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Effects of storage and handling on neutral lipid fatty acid profiles of two woodlice (Isopoda, Crustacea) species differing in size

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ABSTRACT

Neutral lipid fatty acid (NLFA) analysis is a promising tool to investigate energy fluxes and basal resources of soil animal communities. This study aimed at investigating the effect of common handling and storage procedures in soil animal ecology on the NLFA composition and marker NLFAs. First, we tested the effect of the method of killing (by freezing and by drowning in diethylene glycol mixed with water; 1:1) on NLFA composition of the woodlouse *Oniscus asellus*. Additionally, we inspected the effect of two reference methods (direct freezing at −20 °C and storage in methanol at −80 °C with evaporating methanol prior to lipid extraction) after 4 and 12 months on the NLFA profile of *O. asellus*. We investigated the effect of the collection solution used in heat extraction of soil animals (water and glycol), storage fluids (saltwater, glycerine, ethanol and methanol) and storage time (4 and 12 months) on NLFA composition and common marker NLFAs of two Isopoda species differing in body size. The NLFA composition and marker fatty acids (FAs) did not differ between the methods of killing and between the two reference treatments but the amount of saturated FAs and the saturated-to-unsaturated FA ratio generally increased after 12 months indicating oxidation processes. Generally, the collection solution was of minor importance, whereas storage fluid and storage time strongly affected NLFA patterns. The NLFA profile of the larger species *O. asellus* was affected by storage in methanol and ethanol and to some extent in saltwater, while the NLFA profile of the smaller *Trichoniscus pusillus* was affected by each of the treatments. Notably, however, marker FAs of *O. asellus* and *T. pusillus* were not significantly affected by storing in glycerine even after 12 months in *O. asellus*. Therefore, if animals need to be stored prior to NLFA analysis for sorting and identification, storage in glycerine is advisable.

1. Introduction

The main storage compounds of invertebrates are neutral lipids (triglycerides) (O'Connor and Gilbert, 1968; Beenakkers et al., 1985) irrespective of differences in storage organs, i.e. fat body in Insecta and hepatopancreas in Crustacea (Gilbert and O'Connor, 1970). Triglycerides consist of three fatty acids (FAs) bound to a glycerol skeleton via ester bindings. In Arachnida little is known on storage compounds, but triglycerides presumably also are of major importance (Laino et al., 2009, 2015). Besides being part of energy storage and utilization, neutral lipids bear information on the nutritional status and food sources of organisms (Lease and Wolf, 2011). This is because it is energetically more efficient to take up and store FAs from food without modification rather than synthesizing them, a process termed 'dietary routing'. In insects it has been shown that dietary fat from the midgut is directly transferred as diacylglycerol into the fat body during feeding (Canavoso et al., 2001). Thus, the consumer FA profile is reflecting the FA profile of the diet (Haubert et al., 2004; Ruess et al., 2004; Menzel et al., 2017). In a number of ecological systems, but in particular in soil animal communities, the channelling of carbon from basal resources to higher trophic levels still is little understood (Moore et al., 2005). Allowing to trace basal food resources in consumers, neutral lipid fatty acid (NLFA) analysis is a promising tool to fill this gap of knowledge (Ruess and Chamberlain, 2010; Traugott et al., 2013).

The handling and storage procedure of animals prior to the extraction of NLFAs might affect NLFA profiles. FAs are known to be prone to modifications after death of an animal (Petillo et al., 1998; Rudy et al., 2016). These processes include non-enzymatic oxidation (autoxidation) of animals exposed to air as well as hydrolysis by lipases in animal tissue not inactivated after death. These lipases are responsible for FA separation from their glycerol backbone resulting in an increase in free FAs. In addition, exposure to light might lead to photo-oxidation of triglycerides (Frankel, 1984; Kato et al., 2018). Therefore, direct analysis of lipids from animals collected in the field is preferable

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Table 1
Experimental set-up to test the effects of methods of killing (drowning, freezing at −80 °C), heat extraction (no extraction, heat extraction in water for 1 day, heat extraction in glycol for 7 days) and storage fluid (methanol, −80 °C or directly frozen, methanol stored at −20 °C, 70% ethanol stored at −15 °C, glycerine stored at −15 °C, saltwater stored at −10 °C) and storage time (4 or 12 months) on neutral lipid FA profiles of *Oniscus asellus* and *Trichoniscus pusillus*. Numbers represent the number of replicates analysed for the respective treatment; nd, not determined.

Species	Experiment	Killing method	Heat extraction	Storage	4 months	12 months
Oniscus asellus	killing test	−80 °C	no	ethanol, −15 °C	5	nd
Oniscus asellus	killing test	drowning	no	ethanol, −15 °C	4	nd
Oniscus asellus	reference test	−80 °C	no	directly frozen, −20 °C	5	5
Oniscus asellus	reference test	−80 °C	no	methanol, −80 °C	5	5
Oniscus asellus	main experiment	−80 °C	no	methanol, −80 °C	5	nd
Oniscus asellus	main experiment	−80 °C	water,1d	methanol, −20 °C	5	nd
Oniscus asellus	main experiment	−80 °C	water,1d	ethanol, −15 °C	5	nd
Oniscus asellus	main experiment	−80 °C	water,1d	glycerine, −15 °C	5	5
Oniscus asellus	main experiment	−80 °C	water,1d	saltwater, −10 °C	4	5
Oniscus asellus	main experiment	−80 °C	glycol,7d	methanol, −20 °C	4	nd
Oniscus asellus	main experiment	−80 °C	glycol,7d	ethanol, −15 °C	5	nd
Oniscus asellus	main experiment	−80 °C	glycol,7d	glycerine, −15 °C	5	5
Oniscus asellus	main experiment	−80 °C	glycol,7d	saltwater, −10 °C	4	5
Trichoniscus pusillus	main experiment	drowning	water,1d	methanol, −80 °C	5	nd
Trichoniscus pusillus	main experiment	drowning	water,1d	ethanol, 70%, −15 °C	5	nd
Trichoniscus pusillus	main experiment	drowning	water,1d	glycerine, −15 °C	5	nd
Trichoniscus pusillus	main experiment	drowning	water,1d	methanol, −20 °C	5	nd
Trichoniscus pusillus	main experiment	drowning	water,1d	saltwater, −10 °C	5	nd
Trichoniscus pusillus	main experiment	drowning	glycol,7d	ethanol, 70%, −15 °C	5	nd
Trichoniscus pusillus	main experiment	drowning	glycol,7d	glycerine, −15 °C	5	nd
Trichoniscus pusillus	main experiment	drowning	glycol,7d	methanol, −20 °C	5	nd
Trichoniscus pusillus	main experiment	drowning	glycol,7d	saltwater, −10 °C	5	nd

over storage, but often storage at least for some period of time is necessary, e.g. for extraction of animals and for determination of species. Therefore, there is the need to investigate the effect of common handling procedures on the NLFA composition of soil animals.

For NLFA extraction of larger soil animals or small animals from laboratory experiments, single individuals can be picked by hand and kept frozen directly thereafter (Haubert et al., 2011; Ferlian et al., 2012; Buse et al., 2013), or separately transferred into methanol and stored frozen (Haubert et al., 2009). Before lipid extraction the methanol is evaporated and the lipids are further processed. Methanol is known to inactivate lipases responsible for changes in FA profiles (Lotti et al., 2015). For microarthropods, such as Collembola and Acari as well as for Nematoda, numerically dominating soil invertebrate taxa (Schaefer, 1990), a number of individuals have to be pooled for NLFA analysis. In field experiments, a common procedure to extract microarthropods from soil or litter is using a heat gradient for 7–10 days (Kempson et al., 1963) and to collect the animals in diethylene glycol mixed with water (1:1). An alternative is the extraction in water for several days with collection of living animals and directly store them frozen (Pollierer et al., 2012). However, this is only possible for taxa which can be identified quickly. Commonly, after extraction, animals are stored in ethanol, saturated saltwater or glycerine, and inspected under the microscope for identification.

For the efficient use of neutral lipid analysis for soil microarthropods it is important to know which handling procedures are the most protective against lipid degradation. Effects of storage temperature and time on lipids (phospholipids, triglycerides) has been tested mainly in fish (Braddock and Dugan, 1972; Ingemansson et al., 1995; Srikar et al., 2007; Rudy et al., 2016), meat (Zymon et al., 2007) and some vegetables and juice (Lee and Mattick, 1961; Nagy and Nordby, 1970) with the purpose of meeting quality and food safety standards. Storage of soils has been shown to alter phospholipids (Petersen and Klug, 1994; Trabue et al., 2006; Lee et al., 2007; Schnecker et al., 2012). Compared to phospholipids, neutral lipids (triglycerides) are less degraded during storage in fish (Lovern et al., 1959; Bosund and Ganrot, 1969; Hardy et al., 1979; Sasaki and Capuzzo, 1984). However, the effect of heat extraction and capturing soil animals in glycol has never been studied.

We investigated the effect of extraction method (no extraction, heat extraction in water for 1 day, heat extraction in glycol for 7 days), storage fluid (methanol at −20 °C, 70% ethanol at −15 °C, glycerine at −15 °C, saltwater at −10 °C) and storage time (4 and 12 months) on the composition and amounts of marker NLFAs of common soil detritivores of different body size, *Oniscus asellus* Brandt, 1833 and *Trichoniscus pusillus* Linnaeus, 1758 (Isopoda). With decreasing body size, the surface area-to-volume ratio is increasing, which may result in higher reaction rates e.g., due to hydrolysis, as shown in peas (Bengtsson and Bosund, 1966). We aimed at identifying a protocol keeping changes in NLFA composition of soil animals during extraction and storage at a minimum. Further, we inspected the effect of direct freezing at −20 °C and storage in methanol at −80 °C with evaporating methanol prior to lipid extraction after 4 and 12 months on the NLFA profile of *O. asellus* to check if these treatments are suitable as reference. Due to differences in the killing procedure for *O. asellus* and *T. pusillus*, effects of the method of killing (killed by freezing and by drowning in glycol) on NLFA composition of *O. asellus* was inspected. It has been suggested to use chloroform and 0.01% butylated hydroxytoluene, and store animals under nitrogen atmosphere prior to lipid analyses to prevent oxidative processes (Budge et al., 2006). However, this treatment is difficult to apply if animals need to be extracted by heat and determined under the microscope before analysis.

2. Materials and methods

2.1. Experimental set-up

Two Isopoda species differing in body size were used, *T. pusillus* with a length of 3–5 mm and *O. asellus* with a length of 14–18 mm. The animals were sampled from a deciduous forest close to the city of Göttingen (51°31′39.7″N 10°02′23.5″E). Adult *O. asellus* were hand collected from dead wood, whereas *T. pusillus* was extracted from litter and soil using heat (Kempson et al., 1963).

O. asellus either was kept in water for one day (water 1d) or in diethylene glycol (Stockmeier Chemie GmbH, Bielefeld, Germany) mixed with tap water (1:1) for 7 days (glycol 7d) to represent two commonly used extraction methods. Individuals of each extraction method were stored for 4 months in methanol (HPLC grade, VWR, Radnor, USA) at −20 °C, in 70% ethanol [99% denatured with methyl