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PhD Thesis

Impact of vertebrate cadavers on soil communities and soil chemistry to develop new forensic indicators for estimating the time after death

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“Mindig van megoldás, és nem is csak egy.”

(Rubik Ernö)

“There is always a solution and not just one.”

“Es gibt immer eine Lösung und nicht nur eine.”

“Il y a toujours une solution et pas uniquement une seule.”

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Abstract

Decomposing cadavers in terrestrial ecosystem represent discrete ephemeral "hot spots" that introduce water, microbial communities and high amounts of nutrients into the soil. This has a strong, although spatially and temporally limited, impact on soil chemistry and belowground soil organisms. The study of cadaver impacts on the soil environment is at the interface between ecological and forensic sciences, the former focusing on their importance as a resource in ecosystems and the latter on the estimation of the post-mortem interval (PMI) which is the time elapsed since death. However, carrion research is still sparse and especially knowledge about cadaver impacts on soil organisms remains extremely poor. In forensic science the estimation of long post-mortem intervals (after weeks, months or years) is still a challenge, because current established methods such as the medical examination of the deceased or the use of insect evidence (forensic entomology) lose their accuracy after a few days or several weeks respectively. Hence, there is an increasing demand for new and applicable methods in forensic science. This PhD thesis focuses on three key subjects: (1) characterising the changes in soil chemical characteristics in response to decomposing cadavers, (2) assessing the response of organism groups (testate amoebae, nematodes, insects) to these changes and evaluating their potential for PMI estimation (3) testing several methods (analysis of bones, mites, nematodes and micro-Eukaryotes) in a real case investigation, combining different disciplines (^{14}C dating, microscopy, high throughput sequencing). The overall aim is to improve our understanding of cadaver impact on the soil and use this knowledge to develop new methods for PMI estimation.

Therefore we conducted two experiments, using three pig cadavers (*Sus scrofa*) in the first (2009-2010) and ten pig cadavers in the second experiment (2013- 2014) that were placed in forest areas near Neuchâtel, Switzerland. In the first experiment, we compared three treatments (three replicates each): 1) control (bare soil for reference), 2) fake pigs (cotton bags of the same size as the pig cadavers for microclimatic effects) and 3) ground pigs (cadavers directly placed on the ground for microclimatic and cadaveric fluids effects). In the second experiment, we increased the number of replicates (five) and added a fourth treatment i.e. hanging pigs (cadavers hanging 1 m above ground) for cadveric fluids effects only. In both experiments sampling started shortly before the cadavers were put in place and continued in defined intervals after.

The results from this PhD thesis revealed that changes in soil chemistry and in the abundance and community structure of testate amoebae and nematodes show identifiable patterns that can be related to the decomposition process. Neither the chemical constituents of the soil, nor the community composition of the analysed soil organisms had returned to the initial status (i.e. before the decomposition process) after 10 or 12 months. Therefore, these approaches have a high potential for long PMI estimation. Finally, this PhD thesis shows that the combination of disciplines - in this case, acarology, anthropology, entomology and soil science – is especially powerful for real case investigations. In this PhD thesis we have not only successfully shown the importance of soil in forensic science, but also paved the way for additional forensic markers to estimate the PMI.

Chapter 1

Introductory remarks

Soil – a so far neglected tool in forensic science

Ildikó Szelecz

Soil – a so far neglected tool in forensic science
Introductory remarks

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1. Methods for estimating the post-mortem interval (PMI)

Estimating the time elapsed since death, the so-called post-mortem interval (PMI), is crucial when investigating homicides and other untimely death occurrences (Catts, 1992; Wells and Lamotte, 2010). Up to the first 72-96 hours post mortem, medical methods can be used, but the applicability of these methods terminates when the body temperature equals the ambient temperature and initial putrefaction starts (Amendt et al., 2011, 2004; Henssge et al., 2000a, 2000b).

As insects are attracted to a corpse within minutes after death, forensic entomology has been established as a powerful tool to provide valuable information for estimating the minimum PMI (PMImin), which is based on calculating the time of insect colonization and might not coincide with the actual time of death (Amendt et al., 2011, 2004; Wells and Lamotte, 2010). Although Madra et al. (2015) suggested that carrion entomofauna might be useful for long PMIs (> one year), this is not well studied and in most cases entomological evidence for PMImin estimation is used up to several weeks or months only, depending on the circumstances (Amendt et al., 2011, 2004; Wells and Lamotte, 2010).

Hence the estimation of PMIs remains challenging when cadavers are discovered months or years after death, and there is a clear need for new methods (Anderson et al., 2013; Carter et al., 2007; Madra et al., 2015). A combination of several disciplines, including classical and newly emerging methods, are necessary to reconstruct as accurately as possible the circumstances and timeline of death in order to answer a variety of questions: How much time has elapsed since death? How long has the victim been at a particular deposition or burial site? Was the body moved? How did he or she die? etc. (Márquez-Grant and Roberts, 2012).

1.1. Various methods for PMI estimation

Numerous methods, based on analysing the cadaver itself and the crime or depositional environment are in practice for PMI estimation.

Within the first days after death complex medical methods are used that include the temperature based nomogram method, which is supplemented by examining other criteria (Henssge et al., 2000a, 2000b). The temperature method is based on the equilibration of the body temperature according to the ambient temperature (Henssge et al., 2000a; Madea et al., 2015). A relatively precise PMI can be estimated using rectal temperature, ambient temperature, body weight and several correction factors e.g. clothing and surface beneath the corpse (Henssge, 2002). Supplementary criteria are lividity (the hypostatic settling of blood), rigor mortis (the stiffening of the muscle tissue) and supravital reactions (Madea et al., 2015). These include the mechanical and electrical excitability of the skeletal muscles, the degrees of a positive reaction after stimulation of the Orbicularis oculi muscle and the pharmacological excitability of the iris (Henssge et al., 2000b; Madea et al., 2015). In the study of 72 consecutive cases, Henssge et al. (2000b) showed that when using the temperature based nomogram method as a primary method, the estimated PMI could be improved in 49 cases when adding the aforementioned additional criteria. Hence a compound method leads to a more precise PMI estimation. This should be taken into account when developing new methods.

There are a variety of morphological, chemical, physical and histological methods for PMI estimation when the above-mentioned methods are no-longer applicable or need to be accompanied for higher precision for instance luminol tests and the C-14 method (Cappella et al., 2015; Ramsthaler et al., 2009), the citrate content of the bone (Kanz et al., 2014) or volatile organic compound (VOC) analyses for VOC profiling (Stefanuto et al., 2015, 2014). Additional studies are focusing on the Thanatobiome investigating the bacterial diversity on the body before and after death (Guo et al., 2016; Hauther et al., 2015; Johnson et al., 2016; Zhang et al., 2016).

When insects have access to the body, the use of entomological evidence is a well-established method. This method dates back to 13th century China with the earliest record of medico-criminal entomology used to solve the case of a farmer which was killed in a rice field (Amendt et al., 2004; Hall and Huntington, 2010). A Chinese

criminalist investigated the case and villagers were asked to bring their scythes to one spot. The investigator recognized that flies were attracted to one of the scythes due to minute invisible blood traces. The villager was confronted by this evidence and confessed (Hall and Huntington, 2010).

However, it was not until 1855 that forensic entomology was applied in a French courtroom (Amendt et al., 2004; Hall and Huntington, 2010). The skeletonised remains of a child were detected behind a mantle. According to the insect fauna it was concluded, that the child had been dead for two years i.e. before the current inhabitants had moved into the house, so they could not have killed the child (Amendt et al., 2004; Hall and Huntington, 2010). Even though the results would be questionable today, this application of forensic entomology can be seen as a breakthrough and paved the way for further studies (Amendt et al., 2004; Hall and Huntington, 2010).

Towards the end of the 19th century, Yovanovich (1888) and Mégnin (1894) were examining successional insect colonization on corpses, but in Europe it was not until the 20th century that forensic entomology was used to determine the PMI (Amendt et al., 2004). Nowadays forensic entomology is a widely-accepted method in forensic science and from 400 described insect species on a pig cadaver a variety of studies have selected and repeatedly tested taxa according to their usefulness for forensic purposes (Amendt et al., 2004; Matuszewski et al., 2010; Payne, 1965). Forensic entomology is based on two methods for PMI estimation (1) the degree of development of immature insects found on the corpse and (2) the stage of insect succession (Amendt et al., 2004; Wells and Lamotte, 2010). The main driver for insect development is temperature and the rate can differ even between closely related species (Amendt et al., 2011; Reed, 1958). Therefore, insect species need to be clearly identified and crime scene temperature has to be reconstructed before the development rate of the immature insects can be modelled (Amendt et al., 2011).

A variety of insects are attracted to a cadaver, they will not occur at the same time, but rather in a chronological sequence (Grassberger and Frank, 2004; Rodriguez and Bass, 1983; Schoenly and Reid, 1987). For instance, Calliphoridae (blowflies) prefer fresh cadavers and arrive at the early decomposition stages, whereas Dermestidae (larder beetles) are attracted to the later stages of decomposition preferring dry skin, hair and bones (Amendt et al., 2011). Not all insects visiting a cadaver feed on the cadaver and/or use it as a breeding source. Smith (1986) described four different

ecological groups of insects that can be classified on a corpse: (1) necrophagous species that feed on the dead organic matter directly (e.g. Calliphoridae), (2) predators and parasites of insects and other necrophagous species (e.g. Staphylinidae), (3) omnivores (e.g. wasps), (4) adventive species that use the corpse as an extension of their environment (e.g. spiders).

In order to be more accurate, especially when it comes to the estimation of a long PMI (beyond several months post mortem), new methods have started to evolve. These methods need to be continuously verified by adding data from decomposition experiments and case studies. Here especially the potential of mites and their relevance for criminal investigations has already been demonstrated (Braig and Perotti, 2009; Perotti and Braig 2009; Perotti et al., 2009).

2. The cadaver decomposition island (CDI)

Cadaver research is mainly focusing on two perspectives (1) a forensic-based one that focuses on the estimation of the post- mortem interval i.e. the time elapsed since death or (2) an ecological-based one that views carrion as an important resource in ecosystems (Barton et al., 2013).

Cadavers are at the interface between ecological and forensic sciences and cadaver research can help to fill knowledge gaps, with each scientific field benefiting from the other. A cadaver forms a so-called cadaver decomposition island (CDI) defined as a discrete, ephemeral “hot spot” with direct and indirect effects on nutrient cycling and species diversity of an ecological community (Barton et al., 2013; Beasley et al., 2012; Carter et al., 2007) (Figs. 1 & 2). Cadavers can be seen as specialized habitats for a number of species increasing the biodiversity in terrestrial ecosystems (Carter et al., 2007) and serving as a food source and a resource for reproduction (Hall et al., 2011).

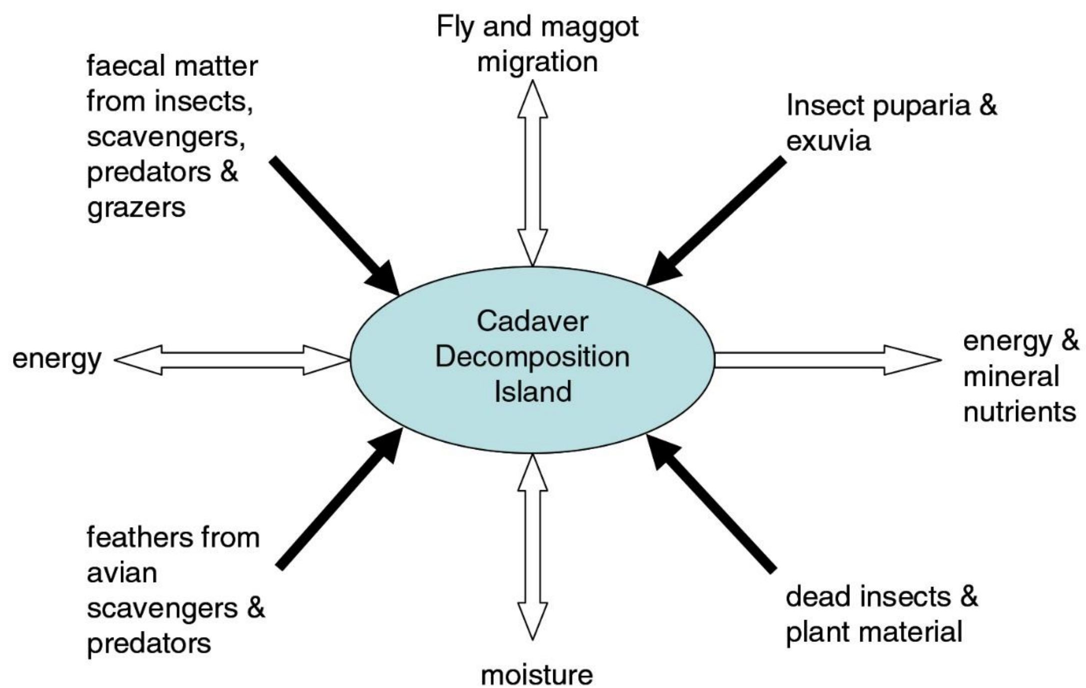

Figure 1. The cadaver decomposition island (CDI). A highly concentrated flux of energy and nutriens that contribute to landscape heterogeneity, physical and chemical complexity and biodiversity in a terrestrial ecosystem (Carter et al., 2007)

Figure 2. Cow cadaver decomposition island in Chilean Tierra-del-Fuego. Picture: E. Mitchell

2.1. The process of decomposition

A human body contains approximately 64% water, 20% protein, 10% fat, 1% carbohydrates and 5% minerals (Dent et al., 2004; Macdonald et al., 2014).
The elemental composition is on average 32g kg^{-1} Nitrogen, 10g kg^{-1} Phosphorous, 4g kg^{-1} Potassium, 15g kg^{-1} Calcium and 1g kg^{-1} Magnesium (Carter and Tibbett, 2008; Tortora and Grabowski, 2000).

After death, enzymes and micro-organisms are involved in the breakdown of proteins, carbohydrates and fats, of which some are destroyed in an earlier stage of decomposition while others are broken down at a later stage (Dent et al., 2004). When the heart stops beating, the internal oxygen concentration is depleted and the aerobic metabolism inhibited (Carter et al., 2007; Madea et al., 2015). Hence, the destruction of cells by enzymatic digestion begins and dissolves the tissues starting in enzyme-rich organs such as adrenal glands, brain, spleen (Carter et al., 2007; Madea et al., 2015). This process is called autolysis and is defined as the hydrolytic splitting of proteins, carbohydrates and fats (Dent et al., 2004).

The continuing depletion of oxygen creates an ideal environment for anaerobic micro-organisms from the gastrointestinal tract and the respiratory system, which leads to putrefaction (Carter et al., 2007; Dent et al., 2004; Madea et al., 2015). Micro-organisms transform carbohydrates, lipids and proteins into organic acids and gases (Carter et al., 2007; Dent et al., 2004; Madea et al., 2015). Putrefactive bloating of the body and maggot activity cause ruptures in the skin, allowing oxygen to enter the cadaver, followed by aerobic microbial activity (Carter et al., 2007; Dent et al., 2004; Madea et al., 2015). Body tissues and organs are more and more softened and degenerate to a mass of unrecognizable tissue that becomes liquefied (Dent et al., 2004).

After the removal of soft tissue, only skeletal remains are left and bones, teeth and cartilage are broken down by physical breaking and weathering (Dent et al., 2004). As mentioned before the breakdown processes do not occur at a uniform rate (Forbes, 2008). The breakdown of proteins (proteolysis) for instance starts in neuronal and epithelial cells of the brain, liver and kidneys, whereas reticulin, epidermis and muscle protein will resist the breakdown for a while and collagen and keratin survive even longer (Forbes, 2008).

The changes during the decomposition process are apparent on the cadaver and lead to the description of different decomposition stages: fresh stage (no visible external changes), bloated stage (accumulation of gases in the abdomen, bloating of the body), active decay (ruptures in the skin, substantial release of cadaveric fluids, extensive loss of mass), advanced decay (most of the flesh has been removed, body starts to dry), dry (only dry skin, cartilage and bones) and remains (skin and flesh had been removed leaving only bones, teeth and hair) (Payne, 1965).

Despite these defined stages, decomposition has to be seen as a continuous process, influenced by a variety of factors, such as temperature, access of insects, scavenging etc. (Campobasso et al., 2001; Carter and Tibbett, 2006; Carter et al., 2008; DeVault et al., 2003; Goff, 2009).

3. Forensic soil science: A new star is born?

3.1. The importance of being a soil

Soils are dynamic systems, play an important role in recycling matter and nutrients, and result from the interactions of climate, organisms, parent material and topology over time (Bardgett, 2005; Coleman et al., 2004). These factors influence ecosystem processes and lead to the development of different soil types with complex interactions of physical, chemical and biological agents (Coleman et al., 2004).

The important functions of soil are indisputable, including: (1) sustaining biological activity, diversity and productivity, (2) regulation and portioning of water, (3) filtering, buffering, degrading, immobilizing and detoxifying inorganic and organic materials, (4) storing and cycling nutrients and other elements, (5) providing support for socioeconomic structures (Seybold et al., 1997).

3.2. Soil chemistry

Soil is a predominantly sand-silt-clay matrix that contains living and dead organic matter, (Coleman et al., 2004). Soil is composed of minerals (that derive from the weathering of rocks), organic constituents (deriving from the decomposition of living organisms), liquid constituents (from precipitation, groundwater) and gaseous constituents (from air, respiration) (Gobat et al., 2004).

The different physical, chemical and biological processes mark the soil with identifiable layers i.e. horizons (Gobat et al., 2004). These horizons differ from each other in constituents, organisation and behaviour. The top organic layer (O-horizon) contains litter i.e. needles, leaves, twigs etc. (more or less transformed) and is identifiable by the naked eye (Gobat et al., 2004).

The A- horizon is next, an organo-mineral surface horizon, that is structured and the site of the clay-humus complex (Gobat et al., 2004). In this PhD thesis, we mainly focus on the upper layers of forest soil as the top 10-15cm of the A-horizon include the majority of plant roots, microbes and fauna and a majority of the biological and chemical activities occur in this layer (Coleman et al., 2004). Plant material in the O-horizon is composed of carbohydrates (mono- and polysaccharides), lignins, lipids and nitrogenous compounds (proteins, amino acids) that consists of 11 major elements i.e. carbon, hydrogen, oxygen, nitrogen, phosphorous, sulfur, calcium, magnesium, potassium, chloride and sodium (Gobat et al., 2004). The proportions of course vary with the type of litter.

The clay-humus complex in the A-horizon is an association of humified organic molecules and clay minerals (Gobat et al., 2004). These two components (both electronegative) are bound together by a cation (usually calcium or iron). Calcium provides a very strong and stable bond (Gobat et al., 2004). Amongst other properties the clay humus complex slows down mineralization of organic matter (Gobat et al., 2004). The clay-humus complex is able to exchange, retain and fix ions with increasing retention intensity i.e. $Na^+ < NH_4^+ < K^+ < H^+ < Mg^{2+} < Ca^{2+} < Al^{3+} < Mn^{2+}$ etc. (Gobat et al., 2004).

In a variety of complex processes, soil fauna and enzymatic reactions degrade organic matter (e.g. carbohydrates, proteins, amino acids, lipids and nucleic acids) into simple molecules, cations and anions (Gobat et al., 2004). They can then undergo (1) an evacuation through the atmosphere (e.g. CO_2, NH_3, N_2), (2) absorption by plants (e.g. cations, anions), (3) absorption by micro-organisms (e.g. NH_4^+, NO_3^-, PO_4^{3-}), (4) retention in the exchange complex (e.g. Ca^{2+}, K^+, NH_4^+, H^+) or (5) removal by leaching (e.g. K^+, Ca^{2+}, NO_3^-) (Gobat et al., 2004).

3.3. Soil biota

Our knowledge about global diversity is still limited with an estimate of 8.7 million (±1.3 million SE) eukaryotic species globally (Fig. 3) (Adl et al., 2012; Mora et al., 2011). In terrestrial ecosystems, soils contain by far the greatest diversity of organisms (prokaryotic and eukaryotic), differing in life cycles and their capacity to resist to and recover from environmental stress (Coleman et al., 2004; Wardle, 2002).

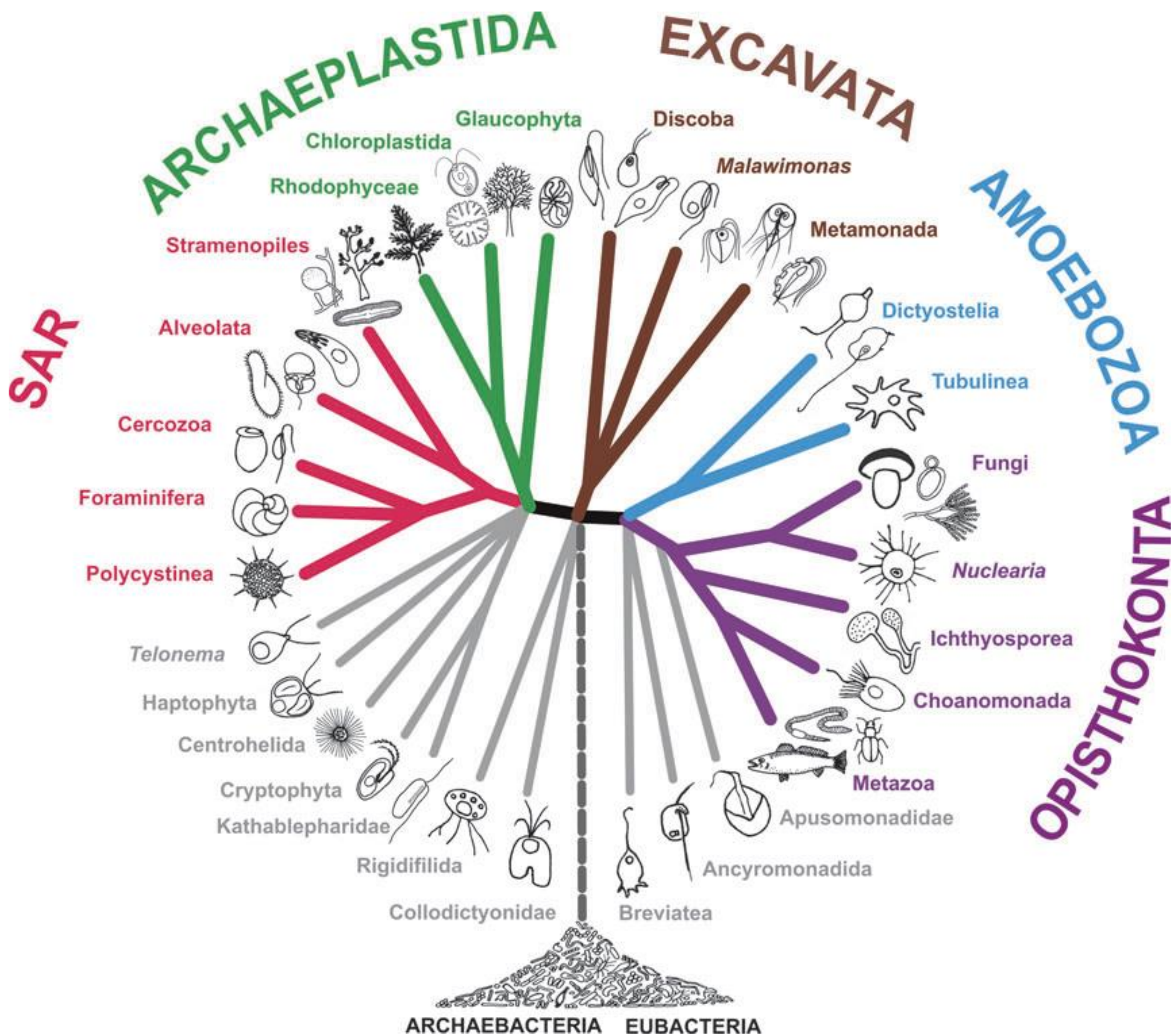

Figure 3. The phylogenetic tree of life (Adl et al., 2012).

Basically, soil fauna can be divided into three main groups according to their body size: (bacteria, fungi and algae) and Microfauna (protozoa and nematodes) with a body width < 0.1 mm, (b) Mesofauna for instance micro-arthropods such as collembola and acari, but also enchytraeids with a body width of 0.1-2.0 mm and (c) Macrofauna with

a variety of taxonomic groups such as isopoda, araneida or mollusca with a body width > 2 mm (Fig. 4) (Bardgett, 2005; Scheffer et al., 2010).

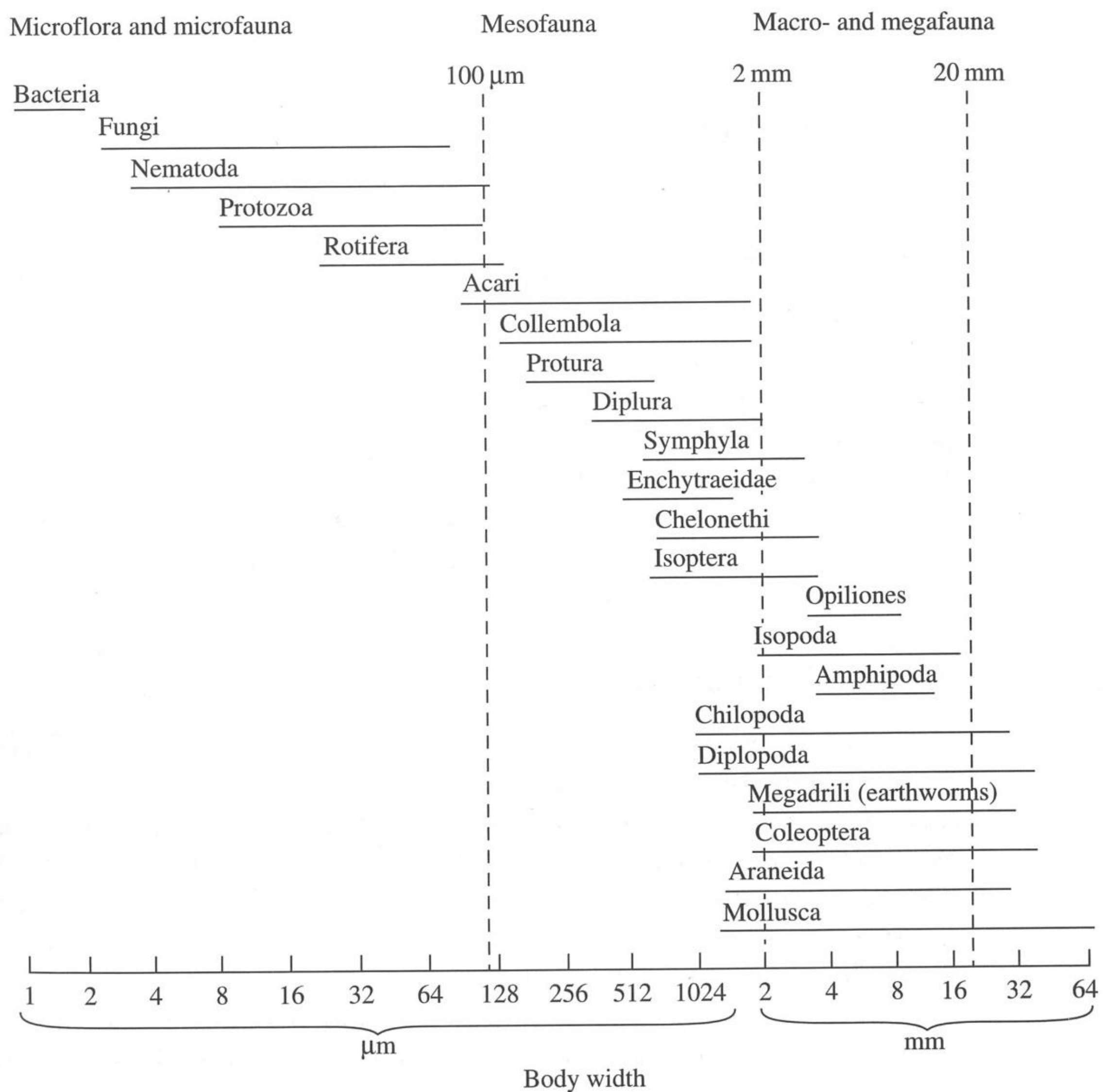

Figure 4. Soil biota classification of soil organisms according to body width (Swift et al., 1979).

Soil micro-organisms offer many advantages for the discovery of potential PMI indicators for two main reasons:

(1) They are numerous and diverse e.g. in terms of abundance and species diversity, prokaryotes (Archaea and Bacteria) dominate the soil community e.g. 4.8.x 10^9 cells cm^{-3} with 10^4 estimated species (Torsvik, 2002). Here bacteria are the most extensively studied microbial group in soil (Fierer et al., 2007).

Nevertheless, protozoa and nematodes are the two most abundant groups in the soil microfauna (Bardgett, 2005). Protozoa are single-celled eukaryotic organisms e.g. testate amoebae, flagellates, naked amoebae, ciliates (Bardgett, 2005). Among them, flagellates and naked amoebae are regarded as the most abundant with up to 10^6 and $2x10^6$ organisms per gram of soil (Foissner, 1999), followed by testate amoebae with up to 10^5 per gram leaf litter and 10^4 per gram soil and ciliates with up to 10^4 per gram litter (Foissner, 1999). Nematodes, multicellular roundworms, can reach up to 50 milliions per m^2 (Bongers and Bongers, 1998).

(2) They play key roles in nutrient cycling e.g. decompose organic matter and release nutrients into plant-available forms and they are known to respond to changes in their environment (Bongers and Ferris, 1999; Coûteaux and Darbyshire, 1998; Foissner, 1999, 1997; Gobat et al., 2004).

3.4. Forensic use of soil

In the need for new and additional forensic tools, soil came into the focus of interest as either trace evidence or the surface beneath a decomposing cadaver. In practice, this led to two main applications in forensic science:

(1) Soil is used for associative evidence as a powerful contact trace helping to reconstruct the circumstances of a crime (Fitzpatrick, 2008). Soil is an easily transferred material and can be recovered from a suspect´s shoe, from a carpet in a vehicle boot or deposited as muddy footprints (Fitzpatrick et al., 2009; Lambert and Chardez, 1978; Morgan et al., 2009; Swindles and Ruffell, 2009).

Here, combined pedological, mineralogical and spectroscopic methods are applied to compare the soil samples (Fitzpatrick et al., 2009).

(2) Soil, associated with above– or below-ground cadaver decomposition, is analysed from a chemical, physical and biological point of view to estimate the post-mortem or post-burial interval (Tibbett and Carter, 2009).

3.5. Classical and upcoming methods- advantages and limitations

As mentioned above, the use of medical and entomological methods for PMI estimation is temporally limited to a few days or several weeks respectively. When

investigating soil for forensic purposes and especially for PMI investigation, soil chemistry and soil fauna might be the keys to success depending on the period in question.

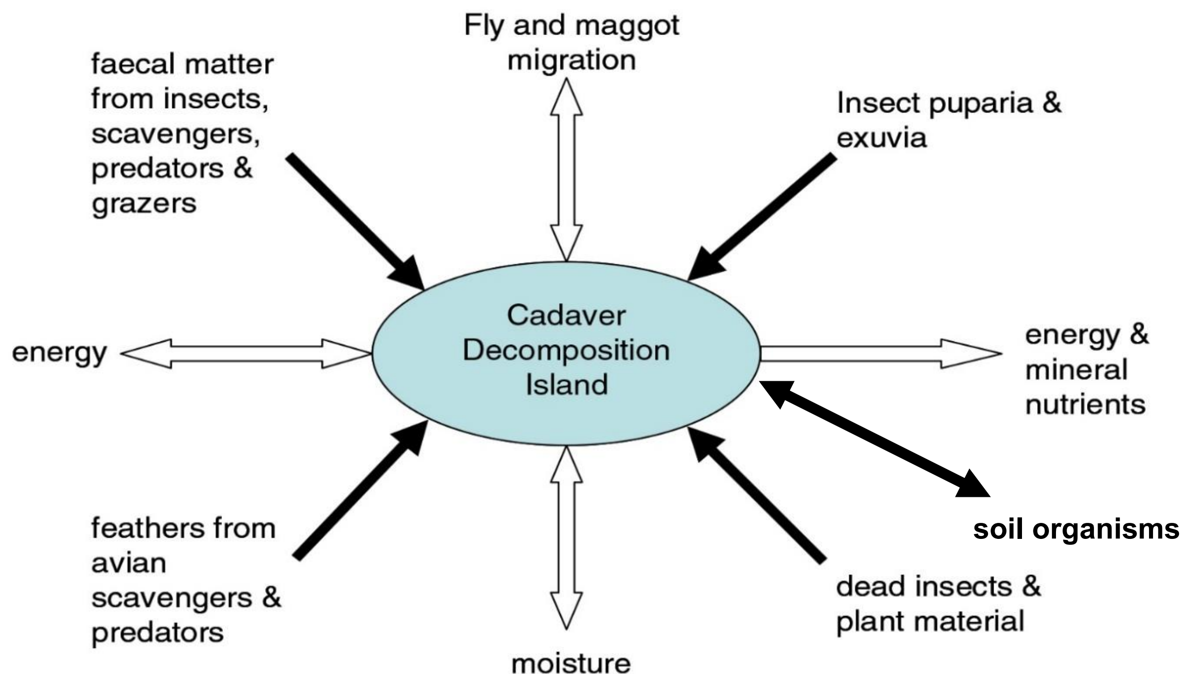

Figure 5. The cadaver decomposition island (CDI).Modified according to Carter et al. (2007).

So far, forensic science has developed relatively few resources to estimate the extended PMI, but one basis is the analysis of gravesoil chemistry (Anderson et al., 2013). Decomposition studies have shown that decomposing cadavers or tissues have an effect on soil chemistry e.g. soil nutrients, trace elements and pH (Aitkenhead-Peterson et al., 2012; Anderson et al., 2013; Benninger et al., 2008; Carter and Tibbett, 2008; Carter et al., 2008, 2007; 2006; Forbes, 2008; Haslam and Tibbett, 2009; Hopkins et al., 2000; Macdonald et al., 2014; Melis et al., 2007; Meyer et al., 2013; Parmenter and MacMahon, 2009; Stokes et al., 2009; Towne, 2000; Vass et al., 1992). These effects and patterns need to be further validated and to be confirmed for PMI estimation.

Cadaver decomposition and the formation of a CDI can not only change edaphic, but also biological characteristics in the soil below and links above- and belowground communities (Carter et al., 2007; Hopkins et al., 2000; Towne, 2000). The effects on belowground organisms could be negative, as temporarily changed conditions

provided by the cadaveric fluids compromise or even kill certain taxa, or positive, by causing an increase in food availability due to the nutrient input related to the fluids (Carter et al., 2007).

Some studies have focused on potential PMI indicators from soil and have investigated the community structure of micro-organisms, mainly bacteria and fungi (Carter and Tibbett, 2003; Finley et al., 2016, 2015; Hawksworth and Wiltshire, 2011; Metcalf et al., 2016; Parkinson et al., 2009; Pechal et al., 2013). In this PhD thesis we show, that decomposing cadavers alter the density and community structure of soil micro-organisms such as testate amoebae (Seppey et al., 2016; Szelecz et al., 2014) and terrestrial nematodes (Szelecz et al., 2016) and extend the CDI concept (Fig. 5).

Morphological approaches reveal promising results for PMI estimation, but they require taxonomic expertise and might be relatively time-consuming. With the rapid development of molecular techniques during the 1990s, the DNA barcoding initiative launched by Hebert et al. (2003) and the increasing use of high-throughput sequencing, additional relevant data will be produced and may pave the way for further applicable forensic tools (Hubert and Hanner, 2015; Pechal et al., 2014; Seppey et al., 2016; Shokralla et al., 2012; Valentini et al., 2009).

For the use in forensic science both approaches provide complementary information and PMI estimations may thus be most accurate when several methods are used in combination. The evidence from a decomposing cadaver in criminal investigations might have to be seen as a mosaic where the whole picture only appears when all the single parts are put together.

4. Experimental design and outline of this PhD thesis

Two field experiments are the basis of this PhD thesis.

4.1. 1st experiment - 2009

The first experiment started in 2009 in a mixed beech and oak forest near the city of Neuchâtel, Switzerland. Litter and soil samples taken beneath pig (*Sus scrofa*) cadavers (placed directly on the ground) were contrasted to two controls: bare litter/soil and litter/soil beneath a fake cadaver (bags filled with soil) (Figs. 6, 7) (for a more detailed description see chapter 3).

Figure 6. 1st experiment, 2009, field site near Neuchâtel, Switzerland.

Figure 7. Treatments in the 1st experiment, 2009, field site near Neuchâtel, Switzerland. Top left: control, top right: fake pig (bags filled with soil), bottom left: pig cadaver placed on the ground, bottom right: moving of the cage for sampling (I.Szelecz & B. Fournier).

4.2. 2nd experiment - 2013

The second experiment started in 2013 in a small spruce (*Picea abies*) forest near Neuchâtel, Switzerland. In addition to the treatments from the 2009 experiment, pig cadavers were also hung one metre aboveground. Soil samples were taken from all four treatments (Figs. 8, 9) (for a more detailed description see chapters 2 and 4).

4.3. The chapters in a nutshell

As mentioned above, changes in soil chemistry due to the nutrient flux of cadaveric fluids were detected and have received increasing attention in the last decade. In "Soil chemistry changes beneath decomposing cadavers over a one-year period" (chapter 2) novel categories for (chemical) PMI markers are proposed and tested.

The impact of decomposing cadavers on soil organisms has not yet received much attention. We have therefore studied two selected groups of soil organisms: testate amoebae and soil nematodes. So far, these groups have been neglected in cadaver research as well as in their possible applicability for forensic science. In the first study "Can soil testate amoebae be used for estimating the time since death? A field experiment in a deciduous forest" we investigated the response of soil testate amoeba communities to decomposing cadavers and "fake" cadavers in comparison to controls (chapter 3). This study was published in Forensic Science International in 2014.

During the analysis of testate amoeba communities, major changes in nematode abundance were anecdotally observed in response to cadaver decomposition. This led to the subsequent topic, the "Effects of decomposing cadavers on soil nematode communities over a one-year period" (chapter 4), which was published in Soil Biology and Biochemistry in 2016. These studies might be the gateway to new disciplines i.e. forensic protistology and forensic nematology.

In a real case investigation, we then demonstrated the possible application of our methods to estimate a long post-mortem interval and present a multiproxy approach bridging different disciplines in a suspected homicide. The "Comparative analysis of mites, soil chemistry, nematodes and soil micro-eukaryotic communities of a suspected homicide to estimate a long post-mortem interval" (chapter 5) not only shows the future possibilities of our methods, but also unites researches from different countries and disciplines which is fundamental to reach our goals in science.

Figure 8. 2nd experiment, 2013, field site near Neuchâtel, Switzerland.

Figure 9. Treatments in the 2nd experiment, 2013, field site near Neuchâtel, Switzerland. Top left: control, top right: fake pig (bags filled with soil), bottom left: pig cadaver placed on the ground, bottom right: pig cadaver hung 1m aboveground.

Forensic indicators and methods need sound research, especially when methods have to provide evidence in court. Forensic entomology is a good example of an already well-established method presenting forensically useful indicators. In "Is *Saprinus semistriatus* (Coleoptera: Histeridae) a useful taxon for PMI (post-mortem interval) estimation?" (chapter 6) *Saprinus semistriatus* data are discussed in relation to other studies to show the importance of repeated data collection.

5. Aims and objectives of this PhD thesis

The increasing development of forensic science in general (and especially forensic pathology) in the past decades relied on its interdisciplinary approach and the inclusion of non-forensic experts such as insect taxonomists and ecologists in the field of forensic entomology. However, there are still borders to cross, as was highlighted by a recently founded working group of the European Network of Forensic Science Institutes (ENFSI), the A(nimal) P(lant) S(oil) T(races) working group. There, for the first time, people from very different branches of science, from universities, museums, forensic medicine institutes, police institutions, etc. came together to answer and discuss a variety of questions, develop new methods and work together.

The aim of this PhD thesis fits perfectly into this novel approach by enhancing our knowledge about the effects of decomposing cadavers on the soil below and by using different methods. This eventually leads to the development of new forensic indicators especially for long PMI estimation and may aid in solving criminal cases. The main focus in this PhD thesis is on (1) establishing forensically important chemical markers, (2) providing key organism groups (testate amoebae and nematodes) that have a high potential for PMI estimation and (3) testing different methods in a real case investigation.

6. References

[1] Adl, S.M., Simpson, A.G.B., Lane, C.E., Lukeš, J., Bass, D., Bowser, S.S., Brown, M. W., Burki, F., Dunthorn, M., Hampl, V., Heiss, A., Hoppenrath, M., Lara, E., Le Gall, L., Lynn, D.H., McManus, H., Mitchell, E.A.D., Mozley-Stanridge, S.E., Parfrey, L.W., Pawlowski, J., Rueckert, S., Shadwick, L., Schoch, C.L., Smirnov, A., Spiegel, F.W., 2012. The revised classification of Eukaryotes. Journal of Eukaryotic Microbiology 59, 429–514. doi:10.1111/j.1550-7408.2012.00644.x

[2] Aitkenhead-Peterson, J.A., Owings, C.G., Alexander, M.B., Larison, N., Bytheway, J.A., 2012. Mapping the lateral extent of human cadaver decomposition with soil chemistry. Forensic Science International 216, 127–134. doi:10.1016/j.forsciint.2011.09.007

[3] Amendt, J., Richards, C.S., Campobasso, C.P., Zehner, R., Hall, M.J.R., 2011. Forensic entomology: applications and limitations. Forensic Science, Medicine, and Pathology 7, 379–392. doi:10.1007/s12024-010-9209-2

[4] Amendt, J., Krettek, R., Zehner, R., 2004. Forensic entomology. Naturwissenschaften 91, 51–65. doi:10.1007/s00114-003-0493-5

[5] Anderson, B., Meyer, J., Carter, D.O., 2013. Dynamics of ninhydrin-reactive nitrogen and pH in gravesoil during the extended postmortem interval. Journal of Forensic Sciences 58, 1348–1352. doi:10.1111/1556-4029.12230

[6] Bardgett, R.D., 2005. The biology of soil: a community and ecosystem approach. Oxford University Press, New York.

[7] Barton, P.S., Cunningham, S.A., Lindenmayer, D.B., Manning, A.D., 2013. The role of carrion in maintaining biodiversity and ecological processes in terrestrial ecosystems. Oecologia 171, 761–772. doi:10.1007/s00442-012-2460-3

[8] Beasley, J.C., Olson, Z.H., Devault, T.L., 2012. Carrion cycling in food webs: comparisons among terrestrial and marine ecosystems. Oikos 121, 1021–1026. doi:10.1111/j.1600-0706.2012.20353.x

[9] Benninger, L.A., Carter, D.O., Forbes, S.L., 2008. The biochemical alteration of soil beneath a decomposing carcass. Forensic Science International 180, 70–75. doi:10.1016/j.forsciint.2008.07.001

[10] Bongers, T., Ferris, H., 1999. Nematode community structure as a bioindicator in environmental monitoring. Trends in Ecology & Evolution 14, 224–228. doi:10.1016/S0169-5347(98)01583-3

[11] Bongers, T., Bongers, M., 1998. Functional diversity of nematodes. Applied Soil Ecology 10, 239–251. doi:10.1016/S0929- 1393(98)00123-1

[12] Braig, H.R., Perotti, M.A., 2009. Carcases and mites. Experimental and Applied Acarology 49, 45–84. doi:10.1007/s10493-009-9287-6

[13] Campobasso, C.P., Di Vella, G., Introna, F., 2001. Factors affecting decomposition and Diptera colonization. Forensic Science International, Forensic Entomology 120, 18–27. doi:10.1016/S0379-0738(01)00411-X

[14] Cappella, A., Gibelli, D., Muccino, E., Scarpulla, V., Cerutti, E., Caruso, V., Sguazza, E., Mazzarelli, D., Cattaneo, C., 2015. The comparative performance of PMI estimation in skeletal remains by three methods (C-14, luminol test and OHI): analysis of 20 cases. International Journal of Legal Medicine. doi:10.1007/s00414-015-1152-z

[15] Carter, D.O., Tibbett, M., 2008. Cadaver decomposition and soil: processes In: Tibett, M., Carter, D.O. (Eds.), Soil Analysis in Forensic Taphonomy: Chemical and Biological Effects of Buried Human Remains. CRC Press, Boca Raton, pp. 29–51.

[16] Carter, D. O., Tibbett, M., 2006. Microbial decomposition of skeletal muscle tissue (*Ovis aries*) in a sandy loam soil at different temperatures. Soil Biology and Biochemistry 38, 1139–1145. doi:10.1016/j.soilbio.2005.09.014

[17] Carter, D.O., Tibbett, M., 2003. Taphonomic Mycota: fungi with forensic potential. Journal of Forensic Sciences 48, 2002169. doi:10.1520/JFS2002169

[18] Carter, D.O., Yellowlees, D., Tibbett, M., 2008. Temperature affects microbial decomposition of cadavers (*Rattus rattus*) in contrasting soils. Applied Soil Ecology 40, 129–137. doi:10.1016/j.apsoil.2008.03.010

[19] Carter, D.O., Yellowlees, D., Tibbett, M., 2007. Cadaver decomposition in terrestrial ecosystems. Naturwissenschaften 94, 12–24. doi:10.1007/s00114-006-0159-1

[20] Catts, E.P., 1992. Problems in estimating the postmortem interval in death investigations. Journal of Agricultural Entomology 9, 245–255.

[21] Coleman, D.C., Crossley, D.A., Hendrix, P.F., 2004. Fundamentals of soil ecology, 2nd ed. Elsevier Academic Press, Amsterdam; Boston.

[22] Coûteaux, M.-M., F. Darbyshire, J., 1998. Functional diversity amongst soil protozoa. Applied Soil Ecology 10, 229–237. doi:10.1016/S0929-1393(98)00122-X

[23] Dent, B.B., Forbes, S.L., Stuart, B.H., 2004. Review of human decomposition processes in soil. Environmental Geology 45, 576–585. doi:10.1007/s00254-003-0913-z

[24] DeVault, T.L., Rhodes, Jr., O.E., Shivik, J.A., 2003. Scavenging by vertebrates: behavioral, ecological, and evolutionary perspectives on an important energy transfer pathway in terrestrial ecosystems. Oikos 102, 225–234. doi:10.1034/j.1600-0706.2003.12378.x

[25] Fierer, N., Breitbart, M., Nulton, J., Salamon, P., Lozupone, C., Jones, R., Robeson, M., Edwards, R.A., Felts, B., Rayhawk, S., Knight, R., Rohwer, F., Jackson, R.B., 2007. Metagenomic and small-Subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi, and viruses in soil. Applied and Environmental Microbiology 73, 7059–7066. doi:10.1128/AEM.00358-07

[26] Finley, S.J., Benbow, M.E., Javan, G.T., 2015. Microbial communities associated with human decomposition and their potential use as postmortem clocks. International Journal of Legal Medicine 129, 623–632. doi:10.1007/s00414-014-1059-0

[27] Finley, S.J., Pechal, J.L., Benbow, M.E., Robertson, B.K., Javan, G.T., 2016. Microbial signatures of cadaver gravesoil during decomposition. Microbial Ecology 71, 524–529. doi:10.1007/s00248-015-0725-1

[28] Fitzpatrick, R.W., 2008. Nature, distribution, and origin of soil materials in the forensic comparison of soils In: Tibbett, M., Carter, D.O. (Eds.), Soil Analysis in Forensic Taphonomy: Chemical and Biological Effects of Buried Human Remains. CRC Press, Boca Raton, pp. 1–28.

[29] Fitzpatrick, R.W., Raven, M.D., Forrester, S.T., 2009. A systematic approach to soil forensics: Criminal case studies involving transference from crime scene to forensic evidence. In: Ritz, K., Dawson, L., Miller, D. (Eds.), Criminal and Environmental Soil Forensics, Springer Netherlands, 2009, pp. 105–127.

[30] Foissner, W., 1999. Soil protozoa as bioindicators: pros and cons, methods, diversity, representative examples. Agriculture, Ecosystems and Environment 74, 95–112.

[31] Foissner, W., 1997. Protozoa as bioindicators in agroecosystems, with emphasis on farming practices, biocides, and biodiversity. Agriculture, Ecosystems & Environment 62, 93–103. doi:10.1016/S0167-8809(96)01142-5

[32] Forbes, S.I., 2008. Decomposition chemistry in a burial environment. In: Tibbett, M., Carter, D.O. (Eds.), Soil Analysis in Forensic Taphonomy: Chemical and Biological Effects of Buried Human Remains. CRC Press, Boca Raton, pp. 202–223.

[33] Gobat, J.-M., Aragno, M., Matthey, W., 2004. The living soil: fundamentals of soil science and soil biology. Science Publishers, Enfield, NH.

[34] Goff, L.M., 2009. Early post-mortem changes and stages of decomposition in exposed cadavers. Experimental and Applied Acarology 49, 21–36. doi:10.1007/s10493-009-9284-9

[35] Grassberger, M., Frank, C., 2004. Initial study of arthropod succession on pig carrion in a Central European urban habitat. Journal of Medical Entomology 41, 511–523. doi:10.1603/0022-2585-41.3.511

[36] Guo, J., Fu, X., Liao, H., Hu, Z., Long, L., Yan, W., Ding, Y., Zha, L., Guo, Y., Yan, J., Chang, Y., Cai, J., 2016. Potential use of bacterial community succession for estimating post-mortem interval as revealed by high-throughput sequencing. Scientific Reports 6, 24197. doi:10.1038/srep24197

[37] Hall, C.L., Wadsworth, N.K., Howard, D.R., Jennings, E.M., Farrell, L.D., Magnuson, T.S., Smith, R.J., 2011. Inhibition of microorganisms on a carrion breeding resource: The antimicrobial peptide activity of burying beetle (Coleoptera: Silphidae) oral and anal secretions. Environmental Entomology 40, 669–678. doi:10.1603/EN10137

[38] Hall, R.D., Huntington, T.E., 2010. Introduction: Perceptions and status of forensic entomology. In: Byrd, J.H., Castner, J.L. (Eds.), Forensic Entomology: The utility of arthropods in legal investigations. CRC Press, Boca Raton, pp. 1–16.

[39] Haslam, T.C.F., Tibbett, M., 2009. Soils of contrasting pH affect the decomposition of buried mammalian (*Ovis aries*) skeletal muscle tissue. Journal of Forensic Sciences 54, 900–904. doi:10.1111/j.1556-4029.2009.01070.x

[40] Hauther, K.A., Cobaugh, K.L., Jantz, L.M., Sparer, T.E., DeBruyn, J.M., 2015. Estimating time since death from postmortem human gut microbial communities. Journal of Forensic Sciences 60, 1234–1240. doi:10.1111/1556-4029.12828

[41] Hawksworth, D.L., Wiltshire, P.E.J., 2011. Forensic mycology: the use of fungi in criminal investigations. Forensic Science International 206, 1–11. doi:10.1016/j.forsciint.2010.06.012

[42] Hebert, P.D.N., Ratnasingham, S., de Waard, J.R., 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society B: Biological Sciences 270, S96–S99. doi:10.1098/rsbl.2003.0025

[43] Henssge, C., 2002. Todeszeitbestimmung an Leichen. Rechtsmedizin 12, 112–131. doi:10.1007/s00194-002-0136-8

[44] Henssge, C., Althaus, L., Bolt, J., Freislederer, A., Haffner, H.-T., Henssge, C.A., Hoppe, B., Schneider, V., 2000a. Experiences with a compound method for estimating the time since death II. Integration of non-temperature-based methods. International Journal of Legal Medicine 113, 320–331.

[45] Henssge, C., Althaus, L., Bolt, J., Freislederer, A., Haffner, H.-T., Henssge, C.A., Hoppe, B., Schneider, V., 2000b. Experiences with a compound method for estimating the time since death. I. Rectal temperature nomogram for time since death. International Journal of Legal Medicine 113, 303–319.

[46] Hopkins, D.W., Wiltshire, P.E.J., Turner, B.D., 2000. Microbial characteristics of soils from graves: an investigation at the interface of soil microbiology and forensic science. Applied Soil Ecology 14, 283–288. doi:10.1016/S0929-1393(00)00063-9

[47] Hubert, N., Hanner, R., 2015. DNA Barcoding, species delineation and taxonomy: a historical perspective. DNA Barcodes 3. doi:10.1515/dna-2015-0006

[48] Johnson, H.R., Trinidad, D.D., Guzman, S., Khan, Z., Parziale, J.V., DeBruyn, J.M., Lents, N.H., 2016. A machine learning approach for using the postmortem skin

microbiome to estimate the postmortem interval. PLOS ONE 11, e0167370. doi:10.1371/journal.pone.0167370

[49] Kanz, F., Reiter, C., Risser, D.U., 2014. Citrate content of bone for time since death estimation: Results from burials with different physical characteristics and known PMI. Journal of Forensic Sciences 59, 613–620. doi:10.1111/1556-4029.12341

[50] Lambert, J., Chardez, D., 1978. Intérêt criminalistique de la microfaune terrestre. Revue Internationale de Police criminelle, 319, 158–170.

[51] Macdonald, B.C.T., Farrell, M., Tuomi, S., Barton, P.S., Cunningham, S.A., Manning, A.D., 2014. Carrion decomposition causes large and lasting effects on soil amino acid and peptide flux. Soil Biology and Biochemistry 69, 132–140. doi:10.1016/j.soilbio.2013.10.042

[52] Madea, B., Jachau, K., Reibe, S., Schmidt, P., Kernbach-Wighton, G., Peschel, O., Henn, V., Meissner, C., Oehmichen, M., Thali, M., Pollak, S., Zollinger, U., 2015. Thanatologie. In: Madea, B. , Rechtsmedizin: Befunderhebung, Rekonstruktion, Begutachtung. Springer Medizin, pp. 33–170.

[53] Madra, A., Fratczak, K., Grzywacz, A., Matuszewski, S., 2015. Long-term study of pig carrion entomofauna. Forensic Science International 252, 1–10. doi:10.1016/j.forsciint.2015.04.013

[54] Márquez-Grant, N., Roberts, J. (Eds.), 2012. Forensic ecology handbook: from crime scene to court. Wiley Blackwell, Chichester, UK; Hoboken, U.S.A.

[55] Matuszewski, S., Bajerlein, D., Konwerski, S., Szpila, K., 2010. Insect succession and carrion decomposition in selected forests of Central Europe. Part 2: Composition and residency patterns of carrion fauna. Forensic Science International 195, 42–51. doi:10.1016/j.forsciint.2009.11.007

[56] Melis, C., Selva, N., Teurlings, I., Skarpe, C., Linnell, J.D.C., Andersen, R., 2007. Soil and vegetation nutrient response to bison carcasses in Bialowieza Primeval Forest, Poland. Ecological Research 22, 807–813. doi:10.1007/s11284-006-0321-4

[57] Metcalf, J.L., Xu, Z.Z., Weiss, S., Lax, S., Van Treuren, W., Hyde, E.R., Song, S.J., Amir, A., Larsen, P., Sangwan, N., Haarmann, D., Humphrey, G.C., Ackermann, G., Thompson, L.R., Lauber, C., Bibat, A., Nicholas, C., Gebert, M.J., Petrosino, J.F., Reed, S.C., Gilbert, J.A., Lynne, A.M., Bucheli, S.R., Carter, D.O., Knight, R., 2016.

Microbial community assembly and metabolic function during mammalian corpse decomposition. Science 351, 158–162. doi:10.1126/science.aad2646

[58] Meyer, J., Anderson, B., Carter, D.O., 2013. Seasonal variation of carcass decomposition and gravesoil chemistry in a cold (Dfa) climate. Journal of Forensic Sciences 58, 1175–1182. doi:10.1111/1556-4029.12169

[59] Mora, C., Tittensor, D.P., Adl, S., Simpson, A.G.B., Worm, B., 2011. How many species are there on earth and in the ocean? PLoS Biology 9, e1001127. doi:10.1371/journal.pbio.1001127

[60] Morgan, R.M., Freudiger-Bonzon, J., Nichols, K.H., Jellis, T., Dunkerley, S., Zelazowski, P., Bull, P.A., 2009. The forensic analysis of sediments recovered from footwear. In: Ritz, K., Dawson, L., Miller, D. (Eds.), Criminal and Environmental Soil Forensics, Springer Netherlands, pp. 253–269.

[61] Parkinson, R.A., Dias, K.-R., Horswell, J., Greenwood, P., Banning, N., Tibbett, M., Vass, A.A., 2009. Microbial community analysis of human decomposition on soil, in: In: Ritz, K., Dawson, L., Miller, D. (Eds.), Criminal and Environmental Soil Forensics, Springer Netherlands, pp. 379–394.

[62] Parmenter, R.R., MacMahon, J.A., 2009. Carrion decomposition and nutrient cycling in a semiarid shrub—steppe ecosystem. Ecological Monographs 79, 637–661.

[63] Payne, J.A., 1965. A summer carrion study of the baby pig *Sus Scrofa* Linnaeus. Ecology 46, 592–602. doi:10.2307/1934999

[64] Pechal, J.L., Crippen, T.L., Benbow, M.E., Tarone, A.M., Dowd, S., Tomberlin, J.K., 2014. The potential use of bacterial community succession in forensics as described by high throughput metagenomic sequencing. International Journal of Legal Medicine 128, 193–205. doi:10.1007/s00414-013-0872-1

[65] Pechal, J.L., Crippen, T.L., Tarone, A.M., Lewis, A.J., Tomberlin, J.K., Benbow, M.E., 2013. Microbial community functional change during vertebrate carrion decomposition. PLoS ONE 8, e79035. doi:10.1371/journal.pone.0079035

[66] Perotti, M.A., Braig, H.R., 2009. Phoretic mites associated with animal and human decomposition. Experimental and Applied Acarology 49, 85–124. doi:10.1007/s10493-009-9280-0

[67] Perotti, M.A., Goff, M.L., Baker, A.S., Turner, B.D., Braig, H.R., 2009. Forensic acarology: an introduction. Experimental and Applied Acarology 49, 3-13.

[68] Ramsthaler, F., Kreutz, K., Zipp, K., Verhoff, M.A., 2009. Dating skeletal remains with luminol-chemiluminescence. Validity, intra- and interobserver error. Forensic Science International 187, 47–50. doi:10.1016/j.forsciint.2009.02.015

[69] Reed, H.B., 1958. A study of dog carcass communities in Tennessee, with special reference to the insects. American Midland Naturalist 59, 213–224.

[70] Rodriguez, W.C., Bass, W.M., 1983. Insect activity and its relationship to decay rates of human cadavers in East Tennessee. Journal of Forensic Sciences 28, 11524J. doi:10.1520/JFS11524J

[71] Scheffer, F., Schachtschabel, P., Blume, H.-P., Thiele-Bruhn, S., 2010. Lehrbuch der Bodenkunde, 16. Auflage. Spektrum, Akademischer Verlag, Heidelberg.

[72] Schoenly, K., Reid, W., 1987. Dynamics of heterotrophic succession in carrion arthropod assemblages: discrete seres or a continuum of change? Oecologia 73, 192–202. doi:10.1007/BF00377507

[73] Seppey, C.V.W., Fournier, B., Szelecz, I., Singer, D., Mitchell, E.A.D., Lara, E., 2016. Response of forest soil euglyphid testate amoebae (Rhizaria: Cercozoa) to pig cadavers assessed by high-throughput sequencing. International Journal of Legal Medicine 130, 551–562. doi:10.1007/s00414-015-1149-7

[74] Seybold, C.A., Mausbach, M.J., Karlen, D.L., Rogers, H.H., 1997. Quantification of soil quality. In: Lal, R., Kimble, J.M., Follett; R.F., Stewart, B.A. (Eds.), Soil Processes and the Carbon Cycle. CRC Press, Boca Raton, pp. 387–404.

[75] Shokralla, S., Spall, J.L., Gibson, J.F., Hajibabaei, M., 2012. Next-generation sequencing technologies for environmental DNA research: Next-Generation Sequencing for environmental DNA. Molecular Ecology 21, 1794–1805. doi:10.1111/j.1365-294X.2012.05538.x

[76] Smith, Kenneth G.V., 1986. A Manual of Forensic Entomology. The Trustees of the British Museum, London.

[77] Stefanuto, P.-H., Perrault, K.A., Lloyd, R.M., Stuart, B., Rai, T., Forbes, S.L., Focant, J.-F., 2015. Exploring new dimensions in cadaveric decomposition odour analysis. Analytical Methods 7, 2287–2294. doi:10.1039/C5AY00371G

[78] Stefanuto, P.-H., Perrault, K., Stadler, S., Pesesse, R., Brokl, M., Forbes, S., Focant, J.-F., 2014. Reading cadaveric decomposition chemistry with a new pair of glasses. ChemPlusChem 79, 786–789. doi:10.1002/cplu.201402003

[79] Stokes, K.L., Forbes, S.L., Tibbett, M., 2009. Freezing skeletal muscle tissue does not affect its decomposition in soil: Evidence from temporal changes in tissue mass, microbial activity and soil chemistry based on excised samples. Forensic Science International 183, 6–13. doi:10.1016/j.forsciint.2008.08.013

[80] Swift, M.J., Heal, O.W., Anderson, J.M., 1979. Decomposition in terrestrial ecosystems, Studies in ecology. University of California Press, Berkeley.

[81] Swindles, G.T. & Ruffell, A., 2009. A preliminary investigation into the use of testate amoebae for the discrimination of forensic soil samples. Science & Justice, 49, 182-190.

[82] Szelecz, I., Fournier, B., Seppey, C., Amendt, J., Mitchell, E., 2014. Can soil testate amoebae be used for estimating the time since death? A field experiment in a deciduous forest. Forensic Science International 236, 90–98. doi:10.1016/j.forsciint.2013.12.030

[83] Szelecz, I., Sorge, F., Seppey, C.V.W., Mulot, M., Steel, H., Neilson, R., Griffiths, B.S., Amendt, J., Mitchell, E.A.D., 2016. Effects of decomposing cadavers on soil nematode communities over a one-year period. Soil Biology and Biochemistry 103, 405–416. doi:10.1016/j.soilbio.2016.09.011

[84] Tibbett, M., Carter, D.O., 2009. Research in forensic taphonomy: A soil-based perspective, in: In: Ritz, K., Dawson, L., Miller, D. (Eds.), Criminal and Environmental Soil Forensics, Springer Netherlands, pp. 317–331.

[85] Torsvik, V., 2002. Prokaryotic diversity-magnitude, dynamics, and controlling factors. Science 296, 1064–1066. doi:10.1126/science.1071698

[86] Tortora, G.J., Grabowski, S.R., 2000. Principles of anatomy and physiology, 9. ed., Wiley, New York.

[87] Towne, E.G., 2000. Prairie vegetation and soil nutrient responses to ungulate carcasses. Oecologia 122, 232–239. doi:10.1007/PL00008851

[88] Valentini, A., Pompanon, F., Taberlet, P., 2009. DNA barcoding for ecologists. Trends in Ecology & Evolution 24, 110–117. doi:10.1016/j.tree.2008.09.011

[89] Vass, A., Bass, W.M., Woldt, J.D., Foss, J.E., Ammons, J.T., 1992. Time since death determinations of human cadavers using soil solution. Journal of Forensic Sciences 37, 1236–1253.

[90] Wardle, D.A., 2002. Communities and ecosystems: linking the aboveground and belowground components, Monographs in population biology. Princeton University Press, Princeton, N.J.

[91] Wells, J.D., Lamotte, L.R., 2010. Estimating the postmortem interval. In: Byrd, J.H., Castner; J. L. (Eds.) Forensic Entomology: The utility of arthropods in legal investigations. CRC Press Boca Raton, pp. 367–388.

[92] Zhang, L, Guo, J.J., Telet-Siyit, Peng, Y.L., Xie, D., Guo, Y.D., Yan, J., Zha, L., Cai, J.F., 2016. Bacterial succession on rat carcasses and applications for PMI estimation. Fa Yi Xue Za Zhi 32, 1–6.

Chapter 2

Soil chemistry changes beneath decomposing cadavers over a one-year period

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Soil chemistry changes beneath decomposing cadavers over a one-year period

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Abstract

In terrestrial ecosystems decomposing vertebrate cadavers have a clear impact on the soil ecosystem. Due to large, localized inputs of nutrients they act as temporarily limited resource patches and affect nutrient cycling. In this study, we investigated the effects of decomposing pig cadavers (*Sus scrofa*) on soil chemistry (pH, ammonium, nitrate, nitrogen, carbon, phosphorous, potassium, magnesium and calcium) over a one-year period. Four treatments were applied: two treatments including pig cadavers (placed on the ground and hung 1m aboveground) and two controls (bare soil and bags filled with soil placed on the ground i.e. "fake pig" treatment). At the beginning (15-59 days) of the experiment cadavers caused significant increases of potassium, nitrogen, ammonium and phosphorous ($P < 0.05$) whereas nitrate significantly increased towards the end of the study (263-367 days; $P < 0.05$). Soil pH increased significantly at first and then decreased significantly at the end of the experiment ($P < 0.05$). Based on these response patterns, we define three categories of forensic chemical markers that may be potentially useful to date the age of cadavers: early peak markers (EPM), late peak markers (LPM) and late decrease markers (LDM). After one year, some markers returned to basal levels, whereas others were still significantly different from the controls. The impact of decomposing cadavers on soil chemistry is relevant to general soil biology as well as in forensic science to estimate the post-mortem interval.

1. Introduction

When it comes to the understanding of vertebrate cadaver decomposition and its impact on soils, there is still a considerable knowledge gap in both soil ecology and forensic taphonomy, the study of post-mortem processes (Carter et al., 2010; Haglund and Sorg, 1997). This is understandable when we consider that 99 % of decomposing organic resources are either plant-derived or faecal matter and most studies focus on these materials (Carter et al., 2007).

So quantitatively the decomposition of a cadaver contributes minimally to the total ecosystem nutrient cycling, but it can have a significant, although temporarily limited, impact on the local patch scale (Parmenter and MacMahon, 2009). The study of these effects enhances our understanding of soil processes and soil diversity and helps to develop new tools for the estimation of a post-mortem interval (PMI) i.e. the time elapsed since death in criminal cases.

Cadavers are unique resources with high nutrient contents that affect soils chemistry, organisms and plants (Barton et al., 2013). During decomposition cadavers release high amounts of water and breakdown products from proteins, fats and carbohydrates which enter the underlying soil (Dent et al., 2004). The changed conditions in soil chemistry then influence the abundance and community structure of soil organisms (Carter et al., 2007; Szelecz et al., 2016, 2014).

Additionally, the decomposition environment comprises two primary habitats each with their own chemical profiles: the cadaver itself and the soil into which the cadaveric fluids are released (Aitkenhead-Peterson et al., 2015).

Death is not an event, it is a process and reduces the decedent to a collection of molecules (Gill-King, 1997). The breakdown of a cadaver, i.e. the transformation of carbohydrates, proteins and lipids into various acids, gases and other products, can be divided into autolysis, putrefaction and decay (Carter and Tibbett, 2008). During autolysis cellular enzymes digest carbohydrates and proteins and to a lesser extent fats (Clark et al., 1997). This does not occur in all tissues at the same time e.g. internal organs are digested before skeletal muscles, connective tissues and integument (Gill-King, 1997). During putrefaction, which is almost entirely an anaerobic process, micro-organisms from the gastrointestinal tract, the respiratory system and surrounding environment further digest the tissues (Carter and Tibbett, 2008; Gill-King, 1997). The

accumulation of gases (produced by micro-organisms) causes a bloating of the cadaver followed by ruptures of the skin, which re-establishes aerobic metabolism and the beginning of decay where the most rapid breakdown takes place due to a peak in maggot activity and the release of cadaveric fluids into the soil (Carter et al., 2007; Carter and Tibbett, 2008).

Major transitions in the decomposition process are apparent on the cadaver and lead to the division into different decomposition stages i.e. fresh, bloated, active decay, advanced decay, dry and remains (Payne, 1965). Nevertheless, decomposition has to be seen as a time-continuous process with overlapping and not clear-cut stages (Goff, 2009). In addition, various factors can influence decomposition and accordingly its impact on soils. These factors include or may include temperature (Carter and Tibbett, 2006, Carter et al., 2008), access of insects (Campobasso et al., 2001), vertebrate scavenging (DeVault et al., 2003), moisture (Carter et al., 2010), soil type (Tumer et al., 2013), season (Meyer et al., 2013), associated material e.g. clothing (Matuszewski et al., 2014), burial (Forbes, 2008), pH (Haslam and Tibbett, 2009), trauma (open wounds) (Carter and Tibbett, 2008), size, age and type of carcass (Spicka et al., 2011; Stokes et al., 2013; Towne, 2000).

However, for some of the aforementioned factors knowledge remains very limited. Nevertheless, even decomposition studies that are different in their experimental design (e.g. using different cadavers, tissues, buried cadavers or placed on the soil surface) show that decomposing cadavers or tissues have an effect on, amongst others, soil pH (Aitkenhead-Peterson et al., 2012; Benninger et al., 2008), the concentration of ammonium (Meyer et al., 2013; Stokes et al., 2009a), nitrates (Anderson et al., 2013; Meyer et al., 2013), total nitrogen (Anderson et al., 2013; Parmenter and MacMahon, 2009), carbon (Hopkins et al., 2000; Macdonald et al., 2014), but also phosphorous (Macdonald et al., 2014; Towne, 2000) as well as cations such as potassium (Aitkenhead-Peterson et al., 2012; Stokes et al., 2013), magnesium (Aitkenhead-Peterson et al., 2012) and calcium (Aitkenhead-Peterson et al., 2012; Melis et al., 2007).

We investigated the changes of selected chemical markers in soil over a one-year period to include seasonal variation and to monitor the changes in soil chemistry beyond the peak decay stages. We compared the effects on soil chemistry of pig cadavers that were placed directly on the ground and pig cadavers that were hung 1m

aboveground and contrasted them with two controls (bare soil and bags filled with soil). Then we addressed the following questions: Can the changes in soil chemistry be related to certain decomposition changes or time points? Are there significant differences between treatments especially between hanging and ground pigs? Can we group chemical markers according to their pattern over time?

We hypothesize, that the breakdown of cadavers would strongly influence soil chemistry and that different chemical markers would show contrasted patterns, in line with the known mobility or lability of each (e.g. nitrate being highly mobile and leachable while ammonium and phoshorous are more stable) (Scheffer, et al., 2010).

2. Material and Methods

2.1. Study site and experimental design

The experiment was conducted in a small spruce (*Picea abies*) forest near Neuchâtel, Switzerland (47°01'05.01 N, 6°52'27.76 E, 775m a.s.l.). The study site is almost flat and covered an area of 1200m^2. Mean temperature and total precipitation (measured in-field with a Decagon Em50 digital data logger) were 10.2 °C and 978 mm as published in Szelecz et al. (2016) (Fig. 1, p. 407).

The topsoil consisted of a litter layer (spruce needles and mosses), a fragmentation layer and a humification layer (O horizon, up to 1 cm) and an umbric horizon with a dark brown colour (A horizon, 1-17 cm).

In total 20 plots (ca. 4 m distant from each other) with four treatments (five replicates each) were set up randomly: 1) control (bare soil), 2) fake pigs (cotton bags filled with soil of the same size as the pig cadavers for microclimatic effects), 3) ground pigs (cadavers directly placed on the ground for microclimatic and cadaveric fluids effects), and 4) hanging pigs (cadavers hanging 1m above ground for cadaveric fluids effects).

Ten domestic pigs (*Sus scrofa*), 8 females and 2 males, 10 weeks old, were bought at a local farm. They were sedated with Stresnil® (Azaperone) and euthanized with T61® by a veterinarian, immediately transported to the experimental site, weighed and placed on the plots. The average cadaver weight was 27.8 kg ± 0.8 kg (SE). All cadavers were placed in cages (140 cm x 95 cm) surrounded by wire mesh fences to keep scavengers and larger animals away. The experimental area was surrounded by

an electric fence for additional protection. Control and fake pig plots were marked with sticks and cords. Fences and cages could be opened at one side for soil sampling and weighing the cadavers. Cadavers were weighed just before placing and on every sampling day until D 331 using a digital hanging scale. Accordingly, soil from inside the fake pig bags was removed to match the weight loss of the pig cadavers.

2.2. Decomposition stages and sampling

Decomposition stages were estimated using the definitions provided by Payne (1965) for arthropod-exposed carrions. From the first day of cadaver placement (July, 01, 2013) until the beginning of the dry stage, each pig cadaver was examined daily to record the state of decomposition (including photographs and written reports) according to physical characteristics and arthropods present. After the beginning of the dry stage the cadavers were examined at longer intervals (> 9 days).

On 11 sampling days from June 2013 until July 2014, a total of 220 soil samples (11 days x 4 treatments x 5 replicates) were collected. Samples were initially taken shortly before the placing of the cadavers (D0), then at one-week intervals i.e. on days D8, D15, D22, and thereafter at increasing intervals on days D36, D59, D84, D123, D263, D331 and D367.

A wooden frame identical in size to the experimental cages with x (letters A-N) and y (numbers 1-8) coordinates was placed on the ground at each site to ensure random sampling based on the coordinates. Before sampling, coordinates for 10 subsamples per plot were selected with raffles, avoiding re-sampling the same place. Subsamples were taken with a bulb planter to a depth of 10 cm, pooled and mixed to obtain one soil sample from each plot at each sampling day. Samples were stored at 4 °C until further processing.

2.3. Chemical analyses

Chemical analyses were conducted at the University of Neuchâtel, Switzerland.
Soil water pH was measured with a pH metre after diluting the sample in water in a 1:2.5 proportion (Pansu and Gautheyrou, 2006).
Ammonium and nitrate analyses were performed directly after sampling using colorimetric determination (Scheiner, 2005). Total nitrogen and carbon were

determined using a CHN analyser (Thermo Finnigan Flash EA 1112) on dry, ground soil. Bioavailable phosphorus content was determined by colorimetric analysis according to the Olsen method (Olsen et al., 1954). Cations (K^+, Mg^{2+} and Ca^{2+},) contents were determined using inductively coupled plasma optical emission spectrometry (Perkin-Elmer Optima 3300 DV ICP-OES) preceded by a cation exchange capacity extraction (CEC, Cobaltihexamine method).

2.4. Grouping of chemical markers

Potential chemical soil markers were classified into the categories described below. To be grouped into one of the categories a chemical marker has to be significantly different from both control treatments (control and fake) in at least one cadaver treatment (ground or hanging).

(1) early peak markers (EPM) that show significantly higher concentrations in the soil beneath cadavers when compared to the controls at a certain point relatively early in the decomposition process (until the end of greatest cadaver mass loss and the end of the main purge of cadaveric fluids).

(2) late peak markers (LPM) that show significantly higher concentrations in the soil beneath cadavers when compared to the controls at a certain point relatively late in the decomposition process i.e. not before the dry and remains stage.

(3) late decrease markers (LDM) that show significantly lower concentrations in the soil beneath cadavers when compared to the controls at a certain point relatively late in the decomposition process i.e. not before the dry and remains stage.

In the case where peaks or decreases are followed by a relatively fast decrease/increase and levels discontinue to be significantly higher or lower than the controls, markers are named EPM, LPM, LDM without any addition. In the case where peaks or decreases continue to be significantly higher/lower than the controls over a certain period of time either (+) EL (elevated levels) or (-) RL (reduced levels) are added. If possible, the duration of EL or RL should be defined. Depending on their pattern chemical markers are applied to one or more groups.

2.5. Data analyses

The duration of each decomposition stage was tested according to treatment (t-test adjusted according to Holm) to determine whether the length of the decomposition stages differ between hanging and ground pigs.

To test the significance of difference between treatments at each sampling day and overall, we used analysis of variance (ANOVA) and Tukey post hoc analysis (TukeyHSD). We assessed the significance over time using one- way ANOVA with repeated measure and post hoc multiple comparison of means (Tukey contrasts) with Bonferroni adjusted p-value. To follow the parametric assumptions of a normal distribution, variables were transformed (log 10 or square root) before the analyses.

Direct gradient analysis was made with a redundancy analysis (RDA) on previously transformed and standardised variables, with day and treatment as explanatory variables. This analysis allows to summarise the variation of the chemical variables and to interpret the evolution of soil characteristics over time and within treatments.

All statistical analyses were performed with R statistical software (version 3.1.0) (R Core Team, 2016), and packages vegan, version 2.4.1 (Oksanen et al., 2016), nlme, version 3.1-128 (Pinheiro et al., 2016), multcomp, version 1.4-6 (Hothorn et al., 2008) and lme4, version 1.1-12 (Bates et al., 2015).

3. Results

3.1. Decomposition stages and weight loss

At the end of the experiment (day 367) four of the ground pigs and one of the hanging pigs had reached the remains stage, while one of the ground and four of the hanging pigs were still in the dry stage. For the ground pigs the bloated stage lasted on average twice as long as for the hanging cadavers (i.e. eight vs. four days; $p < 0.05$, t-test, adjusted p-value according to Holm). However, the active decay stage was significantly longer in the hanging cadavers ($p < 0.01$, t-test, adjusted p-value according to Holm) (Fig. 1).

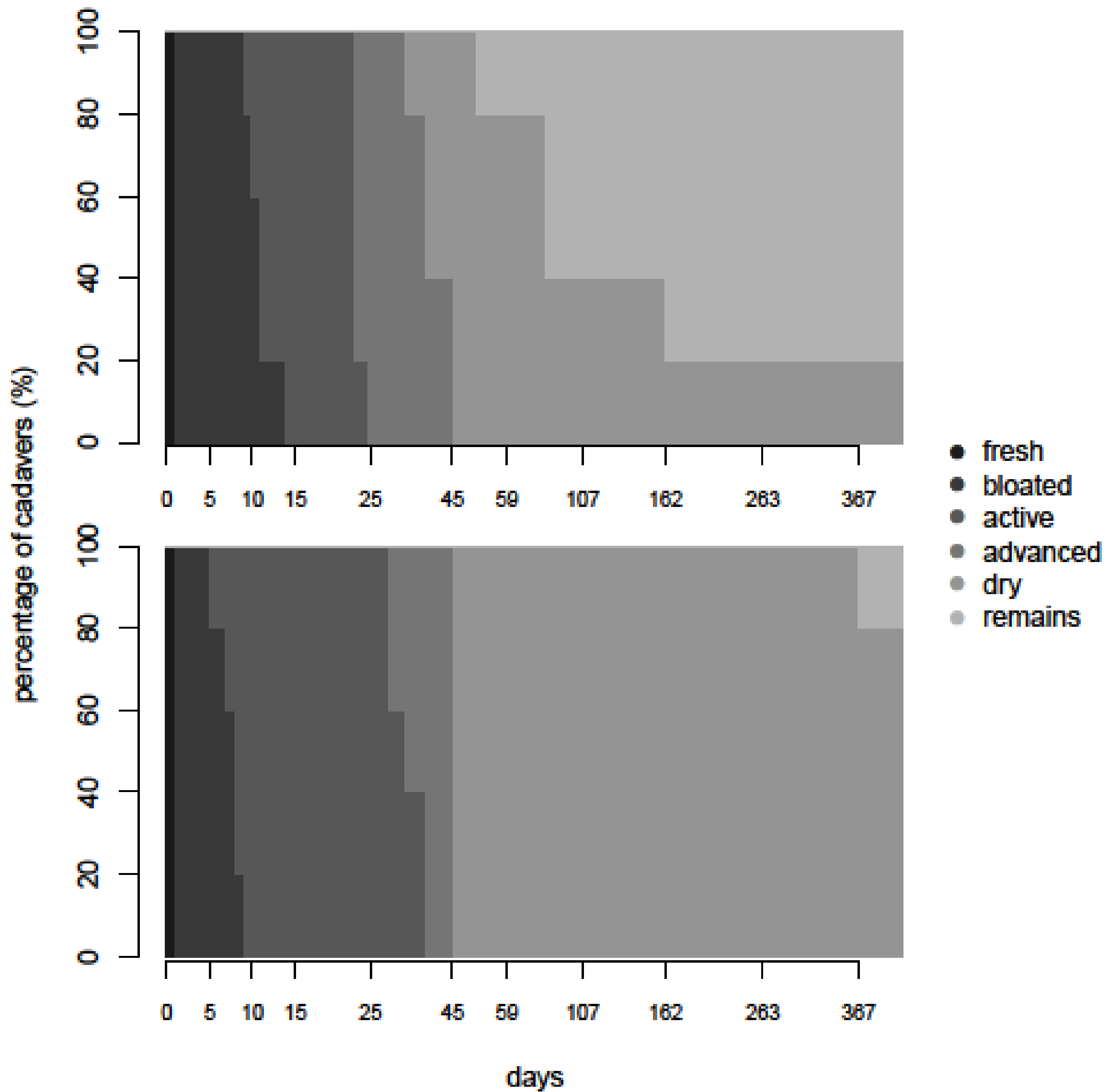

Figure 1. Duration of decomposition stages, and percentage of cadavers representing a given decomposition stage in the ground (top) and hanging pig (bottom) cadaver treatments over time at the Bois-du-Clos spruce forest experimental site (Neuchâtel, Switzerland). Decomposition stages are shown in different shades of grey.

Cadaver mass loss followed a sigmoidal pattern with the greatest mass loss before day 59. At this point all cadavers had gone through the advanced decay stage with only bones and dry skin left. The mass loss from day 59 onwards was more or less stable until the end of the experiment (Fig. 2).

3.2. Soil pH

The pH in soil beneath the control and fake pigs fluctuated over the one-year period ranging from 5.05 to 7.02 (controls) and 4.71 to 6.50 (fake pigs) (Table 1). In contrast, pH beneath the ground pigs increased by 4.13 units (ranging from 4.63 to 8.76) and was significantly different in comparison to the control and fake pig samples from days 15 to 36 ($p<0.05$, ANOVA, TukeyHSD) (Table 1, Fig. 3). Additionally, it was significantly higher to the hanging pig samples on day 22 ($p<0.05$, ANOVA, TukeyHSD). This increase was followed by a decrease reaching significantly lower pH values as compared to the control from day 263 to day 367 ($p<0.05$, ANOVA, TukeyHSD) (Fig. 3). In comparison, the increase in pH in the hanging samples (ranging from 4.68 to 8.70) at the beginning of the experiment was weaker, but the decrease towards the end of the experiment was also significant (day 263 to 367) when compared to the control and fake pig treatment ($p<0.05$, ANOVA, TukeyHSD) (Table 1, Fig. 3).

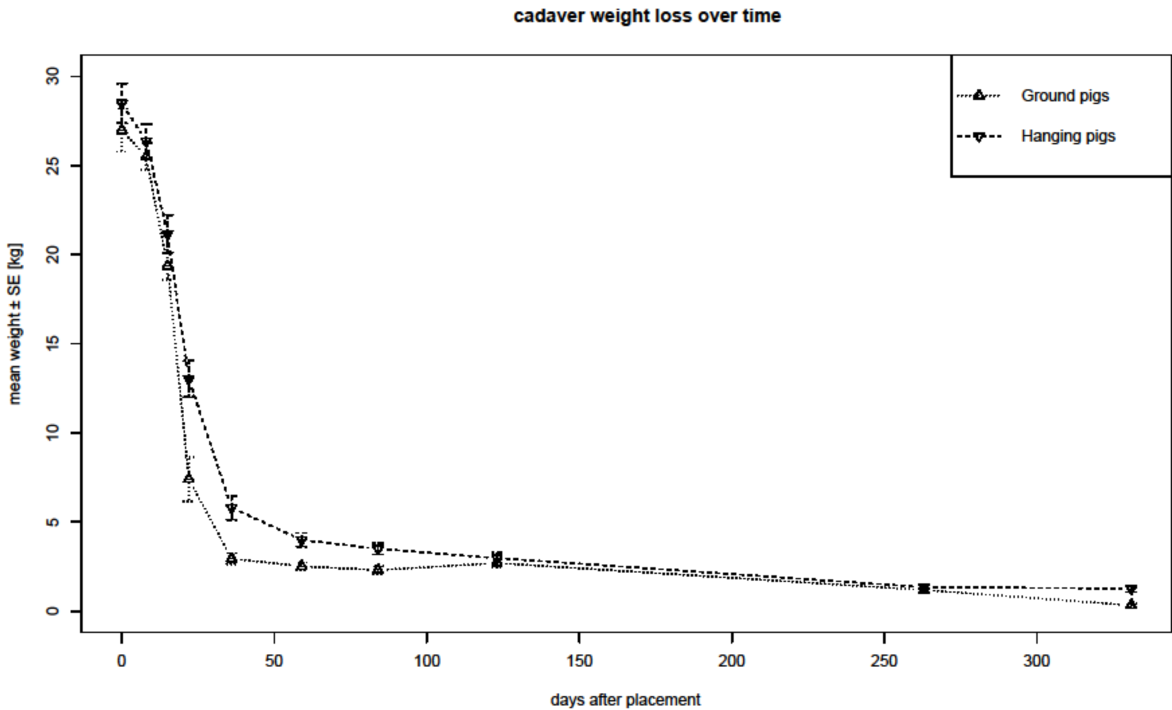

Figure 2. Average cadaver weight loss ± SE [kg] in the ground and hanging pig cadaver treatments over time at the Bois-du-Clos spruce forest experimental site (Neuchâtel, Switzerland).

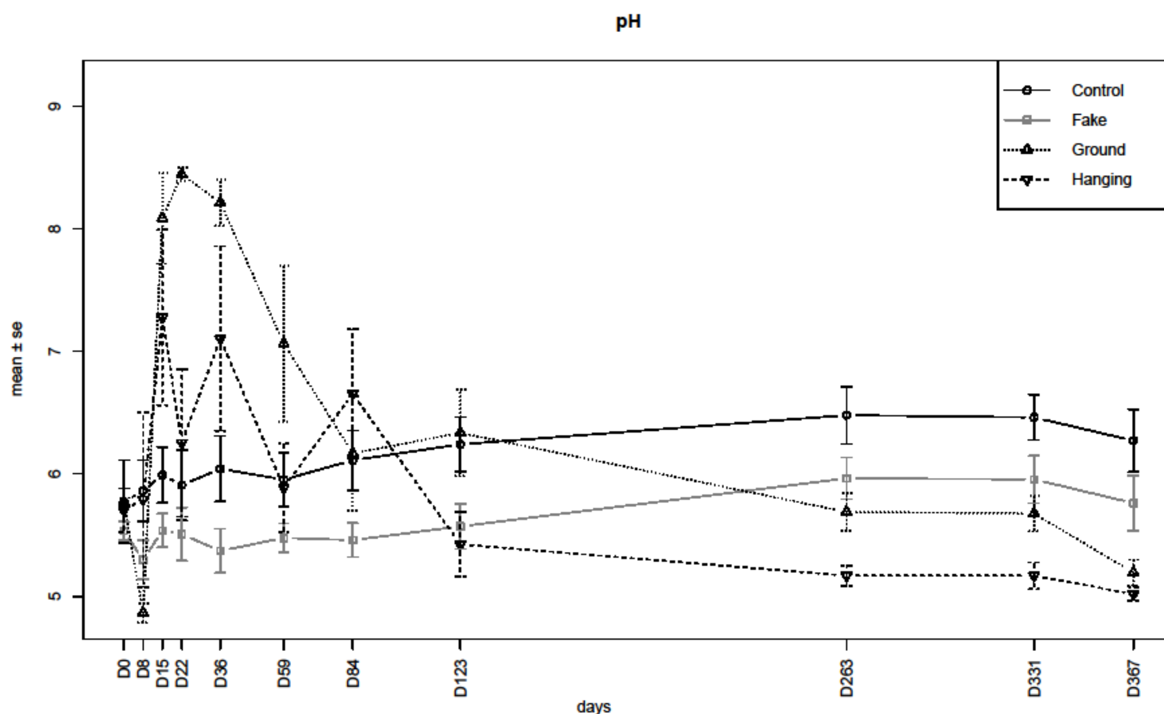

Figure 3. Average pH ± SE in the control, fake pig, ground pig and hanging pig treatments over time at the Bois-du-Clos spruce forest experimental site (Neuchâtel, Switzerland).

3.3. Ammonium (NH_4^+)

Ammonium content in the soil of the control and fake pig samples ranged from 0.92 to 50.57 µg g^{-1} in the control and 1.0 to 62.51 µg g^{-1} in the fake pig samples (Table 1). There was a massive and significant increase in Ammonium content in the ground (ranging from 1.98 to 1561.78 µg g^{-1}) and hanging pig samples (ranging from 0.64 to 1124.71 µg g^{-1}) from day 15 to 123 with a peak on day 59 in contrast to both controls ($p<0.0001$, ANOVA, TukeyHSD) (Fig. 4). Ammonium content returned to basal levels towards the end of the experiment with no significant differences between treatments on days 263, 331 and 367 ($p>0.05$, ANOVA, TukeyHSD) (Fig. 4). Overall ammonium content differed significantly between cadaver treatments and controls ($p<0.0001$, ANOVA, TukeyHSD) but not between hanging and ground pigs or between fake and control ($p>0.5$, ANOVA, TukeyHSD).

3.4. Nitrate (NO_3^-)

Soil nitrate content ranged from 3.12 to 57.26 µg g^{-1} in the control samples, from 3.36 to 235.89 µg g-1 in the fake pig samples and from 3.70 to 321.97 µg g^{-1} in the ground and 3.67 to 164.35 µg g^{-1} in the hanging pig samples (Table 1).

Although fluctuations were observed, no significant differences were recorded between the treatments until day 263 (Fig. 5). Ground pig samples were significantly different from both controls on days 263 and day 367 ($p<0.01$, ANOVA, TukeyHSD) and hanging pig samples accordingly on days 263, 331 and 367 ($p<0.05$, ANOVA, TukeyHSD) (Fig. 5). Overall nitrate content differed significantly between cadaver treatments and controls ($p<0.01$, ANOVA, TukeyHSD) but not between hanging and ground pigs or between fake and control ($p>0.4$, ANOVA, TukeyHSD).

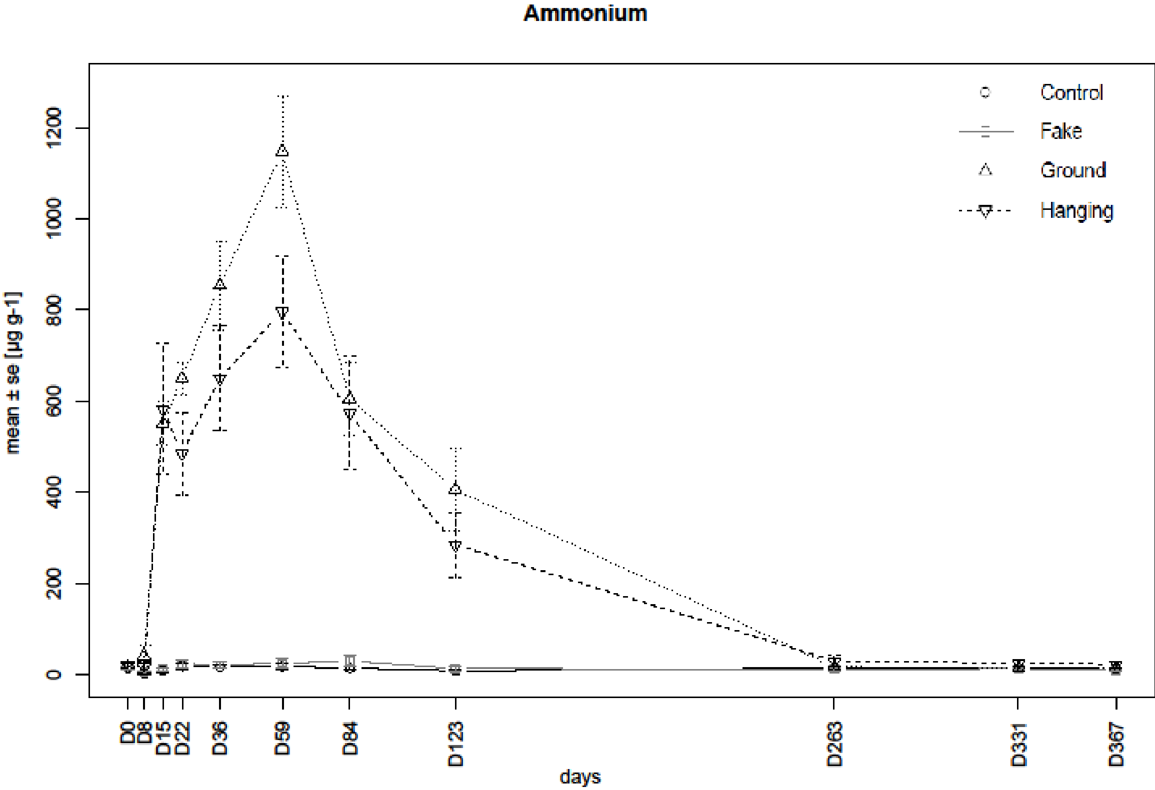

Figure 4. Average Ammonium (NH_4^+) content ± SE [µg g^{-1}] in the control, fake pig, ground pig and hanging pig treatments over time at the Bois-du-Clos spruce forest experimental site (Neuchâtel, Switzerland).

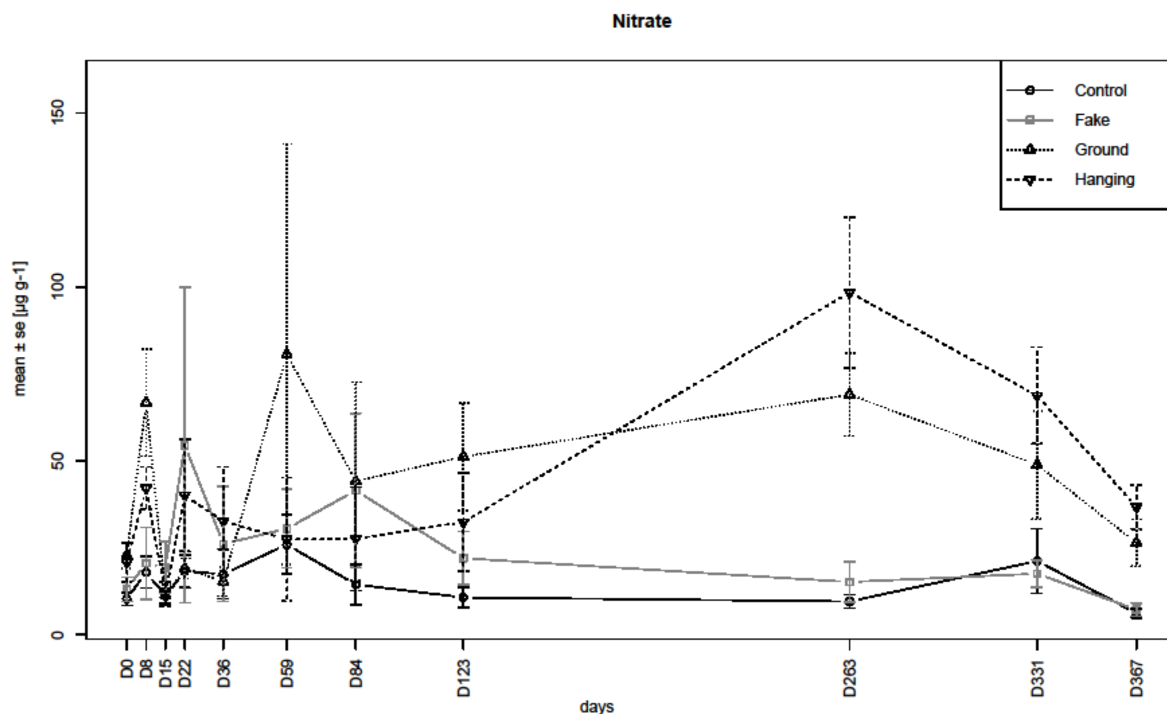

Figure 5. Average Nitrate (NO_3^-) content ± SE [µg g^{-1}] in the control, fake pig, ground pig and hanging pig treatments over time at the Bois-du-Clos spruce forest experimental site (Neuchâtel, Switzerland).

3.5. Nitrogen (N)

Total nitrogen content ranged from 0.45 to 1.95 % in the control, 0.31 to 1.55 % in the fake, 0.58 to 1.81 % in the ground and 0.57 to 2.78 % in the hanging pig treatment (Table 1). In the soil samples from beneath the ground and hanging pigs nitrogen content increased at the beginning of the experiment and was significantly higher as compared to both controls on days 15 and 22 ($p<0.05$, ANOVA, TukeyHSD) (Fig. 6). Nitrogen content in the cadaver samples stayed above the controls until day 331, not significantly and without any clear pattern (Fig. 6). Overall nitrate content differed significantly between cadaver treatments and controls ($p<0.0001$, hanging and ground pigs or between fake and control ($p>0.6$, ANOVA, TukeyHSD).

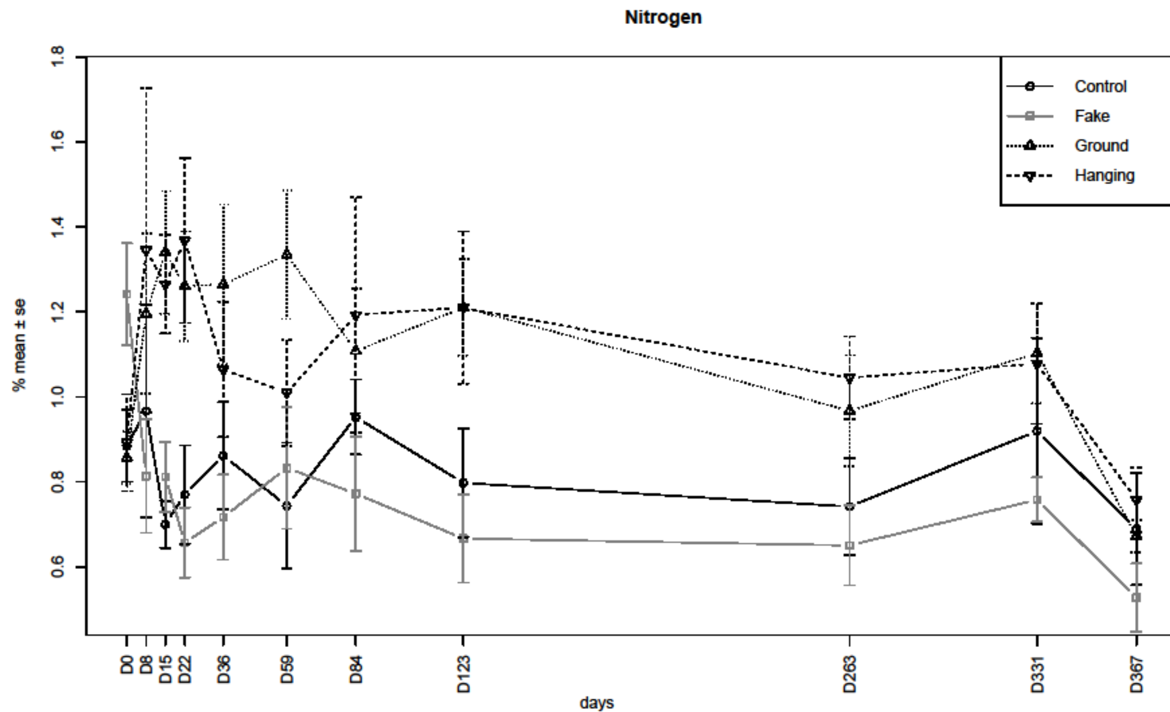

Figure 6. Average total Nitrogen (N) concentration ± SE [%] in the control, fake pig, ground pig and hanging pig treatments over time at the Bois-du-Clos spruce forest experimental site (Neuchâtel, Switzerland).

3.6. Carbon (C)

Soil carbon content ranged from 8.51 to 36.54 % in the control, 5.8 to 35.31 % in the fake, 9.01 to 31.97 % in the ground and 8.78 to 36.68 % in the hanging pig treatment (Table 1). No significant differences between the four sets of samples were observed on any of the sampling days ($P > 0.05$, ANOVA, TukeyHSD; Fig. 7).

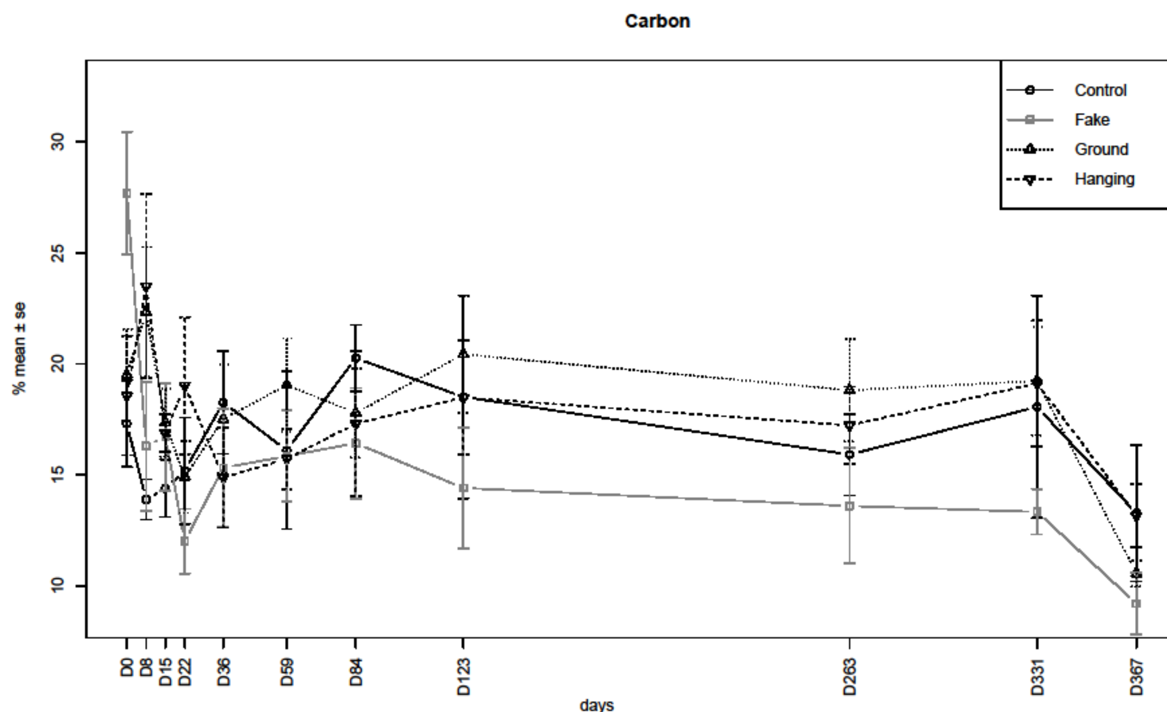

Figure 7. Average total Carbon (C) concentration ± SE [%] in the control, fake pig, ground pig and hanging pig treatments over time at the Bois-du-Clos spruce forest experimental site (Neuchâtel, Switzerland).

3.7. Bioavailable Phosphorous (P_{bio})

Bioavailable phosphorous content in soil ranged from 4.64 to 110.86 µg g^{-1} in the control and from 0.56 to 114.41 µg g^{-1} in the fake pig samples and varied slightly over the course of the experiment. In the ground and hanging pig samples it ranged from 10.96 to 1105.30 µg g^{-1} and 13.77 to 724.42 µg g^{-1} respectively (Table 1).

At the beginning of the experiment (day 15), there was a massive and significant increase in phosphorous content in both cadaver samples with a first peak on D15 and a second peak on D 36 (ground pigs) and 84 (hanging pigs) ($p<0.0001$, ANOVA, TukeyHSD; Fig. 8). Although phosphorous decreased again after the second peaks, the content stayed significantly higher until the end of the experiment (day 367) ($p<0.01$, ANOVA, TukeyHSD; Fig. 8). Overall phosphorous content differed significantly between cadaver treatments and controls ($p<0.0001$, ANOVA, TukeyHSD) but not between hanging and ground pigs or between fake and control ($p>0.5$, ANOVA, TukeyHSD).

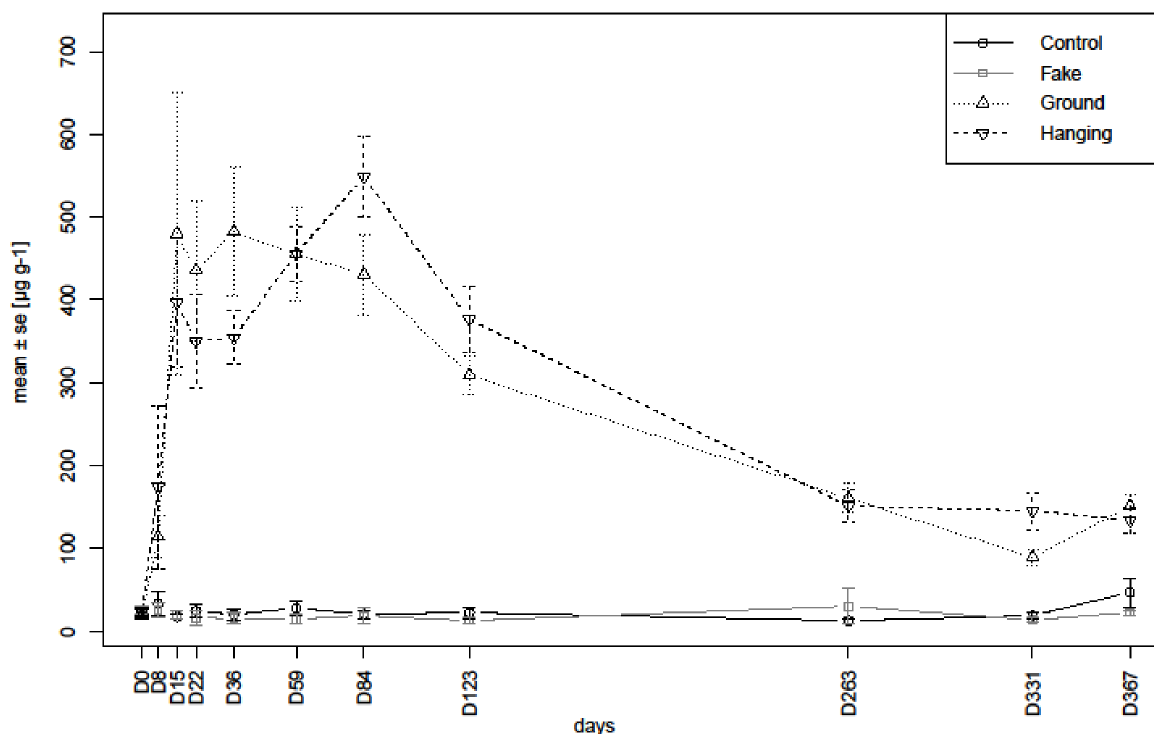

Figure 8. Average bioavailable Phosphorous (P_{bio}) content ± SE [µg g^{-1}] in the control, fake pig, ground pig and hanging pig treatments over time at the Bois-du-Clos spruce forest experimental site (Neuchâtel, Switzerland).

3.8. Potassium (K^{+}), Magnesium (Mg^{2+}) and Calcium (Ca^{2+}) (exchangeable cations)

Potassium concentrations in soil ranged from 0 to 2.2 cmolc kg^{-1} in the control, 0 to 0.34 cmolc kg^{-1} in the fake, 0 to 30.76 cmolc kg^{-1} in the ground and 0 to 22.93 cmolc kg^{-1} in the hanging pig treatment (Table 1). Potassium content in the control and fake pig samples did not change over the course of the experiment (Fig. 9a). However, it increased in the ground and hanging pig samples at the beginning of the experiment and was significantly different from both controls from day 36 until day 59 ($p<0.05$ ANOVA, TukeyHSD). Overall potassium content was significantly different between cadaver treatments and controls ($p<0.001$, ANOVA, TukeyHSD) but not between hanging and ground pigs or between fake and control ($p>0.9$, ANOVA, TukeyHSD).

Magnesium content ranged from 0 to 9.5 cmolc kg^{-1} in the control, 0 to 11.62 cmolc kg^{-1} in the fake, 0 to 14.22 cmolc kg^{-1} in the ground and 0.46 to 14.84 cmolc kg^{-1} in the hanging pig treatment (Table 1). Small fluctuations were observed in all treatments without differences between treatments on the sampling days (Fig. 9b).

Table 1. Chemical components in the control, fake pig, ground pig and hanging pig treatments over the course of the experiment at the Bois-du-Clos spruce forest experimental site (Neuchâtel, Switzerland) showing minimum value (min), maximum value (max), range between min and max, mean and standard error (SE).

	control					fake pig					ground pig					hanging pig				
	min	max	range	mean	se	min	max	range	mean	se	min	max	range	mean	se	min	max	range	mean	se
pH	5.05	7.02	1.97	6.1	0.08	4.71	6.5	1.79	5.58	0.05	4.63	8.76	4.13	6.5	0.18	4.68	8.7	4.02	5.96	0.16
NH_4^+ [µg g^{-1}]	0.92	50.57	49.65	12.57	1.4	1	62.51	61.51	16.04	2.03	1.98	1561.78	1559.8	391.88	54.84	0.64	1124.71	1124.07	316.7	45.88
NO_3^- [µg g^{-1}]	3.12	57.26	54.14	14.82	1.63	3.36	235.89	232,53	24.52	5.07	3.7	321.97	318.27	41.42	6.8	3.67	164.35	160.68	39.87	4.85
N [%]	0.45	1.95	1.5	0.82	0.04	0.31	1.55	1.24	0.77	0.04	0.58	1.81	1.24	1.12	0.05	0.57	2.78	2.22	1.11	0.06
C [%]	8.51	36.54	28.03	16.51	0.85	5.8	35.31	29.52	15.53	0.87	9.01	31.97	22.96	17.96	0.71	8.78	36.68	27.9	17.62	0.78
P [µg g^{-1}]	4.64	110.86	106.22	24.39	2.64	0.58	114.41	113.84	19.89	2.5	10.98	1105.3	1094.33	284.29	29.58	13.77	724.42	710.65	283.03	25.11
K [cmol$_c$ kg^{-1}]	0	2.2	2.2	0.08	0.05	0	0.34	0.34	0.01	0.01	0	30.76	30.76	2.78	0.66	0	22.93	22.93	2.59	0.55
Mg [cmol$_c$ kg^{-1}]	0	9.5	9.5	3.33	0.3	0	11.62	11.62	3.34	0.3	0	14.22	14.22	4.37	0.34	0.46	14.84	14.38	4.41	0.38
Ca [cmol$_c$ kg^{-1}]	14.58	98.31	83.73	42.92	2.85	5.19	92.4	87.21	38.15	2.51	0	83.68	83.68	30.74	1.76	12.1	139.96	127.86	35.98	2.96

content ranged from 14.58 to 98.31 cmolc kg^{-1} in the control, 5.19 to 92.4 cmolc kg^{-1} in the fake, 0 to 83.68 cmolc kg^{-1} in the ground and 12.1 to 139.98 cmolc kg^{-1} in the hanging pig treatment (Table 1).

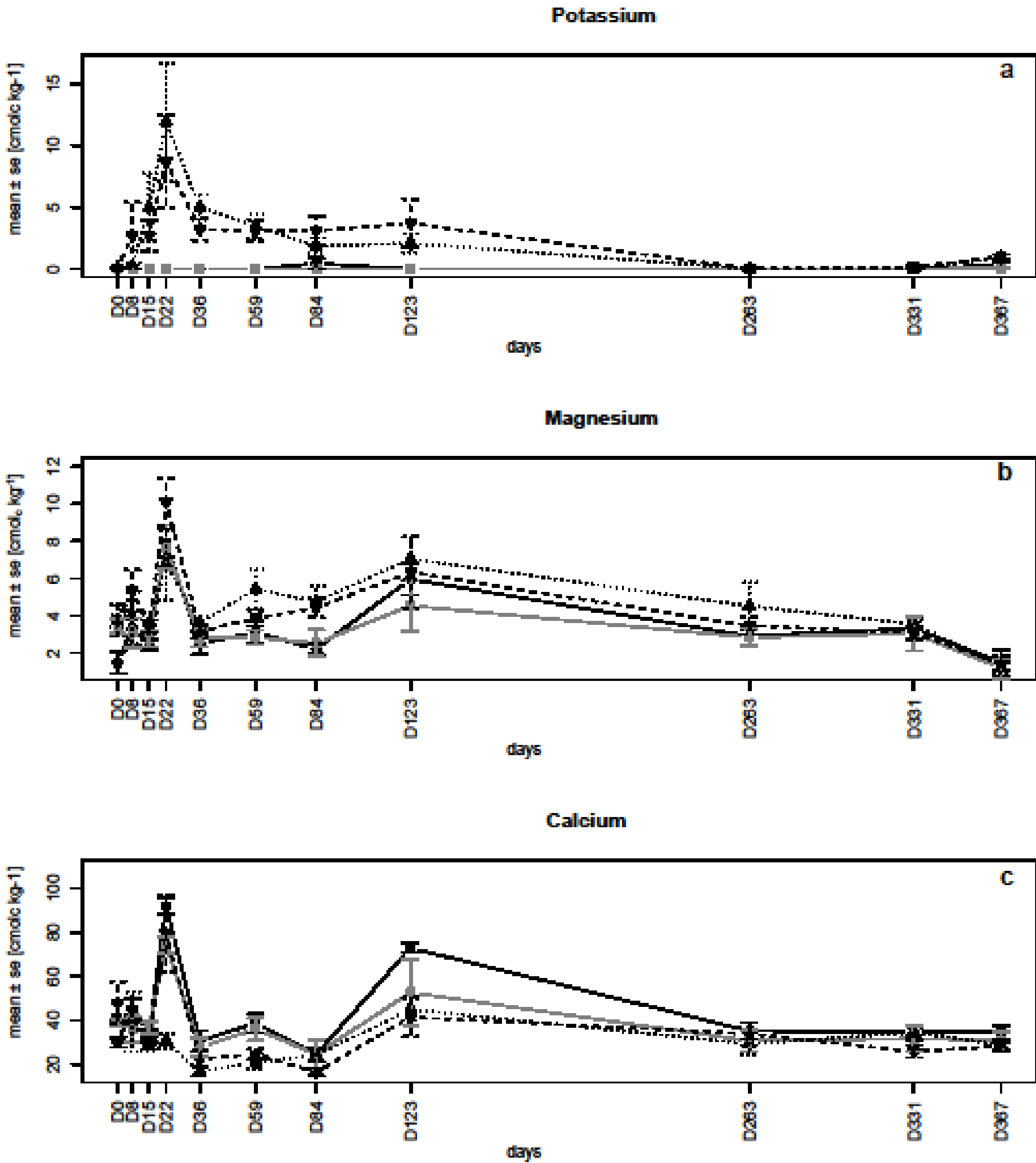

Figure 9. Average Potassium (K^{+}) (a), Magnesium (Mg^{2+}) (b) and Calcium (Ca^{2+}) (c) content ± SE [$cmol_c$ kg^{-1}= centimoles of charge per kg of soil] in the control, fake pig, ground pig and hanging pig treatments over time at the Bois-du-Clos spruce forest experimental site (Neuchâtel, Switzerland).

Calcium content on day 22 ($p > 0.05$, ANOVA, TukeyHSD) was lower in the ground pig treatment and significantly different from all other treatments (Fig. 9c). Overall the ground pig treatment differed significantly from the control ($p<0.01$, ANOVA, TukeyHSD).

3.9. Redundancy analysis (RDA)

The RDA on all the chemical variables (response variables) and all time points (sampling days) with time and treatment as explanatory variables showed a clear difference between the cadaver treatments and the controls (axis 1) as well as temporal changes (axes 1 and 2) (Fig. 10). The ground and hanging pig samples diverged from the control samples from T1 onwards (Fig. 10). Variables most strongly correlated with axis 1 and thus best explaining the difference between cadaver and control samples were P, NH_4^+, total N and K^+. Ca^{2+} was the only variable negatively correlated with the cadaver samples, but weakly so. Starting from T8 - T10 (day 263-367), cadaver-impacted samples started to converge back towards the control and fake pig samples. However, by T10 they clearly remained different, owing mainly to higher nitrate and lower calcium concentrations than under control and fake pigs.

3.10. Grouping according to EPM, LPM and LDM

Nine chemical soil markers (pH, NH_4^+, NO_3^-, N, C, P, K^+, Mg^{2+}, Ca^{2+}) were investigated in all treatments and at all time points.

The turning point from early (< /= day 59) to late markers (> day 59 - < /= day 367) in our study is two months after the cadavers were placed which is after the greatest mass loss (Fig. 2) and the end of the main pulse of cadaveric fluids into the soil (after advanced decay) (Fig. 3).

Based on significant differences between controls and cadaver treatments, chemical markers were grouped into three categories: early peak markers (EPM), late peak markers (LPM) and late decrease markers (LDM) (Table 2). As each chemical marker could be attributed to more than one category, in this analysis five groups of markers could be identified:

(1) Nitrogen and potassium were classified as EPMs alone.

(2) Nitrate was the only LPM.

All other chemical markers belonged to more than one category.

(3) pH belongs to the EPMs first and then to LDMs towards the end of the experiment.

(4) Ammonium is in the EPM category but with continuing elevated levels (ELs)

(5) Phosphorous is as well EPM and LPM also followed by ELs.

Magnesium, calcium and carbon could not be grouped.

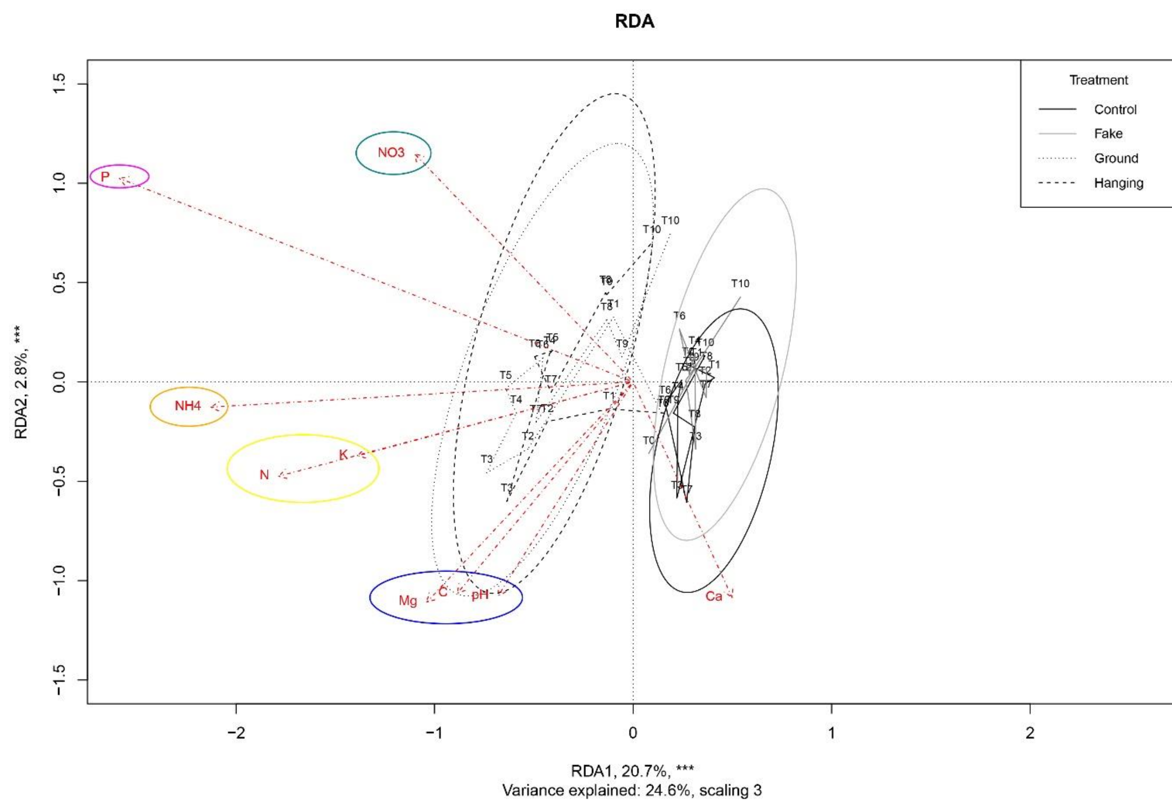

Figure 10. Redundancy analysis (RDA) ordination diagram showing the response of soil chemistry according to treatment (control, fake pig, ground pig and hanging pig treatments) and time in a spruce forest at the Bois-du-Clos experimental site (Neuchâtel, Switzerland). The lines (solid black: control; solid grey: fake; dotted: ground pig; dashed: hanging pig) join the centroids of the five replicates from each sampling day. T0 to T10 represent the mean coordinates of the 5 replicates per treatment (numbers indicating the time since death in days). For better readability D0 was represented by T0, D8 by T1, D15 by T2 and so forth until D367 by T10. Arrows represent the chemical i.e. explanatory variables. The grouping of chemical markers is indicated by different colours: EPM (yellow), LPM (green), EPM+ LDM (blue), EPM+EL (orange) and EPM+LPM+ELs (pink).

Table 2. Grouping of chemical components into EPM (yellow), LPM (green), EPM+ LDM (blue), EPM+EL (orange) and EPM+LPM+ELs (pink).

	T0	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	
days	0	8	15	22	36	59	84	123	263	331	367	Figures
pH	x	x	EPM	EPM	EPM	x	x	x	LDM	LDM	LDM	Fig. 3
NH4+	x	x	EPM	EPM	EPM	EPM	(+) EL	(+) EL	x	x	x	Fig. 4
NO3-	x	x	x	x	x	x	x	x	LPM	LPM	LPM	Fig. 5
N	x	x	EPM	EPM	x	x	x	x	x	x	x	Fig. 6
C	x	x	x	x	x	x	x	x	x	x	x	Fig. 7
P	x	x	EPM	EPM	EPM	EPM	LPM	(+) EL	(+) EL	(+) EL	(+) EL	Fig. 8
K	x	x	x	x	EPM	EPM	x	x	x	x	x	Fig. 9_a
Mg	x	x	x	x	x	x	x	x	x	x	x	Fig. 9_b
Ca	x	x	x	x	x	x	x	x	x	x	x	Fig. 9_c

4. Discussion

In both cadaver treatments mass loss followed a sigmoidal pattern in line with the classical pattern of breakdown of cadaver tissue and release of fluids taking place at the beginning of the decomposition process (Carter et al., 2007; Spicka et al., 2011). The longer active decay stage in the hanging pigs was due to a lower insect activity (especially beetles) on the hanging pigs (unpublished data) and the continuous dripping and loss of maggot masses from the hanging cadavers. However, overall in this study soil chemistry between ground and hanging pigs did not reveal significant differences.

At the beginning of the experiment (>D15) soil pH, NH_4^+, N, P and K^+ (EPMs) increased in at least one of the two cadaver treatments. On day 15 all cadavers were in the active decay stage, skin was ruptured and cadaveric fluids were released into the soil. Due to the degradation of proteins, lipids and carbohydrates from the vertebrate cadaver, carbon-, nitrogen and phosphorous- based products are yielded into the soil below (Stokes et al., 2009b).

During these processes an increase of soil pH in our study was observed beneath the ground pigs causing an increase in soil pH as compared to the controls. In previous studies, soil pH has been shown to either decrease and increase beneath human and other mammal remains (Aitkenhead-Peterson et al., 2012; Benninger et al., 2008). In our study the increase of pH is probably due to an accumulation of ammonium- ions that follow the same pattern as shown by Benninger et al. (2008). Therefore, pH and NH_4^+ can be regarded as EPMs. It is suggested that during and after the release of cadaveric fluids the soil beneath the cadavers becomes more and more anoxic for a while, which would explain why NH_4^+ ions were not further nitrified (Aitkenhead-Peterson et al., 2015).

Although pH beneath the hanging pigs was also elevated at the beginning, it did not reach the significant values from the ground pig treatment. The dripping of the fluids and maggot masses probably did not cause a complete anoxic environment and did not cover the area beneath the cadaver completely, so nitrification took place to some extent. The significant decrease of pH towards the end of the experiment in both cadaver treatments is line with the decline of NH_4^+ after > 2 months and an increase of NO_3^-. This groups pH additionally into LDMs and NO_3^- into LPMs. It suggests that aerobic soil conditions take place again allowing aerobic nitrification after an initial lag phase (Aitkenhead-Peterson et al., 2015; Stokes et al., 2013). This follows a pattern shown by Meyer at al. (2013) for $NH_4^+$and NO^{3-}, who suggested that ammonification is the dominant process up to advanced decay and nitrification after advanced decay. Significantly elevated NO_3^- were described after one and three years beneath decomposing pig cadavers (Anderson et al., 2013).

In our study, total nitrogen (EPM) increased two and three weeks after the beginning of the experiment in the cadaver treatments. Similar findings were observed by Benninger et al. (2008) showing an increase of total N in the first 14 days of the decomposition trial and smaller peaks between days 21 and 42, and could be either the influx of organic or inorganic nitrogen forms. This is not surprising as a cadaver is a rich source for nitrogen for instance 26g kg^{-1} N concentration is reported for pigs (Benninger et al., 2008). The main N moiety from cadavers derives from the breakdown of proteins, this process does not occur at a uniform rate and the degradation products can be released over a longer time- span including more decomposition stages (Macdonald et al., 2014). It might not be straightforward to group N into EPMs alone

because other studies have shown that total N was significantly higher after one year beneath decomposing pigs (Anderson et al., 2013; Parmenter and MacMahon, 2009). Here more data will be necessary.

The input of phosphorous from the cadaver, where P is stored in proteins, coenzymes, sugar phosphates and phospholipids (Dent et al., 2004), may translate into a large increase in soil as available phosphorous (Perrault and Forbes, 2016). In our study, bioavailable phosphorous peaks at the beginning of the experiment (EPM) but also on day 84 (LPM) and shows significantly elevated levels until the end of the experiment (+) EL in the cadaver treatments when compared to the controls. Therefore, it cannot be assigned to just one category. Various studies confirm our results: The presence of a double peak was also noted by Benninger et al. (2008) and Perrault and Forbes (2016). Additionally MacDonald et al. (2014) also described a significant and lasting increase in plant available P control 12 and 24 weeks after carcass addition and extractable P concentrations are described to be higher at carcass-impacted sites than in the surrounding soil one and three years post-mortem (Towne, 2000). P concentrations seem to be a good indicator for either locating the decomposition of remains (Perrault and Forbes, 2016) and for the estimation of longer-term PMIs.

Potassium is also grouped into the EPMs. Assuming that 100 g of pig body tissue contain approximately 280 mg Potassium (Spray and Widdowson, 1950) being released into the soil relatively early in the decomposition process when tissues are broken down. Elevated K levels were also reported in Aitkenhead-Peterson et al. (2012) and Stokes et al. (2013) beneath decomposing cadavers and buried skeletal muscle tissues respectively.

The contents of calcium and magnesium in 100 g of pig body tissue are 1200 mg and 45 mg respectively (Spray and Widdowson, 1950). Although this is released into the soil during decomposition, no clear pattern of peaks or decrease are observed in this study. Therefore, Ca and Mg are not grouped into the proposed categories. As shown by Melis et al. (2007) no differences in Ca concentration were observed during the first year after death but three years after death Ca concentrations were higher at the carcass centre. It is suggested that Ca will be mainly released by bones and probably increase at later time points (Melis et al., 2007). Ca might be a good LPM which could be investigated in a longer-term study (> 1 year).

Although carbon accounts for 20% of the mass of cadavers (Carter et al., 2007) no significant changes were observed in the soil beneath the cadavers, which is in line with other studies (Anderson et al., 2013; Benninger et al., 2008; Meyer et al., 2013). One reason for this might be that the intense pulse of C input caused an increase in micro-organisms that utilize carbon and then release CO_2 into the atmosphere via respiration. Nevertheless, results are conflicting and some studies describe significant increases in total carbon beneath decomposing cadavers (Macdonald et al., 2014).

5. Conclusion

The results from this and other studies indicate that it might be possible to categorize soil chemical markers according to their response pattern to decomposition products over time. As this is the first attempt to group cadaver-impacted soil chemical markers, we correlated the changes to decomposition stages and weight loss of the cadavers. A grouping into defined markers can be highly useful when the changes in soil chemistry are related to changes in the composition of soil organism communities. When applied in a forensic context a marker that shows clear and high peaks and/ or decreases for a short period of time might be more useful than a marker that has elevated levels over a longer time-span to estimate the PMI.

Future studies and the inclusion of results from other research groups should help refine and test the applicability of these categories.

6. References

[1] Aitkenhead-Peterson, J.A., Alexander, M.B., Bytheway, J.A., Carter, D.O., Wescott, D.J., 2015. Applications of Soil Chemistry in Forensic Entomology. In: Tomberlin, J.K., Benbow, M.E. (Eds.), Forensic Entomology: International Dimensions and Frontiers. CRC Press, Boca Raton, pp. 283–296.

[2] Aitkenhead-Peterson, J.A., Owings, C.G., Alexander, M.B., Larison, N., Bytheway, J.A., 2012. Mapping the lateral extent of human cadaver decomposition with soil chemistry. Forensic Science International 216, 127–134. doi:10.1016/j.forsciint.2011.09.007

[3] Anderson, B., Meyer, J., Carter, D.O., 2013. Dynamics of ninhydrin-reactive nitrogen and pH in gravesoil during the extended postmortem Interval. Journal of Forensic Sciences 58, 1348–1352. doi:10.1111/1556-4029.12230

[4] Barton, P.S., Cunningham, S.A., Lindenmayer, D.B., Manning, A.D., 2013. The role of carrion in maintaining biodiversity and ecological processes in terrestrial ecosystems. Oecologia 171, 761–772. doi:10.1007/s00442-012-2460-3

[5] Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67. doi:10.18637/jss.v067.i01

[6] Benninger, L.A., Carter, D.O., Forbes, S.L., 2008. The biochemical alteration of soil beneath a decomposing carcass. Forensic Science International 180, 70–75. doi:10.1016/j.forsciint.2008.07.001

[7] Campobasso, C.P., Di Vella, G., Introna, F., 2001. Factors affecting decomposition and Diptera colonization. Forensic Science International, Forensic Entomology 120, 18–27. doi:10.1016/S0379-0738(01)00411-X

[8] Carter, D.O., Tibbett, M., 2008. Cadaver decomposition and soil: processes. In: Tibett, M., Carter, D.O. (Eds.), Soil Analysis in Forensic Taphonomy. CRC Press, Boca Raton, pp. 29–52.

[9] Carter, D.O., Tibbett, M., 2006. Microbial decomposition of skeletal muscle tissue (*Ovis aries*) in a sandy loam soil at different temperatures. Soil Biology and Biochemistry 38, 1139–1145. doi:10.1016/j.soilbio.2005.09.014

[10] Carter, D.O., Yellowlees, D., Tibbett, M., 2010. Moisture can be the dominant environmental parameter governing cadaver decomposition in soil. Forensic Science International 200, 60–66. doi:10.1016/j.forsciint.2010.03.031

[11] Carter, D.O., Yellowlees, D., Tibbett, M., 2008. Temperature affects microbial decomposition of cadavers (*Rattus rattus*) in contrasting soils. Applied Soil Ecology 40, 129–137. doi:10.1016/j.apsoil.2008.03.010

[12] Carter, D.O., Yellowlees, D., Tibbett, M., 2007. Cadaver decomposition in terrestrial ecosystems. Naturwissenschaften 94, 12–24. doi:10.1007/s00114-006-0159-1

[13] Clark, M.A., Worrell, M.B., Pless, J.E., 1997. Postmortem changes in soft tissue. In: Haglund, W.D., Sorg, M.H. (Eds.), Forensic Taphonomy: The Postmortem Fate of Human Remains. CRC Press, Boca Raton, pp. 151–164.

[14] Dent, B.B., Forbes, S.L., Stuart, B.H., 2004. Review of human decomposition processes in soil. Environmental Geology 45, 576–585. doi:10.1007/s00254-003-0913-z

[15] DeVault, T.L., Rhodes, Jr., O.E., Shivik, J.A., 2003. Scavenging by vertebrates: behavioral, ecological, and evolutionary perspectives on an important energy transfer pathway in terrestrial ecosystems. Oikos 102, 225–234. doi:10.1034/j.1600-0706.2003.12378.x

[16] Forbes, S.I., 2008. Decomposition chemistry in a burial environment. In: Tibbett, M., Carter, D.O. (Eds.), Soil Analysis in Forensic Taphonomy: Chemical and Biological Effects of Buried Human Remains. CRC Press, Boca Raton, pp. 202–223.

[17] Gill-King, H., 1997. Chemical and ultrastructural aspects of decomposition. In: Haglund, W.D., Sorg, M.H. (Eds.), Forensic Taphonomy: The Postmortem Fate of Human Remains. CRC Press, Boca Raton, pp. 93–104.

[18] Goff, L.M., 2009. Early post-mortem changes and stages of decomposition in exposed cadavers. Experimental and Applied Acarology 49, 21–36. doi:10.1007/s10493-009-9284-9

[19] Haglund, W.D., Sorg, M.H., 1997. Introduction to forensic taphonomy. In: Haglund, W.D., Sorg, M.H. (Eds.), Forensic Taphonomy: The Postmortem Fate of Human Remains. CRC Press, Boca Raton, pp. 1–9.

[20] Haslam, T.C.F., Tibbett, M., 2009. Soils of contrasting pH affect the decomposition of buried mammalian (*Ovis aries*) skeletal muscle tissue. Journal of Forensic Sciences 54, 900–904. doi:10.1111/j.1556-4029.2009.01070.x

[21] Hopkins, D.W., Wiltshire, P.E.J., Turner, B.D., 2000. Microbial characteristics of soils from graves: an investigation at the interface of soil microbiology and forensic science. Applied Soil Ecology 14, 283–288. doi:10.1016/S0929-1393(00)00063-9

[22] Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biometrical Journal 50, 346–363.

[23] Macdonald, B.C.T., Farrell, M., Tuomi, S., Barton, P.S., Cunningham, S.A., Manning, A.D., 2014. Carrion decomposition causes large and lasting effects on soil amino acid and peptide flux. Soil Biology and Biochemistry 69, 132–140. doi:10.1016/j.soilbio.2013.10.042

[24] Matuszewski, S., Konwerski, S., Fratczak, K., Szafalowicz, M., 2014. Effect of body mass and clothing on decomposition of pig carcasses. International Journal of Legal Medicine 128, 1039–1048. doi:10.1007/s00414-014-0965-5

[25] Melis, C., Selva, N., Teurlings, I., Skarpe, C., Linnell, J.D.C., Andersen, R., 2007. Soil and vegetation nutrient response to bison carcasses in Bialowieza Primeval Forest, Poland. Ecological Research 22, 807–813. doi:10.1007/s11284-006-0321-4

[26] Meyer, J., Anderson, B., Carter, D.O., 2013. Seasonal variation of carcass decomposition and gravesoil chemistry in a cold (Dfa) climate. Journal of Forensic Sciences 58, 1175–1182. doi:10.1111/1556-4029.12169

[27] Oksanen, J., Guillaume Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2016. Package ´vegan´?: Community Ecology Package. R package version 2.4-1.

[28] Olsen, S.R., Cole, C.V., Watanabe, F.S., Dean, L.A., 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. United States Department of Agriculture, Circular No .939, 1-19.

[29] Pansu, M., Gautheyrou, J., 2006. Handbook of soil analysis: mineralogical, organic and inorganic methods. Springer, Berlin, New York.

[30] Parmenter, R.R., MacMahon, J.A., 2009. Carrion decomposition and nutrient cycling in a semiarid shrub—steppe ecosystem. Ecological Monographs 79, 637–661.

[31] Payne, J.A., 1965. A summer carrion study of the baby pig *Sus Scrofa* Linnaeus. Ecology 46, 592–602. doi:10.2307/1934999

[32] Perrault, K.A., Forbes, S.L., 2016. Elemental analysis of soil and vegetation surrounding decomposing human analogues. Canadian Society of Forensic Science Journal 49, 138–151. doi:10.1080/00085030.2016.1184840

[33] Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R Core Team, 2016. Package ´nlme´: Linear and nonlinear mixed effects models. R package version 3.1-128, http://CRAN.R-project.org/package=nlme>.

[34] R Core Team, 2016. R: A language and environment or statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

[35] Scheffer, F., Schachtschabel, P., Blume, H.-P., Thiele-Bruhn, S., 2010. Lehrbuch der Bodenkunde, 16. Auflage. Spektrum, Akademischer Verlag, Heidelberg.

[36] Scheiner, J.D., 2005. Spéciation du carbone, de l"azote et du phosphore de différentes boues de stations d"épuration au cours de leurs incubations contrôlées dans deux types de sol, thèse pour obtenir le diplôme de docteur de l'2005. Institut National Polytechnique de Toulouse; Toulouse.

[37] Spicka, A., Johnson, R., Bushing, J., Higley, L.G., Carter, D.O., 2011. Carcass mass can influence rate of decomposition and release of ninhydrin-reactive nitrogen into gravesoil. Forensic Science International 209, 80–85. doi:10.1016/j.forsciint.2011.01.002

[38] Spray, C.M., Widdowson, E.M., 1950. The effect of growth and development on the composition of mammals. British Journal of Nutrition 4, 332–353.

[39] Stokes, K.L., Forbes, S.L., Tibbett, M., 2013. Human versus animal: Contrasting decomposition dynamics of mammalian analogues in experimental taphonomy. Journal of Forensic Sciences 58, 583–591. doi:10.1111/1556-4029.12115

[40] Stokes, K.L., Forbes, S.L., Tibbett, M., 2009a. Freezing skeletal muscle tissue does not affect its decomposition in soil: Evidence from temporal changes in tissue mass, microbial activity and soil chemistry based on excised samples. Forensic Science International 183, 6–13. doi:10.1016/j.forsciint.2008.08.013

[41] Stokes, K.L., Forbes, S.L., Benninger, L.A., Carter, D.O., Tibbett, M., 2009b. Decomposition studies using animal models in contrasting environments: Evidence from temporal changes in soil chemistry and microbial activity, in: Criminal and Environmental Soil Forensics. Springer, pp. 357–377.

[42] Szelecz, I., Fournier, B., Seppey, C., Amendt, J., Mitchell, E., 2014. Can soil testate amoebae be used for estimating the time since death? A field experiment in a deciduous forest. Forensic Science International 236, 90–98. doi:10.1016/j.forsciint.2013.12.030

[43] Szelecz, I., Sorge, F., Seppey, C.V.W., Mulot, M., Steel, H., Neilson, R., Griffiths, B.S., Amendt, J., Mitchell, E.A.D., 2016. Effects of decomposing cadavers on soil nematode communities over a one-year period. Soil Biology and Biochemistry 103, 405–416. doi:10.1016/j.soilbio.2016.09.011

[44] Towne, E.G., 2000. Prairie vegetation and soil nutrient responses to ungulate carcasses. Oecologia 122, 232–239. doi:10.1007/PL00008851

[45] Tumer, A.R., Karacaoglu, E., Namli, A., Keten, A., Farasat, S., Akcan, R., Sert, O., Odabasi, A.B., 2013. Effects of different types of soil on decomposition: An experimental study. Legal Medicine 15, 149–156. doi:10.1016/j.legalmed.2012.11.003

Chapter 3

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

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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 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.

Keywords: forensic indicators; soil protozoa; testate amoebae; community ecology; cadaver; post-mortem interval

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 hours 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 phlyogenetic groups according to their feet-like extensions (pseudopodia) and shell characteristics: 1) Arcellinida, with lobose (finger-shaped) pseudopodia, the most 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 478m). Three ca. 25 m^2 sampling sites were selected within a ca. 80 x 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 x 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 meters 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 x 100 x 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 minutes. 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 10-160µm fraction was collected and centrifuged at 2500 rpm for 10 minutes and the supernatant discarded. Rose Bengale (50µl; 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 minutes 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, 10679 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 homogenized 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 400x 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 10679 (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 the effect of covering 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 [ºC] of the soils in control plots (thermologgers) and that measured 5 cm above ground from Neuchâtel meteo station (www.meteosuisse.ch) and precipitations [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).

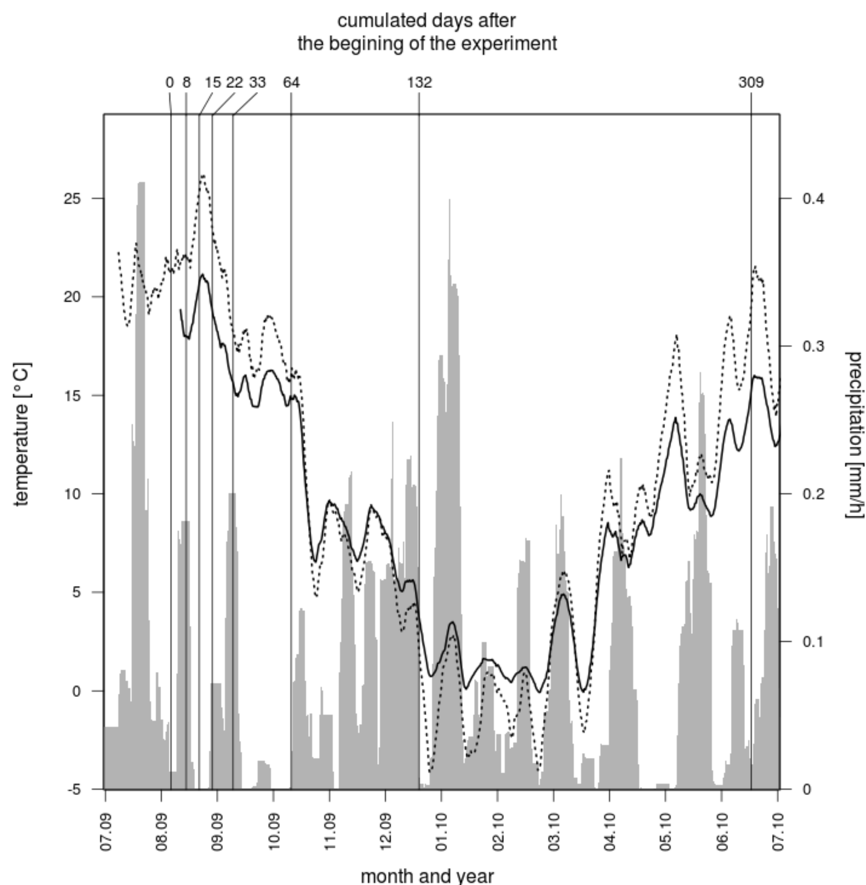

Fig. 1. Climatic data measured in Neuchâtel and at the experimental site from August 2009 until June 2010. Temperature data (left axis) are 7 days running averages of air and soil temperature; dotted lines: Neuchâtel meteorological station; plain lines: data from temperature loggers placed in the litter/soil 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.

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, Fig. 2).

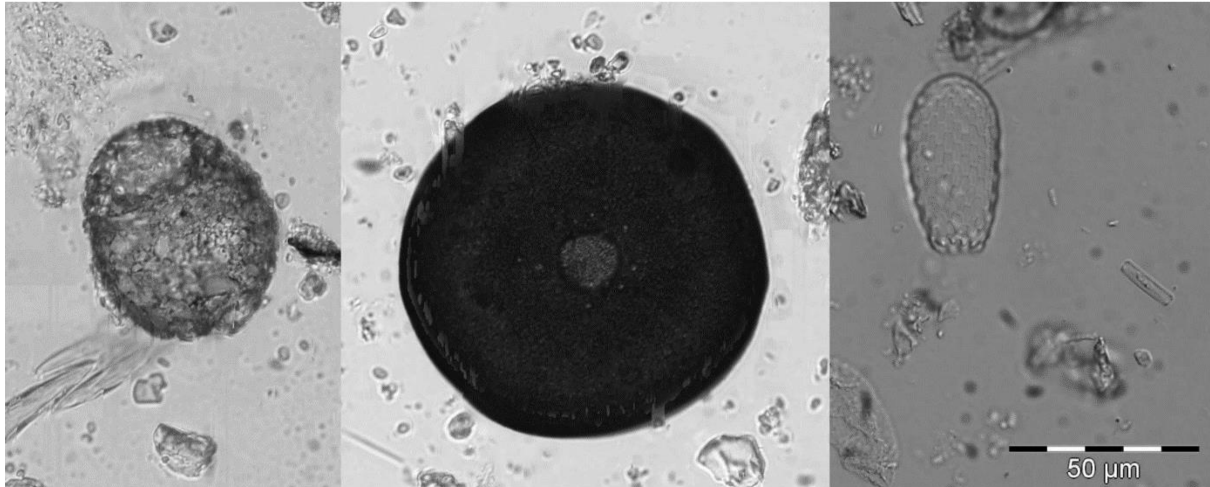

Fig. 2. Illustration of the three most abundant testate amoeba species in this experiment *Centropyxis aerophila* (left) *Arcella arenaria* (middle) and *Euglypha rotunda* (right). (Pictures from the Laboratory of Soil Biology, Neuchâtel, Switzerland)

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. g^{-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 + encys ted 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.

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 (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).

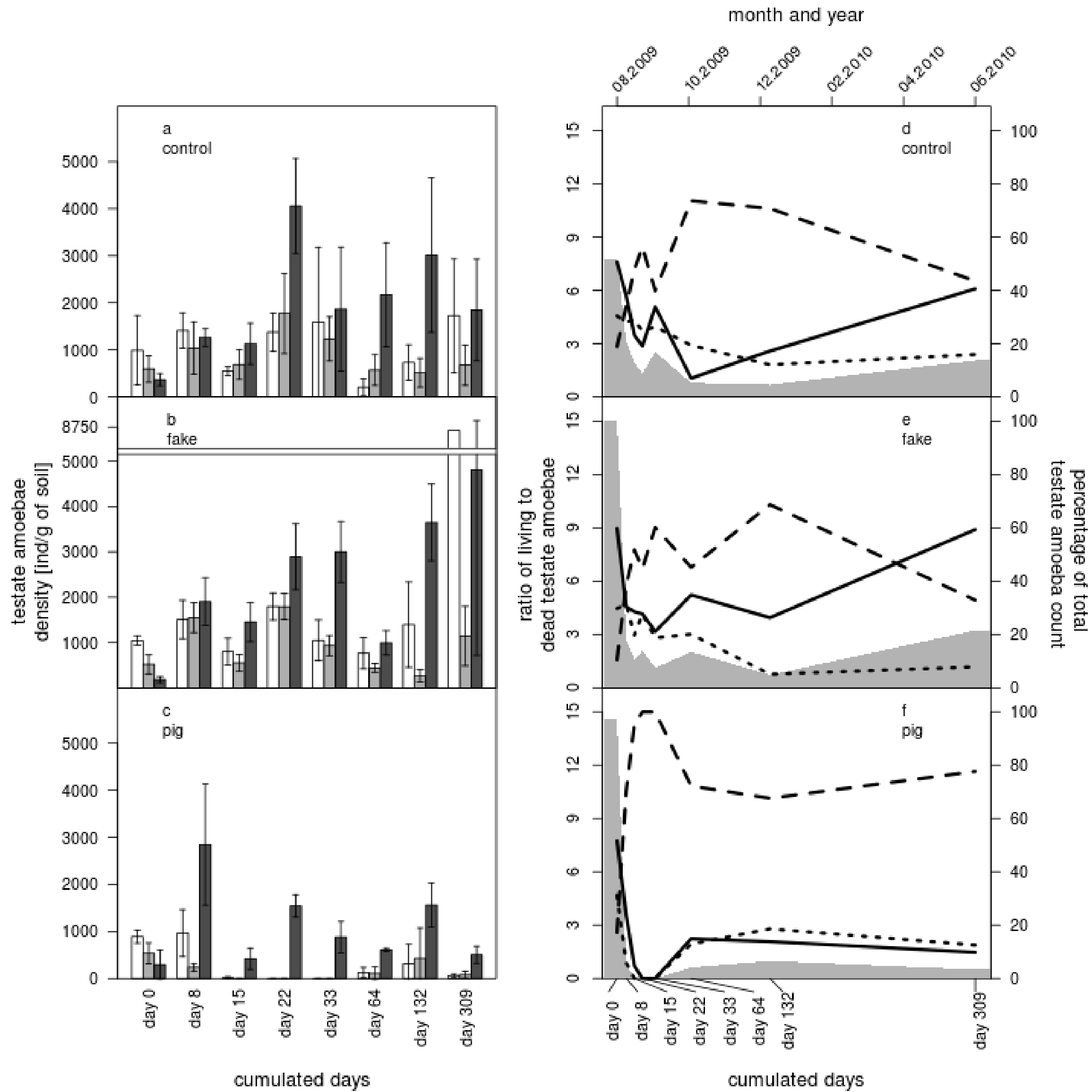

Fig. 3. Patterns of testate amoeba density in response to a pig decomposition experiment in a deciduous forest in Neuchâtel, Switzerland. Density (a-c) (number of individuals per gram litter) and living (active and encysted) to dead ratio (grey area, left scale) and percentage (right scale) (d-f) of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

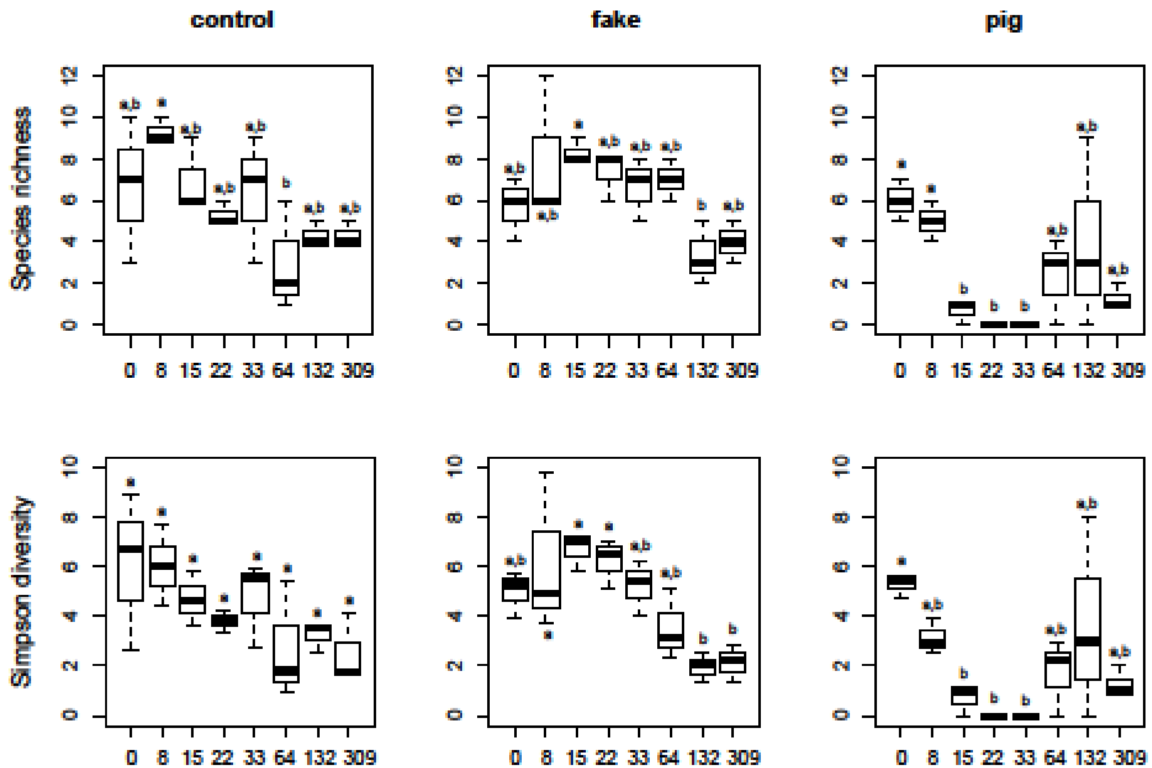

Fig. 4. Temporal patterns of alive testate amoeba species richness (top) and diversity (Simpson's N2 index) (bottom) in the three treatments (control, fake pig, pig cadaver) of a pig decomposition experiment in a deciduous forest in Neuchâtel, Switzerland. Different letters (a,b) indicate significant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

3.3. Species response to treatments

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

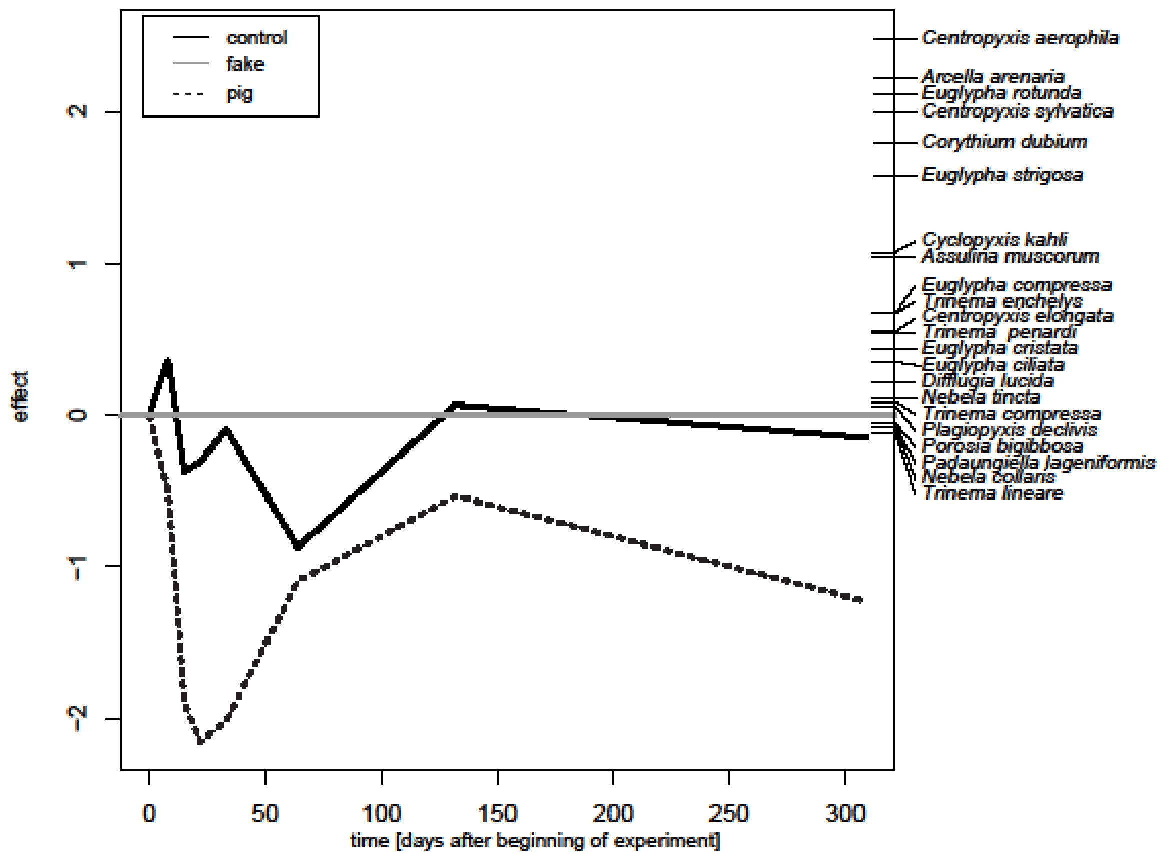

Fig. 5. Principal response curve (PRC) diagram showing the deviations of the pig cadaver treatment (dashed line) and the control (bare soil) treatment (plain line) from the fake pig treatment used as reference (horizontal grey line) over time. The left axis shows the treatment effect (i.e. regression 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.

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, pig, 3 replicates, 8 sampling days

	species	phylogenetic	relative abundance	average species density [ind. g-1]		
testate amoeba taxa	**code**	**group**	**%**	**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 Tarànek 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

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, being present in more than 50% of 696 samples from Eur ope, Russian Asia and North America (Mitchell, unpublished results). 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 to 10^4 individuals 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 ration 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], 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 *T. 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 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.

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 [60-61], 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 [62]. 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. Further studies need to be done to establish testate amoebae analysis as an approach in forensic science to estimate the post-mortem interval and to establish user-friendly methods.

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5. References*

*citation style kept according to the Journal

[1] J. Amendt, C.S. Richards, C.P. Campobasso, R. Zehner, M.J. Hall, Forensic entomology: applications and limitations, Forensic science, medicine, and pathology 7 (2011) 379-392.

[2] J. Amendt, C.P. Campobasso, E. Gaudry, C. Reiter, H.N. LeBlanc, M. Hall, Best practice in forensic entomology—standards and guidelines, Int J Legal Med 121 (2007) 90-104.

[3] W.C. Rodriguez, W. M. Bass, Decomposition of buried bodies and methods that may aid in their location, Journal of Forensic Sciences 30 (3) (1985) 836-852.

[4] J. Prangnell, G. McGowan, Soil temperature calculation for burial site analysis, Forensic Science International 191 (2009) 104-109.

[5] D. Chardez, Thecamoebologie et expertises juridiques, Travaille Laboratoire Unitie Zoologique General Applice Facultie Science Agricole, Gembloux 22 (1990).

[6] R. Fitzpatrick, M. Raven, S. Forrester, A Systematic Approach to Soil Forensics: Criminal Case Studies Involving Transference from Crime Scene to Forensic Evidence, in: K. Ritz , L. Dawson, D. Miller (Eds.), Criminal and Environmental Soil Forensics, Springer Netherlands, 2009, pp. 105-127.

[7] R.M. Morgan, J. Freudiger-Bonzon, K.H. Nichols, T. Jellis, S. Dunkerley, P. Zelazowski, et al., The forensic analysis of sediments recovered from footwear, in: K. Ritz , L. Dawson, D. Miller (Eds.), Criminal and Environmental Soil Forensics, Springer Netherlands, 2009,pp. 253-269.

[8] N. Petraco, T.A. Kubic, N.D.K. Petraco, Case studies in forensic soil examinations, Forensic Science International 178 (2008) e23-e27.

[9] G.T. Swindles, A. Ruffell, A preliminary investigation into the use of testate amoebae for the discrimination of forensic soil samples, Science & Justice 49 (2009) 182-90.

[10] K. Zala, Forensic science. Dirty science: soil forensics digs into new techniques, Science (New York, NY) 318 (2007) 386.

[11] D. Carter, D. Yellowlees, M. Tibbett, Cadaver decomposition in terrestrial ecosystems, Naturwissenschaften 94 (2007) 12-24.

[12] K. Stokes, S. Forbes, L. Benninger, D. Carter, M. Tibbett, Decomposition Studies Using Animal Models in Contrasting Environments: Evidence from Temporal Changes in Soil Chemistry and Microbial Activity, in: K. Ritz , L. Dawson, D. Miller (Eds.), Criminal and Environmental Soil Forensics, Springer Netherlands, 2009, pp. 357-377.

[13] R. Meisterfeld, Testate amoebae with filopodia, The illustrated guide to the protozoa 2 (2002) 1054-1084.

[14] R. Meisterfeld, Order Arcellinida Kent, 1880, The illustrated guide to the protozoa 2 (2002) 827-860.

[15] R. Meisterfeld, Testate amoebae in mosses and forest soils, Protozoological Monographs 4 (2009) 97-110.

[16] F. Gomaa, E.A.D. Mitchell, E. Lara, Amphitremida (Poche, 1913) Is a New Major, Ubiquitous Labyrinthulomycete Clade, PloS one 8 (2013) e53046.

[17] H.G. Smith, A. Bobrov, E. Lara, Diversity and biogeography of testate amoebae, Biodivers Conserv 17 (2008) 329-343.

[18] W. Foissner, Soil protozoa as bioindicators: pros and cons, methods, diversity, representative examples, Agriculture, Ecosystems & Environment 74 (1999) 95-112.

[19] D.M. Wilkinson, E.A.D. Mitchell, Testate Amoebae and Nutrient Cycling with Particular Reference to Soils, Geomicrobiology Journal 27 (2010) 520-533.

[20] W. Schönborn, Comparative studies on the production biology of protozoan communities in freshwater and soil ecosystems, Archiv für Protistenkunde 141 (1992) 187-214.

[21] R. Payne, V. Gauci, D. Charman, The Impact of Simulated Sulfate Deposition on Peatland Testate Amoebae, Microb Ecol 59 (2010) 76-83.

[22] J.D. Lousier, D. Parkinson, The disappearance of the empty tests of litter-and soil-testate amoebae (Testacea, Rhizopoda, Protozoa), Archiv für Protistenkunde 124 (1981) 312-336.

[23] P. Volz, Über Trockenresistenz und Wiederaufleben der Mikrofauna (inbesondere Protozoen) des Waldbodens, Pedobiologia 12 (1972) 156-166.

[24] L. Bonnet, Dékystement, phase trophique et enkystement chez Plagiopyxis minuta Bonnet. Incidences systématiques, CR Soc Acad Sci 249 (1959) 2617-2619.

[25] E.A.D. Mitchell, D. Charman, B. Warner, Testate amoebae analysis in ecological and paleoecological studies of wetlands: past, present and future, Biodivers Conserv 17 (2008) 2115-2137.

[26] M. Todorov, V. Golemansky, Effect of pesticides Fundasol, Fuzamicin and Lavendotricin on the growth of laboratory cultures of Protozoa, Acta zoologica bulgarica 45 (1992) 20-25.

[27] W. Foissner, Protozoa as bioindicators in agroecosystems, with emphasis on farming practices, biocides, and biodiversity, Agriculture, Ecosystems & Environment 62 (1997) 93-103.

[28] B. Fournier, E. Malysheva, Y. Mazei, M. Moretti, E.A.D. Mitchell, Toward the use of testate amoeba functional traits as indicator of floodplain restoration success, Eur J Soil Biol 49 (2012) 85-91.

[29] J. Lousier, Response of soil testacea to soil moisture fluctuations, Soil Biology and Biochemistry 6 (1974) 235-239.

[30] J.W. Murray, S.S. Bowser, Mortality, protoplasm decay rate, and reliability of staining techniques to recognize ‘living’ foraminifera: a review, The Journal of Foraminiferal Research 30 (2000) 66-70.

[31] J. Stockmarr, Tablets with spores used in absolute pollen analysis, Pollen et spores 13 (1971) 615-621.

[32] R.J. Payne, E.A.D. Mitchell, How many is enough? Determining optimal count totals for ecological and palaeoecological studies of testate amoebae, Journal of Paleolimnology 42 (2009) 483-495.

[33] M.O. Hill, Diversity and Evenness: A Unifying Notation and Its Consequences, Ecology 54 (1973) 427-432.

[34] K.J. Gaston, Global patterns in biodiversity, Nature 405 (2000) 220-227.

[35] J.H. Lawton, D.E. Bignell, B. Bolton, G.F. Bloemers, P. Eggleton, P.M. Hammond, et al., Biodiversity inventories, indicator taxa and effects of habitat modification in tropical forest, Nature 391 (1998) 72-76.

[36] D.P. Tittensor, C. Mora, W. Jetz, H.K. Lotze, D. Ricard, E.V. Berghe, et al., Global patterns and predictors of marine biodiversity across taxa, Nature 466 (2010) 1098-1101.

[37] B. Fournier, E. Samaritani, J. Shrestha, E.A.D. Mitchell, R.-C. Le Bayon, Patterns of earthworm communities and species traits in relation to the perturbation gradient of a restored floodplain, Appl Soil Ecol 59 (2012) 87-95.

[38] M. Moretti, M.K. Obrist, P. Duelli, Arthropod biodiversity after forest fires: winners and losers in the winter fire regime of the southern Alps, Ecography 27 (2004) 173-186.

[39] P.J. Van den Brink, C.J.F.T. Braak, Principal response curves: Analysis of time-dependent multivariate responses of biological community to stress, Environ Toxicol Chem 18 (1999) 138-148.

[40] P. Legendre, L. Legendre, Numerical ecology, ed., Elsevier, Amsterdam, 2012.

[41] R Development core team, R: A language and environment for statistical computing, R foundation for statistical computing, Vienna, Austria, 2011.

[42] J. Oksanen, F.G. Blanchet, R. Kindt, P. Legendre, P.R. Minchin, R.B. O'Hara et al., Vegan: Community Ecology Package. R package version 2.0-5., 2012.

[43] W. Foissner, G.A. Korganova, The Centropyxis aerophila complex (Protozoa: Testacea), Acta Protozoologica 39 (2000) 257-274.

[44] C. Wylezich, R. Meisterfeld, S. Meisterfeld, M. Schlegel, Phylogenetic analyses of small subunit ribosomal RNA coding regions reveal a monophyletic lineage of euglyphid testate amoebae (order Euglyphida), Journal of Eukaryotic Microbiology 49 (2002) 108-118.

[45] T.J. Heger, E.A.D. Mitchell, P. Ledeganck, S. Vincke, B. Van de Vijver, L. Beyens, The curse of taxonomic uncertainty in biogeographical studies of free-living terrestrial protists: a case study of testate amoebae from Amsterdam Island, Journal of Biogeography 36 (2009) 1551-1560.

[46] O. Heal, Observations on the seasonal and spatial distribution of testacea (Protozoa: Rhizopoda) in Sphagnum, The Journal of Animal Ecology (1964) 395-412.

[47] B.G. Warner, T. Asada, N.P. Quinn, Seasonal influences on the ecology of testate amoebae (Protozoa) in a small Sphagnum peatland in Southern Ontario, Canada, Microb Ecol 54 (2007) 91-100.

[48] J. Lousier, Effects of experimental soil moisture fluctuations on turnover rates of Testacea, Soil Biology and Biochemistry 6 (1974) 19-26.

[49] C.M. Spray, E.M. Widdowson, The effect of growth and development on the composition of mammals, British Journal of Nutrition 4 (1950) 332-353.

[50] A. Vass, W. Bass, J. Wolt, J. Foss, J. Ammons, Time since death determinations of human cadavers using soil solution, Journal of Forensic Sciences 37 (1992) 1236-1253.

[51] D. Gilbert, C. Amblard, G. Bourdier, A.-J. Francez, Short-term effect of nitrogen enrichment on the microbial communities of a peatland, in: J.-C. Amiard, B. Le Rouzic, B. Berthet, G. Bertru (Eds.), Oceans, Rivers and Lakes: Energy and Substance Transfers at Interfaces, Springer Netherlands, 1998, pp. 111-119.

[52] E.A.D. Mitchell, Response of Testate Amoebae (Protozoa) to N and P Fertilization in an Arctic Wet Sedge Tundra, Arctic, Antarctic, and Alpine Research (2004) 78-83.

[53] D. Gilbert, C. Amblard, G. Bourdier, A.-J. Francez, The microbial loop at the surface of a peatland: structure, function, and impact of nutrient input, Microb Ecol 35 (1998) 83-93.

[54] H. Nguyen-Viet, N. Bernard, E.A.D. Mitchell, P.-M. Badot, D. Gilbert, Effect of lead pollution on testate amoebae communities living in *Sphagnum fallax*: An experimental study, Ecotoxicology and environmental safety 69 (2008) 130-138.

[55] C. Meyer, N. Bernard, M. Moskura, M. Toussaint, F. Denayer, D. Gilbert, Effects of urban particulate deposition on microbial communities living in bryophytes: An experimental study, Ecotoxicology and environmental safety 73 (2010) 1776-1784.

[56] E.A.D. Mitchell, D. Gilbert, Vertical micro-distribution and response to nitrogen deposition of testate amoebae in sphagnum, Journal of Eukaryotic Microbiology 51 (2004) 480-490.

[57] M. Wanner, W. Dunger, Immigration and primary succession of protists (testate amoebae) on recultivated lignite mine spoils in Eastern Germany, Verhandlungen der Gesellschaft für Ökologie 29 (1999) 321-328.

[58] M.-M. Coûteaux, J. F. Darbyshire, Functional diversity amongst soil protozoa, Applied Soil Ecology 10 (1998) 229-237.

[59] M. Wuthrich, W. Matthey, Les Diatomées de la Tourbière du Cachot (Jura neuchâtelois), Schweizerische Zeitschrift für Hydrologie 42 (1980) 269-284.

[60] C. Melis, N. Selva, I. Teurlings, C. Skarpe, J.D. Linnell, R. Andersen, Soil and vegetation nutrient response to bison carcasses in Bialowieza Primeval Forest, Poland, Ecological Research 22 (2007) 807-813.

[61] E.G. Towne, Prairie vegetation and soil nutrient responses to ungulate carcasses, Oecologia 122 (2000) 232-239.

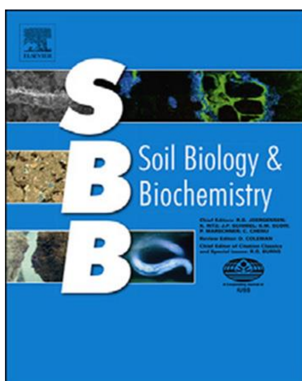
[62] B. Fournier, E. Malysheva, Y. Mazei, M. Moretti, E.A.D. Mitchell, Toward the use of testate amoeba functional traits as indicator of floodplain restoration success, European Journal of Soil Biology 49 (2012) 85-91.

[63] F. Laggoun-Défarge, E.A.D. Mitchell, D. Gilbert, J.R. Disnar, L. Comont, B.G. Warner, et al., Cut-over peatland regeneration assessment using organic matter and microbial indicators (bacteria and testate amoebae), Journal of Applied Ecology 45 (2008) 716-727.

Chapter 4

Effects of decomposing cadavers on soil nematode communities over a one-year period

Ildikó Szelecz, Franziska Sorge, Christophe V.W. Seppey, Matthieu Mulot, Hanne Steel, Roy Neilson, Bryan S. Griffiths, Jens Amendt, Edward A.D. Mitchell



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Effects of decomposing cadavers on soil nematode communities over a one-year period

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Abstract

In terrestrial ecosystems decomposing cadavers act as resource patches affecting nutrient cycling and soil communities, but the effects on soil communities are not well known. In this study, we investigated nematode community response to decomposing pig cadavers (*Sus scrofa*) over a one-year period. As nematodes play key roles in soil food webs and are known to respond to disturbances and nutrient enrichment, we hypothesised that they would respond to decomposing cadavers and that this response would change over time. We compared the temporal patterns of nematode density and community structure under pig cadavers, either placed directly on the ground or hung 1 m aboveground (for effects of cadaveric fluids only), with two controls, i.e., bare soil and bags filled with soil placed on the ground (fake pigs – for microclimatic effects only). In the control and fake pig treatments nematode densities,

community patterns and maturity indices did not change significantly. In contrast, density increased significantly underneath the ground and hanging pigs two weeks after the beginning of the experiment, and nematode family richness, Simpson diversity and maturity index were significantly reduced in the cadaver treatments. Most nematode families responded negatively to cadavers with the notable exceptions of Rhabditidae, Neodiplogasteridae and Diplogasteroididae. The latter two were found exclusively underneath the decomposing cadavers and are promising bioindicators of vertebrate cadaver decomposition. Even though diversity, density and communities were recovering after one year, the impact of cadavers was still significant for the maturity index. These contrasting patterns illustrate how decomposing cadavers contribute to increasing local biodiversity and suggest that soil nematodes could be used as a tool to document the presence of a decomposing cadaver, or to estimate the time elapsed since death (post-mortem interval). Patterns should, however, be compared in different settings and seasons before such a tool can be validated.

1. Introduction

Decomposing cadavers represent an important natural resource in terrestrial ecosystems. By providing food for scavengers (ranging from microbes to vertebrates), they contribute to the heterogeneity of the soil ecosystem at different spatial and temporal scales, depending on the size of the cadaver (DeVault et al., 2003; Carter et al., 2007). A large number of mammals die from other causes than predation, e.g., injuries, starvation, extreme weather conditions, parasites or diseases (Carter et al., 2007; Parmenter and MacMahon, 2009), and their cadavers strongly affect biotic communities and nutrient cycling (Beasley et al., 2012; Barton et al., 2013). Cadavers can be described as patchy "resource pulses" for local consumers, being rare, brief and intense episodes of increased resource availability in space and time (Payne, 1965; Schoenly and Reid, 1987; DeVault et al., 2003; Yang, 2008).

Previous cadaver research has mainly focused on the post-mortem interval (PMI), or time since death, and other aspects directly useful in criminal investigations (Barton et al., 2013), while detailed studies on the impact of cadavers on the diversity and community structure of soil organisms are rare (Carter et al., 2007; Metcalf et al., 2013, 2016). Thus, cadavers offer opportunities to explore natural disturbance patterns, succession and, more generally, soil biodiversity. However, it is often not obvious that cadavers should be left to decompose naturally, at least not close to where humans or farm animals may be exposed to the transmission of diseases (Fielding et al., 2014).

Decomposing cadavers are sources of water (60-80%), lipids, proteins, carbohydrates, and micro-organisms (including pathogens) (Spray and Widdowson, 1950; Swift et al., 1979; Clark et al., 1997; Tortora and Grabowski, 2000). Through decomposition they release large amounts of nutrients and organic carbon (Putman, 1978; Vass et al., 1992; Hopkins et al., 2000; Towne, 2000; Carter et al., 2007; Benninger et al., 2008; Forbes, 2008). The resulting chemical changes in soil may remain measurable for several years (Towne, 2000).

Vertebrate cadaver decomposition can be divided roughly into six stages: fresh, bloated, active decay, advanced decay, dry and remains (Payne, 1965). Directly after the fresh stage the bloated stage begins, characterized by a tightening of the skin and inflation, due to an accumulation of gases (Vass et al., 1992, 2002). In

the active decay stage blood and body fluids escape from body orifices, the skin begins to crack, and the body deflates releasing cadaveric fluids, allowing oxygen to enter the cadaver which stimulates aerobic microbial activity. In the advanced decay stage, most of the flesh is removed and the cadaver begins to dry. When only dry skin, cartilage and bones remain, the dry stage begins. At the remains stage, only bits of skin, hair, bones and teeth are left (Payne, 1965; Vass, 2001; Carter and Tibbett, 2008). The duration of the various stages depends on temperature and insect access to the cadaver (Vass et al., 1992; Campobasso et al., 2001; Megyesi et al., 2005).

This “island of fertility” associated with increased nutrient content, soil microbial biomass and activity on a small terrestrial area has been described as the “Cadaver Decomposition Island” (CDI) (Carter et al., 2007; Benninger et al., 2008). The CDI receives additional organic and inorganic materials, brought by scavengers, grazers and predators (Towne, 2000; Carter et al., 2007). It serves as a food source and a resource for reproduction of blow flies and carrion beetles (Smith and Merrick, 2001; Carter et al., 2007; Hall et al., 2011) within a spatially discrete area. Being a specialised habitat for these organisms, which in turn may affect other trophic levels (bacteria, fungi, protozoa, nematodes) the CDI contributes to overall biodiversity (Carter et al., 2007). Among these groups nematodes have been shown to be stimulated by the increase of bacteria responding to the cadaver nutrient pulse (Metcalf et al., 2013, Carter at al. 2015, Weiss et al., 2016).

Nematodes are one of the most abundant groups of soil metazoans with densities reaching up to 50 million m^{-2} (Bongers and Bongers, 1998; Bongers and Ferris, 1999). They are ubiquitous (Ritz and Trudgill, 1999) and diverse with ca. 30,000 described species, but a total diversity estimated at > one million species (Hugot et al., 2001). Nematodes are recognized as useful biological proxies (bioindicators) of soil conditions (Ritz et al., 2009) due to their abundance, diversity and short generation time (Bongers, 1990; Ettema and Bongers, 1993; Neher and Campbell, 1994; Yeates, 1999; Yeates et al., 2009). Soil nematodes are known to respond to changes in food resource availability and environmental conditions (Boag and Yeates, 1998; Yeates, 1999; Zhang et al., 2012). They can be classified according to feeding types and “coloniser—persister” (c-p) functional groups (Bongers, 1990; Yeates et al., 1993; Bongers and Bongers, 1998; Yeates, 2003). The c-p groups are

based on the life strategies of nematodes ranging from c-p 1 i.e. “colonisers” enrichment opportunists, with an immense population growth under food-rich conditions, to c-p 5 “persisters” with a low reproduction rate and a high sensitivity to disturbance (Bongers and Bongers, 1998). This classification facilitates the interpretation of changes in nematode community structure at family level (Korthals et al., 1996). The use of the c-p classification overcomes a main limitation of the trophic group approach, namely the fact that different responses to stress may exist within a given trophic group (Cesarz et al., 2015).

The response of soil nematodes to a range of perturbations has been studied in various natural and agro-ecosystems (Weiss and Larink, 1991; Korthals et al., 1996; Georgieva et al., 2002) and pasture (Bardgett et al., 1994; Yeates et al., 1994, 1995). However, the impact of vertebrate cadavers on soil nematodes is not well known. In a previous experiment focusing on testate amoebae, Szelecz et al. (2014) anecdotally observed major changes in nematode abundance and community structure in response to cadaver decomposition (the nematode data were not published). In this new study we therefore investigated the temporal patterns of nematode density and community structure associated with the decomposition of pig cadavers over a one-year period. Given the responses of nematodes to perturbations and vertebrate decomposition in soils, we firstly expected to find shifts towards bacterial feeding c-p 1 nematodes during the peak of nutrient input along with a decline of other feeding groups and more sensitive nematodes. We then expected that over time community structure and density would gradually return to the pre-disturbance status.

2. Materials and methods

2.1. Study site and experimental setup

The experiment was conducted in a small spruce (*Picea abies*) forest near Neuchâtel, Switzerland (47°01’05.01 N, 6°52’27.76 E, 775 m a.s.l.). The study site was almost flat and covered an area of 1200 m^2. Mean annual temperature and total precipitation (measured in-field with a Decagon Em50 digital data logger) were 10.2 °C and 978 mm (Fig. 1). The topsoil consisted of a litter layer (spruce needles and mosses), a

fragmentation layer and a humification layer (O horizon, up to 1 cm), and an umbric horizon with a dark brown colour (A horizon, 1-17 cm).

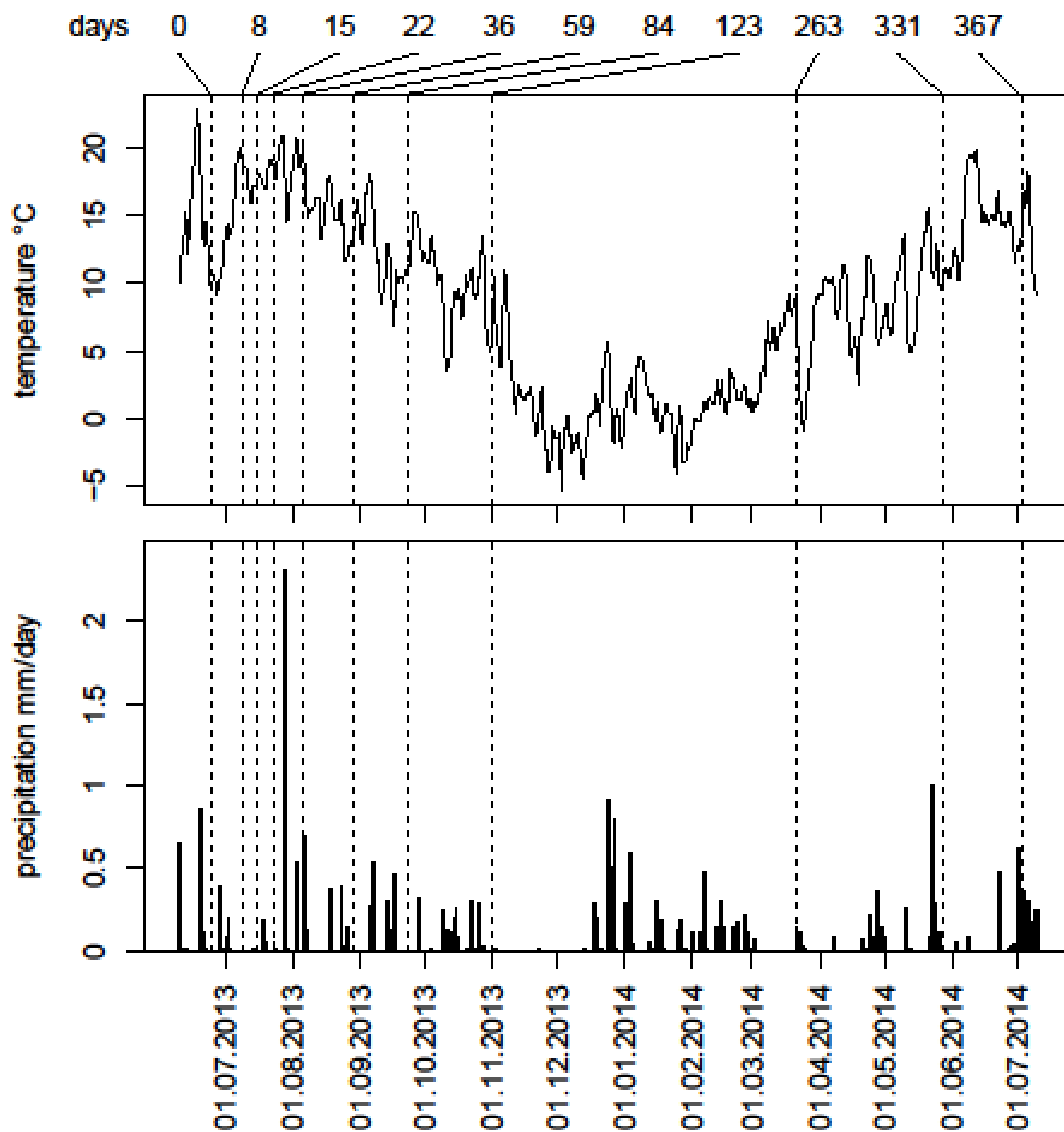

Fig. 1. Climatic data measured at the Bois-du-Clos experimental site Neuchâtel, Switzerland) over the course of the experiment from June 10, 2013 to July 10, 2014: Air temperature (top) in °C, precipitation (bottom) in mm h^{-1} (grey columns). Days (top) indicate the days elapsed since placing the cadavers on July 01, 2013. Day 0 data was taken on June 26, 2013.

In total 20 plots (ca. 4 m distance from each other) with four treatments (five replicates each) were set up randomly: 1) control (bare soil), 2) fake pigs (cotton bags of the same size as the pig cadavers), 3) ground pigs (cadavers directly placed on the ground for microclimatic and cadaveric fluids effects), and 4) hanging pigs (cadavers hanging 1 m above ground). The fake pigs served as an additional control for microclimatic effects (e.g. temperature, humidity, solar insolation). The bags covered an area of the same size, but without any cadaveric fluids seeping into the ground. They were filled with soil of the same weight as the cadavers and were gradually emptied to match the average weight loss of the ground cadavers. The hanging pigs provided cadaveric fluids, but without any direct contact to the soil or covering of an area, and thus with no or very minimal microclimatic effect.

Ten domestic pigs (*Sus scrofa domestica*), eight females and two males, 10 weeks old, were bought at a local farm. They were sedated with Stresnil® (Azaperone) and euthanized with T61® by a veterinarian, immediately transported to the experimental site, weighed and placed on the plots. The average cadaver weight was 28 kg ± 2.5 kg (SD). All cadavers were placed in cages (140 cm x 95 cm) surrounded by wire mesh fences to keep scavengers and larger animals away. The experimental area was surrounded by an electric fence for additional protection. Control and fake pig plots were marked with sticks and cords. Fences and cages could be opened at one side for soil sampling and weighing of the cadavers. At each sampling time, soil from inside the fake pig bags was removed to match the weight loss of the pig cadavers.

2.2. Decomposition stages, sampling and laboratory analyses

Decomposition stages were estimated using the definitions provided by Payne (1965) for arthropod-exposed carrions. From the first day of cadaver placement (July, 01, 2013) until the beginning of the dry stage, each pig cadaver was examined daily to record the state of decomposition (including photographs and written reports) according to physical characteristics and arthropods present. After the beginning of the dry stage the cadavers were examined at longer intervals (> 9 days).

On 11 sampling days from June 2013 until July 2014 a total of 220 soil samples (11 days x 4 treatments x 5 replicates) were collected. Samples were initially taken shortly before the placing of the cadavers (D0), then at one-week intervals i.e. on days D8,

D15, D22, and thereafter at increasing intervals on days D36, D59, D84, D123, D263, D331 and D367. A wooden frame, identical in size to the experimental cages, with x (letters A-N) and y (numbers 1-8) coordinates was placed on the ground at each site to ensure random sampling based on the coordinates. Before sampling, coordinates for 10 subsamples per plot were selected with raffles, avoiding re-sampling the same place. Subsamples were taken with a bulb planter to a depth of 10 cm, pooled and mixed to obtain one soil sample from each plot at each sampling day. Samples were stored at 4 °C until further processing.

Nematodes were extracted from 100 g soil using a modified Baermann funnel technique (Brown and Boag, 1988). Nematodes from all 220 samples were enumerated live using a dissecting microscope (Olympus SZ51), fixed with heated formaldehyde (4%) and heat-killed at 65 °C for 3 minutes. A subset of three replicates per treatment were used for community analysis (n = 132) and one hundred randomly chosen nematodes per sample were identified to family level (Bongers, 1994; Scholze and Sudhaus, 2011) using an upright light microscope at x400 magnification (Axio Lab.A1, Zeiss). All densities were given as mean ± standard deviation (SD) 100 g^{-1} dry soil. For this calculation 10 g of each sample were dried in a drying oven (105 °C, 24 hours) to determine soil moisture.

Soil samples were analysed for selected chemical markers known to vary in response to decomposing cadavers. Soil pH was measured after diluting the sample in water in a 1:2.5 proportion. Ammonium and nitrate analyses were performed directly after sampling using colorimetric determination (Scheiner, 2005). Total nitrogen was determined using a CHN (Carbon, Hydrogen, Nitrogen) analyser (Thermo Finnigan Flash EA 1112) on dry, ground soil. Bioavailable phosphorus content was determined by colorimetric analysis (Olsen et al., 1954).

2.3. Data analyses

The duration of each decomposition stage was tested according to treatment (t-test adjusted according to Holm) to determine whether the length of the decomposition stages differed between hanging and ground pigs. Nematode data were analysed based on the following metrics 1) functional composition according to trophic grouping (Yeates et al., 1993), 2) colonizer-persister (c-p) life-strategy groups (Bongers, 1990),

3) taxonomic composition (family-level community structure) and 4) Simpson diversity, richness, Hill´s evenness and the maturity index (MI), calculated as the weighted mean of the individual c-p values (Bongers, 1990). The c-p structure and MI were calculated using NINJA, the Nematode Indicator Joint Analysis software (Sieriebriennikov et al., 2014).

Temporal patterns and treatment effects were evaluated by ANOVA followed by post-hoc Tukey tests (Chambers et al., 1992). Community patterns (feeding types, c-p groups and family-level communities) were analysed in relation to treatment, sampling day and environmental variables (pH, $NH_4^+NO_3^-$, P and N) using partial redundancy analysis (RDA) calculated on Bray-Curtis distance. Factors and variables were selected using stepwise forward selection (ordistep function: vegan package 2.3-0). Significance of the ordination axes were assessed using permutation tests (1000 permutations, p-value threshold=0.05). All statistical analyses were performed with R statistical software (version 3.1.0) (R Development Core Team, 2013) and the vegan package (2.3-0).

3. Results

3.1. Temporal patterns of decomposition stages

Over the course of the experiment, four out of five ground pigs went through all six decomposition stages by day 367 and had reached the remains stage, whereas one cadaver was still in the dry stage (Fig. 2). The hanging pigs had at least reached the dry stage (4 cadavers), with one cadaver already in the remains stage (Fig. 2). Decomposition was at first slower for the ground pigs where the bloated stage lasted on average twice as long as in the hanging cadavers (i.e. eight vs. four days; $p<0.05$, t-test, adjusted p-value according to Holm). However, the active decay stage was significantly longer in the hanging cadavers ($p<0.01$, t-test, adjusted p-value according to Holm).

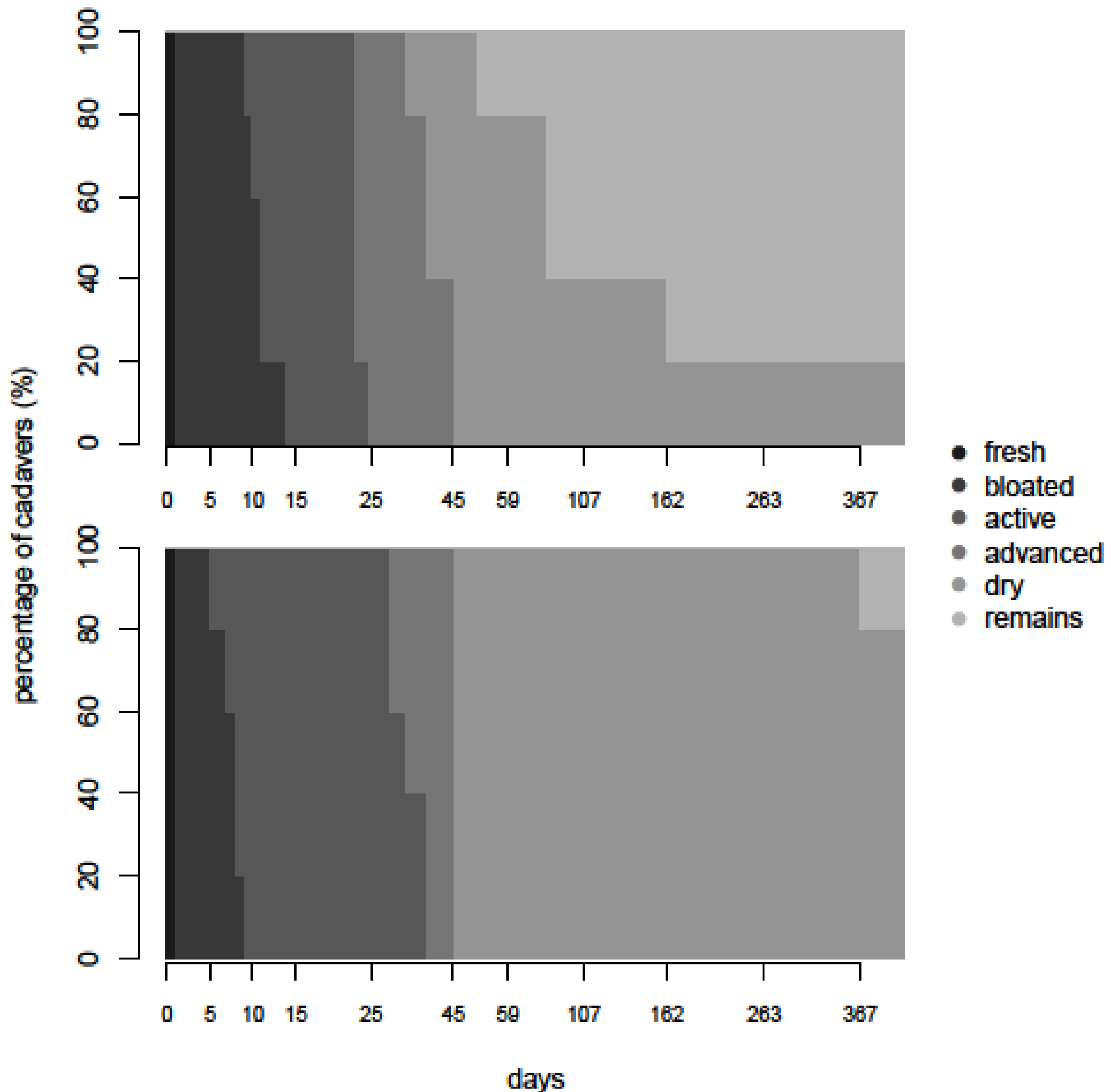

Fig. 2. Duration of decomposition stages, and percentage of cadavers representing a given decomposition stage, in the ground (top) and hanging pig (bottom) cadaver treatments over time at the Bois-du-Clos spruce forest experimental site (Neuchâtel, Switzerland). Decomposition stages are shown in different shades of grey.

3.2. Nematode density

Nematode density (100 g^{-1} dry soil) sharply increased under the ground pigs from D0 (933±260) to D15 (13780±13755) (ANOVA, Tukey HSD, $p<0.01$, Fig. 3, Table S1). Under the hanging pigs the trend was similar, not as strong but also significantly different (D0: 874±274, D15: 8261±10531, ANOVA, Tukey HSD, $p<0.05$, Fig. 3, Table S1). In contrast, there was no significant temporal variation in nematode density in the control and fake pig plots (Fig. 3).

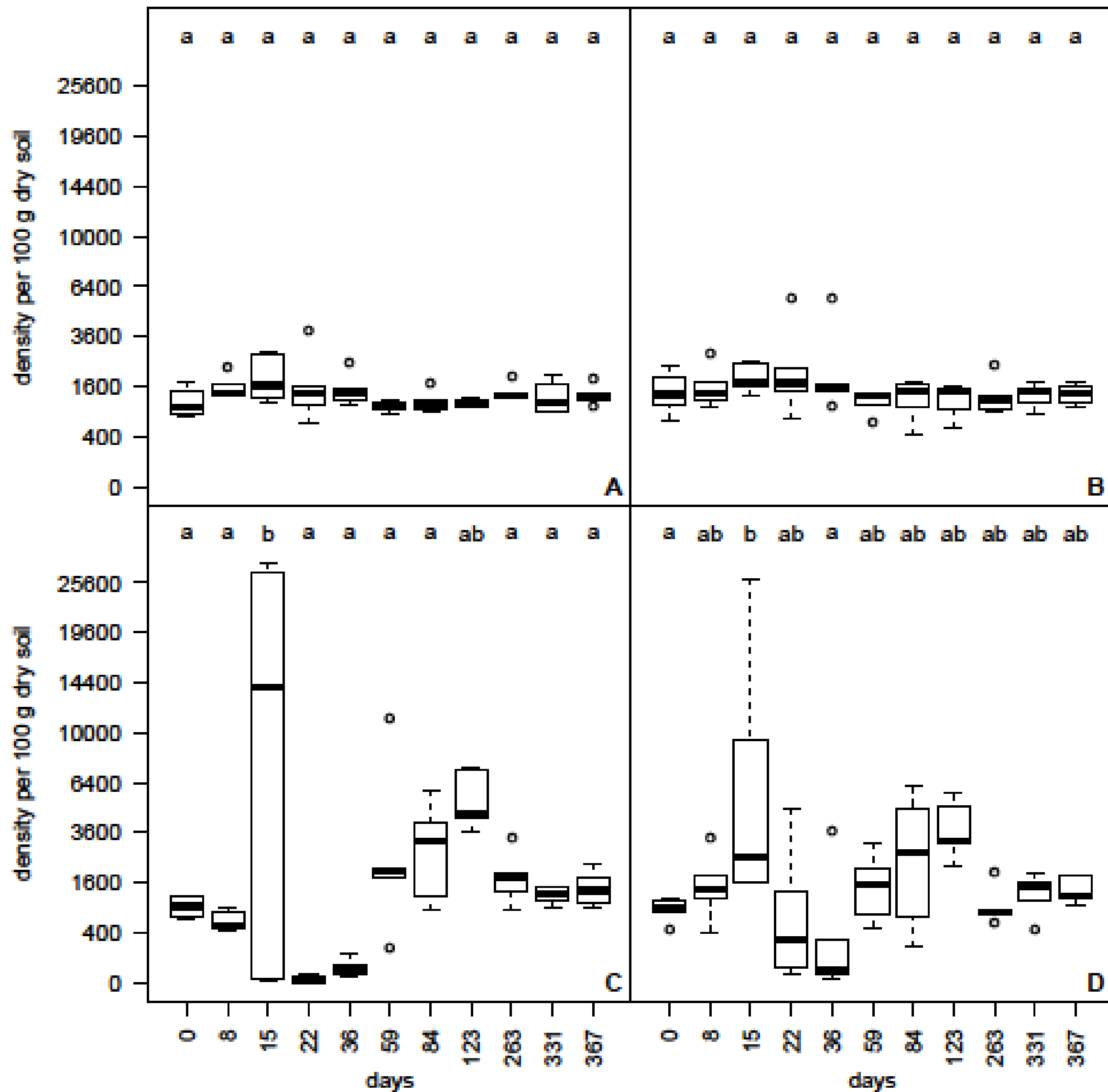

Fig. 3. Box plots showing temporal patterns of soil nematode density in 100 g-1 dry soil in the four treatments (A) control, (B) fake pig, (C) ground pig and (D) hanging pig in a spruce forest at the Bois-du-Clos experimental site (Neuchâtel, Switzerland). Different letters (a and b) indicate significant differences among sampling days (ANOVA, Tukey HSD).White box ¼ interquartile range (IQR); Solid black bar ¼ median; Error bar ¼ maximum (top)/minimum (bottom) value excluding outliers; Open circle ¼ outliers.

3.3. Feeding groups

Bacterivorous nematodes were the most abundant feeding group in all treatments, followed by herbivores and predators (Table 1). Fungivorous nematodes reached somewhat lower densities, and omnivores were least abundant (Table 1). Density of bacterivorous nematodes under the ground pigs was significantly higher than in all other treatments (ANOVA, Tukey HSD, $p<0.05$).

In the RDAs (calculated on raw nematode densities in 100 g-1 dry soil, Fig. 4 a, b ,c) a nematode group/family is correlated to/influenced by an environmental/explanatory variable if its orthogonal projection on the vector of the environmental variable is far from zero. The length of the arrow and its angle to an axis gives the strength of the impact (maximum: length = 1, angle = 0°). The further away a point is located in the direction of an axis, the more it is influenced by the variable pointing in the same direction. The closer the coordinates of nematode groups/families are to a sample centroid, the more abundant they are in the sample group. Sample centroids that are close together in an ordination space are more similar in their community structure than those that are far away from each other. Thus, a strong dominance of bacterivores in the ground and hanging cadaver samples was correlated with changes in soil pH and phosphorous content (permutation test, $p<0.05$; Fig. 4a, Table S2).

3.4. Colonizer-persister (c-p) groups

In the control samples c-p 4, c-p 2 and c-p 3 co-dominated, while in the fake pig treatment c-p 2 was clearly dominant, followed by c-p 1 and c-p 4 (Table 2). In both pig treatments c-p 1 was the most abundant group, followed by c-p 2, then c-p 4 for the ground pig treatment and c-p 3 for the hanging pig treatment (Table 2). Overall the density of c-p 1 in the ground pig treatments differed significantly from the other treatments (ANOVA, Tukey HSD, $p<0.05$). In the fake pig treatment c-p 2 differed significantly from all others (ANOVA, Tukey HSD, $p<0.01$), and c-p 3 to c-p 5 in the fake pig and control treatments differed from both cadaver treatments (ANOVA, Tukey HSD, $p<0.001$). The RDA showed that the samples from the two cadaver treatments diverged due to the strong dominance of c-p 1 associated with increases in soil pH and ammonium content (permutation test, $p<0.05$; Fig. 4b, Table S2).

3.5. Families

In total 28 nematode families were identified (Table 3). The three most abundant families were Tylenchidae, Plectidae and Rhabditidae in the controls, Tylenchidae, Cephalobidae and Rhabditidae in the fake pig samples, Rhabditidae, Diplogasteroididae and Neodiplogasteridae in ground pig samples, and Rhabditidae,

Neodiplogasteridae and Diplogasteroididae in the hanging pig samples (Table 3); the latter two families were exclusively found in the ground and hanging pig treatments.

The RDA showed that the divergence of nematode communities in both pig treatments as compared to the control and fake pig treatments was correlated, first to an increase in soil nitrate and then to increases in pH and phosphorous and ammonium content (permutation test, $p<0.05$; Fig. 4c, Table S2). Most nematode families responded negatively to cadavers with the notable exceptions of Rhabditidae, Neodiplogasteridae and Diplogasteroididae (Fig. 4c). The position of samples in the ordination space showed that, although by D263-D367 communities from both cadaver treatments were clearly converging towards the control and fake pig communities, they still remained different.

3.6. Nematode diversity, richness, evenness and maturity indices

Overall Simpson diversity (Fig. 5a) was significantly highest in the control, lower in the fake pig samples, and lowest in the two cadaver treatments (ground and hanging) (ANOVA, Tukey HSD, $p< 0.001$). In the ground and hanging pig samples diversity decreased at D8 and stayed fairly low until D263, being significantly different from D0 (ground pigs: $p<0.001$, hanging pigs: $p<0.05$; ANOVA, Tukey HSD). After that it increased again in the ground pig treatment at D331, with significant differences to the preceding time points from D8 to D263 (ANOVA, Tukey HSD, $p<0.05$).

Family richness overall was significantly highest in the controls, lower in the fake pig samples (ANOVA, Tukey HSD, $p<0.01$), and lowest in the ground and hanging pig samples (ANOVA, Tukey HSD, $p<0.001$; Fig. 5b). In the control samples family richness remained constant throughout the experiment. In contrast, it decreased in the ground pig samples significantly from 15±2 at D0 to 5±3 at D8 and 1±1 at D22, remaining low until D263 (ANOVA, Tukey HSD, $p<0.001$). Family richness recovered completely by D331 and D367 with no significant differences compared to D0. Hanging pig samples showed the same pattern, but the effect was less pronounced. Richness dropped from 13±2 at D0 to 7±3 at D8, reached a minimum of 2±1 at D59, but did not drop to 0, with D0 being significantly different from the subsequent time points D15 to D123 (ANOVA, Tukey HSD, $p<0.05$), but not from D263-D367 where it had increased again reaching 12±2 at D367.

Family richness declined from an average of 14.7±2.1 at D0 (i.e., before the cadavers were placed) to 1.3±1.0 (ground pigs), and from 12.6±2.3 (D0) to 5.1±2.8 (hanging pigs) during the active decay stage (ANOVA, Tukey HSD, $p<0.05$, Fig. 6). By the end of the experiment, in the dry and remains stage, family richness was on average about half of that of D0 (but no longer significantly different) under both treatments (7.7±4.7 in the ground pigs and 7.7±3.8 in the hanging pigs).

Overall, Hill's evenness in the hanging and fake pig treatments differed significantly from the control and ground pig treatments (ANOVA, Tukey HSD, $p<0.05$). Hill's evenness did not show significant differences over time regardless of the treatment (Fig. 5c).

The maturity index (MI) in the control and fake pig treatments was overall significantly higher compared to the ground and hanging pig treatments (ANOVA, Tukey HSD, $p<0.001$). The MI remained stable (mostly 2.5-3.5) in the control and the fake pig treatments throughout the experiment (Fig. 5d), with no significant differences over time. By contrast, in both the ground and hanging pig treatments it dropped sharply to ca. 1 at D8 and D36 in the ground pig and hanging pig treatments, respectively, and remained low until D123 in both, with significant differences between D0 and all other time points (ANOVA, Tukey HSD, $p<0.001$). Higher values were recorded on the last three sampling dates (D263-D367), but still significantly lower compared to D0 (ANOVA, Tukey HSD, $p<0.001$).

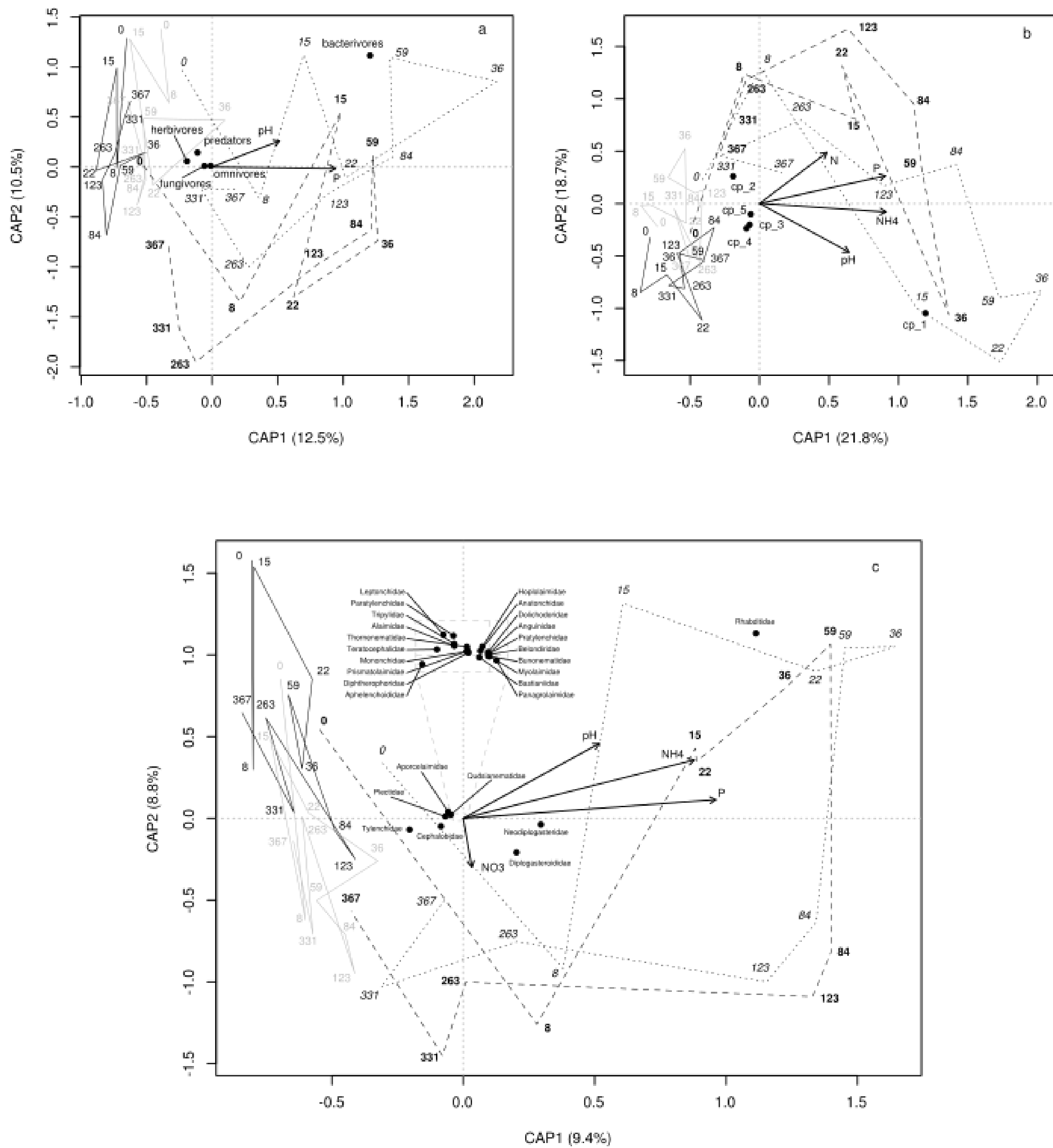

Fig. 4. Redundancy analysis (RDA) ordination diagram showing the temporal patterns of soil nematode (a) feeding groups, (b) c-p groups and (c) families in control, fake pig, ground pig and hanging pig treatments in a spruce forest at the Bois-du-Clos experimental site (Neuchâtel, Switzerland). The lines (solid black: control; solid grey: fake; dotted: ground pig; dashed: hanging pig) join the centroids of replicates from each sampling day (numbers indicating the time since death in days). Arrows represent the environmental/explanatory variables selected by stepwise selection to discriminate the above-mentioned nematode groups (response variables). Stepwise selection alternated between forward selection and backward elimination. This overcame the problem of using either forward selection or backward elimination where all variables that are included/eliminated at a previous step are kept in the model/removed from the model (even though they might not contribute much after other variables are incorporated) (Legendre and Legendre, 1998).

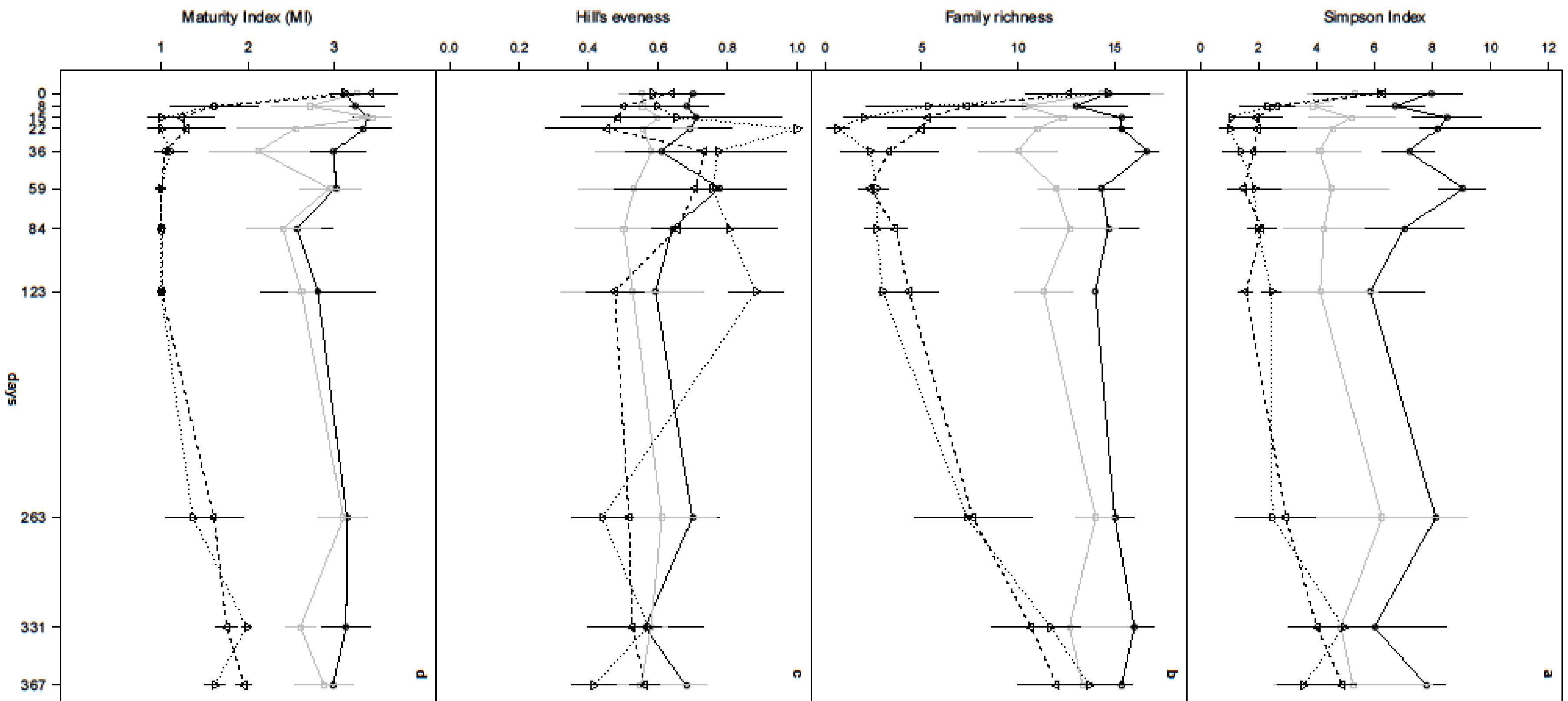

Fig. 5. Temporal patterns of soil nematodes (family level) in the four treatments (control, fake pig, ground pig and hanging pig) in a spruce forest at the Bois-du-Clos experimental site (Neuchâtel, Switzerland) (a) Simpson diversity index, (b) family richness, (c) Hill's evenness, (d) Maturity index (MI). Treatments are indicated by line type: solid/circles: control; grey/squares: fake; dotted/triangle up: ground pig; dashed/ triangle down: hanging pig.

Table 1. Overall density of nematode feeding groups in $100g^{-1}$ dry soil at Bois-du-Clos experimental site (Neuchâtel, Switzerland) in the control, fake pig, ground pig and hanging pig treatments: average (ave), minimum (min), maximum (max) density and standard deviation (SD).

Feeding groups	Control			Fake			Ground			Hanging		
	Min ± sd	Max ± sd	Ave ± sd	Min ± sd	Max ± sd	Ave ± sd	Min ± sd	Max ± sd	Ave ± sd	Min ± sd	Max ± sd	Ave ± sd
bacterivores	272.26 ± 122.97	525.41 ± 192.37	398.06 ± 73.60	378.36 ± 266.31	1934.46 ± 1924.96	751.16 ± 499.55	0.48 ± 0.43	22,824.84 ± 7863.33	3755.58 ± 6635.44	94.4 ± 158.49	2828.40 ± 606.05	1185.65 ± 831.43
fungivores	27.12 ± 23.56	188.19 ± 83.60	116.28 ± 45.39	34.77 ± 39.14	691.65 ± 830.21	187.54 ± 176.83	0 ± 0	109.15 ± 85.06	25.87 ± 35.76	0 ± 0	99.09 ± 77.34	37.94 ± 36.88
herbivores	226.33 ± 72.19	472.03 ± 192.36	364.80 ± 81.25	329.92 ± 133.20	723.72 ± 520.53	523.09 ± 134.53	0 ± 0	437.90 ± 187.88	116.40 ± 152.07	0 ± 0	378.96 ± 308.33	117.65 ± 154.09
omnivores	17.47 ± 19.89	46.35 ± 35.97	26.69 ± 7.85	0 ± 0	66.24 ± 72.00	18.13 ± 17.89	0 ± 0	18.14 ± 7.73	3.77 ± 6.02	0 ± 0	9.37 ± 16.22	1.10 ± 2.86
predators	144.60 ± 83.49	356.71 ± 10.41	242.63 ± 58.04	153.76 ± 56.07	713.99 ± 521.51	284.48 ± 184.73	0 ± 0	265.63 ± 90.05	47.92 ± 80.38	0 ± 0	222.73 ± 115.71	41.48 ± 70.52

Table 2. Overall density of nematode functional cp-groups in $100g^{-1}$ dry soil at Bois-du-Clos experimental site (Neuchâtel, Switzerland) in the control, fake pig, ground pig and hanging pig treatments: average (ave), minimum (min), maximum (max) density and standard deviation (SD).

c-p groups	Control			Fake			Ground			Hanging		
	Min ± sd	Max ± sd	Ave ± sd	Min ± sd	Max ± sd	Ave ± sd	Min ± sd	Max ± sd	Ave ± sd	Min ± sd	Max ± sd	Ave ± sd
c-p 1	18.00 ± 18.94	308.47 ± 169.85	137.01 ± 100.90	12.43 ± 21.53	1503.30 ± 1907.44	294.93 ± 428.28	0.48 ± 0.43	22,871.19 ± 7783.23	3665.92 ± 6094.42	25.58 ± 35.46	2846.27 ± 601.41	1046.83 ± 833.08
c-p 2	226.18 ± 67.48	448.36 ± 207.67	310.74 ± 76.25	288.07 ± 296.99	2065.58 ± 2535.70	748.40 ± 508.23	0 ± 0	488.39 ± 159.76	174.21 ± 205.95	0 ± 0	644.83 ± 384.43	223.87 ± 229.95
c-p 3	131.03 ± 42.13	308.29 ± 34.53	211.47 ± 49.90	152.51 ± 49.50	355.45 ± 400.00	243.35 ± 63.68	0 ± 0	138.21 ± 95.17	29.30 ± 45.45	0 ± 0	227.82 ± 145.36	44.91 ± 68.29
c-p 4	207.85 ± 95.46	446.49 ± 100.98	320.50 ± 79.86	148.38 ± 67.53	493.36 ± 159.36	294.93 ± 428.28	0 ± 0	277.21 ± 57.25	45.16 ± 85.00	0 ± 0	227.74 ± 65.66	41.76 ± 76.93
c-p 5	94.17 ± 40.65	277.12 ± 61.83	168.84 ± 51.58	81.87 ± 31.79	670.81 ± 583.23	216.20 ± 168.88	0 ± 0	178.12 ± 138.43	33.24 ± 55.10	0 ± 0	156.14 ± 117.83	26.66 ± 45.36

Table 3. Overall density of nematode families in $100g^{-1}$ dry soil at Bois-du-Clos experimental site (Neuchâtel, Switzerland) in the control, fake pig, ground pig and hanging pig treatments: average (ave), minimum (min), maximum (max) density and standard deviation (SD).Highest average densities are given in bold.

No.	Families	Control			Fake			Ground			Hanging		
		Min ± sd	Max ± sd	Ave ± sd	Min ± sd	Max ± sd	Ave ± sd	Min ± sd	Max ± sd	Ave ± sd	Min ± sd	Max ± sd	Ave ± sd
1	Alaimidae	20.03 ± 12.39	70.80 ± 20.25	44.90 ± 16.57	7.12 ± 12.34	43.89 ± 43.42	22.02 ± 14.34	0 ± 0	18.88 ± 16.39	5.73 ± 8.08	0 ± 0	29.71 ± 7.06	4.47 ± 8.83
2	Anatonchidae	0 ± 0	27.11 ± 20.79	11.24 ± 9.25	0 ± 0	24.52 ± 23.31	7.65 ± 8.74	0 ± 0	10.16 ± 10.58	1.82 ± 3.50	0 ± 0	25.32 ± 36.40	6.15 ± 9.40
3	Anguinidae	0 ± 0	11.88 ± 11.46	1.53 ± 3.75	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
4	Aphelenchoididae	0 ± 0	81.81 ± 36.92	40.56 ± 24.01	12.86 ± 22.27	628.95 ± 885.93	121.19 ± 175.06	0 ± 0	103.15 ± 85.06	22.32 ± 34.75	0 ± 0	89.67 ± 68.48	31.41 ± 36.11
5	Aporcelaimidae	36.73 ± 24.75	157.44 ± 34.83	85.71 ± 34.57	41.33 ± 42.24	508.89 ± 535.76	129.82 ± 132.20	0 ± 0	95.77 ± 73.48	21.47 ± 36.98	0 ± 0	111.72 ± 78.21	18.34 ± 33.30
6	Bastianiidae	0 ± 0	27.21 ± 29.62	9.54 ± 8.29	0 ± 0	56.16 ± 97.27	11.46 ± 18.01	0 ± 0	10.96 ± 9.64	1.31 ± 3.36	0 ± 0	15.86 ± 16.52	2.48 ± 5.61
7	Belondiridae	0 ± 0	4.67 ± 8.09	0.68 ± 1.57	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	Bunonematidae	0 ± 0	3.72 ± 6.44	0.34 ± 1.12	0 ± 0	15.47 ± 26.79	2.76 ± 5.34	0 ± 0	10.56 ± 11.41	1.75 ± 3.92	0 ± 0	6.53 ± 11.30	0.84 ± 2.05
9	Cephalobidae	0 ± 0	189.99 ± 122.02	53.98 ± 48.42	82.12 ± 39.93	984.90 ± 1610.07	**316.28 ± 281.76**	0 ± 0	233.08 ± 145.70	68.14 ± 90.49	0 ± 0	485.03 ± 384.75	119.32 ± 152.76
10	Diphtherophoridae	6.42 ± 11.13	28.44 ± 4.19	16.58 ± 6.96	4.53 ± 7.84	105.42 ± 182.59	27.73 ± 4.53	0 ± 0	56.02 ± 60.27	7.11 ± 16.74	0 ± 0	13.23 ± 19.19	4.33 ± 6.08
11	Diplogasteroididae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3120.98 ± 1101.67	**313.78 ± 933.26**	0 ± 0	659.23 ± 1141.81	**125.64 ± 219.62**
12	Dolichodoridae	0 ± 0	4.06 ± 7.03	0.37 ± 1.22	0 ± 0	40.74 ± 70.56	3.70 ± 12.28	0 ± 0	11.03 ± 10.59	1.00 ± 3.32	0 ± 0	16.26 ± 28.27	1.48 ± 4.90
13	Hoplolaimidae	0 ± 0	61.86 ± 35.80	8.65 ± 19.69	0 ± 0	38.68 ± 27.06	3.52 ± 11.66	0 ± 0	21.43 ± 22.94	1.95 ± 6.46	0 ± 0	10.33 ± 9.20	0.94 ± 3.11
14	Leptonchidae	5.42 ± 4.71	100.49 ± 71.35	57.61 ± 30.40	0 ± 0	60.99 ± 56.64	27.47 ± 24.19	0 ± 0	12.58 ± 5.86	1.83 ± 4.22	0 ± 0	36.18 ± 25.24	3.35 ± 10.89
15	Mononchidae	13.97 ± 12.95	51.01 ± 51.55	26.60 ± 13.67	0 ± 0	40.59 ± 28.06	17.40 ± 13.84	0 ± 0	54.81 ± 49.85	8.16 ± 16.62	0 ± 0	10.33 ± 9.20	1.24 ± 3.17
16	Myolaimidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	6.85 ± 11.86	0.62 ± 2.06	0 ± 0	17.58 ± 23.19	1.60 ± 5.30
17	Neodiplogasteridae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1185.55 ± 1183.52	**255.27 ± 422.61**	0 ± 0	1524.55 ± 1366.20	**239.23 ± 490.39**
18	Panagrolaimidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	89.39 ± 154.82	8.13 ± 26.95
19	Paratylenchidae	34.04 ± 58.96	122.49 ± 212.16	62.73 ± 25.40	0 ± 0	13.58 ± 23.52	1.77 ± 4.30	0 ± 0	0 ± 0	0 ± 0	0 ± 0	23.49 ± 40.69	4.49 ± 7.82
20	Plectidae	78.34 ± 6.26	157.83 ± 58.51	**119.01 ± 28.19**	66.11 ± 5.78	215.48 ± 142.29	124.64 ± 48.37	0 ± 0	198.16 ± 33.02	44.94 ± 71.12	0 ± 0	74.71 ± 35.67	24.42 ± 29.33
21	Pratylenchidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18.11 ± 31.36	1.65 ± 5.46	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
22	Prismatolaimidae	7.09 ± 12.27	34.55 ± 32.81	23.61 ± 9.17	0 ± 0	43.05 ± 60.58	19.22 ± 12.13	0 ± 0	29.51 ±± 39.02	6.04 ± 9.95	0 ± 0	40.72 ± 12.58	8.57 ± 15.07
23	Qudsianematidae	30.58 ± 34.18	144.87 ± 70.13	81.14 ± 32.85	35.97 ± 34.25	218.23 ± 184.68	90.43 ± 51.12	0 ± 0	102.55 ± 37.43	15.36 ± 31.08	0 ± 0	69.45 ± 39.10	14.18 ± 25.45
24	Rhabditidae	13.76 ± 14.04	256.02 ± 105.68	**91.52 ± 68.39**	6.72 ± 11.64	1255.77 ± 1694.54	**206.95 ± 358.58**	0.48 ± 0.43	22,593.33 ± 8263.91	**3041.63 ± 6597.45**	12.68 ± 18.23	1664.86 ± 2659.80	**635.40 ± 506.46**
25	Teratocephalidae	25.18 ± 8.82	121.81 ± 37.29	54.25 ± 26.12	25.28 ± 9.37	77.52 ± 80.77	50.07 ± 17.95	0 ± 0	39.36 ± 29.51	10.51 ± 15.09	0 ± 0	55.86 ± 59.75	14.40 ± 19.18
26	Thornenematidae	17.47 ± 19.89	41.68 ± 28.82	26.01 ± 6.68	0 ± 0	66.24 ± 72.00	18.13 ± 17.98	0 ± 0	18.14 ± 7.73	3.77 ± 6.02	0 ± 0	9.37 ± 16.22	1.10 ± 2.86
27	Tripylidae	14.03 ± 24.30	78.91 ± 86.53	37.95 ± 18.13	3.36 ± 5.82	96.04 ± 97.66	36.92 ± 26.15	0 ± 0	9.86 ± 17.08	1.11 ± 2.99	0 ± 0	11.42 ± 19.78	1.57 ± 3.71
28	Tylenchidae	153.88 ± 81.94	370.02 ± 96.04	**288.07 ± 68.32**	329.92 ± 133.20	712.90 ± 490.46	**509.90 ± 120.69**	0 ± 0	437.90 ± 187.88	111.86 ± 146.83	0 ± 0	364.89 ± 183.40	

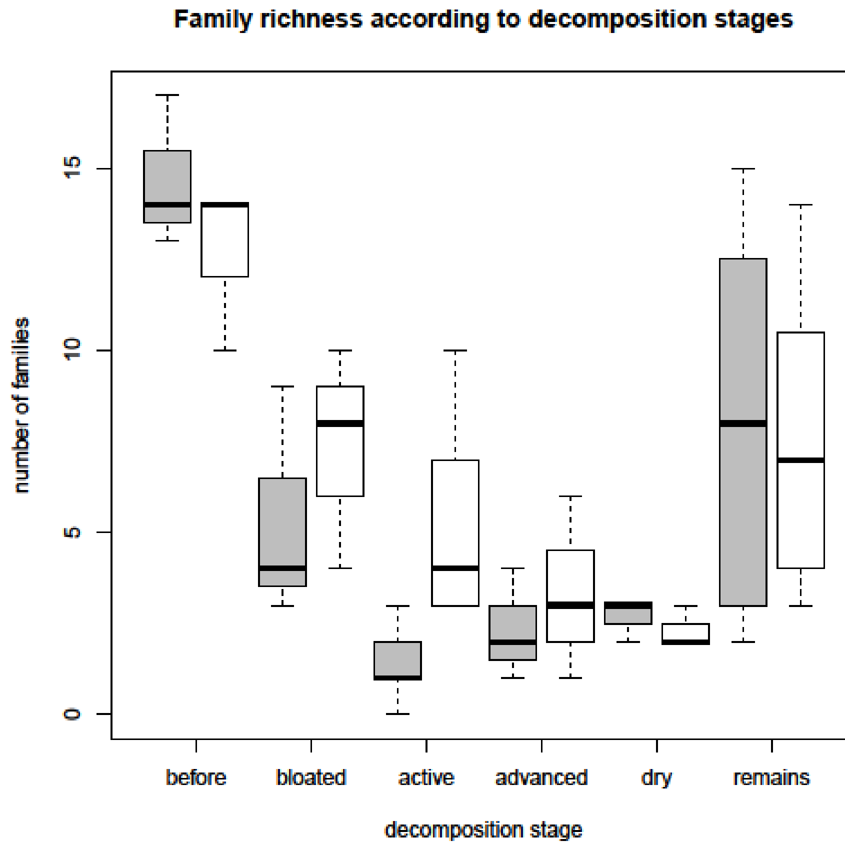

Figure 6. Boxplot showing the number of nematode families according to the decomposition stages in the ground pig treatment (grey boxes) and in the hanging pig treatment (white boxes) at the Bois-du-Clos experimental site (Neuchâtel, Switzerland). White box =interquartile range (IQR); Solid black bar = median; Error bar = maximum (top)/minimum (bottom) value excluding outliers; Open circle = outliers.

4. Discussion

4.1. Shifts in nematode density and community structure in response to cadaver decomposition

Nematode density, diversity and community structure are known to vary among ecosystems in relation to differences in soil type, climate and vegetation (Boag and Yeates, 1998), and we therefore hypothesised that we would observe clear changes in response to decomposing cadavers. In the control and fake pig samples nematode density was somewhat lower than the values reported in a similar habitat by Bongers and Bongers (1998), i.e., ca. 1400 vs. ca. 3000 ind. 100 g^{-1} soil, and did not change during the course of the experiment. The communities in the control and fake pig samples were dominated by bacterial-feeding nematodes (Cephalobidae, Plectidae and Rhabditidae) and herbivores (Tylenchidae) which are frequently dominant in soils

(Yeates, 2003). A similar community structure from a study of spruce stands in south-west Germany was reported by Ruess (1995). Other studies have shown that, amongst bacterial feeders, Cephalobidae were the most abundant group in soils, and Plectidae were indicative of stressed natural environments (Yeates, 2003).

In contrast to the stability of nematode density observed in the control plots, we observed a clear peak in density in both cadaver treatments on D15 which corresponded to the active decay phase. Bacterial feeding nematodes, primarily Rhabditidae, are known to respond positively to nutrient enrichment (e.g., water or organic matter) compared to other bacterivores such as the Cephalobidae (Bongers, 1990; Ferris and Matute, 2003; Georgieva et al., 2005; Blanc et al., 2006, Steel et al., 2010). The increase in Rhabditidae abundance, which we observed in the later stages of decomposition in response to the nutrient pulse and associated enhanced bacterial density, is in line with several recent studies (Metcalf et al., 2013, Carter et al., 2015, Weiss et al., 2016). Rhabditidae (c-p 1 nematodes) are typically r-strategists, with rapid population increases typically recorded two to three weeks after manure addition to soil (Bongers and Ferris, 1999). This rapid increase may also partly be explained by the fact that the Rhabditidae can be transported to new food sources by phoresy on scavenger insects (Bongers and Ferris, 1999). For example, *Rhabditis stammeri* is known to live exclusively on cadavers and is transported by insects, especially the burying beetle *Nicrophorus vespilloides* (Richter, 1993).

Overall, hanging pigs had less impact on soil communities than ground pigs during the active decay phase. Furthermore, the first density peak at D15 was somewhat lower as compared to the ground pigs. The impact of hanging pigs was due to the so-called drip zone, a secondary site of activity after the cadaver itself (Shalaby et al., 2000), which is not in direct contact with the soil.

The decline of nematode density (notably to almost zero in the ground pig treatments) towards the end of the active decay stage (D22) was most likely due to a hostile environment caused by cadaveric fluids and the associated change in microclimatic conditions (i.e., higher temperature). Rhabditidae are known to be vulnerable to thermal stress (Venette and Ferris, 1997), and cadavers with associated insect larval masses can generate temperatures up to 50 °C, with the difference between ambient and cadaver temperatures exceeding 20 °C (Charabidze et al., 2011). When microbial activity decreases, Rhabditidae stop feeding and form survival stages, i.e., dauer

larvae (Bongers and Bongers, 1998). Although the decrease on D22 was observed in all plots of the ground pig treatment, in two of the five plots the peak in nematode abundance was not apparent on D15, explaining the large error bar in Fig. 3. Since an increase in nematode abundance was not observed at later time points, it might have occurred before D15, but would then not have been recorded given our sampling intervals of one week.

In the hanging pig treatment, the active decay stage lasted significantly longer, probably mainly because the larvae that fell to the ground were unable to regain the carcass, which slowed down the removal of biomass (Shalaby et al., 2000). This is in accordance with the delayed density decrease to almost zero under the hanging pig treatment on D36. Shalaby et al. (2000) also observed a similar delay in the progression of decomposition of hanging cadavers compared to ground cadavers due to cooling effects of the air and the inability to form large heat-generating maggot masses, resulting in reduced numbers of Diptera larvae and the inability of ground-dwelling arthropods to access the carcass. This reduced micro-environmental impact of the hanging cadavers has likely also contributed to the weaker responses of nematode communities in our study.

By D36 the density of Rhabdititae had slowly started to increase again in both cadaver treatments. As the active decay stage terminated, Diptera larvae had partly migrated from the carcass, and the impact from the cadavers was clearly declining. Increased rainfall at the end of July and beginning of August 2013 (Fig. 1) further contributed to improved conditions for Rhabditidae (Sohlenius, 1985). A second, though not significant peak in nematode density at D123 in the cadaver treatments corresponded to the remains stage which was dominated by three families Rhabditidae, Neodiplogasteridae and Diplogasteroididae, the latter two were only observed in the cadaver treatments. Like Rhabditidae, the Diplogasteroididae and Neodiplogasteridae may disperse over relatively long distances by phoresy (Steel et al., 2013). Whereas the Rhabditidae are bacterial feeders (Yeates et al., 1993), Neodiplogasteridae and Diplogasteroididae are also known to prey on other nematodes (Von Lieven and Sudhaus, 2000; Steel at al. 2011) which may have hindered a re-occurring dominance of Rhabditidae at D123. In both cadaver treatments, nematode density decreased after the second peak (D263) and thereafter was more or less stable until the end of the experiment.

4.2. Feeding groups

Apart from bacterial feeders the other trophic groups disappeared completely in the ground pig treatment (omnivores at D8, fungivores at D15, and herbivores and predators at D22) and remained absent until D123. Higher-trophic level nematodes such as predators and omnivores are considered more sensitive to environmental changes than lower-trophic level nematodes such as bacterial and plant-feeders (Bongers and Bongers, 1998; Šalamún et al., 2012; Thakur et al., 2014). By D263 all trophic groups were again present, indicating that the communities were recovering from the stressed environment.

4.3. Colonizer-persister (c-p) groups

The above-mentioned findings were reflected in the c-p groups, i.e., in the ground pig treatment c-p groups 2-5 were only present at the beginning (D0 and D8) and the end of the experiment (D263-D367), and were completely absent in between (i.e. D15-D123 for c-p 2-4 and D22-D123 for c-p 5). Steel et al. (2010) found a shift in dominance from c-p 1 to c-p 2 in compost succession between 22 and 29 days. In our study the initial highly enriched and then unfavourable conditions probably made it difficult for c-p 2 nematodes to become established, even after the collapse of the c-p 1 group. The comparatively longer generation time of c-p 2 taxa likely also prevented them from reaching high abundance. The absence of c-p 3-5 (persisters) is unsurprising given their known sensitivity to disturbance, e.g., the release of cadaveric fluids and increase in temperature (Ferris and Bongers, 2009).

4.4. Nematode diversity and maturity indices

Whereas diversity, richness and maturity indices did not change over time in the control and fake pig samples, they were all clearly affected by the presence of the cadavers. Nematode diversity, family richness and maturity index (MI) were significantly reduced in the cadaver treatments. Though caution should be exercised in the use of univariate statistics to describe nematode communities (Wall et al., 2002), high nematode diversity is considered to be indicative of relatively stable conditions (Bongers and Bongers, 1998), while stress causes a decrease in nematode family richness and diversity resulting in the dominance of a few taxa (Georgieva et al., 2002). Previous

studies have observed a decrease in MI under disturbed conditions (Bongers, 1999; Ettema and Bongers, 1993; Georgieva et al., 2002). In the cadaver treatments, MI was significantly reduced until the end of the experiment, in contrast to the other nematode indices (family, trophic group and c-p group) which showed signs of recovery.

4.5. Composts as analogues to cadaver impacts?

To our knowledge this is the first study exclusively focusing on cadaver impacts on soil nematodes, which makes it difficult to compare our results to previous studies. However, shifts in nematode communities have been well studied in composts and plant litter decomposition (Georgieva et al., 2005; Steel et al., 2010; 2012; 2013). Decomposing cadavers and composts are similar in that they both release considerable amounts of nutrients and are characterised by well-defined phases in the decomposition process. We would thus expect some similarities between temporal patterns of nematode community changes in response to cadavers and compost. However, the higher nutrient content of cadavers as compared to wood and plant litter, and the difference in the stoichiometry of the released fluids, can be expected to drive contrasting patterns of community changes.

At the beginning of the composting process there is a clear dominance of bacterivorous enrichment opportunists (Rhabditidae, Panagrolaimidae) and bacterivorous/ predacious Diplogasteridae, followed by bacterivorous (Cephalobidae) and fungivorous general opportunists (Aphelenchoididae, Anguinidae) in the subsequent stages (Steel et al., 2010; 2013). The dominance of Rhabditidae at the beginning of the decomposition process, and the later occurrence of diplogasterids, was also observed in our study. In the ground pig treatment Neodiplogasteridae were first observed on D15, then disappeared and reappeared on D59 after the end of the active decay phase. Delayed occurrence of Neodiplogasteridae in composts, compared to soil where they are abundant from the beginning of the decomposition processes (Georgieva et al., 2005), is due to the relatively high and lethal temperatures (ca. 40 °C) at initiation of the composting process (Steel et al., 2010).

Thus, despite similarities in the community dynamics there are also clear differences. In our study, there was no increase or replacement of Rhabditidae by Cephalobidae beneath the decomposing cadavers. Cephalobidae are, apart from soil, rather

common in litter and rotting plant tissues and might not favour the altered soil conditions caused by cadaveric fluids. Additionally, the increase in abundance of fungal feeders during the maturation phase of the composting process (Steel et al., 2010; 2013) was not documented in our study where no increase in fungal feeders was observed, be it in the ground or hanging pig treatment over the course of the experiment. This comparison shows that the patterns of change in nematode communities are only partly similar between composts and cadaver-impacted soils.

4.6. Conclusions

We expected to observe very clear responses of soil nematodes to decomposing cadavers, and this was indeed the case. Decomposing cadavers modified the soil environment and allowed previously absent or rare taxa to reach high densities, thus clearly altering the communities of soil nematodes. Interestingly, such changes were already obvious at the relatively crude taxonomic resolution we used (i.e. family level), but may be even clearer if a higher resolution were used (e.g. using molecular approaches). The clear contrast in all metrics used suggests that nematodes could be used as a tool to document the presence of cadavers and the time since death. Further studies in different climatic conditions, seasons, vegetation and soil types will, however, be necessary before such a tool can be validated. It would also be useful to assess if a higher taxonomic resolution could give more precise results. Also, the impact of different nutrient sources (e.g. faeces, urine) and perturbation (e.g. fire, physical disturbance) should be compared more systematically to determine if the indicators we identified are indeed specific to decomposing cadavers.

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5. References*

*citation style kept according to the Journal

[1] Bardgett, R.D., Speir, T.W., Ross, D.J., Yeates, G.W., Kettles, H.A., 1994. Impact of pasture contamination by copper, chromium, and arsenic timber preservative on soil microbial properties and nematodes. Biology and Fertility of Soils 18, 71–79.

[2] Barton, P.S., Cunningham, S.A., Lindenmayer, D.B., Manning, A.D., 2013. The role of carrion in maintaining biodiversity and ecological processes in terrestrial ecosystems. Oecologia 171, 761–772.

[3] Beasley, J.C., Olson, Z.H., Devault, T.L., 2012. Carrion cycling in food webs: comparisons among terrestrial and marine ecosystems. Oikos 121, 1021–1026.

[4] Benninger, L.A., Carter, D.O., Forbes, S.L., 2008. The biochemical alteration of soil beneath a decomposing carcass. Forensic Science International 180, 70–75.

[5] Blanc, C., Sy, M., Djigal, D., Brauman, A., Normand, P., Villenave, C., 2006. Nutrition on bacteria by bacterial-feeding nematodes and consequences on the structure of soil bacterial community. European Journal of Soil Biology 42, S70–S78.

[6] Boag, B., Yeates, G.W., 1998. Soil nematode biodiversity in terrestrial ecosystems. Biodiversity and Conservation 7, 617–630.

[7] Bongers, T., 1990. The maturity index: an ecological measure of environmental disturbance based on nematode species composition. Oecologia 83, 14–19.

[8] Bongers, T., 1994. De nematoden van Nederland: een identificatietabel voor de in Nederland aangetroffen zoetwater- en bodembewonende nematoden. Koninklijke Nederlandse Natuurhistorische Vereniging, Utrecht.

[9] Bongers, T., 1999. The Maturity Index, the evolution of nematode life history traits, adaptive radiation and cp -scaling. Plant and Soil 212, 13–22.

[10] Bongers, T., Bongers, M., 1998. Functional diversity of nematodes. Applied Soil Ecology 10, 239–251.

[11] Bongers, T., Ferris, H., 1999. Nematode community structure as a bioindicator in environmental monitoring. Trends in Ecology & Evolution 14, 224–228.

[12] Brown, D.J.F., Boag, B., 1988. An examination of methods used to extract virus-vector nematodes (Nematoda: Longidoridae and Trichodoridae) from soil samples. Nematologia mediterranea 16, 93-99.

[13] Campobasso, C.P., Di Vella, G., Introna, F., 2001. Factors affecting decomposition and Diptera colonization. Forensic Science International 120, 18–27.

[14] Carter, D.O., Tibbett, M., 2008. Cadaver decomposition and soil: processes. In: Tibett, M., Carter, D. (Eds.), Soil analysis in forensic taphonomy. CRC Press, Boca Raton, pp. 29–52.

[15] Carter, D.O., Yellowlees, D., Tibbett, M., 2007. Cadaver decomposition in terrestrial ecosystems. Naturwissenschaften 94, 12–24.

[16] Carter, D.O., Metcalf, J.L., Bibat, A., Knight, R., 2015. Seasonal variation of postmortem microbial communities. Forensic Science, Medicine and Pathology 11, 202-207.

[17] Cesarz, S., Reich, P.B., Scheu S., Ruess, L., Schaefer M., Eisenhauer, N., 2015. Nematode functional guilds, not trophic groups, reflect shifts in soil food webs and processes in response to interacting global change factors. Pedobiologia 58, 23-32.

[18] Chambers, J.M., Freeny, A., Heiberger, R.M., 1992. Analysis of variance; designed experiments. In: Chambers, J.M., Hastie, T.J. (Eds.), Statistical Models in S. CRC Press, Boca Raton, pp. 145–193.

[19] Charabidze, D., Bourel, B., Gosset, D., 2011. Larval-mass effect: characterisation of heat emission by necrophageous blowflies (Diptera: Calliphoridae) larval aggregates. Forensic Science International 211, 61–66.

[20] Clark, M.A., Worrell, M.B., Pless, J.E., 1997. Postmortem changes in soft tissues. In: Haglund, W.D., Sorg, M.H. (Eds.), Forensic taphonomy: the postmortem fate of human remains. CRC Press, Boca Raton, pp. 151–164.

[21] DeVault, T.L., Rhodes, Jr., O.E., Shivik, J.A., 2003. Scavenging by vertebrates: behavioral, ecological, and evolutionary perspectives on an important energy transfer pathway in terrestrial ecosystems. Oikos 102, 225–234.

[22] Ettema, C.H., Bongers, T., 1993. Characterization of nematode colonization and succession in disturbed soil using the Maturity Index. Biology and Fertility of Soils 16, 79–85.

[23] Ferris, H., Bongers, T., 2009. Indices developed specifically for analysis of nematode assemblages. In: Wilson, M.J., Khakouli-Duarte, T. (Eds.), Nematodes as environmental indicators. CABI, Oxfordshire, pp. 124–145.

[24] Ferris, H., Matute, M.M., 2003. Structural and functional succession in the nematode fauna of a soil food web. Applied Soil Ecology 23, 93–110.

[25] Fielding, D., Newey, S., van der Wal, R., Irvine, R.J., 2014. Carcass provisioning to support scavengers: evaluating a controversial nature conservation practice. AMBIO 43, 810–819.

[26] Forbes, S.I., 2008. Decomposition chemistry in a burial environment. In: Tibbett, M., Carter, D.O. (Eds.), Soil analysis in forensic taphonomy. CRC Press, Boca Raton, pp. 203–223.

[27] Georgieva, S., Christensen, S., Stevnbak, K., 2005. Nematode succession and microfauna–microorganism interactions during root residue decomposition. Soil Biology & Biochemistry 37, 1763–1774.

[28] Georgieva, S.S., McGrath, S.P., Hooper, D.J., Chambers, B.S., 2002. Nematode communities under stress: the long-term effects of heavy metals in soil treated with sewage sludge. Applied Soil Ecology 20, 27–42.

[29] Hall, C.L., Wadsworth, N.K., Howard, D.R., Jennings, E.M., Farrell, L.D., Magnuson, T.S., Smith, R.J., 2011. Inhibition of microorganisms on a carrion breeding resource: the antimicrobial peptide activity of burying beetle (Coleoptera: Silphidae) oral and anal secretions. Environmental Entomology 40, 669–678.

[30] Hopkins, D.W., Wiltshire, P.E.J., Turner, B.D., 2000. Microbial characteristics of soils from graves: an investigation at the interface of soil microbiology and forensic science. Applied Soil Ecology 14, 283–288.

[31] Hugot, J.-P., Baujard, P., Morand, S., 2001. Biodiversity in helminths and nematodes as a field of study: an overview. Nematology 3, 199–208.

[32] Korthals, G.W., Alexiev, A.D., Lexmond, T.M., Kammenga, J.E., Bongers, T., 1996. Long-term effects of copper and pH on the nematode community in an agroecosystem. Environmental Toxicology and Chemistry 15, 979–985.

[33] Legendre, P., Legendre, L., 1998. Numerical Ecology, Elsevier Science, Amsterdam.

[34] Megyesi, M.S., Nawrocki, S.P., Haskell, N.H., 2005. Using accumulated degree-days to estimate the postmortem interval from decomposed human remains. Journal of Forensic Sciences 50, 618-626.

[35] Metcalf, J.L., Parfrey, L.W., Gonzalez, A., Lauber, C.L., Knights, D., Ackermann, G., Humphrey, G.C., Gebert, M.J., Van Treuren, W., Berg-Lyons, D., Keepers, K., Guo, Y., Bullard, J., Fierer, N., Carter, D.O., Knight, R., 2013. A microbial clock provides an accurate estimate of the postmortem interval in a mouse model system. eLife 2, e01104.

[36] Metcalf, J.L., Xu, Z.Z., Weiss, S., Lax, S., Van Treuren, W., Hyde, E.R., Song, S.J., Amir, A., Larsen, P., Sangwan, N., Haarmann, D., Humphrey, G.C., Ackermann, G., Thompson, L.R., Lauber, C., Bibat, A., Nicholas, C., Gebert, M.J., Petrosino, J.F., Reed, S.C., Gilbert, J.A., Lynne, A.M., Bucheli, S.R., Carter, D.O., Knight, R., 2016. Microbial community assembly and metabolic function during mammalian corpse decomposition. Science 351, 158–162.

[37] Neher, D.A., Campbell, C.L., 1994. Nematode communities and microbial biomass in soils with annual and perennial crops. Applied Soil Ecology 1, 17–28.

[38] Olsen, S.R., Cole, C.V., Watanabe, F.S., Dean, L.A., 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. United States Department of Agriculture, Circular No .939, 1-19.

[39] Parmenter, R.R., MacMahon, J.A., 2009. Carrion decomposition and nutrient cycling in a semiarid shrub-steppe ecosystem. Ecological Monographs 79, 637–661.

[40] Payne, J.A., 1965. A summer carrion study of the baby pig *Sus scrofa* Linnaeus. Ecology 46, 592–602.

[41] Putman, R.J., 1978. Patterns of carbon dioxide evolution from decaying carrion decomposition of small mammal carrion in temperate systems 1. Oikos 31, 47–57.

[42] R Development Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org

[43] Richter, S., 1993. Phoretic association between the dauerjuveniles of Rhabditis stammeri (Rhabditidae) and life history stages of the burying beetle Nicrophorus vespilloides (Coleoptera: Silphidae). Nematologica 29, 346-355.

[44] Ritz, K., Trudgill, D.L., 1999. Utility of nematode community analysis as an integrated measure of the functional state of soils: perspectives and challenges. Plant and Soil 212, 1–11.

[45] Ritz, K., Black, H.I.J., Campbell, C.D., Harris, J.A., Wood, C., 2009. Selecting biological indicators for monitoring soils: a framework for balancing scientific and technical opinion to assist policy development. Ecological Indicators 9, 1212–1221.

[46] Ruess, L., 1995. Nematode fauna in spruce forest soils: a qualitative/quantitative comparison. Nematologica 41, 106–124.

[47] Šalamún, P., Renco, M., Kucanová E., Brázová, T., Papajová, I., Miklisová D., Hanzelová, V., 2012. Nematodes as bioindicators of soil degradation due to heavy metals. Ecotoxicology 21, 2319-2330.

[48] Scheiner, J.D., 2005. Spéciation du carbone, de l'azote et du phosphore de différentes boues de stations d'épuration au cours de leurs incubations contrôlées dans deux types de sol, thèse pour obtenir le diplôme de docteur de l' Institut National Polytechnique de Toulouse, Toulouse, pp 60-64.

[49] Schoenly, K., Reid, W., 1987. Dynamics of heterotrophic succession in carrion arthropod assemblages: discrete seres or a continuum of change? Oecologia 73, 192–202.

[50] Scholze, V.S., Sudhaus, W., 2011. A pictorial key to current genus groups of "Rhabditidae." Journal of Nematode Morphology and Systematics 14, 105–112.

[51] Shalaby, O.A., DeCarvalho, L.M.L., Goff, M.L., 2000. Comparison of patterns of decomposition in a hanging carcass and a carcass in contact with soil in a xerophytic habitat on the Island of Oahu, Hawaii. Journal of Forensic Sciences 45, 1267–1273.

[52] Sieriebriennikov, B., Ferris, H., de Goede, R.G.M., 2014. NINJA: an automated calculation system for nematode-based biological monitoring. European Journal of Soil Biology 61, 90–93.

[53] Smith, R.J., Merrick, M.J., 2001. Resource availability and population dynamics of Nicrophorus investigator, an obligate carrion breeder. Ecological Entomology 26, 173–180.

[54] Sohlenius, B., 1985. Influence of climatic conditions on nematode coexistence: a laboratory experiment with a coniferous forest soil. Oikos 44, 430-438.

[55] Spray, C.M., Widdowson, E.M., 1950. The effect of growth and development on the composition of mammals. British Journal of Nutrition 4, 332–353.

[56] Steel, H., de la Peña, E., Fonderie, P., Willekens, K., Borgonie, G., Bert, W., 2010. Nematode succession during composting and the potential of the nematode community as an indicator of compost maturity. Pedobiologia 53, 181–190.

[57] Steel, H., Moens, T., Scholaert, A., Boshoff, M., Houthoofd, Bert, W., 2011. Mononchoides composticola n.sp. (Nematoda: Diplogastridae) associated with composting processes: morphological, molecular and autecological characterization. Nematology 13, 347-363.

[58] Steel, H., Vandecasteele, B., Willekens, K., Sabbe, K., Moens, T., Bert, W., 2012. Nematode communities and macronutrients in composts and compost-amended soils as affected by feedstock composition. Applied Soil Ecology 61, 100–112.

[59] Steel, H., Verdoodt, F., Čerevková, A., Couvreur, M., Fonderie, P., Moens, T., Bert, W., 2013. Survival and colonization of nematodes in a composting process. Invertebrate Biology 132, 108–119.

[60] Swift, M.J., Heal, O.W., Anderson, J.M., 1979. Decomposition in terrestrial ecosystems. University of California Press, Berkeley and Los Angeles.

[61] Szelecz, I., Fournier, B., Seppey, C., Amendt, J., Mitchell, E., 2014. Can soil testate amoebae be used for estimating the time since death? A field experiment in a deciduous forest. Forensic Science International 236, 90–98.

[62] Thakur, M.P., Reich, P.B., Fisichelli, N.A., Stefanski, A., Cesarz, S., Dobies, T., Rich, R.L., Hobbie, S.E., Eisenhauer, N., 2014. Nematode community shifts in response to experimental warming and canopy conditions are associated with plant community changes in the temperate-boreal forest ecotone. Oecologia 175, 713–723.

[63] Tortora, G.J., Grabowski, S.R., 2000. Principles of anatomy and physiology, 9th ed. Wiley, New York.

[64] Towne, E.G., 2000. Prairie vegetation and soil nutrient responses to ungulate carcasses. Oecologia 122, 232–239.

[65] Vass, A.A., 2001. Beyond the grave - understanding human decomposition. Microbiology Today 28, 190–192.

[66] Vass, A.A., Bass, W.M., Woldt, J.D., Foss, J.E., Ammons, J.T., 1992. Time since death determinations of human cadavers using soil solution. Journal of Forensic Sciences 37, 1236–1253.

[67] Vass, A.A., Barshick, S.-A., Sega, G., Caton, J., Skeen, J.T., Love, J.C., Synstelien, J.A., 2002. Decomposition chemistry of human remains: a new methodology for determining the postmortem interval. Journal of Forensic Sciences 47, 542–553.

[68] Venette, R.C., and Ferris, H., 1997. Thermal constraints to population growth of bacterial-feeding nematodes. Soil Biology & Biochemistry 29, 63–74.

[69] Von Lieven, A.F., Sudhaus, W., 2000. Comparative and functional morphology of the buccal cavity of Diplogastrina (Nematoda) and a first outline of the phylogeny of this taxon*. Journal of Zoological Systematics and Evolutionary Research 38, 37-63.

[70] Wall, J.W., Skene, K.R., Neilson, R., 2002. Nematode community and trophic structure along a sand dune succession. Biology and Fertility of Soils 35, 293–301.

[71] Weiss, B., Larink, O., 1991. Influence of sewage sludge and heavy metals on nematodes in an arable soil. Biology and Fertility of Soils 12, 5–9.

[72] Weiss, S., Carter, D.O., Metcalf, J.L., Knight, R., 2016. Carcass mass has little influence on the structure of gravesoil microbial communities. International Journal of Legal Medicine 130, 253-263.

[73] Yang, L.H., 2008. Pulses of dead periodical cicadas increase herbivory of American bellflowers. Ecology 89, 1497–1502.

[74] Yeates, G.W., 1999. Effects of plants on nematode community structure. Annual Review of Phytopathology 37, 127–149.

[75] Yeates, G.W., 2003. Nematodes as soil indicators: functional and biodiversity aspects. Biology and Fertility of Soils 37, 199–210.

[76] Yeates, G.W., Orchard, V.A., Speir, T.W., 1995. Reduction in faunal populations and decomposition following pasture contamination by a Cu-Cr-As based timber preservative. Acta Zoologica Fennica 196, 297–300.

[77] Yeates, G.W., Ferris, H., Moens, T., Van der Putten, W.H., 2009. The role of nematodes in ecosystems. In: Wilson, M.J., Kakouli-Duarte, T. (Eds.), Nematodes as environmental indicators. CABI, Oxfordshire, pp. 1–44.

[78] Yeates, G.W., Bongers, T., De Goede, R.G.M., Freckman, D.W., Georgieva, S.S., 1993. Feeding habits in soil nematode families and genera-an outline for soil ecologists. Journal of Nematology 25, 315–331.

[79] Yeates, G.W., Orchard, V.A., Speir, T.W., Hunt, J.L., Hermans, M.C.C., 1994. Impact of pasture contamination by copper, chromium, arsenic timber preservative on soil biological activity. Biology and Fertility of Soils 18, 200–208.

[80] Zhang, M., Liang, W.-J., Zhan, X.-K., 2012. Soil nematode abundance and diversity in different forest types at Changbai Mountain, China. Zoological Studies 51, 619–626.

Table S1. Density of all nematodes in 100g -1 dry soil at the Bois-du –Clos experimental site (Neuchâtel, Switzerland) in the control, fake, ground and hanging pig treatments per sampling day: minimum (min), maximum (max) and average (ave) density ± standard deviation (SD).

	control			fake			ground			hanging		
day	min	max	ave±sd	min	max	ave±sd	min	max	ave±sd	min	max	ave±sd
0	776	1765	1162±421	707	2320	1475±651	642	1197	933±260	446	1119	874±274
8	1323	2308	1615±412	1008	2806	1624±710	452	895	631±206	410	3352	1639±1095
15	1143	2861	1939±824	1328	2483	1918±507	1	28144	13780±13755	1607	26175	8261±10531
22	621	3936	1720±1291	715	5688	2360±1940	0	9	4±4	13	4879	1313±2065
36	1053	2407	1505±533	1016	5616	2258±1894	6	137	47±54	4	3690	804±1618
59	863	1200	1029±131	675	1370	1155±294	195	11156	3442±4381	491	3103	1600±1057
84	889	1711	1168±327	426	1764	1270±561	829	5974	3063±2133	211	6127	2914±2554
123	1011	1251	1125±104	563	1630	1225±452	3666	7382	5453±1753	2154	5737	3867±1460
263	1304	1922	1454±264	870	2331	1330±589	829	3306	1838±931	571	1940	992±542
331	877	1963	1312±491	859	1762	1339±345	905	1479	1235±251	444	1924	1305±565
367	1040	1862	1346±309	989	1757	1381±324	903	2266	1457±555	979	1880	1405±425

Supplementary material

Table S2

Range (lowest and highest value) of soil properties (pH, Phosphorous (P), Nitrogen (N), Ammonium (NH_4^+), Nitrate (NO_3^-) at the Bois-du-Clos experimental site (Neuchâtel, Switzerland) in the control, fake, ground and hanging pig treatments.

treatment	pH	P [$\mu g\, g^{-1}$]	N (%)	NH_4^+ [$\mu g\, g^{-1}$]	NO_3^- [$\mu g\, g^{-1}$]
control	5.17 - 7.02	1.9 - 34	0.45 - 0.95	0.21 - 5.59	2.42 - 9.43
fake	4.71 - 6.5	0.56 - 114.41	0.31 - 1.55	0.2 - 11.25	2.31 - 160.04
ground	4.63 - 8.76	11.2 - 713.41	0.57 - 2.17	0.4 - 312.36	3.03 - 52.74
hanging	4.79 - 8.7	16.27 - 724.42	0.57 - 2.78	0.2 - 224.94	1.44 - 62.34

Chapter 5

Comparative analysis of bones, mites, soil chemistry, nematodes and soil micro-eukaryotic communities of a suspected homicide to estimate a long post-mortem interval

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Comparative analysis of bones, mites, soil chemistry, nematodes and soil micro-eukaryotic communities of a suspected homicide to estimate a long post-mortem interval

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Abstract

Criminal investigations of suspected murder cases require estimating the post-mortem interval (PMI, or time after death) which is challenging for periods longer than a few weeks. Here we present the case of human remains found in a Swiss forest. We have used a multidisciplinary approach involving the analysis of bones, soil chemical characteristics, mites and nematodes (by microscopy) and micro-Eukaryotes (by Illumina high throughput sequencing). We analysed soil samples collected beneath the remains of the head, upper and lower body and “control” samples taken a few meters away in four cardinal directions. The PMI estimated on hair ^{14}C-data via bomb peak radiocarbon dating gave a time range of 1 to 2 years before the finding of the remains on site. Cluster analyses for chemical constituents, nematodes, mites and micro-

Eukaryotes revealed two clusters 1) head and upper body and 2) lower body and controls. From mite evidence, we conclude that the body was likely to have been brought to the site after death. However, chemical analyses, nematode community analyses and the analyses of micro-Eukaryotes indicate that decomposition took place at least partly on site. This study illustrates the usefulness of combining several lines of evidence for the study of homicide cases and calls for more studies on cadaver impacts on soil organisms to better calibrate PMI inference tools.

1. Introduction

The estimation of a long-term post-mortem interval (PMI) i.e. the time since death has been a research priority in forensic science for over a century since the pioneering work of Mégnin (1894), who defined the decomposition stages of corpses for the first time. However, precise PMI estimation is still a challenge and there is a demand for reliable tools.

Cadaver decomposition can be seen as a continuum of several stages: fresh, bloated, active decay, advanced decay, dry and remains (Payne, 1965). The duration of each stage depends mainly on temperature, humidity and scavenger access to the cadaver (Campobasso et al., 2001; Mann et al., 1990; Megyesi et al., 2005). Currently PMI is mainly estimated based on a medical assessment that relies on the physical changes of the dead body occurring in the first hours up to days and/or entomological evidence, a well-established method applied to periods of up to several weeks or months (Amendt et al., 2007). Although insects can be related to all decomposition stages (Anderson and VanLaerhoven, 1996; Sharanowski et al., 2008), the accuracy of PMI estimation decreases over time (Amendt et al., 2011). Chemical- based methods such as citrate content of the bone or radiocarbon dating can also be useful (Schwarcz et al., 2010). However, currently there is no reliable PMI estimation method for human remains that have already reached the dry and remains stages.

Additionally, there is a demand for more precise PMI estimation methods focusing on outdoor environments e.g. in the Institute of Legal Medicine in Frankfurt am Main, Germany, 51 corpses with a long post-mortem interval were checked for entomological evidence in the years 2014-2016 and 20 % originated from outdoor environments (V. Bernhardt personal communication). Therefor various studies have focused on the effects of cadaver decomposition on the underlying soil as new venues for gathering forensic evidence. These studies monitored the changes in soil chemistry (Benninger et al., 2008; Carter et al., 2007; Melis et al., 2007; Towne, 2000) and in the community structure of soil micro-organisms (Metcalf et al., 2016; Pechal et al., 2013), bacteria (Cobaugh et al., 2015; Parkinson et al., 2009; Pechal et al., 2014), fungi (Carter and Tibbett, 2003; Hawksworth and Wiltshire, 2011; Parkinson et al., 2009), testate amoebae (Seppey et al., 2016; Szelecz et al., 2014), nematodes (Szelecz et al., 2016) and micro-arthropods (Braig and Perotti, 2009; Merritt et al., 2007; Perez et al., 2014; Perotti and Braig, 2009; Perotti et al., 2010; Perotti et al., 2009;). While the relevance

of mites for criminal investigations is well-established (Braig and Perotti, 2009; Perotti and Braig, 2009), studies of other potential forensic indicators are still rare and comparative studies are lacking. Soil arthropods such as springtails (Collembola) (Merritt et al., 2007; Perez et al., 2014) and ants (Moretti and Ribeiro, 2006) were also proven useful for PMI estimation. More recently, high-throughput sequencing (HTS) of soil organisms has been used to develop new forensic tools (Finley et al., 2015; Metcalf et al., 2013; Seppey et al., 2016). These methods may each provide complementary information and PMI estimations should thus become more robust when several methods are combined. Indeed, inferring forensic evidence from a decomposing cadaver is challenging and the whole picture may only appear clearly when several independent lines of evidence are combined.

In the present work, five different approaches were applied to a case study, aiming to reconstruct the crime scene. Human bones were found in a forest area in the Swiss midlands. Bones were examined to determine gender and age of the deceased and to suggest a minimum PMI. Soil samples were collected and analysed for selected chemical markers, for nematode and mite diversity (based on morphology), and micro-Eukaryotes (using high throughput DNA sequencing: metabarcoding) to assess possible differences in community structure.

2. Material and methods

2.1. Case history and sampling

Human bones were found in a small forest in the Swiss midlands (open case: exact date and site omitted). The site is located approximately 20 meters away from a vehicle-accessible forest track and has sparse tree cover. A village is located approximately 2 km in linear distance.

The ground was covered with dry leaves and the bones were partly covered with leaves and small branches. After their removal, the bones showed signs of thermal destruction. Some burned tree trunks with a diameter of approx. 20 cm to 30 cm and bigger branches were arranged in a square approximately 2 m x 2.5 m around the bones. These branches -originally covering the bones showed signs of charring and had been removed by the finder. The burnt branches were partially overgrown by moss. The surrounding area was searched for further bones and everything was

transferred to the Department of Physical Anthropology (University of Bern) for examinations. Additionally, some dark hair and residues of different fabrics, a key, some coins and jewellery were found.

Soil samples (soil cores of 10 cm in diameter by 10 cm depth) were taken from the area where the bones were found i.e. head (H), upper body (UB) and lower body (LB) and from control areas not impacted by the cadaver or the fire in the four cardinal directions, 4-6.5m from the body: North (CN), South (CS), East (CE) and West (CW) (Fig. 1).

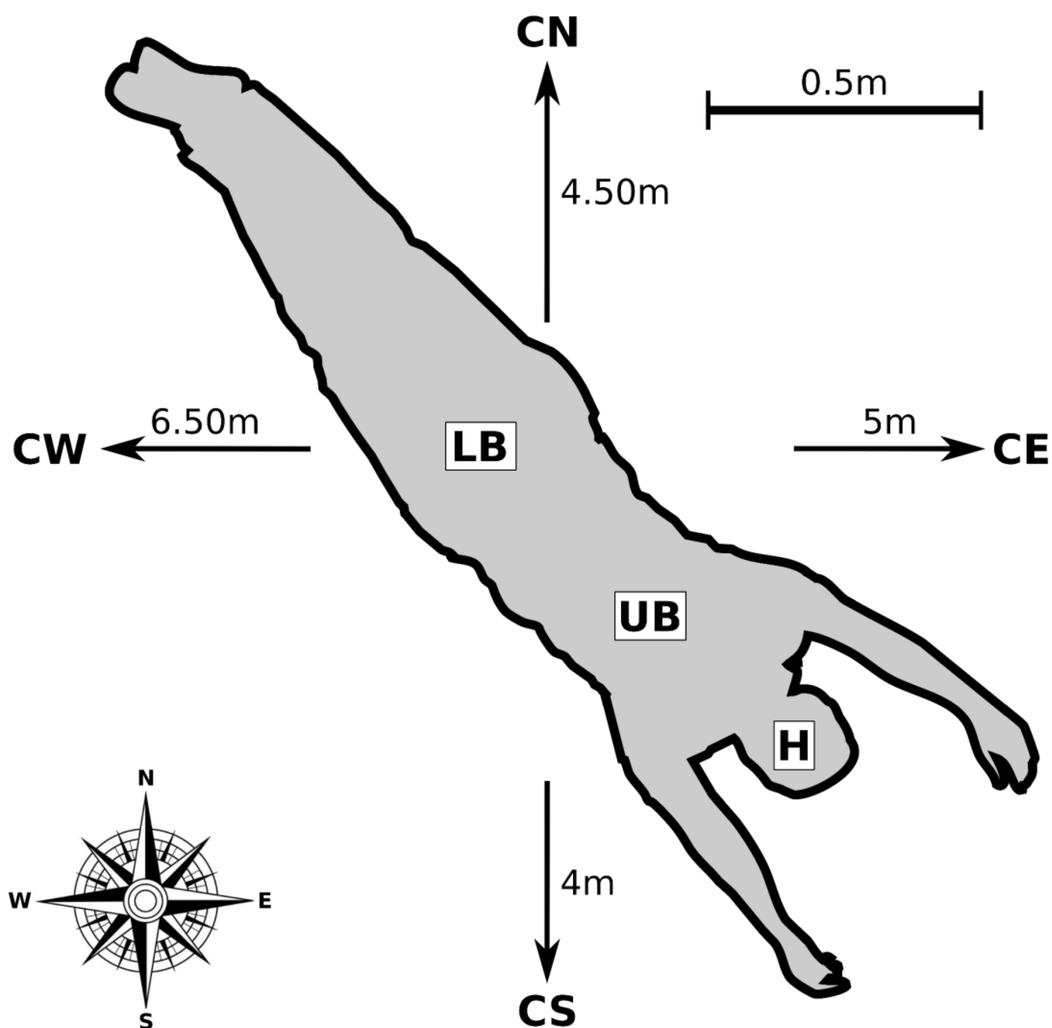


Figure 1. Soil sampling area from a criminal case investigation in Switzerland.

2.2. Bone analyses

The human bones which did not show severe thermal destruction were investigated anthropologically in the Department of Physical Anthropology at the Institute of Forensic Medicine in Bern, Switzerland according to Acsádi and Nemeskéri (1970), Rösing et al. (2007), Sjøvold (1988) and Szilvássy (1988).

A 5.1 g sample of the femoral diaphysis without any visible thermal destruction was sent for radiocarbon dating to CEZ Archäometie gGmbH, Mannheim, Germany. Additionally, 2.0 gram of hair was also sent for radiocarbon dating in order to perform a bomb peak application (Wild et al., 2000). Bone collagen was extracted from the femur (Ambrose, 1990; Longin, 1971) and the hair was carefully cleaned and measured via MICADAS-accelerator. The calibration was performed with CALIBomb (Reimer et al. 2004).

2.3. Soil chemical analyses

Soil pH was measured with a pH metre after diluting the sample in water in a 1:2.5 proportion. Ammonium and nitrate analyses were performed directly after sampling using colorimetric determination (Scheiner, 2005). Total carbon and nitrogen was determined using a CHN analyser (Thermo Finnigan Flash EA 1112) on dry, ground soil. Bioavailable phosphorus content was determined by colorimetric analysis according to the Olsen method (Olsen et al., 1954). Mg^{2+} and Ca^{2+} contents were determined using inductively coupled plasma optical emission spectrometry (Perkin-Elmer Optima 3300 DV ICP-OES) preceded by a cation exchange capacity extraction (CEC, Cobaltihexamine method). Potential chemical soil markers were classified into (1) early peak markers (EPM) that show the highest concentrations early in decomposition until advanced decay following degradation to (2) late peak markers (LPM), during the dry and remains stage. Some of them might show (3) late elevated levels (LEL).

2.4. Nematodes

Nematodes were extracted from 100 g of soil using a modified Baermann funnel technique according to the protocol of Brown and Boag (1988). Nematodes were

counted alive using a dissecting microscope (Olympus SZ51) and then fixed with heated formaldehyde (4%) and heat-killed at 65 °C for 3 minutes. One hundred randomly chosen nematodes per sample were identified to family level using an upright light microscope at 400x magnification (Axio Lab.A1, Zeiss). Identification was based on the identification guides of Bongers (1994) and Scholze and Sudhaus (2011). All nematode densities are given in individuals per 100gdw.

2.5. Mites

A 500 ml soil sample was collected from each sampling site and placed on Tullgreen-Berlese funnels to extract moving mites into a collector jar with 70% Ethanol (following the method use by Saloña et al., 2010). Mites were cleaned from the collector jars, cleared for permanent mounting in Hoyer (Krantz, 1978) and identified using a variety of keys and descriptions (Bregetova et al., 1977; Evans and Browning, 1956; Evans et al., 1961; Evans and Till, 1979; Hughes, 1976; Hyatt, 1980; Hyatt, 1990). A voucher number was assigned to all microscope slides and they were deposited in the Forensic Acarology Reference Collection, Acarology Lab, University of Reading.

2.6. Soil micro-Eukaryotes

To screen the eukaryotic diversity of the seven soil samples, the V9 fragments of the small sub-unit ribosomal RNA (SSU rRNA) was amplified and sequenced. The DNA was extracted using the MoBio PowerSoil isolation kit following the manufacturer`s instruction. The V9 fragments were then amplified following the protocol of Amaral-Zettler et al. (2009) and the amplicons sequenced with an Illumina HiSeq2000® sequencer (Fasteris, Geneva, Switzerland).

The resulting V9 sequences were then processed through the bioinformatic pipeline. Sequences were removed when the average phred score of a 50 nucleotides window was below 20. Chimera were also discarded using the program Uchime (v. 7.0.1090) (Edgar et al., 2011) by comparing sequences between them and between the PR2 database (Guillou et al., 2013). The sequences were then clustered into OTUs using the software Swarm (v. 1.2.12) (Mahe et al., 2014) with the default parameters. The OTUs were finally taxonomically assigned by aligning the dominant sequence of each OTU to the PR2 database using the program Ggsearch36 (Fasta package v. 36.3.6

http://faculty.virginia.edu/wrpearson/fasta/CURRENT/) with the default parameters. OTUs assigned to prokaryotes were removed from the analysis as well as those assigned to Metazoa or Embryophyceae in order to avoid bias which would be caused by a piece of those macro-organisms in the soil sample.

2.7. Numerical analysis

Chemical variables were scaled and community matrices (eukaryotes, nematodes and mites) were Hellinger-transformed (Legendre and Gallagher, 2001). As metabarcoding data are prone to yield a huge proportion of rare OTUs, we discarded OTU representing less than 0.5‰ of the Hellinger-transformed matrix. A multiple factor analysis (MFA) (package FactoMineR v. 1.31.4 (Husson et al., 2015) was then computed with the four data matrices to assess the correlative structure of the data. A hierarchical clustering was also performed with Euclidean distances on the chemical variables and with Bray-Curtis distances on each of the three community matrices.

Assessed the bioindicator value of each taxon or OTU in the three communities data set using an indicator species analysis (function indval; package labdsv 1.6-1) (Dufrene and Legendre, 1997). For this analysis, we used two groups based on the results of the four clusterings and the first dimension of the MFA: 1) head and upper body, and 2) lower body and controls. OTUs were selected as bioindicator if the p-value of their indicator value was under 0.05 after 10000 iterations. As the number of OTUs was high, many were significant in the IndVal analysis. To keep the number manageable, we only retained those that have both a significant Indval and showed a clear pattern in the first dimension of the MFA.

3. Results

3.1. Bone analyses

The condition and the preservation of the bones were low due to signs of thermal destruction caused by a fire (Fig. 2). Particularly affected by the fire were the arms including hands and the legs including feet. Parts of the pelvis and skull were present allowing a sex determination as male. An age at death was estimated to be between 18 to 25 years using combined methods (Acsádi and Nemeskéri, 1970; Brothwell,

1981; Rösing et al., 2007; Ubelaker et al., 2006). The ^{14}C in the collagen of the femur was integrated approximately between the years 2002-2004 (1 sigma). The ^{14}C in the hair was integrated approximately in the last 3 years before the investigation (approximately 2012-2014, 1 sigma).

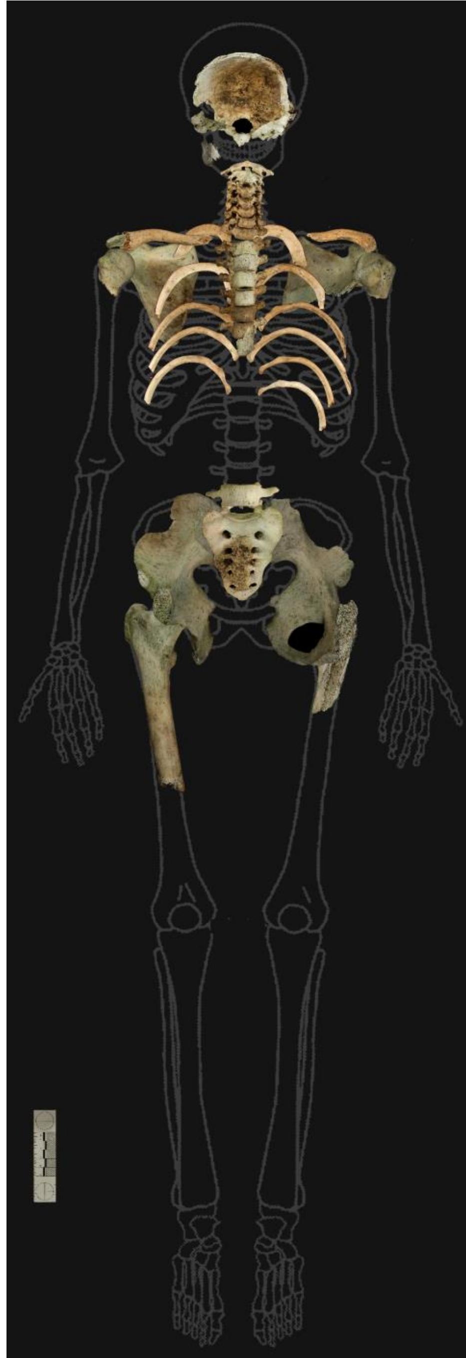


Figure 2. Identified bones from a criminal case investigation in Switzerland.

3.2. Chemical analyses

Soil bioavailable P content was higher beneath the head (192.54 µg/g) and upper body (147.99 µg/g) than under the lower body (LB) and control samples (ranging from 17.52µg/g in control West to 65.87µg/g in control South, Table 1). Total N was at least twice higher under the head as compared to other samples (Table 1). Other soil chemical markers (pH, NH_4^+, NO_3^-, Mg^{2+}, Ca^{2+} and C content) did not differ between the control and cadaver samples (Table 1). The cluster analysis separated the samples into two clusters: 1) head and upper body and 2) controls and lower body (Fig. 3a).

Table 1. Chemical constituents in soil samples taken beneath human remains and in controls (open case: site and year omitted)

chemical consituents	Head H	Upper Body UB	Lower Body LB	Control North CN	Control South CS	Control West CW	Control East CE
pH	7.65	7.25	7.34	7.17	7.34	7.24	6.97
NH_4^+ [ug/g]	3.2	0.6	1.1	1.3	3.2	1.3	0.9
NO_3^- [ug/g]	47.52	52.88	44.81	65.07	54.12	47.43	61.52
N [%]	2.86	1.15	1.09	1.19	1.35	0.54	0.61
C [%]	27.79	15.22	18.5	22.74	22.69	9.4	10.71
H [%]	2.19	1.52	0.65	0	0.99	1.17	1.23
Pbio [ug/g]	192.54	147.99	31.41	50.05	65.87	17.52	22.86
Mg^{2+} [mg/l]	0.03	0.03	0.04	0.04	0.04	0.01	0.01
Ca^{2+} [mg/l]	0.66	0.518	0.802	0.816	0.879	0.386	0.5

3.3. Nematodes

Nematode density under the cadaver was highest beneath the head (2488 ind./100g dry soil), lower under the upper body (1653 ind./100g) and lowest under the lower body (1192 ind./100g), the latter value being within the range of the control samples (982-1402 ind./100g). In total 19 nematode families were identified (Table 2). Diversity was highest under the lower body and in the control samples (12-14 families), 9 of which being present in all of these samples (Table 2). By contrast, only 5 and 6 families were present beneath the head and the upper body respectively (Table 2). Bacterial feeding Cephalobidae, Rhabditidae and Plectidae were the three most abundant families and occurred in all samples (Table 2). The cluster analysis separated the samples into two clusters: 1) head and upper body and 2) controls and lower body (Fig. 3c). In the PCA the sample from beneath the head was separated from all other samples (Fig. 3d). Bacterial feeding Cephalobidae and Myolaimidae were most abundant in the sample

beneath the head. The Myolaimidae were only found beneath the head and were further identified as *Myolaimus sp.* (Figs. 3i, j). Tylenchidae (herbivorous), Qudsianematidae (predators, omnivorous) and Prismatolaimidae (bacterivorous) were associated with the controls North and South and the lower body samples (Figs. 3i, j). Control West and East were associated with Teratocephalidae (bacterivorous) and Leptonchidae (fungivorous) (Figs. 3i, j). Control West was associated with the bacterivorous Alaimidae. Four nematode families (Alaimidae, Prismatolaimidae, Qudsianematidae, Tylenchidae) were considered as indicators for controls and lower body (Fig. 4).

Table 2. Density of nematode families in $100g^{-1}$ dry soil beneath human remains and in controls (open case: site and date omitted).

nematode taxa	feeding groups	Head H	Upper Body UB	Lower Body LB	Control North CN	Control South CS	Control West CW	Control East CE
Alaimidae	bacterial feeding	0	0	36	20	14	20	84
Bastianiidae	bacterial feeding	0	0	0	20	0	10	14
Bunonematidae	bacterial feeding	0	0	12	0	0	0	14
Cephalobidae	bacterial feeding	1319	727	489	150	389	147	140
Myolaimidae	bacterial feeding	547	0	0	0	0	0	0
Plectidae	bacterial feeding	75	463	167	120	209	79	112
Prismatolaimidae	bacterial feeding	0	0	72	20	14	39	28
Rhabditidae	bacterial feeding	522	281	60	100	83	128	280
Teratocephalidae	bacterial feeding	0	66	24	120	111	157	168
Aphelenchoididae	fungal feeding	0	0	36	0	0	0	28
Leptonchidae	fungal feeding	0	99	107	50	139	128	252
Thornenematidae	omnivorous	0	0	0	20	28	10	0
Aporcelaimidae	animal predator, omnivorous	25	17	48	0	125	10	28
Qudsianematidae	animal predator, omnivorous	0	0	95	120	28	88	98
Mononchidae	animal predator	0	0	0	0	0	20	42
Diphtherophoridae	plant feeding	0	0	0	0	28	39	0
Pratylenchidae	plant feeding	0	0	12	0	14	0	0
Tylenchidae	plant feeding	0	0	24	230	181	108	112
Tylodoridae	plant feeding	0	0	12	30	28	0	0

3.4. Mites

A total of 391 mites belonging to four Acari orders (Astigmata, Mesostigmata, Prostigmata and Oribatida) were identified from the seven sampling sites. The most diverse and numerous were the Mesostigmata (N = 130, 13 species) followed by the Astigmata (N = 126, 5 species). A number of soil mites found in the control samples, such as Oribatida and Prostigmata were only discerned as morphotypes (Table 3).

Most mites were found in the adult stage, with the exception of members of the Astigmata. The Acaridae *Sancassania berlesei* (= *Caloglyphus berlesei*), *S. oudemansi* (= *Caloglyphus oudemansi*), and the Histiostomatidae unidentified sp. 1,

which sum up 123 specimens, were sampled in their immature, phoretic hypopial form. Interestingly, Astigmata were extracted only from the sample sites associated with bones i.e. from the soil beneath the head, upper body and lower body (Table 3). These samples also contained the greatest richness of Mesostigmata.

Considering all seven samples, three Mesostigmata species frequently found in forest soil were the most numerous, the detritivorous Uropodidae unidentified sp. 1 (N = 55), *Prozercon traegardhi* (N = 13) and the predaceous *Vulgarogamasus sp.* (N = 23). A particular soil mite morphospecies of the Oribatida, Oppiidae, Oppiella was highly abundant and 100% prevalent.

In terms of the singularity of sample diversity, sample sites and mite species aggregated into three defined clusters. Upper body and head were different from each other and from the remaining five samples, which group together (Fig. 3f). The clustering further indicated that the upper body and head were the two most distinctive samples (Fig. 3e). These two samples contained rare species, which are not typical inhabitants of forest soil. The taxa found in these samples rather correspond to poultry farm or granary species such as *S. berlesei* (hypopi), which is also a predominant species in corpses (especially in advanced decay or mummification stages), accompanied by a specialised predator of these unique farming environments, *Macrocheles matrius* (Figs. 3i, j).

Table 3. Total counts of mites from soil samples (500ml) beneath human remains and in controls (open case: site and date omitted).

mites taxa			Head H	Upper Body UB	Lower Body LB	Control North CN	Control South CS	Control West CW	Control East CE	total
Astigmata	Acaridae	*Sancassania berlesei*	13	108	0	0	0	0	0	121
		Sancassania oudemansi	1	0	0	0	0	0	0	1
		unidentified sp.	1	0	0	0	0	0	0	1
	Glycyphagidae	*Glycyphagus bicaudatus*	1	0	1	0	0	0	0	2
	Histiostomatidae	unidentified sp.	0	1	0	0	0	0	0	1
Mesostigmata	Gamasida	unidentified sp.	0	2	0	0	0	0	0	2
	Laelapidae	*Stratiolaelaps (Hypoaspis) miles*	3	0	5	0	0	0	0	8
	Macrochelidae	*Macrocheles matrius*	1	0	0	0	0	0	0	1
	Melicharidae	unidentified sp.	0	1	0	0	0	0	0	1
	Pachylaelapidae	*Pachylaelaps longisetus*	2	0	0	0	0	0	0	2
		Pachylaelaps pectinifer	0	0	1	0	0	0	0	1
	Parasitidae	*Leptogamasus sp.*	3	0	4	0	0	0	0	7
		Vulgarogamasus sp.	4	0	0	16	0	3	0	23
		Parasitus unidentified	0	0	0	6	0	0	0	6
	Rhodacaridae	*Rhodacarellus*	4	0	0	0	0	0	0	4
	Uropodidae	unidentified sp.	0	0	18	17	17	2	1	55
	Zerconidae	*Prozercon traegardhi*	1	3	0	7	0	0	2	13
		Mixozercon sellnicki	5	0	0	0	0	2	0	7
Prostigmata	unidentified	unidentified sp.	0	0	0	11	8	0	0	19
Oribatida	unidentified	unidentified sp.	0	0	0	4	1	5	0	10
	Oppiidae	*Oppiella*	1	12	17	33	11	24	8	106

3.5. Micro-Eukaryotes

The metabarcoding revealed 386 OTUs on a total of 648'344 sequences. Globally, soils were dominated by Fungi, with a majority of Basidiomycota in the control/lower body samples versus a large dominance of Mucoromycotina in the head/upper body samples. Likewise, taxa containing many small bacterivores (Heterolobosea, Chrysophyceae, Tubulinea, Cercozoa) were relatively more abundant in these samples. Variosea (Amoebozoa) as well as the parasitic Apicomplexa were in turn more abundant under the control/lower body samples.

3.6. Multiple Factor Analysis (MFA), hierarchical clustering and Indicator Value

The first dimension showed a clear dichotomy between the head/upper body and control/ lower body sample groups (Fig. 3i). This pattern is in line with the separate analyses of the three community matrices (Figs. 3d, f, h) and by the cluster analyses (Figs. 3a, c, e, g). This similarity among results is also shown by the RV coefficients which reveal a significant correlation among the three matrices (Table 4). By contrast, the chemical variables were only correlated with the nematodes data.

A total of 97 variables (three chemical variables, 79 eukaryotic OTUs, seven mite taxa and eight nematode families) were characteristic of the first dimension (Fig. 3j). From the 97 variables characteristic of the first dimension, the majority were well associated with the head/upper body samples (3/3 chemical variables, 61% of micro-eukaryotic OTUs, 2/8 nematodes families and 86% of mite taxa).

The IndVal analyses revealed 18 eukaryotic OTUs and the mite species *Sancassania berlesei* as head/upper body indicators, while indicators for controls/lower body indicators, included 53 eukaryotic OTUs, four nematode families (Alaimidae, Prismatolaimidae, Qudsianematidae, Tylenchidae) and two mite families (Uropodidae, Oppiidae) (Fig. 4).

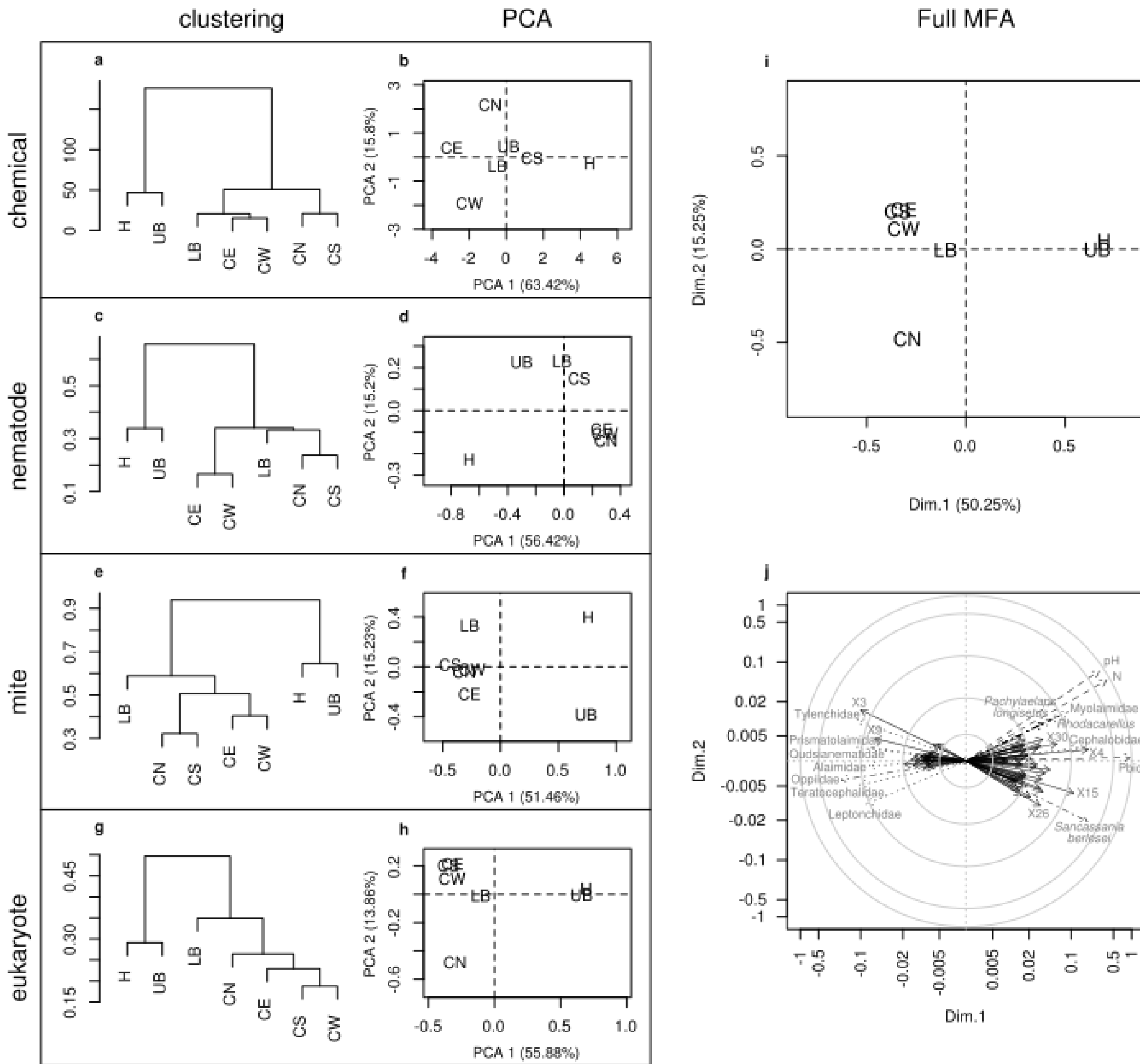


Figure 3. Multiple factor analysis (MFA) on chemical variables and community matrices of nematodes, mites and of micro-Eukaryotes from soil samples taken beneath human remains and controls in a forest area in Switzerland. On the left part the projections of the samples according to the clustering (a,c,e,g) and the PCA (b,d,f,h) calculated on chemical variables (a,b), nematode families (c,d), mites taxa (e,f) and micro-eukaryotic OTUs (g,h) are represented. On the right part of the figure the projection of the samples according to the global analysis (i) and the correlation circle (j) of the most characteristic variable ($P < 0.05$) of the first and second dimensions are shown. The four variable types are represented with bold italic font and dashed arrows for chemical variables, bold font and dotted arrows for nematodes families, italic font and dot dash arrows for mite taxa and normal font and plain arrows for micro-eukaryotic OTUs. In order to improve readability, only names of variables with a distance between the origin and their coordinate higher than 0.05 were shown. The correlation circle is shown on a log scale because of the difference between coordinates from eukaryotic and chemical data.

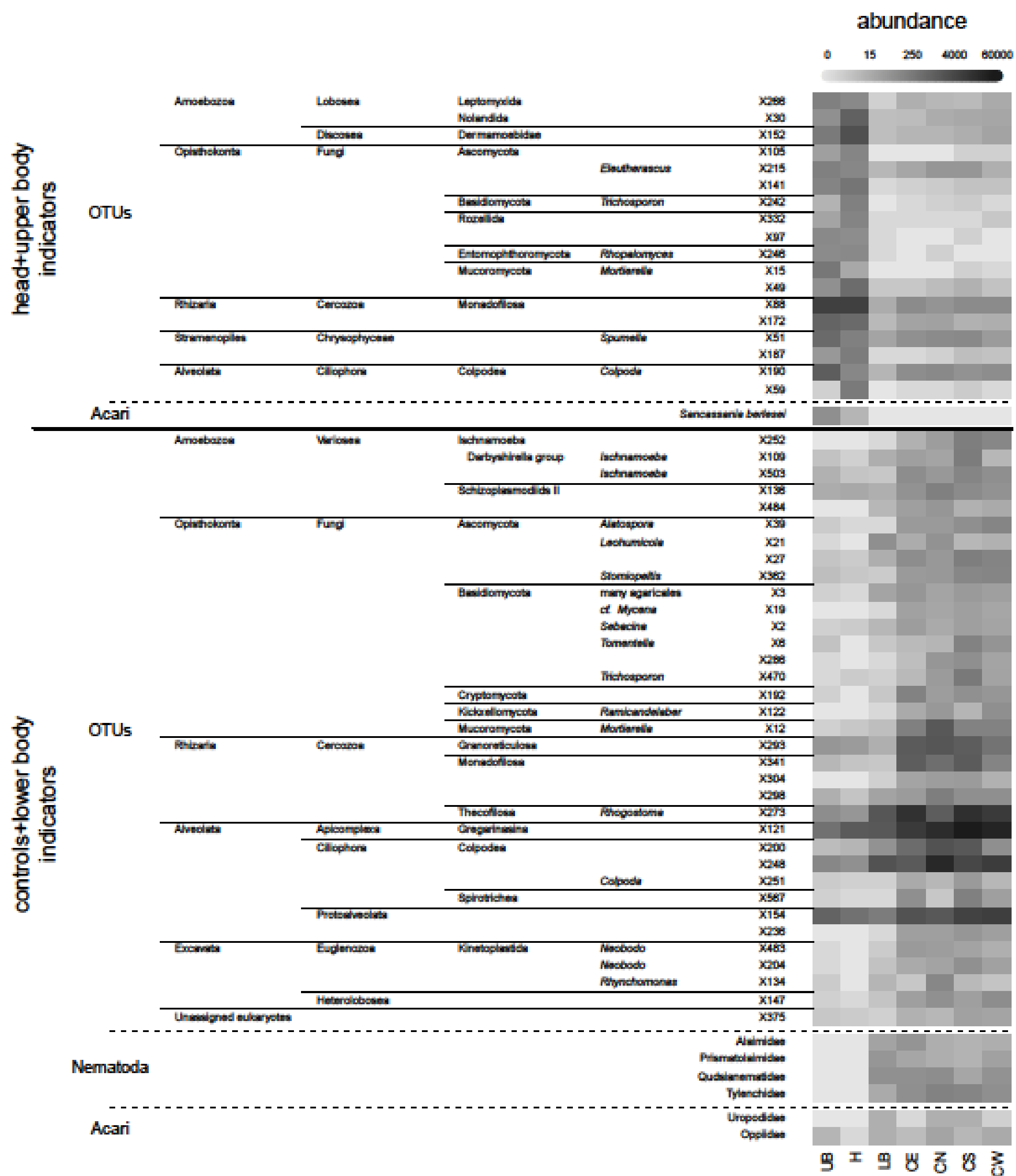


Figure 4: Abundance of the bioindicators of head and upper body (H/UB), and controls and lower body (C/LB) from a criminal case investigation in Switzerland. For each group the eukaryotic bioindicators are sorted according to their taxonomic assignation followed by nematodes families and mite taxa.

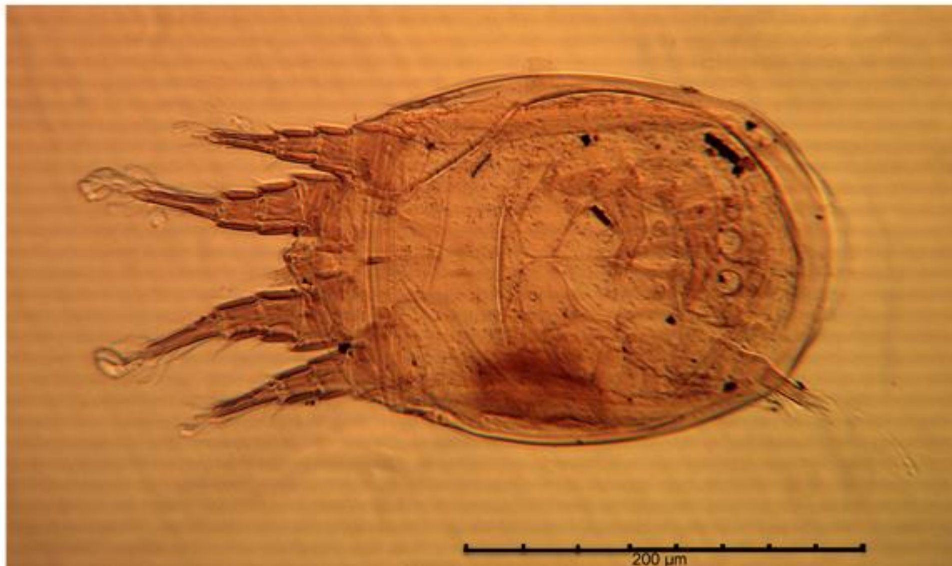


Figure 5. A hypopus of *Sancassania berlesei* from the head sample site (H) from a criminal case investigation in Switzerland.

Table 4. RV coefficients from multifactor analysis (MFA) calculated on standardized chemical variables and Hellinger transformed matrices of micro-eukaryotes, nematodes and mites taken from soil samples beneath human remains and controls in a forest area in Switzerland. The lower half matrix shows the RV coefficients (in bold) between pairs of matrices while the upper right half matrix (not bold) shows the significance of the corresponding coefficient. “MFA” row and column represent the RV coefficient and p-value between each group of variables and the global model.

RV coefficients	chemical components	nematodes	mites	eukaryotes	MFA
chemical components	-	0.0169	0.1297	0.0949	0.0020
nematodes	**0.7741**	-	0.0097	0.0075	0.0123
mites	**0.5774**	**0.8755**	-	0.0103	0.0851
eukaryotes	**0.5938**	**0.8949**	**0.9446**	-	0.0613
MFA	**0.8036**	**0.9709**	**0.9329**	**0.9421**	-

4. Discussion

The bone analyses (PMI estimated via bomb peak radiocarbon dating) gave a time range of 1 to 2 years before the finding of the remains on site (hair ^{14}C-data). From the anthropological investigation, it was also assumed that the bones could not have been lying outside for less than one year due to the lack of fatty appearance, no filling of the medullary cavity of the femur, no smell and no soft tissue (Berg and Protsch von Zieten, 1998) except of the hair, which was found in the earth right beneath the occipital bone (Os occipetale). Although changes in taphonomy, in particular a faster decomposition due to the thermal destruction (fire) cannot be excluded.

When interpreting the $F^{14}C$-data of the collagen, the turnover rates of human bones were based on previous studies (Geyh 2001; Hedges et al. 2007; Ubelaker et al. 2006; Wild et al. 2000). Since this individual was a male with an age of death in the late adolescence or young adult age (based on anthropological bone features), a turnover rate of 10-30% per year (between the age of 10 and 15 years) and 3-1.5% per year (after the age of 20 years) has to be considered (Hedges et al. 2007). Therefore, collagen data are always mixed values containing the carbon signal of the last years of life. Nevertheless, the collagen of the femur has its core area between the years 2002 and 2005. Assuming that most of the incorporated carbon derives from the two first life decades the data are compatible with a young adult.

The individual was subsequently identified via a DNA match in the EDNAIS database, Switzerland. A witness interrogation showed that the individual was last seen in June 2012. All data are thus concordant with this information.

In studies on soil chemistry beneath cadavers a sharp increase of soil nutrient content and pH is generally observed during the active and advanced decay phases of cadaver decomposition (Tibbett and Carter, 2009). This is when the most rapid breakdown of a cadaver takes place (Carter et al. 2007). In this study, bones were found at the stage of dry remains, which does not mean that the concentration of nutrients have returned to basal levels (Carter et al., 2007). According to our grouping (see chapter 2) pH and NH_4^+ are described as early peak markers (EPMs) showing significant increases at the beginning of the decomposition process. The absence of these early peak markers in the samples beneath the remains suggests that the timespan since death was sufficient for EPMs to return to basal levels or that the body had started decomposing elsewhere and was transported to the find site when it had already reached a later decomposition stage i.e. advanced decomposition.

At later decomposition stages, Ca^{2+} and other additional late peak markers (LPMs) such as NO_3^- increase (Anderson et al., 2013; Melis et al., 2007). NO_3^- levels were significantly elevated beneath carcasses one year post-mortem (Anderson et al., 2013) which was confirmed by our studies (see chapter 2). The lack of higher NO_3^- concentration under the cadaver in this study suggests that the PMI was > 1 year. The absence of LPMs such as Ca^{2+} might indicate an upper limit for the PMI as shown by

Melis et al. (2007) who observed elevated Calcium levels at carcass sites from three to six years post-mortem probably released from the bones. However, pattern of soil nutrient response to decomposing cadavers vary among studies (Melis et al., 2007) and thus it is currently not possible to develop a precise PMI estimation method from soil chemical characteristics alone.

The presence of elevated levels for P (head and upper body) and N (head) indicate that the time elapsed since peak decomposition was not long enough for these markers to return to basal levels. Significantly elevated P levels were described one and three years post-mortem (Towne, 2000). N levels beneath large ungulate carcasses were significantly higher two years post-mortem and 10 times higher than in the surrounding soil even three years post-mortem (not significant) (Towne, 2000). Nevertheless, elevated levels of P beneath the head and upper body, plus a higher N concentration beneath the head and the clustering of head and upper body samples might indicate that the decomposition process had at least partly taken place on site. In contrast, the lower body part groups with the controls indicate that decomposition may have been hindered or not taken place in this area. This is in accordance with the findings that the lower body parts were burnt much stronger than the upper body parts (excluding arms). However, this interpretation should be taken cautiously given the small sample size of our study. When applying our marker categories to this case study we have to bear in mind that, despite the fact that pig cadavers have been widely accepted as surrogate human decomposition, differences in anatomy, intestinal bacteria, living conditions and diseases can lead to variations in the decomposition process (Keough et al., 2016; Stokes et al., 2013).

Nematode density in all samples was within the range reported elsewhere for terrestrial nematodes (Bongers and Bongers, 1998; Szelecz et al., 2016) and the three most abundant families that occurred in all samples i.e. Cephalobidae, Rhabditidae and Plectidae are common in soil (Yeates, 2003). The clustering grouped nematode communities from controls and lower body samples together and revealed four indicators: Alaimidae, Prismatolaimidae, Qudsianematidae and Tylenchidae. Nematodes can be classified according to their feeding habits and life-history characteristics (Bongers and Bongers, 1998; Yeates, et al., 1993). Among the indicators Alaimidae and Qudsianematidae are typical K-strategists (persisters) with a

high sensitivity to disturbances (Ferris and Bongers, 2009). Tylenchidae are very tolerant to disturbances, they are r-strategists (colonizers) and frequent in soils (Yeates, 2003). Prismatolaimidae can be considered as intermediate between these two groups being more sensitive than Tylenchidae (Ferris and Bongers, 2009). The presence of nematodes that are sensitive to disturbances in line with a higher family richness indicates that the control and lower body samples might not have been exposed to stress (Szelecz et al., 2016).

On the contrary head and upper body samples were characterized by a low family richness and were dominated by r-strategists i.e. bacterial feeding nematodes that are tolerant to pollutants and organic matter decomposition (Ferris and Bongers, 2009). Especially Myolaimidae which only occur in the head samples were shown to be enrichment opportunists (Bongers et al., 1995). Myolaimidae were further determined as *Myolaimus sp.*, which is rarely recorded in samples from terrestrial habitats (Bärmann et al., 2009). During the composting process *Myolaimus sp.* was restricted to the last phase of the composting process (maturation) indicating that it might have special requirements (Steel et al., 2013). Therefore, *Myolaimus sp.* might be an indicator for a late decomposition stage suggesting that only part of the decomposition process took place in-situ and bones and some flesh remained after the fire to decompose. Nevertheless, in a study on the effect of decomposing pig cadavers on soil nematode communities in the same general region (albeit in a different forest type) Myolaimidae did not occur at all during the whole decomposition process within a one-year post-mortem period (Szelecz et al., 2016). In that study (for details see chapter 3) it was also shown that family richness was still significantly lower 263 post-mortem (Szelecz et al., 2016). Here further studies are necessary. Bearing in mind that comparable data are sparse, a PMI of at least 8-9 months but rather > 1 year is suggested based on the nematode data.

Of the seven samples analysed in this study two stand out due to their unique mite fauna composition, which is exogenous to the forest soil and comprised species of forensic importance (previously found on corpses) and used as trace evidence. Communities from the upper body, followed by head are unique in that they contain the only and highest number of *Sancassania berlesei*, and the only specimens of *Macrocheles matrius*. Both, *S. berlesei* and its main predator, *M. matrius* are

inhabitants of synanthropic habitats, particularly those related to granaries, farms (poultry and pig hay-beddings) and less often agricultural lands (cereal fields). In Europe, these species are found in compost, poultry litter, decayed bulbs and tubers, especially deep litter broiler houses, and have been originally found in stored grains or food products (Emberson, 1972; Hughes, 1976; O´Conner, 2009). Interestingly, *S. oudemansi* (found in the head sample) is even more restricted to synanthropic habitats, poultry settlings or granaries, wherever wheat or other grains abound (Hubert et al., 2006; Hughes, 1976; O'Conner, 2009; White, 1995).

The large numbers of Sancassania all found in hypopial stages, in upper (the body and head) samples indicate: 1) that the human remains and their associated Sancassania (and Macrocheles) mites have originated in one of the aforementioned habitats (e.g. a farm); 2) that a massive *Sancassania* population was living out of the remains; and 3) that this well-developed population was suddenly exposed to very unfavourable environmental conditions (e.g. draught, fire). These facts are strong indicators of movement or relocation of the remains. From the data of the two key sites (upper body and head) it is possible to predict that the original massive mite population very likely built up from (early) active to advance decay, and that at this stage the remains were transported and deposited in their final resting place, the forest soil, more precisely on a limited space or position where the upper body and head bones were later found. This is also explained by the lack of any other forensic markers, characteristic of decay on forest soil. Indeed, there is no acarological evidence indicative of decomposition having taken place on the forest soil.

Sancassania berlesei, like the majority of stored-product mites (Astigmata) are sensitive to humidity; exposure to low humidity levels leads either to death or to moulting into more resistant immature stages commonly known as hypopi (Hughes, 1976; Timms et al., 1982; White, 1995). Optimal conditions imply very high humidity levels; water saturated habitats or exceptionally damp conditions, such as enclosed or sealed habitats. A good example would be a human corpse wrapped, concealed in clothes, inside bags or inside any sort of sealed container able to maintain the damp conditions needed for the mites to thrive (Braig and Perotti, 2009; Early and Goff, 1986; Goff et al., 1986; Leclerq and Verstraeten, 1993; Leclercq and Verstraeten, 1988; Mégnin, 1894; Perotti and Braig, 2009; Saloña et al., 2010). This might explain the well-developed colony of S. berlesei found only in a reduced area or particular patch

of soil (upper body and head sites). Soil under the lower body bones did not contain any forensically relevant mite species. Mites in this area are the same as those found in controls, lower body and clustered together with most controls. This suggests that the lower body site was not the place or location of the 'container' or 'bag' with human remains, and perhaps the bones observed on this area were the product of the movement of the bones by forest scavengers (foxes, dogs, birds, etc.).

Under unfavourable conditions such as draught and lack of food, or food lacking nutritional content, *S. berlesei* will always produce hypopi and most of these hypopi are active forms. Hypopi are second instar nymphs able to disperse mainly (not exclusive) as phoretic on other animals to new, optimal environmental conditions (Houck and O´Connor, 1991; Hughes; 1976; O´Connor, 1982; Perotti and Braig; 2009).

Only a handful of insects have been described as hosts of *S. berlesei* hypopi, particularly Scarabaeidae (chaffers) and Tenebrioniidae (stored product beetles) (O'Connor, 1982; Sarwar et al., 2013; Zakhvatkin, 1941). The unique association with the flour beetle, *Tenebrio molitor*, confirms its habitat specificity to granaries or stored grain facilities. Termination of hypopial forms will occur when both high humidity and food are restored, moulting to the next stage or trinonymph; otherwise they will die as hypopi (Timms et al., 1982). Experiments designed to produce hypopi considered exposure to dry cultures and lack of food (Timms et al., 1982). All 121 *S. berlesei* collected in this case were hypopi (Fig. 5), no adult was found. It means that the massive population went through a bottleneck of unfavourable conditions, like exposure to extreme draught or lack of food. In addition to mites, the two sample sites upper body and head contained the majority of charcoal particles, indicative of fire and the site where the remains might have been burnt. The remains must have been burned exactly in that patch of forest soil. The fire most likely consumed the external layers of the wrapped remains killing most mites of the outer parts. The thickness of the massive population of perhaps thousands to millions of mites, which probably reached several cm, protected the most internal part of the colony from fire. Those hundreds of *S. berlesei* that survived the fire were then exposed to a foreign environment with no food, not enough humidity, and were surrounded by soil predators producing resistant offspring or hypopi.

Remarkably, the presence of a second mite species, a strict inhabitant of farm/granary habitats confirms the origin of the fauna associated with upper body and head.

Macrocheles matrius is a predator, a foreign species to the forest soil and to carcasses/corpses, but specialized in hunting and consuming the acarids of granaries or poultry litter (Emberson, 1972; Evans and Browning, 1956; White, 1995).

In terms of PMI estimation, the massive colony of mites inhabiting the original human remains that reached the forest soil are similar to that of a concealed corpse in its later stage of decomposition, very likely a mummified corpse. Judging by the size of the surviving population of Sancassania hypopi, and the time it takes to mummification of an adult inside a sealed enclosure, it is possible that the killing and concealment of the body happened at least 8-12 months before the remains were disposed on the forest soil. This is due to the life-span of these hypopi which might have struggled to survive in the new habitat and conditions. Mite traces are good indicators of what might have happened from the moment of death until decomposition reached advanced stages, in this case indicating the corpse location and confirming the use of fire.

The presence of a decomposing corpse has clearly modified the micro-Eukaryote communities. The combination of our two statistical analyses (IndVal and MFA) allowed determining a limited number of OTUs that were either typical for (1) head and upper body (H/UB) samples or (2) controls and lower body (C/LB).

General communities differ considerably between head/upper body samples and control/lower body. Basidiomycetes, which dominated the control and lower body samples, are virtually replaced by Mucoromycota in the head and upper body samples. While the first group includes many saprotrophs and mycorrhizae, the second includes typically r-strategists such as *Mortierella spp.* This shift has been observed also in the case of perturbations such as disruption of connections between the root and the fungus (Lindahl et al., 2010). The higher incidence of mainly bacterivorous taxa is associated with higher values of P and N, which are expected to increase bacterial densities.

Soil fungi are the best-studied soil micro-Eukaryotes and thus their community patterns are easiest to interpret. For instance, OTU X242 is affiliated with genus Trichosporon, a fungus which belongs to the normal skin microbiota (Middelhoven, 2004). Members of this genus have been found associated to late stages of decomposition of human bodies (Martinez-Ramirez et al., 2013). OTU X246 (Rhopalomyces) is an exclusive

parasite of nematode eggs (Barron, 1973); its abundance in the head and upper body samples is thus coherent with the high number of nematodes found in these samples. Likewise, the prevalent presence of ectomycorrhizal fungi among the controls and lower body (C/LB) indicators (i.e. OTUs X3, representing several tree mycorrhizae in forests and X362, a Pezizomycotina affiliated to genus Stomiopeltis) was to be expected as these organisms are ubiquitous in forest soils.

Several species of amoeboid protists, known to occur only in stable systems, were found amongst the indicator OTUs of the controls and lower body samples. Amongst them, Variosea (Amoebozoa) from the Ischnamoeba/Darbyshirella clade (X109, X503) are reticulate organisms with very thin and delicate pseudopodia which are typical slow-growing organisms (Berney et al., 2015). Rhogostoma (X273), like many other testate amoebae are also typically K-selected organisms that are supposed to perish underneath cadavers (Seppey et al., 2016; Szelecz et al., 2014). On the other hand, the presence of OTU X152, affiliated to the amoebozoan genus Mycamoeba suggests a beginning of recovery, as these organisms were showed to be negatively influenced by the presence of a cadaver in an experimental setup containing three pig corpses laid on forest soils (Blandenier et al., 2016). The presence of this particular OTU therefore suggests a PMI of over one year (Blandenier et al., 2016).

4.6. Synthesis and outlook

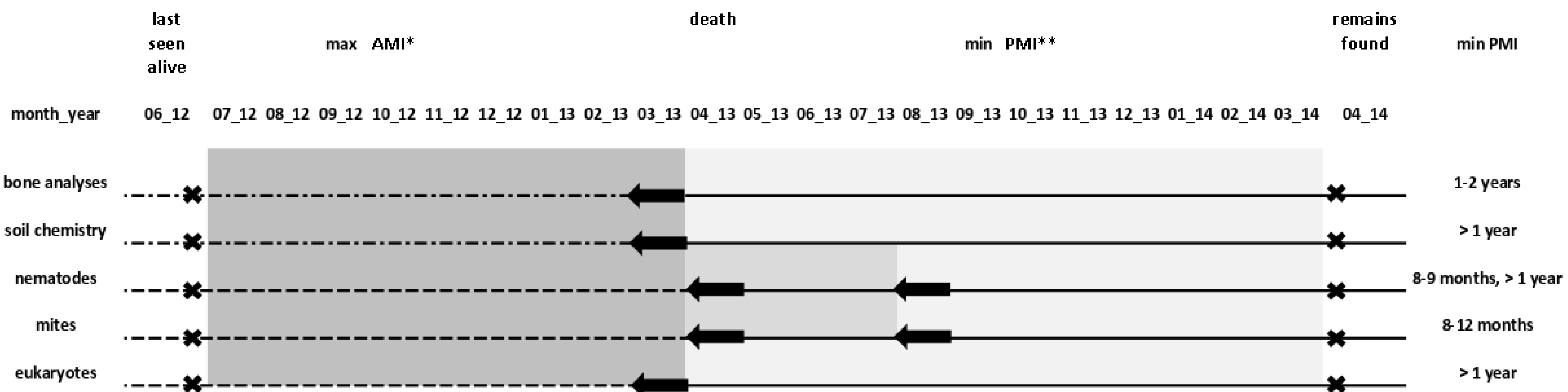
The following story can be told:

Bones were found in a Swiss lowland forest. The examination by a forensic anthropologist revealed that the bones were from a young human male adult who must have deceased 1-2 years before the discovery of the bones. Later this person was identified and he was last seen 22 months before his remains were found. The bones showed signs of scavenging and exposure to fire, likely the remains were burnt in-situ. The analyses of chemical constituents, nematodes and micro-eukaryotes revealed that the decomposing remains probably have been on the site for at least 8-9 months more likely more than one year (Fig. 6). This means at least part of the decomposition process had taken place on site. Mite evidence, however suggests that the corpse was moved from the original crime scene; and that the body had already reached later stages of decomposition before being moved to the forest. If the decomposing remains

had been transported to the site at least 12 months before the findings the person must have been killed between July 2012 and April 2013. Bearing in mind that decomposition is accelerated by temperature and insect access to the body (Campobasso et al., 2001; Carter et al., 2008) no tissue would have been left if the person had started to decompose at a different location in summer 2012. Instead, our findings suggest that the person was killed probably in autumn or winter 2012, started to decompose in a confined environment, likely on a farm, and was brought to the finding site in April 2013 the latest where it was partially burned.

Although some of the methods shown are still in the process of being developed (e.g. nematodes as forensic indicators for PMI estimation), others are already well-established (e.g. forensic anthropology). Nevertheless, despite obvious shortcoming, the interpretation that can be given based on this multiproxy study illustrates the added value of including several independent lines of evidence in forensic ecology (Fig. 6). We believe that this study should provide a strong incentive for conducting experimental studies including several groups of soil organisms to further develop a diverse toolbox for forensic investigations and to apply this approach in suspected homicide scenes.

Figure 5: Summary timeline of the PMI estimates revealed from the different analyses (bones, soil chemistry, nematodes, mites, micro-Eukaryotes) in a criminal case investigation in Switzerland



5. References

[1] Acsádi, G., Nemeskéri, J., 1970. History of human lifespan and mortality. Akadémiai Kiodó, Budapest.

[2] Amaral-Zettler, L. A., McCliment, E. A., Ducklow, H. W., Huse, S. M., 2009. A method for studying protistan diversity using massively parallel sequencing of v9 hypervariable regions of small-subunit ribosomal RNA genes. Plos One, 4(7):e6372.

[3] Ambrose, S. H., 1990. Preparation and characterization of bone and tooth collagen for isotopic analysis. Journal of Archaeological Science 17, 431-451.

[4] Amendt, J., Richards, C.S., Campobasso, C.P., Zehner, R., Hall, M.J.R., 2011. Forensic entomology: applications and limitations. Forensic Science, Medicine, and Pathology 7, 379–392. doi:10.1007/s12024-010-9209-2

[5] Amendt, J., Campobasso, C.P., Gaudry, E., Reiter, C., LeBlanc, H.N., J. R. Hall, M., 2007. Best practice in forensic entomology—standards and guidelines. International Journal of Legal Medicine 121, 90–104. doi:10.1007/s00414-006-0086-x

[6] Anderson, B., Meyer, J., Carter, D.O., 2013. Dynamics of ninhydrin-reactive nitrogen and pH in gravesoil during the extended postmortem interval. Journal of Forensic Sciences 58, 1348–1352. doi:10.1111/1556-4029.12230

[7] Anderson, G.S., VanLaerhoven, S.L., 1996. Initial studies on insect succession on carrion in southwestern British Columbia. Journal of Forensic Sciences 41, 617–625.

[8] Bärmann, E.V., Fürst von Lieven, A., Sudhaus, W., 2009. Revision and phylogeny of Myolaimus Cobb, 1920 (Secernentea, Nematoda) with the description of four new species. Journal of Nematode Morphology and Systematics, 12, 145-168.

[9] Barron, G. L., 1973. Nematophagous fungi: *Rhopalomyces elegans*. Canadian Journal of Botany- Revue Canadienne De Botanique, 51, 2505-2507.

[10] Benninger, L.A., Carter, D.O., Forbes, S.L., 2008. The biochemical alteration of soil beneath a decomposing carcass. Forensic Science International 180, 70–75. doi:10.1016/j.forsciint.2008.07.001

[11] Berg, S., Protsch von Zieten, R., 1998. Datierung von Skelettfunden. In: Leopold, D. (ed), Identifikation unbekannter Toter: Interdisziplinäre Methodik, Forensische

Osteologie Arbeitsmethoden der Medizinischen und Naturwissenschaftlichen Kriminalistik, Schmidt-Römhild, Lübeck, pp. 107-162

[12] Berney, C., Geisen, S., Van Wichelen, J., Nitsche, F., Vanormelingen, P., Bonkowski, M., Bass, D., 2015. Expansion of the ‚Reticulosphere‘: Diversity of novel branching and network-forming amoebae helps to define Variosea (Amoebozoa). Protist, 166, 271-95.

[13] Blandenier, Q., Seppey, C.V.W., Singer, D., Vlimant, M., Simon, A., Duckert, C., Lara, E., 2016. *Mycamoeba gemmipara* nov. gen., nov. sp., the first cultured member of the environmental Dermamoebidae Clade LKM74 and its unusual life cycle. Journal of Eukaryotic Microbiology. doi:10.1111/jeu.12357

[14] Bongers, T., 1994. De nematoden van Nederland: een identificatietabel voor de in Nederland aangetroffen zoetwater- en bodembewonende nematoden. Stichting Uitgeverij Koninklijke Nederlandse Natuurhistorische Vereniging 46, Pirola, Schoorl, The Netherlands.

[15] Bongers, T., Bongers, M., 1998. Functional diversity of nematodes. Applied Soil Ecology 10, 239–251. doi:10.1016/S0929-1393(98)00123-1

[16] Bongers, T., de Goede, R.G.N., Korthals, G.W., Yeates, G.W., 1995, Proposed changes of c-p classification for nematodes. Russian Journal of Nematology 3, 61-62.

[17] Braig, H.R., Perotti, M.A., 2009. Carcases and mites. Experimental and Applied Acarology 49, 45–84. doi:10.1007/s10493-009-9287-6

[18] Bregetova N.G., Vainshtein B.A., Kadite B.A., Koroleva E.V., Petrova A.D., Tikhomirov S.I., Shcherbak G.I., 1977. A key to the soil-inhabiting mites of the mesostigmata. Leningrad.

[19] Brothwell, D.R., 1981. Digging up bones: the excavation, treatment, and study of human skeletal remains, Cornell University Press, N.Y.

[20] Brown, D.J.F., Boag, B., 1988. An examination of methods used to extract virus-vector nematodes (Nematoda: Longiridae and Trichodoridae) from soil samples. Nematologia Mediterranea 16, 93–99.

[21] Campobasso, C.P., Di Vella, G., Introna, F., 2001. Factors affecting decomposition and Diptera colonization. Forensic Science International 120, 18–27. doi:10.1016/S0379-0738(01)00411-X

[22] Carter, D.O., Tibbett, M., 2003. Taphonomic mycota: fungi with forensic potential. Journal of Forensic Sciences 48, 2002169. doi:10.1520/JFS2002169

[23] Carter, D.O., Yellowlees, D., Tibbett, M., 2008. Temperature affects microbial decomposition of cadavers (*Rattus rattus*) in contrasting soils. Applied Soil Ecology 40, 129–137. doi:10.1016/j.apsoil.2008.03.010

[24] Carter, D.O., Yellowlees, D., Tibbett, M., 2007. Cadaver decomposition in terrestrial ecosystems. Naturwissenschaften 94, 12–24. doi:10.1007/s00114-006-0159-1

[25] Cobaugh, K.L., Schaeffer, S.M., DeBruyn, J.M., 2015. Functional and structural succession of soil microbial communities below decomposing human cadavers. PLOS ONE 10, e0130201. doi:10.1371/journal.pone.0130201

[26] Dufrene, M., Legendre, P., 1997. Species assemblages and indicator species: The need for a flexible asymmetrical approach. Ecological Monographs 67(3), 345–366.

[27] Early M., Goff M.L., 1986. Arthropod succession patterns in exposed carrion on the island of O‘ahu, Hawaiian Islands, USA. Journal of Medical Entomology 23, 520-531.

[28] Edgar, R. C., Haas, J B., Clemente, J. C., Quince, C., Knight, R., 2011. Uchime improves sensitivity and speed of chimera detection. Bioinformatics, 27(16), 2194–2200.

[29] Emberson R.M., 1972. Macrochelid mites in N.Z. (Acarina: Mesostigmata: Macrochelidae). New Zealand Entomologist 5, 118-127.

[30] Evans G.O., Till, W.M., 1979. Mesostigmatic mites of Britain and Ireland (Chelicerata: Acari-Parasitiformes): an introduction to their external morphology and classification. Transactions of the Zoological Society of London 35, 139-270.

[31] Evans G.O., Browning E., 1956. British mites of the subfamily Macrochelinae Trägårdh (Gamasina, Macrochelidae). Bulletin of the British Museum (Natural History). Zoology 4, 1-55.

[32] Evans G.O., Sheals J.G., MacFarlane D., 1961. The terrestrial acari of the British Isles: And introduction to their morphology, Biology and Classification, Dorking.

[33] Ferris, H., Bongers, T., 2009. Indices developed specifically for analysis of nematode assemblages In: Wilson, M.J., Khakouli-Duarte, T. (Eds.), Nematodes as Environmental Indicators. CABI, Oxfordshire, pp. 124–145.

[34] Finley, S.J., Benbow, M.E., Javan, G.T., 2015. Potential applications of soil microbial ecology and next-generation sequencing in criminal investigations. Applied Soil Ecology 88, 69–78. doi:10.1016/j.apsoil.2015.01.001

[35] Geyh, M.A., 2001. Bomb radiocarbon dating of animal tissues and hair. Radiocarbon 43, 723-730.

[36] Goff M.L., Early M., Odom C.B., Tullis, K., 1986. A preliminary checklist of arthropods associated with exposed carrion in the Hawaiian Islands, USA. Proceedings of the Hawaiian Entomological Society 26, 53-58.

[37] Guillou, L., Bachar, D., Audic, S., Bass ,D., Berney, C., Bittner, L., Boutte, C., Burgaud, G., de Vargas, C., Decelle, J., del Campo, J., Dolan, J.R., Dunthorn, M., Edvardsen, B., Holzmann, M., Kooistra, W. H. C. F., Lara, E., Le Bescot, N., Logares, R., Mahe, F., Massana, R., Montresor, M., Morard, R., Not, F. , Pawlowski, J., Probert, I., Sauvadet, A. L. , Siano, R., Stoeck, T., Vaulot, D., Zimmermann, D. Christen, R. , 2013. The protist ribosomal reference database (pr2): a catalog of unicellular eukaryote small sub-unit rrna sequences with curated taxonomy. Nucleic Acids Research, 41(1), D597–D604.

[38] Hawksworth, D.L., Wiltshire, P.E.J., 2011. Forensic mycology: the use of fungi in criminal investigations. Forensic Science International 206, 1–11. doi:10.1016/j.forsciint.2010.06.012

[39] Hedges RE, Clement JG, Thomas CD, and O'Connell T C. 2007. Collagen turnover in the adult femoral mid-shaft: modeled from anthropogenic radiocarbon tracer measurements. American Journal of Physical Anthropology 133(2), 808-816.

[40] Houck, M.A., O´Connor, B.M., 1991. Ecological and evolutionary significance of phoresy in the Astigmata. Annual Review of Entomology 36, 611-636.

[41] Hubert, J., Munzbergova, Z., Kucerova, Z., Stejskal, V., 2006. Comparison of communities of stored product mites in grain mass and grain residues in the Czech Republic. Experimental & Applied Acarology 39, 149-158.

[42] Hughes, A.M., 1976. The Mites of Stored Food and Houses. Her Majesty's Stationary Office, London.

[43] Husson, F., Josse, J., Lê, S., Mazet, J., 2015. FactoMineR: Multivariate Exploratory Data Analysis and Data Mining, 2015. URL http://CRAN.R-project.org/package=FactoMineR. R package version 1.31.4.

[44] Hyatt, K., H., 1990. Mites associated with terrestrial beetles in the British Isles. Entomologist's Monthly Magazine 126, 1512-1515.

[45] Hyatt, K.H., 1980. Mites of the subfamily Parasitinae (Mesostigmata: Parasitidae) in the British Isles. Bulletin of the British Museum (Natural History) Zoology series 38, 237–378.

[46] Keough, N., Myburgh, J., Steyn, M., 2016. Scoring of decomposition: a proposed amendment to the method when using a pig model for human studies. Journal of Forensic Sciences. doi:10.1111/1556-4029.13390

[47] Krantz, G.W., 1978. A manual of acarology: Oreon State University Book Stores

[48] Leclercq, M., Verstraeten, C., 1993. Entomologie et médecine légale. L'entomofaune des cadavres humains: sa succession par son interprétation, ses résultats, ses perspectives. Journal de Médecine Légale et Droit Médical 36, 205-222

[49] Leclercq, M., Verstraeten, C., 1988. Entomologie et Médecine légale. Datation de la mort. Acariens trouvés sur des cadavres humains. Bulletin et Annales de la Société Royale Belge d'Entomologie 124, 195-200.

[50] Legendre; P., Gallagher, E.D., 2001. Ecologically meaningful transformations for ordination of species data. Oecologia, 129(2), 271–280.

[51] Lindahl, B. D., de Boer, W., Finlay, R. D. 2010. Disruption of root carbon transport into forest humus stimulates fungal opportunists at the expense of mycorrhizal fungi. Isme Journal, 4,872-881.

[52] Longin R. 1971. New method of collagen extraction for radiocarbon dating. Nature 230(5291), 241-242.

[53] Mahe, F., Rognes, T., Quince,C., de Vargas, C., M. Dunthorn, 2014. Swarm: robust and fast clustering method for amplicon-based studies. PeerJ, 2:e539.

[54] Mann, R., Bass, W. & Meadows, L. Time since death and decomposition of the human body: variables and observations in case and experimental field studies. Journal of Forensic Sciences 35, 103-111 (1990).

[55] Martinez-Ramirez, J. A, Strien, J., Sanft, J., Mall, G., Walther, G., Peters, F. T., 2013. Studies on drug metabolism by fungi colonizing decomposing human cadavers. Part I: DNA sequence-based identification of fungi isolated from postmortem material. Analytical and Bioanalytical Chemistry 405 (26), 8443–8450.

[56] Mégnin, J.P., 1894. La faune des cadavres-application de l'entomologie a la médecine légale. Gauthier-Villars et fils, Paris.

[57] Megyesi, M.S., Nawrocki, S.P., Haskell, N.H, 2005. Using accumulated degree-days to estimate the postmortem interval from decomposed human remains. Journal of Forensic Sciences 50, 618-626.

[58] Melis, C., Selva, N., Teurlings, I., Skarpe, C., Linnell, J.D.C., Andersen, R., 2007. Soil and vegetation nutrient response to bison carcasses in Bialowieza Primeval Forest, Poland. Ecological Research 22, 807–813. doi:10.1007/s11284-006-0321-4

[59] Merritt, R.W., Snider, R., De Jong, J.L., Benbow, M.E., Kimbirauskas, R.K., Kolar, R.E., 2007. Collembola of the grave: A cold case history involving arthropods 28 years after death. Journal of Forensic Sciences 52, 1359–1361. doi:10.1111/j.1556-4029.2007.00568.x

[60] Metcalf, J.L., Xu, Z.Z., Weiss, S., Lax, S., Van Treuren, W., Hyde, E.R., Song, S.J., Amir, A., Larsen, P., Sangwan, N., Haarmann, D., Humphrey, G.C., Ackermann, G., Thompson, L.R., Lauber, C., Bibat, A., Nicholas, C., Gebert, M.J., Petrosino, J.F., Reed, S.C., Gilbert, J.A., Lynne, A.M., Bucheli, S.R., Carter, D.O., Knight, R., 2016. Microbial community assembly and metabolic function during mammalian corpse decomposition. Science 351, 158–162. doi:10.1126/science.aad2646

[61] Metcalf, J.L., Wegener Parfrey, L., Gonzalez, A., Lauber, C.L., Knights, D., Ackermann, G., Humphrey, G.C., Gebert, M.J., Van Treuren, W., Berg-Lyons, D., Keepers, K., Guo, Y., Bullard, J., Fierer, N., Carter, D.O., Knight, R., 2013. A microbial clock provides an accurate estimate of the postmortem interval in a mouse model system. eLife 2, e01104. doi:10.7554/eLife.01104

[62] Middelhoven, W. J., 2004. The yeast flora of some decaying mushrooms on trunks of living trees. Folia Microbiologica (Praha), 49(5),569–573.

[63] Moretti, T. de C., Ribeiro, O.B., 2006. *Cephalotes clypeatus* Fabricius (Hymenoptera: Formicidae): nesting habits and occurrence in animal carcass. Neotropical Entomology 35, 412–415. doi:10.1590/S1519-566X2006000300019

[64] O´Connor, B.M., 2009. Astigmatid mites (Acari: Sarcoptiformes) of forensic interest. Experimental and Applied Acarology 49, 125-133.

[65] O´Connor, B.M., 1982. Evolutionary ecology of astigmatid mites. Annual Review of Entomology 27: 385-409.

[66] Olsen, S.R., Cole, C.V., Watanabe, F.S., Dean, L.A., 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. United States Department of Agriculture, Circular No .939, 1-19.

[67] Parkinson, R.A., Dias, K.-R., Horswell, J., Greenwood, P., Banning, N., Tibbett, M., Vass, A.A., 2009. Microbial community analysis of human decomposition on soil, in: Criminal and Environmental Soil Forensics. Springer, pp. 379–394.

[68] Payne, J.A., 1965. A summer carrion study of the baby pig *sus scrofa* Linnaeus. Ecology 46, 592–602. doi:10.2307/1934999

[69] Pechal, J.L., Crippen, T.L., Benbow, M.E., Tarone, A.M., Dowd, S., Tomberlin, J.K., 2014. The potential use of bacterial community succession in forensics as described by high throughput metagenomic sequencing. International Journal of Legal Medicine 128, 193–205. doi:10.1007/s00414-013-0872-1

[70] Pechal, J.L., Crippen, T.L., Tarone, A.M., Lewis, A.J., Tomberlin, J.K., Benbow, M.E., 2013. Microbial community functional change during vertebrate carrion decomposition. PLoS ONE 8, e79035. doi:10.1371/journal.pone.0079035

[71] Perez, A.E., Haskell, N.H., Wells, J.D., 2014. Evaluating the utility of hexapod species for calculating a confidence interval about a succession based postmortem interval estimate. Forensic Science International 241, 91–95. doi:10.1016/j.forsciint.2014.05.007

[72] Perotti, M.A., Braig, H.R., 2009. Phoretic mites associated with animal and human decomposition. Experimental and Applied Acarology 49, 85–124. doi:10.1007/s10493-009-9280-0

[73] Perotti, M.A., Braig, H.R., Goff, M.L., 2010. Phoretic mites and carcasses. In: Amendt, J., Campobasso, C.P., Grassberger, M., Goff, M.L. (Eds.), Current Concepts in Forensic Entomology: Novel Arthropods, Environments and Geographical Regions Springer, Amsterdam, pp. 69-92.

[74] Perotti, M.A., Goff, M.L., Baker, A.S., Turner, B.D., Braig, H.R., 2009. Forensic acarology. Experimental and Applied Acarology 49: 3-13.

[75] Reimer, P.J., Brown, T.A., Reimer, R.W., 2004. Discussion: Reporting and calibration of post-bomb C-14 data. Radiocarbon 46 (3),1299-1304.

[76] Rösing, F.W., Graw, M., Marré, B., Ritz-Timme, S., Rothschild, M.A., Rötzscher, K., Schmeling, A., Schröder, I., Geserick, G., 2007. Recommendations for the forensic diagnosis of sex and age from skeletons. HOMO - Journal of Comparative Human Biology 58, 75–89. doi:10.1016/j.jchb.2005.07.002

[77] Saloña, M.I., Moraza, M.L., Carles-Tolrá, M., Iraola, V., Bahillo, P., Yélamos, T., Outerelo, R., Alcaraz, R., 2010. Searching the soil: Forensic importance of edaphic fauna after the removal of a corpse. Journal of Forensic Sciences 55, 1652-1655

[78] Sarwar, Z.M,. Bashir, M.H., Khan, B.S., Khan, M.A. 2013. Description of two new species of genus Caloglyphus berlese (Acari: Acaridae) from Pakistan. Pakistan Journal of Zoology 45, 101-106.

[79] Scheiner, J.D., 2005. Spéciation du carbone, de l"azote et du phosphore de différentes boues de stations d"épuration au cours de leurs incubations contrôlées dans deux types de sol, thèse de doctorat. Institut National Polytechnique de Toulouse; Toulouse.

[80] Scholze, V.S., Sudhaus, W., 2011. A pictorial key to current genus groups of "Rhabditidae." Journal of Nematode Morphology and Systematics 14, 105–112.

[81] Schwarcz, H.P., Agur, K., Jantz, L.M., 2010. A new method for determination of postmortem interval: citrate content of bone *. Journal of Forensic Sciences 55, 1516–1522. doi:10.1111/j.1556-4029.2010.01511.x

[82] Seppey, C.V.W., Fournier, B., Szelecz, I., Singer, D., Mitchell, E.A.D., Lara, E., 2016. Response of forest soil euglyphid testate amoebae (Rhizaria: Cercozoa) to pig cadavers assessed by high-throughput sequencing. International Journal of Legal Medicine 130, 551–562. doi:10.1007/s00414-015-1149-7

[83] Sharanowski, B.J., Walker, E.G., Anderson, G.S., 2008. Insect succession and decomposition patterns on shaded and sunlit carrion in Saskatchewan in three different seasons. Forensic Science International 179, 219–240. doi: 10.1016/j.forsciint. 2008.05.019

[84] Sjøvold, T., 1988). Geschlechtsdiagnose am Skelett. In Knussmann, R. (ed) Anthropologie, Fischer, Stuttgart, pp. 444–480.

[85] Steel, H., Buchan, D., De Neve, S., Couvreur, M., Moens; T., Bert, W., 2013. Nematode and microbial communities in a rapidly changing compost environment: How nematode assemblages reflect composting phases. European Journal of Soil Biology 56, 1-10.

[86] Stokes, K.L., Forbes, S.L., Tibbett, M., 2013. Human versus animal: contrasting decomposition dynamics of mammalian analogues in experimental taphonomy. Journal of Forensic Sciences 58, 583–591. doi:10.1111/1556-4029.12115

[87] Szelecz, I., Sorge, F., Seppey, C.V.W., Mulot, M., Steel, H., Neilson, R., Griffiths, B.S., Amendt, J., Mitchell, E.A.D., 2016. Effects of decomposing cadavers on soil nematode communities over a one-year period. Soil Biology and Biochemistry 103, 405–416. doi:10.1016/j.soilbio.2016.09.011

[88] Szelecz, I., Fournier, B., Seppey, C., Amendt, J., Mitchell, E., 2014. Can soil testate amoebae be used for estimating the time since death? A field experiment in a deciduous forest. Forensic Science International 236, 90–98. doi:10.1016/j.forsciint.2013.12.030

[89] Szilvássy, J., 1988. Altersdiagnose am Skelett. In Knussmann, R., (ed.), Anthropologie: Handbuch der vergleichenden Biologie des Menschen Gustav Fischer Verlag, Stuttgart, New York, pp. 421-435.

[90] Tibbet, M., Carter, D. O., 2009. Research in Forensic taphonomy: A soil-based perspective. In Ritz, K., Dawson, L., Miller, D. (Eds.) Criminal And Environmental Soil Forensics, Springer, Netherlands, 317-331.

[91] Timms S, Ferro DN & Emberson RM (1982) General biology and nomenclature of Sancassania berlesei (Michael). Acarologia 22, 385-390.

[92] Towne, E.G., 2000. Prairie vegetation and soil nutrient responses to ungulate carcasses. Oecologia 122, 232–239. doi:10.1007/PL00008851

[93] Ubelaker, D.H., Buchholz, B.A., Stewart, J.E.B., 2006. analysis of artificial radiocarbon in different skeletal and dental tissue types to evaluate date of death. Journal of Forensic Sciences 51, 484–488. doi:10.1111/j.1556-4029.2006.00125.x

[94] White, N.D.G., 1995. Insects, mites and insecticides in stored-grain ecosystems. In: Jayas, D.S., White, D.G., Muir, W.E., Stored-grain ecosystems, Marcel Dekker, New York, pp. 123-168.

[95] Wild, E.M., Arlamovsky, K.A., Golser, R., Kutschera, W., Priller, A., Puchegger, S., Rom, W., Steier, P., Vycudilik, W., 2000. C-14 dating with the bomb peak: An application to forensic medicine. Nuclear Instruments and Methods in Physics Research Section B 172, 944-950.

[96] Yeates, G.W., 2003. Nematodes as soil indicators: functional and biodiversity aspects. Biology and Fertility of Soils 37, 199–210.

[97] Yeates, G. W., Bongers, T., De Goede, R. G. M., Freckman, D. W., Georgieva, S. S., 1993. Feeding habits in soil nematode families and genera—an outline for soil ecologists. Journal of Nematology 25(3), 315–331.

[98] Zakhvatkin, A.A., 1941. Fauna of U.S.S.R. Arachnoidea Tyroglyphoidea (Acari). Zoological Institute of the Academy of Sciences of the U.S.S.R., Moscow.

Chapter 6

Saprinus semistriatus (Coleoptera: Histeridae) a useful taxon for the estimation of the post-mortem interval in pigs

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Abstract

In forensic science, entomological evidence to estimate the minimum post- mortem interval can be crucial. However, as not all cadaver-visiting insects are equally useful for forensic purposes, it is important to establish criteria that define and test the forensic relevance of each species. Our focus is on the histerid *Saprinus semistriatus* (Scriba 1790) (Histeridae; Coleoptera) that has been described as being forensically useful. Histeridae are typical predators that feed mainly on Diptera larvae on carrion and dung. We review 23 published papers mentioning this species and provide new experimental data on the temporal pattern of this species beneath and on hanging pig cadavers. In a field experiment near Neuchâtel, Switzerland, we recorded the abundance of *S. semistriatus* on ten decomposing pig cadavers (*Sus scrofa*) over a 32-day period. Five cadavers were placed directly on the ground and five cadavers were hung one metre above ground. Insects were collected from pitfall traps and manual sampling. The abundance of *S. semistriatus* was significantly higher during the active stage than during the fresh and the bloated stages of decomposition in both, ground and hanging pigs. However, *S. semistriatus* was more abundant on the ground than on the hanging cadavers. The literature and our new data show that *S. semistriatus* is present during a relatively short period of time on cadavers, mainly during the active decay stage, but

it already appears in low numbers in the bloated stage. Identifying key indicators such as *S. semistriatus* can help optimise forensic research by focusing on the most informative taxa. We argue that a few key indicators for each decomposition stage may constitute an optimal toolbox for forensic entomologists.

1. Introduction

In forensic science, the estimation of the minimum post-mortem interval (PMImin) using entomological evidence is a common practice. Four different ecological groups of insects can be classified on a corpse: (1) necrophagous species that feed on the dead organic matter directly, (2) predators and parasites of insects and other arthropods, (3) omnivores and (4) adventive species [1]. The first two categories are most useful for forensic purposes [2]. However, within these two groups not all species are equally suitable [3]. Matuszewski et al. [3] proposed four criteria to characterise the residency pattern of taxa on a carrion: (1) the length of the presence period (LPP), (2) the number of breaks inside the presence period (NBPP), i.e. the absence of the taxon within the presence period and the location of breaks, (3) the length of the longest unbroken period of presence (LLUP) and (4) the strength of the relationship between the appearance time (AT) of a taxon and the onset of a decomposition stage. Of these, criteria 1 and 4, are considered to be the most important [3]. Two types of taxa can be distinguished: (1) reoccurring taxa with break(s) in their presence period and (2) non-reoccurring taxa with an unbroken presence period [3]. To ensure that a taxon can be used for PMI estimation, it should be abundant and its occurrence in a given habitat should be predictable [3]. In addition, a promising approach to determine the PMI is the pre- appearance interval (PAI), which is the time that precedes the appearance of a taxon on a carcass [4]. This can be estimated from temperature and is strongly related to it [4].

We focus our study on *Saprinus semistriatus* (Scriba 1790), a carrion-visiting Histeridae (Coleoptera) species that is collected regularly and abundantly on pig cadavers [4,5]. *S. semistriatus* meets criteria 1 and 4 of Matuszewski et al. [3] and is thus considered highly useful for PMI estimation. It also shows a close relationship between its PAI and temperature [4]. *S. semistriatus* has been found on pig carcasses of different sizes (5-70 kg), on clothed and unclothed carcasses [6], in a variety of study sites and habitats (e.g. different types of forests [3,7], in indoor as well as outdoor environments [8]. Histeridae prey on mites, insects and insect larvae and some species feed specifically on larvae of a single insect species [9]. On decomposing cadavers, they arrive in large numbers when fly larvae develop massively [10]. Here, most carrion beetles are attracted by volatile organic compounds (VOCs) released by the cadaver or the insects feeding on it [4]. However, it is currently unclear if *S. semistriatus* is also a useful indicator in the case of hanging cadavers.

In this study, we address the following questions: Is the presence of *S. semistriatus* related to a certain decomposition stage? Does the abundance of *S. semistriatus* differ between cadavers that were placed on the ground and cadavers hanging 1 m aboveground? Does *S. semistriatus* data from our study meet criteria 1 and 4 of Matuszewski et al. [3]? We hypothesized that *S. semistriatus* is highly useful for forensic purposes and can be used to mark the onset of a decomposition stage.

2. Material and methods

2.1. Study site

The experiment was conducted in a spruce (Picea abies) forest near Neuchâtel, Switzerland (47°01'94,02" N, 6°87'61,11" E, 775 m a.s.l.). Mean daily temperature (measured in-field with a Decagon Em50 digital data logger) ranged from 13.3 °C to 20.9 °C over the course of the sampling period i.e. July, 01- August; 02, 2013 (Fig. 1).

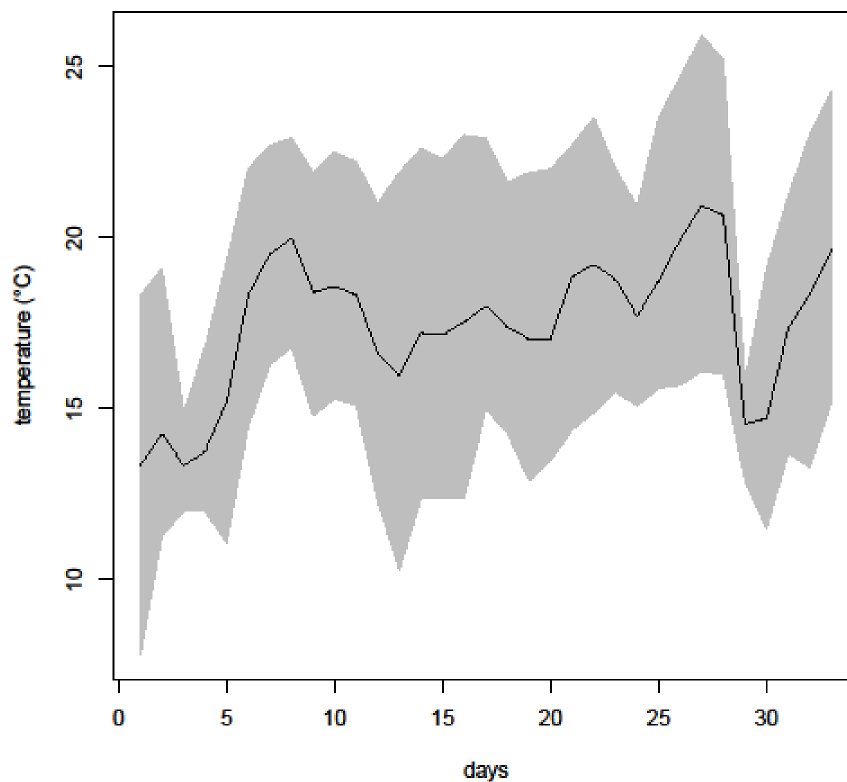

Figure 1. Ambient temperature data in °C measured at the Bois-du-Clos experimental site (Neuchâtel, Switzerland) over the course of the sampling period from July, 01 to August, 02, 2013. Days indicate the days elapsed since placing the cadavers on July 01, 2013.

2.2. Animal model and experimental setup

Ten domestic pigs (*Sus scrofa*, eight females and two males) were bought at a local farm. They were sedated with Stresnil® (Azaperone) and euthanized with T61® by a veterinarian, immediately transported to the experimental site, weighed and placed on the plots. The pigs showed no visible wounds or injuries. The average cadaver weight was 28 kg ± 2.5 kg (SD). All cadavers were placed in cages (140 cm x 95 cm) surrounded by wire mesh fences to keep scavengers and larger animals away. Two different carcass treatments (five replicates each) were used: (1) ground pigs (placed directly on the ground) and (2) hanging pigs (1 m above ground placed in cages). Plots were distributed randomly on the experimental site with 4 m distance between each other. For additional protection the experimental site (1200m^2) was surrounded by an electric fence. Fences and cages could be opened at one side for sampling, weighing of the cadavers and photo documentation.

2. 3. Arthropod sampling and identification

Sampling started a few hours after the placement of the carcasses on July, 01, 2013 (day 0) and continued until August, 02, 2013 (day 32). Sampling took place on a daily basis until day 26 and on three additional days the week after (day 29, 30 and 32). Adult Coleoptera were collected using pitfall traps and manual sampling. Two pitfall traps (1000 ml plastic containers) were buried next to the carcass (dorsally and ventrally). The traps were filled with a small litter layer and protected with a rain cover (plastic plate on wooden sticks). On each sampling day Coleoptera were collected from the traps, killed by freezing for several hours and then stored in 70 % EtOH. In addition, manual sampling was done by 2-3 people per sampling day and lasted 10 minutes per carcass. The collected Coleoptera were killed with ethyl acetate on the experimental site and were identified using the identification key from Freude et al. [11].

2.4. Decomposition stages

On all sampling days each pig carcass was examined to record the state of decomposition (including photographs and written reports). Decomposition stages were estimated using the definitions provided by Payne [12] for arthropod-exposed carrions.

2.5. Data analyses

Differences in the duration of decomposition stages between ground and hanging cadavers was tested using t-tests (adjusted p-value according to Holm). The criteria describing the residence pattern of *Saprinus semistriatus* were calculated according to Matuszweski et al. [3]. Treatment effects of ground and hanging pigs according to decomposition stages were evaluated by ANOVA followed by post-hoc Tukey tests on log transformed data [13]. All statistical analyses were performed with R statistical software (version 3.1.0) [14].

To compare our results with previous studies on the forensic relevance of *Saprinus semistriatus* we searched the ISI Web if Knowledge for studies published from 1950 to 2016. In the topic search section, we used the following terms "coleoptera AND forensic*" for a more general overview and then for a more refined search "saprinus semistriatus AND forensic*", "saprinus semistriatus AND carrion", "saprinus semistriatus AND cadaver", "saprinus semistriatus AND carcass", "saprinus semistriatus AND decomposition" and "saprinus semistriatus AND pmi". From this pool of papers, we first omitted papers that focused on selected Coleopteran families (except Histeridae). Then we checked the remaining pool for those describing the occurrence of Histeridae and finally from this smaller pool of papers, we selected the ones that mentioned the presence of *Saprinus semistriatus*. These data were compared with the data from our experiment.

3. Results

3.1 Decomposition stages and fly activity

Over the course of the sampling period (until day 32), all pigs went through the fresh, bloated and active decay stages (Fig. 2). The bloated stage (inflation of the abdomen) was visible at all cadavers on day two after placing the pigs. Fly oviposition was visible on day two mainly around the nozzle, but also on the chin and mouth. On day five small maggots were found on three ground and three hanging pigs. For the ground pigs the bloated stage lasted on average twice as long as in the hanging cadavers (i.e. eight vs. four days; $p<0.05$, t-test, adjusted p-value according to Holm). By day 9 (hanging pigs) and day 14 (ground pigs) all cadavers had reached the active decay stage (Fig. 2) accompanied by a cracking of the skin and an invasion of the cadavers by maggot

masses. Beneath the hanging pigs, piles of maggots had fallen on the ground. The active decay stage was significantly longer in the hanging cadavers ($p<0.01$, t-test, adjusted p-value according to Holm). After 25 days (ground pigs) and 36 days (hanging pigs) all cadavers had reached the advanced decay stage (Fig. 2) where most of the flesh had been removed and Diptera larvae had begun to migrate from the cadavers.

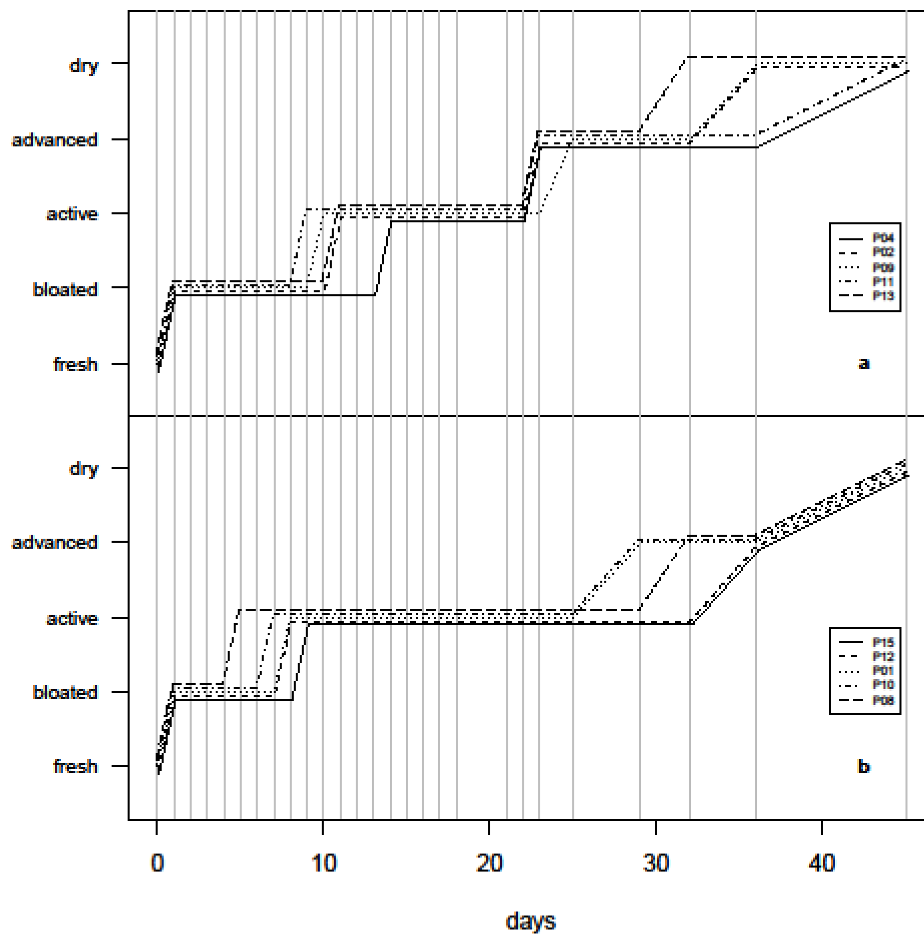

Figure 2. Duration of decomposition stages in ground (a) and hanging (b) pig treatments for each cadaver (5 per treatment) from day 0 (placing of the cadavers) until day 45 at the Bois-du-Clos spruce forest experimental site (Neuchâtel, Switzerland). Different lines indicate different cadavers.

3.2. Residency pattern of *Saprinus semistriatus*

A total of 138 *S. semistriatus* adult individuals were found in our study, 103 on the ground pigs and 35 on the hanging pigs (Table 1). There was no significant difference between treatments. The length of the presence period (LPP) ranged from 7-20 days on the ground pigs (average 12.8 ± 4.9 SD) and 1-21 days (average 12 ± 9.4 SD) on

the hanging pigs (Table 1). The average relative length of presence period in relation to the sampling period was 40% for the ground and 37.5 % for the hanging pigs (Table 1). Average number of breaks was 2.4 (ground) and 2.2 (hanging) (Table 1). The average relative length of the longest unbroken period in relation to the presence period was 35.6 % on the ground pigs and 34.6 % on the hanging pigs (Table 1).

3.3. Appearance time (AT) in relation to decomposition stages

S. semistriatus appeared on average earlier on the ground pigs than the hanging pigs. On the ground pigs the species appeared in the bloated stage for 3 carcasses and in the active decay stage for the other two, while for hanging cadavers it appeared in the bloated stage only for one carcass and in the active decay stage for the four others. According to field reports and photo documentation, Diptera larvae were visible on 70 % of the carcasses when *S. semistriatus* appeared, the remaining 30 % of the carcasses showed Diptera eggs and two of these were additionally reported as having a strong fly activity. In both treatments the majority of individuals were present in the active decay stage (ground: 94; hanging: 32) with only a few in the bloated stage (ground: 9; hanging: 3) and none in the fresh stage. The abundance of *S. semistriatus* in the active decay stage on the ground and hanging pigs differed significantly from the fresh and bloated stage respectively (Fig. 4, ANOVA, TukeyHSD, $p < 0.05$). When the sampling period had stopped on day 32, the advanced decay was not completed for all the cadavers, so we cannot correctly compare the advanced decay to the other stages.

3.4. Pre-appearance interval (PAI) in relation to ambient temperature

The PAI increased according to the averaged ambient temperature (Fig. 3). PAI ranged from 3 to 14 days on the ground pigs with an average of 8±3.8 (SD) days and from 6 to 14 days for the hanging pigs with an average of 10.4±3.6 (SD) days (Fig. 3). Ambient temperature averaged on the duration of the PAI ranged from 13.7 to 16.6°C (Fig. 3).

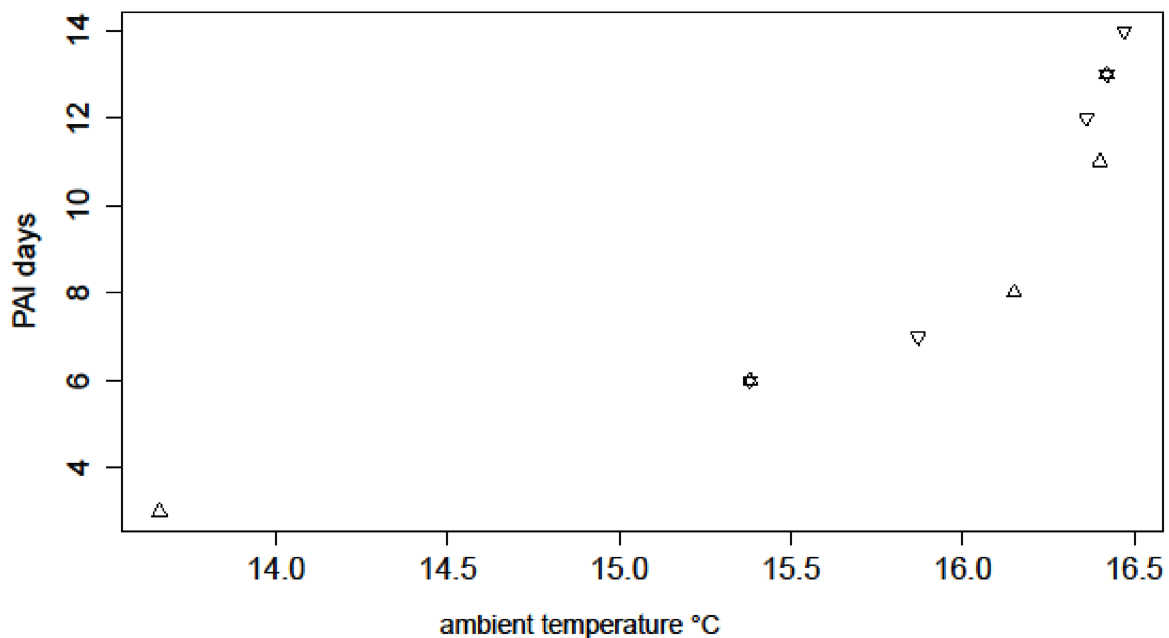

Figure 3. Pre-appearance interval (PAI) of *S. semistriatus* in relation to ambient temperature averaged on the duration of the PAIs at the Bois-du-Clos experimental site (Neuchâtel, Switzerland) for ground (∆) and hanging pigs (∇). Picture *S. semistriatus* U. Schmidt.

3.5. *Saprinus semistriatus* in other studies

From a total of 93 publications that mentioned the presence of Histeridae on decomposing cadavers, 23 described the occurrence of *S. semistriatus* (Table 2). In these studies, different types of cadavers were used and studies were conducted in different seasons (Table 2). Most of these studies describe the occurrence of *S. semistriatus* in spring and summer, some in autumn and only one in winter, but the latter study was conducted in Pakistan in December with an average temperature of 20 °C [15] (Table 2). All studies except two were conducted in Europe. Eight studies directly relate *S. semistriatus* to one or more decomposition stages: bloated (4x), active decay (7x), advanced decay (3x), dry (2x) and remains (2x) (Table 2). *S. semistriatus* was also reported in forensic entomology cases [16] (Table 2).

Table 1. Presence pattern of *Saprinus semistriatus* (numbers of individuals) on ground and hanging pig cadavers in a spruce forest at the Bois-du-Clos experimental site Neuchâtel, Switzerland. Days indicating the days elapsed since cadaver placement. Length of sampling interval (LSI)= 32 days, TNI= total number of individuals collected, LPP= length of presence period , NBPP= number of breaks in presence period, LLUP= length of the longest unbroken period, RLPP= relative length of presence period in relation to sampling interval, RLLUP= relative length of the longest unbroken period in relation to presence period, ARLPP= average relative length of presence period, ANBPP= average number of breaks in presence period, ARLLUP= average relative length of longest unbroken period.

plot no.	treatment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	TNI	LPP	NBPP	LLUP	RLPP	RLLUP	ARLPP	ANBPP	ARLLUP
														days																					days	n	days	%	%	%	n	%
2	ground			1			1		2		2	4	1	1	12	4	1	5	4				13											51	20	4	9	62.5	45			
4	ground								1	1					1	2	2		1			2												10	14	3	3	43.8	21.4			
9	ground													5	1	3	2	9	5				2											27	10	1	6	31.2	60			
11	ground						1			2				1	2		1	1	3															11	13	3	3	40.6	23.1			
13	ground											1					1	2																4	7	1	2	21.9	28.6	40	2.4	35.6
1	hanging						2	1		1					2		2									2								10	20	4	2	62.5	10			
8	hanging														1		1																	2	3	1	1	9.3	33.3			
10	hanging							1					2	1	1		4	1	2							1								13	15	2	3	46.9	20			
12	hanging												1		1		3					1	1										2	9	21	4	2	65.6	9.5			
15	hanging													1																				1	1	0	1	3.1	100	37.5	2.2	34.6

Table 2. Summary of selected studies with occurrences of adult Saprinus semistriatus associated with decomposition stages and seasons (country= origin of study, cadaver=model organisms used for decomposition).ARLPP= average relative length of presence period as % of sampling interval, ANBPP= average number of breaks in presence period, ARLLUP= average relative length of longest unbroken period as % of presence period

no.	reference	country	cadaver	season with *S. semistriatus* presence	decomposition stages associated with the the appearance of *S. semistriatus*	ARLPP (%)	ANBPP no.	ARLLUP (%)
1	Anton et et al. [8]	Germany	8 domestic pigs	spring, summer, late summer	active, advanced, dry, remains	—	—	—
2	Arnaldos et. al. [27]	Spain	chicken	spring, summer, autumn	decomposition, advanced, skeletonized	—	—	—
3	Bajerlein et al.* [5]	Poland	36 domestic pigs	spring, summer	—	—	—	—
4	Bourel et al. [28]	France	6 rabbits	spring, summer	—	—	—	—
5	Chapman and Sankey [22]	UK	3 rabbits	summer	—	—	—	—
6	Deikersschieter et al. [16]	Belgium, France	132 cases from Leclercq	—	—	—	—	—
7	Diaz-Martin and Salona-Bordas [29]	Spain	10 piglets	summer	fresh, bloated, active, advanced decay	—	—	—
8	Grosso-Silva and Soares-Vieira [30]	Portugal	27 mammals, 3 birds, 4 reptiles	—	—	—	—	—
9	Kočárek [31]	Czech Republik	rats	spring, summer	bloated to decay	—	—	—
10	Mądra et al. [17]	Poland	10 domestic pigs	spring, early summer, late summer	—	—	—	—
11	Matuszewski et al.[a] [7]	Poland	3 domestic pigs	end of summer - beginning of autumn	active decay	—	—	—
12	Matuszewski et al.* [3]	Poland	36 domestic pigs	spring, summer	onset of bloating	33.8	1.8	60.8
13	Matuszewski et al.* [21]	Poland	36 domestic pigs	spring, summer	—	—	—	—
14	Matuszewski and Szafałowicz** [4]	Poland	30 domestic pigs	spring, summer	—	—	—	—
15	Matuszewski et al.** [18]	Poland	26 domestic pigs	spring, summer, autumn	—	—	—	—
16	Matuszewski et al. [6]	Poland	24 domestic pigs	—	—	—	—	—
17	Matuszweski and Mądra** [26]	Poland	30 domestic pigs	—	—	—	—	—
18	Matuszewski and Mądra-Bielewicz[c] [32]	Poland	domestic pigs (data from various experiments)	—	—	—	—	—
19	Palanco et al.[b] [33]	Spain	dogs	spring, summer	bloated, liquefaction, dry, remains	—	—	—
20	Reed [34]	Tennessee, USA	43 dogs	—	—	—	—	—
21	Schlechter [35]	Luxembourg	rabbits	spring, summer, autumn	—	—	—	—
22	Vanin et al. [36]	Italy	4 domestic pigs	summer	—	—	—	—
23	Zaidi and Chen [15]	Pakistan	livestock sacrifices from 23 animals	winter	bloated, active	—	—	—

*studies refer to the same experiment (2006 & 2007)

**studies refer to the same experiment (2011)

[a] *S. semistriatus* (present at all sites) is grouped together with *S. planiusculus* (Motschulsky, 1849) (present at one site)

[b] publication in Spanish language

[c] data from 2005, 2006-2007, 2012 and unpublished data

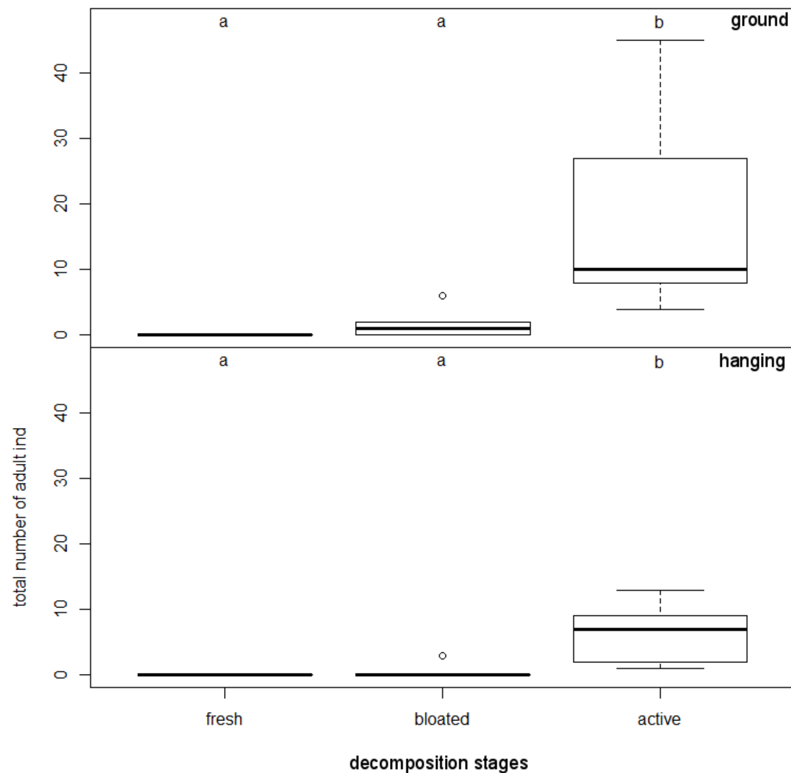

Figure 4. Boxplot showing the total number of adult *S.semistriatus* on the ground pig treatment (top) and hanging pig treatment (below). Different letters (a and b) indicate significant differences among decomposition stages (ANOVA, TukeyHSD).

4. Discussion

In our study, *S. semistriatus* can be described as a stable component of the carrion fauna in summer, which strengthens its relevance for forensic purposes. The average length of the presence period from our study (days ground: 12.8±4.9; hanging: 12±9.4 days) is comparable to a study from Matuszewski et al. [17] where the average of three forest types was 10.33±4.91 days (average and standard deviation) in summer 2006 and 13.35±7.01 days in summer 2007. This was also shown in another study, where *S. semistriatus* was one of the most abundant beetles, it arrived on day four and was present during 10 days (numbers remained constant with an exception on days 9 and 10 when it was quite cold) [18]. The presence period in relation to the sampling interval (ARLPP) is described to be > 60% for Coleoptera (for some species > 80%) and is negatively correlated to the accuracy of the PMI estimate [3]. In our study the ARLPP is 40% (ground pigs) and 37.5 % (hanging pigs) which is in line with 35 % given by Matuszewski et al. [3]. It has been suggested by Bayerlein et al. [5] that differences in residence period between histerid genera (e.g. Margarinotus and Saprinus) might be

due to the release of volatile organic compounds (VOCs) they are attracted to or different food preferences. The shorter presence period of *S. semistriatus* might reflect that a release of VOCs as well as the availability of larvae they feed on occur only for a short time [5]. The use of decomposition stages makes it easier to scale it and use it as a reference but decomposition is a continuous process, hence stages are overlapping and clear-cut, discrete stages do not exist in nature [19]. It is important to note, that the relation of *S. semistriatus* to a decomposition stage has to be described from two angles: First by defining the decomposition stage corresponding to the arrival of *S. semistriatus* on the cadaver and the stage that shows its highest abundance – and these might not be the same stages. Second and more useful for PMImin estimation, is identifying the drivers for *S. semistriatus* appearance and presence at a certain decomposition stage (e.g. temperature, food availability, season, habitat type, or the accessibility of the cadaver). Thereafter key drivers have to be selected and the relation between decomposition stage, drivers, the appearance of *S. semistriatus* and PMImin has to be estimated. Here *S. semistriatus* can provide additional information to narrow PMImin estimates. In our study the AT ranged from day 3 to 14 (both treatments) which is in line with Matuszewski et al. [17] who reported AT on day 3 to 9 in summer. *S. semistriatus* arrived in the bloated and active decay stage which is in accordance with other studies. When comparing studies, it has to be taken into account that not all studies refer to the same definitions and numbers of decomposition stages. In an initial study (3 pig cadavers) Matuszewski et al. [7] showed that the occurrence of *S. semistriatus* was characteristic for the active decay stage having a close relation to Diptera larvae on which they feed. This was confirmed by Anton et al. [8] who observed large numbers in spring to late summer, but not before the start of the active decay stage. In a later study (36 cadavers) a strong positive significant relationship ($p < 0.001$) for *S. semistriatus* with the onset of bloating was found [3]. The differences in relating *S. semistriatus* to a stage of decomposition may result from the fact that the continuous nature of decomposition and the fact that not all parts of a cadaver decompose simultaneously are often ignored [20]. As *S. semistriatus* is a predator of Diptera larvae, the presence of these larvae (possible main driver) is more important than the actual decomposition stage. Nevertheless, the highest presence of Diptera larvae is found during the active decay stage but also during the bloated stage during which larvae could be found around the orifices. In a repeated experiment (2006 and 2007), factorial design with two factors (three seasons and three forest types),

Matuszewski et al. [17] showed that for most taxa the pattern of differences in appearance between seasons, forests and years mirrored the pattern of differences in temperature. Therefore, it makes sense not only to investigate the AT, but also the PAI i.e. duration of the period before the species appeared. A strong exponential relationship was also observed between *S. semistriatus* and PAI (range 2.4-23.3 days) as well as temperature (range 12.3-23.5 °C), while the PAI decreased with increasing temperature, it remained nearly constant above a certain temperature [4]. This is not in accordance with our study where the PAI increased with increasing temperature, ranging from 3 to 14 days at temperatures from 13.7 to 16.6°C (Fig. 3), although the number of data points measured within this small temperature range between the two studies is almost the same (this study n=10, Matuszewski and Szafalowicz [4] n =12). The discrepancy between the two studies may be due to the fact that our study did not meet all the criteria suggested by Matuszewski and Madra [21] to assure the quality of the temperature model for PAI. Indeed, although the frequency of insect sampling and the combination of pitfall traps and hand collections was carried out, one of the most important factors according to Matuszewski and Madra [21], i.e. the broad range of temperature data that should be covered, was not realized in our study. In addition, we related the PAI to the ambient temperature in our study (measured directly on the field) whereas Matuszewski and Szafalowicz [4] measured the temperature on the ground (uncovered temperature data loggers). Nevertheless, if our temperature range represents a subset from the one described in Matuszewski and Szafalowicz [4], further studies are necessary, especially to analyse data on a finer scaled level to improve the accuracy for PMI estimation. Although differences between hanging and ground pigs were not significant, the abundance of *S. semistriatus* on the ground pigs was on average three times higher than on the hanging pigs. Diptera larvae were not able to form huge maggot masses on the hanging pig cadavers as they fell to the ground. This is probably one of the reasons why 85.2% of the collected Coleoptera in the hanging pig treatment were collected in the pitfall traps and not by manual sampling (data not shown) as most Coleoptera preferred the easily accessible larvae on the ground. *S. semistriatus* was the only histerid beetle that was collected manually on the hanging pigs (corresponding to 25% of all the beetles collected on the hanging pigs with manual sampling). In general, Histerid beetles are good fliers which makes the migration between habitats easy [22], but *S. semistriatus* is especially fast and agile [23] and is possible a good indicator for ground and hanging cadavers. *S. semistriatus*

was mentioned in 25% of the published studies and was described as highly abundant [5]. Some studies only list Saprinus spp. and could thus also include *S. semistriatus*. When it was found, *S. semistriatus* was an important part of the carrion fauna in summer [3, 8, 24, 25]. When sampling manually it is also possible that Histeridae were not caught because they hide quickly underneath carcasses and when in danger they can fold into a little pellet that can easily be overlooked. However, the identification to species level is crucial because compared to for instance Saprinus aeneus who is a typical coprophilous species (i.e. feeding on dung), *S. semistriatus* is more frequent on carcass and rather accidental on cow dung [22].

Conclusion

According to our study and the finding by other authors the usefulness of *S. semistriatus* for forensic purposes can be confirmed. *S. semistriatus* is highly abundant on decomposing cadavers and is present in a variety of habitats. It can target a decomposing cadaver even though it is not easily accessible (hanging cadavers). Its relatively short presence period is an additional tool to make PMImin estimation more precise. Its appearance time and presence is related to bloated and active decay. So far, temperature and the availability of food (prey) seem to be the most important factors (drivers) for the presence of *S. semistriatus*. Despite our findings, the bias of this experiment is, that it is only conducted in one year and one season and the distance between carcasses might be too short (4 meters). However, the adequate distance between carcasses to ensure independent experimental units is not clearly defined yet and so far, the effects of carcass cross contamination have not been tested experimentally (e.g. using different distances) to provide clear-defined criteria [26]. In the past, distances from a few meters up to hundreds of meters have been chosen by researches [26]. Even though a distance of approximately 50 meters to avoid cross-contamination has been emerged recently, experimental designs are also due to practical and economical issues and should not be dismissed as long as they provide valuable data [26]. Even though these points are worth criticizing, our study provides useful data on *S. semistriatus* on decomposing vertebrate cadavers.

5. References

*citation style kept according to the Journal

[1] K.G.V. Smith, A Manual of Forensic Entomology, The Trustees of the British Museum, 1986.

[2] J. Amendt, R. Krettek, R. Zehner, Forensic entomology, Naturwissenschaften. 91 (2004) 51–65.

[3] S. Matuszewski, D. Bajerlein, S. Konwerski, K. Szpila, Insect succession and carrion decomposition in selected forests of Central Europe. Part 2: Composition and residency patterns of carrion fauna, Forensic Science International 195 (2010) 42–51.

[4] S. Matuszewski, M. Szafalowicz, Temperature-dependent appearance of forensically useful beetles on carcasses, Forensic Science International 229 (2013) 92–99.

[5] D. Bajerlein, S. Matuszewski, S. Konwerski, Insect succession on carrion: seasonality, habitat, preference and residency of histerid beetles (Coleoptera: Histeridae) visiting pig carrion exposed in various forests (Western Poland), Polish Journal of Ecology Ecol. 59 (2011) 787–797.

[6] S. Matuszewski, K. Fratczak, S. Konwerski, D. Bajerlein, K. Szpila, M. Jarmusz, M. Szafalowicz, A. Grzywacz, A. Madra, Effect of body mass and clothing on carrion entomofauna, International Journal of Legal Medicine 130 (2016) 221–232.

[7] S. Matuszewski, D. Bajerlein, S. Konwerski, K. Szpila, An initial study of insect succession and carrion decomposition in various forest habitats of Central Europe, Forensic Science International 180 (2008) 61–69.

[8] E. Anton, S. Niederegger, R.G. Beutel, Beetles and flies collected on pig carrion in an experimental setting in Thuringia and their forensic implications, Medical and Veterinary Entomology 25 (2011) 353–364.

[9] D.G.H. Halstead, Handbooks for the identification of British insects Coleoptera: Histeroidae, Royal Entomological Society of London, London, 1963.

[10] L.E.O. Braack, Community dynamics of carrion-attendant arthropods in tropical African woodland, Oecologia. 72 (1987) 402–409.

[11] H. Freude, K.W. Harde, G.A. Lohse, Die Käfer Mitteleuropas, Goecke & Evers Verlag, Krefeld, 1971.

[12] J.A. Payne, A Summer Carrion Study of the Baby Pig *Sus Scrofa* Linnaeus, Ecology. 46 (1965) 592–602.

[13] J.M. Chambers, A. Freeny, R.M. Heiberger, Analysis of variance; designed experiments, in: J.M. Chambers, T.J. Hastie (Eds.), Stat. Models S, 1992: pp. 145–193.

[14] R Core Team, R: A language and environment for statistical computing., R Foundation for Statistical Computing, Vienna, Austria., 2016. URL https://www.R-project.org/.

[15] F. Zaidi, X. Chen, A preliminary survey of carrion breeding insects associated with the Eid ul Azha festival in remote Pakistan, Forensic Science International 209 (2011) 186–194.

[16] J. Dekeirsschieter, C. Frederick, F.J. Verheggen, P. Boxho, E. Haubruge, Forensic entomology investigations from doctor Marcel Leclercq (1924–2008): A review of cases from 1969 to 2005, Journal of Medical Entomology 50 (2013) 935–954.

[17] S. Matuszewski, D. Bajerlein, S. Konwerski, K. Szpila, Insect succession and carrion decomposition in selected forests of Central Europe. Part 3: Succession of carrion fauna, Forensic Science International 207 (2011) 150–163.

[18] R.F. Chapman, J.H.P. Sankey, The larger invertebrate fauna of three rabbit carcasses, Journal of Animal Ecology. 24 (1955) 395–402.

[19] L.M. Goff, Early post-mortem changes and stages of decomposition in exposed cadavers, Experimental and Applied Acarology. 49 (2009) 21–36.

[20] S. Matuszewski, D. Bajerlein, S. Konwerski, K. Szpila, Insect succession and carrion decomposition in selected forests of Central Europe. Part 1: Pattern and rate of decomposition, Forensic Science International 194 (2010) 85–93.

[21] S. Matuszewski, A. Madra, Factors affecting quality of temperature models for the pre-appearance interval of forensically useful insects, Forensic Science International 247 (2015) 28–35.

[22] D. Bajerlein, Coprophilous histerid beetle community (Coleoptera: Histeridae), Polish Journal of Entomology 78 (2009) 201–207.

[23] W. Lindner, Ökologie und Larvalbiologie einheimischer Histeriden, Zeitschrift Morphologie und Ökologie der Tiere. 59 (1967) 341–380.

[24] A. Madra, K. Fratczak, A. Grzywacz, S. Matuszewski, Long-term study of pig carrion entomofauna, Forensic Science International 252 (2015) 1–10.

[25] S. Matuszewski, M. Szafalowicz, M. Jarmusz, Insects colonising carcasses in open and forest habitats of Central Europe: Search for indicators of corpse relocation, Forensic Science International 231 (2013) 234–239.

[26] J.-P. Michaud, K.G. Schoenly, G. Moreau, Sampling flies or sampling flaws? Experimental design and inference strength in forensic entomology, Journal of Medical Entomology 49 (2012) 1–10.

[27] M.I. Arnaldos, E. Romera, J. J. Presa, A. Luna, M. D. García, Studies on seasonal arthropod succession on carrion in the southeastern Iberian Peninsula, International Journal of Legal Medicine 118 (2004) 197– 205.

[28] B. Bourel, L. Martin-Bouyer, V. Hedouin, J.-C. Cailliez, D. Derout, D. Gosset, Necrophilous insect succession on rabbit carrion in sand dune habitats in Northern France, Journal of Medical Entomology 36 (1999) 420-425.

[29] B. Díaz-Martín, M.I. Saloña-Bordas, Arthropods of forensic interest associated to pig carcasses in Aiako Harria Natural Park (Basque Country, Northern Spain), Ciencia Forense, 12 (2015) 207–228.

[30] J. M. Grosso-Silva, P. Soares-Vieira, Beetles (Coleoptera) associated with animal carcasses in Portugal: Data collected since 1995 and a survey of earlier publications, Boletín Sociedad Entomológica Aragonesa, 44 (2009) 481–491.

[31] P. Kocárek, Decomposition and Coleoptera succession on exposed carrion of small mammal in Opava, the Czech Republic, European Journal of Soil Biology 39 (2003) 31–45.

[32] S. Matuszewski, A. Madra–Bielewicz, Validation of temperature methods for the estimation of pre-appearance interval in carrion insects, Forensic Science, Medicine and Pathology 12 (2016) 50–57.

[33] J. L. Romero Palanco, F. Munguía Girón, J. Gamero Lucas, Entomología cadavérica en la Provincia de Cádiz (S. de España), Ciencia Forense, 8 (2006) 83-106.

[34] H.B. Reed, A study of dog carcass communities in Tennessee, with special reference to the insects, American Midland Naturalist 59 (1958) 213-245.

[35] J. Schlechter, Beetle fauna found on carrion in three woodland sites in Luxembourg (Insecta, Coleoptera), Bulletin de la Société des naturalistes luxembourgeois 109 (2008) 97-100.

[36] S. Vanin, E. Zanotti, D.Gibelli, A.Taborelli, S. Andreola, C. Cattaneo, Decomposition and entomological colonization of charred bodies–a pilot study, Croatian Medical Journal 54 (2013) 387-93.

Chapter 7

Concluding remarks

Ildikó Szelecz

Concluding remarks

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1. Background

The development of tools to determine the post-mortem interval or to locate decomposition sites, for instance when a corpse has been removed, are key aspects in forensic science. Most of the established tools lose their precision several weeks post mortem (Amendt et al., 2011, 2004; Wells and Lamotte, 2010). Studying cadaver decomposition in terrestrial ecosystem helps us to understand to which extent and how long cadavers have an impact on the soil below. Hence, a detailed documentation of soil changes (chemistry and soil organisms) during disturbance and recovery phases enhances our knowledge in fundamental soil ecology and applied forensic science.

The release of cadaveric fluids during the decomposition process adds high amounts of water, nutrients and microbial communities into the soil below (Carter et al., 2007; Carter and Tibbett, 2008; Parmenter and MacMahon, 2009; Towne, 2000). Soil organisms will have to deal with the changes in soil pH, moisture content, anoxic conditions, nutrient concentrations, food resources etc. Changes in the soil bacterial community due to bacterial growth and the leaching of bacteria present in the body, for instance, will increase the food availability for bacterivorous soil organisms (Bergmann et al., 2014; Javan et al., 2016; Moreno et al., 2011; Olakanye et al., 2014; Sender et al., 2016).

The breakdown of a cadaver and its related impacts on the soil below do not occur at the same time (Gill-King, 1997). Hence, over the course of the decomposition process different response pattern evolve, until at some point cadaver impacts will be negligible or terminated.

2. Achievements

This PhD thesis has covered a variety of disciplines in order to give an insight into the diversity of methods and approaches possible and necessary when it comes to solving

crimes. Our main focus was on the (further) development of new PMI estimation methods using soil chemistry and soil organisms. In addition, we have also used well-established methods such as forensic entomology, forensic acarology and forensic anthropology.

Our experiments covered time spans of ca. 1-3 years aiming at the estimation of long PMIs, beyond several weeks or months. Nevertheless, soil chemistry and the changes in testate amoeba and nematode communities were monitored from the beginning of the experiments to understand the different patterns. We have shown that there are significant differences between cadaver impacted and control soil samples. In the following we will summarize the main findings from the different approaches used in our experiments. In addition, we were able to demonstrate the validity of our approach in a real case study.

2.1. Soil chemical markers

The value of soil was long associated with evidence and soil in forensic science was seen more or less as a passive medium (Carter and Tibbett, 2008). This neglects the fact that soil is dynamic and responds to environmental change and disturbance (Carter and Tibbett, 2008). The changes in soil chemistry observed in our study revealed response patterns and led to the definition of three categories for forensic chemical markers. Some markers responded with an increase at the beginning of the decomposition process (EPM), other increased (LPM) or decreased late (LDM) during decomposition. At the end of the second experiment (after 367 day) some chemical soil markers were still significantly different from the controls. This illustrates their usefulness for the estimation of long post-mortem intervals.

2.2. Soil organisms

Our selected groups of soil organisms (testate amoebae and nematodes) share advantages that make them good candidates as forensic indicators for PMI estimation: They are diverse, occur in high numbers, are easily sampled and can be identified morphologically (if not at species level at least at ecologically- and forensically-meaningful taxonomic/functional levels), have relatively short generation times (thus

making it possible to detect changes in community structure fast), can be sampled in all seasons and react to disturbances.

The morphology-based testate amoeba analyses revealed that cadavers negatively affected testate amoeba density, species richness and diversity (8-33 days after the cadaver placement). Cadaver impacts were massive during active and advanced decay and lasted until the beginning of the dry stage (D33). In comparison to climatic effects (fake cadavers), cadaver impacts were clearly stronger. After four months, in the dry stage, cadaver effects started to increase again (D132). Testate amoeba communities had not recovered 10 months after (D309). This was also shown in the meta-barcoding analyses on euglyphid testate amoebae. Here cadaver treatments diverged from the controls until D309. Although most OTUs responded negatively, two OTUs responded positively from D33 to D309. Euglyphid communities had not recovered by D309. The combination of these approaches demonstrated that testate amoebae have the potential to indicate the presence of a cadaver.

Nematode abundance peaked on D15, which corresponds to the active decay, followed by a decline in density to almost 0 on D22. A second increase was observed four months after the cadavers were placed. Most nematode families respond negatively to the presence of the cadavers with the exceptions of Rhabditidae, Neodiplogasteridae and Diplogasteroididae. The latter two were only found in the cadaver samples and are promising candidates for long PMI estimation.

In both, testate amoebae and nematodes, we have shown that cadavers can influence soil organisms negatively (i.e. “cadaver haters” that decline in numbers or disappear) or positively (i.e. “cadaver lovers” that increase in numbers or appear in high numbers). Both responses are important for PMI estimation as they occur at different time points during the decomposition process.

2.3. Real case investigation

When a dead person is found, we have a snapshot instead of a pattern over time. Such “real cases” provide opportunities to assess if our approaches are reliable and also to identify further research questions.

Our real case investigation has shown that the combination of well-established methods and methods that are in the process of being developed may add the missing

information in suspected homicides. A cooperation with police and crime scene investigators that provides us with soil samples from an outdoor crime scene will be necessary to test our methods. Here we need to set standards for the sampling procedure (sample size, depths, number of samples etc.). As developed for forensic entomology (Amendt et al., 2007), a “best practice manual” for soil sampling will give the necessary instructions.

2.4. Forensic entomology

Not all species used in forensic entomology are equally relevant for PMI estimation (Matuszewski et al., 2010). In this PhD thesis, we have focused on *Saprinus semistriatus* to investigate, whether it meets the criteria proposed by Matuszewski et al. (2010) for forensically useful insect taxa. These criteria can serve as a template that, after adjusting it to soil organisms, can be used to categorize their forensic usefulness. In accordance with other studies we have shown that *S. semistriatus* is abundant on cadaver decomposition sites (hanging and ground cadavers), has a relatively short presence time and can be related to the bloated and active decay stages.

2.5. Forensic markers

The marker categories for soil chemistry markers that were developed in this PhD thesis are also applicable for grouping the analysed soil organisms. To be identified as a marker, potential indicators must show a significantly different response in increase (peak) or decrease in comparison to the controls. In addition, a transition line between early and late has to be defined (here: after the greatest weight loss / beginning of the dry stage) (Figure 1).

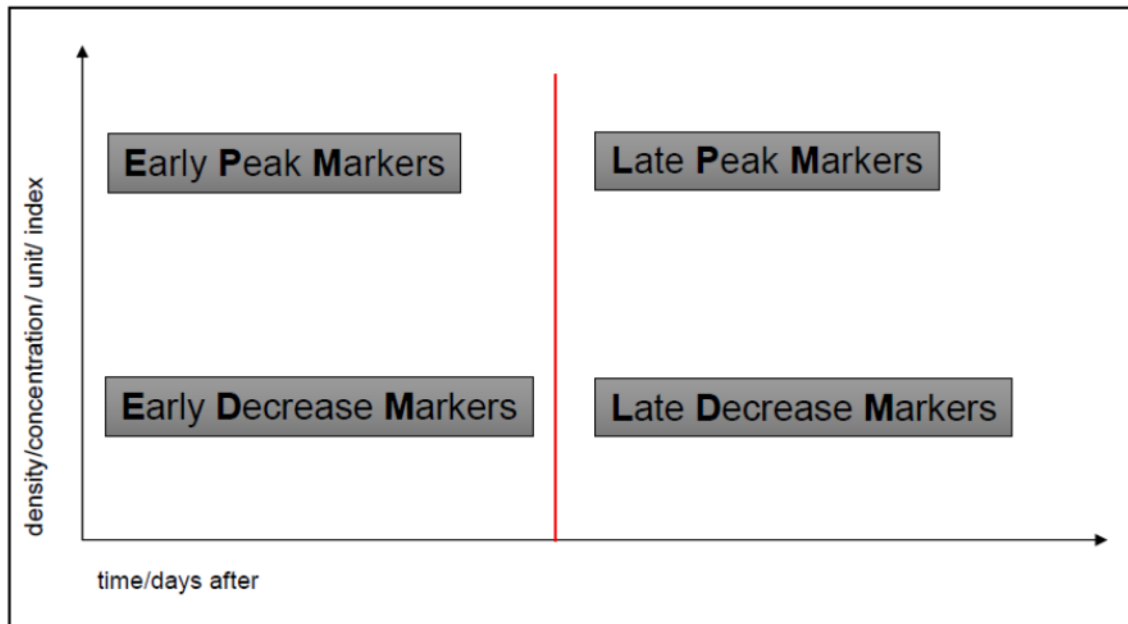

Figure 1. Forensic marker categories

The results from this PhD thesis reveal the following groups:

1. **E**arly **D**ecrease **M**arkers (**EDM**s) i.e. showing significantly lower values in the soil beneath cadavers when compared to the controls at a certain point relatively early in the decomposition process.

2. **E**arly **P**eak **M**arkers (**EPM**s) i.e. showing significantly higher values in the soil beneath cadavers when compared to the controls at a certain point relatively early in the decomposition process.

(3) **L**ate **D**ecrease **M**arkers (**LDM**s) showing significantly lower values in the soil beneath cadavers when compared to the controls at a certain point relatively late in the decomposition process.

(4) **L**ate **P**eak **M**arkers (**LPM**s) showing significantly higher values in the soil beneath cadavers when compared to the controls at a certain point relatively late in the decomposition process.

Soil chemistry, testate aoebae and nematodes are grouped into the following markers categories. Some markers are attributed to more than one category*.

category	marker
EDM	• testate amoebae
EPM	• nematodes • nitrogen • potassium • ammonium • pH* • phosphorous*
LDM	• pH*
LPM	• nitrate • phosphorous*

3. Follow-up research

Even though we have presented relevant results, the project is far from being completed. Follow-up research is needed to validate and complement our findings.

(1) Repeat analyses.

The results described in this PhD thesis showed the potential of the approach and provide valuable data that serve as a basis for further studies. These should investigate, by repeating the same or similar experiments, whether the changes induced by decomposing cadavers are predictable.

(2) Add other groups of soil organisms.

Other groups of soil organisms might be worth analysing. One possibly interesting group would be Rotifera. Indeed, we observed high numbers of rotifers beneath the decomposing cadavers in the first experiment (data not published). Rotifers are also conditioned by temperature, pH, ions, organic compounds, prey-predator relationships, competition, food supply parasites etc. and many species play an important role in the food webs because of their large population size and rapid

turnover rate (Fontaneto and De Smet, 2015). Additional soil organism groups might also lead to more potential markers within the marker categories.

(3) Extend the sampling period.

Often practical and financial limitations make it impossible to conduct experiments over long time spans i.e. a few years. Especially for long PMI estimation sampling beyond a one-year period is crucial and our results have shown that neither soil chemistry nor community composition of testate amoebae or nematodes have returned to the initial status (before the cadavers were placed).

4. Open questions

Every experiment, every study not only gives answers, but also creates new questions. This is the nature of science. During the experiments and in discussions two key questions have emerged:

(1) Are the changes described above only caused by the presence of cadavers or would other nutrient input cause similar impacts?

So far, our data has shown that microclimatic effects had little (testate amoebae) or no (nematodes) effect on the selected soil organisms when compared to cadaver impacts. Fluctuations in soil chemistry over the one-year period in the controls were also negligible in comparison to the impact of cadavers. The cadaver impact was strong enough to be distinguished from both control treatments. Nevertheless, it should be tested whether e.g. urine, blood or faeces could cause similar effects. Faeces and urine from animals and humans can add a variety of components e.g. phosphorous, potassium, ammonia, nitrogen, ammonium, nitrate into the soil (Chapuis-Lardy et al., 2004; Kirchmann and Pettersson, 1995; Leslie et al., 2008; Rose et al., 2015). Omnivorous and microbivorous nematodes for instance also increased with the application rate in manure-treated soils. Microbivores increased probably due to the bacterial blooming in highly enriched soils and omnivores increased with more numerous prey (Bernard, 1992).

(2) Do we find similar response patterns (soil chemistry and soil organisms) in different soil types?

It has already been shown that for instance soil type had a considerable effect on the decomposition of skeletal muscle tissue (SMT) buried in soil. Differences in the rate of decomposition (SMT mass-loss) were over three times greater in the Podsol compared with the Rendzina after 21 days (Haslam and Tibbett, 2009). Faster decomposition rates may reveal changed response patterns.

5. New experiments

An on-going Swiss SNF project is addressing some of the questions identified as a result of our first two experiments. The impact of pig cadavers, blood, urine and faeces on soil chemistry and organisms are currently being studied in a field experiment (started June 2016) (Fig. 2). In this one year experiment analyses from this PhD thesis e.g. soil chemical analyses and soil nematode community analyses will be repeated to show whether our results can be confirmed.

control

urine

blood

tissue, skin, bones, internal organs (*Sus scrofa*)

faeces

Figure 2. Field site and experimental set-up of the 2016 experiment in Neuchâtel, Switzerland (below). The five different treatments (control, urine, blood, *Sus scrofa* (tissue, skin, bones, internal organs), faeces). Each treatment with three replicates.

References

[1] Amendt, J., Richards, C.S., Campobasso, C.P., Zehner, R., Hall, M.J.R., 2011. Forensic entomology: applications and limitations. Forensic Science, Medicine, and Pathology 7, 379–392. doi:10.1007/s12024-010-9209-2

[2] Amendt, J., Campobasso, C.P., Gaudry, E., Reiter, C., LeBlanc, H.N., J. R. Hall, M., 2007. Best practice in forensic entomology—standards and guidelines. International Journal of Legal Medicine 121, 90–104. doi:10.1007/s00414-006-0086-x

[3] Amendt, J., Krettek, R., Zehner, R., 2004. Forensic entomology. Naturwissenschaften 91, 51–65. doi:10.1007/s00114-003-0493-5

[4] Bergmann, R.C., Ralebitso-Senior, T.K., Thompson, T.J.U., 2014. An RNA-based analysis of changes in biodiversity indices in response to *Sus scrofa domesticus* decomposition. Forensic Science International 241, 190–194. doi:10.1016/j.forsciint.2014.06.001

[5] Bernard, E.C., 1992. Soil nematode biodiversity. Biology and Fertility of Soils 14, 99–103. doi:10.1007/BF00336257

[6] Carter, D.O., Tibbett, M., 2008. Cadaver decomposition and soil: processes. In: Tibett, M., Carter, D.O. (Eds.), Soil Analysis in Forensic Taphonomy. CRC Press, Boca Raton, pp. 29–51.

[7] Carter, D.O., Yellowlees, D., Tibbett, M., 2007. Cadaver decomposition in terrestrial ecosystems. Naturwissenschaften 94, 12–24. doi:10.1007/s00114-006-0159-1

[8] Chapuis-Lardy, L., Fiorini, J., Toth, J., Dou, Z., 2004. Phosphorus concentration and solubility in dairy feces: variability and affecting Factors. Journal of Dairy Science 87.

[9] Fontaneto, D., De Smet, W.H., 2015. Rotifera. In: Schmidt-Rhaesa, A. (Ed.). Handbook of Zoology, Gastrotricha, Cycloneuralia and Gnathifera, Walter de Gruyter, Berlin, pp. 217-286.

[10] Gill-King, H., 1997. Chemical and ultrastructural aspects of decomposition. In: Haglund, W.D., Sorg, M.H. (Eds.), Forensic Taphonomy: The Postmortem Fate of Human Remains. CRC Press, Boca Raton, pp. 93–104.

[11] Haslam, T.C.F., Tibbett, M., 2009. Soils of contrasting pH affect the decomposition of buried mammalian (*Ovis aries*) skeletal muscle tissue. Journal of Forensic Sciences 54, 900–904. doi:10.1111/j.1556-4029.2009.01070.x

[12] Javan, G.T., Finley, S.J., Abidin, Z., Mulle, J.G., 2016. The Thanatomicrobiome: A missing piece of the microbial puzzle of death. Frontiers in Microbiology 7. doi:10.3389/fmicb.2016.00225

[13] Kirchmann, H., Pettersson, S., 1995. Human urine- chemical composition and fertilizer use efficiency. Fertilizer Research 40, 149–154.

[14] Leslie, D.M., Bowyer, R.T., Jenks, J.A., 2008. Facts from feces: nitrogen still measures up as a nutritional index for mammalian erbivores. Journal of Wildlife Management 72, 1420–1433. doi:10.2193/2007-404

[15] Matuszewski, S., Bajerlein, D., Konwerski, S., Szpila, K., 2010. Insect succession and carrion decomposition in selected forests of Central Europe. Part 2: Composition and residency patterns of carrion fauna. Forensic Science International 195, 42–51. doi:10.1016/j.forsciint.2009.11.007

[16] Moreno, L.I., Mills, D., Fetscher, J., John-Williams, K., Meadows-Jantz, L., McCord, B., 2011. The application of amplicon length heterogeneity PCR (LH-PCR) for monitoring the dynamics of soil microbial communities associated with cadaver decomposition. Journal of Microbiological Methods 84, 388–393. doi:10.1016/j.mimet.2010.11.023

[17] Olakanye, A.O., Thompson, T., Komang Ralebitso-Senior, T., 2014. Changes to soil bacterial profiles as a result of *Sus scrofa domesticus* decomposition. Forensic Science International 245, 101–106. doi:10.1016/j.forsciint.2014.10.002

[18] Parmenter, R.R., MacMahon, J.A., 2009. Carrion Decomposition and Nutrient Cycling in a Semiarid Shrub—Steppe Ecosystem. Ecological Monographs 79, 637–661.

[19] Rose, C., Parker, A., Jefferson, B., Cartmell, E., 2015. The characterization of feces and urine: a review of the literature to inform advanced treatment technology. Critical reviews in environmental science and technology 45, 1827–1879. doi:10.1080/10643389.2014.1000761

[20] Sender, R., Fuchs, S., Milo, R., 2016. Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans. Cell 164, 337–340. doi:10.1016/j.cell.2016.01.013

[21] Towne, E.G., 2000. Prairie vegetation and soil nutrient responses to ungulate carcasses. Oecologia 122, 232–239. doi:10.1007/PL00008851

[22] Wells, J.D., Lamotte, L.R., 2010. Estimating the postmortem interval. In: Byrd, J.H., Castner; J. L. (Eds.) Forensic Entomology: The utility of arthropods in legal investigations. CRC Press Boca Raton, pp. 367–388.

Appendices

Appendix A

Response of forest soil euglyphid testate amoebae (Rhizaria: Cereozoa) in pig cadavers assessed by high-throughput sequencing

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ORIGINAL ARTICLE

Response of forest soil euglyphid testate amoebae (Rhizaria: Cercozoa) to pig cadavers assessed by high-throughput sequencing

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Abstract Decomposing cadavers modify the soil environment, but the effect on soil organisms and especially on soil protists is still poorly documented. We conducted a 35-month experiment in a deciduous forest where soil samples were taken under pig cadavers, control plots and fake pigs (bags of similar volume as the pigs). We extracted total soil DNA, amplified the SSU ribosomal RNA (rRNA) gene V9 region and sequenced it by Illumina technology and analysed the data for euglyphid testate amoebae (Rhizaria: Euglyphida), a common group of protozoa known to respond to micro-environmental changes. We found 51 euglyphid operational taxonomic units (OTUs), 45 of which did not match any known sequence. Most OTUs decreased in abundance underneath cadavers between days 0 and 309, but some responded positively after a time lag. We sequenced the full-length SSU rRNA gene of two common OTUs that responded positively to cadavers; a phylogenetic analysis showed that they did not belong to any known euglyphid family. This study confirmed the existence of an unknown diversity of euglyphids and that they react to cadavers. Results suggest that metabarcoding of soil euglyphids could be used as a forensic tool to estimate the post-mortem interval (PMI) particularly for long-term (>2 months) PMI, for which no reliable tool exists.

Keywords Environmental DNA · Euglyphid testate amoebae · Illumina high-throughput sequencing · Metabarcoding · SSU rRNA gene V9 region · Forensic ecology

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Introduction

The estimation of time since death and more generally the ability to detect the presence of cadavers even when the remains are no longer present are the two major objectives in forensic research [1, 2].

Calculation of the post-mortem interval (PMI), an essential element of legal medicine and criminal investigation to establish the timing of events that led to the death of a person, becomes less precise with the advance of the decomposition process. Until now, two main approaches are used to estimate the PMI. The medical method provides information ranging from a few hours to several days after death [3–5]. The second method, forensic entomology, is based on the observation of larval stages of necrophagous flies and beetles and can be used to establish a PMI up to some weeks [4, 5]. Although well established, the accuracy of entomological methods has been questioned after the full development of the first generation of necrophagous insects [5]. Therefore,

the development of additional indicators for PMI estimates beyond 1 month would constitute a welcome addition to the toolkit of forensic criminal investigators.

According to Payne [6], the decomposition of cadavers can be separated into six stages: fresh, bloated, active decay, advanced decay, dry and remain stages. During the 'bloated' and 'active decay' stages [6, 7], the release of cadaver liquids into the soil changes the chemical parameters drastically [8]. This perturbation of the soil environment has been referred to as 'ephemeral resource patches' [9] leading to the development of 'cadaver decomposition islands' (CDI) [7]. Although most of the decomposition takes place in the first few weeks under optimal conditions, cadaver effects on the soil environment can be long lasting. For example, Towne [10] showed that nitrogen and phosphorus concentration and pH were still significantly enhanced in soil samples taken under cadavers 2 years after laying ungulate cadavers on a prairie, while Melis et al. [11] reported enhanced soil calcium content and pH as late as 7 years post mortem in a CDI. Such environmental changes were shown to have an effect on the soil fauna [12], bacteria [13–15] and fungi [16, 17]. However, knowledge about cadaver effects on soil communities remains very limited, and almost nothing is known about the response of soil protists [18].

In this study, we focused on euglyphid testate amoebae (Rhizaria: Cercozoa), a highly diverse and abundant group of protists that reacts rapidly to environmental changes by shifts in community structure and abundance [19]. Euglyphids include about one quarter of the ca. 300 testate amoeba morphospecies known to occur in soils [19]. These amoeboid unicellular protists range mostly between 20 and 150 μm in length, and their densities typically range between ca. 10^6 and 10^8 individuals per square metre [20]. They build a shell (test) reinforced with ornamented self-secreted siliceous plates, and these shells allow species identification even after the death of the organism [21, 22]. Most euglyphids are heterotrophs and feed mainly on bacteria and fungi [23]. The distribution patterns of soil testate amoebae along environmental gradients and their response to environmental changes have been well studied, including soil humidity [24–26]; temperature [27, 28]; pH [29–32]; and pesticide [33], nitrogen, phosphorus and sulphate concentration [34–37]. They can thus be expected to also respond to the presence of decomposing cadavers. The generation time of euglyphids, which ranges from ca. 2 days to 1 week under natural conditions [38], is considerably longer than that of bacteria or smaller protozoa such as nanoflagellates, and this represents an advantage regarding their use as bioindicators. It is indeed short enough to allow them to (re)colonise rapidly suitable habitats [39, 40] and respond to environmental change over a period of weeks. However, as euglyphids are highly sensitive to environmental conditions, the effects on communities can be expected to be long lasting under continuous environmental stress. So especially for estimating longer PMIs, euglyphids might be a group to consider for forensic applications.

However, a current limitation to the development of euglyphid analysis (or that of other soil protists) as a standard tool for PMI estimates is taxonomy. Sound taxonomy is indeed a prerequisite for the use of a group of organisms as bioindicators. Up to now, all ecological studies on testate amoebae were based on morphology-based species identifications. The morphological identification of testate amoebae requires taxonomic expertise and is time-consuming. Furthermore, recent molecular taxonomy studies on euglyphids have revealed the existence of a substantial higher diversity than estimated based only on morphology [41, 42], and this hidden/unknown diversity may prove to have bioindication value. The molecular approach presented in this study overcomes the current limitation of morphology-based taxonomy and is also faster (i.e. weeks instead of months for the number of samples analysed here).

Analysis of environmental samples (e.g. soil, water, faeces) targeting a specific DNA barcode gene and aiming at characterising the entire community is referred to as metabarcoding [43]. The V9 region of the 18S rRNA gene has sufficient variability for obtaining reasonably high taxonomic resolution [44, 45] and two main advantages for biodiversity surveys as follows: (1) it is short and thus likely to be well preserved in environmental DNA samples and (2) it contains highly conserved sites allowing to designing primers for virtually all eukaryotes [46]. The advent of high-throughput sequencing (HTS) now allows using the metabarcoding approach in ecological studies with high sample numbers (e.g. spatial and/or temporal sampling).

In this study, we used a DNA metabarcoding approach applied to the V9 region of the 18S rRNA gene to assess the temporal response of soil euglyphid testate amoebae to decomposing cadavers over a period of 35 months (1051 days). Given the sensitivity of the technique and the well-documented response of testate amoebae to ecological gradients, changes and disturbances, we expected to find (1) a higher diversity of soil Euglyphids than generally inferred from microscopic analyses and (2) a strong generally negative response of communities to decomposing cadavers with i) rapid disappearance of the majority of taxa following the massive release of cadaver fluids in the soil and ii) slow recovery after the end of the active decay phase, ca. 1–2 months after the peak of cadaver fluid input in the soil. If such patterns were indeed found, this may lead to the development of new PMI indicators in the future.

Materials and methods

Sampling site

The experimental site is situated in a beech- (*Fagus sylvatica*) and oak- (*Quercus robur*, *Q. petraea*) dominated forest near Neuchâtel (Switzerland 47° 00′ N; 06° 56′ E, elevation 478 m). The overall average temperature measured over the course of the experiment was 10.4 °C (SD 6.09 °C). The mean annual

precipitation of the nearest meteorological station (Neuchâtel) for 1993 to 2013 was 974 mm per year (MétéoSuisse).

Experimental setup

The experiment included three treatments: control (plots of forest soil left under natural conditions), fake pig (plastic bags filled with a volume of soil similar to that of the pigs placed in a cotton cloth) and pig (*Sus scrofa*). The fake pig treatment was used to differentiate the chemical effect of pig cadaveric liquids from the physical effects (i.e. humidity, soil compaction) due to the presence of a carcass on the soil. The bag volume was kept approximately similar to that of the pigs by removing soil from the bag to mirror the volume loss of the pig cadavers over time. The pigs (20 kg±1 kg) were killed on the farm with captive bolt stunning and the cadavers immediately brought to the experimental site. The cadavers were placed in strong metal wire cages (90×100×50 cm) to protect them from scavengers. The cages also allowed moving the cadavers for sampling. Pigs are commonly used in comparable forensic studies due to the similarities with humans, comparable thoracic cage size and almost naked skin [47]. Each treatment was run in triplicate. The sampling plots were organised into three randomised blocks (15–34 m apart). Within each block, the plots were at least 4 m apart.

Sampling and chemical analyses

Eight sets of samples were collected from the onset of the study (August 5th, 2009=D0) until June 21st, 2012 (Table 1). At the onset of the experiment (D0, before the pigs and fake pigs were placed), initial control samples were collected from all sampling plots and pooled for each block (i.e. three pooled samples in total). Sampling days were scheduled according to decomposition stages (Table 1) [6]. On each sampling day, ca. 25 g of soil was taken to a depth of 10 cm in each plot and stored at −80 °C.

Soil subsamples (3 g) were dehydrated (40 °C, 12 h), ground to powder and analysed for total organic carbon (Soil_C) and total nitrogen (Soil_N) using combustion infrared spectroscopy (CHNEA1108-Elemental analyser, Carlo Erba Instrument) after decarbonation with HCl [48].

Table 1 Sampling dates and corresponding decomposition stages of the pig cadavers in a *Fagus-Quercus* forest above Neuchâtel, Switzerland

Sample code	Sampling date	Decomposition stage
D0	05/08/2009	Fresh
D8	13/08/2009	Active decay
D15	20/08/2009	Dry stage
D22	27/08/2009	Dry stage
D33	07/09/2009	Dry stage
D64	08/10/2009	Dry stage
D309	10/06/2010	Dry stage
D1051	21/06/2012	Dry stage

Molecular analyses

DNA was extracted from soil samples using the MoBio PowerSoil DNA Isolation Kit (Carlsbad, CA, USA) following the manufacturer instructions. The SSU rRNA V9 region was amplified by PCR using the specific eukaryotic primers 1380f/1510r (CCCTGCCHTTTGTACACAC/CCTTCYGCAGGTTCACCTAC) [44]. Forward primers were tagged on the 5′ end with a 10 nucleotides strand, specific to each sample. PCR reactions were run in triplicate with a PTC-200 Peltier Thermo Cycler (BioConcept, Allswill, Switzerland) with 1 ng of environmental DNA, 6 μL of 10x PCR buffer, 0.6 μl of each primer, 0.6 μl of each dNTP 400 μM (Promega) and 0.2 μl of 0.05 U/μl Go Taq (Promega). The volume was adjusted to 30 μL with ultra-pure water. Amplification was conducted with the following conditions: denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 57 °C for 60 s and 72 °C for 90 s and final extension at 72 °C for 10 min [44]. PCR products were purified through QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and pooled together with a 4-ng DNA of each sample. A DNA library was prepared using the New England Biolabs's kit NEBNext DNA Sample Prep Master Mix Set 1 (http://www.neb.com/nebecomm/ManualFiles/manualE6040.pdf) except for the size selection step. Sequencing was done by the Genomics Core Facility at Brown University (Providence, USA) with an Illumina ® HiSeq 2000 sequencer to obtain paired-end reads covering the full length of the V9 region.

Sequence treatment

A database was constructed by selecting 44 complete euglyphid V9 sequences from the GenBank database, using sequences derived both from identified organisms and from related environmental sequences retrieved from GenBank. Each environmental V9 read was compared to the database using the

Table 2 Summary of the sequence filtering of euglyphid testate amoeba from the control, fake pig and pig cadaver plots in a *Fagus-Quercus* forest above Neuchâtel, Switzerland

Analysis steps	Total reads	Euglyphid sequences	Euglyphid dereplicated reads	Euglyphid OTUs
Raw fastq	247366905	–	–	–
Blast selection	187566	187566	–	–
Reads >= 5 times	57533	57533	2621	–
OTU building	57724	57724	2621	198
OTU selection	52860	52860	–	51
Triplication D0	57640	57640	–	51

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Table 3 Summary of total euglyphid testate amoeba OTU abundance in the control, fake pig and pig cadaver plots in a *Fagus-Quercus* forest above Neuchâtel, Switzerland

Euglyphid OTUs	Total abundance
eugly_59	4234
eugly_13	4205
eugly_2	4149
eugly_12	3873
eugly_666	3161
Euglypha rotunda AJ418783.1	3056
Euglypha filifera AJ418786.1	2583
eugly_66	2530
Uncultured eukaryote EF025028.1	2279
eugly_81	2048
eugly_151	1949
eugly_156	1933
eugly_5	1916
eugly_136	1630
eugly_183	1451
eugly_322	1400
eugly_38	1227
eugly_307	1199
eugly_54	1186
Assulina muscorum AJ418791.1	1162
eugly_113	1086
eugly_234	946
Cercomonadida env sample EF024983.1	858
eugly_79	675
eugly_33	594
eugly_41	514
eugly_60	417
eugly_290	411
eugly_991	402
eugly_98	382
eugly_320	367
eugly_992	349
eugly_16	314
eugly_473	273
eugly_862	253
eugly_82	238
eugly_76	211
eugly_80	203
eugly_973	195
eugly_233	182
eugly_1245	177
eugly_854	176
Tracheleuglypha dentata X77698.1	176
eugly_282	172
eugly_885	170
eugly_371	165
eugly_250	141
eugly_120	134
eugly_1777	105
eugly_1716	96
eugly_1890	87

BLASTn algorithm [49] in order to select euglyphid sequences. Before the BLASTn, each nucleotide with a Phred score below 28 was changed to an unknown nucleotide 'N' in order to avoid unreliable nucleotides. The BLASTn algorithm was setup with a match/mismatch ratio of 1:−1, gap open and extend penalty, respectively, of 0 and 2 and a word size of 32 nucleotides.

We used an empirically determined e-value threshold as the criterion for classifying a read as belonging to the euglyphids. To determine the appropriate e-value threshold, a subset of eukaryotic V9 sequences (sample D309, block 2, pig treatment) was compared by BLASTn to the previously established euglyphid V9 sequence database with a permissive e-value (i.e. 10). The hit results were sorted by increasing e-value and compared to the GenBank database by BLASTn, using the previous setup, until sequences corresponding to taxa other than euglyphids were found. Once the e-value threshold was found (i.e. $8e^{-29}$), each environmental sequence was compared against the V9 euglyphid database using BLASTn.

Only sequences over 130 nucleotides long and occurring at least five times in the 66 samples were retained, in order to remove possible false-positive sequences. As our database showed that some closely related but nevertheless morphologically and genetically (e.g. COI gene or full SSU) distinct euglyphid morphospecies shared exactly the same V9 sequence (e.g. *Euglypha penardi* (EF456753) and *Euglypha* cf. *ciliata* (EF456754) [50]), we considered each unambiguous difference in the nucleotide sequence as sufficient for discriminating two OTUs. Conversely, when two sequences differed only in ambiguous nucleotides, they were considered as belonging to the same OTU. The resulting OTU sequences were then counted in each sample.

Numerical analyses

We assessed the response of the 51 OTUs found in the 66 samples to the different treatments using partial redundancy analysis (RDA) on Hellinger-transformed data [51] with the blocks used as conditional variable. Rare OTUs (present less than three times in a minimum of ten samples) were removed to reduce noise in the model and optimise the adjusted R^2 [52]. These thresholds were selected after testing several options (presence threshold 1, 3, 5, 7; minimum number of presence 7, 10, 12, 14). The significance of variables (Soil_C, Soil_N, treatment) and ordination axes (first, second and third) were assessed using Monte Carlo tests (999 permutations, p value threshold=0.05).

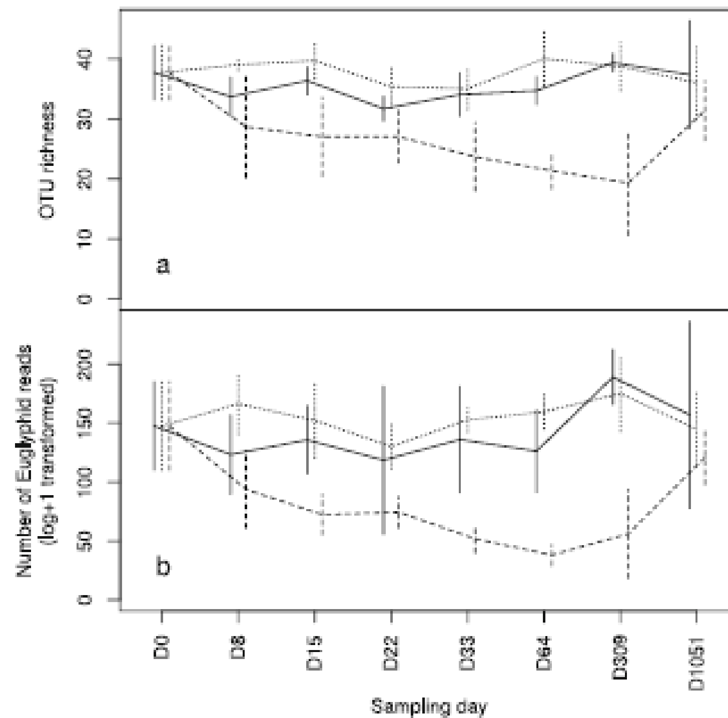

Fig. 1 Temporal patterns of euglyphid OTU richness (a) and number of reads log +1 transformed (b) in soil samples from control, fake pig and pig cadaver plots in a *Fagus-Quercus* forest above Neuchâtel, Switzerland. Treatments are represented by line type (*plain*: control, *dashed*: pig, *dotted*: fake pig). The *vertical lines* show the standard deviation of the richness and number of reads for each treatment and sampling date. The lines for the three treatments are slightly offset to improve readability

We assessed the effect of the treatments, relative to control, on the OTU responses over time using a principal response curve (PRC) [53]. The model was also tested using a Monte Carlo procedure (999 permutation, p value threshold=0.05).

All statistical analyses were performed with R-2.13.1 [54] using package 'vegan' [55] for the Hellinger transformation, RDA and PRC analyses.

Retrieval of full-length SSU rRNA gene sequences of selected taxa and phylogenetic analysis

Because sequences of the V9 variable region of the SSU rRNA gene are short (i.e. generally less than 200 bp), they are not suited for inferring the position of OTUs in phylogenetic trees. This is especially problematic if the considered sequences are suspected not to cover a large part of the diversity of the group of interest [56]. In order to place the OTUs of interest (i.e. showing a strong response to cadavers) in a phylogenetic tree, we used the sequence information included in the V9 region to design specific reverse primers and amplified the rest of the SSU rRNA gene. We designed specific primers to amplify specifically two phylotypes that responded positively to the pig treatment: eugly_13R (CACGAACTGAAGGCAAGCCCA) and eugly_666R (TTCACTTCCAATCACAGGAG). The newly designed primers were used in combination with the euglyphid-specific forward primer Eugly1SSUF (GCGTACAGCTCATTATATCA GCA [41]) located at the beginning of the SSU rRNA gene. DNA extractions, where the OTU was most abundant, were selected for specific amplification of the SSU rRNA gene of interest. Cycling profile was the same as described above (with 40 cycles). PCR products were cloned into pCR2.1 Topo TA cloning vector (Invitrogen) and used to transform *E. coli* TOP10' OneShot cells (Invitrogen) according to the manufacturer's instructions. Up to five clones per PCR product were chosen for sequencing. Sequencing was performed with an ABI-3130xl DNA Sequencer (Applied Biosystems). The new sequences obtained were placed into an alignment that comprised all euglyphid sequences retrieved from GenBank, which included both environmental clones and sequences derived from identified organisms. The alignment was performed using MUSCLE [57]. A maximum likelihood tree was built using the RAxML v7.2.8 algorithm [58] as proposed on the portal (http://phylobench.vital-it.ch/raxml-bb/) using a general time-reversible model. Rate heterogeneity was estimated using a CAT model.

Results

Diversity and structure of euglyphid OTU assemblages

Of the 247,366,905 raw Eukaryote reads, 187,566 were identified as euglyphids and 57,533 of these were found at least five times overall (Table 2). These 57,533 reads were divided

into 198 OTUs. Of these, 51 OTUs respected the thresholds and were thus retained for further analyses. Six of these OTUs matched exactly with sequences from our database. Total OTU abundance data are summarised in Table 3, and OTU richness and number of euglyphid reads along time for the three treatments are shown in Fig. 1.

The partial redundancy analysis (RDA, Fig. 2) with the blocks used as conditional variable revealed a significant correlation between euglyphid communities and Soil_N and Soil_C (Monte Carlo test, 999 permutations, both p=0.01). Axes 1 and 2 were significant (p=0.005 for both). The RDA ordination showed that the pig treatment samples diverged from the control and fake pigs along the soil nitrogen content gradient until day 309 after which they converged again with the samples of the other two treatments. The RDA also showed that most OTUs responded negatively to the pig treatment. However, some OTUs responded positively to the pig treatment (e.g. eugly_13, eugly_666).

The principal response curve (Fig. 3) summarises the treatment effects on OTUs over time and shows the average responses of individual OTUs. The first PRC axis explained significantly (p< 0.03) 42 % of the model variance, while time and treatments explained, respectively, 10 and 27 % of the variance. Qualitatively, the PRC diagram showed an overall negative effect of the pig treatment (D8 to D1051) on the majority of euglyphid OTUs and the positive response of a few OTUs, especially eugly_666 and eugly_13, which were therefore further studied.

Retrieval of full-length SSU rRNA gene sequences of eugly_13 and eugly_666

All clone sequences obtained were identical (n=5 and 8, respectively). Phylogenetic analyses confirmed the position of the two phylotypes within euglyphid testate amoebae (supported with 100 % bootstrap value) and showed that they did not belong to any barcoded family (Fig. 4) [59]. They were basal to all known euglyphid families. Eugly_13 branched robustly (80 % bootstrap) with an environmental sequence from freshwater sediments (freshwater 13_2.2 AY620297). By contrast, eugly_666 did not branch robustly with any sequence—be it from environmental samples, cultures, or isolated cells.

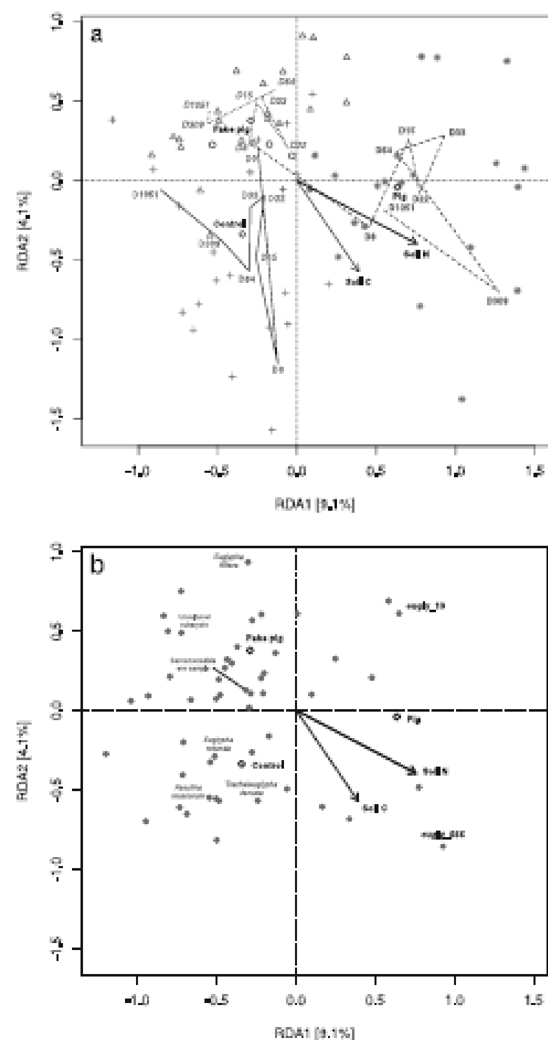

Fig. 2 Partial redundancy analysis (RDA) ordination diagram showing the temporal patterns of soil euglyphid testate amoeba communities (OTUs) in control, fake pig and pig cadaver plots in a *Fagus-Quercus* forest above Neuchâtel, Switzerland. *Diamonds* represent treatment centroids, and *arrows* represent weight percentage of total organic carbon (soil C) and total nitrogen (soil N). Percentages of variance explained by axes are shown in *brackets*. In **a**, successive sampling dates for each treatment (days 0, 8, 15, 22, 33, 64, 309, 1051) are connected by *lines*. The *line corners* correspond to the centroid (average for RDA1 and RDA2 coordinates) of three samples of the same treatment and same sampling day. Treatments are indicated by line type (*plain*: control, *dashed*: pig, *dotted*: fake pig), fonts of the sample labels (*plain*: control, *bold*: pig, *italic*: fake pig) and symbols ('+': control, '*Δ*': fake pig, 'stars': pig). *Empty circles* represent day 0 for each block. In **b**, OTU responses are represented by *dots*. The two OTUs for which full SSU sequences were obtained are represented in *bold*. The OTUs with a perfect match with a database sequence are represented with their *names*

Discussion

Euglyphid community responses to decomposing pigs

This study showed that the presence of decomposing pig cadavers significantly affected the community structure of euglyphid testate amoebae, showing a drastic decrease in sequence abundance and in OTU richness (Fig. 1). This result is in agreement with our general working hypothesis. The

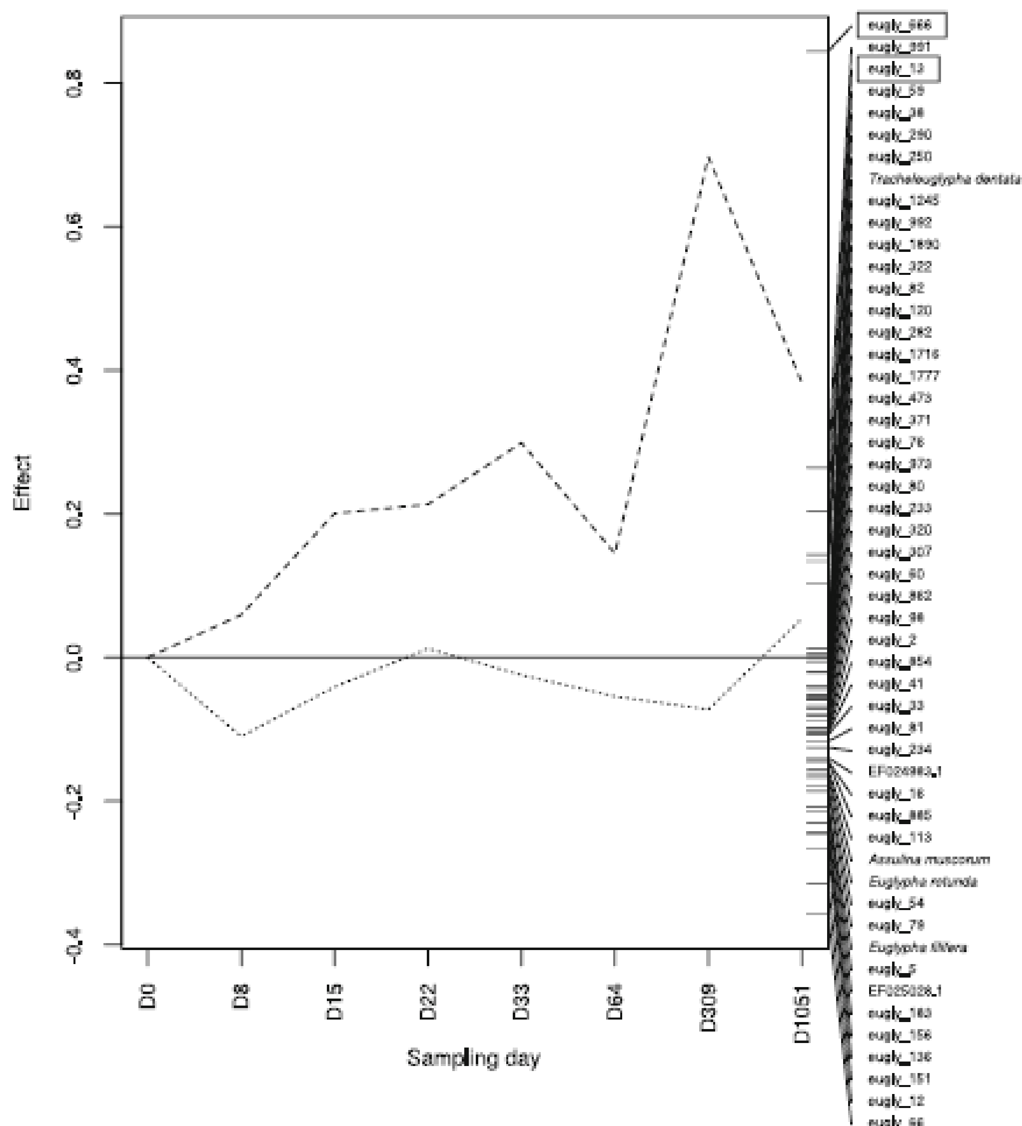

Fig. 3 Principal response curve (PRC) diagram showing the effects of pig (*dashed line*) and fake pig (*dotted line*) treatments relative to control treatment over time on soil euglyphid testate amoeba communities in a *Fagus-Quercus* forest above Neuchâtel, Switzerland. The average response of individual OTUs is shown on the *right axis*. The two OTUs for which full SSU sequences were obtained are framed. GenBank accession numbers represent sequences that matched perfectly with the database

negative effect of a cadaver on euglyphid communities was correlated to the large input of nitrogen and organic carbon in the soil. This result was consistent with previous studies, which show that inputs of nitrogen strongly and negatively influenced testate amoeba communities [34–36]. It is probable that most euglyphids died because of anoxic conditions, but a direct or indirect effect of high nitrogen content is also possible.

However, two well-represented OTUs, namely eugly_13 and eugly_666 (eugly_991 also responded positively but was less abundant), responded positively to the presence of cadavers, but only in the late decomposition stage (i.e. after 1 month to 1 year). These OTUs were present but rare at the beginning of the decomposition process as well as in the control and fake pig treatment, but their abundance peaked, respectively, at D33 and D309 in the pig treatments only and in the three replicates simultaneously (Fig. 5). This suggests that they did not benefit from the initial perturbation brought by the release of cadaveric fluids but rather found optimal conditions (i.e. abiotic, e.g. soil water chemistry, and biotic, e.g.

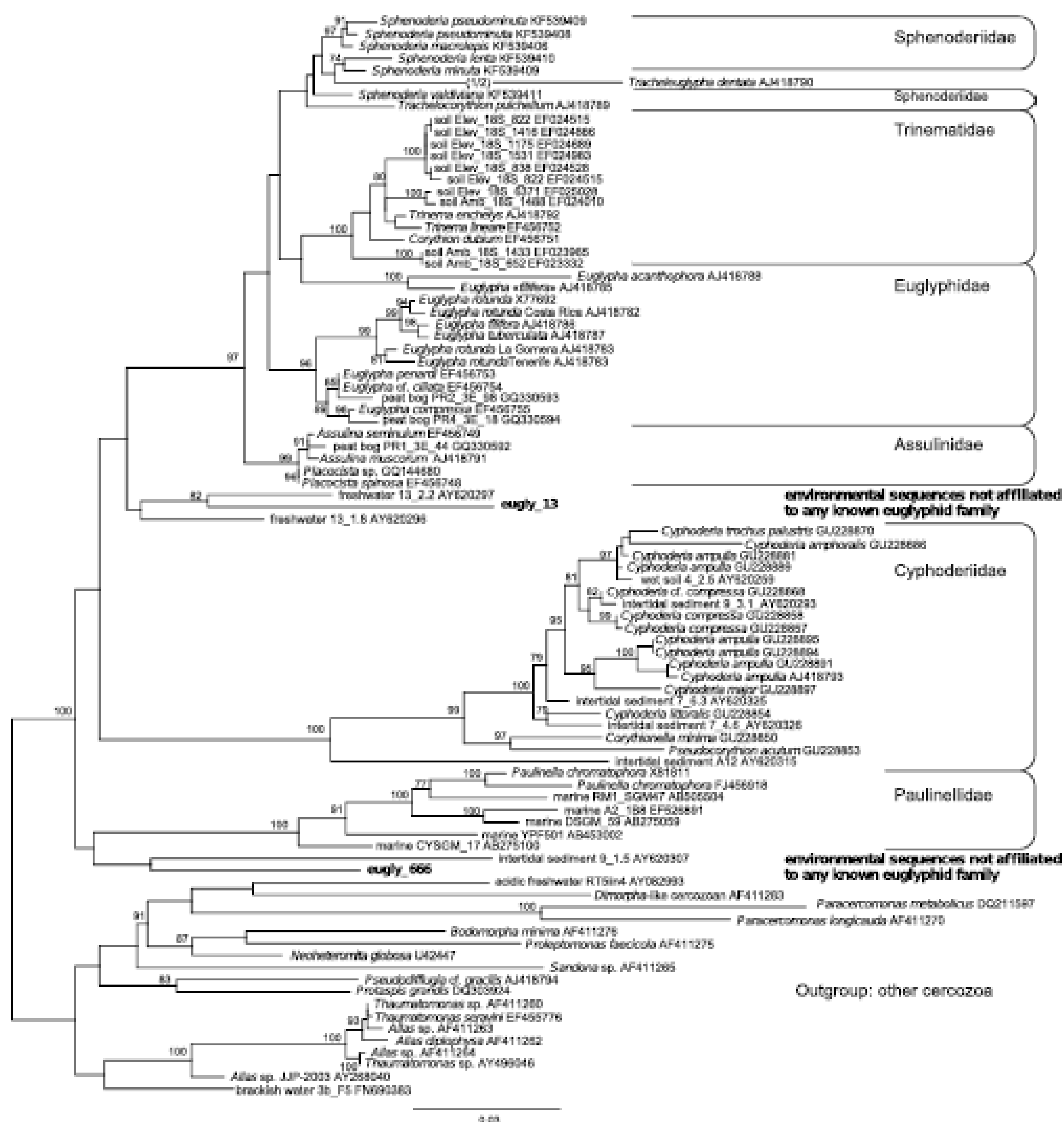

Fig. 4 Maximum likelihood tree built on full SSUrRNA gene sequences of Euglyphida showing the phylogenetic position of eugly_13 and eugly_666 full-length sequences (in *bold*) obtained from pig cadaver plots in a *Fagus-Quercus* forest above Neuchâtel, Switzerland. The tree was built using RaxML on 1440 positions and rooted with several cercozoan sequences retrieved from GenBank. Robustness of nodes was evaluated by bootstrapping (1000 replicates)

prey and/or low level of competition or predation) for their growth in later stages. These organisms probably benefited from changes in the bacterial communities, as these are supposed to change deeply and progressively underneath a cadaver [60–62]. Indeed, previous studies have shown that decomposing carcasses cause an increase in soil bacterial biomass [9] but also drastically change bacterial community structure [14, 15]. As bacteria constitute a large part of euglyphid food regime [22], any change in the abundance or community structure of bacteria is likely to also influence the abundance and community composition of euglyphids. It may also be that these taxa represent nutrient-tolerant organisms that benefit well from high abundance of prey organisms, but with low competitive ability in the normally more

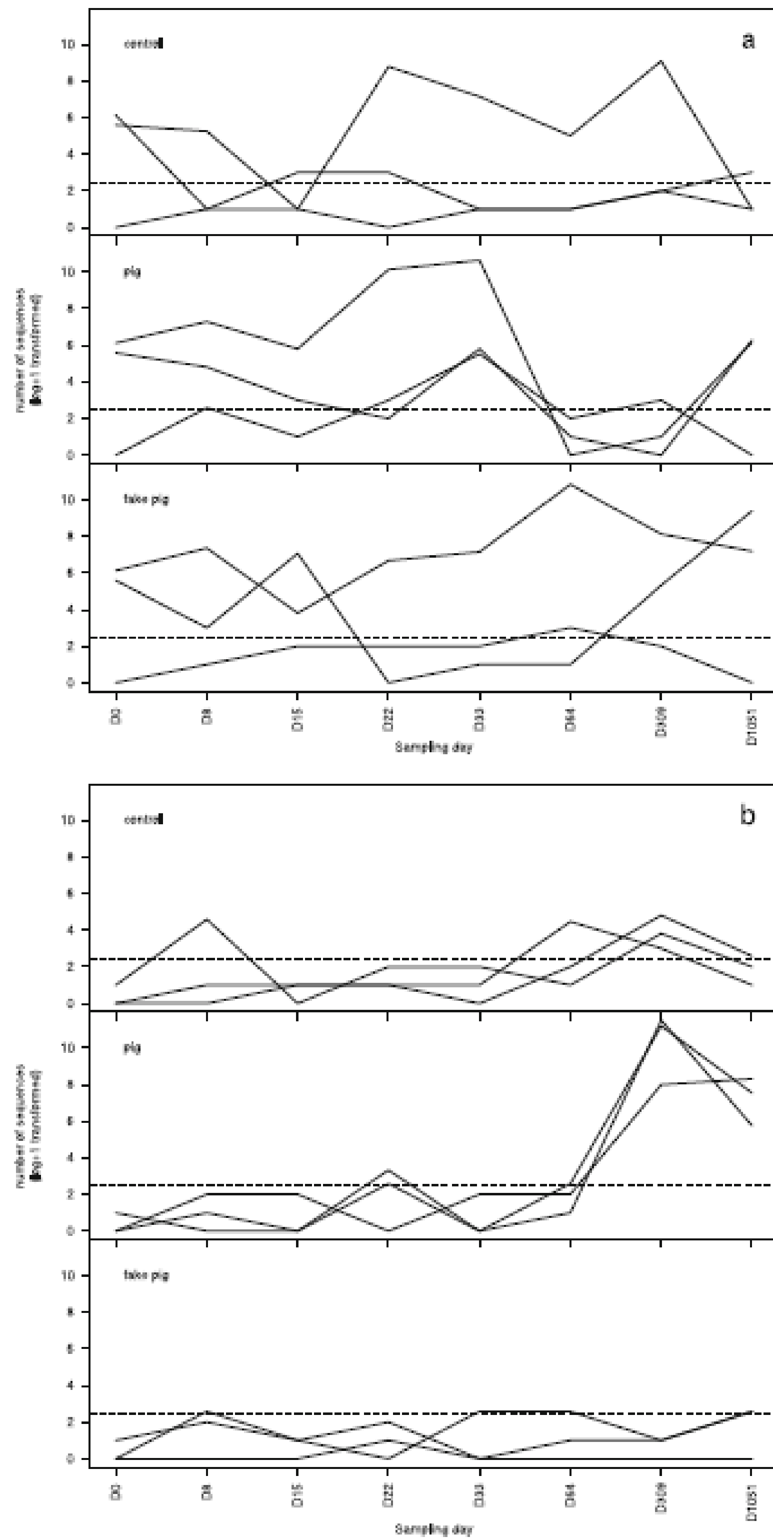

Fig. 5 Temporal pattern of number of sequences (log +1 transformed) over time in control, fake pig and pig cadaver plots in a *Fagus-Quercus* forest above Neuchâtel, Switzerland, for euglyphid testate amoeba OTUs eugly_13 (a) and eugly_666 (b). *Full lines* represent the number of sequences in each block. The *dashed lines* represent the average sequence number in a sample independently of the treatment, block or sampling

oligotrophic conditions. The precise mechanism for this response however remains to be elucidated.

A possible bias could have been due to the import of euglyphids with the cadavers, either from the farm or during transport. However, at D8, cadaver samples were less different from the control than samples from fake cadavers (which could not have been contaminated by the new plastic bags and cotton cloth), and we therefore conclude that such contamination was negligible.

The effect of cadavers on euglyphids peaked at D309 (Fig. 3). This time interval seemed quite long in comparison with the results obtained by Szelecz et al. [18] from the same field experiment (i.e. complete die-out of testate amoebae 22 days post mortem). However, Szelecz et al. [18] studied litter and not the underlying mineral soil horizon as done here, and they used a direct observation (microscopy) approach, which most likely underestimated diversity. Indeed, as OTUs eugly_13 and eugly_666 did not belong to any known euglyphid family (Fig. 4), their morphology may differ significantly from known forms, they may be very small and thus overlooked or lost in classical sample preparation protocols using 10–20-μm filters and/or they may represent naked forms (i.e. without test), as documented in foraminiferans [63].

The RDA and PRC both showed that euglyphid communities had not completely recovered from the influence of cadavers by the end of the experiment (i.e. D1051). This pattern is in line with the observations of Szelecz et al. [18], who did not observe a full recovery by D309 (end of their experiment). This long resilience time suggests that euglyphid communities (and probably testate amoebae in general) could be used as indicators of cadaver presence over very long periods. The fact that euglyphid communities still indicated an effect of cadavers either shows a lag in return to pre-disturbance community structure or that they still responded to other differences (e.g. soil chemistry, abundance and composition of prey).

In addition—and this is in itself an unexpected result—the positive response of certain euglyphid OTUs at certain time points (eugly_13 at D33, eugly_666 at D309) (Fig. 5) suggests that individual taxa may respond specifically and positively to some decomposition stages. Such patterns suggest the possibility to use soil testate amoebae as bioindicators for estimating the time elapsed since death (post-mortal interval, PMI), a parameter of considerable importance in forensic sciences.

Unknown diversity of soil euglyphid testate amoebae

Even after removing rare OTUs, we still found 51 OTUs, 45 of which did not match any sequence in the database. The V9 region does not allow discrimination between close-related species, and it is unclear to how many morphologically and genetically different taxa these 51 OTUs correspond. Regardless of the short length of the barcode, these results reveal the existence of a very high overall diversity of euglyphids in forest soils. This technique yields large amounts of data from small sample volumes, requires much less taxonomic expertise than classical morphological analyses and does not depend on the existence of a reliable taxonomy (which is often lacking for protists).

Perspectives and potential future application

Focusing our study on a specific taxonomic group allowed us to define OTUs at high resolution, using a threshold adapted to already barcoded morphospecies. This approach allowed us to use metabarcoding at a taxonomic resolution close to morphological analysis—much more than what is generally achieved in studies using general eukaryotic marker. Indeed in most studies, more sequences are pooled into OTUs, each of which corresponds to broader taxonomic units than what we achieved in this study. The approach we used to study the response of euglyphid testate amoebae to the impact of decomposing cadavers can also be used to study the responses of any other group of soil eukaryotes. It is indeed very likely that many other taxonomic groups will also show comparable responses to those documented here for euglyphids. Our study shows that some of this unknown diversity could be of potential use for applied purposes such as forensic science. If such patterns can be explored in details, we believe that it will be possible to develop accurate and reliable new molecular bioindicator tools for PMI estimations and other applications.

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Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare no conflict of interest.

References

1. Prangnell J, McGowan G (2009) Soil temperature calculation for burial site analysis. Forensic Sci Int 191:104–109
2. Rodriguez WC, Bass WM (1985) Decomposition of buried bodies and methods that may aid in their location. J Forensic Sci 30:836–852

3. Henssge C, Althaus L, Bolt J, Freislederer A, Haffner HT, Henssge CA, Hoppe B, Schneider V (2000) Experiences with a compound method for estimating the time since death. I. Rectal temperature nomogram for time since death. Int J Legal Med 113:303–319
4. Amendt J, Krettek R, Zehner R (2004) Forensic entomology. Naturwissenschaften 91:51–56
5. Wyss C, Cherix D (2006) Traité d'entomologie forensique. Les insectes sur la scène de crime. Presses polytechniques et universitaires romandes, Lausanns
6. Payne JA (1965) A summer carrion study of the baby pig Sus scrofa Linnaeus. Ecology 46:592–602
7. Carter DO, Yellowlees D, Tibbett M (2007) Cadaver decomposition in terrestrial ecosystems. Naturwissenschaften 94:12–24
8. Vass AA, Bass WM, Wolt JD, Foss JE, Ammons JT (1992) Time since death determinations of human cadavers using soil solution. J Forensic Sci 37:1236–1253
9. Barton PS, Cunningham SA, Lindenmayer DB, Manning AD (2013) The role of carrion in maintaining biodiversity and ecological processes in terrestrial ecosystems. Oecologia 171:761–772
10. Towne EG (2000) Prairie vegetation and soil nutrient responses to ungulate carcasses. Oecologia 122:232–239
11. Melis C, Selva N, Teurlings I, Skarpe C, Linnell JDC, Andersen R (2007) Soil and vegetation nutrient response to bison carcasses in Bialeowieza Primeval Forest, Poland. Ecol Res 22:807–813
12. Bornemissza GF (1957) An analysis of arthropod succession in carrion and the effect of its decomposition on the soil fauna. Aust J Zool 5:1–12
13. Horswell J, Cordiner SJ, Maas EW, Martin TM, Sutherland BW, Speir TW, Nogales B, Osborn AM (2002) Forensic comparison of soils by bacterial community DNA profiling. J Forensic Sci 47:350–353
14. Howard GT, Duos B, Watson-Horzelski EJ (2010) Characterization of the soil microbial community associated with the decomposition of a swine carcass. Int Biodeterior Biodegrad 64:300–304
15. Moreno LI, Mills D, Fetscher J, John-Williams K, Meadows-Jantz L, McCord B (2011) The application of amplicon length heterogeneity PRC (LH_PCR) for monitoring the dynamics of soil microbial communities associated with cadaver decomposition. J Microbiol Meth 84:388–393
16. Hawksworth DL, Wiltshire PEJ (2011) Forensic mycology: the use of fungi in criminal investigations. Forensic Sci Int 206:1–11
17. Carter DO, Tibbett M (2003) Taphonomic mycota: fungi with forensic potential. J Forensic Sci 48:168–171
18. Szelecz I, Fournier B, Seppey CVW, Amendt J, Mitchell EAD (2014) Can soil testate amoebae be used for estimating the time since death? A field experiment in a deciduous forest. Forensic Sci Int 236:90–98
19. Foissner W (1999) Soil protozoa as bioindicators: pros and cons, methods, diversity, representative examples. Agric Ecosyst Environ 74:95–112
20. Foissner W (1987) Soil protozoa: fundamental problems, ecological significance, adaptations in ciliates and testaceans, bioindicators, and guide to the literature. In: Corliss JO and Patterson DJ (ed) Progress in Protistology, vol 2. Bristol, Biopress, pp 69–212
21. Meisterfeld R (2000) Order Arcellinida Kent, 1880. In: Lee JJ, Leedale GF, Bradbury P (eds) An illustrated guide to the Protozoa Society of Protozoologists. Lawrence, Kansas, pp 827–859
22. Meisterfeld R (2000) Testate amoebae with filopodia. In: Lee JJ, Leedale GF, Bradbury P (eds) An illustrated guide to the Protozoa Society of Protozoologists. Lawrence, Kansas, pp 1054–1084
23. Gilbert D, Amblard C, Bourdier G, Francez AJ, Mitchell EAD (2000) Le régime alimentaire des Thécamoebiens (Protista, Sarcodina). Annee Biol 39:57–68
24. Bobrov AA, Charman DJ, Warner BG (1999) Ecology of testate amoebae (Protozoa: Rhizopoda) on peatlands in Western Russia with special attention to niche separation in closely related taxa. Protist 150:125–136
25. Booth RK (2008) Testate amoebae as proxies for mean annual water-table depth in Sphagnum-dominated peatlands of North America. J Quat Sci 23:43–57
26. Swindles GT, Ruffell A (2009) A preliminary investigation into the use of testate amoebae for the discrimination of forensic soil samples. Sci Justice 49:182–190
27. Beyens L, Ledeganck P, Graae BJ, Nijs I (2009) Are soil biota buffered against climatic extremes? An experimental test on testate amoebae in arctic tundra (Qeqertarsuaq, West Greenland). Polar Biol 32: 453–462
28. Tsyganov AN, Nijs I, Beyens L (2011) Does climate warming stimulate or inhibit soil protist communities? A test on testate amoebae in high-arctic tundra with free-air temperature increase. Protist 162: 237–248
29. Booth RK (2001) Ecology of testate amoebae (Protozoa) in two lake superior coastal wetlands: implications for paleoecology and environmental monitoring. Wetlands 21:564–576
30. Charman DJ (2001) Biostratigraphic and palaeoenvironmental applications of testate amoebae. Quat Sci Rev 20:1753–1764
31. Mitchell EAD, Buttler AJ, Warner BG, Gobat JM (1999) Ecology of testate amoebae (Protozoa: Rhizopoda) in Sphagnum peatlands in the Jura mountains, Switzerland and France. Ecoscience 6:565–576
32. Mitchell EAD, Charman DJ, Warner BG (2008) Testate amoebae analysis in ecological and paleoecological studies of wetlands: past, present and future. Biodivers Conserv 17:2115–2137
33. Petz W, Foissner W (1989) The effect of mancozeb and lindane on the soil microfauna of a spruce forest—a field study using a completely randomized block design. Biol Fertil Soils 7:225–231
34. Gilbert D, Amblard C, Bourdier G, Francez AJ (1998) Short-term effect of nitrogen enrichment on the microbial communities of a peatland. Hydrobiologia 374:111–119
35. Gilbert D, Amblard C, Bourdier G, Francez AJ (1998) The microbial loop at the surface of a peatland: structure, function, and impact of nutrient input. Microbial Ecol 35:83–93
36. Mitchell EAD (2004) Response of testate amoebae (Protozoa) to N and P fertilization in an arctic wet sedge tundra. Arct Antarct Alp Res 36:78–83
37. Payne R, Gauci V, Charman DJ (2010) The impact of simulated sulfate deposition on peatland testate amoebae. Microbial Ecol 59: 76–83
38. Heal OW (1964) Observations on the seasonal and spatial-distribution of testacea (Protozoa: Rhizopoda) in Sphagnum. J Anim Ecol 33:395–412
39. Wanner M, Elmer M, Kazda M, Xylander WER (2008) Community assembly of terrestrial testate amoebae: how is the very first beginning characterized? Microbial Ecol 56:43–54
40. Wanner M, Elmer M (2009) "Hot spots" on a new soil surface—how do testate amoebae settle down? Acta Protozool 48:281–289
41. Chatelain AP, Meisterfeld R, Roussel-Delif L, Lara E (2013) Sphenoderiidae (fam. nov.), a new clade of euglyphid testate amoebae characterized by small, round scales surrounding the aperture. Protist 164:782–792
42. Heger TJ, Pawlowski J, Lara E, Leander BS, Todorov M, Golemansky V, Mitchell EAD (2011) Comparing potential COI and SSU rDNA barcodes for assessing the diversity and phylogenetic relationships of cyphoderiid testate amoebae (Rhizaria: Euglyphida). Protist 162:131–141
43. Taberlet P, Coissac E, Pompanon F, Brochmann C, Willerslev E (2012) Towards next-generation biodiversity assessment using DNA metabarcoding. Mol Ecol 21:2045–2050
44. Amaral-Zettler LA, McCliment EA, Ducklow HW, Huse SM (2009) A method for studying protistan diversity using massively parallel sequencing of v9 hypervariable regions of small-subunit ribosomal RNA genes. Plos One 4:e6372
45. Adl SM, Habura A, Eglit Y (2013) Amplification primers of SSU rDNA for soil protists. Soil Biol Biochem 69:328–342
46. Valentini A, Pompanon F, Taberlet P (2009) DNA barcoding for ecologists. Trends Ecol Evol 24:110–117

47. Stokes KL, Forbes SL, Tibbett M (2013) Human versus animal: contrasting decomposition dynamics of mammalian analogues in experimental taphonomy. J Forensic Sci 58:583–591
48. Harris D, Horwath WR, van Kessel C (2001) Acid fumigation of soils to remove carbonates prior to total organic carbon or carbon-13 isotopic analysis. Soil Sci Soc Am J 65:1853–1856
49. Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ (1997) Gapped blast and psi-blast: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
50. Lara E, Heger TJ, Mitchell EAD, Meisterfeld R, Ekelund F (2007) SSU rRNA reveals a sequential increase in shell complexity among the euglyphid testate amoebae (Rhizaria: Euglyphida). Protist 158: 229–237
51. Legendre P, Gallagher ED (2001) Ecologically meaningful transformations for ordination of species data. Oecologia 129:271–280
52. Borcard Daniel, Legendre Pierr, Gillet François (2011) Numerical ecology with R. Springer, New York. URL http://www.worldcat.org/search?qt=worldcat_org_all&q=9781441979759.
53. Van den Brink PJ, Ter Braak CJF (1999) Principal response curves: analysis of time-dependent multivariate responses of biological community to stress. Environ Toxicol Chem 18:138–148
54. Team RCore (2013) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/
55. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H (2012) vegan: Community Ecology Package. URL http://CRAN.R-project.org/package=vegan
56. Dunthorn M, Otto J, Berger SA, Stamatakis A, Mahe F, Romac S, Vargasde C, Audic S, Stock A, Kauff F, Stoeck T, Consortium BioMarKs (2014) Placing environmental next-generation sequencing amplicons from microbial eukaryotes into a phylogenetic context. Mol Biol Evol 31:993–1009
57. Edgar RC (2004) Muscle: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5:1–19
58. Stamatakis A (2006) Raxml-vi-hpc: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690
59. Heger TJ, Mitchell EAD, Todorov M, Golemansky V, Lara E, Leander BS, Pawlowski J (2010) Molecular phylogeny of euglyphid testate amoebae (Cercozoa: Euglyphida) suggests transitions between marine supralittoral and freshwater/terrestrial environments are infrequent. Mol Phylogenet Evol 55:113–122
60. Allison SD, Martiny BH (2008) Resistance, resilience, and redundancy in microbial communities. Proc Natl Acad Sci U S A 105:11512–11519
61. Fierer N, Lauber CL, Ramirez KS, Zaneveld J, Bradford MA, Knight R (2012) Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. ISME J 6:1007–1017
62. Ramirez KS, Lauber CL, Knight R, Bradford MA, Fierer N (2010) Consistent effects of nitrogen fertilization on soil bacterial communities in contrasting systems. Ecology 91:3463–3470
63. Pawlowski J, Bolivar I, Fahrni J, de Vargas C, Bowser SS (1999) Naked foraminiferans revealed. Nature 399:27

Appendix B

Pictures from the experiments

Ildikó Szelecz

1. First experiment 2009 near Neuchâtel, Switzerland. Decomposition stages *Sus scrofa*

a.

b.

c.

d.

e.

Decomposition stages a: fresh stage (open cage), **b:** fresh stage (cage closed), **c:** bloated stage, **d:** active decay, **e:** advanced decay, **f:** dry stage.

1. First experiment 2009 near Neuchâtel, Switzerland. Decomposition stages *Sus scrofa*

f.

cage for cadaver

2. Second experiment 2013 near Neuchâtel, Switzerland. Decomposition stages *Sus scrofa*

a.

b.

c.

d.

e.

f.

Decomposition stages a: fresh stage, **b:** bloated stage **c:** skin ruptures, **d:** active decay starts, **e:** active decay, **f:** advanced decay starts.

2. Second experiment 2013 near Neuchâtel, Switzerland. Decomposition stages *Sus scrofa*

g.

h.

I.

j.

k.

Decomposition stages g: advanced decay, starts to dry, **h:** dry stage, **i:** remains.
j: dripping zone beneath hanging pig, **k:** soil sampling frame.

Appendix C

Curriculum vitae

Ildikó Szelecz

Ildikò Szelecz – Curriculum Vitae

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ildiko.szelecz@unine.ch or szelecz@web.de

My main research interest is Forensic Science especially PMI estimation.
Here I am using different approaches
to understand the complex changes above and below ground.

Education

12.2012-12.2016 PhD

Impact of vertebrate cadavers on soil communities and soil chemistry to develop new forensic indicators for estimating the time after death

University of Neuchâtel, Switzerland

09.2010- 10.2012 Research assistant

Institute of Legal Medicine, Frankfurt am Main, Germany

10.2003–07.2010 Diploma in Biology

Soil organisms beneath a cadaver- a tool for estimating the time of death

University of Frankfurt am Main, Germany

Scientific work

1. Publications

Peer-reviewed publications

Szelecz I, Sorge F, Seppey C, Mulot M, Steel H, Neilson R, Griffiths B, Amendt J, Mitchell E. A.D. Seppey CVW (2016). Effects of decomposing cadavers on soil

nematode communities over a one-year period. Soil Biology & Biochemistry 103 (2016): 405-416.

Szelecz I, Fournier B, Seppey C, Amendt J, Mitchell EAD (2014). Can soil testate amoebae be used for estimating the time since death? A field experiment in a deciduous forest. Forensic Science International 236: 90-98.

Submitted

Szelecz I, N. Feddern N, Seppey CVW, Amendt J, Mitchell EAD (2016). Is Saprinus semistriatus (Coleoptera: Histeridae) a useful taxon for the estimation of the post-mortem interval? Forensic Science International

Contributed

Seppey CVW, Fournier B, Szelecz I, Singer D, Mitchell EAD, Lara E (2015). Response of forest soil euglyphid testate amoebae (Rhizaria: Cercozoa) to pig cadavers assessed by high-throughput sequencing. International Journal of Legal Medicine 1: 1149-1157.

Other publications

Szelecz I, Mitchell EAD (2014) Les îles de décomposition. L'Ermite herbu. Journal de l`Association des amis du Jardin botanique de l'Ermitage, N° 48.

Szelecz I, Amendt J, Sorge F, Seppey C, Mulot M, Mitchell M (2014): Soil beneath cadavers – Influence of decomposition on selected chemical markers and free-living terrestrial nematodes. Rendiconti Online della Societa Geologica Italiana 30, Suppl. n. 1: 18

Szelecz I, Sorge F, Feddern N, Seppey C, Mulot M, Amendt J, Mitchell EAD (2014) Invertebrates: Above ground, below ground and in court?. Special issue of Antenna, Bulletin of the Royal Entomology Society, United Kingdom: 116-117.

Szelecz I, Amendt J (2010). Leichenfund im Wald. Bodenökologische Untersuchungen zur Eingrenzung der Leichenliegezeit Forschung Frankfurt 2/2010.

2. Presentations and posters (underlined: person presenting)

National and international meetings

2016

Szelecz I, Lara E, Seppey C, Sorge F, Singer D, Perotti MA, Loesch S , Tschui J , Mitchell EAD. The lament of scattered bones-a multiproxy approach in a real case investigation in forensic science,
DGP (German Society for Protozoology) meeting, Saignelégier, Jura, Switzerland; 23-26 February 2016 (2nd prize poster award).

2015

Szelecz I, Sorge F, Mulot M, Seppey C, Amendt J, Mitchell EAD.2015. Effects of decomposing cadavers on soil nematodes- a novel indicator for longer post-mortem intervals? Sommertagung der Schweizerischen Gesellschaft für Rechtsmedizin SGRM, Locarno, Switzerland, 12-13 June 2015.

Szelecz I, Feddern N, Fournier B, Seppey C, Amendt J, Mitchell EAD. 2015. *Saprinus semistriatus*- a lazy little fellow? 12th Meeting of the European Association for Forensic Entomolgy (EAFE), Huddersfield, UK, 6-9 May 2015.

Reczuga MK, Seppey C, Szelecz I, Fournier B, Singer D, Lara E, Mitchell EAD. 2015. Response of soil micro-eukaryotes to cadaver decomposition as assessed by high throughput sequencing. 34nd Meeting of the German Society for Protistology. Magdeburg, Germany, 3-6 March 2015.

2014

Szelecz I, Seppey C, Mulot M, Kohler R, Sorge F, Feddern N, Amendt J, Mitchell EAD. 2014. Influence of decomposing cadavers on soil: A field experiment in Switzerland. Soil Science Society (BGS/SSP) meeting in Changins, Switzerland, 13- 14 February 2014.

Szelecz I, Amendt J, Sorge F, Seppey C, Mulot M, Mitchell EAD. 2014. Soil beneath cadavers –Influence of decomposition on selected chemical markers and free-living terrestrial nematodes, 3rd ENFSI APST WG 8 (Animal Plant and Soil Traces meeting of the European Network of Forensic Science Institutes), Rome, Italy, 2- 4 April 2014.

Seppey C. 2014. Euglyphid communities in Cadaver Decomposition Island by a metabarcoding approach, DNA watch meeting, Geneva, Switzerland, 24-25 April 2014.

Szelecz I, Sorge F, Feddern N, Seppey C, Mulot M, Amendt J, Mitchell EAD. 2014. Impact of cadavers on above- and below-ground invertebrates and soil function. European Congress of Entomology (ECE 2014), York, UK, 3- 8 August 2014.

Feddern N, Szelecz I, Seppey C, Mulot M, Amendt J, Mitchell EAD. 2014. Comparison of the insect fauna of hanging and ground pig cadavers, European Congress of Entomology (ECE 2014), York, UK, 3-8 August 2014.

Sorge F, Szelecz I, Amendt J, Seppey C, Mulot M, Mitchell EAD. 2014. Soil beneath cadavers – Influence of decomposition on selected chemical markers and free-living terrestrial nematodes. 93. Jahrestagung Deutsche Gesellschaft für Rechtsmedizin (DGRM), Heringsdorf, Germany, 9 – 13 September 2014.

Feddern N, Szelecz I, Mulot M, Sorge F, Mitchell EAD, Amendt J. 2014. Insektenbesiedlung und Verwesung von hängenden und aufliegenden Schweinekadavern. 93. Jahrestagung Deutsche Gesellschaft für Rechtsmedizin (DGRM), Heringsdorf, Germany, 9-13 September 2014.

Koenig I, Mulot M, Szelecz I, Fournier B, Lara E, Mitchell EAD. 2014. Response of testate amoeba functional diversity to environmental gradients, stress and perturbation. 7th International Symposium on Testate Amoebae, Poznań, Poland, 8 - 12 September 2014.

Szelecz I, Sorge S, Mulot M, Fournier B, Seppey C, Amendt J, Mitchell EAD. 2014. Cadaver effects on soil biodiversity- results from two field experiments. First Global Soil Biodiversity Conference, Dijon, Franc, 2-5 December 2014.

Seppey C, Szelecz I, Fournier B, Tarnawski SE. 2014. Metabarcoding of micro-eukaryotes – multiple applications from biodiversity assessment to applied ecological research First Global Soil Biodiversity Conference, Dijon, Franc, 2-5 December 2014.

2013

Seppey, C. 2013. Euglyphida (Cercozoa; Rhizaria; Eukaryota) communities under pig cadavers: study by high throughput sequencing, 5th Swiss Microbial Ecology Meeting, Murten, Switzerland, 4-6 February 2013.

Mitchell EAD, Szelecz I, Klang C, Seppey C, Fournier B, Mulot M, Amendt J, Heurich M, Lara E. 2013. Response of soil testate amoebae to the presence of a decomposing pig cadaver – a study using morphological and molecular approaches. 32nd Meeting of the German Society for Protistology. Zurich, Switzerland, 27 February – 2 March 2013.

Seppey C, Fournier B, Mulot M, Szelecz I, Lara E, Mitchell EAD. 2013. Euglyphida communities under pigs cadavers assessed by high throughput sequencing: a new indicator of the post mortem interval (PMI)? 32nd Meeting of the German Society for Protistology, Zurich, Switzerland, 27 February – 2 March 2013.

Szelecz I, Klang C, Fournier B, Amendt J, Heurich M, Mitchell EAD. 2013. Testate amoebae in soil – their potential as an indicator of time since death. 32nd Meeting of the German Society for Protistology, Zurich, Switzerland, 27 February – 2 March 2013.

2009-2012

Szelecz I, Fournier B, Amendt J, Mitchell E. 2010. Testate amoebae as bioindicators of cadaver decomposition. 89th Annual conference of the German Society of Legal Medicine, Berlin, Germany, 22- 25 September 2010.

Szelecz I, Amendt J. 2012. Nematodes in soil- their potential use as an indicator of time since death. ENFSI- APST (Animal Plant and Soil Traces meeting of the European Network of Forensic Science Institutes), Madrid, Spain, 23-25 May, 2012.

Amendt J, Szelecz I. 2011. Going down, down, down… Soil organisms as a tool in forensic science. ENFSI -APST (Animal Plant and Soil Traces meeting of the European Network of Forensic Science Institutes), Brussels, Belgium, 10 March 2011.

Szelecz I, Fournier B, Amendt J, Mitchell E. 2009. Forensic science goes underground: Testate Amoebae as bioindicators of cadaver decomposition. ISTA-5 5th International Symposium on Testate Amoebae, Montbéliard, France, 14-17 September 2009.

Amendt J, Szelecz I. 2009. Entomologische und Bodenbiologische Untersuchungen zur Liegezeitabschätzung. IX. Forensische Gespräche, Kiel-Lübeck, Germany, 4-5 December 2009.

3. Seminars and internal workshops

2015

Feddern N, Szelecz I, Seppey C, Mulot M, Amendt J, Mitchell EAD. 2015. Comparison of the insect fauna of hanging and ground pig cadavers. Forensic Ecology workshop, University of Neuchâtel, Neuchâtel, Switzerland 18-19.02.2015 (poster).

2014

Mitchell EAD. 2014. Des cochons, des sphaignes et des amibes: tour d'horizon de quelques recherches en cours au laboratoire de biologie du sol. Société Neuchâteloise de Sciences Naturelles. 5.2.2014.

Szelecz I, Sorge F, Seppey C, Mulot M, Amendt J, Mitchell EAD. 2014. Impact of decomposing cadavers on soil with focus on soil nematodes. University of Ghent, Belgium, 07 January 2014 (oral presentation).

Szelecz I, Seppey C, Feddern N, Sorge F, Mulot M, Amendt J, Mitchell EAD. 2014. Impact of cadavers on soil community and function. The search for new tools in Forensic science. Institute of Forensic Medicine, Bern, Switzerland, 29 January 2014 (oral presentation).

Szelecz I, Seppey C, Sorge F, Mulot M, Amendt J, Mitchell EAD. 2014. Decomposing cadavers on soil. Institute of Legal Medicine, Frankfurt/Main, Germany, 17 March 17 2014 (oral presentation).

Seppey C. 2014. Micro-eukaryotic biodiversity and ecology assessed by metabarcoding. Annual PhD meeting, University of Neuchâtel, Switzerland, 08 May 2014 (poster).

Szelecz I, Seppey C, Mulot M, Kohler R, Sorge F, Feddern N, Amendt J, Mitchell EAD. 2014. Influence of decomposing cadavers on soils- a field experiment in Switzerland. Annual PhD meeting, University of Neuchâtel, Switzerland, 08 May 2014 (poster).

Seppey C. 2014. Euglyphid communities in Cadaver Decomposition Island by a metabarcoding approach. Meeting of the Swiss Institute of Bioinformatic Training Network, 07 July 2014 Bern, Switzerland (oral presentation).

2013

Szelecz I. 2013. Testate amoebae in soil – their potential as an indicator of time since death. PhD meeting, University of Neuchâtel, Switzerland, 02 May 2013 (poster).

Szelecz I, Seppey C, Mitchell EAD. 2013. Impact of cadavers on soil community and function - an experiment in Switzerland. University of Reading, UK, 06 November 2013 (oral presentation).

Szelecz I, Klang C, Fournier B, Amendt J, Heurich M, Mitchell EAD. 2013. Potential of soil testate amoebae as an indicator of time since death. Interuniversity testate amoeba seminar, Frasne, France 08 March 2013 (oral presentation).

2009-2012

Amendt J, Szelecz I. 2012. Tierkadaver und Verwesung als möglicher Motor von Biodiversität? University of Bochum, Bochum, Germany, 19 December 2012 (oral presentation).

4. Teaching

2015

Szelecz I, Seppey C, Mitchell EAD, 2015. Effets des perturbations et du stress sur les communautés d'organismes du sol. Testate amoebae and their response to blood, manure and decomposing cadavers. APP (Problem Based Learning for 3rd year biology bachelor students), University of Neuchâtel, Neuchâtel, Switzerland, 30 April- 28 May 2015.

Szelecz I, Mitchell EAD (organizers). 2015. Forensic Ecology workshop (Scientific meeting). University of Neuchâtel, Neuchâtel, Switzerland 18-19 February 2015.

2014

Sorge F. 2014. Effect of cadavers on soil nematode communities – developing a new indicator for post-mortem interval, Institute of legal Medicine, Frankfurt am Main, Germany, Johannes Gutenberg University Mainz, Germany and University of Neuchâtel, Switzerland (Master`s Thesis), May 2014 (co-supervisor of Master's Thesis).

Szelecz I, Seppey C, Mitchell EAD. 2015. Effects of mice cadavers' decomposition on the composition of testate amoebae communities. APP (Problem Based Learning for 3rd year biology bachelor students), University of Neuchâtel, Neuchâtel, Switzerland, 28 April- 27 May 2014.

5. Outreach – the experiments in press, radio and television

2016

Swiss TV (SRF Schweizer Radio und Fernsehen), 15.09.2016 (21 Uhr), Einstein: Mumien-Kampf gegen den Zerfall, Wie tote Schweine helfen, künftige Kriminalfälle aufzuklären (www.srf.ch)

Articles in four Swiss newspapers:

Mit Schweinen Kriminalfälle lösen, Nordwestschweiz, 04.03.2016

Tote Schweine sollen helfen, Kriminalfälle zu lösen, Der Landbote, 04.03.2016

Tote Schweine sollen helfen, Kriminalfälle zu lösen, Südostschweiz, 05.03.2016

Tote Schweine sollen helfen, Kriminalfälle zu lösen, Berner Oberländer/Thuner Tagblatt, 09.03.2016

2014

Scientific American Magazine: "Amoebas on Deathwatch", October 2014 Issue. http://www.nature.com/scientificamerican/journal/v311/n4/full/scientificamerican1014-24.html

Neue Zürcher Zeitung: "Schalenamöben helfen Forensikern. Verräterische Einzeller", 01.05.2014. http://www.nzz.ch/wissenschaft/uebersicht/verraeterische-einzeller-1.18292863

RTN (Radio Television Neuchâtel): "Porcs morts pour la science ", 07.04.2014. http://www.rtn.ch/rtn/Programmes/emissions/La-Matinale/Porcs-morts-pour-la-science.htm

2013

RTS (Swiss National Television) : "Des scientifiques datent les morts avec une methode inédite", 23.10.2013. http://www.rts.ch/play/tv/le-19h30/video/ne-des-scientifiques-datent-les-morts-avec-une-methode-inedite?id=5317300

6. Other skills and interests

Languages: English (fluent), German and Hungarian (fluent), French (beginner), Spanish (beginner)

Computer skills: R (basic), Inkscape, InDesign, Office.

Other qualifications: M.A. in German language and literature, certified teacher "German for foreigners and immigrants"

Interests and hobbies: travelling, modern art, running, goats, theatre and comedy.

Appendix D

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Onwards and upwards !

Final figure: Detektivski by Nina Feddern.