Forensic Science International 236 (2014) 90–98

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Can soil testate amoebae be used for estimating the time since death? A field experiment in a deciduous forest

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ARTICLE INFO

Article history:
Received 22 August 2013
Received in revised form 29 November 2013
Accepted 20 December 2013
Available online 6 January 2014

Keywords:
Forensic indicators
Soil protozoa
Testate amoebae
Community ecology
Cadaver
Post-mortem interval

ABSTRACT

Estimation of the post-mortem interval (PMI, the time interval between death and recovery of a body) can be crucial in solving criminal cases. Today minimum PMI calculations rely mainly on medical and entomological evidence. However, beyond 4–6 weeks even entomological methods become less accurate. Thus additional tools are needed. Cadaveric fluids released by decomposing cadavers modify the soil environment and thus impact soil organisms, which may thus be used to estimate the PMI. Although the response of bacteria or fungi to the presence of a corpse has been studied, to the best of our knowledge nothing is known about other soil organisms. Testate amoebae, a group of shelled protozoa, are sensitive bioindicators of soil physico-chemical and micro-climatic conditions and are therefore good potential PMI indicators. We investigated the response of testate amoebae to three decomposing pig cadavers, and compared the pattern to two controls each, bare soils and fake cadavers, in a beach-oak forest near Neuchâtel, Switzerland. Forest litter samples collected in the three treatments over 10 months were analysed by microscopy. The pig treatment significantly impacted the testate amoeba community: after 22 and 33 days no living amoeba remained underneath the pig cadavers. Communities subsequently recovered but 10 months after the beginning of the experiment recovery was not complete. The fake cadavers also influenced the testate amoeba communities by altering the soil microclimate during a dry hot period, but less than the cadavers. These results confirm the sensitivity of soil testate amoebae to micro-climatic conditions and show that they respond fast to the presence of cadavers – and that this effect although decreasing over time lasts for months, possibly several years. This study therefore confirms that soil protozoa could potentially be useful as forensic indicators, especially in cases with a longer PMI.

1. Introduction

The estimation of time since death (or post-mortem interval – PMI) is one of the most important tasks whenever events and circumstances of a death need to be reconstructed for legal investigations. Here, forensic medicine reaches its limits already after 24–48 h post-mortem [1,2]. Additional methods are therefore needed beyond this time for PMI estimation. Forensic entomology is the method of choice in cases where insects had access to the body, and is most useful in the first weeks after insect colonisation [2]. Beyond 4–6 weeks entomological PMI estimates become less reliable. New forensic tools, complementary to existing ones especially with respect to a longer PMI are therefore necessary.

In the last decades forensic researchers have started to study changes in the soil beneath a decomposing cadaver. So far, soil investigations in crime scenes have focused mainly on locating burial sites [3,4] and on the identification of soil samples recovered from suspects' footwear, clothes, vehicles or weapons [5–10]. However, a decomposing cadaver strongly modifies the soil environment and as a result affects the soil organisms [11,12]. Soil abiotic or biotic characteristic are therefore potentially useful sources of information for the presence of cadavers and PMI estimates. Our focus here is on a common group of soil protozoa, the testate amoebae.

Testate amoebae, also known as shelled amoebae, or testaceans, are a polyphyletic group of shelled unicellular protists which are found in various habitats, such as mosses, soils, peatlands, lakes, rivers and even estuarine environments all around the world. They are subdivided into three main phylogenetic groups according to their feet-like extensions (pseudopodia) and shell characteristics: (1) Arcellinida, with lobose (finger-shaped) pseudopodia, the most

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http://dx.doi.org/10.1016/j.forsciint.2013.12.030

diverse group, comprising three quarters of all known species, (2) Euglyphida, with filose (thin and filament-like) pseudopodia [13–15], and (3) Amphitremida, with anastomosing pseudopodia and symmetrical shells with two apertures [16]. Of the ca. 2000 described taxa [13,14,17], about 300 species have been found in soils [18].

Soil testate amoebae can be very abundant reaching 10^6–10^8 individuals per m^2 and 10^2–10^5 individuals per gram dry mass in soils and leaf litter [18]. They reproduce relatively slowly, by microbial standards, with generation time of a few days to over a week [19,20]. Their shell can persist after the death of the organism for months to millennia (e.g. in peat or sediments), making long-term studies possible [21,22]. They are able to encyst (form a cyst) under unfavourable conditions and excyst when conditions improve, a behaviour that can indicate changes in the environmental conditions [23,24].

Testate amoebae are used as indicators in a variety of research fields (ecology, paleoecology, limnology, paleolimnology, paleoclimatology, peatland regeneration, soil and air pollution monitoring and ecotoxicology) because they respond to biotic and abiotic factors by abundance, community composition or even shell morphology [25,26]. Despite the strong potential of testate amoebae and other soil protozoa as bioindicators in agro-ecosystem and natural ecosystems (e.g. for soil nutrient content, moisture, pH, various types of pollution, agricultural practices) [18,27], forensic application of testate amoeba analysis is currently limited to correlative approaches where the soil from the crime scene is compared with soil attached to shoes from suspects [5,9]. Soil organisms, including testate amoebae, respond to spatial gradients [28] and temporal changes [29] in micro-environmental conditions and can be expected to respond also to the presence of a cadaver. As their generation times are relatively short – typically a few days in good conditions – compared to the insects that are commonly used as indicators, the communities can be expected to recover once the cadavers have decomposed completely, albeit with possible longer term effects related to long-lasting changes in soil chemistry. From these responses, a forensic PMI tool could be developed. However, in order to develop such a tool, experimental work is required. We therefore conducted a field experiment aiming at assessing the spatial and temporal variation of soil testate amoeba assemblages in response to the presence and decomposition of cadavers in a beech and oak forest. We hypothesised that their density, diversity and community structure would (1) be strongly affected (i.e. they would die and/or encyst) by the effect of decomposing pig cadavers during the active phase of decay and (2) would subsequently slowly recover over time.

2. Materials and methods

2.1. Field experiment

The study area was located in a mixed beech and oak forest near the city of Neuchâtel, Switzerland (47°00′11.90–12.26″ N/ 6°56′6.45–8.05″ E, elevation 478 m). Three ca. 25 m^2 sampling sites were selected within a ca. 80 m × 80 m area fenced to keep out deer and allow the regeneration of oak trees. Inter-site distance ranged from 15 to 33 m. Within each site three 90 cm × 100 cm surfaces were selected for (1) a control, (2) a fake cadaver (plastic bags filled with soil and covered with a cotton cloth) to investigate microclimatic effects without cadaveric fluids and (3) a pig cadaver, to investigate combined effect of cadaveric fluids and microclimate. The amount of soil used to fill the fake cadavers corresponded to the initial weight of cadavers at the onset of the experiment and soil was gradually removed at each sampling day so as to approximately match the declining cadaver weight over the course of decomposition. This set-up allowed us to separate the influences of the decomposing cadaver from the normal seasonal change in testate amoeba communities (control plots) and from the microclimatic effects + seasonal changes (fake cadavers). Within each site, the three plots were at least four metres distant from each other.

Three pigs (*Sus scrofa* Linnaeus) all females and 20 kg (±1 kg) were killed with captive bolt stunning and were immediately delivered post-mortem to the study site. To enable sampling underneath the decomposing cadavers and to prevent the disturbance by large scavenger vertebrates, each pig cadaver was kept in a cage 90 cm × 100 cm × 50 cm built of a compost frame and closed at both ends with strong wire mesh. The position of the cages was marked with a stick at each corner to ensure that the cages are always placed in the same spot. The cages were lifted and placed nearby during sampling and placed back in the exact same place afterwards.

On each sampling day from August 2009 until June 2010 soil litter was sampled (down to the litter-mineral soil contact) from the surface area of the control plot, underneath the fake pig and from the contact area underneath the pig cadaver impacted by cadaveric fluids. The samples were taken in at least five random points within each sampling area at each sampling time in order to obtain representative samples from each plot at each time. The first sampling took place at the start of the experiment (day 0, 05.08.2009) just before the dead and fake pigs were put in place. Then samples were taken at defined intervals 8, 15, 22, 33, 64, 132 and 309 days after day 0. Soil temperature was recorded every hour between August 2009 and June 2010 using thermologgers (HOBO Pendant® temp/Alarm 64K UA-001-64), one per treatment and replicate. The data loggers were placed at the interface between the litter and the mineral soil. Precipitation data were obtained from the local meteo agency (www.meteosuisse.ch).

2.2. Laboratory analyses

To extract testate amoebae each sample was cut with scissors, mixed, and 5 g was put into a plastic flask and deionised water was added. The flask was closed and shaken manually for approximately 3 min. The water was then sieved through a 160-μm mesh size in order to remove coarse particles. The filtrate was then sieved again through a 10-μm mesh to remove clay and fine silt particles. The 10–160 μm fraction was collected and centrifuged at 2500 rpm for 10 min and the supernatant discarded. Rose Bengale (50 μl; C.I. #45440, BBLTM, U.S.A.) was added to differentiate living from dead cells (i.e. empty shells) [30] by staining. The samples were left at room temperature for 30 min to colour the living testate amoeba cells. Water was then added up to 50 ml to remove excess Rose Bengale and the sample was centrifuged again. The supernatant was discarded leaving approximately 5 ml in the tube. For fixation 1.5 ml glutaraldehyde was added (2.5% final concentration). A *Lycopodium* spore tablet (batch no. 938934, 53394 spores ±953 per tablet, Department of Quaternary Geology, Lund, Sweden) was then added to allow the calculation of test concentration [31]. The tube with the spore tablet was homogenised for 1 min with a vortex and stored overnight to allow the tablet to completely dissolve. Slides were prepared by mixing two drops of the preparation with one drop of glycerol.

Testate amoebae were identified to morpho-species and counted using a light microscope at 400× magnification. Living, encysted, and dead individuals were tallied separately. When staining with Rose Bengale the living and encysted testate amoebae are coloured red and can be targeted. Dead testate amoebae (empty shells) are not coloured and therefore can easily be separated from living and encysted ones. A count of 150 testate amoebae was aimed for (total of living, encysted and dead), which is the number of individuals that most studies use although counts

of 100 or even 50 were shown to also be informative [32]. The density of testate amoebae in the sample was calculated by multiplying the total number of testate amoebae by 53394 (i.e. average number of spores per tablet) divided by the number of spores counted.

2.3. Numerical analyses

The temporal changes in soil testate amoeba assemblages during decomposition were examined at two levels.

First we used simple indices describing key aspects of biodiversity: Species richness (N0) and Simpson diversity (N2) as defined by Hill [33]. High N0 indicates a large number of species and high N2 a large number of species evenly distributed. Strong relations between N0 and N2 and environmental conditions were demonstrated at various spatial scales [34] and in a broad range of ecosystems such as tropical forest [35], marine ecosystems [36] and flood or fire prone ecosystems [28,37,38]. N0 and N2 were calculated for each treatments and time step. The differences among and within treatments in N0, N2, testate amoeba density and count data were analysed using Tukey's Honest Significant Difference method (TukeyHSD) and p corrected for multiple comparisons.

Second, we investigated the changes in the species composition of testate amoeba communities using Principal Response Curves (PRC) [39]. PRC are a variant of the broadly used redundancy analysis [40] that was specifically designed for the analysis of multivariate responses in repeated observation design as is the case in the present study. PRC were used to assess the effect of the pig and control treatments as compared to the fake pig treatment. Doing so allowed discriminating between effects of covering the soil surface (fake pig versus control treatments) from the effect of the decomposition process (fake pig versus pig treatments).

Finally, the changes in climatic conditions were assessed using both the temperature [°C] of the soils in control plots (thermologgers) and that measured 5 cm above ground from Neuchâtel meteo station (www.meteosuisse.ch) and precipitation [mm h^{-1}] to further discriminate the effect of the decomposition process from that of climatic trends that may influence all treatments. In order to smooth the climatic trends moving averages were computed on 7 days and hourly measurements for temperature and precipitation respectively. All statistical analyses were conducted using the R software for statistical computing [41] and the "vegan" packages [42].

3. Results

3.1. Temperature and precipitation

After a warm period in August with a mean temperature of 19.6 ± 3.8 °C (day 0 = start of the experiment: 21.6 ± 2.6 °C; day 8: 19.1 ± 2.9 °C; day 15: 22.6 ± 3.9 °C) mean temperature continually decreased to 15.4 ± 3.9 °C on day 33 and 15.5 ± 0.9 °C on day 64 until December (day 132: 1.4 ± 0.3 °C). It then increased again and reached 18.9 ± 3.3 °C on day 309 (Fig. 1). After a short period of rain in July 2009, a fairly dry season, mean precipitation in August 0.06 mm h^{-1} (±0.44), with only short rain intervals followed up to November 2009 (day 64). Precipitation increased in November and December. The last sampling day (309) took place in June 2010 after a rainy period in May followed by less rain in June (Fig. 1).

3.2. Testate amoeba taxa and density

A total of 23 testate amoeba taxa were identified, 12 Arcellinida (Amoebozoa) and 11 Euglyphida (Rhizaria) (Table 1). The three most abundant taxa *Centropyxis aerophila*, *Arcella arenaria* and *Euglypha rotunda* together accounted for 53.4% of the community on average (Table 1 and Fig. 2).

Testate amoeba density (alive, dead and encysted) averaged 3623 individuals per gram litter over all samples. Living, encysted and dead amoebae accounted respectively for 32.2%, 18.0% and 49.8% in the control, fake pig and pig cadaver samples. Density changed over time and among the three treatments (Fig. 3). Compared to the beginning of the experiment (day 0 and day 8) density decreased significantly from day 8 onwards up to day 22 in the pig treatment (TukeyHSD, $p < 0.01$), whereas no significant difference was observed in the fake and the control treatment. In the pig samples the average density of living testate amoebae decreased to 21 ind. g^{-1} at day 15 and to 0 ind. g^{-1} at days 22 and 33. Recovery was first observed in October, two months after the beginning of the experiment (i.e. between days 33 and 64, Fig. 3), but density was still significantly lower than at the start (day 0) ($p = 0.02$). By contrast, the lowest densities of living testate amoebae recorded on day 64 in the control (207 ind. g^{-1}) and on day 64 in the fake pig plots (766 ind. g^{-1}) were not significantly different from day 0. Nine months later, in June 2010 (day 309) the density of living testate amoebae had increased again in the control (1725 ind. g^{-1}) and reached its highest value in the fake (8674 ind. g^{-1}) (not significant). By contrast the density of living testate amoebae in the pig treatment was unsignificantly lower on day 309 (64 ind. g^{-1}) than on both day 64 (126 ind. $^{-1}$) and day 132 (319 ind. g^{-1}) (Fig. 3).

Assuming that encysted testate amoebae may excyst when conditions improve we also show the results of the living + encysted vs. dead, hereafter L/D ratio (Fig. 3). The L/D ratio was >1 in the control in the first two weeks (3 sampling days) as well as in September and <1 at the end of August, in October and most clearly in the winter. In the fake pig samples the pattern at the beginning of the experiment was similar but not identical to the control. By contrast, in the pig samples the L/D ratio was only >1 at the beginning of the experiment (day 0) and <1 thereafter and until the end of the experiment.

3.3. Species richness and species diversity

Testate amoeba species richness varied in all treatments (Fig. 4). In the control (Fig. 4) species richness was only significantly lower between day 8 and day 64 (TukeyHSD; $p = 0.03$), and then increased again at days 132 and 309 but without reaching the value of day 0. In the fake treatment (Fig. 4) species richness was only significantly lower between day 15 and day 132 ($p = 0.04$) – increased until day 15, after that declined until day 132 and increased again on day 309. In the pig treatment (Fig. 4) species richness declined significantly between day 0 to day 15 ($p = 0.005$), reached the lowest point on days 22 and 33. It then increased at day 64 and until day 132 and declined again at day 309 (all not significant), but remained lower than on day 0.

Simpson diversity (N2) tended to decline in all three treatments over the course of the experiment (Fig. 4). This was not significant in the control samples. In the fake samples the diversity on day 132 and 309 was significantly lower than on day 8, 15 and 22 (Fig. 4). In the pig treatment diversity decreased rapidly being significantly different on day 15, 22 and 33 compared to the beginning of the experiment (day 0) (Fig. 4). Testate amoeba communities started to recover from the cadaver-induced disturbance on day 64 (i.e. diversity increased again), but never reached values comparable to day 0 (Fig. 4).

3.4. Species response to treatments

The principal response curve analysis (PRC) shows how the testate amoeba communities in the control and the pig treatment

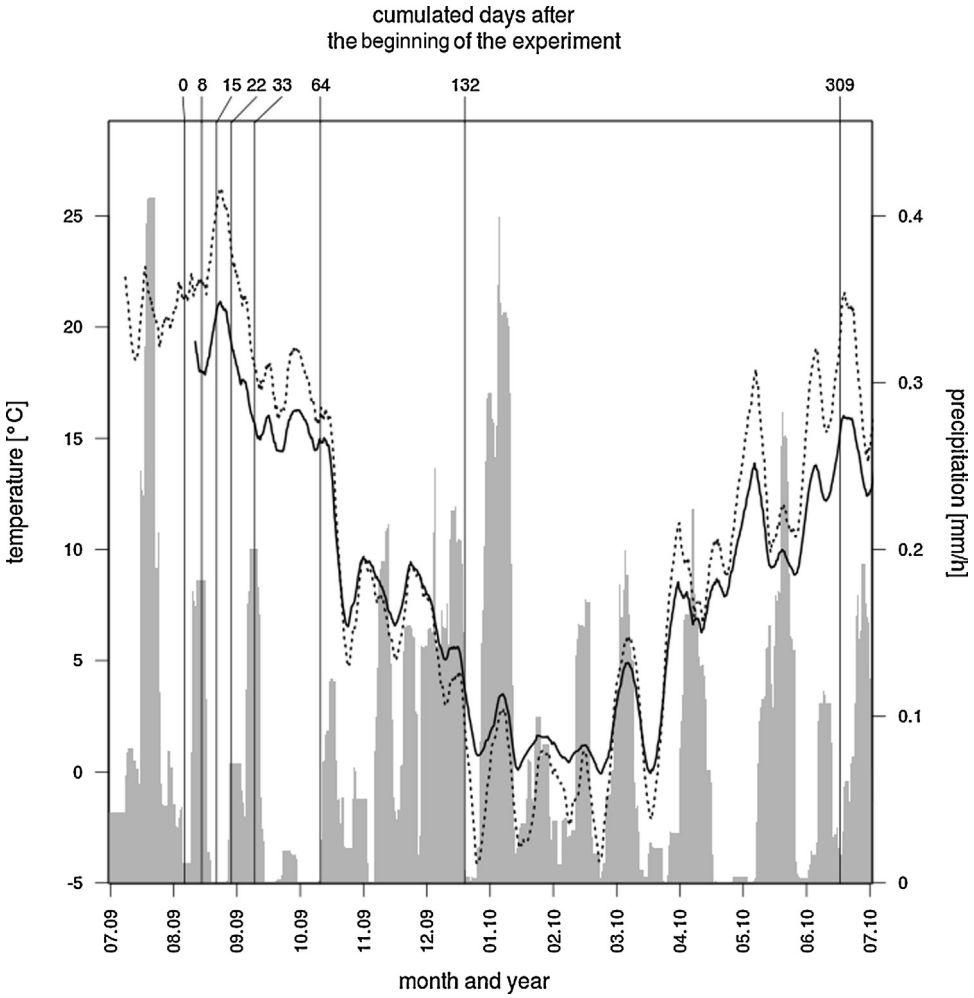

Fig. 1. Climatic data measured in Neuchâtel and at the experimental site from August 2009 until June 2010. Temperature data (left axis) are 7 days running averages of air and soil temperature; dotted lines: Neuchâtel meteorological station; plain lines: data from temperature loggers placed in the litter/soil interface in the experimental plots. Grey field: precipitation corresponds to 7 days running average of hourly precipitation (right axis). Vertical lines indicate time points for sampling 0, 8, 15, 22, 33, 64, 132 and 309 days after the beginning of the experiment.

varied over time in comparison to the fake pig, used here as a reference (Fig. 5). The difference between control and fake pig was maximal at day 64, which corresponded to the end of a warm and dry period (Fig. 1).

This difference was relatively low otherwise. The effect of the pig treatment increased up to day 33 and then declined, almost reaching the level of the control on day 64. The difference of both pig and control treatments and fake pig then declined at day 132. The pig effect increased again at the last sampling date (day 309) (Fig. 5).

4. Discussion

4.1. Testate amoeba community composition, species richness and density

The testate amoeba species richness and community composition we found in the litter samples agrees with previous studies in comparable habitats [15]. The dominant morpho-taxon in our study, *Centropyxis aerophila*, is also one of the most abundant and frequent testate amoeba taxon worldwide [43]. *Arcella arenaria* and *Euglypha rotunda*, the next most dominant morpho-taxa are also very frequent globally. However it should be noted that these morphological species certainly hide numerous cryptic or pseudo-cryptic species that may have more restricted distribution or ecological preferences [44,45].

The density of testate amoebae was relatively low at the beginning of the experiment (10^3–10^4 ind. g^{-1}), but within the range usually reported in soils [19]. This relatively low density is probably due to the fairly dry conditions in the period preceding the experiment (Fig. 1). Indeed testate amoeba density and community structure were previously shown to respond to soil moisture fluctuations [25,29,46–48].

4.2. Effect of decomposing pigs on soil testate amoebae

This is the first study assessing the effect of cadavers on soil testate amoebae. As predicted, testate amoebae responded very clearly to the presence of decomposing cadavers in density, species richness, diversity and community structure. The strongest response was observed 22 days post mortem (day 22), by which time no living or encysted testate amoeba was present under the pig.

The chemical composition of a domestic pig aged two months is ca. 80% water, 26 g kg^{-1} nitrogen, 6.5 g kg^{-1} phosphorus, 2.9 g kg^{-1} potassium, 10 g kg^{-1} calcium and 0.4 g kg^{-1} magnesium and a C/N ratio of 7.7 [49]. As cadaveric fluids add ammonium, calcium, chloride, magnesium, nitrogen, potassium, sodium, sulphate and volatile fatty acids to the underlying ground [50],

Table 1
Relative abundance (% total tests) and average species density (number of alive, dead and encysted individuals per gram litter) of testate amoebae in all 72 samples in the 3 treatments (control, fake pig and pig cadaver, 3 replicates, 8 sampling days).

Testate amoeba taxa	Species code	Phylogenetic group	Relative abundance (%)	Average species density (ind. g^{-1})		
				Control	Fake	Pig
Centropyxis aerophila Deflandre 1929	CENTae	Arcellinida	22.5	304.4 ± 49.9	1020.3 ± 501.8	87.1 ± 29.7
Arcella arenaria Greef 1866	ARCar	Arcellinida	16.0	285.3 ± 98.2	186 ± 41.1	77.2 ± 37.8
Euglypha rotunda Wailes 1911	EUGrot	Euglyphida	14.9	84.5 ± 39.1	87 ± 21.8	10.1 ± 5.8
Centropyxis sylvatica Deflandre 1929	CENTsy	Arcellinida	7.0	92 ± 18.7	228.8 ± 62.4	86.4 ± 39.3
Euglypha strigosa Ehrenberg 1872	EUGstri	Euglyphida	6.2	101.8 ± 36.6	107.5 ± 30.3	46.2 ± 24.7
Corythium dubium Taránek 1881	CORdu	Euglyphida	5.4	91.9 ± 23.9	94.9 ± 30.6	27.9 ± 20.9
Assulina muscorum Greef 1866	ASSmu	Euglyphida	4.6	90 ± 33.6	231 ± 130.6	63.6 ± 31.6
Cyclopyxis kahli Deflandre 1929	CYCka	Arcellinida	4.0	51.5 ± 15.6	158.3 ± 103.6	26.1 ± 15
Euglypha compressa Carter 1864	EUGcom	Euglyphida	3.4	54.9 ± 20.1	55 ± 21.2	32.4 ± 15
Trinema enchelys Ehrenberg 1838	TRIenc	Euglyphida	2.9	34.5 ± 12.4	43.9 ± 14	16.2 ± 7.8
Euglypha ciliata Ehrenberg 1848	EUGcil	Euglyphida	2.0	51.3 ± 27	62.8 ± 27.7	44.5 ± 27
Trinema penardi Thomas and Chardez 1958	TRIpen	Euglyphida	1.9	21 ± 12.3	21.3 ± 11	12.3 ± 9.8
Trinema lineare Penard 1890	TRIlin	Euglyphida	1.6	0 ± 0	4.5 ± 4.5	7.1 ± 5.6
Padaungiella lageniformis Penard 1890	PADlag	Arcellinida	1.2	0 ± 0	0 ± 0	3.8 ± 3.8
Nebela collaris Ehrenberg 1848 sensu Kosakyan et al., 2013	NEBcol	Arcellinida	1.2	2.9 ± 2.9	0 ± 0	1.2 ± 1.2
Centropyxis elongata Penard 1890	CENTel	Arcellinida	1.2	11.5 ± 7.9	27.2 ± 14.2	0 ± 0
Euglypha cristata Leidy 1879	EUGcris	Euglyphida	1.0	9.9 ± 7.8	11.6 ± 8	0 ± 0
Trinema complanatum Penard 1890	TRIcom	Euglyphida	0.8	4.1 ± 4.1	6.1 ± 4.5	4.1 ± 4.1
Porosia bigibbosa Penard 1890	PORbig	Arcellinida	0.7	0 ± 0	4.5 ± 4.5	1.7 ± 1.7
Nebela tincta Deflandre 1936	NEBtin	Arcellinida	0.6	12 ± 12	1.3 ± 1.3	0 ± 0
Difflugia lucida Penard 1890	DIFFlu	Arcellinida	0.6	3.7 ± 3.7	6.8 ± 5.1	0 ± 0
Trigonopyxis arcula Leidy 1879	TRIGarc	Arcellinida	0.2	0 ± 0	0 ± 0	0 ± 0
Plagiopyxis declivis Bonnet et Thomas 1955	PLAdec	Arcellinida	0.1	7.5 ± 5.3	4.6 ± 4.6	4.6 ± 4.6

the cadaveric fluids strongly modify the soil environment. Our results show that testate amoebae clearly do not tolerate these changes.

Although we cannot compare our results with any other forensic study, several experiments have assessed the effects of soil chemistry on testate amoebae in *Sphagnum* and other mosses. Deposition of sulphate [21], nitrogen [51], nitrogen and phosphorus [52], PKCa and NPKCa [53], lead [54], and exposure to urban pollution [55] significantly reduced the testate amoeba density (and, where reported, also species richness). Sulphate deposition also affected the abundance of some species, with positive effect recorded for *Hyalosphenia papilio*, *Arcella arenaria* and *Cryptodifflugia oviformis* and negative effects for *Euglypha rotunda* type, *Corythion dubium*, *Trinema complanatum* and *Trinema lineare*. [21]. Nitrogen and phosphorus addition decreased the density of *Assulina muscorum* and *Difflugia oviformis* [52], while nitrogen addition increased the density of *Bullinularia indica* [52,56].

Changes in testate amoeba density in the control samples paralleled the changes in climatic conditions. Indeed, the very low soil moisture content resulting from the warm and dry period in August and September (day 64; Fig. 1) induced the lowest observed density of testate amoebae. By contrast, environmental conditions did not induce a similar decrease underneath the fake pig where it was less strong than in the control, most likely because the bag used to simulate the presence of a fake pig reduced evaporation and thus maintained a somewhat higher soil moisture content, as we indeed noticed during sampling. This hypothesis is further supported by the overall higher average density of testate amoebae in the fake (5388 ind. g^{-1}) as compared to the control treatment (3926 ind. g^{-1}). This interpretation agrees with experimental evidence for increased testate amoeba density in response to water addition in a relatively dry aspen forest [29].

Given these patterns, we compared the community changes under the pig and in the control plots to the fake treatment in the Principal Response Curve analysis. This allowed us to show (1) the

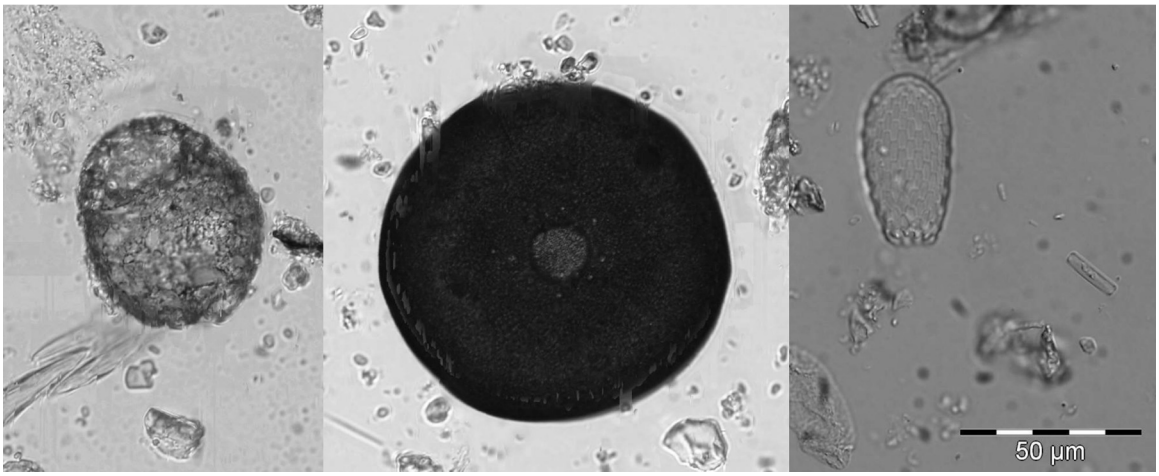

Fig. 2. Illustration of the three most abundant testate amoeba species in this experiment *Centropyxis aerophila* (left) *Arcella arenaria* (middle) and *Euglypha rotunda* (right). Pictures from the Laboratory of Soil Biology, Neuchâtel, Switzerland.

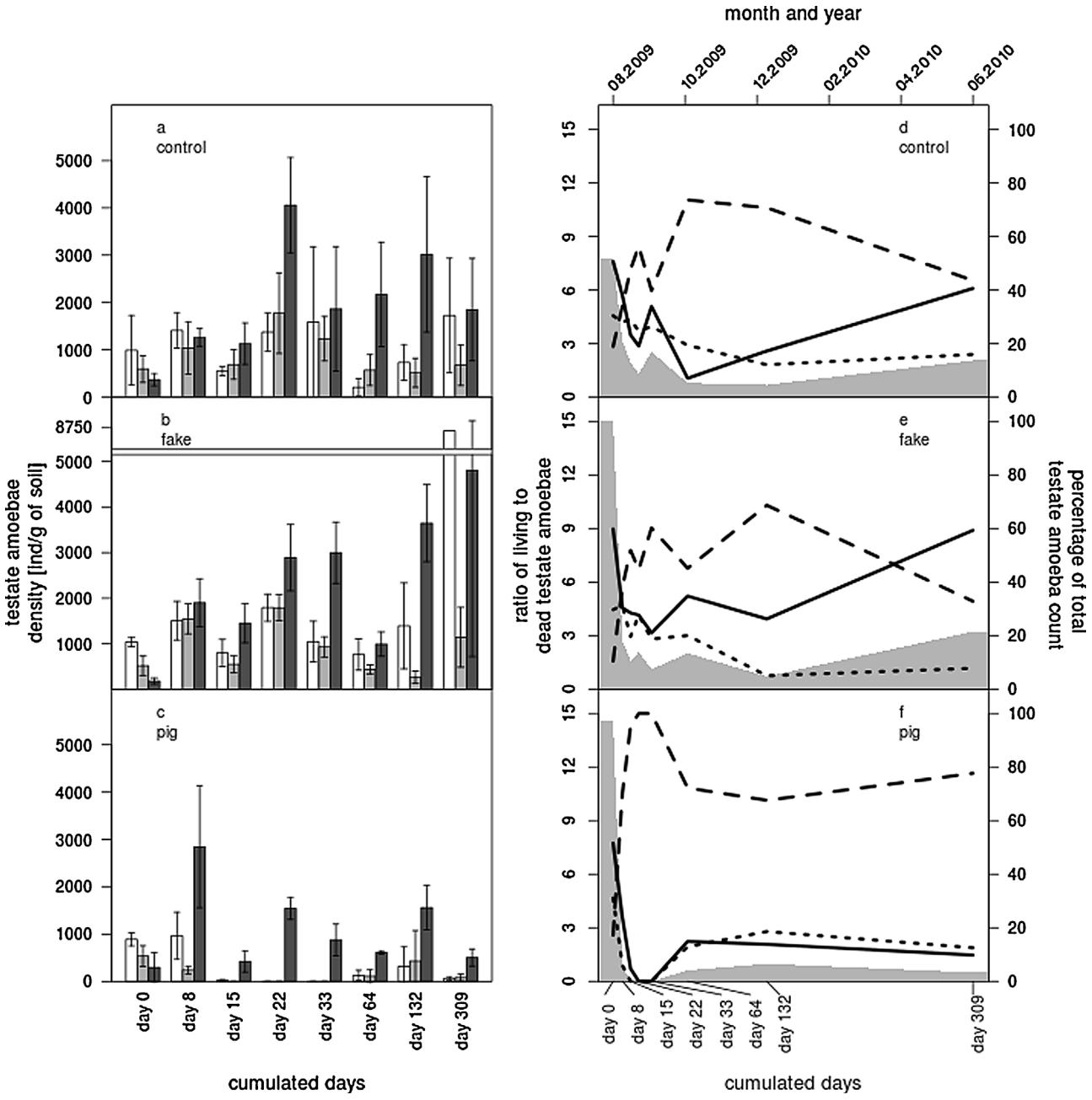

Fig. 3. Patterns of testate amoeba density in response to a pig decomposition experiment in a deciduous forest in Neuchâtel, Switzerland. Density (a–c) (number of individuals per gram litter) and living (active and encysted) to dead ratio (grey area, left scale) and percentage (right scale) (d–f) of living (white bars/plain line), encysted (grey bars/short dashed line) and dead (black bars/dashed line) testate amoebae from control (a and d), fake pig (b and e) and pig cadaver (c and f) treatment.

effect of differences in microclimatic conditions between the fake pig and the control samples, and (2) the effect of the pig but controlling for these microclimatic effects. The results clearly show that microclimatic conditions had an impact on testate amoeba communities. During the course of our experiment climatic conditions were quite contrasted, with extensive dry periods and more predictable seasonal changes in temperature (Fig. 1). The testate amoeba communities clearly responded to these patterns in the control treatment. For example the dry period between days 33 and 64 caused a change in community structure. Nevertheless, the effect of the decomposing cadaver was much stronger than that of climatic conditions (Fig. 5). Although the effect of the pig treatment decreased over time, it remained strong during the whole duration of the experiment and even increased again after 132 days in contrast to the effect of climatic conditions that was almost null from day 132 onward, in line with the removal of soil from the plastic bags used to mimic the cadavers.

Testate amoebae are believed to be transported over long distances mostly passively (e.g. by wind) [57,58], but transport by phoresy (passive transport by animals), although not studied for testate amoebae, is well documented for other protists such as diatoms transported by birds [59]. Over short distances (cm-m), active migration is probably the main colonisation mechanism. Passive migration allows potentially any species to reach a given point but the survival of individual amoebae and the build-up of measureable populations will depend on local conditions. Active dispersal by contrast will only take place if conditions are favourable. We therefore consider that recolonisation of perturbed habitats such as cadaver decomposing sites reflects mainly active dispersal at the scale of a cadaver decomposition site from the surrounding soil.

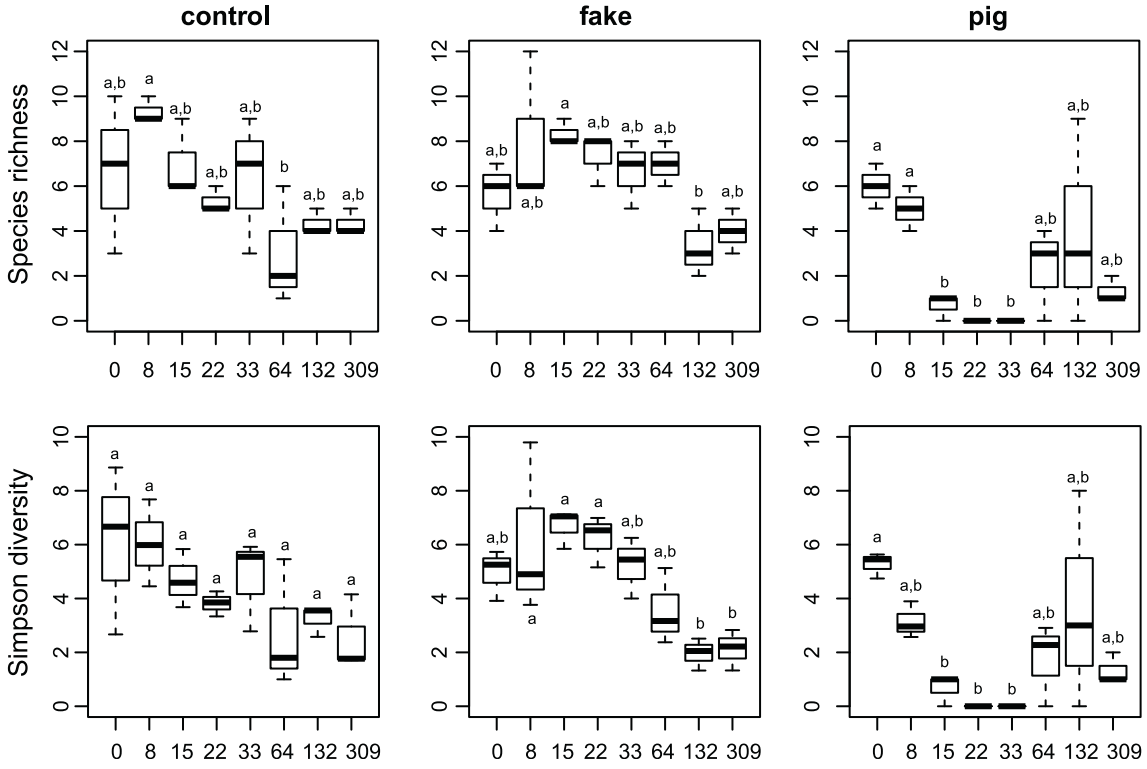

Fig. 4. Temporal patterns of alive testate amoeba species richness (top) and diversity (Simpson's N2 index) (bottom) in the three treatments (control, fake pig, pig cadaver) of a pig decomposition experiment in a deciduous forest in Neuchâtel, Switzerland. Different letters (a and b) indicate significant differences among sampling days.

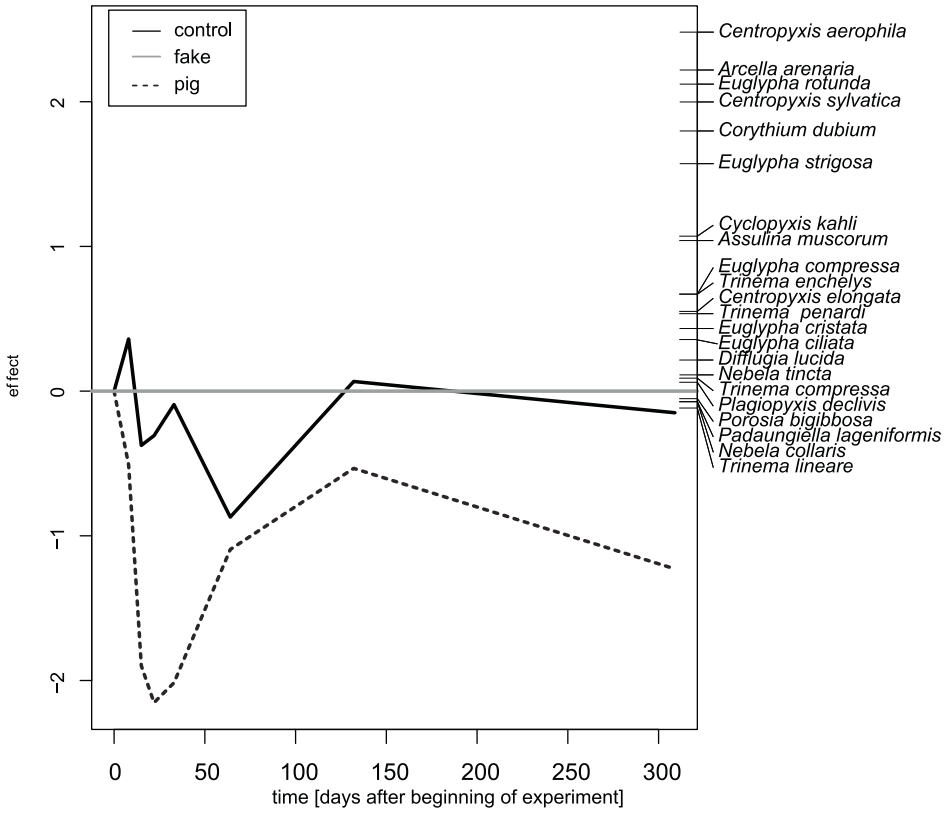

Fig. 5. Principal response curve (PRC) diagram showing the deviations of the pig cadaver treatment (dashed line) and the control (bare soil) treatment (plain line) from the fake pig treatment used as reference (horizontal grey line) over time. The left axis shows the treatment effect (i.e. regression coefficient). Species scores are shown on the right vertical axis. High values indicate that the response of the species is strongly positively correlated to the pattern in the PRC; low values the opposite and values close to zero show that the species response and PRC pattern are un-related.

For forensic purposes it shall be highly interesting to look at the testate amoeba community and at what time the population recovers totally and whether this follows a succession pattern. As nutrient levels can remain high up to several years in soil influenced by cadavers [60–62], the influence of long-gone cadavers on soil communities can also be expected to remain visible for over one year, as indeed suggested by our results. Longer-term experiments are required to study in further detail the re-colonisation patterns (which species re-colonise first, how do these patterns relate to food sources such as bacteria, fungi, other protists, etc.) and results from such studies could potentially lead to developing a tool for extended PMI.

Morphological trait analysis could also potentially be useful to develop such an index. Indeed as testate amoeba taxonomy is currently not satisfactory [45] species traits such as shell morphology and biovolume can be used. Such an approach has only been used once for testate amoebae [28]. In the context of peatland regeneration it has been suggested that larger testate amoebae might be slower to re-colonise secondary habitats owing to (1) lower population densities, and (2) lower probability to travel passively over long distances [63]. We therefore expect that longer-term studies combined with trait analysis of testate amoeba communities will allow to reconstructing the stage of active decay and the post-mortem interval. Rather than identifying all taxa to species level simple morphological trait approaches might prove sufficiently robust and would also allow forensic scientists to become trained for such an analysis. Alternatively or in addition environmental DNA approaches could be developed.

In this study we have shown that testate amoebae were clearly affected by decomposing cadavers in comparison to controls and fake cadavers. Given the strength of the cadaver effect (no living or encysted testate amoebae 22 days post mortem) we expect that the response will be similar in a broad range of conditions (i.e. different forest, litter or soil types). Nevertheless it would be interesting to compare patterns across ecosystems types, under different climates or seasons. Further studies are therefore needed to establish testate amoeba analysis as an approach in forensic science to estimate the post-mortem interval and to establish user-friendly methods.

Acknowledgements

This work was funded by the Swiss National Science Foundation (project no. 31003A_141188 to EM), the University of Neuchâtel, Switzerland and the Stiftung Forensisches Forum Frankfurt/Main, Germany and the Vereinigung von Freunden und Förderern der Goethe-Universität, Frankfurt/Main, Germany. We thank Nicolas Derungs, Cécile Genetti, and Enrique Lara for help in field and lab work.

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