

Unravelling the Feeding Habits of Fungivores

Interactions between Soil Fauna and Ectomycorrhizal
Fungi

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Cover: The oribatid mite *Ceratozetes gracilis* (Michael 1884).
(Photo: Vitezslav Manak)

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Unravelling the Feeding Habits of Fungivores. Interactions between Soil Fauna and Ectomycorrhizal Fungi.

Abstract

The aim of this project was to investigate and clarify the feeding habits of fungivorous soil fauna, with the overall objective of improving our understanding of their functional roles in the ecosystem. Special focus was given to symbiotic ectomycorrhizal (EM) fungi, that, owing to their abundance and diversity, are considered to constitute an important food source for fungivorous soil fauna and may be a factor regulating these faunal communities. Previous studies on soil animal feeding habits have been strongly dependent on the methodology used; therefore an additional aim was to find the technique most suitable for studying fungivorous feeding. The results in this thesis confirm that it is necessary to combine a number of different methods in order to determine the feeding habits of fungivorous soil fauna. The total abundance of oribatid mites was significantly reduced in spruce forest stands that were girdled to restrict flow of photoassimilates to roots and ectomycorrhizal fungi, and *Oppiella nova* was the species that showed the most marked reduction in abundance, especially in spruce forest soils. This reduction in abundance could be explained by the results from a microcosm study, in which *O. nova* was clearly favoured by the presence of certain EM fungi and increased its abundance of both adults and juveniles in microcosms with the two EM species *Suillus variegatus* and *Paxillus involutus*. In the same study, *Cognettia sphagnetorum* was suppressed by the presence of EM fungi, which partly explains why this species reach high populations after clear-cutting.

PCR in combination with washing of the body surface and dissection was a successful method for analysing diets of fungal feeding oribatid mites. This method will be considered for future analyses of field collected animals. We could also demonstrate that the isotopic composition of fungivorous animals should be interpreted with great care, when used as a method for studying fungivore feeding habits. The range of values recorded from field collected Diptera larvae utilising a single food source was large enough to span across several trophic levels when these were determined using fixed enrichment values from literature data. The $\delta^{15}\text{N}$ of the animals was higher than that of the ingested fungal tissue, but similar to the $\delta^{15}\text{N}$ of the protein and amino acids of the food.

Keywords: oribatid, feeding, fungi, PCR, stable isotopes, girdling, mycorrhiza, soil.

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Dedication

Till Tomas, Frode och mini-hopp

Ingenting är omöjligt, bara mer eller mindre besvärligt.

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List of Publications

This thesis is based on the work in the following papers, referred to by Roman numerals in the text:

- I Remén, C., Persson, T., Finlay, R., Ahlström, K. (2008). Responses of oribatid mites to tree girdling and nutrient addition in boreal coniferous forests. *Soil Biology and Biochemistry* 40, 2881-2890.
- II Remén, C., Fransson, P., Persson, T. (2010). Population responses of oribatids and enchytraeids to plant-soil microcosms. *Soil Biology and Biochemistry* 42, 978-985.
- III Remén, C., Krüger, M., Lundhagen, A. (2010). Successful analysis of diets in fungal feeding oribatid mites by combining body surface washing and PCR analysis. (submitted manuscript)
- IV Remén, C., Ekblad, A., Persson, T., Taylor, A.F.S. (2010). Variable shifts in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between insect larvae and their fungal substrates. (Manuscript)

Papers I and II are reproduced with the permission from Elsevier.

The contribution of Cecilia Remén to the papers included in this thesis was as follows:

- I Main author. Collection of field samples in one of the two field seasons. Identification of oribatid mites with the aid of Kerstin Ahlström. Interpretation of data together with co-authors.
- II Main author and maintenance of the experiment. Identification of oribatid mites and statistical analysis. Idea development and interpretation of data together with co-authors.
- III Main author and molecular work. Idea, method development and interpretation of data in cooperation with Anna Lundhagen.
- IV Main author, compilation of data and statistical analysis. Collection of material together with Andy Taylor. Idea development together with co-authors.

Abbreviations

EM Ectomycorrhiza

PCR Polymerase chain reaction

NPK Nitrogen (N), phosphorus (P) and potassium (K)

Bp Base pairs

rDNA Ribosomal DNA

ITS Internal transcribed spacer

1 General Introduction

1.1 The soil system

Terrestrial ecosystems consist of both an aboveground and a belowground compartment. Most plants depend on soil for nutrient and water supply and in return the soil system relies directly or indirectly on the aboveground photosynthesis for carbon input. Carbon is allocated to the soil system through plant roots and mycorrhizal symbionts and through aboveground inputs of litterfall. The organic matter content of soil varies both with regard to its chemical composition and its quantity. This is due to the interaction of different factors such as vegetation type, climate, bedrock and the activity of soil biota.

In Sweden, coniferous forests are the dominating vegetation type. Needles contain large complex structures such as lignin, cellulose and polyphenols which create a recalcitrant and acidic litter that decomposes slowly. Fungi and bacteria are the main decomposer organisms, but fungi are best adapted for utilising poor-quality litter and account for most of the organic matter breakdown in coniferous forest soils (Wardle, 2002). However, soil fauna e.g. enchytraeids, protozoans and microarthropods contribute to decomposition by fragmenting organic matter into smaller pieces with a larger surface area to volume ratio, facilitating microbial attack. In addition, the soil fauna indirectly regulates the growth and activity of the microbes through feeding and by excreting nutrients into the soil.

1.2 Diversity of soil animals

The diversity of soil animals may exceed above-ground diversity in many habitats (Anderson, 2009). There is however a clear negative correlation between population density and the body size of the soil organisms (Giller,

1996). Due to their small size, inaccessible habitat and sometimes problematic identification they have not gained as much attention as aboveground organisms.

Soil fauna are often classified into micro-, meso-, and macrofauna on the basis of their body size (Swift *et al.*, 1979). The small microfauna (body width <0.1mm) include, e.g., nematodes and protozoans, the mesofauna (body width 0.1–2.0 mm) includes the mites, springtails and enchytraeids, and the macrofauna (body width >2.0 mm) contains well-known groups such as earthworms and beetles. Soil animals are also separated into animals living in air filled pores or those inhabiting the water film. In addition, soil fauna often show a vertical differentiation, with different groups or species preferring different depths in the soil profile. Species living in deeper horizons lack pigmentation, eyes, and antennae and are often smaller in size than the surface dwellers. Many of these adaptations can be explained by the lack of UV-light deep down in soil. Another factor that contributes to the structure and functioning of communities in the soil is the diversity of food resources.

Soil animals are found in a range of trophic levels and in soil food webs they are often allocated to functional groups based on their feeding habits. Some feed primarily on microbes or litter, whereas others feed principally on plant roots or other animals. The soil food web is very complex with many interactions resulting in food chain lengths up to 8 links: roots – bacteria – flagellates – amoebae – omnivorous nematodes – predatory nematodes – nematophagous mites – predatory mites (Coleman, 1985). In fact the food chains can be even longer if these mites are eaten by e.g. ants or predatory beetles on the soil surface.

The soil ecosystem may seem to harbour too many species in relation to the number of possible niches. A possible explanation of this could be that the soil contains more niches than we have been able to see and that each soil fauna species actually has its own specific way of using the complex soil environment (Petersen, 2002). One example of being specific could be to feed on a particular food source. However, many scientists believe that soil animals are generalists or opportunists in their feeding behaviour (Ponsard & Ardit, 2000; Scheu & Falca, 2000) and the degree of specialisation is also under debate (Bolnick *et al.*, 2003; Maraun *et al.*, 2003b). A species specialising on fungi can be described as fungivorous, but a fungivore may also show feeding preferences among different species of fungi. By studying the gut contents of soil fauna, Ponge (1991) classified different animal groups according to their food specialisation: Lumbricidae < Enchytraeidae

< Sciaridae < Collembola < Oribatida with earthworms being the least specialised group and oribatid mites the most specialised.

1.3 Oribatid mites



Figure 1. Oribatid mites are small arthropods that are abundant in coniferous forest soils. The species in the photo is *Oribatella calcarata*(C.L. Koch, 1835). Photo by V. Manak 2009.

Mites, small (body width, 80µm – 2mm) members of the Arachnidae (Swift *et al.*, 1979), are the most abundant microarthropods in many types of soils (Norton, 1994)(Fig. 1). They are generally separated into several orders and groups of which one is called the oribatid mites. Oribatids are commonly regarded as detritivores or fungal feeders and as many as 430 000 individuals m⁻² have been found in Scots pine forest soil (Persson *et al.*, 1980). They are also very species rich with about 11 000 named species worldwide (Walter & Proctor, 1999), and about 280 species found in Sweden (Lindberg *et al.*, 2004; Lundqvist, 1987).

Oribatid mites are particulate feeders which means that structures of the mouthparts are used together to cut or tear particles into sizes suitable for intake (Norton, 1990). Several researchers have aimed at separating oribatid mites into different feeding guilds. The most commonly used classification is based on the food preferences and enzyme complexes described by Luxton (1972) and Schuster (1956). They define three main categories of microarthropods: macrophytophages (feeding on higher plant material), microphytophages (feeding on microflora) and panphytophages (feeding on both plant material and microflora). The classifications have been redefined several times, for example by Siepel and de Ruiter-Dijkman (1993). These scientists used the analysis of gut enzyme complements and classified the former macrophytophages as herbivorous grazers and browsers, the

microphytophages as fungivorous grazers and browsers and the panphytophages as herbivorous grazers.

1.4 Enchytraeidae

Enchytraeids are small, white worms belonging to the Oligochaeta. They are anatomically similar to earthworms but much smaller with an adult length of 10-20 mm (Coleman & Crossley, 1996). These worms are generally more abundant in highly organic forest and moorland soils than in alluvial and sandy grassland. In the acid soils of heathlands and conifer forests they can reach densities of 200 000 m⁻² compared to 1000-100 000 m⁻² in grassland soils (Wallwork, 1970).

Enchytraeids have been found to ingest plant material, particles of silica, detritus, fungal mycelium, faeces, algae, bacteria and nematodes. In some species the feeding mechanism seems to involve a preliminary softening of the food material before ingestion (O'Connor, 1967). Enchytraeids have been suggested to feed predominantly on fungi, at least in arable soil (Didden, 1990), but mycorrhizal hyphae have also been found in faecal pellets from enchytraeids in pine litter (Ponge, 1991).

1.5 Diptera

Although some Diptera families occur as adults in soil, it is mainly the larval stages that contribute to the soil community. In general, they prefer moist habitats and are commonly found in wet-to-damp environments, usually in soils with high organic matter. Since the group is very diverse, the feeding habits are so diverse too. Some species feed on organic litter material, some are fungivorous, some are predacious and some feed on dung. Fungivorous species often infest fungal fruit bodies, but they are also in most cases assumed to be true soil dwellers that feed on fungal mycelium in soil (Teskey, 1990).

1.6 Fungi

Fungi are the most abundant microorganisms in soil on a mass basis (500-5000 kg wet mass ha⁻¹) (Metting, 1993) and form the food source for many animals among the micro- meso- and macrofauna. The majority of fungi are multicellular organisms with a filamentous, vegetative body, but they can also be single-celled, as for example yeasts. Fungi lack chlorophyll and are heterotrophic, which means that they obtain their carbon by degrading

organic compounds, or through symbiotic associations with plants. Most fungi have cells organized into filamentous hyphae, with cell walls containing chitin. The hyphae grow at the tip in their search for organic substrate and individual hyphae growing and forming branches can result in a compact mat of mycelia (Madigan *et al.*, 2000). The kingdom Fungi consists of two divisions, the Eumycota and Myxomycota, or the true fungi and slime moulds. The true fungi are divided into five phyla; the Chitridiomycota, the Zygomycota, the Glomeromycota, the Ascomycota and the Basidiomycota. These groups are immensely diverse, both structurally and functionally with an estimated number of 1.5 million fungal species (Hawksworth, 2001). Depending on the organic substrate used for nutritional supply, fungi have adopted different trophic strategies and occur as saprotrophs, symbionts and pathogens. Saprotrophs obtain their carbon by degrading nonliving organic matter, while pathogens obtain it by parasitizing or killing a living host. Symbionts obtain their carbon by living in symbiosis with autotrophic host organisms. One example is the mycorrhizal symbiosis in which a plant and a fungus exchange nutrients, water and minerals (Petersen, 1995). Photosynthetically fixed carbon from the plant is generally transferred to the fungus and nutrients taken up by the fungus are transported to the plant. Approximately 95% of all plant species belong to genera that characteristically form mycorrhiza, but mycorrhizal associations differ extensively in structure and function (Sylvia, 1999). Ectomycorrhiza (EM) are the associations mostly formed between basidiomycetes and long-lived woody perennials. There are over 7000 species of EM fungi (Taylor & Alexander, 2005) colonising over 8000 species of plants. The symbiosis is characterised by the so-called Hartig net. This is the name of the net-like structure of hyphae occurring between the root cortical cells. Another characteristic feature is the presence of a mantle, which, as the name implies, is a sheath of fungal tissue surrounding the terminal, nutrient-absorbing rootlets. The rootlets also lack root hairs and are often short and stumpy (Deacon, 1997).

1.7 Interactions between soil fauna and fungi

The interactions between fungi and soil animals can be both beneficial and harmful. Direct feeding of animals on fungal hyphae reduces the fungal biomass, but it may also stimulate re-growth by removal of dead or senescent mycelia (Hanlon, 1981). Grazing can also increase nutrient release which stimulates growth of plant and fungi (Gormsen *et al.*, 2004; Setälä, 2000). It has also been shown that a reduced biomass of EM fungi due to

soil fauna can be beneficial to the plant and increase the aboveground biomass. This occurs if the community composition of soil organisms is complex enough to ensure efficient mobilization of nutrients (Setälä, 1995). Another beneficial interaction between soil fauna and fungi is the animals' dispersal of fungal spores (Dromph, 2003; Hubert *et al.*, 2003; Lilleskov & Bruns, 2005; Renker *et al.*, 2005). Fungivorous animals are also valuable for plants, since many animals have been shown to feed on and reduce fungal plant pathogens (Friberg *et al.*, 2005).

Despite the beneficial aspects mentioned above, many fungal species have developed potential defensive strategies to prevent grazing. Many fungal species contain toxic chemicals, or have developed specialised cells on the surface of the mantle which can be thick-walled, pointed and melanised. A number of species can produce ornamented hyphae that are coated with crystals of calcium oxalate or other crystalline deposits (Taylor & Alexander, 2005). Some ectomycorrhizal fungal species such as *Laccaria bicolor* may even act as predators on animals such as Collembola, transferring N derived from killed animals to their host plants (Klironomos & Hart, 2001). The authors found that less than 5 % of the added springtails (*Folsomia candida*) survived after two weeks' exposure to *L. bicolor* during a routine feeding study in microcosms. All dead animals were internally infected by the fungus and careful observation of the microcosms revealed that the *L. bicolor* immobilized the animals before infecting them. To determine the fate of the arthropod derived nitrogen, springtails were labelled with ^{15}N of which 25% eventually ended up in the host plant tissue of the mycorrhizal fungi.

1.8 Methods to investigate soil faunal feeding habits

Numerous studies have investigated soil faunal feeding habits with classic methods like laboratory food preference tests, gut content analysis (Mitchell & Parkinson, 1976; Ponge, 1991) and the studying of gut enzyme complements (Berg *et al.*, 2004; Luxton, 1972; Siepel & Ruiter-Dijkman, 1993). Animals have also been categorised into different feeding groups based on their mouthpart morphology (Buryn & Brandl, 1992; Chen *et al.*, 1997; Kaneko, 1988). Even though these are rigorous studies, the methods have limitations. Feeding preferences in the laboratory may not represent feeding habits *in situ* since it is not practical to offer all possible food sources. Gut content analyses may only show the least digestible material, which may constitute a less important part of the animal's requirements. Finally the comparison of gut enzymes and mouthparts is a useful approach but can only categorize the animals into broad groups.

In recent years, stable isotope techniques (Scheu & Falca, 2000; Schneider *et al.*, 2004) and the analysis of fatty acids (Chamberlain *et al.*, 2006; Haubert *et al.*, 2009; Ruess *et al.*, 2005), have become popular methods to separate soil fauna into trophic niches. Biochemical and molecular tools such as ELISA (Enzyme-linked immunosorbent assay) (Bohan *et al.*, 2000) and FISH (fluorescence *in situ* hybridization) (Fischer *et al.*, 1995) have also gained interest among soil biologists. A very promising tool for the study of feeding habits is the use of PCR (polymerase chain reaction) based methods to amplify small amounts of DNA in food materials. It is a method commonly used for molecular analysis of predator-prey relationships (King *et al.*, 2008) but has also been successfully applied to study collembolan gut contents (Bracht Jørgensen *et al.*, 2005).

2 Aims

The aim of my doctoral project was to investigate and clarify the feeding habits of fungivorous soil fauna, with the overall objective of improving the understanding of their functional roles in the ecosystem. Special focus was given to ectomycorrhizal (EM) fungi, that, owing to their abundance and diversity, are considered to constitute an important food source for fungivorous soil fauna and may be a factor regulating these faunal communities.

When studying fungivorous feeding by soil animals the results obtained may be strongly dependent on the methodology used (see section 1.8). Therefore, the papers included in this thesis contain the results from four studies using different methods, with the additional aim of finding the best approach to solve the riddle of fungivorous feeding. The thesis can be divided into two parts where the main objective of papers I and II was to determine the role of EM fungi for mites and enchytraeids. The objectives of papers III and IV were to find suitable methodology to clarify the feeding habits of fungivores.

The specific aims of the different studies were to:

1. Determine the effect of restricted carbon allocation to mycorrhizal fungi on soil-living oribatid mites, in order to identify species that are susceptible to a presumed decline in EM fungi (*Paper I*).
2. Test whether the presence of EM fungi is a major factor in explaining population changes of oribatid mites and enchytraeid worms (*Paper II*) based on the results in *Paper I*.

3. Analyse whether a body surface washing in combination with PCR can be a successful approach for identifying the food source of fungivorous mites (*Paper III*).
4. Examine whether the analysis of stable isotope ratios of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ can be useful for the study of fungivorous soil animals (*Paper IV*).

3 Animal and fungal species studied

3.1 Oribatids

Resource partitioning and feeding specialisation are discussed as possible explanations for the high diversity of soil animals, including oribatid mites. So far the true nature of the feeding habits of oribatids has not yet been explained, and though earlier studies point to a low level of feeding specialisation, these results may be partly due to the methodology used. This, in combination with the fact that oribatid mites are the most numerous arthropods in temperate coniferous forest soil, makes their feeding habits very interesting to examine further, and they were the main fungivorous organisms studied in this thesis, specifically in *Papers I-III*.

In *Paper I* oribatids from three different field sites were sampled in order to determine if any species responded to a presumed decline in EM fungi. The EM fungi were assumed to decline because of an experimental tree girdling. Girdling inhibits the supply of carbohydrates to the tree roots and mycorrhizal fungi and the treatment has been shown to eliminate EM fruit bodies and reduce soil respiration by as much as 54 % within 1-2 months, suggesting a negative effect on EM fungi (Bhupinderpal-Singh *et al.*, 2003; Högberg *et al.*, 2001; Subke *et al.*, 2004).

Based on the results in *Paper I* we investigated whether the presence of EM fungi is a major factor in explaining population changes of oribatid mites (*Paper II*). This was tested by measuring the population response of three oribatid mite species (*Oppiella nova* (Oudemans), *Nothrus silvestris* (Nicolet) and *Tectocephus velatus* (Michael)) to different fungal species in plant-soil microcosms. The mites were collected from the humus layer of a mixed coniferous forest and were all added as adults to the plant-soil microcosms. *O. nova*, which is referred to in the literature as being fungivorous (Luxton, 1972; Ponge, 1991), was chosen as a study organism

because earlier results pointed to an interaction between this species and EM fungi (*Paper I*). In the same study *T. velatus* did not respond to an EM fungal decline but was chosen for the microcosm study as it is generally referred to as being fungivorous (Norton, 1985). *N. silvestris* is referred to as feeding on plant-litter (Luxton, 1972) and did not decrease in *Paper I*, but was interesting to study since we hypothesised that the response of a suggested plant-litter feeder would differ from the fungivorous species. All three species reproduce through parthenogenesis (Domes *et al.*, 2007; Maraun *et al.*, 2004; Norton & Palmer, 1991). *O. nova* and *T. velatus* are fairly small species (220–365 μm) and *N. silvestris* is relatively large (710–810 μm) (Weigmann, 2006).

In *Paper III* we analysed whether PCR in combination with a body surface washing could be a successful approach for identifying the food source of fungivorous mites. As a study organism we used the oribatid species *Archegozetes longisetosus* (Aoki) originating from a laboratory culture isolated by R. A. Norton in 1993. *A. longisetosus* is relatively large (800–1100 μm) (Senizcak *et al.*, 1998) and reproduces through parthenogenesis (Palmer & Norton, 1990; Palmer & Norton, 1992). The species has a pan-tropical distribution (Palmer & Norton, 1991) and has been found to feed on algae, (Smrž & Norton, 2004) fungi and decaying leaves (Haq, 1982; Haq & Prabhoo, 1977). Mites were reared in plastic jars on a plaster-of-Paris/charcoal mixture that was moistened with some drops of fresh water at 3-day intervals. The jars were kept in constant darkness at room temperature (20–22°C). Algae growing on bark from different trees were provided as food in the cultures.

3.2 Enchytraeids

Enchytraeids have been shown to increase in abundance (T. Persson pers. obs.) at sites with limited carbon allocation to the mycorrhizal fungi e.g. clear-cuts and plots with experimental girdling. The response is thus opposite to that of some of the oribatid mite species (*Paper I*). We therefore examined whether the response of the enchytraeid worm *Cognettia sphagnetorum* (Vejd.) was different from that of oribatid mites when reared in the presence of different fungal species in plant-soil microcosms (*Paper II*). As for the mites, the individuals used in the experiment were collected from the humus layer of a mixed coniferous forest. *C. sphagnetorum* reproduces through fragmentation (Dash, 1990) and only large to medium-sized individuals were used.

3.3 Diptera larvae

To test whether the stable isotopic signatures of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ could be useful for studying the feeding habits of fungivores, we analysed field-collected fungivorous animals that had fed on the same fungal tissue during their entire lifetime (*Paper IV*). We found the ideal study organisms to be Diptera larvae of the families Anthomyiidae and Mycetophilidae, which had been deposited as eggs on fungal fruit bodies by the adult female. The larvae had spent their entire larval stage in the fruit body and were collected from different fruit body compartments using forceps.

3.4 Fungal species in the experiments

Six basidiomycetous fungal species were used in the plant-soil microcosm experiment (Table 1), where we tested whether the presence of EM fungi is a major factor in explaining population changes of oribatid mites and enchytraeid worms (*Paper II*).

The ectomycorrhizal fungus *Laccaria laccata* (Scopoli, Cooke) was used in the feeding experiment in *Paper III*, where we analysed ingested fungal DNA from the guts of oribatid mites with PCR. DNA isolates of 14 other fungal species (Table 1) were used for a specificity test of the primers.

Fungal fruit bodies of four basidiomycetous species (Table 1) were collected for analysis of stable isotopic signatures of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (*Paper IV*).

Table 1. *Fungal species used in Papers II-IV.*

Used in the following experiment	Fungal species	Functional group
Microcosm experiment (<i>Paper II</i>)	<i>Hebeloma velutipes</i> Bruchet (1970)	EM
"	<i>Paxillus involutus</i> (Batsch) Fr. 1838	EM
"	<i>Piloderma fallax</i> Lib. Stalpers (1984)	EM
"	<i>Rhizopogon roseolus</i> (Corda) Th. Fr. (1909)	EM
"	<i>Suillus variegatus</i> (SW.) Kuntze (1898)	EM
"	<i>Hypholoma capnoides</i> Fr. P. Kumm. (1871)	Saprotrophic
PCR and washing experiment (<i>Paper III</i>)	<i>Amanita muscaria</i> (L.) Lam. (1783)	EM
"	<i>Cortinarius triumphans</i> Fr. (1838)	EM
"	<i>Hebeloma sinapizans</i> Fr. Sacc. 1887	EM
"	<i>Kuehneromyces mutabilis</i> (Schaeff.) Singer & A.H. Sm. (1946)	EM
"	<i>Laccaria amethystina</i> Cooke (1884)	EM
"	<i>L. bicolor</i> (Maire) P. D. Orton (1960)	EM
"	<i>L. laccata</i> (Scop.) Cooke (1884)	EM
"	<i>Lactarius blennius</i> (Fr.) Fr. (1838)	EM
"	<i>Russula aurea</i> Pers. (1796)	EM
"	<i>R. foetens</i> (Pers.) Pers. (1796)	EM
"	<i>Tricholoma atrovioleaceum</i> A.H. Sm. (1944)	EM
"	<i>T. flavovirens</i> (Pers.) S. Lundell (1942)	EM
"	<i>T. fulvum</i> (Fr.) Bigeard & H. Guill. (1909)	EM
"	<i>T. sudum</i> (Fr.) Quél. (1873)	EM
"	<i>T. terreum</i> (Schaeff.) P. Kumm. (1871)	EM
Analysis of stable isotopes in Diptera larvae living in fruit bodies (<i>Paper IV</i>)	<i>Boletus aestivalis</i> (Paulet) Fr. (1838)	EM
"	<i>Suillus granulatus</i> (L.) Roussel (1806)	EM
"	<i>S. grevillei</i> (Klotzsch) Singer (1945)	EM
"	<i>Clitocybe nebularis</i> (Batsch) P. Kumm. (1871)	Saprotrophic

4 Methods

4.1 Field experiment with girdling and nutrient addition (I)

Girdling is a method whereby the stem bark and phloem of a tree is removed around the tree's outer circumference to the depth of the current xylem (Fig. 2). Since the tree, at least in the short term, can continue to take up water and nutrients, girdling results in a less immediate disturbance of the abiotic conditions in the soil than clear-cutting, but the tree will eventually die. Girdling is not commonly used for thinning of forests, but can be used for creating gaps in the tree canopy (Romell *et al.*, 2009) or to remove individual trees at a relatively low cost (Ohlson-Kiehn *et al.*, 2006).

Since girdling stops the transport of carbohydrates from the canopy to the roots it also selectively removes the supply of host-derived assimilates that EM fungi are dependent on. Saprotrophic fungi are not directly affected by girdling as their supply of carbon compounds comes from humus, litter and decomposition. The method can thus be used to manipulate EM fungi and saprotrophic fungi selectively in order to study responses of soil animals to these two functional groups of fungi.

Both oribatid mites and EM fungi have been shown to respond with decreased species richness and diversity or changed species composition to nitrogen fertilisation (Fransson *et al.*, 2000; Jonsson *et al.*, 2000; Lindberg & Persson, 2004; Lohm *et al.*, 1977). A simultaneous reduction in EM fungal biomass and oribatid mites would be consistent with the hypothesis that there is an interaction between these two groups of organisms.



Figure 2. Girdled trees of Scots pine at Åheden. Photo by Tryggve Persson (2001).

4.1.1 Field sites

To investigate whether girdling changes the community of oribatid mites as an effect of changes in EM fungal composition, we collected samples from three already established girdling experiments in northern Sweden, Åheden (lat. 64°14'N, long. 19°46'E, alt. 175 m a.s.l.) (Fig. 2), Storskogberget (lat. 64°00'N, long. 20°35'E, alt. 75 m a.s.l.) and Flakaliden (lat. 64°07'N, long. 19°27'E, alt. 310–320 m a.s.l.) (*Paper I*).

The effect of nutrient addition in combination with girdling was only tested at the Flakaliden site. The three sites are the only sites in Sweden in which tree girdling has been used as an experimental treatment.

4.1.2 Experimental design

The experiment at Åheden consisted of nine 30 x 30 m² plots arranged in three separate blocks (each block containing three plots with different treatments) with 1300 ± 130 trees per hectare (Högberg *et al.*, 2001). The trees were girdled in early June 2000 in three plots and in mid August 2000 in three other plots. Thus, there were early-girdled (EG) plots, late-girdled plots (LG) and control (C) plots.

The experiment at Storskogberget consisted of six circular plots with a radius of 15 m (707 m²) and a tree density of c. 1080 per hectare (Göttlicher *et al.*, 2008). Three of these plots were girdled in June 2002. Three pairs of girdled and non-girdled plots formed three blocks.

The Flakaliden experiment started in 1987 with several different treatments e.g. fertilisation (Bergh *et al.*, 1999). Solid fertilisers including NPK and other macro- and micronutrients were added annually to the fertilised plots between 1987 and 2001, and at the time of sampling they had received a cumulative dose of 1200 kg N ha⁻¹. In June 2002, a total of 1412 trees in three fertilised and three non-fertilised plots were girdled. Half of the 50 x 100 m non-fertilised and fertilised plots were divided into one 50 x 50 m girdled and one 50 x 50 m control subplot. The treatments were non-fertilised control plots (C), non-fertilised girdled plots (G), fertilised control plots (F) and fertilised girdled plots (FG). The experiment was originally not designed as a block experiment, but nearby plots were later combined to form blocks.

4.1.3 Sampling and identification

Soil fauna were sampled 9–16 months after girdling, depending on the site. In addition, to gain an indication of long-term effects, Flakaliden was also sampled 28 months after girdling. Three soil samples per plot were collected by pressing a 100 cm² metal frame into the forest floor and mineral soil after cutting off the roots with a knife (Fig. 3, left). The animals were extracted in modified Tullgren funnels (Fig. 3, right) equipped with a 20 x 20 cm net (4-mm mesh) basket on which the samples were evenly spread out to a thickness of 1–2 cm. The samples were heated (up to 45 °C) and dried for 4 days by means of light bulbs, and the mites extracted were collected in 70–80 % ethanol. The mites were counted and determined to species level under a binocular microscope. A transmission microscope with 40–400 x magnification was used for identification of smaller animals.

Unfortunately, no direct measurements were made of fungal biomass on the sampled plots. However, since soil respiration can decrease by as much as 54 % within 1–2 months and that EM fruit bodies are virtually eliminated after girdling (Bhupinderpal-Singh *et al.*, 2003; Högberg *et al.*, 2001; Subke *et al.*, 2004), we made the assumption that girdling has an irreversible negative effect on the fungi.



Figure 3. Soil samples were collected by pressing a 100 cm² metal frame into the forest floor and mineral soil after cutting off the roots with a knife (left). The animals were extracted in modified Tullgren funnels (right). Photo by C. Remén

4.2 Microcosm experiment (II)

4.2.1 Experimental system

Based on the results in *Paper I* we investigated whether the presence of EM fungi is a major factor in explaining population changes of oribatid mites and enchytraeid worms (*Paper II*). The population responses of three species of oribatid mites (*N. silvestris*, *O. nova*, and *T. velatus*) and one species of enchytraeid worms (*C. sphagnetorum*) to the presence of different species of fungi were compared in plant-soil microcosms. We hypothesized that *O. nova* should be favoured by the presence of specific EM fungi, while *C. sphagnetorum* should be suppressed by EM fungi but probably increase in treatments with the saprotrophic fungus. We also assumed that *T. velatus* and *N. silvestris*, which are referred to as being non specialist fungivores or microbi-detritivores, would show good survival and reproduction in the presence of high amounts of fungi and high amounts of organic matter rather than to selected species of soil fungi. These hypotheses were tested in pot microcosms which consisted of eight plant/fungal treatments; Scots pine seedlings colonised with one of five different EM fungi (*H. velutipes*, *P. involutus*, *P. fallax*, *R. roseolus*, *S. variegatus*), and the wood-living fungus *H. capnoides* grown on a 5 x 2 x 0.5 cm piece of pine wood, Scots pine seedlings without inoculated mycorrhizal fungi (non-mycorrhizal control, NM) and Scots pine seedlings without inoculated mycorrhizal fungi in fresh mor humus (forest soil, FS).

In order to ensure a sufficient inoculation of mycorrhiza on the roots, mycorrhizal syntheses were established in a controlled environment prior to placing the plants in the pots. Four-week-old pine seedlings were placed in sterile Petri dishes with a 1:4 v/v moistened mixture of peat and vermiculite, together with agar plugs from six-week-old EM fungal cultures (Fig 4, left). The Petri dishes were covered with aluminium foil to protect the roots and mycorrhizas from light, and were placed vertically in a small plastic propagator in a plant growth facility at 14–16 °C during a 16 h photoperiod at a photon flux density of 250–300 $\mu\text{mol mm}^{-2} \text{s}^{-1}$ and 6–8 °C during the 8h dark period. After 12 weeks >90% of the root system was colonised and the inoculated plants were transferred to pots (Fig 4, right).



Figure 4. *Pinus sylvestris* L. seedlings were inoculated with EM fungal cultures in sterile Petri dishes (left) with a 1:4 mixture of peat, vermiculite and MMN medium (Marx, 1969). After 12 weeks, the seedlings were transferred to the pot microcosms (right). Photo by C. Remén

Five replicates of each plant/fungus treatment received either 11 individuals of *N. silvestris*, 55 individuals of *O. nova*, 70 individuals of *T. velatus* or 20 individuals of *C. sphagnetorum*. The animals were collected from field samples and placed in the pots manually with a small brush or pipette. During this period, all treatments stepwise received the same addition of animals.

The pots were placed for 70–84 days in a greenhouse with additional light of $250\text{--}300\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ PAR and 18/6 h day/night cycle. When the experiment ended, the mites were extracted in modified Tullgren funnels (Fig. 3, right) and collected in 80 % ethanol. The enchytraeids were extracted using modified Baermann wet funnels (O'Connor, 1962) and enumerated and determined to species level within 24 h after extraction.

4.2.2 Chitin analysis

To examine whether fungal biomass would affect the animals' feeding we determined the amount of fungal biomass by measuring the amount of chitin in the pots. Chitin was used as an indicator of both living and dead fungal biomass, and was determined by analysing glucosamine (Glu-NH_2), the building block of chitin originating from fungal cell walls. The extraction was done on separately dried and milled samples of soil and roots with or without mycorrhizal fungi, according to the protocol developed by Ekblad and Näsholm (1996).

4.3 PCR and body surface washing (III)

4.3.1 Detection of ingested fungal DNA using PCR

DNA-based methods, in particular polymerase chain reaction (PCR) techniques, are commonly used when studying arthropod predator-prey interactions (King *et al.*, 2008). PCR is a molecular method used to multiply a piece of a DNA molecule. The region to be copied needs to be known in advance because two complementary oligonucleotides, so called primers, are used to bind to the sequences of interest. In a repetitive reaction an enzyme that copies DNA molecules, DNA polymerase, together with the primers then amplify the target fragment during cycles of repeated heating and cooling.

PCR has rarely been used on fungivorous animals, but see Bracht Jørgensen *et al.* (2005). The main reason for this is the problem of body surface contamination. When fungivorous animals feed on fungi, fungal spores and mycelia attach to their body surface. Since PCR is a very sensitive method, the slightest amount of contaminant DNA from non-target organisms can be amplified in the reaction, creating a misleading result.

Since PCR would be very useful for studying the feeding habits of fungivorous animals, we conducted a laboratory experiment (Fig. 5) to test whether it is possible to eliminate body surface contamination and use PCR on gut contents of fungivorous mites.

4.3.2 Laboratory feeding experiments

Adult individuals of the oribatid mite *A. longisetosus*, reared in a laboratory culture, were placed to feed on agar plates with *L. laccata* colonies during 24 to 72 h. After feeding, the mites were collected and killed either in 95% ethanol or by freezing in -80 °C depending on the following treatment. The mites had, thus, a high likelihood of containing *L. laccata* DNA both in their guts and on the body surface. Another set of mites that initially had not come into contact with *L. laccata* were killed by freezing and then rolled in the *L. laccata* mycelium. These mites, with *L. laccata* DNA on the body surface but not in their guts, were compared as to the presence of DNA with the former group of mites. The whole experimental setup is described in detail below and the experimental steps are shown in Fig. 5. All treatments were replicated five times.

4.3.3 Washing/no washing

To test if it was sufficient to wash the mites in order to eliminate surface contamination, half of the samples were washed with a washing technique modified from Meyer and Hoy (2008). The mites were placed in separate microcentrifuge tubes and vortexed (1 minute/step) two times in 1 ml 3.7 % NaOCl (Colgate-Palmolive) and three times in 1 ml sterile water. After each vortex step, the mites were transferred to a new microcentrifuge tube with a sterile pipette tip and new solution was added. Individual mites were pooled into samples of one, five and ten after the washing treatment and they were compared with non-washed mites.

4.3.4 Dissection

To further eliminate contamination of fungal DNA from the body surface, the mites were dissected. Washed and non-washed individuals were killed and fixed on a microscopic slide with the glue Expresslim® (Casco). The gut was revealed and could be removed by making a gentle cut with an insect needle size 0-000 in the mite's body shield (notogaster). Until the required amount of animals (one, five or ten) had been dissected the guts were placed in sodium phosphate buffer provided in the DNA extraction kit.

4.3.5 DNA extraction and PCR

Total DNA of whole and dissected mites was extracted using the Fast DNA® SPIN Kit for Soil Protocol (MP Biomedicals, LLC, Solon, USA) following the manufacturer's instructions. All samples were stored at -20 °C before PCR amplification.

When searching for *L. laccata* in the mite samples, we used group-specific primers that amplify a 278-bp fragment of the nuclear internal transcribed spacer (ITS) rDNA region in *Laccaria*. We designed the primers by aligning sequences of the ITS region including 53 target sequences of closely related *Laccaria* species and 35 sequences of non-target species, all published on Genbank.

We used the ITS region, because it is a region of great taxonomic variability in fungi and because it is essential to use a specific DNA region when designing primers for a restricted range of organisms (Anderson *et al.*, 2003; Lord *et al.*, 2002). The non-coding ITS region incorporates the 5.8S rRNA gene and is located between the more conserved and less species-specific coding regions 18S rRNA and 28S rRNA genes (Anderson & Cairney, 2004). The optimum annealing temperature for the primers was determined by a gradient PCR and tested for specificity on three target and

12 non-target species. The PCR products were separated by agarose gel electrophoresis. The gels were stained with ethidium bromide and the PCR products were visualized under UV light. In order to confirm that the PCR products originated from the target organisms, a selected number of PCR products were purified using ExoSAP-IT® (USB Corporation) and were directly sequenced by Macrogen Inc. (Seoul, South Korea).

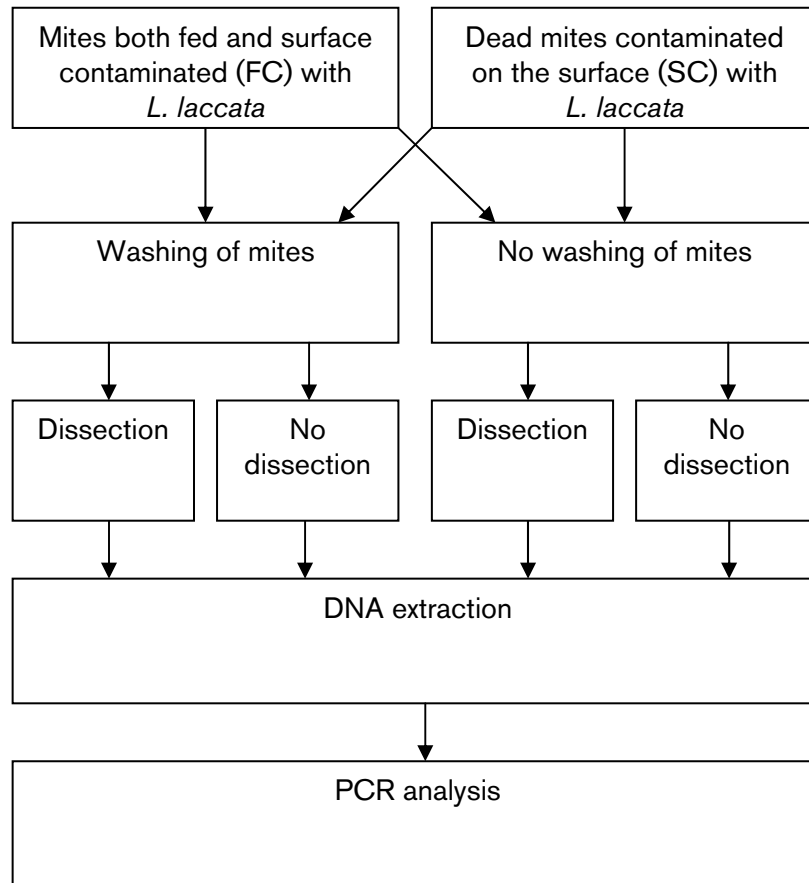


Figure 5. Diagram showing the different experimental steps prior to PCR analysis of the mite samples.

To check for false negative results, samples that tested negative for fungal DNA were tested with the forward primer D3A and the reverse primer D3B (Litvaitis *et al.*, 1994) previously used to amplify DNA from oribatid mites (Laumann *et al.*, 2007; Maraun *et al.*, 2003a). Amplifications were performed in a real-time PCR thermocycler (Bio-Rad Laboratories).

4.4 Analysis of stable isotopic ratios, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, in insect larvae and their fungal substrates (IV)

4.4.1 Stable isotopes

Isotopes are atoms of the same element that have the same number of protons and electrons but differing numbers of neutrons. An isotope can be either radioactive or stable. Stable isotopes, in contrast to radioactive isotopes, do not decay spontaneously into other nuclides once they are formed. Most stable isotopes of one element have one dominating isotope and one or two isotopes of relatively minor abundance. As an example, the common carbon isotope ^{12}C has a 98.98 % abundance in terrestrial environments, while the abundance of ^{13}C is 1.11 % (Dawson *et al.*, 2002). In different components of the biosphere there are small variations in the levels of most isotopes. These variations also known as delta (δ) values and are usually referred to as parts per thousand (‰) divergences from internationally accepted standards. Delta values are calculated as: $\delta = ((R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}}) \times 1000$, where R is the ratio of the heavy to light isotope (e.g., $^{15}\text{N}/^{14}\text{N}$). The δ value can be either positive or negative depending on whether the sample has more or less of the heavier isotope compared with the standard (Dawson & Brooks, 2001).

4.4.2 Carbon and nitrogen isotopes and diet

The isotopic composition of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ in animals is often used in food web studies. A presumption is that the stable isotopic ratio of the animals reflects those of their diets to within a few ‰, and that the consumer is enriched in $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ relative to its food source. Stable isotope ratios of carbon ($\delta^{13}\text{C}$) are often assumed to show little or no trophic enrichment (0.5–1 ‰) (Peterson & Fry, 1987) in animals relative to their diet, while the enrichment of $\delta^{15}\text{N}$ in a consumer is often assumed to be around 3 ‰ compared with the food (Minagawa & Wada, 1984). Generally $\delta^{13}\text{C}$ have been used to determine animals' diets while $\delta^{15}\text{N}$ has more often been used to determine their trophic position in the food web (Tiunov, 2007).

4.4.3 Constraints

The use of stable isotope ratios in food web studies may seem to be rather uncomplicated when using the assumed enrichment values in the literature (Minagawa & Wada, 1984; Peterson & Fry, 1987). However, enrichment values have been shown to vary considerably, for example with regard to the animal tissue analysed (e.g. liver or muscle), in which environment the

animals have lived, their type of excretion (e.g. urea or ammonia), the quality of the food, and also due to taxonomical differences (McCutchan *et al.*, 2003; Vander Zanden & Rasmussen, 2001; Vanderklift & Ponsard, 2003).

In studies on trophic structure of soil invertebrate communities the isotope data of the animals have delimited trophic level boundaries by first determining a baseline value of the primary food source (e.g. litter) and then establishing margins of the trophic levels by using mean enrichment values. Since the enrichment values can vary as mentioned above, and it is not always clear what to set as a primary food source, these studies are often difficult to interpret.

An important aspect in studies of trophic structure of soil invertebrates is that the isotopic signatures of fungivores are seldom compared with the isotope signatures of fungal mycelia in soil, probably as these data are difficult to obtain. However the values for the fungal fruit bodies are well documented (Kohzu *et al.*, 1999; Taylor *et al.*, 2003; Trudell *et al.*, 2004) and show that even within a single forest ecosystem, the $\delta^{15}\text{N}$ signatures of fungal fruit bodies may vary considerably (-4 to +22‰) (Kohzu *et al.*, 1999; Taylor *et al.*, 2003; Trudell *et al.*, 2004). If the $\delta^{15}\text{N}$ signatures of fungal mycelia vary to the same extent, fungivorous animals can cover the entire range of trophic positions, yet only feeding on the same substrate – fungi.

4.4.4 Collecting and handling of fruit bodies and Diptera larvae

In order to examine the usefulness in working with stable isotopes when studying fungivore/fungal interactions we wanted to answer the following questions: 1) Will the same animal group show a constant or variable enrichment value in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ when the animals have fed on different fungal substrates (i.e. species and compartment)? (2) Will different animal groups feeding on the same fungal compartment have different isotopic signatures? (3) What determines the ^{15}N enrichment of the animal relative to the nitrogenous compounds in its food? (4) What is the range of stable isotope values recorded from fungivorous Diptera larvae found in field-collected fruit bodies?

Since field-collected soil fauna could have eaten a number of food items during their lifetime, and it is difficult to analyse all fungal species present as mycelia in the studied soil, we used Diptera larvae collected from fungal fruit bodies as our study organisms. Diptera larvae are commonly found in fungal fruit bodies, where they are deposited as eggs by the adult females; hence they have eaten the same food source during their whole larval stage.

Four fungal species including both mycorrhizal (*Suillus grevillei* (Klotzsch) Singer, *Suillus granulatus* (L.) Roussel, *Boletus aestivalis* (Paulet) Fr. and saprotrophic *Clitocybe nebularis* (Batsch) P. Kumm. fungi were collected from four sites within 1 km radius of Uppsala, Sweden (lat. 59°49' N, long. 17°40' E). The fruit bodies were separated into different parts – the stem or stipe, cap, and tubes/gills and Diptera larvae from the families Anthomyiidae and Mycetophilidae were collected from each fruit body compartment using forceps. If no visible tracks were found between the different fruit body compartments (e.g. cap or tubes), the collected larvae were assigned to the specific part in which they were found. The larvae were examined in different parts of the fruit body, because these are known to vary with respect to isotope signatures and N and C concentrations (Taylor *et al.*, 1997). In addition, N concentrations and $\delta^{15}\text{N}$ values were determined in dominant N-containing compounds (proteins, free amino acids and chitin) in the fruit-body compartments, to gain an indication of which fraction that was preferably assimilated by the consumer and to test if the food quality, in this case nitrogen concentration will have an effect on the enrichment level. The collected materials, both larvae and fungi, were either stored at -20 °C followed by freeze-drying or dried directly at 37 °C. The dried material was weighed in tin capsules and stored in desiccators until analysed.

4.4.5 Stable isotope analysis

The isotopic signatures of both fungal and animal materials were determined using an elemental analyzer coupled on line to an Isoprime isotope-ratio mass spectrometer. For dual C and N stable isotope ratio analysis, samples of 1.5 ± 0.5 mg dried and milled fungal materials and 1.4 ± 0.6 mg animal materials were used. Each animal sample comprised, in most cases, one whole individual, but to minimise the range in sample weights, small individuals were pooled and large individuals were fragmented. The C and N concentration and isotopic composition of the fungal and animal materials were determined as described by Boström *et al.* (2007). The isotopic values are given in parts per thousand deviations from the international standards Vienna Pee Dee Belemnite (V-PDB) and atmospheric N_2 [$\delta^{13}\text{C}$ or $\delta^{15}\text{N} = ((R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}) \times 1000$ (‰), where R is the ratio of $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$]. The standard deviation of 10 replicated samples was ≤ 0.10 ‰ for $\delta^{13}\text{C}$ and ≤ 0.20 ‰ for $\delta^{15}\text{N}$.

4.4.6 ^{13}C and ^{15}N abundance in different N pools of fruit bodies

The isotopic composition and the amount of N in different pools within the fruit bodies were determined. Fruit body material was fractionated into four classes of nitrogenous compounds: HCl-soluble, NaOH-soluble, HCl-hydrolysable and insoluble N. The HCl fraction contains low molecular-weight soluble N, mainly free amino acids, amines and amides and the NaOH fraction contains mainly polypeptides and proteins (Taylor et al., 1997). The HCl-hydrolysable fraction contains only glucosamine N (tested with HPLC analysis, see below), while the composition of the insoluble material remaining after the HCl hydrolysis is unknown.

Glucosamine concentrations were determined on the 200 μl volume of the supernatant after the 6N HCl hydrolysis. The protocol was a modification of the method used by Ekblad and Näsholm (1996). Further details of the analyses are given in *Paper IV*.

5 Results and discussion

5.1 Effect of girdling and nutrient addition (I)

5.1.1 Girdling

Tree girdling significantly reduced the oribatid abundance at the two sites planted with Norway spruce, Storskogberget and Flakaliden (Figs. 6 and 7) but not at the pine forest site Åheden (Fig. 8).

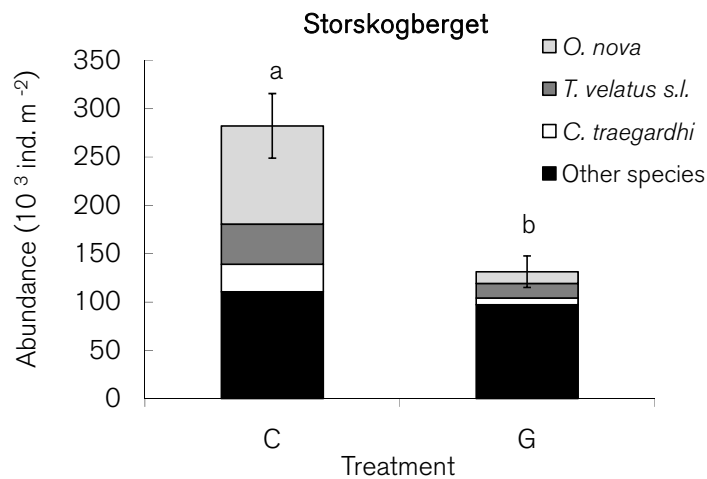


Figure 6. Mean total abundance of Oribatida (\pm SE) including the three most abundant species *Oppiella nova*, *Tectocephus velatus* and *Conchogneta traegardhi* in control (C) and girdled (G) plots at Storskogberget, 16 months after girdling. SE includes both treatment and plot variation. Different letters indicate statistically significant differences ($P < 0.05$) between treatment means.

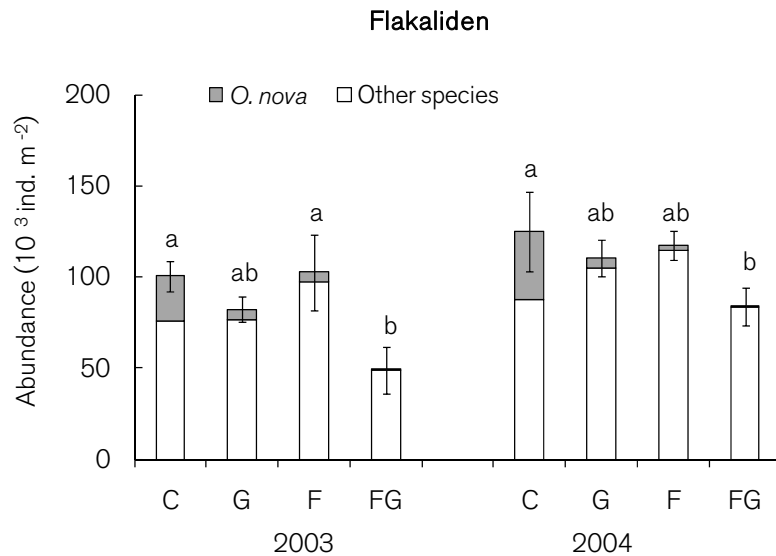


Figure 7. Mean total abundance (\pm SE) including the most abundant species *Oppiella nova* at non-fertilised control (C), non-fertilised girdled (G), fertilised control (F) and fertilised girdled (FG) plots at Flakaliden, one and two years after girdling. SE includes both treatment and plot variation. Different letters indicate statistically significant differences ($P < 0.05$) between treatment means.

At Storskogberget, tree girdling resulted in a marked reduction (53 %) of the mean total abundance of oribatids in comparison with non-girdled plots (Fig. 6). At Flakaliden, the mean total abundance of oribatid mites was significantly lower (52 %) in fertilised and girdled plots (FG) than in plots that had only been fertilised (F) in 2003 (Fig. 7). Girdling in non-fertilised plots (G) did not show any significant effect on total oribatid abundance compared to the non-fertilised control plots (C).

The decrease at the girdled spruce sites was primarily due to the negative response of the common species *O. nova*. Three other species, *Oppiella maritima*, *Scheloribates pallidulus*, *Eulohmannia ribagai* and the family of Brachychtoniidae, had significantly lower abundances after girdling in one of the sites (Table 2). *O. nova*, *O. maritima*, and *S. pallidulus* are considered fungivorous (Hartenstein, 1962; Kaneko, 1988; Luxton, 1972; Ponge, 1991; Schneider *et al.*, 2004; Wallwork, 1958), whereas the ecology and feeding

habits of *E. ribagai* and Brachychtoniidae are not well understood. The decline of fungivorous species in plots, where the EM fungi had been negatively affected (Göttlicher *et al.*, 2008) could indicate a dependence on EM fungi as a food source.

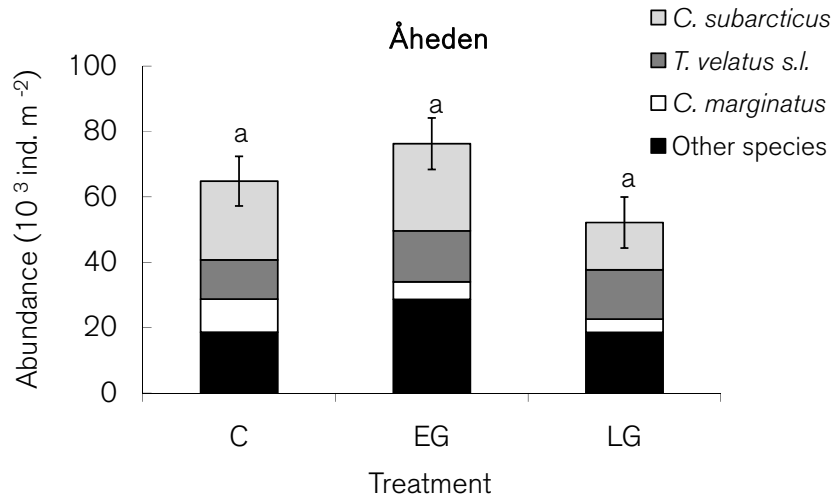


Figure 8. Mean total abundance (\pm SE) including the three most abundant species *Carabodes subarcticus*, *C. marginatus* and *Tectocephus velatus* in control (C), early girdling (EG) and late girdled (LG) plots at Åheden, 11 and 9 months after girdling, respectively. SE given here includes both treatment and plot variation. The same letters indicate no significant differences ($P > 0.05$) between treatment means.

There was no clear effect of girdling on oribatid mite abundance in the Scots pine site Åheden (Fig 8). One explanation might be that tree girdling was not detrimental to all EM fungi at that site because of the presence of dwarf-shrubs. It has been shown that the common mycorrhizal fungus on ericaceous dwarf-shrubs, *Rhizoscyphus ericae*, can form ericoid mycorrhiza on *Vaccinium myrtillus* but also EM mycorrhiza on *P. sylvestris* (Villareal-Ruiz *et al.*, 2004). Thus there might be viable EM mycelia left for the mites to consume on *P. sylvestris* roots after girdling. It is also possible that the mites at Åheden survived by feeding directly on the ericoid mycorrhiza formed by *R. ericae* on dwarf-shrub roots. Another explanation as to why there was no clear response to girdling in total oribatid abundance can be that none of the dominant oribatid species at Åheden was entirely dependent on EM fungi as a food source. *O. nova*, the most abundant species in ungirdled spruce plots (Flakaliden and Storskogberget) made up only 3.6 % of total oribatid

abundance in the ungirdled plots at Åheden. The low abundance of *O. nova* at the pine site as compared to the spruce sites indicates that this species was restrained by some factor at the pine site. One possible factor might be the lack of palatable EM fungi at Åheden, indicating that the species of EM fungi can matter for fungivorous oribatids.

Table 2. Significant ($P<0.05$) effects of tree girdling on the abundance of oribatid mites at Åheden, Storskogberget and Flakaliden. C=control and non-fertilised, control, G=girdling and non-fertilised girdling, EG=early girdling, F=fertilisation and FG=fertilisation + girdling. Overall effects of girdling at Flakaliden take both non-fertilised and fertilised plots and time (2003 and 2004) into consideration.

	Åheden	Storskog- berget	Flakaliden 2003	Flakaliden 2004	Overall effects at Flakaliden
Species decreasing after girdling					
<i>Oppiella nova</i>		C>G	F>FG		Girdling
<i>Brachychthoniidae</i> spp.			C>G F>FG		Girdling
<i>Eulohmannia ribagai</i>			F>FG	C>G F>FG	Girdling
<i>Schelorbates pallidulus</i>		C>G			
<i>Oppiella maritima</i>					Girdling
<i>Edwardzetes edwardsi</i>			F>FG		
Species increasing after girdling					
<i>Suctobelbidae</i> spp.	C<EG				
<i>Heminothrus longisetosus</i>	C<EG				
<i>Belba compta</i>				C<G	

5.1.2 Nutrient addition

We hypothesised that nutrient addition would decrease the diversity and species richness of EM fungi (Fransson *et al.*, 2000; Jonsson *et al.*, 2000), which would result in a decline in the number of oribatid mites. However, this was not confirmed since there was no overall effect of fertilisation on total abundance of oribatid mites at Flakaliden (Fig. 7). Several oribatid species were negatively affected by fertilisation, but there were also some species that were positively affected by this treatment, resulting in higher abundance at fertilised plots (Table 3). Most species that were affected by fertilisation had shown the same pattern in a previous fertilisation study carried out in the same area in 1999 (Lindberg & Persson, 2004). Knowledge about why these animals were affected by fertilisation is still poor. The decline in mite abundance can have been caused by high salt concentration (Heungens & van Daele, 1981), a toxic effect of ammonia (Moursi, 1962) or because fertilisation changes the species composition of the fungal community (Fransson *et al.*, 2000). The opposite situation, when the mites were favoured by fertilisation, can be explained by higher nutrient concentration in the litter (Tamm, 1991), increased litterfall (Iivonen *et al.*, 2006; Tamm, 1991), or higher abundance of saprophytic fungi. An increase in abundance of saprophytic fungi could be due to the higher amount of litter in the fertilised plots, or due to less competition from mycorrhizal fungi for nutrients or space (Gadgil & Gadgil, 1975; Leake *et al.*, 2002).

Table 3. Significant ($P<0.05$) effects of fertilisation on oribatid mites abundance at Flakaliden. C=non-fertilised control, F=fertilisation. Overall effects of girdling at Flakaliden take both non-fertilised and fertilised plots and time (2003 and 2004) into consideration.

	Flakaliden 2003	Flakaliden 2004	Overall effects at Flakaliden
Species decreasing after fertilisation			
Suctobelbidae spp.	C>F	C>F	Fertilisation
<i>Oppiella nova</i>		C>F	Fertilisation
<i>Camisia biurus</i>	C>F		Fertilisation
<i>Tectocepheus velatus</i> s.l.	C>F		Fertilisation
<i>Heminothrus longisetosus</i>	C>F		Fertilisation
<i>Edwardzetes edwardsi</i>		C>F	Fertilisation
<i>Pergalumna nervosa</i>		C>F	Fertilisation
<i>Melanozetes mollicomus</i>	C>F	C>F	Fertilisation
<i>Carabodes marginatus</i>	C>F		Fertilisation
<i>Carabodes tenuis</i>	C>F		
<i>Carabodes subarcticus</i>	C>F		
<i>Ceratoppia bipilis</i>		C>F	
<i>Scheloribates initialis</i>		C>F	
<i>Nanhermannia sellnicki</i>		C>F	
Species increasing after fertilisation			
<i>Oribatella calcarata</i>	C<F	C<F	Fertilisation
<i>Oppiella subpectinata</i>	C<F		Fertilisation
<i>Oribatula tibialis</i>	C<F	C<F	Fertilisation
Brachychthoniidae spp.		C<F	Fertilisation
<i>Eupelops acromios</i>		C<F	Fertilisation
<i>Belba compta</i>	C<F	C<F	Fertilisation
<i>Oppiella maritima</i>	C<F		
<i>Oppiella</i> sp.		C<F	
<i>Dissorhina ornata</i>	C<F		
<i>Platynothrus capillatus</i>	C<F		
<i>Autogneta parva</i>		C<F	
<i>Scheloribates latipes</i>		C<F	
<i>Eulohmannia ribagai</i>	C<F		
<i>Adoristes ovatus</i>	C<F		

5.2 Species depending on or favoured by the presence of EM fungi. (II)

5.2.1 *Oppiella nova*

The soil animals added to the plant-soil microcosms (*Paper II*) responded to the different treatments in a variable manner (Figs. 9–13). In agreement with the hypothesis, *O. nova* was clearly favoured by the presence of certain EM fungi and increased in abundance of both juveniles and adults in the microcosms with two EM species (*S. variegatus* and *P. involutus*) (Fig. 9, Table 4).

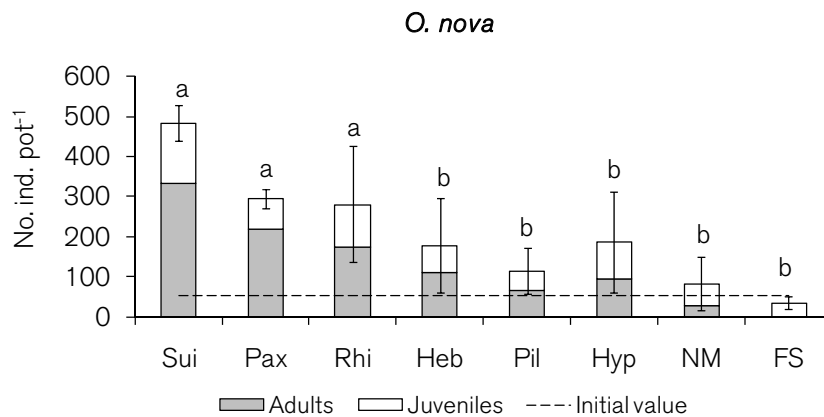


Figure 9. Mean total abundance (\pm SE) of *O. nova* in microcosms with different plant/fungus treatments. Sui=*S. variegatus*, Pax=*P. involutus*, Rhi=*R. roseolus*, Heb=*H. velutipes*, Pil=*P. fallax*, Hyp=*H. capnoides*, NM=non-mycorrhizal plant and FS=forest soil. Hyp lacks plants. NM and FS lack active inoculation of fungi. Broken line indicates the no. of specimens added. Only adult oribatids (ad) were added, whereas juveniles (juv) hatched during the experiment. Different letters indicate statistically significant differences ($P < 0.05$) between total (ad+juv) treatment means.

Adults of *O. nova* showed a decline in abundance in the forest soil treatment (FS), which probably contained a mixture of EM and saprotrophic fungi. When expressing the mite abundance per unit of chitin (fungal biomass) (Fig. 10) the data showed that *O. nova* had the highest abundance in the treatment with the EM fungus *S. variegatus*, followed by the EM fungi *R. roseolus*, *P. involutus* and *H. velutipes*. These species enhanced *O. nova* populations compared to the EM fungus *P. fallax*, the saprotrophic fungus *H. capnoides* and the undetermined fungi in the NM and

FS treatments. According to the literature, *O. nova* primarily feeds on fungi (Luxton, 1972; Ponge, 1991). However, since *O. nova* is found in all kinds of habitats (Behan-Pelletier, 1999; Skubala & Gulvik, 2005), many of them lacking EM fungi, it is clear that it cannot be restricted to feed on single EM species. One explanation could be that there is a wide range of fungi on which *O. nova* can survive although the fungi may not be highly preferred (Kaneko *et al.*, 1995).

Table 4. Statistically significant ($P < 0.05$) increases (+) or decreases (-) in juvenile, adult and total abundances of soil animals in different treatments after addition of a fixed number of adults and a response period of 70-84 days at 15 ± 2 °C. Sui=S. variegatus, Pax=P. involutus, Rhi=R. roseolus, Heb=H. velutipes, Pil=P. fallax, Hyp=H. capnoides, NM=non-mycorrhizal plant, FS=forest soil and PV=peat+vermiculite. Hyp and PV lack plants. NM, FS and PV lack active inoculation of fungi. Mean abundances are given in Figs.9, 11,12 and 13. n.s.=not significant.

Treat	<i>O. nova</i>			<i>T. velatus</i>			<i>N. silvestris</i>			<i>C. sphagnetorum</i>
	juv	ad	tot	juv	ad	tot	juv	ad	tot	tot
Sui	+	+	+	+	-	n.s.	+	-	n.s.	n.s.
Pax	+	+	+	n.s.	-	-	+	-	-	n.s.
Rhi	+	n.s.	n.s.	+	-	-	+	-	n.s.	n.s.
Heb	n.s.	n.s.	n.s.	+	-	n.s.	n.s.	-	n.s.	n.s.
Pil	+	n.s.	n.s.	+	-	-	+	-	-	n.s.
Hyp	+	n.s.	n.s.	+	-	-	+	-	n.s.	n.s.
NM	+	n.s.	n.s.	+	-	-	+	-	-	n.s.
FS	+	-	n.s.	+	-	+	+	-	n.s.	+
PV	no data	no data	no data	no data	no data	no data	no data	no data	no data	+

The FS treatment was the least favourable for *O. nova*. One possible explanation could be that the forest soil contained relatively low amounts of living EM hyphae or spores. This was indicated by low amounts of chitin attached to the roots (Table 5). Soil from the FS treatment always had the highest amount of chitin, which indicates a high fungal biomass, presumably saprophytic fungi, although it seems as if the fungal species present were not the ones most conducive for the growth of *O. nova*. The chitin analyses showed that there were 2-6 times higher amount of chitin in the NM (without EM inoculation), Hyp (wood piece without plant) and FS treatments than in the EM treatments. Since EM fungi (e.g., *S. variegatus* and *P. involutus*) have been shown to inhibit the growth of saprotrophic fungi (Lindahl *et al.*, 1999), an interaction between the two types of fungi may drastically reduce growth of saprotrophic soil mycelium (Lindahl *et al.*,

2002). We therefore assume that the five species of EM fungi inoculated and pre-grown on the roots in our experiment had the capacity to compete with and negatively affect the biomass of saprotrophic fungi in the peat-vermiculite substrate, whereas the saprotrophic fungi could develop without severe competition in the Hyp, NM, FS and PV (peat + vermiculite without plant) treatments. Thus, we assume that the latter four treatments were dominated by saprotrophic fungi, whereas the five EM treatments were dominated by mycorrhizal fungi.

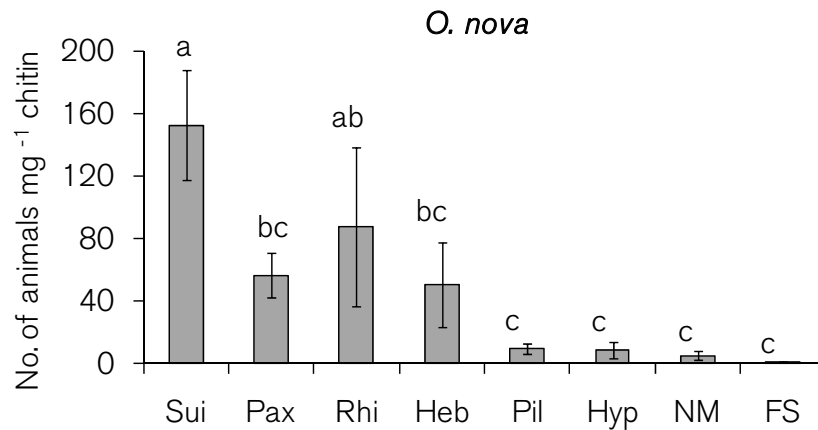


Figure 10. Mean total abundance (\pm SE) of *O. nova*, mg⁻¹ chitin in different plant/fungus treatments. Different letters indicate statistically significant ($P < 0.05$) differences between total treatment means. Notation as in Fig. 9.

5.2.2 *Tectocephus velatus*

T. velatus had the highest abundance in the forest soil treatment (Fig. 11), which is consistent with earlier studies showing that it feeds on both litter and fungi (Norton, 1985; Schuster, 1956; Wallwork, 1958). The total population size increased only in the FS treatment and decreased in most of the EM treatments with the exception of *S. variegatus* (Table 4). However, the abundance of adult *T. velatus* decreased in all treatments, which can be explained by mortality of the introduced adults and too short a time (70–84 days at 15 ± 2 °C) to reach maturity for juveniles emerging from eggs. Consequently, the number of juveniles produced is probably a better measure of performance than the total abundance. More *T. velatus* juveniles were produced in the FS treatment than in any of the other treatments. The FS and Hyp treatments contained high amounts of chitin, but the numbers

of juveniles were three times higher per unit of chitin in the FS than in the Hyp treatment. Both treatments probably had a dominance of saprotrophic fungi, but some chitin in the FS treatment probably originated from pieces of EM fungi left after the preparation of the humus. Thus, it is possible that *T. velatus* prefers saprotrophic fungi over EM fungi. It could also indicate that it prefers dying or senescent fungi over actively growing fungi, but much work remains to be done to elucidate this possibility.

Table 5. Mean amount of chitin ($n=5$) (mg pot^{-1}), in roots and soil in different plant/fungus treatments. Treatment codes are given in Table 4. Different letters indicate statistically significant differences ($P<0.05$) between treatment means within animal species. Total means for all microcosms including all animal species are based on $n=20$ samples.

Treat	Chitin in roots				Chitin in soil				Chitin (mean)			
	<i>O. nova</i>	<i>T. velatus</i>	<i>N. silvestris</i>	<i>C. sphagn.</i>	<i>O. nova</i>	<i>T. velatus</i>	<i>N. silvestris</i>	<i>C. sphagn.</i>	Roots	Soil	Roots+Soil	% in roots
Sui	0.26 b	0.24 ab	0.16 b	0.24 abc	3.7 d	4.2 d	5.3 c	3.2 cd	0.23	4.1	4.3	5.5
Pax	0.48 a	0.34 a	0.86 a	0.33 ab	7.5 d	6.4 d	4.0 c	6.5 c	0.50	6.1	6.6	8.4
Rhi	0.20 b	0.18 ab	0.24 b	0.43 a	4.1 d	3.2 d	3.0 c	2.9 cd	0.26	3.3	3.6	7.5
Heb	0.14 b	0.32 ab	0.23 b	0.34 ab	3.7 d	3.0 d	2.2 c	2.5 d	0.26	2.8	3.1	8.7
Pil	0.12 b	0.15 bc	0.14 b	0.16 bc	12.2 c	7.4 d	5.2 c	8.9 c	0.15	8.4	8.6	1.9
Hyp	no plant	no plant	no plant	no plant	23.9 b	23.8 b	21.6 b	21.5 b	0	22.7	22.7	0
NM	0.25 b	0.16 bc	0.36 b	0.16 bc	16.7 c	16.3 c	17.3 b	18.7 b	0.23	17.3	17.5	1.3
FS	0.13 b	0.06 c	0.08 b	0.05 c	36.9 a	32.7 a	34.1 a	31.2 a	0.08	33.7	33.8	0.2

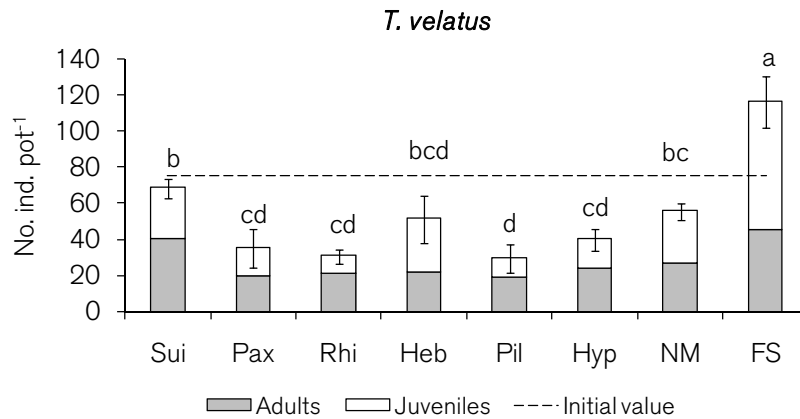


Figure 11. Mean total abundance (\pm SE) of *T. velatus* in microcosms with different plant/fungus treatments. Notation as in Fig. 9. Broken line indicates the no. of specimens added. Only adult oribatids (ad) were added, whereas juveniles (juv) hatched during the experiment. Different letters indicate statistically significant differences ($P < 0.05$) between total (ad+juv) treatment means

5.2.3 *Nothrus silvestris*

Similar to *T. velatus*, *N. silvestris* also showed a decline in adult abundances during the experiment in all treatments (Fig. 12). The highest abundance of juveniles was found in the FS treatment, which contained organic matter and probably a mixture of saprotrophic and EM fungi (see above). This is in line with previous studies, in which *N. silvestris* is regarded to be a generalist feeder on higher plant material and on fungi (Anderson, 1975; Luxton, 1972; Siepel & Ruiter-Dijkman, 1993).

N. silvestris

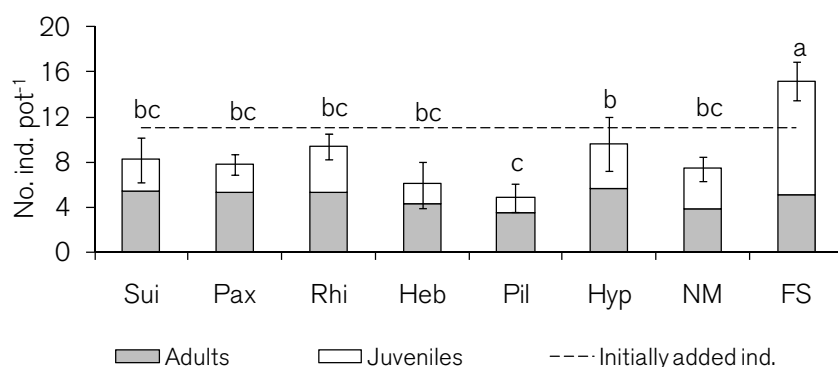


Figure 12. Mean total abundance (\pm SE) of *N. silvestris* in microcosms with different plant/fungus treatments. Notation as in Fig. 9 and 11.

5.2.4 *Cognettia sphagnetorum*

C. sphagnetorum was not favoured by the presence of EM fungi (Fig. 13). The number of individuals decreased or remained unchanged in all EM treatments during the experiment. The abundance of *C. sphagnetorum* increased markedly in the FS and PV (peat + vermiculite without plant) treatments, and the increase was close to significant ($P=0.06$) in the Hyp treatment containing the saprotrophic fungus (Table 4). According to our initial hypothesis, *C. sphagnetorum* should have responded more positively to the Hyp treatment than it actually did. The population increase was five times higher in the PV than in the Hyp treatment. The only difference between the setup of these two treatments was the piece of wood and the inoculation of a decomposer fungus in the Hyp treatment. A possible explanation is that *C. sphagnetorum* was suppressed in its growth by the presence of the *H. capnoides* fungus, at least to a certain extent. Both this saprotrophic fungus and the EM fungi pre-inoculated on the host roots have an abundant supply of carbon, either from the wood block or via photo-assimilates. It seems that *C. sphagnetorum* may be sensitive to actively growing fungi, but the nature of the agents suppressing the growth of the enchytraeid is not known. In general, very little is known about the chemical ecology of fungi with regard to both permanent and wound-activated defence substances (Spiteller, 2008).

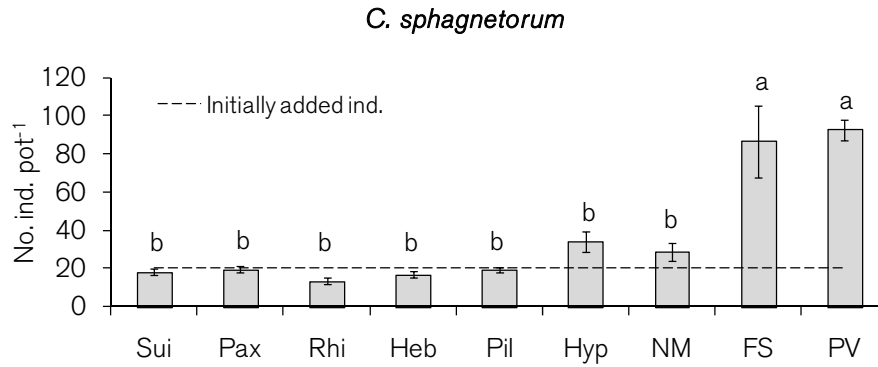


Figure 13. Mean total abundance (\pm SE) of *C. sphagnetorum* in microcosms with different plant/fungus treatments. Sui=*S. variegatus*, Pax=*P. involutus*, Rhi=*R. roseolus*, Heb=*H. velutipes*, Pil=*P. fallax*, Hyp=*H. capnoides*, NM=non-mycorrhizal plant and FS=forest soil. Hyp and PV lack plants. NM, FS, and PV lack active inoculation of fungi. Broken line indicates the no. of specimens added. Different letters indicate statistically significant differences ($P < 0.05$) between total (ad+juv) treatment means.

The roots of the plants subjected to EM inoculation were still heavily colonised by mycorrhizal fungi at harvest (Fig. 14) but apart from the chitin analyses, the degree of colonisation was not determined quantitatively.



Figure 14. The roots of the plants subjected to EM inoculation were still heavily colonised by mycorrhizal fungi, (see arrow) at harvest.

5.3 The use of molecular tools when studying oribatid gut contents. (III)

The primers designed to amplify *Laccaria* species resulted in clear products in the specificity test, and none of the nontarget species gave a visible band of the correct size. Thus, the primers could be defined as *Laccaria* group specific (*Paper III*).

When analysing single fed individuals, fungal DNA was only amplified to detectable levels in the samples with unwashed mites (Figs. 15 and 16). However, the results were similar for surface-contaminated mites, which demonstrated the need for a washing step prior to PCR analysis. Since DNA of *Laccaria* was not amplified in the samples with one washed mite we pooled five and ten whole-washed individuals in each sample. This resulted in amplification of *Laccaria* DNA, but the amount of surface contamination was also increased to such a level that *Laccaria* DNA was amplified in samples with surface-contaminated washed individuals (Fig. 16). We concluded that the washing was not sufficient to remove all fungal DNA from the body surface and that dissection could be a better way to remove the contamination. Fungal DNA was amplified to detectable levels in samples with one dissected gut from fed mites, but only if the mite had not been washed prior to dissection (Figs.15 and 16). On the other hand, the results were still similar for surface contaminated mites.

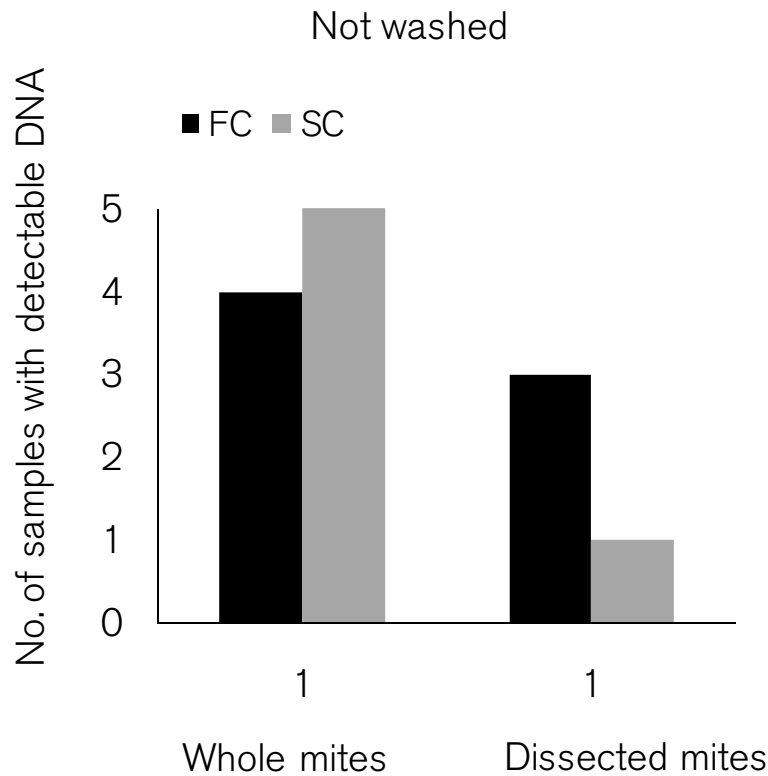


Figure 15. Number of samples (n=5) with detectable (amplified) *L. laccata* DNA in unwashed *A. longisetosus* mites either after feeding and walking (FC) in *L. laccaria* mycelium or being rolled as dead specimens in *L. laccaria* mycelium (SC, surface contaminated only). Each sample included one whole individual or a dissected gut from one individual. No significant differences were detected between the different treatments FC and SC.

Since neither washing nor dissection could eliminate surface contamination when used separately, we combined the two treatments. Finally, by pooling at least five guts from washed mites it was possible to detect *Laccaria* DNA from fed but not from surface-contaminated animals (Fig. 16), but to get a significant difference between the two treatments, ten guts were needed in the samples ($P<0.05$). Consequently, in order to exclude surface contamination and at the same time obtain target DNA of detectable levels, the mites had to be washed, pooled and dissected.

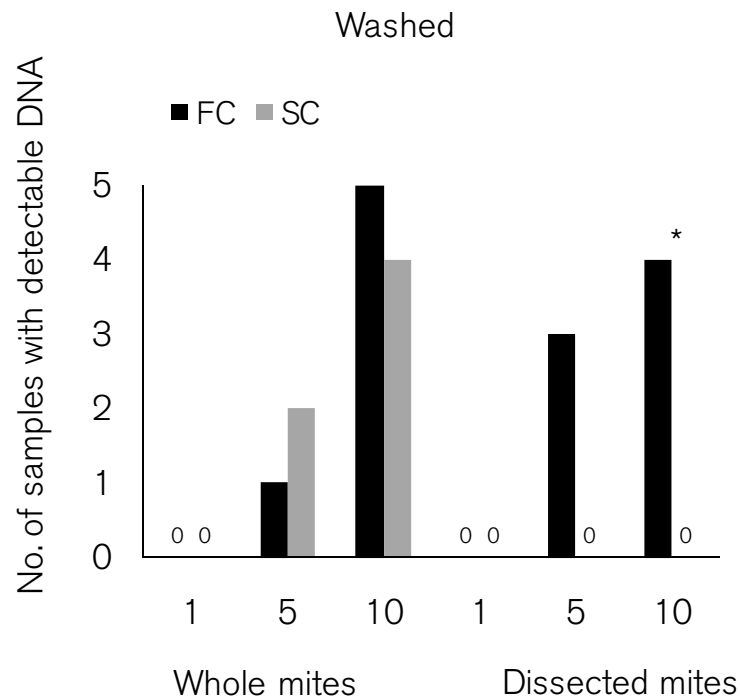


Figure 16. Number of samples (n=5) with detectable (amplified) *L. laccata* DNA in NaOCl-washed *A. longisetosus* mites either after feeding and walking (FC) in *L. laccaria* mycelium or being rolled as dead specimens in *L. laccaria* mycelium (SC, surface contaminated only). Each sample included one, five or ten whole individuals as well as one, five or ten dissected guts. The star indicates a significant difference ($P<0.05$) between the fed (FC) and contaminated (SC) mites with samples containing ten guts. No significant differences were detected between the other treatments. The zeros indicate that no *L. laccata* DNA was amplified in these samples.

The problem with surface contamination when doing molecular analysis on whole-body microarthropods has been demonstrated earlier, but a reliable solution was not proposed (Bracht Jørgensen *et al.*, 2005; Renker *et al.*, 2005). Renker *et al.*, (2005) found 15 fungal species in a sample containing four whole oribatid mites, but the authors did not distinguish between surface contamination and gut contents. Bracht-Jørgensen *et al.*, (2005), found eight fungal species in samples with whole collembolans but only one fungal species in samples with 20 dissected guts, however the importance of washing prior to dissection was not evaluated.

The feeding habits of other soil-dwelling meso- and macroinvertebrates are in many cases as unexplored as for the oribatid mites. The present

protocol has a high potential to work for them as well, as long as the methods included are tested under controlled conditions. The washing technique may have to be modified if, e.g., the animals have more setae or other structures that can accumulate fungal material, and all species are not as easy to dissect as *A. longisetosus*.

The animal and fungal species combined in this experiment, i.e., *A. longisetosus* and *L. laccata*, do not occur together in their natural environments. Nevertheless, the experiment was mainly conducted to develop a reliable method and not to study the feeding habits of *A. longisetosus*. To ensure that the food source was the only fungal contaminant in our experiment, we used *Laccaria* specific primers. The species were also used because they are easy to keep under laboratory conditions, and because PCR primers for *L. laccata* were available.

One concern when analysing predator-prey interactions with PCR analysis is the relatively short length of time between the feeding event and when the DNA of the prey is no longer detectable in the gut due to digestion (MacMillan *et al.*, 2007). This issue has not been dealt with in the present protocol but needs to be considered when analysing field-collected soil animals, especially when collecting and extracting the animals. Soil animals are commonly extracted by heating/drying of a soil sample (0–48 h), and the animals are collected as soon as they reach the collection vessel (see section 4.1.3 and Fig. 3). The animals will then have access to the food present in the soil on their way through the sample, but since they will be highly stressed they will most probably not feed during extraction. Since the increased temperature during extraction can speed up the digestion rate and lead to unwanted starving, this will probably have negative effects on the detection rate and should be controlled for when designing sampling strategies.

Another important issue that was not considered in this study was the inclusion of negative controls during DNA extraction. These controls screen for potential contamination by target DNA between samples, which is addressed in the review by King *et al.*, (2008). In the present study we did not consider cross-contamination between samples as a major problem since the results obtained show a logical pattern, nevertheless the matter should not be neglected in future studies.

Screening for a multitude of fungal food items simultaneously in field-collected microarthropods will probably need the use of fungal-specific primers in combination with sequence analysis. Alternatively, fungal-specific primers can be combined with species-specific primers in order to reduce the number of sequence analyses. Fungal-specific primers (EF 4, EF3 and

fung 5), were used in a nested PCR when searching for fungal DNA in guts of Collembola (Bracht Jørgensen *et al.*, 2005). The primers amplify a 550 bp product, which according to some researchers may be more difficult to amplify, since the DNA is being broken into smaller fragments during digestion (King *et al.*, 2008). The same primers could still, theoretically, be used when analysing oribatid gut contents, when combined with the presented washing and dissection technique.

The procedures in this protocol are relatively time-consuming and may not be feasible for screening large numbers of mites. However, by using real time PCR the time of analysis can be reduced, since there is no need for gel visualisation. The protocol could also be improved by washing pooled mites instead of individual mites in separate tubes and more than one food source can be analysed simultaneously if multiplexes of different primer pairs are developed (Harper *et al.*, 2005). After all, since there is no other method that can produce such a detailed answer on what the animals have actually consumed, a small number of samples with mites from different environments can still answer many questions about their feeding habits and their functional role in the soil ecosystem.

5.4 Can the analysis of stable isotope ratios of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ be useful when studying fungivorous animals? (IV)

In *Paper IV*, we investigated the natural abundance of the stable isotopes ^{15}N and ^{13}C of fungal fruit bodies and the associated fungivorous Diptera larvae. Specifically, we examined the following questions: 1) Will the same animal group show a constant or variable enrichment value in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ when the animals have fed on different fungal substrates (i.e. species and compartments)? (2) Will different animal groups feeding on the same fungal compartment have different isotopic signatures? (3) What determines the ^{15}N enrichment of the animal in relation to the nitrogenous compounds in the food? (4) What is the range of stable isotope values recorded from fungivorous Diptera larvae found in field-collected fruit bodies?

In general, the animals were enriched in ^{15}N and ^{13}C compared to the fruit body parts they lived in, but the enrichments were variable. Delta ^{15}N signatures were much higher in fruit bodies of the ectomycorrhizal (EM) fungi *Suillus* and *Boletus* than in the saprotrophic fungus *Clitocybe*, whereas $\delta^{13}\text{C}$ was generally lower (Figs. 17 and 18, Tables 6 and 7). Tubes had higher $\delta^{15}\text{N}$ values than caps and stems in *S. grevillei*, but such differences

could not be found for the other fungal species (Table 8). The $\delta^{13}\text{C}$ values were generally higher in tubes and gills than in caps and stems.

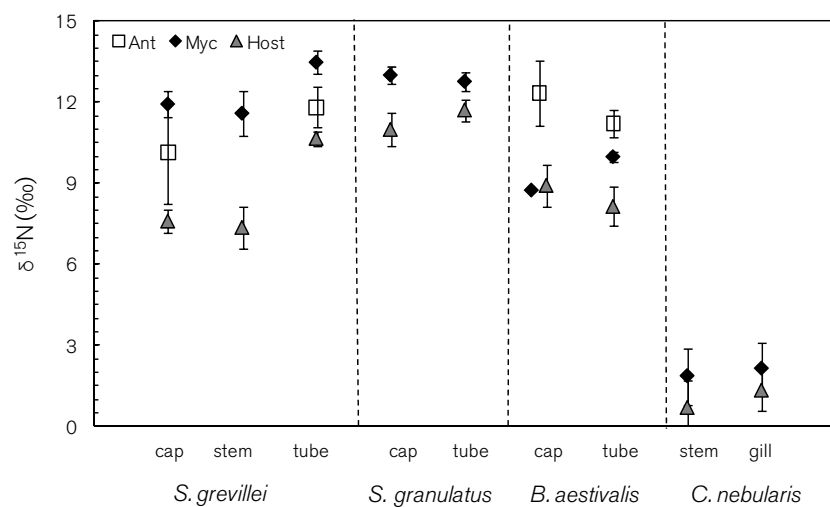


Figure 17 and 18. The value of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ (mean \pm SE) in Anthomyiidae (Ant) and Mycetophilidae (Myc) living in different parts of the fungal species *Suillus grevillei*, *Suillus granulatus*, *Boletus aestivalis* and *Clitocybe nebularis*.

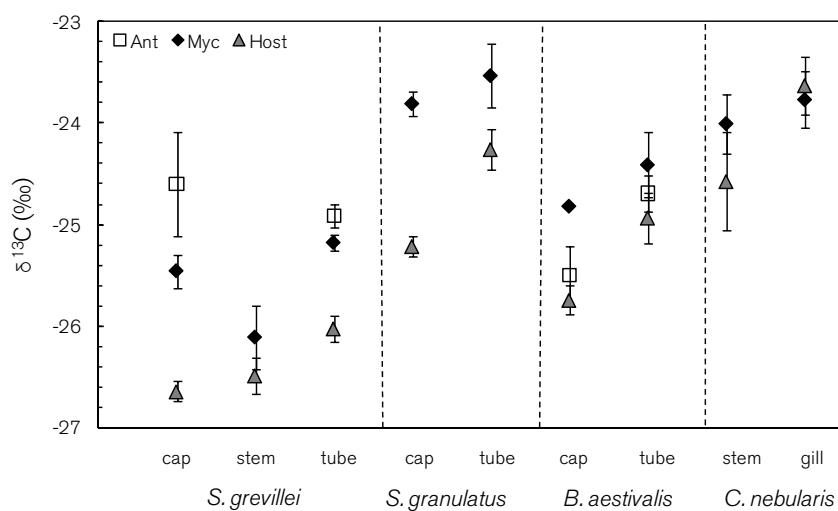


Table 6. Mean values of $\delta^{15}\text{N}$ (‰) in fungal hosts (H) and Diptera larvae (Mycetophilidae, M, and Anthomyiidae, A) living in the same tissues. Mean difference (Δ) in $\delta^{15}\text{N}$ between larvae and host and between the two larval species are also given. Significant differences are indicated by P values $\star < 0.05$, $\star\star < 0.01$ and $\star\star\star < 0.001$. n.s. = not significant. n indicates the number of pairs of comparisons. Note the different numbers of comparisons of the two animal groups

$\delta^{15}\text{N}$ (‰)										
Fungal species	Part	n	H	M	Δ M-H	n	H	A	Δ A-H	Δ M-A
<i>S. grevillei</i>	cap	6	7.6	12.0	4.4 $\star\star\star$	2	6.7	10.2	3.3 ^{n.s.}	1.7 ^{n.s.}
”	stem	4	7.4	11.6	4.2 \star					
”	tube	9	10.7	13.5	2.8 $\star\star$	8	10.7	11.8	1.1 ^{n.s.}	1.7 \star
<i>S. granulatus</i>	cap	3	11.0	13.0	2.0 \star					
”	tube	3	11.7	12.8	1.1 ^{n.s.}					
<i>B. aestivalis</i>	cap	1	8.1	8.7	0.7 ⁿ⁼¹	2	9.3	12.3	3.0 \star	
”	tube	3	7.9	10.0	2.1 \star	4	8.1	11.2	3.1 $\star\star\star$	-0.7 ⁿ⁼¹
<i>C. nebularis</i>	stem	4	0.7	1.9	1.1 $\star\star$					
“	gill	4	1.4	2.1	0.8 \star					

Table 7. Mean values of $\delta^{13}\text{C}$ (‰) in fungal hosts (H) and Diptera larvae (Mycetophilidae, M, and Anthomyiidae, A) living in the same tissues. Mean difference (Δ) in $\delta^{13}\text{C}$ between larvae and host and between the two larval species are also given. See Table 6 for further explanations.

$\delta^{13}\text{C}$ (‰)										
Fungal species	Part	n	H	M	Δ M-H	n	H	A	Δ A-H	Δ M-A
<i>S. grevillei</i>	cap	6	-26.7	-25.6	1.1 $\star\star$	2	-26.6	-24.6	2.0 ^{n.s.}	-1.0 ^{n.s.}
”	stem	4	-26.5	-26.1	0.4 ^{n.s.}					
”	tube	9	-26.0	-25.2	0.8 $\star\star\star$	8	-25.9	-24.9	1.0 $\star\star\star$	-0.2 ^{n.s.}
<i>S. granulatus</i>	cap	3	-25.2	-23.8	1.4 $\star\star$					
”	tube	3	-24.3	-23.5	0.7 \star					
<i>B. aestivalis</i>	cap	1	-25.8	-24.8	1.0 ⁿ⁼¹	2	-25.7	-25.5	0.2 ^{n.s.}	
”	tube	3	-25.0	-24.4	0.6 \star	4	-24.8	-24.7	0.1 ^{n.s.}	0.6 ⁿ⁼¹
<i>C. nebularis</i>	stem	4	-24.6	-24.0	0.6 ^{n.s.}					
“	gill	4	-23.6	-23.8	-0.1 ^{n.s.}					

Table 8. Mean difference (Δ) in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values and N and C concentration between tubes (T) and caps (CP) and gills (G) and stems (S) in fungal fruit bodies. See Table 6 for further explanations.

		n	$\Delta \delta^{15}\text{N}$ (‰)	$\Delta \delta^{13}\text{C}$ (‰)	ΔN (%)	ΔC (%)
<i>S. grevillei</i>	T-CP	6	2.8***	0.6 *	2.4***	6.5 ***
<i>S. granulatus</i>	T-CP	3	0.7 ^{n.s.}	1.0*	2.3**	-0.6 ^{n.s.}
<i>B. aestivalis</i>	T-CP	3	-0.2 ^{n.s.}	0.7*	3.5*	3.0*
<i>C. nebularis</i>	G-S	4	0.6 ^{n.s.}	0.9*	1.5 ^{n.s.}	-0.4 ^{n.s.}

5.4.1 Will the same animal group show a constant or variable enrichment value of ^{15}N and ^{13}C when the animals have fed on different fungal substrates?

The relationship between substrate and animal isotope signatures was complex as the enrichment was both animal and substrate dependent. Mycetophilidae showed high ^{15}N enrichment relative to the host in caps and stems of *S. grevillei* (>4 ‰), in tubes of *S. grevillei* and *B. aestivalis* and caps of *S. granulatus* (2.0–2.8 ‰), while there were minor differences between Mycetophilidae and its host in *S. granulatus* tubes, *B. aestivalis* caps and in *C. nebularis* (Fig. 17, Table 6). In contrast, Anthomyiidae showed high ^{15}N -enrichment in both caps and tubes of *B. aestivalis* (3 ‰) but not in tubes of *S. grevillei* (Fig. 17, Table 6). The larvae of Mycetophilidae showed a mean enrichment of $\delta^{13}\text{C}$ in relation to caps and tubes in Boletales (*Suillus* and *Boletus*) of 1.15 ± 0.07 ‰ and 0.73 ± 0.10 ‰, respectively (Fig. 18, Table 7). However, Mycetophilidae larvae living in *C. nebularis* did not show any significant enrichment in $\delta^{13}\text{C}$ (Table 7). The data on Anthomyiidae larvae were limited, and a significant enrichment in $\delta^{13}\text{C}$ (1 ‰) was only found for larvae living in *S. grevillei* tubes (Table 7).

Stable isotopic ratios of animals have previously been shown to reflect that of their diets. One explanation is that the lighter isotope is preferentially excreted, which leads to an accumulation of the heavier isotope in consumers (DeNiro & Epstein, 1978; Peterson & Fry, 1987), but the relationship is not fully understood. Early studies on ^{15}N in consumers relative to their diets estimated the mean enrichment to be 3.4 ‰ (Minagawa & Wada, 1984). This value was later modified and according to the meta-analysis by Vanderklift and Ponsard (2003), the enrichment in ^{15}N

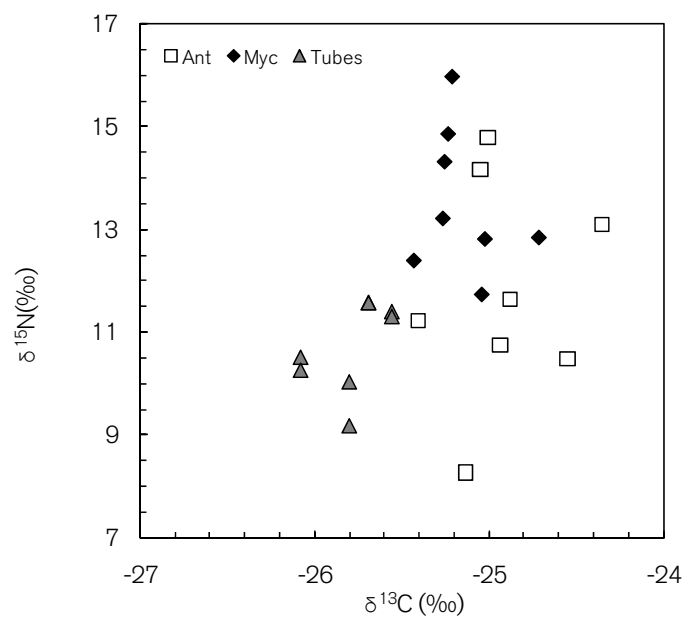
is 2.54 ± 0.11 ‰, which is close to the mean value of 2.4 ± 0.2 (\pm SE, $n=53$, $P<0.001$), in the present study. The mean isotopic shift for carbon has been estimated to be $+0.5 \pm 0.13$ ‰ (McCutchan *et al.*, 2003), which again is close to the mean value of 0.7 ± 0.08 ‰ ($n=53$, $P<0.001$), in this study. Even though the mean enrichment values from the literature agree closely with our findings, we could show that the enrichment of the same animal group (Mycetophilidae) varied depending on which fungal species or compartment they had ingested. In addition, it is also important to recognise that fungi do not represent a single homogeneous diet, since the isotopic signatures of both species and different fungal tissues can vary significantly.

5.4.2 Will different animal groups feeding on the same fungal compartment have different isotopic signatures?

The tubes of individual *S. grevillei* fruit bodies were often colonised by both Anthomyiidae and Mycetophilidae. Despite the two animal groups having fed on the same tissue, there were significant differences. Both animals covered a much larger range in $\delta^{15}\text{N}$ values than that of the tubes, which was only 9.2 to 11.6 ‰ (Figs. 19). The range of Anthomyiidae was 8.3 to 14.8 ‰, and the Mycetophilidae had higher values but a slightly narrower range 11.7 to 16 ‰. Mycetophilidae were significantly enriched in ^{15}N in comparison with the fungal tubes (2.8 ‰; $P<0.01$, Table 6) while the Anthomyiidae were not. Mycetophilidae also had 1.7 ‰ higher $\delta^{15}\text{N}$ ($P<0.05$) than the Anthomyiidae (Table 6, Figs. 19 and 20). Nitrogen concentrations were however lower in the Mycetophilidae than in the Anthomyiidae ($P<0.01$) (Table 9). The ^{13}C values were fairly similar in the two animal groups. The range of individual $\delta^{13}\text{C}$ values was -25.4 to -24.7 ‰ in Mycetophilidae and -25.4 to -24.4 ‰ in Anthomyiidae, whereas the *S. grevillei* tubes range was -26.3 to -25.6 ‰ (Fig. 19). The ^{13}C enrichment in Mycetophilidae and Anthomyiidae relative to the fungal tubes was significant ($P<0.001$) (Table 7).

The fact that there were significant differences in $\delta^{15}\text{N}$ between the two animal groups that had been sampled from exactly the same tissue suggests that other factors than isotopic differences in the food may be of importance for the isotopic signature of the animals. In comparison, a 3 ‰ difference in mean $\delta^{15}\text{N}$ was found between two different species of weevils, when both were feeding on wheat seeds (DeNiro & Epstein, 1981). In both these cases differences in the physiology of the animals may be the main reason for these differences. It has been suggested that taxonomic differences in which form the nitrogen is excreted may contribute to between species differences

in isotopic fractionation (Vanderklift & Ponsard, 2003). Another possibility is that the animals differ in their ability to utilize the different nitrogenous components of the food. Since there are large N isotopic differences between some amino acids (Chikaraishi *et al.*, 2007) and between chitin and the two other major nitrogenous components, relatively small differences in utilization efficiency may cause significant differences in $\delta^{15}\text{N}$. In this study, when the two animal groups were feeding on the same *S. grevillei* tubes, Mycetophilidae larvae not only had significantly higher $\delta^{15}\text{N}$ than Anthomyiidae, but they also had 1.5 % lower N concentration. This suggests differences between the two animal groups in their ability to utilize the various nitrogenous compounds of the food e.g., having different enzymatic capacity.



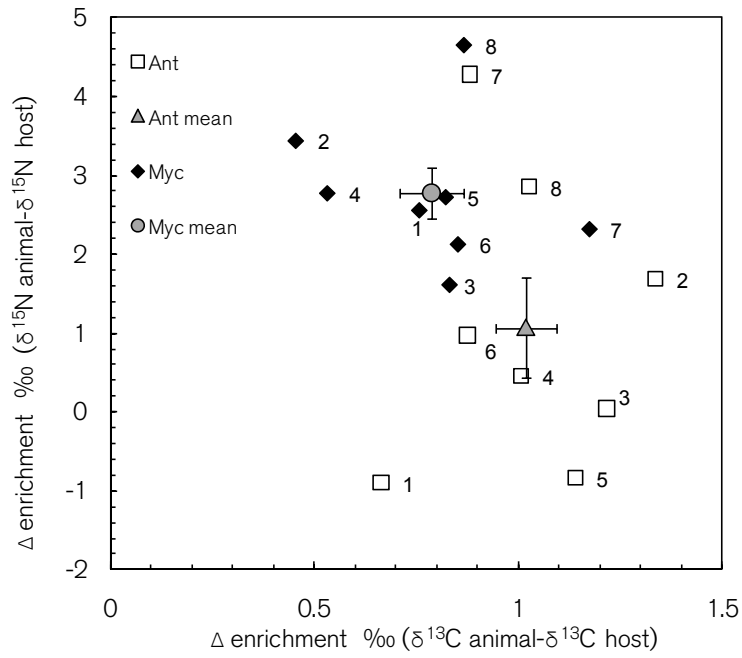


Figure 20. Enrichment (Δ) of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in individuals of Anthomyiidae (Ant) and Mycetophilidae (Myc) found in the tubes of *S. grevillei* fruit bodies. The numbers indicate the individual fruit body from where the animals were collected. Error bars are given as SE.

Table 9. Mean values of N (%) in fungal hosts (H) and Diptera larvae (Mycetophilidae, M, and Anthomyiidae, A) living in the same tissues. Mean difference (Δ) in N concentrations between larvae and host and between the two larval species are also given. See Table 6 for further explanations.

Fungal species	Part	n	N (%)					H	A	Δ A-H	Δ M-A
			H	M	Δ M-H						
<i>S. grevillei</i>	cap	6	1.9	8.0	6.1***	2	1.7	10.1	8.4*	-2.4 ^{n.s.}	
"	stem	4	1.9	7.6	5.7***						
"	tube	9	4.5	8.3	3.7***	8	4.7	9.8	5.1***	-1.5**	
<i>S. granulatus</i>	cap	3	2.2	7.9	5.7**						
"	tube	3	4.5	9.8	5.3*						
<i>B. aestivalis</i>	cap	1	3.0	9.1	6.1 ⁿ⁼¹	2	3.7	7.5	3.9*		
"	tube	3	6.1	10.3	4.2**	4	6.1	10.3	4.2**	1.0 ⁿ⁼¹	
<i>C. nebularis</i>	stem	4	3.8	8.2	4.4**						
"	gill	4	5.3	8.9	3.6**						

5.4.3 What determines the ^{15}N enrichment of the animal in relation to the nitrogenous compounds in the food?

Most of the N content in the fruit bodies was made up by proteins followed by amino acids and chitin (Fig. 21). The protein and amino acid fractions had generally higher $\delta^{15}\text{N}$ signatures than chitin (Fig. 22). A comparison of the $\delta^{15}\text{N}$ values in animals and protein (Fig. 22) showed that these were closely related, which resulted in enrichment values being considerably less than when compared with the bulk fungal material ingested. The fact that the $\delta^{15}\text{N}$ values of the animals was similar to that of the protein and amino acids of the food (Fig. 22), suggests that an enrichment of $\delta^{15}\text{N}$ in the animals relative to the food is apparent and probably a result of preferential uptake of protein and amino acid N in favour of chitin N. However, in tissues with low amounts of proteins and amino acids, there was a significant enrichment in ^{15}N in the Mycetophilidae larvae in relation to the nitrogenous compounds.

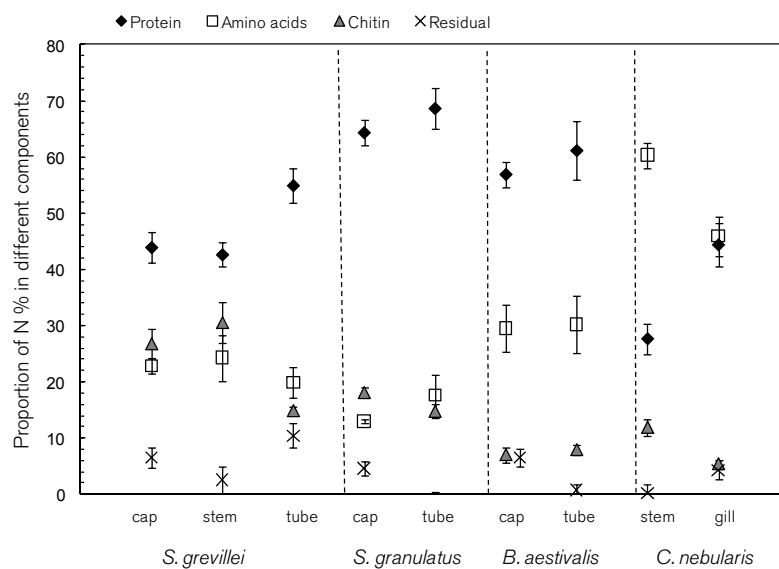


Figure 21. Proportion of N (%) (mean \pm SE) in different compartments of the fungal fruit body of *Suillus grevillei*, *S. granulatus*, *Boletus aestivalis* and *Clitocybe nebularis*.

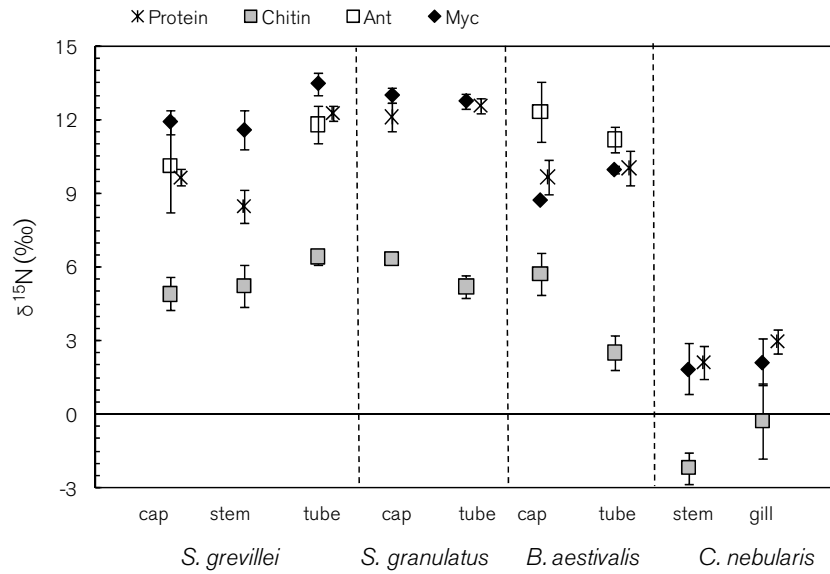


Figure 22. Mean (\pm SE) $\delta^{15}\text{N}$ (mean) in Anthomyiidae (Ant) and Mycetophilidae (Myc) living in different parts of the fungal species *Suillus grevillei*, *S. granulatus*, *Boletus aestivalis* and *Clitocybe nebularis*. The $\delta^{15}\text{N}$ values (mean \pm SE) in the protein and chitin fractions of the different fungal parts are given as a comparison.

The ^{15}N enrichment in the animals relative their food is not constant but possibly dependent on variations in the nitrogen quality of the food, as well as variations in the size of the isotopic shifts between the various nitrogen components. The significant negative correlation between the nitrogen concentration in the animals and the ^{15}N enrichment in the animals relative to the fungal materials (Fig. 23), supports this view. We argue that the higher the content of available proteins and amino acids of the food is, the smaller the isotopic enrichment would be. This is because the $\delta^{15}\text{N}$ of the fungal tissue will be closer to that of the proteins and amino acids when they contribute more to the total nitrogen of the material. This model can be tested in the present study by comparing animals from tubes and gills (food with high protein and amino acid content) with those from caps and stems (relatively low protein and amino acid content). All fungal species except *B. aestivalis* followed the predicted pattern, i.e., the ^{15}N enrichment in the animals relative to the food was higher in caps and stems than in tubes and gills. A probable explanation for this is the relatively high nitrogen content of the cap in *B. aestivalis*. Similar results, where a low quality diet corresponds to a larger enrichment than a high quality diet have also been

found in laboratory feeding studies of locusts feeding on maize or wheat (Webb *et al.*, 1998).

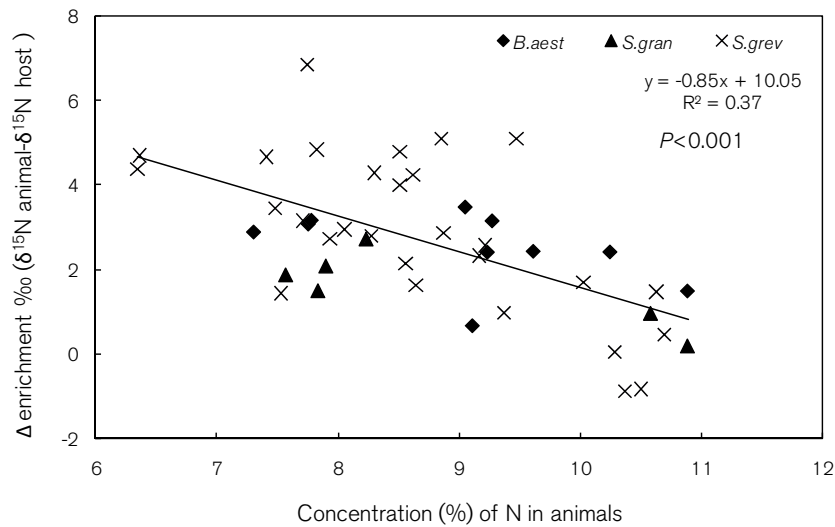


Figure 23. Relation between the N concentration (%) in animal and the enrichment of $\delta^{15}\text{N}$ in individual Diptera larvae (both Mycetophilidae and Anthomyiidae) vs. corresponding EM fruit bodies.

5.4.4 What is the range of stable isotope values recorded from fungivorous Diptera larvae found in field-collected fruit bodies?

Studies on the trophic structure of soil invertebrate communities have delimited trophic level boundaries by first determining a baseline value of the primary food source (e.g. litter) and then establishing margins of the trophic levels by using mean enrichment values such as $2.54 \pm 0.11\text{‰}$ for $\delta^{15}\text{N}$ (Vanderklift *et al.*, 2003) and $0.50 \pm 0.13\text{‰}$ for $\delta^{13}\text{C}$ (McCutchan *et al.*, 2003). Applying these values to the data obtained in the present study shows that the Mycetophilidae larvae would effectively span across at least four trophic levels – even though they all had fed on fungi. If the data from only *S. grevillei* are considered, the combined animal ranges are 7.7 ‰ for $\delta^{15}\text{N}$ and 2.8 ‰ for $\delta^{13}\text{C}$, which together create an imaginary shift across three trophic levels. The wide range of values found in fungal material clearly result in an equally wide range of values in the fungivores feeding on them, which demonstrates that the range of values recorded from field-collected animals utilising a single food source (i.e. fungi) is sufficient to span across trophic levels.

The substantial variation in isotopic composition found in the present study between and within fruit bodies and the consumers is important to consider when studying field-collected fungivorous animals. Variation in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values is not only dependent upon the variability of diets, but also upon the amount of isotopic variation within the food sources (Bennett *et al.*, 2009; Matthews & Mazumder, 2004). In the study by Taylor *et al.* (2003), the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ signatures of fungal fruit bodies spanned almost as many units as the soil living animals in the studies by Pollierer *et al.* (2009) and Schneider *et al.* (2004) indicating that all the animals studied could theoretically be fungivorous. In addition, since different parts of a fruit body can have significantly different isotopic composition, and the growing mycelia can differ in isotopic ratio compared to fruit bodies (Boström *et al.*, 2007), it is important to determine which component of the fungus actually consumed by the fungivore.

6 Main conclusions

- PCR in combination with body surface wash and dissection can be used for the analysis of diet in fungal-feeding oribatid mites. Surface contamination was shown to be a serious problem when using PCR on small fungal feeding animals, but it could be eliminated with the combined treatment of surface washing and dissection.
- The common oribatid species in many soils, *Oppiella nova*, is favoured by the presence of EM fungi and seems to prefer *Suillus variegatus* and *Paxillus involutus* over other EM fungi. These findings explain why *O. nova* shows a massive decline in abundance in boreal forest soils after clear-cutting and experimental tree girdling, when the preferred food is almost eliminated.
- The common enchytraeid worm *Cognettia sphagnetorum* is suppressed by the presence of EM fungi, which partly explains why this species reach much higher populations after clear-cutting than in the growing forest with EM fungi on the tree roots.
- The isotopic composition of fungivorous animals should be interpreted with great care, when used as a method for studying fungivore feeding habits. It is important to recognise that fungi do not represent a single homogenous diet and that there is substantial variation in isotopic composition between and within fruit bodies and among the animals feeding on them.

7 Future challenges

Based on the different studies in this thesis, the most promising way forward is to use molecular tools for investigating the feeding habits of fungivorous soil fauna. In *Paper III* we could show that molecular gut-content analyses with PCR, in combination with dissection and washing was a successful method for this purpose. However, the method has not been tested on field-collected mites which would be a very interesting challenge to undertake, as well as testing the protocol on other fungivorous animals such as collembolans. It would also be interesting to compare the feeding habits of the same species from different environments, e.g., *O. nova* from forest and agricultural soils.

Fungal-specific primers in combination with cloning and sequence analysis will be necessary tools in order to screen the large number of fungal species present in soil. Alternatively fungal-specific primers in combination with group-specific primers could be used in order to reduce the number of sequence analyses. An important factor to consider when using the method with field-collected animals is how long time after the feeding event it is possible to detect the ingested food. The extraction technique may need to be modified in order to limit the time of food digestion.

Results of field collected soil animals may, in combination with visual observation in the lab or, microcosm studies, be a promising way forward when trying to explain the unknown feeding habits of fungivorous fauna.

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8 Svensk sammanfattning

Skogsmarkens biologiska mångfald är rik men mycket ofullständigt känd både vad gäller artdiversitet och funktionell diversitet. Två mycket artrika funktionella grupper i barrskogsmark är mykorrhizasvampar och svampkonsumerande markdjur. Tidigare studier har visat att det kan finnas ett samband mellan vissa markdjur t.ex. pansar- eller oribatidkvalster och mykorrhizasvampar, eftersom båda dessa organismgrupper har minskat i antal vid kvävegödsling. En möjlig förklaring kan vara att mykorrhizasvamparna är en viktig föda för dessa djur. Det kan till och med vara så att vissa djur föredrar somliga mykorrhizasvamparter framför andra.

Syftet med mitt doktorandprojekt har varit att undersöka och klargöra vad svampätare i marken egentligen äter, med det övergripande målet att öka förståelsen för deras funktion i ekosystemet. Interaktionen mellan svampätare och ektomykorrhiza (EM) har varit särskilt intressant att undersöka närmare, eftersom dessa svampar har hög biomassa och artrikedom i skogsmark. Tidigare studier av markdjurs födoval har varit starkt beroende av den befintliga metodik som har använts. Därför har doktorandprojektet dessutom syftat till att jämföra olika metoder, för att på så vis hitta den teknik som är bäst lämpad för att undersöka vad svampätarna verkligen äter. Det är till exempel svårt att studera födopreferens i labbmiljö, eftersom det är praktiskt omöjligt att presentera alla tänkbara födokällor som finns i marken för det djur som studeras.

Den första studien i avhandlingen är ett fältförsök som syftade till att undersöka om oribatidkvalster i skogsmark påverkas av ringbarkning och kvävegödsling. Ringbarkningen antas minska tillgången och artsammansättningen på EM eftersom kolhydrater från trädkronan inte längre kan nå rötterna och den i symbios levande mykorrhizasvampen. Om EM svampen är en viktig födokälla för oribatiderna kan en minskad tillgång på denna påverka kvalstrens populationer och artsammansättning.

Den andra studien gjordes i mikrokosmsystem i växthusmiljö och baseras på resultatet från ringbarkningsförsöket. Här undersöktes om populationerna av några utvalda arter av oribatider samt en art av småringmask påverkades av vilken art av EM som fanns närvarande.

Studie nummer tre är en metodstudie som syftade till att undersöka om det är möjligt att använda sig av molekylära metoder som PCR för att detektera svamp från oribatiders magar. Framför allt undersöktes om det är möjligt att använda tekniken trots att djuren även kan ha spår av svamp som inte ätits på utsidan av kroppen.

Den fjärde och sista studien är också en metodstudie där vi undersökte möjligheten att använda stabila isotoper för att avgöra vad svampätande djur har ätit. Här jämfördes halten av de stabila isotoperna ^{15}N och ^{13}C i olika fruktkroppar av svamp med halten i de flug- och mygglarver som levde inuti dem.

Att kombinera PCR med tvättning av djurens yta gav lovande resultat för att i framtiden kunna använda denna metod på svampätande oribatidkvalster från fält, men även på andra svampätande djur i marken. Vi kunde också visa att den totala abundansen av oribatidkvalster minskade signifikant på ringbarkade granytor och att en av barrskogens vanligaste kvalsterarter, *Oppiella nova*, var den art som visade tydligast nedgång. I mikrokosmsystemet visades att *O. nova* också gynnas av vissa EM-arter medan andra EM-arter var mindre attraktiva som föda. Resultaten från undersökningen av stabila isotoper i svampfruktkroppar och flug- och mygglarver visade att det är viktigt att tolka denna typ av data mycket noggrant, speciellt när den används för att studera svampätares födoval. Avståndet mellan isotopvärdena från de flug- och mygglarver som alla ätit svamp var så stort, att det skulle sträcka sig över flera trofiska nivåer om dessa hade uppskattats med hjälp av de medelvärden på anrikning som används i litteraturen.

Resultaten som presenteras i den här avhandlingen bekräftar att det är nödvändigt att kombinera flera olika metoder för att kunna få svar på vad markdjur äter. Olika metoder passar olika bra för att besvara olika frågor. Min studie kan förhoppningsvis ge viss vägledning när man skall gå vidare med att förstå markens komplicerade näringsvävar.

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