

**Western hemlock regeneration on coarse woody debris is facilitated  
by linkage into a mycorrhizal network in an old-growth forest**

by

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## **Abstract**

Western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) is the climax tree species in most of the low elevation Pacific northwest coastal forests. Regeneration of western hemlock under a closed canopy is tightly associated with coarse woody debris (CWD). Nurse-logs contain physical, chemical and biological features that make them the most suitable seedbed for hemlock in old-growth forests. However, the preference of western hemlock for CWD is still not completely understood. Few studies have investigated belowground dynamics related to western hemlock growing on decayed wood, specifically ectomycorrhizal (EM) associations and the potential access to a mycorrhizal network (MN). My research addressed two objectives: 1) To compare establishment and survival of western hemlock germinants among different microsites (CWD with different wood breakdown levels and forest floor) in the forest understory; and 2) To investigate whether carbon is transferred through mycorrhizal networks from mature trees to seedlings established on CWD. To accomplish this, I pulse-labeled  $^{13}\text{C}$ -glucose solution into the phloem of mature trees. I found that western hemlock successful recruitment was associated with the presence of medium to advanced decay classes of CWD. I also found the first evidence that there is carbon transfer from mature trees to regenerating seedlings established on CWD. I conclude that western hemlock regeneration on nurse-logs is facilitated by mycorrhizal networks of canopy trees in an old-growth forest of coastal British Columbia.

## **Lay summary**

Western hemlock is the climax tree species in most of the low elevation Pacific northwest coastal forest. In a close canopy, seedlings of this conifer are highly dependent of the presence of decay logs to establish. Nurse-logs contain physical, chemical and biological features that make them the most suitable seedbed for western hemlock in old-growth forests. However, the preference of western hemlock for CWD is still not completely understood. Few studies have investigated belowground dynamics related to western hemlock growing on decayed wood, specifically ectomycorrhizal (EM) associations and the potential access to a mycorrhizal network (MN). The key goal of this research was to determine if western hemlock regeneration established on decay logs in an old growth forest, is linked to the forest mycorrhizal network. More specifically, we tested if there is an inter-plant carbon transfer from mature western hemlock trees towards its regeneration growing on nurse logs.

## **Preface**

This thesis is an original, unpublished product of the author, Gabriel Orrego. The overall project was identified and designed by Gabriel Orrego with key advice from Dr. Suzanne Simard and Dr. Brian Pickles. The project was part of an NSERC Strategic Project Grant known as “The Mother Tree Project”. The specific experiments were designed by Gabriel Orrego, Dr. Suzanne Simard and Dr. Brian John Pickles. All the experiments and field work were conducted at Malcom Knapp Research Forest. Gabriel Orrego established and led both the field experiment and sample processing, with the exception sample analysis using mass spectrometry, which was conducted by the Stable Isotope Facility at UBC-Vancouver. Statistical analyses were conducted by Gabriel Orrego with constant suggestions and support from Dr. Suzanne Simard and Dr. Brian John Pickles.

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Western hemlock, you beautiful tree. Wherever I establish in the future, I'll plant you on the most suitable nurse logs I can find.

For regeneration, mother trees and life in death.

## **Chapter 1: Introduction**

Old trees are nature's luminaries that hold crucial ecological roles in forest ecosystems (Carey, 1989; Gutiérrez, 2016; Lindenmayer et al., 2009). Many of these functions cannot be replaced, making them "keystone structures" (Lindenmayer et al., 2014; Sutherland et al., 2016). Primary forest accounts for only 36% of the total world forest area, and it is decreasing annually at a rate of 0.4% (FAO, 2010). Gutierrez (2016), maintains that these "monumental trees" should be conserved because they: 1) foster natural fascination, 2) are rarities of nature, 3) provide ecosystem services, 4) allow the study of individual adaptation, 5) represent a biocultural heritage, and 6) have been strongly exploited. Beyond ecological services, old trees are important to different cultures around the world (Dafni, 2006; Rozzi et al., 2013). Large old trees are not simple enlarged versions of young trees (Lindenmayer et al., 2014); they represent a standing reservoir of biological and genetic information (Gutiérrez, 2016). Also, their long-lived and extensive root systems generate more physical belowground connections than any other tree in a forest (Beiler et al., 2010). Finally, their large biomass has substantial relevance for carbon storage, both above and below ground (Laurance et al., 1997). Old growth forests contain complex structural and biological features that may engender resilience under a changing climate (Simard, 2009). The Pacific Northwest coast contains some of the most enormous, stunning and oldest forests in the world, with an invaluable iconic worth. Given the alarming rate of disturbance, the remaining old growth forest should be preserved.

### **1.1 Mycorrhizal networks**

Research on forest ecosystems has resulted in an increased understanding of their seemingly infinite complexity. It has been suggested that the mycorrhizal network (MN), the

connection between roots of at least two plants by mycorrhizal fungal hyphae, plays a determinant role in the functioning of ecosystems as complex adaptive systems (Simard, 2009). This network, based on a mutualistic symbiotic association between plants and mycorrhizal fungi, is understood as a highly important evolutionary driver for plants and fungal species (Archibald, 2011); moreover, it challenges assumptions about the role of competition versus mutualism in ecology and evolution. Fossil evidence shows that the plant-fungus symbiosis enabled land colonization of plants, after living exclusively in aquatic habitats (Humphreys et al., 2010; Trappe, 1987). Plants in a symbiotic association with fungi were able to access to larger pools of water and nutrients in soil, and in exchange, plant photosynthates were transferred to the mycorrhizal fungi. This successful symbiotic association occurs in approximately 90% of terrestrial plants species (Smith & Read, 2008).

Mycorrhizal networks are composed of a common mycelium that can physically link different plant individuals together. In a forest ecosystem, the spatial extent over which individual plants are linked in an MN is extremely difficult to quantify. However, Beiler et al. (2010) conducted a “ground-breaking” study where they mapped the approximate architecture of the MN in an interior Douglas-fir forest in British Columbia. In a 30 X 30 m plot, they found that two sister species, *Rhizopogon vesiculosus* and *Rhizopogon vinicolor* (2 out of the 65 ectomycorrhizal fungal species previously described in this forest (Twieg et al., 2007)) linked almost all trees together. One of the trees was directly linked through its mycorrhizal fungi to 47 other trees out of a total of 67 present in the plot. Furthermore, 19 trees from all age classes were linked together by a single *Rhizopogon* genet.

Theoretically, a network is a system of nodes connected by links (Southworth et al., 2005). In Beiler et al. (2010), when presenting trees as nodes and fungi as links, they discovered

tree size was positively correlated with connectivity, or node degree distribution. While mature trees have a well-established MN (Beiler et al., 2010), young seedlings can become included in the MN as their roots encounter the mycelial front (Simard et al., 2012). Regeneration establishment and growth have improved when seedlings linked in to the MN (Bingham & Simard, 2012; Horton et al., 1999; Nara, 2015; Teste et al., 2009; van der Heijden & Horton, 2009). The MN has also been shown to serve as a conduit for transmission of chemical compounds from one plant to another. Water, nutrients, organic molecules and other compounds have been found to flow through the mycorrhizal network (Egerton-Warburton et al., 2007; Selosse et al., 2006; Simard et al., 1997; Teste et al., 2009; van der Heijden & Horton, 2009). Resource transfer through MNs is not susceptible to interruption by other organisms because it is compartmentalized and protected, enhancing its efficiency (Simard et al., 2012).

Carbon and nutrient fluxes through MNs appear to be driven by a source-sink gradient, where compounds travel via mass flow from zones of high to low concentration (Francis & Read, 1984). Inter-plant transfer of nitrogen, for example, has been observed to occur from a nitrogen-fixing tree (corresponding to a source) to a non-nitrogen-fixing tree (corresponding to a sink) (He et al., 2005; Ribeiro et al., 2015), or even from one non-N<sub>2</sub> fixing plant to another provided their tissues differ in nitrogen concentration. The transfer of carbon or nitrogen appears to be more strongly controlled by the strength of the sink than the source (Teste et al., 2009). A rapidly growing seedling, for instance, is a stronger sink than more slowly growing seedlings; therefore, it is predicted that more carbon would be transferred to it (Simard et al., 2012). Conversely, shading or other abiotic stresses that impose greater sink strength in seedling establishment can also modulate MN resource fluxes (Bingham & Simard, 2012; Simard et al., 1997).

In the understory of old growth forests, as examined in this thesis, seedlings with limited resource access are considered carbon sinks. Canopy trees, on the other hand, have well-established root systems and a surplus of light, and thus are considered carbon sources within an existing MN. Thus, seedling establishment is thought to be facilitated by association with and proximity to carbon donors. Canopy trees have the potential to transfer carbon via the MN to establishing seedlings, thus enhancing regeneration (Bingham & Simard, 2012; Selosse et al., 2006; Teste et al., 2009). Both Teste et al. (2009) and Bingham and Simard (2012) found that interior Douglas-fir seedling survival increased with greater access to a MN associated with older trees. Old mature trees, identified as hub trees by Beiler et al. (2010) and informally called “mother trees” (Simard, 2017), may have facilitated regeneration by acting as a carbon source.

## **1.2 Coarse Woody Debris**

Decaying logs and larger branches in a forest are referred to as coarse woody debris (CWD). It is a common and substantial component of a natural forest, providing structural features and several ecological functions. For example, CWD provides important substrate for seedling establishment, habitat for animals, and resources for decomposers and other organisms, including autotrophs and heterotrophs (Harmon et al., 1986; Tedersoo et al., 2003). Decayed CWD with certain features are designated as “nurse logs”, referring to their facilitative effects in regeneration (Kirk, 1966). Harmon et al. (1986), defines CWD as any dead woody material > 2.5 cm in diameter. For this study, we considered CWD as large fallen dead trees suitable for tree-seedling establishment with width greater than 15 cm.

Coarse woody debris has several beneficial qualities for tree regeneration. In some forests, competition with bryophytes, herbs and shrubs on the forest floor (ff) dramatically

reduces tree-seedling survival (Harmon et al., 1986; Harmon & Franklin, 1989). Rotting wood can elevate seedlings above this community, thus increasing light availability and reducing soil resource competition. Further, large logs or stumps may provide refuge for seedlings against herbivory (Harmon et al., 1986). Also, rotting wood has higher moisture holding capacity than soil, therefore enhancing survival of seedlings as well as providing refuge to ectomycorrhizal (EM) fungal communities (Harvey et al., 1978; Walker & Jones, 2013). However, there are other aspects of CWD that can be considered detrimental for plant growth, such as its very low pH and poor availability of nutrients such as nitrogen (Cowling & Merrill, 1966; Minore, 1972).

During early stages of CWD decay, while wood density is still relatively high, root growth of the establishing seedlings is difficult, thus negatively influencing survival. As CWD decay proceeds, wood density declines while nutrient availability and water holding capacity increase, and CWD therefore becomes a better substrate for seedling establishment. Likewise, while mass is lost through leaching of soluble compounds and mineralization of carbon compounds to CO<sub>2</sub>, nutrients such as N and P are retained and their relative concentration and availability increase (Boddy & Watkinson, 1995; Keenan, et al., 1993; Means et al., 1992; Preston et al., 1998; Sollins et al., 1987). Grier (1978) suggests, however, that most nutrient increases over time are due to litterfall and throughfall input. Additionally, N<sub>2</sub>-fixation by asymbiotic bacteria in CWD has been associated with an N increase in the decay process (Brunner & Kimmins, 2003; Hendrickson, 1991). Despite of all this, CWD is still a very poor nutrient substrate for plant growth compared with mineral soil or forest floor (Harmon et al., 1986; Keenan et al., 1993; Marx & Walters, 2006).

### **1.3 Western hemlock and the fungal community on CWD**

In the rainforests of the Pacific Northwest, trees, especially western hemlock, frequently establish on logs and stumps. Western hemlock is a highly shade tolerant species, enabling seedlings to establish in the understory, mainly on CWD (Fowells, 1965; Kirk, 1966). Gray & Spies (1997) found that, in closed-canopy areas, western hemlock seedling establishment was greater on decayed wood than on the forest floor or mineral soil. In another experiment in an old-growth forest of western hemlock, 98% of western hemlock seedlings occurred on CWD, which covered only 6% of the forest floor (Christy & Mack, 1984). Western hemlock seedlings are sensitive to understory plant competition; thus, they are typically limited to the surface of decaying wood where other plants generally cannot grow (Christy & Mack, 1984; Scott et al., 1976). Also, western hemlock has smaller seed compared to other co-occurring conifers (Schopmeyer, 1974); their survival is therefore enhanced by the higher moisture content and lack of litter on CWD, protecting germinants against competition and drought (Haig et al., 1941).

As previously mentioned, rotting wood is poor in nutrient availability (Cowling & Merrill, 1966); therefore, tree-seedling nutritional strategies such as EM associations might be crucial. It is well known that in soil with limited resources, mycobionts can extract enough nutrients to support plant growth (Poznanovic et al., 2015). Mycorrhizal fungal hyphae have 60 times more absorption area than fine roots and are able to decompose larger organic molecules (Selosse et al., 2006; Talbot et al., 2008).

Western hemlock seedlings regenerating in CWD have been shown in several studies to associate with a broad range of ectomycorrhizal fungal species (Christy et al., 1982; Durall et al., 1999; Horton et al., 2005; Kropp, 1982; Kropp & Trappe, 1982). Moreover, successful regeneration of western hemlock can be explained in large part by its symbiotic associations.

Kropp & Trappe (1982) suggest that, on the forest floor, there is strong competition between western hemlock-specific mycorrhizal fungi and generalist mycobionts that are associated with established early successional plants. Consequently, western hemlock seedlings that establish on CWD avoid this competition, enabling them to form ectomycorrhizas. Similar EM fungal associations have been described for eastern hemlock (*Tsuga canadensis* (L.) Carr) (Poznanovic et al., 2015).

Remarkably, western hemlock can survive its first year of life growing on rotten wood without an EM association (Christy et al., 1982). This may help explain its success at establishing on CWD. Nevertheless, growth performance improved once it was colonized by symbiotic fungi (Christy et al., 1982). In another study Kropp (1982b), found that 3 month-old seedlings were colonized. Since these earlier studies, substantial improvements have been developed in molecular analysis techniques (Horton & Bruns, 2001; Lindahl et al., 2013) opening the door for new and more accurate research on mycorrhizal development in western hemlock germinants.

In addition to the poor nutrient availability in CWD, western hemlock seedlings grow in shaded conditions, resulting in low photosynthetic activity. This results in low photosynthate availability for their symbiotic fungi. The role of MNs in regeneration of western hemlock within these microsites is not well studied or understood. It is suggested, though, that seedling survival under these conditions may depend on alternative sources of carbon. Carbon potentially transferred from “source” canopy trees through MN might be crucial for the recruitment of suppressed seedlings, particularly on nutrient-limited substrates (Bingham & Simard, 2012; Kennedy, Izzo, & Bruns, 2003; Simard et al., 2012). Tedersoo et al. (2008), suggest that the success of seedlings growing in elevated dead wood may result from both carbon transfer from MN, and the lack of direct root competition in CWD. The work of Christy & Mack’s (1984)

could strengthen the hypothesis of Tedersoo et al. (2008), since they have shown that western hemlock seedlings typically establish on moderately decayed wood, and become less common with progressing decay, possibly due to litter accumulation and development of root competition with mature trees.

Ectomycorrhizal fungi may also directly transport carbon from decaying wood to seedlings, providing yet another mechanism explaining the success of western hemlock seedlings on CWD. Mycorrhizal fungi are often described as biotrophs, which obtain photosynthetic carbon compounds from plants. However, EM fungi evolved from saprotrophic fungi, and many of them kept distinct genes that encode enzymes associated with plant cell wall degradation or other complex polymers (Kohler et al., 2015; Read & Perez-Moreno, 2003). Therefore, EM fungi inhabiting CWD might be also decomposing cellulose and lignin. It has been shown that in conditions where soils are highly organic or where there is low light availability, some species of EM fungi act as decomposers of larger organic molecules through the production of these enzymes (Talbot et al., 2008). More evidence is available for orchid mycorrhizal fungi, which have been observed to transfer carbohydrates from organic substrates to juveniles of host plant species (Dearnaley, et al. 2012). Similarly, some plants (e.g., myco-heterotrophic plants) receive carbohydrates from saprotrophic fungi (Leake et al., 2001; Lindahl et al., 1999; Merckx et al., 2009; Ogura-Tsujita et al., 2009). Studies have examined the role of EM in soil organic matter decomposition through the measurement of extracellular enzymes (Talbot et al., 2013); however, this kind of research has not been done on EM fungi growing in rotten wood. Aligning with this possibility is the potential role of ectomycorrhizal fungi serving as saprotrophs in wood decay, and then acting as facilitators of regeneration on CWD through direct carbon supply.

Despite many studies examining the tight hemlock-log association, few agree on which factors are most important for hemlock successful establishment. In an old growth forest dominated by western hemlock, where CWD are a common component of the understory, I reevaluated the effect of forest microsites on hemlock recruitment, and how mature trees and mycorrhizal networks may enhance the facilitative effect of decaying logs. Since western hemlock is the climax species of the Pacific Northwest rainforest, exploring the facilitative effects of belowground pathways on seedling regeneration could provide excellent insights into old growth forest ecology and management.

#### **1.4 Thesis objectives**

The main objective of this study was to determine whether, in an old-growth forest, *Tsuga heterophylla* (Raf.) Sarg. regeneration on CWD is linked to the mycorrhizal network. The following hypotheses were tested: (1) western hemlock recruitment is mediated by presence of CWD and its level of decay; (2) western hemlock establishing on CWD receives carbon transferred from mature trees through a MN; and (3) the size of mature donor trees is positively correlated with the amount of carbon transferred to seedlings.

## **Chapter 2: Carbon transfer from mature western hemlock to seedlings regenerating on different forest microsites**

### **2.1 Introduction**

Western hemlock growing on CWD has long been studied to improve our understanding of the main factors involved in the tight hemlock-log association (Christy et al., 1982; Christy & Mack, 1984; Durall et al., 1999; Fowells, 1965; Gray & Spies, 1997; Harmon & Franklin, 1989; Kirk, 1966; Kropp, 1982b; Kropp & Trappe, 1982; Scott et al., 1976).

Decayed wood is very poor in nutrients compare to soil or the forest floor (Harmon et al., 1986; Keenan et al., 1993; Marx & Walters, 2006); still, western hemlock germinants prefer it above other forest microsites (Christy & Mack, 1984). Western hemlock's specificity for establishing on woody substrates is particularly important in a closed canopy (Gray & Spies, 1997). Thus, the typical conditions where western hemlock establishes are on poor nutrient substrates and under deep shade. Based on this, we focused on belowground dynamics involved in western hemlock establishment on CWD. It has been well documented that western hemlock forms EM with a broad range of mycobionts (Christy et al., 1982; Durall et al, 1999; Horton et al., 2005; Kropp, 1982; Kropp & Trappe, 1982). However, the direct contribution of the forest MN to hemlock seedlings establishment on CWD remains unknown.

Studying belowground dynamics in forest ecosystems has improved with the availability of new innovative technologies and methodologies. Stable isotope techniques, for example are both natural integrators and tracers of ecological processes that have been intractable using other methods (Craine et al., 2015; Dawson et al., 2002). Natural abundance of  $\delta^{15}\text{N}$  has been useful in examining the function of the mycorrhizal symbiosis in N-limiting ecosystems. Fungi

discriminate against  $^{15}\text{N}$  during the synthesis of transferred compounds to the host plant, causing  $^{15}\text{N}$ -enrichment in the fungal body and  $^{15}\text{N}$ -depletion in the plant (Hobbie et al., 2005; Hobbie & Colpaert, 2003; Hobbie et al., 2000). This pattern is more noteworthy in ecosystems where N is limiting, it is immobilized in organic compounds, and mineralization is insufficient to support plant demand. Therefore, plants in N-limited ecosystems sometimes rely on the enzymatic capabilities of some EM fungi to breakdown recalcitrant organic N pools (Hobbie & Hobbie, 2008; Read & Perez-Moreno, 2003; Schimel & Bennett, 2004). Thus, mycorrhizal fungal biomass associated with plant hosts is positively correlated with plant  $^{15}\text{N}$  depletion (Hobbie & Colpaert, 2003).

The stable isotope  $^{13}\text{C}$  also has important ecological applications (Dawson et al., 2002). During photosynthetic assimilation,  $^{13}\text{CO}_2$  is discriminated against over the lighter  $^{12}\text{CO}_2$  (Farquhar et al., 1982). When plants are under water stress, water use efficiency is associated with stomatal closure, manifesting a decrease in  $^{13}\text{CO}_2$  discrimination during photosynthetic assimilation. Thus, under natural conditions,  $^{13}\text{C}$  enrichment is positively correlated with water stress (Farquhar et al., 1989).

Pulse labeling with stable isotopes provides a means to trace the fate of resources. For example, pulse-labeling with  $^{13}\text{C}$  enables tracing of carbon compounds within plants, through soil pools and even to other plants (Mordacq et al., 1986; Olsson & Johnson, 2005; Simard et al., 1997). Pulse labeling  $^{13}\text{C}$  in trees is often executed utilizing  $^{13}\text{CO}_2$  (see Epron et al., 2012 for a review). In this study, we used a novel methodology of injecting  $^{13}\text{C}$ -Glucose dissolved in water into mature-tree phloem.

The main objective of this study was to determine whether regeneration of western hemlock on CWD was associated with the ability of seedlings to link into the mycorrhizal

network of old trees. To achieve this, we first assessed regeneration success across a range of forest microsites. Next, we analyzed  $\delta^{15}\text{N}$  natural abundance of hemlock seedlings in order to estimate the significance of the potential EM associations. And finally, we carried out  $^{13}\text{C}$  pulse labelling of mature trees followed by sampling of surrounding hemlock seedlings on the forest floor and CWD to determine whether carbon subsidies were provided to seedlings through mycorrhizal networks. The experiment was conducted in an old growth forest dominated by western hemlock on the coast of British Columbia, Canada.

The following hypotheses were tested: (1) western hemlock recruitment is mediated by presence of CWD and its level of decay; (2) western hemlock establishing on CWD receives carbon transferred from mature trees through a MN; and (3) the size of mature donor trees is positively correlated with the amount of carbon transferred to seedlings.

## **2.2 Methods**

### **Study area**

This project was conducted at the UBC Malcom Knapp Research Forest, located near Maple Ridge, British Columbia, Canada. The experiment was carried out in a single forest, next to Eunice Lake at the foot of Golden Ears Provincial Park (49°20'21.97''N, 122°33'37.24''W) (Figure 1). The study site is occupied by an unmanaged, uneven-aged old-growth forest (250+ years old) dominated by Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco, (Fd)); western hemlock (*Tsuga heterophylla* (Raf.) Sarg, (Hw)) and western redcedar (*Thuja plicata* Donn ex D.Don, (Cw)), with some amabilis fir (*Abies amabilis* Douglas ex J.Forbes, (Ba)). The forest falls within the Submontane Very Wet - Maritime variant of the Coastal Western Hemlock biogeoclimatic zone (CWHvm1) (Green & Klinka, 1994). In the CWHvm1, mean annual precipitation is 2787 mm (75mm during the driest month and 436 during the wettest month) and

mean annual temperature is 8.2°C (16°C during the warmest month and 0.3°C during the coldest month) (Pojar et al., 1991). The elevation of the site ranges from 500 – 570 meters above sea level. The main slope faces south-west and has an average inclination of 30%.



Figure 1. Google map (© 2017 Google) image of Malcom Knapp research forest next to Pitt river outside of Maple Ridge, BC, Canada, with the research sites polygon next to Eunice lake inside a blue circle.

This primary forest has an uneven age structure forest with a large range of trees sizes. Crown cover is approximately 50%. There is abundant regeneration and saplings of the shade tolerant tree species, western hemlock. The largest stratum is dominated by western hemlock (>80 cm DBH), which was a determinant factor in selection of the site. Most of the veteran trees in the wetter areas, close to a small creek that divided the site in two, are Douglas-fir and western redcedar. This vibrant old growth forest is full of snags and canopy gaps, developed through gap

phase dynamics, and is home to abundant wildlife. This ecosystem is habitat for a wide variety of birds, including woodpeckers and owls, as well as mammals such as black-tailed deer, black bear and lynx, along with amphibians and fish (based on two years of field observations). The CWD is an important component of the forest floor surface, due to its high abundance in the site. Cover of CWD averages 15%, where approximately 1% is decay class 1, 2% class 2, 3% class 3, 5% class 4 and 4% class 5.

The undergrowth is mainly composed of shrub species from the Ericaceae family, including oval-leaf blueberry (*Vaccinium ovalifolium* Sm.), Alaskan huckleberry (*Vaccinium alaskaense* Howell), red huckleberry (*Vaccinium parvifolium* Sm.), salal (*Gaultheria Shallon* Pursh) and false azalea (*Menziesia ferruginea* Sm.).

The soil is a Humo-Ferric Podzol with loamy sand texture (Canadian System of Soil Classification 1998) and the humus form is Hemihumimor (Green et al., 1993). Based on the assessment of soil moisture regime as 2-3 and soil nutrient regime as P-M, as well as plant species composition, the site series was transitional between 01 (zonal; HwBa – Blueberry) and 03 (HwCw – Salal) (Green & Klinka, 1994).

### **Experimental design and treatments**

A total of 50 horizontal logs (CWD) with diameters ranging from 18 to 80 cm were selected within the study area (Figure 2) using a number of selection criteria described below. This total included 10 logs from each decay classes (I to V), utilizing the classification from the Field Manual for Describing Terrestrial Ecosystems of BC (B.C. Ministry of Forests and Range & B.C. Ministry of Environment, 2010) (Figure 3). Wood hardness, was measured with a penetrometer and a knife blade to corroborate the log decay class. The selection criteria included:

(1) there was a minimal distance of 10 meters between logs; (2) western hemlock regeneration was naturally regenerating on top of the logs, except for logs of class 1; and (3) the logs were of consistent shade level (high shade), species (western hemlock) and aspect (southwest). A control treatment was also located adjacent to each log (1-m away and southwest direction from the log). In these controls, the forest floor was considered a decay class beyond decay class V of CWD. In many of the forest floor microsites, buried decayed wood was found. The logs and forest floor were thusly organized in a completely randomized design with six substrate classes and uneven replication of 10 log and 50 forest floor replicates.

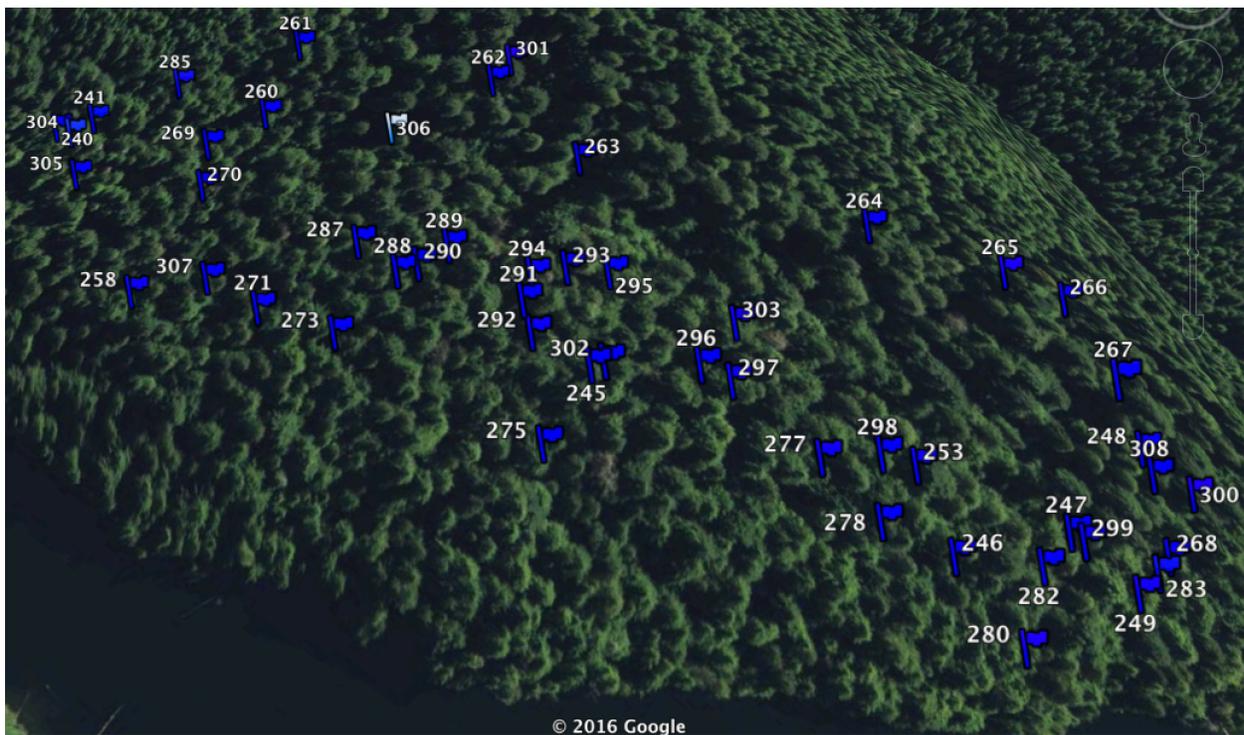


Figure 2. Google map (© 2016 Google) image of the research site with the 50 selected CWD. Malcom Knapp research forest, BC, Canada.

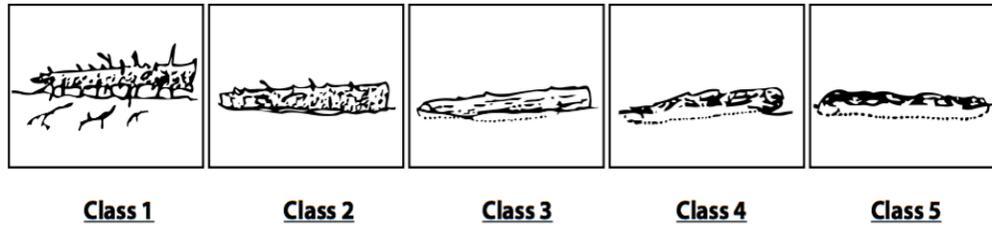


Figure 3. CWD decay classification modified from (B.C. Ministry of Forests and Range & B.C. Ministry of Environment, 2010).

In April of 2016, 50 stratified western hemlock seeds were sown on each of the 50 logs and 50 adjacent forest floor microsites (total of 5000 seeds). A small dome-cage (20cm x 20cm) made of chicken wire was installed above the seeds in order to exclude any possible predators and to provide a long-term marker for the experimental units.

#### *Observational data, sampling and elemental analysis*

For each log, the following variables were measured: diameter (cm); dominant moss species; moss cover (%); and water content ((wet weight-dry weight)/wet weight) ( $\text{g g}^{-1}$ ). The latter three variables were also measured for the forest floor experimental units.

The number and height (cm) of all naturally established seedlings growing on each log and the forest floor were measured. Three of the seedlings growing on the log were collected (when found). The collections were made to represent the range of seedling heights present, and these were put together in the same Ziploc bag (small, 2-5cm; medium, 6-12cm and large, 13-25cm) for later foliar and mycorrhizal root analyses. The same procedure was applied to each adjacent forest floor microsite (when found).

The roots of the collected seedlings were cleaned from the substrate they were growing on (wood or forest floor) and later ground in the lab using a mortar and pestle. The mycorrhizal

fungal DNA was extracted utilizing the DNeasy Plant Mini Kit, amplified and sequenced (unfortunately these results were not yet available for inclusion in this thesis).

The foliage from the same seedlings were analyzed for  $^{13}\text{C}$  and  $^{15}\text{N}$ . To prepare the samples, the foliage was: (1) dried inside paper bags at  $80^\circ\text{C}$  for >48 hours; (2) ground to a homogenous particle size using a mortar, pestle and liquid nitrogen; (3) weighed,  $0.3\pm 0.03$  milligram samples using an analytical balance and placed them into tin capsules, which were then arranged on plates; and (4) analyzed using an elemental analyzer and isotope-ratio mass spectrometer at the UBC Stable Isotope Facility. Stable isotopes values were given as  $^{13}\text{C}/^{12}\text{C}$  and  $^{15}\text{N}/^{14}\text{N}$  ratio, which are was calculated as:

$$\text{(eq.1)} \quad \delta^{13}\text{C} \text{ or } \delta^{15}\text{N} = 1000 \times \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}}$$

Where  $R_{\text{standard}} =$  Vienna-PeeDee Belemnite (VPDB) standard ( $1.1237 \times 10^{-2}$ ) for C, and  $\text{N}_2$  atmospheric gas ( $3.677 \times 10^{-3}$ ) for N.

For sown seed, germinant emergence was monitored 5 times through the summer of 2016, and survival was measured on October 15<sup>th</sup>, 2016. Emergence results from August were used for analysis since germination was greatest at this time.

### *$^{13}\text{C}$ labeling*

Pulse-labeling with  $^{13}\text{C}$  was used to determine whether mature hemlock trees provided carbon subsidies to seedlings establishing on the logs and forest floor through the MN. To do this, eight of the 50 logs were selected (8 plots): five with medium wood breakdown and three with high wood breakdown. All included a mature western hemlock tree within 10 m, and had western hemlock seedlings established on the log and the adjacent forest floor (Figure 4). The

eight mature trees were assigned as “mother-trees” and named (Table 1). To establish a control that severed any existing MN connection between the mother tree and seedlings on the log, a portion of each of the 8 logs was cut with a chainsaw, lifted from the ground and placed on two 10cm x 10 cm wooden beams. Large nails kept the isolated logs fixed in place. Since the wood of most logs was very loose, lifted log portions were wrapped in chicken wire to maintain structure and morphology (Figure 5).

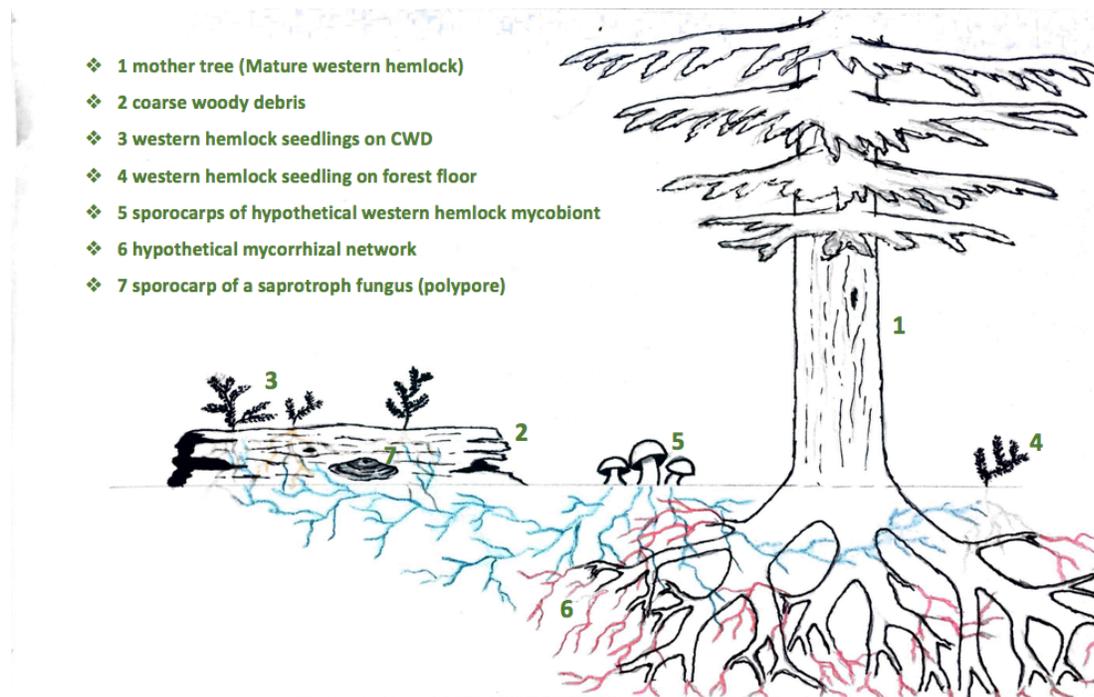


Figure 4. Illustration of plots. Components of interest for this research are numbered.

Table 1. Features of the eight pulse labelled trees.

<b>Plots</b>	<b>Name</b>	<b>Height (m)</b>	<b>dbh (cm)</b>	<b>Estimated live crown percent</b>	<b>Crown class</b>	<b>Mistletoe</b>	<b>Est. age</b>
<b>1</b>	Gina	27.3	67.7	50%	intermediate	yes	125-250
<b>2</b>	Robertina	40.6	83.2	50%	dominant	no	150-225
<b>3</b>	Nala	38.3	76.8	40%	codominant	no	150-225
<b>4</b>	Betty	35.6	69.6	50%	codominant	no	125-175
<b>5</b>	Carlita	37.1	88.4	60%	codominant	no	175-250
<b>6</b>	Kalu	40.3	86.1	50%	codominant	no	175-250
<b>7</b>	Carlota	37.2	64.9	50%	codominant	no	175-200
<b>8</b>	Rosita	44.4	91.7	50%	codominant	no	175-275



Figure 5. Log isolated from forest floor, thus serving as a control for carbon transfer through the MN. Control seedlings are abundant on top of the isolated log.

On November 21<sup>st</sup> 2016, 15 grams of 99 atom%  $^{13}\text{C}$ -D-GLUCOSE (Cambridge Isotopes Laboratory, Inc. Tewksbury, MA, USA) diluted at 5% in deionized water, were injected into the phloem of each mother-tree. The 75ml of solution was split into four aliquots (approx. 19 ml each) in order to reduce and distribute the volume and allow the tree phloem tissue to absorb it without leakage. To label the mother trees, four holes (10 cm in diameter, <0.5 cm deep, fitted to the shallow phloem of Hw) were dug into the base of the tree (at the aspect facing the CWD of interest) and fitted with a 10cm diameter plastic pipe (glued in place with silicon) (Figure 6). The four aliquots were carefully poured into one pipe each. On day 9 and 15 following the injections, seedling on the logs and forest floor, plus their respective substrates, were collected for  $^{13}\text{C}$ -enrichment analysis. Western hemlock seedlings established on logs and forest floor

were also sampled as controls prior to the injections to determine  $^{13}\text{C}$  natural abundance for comparison.

Pulse labelling of diluted  $^{13}\text{C}$ -glucose, is a novel methodology that was employed for this research. Most of the experiments done in this area of ecology, utilize  $^{13}\text{CO}_2$  (Bingham & Simard, 2012; Deslippe & Simard, 2011; Francis & Read, 1984; Pickles et al., 2017; Simard et al., 1997; Teste & Simard, 2008) or other soluble carbohydrates (Churchland, 2012).



Figure 6. Mature western hemlock with pipe glued to the tree phloem for pulse-labeling.

### **Statistical analysis**

All statistical analyses were performed in R 3.3.3 (R core team, 2017). Linear models were used to examine the effect of CWD decay classes and forest floor on seedlings isotopic

signatures, carbon and nitrogen estimates, and water content of the corresponding substrate. Linear mixed effect models were used for  $^{13}\text{C}$  enrichment analysis. Generalized linear models were used for natural established hemlock emergence and survivorship. For ANOVAs, pair-wise comparisons among levels were estimated through differences in least square means (lsmeans package), with Tukey's adjustment when GLM was used, and "false discovery rate" (FDR) adjustment (Benjamini & Hochberg, 1995; Lenth, 2016), when linear and linear mixed effect models were used. Equal residuals variance assumption, was visually met for every analysis. Normality assumption was tested and accepted in the corresponding models, with a Shapiro-wilk test. Significance level in this thesis was set *a priori* for all statistical test as  $p < 0.05$ .  $p < 0.1$  was considered to be of interest for further research.

### *Survivorship experiment*

Due to low establishment and survival rates, some of the decay classes were combined to increase the power of the statistical tests. The combining criteria focused on breakdown of the woody substrate on which hemlock seedlings were growing, instead of other CWD morphology aspects (B.C. Ministry of Forests and Range & B.C. Ministry of Environment, 2010) (Figure 3). Classes with similar wood structure were combined into four new "wood breakdown" classes as follows: decay class I and II were grouped into the "Low Breakdown"; decay class III and IV into "Medium Breakdown"; and class V into "High Breakdown"; and forest floor remained simply as "Forest floor". The descriptions and equivalences of the new classes compared to the original conventional classes are shown in Table 2. Additionally, as a further test, all CWD decay levels were nested into one class named "nurse-log" in order to contrast with forest microsites.

Table 2. Description of methods used in the field for identifying different wood breakdown level where western hemlock regeneration establishes.

Wood breakdown (new classes)	Main features for classification	CWD decay classes + forest floor (initial classes)
Low breakdown (LB)	No superficial wood falling apart or cracks. Intact outer log. Knife blade penetrates 1 or 2 cm into the wood. Occasionally with moss colonization and tree seedlings.	1 and 2
Medium breakdown (MB)	Irregular surface with cracks. Looser wood that can be easily removed using fingers. 10 cm knife blade penetrates from half to completely through with little effort. Original log shape still distinguishable.	3 and 4
High breakdown (HB)	Collapsed log, merged with forest floor. Highly irregular and very loose surface. Knife blade penetrates completely with no effort.	5
Forest floor (ff)	No distinguishable log. High content of litter. Often buried wood found. No resistance to knife blade.	forest floor

To estimate the impact of wood breakdown on naturally established seedlings, emergence and survival, generalized linear models (GLM) were used, with a negative binomial distribution and a logit link function (Mass package; Venables & Ripley, 2002). Although germination was relatively good, there were many experimental units where the count of germinants was zero. Thus, the distribution of the residuals was non-normal and GLM therefore appropriate. The negative binomial fits the data best because first, the dependent variable is a count rather than a continuous real number, and second, the data was over-dispersed. To test over-dispersion, a likely ratio test was done between the model with negative binomial distribution against the same

model run as Poisson. Negative binomial regression model is a proposed modification from Poisson (Greene, 1994; King, 1989).

GLM:

(eq. 2) *Naturally established* ~ *Wood breakdown*

(eq. 3) *Emergence* ~ *Wood breakdown*

(eq. 4) *Survivorship* ~ *Wood breakdown*

Substrate water content, moss cover and log height were independently tested as potential covariates for the above models (eq. 2; eq. 3; eq. 4) but none of them improved the explanatory power of the models and were removed.

### *<sup>13</sup>C transfer*

For examining isotope enrichment by substrate treatment, linear mixed effects (LME) models were constructed using the lme4 package (Bates *et al.*, 2015). <sup>13</sup>C enrichment was tested by comparing  $\delta^{13}\text{C}$  of control seedlings with pulsed isotope content of treatment seedlings after pulse-labeling. In all the models, the variable “Plots” (8 pulse labelled trees) was used as random effect. The CWD and forest floor seedlings were analyzed separately, since the controls to test enrichment were different. For seedling enrichment on logs, the control seedlings were the ones established on the lifted log portions (eq. 5). For seedlings enrichment on forest floor, the control seedlings were the ones harvested from forest floor prior to pulse labeling (eq. 6).

(eq. 5)  $\delta^{13}\text{C}_{\text{logs}} \sim \text{Log isolation, random} = 1|\text{Plots}$

(eq. 6)  $\delta^{13}\text{C}_{\text{ff}} \sim \text{Pulse label, random} = 1|\text{Plots}$

Nevertheless, forest floor and log seedling were later analyzed together when delta ( $\delta^{13}\text{C}$  enriched -  $\delta^{13}\text{C}$  control labelling) was used as the dependent variable. Time (day 9 and 15); distances from mother tree; mother tree size (DBH) were also used as predictors in this same model (eq. 7).

$$\text{(eq. 7) } \delta^{13}\text{C delta} \sim \text{Microsite} + \text{Time} + \text{Distance} + \text{DBH} + \text{Microsite*Time} + \text{Microsite*DBH, random} = 1|\text{Plots}$$

Finally, the data was decomposed by plots, and linear models were run for each mother-tree. Seedlings on CWD and forest floor were analyzed separately.

## 2.3 Results

### *Observational data*

Natural abundance of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  in naturally regenerating western hemlock seedlings differed between decaying logs and forest floor (Table 3). Total nitrogen and C:N ratio, on the contrary, did not vary among substrates (Table 3). Both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were generally more depleted in the advanced decay classes (MB and HB), compared to the forest floor and low breakdown decay classes (Figure 7). In this test, the lowest breakdown class included seedlings only from decay class 2 since seedlings were absent from decay class 1.

Water content was lower in the lowest compared to the greater decay classes (medium, high breakdown, and forest floor) (Table 3) (Figure 7) ( $P < 0.0001$ ).

Table 3. ANOVA results for the effect of wood breakdown and forest floor, on seedling  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  natural abundance; estimated nitrogen %, estimated carbon-nitrogen ratio and water content from the respective substrates ( $p < 0.05$ ).

	$R^2$	F	<i>df</i>	p-value
$\delta^{15}\text{N}$	0.244	4.5191	3	<b>0.0078</b>
$\delta^{13}\text{C}$	0.2646	5.0383	3	<b>0.0045</b>
Est. N	0.0569	0.8445	3	0.4773
Est. C:N	0.0381	0.5544	3	0.648
Water Content (%)	0.5614	40.957	3	<b>0.0001</b>

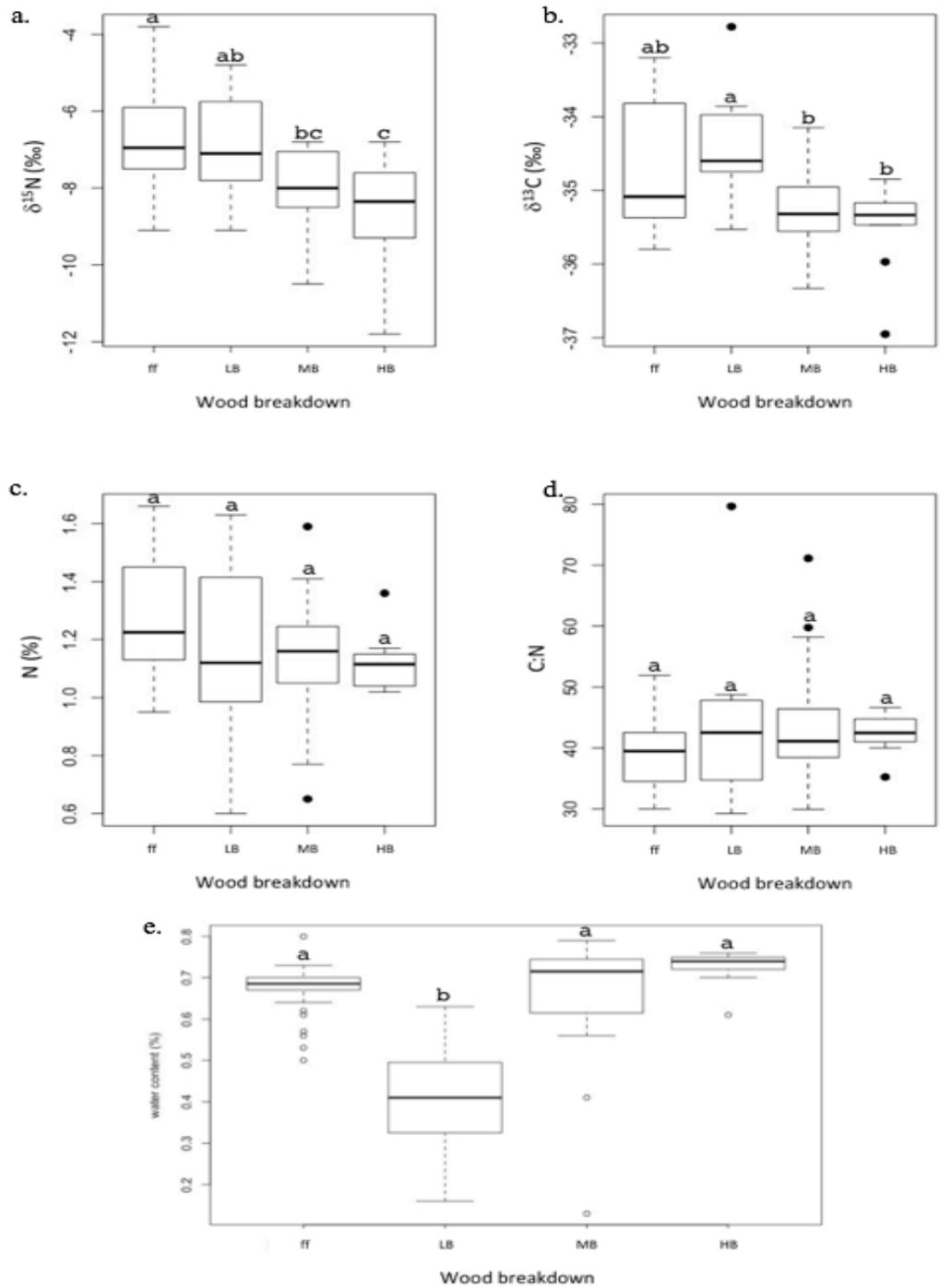


Figure 7. Box and whisker plots of  $\delta^{15}\text{N}$  (a),  $\delta^{13}\text{C}$  (b) natural abundance, estimated nitrogen (%) (c), and carbon-nitrogen ratio (d) of seedlings established on CWD with different decay classes and forest floor, plus water content (%) (e) of the respective substrates. The dark line represents the median value, the box indicates the upper and lower quartile of the spread of the values (interquartile range), and the whiskers indicate the extent of the extreme values of the data. Dark circles represent potential outliers, denoted by a value outside 1.5x the interquartile range. Different lower-case letters indicate averages that have a significant difference.

### *Survivorship experiment*

Naturally regenerated hemlock seedlings were most abundant on logs with advanced levels of decay. The greatest number of seedlings occurred in the 'medium breakdown class' (decay classes 3 and 4), but the numbers in the highest class (class 5) were not significantly lower (Figure 8) (Table 5). Forest floor seedlings were not included in this analysis because they were insufficient in numbers or were growing in buried rotten wood.

Of the 5000 seeds sown in the survivorship experiment, 473 seedlings germinated for an overall emergence rate of 9.46% (Table 4). There were no significant differences in emergence among substrate decay classes (Table 5) (Figure 8), however, the total emergence on logs (11.92%) was greater than on forest floor (7.00%) (Table 5).

Survival on the other hand, was highly affected by decay class as well as forest microsite (Table 5). The rank order of survival by decay class and forest floor was: HB > MB > LB > ff. Survival on the HB and MB classes were each significantly greater than on the forest floor (Figure 8).

Table 4. Emergence (August 2nd) and survival (October 2nd) count (n°); percentage (%); and mean and standard deviation for the effects of five decay class and forest floor (ff) (initial classes) on western hemlock germinants.

Classes	Emergence (August 2 <sup>nd</sup> )			Survival (October 15 <sup>th</sup> )		
	n°	%	Mean±SD	n°	%	Mean±SD
<b>1</b>	48	9.60%	4.80±6.21	0	0.00%	0
<b>2</b>	58	11.60%	5.80±6.86	12	20.69%	1.20±1.69
<b>3</b>	48	9.60%	4.80±7.66	14	29.17%	1.40±1.65
<b>4</b>	69	13.80%	6.90±9.00	14	20.29%	1.40±1.65
<b>5</b>	75	15.00%	7.50±7.82	18	24.00%	1.80±2.25
<b>ff</b>	175	7.00%	3.50±5.22	17	9.71%	0.34±0.77
<b>Total</b>	473	9.46%		75	15.86%	

Table 5. Negative binomial generalize linear models results for: 1 the effect of wood breakdown on naturally established hemlock; 2 and 4 The effects of forest microsites (ff/nurse-log) on the number of emerging and surviving seedlings respectively; 3 and 5 the effect of wood breakdown and forest floor on the number of emerging and surviving seedlings respectively. ( $p < 0.05$ ).

		$\chi^2$	<i>df</i>	p-value
1. Natural seedlings	Wood breakdown	15.7513	2	<b>0.0004</b>
2. Emergence	ff/nurse-log	4.7048	1	<b>0.0301</b>
3. Emergence	Wood breakdown	5.3115	3	0.1504
4. Survival	ff/nurse-log	8.6266	1	<b>0.0033</b>
5. Survival	Wood breakdown	11.9461	3	<b>0.0076</b>

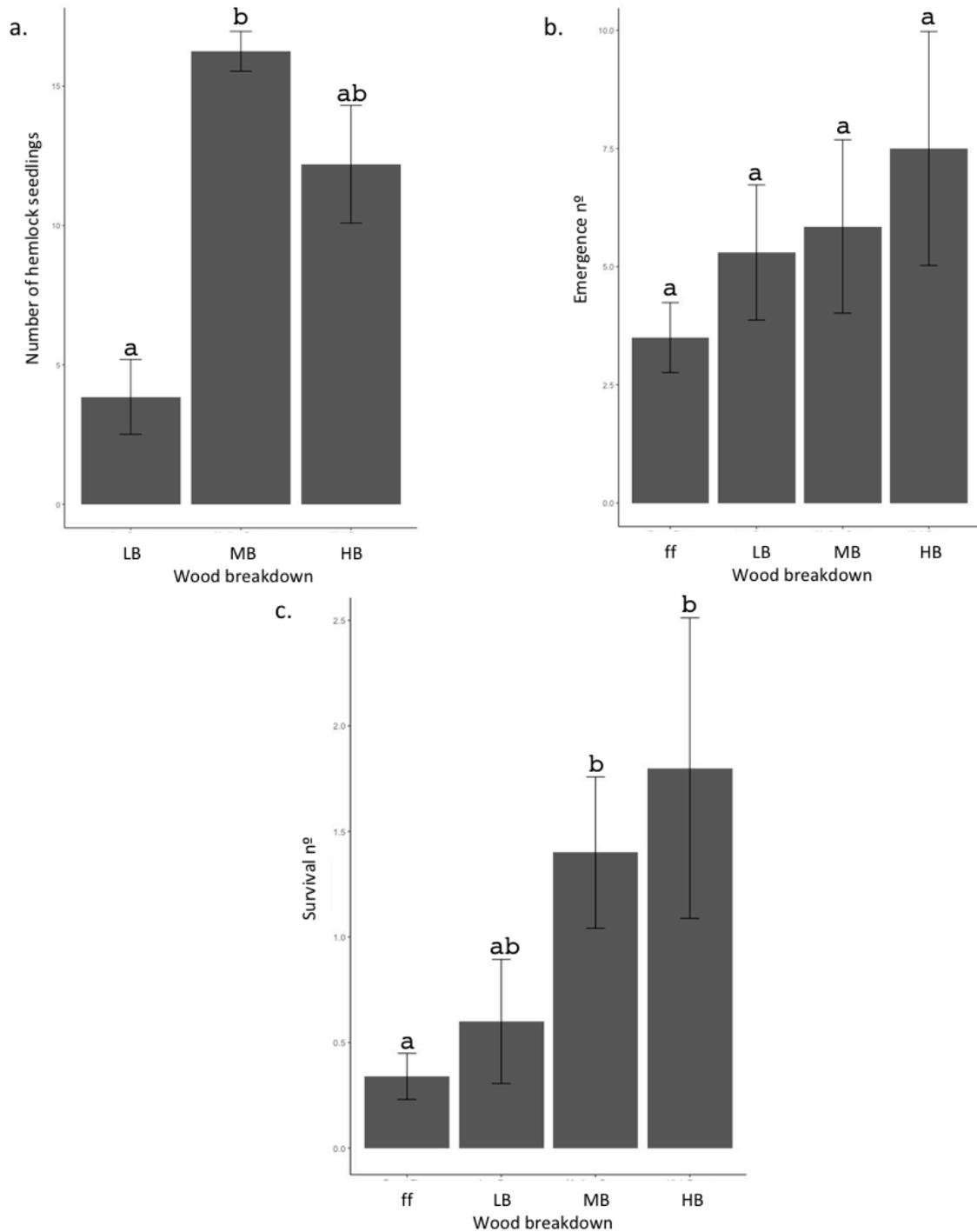


Figure 8. Average count of: natural established western hemlock (a); seedling emergence (b); seedling survival (c) by decay classes and forest floor. Low breakdown (LB); medium breakdown (MB); low breakdown (LB) and forest floor (ff). Bars represent standard error of the mean. Different lower-case letters indicate averages that have a significant difference.

### *Pulse labelling experiment*

No significant  $^{13}\text{C}$  enrichment occurred either 9 or 15 days post-labeling among seedlings growing on logs isolated from the forest floor (Table 6). However, seedlings established on intact CWD and forest floor were significantly enriched (Table 6), and those on CWD had higher delta  $\delta^{13}\text{C}$  delta values than seedlings growing on the forest floor (Table7). For seedlings growing on logs isolated from forest floor, mean  $\delta^{13}\text{C}$  and SE was  $-34.953 \pm 0.155\%$  (n= 25) versus  $-33.899 \pm 0.128\%$  (n=63) in seedlings on intact logs.  $^{13}\text{C}$  enrichment of seedling on forest floor was less noteworthy than seedlings on CWD, but still significant (Table 6) (Figure 9). Means and SE's from before and after labelling were the following:  $-34.79448 \pm 0.288$ , n=10 and  $-34.01844 \pm 0.216$ , n=33.

Table 6. ANOVA results and marginal and conditional  $R^2$  of general linear mixed models on seedling  $^{13}\text{C}$  enrichment. Plots are the random factor. 1: consistency on  $\delta^{13}\text{C}$  between control and baseline seedlings. 2 and 3: differences in seedlings  $\delta^{13}\text{C}$  between their respective controls and pulse labelling treatment on logs and forest floor (ff).

LME models	Marginal $R^2$	Conditional $R^2$	n	$\chi^2$	df	p-values
1. Control/Baseline (logs)	0.061	0.061	25	1.512	1	0.219
2. Enrichment in Logs	0.195	0.454	88	36.383	3	<b>0.0001</b>
3. Enrichment in forest floor	0.102	0.358	43	5.934	1	<b>0.015</b>

For both, CWD and forest floor seedlings, enrichment strongly varied among replicated plots, validating the choice of assigning plots as random effect in the models (Figure 9).

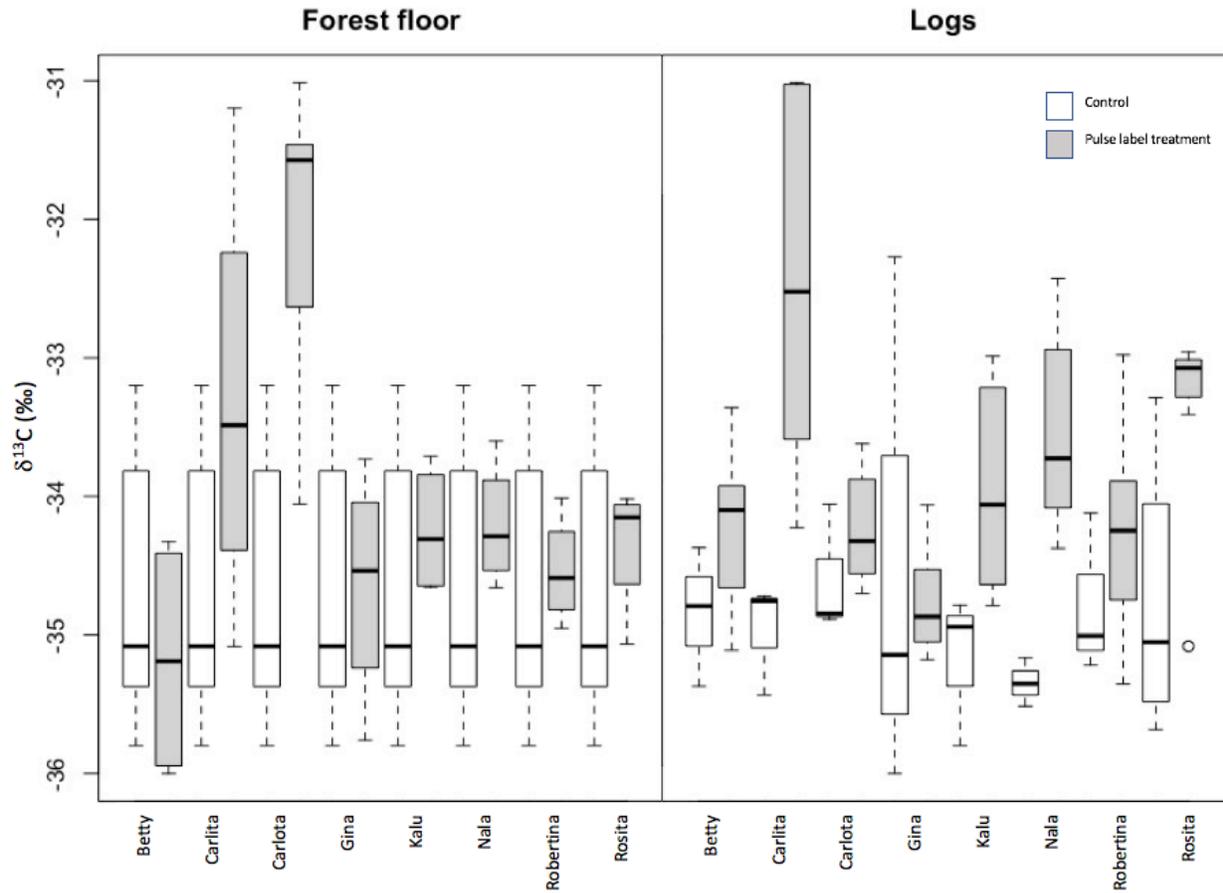


Figure 9. Variation in seedlings  $\delta^{13}\text{C}$  due to pulse labelling, by plots, on forest floor and logs. White bars represent seedlings harvested from their respective controls. Shaded bars represent seedlings exposed to pulse label treatment. The dark line represents the median value, the box indicates the upper and lower quartile of the spread of the values (interquartile range), and the whiskers indicate the extent of the extreme values of the data. Dark circles represent potential outliers, denoted by a value outside 1.5x the interquartile range.

The interaction between time since labelling and microsite ( $p=0.052$ ) was only significant at the  $\alpha=0.1$  level but not 0.05. The  $\delta^{13}\text{C}$  values tended to decline from day 9 to 15 among seedlings on forest floor, but increase among seedlings on logs (Table 7) (Figure 10).

The interaction effect between “mother tree” size (DBH) and microsite was also significant for seedling enrichment. The bigger the tree, greater the enrichment of seedlings,

particularly for those growing on CWD compared with forest floor (Table 7) (Figure 10).

Seedling enrichment was not significantly explained by distance from mother-trees (Table 7) (Figure 10).

Table 7. ANOVA result of general linear mixed model of change in seedling  $\delta^{13}\text{C}$ .

	Chisq	Df	p-values
(Intercept)	1.814	1	0.178
Distance	0.207	1	0.649
DBH	0.731	1	0.393
Time	3.282	1	0.07
Microsite	15.001	1	<b>0.0001</b>
Time*microsite	3.784	1	<b>0.052</b>
DBH*microsite	16.215	1	<b>0.0001</b>

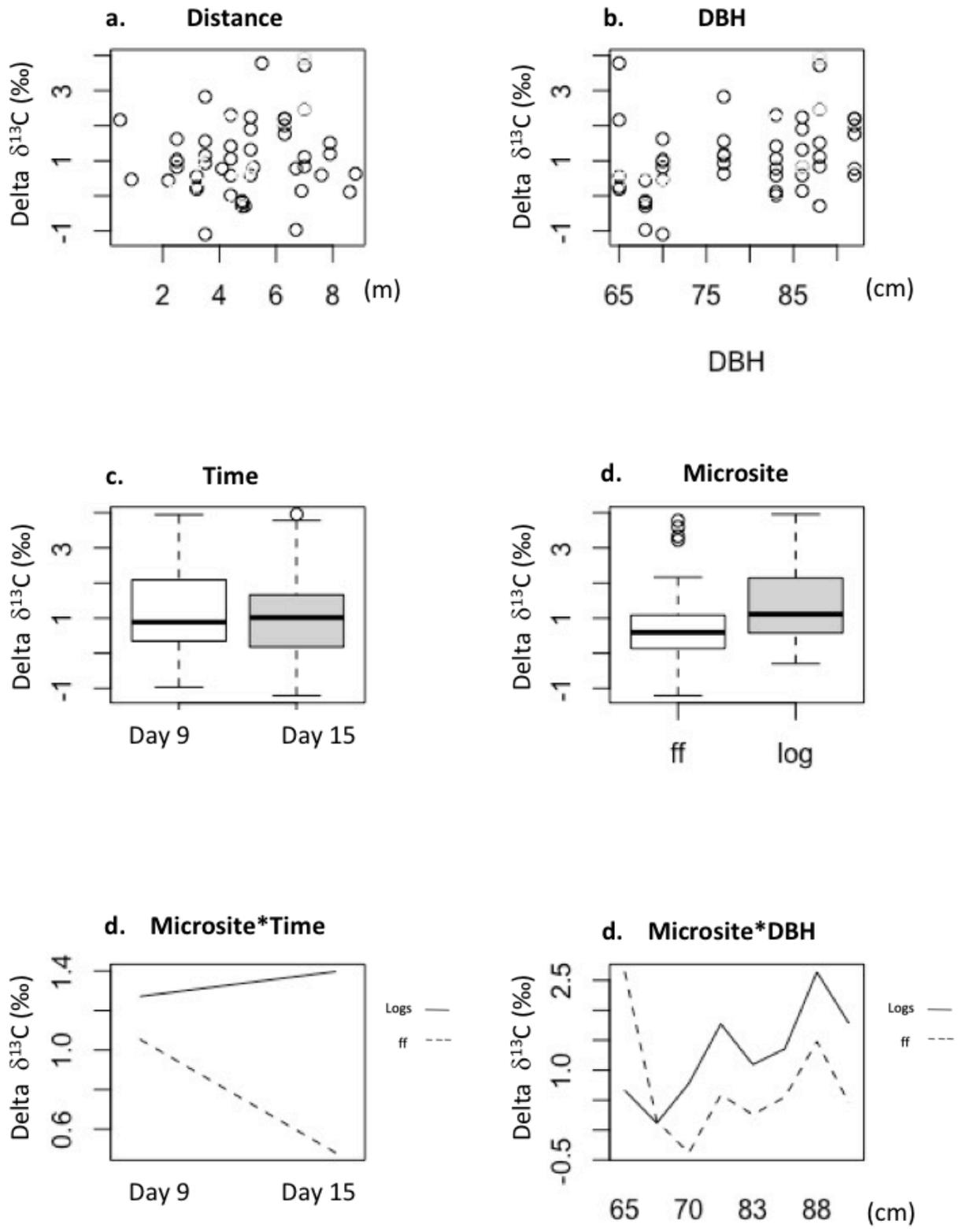


Figure 10. General linear mixed model of change in seedling  $\delta^{13}\text{C}$ . Y-axes correspond to the differences between seedlings exposed to pulse label treatment and their respective controls.

When examined separately, labeling of mother trees in four out of the eight cases resulted in significant enrichment of seedlings established on CWD. The  $\delta^{13}\text{C}$  enrichment of forest floor seedlings also tended to increase after labelling, but enrichment was significant in only two out of the eight plots (Figure 11). Overall, in all cases except “Betty’s” forest floor seedlings,  $\delta^{13}\text{C}$  tended to increase with labelling (Figure 11).

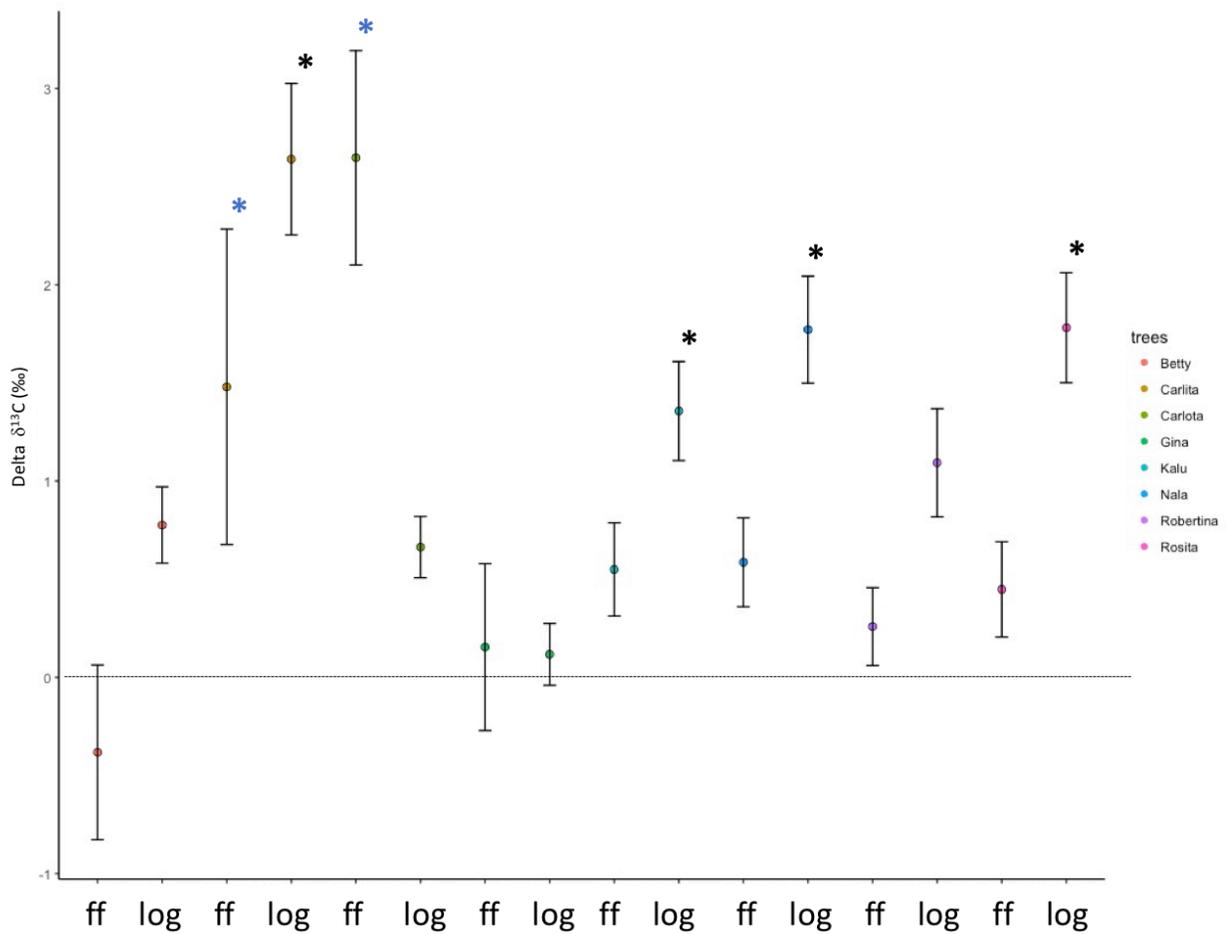


Figure 11. Observational data showing means and SE of forest floor (ff) and logs seedlings change in  $\delta^{13}\text{C}$ . Y-axis correspond to the differences between seedlings exposed to pulse label treatment and their respective controls. Blue and black stars represent significant enrichment in forest floor and logs seedlings respectively.

## 2.4 Discussion

The main objective of this study was to determine whether, in an old-growth forest, *Tsuga heterophylla* (Raf.) Sarg. regeneration on CWD is linked to the mycorrhizal network. The following hypotheses were tested: (1) western hemlock recruitment is mediated by presence of CWD and its level of decay; (2) western hemlock establishing on CWD receives carbon transferred from mature trees through an MN; and (3) the size of mature donor trees is positively correlated with the amount of carbon transferred to seedlings.

### *Western hemlock recruitment*

Successful establishment of western hemlock was highly mediated by the presence of CWD, with 77.3% of seedlings that survived the summer occurring on CWD (Table 4). Within CWD, advance wood breakdown of logs was a determinant factor for western hemlock success, agreeing with the first hypothesis. Previous research also corroborates these findings (Christy & Mack, 1984; Gray & Spies, 1997; Harmon & Franklin, 1989; Minore, 1972). The results of this study support previous research showing that the intimate seedling-log association is decisive in closed canopy forests (Gray & Spies, 1997).

In the climax old-growth forest of our experiment, we clearly observed western hemlock's high shade tolerance. Seedling emergence was high and surviving seedlings were abundant in many different microsites (forest floor, CWD, rock boulders, trees bark, fungi polypores). Gray & Spies (1997) suggest that western hemlock is able to establish under a dense closed canopy as long as there is a sufficient heterogeneity of microsites present in the forest. There were no significant differences in seedling emergence across wood breakdown class and

forest floor (Table 5). However as mentioned above, later survivorship was strongly favored by advanced wood decay.

Literature describes hemlock as a small seeded (1.7 mg)/shade tolerant conifer that produces at least some seeds every year (Fowells, 1965; Schopmeyer, 1974). A large annual seed rain and high emergence rates (as showed in our results), increases the probability that hemlock seeds fall and root on at least some favorable microsites. Contrarily, the co-occurring shade tolerant tree species *Abies amabilis*, has a larger seed (41 mg) that is shed in mast years, usually every 3-5 years (Schopmeyer, 1974). *Abies amabilis* is a late successional species like hemlock, however it commonly establishes on the forest floor in closed-canopy forest (Mori et al., 2004; Wilson, 1991). This suggests that seed size is an important determinant of seedling survival on the forest floor. Fukasawa (2012) and Lusk & Kelly (2003), observed the same pattern of small and large seeded tree species establishment in a Japanese and Chilean forest respectively. Nurse logs are an important seed bed for trees in many different forest around the world, but particularly for small/light seeded pioneer species (Grime & Jeffrey, 1965; Lawton & Putz, 1988; Veblen, 1985; Brokaw, 1985). Thus, hemlock has certain pioneer attributes for a late successional species; we suggest that under a closed canopy, the requirement of those pioneer attributes might be fulfilled by the features found in CWD with advance wood breakdown. Gray & Spies (1997) made a similar observation, pointing out that hemlock seems to have a small seed for a shade tolerant tree.

Lower litter accumulation on log surfaces compared with forest floor might be an important factor explaining why hemlock's small seed survives more on CWD than forest floor (Haig et al., 1941; personal observations). Small seeded trees with lower carbohydrate reserves probably need to root faster into more favorable environments, such as highly decayed

substrates; otherwise they are prone to desiccation (Gray & Spies, 1997). Additionally, highly decayed CWD has better water holding substrate than soil (Harmon et al., 1986; Walker & Jones, 2013), and western hemlock is particularly sensitive to substrate moisture (Williamson & Ruth, 1976). In fact, Minore (1972), in a greenhouse experiment, found no differences in seedling survival between wood and litter, when moisture levels were kept high, implying that western hemlock was sensitive to substrate moisture. However, in our research despite the marked survival differences among substrates (Figure 8c), we found no significant differences in water content between substrates with advanced wood breakdown and forest floor (Figure 7e). This probably explains why water content did not improve the explanatory power of the survivorship model (eq. 4). Based on this, we suggest that water does not determine hemlock's better survivorship in these forest ecosystems (at least in the particular year and in those particular substrates of this study). It is important to highlight that substrates were sampled for water content analysis only once during the summer; therefore, it is possible that water content from advanced wood breakdown and forest floor could have differed if samples were taken periodically during the dry season.

The natural  $\delta^{13}\text{C}$  signatures of hemlock seedlings established on different treatments support our substrate water content analysis.  $\delta^{13}\text{C}$  was not significantly different among MB, HB and forest floor (Figure 7b). Correspondingly, logs with the lowest decay had the lowest water content of all substrates, and thus the higher  $\delta^{13}\text{C}$  values in seedlings (significantly higher than MB and HB (Figure 7b), suggesting higher water stress of seedling under those conditions (Dawson et al., 2002; Farquhar, et al., 1989).

Within the crowded understory, CWD are elevated microsites with more access to light and less plant competition (Harmon et al., 1986). Harmon & Franklin (1989) conducted an

exceptional experiment to unveil the tight hemlock-log association. They examined pathogens, predation, plant competition and standing water in logs and soil, and concluded that competition with herbs and mosses accounted for lower western hemlock establishment on the forest floor. Apparently moss mats hinder hemlock seed penetration considerably. These might explain germination variation for some forests like the *Picea sitchensis*–*Tsuga heterophylla* forests, but on our sites, most logs and forest floor were equally and completely covered by moss (personal observations). It could be possible, however, that moss species differentially affect seedling establishment. For example, the moss species growing on CWD may be less aggressive than moss species on the forest floor. These observations suggest a potential facilitative interaction between moss and hemlock on wood, but a more competitive interaction in the forest floor. This could be a subject for further research.

One explanation for increased seedling establishment on decayed wood, which we can explore with our pending fungal DNA results, is lower abundance of pathogens compared with the forest floor. Harmon & Franklin (1989) did not find consistent increases in survival when seedlings were grown on sterilized soils, but other authors have found that logs provided a pathogen-free space (O’hanlon-Manners & Kotanen, 2004). The CWD in forest is a highly active, combative and antagonistic medium for saprotrophic fungi; hence, pathogenic intruders might be efficiently controlled (Boddy, 1993, 2000). Additionally, logs are elevated microsites and often free from direct mature tree root competition (Tedersoo et al., 2008), reducing potential for asymmetric competition with mature trees (Grubb, 1977). Moreover, soil pathogens can be found more abundantly near conspecific adults (Dickie et al., 2002), suggesting that CWD seedlings may be freer of pathogens than seedlings growing on forest floor.

Tedersoo et al. (2008) suggest that tree seedlings typically establish on moderately decayed wood, and become less common with progressing decay, possibly due to development of root competition with mature trees. We observed this in our research; hemlock natural establishment tended to be more abundant in moderately decayed wood (MB) than other classes (Figure 8a). Christy & Mack's (1984) results align with our observations of most hemlock growing on logs of medium decay class. As wood decay proceeds, it probably comes to resemble forest floor, gaining substrate features that might be detrimental to western hemlock regeneration. On the other hand, this could also be explained by the potential competitive exclusion among hemlock seedlings on the same log. CWD of high decay classes has resided longer in the understory, with fewer and larger seedlings remaining due to intraspecific competition.

#### *Inter-plant carbon transfer*

Our results provide the first evidence for carbon transfer from mature trees to small seedlings established on CWD, supporting our second hypothesis. These findings indicate there exists an underground flux between western hemlock canopy trees and regeneration, whether it is growing on forest floor or decayed wood.

Moreover, our pulse-labeling result suggests that seedlings on CWD are linked to the mature trees via a mycorrhizal network. In support of this, more seedlings established on logs received carbon from mature trees than on forest floor (4/8 and 2/8 plots respectively). Also, the differences in  $\delta^{13}\text{C}$  (enriched sample – respective control), was greater among seedlings on logs than forest floor.

Mature trees allocate significant amounts of photosynthates to their roots, and there are different possible pathways through which injected  $^{13}\text{C}$ -glucose could be transferred to seedlings: 1) Labelled glucose released through fine root surfaces through exudation, and flowing indirectly to seedlings through the soil and wood matrices (Mencuccini & Hölttä, 2010); or 2) Labelled glucose was delivered to its fungal symbionts (Jones et al., 2004; Kaiser et al., 2014) and then directly transferred through the mycorrhizal network (Francis & Read, 1984; Selosse et al., 2006; Simard & Durall, 2004). Kaiser et al. (2014) point out that most released recent photosynthate leaves the plant via mycorrhiza. The MN provides direct pathways for compounds to travel through soil, compartmentalizing and sustaining a more efficient and effective carbon transfer from mature-donor trees to regenerating seedlings (Finlay, 2008; Simard et al., 2012). Our results indicate that carbon transferred to seedlings on logs was likely in the form of glucose through the mycorrhizal network, and possibly via fungal species forming long distance exploration types (Agerer, 2001).

Seedlings established on the forest floor were spatially located in-between donor trees and the logs, and thus closer to the source of enriched carbon. Consequently, we expected to find clearer signals of carbon transfer in those seedlings than in logs, but we did not. Interplant carbon transfer also occurred to seedlings growing in the forest floor, but to a lesser extent. Furthermore, by day 15 after pulse labeling, the enrichment in forest floor seedlings was smaller than on day 9; by contrast, signals in seedlings on logs increased from day 9 to day 15 (Figure 10e). Mature tree roots and understory plants crowd the forest floor, possibly competing for the transferred carbon and diluting the signal. Similar experiments (Deslippe & Simard, 2011; Teste et al., 2009) utilizing mesh bags to control access to MN, found increased inter-plant carbon transfer when seedlings were able to form networks; however, seedlings isolated from MN also received

carbon from neighbors, but to a lesser extent. The weaker signal was attributed to carbon moving through soil solution or discontinuous mycelial pathways. This could have happened in the forest floor seedlings of our experiment, where  $^{13}\text{C}$  enriched compounds were more rapidly turned over by soil matrix microorganism than EM fungi (Högberg et al., 2007). This could explain the lower carbon uptake among seedlings on the forest floor than logs on day 15.

To test assumptions of different possible nutrient transfer pathways to seedlings on the forest floor compared to logs, we compared  $\delta^{15}\text{N}$  natural abundance of seedlings established on different substrates. We observed greater  $\delta^{15}\text{N}$  depletion in seedlings on MB and HB logs and we associated this with a more intimate EM association in those seedlings (more abundant fungal biomass in roots systems and higher fungal partitioning of  $^{15}\text{N}$  on transferred N to host plant). Forest floor seedlings on the other hand, were significantly more enriched, suggesting that more nitrogen may have been taken directly from roots, without as much EM mediation (resembling to a non-mycorrhizal  $\delta^{15}\text{N}$  signature) (Hobbie & Hobbie, 2006; Hobbie & Colpaert, 2003; Hobbie et al., 2000). Depleted and enriched  $\delta^{15}\text{N}$  signatures in plants can be used in limited N ecosystems as estimators of fungal biomass associated with host. In this case we could suggest that  $\delta^{15}\text{N}$  depleted seedlings acquired more of their N through mycobionts. Seedlings with better established EM associations, as we found on medium and advanced wood decay classes, might be more capable of tapping into the MN of nearby trees.

In mature forests, decayed logs often support an EM fungal community that is distinct from those in organic or mineral soils (Goodman & Trofymow, 1998; Tedersoo et al., 2003). Kropp & Trappe (1982) propose that if there are any specific fungi for hemlock, rotten wood seems the most likely substrate for it to occur in. We suggest that there might be a more specific

mycobionts for hemlock in advanced wood decay than forest floor. We will explore this hypothesis once our molecular data is obtained.

Another possible explanation for higher transfer and natural abundance isotopic signatures in decayed wood is that, since logs are poorer substrate than forest floor (Harmon et al., 1986; Keenan et al., 1993; Marx & Walters, 2006), seedlings established on logs might be stronger sinks within the MN (Simard et al., 2012). The higher nutritional stress in logs than forest floor suggest a greater source-sink gradient and thus a greater facilitation role of MN on those substrates (Bingham & Simard, 2012).

In mycorrhizal networks, mature canopy trees are the major photosynthate producers; therefore, carbon transfer could have occurred along a strong source-sink gradient from source trees towards regeneration sinks (Bingham & Simard, 2012; Kennedy et al., 2003; Teste et al., 2009). In our model of seedling  $\delta^{13}\text{C}$ , we found a significant interaction between tree size (DBH) and microsite (Table 7), supporting our third hypothesis. This interaction is mostly accounted for by the increase in carbon transferred to seedlings on logs with increasing DBH of the donor tree (Figure 10e). The bigger the tree, the more resource accessibility, and greater amount of carbon available to potentially transfer to seedlings. For example, greater light availability increases plant C surpluses, or “luxury of consumption”, with greater potential allocation to MNs (Turner et al., 2009). Since, carbon transferred varied with donor size, we assume that there was a significant “source strength” acting in our forest. Our results contrast with Teste et al. (2009), who found carbon transferred to seedlings decreased with increasing donor size. However, Teste et al. (2009) was examining sapling rather than mature tree donors, and where source-sink gradients would have been much weaker than in our study. If more C is transferred to seedlings

on CWD when a nearby networked tree is larger, those seedlings might be particularly advantaged. Further research is needed to explore this association.

In support of our findings, there is considerable evidence that MNs affect seedling establishment (Nara, 2015), survival (McGuire, 2007; Teste et al., 2009) and growth (Booth, 2004). Once germinants tap into a much larger resource pool than what they could access on their own, greater performance is expected (Bingham & Simard, 2012; Teste et al., 2009). Hemlock regeneration established on CWD is often growing under deep shade and in an extremely poor substrate, increasing the importance of EM and MNs on performance of those seedlings. Under such harsh conditions, the symmetry of the mycorrhizal symbiosis is questionable, pointing even further to the crucial role of mature tree MNs for successful establishment. If the network developed in CWD is supported by the earlier established mature hemlocks, then seedlings may be able to allocate fewer resources to their direct mycobiont while gaining from the association (Newman, 1988). Poznanovic et al., (2015), proposed something similar. They assessed an eastern hemlock EM community established in wood under a closed canopy, suggesting that persistence of eastern hemlock seedlings in the highly shaded understory may have been enhanced by carbohydrate subsidy to the mycorrhizal network from canopy trees.

Our finding that more  $^{13}\text{C}$  was transferred to seedlings where logs were intact than isolated suggests there existed a MN between seedlings established on CWD and mature trees, and that this MN increased the absorptive network of hyphae of the seedlings with minimal investment of carbon (Finlay & Söderström, 1992).

Tedersoo et al. (2008) suggest that the success of seedlings growing in elevated dead wood may result from both carbon transfer from MN, and the lack of direct root competition in CWD. Thus, hemlock regeneration in wood might be obtaining the benefits of MN, but not the

competitive interaction with mature tree roots and understory plants growing on the forest floor (Booth & Hoeksema, 2009; Coomes & Grubb, 2000; Harmon et al., 1986; Simard et al., 2012; Teste & Simard, 2008). This perfectly aligns with our results: 1) the preference of western hemlock to establish on advance wood breakdown CWD rather than forest floor; 2) the more intimate EM association in logs than forest floor, supported by  $\delta^{15}\text{N}$  natural abundance; and 3) the stronger and longer inter-plant C transfer in seedlings on CWD than in forest floor.

## 2.5 Conclusions

In conclusion, we found evidence that western hemlock regeneration established on CWD is linked to mature conspecific trees through a common MN. The amount of transferred carbon increased with tree size. These connections might facilitate hemlock establishment in logs, which adds valuable information to our understanding of the hemlock-log association and forest stand dynamics.

Successful establishment of western hemlock regeneration in old-growth climax forests requires the presence of logs in the understory with a medium to advanced decay level. Few germinants survived when seeds were sown in the forest floor. Furthermore, signals of inter-plant carbon transfer in this substrate were weaker and shorter than for seedlings on CWD. We conclude that seedlings on forest floor might also be linked to the MN, but to a lesser extent. They are also more susceptible to strong competition for resources with mature tree roots and other understory plants, compared to logs seedlings.

Nurse logs are the safest sites for western hemlock seedlings to establish. We predict that seedlings on logs will continue to grow, facilitated by the MN and by the absence of mature tree

roots, until their roots reach the forest floor and they become strong and well established seedlings.

Western hemlock complex demography is mediated by moderated to advanced decayed CWD and access to the mycorrhizal network.

### **Chapter 3: Conclusion**

Western hemlock is the climax tree species across its distribution range (Franklin & Dyrness, 1973). This must be because of its remarkable ability to regenerate in the understory. We observed high rates of seed emergence in a variety of substrates. However, it has been documented that western hemlock seedling survival can be quite sensitive to microsite heterogeneity under close canopy forests (Gray & Spies, 1997). The availability of proper seedbed is especially necessary for hemlock establishment, compared to other late successional co-occurring conifers (Mori et al., 2004; Wilson, 1991), as evidenced by hemlock's low survivorship on the forest floor. Thus, CWD with medium to advanced decay is required in an old growth forest for hemlock to succeed as climax species.

We conclude that decay wood gathers a number of attributes that are beneficial and crucial for western hemlock regeneration in old growth forests. In light of previous literature, CWD is a key component of the forest, and hemlock's success might be explained by: lower litter accumulation and plant competition, higher moisture content and sun radiation, and protection against herbivores and pathogens compared to the forest floor (Boddy, 1993; Dickie, 2002; Harmon & Franklin, 1989; Harvey et al., 1978; Long et al., 1998; O'hanlon-Manners & Kotanen, 2004; Walker & Jones, 2013). Additionally, we found that seedlings on medium and advanced decayed CWD showed evidence of being linked to old trees via a mycorrhizal network, and they received direct carbon transfer from mature canopy trees. Based on differences in carbon transfer evidence and stable isotope partitioning between seedlings established on logs and the forest floor, we propose there is a more intimate and less disrupted access to MNs among hemlock seedlings growing in CWD than in forest floor. This opens numerous research opportunities addressing the potential facilitative effect of MN in CWD

regeneration. Furthermore, the contribution of MN in the continuity of the climax forest is now well established in coastal BC.

### **3.1 Review of objectives**

The main objective of this study was to determine whether, in an old-growth forest, *Tsuga heterophylla* (Raf.) Sarg. regeneration on CWD is linked to the mycorrhizal network. The following hypotheses were tested: (1) western hemlock recruitment is mediated by presence of CWD and its level of decay; (2) western hemlock establishing on CWD receives carbon transferred from mature trees through an MN; and (3) the size of mature donor trees is positively correlated with the amount of carbon transferred to seedlings.

### **3.2 Summary of main findings**

#### *Hypothesis 1: Hemlock on CWD*

We found that western hemlock emergence and survivorship was higher on CWD than the forest floor. Furthermore, logs with medium to advanced wood decay had higher recruitment. For these reasons, we accept our first hypothesis that successful recruitment of western hemlock seedlings is mediated by the presence of CWD and its level of decay. These results verify findings of previous studies (Christy et al., 1982; Christy & Mack, 1984; Durall et al., 1999; Gray & Spies, 1997; Harmon & Franklin, 1989; Kropp, 1982b; Kropp & Trappe, 1982).

#### *Hypothesis 2: Inter-plant carbon transfer*

We found significant signals of carbon transferred from mature trees to regenerating seedlings established on CWD.  $^{13}\text{C}$  enrichment was found on day 9 after pulse labelling and then higher

enrichment on day 15. Furthermore, carbon transfer occurred solely where connections between mature trees and logs were kept intact, suggesting transfer was mediated by the mycorrhizal network. We thus accept our second and most novel hypothesis. These results are the first evidence of any type of resource transfer from mature trees to seedlings established on logs. Furthermore, it represents the first evidence of carbon transfer among hemlock trees.

*Hypothesis 3: Source strength in inter-plant carbon transfer*

We found that  $^{13}\text{C}$  enrichment in seedlings increased with mature donor tree size (DBH). This trend was clearer in seedlings established on CWD than in forest floor; therefore the significance of these results was found in the interaction between tree size and seedling microsite. These results suggest source strength of the donor affected carbon transfer, leading us to accept our third hypothesis.

### **3.3 Contributions to the field of study**

These findings significantly contribute to a better understanding of regeneration dynamics in old-growth western hemlock forests, and specifically the tight hemlock-log association and the contribution of the mycorrhizal network. The results indicate a direct belowground pathways from mature trees to seedlings on logs, and may inspire the re-examination of previous studies addressing factors that determine hemlock's preference for CWD on their establishment.

While we conclude positive effects of the MN on seedling survival, simultaneous negative effects of tree roots in the understory also occurs (Booth & Hoeksema, 2009). Thus, the

lack of root competition in CWD, and simultaneous facilitation by the MNs, offer an ideal scenario for studying simultaneous positive and negative interactions between mature trees and regeneration.

This research highlights the key role of mature trees in forest ecosystems, particularly their role in the MN as “hub-trees”, and their potential to facilitate regeneration. The larger the mother tree, the more carbon is potentially transferred to small seedlings.

### **3.4 Study limitations**

Unfortunately, molecular techniques for fungal community analysis were not included in this thesis due to time constraints. However, our ongoing study will incorporate it, providing us valuable information of niche partitioning of hemlock’s EM associations.

Hemlock seedlings established on forest floor were scarce, therefore unbalanced statistical analysis were made when forest floor and logs seedlings  $^{13}\text{C}$  enrichments were compared.

Likewise, sporocarp fructification was very low during field work, therefore we were not able to collect enough for a proper  $\delta^{15}\text{N}$  natural abundance analysis. This would have given us stronger proofs to estimate fungal transferred N to plant host.

Ideally the log isolation from our experimental design, should have been done years before pulse labeling. That way we could have been able to analyze stable isotopes and EM fungal communities associated with hemlock, and long-term effect of MN on regeneration established on CWD.

Finally, carbon transfer varied considerably through plots, and enrichment was not found in all of them. This gives us novel insights into the significant complexity involved in coastal forest mycorrhizal networks.

### **3.5 Applications**

CWD in hemlock regeneration dynamics is of such importance that the removal of this key component from forests could threaten hemlock's continuity in the northwest Pacific climax forest. Any type of forest management should consider the tight hemlock-log association.

Acknowledging that western hemlock regeneration established on CWD is linked to MNs and that big trees are carbon sources for seedlings, may also influence forest management. Therefore, it is crucial to motivate decision makers to maintain soil health in forest ecosystems, and to keep big trees.

### **3.6 Further research**

Further research is needed on the interaction between saprotrophic and EM fungi in wood where hemlock regenerates. There exists a solid literature on saprotrophic and mycorrhizal fungi interactions, some with contradictory results (positive and negative interactions) (Baar & Stanton, 2000; Fernandez & Kennedy, 2016; Gadgil & Gadgil, 1975; Gadgil & Gadgil, 1971; Hobbie & Horton, 2007; Leake et al., 2002; Leake et al., 2001; Lindahl, Stenlid, & Finlay, 2001; Lindahl et al., 1999; Mucha et al., 2008; Pereira et al., 2012; Shaw et al., 1995). Also, more attention is needed on the potential direct interaction between hemlock and decay wood fungi. There is evidence of wood decay fungi interacting with conifer seedlings by colonizing their

fine roots as mycobionts (Vasiliauskas et al., 2007). This possibility needs to be studied in western hemlock established on CWD. Additionally, further research is needed to test the enzymatic potential of EM fungi associated with hemlock in wood. A better understanding of how saprotrophic and symbiotic fungi interact in CWD where hemlock is established, might give us relevant knowledge for understanding better hemlock-log association and MN in coastal old-growth forest.

Similarly, the association between western hemlock regeneration and the moss community in different forest microsites needs to be explored. We observed distinct composition of moss species on wood decay classes and forest floor. Therefore, further research should study the potential facilitative interaction between moss and western hemlock on wood and the more competitive interaction in the forest floor.

Long-term research should explore: the effect and behavior of EM fungi in hemlock establishment on wood, the benefits seedlings gain in having access to the forest MN and the lack of competition with roots of mature trees, and finally, the specific role of old trees in the MN and in tree regeneration in the rainforests of the Pacific Northwest.

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