Drought effects on carbon and nutrient dynamics in Scots pine

Homeostasis, thresholds and tipping points

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Homeostasis, thresholds and tipping points

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A tree with strong roots laughs at the wind

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Preface

With forests covering 30% of the world land surface and offering humans so many ecological services – wood, oxygen, food, recreation, safety, biodiversity – it is astonishing how much we still have to learn about their functioning. Especially in times of relatively quick global change, it is important how these changes affect forests worldwide. To understand the effects of environmental stresses on forests, I study very small processes, on molecular level, and use those processes to explain larger-scale ecological ones. I find it fascinating to see how much we need to understand the small processes before we can understand what is happening on a larger scale.

‘A tree with strong roots laughs at the wind’ is a Malaysian saying. Having a good and strong basis gives you resilience against disturbances and stress. In a tree perspective, I think this saying explains the message of this thesis quite well. From the results of the different chapters it appears that the roots, the basis of the tree, are very sensitive to environmental changes, but they are also the driving factor behind many tree processes. It becomes clear that, as long as a tree has a well-functioning root system, it might be able to overcome drought and other stresses, and recover afterwards.

Not only does this saying appeal to me in a tree perspective, it has also been an important saying for myself. Doing a PhD comes, like everything in life, with bumps in the road, and with ups and downs. If you have a strong basis, you also have the resilience to overcome any kind of bump in the road. I turned into a stronger person and know more and more what I want, and I developed resilience during the last few years. The strong personal basis lies at my own roots, my family, but the strong basis for this PhD may refer to my supervisors. I had the honor to be supervised by six of them, all great scientists with their own specialization and focus. Although I sometimes struggled to involve them all in my thoughts, discussions and work, in the end they were all incredibly involved, (hopefully) resulting in a, to cite one of them, “Bulletproof thesis”.

I wish you happy reading.

Birmensdorf, January 2019

Summary

Species of the tree genus Pine (*Pinus* L.) exist all over the world and no other group contains so many attractive forms (Curtis & Bausor, 1943)[1]. Scots pine (*Pinus sylvestris* L.) is currently the most widely distributed pine and occurs throughout all of Eurasia. In the central alpine valleys, Scots pine is growing at the dry border of its distribution range, which involves overcoming periods with extreme low water availability. Although the species is known for its ability to grow on dry and nutrient poor soils, several extreme droughts during the last two decades have caused a 50% dieback of Scots pine in the dry valleys of the Central Alps in Switzerland. The ability of trees to survive drought is determined by their initial health and their resilience to drought, as well as on the characteristics of a drought event – i.e. timing, duration and intensity. The mechanisms underlying drought-induced mortality are still unclear, as well as the recovery process after soil rewetting. Furthermore, possible mitigation or aggravation of drought effects by elevated nutrient availability in the soil has not been studied before. The carbon (C) balance in trees is used as an indicator for C assimilation, growth, defense and storage processes. When trees are exposed to drought, to changes in soil nutrition or sudden defoliation, the C balance may change. In this thesis, the main objective was thus to combine effects of drought and fertilization to study the C and nitrogen (N) dynamics in Scots pine trees.

In the **first chapter**, I give an overview of the state-of-the-art in research on drought-affected C and N dynamics in trees. The aim of the **second chapter** was to assess the effects of long-term drought release on growth and non-structural carbohydrate (NSC) concentrations of adult *P. sylvestris* trees. A long-term (13 years) irrigation experiment was conducted in the Pfynwald, a Scots pine dominated forest located at the dry distribution margin of the species in southern Switzerland. I measured growth, NSC, N and phosphorus (P) concentrations, as well as the natural abundance of ^{13}C isotopes on trees with different leaf area in control and irrigation plots. Irrigation resulted in higher growth rates and carbon isotope discrimination, but did not alter NSC levels. Growth and NSC decreased with lower leaf area in both control and irrigated trees, but NSC did not correlate with leaf-level gas exchange indices such as foliar δ13C, which is an indicator for water use efficiency, N or P, which are both stimulants of photosynthesis. Trees with initially low leaf area had limited ability to respond to the long-term irrigation, indicating a legacy effect of previously low crown condition. The NSC constancy across treatments suggests that carbohydrate storage may stay constant when changes in climate are slow enough to allow acclimation. Moreover, total leaf area, rather than leaf gas exchange per unit leaf area, drives variation in whole-tree carbohydrate dynamics in this system.

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[1] **Curtis CC, Bausor SC. 1943**. The complete guide to North American trees. *New Home Library*

The main focus of the **third chapter** was the mitigation or aggravation of drought effects by nutrient availability in the soil. Three year-old *P. sylvestris* saplings were exposed to drought during two subsequent years, using four different water and two soil nutrient regimes, and drought was released thereafter. In addition, partial and full needle removal was performed in order to assess effects of changes in source:sink ratio. Biomass, leaf gas exchange and tissue NSC were measured during and after the first and second growing season. Extreme drought reduced stomatal conductance, photosynthesis, biomass and NSC, whereas intermediate drought only slightly affected biomass and NSC. Defoliation stimulated photosynthesis and fertilization increased growth and root biomass fraction, but mainly in the two intermediate drought levels. Only extreme drought pushed P. sylvestris trees to mortality. The third chapter concludes that tree mortality under severe drought periods will not be mitigated, but that the effects of low intensity drought stress could be compensated by increased nutrient availability and decreased source:sink ratio.

The aim of the **fourth chapter** was to assess the C and N allocation underlying the biomass changes that were found in chapter 3. I hypothesized that, during drought, increased soil nutrient availability stimulates root metabolism and carbon allocation to belowground tissues under drought stress. I therefore conducted a ^{15}N and ^{13}C labelling experiment in July and August 2016 respectively, on the saplings described above. ^{15}N labelling was conducted with fertilized saplings from all water regimes, while ^{13}C labelling was only conducted with saplings (both nutrient regimes) from two out of four water regimes (well-watered and mild drought). I assessed the abundance of ^{15}N and ^{13}C in the roots, stem and needles after the first growing season and during the second year. C uptake was slightly lower in drought stressed trees, and extreme drought inhibited largely the N uptake and transport. Carbon allocation to belowground tissues was decreased under drought, but not in combination with fertilization. The results indicate a potential positive feedback loop, where fertilization improved the metabolism and functioning of the roots, stimulating source activity and hence C allocation to belowground tissues. We can thus conclude that soil nutrients might play an important role in mitigating drought stress of trees.

Overall this thesis shows that the impairment of tree functioning and mortality can be explained with thresholds: long-term drought causes a reduction in tree vigor and leaf area, and if a threshold of approximately 60 – 70% loss of leaf area is reached, trees may follow a trajectory towards mortality, even if drought is released in the soil. In the controlled experiment, soil moisture thresholds were visualized. The impairment of C allocation belowground under mild drought, the reduction of NSC in and impairment of ^{15}N uptake by the roots under extreme drought indicate that roots might be the first tissue to lose function and eventually die off during drought stress. Additional nutrient supply can sustain root functioning under drought, indicating that soil moisture tipping points are not fixed, but can be modified. In general, trees have a strongly coordinated supply – demand regulation for C and N, enabling homeostatic C balances as long as changes in climate are slow or mild enough for trees to acclimate.

1

General introduction

General introduction

Background

Forests and climate change

Trees are sturdy organisms that, because of their lifespan and immobility, must be able to adjust to changing environmental conditions. Covering approximately 30% of the world's land surface, forests act as important carbon (C) sinks and fulfil several important ecosystem services like wood production, protection and biodiversity (Bonan, 2008; IPCC, 2013). The functioning of forests during frequent and severe environmental stresses is largely unknown, because of the immense complexity and diversity of forest ecosystems around the globe. Whilst temperatures are expected to increase globally, precipitation models predict varying changes in precipitation pattern among ecosystems. Temperate forests are expected to experience more frequent periods of extreme drought (IPCC, 2013; Allen *et al.*, 2015). Not only do they need to be adapted to longer and more intense periods of water deficit, they also need to immediately capitalize on soil rewetting after subsequent rainfall events. In Switzerland, the effects of such extreme droughts are especially visible in the dry valleys of the Alps. Several extreme droughts during the last two decades have caused a 50% dieback of Scots pine (*Pinus sylvestris* L.) (Dobbertin *et al.*, 2005; Bigler *et al.*, 2006), and a transition towards more drought tolerant species like downy oak (*Quercus pubescens*) (Rigling *et al.*, 2013).

Physiological processes during drought

Drought reduces tree growth and productivity, and increases tree mortality and forest decline (Ciais *et al.*, 2005; Bigler *et al.*, 2006; Sánchez-Salguero *et al.*, 2012b), but the mechanisms behind drought-induced mortality remain largely unanswered (McDowell *et al.*, 2008). Most probably, the answer lies in an interaction between many different biotic and abiotic processes, like duration and intensity of drought, the combination of drought with increasing temperatures, the occurrence of insect outbreaks, and the survival strategy of trees. A general accepted theory assumes two strategies for plants responding to drought. Isohydric species close stomata rapidly under drought conditions, so that leaf water potential is kept within a narrow range that prevents embolisms and hydraulic failure, but simultaneously reduces C uptake (McDowell *et al.*, 2008). If the C demand is higher than the C supply, trees are C limited and consequently C depletion or even starvation could occur. Anisohydric species maintain open stomata during drought, enabling C uptake but increasing the risk of xylem cavitation (Parolari *et al.*, 2014). If the tension on the root-to-leaf water column exceeds a certain threshold, air bubbles occur and the water column breaks, inhibiting the water transport and causing hydraulic failure. The isohydry / anisohydry theory is debatable, as recent studies show that stomatal behavior seems disconnected to water potential in many different plant species (Martínez-Vilalta & Garcia-Forner, 2017).

Trees store C in the form of non-structural carbohydrates (NSCs). NSCs include starch, fructans and lipids, that make up the accessible C storage, and mobile soluble sugars and sugar alcohols that are used for osmotic adjustment and are inaccessible to metabolic processes and growth (Quick *et al.*, 1992; Arndt *et al.*, 2008). Theory and data both suggest that the higher sensitivity of growth (sink activity) than photosynthesis (source activity) to drought leads growth to cease first during drought, followed by photosynthetic decline (Körner, 2015). This suggestion comes forth from the observation that growth requires turgor, which is at its highest when a tree is fully hydrated (Lockhart, 1965). Reduction of growth while photosynthesis remains active probably results in (passive) C accumulation within the tree (McDowell, 2011). On the other hand, energy demanding processes such as metabolism and defense, and the need for osmotic adjustment during stress, might eventually result in a depletion of NSC. The amount of NSC in a plant thus seems a good indicator for the balance between C gain and C utilization (Li *et al.*, 2002; Hoch *et al.*, 2003; Körner, 2003), and for the condition of a tree, but this view relies on the assumption that NSC storage is solely a passive mechanism that occurs mainly as accumulation. Yet, an increasing number of studies shows the presence of active storage mechanisms in trees (Wiley *et al.*, 2013; Dietze *et al.*, 2014), which would not support a one-to-one comparison between C gain and C utilization in determining tree condition. Moreover, it is unknown whether trees can use up their total pool of NSC (Sala *et al.*, 2012; Hartmann, 2015). Alternatively, a certain non-zero level of NSC could be actively maintained, NSC transport might be inhibited, or access and conversion of NSC could be inhibited due to a lack of photosynthetic energy (Chapin *et al.*, 1990; Sala *et al.*, 2012; Hartmann, 2015). Until now, an absolute zero level of NSC during drought stress has not been proven, and thus the question remains whether trees could die from C starvation.

Temperate trees shed their leaves during drought stress to reduce the transpiring area and to adjust to imbalances between water loss and supply (Sánchez-Salguero *et al.*, 2017). Although this might be temporarily beneficial, trees might eventually reach a point of no return when drought induced defoliation progresses, and might not be able to further adjust to, and recover from drought. The C uptake capacity significantly decreases with a reduction in leaf area. Moreover, extreme defoliation results in the loss of nutrients like nitrogen (N) and phosphorus (P), which might even get more limited than C (Chapin *et al.*, 1990). Decreasing leaf area is correlated with long-term growth decrease (Timofeeva *et al.*, 2017) and with stem sapwood NSC after a drought event (Galiano *et al.*, 2011; Camarero *et al.*, 2015b). Hence, crown condition largely determines the health status of trees (Dobbertin *et al.*, 2004; Eilmann *et al.*, 2013).

Drought duration, intensity and release

The characteristics of a drought might strongly influence the physiological response of trees, which explains the numerous different outcomes of drought experiments on for example NSC responses (Adams *et al.*, 2017; Hartmann *et al.*, 2018).

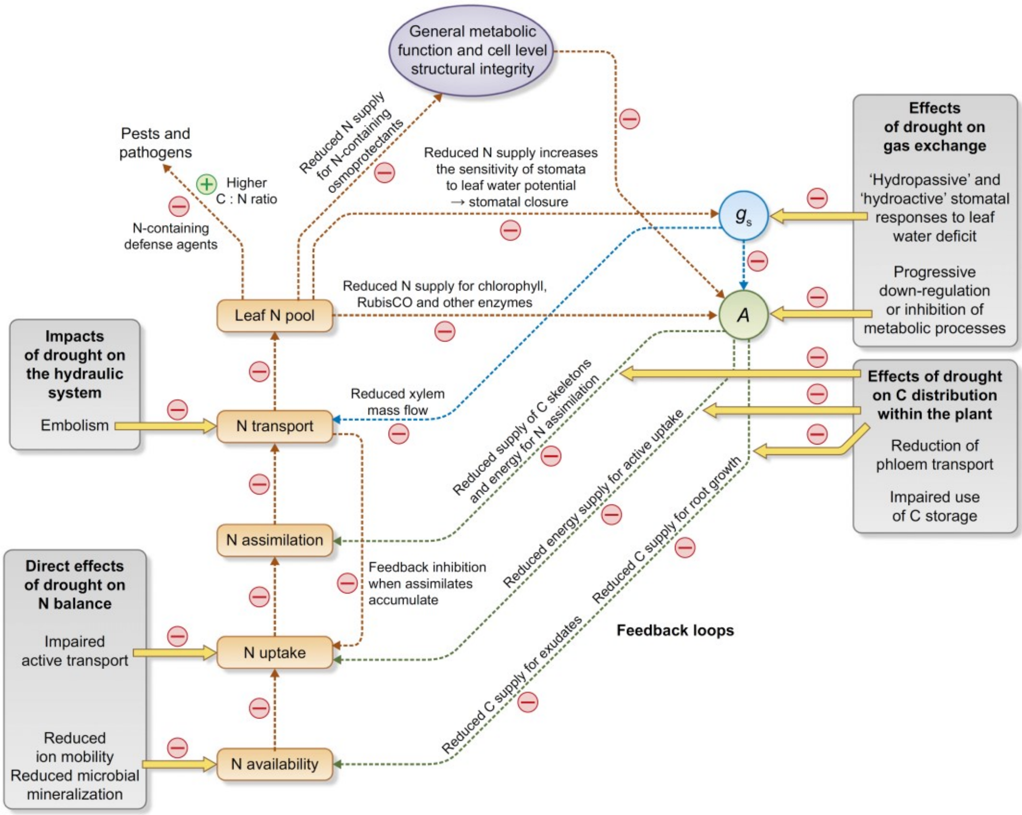

Figure 1.1: Impacts of drought on the nitrogen (N) balance of plants during a drought event. Figure from Gessler *et al.* (2017).

It has been shown, for example, that the timing of drought strongly affects the sensitivity of trees to drought (Camarero *et al.*, 2015a; Maxwell *et al.*, 2018). Similarly, drought duration and intensity might trigger different responses in trees. A short, intense drought is likely to cause hydraulic failure, whereas a longer, less intense drought is thought to increase the chance for C starvation (McDowell *et al.*, 2008). Moreover, while mild drought has been shown to increase the transport of new assimilates to the roots and thus to allow the production of larger water absorbing surfaces, more intensive drought events seem to reduce and delay the C supply to roots (Hommel *et al.*, 2016) and deplete C storage pools (Hartmann *et al.*, 2013; Li *et al.*, 2018a).

Rewetting allows trees to recover root water and nutrient uptake, as well as photosynthetic activity. Rewetting experiments resulted in a high root or sink driven C allocation belowground (Hagedorn et al., 2016; Galiano Pérez et al., 2017), to recover root biomass or to refill the depleted NSC pools (Piper et al., 2017). Recovery after drought might be, comparable to survival during, strongly dependent on the intensity and duration of a drought.

Soil nutrient availability and drought

Whilst direct effects of drought on C allocation have been studied rigorously, little is known about factors that could mitigate or intensify the negative effects of drought, such as nutrients (Vitousek *et al.*, 2002; Fisher *et al.*, 2012). When water is not the main growth limiting factor, high and long-term nutrient availability can decrease the root biomass fraction, increase the aboveground biomass, reduce the sapwood:leaf area ratio and increase vessel size. Nitrogen, the most important limiting nutrient, is an important constituent of Rubisco and thus improves photosynthetic capacity (Andersson & Backlund, 2008). These 'predisposing traits' ultimately lead to a disadvantage when a tree is subjected to drought (Gessler *et al.*, 2017). The lower ratio of root to leaf inhibits sufficient water transport to the leaves (Kozlowski & Pallardy, 2002), and wide vessels increase the chance for embolisms. On the other hand, large nutrient reserves acquired before a drought event can promote a plant's ability to survive during or to recover after a drought (Waring, 1987; Gessler *et al.*, 2017). Nutrients reduce the vulnerability to embolisms by decreasing stomatal conductance, and increase the production of N-based defense compounds.

Higher soil nutrient availability during drought might compensate for impaired active transport and lower ion mobility in the soil, allowing the plants still to achieve sufficient nutrient uptake (Gessler et al., 2017). A feedback loop arises though (Fig. 1.1), when drought impairs N uptake and transport. N deficiency in the crown negatively influences stomatal sensitivity and photosynthetic activity, ultimately increasing the risk for C starvation when C supply does not meet the demands.

Main research objectives

This study was developed to assess the interacting effects of drought and **nu**trient availability on the **ca**rbon dynamics in Scots **pi**ne (CaNuPine). Although the effects of drought on pine functioning and C allocation have been studied rigorously, the interaction between nutrient availability and drought has largely been left unstudied. Furthermore, changing source:sink relationships during drought might give us insights on source and sink activity during stresses. The project consisted of two experiments with different characteristics, both focused on assessing the C dynamics during drought, in interaction with fertilization and defoliation. In addition, the aim was to distinguish between different drought durations and intensities. The first experiment (Pfynwald) consisted of a 13 year long irrigation experiment with 100 year old Scots pine trees growing at the dry edge of their distribution range. Here, the focus was on the effect of long term drought release on trees, their acclimation potential to changing conditions. The second experiment consisted of a two-year long semi-controlled experiment in the open-top chamber facility at the WSL (MODOEK), including 480 Scots pine saplings which were three years old at the start of the experiment. Here, tree saplings were exposed to different levels of drought, nutrient availability and manual defoliation. The main aims of the CaNuPine project were:

1) To assess the effects of short- and long-term drought and subsequent short- and long-term rewetting on growth, crown condition and tissue NSC levels.
2) To assess the differences between mild and extreme drought on tree functioning and mortality.
3) To find out whether fertilization and changing source/sink balances can mitigate the negative effects of drought on growth, NSC balance, and survival.
4) To assess the reallocation of C and N using stable isotope tracers, to find differences in C and N allocation between well-watered and drought-exposed trees.

Study design

Species

Scots pine is currently the most widely distributed pine and is found throughout all of Eurasia. The genetic variety is immense and several different subspecies exist across its distribution. The tree is tolerant to poor soils, drought and frost and is found in various climatic conditions and ecological habitats (Houston Durrant et al., 2016). It is a pioneer species, able to colonize nutrient-poor soils in disturbed areas, and soils with a low water retention capacity (Matías & Jump, 2012; Lévesque et al., 2016). The tree grows in altitudes ranging from sea level up to 1000 m a.s.l. in the north of its range, and from 1200 to 2600 m in the south of its distribution (Matías & Jump, 2012). Scots pine is, especially in the north of Europe, an economically important species. In the Swiss inner alpine valleys, Scots pine is growing not very far from the southern border of its geographic distribution (Fig. 1.2), and at the border of its dry distribution limit. In northern Spain (Martínez-Vilalta & Piñol, 2002; Castro *et al.*, 2004; Galiano *et al.*, 2010) and the Alpine valleys (Dobbertin *et al.*, 2005; Schuster & Oberhuber, 2013; Rigling *et al.*, 2013), Scots pine trees increasingly suffer from extreme droughts and mortality. It is known as an isohydric species, one that tries to avoid drought stress by closing its stomata early and keep its water potential within a small range, which, as discussed before, might make it susceptible for C starvation.

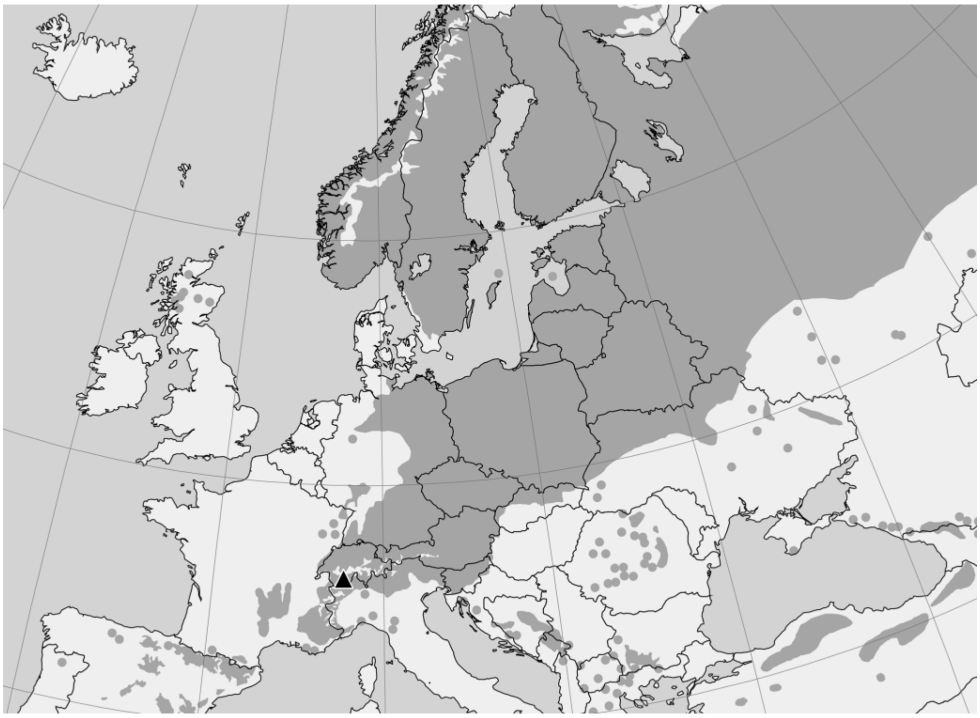

Figure 1.2: Distribution of Scots pine (*Pinus sylvestris*) in Europe (dark grey areas). The black triangle indicates the Swiss inner alpine valley, hosting the Pfynwald. (EUFORGEN 2009, www.euforgen.org).

Study site Pfynwald

The Pfynwald (46°18’ N, 7°36’ E, 615 m a.s.l.) is the largest Scots pine dominated forest in Switzerland, located in the dry inner-Alpine valley of the river Rhone, close to the dry edge of the natural distribution of Scots pine. The Pfynwald is a naturally regenerated forest, but past forest practices have favored regeneration of Scots pine over other species such as *Quercus pubescens*. During the past decades, extreme droughts have put a higher pressure on the survival of Scots pine, and between 1995 and 2000, half of the Scots pine population died as a consequence of drought (Rebetez & Dobbertin, 2004; Dobbertin & Rigling, 2006). In 2003, the Swiss Federal Institute for Forest, Snow and Landscape Research (WSL) launched a long-term irrigation experiment to compare growth and recovery of pines in irrigated forest plots with trees that only receive the natural amount of precipitation. This unique long-term experimental monitoring plot gives the possibility to study the drought resistance and recovery ability of Scots pine in competition with other tree species such as *Quercus pubescens* on the large range from cell to forest stand level.

The experimental site (1.2 ha; 800 trees) consists of eight plots of 25 m x 40 m each, separated by a 5 m buffer zone (Fig. 1.3). The average tree age is approximately 100 years and the forest has a mean canopy height of 10.8 m, a stand density of 730 stems ha^{-1} and a basal area of 27.3 m^{2} ha^{-1} (Dobbertin et al., 2010). The forest receives annually approximately 600 mm of precipitation and has a soil with very low water retention and high vertical drainage (Brunner et al., 2009). Between April and October, irrigation is applied at night on four out of eight plots, corresponding to ~600 mm/year, thus a doubling of the ambient situation. The irrigation is applied with 1 m high sprinklers using water from a nearby channel parallel to the experimental plot, fed by the Rhone river.

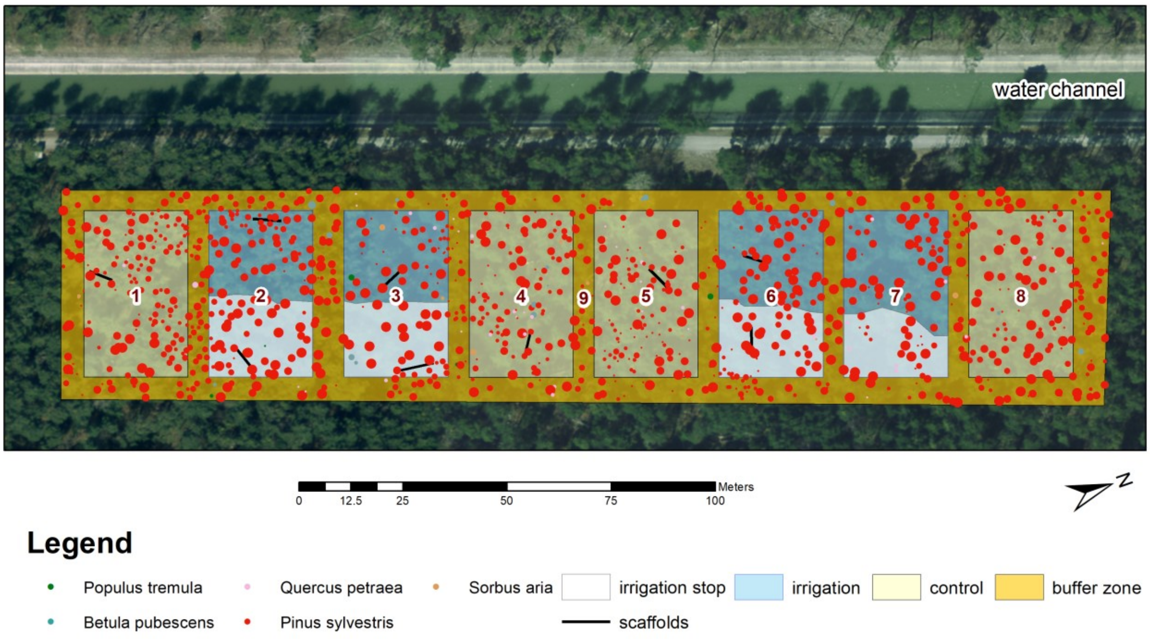

Figure 1.3: The experimental forest plot in Pfynwald. Control plots are indicated by yellow, and irrigated plots by blue colors. The orange area around the plots serves as buffer area between treatments. The bottom parts of the irrigated plots, the light blue areas, did not receive irrigation anymore from 2013 onwards. Red dots show every single pine tree.

Volumetric soil water content, air temperature, relative humidity and precipitation are measured continuously with 10 minute intervals. Tree height and stem diameter (DBH) were measured in 2003, 2009 and 2014 and crown transparency is measured once a year since 2003. Several studies have exploited this experimental site for growth, regeneration, understory growth, biodiversity and soil dynamic assessments.

Study site MODOEK

The Open-top chamber facility of the Swiss Federal Research Institute WSL (47°21'48'' N, 8°27'23'' E, 545 m a.s.l.), Birmensdorf, Switzerland, consists of 16 hexagonal open-top chambers (OTCs) of 3-m height and a plantable area of 6 m2 each (Fig. 1.4). Mobile roofs can automatically control incoming rainfall, but were kept closed during the entire experimental period. Belowground, the chambers are divided into two semicircular lysimeters (1.5 m deep) with concrete walls. The lysimeters were filled with a 1 m deep layer of gravel for fast drainage, covered with a fleece layer that is impermeable for roots but permeable for water, and on top a 40 cm layer of calcareous sandy loam soil (Kuster et al., 2013). Every lysimeter was planted with 15 three years-old saplings of *Pinus sylvestris* (55.61 cm +/- 5.41 cm height) in April 2015. Temperature and air humidity inside and outside the OTC, as well as soil moisture and soil temperature inside (5, 20, 35 cm deep) were automatically monitored (5TM soil moisture and temperature logger, Metergroup, Munich, Germany). Six sprinklers (1 m high) per lysimeter were evenly distributed, and irrigation was programmed for every lysimeter separately. Every chamber was assigned one of four different water regimes as whole-plot treatment (four chambers / replicates per regime, in a Latin Square design) (Fig. 1.4). The amount of water to be applied was controlled by means of the soil moisture measurements and previously measured pF curves.

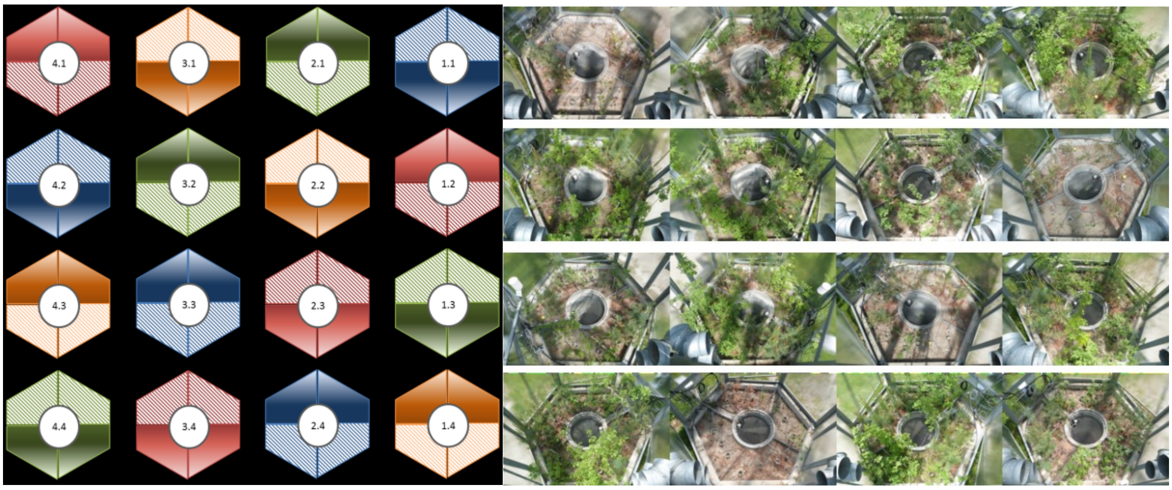

Figure 1.4: Left: Experimental setup of the Open top chambers. Numbers indicate the column and row number. Colors indicate the four different drought regimes. Blue = W100, Green = W50, Orange = W20, Red = W0. Nutrient regime is indicated with solid (unfertilized) and dashed (fertilized) fill. Right: Photographs of the 16 Open top chambers in May 2017 in the same order as indicated in the scheme.

These measurements allowed for four different levels of VWC: close to field capacity, W100 (approximately 25% VWC), wilting point, W0 (approx. 6%; achieved by no irrigation at all) and two intermediate levels, corresponding to approximately 50% (W50) and 20% (W20) of FC. Water treatments started a year after planting. They ran from April to October in 2016 and from April to mid-July 2017, after which all chambers were (re)watered until field capacity in order to study the recovery process in the trees. Twice a year, one of the two lysimeters (split-plot) in the OTC's were fertilized with liquid fertilizer (Wuxal, Universaldünger, NPK 4:4:3), corresponding to 50 kg / ha N per year. Lastly, in each lysimeter (i.e. across all water and nutrient treatments) individual trees (split-split plot) were randomly assigned a control, debudding, 1/3, 2/3 or 3/3 needle removal treatment (in grams fresh biomass, n = 3). The detailed methodology is described in chapter 3 and 4.

Thesis overview

Chapter 2

During long-term drought stress, tree growth is inhibited and trees shed leaves or needles to reduce the transpiring area, causing increased crown transparency / loss of leaf area. Patterns of NSC concentration under long-term drought *vs*. the release of such drought exposure under field conditions have not been extensively studied. We also lack information on the interrelationship between NSC, growth, tissue nutrient concentrations and crown conditions under such long-term drought *vs*. non-drought exposed conditions. In Chapter 2, we focused on the interrelationship between long-term drought and drought release, crown leaf area and growth and NSC concentrations. We sampled root, wood and needle tissues of 60 trees in the Pfynwald throughout the year, and measured NSC levels in these tissues. We combined our findings on NSC levels with growth, crown condition (leaf area) and gas exchange data to discuss the following hypotheses:

1) Trees are source-limited during long-term drought, resulting in lower NSC concentrations in dry (control) compared to irrigated trees after 13 years of treatment.
2) NSC is correlated with relative leaf area. Alternatively, NSC may be better correlated with indices of leaf-specific gas exchange (we note this is possibly a false-dichotomy as both total photosynthetic leaf area and gas exchange per unit leaf area may influence NSC).
3) Legacy effects of low leaf area prior to irrigation could constrain growth and NSC responses to irrigation after 13 years.

Chapter 3

Trees respond to drought depending on the droughts' duration and intensity. Whilst not only the duration and intensity of summer droughts are predicted to increase, but also intermittent high intensity rainfall events, trees have to withstand drought periods and be able to immediately capitalize on soil rewetting, in order to secure survival and competitiveness. In addition, little is known about the biotic or abiotic influences that can mitigate or intensify negative drought effects on tree functioning, like nutrient availability.

In Chapter 3, we combined different drought regimes with fertilization and changing source:sink balances to address the following hypotheses:

1) The relationship between drought and C allocation is a threshold response, where trees can adjust to and survive mild drought, but will succumb to mortality under severe drought.
2) Fertilization and thus increased N availability will mitigate the negative effects of intermediate drought but under severe drought nutrient uptake and transport will be inhibited independent of nutrient availability and thus no mitigation effect is suspected.
3) Decreased source:sink ratio due to mild defoliation will lead to lower susceptibility in response to drought, whilst severe defoliation will not compensate drought effects
4) Recovery after drought strongly depends on the available C and nutrient reserves that will be affected by drought intensity subjected to before.

Chapter 4

C allocation is generally prioritized to tissues that increase the uptake potential for limiting resources. Mild drought has been shown to increase the transport of new assimilates to the roots, but very intensive drought might inhibit transport of C and N. Fertilization might stimulate root water uptake, stomatal conductance and improve general metabolic functions, and thus promote a plant's ability to survive during drought. In Chapter 4, we conducted a labelling experiment with ^{13}C and ^{15}N, to assess C and N allocation during, and reallocation after drought, in combination with fertilization. We hypothesized that

1) C allocation to the roots increases relative to other tissues under drought but that C allocation to belowground tissues is inhibited if the drought gets too intensive.
2) Fertilization results in less C being invested in roots and more in aboveground biomass under optimal water supply, but that with drought, fertilization can improve the C allocation to belowground tissues, especially under more intensive drought.
3) Drought stressed trees have a strongly coordinated supply – demand regulation for C and N and thus do not deplete C and N reserves for needle growth early in the season.
4) Rewetting results in enhanced uptake and (re-)allocation of N to the needles when trees grew before under severe water limitation, while at the same time C allocation is prioritized for the restoration of the root system.

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2

Homeostatic levels of non-structural carbohydrates after 13 years of drought and irrigation in *Pinus sylvestris* L.

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Summary

- Non-structural carbohydrates (NSCs) are important for growth and survival of trees. Drought might lead to a decrease in tree growth and to NSC depletion, while increased soil moisture in otherwise dry ecosystems might increase growth and NSC concentrations.
- A long-term (13 years) irrigation experiment was conducted in a *Pinus sylvestris* L. dominated forest located at the dry margin of the species in southern Switzerland. We measured relative leaf area, growth, NSCs, needle $\delta^{13}C$, [N] and [P] in trees on control and irrigated plots.
- Irrigation resulted in higher growth rates and carbon isotope discrimination, but did not alter NSC levels. Growth and NSC decreased with decreasing leaf area in both treatments, but NSC did not correlate with leaf-level gas exchange indices such as foliar $\delta^{13}C$, [N] or [P]. A legacy effect was shown as trees with initially low leaf area had limited ability to respond to the prolonged irrigation.
- The NSC constancy across treatments provides evidence that carbohydrate storage may stay constant when climate changes are slow enough to allow acclimation. Moreover, we speculate that total leaf area, rather than leaf gas exchange per unit leaf area, drives variation in whole-tree carbohydrate dynamics in this system.

Keywords: acclimation, homeostasis, irrigation, growth, non-structural carbohydrates (NSCs), *Pinus sylvestris* L., starch, sugar

Introduction

Drought causes reduced forest productivity, and increasing duration and frequency of drought events can turn forest ecosystems from carbon (C) sinks into sources (Ciais *et al.*, 2005). Trees store large amounts of assimilated C in their sapwood and roots, as well as in needles in the case of conifers (Hoch, 2015). These storage pools can be used for growth, maintenance metabolism and defense (Chapin *et al.*, 1990). As drought impairs photosynthetic C assimilation, C storage pools might play a major role in supporting tree functions during such periods. Although our general knowledge on C storage and remobilization dynamics in trees is increasing, there is still an ongoing debate about how, and to what extend C reserves play a role in sustaining functioning and growth under stressful conditions as well as during tree recovery after stress (McDowell, 2011; Sala *et al.*, 2012; Palacio *et al.*, 2014; Gessler & Treydte, 2016).

For trees, C storage mostly occurs in the form of sugars and starch that make up non-structural carbohydrates (NSCs) (Dietze *et al.*, 2014) whilst other compounds such as lipids play a minor role in most species (Hoch *et al.*, 2003). NSCs buffer the imbalance between supply and demand of C across plant tissues on diurnal and seasonal scales (Li *et al.*, 2002; Hoch *et al.*, 2003; Hartmann & Trumbore, 2016). In periods of drought, NSC pools in different plant organs can serve as a buffer to compensate for the decreased C assimilate supply due to decreased photosynthesis (McDowell, 2011; Sala *et al.*, 2012; Hoch, 2015). C storage has been considered both a passive process that only occurs when the C supply is higher than the demand (Sala *et al.*, 2012) and is also thought to be actively regulated in balance with other C sinks such as growth and defense (Chapin *et al.*, 1990; McDowell, 2011; Dietze *et al.*, 2014). There is presently intensive discussion whether C storage is ‘passive’ or ‘active’ or both (Sala *et al.*, 2012; Wiley & Helliker, 2012; Hartmann & Trumbore, 2016) and whether NSC depletion under extreme conditions, and thus C starvation is a driving mechanism for reduced tree survival under drought (Sala *et al.*, 2012; Klein, 2015).

Theory and data both suggest that the higher sensitivity of growth (sink activity) than photosynthesis (source activity) to long-term drought (Körner, 2015) leads growth to cease first during drought followed by photosynthetic decline (McDowell, 2011). These differing temporal patterns in photosynthetic supply and demand allow accumulation of NSC during the initial phase of a drought period or at very mild drought stress (trees are sink limited), and might eventually result in NSC depletion if drought persists over longer periods when respiratory, metabolic, and perhaps hydraulic C demands exceed the supply by photosynthesis (trees get source limited) (McDowell *et al.*, 2013; Hoch, 2015). This hypothesis can possibly explain the seemingly contradicting results found in literature, ranging from NSC decrease during drought, as repeatedly shown in Scots pine (*Pinus sylvestris* L.), to no changes at all or even increases of NSC (Adams *et al.*, 2017).

Patterns of NSC concentration under long-term drought *vs*. the release of such drought exposure under field conditions have not been extensively studied. We also lack information on the interrelationship between NSC, growth, tissue nutrient concentrations and crown conditions under such long-term drought *vs*. non-drought exposed conditions (but see von Arx *et al.*, 2017). Decreasing leaf area (in some literature referred to as 'crown transparency', a surrogate for relative leaf area (Dobbertin, 2005; Gottardini *et al.*, 2016)), is correlated with long-term growth decrease (Timofeeva *et al.*, 2017), and also with stem sapwood NSC after a drought event (Galiano *et al.*, 2011; Camarero *et al.*, 2015b). In the first years of release from long-term drought, high crown defoliation impeded the growth recovery of trees (Eilmann *et al.*, 2013), thus reduced relative leaf area (relative, compared to a tree with maximum leaf area in a forest) may also limit NSC storage dynamics after irrigation was commenced. Moreover, tissue nutrient depletion resulting from impaired nutrient uptake during drought might exacerbate the adverse effects of long-term reduced water availability by impairing leaf-level photosynthesis (Gessler *et al.*, 2017).

At the Pfynwald, the largest Scots pine dominated forest in Switzerland, located in a dry inner alpine valley, several drought events within the last decades have caused large-scale mortality of Scots pine trees (Allen *et al.*, 2010; Rigling *et al.*, 2013). Together with low growth rates (Dobbertin *et al.*, 2010), this indicates that the stand grows at the dry edge of the distribution of Scots pine. A unique long-term (13 year) irrigation experiment at this forest site allowed us to study long-term effects of chronically increased soil water content on seasonal NSC dynamics in Scots pine, compared to many studies that used short-term treatments or single extreme (natural) drought events (Adams *et al.*, 2017).

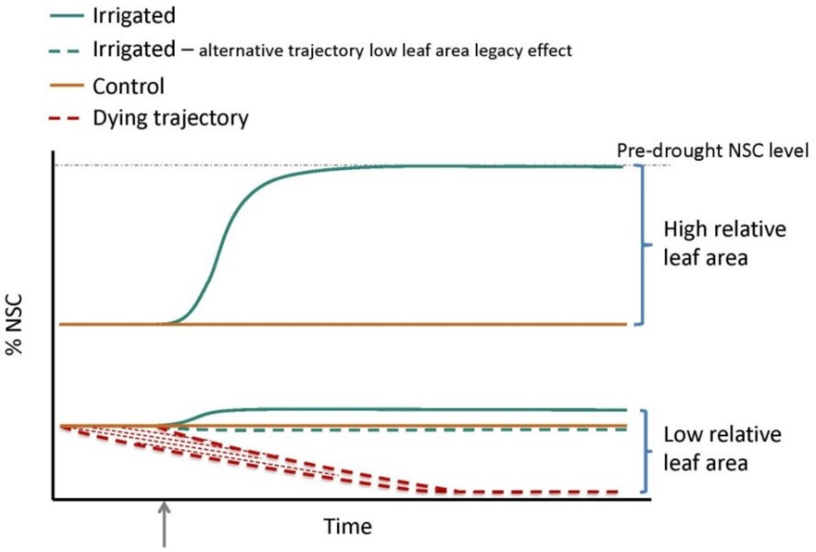

Figure 2.1: Possible trajectories of long-term tree non-structural carbohydrate (NSC) concentrations in irrigated and control plots, as an elaboration on the modeled representation of short-term NSC dynamics during drought for generic trees posed in (McDowell, 2011). We hypothesize that NSC will be lower in dry (control) compared to irrigated trees, but that NSC is also correlated with relative leaf area. This interaction may result in a wide spectrum of NSC levels in trees with or without drought stress and with different crown conditions. Drought release may bring a trees' NSC pools back to pre-drought levels but historical crown legacy might reduce the capability to recover C reserves fully (green solid line) or even at all (green dashed line) after drought release. Lastly, trees might reach a point of no return that only leads to mortality, caused by hydraulic failure, C starvation or biotic factors.

The conceptual framework on NSC trajectories proposed by McDowell (2011) was based on evidence from short-term droughts (e.g. seasonal to a few years), but NSC dynamics may differ after 13 years of treatment that allow acclimation to new soil-water conditions. We based our *a priori* hypotheses on the assumption that despite any acclimation, NSC dynamics would respond to treatments and crown conditions. First (I), we hypothesized that trees would be source-limited during long-term drought, resulting in lower NSC concentrations in dry (control) compared to irrigated trees after 13 years of treatment based on their presumed locations within the hypothesized NSC curve (Fig. 2.1). Second (II), we predicted that NSC would be correlated with relative leaf area. Alternatively, NSC may be better correlated with indices of leaf-specific gas exchange (we note this is possibly a false-dichotomy as both total photosynthetic leaf area and gas exchange per unit leaf area may influence NSC). Lastly (III), we hypothesized that legacy effects of low leaf area prior to irrigation could constrain growth and NSC responses to irrigation after 13 years. As most studies determined the carbohydrate levels when drought reaches its maximum, but neglected the role of winter NSC in growth and survival of drought stressed trees, we sampled multiple tissues (needles, roots and sapwood) throughout multiple seasons to better test our hypotheses at both the whole-tree and seasonal scales.

Materials and Methods

A 13-year irrigation experiment was conducted in the Pfynwald forest (46°18N, 7°36’ E, 615 m a.s.l.), the largest Scots pine (*Pinus sylvestris* L.) dominated forest in Switzerland, located in the dry inner-Alpine valley of the river Rhone, close to the dry edge of the natural distribution of Scots pine. The Pfynwald is a naturally regenerated forest, but past forest practices have favored regeneration of Scots pine over other species such as *Quercus pubescens*. Climatic conditions are characterized by a mean annual temperature of 10.1 °C and an annual precipitation sum of approximately 600 mm. Scots pine forests in the Valais are regularly subjected to drought- and heat-induced mortality (Bigler *et al.*, 2006; Allen *et al.*, 2010; Rigling *et al.*, 2013). The average tree age is approximately 100 years and the forest has a mean canopy height of 10.8 m, a stand density of 730 stems ha^{-1}, and a basal area of 27.3 m^2 ha^{-1} (Dobbertin *et al.*, 2010). The soil is shallow parendzina, characterized by very low water retention and high vertical drainage (Brunner *et al.*, 2009).

The experimental site (1.2 ha; 800 trees) is divided into eight plots of 25 m x 40 m each, separated by a 5 m buffer zone. The irrigation of ~600 mm/year is applied at night on four plots between April and October, from the year 2003 onwards, with 1 m high sprinklers using water from a nearby channel parallel to the experimental plot, fed by the Rhone river. Nutrient input through irrigation was proven to be minor (Thimonier *et al.*, 2005, 2010). The control plots thus represent the drought exposed situation, whereas the treatment results in a drought release. The volumetric soil water content was monitored hourly in one control and one irrigated plot using time domain reflectometry (Tektronix 1502B cable tester, Beaverton, OR), at a soil depth of 10, 40 and 60 cm at four different locations per plot (Fig. S2.1).

Air temperature, relative humidity (Sensirion SHT-21, Sensirion AG Switzerland) and precipitation (Tipping Bucket Rain Gauge, R.M. Young, Michigan USA) were measured and stored with a time resolution of 10 min. Tree height and stem diameter (DBH) were measured in 2003, 2009 and 2014 by manual calipering. Crown transparency (the surrogate for relative leaf area) was measured once a year since 2003. Crown transparency assessment was performed as described by Dobbertin *et al.* (2004) by visual rating of the crown transparency (also termed defoliation) using reference photographs ranging from 0% (= a fully foliated tree) to 100% (= a dead tree; for more detail, see Dobbertin *et al.* (2004). This assessment is not a strict crown transparency assessment as tree crown foliage is judged relative to the optimum foliage a tree of the same species can achieve. Relative leaf area (hereafter called shortly 'leaf area') was calculated by 100% - % crown transparency.

Sampling

Thirty control and 23 irrigated trees from 5 different relative tree leaf area classes (1 = 100-80% leaf area, 2 = 80-60%, …, 5 = 20—0% leaf area) as determined in 2014 were randomly selected (n=6 per class, except for the irrigated plots, where n was 2 and 1 for classes 4 and 5, respectively). The trees were randomly distributed over the four plots per treatment. Only trees that were alive and carried at least 10% green needles in February 2015 were included. Sampling took place on February 24-25, June 8-9 and October 26-27, 2015. Sapwood samples of the trunk of all 60 trees were taken with an increment corer (5 mm in diameter) 1 m, 3 m and 5 m above the ground, on the south side of the tree. Two cores were taken per height, with a distance of 20 cm above each other. The first 2 cm of sapwood from the cambium was used and the bark and phloem were removed. The two sapwood cores were pooled. At 7 m and 9 m above the ground, small sun exposed branches were cut. A small piece of branch wood was collected, and 1 yr, 2 yr and 3 yr old needles were separated and pooled for the 7 m and 9 m collection. A main root located close to the trunk with a diameter of approx. 1 cm was collected at a distance of ~30cm from the stem base. Fine roots (< 2 mm) were collected by following the root to a distance of ~60 cm from stem base. All samples were immediately put on dry ice to stop enzymatic activities.

NSC measurements

All stem, root and needle materials were dried at 60°C until stable weight was achieved and then ground to a fine powder. NSCs were analyzed following the protocol as described in Wong (1990) adapted according to Hoch *et al.* (2002). NSCs are defined here as low molecular weight sugars (glucose, fructose and sucrose) plus starch. 10-12 mg of ground material was boiled in 2 ml distilled water for 30 minutes. After centrifugation, an aliquot of 200 µl was treated with Invertase and Isomerase from baker's yeast (Sigma-Aldrich, St. Louis, MO, USA) to degrade sucrose and convert fructose into glucose. The total amount of glucose (sugars) was determined photometrically at 340 nm in a 96-well microplate photometer (HR 7000, Hamilton, Reno, NE, USA) after enzymatic conversion to gluconate-6- phosphate (hexokinase reaction, hexokinase from Sigma Diagnostics, St. Louis, MO, USA). Total amount of NSC was measured by taking 500 µl of the extract (including sugars and starch) incubated with a fungal amyloglucosidase from *Aspergillus* *niger* (Sigma-Aldrich, St. Louis, MO, USA) for 15 h at 49°C to digest starch into glucose. Total glucose (corresponding to NSC) was determined photometrically as described above. The concentration of starch was calculated as NSC minus free sugars. Pure starch and glucose-, fructose- and sucrose- solutions were used as standards and standard plant powder (Orchard leaves, Leco, St. Joseph, MI, USA) was included to control reproducibility of the extraction. NSC concentrations are expressed on a percent dry matter basis. Because all samples were run in a single laboratory with no change in protocol during the laboratory processing of samples, issues with comparison of results across methods or labs were obviated (Quentin *et al.*, 2015).

Needle $\delta^{13}C$ and C, N and P content

2 mg (+/- 0.1 mg) ground needle material was weighed into tin cups and converted to CO_2 and N_2 in an elemental analyzer *Euro EA* (Hekatech GmbH) connected to an Isotope Ratio Mass Spectrometer (IRMS Delta V Advantage, Thermo Scientific) to determine C and N contents and the isotopic compositions. Laboratory standards and international standards with known $\delta^{13}C$ values were used for calibration of the measurements resulting in a precision of 0.2 ‰. The isotopic ratios in all samples were expressed in δ notation (‰) relative to the international standard Vienna Pee Dee Belemnite (VPDB). C and N content were assessed as percentage relative to dry weight, and total P as per mille relative to dry weight. Total P was determined on acidified samples and measured by inductive coupled plasma (ICP-OES) spectrometry Optima 7300 DV analysis (Perkin Elmer Inc, Waltham, MA, USA) after pressure digestion with HNO_3 and HF.

Growth

The length-increment of the sun exposed shoots was measured for the last 5 years on 2 branches per tree. A subsample (approximately 30 to 50 needles) of the last 3 generations of needles from one sun-exposed branch were scanned with a flatbed scanner and their respective fresh and dry weights were measured. Specific leaf area, total leaf area, length and width were calculated from the scans using the free software Pixstat (vs 1.2.0.0, Schleppi, 2011).

Gas exchange and leaf water potential

In the summer of 2016, one year after the extensive sampling campaign for NSC measurements (due to time restrictions the year before), pre-dawn leaf water potential and gas exchange measurements were carried out. These measurements were taken on 24 of 53 trees sampled in 2015. In both treatments, 12 trees were selected, evenly divided over leaf area class 1, 3 and 4-5. Leaf water potential was measured before sunrise, between 3.30 am and 5.30 am. With a pole pruner, a small twig was cut from a branch at approx. 9 m above ground. The bark was removed to make it easier to distinguish between water and resin. Leaf/twig water potential was measured using a Scholander bomb (Model 600 pressure bomb, PMS Instrument Company, Albany, USA), in steps of 0.05 mPa. Gas exchange (stomatal conductance and A_{max}) was measured using a LI6400 Portable Photosynthesis System (LiCor, Inc., Lincoln, NE, USA) in June, July and October of 2016. A branch of approx. 50 cm length was cut with a pole pruner and immediately put in water.

Approximately 20 needles were enclosed in the 2x3 chamber and photosynthesis (A_{max}) was measured under 400 µmol mol^{-1} CO_2, 1200 PAR, 60-70% RH and 22°C.

Statistical analysis

To validate that the selected trees were a good representation of the population, DBH and diameter growth rate of the whole population and the subset used for NSC and other analyses were compared in a paired Student t-test, separately for the different combinations of treatment (irrigation (I), control (C)) and leaf area class groups. In addition, initial DBH in 2002 was compared between control and irrigated plots with a Student t-test, and it turned out to be significantly different from each other ($p < 0.001$, C=19.95, I=22.333). To correct for these differences, we calculated relative DBH increment from the start of the experiment [$(DBH - DBH_0)/DBH_0$ x 100%], where DBH is diameter at breast height in 2015 and DBH_0 is DBH at the onset of irrigation in 2003.

Concentrations were log-transformed and where necessary, other transformations were carried out to obtain normality of residuals (for transformations, see supplementary materials). Sugars, starch, $\delta^{13}C$, nitrogen (N) and phosphorus (P) content were analyzed using linear mixed effect models using maximum likelihood (lmer function; lme4 package, R version 3.2.2) and p-values were calculated based on Satterthwaite's approximations. Fixed factors were Treatment (Tr), Leaf area class (C), Season (S), Tissue (T) and all 2-way interactions. DBH was also considered a factor in the complete model, but because it gave non-significant results in all analyses (Table S2.1), we simplified the model. Tree individual was used as repeated measure factor. Separate analyses were carried out for three tissue groups: roots (2 tissues) stem wood (3 tissues/heights) and needles (3 generations). Normality of residuals was checked and corrections were made by transformation of the data. Least square means were calculated for specific differences of significant factors. Correlation analyses were carried out for NSC and growth, with $\delta^{13}C$, N and P.

Leaf area and tree diameter data were analyzed for all trees in the experimental plot for the years 2002-2014, with linear mixed effects models as indicated above. Fixed factors were year (Y) (for DBH: 2002, 2009, 2015; for leaf area: yearly 2003-2016), treatment (TR), current leaf area class (C) (as indicated in 2014) and their interactions. Tree individual was used as a random factor for repeated measures. Shifts of trees to higher or lower leaf area classes were not taken into account.

Gas exchange and leaf water potential data were also analyzed with linear mixed effect models, with Treatment, Class and Date as fixed factors and Tree individual as random factor.

Tree biomass was estimated using allometric equations developed for Scots pine (Forrester *et al.*, 2017). For all tissues (foliage, branch, stem and root mass), the same equation type was used: [ln(biomass) = ln(b0) + b1*ln(DBH))]. The parameter values used can be found in Table S2.2. As these equations were developed for average trees and trees with different crown conditions were included in this study, needle biomass was scaled according to the crown condition. For this, the assumption was made that the average leaf area is ~80%, based on the average leaf area (between 2005 and 2013) for Scots pine in whole Switzerland as measured in the Sanasilva forest inventory (A. Gessler, unpublished data). Total NSC pools of needles, branch, stem and roots were determined using the calculated biomass and measured NSC concentrations of 1-year old needles (N1), 9 m branch wood, 1 m stem wood and coarse roots (CR) and total tree NSC pool was calculated as the sum of the 4 tissues. For stem wood, we considered only the outer 2 cm of sapwood in the calculation, as it is shown that NSC concentrations decline radially from the cambium towards the pith (Hoch *et al.*, 2003). These are estimates and we don't know the exact amounts of NSC in the entire stem. A student's t test was used to test the treatment difference for total NSC pools of the trees.

Linear regression models were carried out to compare growth to starch and sugar concentrations in winter in all tissues. If necessary, growth was log-transformed. All statistical calculations were performed with R (R Core Team, 2015).

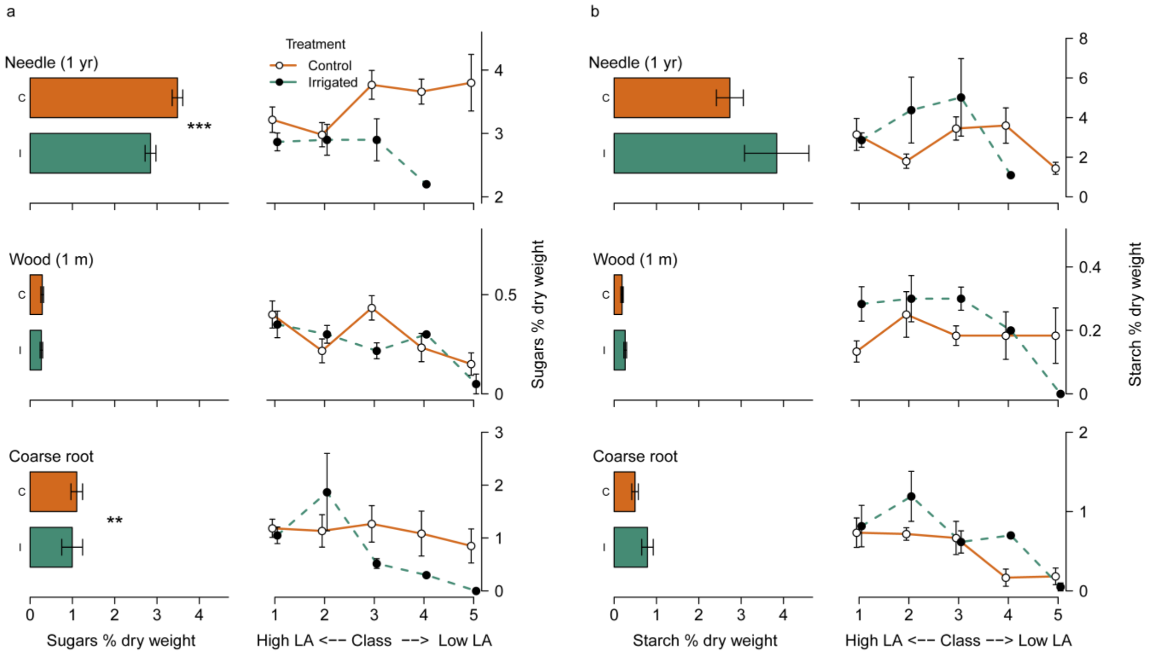

Figure 2.2: Sugar (a) and starch (b) concentrations in 1 yr old needles, stem wood at 1 m height and coarse roots of *Pinus sylvestris* in summer. In the left panels, the comparison between control (C) and irrigated (I) plots is shown, on average for all leaf area classes. Asterisks show significant differences between control and irrigated trees (**, $p < 0.01$, ***, $p < 0.001$). Right panels show concentrations in the 3 tissues in summer, plotted against relative leaf area classes for irrigated (green) and control (orange) plots. Bars show SE of the mean. n=6 except for irrigated class 4 (n=1) and 5 (n=2). In summer, the irrigated trees in class 5 had no needles resulting in a missing data point.

Results

Irrigation did not significantly affect starch concentrations in any tissue examined in the summer season (needles, wood, roots, Fig. 2.2b, Table S2.3). Sugar levels on the other hand, were lower with irrigation in the needles and roots (Fig. 2.2a, Table S2.3). In stem wood, irrigated and control trees had comparable sugar levels. Needle δ^{13}C values were significantly ($p < 0.001$) higher in control trees than in irrigated trees in summer in all three needle age classes examined (Fig. 2.3, Table S2.4). No significant treatment effect was found for needle N or P concentrations (Fig. 2.3, Table S2.4).

Leaf area was positively related to sugars and starch levels in wood and roots in summer (Fig. 2.2, Table S2.3). This trend was largely driven by lower values in two classes with the lowest leaf area (classes 4 and 5). In the needles of control trees, however, decreasing leaf area was associated with increasing sugar levels (Fig. 2.2, Table S2.3). No significant effects of leaf area were found for needle N or P concentrations (Fig. 2.3, Table S2.4), but δ^{13}C was higher in trees with low leaf area than those with high leaf area ($p = 0.007$), and δ^{13}C values increased faster with decreasing leaf area in irrigated trees, compared to control trees ($p = 0.017$, Fig. 2.3, Table S2.4). NSC was mostly negatively correlated with needle nutrients and positively correlated with δ^{13}C (Fig. 2.4). Growth was positively correlated with needle nutrients (N and P) and negatively correlated with δ^{13}C values (Fig. 2.4). For all treatment and leaf area effects, similar patterns were found during autumn and winter season, with some exceptions (Fig. S2.2, S2.3).

Irrigation increased leaf area especially during the first 4 years after the start of the treatment (Fig. 2.5a, S2.4, Table S2.5a), but only trees that are currently in class 1, 2 and 3 showed a clear positive treatment response in the first 4 years after irrigation, and this stabilized after 2007 (Fig. 2.5a, Table S2.5a).

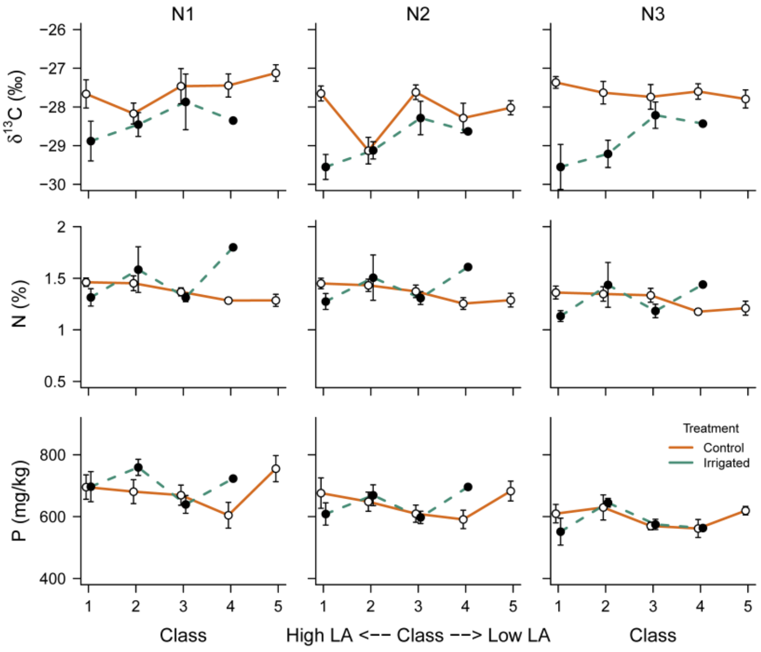

Figure 2.3: Summer levels of δ^{13}C, nitrogen and phosphorus in 3 generations needles of *Pinus sylvestris*, plotted against leaf area classes for control and irrigated plots. Bars show SE of the mean. n=6 except for irrigated class 4 (n=1) and 5 (n=2). In summer, the irrigated trees in class 5 had no needles anymore resulting in a missing data point.

Trees from classes 4 and 5 in 2014 had an average initial leaf area of ~40%, and independent of treatment, their leaf area decreased (Fig. 2.5a, Table S2.5a). Relative DBH increment increased significantly with irrigation in leaf area class 1 and 2, but in class 3-5, the effect of irrigation was not significant (Table S2.5b), showing that the differences between control and irrigated trees (hence the positive effect of irrigation) became smaller with decreasing leaf area (Fig. 2.5b, Table S2.5a). Relative DBH increment was lower in trees with low leaf area in both treatments (Fig. 2.5b, Table S2.5b). Shoot growth also decreased with decreasing leaf area in both treatments, but was not influenced by irrigation (Fig. 2.5c, Table S2.5a).

Irrigation decreased leaf water potential in all trees but no treatment effect was seen in A_{max} and g_s. No leaf area class effect was found for leaf water potential, A_{max} and g_s (Fig. S2.5, Table S2.6).

Shoot growth was positively correlated with total NSC (sugars + starch) and starch levels in winter in fine roots ($p = 0.040$, $R^2 = 0.1$ for NSC, $p = 0.001$, $R^2 = 0.23$ for starch, Fig. S2.6) and wood taken from 5 m height ($p = 0.004$, $R^2 = 0.18$ for NSC; $p = 0.048$, $R^2 = 0.11$ for starch; Fig. S2.6). In the fine roots, the correlations were stronger in control trees ($p < 0.001$, $R^2 = 0.48$), whereas in sapwood from 5 m stem height, the irrigated trees showed a better fit ($p = 0.018$, $R^2 = 0.3$). In irrigated trees, starch levels in needles ($p = 0.033$, $R^2 = 0.26$) were positively correlated with shoot growth (Fig. S2.6). Total NSC pools of the trees in irrigated plots was significantly higher than those of trees in control plots in all seasons (Fig. S2.7, Table S2.7).

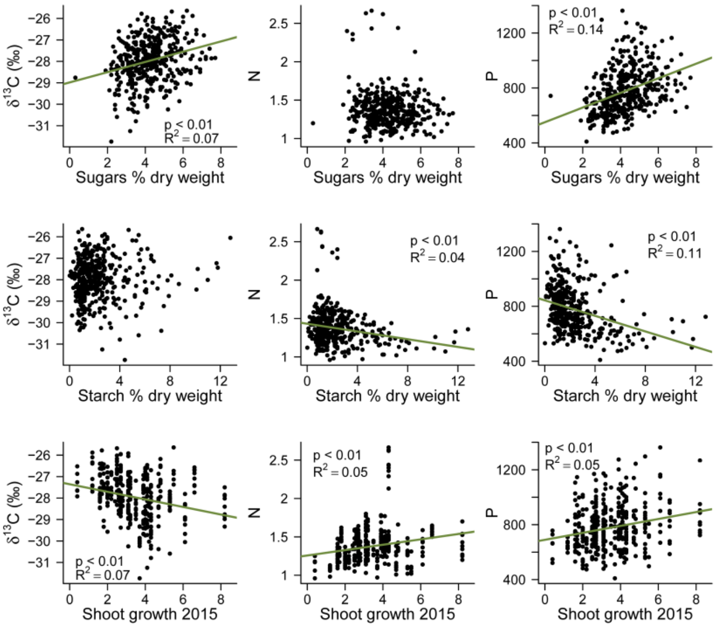

Figure 2.4: Correlation between sugars, starch and shoot growth in 2015 on the one hand (x-axes) and $\delta^{13}C$, nitrogen and phosphorus on the other (y-axes). Regression lines are shown when significant.

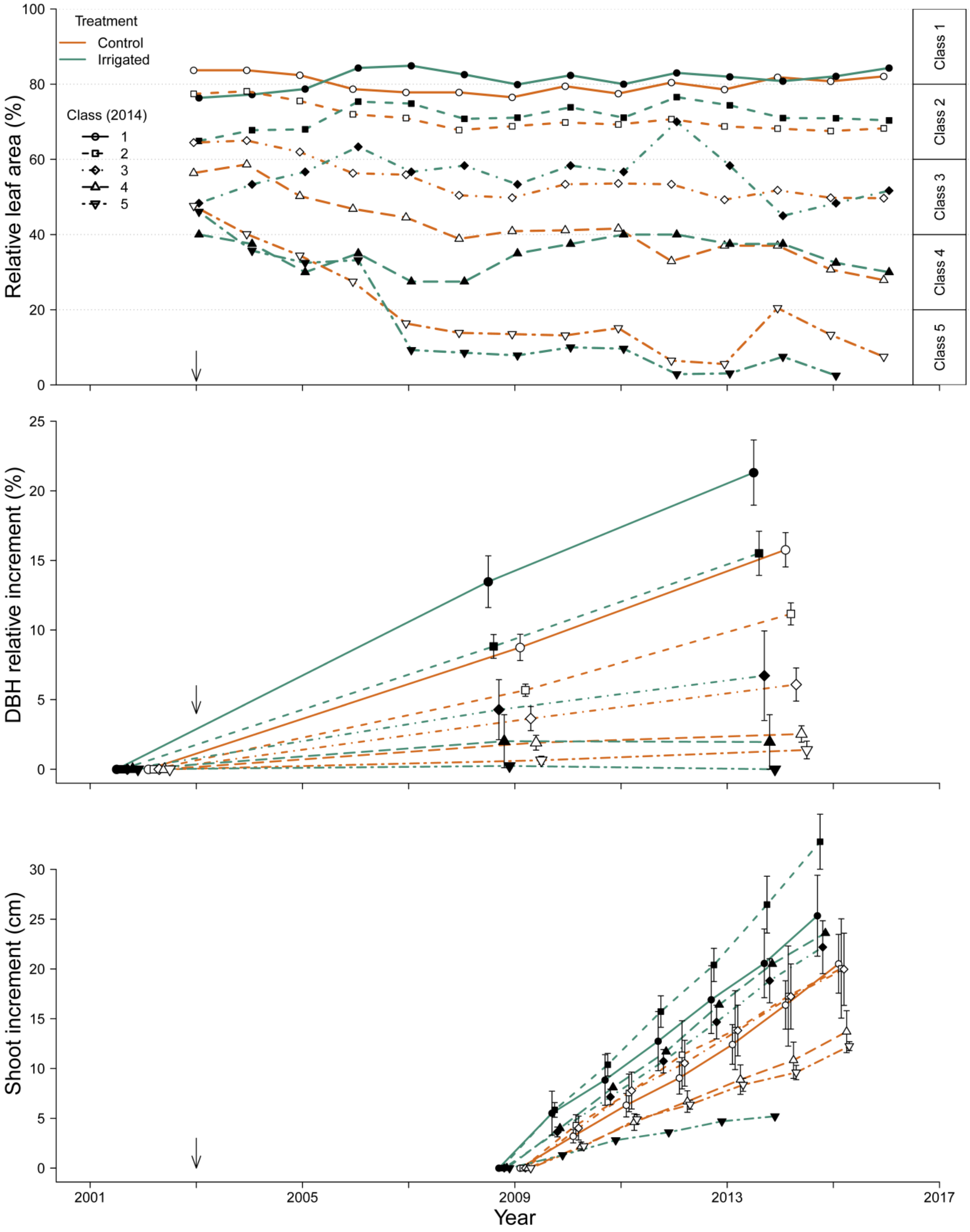

Figure 2.5: (top) Development of relative leaf area of *Pinus sylvestris* trees from 2003-2015 in control and irrigated plots, divided in 5 different leaf area classes; (middle) cumulative relative DBH increment of trees in each treatment:leaf area class group; (bottom) cumulative shoot growth of trees in each treatment:leaf area class group. Bars show SE of the mean. Arrows show the onset of irrigation in 2003.

Discussion

Homeostatic levels of NSC after 13 years of irrigation

We tested the hypothesis that 13 years of irrigation and thus drought release increases the trees' C storage pool in drought exposed *P. sylvestris*. In contrast to our hypothesis, NSC concentrations did not differ between control and irrigated trees in most cases. Instead, after 13 years of irrigation homeostatic NSC levels were found. Needles and root sugars showed even higher concentrations in control trees compared to irrigated trees, which is likely to be caused by osmotic adjustments (Brodribb & Cochard, 2009; Li *et al.*, 2013; Brunner *et al.*, 2015). We speculate that irrigated trees invested new C preferentially into growth but not in higher C reserve tissue concentrations; i.e. with increased growth the absolute NSC pool increased proportionally, which is in line with Von Arx et al. (2017) who studied in the same experiment the NSC-pools in relation to tree-ring growth and storing tissues in the sapwood. Due to the increased biomass production, the total NSC pools of irrigated trees were consequently larger than those from control trees. In contrast, seedlings of the same species prioritized the allocation of new assimilates to storage pools at the expense of growth shortly after drought has stopped, even though the drought did not cause any NSC depletion (Galiano Pérez *et al.*, 2017). This process might have taken place in our adult trees as well, in the first months or maybe years after the onset of irrigation, but acclimation to long-term irrigation eliminated the short-term need for higher storage input. This is in line with other experiments with Scots pine seedlings that were exposed to 2 subsequent summer droughts. They also had homeostatic NSC levels at the end of the 2nd summer (Bachofen *et al.*, 2017). Both our growth and leaf area data support the hypothesis that adult Scots pine acclimates to the new growing conditions reaching a new equilibrium: relative growth rates decreased in 2009-2015 relative to the previous period 2003-2009 in irrigated plots, and the increase in leaf area stabilized after approximately four years. The increase in soil water content due to irrigation (Dobbertin *et al.*, 2010) indicates a decrease in the soil-to-leaf water potential difference which allowed trees to increase their total leaf area to maintain high canopy scale water conductance, as Darcy's law predicts (McDowell & Allen, 2015). Trees grew towards a new hydraulic limit, with higher growth rates and a higher leaf area. As a result, soil water availability data for the study site showed that irrigated and control plots slowly converged in terms of soil relative water content over the 13-years duration of the experiment (M. Dawes, personal communication). We do acknowledge that high leaf area may also offer an increase in drought risk depending on the trees' growth stage, because evapotranspiration loss increases with increasing leaf area.

Defoliation was related to NSC and tree growth

While there was no direct effect of drought release on NSC concentrations, leaf area was clearly related to NSC. We found that irrespective of the treatment, trees with higher leaf area grew faster, had higher NSC concentrations in their roots, and in most seasons also in leaves and wood. Decreased leaf area may be a risk factor reducing tree growth under drought and is also an indicator for mortality (Dobbertin & Brang, 2001; Eilmann *et al.*, 2013). The negative influence of defoliation on growth has been reported from several other natural and experimental studies (Galiano *et al.*, 2011; Piper *et al.*, 2015; Puri *et al.*, 2015) and is supported with the growth efficiency theory by Waring (1983). Here, we show that decreasing leaf area not only negatively affects growth but also C storage. Other studies also found decreasing NSC levels during defoliation in evergreen and deciduous species (Ericsson *et al.*, 1980; Kolb *et al.*, 1992; Li *et al.*, 2002), but short-term experimental studies normally find a fast recovery after defoliation (Palacio *et al.*, 2012; Puri *et al.*, 2015). However, the trees in such experiments are not pre-exposed to any other stress factor like drought and are not naturally defoliated, like in our study.

Irrigation did not result in higher leaf nutrient concentrations

Leaf-level indices of gas exchange such as N and P, and leaf δ^{13}C were only weakly correlated with NSCs and growth (Fig. 2.4), suggesting that leaf-level gas exchange was only a weak driver of carbohydrates and growth, which is supported by Feichtinger et al. (2017), who found higher growth rates in response to irrigation, but a high plasticity in gas-exchange responses to short- and long-term changes in water availability in Scots pine and European Larch. Our results indicate that tree level leaf area rather than gas exchange per unit leaf area best explains the NSC patterns observed. Nutrient availability in the soil was almost unchanged by irrigation (Thimonier *et al.*, 2005, 2010). However, drought can impair soil nutrient uptake and transport to the crown (Rennenberg *et al.*, 2006), and irrigation could have thus led to an improved uptake and transport of nutrients (Feichtinger *et al.*, 2014; Gessler *et al.*, 2017). However, irrigation and leaf area had no impact on the N and P concentrations in needles. We might assume that irrigation may have in fact led to improved soil nutrient uptake, but this increase was balanced by the demand for higher growth compared to the drier control trees leading to constant tissue concentrations.

Leaf area, not irrigation, determines drought stress

A decrease of leaf δ^{13}C was observed due to irrigation. In addition, in irrigated trees, δ^{13}C increased with decreasing leaf area. The first observation indicated that control trees indeed were exposed to higher drought stress than irrigated trees, confirming the positive effect of additional water on gas exchange (Eilmann *et al.*, 2010). The second observation indicated that even irrigated trees showed signs of drought stress when strongly defoliated. Increasing δ13C values could be caused by various factors, including increased leaf area and increasing N concentrations. Higher leaf area would lead to lower stomatal conductance per leaf area if other factors were constant, leading to an increase of δ13C (Mencuccini & Comstock, 1999; Levanič et al., 2011). Most of foliar N is stored in Rubisco and therefore directly related to a tree's photosynthetic capacity, reducing the internal CO_2 concentration of the leaf and increasing δ^{13}C. But as shown above, neither leaf area, stomatal conductance nor N could have caused these increasing δ^{13}C levels, strengthening the conclusion that these irrigated trees with lower leaf area still suffered from drought stress.

Historical crown legacies determine tree recovery after drought

We find large within-population differences in growth and C dynamics that are related back to the beginning of the irrigation treatment. Irrigation stimulated growth rates, increased crown cover and reduced the number of trees with strong defoliation symptoms (Fig. S2.4). Within different leaf area classes, we showed that irrigation mostly positively affected trees with a leaf area higher than 40% (at the onset of the treatment), whereas trees with lower leaf area did not benefit from irrigation, both in terms of growth and crown improvements. The initial leaf area in the beginning of the experiment seems to be a good predictor of growth and C dynamics after 13 years. In a recent study, Timofeeva *et al.* (2017) studied living and recently died trees at the Pfynwald site and found that trees that died around the year 2013 with low leaf area exhibited reduced growth since the 1980's. Thus, the process of tree weakening in our study site is a long-lasting and slow process, rather than being triggered by a fast and singular event. Similar to our study, Galiano *et al.* (2011) investigated mature pines under drought stress which showed different stages of needle loss. In their study, several possible pathways were discussed that can relate NSC to crown foliage loss, and the most likely pathway would be that previous drought reduces the amount of green leaves, which in turn results in lower NSC pools. This pathway leads to a negative feedback (e.g. Manion, 1991), causing trees with a reduced needle area to experience decreasing growth and therefore limiting the recovery of trees, ultimately resulting in a 'point of no return' after which trees are unable to recover. This idea is corroborated by the finding of a positive correlation between winter NSC storage in fine roots and stem wood (at 5 m height), and shoot growth in the following season (Fig. S2.6). Overwinter-storage is thus crucial for next season growth and reduced NSC accumulation in one year negatively affects growth in the following (Li *et al.*, 2008, 2018b). In fact, we found that the trees that were most defoliated in 2015 (all trees with a relative leaf area of < 25% and some with a relative leaf area of 25% < 35%), died by 2017. Given the relationship between relative leaf area and NSC, our results also indicate that reduced NSC concentrations are related to, but not necessarily causing, tree death in Scots pine. This is in agreement with findings of Adams *et al*. (2017) who showed that C starvation is a common (but not universal) process associated with tree mortality.

Our results showed that after 13 years of irrigation, the treatment lead to higher growth rates and improved crown conditions until new hydraulic limits were reached, but homeostatic NSC concentrations on tree individual and stand level occurred. Reduced NSC was related to decreased leaf area, but not to gas exchange per unit leaf area suggesting that tree leaf area drives variation in whole-tree carbohydrate dynamics in this study. Historical crown legacies might drive the within-population variability in growth and survival; the process of tree decline is a slow and long-term process, and present crown cover data predicts tree growth and functioning in the future. The NSC constancy across treatments provides evidence that growth:storage ratios may be kept constant, either actively, passively or both (Li *et al.*, 2018b), when climate changes are slow enough to allow acclimation.

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Supporting information

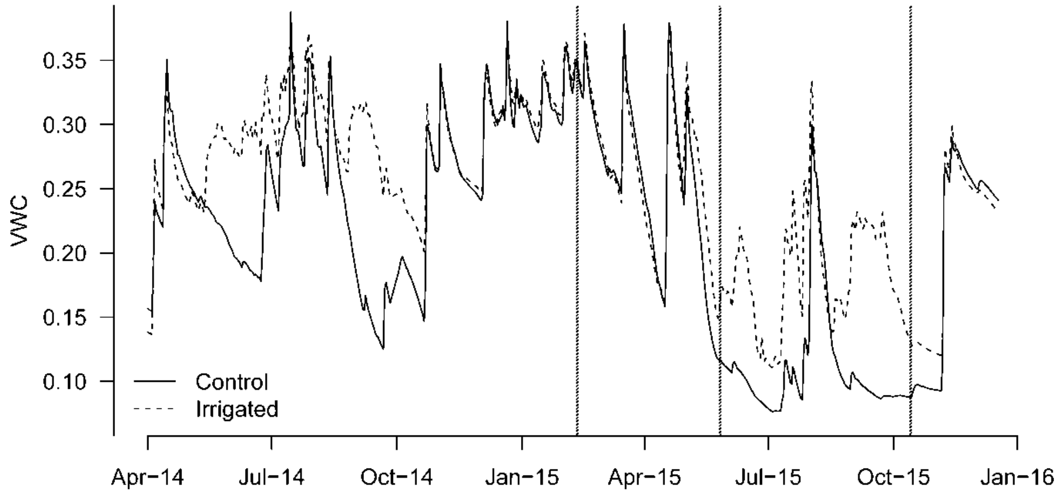

Figure S2.1: Volumetric water content of the soil (VWC) in the year 2014 and 2015 in control (solid lines) and irrigated plots (dashed lines) of the Pfynwald experiment. Vertical lines indicate the 3 sampling campaigns in 2015.

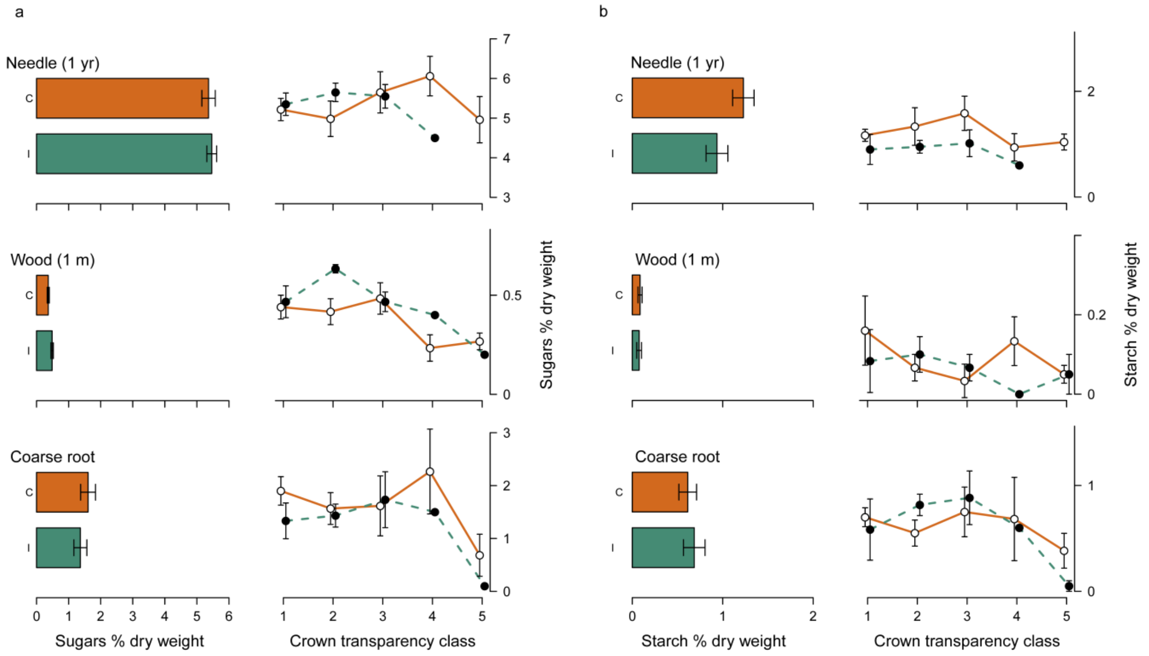

Figure S2.2: Winter levels of sugars (a) and starch (b) in 1 yr needles, stem wood at 1 m height and coarse roots of *Pinus sylvestris* trees. Left panels show differences between control and irrigated plots. Right panels show the starch and sugar levels against leaf area classes for control (orange) and irrigated (green) plots. Bars indicate SE of the mean.

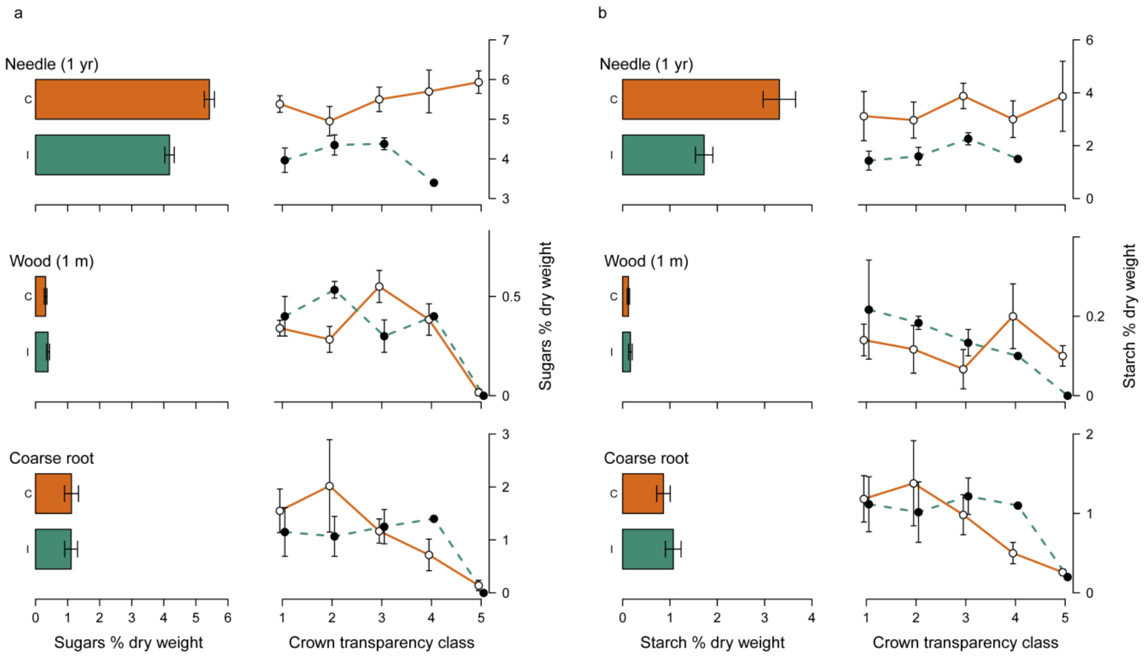

Figure S2.3: Autumn levels of sugars (a) and starch (b) in 1 yr needles, stem wood at 1 m height and coarse roots of *Pinus sylvestris* trees. Left panels show differences between control and irrigated plots. Right panels show the starch and sugar levels against leaf area classes for control (orange) and irrigated (green) plots. Bars indicate SE of the mean.

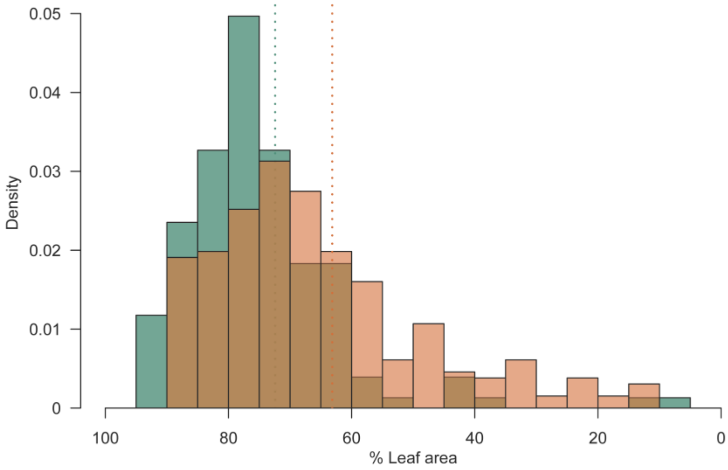

Figure S2.4: Distribution of *Pinus sylvestris* trees with different relative leaf area in irrigated (green) and control (orange) plots in 2014, in steps of 5%. Vertical dotted lines show the mean relative leaf area in irrigated and control plots.

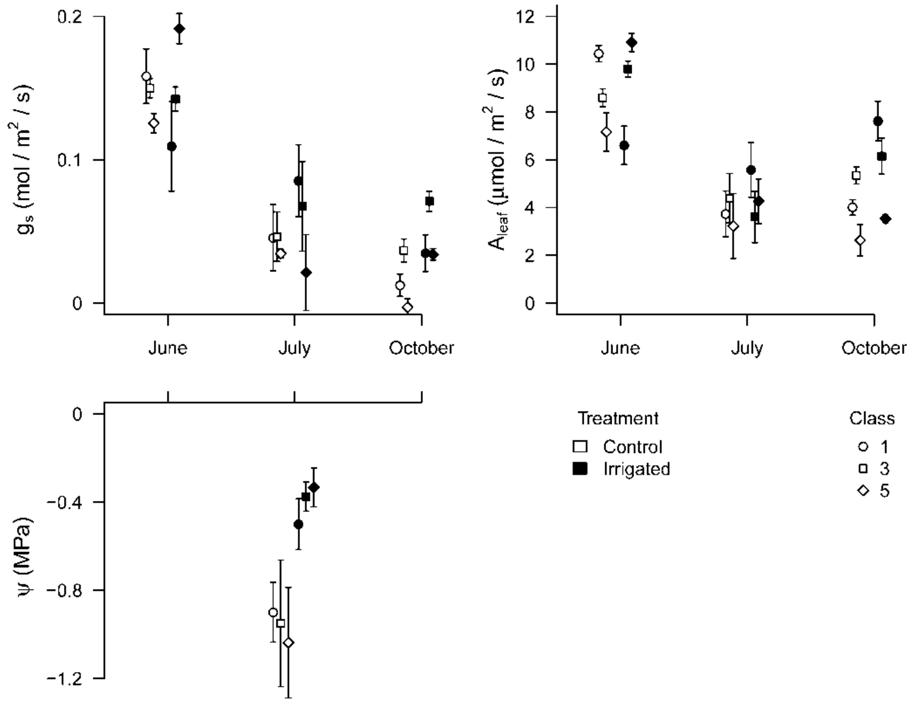

Figure S2.5: Stomatal conductance (g_s) and photosynthesis (A_{leaf}) in June, July and October 2016 (upper graphs), and leaf water potential in July 2016 (lower graph). Open symbols indicate control, and closed symbols irrigated trees. Three leaf area classes were measured (1 = 100%-60%, 3 = 60-40%, 5 = 40-0%); Bars show SE of the mean.

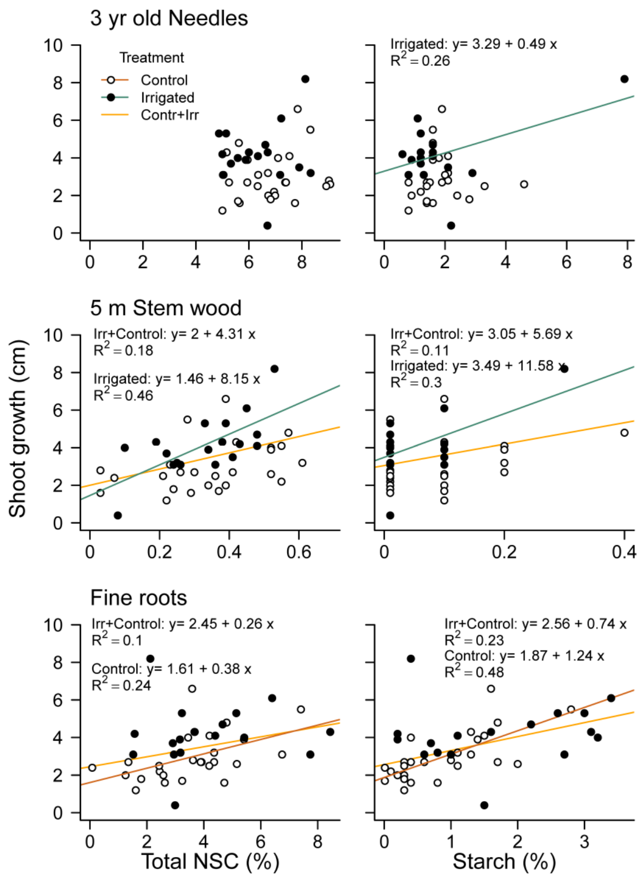

Figure S2.6: Shoot growth (y-axis) correlated to winter total NSC (sugars + starch) and starch levels (x-axis) in fine roots, 5 m stem wood and 3 year old needles. Sugars alone are not shown, as no significant correlations were found. Significant correlations are indicated by regression lines for control (brown), irrigation (blue) and average of control and irrigated plots (yellow).

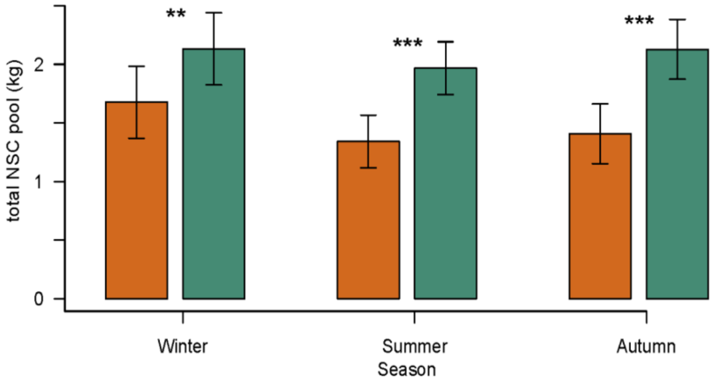

Figure S2.7: Total NSC pools in control (orange) and irrigated (green) trees in kg. Asterisks show significant differences between control and irrigated trees (**, $p < 0.01$, ***, $p < 0.001$). Bars show SE of the mean.

Table S2.1: Results of the correlation analysis between DBH in 2002 and 2014, and NSC, Sugars and Starch. (r = correlation coefficient).

	Total NSC		**Sugars**		**Starch**	
	r	***p***	***r***	***p***	***r***	***p***
DBH-02	-0.012	0.660	-0.008	0.780	-0.017	0.540
DBH-14	0.015	0.600	0.012	0.660	0.011	0.680

Table S2.2: Parameters derived from Forrester et al (2017) for calculation of foliage, branch, stem and root biomass of P. sylvestris, for the equation $\ln(Y) = \ln(\beta 0) + \beta 1 * \ln(d)$ where d is diameter at breast height.

Tissue type	**ln(β0)**	**β for ln(d)**
Foliage	-3.5276	1.7471
Branch	-3.8377	2.1775
Stem	-2.3583	2.308
Root	-3.6347	2.3038

Table S2.3: ANOVA results of differences in starch, sugars and total NSC in needles (3 generations), stem (3 heights) and roots (coarse and fine) between seasons, water treatments, leaf area classes and tissues (generation, height or type). Significant effects are shown in bold. Transformations used to reach normality of residuals are indicated in italics.

		Starch		**Sugars**		**Total NSC**	
	df	***F***	***p***	***F***	***p***	***F***	***p***
Needles		*Log(x+1)*		*Log(x+1)*		*Log(x+1)*	
Season (S)	2	100.7	**< 0.001**	288.3	**< 0.001**	4.3	**0.014**
Treatment (Tr)	1	1.7	0.198	13.0	**0.001**	6.2	**0.014**
Class (Cl)	4	2.3	0.076	0.7	0.570	2.1	0.098
Tissue (Ti)	2	0.1	0.919	12.5	**< 0.001**	2.7	0.066
S x Tr	2	21.8	**< 0.001**	14.0	**< 0.001**	25.7	**< 0.001**
S x Cl	8	3.9	**< 0.001**	2.8	**0.005**	3.0	**0.003**
S x Ti	4	14.9	**< 0.001**	10.1	**< 0.001**	16.8	**< 0.001**
Tr x Cl	4	0.7	0.584	2.6	**0.047**	2.6	0.051
Tr x Ti	2	1.2	0.293	0.9	0.428	1.2	0.298
Cl x Ti	8	0.4	0.910	0.5	0.827	0.6	0.777
Wood		*Sqrt(log(x+1)*		*Log(x+1)*		*Log(x+1)*	
Season (S)	2	20.1	**< 0.001**	37.5	**< 0.001**	1.6	0.204
Treatment (Tr)	1	0.4	0.523	0.0	0.873	0.0	0.936
Class (Cl)	4	1.2	0.316	10.3	**< 0.001**	8.2	**< 0.001**
Tissue (Ti)	2	3.6	**0.029**	40.2	**< 0.001**	39.0	**< 0.001**
S x Tr	2	1.2	0.313	0.9	0.395	0.8	0.430
S x Cl	8	2.0	**0.045**	2.1	**0.033**	2.2	**0.024**
S x Ti	4	2.5	**0.042**	1.1	0.355	0.6	0.653
Tr x Cl	4	0.5	0.706	3.4	**0.017**	2.7	**0.042**
Tr x Ti	2	0.3	0.741	0.2	0.803	0.6	0.557
Cl x Ti	8	1.3	0.254	3.2	**0.002**	2.0	**0.046**
Roots		*Sqrt(log(x+1))*		*Log(x+1)*		*Log(x+1)*	
Season (S)	2	4.4	**0.014**	3.2	**0.041**	0.1	0.937
Treatment (Tr)	1	0.1	0.823	5.8	**0.020**	2.4	0.100
Class (Cl)	4	17.1	**< 0.001**	10.9	**< 0.001**	15.	**< 0.001**
Tissue (Ti)	1	50.0	**< 0.001**	59.9	**< 0.001**	67.9	**< 0.001**
S x Tr	2	0.7	0.489	0.3	0.715	0.4	0.683
S x Cl	8	0.7	0.662	0.5	0.870	0.4	0.916
S x Ti	2	5.3	**0.006**	0.7	0.479	2.1	0.130
Tr x Cl	4	3.1	**0.026**	1.2	0.308	2.0	0.117
Tr x Ti	1	0.0	0.964	0.4	0.505	0.3	0.556
Cl x Ti	4	0.3	0.901	0.2	0.961	0.2	0.909

Table S2.4: ANOVA results of differences in summer δ13C, nitrogen and phosphorus levels in needles, between water treatment, leaf area class and tissue (generation). Significant effects are shown in bold. Transformations used to reach normality of residuals are indicated between brackets.

Factor		**δ^{13}C (log(abs(x)))**		**N (log)**		**P (log)**	
	df	***F***	***p***	***F***	***p***	***F***	***p***
Treatment (Tr)	1	17.7	**< 0.001**	3.1	0.084	0.1	0.777
Class (Cl)	4	8.0	**0.007**	0.0	0.875	0.0	0.974
Tissue (Ti)	2	0.7	0.497	12.7	**< 0.001**	7.0	**0.002**
Tr x Cl	4	6.2	**0.017**	2.9	0.098	0.1	0.811
Tr x Ti	2	2.1	0.126	5.4	**0.006**	1.3	0.277
Cl x Ti	8	0.9	0.412	0.3	0.723	0.0	0.976

Table S2.5a: ANOVA results of differences in relative leaf area, shoot growth, absolute DBH and relative increment DBH between treatments, years and leaf area classes. Significant effects are shown in bold.

Factor		**Relative LA**		**Shoot growth**		**DBH**		**Relative increment DBH**	
	df	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
Treatment (Tr)	1	14.1	**<0.001**	0.5	0.490	4.6	**0.033**	0.3	0.556
Year (Y)	1	234.5	**<0.001**	9.7	**0.002**	317.4	**<0.001**	272.9	**<0.001**
Class (Cl)	4	72.4	**<0.001**	1.0	0.395	2.8	**0.040**	73.8	**<0.001**
Y x Tr	1	34.6	**<0.001**	0.0	0.866	71.9	**<0.001**	1.0	0.376
Tr x Cl	4	2.7	**0.029**	0.3	0.895	2.2	0.084	1.4	0.217
Y x Cl	4	172.4	**<0.001**	2.5	**0.043**	27.9	**<0.001**	55.8	**<0.001**
Tr x Y x Cl	4	8.1	**<0.001**	1.2	0.328	5.4	**0.001**	1.2	0.311

Table S2.5b: Contrasts for relative DBH increment, calculated using least square mean differences Control – Irrigated, for the period 2002 – 2009 and 2009 - 2014. Significant effects are shown in bold.

Class	**1**		**2**		**3**		**4**		**5**	
Year	***Diff.***	***p***	***Diff.***	***p***	***Diff.***	***p***	***Diff.***	***p***	***Diff.***	***p***
2009	-0.31	**0.004**	-0.30	**<0.001**	-0.18	0.599	-0.06	0.898	0.12	0.552
2014	-0.18	0.122	-0.27	**<0.001**	-0.14	0.682	0.23	0.654	0.53	0.100

Table S2.6: ANOVA results of differences in predawn leaf water potential (LWP), photosynthesis (A_{max}) and stomatal conductance (g_s) between water treatments, leaf area classes and dates. Significant effects are shown in bold.

Factor		**LWP**		**A_{max}**		**g_s**	
	Df	***F***	***p***	***F***	***p***	***F***	***p***
Treatment (Tr)	1	15.30	**< 0.001**	1.5	0.230	0.3	0.610
Class (Cl)	2	0.007	0.932	0.8	0.470	0.1	0.870
Date (D)	2	--	--	69.5	**< 0.001**	88.8	**< 0.001**
Tr x Cl	2	0.8	0.378	0.5	0.640	0.2	0.850
Tr x D	2	--	--	1.9	0.150	0.8	0.460
Cl x D	2	--	--	2.9	**0.030**	2.7	**0.040**
Tr x Cl x D	2	--	--	9.1	**< 0.001**	3.3	**0.010**

Table S2.7: Results of the students t-test comparing total NSC pools (in kg) in irrigated and control trees, in winter, summer and autumn. Mean NSC pool for control and irrigated are given, as well as the p value. Significant effects are shown in bold.

Season	**Mean control**	**Mean irrigated**	**p value**
Winter	1.71	2.18	**0.02**
Summer	1.27	1.91	**< 0.001**
Autumn	1.48	2.17	**< 0.001**

3

Soil nutrients and lowered source:sink ratio mitigate effects of mild but not of extreme drought in trees

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Summary

- Little is known about factors that mitigate or intensify negative drought effects on tree functioning, like nutrient availability. We hypothesized that higher nutrient availability and subtle manipulations of source-sink relationships can partially compensate negative drought effects when rought is not too severe, whilst too extreme drought can inhibit carbon and nutrient uptake and allocation irrespective of nutrient availability or the plant source:sink balance.
- We exposed three year-old *Pinus sylvestris* saplings during two subsequent years to drought using four different water supply regimes (from no drought to extreme drought) and released drought thereafter. Trees were exposed to two soil nutrient regimes. In addition, partial and full needle removal was performed. We assessed biomass, leaf gas exchange and tissue non-structural carbohydrates (NSCs).
- Extreme drought reduced stomatal conductance, photosynthesis, biomass and NSC, whereas intermediate drought levels only slightly affected biomass and NSC. Defoliation stimulated photosynthesis and fertilization increased growth and root biomass fraction, but mainly in the two intermediate drought levels. Only extreme drought pushed *P. sylvestris* trees to mortality.
- We conclude that tree mortality under severe drought periods will not be mitigated, but that the effects of low intensity drought stress could be compensated by increased nutrient availability and decreased source:sink ratio.

Keywords: biomass, drought, leaf gas exchange, NSC, nutrients, open-top chambers, *Pinus sylvestris,* source:sink

Introduction

The duration and intensity of summer droughts are predicted to increase and climate projections assume a higher frequency of droughts as well as a higher probability of intermittent high intensity rainfall events (IPCC, 2013). For plants, both the ability to withstand drought periods of different duration and intensity, as well as to immediately capitalize on soil rewetting from subsequent rainfall events will be crucial for their survival and competitiveness (Dietrich and Kahmen, 2019; Hommel et al., 2016; Volkmann et al., 2016).

Depending on source (delivery of new assimilates via photosynthesis) and sink (use of assimilates in heterotrophic tissues) activity during drought, the storage pool of non-structural carbohydrates (NSC) within a tree can increase and decrease during drought events (McDowell, 2011), and C allocation can be prioritized to particular C pools or tissues (Hartmann et al., 2015; Huang et al., 2018). C allocation might be affected differently depending on drought intensity, and intensity thresholds may occur, which when exceeded cause an impairment of tree functioning and finally cause mortality (Choat et al., 2018; Sala et al., 2010). While mild drought has been shown to increase the transport of new assimilates to the roots and thus allow the production of larger water absorbing surfaces, more intensive drought events seem to reduce and delay the C supply to roots (Hommel et al., 2016), and deplete C storage pools (Hartmann et al., 2013; Li et al., 2018), leading to C restriction and thus loss of root functioning. Moreover, trees are able to prioritize C allocation, e.g., towards defense and storage pools at the expense of growth and respiration when C supply gets limited (Huang et al., 2018). In addition, recovery after drought has been shown to trigger belowground C allocation, indicating a strong sink driven C allocation strategy that allows fast regain of root functioning (Hagedorn et al., 2016). But also preferential allocation of new assimilates to storage pools at the expense of growth has been observed during recovery, suggesting the presence of ‘drought memory effects’, possibly to ensure future growth and survival (Galiano Pérez et al., 2017; Trugman et al., 2018). Such ambiguous findings underline the necessity for more research on C allocation and prioritization for growth and storage in changing conditions.

Source:sink relationships might change during drought stress. Apart from the uncoupling of growth from photosynthesis (Muller et al., 2011), trees adjust to hydraulic stress by reducing their transpiring area (Sánchez-salguero et al., 2017). This reduces water loss, but might limit growth by C limitation. Conversely, stresses like drought and insect outbreaks are very likely to occur concomitantly in the future (Allen et al., 2010) causing sudden extreme defoliation events that might limit trees in the recovery from drought or even push them to a point of no return (Schönbeck et al., 2018). The importance thus rises to disentangle the effects of combined stresses on C source:sink relations and tree survival.

Whilst direct effects of drought on C allocation have been studied more rigorously, little is known on how these effects could be intensified or mitigated by nutrient availability in the soil and plant (Gessler et al., 2016; Kreuzwieser and Gessler, 2010). Before the direct impact of a drought event, nutrients might on the one hand have a negative predisposing effect on tree architecture: high and long-term nutrient availability can decrease the root biomass fraction, increase the aboveground biomass and reduce the sapwood:leaf area ratio, ultimately leading to a disadvantage when a tree is subjected to drought (Gessler et al., 2016). On the other hand, large nutrient reserves acquired before a drought event might compensate for lower uptake during drought thus promoting a plant's ability to survive during or to recover after a drought (Gessler et al., 2016; Waring, 1987). Higher soil nutrient availability during drought might compensate for impaired active nutrient transport and lower ion mobility in the soil, allowing the plants still to achieve sufficient nutrient supply (Gessler et al., 2016). Nitrogen (N) is a major growth limiting nutrient and thus drought effects on the plant N uptake and transport of this element is of central importance (Kreuzwieser and Gessler, 2010). Since drought affects the N partitioning between roots and shoots (Fotelli et al., 2002; Grossiord et al., 2018) an increased N availability might allow for a sustainable N supply of all plant tissues. N uptake requires large amounts of photosynthetic energy but is in turn necessary to convert light into chemical energy. It is therefore strongly connected to the performance of photosynthesis (Kreuzwieser and Gessler, 2010; Lloyd, 1999; Rennenberg et al., 2006) and N deficiency has been shown to increase the sensitivity of the stomata to negative leaf water potentials (Ghashghaie and Saugier, 1989; Radin and Ackerson, 1981), which in turn could increase the risk of C starvation (McDowell, 2011).

This study was designed to test if higher nutrient availability and manipulations of source:sink relationships can partially compensate negative drought effects on gas exchange, biomass accumulation and C allocation in *Pinus sylvestris* saplings, and to study the recovery potential after release of drought. In our experiment we exposed three year-old *P. sylvestris* saplings during two subsequent years to drought using four different water regimes (field capacity, no water, two intermediate levels) and two soil nutrient regimes, and released drought thereafter. In addition, partial and full needle removal was performed before budbreak adjust the source:sink ratio and simulate extreme defoliation due to insect attacks. We hypothesized that 1) the relationship between drought and C allocation is a threshold response, where trees can adjust to and survive mild drought, but will succumb to mortality under severe drought; 2) fertilization and thus increased N availability will mitigate the negative effects of intermediate drought but under severe drought nutrient uptake and transport will be inhibited independent of nutrient availability and thus no mitigation effect is suspected; 3) decreased source:sink ratio due to mild defoliation will lead to lower susceptibility in response to drought, whilst severe defoliation will not compensate drought effects; 4) recovery after drought strongly depends on the available C and nutrient reserves that will be affected by drought intensity subjected to before. Specifically, we expected to see increased C allocation to roots at intermediate drought levels. However, at extreme drought we expected to see ceasing of photosynthesis, growth and C allocation belowground for root development at extreme drought (cf. H1); higher stomatal conductance, photosynthesis, root water uptake and C allocation belowground in drought stressed fertilized trees compared to unfertilized trees with the exception of the extreme drought treatment (cf. H2); compensatory higher photosynthesis per leaf area after mild defoliation, even in drought stressed trees, but high nutrient losses, lower growth and

NSC levels due to insufficient C supply after extreme defoliation (cf. H3); quick recovery of photosynthesis and NSC in surviving trees, but mortality in trees that were too heavily defoliated or passed the minimum NSC threshold, and a high investment in root restoration after rewetting.

Materials and methods

Study site

This study was conducted in the model ecosystem facility of the Swiss Federal Research Institute WSL (47°21’48’’ N, 8°27’23’’ E, 545 m a.s.l.), Birmensdorf, Switzerland, which consists of 16 hexagonal open-top chambers (OTCs) of 3 m height and a plantable area of 1.5 m^2 each (Fig. 3.1). Mobile roofs can automatically control incoming rainfall, but were kept closed during the entire experimental period. Belowground, the chambers are divided into two semicircular lysimeters (1.5 m deep) with concrete walls. The lysimeters were filled with a 1 m deep layer of gravel for fast drainage, covered with a fleece layer that is impermeable for roots but permeable for water, and on top a 40 cm layer of calcareous sandy loam soil (Kuster et al., 2013). Every lysimeter was planted with 12 three years-old saplings of *Pinus sylvestris* (55.61 cm +/- 5.41 cm height) in April 2015. Temperature and air humidity inside and outside the OTC, as well as soil moisture and soil temperature inside (5, 20, 35 cm deep) were automatically monitored (5TM soil moisture and temperature logger, Metergroup, Munich, Germany). Six sprinklers (1 m high) per lysimeter were evenly distributed, and irrigation was programmed for every lysimeter separately.

Treatments

The experiment was set up as a split-split plot design. Every chamber was assigned one of four different water regimes as whole-plot treatment (four chambers / replicates per regime, in a Latin Square design) (Fig. 3.1).

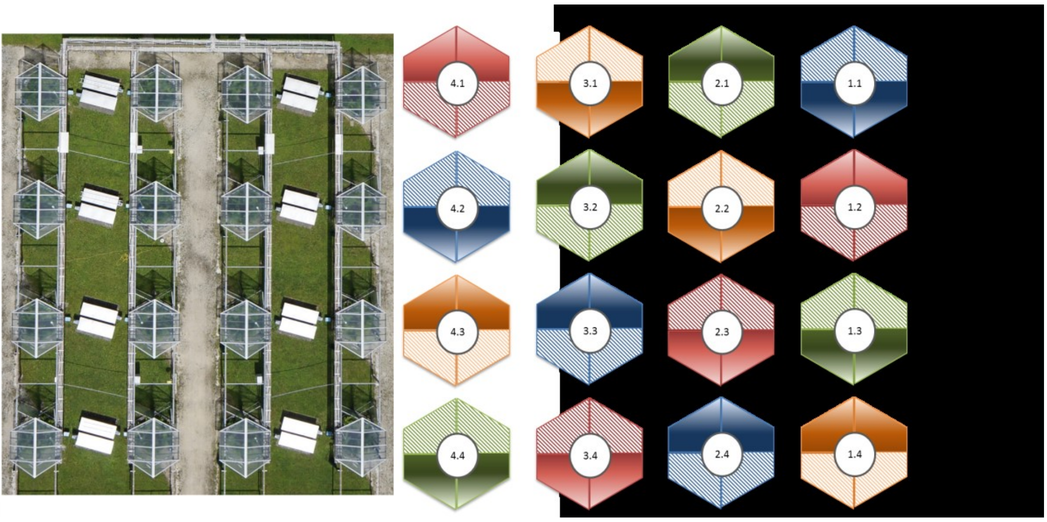

Figure 3.1: Left: Aerial picture of the open-top chambers. Right: Experimental setup. Every chamber was assigned one of four different water regimes. Blue = W100 (close to field capacity), Green = W50 (50% of field capacity), Orange = W20 (20% of field capacity), Red = W0 (close to wilting point). North and south lysimeters were assigned an unfertilized (filled) or fertilization (dashed) treatment. Numbers indicate column and row numbers of the chambers.

The amount of water to be applied was controlled by means of automated soil moisture measurements. Field capacity (FC) and wilting point (WP) were determined by pF curves (Supplementary material Fig. S3.1), and volumetric water content (VWC) for the irrigation regimes was adjusted accordingly, allowing for the following four different levels of VWC: close to FC (approximately 25% VWC), WP (approx. 6%; achieved by no irrigation at all) and two intermediate levels, corresponding to approximately 50% and 20% of FC. The regimes will be referred to as W100 (FC), W50, W20 and W0 (WP) from now onwards. Water treatments started a year after planting. The irrigation system was in function from April to October in 2016 and from April to mid-July 2017, to prevent frost damage in winter. In winter and early spring, watering was done by hand (in W100, W50 and W20) to maintain stable soil water levels. From the 13th of July 2017 until the last harvest in the beginning of November, all chambers were (re)watered to field capacity in order to study the recovery process in the trees.

Twice a year, in April and July one of the two lysimeters (split-plot) in the OTC's were fertilized with liquid fertilizer (Wuxal, Universaldünger, NPK 4:4:3), corresponding to 50 kg N / ha / year. In April 2016 and April and July 2017, the fertilizer was applied using 3 L water per lysimeter, and the ambient treatment was given 3 L water without nutrients. The applied moisture was equal to 2 mm precipitation. In July 2016, fertilizer was applied using only 1 L water per lysimeter, and injected with a needle with four lateral holes in the soil, at three different depths (5, 15 and 25 cm), evenly distributed over the planted area (20 cm grid) according to Jesch *et al.* (2018). This procedure was applied in order not to strongly change the water supply of the drought treatments but still to distribute the nutrients evenly across the soil volume. In July 2017, the fertilizer coincided with the rewetting of all lysimeters, allowing more water to be applied for fertilization.

In each lysimeter (i.e. across all water and nutrient treatments) individual trees (split-split plot) were randomly assigned to a no defoliation, 1/3, 2/3 or 3/3 needle removal treatment (in grams fresh biomass, n = 3). The amount of needles removed for the 1/3 and 2/3 treatment was determined by weighing the biomass of the needles of trees that were treated with 3/3 removal. Defoliation was done in March 2016 and 2017, before bud break and fertilization, and was evenly distributed over all needle generations.

Measurements

Net-photosynthesis (A_{leaf}) was measured at the beginning (May / June), middle (July) and end (October) of the growing seasons in 2016 and 2017. In 2017, photosynthesis was also measured 1, 2, 4 and 8 weeks after onset of rewetting. The last measurement was on 13th of September 2017. Measurements were done using a LiCor 6400 system (LI-COR, Lincoln, USA). Approximately 20 needles were enclosed in the 2 x 3 cm broadleaf chamber and A_{leaf} was measured with 400 μmol mol^{-1} CO_2, 1200 μmol m^{2} s^{-1} photosynthetically active radiation, 60% relative humidity and 22 °C air temperature. The enclosed needles were later scanned with a flatbed scanner to determine leaf area. On the same days, predawn leaf water potential (ψ_{pred}) was measured on current-year twigs between 03:30 and 05:30 h, using a Scholander bomb (Model 600 pressure bomb; PMS Instrument Company, Albany, NY, USA) in steps of 0.05 MPa. Only the chambers with W100, W20 (October 2016 -

October 2017) and W0 treatments and 3 out of 4 chambers per treatment were measured due to time restrictions.

Full tree harvests took place in October 2016, July 2017 and, 3 months after rewetting, i.e, in November 2017. Four trees per lysimeter (1 tree for every defoliation treatment, with chamber as replicate, n=4) were taken out including the roots. In addition, needle and root samples were taken in February 2017, and more needle samples were taken in June 2016, and 1, 2, 4 and 8 weeks after onset of rewetting, always from four randomly selected trees per lysimeter, one per defoliation treatment. In July 2017, after a high mortality in the W0 treatment, no full harvest was done in W0, to keep the surviving trees for the last harvest in November, but needle samples were taken from those trees.

After the three full harvests, tree diameter and height, fresh and dry weight (after drying at 60 °C until stable weight) of root, stem, shoot and needle (2016 and 2017 generations separately) biomass were measured. In July 2017, W0 biomass measurements consisted only of the trees that had died between February and July 2017. Root, stem and needle tissues (approximately 100 mg of the dry tissue) were ground to fine powder and NSC's were analyzed following the protocol of Wong (Wong, 1990) adapted according to Hoch et al. (Hoch et al., 2002). NSCs are defined here as low molecular weight sugars (glucose, fructose and sucrose) plus starch. In short, 10-12 mg of ground material was boiled in 2 ml distilled water for 30 minutes. After centrifugation, an aliquot of 200 µl was treated with Invertase and Isomerase from baker's yeast (Sigma-Aldrich, St. Louis, MO, USA) to degrade sucrose and convert fructose into glucose. The total amount of glucose (sugars) was determined photometrically at 340 nm in a 96-well microplate photometer (HR 7000, Hamilton, Reno, NE, USA) after enzymatic conversion to gluconate-6- phosphate (hexokinase reaction, hexokinase from Sigma Diagnostics, St. Louis, MO, USA). Total amount of NSC was measured by taking 500 µl of the extract (including sugars and starch) incubated with a fungal amyloglucosidase from Aspergillus niger (Sigma-Aldrich, St. Louis, MO, USA) for 15 h at 49°C to digest starch into glucose. Total glucose (corresponding to NSC) was determined photometrically as described above. The concentration of starch was calculated as NSC minus free sugars. Pure starch and glucose-, fructose- and sucrose- solutions were used as standards and standard plant powder (Orchard leaves, Leco, St. Joseph, MI, USA) was included to control reproducibility of the extraction. NSC concentrations are expressed on a percent dry matter basis. Because all samples were run in a single laboratory with no change in protocol during the laboratory processing of samples, issues with comparison of results across methods or labs were obviated (Quentin et al., 2015).

Two milligrams (±0.1 mg) of ground tissue material were weighed into tin cups and converted to N_2 in an elemental analyzer *Euro EA* (Hekatech GmbH, Wegberg, Germany) to determine N contents as a percentage relative to dry weight.

Soil

Soil samples were taken in October 2016, after the first harvest. Soil was ground to powder, weighed in tin capsules and total N concentration was measured using IRMS-EA

(IRMS Delta V Advantage, Thermo Scientific). In addition, 7.5 g dry soil was extracted with 30 ml 1M KCl and filtered through filer paper (Albet-Hahnemuehle, Dassel, Germany) into 50 mL PE bottles. NH_4 concentration in the extract was measured photometrically with flow-injection (FIAS-400) and UV/VIS spectrometer (Lambda 2s, Perkin-Elmer, Schwerzenbach, Switzerland), NO_3 was measured by colorimetric analysis (Cary-UV50 spectrophotometer), using the absorption of nitrate at a wavelength of 210 nm. Soluble P was extracted using the method of Hedley (1982), modified by Tiessen and Moir (Tiessen and Moir, 2006).

Statistical analyses

Dry weight, A_{leaf}, ψ_{pred}, sugars, and starch concentrations were all analyzed using linear mixed effect models employing maximum likelihood (*lmer* function, LME4 package, R v.3.2.2, R Core Team, Vienna, Austria), and *P* values were calculated based on Satterthwaite's approximations. Fixed factors were water (four levels), nutrients (two levels) and defoliation (four levels) and their two-way interactions. Three-way interactions were left out because the main focus was on the two-way interactions drought-nutrients and drought-defoliation. The four chambers per water treatment function as replicates in this split-plot analysis, resulting in n = 4. Chamber and lysimeter were included as random factors to control for the split-plot design of the experiment. Row and column number (see Fig. 3.1) of the chambers were included in the complete model to check for possible spatial patterns, but were never significantly different and afterwards omitted from of the model. Where necessary, log- and sqrt-transformations were carried out to obtain the normality of residuals. Post-hoc tests for pairwise comparisons were carried out with the general linear hypothesis function (glht) in the package "multcomp" (Hothorn et al., 2019). All statistical calculations were performed with R v.3.2.2 (R Core Team, 2015).

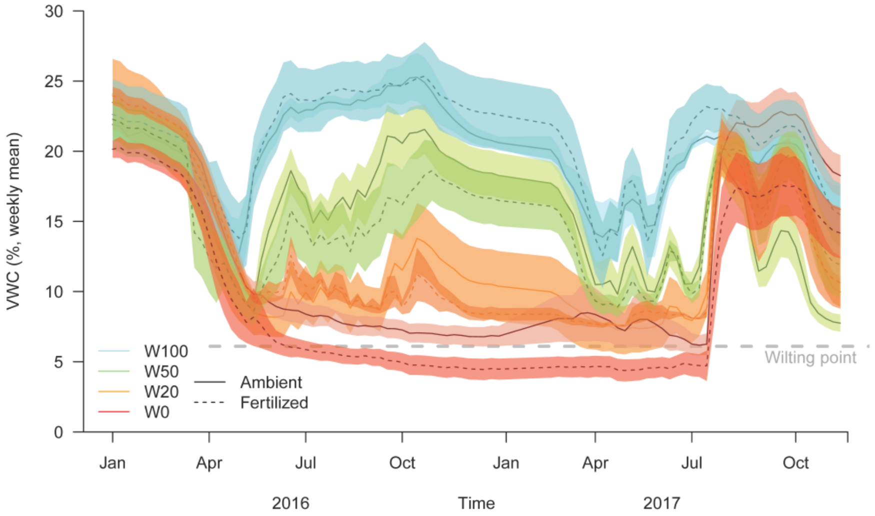

Figure 3.2: Volumetric soil water content in the four different water (colors) and two different nutrient (solid = ambient, dashed = fertilized) treatments. Lines show the average of four chambers and weekly means, shaded bands show the SE over the four chambers. The horizontal dashed line shows soil wilting point as determined by means of pF curves. The steep increase in July clearly indicates the rewetting period.

Results

Soil

Volumetric soil water content (VWC) decreased steeply in early spring 2016 and the four different water regimes reached the anticipated level by end of June. Maximum (W100) mean VWC was ~ 24% and minimum (W0) ~ 6% (Fig. 3.2, Table S3.1). During the growing seasons, the W50 (~ 50% of field capacity) and W20 (~ 20% of field capacity) levels contained ~ 14% and ~ 9% VWC (Fig. 3.2, Table S3.1). VWC did not differ between fertilized and ambient plots, except for the W0 regime, where VWC of fertilized plots was on average 3% lower than on ambient plots (paired t.test, W100: $p = 0.060$; W50: $p = 261$; W20: $p = 0.765$, W0: $p < 0.001$). After rewetting in July 2017, VWC was brought to approximately 20% in all treatments.

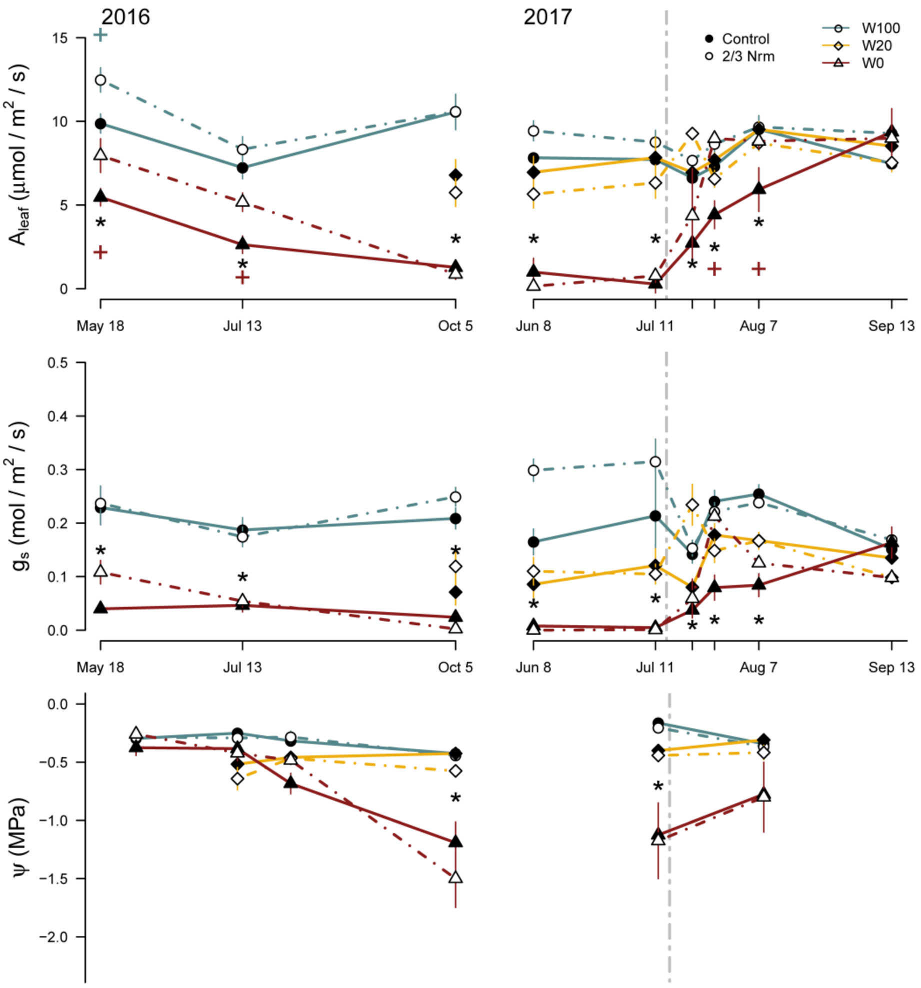

Figure 3.3: Net-photosynthesis (A_{leaf}), stomatal conductance (g_s) and predawn water potential (ψ) throughout the growing seasons of 2016 and 2017. Colors indicate water treatment, solid lines show undefoliated control trees, dashed lines show trees treated with 2/3 needle removal. Grey vertical dashed line indicate the moment of rewetting in 2017. Only W100, W20 and W0 are shown, as W50 was very similar to the W100 treatment. Only undefoliated trees and trees with 2/3 needle removal were measured. Nutrients were not significantly influencing gas exchange and thus the data was pooled over both nutrient levels.

Mortality

Only extreme drought (W0) caused tree mortality. Approximately 60% of the trees in W0 died, 90% of which died between February 2017 and October 2017, i.e. in the second growing season (Fig. S3.2) and 10% died between October 2016 and February 2017. Fertilized plots had a slightly higher mortality than unfertilized, but this difference was not significant (results not shown). Defoliation did not change mortality rates.

Gas exchange

Net-photosynthetic rates (A_{leaf}), stomatal conductance (g_s) and predawn water potential (ψ_{pred}) decreased significantly during the first four months in the W0 chambers (Fig. 3.3, Table S3.2). W50 and W20 trees did not significantly reduce gas exchange parameters, but W20 trees showed significantly lower ψ_{pred} compared to W100 trees in midsummer. Fertilization did not have a significant effect on gas exchange or water potential parameters (Table S3.2). Defoliation resulted in an increase of A_{leaf} and g_s, but did not affect ψ_{pred}. The defoliation effect disappeared in W0 treatments from October 2016 onwards and became significant again at the time of rewetting (13th of July 2017 onwards). Rewetting from 13th July, 2017 resulted in a sharp increase of A_{leaf} and g_s in the W20 and W0 treatments, as well as in an increase of ψ_{pred} (Fig. 3.3, Table S3.2). Trees from all treatments reached similar A_{leaf}, g_s and ψ_{pred} approximately 10 (2/3 removal) and 30 (undefoliated) days after rewetting (Fig. 3.3).

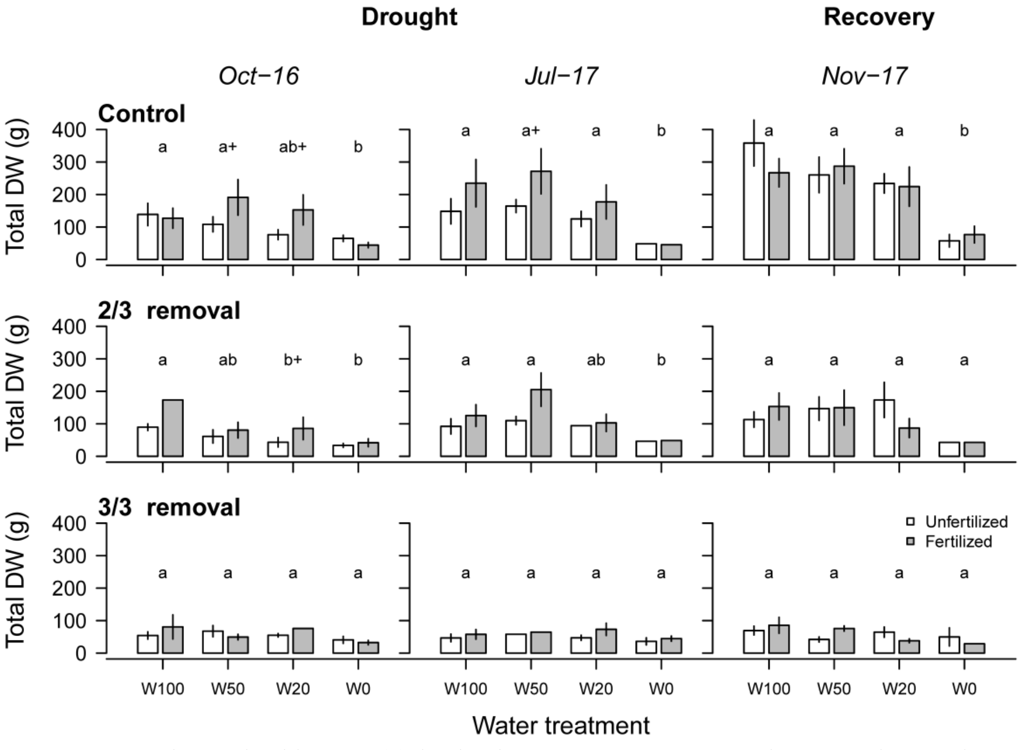

Figure 3.4: Total tree dry biomass (g) in the four water treatments and two nutrient regimes in October 2016, July 2017 (during drought), and November 2017 (after rewetting). Trees with 1/3 needle removal are not shown because they were very similar to undefoliated trees. White bars show unfertilized, and grey bars fertilized trees. Letters show the significant differences between water treatments. + signs show significant differences between unfertilized and fertilized trees within the water treatment. Error bars indicate the SE of the mean. Tissue specific dry weights are shown in Supplementary Data Fig. S3.3.

Biomass

Total tree biomass decreased with drought intensity, but only the W0 treatments differed significantly from W100 (Fig. 3.4, Table S3.3). Fertilization significantly increased biomass until July 2017. The effect size of fertilization differed among the water regimes, especially in fully foliated trees (Fig. 3.4; Table S3.3). Unfertilized trees showed a more or less continuous biomass reduction for all tissues with increasing drought level (additional information on tissue specific weights in Fig. S3.3). The fertilized trees in contrast did not show such pattern but rather a biomass increase under W50 and W20 and only a strong reduction at W0. With the exception of the W0 treatment, higher biomass in fertilized vs. unfertilized trees was still seen in July 2017 (Fig. 3.4), but these patterns disappeared at the last harvest in November 2017, 3 months after drought release (Fig. 3.4). The root biomass fraction (root biomass / total biomass) did not increase with drought intensity during the first growing season in 2016, but an increase with drought intensity was found in the fertilized plots in July 2017 and in all plots in November 2017 (Fig. S3.4). Defoliation reduced not only needle biomass, but also stem and root biomass, mainly in the W100, W50 and W20 regimes (Fig. S3.3). All trees in the W0 treatment had comparable low biomass, independent of defoliation level.

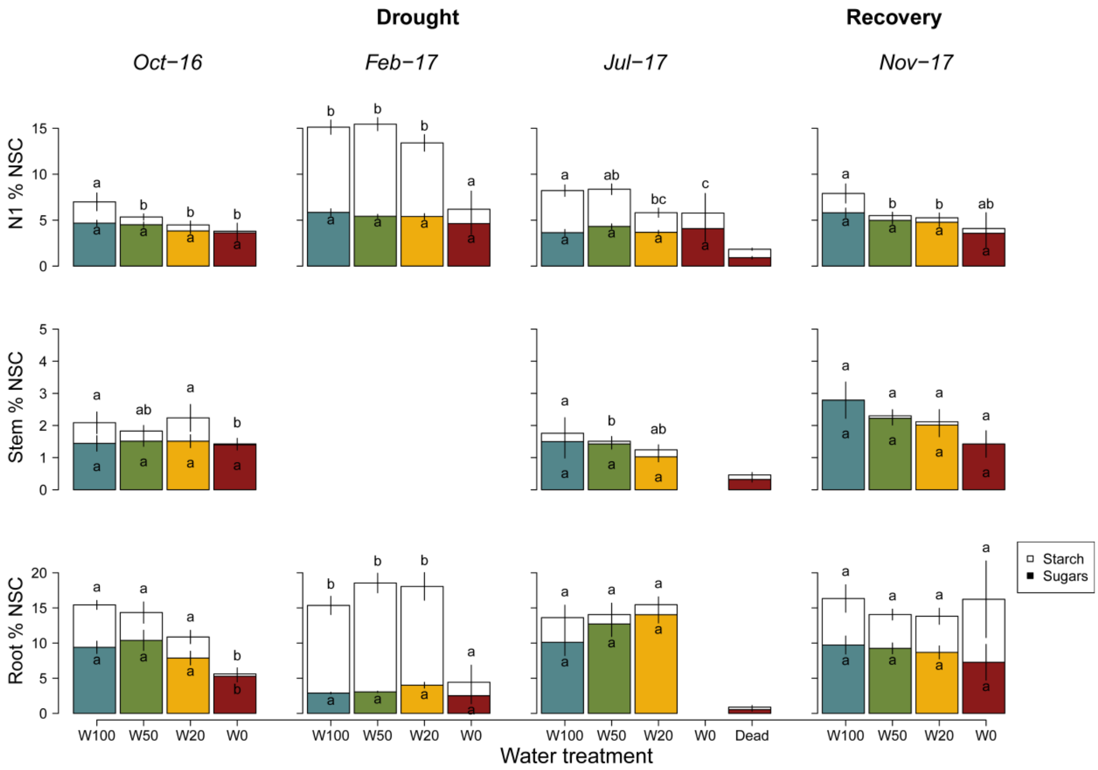

Figure 3.5: Total NSC, split up in sugars (colored parts) and starch (white parts), in current year needles (N1), stem and roots of undefoliated trees on four different harvesting times. Fertilization did not have an effect on NSC and thus unfertilized and fertilized trees were pooled. In February 2017, only needles and roots were harvested without taking out entire trees. Dead trees were only found in the extreme drought, and mortality only occurred between February and July (before rewetting) in the second growing season, and thus shown in the July 2017 plots. Error bars indicate the SE of the mean. Different letters indicate significant differences in starch (upper letters) and sugars (lower row, within the colored bars) between water treatments on the specific harvest date. Sugar and starch concentrations in different defoliation regimes can be found in Supplementary Data Fig. S3.5.

Non-structural carbohydrates

Significant NSC reductions due to drought were seen mostly in the W0 treatment (Fig. 3.5, Table S3.3). These reductions were mainly due to sharp starch reductions, whereas sugar levels were reduced in lesser amounts. Whilst in October 2016, NSC also showed reductions in the W50 and W20 treatments in roots and needles, in July 2017 NSC concentrations, mostly sugars, were largely restored to W100 levels in those tissues (Fig. 3.5, Table S3.3). Also the needles of the surviving trees in W0 showed an increase in sugars compared to October 2016. Dead trees always ended up with significantly lower amounts of NSC in all tissues at the time of death (Fig 3.5).

Fertilization did not significantly induce changes in NSC concentrations for any drought level (Table S3.3). Trees with reduced leaf area due to defoliation, mainly the trees with 100% needle removal, had lower starch concentrations, especially in the roots sampled in summer 2017 (Fig. S3.5, Table S3.3). In the case of an interaction between drought and defoliation, W100, W50 and W20 generally showed constant NSC with lower leaf area, whereas W0 trees showed an NSC decline after extreme defoliation (Table S3.3). Seasonal NSC trends were similar in every treatment, and were especially visible in the roots and needles, where sugars dominated in summer and starch in winter (Fig. S3.5).

Rewetting resulted in an increase of sugars and starch in the needles the saplings, especially in trees from the W20 and W0 drought treatments (Fig. 3.6), and NSC levels were comparable between drought treatments after the last harvest in November 2017 (Fig. 3.5, Fig. 3.6).

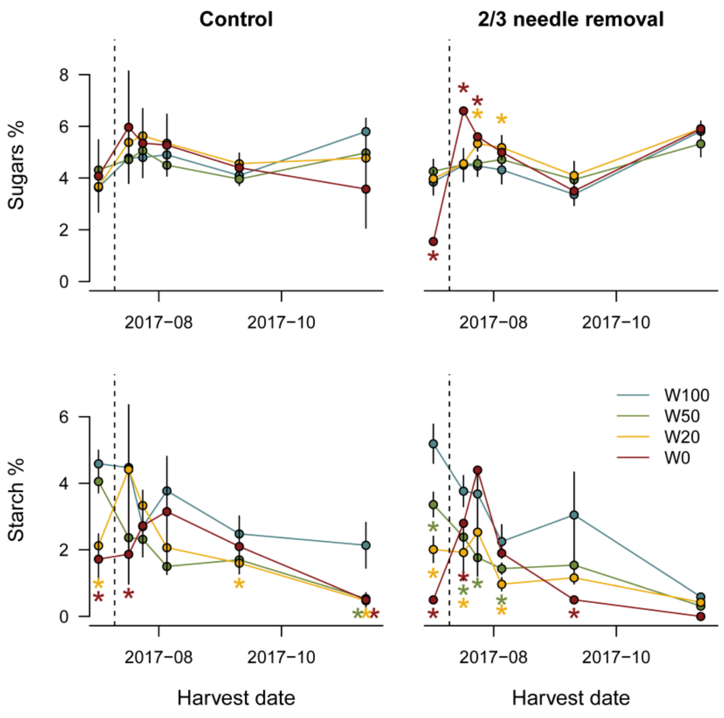

Figure 3.6: Sugar and starch concentrations in current year needles (N1) during the time of rewetting. The dotted vertical line indicates the time where rewetting started. Only trees without needle removal and with 2/3 needle removal were measured. Error bars indicate the SE of the mean. Colored asterisks show which treatments significantly differed from the control (W100) treatment at every measuring date.

Nutrients in soil and plants

In general, fertilization increased nitrate, ammonium and phosphorus concentrations in the soil after the first growing season, although significance differed between water treatments (Fig. S3.6). Total N pools in the system (soil + plant) were significantly higher in the W50 and W20 fertilized treatments, whilst W100 and W0 treatments also trended towards an increase due to fertilization. The concentration of N in plant tissues was not significantly affected (Fig. S3.7), although slight increases were seen in the stem and roots, and needles of well-watered (W100) trees (Fig. S3.7).

Discussion

Extreme drought leads to ceasing of photosynthesis, growth and C allocation belowground

Extreme drought (W0) was the only regime that significantly reduced gas exchange per unit leaf area, biomass and NSC concentrations in all seasons, and resulted in drought induced mortality. A decreasing trend in biomass and NSC was also seen in mild drought levels W50 and W20, especially in October 2016 but biomass was not clearly different between W100, W50 and W20 at the end of the drought period in July 2017. These results support our first hypothesis that across increasing drought intensity, assimilation and – after an adjustment period – C allocation to biomass is kept constant and only when a threshold is exceeded (here seen in the W0 treatment) photosynthesis and growth would cease and eventually also lead to mortality. A common theory is that plants tend to mitigate stress levels by increasing the uptake potential for the limiting resource (Freschet et al., 2018). In the case of drought stress, plants would thus invest energy belowground to expand the root system and to improve the water uptake capacity (hypothesis 1). In the current study, increased biomass allocation to roots was indeed observed in fertilized trees during drought stress in the first half of the second growing season (2017), even in extreme drought, supporting our hypothesis, while in unfertilized trees, increased root investment occurred only after rewetting. The earlier occurrence of this strategy (i.e. higher investments in root production) in fertilized plants suggests that nutrients could help trees to adjust to drought by stimulating root growth. The fact that unfertilized trees showed an increase in root investment after rewetting could be due to increased belowground transport of C after rewetting (Hagedorn et al., 2016), and might thus be more a rewetting than a drought effect.

As expected, the most extreme reductions in NSC were seen in the W0 water regime, whilst mild drought also induced decreases in NSC in some tissues and seasons, mostly in starch concentration. Sugar concentrations were less affected, because constant sugar concentrations are important for maintaining a high osmotic potential under drought (Brodribb and Cochard, 2009; Brunner et al., 2015; Li et al., 2013). Interestingly, root NSC concentrations were re-adjusted to levels comparable to W100 during the first half of the second growing season in trees experiencing mild drought, whereas growth and photosynthesis rates were comparable to the first growing season. Even in the surviving trees in the extreme drought, NSC levels were slightly recovered. The fact that NSC dynamics changed irrespective of photosynthesis or growth dynamics suggests that trees keep homeostatic NSC tissue concentrations on the longer term when they have the possibility and time to adapt to changing conditions (Schönbeck et al., 2018), and that they prioritized homeostatic NSC pools over growth, a process that has been reported in several experiments using different stressors like drought, defoliation, shading and girdling (Oberhuber et al., 2017; Piper et al., 2015; Puri et al., 2015; Weber et al., 2019). Interesting to note is that dying trees had significantly lower NSC levels than surviving trees, which indicates that there is a relation between the C storage pool and a ‘point of no return’ for trees experiencing extreme drought stress. Further this result shows that trees are able to use almost all stored C (incl. substantial parts of the free sugar pool) before death, like it has been previously documented for darkened trees (Weber et al., 2018; Wiley et al., 2017).

Nutrients stimulate higher root biomass fraction but do not mitigate extreme drought stress
Fertilization did not affect gas exchange and NSC concentrations in any tissue and drought regime. For gas exchange, this is unexpected, and not in line with our second hypothesis, as especially N is thought to play an important role in adjusting stomatal conductance to water potential (Gessler et al., 2016), and high N availability is known to generally increase stomatal conductance (Fangmeier et al., 1994). However, the N concentrations in needles were not affected by the fertilization treatment even though the total N stock per plant was higher, thus explaining that lack of difference in the area normalized rates of photosynthesis and stomatal conductance. In the case of some sporadic significant differences, fertilized trees had even lower gas exchange rates than ambient trees. This could be due to the fact that fertilized trees had in general higher needle biomass, and thus higher leaf area, which allows lower gas exchange per unit leaf area to reach comparable C uptake.

Fertilization did in general increase biomass, but the fertilization effect, i.e. the difference between the biomass of fertilized and unfertilized trees, increased with mild drought and was almost absent in W100 and W0 regimes (Fig. 3.4). For extreme drought, reduced ion mobility in the soil, and impaired active transport, as well as reduced transpiration and thus reduced water transport to the needles and other sinks probably prevented fertilization from having an effect (Gessler et al., 2016). For well-watered plots, the absence of a fertilization effect was most likely due to the fact that here, unfertilized trees were not nutrient limited, and only a drying soil prevents nutrient mobility and causes nutrient limitation in trees. Moreover, whilst biomass decreased with drought intensity in unfertilized plots, mild drought supported higher growth than well-watered conditions in fertilized plots – mainly visible in October 2016 and July 2017 in undefoliated trees. The very regular watering might have been not beneficial for pines. Scots pine is known to be in a competitive advantage on nutrient-poor and dry sites (Lévesque et al., 2015), which does not imply that it never experiences nutrient limitation though (e.g. Matías and Jump, 2012). Although interspecific competition was not present in this study, the adaptation of pine to soils with low soil water retention is well known and could probably explain the disadvantages on wetter soils (cf. Heiskanen, 1995). Washing out of nutrients due to heavy watering seems not the case, as whole ecosystem total N, or in the form of Ammonium and Nitrate, were similar in the W100, W50 and W20 treatments (Supplementary Data Fig. S3.6). To summarize, under high water supply, rather the potentially unfavorable (i.e., too high) soil moisture conditions and not nutrient availability might have limited biomass production.

During the period July to November 2017, the previously visible fertilization effects disappeared. A change in the intraspecific competition, after removal of 2/3 of the trees, in combination with the rewetting might have strongly changed the growth conditions especially in the drought treatments. None of these results give evidence that nutrients were significantly mitigating the negative effects of extreme drought. The fact that mortality rates were slightly but not significantly higher than in unfertilized plots strengthens this conclusion.

Extreme drought and defoliation result in too little C supply to keep NSC levels stable

We hypothesized that changes in source:sink balance between mild versus severe defoliation would lead to differences in the susceptibility and response to drought (cf. hypothesis 3). More specifically, we expected to see higher photosynthesis per leaf area after mild defoliation, even in drought stressed trees, to compensate for the reduced leaf area, but lower growth and NSC levels due to insufficient C supply after extreme defoliation. We found that trees with partial needle removal had higher photosynthesis rates than untreated trees until October 2016, and as a result had comparable leaf water potential levels as untreated trees. Defoliation did thus not result in lower water loss but in gas exchange compensation for lower leaf area and thus C supply (Eyles et al., 2009; Kruger et al., 1998). However, extreme drought inhibited photosynthetic activity in trees with and without defoliation after October 2016.

Partial and extreme defoliation led to lower biomass, despite of higher gas exchange rates, and mainly in the second growing season (2017), extreme defoliation and drought led to lower NSC levels, although NSC levels were not depleted even after repeated defoliation before the second growing season, similar to what was found in Li *et al.* (2002) in treeline trees. Lower growth rates due to a sudden stressor like extreme defoliation have been reported by several other studies (Eyles et al., 2009; Jacquet et al., 2014; Kruger et al., 1998; Li et al., 2002; Piper et al., 2015; Schmid et al., 2017; Wiley et al., 2013). Trees did not use stored NSC to restore growth during drought but seemed to prioritize stable NSC pools as far as possible, which has been shown earlier in other defoliation experiments (Jacquet et al., 2014; Piper et al., 2015; Weber et al., 2019). The combination of extreme drought and extreme defoliation caused, as we show in this study, too little C supply to keep stable NSC levels.

In our study, defoliated trees had slightly higher N concentrations in their needles than undefoliated trees, in line with Piper *et al.* (2015), who found constant N in leaves after defoliation. The trees might need to keep NSC and N levels on a particular level to stay functional, leaving less C for growth, suggesting a highly regulating C and N conservation strategy and indicating C limitation for growth and prioritization of storage over growth (Piper et al., 2015; Puri et al., 2015; Weber et al., 2019). Alternatively, it was hypothesized that the reduction in wood growth could have been caused by a reduced demand for water transport due to allometric relationships (Schmid et al., 2017).

Rewetting results in a recovery and overshoot of photosynthesis and NSC

We expected quick recovery of photosynthesis and NSC in surviving trees after rewetting, but mortality in trees that were too heavily defoliated of passed the minimum NSC threshold. After rewetting from mid-July, 2017, A_{leaf} increased steeply, especially in trees that experienced extreme drought before, and trees with lower leaf area increased their photosynthesis faster than trees without needle removal, indicating the compensation reaction for a reduced leaf area reappeared as soon as environmental conditions allowed for. Moreover, fertilized trees showed a slower increase of A_{leaf} than unfertilized trees, which could be explained by the difference in VWC between the fertilization treatments in W0 instead of fertilization itself. Soon after rewetting, surviving trees in the W0 regime developed very short shoots with a very dense needle cover, to increase their leaf area and thus C uptake (Supplementary Data Fig. S3.8).

The few dying trees after rewetting showed lower NSC levels than any surviving tree (Supplementary material Fig. S3.5), indicating their limits in recovering from extreme drought (cf. hypothesis 4). Surviving trees showed increasing sugar levels in the needles, and an overshoot within one to two weeks after rewetting, reaching higher levels than previously well-watered trees, supporting our fourth hypothesis. Starch levels in the roots also showed this overshooting four months after rewetting, which could suggest that trees prioritized NSC storage over growth (Galiano Pérez et al., 2017). However, although it might be too soon to draw conclusions from biomass harvested in November 2017 (i.e. only 4 months after rewetting), trees in W20 and W0 seemed to have increased root biomass fraction between July and November 2017 more than trees from the W100 and W50 trees. Thus, trees that had experienced more intense drought might have allocated more C into root restoration after rewetting. These results are speculative and rewetting earlier in the season could have resulted in more pronounced results. However, growth later in the season is possible for Scots pine, as it is the species which has among the longest growing seasons and utilizes beneficial growing conditions even if it is late in the season (Etzold and Zweifel, 2018).

We subjected Scots pine saplings to drought, fertilization and defoliation to unravel the C dynamics under those interacting conditions, and expected to see mitigating effects of fertilization and mild defoliation on the negative consequences of drought. We showed in this study that drought stressed saplings show a strong prioritization of C and N allocation to storage over growth. Fertilization did play a role on allometric adaptations, stimulating a higher root biomass fraction for improving the uptake capacity of limited resources such as water, but did not mitigate the negative effects of drought by reducing ψ_{leaf} or mortality under intensive drought. Comparably, partial defoliation increased photosynthesis rates at the needle area basis but did not decrease water consumption or mortality, and extreme defoliation resulted in reduced NSC concentrations. However, we showed that partial defoliation stimulated recovery after drought. Although 60% of the trees died in the extreme drought treatment, the surviving Scots pine saplings were able to recover photosynthesis and NSC reserves very fast, even after two years of drought. This experiment was one of only a few studies so far that combined drought stress with gradual defoliation and nutrition. We demonstrated that tree mortality under severe drought periods will not be mitigated, but that the effects of low intensity drought stress could be compensated by increased nutrient availability and changing source:sink balances.

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Supporting information

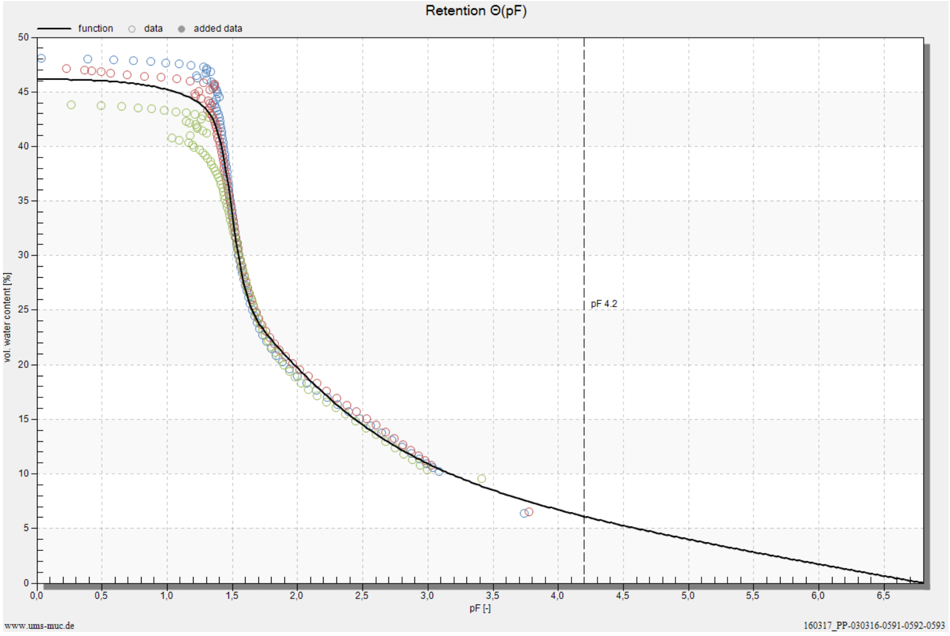


Figure S3.1: pF curves of four samples of the soil for determination of field capacity and wilting point.

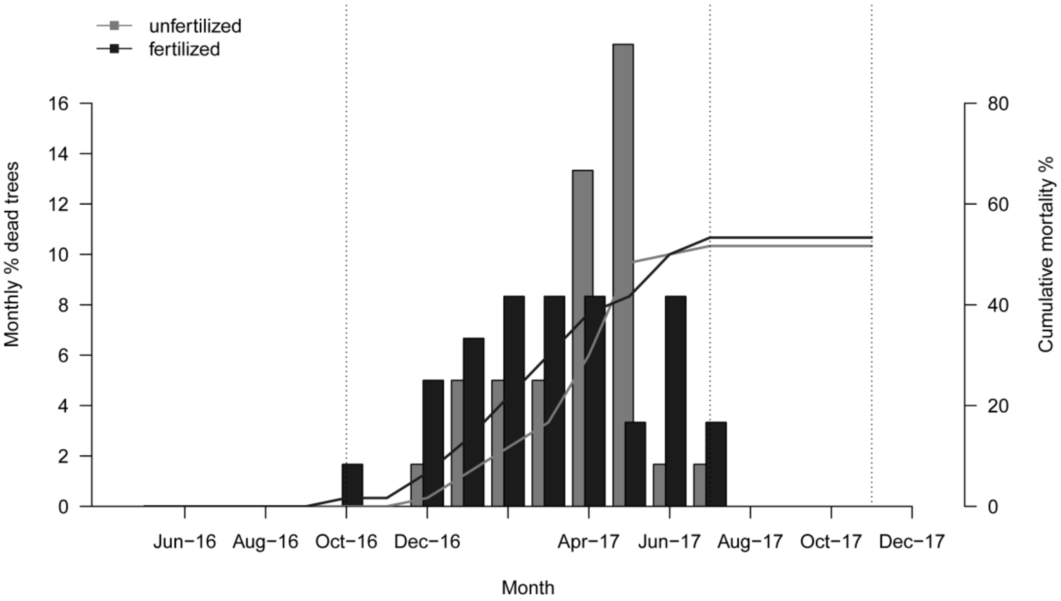


Figure. S3.2: Mortality in the extreme dry plots (W0) during the experiment. Bars show the percentage out of 120 trees, the line shows the cumulative mortality over time. Light grey bars and line show unfertilized trees, dark grey bars and line show fertilized trees.

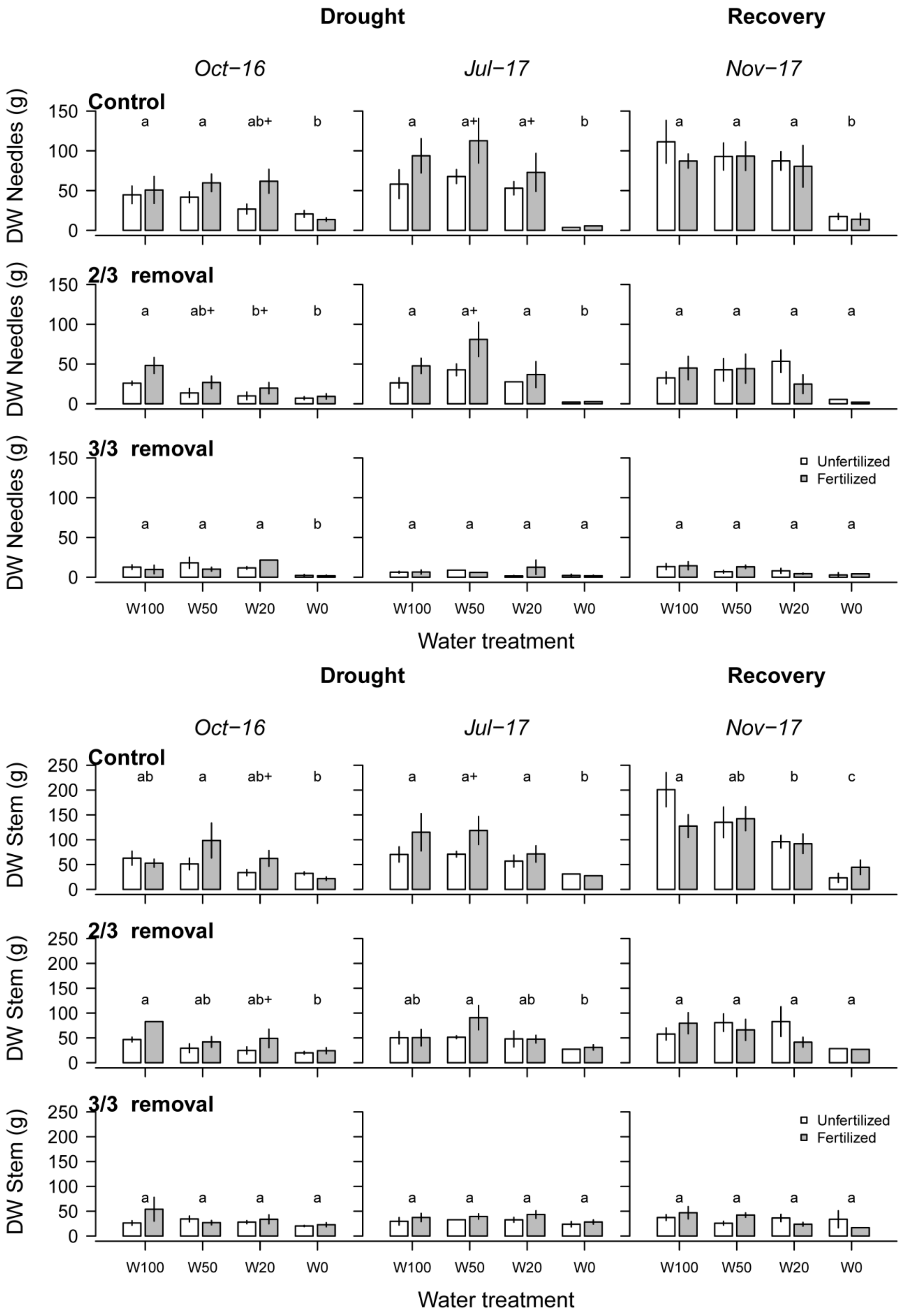
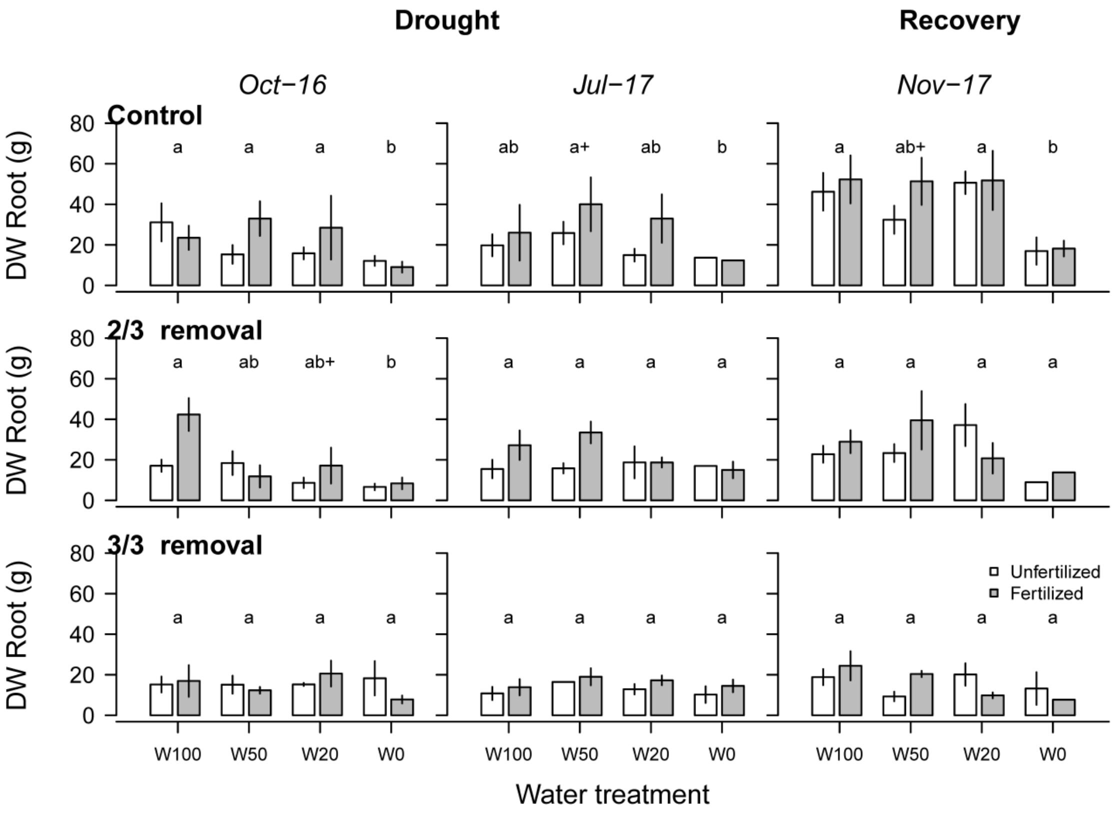


Figure S3.3: Dry biomass of needles, stem and roots (next page) in October 2016, July 2017 and November 2017. White bars show the unfertilized trees, grey bars show fertilized trees. Trees with a control, 2/3 and 3/3 needle removal treatment are shown. Trees with 1/3 needle removal were very similar to the controls. Error bars show the SE of the mean.



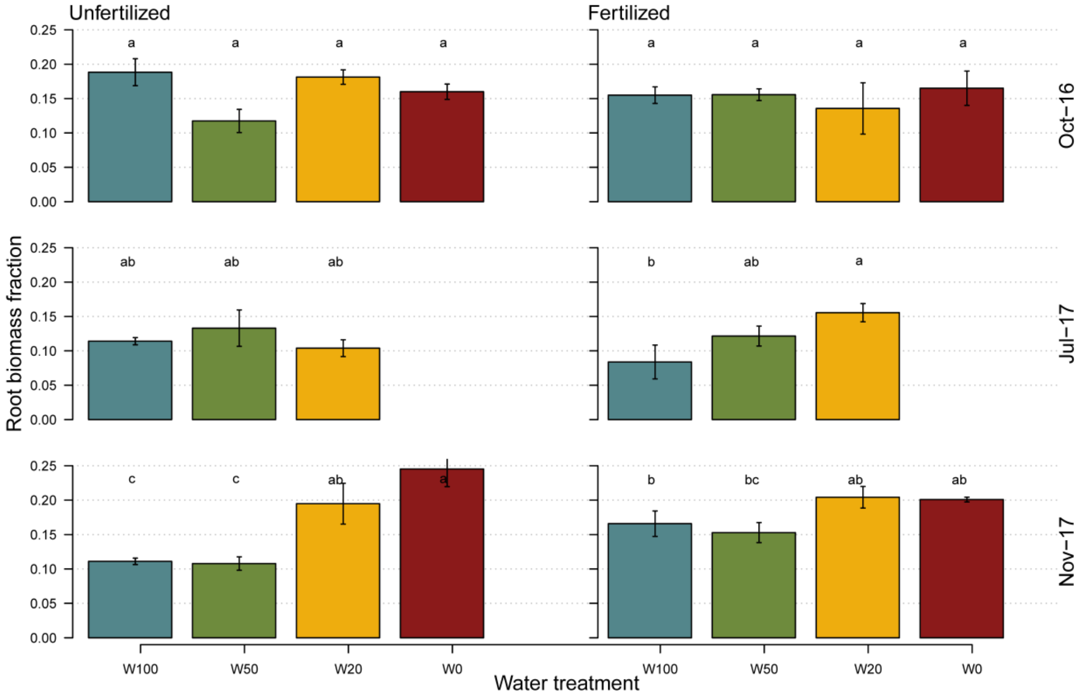


Figure S3.4: Root biomass fraction in undefoliated trees in October 2016, July 2017 (drought) and November 2017 (after rewetting). Colours show the four water treatments. Left panels show unfertilized, right panels fertilized trees.

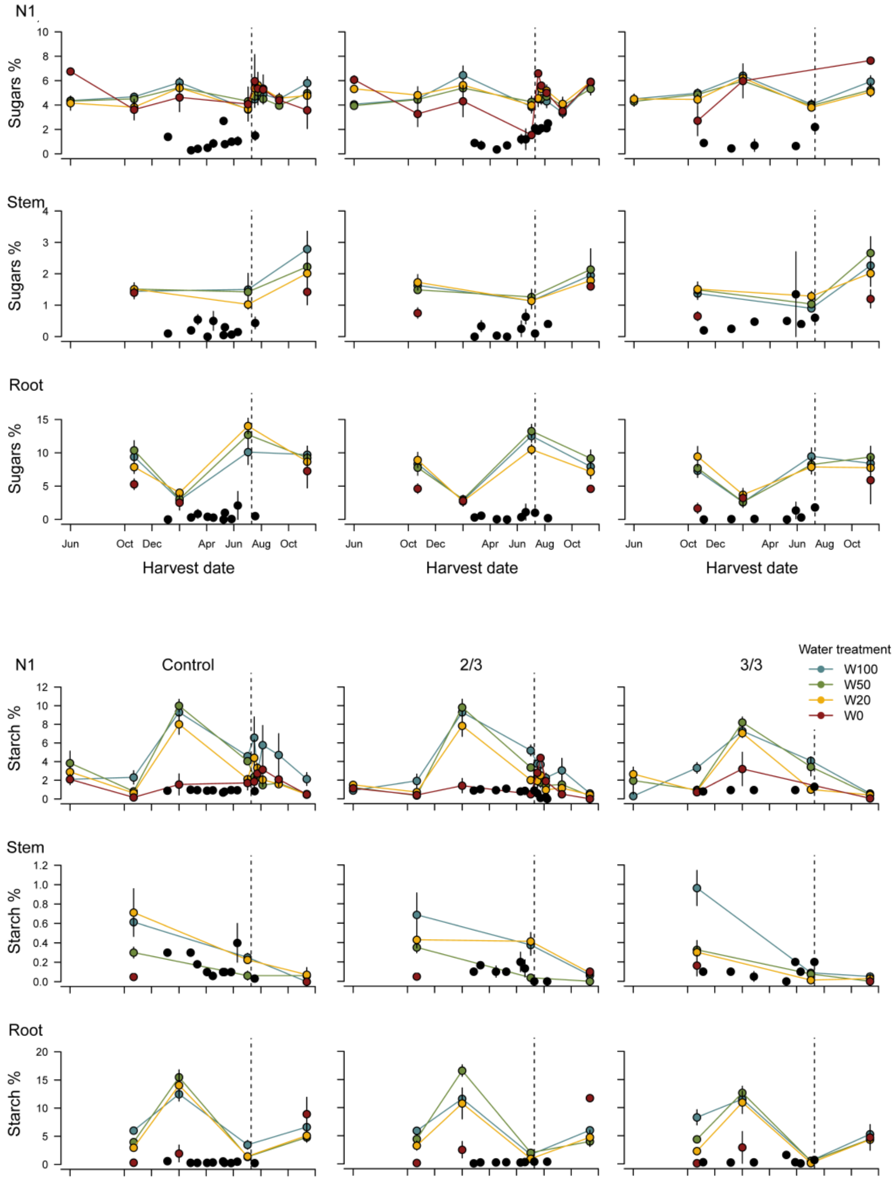


Figure S3.5: Sugar (upper) and starch (lower) concentrations in needles (N1), stem and root over time, in undefoliated trees and trees with 2/3 and 3/3 needles removed. Black dots show the concentrations in dead trees at the time of death.

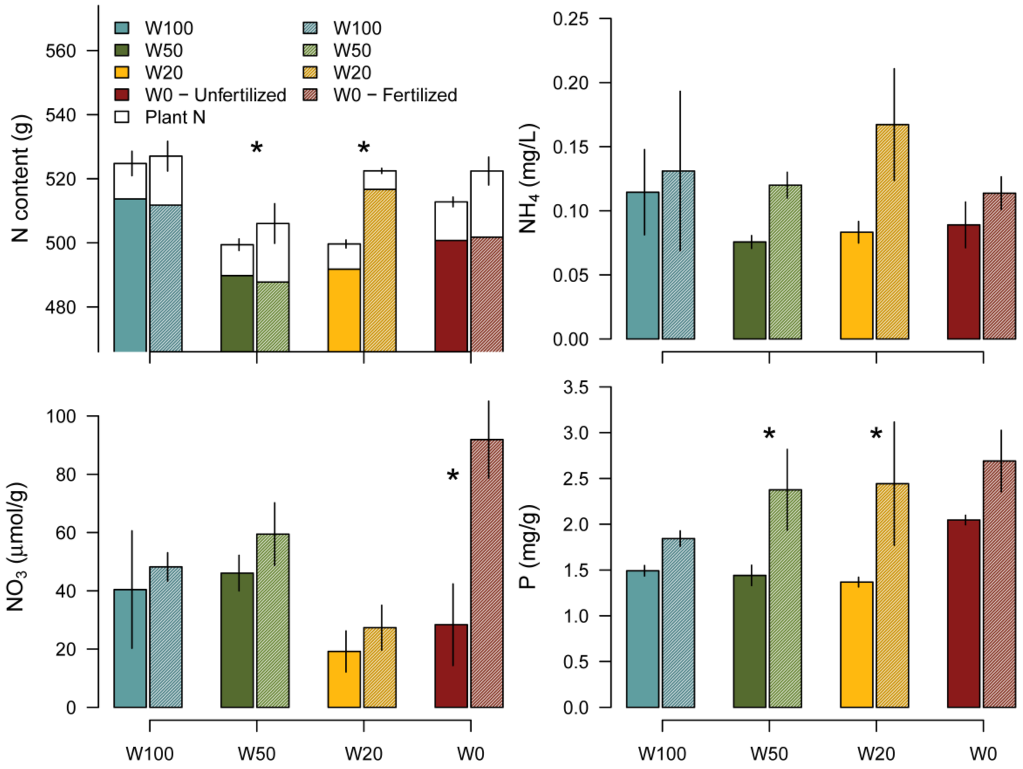


Fig. S3.6: Total Nitrogen in grams in the ecosystem soil (colors) and plants (white bars), ammonium (NH_4) concentration (mg/L), nitrate (NO3) concentration (µmol/g) and phosphorus (P) concentration (mg/g) in the soil in October 2016. Colors represent the four drought treatments, W100 (blue), W50 (green), W20 (orange) and W0 (red). Patterns indicate fertilization treatments. Asterisks indicate significant differences between unfertilized and fertilized treatments within a water treatment. Error bars indicate the SE of the mean.

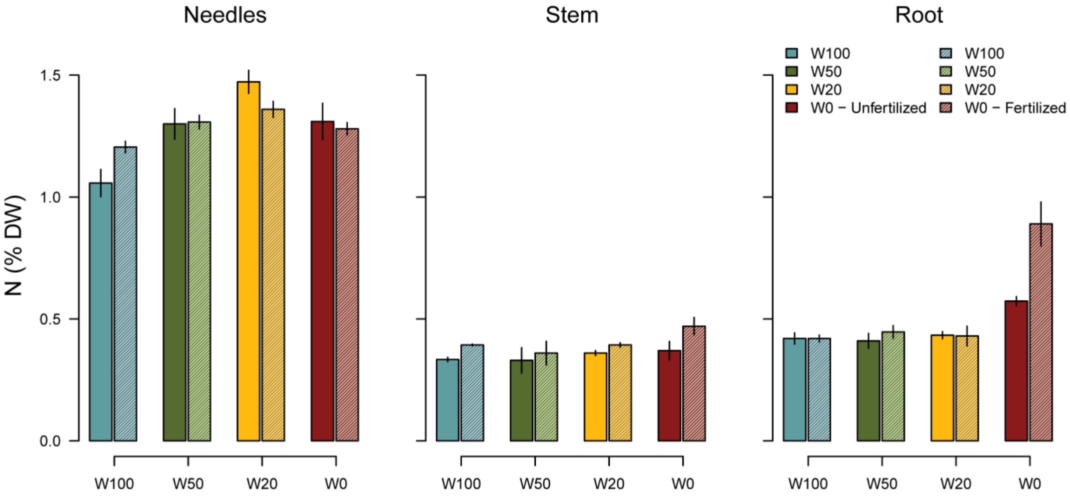


Fig. S3.7: Nitrogen concentrations in needles, stem and root in October 2016 as a percentage of the dry weight of the tissue. Error bars indicate the SE of the mean. Colors indicate water treatments, patterns indicate the fertilization treatment.

Figure S3.8 Emergence of needles 8 weeks after rewetting

Table S3.1: Volumetric water content of the soil in the growing season of 2016 (from the moment of stable soil conditions) and the growing season of 2017 (until the moment of rewetting in July). Relative changes on a scale from ~field capacity (100%) to ~wilting point (0%) are also given.

VWC Jun-Oct 2016		**VWC 2017 Jun-Jul 2017**	
23.9% +- 1.5%	~ 100% (W100)	20.5% +- 1.3%	~ 100% (W100)
16.3% +- 2.1%	~ 55% (W50)	11.5% +- 0.9%	~ 40% (W50)
10% +- 1.3%	~ 20% (W20)	8.4% +- 0.9%	~ 20% (W20)
6.7% +- 0.7%	~ 0% (W0)	5.6% +- 0.8%	~ 0% (W0)

Table S3.2: ANOVA results of differences in photosynthesis (A_{leaf}), stomatal conductance (g_s) and predawn leaf water potential (ψ) between water, nutrient and needle removal treatments. F-values and p-values (between brackets) are given. Significant results are indicated in bold.

	2016	**2016**	**2016**	**2017**	**2017**	**2017**	**2017**	**2017**	**2017**
	05/18	**07/13**	**10/05**	**06/08**	**07/11**	**07/21**	**07/27**	**8/8**	**09/13**
A_{leaf}									
Water	**10.09**	**16.41**	**21.33**	**8.65**	**14.11**	**12.90**	1.55	2.67	0.88
	(0.019)	**(0.001)**	**(<0.001)**	**(0.015)**	**(0.002)**	**(<0.001)**	(0.258)	(0.158)	(0.473)
Nutrients	0.32	0.00	0.58	1.56	1.89	2.24	0.00	5.80	1.22
	(0.589)	(0.981)	(0.469)	(0.249)	(0.210)	(0.156)	(0.976)	(0.052)	(0.287)
Nrm	**23.43**	**22.07**	1.52	0.04	**14.38**	**39.01**	1.05	0.45	1.96
	(<0.001)	**(< 0.001)**	(0.223)	(0.847)	**(<0.001)**	**(<0.001)**	(0.352)	(0.637)	(0.144)
W:N	0.80	0.06	0.58	0.52	**4.51**	1.91	0.48	**8.48**	1.02
	(0.403)	(0.811)	(0.582)	(0.620)	**(0.046)**	(0.174)	(0.701)	**(0.025)**	(0.414)
W:Nrm	0.37	**9.17**	0.41	**5.83**	**3.83**	**5.69**	**2.33**	**4.11**	**3.59**
	(0.545)	**(0.004)**	(0.669)	**(0.007)**	**(0.001)**	**(<0.001)**	**(0.036)**	**(<0.001)**	**(0.002)**
N:Nrm	1.13	**35.72**	**5.91**	0.39	2.46	**12.85**	0.55	**14.51**	0.59
	(0.294)	**(< 0.001)**	**(0.019)**	(0.536)	(0.089)	**(<0.001)**	(0.577)	**(<0.001)**	(0.556)
g_s									
Water	**9.28**	**27.97**	**14.35**	**9.61**	**7.94**	1.89	2.78	**12.71**	1.35
	(0.023)	**(0.001)**	**(0.002)**	**(0.009)**	**(0.012)**	(0.207)	(0.089)	**(<0.001)**	(0.298)
Nutrients	0.003	0.41	0.14	0.01	0.88	0.18	0.00	0.12	1.48
	(0.957)	(0.550)	(0.721)	(0.909)	(0.376)	(0.685)	(0.946)	(0.735)	(0.243)
Nrm	**4.35**	0.00	2.85	2.53	**9.43**	**20.58**	3.00	0.75	1.36
	(0.043)	(0.945)	(0.097)	(0.121)	**(<0.001)**	**(<0.001)**	(0.053)	(0.475)	(0.260)
W:N	0.89	1.16	2.89	0.38	**7.95**	2.72	0.21	1.67	2.75
	(0.383)	(0.328)	(0.107)	(0.700)	**(0.013)**	(0.115)	(0.890)	(0.218)	(0.082)
W:Nrm	1.83	2.55	1.92	**8.67**	**3.71**	**12.64**	**4.89**	**3.27**	**9.83**
	(0.184)	(0.117)	(0.156)	**(<0.001)**	**(0.002)**	**(<0.001)**	**(<0.001)**	**(0.005)**	**(<0.001)**
N:Nrm	0.49	**16.53**	1.89	**4.35**	**11.69**	**7.30**	3.06	**3.46**	**3.30**
	(0.486)	**(<0.001)**	(0.175)	**(0.045)**	**(<0.001)**	**(<0.001)**	(0.050)	**(0.034)**	**(0.039)**
ψ ***(MPa)***									
Water	0.37	**12.86**	**41.72**		**44.92**			3.43	
	(0.562)	**(0.001)**	**(<0.001)**		**(<0.001)**			(0.191)	
Nutrients	0.48	0.13	0.24		1.65			**23.27**	
	(0.494)	(0.719)	(0.631)		(0.212)			**(0.011)**	
Nrm	3.84	1.62	3.13		1.18			0.02	
	(0.063)	(0.208)	(0.099)		(0.290)			(0.904)	
W:N	0.67	0.72	0.14		0.889			**10.98**	
	(0.420)	(0.491)	(0.874)		(0.426)			**(0.048)**	
W:Nrm	2.50	0.23	0.60		0.01			2.72	
	(0.128)	(0.799)	(0.561)		(0.986)			(0.100)	
N:Nrm	0.01	1.11	2.61		0.37			1.61	
	(0.930)	(0.297)	(0.129)		(0.549)			(0.226)	

Table S3.3: ANOVA results of differences in biomass, sugars and starch between water, nutrient and needle removal treatments. F-values and p-values (between brackets) are given. Significant results are indicated in bold.

	Biomass			**Sugars**			**Starch**		
	Oct16	Jul17	Nov17	Oct16	Jul17	Nov17	Oct16	Jul17	Nov17
Needles									
Water	**17.19** **(<0.001)**	**37.09** **(<0.001)**	**8.58** **(0.002)**	1.09 (0.392)	17.59 (<0.001)	0.68 (0.575)	**12.56** **(<0.001)**	**23.35** **(<0.001)**	3.26 (0.058)
Nutrients	2.52 (0.126)	**7.04** **(0.022)**	0.06 (0.800)	0.31 (0.590)	1.00 (0.334)	0.27 (0.609)	1.87 (0.175)	0.05 (0.825)	0.65 (0.430)
Nrm	**33.82** **(<0.001)**	**56.08** **(<0.001)**	**38.84** **(<0.001)**	0.71 (0.550)	9.89 (<0.001)	2.62 (0.058)	**3.53** **(0.018)**	**5.46** **(0.002)**	**6.58** **(<0.001)**
W:N	1.45 (0.254)	0.84 (0.498)	0.37 (0.774)	0.17 (0.917)	1.08 (0.393)	1.64 (0.221)	**9.43** **(<0.001)**	1.33 (0.294)	0.69 (0.577)
N:Nrm	**2.86** **(0.042)**	0.48 (0.696)	1.02 (0.388)	0.56 (0.641)	1.46 (0.237)	**2.95** **(0.041)**	0.83 (0.482)	0.36 (0.782)	1.24 (0.303)
W:Nrm	**2.55** **(0.013)**	**6.54** **(<0.001)**	1.33 (0.237)	1.97 (0.055)	6.14 (<0.001)	1.96 (0.061)	1.86 (0.068)	1.28 (0.269)	**2.16** **(0.037)**
Stem									
Water	**10.51** **(<0.001)**	**16.98** **(<0.001)**	**8.48** **(0.004)**	**3.60** **(0.046)**	0.13 (0.877)	1.99 (0.183)	**28.91** **(<0.001)**	**6.72** **(0.016)**	0.15 (0.930)
Nutrients	2.75 (0.112)	**9.03** **(0.006)**	0.10 (0.756)	0.02 (0.900)	2.94 (0.091)	0.00 (0.974)	0.84 (0.361)	**9.67** **(0.003)**	0.08 (0.781)
Nrm	**6.04** **(<0.001)**	**13.46** **(<0.001)**	**17.65** **(<0.001)**	1.76 (0.161)	0.16 (0.921)	0.14 (0.938)	0.59 (0.621)	**9.45** **(<0.001)**	0.18 (0.910)
W:N	0.41 (0.750)	1.66 (0.206)	0.28 (0.842)	0.07 (0.977)	0.96 (0.389)	2.20 (0.169)	2.28 (0.085)	2.09 (0.132)	0.77 (0.524)
N:Nrm	1.78 (0.158)	0.20 (0.898)	0.64 (0.589)	**3.67** **(0.015)**	0.21 (0.888)	1.14 (0.341)	0.67 (0.570)	1.07 (0.367)	1.28 (0.288)
W:Nrm	1.55 (0.146)	**2.36** **(0.018)**	1.66 (0.112)	1.35 (0.225)	1.399 (0.227)	0.50 (0.868)	1.97 (0.052)	**2.36** **(0.039)**	0.60 (0.794)
Root									
Water	**7.11** **(0.005)**	**6.48** **(0.002)**	**3.80** **(0.013)**	**12.55** **(<0.001)**	1.56 (0.216)	1.14 (0.356)	**50.42** **(<0.001)**	2.69 (0.123)	2.09 (0.148)
Nutrients	0.73 (0.395)	**8.77** **(0.006)**	0.55 (0.461)	0.08 (0.775)	0.49 (0.487)	0.00 (0.950)	0.42 (0.526)	**5.61** **(0.043)**	0.00 (0.945)
Nrm	1.65 (0.184)	**3.39** **(0.020)**	**11.94** **(<0.001)**	2.14 (0.102)	**5.46** **(0.002)**	0.69 (0.564)	0.15 (0.932)	**11.56** **(<0.001)**	1.69 (0.179)
W:N	0.87 (0.460)	1.23 (0.320)	1.95 (0.128)	1.81 (0.197)	0.35 (0.708)	0.63 (0.606)	0.70 (0.567)	**6.65** **(0.018)**	0.51 (0.680)
N:Nrm	0.91 (0.439)	0.07 (0.974)	0.23 (0.877)	0.61 (0.611)	1.29 (0.285)	0.065 (0.978)	0.69 (0.558)	0.66 (0.578)	0.14 (0.937)
W:Nrm	**2.09** **(0.038)**	0.57 (0.817)	0.68 (0.724)	**2.14** **(0.035)**	1.88 (0.095)	0.40 (0.933)	1.22 (0.295)	1.79 (0.117)	0.67 (0.735)
Total biomass									
Water	**15.80** **(<0.001)**	**16.65** **(<0.001)**	**7.71** **(<0.001)**						
Nutrients	3.33 (0.052)	**7.96** **(0.016)**	0.01 (0.928)						
Nrm	**9.98** **(<0.001)**	**24.20** **(<0.001)**	**23.20** **(<0.001)**						
W:N	1.22 (0.326)	1.05 (0.409)	0.56 (0.644)						
N:Nrm	1.69 (0.175)	0.16 (0.923)	0.62 (0.602)						
W:Nrm	2.01 (0.050)	**3.39** **(0.001)**	1.28 (0.260)						

4

Soil nutrient availability alters tree carbon allocation dynamics during drought

Submitted to a peer reviewed journal as:
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Summary

- Carbon (C) and nutrient allocation in trees changes or gets impaired during drought. Elevated soil nutrient availability might alter the response of trees to drought. We hypothesize that increased soil nutrient availability stimulates root metabolism and carbon allocation to belowground tissues under drought stress.
- To test this hypothesis, we subjected three-year-old *Pinus sylvestris* saplings during two subsequent years to drought using three different water treatments (100%, 20% and 0% plant available water in the soil) and two soil nutrient regimes (ambient and nitrogen-phosphorus-potassium (N-P-K) fertilization corresponding to 5 g N/m^2/yr) and released drought thereafter. We conducted a ^{15}N and ^{13}C labelling experiment during the peak of the first-year drought by injecting ^{15}N labelled fertilizer in the soil and exposing the tree canopies to ^{13}C labelled CO_2 in growth chambers. The abundance of the N and C isotopes in the roots, stem and needles was assessed during the following year.
- C uptake was slightly lower in drought stressed trees, and extreme drought inhibited largely the N uptake and transport. Carbon allocation to belowground tissues was decreased under drought, but not in combination with fertilization.
- Our results indicate a potential positive feedback loop, where fertilization improved the metabolism and functioning of the roots, stimulating the source activity and hence C allocation to belowground tissues. We conclude that soil nutrients might play an important role in mitigating drought stress of trees.

Keywords: carbon allocation, ^{13}C, drought, isotopes, ^{15}N, nitrogen allocation, *Pinus sylvestris*

Introduction

Carbon allocation is an important determinant of the C budget of forests and their response to changing environmental conditions. During the growing season, trees actively take up C and allocate it to growth, defense, respiration or storage (Chapin et al. 1990, Körner 2003). Seasonal fluctuations in C storage pools occur, with refilling of the pools in preparation for early spring growth, or depletion of pools when peak growth requires more C than is being assimilated (Oberhuber et al. 2011). The C used for early spring development of new shoots and leaves in deciduous trees may consist of 10% to even 50% from previously stored C (Hansen 1967, Kagawa et al. 2006), whilst in evergreen coniferous trees, the role of stored C is assumed to be smaller due to photosynthetic activity of the older needles. Drought might affect the use of stored C for the production of new foliage, considering the C limitation and its potential negative effect on phloem transport of stored C. Klein et al. (2014) assumed a close coordination between C supply and demand for the development of new needles in drought exposed Pinus halepensis leading to smaller needles rather than to stronger use of carbohydrate storage.

C allocation is generally prioritized to tissues increasing the uptake of limiting resources (Freschet et al. 2018). Changing environmental conditions can thus alter the C allocation strategy of trees. While mild drought has been shown to increase the transport of new assimilates to the roots for the production of larger water absorbing surfaces (Kozlowski and Pallardy 2002), extreme drought events seem to reduce the C supply to roots (Hommel et al. 2016, Salmon et al. 2019), either due to lower water use and photosynthesis, or due to reduced belowground sink strength, both leading to reduced phloem transport (Hagedorn et al. 2016, Hesse et al. 2018). The tipping points where a further increase in drought duration or intensity leads to a switch from increased to reduced belowground allocation of C, are however not well described.

Given the fact that the intensity but also the frequency of drought and subsequent rewetting events is predicted to increase in future (Easterling et al. 2000), it is important to better understand the ability of plants to recover from restricted water supply. Recent studies have shown that trees are able to prioritize C storage over immediate growth during recovery (Sala et al. 2012, Galiano Pérez et al. 2017). Moreover, plant C allocation after drought recovery has been found to be sink-driven, and shortly after rewetting, trees allocate C belowground, probably for restoration of drought-impaired roots (Hagedorn et al. 2016). In general, however, the mechanisms of C allocation that determine the recovery after drought are still far from being resolved.

Not only carbon, but also nutrients are indispensable for growth (Millard and Proe 1992) and survival (Gessler et al. 2016). Newly developing leaves are often supplied by both, stored and newly taken up nutrients (Millard et al. 2001). For evergreen trees, remobilization of stored nitrogen (N) can contribute up to 50% of the total N needed for new foliage, and there are indications that lower N storage can reduce the production of new leaves (Millard et al. 2001). Later on, during the growing season, trees rely mostly on root nutrient uptake. An interaction between drought and soil nutrient availability on tree functioning is likely to occur as the allocation of C and N are tightly related (Gessler et al. 2004, He and Dijkstra 2014). If no other resources are limiting, long-term high N availability is assumed to affect the stature of plants making them more susceptible to drought events, due to reductions in the root to aboveground biomass fraction and increasing assimilation rates, stomatal conductance and thus water loss (Gessler et al. 2016). On the other hand, drought might impair N uptake by the roots, increasing the C:N ratio and inducing nutrient limitation, eventually affecting many processes including stomatal sensitivity and root cell integrity. Furthermore, N allocation might be altered by drought, due to allocation of soluble N in the form of amino acids to the roots, for tolerance to dehydration (Fotelli et al. 2002). As ion mobility and nutrient uptake capacity become both impaired when water availability decreases (Kreuzwieser and Gessler 2010), sufficient soil nutrients could increase the available N to the rhizoplane, maintain or even improve general metabolic functions and cell integrity and thus promote a plant's ability to survive or to recover after a drought (Waring 1987, Gessler et al. 2016). Higher N availability for example might then allow to more efficiently synthesize N-containing osmoprotectants such as proline, which have positive effects on enzyme and membrane integrity (Ashraf and Foolad 2007) and thus might sustain root metabolism under drought. Severe drought, however, might fully inhibit the uptake of nutrients and their transport from the roots to the leaves independent of the soil nutrient supply.

In this study, we tested how a trees' C and N allocation during drought and the recovery from drought is influenced by the availability of nutrients in the soil. For this purpose, we combined $^{13}C\text{-}CO_2$ pulse labelling of the crowns of three-year-old Scots pine (*P. sylvestris* L.) trees with $^{15}N\text{-}NH_4NO_3$ labelling. We hypothesized that (1) C allocation to the roots increases relative to other tissues under drought but that C allocation to belowground tissues is inhibited if the drought gets too intensive, (2) fertilization results in less C being invested in roots and more in aboveground biomass under optimal water supply, but that with drought, fertilization can improve the C allocation to belowground tissues, especially under more intensive drought, (3) drought stressed trees have a strongly coordinated supply – demand regulation for C and N and thus do not deplete C and N reserves for needle growth early in the season, and (4) rewetting results in enhanced uptake and (re-)allocation of N to the needles when trees grew before under severe water limitation, while at the same time C allocation is prioritized for the restoration of the root system.

Materials and methods

Study site

This study was conducted in the model ecosystem facility of the Swiss Federal Research Institute WSL, Birmensdorf, Switzerland (47°21'48'' N, 8°27'23'' E, 545 m a.s.l.), which consists of 16 hexagonal open-top chambers (OTC) of 3 m height and a plantable area of 6 m^2 each. 12 of those chambers were used for this experiment. The roofs were kept closed during the entire experiment to exclude natural precipitation. Belowground, the chambers are divided into two semicircular lysimeters (1.5 m deep) with concrete walls. The lysimeters were filled with a 1 m deep layer of gravel for fast drainage, then a fleece layer that is impermeable for roots but permeable for water, and on top a 40 cm layer calcareous sandy loam soil (Table S4.1, Kuster et al. 2013). Each lysimeter was planted with 15 three years-old individuals of *Pinus sylvestris* saplings (55.61 cm +/- 5.41 cm height). Temperature and air humidity inside and outside the OTC, as well as soil moisture and soil temperature inside (at 5, 20, 35 cm depth) were automatically monitored (5TM soil moisture and temperature logger, Metergroup, Munich, Germany).

Water and fertilization/nutrient treatments

The experiment was set up as a split-split plot design. Each chamber was assigned one of three different water regimes as whole-plot treatment (four chambers / replicates per regime). Six sprinklers (1 m high) per lysimeter were evenly distributed, and irrigation was programmed for each lysimeter separately. The amount of water to be applied was controlled by means of the automated soil moisture measurements. Field capacity (W100 – 100% water) and wilting point (W0 – achieved by no irrigation at all), the two most extreme regimes, were determined by pF curves, and volumetric water content (VWC) for the irrigation regimes was adjusted accordingly, allowing for an additional ‘mild drought’, W20 regime, with 20% of the water available compared to W100. Water treatments started a year after planting. The irrigation system was in function from April to October in 2016 and from April to mid-July 2017, to prevent frost damage in winter. In winter and early spring, watering was done by hand (in W100 and W20) to maintain stable soil water levels. From the 13th of July 2017, all chambers were (re)watered until field capacity was reached in order to study the recovery process in the trees.

Twice a year, in April and July, one of the two lysimeters (split-plot) in every OTC was fertilized with liquid fertilizer (Wuxal, Universaldünger, NPK 4:4:3), corresponding to 5 g N/m^2/year. In April 2016 and in April and July 2017, the fertilizer was applied using 3 L water per lysimeter, and the unfertilized treatment was given 3 L water without nutrients, to prevent differences in water content between fertilization treatments. In July 2016, fertilizer was applied in combination with ^{15}N pulse labelling described below.

^{13}C and ^{15}N pulse labelling

In July 2016 (i.e. in the first year of treatment), a ^{15}N pulse labelling experiment was carried out in all irrigation regimes, but only for fertilized plots. Per lysimeter, 34.5 mL of the liquid fertilizer was mixed with 0.85 g ^{15}N labelled N (98 atom% ^{15}N, in the form of $^{15}NH_4{}^{15}NO_3$, Sigma-Aldrich, Buchs, Switzerland). The amount of ^{15}N corresponded to 8% of the total N given, and the total N corresponded to 2.5 g N/m, half of the yearly added amount. 900 mL water was added and the solution was injected with a needle (Ø 2 mm) with four lateral holes in the soil, at three different depths (5, 15 and 25 cm), evenly distributed over the planted area (20 cm grid) according to (Jesch et al. 2018). The labeling technique allowed to introduce ^{15}N into the lysimeter without significantly affect the actual water and fertilization treatment.

On 10 and 16 August 2016, a ^{13}C pulse labelling experiment was conducted. For feasibility, only the W100 and W20 water regimes were selected (4 chambers each). The W20 treatment was chosen above the W0 to ensure photosynthetic activity and thus uptake of CO_2. Two W100 and two W20 chambers were simultaneously labelled per day. The trees in the chambers were covered with a tall tent of transparent plastic foil. For the labelling application, per chamber, 7.5 g 99% ^{13}C sodium bicarbonate (Sigma Aldrich, Buchs, Switzerland) was mixed with 7.5 g standard ^{12}C sodium bicarbonate and hydrochloric acid in an airtight sealed beaker outside the chamber to generate the 50% labelled $^{13}CO_2$ gas. CO_2 concentration was measured using a Los Gatos Carbon Dioxide Analyzer (Los Gatos Research, San Jose, USA), which is able to detect both $^{12}C\text{-}CO_2$ and $^{13}C\text{-}CO_2$. The labelled gas was pumped into the chamber as soon as the CO_2 concentration inside reached approx. 300 ppm due to photosynthetic CO_2 uptake, and was brought and kept at ~ 500 ppm for approx. 1.5 hours. Fans inside ensured an even mixing of the air.

Tree harvests and stable isotope analyses

Whole tree harvests took place during the drought treatment in October 2016, July 2017 and, 3 months after rewetting, in November 2017. In general, one complete tree per lysimeter (with chamber as replicate, n=4) was sampled including the roots. In July 2017, after a high mortality in the W0 treatment, we decided to not harvest the surviving trees in W0. Root, stem and needle tissues were separated, dried at 60 ºC until stable weight and ground to fine powder. 1 mg (±0.1 mg) of the ground material was weighed in tin capsules and converted to CO_2 and N_2 in an elemental analyzer *Euro EA* (Hekatech GmbH, Wegberg, Germany) connected to an Isotope Ratio Mass Spectrometer (IRMS Delta V Advantage, Thermo Scientific, Bremen, Germany) to determine C and N contents and the isotopic compositions. C and N content were assessed as percentage relative to dry weight. Laboratory standards and international standards with known $\delta^{13}C$ and $\delta^{15}N$ values were used for calibration of the measurements, resulting in a precision of 0.1 ‰ for both elements. The isotopic ratios in all samples were expressed in δ notation (‰) relative to the international standard Vienna Pee Dee Belemnite (VPDB). To calculate the total amount of ^{13}C and ^{15}N added by pulse-labelling, δ notations were expressed in atom%, as follows:

$$atom\% = \frac{100\ \times RVPD\ \times (\frac{\delta}{1000} + 1)}{1 + RVPD \times\ (\frac{\delta}{1000} + 1)}$$

Where RVPD is the standard value for the isotope ratio of VPDB – 0.0111802 for ^{13}C and 0.0036765 for ^{15}N and δ is the $\delta^{13}C$ and $\delta^{15}N$ value, respectively. To calculate the excess ^{13}C and ^{15}N in the plant compartments in µg / g dry biomass, we used

$$excess\% = \frac{atom\%_s -\ atom\%_n}{100} \times \frac{Conc}{100}$$

Where atom$\%_s$ is the atom$\%_s$ in the labelled sample, atom$\%_n$ is the natural abundance of the isotope average per treatment (water / nutrients) before labelling, and Conc is the concentration of C or N in the sample.

Lastly, we calculated the proportion of the total added ^{13}C and ^{15}N in the plant compartments relative to the total plants' biomass using

$$Proportion = \frac{excess\% * DW_s}{DW_t}$$

Where proportion is the proportion of ^{13}C or ^{15}N in a certain plant compartment, DW_s is the dry weight of the plant compartment and DW_t is the dry weight of the tree individual.

Soil sampling

Soil samples were taken in October 2016, after the first tree harvest. Soil was dried at 60°C, ground to powder, weighed in tin capsules and total N concentration was measured using the EA-IRMS as described above. In addition, 7.5 g dry soil was extracted with 30 ml 1M KCl and filtered through filer paper (Albet-Hahnemuehle, Dassel, Germany) into 50 mL PE bottles. NH_4 concentration in the extract was measured photometrically with flow-injection (FIAS-400) and UV/VIS spectrometer (Lambda 2s, Perkin-Elmer, Schwerzenbach, Switzerland), NO_3 was measured by colorimetric analysis (Cary-UV50 spectrophotometer), using the absorption of nitrate at a wavelength of 210 nm. Soluble and exchangeable and microbial P were extracted using the method of Hedley (1982), modified by Tiessen and Moir (Tiessen and Moir 2006).

Statistical analysis and isotopic calculations

The effects of fertilization were tested with ANOVA using the measurements of soil P, NO_3 and NH_4, with both water and fertilization treatments as factors, and fertilization significantly increased P and NO_3 concentration, and the total N pool of plant and soil together (Fig. S4.1, Table S4.2). Linear mixed effect models were used to test the ^{13}C excess, and the proportions within a tree individual, against water treatments and fertilization and their interaction. The individual OTC's were taken as random factor. ^{15}N excess and distribution in the plant was tested for water treatment differences with LMER with individual OTC's as a random factor. Pairwise differences for both elements were tested with Tukey multiple comparison tests (package "multcomp" (Hothorn et al. 2019)). Every plant tissue and every harvest time were analyzed separately. All analyses were carried out with R v.3.5.1 (R Core Team, 2019).

Results

^{13}C incorporation and distribution

Water and fertilization regimes did not affect the ^{13}C excess in any tissue shortly after pulse-labeling during the first year of drought (October 2016), but clear treatment or interaction effects of both factors on ^{13}C excess on needles produced in 2017 (N17), roots, and stem were observed in the second year of drought (July 2017) (Fig. 4.1, Table S4.2). Drought caused an increase in ^{13}C in N17 needles independent of the fertilization treatment. In contrast, an interaction between the water regime and fertilization was observed in stems and roots, where the combination of drought with fertilization stimulated the allocation of larger amounts of ^{13}C to these two organs. After rewetting, the effects of the previous water regime of and fertilization were absent (November 2017) (Fig. 4.1, Table S4.2).

Also the relative distribution pattern of ^{13}C within the tree was affected by an interaction between drought and fertilization (Fig. 4.2). In line with the absolute ^{13}C excess results, only in unfertilized trees, the proportion of ^{13}C in the roots decreased from 30% ±4% in W100 to 12% ±5% in W20, whilst fertilized trees had 23% ±4% of the ^{13}C in the roots in both W100 and W20 trees (October 2016). In July 2017, these patterns were even stronger, and fertilization resulted in higher allocation to roots in W20 compared to W100 trees (Fig. 4.2). An interaction effect of drought and fertilization was also found in the allocation to new grown (i.e. N17) needles. Unfertilized trees allocated relatively more 'old C' (^{13}C assimilated in 2016) to needle growth in 2017 when affected by drought (8% in W100, 14% in W20), whereas fertilized trees allocated relatively less 'old' C in W20 compared to W100 trees (Fig. 4.2). There were no treatment differences in the proportion of ^{13}C ending up in the stem, but over time, the proportion of ^{13}C that was found in the stem gradually increased in every treatment. In summary, fertilization stimulated carbon allocation to belowground under drought, whilst ^{13}C stayed in aboveground tissues/needles in unfertilized trees under drought.

^{15}N incorporation and distribution

After the first year of extreme drought (W0) in October 2016, the ^{15}N excess was significantly reduced in the stem and needles but only slightly (and not significant) in the roots, compared to well-watered W100 trees (Fig. 4.3, Table S4.2), whilst W20 trees did not differ from W100. During the second drought year in July 2017, ^{15}N excess in the needles and roots was much higher in W20 trees than in W100 trees. After rewetting, the ^{15}N incorporation in previously W0 trees increased steeply in needles and stem, resulting in comparable amounts of ^{15}N in all treatments, and decreased in the roots, resulting in lower amounts of ^{15}N in the roots of W0 compared to W100 or W20 trees (Fig. 4.3). The very high variance in the ^{15}N excess in tissues of W0 trees were due to a lower amount of replicates after high mortality events, and probably also due to high variation in recovery potential of previously drought stressed trees.

By October 2016, trees in W100 and W20 transported 57% (± 7%) and 70% (± 2%), respectively, of the total ^{15}N taken up to their needles (ns between water treatments), whilst in W0 trees, the majority (72% ± 4%) of the ^{15}N stayed in the roots (Fig. 4.4). Only after rewetting, trees from the extreme drought treatment transported a significant amount of N towards needles, causing similar distribution patterns in W0 trees compared to W100 and W20 trees (between 46% - 62% in needles and 9% - 14% in roots), with the exception of the newly grown needles, that received only a minor percentage of ^{15}N. The proportion of ^{15}N recovered in the stem was generally constant between harvest dates and water treatments (Fig. 4.4).

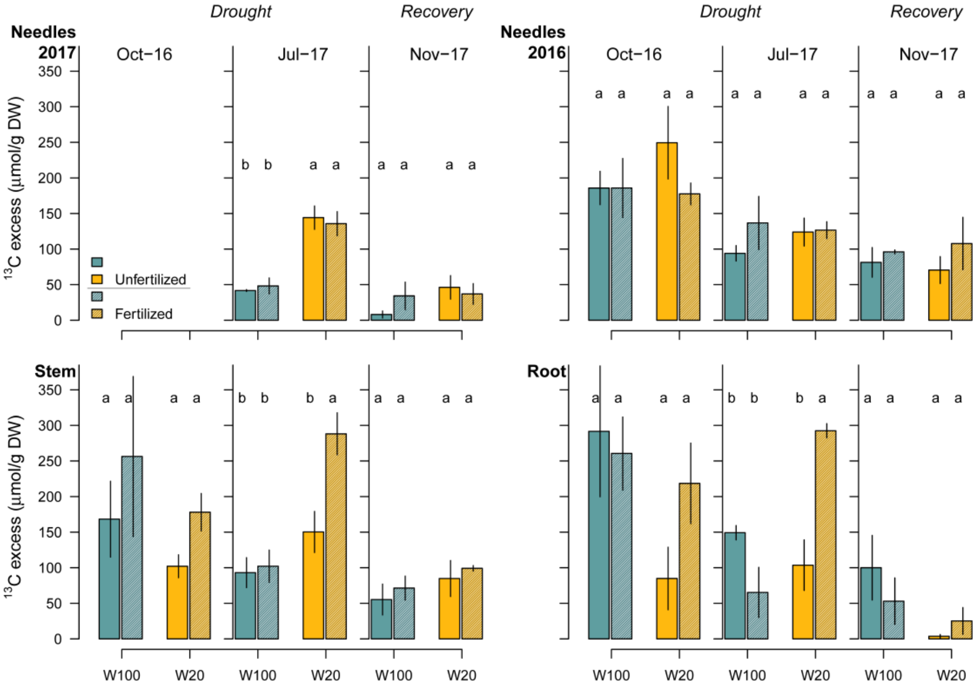

Figure 4.1: ^{13}C excess in needles produced in 2017 and 2016, stem and roots in October 2016, July 2017 and November 2017 (three months after rewetting). ^{13}C label was applied in August 2016. Water regimes are indicated by colors, fertilization is indicated by shading (solid = unfertilized, pattern = fertilized). Letters indicate significant differences between water and fertilization treatment within every tissue and harvest date. Error bars indicate standard error of the mean (SE).

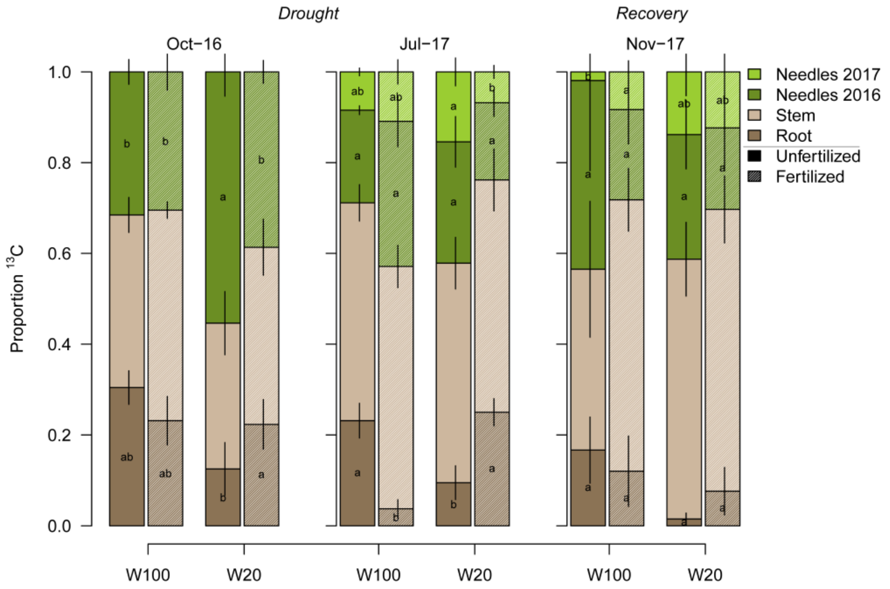

Figure 4.2: Proportion of total ^{13}C found in the different tree compartments – needles produced in 2017 and 2016, stem and roots (indicated by colors). Solid bars indicate unfertilized, and bars with pattern indicate fertilized trees. Letters in the bars indicate significant differences between water and fertilization treatments within every tissue and harvest date. Error bars show the standard error of the mean.

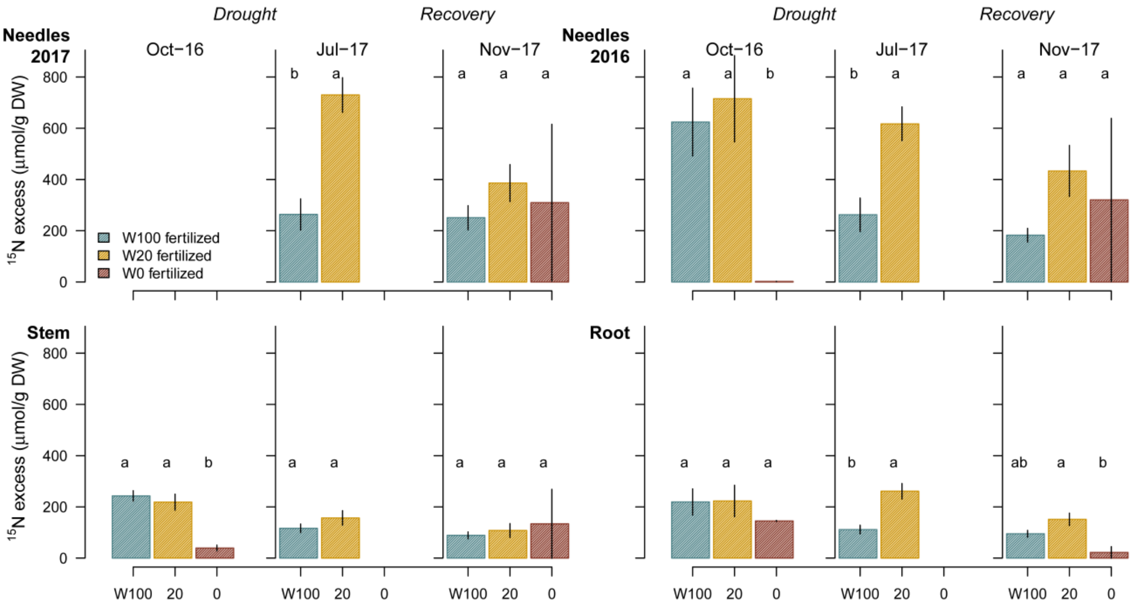

Figure 4.3: ^{15}N excess in needles produced in 2017 and 2016, stem and roots in October 2016, July 2017 and November 2017 (three months after rewetting). In July 2017, no samples were taken in the W0 treatment. Water regimes are indicated by colors, the shading indicates that only fertilized trees were tested. Letters indicate significant differences between water treatments within every tissue and harvest date. Error bars indicate standard error of the mean (SE).

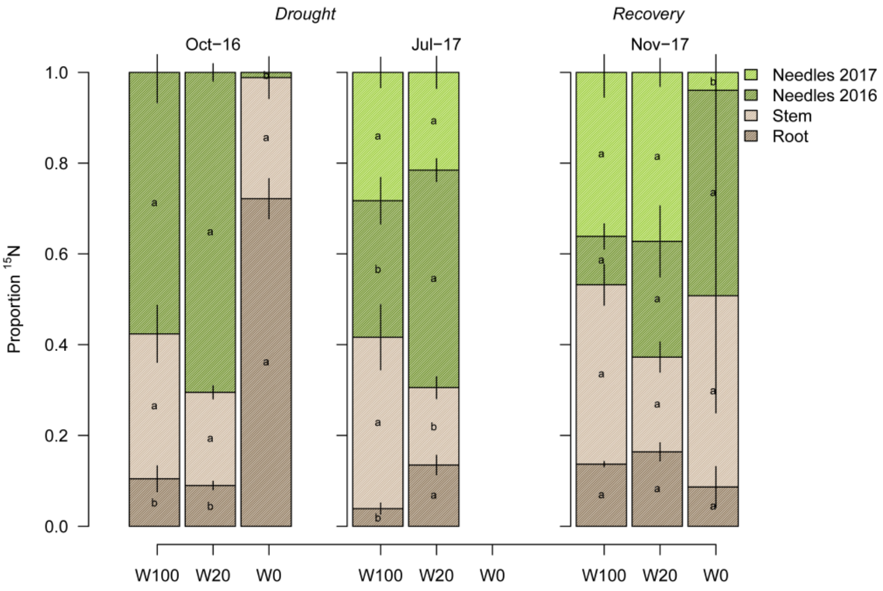

Figure 4.4: Proportion of total ^{15}N found in the different tree compartments – needles produced in 2017 and 2016, stem and roots (indicated by colors). Pattern shows that only fertilized trees were labelled and measured. Letters indicate significant differences between water treatments within every tissue and harvest date. Error bars show the standard error of the mean.

Discussion

Interaction between water and nutrition drives changes in belowground C allocation

We hypothesized an increase of C allocation to the roots relative to other tissues under mild drought, to improve the water uptake potential (Kozlowski and Pallardy 2002, Freschet et al. 2018). But the ^{13}C allocation to roots was much lower in the drought treatment compared to well-watered trees when no fertilization was applied (Fig. 4.1, Fig. 4.2). Hence, we had to reject our first hypothesis. We assumed that our W20 treatment would not impair C allocation to the roots. Although we considered the W20 drought regime as mild drought – photosynthesis and growth (data not shown), as well as N transport to aboveground tissues (Fig. 4.3) were not affected, and no mortality occured – the soil water restriction might already have been severe enough to disable transport of new assimilates to the roots. One possibility would be that root biomass still increased but with the use of older C reserves instead of newly assimilated C. In an earlier study, we found that C reserves such as starch and mobile sugars indeed decreased with drought in the roots in October 2016, but were restored in July 2017, and total root biomass was not increased but rather decreased in the W20 treatment if not fertilized (Schönbeck et al. 2020). Alternatively, the metabolic activity of the roots might have been impaired by drought and thus C demand was restricted (Hagedorn et al. 2016). Considering that root embolisms are probably the first to occur during severe drought stress (Rodríguez-Calcerrada et al. 2017) and root NSCs are the most sensitive and variable compared to NSC in all other tissues (Hartmann et al. 2013, Choat et al. 2018), we can speculate that the root system might be the first to lose function and eventually die off during extreme drought stress.

We hypothesized that fertilization decreases assimilate allocation to roots compared to aboveground biomass in well-watered trees, but that fertilization in combination with drought increases C allocation belowground, due to the maintenance of root metabolism by improved nutrient uptake. Assimilate allocation to roots was slightly but not significantly reduced due to fertilization under well-watered conditions, which also has been described in earlier studies (Kozlowski and Pallardy 2002, Gessler et al. 2016). Under non-limiting water conditions and increased nutrient availability, trees do invest more in aboveground biomass, causing lower root:shoot ratios. Under limiting water conditions, in accordance with our hypothesis, fertilization seemed to increase allocation of new assimilates to the roots. Nitrogen uptake of plants depends on the N availability to the roots, which is partially determined by the water mass flow and the nitrogen transported with it. Thus drought can, under constant soil nutritional conditions, cause nutrient limitation within plants (Fig. 4.5). We speculate that increased nutrient availability in the soil improved the root nutrient uptake and released the nutrient limitation (Fig. 4.5). The higher nutrient uptake could then trigger plant responses to drought by stimulating e.g. the synthesis of drought-responsive amino acids and proteins (e.g. Alam et al. 2010). These compounds play a central role in osmoprotection (Nguyen and Lamant 1988, Rathinasabapathi 2000, Ashraf and Foolad 2007, Galiano Pérez et al. 2017) and might strengthen the C sink function of the roots. We thus expect that as a consequence of improved root activity and cell integrity, sink activity was increased as indicated by increased C allocation belowground (Fig. 4.5).

Interaction effects between water and nutrition alters the C and N source of new needles

We hypothesized a strongly coordinated supply – demand regulation of C and N and thus expected that drought stressed trees do not use more stored C (i.e. ^{13}C) and N (i.e. ^{15}N) for growth of new needles than well-watered trees. The incorporation of ^{13}C in new needles per dry weight was, however, higher in W20 than in W100 trees in July 2017 (Fig. 4.1).

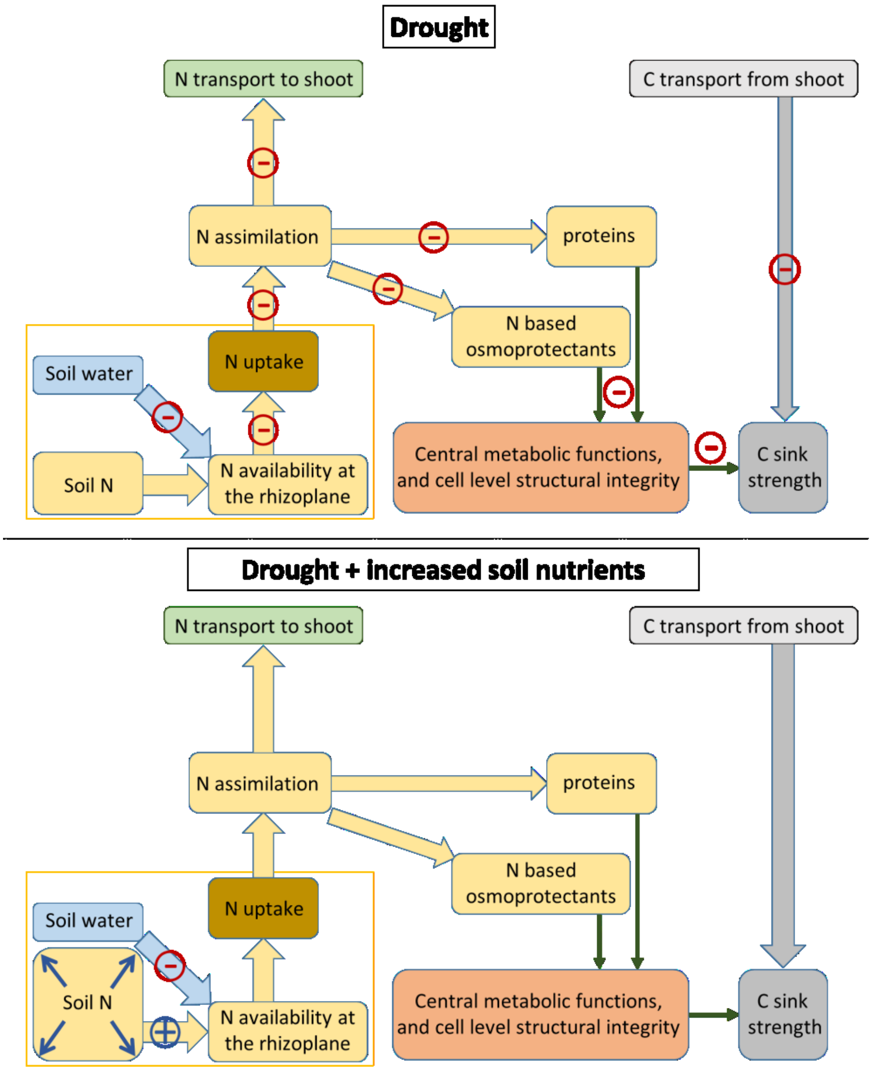

Figure 4.5: Conceptual framework on the role of soil N and drought in the allocation of assimilates. As both soil water availability (via water mass flow and thus transport of nitrogen) and soil nitrogen concentration influence the N availability at the rhizoplane, both can induce nutrient limitation to the plant. During drought, N based osmoprotectants might play an important role in maintaining central metabolic functions, sustaining or increasing the C sink strength and the C transport from the shoots to the roots. Drought might induce a nitrogen limitation because of decreasing transport of nutrients to the root surface (rhizoplane). An increase in soil N concentration could mitigate such N limitation due to reduced water mass flow and induce a positive feedback.

The difference in turnover rate of ^{13}C as well as a dilution of ^{13}C due to higher needle biomass in W100 trees could have led to the differences found in the absolute values of incorporation. But when looking at the proportional distribution within the tree, it became clear that indeed W20 trees allocated relatively more ‘old’ C into new needles, at least when unfertilized (Fig. 4.2). Fertilization cancelled out this allocation pattern and the opposite as in unfertilized trees was observed in reaction to drought. Although fertilization in well-watered conditions resulted in higher C allocation to the new needles, increasing the aboveground biomass and photosynthetic active area as postulated by Gessler *et al.* (2016), fertilized trees under drought had the lowest relative amount old C out of all treatments in the new needles. Considering nitrogen, we found that the ^{15}N amount per g needle was much higher in W20 compared to W100, while the proportion of total ^{15}N in the needles was similar between well-watered and mildly drought stressed (W20) trees, indicating that needle contribution to the total ^{15}N pool in W20 trees was only small, likely caused by small needle biomass. These findings do not lead to an acceptance of our third hypothesis, especially regarding C in unfertilized conditions, probably due to transport failure. Moreover, ceasing of the root system in unfertilized drought-stressed trees might have increased the importance of needles (and stem) as a storage tissue. At the same time, spatial imbalances might have occurred (Klein et al. 2014), where root but not needle functionality were affected by drought, not inducing any stress related changes in prioritization of C into growth or storage. Furthermore, coniferous trees are thought to be less dependent on stored C for spring regrowth than deciduous trees – in this experiment, only 10% of stored C ended up in new grown needles, compared to levels up to 50% found in deciduous trees (Hansen and Beck 1990, Kagawa et al. 2006). Hence the risk and the consequences of maintaining or changing the relative amount of reallocated C to new-grown needles under drought are relatively low.

Water availability after drought stress alters C and N allocation

For the recovery period, we expected that previously extreme drought stressed trees show an enhanced uptake and (re)allocation of ^{15}N and a prioritization of C allocation belowground in response to rewetting, in order to restore the root system. Indeed, a shift was found in the allocation of ^{15}N in previously extreme drought-stressed trees. Extreme drought (W0) initially caused almost no ^{15}N incorporated into needles after the first year of drought and most N was concentrated in the roots (Fig. 4.4). On the one hand, ceasing of phloem transport probably influenced the N distribution between below- and aboveground tissues. On the other, N allocation to the roots during drought is important to support drought tolerance in the form of osmoprotective aminoacids, as was previously shown in beech (Fotelli et al. 2002). Rewetting recovered N transport to the needles, and the distribution of ^{15}N was comparable between all drought regimes in November 2017. N transport from the roots to the shoot is important to restore the photosynthetic system and support aboveground metabolism and / or growth (Palacio et al. 2018). Moreover, rewetting caused extremely low ^{13}C-label allocation to roots of previously drought stressed trees (Fig. 4.1), and thus does not directly point to a prioritized C allocation belowground to restore the root system. We can thus not accept our last hypothesis regarding C allocation. However, assuming a recovery of the gas exchange in previously drought-stressed trees, we expect that the isotopic signal has been diluted by (non-labelled) new assimilates that have been allocated to regeneration of the root system. This is in agreement with findings of Hagedorn et al. (2016) of a strong prioritized transport of new rather than stored assimilates to the root system after drought release in beech.

Conclusion

We show that mainly the root system was affected by drought and fertilization, while the expected alterations in C allocation to aboveground tissues such as needles could not be proven. We speculate that the root system might have already been impaired by the 80% reduction of water availability when no fertilization was applied, indicated by reduced C allocation to the root system during drought. Nutrients might restore drought-induced alterations in C and N allocation, by contributing to the maintenance of cellular functions (e.g., via osmotic adjustment), consequently strengthening C sinks. Thus, an increased nutrient supply under drought does not only improve leaf metabolic functioning and cell structural integrity as suggested by Gessler et al. (2016) but might also be compensating for drought-induced loss of root functioning. Soil nutrients might thus play an important role in mitigating drought stress of trees.

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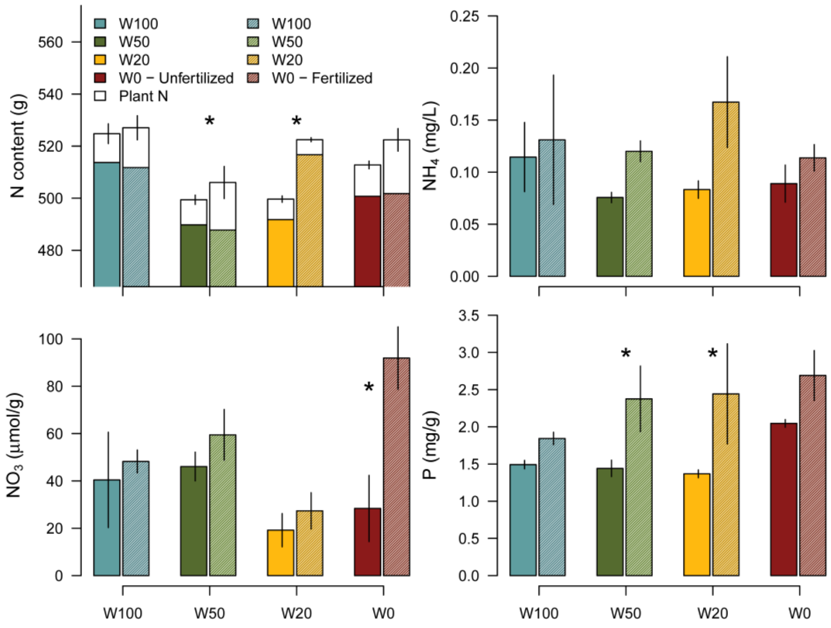

Figure S4.1: Total Nitrogen in grams in the ecosystem soil (colors) and plants (white bars), ammonium (NH_4) concentration (mg/L), nitrate (NO3) concentration (µmol/g) and phosphorus (P) concentration (mg/g) in the soil in October 2016. Colors represent the four drought treatments, W100 (blue), W50 (green), W20 (orange) and W0 (red). Patterns indicate fertilization treatments. Asterisks indicate significant differences between unfertilized and fertilized treatments within a water treatment. Error bars indicate the SE of the mean.

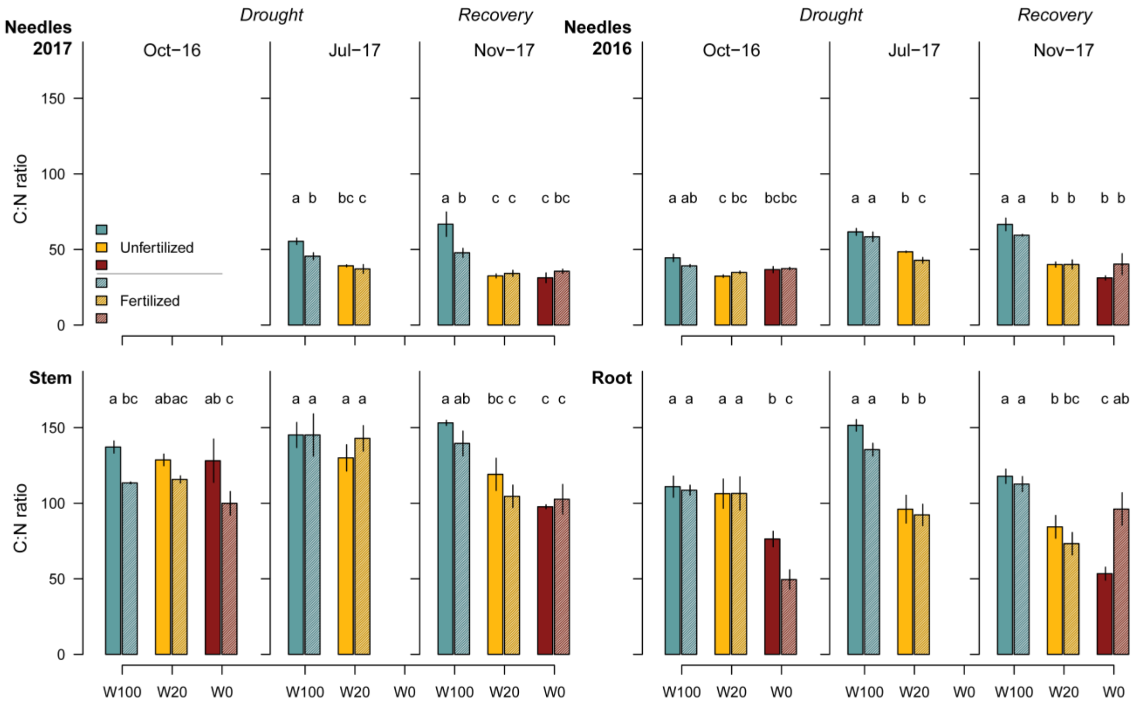

Figure S4.2: C:N ratios in needles produced in 2017 and 2016, stem and roots in October 2016, July 2017 and November 2017 (three months after rewetting). Water regimes are indicated by colors, fertilization is indicated by shading (solid = unfertilized, pattern = fertilized). Letters indicate significant differences between water and fertilization treatment within every tissue and harvest date. Error bars indicate standard error of the mean (SE).

Table S4.1: Original soil characteristics before start of treatment in the open top chambers (OTCs)

Characteristic	Calcareous sandy-loam
Origin	Brugg (Fluvisol)
Texture (% sand, silt, clay)	71, 18, 12
pH (0.01 $_M$ $CaCl_2$)	6.88
C_{tot} *(%)*	1.97
N_{tot} *(%)*	0.05
P_{tot} *($mg \cdot kg^{-1}$)*	357.96
$Ca_{exch.}$ *($mg \cdot kg^{-1}$)*	1629.46
$Mg_{exch.}$ *($mg \cdot kg^{-1}$)*	21.87
$K_{exch.}$ *($mg \cdot kg^{-1}$)*	25.75
$Mn_{exch.}$ *($mg \cdot kg^{-1}$)*	1.44
CEC ($mmol_c \cdot kg^{-1}$)	84.69
Base saturation (%)	99.45

Table S4.2: Anova table of the linear mixed effect model testing ^{13}C and ^{15}N excess in the different tissues against water and fertilization treatment and their interaction. Separate models were made per harvest date. Values written in bold indicate significant effects. Pairwise comparisons can be found in figure 4.1 and 4.3. For ^{15}N, only the water effect was tested, as only fertilized plots were labelled with ^{15}N.

		Root		**Stem**		**N16**		**N17**	
^{13}C	**Df**	**F**	**p**	**F**	**p**	**F**	**p**	**F**	**p**
October									
Water	1	3.50	0.124	1.75	0.213	0.45	0.526		
Fertilization	1	0.79	0.418	2.25	0.162	1.52	0.264		
W:F	1	1.85	0.24	0.01	0.914	1.52	0.264		
July									
Water	1	13.35	**0.004**	19.12	**0.008**	0.12	0.741	32.02	**0.001**
Fertilization	1	4.46	**0.058**	11.33	**0.022**	2.53	0.163	0.02	0.901
W:F	1	30.12	**<0.001**	8.79	**0.034**	1.97	0.210	0.93	0.373
November									
Water	1	4.36	0.082	1.99	0.186	0.00	0.988	1.35	0.29
Fertilization	1	0.19	0.679	0.56	0.470	3.05	0.131	0.54	0.488
W:F	1	1.37	0.286	0.00	0.966	0.57	0.480	2.42	0.171
^{15}N (only water tested)									
October	2	0.24	0.795	24.68	**0.003**	207.04	**<0.001**		
July	1	21.20	**0.006**	1.29	0.320	11.94	**0.014**	21.84	**0.003**
November	2	6.01	**0.030**	0.73	0.529	1.46	0.296	1.39	0.309

Additional work:
Phenology of Scots pine affected by drought, fertilization and changes in source:sink balance

Thesis written in order to obtain the
Diploma of Advanced studies 'Applied statistics' at the ETH
(condensed version)

Summary

- Drought reduces tree growth and productivity, and increases tree mortality and forest decline. Responses to drought may however differ depending on the characteristics of drought. Here, we assessed weekly growth patterns and hypothesized a gradual growth decline with decreasing water availability. The physiological mechanisms underlying this drought-induced tree mortality are however not clear and intensively debated.
- We exposed three year-old *Pinus sylvestris* saplings to drought using four different water and two soil nutrient regimes. In addition, debudding and partial needle removal were performed. During the growing season of 2016, we measured shoot and needle growth weekly.
- We found that growth was limited by both drought and defoliation treatments. Only extreme drought reduced needle length, whereas mild drought already caused a reduction in shoot length. Needle length, not shoot length, was positively influenced by fertilization. Drought and nutrients did not have an interacting effect on tree growth, but rather did they have an accumulative effect.
- The onset of drought before budbreak and growth resulted in immediate growth adjustment during the growing season. The direct effects of manual defoliation in well-watered as well as drought conditions show that slight defoliation causes C-limitation in saplings.

Introduction

The duration and intensity of summer droughts are predicted to increase and climate projections assume a higher frequency of droughts as well as a higher probability of intermittent high intensity rainfall events (IPCC, 2013). Drought reduces tree growth and productivity, and increases tree mortality and forest decline (Ciais *et al.*, 2005; Bigler *et al.*, 2006; Sánchez-Salguero *et al.*, 2012a; Balducci *et al.*, 2014). In Switzerland, the effects of extreme drought have been observed in the dry Rhone Valley, half of the Scots pine (*Pinus sylvestris* L.) died as a consequence of drought in the previous two decades (Rebetez & Dobbertin, 2004; Dobbertin & Rigling, 2006). Similar drought-induced declines of Scots pine have been observed in other parts of the European Alps and in southern France and Spain (Vila-Cabrera *et al.*, 2011; Aguade *et al.*, 2015).

Drought decreases turgor, which is required for cell expansion and thus growth (Lockhart, 1965). Hence, theory and data suggest that growth is the first to cease during drought, before photosynthesis (Körner, 2015). Shoot and stem growth may respond immediately to drought when drought occurs early in the season, but growth responses with one year delay have also been found to drought or drought release in young and old trees (Dobbertin *et al.*, 2010; Kuster *et al.*, 2013). Young trees grow by a factor of 2-10 each year, and this factor reduces with maturation. The turnover rate of tissues thus decreases, and adjustment to short-term stresses gets complicated with increasing size. It could thus be expected that young trees are able to adjustment to stresses rather quick.

Most vegetation is limited in productivity in nutrient availability (Fisher *et al.*, 2012). Increased nutrient levels in the soil lead to higher above-ground growth and a lower root biomass fraction and sapwood:leaf area ratio (Gessler *et al.*, 2017). Furthermore, nutrients might mitigate the negative effects of drought on tree functioning. Nitrogen (N) intensively controls stomatal conductance with low N availability increasing stomatal sensitivity towards drought (Radin & Ackerson, 1981; Ghashghaie & Saugier, 1989), and phosphorus (P) stimulates carbon assimilation (Mengel & Kirkby, 1987). Whilst extreme drought might inhibit the nutrient uptake of roots, a sufficient amount of stored nutrients before a drought could be beneficial to overcome drought. Whilst growth is influenced by both drought and nutrients, it is unclear how soil nutrients might influence growth response of trees to drought is unclear (Lévesque *et al.*, 2016; Gessler *et al.*, 2017).

Leaf shedding is a mean for both deciduous and evergreen trees to reduce the transpiring area during drought stress and thus to adjust to imbalances between water loss and supply (Sánchez-Salguero *et al.*, 2017). While leaf shedding can be a measure of trees to adjust unfavorable water balance, crown transparency can reduce shoot length and needle weight (Gottardini *et al.*, 2016). Drought stress might make trees more vulnerable for sudden extreme defoliation due to insect outbreaks. Extreme defoliation changes the C source-sink relationship of a tree, and might result in the loss of nutrients and a reduction in growth. As stresses like drought and insect outbreaks are very likely to occur concomitantly in the future, the importance rises to disentangle the effects of combined stresses on the C source-sink relations and tree survival.

In this experiment, I studied the interacting effects of drought, nutrient availability and leaf area. I hypothesized that: 1) Needle removal results in lower tree growth, as trees get C-source limited. 2) Bud removal increases shoot and needle length, as the C-sink and thus the C-demand gets smaller than the supply 3) Drought negatively influences shoot and needle growth, but has lower impact on more defoliated trees than on trees with a full crown cover. 4) Fertilization increases tree growth, but more in the well-watered than in drought stressed trees.

Materials and methods

Study site and treatments

Please refer to Chapter 3 of this thesis for a detailed description of study site and treatments.

Measurements

From 13 May 2016 onwards, shoot and needle growth of all trees was measured on a weekly basis until 9 August 2016. Shoot length was measured on the main (terminal) shoot of the tree. Needle length was measured on the same shoot and registered in steps of 1 cm.

Statistical analysis

For statistical analysis, only the final shoot and needle length were used, measured on 9. August 2016, as final shoot length and needle length are of highest interest. A mixed effects linear model was used to analyze the effects of water, nutrients and defoliation on final shoot length and needle length. Water treatment, nutrients and defoliation were used as fixed factors. In addition, chamber numbers were separated into row and column numbers, and these factors were added as block factors. Random factors were whole-plot error (chamber) and split-plot error (lysimeter). Spatial correlation between different trees was not accounted for. As this was a planned fully controlled experiment, no model selection was done but all results were interpreted according to the full model. The normality of the residuals were considered by looking at qq-plots at the split-plot level and the split-split-plot level. Pairwise differences for all significant factors and their interaction were calculated with Least Squares means. All analyses were done in the Statistical open-source program R (R Development Core Team, 2008).

Results

The time course of growth looks very similar among the different water regimes (Fig. A1). Shoot growth stopped after June 10th in all treatments (dashed vertical line in Fig. A1). Both defoliation and drought had a significant negative effect on final shoot length (Fig. A2, Table A1). All drought regimes (W50, W20, W0) caused significant reduction in shoot length compared to control, but were similar amongst each other. Fertilization did not affect shoot length (Fig A2).

Extreme drought (W0) reduced needle growth with 50% compared to control trees (Fig. A2) and caused growth to stop earlier than the other three drought regimes (Fig. A1). Defoliation caused additional reduction of needle length and did not pose any interaction with drought on the time course of growth or final length (Table A1). Debudding led to longer needles than control trees (Fig. A2). A significant interaction was found between water and defoliation treatments, which is mainly due to the fact that undefoliated and 2/3 needle removal trees reacted stronger on drought than trees with debudding and 1/3 and 3/3 needle removal. Fertilization increased needle length significantly with approximately 9% compared to ambient nutrient availability in all drought and defoliation regimes (Fig. A2, Table A1).

Discussion

As expected, needle removal indeed resulted in lower shoot and needle length in all water and nutrient treatments, but only extreme defoliation resulted in significant reduction in growth (2/3 and 3/3 removal). The reduction in growth due to defoliation has been shown in earlier studies (Puri *et al.*, 2015) and indicates that severe needle removal can cause C limitation in trees. Slight defoliation might not result in reduced growth yet because trees can compensate for reduced leaf area by increasing photosynthesis rates, or because trees use higher amounts of stored carbon reserves (NSC) for growth compensation (Wiley *et al.*, 2013; Puri *et al.*, 2015). This way, sufficient C uptake in the current and next growing season can be enabled.

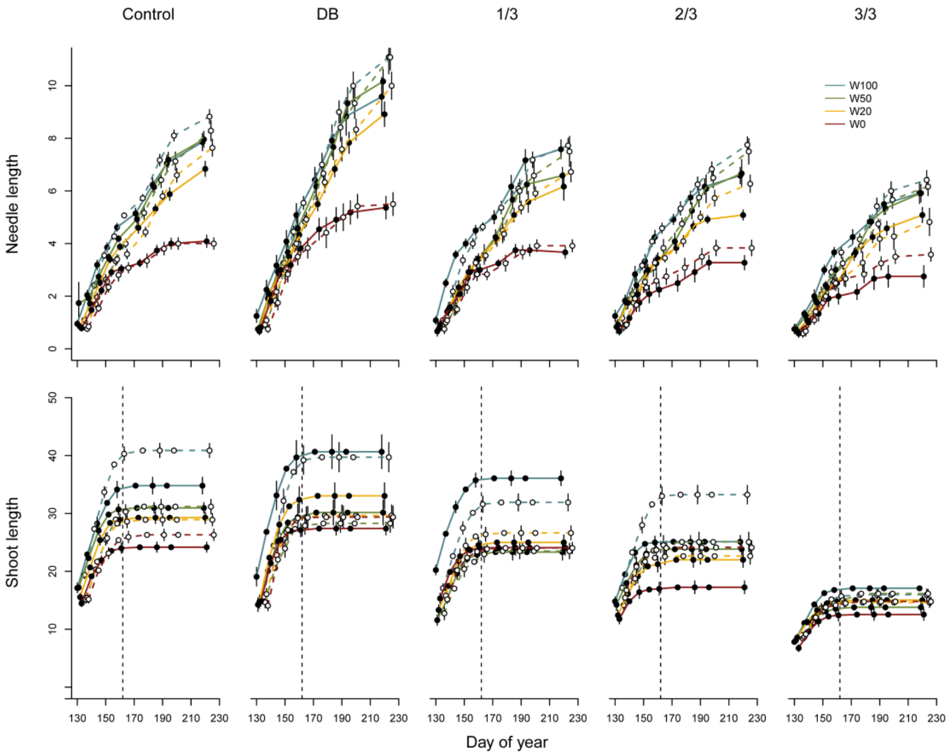

Figure A1: Needle and shoot growth over time in 2016, for all water, nutrient and defoliation treatments. Solid lines and closed circles show unfertilized, dashed lines and open circles the fertilized trees. Colours show the 4 water regimes. Headings above the graphs indicate the control, debudding (DB) or the amount of needle removal.

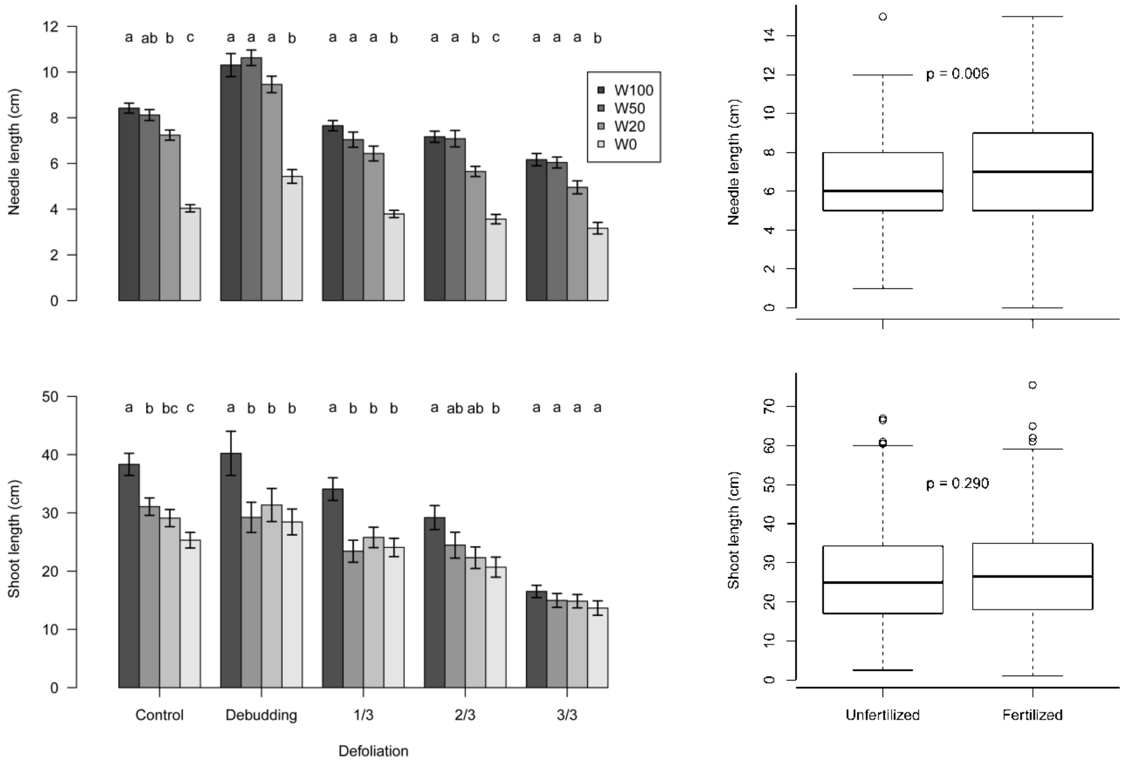

Figure A2: Final needle and shoot length on the last measuring day. Bar plots show the needle and shoot length in the drought (grey tones) and defoliation (x-axis) regimes. Letters above the bars indicate significant group differences between water treatments within a defoliation group. Boxplots show the overall difference between nutrient treatments, Error bars show the SE of the mean.

Bud removal indeed increased final shoot and needle length, corresponding to hypothesis 2. With a lower amount of buds, energy can be concentrated to the one bud standing. Interestingly, debudding had a bigger effect on needle length than on shoot length. Probably, a shoot has a certain maximum possible height. The amount of needles on one long shoot can probably not compensate for the amount that would have been present when all buds would have grown, and thus longer needles must be produced to ensure sufficient leaf area and C supply.

Drought reduced shoot and needle growth. The expected lower impact of drought on defoliated trees, compared to undefoliated trees, was however not observed. Manual defoliation did not lead to a lower sensitivity to drought. It is possible that defoliated trees compensated for lower leaf area by increasing photosynthesis, which in turn resulted in the same amounts of water loss and thus similar susceptibility to drought. Interestingly, shoot growth and needle growth behave differently in response to drought. Where shoot growth decreased significantly from W100 to W50, and stayed relatively similar with increasing drought stress, needle length was only significantly reduced in extreme drought (W0), whereas the mild drought regimes did not significantly affect needle growth. The possibility to shed needles, and thus adjust leaf area when necessary, allows for a higher investment in those tissues compared to structural woody tissues like stem and branches.

Fertilization only slightly increased shoot length but significantly increased needle length. This could be due to the fact that fertilization was applied in mid-April, when shoot growth was already advanced but needle growth had not started yet. Newly developing leaves are often supplied by both, stored and newly taken up nutrients (Millard *et al.*, 2001), and thus, it is likely that these new needles already benefited from the elevated nutrients in the soil. As nitrogen and phosphorus are mainly beneficial for the photosynthetic apparatus, fertilization might be more beneficial to needles than to shoots (Mengel & Kirkby, 1987; Brown & van den Driessche, 2005), which would explain the different response to fertilization.

This study shows that indeed, young trees are able to quickly adjust their growth to stresses when these stresses occur early in the growing season. Only weeks after onset of drought and application of fertilization and defoliation, shoot and needle growth got adjusted to the changing conditions. In adult trees, such rapid adjustments are not feasible, and probably other mechanisms play a role in prevention of stress, e.g. deeper rooting systems against drought stress and higher nutrient storage as buffer. I show here that the direct effects of manual defoliation in well-watered as well as drought conditions indicate C-limitation in saplings. Concomitant stresses might induce a combination of C-source and –sink limitation in saplings.

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Table 1: ANOVA results of differences in final needle and shoot length on the last measuring date, between row, column, water, nutrient and needle removal treatments..

		Needle		**Shoot**	
	df	***F***	***p***	***F***	***p***
Row	3	0.082	0.969	0.436	0.729
Column	3	1.144	0.358	2.131	0.132
Water (W)	3	78.667	**0.000**	17.107	**0.000**
Nutrients (N)	1	9.45	**0.006**	1.178	0.290
Nrm (Nr)	4	106.799	**0.000**	46.134	**0.000**
*W*N*	3	0.221	0.880	0.467	0.708
*N*Nr*	4	1.169	0.324	1.019	0.397
*W*Nr*	12	2.487	**0.004**	1.120	0.341
*W*N*Nr*	12	0.646	0.802	0.441	0.947

5

General discussion and conclusions

General discussion

Trees face several stresses during their lifetime, often related to water or nutrient limitation, or biotic attacks. Drought reduces tree growth and productivity, but the exact mechanisms behind drought-induced impairment of tree functioning, the pathways that lead to mortality, and the requirements for recovery after drought are still unclear. With this thesis I aim to shed a light on those mechanisms playing a role in tree mortality during drought. Firstly, I wanted to unravel the carbon and nutrient dynamics in Scots pine trees during drought. Secondly, whilst the potential role of nutrients before, during and after drought has been underexposed in literature and only recently been described (Gessler *et al.*, 2017), I aimed to find interactions between the effects of nutrient availability and drought on the before mentioned dynamics. Lastly, by changing the source:sink balance in trees, I wanted to unravel the supply and demand mechanisms of C in trees during and after drought.

The project was divided into two experiments. The first experiment, 'Pfynwald', gave insights in the long-term growth and C dynamics in adult Scots pine trees in a natural dry environment compared to trees irrigated with +100% precipitation during the growing season. The second experiment, 'MODOEK', allowed for a more detailed, whole-tree approach to study the C budget, allocation and utilization in saplings under different drought and nutrition levels. The two experiments cover a timescale from seasonal to multiyear (13 years) and together form a complementary view on C allocation, survival and mortality of Scots pine saplings and adults during drought. In this discussion, I synthesize the obtained results from the two experiments and reflect specifically on the questions:

- What are the effects of drought, rewetting and long term irrigation on growth, crown condition and tissue NSC levels?
- Can we identify tipping points in drought, tree functioning or vigor, beyond which mortality is inevitable?
- Do fertilization and changing source/sink relations mitigate the negative effects of drought on growth, C and N allocation and survival?
- How are C and N allocation and next years' reallocation influenced by drought stress and fertilization?

Drought and (subsequent) defoliation reduce tree growth

In Chapter 2 (Pfynwald), I showed that drought stressed trees had lower growth rates than trees that were released from drought since 13 years. In Chapter 3 (MODOEK) the biomass of extreme drought stressed saplings was significantly lower than well-watered saplings after one growing season (see also Additional work). In addition, leaf area was an important determinant of tree growth in both experiments. Trees shed their leaves in order to reduce the transpiring area during drought stress and thus to adjust to imbalances between water loss and supply (Sánchez-Salguero *et al.*, 2017). However, reduction in leaf area came with the cost of even more growth reduction both with natural and manual defoliation (cf. Chapter 2 and Chapter 3), even though photosynthesis increased after manual defoliation. From the viewpoint of sink limitation of tree growth, reduced sink activity would rather allow trees to reduce their leaf area corresponding to the lower C demand, than that defoliation would cause growth reduction. I argue that there is a threshold beyond which defoliation is rather a clear indicator of tree weakening than just an adjustment to growth demands, and that source limitation could get stronger than sink limitation. Manual defoliation led to reductions in shoot (Additional work) and biomass growth (Chapter 3), both in well-watered and drought conditions, indicating C source limitation also when defoliation could have been beneficial in combination with the drought treatment. In the Pfynwald, defoliation above approximately 60% led to a 'point of no return', where trees were not able to further adjust to, and recover from drought (Chapter 2). Similar threshold levels of 50-60% drought-induced defoliation were found leading to growth decline in other studies across Europe (Fischer *et al.*, 2004; Drobyshev *et al.*, 2007; Sánchez-Salguero *et al.*, 2012a). Consequently, it has been argued that defoliation might be rather an inevitable consequence of drought than a strategy to cope with stress (Poyatos *et al.*, 2013).

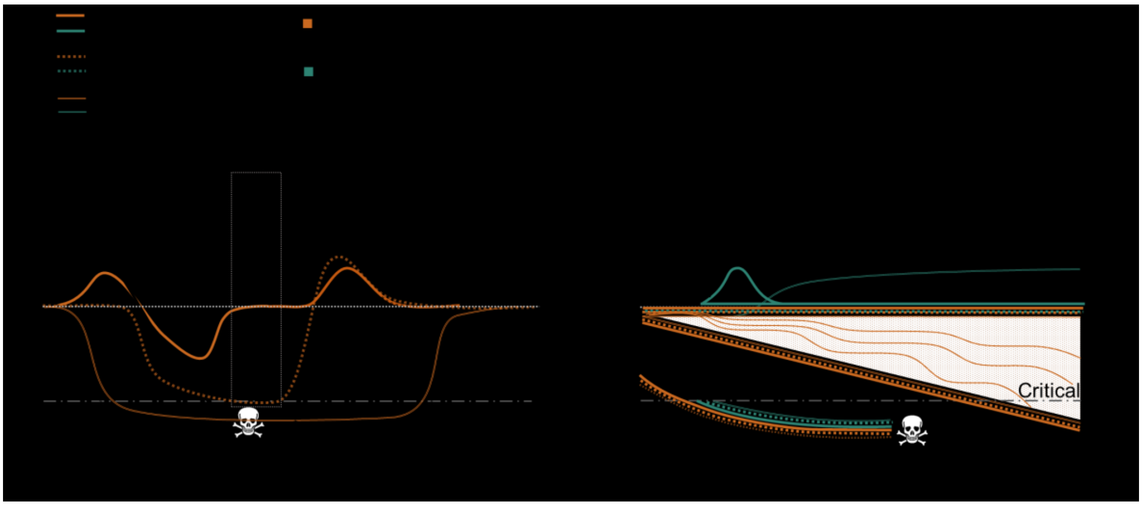

Figure 5.1: Visual summary of the findings of this thesis, adapted from McDowell *et al.* 2011 and Figure 2.1. On the short term (a), mild drought (orange) reduces growth and photosynthesis, but only causes a slight deviation of NSC before reaching homeostatic levels again. Extreme drought (black) causes stronger reductions in growth, photosynthesis and NSC, and when NSC levels sink below the critical threshold, recovery is inhibited and mortality occurs. In figure (b), the long-term trajectories of drought and rewetting are compared, as a prolonging of the grey dashed rectangle in (a). From the 'starting point', trees with high leaf area show generally stable growth and NSC during mild drought, but sudden extreme drought or biotic stresses might push a tree towards a weakening process that causes loss of leaf area and a loss of NSC, resulting in the large orange range in the figure. Recovery is possible as long as the tree did not cross the 'critical NSC or leaf area threshold'. Trees with initial low leaf area are already close to that threshold and rewetting does not benefit them anymore. Rewetting results in steep increases of NSC and growth (a). Long term rewetting (a&b) results in homeostatic NSC and photosynthesis, while growth rates might stay higher than before (b).

Acclimation to long-term mild stress

Trees might acclimate to reductions in water availability. In adult Scots pine for example, sudden but long term reduction in water availability caused six years of growth reduction before they adjusted growth back to previous rates (Feichtinger *et al.*, 2014). However, trees in the Pfynwald rather seem to show continuous growth decline, and it was shown that this decline is an early indicator of tree mortality (Timofeeva *et al.*, 2017). In ca. 84% of the mortality events across the world, a decrease in radial growth before death was observed (Cailleret *et al.*, 2017). This decline and thus the actual mortality process can last decades before the final point of death (Timofeeva *et al.*, 2017).

The fact that growth decreased with drought, whereas photosynthesis was unaffected or only slightly affected (resp. Chapter 2 and 3) points to a confirmation of the proposed theory that growth has higher sensitivity to drought than photosynthesis (Körner, 2015). McDowell *et al*. (2011) speculated that if growth ceases but photosynthesis does not, NSC should accumulate in the tree during a short mild drought. Enduring drought would result in depletion of NSC levels due to reduction in photosynthesis and higher demand than supply for e.g. defense, respiration or osmotic adjustments. However, a central conclusion that can be drawn from this thesis is actually that trees aim for homeostatic NSC reserves, but that both very extreme and long-term drought can reduce NSC levels to very low levels (see Fig. 5.1). Apart from the seasonal NSC fluctuations (cf. Hoch *et al.*, 2003; Oberhuber *et al.*, 2011), short-term disturbances cause deviations from the generally stable NSC pool level. After the first growing season in the MODOEKs, drought caused a reduction in starch levels in the roots, and an increase in needle sugar concentrations (Chapter 3). When drought was not too extreme, the trees prioritized energy allocation to storage over growth, resulting in a recovery of NSC concentrations to control levels (Chapter 3, Fig. 5.1a – orange lines). In the Pfynwald, the long-term higher availability of water in the irrigation treatment did not lead to higher levels of NSC, instead to higher growth rate and maintenance of NSC levels between the treatments. Similar to the saplings acclimating to drought, C allocation in the adult Pfynwald trees was balanced between growth and storage in such a way that stable NSC levels were maintained. In contrast, extreme drought (Chapter 3) and enduring drought (Chapter 2) not only reduced growth but also photosynthesis, NSC and leaf area (Chapter 2). Such a reduction in tree vigor ultimately leads to mortality in trees and saplings (Chapter 2 and 3).

Thresholds and tipping points

Acclimation to mild drought is possible - trees in the Pfynwald have been growing in relatively dry conditions for 100 years. Many trees are showing constant growth and photosynthesis rates and stable NSC concentrations. However, increasing drought and a higher frequency of extreme droughts on top of that, is probably the trigger that causes the tipping point from trees following the ‘surviving trajectory’, to the ‘mortality trajectory’ (orange decreasing curves in Fig. 5.1). These extreme droughts on top of the long-term mild drought, weaken trees such that leaf area – and, correlated with this, the NSC concentration – decreases below the threshold for survival. This was corroborated by the observation that trees with initially low leaf area had limited ability to respond to prolonged irrigation (Chapter 2). The defoliation process continued in those trees, ultimately leading to mortality.

In Chapter 3, it was the threshold in drought intensity that determined reductions in growth, photosynthesis and the occurrence of tree mortality. Only extreme drought led to tree mortality during the second growing season of drought. NSC concentrations of dead trees were close to zero at the point of death. This could be one of the reasons for mortality. However, the possible occurrence of hydraulic failure cannot be excluded, as extreme drought stressed trees experienced predawn twig water potential between -1.5 to -1.9 MPa, which is at the limit of the hydraulic safety margin of Scots pine. Scots pine is known as a species that preserves needle water potentials above approximately -1.5 MPa to prevent xylem embolisms (Irvine *et al.*, 1998; Salmon *et al.*, 2015). It is very likely that a combination of rapid tree weakening in terms of C balance (i.e. ceasing of photosynthesis and consequently a decrease in NSC) and embolisms caused the relatively fast mortality of these saplings in the extreme drought regime.

Carbon and nitrogen allocation are influenced by drought and nutrient availability

Mild drought has been shown to increase the transport of new assimilates to the roots and thus allow the production of larger water absorbing surfaces (Kozlowski & Pallardy, 2002). I expected therefore to see increased root biomass fraction in drought stressed trees of regime W50 and W20, assuming that W20 was still a mild drought – photosynthesis and (aboveground) growth were not influenced and mortality did not occur. However, in unfertilized trees, root biomass fraction did not increase with drought, whilst fertilization enabled this increase. These results indicate that soil moisture tipping points seem to be variable and can be modified by the addition of nutrients.

In Chapter 4, I made use of isotopic tracers to find out the mechanisms behind these allometric adjustments during drought. Here I found that drought might already impair tree functioning earlier than was assumed in Chapter 3. Although I considered the W20 drought regime as a mild drought the soil water restriction might have already been severe enough to impair root metabolism and disable transport of new assimilates. The ^{13}C label that we had applied was not allocated to the roots in the W20 drought regime when no fertilization was applied. When fertilization was applied, C allocation to the roots was similar in the drought stressed and well-watered trees. Under fertilization, N-based osmoprotectants and drought-responsive proteins are likely to maintain root functioning and sink activity, which allows for greater root growth to forage for water. Nutrients, for example nitrogen, are known to reduce the root biomass fraction under non-limiting water conditions, as trees do not have the urge to forage for water or nutrients. In Chapter 4, I show how nutrients can also stimulate the opposite process when other sources get limited. The fact that many physiological processes (e.g. photosynthesis, NSC dynamics) did not seem to be affected by drought (Chapter 3) whilst C allocation belowground was (Chapter 4), suggests that root growth and functioning might be the first processes to cease during an intense drought. This speculation is strengthened by the fact that root NSC levels are fluctuating the most in response to drought, compared to other tree tissues, both in mature trees in Pfynwald and saplings in the MODOEKs (Chapter 2 and 3).

Recovery

Although the term recovery has been mentioned a few times in this discussion already, this was mostly related to the recovery ability of the Pfynwald trees during 13 years of irrigation. The short-term recovery process after drought was assessed using the saplings in the MODOEKs, where a more mechanistic understanding of the processes could be gained. Rewetting resulted in a very steep increase of photosynthesis, and in an overshoot of photosynthates (NSC). Also root starch levels recovered or got even slightly higher than in trees that have been watered constantly, whereas root sugar levels stayed slightly lower than control trees (cf. Chapter 3, Fig. 5.1). In Chapter 4 I showed that rewetting restored the N uptake and allocation, resulting in a similar incorporation of ^{15}N in the needles and stem in all water treatments. This all indicates that, whilst the root system was dysfunctional during relative mild drought, the function recovered soon after rewatering. Probably a lot of C was invested belowground to recover root biomass and functioning (Hagedorn *et al.*, 2016).

Concluding this part of the discussion, impairment of tree functioning and thus tree mortality can be explained by thresholds: long term drought causes a reduction in tree vigor and relative leaf area, and I could show that trees that cross the threshold of approximately 60 – 70% loss of foliage will follow the trajectory towards mortality, independent if they get rewatered. Extreme drought leads to a fast mortality process, but addition of nutrients might influence the soil moisture thresholds for tree functioning and mortality. Drought might affect the root system already quite early, which is proven by the impairment of C allocation belowground, the reduction in NSC and the lack of ^{15}N uptake in extreme drought. C allocation during recovery after drought seems to be strongly sink driven (e.g. Hagedorn *et al.*, 2016; Galiano Pérez *et al.*, 2017), ensuring a rapid recovery of the root system. Trees have a strong coupling of demand and supply mechanisms that balances C and nutrient allocation to growth, storage and defense. This results in homeostatic NSC levels when trees acclimate to changing environmental conditions.

Methodological aspects

Old versus young trees

Due to the size and lifespan of adult trees, the majority of physiological experiments are done on seedlings and saplings and extrapolated to adult trees. However, it is important to study both saplings and mature trees, and to find similarities between them, before extrapolation of physiological processes from saplings to adults becomes trustworthy. The development from seedling to adult requires different allocation patterns and changes in allometry, photosynthetic capacity, and growth during maturation suggest that allocation of carbohydrates may be different between seedlings and mature trees (Hartmann *et al.*, 2018). For example, saplings can perform more anisohydric behavior and take more risk than the mature individuals of the same species (Oberhuber *et al.*, 2015). Moreover, the absolute concentrations of NSC have been shown to be very different between seedling and adult (Hartmann *et al.*, 2018) and also in this study, total NSC concentration was two to three times higher in all tissues of saplings compared to adult trees. This has mainly to do with the fact that NSC in young trees is much less diluted – there is a lower proportion of old and non-storing tissue in young trees.

The total biomass of the saplings (well-watered and not defoliated) in the MODOEK increased with factor 2 over one growing season, so new grown tissue accounts for 50% to the total biomass. In contrast, the needles, for example, of mature trees in Pfynwald have a lifespan of three to four years, and thus new grown needles only account for approximately 25% of the total leaf area. Hence, it can be expected that saplings have a much higher short-term adjustment potential to environmental changes than mature trees. On the one hand, this hypothesis is rejected by several studies that find that seedlings and saplings are actually more sensitive to small scale fluctuations of the environment, as mature trees might be able to buffer changes better due to larger above- and belowground biomass to access resources (Bazzaz, 1996; Oberhuber *et al.*, 2015). Moreover, mature trees appear to be able to increase their water use efficiency to a much greater extent during drought than seedlings (Cavender-Bares & Bazzaz, 2000). On the other hand, it is argued that the larger belowground biomass does not necessarily imply deeper roots, which is mainly needed during drought stress. Furthermore, it has been shown that hydraulic vulnerability increases with tree height, as the distance over which water has to be transported and the tension this creates on the water column increases (McDowell & Allen, 2015). In conjunction with these results, a largescale meta-analysis showed that worldwide, larger trees suffer most during drought (Bennett *et al.*, 2015).

The differences between adult and young trees still pose many challenges for the improvement of forest and vegetation models, and the definition of generalized thresholds or tipping points seems still far from solved. Comparative studies between adults and saplings, as presented in this thesis, are necessary to fill the gap between physiological and modelling studies. Although also from this study, much can still only be speculated, the similarity in the general C supply and demand strategy during and after drought in young and mature *P. sylvestris* trees might be a promising result for the extrapolation of physiological studies to large-scale ecological models.

NSC – what does it tell?

The paradigm on NSC has been developing and changing over the years. NSC is still considered to have the lowest priority for a trees' C utilization, with accumulation occurring only when other sinks are saturated (Hartmann *et al.*, 2018), but an increasing number of studies, including this one, finds that NSC storage is not just pure C accumulation, but rather C reserve formation, after the concepts of Chapin *et al.* (1990), who distinguished between accumulation, reserve formation and recycling in defining 'storage'. Moreover, considering the fact that nobody has found an absolute zero-level of NSC concentration yet, it is clear that NSC concentration is not a direct indicator of the C balance and health of a tree, but rather the whole tree carbon mass balance should be considered to unravel the mechanisms underlying drought-induced tree mortality (Klein & Hoch, 2014). Although this study shows that declining NSC levels are correlated with tree mortality, it is still a challenge to identify the causes and the consequences of the one and the other.

The fact that absolute zero-levels of NSC cannot be demonstrated has partly physiological reasons – it is speculated that a tree will never use up the entire C reserve pool, similar to the fact that starvation in mammals occurs before blood sugar levels are down to zero (Hoch, 2015). Another reason lies in the fact that the methodology of NSC extraction is not standardized and knows many different versions. Recently it was shown that these different methods generate a wide range of absolute sugar and starch vaulues (Quentin *et al.*, 2015). As such differences reduce the replicability, prohibit the comparison of absolute values between studies, and thus will never generate a trustworthy 'absolute zero-level' of NSC, new efforts have been made to standardize the methodology for NSC measurements (Landhäusser *et al.*, 2018).

Outlook

In this thesis, I answered a number of questions on the physiological mechanisms behind drought-induced mortality. New findings generally lead to new questions and challenges in research, of which I name a few:

The Black box

I realize how much we still don't know about tree functioning, partly because the root system of the tree is still a 'black box', difficult to open and look into. Just recently, researchers emphasized the fact that even some fundamental information on the relationship between root biomass, nutrient availability and nutrient uptake were unknown (Dybzinski *et al.*, 2019). Also, in this thesis, it is shown that roots are incredibly dynamic and very sensitive to changes in soil moisture and nutrition. However, roots are not often considered in studies on for example NSC. That is why a very important outlook point is to deepen our understanding of root functioning under changing environmental conditions. This includes studying the turnover rate of roots, the speed of deterioration of roots during drought, and the uptake rate of water and nutrients under different environmental conditions. An improved knowledge on root functioning will also improve modelled predictions on the effects of global change on terrestrial ecosystems.

Deepening our knowledge on the role of nutrients during drought-induced mortality

Following up on the previous outlook, the role of nutrients in drought-induced tree mortality is still far from resolved, as this thesis is giving new insights but is also opening doors towards new questions. In this thesis, we speculate how the stimulation of nitrogen containing osmoprotectants might sustain root functioning under drought. This in turn might shift the tipping point of soil moisture where plant mortality increases. Not only the availability of nutrients, but also the timing of fertilization can play a role here. In this study, the role of nutrients seemed mostly mitigating, but early exposure to high nutrient levels might have the negative predisposing effects on tree posture as discussed in Gessler *et al.* (2017). Thus, where is the tipping point from the predisposing to mitigating role of nutrients? And can we define specific tipping points for drought to belowground C allocation, over a range of nutrient availability in the soil?

Hydraulic failure vs carbon starvation

The results given in this thesis shed new lights on the allocation of C and N in trees, the prioritization mechanisms, but it remains extremely difficult to define the immediate cause of death of the trees. This is partly due to the fact that mortality is most probably a result of abiotic and additional biotic stresses, like infestation of bark beetles, a phenomenon that is known to occur especially in already weakened trees. Another aspect is the fact that it is still very difficult to measure hydraulic failure due to embolisms in the xylem, on intact plants. This results in a never-ending question whether trees die from hydraulic failure, carbon starvation, or both. Being able to simultaneously measure C and water transport and utilization would bring us closer to answering this fundamental question. Techniques to measure embolism on intact plants are being developed and are improving, among which microcomputed tomography and magnetic resonance imaging are promising but still expensive and time-consuming methodologies (Windt & Blümler, 2013). Combining such measurements with short- and long-term NSC dynamics will generate a better understanding on the whole plant C and water utilization.

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Thesis: Temperature and precipitation determine tree establishment in *Sphagnum* dominated peatlands. (Grade: 8.5/10)

2001 – 2007 **Gymnasium**
Agnieten College, Carolus Clusius, Zwolle, the Netherlands
Major: Nature and Health
Additional: Latin, Greek, Sociology

Work experience

09/2014 – 02/2015 **Research intern**
WSL and Wageningen University

9/2011 – 09/2012 **Student representative**
Wageningen UR Student Council (co-participation council)

Teaching experience

09/2018	**Teaching assistant during school visit “Junior research”** *WSL and MINT Lernzentrum (ETH)*
04/2018	**Teaching assistant “Scientific visualisations using R”** *WSL*
11/2017	**Teaching assistant in statistics course “Mixed effect modelling”** *WSL and Oikostat*

Extracurricular activities

2016-2018	**PhD representative** *WSL* Organizing activities for PhD students at WSL: Inhouse data day, Statistics course on mixed effect models, Presentation skills course.
08/2015	**Participant NFZ summerschool ‘ForeScale’** *Organized by INRA Nancy, University Freiburg and WSL*
2010 – present	**Violinist** *Several orchestra’s, including the Dutch Student Orchestra, Dutch Student Chamber Orchestra and the Academic Orchestra of Zürich*
09/2007 – 06/2008	**Travelling** through Australia and New Zealand

Peer-reviewed publications

Schönbeck L, Gessler A, Schaub M, Rigling A, Hoch G, Kahmen A, Li M-H. 2020. Soil nutrients and lowered source:sink ratio mitigate effects of mild but not of extreme drought in trees. *Environmental and Experimental Botany* **169:** 103905

Grossiord C, Gessler A, Reed SC, Borrego I, Collins AD, Dickman LT, Ryan M, Schönbeck L, Sevanto S, Vilagrosa A, McDowell, NG. 2018. Reductions in tree performance during hotter droughts are mitigated by shifts in nitrogen cycling. *Plant, Cell & Environment* **41**: 2627–2637

Gessler A, Cailleret M, Joseph J, Schönbeck L, Schaub M, Lehmann M, Treydte K, Rigling A, Timofeeva G, Saurer M. 2018. Drought induced tree mortality - a tree-ring isotope based conceptual model to assess mechanisms and predispositions. *New Phytologist,* **219**: 485–490

Schönbeck L, Gessler A, Hoch G, McDowell NG, Rigling A, Schaub M, Li M-H. 2018. Homeostatic levels of nonstructural carbohydrates after 13 yr of drought and irrigation in *Pinus sylvestris*. *New Phytologist*, **219** (4), 1314-1324

Li M-H, Yong J, Wang A, Li X, Wanze Z, Yan C-F, Du Z, Shi Z, Lei J, Schönbeck L, He P, Yu, F-H, Wang X. 2018. Active summer carbon storage for winter persistence in trees at the cold alpine treeline. *Tree Physiology*, **38** (9), 1345-1355

Schönbeck L, Lohbeck M, Bongers F, Ramos MM, Sterck F. 2015. How do light and water acquisition strategies affect species selection during secondary succession in moist tropical forests? *Forests*, **6** (6), 2047-2065

Talks

Schönbeck L, Gessler A, Rigling A, Schaub M, Hoch G, Kahmen A, Li M-H. 2018. Drought stress in Scots pine: Could nutrients help to survive? *GfÖ Meeting, Vienna, Austria.* 03.10.2018

Schönbeck L, Gessler A, Rigling A, Schaub M, Li M-H. 2017. The winner takes it all? Who benefits from drought release? *LWF – Pfynwald Workshop, WSL, Birmensdorf, Switzerland.* 31.10.2017

Schönbeck L, Gessler A, Rigling A, Schaub M, Li M-H. 2017. Drought stress release increased growth rate but did not affect levels of storage carbohydrates in Scots pine trees. *IntEcol, Beijing, China.* 22.08.2017

Schönbeck L, Gessler A, Rigling A, Schaub M, Li M-H. 2015. Carbon and nutrient dynamics in response to drought. *LWF- Pfynwald Workshop, WSL, Birmensdorf, Switzerland*

Posters

Schönbeck L, Gessler A, Hoch G, Kahmen A, Rigling A, Schaub M, Li M-H. 2018. Mitigating effects of nutrients on drought stress in Scots pine. *SwissForestLab Science day, Birmensdorf, Switzerland.* November 2018

Schönbeck L, Gessler A, Rigling A, Schaub M, Li M-H. 2017. Drought stress release increased growth rate but did not affect levels of storage carbohydrates in Scots pine trees. *EGU General Assembly, Vienna, Austria*. April 2017

Schönbeck L, Li M-H, Gessler A, Rigling A, Schaub M, Hoch G, Kahmen A. 2015. Drought induced mortality in the Swiss Rhone Valley – A study on the coupling of carbon dynamics and nutrients in Scots pine. *NFZ Summerschool 'ForeScale'.* August 2015

Media

Schönbeck L, Kaennel Dobbertin M, De Girardi N. 2018. Surviving drought (3m07s)
https://www.youtube.com/watch?v=OA7A-xWhDeo

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