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

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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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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