wind speed was very low for the whole period (mean = 2.4 ± 0.9 m/s; data from Meteo France). These data were similar to those usually observed at the beginning of spring in Besançon.

**Sampling for testate amoebae.** For each site, two individual moss cushions were collected on hard substrate and in non-trampled places (walls, large rocks, roofs). The same bryophyte species, *Tortula ruralis*, a cosmopolitan and common bryophyte growing on rocks, walls, and calcareous-rich substrates (Jahns 1996), was sampled in all sites. The top part of the mosses (living, green) was separated from the lower part (brown, dead) in the laboratory. Only the top part was used for testate amoebae analyses. For each sample we measured 20 shoots to determine the average thickness of the green part. For each sample, approximately 0.3 g (fresh weight) of the living part was inserted in a glass vial with 7 ml of a 4% formaldehyde solution.

**Testate amoebae extraction and analyses.** To extract testate amoebae, the moss samples were shaken with a vortex mixer, filtered through a 40 µm mesh, and washed with deionised water. The fraction remaining on the filter contained no testate amoebae. Testate amoebae larger than 40 µm were recovered in the filtrate. This suggests that the tests of species such as *Arcella catinus*-type were flexible enough to be forced through the filter by the water pressure, and/or that the filter itself was flexible. The fraction remaining on the filter was dried at 80°C during 48h and weighed. The filtrate containing the testate amoebae was placed in a plankton-settling chamber and left to sediment for 24h. The slides were then analysed at a magnification of 200× and 400× with an inverted microscope following Uthermöhls method (Uthermöhls 1958). The whole slide was analysed for testate

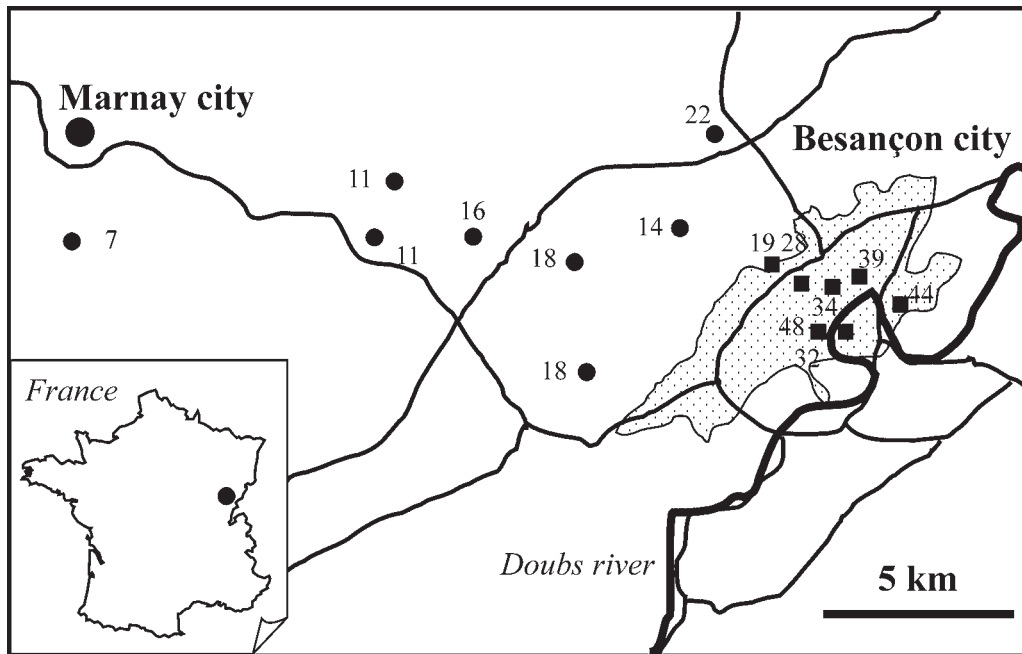
amoebae. The total number of tests counted varied between 23 and 2757 individuals. Living and dead (empty shells) individuals were counted separately.

**NO<sub>2</sub> sampling and measurements.** Passive samplers (Palmes *et al.* 1976) are calibrated tubes, 7 cm long, with an inside diameter of 1 cm, in which gases move only by molecular diffusion (Gradko International, Winchester, Great Britain). A triethanolamine solution, which was deposited on the grid at one end of the tube, fixed the NO<sub>2</sub>. The other end of the tube remained open for diffusion of gases. At 21.1°C and at a pressure of 1 atmosphere, the diffusion coefficient for NO<sub>2</sub> is 0.154 cm<sup>2</sup>/s, which means that the collection rate for our passive sampler could be calculated at 72 cm<sup>3</sup>/h. Mean hourly concentration of NO<sub>2</sub> (in µg/m<sup>3</sup>, hereafter [NO<sub>2</sub>]) in the air sample was calculated on the basis of the amount of pollutant collected, exposure time, and gas collection rate in the tube. Absorbed NO<sub>2</sub> was measured by spectrophotometry using a variant of the Griess-Saltzman method (Atkins *et al.* 1986). NO<sub>2</sub> concentrations were expressed in µg/m<sup>3</sup>, (1 ppb = 1.91 µg/m<sup>3</sup> at 21.1°C). In an earlier study, the passive samples were validated on chemiluminescence analysers (Bernard *et al.* 1997). Each passive sampler tube was exposed and allowed an integration of the NO<sub>2</sub> pollution level over a period of 14 days (the recommended exposure time for the model we used was of less than 20 days). For each sampling location, two tubes were placed on April 20<sup>th</sup> 2001 and removed 14 days later. Passive samplers were set up vertically on freestanding poles at 2 m from the ground and at over 2 m from vertical surfaces. The samples were fixed on wooden blocks, which kept them at a distance of 80 mm from the sides of the support, thus allowing air to circulate freely around them.

## RESULTS

**Atmospheric NO<sub>2</sub> concentrations and thickness of the green part of the mosses.** Atmospheric NO<sub>2</sub> concentrations levels varied between 7 µg/m<sup>3</sup> (Marnay, site most distant from the city) and 48 µg/m<sup>3</sup> (Besançon, city centre) (mean: 24 ± 12.5 µg/m<sup>3</sup>) (Fig. 1). The [NO<sub>2</sub>] increased from the west to the east, which corresponds to the gradient of increasing urbanization. However, a clear distinction also appears between the sites located within the city zone, with values of 30 and above (mean: 34.8 ± 9.5 µg/m<sup>3</sup>), and the periphery zone, with values mostly between 10 and 20 (mean: 14.6 ± 4.7 µg/m<sup>3</sup>). The NO<sub>2</sub> concentrations were significantly different between these two zones (Mann-Whitney-test, p = 0.012). By contrast, the length of the green part of the mosses did not differ between the two zones (Mann-Whitney-test, p = 0.631).

**Testate amoebae density.** The total density of testate amoebae tests (living + dead) varied between 990 and 26225 ind/g dry weight of moss in the samples (mean: 6767 ± 6279 ind/g). The density of living amoebae was correlated with that of empty tests (0.001 < p < 0.043 depending on the species). Furthermore, the proportion of tests containing a living cell



**Fig. 1.** Location of the study site and average atmospheric NO<sub>2</sub> concentrations [µg/m<sup>3</sup>] measured at the sampling locations (squares: city; circles: periphery). Stippled area: Besançon agglomeration. Continuous lines: principal roads.

**Table 1.** Comparison of environmental and testate amoebae variables in the periphery (8 samples) and city (7 samples) zones.

	Periphery			City limits			P-value #
	n	Mean	SE	n	Mean	SE	
NO <sub>2</sub> concentration [µg/m <sup>3</sup> ]	8	14.6	1.7	7	34.8	3.6	<b>0.001</b>
Thickness of the living part of mosses [cm]	8	0.6	0.1	7	0.6	0.0	0.631
Total testate amoeba density [ind/g]	8	7218	2115	7	6252	2462	0.487
Testate amoeba species richness	8	6.0	0.53	7	4.71	0.48	<b>0.016</b>
<i>Paraquadrula irregularis</i>	7	176	78	0	0	0	<b>0.017</b>
<i>Nebela tinctoria</i> -type	2	3	2	3	121	87	0.270
<i>Euglypha ciliata</i>	8	4260	1447	7	3147	1145	0.487
<i>Diffugia pristis</i> -type	8	255	65	5	132	80	0.487
<i>Assulina muscorum</i>	8	1447	790	6	2527	2361	0.563
<i>Arcella catinus</i> -type	8	327	239	7	183	52	0.728
<i>Trinema complanatum</i>	7	749	468	5	137	51	0.907

# Mann Whitney-test between the values for the two zones.

(between 13.2 and 64.2% of the total) was similar in the two studied zones (respectively,  $40.3 \pm 11.0\%$  and  $38.8 \pm 13.7\%$  in zones 1 and 2) and was not significantly different between the two zones. Therefore we used the

total of dead and living amoebae in all further analyses. The total density of testate amoebae was neither significantly correlated with [NO<sub>2</sub>], nor with the length of the green part of the mosses, and was only slightly, but not

significantly, lower in the city than in the surrounding areas (Table 1).

**Testate amoebae species richness and diversity.** A total of nine testate amoebae species were identified in the 15 samples. However, two taxa (*Centropyxis minuta*-type and *Corythion dubium*-type) each occurred in only sample with one and four empty tests. Thus, we decided to remove these two taxa from the data set for further analyses. The species richness of individual samples varied between four and six (mean:  $5.4 \pm 0.8$ ) and was significantly lower in the city ( $4.7 \pm 0.48$ ) than in the surrounding areas ( $6.0 \pm 0.53$ ; Mann-Whitney test,  $p = 0.016$ ; Table 1). Furthermore, the species richness was significantly correlated with the  $\text{NO}_2$  concentration ( $n = 15$ ,  $r = -0.651$ ,  $p = 0.007$ ).

## DISCUSSION

The  $\text{NO}_2$  concentrations values measured are similar to those that are regularly published by the service of air pollution monitoring (ASQAB 2001) and are well under the official EC norm (Mean emission limit for 1 h:  $200 \mu\text{g}/\text{m}^3$ ). This indicates an overall good quality of air in the region, at least for  $\text{NO}_2$  pollution. Furthermore, this shows that the gradient we have chosen to study is not an extreme case.

The diversity of testate amoebae found in our samples is lower than usually found in mosses. However, our sampling protocol excluded the lower part of the mosses, richer in humus and where conditions are likely more favourable to a higher diversity of testate amoebae and other microorganisms. In the deeper parts of the moss cushion, other environmental factors than atmospheric pollution would be likely to influence the structure of communities and the response of testate amoebae to atmospheric pollutants would most likely be less clear.

In accordance with our hypothesis, the testate amoebae species richness was significantly higher in the peripheral zone where  $\text{NO}_2$  concentrations were lower. Furthermore, a significant relationship was found between the testate amoebae data and  $[\text{NO}_2]$ .

One species, *Paraquadrula irregularis*, appeared to be sensible to the atmospheric pollution characterized by  $\text{NO}_2$  in the city centre. This species, previously reported in moss ecosystems (Cash and Hopkinson, in Decloître 1961), has an endogenic calcareous test (Bonnet 1989). The sensitivity of this species might reflect an indirect impact of pollution through an acidification effect causing a leaching of  $\text{Ca}^{2+}$  through a cation ex-

change mechanism ( $\text{H}^+$  replacing  $\text{Ca}^{2+}$ ) in the mosses. If true, this would mean that *P. irregularis* is rare or totally absent from the city centre of Besançon.

Contrary to our expectations, the density of testate amoebae does not appear to be a good indicator of  $\text{NO}_2$  pollution within the range of concentrations covered by this study. The variability of density may be due in great part to other variables, such as the physical characteristics of the sampling location. Indeed the growth of mosses depends on the micro-climatic conditions and the production of amoebae is higher under high humidity conditions (Van Kerckvoorde *et al.* 2000). Our results agree with those of Balik (1991) regarding the relationship between atmospheric pollution and species richness, but not the density.

Despite the well-established relationship between testate amoebae and micro-environmental conditions, no significant correlation emerged between the thickness of the moss cushion and the testate amoebae data. This variable was chosen because it was likely to be correlated to variables related to the structure of their habitat such as the range of moisture, water holding capacity, or temperature fluctuations to which testate amoebae are likely to be sensitive (Lousier 1974a, b; Charman and Warner 1992; Tolonen *et al.* 1994). The absence of such a relationship in our data set does not necessarily contradict these findings but rather suggests that the range of variability of moss cushion thickness (and of other variables related to this variable) present in our samples was not enough to influence significantly the testate amoebae (Mitchell *et al.* 2000a, b).

Our preliminary results suggest testate amoebae have a potential as biomonitors of atmospheric pollution. These results are especially interesting because the pollution level recorded was very low. This tool could prove very useful in two cases: (1) where the temporal resolution of measurements is not a high priority and a more integrative measurement is preferable, (2) where financial resources are not available to undertake continuous direct measurements of air pollution. This second case is certainly common in developing countries, where urban pollution problems are often the most acute.

Further work is needed to assess the full value of testate amoebae as bioindicators of atmospheric pollution before they can be used as a monitoring tool. The correlation between testate amoebae diversity and community structure and  $[\text{NO}_2]$  does not allow us yet to establish a causal relationship. It is possible that some other variables, such as another atmospheric pollutant, or other environmental variables, were responsible for the

observed differences. Future work should focus on (1) a calibration of the response of testate amoebae along a broader pollution gradient, and under controlled conditions, 2) the effects of other pollutants, such as ozone or heavy metals, and interactions among pollutants and (3) the effects of the urban microclimate on mosses and testate amoebae.

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