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Effect of multitrophic interactions in soil on the carbon and nutrient exchange between plants and the rhizosphere microbiome

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1 Introduction

Soils are central to nutrient recycling in terrestrial ecosystems. Organic matter accumulates in soils, forming large amounts of varied organic compounds which need to be decomposed to free the nutrients it contains (Sollins and Gregg, 2017). This is done by the diverse community of both macro- and microorganisms that populate soils. They can decompose this organic matter, breaking down the complex and varied molecules and releasing mineralized nutrients that can then be absorbed by plants (Bonkowski et al., 2001). As we investigated the impact of soil organisms and their interactions on the nitrogen and carbon cycles in soils, we created mesocosms where their effects could be studied, focusing on plants and their mycorrhizal partners as well as rhizosphere bacteria and their protozoan predators, four central actors in soil ecosystems and nutrient cycles (Bonkowski, 2004).

1.1 Plant productivity and growth strategy

The functions of the different organisms in soil ecosystem results from their resources availability and needs: typically, the development of soil organisms is limited by the availability of assimilable energy, in the form of reduced carbon (Clarholm, 1989). On the other hand, plants use photosynthesis to reduce atmospheric carbon and are therefore not limited by this element, but are limited by resources such as water, nitrogen or phosphorus. This pattern shapes how these organisms interact, as well as the productivity of the ecosystem as a whole, as plants are dependent on the intake of nutrients released by soil organic matter decomposition for their development and to be able to fix of more atmospheric carbon (Steinauer et al., 2015, Kaštovská et al., 2015). As with any other terrestrial ecosystem, plants are the central producer, with all other organisms relying on plant-derived carbon for their development (Bonkowski, 2004).

Interestingly, the plant-derived carbon feeding soil organisms does not only originate from litterfall but comes in part from root exudates which are consumed by rhizosphere microorganisms with no direct benefit for the plant. Through these exudates alone, plant-derived carbon input to soil adds up to an important fraction of the total carbon fixed by photosynthesis: from 5 to 20% in most cases (Whipps and Lynch, 1983, Vives-Peris et al., 2020), with up to 40% reported (Kumar et al., 2006). The investment of such

an important fraction of their production in rhizosphere microorganisms suggests that plants receive a significant benefit in return. In typically carbon limited soil (Clarholm, 1989), this significant input from plant root promotes microbial activity, which in turn increase the decomposition of organic matter and nutrient mobilization (Kuzyakov and Domanski, 2000).

Missing from most of the available data are tests on the impact of the carbon flow itself on the nutrient uptake by plants (Chowdhury et al., 2022). As plants exchange carbon for soil nutrients, it is generally assumed that the flow of carbon and that of other nutrients will evolve proportionally (Koller et al., 2013). Contrary to soil nutrients that are taken up by roots, the carbon flow going to rhizosphere organisms has been shown to be mostly source-regulated rather than consumer-regulated (Wamberg et al., 2003). In that context, an important determinant of carbon flow is the plant strategy in its carbon allocation, whereas the presence of certain microorganisms can only have a localized impact on the amount of carbon exudated by roots (Vives-Peris et al., 2020).

Plants have indeed been shown to limit their growth below what available resources allow (Parsons et al., 2013), with potential for additional nutrients use and plant productivity under different plant growth strategy (Chen et al., 2016, Williams and de Vries, 2020). Here, the plant growth strategy was used to create productivity treatment, by planting either a plant species considered fast-growing and resource exploitative or another more conservative in its growth strategy (Lambers and Poorter, 1992). To have two plant species that are both significantly different in their strategies but still comparable in all other aspects, we used the closely related *Plantago lanceolata* as the fast-growing, exploitative species, and *Plantago media* as the slower-growing, more conservative species. *Plantago* species are fast-growing generalists that could tolerate well the growing conditions we provided and associate well with myccorhiza (Thakur et al., 2019). As non-legume plants, they can not use interaction with nitrogen-fixing bacteria and have to source their nitrogen from the soil, and we expect this intake to be affected by our other treatments.

Once growing, the more exploitative *P.lanceolata* is expected to exude a higher quantity of organic compounds to accelerate the increase in its nutrient intake (Henneron et al., 2020). In our mesocosms, we would see a faster flow of both carbon and nitro-

gen as the plants productivity increased, and therefore a significant difference between *P.lanceolata* and *P.media* (Chen et al., 2016, Williams and de Vries, 2020).

1.2 Rhizosphere bacteria

The interaction between plant roots and the rhizosphere community and its potential benefits for the plant do not stop at nutrient exchanges. Rhizosphere bacteria have been found to promote plant growth through an array of mechanisms. One aspect is the capacity of different bacteria taxa to either directly produce auxins, cytokinins, ethylene, and other phytohormones or indirectly influence the production by plant cells of these hormones (Kudoyarova et al., 2019), resulting in promoting root growth or modification of the root architecture, notably by promoting the development of lateral roots (Kurepin et al., 2014) and the production of root exudates (Oppenheimer-Shaanan et al., 2022), as well as the adaptation of the root system to their environment, typically by shaping the development of different plant tissues in response to abiotic stress such as drought or high concentration of salts or heavy metals (Egamberdieva et al., 2017). Similar shifts in root architecture can be attributed to the local availability of nutrients, particularly ammonia, which in turn can also be affected by microbial activity leading to the release of more nutrient (Mantelin and Touraine, 2004).

These interactions between plants and bacteria are often described as mutualistic (Saeed et al., 2021), meaning that the fitness of both organisms sees a net increase (Bronstein, 1994). These microorganisms are reliant on carbon from the plants for their energy, mostly in the form of exudates, which are produced in higher quantities at the tips of growing roots (Rovira, 1969). This thriving rhizosphere microbial community then improve significantly the nutrient uptake by the plant, and experiments testing the presence of common rhizosphere bacteria often found a positive impact on plant growth and, in the case of crops, yield (Mendes et al., 2013). Another indirect benefit for plants is the biocontrol of phytopathogens by rhizosphere microbes, either through niche occupation, resource competition, or direct antagonistic interaction (Sindhu et al., 2009, Gómez Expósito et al., 2017).

1.3 Bacteria grazers

The most important aspect of the nutrient cycles for plant nutrition, the mineralization of nutrients, is not yet convincingly shown to be a benefit of rhizodeposition when looking only at interactions between root and bacteria (Bonkowski, 2004). As carbon released through root exudates increases the C:N ratio, nitrogen is increasingly competed for, and instead of being available to plants, it is immobilized by bacteria in a mechanism called the "microbial loop", a concept originally described in marine microbiology (Azam et al., 1983) since found to be also applicable in soil ecosystems, where nutrients found in bacterial biomass are constantly re-absorbed by other bacteria instead of moving up the food chain. This is where bacteria predators play a critical role in nutrient cycles. These organisms, either protozoa or small animals like nematodes, have a similar physiological C:N ratio to bacteria (Clarholm, 1981). When they consume bacterial biomass, which they do in important quantities (Clarholm, 1985), they accumulate an excess of nitrogen as a fraction of the absorbed carbon is used to produce energy through respiration rather than to build up their own biomass. That surplus of nitrogen is then made available to plants when it is released as ammonia (Zwart, 1994). The accelerated life cycle of bacteria as their growth is stimulated by grazing and they are rapidly consumed by predators that recycle the nutrients they contain is also thought to promote resource transfer through this food web (Morriën et al., 2017).

As in the case of plant-bacteria interactions, this effect on nitrogen flow is not the only way that bacteria grazers can interact with plant roots. Protozoan bacteria grazers have been shown to promote root development through their impact on the activity of the bacteria community, indirectly affecting the production of phytohormones by these bacteria (Bonkowski and Brandt, 2002, Krome et al., 2010). One of the mechanisms at play is grazing-induced stimulation of auxin- producing bacteria, as consumption of parts of the population of bacteria will free space and resources that promote the growth of that same bacteria population, resulting in a significant increase in its metabolic activity (Hunt et al., 1977).

Another effect of bacteria grazers on rhizosphere bacteria is the modification of their community composition. In previous studies, the impact of this grazing pressure on soil bacteria community composition was found to be important and rapid (Mielke et al., 2022, Rosenberg et al., 2009, Rønn, McCaig, et al., 2002). This is due to the selective

nature of this pressure, with bacteria that are smaller or sporting defense mechanisms being more grazing-tolerant (Matz and Kjelleberg, 2005). They are therefore advantaged by the integration of bacteria grazers in an environment, which quickly reduces the population of their bigger, faster growing competitors.

Among the predators of bacteria in soils, amoeba are of particular importance. They are known to be very efficient, their presence having a high impact on the bacteria community's diversity and total biomass (Clarholm, 1981). This is due to their physiology, which makes them great bacteria hunters in the water films around soil particles, where their pseudopod can penetrate pores as small as 1 µm, where bacteria are typically protected from other predators (Ekelund and Rønn, 1994). Additionally, their short generation time, close to that of their prey, allows them to keep a strong grazing pressure on bacteria (Clarholm, 1981).

And among amoeba found in soil, *Acanthamoeba castellanii* is very common and widely distributed, and was previously shown to be an efficient bacteria grazer (Page, 1976). Here, it was inoculated in some of our mesocosms to create a treatment. In the rest of the mesocosms, all bacteria grazers were excluded. In the absence of predation, we expect the bacterial loop to be a significant obstacle to nutrient uptake by plants, and therefore the *Acanthamoeba castellanii* treatment to make a clear impact on the flow of nutrients. A strong increase in nutrient availability could stimulate plant growth significantly, with a potentially disproportionate effect on roots. If this treatment indeed improves plant development, we would also expect a proportionate increase in carbon flow from plants to the soil.

1.4 Mycorrhizal fungi

Another plant-microbe interaction that significantly improve the availability of nutrients for plant roots is the mutualistic relationship with mycorrhizal fungi (Ingraffia et al., 2019). Some fungi, from the Glomeromycota taxon (Tedersoo et al., 2018), have the capacity of infesting plant roots with their hyphae without directly causing harm to these tissues. In typical natural conditions, this interaction increases plant fitness instead (Thakur et al., 2019, M. Wang et al., 2023), and currently most terrestrial vascular plants form mutualistic interactions with mycorrhizal fungi (Averill et al., 2019), with

evidence that it has been the case since the start of the evolution of land plants (Redecker et al., 2000). When a mycorrhize colonize a root, their close connection allows the plant and the fungus to exchange resources directly. This exchange is based on the complementary nature of plants and fungi, as their limiting resources are different. Plant roots have access to abundant carbon photosynthesized above-ground, and forage for water and soil nutrients, and although mycorrhizal fungi cannot digest the soil organic matter themselves (Tisserant et al., 2013), they have a much better access to the soil nutrient once decomposed by other microbes.

This is due to their mycelium requiring fewer resources to grow than plant roots (Bonfante and Genre, 2010). Whereas a root cross section typically shows hundreds of cells and a diameter of at least 300 µm, the mycelium network is formed of strings of single hyphae cells measuring around 10 µm in diameter. With fewer resources invested in structural tissue than plant roots, they can cover a larger volume of soil, creating a denser network that can also be resorbed once resources have been collected. With this rapid and "cheap" access to soil resources, they can compete with other rhizosphere microorganisms for nutrient uptake, which plant roots cannot do (Bunn et al., 2019). Their impact on the nutrient availability for plants is such that mycorrhiza can also have an important effect on root development. Previous experiments have even reported reduced root biomass in the presence of mycorrhiza, as the fungal partner took over some of the root function (Henkes et al., 2018). But without their plant hosts, the typically low amount of carbon in soils would limit their development, as is the case for other soil organisms.

Additionally, the presence of myccorhiza has also been shown to create shifts in the rhizosphere microbial community composition, presumably by out-competing other fungi, changing the availability of both soil nutrients and plant carbon, or more directly through hyphal exudates (Nuccio et al., 2013). Although the feedback of this phenomenon on other plant-microbe interactions is not clear, such findings suggest that myccorhiza has an important impact on other rhizosphere microbes. This mechanism might also play a role in shaping the plant-myccorhiza association effect on nutrient cycles in soils (Chowdhury et al., 2022).

Similarly to the protozoa treatment, a mycorrhiza treatment was created by excluding

these organisms and inoculating them only in some of the mesocosms. *Plantago* species associating with Arbuscular Mycorrhizal Fungi (AMF) (Averill et al., 2019), we used the typical model organism of AMF, *Rhizophagus irregularis*. This species has been shown to be particularly beneficial to the growth of *Plantago* (Thakur et al., 2019). For this reason, we also expected this treatment to significantly improve nutrient uptake by the plants, potentially leading to a notable increase in plant growth and carbon flow. Here, we are particularly interested in the interactions between treatments. Available data suggest that this effect of AMF association could be stronger with *Plantago lanceolata* (Thakur et al., 2019), as well as in the presence of our bacteria grazer treatment (Koller et al., 2013). According to these previous findings, we would see an increase in nutrient exchange in the interaction of these treatments that would be higher than the simple sum of their individual effects. But contrary to the protozoa treatment, we do not expect mycorrhiza to promote the growth of roots proportionally to the rest of the plant; rather, previous data has shown that they can reduce the development of the root system as they take over some of the functions of the roots (Henkes et al., 2018).

1.5 Experiment goal and design

Although numerous studies have shown different positive impacts of plant-mycorrhiza (Asghari and Cavagnaro, 2012, Bender and van der Heijden, 2015) or plant-protozoa (Bonkowski and Brandt, 2002, Gao et al., 2019) relations on plant nutrient intake and growth, only a limited number of experiments have combined both effects to measure their interactions and their relative importance. In his 2004 review, Bonkowski hypothesized that, in natural conditions, having both associated mycorrhiza and protozoa maximizes plant growth as mycorrhiza increase uptake of nutrients mobilized by protozoa, while the respective effects of each interaction on root architecture are counterbalanced (Bonkowski et al., 2001, Bonkowski, 2004). Later experiments tended to confirm an added benefit to plant development when both mycorrhiza and protozoa were present in the rhizosphere (Koller et al., 2013, Henkes et al., 2018). Although contrasting evidence suggests that inhibition effects exist (Rønn, Gavito, et al., 2002) or, in some cases, negative impacts on plant growth are found, for example, when ecological conditions mean that mycorrhiza turn into functional parasites of plant roots (Herdler et al., 2008). There is still a limited amount of data available to understand the complex

rhizosphere food webs (Geisen and Quist, 2021), and the impact of interactions between roots, mycorrhiza, and protozoa is typically dependent on the ecological conditions and the species involved. And data from experimental studies currently available usually tackle only one set of these conditions at a time. There is also limited data on the impact of the carbon flow itself on this complex network of interactions (Chowdhury et al., 2022).

To test the impact of these different trophic levels, from plant producers to protozoa and myccorhiza, on the nutrient cycles in the rhizosphere, we designed an experiment where the presence of these organisms was made a treatment in a full factorial design, allowing us to check for their individual effects as well as those of their interactions. A controlled environment was created in rhizosphere mesocosms, starting from sterile soil, where it was possible to create treatments involving the absence or presence of protozoa and myccorhiza. The three treatments selected (plant growth strategy, protozoa inoculation, myccorhiza inoculation) were applied in a full factorial design (see Figure 2), so that both their individual impact and that of their interactions could be studied.

To follow and quantify the nutrients flow through the rhizosphere of these mesocosms, we used Stable Isotope Probing (SIP; Dumont and Murrell, 2005) and amended the isotope ratio of certain pools of elements to later measure their diffusion into other pools. We chose to focus on the carbon and nitrogen cycles. Here, carbon represents the amount of energy flowing from above-ground tissues of the plant to the roots and then to soil organisms. And among the nutrients which are flowing from the soil to plant tissues, nitrogen is often the primary limiting element for plant growth (Vitousek and Howarth, 1991), and was therefore preferred to other soil nutrients. By tracking both the carbon and nitrogen flow this way, we were able to measure the amounts of each element that were absorbed by the different organisms present.

In the case of nitrogen, this probing was done by amending a patch of bacterial necromass in the soil with ^{15}N and later measuring how much the plant biomass had been enriched in this isotope.

For the carbon, lipid markers were extracted and identified so that they could be attributed to certain soil microorganisms. These compounds, either part of the cell mem-

brane of organisms (phospholipid fatty acids, PLFA) or storage lipids (neutral lipid fatty acids, NLFA), have been found to be specific to some large taxonomic and functional groups of soil organisms (Frostegård et al., 1993). As fatty acids are quickly degraded in natural conditions, they are considered a quantitative representation of the microbial community at a given time, whereas molecules such as DNA can indicate the presence of an organism but have a slow turn-over rate in soil and can accumulate in organic matter, limiting their sensitivity to temporal shifts in community composition (Willers et al., 2015, Joergensen, 2022).

More recently, this technique has been combined with carbon labeling, with the isotopic enrichment of individual biomarkers bringing more insight into the carbon flow (Yao et al., 2015). Here, the flow of carbon from plants to the soil was probed by placing the mesocosms in labeling chambers containing a high amount of $^{13}CO_2$. Later, the enrichment in ^{13}C of lipid markers was measured. A high enrichment in a marker was interpreted as the consequence of the metabolic activity of organisms that had been feeding on plant-derived carbon.

2 Material and methods

2.1 Mesocosms setup

Testing the effect of microorganisms required to exclude them from certain treatment. This was achieved by creating soil mesocosms containing sterile soil that could then be re-inoculated with some microorganisms but kept hermetically closed to prevent contamination. Inoculum of these organisms were prepared in sterile conditions for the same reason. Substrate amended with ^{15}N was also added to the soil during the setup of these mesocosms.

2.1.1 Soil collection

The soil used in this setup was sourced from the Jena Experiment site (Roscher et al., 2004). The relatively high sand content of this soil would promote the development and interaction of the different organisms present. Such soil texture leads to larger pore spaces with a high available surface area, allowing all soil organisms to move freely and have good access to resources (Rutherford and Juma, 1992). This ensured the proper and homogeneous development of all organisms present (bacteria, amoeba, AMF, and plant roots) in our mesocosms.

The soil on the Jena Experiment site is classified as an Eutric Fluvisol, with texture ranging from silty clay to sandy loam as the distance to the river decreases. A segment of this site, closer to the river, was chosen for soil collection. This soil has been reported to have a sand content above 40% and a density of 1.25 g cm^{-3}, with a relatively low organic matter content of 18.2 g kg^{-1} and nitrogen content of 1.9 g kg^{-1}. On site, the presence of numerous molehills was noted, and soil was collected directly there. Not only is it a practical way to collect a large volume of soil compared to digging, but this also meant that less plant and mineral matter were mixed with the soil and that it had already been stirred up, decompacted, and freed of bigger aggregate.

The collected soil was then sieved through a 2 mm mesh to remove fragments of roots and other debris and keep the aggregate size to a minimum. This improved the surface area available to soil organisms and plant roots. This step also further homogenized the soil.

2.1.2 Soil sterilization

To ensure that only selected organisms would be present in the mesocosms, this soil was sterilized by autoclaving. As some soil microorganisms, including sporulating fungi and bacteria, are capable of surviving a single autoclave cycle (Wolf et al., 1989), two cycles of 30 minutes at 121°C and 210 kPa, separated by a week of incubation at room temperature, were used. This sterile soil was later used to fill our mesocosms before being inoculated using cultures of soil organisms.

2.1.3 Plants seedlings

The seeds of *Plantago lanceolata* and *Plantago media* that were used to germinate plants for the mesocosms also needed to be sterilized and kept in sterile conditions. All seeds were surface sterilized by a cycle of washing in 70% ethanol, followed by 5% Na-Hypochlorid, and then cleaned with sterile deionized water (Davoudpour et al., 2020).

They were then germinated in sterile conditions on a wet paper filter inside Petri dishes that were closed with paraffin. These dishes were then placed in a climate chamber for 4 days. As *Plantago* germinates during the summer in the wild, summer-like conditions were recreated in the chamber to stimulate germination and fast growth (Blom, 1978). Throughout the development of the plants, they had a 16-hour long day, 18°C (+7°C with light on during the day period), 65% humidity, and 280 μmol m^{-2} s^{-1} light photon flux density.

At the end of this germination period, 80 seedlings (40 of each species) were selected to be planted in the mesocosms. After choosing individuals of similar size for homogeneous starting conditions, seedlings were planted in small tubes filled with autoclaved sand, which were then placed in Magenta vessels (Sigma-Aldrich, St Louis, MO, USA). Seedlings were kept in these boxes, which were hermetically sealed and transparent, while they developed further. They were again placed in a climate chamber with similar conditions for 4 more days, until they had developed enough to be transferred to mesocosms.

2.1.4 Bacteria suspension

Before growing any other organisms in our mesocosms, the sterile soil needed to be populated with a bacteria community that would naturally occur in the same soil, while excluding any protozoa or fungi. A bacteria inoculum was prepared by taking the microbial community of the original soil and filtering out the bigger organisms. First, a slurry was created by suspending a small amount of non-sterile soil in tap water. This slurry was filtered first through a paper filter (595½ Schleicher & Schuell, Dassel, Germany) to remove the bigger soil particles, then through 5 µm water filters, and finally through 3 µm sterile filters. This successive filtration guaranteed the exclusion of larger organisms, until only bacteria were left in the solution. This operation was repeated to create four individual suspensions.

These suspensions were then checked under a microscope for contamination by protozoan or fungal organisms after 1 week of cultivation in NB-NMAS (modified from Page, 1976). This liquid growth medium was modified to be poorer in nutrients, using 0.26 g of nutrient broth (CM0001 Nutrient Broth; Oxoid, Basingstoke, UK) for 1 L of NMAS buffer, or one tenth of the usual recommended quantity. This limited amount of nutrients ensures slower development of bacteria, thus preventing them from filling the space in the culture vessels, which would have made the detection of the potential eukaryotic contaminants harder. In such conditions, after 1 week of growth, it was still possible to clearly distinguish every single organism present in the culture medium under a microscope. Only when no contamination could be observed were the bacteria suspensions selected to be used as inoculum for the mesocosms, each receiving 2 ml of this solution.

2.1.5 Bacteria grazer: protozoa treatment

The *Acanthamoeba castellanii* inoculum was prepared using preexisting culture from the Department of Terrestrial Ecology of the University of Cologne (Rosenberg et al., 2009). After being checked for contamination under a microscope, the *Acanthamoeba* culture was concentrated using two cycles of centrifugation at 1000 rpm for 3 minutes and re-suspension in NB-NMAS. The resulting solution was estimated to contain roughly 50'000 organisms per ml by using a Neubauer counting plate, also confirming that live

Acanthamoeba were present at that stage, and was further diluted by doubling its volume, to 48 ml total. This solution was used as the *Acanthamoeba* inoculum, using a volume of 0.5 ml in each mesocosm that received this treatment. This amount contained approximately 12'500 individual *Acanthamoeba*.

2.1.6 Mycorrhiza: arbuscular mycorrhizal fungi (AMF) treatment

The Arbuscular Mycorrhizal Fungi (AMF) that was used as a treatment in this setup, *Rhizophagus irregularis*, was received as spores in liquid media (Symbiom, Lanskroun, Czech Republic). This media was used directly as inoculum by injecting 0.5 ml into the sand tubes before planting the seedlings. Half the tubes received this treatment, so that half the plants were in contact with AMF spores within days of their germination. This ensured maximal colonization of their root system.

2.1.7 ^{15}N labeling

Tracking of nitrogen flow from the rhizosphere to plants was done using a ^{15}N label added to the soil of the mesocosms in the form of bacterial necromass. This label would thus first be available to microorganisms in the soil before it could be taken up by plants. To create this labeled bacterial necromass, a liquid media fitting for general bacteria culture (with a C:N ratio of 8, neutral pH and glucose as the sole energy source) was prepared (Vrede et al., 2002). The total ^{15}N fraction of this media was brought to 1 atom%, by the addition of 10 atom% $K^{15}NO_3$ salt. One litter of this solution was produced; its detailed composition can be found in Appendix A.1 (modified from C. Wang et al., 2020). Its pH was brought to 7.4 using NaOH before it was autoclaved at 121°C for 20 min at 210 kPa. Using the same bacterial suspension that had been created for the soil of the mesocosms, this solution was inoculated and left to incubate at room temperature for one week. The result was a solution where all nutrients had been digested by bacteria and turned into bacterial biomass. Before it could be used in the mesocosms, this one-liter solution was concentrated by centrifugation (3500 rpm for 15 minutes) and re-suspension in 200 ml of deionized water, and then autoclaved again. This resulted in a concentrated solution of bacterial necromass, enriched in ^{15}N, that could later be used as a labeled substrate in the soil of the mesocosms.

Of this solution, 20 ml were kept aside to be freeze-dried and measured using an Element Analyzer (NA 1110, CE Instruments, Milan, Italy) coupled to an IRMS (Delta+XL, Thermo Finnigan, Bremen, Germany) to get the precise δ^{15}N-value. This analysis found that nitrogen made 9.74 ± 0.26 % of the dry weight of this solution, and this nitrogen had δ^{15}N-value 1216.81 ± 4.57, more than double the natural isotope abundance with approximately 0.815 atom% ^{15}N.

During setup, each mesocosm received 2 ml of this solution, which was added to a small layer of soil at the bottom of the flask. This concentrated the nutrients in the necromass into a small volume of soil, creating a patch of labeled resources. These resources would then be first absorbed by bacteria and kept concentrated in a small part of the mesocosm, making them hard to access by plants in the absence of other organisms such as bacteria grazer or mycorrhiza.

2.1.8 Mesocosms assembly

The 80 mesocosms consisted of single 250 ml culture flasks (CELLSTAR® 250 ml Cell Culture Flasks, Greiner bio-one, Frickenhausen, Germany) filled with sterilized soil, having the advantages of being practical, cheap, coming in pre-sterilized, and being transparent so root development could be monitored throughout the growth of the plants (see Figure 1). After filling these flasks with soil and having added the ^{15}N labeled necromass, mesocosms were inoculated with our bacteria suspension, and left to incubate for 24 hours at room temperature. The 40 mesocosms in the protozoa treatment group then received 0.5 ml of the *Acanthamoeba castellanii* inoculum, while the rest received the same volume of sterilized water.

After another day of incubation, plant seedlings were transferred into the mesocosms by simply placing their sand tube in the neck of the flask, sitting on the soil. This allowed the plant roots to continue their development in the mesocosm soil without the risk of damaging them by transplanting the seedling directly into the soil. At this point, all mesocosms had been inoculated with the required organisms for their respective treatments (see Figure 2), as the sand tubes had already received AMF spores.

To prevent any contamination from external microorganisms, the neck of the flasks was

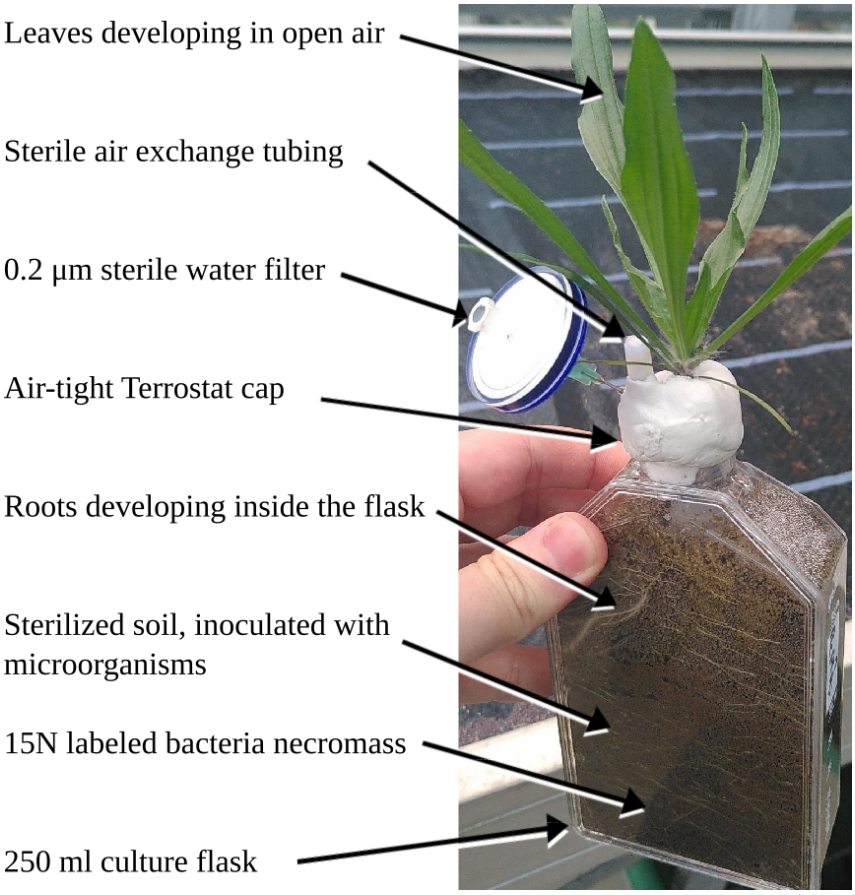

Figure 1: A mesocosm, fully grown stage

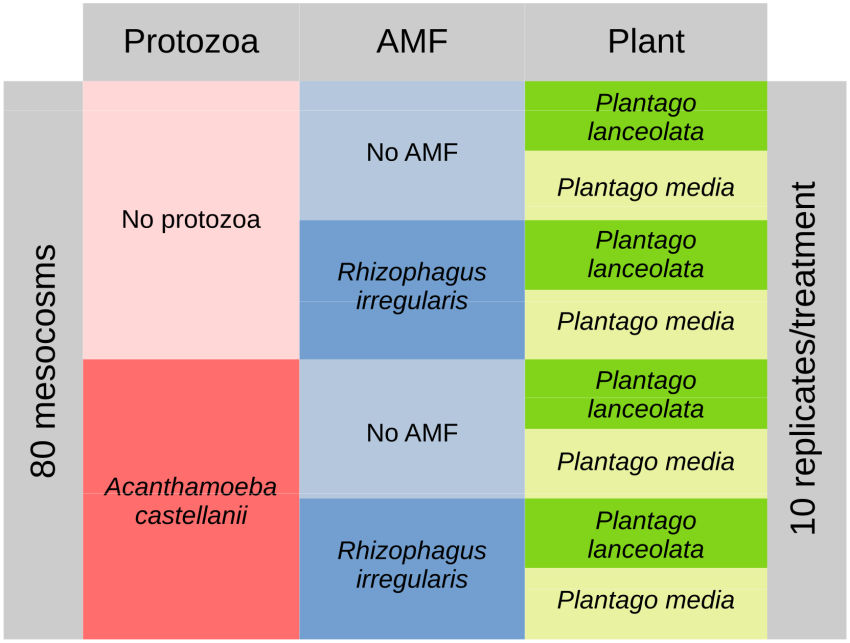

Figure 2: Experimental design

filled with autoclaved cotton wool and covered with a layer of Terostat, creating an air-tight seal around the stem of the plants. This allowed the leaves of the plants to develop outside of the flask and their roots to access the soil in the flask while allowing the stem to grow in thickness. To prevent anoxic conditions inside the mesocosms, the necks of the flasks were also fitted with a short tube filled with cotton wool, allowing for sterile gas exchange. Similarly, to allow watering of the mesocosms without risking contamination, a long needle was installed that went through the neck of the flask into the soil and was fitted with a 0.2 μm water filter (Filtropur S 0,2; Sarstedt, Nübrecht, Germany). Throughout the development of the plants, mesocosms were watered with a simple syringe filled with non-sterile deionized water.

2.2 Growth period

During eight weeks, mesocosms were placed in a climate chamber, again in summer-like conditions, to promote the growth of the *Plantago* species, typically germinating during the summer months (Grey et al., 2019). Similarly to conditions during seed germination, plants had a 16-hour long day, with 18°C at night and 25°C during the day, constant humidity of 65% and luminosity of 280 μmol m^{-2} s^{-1} light photon flux density. To ensure that variation in light intensity and air flow across the chamber did not compromise the homogeneity of their development, mesocosms had their positions changed every week.

Soil water content measurement on the Jena Experiment site (Wetterstation Saaleaue, www.bgc-jena.mpg.de/wetter/, n.d.) showed that winter, the wetter season in this climate, saw an average of 35% water content, reaching only 30% on the driest year, 2019, when soil in the summer had around 20%, with drier years as low as 15%. Given that these measurements were done on silt loam, the estimation was that the natural wet condition on site reached a maximum of 80% of the field capacity (Novák and Hlaváčiková, 2019).

Therefore, to provide optimal growth conditions to plants, this 80% water content was also used as a maximum for moisture level inside the mesocosms. Through frequent rewetting (first weekly when plants were still small, then every 48 hours during the last 2 weeks), it was maintained between 80% and 60% of field capacity by weighting the

mesocosms and adding missing water weight. The Field Capacity of the soil was estimated by wetting to saturation 250 ml of soil with 250 ml of water and letting it to drain for 24 hours on top of a sand column, then weighting it before and after oven-drying at 65°C for 6 hours. The difference in weight corresponded to 100% field capacity, here 82 g of water for 250 ml of dried soil, or around 30% of the Volumetric Water Content, which is coherent with the high sand content of this soil (Chapin et al., 2011).

2.3 ^{13}C pulse labeling

At the end of the eight weeks of growth, the plants had developed enough to fully root the soil in the mesocosms (see Figure 1). From this point, all of the soil inside the flasks was considered to be rhizosphere soil, as it was in direct contact with plant roots and their exudates. Before the plants outgrew their containers and slowed their growth, they went through the pulse labeling procedure, using 95 atom% $^{13}CO_2$ to mark the carbon that would be metabolized by the plants over a 2-hour long period.

This required an airtight container in which we could place our mesocosms and that we could fill with $^{13}CO_2$ gas (see Figure 3). Two interconnected labeling chambers made of transparent acrylic glass were used. They formed a closed system into which we could pump $^{13}CO_2$ gas and then monitor its abundance during the whole process. This was measured using a Picarro CRDS analyzer (G2101-i analyzer, Picarro, Santa Clara, USA) and Vaisala CO_2 Probe (CARBOCAP® Carbon Dioxide Probe GMP343, Vaisala, Vantaa, Finland). Other variables being monitored were temperature, humidity, and light intensity, to ensure that environmental conditions would not be detrimental to plant growth.

During the two hours of labeling, the temperature inside the chambers increased from 24 to 28°C and the humidity from 70% to 85%, with a light intensity of approximately 600 μmol m^{-2} s^{-1}. Two pumps constantly circulated air between the two chambers to homogenize the conditions inside, as well as the CO_2 and $^{13}CO_2$ levels. Other measures taken to prevent adverse or heterogeneous conditions inside the chambers included adding ice packs to help limit the temperature increase, water dishes to help maintain humidity levels, and fans to create air flow. Preventing sunlight from creating heterogeneous luminosity inside was done by covering the chambers with opaque panels,

with LED lighting providing homogeneous luminosity to all plants.

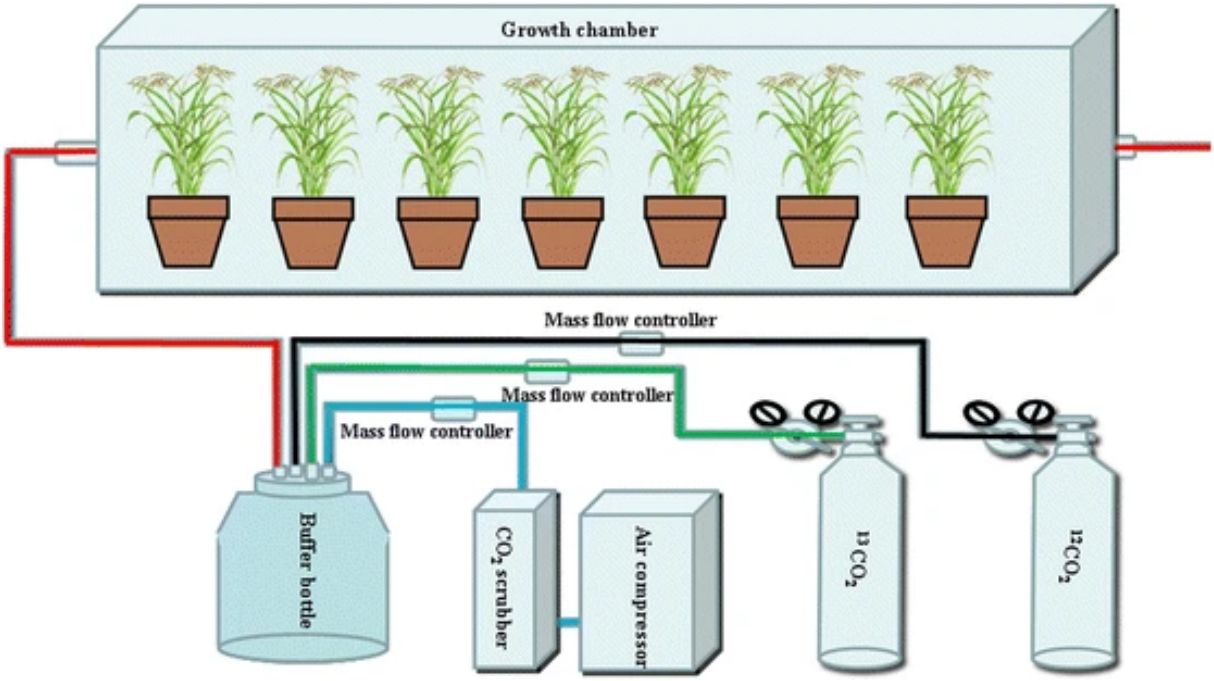

Figure 3: $^{13}CO_2$ labeling chamber principle (Yao et al., 2015)

Once the chambers were ready and our mesocosms were placed inside, natural CO_2 inside the chambers was flushed with synthetic air, from approximately 600 ppm down to 70 ppm. $^{13}CO_2$ level was then increased to 500 ppm by adding 360 ml of 95 atom% $^{13}CO_2$ gas. This was considered the starting point of the 2-hour-long labeling. During this period, $^{13}CO_2$ concentration level was monitored (see Figure 4) and was increased back to 550 ppm when it was measured below 400 ppm by adding 45 ml of $^{13}CO_2$ gas. This happened roughly every 6 to 7 minutes. This strong and constant decrease of $^{13}CO_2$ was caused by plant photosynthesis and showed that the label was being metabolized. During these 2 hours, the $^{12}CO_2$ level was measured to stay between 120 and 140 ppm, meaning that at all times the $^{13}CO_2$ level was 3- to 5-fold higher.

After the 2 hours of labeling, the chambers were again flushed with synthetic air to remove the remaining $^{13}CO_2$ before opening and removing the plants. The mesocosms were then placed in a greenhouse with the same growing conditions that they had before the labeling while waiting to be sampled 48 hours later.

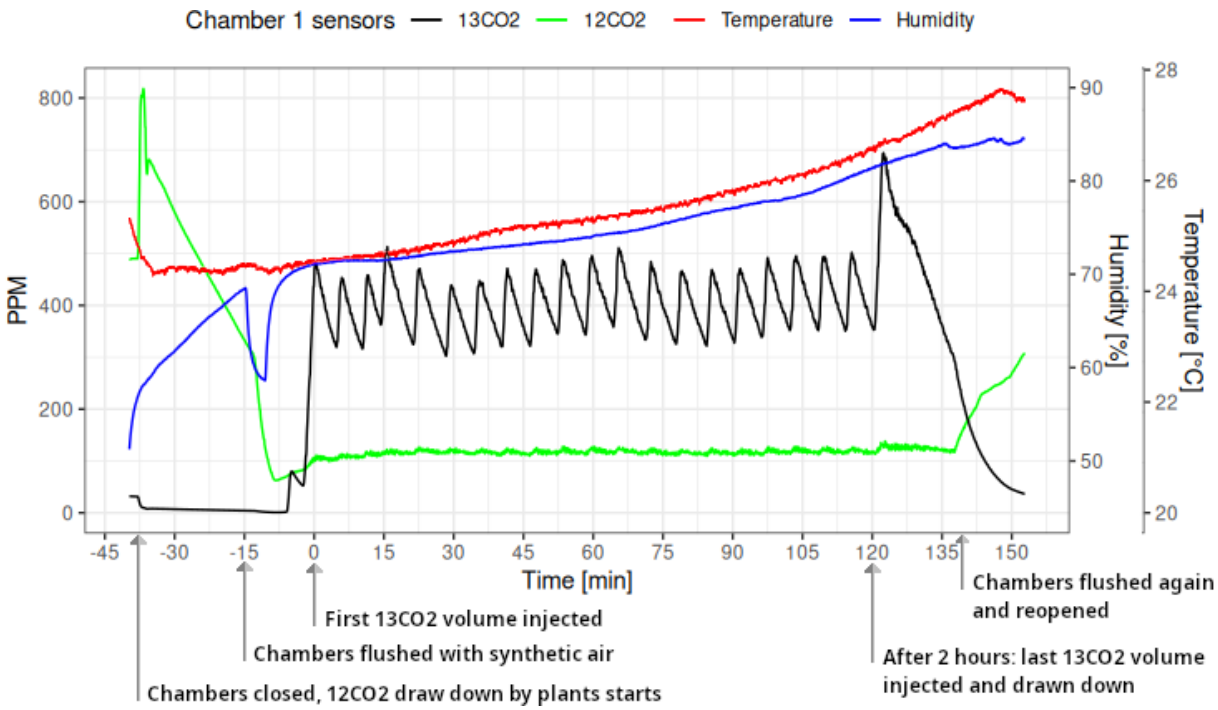

Figure 4: $^{13}CO_2$ pulse labeling log

2.4 Sampling

At this stage, the smaller plants were discarded, while taking care to keep at least six replicates per treatment. The mesocosms that were kept were then sampled. Their soil was extracted and separated from roots using 2 mm sieves and frozen at -20°C to prevent any alteration to the biomarkers (here fatty acids). A sample of 5 g of soil from each mesocosm was taken to be oven-dried at 60°C for a day and weighted again to determine their water content. The roots were washed from soil particles and then dried at 60°C for 3 days, along with the leaves and flowers of the plants, all kept separated.

2.5 Plants matter

After the plant matter had dried, roots, leaves, and flowers were weighted separately to measure the biomass of each plant and of each tissue type. From these weights, it was also possible to calculate the root-to-shoot ratio of each plant. After weighting, plant matter was ground into a powder for measurement of the nitrogen content and ^{15}N atom%, to quantify the absorption of the ^{15}N label by the plants. The powdered plant matter was split between root and shoot matter only, as only a limited number of plants had developed big enough flowers to be measured on their own; thus, leaves and flowers were gathered into "shoot matter". The measurements were conducted us-

ing an Element Analyser (NA 1110, CE Instruments, Milan, Italy) coupled to an IRMS (Delta+XL, Thermo Finnigan, Bremen, Germany).

As a non-labeled control was missing for ^{15}N atom% of plant matter, mean values from a previous study in the Jena Experiment were used instead (unpublished data).

2.6 PLFA/NLFA extraction

To extract lipids from the soil samples, a Blight-Dyer-Extraction (modified from Bligh and Dyer, 1959) was run using a Büchi Speed Extractor (BÜCHI Labortechnik GmbH, Essen, Germany). Soil samples used for extraction were weighted while taking into account their differences in water content, so that all samples contained 7 g of dry matter. Although this dry matter content was the same across the samples, the amount of water present in each cell during the extraction process varied significantly.

To control for potential impacts on the efficiency of the extraction process (Fu et al., 2021), a water content control was created. From the original soil collected from the Jena Experiment site, six samples were taken to be extracted. This soil was measured at 13% water content. Two of these control samples had water added to 25%, two others only to 19%, and the two last control samples were kept at 13%. This produced three treatments: low, medium and high water content control samples, all of the same soil, that we extracted and measured along with the other samples. These water content control samples were also later used to check for extraction bias in the relative abundance of the yielded compounds, as well as references for the natural abundance of ^{13}C in lipid markers, as this soil had not gone through pulse labeling.

During the extraction process, cells containing the soil samples were heated to 70°C, filled with a solvent mixture of chloroform, methanol, and phosphate buffer (2:1:0.8), and put under 120 bar pressure for 10 minutes before being flushed. This cycle is repeated three times, aiming for total extraction of both water-soluble and lipidic compounds from the sample. C19:0 phospholipid was added as an external standard used for the determination of the extraction efficiency. 50 µg were added to the soil samples at the start of the extraction, and the proportion of this amount found in the samples during analysis indicated how efficient the extraction process had been.

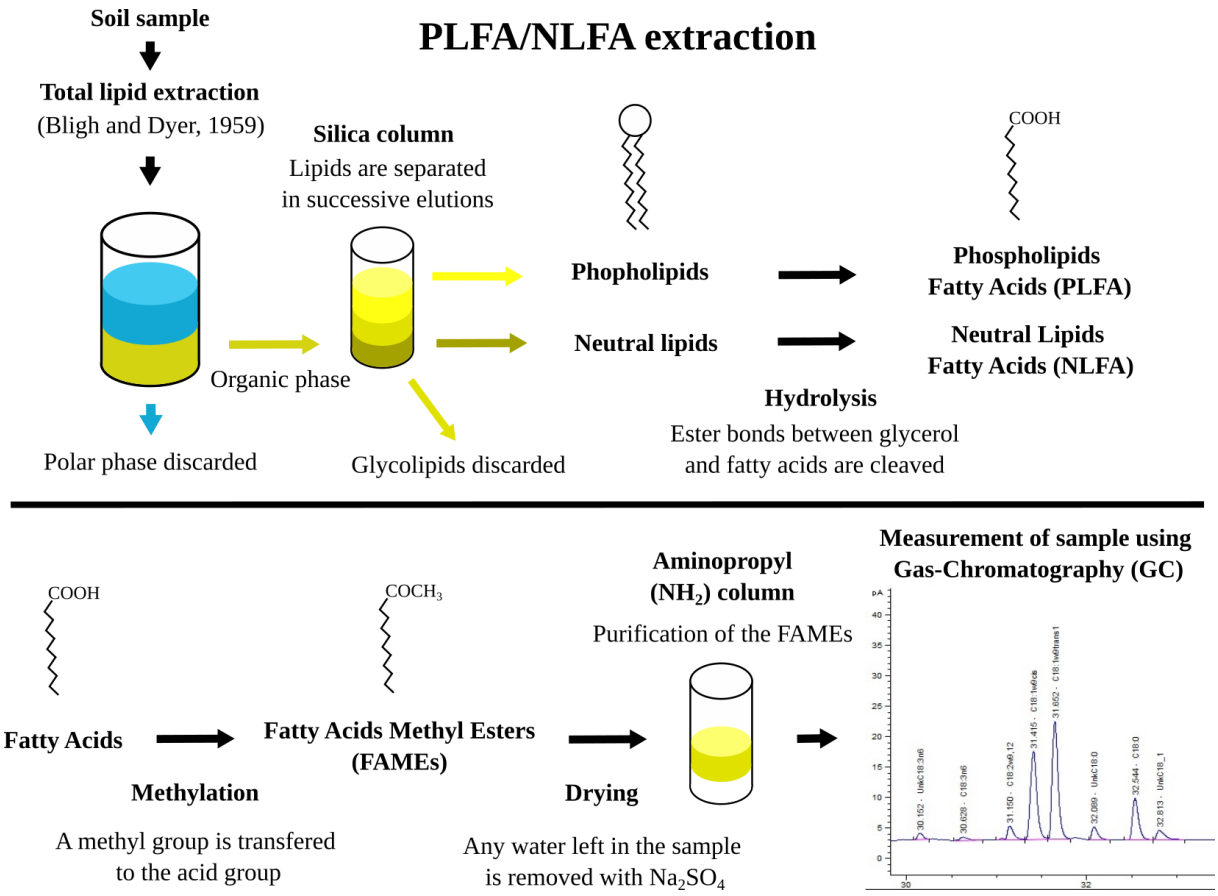

Figure 5: PLFA/NLFA extraction principle

After exclusion of water-soluble compounds by phase separation, phospholipids and neutral lipids were isolated from other lipidic compounds by elution through a Chromabond® silica column (Macherey Nagel GmbH & Co. KG, Düren, Germany), using successively chloroform, acetone, and methanol as solvents to elute respectively, neutral lipids, glycolipids, and phospholipids (see Figure 5). Glycolipids were not analyzed here and were discarded at that point.

From the neutral and phospholipids, only their fatty acids were kept (respectively Phospholipids Fatty Acid, PLFA, and Neutral Lipids Fatty Acid, NLFA). By hydrolysis, ester bonds between the glycerol and the fatty acids were cleaved, freeing the fatty acids that were then methylated, forming Fatty Acids Methyl Esther (FAME). This prevented fatty acids from reacting with other molecules during the rest of the process. Hydrolysis and methylation were simultaneous, injecting the lipid samples into a 0.2 M KOH methanol solution before adjusting it to a pH of 6 with acetic acid. A sample of the methanol used during this step was kept for later measurement of its ^{13}C atom%, which

was needed for correcting its impact on the δ^{13}C-value of the FAMEs. During methylation, the carbon atom from the methyl group added to the fatty acids to form the FAME, which originated from this methanol, and participated in the δ^{13}C-value of the molecule. Therefore, to know the δ^{13}C-value of the original fatty acid molecule, correcting for the addition of this individual carbon was required.

Before analysis, these FAMEs were purified by elution through Na_2SO_4 powder to remove any remaining water, and then through a Chromabond® aminopropyl column (Macherey Nagel GmbH & Co. KG, Düren, Germany) to remove potential contaminants. Samples were then reduced to a small volume (less than 50 µl) and put into 500 µl GC-vial inserts, along with 300 µl of 100 ng/µl C12:0 FAME as an internal standard, used to correct for the variations in total volume between samples.

2.7 PLFA/NLFA abundance and ^{13}C enrichment

Measurement of the concentration of each individual FAME in samples was done with GC-FID (Gas Chromatography-Flame Ionization Detector; GC: HP 6890 Series, AED: G 2350 A, Agilent Technologies Inc., Santa Clara, USA). By gas chromatography, each compound is isolated to allow its quantification by ionization, as the column inside the gas chromatograph is gradually heated from 45°C to 325°C, with a slow increase from 140°C to 242°C over 50 minutes. Lighter compounds, with a lower boiling point, vaporize first. Then, a carrier gas (here hydrogen) pushes them through a polar capillary, which forms a 60-meter-long column. Compounds that are apolar migrate at a slower speed through this column. When compounds reach the FID at the end of the column, they are thus temporally separated according to their molecular weight and polarity and can then be measured individually. On the resulting chromatogram, individual compounds appear as successive peaks.

These peaks were identified by matching their retention time (the time between sample injection and measurement) to the peaks measured in two standards: the BAME Mix (Bacterial Acid Methyl Ester (BAME) Mix 47080-U, Sigma-Aldrich, St Louis, MO, USA) and FAME Mix (37 Component FAME Mix CRM47885, Sigma-Aldrich, St Louis, MO, USA). A few compounds found in the samples but absent from these standards were identified using GC-MS (Gas Chromatography-Mass Spectrometry; GC: 7890A, MS:

5977B, Agilent Technologies Inc., Santa Clara, USA) and matching the fraction profile of the compounds to standard profiles. For each peak, the GC-FID returned an intensity, corresponding to a quantity of matter that could be calculated by finding the response factor of the machine when running a standard with a known concentration of compounds.

Once the concentration of the compounds of interest for this experiment was known, samples were reduced using nitrogen flow to increase their concentration to 20 to 30 ng/µl. This was the estimated optimal accuracy range of the GC-IRMS (Gas Chromatography-Isotope Ratio Mass Spectrometer; GC: 7890A, Agilent Technologies Inc., Santa Clara, USA; IRMS: Delta V Plus, Thermo Fisher Scientific, Waltham, USA) used to measure the $^{12}C/^{13}C$ ratio of the compounds. Similarly to GC-FID, the samples were injected into the Gas Chromatograph, where individual compounds were separated, but at the end of the column, they first passed through a combustion oven, where they were heated at 980°C and fully oxidized (see Figure 6). Every organic compound was broken down, and each atom of carbon turned into CO_2. This CO_2 was then measured in the IRMS part (Isotope Ratio Mass Spectrometer), where molecules of three different molecular masses are quantified. Here, to measure the isotopic abundance of CO_2, the three molecular masses are 44, 45, and 46, corresponding to the weight of $^{12}CO_2$, $^{13}CO_2$ and $^{14}CO_2$. For each compound isolated by the GC, the IRMS returned a ratio between these three isotopes of carbon.

Because of the potential limited accuracy of this system, each sample was measured in triplicate. Furthermore, because of a significant drift in the measurement over the measuring period, each day of measurement started and ended with triplicates of a FAME standard, later used to correct for this daily shift. These standards, having known $\delta^{13}C$-values, were also used to correct for the average shift in ratio returned by the IRMS.

As the GC in both analyses had similar parameters, the peaks of the different compounds arrived in the same sequence, and the identification of the peaks done on the GC-FID sufficed to identify the peaks from the GC-IRMS. From these two analyses, the results obtained for each FAME in each sample gave a measure of their concentration and their $\delta^{13}C$-values.

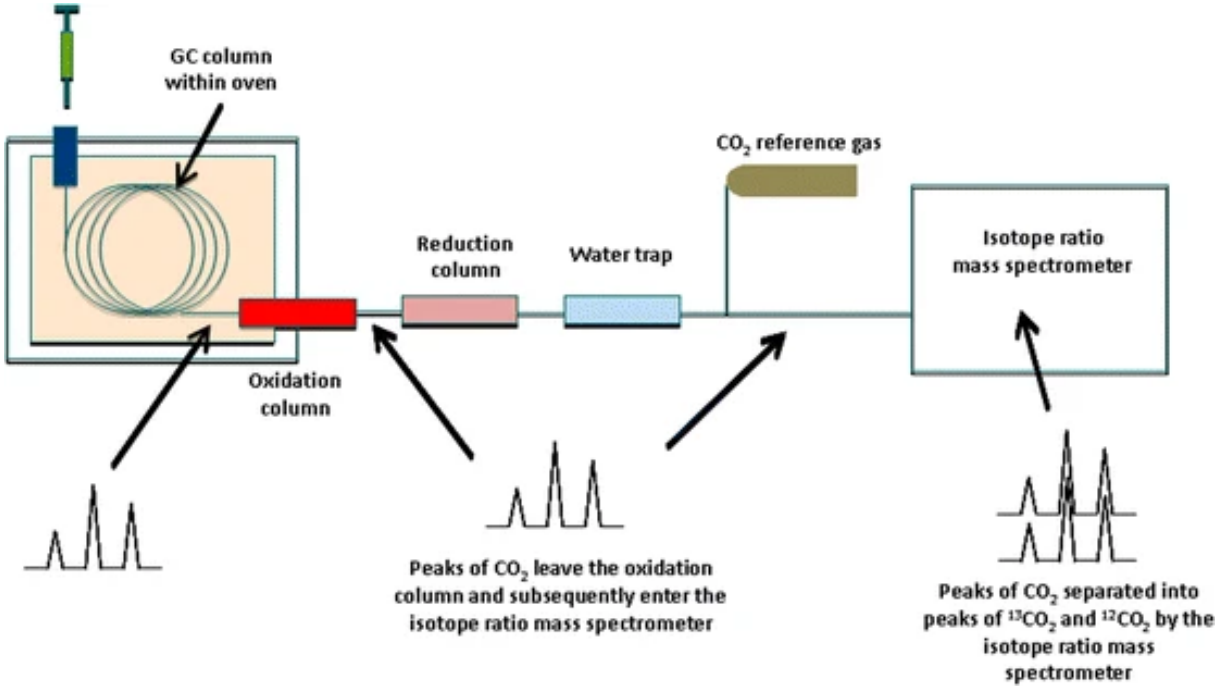

Figure 6: GC-IRMS principle (Yao et al., 2015)

2.8 Fatty acid markers attribution

From these identified compounds, only those that were fatty acids and were present in at least 80% of our samples were considered. Out of these, the known biomarkers of specific organic groups were selected. In phospholipids, saturated fatty acids (here C14:0, C16:0, and C18:0) are generally attributed to bacterial metabolism when found in soil (Zelles, 1997) although they are found in all microorganisms (Zabeti et al., 2010). Mono-unsaturated fatty acids can be linked to Gram- bacteria activity (Veum et al., 2019). From our samples, commonly found were C16:1ω5, C16:1ω7, C18:1ω9c, and two more compounds for which the position of the double-bound on the carbon chain could not be confirmed with certainty, that were only identified here as C18:1 and C19:1. Other markers of Gram- activity are cyclic fatty acids, which are specifically attributed to metabolic activity under environmental stress (Breulmann et al., 2014, Lange et al., 2014). Here, only one was selected: cyC17:0. Gram+ bacteria activity was attributed another family of molecules, the branched fatty acids, sporting an additional methyl group on their carbon chain (Vestal and White, 1989). It was possible to differentiate between fatty acids with a methyl group at the tail end of their carbon chain, in either iso or anteiso position, of which six compounds were selected: iC14:0, iC15:0, aC15:0, iC16:0, iC17:0, and aC17:0, and those with a methyl group on the tenth carbon of the chain, which are more specifically linked to the *Actinobacteria* group of Gram+ bacte-

ria (Högberg et al., 2013). From these, 10MeC16:0, 10MeC17:0, and 10MeC18:0 were kept for further analysis. Another fatty acid, the double-unsaturated C18:2ω6, was attributed to plant root metabolism. This fatty acid is typically attributed to saprophytic fungi when found in soil (Willers et al., 2015), but was found here despite the exclusion of saprophytic fungi by the sterilization of the soil. In these conditions, plants are the most likely source of this compound (Frostegård et al., 2011, Joergensen, 2022).

In the neutral lipid fraction, only one marker was kept for further analysis, the C16:1ω5. When found in NLFA, it is considered a marker for arbuscular mycorrhizal fungi (AMF) (Olsson and Lekberg, 2022). The other eukaryotic microbe inoculated as a treatment, *Acanthamoeba castellanii*, has no known specific lipidic biomarkers that could be used in this setup (Willers et al., 2015).

2.9 Data treatment and corrections

Data collected during these GC-FID and GC-IRMS analyses were corrected for several bias causes, first for extraction bias affecting the measured concentration of the fatty acids in the samples. This was done using the C12:0 FAME standard, as it had a fixed concentration of 100 ng/µl in the solvent used in the final transfer. Variations in the measured C12:0 concentration in samples were therefore assumed to be due to differences in total volume in sample GC-flasks, and the measured concentration of all compounds was corrected accordingly:

$$\text{Vol Corr FAME concentr} = \frac{\text{FAME concentr}}{\text{C12:0 stand concentr}} * 100 \text{ [ng/µl]}$$

Similarly, the impact of the efficiency of the extraction process on the concentration of individual FAME was corrected using the C19:0 phospholipid standard. As 50 µg, or 0.0611 µmol of this standard were added to all samples, a 100% efficient extraction would have resulted in the presence of 0.1222 µmol of C19:0 FAME in the final sample (one molecule of this phospholipid having two fatty acid chains that are freed during the hydrolysis), for a mass of 38.192 µg dissolved in 300 µl of sample. Therefore, in the case of a 100% efficient extraction, the GC-FID would have found 127.31 ng/µl of C19:0 FAME. The fraction of this standard found in each sample was taken as its extraction

efficiency, and the concentration of all other compounds was corrected by assuming that this efficiency had the same impact:

$$\text{Extract Corr FAME concentr} = \frac{\text{Vol Corr FAME concentr}}{\text{Vol Corr C19:0 stand concentr}} * 127.31 \text{ [ng/µl]}$$

Using this corrected concentration of FAME, the concentration of each compound in the original soil sample is calculated by dividing the total amount of compound (the volume in the GC-flask multiplied by the corrected FAME concentration) by the mass of the soil sample used to get the amount found in 1 gram of soil. Additionally, the mass of the carbon from the original fatty acid compound is calculated by removing the mass of carbon added by methylation, using the known difference in molar mass between FA and FAME:

$$\text{FA C µg/soil g} = \frac{\text{FAME concentr} * \text{FAME molar mass} * \text{FA C ratio}}{\text{FA molar mass} * \text{soil sample dry mass}} * 300 \text{ µl}$$

This result is the mass of carbon represented by each identified fatty acid in the soil of our mesocosms. Because of the high variability in the total amount of fatty acids in the different samples, their relative abundance was calculated:

$$\text{FA rel abundance} = \frac{\text{FA absolute abundance}}{\text{total amount FA}}$$

The $^{12}C/^{13}C$ ratio data returned by the GC-IRMS analysis was also corrected for several measurement biases. First, potential mistakes in the identification of peaks due to a shift in the retention time of the compounds over time were corrected by calculating a standard deviation in the retention time of each identified compound, allowing the detection of abnormally high, potentially problematic shifts, and their manual reassignment when necessary. Once the identification of the peaks had been checked, a mean was calculated between the triplicates of each sample and standard. From there, these means were used, first for correcting for the difference between the average measured $^{12}C/^{13}C$ ratio and the known ratio in standards, and subtracting this difference from all sample measures. Next was the daily drift correction, where the drift was assumed to be linear between the standard measured at the start and at the end of the

day of measurement. The difference between these measures was the daily drift for the day, and a fraction of this difference was subtracted from the ratio measured in samples depending on their running order. Finally, this data was corrected for the addition of the carbon in the methyl group during the methylation step. The $^{12}C/^{13}C$ ratio of the methanol used being known, its impact on the measured $^{12}C/^{13}C$ ratio of the FAME is calculated based on the amount of carbon of the FA molecule:

$$\text{FA } ^{12}C/^{13}C \text{ ratio} = \frac{\text{FAME C number} * \text{FAME } ^{12}C/^{13}C \text{ ratio} - \text{Me } ^{12}C/^{13}C \text{ ratio}}{\text{FA C number}}$$

This corrected data had, for each fatty acid marker in each sample, a measure of its concentration in the soil of the mesocosm and a measure of its $\delta^{13}C$. This last value is the expression of the total amount of isotope, and although a high incorporation of the ^{13}C label in a particular compound would lead to a clearly unnatural, isotopically enriched ratio, this has several problems. First, it does not account for the relative concentration of the fatty acids in soil; a small increase in the isotope ratio of a very common compound can be a sign of the incorporation of a significant amount of label, that is "dissolved" in an already large pool of compounds. Secondly, it does not account for the potential differences in the natural abundance of the isotope between the different compounds, which could lead to faulty analysis. For an easier interpretation of this data, the APE (Atom Percent Excess) was calculated for each marker. This number expresses the absolute amount of isotope that can be linked to label incorporation. The measured $\delta^{13}C$ in the non-labeled water content control samples was used as the baseline natural isotope abundance for each compound, and the difference between this value and the $\delta^{13}C$ value (Δ atom%) measured in labeled samples was considered due to enrichment by label integration. The fatty acid concentration measured by the GC-FID was then used to get the absolute amount of matter corresponding to this enrichment:

$$\text{APE} = \frac{\Delta \text{ atom\%} * \text{FA concentration} * \text{FA C number}}{100\%} * 1000$$

2.10 Statistical analysis

The effects of the AMF, protozoan bacteria grazer, and plant productivity treatments on the flow of the labeled carbon and nitrogen in the mesocosms were tested using the data yielded by the different measurements presented above. First, the biomass of

the plants, used as a proxy for plant activity, was tested both as a response variable affected by environmental and treatment effects and as a predictor of the assimilation rate of both the ^{15}N and ^{13}C labels. Analyzing the effect of different factors on plant growth was done by fitting a linear model of the total plant dry weight with the treatments, as well as the position of the mesocosms during the first week of growth, as explanatory variables added stepwise to extend the model and test alternative model hypotheses. From this point, plant biomass was used as an explanatory variable in all models used to analyze other collected measures, but was then log-transformed for distribution improvement. Looking further into the characteristics of the collected plant matter, the total nitrogen content measured by the Element Analyzer was similarly tested by fitting a linear model in a stepwise succession for effects from plant biomass and/or treatments, before the same method was used for the $\delta^{15}N$ values of the plant matter. Here, data yielded from root matter and shoot matter were analyzed in separate models, as potential differences in nutrient attribution mean that they do not constitute exact measure repetition. Rather, they were analyzed in parallel, resulting in two different models being selected.

When analyzing data from soil lipids, the first test to be run was the sum of the concentration of all lipid markers yielded by the extraction process against the plant biomass and the soil water content, as they could both cause variation in the total amount of lipid extracted from the soil samples. This was done by comparing data from the six water content control soil samples, that had the same organic matter content but had their water content manipulated. The relative abundance of the different lipid markers was then compared across treatments, while keeping plant biomass as the most important single effect in all models. For this part of the analysis, microbial groups attributed to a single marker (cyC17:0 for Cyclic Gram-, C18:2n6 for plant roots here, and AMF with NLC16:1ω5) had their data fitted in linear models, but in groups that were linked to multiple markers (Gram- bacteria, Gram+ bacteria, Actinobacteria) Linear Mixed Models were used, considering that each marker was a replicate of the measure in a single mesocosm and using the identity of the mesocosms as a random effect. Both LM and LMM were similarly extended stepwise and tested for significance using ANOVA to perform Chi^2 tests. A single ordered list of fixed effects was used when creating these models, consisting of log-transformed plant biomass, plant species treatment, AMF treatment, amoeba treatment and then four interaction terms between plant biomass

and the three treatments and between the AMF and amoeba treatments. Models used to compare the ^{13}C enrichment of the markers were created similarly, also using LM and LMM with mesocosm identity as random effect to fit ^{13}C APE data with the same fixed effects list.

All statistical analyses were done in R version 4.3.3 (R Core Team, 2024). For linear models, *lm* function from *stats* package (R Core Team, 2024) was used, and for linear mixed models function *lmer* of the *lme4* package (Bates et al., 2015). Graphical representation of the correlations found was done using the gglot2 package (Wickham, 2016).

3 Results

3.1 Plant growth and nitrogen intake

3.1.1 Plant biomass

During the growth of the plant, strong variation appeared. This also appeared in their water consumption, causing as much as a 4-fold increase in watering needs by the end of the growth period. During sampling, as the bigger plants had used much more of the available water, with soil moisture measured around 13% water content, but smaller plants had a very damp soil, as high as 25%. When comparing the total dry weight of the plants, the species of the plants showed a clear difference with *Plantago lanceolata* on average, more than double the weight of *P. media*: 1405 ± 626 g against 637 ± 313 g (see Figure 7). In both groups, biomass varied greatly, with a standard deviation roughly half the value of the mean. Neither protozoa and AMF treatments nor any interaction between treatments did show significant impact (see Table 1). The position of the mesocosm in the climate chamber during the first week of the growing period, although seemingly impacting the survival rate of the plants at the start of their development, did not have a significant effect on the final biomass of the surviving plants that were selected for sampling.

	Estimate	**Std. Error**	**t value**	***P*-value**
(Intercept)	1370.99	145.34	9.43	<0.001 ***
Plant species	-767.59	130.42	-5.89	<0.001 ***
Position week 1	52.09	129.70	0.40	0.689
AMF inoculation	35.91	129.41	0.28	0.782
Amoeba inoculation	-26.59	129.48	-0.21	0.838

Table 1: Linear model of plant growth

3.1.2 Nitrogen content of plant tissues

The total nitrogen content of plant tissues, split between above- and below-ground, showed a strong decrease with increasing total biomass of the plant, with on average a lower amount of nitrogen below-ground (see Figure 8). No effect from either treatment was found to be significant (see Table 2).

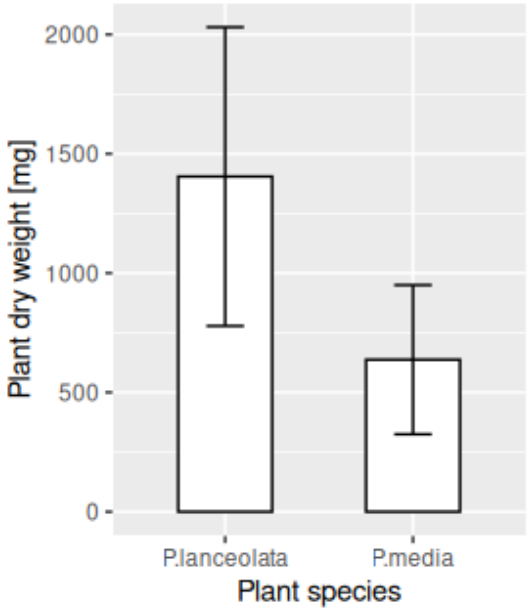

Figure 7: Whole Plant Biomass

	Estimate	**Std. Error**	**t value**	***P*-value**
(Intercept)	13.65	0.92	14.85	<0.001 ***
Plant biomass (log-transformed)	-1.60	0.13	-12.32	<0.001 ***
Plant species	0.47	1.53	0.31	0.760
AMF inoculation	<0.01	0.12	0.03	0.974
Amoeba inoculation	0.18	0.12	1.51	0.137
Plant biomass x species	-0.07	0.23	-0.32	0.753

Table 2: Linear model of nitrogen content in plant leaf tissue

3.1.3 ^{15}N in plant tissues

The δ^{15}N value of plant biomass, both above- and below-ground and excluding outliers, was measured between 11 and 16, meaning that all plants showed values clearly above non-labeled conditions, with plants growing on the Jena Experiment site having a value of 2 on average (unpublished data). Across the data set, a very strong correlation was found between the above- and below-ground matter of an individual plant ($r = 0.613$, $p < 0.001$). Although neither of our treatments had by themselves any significant effects on the measured δ^{15}N values (see Figure 9), linear models built using stepwise regression (see Tables 3 and 4) showed significant increase of ^{15}N enrichment in both

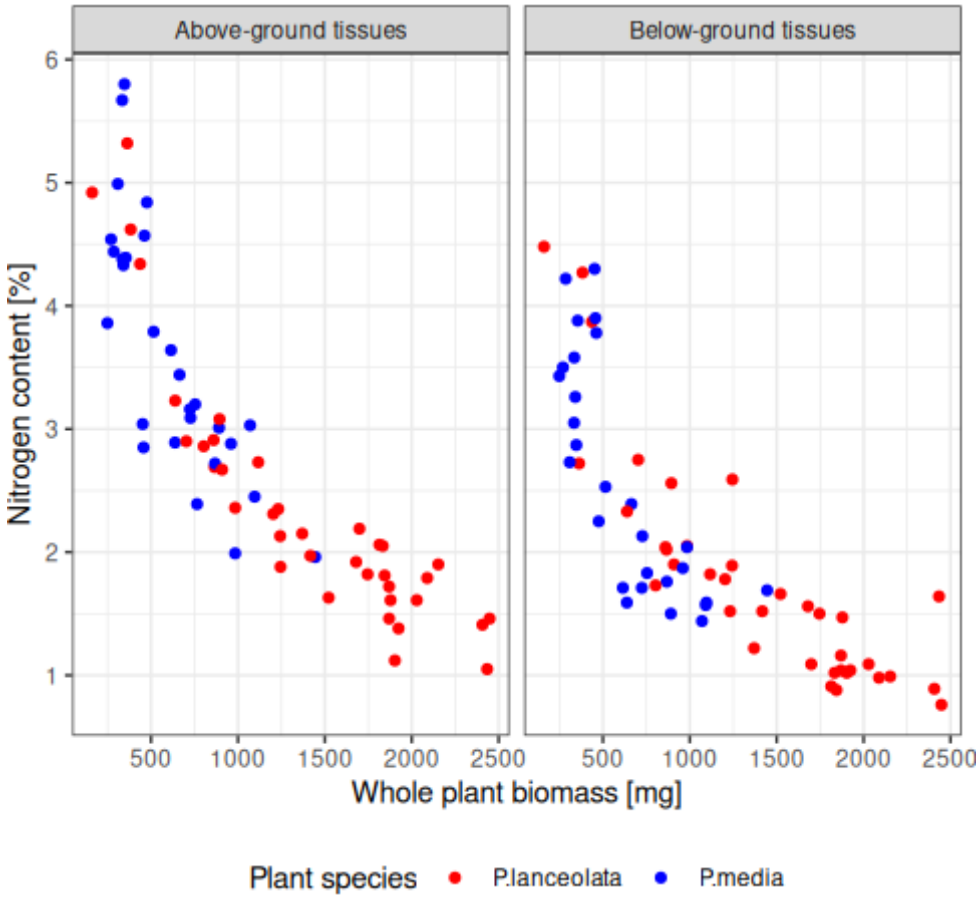

Figure 8: Nitrogen content of plant tissue against plant growth

above- and below-ground tissues. This effect seems to affect mostly smaller plants of the *P.lanceolata* species (see Figure 10). The same models also consistently showed that plants that were smaller or of the *P.media* species were less enriched.

	Estimate	**Std. Error**	**t value**	***P*-value**
(Intercept)	4.87	2.15	2.26	0.028 *
Plant biomass (log-transformed)	1.32	0.31	4.27	<0.001 ***
Plant species	-6.28	3.28	-1.92	0.061 .
AMF inoculation	5.78	2.57	2.25	0.029 *
Plant biomass x species	0.97	0.50	1.95	0.056 .
Plant biomass x AMF	-0.82	0.38	-2.17	0.035 *

Table 3: Linear model of $\delta^{15}N$ in plant above-ground tissues

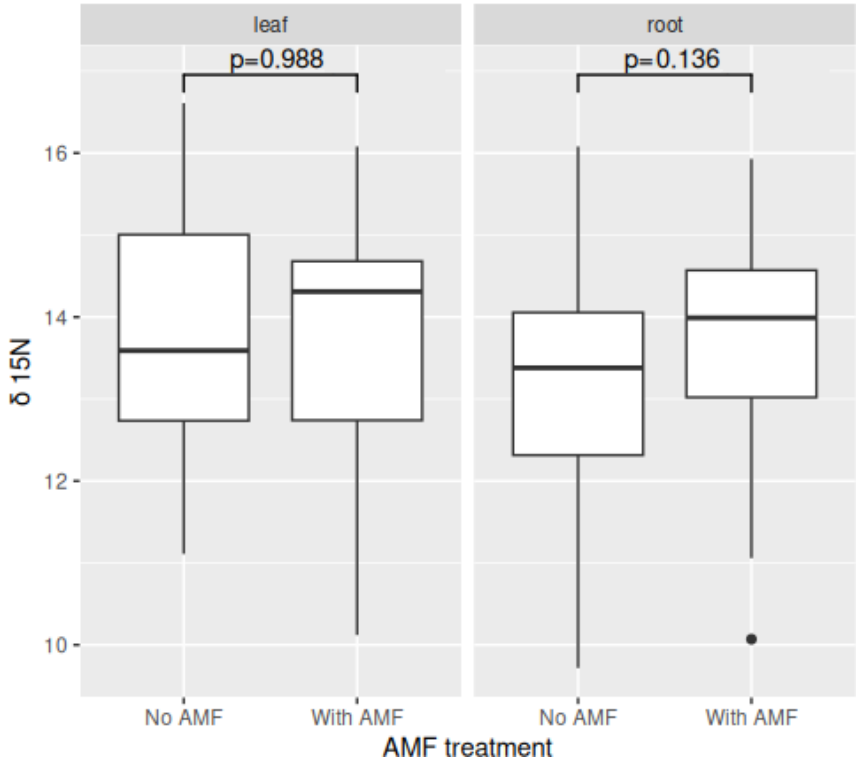

Figure 9: δ^{15}N in leaf and root tissue

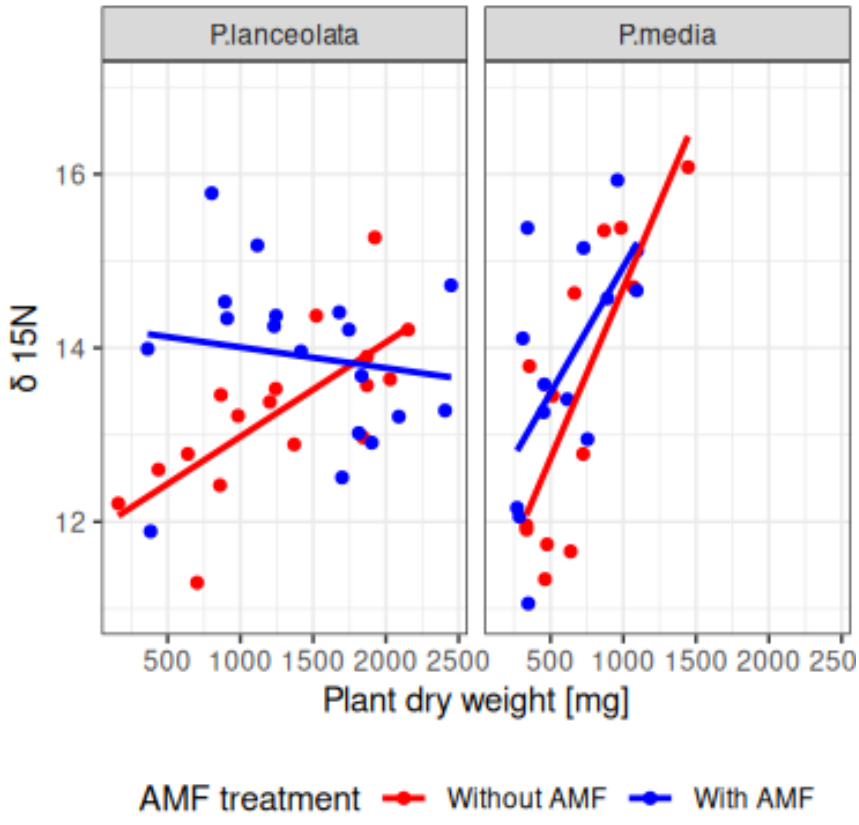

Figure 10: 15N δ in root content

	Estimate	Std. Error	t value	*P*-value
(Intercept)	7.54	2.22	3.39	0.001 **
Plant biomass (log-transformed)	0.87	0.32	2.70	0.009 **
Plant species	-9.92	3.21	-3.09	0.003 **
AMF inoculation	5.35	2.57	2.08	0.042 *
Amoeba inoculation	-0.48	0.25	-1.94	0.058 .
Plant biomass x species	1.61	0.48	3.33	0.002 **
Plant biomass x AMF	-0.70	0.38	-1.86	0.067 .

Table 4: Linear model of δ^{15}N in plant root tissues

3.2 Microbial community and carbon flow

3.2.1 Lipid markers concentration in soil

Important variations were observed in the total amount of lipid marker in individual samples (see Figure 11). Both plant biomass and the soil water content were very good predictors of this total FA concentration (both with p-values < 0.001 when tested individually in linear models, r-values respectively 0.352 and -0.371), as well as being strongly negatively correlated (p < 0.001, r = -0.787), with high biomass being linked to high FA concentration and low soil water content.

Our six water content controls showed a similar decreasing fatty acid concentration with increasing water content (see Figure 11, r = -0.901, p = 0.014).

Linear models only found correlations between single markers and the biomass of the plant (see Table 5), except for AMF showing some effect from both the plant biomass and species. Additionally, a strong correlation was found between the cyclic Gram- marker and the interaction effect of plant biomass and the *Acanthamoeba* treatment.

In the other microbial groups, requiring Linear Mixed Models for similar analysis (see Table 6), no significant effect could be found.

Following these LMM analyses, markers were also tested individually with linear models. In these models, similarly to LMMs, most of the few correlations found were linked to either plant biomass (10MeC16:0 p = 0.009, aC17:0 p = 0.016) or plant species (aC15:0 p = 0.007, aC17:0 p = 0.045, iC14:0 p = 0.027, iC15:0 p = 0.021). The only other ef-

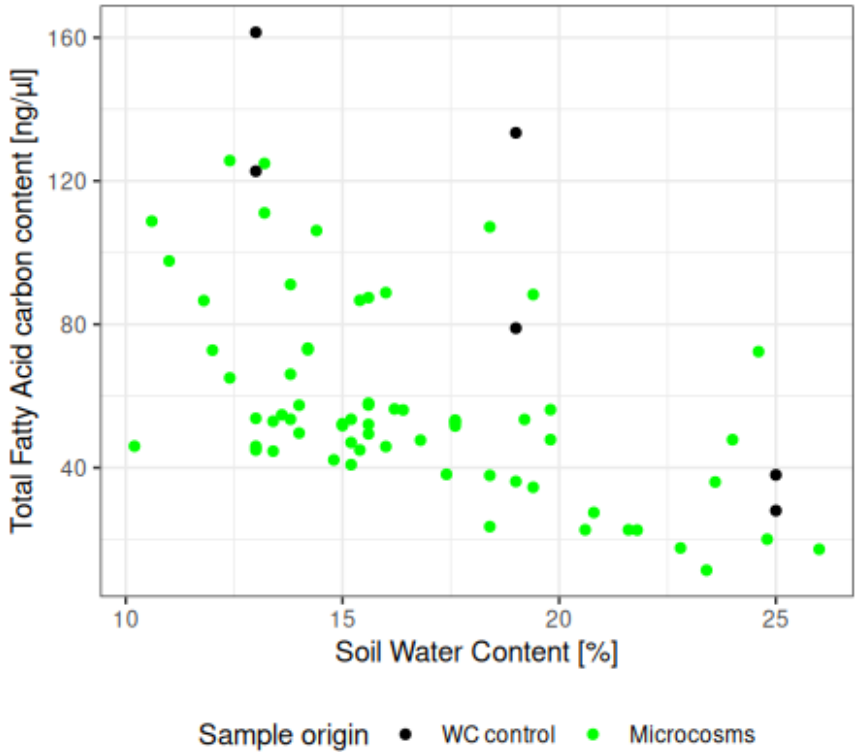

Figure 11: Fatty acid yielded by the extraction process, against sample water content

	Cyclic Gram-		**C18:2n6**		**AMF**	
Added fixed effect	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Plant biomass (log-transformed)	8.21	0.007 **	19.44	<0.001 ***	7.47	0.009 **
Plant species	0.49	0.486	0.07	0.797	4.87	0.033 *
AMF inoculation	<0.01	0.934	1.00	0.324	<0.01	0.949
Amoeba inoculation	0.89	0.352	1.25	0.269	0.01	0.910
Plant biomass x species	<0.01	0.971	0.03	0.868	2.90	0.097 .
AMF x Amoeba	0.06	0.802	1.44	0.237	0.02	0.880
Plant biomass x AMF	0.44	0.511	1.02	0.319	3.08	0.087 .
Plant biomass x Amoeba	11.02	0.002 **	0.03	0.853	0.23	0.636

Table 5: ANOVA of the linear models of FA markers relative abundance

	Gram-		**Gram+**		**Actinobacteria**	
Added fixed effect	Chi^2	*P*-value	Chi^2	*P*-value	Chi^2	*P*-value
Plant biomass (log-transformed)	<0.01	0.944	<0.01	0.966	0.24	0.625
Plant species	<0.01	0.929	1.65	0.198	0.03	0.868
AMF inoculation	0.06	0.801	0.03	0.853	0.53	0.465
Amoeba inoculation	0.03	0.854	0.03	0.869	0.01	0.898
Plant biomass x species	0.09	0.768	<0.01	0.985	<0.01	0.963
AMF x Amoeba	<0.01	0.990	0.24	0.622	0.04	0.840
Plant biomass x AMF	0.04	0.836	1.39	0.238	0.01	0.910
Plant biomass x Amoeba	0.11	0.739	0.10	0.748	<0.01	0.973

Table 6: ANOVA of the linear mixed models of FA markers relative abundance, individual mesocosms as random effect

fects found were C16:1ω7 being slightly less abundant in AMF treatment (p = 0.03) and 10MeC18:0 being found in a higher amount in AMF treatment (p = 0.034) and a lower amount in *Acanthamoeba* treatment (p = 0.052).

3.2.2 ^{13}C in lipid markers

Across the different fatty acids identified, the measured δ^{13}C-values varied strongly, with some compounds showing high ^{13}C enrichment (see Figures in A.3). Phospholipid markers such as C18:1ω7 and C16:1ω7 had δ^{13}C-values measured over 100 on average, and C18:2ω6 with a mean delta of almost 500. Others were close to natural abundance, which was measured at values between -25 and -30 in non-labeled samples. When looking at the APE calculated from these values for each fatty acid, the highly enriched compounds tend to be attributed to Gram- bacteria, with the addition of C18:2ω6. Markers showing low to no enrichment were generally those attributed to Gram+ bacteria, including the *Actinobacteria* markers, and one cyclic Gram- marker (cyC17:0). In the neutral lipids, C16:1ω5, which is attributed to AMF organisms, showed no enrichment on average, with APE in most samples calculated to be below 1.

Comparing ^{13}C enrichment across our treatments was done with Linear Mixed Model again, this time to predict the APE values of the Gram-, Gram+ and *Actinobacteria* groups (see Table 8). In all three groups, correlations are found to the biomass of the

plant, with Gram- and Gram+ having higher APE in the presence of bigger plants and *Actinobacteria* lower APE. *Actinobacteria* also showed significantly higher APE in the AMF treatment, knowing that this group on average showed no enrichment at all. The models fitting data from Gram+ bacteria also found significant effects from two interactions of the AMF treatment, one with the amoeba treatment and one with the plant biomass.

In the three other groups too, linear models showed very little significant effects (see Table 7). Only on C18:2ω6 and NLC16:1ω5 was plant biomass found to have significant effects, with bigger plants leading to higher APE.

	Cyclic Gram-		**C18:2n6**		**AMF**	
Added fixed effect	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Plant biomass (log-transformed)	0.19	0.668	11.43	0.001 **	4.29	0.044 *
Plant species	1.08	0.305	0.06	0.812	0.04	0.851
AMF inoculation	2.22	0.143	0.23	0.633	0.84	0.365
Amoeba inoculation	0.60	0.444	0.66	0.422	0.13	0.723
Plant biomass x species	0.86	0.358	0.23	0.636	0.50	0.482
AMF x Amoeba	0.70	0.409	<0.01	0.944	0.21	0.653
Plant biomass x AMF	0.65	0.423	0.18	0.676	0.01	0.913
Plant biomass x Amoeba	1.35	0.252	2.06	0.159	0.71	0.404

Table 7: ANOVA of the linear models of FA markers ^{13}C APE

	Gram-		**Gram+**		**Actinobacteria**	
Added fixed effect	Chi^2	*P*-value	Chi^2	*P*-value	Chi^2	*P*-value
Plant biomass (log-transformed)	15.98	<0.001 ***	27.31	<0.001 ***	7.54	0.006 **
Plant species	1.37	0.243	0.82	0.367	0.15	0.700
AMF inoculation	2.01	0.157	1.90	0.168	6.02	0.014 *
Amoeba inoculation	0.02	0.876	0.09	0.759	1.02	0.312
Plant biomass x species	1.78	0.182	1.49	0.222	0.80	0.370
AMF x Amoeba	0.70	0.404	3.86	0.049 *	0.43	0.513
Plant biomass x AMF	1.13	0.288	5.01	0.025 *	0.08	0.772
Plant biomass x Amoeba	2.42	0.120	0.51	0.477	0.19	0.664

Table 8: ANOVA of the linear mixed models of FA markers ^{13}C APE, individual mesocosm as random effect in LMM

4 Discussion

In this experiment, the impact of plant productivity, AMF, and bacteria grazers on carbon and nitrogen flow was tested over a period of two months in a mesocosms experiment. Sterile soil was inoculated with a naturally occurring bacteria community, and then, following a full factorial design, some mesocosms were inoculated with protists (*Acanthamoeba castellanii*) and/or AMF (*Rhizophagus irregularis*) and planted with either the fast-growing *Plantago lanceolata* or the slower, more conservative *Plantago media*. Results consistently showed that the strongest effect on both carbon and nitrogen flow was the biomass of the plant, which in turn was caused by the difference in growth strategy from the plant species, and that our microbial treatments had no significant effect, except for the AMF inoculation being linked to a slight increase in nitrogen content of the some of the plants. One of the main issues encountered during this experiment was the discrepancy in plant growth.

4.1 Plant biomass effect

As the plants grew, a stark difference in their growth rates became apparent. Research in plant growth did show a potential for healthy growth passed the limit observed in nature, suggesting that this limit in growth is not due to resource availability (Parsons et al., 2013), and therefore that our plant species, which had access to the same resources, could show differences in their growth. But after sampling, substantial variations in total biomass were measured, not only between the two *Plantago* species, which was expected, but also among individual plants of the same species. This is thought to be in part due to the harsh conditions for the young seedlings when they were transferred in the climate chamber, where a high flow of dryer air caused leaves to suffer drought stress, in some cases showing visible damage and even causing some seedlings to die at this stage. This likely created heterogeneity in the conditions at the start of the development of the plants, with some having to lag behind in their growth due to these initial damages, as was previously shown in trials on seedlings (Hanley and May, 2006).

Alternatives include waiting longer to transplant bigger seedlings and covering the mesocosms with a transparent container, such as plastic bags, during their first days in the climate chambers. Under this cover, humidity could have been kept high, and

the effect strong air flow inside the chamber would have been avoided. This would have created a transition phase for the seedlings, during which they could have developed further while adapting progressively to the lower humidity inside the chamber compared to the closed Magenta vessels where they started their growth. With an easier and more equal starting condition and a higher survival rate, the final batch of sampled mesocosms could have constituted more comparable and numerous replicates.

This high variation in biomass of the plants had an array of consequences for the rest of the experiment. Higher production of exudates is expected from plants with higher biomass, and more capacity to absorb the $^{13}CO_2$ label. It also impacted the water consumption of the plants, which caused our soil samples to be strongly dissimilar in their moisture level. This difference in water content in turn affected the efficiency of our lipid extraction, despite the amount of dry soil matter being kept equivalent. This was shown by our "water content control samples", where several samples of the same soil at different moisture level yielded variable amount of fatty acid (see Figure 11), which is coherent with previous findings (Fu et al., 2021) and demonstrate that this effect was not due to higher quantity of exudation by bigger plants.

One change might have improved the consistency of the lipid extraction efficiency: by adapting the amount of phosphate buffer (mostly made up of water) in the solvent mixture used during this step. When soil samples with higher water content were being processed, the proportion of buffer injected should have been lowered so that the total amount of water in the extraction cells would have remained similar across samples with different soil moisture. This correction would have kept the conditions inside the extraction cells more homogeneous between the samples and potentially reduced the variability brought to the lipid marker analysis by the difference in water content, itself caused by the discrepancy in plant growth.

In nitrogen measurements of plant matter, too, this wide variability in plant biomass had a clear impact. Results show that plant matter in all treatments were measurably enriched, but that the bigger plants were able to absorb more ^{15}N label as their well-developed root systems had better access to the labeled resource patch.

4.2 Plant growth strategy effect

Beside this clear effect of plant biomass, an increase from *P.media* to *P.lanceolata* suggested that the difference in strategy regarding resource foraging between the two species did make a small but significant change to the nitrogen flow. This was limited to a subset of smaller plants, whereas the total exclusion of AMF organisms by soil sterilization was expected to have much more noticeable effect on plant nutrition and exudation (Chen et al., 2016). Interestingly, the AMF treatment seemed to compensate the decrease in labeled nitrogen intake seen in other smaller-sized *P.lanceolata* that did not receive inoculum (see Figure 10). This might suggest that in this plants the AMF functionally replaced the roots that the plant had not grown yet (Henkes et al., 2018), but this effect is absent in *P.media* which did not show any impact from the AMF treatment. Being more conservative in its resource management, *P.media* might not be as inclined to use AMF to intensify its foraging (as shown in Thakur et al., 2019). Overall, the impact of our AMF treatment on the nitrogen flow was very limited compared to similar setup (Nuccio et al., 2013, Ingraffia et al., 2019).

4.3 Mycorrhiza treatment effect

When looking at the result of our soil lipid analysis, the abundance of AMF organisms or their activity did not seem to be impacted by the AMF inoculation, including in the *P.lanceolata* subset that showed a relative increase in nitrogen uptake in the presence of AMF. This suggests that AMF did not develop in any significant manner in the mesocosms and that it was not feeding on plant-derived carbon at the time of the labeling, despite being inoculated right at the start of the growth of the plants. This was totally unexpected, knowing that *Plantago* is often used in plant-AMF experiments and showing good root colonization (Thakur et al., 2019), and that in previous studies AMF showed high enrichment, consistent with their strong reliance on plant-derived carbon for nutrition (Blažková et al., 2021, Mielke et al., 2022).These results lead us to the conclusion that either the ecological conditions inside the mesocosms did not allow AMF to develop or the water and nutrient availability in the soil were such that plants did not need to rely on AMF for foraging, as it has been shown that lower nutrient amount and drier conditions in soil increase the colonization of root by AMF (Tahat and Sijam, 2012, Monokrousos et al., 2020, M. Wang et al., 2023). In both cases, this finding sug-

gests that our mesocosms setup did not allow the AMF treatment to play its part fully, limiting the insight that could be gained from such experiment.

4.4 Bacteria grazer effect

The amoeba treatment had no significant impact on nitrogen acquisition by the plants, which contradict previous research on the role of this bacteria grazer (Bonkowski, 2004, Zwart, 1994, Clarholm, 1989, Hunt et al., 1977). Similarly, there was no convincing evidence of any impact from the *Acanthamoeba* treatment on the bacteria community structure or its metabolic activity, contrary to our expectation (Clarholm, 1981, Griffiths, 1994). As there is no direct proof of presence or absence available, because no specific biomarkers could be found for this organism, it is not possible to say whether this is due to the amoeba not being able to establish itself and develop enough in our mesocosms to have an impact, or if it was indeed present but still did not have an impact. Another question is left unanswered because of this lack evidence. Contamination might have prevented this treatment from showing any shift in the bacteria community or difference in the nutrient flow. Although contamination is always possible in such a setup, it seems unlikely that a protozoan contamination would have affected a high number of mesocosms (Ali, 2017) to the point of preventing any effect from the inoculation.

Contrary to the hypothesized potential impact of the AMF-protozoa treatments interaction on nutrient flow (Koller et al., 2013, Griffiths, 1994, Bonkowski, 2004), no effect of this particular interaction appear in our results. This can be easily explained by the lack of effects from these two treatments individually.

4.5 Setup limitation and proposed improvements

When looking for possible explanations for the lack of significant impact from the AMF and the Amoeba treatments, several suspicions of unsuitable conditions inside the mesocosm flasks arose. Keeping the mesocosms free of external contaminants required airtight sealing of the flasks, with only a narrow tubing allowing sterile gas exchange. A limited amount of oxygen could have prevented the development of both AMF and *Acanthamoeba*, but it would also have affected the plant roots which did de-

velop normally. Studies on their metabolic activity have shown that low oxygenation conditions are well tolerated by both amoeba (Cometa et al., 2011) and fungi (Pavarina and Durrant, 2002) but not by plant roots (Geigenberger, 2003) which have the additional constrain of having thick layers of cells that need to be provided with oxygen, contrary to microorganisms. We can therefore assume that as long as the roots had enough oxygen to develop, soil microorganisms did too.

Another problem with enclosed soil was keeping a suitable water level inside. Although sterile watering was easily done, the absence of draining holes at the bottom of the flasks meant that they could also easily be overwatered. During sampling of the soil, it was clear that the 80% Field Capacity chosen as the maximum amount for watering was too high, as a portion of soil at the bottom of some of the mesocosms was found to be water-logged. Again, given that plant roots did develop normally in all mesocosms, including in the wetter bottom of the flasks, this can not explain the absence of the inoculated organisms. But more limited soil moisture might have promoted AMF root colonization during plant growth (Monokrousos et al., 2020, Williams and de Vries, 2020)).

Nutrition is another factor that might have promoted plant interaction with AMF. The arguably relatively low luminosity in the climate chamber of 280 μmol m^{-2} s^{-1} (Chiang et al., 2020) could have limited the amount of available photosynthesized carbon that the plants could spend on root exudates and transfer to the AMF (Rovira, 1959). Guaranteeing good lighting for plants might have helped promote carbon availability in the rhizosphere, which could have positively influenced the establishment of the inoculation of AMF and *Acanthamoeba* at the start of the experiment (Cortés-Pérez et al., 2023). These different parameters, although they might have played a role in limiting the potential growth of AMF, are not sufficient explanations for the lack of development that has been observed in our soil lipid analysis.

Although the $^{13}CO_2$ pulse labeling was found to produce the needed effect for our analyses, with the difference in lipid marker enrichment coherent and varying with the conditions inside individual mesocosms, the ^{15}N labeling led to a limited variability in plant matter enrichment, making the potential difference caused by the treatment effect harder to detect. The ^{15}N labeled necromass patch, which was positioned at the bottom

of the flask to allow root access only later in the plant growth, seems to have been a source of nitrogen for the plant roughly similarly throughout their development, only showing a slight increase as the plants grow. Previous experiments using ^{15}N labeled substrate created distinct compartments, physically allowing or limiting the access of plant roots, mycorrhizal hyphae, or protozoa to labeled resources (Koller et al., 2013, Chowdhury et al., 2022). Although more complex, such setups have the advantage of offering a straight-forward indication of the role played by the different organisms. Here, the measured nitrogen flow did not fluctuate strongly with the treatment effects. Improvements to this setup might include placing the necromass pellet higher in the flask to reduce the bias in absorption by plants with differently developed root systems, or a later addition of the labeled substrate. Additionally, a washing of the soil can be performed during the sterilization steps to reduce the amount of available nutrients at the start of the growth period. This could potentially promote the use by plants of the labeled resources, as well as increasing their reliance on organisms such as mycorrhiza and protozoa for nutrition, as nutrients would be harder to forage from soil (Pankoke et al., 2015).

Future experimentation with a similar setup might also need to multiply the model organisms used, as previous research has found that using different species categorized as mycorrhizal fungi or bacteria grazers can lead to very different results, as they do not all have the same interactions with other soil microbes or plants (Powell and Rillig, 2018, Thakur et al., 2019).

4.6 Significant contribution from plants to the C18:2ω6 PLFA marker

One lipid marker used here showed a very unexpected pattern: C18:2ω6. This compound is typically used as a marker for saprophytic fungi (Willers et al., 2015), but here was found t be highly correlated to the plant biomass, both in its quantity and its ^{13}C enrichment, while being systematically much more enriched than any other markers (see Figure 13). In similar experiments where saprophytic fungi are present, this marker is found in higher concentrations, but typically shows little enrichment (Mielke et al., 2022, Denef et al., 2007, Elias et al., 2017). The very high ^{13}C enrichment measured here is confirmation that plants are the most likely source of C18:2ω6 in our samples, as was

previously suspected (Frostegård et al., 2011, Joergensen, 2022). Given this observation, more attention should be paid to the use of this marker to estimate saprophytic fungi metabolism. In a natural setting, it is still highly likely that most of this fatty acid will originate from fungi, hence its use as a marker in the literature (Willers et al., 2015). But in future research, attention must be paid to the fact that a portion of this compound, when found in soil, actually originates from root metabolism. Especially when using $^{13}CO_2$ labeling as we did, as it is likely that the plant-derived C18:2ω6 will have a disproportionate impact on the overall enrichment of this marker, making it unreliable as an indication of the flow of plant-derived carbon to saprophytic fungi.

5 Conclusion

In contrast to our expectations, the AMF and amoeba inoculation treatments did not have any profound impact on the flow of labeled nitrogen or carbon through our rhizosphere mesocosms. An important doubt remains as to whether these organisms did actually develop and had the possibility of producing such effects. Particularly, the AMF treatment, despite correlating to a small increase in nitrogen uptake by some of the plants, did not lead to any enrichment of the AMF biomarker, which means that this organism was not metabolizing any significant amount of plant-derived carbon at the time. This suggests that the AMF would not have been active during the labeling period. Our plant treatment, the two *Plantago* species, only showed a very limited difference in their impact on these nutrient flows, with *P.lanceolata* taking up more labeled nitrogen in its root tissue when compared to *P.media* of the same biomass. This lack of observable effect from the plant growth strategy might have been caused by the wide variation in total plant growth over the duration of this experiment. Throughout our results, this difference in plant production was systematically found to be the most important cause of variation in the measured variables by our statistical analyses. It is therefore possible that the same analyses would have had a better chance of detecting treatment effects if the plant growth had been more homogeneous across our samples.

One valuable insight gained during this experiment has been the unexpected enrichment of the phopholipid C18:2ω6. Typically used as a marker for saprophytic fungi, this compound was still found to be ubiquitous in our samples despite the exclusion of any other fungi than AMF. Contrary to previous data from setup that included saprophytic fungi, C18:2ω6 was here found in relatively small concentration, but with an extremely high ^{13}C enrichment compared to any other compound measured here. This confirms previous suggestions that this lipid marker is not specific to saprophytic fungi but can also originate from plant roots. Future experiments using isotope probing in combination with PLFA markers will need to take this into account before drawing conclusions when measuring ^{13}C enrichment of this compound.

Although such experiments can be complicated to setup and require good attention to details to yield usable results, lipid markers and stable isotope probing still have the potential to bring unique insight on the role that the huge diversity of interactions between soil organisms play on the nutrient cycles in nature. Manipulating these interactions

might necessitate convoluted experimental setups, but by learning from unsuccessful trials, they can be improved to become more reliable.

6 Summary

Soil bacteria are essential to nutrient recycling and plant nutrition, but they tend to immobilize nutrients, making them unavailable to plant roots. Other microorganisms, such as arbuscular mycorrhizal fungi (AMF) and protozoan bacteria grazers, play an important role in improving plant access to soil resources. Although their individual impacts on plant nutrition have been well studied, there is still limited data on their combined effects and interactions. Creating rhizosphere mesocosms where these organisms were excluded or inoculated, we tested their effects on the carbon and nitrogen exchange between plant roots and rhizosphere microorganisms, using either fast- or slow-growing plant species to manipulate the carbon input by roots.

The flows of both carbon and nitrogen through our mesocosm soil was measured using stable isotope probing (SIP). A patch of ^{15}N enriched bacterial necromass was added to the soil, and the isotopic abundance of nitrogen was later measured in plant biomass. For carbon, we placed our mesocosms in chambers with a high amount of $^{13}CO_2$ to do a pulse labeling and measured the resulting ^{13}C enrichment in lipid biomarkers found in soil. The enrichment rate in both ^{15}N and ^{13}C is directly linked to the nutrient exchange in the soil.

The lack of significant effect from any of our treatments, due to the highly heterogeneous plant growth and the apparent lack if development of our AMF and protozoa inoculum, meant that no hindsight was gained on the questions of the nutrient flows in the rhizosphere that we hoped to investigate. But several recommendations for potential improvements are made, with optimistic expectations for a future iteration of a similar setup. Unexpectedly, sterile conditions in the mesocosms allowed for an interesting observation on the abundance and ^{13}C enrichment of one lipid marker, the phopholipid C18:2ω6, despite the absence of saprophytic fungi, to which it is usually associated. We conclude that this compound originated from plant roots, leading us to advise caution in any future use of this biomarker, especially in the context of carbon

labeling.

7 Zusammenfassung

Bodenbakterien spielen für die Nährstoffkreisläufe und die Nährstoffaufnahme durch Pflanzen eine entscheidende Rolle. Sie neigen aber auch dazu Nährstoffe zu binden, sodass sie den Pflanzen zur Aufnahme über die Wurzeln nicht zur Verfügung stehen. Andere Mikroorganismen wie arbuskuläre Mykorrhizapilze (AMF) und bakterienfressende Protozoen hingegen können die Nährstoffverfügbarkeit für Pflanzen wesentlich verbessern. Während der individuelle Einfluss dieser Organismen auf die Nährstoffaufnahme von Pflanzen bereits gut erforscht ist, ist die Datenlage zu kombinierten Effekten und Interaktionen zwischen den Organismengruppen noch begrenzt. In unserem Versuchsaufbau mit Mesokosmen in deren Rhizosphären bestimmte Organismen hinzugefügt oder ausgeschlossen wurden, untersuchten wir deren Einfluss auf den Austausch von Kohlenstoff und Stickstoff zwischen den Pflanzenwurzeln und Rhizosphären-Mikroorganismen. Dabei wurden entweder schneller oder langsam wachsende Pflanzen verwendet, um den Kohlenstoffeintrag über die Wurzeln zu manipulieren.

Zur Untersuchung der Kohlenstoff- und Stickstoffflüsse zwischen Pflanzenwurzeln und Rhizosphären-Mikroorganismen verwendeten wir in unseren Mesokosmen-Versuchen stabile Isotopenmarkierungen (SIP). Dem Boden wurde ^{15}N-angereichertes Material aus bakterieller Nekromasse zugesetzt, um später die Isotopenhäufigkeit in der Pflanzenbiomasse zu messen. Zur Verfolgung des Kohlenstoffflusses erfolgten Pulsmarkierungen der Mesokosmen in Kammern mit erhöhter $^{13}CO_2$-Konzentration, als auch Messungen der resultierenden ^{13}C Anreicherung in Lipid-Biomarkern im Boden. Die Anreicherungsraten sowohl von ^{15}N als auch ^{13}C stehen in direktem Zusammenhang mit dem Nährstoffaustausch im Boden.

Das Ausbleiben signifikanter Effekte unserer Behandlungen aufgrund des stark heterogenen Pflanzenwachstums und des fehlenden offensichtlichen Entwicklungsfortschritts unseres AMF- und Protozoen-Inokulums bedeutete, dass keine Erkenntnisse zu den Fragen des Nährstoffflusses in der Rhizosphäre gewonnen werden konnten. Es werden

deshalb mehrere Verbesserungsvorschläge erörtert, deren Einbezug in zukünftige Versuchsplanungen mit ähnlichem Aufbau eindeutigere Ergebnisse hervorbringen könnte.

Allerdings ergaben sich aus den sterilen Bedingungen in den Mesokosmen interessante Beobachtungen zur Häufigkeit und ^{13}C-Anreicherung eines Lipidmarkers, des Phospholipids C18:2ω6, dessen Vorkommen normalerweise mit saprophytischen Pilzen assoziiert ist. Daraus schließen wir, dass diese Verbindung möglicherweise auch aus Pflanzenwurzeln stammen kann, was bei zukünftiger Verwendung dieses Biomarkers berücksichtigt werden sollte, insbesondere im Kontext von Kohlenstoffmarkierungen.

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9 References

Ali, S. (2017). Microbial and viral contamination of animal and stem cell cultures: Common contaminants, detection and elimination. *Journal of Stem Cell Research & Therapeutics*, *2*(5). https://doi.org/10.15406/jsrt.2017.02.00078

Asghari, H. R., & Cavagnaro, T. R. (2012). Arbuscular mycorrhizas reduce nitrogen loss via leaching. *PLOS ONE*, *7*(1), e29825. https://doi.org/10.1371/journal.pone.0029825

Averill, C., Bhatnagar, J. M., Dietze, M. C., Pearse, W. D., & Kivlin, S. N. (2019). Global imprint of mycorrhizal fungi on whole-plant nutrient economics. *Proceedings of the National Academy of Sciences*, *116*(46), 23163–23168. https://doi.org/10.1073/pnas.1906655116

Azam, F., Fenchel, T., Field, J., Gray, J., Meyer-Reil, L., & Thingstad, F. (1983). The ecological role of water-column microbes in the sea. *Marine Ecology Progress Series*, *10*, 257–263. https://doi.org/10.3354/meps010257

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01

Bender, S. F., & van der Heijden, M. G. (2015). Soil biota enhance agricultural sustainability by improving crop yield, nutrient uptake and reducing nitrogen leaching losses. *Journal of Applied Ecology*, *52*(1), 228–239. https://doi.org/10.1111/1365-2664.12351

Blažková, A., Jansa, J., Püschel, D., Vosátka, M., & Janoušková, M. (2021). Is mycorrhiza functioning influenced by the quantitative composition of the mycorrhizal fungal community? *Soil Biology and Biochemistry*, *157*, 108249. https://doi.org/10.1016/j.soilbio.2021.108249

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917. https://doi.org/10.1139/o59-099

Blom, C. W. P. M. (1978). Germination, seedling emergence and establishment of some plantago species under laboratory and field conditions. *Acta botanica neerlandica*, *27*(5/6), 257–271. https://natuurtijdschriften.nl/pub/540234

Bonfante, P., & Genre, A. (2010). Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. *Nature Communications*, *1*(1), 48. https://doi.org/10.1038/ncomms1046

Bonkowski, M., & Brandt, F. (2002). Do soil protozoa enhance plant growth by hormonal effects? *Soil Biology and Biochemistry*, *34*(11), 1709–1715. https://doi.org/10.1016/S0038-0717(02)00157-8

Bonkowski, M., Jentschke, G., & Scheu, S. (2001). Contrasting effects of microbial partners in the rhizosphere: Interactions between norway spruce seedlings (*picea abies* karst.), mycorrhiza (*paxillus involutus* (batsch) fr.) and naked amoebae (protozoa). *Applied Soil Ecology*, *18*(3), 193–204. https://doi.org/10.1016/S0929-1393(01)00165-2

Bonkowski, M. (2004). Protozoa and plant growth: The microbial loop in soil revisited. *New Phytologist*, *162*(3), 617–631. https://doi.org/10.1111/j.1469-8137.2004.01066.x

Breulmann, M., Masyutenko, N. P., Kogut, B. M., Schroll, R., Dörfler, U., Buscot, F., & Schulz, E. (2014). Short-term bioavailability of carbon in soil organic matter fractions of different particle sizes and densities in grassland ecosystems. *Science of The Total Environment*, *497–498*, 29–37. https://doi.org/10.1016/j.scitotenv.2014.07.080

Bronstein, J. L. (1994). Our current understanding of mutualism. *The Quarterly Review of Biology*, *69*(1), 31–51. https://doi.org/10.1086/418432

Bunn, R. A., Simpson, D. T., Bullington, L. S., Lekberg, Y., & Janos, D. P. (2019). Revisiting the 'direct mineral cycling' hypothesis: Arbuscular mycorrhizal fungi colonize leaf litter, but why? *The ISME Journal*, *13*(8), 1891–1898. https://doi.org/10.1038/s41396-019-0403-2

Chapin, F. S., Matson, P. A., & Vitousek, P. M. (2011). Water and energy balance. In F. S. Chapin, P. A. Matson, & P. M. Vitousek (Eds.), *Principles of terrestrial ecosystem ecology* (pp. 93–122). https://doi.org/10.1007/978-1-4419-9504-9_4

Chen, W., Koide, R. T., Adams, T. S., DeForest, J. L., Cheng, L., & Eissenstat, D. M. (2016). Root morphology and mycorrhizal symbioses together shape nutrient foraging strategies of temperate trees. *Proceedings of the National Academy of Sciences*, *113*(31), 8741–8746. https://doi.org/10.1073/pnas.1601006113

Chiang, C., Bånkestad, D., & Hoch, G. (2020). Reaching natural growth: Light quality effects on plant performance in indoor growth facilities. *Plants*, *9*(10), 1273. https://doi.org/10.3390/plants9101273

Chowdhury, S., Lange, M., Malik, A. A., Goodall, T., Huang, J., Griffiths, R. I., & Gleixner, G. (2022). Plants with arbuscular mycorrhizal fungi efficiently acquire nitrogen from substrate additions by shaping the decomposer community composition and their net plant carbon demand. *Plant and Soil*, *475*(1), 473–490. https://doi.org/10.1007/s11104-022-05380-x

Clarholm, M. (1989). Effects of plant-bacterial-amoebal interactions on plant uptake of nitrogen under field conditions. *Biology and Fertility of Soils*, *8*(4), 373–378. https://doi.org/10.1007/BF00263171

Clarholm, M. (1981). Protozoan grazing of bacteria in soil—impact and importance. *Microbial Ecology*, *7*(4), 343–350. https://doi.org/10.1007/BF02341429

Clarholm, M. (1985). Interactions of bacteria, protozoa and plants leading to mineralization of soil nitrogen. *Soil Biology and Biochemistry*, *17*(2), 181–187. https://doi.org/10.1016/0038-0717(85)90113-0

Cometa, I., Schatz, S., Trzyna, W., & Rogerson, A. (2011). Tolerance of naked amoebae to low oxygen levels with an emphasis on the genus acanthamoeba. *Acta Protozoologica*, *Vol. 50.1*, 33–41. https://scholarworks.calstate.edu/concern/publications/1z40kt51r

Cortés-Pérez, S., Ferrera-Cerrato, R., Rodríguez-Zaragoza, S., & Alarcón, A. (2023). Short-term evaluation of the spatial distribution of trophic groups of amoebae in the rhizosphere of zea mays inoculated with rhizophagus intraradices. *Microbial Ecology*, *86*(2), 997–1009. https://doi.org/10.1007/s00248-022-02132-3

Davoudpour, Y., Schmidt, M., Calabrese, F., Richnow, H. H., & Musat, N. (2020). High resolution microscopy to evaluate the efficiency of surface sterilization of zea mays seeds. *PLOS ONE*, *15*(11), e0242247. https://doi.org/10.1371/journal.pone.0242247

Denef, K., Bubenheim, H., Lenhart, K., Vermeulen, J., Van Cleemput, O., Boeckx, P., & Müller, C. (2007). Community shifts and carbon translocation within metabolically-active rhizosphere microorganisms in grasslands under elevated co_2. *Biogeosciences*, *4*(5), 769–779. https://doi.org/10.5194/bg-4-769-2007

Dumont, M. G., & Murrell, J. C. (2005). Stable isotope probing — linking microbial identity to function. *Nature Reviews Microbiology*, *3*(6), 499–504. https://doi.org/10.1038/nrmicro1162

Egamberdieva, D., Wirth, S. J., Alqarawi, A. A., Abd_Allah, E. F., & Hashem, A. (2017). Phytohormones and beneficial microbes: Essential components for plants to balance stress and fitness. *Frontiers in Microbiology*, *8*. https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2017.02104

Ekelund, F., & Rønn, R. (1994). Notes on protozoa in agricultural soil with emphasis on heterotrophic flagellates and naked amoebae and their ecology. *FEMS Microbiology Reviews*, *15*(4), 321–353. https://doi.org/10.1111/j.1574-6976.1994.tb00144.x

Elias, D. M. O., Rowe, R. L., Pereira, M. G., Stott, A. W., Barnes, C. J., Bending, G. D., & McNamara, N. P. (2017). Functional differences in the microbial processing of recent assimilates under two contrasting perennial bioenergy plantations. *Soil Biology and Biochemistry*, *114*, 248–262. https://doi.org/10.1016/j.soilbio.2017.07.026

Frostegård, Å., Bååth, E., & Tunlio, A. (1993). Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. *Soil Biology and Biochemistry*, *25*(6), 723–730. https://doi.org/10.1016/0038-0717(93)90113-P

Frostegård, Å., Tunlid, A., & Bååth, E. (2011). Use and misuse of plfa measurements in soils. *Soil Biology and Biochemistry*, *43*(8), 1621–1625. https://doi.org/10.1016/j.soilbio.2010.11.021

Fu, Z., Hu, W., Beare, M. H., Müller, K., Wallace, D., & Wai Chau, H. (2021). Contributions of soil organic carbon to soil water repellency persistence: Characterization and modelling. *Geoderma*, *401*, 115312. https://doi.org/10.1016/j.geoderma.2021.115312

Gao, Z., Karlsson, I., Geisen, S., Kowalchuk, G., & Jousset, A. (2019). Protists: Puppet masters of the rhizosphere microbiome. *Trends in Plant Science*, *24*(2), 165–176. https://doi.org/10.1016/j.tplants.2018.10.011

Geigenberger, P. (2003). Response of plant metabolism to too little oxygen. *Current Opinion in Plant Biology*, *6*(3), 247–256. https://doi.org/10.1016/S1369-5266(03)00038-4

Geisen, S., & Quist, C. W. (2021). Microbial–faunal interactions in the rhizosphere. In V. V. S. R. Gupta & A. K. Sharma (Eds.), *Rhizosphere biology: Interactions between*

microbes and plants (pp. 237–253). https://doi.org/10.1007/978-981-15-6125-2_12

Gómez Expósito, R., de Bruijn, I., Postma, J., & Raaijmakers, J. M. (2017). Current insights into the role of rhizosphere bacteria in disease suppressive soils. *Frontiers in Microbiology*, *8*. https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2017.02529

Grey, T. L., Eason, K. M., Wells, L., & Basinger, N. T. (2019). Effects of temperature on seed germination of plantago lanceolata and management in carya illinoinensis production. *Plants*, *8*(9), 308. https://doi.org/10.3390/plants8090308

Griffiths, B. S. (1994). Microbial-feeding nematodes and protozoa in soil: Their effects on microbial activity and nitrogen mineralization in decomposition hotspots and the rhizosphere. *Plant and Soil*, *164*(1), 25–33. https://doi.org/10.1007/BF00010107

Hanley, M. E., & May, O. C. (2006). Cotyledon damage at the seedling stage affects growth and flowering potential in mature plants. *New Phytologist*, *169*(2), 243–250. https://doi.org/10.1111/j.1469-8137.2005.01578.x

Henkes, G. J., Kandeler, E., Marhan, S., Scheu, S., & Bonkowski, M. (2018). Interactions of mycorrhiza and protists in the rhizosphere systemically alter microbial community composition, plant shoot-to-root ratio and within-root system nitrogen allocation. *Frontiers in Environmental Science*, *6*. https://doi.org/10.3389/fenvs.2018.00117

Henneron, L., Kardol, P., Wardle, D. A., Cros, C., & Fontaine, S. (2020). Rhizosphere control of soil nitrogen cycling: A key component of plant economic strategies. *New Phytologist*, *228*(4), 1269–1282. https://doi.org/10.1111/nph.16760

Herdler, S., Kreuzer, K., Scheu, S., & Bonkowski, M. (2008). Interactions between arbuscular mycorrhizal fungi (*glomus intraradices*, glomeromycota) and amoebae (*acanthamoeba castellanii*, protozoa) in the rhizosphere of rice (*oryza sativa*). *Soil Biology and Biochemistry*, *40*(3), 660–668. https://doi.org/10.1016/j.soilbio.2007.09.026

Högberg, M. N., Högbom, L., & Kleja, D. B. (2013). Soil microbial community indices as predictors of soil solution chemistry and n leaching in picea abies (l.) karst. forests in s. sweden. *Plant and Soil*, *372*(1), 507–522. https://doi.org/10.1007/s11104-013-1742-9

Hunt, H. W., Cole, C. V., Klein, D. A., & Coleman, D. C. (1977). A simulation model for the effect of predation on bacteria in continuous culture. *Microbial Ecology*, *3*(4), 259–278. https://doi.org/10.1007/BF02010735

Ingraffia, R., Amato, G., Frenda, A. S., & Giambalvo, D. (2019). Impacts of arbuscular mycorrhizal fungi on nutrient uptake, n2 fixation, n transfer, and growth in a wheat/faba bean intercropping system. *PLoS ONE*, *14*(3). https://doi.org/10.1371/journal.pone.0213672

Joergensen, R. G. (2022). Phospholipid fatty acids in soil—drawbacks and future prospects. *Biology and Fertility of Soils*, *58*(1), 1–6. https://doi.org/10.1007/s00374-021-01613-w

Kaštovská, E., Edwards, K., Picek, T., & Šantrůčková, H. (2015). A larger investment into exudation by competitive versus conservative plants is connected to more coupled plant–microbe n cycling. *Biogeochemistry*, *122*(1), 47–59. https://doi.org/10.1007/s10533-014-0028-5

Koller, R., Rodriguez, A., Robin, C., Scheu, S., & Bonkowski, M. (2013). Protozoa enhance foraging efficiency of arbuscular mycorrhizal fungi for mineral nitrogen from organic matter in soil to the benefit of host plants. *New Phytologist*, *199*(1), 203–211. https://doi.org/10.1111/nph.12249

Krome, K., Rosenberg, K., Dickler, C., Kreuzer, K., Ludwig-Müller, J., Ullrich-Eberius, C., Scheu, S., & Bonkowski, M. (2010). Soil bacteria and protozoa affect root branching via effects on the auxin and cytokinin balance in plants. *Plant and Soil*, *328*(1), 191–201. https://doi.org/10.1007/s11104-009-0101-3

Kudoyarova, G., Arkhipova, T., Korshunova, T., Bakaeva, M., Loginov, O., & Dodd, I. C. (2019). Phytohormone mediation of interactions between plants and non-symbiotic growth promoting bacteria under edaphic stresses. *Frontiers in Plant Science*, *10*. https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2019.01368

Kumar, R., Pandey, S., & Pandey, A. (2006). Plant roots and carbon sequestration. *Current Science*, *91*(7), 885–890. https://www.jstor.org/stable/24094284

Kurepin, L. V., Zaman, M., & Pharis, R. P. (2014). Phytohormonal basis for the plant growth promoting action of naturally occurring biostimulators. *Journal of the Science of Food and Agriculture*, *94*(9), 1715–1722. https://doi.org/10.1002/jsfa.6545

Kuzyakov, Y., & Domanski, G. (2000). Carbon input by plants into the soil. review. *Journal of Plant Nutrition and Soil Science*, *163*(4), 421–431. https://doi.org/10.1002/1522-2624(200008)163:4<421::AID-JPLN421>3.0.CO;2-R

Lambers, H., & Poorter, H. (1992, January 1). Inherent variation in growth rate between higher plants: A search for physiological causes and ecological consequences. In M. Begon & A. H. Fitter (Eds.), *Advances in ecological research* (pp. 187–261, Vol. 23). https://doi.org/10.1016/S0065-2504(08)60148-8

Lange, M., Habekost, M., Eisenhauer, N., Roscher, C., Bessler, H., Engels, C., Oelmann, Y., Scheu, S., Wilcke, W., Schulze, E.-D., & Gleixner, G. (2014). Biotic and abiotic properties mediating plant diversity effects on soil microbial communities in an experimental grassland. *PLOS ONE*, *9*(5), e96182. https://doi.org/10.1371/journal.pone.0096182

Mantelin, S., & Touraine, B. (2004). Plant growth-promoting bacteria and nitrate availability: Impacts on root development and nitrate uptake. *Journal of Experimental Botany*, *55*(394), 27–34. https://doi.org/10.1093/jxb/erh010

Matz, C., & Kjelleberg, S. (2005). Off the hook – how bacteria survive protozoan grazing. *Trends in Microbiology*, *13*(7), 302–307. https://doi.org/10.1016/j.tim.2005.05.009

Mendes, R., Garbeva, P., & Raaijmakers, J. M. (2013). The rhizosphere microbiome: Significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiology Reviews*, *37*(5), 634–663. https://doi.org/10.1111/1574-6976.12028

Mielke, L., Taubert, M., Cesarz, S., Ruess, L., Küsel, K., Gleixner, G., & Lange, M. (2022). Nematode grazing increases the allocation of plant-derived carbon to soil bacteria and saprophytic fungi, and activates bacterial species of the rhizosphere. *Pedobiologia*, *90*, 150787. https://doi.org/10.1016/j.pedobi.2021.150787

Monokrousos, N., Papatheodorou, E. M., Orfanoudakis, M., Jones, D.-G., Scullion, J., & Stamou, G. P. (2020). The effects of plant type, amf inoculation and water regime on rhizosphere microbial communities. *European Journal of Soil Science*, *71*(2), 265–278. https://doi.org/10.1111/ejss.12882

Morriën, E., Hannula, S. E., Snoek, L. B., Helmsing, N. R., Zweers, H., de Hollander, M., Soto, R. L., Bouffaud, M.-L., Buée, M., Dimmers, W., Duyts, H., Geisen, S., Girlanda, M., Griffiths, R. I., Jørgensen, H.-B., Jensen, J., Plassart, P., Redecker, D., Schmelz, R. M., … van der Putten, W. H. (2017). Soil networks become more con-

nected and take up more carbon as nature restoration progresses. *Nature Communications*, *8*(1), 14349. https://doi.org/10.1038/ncomms14349

Novák, V., & Hlaváčiková, H. (2019). *Applied soil hydrology* (Vol. 32). https://doi.org/10.1007/978-3-030-01806-1

Nuccio, E. E., Hodge, A., Pett-Ridge, J., Herman, D. J., Weber, P. K., & Firestone, M. K. (2013). An arbuscular mycorrhizal fungus significantly modifies the soil bacterial community and nitrogen cycling during litter decomposition. *Environmental Microbiology*, *15*(6), 1870–1881. https://doi.org/10.1111/1462-2920.12081

Olsson, P. A., & Lekberg, Y. (2022). A critical review of the use of lipid signature molecules for the quantification of arbuscular mycorrhiza fungi. *Soil Biology and Biochemistry*, *166*, 108574. https://doi.org/10.1016/j.soilbio.2022.108574

Oppenheimer-Shaanan, Y., Jakoby, G., Starr, M. L., Karliner, R., Eilon, G., Itkin, M., Malitsky, S., & Klein, T. (2022). A dynamic rhizosphere interplay between tree roots and soil bacteria under drought stress (M. C. Schuman & A. Weinhold, Eds.). *eLife*, *11*, e79679. https://doi.org/10.7554/eLife.79679

Page, F. C. (1976). *An illustrated key to freshwater and soil amoebae* (Freshwater Biological Association, Ambleside, UK).

Pankoke, H., Höpfner, I., Matuszak, A., Beyschlag, W., & Müller, C. (2015). The effects of mineral nitrogen limitation, competition, arbuscular mycorrhiza, and their respective interactions, on morphological and chemical plant traits of *plantago lanceolata*. *Phytochemistry*, *118*, 149–161. https://doi.org/10.1016/j.phytochem.2015.07.014

Parsons, A. J., Rasmussen, S., Liu, Q., Xue, H., Ball, C., & Shaw, C. (2013). Plant growth – resource or strategy limited: Insights from responses to gibberellin. *Grass and Forage Science*, *68*(4), 577–588. https://doi.org/10.1111/gfs.12035

Pavarina, E. C., & Durrant, L. R. (2002). Growth of lignocellulosic-fermenting fungi on different substrates under low oxygenation conditions. *Applied Biochemistry and Biotechnology*, *98*(1), 663–677. https://doi.org/10.1385/ABAB:98-100:1-9:663

Powell, J. R., & Rillig, M. C. (2018). Biodiversity of arbuscular mycorrhizal fungi and ecosystem function. *New Phytologist*, *220*(4), 1059–1075. https://doi.org/10.1111/nph.15119

R Core Team. (2024). *R: A language and environment for statistical computing*. https://www.R-project.org/

Redecker, D., Kodner, R., & Graham, L. E. (2000). Glomalean fungi from the ordovician. *Science*, *289*(5486), 1920–1921. https://doi.org/10.1126/science.289.5486.1920

Rønn, R., Gavito, M., Larsen, J., Jakobsen, I., Frederiksen, H., & Christensen, S. (2002). Response of free-living soil protozoa and microorganisms to elevated atmospheric co2 and presence of mycorrhiza. *Soil Biology and Biochemistry*, *34*(7), 923–932. https://doi.org/10.1016/S0038-0717(02)00024-X

Rønn, R., McCaig, A. E., Griffiths, B. S., & Prosser, J. I. (2002). Impact of protozoan grazing on bacterial community structure in soil microcosms. *Applied and Environmental Microbiology*, *68*(12), 6094–6105. https://doi.org/10.1128/AEM.68.12.6094-6105.2002

Roscher, C., Schumacher, J., Baade, J., Wilcke, W., Gleixner, G., Weisser, W. W., Schmid, B., & Schulze, E.-D. (2004). The role of biodiversity for element cycling and trophic interactions: An experimental approach in a grassland community. *Basic and Applied Ecology*, *5*(2), 107–121. https://doi.org/10.1078/1439-1791-00216

Rosenberg, K., Bertaux, J., Krome, K., Hartmann, A., Scheu, S., & Bonkowski, M. (2009). Soil amoebae rapidly change bacterial community composition in the rhizosphere of arabidopsis thaliana. *The ISME Journal*, *3*(6), 675–684. https://doi.org/10.1038/ismej.2009.11

Rovira, A. D. (1959). Root excretions in relation to the rhizosphere effect. *Plant and Soil*, *11*(1), 53–64. https://doi.org/10.1007/BF01394753

Rovira, A. D. (1969). Plant root exudates. *The Botanical Review*, *35*(1), 35–57. https://doi.org/10.1007/BF02859887

Rutherford, P. M., & Juma, N. G. (1992). Influence of texture on habitable pore space and bacterial-protozoan populations in soil. *Biology and Fertility of Soils*, *12*(4), 221–227. https://doi.org/10.1007/BF00336036

Saeed, Q., Xiukang, W., Haider, F. U., Kučerik, J., Mumtaz, M. Z., Holatko, J., Naseem, M., Kintl, A., Ejaz, M., Naveed, M., Brtnicky, M., & Mustafa, A. (2021). Rhizosphere bacteria in plant growth promotion, biocontrol, and bioremediation of contaminated sites: A comprehensive review of effects and mechanisms. *International Journal of Molecular Sciences*, *22*(19), 10529. https://doi.org/10.3390/ijms221910529

Sindhu, S. S., Rakshiya, Y. S., & Sahu, G. (2009). Biological control of soilborne plant pathogens with rhizosphere bacteria. *Pest Technol*, *3*(1), 10–21.

Sollins, P., & Gregg, J. W. (2017). Soil organic matter accumulation in relation to changing soil volume, mass, and structure: Concepts and calculations. *Geoderma*, *301*, 60–71. https://doi.org/10.1016/j.geoderma.2017.04.013

Steinauer, K., Tilman, D., Wragg, P. D., Cesarz, S., Cowles, J. M., Pritsch, K., Reich, P. B., Weisser, W. W., & Eisenhauer, N. (2015). Plant diversity effects on soil microbial functions and enzymes are stronger than warming in a grassland experiment. *Ecology*, *96*(1), 99–112. https://doi.org/10.1890/14-0088.1

Tahat, M. M., & Sijam, K. (2012). Mycorrhizal fungi and abiotic environmental conditions relationship. *Research Journal of Environmental Sciences*, *6*(4), 125–133. https://doi.org/10.3923/rjes.2012.125.133

Tedersoo, L., Sánchez-Ramírez, S., Kõljalg, U., Bahram, M., Döring, M., Schigel, D., May, T., Ryberg, M., & Abarenkov, K. (2018). High-level classification of the fungi and a tool for evolutionary ecological analyses. *Fungal Diversity*, *90*(1), 135–159. https://doi.org/10.1007/s13225-018-0401-0

Thakur, M. P., Quast, V., van Dam, N. M., Eisenhauer, N., Roscher, C., Biere, A., & Martinez-Medina, A. (2019). Interactions between functionally diverse fungal mutualists inconsistently affect plant performance and competition. *Oikos*, *128*(8), 1136–1146. https://doi.org/10.1111/oik.06138

Tisserant, E., Malbreil, M., Kuo, A., Kohler, A., Symeonidi, A., Balestrini, R., Charron, P., Duensing, N., Frei dit Frey, N., Gianinazzi-Pearson, V., Gilbert, L. B., Handa, Y., Herr, J. R., Hijri, M., Koul, R., Kawaguchi, M., Krajinski, F., Lammers, P. J., Masclaux, F. G., … Martin, F. (2013). Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *Proceedings of the National Academy of Sciences*, *110*(50), 20117–20122. https://doi.org/10.1073/pnas.1313452110

Vestal, J. R., & White, D. C. (1989). Lipid analysis in microbial ecology. *BioScience*, *39*(8), 535–541. https://doi.org/10.2307/1310976

Veum, K. S., Lorenz, T., & Kremer, R. J. (2019). Phospholipid fatty acid profiles of soils under variable handling and storage conditions. *Agronomy Journal*, *111*(3), 1090–1096. https://doi.org/10.2134/agronj2018.09.0628

Vitousek, P. M., & Howarth, R. W. (1991). Nitrogen limitation on land and in the sea: How can it occur? *Biogeochemistry*, *13*(2), 87–115. https://doi.org/10.1007/BF00002772

Vives-Peris, V., de Ollas, C., Gómez-Cadenas, A., & Pérez-Clemente, R. M. (2020). Root exudates: From plant to rhizosphere and beyond. *Plant Cell Reports*, *39*(1), 3–17. https://doi.org/10.1007/s00299-019-02447-5

Vrede, K., Heldal, M., Norland, S., & Bratbak, G. (2002). Elemental composition (c, n, p) and cell volume of exponentially growing and nutrient-limited bacterioplankton. *Applied and Environmental Microbiology*, *68*(6), 2965–2971. https://doi.org/10.1128/AEM.68.6.2965-2971.2002

Wamberg, C., Christensen, S., & Jakobsen, I. (2003). Interaction between foliar-feeding insects, mycorrhizal fungi, and rhizosphere protozoa on pea plants. *Pedobiologia*, *47*(3), 281–287. https://doi.org/10.1078/0031-4056-00191

Wang, C., Wang, X., Pei, G., Xia, Z., Peng, B., Sun, L., Wang, J., Gao, D., Chen, S., Liu, D., Dai, W., Jiang, P., Fang, Y., Liang, C., Wu, N., & Bai, E. (2020). Stabilization of microbial residues in soil organic matter after two years of decomposition. *Soil Biology and Biochemistry*, *141*, 107687. https://doi.org/10.1016/j.soilbio.2019.107687

Wang, M., Wang, Z., Guo, M., Qu, L., & Biere, A. (2023). Effects of arbuscular mycorrhizal fungi on plant growth and herbivore infestation depend on availability of soil water and nutrients. *Frontiers in Plant Science*, *14*, 1101932. https://doi.org/10.3389/fpls.2023.1101932

Wetterstation Saaleaue, www.bgc-jena.mpg.de/wetter/. (n.d.). *Max-planck-institut fuer biogeochemie - wetterdaten*. https://www.bgc-jena.mpg.de/wetter/

Whipps, J. M., & Lynch, J. M. (1983). Substrate flow and utilization in the rhizosphere of cereals. *New Phytologist*, *95*(4), 605–623. https://doi.org/10.1111/j.1469-8137.1983.tb03525.x

Wickham, H. (2016). *Ggplot2: Elegant graphics for data analysis*. https://ggplot2.tidyverse.org

Willers, C., Jansen van Rensburg, P., & Claassens, S. (2015). Phospholipid fatty acid profiling of microbial communities–a review of interpretations and recent applications. *Journal of Applied Microbiology*, *119*(5), 1207–1218. https://doi.org/10.1111/jam.12902

Williams, A., & de Vries, F. T. (2020). Plant root exudation under drought: Implications for ecosystem functioning. *New Phytologist*, *225*(5), 1899–1905. https://doi.org/10.1111/nph.16223

Wolf, D. C., Dao, T. H., Scott, H. D., & Lavy, T. L. (1989). Influence of sterilization methods on selected soil microbiological, physical, and chemical properties. *Journal of Environmental Quality*, *18*(1), 39–44. https://doi.org/10.2134/jeq1989.00472425001800010007x

Yao, H., Chapman, S. J., Thornton, B., & Paterson, E. (2015). 13c plfas: A key to open the soil microbial black box? *Plant and Soil*, *392*(1), 3–15. https://doi.org/10.1007/s11104-014-2300-9

Zabeti, N., Bonin, P., Volkman, J. K., Guasco, S., & Rontani, J.-F. (2010). Fatty acid composition of bacterial strains associated with living cells of the haptophyte *emiliania huxleyi*. *Organic Geochemistry*, *41*(7), 627–636. https://doi.org/10.1016/j.orggeochem.2010.04.009

Zelles, L. (1997). Phospholipid fatty acid profiles in selected members of soil microbial communities. *Chemosphere*, *35*(1), 275–294. https://doi.org/10.1016/S0045-6535(97)00155-0

Zwart, K. (1994). Rhizosphere protozoa: Their significance in nutrient dynamics. *Soil Protozoa*, 93–121. https://cir.nii.ac.jp/crid/1572261549640919296

A Appendix

A.1 Culture media for ^{15}N labeled necromass

Composition	Content (mg L^{-1})
Na_2HPO_4	6000
Glucose	5000
KH_2PO_4	3000
NH_4Cl	1000
NaCl	500
$MgSO_4 \cdot 7H_2O$	246.5
$K^{15}NO_3$ (10 atom%)	122.9
$CaCl_2$	14.7
H_3BO_3	0.15
$CoCl_2 \cdot 6H_2O$	0.10
$ZnSO_4 \cdot 7H_2O$	0.05
$MnCl_2 \cdot 4H_2O$	0.015
$Na_2MoO_4 \cdot 2H_2O$	0.015
$NiCl_2 \cdot 6H_2O$	0.010
$CuCl_2 \cdot 2H_2O$	0.005

Table 9: Composition of the culture media, modified from C. Wang et al., 2020

A.2 Relative abundance of phospholipid markers

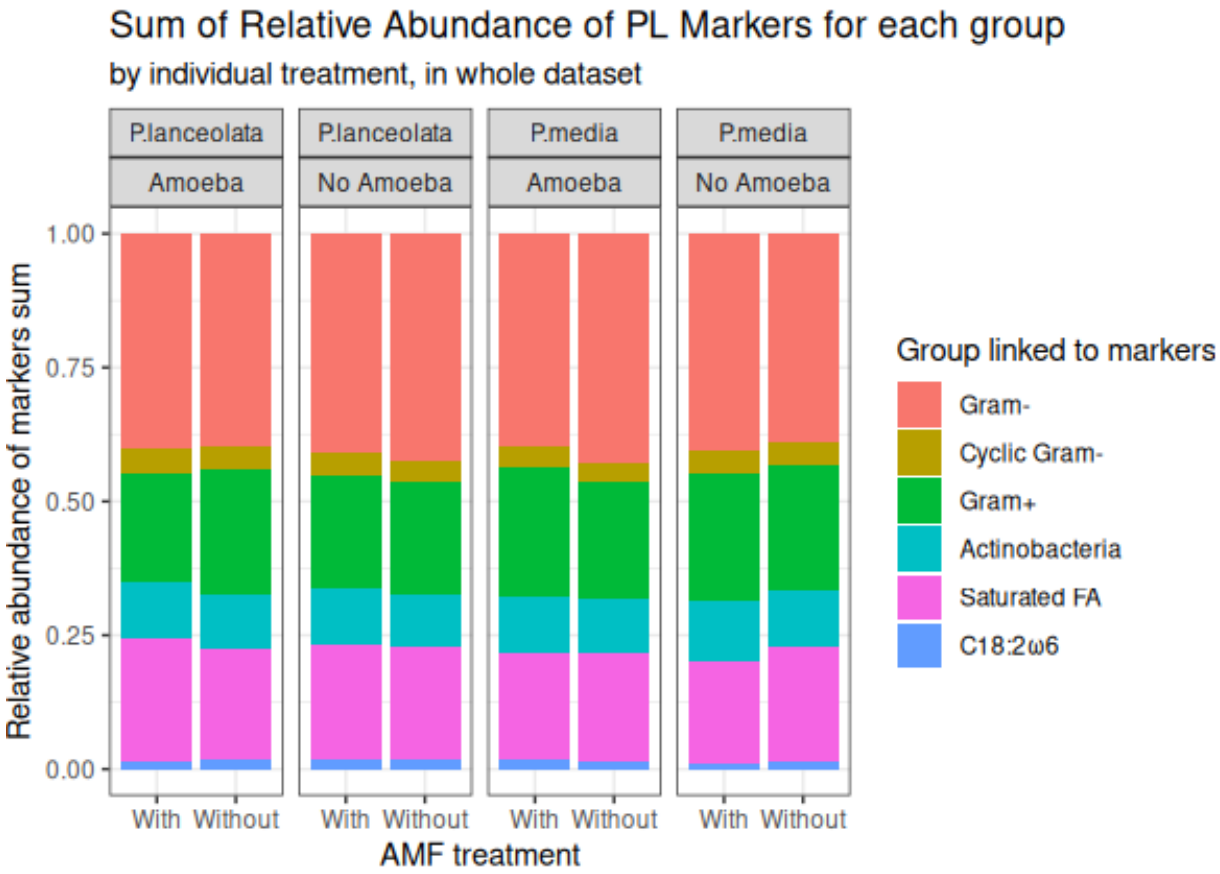

Figure 12: Representation of relative abundance of lipid markers in each treatment

A.3 Phospholipid markers ^{13}C enrichment (δ-values)

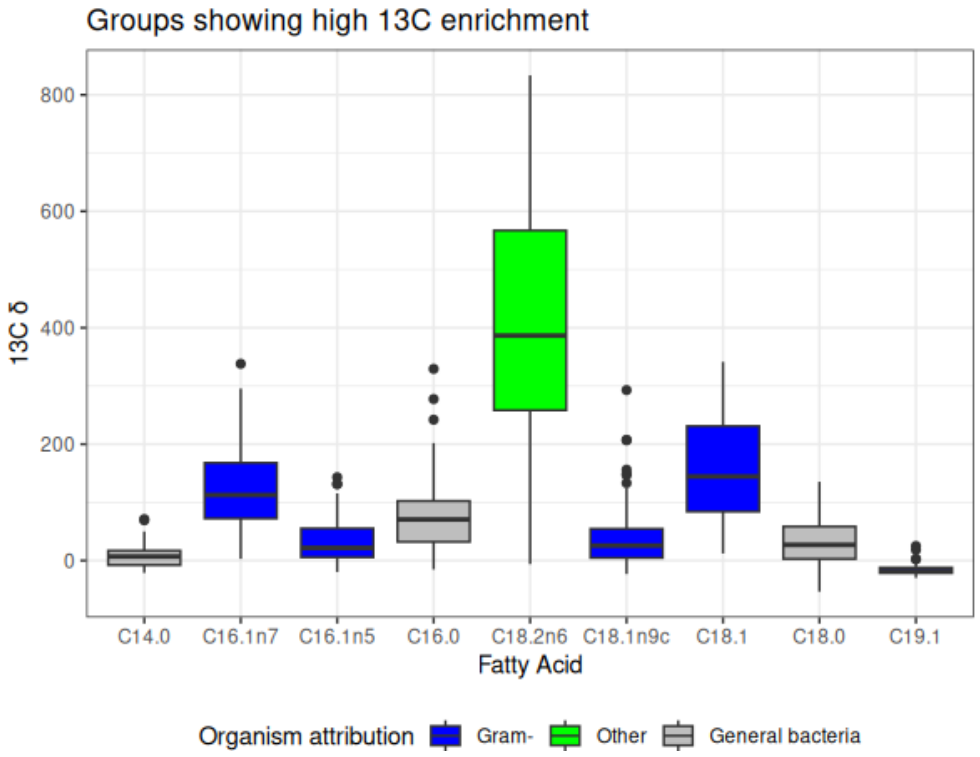

Figure 13: ^{13}C δ-values of lipid markers showing high enrichment

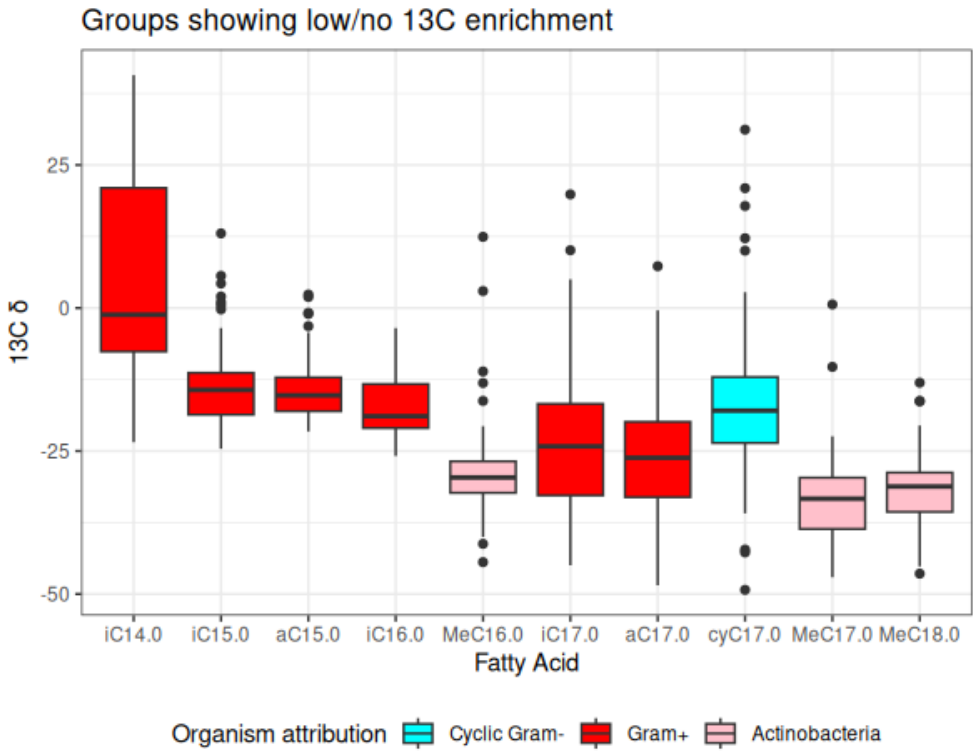

Figure 14: ^{13}C δ-values of lipid markers showing low or no enrichment

A.4 Phospholipid markers ^{13}C enrichment (APE)

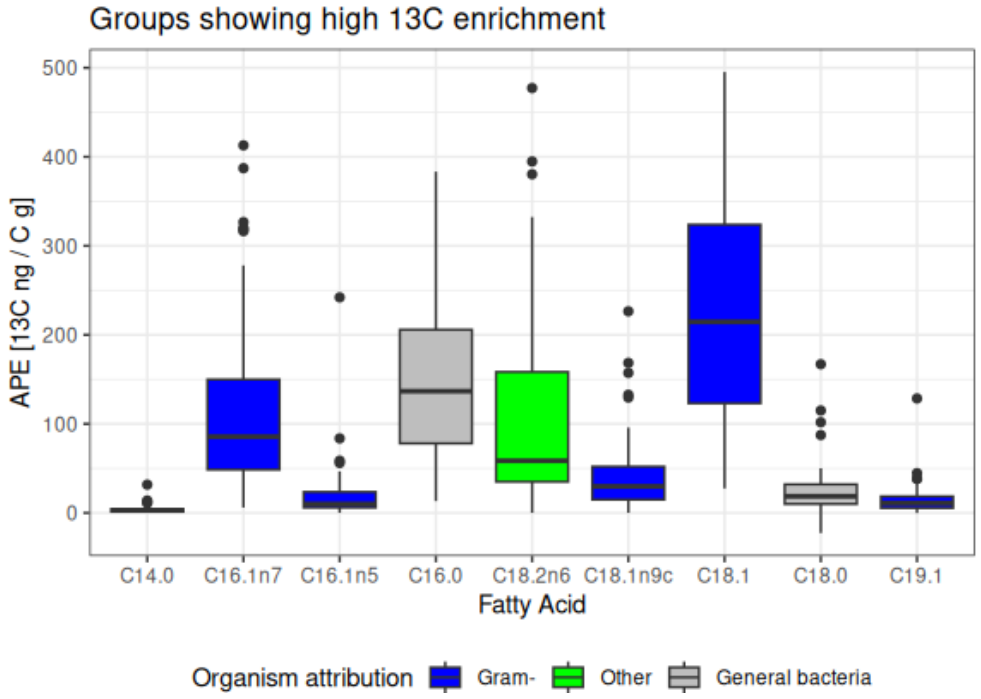

Figure 15: ^{13}C APE of lipid markers showing high enrichment

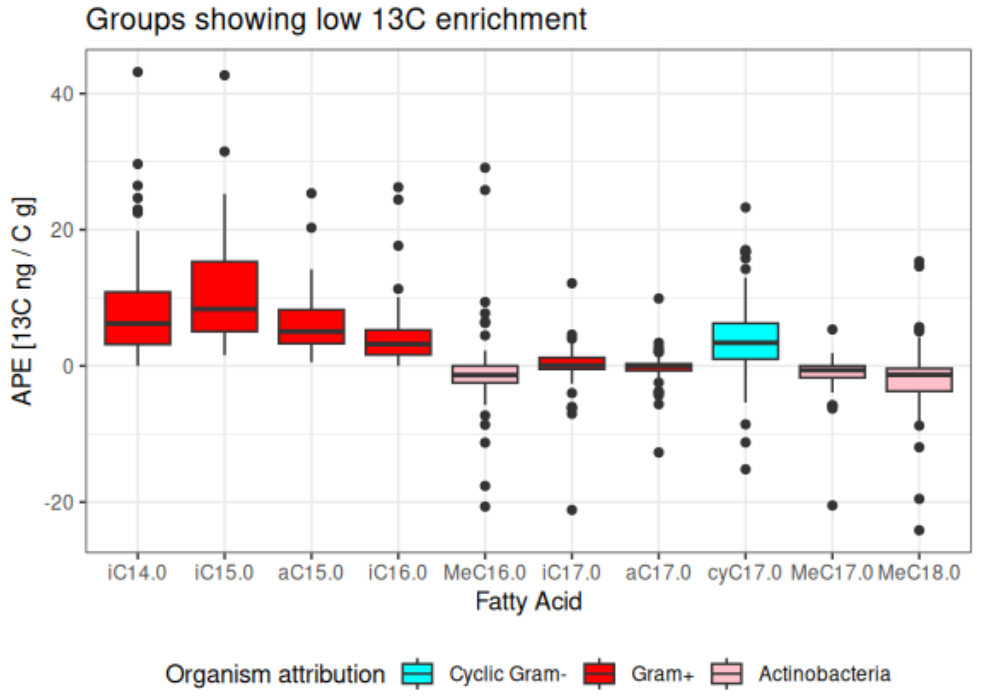

Figure 16: ^{13}C APE of lipid markers showing low or no enrichment

B Declaration of self-dependence

Herewith I declare that I prepared this thesis on my own, that I did not use any other sources and resources than those that are specified, that all arguments and ideas that were literally or analogously taken from other sources are sufficiently identified, and that the thesis in identical or similar form has not been use as part of an earlier course achievement or examination procedure.

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Place, Date Signature