Boron interactions with poplars in deficient and contaminated soil

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Publication Date:
2011

Permanent Link:

https://doi.org/10.3929/ethz-a-006833935 →

DISS. ETH NO. 19823

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A dissertation submitted to the

ETH, Zurich
Eidgenössische Technische Hochschule, ETH Zürich

for the degree of

Doctor of Sciences
(Dr. sc. ETH Zürich)

presented by

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2011

Acknowledgements

I would never have been able to finish this piece of work all alone and many people contributed in one or the other way to it. Therefore I would like to thank the following people:

First and foremost I would like to express my gratitude to my supervisor Prof. Dr. Rainer Schulin, who has given me so much freedom with regards to my scientific work and working environment. However, this freedom would have been worthless without the guidance he provided. Without it, I would have kept turning in circles around single trees instead of seeing the poplar forest growing around me. I am also thankful to Rainer for always having an open office and for giving me to opportunity to promote my scientific work.

I would like to thank P.D. Dr. Brett Robinson for his enthusiasm and the inspiring ideas he had about this work. Thank you very much again for coming over last June; this visit was really important to me and this thesis, as it finally really launched the writing process. I would also like to thank Prof. Dr. Walter Wenzel from BOKU, Vienna for accepting to act as co–advisor of my thesis.

My thanks also go to Dr. Eberhard Lehmann from the Paul Scherrer Institute for introducing me to neutron radiography and giving me the opportunity to work with this amazing technique. I would also like to thank the members of the Neutron Imaging and Activation Group at PSI especially Peter Vontobel, Stefan Hartmann and Anders Kästner for their support and technical help.

I am grateful to Chris Rog and Bart Sexton from Sand Creek Consultants for giving me the opportunity to carry out my field work at the site in Rhinelander, WI, USA. They supported me with all kinds of technical and logistical help and data and showed me how and that phytomanagement works in the “real” world. I want to thank them for the pleasant collaboration.

At ETH Zürich technical assistance came from Réne Saladin, who did a great job building the containers for me and from Björn Studer. I am sorry for not having had his great lab skills for support in an earlier stage of this work already. Big thanks go to Judit Valentini and Stephanie Conrad for sharing the pleasure of week-long root washing with me. Andreas Papritz helped me a lot with all kinds of statistical questions and problems.

I would also like to thank all my colleagues at ETH Zürich and especially those from the soil protection group who supported and accompanied me in an (at least most of the time) unscientific way. However, I had some great and inspiring scientific discussions with my desk neighbor Bernd Felderer, and even though they used to turn on his work most of the time, they were of great help for me as well, thank you Bernd. I would also like to thank Minimum boulder club for helping me to hang on the holds and on “mens sana in corpore sano” and my colleagues Anja Gramlich and Erika Fässler for joining me there more and more often lately. I am also grateful to all my friends outside ETH who supported me in one way or another during my PhD.

Finally, I would like to express my gratitude towards my whole family for their love, their unconditional support and encouragement in good times as in bad. There is one last person I have to mention here: My wife Eva. My deepest appreciation goes to Eva for her patience, support and understanding and everything else.

Contents

Summary

Boron (B) is an essential micronutrient for plants and higher animals. The optimum range of B for plant growth is narrower than for any other micronutrient. Boron deficiency as well as toxicity limit plant growth on agricultural lands worldwide. Furthermore, B is a very mobile trace element in soils, and B leaching from contaminated sites threatens ground and surface waters at many places. Major anthropogenic sources of B in soils are the irrigation of agricultural lands with B rich waters, mining and fly ash deposition. For the clean-up of B contaminated sites phytoremediation could be an option. Hybrid poplars already find widespread use in phytoremediation and as they accumulate inordinate amounts of B in their aerial tissues, they are in particular considered candidate plants for the remediation of B contaminated soils and have been successfully used for the phytomanagement and hydraulic control of B contaminated sites. The objectives of this study were (I) to investigate the B accumulation potential and tolerance of hybrid poplars under controlled conditions; (II) to elucidate how B tolerance and accumulation of poplars are affected by heterogeneous distributions of B in soil and (III) to examine to what extent such effects are relevant under field conditions.

To assess the B tolerance and accumulation potential of poplars we performed a pot experiment in which hybrid poplar *Populus nigra* × *euramericana* were grown on a substrate with B concentrations ranging from 13 to 280 mg kg^{-1}. *Salix viminalis, Brassica juncea* and *Lupinus albus* were grown for comparison. To determine critical leaf B concentration thresholds, the spatial distribution of B within individual poplar leaves was determined by neutron radiography (NR) and related to leaf toxicity symptoms. Poplar growth was not affected at treatment levels ≤93 mg kg^{-1}, but significantly reduced at levels of 168 and 280 mg kg^{-1}, respectively. None of the other species survived at the latter two soil B concentrations. At whole leaf B concentrations <900 mg kg^{-1} at most 10% of the leaf surface was chlorotic or necrotic. Neutron radiography revealed that chlorotic leaf tissues had B concentrations of 1000 – 2000 mg kg^{-1}, while necrotic tissues had >2000 mg kg^{-1}.

Some portions of the leaves had concentrations >7000 mg kg^{-1}, indicating that necrotic leaf tissue still received B with the stream of transpiration water.

As with other trace elements, B is heterogeneously distributed in soils in typical field situations. As it is notoriously difficult to determine the spatial distribution of all relevant factors in soil with sufficient accuracy under field conditions, we compared the growth and B accumulation of young poplar plants growing on soil with constructed B heterogeneity to poplars growing on soils with a homogenous B-distribution. In a first experiment we varied the concentration of soil B by mixing artificially B-spiked soil at various ratios with unspiked soil. In the heterogeneous treatment one half of each container was filled with B-spiked soil, the other with unspiked soil. A homogeneous treatment with similar average soil B concentrations was set up for comparison. Poplar growth and B accumulation were unaffected by the spatial distribution of B in the soil and only dependent on the average soil B concentrations in the containers. The reduction in root growth correlated with a reduction in shoot growth with increasing shoot B concentrations. In soil where B concentrations exceeded 20 mg kg^{-1}, we found also local toxicity effects on root growth. In a second experiment we investigated root growth at a given level of average soil B concentration for homogeneous and heterogeneous B distribution in more detail. Fine root (FR) length growth appeared to be more inhibited under B stress than growth in thickness, as indicated by a shift in root diameter length distribution. Using NR we found depth growth of roots in B contaminated soil to be slowed down compared to an unspiked control, independent of the homogeneity of spiking.

To compare the results from the two aforementioned studies with a real-world field situation, we conducted a field survey on a paper mill waste landfill. The mixture of paper mill wastes consisted of paper mill sludge, ashes and cinders, all of them by-products from pulp and on-site energy production of a nearby paper mill. As these wastes contained high concentrations of B and heavy metals, placing them in a land fill poses risks for ground and surface waters. Poplars were planted to reduce these risks. We assessed the growth and survival of four different poplar clones, investigated the accumulation of B by the trees and explored the relationship between local-scale root growth and substrate properties. For this purpose, we sampled leaf and root tissue of the poplars, analyzed them for their chemical properties and root traits and recorded tree growth parameters in the field. A soil core was taken to a total depth of 20 cm next to each tree, divided in to 4 sections and analyzed for various element concentrations and other properties.

Based on these parameters a principal component analysis was conducted, followed by a hierarchical cluster analysis, which resulted in the identification of 3 different substrate types. Despite the complexity of factors and interactions at the site, this method allowed the differentiation of the effects of the substrate types on tree survival and growth. Although B was accumulated in the foliage of some trees up to toxic levels, it was not limiting growth. Instead, we found that growth was primarily limited by an imbalance in the leaf Mg:Ca ratio, inducing Ca deficiency in the trees. There was no indication of root growth limitation due to toxicity of the contaminants.

The B accumulation ability of all 6 poplar clones employed in the three parts of this study fits some of the criteria for B hyperaccumulation. However, because of the passive nature of the B accumulation in poplars we concluded that poplars rather hypertolerate than hyperaccumulate B. The first experiment showed that the B hypertolerance of poplars was associated with a high B tolerance in the living tissue and the storage of B in dead leaf tissue. Our results suggest that in poplars root growth reduction is primarily a systemic top-down response to B toxicity in the shoots caused by exposure to high soil B concentrations. Only at soil B concentrations >20 mg kg^{-1} also local toxicity effects were found to stop root growth. We conclude from these results that local heterogeneity in soil B should have little influence on the phytoremediation of contaminated sites using poplar, as long as the contamination allows root growth. Our lab and field results on B accumulation and tree growth show that poplars are well suited to be used in phytomanagement and for establishing vegetation on B contaminated soils.

Zusammenfassung

Bor (B) ist ein essentielles Spurenelement für Pflanzen und höher entwickelte Tiere. Der Bereich der Optimumkonzentration von B für das Pflanzenwachstum ist enger als für alle anderen Spurenelement und sowohl B-Mangel als auch B-Toxizität hemmen das Pflanzenwachstum auf landwirtschaftlichen Flächen weltweit. Wenn B in hohen Konzentrationen von belasteten Flächen ausgewaschen wird, gefährdet es Grund- und Oberflächengewässer. Die wichtigsten anthropogenen B-Quellen für Böden sind das Bewässern von landwirtschaftlichen Flächen mit belastetem Wasser, Bergbau und die Ausbringung von Flugaschen auf Landwirtschaftsflächen. Pappelhybride werden verbreitet in der Phytoremediation eingesetzt und da sie aussergewöhnlich grosse Mengen von B in ihrer oberirdischen Biomasse aufnehmen, könnten sie auch für die Phytoremediation von B-belasteten Flächen geeignet sein. Die wichtigsten Ziele der vorliegenden Arbeit waren (I) unter kontrollierten Bedingungen die B-Akkumulation und die B-Toleranz von Pappelhybriden zu untersuchen; (II) zu untersuchen wie sich die heterogene Verteilung von B im Boden auf die B-Aufnahme und die B-Toleranz von Pappeln auswirkt und mögliche Auswirkungen der heterogenen B-Verteilung auf die Wurzeln zu erforschen und (III) zu testen ob sich die im Bereich B-Toleranz, B-Akkumulation und Wurzelwachstum gewonnenen Erkenntnisse unter realistischen Bedingungen im Feld bestätigen lassen.

Um die B-Toleranz und das Akkumulationspotential von Pappeln zu bestimmen wurde ein Topfversuch durchgeführt, in dem ein Klon von *Populus nigra* × *euramericana* in einem Substrat mit B-Konzentrationen von 13 – 280 mg kg^{-1} gepflanzt wurde. Zum Vergleich wurden *Salix viminalis, Brassica juncea* und *Lupinus albus* gepflanzt. Um den Grenzwert der B-Konzentration im Blattgewebe zu bestimmen bei dem Toxizitätseffekte auftreten, wurde mittels Neutronenradiographie die Verteilung des Bors im Blatt gemessen und mit dem Auftreten von Toxizitätssymptomen verglichen. Das Pappelwachstum wurde bei B-Konzentrationen von bis zu 93 mg kg^{-1} im Substrat nicht beeinträchtigt, wohingegen die Pappelbiomasse bei höheren Konzentrationen stark reduziert war. Keine der Arten, die zum Vergleich gepflanzt wurden, überlebten Konzentrationen von mehr als 93 mg kg^{-1}. Bei Blättern, in denen die durchschnittliche B-Konzentration kleiner als 900 mg kg^{-1} war, war

nur 10% der Blattoberfläche durch B geschädigt. Anhand der Neutronenradiographien konnte bestimmt werden, dass chlorotische Blattoberflächen eine B-Konzentration von 1000 – 2000 mg kg^{-1} hatten, während nekrotische Blattoberflächen Konzentrationen von mehr als 2000 mg kg^{-1} aufwiesen. Einzelne Stellen in den Blättern wiesen Konzentrationen von mehr als 7000 mg kg^{-1} auf, was darauf hinweist, dass im nekrotischen Gewebe weiterhin B abgelagert wurde.

Bor und andere Spurenelemente sind unter Feldbedingungen heterogen im Boden verteilt. Ihre räumliche Verteilung, sowie die Verteilung anderer relevanten Faktoren im Boden mit ausreichender Genauigkeit zu bestimmen, ist sehr schwer. Daher verglichen wir junge Pappeln die auf Böden mit künstlich erzeugter heterogener B-Verteilung wuchsen, mit ebensolchen die auf Böden mit homogener B-Verteilung wuchsen, hinsichtlich ihrer B-Toleranz und Aufnahme. Eine Teilmenge des gesamten benötigten Bodens wurde mit Borsäure gemischt. Durch das Vermischen mit weiteren Teilmengen wurde eine B-Konzentrationsreihe von Böden hergestellt. In der heterogenen Behandlung wurde jeweils eine Hälfte der Container mit B-versetztem Boden gefüllt und die andere Hälfte mit Boden, dem kein B zugesetzt worden war. Das Wachstum und die B-Aufnahme der Pappeln wurden durch die ungleichmässige Verteilung des B im Boden nicht beeinflusst, sondern durch die Durchschnittskonzentration von B im Boden bestimmt. Mit Zunahme der B Konzentration im Spross kam es zu einer parallelen Abnahme der Spross- und Wurzelbiomasse. In der höchsten heterogenen Behandlung, in der die B-Konzentration lokal >20 mg kg^{-1} war, kam es zu einer negativen lokalen Beeinflussung des Wurzelwachstums. Ein zweites Experiment diente der detaillierten Untersuchung des Wurzelwachstum in einem Boden, der zwar mit der gleichen Menge von B versetzt war, diese wurde aber in der heterogenen Behandlung in einem Drittel und in der homogenen Behandlung im ganzen Container appliziert. Das Längenwachstum der Feinwurzeln war unter B-Stress stärker gehemmt als das Dickenwachstum, was durch eine Verschiebung in den Wurzeldurchmesserlängenklassen deutlich wurde. Durch die Anwendung von Neutronenradiographie zeigte sich, dass unabhängig von der B-Verteilung im Boden die vertikale Wachstumsrate der Wurzel reduziert wurde. Um die Ergebnisse der beiden Experimente mit einer realistischen Situation zu vergleichen, führten wir eine Feldstudie auf einer Papiermühlenabfalldeponie durch. Auf der Deponie wurden Papiermühlenschlamm, Aschen und Schlacken abgelagert Diese Abfallstoffe waren mit B und Schwermetallen belastet, was mit Risiken für Grund- und Oberflächengewässern verbunden war.

Das Wachstum und Überleben von 4 Pappelklonen wurde erfasst und die B-Aufnahme der Pappeln wurde ebenso untersucht wie mögliche Zusammenhänge zwischen dem Wurzelwachstum und den Bodenparametern. Proben der Pappelblätter und Wurzeln wurden genommen und auf ihre chemischen Eigenschaften sowie die Parameter des Wurzelwachstums hin analysiert. Bei jedem Baum wurden 4 Bodenproben schrittweise bis zu einer Tiefe von 20 cm genommen und ihre chemischen sowie physikalischen Eigenschaften bestimmt. Anhand dieser Eigenschaften wurde eine Hauptkomponentenanalyse durchgeführt. Die Werte der Bodenproben auf den ersten 3 Hauptkomponenten wurden dann für ein hierarchisches Clustering verwendet, aufgrund dessen eine Einteilung der Proben in 3 Substratklassen erfolgte. Trotz der Komplexität der Faktoren am Standort erwies sich diese Methode als äusserst hilfreich, um einen Zusammenhang zwischen den Bodenparametern, dem Wachstum und dem Überleben der Pappeln herzustellen. Obwohl die B-Konzentrationen in den Blättern einiger Bäume toxische Werte erreichten, war B nicht der wachstumslimitierende Faktor am Standort. Stattdessen zeigte sich, dass ein durch ein hohes Mg:Ca-Verhältnis hervorgerufener Ca-Mangel wachstumslimitierend war. Es gab keine Anzeichen einer Limitierung des Wurzelwachstums durch eine eventuell vorhandene Substrattoxizität.

Die B-Akkumulationsfähigkeit aller 6 untersuchten Pappelarten entsprach einigen der Kriterien für Hyperakkumulation. Die Passivität der B-Aufnahme der Pappeln liess aber lediglich den Schluss zu, dass es sich hier um Hypertoleranz und nicht um Hyperakkumulation im strengen Sinne der Definition handelte. Wie wir in dieser Studie zeigen konnten, wurde die Hypertoleranz von B in Pappeln durch die hohe Toleranz des lebenden Blattgewebes und die Speicherung von B in totem Gewebe ermöglicht. Die Auswirkungen von B auf das Wurzelwachstum schien hauptsächlich durch eine systemische Top-down Regulierung des Wurzelwachstums ausgelöst worden zu sein, die wiederum durch B-Toxizität in der oberirdischen Biomasse ausgelöst wurde. Lediglich bei sehr hohen Konzentrationen von B im Boden wurde auch lokal eine negative Reaktion des Wurzelwachstums beobachtet. Solange das Wurzelwachstum aufrechterhalten werden kann, sollten daher lokal hohe B-Konzentrationen einen geringen Einfluss auf die Phytoremediation von belasteten Flächen mit Pappeln haben. Unsere Ergebnisse hinsichtlich Toleranz und Akkumulation von B unter heterogenen und homogenen Bedingungen und ihre Übertragbarkeit auf einen B-belasteten Feldstandort zeigen, dass Pappeln sehr gut für das Phytomanagement und die Rekultivierung von B-belasteten Flächen geeignet sind.

1 Introduction

1.1 Background

1.1.1 Boron in the environment

Boron (B) occurs in the environment primarily in the form of borates. Major natural sources of B in the atmosphere and aquatic environments are runoff from the weathering of clay-rich rocks, volcanic activity and sea-spray (Howe, 1998; Park et al., 2002). The mean concentration of B in the earth's crust is 10 mg kg^{-1}, but concentrations can be as high as 100 mg kg^{-1} (Evans et al., 1983). Borax ($Na_2[B_4O_5(OH)_4]\cdot 8H_2O$) is the most important mineral in B mining. In 2005, the worldwide B consumption reached 1.8×10^9 kg and was expected to rise further (Kot, 2009). Major uses of B are in glasses, detergent products, fire retardants, agriculture and metallurgy (Parks et al., 2005). Boron used in the glass industry is strongly bound in borosilicate glasses and is therefore immobile and environmentally benign. Recently, B compounds have replaced persistent organochlorine insecticides protecting lumber against insects and fungi (Gentz et al., 2006). Boron used in timber treatment is subject to leaching and may therefore be harmful to the environment (Robinson et al., 2003; Obanda et al., 2008). Wood ash production, which has much increased in recent years due to the popularity of wood combustion for CO_2-"neutral" energy production, is also of concern with regards to B and other micronutrient releases into the environment (Reimann et al., 2008). The oceans are the most important sinks for B, where B is trapped in sediments or co-precipitated with SiO_2 or $CaCO_3$ (Carrano et al., 2009). In aquatic environments B occurs mainly as boric acid (H_3BO_3). Boric acid is a weak Lewis acid with a pK_a of 9.24 and a solubility in water of 0.55 g l^{-1} at 25°C.

High levels of B are found naturally in soils in arid areas and negatively affect crop yields in some areas (Stangoulis et al., 2002). Boron contamination of soils results from a wide range of human activities, including the use of B rich irrigation waters, surface mining of B rich minerals, wood treatment, the excess application of B-rich fertilizers and fly ashes (Nable et al., 1997; Robinson et al., 2003; Parks et al., 2005).

Remediation techniques for B-contaminated soils include, the leaching of B with excess water, the application of amendments for B detoxification and vegetation management, i.e. the use of more tolerant plants have been employed (Nable et al., 1997).

1.1.2 Boron in humans and animals

Boron is an essential micronutrient not only for plants (Marschner, 1999), but also for higher animals and possibly also other organisms. Recent studies have shown beneficial effects on human health (Mastromatteo et al., 1994; Hunt, 2007). Evidence of the essentiality of B for humans came from the discovery of a B transporter protein that is specific for mammals (Park et al., 2004). Major sources of B in human nutrition are water and vegetables. However, conclusive proof that B is essential for humans is not available as a possible biochemical function has not been clearly identified yet. Beneficial effects of B for humans were found for glucose and vitamin metabolism, bone stability and brain function, but not all off these effects could be confirmed in studies following the original reports (Hunt, 2007; Kim et al., 2008; Nielsen, 2008). At high intakes rates, B is toxic to animals and humans. The limit for B in drinking water is 2.4 mg l^{-1} according to the recommendations of the WHO (2009). Guideline values for daily B intake that should not be exceeded in human nutrition are compiled in Jensen (2009) and range from 0.16 to 0.4 mg B kg^{-1} body weight. These guideline values are mainly based on animal experiments with rats where B toxicity led to reduced reproduction rates and fetal weights. However, direct transfer of these results to humans is questionable as no clear evidence for negative influences of B on male fertility was found in a field study where daily B intake rates were10-fold higher than suggested by the guidelines (Scialli et al., 2010). Boron is not biomagnified in terrestrial food-chains because of the low P_{OW} (octanol /water partition coefficient) of boric acid, which leads to rapid excretion (Saiki et al., 1993; Howe, 1998). The B toxicity threshold for livestock fodder is 800 mg kg^{-1} (Underwood et al., 1999).

The risk that B poses to aquatic ecosystems is thought to be low (Howe, 1998). However, aquatic organisms show a wide range of B sensitivity. While fish can stand high B concentrations in water when exposed for a short period of time, chronic exposure to B leads to negative effects at relatively low concentrations. The most sensitive species tested were rainbow trout with a no-observed-effect concentration (NOEC) of 0.009 – 0.103 mg B l^{-1}, depending mainly on the developmental stage of the fish, with earlier developmental stages being more sensitive (Birge et al., 1977). However, most other fish species showed much higher B tolerance in the same study, with EC_{50} (half maximal effective concentration;

induces a response halfway between the baseline and the maximum response, i.e. being fatal) of 50 – 200 mg B l^{-1}. In an experimental ecosystem study with aquatic microorganisms, an NOEC of 2.5 mg B l^{-1} was found for chronic exposure (ECETOC, 1997).

1.1.3 Boron in plants

In plants, B plays an essential role in carbohydrate metabolism and transport, cell division, function of the apical meristem, nucleic acid synthesis and cell-wall crosslinking via pectin components (Matoh, 1997; Howe, 1998; Cosgrove, 2005). Boron deficiency can affect vegetative and reproductive plant growth; depending on the time of the occurrence of the deficiency. Shorrocks (1997) estimates the global area of agricultural land which is treated for B deficiency as 15 million ha and lists cases of positive growth responses after B application for 132 crops in 80 countries over 60 years. Areas with B deficiencies are often characterized by sandy soils with a humid climate and low pH. Major areas were B deficiencies occur are south and southeast Asia, eastern Australia and New Zealand, Africa, North and South America and northern Europe (Shorrocks, 1997; Lehto et al., 2010a). Before macroscopic effects of B deficiency are visible in shoots, root elongation is usually reduced, leading to an increase in the shoot/root ratio (Dell et al., 1997). Deficiency symptoms typically become apparent at tissue concentrations of 10 – 30 mg kg^{-1} (Gupta, 1983). Dicots are less sensitive to B deficiency than monocots. Boron uptake into plants is mainly passive and primarily driven by convection with the stream of transpiration water when soil B concentrations are adequate (Schulin et al., 2010). However, under B deficiency conditions B uptake can become active in plant species that have transporters for the xylem loading of B (Dannel et al., 2002; Miwa et al., 2010). Poplars lack such transports (Tuskan et al., 2006).

Increasing B availability increases the mycorrhizal colonization of plant roots. The reason for this effect remains unknown as experimental evidence is sparse, but increased carbohydrate allocation to the roots under better B supply, reduced concentrations of phenolic compounds in the roots at higher B concentrations and alterations of the plant-mycorrhiza cell wall interactions have been hypothesized to cause this effect (Lehto et al., 2010a). The translocation of B from fungal hyphae into plants seems to be generally possible by transport of B bound to sugar alcohols. However, it remains unclear if mycorrhizal plants are superior to nonmycorrhizal plants with respect to B uptake.

Carbon compounds in leaf litter from B deficient trees are chemically distinct from C compounds from trees with a sufficient B supply (Lehto et al., 2010b). This may influence C cycles in B deficient forest ecosystems.

The range between B deficiency and toxicity in plants is smaller than for any other trace element (Goldberg, 1997). Reid et al. (2004) found B toxicity in plants to be caused by disrupting of cell wall development, disruption of the plant metabolism by binding to NAD^+ and by disrupting cell division and development by binding to RNA. Furthermore, accumulation of B in leaves leads to osmotic imbalances. Yet, it remains unclear which of these processes contributes the most to reduced plant growth. Large gradients of B concentrations within plants and even within single leaves make the definition of critical B levels in foliar analysis difficult (Oertli, 1994; Nable et al., 1997). For barley and wheat the critical B levels range between 10 and 130 mg kg^{-1}. Highly B tolerant plant species such as *Puccinellia distans* survive at shoot B concentrations of up to 6000 mg kg^{-1} (Stiles et al., 2010). However, in this study as the criteria for hyperaccumulation were not met for all B treatment levels of the experiment and the question if *Puccinellia distans* is a B hyperaccumulator could not be answered conclusively. Among tree species, poplars (*Populus spp.*) and willows (*Salix spp.*) have been shown to be especially B tolerant with foliage B concentrations of up to 1200 mg kg^{-1} (Robinson et al., 2003; Bañuelos et al., 2010) and poplars have been successfully employed in B phytomanagement (Robinson et al., 2007). However, it is unknown how poplars tolerate high soil B-concentrations, and what processes control B uptake into the shoots. Tolerance mechanisms of plants towards high B concentrations include exclusion in the root zone as well as internal tolerance mechanisms (Nable et al., 1997). While Schnurbusch et al. (2010) identified an exclusion mechanism for excess B in barley that increased B tolerance, a mechanism for the internal detoxification of B has been found in some halophytes and members of the *Rosaceae* family which are able to inactive excess B by binding to sugar alcohols like sorbitol (Rozema et al., 1992; Brown et al., 1997). Little is known on the influence of mycorrhiza on B toxicity in plants. While in mycorrhizal durum wheat reduced toxicity symptoms and B uptake were found (Sonmez et al., 2009), there was no positive effect of mycorrhizal colonization on B tolerance in *Pinus banksiana* (Calvo Polanco et al., 2008).

1.2 Objectives of this study

The various environmental implications of excess B in the environment makes it necessary to control the release of B from contaminated sites or to clean them up. Poplars have

previously been shown to be especially B tolerant and to be therefore suitable for the phytomanagement of B contaminated sites. However, plant-B interactions among poplars are poorly understood and the limits of B tolerance and accumulation are unknown. Information about the influence of B on the poplar root system is unavailable, especially under heterogeneous conditions.

To fill these knowledge gaps, three key objectives were set up, each of which is specifically addressed in one of the three main chapters of this dissertation:

I. The first objective was to investigate the B tolerance and accumulation potential of poplars.

To address this objective a greenhouse study was conducted (Chapter 2) where we grew *Populus nigra* × *euramericana* and three other species, namely *Salix viminalis*, *Lupinus alba* and *Brassica juncea* at various soil B concentrations and determined the accumulation of B by *P. nigra* × *euramericana* in comparison to the three other species and their tolerance to various concentrations of B in soil. Furthermore, the distribution of B within poplar roots, shoots and leaves was investigated. By means of neutron radiography (NR) the spatial distribution of B within individual poplar leaves was determined to identify the B toxicity threshold concentration in leaf tissue.

As with other trace elements, B occurs heterogeneously in soil (McLaren et al., 1996). Depending on the origin, B-concentrations may change by an order of magnitude or more over just a few centimeters. The heterogeneous distribution of trace elements has been shown to affect plant root growth and is considered to be one of the major constraints in the design of phytoremediation systems (Keller et al., 2003; Robinson et al., 2009). The effects of a heterogeneous soil B distribution on B uptake and tolerance of poplars are, as well as the reactions of the poplar root system unknown and may influence the performance of poplars under typically heterogeneous field conditions when employed in phytoremediation.

II. To elucidate how B tolerance and accumulation of poplars are affected by heterogeneous distributions of B in soil and to investigate possible reactions of the poplar root system to a heterogeneous soil B distribution.

To meet the aims of the second objective we set up a climate chamber study (Chapter 3), in which we planted small poplar cutting into containers that were either homogeneously or heterogeneously filled with different amounts of B. The design of this experiment was developed to investigate B tolerance and uptake over a range of heterogeneous soil B concentrations and to compare them with poplars grown on soil with a homogeneous B distribution. To look more into detail into growth and root traits of poplars roots under heterogeneous conditions a second experiment was conducted. Here, NR was used to monitor the root growth in soil during the experiment.

After investigating B toxicity, accumulation and tolerance in poplars under controlled homogenous or heterogeneous conditions and investigating poplar root growth under such conditions, the question remained whether the obtained results were transferrable to field conditions, where other environmental factors such as climate, co-contaminants and nutrient or water deficiencies might play a major role in B uptake and tolerance and where other soil heterogeneities than B might alter root and shoot growth. Hence objective III:

III. To determine whether the results on B tolerance and accumulation and root growth of poplars obtained under controlled conditions were transferable to typically heterogeneous field conditions where a number of additional constraints might limit poplar growth.

To achieve the third objective a field survey was conducted on a mixed paper mill waste landfill in Rhinelander, WI, US (Chapter 4). To characterize the soil properties and their distribution on the three poplars stands established on the landfill we took soil cores up to 20 cm depth, characterized their chemical and physical properties and classified each soil sample based on these properties into one of three substrate types by means of hierarchical clustering. Growth and survival of the four different poplars clones were examined with regards to the soil properties, the uptake of B and other elements into the poplars were investigated. Finally, the relationship between local-scale root traits and B contamination as well as the influence of other soil factors on root growth was determined.

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2 Boron accumulation and toxicity in hybrid poplar (*Populus nigra* × *euramericana*)

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Environmental Science and Technology, DOI: 10.1021/es201100b, in press

2.1 Abstract

Poplars are known to accumulate high B concentrations and thus used in the treatment of B contaminated soils. Here, we performed pot experiments in which *Populus nigra* × *euramericana* were grown on a substrate with B concentrations ranging from 13 to 280 mg kg^{-1}. *Salix viminalis, Brassica juncea* and *Lupinus albus* were grown for comparison. Poplar growth was not affected at the lower treatment levels up to 93 mg kg^{-1}, but slightly and severely reduced at levels of 168 and 280 mg kg^{-1}, respectively. None of the other species survived at these levels. At B concentrations <900 mg kg^{-1} only 10% of poplar leaf area showed signs of toxicity. Neutron radiography revealed that chlorotic leaf tissues had B concentrations of 1000 – 2000 mg kg^{-1}, while necrotic tissues had >2000 mg kg^{-1}. Average B concentrations of up to 3500 mg kg^{-1} were found in leaves, spots within leaves had concentrations >7000 mg kg^{-1}. Boron accumulation in leaf tissue continued even after the onset of necrosis. The B accumulation ability of *P. nigra* × *euramericana* foliage fits the criteria for a B hyperaccumulator plant and is associated with a high B tolerance in the living and storage of B in dead leaf tissue.

2.2 Introduction

At low concentrations boron (B) is an essential plant and animal micronutrient (Salisbury et al., 1992; Mastromatteo et al., 1994). Recent studies show that it is at least beneficial if not essential also for humans (Hunt, 2007; Kot, 2009). Boron deficiencies in plants have been reported in over 80 countries for a total of 132 crops (Shorrocks, 1997). On the other hand B becomes toxic at elevated concentrations like other trace elements. In fact, the concentration range between B deficiency and toxicity is smaller than for any other nutrient element (Goldberg, 1997). Boron concentrations in plant tissues exceeding 50 mg kg^{-1} are generally considered to be toxic (Jones, 1991). Plant uptake of B is primarily passive and becomes actively metabolic-driven only under B deficiency conditions (Takano et al., 2002). Boron is transported from soil into roots and from there into stems and leaves primarily by convection, with the stream of transpiration water (Schulin et al., 2010). High levels of B occur naturally in many soils of arid regions. Also, human activities can lead to high soil B concentrations, such as the irrigation of agricultural fields with B-laden water, coal mining or fly ash deposition onto agricultural land (Nable et al., 1997; Parks et al., 2005). In soil solution, B is normally present as the non-dissociated species of boric acid H_3BO_3, as the pK_a (9.24) of boric acid is above the pH-range normally found in soils. Soil B is mainly bound to organic matter, clay minerals and Al-hydroxides (Goldberg, 1997).

Poplars (*Populus* spp.) are widely used to protect soils against wind erosion, for wood production, as supplementary stock fodder during times of drought (Hathaway, 1986; Wilkinson, 1999) and for the phytomanagement of contaminated sites (Robinson et al., 2007). Due to their high transpiration rates and B accumulation, poplars have been employed to reduce B leaching from contaminated sites into receiving waters when deployed in B phytoremediation. Boron accumulated in the aboveground biomass of poplar can be removed from the site by harvesting the trees (Robinson et al., 2007). When fed to sheep or cattle, foliage as well as small twigs can be used as supplementary stock fodder (Douglas et al., 1996). Feeding livestock with B-enriched poplar twigs and leaves may provide a source of this essential trace element to livestock, resulting in increased growth and bone density (Mastromatteo et al., 1994).

In an experiment with 8 hybrid poplar clones, the clone OP-367 (*P. deltoides* × *nigra*) accumulated 543 mg B kg^{-1} in its aerial tissue after irrigation with water containing 3 mg B l^{-1} (Bañuelos et al., 1999).

Robinson et al. (2003) found that two *P. deltoides* hybrid clones grown on soil containing just 40 mg B kg^{-1} accumulated up to 1200 mg B kg^{-1} in their leaves, some 20 times more than other tree species grown on the same site. In a lysimeter experiment, where 3 hybrid poplar clones were grown on substrates containing up to 36 mg B kg^{-1}, leaves had an average B concentration of 845 mg kg^{-1}, while the stems contained just 21 mg B kg^{-1} at harvest (Robinson et al., 2007). Leaf B concentrations increased linearly with leaf age, indicating that B translocation within the plants largely occurs via xylem transport and that there was little redistribution via the phloem. Apart from field studies where B accumulation in poplars was a found (Pavlović et al., 2004; Dellantonio et al., 2008), there have been no studies following the original report by Bañuelos et al. (1999) that went deeper into the B accumulation of poplars, including bioaccumulation factors and B threshold concentrations compared to other species.

Dellantonio et al. (2008) reported elevated leaf B concentrations in poplars grown on fly ash disposal sites with soil B concentrations of up to 600 mg kg^{-1}. However, the same authors found even higher leaf B concentrations (>800 mg kg^{-1}) in various *Salix* species grown on the same site, rendering also these species interesting for the purpose of B extraction from contaminated soil. The phytoextraction efficiency of a plant species for a trace element depends on the respective accumulated concentration of the element and the amount of harvestable biomass (Pulford et al., 2003). Lower biomass production can be compensated by higher accumulation (Robinson et al., 2009; Vangronsveld et al., 2009). *Brassica juncea* is widely used in phytoremediation and was reported to exhibit a high B tolerance and phytoremediation potential (Bañuelos et al., 1993b). Therefore, *B. juncea* might be able to achieve the same B phytoextraction efficiency as poplars or willows, despite its lower biomass production.

Boron accumulation varies widely between different parts of a plant (Brown et al., 1997; Grieve et al., 2010). To assess the accumulation potential of a given plant species it is necessary to analyse all plant parts for their B concentration. The increase of leaf B concentration during the growing period makes it difficult to determine toxicity thresholds for leaf B concentrations by foliar analysis, as B concentrations can vary considerably between old and young leaves. Moreover, when toxicity symptoms become visible in leaves, B concentrations can vary over several orders of magnitude even within single leaves (Oertli, 1994; Reid et al., 2009). Therefore, the distribution of B not only among but also within leaves needs to be analysed for the determination of B toxicity thresholds in leaf tissue.

Various techniques have been applied to measure the spatial B concentration in leaves (Loria et al., 1999; Reid et al., 2009; Wu et al., 2009). Loria et al. (1999) applied neutron-capture-radiography to detect B in *Coffee Arabica* leaves. This technique allows tracing of ionizing particles produced by the collisions of ^{10}B atoms and neutrons, but does not provide a complete picture of the B distribution in leaf tissue. Furthermore, this method is very time consuming even for small areas, since both, irradiation as well as neutron tracking by optical microscopy is slow. Excising single necrotic spots gives an incomplete picture of the B distribution within a leaf and is limited by the amount of sampled material required for analysis. Laser ablation ICP-MS is a promising method for low leaf B concentrations (Wu et al., 2009), but its feasibility for high leaf B concentrations has not been shown yet. Here, neutron radiography (NR) was applied for the first time to analyse the spatial distribution of B in leaves.

While the transfer of B from soil into the shoots of poplars is of great interest with respect to potential phytoremediation of contaminated sites as well as for the assessment of toxicity risks for animals feeding on B-enriched poplar foliage, there is little knowledge on B accumulation by poplars. Therefore, the objectives of this study were to determine (1) the accumulation of B by *P. nigra* × *euramericana* in comparison to *S. viminalis*, *B. juncea* and *L. albus* and their tolerance to B in soil under controlled conditions, (2) the partitioning of B among roots, shoots and leaves in these plants and (3) the distribution of B within individual poplar leaves in order to identify critical threshold concentrations for B in leaf tissue. *Populus* was chosen because of its known B accumulation and phytoremediation potential of B contaminated sites. *Salix viminalis* and *B. juncea* were chosen as alternative phytoremediation plants that are often used or proposed for the phytoremediation of contaminated sites (Dickinson et al., 2005; Vamerali et al., 2010), and *L. albus* was selected to compare the other three species with a legume plant.

2.3 Materials and Methods

2.3.1 Plant growth

Populus nigra × *euramericana*, (clone "Dorskamp"), *S. viminalis* (spp.), *B. juncea* (spp.) and *L. albus* (L.) plants were grown on a potting mix (PM) under greenhouse conditions with natural lighting at the Swiss Federal Research Institute WSL, Birmensdorf, Switzerland.

Apart from the control treatment with no added B, three soil B treatments were initially established by spiking the PM substrate with different amounts of ^{10}B-enriched H_3BO_3 (^{10}B >96%, EaglePicher Technologies, Quapaw, USA). Table 1 gives the resulting the HNO_3- and $CaCl_2$-extractable B concentrations of the substrates. They showed a quite close linear relationship ($r^2 = 0.88$; $y = 0.50\ x - 13.1$; $p < 0.001$).

Table 1. Mean ± Standard error (S.E.) (N = 3) nitric-acid and $CaCl_2$-extractable B concentration in the control treatment T_{13} and the initially set-up B treatments T_{93}, T_{168} and T_{280}.

	Boron concentration			
Treatment	HNO_3		$CaCl_2$-extractable	
	[mg kg^{-1}]			
T_{13}	13.01	± 0.68	4.69	± 0.43
T_{93}	92.61	± 6.92	27.70	± 3.36
T_{168}	167.5	± 8.59	51.92	± 4.50
T_{280}	279.6	± 2.35	133.3	± 15.78

The chosen B treatments represent the range of soil B concentrations reported in previous studies on B uptake by poplars from contaminated soils (Robinson et al., 2003; Robinson et al., 2007; Dellantonio et al., 2008). Nitric-acid and $CaCl_2$-extractable concentrations of macro- and micro-nutrients in the PM substrate are given in Table 2. The pH ($CaCl_2$) of the substrate was 5.0, the total carbon concentration was 270.6 g kg^{-1} and the concentration of nitrogen was 6.78 g kg^{-1}. In April 2005, we prepared three replicate pots (5.5 L) for each treatment and plant species and planted 3 plants in each pot. The planting took place immediately after the substrate was prepared. *Populus nigra* × *euramericana* and *S. viminalis* were planted as cuttings (20 cm in length and 1 cm diameter), *L. albus* and *B. juncea* as seeds. Two weeks after planting, the plants were thinned to one plant per pot.

After *S. viminalis*, *L. albus* and *B. juncea* did not grow at substrate B concentrations of 168 and 280 mg kg^{-1} two intermediate treatments were set up on the same occasion with B concentrations of 22 and 45 mg kg^{-1}. *Populus nigra* × *euramericana* was not planted in the two additional treatments. In the following, the control treatment and the five B treatments are denoted as T_{13}, T_{22}, T_{45}, T_{93}, T_{168} and T_{280} according to the total initial B concentration of the respective substrate.

Table 2. Mean ± S.E. concentrations of macro- and micro nutrients in the unaltered PM substrate used as plants growth medium in this study. The concentrations are valid for all treatments together.

	Concentration			
Element	HNO_3		$CaCl_2$-extractable	
	[mg kg^{-1}]			
Ca	23125	± 974.6	N/A	
Fe	2724	± 140.8	1.79	± 0.38
K	2529	± 236.0	1128	± 145.2
Mg	1985	± 108.4	454.9	± 35.8
Mn	734.6	± 32.2	37.52	± 2.11
Na	471.1	± 35.6	N/A	
P	1792	± 123.9	436.4	± 131.9
Zn	141.8	± 9.67	3.14	± 0.30

N = 12, N/A: not applicable

Pots were irrigated 3 - 4 times per week. Leachates were collected and reapplied to the pots. All plants were harvested after four months of growth. The aboveground biomass was separated into leaves, stems, and in the case of *B. juncea,* also into pods. For *P. nigra* × *euramericana* and *S. viminalis*, only the new shoot growth was used for analysis. Roots were separated from the substrate by washing. Plant biomass was dried until constant weight was obtained and the biomass was recorded. For *P. nigra* × *euramericana* we also recorded the position of the leaves in the sequence along the shoot starting with the 1st leaf at the bottom of the plant.

2.3.2 Neutron radiography

We used ^{10}B-enriched B in order to determine the areal distribution of accumulated B within leaves by means of neutron radiography (Zawisky et al., 2004; Menon et al., 2007). The neutron absorption cross section of ^{10}B as determined at ICON is 8720 E^{-24} cm^{-2}. This is several orders of magnitude higher than that of ^{11}B (11.5 E^{-24} cm^{-2}), enabling the visualization of ^{10}B within leaf tissue at high spatial resolution. A preliminary test with NR revealed that only poplar, but none of the other plants accumulated sufficient ^{10}B in their leaves for NR. Neutron radiographs of dried poplar leaves were taken at the ICON (Instrument for Cold Neutron Radiography) facility of the Paul-Scherrer-Institute (Villigen), Switzerland (Kuhne et al., 2005). The NR data were calibrated against ICP-OES measurements of leaf B concentrations.

After neutron imaging, the leaves were scanned with a standard office scanner (Agfa, SnapScan 1236) at 150 dpi. Colour images were analysed using WinRhizo Pro© (Regent Instruments 2009) to assess the ratio between healthy and chlorotic or necrotic leaf area ($R_{h/cn}$) for each leaf.

2.3.3 Chemical analysis

For chemical analysis, aliquots of dried and ground plant samples were digested in a heating block at 130°C in 15 mL of a 65% HNO_3. The digests were analysed for B and other elements by ICP-OES (Vista MPX, Varian, Australia). Samples of PM substrate were analysed for nitric-acid concentrations in the same way. Certified plant reference material NCS DC-73350 (poplar leaves, China National Analysis Centre for Iron and Steel, Beijing, China) was used for quality control. The average recovery rate for B was 98.4 ± 2%. To determine extractable concentrations of B and other elements in the PM substrate, 1:10 mixtures of substrate and 0.01 mol $CaCl_2$ were shaken for 16 h, centrifuged at 2500 r.p.m. for 10 min, filtered through a 0.25 µm membrane filter and analysed by ICP-OES. Carbon and nitrogen contents of the PM substrate were measured using an elemental analyser (CNS-2000, Leco Corp., Saint Joseph, Michigan USA).

2.3.4 Statistics

Mean whole-plant element concentrations were calculated as mass-weighted average of the respective element concentrations of individual plant parts. Kruskal-Wallis-ANOVA was performed to test for differences in biomass and element concentrations between B treatments, followed by the Mann-Whitney U-Test as post-hoc test to compare pair-wise differences between treatments. Values given for correlations between variables represent Pearson's correlation coefficients. All statistical analyses were carried out using PASW Statistics (Release 17.0.2).

2.4 Results and discussion

2.4.1 Biomass

All poplar saplings survived even at the highest B treatment levels, although they showed reduced growth in T_{168} and severe growth reduction in T_{280}. Our results are consistent with the high B tolerance reported by Banuelos et al. (1999) for poplars growing on B contaminated sites. Figure 1 shows the aboveground biomass of the harvested plants,

excluding the part of the stem axis corresponding to the cutting originally planted in the case of *P. nigra* × *euramericana* and *S. viminalis*.

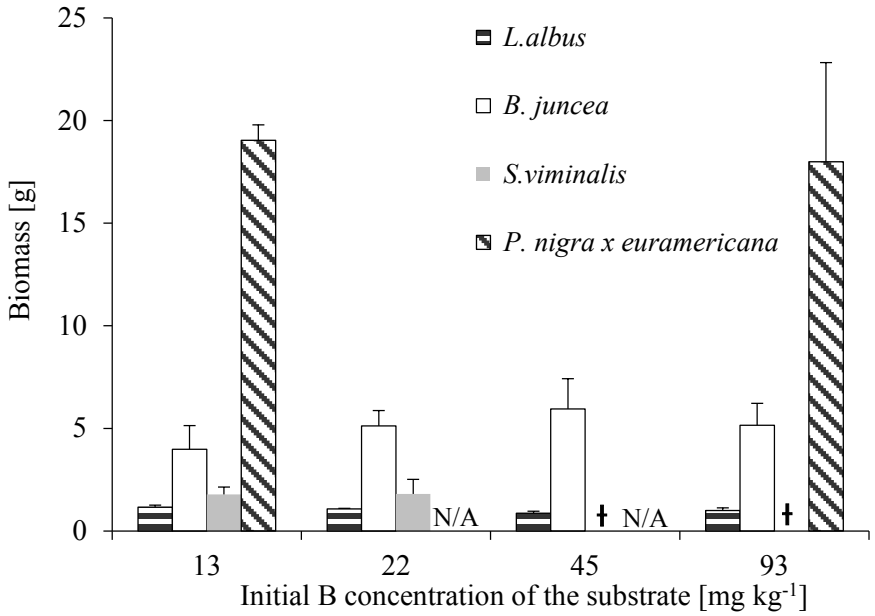

Figure 1. Aboveground biomass production of *P. nigra* × *euramericana*, *S. viminalis*, *L. albus* and *B. juncea* during 4 months of growth on substrate with different initial B concentrations. The lowest B concentration (13 mg kg^{-1}) is the control treatment. Error bars represent standard errors (N = 3). N/A: not applicable. ✝: plant died.

Lupinus albus and *B. juncea* plants survived in the T_{93} treatment without any reduction in growth, but completely failed to grow at higher B concentrations. *Salix viminalis* only grew in the T_{13} and the T_{22} treatment. Its biomass was significantly smaller than that of *P. nigra* × *euramericana* in T_{13} and that of *B. juncea* in T_{13} and T_{22}. Thus, *S. viminalis* turned out to be the least B tolerant of the four species tested while poplar was the most tolerant. This was surprising given that poplars and willows belong to the same family (*Salicaceae*). Plants that do not tolerate elevated soil B concentrations are obviously not suited to remediate B contaminated sites. However, both *Populus* and *Salix* are known to exhibit considerable inter- and intra-specific genetic and phenotypic variability with respect to B uptake and tolerance (Dellantonio et al., 2008; Bañuelos et al., 2010). Therefore, other *Populus* and *Salix* species and genotypes may have different B tolerance characteristics.

Figure 2 shows that the relative decrease in the biomass of the poplar plants was larger in the roots than in leaves and stems in the T_{168} and T_{280} treatments. The shoot:root biomass ratio increased from 6 in the control treatment to 25 and 57 in the T_{168} and the T_{280} treatments, respectively.

High concentrations of soil B are known to inhibit root growth relative to shoot growth (Reid et al., 2004). Reduced growth might be a general response of poplar roots towards contaminants as poplar roots were shown to react in the same way towards elevated soil Zn and Cd concentrations (Dos Santos Utmazian et al., 2007).

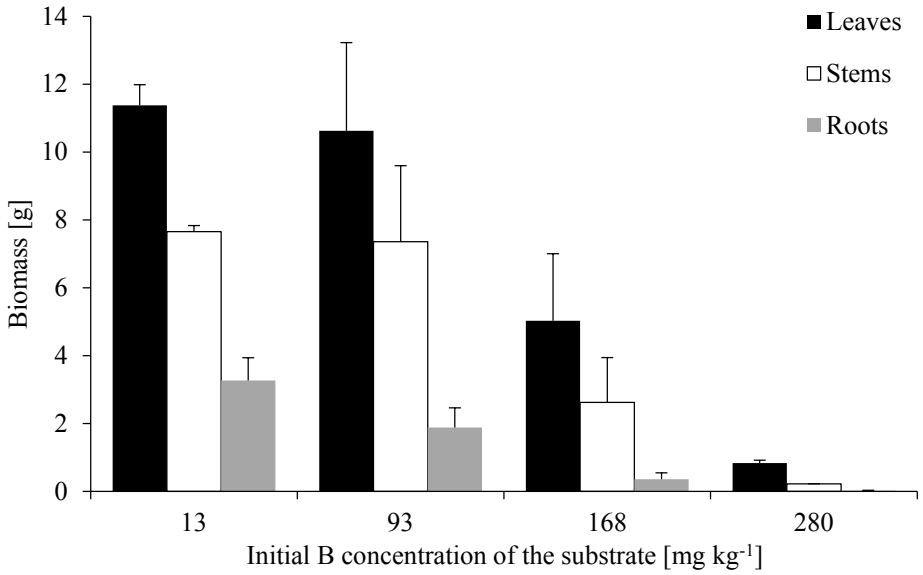

Figure 2. Leaf, stem and root biomass of 5 month old *P. nigra* × *euramericana* saplings grown on substrates with different B concentrations. The lowest B concentration (13 mg kg^{-1}) is the control treatment. The mass of the cutting from which the saplings were grown is not included. Error bars represent S.E. (N = 3).

2.4.2 Boron accumulation and allocation in the plants

Compared to the large differences in B tolerance, there was little variation in B accumulation among the four plant species studied (Table 3). While in the control treatment shoot B concentrations did not differ between species, significant differences emerged at higher B concentrations. The bioconcentration factors (BCF) (plant/soil concentration quotients) ranged between 3.5 – 5 for all species and all treatments, except for *B. juncea* (BCF: 1.5 – 2.7) in the B treatments. The highest BCF values were found for poplar in the T_{168} and T_{280}. *Brassica juncea* was found to exclude B from entering its shoots. Shoot B concentrations in this species did not differ between T_{13}, T_{22} and T_{45} and were still less than half of the surviving L. albus plants in the T_{93} treatment. The B concentrations found in *B. juncea* were in the same range as those reported by Bañuelos et al. (1993a).

Table 3. Mean ± S.E. B accumulation in the aboveground biomass of *L. albus*, *B. juncea*, *P. nigra* × *euramericana* and *S. viminalis* grown on substrate with different B concentrations. T_{13} is the control treatment.

Treatment	B concentration							
	L. albus		*B. juncea*		*P. nigra* × *euramericana*		*S. viminalis*	
	[mg kg^{-1}]							
T_{13}	40.5 ^{a}	± 3.44	43.5 ^{a}	± 4.69	43.8 ^{a}	± 0.29	48.6 ^{a}	± 4.67
T_{22}	114.2 $^{b\,I}$	± 16.6	60.1 $^{a\,II}$	± 4.37	N/A		118.3 $^{b\,I}$	± 11.3
T_{45}	174.6 $^{bc\,I}$	± 27.2	68.1 $^{ab\,II}$	± 17.2	N/A		✝	
T_{93}	304.4 $^{c\,I}$	± 20.7	136.4 $^{b\,II}$	± 19.1	392.4 $^{b\,I}$	± 28.7	✝	

Statistically significant differences between treatments are indicated by characters and differences between plant species within the same treatment by roman numerals (Mann-Whitney U-test, $p < 0.05$, N = 3). N/A: not applicable. ✝: plant died

If the B tolerance of *P. nigra* × *euramericana* was due to B exclusion from uptake by the roots, we would expect non-tolerant plants to have higher shoot B concentrations than B-tolerant poplars grown on the same substrate. We did not find such a relationship between the plant species used in this study. The ability of the poplars to accumulate higher concentrations of B than the other species was apparently due to a greater B tolerance in their leaf tissues, demonstrating that this can be a successful strategy to deal with elevated soil B concentrations. The lower B accumulation in *B. juncea* did not increase its B tolerance compared to *L. albus* and *S. viminalis* and was less successful under the conditions of our study. The results are consistent with findings that B can easily penetrate cell membranes, indicating that regulation of B entry into the symplast and further into the root xylem, by means of membrane transporters is ineffective (Hu et al., 1997). Unlike other nutrient elements, B is taken up by plants as the neutral species H_3BO_3 (Hu et al., 1997). This species has a diameter of only 0.257 nm and thus may easily pass through cell membranes via aquaporins (Tanaka et al., 2008).

Figure 3 shows that there were no significant differences between root and stem B concentrations in the poplar plants. Both increased with the B concentration of the substrate. The leaves accumulated much higher B concentrations. At the highest B concentrations of the substrate, the average leaf B concentration exceeded 1000 mg kg^{-1}. This is in agreement with the notion that B is primarily passively transported with the transpiration stream and deposited in the leaves upon evaporation of the water, consistent with previous reports (Robinson et al., 2003; Robinson et al., 2007).

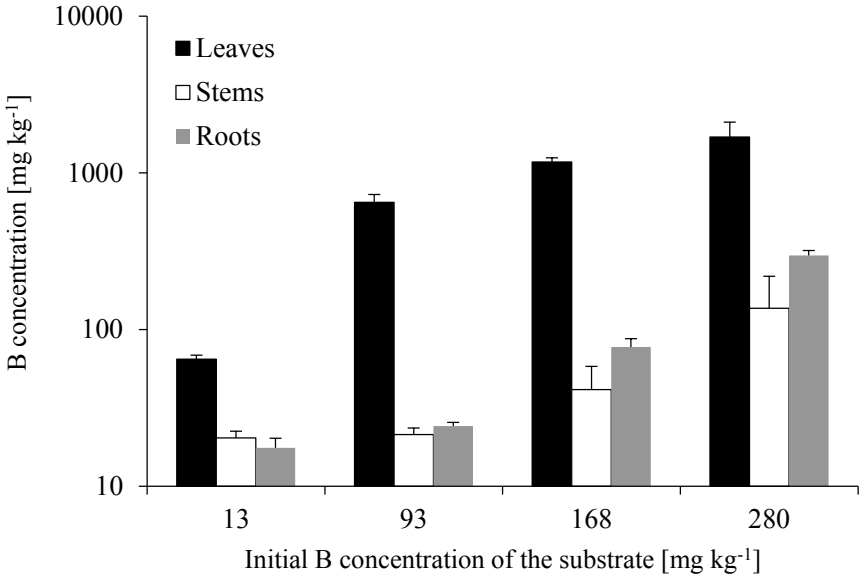

Figure 3. Concentrations of B in roots, stems and leaves of 4 months old of *P. nigra* × *euramericana* plants. The lowest B concentration (13 mg kg^{-1}) is the control treatment. Note that the B concentration is shown on logarithmic scale for better clarity. Error bars represent S.E. (N = 3).

Compared to the other tested species, *P. nigra* × *euramericana* has good potential for the phytomanagement of B contaminated sites. The total uptake of B into the aboveground biomass of *P. nigra* × *euramericana* during 4 months ranged between 1 mg and 8 mg per plant in T_{13} and T_{93}. This represented an extraction of 2.1% of the total B initially present in the pots. In the T_{168} treatment the total uptake of B was 7.2 mg per plant, here the higher plant B concentration nearly compensated the lower plant biomass in comparison to T_{93}. However, in T_{168} the 7.2 mg B extracted were only 1% of the total B in the pot. This uptake was higher than found in *Gypsophila arrostil* and in the same range as reported for *Pucinella distans*, two species considered as potential B hyperaccumulator plants (Stiles et al., 2010). The highest uptake found one of the other species tested in this study was 0.7 mg B per plant in *B. juncea*. With an estimated annual leaf biomass production of 15 t ha^{-1} a^{-1} *P. nigra* × *euramericana* could extract 6.3 kg B ha^{-1} a^{-1} from contaminated topsoil containing 75 kg B ha^{-1}. To prevent the extracted B from returning to the soil via leaf fall, removal of the leaves from the site would be necessary. For that purpose poplars could be coppiced (Robinson et al., 2003). The B rich leaves could be used as an organic fertilizer on B deficient sites or used as stock fodder (Robinson et al., 2005). Only leaves from T_{13} and T_{93} would be suitable as stock fodder, as B concentrations >800 mg kg^{-1} may be toxic to livestock (Underwood et al., 1999). Leaves from the T_{168} and T_{280} treatment could still be used as fodder if mixed with fodder produced on unpolluted soil.

2.4.3 Partitioning of B in *Populus nigra* × *euramericana* leaves

In all treatments, B concentrations decreased exponentially with leaf number from the lower (older) to the upper (younger) leaves of the poplar saplings (Fig. 4). There was a more than tenfold difference in average B concentration between the oldest and the youngest leaves in all B treatments. The B concentration ranges from top to bottom leaves were 22 – 185 (T_{13}), 62 – 1725 (T_{93}), 190 – 3241 (T_{168}) and 298 – 3472 (T_{280}) for the respective treatments, with only small differences between the 2 highest treatments T_{168} and T_{280}.

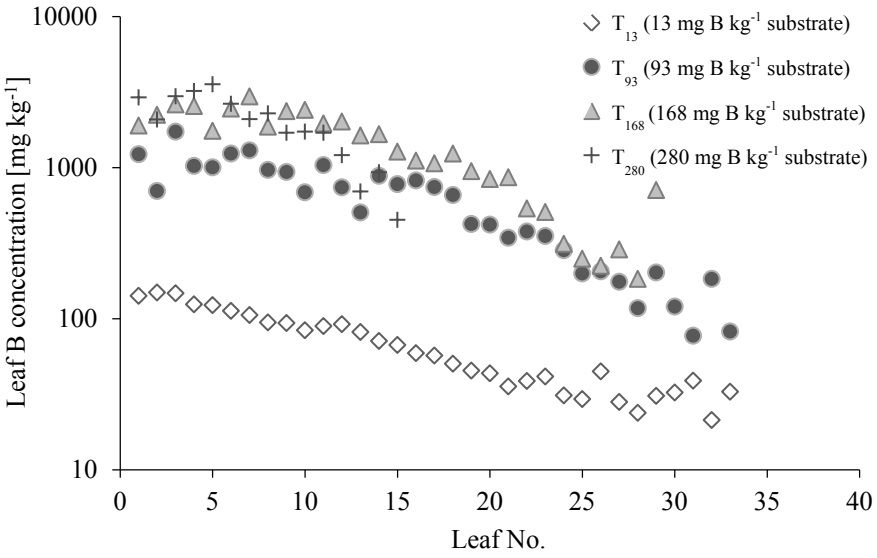

Figure 4. Leaf B concentration as a function of leaf position, counting from bottom to top along the stems of 4 months old poplars grown on substrate with different B concentrations (c. legend). Note that the B concentration is shown on logarithmic scale for better clarity.

These results have implications for the interpretation of data for poplar trees sampled in the field (Oertli, 1994). It is usually only possible to collect and analyse a small number of leaves from a tree. As our results show, B concentration data from leaf samples may vary by an order of magnitude depending on the position of the sampled leaves. Robinson et al. (2007) found that leaf B concentrations also varied considerably with time over a growing season. Again, these findings are consistent with the notion that B accumulation in the leaves is primarily associated with the transpiration water flow and that there is little or no relocation of B in the phloem of poplars. The leaf B concentrations did not depend on the size of the leaves (data not shown). The leaves emerging in the middle of the growing season were larger than the leaves produced at the beginning and the end of the growing season, while the B concentration of the leaves that emerged in the middle of the growing season steadily increased with age.

With increasing leaf B concentrations the fraction of chlorotic and necrotic areas on the sampled leaves increased (Fig. 5). Below a B concentration of 900 mg kg^{-1} $R_{h/cn}$ was always below <10%. The leaf B concentration range 900 – 1199 mg kg^{-1} was found to be a threshold across which $R_{h/cn}$ jumped to values above 30%.

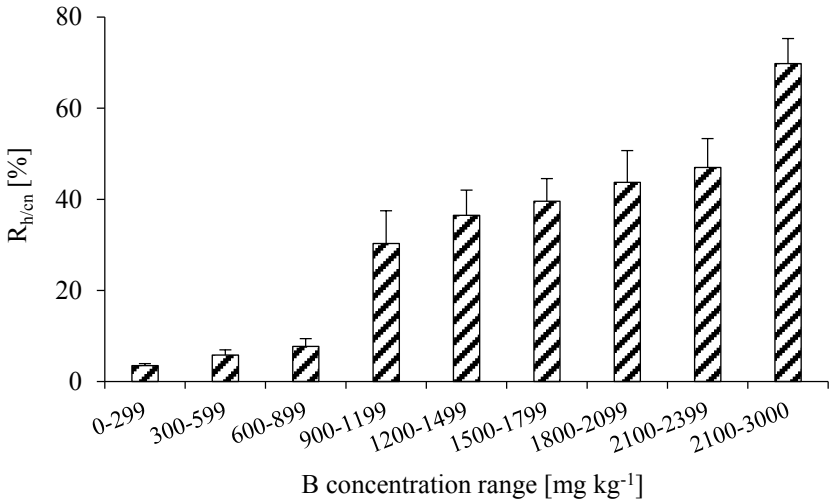

Figure 5. Chlorotic and necrotic leaf area expressed as percentage of total leaf area ($R_{h/cn}$) as a function of leaf B concentration. Note the large increase in chlorotic and necrotic leaf area above 900 mg B kg^{-1}. Error bars represent S.E..

At leaf B concentrations higher than 1200 mg kg^{-1} $R_{h/cn}$ increased linearly ($r^2 = 0.98$; $y = 4.07x + 27.21$; $p < 0.001$), until a second threshold was reached at B concentrations >2100 mg kg^{-1} where $R_{h/cn}$ increased from around 50 to 70%. Tripler et al. (2007) found similar leaf necrosis effects associated with high leaf B concentrations in date palm. Increasing contaminant accumulation and leaf chlorosis/necrosis with leaf age is also known for Zn and Cd, although these metals were stored in different tissues (Vollenweider et al., 2006; Vollenweider et al., 2011).

2.4.4 The distribution of B within *Populus nigra* × *euramericana* leaves

Within individual leaves, the highest B concentrations occurred at the leaf margins and tips. The margins and tips were also the locations where chlorosis and necrosis occurred first and were strongest. At average leaf B concentrations greater than 1000 mg kg^{-1} leaf margins and tips curled. At higher total leaf B concentrations necrotic spots occurred throughout the leaf. These spots contained >2000 mg B kg^{-1}. Figure 6 shows the distribution of B within leaves with average B concentrations of 147, 1216 and 1964 mg kg^{-1}.

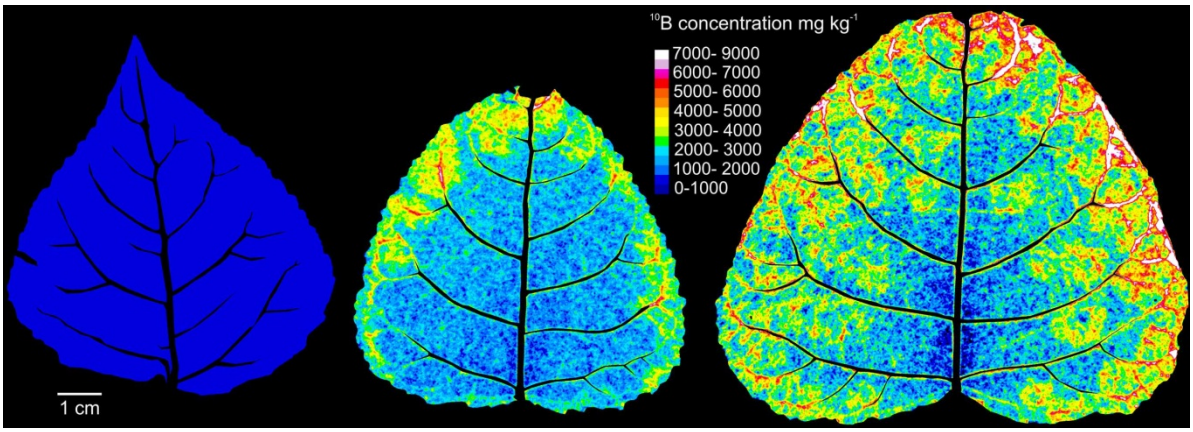

Figure 6. Neutron radiographs showing the distribution of accumulated ^{10}B in *P. nigra* × *euramericana* leaves grown on the PM substrate containing 13 (a), 93 (b) and 168 (c) mg B kg^{-1}. The average leaf B concentrations were 147 (a), 1216 (b) and 1964 (c) mg kg^{-1}. Leaf veins were excluded from the analysis.

Leaf tissue containing between 1000 and 2000 mg B kg^{-1} was chlorotic and tissue containing more than 2000 mg kg^{-1} was necrotic. Our findings indicate that necrotic tissue can still receive B via the transpiration flow, given that B concentrations greater >7000 mg kg^{-1} were reached in some spots within the investigated leaves. Similar findings were reported by Reid and Fitzpatrick (2009) for barley. Deposition of B at high concentrations in discrete patches may be a tolerance mechanism by which a small patch of photosynthetic tissue is sacrificed in order to prevent overloading of the surrounding tissues.

The B accumulation characteristics of *P. nigra* × *euramericana* are consistent with the criteria set by Kramer (2010) and Mc Grath et al. (2003) for hyperaccumulation. The accumulation 20 times higher B concentrations than those considered toxic in tissues of most other plants could be regarded as hyperaccumulation. For Ni the threshold concentration used as criterion for hyperaccumulation is 1000 mg kg^{-1} (Brooks et al., 1977), which corresponds to 17.0 mmol kg^{-1}. The equivalent mass concentration of B is just 172 mg kg^{-1} because of its 80% lower molar weight compared to Ni. This concentration was exceeded in some of the poplar leaves grown in the control treatment and in more than 85% of the leaves in the treatments with higher B concentrations.

Comparison of the ICP-OES measurements and the NR results showed that local tissue B accumulation in leaves was detectable by NR if concentrations in leaves exceeded 300 mg kg^{-1}. The detection limit and the spatial resolution of neutron radiographs (130 µm) thus were sufficient for the determination of toxicity thresholds in *P. nigra* × *euramericana* leaf tissue. Boron concentrations in the leaves of *B. juncea*, *S. viminalis* and *L. albus* were below the detection limit. Here, laser ablation ICP-MS could be an alternative (Wu et al., 2009).

2.5 Conclusions

Populus nigra × *euramericana* tolerated much higher B concentrations in the substrate than *S. viminalis, B. juncea* and *L. albus*. The fact that high soil B concentrations had a stronger negative effect on root than on shoot biomass in *P. nigra* × *euramericana* indicates a higher B sensitivity of the roots. Leaf tissue became chlorotic at B concentrations exceeding >1000 mg kg^{-1} and necrotic at B concentrations >2000 mg kg^{-1}. The finding of B concentrations >7000 mg kg^{-1} in necrotic leaf tissue indicates that B accumulation continued in leaf tissue even after the onset of necrosis. The fact that *L. albus* accumulated similar leaf B concentrations as the poplars as long as they survived, suggests that the ability of *P. nigra* × *euramericana* to accumulate much higher B concentrations in its aerial tissue can be attributed to the high B tolerance of living and storage of B in dead leaf tissue. This poplar hybrid can be considered as woody B hyperaccumulator plant. According to our results it is much better suited for phytoremediation of B contaminated soil than *S. viminalis* or *B. juncea,* which have been proposed for phytoextraction of other trace elements.

2.6 Acknowledgements

Funds for this study came from the Swiss National Science Foundation (SNSF). We would also like to thank Rene Saladin from the Soil Protection lab at ETH, Lidija Josic from PSI for help with the NR analysis and providing the ^{10}B cross section data and Anton Burkart and his team at WSL for the cuttings and tending the plants.

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3 Response of B tolerant hybrid poplar to heterogeneous B distributions in soil

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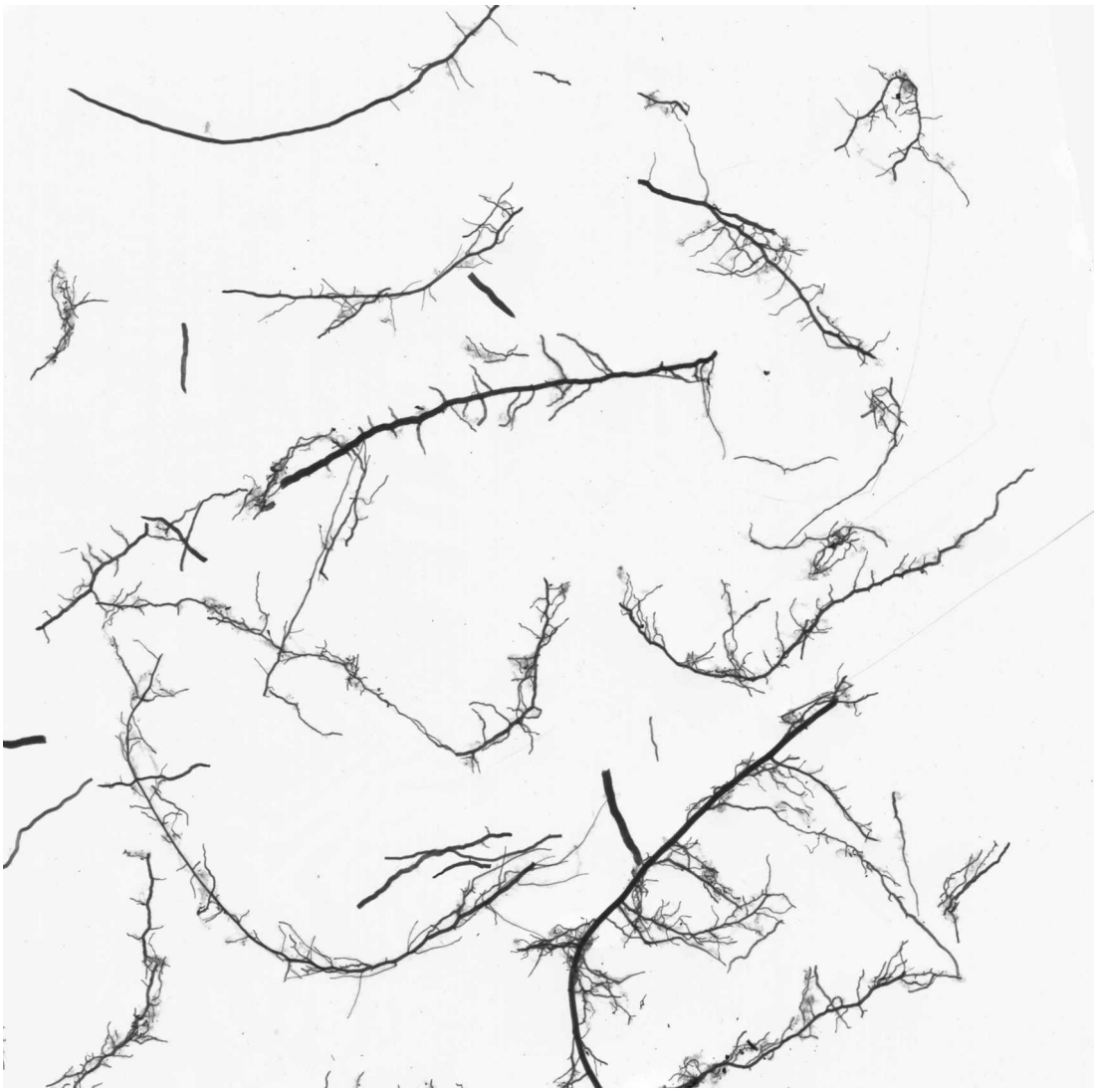
Submitted for publication in *Plant and Soil*



3.1 Abstract

Hybrid poplars find widespread use in phytoremediation. As they can accumulate inordinate amounts of B in their aerial tissues they are in particular considered candidate plants for the remediation of soil contamination by boron (B). In this study, we compared the growth and B accumulation of young poplar plants growing on soil with heterogeneous and homogeneous B distribution. In a first experiment we varied the concentration of soil B by spiking a soil with low B concentrations. In the heterogeneous treatment one half of each container was filled with B-spiked soil of varying concentrations, the other with unspiked soil. The growth of the plants was unaffected by the spatial distribution of soil B over a large range of concentration. Boron accumulation in the shoots only depended on the average soil B concentration in the containers. Root growth was reduced in parallel with shoot growth as shoot B concentrations increased. Local toxicity effects on root growth only occurred at the highest levels of heterogeneous B treatment with >20 mg kg^{-1} B in the spiked container halves. In a second experiment we investigated root growth at a given level of average soil B concentration for homogeneous and heterogeneous distribution in more detail. Fine root (FR) length growth appeared to be more inhibited under B stress than growth in thickness, as indicated by a shift in root diameter length distribution. Our results suggest that in poplars B toxicity on root growth is primarily a systemic response to B toxicity in the shoots and that local soil B toxicity effects on roots become dominant only at high soil B concentrations. We conclude from these results that local heterogeneity in soil B should have little influence on the phytoremediation of contaminated sites using poplar, as long as the contamination allows root growth.

3.2 Introduction

3.2.1 Boron in soils

Boron (B) is an essential plant and animal micronutrient that becomes toxic at already moderately elevated concentrations (Salisbury et al., 1992; Mastromatteo et al., 1994). The concentration range between B deficiency and toxicity in plants is smaller than for any other nutrient element. Boron deficiency symptoms become evident in plants, when tissue concentrations decrease to 10 – 30 mg kg^{-1} (Bell, 1997), while tissue concentrations exceeding 100 mg kg^{-1} are toxic for most plants. However, there is a wide range of toxicity thresholds in plants, sometimes even among varieties of the same species (Gupta et al., 1985; Nable et al., 1997). Boron uptake by plants is closely related to soil B concentrations, which depend on geological parent material, soil type, soil pH, soil organic matter, natural and anthropogenic inputs and soil temperature (Goldberg et al., 1993; Goldberg, 1997). High B concentrations occur naturally in many arid soils. Human activities leading to high soil B concentrations include the irrigation of agricultural fields with B laden waste or ground water, surface mining and the application of fly ashes on agricultural fields (Nable et al., 1997; Parks et al., 2005).

Boron retention in soils generally increases with pH, organic matter and clay content (Goldberg, 1997). The major form of B in the soil solution is boric acid, a weak Lewis acid with a pK_a of 9.24 (Power et al., 1997). Since soils generally have a pH much below 9.24, the un-dissociated, neutral species H_3BO_3 is predominant in the soil solution of most soils (Hu and Brown, 1997). Soil organic matter has a higher capacity for B sorption than mineral soil constituents (Goldberg, 1997). Evans et al. (1983) and Van et al. (2005) found substantial release of B into the soil solution when soil organic matter was decomposed by microbial activity. Boron adsorption on soil mineral surfaces takes place rapidly via ligand exchange (Goldberg, 1997) and can usually be described by Langmuir and Freundlich isotherms (Goldberg et al., 1993; Communar et al., 2004). Aluminum-oxides are generally more important for B retention in soils than Fe-oxides (Hatcher et al., 1967).

3.2.2 Phytomanagement of boron

Boron has a higher mobility in soil than most other trace elements. It is primarily transported by convection with the flow of soil water (Kabata-Pendias et al., 2001; Ishak et al., 2002).

Corresponding to its speciation in the soil solution, B is also taken up by plants as the neutral B species H_3BO_3, which is small enough to pass through cell membranes via aquaporins (Tanaka et al., 2008). Passive uptake and translocation with the stream of transpiration water explains the results of Rees et al. (2011) who found the highest B concentrations in the oldest leaves of their experimental plants. In B-deficient conditions, active uptake may occur in some plant species (Miwa et al., 2006).

Boron contamination should be well suited for phytoremediation, as B is very mobile in soils and easily taken up by plants. Phytoextraction of B from contaminated soils was first proposed and investigated by Banuelos et al. (1993a; 1993b). They tested *Brassica juncea* and *Festuca arundinacea* as candidate plants. While B accumulation in the aboveground biomass of these species was found to be sufficient, their biomass production and rooting depth is relatively low compared to other species, thus limiting B extraction efficiency.

Poplars are widely used for the phytomanagement of contaminated sites (Robinson et al., 2009). Poplars are particularly suited for the revegetation of sites contaminated with B because they are tolerant of high soil B concentrations (Robinson et al., 2007; Bañuelos et al., 2010). Rees et al. (2011) found that their high B tolerance enabled poplars to accumulate B at concentrations of up to 1000 mg kg^{-1} in their leaves, qualifying them as B hyperaccumulator plants.

3.2.3 Heterogeneity and phytomanagement

While test trials in growth chambers or greenhouses provide useful information about the potential use of plants in phytomanagement, the performance of the same plants may be vastly different in the field. One important problem is the heterogeneous distribution of contaminants in field soils, which is usually not represented in pot experiments (Wenzel, 2008; Robinson et al., 2009). This may affect contaminant tolerance and uptake, which are of critical importance in phytomanagement. Soil B concentrations may change by an order of magnitude or more over just a few centimeters (Hall, 1971; McLaren et al., 1996). Plant root growth can respond to soil patches with increased trace element concentrations in three different ways: reduction/avoidance, indifference or proliferation. The roots of some plant species were found to avoid hotspots of trace element concentrations (Breckle et al., 1992; Moradi et al., 2009b), whereas those of others, like the Zn hyperaccumulators *Thlaspi caerulescens* and *Sedum alfredii*, were found to proliferate into Zn-rich soil patches (Schwartz et al., 1999; Whiting et al., 2000; Liu et al., 2010). However, preferential root

proliferation into hot-spots of the hyperaccumulated elements is not a general trait in hyperaccumulator plants, as Moradi et al. (2009b) showed that the roots of Ni hyperaccumulator *Berkheya coddii* were unaffected by patches of elevated Ni. In contrast to macronutrient patchiness effects (Robinson, 1994), little is known about the response of roots to B enriched soil patches. Menon et al. (2007) found that roots of *Lupinus albus* did not grow into patches of high B concentrations in soil.

Poplars are plastic in their responses to variations in soil conditions (Friend et al., 1999; Mulia et al., 2006). Adjusting the morphology of their fine roots (FR) to varying soil conditions enables them to adapt to a wide range of stress conditions in soil (Pregitzer et al., 1996). Fine root diameter and particularly relative FR diameter class length (ratio of FR length per diameter class/ total FR length) are parameters often used to assess the response of FR to changing soil chemical conditions (Borken et al., 2007; Zobel, 2008). Rees et al. (2011) observed that the root: shoot ratio decreased in young poplar plants with increasing soil B concentration, indicating that root biomass responds more sensitively to B stress than the aboveground biomass in poplar. However, there are no studies on the effect of patchy soil B distributions on poplar root growth.

Soil physical and chemical conditions affect the anchorage of trees in soil, thereby influencing their vulnerability towards wind throw (Mayer et al., 2005; Dupuy et al., 2007). Root system properties and the ratio between above- and belowground biomass influence the wind throw risk of poplars (Harrington et al., 1996). Any changes of root system properties in response to soil B could affect wind throw vulnerability of poplar stands on B contaminated sites.

Given the lack of knowledge on root responses to heterogeneous B distribution in soil and the potential implications of these responses for the phytomanagement of B-contaminated sites, the objectives of this study were to investigate (1) whether poplars tolerate higher average soil B concentrations when B is heterogeneously distributed compared to homogeneous distribution, (2) how poplar roots respond to B-enriched soil patches and (3) how B-uptake by poplars is affected by spatial heterogeneity in soil B. Root growth responses to soil heterogeneity are notoriously challenging to assess under field conditions, because it is usually difficult to determine the spatial distribution of all relevant factors around plant roots with sufficient accuracy or to control confounding factors. Therefore, we used pot experiments to assess growth and B uptake of poplars seedlings to well-defined constructed heterogeneities in the soil B distribution.

3.3 Material & Methods

3.3.1 Experiment 1

Two experiments were performed. In the first experiment (Exp. 1) we compared the growth and B uptake of poplars seedlings growing on soil with heterogeneous B distribution to that of poplars growing on soil with homogeneous B distribution. We used an acidic subsoil originating from a Haplic Alisol from Eiken, Switzerland. This soil was chosen for both experiments because it allowed for easy root visualization by means of neutron radiography (NR) in Experiment 2 (Moradi et al., 2009a). The B concentration of this soil was 2.1 mg kg^{-1}. Other physical and chemical soil properties have been described by Nowack et al. (2006) and Moradi et al. (2009a). To facilitate plant establishment, the soil pH was raised from 4.2 to 6.5 by addition of $CaCO_3$. A small portion of this soil was thoroughly mixed with H_3BO_3 powder giving a stock of soil with a total B concentration of 26.4 mg kg^{-1}. By means of stepwise dilution of the stock soil with unspiked soil, we also produced soil batches with B concentrations of 2.7, 3.3, 4.3, 7.3 and 13.5 mg kg^{-1}. One set of Al containers (internal dimensions of 17 × 15 × 1.2 cm) was filled homogeneously with spiked soil from these batches and un-spiked soil for control. Containers of a second set of containers were each filled with unspiked soil on one and spiked soil on the other side. For this purpose they were divided into two compartments by a temporary divider during filling. After filling, the divider was removed, taking care that the two soils did not mix. Each of the resulting 2 × 6 heterogeneous and homogeneous treatments with B-spiked soil as well as the homogeneous treatment with unspiked soil (control) were prepared in triplicates. Then a 5 cm cutting of a *Populus tremula* var. *Birmensdorf* (Qin et al., 2007) clone was planted in the middle of each container. The plants were grown for 86 days in a climate chamber (16 h day (22°C)/7 h night (15°C) cycle, 0.5 h transition time, 75% relative humidity). The pots were irrigated every 2nd to 3rd day to keep the soil water content between 15 and 20%. Water and B-free Hoagland's solution (Hoagland et al., 1938) were alternately used for irrigation to meet the nutrient demands of the plants.

3.3.2 Experiment 2

The second experiment (Exp. 2) focused on FR growth responses to heterogeneous B distribution. Here, Al-containers with internal dimensions of 64 × 67 × 1.2 cm were used. The same soil and cuttings of the same poplar clone were used as in Exp. 1.

Again a control (unspiked soil), a homo- and a heterogeneous B-treatment were established in triplicates. In each container of the heterogeneous treatment, a lateral third of the total soil packing was spiked with the same total amount of H_3BO_3 as the whole packing in the homogeneous treatment. The applied B concentrations were 22.5 mg kg^{-1} in the spiked part of the heterogeneous and 7.5 mg kg^{-1} of the homogeneous treatment. These concentrations were applied in addition to the background soil B concentration of 2.1 mg g kg^{-1}. To avoid soil layering, the containers were laid down and filled from the open side. Before planting, the bare soil was irrigated for two weeks to allow the soil to settle. For the first two months, the plants were grown under greenhouse conditions with natural lighting at the Paul-Scherrer-Institute (PSI), Villigen, Switzerland. Then the containers were transferred for logistic reasons into the climate chamber at ETH, where the experiment was continued for another four months. As in Exp. 1, the gravimetric water content of the soil was kept at 15 – 20%, which was a compromise avoiding water stress on one hand but still giving sufficient root-soil contrast in neutron radiography images on the other hand (Moradi et al., 2009a).

3.3.3 Neutron radiography

Neutron imaging of root growth in Exp. 2 was performed at the NEUTRA facility (Lehmann et al., 2001) of the PSI every 3 to 4 weeks over 6 months. Neutron radiography is a non-invasive and non-destructive technique that can be used to study the development of plant roots in soil (Menon et al., 2007; Luster et al., 2009; Moradi et al., 2009a). Because hydrogen strongly attenuates neutrons, NR can image root structures non-destructively with high spatial resolution if there is sufficient contrast between the moisture content of the surrounding porous medium and the roots. As roots have a higher water content than soil, optimum contrast is achieved when the soil water content is low. The size of the scintillator used was 279 × 234 mm. With 1048 × 879 pixels, the spatial resolution that could be achieved was 0.27 mm. To cover a whole container it was necessary to take 9 neutron radiographs (NRs) per container. For his purpose, the container to be imaged was mounted on an automatically maneuverable sample table in front of the scintillator and moved to the imaging positions. The exposure time was 30 s and the scintillator object distance was 10 cm. The NR images were despeckled by means of a 3 × 3 median filter and corrected for camera noise and beam fluctuations using the flat field correction method described by Moradi (2009a). After correction, the 9 NR partial images of a given recording were assembled to one single picture.

Depth growth of the main root was measured manually by means of an image analysis program. To determine the minimum root diameter of roots visible in the neutron radiographs, the root length in the neutron radiographs were compared with root length measurements by size classes from WinRhizo Pro© (Regent Instruments, Inc. 2009c).

3.3.4 Analysis of plant samples

At the end of each experiment, the aboveground biomass of all seedlings was harvested, separated into stems and leaves and dried until no further weight loss occurred. After harvesting the plants, the containers were opened laterally and in the case of Exp. 1 the soil of each container was sub-divided into two equal portions, representing the two halves of the container. In the case of Exp. 2, the soil of each container was divided into 3 × 3 equal portions, representing the 3 soil depths layers and 3 vertical columns that had been imaged separately. The roots of each soil portion were separated from the soil by washing. In Exp. 2, the roots were scanned in a water bath at 400 dpi with a backlighting scanner (Epson Expression 10000XL) prior to drying and further analysis. The maximum scanning density of 3 cm root lengths cm^{-2} scanner surface recommended by Himmelbauer et al. (2004) was not exceeded. After scanning, root lengths and diameters were determined by means of image analysis software WinRhizo Pro©. The containers were stored at 4°C in a cooling chamber until scanning. In both experiments, total root mass and FR biomass (<2 mm) were determined after drying at 40°C until weight constancy. For chemical analysis all plant samples (stems, leaves, roots) from both experiments were digested in a heating block at 130°C with 65% HNO_3 and analyzed for B and other elements using ICP-OES (Vista MPX, Varian, Australia). To minimize B memory effects we rinsed the instrument with a 2% mannitol solution after each sample.

3.3.5 Statistics

Mean shoot B concentrations were calculated as mass-weighted averages of the B concentrations of the individual plant parts. Analysis of variance was performed to test for treatment effects on biomass and B concentrations, followed by Fisher-LSD post-hoc test to compare pair-wise differences between treatments. Values given for correlations between variables represent Pearson's correlation coefficients. All statistical analyses were carried out using Sigma Plot 11 (Systat Software, Inc. 2008).

3.4 Results and discussion

3.4.1 Shoot and root growth

There was a significant increase in shoot growth compared to the control treatment (2.1 mg kg^{-1}) in the homogeneous 2.7 mg kg^{-1} and the heterogeneous 3.3 mg kg^{-1} treatment, which had the same average soil B concentration, suggesting marginal B deficiency conditions at the lowest soil B level (control) (Fig. 1).

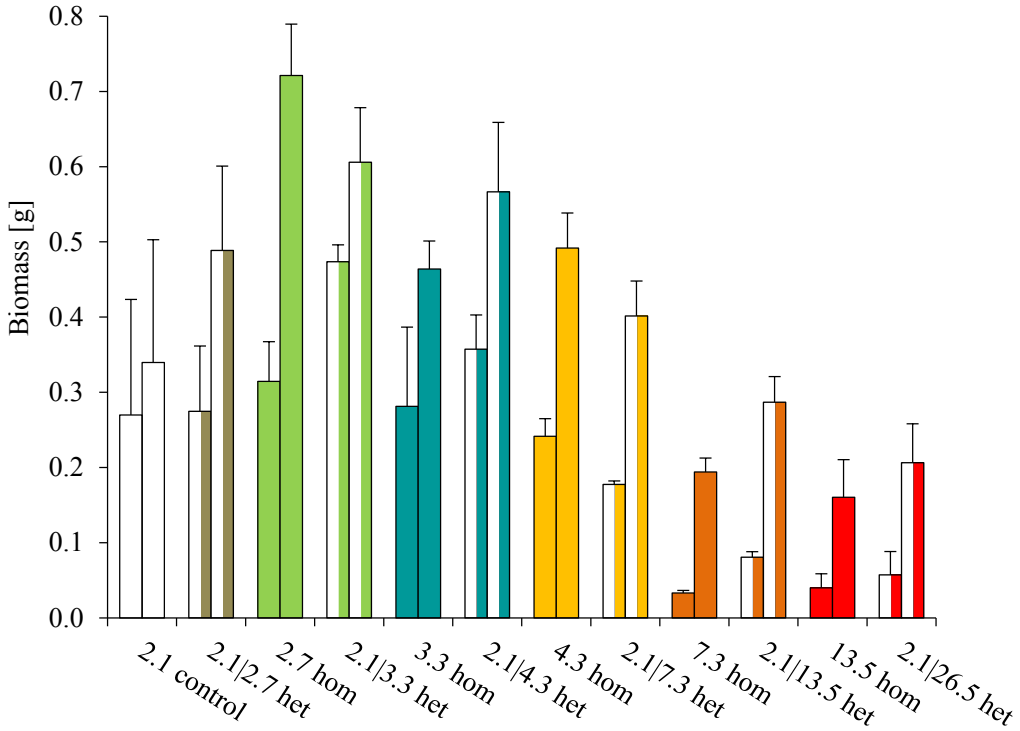


Figure 1. Root (left bars) and shoot (right bars) biomass of 3-month old poplar plants grown on B-spiked soil. The soil was spiked either homogeneously (hom) or heterogeneously (het). The numbers represent the soil B concentrations [mg kg^{-1}]. The two numbers given for the heterogeneous treatments (het) represent the unspiked and spiked compartment. Similar colors indicate similar average soil B concentrations of two treatments. Error bars represent standard errors.

While there was a non-significant trend for decreased growth at soil B concentrations of 4.3 mg kg^{-1}, shoot growth was significantly reduced at soil B concentrations ≥7.3 mg kg^{-1} and symptoms of B toxicity like burned leaf margins became visible. The plants did not survive the highest homogeneous B treatment (26.5 mg kg^{-1}) level in contrast to the highest heterogeneous treatment level (2.1|26.5 mg kg^{-1}), at which the average B concentration was the same as in the homogeneous 13.5 mg kg^{-1} treatment.

Although there was a trend for larger shoot growth in the heterogeneous treatments compared to the respective homogeneous treatments at average soil B concentrations ≥7.3 mg kg^{-1}, these differences were not significant. Apparently, the plants did not substantially benefit from the fact that half of their root system in the heterogeneous treatments was developing in low B soil. While shoot biomass was always larger than root biomass, both were closely correlated to each other ($y = 1.11x + 0.17$; $r^2 = 0.78$; $p<0.001$) and showed similar patterns of dependency on the soil B treatments. However, as shoot growth was more stimulated by B than root growth in the average soil B concentration range from 2.1 to 2.7 mg kg^{-1}, the root: shoot ratio was significantly lower in the homogeneous 2.7 mg kg^{-1} treatment than in the control (data not shown). The maximum root: shoot ratio (0.8) was found in the heterogeneous treatment with an average soil B concentration of 2.7 mg kg^{-1}. At soil B concentrations ≥7.3 mg kg^{-1} the root: shoot ratio significantly declined, indicating a stronger B toxicity in the roots than in the shoots. From a meta-analysis of published data, Audet et al. (2008) concluded that plants tend to allocate relatively more biomass into roots with increasing soil metal concentrations. Here, we did not observe this pattern in poplars exposed to high soil B concentrations, which is consistent with the findings of Reid et al. (2004) for wheat and Rees et al. (2011) for poplars.

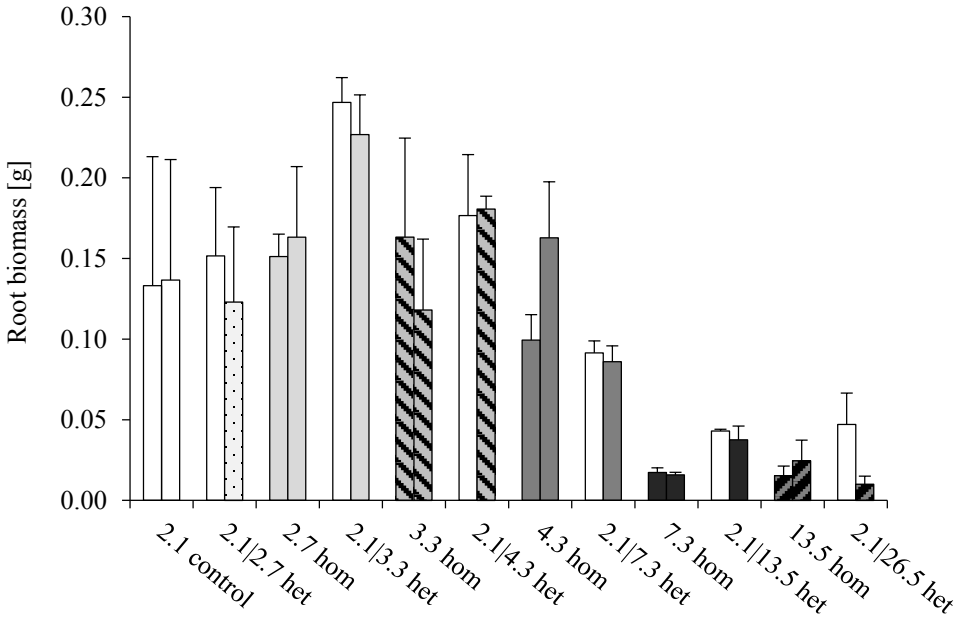


Figure 2. Poplar fine root biomass in the unspiked (left bars) and the spiked (right bars) portions of the soil in Experiment 1. Similar bar shades or fillings indicate similar average soil B concentrations of two treatments. Error bars represent standard errors.

In Exp. 1 we found no significant effects of the heterogeneous distribution of soil B on FR biomass when we compared the spiked with the unspiked portions of the containers (Fig. 2). At the highest B level of the heterogeneous treatment FR biomass was 4 times lower in the spiked than in the unspiked portion, but this difference was not significant, due to the high variability in FR biomass. In the heterogeneous treatments it became obvious that the significantly reduced total FR biomass at average soil B concentrations ≥7.3 mg kg^{-1} was not only due to root growth reduction in the spiked, but in both portions (Fig. 1).

The highest root biomass and coarse root length was always found beneath the stem base, in the top middle compartment of the containers. Coarse root length in this compartment ranged from 16.5 cm in the heterogeneous, to 22.8 cm in the control and 23.9 cm in the homogeneous treatment, without showing significant differences. In all other compartments, the coarse root lengths were <1.5 cm. All treatments with B spiking resulted in a greater proportion of FR length near the soil surface than in the control. At soil depth >21 cm FR lengths were always larger in the central than in the lateral compartments of the containers, independent of B treatments (Fig. 3).

Coarse root lengths were unaffected by B patchiness in our experiment. However, coarse roots develop from FR by secondary thickening. Coarse roots cannot establish in soil where FR cannot establish. Therefore, the inhibition of FR growth in a larger volume of soil will also impair the development of coarse roots and thus reduce the stability of a tree as it grows and needs increasing anchorage in the soil. Depending on the localization of a contaminant hotspot and wind direction trees could be more vulnerable towards leaning and wind throw and might suffer from reduced water supply. Despite the large variability in root growth patterns, the neutron radiographs taken in Exp. 2 revealed faster root system development in the control than in both B-treatments (Fig. 4), and especially in the homogeneous B treatment. This observation is in line with the greater fraction of FR in the uppermost soil in B-spiked than in un-spiked soil mentioned before. After 4 months of growth (24.10.), NR-visible roots had already reached a soil depth of 40 cm in the control treatment, while in the B treatments NR-visible roots did not reach below 25 cm depth (Fig. 5). The bottom of the containers was reached by the roots after 5 months of growth in the control treatment, while it was not reached during the course of the experiment in the homogeneous B treatment. Delayed root depth growth could make stand establishment difficult under field conditions, especially when factors such as water or nutrient deficiencies lead to additional stress.

The total length of the roots that were collected by soil washing was on average 15 times larger than the total root length determined from the NR images. The total length of roots determined by NR imaging was approximately equivalent to the length of roots with a diameter >0.7 mm obtained by soil washing, indicating that roots below that diameter were not detected by NR imaging.

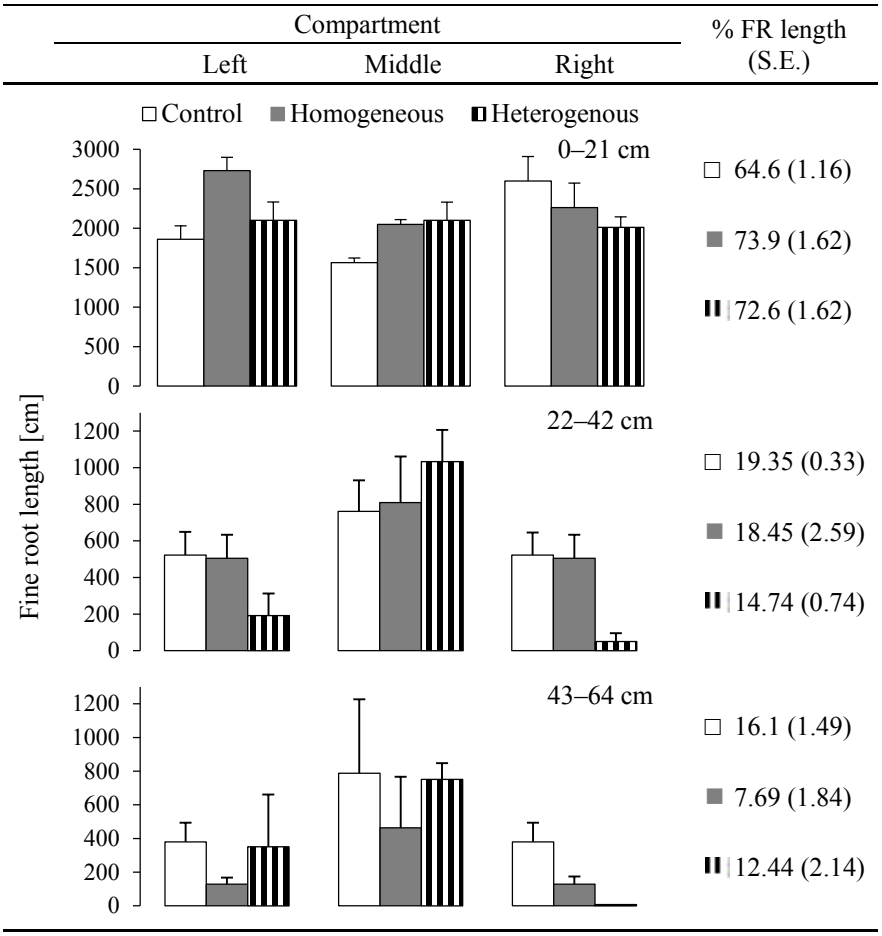


Figure 3. Poplar fine root length after 6 months of growth in containers with no, homogeneous and heterogeneous soil B spiking. The right compartment is spiked in the heterogeneous treatment. The background B concentration of the unspiked soil was 2.1 mg kg^{-1}. In the homogeneous spiking 7.5 mg kg^{-1} B were added to the entire soil, while in the heterogeneous spiking the same total amount of B was added to only one third of each packing. Error bars represent standard errors. The figures on the right give total poplar FR length per depth as percentage of total FR length (S.E. = Standard Error).

This is about 2.5 times more than the pixel size of the scintillator. Only about 6 – 8% of the total root length was contributed by roots with a diameter >0.7 mm. Thus, we considered the NR visible fraction as insufficient for a more detailed analysis of the root system. Nonetheless, there was a close relationship (r^2= 0.77; y = 323.66x + 14.71; p<0.001) between total root biomass and NR-visible root length in the individual compartments, when

we excluded the uppermost central compartment because of the extremely large root mass in this part of the container. Given that roots with diameter <0.7 mm were not detected by NR, one might argue that not FR length growth was inhibited by B stress but only secondary thickening. However, the root diameter distributions presented below do not support this hypothesis.

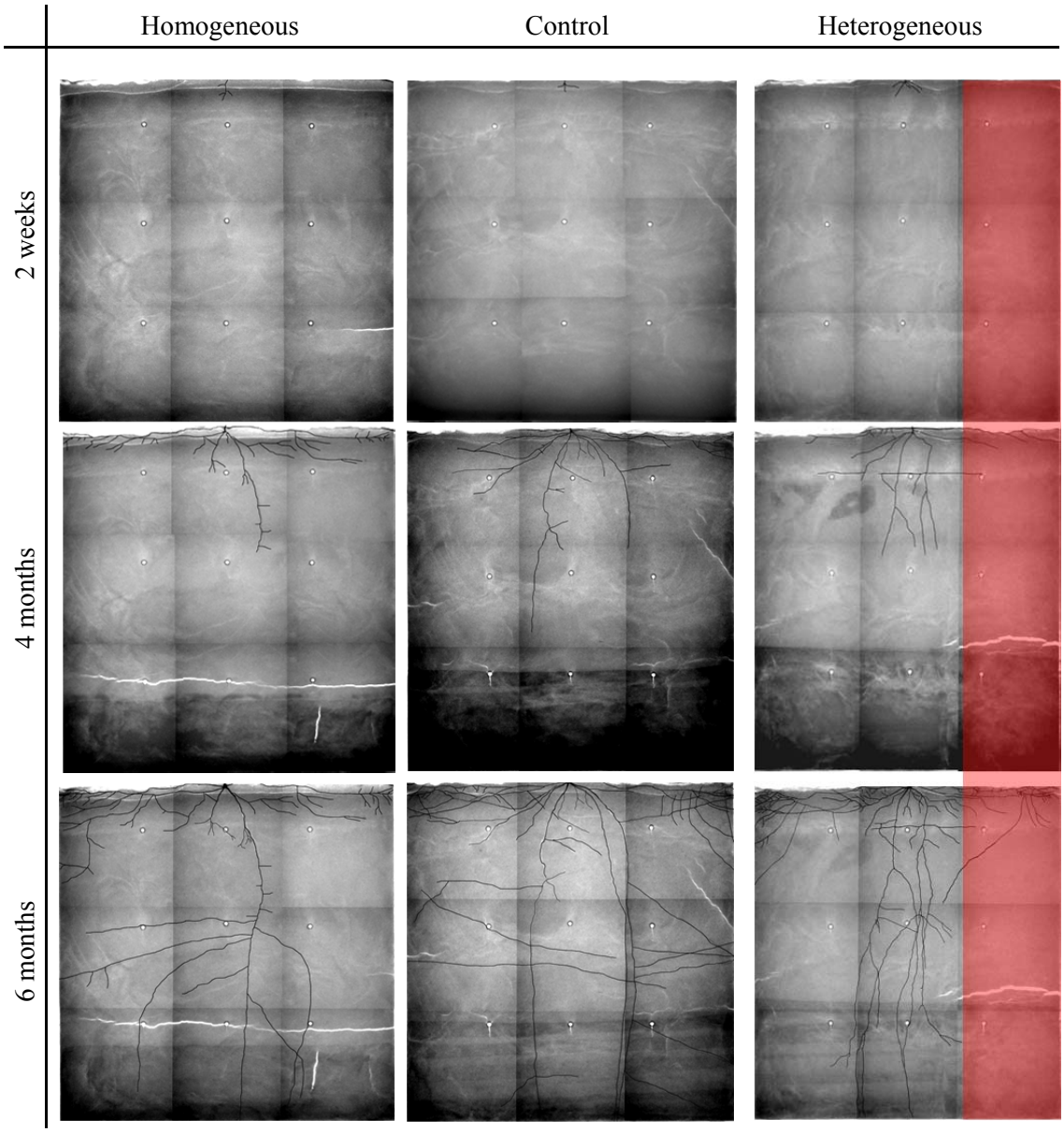


Figure 4. Neutron radiographs of soil and poplar roots from Exp. 2 growing in homogeneously B- spiked soil, un-spiked control and heterogeneously B-spiked soil portions. Roots were re-traced by hand to enhance the contrast to the surrounding soil for improved presentation. The transparent red area represents the B- spiked portion of the heterogeneous treatment.

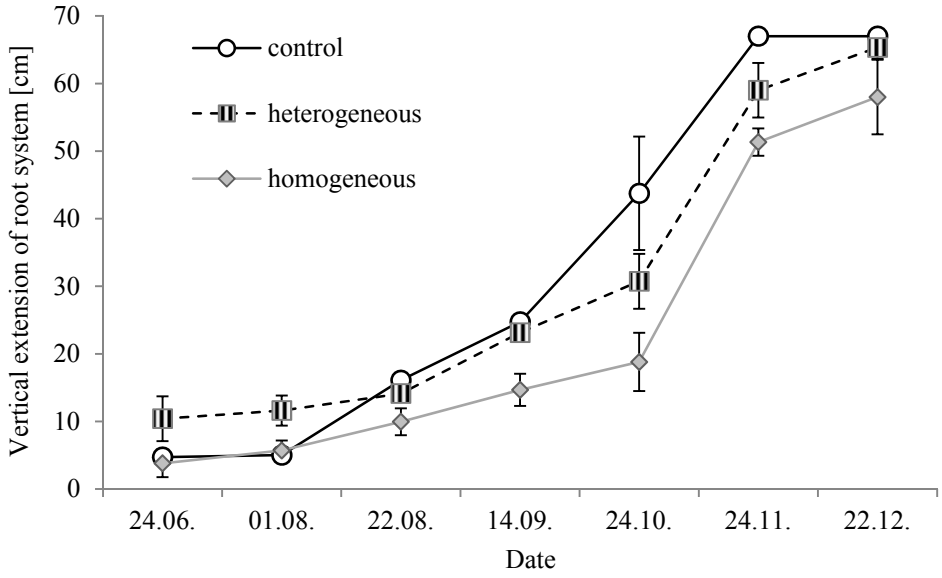


Figure 5. Vertical extension of the main root over time in Experiment 2 as determined by repeated NR imaging. The maximum vertical extension of the roots determined by the size of the containers (64 cm) was reached in all control plants at the 24.11.. Root length was assessed for roots >0.7 mm in diameter. Error bars represent standard errors.

The lower two thirds of the B-treatments differed significantly in their root diameter distributions from the control treatment and the uppermost soil layer of the B-treatments. While in the control treatment and in the uppermost soil layer in the B-treatments, roots had a diameter of 0.1 – 0.3 mm, the peaks of the frequency distributions were broader and shifted to higher diameter classes in B-spiked soil (Fig. 6). These effects increased with soil depth and lateral distance from the stem. They were also found in the unspiked soil of the heterogeneous B treatment, indicating a systemic root system response. An explanation for these changes in diameter class distributions could be that poplars shift to an extensive rooting strategy under stress conditions, in which they invest more assimilates into exudate production and less into increasing specific root length [cm g^{-1}] (Lohmus et al., 2006; Ostonen et al., 2007). As far as the differences in root diameters reflect local toxicity effects, they could also have been due to a higher survival probability of thicker roots as well as to a stronger B toxicity effect on length than on diameter growth. The observed shift to higher FR diameters could be disadvantageous for nutrient and water acquisition because the fraction soil pore space that can be accessed by roots decreases with root diameter.

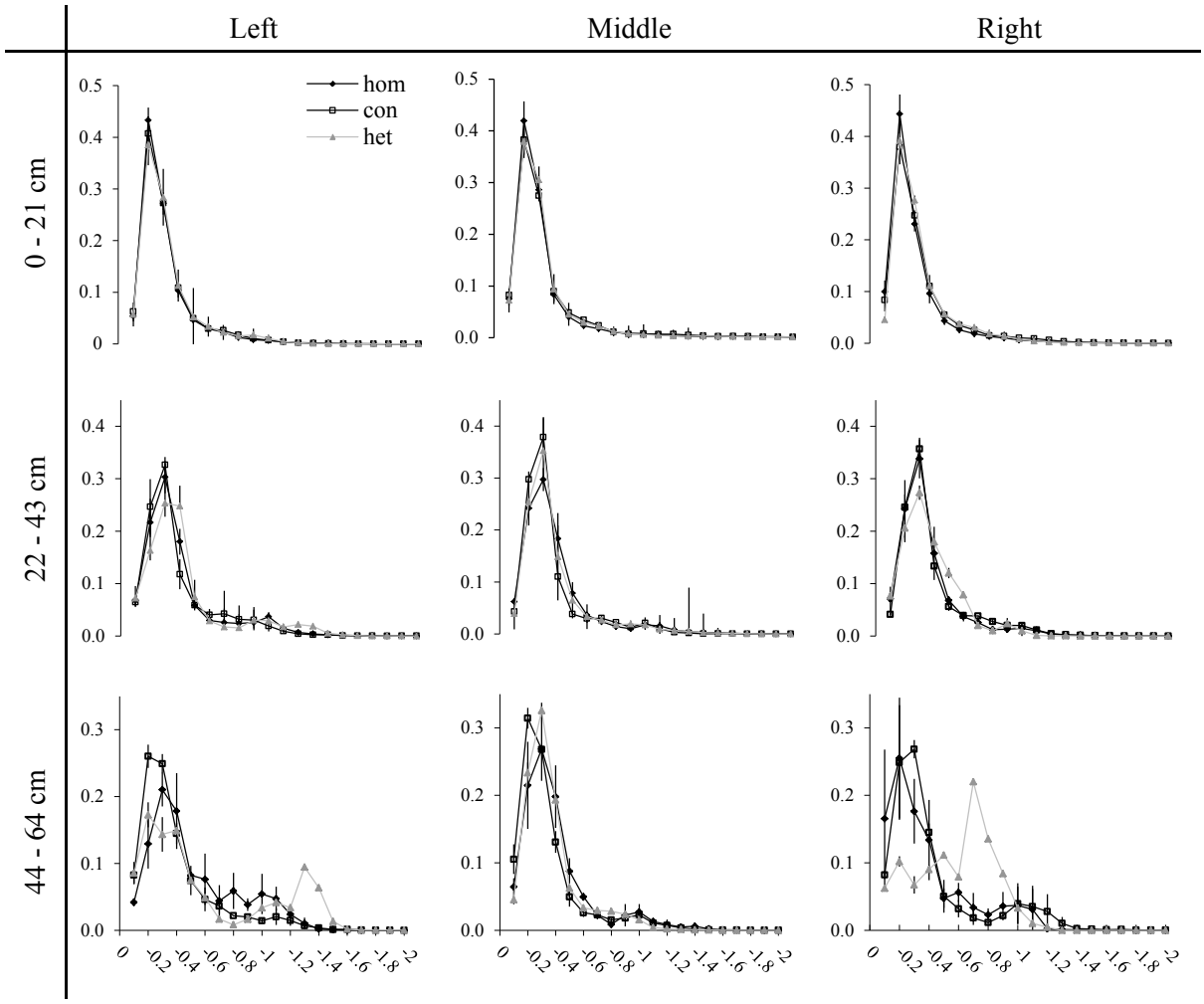


Figure 6. Root diameter distributions of fine roots in Exp. 2. Root length per diameter class is given as fraction of total root lengths. Left, middle and right are the respective portions of the containers. Error bars represent standard errors.

3.4.2 Influence of accumulated plant B on growth

Figure 7 shows high shoot biomass variability at shoot B concentrations <500 mg kg^{-1}. The maximum growth was found in plants with shoot B concentration of 40 to 150 mg kg^{-1}. At shoot B concentrations above 500 mg kg^{-1} shoot growth was reduced, indicating B toxicity. The leaf B concentrations found at average shoot B concentrations >500 mg kg^{-1} were in the range 800 – 1660 mg kg^{-1}, showing an onset of toxicity at similar leaf concentrations as found by Rees et al. (2011) in *Populus nigra* × *euramericana*. The relationship between shoot biomass and shoot B accumulation was the same in heterogeneous and homogeneous treatments, suggesting that any B toxicity effect on shoot growth was only dependent on the shoot B concentrations.

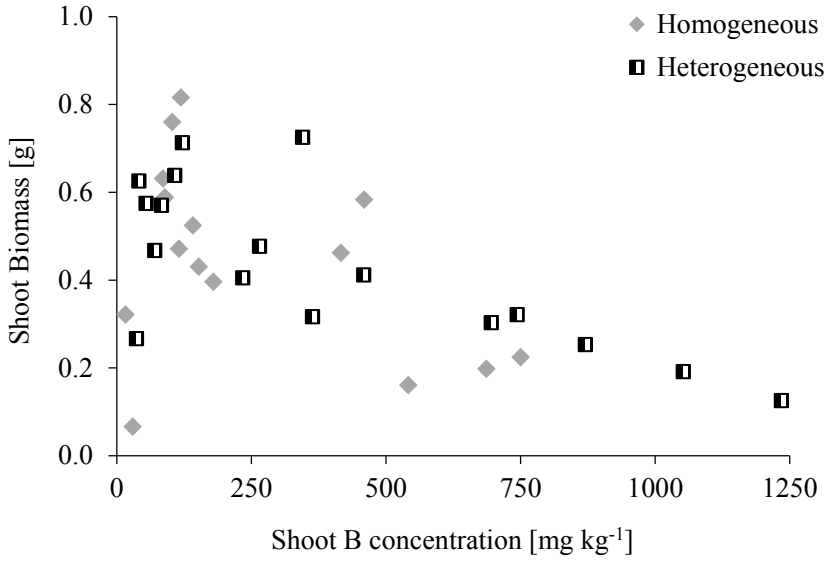


Figure 7. Shoot biomass as a function of shoot B concentration of poplars grown in Experiment 1 in the homo- and in the heterogeneous treatment.

All plants with shoot B concentrations <40 mg kg^{-1} produced less than 0.35 g biomass during the three months growth period of Exp. 1, which was less than 40% of the maximum growth. The corresponding leaf B concentrations of these plants were below 45 mg kg^{-1}. While one of the plants with such a low B concentration was found in the heterogeneous treatment with the lowest level of B spiking, two others were obtained from the homogenous treatment with unspiked soil. Boron deficiency limits for tree foliage are in the range of 4 (*Picea Abies*, *Pinus Sylvestris*) to 16 mg kg^{-1} (*Eucalyptus globulus*) and are generally higher in broadleaf species than in conifers (Lehto et al., 2010). Our results indicate that poplars are more sensitive to low soil B supply than most other plants. This may be due a higher B demand and a lack of transporters for active xylem loading of B under deficiency conditions (Tuskan et al., 2006). The higher B demand may be related to the higher B tolerance of poplars compared to other tree species. The first symptom of B deficiency in plants usually is decreased root growth in relation to shoot growth (Dell et al., 1997). Such an effect was not found here. We also found no enhanced proliferation of roots in B-enriched patches to compensate for low B supply in the heterogeneous treatments.

Paralleling the larger variability in shoot biomass at shoot B concentrations <500 mg kg^{-1}, there was also a large variability in root biomass at root B concentrations <40 mg kg^{-1}, indicating that at lower B concentrations the accumulation of excessive B in the plants was not a growth-limiting factor. The maximum root biomass was significantly reduced when root B concentrations exceeded 35 mg kg^{-1} in B-spiked soil (Fig. 8). This was the case at soil B concentrations of ≥4.3 mg kg^{-1} in the homogeneous treatment (Fig. 9a).

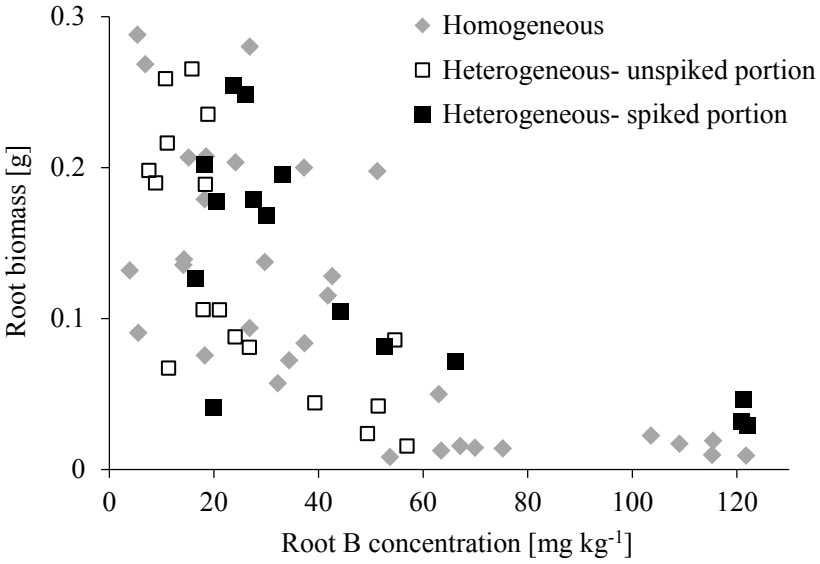


Figure 8. Root biomass as a function of root B concentration of roots grown in Experiment 1. Each point represent one half of an either homogeneously or heterogeneously spiked container and the B concentration of the roots grown there.

In the unspiked soil of the heterogeneous treatments, root growth tended to be reduced at root B concentrations >20 mg kg^{-1}, which again occurred at soil B concentrations >7.3 mg kg^{-1} in the spiked part of the heterogeneous treatment (Fig. 9a). This indicates that the root growth inhibition observed at moderately elevated B treatment levels was not a toxicity response to the accumulation of B in the roots or to soil B, but a systemic response to B in the shoots. This hypothesis also agrees with the observation that shoot biomass was more sensitive to the B treatments at moderately elevated B concentrations than root biomass (Fig. 1).

Figure 9a shows that B accumulation by roots in the unspiked soil parts of the heterogeneous treatments increased proportionally to the B concentrations of the roots in the spiked parts of the soil, with a ratio of approximately 1:2 between the B concentrations in the roots of the unspiked and the spiked soil in a given treatment. This means that there was substantial lateral B transfer from the spiked to the unspiked soil compartments. Such transfer could have occurred through the soil solution as well as through roots extending from the unspiked into the spiked soil. The highest uptake of B into roots took place in the spiked soil at the two highest B levels in the heterogeneous treatments. In these cases, root B concentrations of up to 130 mg kg^{-1} were found. The results from Exp. 2 agreed with the findings of Exp. 1 that root toxicity effects started to occur in B spiked soil when root B concentrations exceeded 35 mg kg^{-1} (data not shown).

Root B concentrations exceeded 35 mg kg^{-1} in Exp. 2 in the homogeneously spiked soil as well as in the spiked portion of the heterogeneous treatment and at the same time also FR length was reduced.

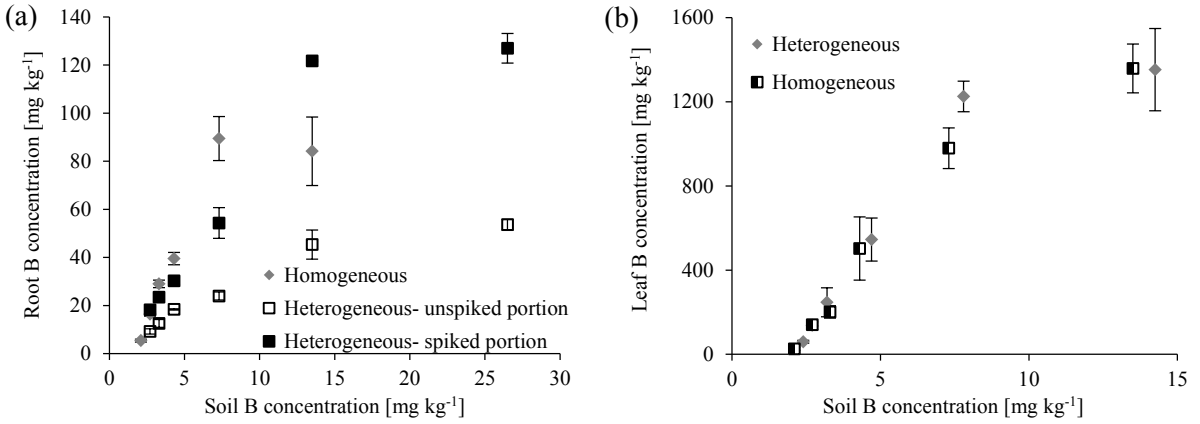


Figure 9 (a). Root B concentrations as function of soil B concentrations. For better clarity the B concentrations of roots from the unspiked soil portions of the heterogeneous treatments are plotted against the respective B concentrations of the spiked soil portions. (b): Leaf B concentration as function of the average B concentration in soil with heterogeneous or homogeneous B spiking in Exp. 1. Error bars represent standard errors.

Leaf B concentrations increased with increasing average soil B concentrations up to 1350 mg kg^{-1} at average soil B concentrations of 13 – 14 mg kg^{-1}, irrespective of the homogeneity of the spiking. Between average soil B concentrations of 3 and 8 mg kg^{-1} this increase was almost linear; then it leveled off (Fig. 9b). The finding that the accumulation of B in the leaves only depended on the average concentration of B in the soil and not on its spatial distribution indicates that the spiked and the unspiked parts contributed equally to B uptake in the heterogeneous treatments This is in agreement with the hypothesis that toxicity effects in the roots were primarily systemic plant responses to B accumulation in the shoots and thus similar in spiked and unspiked soil portions. In this regard B uptake appears to differ from the uptake of other trace elements with heterogeneous distribution in soil (Millis et al., 2004). In Exp. 2 leaf B concentrations ranged between 36.7 (control), 260 (homogeneous treatment) and 380 mg B kg^{-1} (heterogeneous treatment). Significant differences were only found between the control and the two B treatments, but not among the homogeneous and the heterogeneous treatment. This was consistent with the result from Exp. 1 that B uptake was only due to the average soil B concentration. In the B treatments of Exp. 2 leaf B concentrations did not exceed the leaf toxicity threshold found in Exp. 1 (800 mg kg^{-1}), which may explain the absence of significant effects on the shoot biomass (data not shown).

Our results on B tolerance and uptake from heterogeneously contaminated soils have implications for the phytomanagement of B contaminated sites. Long clean-up times are one of the major disadvantages of phytoremediation (Pulford et al., 2003; Robinson et al., 2009). Thus, the finding that patchy soil B distribution did not lead to decreased B accumulation in the shoots, while it tended to result in larger growth compared to a homogenous distribution of the same amount of B is an encouraging result for further field applications of poplars in phytoremediation, as heterogeneous contamination is rather the rule than the exception under field conditions. Breeding of poplar hybrids for B phytoremediation should focus on the B tolerance of poplars leaf tissue as it seems to govern the whole plant status.

3.5 Conclusions

In summary the results of this study suggest that the B toxicity effects observed in the growth of the young poplar plants occurred along two routes. With increasing exposure to soil B, shoot growth became already inhibited at average soil B concentrations of 7 mg kg^{-1}. Root growth was inhibited indirectly by this toxicity effect, i.e. due to a systemic response to B accumulation in the shoots. At soil B concentrations between 13.5 and 22.5 mg kg^{-1} also toxicity effects on root growth due to local exposure became manifest. We conclude from these results that local heterogeneity in soil B should have little influence on the phytoremediation of contaminated sites using poplar, as long as the contamination allows root growth.

3.6 Acknowledgments

Funds for this study came from the Swiss National Science Foundation (SNSF). The technical assistance with neutron radiography measurements provided by Stefan Hartmann and Peter Vontobel from PSI is gratefully acknowledged. We would also like to thank Nadine Grisel from WSL for preparing the poplar cuttings.

3.7 References

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4 Boron accumulation and tolerance of hybrid poplars grown on a B-laden mixed paper mill waste landfill

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A shortened version of this chapter was submitted for publication in *Journal of Hazardous Materials*

4.1 Abstract

Paper mill wastes are a mixture of by-products from pulp production and on-site energy production, consisting of paper mill sludge, ashes and cinders. Landfilling of these highly boron (B) and heavy metal laden wastes products implies environmental risks, especially for receiving ground and surface waters. Poplars have been successfully employed in the phytomanagement and hydraulic control of B contaminated sites. In this paper, we assess the growth and survival of 4 different poplar clones on a mixed paper mill waste landfill site, investigate the accumulation of B by the trees and explore the relationship between local-scale root growth and substrate properties. Leaf and root tissue samples of 40 months old poplars were collected on three test plots and analysed for their chemical properties and root traits. In addition, we sampled 4 soil cores (0 – 20 cm depth, subdivided into 4 sections) in the vicinity of each of the 75 trees and determined chemical and physical properties. Using a principal component analysis, followed by a hierarchical cluster analysis 3 different substrate types were identified. This method proved to be effective in detecting clear soil effects on tree survival and growth, although correlations with individual soil element concentrations and other parameters were weak. Despite the fact that B accumulation reached toxic levels in the leaves, B toxicity was not found to be the key limiting factor for poplar survival and growth at the site. Instead, our data indicate that Ca deficiency, induced through Mg:Ca imbalance was a more important growth limiting factor. The substrates were intensively colonized by fine roots and there was no indication of root growth limitation due to toxicity effects of the soil contaminants. Our results show that poplars in general are well capable to endure under the harsh growing conditions on a multiply contaminated, B-laden substrate in a hemiboreal climate. There were considerable differences in the performance of the four clones. Considering these differences in relation to the three soil types could help to increase revegetation success on the remainder of the study site and on similar landfills.

4.2 Introduction

During paper production many different by-products accrue in paper mills, of which paper mill sludge is the most important by volume. It is used as raw material in other industries, spread on agricultural land as fertilizer, incinerated for energy production in paper mills or otherwise landfilled (Lteif et al., 2007; Monte et al., 2009). In a study by the Environmental Protection Agency (U.S.EPA, 1988), sludge production was found to vary widely among mills, ranging from 14 to 140 kg per 10^3 kg of pulp among 104 mills with a total sludge generation of 2.5×10^9 kg a^{-1}. Other solid wastes from paper mills include various kinds of ashes and cinders, originating from on-site energy production by incineration of wood wastes and paper mill sludge. Fly and other ashes contain high concentrations of boron (B), besides other potentially toxic elements such as Cu and Ni (Dellantonio et al., 2008). Landfilling and agricultural application of paper mill wastes, including ashes, a are major source of B in the environment (Nable et al., 1997; Pavlović et al., 2004). Boron is an essential micronutrient for plants and animals at low concentrations (Salisbury et al., 1992; Mastromatteo et al., 1994), but like other trace elements it becomes toxic at higher concentrations. The concentration range between B deficiency and toxicity is smaller than for any other element (Goldberg, 1997). In addition, B is a rather mobile element in the environment and easily leached from a landfill into ground or surface waters if not contained properly. Thus, landfilling paper mill sludges or their incineration products requires particular attention.

Poplars are used extensively for soil stabilization and erosion control (Wilkinson, 1999; McIvor et al., 2009), for phytomanagement and hydraulic control of contaminated sites (Robinson et al., 2007; Zalesny et al., 2007b; Zalesny Jr et al., 2009), and also to provide supplementary stock fodder for cattle and sheep during times of drought (Hathaway, 1986; Douglas et al., 1996). Poplars have been found to accumulate inordinate concentrations of B compared to other plant species. Bañuelos et al. (1999). Robinson et al. (2003) found B accumulation in poplar leaves up to 1200 mg B kg^{-1}, some 20 times more than in other species grown in the same environment. At average leaf B concentrations of 800 mg kg^{-1} no growth reduction was found in a *Populus nigra* × *euramericana* hybrid clone, while leaf B concentrations higher than 194 and 304 mg kg^{-1} were found to be fatal for *Brassica juncea* and *Lupinus albus* in the same pot experiment (Rees et al., 2011). The capacity of poplars to accumulate extremely high B concentrations in their leaves indicates that they have special mechanisms for B detoxification and storage.

Halophytes have been shown to be able to detoxify B tissue concentrations of up to 300 mg kg^{-1} by complexation of B with *cis*-diol groups (Rozema et al., 1992). However, a concentration of 300 mg kg^{-1} is well below the highest values measured in poplar leaves. Little is known about B detoxification in plants. In some plants, such as *Rosaceae*, B toxicity seems to be controlled by binding of B to alcohol sugars (Brown et al., 1997).

Poplars can reduce B leaching from contaminated soils into receiving waters by extracting large amounts of water for transpiration and simultaneously taking up B from the soil into their aerial parts, whereupon it can be removed from the site by harvesting these parts (Robinson et al., 2003). Complete clean-up of a B-contaminated site is limited in theory only by the depth to which the contamination is accessible by root water uptake and by the tolerance of the tissues to the accumulated contaminant. Our previous pot experiments have shown that poplars are very promising as phytoremediation plants on B-rich soils. However, in phytoremediation and phytomanagement research there is often a discrepancy between results obtained under well-controlled experimental conditions and in real–world field situations. A major problem in the design of phytomanagement systems under field situations is the large spatial variability in soil properties and contaminant distribution (Dickinson et al., 2009), which can strongly affect the development and activity of the root system and thus the success and efficiency of the operation (Keller et al., 2003; Wenzel, 2009). Hot spots of heavy metals contamination in soils were found to be avoided by the roots of various tree species (Breckle et al., 1992), whereas the roots of some hyperaccumulator plants, such as *Thlaspi caerulescens,* were found to proliferate into zinc-rich spots (Whiting et al., 2000). Soil is a heterogeneous medium in general. Not only trace elements but also other soil factors influencing root traits and morphology, such as water, air or macronutrients, often show quite heterogeneous distribution patterns (Heilman et al., 1994; Hodge, 2004; Zobel et al., 2007). Under well-defined conditions, poplar root growth was shown to be inhibited in soil patches with B concentrations >20 mg kg^{-1} (Rees et al. 2011), but there are no studies on the response of poplar roots to a spatially heterogeneous distribution of soil B under field conditions. Here, we report the results of a study on the relationship between root development, tree growth and the concentrations of soil B and other contaminants in landfilled paper mill wastes that had been revegetated with various *Populus deltoids* × *nigra* clones. The aims of this study were: (1) to assess the growth and survival of the different poplar clones (a) in response to soil conditions and (b) in relation to the concentrations of B and other potentially toxic or deficient elements in the tissues of the

trees; (2) to investigate the accumulation of B by the trees in relation to the extent of soil contamination and (3) to determine the relationship between local-scale root growth, B contamination and other soil factors.

4.3 Material & Methods

4.3.1 Site description

The study was carried out on the "Pine Lake Landfill" (PLL) at Rhinelander, Wisconsin, U.S. (45° 38′ 22″ N 89° 24′ 44″ W) (Fig. 1 (a)).

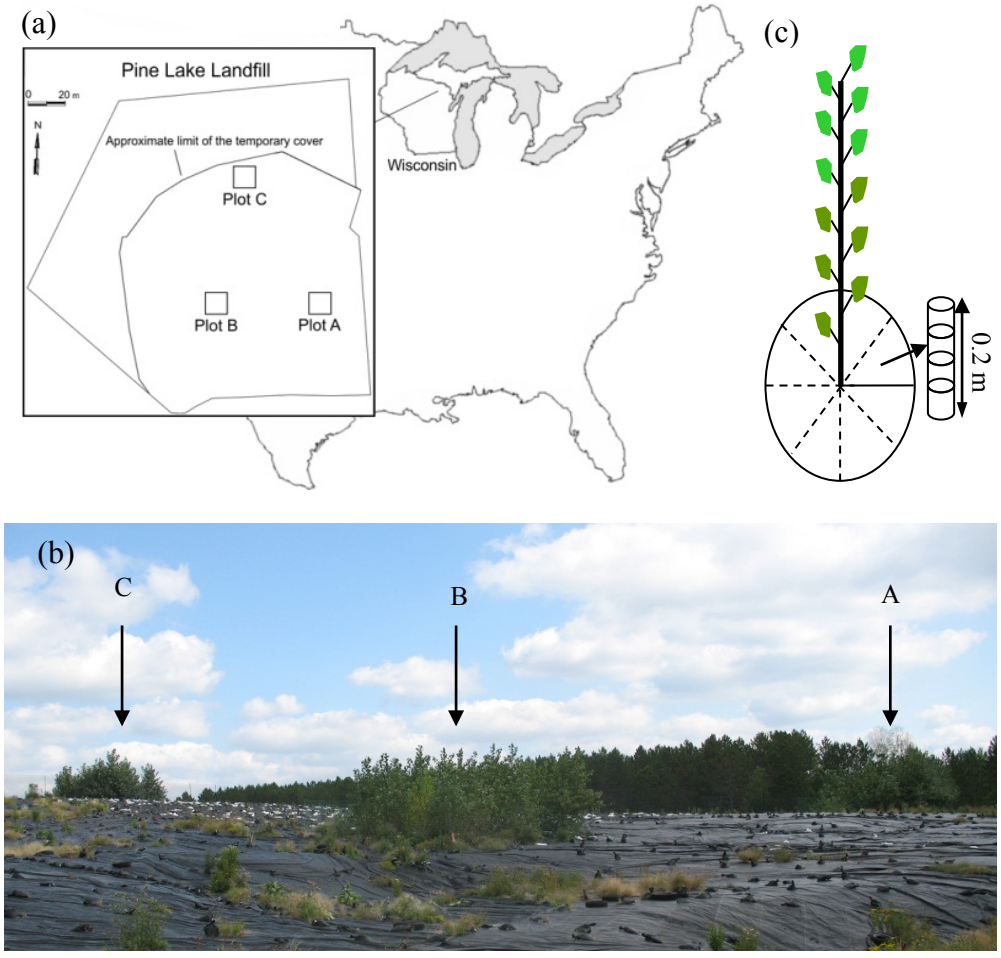

Figure 1 (a). Situation of Pine Lake Landfill in the Eastern United States and the state of Wisconsin and situation of the three plots A, B and C on the part of the landfill that has a temporary cover. (b) View of Pine Lake Landfill from south west showing the three test plots A, B and C (marked by arrows). The black geotextile and the sandbags holding it down are visible. The trees on the three plots were on average around 5 m high. Herbs growing on the site are visible in the foreground. (c) Sampling scheme for soil, root and leaf samples. Soil and root samples were taken with a soil auger at 70 cm distance from each stem in one of the false point directions of the compass. Leaves from the top and bottom half of the poplars were samples separately.

The average annual mean temperature at the Rhinelander climate station (NCDC normal 1971 - 2000) is 4.9 °C. The average monthly mean temperature has a maximum of 19.6 °C in July and a minimum of -11.9 °C in January. The annual precipitation averages 810 mm. The landfill has a total size of approximately 4 ha. A 2 ha area of the landfill had been covered only provisionally with an approximately 10 cm thick layer of subsoil, taken from the adjacent forest, and a geotextile (Fig 1 (c)). This part of the landfill had a slight slope facing southwards. A permanent cover had not been installed here at the time of the study, as this part of the landfill was not full and reserved for further landfilling in later years. The sub-soil layer was partially eroded due to surface runoff, which occurred despite the geotextile. Paper mill sludge, fly ash and cinders had been deposited on the site until 1995. The material had been brought by trucks and the dumped truck loads had created a patchwork pattern of the different materials with an average patch size of approximately 40 m^2. A plastic bottom liner and a leachate collection system had been installed to capture any leachates from the site and to collect them in two tanks, from where they were brought by trucks to a wastewater treatment facility.

In July 2006, a pilot project was started to investigate if a stand of poplar trees could be established and provide a suitable alternative cover to the weakened geotextile for erosion and leachate control. Three 10 × 10 m test plots (A, B and C, Fig. 1 (a), (b)) were established with 4 commercially available poplar clones of *Populus deltoides* Bartr. ex. Marsh × *P. nigra* L. (DN-2, DN-34, DN-182, OP-367). On each of the plots 36 poplar cuttings were planted on a regular 6 × 6 grid with a spacing of 1.7 m (9 cuttings of each clone). While DN-2 was planted as rooted stock, DN-34, DN-182 and OP-367 were planted as 25 cm cuttings. Small holes were made into the geotextile, through which the cuttings were directly planted into the substrate. Weeds were removed two months after planting in 2006 to ease the establishment of the young trees. One of the three plots (C) was situated at the northern (upper) end of the 2 ha area, the other two plots (A, B) on the lower (southerly) side of the area with temporary cover. The distances between the plots were 57 m (A - B), 86 m (A - C) and 76 m (B - C). Nylon mesh fences were installed around each plot to protect the trees from browsing deer. At locations, where the geotextile had been deteriorated with age or accidentally damaged, weeds such as *Cirsium vulgare* Ten and *Verbascum spp*. L. grew spontaneously.

4.3.2 Sampling and sample analysis

The height and survival of all poplar trees was recorded in July ’07, July ’08, October ’08 and September ‘09. In September ’09 also the diameter at breast height (DBH) of each tree was determined, by averaging two perpendicular caliper measurements at 1.3 m above the soil surface. Woody biomass (stem + branches) of the trees was estimated from the DBH using the equation of Riemenschneider *et al.* (2001):

$$\text{Woody biomass [kg]} = [6.16 - (2.23 \times \text{DBH}) + (0.353 \times \text{DBH}^2)] \quad \text{(Equation 1)}$$

In September 2009, also soil and leaf samples were taken during 10 consecutive days with no rainfall. Ten leaves each were taken from the lower and the upper half of the trees. The leaf samples were dried at 60°C until constant weight and then ground, digested in a heating block (DigiPREP MS, 130°C, 65% HNO_3, SCP Science, QC, Canada) and analyzed by means of inductively-coupled plasma optical emission spectroscopy (ICP-OES) using a Varian Vista MPX (Varian, Australia). After each analysis the instrument was rinsed with a solution containing 2% D-Mannit and 0.5% HNO_3 in order to flush out B from the proceeding sample. Carbon and nitrogen contents were measured by means of an elemental analyzer (CNS-2000, Leco Corp., Saint Joseph, Michigan USA). Soils including roots were sampled by taking 4 consecutive cores of 5 cm length and 5 cm diameter down to a depth of 20 cm at a distance of 70 cm from the stem of each tree (Fig. 1 (c)), with the exception of 4 samples from 15 – 20 cm depth, which could not be taken for mechanical reasons. The direction of the sampling point from the respective tree was randomly selected in one of the eight false point directions of the compass (NNE, ENE, ESE, SSE, SSW, WSW, WNW, NNW). The coordinates (relative to the grid) of all sampling locations were calculated from their direction and distance to the stem. The samples were stored in a cool box until they were further processed in the lab. Before the roots were separated from the soil substrate in the lab, a subsample of substrate was taken from each sample for chemical and physical analysis. Roots were separated from the substrate by washing with tab water and then were scanned in a water bath at 400 dpi with a backlighting scanner. The morphology of the roots was analysed from the scanned images by means of WinRhizo Pro© image analysis software (Regent Instruments. Inc., WinRhizo Pro 2009c). Root length and root diameter distributions were also recorded and used for the calculation of specific root length (SRL, cm fine root (FR) length g^{-1} FR dry weight) and root length density (RLD, cm FR length cm^{-3} soil).

After scanning, FR were dried, biomasses were recorded and then digested in the same way as the leaf samples (see above). Only fine root samples >50 mg were analysed chemically. This criterion was met by about half of the samples.

Soil B and other element concentrations were analysed in two different extracts. Extraction with a HNO_3/ H_2O_2 solution (microwave digestion, 0.1 g sample, 180°C, 1 h, 5 ml 69% HNO_3:2 ml 30% H_2O_2) was used to obtain quasi-total concentrations, denoted with the subscript "$_{tot}$". Extraction with a $CaCl_2$ solution was used to characterize the availability of soil B and other elements. To obtain $CaCl_2$-extracts, 1g sample was shaken for 16 h with 10 ml 0.01 mol $CaCl_2$, centrifuged at 2500 rpm, filtered through a 0.25 µm filter and analysed by ICP-OES. The resulting $CaCl_2$-extractable soil element concentrations are denoted here with the subscript "$_{CaCl_2}$". Soil bulk density (ρ [g cm^{-3}]) was calculated from the volume of the cylinders after the soil was dried at 105 °C, gravimetric water content (H_2O_{grav}) and pH (H_2O, soil:H_2O ratio: 1:2.5) were determined. Carbon and N concentrations of soil samples were determined by elemental analyser. Digested of fine root and digests and extracts of soil samples were analysed by ICP-OES as described above.

4.3.3 Statistics

Based on varimax-rotated principal component analysis of the standardized and normalized physical and chemical soil parameters (Reimann et al., 2008) the soil samples were classified into 3 different substrate types (ST) using the method of Ward (1963). Values of trace element concentrations below each elements' detection limit were replaced according to the method proposed by Farnham et al. (2002). The values of the first three principal components (PC1, PC2, PC3), explained 79.9% of the total variance.

To describe the spatial auto-correlation structure of the substrate within and between plots, semi-variograms were computed for the PC scores of the samples and FR length, using the "modulus" estimator proposed by Cressie (1980).

$$\hat{\gamma}(h) = \left\{ \frac{1}{\mathrm{N}(h)} \sum_{x_i - x_j \sim h} \left| z\,(x_i) - z\,(x_j) \right|^{1/2} \right\}^4 \Big/ \left(\frac{0.914 + 0.988}{N(h)} \right) \qquad \text{(Equation 2)}$$

Here, h is the distance between the locations x_i and x_j of a pair of measurement points z (x_i) and z (x_j), and with separation distance h.

The dependence of tree growth, element uptake, and root morphology on soil properties was analyzed by means of multiple analysis of variance and regression analysis. Each tree was assigned to the substrate type of the soil core taken next to it. When the substrate type was not the same for the four segments of a core, then the class of the majority of segments was taken as the type of the entire core. If there was no majority, the type of the uppermost 2 samples was taken. There were no cores which could not be decided with these rules. For the regression analysis of tree growth and element accumulation average values of all four segments of a core were used. For the leaf samples, a 2 - way factorial analysis of variance was conducted using substrate and clone as independent factors. For the root samples, a 2 - way factorial analysis of variance was conducted for each clone × depth and depth × substrate combination. Significant differences between groups were post-hoc tested using the Holm-Sidak (Holm, 1979). All data were tested for normality prior to statistical analysis and transformed if necessary.

To analyse root diameter distributions a cumulative log-normal distribution was fitted to the cumulative frequency distribution of the measured root radii of each soil sample. From μ_{ln} and σ_{ln} parameter values of the best-fit distribution the back-transformed distribution mean and variance were calculated for each distribution (Scanlan et al., 2010).

4.4 Results and Discussion

4.4.1 Substrate properties and spatial structure

The 1st principal component (PC1) explained 50.3% of the total variance of the standardized and normalized soil parameters, the 2nd (PC2) 20.0% and the 3rd (PC3) 9.60%. The highest loadings on PC1 were obtained from B_{tot}, P_{tot} and Zn_{tot} while PC2 was dominated by Pb_{tot} and Ni_{tot} (Table 1). The third component was characterized by a high negative score for Mn_{CaCl_2} and a high positive score for pH. The three substrate types derived by hierarchical grouping on the basis of this analysis were primarily determined by the first two principal components. Substrate Type 1 was defined by high negative scores on the first two principal components, while ST 2 had a high positive score on PC1 only and no substantial scores on the other two. Substrate Type 3 had a high positive score on PC2, a less pronounced negative score on PC1 and negligible score on PC3. Table 2 provides an overview of the association of the ST with the surviving trees on the three plots.

Of the 296 soil samples taken in total, 100 were classified as ST 1, 135 as ST 2, and 61 as ST 3, and of the 75 living poplar trees on the three plots, 26 were associated with a core of ST 1, 33 with ST 2 and 16 with ST 3.The STs were equally distributed among the 4 sampling depths.

Table 1. Factor loadings of the of the analyzed soil parameters on the first three principal components of the varimax-rotated PCA. Values between -0.1 and 0.1 are not shown in the component matrix for better clarity. The highest (lowest) loadings on each component are shown in bold figures.

	Principal Component		
Parameter	PC 1	PC 2	PC 3
	Factor loadings [-]		
B_{CaCl_2}	0.78	0.47	0.22
B_{tot}	**0.92**	0.29	-
C_{tot}	0.77	**0.56**	-0.10
Cu_{tot}	0.86	0.22	-0.20
Fe_{tot}	0.85	0.41	-
H_2O	0.84	0.43	-
K_{CaCl_2}	0.71	0.29	0.22
K_{tot}	0.81	-0.45	-
Mg_{CaCl_2}	0.80	0.32	0.30
Mg_{tot}	0.79	0.13	**0.39**
Mn_{CaCl_2}	-	-	**-0.79**
Mn_{tot}	-	**-0.81**	-
N_{tot}	0.63	0.56	-0.24
Na_{CaCl_2}	0.74	0.36	0.33
Na_{tot}	0.86	0.23	0.13
Ni_{tot}	0.46	**0.82**	-
Pb_{tot}	0.49	**0.82**	-
P_{CaCl_2}	0.42	0.40	-0.43
P_{tot}	**0.92**	-	-0.11
pH	0.14	-	**0.78**
Zn_{tot}	**0.89**	0.35	-

Table 2. Association of surviving trees with the 3 substrate types (ST) for each plot (number of trees (NoTs) per plot and percentage survival per plot).

	Plot						
	A		B		C		
	NoTs	[%]	NoTs	[%]	NoTs	[%]	Sum
ST 1	24	80.0	2	7.1	0	0.0	26
ST 2	6	20.0	20	71.4	7	41.2	33
ST 3	0	0.0	6	21.4	10	58.8	16
Sum	30		28		17		75

The semivariograms presented in Fig. 2 showed spatial autocorrelation of the PCA scores of the soil samples on the first three principal components from the uppermost soil layer (0 – 5 cm) over lag distance ranges of up to 5 – 7 m. Findings from the other soil layers were similar.

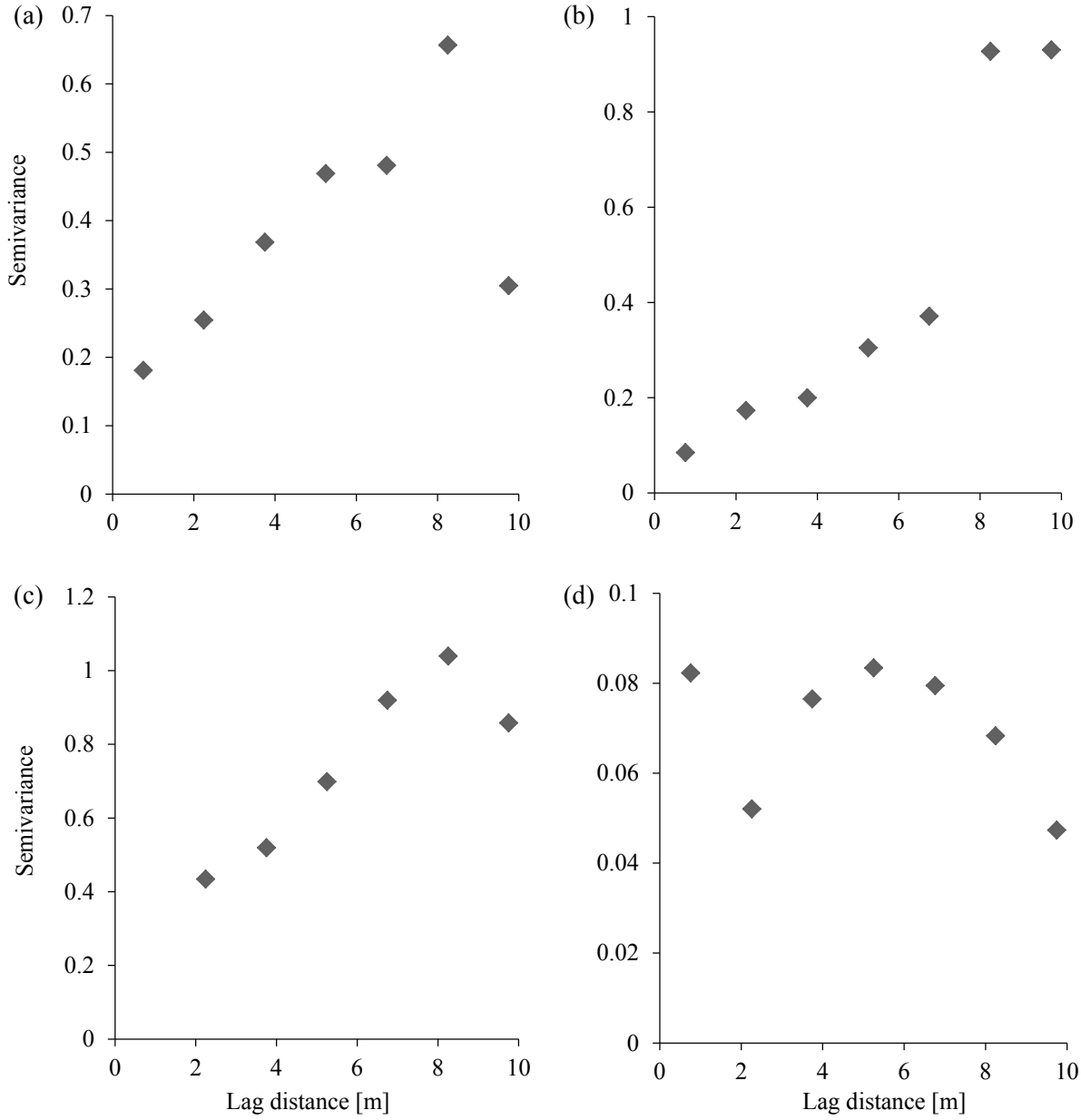

Figure 2. Semivariograms of the scores of the soil samples on (a) PC1, (b) PC2, (c) PC3 and (d) fine root biomass (mg per 100 cm^{-3}) in the topsoil (0 – 5 cm) for lag distances up to 10 m. For the shortest lag distance of 0.76 m there were not enough data points for a robust calculation on PC 3.

These ranges correspond well to expected patch sizes of around 40 m^2 caused by the size of the truck loads.

There was still considerable short range variability though, as the variance at the shortest lag distance was still about half of the sill variance. Nonetheless, the semivariograms show that the soil core samples are in average a good representation of the soil conditions around the neighboring tree. The physical and chemical soil parameters of the three substrate types are given in Table 3.

Table 3. Physical and chemical characteristics of the 3 substrate types identified at Pine Lake Landfill.

		Substrate Type								
		1			2			3		
Parameter		Mean	±	S.D.	Mean	±	S.D.	Mean	±	S.D.
pH_{H2O}	[-]	7.66	±	0.43	7.73	±	0.42	7.54	±	0.50
H_2O	[g kg^{-1}]	11.06	±	10.03	37.52	±	10.82	31.14	±	11.18
Bulk density [ρ]	[g cm^{-3}]	1.13	±	0.27	0.58	±	0.13	0.53	±	0.11
C	[g kg^{-1}]	45.93	±	82.77	283.84	±	78.13	293.16	±	69.77
N	[g kg^{-1}]	1.44	±	2.52	7.56	±	2.31	8.59	±	3.01
B_{tot}	[mg kg^{-1}]	61.14	±	79.57	304.9	±	101.9	173.0	±	79.95
B_{CaCl_2}	[mg kg^{-1}]	6.09	±	6.69	23.16	±	10.26	20.49	±	9.89
Ca_{tot}	[g kg^{-1}]	4.69	±	6.06	16.50	±	7.58	11.23	±	4.01
Cu_{tot}	[mg kg^{-1}]	129.9	±	288.7	496.7	±	212.1	316.1	±	271.4
Cu_{CaCl_2}	[mg kg^{-1}]	0.09	±	0.05	0.13	±	0.11	0.22	±	0.10
Fe_{tot}	[g kg^{-1}]	12.80	±	8.16	35.56	±	8.12	30.54	±	7.50
Fe_{CaCl_2}	[mg kg^{-1}]	1.32	±	2.71	7.43	±	16.43	5.56	±	14.35
K_{tot}	[g kg^{-1}]	2.67	±	1.14	4.96	±	1.26	1.29	±	1.16
K_{CaCl_2}	[mg kg^{-1}]	72.96	±	64.71	196.6	±	110.6	160.0	±	119.3
Mg_{tot}	[g kg^{-1}]	1.94	±	0.84	3.79	±	1.35	2.62	±	1.21
Mg_{CaCl_2}	[mg kg^{-1}]	161.6	±	137.9	603.2	±	308.7	470.9	±	303.7
Mn_{tot}	[mg kg^{-1}]	126.9	±	72.16	151.8	±	66.11	535.2	±	419.1
Mn_{CaCl_2}	[mg kg^{-1}]	1.46	±	1.86	2.71	±	3.83	4.20	±	6.01
Na_{tot}	[g kg^{-1}]	0.83	±	0.47	1.88	±	0.46	1.43	±	0.55
Na_{CaCl_2}	[mg kg^{-1}]	50.64	±	51.99	187.4	±	93.34	154.1	±	105.1
Ni_{tot}	[mg kg^{-1}]	35.39	±	37.10	102.6	±	42.50	204.9	±	79.87
Ni_{CaCl_2}	[mg kg^{-1}]	0.51	±	0.64	0.38	±	0.64	0.84	±	1.63
P_{tot}	[g kg^{-1}]	0.60	±	0.82	2.63	±	0.85	1.23	±	0.80
P_{CaCl_2}	[mg kg^{-1}]	0.46	±	0.46	0.81	±	0.47	0.95	±	0.87
Pb_{tot}	[mg kg^{-1}]	138.0	±	119.5	307.9	±	89.31	606.1	±	252.5
Pb_{CaCl_2}	[mg kg^{-1}]	N/A			N/A			N/A		
Zn_{tot}	[mg kg^{-1}]	60.30	±	79.20	294.0	±	92.41	227.1	±	77.35
Zn_{CaCl_2}	[mg kg^{-1}]	N/A			N/A			N/A		

S.D. = standard deviation; N/A = not applicable

The concentrations of B, C, N, P and metal elements were lowest in ST 1, except for K which was lowest in ST 3. Substrate Types 2 and 3 were also characterized by a much higher water content and lower bulk density than ST 1 samples. The differences between ST 1 and the other two STs in element concentrations were particularly large for C and N. In substrate

Types 2 and 3 they were more than five times higher on average than in Type 1. Most of the other elements including B, Ca, Cu, Fe, Ni, P, Pb and Zn were on average at least twice as concentrated in Type 2 and 3 as in Type 1, in terms of HNO_3-extractability. Substrate Types 2 and 3 differed particularly in their average B, Cu, K, Mn, Ni, P and Pb concentrations. The HNO_3-extractable concentrations of B, Cu K and P were on average highest in Type 2 samples, while Mn, Ni and Pb were most abundant in Type 3 samples. Cadmium was detectable (DL: 0.05 mg kg^{-1}) only in 4 substrate samples. The maximum Cd concentration was 0.73 mg kg^{-1}. Copper was the only heavy metal with higher concentrations in substrate Type 2 than in Type 3 samples. The trace metal concentrations of the substrate samples were all in ranges of values that are frequently found in contaminated soils and fly ash disposal sites. (Pavlović et al., 2004; Domínguez et al., 2008; Fässler et al., 2010). The main source of B on the site was probably paper mill fly ash, which is known to be B rich. The B concentrations were much higher than usually found in contaminated soils, particularly in substrate Type 2, where B_{tot} averaged 305 mg kg^{-1}.

The solubility of the investigated trace metals was low as indicated by the low $CaCl_2$-extractable concentrations, which is consistent with the high pH of the soils (7.5 – 7.7). Only 0.3 – 0.7% of Cu_{tot}, 0.8 – 1.8% of Mn_{tot} and 0.4 – 1.4% of Ni_{tot} were extractable with $CaCl_2$. Solubility of B was much higher in comparison. About 10.0 (substrate 1), 7.6 (substrate 2) and 11.8% (substrate 3) of B_{tot} were $CaCl_2$-extractable. At such pH values B is present as the neutral species H_3BO_3 (Goldberg, 1997). The average electrical conductivity of the leachates from the site during the 40 months of poplar growth was 1.01 dS m^{-1} (min.-max.: 0.61 - 1.5) and thus well below levels of salinity stress.

4.4.2 Tree survival and growth of the surviving trees

Table 4 gives a comparison of tree survival rates, among clones and plots. It was not possible to calculate survival rates for substrate types, because we have no data on the substrates associated with the non-surviving trees. Among the surviving trees, there was no significant correlation between clones and substrate types of the associated core samples. No specimen of DN-34 was found associated with Type 3. Mortality was highest on plot C, where more than 50% of the trees died and only one specimen of DN-34 survived. The survival of DN-2 and DN-34 was lower than that of OP-367 and DN-182. Mortality was highest in the first year. Only 12% of the mortality observed during the 4 years of this study occurred after the first year (data not shown).

Table 4. Tree survival rates by clones and plots, presented as percentage of living trees relative to the total number of trees initially planted per plot.

Survival rate					
	Clone				
Plot	DN-182	DN-2	DN-34	OP-367	Average
	[%]				
A	100.0	33.3	100.0	100.0	83.3
B	55.6	77.8	77.8	100.0	77.8
C	66.7	66.7	11.1	44.4	47.2
Average	74.1	59.3	63.0	81.5	69.4

The mortality rates found in this study are not unusual for poplar stands, no matter if on contaminated or uncontaminated sites (Hansen, 1992; Laureysens et al., 2005). A reason for the high first-year mortality rate could be that the trees had been planted very late in the growing season (July 2006). Thus, they had only about 2 – 3 months to establish before the first frosts typically come in Wisconsin. A second planting of 200 cuttings in the year after completely failed due to early frost. Other factors that may have contributed to tree mortality apparently were the incidental covering of cuttings by the geotextile and leaning of the growing trees, due to insufficient mechanical stability of the substrate to host poplars. Paper mill sludge is a rather soft growth substrate.

On average, surviving trees associated with Type 3 substrate grew higher than trees associated with 1 and 2 (Fig. 3). There were no significant differences in height growth between the clones. Similar substrate effects with no clonal differences as in height growth were observed in the diameter at breast height (DBH) and woody biomass, except that there was no significant difference in woody biomass between trees associated with substrate Type 1 and 3 (data not shown). There was a wide range in biomass production among clones and plots, with a minimum of 1.9 Mg ha^{-1} and a maximum of 20.6 Mg ha^{-1} (Table 5), and also a quite similar pattern as in the survival rates. Thus, OP-367 and DN-182, the clones with the lower mortality also had the higher biomass. On the other hand, trees on plot C produced more biomass than on plot B, even though mortality on C was 30% higher. The mean annual biomass production of all clones and on all plots was 3.4 Mg ha^{-1} a^{-1}, exceeding the averages of respective values given by Hansen (1990) for DN-34 and DN-182 2.5 and 3.3-fold.

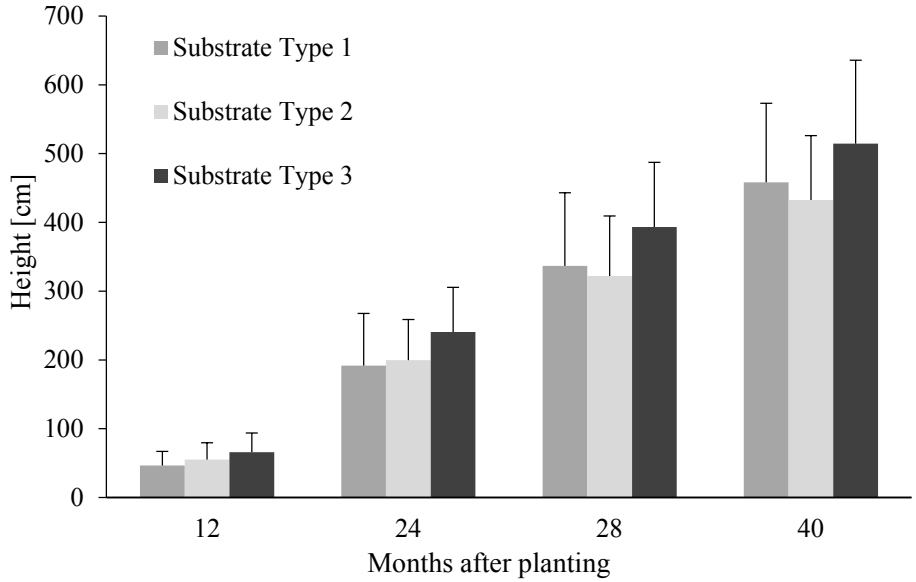

Figure 3. Average tree height (all poplar clones pooled) after 12, 24, 28 and 40 months of growth on the 3 substrate types identified by PC analysis. Error bars represent standard deviations.

Table 5. Woody biomass of the surviving trees on the three plots A, B and C. The standing woody biomass was calculated on a per hectare basis and extrapolated for a complete tree survival.

	Woody biomass				
	Clone				
Plot	DN-182	DN-2	DN-34	OP-367	Average
	[$10^3 \times$ kg ha^{-1}]				
A	20.6	3.5	11.0	15.4	12.6
B	5.4	8.0	8.4	12.5	8.6
C	13.2	13.0	1.9	10.2	9.6
Average	12.7	8.2	7.1	12.7	10.3

4.4.3 Element concentrations of leaves

The concentrations of macro and micro-nutrients in the leaf samples of the four poplar clones are given in Table 6. Leaf element concentrations differed more strongly among clones than among associated substrates. Significant substrate-related differences in leaf element concentrations were only found for Mn and Zn (data not shown). The foliar concentrations of the macronutrients N, K, Mg and P were in the same range as reported for fertilized cultivated poplar cultures (Jug et al., 1999; van den Driessche et al., 2008) and always above the respective sufficiency levels given for poplars by van den Burg (1985; 1990).

Foliar calcium levels were below the recommended concentration of 7 mg g^{-1} in 7 trees, the other trees were well supplied with Ca. However, Ca deficiency threshold concentrations for foliage vary widely and are not well established (Jug et al., 1999), and the Ca concentration of 22 g kg^{-1} which was proposed as optimum level for poplars by Coté et al. (1987) was not reached in any of the leaf samples. The foliar concentrations of the micro-nutrients Fe, Mn and Na showed no peculiarities.

Table 6. Concentrations of macro- and micro-nutrients in the leaves of the different poplar clones planted on the experimental site.

	Element concentration											
Clone	C		N		Ca		K		Mg		P	
	[g kg^{-1}]											
DN-182	449.3	(28.37)	30.76	(3.37)	13.32^{a}	(3.66)	26.24^{a}	(3.36)	3.77^{bc}	(1.32)	2.82	(0.45)
DN-2	451.5	(13.59)	33.45	(2.38)	11.25^{ab}	(5.48)	25.68^{ab}	(3.84)	4.59^{a}	(1.33)	2.59	(0.43)
DN-34	460.6	(21.19)	31.24	(4.67)	9.47^{b}	(2.01)	26.66^{a}	(2.86)	3.55^{b}	(0.62)	2.91	(0.48)
OP-367	454.2	(19.72)	32.30	(3.32)	11.29^{ab}	(2.96)	24.00^{b}	(2.34)	4.35^{ac}	(1.25)	2.59	(0.39)
	B		Cu		Fe		Mn		Na		Zn	
	[mg kg^{-1}]											
DN-182	636.9^{a}	(143.9)	25.19	(6.13)	67.01	(14.93)	127.1^{a}	(48.87)	39.74^{b}	(7.19)	239.5^{ab}	(104.2)
DN-2	830.5^{b}	(220.1)	29.91	(7.58)	69.56	(27.28)	85.73^{ab}	(51.05)	74.00^{b}	(56.31)	313.6^{a}	(124.4)
DN-34	636.3^{a}	(99.43)	28.60	(6.26)	63.26	(11.70)	72.38^{b}	(27.24)	87.88^{b}	(75.72)	181.2^{b}	(67.59)
OP-367	1017.8^{c}	(180.9)	27.17	(8.04)	65.89	(15.54)	103.8^{ab}	(46.27)	193.5^{a}	(130.1)	253.2^{ab}	(81.38)

Different letters indicate statistically significant differences in leaf element concentrations between clones at the $p<0.05$ level (Holm-Sidak-Test). Numbers in brackets represent standard deviations.

The concentrations of B were extraordinarily high in the leaves of all clones. The clones DN-34 (636 mg kg^{-1}) and DN-182 (637 mg kg^{-1}) had a similar leaf B concentration, differing significantly from DN-2 (831 mg kg^{-1}) and OP-367 (1018 mg kg^{-1}). Leaf B concentrations were weakly but significantly correlated to the $CaCl_2$-extractable soil B concentrations of the associated soil cores (r^2= 0.061; y = 5.21x + 702.14; $p<0.05$), but not to the HNO_3-extractable soil B-concentrations. The leaf B concentrations measured here are comparable in magnitude to those found by Banuelos (1999) and Robinson (2003). Zalesny et al. (2007a) found similar B accumulation in the clones DN-34 and DN-182, but at much lower soil concentrations than in our study. Despite the large variability within clones with relative standard deviations of 70 – 140%, the average bioconcentration factors (BCF), i.e. the ratios between element concentrations of plant and associated soil samples differed significantly between the poplar clones, ranging from 4.52 in DN-2 to 20.6 in DN-182. These values are

in the same range as those reported for poplars by Robinson et al. (2007) and Rees et al. (2011).

In some leaves, symptoms such as necrotic leaf margins were observed, indicating that B had been accumulated to toxic levels. In a pot experiment leaf B concentrations were found to decrease with leaf position along the axis of poplar shoots from base to the top (Rees et al. 2011). Here we found no such relationship between leaf position and B concentration. This might have been due to early senescence and fall of older leaves. Some of the trees were strongly defoliated at the time of sampling, especially in their lower parts. Of the 150 leaf samples taken 43% exceeded the threshold B concentration of 800 mg kg^{-1}, above which fodder becomes toxic for livestock (Underwood et al., 1999).

Also other trace elements, such as Zn, were present in the leaves at elevated concentrations compared to uncontaminated sites (Vandecasteele et al., 2003). This was especially true for clone DN-2, in which leaf Zn concentrations averaged 314 mg kg^{-1} and reached a maximum concentration of 508 mg kg^{-1}. Such concentrations are not uncommon in poplars, even on uncontaminated soil and not known to be phytotoxic in poplar leaves (Robinson et al., 2005; Dos Santos Utmazian et al., 2007; Hermle et al., 2007). The Cu concentrations of the leaf samples varied between 25.2 and 30.0 mg kg^{-1}, which is within the range where Cu toxicity can occur (Kramer, 2010). Cadmium was detected in only 14 leaf samples (DL: 1 mg kg^{-1}). The average Cd concentration was 4.3 mg kg^{-1} in these samples, which is in the range of Cd concentrations that have been reported for poplar leaves on uncontaminated sites (Vandecasteele et al., 2003). Lead was detected in only 2 and Ni in 9 samples. Their concentrations were below respective toxicity levels reported for Pb and Ni in plants (Kramer, 2010).

4.4.4 Regression analysis of tree growth and leaf element concentrations

There were significant correlations between the average PC scores of the soil cores taken next to each tree and tree growth parameters (Table 7). PC1 and PC3 were found to be negatively correlated with tree height and biomass, while PC2 showed a positive correlation with tree height but no correlation with biomass. This agrees with the finding of a positive influence of substrate Type 3 on tree growth, as this substrate type had a high positive score on PC2, and the rather negative influence on tree growth associated with substrate Type 2, which scored high on PC1.

Tree height and biomass showed strong correlations with the accumulation levels of some elements in the leaves, in particular with leaf Ca, Mn and to a lesser degree also with Mg and Zn (Table 7). The strongest positive correlation between nutrient element concentration in foliage and tree growth parameters was found for Ca and the strongest negative correlation was found for Mg. Calcium and B play an important role in the formation of pectic polysaccharides in cell wall constituents and Ca is especially important for wood formation in poplars (Matoh et al., 1998; Lautner et al., 2007).

Table 7. Linear correlations (Pearson's coefficients) between the average scores of the first 3 principal components (PC) of the soil cores taken next to each tree, tree growth parameters (height and biomass after 40 months), leaf nutrient concentrations (N, B, Ca, Cu, Fe, K, Mg, Mn, Na, P, Zn) and the Mg:Ca ratio. Elements such as C, which did not show any significant correlation with the growth parameters or other nutrients are not included.

	Height	Biomass	N	B	Ca	Cu	Fe	K	Mn	P
PC 1	-0.40***	-0.35**								
PC 2	0.25*	-								
PC 3	-0.24*	-0.44***								
Mg:Ca ratio	-0.58***	-0.5***								
B	-	-	0.38**							
Ca	0.627***	0.604***	-	-						
Cu	-	-	0.36**	0.35**	0.23*					
Fe	-	-	0.36**	-	0.45***	0.24*				
K	-	-	-	-	-	0.33**	0.35**			
Mg	-0.37**	-0.36**	-	0.60***	-	0.32**	-	-		
Mn	0.653***	0.443***	-	-	0.79***	0.31**	-	-		
Na	-	-	-	0.60***	-	-	-	-	-	
P	-	-	0.36**	-	0.28	0.47***	0.25*	0.42***	0.31**	
Zn	0.29*	0.31**	-	-	0.61***	0.28*	0.27*	-	0.50***	0.28**

Significance levels are indicated by asterisks: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; - = non-significant

Using a model that fitted well to their experimental data, Teasdale et al. (1986) showed that at a high Mg:Ca ratio, Mg is able to displace Ca from the binding sites of an acceptor molecule that is only activated when both, Ca and B, are bound to it. At high Mg:Ca ratios even high B concentrations were found to promote growth in cell cultures. The Mg:Ca ratio was as high as 1 in some of our leaf samples. Under these conditions, the high B concentrations found in the leaves might have partially compensated growth reduction induced by low Ca supply to the leaves. However, this was not expressed by a positive

correlation of tree growth with leaf B accumulation as B was present in excess in all leaf samples.

Low foliage Ca concentrations were not related to low soil Ca concentrations, as substrate Ca concentrations were lowest in substrate Type 1, whereas most of the trees (5 out of 7) with foliage Ca concentrations <7 g kg^{-1} were associated with substrate Type 2. Since the leaf Zn and Mn concentrations were far above the respective deficiency levels, their positive correlation with tree growth may have been co-incidental due to their correlation with leaf Ca. Negative correlations between tree growth and elements that were present in potentially toxic concentrations in the leaves, such as Cu, were not observed.

4.4.5 Fine roots traits

All three substrates were intensively colonized by roots (Fig. 4). The semivariograms for fine root (FR) biomass and FR length show that these parameters were spatially independent, as there was a pure nugget effect (Fig. 2 d). Fine root length densities were found to vary between 1.9 and 16.8 cm cm^{-3} (Fig. 4).

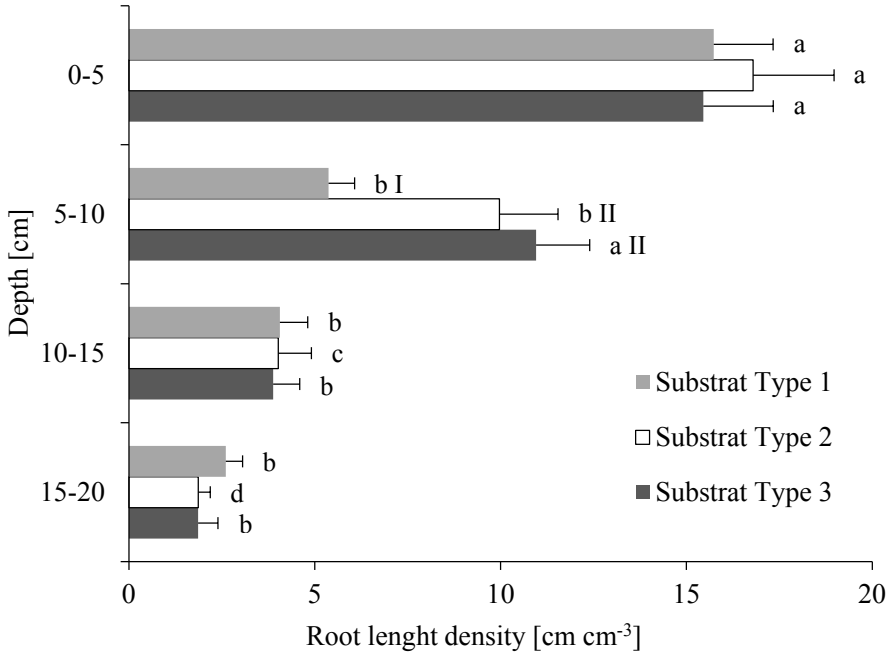

Figure 4. Average root length density in the 3 substrates types after 40 months growth. The sample volume was 98 cm^3. Statistically significant differences within substrates, between depths are indicated by characters ($p < 0.05$) and between substrates for a given depth by Roman numbers. Error bars represent standard errors.

These values were 2 – 6 times higher than those found by Al Afas et al. (2008) in a poplar coppice culture. No significant differences in RLD were found among clones and substrate

types when all soil samples were pooled. However, pair-wise comparisons revealed differences in RLD among substrate types collected at 5 – 10 cm depth. Trees associated with substrate Type 1 developed fewer roots at this depth than in association with substrate Types 2 and 3. For all substrates and clones RLD decreased significantly with depth. The RLD was closely correlated to the fine root biomass per soil sample (RLD = 117.1× FR biomass + 0.85; $r^2 = 0.73$; $p < 0.001$) (data not shown).

The average FR diameter generally increased with substrate depth (Fig. 5 (a)). The FR diameter was the only FR parameter for which we found significant differences between clones. Fine root diameters were not significantly different between substrate types. Specific root length decreased with depth and was higher in substrate Type 3 than in Type 1 and 2 (Fig. 5 (b)). The SRL values found here are twice as high as those reported by Ostonen et al. (2007) and Brunner et al. (2008), but in the same order of magnitude as those reported by Pregritzer et al. (2002) for *Populus balsamifera*. Also our FR diameter values agreed well with those of Pregritzer et al. (2002).

The cumulative frequency distributions of the FR diameters were well described by log-normal distributions. The average r^2 of all samples was 0.993 for all models, with a maximum of 1 and a minimum of 0.875. Despite the good model fits, the average diameter values determined from the back transformed best-fit model distributions generally underestimated the average diameters determined directly by means of WinRhizo Pro© by about a factor 0.5 ($r^2 = 0.71$; $y = 0.53x + 0.02$; $p<0.01$). However, the back-transformed model distributions parameters (mean and variance) agreed well with the respective parameters compiled by Scanlan (2010) from 96 data sets for fine roots of herbaceous as well as tree species grown under a wide variety of conditions (data not shown).

Interactions between clone and depth or substrate and depth were not found in the parameters of the back-transformed model distributions (data not shown). Differences between sample average diameters determined by model-fitting were significant for all three factors: substrate, clone and depth. The averages of measured root diameters determined directly by WinRhizo Pro © only differed significantly between clones and depth but not between the substrates. The sample variances in root diameter were independent of the clone, but showed the same trends as the distribution mean otherwise. Pair-wise comparison showed that the uppermost substrate samples (0 – 5 cm) significantly differed in variance from the deeper samples. The latter had the highest distribution variance.

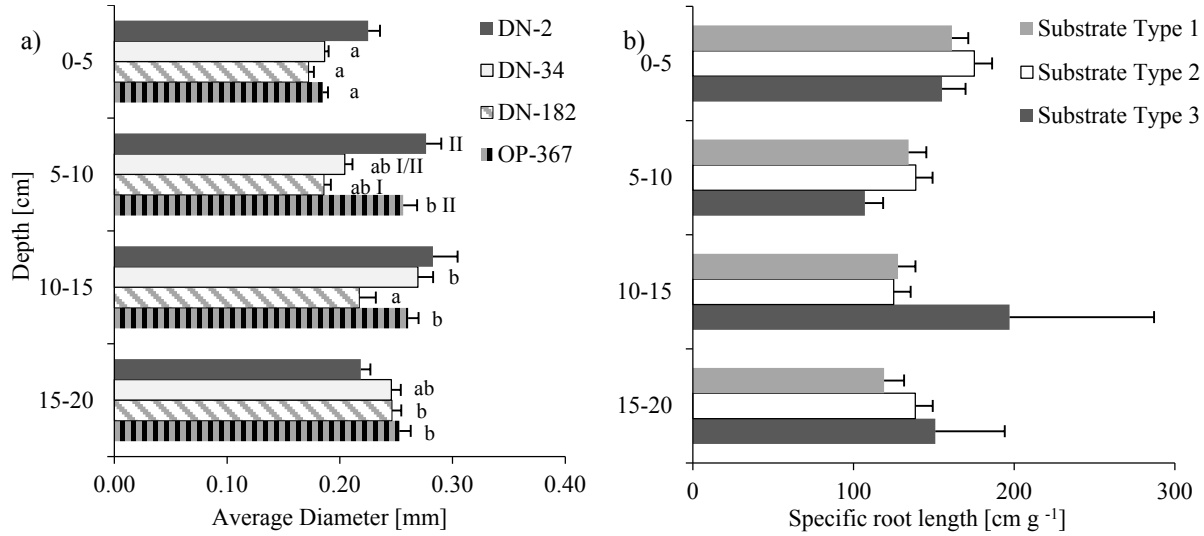

Figure 5. Depth distribution of (a) Average root diameters for the 4 poplar clones and (b) specific fine root length found in the 3 substrate types associated with the trees after 40 months of growth. Bars within a given clone with the same letter are not significantly different. Likewise, bars within a given depth do not differ significantly if they are marked with the same Roman number. Error bars represent standard errors.

A higher SRL is associated with a higher root surface area for a given biomass and thus with an improved capacity for nutrient and water uptake. Specific root length is known to decrease under abiotic stresses such as Al or heavy metal stress, because roots thicken under such conditions (Ostonen et al., 2007). Under abiotic stress conditions black alder FR were found to avoid direct soil contact, as reported by Lohmus et al. (2006), investing more into exudation than into root growth. Poplars were found to react to high concentrations of heavy metals in nutrient solutions by exuding organic acids (Qin et al., 2007). The composition of the exudates differed for different heavy metals. Metals bound to organic acids are less reactive than free metal ions. The combination of such morphological and chemical responses might enable FR to deal flexibly with different stress conditions in soils.

4.4.6 Element concentrations of fine roots

Table 8 shows that there were significant differences among substrate types in the concentrations of various elements in the FR, but no significant differences between clones, except for Na in DN-34 and DN-182 roots.

Fine-root B concentrations varied between 134 and 187 mg kg^{-1}. Boron concentrations in roots are typically in the same magnitude as soil B concentrations and below leaf B concentrations, which was the case here as well (Rees et al., 2011).

Fine-root Ca concentrations were higher than soil and leaf Ca concentrations, suggesting that the low leaf Ca concentrations were not due to insufficient supply of soil Ca to the roots. The Zn and Mn concentrations found here in the FR were in the same order of magnitude as those reported by Brunner et al. (2008) for poplar roots grown in uncontaminated soil. The FR Fe concentrations, however were far above the concentrations reported by Zalesny et al. (2008) for poplar roots. The FR Fe concentrations found here were similar to the substrate Fe concentrations. These extraordinarily high root Fe concentrations might have been due to temporarily anaerobic conditions in the Fe-rich substrates.

Table 8. Mean concentrations of elements in poplar fine roots for the 3 substrate types at Pine Lake Landfill. Different letters indicate statistically significant differences in leaf element concentrations between clones.

	Element concentration											
Substrate	Ca		Fe		K		Mg		Na		P	
	[g kg^{-1}]											
Type 1	14.81^{a}	(1.20)	13.24	(1.40)	5.71	(0.54)	2.65^{a}	(0.14)	2.31^{a}	(0.28)	1.82^{a}	(0.09)
Type 2	19.13^{ab}	(1.73)	15.32	(1.22)	4.28	(0.38)	2.50^{a}	(0.12)	1.66^{ab}	(0.13)	2.47^{b}	(0.11)
Type 3	21.85^{b}	(1.97)	17.13	(1.77)	4.47	(0.41)	1.77^{b}	(0.12)	1.29^{b}	(0.18)	2.31^{b}	(0.11)
	B		Cu		Mn		Ni		Pb		Zn	
	[mg kg^{-1}]											
Type 1	134.4^{a}	(9.49)	393.6^{a}	(36.61)	383.4^{a}	(53.35)	28.99	(2.78)	141.4^{a}	(19.01)	149.0^{a}	(16.45)
Type 2	186.5^{b}	(8.59)	569.9^{b}	(31.95)	367.9^{a}	(48.18)	39.21	(2.48)	221.0^{b}	(16.13)	222.5^{b}	(11.90)
Type 3	150.1^{a}	(8.56)	533.2^{ab}	(50.83)	208.6^{b}	(33.01)	69.72	(12.82)	132.4^{a}	(9.23)	214.5^{b}	(17.20)

Different letters indicate statistically significant differences in leaf element concentrations between clones at the $p<0.05$ level (Holm-Sidak Test). Numbers in brackets represent standard errors.

In fact, we found very high soil water contents during sampling, and in some locations the soil had the typical smell of anaerobic conditions. Some roots were completely red on the surface due to precipitation of Fe-oxides. Changes in redox conditions would have also affected redox state and thereby the mobility of Mn and possibly Cu. The Cu concentrations of the FR were indeed high. They ranged between 400 and 570 mg kg^{-1} and even exceeded soil Cu concentrations. In roots, Cu is primarily bound in epidermis cell walls to pectins and thus hindered to be translocated into the shoots (Brunner et al., 2008). Copper concentrations of 3 mg l^{-1} were shown to be toxic to roots of *Populus tremula* in a 0.2 mmol $CaCl_2$-solution (Qin et al., 2007). In our study, $CaCl_2$-extractable Cu concentrations were always more than an order of magnitude lower than 3 mg l^{-1} and should therefore not be toxic to poplar roots. Also Pb concentrations in roots were high. Like Cu, Pb is known to accumulate in tree roots

and not to be translocated into stems and leaves readily (Pulford et al., 2003; Marmiroli et al., 2005). Root Ni concentrations were elevated only in substrate Type 3.

4.4.7 Regression analysis of fine root traits

Various mineral element concentrations of the sampled FR were significantly correlated with SRL (Table. 9). While the macro-nutrient elements K and Ca were negatively correlated to SRL, Na and Mg and the micronutrient elements B, Cu and Zn showed a positive correlation with SRL. Copper was the only mineral element that was significantly correlated to the average root diameter determined directly by WinRhizo Pro©. In contrast to expectation, the correlation was negative, meaning that diameters decreased with increasing root Cu concentration. In addition, there was a positive correlation between root Cu and SRL. This is in line with the negative correlation between root Cu and root diameter, as thinner roots lead to a higher SRL. SRL also showed positive correlations with root B, Mg, Na and Zn (as well as negative correlations with Ca and K), although all these elements were not significantly correlated with root diameter, suggesting that these relationships to SRL were of a rather indirect nature. The finding that SRL increased with the concentrations of some trace elements in the roots is in contrast to the opposite conclusion of a negative metal influence on SRL that Ostonen et al. (2007) drew from a meta-analysis of published root data. Root B, Mg and Na were also positively correlated with root length density. In the case of B, this was accompanied by a positive correlation with FR biomass. In fact, B was the only element, for which we found a significant correlation between its concentration in the FR and the FR biomass.

There was a good agreement between the correlations of PC scores and root traits and the correlations of these traits with those soil parameters showing high negative or positive loadings on the respective PCs: PC1 scores correlated positively to the average root diameters of the samples and so were all soil parameters with a high score on PC 1, PC 2 scores showed only a weak negative correlation with to the parameters of the back-transformed model; and PC3 showed a weak negative correlation to the WinRhizo Pro© measured average root diameters, but no correlation with mean diameter of the best-fit model distribution.

Table 9. Linear correlations (Pearson's coefficients) between root traits and root element concentrations, between root traits and principal component (PC) scores and between root traits and soil parameters of the substrate samples. Arrows indicate the soil parameters with the highest scores on the respective PC. Only coefficients of significant correlations are given.

		FR Biomass	RLD	SRL	Avg. Diam.[1]	Dist. Mean[2]	Dist. Variance[3]
Root element concentration	B	0.22*	0.29**	0.30***	-	0.20*	-
	Ca	-	-	-0.19*	-	-	-
	Cu	-	-	0.31***	-0.22*	-	-
	K	-	-	-0.20*	-	-	-
	Mg	-	0.27**	0.23*	-	-	-
	Na	-	0.19*	0.19*	-	-	-
	Pb	-	-	-	-	0.37***	0.35**
	Zn	-	-	0.23*	-	-	-
Principal components	PC 1	-	-	-	0.17**	0.20***	0.15*
	PC 2	-	-	-	-	-0.13*	-0.17**
	PC3	0.25***	0.39***	0.31***	-0.13*	-	-
Soil parameters, PC 1 ↑	P_{tot}	-	-	-	0.15*	0.13*	-
	B_{tot}	-	-	-	0.17**	0.15**	-
	Zn_{tot}	-	-	-	0.17**	0.13*	-
	Na_{tot}	-	-	-	0.18**	0.17**	-
	H_2O	-	-	-	0.17**	0.23***	0.14*
	K_{tot}	-	-	-	0.14**	0.21***	0.18**
	K_{CaCl_2}	-	-	-	0.17**	0.19**	-
	C	-	-	-	0.12*	-	-
PC 2 ↑	Ni_{tot}	-	-	-	-	-	-
	Pb_{tot}	-	-	-	0.12*	-	-
	Mn_{tot}	-	-	-	-	-0.15*	-0.14*
PC 3 ↑	pH	0.19**	0.29***	0.115*	-	-	-
	Mn_{CaCl_2}	-0.16**	-0.26***	-0.15***	-	-	-
	Na_{CaCl_2}	-	0.11*	-	-	-	-
	B_{CaCl_2}	0.14*	0.22***	-	-	-	-
	Ca_{tot}	-	-	-	-	0.13**	-
	Cu_{CaCl_2}	-	-	-0.12*	-	-	-
	Ni_{CaCl_2}	-	-0.13*	-	-0.15*	0.13*	-
	P_{CaCl_2}	-	-	-	-	-	-

[1] Average FR diameter directly determined by means of WinRhizo Pro© for each sample.
[2]Mean and variance of the best-fit log-normal distribution of sample FR diameters.

PC3 was also weakly correlated with FR biomass, SRL and RLD, as were the two main factors loading on PC3, i.e. pH and $CaCl_2$-extractable Mn. The correlation coefficients between root traits and soil chemical parameters were generally low, ranging from -0.26 to 0.3, suggesting that in our experimental field soil root growth was not simply governed by a few dominant factors, but by rather complex interactions among a large variety of factors.

4.5 Conclusions

Survival and growth of the poplars trees growing on the experimental plots of the studied landfill site were normal for poplar plantations after 3 years, despite the elevated concentrations of various trace elements in the substrate. However, there were significant differences in survival and growth parameters among the four clones and the three substrate types associated with the trees. Taking due account of these differences, e.g. by surveying the substrates before planting, could greatly enhance the chances of a successful establishment of poplars on the remainder of the site or on other sites with similar conditions. Element uptake in the roots and leaves of the poplars was high, especially for B, which reached potentially toxic concentrations in the leaves of some trees. Boron accumulation in the leaves was weakly related to the $CaCl_2$-extractable B concentrations, but not to the total concentrations of the associated soil cores. The results suggest that the major constraint for tree growth at the site was not B toxicity but Ca deficiency in the leaves, as indicated by the Mg:Ca ratio in the foliage. This imbalance was not caused by Ca deficiency in the soil, but by insufficient Ca transfer from the roots to the shoots. All three substrates were intensively colonized by poplar FR and there was no indication of root growth limitation due to toxicity effects of the soil contaminants. In contrast, there was a positive relationship between FR B concentration and FR biomass and length. Substrate type classification based on PC analysis proved to be an efficient method to detect soil effects on poplar survival and growth, despite of the complexity of factors and interactions.

In summary, the results show that poplars are well capable to cope with the harsh growing conditions on a multiply contaminated and in particular B-laden substrate in a hemiboreal climate, as represented by the PLL site, and thus are well suited to be used for establishing vegetation on such landfill sites.

4.6 Acknowledgments

Funds for this study came from the Swiss National Science Foundation (SNSF). We would like to thank Wausau Paper Specialty Products for giving us the opportunity to work on their site. We are thankful for the great support in field work and organisation that Bart Sexton and the staff of Sand Creek Consultants provided. We are grateful to Judit Valentini and Stéphanie Conrad for their assistance in root washing. We would also like to thank Rene Saladin and Björn Studer from the Soil Protection lab at ETH for their help with the analysis.

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5 Conclusions and Outlook

The main objectives of this study were (I) to investigate the B accumulation potential and tolerance of poplars under controlled conditions; (II) to elucidate how B tolerance and accumulation in poplars are affected by a heterogeneous distribution of B in soil and to investigate possible reactions of the poplar root system to a heterogeneous soil B distribution and (III) to determine whether the results on B tolerance, accumulation and root growth of poplars obtained under controlled conditions were transferable to typically heterogeneous field conditions where a number of additional constraints might limit poplar growth.

Independent of the growth conditions during the experiments and also in the field survey conducted, the B accumulation of the different poplar clones used was high. However, a comparison of the B accumulation obtained in the different clones in the different experiments is difficult. While we found a close relationship between B accumulation and the total soil B concentration in controlled conditions, only a weak correlation between B accumulation and the $CaCl_2$-extractable B existed in the field situation. This might have been due to the high short-range variability found at the field site, being nearly half as high as the sill semivariance for all substrate types. Furthermore, the B heterogeneity in the Chapter 2 was only applied in a 2-dimensional plane, while soil heterogeneity was 3-dimensional and multi-factorial in the field study, which strongly complicated the relationship between soil B and plant B accumulation. The maximum leaf B concentrations (1000 – 1700 mg kg^{-1}) found in the three studies were much more uniform, than the maximum B concentrations in the soils (14 – 304 mg B kg^{-1}). This was probably due to the high mobility of B in the soil used in the Chapter 2. Another reason for the more uniform maximum concentration in the leaves is that the maximum leaf B concentrations found in all three studies were close to the B accumulation limit of poplar leaves.

Poplars proved to be very tolerant towards high soil B concentration in all three studies. The finding that leaf B concentrations >1000 mg kg^{-1} caused chlorosis and >2000 mg kg^{-1} caused necrosis, with B accumulation continuing in leaf tissue even after the onset of necrosis explains the high B accumulation potential of poplars. According to the bioconcentration factors and the leaf B concentrations found in the three studies, all poplar clones utilized can be considered as woody B hyperaccumulators. However, in contrast to other hyperaccumulator plants which take up the hyperaccumulated element actively, the B accumulation of poplars seems to be passive. Therefore, as discussed in chapter 1, it is questionable whether the passive accumulation of B found in poplars is hyperaccumulation in the strict sense of the original concept, even though most of the technical criteria are met. Hypertolerance would be the term that fits the B accumulation potential of poplars much better. The hypertolerance of poplars to B is possible through the high tolerance of the living and the storage of B in dead leaf tissue. The tolerance mechanism of poplar leaf tissue towards B is unknown and would be an interesting research objective for further studies. Since the entire genome of *Populus triocharpa* has been sequenced gene expression studies could be very helpful to identify detoxification mechanisms.

From the results of the Chapter 2 we concluded that roots were more sensitive to B than the shoots, because of a stronger negative effect of B on root than on shoot biomass. However, this conclusion is in contradicted by Chapter 3, where we found the stronger reduction of root compared to shoot growth to be a systemic effect of B toxicity on shoot growth. This systemic response could not have been observed with the experimental design used in Chapter 1. Only the comparison of tolerance under heterogeneous and homogeneous soil B conditions enabled us to identify the two routes along which the B toxicity effects the growth of poplar plants, disproving our finding of higher root sensitivity. If the second route, namely the local effects on root growth were caused by direct B toxicity in the roots remains unknown. However, if B toxicity in the root tissue was the reason for the negative growth effect, the B toxicity threshold in root tissue would be about 10 times smaller than in the leaf tissue. To further investigate the B toxicity threshold in root tissue hydroponic experiments could be conducted; here it would be advantageous to minimize the B uptake into the shoots to avoid or at least delay a systemic response. This could be achieved by exposing only small portions of the poplar root systems to high B concentrations in solution and by minimizing the transpiration of the poplars by manipulating the environmental conditions during B exposure. Boron deficiency was only found in some of the poplars in Chapter 3. The B

concentrations found in these plants were in the range where B deficiency normally occurs in broadleaf tree species. A comparison of poplar growth to other species at low soil B concentrations would be helpful to give further insights into B deficiency limits in poplars.

Regarding the transferability of the results obtained under controlled conditions in Chapter 2 and 3 to field conditions, we concluded that our results are very comparable. Even though the B accumulation was lower under field conditions, it was in the same range as in Chapter 2 at a similar range of soil concentrations. A reason for the lower foliage B concentrations found in the field study might have been B leaching from the leaves by rain. Additionally, the higher B concentrations of the leaves from the upper part of the trees in Chapter 4 indicated early senescence and subsequent loss of older leaves with high B concentrations; this might have led to an underestimation of the real B concentrations of the leaves.

We conclude from Chapter 3 that local heterogeneity in soil B should have little influence on the success of phytoremediation using poplar. This conclusion was confirmed in Chapter 4 as the influence of soil B contamination and co-contaminates on root growth were minor and the performance of the poplars actually exceeded the performance of poplars from non-contaminated sites with a similar climate. As we could not determine the whole root biomass of the poplars under field conditions, conclusions on systemic responses were not possible for the poplar stands of Chapter 4.

Due to the depth of the material the complete clean-up of the B contamination at the landfill from Chapter 4 is impossible; therefore the major aim of phytomanagement should be to control the leaching of contaminants from the site. A better performance of the poplars with regard to growth and survival would increase the amount of water transpired from the site and thus reduce the amount of leachate. As our results suggested, the major constraint for tree growth in Chapter 4 was Ca deficiency in the leaves. The first measure to improve the performance of the trees could be foliar application of Ca, a commonly used method to correct Ca deficiency in apple orchards. However, the costs of this technique would offset one of the main advantages of phytomanagement, namely its cost efficiency. Another, much simpler way of increasing the poplars performance without improving Ca nutrition status, would be to use the gained knowledge (Chapter 4) on the relationship between the growth of the different poplar clones and the substrate types, by planting the best suited clones into the respective substrate type. At least a rough surveying of the distribution of substrate types before planting should be possible under field conditions.

Meanwhile, the whole landfill has been planted with poplars, making a study on the long term fluxes of B in the system interesting. Here, a major question could be the fate of B from the poplar leaves. After litterfall, B is released from litter and depending on the speed of the release there might be a strong peak in the leachate B concentration in fall. However, release of B from high B leaf litter has not been studied under natural conditions. The speciation of B leached from leaf litter may profoundly affect its fate. If B was bound to organic matter and transported into the soil it might be adsorbed and deposited, while B in the form of boric acid would be transported more rapidly through the soil profile. To avoid the return of B to the soil, coppicing of the poplars would theoretically be an option; however, due to the instability of the substrate at the site the utilization of machinery would be impractical, making the coppicing so, too.

If poplars are utilized for phytomanagement the fundamental knowledge gained in this study on the interactions of poplars with B, such as B toxicity limits in leaf tissue and the top-down regulation of root growth by shoot B status is very valuable, showing that the clean-up time would not be prolonged by soil B heterogeneity and setting B tolerance of poplars leaf tissue as major objective in poplar breeding for B phytomanagement. Our results on B tolerance and accumulation of B from both, homogeneously and heterogeneously contaminated soils and their transferability to a multiply contaminated but particularly B-laden field site showed that poplars are well suited for use in phytomanagement and for establishing vegetation on such landfill sites.

A. Neutron Radiography

A.1 Principles and Facilities

Neutron radiographs (NR) is an imaging technology that shares some properties with X-radiography, but while X-rays are electromagnetic radiation with a wavelength of 10^{-8} to 10^{-12} m, a neutron beam typically utilized in NR consists of thermal (<2200 m s^{-1}) or cold (152 - 1515 m s^{-1}) neutrons with a wavelength of about 10^{-10} m. As any particle, neutrons also exhibit wave characteristics, but these are of minor importance in NR. X-radiography and neutrons significantly differ with respect to their interactions with atoms. While X-rays interact with the electrons in the atomic shell, neutrons are uncharged and do only interact with the nuclei of atoms. These interactions are independent of the atomic weight of an element. Elements whose nuclei strongly interact with neutrons are e.g. boron (B), hydrogen (H) or gadolinium (Gd).

Neutron radiography is mainly used in physics and engineering sciences (Lehmann et al., 2010). Here it was used to measure the concentration of ^{10}Boron (^{10}B) in steel with an accuracy of 5×10^{-3}% weight (Zawisky et al., 2004). Neutron radiography has also been employed for biological samples (e.g. wood) and in soil sciences (Mannes et al., 2009; De Ridder et al., 2011). In soil science the high neutron cross section of hydrogen (H) has been made use of by studying evapotranspiration (Shokri et al., 2010), the distribution and flow of water between soil aggregates and in the rhizosphere (Carminati et al., 2007; Kaestner et al., 2008; Esser et al., 2010) and to visualize and study root growth in porous media and soil (Willatt et al., 1978; Kaestner et al., 2006; Moradi et al., 2009). In this thesis we also made use of the high neutron cross section of ^{10}B (Chapter 2) and H (Chapter 3).

The two neutron radiography experiments conducted were both carried out at the Paul-Scherrer Institute (PSI), Villigen, Switzerland. At the PSI thermal and cold neutrons are produced at SINQ (Swiss Spallation Neutron Source) (Bauer, 1998). At SINQ neutrons are emitted from a heavy metal target which is constantly hit by a 570 MeV proton beam. The two neutron imaging beamlines NEUTRA (Neutron Radiography facility) and ICON

(Instrument for Cold Neutron Radiography) currently existing at PSI receive neutrons from SINQ after they passed the moderator tank where the neutrons are slowed down to <2200 m s^{-1}. While NEUTRA receives the thermal neutrons emerging from the moderator tank, ICON receives cold neutrons that are further decelerated in a tank filled with liquid D_2 at 25K. The neutron beam is parallelized at both facilities by a collimator tube. After passing the collimator tube, the neutron beam hits the sample. The spatial structure of the transmitted neutron beam neutron is then recorded by a charge-coupled device (CCD) camera after conversion into light by a scintillator. Detailed information on the facilities is given in Lehmann et al. (2001) and Kühne et al. (2005).

A.2 Imaging procedure and basic image processing

For our experiments we used a CCD camera at ICON (Chapter 2) with a spatial resolution of 0.013 cm (field of view (FOV): 26.6 × 26.6 cm) and at NEUTRA (Chapter 3) with 0.026 cm (FOV: 27.9 × 27.9 cm). In Chapter 2 the leaves were directly mounted to the scintillator screen with aluminum tape. The containers in Chapter 3 were set-up in 10 cm distance from the scintillator screen on an automatic maneuverable sample table, on which they were moved to the imaging positions. Exposure time was set to 40 sec for the leaves in Chapter 2 and 80 sec for the containers in Chapter 3. Besides the images of the actual samples open beam images and CCD dark current images were taken for flat field correction.

All images including open beam and CCD dark current were despeckled by a 3 × 3 median filter. The images were normalized to overcome the problem of beam fluctuations. In Chapter 2 the normalization was done via an empty area in the images and in Chapter 3 via the aluminum frames of the containers. The transmission images (I') were calculated by flat field correction:

$$I' = \frac{I_{raw}(x,y) - I_{DC}(x,y)}{I_{OB}(x,y) - I_{DC}(x,y)} \qquad \text{Equation 1}$$

In Equation 1 I_{raw} is the raw image, I_{DC} is the CCD dark current and I_{OB} is the open beam image, while (x,y) represents each pixels' positions in the respective image. The 9 pictures taken from each container in Chapter 3 were assembled by hand as semi-automatic image assembling with MosaicJ (Thevenaz et al., 2007) failed due to insufficient contrast in the images. Another problem with the NR images of the containers (Chapter 3) was that the flat field correction did not yield completely flat images. The beam structure, as seen in an open beam image (Fig. 1a) should theoretically not persist in I' anymore.

(a) (b)

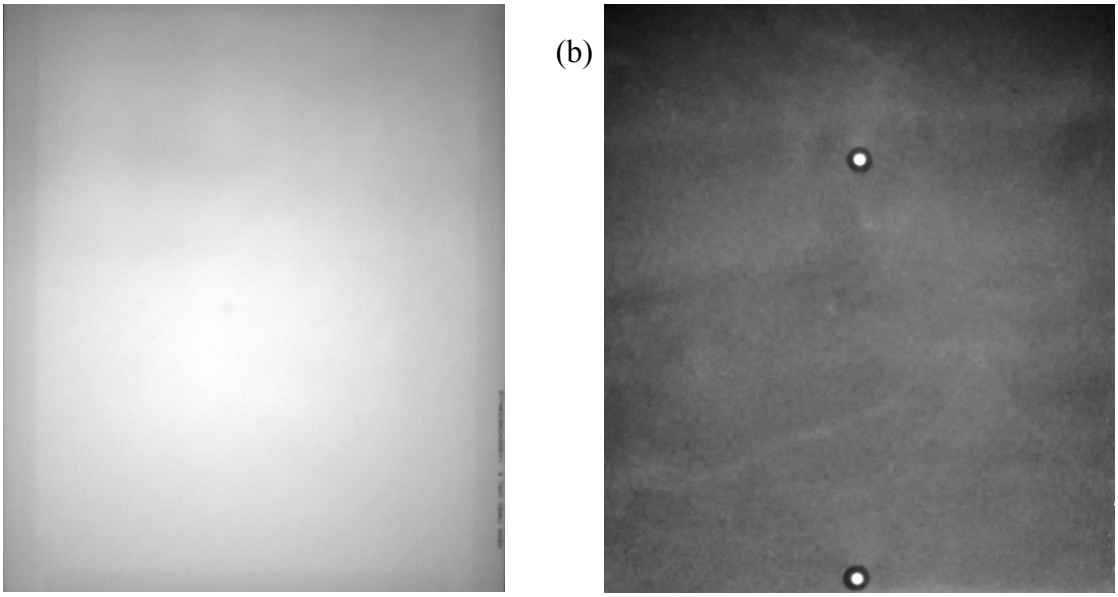

Figure 1. Open beam image (a) taken at NEUTRA with an exposure time of 40 sec. The beam structure and the brightness loss towards the edges are clearly visible. An image (b) of the center of a soil-filled container taken at the 04.06.2008 does still show brightness loss towards the edges after flat field correction.

However, this was not the case for the corrected images in Chapter 3 where the structure of the beam was still apparent in I' as the images were still brighter in the center (Fig. 1b). The reason for the insufficient correction remained unknown, but could have been caused by the longer exposure time that was used for the actual samples than for the open beam in Chapter 3. The use of different beam times was necessary to make use of the full dynamic range of the camera when imaging the samples and not to produce a signal overflow at the detector (CCD) when taking the open beam images. However, the failure of the flat field correction did not negatively influence the evaluation of the NR images done in Chapter 3.

A.3 Calculation of the spatial distribution of ^{10}B in the leaves

Areas of higher neutron attenuation are indicated in the NR images by lower grey values. From comparing the NR images with pictures of the leaves we found the locations of chlorotic and necrotic spots to coincide with the darker areas in the NR images (Fig. 2). The first step to evaluate whether the ^{10}B concentrations in the poplars leaves were measurable with NR was to cut up several leaves according to a grid projected on the NR images of these leaves and measure the ^{10}B concentration of each peace individually by means of ICP-OES (c. Chapter 2).

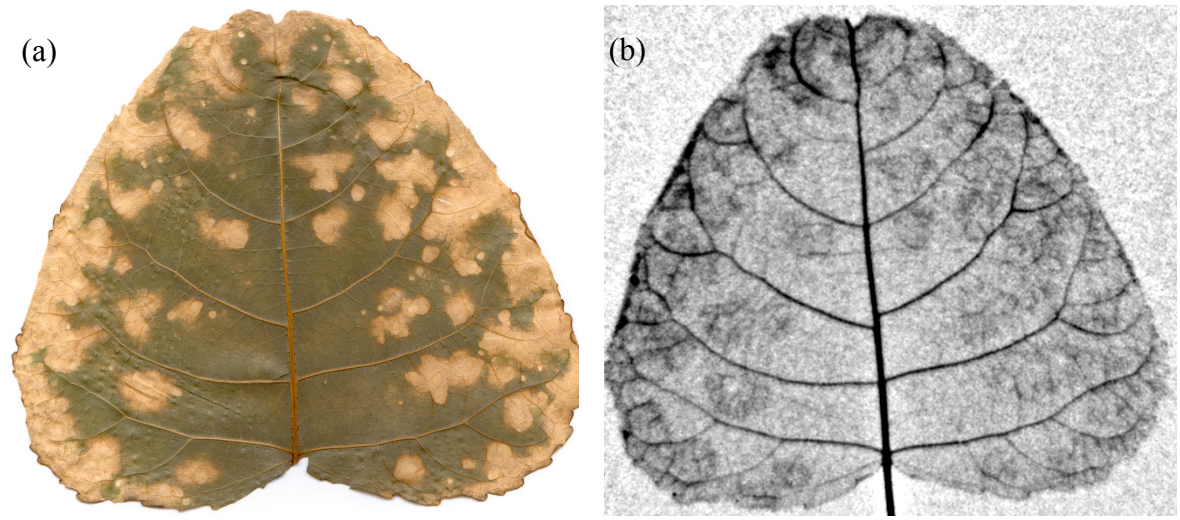

Figure 2. Scanned poplar leaf (a), showing severe signs of necrosis at the leaf margins and also within the leaf area. Neutron radiography image (b) of the same leaf. Darker areas indicate stronger neutron attenuation.

The advantage of this measure was that leaf thickness within an individual leaf is more homogenous than between different leaves and so leaf thickness differences were less likely to conceal the expected correlation between the ICP-OES measured ^{10}B concentrations and the neutron attenuation. To further homogenize the leaf thickness the leaf veins were cut from the NR images. Pearson's r-values for the correlations between the ICP-OES ^{10}B concentrations and the neutron attenuations of the pieces of the cut-up leaves ranged from -0.374 to -0.965 (Fig. 3). The cut-up leaves from the control treatment with a ^{10}B concentration in the soil <5 mg kg^{-1} did only partially show a significant correlation between the neutron attenuation and the ^{10}B concentration of the pieces. In these leaves the maximum ^{10}B concentrations reached were <100 mg kg^{-1}. Because of these low ^{10}B concentrations these leaves were furthermore excluded from the analysis of the ^{10}B distribution. The total theoretical attenuation coefficient Σ_{tot} of a sample, consisting of more than one element is the sum of the attenuations coefficients of the single elements' Σ_i:

$$\sum_{tot} = \sum_{i=1}^{n} \Sigma_i = \sum_{i=1}^{n} (\sigma_i \cdot N_i) \qquad \text{Equation 2}$$

which is the product of the microscopic cross-section (σ_i) and the atomic density (N_i), which is calculated as:

$$N_i = \ln\left(\frac{\rho}{A}\right) \cdot N_A \qquad \text{Equation 3}$$

Here ρ is the material density, A the atomic weight and N_A Avogadro's constant.

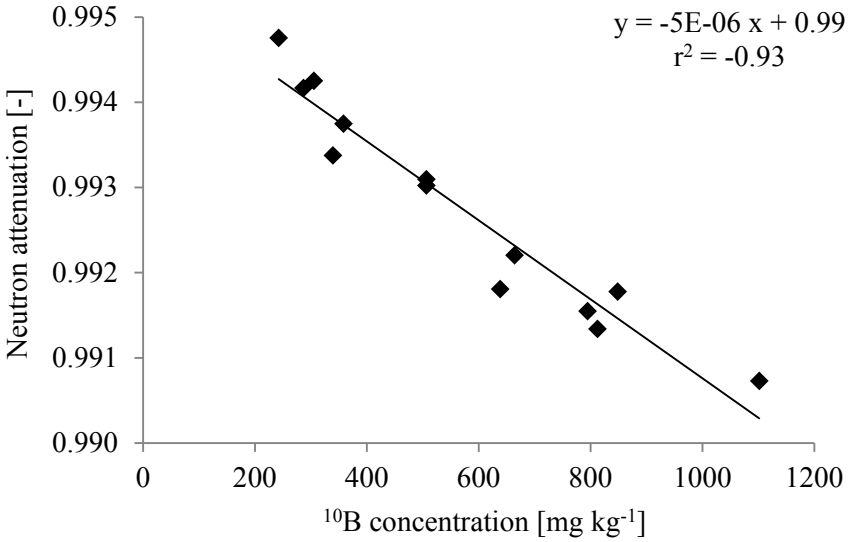

Figure 3. Correlation between the ^{10}B concentrations and the neutron attenuation of the pieces of one leaf of a *Populus nigra* × *euramericana* grown on a soil with a ^{10}B concentration of 75 mg kg^{-1} (total: 93 mg kg^{-1}).

Based on the microscopic cross-sections and atomic densities of C, O and H Mannes et al. (2009) calculated the theoretical attenuation coefficients of wood. They found other elements to be negligible for the calculation of the theoretical attenuation coefficient as a comparison to the measured attenuation coefficients showed. Either the element concentrations in the leaves (e.g. Li, Fe) or the macroscopic cross sections (e.g. Ca, P) are too small to contribute significantly to the total attenuation.

To calculate the spatial distribution of ^{10}B in the leaves, we used Equation 2 with oxygen (O), carbon (C) and hydrogen (H) as elements Σ_i and added a term for ^{10}B. The equation was then solved for the ^{10}B concentration by calculating the proportion of ^{10}B to the total attenuation from the difference between the measured and theoretical attenuation coefficient. Since the thickness of the leaves could not be measured pixel-wise it was estimated. The estimation was done by setting the thickness of each leaf to a value that led to a slope close to 1 for the correlation between ICP-OES measured and calculated ^{10}B concentrations. The optimum thickness values obtained ranged between 37 and 62 µm which was in the range of real leaf thicknesses as caliper measurements showed. The microscopic cross-sections for C, H and O were derived from Mannes et al. (2009), while that of ^{10}B was directly measured at ICON. Based on this calculation ^{10}B concentration maps of the NRs were created (c. Chapter 2).

A.4 Error estimation of the spatial distribution of ^{10}B in the leaves

Generally there are two main sources of error in NR images: the statistical and the systematic error (Hassanein, 2006). The statistical error in a NR image of a sample of constant composition and varying thickness is large for low and large thickness values. For water the statistical error is <2% for sample thickness values of about 0.07 – 1 cm at ICON (Hassanein, 2006). Taking into account the differences in sample composition and density between water and our leaf samples the statistical error is assumed to be <3%.

The pattern of a high error for very low and large thickness values holds true for the systematic sources of error, background and sample scattering as well. Mannes et al. (2009) showed that for low material densities (<0.5 g cm^{-3}) the measured attenuation coefficient of wood is very close to theoretical attenuation and scattering correction lead to an overestimation. Due to the experimental set-up, with the leaves directly attached to the scintillator the path length of scattered neutrons was very short, thereby reducing the underestimation of the ^{10}B concentration caused by non-detected neutrons. Another source of error is beam hardening. Because of the low thickness of the leaf samples beam hardening should not contribute significantly to the relative error in our study (Zawisky et al., 2004).

Besides the NR inherent errors like scattering and beam hardening there are also sources of error occurring from the biological nature of the samples and measurement limitations. Here, the main sources of error are pixel-wise deviations from the approximated elemental composition and the sample thickness. Deviations from the elemental composition with regards to C and O play a minor role in NR (Mannes et al., 2009).

However, NR is very sensitive to H and relatively small local deviations from the used values could have contributed strongly to the relative error.

Likewise, local differences from the approximated thickness values might have had a strong influence and are probably the cause for the main part of the deviation in the correlation between the ICP-OES measured and the NR imaging derived ^{10}B concentrations (Fig. 4).

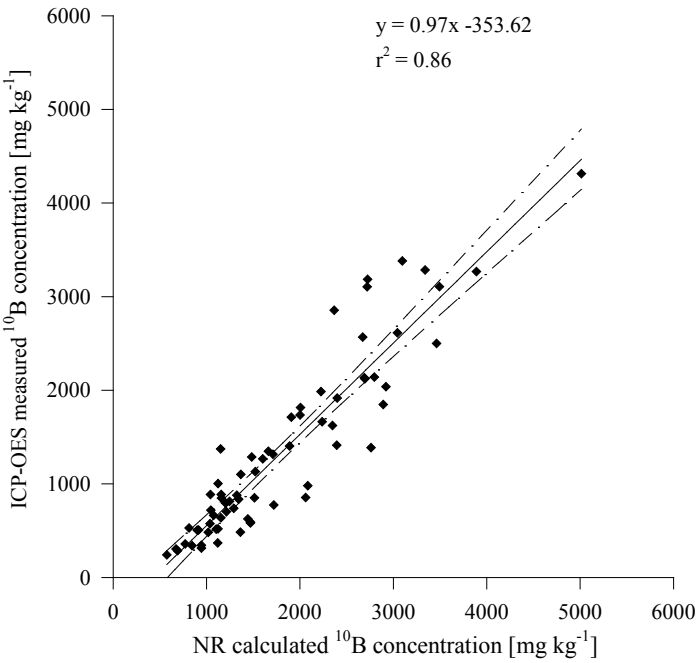

Figure 4. Correlation between the ICP-OES measured ^{10}B concentrations of the cut-up leaf pieces and the average ^{10}B concentrations of the same pieces derived from NR imaging. The dashed lines represent the 95% confidence interval.

However, the sample thickness was not measurable at the same spatial resolution as the neutron attenuation. Even though the relative error of the ^{10}B concentrations maps derived from the NR images (c. Chapter 2) might exceed 10% for some pixels, the general trends of the ^{10}B distribution and the concentrations are reliable as the correlation with the ICP-OES measured data shows.

A.5 Additional NR images

Due to space limitations in Chapter 2 and Chapter 3 of this thesis, which were prepared for publication as journal articles, more NR images are shown here to make them accessible for the scientific community.

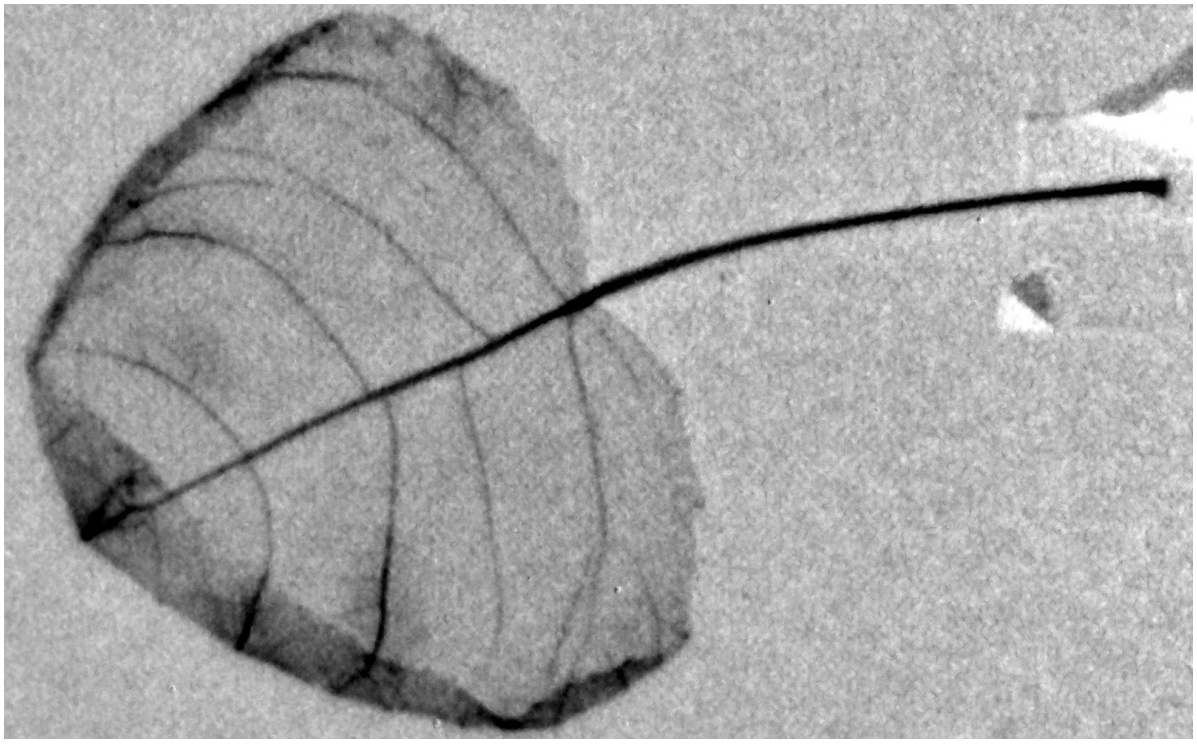

Figure 5. Transmission image of a leaf of *Populus nigra* × *euramericana* grown at a soil B concentration of 280 mg kg^{-1}. The total B concentration of the leaf was 1010 mg kg^{-1}. The aluminum tape that fixed the leaf to the scintillator during the exposure is visible as brighter structure on the right hand side. The leaf edge on the left hand side is curled due to the high B concentration.

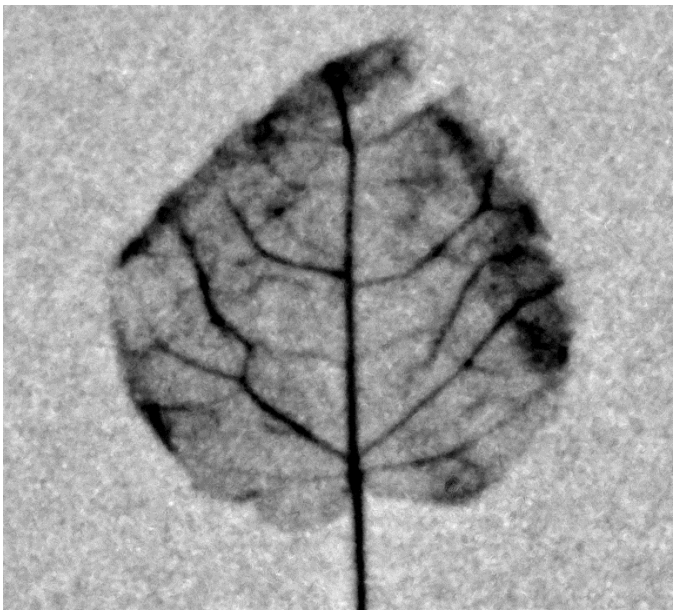

Figure 6. Transmission image of a leaf of *Populus nigra* × *euramericana* grown at a soil B concentration of 168 mg kg^{-1}. The total B concentration of the leaf was 1307 mg kg^{-1}. The leaf edge of nearly the whole leaf was curled due to the high B concentration.

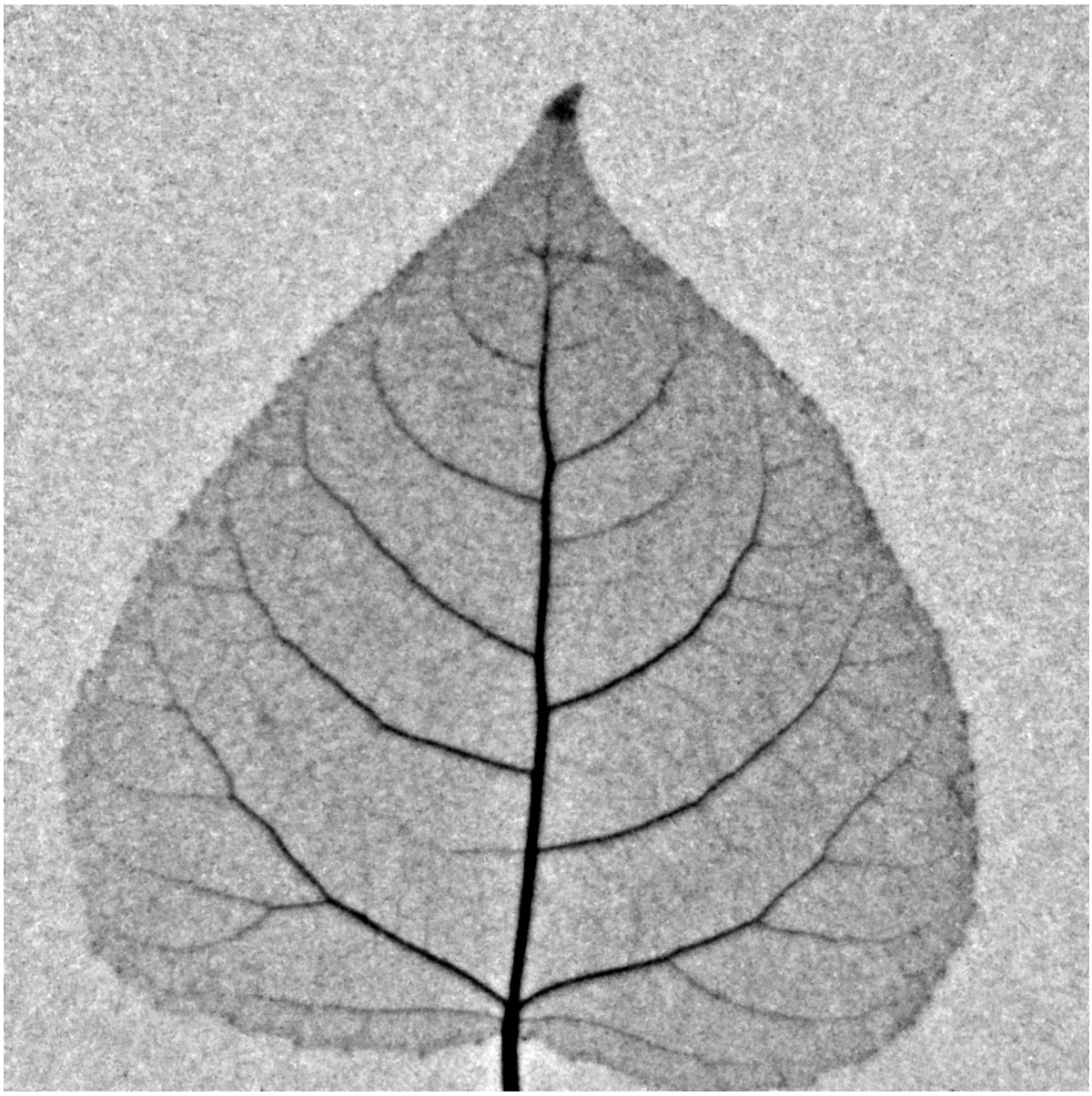

Figure 7. Transmission image of a young leaf of *P. nigra* × *euramericana,* grown at a soil B concentration of 168 mg kg^{-1}. The total B concentration of the leaf was 537 mg kg^{-1}. The tip was the only necrotic area of the leaf.

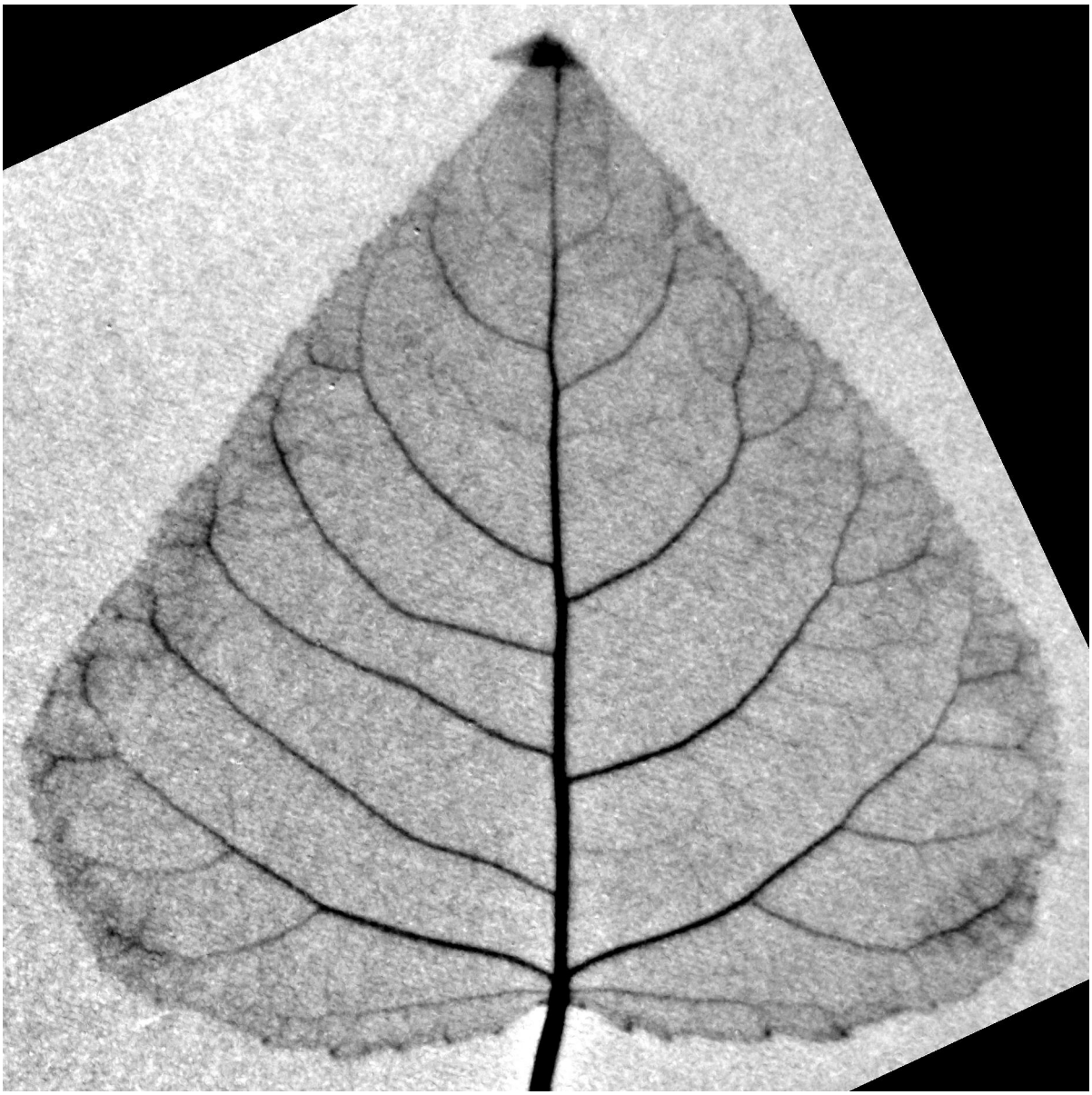

Figure 8. Transmission image of a young leaf of *P. nigra* × *euramericana,* grown at a soil B concentration of 93 mg kg^{-1}. The total B concentration of the leaf was 1041 mg kg^{-1}. Darker grey values, indicating higher ^{10}B concentrations are visible at the leaf edges.

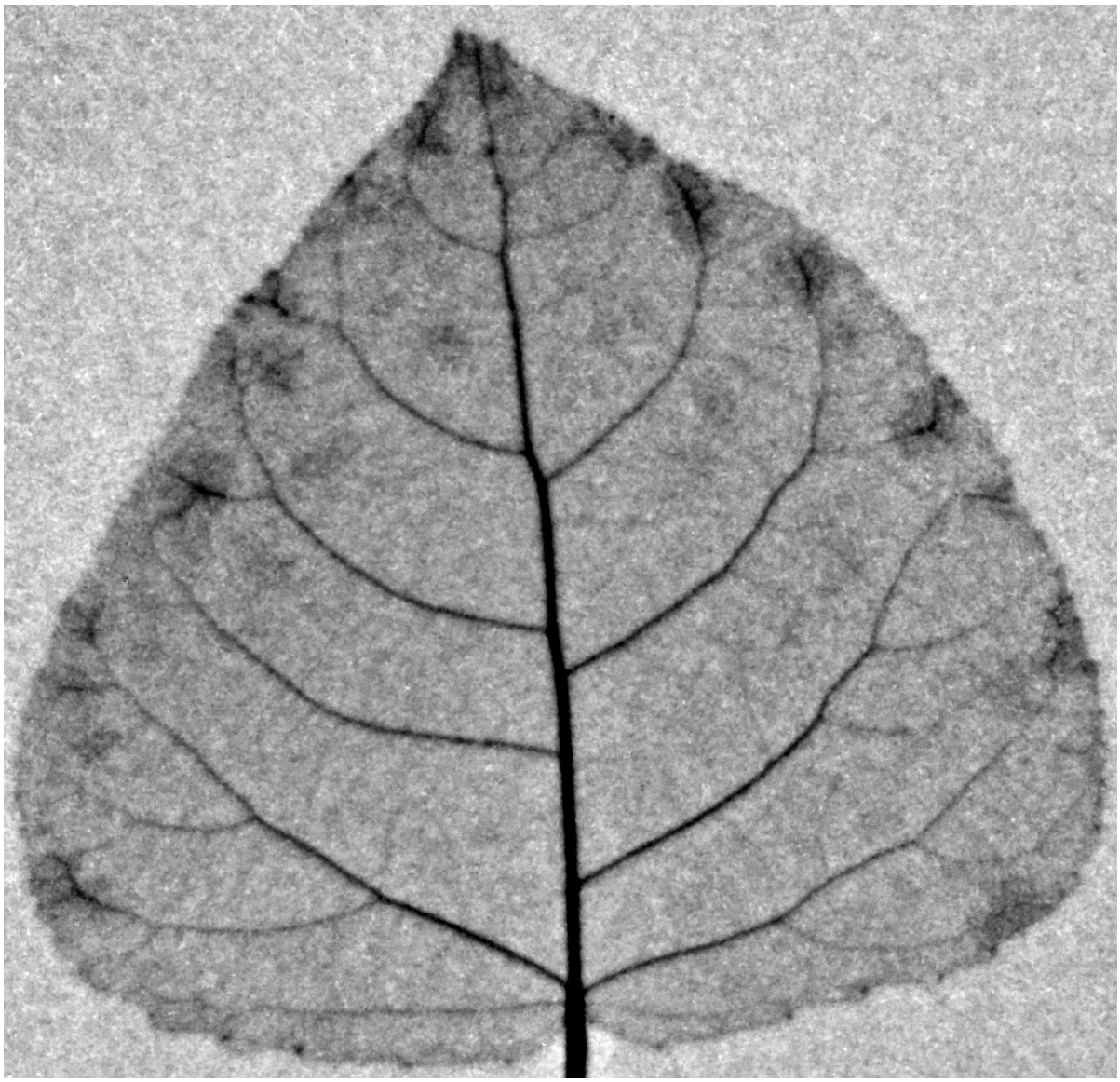

Figure 9. Transmission image of a young leaf of *P. nigra* × *euramericana,* grown at a soil B concentration of 93 mg kg^{-1}. The total B concentration of the leaf was 1037 mg kg^{-1}. Darker grey values, indicating higher ^{10}B concentrations are visible at the leaf edges. The necrotic leaf tip had fallen off at the time of the sampling.

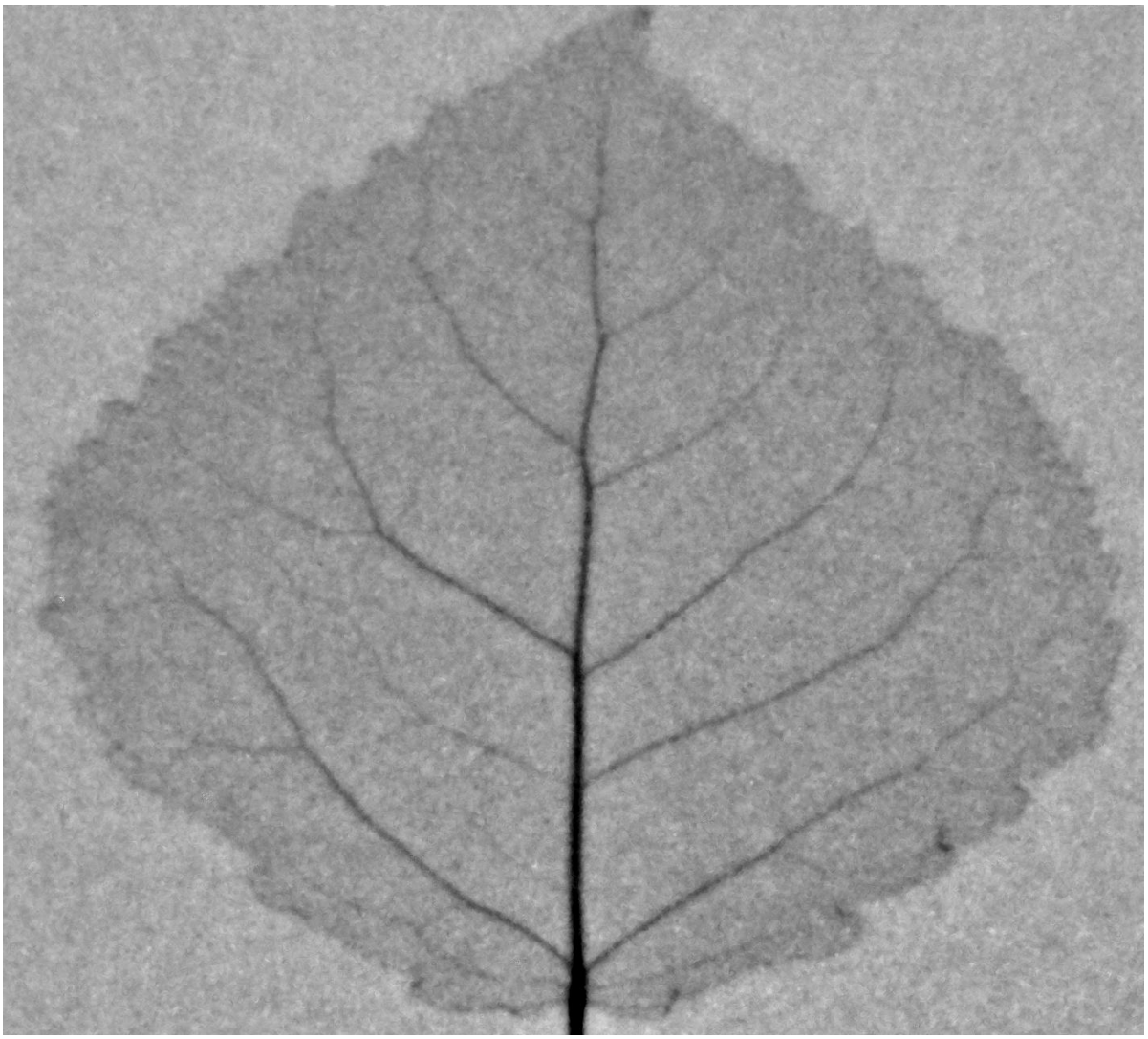

Figure 10. Transmission image of a young leaf of *P. nigra* × *euramericana,* grown at a soil B concentration of 13 mg kg^{-1}. The total B concentration of the leaf was 100 mg kg^{-1}.

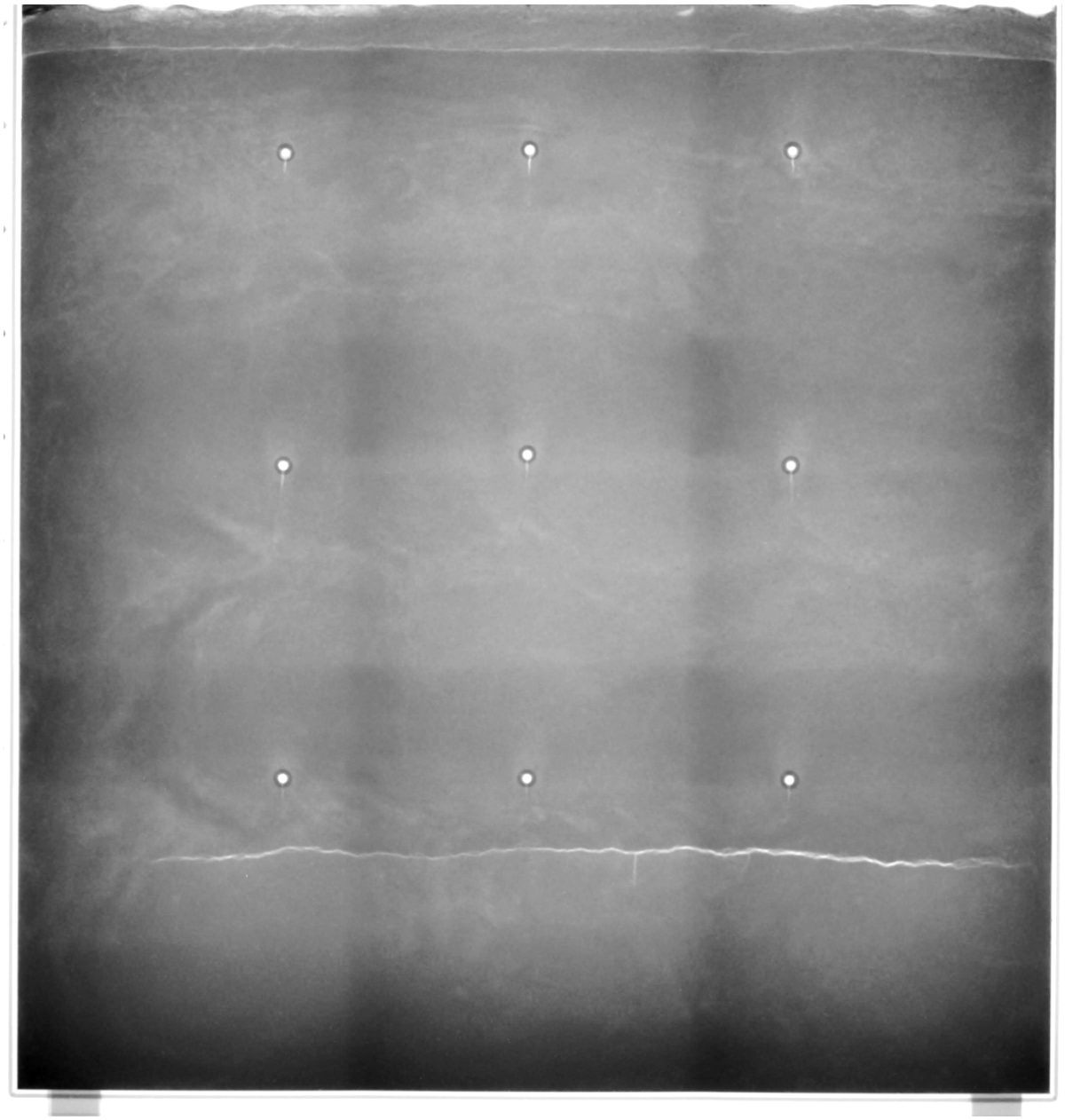

Figure 12. Transmission image of one of the soil filled containers used for poplar growth in Chapter 3. Shown is one of the containers from the homogeneous B treatments after 2 weeks of plant growth. The nine bright spots in the soil are aluminum screws that prevented the containers from bulging. One can also see the aluminum frame of the container around the edges of the picture. The bright structure in the lower third of the soil is a crack. The darker linear structures in the soil are caused by the failed flat field correction. Other soil structures are due to differences in water content or density.

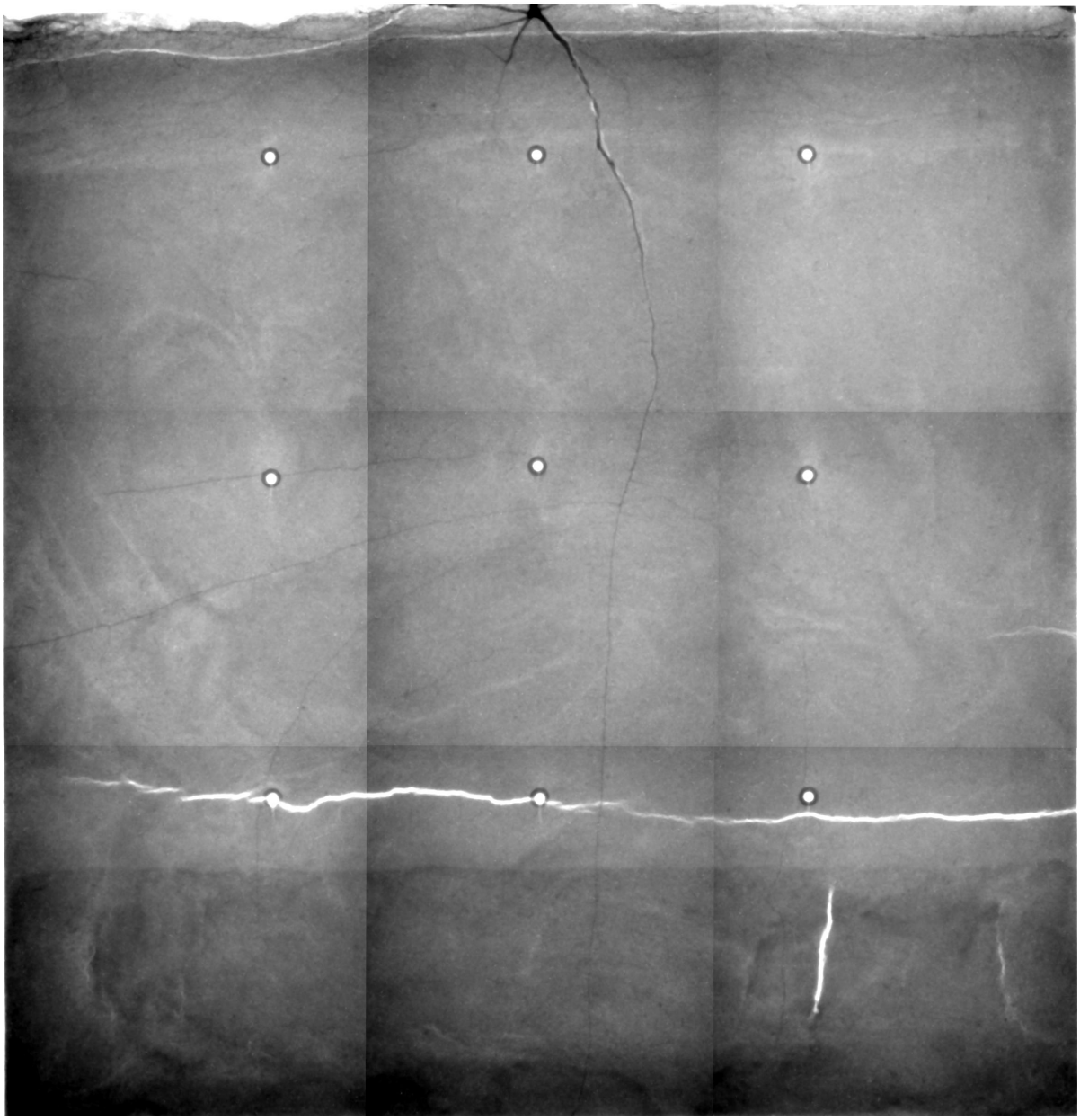

Figure 13. Transmission image of one of the soil filled containers used for poplar growth in Chapter 3. Shown is one of the containers from the homogeneous B treatments at the end of the experiment (6 months). The screws and some cracks are clearly visible as well as the root.

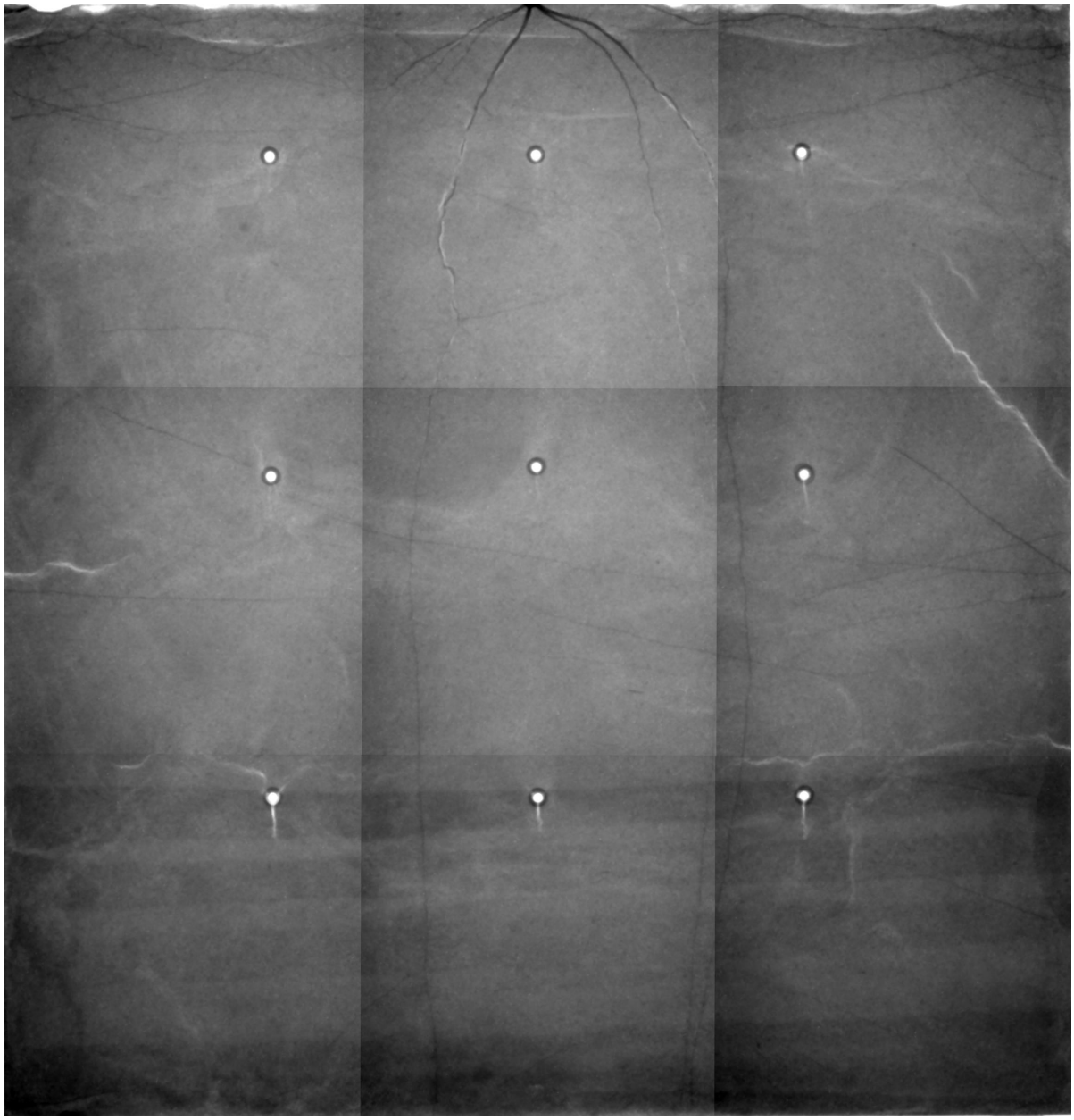

Figure 14. Transmission image of one of the soil filled containers used for poplar growth in Chapter 3. Shown is one of the containers from the control treatments without B-spiking at the end of the experiment (6 months). The screws and some cracks are clearly visible as well as the root.

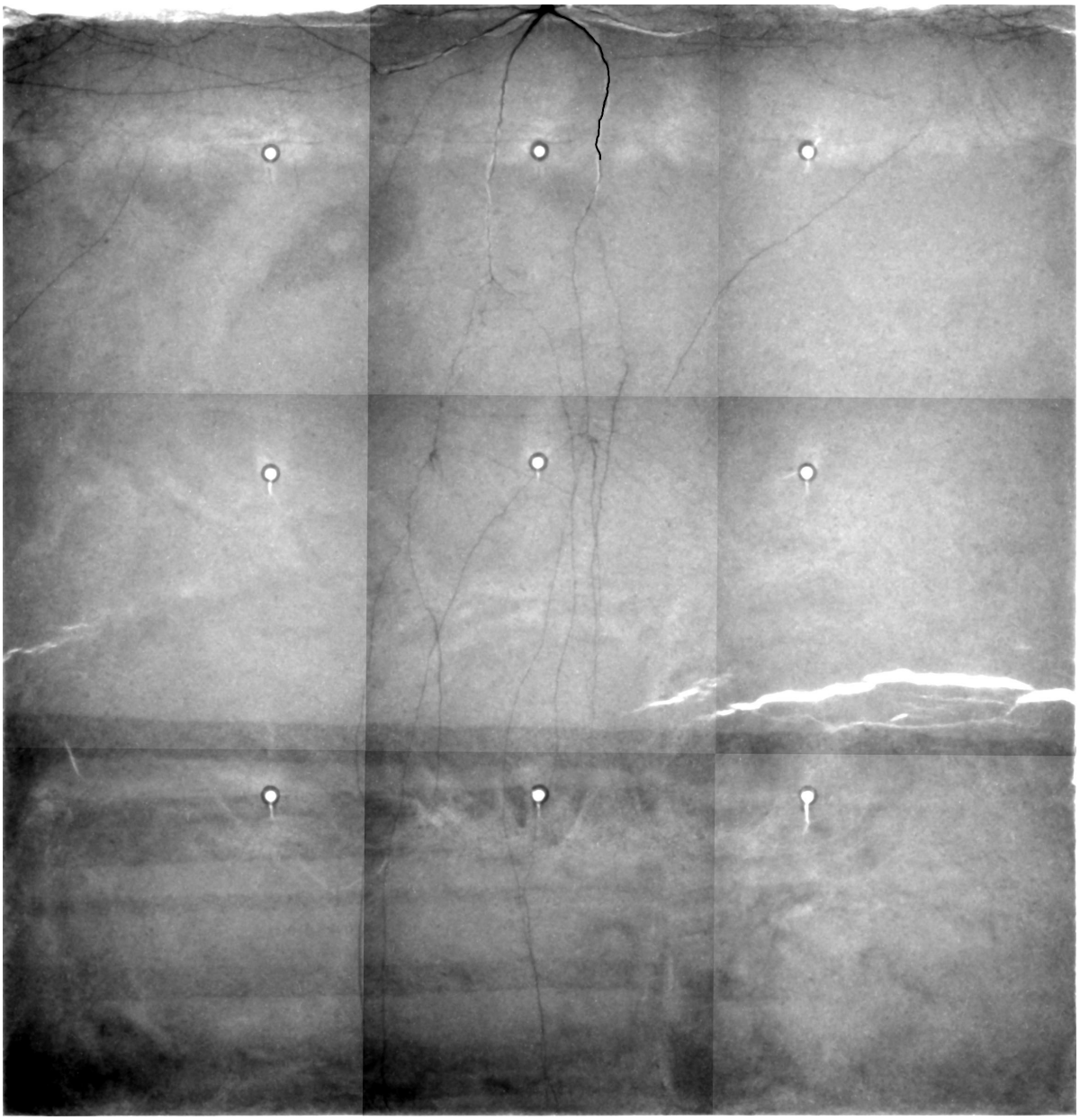

Figure 15. Transmission image of one of the soil filled containers used for poplar growth in Chapter 3. Shown is one of the containers from the heterogeneous B treatment at the end of the experiment (6 months). The screws and some cracks are clearly visible as well as the root.

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