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博士論文

Effect of waterlogging on plant carbon isotope discrimination  
(湛水が植物炭素同位体分別に及ぼす影響)

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## Abstract

Soil moisture is a major factor controlling carbon isotope discrimination ( $\Delta^{13}\text{C}$ ), which has been demonstrated to decrease under dry conditions in many studies; however, few studies on  $\Delta^{13}\text{C}$  under waterlogging condition have been conducted. In this study, a pot experiment was conducted with *Larix gmelinii*, a major larch species in the east Siberian Taiga, to investigate the effect of waterlogging on  $\Delta^{13}\text{C}$  during photosynthesis. Assimilation rate and  $\Delta^{13}\text{C}_{\text{RD}}$  (instantaneous  $\Delta^{13}\text{C}$  calculated with Rayleigh Distillation equation) decreased drastically soon after waterlogging, followed by recovery in their values, which was caused by a change in stomatal conductance. Thereafter, assimilation rate decreased gradually, whereas  $\Delta^{13}\text{C}_{\text{RD}}$  decreased more gently. These results were thought to be caused by the decrease in both stomatal conductance and carboxylation. The results indicate that extreme wet events may cause a decrease in  $\Delta^{13}\text{C}$ , which is important information for detecting flooding events in the past using tree-ring isotope analyses and for studying impacts of flooding on plants in areas where waterlogging might occur.



## Chapter 1 Introduction

### 1.1. Plant or ecosystem $\delta^{13}\text{C}$ as a useful tool

The terrestrial ecosystem is thought to play an important role for the variation of  $\text{CO}_2$  concentration of annual and seasonal scale (Conway et al., 1994). The terrestrial ecosystems influence the  $\text{CO}_2$  in atmosphere mainly through the photosynthesis, respiration to assimilate and release  $\text{CO}_2$  and respiration from the soil, which release  $\text{CO}_2$  also. From 1950s, the carbon isotope ratio of atmosphere  $\text{CO}_2$  ( $\delta^{13}\text{C}$ ) began to be used for research, and it was developed well in 1990s. Combination study of carbon dioxide concentration and stable carbon isotope ratios of atmosphere to study the carbon cycle has caused people's interest (Canadell et al., 2000).

Plant foliar  $\delta^{13}\text{C}$  or carbon isotope discrimination ( $\Delta$ ) depends on the processes such as photosynthesis, stomatal conductance, and respiration, which respond to variable environmental conditions such as air humidity, temperature, light and salinity (Farquhar et al., 1989; Aranibar et al., 2006; Matsuo et al., 2013). Isotope signatures of plants are useful for ecological studies to understand and predict the potential impacts of climate change on trees and forest ecosystems. Saurer et al (2004) found a reduction of the stomatal conductance and an improvement of intrinsic water-use efficiency under climate change and increasing  $\text{CO}_2$  over the last century through study on carbon isotope ratio in tree rings of conifer trees in northern Eurasia. Elevated  $\text{CO}_2$  concentration levels increased  $^{13}\text{C}$ -derived water-use efficiency of *Pinus taeda* and *Populus sp.* significantly across three forest FACE (Free-Air Carbon dioxide Enrichment) sites (Battipaglia et al., 2013). Phloem  $\Delta^{13}\text{C}$  of Scots pine was assumed to

be concertedly influenced by  $G_s$  (canopy stomatal conductance) and photosynthetic photon flux density (PPFD) (Brandes et al., 2007). Increasing carbon isotope discrimination ( $\Delta$ ) derived from tree-ring  $\delta^{13}\text{C}$  of the deciduous and coniferous species over the 1992-2010 period was attributed to increasing annual and summer water availability identified at Harvard Forest and across the region (Belmecheri et al., 2014). Insufficient moisture during the previous autumn and the current growing season was the dominant climatic factor that controlled tree-ring carbon isotope discrimination in the semi-arid Xinglong Mountains (Wang et al., 2012). The stable isotope composition of respired  $\text{CO}_2$  is a useful ecosystem-scale tool to help study constraints to photosynthesis and acclimation of ecosystems to environmental stress (Ponton et al., 2006). Carbon isotope discrimination ( $\Delta$ ) has been suggested as an indirect selection tool for plant water use efficiency and yield potential in wheat (*Triticum aestivum* L.) (Xue et al., 2002). The non-destructive and time-integrating  $\delta^{13}\text{C}$  measurement is a useful tool in monitoring responses of *P. australis* to environmental conditions in saline wetlands (Choi et al., 2005).

On the other hand, the isotopic signal of  $\text{CO}_2$  exchanged between ecosystems and the atmosphere has been used for studies on the global carbon cycle, and the carbon isotope discrimination of terrestrial ecosystems is essential information for such studies (Tans et al., 1993; Fung et al., 1997; Canadell et al., 2000; Yakir and Sternberg, 2000). The atmospheric inversion models rely on estimates of carbon isotope discrimination and the  $\delta^{13}\text{C}$  of ecosystem respired  $\text{CO}_2$  (Tans et al., 1993; Fung et al., 1997). Estimates of terrestrial carbon isotope discrimination are useful to quantify the terrestrial carbon sink

(Aranibar et al., 2006). Ciais et al. (1995) put forward a model using  $\delta^{13}\text{C}$  of atmospheric  $\text{CO}_2$ , and reported that the terrestrial ecosystem of the northern hemisphere is an enormous carbon sink and it assimilated half of the  $\text{CO}_2$  released from the combustion of fossil fuel that consumed by human beings. A 3% overestimate of the global carbon isotope discrimination can translate into 20% underestimate in the magnitude of the biospheric sink (Fung et al., 1997; Chen bao zhang et al., 2007).

The level of stomatal conductance and the rate of carboxylation are the two main factors that control the plant foliar  $\delta^{13}\text{C}$  and carbon isotope discrimination. When diffusing from atmosphere to intercellular space, the light carbon isotope  $^{12}\text{C}$  diffuses faster than the heavy carbon isotope  $^{13}\text{C}$ . When  $\text{CO}_2$  is assimilated by carboxylation reaction of photosynthesis,  $^{12}\text{C}$  reacts faster than  $^{13}\text{C}$ . As a result,  $^{12}\text{C}$  is assimilated faster than  $^{13}\text{C}$  during photosynthesis and carbon isotope discrimination occurs. That's why during summer when the photosynthesis rate of ecosystems in the northern hemisphere increases,  $\delta^{13}\text{C}$  of  $\text{CO}_2$  in the atmosphere increases (Trolier et al., 1996).

Generally,  $\delta^{13}\text{C}$  value, defined below, is used to express carbon isotope ratios:

$$\delta^{13}\text{C}=(R_{\text{sa}}/R_{\text{st}}-1)\times 1000 \text{ [‰]}, \quad (1)$$

where  $R_{\text{sa}}$  and  $R_{\text{st}}$  are ratios of  $^{13}\text{C}$  to  $^{12}\text{C}$  in the sample and standard against PDB (Peedee Belemnite), respectively. Carbon isotope discrimination,  $\Delta^{13}\text{C}$ , is defined by the following equation (Farquhar et al., 1982):

$$\Delta^{13}\text{C}=(1-R_{\text{p}}/R_{\text{a}})\times 1000 \text{ [‰]}, \quad (2)$$

and can be written with  $\delta$  values as follows:

$$\Delta^{13}\text{C} = (\delta^{13}\text{C}_a - \delta^{13}\text{C}_p) / (1 + \delta^{13}\text{C}_a / 1000) \approx \delta^{13}\text{C}_a - \delta^{13}\text{C}_p, \quad (3)$$

where  $R_a$  and  $R_p$ , and  $\delta^{13}\text{C}_a$  and  $\delta^{13}\text{C}_p$  are the ratios of  $^{13}\text{C}$  to  $^{12}\text{C}$ , and  $\delta^{13}\text{C}$  of atmospheric  $\text{CO}_2$  and plant, respectively.

## 1.2. Environmental factors controlling $\delta^{13}\text{C}$ of plants

Carbon isotope discrimination has been traditionally related to intercellular  $\text{CO}_2$  partial pressure, which depends on the stomatal conductance and the rate of carboxylation (Farquhar and Richards, 1984). Carbon isotope discrimination during photosynthesis is described as below:

$$\Delta^{13}\text{C} = a + (b - a) \times \frac{C_i}{C_a}, \quad (4)$$

where  $a$  and  $b$  are fractionations during diffusion in the air and carboxylation, respectively, and  $\frac{C_i}{C_a}$  is the ratio of  $\text{CO}_2$  mole fractions of the intercellular space and ambient air.

When stomatal conductance decreases (carboxylation increases),  $\Delta^{13}\text{C}$  decreases ( $\delta^{13}\text{C}$  increases). In many recent works on photosynthesis, in addition to the stomatal conductance, the importance of mesophyll conductance and effect of respiration have been pointed out, and carbon isotope discrimination has been used to determine mesophyll conductance (Evans et al., 1986; Wingate et al., 2007; Flexas et al., 2008; Tazoe et al., 2011; Flexas et al., 2012; Farquhar et al., 2012; Cernusak et al., 2013; Evans et al., 2013; Gentsch et al., 2014); however, those parameters are not easily measured and therefore they are not taken into consideration in many cases (Flexas et al., 2012).

Using the equations (2) and (3), relationship of the assimilation rate ( $A$ ), stomatal conductance for  $\text{CO}_2$  ( $g$ ) and carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) can be described as below:

$$\frac{A}{g} = C_a \times \left(1 - \frac{C_i}{C_a}\right), \quad (5)$$

$$\frac{A}{g} = C_a \times \frac{b - \Delta^{13}\text{C}}{b - a}, \quad (6)$$

Generally, stomatal closure induced by dry conditions results in a decrease in  $\Delta^{13}\text{C}$  and an increase in  $\delta^{13}\text{C}$  in plants (Farquhar et al., 1984; Ehleringer and Cooper, 1988). On the other hand, increase in assimilation rate with photosynthetic photon flux density (PPFD) also lowers  $\Delta^{13}\text{C}$  and increases  $\delta^{13}\text{C}$  (Farquhar et al., 1989; Klein et al., 2005; Loader et al., 2013). Using these phenomena, tree-ring  $\delta^{13}\text{C}$  has been widely used to reconstruct various environmental parameters such as precipitation, relative humidity, irradiance, temperature, and soil moisture, with resolutions as high as the annual time scale (McCarroll et al., 2004; Gebrekirstos et al., 2009; Kress et al., 2010; Haupt et al., 2011; Heinrich et al., 2013; Hafner et al., 2014; Xu et al., 2014; Tei et al., 2015)

Many studies have demonstrated an increase in plant  $\delta^{13}\text{C}$  under dry conditions. For example, the mean tree-ring  $\delta^{13}\text{C}$  of *Pinus radiata* from a dry site was 3‰ higher than that from a wet site in New Zealand (Walcroft et al., 1997). Tree species aggregated in the upper site of a slope where water availability was low had a more positive leaf  $\delta^{13}\text{C}$  than the valley species growing in the lower site in an evergreen forest (Hanba et al., 2000). Martinon-Martinez et al. (2011) reported higher  $\delta^{13}\text{C}$  in *Pinus pinceana*

needles under low soil moisture conditions in a greenhouse experiment. In a field experiment, rainfed *Olea europaea* trees were always found to have more positive tree-ring  $\delta^{13}\text{C}$  than the irrigated trees (Rossi et al., 2013). Drought treatment caused a significant decrease in carbon isotope discrimination of *Picea asperata* Mast. when compared with well-watered treatment (Duan baoli et al., 2011). Drought-stressed cork oak (*Quercus suber* L.) seedlings showed higher leaf  $\delta^{13}\text{C}$  than well-watered seedlings (Aranda et al., 2007). On the other hand, few studies have been conducted on  $\Delta^{13}\text{C}$  in plants under waterlogged conditions (e.g. Guy et al., 1984; Anderson et al., 2005).

Anderson et al. (2005) found that tree ring  $\delta^{13}\text{C}$  of pond cypress showed a positive correlation with the total amount of annual precipitation. Extreme wet events are expected to occur more frequently in certain areas as a consequence of global warming (IPCC, 2012; Donat et al., 2016) and may affect plants and terrestrial ecosystems. It has been reported that the extreme wet event led to the yellowing and browning of larch trees and caused a decline in the forest cover in the taiga forest (Iwasaki et al., 2010; Iijima et al., 2014) and mortality of trees in the taiga-tundra boundary in eastern Siberia. Paludification (the process of shift from a drier ecosystem to a peat-forming wet ecosystem) has also been reported in the past; it caused forest retreat in the subarctic Canadian forests and western Siberia about 6000 years ago (Kremenetski et al., 1998; Crawford et al., 2003). To detect such events and/or environmental changes, it is essential to understand the changes in carbon isotope discrimination.

Although the effect of waterlogging on plant  $\delta^{13}\text{C}$  has rarely been investigated, some experimental studies on physiological responses of plants to waterlogging do

exist. Oxygen depletion in the rhizosphere occurs soon after waterlogging, and stomatal conductance and assimilation rates decrease quickly (within hours to days) in waterlogging-susceptible plants (Gomes et al., 1980; Terazawa et al., 1992; Olivella et al., 2000; Copolovici et al., 2010), unlike in waterlogging-tolerant plants, for which a longer time is required for a significant decrease (Mielke et al., 2005; Kissmann et al., 2014). Terazawa et al (1992) observed decrease of stomatal conductance and assimilation rate in larch trees under waterlogging condition (Fig 1.1). Reduction in root hydraulic conductance and a consequent decrease in leaf water potential, supposedly caused by oxygen deficit in root membranes, have also been reported (Olivella et al., 2000; Islam et al., 2004). Such responses may decrease the stomatal conductance (Else et al., 2001).

Ethanol and acetaldehyde, products of anaerobic metabolism, were reported to increase significantly after waterlogging, and stress markers, such as nitric oxide (NO), lipoxygenase (LOX), and methanol were also detected (Copolovici et al., 2010). Reactive oxygen species ( $O_2^-$  and  $H_2O_2$ ) were also produced under such conditions (Copolovici et al., 2010; Yordanova et al., 2004; Bertolde et al., 2012). These compounds are phytotoxic for plant roots. Moreover, phytohormones such as abscisic acid (ABA) (Olivella et al., 2000; Castonguay et al., 1993; Else et al., 2009) and ethylene (Jackson et al., 1999) have been reported to control stomatal conductance. Waterlogging can reduce the carboxylation rate (catalyzed by Rubisco) by reducing the amount or activity of the carboxylation enzymes (Islam et al., 2004; Vu et al., 1991; Vu et al., 1992). It can also decrease the chlorophyll content, leading to a lowering of

the maximum quantum yield of PSII (photosynthesis system II), measured in terms of the ratio of variable fluorescence to maximum fluorescence ( $F_v/F_m$ ) (Bertolde et al., 2012; Smethurst et al., 2003). Plants also respond to waterlogging with morphological changes, including the formation of lenticels, aerenchyma tissues, and adventitious roots (Gomes et al., 1980; Kissmann et al., 2014; Bertolde et al., 2012; Tsukahara et al., 1984; Iwanaga et al., 2008).

Several physiological responses of plants to waterlogging, as mentioned above, affect stomatal conductance and carboxylation rate, which may, in turn, affect carbon isotope discrimination.

### **1.3 Objective**

As introduced in the former part, there are many studies on plant  $\delta^{13}\text{C}$  under dry condition, and there are also many studies on plant physiological responses to waterlogging condition. However few studies have been conducted on  $\Delta^{13}\text{C}$  in plants under waterlogged condition.

The objective of the present study was to decipher the effect of waterlogging on carbon isotope discrimination during photosynthesis in *Larix gmelinii* by pot experiments. *Larix gmelinii* forests constitute a large part of the total forested area in eastern Siberia and play an important role as carbon reservoirs in the global carbon cycle (Schulze et al., 1999; Kagawa et al., 2006). This species is well adapted to the dry climate of eastern Siberia, a region that has previously been subjected to severe drought and is expected to face wet conditions owing to global warming (Tei et al., 2013).



To achieve the objective, pot experiment with larch trees was conducted to measure instantaneous carbon isotope discrimination. Experimental chamber system and analytical line of concentration and carbon isotope composition of CO<sub>2</sub> were developed to obtain the instantaneous carbon isotope discrimination, Plant tissue  $\delta^{13}\text{C}$ , which can indicate the integrated carbon isotope discrimination, were also measured.

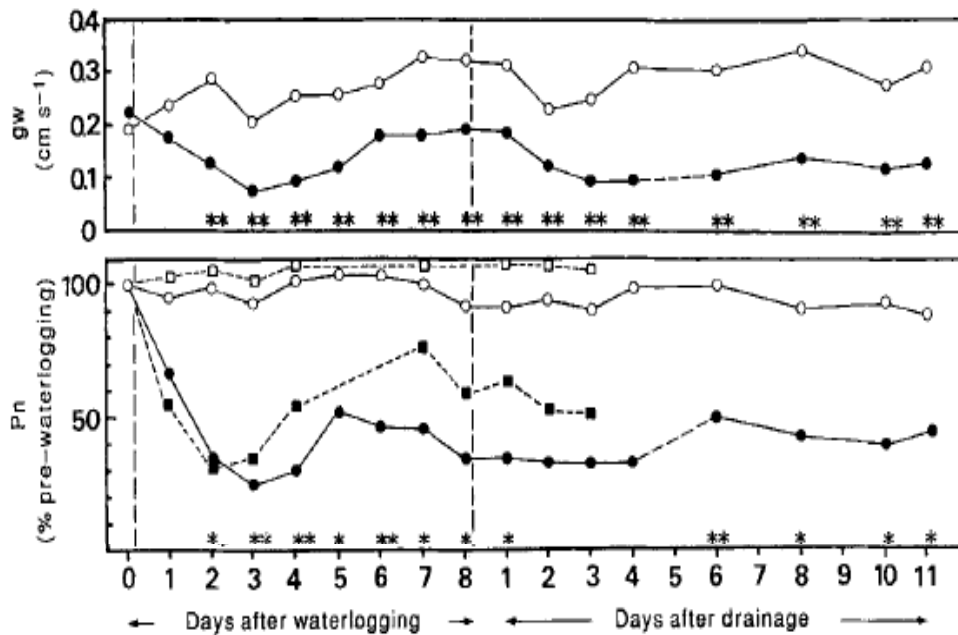


Fig. 1.1 Effects of waterlogging on  $g_w$  (stomatal conductance), and  $P_n$  (assimilation rate) of *Larix Kaempferi* seedlings. Each point represents the mean of three plants for waterlogged (●) and of two plants for non-waterlogged (○) treatments, respectively. Data of waterlogged plants after the 6th day of drainage represent the mean of two plants, because one waterlogged seedling shed leaves severely. Data of the preliminary experiment under the same experimental procedure were also included for  $P_n$ , where squares (■) represents the mean of two waterlogged plants and (□) represents the mean of two non-waterlogged plants. Statistical differences between treatments are denoted by asterisks: \* $P < 0.05$ ; \*\* $P < 0.01$ . (Modified from Terazawa et al., 1992)

## **Chapter 2    Development of experimental and analytical lines**

### **2.1 Development of experimental line: Chamber system**

Generally chamber systems are used to obtain the instantaneous carbon isotope discrimination. In this study, a specially made chamber that is suitable for conifer trees is used. Discrimination is generally calculated according to the CO<sub>2</sub> concentration and carbon isotope composition difference between the entrance and the exit, that's to say before and after photosynthesis respectively.

A chamber system (Fig 2.1) was used to obtain the instantaneous carbon isotope discrimination during photosynthesis. The air was sent from the entrance bag into the line by a pump with flow rate of approximately  $5.0 \times 10^{-4} \text{ L s}^{-1}$ . The air passed through the chamber in which an intact stem of experimental tree (about 8 cm) was enclosed, and flowed into the exit bag by which back diffusion of the air was avoided. During the experiment, the photosynthetic photon flux density (PPFD) of light emitted from a LED source was set to  $1500 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ , a flux density at which the assimilation of trees becomes almost saturated (Fig. 2.2). Air samples were taken 3–5 times every 15 minutes at the exit using a gas-tight syringe, and the concentration and  $\delta^{13}\text{C}$  of CO<sub>2</sub> in the air were analyzed. Fig. 2.3 showed photographs of the chamber system with *Larix gmelinii*.

### **2.2 Development of line for concentration and $\delta^{13}\text{C}$ of CO<sub>2</sub> measurement**

Generally MAT 253, stable isotope ratio mass spectrometer from Thermo Electron Corporation or tunable diode laser absorption spectrometry (TDLAS) is used for measurement of carbon isotope composition of CO<sub>2</sub>. However compared to TDLAS,

analytical accuracy and stability of MAT 253 is much better. Concentration and  $\delta^{13}\text{C}$  of  $\text{CO}_2$  in the air were analyzed with a mass spectrometer (MAT253) coupled to a gas chromatograph (GC) isolink unit via a ConFlo IV interface (Thermo Fisher Scientific, USA) at the Faculty of Environmental Earth Science, Hokkaido University. The photographs are shown as Fig. 2.4.

$\text{CO}_2$  concentration of sample is low, thus necessary volume of sample is too large to use GC isolink which is generally used for measuring stable isotope of gas phase, I had to develop the line for concentration and  $\delta^{13}\text{C}$  of  $\text{CO}_2$  measurement. I made some attempts to measure concentration and  $\delta^{13}\text{C}$  of  $\text{CO}_2$  in the air samples with high precision.

### **2.2.1 Line with a sampler part to inject the sample under vacuum condition.**

First I added a sampler part to inject the sample under vacuum condition.

Figure 2.5 showed the line for measurement of concentration and  $\delta^{13}\text{C}$  of  $\text{CO}_2$  with sample injected under vacuum condition. The test results with laboratory standard  $\text{CO}_2$  (300 ppm air balance, Ls) of this line are shown as Table 2.1.

Peak area of  $\text{CO}_2$  for Ls-3 is apparently larger than that of the other 4 samples and  $\delta^{13}\text{C}$  of  $\text{CO}_2$  is much higher than the others. This means that the ambient atmosphere,  $\delta^{13}\text{C}$  of which is around -8‰ contaminated the laboratory standard gas sample. It is much higher than that of laboratory standard gas. After careful checking, I found that the seal ability of the septum (RUBBER SEPTUM, INJ.PORT 20/PKT, SHIMADZU) is not qualified enough. And the ambient air entered into the sampler part and the line when the samples are injected through the septum under vacuum condition. I still failed

to avoid the contamination after some modifications to the system. So I decided to change the vacuum line to a high-pressure line flushing with Helium gas. And I also changed the septum to a kind of more qualified septum (Silicone seal 12 × 6 mm, Thermo)

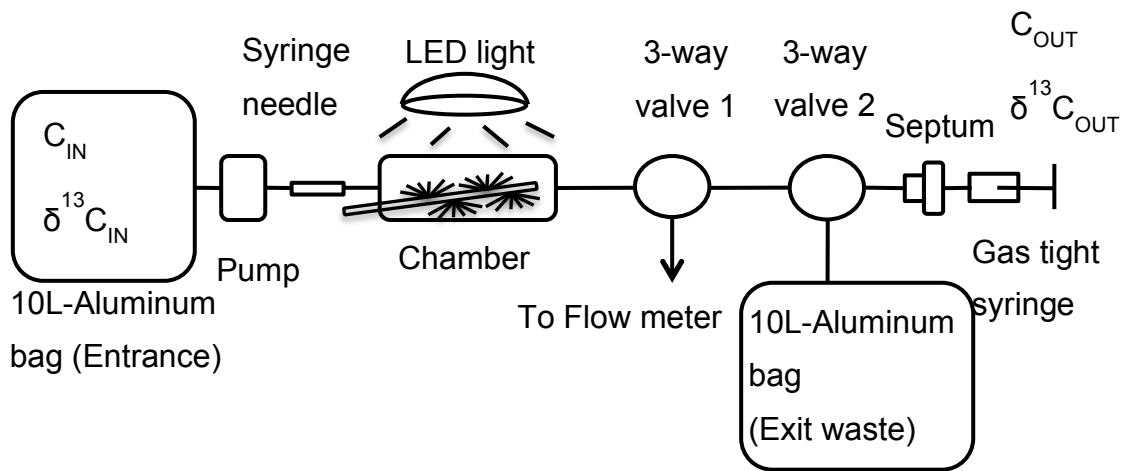
### **2.2.2 Line with a sampler part to inject the sample under high-pressure condition.**

Finally a gas sampler with two cryogenic traps was developed for this study and was attached to the system (Fig 2.6). Because of transpiration, the air samples taken at the exit contain a lot of water vapor. To remove the water vapor, a specially made sampler is used as water trap. Removal of water vapor is more complete and the data are more reliable. Helium gas was led to the sampler part to make sample injection under high-pressure condition. Thus air contamination is avoided. The line was tested with laboratory standard gas: 300 ppm CO<sub>2</sub> with air balance with volume of 1~2.5ml.

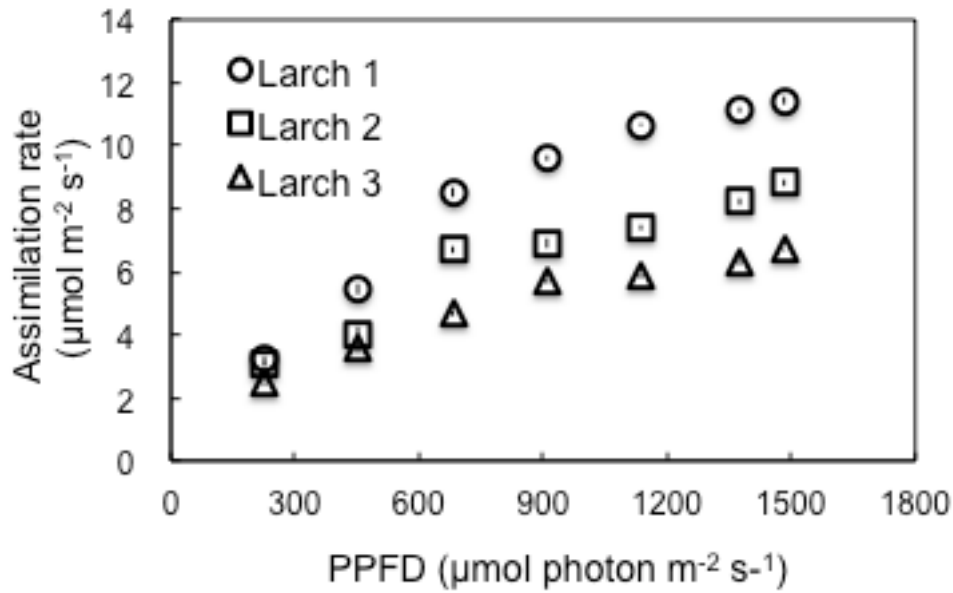
The GC column used was a Varian Capillary Column CP-PoraPLOT Q, 27.5-m long, with an inner diameter of 0.32 mm and wall thickness of 10 μm. Temperature of the column was 30°C. A high temperature conversion (HTC) oven was not used in this study, but CO<sub>2</sub> was introduced to the mass spectrometer through HTC oven at room temperature.

Laboratory standard CO<sub>2</sub> (300 ppm air balance) was analyzed at least 4 times in each measurement for CO<sub>2</sub> concentration and δ<sup>13</sup>C. The concentration of CO<sub>2</sub> was calculated from the peak area of the chromatogram obtained by the mass spectrometer, and the δ<sup>13</sup>C of CO<sub>2</sub> was calibrated against PDB using the laboratory standard CO<sub>2</sub>. Analytical error (reproducibility) for CO<sub>2</sub> concentration was less than 2%, but it was

about 1% for most samples, and that for  $\delta^{13}\text{C}$  was less than 0.1‰.



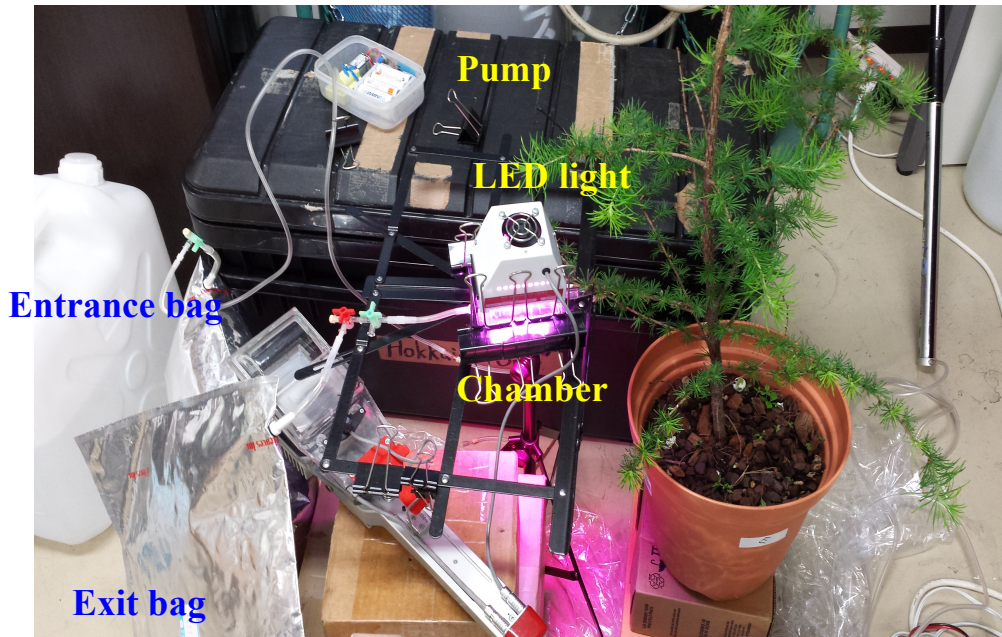
**Figure 2.1.** Chamber system used for assimilation and discrimination measurements in the waterlogging experiment. Air was pumped from a 10-L entrance aluminum bag to an exit waste bag through a chamber in which the larch stem was enclosed. Air samples were taken 3–5 times every 15 minutes at the exit using a gas-tight syringe. Flow rate was controlled at around  $5.0 \times 10^{-4} \text{ L s}^{-1}$  using a pump coupled with syringe needles. Photosynthetic photon flux density (PPFD) of light from an LED source was set at  $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ .



**Fig. 2.2.** Relationship between the assimilation rate and photosynthetic photon flux density (PPFD) for three larch trees. This measurement was made as preparatory measurement with different trees from those used in the present experiments.



(A)



(B)



Fig. 2.3. Photographs of a chamber system with *Larix gmelinii*, for control treatment (A) and for waterlogging treatment (B).

(A)



(B)

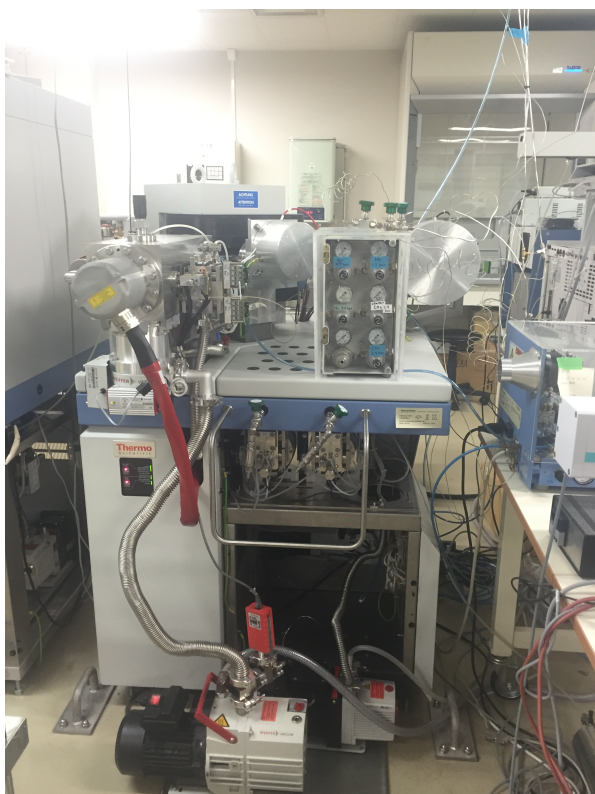
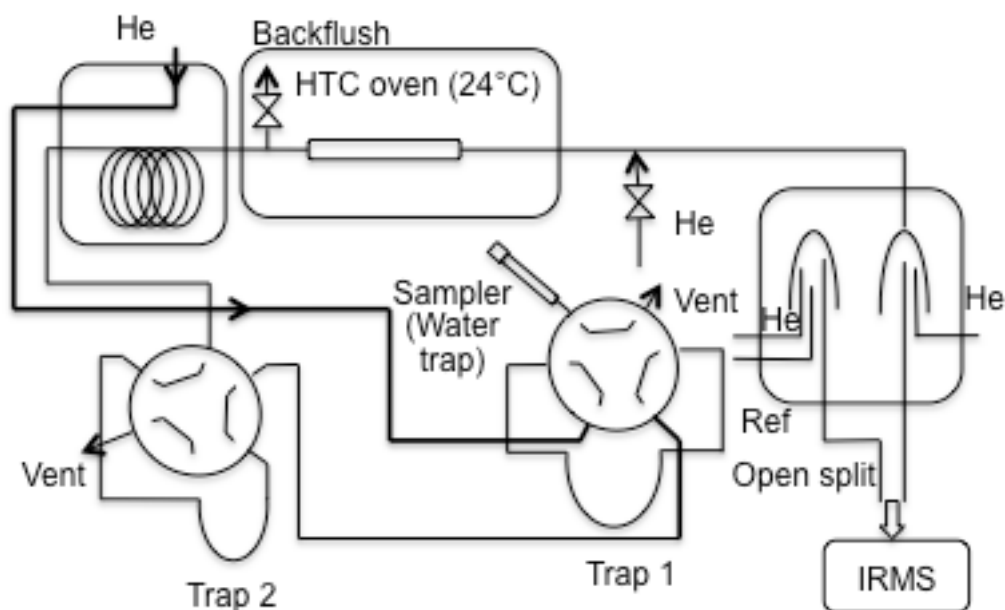


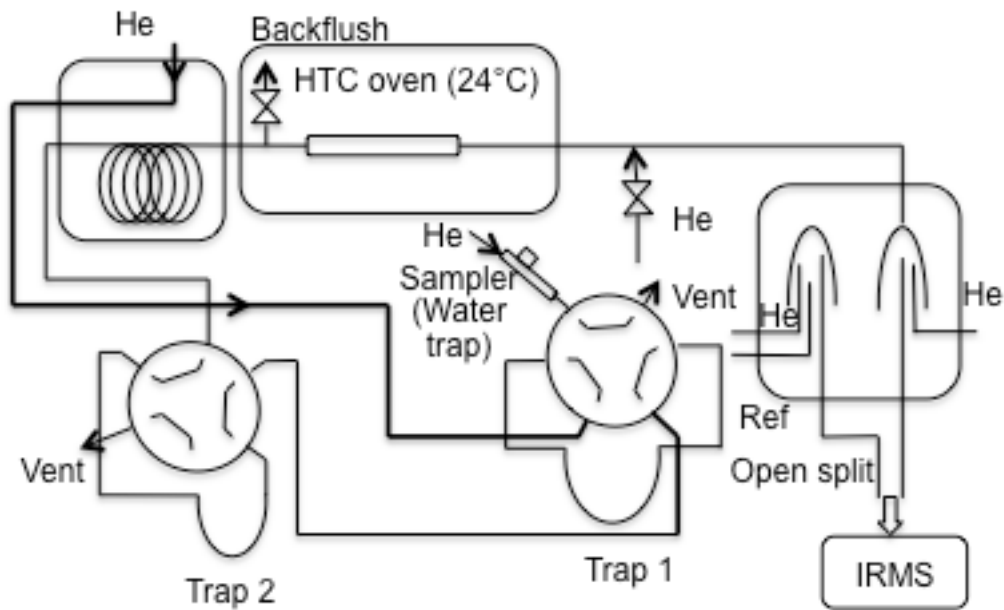
Fig. 2.4. Photographs of gas chromatograph (GC) isolink (A) and MAT253 (B).



**Figure 2.5.** Line with a sampler part to inject the sample under vacuum condition. The sampler is attached to a valve. The vent of the valve is attached to a pump, which is used to vacuum the line. When the valve is set at “charge”, the sampler is connected to Trap 1. After evacuation, put liquid N<sub>2</sub> at Trap 1, and then inject the sample. CO<sub>2</sub> and water vapor is trapped at Trap 1 and the other gases are pumped out of the line. Then the valve is set at “discharge” and CO<sub>2</sub> is released by changing liquid N<sub>2</sub> to liquid N<sub>2</sub> and ethanol slush of about -80°C. Simultaneously, CO<sub>2</sub> is flushed by Helium gas and collected at Trap 2 by liquid N<sub>2</sub> and finally released to the gas chromatography (GC) column and mass spectrometer at room temperature by removing liquid N<sub>2</sub> at Trap 2.

**Table 2.1.** The test results with laboratory standard CO<sub>2</sub> (300 ppm air balance, Ls) of the vacuum line.

Sample	Peak area	$\delta^{13}\text{C}$
Ls-1	15.330	-33.30
Ls-2	15.299	-34.28
Ls-3	18.755	-28.74
Ls-4	15.663	-33.97
Ls-5	14.943	-34.26



**Figure 2.6.** Line used for measurement of CO<sub>2</sub> concentration and δ<sup>13</sup>C. The air sample was injected from the sampler, which was filled with anhydrous magnesium perchlorate and quartz turnings as a water trap. CO<sub>2</sub> was lead to mass spectrometer after removing the water vapor. CO<sub>2</sub> and the remaining water vapor in the air sample was trapped using liquid N<sub>2</sub> at Trap 1; thereafter, CO<sub>2</sub> was released and water vapor was trapped by changing liquid N<sub>2</sub> to liquid N<sub>2</sub> and ethanol slush of about -80°C at Trap 1. Simultaneously, CO<sub>2</sub> was collected at Trap 2 using liquid N<sub>2</sub> and finally released to the gas chromatography (GC) column and mass spectrometer at room temperature by removing liquid N<sub>2</sub> at Trap 2. The outer diameters of both the traps were 1/16 inch.



## Chapter 3 Material and method

### 3.1 Waterlogging experiment

A pot experiment was conducted using small trees of *Larix gmelinii*. Six trees, with heights ranging from 80 to 91 cm and trunk diameter of about 3.5 cm, were grown outside after being transplanted to plastic pots (5 L, 25 cm in diameter and 30 cm in height). Three trees were treated as controls (C1, C2 and C3), whereas the others were used for the waterlogging treatments (W1, W2 and W3). Composted bark, with tuff loam and volcanic gravel, was used as the soil. For the control treatment, the trees were watered every day as needed; the extra water was drained out through holes in the bottom of the pots. For the waterlogging treatment, after the day 0 measurements, the pots were placed in buckets filled with water to submerge the surface soil of the pots. During the experiment, the pots were kept outside and were brought into the laboratory when it rained or in order to take measurements. As instantaneous carbon isotope discrimination cannot be accurately calculated when assimilation rate becomes very small, the assimilation was monitored by a portable photosynthesis system (LCpro+, ADC BioScientific Ltd, UK), prior to the measurement of carbon isotope discrimination. Leaf surface temperature ranged from 25.7 to 28.4°C, and vapor pressure deficit (VPD) of the chamber was between 13.6 and 18.9 mbar. In general, measurements were taken every 4 days for control trees, whereas for trees subjected to the waterlogging treatment, they were taken more frequently when the assimilation rate decreased. The experiments were carried out from June 23 to July 19, 2014, when the waterlogging treatment trees ceased photosynthesis.

### 3.2 Needle area and $\delta^{13}\text{C}$ of plant tissue

Needles in the chamber were sampled on last day of the experiment for needle area and  $\delta^{13}\text{C}$  measurement. Needle area was scanned promptly after sampling, and calculated using the software Image J (<http://rsb.info.nih.gov/ij/>). The needle area for each tree varied between 14.6 and 53.8 cm<sup>2</sup>. For each tree, three current year shoots, formed during the period of experiment, were sampled for  $\delta^{13}\text{C}$  analysis. Average  $\delta^{13}\text{C}$  of current year shoot for each tree was determined from the three sampled shoots and then the difference in  $\delta^{13}\text{C}$  between current year shoot and needles was obtained for each tree.

Needles and shoot samples were dried at 60°C, milled, and wrapped in tin capsules. The  $\delta^{13}\text{C}$  was analyzed with a continuous flow system of isotope ratio mass spectrometer (DELTA V Plus, Thermo Fisher Scientific, USA) coupled to an elemental analyzer (Flash EA1112, Thermo Fisher Scientific, USA) at the Faculty of Environmental Earth Science, Hokkaido University. The  $\delta^{13}\text{C}$  values against PDB were obtained with laboratory standards (two different amino acids) which have been calibrated by NBS 18 and 19. The reproducibility (standard deviation) for  $\delta^{13}\text{C}$  of the organic matter (laboratory standard) was  $\pm 0.04\%$ .

### 3.3 Calculations

Carbon isotope discrimination during photosynthesis was determined using the following equation (Guy et al., 1989):



$$\Delta^{13}C_{RD} = -\frac{\ln \frac{R_{OUT}}{R_{IN}}}{\ln \frac{C_{OUT}}{C_{IN}}} \times 1000 \text{ ‰} \quad (7)$$

where  $\Delta^{13}C_{RD}$  is the instantaneous carbon isotope discrimination calculated with Rayleigh Distillation equation.  $R_{IN}$  and  $R_{OUT}$  and  $C_{IN}$  and  $C_{OUT}$  are carbon isotope ratios ( $^{13}\text{C}/^{12}\text{C}$ ) and concentration of  $\text{CO}_2$  in the air at the entrance and exit, respectively.

For each measurement, 3–5 air samples were taken at the exit of the chamber system and the average carbon isotope discrimination was calculated. When  $\text{CO}_2$  concentration difference at the entrance and exit was lower than 40 ppm, discrimination could not be calculated owing to the large error.

The assimilation rates were calculated using the following equation:

$$A = F \times (C_{IN} - C_{OUT}) / (S \times R \times T) \quad (8)$$

where  $A$  is the assimilation rate ( $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ) and  $F$ ,  $S$ ,  $R$ , and  $T$  are the flow rate ( $\text{L s}^{-1}$ ), leaf area ( $\text{m}^2$ ), gas constant ( $\text{L atm K}^{-1} \text{ mol}^{-1}$ ), and absolute temperature (K), respectively. The flow rate was approximately  $5.0 \times 10^{-4} \text{ L s}^{-1}$ .

The differences in the average  $\delta^{13}\text{C}$  values between the different plant tissues for control and waterlogging trees were assessed using Mann-Whitney U test and significant level was  $p < 0.1$  (two-tailed), which is the smallest value for small sample size ( $n=3$ ), when  $U=0$ .



## Chapter 4 Results

### 4.1 Assimilation rate

We observed a decrease in CO<sub>2</sub> concentration and an increase in its  $\delta^{13}\text{C}$  from the entrance to the exit due to photosynthesis by larch needles present in the chamber. Representative values of the observed concentration and  $\delta^{13}\text{C}$  of CO<sub>2</sub> and examples of calculated assimilation rate and carbon isotope discrimination of C2 and W2 are shown in Table 4.1.

Assimilation rates of trees in the control treatment (C1–C3) did not change during the experimental period, whereas those of the trees exposed to waterlogging conditions (W1–W3) decreased, showing characteristic variations, as shown in Fig 4.1. The assimilation rates of W1 and W3 decreased dramatically on day 4; thereafter, they recovered to some extent but decreased subsequently. This result was consistent with that reported by Terazawa et al (1992). On the other hand, one of the waterlogging treatment trees, W2, did not show such drastic decrease in assimilation rate as W1 and W3, although the photosynthesis measurement prior to the measurements of isotope discrimination showed a decrease in A on day 4. Assimilation rate of W2, exhibited a gradual decrease from the beginning of the experiment. Assimilation ceased in the waterlogging treatment trees W1, W2, and W3 on day 26, 19, and 16, respectively.

### 4.2 Instantaneous carbon isotope discrimination

Temporal variation in the carbon isotope discrimination,  $\Delta^{13}\text{C}_{\text{RD}}$ , is shown in Fig 4.2. The values of  $\Delta^{13}\text{C}_{\text{RD}}$  for control trees showed no obvious temporal variation during the experiment. The average values of  $\Delta^{13}\text{C}_{\text{RD}}$  were 14.0, 11.2, and 15.0‰ for C1, C2,

and C3, respectively. In contrast, the waterlogging treatment trees showed characteristic temporal variation, corresponding to the change in assimilation rate. The values of  $\Delta^{13}\text{C}_{\text{RD}}$  for W1 and W3 decreased by about 3.8‰ on day 4 and recovered, thereafter. After the decrease on day 4,  $\Delta^{13}\text{C}_{\text{RD}}$  of W1 increased from 6.0 to 10.8‰ until day 8 and then decreased to 7.7‰ slowly. Recovery of  $\Delta^{13}\text{C}_{\text{RD}}$  of W3 after the decrease on day 4 continued until day 12. However, the recovery was not complete, and the discrimination values were lower (12.7‰ in average for days 5, 8 and 12) than the initial value (13.5‰). Discrimination  $\Delta^{13}\text{C}_{\text{RD}}$  of W2 decreased gradually from 11.8 to 8.6‰.

#### 4.3 $\delta^{13}\text{C}$ of plant tissues

The  $\delta^{13}\text{C}$  of needles and current year shoots are shown in Fig 4.3a. The average and SD (standard deviation) values for  $\delta^{13}\text{C}$  of needles used for the measurements were  $-28.2 \pm 0.7$  and  $-27.0 \pm 0.6$ ‰ for the control and waterlogging treatment trees, respectively, and there was no significant difference between them. In contrast, the  $\delta^{13}\text{C}$  in the current year shoot of waterlogging treatment was significantly higher than that of control treatment. The average  $\delta^{13}\text{C}$  of the current year shoots in the control treatment was  $-29.5 \pm 0.5$ ‰, which was lower than that of the needles, whereas the average  $\delta^{13}\text{C}$  of the current year shoots of waterlogging treatment was  $-26.5 \pm 1.2$ ‰, which was higher than that of the needles.

Differences in  $\delta^{13}\text{C}$  between the current year shoots and needles ( $\delta^{13}\text{C}$  of shoot  $-\delta^{13}\text{C}$  of needles) were  $-1.3 \pm 0.3$ ‰ for the control treatment trees, whereas those for the waterlogging treatment trees ( $0.5 \pm 0.6$ ‰) were significantly higher (Fig 4.3b). Among

the three waterlogging treatment trees, the  $\delta^{13}\text{C}$  difference for W2 (1.1‰) was higher than those for W1 (0.5‰) and W3 (-0.2‰).

**Table 4.1.** Examples of average CO<sub>2</sub> concentration and δ<sup>13</sup>C of CO<sub>2</sub> at the entrance and exit and calculated assimilation rate and carbon isotope discrimination for C2 and W2.

C2

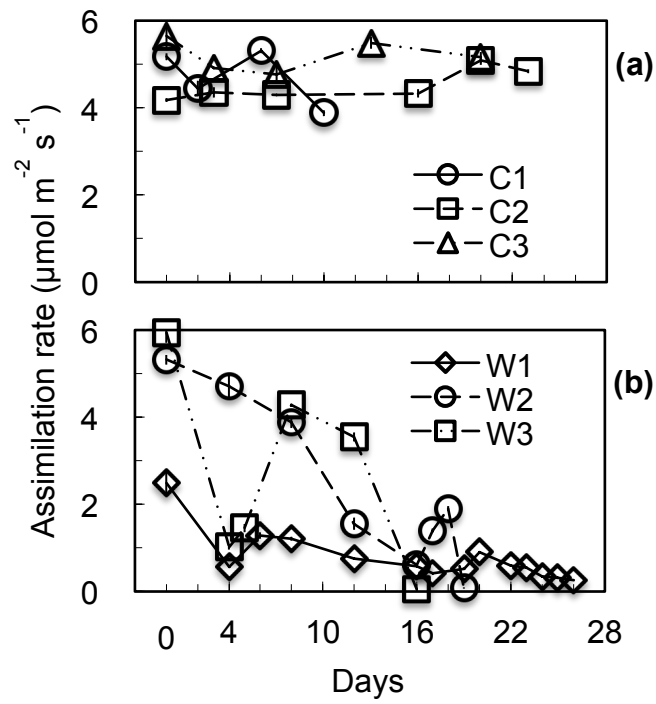
Day	C <sub>IN</sub> (ppm)	δ <sup>13</sup> C of CO <sub>2</sub> at entrance (‰)	C <sub>OUT</sub> (ppm)		δ <sup>13</sup> C of CO <sub>2</sub> at exit (‰)		Assimilation rate (μmol m <sup>-2</sup> s <sup>-1</sup> )		Δ <sup>13</sup> C <sub>RD</sub> (‰)	
			Average	SD	Average	SD	Average	SD	Average	SD
			0	393	-8.6	229	2	-2.3	0.2	4.2
3	396	-9.1	216	5	-1.9	0.4	4.4	0.12	11.9	0.2
7	419	-9.0	215	1	-1.7	0.2	4.3	0.02	10.9	0.2
16	403	-8.8	201	4	-1.6	0.3	4.3	0.10	10.5	0.2
20	406	-9.0	184	4	-0.1	0.2	5.1	0.10	11.3	0.2
23	409	-9.3	182	2	-0.3	0.1	4.8	0.05	11.2	0.2

**Table 4.1. Continued**

W2

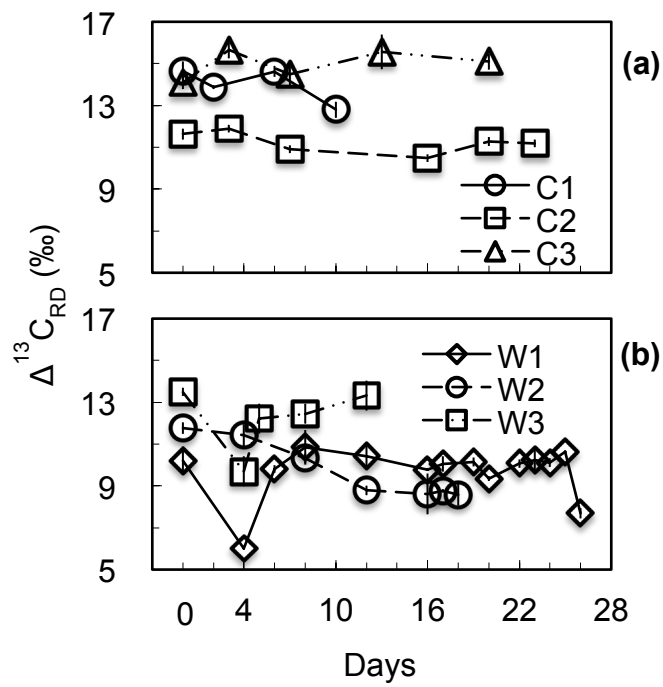
Day	C <sub>IN</sub> (ppm)	$\delta^{13}\text{C}$ of CO <sub>2</sub> at entrance (‰)	C <sub>OUT</sub> (ppm)		$\delta^{13}\text{C}$ of CO <sub>2</sub> at exit (‰)		Assimilation rate ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )		$\Delta^{13}\text{C}_{\text{RD}}$ (‰)	
			Average	SD	Average	SD	Average	SD	Average	SD
			0	397	-8.7	207	4	-2.4	0.4	5.3
4	408	-9.0	270	8	-4.2	0.3	4.7	0.27	11.4	0.5
8	398	-8.7	165	8	0.3	0.5	3.9	0.13	10.3	0.2
12	394	-8.6	302	8	-6.3	0.3	1.6	0.14	8.8	0.2
16	394	-8.5	335	4	-7.2	0.3	0.6	0.04	9.8	0.9
17	424	-9.7	230	7	-4.4	0.3	1.4	0.05	8.8	0.1
18	408	-9.1	224	6	-4.0	0.4	-4.0	0.06	8.6	0.5

C<sub>IN</sub> and C<sub>OUT</sub> are the CO<sub>2</sub> concentration at the entrance and exit, respectively. SD is the standard deviation for each measurement.  $\Delta^{13}\text{C}_{\text{RD}}$  is carbon isotope discrimination calculated with Rayleigh Distillation equation.

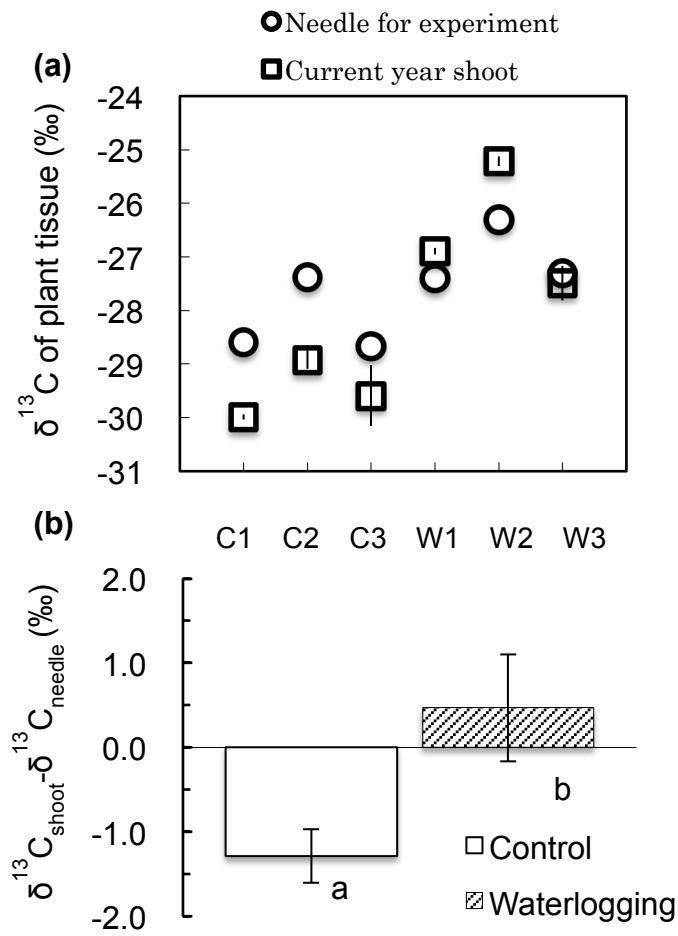


**Figure 4.1.** Assimilation rate calculated from equation (8) for control trees C1, C2, and C3 (a) and waterlogging trees W1, W2, and W3 (b). Error Bars are standard deviations of repeated measurements.





**Figure 4.2.** Carbon isotope discrimination during photosynthesis calculated with Rayleigh Distillation equation ( $\Delta^{13}C_{RD}$ ) for control trees (a) and waterlogging trees (b). Error Bars represent the standard deviations of repeated measurements.



**Figure 4.3.**  $\delta^{13}\text{C}$  values of needles used for measurements and current year shoot formed during the experiment (a) and their difference (b) for control and waterlogging treatment. The error bars show the standard deviation. Different characters a and b denote statistically significant differences ( $p < 0.1$ ).

## Chapter 5 Discussion

### 5.1 Response of larch to waterlogging

A drastic decrease in the assimilation rate and  $\Delta^{13}\text{C}_{\text{RD}}$  of W1 and W3 was observed soon after waterlogging, and the decrease was followed by a slight recovery (Fig 4.1 and 4.2). The decrease in assimilation rate resulted either from stomatal closure or from non-stomatal limitation (Pezeshki et al., 1993). Stomatal closure causes a decrease in  $\Delta^{13}\text{C}$  and an increase in  $\delta^{13}\text{C}$ . In contrast, the assimilation rate decreases, but  $\Delta^{13}\text{C}$  increases, leading to a decrease in  $\delta^{13}\text{C}$ , when the carboxylation rate decreases. Therefore, from the drastic decrease in both assimilation rate and  $\Delta^{13}\text{C}_{\text{RD}}$  and the subsequent recovery, it can be deduced that the stomatal conductance of *Larix gmelinii* decreased soon after waterlogging and then recovered slightly. This is the most common pattern of stomatal conductance and also assimilation rate for trees under waterlogging (e.g. Terazawa et al., 1992; Islam et al and Macdonald, 2004).

After the recovery, the assimilation rate decreased gradually, whereas the decrease in  $\Delta^{13}\text{C}_{\text{RD}}$  was much gentler than that in the assimilation rate (Fig 4.1 and 4.2). These were explained by the reduction in both the stomatal conductance and carboxylation rate. The observed results on time course of assimilation and stomatal conductance were consistent with the results reported by Terazawa et al. (1992) for waterlogging experiment using larch trees. The simultaneous reduction of stomatal conductance and carboxylation rate was also reported for the responses of black spruce and tamarack to waterlogging (Islam and Macdonald, 2004).

Stomatal conductance reduction soon after waterlogging may have been caused by

the decrease in root hydraulic conductance and leaf water potential and the increase in ABA amount. Although precise mechanisms could not be inferred from my study, these responses might affect the discrimination and assimilation. Recovery of stomatal conductance after a drastic decrease has also been observed in several previous studies (Gomes et al., 1980; Terazawa et al., 1992; Copolovici and Niinemets, 2010; Islam and Macdonald, 2004; Gong et al., 2007; Polacik et al., 2013). Stomatal reopening has been related to the formation of adventitious roots under waterlogging conditions in some studies (Gomes et al., 1980; Copolovici and Niinemets, 2010); however, in the present study, no morphological changes were observed, and the mechanism of the recovery of stomatal conductance remains unclear.

Under prolonged waterlogging periods, reduction in carbon assimilation rates may depend upon the alterations in the amount or activity of carboxylation enzymes and degradation of chlorophyll (Bertolde et al., 2012; Pezeshki et al., 2001).

According to a conceptual model by Anderson et al. (2005), variation in relative contribution between stomatal and mesophyll resistances may lead to changes in the assimilation rate and carbon isotope discrimination. Since my purpose was not to investigate the process of photosynthesis, I do not consider these effects in detail, and, instead, emphasize the fact that waterlogging surely lead to a decrease in  $^{13}\text{C}$  discrimination.

Waterlogging caused a decrease in  $\Delta^{13}\text{C}_{\text{RD}}$  of *Larix gmelinii* and an increase in  $\delta^{13}\text{C}$  of C fixed during photosynthesis. This caused a difference in  $\delta^{13}\text{C}$  of plant tissues between the control and waterlogging treatment. The average  $\delta^{13}\text{C}$  in the current year

shoot that formed during the waterlogging treatment was significantly higher than those of the control trees (Fig 4.3). In contrast, the average  $\delta^{13}\text{C}$  of needles showed no significant difference between the control and waterlogging treatment (4.3). Needles rely mostly on stored material fixed during the previous year (Kagawa et al., 2006). Therefore, it is reasonable that a significant difference in  $\delta^{13}\text{C}$  was observed only for the current year shoot, in which the contribution of C fixed during the experiment was much greater than that in the needles. Difference in  $\delta^{13}\text{C}$  between current year shoot and needles (Fig 4.3b) was more distinct, because the effect of individual difference was removed.

The difference in  $\delta^{13}\text{C}$  between the current year shoot and needles of W2 (1.1‰) was the largest among three trees of waterlogging treatment (Fig 4.3a). This result appears to be consistent with the observed fact that a drastic decrease in the assimilation rate (A) and  $\Delta^{13}\text{C}_{\text{RD}}$  soon after waterlogging was not observed, but gradual and steady decreases in A and  $\Delta^{13}\text{C}_{\text{RD}}$  were observed (Fig 4.1 and 4.2). Although W2 did not show drastic decreases in A and  $\Delta^{13}\text{C}_{\text{RD}}$ , I detected the decrease in photosynthesis rate for W2 in the measurement prior to the isotope measurement. We, therefore, interpret that stomatal closure in W2, soon after the waterlogging, may be gentle and much shorter period than W1 and W3.

The obtained  $\Delta^{13}\text{C}_{\text{RD}}$  was the instantaneous discrimination, reflecting the conditions during the measurement. In contrast, plant tissue  $\delta^{13}\text{C}$  reflects what was integrated over the entire period of photosynthesis. It is not surprising that values of  $\Delta^{13}\text{C}_{\text{RD}}$  (11.2–15.0‰, for control trees) were much lower than those estimated from the plant

tissue  $\delta^{13}\text{C}$  (19.4–22.1‰, assuming  $-8.0\text{‰}$  for ambient  $\text{CO}_2$ ) because the  $\Delta^{13}\text{C}_{\text{RD}}$  measurement was conducted under high and constant light conditions. It is known that the plant carbon isotope discrimination decreases as the levels of PPFD increase (Farquhar et al., 1989; Loader et al., 2013). The observed  $\Delta^{13}\text{C}_{\text{RD}}$  (11.2–15.0‰) for control trees in the present study corresponded with the lower half of the reported  $\Delta^{13}\text{C}$  values for *Picea sitchensis* and maritime pine trees in the field, which ranged from 10 to 35‰ (Wingate et al., 2007; Wingate et al., 2010).

## 5.2 Comparison with field data

Although many researchers have pointed out the effect of wet events and/or paludification on trees and ecosystem, few isotope data is available to confirm the decrease in  $\Delta^{13}\text{C}$  and the increase in  $\delta^{13}\text{C}$  under waterlogging condition in a field, other than the observations reported from eastern Siberia. Liang et al. (2014) observed needle  $\delta^{13}\text{C}$  of several larch trees in dry and wet sites at the taiga-tundra boundary in eastern Siberia and showed that  $\delta^{13}\text{C}$  values of larch needles at the wet site were usually lower than those of needles at the dry site. However, they also reported an increase of 0.3‰ in the needle  $\delta^{13}\text{C}$  for larch trees at the wet site in 2012, following a very wet year 2011, whereas a decrease of 0.2‰ was observed at the dry site (Liang in preparation). An extreme wet event was also reported by Tei et al. (2013) for the larch forest near Yakutsk in eastern Siberia. An increase in  $\delta^{13}\text{C}$  of the tree rings of trees that died after the wet event was also observed; in contrast,  $\delta^{13}\text{C}$  of the healthy trees decreased (Tei et al., manuscript in preparation). The above-mentioned phenomena observed for larch trees in eastern Siberia are consistent with my results.

## Chapter 6 Concluding remarks

We conducted pot experiments to clarify the effect of waterlogging on carbon isotope discrimination using small trees of *Larix gmelinii*. Assimilation rate and  $\Delta^{13}\text{C}_{\text{RD}}$  (discrimination) of *Larix gmelinii* decreased drastically soon after waterlogging and subsequently recovered to some extent. This was caused by a decrease in stomatal conductance and the subsequent recovery. After recovery, the assimilation rate decreased gradually whereas the decrease in  $\Delta^{13}\text{C}_{\text{RD}}$  was much gentler. This was caused by a decrease in both the stomatal conductance and carboxylation rate.

Generally, wet conditions cause an increase in discrimination (decrease in  $\delta^{13}\text{C}$ ) (Farquhar et al., 1989; Tei et al., 2013; Belmecheri et al., 2014). However, my results confirm that extreme wet conditions that can damage trees may cause a decrease in carbon isotope discrimination (increase in  $\delta^{13}\text{C}$ ) during photosynthesis. There may be a threshold for soil moisture to turn the physiological condition from healthy to stressful or even fatal for trees. Discrimination would be the highest at this soil moisture threshold and it decreases with both the increasing and decreasing soil moisture.

Extreme wet events are expected to occur more frequently in certain areas due to global warming. My results indicate that not only drought but also extreme wet events might also cause a decrease in discrimination and an increase in  $\delta^{13}\text{C}$  of the plants. In eastern Siberia, extreme wet events occurred more frequently nowadays. Tree mortality was found in areas where have been subjected to waterlogging condition.

The tree-ring  $\delta^{13}\text{C}$  of the dead trees increased during the extreme wet year (Tei et al., in preparation). It is consistent with my experiment result.

In this study, pot experiment with small larch trees was conducted. While in the field, the larch trees are much older, taller and stronger. We expect that the small trees are more sensitive to waterlogging condition than old and large trees and the responses of small trees to waterlogging condition are quicker.



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