Growth and Decay of Scots Pine Roots

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GROWTH AND DECAY OF SCOTS PINE ROOTS

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presented by

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1 Summary

Terrestrial carbon (C) uptake is directly affected by the future climate. A likely scenario for the inner-Alpine valleys implies shifts in precipitation regimes and more frequent and longer-lasting summer droughts. Dry summers occurred in the recent past and have affected mortality rates of Scots pines (*Pinus sylvestris* L.) to increase. The better drought-adapted pubescent oaks (*Quercus pubescens* Willd.) have been observed to occur at higher abundance mainly in the understory of the mature pine forests. In a drought-prone mature pine forest in Valais (Switzerland) an irrigation experiment was installed in 2003 to identify the drought induced changes of the forest. Nine years after irrigation start the vegetation was assessed and revealed clear abundance increase in Scots pine, and a clear reduction of pubescent oak due to irrigation. The assessment of the below-ground changes revealed no short-term (3 years) root growth adaptation, hence after long-lasting irrigation (9 years) an increased fine root standing biomass by 56% was observed. A significant decrease in the ^{13}C signal of the fine root was detected indicating increased photosynthetic activity with irrigation. Moreover, the fine root age, measured by radio carbon (^{14}C), was reduced by irrigation from 10.4 to 5.7 years. This strong but lagged increase in fine root biomass indicates a prioritized investment in above-ground structures after drought relief. Nonetheless, on a long run increased fine root biomass production in combination with reduced longevity due to irrigation will strongly increase litter input.

Litter input is a main flux of the soil C cycle. While most of the needle litter is rapidly decomposed and a minor fraction will reach deeper soil layers, root-litter derived C can be traced to deep mineral soil layers, where C stabilization is more likely to occur. This effect is mainly driven by higher protection from decomposition by physical (adsorption, aggregate formation) and chemical (high percentage of persistent macromolecules) factors. Decomposition is driven by abiotic environmental factors (e.g. moisture) and biotic factor, mainly the microbial community. To assess root litter decomposition under two precipitation regimes and the succession of the microbial communities during decomposition, a litterbag study was performed. Litterbags filled with pine roots were buried and sequentially sampled over two years. Decomposition was not affected by the irrigation treatment, hence, the microbial communities differed significantly from each other. The root composition did shift over time with progressing degradation, nitrogen content increased over the whole study period and lignin content increased during the first year and decreased in the second year. Time was the strongest factor shaping the microbial communities, indicating a strong selection pressure by the progressing decay of the substrate. The early successional stage (first year) was dominated by fast-growing copiotrophic bacteria and fungi (e.g. Proteobacteria, yeasts and molds), whereas the late stage (second year) was characterized by mainly specialized litter associated or parasitic bacteria and fungi (white-rot fungi, Planctomycetes and Pleosporales). Monitoring root litter decomposition during two years in soils revealed a clear succession of microorganism in accordance with the shifting litter composition, however, initial microbial communities did not change the following succession pattern strongly, and overall decomposition rate was stable irrespective of the irrigation treatment applied.

Distinguishing between active and passive microbial community in respect to C assimilation is a main issue for understanding C fluxes in soils. Combining two novel techniques, next-generation

DNA sequencing and stable isotope probing (SIP), enables to identify the vast complex diversity of microorganisms in soils. By providing labelled C sources the C incorporating microbial communities can be discriminated from the passive background. In a laboratory study, soils from the irrigation experiment were incubated with ^{13}C-labelled cellulose or lignin. Carbon dioxide (CO_2) gas fluxes were sequentially measured during one month revealing no increase in total respiration rate after addition of the C sources. However, a significant increase in the ^{13}C signal of the CO_2 was measured, where ^{13}C-labelled sources were used. The DNA from the soils was extracted and used to perform SIP. The reactive community was divided into cellulose specialists, lignin specialists and generalists (degrading cellulose and lignin), according to their abundance increase after C addition compared to the No C control. Cellulose C was incorporated by members of the order Rhizobiales and the ectomyorrhizal order Sebacinales. Ectomycorrhizal fungi have been known to degrade plant derived macromolecules during the process of nitrogen mining. Novel insights were found in lignin C assimilation by microorganisms, either well-known fungal lignin degraders were identified (e.g. Xylariales, Auriculariales, Helotiales), or in case of the bacteria genera *Sphingopyxis* (Sphingomonadales) and *Paucibacter* (Burkholderiales), novel groups were detected, of which *Paucibacter* has never been reported to degrade lignin. The orders Orbiliales and Hymenochaetales were identified as generalist fungi, degrading cellulose and lignin, and both known to be abundant on woody plant debris in nature. A single generalist bacterial genus, *Caulobacter* (Caulobacterales) was identified as lignin degrader, hence, cellulolytic capacity and the genetic foundation for expression of lignin degrading enzymes were previously reported.

In conclusion, the microbial communities are clearly affected by the irrigation treatment, nonetheless, the degradation capacity by the different communities did not differ, thus a functional redundancy by the two distinct microbial communities in root litter degradation is likely. The two microbial communities reacted in a similar way to C addition, hence, the type of C added, selected for specialized cellulose, specialized lignin, or generalist degraders. In addition, similarities in successional patterns of microbial degraders between the root decomposition study and the incubation experiment were detected, in particular for fungi. The C cycle might be strongly affected by a precipitation shift since the flux of litter input has increased. Hence, the flux by the degradation of the root litter was not affected by irrigation, which potentially results in an increase in C stocks on the long run.

2 Zusammenfassung

Die terrestrische Aufnahme von Kohlenstoff (C) ist direkt vom zukünftigen Klima betroffen. Ein wahrscheinliches Szenario für die inneralpinen Täler sagt Verschiebungen in Niederschlagsmustern und häufigere sowie länger anhaltende Sommerdürren voraus. Trockene Sommer traten in der jüngsten Vergangenheit vermehrt auf und haben die Sterblichkeitsrate der Waldföhre (*Pinus sylvestris* L.) erhöht. Bei der an Trockenheit besser angepassten Flaumeiche (*Quercus pubescens* Willd.) wurde ein erhöhtes Vorkommen beobachtet, vor allem in der Strauchschicht der Föhrenwälder. Bereits im Jahr 2003 wurde in einem trockenheitsgefährdeten Föhrenwald im Wallis (Schweiz) ein Bewässerungsexperiment installiert, um die dürreinduzierten Symptome der Bäume zu lindern. Neun Jahre nach Beginn der Bewässerung wurde die Vegetation erfasst, und sie zeigte eine deutliche Zunahme der Waldföhre in Bezug auf den Kronenschluss und eine deutliche Verringerung der Flaumeiche in der Strauchschicht. Das Erfassen der Feinwurzeln der Föhren ergab keine kurzfristige (3 Jahre) Anpassung an die Bewässerung, hingegen zeigte sich, dass nach einer mehrjährigen Bewässerung (9 Jahre) die Biomasse der Feinwurzeln um 56% im Vergleich zu den Kontrollplots zunahm. Eine signifikante Abnahme des $\delta^{13}C$-Signals der Feinwurzeln weist auf eine erhöhte fotosynthetische Aktivität der Föhren aufgrund der Bewässerung hin. Das mittlere Wurzelalter wurde mittels Radiokarbonmethode (^{14}C) bestimmt, und es zeigte sich, dass durch die Bewässerung das mittlere Alter von 10.4 auf 5.7 Jahre sank. Der starke, aber verzögerte Anstieg der Feinwurzel-Biomasse zeigt eine priorisierte Investition der Bäume in ihre oberirdischen Strukturen nach dem Aussetzen des Trockenheitsstresses. Dennoch wird auf lange Sicht durch die Bewässerung die erhöhte Feinwurzel-Biomasse-Produktion in Kombination mit reduzierter Langlebigkeit zu einem erhöhtem Streueintrag führen.

Der Eintrag von Streu ist eine Hauptquelle des Boden-Kohlenstoff-Kreislaufs. Während der Grossteil der Nadelstreu schnell zersetzt wird und nur eine kleiner Teil davon die tieferen Bodenschichten erreicht, gelangt der Kohlenstoff der Wurzelstreu in tiefen Mineralbodenschichten, in welchen eine erhöhte Kohlenstoff-Stabilisierung stattfindet. Dieser Effekt wird vor allem durch einen höheren Schutz vor Zersetzung durch physikalische (Adsorption, Aggregatbildung) und chemische (hoher Prozentsatz an persistenten Makromolekülen) Faktoren begünstigt. Die Zersetzung wird durch abiotische Umwelteinflüsse (z. B. Feuchtigkeit) und biotischen Faktoren, insbesondere die mikrobielle Gemeinschaft, angetrieben. Um die Zersetzung von Wurzelstreu unter zwei Niederschlagsregimen und die Sukzession der mikrobiellen Gemeinschaften während der Zersetzung zur verfolgen, wurde eine Streusäcklein-Studie durchgeführt. Mit Föhrenwurzeln gefüllte Streusäcklein wurden im Boden vergraben und Proben davon über zwei Jahre sukzessiv entnommen. Die Resultate zeigten, dass die Zersetzung durch die Bewässerung nicht beeinträchtigt wurde, jedoch zeigte sich, dass die mikrobiellen Gemeinschaften sich signifikant unterschieden. Die chemische Zusammensetzung der Wurzeln verschob sich im Laufe der Zeit mit fortschreitendem Abbau, wobei im ersten Jahr ein Anstieg der Stickstoffkonzentration und eine Erhöhung des Ligningehalts festgestellt wurden, jedoch der Ligningehalt im zweiten Jahr rückläufig war. Zeit war der stärkste Faktor, welcher die mikrobiellen Gemeinschaften beeinflusste, was auf einen starken Selektionsdruck durch den fortschreitenden Zerfall des Substrats hindeutet. Die frühe Phase der mikrobiellen Sukzession (erstes Jahr) wurde von schnell wachsenden

copiotrophen Bakterien und Pilzen (z. B. Proteobakterien, Hefen und Schimmelpilzen) dominiert, während die späte Phase (zweites Jahr) durch vorwiegend spezialisierte stressassoziierte oder parasitäre Bakterien und Pilze (Weissfäulepilze, Planctomyceten und Pleosporales) gekennzeichnet war. Das Verfolgen der Zersetzung der Wurzelstreu im Boden über die zwei Jahre zeigte eine klare Sukzession von Mikroorganismen, dies in Übereinstimmung mit der sich verändernden chemischen Zusammensetzung der Streu. Die anfängliche mikrobielle Gemeinschaft hat das folgende Muster der Abfolge nicht stark verändert, und die gesamte Zersetzungsrate blieb unabhängig von der Bewässerungsbehandlung stabil.

Das Unterscheiden zwischen aktiver und passiver mikrobieller Gemeinschaft in Bezug auf die Kohlenstoff-Assimilierung ist ein wichtiger Bestandteil für das Verständnis der Kohlenstoff-Flüsse im Boden. Durch die Kombination von zwei neuartigen Techniken der DNA-Sequenzierung und der Beprobung von stabilen Isotopen (SIP) kann die umfangreiche komplexe Vielfalt von Mikroorganismen im Boden identifiziert werden. Zudem, durch das Zurverfügungstellen von markierten unterschiedlichen C-Substraten, kann der aktive Teil der mikrobiellen Gemeinschaft vom passiven Hintergrundrauschen unterschieden werden. In einer Laborstudie wurde der Boden aus dem Bewässerungsexperiment mit ^{13}C-markierter Zellulose oder Lignin inkubiert. Kohlendioxid (CO_2) Gasflüsse wurden sequentiell während des Verlaufs der Inkubation gemessen, und es zeigte sich, dass durch die Zugabe der verschiedenen C-Substrate keine erhöhten Atmungsraten resultierten. Jedoch wurde eine signifikante Erhöhung des ^{13}C-Signals des CO_2 gemessen, was darauf hinweist, dass die hinzugefügten C-Quellen abgebaut und aufgenommen wurden. Die DNA der Mikroorganismen wurde daraufhin aus den Böden extrahiert und zur Durchführung von SIP verwendet. Die aktive mikrobielle Gemeinschaft wurde in Zellulosespezialisten, Ligninspezialisten und Generalisten (Zellulose- und Lignin-Abbauer) unterteilt, dies aufgrund ihrer Häufigkeitszunahme im Vergleich zu der Kontrolle ohne zusätzliche C-Quelle. Der Kohlenstoff der Zellulose wurde von Vertretern der Ordnung Rhizobiales und der ektomyorrhiza-bildenden Ordnung Sebacinales aufgenommen. Es ist bekannt, dass ektomykorrhizierende Pilze pflanzliche Makromoleküle während des Prozesses der Stickstoffgewinnung abbauen. In der Lignin-Behandlung wurden sowohl bekannte Lignin spezialisierte Pilze (z.B. Xylariales, Auriculariales, Helotiales) als auch Bakterien-Gattungen wie *Sphingopyxis* (Sphingomonadales) und *Paucibacter* (Burkholderiales) nachgewiesen, von denen *Paucibacter* als Ligninabbauer bisher noch nicht bekannt war. *Paucibacter* wurde jedoch in grosser Häufigkeit in der späten Phase der Streuabbaustudie nachgewiesen. Die Ordnungen Orbiliales und Hymenochaetales wurden hier als generalistische Pilze eingeteilt, wobei von ihnen bereits bekannt ist, dass sie vorzugsweise in verholzter Streu in Wäldern vorkommen. Als einzige generalistische bakterielle Gattung wurde *Caulobacter* (Caulobacterales) identifiziert. Diese Gruppe wurde noch nie als Lignin-Abbauer beschrieben, ihre genetische Grundlage für die Bildung von Lignin-abbauenden Enzymen wurde aber bereits früher beschrieben.

Zusammenfassend kann gesagt werden, dass die mikrobiellen Gemeinschaften durch die langfristige Bewässerungsbehandlung verändert wurde. Dennoch blieb die Abbaukapazität trotz der verschiedenen Gemeinschaften stabil, was auf eine funktionelle Redundanz der verschiedenen mikrobiellen Gemeinschaften in Bezug auf den Abbau von Wurzelstreu hindeutet. Die beiden mikrobiellen Gemeinschaften reagierten in ähnlicher Weise auf die Kohlenstoffzugabe. Jedoch

spielte die Art des Kohlenstoffs eine wichtige Rolle für die Zusammensetzung der Gemeinschaften, welche in Zellulose spezialisierte, Lignin spezialisierte oder generalistische Abbauer unterteilt werden konnte. Darüber hinaus wurden Ähnlichkeiten in den sukzessiven Mustern der mikrobiellen Gemeinschaften, zwischen der Wurzelzersetzungsstudie und dem Inkubationsexperiment, insbesondere für Pilze, nachgewiesen. Der Kohlenstoffkreislauf könnte durch eine Niederschlagsverschiebung stark beeinflusst werden, da der Streueintrag erhöht würde, jedoch der Wurzelabbau durch die erhöhten Niederschläge nicht beeinträchtigt würde. Dies könnte langfristig zu einer Zunahme der Kohlenstoffvorräte im Waldboden führen, allerdings nur unter der Annahme, dass die Sommerniederschläge zunehmen würden.

3 General Introduction

3.1 Forests in a drying world

3.1.1 Droughts affecting forest growth and mortality

Forest biomes worldwide provide a carbon (C) sink of approximately 1.1±0.8 Pg of C per year over the recent decades (Pan *et al.*, 2011). In our changing world, more frequent climate extremes are predicted and may directly affect terrestrial C uptake (Reichstein *et al.*, 2013). With increasing frequencies and prolonged summer droughts, forests, and in particular temperate forests, will be put to the test (Millar and Stephenson, 2015). Such climatic shifts, are hypothesized to result in an increase of tree mortality (Anderegg *et al.*, 2013; Hartmann *et al.*, 2013), especially in southern Europe (Carnicer *et al.*, 2011). These diebacks of local key tree species may coincide with replacement by more drought adapted tree species (Rigling *et al.*, 2013). Assessment of drought events are still insufficient and there is a demand for adequate and long-term studies (Smith, 2011a). In addition definition of severe drought events and standardization of climate extreme studies are needed (Smith, 2011b). Focusing on the inner-Alpine valleys of Switzerland, more frequent and longer-lasting drought periods have been recorded in the past decades (Rebetez and Dobbertin, 2004; Bigler *et al.*, 2006), and the most recent predictions propose that even an increase of severe drought events can be expected (Forzieri *et al.*, 2014).

3.1.2 Scots pine

Scots pine (*P. sylvestis* L.) natural occurrence spreads from Western Europe to Eastern Siberia, and from the Arctic Circle to the Southern Caucasus region. Its niche is limited to nutrient poor and often sandy soils (Mátyás *et al.*, 2004), while it can cope with very little precipitation (300 mm/year). However, extended drought periods can increase the pine's mortality rates (Bigler *et al.*, 2006). Scots pines are not strong competitors and, thus, they are increasingly threatened to be replaced by broad-leaf trees such as pubescent oaks (*Q. pubescens* Willd.) (Dobbertin *et al.*, 2005; Dobbertin and Rigling, 2006).

In order to investigate the increasing occurrence of Scots pine mortality, an irrigation study had been installed more than a decade ago in a mature pine forest of a drought-prone valley in Valais, Switzerland (Figure 3.1). It was the intention that the irrigation treatment should mitigate negative effects of drought on pines during the summer month. The irrigation was installed in spring 2003 and is still running in 2017 (14 years). This long-term study should investigate whether Scots pine mortality is directly caused by the long-lasting summer droughts, or whether it is rather caused by competition by other plants (Rigling *et al.*, 2013) or by pests such as insects or fungi (Dobbertin and Rigling, 2006).

Early results showed a fast and strong response of the irrigated pines in needle and stem growth, moreover, the isotopic C signal indicated an increase in photosynthetic activity (Eilmann *et al.*, 2010). Interestingly, root growth seemed not to be affected by the short-term irrigation (Brunner *et al.*, 2009).

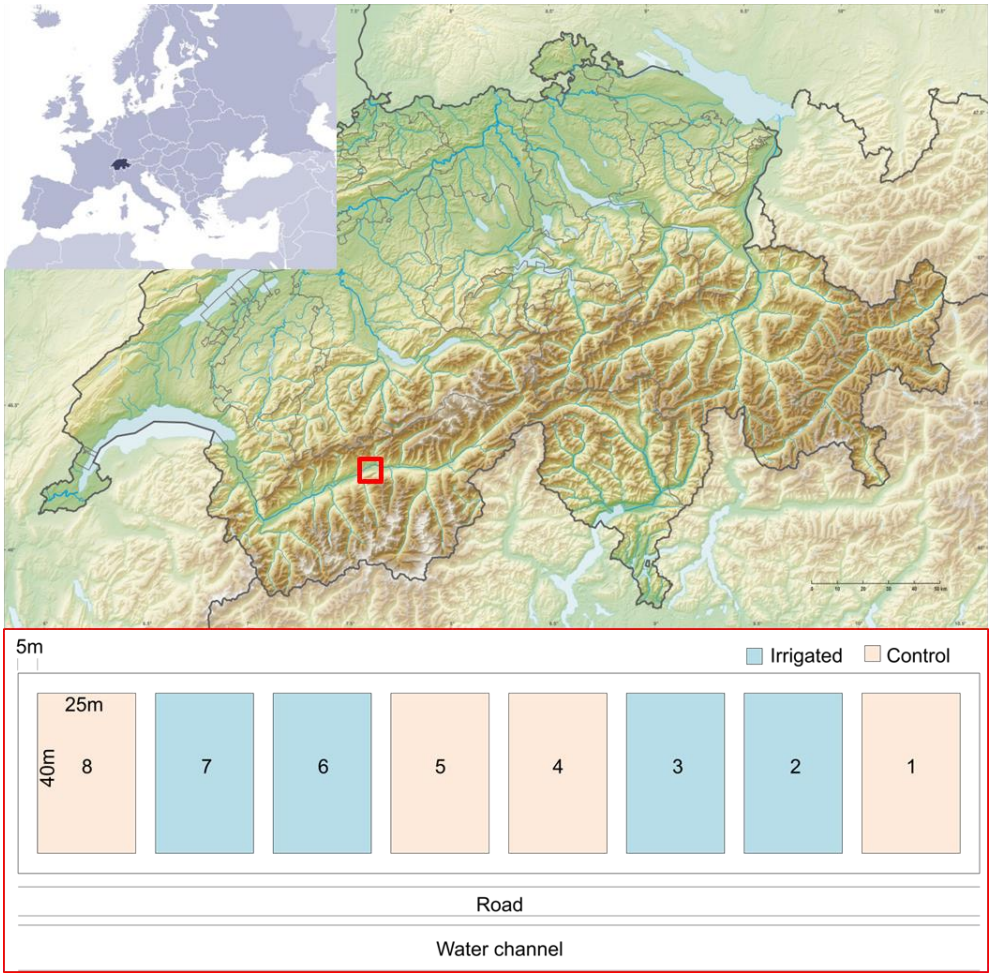

Figure 3.1: Study site *Pfynwald* location in an inner Alpine valley in Valais, Switzerland and study set-up with 8 plots; 4 dry control (orange), 4 irrigated (blue).

3.1.3 Root adaptations to drought

A review by Brunner *et al*. (Brunner *et al.*, 2015) summarizes the known strategies which trees execute to circumvent negative drought induced effects. Tree roots are able to acclimate to dry conditions from small responses on a molecular level (e.g. increase in abscisic acid production, incorporation of aquaporins in cell membranes) to large growth acclimations (e.g. reducing root biomass). Effects on root lifespan are not clearly resolved, but it seems that dry conditions increase root lifespan. Probably maintaining the root system is key to acquire minimal amounts of water, but comes at a cost of reduced plasticity. In certain cases, a change of the composition of the root compounds could be detected, e.g. drought induced an increase of lignin and suberin (Moura *et al.*, 2010). Lignin not only physically strengthens the cell walls but additionally reduces water loss of roots (Cabane *et al.*, 2012). Suberin is an aliphatic plant cell wall component and directly increases water-use efficiency (Baxter *et al.*, 2009) and reduces water loss to the surrounding soil (Steudle, 2000). How fast these adaptations can be induced is not clear. For structural investments, the plants often use stored C such as starch, since under drought condition acquisition of C is limited (Galiano *et al.*, 2011). Despite of these acclimation strategies, long-term or consecutive drought events can lead to direct or delayed tree mortality.

3.2 Carbon cycle

In European temperate forests C pools are estimated to contain about 24 Pg of C, of which 2 Pg C is present as litter (Pan *et al.*, 2011). The main driving fluxes are primary production and litter decomposition (De Deyn *et al.*, 2008), however, effects of climate change on these fluxes are difficult to assess. The agreement to the Kyoto Protocol (1997) resulted a commitment to report to the greenhouse gas inventory, such as fluxes of CO_2 uptake and emissions. Therefore, it is key to address the change of these fluxes under environmental change. It is stated, that the C cycle is strongly affected by precipitation, and Hartmann *et al.* (2017) concluded an increase of the C turnover rate. In the end, the ratio between the respired feedback to the atmosphere compared to the fixed (sequestered) C in soil or biomass is decisive. A positive C respiration/sequestration ratio is called a C source, while a higher sequestration compared to respiration is called a C sink. Increased water availability can affect this ratio in both ways, while irrigation increases plant biomass production (Eilmann *et al.*, 2010) and litter input (Hartmann *et al.*, 2017), respiration from soil is increased as well (Hartmann *et al.*, 2017). Whether this increase in respiration originates from the increase in root biomass or from increased microbial activity in the *Pfynwald* soil has not yet been solved. Additionally, the shift of the microbial community from oligotrophs to copiotrophs indicates an increased C turnover rate due to irrigation (Hartmann *et al.*, 2017), nonetheless, direct evidence for an improved degradation capacity of plant residues by soil microorganisms has not been provided so far.

A simplified C cycle is shown in Figure 3.2 summarizing the major C pools and fluxes. An increase in turnover rate would not only increase the input into the system but all other fluxes likewise, resulting in an increase in decomposition, respiration, leaching, and aggregation/adsorption. During degradation of litter, C has several optional fates, either C is decomposed (oxidized, humified) and ends up as humus in the slow-cycling C pool, part of which will be stabilized (sequestered) in the soil, or C can be mineralized and taken up by microorganisms, which then is fixed within the biomass and will enter the C cycle again after the biomass becomes necromass. During both pathways, C is constantly respired as CO_2 and will eventually leave the soil to the atmosphere. An alternative route is when C is leaving the system by leaching and erosion.

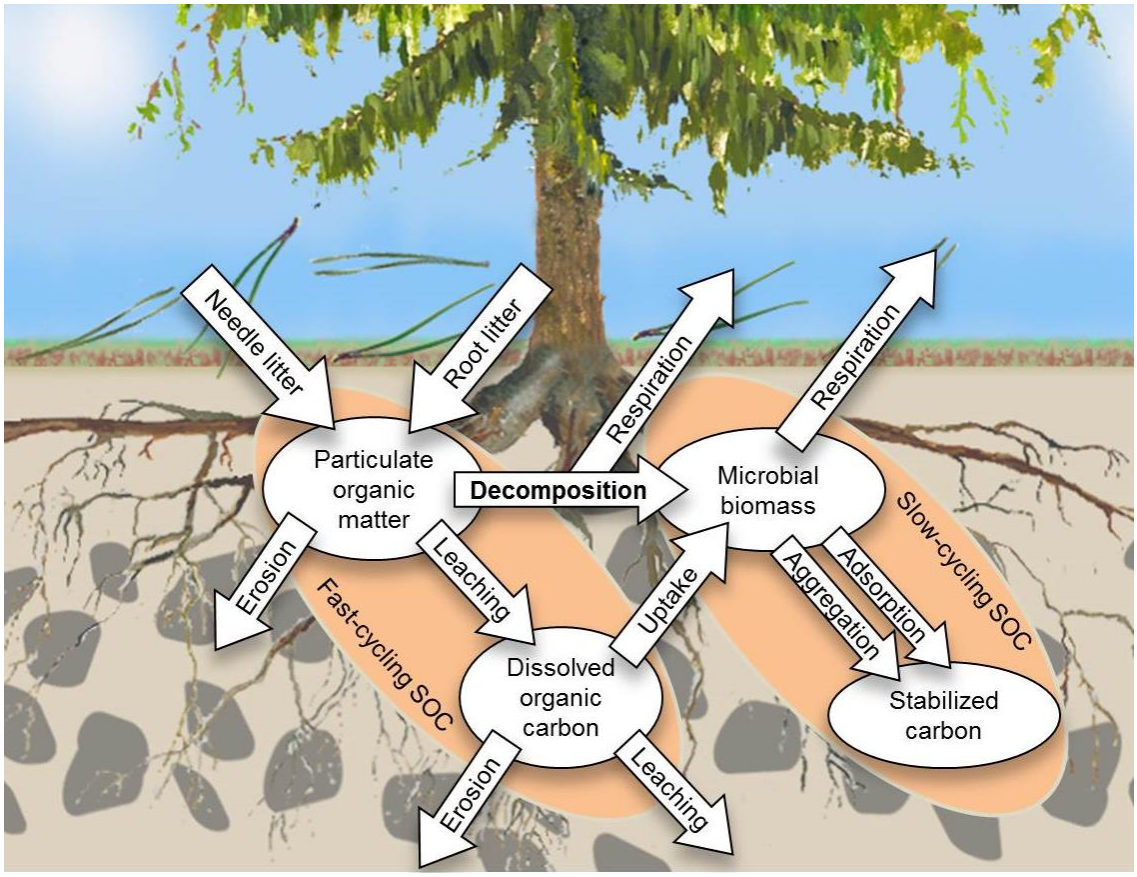

Figure 3.2: A simplified carbon cycle of the major carbon pools (circles) and fluxes (arrows) of a mature forest. Tree painted by Hanspeter Läser.

3.2.1 Fine root turnover

Fine root turnover is a main source of C input to forest soils. While fine-root turnover is mainly dominated by the three processes growth, death, and decomposition, many other factors influence these main processes. In Figure 3.3, an overview of the involved main processes are schematically drawn. In general, fine root turnover has been calculated by addressing standing biomass and yearly root production, root age, or growth and mortality of roots. While the methods for measuring the living root biomass are generally agreed on, root age and the amount of the dead root mass (=”necromass”) remain difficult to assess. A rather new approach uses ^{14}C radiocarbon measurements to estimate the root age by using the radiocarbon bomb-peak as reference curve (Gaudinski *et al.*, 2001). Alternative methods to estimate root turnover are sequential coring of standing biomass, minirhizotrons, or ingrowth cores (Brunner *et al.*, 2013). The amount of dead roots is usually estimated by separating dead from living roots after soil coring (Waisel, 2002), which has been proven to be time consuming using either microscopes, UV-light, or floating. Using minirhizotrons, the disappearance or shrinkage of roots is counted as root death. Hence, even more challenging is to quantify decomposition, in nature nearly impossible, since the shift from a living to a dead root, and from a dead to a decaying root potentially can be a long-lasting transition. From soil coring samples it is almost impossible to discriminate between slowly dying but still living roots, just recently died roots, and roots which are dead but only have partially degraded. In a

review, Majdi *et al.* (2005) compared the various methods and described advantages and drawbacks of each method, with the result that most likely the research question defines the method.

3.2.2 Decomposition

With increasing evidence, the theory consolidates that one of the main inputs of plant litter which ends up in the soil C pool derived from roots (Rasse *et al.*, 2005; Fan and Guo, 2010; Sanaullah *et al.*, 2011b; Amin *et al.*, 2014). Since assessment of root decomposition is work intensive and lacks consensual methods (Goebel *et al.*, 2011; Berg and McClaugherty, 2014b), few *in situ* studies have been published. The reasons why root litter seems to contribute more to the C pools in soils than aboveground litter is hypothesized to be a combination of higher physical and chemical protection (Rasse *et al.*, 2005). Physical protection is given by the surrounding complex soil structure, where particulate organic matter can be included into soil aggregates, or the porous structure of the soil matrix limits microbial accessibility (Goebel *et al.*, 2009; Schmidt *et al.*, 2011). The chemical protection is associated with a higher content of persistent (="recalcitrant") lignin, compared to leaf litter, and may be a key-component controlling soil organic matter (SOM) stabilization (Hofmann *et al.*, 2009; Walela *et al.*, 2014). Hence, in recent years lignin's recalcitrant nature has become debated, and while lignin is in its natural state clearly persistent to degradation by even specialized fungi, its effect on SOM stabilization seems minor (Schmidt *et al.*, 2011; Cotrufo *et al.*, 2013). A conceptual model for lignin degradation (Klotzbücher *et al.*, 2011) revealed that lignin is persistent, but only at a stage where other easy degradable C sources become limited. The main issue in this context is having two perspectives on the matter, on the one hand the 'soil scientists' thinking in decades and trying to decipher of where this slow cycling SOM pool is actually derived from, and on the other hand 'the biochemists' who see this lignin structure with the complex structure and the interlinkage to hemicellulose which obviously is persistent to degradation as a structure. Who is right? Most likely, both groups are right to a certain degree. In a recent study, trying to solve the riddle of "the contentious nature of soil organic matter" Lehmann and Kleber incorporate the classical view with new findings in the field (Lehmann and Kleber, 2015). They conclude that at every stage of degradation some part of the C gets aggregated or attached to mineral surface, therefore, leaving the fast SOM pool and entering into the slow SOM pool. Figure 3.3 displays the soil organic matter cycles (Lehmann and Kleber, 2015) adapted according to our understanding of root turnover in forest soils. In our view, the importance of microbes is crucial, and with increasing evidence, it has been shown that the persistent molecules in soils are of microbial origin and not plant-derived (Cotrufo *et al.*, 2013; Kallenbach *et al.*, 2016). Thus, microbial trophic cascades during the decay of root litter might influence C stabilization more than was thought before, and consequently, microorganisms should be given more emphasis in terms of the C cycle.

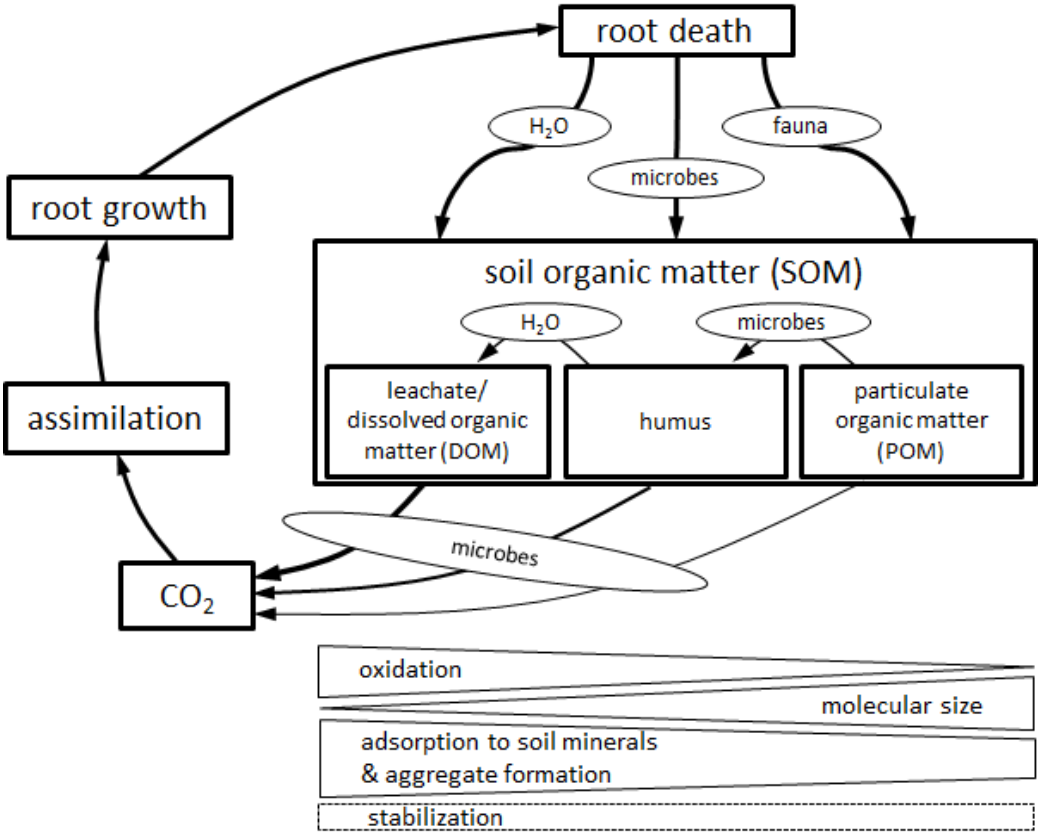

Figure 3.3: Fine root turnover cycle in soil, adapted from Figure 2 in "*The contentious nature of soil organic matter*" (Lehmann and Kleber, 2015). States of root or root derived matter as boxes, fluxes as arrows and main processing agent as circles. Below properties (oxidation state, molecular size) of soil organic matter (SOM) for its different states (POM, Humus, DOM) and potential interactions with surrounding soil (adsorption, aggregation, stabilization)

3.2.3 Lignin

Lignin is one of the major compounds of woody plant tissues. Depending on the extraction method, it can make up to 80% of stem wood, or 30% of fine root biomass (Berg and McClaugherty, 2014b). Its chemical recalcitrance and claimed resistance to degradation makes it interesting to study its fate in soil in the context of C sequestration. The chemical recalcitrance is mainly given by the linkage heterogeneity (Abdel-Hamid *et al.*, 2013) of phenolic and non-phenolic bonds. In addition, the close interlinkage with hemicellulose in the cell wall fibrils (Figure 3.4) makes it hard to access, thus, lignin degradation is always coupled to partial degradation of cellulose and hemicellulose. In general, many fungi and some bacteria are capable to degrade the lignin partially using laccase (Abdel-Hamid *et al.*, 2013). However, a full lignin degradation is limited only to a specialized group of fungi, the so-called "white rot fungi" (="white rots"). The main enzymes used by the white rots are peroxidases, such as manganese peroxidase (MnP), lignin peroxidase (LiP), or versatile peroxidase (VP). These large enzymes are often not capable to penetrate the dense cell wall structure, therefore, loosening of the structure is conducted by hydroxyl radical formed by laccases (Mishra and Thakur, 2015).

White rot fungi are the most efficient lignin degraders, nonetheless, also brown rot and soft rot fungi are capable of degrading lignin. In case of brown rots, lignin is not preferentially degraded,

cellulose and hemicellulose are degraded first, lignin is often oxidized and not fully degraded (Abdel-Hamid *et al.*, 2013). Brown rot and white rot fungi are often dominated by basidiomycetes, while soft rot fungi are mostly ascomycetes dominated (Worrall *et al.*, 1997). Molecular structure of soft rot is morphologically quite similar in appearance to white rot, hence, soft rot often occurs on herbaceous plant debris, while white rot is very specific for woody debris (Worrall *et al.*, 1997). The strength of the phylogenetic link between the different lignin decay types is not fully understood (Floudas *et al.*, 2012), which leads to a challenge of classifying the decay types (Riley *et al.*, 2014). The strong oxidative potential of the peroxidases produced by white rot fungi remains unique and seems to have evolved only once, and brown rot fungi evolved from this common white rot ancestor (Floudas *et al.*, 2012). These results are gathered from genomic data, which identify potential lignin degradation capacity. Less clear is which organisms actually degrade and incorporate lignin under natural conditions. Since lignin degradation happens extracellularly and in more than one step, symbiotic interactions (mutualistic, parasitic, competition) among microorganisms are very likely in soil.

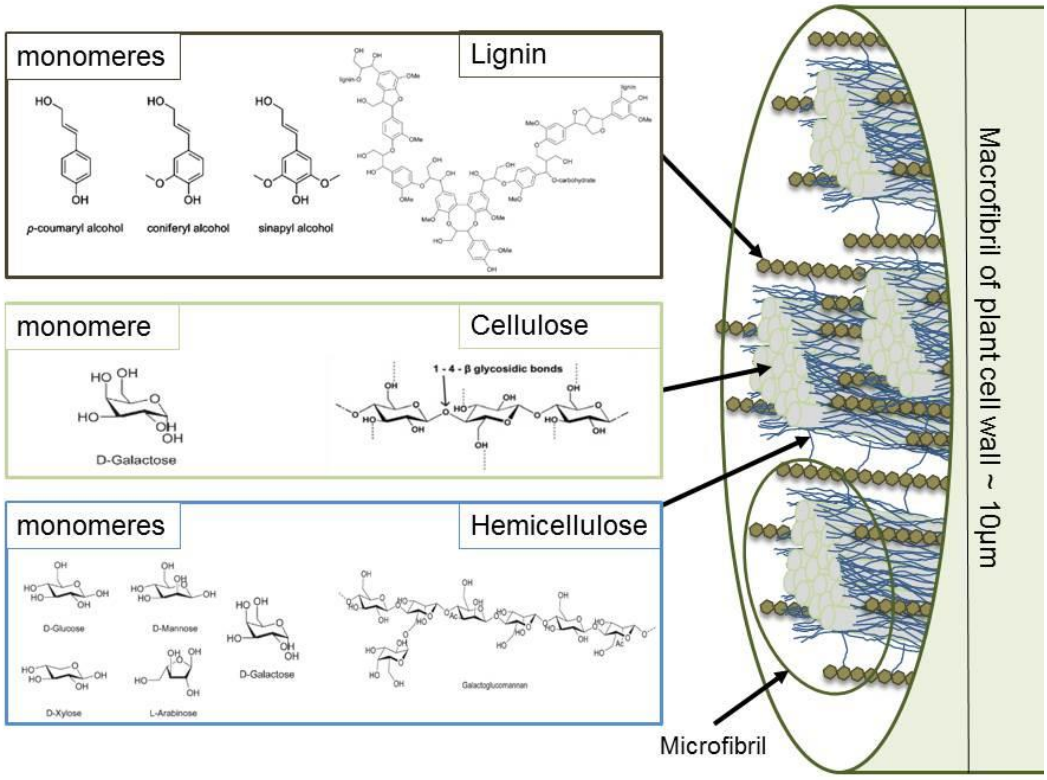

Figure 3.4: The chemical structure of the main plant cell wall components cellulose, hemicellulose and lignin as polymer and their subunits, with the schematic cross-section of a macrofibril of a plant cell wall. The many microfibrils (subunits) consist of cellulose chains surrounded by hemicellulose connected to lignin sheets. Microfibrils are cross-linked by hemicellulose to each other. Adapted from (Zakzeski *et al.*, 2010; Brandt *et al.*, 2013).

3.3 Forest microbiome

3.3.1 Methodological progress in identifying soil microbial complexity

Recent efforts in addressing the complex systems of microorganism in soils quickly evolved in the past years due to the application of next-generation sequencing techniques to address the soil microbiome (Baldrian, 2017). Before the 20th century many studies were limited to laboratory intensive and less sensitive techniques, such as phospho-lipid fatty acids (PLFA), or terminal restriction fragment length polymorphism (T-RFLP). While analyzing the PLFAs many studies successfully applied isotopic labelling to trace nutrient flows to the soil microbiome (Radajewski *et al.*, 2000), the resolution was limited to highly variable and diverse taxonomic groups, which often not share a common ecological niche. With T-RFLP and the beginning of the 20th century the use of polymorphic chain reaction (PCR) to amplify low concentration of DNA to many hundred copies enabled researchers to approach the soil microbial diversity with higher resolution (Marsh, 1999). While from a cost perspective it remains a good tool for answering many studies research questions, its missing the in-depth and holistic information, which is provided by more modern next-generation sequencing (NGS) tools. Hence, with NGS we still face the problem of PCR bias and it remains a challenge to properly correct for these. In near future these PCR-based tools will partly be replaced by metagenomic approaches, hence, at the moment they remain very costly and tend to lack in well-established databases needed to mirror measured sequences to fully sequenced genomes (Scholz *et al.*, 2012). Therefore, to keep up with the classical biological research paradigm of replication in time and space, paired-end amplicon sequencing is an adequate solution to study in-depth the microbial soil community. The functional component can be approached by using stable isotope probing (SIP), were labelled material is added as a source for microbial incorporation in their DNA (Neufeld *et al.*, 2007). These two coupled, amplicon-sequencing and DNA-SIP, result in a very powerful tool to detect microbial activity over an integrated time period.

3.3.2 Forest soil as habitat

The organisms living in forest soils (edaphon) are clearly dominated by the microflora (e.g. bacteria, fungi) which account for approximately 80% of total organisms biomass in soils (Blume *et al.*, 2016); while bacteria approximately account for 50% and fungi 30% of the biomass. All other soil organisms make the remaining 20%, in which earthworms (8%) and algae (5%) are the most relevant. In 1 g of soil it is estimated that >7000 bacterial species and >2000 fungal species are present, this reveals the clear dominance and huge functional range which these microorganisms represent. The bacterial and fungal dominance is a main driver of plant derived organic matter degradation, additionally their turnover time has become a study focus as well, since they themselves represent a large source of nutrients (e.g. nitrogen, phosphorus). Multicellular fungi express a turnover time of few weeks or months (Rousk and Bååth, 2011; Ekblad *et al.*, 2013), whereas single cellular organisms such as yeasts and bacteria turn over within few hours or days (Hagerty *et al.*, 2014). Moreover, the microbial turnover rate is influenced by environmental parameters (e.g. temperature, moisture) and soil properties such as clay content which provide protection and stabilize the soil pH (Six *et al.*, 2006). Recently, two reviews compiled the overall knowledge of the diversity and function of the bacterial and fungal microbiome in forest soils

(Baldrian, 2017; Lladó *et al.*, 2017). These reviews emphasize the importance of the high diversity and ecosystem functions of the forest soil microbiome, especially for bacteria since they were often not investigated. One main conclusion is that even though in the past bacteria and fungi have often been studied separately, there is strong enough evidence that soil functioning cannot be accounted to only one of these kingdoms, since in soil there interactions are versatile and ubiquitous (Haq *et al.*, 2014). Symbiotic interactions from parasitism to tightly coupled mutualistic symbiosis and all intermediated stages can be found. Additionally, the lack of *in situ* studies which include bacteria and fungi are scarce, and Lladó *et al.* (2017) specifically addressed the lack of studies investigating the active microbial community and the interactions therein.

3.3.3 Root as habitat

Roots, especially in forest, are known to be colonized by microorganisms of many kinds. Ectomycorrhizal fungi are likely the most studied group due to big scientific efforts, supporting trees in not only acquiring nutrients but also with water during dry conditions. Nonetheless, many other important symbionts have been detected, from mutualistic endophytes to parasites (Eshel and Beeckman, 2013). Healthy soil with a functional microbiome is key for tree growth, which can for example be impaired by soil compaction (Hartmann *et al.*, 2014) or over-fertilization (Phillips and Fahey, 2007). Many of these soil organisms support growth, nutrient acquisition, or colonization (Kottke and Kovács, 2013), hence, some fungal pathogens also inflict a reduction in root lifespan (Eissenstat *et al.*, 2013). With death of the root not only the parasitic but also former ectomycorrhizal symbionts start to degrade the roots (Churchland and Grayston, 2014; Kuyper, 2016). Not much is known about the following succession of microbiota on the decomposing root in forest soils. In some studies the succession of fungi on aboveground litter has been analyzed (Treseder *et al.*, 2014; Haňáčková *et al.*, 2015), or microbial succession on root litter in agricultural soil (Sanaullah *et al.*, 2016). A research gap remains the interaction of bacteria and fungi during progressing root decomposition in forest soils. Who does the tough work and who benefits from degradation efforts of others?

3.4 Thesis objectives

The main objectives of the thesis were to assess growth and decay of Scots pine roots under different soil moisture conditions. Specifically it was of interest how Scots pine root acclimate to a changing climate, how increased water availability effects root growth, and which are the main microbial players during root decomposition.
To approach these objectives, the study was performed in a long-term irrigation experiment started in 2003 in the study site *Pfynwald* (Valais, Switzerland). The irrigation treatment was set to repeal the frequent summer droughts causing increased pine mortality in the area.

Sequential soil coring was performed and the roots were analyzed for dry weight biomass and their radiocarbon (^{14}C) signal to approximate the mean root age. Additionally, the effect on the surrounding vegetation was addressed by a vegetation assessment in the irrigated and control plots, to answer the following research questions:

- How did the vegetation in the drought-prone *Pfynwald* shift after nine years of irrigation?
- How do drought adapted Scots pine roots acclimate to long-lasting irrigation treatment?

Fresh fine roots were sampled, air dried, and chemically analyzed. Then, during a two-year period, the dried roots were exposed in litter bags in the forest soils to dacay. Decomposing roots were sequentially sampled during the two years and analyzed for mass loss, change in elemental composition, and shifts of the microbial communities to answer the following research questions:

- How does the irrigation treatment affect root mass, elemental composition, and lignin characteristics in the two year period?
- Who are the bacterial and fungal colonizers of decomposing roots and how do the microbial communities change over time (“microbial succession”)?

Using control and irrigated soils, an incubation study was installed in an incubation chamber. Two main carbon components of root litter (cellulose and lignin) were added to the soils, consisting of stable isotopic labeled ^{13}C-cellulose and ^{13}C-lignin, natural ^{12}C-cellulose and ^{12}C-lignin, and No C control. During the incubation period of 28 days the soil was sequentially sampled and the DNA extracted. Only active microorganisms should be able to degrade the ^{13}C-labeled plant compounds and incorporate the ^{13}C-label into their DNA. In addition, the ^{13}C signatures and CO_2 fluxes were measured, to answer the following research questions:

- How does the addition of the C sources change the CO_2 fluxes and its isotopic compositions, and how is the difference between the irrigated and the dry control soil?
- Which microorganisms are active decomposers of cellulose and lignin in forest soils, and how do the microbial communities change over time?

3.5 Thesis outline

The long-term acclimation to irrigation of a drought-prone Scots pine forest with a focus on root growth response is presented in section 4.

Herzog C, Steffen J, Pannatier EG, Hajdas I, Brunner I (2014). Nine years of irrigation cause vegetation and fine root shifts in a water-limited pine forest. PloS one 9: e96321.

A two-year succession of the microbial community on decomposing roots in a drought-prone Scots pine forest is presented in section 5.

Herzog C, Hartmann M, Frey B, Rumpel C, Buchmann N, Brunner I (2017). Microbial succession on decomposing root litter in a drought-prone Scots pine forest. Submitted to ISME Journal.

The degradation and uptake of cellulose and lignin carbon by the bacterial and fungal communities in a forest soil incubation experiment is presented in section 6.

Herzog C, Brunner I, Hartmann M, Stierli B, Buchmann N, Frey B (2017). Who eats the tough stuff – DNA stable isotope probing (SIP) of bacteria and fungi degrading ^{13}C-labelled lignin and cellulose in forest soil. In preparation.

4 Nine years of irrigation cause vegetation and fine root shifts in a water-limited pine forest

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4.1 Abstract

Scots pines (*Pinus sylvestris L*.) in the inner-Alpine dry valleys of Switzerland have suffered from increased mortality during the past decades, which has been caused by longer and more frequent dry periods. In addition, a proceeding replacement of Scots pines by pubescent oaks (*Quercus pubescens* Willd.) has been observed. In 2003, an irrigation experiment was performed to track changes by reducing drought pressure on the natural pine forest. After nine years of irrigation, we observed major acclimations in the vegetation and shifts in Scots pine fine root abundance and structure. Irrigation permitted new plant species to assemble and promote canopy closure with a subsequent loss of herb and moss coverage. Fine root dry weight increased under irrigation and fine roots had a tendency to elongate. Structural composition of fine roots remained unaffected by irrigation, expressing preserved proportions of cellulose, lignin and phenolic substances. A shift to a more negative ^{13}C signal in the fine root C indicates an increased photosynthetic activity in irrigated pine trees. Using radiocarbon (^{14}C) measurement, a reduced mean age of the fine roots in irrigated plots was revealed. The reason for this is either an increase in newly produced fine roots, supported by the increase in fine root biomass, or a reduced lifespan of fine roots which corresponds to an enhanced turnover rate. Overall, the responses belowground to irrigation are less conspicuous than the more rapid adaptations aboveground. Lagged and conservative adaptations of tree roots with decadal lifespans are challenging to detect, hence demanding for long-term surveys. Investigations concerning fine root turnover rate and degradation processes under a changing climate are crucial for a complete understanding of C cycling.

4.2 Introduction

In the inner-Alpine valleys of Switzerland, increased temperatures and drought periods have become more frequent and have reached a greater extent during the last few decades (Rebetez and Dobbertin, 2004; Bigler *et al.*, 2006). The most recent predictions reveal no improvement of the situation, contrariwise an increase of severe drought events is expected (Forzieri *et al.*, 2014). The most abundant tree species, Scots pine (*P. sylvestis* L.), has suffered from limited water and from increasing competition from pubescent oak (*Q. pubescens* Willd.), leading to a drastic increase in tree mortality (Dobbertin *et al.*, 2005; Dobbertin and Rigling, 2006). Recent publications concerned with drought induced tree mortality, explaining the interrelation of loss of hydraulic function and carbohydrate depletion, and concluding that the direct lack of water exceeds the carbon failing (Hartmann *et al.*, 2013; Mitchell *et al.*, 2013). Tree defoliation and dieback is not restricted to the Swiss alpine region. In all southern Europe forests, degeneration due to severe droughts has been recorded (Carnicer *et al.*, 2011). In a review, Anderegg *et al*. (Anderegg *et al.*, 2013) depicted the global significance of forest dieback as a result of drought and temperature stress. Assessment of drought events are still insufficient and demand for adequate study length (Smith, 2011a). In addition definition of severe drought events and standardization of climate extreme studies are needed (Smith, 2011b).

In order to reduce tree mortality, an experiment was started in 2003 with the installation of an irrigation set-up in a mature Scots pine stand in Pfynwald (Valais). After a few years of irrigation treatment, therefore reducing drought events, the forest showed explicit changes in Scots pine growth patterns. Adaptations in needle morphology, and shoot and stem growth were detected after four years of irrigation (Dobbertin *et al.*, 2010), as well as in the $\delta^{13}C$ signal in tree rings (Eilmann *et al.*, 2010).

Water uptake in trees is regulated by their roots (e.g. (Steudle, 2000)). However, only a limited number of studies deal with fine root adaptations in forests (Pronk *et al.*, 2002; Genenger *et al.*, 2003; Konôpka *et al.*, 2008; Montagnoli *et al.*, 2012). A few studies have focused on root adaptation after irrigation combined with a fertilization treatment (e.g. (Kätterer *et al.*, 1995; Coleman, 2007; Bakker *et al.*, 2009; Cubera *et al.*, 2012)). In the irrigation study of the forest Pfynwald, Scots pine fine root morphology changed after four years of irrigation only slightly (Brunner *et al.*, 2009), more precisely, an increase in specific root length (SRL) (Ostonen *et al.*, 2007) and a significant decrease in root tissue density (RTD) (Brunner *et al.*, 2009) were noted. In

2007, Ostonen et al. (Ostonen *et al.*, 2007) reviewed the importance of SRL as a morphological parameter in fine roots to address transformations induced by changing environmental factors. Root Area Index (fine root area relative to soil area, RAI) was promoted as a useful morphological parameter to detect changes between soil types (Hacke *et al.*, 2000) or approach the effect of irrigation and fertilization on root morphology (Ewers *et al.*, 2000). In order to detect structural changes, biochemical approaches are feasible. Several fine root compounds are known to increase with parasitic pressure (Cahill and McComb, 1992; Oßwald *et al.*, 2012).

After death, fine roots become fine root litter. Nowadays, with global warming plant litter plays a crucial role in the carbon (C) cycle. The turnover of C in soils highly depends on the residence time of the litter. The underlying factors for litter degradation are biotic (decomposer community) and abiotic (e.g. litter quality, lignin contents, soil temperature, soil water content, pH) (Prescott, 2010). Litter is mostly considered to be foliar litter input on the forest floor. The fine roots of trees are an often overlooked part of litter, even though they account for 13% of the net primary production (NPP) but only account for 3% of trees biomass (Ostonen *et al.*, 2005). Fine root turnover tends to be slower than pine needle litter (Bird and Torn, 2006). Importantly, it should be considered that in contrast to foliar litter, fine root litter is less exposed to weathering (e.g. water, temperature). In any case, Fujii & Takeda (Fujii and Takeda, 2010) showed that the position of the litter above or below the organic layer is of minor importance. The major factors of slow degradability of fine root litter are low water content (De Santo *et al.*, 1993) and higher lignin content (Berg, 1984; Fujii and Takeda, 2010); (Prescott, 2010). Rasse et al. (Rasse *et al.*, 2005) stated a faster degradation of fine root litter than shoot-derived litter in an isotope incubation experiment. The lower degradation rate is biased in nature by the physico-chemical protective properties of the soil and protection by mycorrhiza and root-hair activity. These protective mechanisms highly depend on environmental conditions such as moisture. Nevertheless, the decomposition rate of plant litter material correlates positively with nutrients but negatively with CN ratio and lignin content (Zhang *et al.*, 2008). Hence, the question remains: can a changing environment (e.g. increased rainfall) affect the chemical structure of roots directly, or does the moisture only act as a degradation stimulant without modifying root chemical composition beforehand? The C storage potential of temperate forests therefore depends on degradability and mean lifespan of fine roots.

Natural radioactive isotope of C is a cosmogenic isotope produced in the atmosphere in reaction of thermal neutrons and ^{14}N. A steady-state condition of atmospheric concentrations is maintained

between production and decay. However, due to the above ground nuclear test in 1950s superficial ^{14}C was produce and its concentration doubled. Presence of this so called 'bomb peak' global ^{14}C tracer in the atmosphere has been monitored and applied during the last 50 years (for review see (Hajdas, 2008)). Broecker et al. (Broecker *et al.*, 1982) were pioneers in using this tracer to analyse C cycling in aquatic environments. Later, Trumbore (Trumbore, 2000) adapted the ^{14}C approach used in the terrestrial ecosystem to analyse soil organic matter (SOM) dynamics. Gaudinski et al. (Gaudinski *et al.*, 2001) introduced a new application possibility for ^{14}C isotope measurement to investigate mean fine root age. Further investigation and testing showed that not only new assimilated C is used for root growth but also stored C which can be older. This can lead to a mean age of C of 0.4 yr at the point of integration (Gaudinski *et al.*, 2009). Several recent studies detected discrepancies in mean fine root age and postulated two pools of fine roots: one, a fast but smaller turnover pool with a mean turnover time of <1 yr, the other, larger, with a decadal turnover time (Riley *et al.*, 2009; Gaudinski *et al.*, 2010). In many studies, fine root thickness is or was arbitrary (Pregitzer *et al.*, 2002). In root age estimation and turnover studies this is not the case and is suggested for partitioning (Gaudinski *et al.*, 2001). Recently, Sah and co-workers (Sah *et al.*, 2011) tested the reliability of the radiocarbon method for determining root age whereby fine roots from ingrowth cores with a known maximum age were analyzed. Only for fine roots (<0.5 mm), the measured ^{14}C age was in agreement with the ingrowth core age, thicker roots tended to be older with ^{14}C measurement. Fine root age can vary largely among stands and tree species and there is a tendency for older fine root ages to be in less fertile soil (Sah *et al.*, 2013). Alongside increasing fine root diameters, increasing soil depth as well reveals a positive correlation with fine root age (Gaul *et al.*, 2009).

Benefiting from our nine-year irrigation study site, we attempt to fill the research gap regarding long-term adaptations of Scots pine fine root structure and composition. Is the associated vegetation of the mature pine forest affected by irrigation? Furthermore, our results will contribute to the ongoing discussion on fine root biomass increase or decrease in the topsoil after excessive water addition. Finally, is the longevity of fine roots, measured by radiocarbon dating, influenced by the water availability?

4.3 Materials and methods

Site description

The irrigation experiment was situated in the Rhone Valley near Leuk (Valais, Switzerland, 46°18' N, 7°37' E, 615 m a.s.l.) in a Scots pine (*P. sylvestris*) forest with occasional interspersed pubescent oak (*Q. pubescens*). Permission for the field experiment was issued by the forest service of the canton Wallis (CH) (Kantonaler Forstdienst, Kreis Oberwallis, Kantonsstrasse 275, 3902 Brig-Glis). Additionally, the permission for use of the forest for research purpose was approved by the owner of the forest, the Burgerschaft Leuk (http://www.burgerschaft-leuk.ch). The geological properties are dominated by gravel input from the Rhone river and from the Illgraben alluvial cone. A more pristine pedogenic event was the landslide from Siders. The mean annual precipitation measured in Sion (20 km) was 518 mm and the mean annual temperature 10.7°C from 2003 to 2012 (Schweiz). The irrigation experiment had 8 plots (25 x 40 m) of which four were randomly chosen for irrigation, whereas the remaining four served as control. The plots were separated by a buffer zone of 5 m (Figure 4.1). From 2003 to 2012, the irrigation system was activated in rainless nights during the vegetation period (May-October), doubling the annual rainfall amount. Water from the Rhone-channel situated along the experiment site (Figure 4.1) was used for irrigation. Nutrient input through irrigation was minor: phosphate was below the detection threshold (PO_4 <0.15 kg ha^{-1} yr^{-1}) and the input of nitrogen (2.4- 3.3 kg ha^{-1} yr^{-1}) was less than the amount that could be expected to be deposited by a doubling of rainfall (N ≤ 3.5 kg ha^{-1} yr^{-1}) (Thimonier *et al.*, 2005; Thimonier *et al.*, 2010). Three identical trees per plot with the lowest crown transparency value, which refers to trees with the highest foliation, were chosen for our study (Brunner *et al.*, 2009). In the first two plots, the volumetric soil water content was monitored hourly at a soil depth of 10 cm at four different locations using time domain reflectometry (Tektronix 1502B cable tester, Beaverton, OR).

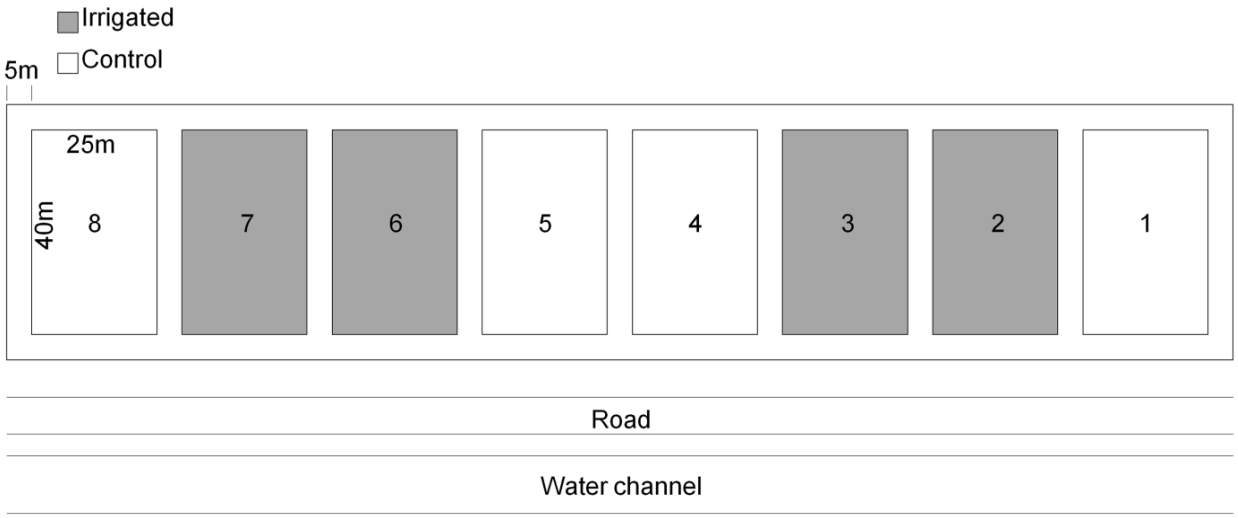

Figure 4.1: Experimental setup of the irrigation experiment in Pfynwald (VS). Irrigated plots (grey) and control plots (white) with the nearby water channel which was used for irrigation.

Vegetation

Vegetation assessment was executed to identify the vegetation shift after the nine years of irrigation. Sampling was done from 23th/24th May 2012. The coverage of vascular plant species of forbs, of shrubs and of the tree layer, were estimated in each plot using the Londo scale (Londo, 1976). The nomenclature used to describe the vegetation is based on Aeschimann et al. (Aeschimann *et al.*, 2004). The Landolt ecological indicator values for Swiss flora were calculated (Landolt, 1977). The indicator values ranged from 1 (low) to 5 (high). The nine indicators are D (aeration value), F (moisture value), H (humus value), K (continentality value), L (light value), N (nutrient value), R (reaction value (pH)), T (temperature value), W (moisture variability value). More detailed information about the indicator values is listed in Table A4.1 of the appendix.

Fine root sampling

Fine root sampling was executed before the irrigation experiment started, on the 24th April 2003 (Brunner *et al.*, 2009) and a second time after 9 years of irrigation on the 10th May 2012. For the 10th May 2012 sampling four soil cores at a distance of 1 m from each of the three trees per plot, were taken with an incremental borer (Ø 45 mm). The soil cores were kept refrigerated until further processing. Soil cores were washed in a sieve (mesh size 0.5 mm), and the roots were collected and separated into pine-roots, oak-roots and remaining root types. The separation of the fine roots (Ø ≤ 2 mm) was executed after following criteria: lignification (shrubs and tree roots), dichotomic

branching of root tips (oak and pine only), mycorrhizal root tip size (oak < 1mm, pine > 1mm). This separation is merely valid for the forest site at Pfynwald. Fine roots were used for all examinations, exclusively.

Fine root morphological and chemical analyses

The fine roots of *P. sylvestris* and *Q. pubescent* were scanned using the WinRhizo version 4.1b software package (Régent Instruments, Inc., Quebec, Canada) to obtain data regarding fine root morphology. All collected fine roots were dried (72 h, 60°C), weighted and grinded for 2 min at 80% intensity using a Retsch Mixer Mill (MM 2000, Haan, Germany).

Klason lignin of Scots pine fine roots was extracted using an adaptation of a Hiltbrunner et al. (Hiltbrunner *et al.*, 2013) protocol. Briefly described, our procedure was as follows: 200 mg of grinded fine root was weighed in falcon tubes (50 ml). Water extraction was run three times with 80°C water and 15 min incubation time per run. Centrifugation (5000 rpm) was done for 10 min. A fourth water washing step was done with ambient MilliQ water. The supernatant of the four water washing steps was then collected in order to determine the presence of phenolic substances. The washing steps were the same for the ethanol extraction. Ethanol (96%) was used at room temperature for all three rounds. Pellets were resolved in ethanol and filtered (paper filter Ø 70 mm, Schleicher Schuell, Blauband 589^3) and dried overnight (105°C). Acid soluble lignins were extracted using H_2SO_4 (72%) for 1 h in a shacking bath (30°C). After having added 16.8ml MilliQ water, the samples were autoclaved at 120°C for 1 h. Samples were filtered through porcelain filter caps (40 mL, Ø 40 mm). After weighing, the retained solid phase was burned in a muffle kiln (4 h, 550°C). The non-acid-soluble lignins were equal to the difference between the retained sample and the ash content. The acid-soluble lignins were measured with a photometric approach (Dence, 1992). Therefore, the filtrate was measured at a wavelength of 205 nm in a Varian Cary 50 UV-visible spectrophotometer (Varian, USA). Overall lignin content was equal to the sum of the percentages of the dry mass acid-soluble and the non-acid-soluble lignins.

Cellulose was extracted using an adaptation of a protocol implemented by Endrulat et al. (Endrulat *et al.*, 2010). Our procedure is described here briefly: 50 mg of dried and powdered Scots pine fine roots were sealed into Teflon filter bags (F57; ANKOM Technology, Macedon, NY, USA), followed by an incubation for 2 h in 5% NaOH at 60°C to extract fats, oils, tannins and hemicelluloses, and three washing steps with deionised boiling water. To remove the lignin, a washing step at 60°C with a 7% $NaClO_2$ solution for 30 h was conducted. Another three washing

steps with deionised boiling water were executed before drying over night at 50°C. Using the water solubles of the lignin extraction, the phenolic substances could be quantified (Swain and Hillis, 1959; Kakáč and Vejdělek, 1977). In a 10 ml test tube, a 900-µl water extract or calibration solution was mixed with 300 µl Folin-Denis reagent (purum, Fluka, 47742) and incubated at room temperature for 3 min. 600 µl of sodium carbonate (waterfree, puriss p.a. Fluka, 71350) was added and set to rest for 3 h. With a 2 ml syringe, the 1 ml solution was filtered with a 0.2 µm syringe filter directly in a cuvette. The absorption was measured at 725 nm with a Varian Cary 50 UV spectrophotometer (Varian Com. US). $\delta^{13}C$ of the extracted *P. sylvestris* fine root cellulose and bulk material was analyzed with an elemental analyser–continuous flow isotope ratio mass spectrometer (Euro-EA, Hekatech GmbH, Germany, interfaced with a Delta-V Advanced IRMS, Thermo GmbH, Germany) (Endrulat *et al.*, 2010). The $^{13}C/^{12}C$ sample values were divided by the Vienna Pee Dee Belemnite international standard (VPDB), resulting in the ratio of the $^{13}C/^{12}C$ ratio of the sample relative to the preindustrial standard VPDB ($\delta^{13}C$). C and nitrogen (N) content and C/N ratios were analyzed with gas chromatography (NC-2500, Carlo Erba Instruments, Wigan, UK). Similar to the procedure used by Richter et al. (Richter *et al.*, 2013) the extracted *P. sylvestris* fine root cellulose was combusted and graphitized (Wacker *et al.*, 2010) The $^{14}C/^{12}C$ ratio was measured using the dedicated acceleretor mass spectrometry AMS system of MICADAS at the ETH facility (Synal *et al.*, 2007).

Statistical analysis

All statistical analyses were executed with the open source tool R (R Development Core Team, 2011). For multivariate analyses, the add-in package VEGAN (Oksanen *et al.*, 2013a) and BiodiversityR (Kindt and Coe, 2008) were required. A non-metric multidimensional scaling (NMDS, NMSrandom function) ordination technique was used for visualization of the similarity of equally treated plots. The indicator values were chosen as environmental variables to the NMDS. Their explanatory significance was tested with a permutation test comparing variables separately. A PERMANOVA (adonis function) was used for testing the variation of the vegetation in differently treated plots. To test for treatment effects, a mixed model was computed with n=12 and plot as random effect by using the add-in package lme4 (Bates, 2005). A likelihood ratio test was performed using the ANOVA function. Residuals were checked for normal distribution with the Kolmogorov-Smirnov test (Massey Jr, 1951). A homoscedasticity test based on Levene (1960) (Levene, 1960) was computed. For the statistical tests the difference between 2012 and 2003 was

used (T_0 reduction), thus reducing individual tree disparity. In case of vegetation assessment, one-way ANOVA variance analysis was executed to detect treatment-induced shifts. To estimate the age of roots based on ^{14}C measurements, the data was compared to the ^{14}C data measured in the atmosphere by Levin and Kromer (Levin and Kromer, 2004) which were fitted using a polynomial regression ($y=-476.5x^2+1'222.5x-754.4$, $R^2=0.977$) (Table 4.4).

4.4 Results

Volumetric water content in soil

The amount of water added for each irrigation period was on average 587 mm, which corresponds to a doubling of the annual precipitation amount. This resulted in a significant effect of irrigation on the volumetric water content (VWC) of the soil at 10 cm depth (p = 0.022). Figure 4.2 illustrates the monthly mean (VWC) during the nine years of irrigation. The mean value of VWC over the nine-year period was 27.8% in the control plot and 34.3% in the irrigated plot. In some cases, the VWC were similar in both plots during failures of the irrigation system. In the wintertime and before irrigation starts in the spring, the VWC was similar in both control and irrigated plots.

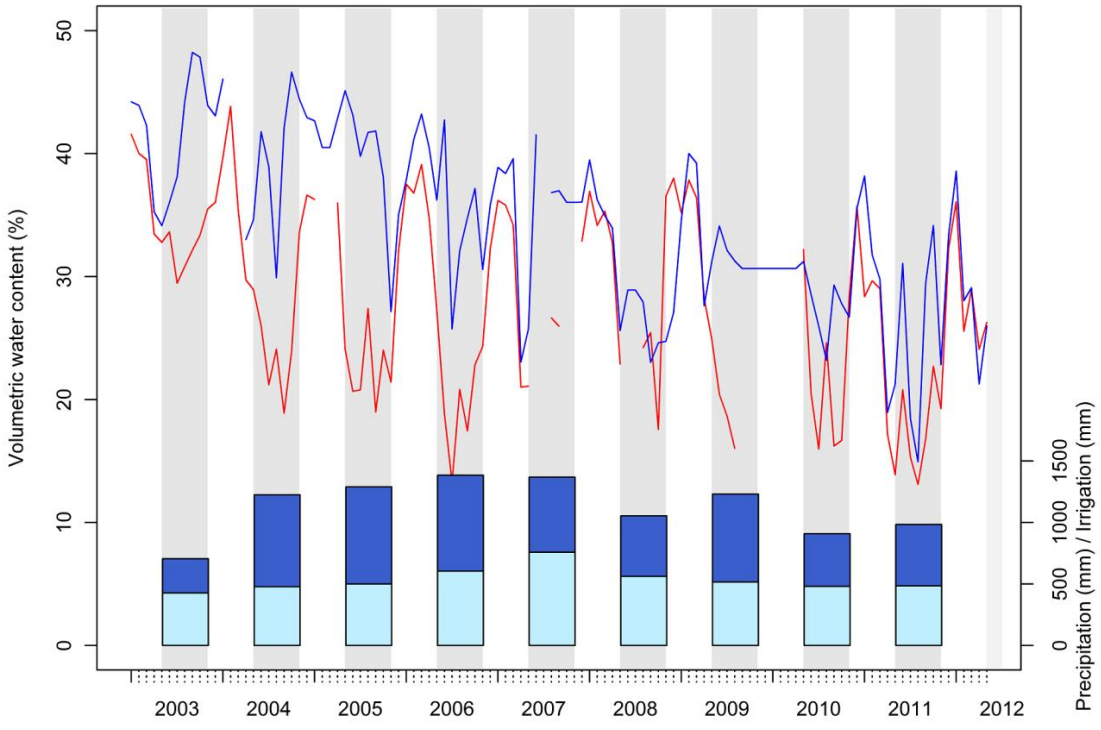

Figure 4.2: Monthly mean volumetric water content (%) of the irrigated (blue) and the control (red) plots over the experiment period (2003-2012). The annual precipitation (light blue bars) and the applied annual irrigation (blue bars) in millimetres are plotted on the second y-axis. Irrigation periods are indicated as grey bars.

Vegetation

The vegetation type of the pine forest belongs to the *Erico-Pinetum caricetosum albae* Br.-Bl. (Werner, 1985). The species richness and the coverage rate of vegetation assessment are listed in Table 4.1 and the full plant list and plant species mean abundance is illustrated in table A4.2 of the supplemental material. Species richness did not differ between control and irrigated plots. The mean number of species in irrigated plots was 41.8, whereas control plots showed a mean number of species of 39.3. Mean vegetation cover as well as the coverage of trees showed a tendency for increase at irrigated sampling sites compared to control plots, although not significantly different. In contrast, the coverage of herbs, mosses, and dead wood showed a decrease at irrigated sampling sites compared to control plots, with significant differences for herbs and dead wood. The shrub cover didn't show any differences between the treatments. The calculated Landolt indicator values were significantly higher for moisture (p = 0.004), moisture variability (p = 0.037) and nutrient value (p = 0.041), but significantly lower for continentality (p = 0.019), and the reaction value (p = 0.032). These five values were significantly explanatory for the NMDS distribution of the sampling plots (Figure 4.3). Using a NMDS technique, the plots that experienced identical treatment showed significant clustering (PERMANOVA: p = 0.023, Figure 4.3).

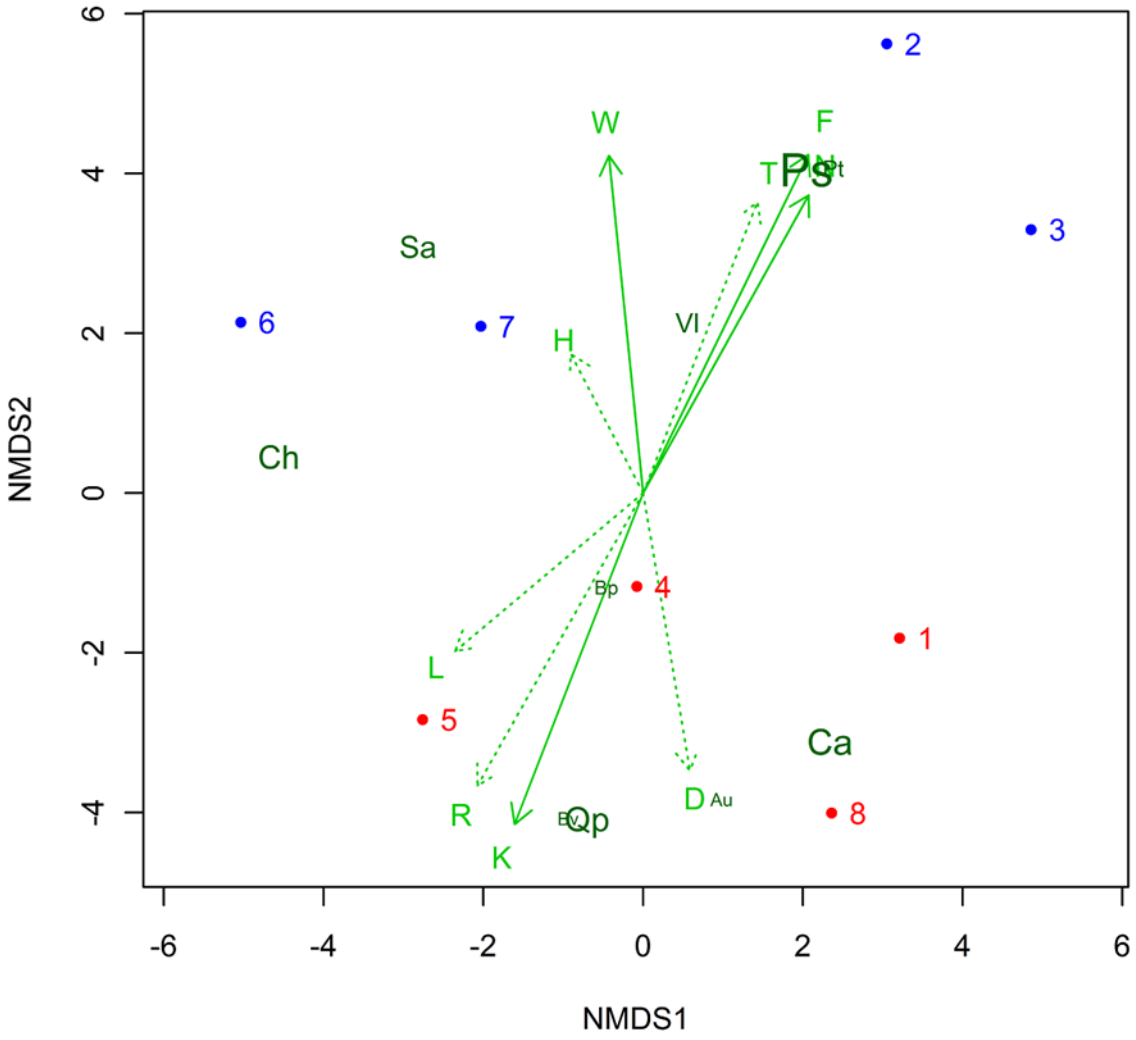

Figure 4.3: Non-metric multidimensional scaling (NMDS) of the eight plots after nine year of irrigation of the ten most abundant plant species (control=red dots, Irrigated=blue dots). The plant species are plotted with logarithmic abundance indicated by font size and orientation of their highest abundance in the plots indicated by the arrows (dark green; Ps= *Pinus sylvestris,* Ca= *Carex alba,* Qp= *Quercus pubescens,* Ch= *Carex humilis,* Sa= *Sorbus aria,* Vl= *Viburnum lantana,* Pt= *Populus tremula,* Bp= *Betula pendula,* Au= *Arctostaphylos uva-ursi,* Bv= *Berberis vulgaris*). The Landolt indicator values are fitted to the plot (light green arrows; significant= solid; non-significant= dotted) for the following properties: D (aeration value), F (moisture value), H (humus value), K (continentality value), L (light value), N (nutrient value), R (reaction value (pH)), T (temperature value), and W (moisture variability value).

Table 4.1. Vegetation assessment results for mean number of species per plot (species richness) and mean percentage coverage of mean vegetation, dead wood, trees, shrubs, herbs, and mosses after nine years of irrigation and their standard deviation (SD). One-way ANOVA: *p*-values in bold are significant ($p < 0.05$), ($n = 4$).

		Control		Irrigated		*p*
		mean	SD	mean	SD	
Species richness		39.3	1.9	41.8	5.1	0.395
Cover (%)	Mean vegetation	29.3	18.7	50.5	22.2	0.193
	Dead wood	9.5	3.3	4.5	1.9	**0.040**
	Trees	55.0	10.8	70.0	8.2	0.069
	Shrubs	16.3	7.5	16.3	4.8	1.000
	Herbs	28.8	8.5	13.8	7.5	**0.039**
	Mosses	50.8	14.2	39.5	18.9	0.378

Fine root morphology

Fine root morphology was altered after the nine-year irrigation period (Table 4.2). The dry weight per soil volume of fine roots developed in the irrigated plots increased for *P. sylvestris* and *Q. pubescens*. The remaining roots derived from other species were not affected by irrigation in their dry weight per soil volume (data not shown). Fine root tips were less frequent under irrigation, significantly for pubescent oak and slightly for Scots pine. Regarding specific root length (SRL) and root tissue density (RTD), we detected contradictory results for the two tree species. For Scots pine, SRL increased and RTD decreased whereas pubescent oak tended to react in the opposite way. Agreement in the trends between the two species was detected in the RAI. Both pine and oak increase their root area index (RAI) with irrigation, though both trends are not significant.

Table 4.2. Morphological properties of *Pinus sylvestris* and *Quercus pubescens* fine roots after nine years of irrigation. Mean values are listed for fine root dry weight per soil volume (DW) and fine root morphological traits: Average diameter (diameter), root length per soil volume (root length), specific root length (SRL), root tissue density (RTD), root tips per root length (root tips). Mixed model followed by ANOVA: *p*-values in bold are significant ($p < 0.05$), ($n = 12$).

	Pinus sylvestris			*Quercus pubescens*		
	Control	Irrigated	*p*	Control	Irrigated	*p*
DW [mg/cm^3]	2.66	3.92	**0.026**	0.09	0.25	**0.040**
Diameter [mm]	0.65	0.63	0.535	0.45	0.53	0.274
Root length [cm/cm^3]	1.74	2.94	0.078	0.19	0.28	0.206
SRL [cm/mg]	0.67	0.78	0.26	2.81	1.93	0.078
RTD [mg/cm^3]	499	451	0.306	288	351	0.236
RAI [m^2/m^2]	5.42	8.89	0.114	0.39	0.73	0.065
Root tips [cm^{-1}]	4.39	3.73	0.063	5.19	4.06	**0.026**

Fine root chemical properties

The irrigation treatment failed to induce significant differences in cellulose, phenol or lignin (Table 4.3). Therefore, coarse structure and the composition of the fine root chemistry persisted. There was a slight increase in cellulose under irrigation, but significant differences due to irrigation in root composition was limited to the amount of N in bulk roots. The amount of N in fine roots was reduced by irrigation. The C/N ratio appeared to increase as a result of the N increase under irrigation. A highly significant result occurred in the ^{13}C signal of extracted cellulose, as well as in the bulk fine root data. The $^{13}C/^{12}C$ ratio was diminished by the induced irrigation.

The correlation of the $\delta^{13}C$ values of cellulose and bulk fine root material showed a strong overall correlation ($p < 0.001$; Figure 4.4). However, the individual treatments control ($p < 0.001$) and irrigation ($p = 0.023$) showed a positive $\delta^{13}C$ values correlation of cellulose and bulk likewise.

The obvious treatment effect on the $\delta^{13}C$ was visible as a shift to a lower $\delta^{13}C$ ratio of bulk and cellulose (Figure 4.4, Table 4.3).

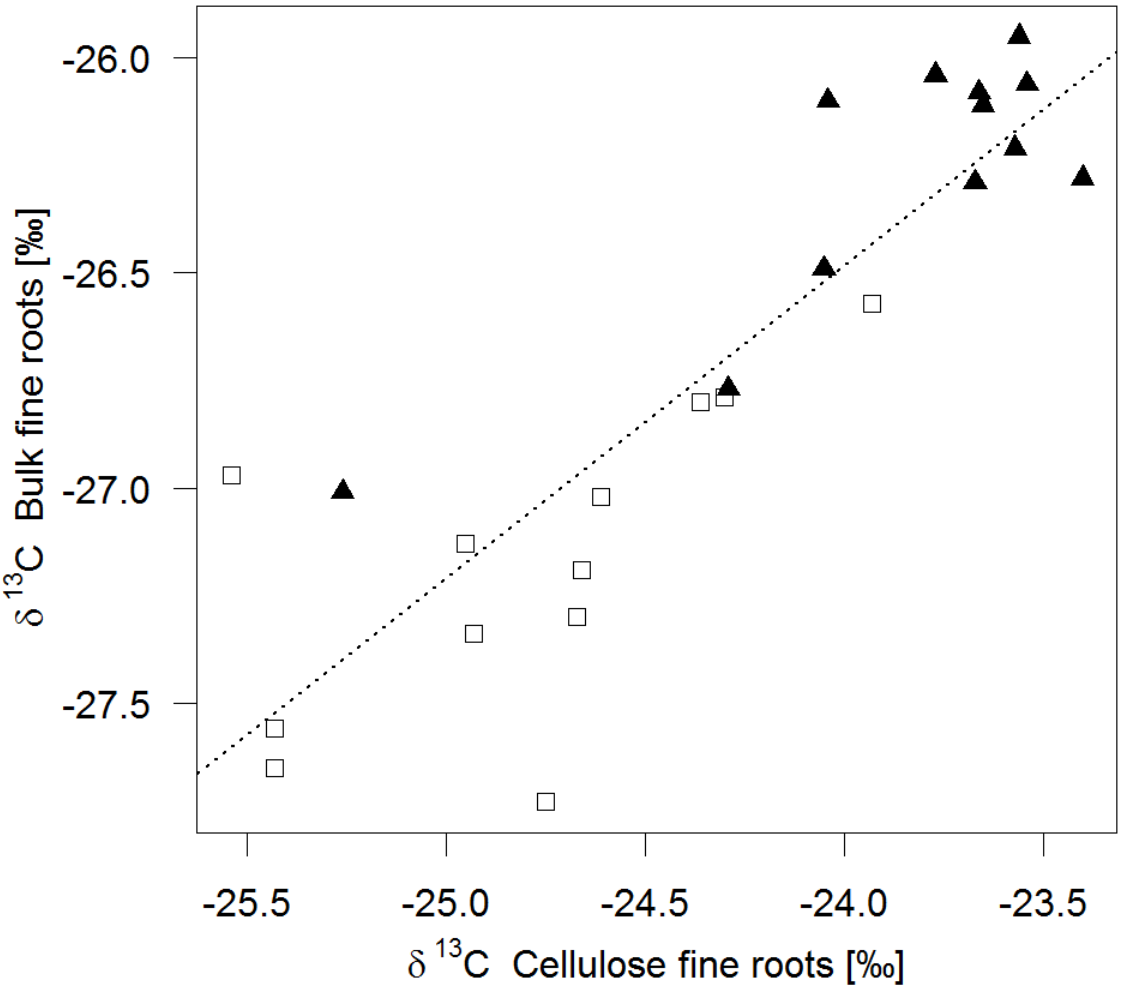

Figure 4.4: The $\delta^{13}C$ values of bulk fine root are plotted against cellulose extracts of the fine roots after nine years of irrigation. Irrigated values (open squares) show a reduction of $\delta^{13}C$ compared to the control (closed triangles). Dotted linear regression shows the overall correlation ($p < 0.001$), the dashed line is the linear regression of the irrigated ratios ($p = 0.023$) and the solid regression line belongs to the control ($p < 0.001$), ($n = 4$).

Results of ^{14}C analysis performed on roots collected in 2003 estimated fine root ages reflected similar results between the irrigation and the control plots (one-way ANOVA $p = 0.674$; Table 4.4). After the nine-year irrigation period, the results showed a significant difference in fine root ages (mixed model $p = 0.044$). The variation in the data was larger in 2012 than the one in 2003. The mean fine root age was 11.2 yr in 2003 and 7.9 yr in 2012. In 2012, the mean fine root age of the irrigated plots was strongly diminished (5.5 yr) in contrast to the control (10.4 yr).

Table 4.3. Chemical properties of *Pinus sylvestris* fine roots before and after nine years of irrigation. Mean values are listed for amount of cellulose, Klason lignin, total phenol, amount of C in bulk roots (C-bulk), amount of nitrogen in bulk roots (N-bulk), the ratio of carbon to nitrogen in bulk roots (C/N) and the $\delta^{13}C$ of cellulose and bulk root. Mixed model followed by ANOVA: *p*-values in bold are significant ($p < 0.05$), ($n = 12$).

	2003			2012		
	Control	Irrigated	*p*	Control	Irrigated	*p*
Cellulose (%)	15.5	16.5	0.719	9.5	11.3	0.430
Lignin (%; *n*=4)	37.1	37.7	0.865[a]	53.0	47.2	0.284[a]
Phenol (%)	0.17	0.17	0.933	0.18	0.18	0.983
C-bulk (%)	45.6	45.0	0.225	43.9	43.4	0.102
N-bulk (%)	0.96	1.03	0.605	0.81	0.75	**0.040**
C/N	50.5	46.9	0.519	56.1	60.5	0.080
Lignin/N (*n*=4)	42.3	36.9	**0.040**	70.3	63.7	0.484
$\delta^{13}C$-cellulose	-25.1	-25.1	0.947	-23.9	-24.8	**0.001**
$\delta^{13}C$-bulk	-26.9	-26.8	0.773	-26.3	-27.2	**<0.001**

[a] calculated from one-way ANOVA, only one sample was extracted per plot.

Table 4.4: Measured ^{14}C and estimated fine root age before and after nine years of irrigation. ^{14}C was analysed with AMS system of MICADAS at the ETH facility. Age estimation using a polynomial regression ($y=-476.5x^2+1'222.5x-754.4$) of atmospherical ^{14}C data (Levin & Kromer 2004). Mixed model followed by ANOVA: *p*-values in bold are significant ($p < 0.05$).

	2003			2012		
	Control	Irrigated	*p*	Control	Irrigated	*p*
^{14}C bulk	1.14	1.15	0.591[a]	1.08	1.06	0.059
Fine root age	10.80	11.61	0.674[a]	10.42	5.66	**0.044**

[a] calculated from one-way ANOVA, only one sample was analyzed per plot.

4.5 Discussion

Water shortage in inner-Alpine valleys force trees to reduce their crowns, shorten their needles, and cause hydraulic failure in extreme drought events (Bréda *et al.*, 2006; McDowell *et al.*, 2008; Dobbertin *et al.*, 2010). Simultaneously, damage caused by nematodes, insects, fungi, and mistletoes increase because the ability to resist such pests is reduced (Polomski *et al.*, 2006; Rouault *et al.*, 2006; Wermelinger *et al.*, 2008; Giordano *et al.*, 2009; Rigling *et al.*, 2010; Heiniger *et al.*, 2011; Zweifel *et al.*, 2012). As a consequence, Scots pines die at a higher rate than the average (Dobbertin *et al.*, 2007). It has thus been predicted that there will be a shift from sub-boreal Scots pine forests towards the sub-Mediterranean pubescent oak forests in the long-term (Rigling *et al.*, 2013). However, the alleviation of water shortage by water addition alters the competition situation among all the plants within the Scots pine forest.

Vegetation shift

After nine years of irrigation, an increase in vegetation cover, mainly due to the better closure of Scots pine crowns, was observed. In contrast, pubescent oak and other drought adapted shrubs such as *Ligustrum vulgare*, *Berberis vulgaris*, and *Arctostaphylos uva-ursi* decreased. Subsequently, with the increase in tree cover, a decrease of the herb (e.g., *Carex alba*) and moss cover was observed, whereas the shrub cover remained unaffected. A change in species richness, however, was not observed, although new species came in and a few species disappeared. Among the new species, some are known to predominantly prefer wet or variably moist conditions, e.g., *Populus tremula*, *Clematis vitalba*, or *Acer* spp. (Worrell, 1995; Lemoine *et al.*, 2001). Species such as *Viola pyrenaica, Trifolium montanum*, *Ligustrum vulgare*, and *Teucrium chamaedris* were naturally present in the control but strongly reduced in the irrigation plots. The reason for this exclusion is most likely caused by fast changes between wet and dry conditions, and competition with better adapted pioneers (e.g. (Glaser *et al.*, 1990)). These effects are limited to annual or herbaceous perennial plants with a short lifespan. Nevertheless, the irrigation also affected, in addition to crown transparency, the mortality of the Scots pine (Rebetez and Dobbertin, 2004; Bigler *et al.*, 2006). Rather surprising is the fact that there is a lack of literature using plant indicator values to demonstrate shifts in vegetation upon environmental change, as has been shown earlier for mires or alpine meadows (Peter *et al.*, 2009; Graf *et al.*, 2010). Undoubtedly, the water availability indicated by moisture variability and moisture values are consistent. However, the

assessment of the plant community additionally reveals subtle changes like a decrease in the light value or an increase in nutrient availability. The light value is reduced by the effect of a closing canopy in the irrigated plots, whereas the nutrient availability seems to be triggered directly by the increase in soil moisture content (Barber, 1995). The decrease in the light value was also detected earlier by Dobbertin et al. (Dobbertin *et al.*, 2010) having recorded a decrease in crown transparency and an increase in needle length in the irrigated plots.

Root morphology

Nine years after the start of the irrigation, the treatment resulted in a significant increase in the fine root biomass to a near-to-significant increase of the fine root length for *P. sylvestris*. Such a trend was observed as well in hardwood forests in the US (Joslin *et al.*, 2001; Tierney *et al.*, 2003), indicating that water seems to stimulate root elongation after suffering from long lasting dry periods (Joslin and Wolfe, 1998). The measured decrease in fine root tips, which was significant for pubescent oak and a tendency for Scots pine, appears to be caused by the detected elongation of the fine roots. The total increase in fine root dry weight per soil volume after irrigation is unsurprising (e.g. (Cavelier *et al.*, 1999)). Interestingly this change wasn't detected immediately. In the initial years of the irrigation experiment no significant change could be detected (Brunner *et al.*, 2009). Comparing the results from Brunner et al. (Brunner *et al.*, 2009) with ours evidently demonstrates the slow acclimation capacity of the Scots pine fine root system in the drought afflicted Pfynwald (Figure 4.5). Bakker et al. (Bakker *et al.*, 2009) demonstrated a shift of fine roots to shallower soil layers with increasing soil moisture, and consequently, the overall biomass was increased in the top layers (0-10 cm). Moreover, Leuschner et al. (Leuschner *et al.*, 2004) found in their driest stand of *Fagus sylvatica* (precipitation < 520 mm yr^{-1}), which is equivalent to our study site, the lowest fine root biomass. In a comparable study in a semi-deciduous forest in Panama, similar results were observed with irrigation in a water limited situation, with an increase of the fine root biomass in the upper most soil layers (Cavelier *et al.*, 1999). This drought effect often referred to as *deep rooting strategy* (e.g. (Schenk, 2008)) appears to be suspended by the irrigation treatment, resulting in the detected fine root biomass increase.

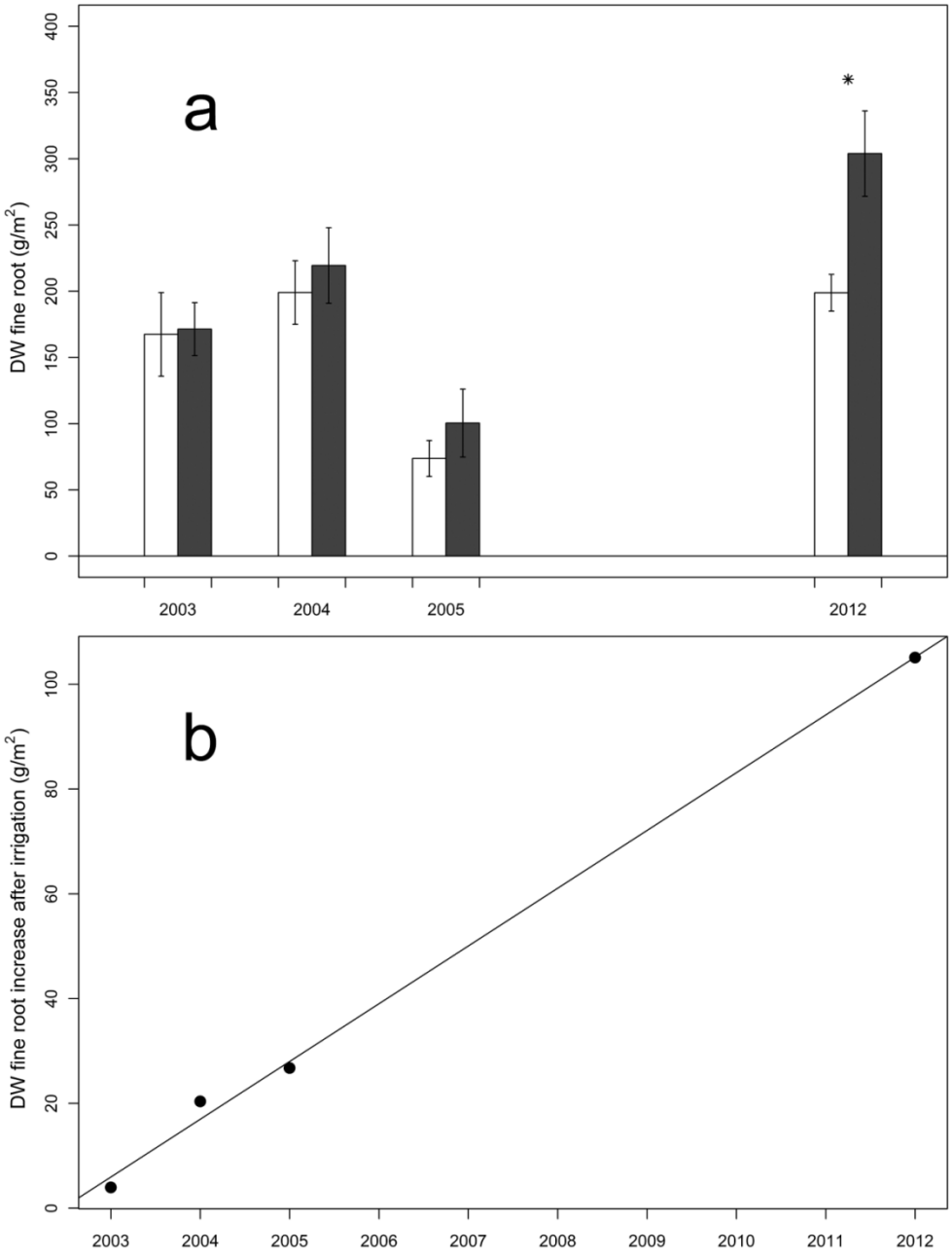

Figure 4.5: Fine root dry weight compared to data from Brunner et al. (Brunner *et al.*, 2009) (error bars = standard error), (*n* = 4). (a) Fine root dry weight (g/m^2) are shown as bar plots from 2003, 2004 and 2005 (Brunner *et al.*, 2009) compared to the 2012 detected. *P*-values < 0.05 calculated with a mixed model followed by ANOVA are marked with *. (b) A significant positive linear correlation of the Scots pine fine roots increased means could be shown (R2= 0.9958, *p*-values= 0.0014).

With an increase of root length and root biomass, a change in the SRL obviously cannot be expected. Indeed, Ostonen et al. (Ostonen *et al.*, 2007), in their meta-analysis of SRL, did not record a change in SRL, either due to irrigation or due to drought. While only a few studies have detected changes in SRL mediated by irrigation, they were also combined with fertilization (e.g. (De

Visser *et al.*, 1994)). However, SRL is best known for differentiating between species or plant strategies (Cornelissen *et al.*, 2003) as it is known for leaf and needle traits such as specific leaf area or leaf dry matter content (e.g. (Cornelissen *et al.*, 1996; Wilson *et al.*, 1999; Cornelissen *et al.*, 2003)). The comparison of the two dominating tree species in our study revealed large differences, with the pubescent oak having a much higher SRL (2.37±0.23 cm/mg) than the Scots pine (0.73±0.04 cm/mg). Similar values were recorded by Ostonen et al. (Ostonen *et al.*, 2007) for *Q. robur* (1.29±1.79 cm/mg) and *P. sylvestris* (0.75±1.05 cm/mg), leading to the conclusion that oaks with 2-3x longer roots per weight unit might have an advantage in water absorbance compared to pines. Overall, this evidence supports the notion that the pubescent oak has greater competitiveness compared to the Scots pine under a drought situation (Dobbertin *et al.*, 2005). Hertel et al. (Hertel *et al.*, 2013) recently showed a positive correlation of the RAI to annual precipitation in beech forests. In our study the RAI reacts positively to the increased water availability. These trends indicate a marginal and tardy morphological plasticity of mature pine roots with the same evolutionary background.

In contrast, air warming or drought treatments are known to decrease the root length of *Quercus* sp. (Collet *et al.*, 1997; Saxe *et al.*, 2001; Arend *et al.*, 2011). These results are potentially triggered by an accompanying nutrient deficiency (Trubat *et al.*, 2010; Grossman and Rice, 2012), taking into consideration the fact that nutrients and water availability are often highly linked. Due to known drought tolerance following a deep rooting strategy (Kuster *et al.*, 2012), the fine roots of *Q. pubescens* in Pfynwald perhaps neglect the upper soil layer (0-10 cm).

Root chemical properties

The fine root structure of *P. sylvestris* reacted strongly to the irrigation treatment. On the one hand, the amount of N in bulk roots was reduced even if the irrigation increased the N input slightly. This phenomenon is known and is described in the literature, where the increase in N results in plant growth stimulation (e.g. (Magnani *et al.*, 2007; Pregitzer *et al.*, 2008; Thomas *et al.*, 2009)). Eilmann et al. (Eilmann *et al.*, 2010) clearly demonstrated the increased pine growth at our study site. The N in the tree fine roots is therefore diluted by the boost of fixated C under irrigation. In addition, the priority of biomass accumulation, including N integration, is based on aboveground structures (regenerative organs, needle production, stem growth) (Lacointe, 2000). Meanwhile, the increased irrigation in our study potentially enhanced N leaching. These results agree with the assumption of a water limited forest in the Pfynwald rather than N limitation. On the other hand, the

isotopic ratio of $\delta^{13}C$ is strongly influenced by additional water supply. The comparison of fine root bulk with cellulose showed a significant correlation. This indicates, similar to the results of Eilmann et al. (Eilmann *et al.*, 2010), that photosynthates were used in a coupled ratio. The shift to overall reduced $\delta^{13}C$ merely demonstrates an increased photosynthetic activity of Scots pine by irrigation due to the discrimination of the ^{13}C isotope (Farquhar *et al.*, 1982). There is no enhanced cellulose production in relation to other C-associated root compounds. The fine root composition of cellulose, lignin, and phenols under irrigation was unchanged. The fine root rough structure was preserved, and revealed a conservative nature similar to King et al. (King *et al.*, 2005) who detected marginal changes with altered nutrient availability. In addition, it is possible that, due to the slow turnover of tree roots, adaptations are still lagging after nine years of irrigation. This is rather surprising, keeping in mind that phenolic compounds (Oßwald *et al.*, 2012) or lignin (Cahill and McComb, 1992) can increase with stress caused by pathogens.

Radiocarbon

Radiocarbon analysis revealed a decrease in mean fine root age by different treatments of *P. sylvestris* after nine years of irrigation. Up to now, a treatment induced alteration of the mean fine root age has not been demonstrated using this new technique of ^{14}C dating. Nonetheless, appropriate caution is needed here. First, the total root age varies considerably depending on the root lifespan assessment method (Eissenstat and Yanai, 2002), and second, considering the increased fine root biomass with irrigation, it is possible that we have detected more young and newly produced roots in relation to old roots. Black et al. (Black *et al.*, 1998) and Chapin et al. (Chapin III *et al.*, 2012) observed in their studies that root elongation tends to be negatively correlated with their longevity. Interestingly, water increase as well as decrease can have negative effects on fine root lifespan (Pregitzer *et al.*, 1993; Jones *et al.*, 2003). However, both studies are barely comparable to our findings; not only was the detection period very short (100 days and 1 year, respectively), but also additional factors such as competition or logging could have had crucial effects on the fine root lifespan (Eissenstat *et al.*, 2013). A similar trend, as in our results, was observed by Yuan and Chen (Yuan and Chen, 2010) and Finér et al. (Finér *et al.*, 2011), who recorded an increase in the turnover rate caused by an increase in the mean annual precipitation. It also appears that, using the fine root lifespan as a fine root turnover approximation, is risky in a temperate zone due to phenology (Eissenstat *et al.*, 2013). In our case, the samples were collected in the same spring period, which, accordingly, reduces the phenological influence. Subsequently,

we postulate an increase in fine root turnover rate in the presence of increasing water availability. Furthermore, it is obvious that the ^{14}C bomb peak model is more uncertain the more recent the samples are because the bomb peak effect is flattening out due to the ocean uptake and recently fossil fuel combustion (Levin *et al.*, 2008). Moreover, the turnover rate of fine roots is a highly discussed topic and represents a major factor for C sequestration which will trigger future climatic conditions (Yuan and Chen, 2010; Brunner *et al.*, 2013). Precipitation and drought, amongst others, are relevant factors which will directly influence future climate and CO_2 feedback cycles (Heimann and Reichstein, 2008; Reichstein *et al.*, 2013).

In conclusion our data imply that the responses belowground to irrigation are less conspicuous than the more rapid adaptations aboveground. Lagged and conservative acclimation of tree roots with decadal lifespans are challenging to detect, hence demanding for long-term surveys. Furthermore, interactions of treatments with biogeochemical processes operate on longer time scales and cannot be detected in short-term studies, which highlights the importance of long-term experiments at natural forest sites (e.g. (Niklaus *et al.*, 2003; Dawes *et al.*, 2011; Bader *et al.*, 2013)). Investigations concerning fine root turnover rate and degradation processes under a changing climate are crucial for a complete understanding of C cycling.

4.6 Acknowledgements

We thank Andreas Rigling for being responsible for the whole treatment experiment in the ‘Pfynwald’, Peter Bleuler and Werner Landolt for regulating the irrigation and providing the striking image, Magdalena Nötzli for the help with lignin and cellulose extraction, the team of the Central Laboratory of WSL for the elemental analyses, and Curtis Gautschi for correcting the English language. The ’Pfynwald’-site belongs to the WSL Long-Term Forest Ecosystem Research Programm LWF.

5 Microbial succession on decomposing root litter in a drought-prone Scots pine forest

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5.1 Abstract

Decomposition is a major flux of the carbon cycle in forest soils and understanding the involved processes is key for budgeting carbon turnover. Decomposition is constrained by the presence of biological agents such as microorganisms and the underlying environmental conditions such as water availability. A metabarcoding approach of ribosomal markers was chosen to study the succession of bacterial and fungal decomposers on root litter. Litterbags containing pine roots were buried in a pine forest for two years and sequentially sampled. Decomposition and the associated communities were surveyed under ambient dry and long-term irrigation conditions. Early decomposition stages were characterized by the presence of fast-cycling copiotrophic microorganisms such as Proteobacteria, yeasts and molds, and were replaced by more oligotrophic bacteria and specialized litter-associated or parasitic fungi such as white-rots, Planctomycetes and Pleosporales. This succession was probably driven by a decrease of easily degradable carbohydrates and a relative increase in persistent compounds such as lignin. We hypothesize that functional redundancy among the resident microbial taxa caused similar root decomposition rates in control and irrigated forest soils. These findings have important implications for drought-prone Alpine forests as frequent drought events reduce litter fall, but not litter decomposition, potentially resulting in lower carbon stocks.

5.2 Introduction

The carbon cycle in forest ecosystems

Forest soils have sequestered approximately 1.1±0.8 petagrams of carbon per year over the past decades (Pan *et al.*, 2011). Future predictions for terrestrial carbon uptake remain uncertain and will be strongly affected by more extreme climatic events (Reichstein *et al.*, 2013). Temperate forests, in particular, will face increasing frequencies and longer lasting periods of summer drought (Millar and Stephenson, 2015), which may result in increased tree mortality (Anderegg *et al.*, 2013; Hartmann *et al.*, 2013) and establishment of more drought-adapted key tree species (Rigling *et al.*, 2013). Forest soil carbon pools are mainly driven by primary production, litter decomposition and stabilization, but it remains unclear whether there will be an increase or decrease in soil carbon with altered climatic conditions (De Deyn *et al.*, 2008). While primary production can be well monitored and modeled (e.g. SILVA (Pretzsch, 2001), ANAFORE (Deckmyn *et al.*, 2008)), modeling carbon stocks in soils is more difficult and, among other factors, hampered by the lack of data on root decomposition (McGuire and Treseder, 2010; Didion *et al.*, 2014). In the past decade, various attempts have been made to quantify and understand the complex process of litter decomposition (Berg and McClaugherty, 2014b; Solly *et al.*, 2014). Nevertheless, direct *in vivo* manipulation studies are rare, and and it remains unclear how climatic factors affect litter decomposition.

Root decomposition

While leaf and needle production as well as foliar litter decomposition can be successfully and routinely assessed, root production and decomposition still lack suitable and widely accepted measurement methods (Goebel *et al.*, 2011; Berg and McClaugherty, 2014b). Measuring root decomposition is important because there is increasing evidence that root carbon may contribute more to forest soil carbon pools than aboveground biomass (Rasse *et al.*, 2005; Fan and Guo, 2010; Sanaullah *et al.*, 2011b; Amin *et al.*, 2014). Root composition and characteristics may also be key factors influencing litter decomposition in soils (Prescott, 2010; Solly *et al.*, 2014). Compared to foliar litter, roots experience greater physical and chemical protection from decomposition (Rasse *et al.*, 2005). Physical protection is directly associated with the complex soil structure, where particulate organic matter (POM) can be included in soil aggregates, or the porous structure of the soil matrix, which generally limits microbial accessibility (Goebel *et al.*, 2009; Schmidt *et al.*, 2011). Greater chemical recalcitrance of roots compared with that of leaf litter, related to a higher lignin and lower nutrient concentration, may impede their microbial degradability (Kögel-Knabner, 2002; Berg and McClaugherty, 2014b). This chemical protection by a high amount of persistent lignin has become a major area of research because of the prospect of being able to identify one of the key components controlling SOM stabilization (Hofmann *et al.*, 2009; Walela *et al.*, 2014). Nonetheless its importance is contested (Cotrufo *et al.*, 2013).

Silver and Miya (Silver and Miya, 2001) concluded from their meta-analyses that the lignin to nitrogen ratio (lignin:N) is one of the main drivers of litter decomposition, and this finding has been confirmed by many others in later studies (Moore *et al.*, 1999; Heim and Frey, 2004; Walela *et al.*,

2014). While nitrogen often acts as a driver of decomposition, lignin, because of its highly branched structure of interlinked aromatic rings (monophenols) (Abdel-Hamid *et al.*, 2013), impedes decomposition. The measurement of lignin monophenols evolved as a state-of-the-art biomarker to determine the quality, quantity and decomposition state of litter (Thevenot *et al.*, 2010; Duboc *et al.*, 2014). Some researchers have followed the decomposition state at different depths along the soil profile (Rumpel *et al.*, 2002; Heim and Frey, 2004; Thevenot *et al.*, 2010), whereas others have combined measurement of microbial biomarkers with cupric oxide lignin characterization (Duboc *et al.*, 2014). Unfortunately, only a limited number of long-term studies have been performed, particularly regarding lignin, that could help trace the plant-derived compounds to their final preserved state (Heim and Frey, 2004).

Effects of irrigation on degradation and soil communities

In addition to effects of litter composition, climatic variables such as temperature and water availability seem to strongly affect decomposition in soils (Prescott, 2010; Solly *et al.*, 2014), hence increased water availability is not directly affecting the tree litter composition (Herzog *et al.*, 2014). If litter quality is not directly affected by water availability, the positive relationship between water availability and decomposition could be derived indirectly from biological factors. Studies investigating drought effects on microbial communities show a decrease in microbial biomass and activity with long-lasting droughts (Schimel *et al.*, 1999). Additionally, severe droughts can decrease enzyme production in the rhizosphere (Sanaullah *et al.*, 2011a). In the soil of a Scots pine forest in a drought-prone environment, a long-term irrigation treatment suspended summer droughts, resulting in a stimulation of microbial activity (Hartmann *et al.*, 2017). Further, the authors showed a clear shift from predominantly oligotrophic microbial communities in the dry control plots to more copiotrophic communities in the resource-rich irrigated plots. This shift could be explained by a higher turnover rate of carbon in the irrigated plots, in which case enhanced degradation of easily degradable components of roots with irrigation would be expected. Overall, irrigation in a drought-prone forest should result in increased microbial degradation of root litter.

Role of the microbiome in root decomposition

Little is known about how the microbial community affects the degradation of root litter in forest soils. Reed and Martiny (2007) reviewed several common garden and reciprocal transplant studies and concluded that microbial community composition affected decomposition only in some cases. In another review, McGuire and Treseder (2010) summarized the relevance of shifts in microbial community structure for the decomposition process; they reported that the presence of certain functional groups, rather than microbial biomass, seems decisive for decomposition. Several studies have been conducted to assess litter decomposition in agricultural systems (Dilly *et al.*, 2004; Allison *et al.*, 2013), but often with a limited taxonomic resolution (Duboc *et al.*, 2014; Treseder *et al.*, 2014) or considering short timescales (Purahong *et al.*, 2016). Two studies using next-generation sequencing techniques (Clemmensen *et al.*, 2013; Sanaullah *et al.*, 2016) detected a vertical succession of fungal functional groups within the soil profile. With the application of modern sequencing techniques, limitations have shifted from the relatively low taxonomic

resolution of the acquired results to the lack of useful and updated comparison data (McDonald *et al.*, 2012; Gilbert *et al.*, 2014).

Microbial succession during decomposition

Specialized lignin degraders such as the white-rot fungi seem to be the only organisms capable of complete mineralization of the lignin molecule (Abdel-Hamid *et al.*, 2013). If and how bacteria play a role in lignin degradation is not entirely resolved (Brown and Chang, 2014; De Gonzalo *et al.*, 2016). Baldrian (2017) pointed out that studies addressing the combined role of bacteria and fungi during decomposition are lacking. Whereas reports on agonistic (e.g. lichen formation, fungal highways) and antagonistic interactions (e.g. parasitism, competition) between bacteria and fungi are numerous, it is less clear how positive and negative interactions between bacteria and fungi affect degradation of carbon sources in soils (Meidute *et al.*, 2008; Haq *et al.*, 2014). Whereas theoretical models about cheaters and producers or opportunists and miners have been developed (Allison, 2005; Moorhead and Sinsabaugh, 2006), it is certain that all these interactions are highly regulated by environmental conditions such as nutrient availability, by costs of enzyme production, and by substrate accessibility.

Succession of microbes on naturally occurring persistent macromolecules has become a focus of many microbiological studies under controlled laboratory conditions, e.g. rapid micro-scale succession on chitin particles in seawater (Datta *et al.*, 2016). Microbial succession studies investigating degradation of different carbon sources have been conducted in various biomes, such as aquatic systems (Bengtsson *et al.*, 2012; Kalenitchenko *et al.*, 2016) and grasslands (Kuramae *et al.*, 2010). However, similar studies in forest soils are still rare. Controlled laboratory studies have been conducted to decipher degradation of various wood types (Prewitt *et al.*, 2014), and a few *in situ* experiments of fungal succession on tree litter have been carried out (Voříšková and Baldrian, 2013; Haňáčková *et al.*, 2015), hence, bacteria are often not addressed in this context. Nonetheless, as Schimel and Schaeffer (2012) stated: “The largest uncertainty about the role of community composition probably exists for dead roots.”

To address this gap, we studied the decomposition of fine and coarse roots in a forest, traced the fate of lignin in these roots, and identified bacterial and fungal succession throughout the course of root decomposition. These investigations were carried out in a drought-prone Scots pine forest in Switzerland, where half of the experimental plots are irrigated with the intention of relieving the trees from summer drought stress. We combined chemical assays with a metabarcoding approach of bacterial and fungal ribosomal markers to address the following questions: (1) Do chemical and biochemical traits of decomposing roots change over a 2-year period, and which traits are affected by the irrigation treatment? (2) Do bacterial and fungal communities in soils and roots differ from each other, and do they differ between root types? (3) Can we observe successional patterns of bacterial and fungal taxa within the decomposing roots over a period of two years, and how are the patterns affected by irrigation? (4) Do lignin-decomposing fungi enter the decomposing roots only at a later stage of decomposition or are they present from the beginning, and how does irrigation affect the succession patterns of these fungi?

5.3 Material and methods

Site description

The root decomposition study has been performed in the Pfyn forest (*Pfynwald*). This forest is situated in the Rhone Valley of Switzerland (46°18'N, 7°37'E, 615 m a.s.l.) in a drought-prone Scots pine forest (*Pinus sylvestris*). In this mature forest, eight plots (25x40 m each) were installed, and four of these plots have been irrigated during the summer months (April – October) since 2003 (Herzog *et al.*, 2014). A reduction of severe and long-lasting summer drought periods has been achieved by approximately doubling precipitation from about 500 to 1000 mm per year, resulting in an increase of the mean crown cover from 57% to 71% within a decade (Hartmann *et al.*, 2017). The irrigation treatment resulted in a significant increase in mean volumetric soil water content from 27.8% to 34.3% (Herzog *et al.*, 2014).

Root decomposition experiment

Roots for the decomposition study were extracted with a spade from each of the *Pfynwald* plots in autumn 2013 and transported in plastic bags to the lab. There, the roots in each sample were washed in a sieve under running water and pine roots were picked out and separated into two size fractions, i.e. fine roots (Ø <2 mm) and coarse roots (Ø 2-5 mm). Roots with a greater diameter were discarded. Subsequently, the roots were air dried and stored at room temperature. In February 2014, litterbags with a mesh size of 1 mm and a size of 10 x 10 cm were filled with 1 g of air-dried fine root or coarse root material. The litterbags were made of a robust nylon mesh (Sefar Petex®, Sefar AG, Heiden, Switzerland). At the end of March 2014, before the start of the irrigation, each root-filled litterbag was buried in the same plot from which roots were originally excavated. In order to destructively sample the litterbags at five different time points, five litterbags per plot were tied together with a nylon cord and buried horizontally at 5 cm soil depth. The sampling time points for the litterbags containing fine roots were 0, 3, 6, 9, 12 and 24 months, and the time points for the coarse roots were 0, 12 and 24 months after installation. In total, 240 litterbags with fine roots and 120 litterbags with coarse roots were collected. Roots from the first time point (T0) were not buried in the soils. Litterbags of later time points were excavated carefully and stored in plastic bags and cool boxes during transportation to the laboratory. In the laboratory, the roots were carefully cleaned with a fine brush to remove adhesive soil, frozen in liquid nitrogen, and freeze-dried. Subsequently, roots from the same root class (fine or coarse), plot, and time point were pooled, dry weights were measured, and samples were then stored at -20°C until further processing. From the remaining root litter mass, the decomposition rate constant k was calculated according to the reaction rate formula:

$$\ln(\frac{M_t}{M_0}) = -k * t$$

Where M_0 is the initial mass and M_t is the remaining mass after decomposition time t.

At the time of litterbag collection, soil samples were also collected next to the litterbags. Three soil samples were taken with a hand spade from the topsoil (0-10 cm) and pooled per plot. Soil samples were transported in plastic bags and cool boxes to the laboratory. The soil samples were

sieved through a 2 mm sieve, and about 1-2 g soil aliquots were frozen in liquid N_2, lyophilized, and stored at -80°C until DNA extraction. About 5-10 g soil was dried at 105°C for soil water content calculations, and the remaining soil was dried at 60°C for final storage. During the whole study period, soil water content was recorded with EC-5 sensors (Decagon Devices, Pullman, WA, USA) and soil temperature with RT-1 sensors (Decagon Devices, Pullman, WA, USA) at 5 cm depth near the litterbags within each plot. Data were stored with EM50 digital data loggers (Decagon Devices, Pullman, WA, USA).

Fine and coarse root chemical analyses

Carbon (C), nitrogen (N), and the stable C and N isotopes of the milled root material were analyzed with an elemental analyzer-continuous flow isotope ratio mass spectrometer (Euro-EA, Hekatech GmbH, Germany, interfaced with a Delta-V Advanced IRMS, Thermo GmbH, Germany). The Vienna Pee Dee Belemnite international standard (VPDB) was used for the calculation of the ratio between ^{13}C and ^{12}C ($\delta^{13}C$), and the isotope ratio ^{15}N to ^{14}N of the air was used as a standard for the calculation of the $\delta^{15}N$. Both $\delta^{13}C$ and $\delta^{15}N$ were expressed in per mil (‰) with a measurement precision of ± 0.20 ‰ and ± 0.30 ‰, respectively.

Lignin monomers from the milled root material were extracted with the cupric oxide oxidation method introduced by Hedges and Ertel (1982) and modified by Kögel-Knabner and Bochter (1985). Briefly, the root material was oxidized by cupric oxide in a pressure oven (175°C, 2.5 hours). A recovery standard was added (ethyl vanillin) and the solution was acidified with HCl (pH 2). After precipitation of the humic acids, lignin monomers were extracted using solid-phase extraction with a C18 absorbent column (SUPELCO, Sigma-Aldrich, Buchs, Switzerland) with one drop per second at 20°C. The monophenols were eluted with ethylacetate and dried under N_2 and re-dissolved in pyridine. An internal standard (phenyl acetic acid) was added to the extracted monophenols. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TCMS; 99:1) were used as derivatization agents (Sigma-Aldrich, Buchs, Switzerland). The monophenols were characterized on a Thermo Scientific™ TRACE™ 1300 Gas Chromatograph coupled to a Thermo Scientific™ ISQ™ Single Quadrupole Mass Spectrometer (GC-MS) and quantified on a Thermo Scientific™ Flame Ionization Detector (FID for TRACE™ 1300 GC Series). For quantification, external standards of the monophenols present in lignin were measured alongside the samples (Table A 5.1 in appendix).

DNA extraction from roots and soils

For DNA extraction, either lyophilized root material, which was hand milled with a mortar under liquid nitrogen, or frozen soil material was used. DNA was extracted using the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). For the bead beating procedure according to Frey (Frey *et al.*, 2008), either 0.5 g soil or 0.1 g root material was used. DNA concentration was determined using PicoGreen (Molecular Probes, Eugene, OR, USA). Ten ng of DNA were used to perform PCR amplification of the ribosomal small-subunit RNA gene (region V3-V4) and the internal transcribed spacer (region ITS2). PCR amplification was done in triplicate and pooled as previously described (Rime *et al.*, 2015; Frey *et al.*, 2016a; Frossard *et al.*, 2017;

Hartmann *et al.*, 2017). Pooled DNA samples were sent to the Génome Québec Innovation Center at McGill University (Montréal, Canada) for barcoding using the Fluidigm Access Array technology (Fluidigm) and paired-end sequencing on the Illumina MiSeq v3 platform (Illumina Inc., San Diego, CA, USA).

Sequence quality control, OTU clustering and taxonomic assignments

Quality filtering and clustering into operational taxonomic units (OTU) were performed in a manner similar to that reported earlier (Frey *et al.*, 2016b). UPARSE (Edgar, 2013; Edgar and Flyvbjerg, 2015), a customized pipeline integrated in USEARCH v.8 (Edgar, 2010), has been used with some modifications. The fastq_mergepairs algorithm, implemented in USEARCH (Edgar and Flyvbjerg, 2015), was used for merging paired-end reads. For substitution errors, originated during the Illumina sequencing procedure, the correction tool BayesHammer (Nikolenko *et al.*, 2013) was applied. For PCR primers trimming Cutadapt (Martin, 2011) was run, with one mismatch allowed. Bacterial sequences ($16S_{V3V4}$) shorter than 300 bp, fungal sequences (ITS2) shorter than 200 bp, and sequences without matching primers were discarded. USEARCH fastq_filter function enabled quality filtering of the trimmed reads with a maximum expected error threshold of one. Singletons were removed to reduce artificial OTU inflation while clustering (Edgar, 2013). A 97% similarity threshold was applied for clustering the sequences into OTU. USEARCH cluster_otu function was used for the clustering and includes an 'on-the-fly' chimera removal algorithm (Edgar, 2013). Metaxa2 (Bengtsson-Palme *et al.*, 2015) and ITSx (Bengtsson-Palme *et al.*, 2013) were used to test the centroid OTU sequences for presence of ribosomal signatures, and unsupported centroid sequences were discarded. Remaining reads which passed the quality filtering were mapped with USEARCH usearch_global algorithm (parameters: maxrejects 0, maxaccepts 0 and top_hit_only) against the final OTU list. Raw sequences were deposited in the European Nucleotide Archive under the accession number PRJEB21241. MOTHUR, with its implemented naïve Bayesian classifier (minimum bootstrap support 60%), was used to query the centroid sequences to the reference database. GREENGENES was used as a reference database for the prokaryotic ($16S_{V3V4}$) sequences. Eukaryotic (ITS2) sequences were queried against the two databases of NCBI GenBank (Benson *et al.*, 2015) and UNITE (Abarenkov *et al.*, 2010). OTU assigned to organelle structures (chloroplast, mitochondria), soil animals (metazoa) and plants (viridiplantae) were removed prior to statistical analysis.

Statistics

All statistical tests were performed with R (Core Team, 2014). A P-value <0.05 was considered significant in all statistical tests. Alpha-diversity was estimated by calculating the Shannon-index implemented in the 'diversity' function of the vegan package (Oksanen *et al.*, 2013b) of rarefied data by smallest site maximum (number of sequences) using 'rarefy' implemented in vegan. Beta-diversity was calculated by Whittaker dissimilarity (Whittaker, 1960) between substrates using 'betadiver' implemented in vegan (R-package). Effects of factors on the relative abundance within the microbial community were assessed by univariate permutational ANOVA based on Euclidian distances using the 'adonis' function in vegan with 10^5 permutations. To correct for multiple testing,

the ‘p-adjust’ function (method: ‘fdr’, (Benjamini and Hochberg, 1995)) was used, resulting in a q-value (corrected P-value), with a q-value <0.05 considered significant. For assessing similarity in microbial community structure, Mantel testing based on the Bray–Curtis dissimilarity matrices and Procrustes analysis based on principle coordinate analysis (PCoA) (Gower, 1966) using the mantel (Legendre and Legendre, 2012) and protest function (Peres-Neto and Jackson, 2001), respectively, were implemented in vegan with 10^5 permutations (Figure 5.1). A partial constrained redundancy analysis (pRDA) using the ‘rda’ function implemented in vegan (Legendre and Legendre, 2012) was calculated from the relative abundance values against factors (treatment, time point) and explanatory variable (total lignin, C/N, H_2O in soil, H_2O in root, weight remaining) and plotted for bacteria and fungi in Figure 5.2. The arrows and statistical legend table in Figure 5.2 were calculated using the function ‘envfit’ implemented in vegan. A heatmap was calculated from Z-score transformed relative abundance data using basic R (Core Team, 2014).

5.4 Results

Long-term irrigation strongly increased the soil volumetric water content (VWC) compared to ambient conditions (irrigated: 0.117 (± 0.016 SE) m^3m^{-3}, control: 0.077 (± 0.006 SE) m^3m^{-3}, appendix figure A5.1). In contrast, soil temperature was not affected by irrigation (irrigated: 10.28 (± 0.10) °C, control: 10.20 (± 0.21) °C; appendix figure A5.1). The first summer of the root litter decomposition study was exceptionally wet. Nonetheless, VWC was lower in naturally dry control plots during the whole irrigation period (Mai – October), and the difference between the two treatments did not disappear until late winter (February). Similar measurements were recorded in the second year. However, the summer (July to September) was much drier during the second year (126 mm) compared with the first year (208 mm), as recorded by Meteo Swiss in Sion, Valais (2017).

After two years of decomposition, 55% (± 2%) and 58% (± 2%) of the fine root litter mass remained in the control and irrigated plots, respectively. Slightly more coarse root litter material remained compare with fine roots, i.e. 64% (± 2%) and 62% (± 2%) in the control and irrigated plots, respectively. A clear decrease by approximately one fourth of the mass was detected after three months for fine root litter. Coarse roots showed a similar tendency, with a greater reduction during first year compared with the second year. Decomposition rates (k) were not altered by the irrigation treatment for fine roots (control: 0.302 ± 0.015; irrigated: 0.282 ± 0.018) or for coarse roots (control: 0.224 ± 0.014; irrigated: 0.245 ± 0.019), even though root litter from irrigated plots showed a significantly higher root water content during the irrigation period (early and late summer) compared to the non-irrigated period. In contrast, during the non-irrigated period, root litter water content was mostly higher in the control than in the irrigated plots.

Shifts in litter chemical properties

For most of the measured elements, similar patterns were observed for fine and coarse root litter (Table 5.1a and 5.1b). Hence, if not specially stated, the term *root* corresponds to both fine and coarse roots. The total carbon (C) in the root litter was not affected by irrigation. Fine root C was affected by time, showing a clear increase after 6 and 12 months, then decreasing back to the starting amount after 18 months. Coarse root C was less affected by time but tended to decrease over time. Over the whole study period, fine root litter showed a lower nitrogen (N) concentration in the irrigated plots compared with in the control plots. Total N increased significantly over the study period in both control and irrigated plots. Therefore, the C to N ratio (C/N) decreased with progressing decomposition and C/N increased significantly under the irrigation treatment. $\delta^{13}C$ decreased significantly with irrigation, from -26.0 to -26.5‰ over the whole study period. In contrast, $\delta^{15}N$ increased under irrigation, from -9.7 to -9.1‰.

The extracted lignin monophenols relative to root dry weight are presented in Table 5.1a and 5.1b. The overall sum of monophenols (V.S.C.) changed significantly during progressing decomposition for both root litters. V.S.C. remained stable during the first 6 months then showed a significant increase, with a peak at 12 months, then decreased again for the time points 18 and 24 months. With progressing decomposition, increasing ratios of acid to aldehyde vanillyl phenols (Ad/Al_V)

were found. The irrigation treatment showed a decreasing Ad/Al_V ratio in fine root litter samples. The acid to aldehyde ratio of syringyl units (Ad/Al_S) did not change over time but was slightly higher in fine root litter from the irrigation treatment compared with that from the control. With progressing decomposition, the lignin to N ratio increased in coarse root, but not in fine root litter.

Table 5.1a: Means of fine root chemical properties over time of decomposition with the standard error in parentheses (n=4). Lignin monomers in milligram per gram root litter dry weight. Overall lignin given as the sum over the vanillyl, syringyl, and cinnamyl monomers (V.S.C.), the ratio of acid to aldehyde vanillyl monomers (Ad/Al_V), and the ratio of acid to aldehyde syringyl monomers (Ad/Al_S). ANOVA P-values <0.05 are given in bold.

Fine root																					
Timepoint	**0 Month**		**3 Month**		**6 Month**		**12 Month**		**18 Month**		**24 Month**		**ANOVA**								
Treatment	**Control**	**Irrigated**	**Control**	**Irrigated**	**Control**	**Irrigated**	**Control**	**Irrigated**	**Control**	**Irrigated**	**Control**	**Irrigated**	**Treatment**	**Timepoint**	**Interaction**						
Mass remaining [g]	1	1	0.77 (0.02)	0.78 (0.01)	0.74 (0.02)	0.73 (0.01)	0.70 (0.00)	0.70 (0.01)	0.65 (0.06)	0.65 (0.02)	0.55 (0.02)	0.58 (0.02)	0.894	**6.0E-16**	0.785						
Root water content [g]	0	0	0.93 (0.16)	1.90 (0.05)	0.73 (0.12)	1.70 (0.22)	0.56 (0.05)	0.41 (0.04)	0.30 (0.03)	0.36 (0.01)	0.45 (0.06)	0.30 (0.02)	0.377	**1.2E-15**	**3.3E-05**						
Carbon content [%]	44.91 (0.46)	45.22 (0.94)	46.18 (0.48)	43.26 (2.05)	46.12 (0.63)	44.63 (0.48)	46.20 (1.17)	45.71 (0.72)	45.31 (1.06)	44.10 (0.97)	45.61 (0.71)	44.22 (1.93)	**2.8E-04**	0.130	0.092						
$\delta^{13}C$ [‰]	-25.91 (0.19)	-26.57 (0.31)	-26.00 (0.18)	-26.63 (0.32)	-26.07 (0.15)	-26.62 (0.41)	-26.00 (0.17)	-26.69 (0.36)	-25.93 (0.39)	-26.63 (0.43)	-26.06 (0.24)	-26.44 (0.36)	**2.3E-09**	0.959	0.829						
Nitrogen content [%]	0.78 (0.03)	0.65 (0.05)	0.82 (0.08)	0.78 (0.06)	0.91 (0.08)	0.76 (0.03)	0.90 (0.06)	0.79 (0.04)	0.89 (0.09)	0.79 (0.04)	0.86 (0.11)	0.77 (0.03)	**1.9E-06**	**0.003**	0.564						
$\delta^{15}N$ [‰]	-9.31 (0.70)	-9.18 (0.44)	-10.12 (0.45)	-9.41 (0.67)	-10.28 (0.19)	-9.45 (1.07)	-10.05 (0.64)	-8.97 (0.74)	-9.09 (1.20)	-8.95 (1.23)	-9.41 (0.63)	-8.75 (0.98)	**0.009**	0.053	0.662						
Carbon/Nitrogen	57.72 (2.73)	69.45 (6.26)	56.71 (4.84)	55.52 (5.83)	50.74 (4.73)	59.08 (2.67)	51.63 (2.99)	58.09 (3.78)	51.08 (5.50)	55.92 (3.02)	53.44 (5.86)	57.79 (4.12)	**9.5E-05**	**0.001**	0.133						
Lignin Monomere [mg/g]																					
V.S.C.	15.81 (1.66)	12.53 (0.81)	15.27 (2.22)	16.95 (1.08)	15.74 (1.74)	13.69 (2.05)	24.58 (4.28)	23.64 (3.14)	24.21 (6.89)	16.82 (3.72)	19.42 (3.57)	15.10 (1.85)	0.119	**0.028**	0.461						
Ad/Al_V	0.32 (0.05)	0.26 (0.02)	0.29 (0.03)	0.24 (0.02)	1.20 (0.60)	0.98 (0.09)	0.46 (0.28)	0.27 (0.05)	0.86 (0.18)	0.61 (0.14)	1.14 (0.65)	0.57 (0.14)	**3.0E-05**	**3.7E-13**	0.432						
Ad/Al_S	0.52 (0.56)	0.91 (0.57)	1.53 (0.76)	1.13 (1.23)	0.70 (0.73)	1.74 (0.24)	0.55 (0.58)	2.42 (1.44)	0.13 (0.14)	0.23 (0.06)	0.44 (0.39)	1.30 (0.36)	**0.001**	**0.001**	0.088						
Lignin/Nitrogen	20.23 (3.59)	19.22 (2.79)	19.07 (7.11)	21.78 (3.64)	17.29 (3.86)	18.01 (4.85)	27.42 (9.82)	30.08 (8.47)	27.98 (17.93)	21.18 (8.94)	22.68 (8.94)	19.60 (4.10)	0.808	0.178	0.894						

Table 5.1b: Means of coarse root chemical properties over time of decomposition with the standard error in parentheses (n=4). Lignin monomers in milligram per gram root litter dry weight. Overall lignin given as the sum over the vanillyl, syringyl, and cinnamyl monomers (V.S.C.), the ratio of acid to aldehyde vanillyl monomers (Ad/Al_v), and the ratio of acid to aldehyde syringyl monomers (Ad/Al_s). ANOVA P-values <0.05 are given in bold.

Coarse root													
Timepoint	**0 Month**		**12 Month**		**24 Month**		**ANOVA**			**Correlation Fine to Coarse root**			
Treatment	**Control**	**Irrigated**	**Control**	**Irrigated**	**Control**	**Irrigated**	**Treatment**	**Timepoint**	**Interaction**	**P-value**	**adjR²**	**Correlation**
Mass remaining [g]	1	1	0.76 (0.01)	0.76 (0.01)	0.64 (0.02)	0.62 (0.02)	0.319	**2.2E-11**	0.371	**4.2E-16**	0.951	0.976
Root water content [g]	0	0	0.92 (0.07)	0.65 (0.07)	0.75 (0.08)	0.49 (0.04)	**0.001**	**5.9E-11**	0.036	**3.4E-12**	0.889	0.945
Carbon content [%]	50.53 (6.31)	46.89 (0.10)	48.41 (0.33)	47.51 (0.25)	47.99 (0.60)	45.16 (1.85)	**0.039**	0.301	0.590	0.162	0.045	0.295
$\delta^{13}C$ [‰]	-26.04 (0.16)	-26.58 (0.36)	-26.07 (0.20)	-26.63 (0.29)	-26.06 (0.46)	-26.51 (0.29)	**0.001**	0.928	0.940	**6.8E-08**	0.729	0.861
Nitrogen content [%]	0.59 (0.30)	0.42 (0.21)	0.73 (0.37)	0.63 (0.32)	0.65 (0.04)	0.57 (0.04)	**0.001**	**0.003**	0.285	**2.4E-05**	0.663	0.827
$\delta^{15}N$ [‰]	-9.21 (4.65)	-9.13 (4.57)	-9.98 (4.99)	-8.95 (4.54)	-9.82 (0.47)	-9.35 (0.83)	0.054	0.071	0.933	**1.6E-04**	0.576	0.775
Carbon/Nitrogen	86.48 (43.94)	111.67 (55.83)	67.13 (33.99)	75.11 (37.61)	74.59 (4.03)	79.29 (7.52)	0.210	**0.024**	0.878	**0.001**	0.471	0.709
Lignin Monomere [mg/g]												
V.S.C.	14.53 (1.15)	15.87 (2.59)	23.36 (1.26)	43.18 (5.96)	24.35 (1.37)	26.73 (2.62)	**0.010**	**2.4E-06**	0.053	0.140	0.055	0.311
Ad/Al_v	0.28 (0.05)	0.26 (0.05)	0.23 (0.02)	0.19 (0.02)	0.29 (0.03)	0.32 (0.12)	0.307	**0.017**	0.789	0.251	0.017	0.244
Ad/Al_s	1.51 (0.96)	1.45 (1.06)	3.17 (0.93)	3.67 (2.73)	0.61 (0.20)	0.84 (0.25)	0.948	**0.011**	0.800	**2.3E-04**	0.444	0.684
Lignin/Nitrogen	22.83 (11.49)	27.83 (13.92)	33.79 (17.23)	69.16 (40.33)	37.83 (4.65)	47.16 (10.98)	**0.021**	0.420	0.125	0.350	-0.004	0.234

Microbial response to substrate

Microbial diversity differed strongly between substrates (root sizes vs. soil), with bacteria and fungi showing similar response patterns (Table 5.2). Soil showed the highest, coarse root litter the lowest and fine root litter an intermediate diversity, quantified as OTU richness and Shannon diversity. The highest beta-diversity, comparing the dissimilarity between the different substrates, and therefore unique OTU, was measured on coarse root litter. Overall, microbial communities differed considerably between soil and coarse root litter samples. Mantel tests showed no significant correlation between these two substrates (Table 5.2). Community structure of the fine root litter

was significantly correlated with that of the coarse root litter, for both bacteria and fungi. Additionally, correlations were observed between fine root litter and soil for bacteria, but to a smaller extent than for coarse root litter. For fungi, the correlation between fine root litter and soil was slightly above the significance threshold (P-value: 0.064).

Microbial succession on fine root litter

A clear succession of the fungal and bacterial communities was detected at the OTU level (Figure 5.1, Procrustes plot). Time was the primary factor explaining the shifts in community structure for bacteria (Adonis F-value: 3.32, P-value: <0.001) and fungi (Adonis F-value: 2.80, P-value: <0.001). The irrigation treatment had a small, though still significant, impact on microbial community structure (bacteria: Adonis F-value: 2.86, P-value: 0.006; fungi: Adonis F-value: 2.91, P-value: 0.008). Both Procrustes analysis of the PCoA ordinations and the Mantel test of the underlying similarity matrices revealed a significant similarity between bacterial and fungal community structures (Figure 5.1).

The strongest community shift for fungi and bacteria was detected between months 6 and 12 (Figure 5.2). This strong shift determined the successional stages: 0-6 months represented the early stage and 12-24 months represented the late stage of succession. Fine root litter weight remaining in the litterbags (weight) clearly and significantly corresponded to early successional stage, whereas overall lignin content (LigTot) corresponded to the late stage. Soil water content explained around 10-12% of the variance of the correlation with the microbial RDA scores, whereas the C/N ratio explained around 6-8%. The distribution and the environmental fitting were highly similar between fungi and bacteria.

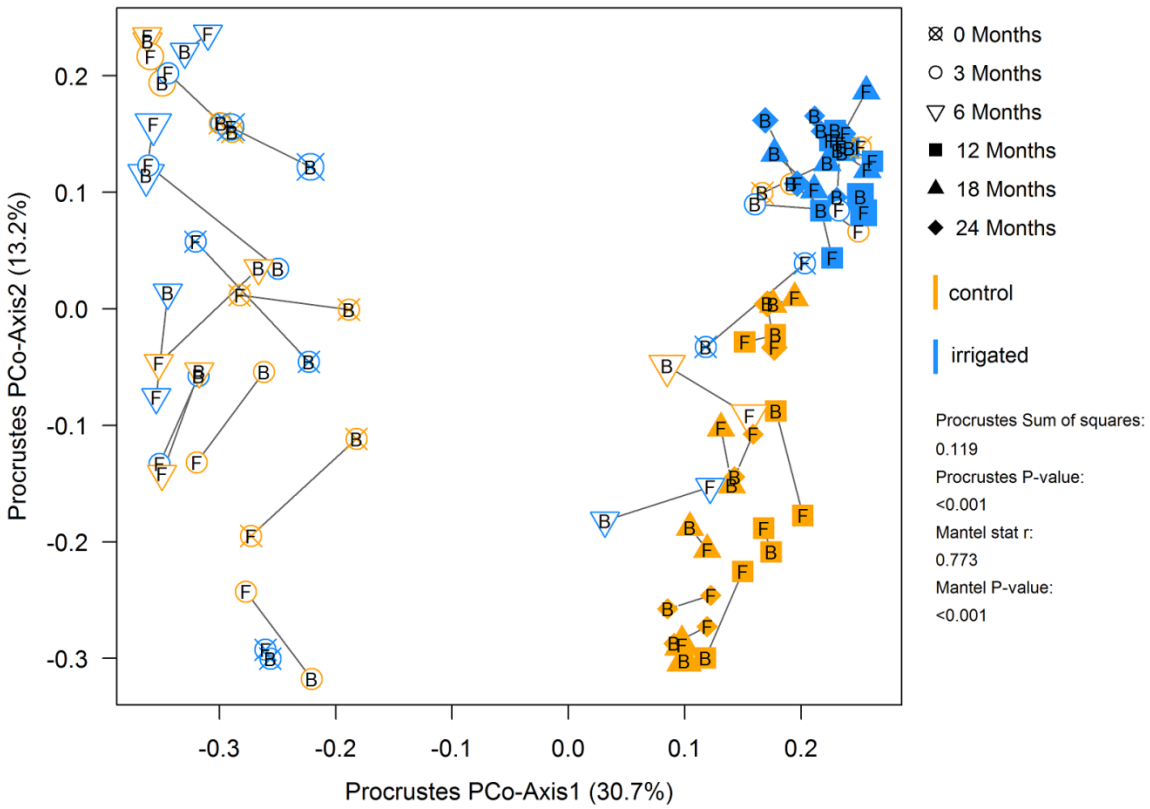

Figure 5.1: Differences in fungal (F) and bacterial (B) community structure surveyed on decomposing fine roots over a two-year period in irrigated (blue) and control (orange) plots. The graph shows a Procrustes overlay of the fungal and bacterial principle coordinate analysis ordinations. Different symbols indicate different stages of decomposition (time), whereas the color-coding refers to the communities in irrigated (blue) and control (orange) plots. Statistical significance of Procrustes, Mantel and environmental variable correlation analysis are provided in the legend.

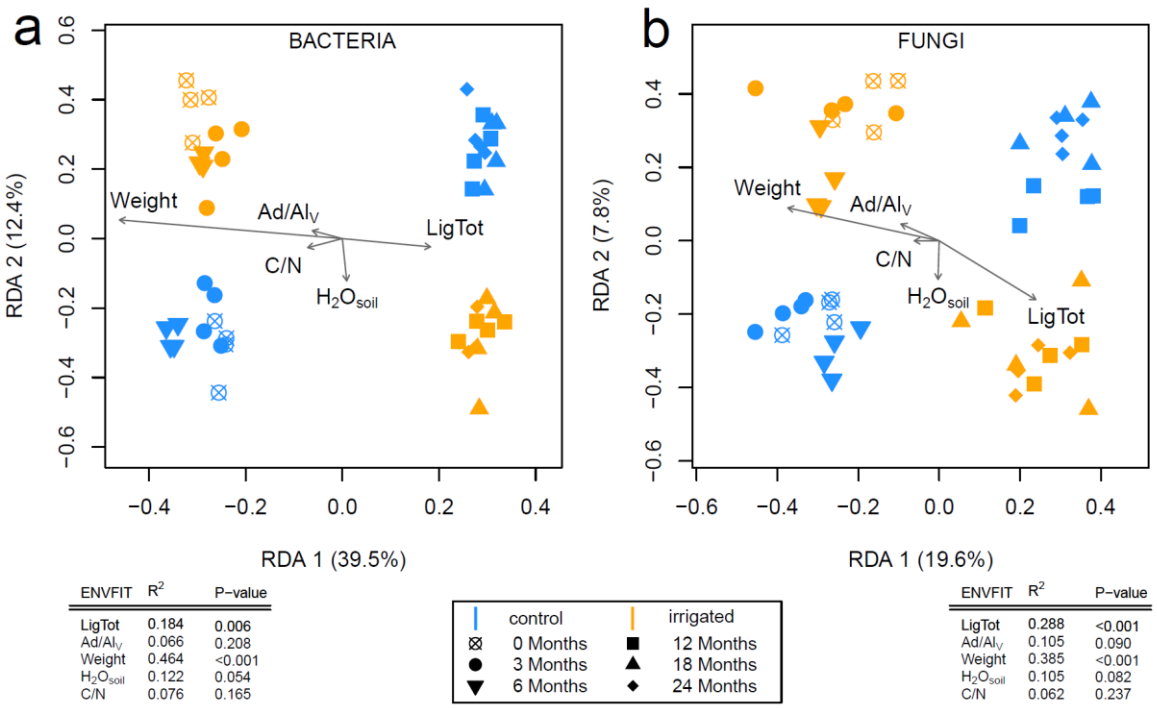

Figure 5.2: Redundancy analysis (RDA) of a) bacterial and b) fungal communities on decomposing fine roots. Different symbols indicate different stages of decomposition (time), whereas the color-coding refers to the communities in irrigated (blue) and control (orange) plots. Grey vectors show the correlation between the measured environmental variables (total lignin, Ad/Al_V, remaining weight, soil water content, C/N) and the ordinations scores, with the length of the vector corresponding to the square root of R^2. Statistical significance of the explained variance (R^2) and the level of significance (P-value) of the environmental variable fit are provided in a legend table.

Table 5.2: Metrics for alpha and beta diversity of bacterial and fungal communities, i.e. number of OTU and mean OTU per sample with the standard error in brackets, Shannon diversity index (Alpha-diversity) with standard error, and beta-diversity (mean distance to centroid) for control and irrigated plots. Similarities between habitats were calculated by a Mantel test ('mantel') implemented in vegan and are given by R and P-value, P-value <0.05 are given in bold.

		Control				Irrigated				Mantel (R)		Mantel (P-value)	
		Richness	OTU/ Sample	Alpha-diversity	Beta-diversity	Richness	OTU/ Sample	Alpha-diversity	Beta-diversity	Fine root	Coarse root	Fine root	Coarse root
Bacteria	Soil	14892	3326.67 (116.99)	4.27 (0.13)	0.39 (0.01)	15121	3654.53 (126.74)	4.27 (0.08)	0.41 (0.01)	0.135	-0.042	0.035	0.599
	Fine root	10857	2847.42 (154.87)	3.16 (0.08)	0.36 (0.01)	12010	3381.63 (196.53)	3.17 (0.09)	0.42 (0.01)	1	0.651	0	0.001
	Coarse root	7807	2300.50 (486.20)	2.39 (0.46)	0.46 (0.06)	8362	2398.67 (522.60)	2.33 (0.57)	0.52 (0.06)	1	1	0	0
Fungi	Soil	3147	563.46 (15.02)	4.25 (0.07)	0.36 (0.01)	3047	560.74 (12.07)	4.26 (0.06)	0.40 (0.01)	0.070	0.158	0.064	0.140
	Fine root	1952	464.17 (20.98)	3.15 (0.11)	0.36 (0.03)	2009	456.71 (18.84)	3.17 (0.08)	0.40 (0.02)	1	0.697	0	0.001
	Coarse root	1008	256.42 (45.13)	2.31 (0.23)	0.46 (0.07)	921	234.17 (46.25)	2.24 (0.23)	0.51 (0.07)	1	1	0	0

Response of bacterial groups

Proteobacteria was the predominant bacterial phylum, yet it was not affected by irrigation and did not change along the stages of decomposition (Figure 5.3a). In contrast, the relatively abundant phyla OD1 and Planctomycetes responded significantly to irrigation and stage of decomposition. However, their change in relative abundance over time was in opposite directions: while the OD1 phylum was mainly abundant during early succession (0-6 months), Planctomycetes became more abundant in the later stages of succession (12-24 months). Chlamidiae showed a significant response and were more abundant during late succession.

Alpha- and Betaproteobacteria, representatives of some selected groups of bacterial classes, changed significantly over time (Figure 5.3b). However, while Alphaproteobacteria were highly abundant during early succession, Betaproteobacteria showed an increase in abundance in late succession. The other proteobacterial classes did not show significant changes. The class Actinobacteria responded significantly to the factor time, exhibiting higher abundances in the early decomposition phase. Thermoleophilia, another class of the Actinobacteria, increased significantly in abundance during late succession. The same was true for Sphingobacteriales, a class of the Bacteroidetes. The two planctomycete classes Plactomycetia and Phycisphaerae reacted in a similar manner to the factor time, increasing significantly in relative abundance during late succession.

Response of fungal groups

Overall, there was a tendency of increasing Basidiomycetes and decreasing Ascomycetes abundances with progressing decomposition. The phylum of Zygomycota disappeared after month 12. Regarding the fungal orders, early succession was clearly dominated by members of the functional group yeasts/molds for both control and irrigated treatments (Figure 5.3c). The most abundant order was Agaricales, which was mainly present during late succession. Helotiales were highly abundant in both treatments, although with no clear trend over time. Chaetothyriaceae, categorized as an ericoid mycorrhiza, showed a significant shift with the irrigated treatment and over time, being more abundant in the later stages of decomposition. Eurotiales were also significantly affected by irrigation, but being more abundant during early decomposition stages. Pezizales, known litter decomposers, showed a tendency to increase in abundance during late succession. Pleosporales also showed a clear but not significant change along the stages of decomposition, being abundant at the beginning (months 0), and at late successional stage (12-24 months). The well-known white-rot fungi orders Hymenochaetales and Polyporales were present in irrigated as well as in control plots. Although they did not show a significant change over time, they tended to increase in abundance during late succession. In general, late succession stages were dominated by members of litter-associated fungi. Moreover, Eurotiales showed a negative correlation with total lignin in the fine roots (P: 0.032, cor: -0.31), while positive correlations with C/N were detected for Hysteriales (P: 0.001, cor: 0.47) and Chaetothyriales (P: 0.027, cor: 0.32).

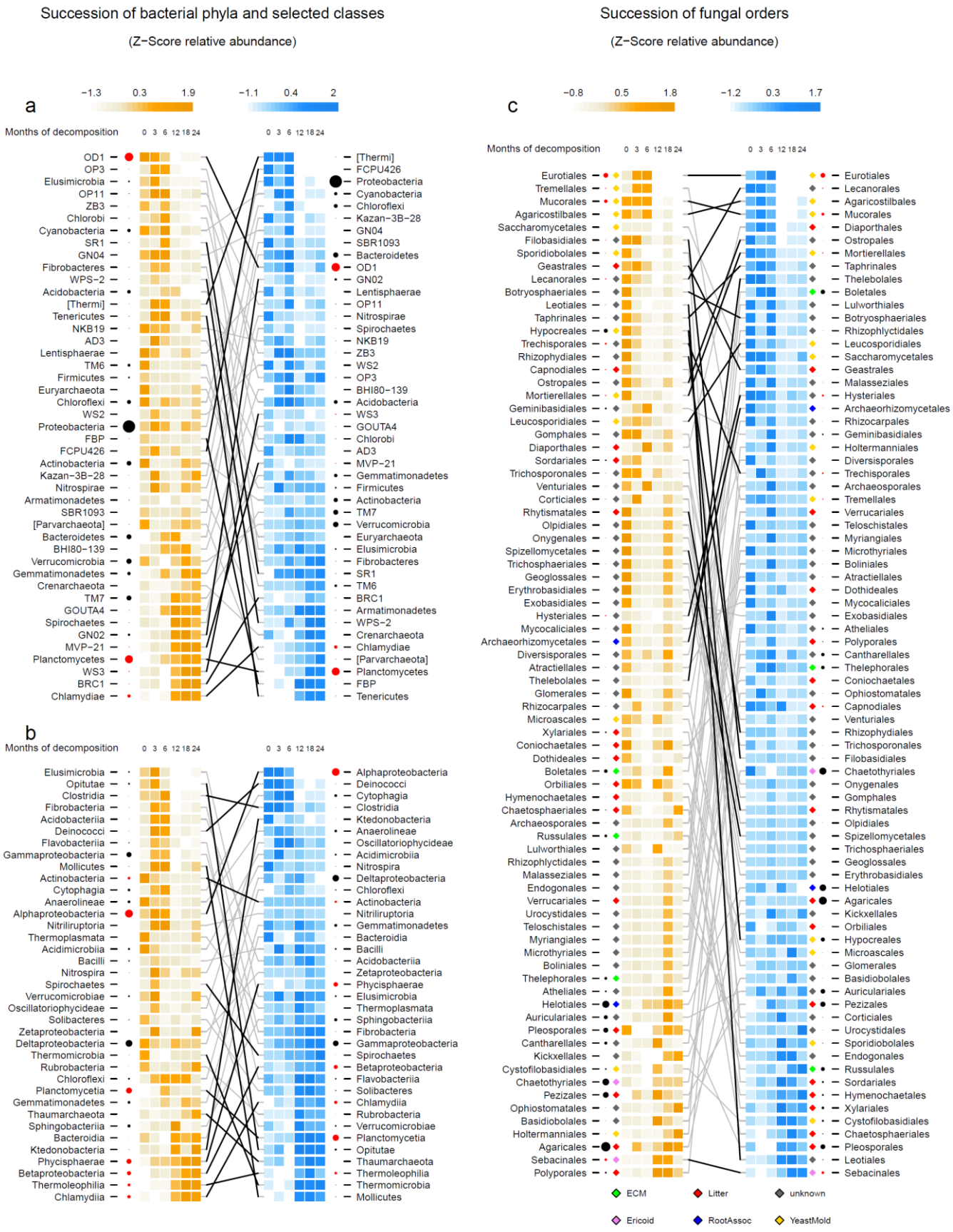

Figure 5.3: Heat map of Z-scores centered over time in control (orange) and irrigated plots (blue) for a) all bacterial phyla and b) selected bacterial classes, as well as for c) all fungal orders. The size of the circles corresponds to the relative abundance (square-root) of each taxon. Taxa that changed significantly over time are highlighted in red. Grey lines connect the same taxa, whereas black lines

connect taxa that changed significantly under irrigation. Colored diamonds represent functional groups of fungi at the order level according to Lindahl *et al.* (2007) and Kyaschenko *et al.* (2017), i.e. yeast and molds (yellow), litter-associated (red), ectomycorrhizae (green), ericoid mycorrhizae (purple), root associated (blue), and unknown (grey).

5.5 Discussion

Root decomposition

Root decomposition is one of the main drivers of the biogeochemical cycle and carbon storage in temperate forest soils (De Deyn *et al.*, 2008). Therefore, assessing and understanding this process is of major importance for accurate carbon budgeting. Under changing environmental conditions, obtained either by experimental manipulation or by using natural gradients caused by global climate change, the rate of root decomposition and therefore carbon sequestration can vary strongly (Reichstein *et al.*, 2013). We expected to find an increase in root decomposition with increasing water availability at the naturally dry *Pfynwald* forest ecosystem, as very dry conditions can lead to a reduction or even impairment in decomposition processes (Sanaullah *et al.*, 2012). In this study, however, no significant difference was observed between irrigated and control plots in the root mass remaining after two years of decomposition. A decrease in root mass of nearly 20% was detected after three months of decomposition in control as well as in irrigated plots. This finding is in agreement with previous decomposition studies and might be caused by leaching of water soluble root components (Berg and McClaugherty, 2008a).

Moreover, our results revealed stable microbial decomposition rates over two years, irrespective of irrigation treatment. Certainly, mortality of Scots pine (Rigling *et al.*, 2013) driven by summer drought must not coincide with limiting conditions for microbial decomposition. Nonetheless, water availability and soil moisture are factors known to be key drivers of decomposition (Prescott, 2010; Solly *et al.*, 2014). Hartmann *et al*. (2017) showed a significant increase in soil respiration due to irrigation in *Pfynwald. F*urthermore, Hagedorn (unpublished data) observed a significant increase in earthworm abundance in the irrigated plots. Although we focused on microbial decomposition in this study and excluded earthworms with the selected mesh size, it is important to note that these soil engineers also play a key role in decomposition (Cadisch, 1997). Water limitation is not as relevant to microorganisms as to larger soil fauna, although the mass loss associated with soil fauna can drastically reduce under dry conditions (Wachendorf *et al.*, 1997). Furthermore, we can assume that the limited water availability to plants is not an equally limiting factor to microorganisms. In drying soil, soil water retention increases and water becomes inaccessible to plant roots, whereas bacteria live *in* water films within soil pores and may be less limited by the increasing soil water surface tension. Moreover, numerous soil bacteria are able to form spores, cysts, or biofilms or can reduce their metabolism to endure unfavorable conditions (Fierer *et al.*, 2003; Barnard *et al.*, 2013; Evans and Wallenstein, 2014), and fungi generally show a high tolerance to desiccation (De Vries *et al.*, 2012; Barnard *et al.*, 2013). Thus, we conclude that the dry conditions in *Pfynwald* did not limit microbial decomposition.

Root litter elements shift during succession

The element analysis of our fine root litter (Table 5.1) showed results similar to those from fine roots measured in 2013 within the same irrigation experiment (Herzog *et al.*, 2014). Both N concentration and the C/N ratio were affected by irrigation. Roots in the irrigated plots had a lower N concentration but an increased C/N ratio compared to those in the dry plots. This reduced N

concentration in the irrigated roots might indicate plant N limitation, since Scots pine had an abundant water supply and growth therefore was not limited. During root decomposition, an increase in N concentration was observed, most likely an indirect effect of increasing colonization by bacteria and fungi, which contain N in their cell walls (Wardle, 1992). The $\delta^{13}C$ values of tree rings increased under dry conditions in the control plots, most likely due to lower stomatal conductance and thus less discrimination against $^{13}CO_2$ during photosynthesis (Timofeeva *et al.*, 2017). This difference between the control and the irrigation treated roots remained stable during the entire course of root litter decomposition. Irrigation led to increased root $\delta^{15}N$ compared to values in the control plots, suggesting that the N mineralization rate increased in irrigated plots (Templer *et al.*, 2007). An increase in N demand of the trees in the irrigated plots coinciding with an increase in N mineralization seems plausible, as the coupling between ectomycorrhizal fungi and trees tends to be highly economical, following the principle of supply and demand (Franklin *et al.*, 2014). Pines in *Pfynwald* limited by low N conditions may therefore lead ectomycorrhizal fungi to augment their investment in N mining.

Lignin

Litter quality, and in particular lignin concentration, is an important factor driving decomposition (Silver and Miya, 2001; Berg and McClaugherty, 2008a). The sum of the measured lignin monophenols vanillyl, syringyl and cinnamyl (V.S.C.) after cupric oxid extraction were within the range of 10–30 mg/g DW, which is in agreement with the values for *Pinus* species reported in the literature (Moingt *et al.*, 2016). Our results showed that irrigation did not significantly influence the lignin fingerprints. A clear temporal pattern over the decomposition period was detected, with a distinct twofold increase in lignin concentration after 12 months and a subsequent decrease after the second year (Table 5.1). A similar pattern, with an initial increase and a long-term decrease in lignin, was detected in a previous study in which concentrations of organic compounds were traced during five years of decomposition (Berg and McClaugherty, 2008a). Duboc et al. (Duboc *et al.*, 2014) confirmed these observations with their findings of slower lignin decomposition rates compared to that of bulk litter C but no difference over longer time scales (two years). This result implies that lignin is selectively preserved during the initial decomposition phase compared to other litter compounds. Interestingly, lignin characterization revealed an increase in the Ad/Al_V ratio, indicating alterations to the molecule even if lignin concentration was not drastically reduced (Table 5.1). The Ad/Al_V ratio corresponded directly to the oxidation state of the lignin (Thevenot *et al.*, 2010), revealing an increase in lignin degradation over time. With respect to irrigation, we observed significantly lower degrees of lignin oxidation during the two-year experiment in irrigated plots compared with in the dry control plots. These patterns were only observed in fine roots and may be due to delayed lignin degradation of coarse roots.

Microbial diversity in forest soils

In theory, dominance of soils by copiotrophic microorganisms leads to higher carbon turnover rates (Fierer *et al.*, 2007). Indeed, in *Pfynwald*, this effect was detected in soils of the irrigated plots, with a hypothesized higher turnover of carbon resulting from a relative increase in copiotrophic

microorganisms such as Proteobacteria and Zygomycota (Hartmann *et al.*, 2017). However, this difference in soil microbial community composition did not lead to a change in root litter degradation. Interestingly, the soil microbial community composition did not differ significantly from the fine root litter community, suggesting that microorganisms recruited from the surrounding soil dominated the microbial community residing in the fine roots (Goldmann *et al.*, 2016). Nonetheless, the selection pressure of the root substrate seemed to be strong, with selection favoring microbes specialized for the given substrate (Cleveland *et al.*, 2014). This was especially apparent for coarse root litter, where the microbial community composition differed significantly from the soil community but was highly correlated with the fine root community. This diversification due to the type of substrate has been observed in a boreal Scots pine forest as a shift from saprotrophic fungi on fresh litter in top soil to nitrogen-assimilating ectomycorrhizal fungi on humus in deeper soil layers (Lindahl *et al.*, 2007). A similar shift was observed in a chronosequence on Swedish islands, where the fungal community shifted alongside a vertical change of the substrate, from litter-associated saprotrophs to mycorrhizal fungi (Clemmensen *et al.*, 2013).

In the present study, the community shift over time, due to progressing litter decay, was similar to that observed in the vertical chronosequence in Sweden (Clemmensen *et al.*, 2013). Early successional groups, mainly dominated by Alphaproteobacteria, yeasts and molds (e.g. Eurotiales, Mucorales) are known to feature largely copiotrophic lifestyles by consuming easily degradable plant components. Late successional groups, mainly dominated by Planctomycetes, Betaproteobacteria, and litter-associated fungi (e.g. Agaricales, Pezizales), show more oligotrophic lifestyles. Hartmann *et al.* (2017) investigated dry and irrigated soils of *Pfynwald* and argued that many members of the phylum Proteobacteria occurring in the irrigated plots exhibited copiotrophic characteristics (Fierer *et al.*, 2007). In our study, however, the Proteobacteria were divided into Alphaproteobacteria, which exhibited a copiotrophic life-style, and Betaproteobacteria, which increased during later succession. Thus, Betaproteobacteria can be considered an exception among the copiotrophic Proteobacteria. Betaproteobacteria may benefit from enzymes released by certain fungal groups that increase in later successional stages (Valášková *et al.*, 2009). Bacteroidetes are known to be abundant litter-associated bacteria (Brabcová *et al.*, 2016a), and this group strongly increased during late succession in the fine root litter. This highlights that not all Bacteroidetes classes behave as copiotrophs, but rather, in case of the order Sphingobacteriales, as cellulose degraders (Eichorst and Kuske, 2012) or mycelium degraders (Chitinophagaceae) (Brabcová *et al.*, 2016a). The candidate phylum OD1, recently renamed to Parcubacteria, which was the second most abundant phylum on the fine root litter, was significantly more abundant in early than in late successional stages. Not much is known about the ecological importance of Parcubacteria, as they have escaped cultivation so far, but members of this group have been characterized by strongly reduced genomes and a preferred occurrence in largely anoxic environments (Peura *et al.*, 2012; Nelson and Stegen, 2015; Frey *et al.*, 2016b). It has been hypothesized that the streamlined genome carrying a limited metabolic capacity might be an indication of a symbiotic lifestyle, either mutualistic or parasitic (Nelson and Stegen, 2015). The importance of bacteria regarding litter decay in soils might have been underestimated. In our study, we detected a strong succession of bacteria over time that could not be explained by a seasonal

variation in the soil. The strong bacterial succession might be the result of versatile functional niche occupation during progressing root degradation.

During fungal succession, a group of ascomycetous fungi, Pleosporales, strongly increased in the late successional stage. This order, known as plant pathogens as well as non-pathogenic saprotrophs, is often found on decaying plant material (Kyaschenko *et al.*, 2017). Some mycorrhizal orders (Sebacinales, Russulales) were observed during the late successional stage in our study, supporting an earlier study where these orders were recorded on humus-like litter in Sweden (Clemmensen *et al.*, 2013). Sebacinales are a functionally diverse order (Vohník *et al.*, 2016) and are able to form ericoid mycorrhizae, but there is also evidence that they follow a facultative saprotrophic lifestyle (Zuccaro *et al.*, 2011; Weiß *et al.*, 2016). Lately, the existence of obligate mycorrhizal fungi has become a matter of debate (Baldrian, 2009; Lindahl and Tunlid, 2015; Kuyper, 2016) because many studies show the potential for mycorrhizal fungi to follow a saprotrophic lifestyle, even competing with litter-specialized saprotrophs (Bödeker *et al.*, 2016). However, the efficacy of mycorrhizal fungi to decompose lignocellulosic components of organic matter might be much lower than that of white- and brown-rot fungi (Uroz et al. 2016). Several authors have proposed that mycorrhizal fungi are able to oxidize organic matter not necessarily to access carbon but rather to scavenge nitrogen (Lindahl and Tunlid, 2015; Shah *et al.*, 2016). Similarly, the significant positive correlation of the C/N ratio with the relative abundance of the ascomycete order Chaetothyriales reveals the relationship of N reduction in the litter with a higher abundance of N miners. Members of this order mostly live on dead plant material and in soils. In general, the most abundant fungal orders in our study were Agaricales, Helotiales, Chaetothyriales and Pezizales. Agaricales and Pezizales are litter-associated fungi and mainly present during late succession, so this result clearly supports the hypothesis of substrate selection by microbial functional groups (Cleveland *et al.*, 2014). Helotiales, here grouped as root-associated fungi, contain various plant parasites; they are known to be able to degrade polysaccharides and therefore are often detected in abundance on decomposing plant material (Zmitrovich *et al.*, 2014).

Lignin degrading microorganisms

Major wood-decaying fungal orders (Auriculariales, Hymenochaetales, Corticiales, Russulales and Polyporales) (Floudas *et al.*, 2012) were present during late successional stages in control and irrigated plots in *Pfynwald*. Similarly, an increase of white-rot fungi with progressing decomposition state was recently detected in a survey on tree logs (Arnstadt *et al.*, 2016). However, these orders have not previously been observed *in situ* on buried roots. For bacteria, lignolytic capabilities have mainly been reported for Actinobacteria (Abdel-Hamid *et al.*, 2013). In this study, the actinobacterial class Thermoleophilia increased significantly during late succession, but other classes of Actinobacteria were more abundant during the early stage of decomposition. Betaproteobacteria including the genus Burkholderia, which strongly increased during the late stage of decomposition, are able to produce lignolytic enzymes (Bandounas *et al.*, 2011). Valášková (Valášková *et al.*, 2009) additionally postulated a co-occurrence of Betaproteobacteria with white-rot fungi profiting from the fungal exuded degradation enzymes. Pseudomonas, Burkholderia and Bacillus species were found on and around fungal structures, most likely profiting from the degradation product of the fungal enzymes or directly from fungal exudates (De Boer *et*

al., 2005) or, in the case of Pseudomonas, directly degrading fungal mycelia (Brabcová *et al.*, 2016a). There is a need for a better understanding of degradation and involved carbon fluxes from plant derived carbon sources through the microbial food web. Most studies dealing with lignin degradation focus on single strains under laboratory conditions, whereas proof of lignin degradation by bacteria under field conditions is currently lacking.

Functional redundancy

In conclusion, our results revealed a clear successional shift, from fast growing to specialized bacteria and fungi, on the decomposing roots independent of soil water conditions. Nevertheless, root-associated microbial communities differed between irrigated and ambient control plots. However, we did not observe a shift in decomposition rates, mediated by these distinct microbial communities, from the dry (control) to the moist environment (irrigated). In microbial studies such as ours, environmental constraints often need to reach very extreme levels, beyond our experimental setup with long-term drought and drought release by irrigation, to exert limiting conditions for microorganisms, exceeding viable conditions for many higher organisms (e.g. vascular plants). Moreover, in recent years, the hypothesis of functional redundancy among microbial communities – suggesting that different species occupying the same niche contribute to the same process at a similar rate – has gained increasing attention (Rousk *et al.*, 2009; Allison *et al.*, 2013). Such functionally redundant species might be dispersed across the taxonomic hierarchy, especially in the case of bacteria. Our data suggest such functional redundancy exists among the microbial communities in terms of root decomposition at the study site *Pfynwald*.

5.6 Acknowledgments

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5.7 Conflict of Interest

The authors declare no conflict of interest.

6 Who eats the tough stuff? DNA stable isotope probing (SIP) of bacteria and fungi degrading ^{13}C-labelled lignin and cellulose in forest soils

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6.1 Abstract

Decomposition and sequestration of plant-derived carbon has become a hot topic in environmental research. Since carbon dioxide has been detected as one of the most relevant drivers of global climate change, the potential storage and fate of carbon in soils has become key for predicting climate scenarios. Unfortunately, studies with a focus on decomposition and its driving factors are still lacking for precise modelling of the fluxes in a changing world. The main actors of degradation of polymeric plant cell wall components in soils have not been fully identified. Especially in regard to bacteria very little is known about their contribution to degradation of persistent plant components. In the present study, the active microbial community involved in degrading ^{13}C-labelled cellulose and lignin in forest soils was identified using a DNA stable isotopic probing (SIP) approach. Microbial activity assessed by measurements of $\delta^{13}C$ in CO_2 increased with labelled substrate addition, during a 28 days incubation period. Within the microbial communities, bacterial and fungal members specialized in degrading cellulose (e.g. *Devosia* (Rhizobiales), *Clitopilus* (Agaricales), Sebacinales), lignin (e.g. *Sphingopyxis* (Sphingomonadales), *Janthinobacterium* (Burkholderiales), Xylariales, Auriculariales, Helotiales), and also degrading both substrates (e.g. *Caulobacter* (Caulobacterales), Xanthomonadales, *Tetracladium* (Helotiales), *Dactylella* (Orbiliales)) have been identified. Fungal OTU dominate the lignin degradation compared to bacteria, revealing ascomycete as the major lignin degrading phyla in soil. Moreover, the ecological importance of ectomycorrhizal fungi, not as plant symbionts, rather as abundant and active cellulolytic and ligninolytic degraders might be underrated. These results provide novel insights into the degradation processes of woody plant debris in soil, disentangling the active microbial degraders from the passive soil microbiome, and potential microbial interactions between the active community in soil.

6.2 Introduction

Cellulose and lignin are two major compounds of plant tissues. Cellulose, the most abundant organic macromolecule on Earth, is the main component in all plant tissues, providing mechanical flexibility and tensile strength, whereas lignin provides rigidity and resistance to compression (Alberts *et al.*, 2002). The fate of these plant-derived components rich in carbon (C) has become of interest due to the important balance of soil respiration (CO_2) feedback to the atmosphere and C sequestration in soils (Pan *et al.*, 2011; Schmidt *et al.*, 2011). At a great extent, carbon stored in forest soils derived from root litter (Rasse *et al.*, 2005). Cellulose, the main components of plant debris, is postulated to degrade at a higher rate than lignin (Berg and McClaugherty, 2014b). While cellulose is a rather simple structure of cross-linked glucose and fructose and most bacteria and fungi are able to rapidly cleave the glycosidic linkages in cellulose, lignin degradation with its cross-linked phenolic structure requires specialized and costly enzymes such as peroxidases or laccases (Abdel-Hamid *et al.*, 2013). Hence, specialized lignin degraders evolved filling the niche on lignin-rich plant litter and are mainly prominent among the fungal basidiomycete order Agaricomycetes (Floudas *et al.*, 2012). Degradation of Lignin was mainly studied in single species incubation assays and knowledge about degradation of lignin under natural soil conditions is restricted.

Much of the carbon stored in forest soils is derived from lignin-rich roots (Rasse *et al.*, 2005); However, the fate of lignin in soils is still poorly understood when compared to cellulose (Baldrian and Valášková, 2008; López-Mondéjar *et al.*, 2016). From incubation experiments, specialized fungi have been identified to produce a range of enzymes that can degrade the persistent lignin structure. In recent years, it has become evident that not only fungi can degrade lignin, but that many bacteria can partially degrade lignin as well (De Boer *et al.*, 2005; De Gonzalo *et al.*, 2016). Several studies detected single bacterial strains capable of lignin degradation (Bandounas *et al.*, 2011; Brown and Chang, 2014; Tian *et al.*, 2014). Under natural conditions, the complex microbial diversity in soil challenges our understanding of who is actually producing the lignin-degrading enzymes and who is finally benefitting from the degradation products. Certain taxa are often found to co-occur during the degradation process, which could be an indication of potential mutualistic interactions of these taxa. However, non-mutualistic interactions have been frequently detected, where secondary organisms either benefit from the enzyme production of the primary degraders (Voříšková and Baldrian, 2013) or directly feed on the biomass of these primary degraders (Brabcová *et al.*, 2016b). The knowledge about interaction between bacteria and fungi in soil is still limited, especially for resource competition and mutualistic interactions during decomposition.

Litter quality as well as contemporary abiotic and biotic factors can strongly affect decomposition (Prescott, 2010; Solly *et al.*, 2014). For example, water availability can directly affect the decomposition process by influencing microbial activity and organic matter fragmentation, but at the same time can also alter microbial community composition, with indirect effects on decomposition (Yahdjian *et al.*, 2006; Hartmann *et al.*, 2017). Such compositional shifts might increase carbon turnover rate by favoring fast growing generalists (copiotrophs) over slow growing and metabolically versatile specialists (oligotrophs). It is not clear to what extent the decomposition of carbon-rich compounds is influenced by the microbial starting community. Readily degradable cellulose could be preferably degraded by a microbial community dominated by copiotrophs,

whereas the persistent lignin would only be degraded by a specialized community dominated by oligotrophic species. Hence, proof for this hypothesis is lacking.

Studies analyzing succession of complex microbial communities decomposing plant-derived C compounds are rare. In the past, incubations of selected species grown under different conditions to study enzyme activity were common. Nowadays, new development of sequencing tools enable studies of systems without limiting the microbial diversity in the incubated soil. Few pioneers in this field started to trace microbial succession on degrading plant (Voříšková and Baldrian, 2013; Haňáčková *et al.*, 2015) or fungal litter (Brabcová *et al.*, 2016b) in forest soils. Nonetheless, to distinguish between the active decomposer and the passive non-decomposer microbial community still remains challenging. To identify the active decomposer community, one prospecting method would be analyzing enzyme gene expressions which are involved in cellulose or lignin degradation (e.g. cellobiohydrolase, peroxidases). However, this approach is mainly limited by the covered time scale, since microbial gene expression can vary within minutes. Alternatively, stable isotope probing (SIP) of the DNA has proven to be a robust, integrating approach to identify the microbial members actively involved in the consumption of a given substrate (Dumont and Murrell, 2005). This promising technique was applied successfully in many environments such as agriculture (Lee *et al.*, 2011), glaciers forefields (Zumsteg *et al.*, 2013; Rime *et al.*, 2015), arctic tundra (Deslippe *et al.*, 2016), and aquatic systems (Gutierrez *et al.*, 2013). Hence, in forest soils, only few studies can be found. One successfully used SIP to identifying the cellulose and xylose, a sugar compound derived from hemicellulose, degrading bacterial community (Pepe-Ranney *et al.*, 2016). In Canadian forest soil, the effects of harvesting was investigated on the active bacterial and fungal community decomposing cellulose (Wilhelm *et al.*, 2017) or hemicellulose (Leung *et al.*, 2016). These very recent findings help to understand the carbon flow through the microbial food web, in the context of forest C cycling, demonstrating the great potential of DNA-SIP. The addition of isotopically labelled substrate to follow the nutrient fluxes might not only reveal active degraders but additionally reveal benefiters (e.g. parasites, predators) of the presence of the degrading microorganism, hence, to distinguish between the two remains challenging. To extend the knowledge of the active microbial degrader community and fill the research gap of the fate of persistent lignin a soil incubation study was performed using ^{13}C-labelled cellulose and lignin. We performed DNA-SIP of the forest soil microbial community identifying the bacterial and fungal taxa involved. This is the first study to follow the fate of lignin derived C during microbial degradation, using DNA-SIP. With this decomposition study we aimed to answer the following research questions:

(1) Do we see differences in CO_2 evolution over time between C-sources or treatments? Can we detect the labelled ^{13}C in the respired CO_2?

(2) Is the labile cellulose utilized faster (more efficient) than persistent lignin?

(3) Do shifts in water availability induce changes in the microbial community and does this influence the decomposition of cellulose and lignin?

(4) Are bacteria able to degrade and utilize C derived from lignin under natural soil conditions?

6.3 Materials and methods

Site description

In the mature Scots pine (*Pinus sylvestris*) forest *Pfynwald* in Valais, Switzerland (46°18'N, 7°37'E, 615 m.a.s.l.), eight plots (25x40 m each) were installed, of which four are irrigated during the summer months (April – October) since 2003 and four act as dry control (for details see Herzog *et al.* (2014)). The aim was to mitigate the drought stress originating from long-lasting and more frequent summer droughts by approximately doubling the yearly precipitation from about 500 mm to approximately 1000 mm. From this study site, soil samples from the topsoil (0-10 cm depth) were collected in November 2016. From each plot, 2x10 cm^3 soil samples were taken with a spade, and stored at 4°C until use for incubation.

Microcosm decomposition experiment

In a climate chamber at the Swiss Federal Research Institute WSL in Birmensdorf (Switzerland) a soil incubation microcosm experiment was installed using soil collected in the *Pfynwald*. This incubation study was designed as a stable isotope probing (SIP) study similar to Rime *et al.* (2016). 3 g of fresh sieved (2 mm) and mixed soil was weighed into screw lid cups (100 ml Polypropylene, Sarstedt AG, Sevelen, Switzerland). After a 24 h equilibration, the cups were closed with perforated screw lids. Four carbon (C) sources, extracted from maize leaves (*Zea mays*), were added: natural cellulose, ^{13}C-labelled cellulose, natural "Klason" lignin, ^{13}C-labelled "Klason" lignin, and No C source as control. For the natural maize material, corncobs were purchased in a grocery (Coop, Zurich, Switzerland), the leaves detached from the corncobs, and cellulose and lignin extracted from the leaves and milled according to Herzog *et al.* (2014). The labelled ^{13}C-cellulose and ^{13}C-lignin (>97 atom%) from maize leaves was purchased from IsoLife (IsoLife, Wageningen, The Netherlands) who used similar extraction techniques (personal communication). 25 mg of each powdered material was added individually (only 1 C source per cup) to the soil and mixed in, resulting in an approximate ^{13}C addition of 12 mg for cellulose and 18 mg for lignin. For maintaining similar soil treatment the No C control was mixed. The soil water content was quantified for the soils from the control (29%) and irrigated (33%) plots and standardized to 30% (of soil mass) and adjusted every second day, using distilled water. The cups were placed in a dark climate chamber and remained at constant 20°C, the mean summer (June to September) temperature at *Pfynwald*. In total, 160 cups were set-up with five C sources (^{13}C "Klason" lignin, ^{13}C cellulose, natural "Klason" lignin, natural cellulose, no C), with soils from eight plots (four non-irrigated and four irrigated plots), and with four replicates per C-source and plot.

CO_2 gas measurements and ^{13}C signature

To measure the gas flux, the cups were put in air-tight 1 L glass jars with a lid containing a septum enabling gas sampling with a syringe. The flux was measured three times during 24 h, at days 0, 1, 2, 4, 6, 8, 10, 16, and 28. For each sampling event, three 1 mL samples were taken each at 0, 12, and 24 h after lid closing. Each 1 mL sample was then released into an Exetainer vial (Labco Limited, Lampeter, United Kingdom) and supplemented with 12 mL of laboratory air leading to a

dilution of 1:12. The CO_2 concentrations and ^{13}C signature were measured with a gas chromatograph (Trace GC Ultra, Thermo Fisher Scientific, Waltham, MA, USA) coupled with an isotope-ratio mass spectrometer (IRMS; Delta V Advantage, Thermo Fisher Scientific, Waltham, MA, USA). CO_2 rates were calculated using a linear regression over the three sampling points during the 24 h period. Due to potential contamination of the Exetainers with too high CO_2 concentrations, two outliers were removed. $\delta^{13}C$ values were expressed as per mil (‰) in relation to the Vienna-Pee Dee Belemnite gauged reference materials with a measurement precision of ± 0.20 ‰.

DNA extraction, fractionation and Illumina paired-end sequencing

Consecutive sampling of soil for DNA-SIP analysis was performed at days 2, 4, 8, and 28. Per sampling day and cup, a soil sample was taken with an ethanol sterilized spatula, put into an Eppendorf tube, and then stored in a freezer at -80°C until DNA extraction. DNA was extracted from frozen soil using the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). For the bead beating procedure according to Frey (Frey *et al.*, 2008), 0.5 g soil was used. DNA concentrations were assessed by PicoGreen (Molecular Probes, Eugene, OR, USA). Enriched ^{13}C-DNA was isolated after ultracentrifugation in a CsCl gradient (Neufeld *et al.*, 2007) and the methodological protocol was adapted in our lab according to Zumsteg *et al*. (2013) and Rime *et al*. (2016). In brief, approximately 5 µg DNA were dissolved in 4.8 mL CsCl buffer. Optical density of the CsCl was adjusted to 1.4029 ± 0.0002 with a Refractometer (Refracto 30PX, Mettler-Toledo, Greifensee, Switzerland) corresponding to the final concentration of 1.723 g mL^{-1}. Samples were filled into 1/2x2 PA tubes (Beckman Coulter, Fullerton, CA, USA) and sealed following manufacturer instructions. For ultracentrifugation, a VTi-65.2 vertical rotor in an optima TM L-80 XP ultracentrifuge (Beckman Coulter, Brea, CA, USA) was used for 40 h at 177 000 g. Eighteen fractions, of approximately 250 µL gradient solution were collected drop-wise from the tube bottom. Optical density of the fractions were measured with the Refracto 30PX, for checking the formation of a gradient. A subset of the samples was used to correlate density with DNA concentration measured by PicoGreen. Fractions 10 + 11 with a density between 1.72 and 1.74 g mL^{-1} CsCl were defined as heavy fraction and fractions 15 + 16 with a density between 1.70 and 1.71 g mL^{-1} CsCl as light fraction, as described earlier (Wang *et al.*, 2015; Rime *et al.*, 2016). The DNA in these fractions were precipitated with 1.2 mL polyethylene glycol buffer (30% PEG6000 and 1.5 M NaCl, Sigma Aldrich, St. Louis, MO, USA) by centrifugation (16 000 g, 30 min), washed with 150 µL 70% ethanol, eluted in 30 µL AE buffer (Qiagen GmbH, Hilden, Germany). Fractions defined as heavy fraction, and fractions defined as light fraction were pooled, respectively. The DNA in the pooled fractions was quantified with PicoGreen. 10 ng of DNA of the pooled fractions were used for PCR amplification, targeting the ribosomal small-subunit RNA genes (region V4-V5) and the internal transcribed spacers 2 (ITS 2). PCR procedures were triplicated and the PCR products pooled, as previously described (Rime *et al.*, 2015; Frey *et al.*, 2016a; Frossard *et al.*, 2017; Hartmann *et al.*, 2017). Sequencing of the pooled DNA samples was performed at the Génome Québec Innovation Center at McGill University (Montréal, Canada). Barcoding was done with the Fluidigm Access Array technology (Fluidigm, South San Francisco, CA, USA) and paired-end sequencing with the Illumina MiSeq v3 platform (Illumina Inc., San Diego, CA, USA).

Sequence quality control, OTU clustering and taxonomic assignments

Quality filtering and clustering into operational taxonomic units (OTU) was performed in similar manner as in Frey (Frey *et al.*, 2016b). UPARSE (Edgar, 2010; Edgar and Flyvbjerg, 2015), a customized pipeline integrated in USEARCH v.8 (Edgar, 2010), has been used with slight modifications. For merging of the paired-end read sequence, fastq_mergepairs algorithm implemented in USEARCH (Edgar and Flyvbjerg, 2015) was used. For substitution errors, which may occur during Illumina sequencing procedure, the BayesHammer (Nikolenko *et al.*, 2013) correction tool had been applied. Cutadapt was run to remove the sequences PCR primers with one mismatch allowed (Martin, 2011). Bacterial sequences ($16S_{V3V4}$) shorter than 300 bp, fungal sequences (ITS2) shorter than 200 bp, and sequences without matching primers were discarded. USEARCH fastq_filter function enabled quality-filtering of the trimmed reads with a maximum expected error threshold of one. Singletons were cleared prior to clustering by de-replicating (Edgar, 2013). For sequence clustering in OTU a 97% similarity threshold was chosen. USEARCH cluster_otu function, including an 'on-the-fly' chimera removal algorithm, was used for the clustering (Edgar, 2013). To test the centroid OTU sequences for presence of ribosomal signatures, Metaxa2 (Bengtsson-Palme *et al.*, 2015) and ITSx (Bengtsson-Palme *et al.*, 2013) were used, and non-sufficient centroid sequences were discarded. Remaining quality-filtered reads were mapped against the final OUT list with USEARCH usearch_global algorithm (parameters: maxrejects 0, maxaccepts 0 and top_hit_only). Raw sequences have been deposited in the European Nucleotide Archive under the accession number PRJEB22281. OTU were compared to a reference database using MOTHUR with its implemented naïve Bayesian classifier (minimum bootstrap support 60%). Reference database for the prokaryotic ($16S_{V3V4}$) sequences GREENGENES was chosen for extracting taxonomic information by mirroring the OUT sequences to the database. For eukaryotic (ITS2) sequences, two databases NCBI GenBank (Benson *et al.*, 2015) and UNITE (Abarenkov *et al.*, 2010) were used and compared against each other. Before statistical analysis, cell organelle structures (chloroplast, mitochondria), eukaryotic soil animals (metazoa), and plant (viridiplantae) centroid sequences were removed from the final OTU list.

Statistics

All statistical tests were performed with R (Core Team, 2014). A P-value <0.05 was considered as significant in all statistical tests, if not stated otherwise. Differences between gas measurements and DNA concentrations were assessed by multifactorial ANOVA. Rank transformation was conducted beforehand, if normality (shapiro.test) or homogeneity of variance across groups did not apply (leveneTest). Tukey multiple comparisons test of means between the groups were tested using 'TukeyHSD'. For extraction of the responsive community and correction of naturally occurring discrepancies of DNA weight (GC content) following steps were performed (Figure A6.1). For all ratios calculated to all numerator and denominator 1^{-10} was added to keep unique OTU in the samples. The ratios of the relative abundance in heavy to the light fractions was calculated over all sequencing reads. OTU in the labelled samples with a lower relative abundance in heavy fraction compared to light fraction were discarded and the absolutes ratios for natural samples were calculated. The OTU expressing a $\log_2$-fold change in relative abundance between the positive ^{13}C-

labelled ratio and the absolute ratio of the natural control samples were extracted, resulting in the responsive community. Effects of factors on the relative abundance of microbial community were assessed by univariate permutational ANOVA based on Euclidian distances using the 'adonis' function in vegan with 10^5 permutations. A P-value <0.05 was considered significant. A heatmap was calculated from Z-score transformed relative abundance data using basic R (Core Team, 2014). The positive $\log_2$-fold generalist, cellulose specialist and lignin specialist taxa were mapped as a taxonomic network against the No C control using Cytoscape v.3.0.2 (Shannon *et al.*, 2003).

6.4 Results

CO_2 emissions, $\delta^{13}C$ values

The CO_2 emitted from soils treated with cellulose and lignin additions were not significantly higher compared to No C control (Table A6.1). Over the incubation time, CO_2 rate was strongly fluctuating with an increase until day 16, followed by a clear decrease until end of the study (day 28) (Figure 6.1a). Hence, the development of the CO_2 rate were very similar between the different treatments (C sources, irrigation treatment) (Table A6.1). The label increased CO_2 rate compared to the non-labelled material, thus this difference disappear if No C control is omitted (P=0.171). Comparing the non-irrigated control soils with the soils of the irrigation treatment revealed that the non-irrigated soils showed higher ^{13}C signatures, hence, missing the significance threshold (P=0.087, Table A6.1). $\delta^{13}C$ values measured after ^{13}C-labelled lignin addition were significantly higher compared to the ^{13}C-labelled cellulose addition (P<0.001, Table A6.1), the natural C sources did not affect ^{13}C signature. Incubation time did affect the ^{13}C signatures of labelled lignin samples with a peak around day 10 and the ^{13}C signatures of labelled cellulose samples strongly increased to the end of the study (day 28) (Figure 6.1b, Table A6.1). Interaction between the factors incubation time and C source was detected for the CO_2 rate (Table A6.1), mainly driven by the strong decrease on day 28. Interactions between the factors label, incubation time, and C source were detected for the $\delta^{13}C$ values (Table A6.1), mainly driven by the clear difference between natural and labelled C sources (factor label) and the fluctuations of the $\delta^{13}C$ values from the labelled C sources over time.

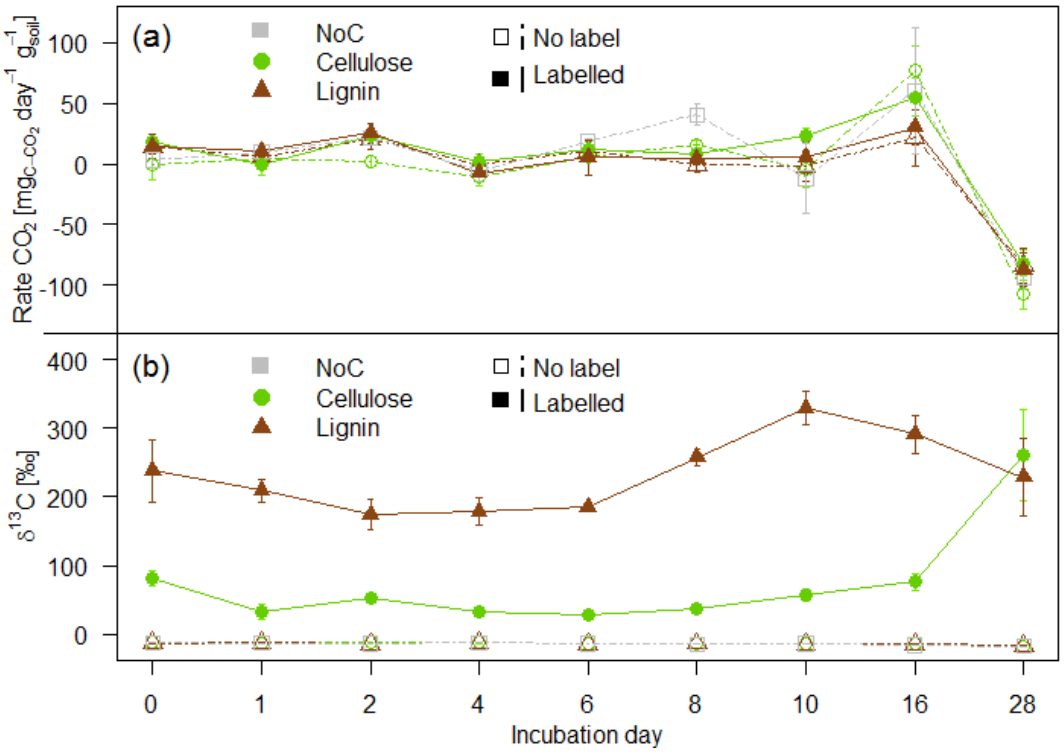

Figure 6.1: Gas rates of CO_2 (a) and (b) $\delta^{13}C$ values over the 28 days of incubation. In grey No C control, in green cellulose, in brown lignin. Open and dash-dot represent non labelled C, filled and solid represent labelled C sources. Error bars represent standard error (n=8).

Factors affecting DNA concentrations and microbial community structures

DNA concentrations did shift significantly over time (Table A6.1). While for No C control just a slight increase up to day 4 was measured, DNA concentrations in cellulose and lignin addition soils peaked on day 8. The natural cellulose showed a lower DNA concentration compared to the other treatments. DNA concentration was highest in soils with lignin addition. The irrigation treatment had no effect on DNA concentrations for all C source treatments.

The effects on the microbial community of the two C sources lignin and cellulose are compared against the No C control for bacteria and fungi (Figures 6.2a and 6.2b). Permutational multivariate analysis of variance revealed that all factors (C source, irrigation treatment, fractions, incubation time) did significantly affect bacterial and fungal community compositions (Table 6.1). The factors irrigation treatment and fraction explained the highest proportion of the variance of the bacterial and treatment of the fungal microbial communities. The factor incubation time and C source were of lower significance as driver of the microbial community composition. Interactions of the factor fraction with incubation time and irrigation treatment were detected for both communities. The MDS reveals the strongest shift by factor fraction for bacteria and treatment for fungi (Figures 6.2a and 6.2b) on the x-axis separation.

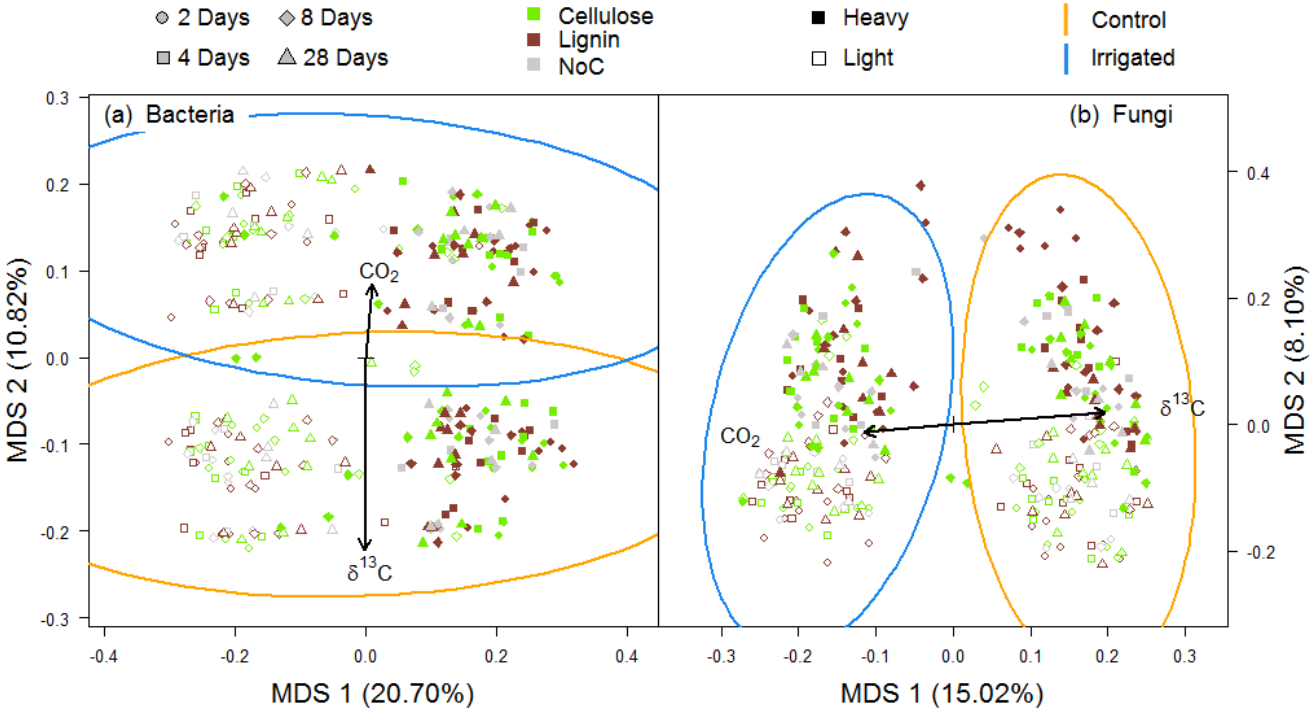

Figure 6.2: Principle Coordinate analysis (PCoA) of (a) bacterial and (b) fungal OTU. Grey vectors show the correlation between the measured gas values (CO_2 and $\delta^{13}C$) and the ordinations scores, with the length of the vector corresponding to the square root of R^2. Colored cycles indicate the confidence interval (< 0.95) of the C source distribution among the microbial community.

Table 6.1: Adonis result table for the statistical test of factors (C source, Fraction, Treatment, Time) and their interactions affecting the bacterial and fungal communities (Permutations 10^5). C sources added to soil are lignin, cellulose and No C control. Factor fraction represents heavy and light fractions of the samples. Factor treatment represents the irrigation treated and dry control soils. Factor time represents the incubation time. Significant P-values are given in bold.

	Bacterial community		Fungal community	
	F	P-value	F	P-value
C source	1.394	0.071	1.709	**0.008**
Fraction	65.819	**<0.001**	19.888	**<0.001**
Treatment	42.455	**<0.001**	55.496	**<0.001**
Time	2.906	**<0.001**	4.322	**<0.001**
C source:Fraction	1.214	0.165	0.883	0.679
C source:Treatment	0.791	0.814	0.653	0.975
Fraction:Treatment	4.545	**<0.001**	1.953	**0.012**
C source:Time	1.1	0.217	1.021	0.401
Fraction:Time	2.303	**<0.001**	1.341	**0.045**
Treatment:Time	1.043	0.343	1.215	0.120

Identification of specialized cellulose or lignin degrading bacterial and fungal taxa

The responsive OTU of the microbial community were detected by comparing heavy to light fractions and comparing the relative abundance increase compared to the No C control. 21.8% of the bacterial OTU responded positively to C addition, of which 4096 to cellulose, 3342 to lignin, and 819 generalistic (to cellulose and lignin). For fungi 23.3% of the fungal OTU responded to C addition, of which 457 to cellulose, 383 to lignin, 122 generalistic (to cellulose and lignin). A higher number of responsive OTU coupled with a high relative abundance were detected in the kingdom of fungi compared to bacteria (Figure 6.3a and 6.3b) this is supported by significance of C source effect on the fungal community compared to bacteria (Table 6.1). The specialization for the respective C source is not randomly distributed over the taxonomic tree (Figure 6.3a and 6.3b), rather clusters of responsive OTU are clustering within taxonomic groups. The individual responsive taxonomic groups will be discussed in detail in the discussion section.

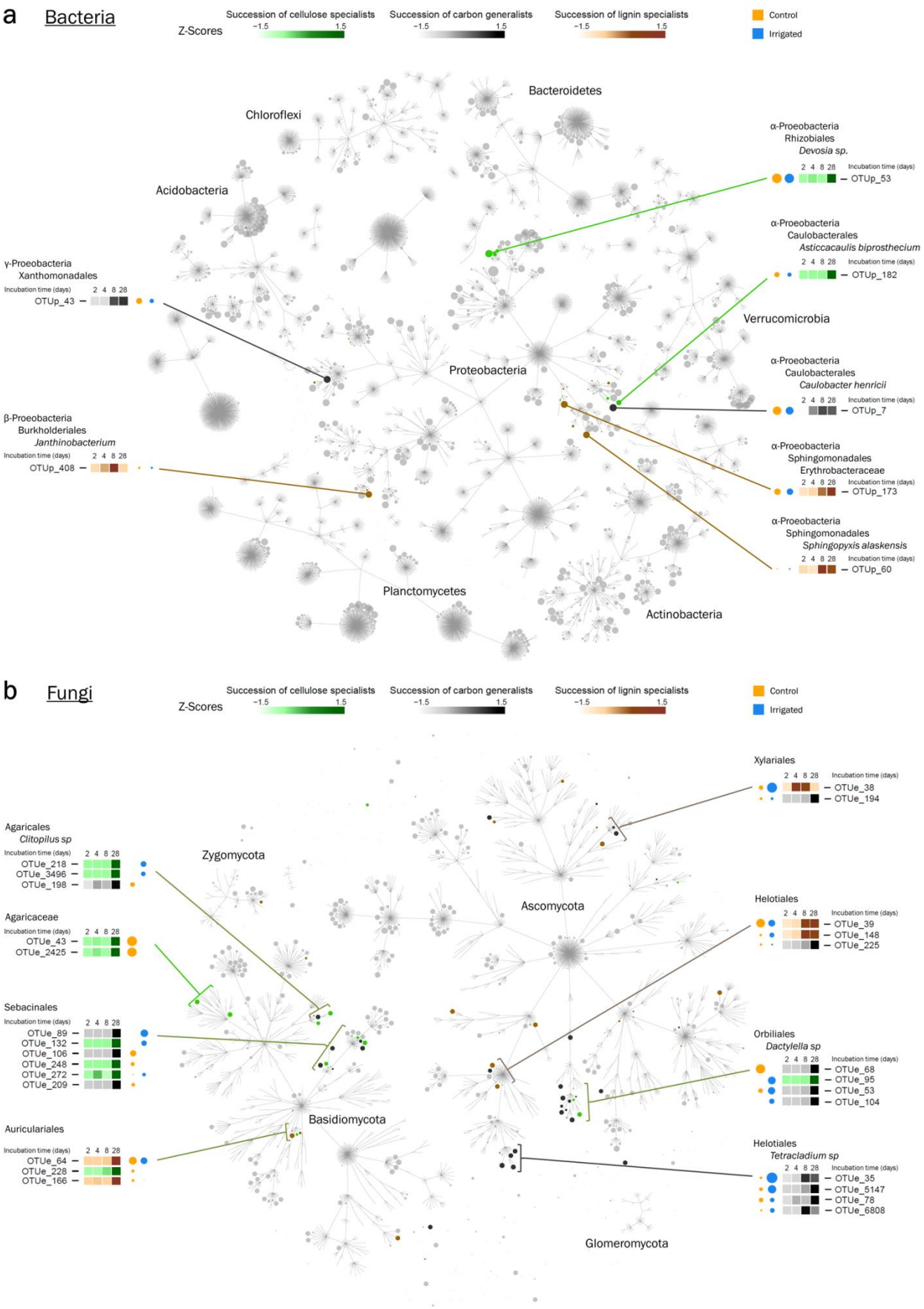

Figure 6.3: Taxonomic network of bacterial (a) and fungal (b) OTU grouped in the detected phyla. OTU which revealed a $\log_2$-fold increase in abundance compared to non-labelled control and a higher abundance compared to No C control are represented in color. Color code indicates cellulose (green),

lignin (brown), or generalistic (both C sources, black) association. Node size is relative to the square root relative abundance of the OTU. Consistently affected taxonomic levels are labelled with the lowest assigned taxonomic classification. Heatmaps show Z-scores of relative abundance for the responsive OTU at incubation time points (2, 4, 8, 28 days). The point size indicating square root of relative abundance in irrigated (blue) and control (orange) samples.

6.5 Discussion

The soil respiration shifts and ^{13}C signatures

The addition of ^{13}C-labelled lignin and cellulose to the forest soil led to a strong increase in $\delta^{13}C$ values in the respired CO_2, indicating respiration of the labelled C sources by microorganisms. The concentration of bulk soil C was higher in the irrigated soils (19% C) compared to the dry control (13% C) (Hartmann *et al.*, 2017), nonetheless, this did not affect the overall respiration rates (Figure 6.1). Therefore, the $\delta^{13}C$ signatures were lower in the irrigation treated soil, since the ratio of added ^{13}C to native soil ^{12}C was lower for irrigated soils (2.63% C) compared to the control (3.85% C). The decrease of the $\delta^{13}C$ signature due to irrigation treatment was not significant for the individual C sources cellulose (P=0.08) and lignin (P=0.20). The microbial activity, assessed by soil respiration, was not affected by the cellulose and lignin addition and decomposition of these C sources seemed to be executed at similar rates. DNA concentration, as a proxy for microbial biomass, revealed highest values with the more persistent lignin compared to cellulose addition and was not different between irrigated and control soils. These finding do not support the hypothesized effects of the fast-growing copiotrophic microbial community on microbial activity and turnover rate in the irrigated soils of Pfynwald (Hartmann *et al.*, 2017), at least not for the two C sources cellulose and lignin.

Utilization of the cellulose and lignin

The utilization of cellulose and lignin was estimated by the $\delta^{13}C$ signature in the respired CO_2 from the soil. The $\delta^{13}C$ signature was increased from the earliest sampling day (day 1) for lignin and cellulose (Figure 6.1) indicating present and fast responding cellulose and lignin degraders in the native soil. Labelled lignin showed a higher $\delta^{13}C$ signature compared to labelled cellulose samples, this shift can be explained by the increased microbial biomass and a higher C concentration (74% C) in lignin and compared to cellulose (46% C) (Figure 6.1). Cellulose degradation is a ubiquitous trait among bacteria and fungi (Berg and McClaugherty, 2014a), this corresponds with the early start of $\delta^{13}C$ signature detection in cellulose samples. The peak at incubation day 28 might be a combination of ongoing degradation of cellulose accompanied by carbon recycling within the food web. Lignin has been postulated do be chemically more recalcitrant to degradation compared to cellulose, due to the linkage heterogeneity of non-phenolic and phenolic bonds (Abdel-Hamid *et al.*, 2013). These results were supported by a long-term decomposition experiment of litter in a Scandinavian forest (Berg and McClaugherty, 2008a), where lignin degradation was occurring not until one year of soil exposition. Newer studies question these findings and provide a lignin degradation model which reveals limitations in bioavailable and soluble carbon compounds as the main limiting factor for lignin degradation in litter (Klotzbücher *et al.*, 2011). In our study, lignin is degraded at high rate and without delay, therefore, contradicting the classical view of recalcitrance nature of lignin.

Shifts in microbial community after C addition

The overall microbial community was most strongly affected by the irrigation treatment (P<0.001, Table 6.1) and the factor fraction (P<0.001, Table 6.1). The starting soil bacterial and fungal

communities were clearly different between irrigated and control soils and remained different over the time of incubation (Figure 6.1), the irrigation treatment effect was stronger for the fungal community. This finding corresponds with the detected differentiation of the soil microbial community due to long-term irrigation (Hartmann *et al.*, 2017). The fractionation process of the SIP procedure did clearly divide the microbial communities, this was more prominent for bacteria compared to fungi (Figure 6.1). The SIP does result in a heavy fraction which include the DNA with a high amount of incorporated ^{13}C and a light fraction containing ^{12}C-DNA. Additionally natural discrepancies in the weight of the DNA (GC content) can additionally drive the separation between the communities detected in the heavy and light fractions. We were able to correct for this natural DNA weight discrepancies, by comparing the heavy and light fractions between the labelled and natural samples (as described in the methods section).

Specialized microbial degraders of cellulose and lignin

The responsive community to the C addition was mainly affected by the C source ($P<0.001$, Adonis $F=13.3$) and less by the irrigation treatment ($P<0.001$, Adonis $F=4.1$, Table A6.2). 21.8% of the bacterial OTU and 23.3% of the fungal OTU responded with a $\log_2$-fold increase in relative abundance to C addition. The most abundant and responsive OTU are presented in a taxonomic tree with the corresponding heatmap for bacteria (Figure 6.3a) and fungi (Figure 6.3b). Only few abundant and responsive bacterial OTU were detected in comparison to fungal OTU. The responsive OTU seem to cluster on the taxonomic tree, implying that functional traits, such as lignin degradation are taxonomically linked, which is supported by the phylogenetical coupling of fungal lignin degradation (Floudas *et al.*, 2012). Irrigation treatment did affect the responsive community (Table A6.2), this is apparent mainly for fungi, some fungal OTU exclusively occur in the irrigation treated or control soils (Figure 6.3b). Responsive and abundant bacterial OTU were only detected in the Proteobacteria phylum, this is surprising, since literature would suggest the phylum Actinobacteria as most important polysaccharide and lignin degrading phylum among bacteria (Berg and McClaugherty, 2014a).

Specialized bacterial degraders

We detect many cellulose degradation specialists but the abundant OTU are found in the Rhizobiales and Caulobacterales order exclusively (Figure 6.3a). The most prominent function among Rhizobiales is nitrogen fixation in root nodules, here, we detected the genus *Devosia* as cellulose specialist, this genus has been previously identified as cellulose degrader in Canadian forest soils (Wilhelm *et al.*, 2017) and strongly increased in abundance with progressing wood degradation (Hoppe *et al.*, 2015). The oligotrophic species *Asticcacaulis biprosthecium*, the species detected to incorporate cellulose C in the order of Caulobacterales, has been shown to utilize glucose and hydrolyze starch (Brenner *et al.*, 2012a), a polysaccharide of glucose linked by glycosidic bonds very similar to cellulose.

Little is known about lignin degrading bacteria, here, we were able to identify three abundant OTU in the order of Burkholderiales and Sphingomonadales specialized on lignin as substrate. For many Sphingomonadales species, members of the genus *Sphingomonas* are known to produce ligninolytic enzymes (Tian *et al.*, 2014), therefore, it is likely that the closely related genus

Sphingopyxis (Brenner *et al.*, 2012a) identified in this study is likewise able to degrade lignin. The OTU identified as member of Erythrobacteraceae (Sphingomonadales) revealed lignin C incorporation coupled with a high relative abundance irrespective of irrigation treatment. This novel ligninolytic member of Erythrobacteraceae might proof, due to its abundance and indifference to irrigation treatment, as an important degrader of lignin in soil. The detected OTU of the genus *Janthinobacterium* (Burkholderiales) incorporated lignin C, but has never been shown to degrade lignin, hence, *Janthinobacterium* was described to produce chitinases (Brenner *et al.*, 2012a). In addition, a *Janthinobacterium* species was identified to be the pathogen involved in soft rot of *Agaricus bisporus* (Lincoln *et al.*, 1999) this rather might indicate C incorporation of lignin degrading fungal chitin, than direct lignin degradation.

An OTU identified as *Caulobacter henricii* has incorporated cellulose and lignin C, and was present at very high abundance in irrigated and control soils. High abundance is an indication for the importance of *Caulobacter* for cellulose and lignin degradation in soil and is supported by other studies, where close relatives in the family of Caulobacteraceae have been detected expressing ligninolytic (Danon *et al.*, 2008) and cellulolytic (Wilhelm *et al.*, 2017) potential. An additional proof is presented by the genetic potential for producing cellulose and lignin degrading enzymes in the genome of *Caulobacter crescentus* (Nierman *et al.*, 2001). The second generalistic OTU present in high abundance was identified as a member of the order Xanthomonales, revealing a higher abundance in the control soil compared to the irrigation treatment. Most members of the order Xanthomonales are plant parasites revealing cellulolytic and pectolytic potential (Brenner *et al.*, 2012b), and on decomposing wood a relative abundance of nearly 50% has been recoded for Xanthomonaceae (Hervé *et al.*, 2014), demonstrating the strong affinity for decomposing wood. Ligninolytic capacity was detected in isolation only (Zimmermann, 1990), this is the first study providing evidence for significant lignin degrading activity by Xanthomonales member in soil.

Specialized fungal degraders

Fungal cellulose degraders were mainly found among the phylum Basidiomycota, and the dominant order in terms of relative abundance was the mainly saprotrophic Agaricales (Figure 6.3b). In the order of Agaricales, the family Agaricaceae dominated the cellulose degradation in control soils, whereas the family Entolomataceae (e.g. *Clitopilus*) were more dominant in the irrigated soils. Several cellulose specialist OTU have been detected in the ectomycorrhizal dominated order Sebacinales of which some were exclusively found in irrigated soil or control, respectively.

The OTU specialized on lignin degradation were mainly found among the Ascomycota and few in the white-rot order Auriculariales (Basidiomycota). The irrigation treatment increased the abundance of the OTU in the order Xylariales, while Helotiales and Auriculariales revealed higher abundance in the control samples. This difference might reveal that the irrigation shifts the competitive balance among the lignin degrading fungi. Xylariales have been detected to not only degrade wood very efficiently, but also revealed morphological properties of the wood-rot similar to those produced by white-rot fungi (Worrall *et al.*, 1997). Helotiales were detected in fresh litter and at several decay stages in high abundance on oak tree stumps (van der Wal *et al.*, 2015), thus, their involvement in wood degradation could only be hypothized, since most species were known to

follow an endophytic or parasitic lifestyle (Tedersoo *et al.*, 2009). Our data reveals, uptake of lignin C by Helotiales OTU present in high abundance, suggesting saprotrophic activity after host death. Generalistic fungi, degrading cellulose and lignin, were detected in the two Ascomycete orders Orbiliales and Helotiales, as well as in the Basidiomycete order Sebacinales. Orbiliales show a tendency towards control soils and Helotiales reveal a higher relative abundance in irrigated samples. The genus *Tetracladium* (Helotiales) has often been recorded as litter decomposer in river systems (Bärlocher, 1992), therefore, generalistic saprotrophic activity and increased abundance under irrigated conditions is coherent. The saprotrophic genus *Dactylella* (Orbiliales) are known to be nematophagic in addition of plant debris degraders to compensate for low N content of the plant litter (Vaz *et al.*, 2017). Sebacinales are known to be almost exclusively ectomycorrhizal fungi, their generalistic saprotrophic activity revealed in this study, has been detected previously and has been hypothesized to be a side product during acquisition of nutrients (e.g. nitrogen, phosphorus) bound in plant debris (Baldrian, 2009; Rineau *et al.*, 2012).

Irrigation induced shift in microbial community but not function

While the effect of irrigation on the microbial community composition is apparent (Figure 6.2) it does not change the activity of cellulose and lignin degradation significantly (Table A6.1). Likewise, the responsive community was affected by the irrigation treatment in terms of composition, more pronounced for fungi than bacteria (Table A6.2). Hence, on a OTU level as indicated in figure 6.3b, often the OTU replace each other for a given substrate depending on the irrigation treatment, either within the same order (e.g. Sebacinales or Agaricales), or between different orders (e.g. Xylariales and Auriculariales). This effect might be driven by substrate competition, filtering for the optimal species under given moisture condition. In recent years, functional redundancy was postulated for some microbial community functions (Rousk *et al.*, 2009; Allison *et al.*, 2013), suggesting occupation of the same niche by different species performing at a similar process rate. We may conclude functional redundancy among the irrigated and control microbial communities for degradation of cellulose and lignin.

6.6 Acknowledgements

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7 Synthesis

7.1 Growth

7.1.1 Aboveground species shift

Drought relief by long-term irrigation had a significant impact on the Scots pine (*Pinus sylvestris*) forest ecotone in the Valais. With changing water availability, many new plant species started to emerge in the *Pfynwald* understory vegetation. While control plots were dominated by *Quercus pubescens*, relative abundance of *Q. pubescens* in irrigated plots decreased significantly at the expense of *Sorbus aria* and *Viburnum lantana*. Closure of the canopy by increased pine crown growth and increased competition of various fast growing broad-leaf species might be the reason why the predicted replacement of *P. sylvestris* by *Q. pubescens* is hindered. Scots pine suffering from increased mortality rates in the Swiss Alps (Rebetez and Dobbertin, 2004; Dobbertin *et al.*, 2005) showed significant growth increase of needles, shoot and stem with the applied irrigation treatment (Dobbertin *et al.*, 2010). Therefore, the fate of Scots pine in the dry Alpine valleys might not yet been decided because it is strongly dependent on the future precipitation regimes.

7.1.2 Belowground changes

Fine-root growth significantly increased by the long-term irrigation. Interestingly on a shorter time scale, no increase of root growth was detected (Brunner *et al.*, 2009), despite of increased growth of yearly increment and needles (Dobbertin *et al.*, 2010). These results show a clear trend towards preferential investment in aboveground biomass after drought stress release. A significant increase in root dry weight was detected after 9 years of irrigation (Figure 7.1), however, this increase remained stable in the following years (11 and 13). Long-lasting drought release and increased investment in root growth seem to be correlated with a relative shortage in nitrogen (N). Therefore, soil nutrient limitation might increase after removal of water limitation and consequential assimilation of carbon (C). After the very warm and dry summers in 2003 and 2015, an overall reduced fine root biomass in the following years 2005 and 2016 was observed in control as well as in irrigated plots.

The isotopic ratio $\delta^{13}C$ was measured in the fine roots of pine and showed a significant decrease in the irrigated roots. Increased $\delta^{13}C$ is an indication for an increase of water use efficiency (Farquhar *et al.*, 1982) or reduced stomatal conductance. From the reduced $\delta^{13}C$ values in the irrigated tree roots compared to the control roots we can increased stomatal conductance due to reduction of water limitation. This increase in stomatal conductance enables longer periods of carbon assimilation. Nonetheless, the C assimilated at higher rates with irrigation was not proprietarily invested in root growth (Figure 7.1).

Mean fine root age, measured by radiocarbon, significantly decreased due to long-term irrigation. On the one hand, this difference might have evolved by an increased turnover rate, on the other hand, it might reveal an increase in use of recently assimilated C for root production. Since trees store C for many years, drought-prone trees may have acclimated to a strategy of long lasting storage of C for survival of severe drought periods (Eilmann *et al.*, 2010). Nonetheless, we could show that with the drought release the strategy of the pines shifted towards higher fine root

plasticity, and with a reduced mean age, this indicates an increase of fine root litter input. The mean fine root age is an approximation for fine root longevity, revealing that with long-term irrigation root have a tendency to die earlier. This might sound counterintuitive, since common ecological theory would predict increase in live expectancy under favorable environmental conditions. Hence, especially for perennial plants, such as trees, growth plasticity seems key to exploit nutrients from their environment leading to higher nutrient acquisition (Hutchings and de Kroon, 1994; Bardgett et al., 2014). This growth plasticity requires partial replacements of former produced roots leading to higher turnover. All growth related measures of C fluxes, assimilation, root growth, and consequent root litter production, strongly increased due to long-lasting irrigation.

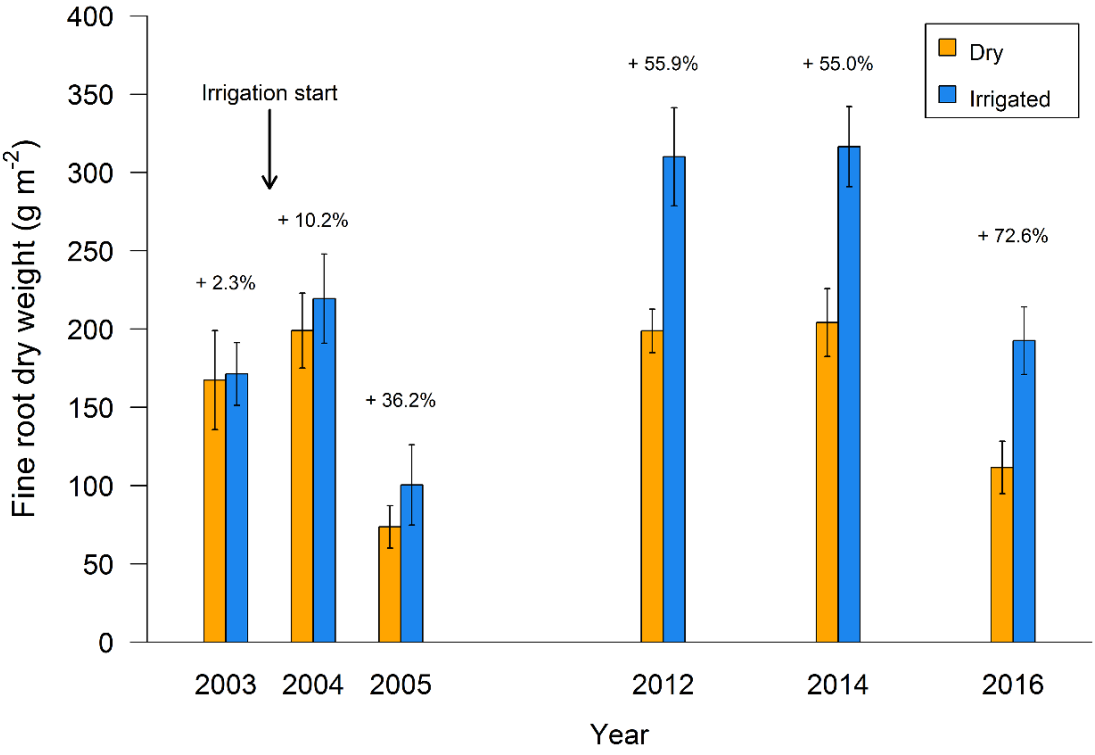


Figure 7.1: Fine root dry weight from sequential coring in Pfynwald, before start of irrigation (2003) and the irregular sampling years until 13 years after irrigation start. Error bars representing standard error (n=12).

7.2 Root decay

7.2.1 Mass loss and root litter composition

Decay starts right after root death, while decay is closely linked to the term decomposition, decay refers to the morphological state of the litter during decomposition. and in nature most likely with ectomycorrhizal fungi (Cullings and Courty, 2009). Hence, root decay remains a very slow process, and a complete degradation can last decades (Berg and McClaugherty, 2014b). In our two-year degradation study of root litter, only a partial degradation was observed, with a mass loss of 45% for fine roots and 37% for coarse roots. Even though decomposition seems to be affected by soil moisture (Prescott, 2010; Solly *et al.*, 2014), we did not measure an irrigation effect on litter mass loss. Initial decomposition is dominated by leaching, which leads to an early drop of the initial mass and which is accompanied by increased occurrence of rapidly growing opportunistic

microorganisms (Berg and McClaugherty, 2008b). We detected this strong mass loss of >20% after 3 months, irrespective of root size or treatment. Simultaneously, the most abundant fungi and bacteria present on the root litter are fast growing organisms specialized on competitive use of labile C sources, such as sugars and amino acids. While the concentration of labile C quickly drops, the relative amount of complex polymeric plant structures, such as cellulose, hemicellulose, and lignin increase over time, even though polymeric degradation starts early likewise (Berg and McClaugherty, 2008b), but at a slower rate. By following the concentration of lignin over two years' time we detected in our study a relative increase of the lignin monomers during the first year and a decreasing trend only in the second year. This loss of lignin concertation during the second year is accompanied by increasing relative abundance of lignin degrading microorganisms such as white rot fungi. Additionally, this specialization for lignin degradation was proven by incorporation of labelled lignin C by many abundant lignin degrading fungi (e.g. Auriculariales, Helotiales, Xylariales) using stable isotope probing.

7.2.2 Microbial succession on root litter

In our two-year decomposition study of bacteria and fungi detected on root litter, the factor 'time' was the most explanatory factor for the composition of the microbial community. This is a strong indication that the ongoing change of substrate, due to degradation, is driving the diversification of the fungal and bacterial communities. A very strong shift in the community was detected between autumn (end of the first year) and spring (beginning of the second year) on the buried root litter. In the first year fast growing bacteria and fungi dominated, more litter specialized bacteria and fungi were detected in the second year. Even though succession of bacteria and fungi during woody root decomposition in soil had never been studied before, many comparable results were detected with alternative approaches, such as microbial succession within the vertical distribution along the soil profile (Clemmensen *et al.*, 2013) or the succession on aboveground litter (Voříšková and Baldrian, 2013; Haňáčková *et al.*, 2015; Arnstadt *et al.*, 2016). Vertical succession of fungi in soils of boreal islands (Clemmensen *et al.*, 2013) revealed similar succession patterns as we observed over time. One striking finding is the degradation of plant-derived C by ectomycorrhizal fungi during mining for N. Ectomycorrhizal species increase in abundance at a later successional stage and display their saprotrophic activity as indicated by Baldrian (2009). Saprotrophic activity by ectomycorrhizal fungi seems to be a required activity to reach N-rich nutrients embedded in plant debris. These finding are confirmed, by the identification of the ectomycorrhizal fungal order Sebacinales among the main cellulose C and even lignin C utilizer in our incubation study. Hence, the actual specialists in degrading woody plant components are not ectomycorrhizal fungi. White, brown and soft rot fungi are the most efficient fungi degrading complex polymeric plant structures (e.g. cellulose, hemicellulose, lignin) (Berg and McClaugherty, 2014a), and are often detected to dominate coarse woody debris (Arnstadt *et al.*, 2016) as observed in our study as well. Hence, in our study the succession of bacteria was assessed in addition to the fungal succession. The shift from early (first year) to late (second year) of fungi and bacteria is strikingly similar between the two kingdoms and might mainly be driven by the degrading substrate (Clemmensen *et al.*, 2013). This dominance of the substrate shift becomes additionally apparent, since we were able to detect it regardless of the two very distinct microbial starting communities due to the decade-long irrigated soil (Hartmann *et*

al., 2017). The two initial soil microbial communities colonizing the root litter were still distinguishable during the succession, hence, was overshadowed by the shift in substrate and did not result in a change of the microbial decomposition rate. The functional spectrum of the two distinct soil microbial communities (Hartmann *et al.*, 2017) regarding degradation of cellulose and lignin was not altered. The overall community found in the soil of the incubation pots remained different between the dry and the irrigated soils over the time of incubation, however, this difference became minor when focusing on the community responding to the added C sources. This supports our finding of substrate dominated shift of the microbial community composition. These novel findings of unchanged microbial root decomposition rate and the similarity of the responsive community in dry and irrigated forest soils might indicate functional redundancy regarding future precipitation shifts.

7.2.3 Specialized microbial degraders

Stable isotope probing (SIP) coupled with next-generation sequencing technics enables not only to assess the microbial diversity, but also to further identify the microbial benefiters of a given substrate. The SIP technique is restricted to identifying organisms incorporating a high amount (approx.. 30%) of labelled C into their DNA. Therefore, we identify either organisms specialized organisms directly degrading and incorporating the labelled substrate, or organisms witch are very efficient in in benefiting from the presence of these degraders. These benefiters might be parasites, predators, or feeders on the pre-degraded substrate or enzymes of other organisms (De Boer *et al.*, 2005; Voříšková and Baldrian, 2013). The discrimination between primary degrader and benefiters is not straightforward and demands for knowledge of the species ecological function or at least potential function (genomic potential). The time line might support a claim of primary decomposer or benefiters, since incorporation of these macromolecules demands for previous enzymatic degradation, but would only be evident with continuous measurements.

Degradation and incorporation of many important plant derived polysaccharides have been successfully studied earlier, such as cellulose (Eichorst and Kuske, 2012; Šťursová *et al.*, 2012), hemicellulose (Leung *et al.*, 2016), or xylose (Pepe-Ranney *et al.*, 2016). In the present study, microbial degradation of lignin, one of the most abundant and persistent plant derived macromolecules, has been analyzed for the first time in roots. The comparison of our findings for cellulose degrading bacteria showed a clear involvement of Rhizobiales and Caulobacterales species. These Alphaproteobacterial orders were also detected in the early succession on buried root litter which supports the involvement in degradation of labile cellulose. The order Burkholderiales used predominantly lignin C in our study, thus was identified as cellulose, or hemicellulose degraders in comparable studies. This indicates functional versatility amongst the order Burkholderiales and reveals high specialization on macromolecule degradation. This novel finding of bacterial involvement in lignin degradation under natural soil conditions lacks comparable studies and is strongly supported by the detection of Burkholderiales in the late succession on buried root litter. The DNA-SIP study reveals a tendency for clustering of responsive microorganisms on the taxonomic tree, which would imply that functional traits, such as lignin degradation are taxonomically linked, which is supported by the phylogenetical coupling of lignin degradation (Floudas *et al.*, 2012). Therefore, we hypothesize that the Erythrobacteraceae, a sister

family to lignolytic Sphingomonadaceae, are a potential lignin degrading bacteria family as well. The genus *Caulobacter* (Caulobacteraceae) found in all known DNA-SIP studies as a dominant degrader of polysaccharide, was found here to utilize cellulose as well as lignin C. Members of the genus *Caulobacter* seem to be highly specialized in polysaccharide degradation and certainly hold the needed genetic foundations for lignin degradation (Nierman *et al.*, 2001). Among the fungal taxa we were able to identify well-known lignin degrading fungi, such as Auriculariales (Floudas et al., 2012) or Xylariales (Morgenstern et al., 2008) by using the DNA-SIP tool, this strongly underpins the capability of detecting active microbial degraders by DNA-SIP.

7.3 Conclusion and outlook

7.3.1 Conclusion

Two main questions remain to be discussed. One question concerns the approach of two different methods to assess the relevant microbial communities of litter decomposition, and the other question deals with the fate of C in the soil under a dry or a moist environment.

Are the two experimental approaches, the litterbag root decomposition experiment and the laboratory incubation SIP study using labelled C sources, comparable?

Lignin degradation by bacteria is still a novel field of study. In the present study, two orders Burkholderiales and Sphingomonadales, in the class Alphaproteobacteria, were detected as lignin C users, in the SIP study. Looking at the succession they tended to be present at early incubation stages. The early occurrence of these lignin degraders on the root litter and the fast degradation of lignin in the incubation study provide evidence for fast degradation of lignin in soil supporting novel findings of lignin decomposition models (Klotzbücher *et al.*, 2011). The main cellulose C incorporator genus *Devosia* (Rhizobiales) in the incubation study revealed a high relative abundance on the root litter but nearly exclusively during the first year. This is coherent with the rapid early degradation of cellulose of litter under natural condition in a Swedish Scots pine long-term decomposition study (Berg and McClaugherty, 2008b). The only generalist C user (lignin and cellulose C) was the genus *Caulobacter* (Caulobacterales), but Caulobacterales did not show a clear successional trend on root litter and not particularly abundant, neither. Degradation of C sources by bacteria is difficult to identify, especially with the immense background noise of bacterial diversity under natural soil conditions.

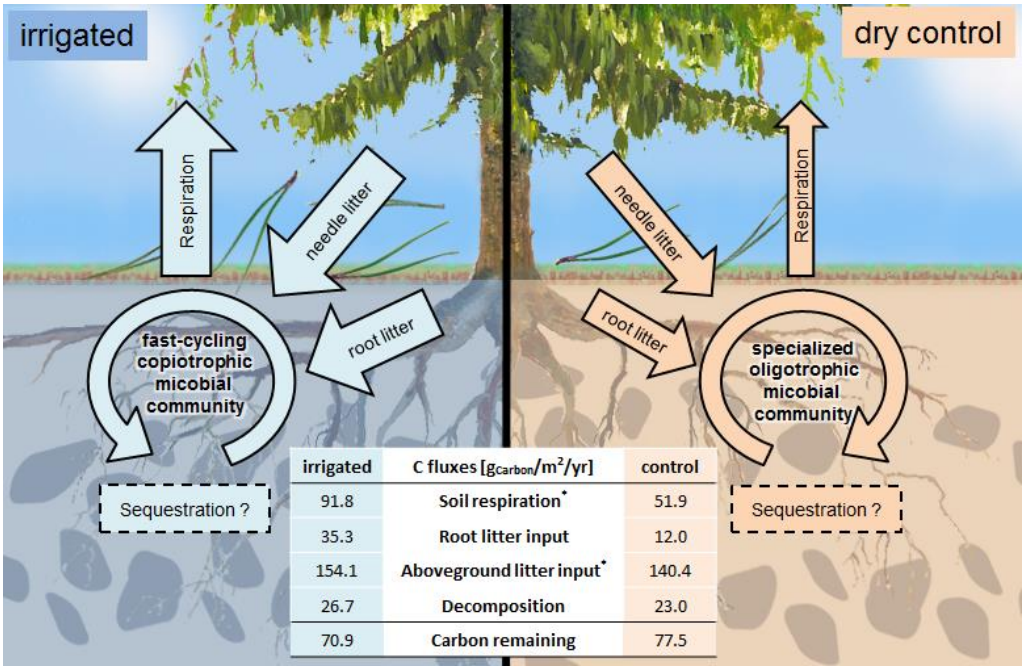
A clear picture is revealed for the fungal lignin degrading orders detected in the SIP study (e.g. Xylariales, Auriculariales, Helotiales). All of them were detected in the late successional stage (year 2) of root litter study. The strength of combining the two approaches lies in the direct identification of lignin C incorporation by the SIP study and the late stage relative abundance increase in the degrading root litter under natural conditions which was accompanied by a decreasing root lignin content. A similar picture can be seen for SIP identified cellulose degraders (e.g. Sordariales, Taphrinales), hence they were more abundant during first year of the litterbag study. The generalist order (Orbiliales) shows a more even relative abundance distribution over the two-years on the root

litter. The order Orbiliales seem to be polysaccharide degradation specialists, hence they do not express the same level of relative abundance as the lignin specialists on the root litter.

The similarities between the very different experimental approaches are striking, hence without the DNA-SIP proof, the involvement in root degradation revealed by the succession pattern could only be presumed. However, the comparison of the two methods was more successful for fungi, than for bacteria; either the diversity of bacteria is much too big and the responsive taxa are clouded by the background noise (sequencing and PCR errors), or the majority of sequences cannot be assigned on lower taxonomic level resulting in a majority of unassigned sequences. For example assignment on a genus level was restricted to 49.3% of the fungal sequences and 12.3% of the bacterial sequences in the litterbag study. To a certain extent, these very diverging assignment percentages reveal the discrepancy between the two kingdoms in terms of accessible information. While the background noise from the methodological errors can be partly filtered, better assignment can only be achieved by increasing efforts in expanding the DNA databases.

Do we detect increases of C fluxes and stocks due to irrigation treatment?

Most of the main fluxes of the soil C cycle are strongly affected by the irrigation treatment in the *Pfynwald* (Figure 7.2). We can conclude that litter input has increased with irrigation. However, its degradation in the soil by the microbial community is not influenced by the treatment. Even so, soil respiration did increase with irrigation, which can be partly explained by the increase of fine root standing biomass. Thus, we find increased litter inputs, of which the fate of C cannot fully be explained, leading to the hypothesis of an increase in C stocks due to irrigation. However, Hartmann *et al.* (2017) did not find a change of the overall C stock after a decade of irrigation. But they found that the distribution of C varied significantly within the soil profile. Whereas in the control plots the C concentration in the organic layer was around 37% and in the lower mineral horizon (5-10 cm) 4%, it changed in the irrigated plots to 20% and 6%, respectively. Thus, there is evidence, that C has been translocated within the soil profile to deeper mineral layers, or litter input at deeper soil layers were higher, and, thus, is better conserved and less susceptible to decomposition compared to C in organic layers.



irrigated	C fluxes [$g_{Carbon}/m^2/yr$]	control
91.8	Soil respiration*	51.9
35.3	Root litter input	12.0
154.1	Aboveground litter input*	140.4
26.7	Decomposition	23.0
70.9	Carbon remaining	77.5

Figure 7.4: Fluxes of the carbon cycle in the *Pfynwald* indicated by arrows. Calculated carbon fluxes from this thesis or Hartmann et al. (2017)(*) in *Pfynwald*. Tree painted by Hanspeter Läser.

7.3.2 Outlook

Environmental research strongly focused on understanding the effect of environmental change on the Earth biomes. These studies are key to increase the prediction power of models, which forecast the future climate and its impact on the environment. Since, some systems (e.g. soils) are very slow in responding to treatments, long-term studies are indispensable; moreover it is often difficult to find the right severity of treatment. In general, scientists might be too sensitive in respect to severity in experimentation, not driving the environment of interest to the edge of viability, therefore falling short of detecting the important thresholds of ecosystem functioning and resilience.

The field of environmental microbiology has gained a lot of interest in recent years, mainly due to the fast developing sequencing techniques. To this trend no end is in sight. On the contrary, with this fast development and especially the progression of dropping sequencing costs, more scientists will join this field of research. When the proposal was written for this thesis, barcode amplicon sequencing on the Roche 454 sequencing platform was the state-of-the-art technique to address microbial communities. Already today, we can see a shift from the subsequent Illumina platform towards whole metagenome sequencing, where PCR biases can be avoided. In terms of function of the microbial community, which still remains a challenge to be fully grasped under the vast diversity in soils, SIP is already in use and might become more powerful if combined with metagenome sequencing. An increasing effort has been established in the transcriptomics field, a method to analyze the expressed genes of all present organisms. On the one hand, it is possible to obtain the information on the diversity of the microbial communities, on the other hand, the expressed genes are describing the functions performed by the present community in soils. Drawbacks with the metatranscriptome might still be the costs, but the very fast shifting gene expressions by microorganisms might be the larger limitation, since the information gained is representative only to a single time point of less than an hour. This implies that the amount of

replication in time must be set high to be able to distinguish between intra- and inter-daily variability in gene expression.

In general, for PhD projects, lasting typically three to four years, this immense speed of methodological progress is hard to cope with, since what was novel at the start of the PhD project becomes yesterday tools of today. This will be a major challenge for PhD supervisors and demands for high flexibility and adaptation capacity to tackle this fast shifting progress.

But let's finish with some positive thoughts for the future – scientists produce enormous amount of sequencing data and all is published on open access databases, these data treasures should be mined for in respect to combining sequencing data sets to answer new research questions. Many studies focus on small taxonomic or enzyme groups of interest by using meta-genomic or -transcriptomic approaches. This will deliver the data of almost all present organisms and enzymes and most of them will remain unattributed. The field of bioinformatics will become an even hotter field of research, and researchers need tools for swift and precise mining of these piles of data. There are many interesting questions which could be approached with not spending a day and any financial resources in the lab.

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The FE, I'd say boys first since there are just too many girls now. Dominik – the Swiss from Germany (-: keep your keen, interested eye and open heart for the world around you, I will miss our football journey, our beer smuggling, and especially your visits always trying to think of something why you wanted to come around in the first place(-; Martin, the almost Dr., if it would just be about how much one knows you would be multiple Professor already, good finishing and say 'Hi' to *Baaasel* from me. Hey ladies – Yes inseparable, almost, but just almost, Sia – always up for a dance, a chat, a hug, and the mom of the PhDs -this is it – maybe we'll bump into each other again(-: Sonia, Radość filozofowania Boga i świata była radością. Człowiek, który nie lubi ludzi, nie istnieje, potrzebuje tylko prawa (-: Carissima Emily, sono molto contento che tu hai scelto la WSL e spero che tu guarderai indietro e sentirai il tempo alla WSL come arricchimento personale e scientifico. Ti auguro futuro prolifico (-;) e decorato con successo, siccome tu sei una buona e vera scienziata! Maggy und Jasmin nur kurz hatten wir das Vergnügen, doch ist es sehr schön was für lustige und lebensfrohe PhD nachrücken. Und an den ganzen Rest der FE, es war super bei Euch! Ich habe es unglaublich genossen mit Euch auf die Exkursionen zu

kommen und den Chlaushöck zu geniessen! Es wird sehr schwierig sein ein Arbeitsort zu finden an dem die Atmosphäre so gut ist wie bei Euch!

De René und d'Sarah the sweetening agents who made it much easier to drink this sometimes bitter coffee!

Family – Herzlichsten Dank geht an meine Eltern welche mir mein Studium überhaupt ermöglicht haben, immer unterstützend und nun endlich froh, dass es eine Ende gefunden hat(-; Meine Schwestern, die Liebe und die Böse, durch dick und dünn sind wir gegangen und ich denke nicht daran damit aufzuhören.

Nina –the love – thanks for all your support and patience, I certainly would have starved (in many ways) by now if it weren't for you, but now let's take our time and enjoy the world together.

The Marchel-clan unglaublich wie warm ich von Euch empfangen wurde! Nach dem ich die ersten 'Marchelblicken' überstanden habe, fühlte ich mich sehr zuhause bei Euch!

The Rüschis – no sympathy or understanding for this idea of making a PhD (-: and that's why they were so precious (– *precious*) to talk about everything else but work, surfing on the waves of the past, kayaking through the roughs of the present and flying (first - what else) back to the future.

Dr. Martin, here I was able to give you last position; last but not least, and almost family so perfect positioned!(-: what would I have done without you? Maybe still sitting in front of my computer wondering how big a single text file can become… I wish you all the best with Manon, you will be a marvelous supervisor! Amazing how much that man can bear – just on the football pitch we realize he's not unbreakable!(-:

10 Curriculum vitae

Claude Philippe Herzog

Date and place of birth: 28 December 1985 in Zurich (ZH)

Nationalities: Swiss

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Education

2014– 2017	PhD student, Swiss Federal Research Institute WSL Birmensdorf, Switzerland
2009 – 2011	Master of Science (MSc) in Biology, ETH Zurich, Switzerland
2005 – 2009	Bachelor of Science (BSc) in Biology, ETH Zurich, Switzerland
2000 – 2004	Matura, Kantonsschule Stadelhofen, Zurich, Switzerland
1998 – 2000	Sekundarschule, Schulhaus Hirschengraben, Zurich, Switzerland
1992 – 1998	Primarschule, Schulhaus Schanzengraben, Zurich, Switzerland

Publications

ISI-indexed articles

Hartmann M, Brunner I, Hagedorn F, Bardgett RD, Stierli B, Herzog C, Chen X, Zingg A, Graf-Pannatier E, Rigling A, Frey B (2017) A decade of irrigation transforms the soil microbiome of a semi-arid pine forest. Molecular Ecology 26:1190-1206.

Brunner I, Herzog C, Dawes MA, Arend M, Sperisen C (2015). How tree roots respond to drought. Frontiers in Plant Science 6:547.

Herzog C, Steffen J, Pannatier EG, Hajdas I, Brunner I (2014) Nine years of irrigation cause vegetation and fine root shifts in a water-limited pine forest. PloS one 9: e96321.

Herzog C, Peter M, Pritsch K, Günthardt-Goerg M, Egli S (2013). Drought and air warming affects abundance and exoenzyme profiles of *Cenococcum geophilum* associated with *Quercus robur*, *Q. petraea* and *Q. pubescens*. Plant Biology 15: 230-237.

Conference contributions

2017

Herzog C, Hartmann M, Frey B, Buchmann N, Brunner I. Fungal succession on decomposing pine roots. Oral presentation. Zurich mycological symposium 2017, Zurich, Switzerland.

2016

Herzog C, Frey B, Brunner I, Hagedorn F, Rigling A, Hartmann M. A decade of irrigation transforms the soil microbiome of a semi-arid pine forest. Poster presentation. ISME 2016, Montreal, Canada.

Herzog C, Frey B, Hartmann M, Buchmann N, Brunner I. Identifying microbial succession during root litter decomposition. Poster presentation. Proclim 2016, Bern, Switzerland.

2015

Herzog C, Frey B, Hartmann M, Buchmann N, Brunner I. Long-term irrigation affects root growth and decomposition in a drought stressed Alpine Scots pine forest. Oral presentation. ISRR 9, Canberra, Australia.

Herzog C, Frey B, Hartmann M, Buchmann N, Brunner I. Long-term irrigation affects root growth and decomposition in a drought stressed Alpine Scots pine forest. Oral presentation. Soil Organic Matter: SOM 2015, Göttingen, Germany.

2014

Herzog C, Steffen J, Graf Pannatier E, Hajdas I, Brunner I. Nine years of irrigation cause vegetation and fine root shifts in a water-limited pine forest. Poster presentation. Biogeomon 2014, Bayreuth, Germany.

Herzog C, Steffen J, Graf Pannatier E, Hajdas I, Brunner I. Nine years of irrigation cause vegetation and fine root shifts in a water-limited pine forest. Poster presentation. COST action FP0803 meeting, Bordeaux, France.

11 Appendix

Table A4.1: Translation and specified classification for the indicator values by Landolt (1977).

Indicator values	Aeration value (D)	Moisture value (F)	Humus value (H)	Continentality value (K)	Light value (L)	Nutrient value (N)	Reaction value (R)	Temperature (T)	Moisture variability value (W)
1	coarse rock	arid	immature soil	oceanic climate	pronounced shade	pronounced nutrient-poor	acid soil (pH 3-4,5)	high-altitude, arctic	constant moisture
2	ruble, gravel, boulder	dry	mineral soil	suboceanic climate	shade favoring	nutrient-poor	moderately acid soil (pH 3,5-5,5)	mountain, boreal	infrequent moisture changes
3	light ground	average	average humus layer	everywhere present	half shade	average	marginal acid soil (pH 4,5-7,5)	montane	average
4	heavy soil	wet	humus	continental climate	light	eutrophic	basic soil (pH 5,5-8)	colline	periodically wet
5	clay, peat	saturated	duff, raw humus, peat	distinct continental climate	pronounced light	over fertilized	pronounced basic soil (pH >6,5)	pronounced warm	frequent wet-dry alternating

Table A4.2: Species list with the mean abundance (%) in the control and irrigated plots. One-way ANOVA: p < 0.05 =significant, denoted; p ≤ 0.05-0.1 = non-significant, denoted; p > 0.1 = non-significant (ns)

Genus	Species	Control	Irrigated	*p*	Genus	Species	Control	Irrigated	*p*
Pinus	*sylvestris*	47.50	58.00	0.040	*Rubus*	*caesius*	0.005	0.005	ns
Carex	*alba*	17.50	10.00	ns	*Viola*	*pyrenaica*	0.010	0.000	<0.001
Quercus	*pubescens*	13.75	7.50	0.017	*Centaurea*	*scabiosa*	0.003	0.005	ns
Carex	*humilis*	5.75	12.50	ns	*Cephalanthera*	*longifolia*	0.005	0.003	ns
Sorbus	*aria*	5.00	8.75	0.046	*Cirsium*	*arvense*	0.000	0.008	0.024
Viburnum	*lantana*	3.00	5.75	ns	*Coronilla*	*vaginalis*	0.005	0.003	ns
Populus	*tremula*	0.00	4.75	0.083	*Bromus*	*inermis*	0.003	0.003	ns
Betula	*pendula*	2.00	1.25	ns	*Clematis*	*vitalba*	0.000	0.005	ns
Arctostaphylos	*uva-ursi*	2.50	0.25	0.080	undefined	undefined	0.003	0.003	ns
Berberis	*vulgaris*	2.00	0.75	0.002	*Euphorbia*	*cyparissias*	0.003	0.003	ns
Polygala	*chamaebuxus*	1.26	1.00	ns	*Fraxinus*	*excelsior*	0.003	0.003	ns
Juniperus	*communis*	2.00	0.26	ns	*Hieracium*	*pilosella*	0.003	0.003	ns
Hieracium	*murorum*	0.76	1.00	ns	*Solidago*	*virgaurea*	0.003	0.003	ns
Melica	*nutans*	0.01	1.26	ns	*Trifolium*	*montanum*	0.005	0.000	ns
Lonicera	*xylosteum*	0.01	1.00	ns	*Tussilago*	*farfara*	0.000	0.005	ns
Rosa	*pendulina*	0.50	0.51	ns	*Acer*	*campestre*	0.000	0.003	ns
Teucrium	*chamaedrys*	0.76	0.01	ns	*Acer*	*pseudoplatanus*	0.000	0.003	ns
Ligustrum	*vulgare*	0.75	0.01	0.024	*Campanula*	*rotundifolia*	0.003	0.000	ns
Cotoneaster	*tomentosa*	0.01	0.50	ns	*Carex*	*ornithopoda*	0.003	0.000	ns
Viscum	*album*	0.26	0.01	ns	*Cirsium*	*acaule*	0.003	0.000	ns
Fragaria	*vesca*	0.01	0.26	ns	*Eupatorium*	*cannabinum*	0.000	0.003	ns
Cornus	*sanguinea*	0.01	0.26	ns	undefined	undefined	0.000	0.003	ns
Platanthera	*bifolia*	0.003	0.258	ns	*Hedera*	*helix*	0.000	0.003	ns
Prunus	*avium*	0.008	0.015	0.097	*Hieracium*	sp.	0.003	0.000	ns
Melanopyrum	sp.	0.010	0.010	ns	*Hieracium*	*umbellatum*	0.000	0.003	ns
Peucedanum	*oreoselinum*	0.010	0.010	ns	*Leucanthemum*	*vulgare*	0.003	0.000	ns
Polygonatum	*odoratum*	0.010	0.008	ns	*Listera*	*ovata*	0.000	0.003	ns
Pulsatilla	*alpina*	0.010	0.008	ns	*Oenothera*	sp.	0.000	0.003	ns
Epipactis	*atrorubens*	0.008	0.008	ns	*Picea*	*abies*	0.003	0.000	ns
Euphorbia	*seguieriana*	0.010	0.005	ns	*Pimpinella*	sp.	0.003	0.000	ns
Galium	*lucidum*	0.010	0.005	ns	*Polygonatum*	*alpina*	0.000	0.003	ns
Lathyrus	*pratensis*	0.005	0.010	ns	*Polypodium*	*vulgare*	0.000	0.003	ns
Lotus	*corniculatus*	0.008	0.008	ns	*Potentilla*	*neumanniana*	0.003	0.000	ns
Potentilla	*erecta*	0.008	0.008	ns	*Prunus*	*mahaleb*	0.003	0.000	ns
Saponaria	*ocymoides*	0.010	0.005	ns	*Pyrola*	*chlorantha*	0.003	0.000	ns
Taraxacum	*officinale*	0.005	0.010	ns	*Pyrola*	*secunda*	0.000	0.003	ns
Coronilla	*minima*	0.008	0.005	ns	*Thalictrum*	*aquilegiifolium*	0.003	0.000	ns
Crataegus	*monogyna*	0.003	0.008	ns	*Viola*	*collina*	0.000	0.003	ns

Table A5.1: List of monophenols with abbreviations and amounts needed for quantification on GC-FID by external standard mix and internal standard phenyl acetic acid (PAA).

Standard Mix	**Abbreviation**	**Amount**
p-hydroxybenzaldehyde	PAL	3 mg
p-hydroxyacetophenon	PON	3 mg
vanillin	VAL	150 mg
ethylvanillin	EVAL	3 mg
p-hydroxy benzoic acid	PAD	20 mg
acetovanillon	VON	20 mg
syringaldehyde	SAL	3 mg
vanillic acid	VAD	20 mg
acetosyringone	SON	3 mg
syringic acid	SAD	3 mg
p-coumaric acid	CAD	3 mg
ferulic acid	FAD	20 mg

PAA Standard	**Abbreviation**	**Amount**
phenyl acetic acid	PAA	3 mg

Figure A5.1: Soil volumetric water content and temperature development over the study period at 5 cm soil depth. The hourly mean is plotted with the standard error (shaded areas) for the irrigated plots (blue lines) and the control plots (orange lines). Light blue boxes mark irrigation periods.

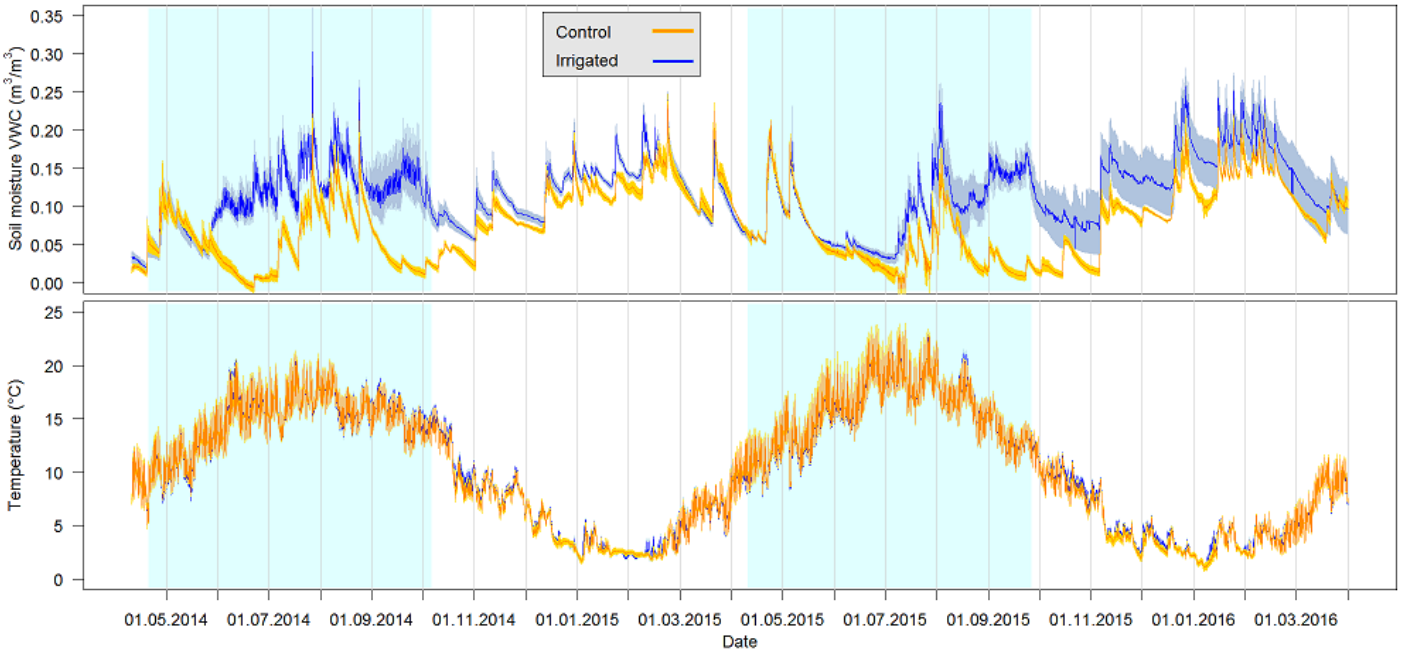

Figure A6.1: Flow chart of the calculations performed to extract the responsive community to the applied C sources in the stable isotope probing (SIP) study. Explanations for abbreviations: Relative abundance (RelAbu), Absolute value (ABS), Operational taxonomic units (OTU).

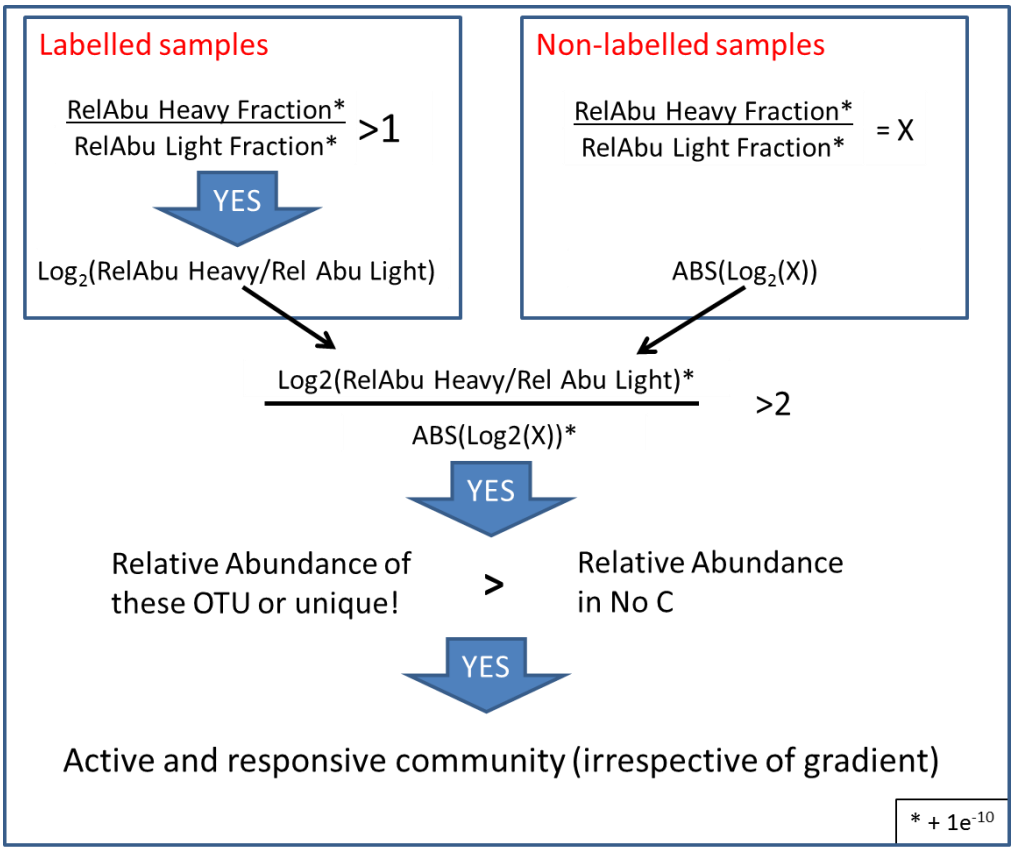

Table A6.1: ANOVA table with F- and P-value for all factors effect on DNA concentration, rate of CO_2, and rate of Rate $\delta^{13}CO_2$ and their interactions.

	DNA concentration		**Rate CO_2**		**Rate $\delta^{13}CO_2$**	
	F-value	P-value	F-value	P-value	F-value	P-value
Timepoint	5.71	**0.001**	18.42	**<0.001**	9.85	**<0.001**
Treatment	0.24	0.624	0.86	0.355	2.95	0.087
C source	3.33	**0.039**	0.57	0.564	159.09	**<0.001**
Label	5.96	**0.016**	7.26	**0.007**	1070.50	**<0.001**
Timepoint:Treatment	1.53	0.211	0.32	0.959	0.60	0.779
Timepoint:C source	2.43	**0.030**	1.74	**0.039**	2.42	**0.002**
Treatment:C source	0.61	0.544	0.03	0.968	0.40	0.674
Timepoint:Label	2.23	0.088	0.90	0.513	5.44	**<0.001**
Treatment:Label	0.10	0.759	1.09	0.297	1.73	0.190
C source:Label	0.71	0.401	1.03	0.310	31.04	**<0.001**
Timepoint:Treatment:C source	0.48	0.822	1.09	0.362	1.05	0.410
Timepoint:Treatment:Label	0.43	0.732	1.47	0.168	1.09	0.371
Timepoint:C source:Label	5.43	**0.002**	0.99	0.443	1.19	0.303
Treatment:C source:Label	3.16	0.078	0.95	0.331	0.00	0.977
Timepoint:Treatment: C source:Label	0.53	0.666	0.89	0.527	0.96	0.465

Table A6.2: Adonis result table for the statistical test of factors (C source, Treatment, Time) and their interactions affecting the bacterial and fungal responsive communities to C sources (Permutations 10^5). C sources added to soil are lignin, cellulose and No C control. Factor treatment represents the irrigation treated and dry control soils. Factor time represents the incubation time. Significant P-values are given in bold.

	Bacterial community		Fungal community	
	F	P-value	F	P-value
C source	13.262	**<0.001**	9.053	**<0.001**
Treatment	4.133	**<0.001**	3.779	**<0.001**
Time	1.754	**0.003**	1.656	**<0.001**
C source:Treatment	1.4936	**0.037**	1.7278	**0.003**
C source:Time	1.4969	**0.003**	1.288	**0.008**
Treatment:Time	0.905	0.666	1.104	0.192
C source:Treatment:Time	0.8601	0.881	0.8686	0.9311