Fatty acid patterns of soil decomposers and predators as affected by plant species richness

Master Thesis

presented by

GERMÁN AUGUSTO MURRIETA MOREY

from

Lima – Perú

conducted at

Johann Friedrich Blumenbach Institute of Zoology and Anthropology

Department of Animal Ecology

Biology Faculty

Georg August University Göttingen

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Prof. Dr. Stefan Scheu

PD Dr. Mark Maraun

1 Abstract

Relationships between organisms in the soil are very important to control the equilibrium of an ecosystem. Many factors and conditions play a crucial role for the development and functioning of decomposers and predators, i.e., plant species richness have an effect on soil biota, increasing the diversity of organisms. During recent years, fatty acid (FA) analysis has become a very useful tool to reveal food webs and feeding preferences of different organisms. The present study took place in the framework of the "Jena Experiment", located in the floodplain of the Saale River near the city of Jena, Thuringia, Germany. Animal samples were collected from 76 plots with different plant diversity levels: 1, 2, 4, 8 and 16 and different functional groups: grasses, legumes, small herbs and tall herbs. Soil animals were extracted by heat, collected in diluted glycerol and transferred into 70% ethanol for storage under room temperature. The most abundant decomposers were: *Allolobophora* chlorotica and *Aporrectodea caliginosa*, while the most abundant predators were: *Geophilus flavus*, *Lithobius microps*, *Amara aenea* and Aleocharinae beetles. Neutral lipid fatty acids (NLFAs) were used to test if plant species richness affect the presence and amount of FAs in the animals and to expose their feeding habitats and preferences in the field. Results indicated that plant species richness has no effect on FA patterns in decomposers and predators, being the presence and amount of FAs only influenced by the species itself. Although, more species and more individuals were found in plots with more plant species richness, the results were not statistically significant. The presence of bacteria, fungi and plant markers in the FA composition of the decomposers and predators suggests trophic transfer and incorporation of intact soil microbial FAs into the FA profiles of the animals, proving the effectiveness of this method not only for laboratory experiments but also for investigations in the field. The plant marker 18:1ω9 was the most abundant FA in all animals, suggesting plant and plant feeding prey species preferences.

2 Introduction

The soil system

Soil is a very interesting and heterogeneous system; where in a minimal space a high diversity of small organisms interacting each other (SCHEU & SETÄLÄ, 2002). These organisms take part in many direct and indirect relations between them, being the direct transfer of matter and energy to control the equilibrium of the system (ELTON, 1927). Soil has been and still represents an important system for investigations, due to its significant features (DORAN & ZEISS, 2000) developing productive, environmental, ecological and social roles on ecosystems (RODA *et al.,* 2003). Soil process and the organisms living in the soil need to be understood for a proper management and conservation of terrestrial ecosystems (VALLADARES, 2004a).

Role of plant diversity and plant functional groups in terrestrial ecosystems

Nowadays there is a discussion about the role and how much plant species richness and plant functional groups affect the functioning of organisms in the soil (EISENHAUER *et al.,* 2010). The loss of plant diversity has a negative impact on soil organisms, affecting their density, diversity and functioning (HOOPER *et al.,* 2005; EISENHAUER *et al.,* 2010). In contrast the increase of plant diversity has a positive effect, increasing the diversity of soil animals and other components in a soil food web (SULKAVA & HUHTA, 1998) making the soil more resistant against environmental disturbances (JEFFERY *et al.,* 2010) and pest attacks (LANDIS *et al.,* 2000).

Soil biota require nutrients and micronutrients for their metabolism. The lack of some of them can affect the correct functioning in the ecosystems (MULDER *et al.,* 2002). Although some functional groups such as legumes are good for the soil by providing nitrogen (HOOPER *et al.,* 2005; MULDER *et al.,* 2002; TEMPERTON *et al.,* 2007) they have less importance than plant species richness (EISENHAUER *et al.,* 2010, 2011).

Diversity of soil invertebrates

The biodiversity of soil invertebrates is represented by organisms classified according to their body size: Microfauna are the smallest, with dimensions < 0.1 mm. Nematodes, rotifers and protozoans belong to this group and they act as nutrients recyclers, regulating the bacteria and fungus populations. Mesofauna includes animals with dimensions between 0.1 – 2.0 mm. Collembolans, mites and enchytraeids are examples of this group. They take part as microbial populations regulators and fragmenting vegetal residues. The biggest ones are macrofauna with dimensions > 2.0 mm. In this group are included earthworms, molluscs, crustaceans, spiders and insects, developing several functions that contribute directly with the soil structure (SWIFT *et al.,* 1979). The feeding preferences for soil animals, distribute them in different groups or categories, having decomposers, herbivores and predators which interact with each other building food chains (COLEMAN, 1985).

Earthworms

Earthworms are a very important group of animals in terrestrial ecosystems (EDWARDS & BOHLEN, 1996), processing through their bodies circa 250 tonnes of soil per hectare every year (LAVELLE *et al.*, 1981). This work has a significant influence on the physical, chemical and biological soil properties, making these animals fundamental in the modification of the soil structure, accelerating the organic matter decomposition and the recycling of nutrients through their interactions with other decomposers (DOMINGEZ *et al.*, 2010).

As earthworms feed on plant tissue and organic matter in different stages of decay, living microorganisms and their dead remains are also ingested (DOUBE *et al.*, 1997). Earthworms have different feeding manners that are used to classify them in some groups: Detritivores that feed on plant litter and mammalian dung at or near the soil surface. Earthworms that feed on soil organic matter and dead roots ingested with soil are found in deeper places and are classified as geophages (LEE, 1985). There are other sub-classifications for detritivores, having epigeics, restricted to the surface, and anecics which feed preferentially on surface but they live borrowed in the soil (BOUCHE, 1977). Geophages earthworms are also sub-divided into groups such as polihumics, mesohumics and oligohumics (LAVELLE, 1981). In this study we used two geophages earthworm species: *Allolobophora* chlorotica and *Aporrectodea caliginosa.*

Centipedes

Centipedes are hunters living in soil and litter. The species are very small with just a few millimeters or they can growth up to 30 cm. As a lethal weapon to hunt they inject poison to their prey using the maxillipedes (JEFFERY *et al.*, 2010). The two centipede subgroups prevailing in Germany are Lithobiomorpha and Geophilomorpha which differ in life history, development, nutrition and microhabitat. Lithobiomorpha are sit-and-wait predators while Geophilomorpha search actively for prey in litter and soil (WOLTERS & EKSCHMITT, 1997).

Centipedes from the order Lithobiomorpha have long and strong legs used to move fast on the soil surface with a few number of body segments, while Geophilomorpha species are usually smaller and narrower with a high number of body segments. Different soil invertebrates such as: mites, nematodes, collembolans, enchytraeids, small insects, spiders and earthworms represent potential prey for centipedes (JEFFERY *et al.*, 2010).

The two centipede species used were *Lithobius microps* (Lithobiomorpha) and *Geophilus flavus* (Geophilomorpha).

(Coleoptera – Carabidae)

Carabidae is one of the most numerous and abundant families of the order Coleptera (DILLON & DILLON, 1972), being distributed all the terrestrial ecosystems, excluding the Antarctic (ERWIN, 1985). Carabids are represented in the planet with more than 30,000 species (REICHARDT, 1977). In the present study we worked with *Amara aenea* a common carabid beetle considered as an omnivorous species, feeding on pollen, fungi, and insects highlighting its preference on plant seeds (MENALLED *et al.*, 2007).

Aleocharinae (Coleoptera – Staphylinidae)

Beetles from the Aleocharinae family are the most varied group from the order Coleoptera described around the whole world. More than 1,000 described genera and more than 12,000 species are registered. Their size can vary between 2.5 to 5 mm. Although, they are considered generalist predators, hunting in the leaf litter and soil communities (ASHE, 1998), reports show them as parasites (MAUS *et al.*, 2001), mycophagous (ASHE, 1984) and also as pollinators (BERNAL & ERVIK, 1996).

Soil food web analysis

For many years researchers have worked with different techniques such as direct feeding observations, macroscopic gut dissection and examination of prey remains for the analysis of soil food webs and the study of trophic relationships in the field (SUNDERLAND, 1987). Early studies on the diet of soil invertebrates focused on food choice experiments under laboratory conditions (MARAUN *et al.*, 2003; RUESS *et al.*, 2004), further studies were based on the analysis of faeces (CHAUVAT *et al.*, 2007). Some investigations focused on the analysis of gut contents (PONGE, 2000; ADDISON *et al.*, 2003;TRAUGOTT, 2003; JUEN & TRAUGOTT, 2005; EITZINGER & TRAUGOTT, 2011). With success stable isotope analysis based mainly on carbon and nitrogen isotopes were used to reveal the transfer of matter in food webs and relationships between soil organisms (MINAGAWA & WADA 1984; NEILSON *et al.*, 1998; SCHAEFER, 2003).

Further procedures have also expanded the attention among soil biologists, using electrophoretic or serological techniques (TRAUGOTT, 2003). Quantitative polymerase chain reaction (qPCR) is also an excellent instrument to describe trophic relationships within subterranean food webs (LUNDGREN, *et al.*, 2009) and to analyze diets of fungal feeding invertebrates (REMÉN, 2010). Another approach is the use of pyrosequencing of prey DNA in faeces that is a practical and economical method to know about the feeding habits of animals (BROWN *et al.*, 2012).

In recent times, fatty acids (FAs) have been used to track certain markers from different trophic levels, revealing diets and food web links (RUESS *et al.*, 2002; 2004, 2005a; CHAMBERLAIN & BLACK, 2005; POLLIERER *et al.*, 2010; FERLIAN *et al.*, 2012).

Fatty acids as trophic biomarkers in soil food webs

The knowledge of crucial animals like decomposers and predators in the soil is key to understand the mechanisms of degradation and treatment of organic wastes based on the action of the organisms (DOMINGUEZ, 2004). To study those interactions, efficient techniques that characterize the structure and function of microbial communities are required, being FA analysis a good choice to make it possible (ZELLES, 1999).

Lipids play a crucial function in organisms providing sources of energy (neutral lipids) and making part of the structural component of cell membranes (phospholipids). Fatty acids are the predominant components of the lipids, having a mixed structure of saturated or unsaturated carbon chains (IUPAC-IUB, 1978).

Some FAs are specific for certain diets; e.g. branched chain (iso, anteiso) and cyclic FAs are specific markers for bacterial diets (WELCH, 1991; ZELLES, 1999; HAUBERT *et al.,* 2006), whereas linoleic acid (18:2ω6,9) is known as a fungi marker (FROSTERGARD & BAATH, 1996), oleic acid (18:1ω9) is known as a plant marker (RUESS *et al*., 2005).

The use of FAs was recorded many years ago to investigate the relationships between predator and prey in aquatic food webs (DESVILETTES *et al.,* 1997; LEVEILLE *et al.,* 1997). The application of this method in soil organisms revealed the trophic transfer of FAs from different levels (RUESS *et al.,* 2002; 2004, 2005a; CHAMBERLAIN & BLACK, 2005; POLLIERER *et al.,* 2010; FERLIAN *et al.,* 2012).

Aims of the study

As plant species richness affects soil biota, it was tested if FA patterns are influenced for this condition. Fatty acids can be tracked over more than one trophic level. With this approach it is possible to reconstruct trophic webs and to place different organisms living in the soil according to their feeding preferences; so FAs were used to find out the feeding habits and trophic positions of the animals in a terrestrial ecosystem. The hypotheses of the study were:

- Plant species richness influences FA patterns of decomposers and predators, since they contribute to increase the diversity of organisms and the components in soil food webs, fundamental for the equilibrium of soil process.
- Using FA analysis it is possible to reconstruct trophic webs, since they have been used as a tool to reveal feeding habits and trophic transfer from decomposers to higher levels in a soil food web.
- Plant species richness has a positive effect by increasing the diversity and number of animals in the soil.

We conducted the experiment, analyzing the macrofauna presented in 76 soil samples which are part of the Jena Experiment (Table A1), in order to find the most abundant decomposers and predators present in the samples. The animals were taxonomically identified and their FA profiles were analyzed.

3 Material & Methods

3.1 Study Area

The Jena Experiment is located in the floodplain of the Saale river near the city of Jena, Thuringia, Germany, $50^0$55´N, $11^0$35´E, 130 m a.s.l. The mean annual temperature in the area is 9.3^0 C with annual precipitations reaching 587 mm (KLUGE & MÜLLER-WESTEMEIER, 2000). This initiative is coordinated by a number fo institutions including the University of Jena and the Max-Planck-Institute for Biogeochemistry, working in conjunction to other universities and research centers in Germany and Switzerland. In this territory, 82 plots of 20 x 20 m were distributed in four blocks with equal number of plots. The Jena Experiment is considered an excellent place to settle experiments using many resources, factors, conditions and parameters in order to provide knowledge about the impact of plant diversity in many ecosystems. The high plant diversity levels in the area, ranging from monocultures to 2, 4, 8, 16 and 60 plant species make this experiment ideal to test questions related to diversity levels and plant functional groups. The functional groups include grasses, legumes, small and tall herbs. The size, distribution and characteristics of each plot, allows the establishment of several investigations, highlighting the interactions between decomposers, herbivores and predators, used to understand the functioning and the equilibrium of the ecosystems. (ROSCHER *et al.,* 2004)

Fig. 1.Aerial view of the experimental area in May 2003 to recognize the arrangement of the individual plots. Picture: Jussi Baade, Jena University.

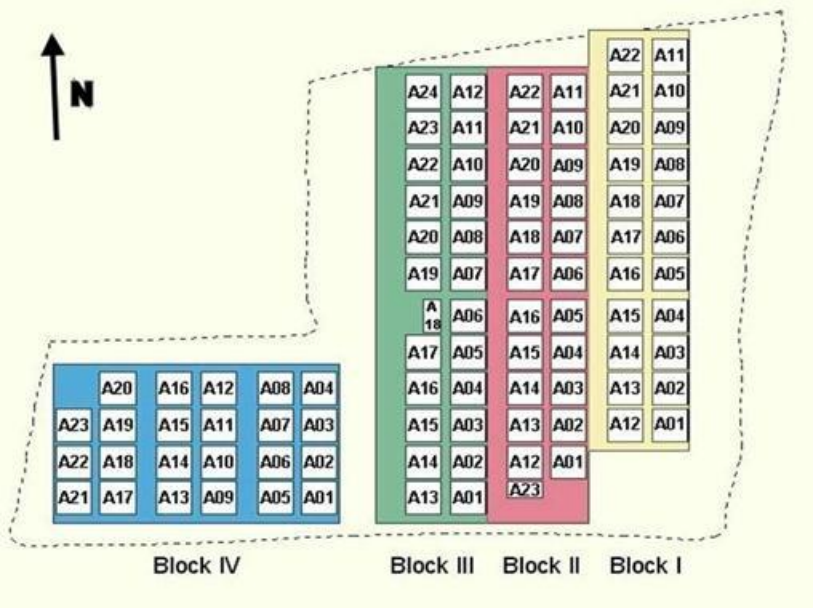

Fig. 2. Distribution of the different blocks and plots of the experimental area

3.2 Plot selection

76 samples from 76 different plots were used for our study. The criteria of selection were based in the diversity level (plant species richness) present in the plots. In this experiment there we selected the diversity levels 1, 2, 4, 8 and 16. They were considered as well four different functional groups: grasses, small herbs, tall herbs and legumes.

3.3 Experimental design

The plant functional groups grasses, small herbs, tall herbs and legumes are distributed in the plots. The presence of these functional groups is balanced, having 40 plots with grass species, 39 with small herbs, 40 with tall herbs and 39 with legumes (Table A1).

3.4 Soil sampling

There were taken soil cores of 5 cm diameter and 5 cm depth on plots with plant species richness 1, 4 and 16 (20 plots in total) in December 2011. Three samples per plot were mixed roughly, stored in a cooler and once arriving to the institute, they were stored at -20 °C.

3.5 Sampling of Macrofauna

Soil macrofauna was collected form soil cores taken to a depth of 10 cm in October 2011. Soil cores were taking using a steel corer (20 cm diameter). One core per plot was taken (80 plots), soil animals were extracted by heat (KEMPSON *et al.*, 1963), collected in diluted glycol, and transferred into 70% ethanol for storage.

3.6 Identification of the animals

For earthworms I used SIMS & GERARD (1985), SCHAEFER (1994), and BAEHRMANN (2011). Chilopods were identified with the books of SCHAEFER (1994), BAEHRMANN (2011). For beetles representing the Family Staphylinidae I used FREUDE *et al.* (1964), HARDE & SEVERA (2000) and for the family Carabidae were used HARDE & SEVERA (2000) and TRAUTNER & GEIGENMULLER (1987).

3.7 Selection of the samples for the fatty acid extraction

For the fatty acid analyses, there were chosen the most abundant decomposer and predator taxa. Animal samples were tested for their NLFA patterns and microorganisms for PLFA.

3.8 Phospholipid fatty acid extraction

To start with the extraction, we weighted the soil samples, putting 4 g of soil in each tube (50ml) then we followed the next steps:

For extraction

18.5 ml of Bligh & Dyer reagent were added to the samples and vortexed (1 x 10 ml and 1 x 8.5 ml). Then all the samples were transferred to a shaker, shaking them for two hours (vortexed after one hour shaking). The next step was to vortex the samples and centrifuge them for 10 minutes at 2500 rpmat 10 ^{0}C. After that, the supernatant was put in new 50 ml tubes, using a Pasteur pipette. At the end the residue was washed with 2.5 ml of Bligh & Dyer, vortexed, centrifuged and the supernatant was transferred like before. This procedure was repeated two times.

For phases-separation

6.0 ml chloroform and 6.0 ml citrate-buffer were added to the supernatant. The mixture was vortexed for one minute (both phases need to be mixed well and the twirl needs to reach down to the bottom) then centrifuged for ten minutes at 2500 rpm in 10 ^{0}C. The next step was to take the upper phases out using a Pasteur pipette. Then the bottom (organic) phase was taken out with a new Pasteur pipette. After that a defined volume from the bottom (organic) phase was taken (between one and three milliliters). The last step was to evaporate the organic phase by using a rotary evaporator (40 minutes at 40 ^{0}C; with a defined volume of 2 ml. The tubes had to be closed immediately.

For lipid-fractionation

Silicic acid columns were conditioned with 2 x 1 ml chloroform, and then all samples were dissolved in 300 µl chloroform separately. Samples were vortexed and put on the columns with a Pasteur pipette. Samples were washed again with 2 x 300 µl chloroform and put again on the columns. For the elution of the lipid fractions were used: 5 ml chloroform to collect the neutral lipids, 10 ml acetone for glycolipids and 5 ml methanol for phospholipids. At the end the chloroform phase (NLFAs) and the methanol phase (PLFAs) were evaporated using a rotary evaporator (90 minutes at 40 ^{0}C; chloroform phase finished after 60 minutes).

For alkaline methanolysis

30 µl of the internal standard (19:0) was added to the samples, then 1 ml methanol-toluene-solution (1:1, v/v) and vortex. After that, 1 ml 0.2 M methanolic potassium hydroxide was added and vortexed again, proceeding with the incubation in water (15 minutes at 37 ^{0}C). Next 2 ml hexane-chloroform-solution, 0.3 ml 1 M acetic acid and 2 ml deionized water were added and all vortexed. The samples had to be centrifuged for ten minutes at 2500 rpm and 10 ^{0}C. Then the upper organic phase was transferred to a new tube with a Pasteur pipette. The bottom phase was washed again with 2 ml hexane-chloroform-solution, vortexed and then centrifuged. The last step was to transfer the upper organic phase again in a new tube and to evaporate the organic phase by using a rotary evaporator (40 ^{0}C/60 minutes).

Finally, samples were put in 100 µl isooctane and vortexed three times holding the tube horizontal to get sure everything was solved. At the end FAs were put into vials by using a Pasteur pipette and the samples were stored at -20 ^{0}C until the measurement.

3.9 Neutral lipids fatty acid extraction

Before starting the extraction of the FAs for the animals selected, a length measurement was made. There was only one special step with earthworms included. A longitudinal incision was made along the body wall and the whole gut was meticulously removed from the earthworm body. The following steps were:

Extraction and fractionation

Ten samples and two blanks were extracted at the same time. Each sample was put in a 10 ml screw cap tube. Five milliliter of a single phase extraction solvent (chloroform, methanol and 0.05 M phosphate buffer at the ratio of 1:2:0.8) were added and shaken overnight. The next day the extraction solvent was transferred into new tubes, 2.5 ml new extraction solvent was added to each sample, shaken for two hours and transferred to the previous solvent. Distilled water and chloroform were added (both 0.8 ml). The mixes were centrifuged for five minutes (1500 rpm) and allowed to separate for 45 minutes. The top two phases were removed. The bottom phases were transferred to silicic acid columns after washing them with chloroform (two times 2.5 ml). With the next steps the FAs in the solutions were fractionated into NLFAs, glycolipid FAs and PLFAs. The NLFA fractions were collected by eluting them with chloroform (two times 2.5 ml) and collecting them in new tubes. Glycolipid FAs were eluted with acetone (three times 2.5 ml) and discarded. PLFAs were eluted with methanol (two times 2.5 ml) and collected in new tubes. Both NLFA and PLFA fractions were dried in a rotational vacuum concentrator (50°C).

Saponification

We added 1.0 ml of reagent 1 to the samples, and then vortexed and the tubes were put in 70 ^{0}C. After 20 minutes the samples were vortexed again and kept under 70 ^{0}C for 70 minutes. So in total it was used 70 ^{0}C for 90 minutes as it was recommended by (van Dooremalen *et al.,* 2009) then the samples were cooled for 40 seconds using ice.

Methylation

2.0 ml of reagent 2 was added to the samples and then vortex and put in 80 ^{0}C for 10 minutes. After 10 minutes the samples were cooled immediately for 40 seconds with ice.

Extraction

1.25 ml of reagent 3 was added and then, the samples were rocked for 10 minutes. Then the samples were centrifuged for 5 minutes at 1500 rpm in 4 ^{0}C**.** The next step was to remove the bottom phase, saving the top phase.

Wash

3.0 ml of reagent 4 was added and then, the samples were rocked for 5 minutes, following the centrifugation for 5 minutes at 1500 rpm in 4 ^{0}C. The last step was to remove the top phase and to transfer the fatty acids to a gas chromatography (GC) vial. Finally the vials were caped and stored at -20 ^{0}C until analysis.

3.10 Quantification and identification of fatty acids

Fatty acid methyl esters (FAMEs) were analyzed by gas chromatography (GC) using a Perkin Elmer CLARUS 500 GC with a flame ionization detector, equipped with a PE-5 capillary column (30 m x 0.32 mm i.d., film thickness 0.25 µm). The temperature program started with 60 ^{0}C (held for 1 minute) and increased by 30 ^{0}C per minute to 160 ^{0}C followed by 3 ^{0}C per minute to 260 ^{0}C. The injection temperature was 250 ^{0}C and helium was used as carrier gas. FAMEs were identified by chromatographic retention time comparison with a standard mixture composed of 37 different FAMEs ranging from C11 to C24 (Sigma-Aldrich, St Louis, USA). Modern gas chromatographic (GC) methods facilitate the separation of individual fatty acids, and with the aid of commercially available standards about 40 fatty acids can by identified by retention time and mass spectra (BRONDZ, 2002).

3.11 Statistical analysis

All percentage values of FAs were arcsine-square root transformed prior to analysis to achieve normal distribution of the data. For the analysis of the FA profiles via discriminant function analysis (DFA) in each experiment the dimensions of the data set were reduced using nonmetric multidimensional scaling (NMDS). For testing differences between the individual FAs in each experiment multivariate analysis of variance (MANOVA) was used. All statistical analyses were conducted using STATISTICA 7.1 for Windows (StatSoftInc, Tulsa, OK, USA).

4 Results

4.1 Influence of plant species richness on the number of individuals and species of decomposers and predators

The number of individuals and species of decomposers and predators was not influenced by plant diversity (ANOVA $F_{4,71}$ = 1.83, p> 0.05, $F_{4,71}$ = 2.37, p> 0.05), finding no significant difference between all treatments (Tukey´s Honestly Significant Difference test; p> 0.05; Table 1 and 2).

Table 1.P-values of Tukey´s Honestly Significant Difference Test; p < 0.05, comparing the plant diversity levels against each other in relation to the number of individuals.

Plant diversity level	1	2	4	8	16
1	1.00	0.85	0.63	0.17	0.17
2	0.85	1.00	0.99	0.71	0.71
4	0.63	0.99	1.00	0.91	0.91
8	0.17	0.71	0.91	1.00	1.00
16	0.17	0.71	0.91	1.00	1.00

Table 2.P-values of the Tukey´s Honestly Significant Difference Test; p < 0.05, comparing the plant diversity levels against each other in relation to the number of species.

Plant diversity level	1	2	4	8	16
1	1.00	0.97	0.36	0.28	0.08
2	0.97	1.00	0.73	0.64	0.28
4	0.36	0.73	1.00	1.00	0.94
8	0.28	0.64	1.00	1.00	0.97
16	0.08	0.28	0.94	0.97	1.00

4.2 Principal components analysis (PCA) of the main decomposers and predators

The PCA analysis showed the position of the different groups according to their fatty acid composition (Fig. 3). The decomposer group (*Aporrectodea caliginosa* and *Allolobophora chlorotica*) were positioned close to each other, having in their fatty acid composition most of the bacteria markers. The predator group (*Geophilus flavus, Lithobius microps* and Alleocharinae beetles) were close positioned, showing the plant marker 18:w9c as the common fatty acid in the group. High values in the ratio of the animals were found (see Table 3).

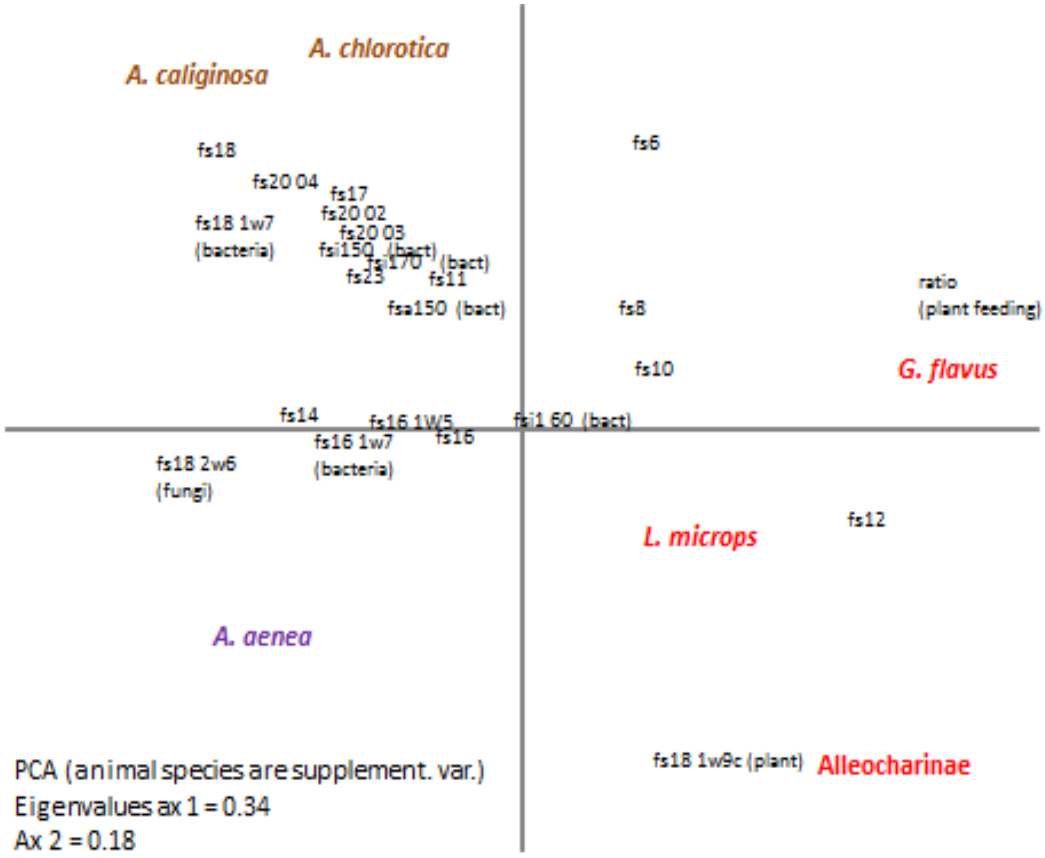

Fig. 3. PCA analysis of decomposers (*Allolobophora chlorotica, Aporrectodea caliginosa*) and predators (*Geophilus flavus, Lithobius microps, Amara aenea* and Aleocharinae).

Table 3. Independent factors of the multivariate analysis of variance (MANOVA) of the experiment. (Sowndiv: Plant species richness, Funcgr: Functional groups, Leg: legumes, Grass: grasses, Sherb: small herbs, Therb: tall herbs, 0: absence, 1: presence).

species	ratio
Allolobophora chlorotica	3.05
Aporrectodea caliginosa	2.17
Geophilus flavus	4.86
Lithobius microps	4.08
Aleocharinae	7.31
Amara aenea	1.61

4.3 Multivariate analysis of variance (MANOVA) of the decomposers and predators

We tested the effect of animal species, plant species richness (one, two, four, eight and sixteen plant species) and plant functional groups (legumes, grasses, small herbs and tall herbs) in the fatty acid composition of decomposers and predators (Table 4).

Table 4. Independent factors of the multivariate analysis of variance (MANOVA) of the experiment. (Sowndiv: Plant species richness, Funcgr: Functional groups, Leg: legumes, Grass: grasses, Sherb: small herbs, Therb: tall herbs, 0: absence, 1: presence).

Class	Levels	Values
Species	6	*A. chlorotica, A. caliginosa, G. flavus, L. microps*, *A. aenea,* Alleocharinae
Sowndiv	5	1, 2, 4, 8, 16
Funcgr	4	1, 2, 3, 4
Leg	2	0, 1
Grass	2	0, 1
Sherb	2	0, 1
Therb	2	0, 1

Before starting the test, the data were log transformed and reduced to two axes. MANOVA showed that the models were statistically significant ($p< 0.05$). The fatty acid compositions of the animals differed highly significant ($p< 0.001$) just for animal species. Factors like plant species richness (Sowdiv) and plant functional groups (Funcgr) were not significant collectively and/or individually in the presence and amount of the fatty acids composition of decomposers and predators ($p < 0.05$; Table 5).

Table 5. P-values of the two models for decomposers and predators. (level of significance: * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Axis 1	***p***	**Axis 2**	***p***
Model	0.0054 **	Model	0.0009 ***
Species	0.0004 ***	Species	< 0.0001***
Sowdiv	0.4926	Sowdiv	0.9812
Funcgr	0.3004	Funcgr	0.0926
Leg	0.3381	Leg	0.9729
Grass	0.3053	Grass	0.8863
Sherb	0.4343	Sherb	0.3526
Therb	0.4541	Therb	0.3702

Analyzing the fatty acids (FA) individually, we observed that most of them were statistically significant, supporting the general results of the model. Only the FAs: 8:0, 11:0, i16:0 and 16:0 were not significant. All bacteria markers with exception of i16:0 were significant. The fungi marker 18:2w6 and the plant marker 18:1w9c were highly significant (Table 6).

Table 6.F-values (F) and p-values (p) of the NLFAs of *Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Geophilus flavus*, *Lithobius microps*, *Amara aenea* and Aleocharinae.(Level of significance $\alpha = 0.05$).

		A. chlorotica	*A. caliginosa*	*G. flavus*	*L. microps*	*Aleocharinae*	*A. aenae*	
NLFA	**Marker FA**	***F***	***F***	***F***	***F***	***F***	***F***	***p***
08:00		1.35	2.97	2.33	5.03	3.3	0.62	0.1171
10:00		1.26	1.08	1.07	1.18	1.29	0.11	<.0001 ***
11:00		0.19	0.12	0.02	0.07	0	0	0.0925
12:00		1.21	3.34	8.12	5.05	12.37	0.42	<.0001 ***
14:00		0.46	0.73	1.03	2.34	0.71	1.11	<.0001 ***
i15:0	Bacteria	1.13	0.97	0.1	0	0	0	<.0001 ***
a15:0	Bacteria	0.09	0.05	0.1	0	0	0	0.0160 *
i16:0	Bacteria	0.32	0.39	20.54	0	0	0	0.5330
16:1w7	Bacteria	0.68	0.45	1.84	0.66	0.41	7.39	<.0001 ***
161:W5		0.07	0.59	0.05	0	0	1.7	<.0001 ***
16:00		17.21	14.94	0	18.04	18.8	12.73	0.2482
i17;0	Bacteria	0.3	0.39	0	0	0	0	0.0012 **
17:00		1.53	1.95	0.51	0	0	0	<.0001 ***
18:2w6	Fungi	5.05	8.26	4.97	5.43	2.41	22.54	<.0001 ***
18:1w9c	Plant	37.44	34.17	52.54	54.79	55.92	50.65	<.0001 ***
18:1w7	Bacteria	9.57	12.86	6.3	4.13	6.29	1.12	<.0001 ***
18:00		10.56	11.47	3.81	1.97	1.37	1.83	<.0001 ***
20:04		3.8	7.03	1.39	0	0	0.29	<.0001 ***
20:03		0.23	0.92	0	0	0	0	<.0001 ***
20:02		2.21	3.46	1.08	1.41	1.69	0	<.0001 ***
23:00		2.44	0.39	0.23	0	0	0	<.0001 ***

4.4 NLFAs of decomposers and predators

The FA composition differed significantly between each species (DFA after NMDS; reduced to two dimensions). The position of the different taxa according to their fatty acid composition placed the decomposers *Allolobophora chlorotica* and *Aporrectodea caliginosa* close to each other. *Geophilus flavus, Lithobius microps* and the Alleocharinae beetles were close related, representing the predator group. *Amara aeneae* took an intermediate position (Figure 4).

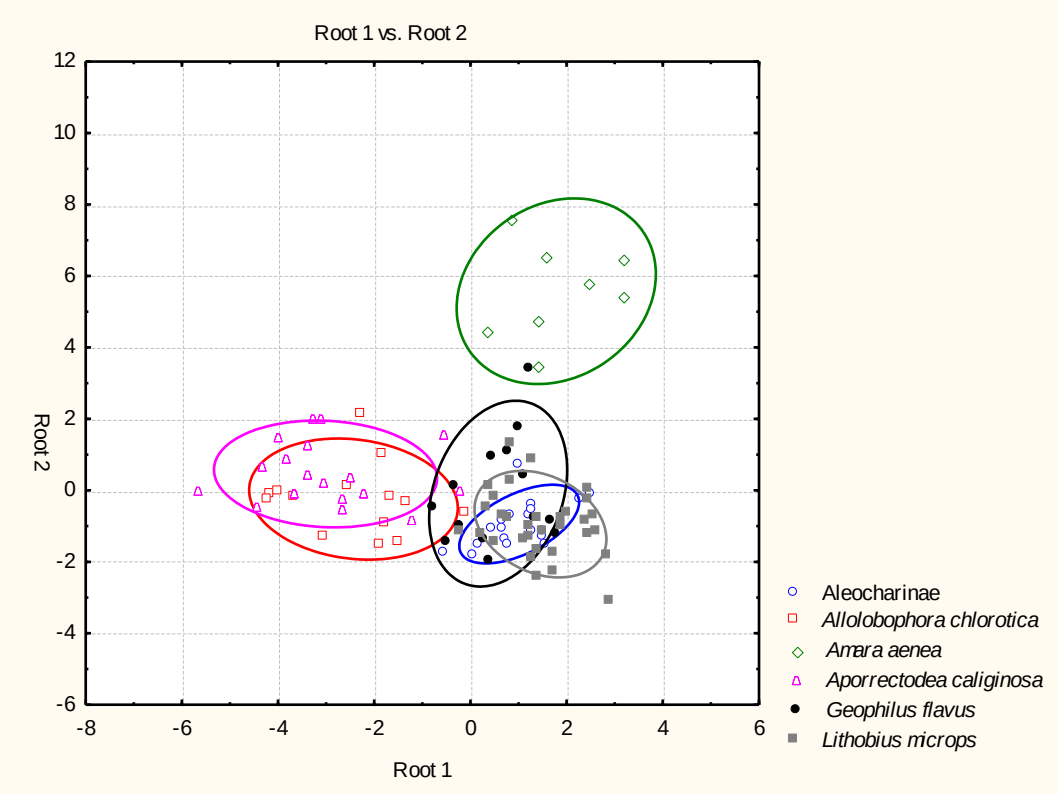

Fig. 4. Discriminant function analysis of NLFAs of *Allolobophor achlorotica, Aporrectodea caliginosa, Geophilus flavus, Lithobius microps, Amara aenea* and Aleocharinae. Ellipses represent confidence ranges p = 0.08

The most abundant FA was the plant marker 18:1ω9. In the predators, we observed the absence of the bacterial marker i17:0. The bacterial markers i15:0 and a15:0 were present in low proportions in *G. flavus* but completely absent in the other predators. The most abundant bacterial marker present in predators was 18:1ω7. It was noticeable the higher amount of bacterial markers in the decomposers compared with the predators. The fungi marker 18:2ω6 was present in all the taxa but in consider higher proportions in *Amara aenea*. The C20 PUFAs were present in small amounts (Fig. 5).

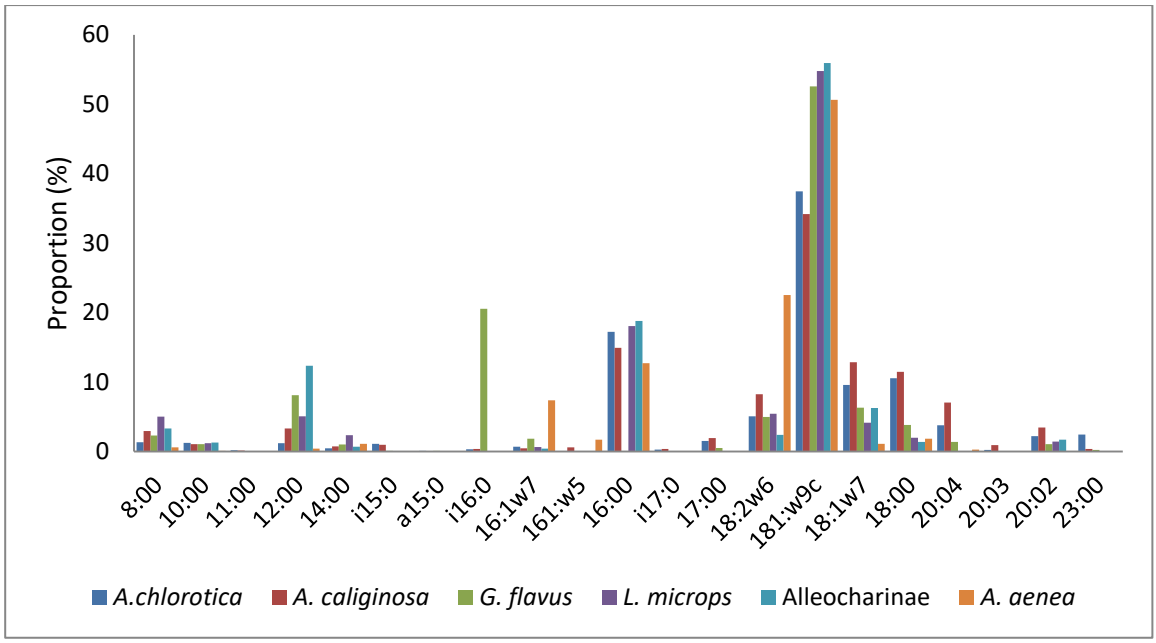

Fig. 5. Concentrations (percentages of total ± SD; note log scale) of NLFAs comparing the decomposers and predators: *Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Geophilus flavus*, *Lithobius microps*, *Amara aenea* and Aleocharinae.

Allolobophora chlorotica

The most abundant FA was the plant marker 18:1ω9c (37.44%). The fungi marker contributed with 5.05 %. Total bacteria marker was 12.09 % and C20 PUFAs were registered with 8.68 % of the total FA composition. The remaining composition is represented by other FAs.

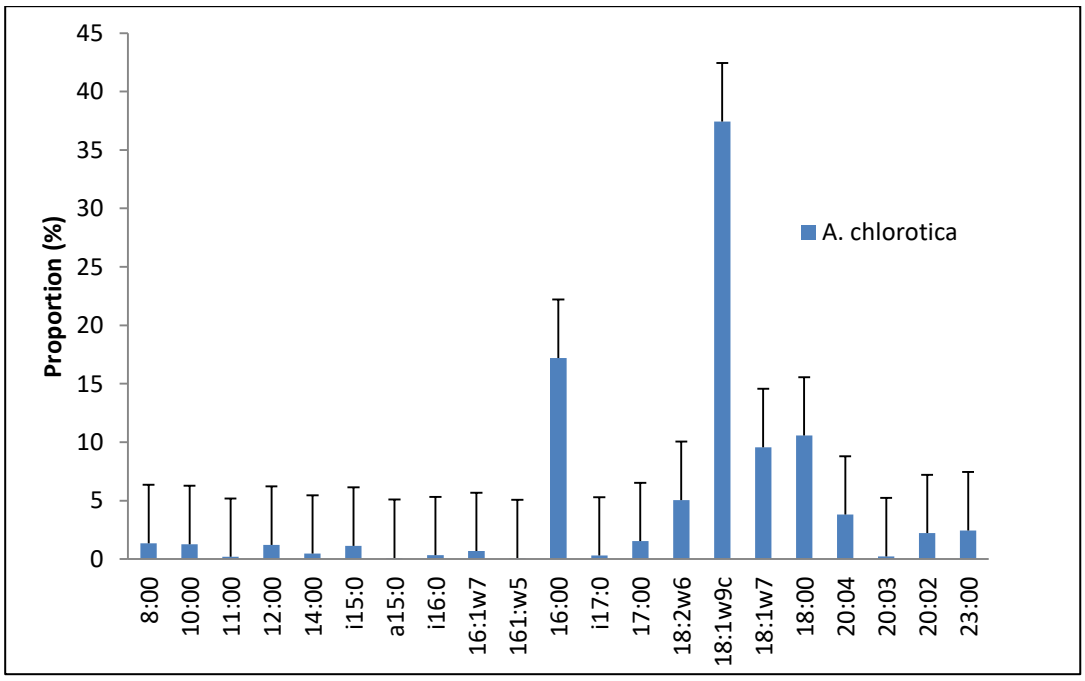

Fig. 6. Concentrations of NLFAs of *Allolobophora chlorotica*. Error bars with Standard error is shown

Aporrectodea caliginosa

The most abundant FA registered was the plant marker 18:1ω9c (34.17%). The total amount of bacteria markers contributed with 15.11%, belonging 12.86% to the FA 18:1ω7. A considerable amount of the fungi marker 18:2ω6 was reported with 8.26%. C20 PUFAs contributed with 11.8% of the total amount of FAs.

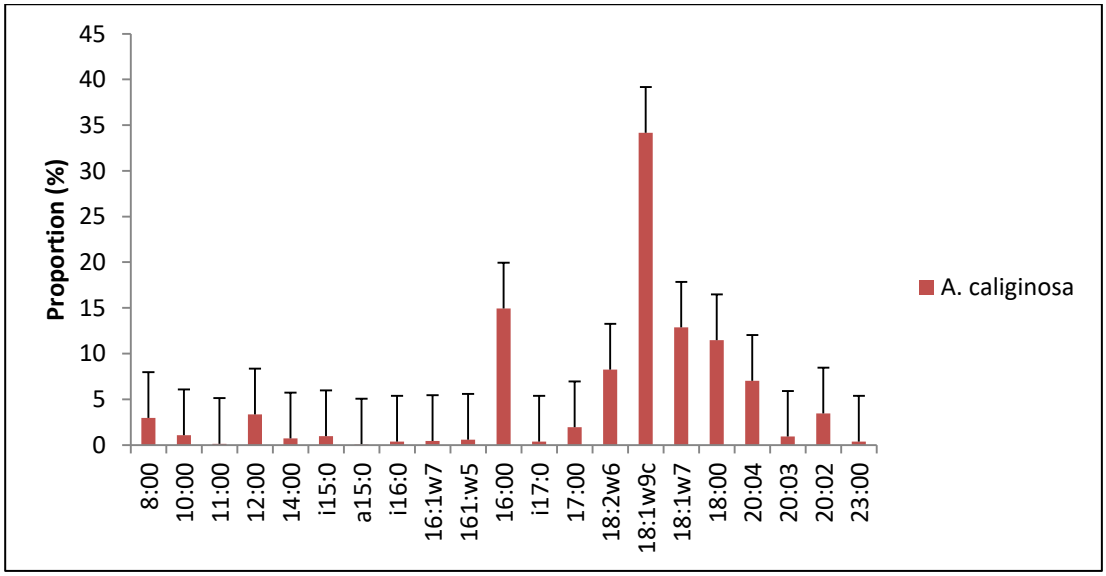

Fig. 7. Concentrations of NLFAs of *Aporrectodea caliginosa*. Error bars with Standard error is shown.

Geophilus flavus

The most abundant FA was 18:1ω9 (52.54%). Bacterial marker contributed with 29.39%, belonging 20.54% to the FA i16:0. The fungi marker 18:2ω6 was presented with ca. 5% of the total FA composition. C20 PUFAs, contributed with just 2.7%.

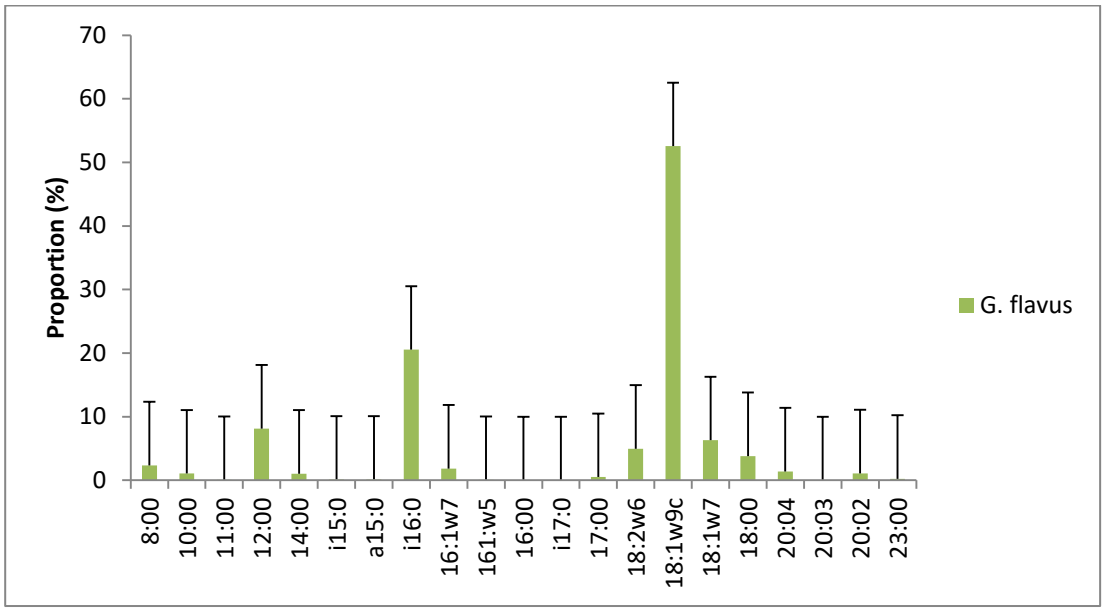

Fig. 8. Concentrations of NLFAs of *Geophilus flavus*.Error bars with Standard error is shown

Lithobius microps

The most abundant FA was 18:1ω9c (54.79%). The total amount of the bacteria marker was 4.79%, belonging 4.13% to the FA 18:1w7. The fungi marker 18:2ω6 contributed with 5.43% and the C20 PUFAs with just 1.41% of the total amount of FAs presented in this animal.

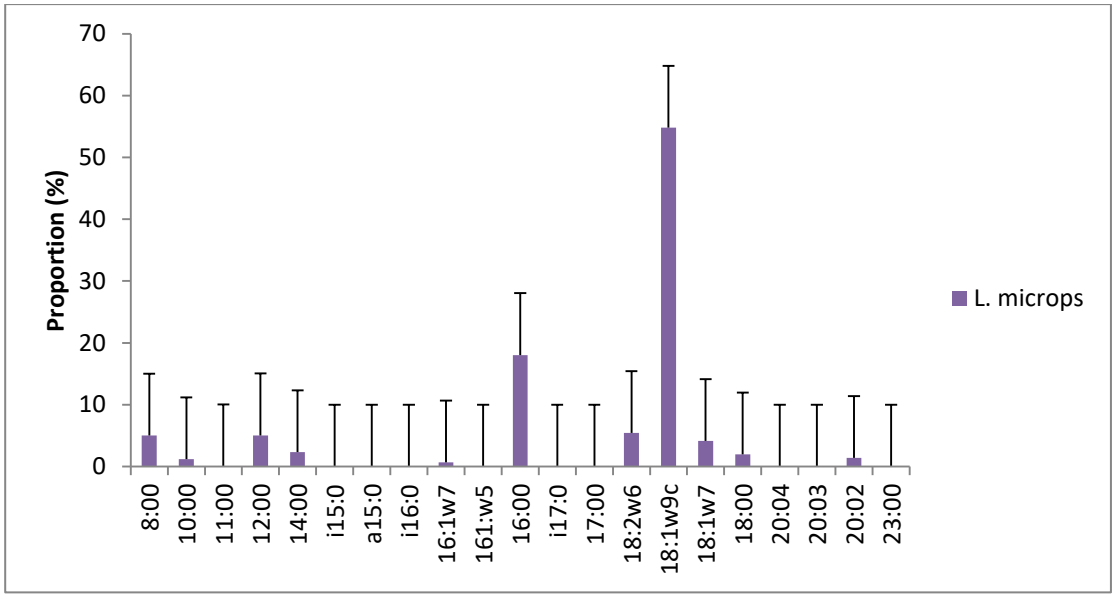

Fig. 9. Concentrations of NLFAs of *Lithobius microps*. Error bars with Standard error is shown

Amara aenea

The most abundant FA was 18:1ω9c with 50.65%. The total amount of bacteria markers was 8.51%, belonging 7.39% to the FA 16:1ω7. The fungi marker 18:2ω6 was registered in high concentrations as well with 22.54%. Total C20 PUFAs have just contributed with 0.29% of the total FA amount for this beetle.

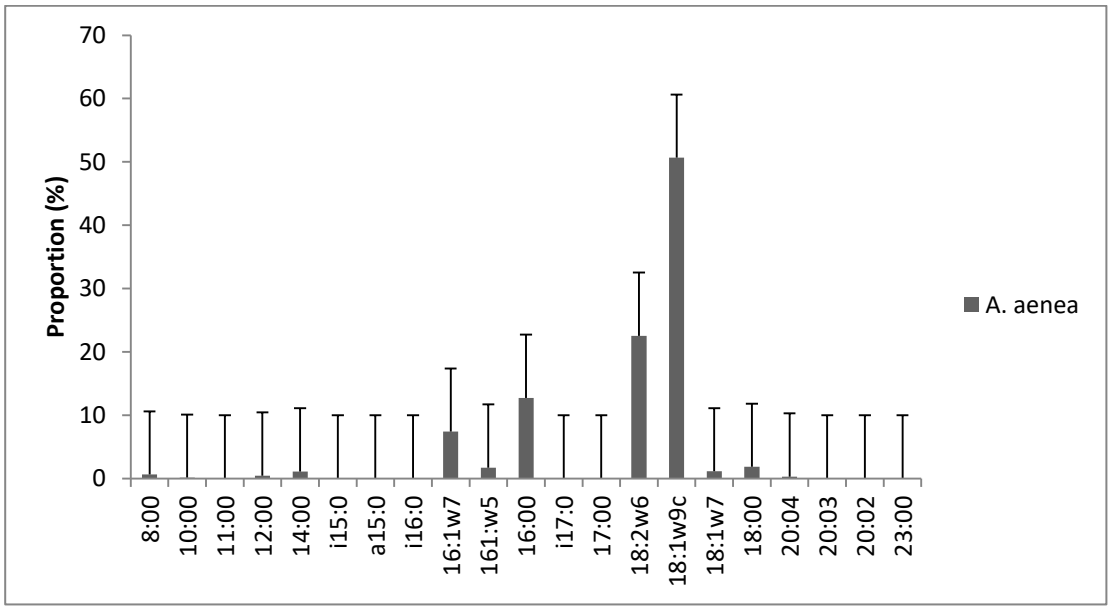

Fig. 10. Concentrations of NLFAs of *Amara aenae.* Error bars with Standard error is shown

Aleocharinae

The most abundant FA was 18:1ω9c with 55.92%. The bacteria marker just contributed with 6.7% of the total FA amount, having in 18:1ω7 6.29%. The fungi marker 18:2ω6 was registered in low concentrations (2.41%) and the total C20 PUFAs was 1.69%.

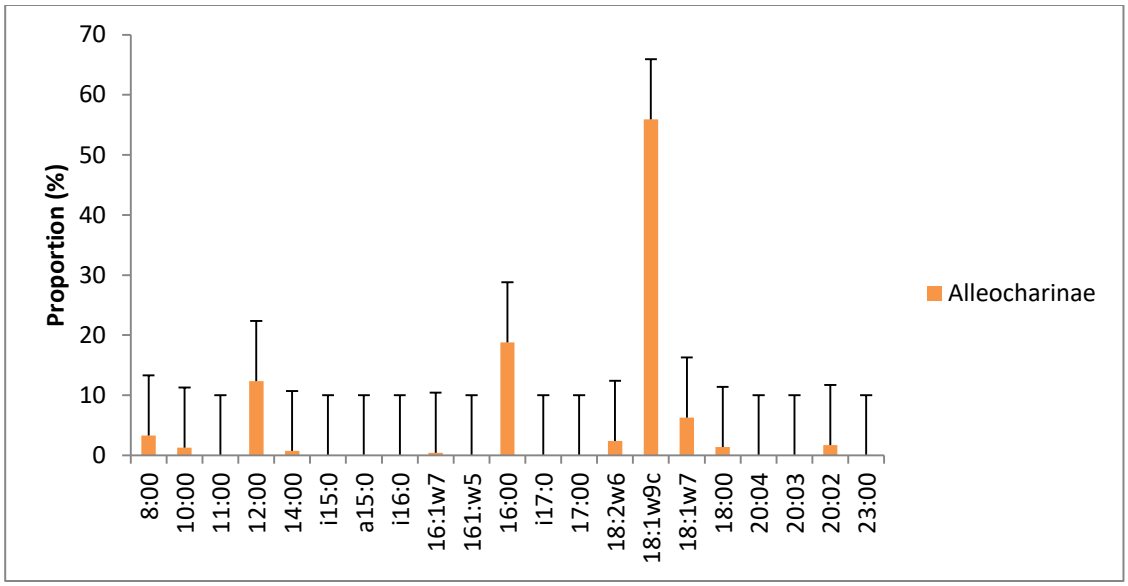

Fig. 11. Concentrations of NLFAs of Aleocharinae. Error bars with Standard error is shown

5 Discussion

5.1 Fatty acid patterns related to plant species richness

Our results show that plant species richness has no effect on the fatty acid composition of decomposers or predators. The composition and amount of fatty acids only depends of each species.

Although we found less individuals and less species in monocultures, having an increase of both factors while the number of plant species increase, the results were not statistically significant, rejecting the hypothesis that the number of individuals and species is higher in plots with more plant species. This result is consistent with HEDLUND *et al.* (2003) who did not find any significant difference between plant species richness and abundance of soil organisms. In contrast, HADDAD *et al.* (2009) found that plant species richness has highly significant overall effects on the abundances of other organisms and on trophic interactions. Supporting those findings BENSON & HARADA (1988) and FERREIRA & MARQUEZ (1998) stated that plant species richness propitiates a very well structured litter, keeping the availability of ecological niches for many species, offering many food sources and increasing the diversity of animals in the soil.

5.2 NLFAs of decomposers and predators

The low concentration of FAs registered in the animals may be explained due to way and time of sample storage. We used 70% ethanol under room temperature for a period superior to four months, so many of the fatty acids and part of the amount may have been lost during that time. This can be support by VINK *et al.*, (2005) who observed that DNA degradation occurs in tissue stored in ethanol at room temperature over six weeks. In addition, POST *et al.* (1993), RIESS *et al.* (1995) and DILLON *et al.* (1996) recommend preserving invertebrates at -80^0C to avoid the loss and degradation of molecular contents.

In the present study, we found twenty-two fatty acids, ranging from 6:00 to 23:00, finding different bacterial markers, highlighting the 18:1ω7 as the most abundant. The fungi and plant markers 18:2ω6 and 18:1ω9 respectively were identified, being 18:1ω9 the most abundant FA detected in all taxa. The ratio of 18:1ω9 to 18:2ω6, used for distinguishing between plant feeders and fungal feeders (RUESS *et al.*, 2007) was high, suggesting that the animals feed more on plants or on plant feeding prey species.

Since the diet of earthworms consists mainly of dead plant material (HANSEN and CZOCHANSKA, 1975), the results support the findings of DUNGAIT *et al.* (2008) that FA 18:1ω9 as the most abundant in *Allolobophora chorotica* and fungi and bacteria markers are registered in low concentrations. Indeed, *Allolobophora chlorotica* and *Aporrectodea caliginosa* are root feeders (BOUCHE & KRETZSCHMAR, 1974), ingesting bacterial colonies while feeding on dung organic matter (HANSEN and CZOCHANSKA, 1975; LATTAUD *et al.*, 1998; DUNGAIT *et al.*, 2008). The presence of the FA 18:2ω6 cannot be used to demonstrate that earthworms feed on fungi because this FA is abundant in the tissues of soil microfauna and may have been assimilated in the earthworm gut from ingested soil microfauna such as nematodes. In addition, it has been shown that this FA can be synthesized by several insects (STANLEY-SAMUELSON *et al.*, 1988; CANAVOSI *et al.*, 2001).

In *Lithobius microps*, we did not find the bacterial markers i15:0 and 15:0. This result is similar to the one found by POLLIERER *et al.* (2010), who fed Collembola species with bacteria, registering very low concentrations of these FA fewer than 1%, pointing out these FAs as very difficult to transfer from one level to another. In contrast, the bacterial marker 18:1ω7 was found in concentrations higher than 4%, results similar to FERLIAN *et al.* (2012), who also found in *Lithobius* species, values higher than 4%, suggesting these FA as a good one to trace bacteria based food chains and energy channels in the field.

The high ratio between 18:1ω9 to 18:2ω6 and the highest concentration registered in the plant marker 18:1ω9, suggest that *Lithobius microps* feeds on plant or on plant feeding prey. This result can be explained as lithobiids hunt in the litter and upper soil layers (POSER, 1990) where potential prey for this predator such as enchytraeids that feed on plant material (BONKOWSKI *et al.*, 2000; BRIONES *et al.*, 2005) and collembolans that feed mainly on fungi (MARAUN *et al.*, 2011; POLLIERER *et al.*, 2010), but can feed as well on leaves (POLLIERER *et al.*, 2010). In addition, centipedes are generalist predators whuch when starving may feed on leaf litter (JEFFERY *et al.*, 2010).

The high concentrations of plant and fungal markers and low concentrations of bacterial markers in *Lithobius microps* were similar to FERLIAN *et al.*, (2012), suggesting that bacterial feeding prey species are of minor importance for lithobiids.

In this study the high ratio and high concentration of the plant marker 18:1ω9 and low concentrations of fungi and bacterial markers, suggest that the diet of *G. flavus* comprises mainly on prey that feed on plant with a small consumption of fungi and bacteria. This result can be support by BOUCHÉ, (1997) who proposed that Geophilomorpha species feed on lumbricids and enchytraeids, which consume plant detritus, incorporating high amounts of plant marker FAs that can be transferred to one trophic level to another. A contrasting result were found by FERLIAN *et al.*, (2012), who suggest that *Strigamia acuminate* (Geophilomorpha) feed on bacterial and fungi feeding prey species and *Geophilus ribauti* feed on decomposers consuming mainly plant litter colonized by fungi.

Since *Amara aenea*, consumes pollen, fungi, insects and mainly plant seeds (MENALLED *et al.* 2007), our results confirm the feeding habits of this species, showing the presence of two bacterial markers: 16:1ω7 and 18:1ω7, high proportions of the fungi marker 18:2ω6 and the highest amount of the plant marker 18:1ω9. In addition, the ratio between 18:1ω9 and 18:2ω6 suggest the preference of this beetle for plant or plant feeding prey.

In Aleocharinae we found high concentration of the fungi marker 18:2ω6 but highest concentration on the plant marker 18:1ω9 with a high ratio between 18:1ω9 and 18:2ω6, suggesting the consumption of fungal feeding prey species but highlighting the preference for plant feeding prey species. This result can be explained since these beetles are mainly generalist predators in leaf litter and soil communities (ASHE, 1998); where fungal feeding prey species are abundant (BERG & BENGTSSON, 2007). In addition, numerous Aleocharinae groups can feed directly on fungi (ASHE, 1992) and others on pollen (BERNAL & ERVINK, 1996).

5.3 Conclusions

Results of this study indicated that plant species richness has no effect on FA patterns in decomposers and predators. The presence of bacteria, fungi and plant markers in the FA composition of the animals show trophic transfer from one level to the other and provide hints on the feeding habits of the animals and their trophic position within the soil. The profile of the NLFAs of the animals comprised mainly the plant marker 18:1ω9 with little concentration of other FAs, suggesting that decomposers and predators feed mainly on plant and/or on plant feeding prey species, with low incorporation of fungi and bacteria. Interestingly, plant species richness does not increase the number of individuals and species in the soil samples; however, more studies are needed taking into consideration the storage methods and time of FAs extraction from animals.

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8 Appendix

Table A1: Selected plots with different diversity levels (sowndiv) and functional groups (funcgr). Legumes (leg), grasses (grass), small herbs (sherb) and tall herbs (therb).

plotcode	sowndiv	funcgr	leg	grass	sherb	Therb
B4A09	1	1	1	0	0	0
B2A05	1	1	0	1	0	0
B3A06	1	1	0	1	0	0
B1A18	1	1	0	0	1	0
B2A04	1	1	0	0	0	1
B4A13	1	1	0	0	0	1
B1A08	1	1	1	0	0	0
B2A15	1	1	1	0	0	0
B4A12	1	1	0	1	0	0
B2A13	1	1	0	0	1	0
B3A17	1	1	0	0	1	0
B1A15	1	1	0	0	0	1
B3A01	1	1	0	0	0	1
B1A05	2	1	1	0	0	0
B1A07	2	1	0	0	0	1
B1A16	2	2	0	1	1	0
B1A17	2	2	0	1	0	1
B2A02	2	1	0	1	0	0
B2A08	2	2	1	0	0	1
B2A19	2	1	0	0	1	0
B2A20	2	2	1	0	1	0
B3A02	2	2	0	1	0	1
B3A08	2	1	0	1	0	0
B3A19	2	2	0	1	1	0
B3A21	2	1	1	0	0	0
B4A14	2	1	0	0	1	0
B4A15	2	2	1	0	1	0
B4A17	2	1	0	0	0	1
B4A21	2	2	1	0	0	1
B1A13	4	1	1	0	0	0
B3A13	4	1	0	1	0	0
B2A06	4	2	1	0	1	0
B4A07	4	2	1	0	0	1
B4A11	4	3	1	1	0	1
B1A19	4	3	0	1	1	1
B3A23	4	4	1	1	1	1
B2A09	4	1	0	0	1	0

B3A11	4	2	0	1	1	0
B3A03	4	3	1	1	1	0
B4A22	4	1	0	0	0	1
B1A21	4	2	0	1	0	1
B2A16	4	3	1	0	1	1
B1A04	4	4	1	1	1	1
B2A01	4	4	1	1	1	1
B4A04	4	4	1	1	1	1
B1A02	8	2	0	1	0	1
B1A03	8	3	1	1	1	0
B1A12	8	1	1	0	0	0
B1A14	8	4	1	1	1	1
B2A12	8	1	0	0	0	1
B2A14	8	4	1	1	1	1
B2A17	8	2	1	0	1	0
B2A21	8	3	1	0	1	1
B3A04	8	1	0	1	0	0
B3A05	8	3	1	1	0	1
B3A07	8	4	1	1	1	1
B3A20	8	2	1	0	0	1
B4A06	8	1	0	0	1	0
B4A08	8	2	0	1	1	0
B4A10	8	3	0	1	1	1
B4A16	8	4	1	1	1	1
B3A09	16	1	0	1	0	0
B4A20	16	2	1	0	0	1
B1A06	16	2	0	1	0	1
B2A22	16	3	1	1	0	1
B1A20	16	3	1	0	1	1
B2A18	16	4	1	1	1	1
B3A22	16	4	1	1	1	1
B3A16	16	2	1	0	1	0
B2A10	16	2	0	1	1	0
B3A24	16	3	1	1	1	0
B1A11	16	1	0	0	0	1
B4A02	16	3	0	1	1	1
B1A01	16	4	1	1	1	1
B3A22	16	4	1	1	1	1
B4A18	16	4	1	1	1	1

Table A1 Part 2: Selected plots with different diversity levels (sowndiv) and functional groups (funcgr). Legumes (leg), grasses (grass), small herbs (sherb) and tall herbs (therb).