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TITOLO TESI

EFFECTS OF N FERTILIZATION ON FOREST TREES AND ECOSYSTEMS: WATER USE, WUE, GROWTH

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Abstract

During my three academic years, I focused on the effects of N fertilization on growth and function of plants and forest stands. The study had the dual objective of estimating the effects of atmospheric N deposition and evaluating the potential management value of N fertilization itself. In particular, the analysis took into account the changes induced in water use and intrinsic transpiration efficiency (ITE), an aspect often overlooked in world literature but of great importance especially in Mediterranean environment, where the positive effects of N fertilization may be denied by the parallel increased transpiration and exacerbated water stress.

The study consisted of three phases: (i) a first experiment studied in a semi-controlled environment the effects of N fertilization on growth and ITE in semination of poplar (*Populus* × *euroamericana*), a widely grown hybrid species in the Italian landscape, (ii) a meta-analysis of the literature on the effects of N fertilization on the ITE of woody plants (through gas exchange measurements and carbon isotope discrimination ($\Delta^{13}C$) analysis and Farquhar model application), (iii) a meta-analysis on the effects of fertilization (not just nitrogen) on the growth of forest stands in China. The analysis was preceded by (i) a careful discussion for the definitions in the literature of water-use efficiency (WUE), a term which has often been attributed different meanings with potential confounding effects, (ii) an in-depth statistical techniques for meta- analysis.

The experimental study has demonstrated the sensitivity of poplar, a species with indefinite growth, following N fertilization, and remarkable effects on water use and ITE. Particularly, interesting were the results of the re-analysis of literature data on the effects of N fertilization on the ITE at leaf level, which has been shown to respond strongly (and in a consistent manner between functional types) to the addition of N due to the absence of an increase in stomatal conductance and transpiration as compared with the sharp rise in photosynthetic rates. ITE obtained by gas exchanges measurements have proved completely consistent with what estimated by $\Delta^{13}C$. In consideration of the N fertilization experiment, it is worthwhile to note that the effects are overestimated in controlled environment (i.e. lab) as compared with measurements in field.

The analysis of growth changes in response to fertilization in Chinese forests also proved, of considerable utility, but the results were not as robust as originally hoped, due to the low number of available studies and the similarly short duration, highlighting the need for more attention to this topic and for further studies in the future.

Chapter 1

General introduction

1.1 Background

Over the last century, human activities, such as fossil fuel combustion and agricultural fertilizer use, have caused a great increase in nitrogen emissions and elevated nitrogen depositions toward land and oceans. The impacts of N deposition on terrestrial ecosystems vary with the status of soil N availability and vegetation types. In N limited ecosystems, N deposition enhances plant growth; the associated increase in net primary productivity (NPP) on temperate and boreal forest ecosystems has been the subject of intense research (Magnani et al. 2007; Thomas et al. 2010). However, when exceeding the critical loads (defined as a quantitative estimate of an exposure to one or more pollutants below which significant harmful effects on specified sensitive elements of the environment do not occur according to present knowledge; UBA 2004), N deposition exerts negative impacts on the health and function of ecosystems, such as loss of biodiversity (Sala et al. 2000; Stevens et al. 2004; Clark & Tilman 2008; Bobbink et al. 2010), soil acidification (Richter & Markewitz 2001; de Vries et al. 2007), eutrophication and nutrient imbalances (Aber et al. 1998), and increased susceptibility to environmental stress (Aerts & Bobbink 1999; Witzell & Shevtsova 2004).

Since the late 1980s or early 1990s, elevated N deposition has aroused great concerns for its ecological impacts on terrestrial and aquatic ecosystems. Cross-site N manipulation experiments, such as the NITREX and EXMAN have been established to assess the impacts of N deposition on forest ecosystems in Europe (Wright & van Breeman 1995; Wright & Rasmussen 1998), and there have also been several chronic nitrogen addition experiments in North America (Aber et al. 1993; Magill et al. 1996, 2004). In the meanwhile, rates of N deposition have leveled off or stabilized in the US and Europe with the implementation of stricter legislation to limit atmospheric pollution (Goulding et al. 1998; NADP 2000). On the contrary, emissions of N_r species in China have been increasing continuously since the 1980s mainly due to growing agricultural and industrial activities (Klimont et al. 2001; Zhang et al. 2007, 2009). These increased N_r emissions to the atmosphere have aroused widespread concern on air pollution in China (Fig. 1). Although there have been several N deposition monitoring programs and N deposition simulation experiments since the late 1990s (Tab. 1), there are still large gaps in knowledge of the magnitude and potential impacts of atmospheric N deposition on different ecosystems across China.

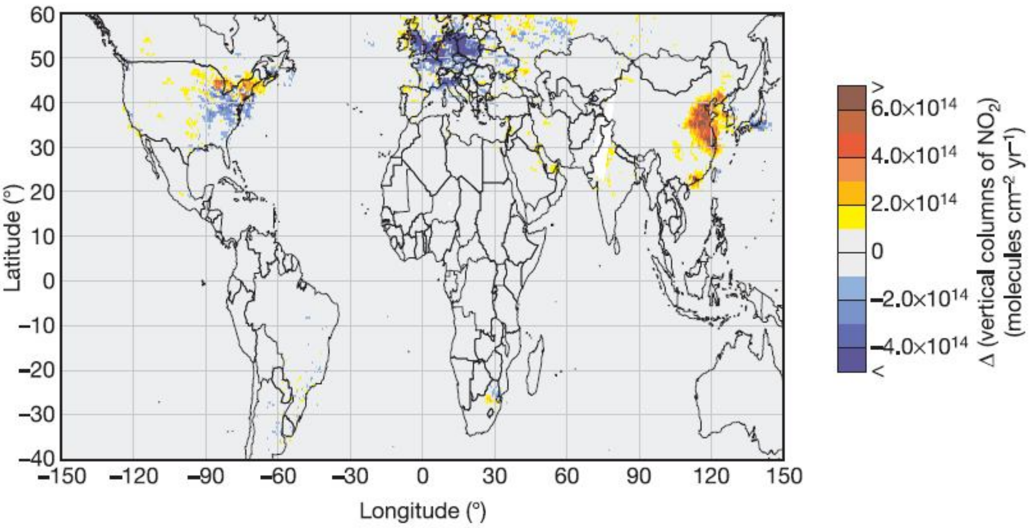
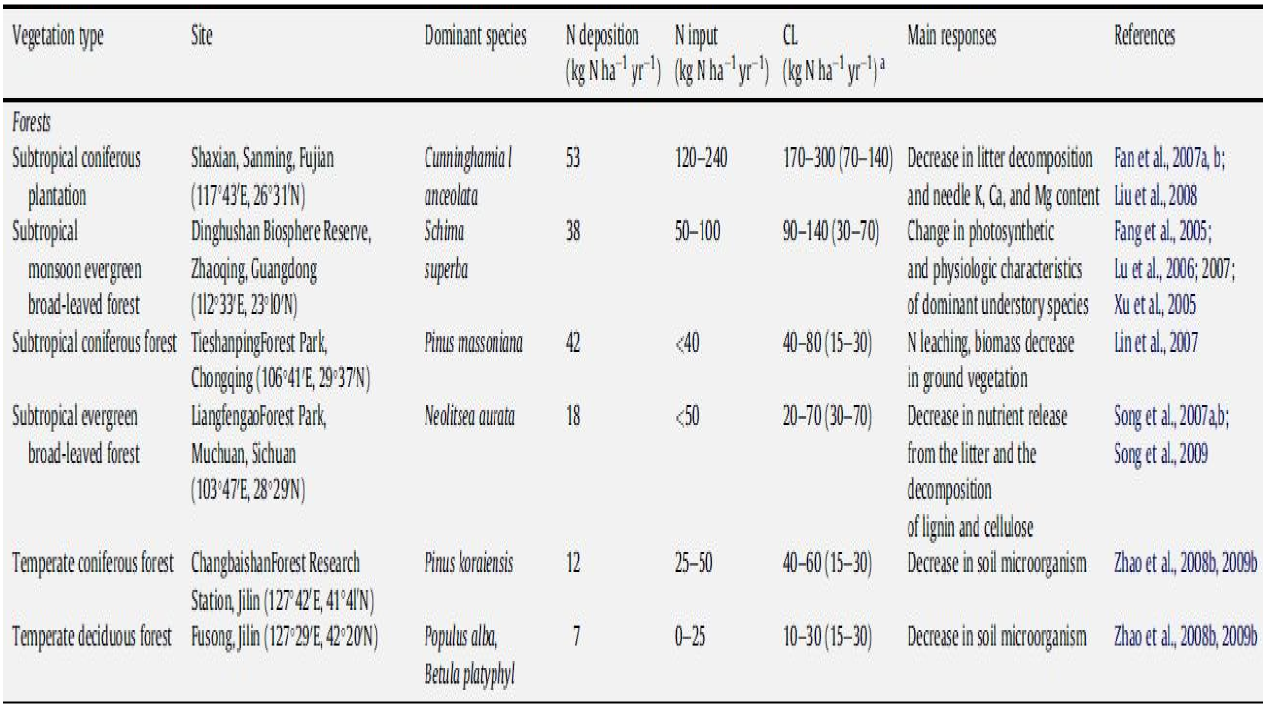


Figure 1 Average annual changes in tropospheric NO_2 as observed by GOME from 1996 to 2002. The gradient obtained from a linear regression of the annual averages of tropospheric GOME NO_2 vertical columns, retrieved close to 10.30 a.m.LT from 1996 to 2002 is shown. Reductions in NO_2 are observed over Europe and the Central East Coast of the United States, while large increases are evident over China (Richter et al. 2005).

Table 1 Summary of simulated N deposition experiments for some forests in China (Liu et al. 2011).



Vegetation type	Site	Dominant species	N deposition ($kg\ N\ ha^{-1}\ yr^{-1}$)	N input ($kg\ N\ ha^{-1}\ yr^{-1}$)	CL ($kg\ N\ ha^{-1}\ yr^{-1}$)[a]	Main responses	References
Forests							
Subtropical coniferous plantation	Shaxian, Sanming, Fujian (117°43′E, 26°31′N)	*Cunninghamia l anceolata*	53	120–240	170–300 (70–140)	Decrease in litter decomposition and needle K, Ca, and Mg content	Fan et al., 2007a, b; Liu et al., 2008
Subtropical monsoon evergreen broad-leaved forest	Dinghushan Biosphere Reserve, Zhaoqing, Guangdong (112°33′E, 23°10′N)	*Schima superba*	38	50–100	90–140 (30–70)	Change in photosynthetic and physiologic characteristics of dominant understory species	Fang et al., 2005; Lu et al., 2006; 2007; Xu et al., 2005
Subtropical coniferous forest	TieshanpingForest Park, Chongqing (106°41′E, 29°37′N)	*Pinus massoniana*	42	<40	40–80 (15–30)	N leaching, biomass decrease in ground vegetation	Lin et al., 2007
Subtropical evergreen broad-leaved forest	LiangfengaoForest Park, Muchuan, Sichuan (103°47′E, 28°29′N)	*Neolitsea aurata*	18	<50	20–70 (30–70)	Decrease in nutrient release from the litter and the decomposition of lignin and cellulose	Song et al., 2007a,b; Song et al., 2009
Temperate coniferous forest	ChangbaishanForest Research Station, Jilin (127°42′E, 41°41′N)	*Pinus koraiensis*	12	25–50	40–60 (15–30)	Decrease in soil microorganism	Zhao et al., 2008b, 2009b
Temperate deciduous forest	Fusong, Jilin (127°29′E, 42°20′N)	*Populus alba, Betula platyphyl*	7	0–25	10–30 (15–30)	Decrease in soil microorganism	Zhao et al., 2008b, 2009b

[a] Values in bracket are critical loads of nutrient N calculated by the steady state mass balance (SSMB) method (Duan et al., 2001).

1.2 Water balance

Water is an essential resource for plant growth in all terrestrial ecosystems, its availability is determined by environmental factors, along with the process by which water moves from the soil, through the plant, and into the atmosphere (Fig. 2).

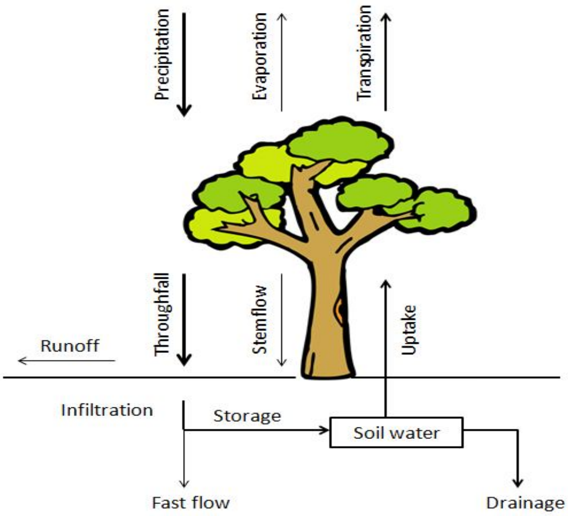


Figure 2 The hydrological cycle in terrestrial ecosystems. The width of the arrows approximates the relative volume of water following each pathway in a humid forest ecosystem. Inputs are mainly as precipitation in the form of rain or snow. At high elevations or in coastal areas (in any place where clouds meet the ground and become fog), additional inputs of water occur through condensation on leaves and stems. Water intercepted by surfaces can evaporate, drip through to the soil (throughfall), or run down the surface of stems (stemflow). Water that reaches the soil surface in liquid form can run off over the soil surface, a process that is important in soil erosion. Water that infiltrates into the soil can pass quickly below the rooting zone through large space called macropores (fast or saturated flow) or can adhere to soil particle surfaces and become part of soil water content (storage). Water on soil particle surfaces can also drain more slowly below the rooting zone in response to gradients of concentration in a process called unsaturated flow (drainage). Finally, water held on soil particle surface can be taken up by plants, transported to leaves, and evaporated back to the atmosphere (transpiration) (Aber & Melillo 2001).

Transpiration can be an important component of leaf energy balance. However, transpiration requires the constant movement of water to the leaf to replace that lost by evaporation. If the plant is unable to take up enough water from the soil, water stress develops, leading eventually to stomatal closure and a great reduction in both water loss and CO_2 uptake for photosynthesis (Fig.3).

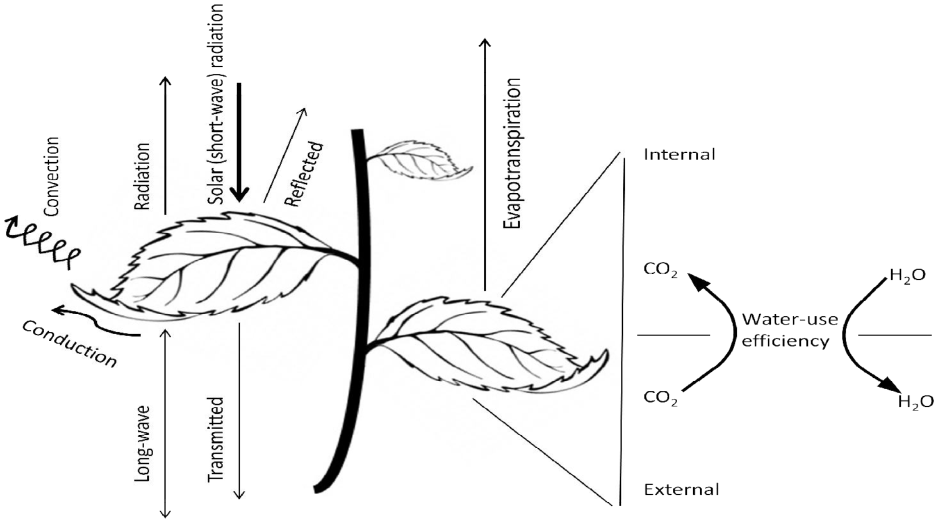


Figure 3 Energy, water, and CO_2 balances over a leaf. Short-wave solar radiation is transmitted, reflected, or absorbed. The energy in absorbed radiation can be re-emitted as thermal (long-wave radiation), lost through conduction and convection (sensible energy), lost through evaporation of water for

> transpiration (latent energy), or converted to chemical energy in the products of photosynthesis. Note that evapotranspiration is an important component of both the energy and water balance and its an inevitable consequence of CO_2 uptake. Water-use efficiency is the ratio of carbon gained to water lost. Rates of both water and carbon exchange are related to the conductance of the leaf or the degree to which the stomata are open (Aber & Melillo 2001).

Stomata opening, leading to the CO_2 uptake that is necessary for photosynthesis, results in an inevitable loss of water. This trade-off between water loss and carbon gain, along with the fact that a lack of available water limits plant growth over a large part of the earth, has generated interest in the concept of water-use efficiency (WUE), which has been recognized as an important characteristic of vegetation productivity in various natural scientific disciplines for decades (Fig. 4, Kuglitsch et al. 2008).

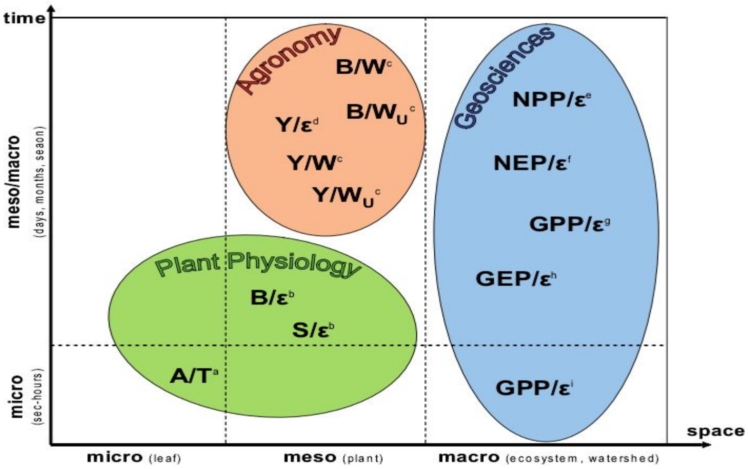


Figure 4 Different calculation types of water-use efficiency (WUE) depending on the scientific discipline (e.g. Plant Physiology, Agronomy, Geosciences) together with temporal and spatial scale (Kuglitsch et al. 2008). A=net CO_2 assimilation, B=total biomass or above ground biomass, S=stem biomass, Y=yield, NPP=net primary production, NEP=net ecosystem production, GPP=gross primary production, GEP=gross ecosystem production, T=transpiration, ε=transpiration or evapotranspiration, W=total admitted water including precipitation, W_U=amount of irrigated water. [a](e.g. Denmead et al., 1993), [b](e.g. Loomis & Connor, 1992), [c](e.g. Jones, 2004), [d](e.g. de Wit, 1958), [e](e.g. Schimel et al., 1997), [f](e.g. Mingkui & Woodward, 1998), [g](e.g. Reichstein et al., 2007), [h](e.g. Law et al., 2000), [i](e.g. Ponton et al., 2006).

Although various definitions of WUE are applied in different scientific disciplines, the common characteristic is that WUE is always a ratio of carbon gain to water loss. As described in Eq. 1, WUE can be defined either on a mass basis or on a molar basis (the value of WUE on a mass basis is 2/3 of the value on a molar basis):

$$WUE = \frac{mass\ C\ fixed}{mass\ H_2O\ transpired} \qquad \text{or} \qquad WUE = \frac{mol\ C\ fixed}{mol\ H_2O\ transpired} \tag{1}$$

Both numerator (the obtained product) and denominator (water invested in the process) of the ratio WUE may be considered at several levels and temporal and spatial scales, consequently, different definitions of WUE can be made. To remove the confusion that arises from the misuse of the terminology in WUE, we will use the definitions summarised in Tab. 2.

Table 2 Definition of WUE at different measurement levels and temporal and spatial scales.

Measurement levels	Equations	Notes
Gas exchange	ITE (Intrinsic WUE) $= \dfrac{A\ (\mu mol\ CO_2\ m^{-2}\ s^{-1})}{g_s\ (mol\ H_2O\ m^{-2}\ s^{-1})}$	Denotes the efficiency of stomatal conductance to water vapour in relation to assimilation of CO_2. (A: CO_2 assimilation rate; g_s: stomatal conductance to water vapour.)
	TE (Instantaneous WUE) $= \dfrac{A_{max}\ (\mu mol\ CO_2\ m^{-2}\ s^{-1})}{E\ (mmol\ H_2O\ m^{-2}\ s^{-1})}$	Represents the amount of evapotranspired water per maximum assimilated carbon. (A_{max}: maximum carbon assimilation rate at saturating light; E: leaf evapotranspiration rate.)
Integrated	WUE_B (Biomass WUE) $= \dfrac{\text{Accumulated dry matter (kg yr}^{-1})}{H_2O \text{ transpired (kg)}}$	Used at plant level.
	WUE_Y (Yield WUE) $= \dfrac{\text{Yield (kg)}}{(E)T_c\ (m^3)}$	Represents water productivity. Used at stand level.
Ecosystem	WUE_e (Ecosystem WUE) $= \dfrac{NPP / NEP / GEP / GPP}{ET}$	Represents ecosystem WUE from eddy-covariance measurements. Used at ecosystem level. (NPP: net primary production; NEP: net ecosystem production; GEP: gross ecosystem production; GPP: gross primary production; ET: water losses.)

At photosynthetic organ (e.g., leaf) scale, WUE is defined as the net CO_2 assimilated by photosynthesis (A), divided by the water transpired in the same time period (E or T); being an instantaneous definition of WUE (TE), the needed measurements are carried out by gas exchange methods (infrared gas analyser and porometry; Polley 2002; Tambussi et al. 2004). One related parameter, the intrinsic water-use efficiency (ITE), is defined as the ratio between CO_2 assimilation rate (A) and stomatal conductance (g_s); carbon isotope discrimination ($\Delta^{13}C$) has been widely accepted as an indicator of ITE (Leonardi et al. 2012). In a broad sense, assimilated dry matter can be considered as the total biomass, commonly referring, to aboveground parts only. Thus, we may also define a biomass water-use efficiency (WUE_B) and a water productivity (WUE_Y) (Pereira et al. 2002; Passioura 2004). Although strongly linked, WUE_B and WUE_Y may indicate different concepts; whilst both may be severely affected by growth, WUE_Y is also influenced by the partition of assimilated in the economical product (i.e., the harvest index (HI) of the crop). However, the denominator (i.e., the consumed water) of WUE_Y can be considered in two ways. Water losses may include, despite water transpired by the plant, the direct evaporation from the soil (E_s; e.g. Ripullone et al. 2004). Thus, the estimation of WUE in experiments carried out in pots (where soil evaporation is commonly eliminated) may be sensibly different with respect to plants grown in the field (Tambussi et al. 2007). Following the application of the eddy covariance technique for the continuous determination of carbon and water fluxes, currently deployed as a network throughout the world enabling scientists to study both the temporal and the large-scale spatial variability of WUE_e, geoscientists and ecologists commonly use the ratio of the main ecosystem fluxes such as net primary production (NPP), net ecosystem production (NEP), gross ecosystem production (GEP) or gross primary production (GPP) to the water losses (ET) (Law et al. 2002; Reichstein et al. 2002), where ET is defined as the total water vapour flux between the canopy and the atmosphere consisting of evaporation from soil (E_S) and plant transpiration and evaporation of the intercepted fraction; the study of such properties has been applied to understanding how future climate change accompanied by hydrological changes will affect the carbon and energy budgets of ecosystems (Kuglitsch et al. 2008).

1.3 Carbon balance

Carbon is one of the most important elements in all life forms, and the carbon cycle is one of the most important processes in all life forms. Forests are the main terrestrial ecosystems, comprising 46.3% of the total carbon pool on land, and forest vegetation carbon pools constitute as much as 77.0% of the terrestrial vegetation carbon pools; the cycling of the forest carbon is the world's most important carbon cycle, and it plays an important role in maintaining global ecological security and the sustainable development of human society. As shown as Fig. 5, nearly all of the primary production in terrestrial ecosystems results from photosynthesis, which is the process by which plants use energy from sunlight to combine carbon dioxide (CO_2) with water to form simple carbohydrates.

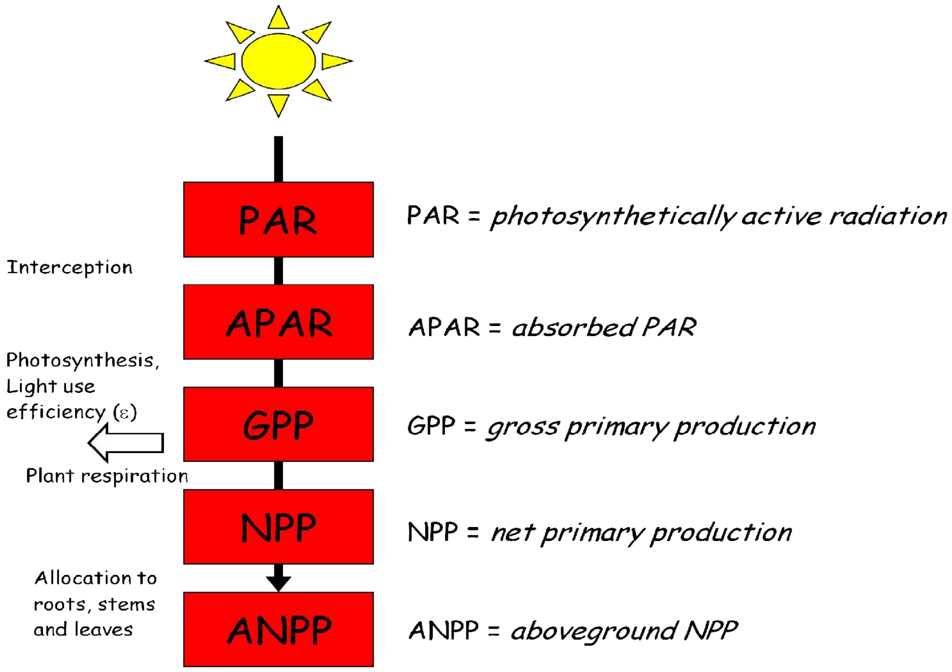


Figure 5 A schematic diagram of the carbon balance.

CO_2 enters the leaf through tiny pores called stomata and the reaction that follows can be generalized as follows:

$$6CO_2 + 6H_2O \xrightarrow{\text{light}} C_6H_{12}O_6 + 6O_2$$

Gross primary production is primarily a function of the total amount of radiation absorbed by the canopy (APAR, absorbed photosynthetically active radiation), which will depend upon PAR (photosynthetically active radiation). Plant gross primary productivity (GPP) can be defined as the sum of photosynthesis over all the leaves in the canopy. GPP is the primary determinant of plant growth and C sequestration by plants, although it is just the first step towards the quantification of the carbon cycle. But an equally important factor is respiration, which is the energy plants must spend to produce new growth and maintain existing live tissue. The overall flux of C at the ecosystem scale (NEP, net ecosystem production) is also a function of the respiration not just by autotrophic green plants (R_a) but also by heterotrophs (R_h):

$$\mathrm{NEP} = \mathrm{GPP} - R_a - R_h$$

Although rates of photosynthesis vary considerably among plant species and even among leaves of a single plant, the necessities of respiration require that the total energy gained via photosynthesis must be greater, on average, than that needed to perform these basic functions. The challenge for plants, then, is attaining an optimal balance between photosynthesis and respiration, which, as we will see, is complicated by the fact that these processes respond differently to environmental variables. To understand primary production in forests, we need to consider the availability of light, water, nutrients and on the physiological composition of a leaf as well as interactions among these factors.

1.4 Effects of N fertilization on water use, WUE and growth

Nitrogen is often the most limiting nutrient for tree growth and carbon sequestration (Kimmins et al. 2010). Approximately 18×10^6 kg of reactive N is globally deposited into forests each year, with marked regional differences in annual deposition rate (Schlesinger 2009). In recent years, a beneficial role of N deposition on forest productivity, and on the terrestrial C sink has been suggested, although the magnitude of this effect is the subject of an intense debate (Pregitzer et al. 2008). It is very important to assess the effects of N deposition on the ecosystem C cycling in order to reduce the uncertainty in ecosystem C sequestration (Heimann & Reichstein 2008; Hungate et al. 2003; Reay et al. 2008; Reich et al. 2006). Increased N fertilization through deposition can affect forest ecosystems in different ways. On one hand, N deposition can increase foliar biomass and photosynthetic efficiency, and thus increase forest ecosystem biomass. On the other hand, N deposition can reduce allocation to fine roots (Litton et al. 2007), reduce respiration rates (Janssens et al. 2010), accelerate N saturation in areas of high nitrogen in soils, and consequently cause leaching loss of N and other nutrients (e.g. base cations) to aquatic systems (Kimmins et al. 2010). Högberg (2007) estimated that input of 1 kg N to the temperate forest ecosystem would increase biomass carbon sequestration by 30 kg C and soil organic matter by 10 kg C, causing a total effect value of 40:1 (C:N). Thomas et al. 2010 reported that the average effect value of N deposition on C sequestration of aboveground tree biomass was C:N=61:1 (51~82:1) and that of the total C sequestration (above and below ground biomass) was C:N=73:1 (61~98:1). In fact, not only the N in forest ecosystems greatly influences total effect of N deposition because of much higher effect value in biomass than that in soil, but also Nadelhoffer et al. (1999) found that soil rather than tree biomass was primary sink of N input that indicated a minor contribution of N deposition to C sequestration by analyzing the data of multisite ^{15}N labeling experiments in temperate forests. In addition, modeling estimation also showed large variation in the contribution of N deposition to the global forest C sequestration, ranging from 0.1 Pg C year^{-1} to more than 2 Pg C year^{-1} (Schindler & Bayley 1993; Townsend et al. 1996; Holland et al. 1997; Ollinger et al. 2002; Churkina et al. 2007; Jain et al. 2009; Dezi et al. 2010; Zaehle et al. 2010). Further, most research on N deposition focuses on boreal and temperate regions where N is generally limited, with much less focus on tropical and sub-tropical regions (de Vries et al. 2008; Hall & Maston 1999; Thomas et al. 2010) or different tree species may have different responses to N deposition even in the same region (Thomas et al. 2010), which are likely to be uncertainty caused by these factors.

Moreover, differences in the response to N deposition could be mediated by C and water interactions under conditions of limited water availability, raising the issue of N effects on water use and water-use efficiency.

In general, increased nutrient availability, due to fertilization, may have potential effects on tree hydraulic properties and ecosystem-level hydrological processes. Water and nutrients often interact to affect tree physiology and growth (Oren & Sheriff 1995). It is likely that increased productivity and foliar biomass, as a result of increased nutrient availability, will increase evapotranspiration (E) due to increased transpiration and rainfall interception rates (Linder et al. 1987). For further assessment of N fertilization on water-use efficiency (WUE), we should combine information about intrinsic water-use efficiency (ITE), mesophyll conductance, carbon isotope discrimination (CID) of specific C pool (sugars, starch, cell walls), estimates of leaf-N pool sizes, leaf morphology and WUE_B; especially the measurement of WUE_B appears to be essential in order to clarify the relevance of apparent N effects on ITE (Brueck 2008).

Some evidence indicates that the portion of respiratory carbon loss is increasing under low N fertilization (van der Werf et al. 1992; Guehl et al. 1995; Poorter et al. 1995; Li et al. 2003). Similarly, low N fertilization will increase the portion of unproductive water loss (Hobbie & Colpaert 2004; Guo et al. 2002, 2006), particularly with regard to bare-soil evaporation. Positive effects of N fertilization on ITE are indicated by both direct measurement of leaf gas exchange (Ripullone et al. 2004; Salifu et al. 2007; Watanabe et al. 2008; Howard & Donovan 2009; Wang et al. 2012) and C-isotope discrimination (Lovelock et al. 2006; Martin et al. 2010; Yin et al. 2012; Pascual et al. 2013). The relevance of the effects of N fertilization on WUE via respiratory carbon loss, ITE and unproductive water losses is illustrated in detail below.

- Respiratory carbon loss

A quantification of N effects on the portion of carbon loss in conjunction with instantaneous water-use efficiency (TE) is highly required, and biomass-partitioning parameters and leaf size should be recorded in order to separate morphological responses from physiological processes which both interactively contribute to variation of TE. For example, Ripullone et al. (2004) showed that instantaneous water-use efficiency (TE) generally increased with increasing N supply in both Douglas-fir and poplar (Fig. 6). When considering WUE at the plant level, additional carbon and water losses have to be considered in addition to those occurring during the photosynthetic carbon gain and the transpiration process, because respiration of non-photosynthetic organs throughout the day and respiration of autotrophic tissues in the dark affect plant carbon balance and non-transpirative (cuticular and lenticellular) water losses influence plant water economy (Farquhar et al. 1989).

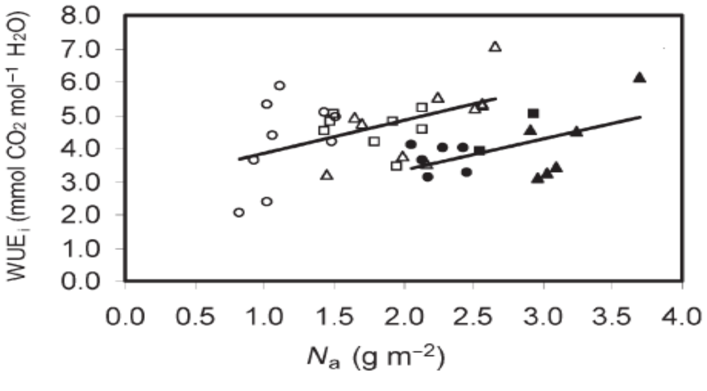


Figure 6 Relationship between instantaneous water-use efficiency (WUE$_i$*=mmol CO$_2$ mol^{-1} H$_2$O) and leaf N concentrations (N$_a$*) in Douglas-fir (open symbols) and poplar (filled symbols). Symbols represent different N treatments: LN (low N supply), circles; MN (intermediate N supply), squares; and HN (high N supply), triangles. The regression relationships are: Douglas-fir, $y=0.97x+2.89$ ($r^2=0.22$, $p<0.02$); Poplar, $y=0.93x+1.48$ ($r^2=0.28$, $p<0.05$). Each value represents an individual plant in panel.

*In this thesis, instantaneous water-use efficiency, abbreviation expressed as TE; leaf N concentration, abbreviation expressed as $N_{\%}$.

- Intrinsic water-use efficiency

The frequently postulated tight coupling between photosynthetic capacity (A) and stomatal conductance (g_s) such that c_i/c_a (c_i and c_a are the ambient and intercellular CO_2 partial pressures) and ITE (ratio A/g_s) remain nearly stable over a range of leaf N concentration ($N_{\%}$) appears to be the exception rather than the rule. However, the increase of ITE with increasing N supply likely exhibits interaction with the light regime (self-shading with increasing leaf-area index), leaf age (N remobilization among leaf layers), leaf morphology (N effects on specific leaf area), leaf carbohydrate metabolism (C metabolite effects on stomatal conductance, repression of enzymes involved in photosynthesis), transient, hidden water stress (higher water consumption of high-N plants), and leaf-to-air vapor-pressure deficit (N effects on surface roughness, de-coupling). Until the relevance of and interaction among these processes is analyzed, it remains open if single-leaf data are representative for whole-plant and canopy processes. For instance, Guehl et al. (1995) showed that the clearly distinct A vs. g_s linear relationships arose for the different nutritional treatments (Fig. 7) reflecting differences in plant ITE (ratio A/g_s); however, the within-treatment variability of A and g_s was large at the individual plant level between oak and pine. The constant (in pine) or decreasing pattern (in oak) of A values with increasing N deficiency were associated with increasing c_i. But increasing g_s in response to N deficiency in pine, constitutes an original result that does not conform with the general observation that g_s is reduced in response to N deficiency while ITE is decreased or maintained constant. The results indicated that these mechanisms do not always bring about a positive coupling between g_s and the CO_2 assimilation capacity.

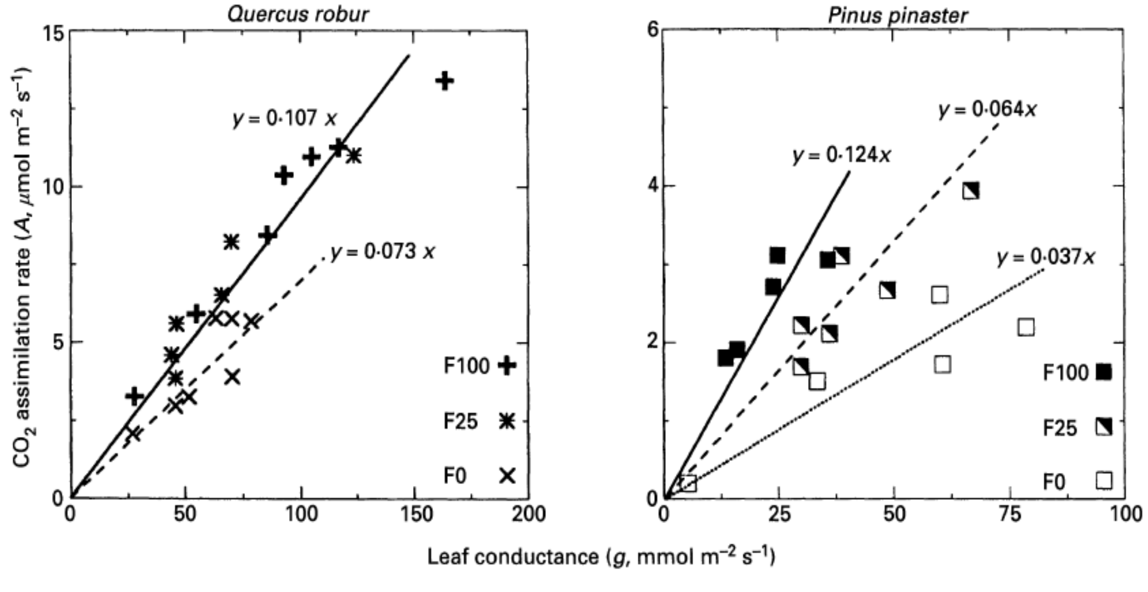


Figure 7 Relationship between CO_2 assimilation rate (A) and leaf conductance for water vapour (g_s) in the different fertilization treatments (F100, optimal fertilization; F25, 25% of optimal fertilization; F0, no fertilization). Regression lines were forced through the origin; their slopes represent average treatment values of instantaneous plant intrinsic water-use efficiency.

- Unproductive water loss

The portion of unproductive water loss appears to be increasing with N limitation. Work on N-induced changes of biomass water-use efficiency (WUE_B) should avoid bare-soil evaporation and include the measurement of nighttime water loss in order to elucidate the relevance of N supply and carbohydrate metabolism on stomatal conductance. For example, Brueck (2008) summarized the effect of different portions of unproductive water loss on WUE_B under variable N supply (Fig. 8). The decrease of WUE_B for a given portion of unproductive water loss is increasing with (N-induced) dry-mass decrease. For a given N-induced growth reduction, the effect of an increase of the portion of unproductive water loss on WUE_B is more pronounced under severe N limitation than under moderate N stress.

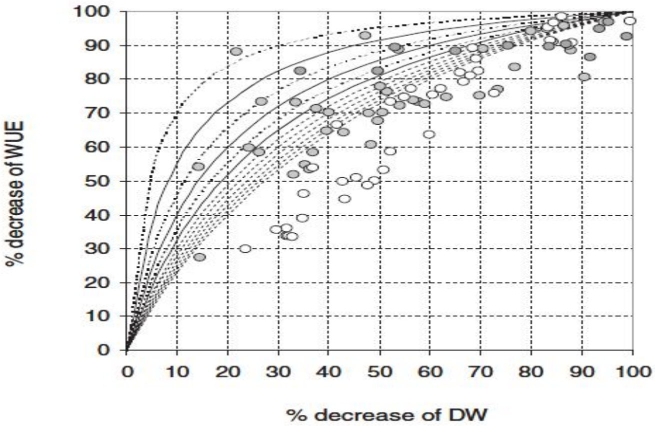


Figure 8 Relationship between percent decrease of biomass and WUE_B of plants under variable N supply. Curves present the expected decrease of WUE_B with increasing portion of unproductive water loss, which is expressed in terms of the total water consumption of plants with a constant internal WUE_B when dry mass is 100%.

Although experiments mostly indicated consistent N effects on these three functional components of WUE, species differences in response to N fertilization indicate that N effects are not straightforward and plants interactively control water-use efficiency by physiological and morphological processes in a very complex manner.

1.5 Meta-analysis

Long gone are the days when tracking a handful of journals would suffice to keep one abreast of important research developments (Curtis & Qeenborough 2012). Over the last 30 years, review papers have shifted from a narrative-driven to a data-driven approach, taking the results from primary research articles and quantitatively analyzing and synthesizing these data in an attempt to arrive at more robust conclusions in a certain research area, in the form of so-called ‘meta-analysis’. It has become the methodological platform of choice in many areas of empirical science, including ecology. In the process, the statistical tool kit available to the ecological meta-analyst has become more sophisticated (Tab. 3) and better adapted to the diverse array of experimental designs and reporting formats favored by field biologists (Hedges & Olkin 1985, Lajeunesse 2011, Koricheva et al. 2012). Concurrent with widening use and increased statistical utility in ecology, the standards of meta-analysis also have been raised. Lajeunesse (2010) put forward general recommendations for a high-quality ecological meta-analysis. Of central importance is transparency in the criteria used to select studies for inclusion in the meta-analysis, with a premium on broad inclusivity.

Table 3 Potentially available meta-analysis tools.

Software	Website	Note
Comprehensive meta-analysis (CMA)	http://www.meta-analysis.com/	Business/Windows
MetaWin	http://www.metawinsoft.com	Business/Windows
DSTAT	http://www.erlbaum.com	Business/Dos
Weasyma	http://www.weasyma.com/	Business/Windows
Review Manager (RevMan)	http://www.cc-ims.net/RevMan/	Free/Windows
Meta-DiSc	http://www.hrc.es/investigacion/metadisc_en.htm	Free/Windows
Meta	http://userpage.fu-berlin.de/-health/meta_e.htm	Free/Dos
EasyMA	http://www.spc.univ-lyon1.fr/easyma.dos/	Free/Dos
MetaTest	http://www.medepi.net/meta/MetaTest.html	Free/Dos
Meta calculator	http://www.lyonsmorris.com/lyons/metaAnalysis/index.cfm	Free/Online
SAS/S-plus/STAT/SPSS	http://www.sas.com, http://www.insightful.com/, http://www.stata.com, http://www.spss.com	Common

As a background, the first goal of meta-analysis is to generalize in a formal way across a number of independent experiments in order to define a suitable effect-size metric (Poorter et al. 2010), such as the value of the variable of interest measured in A divided by its value in environment B (Osenberg et al. 1997, Hedges et al. 1999). For example, the response of various plant traits to specific enviromental factors has been analysed by Searles et al. (2001) for UVB, Morgan et al. (2003) for ozone, and Poorter & Navas (2003) for CO_2. The second goal of a systematic analysis of the literature that could significantly advance our understanding of plant responses to their environment is the quantitative parameterization of such response curves (Poorter et al. 2010). In natural habitat, Wright et al. (2004) described quantitative relationships between leaf morphology, photosynthetic capacity and leaf nitrogen across a wide range of wild species. Subsequently, in the laboratory, Hikosaka & Osone (2009) used the approach to develop further theory on the relationship between growth and nitrogen uptake. The third goal of such an analysis is to analyze retrospectively whether variation in the response to the environment can be ascribed to differences in the experimental design, or to differences between functional groups of species (Poorter et al. 2010). In the case of elevated CO_2, such an approach has been fruitfully used to show that plants grown in small pots were restricted in their response to CO_2 (Arp, 1991) and that C_4 species, although responding less strongly than C_3 species, nonetheless increase biomass at elevated CO_2 concentrations (Poorter, 1993).

There are several inherent steps to meta-analysis, the important ones being summarized in Fig. 9. An important aspect of this type of analysis is the iteration process, which is under the control of the analyst. This circular pattern where prior steps are re-visited and refined is an important aspect of meta-analysis and contribute much to their heuristic characteristic (St-Pierre 2007).

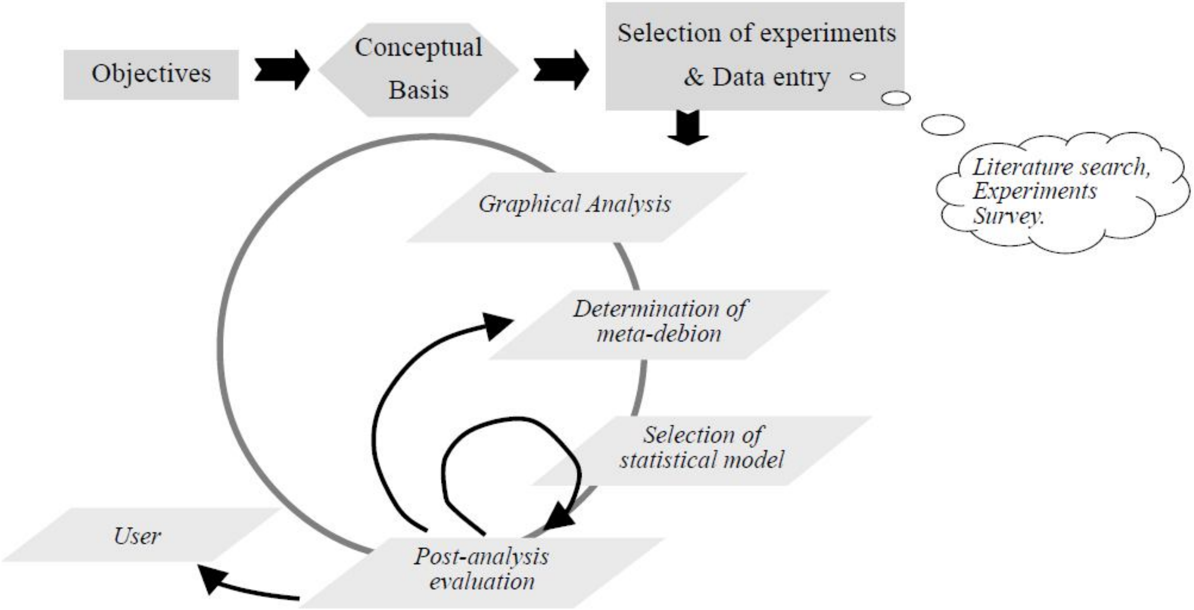


Figure 9 Schematic representation of the meta-analytic process.

Several steps are involved in the process:

(i) Establishing a clear set of study objectives is a critical step that guides most ulterior decisions such as the database structure, data filtering, weighing of observations, and choice of statistical model. Objectives can cover a wide range of targets, ranging from preliminary analyses to identify potential factors affecting a system, thus serving an important role to the formulation of research hypotheses in future experiments, to the quantification of the effect of a nutritional factor such as a specific nutrient fertilization.

(ii) Results from prior research found in the literature must be entered in a database. The structure and coding of the database must include numerous variables identifying the experimental objectives. Hence numerous columns are added to code the objectives of each study which to avoid the improper aggregation of results across studies with very different objectives. The important point is that the sum of these descriptive columns must entirely characterize the objectives of all studies used.

(iii) Data filtering must ensure firstly that the study under consideration is coherent with the objectives of the meta-analysis which dictate that some variables must have been measured and reported. Second, a thorough and critical review of each publication under consideration, focusing on the detection of errors in the reporting of results. This underline the importance of having a highly trained professional involved in this phase of the study. Only after publications have passed this expert quality filter should their results be entered in the database. Third, verification of data entries is important to ensure that a selected publication does not appear to be an outlier with respect to the characteristics and relations under consideration.

(iv) Preliminary graphical analysis is a thorough visual analysis of the data in the essential step to the meta-analytic process. A global view can be formed, regarding the coherence and heterogeneity of the data, as well as to the nature and relative importance of the inter-study and intra-study relationships of prospective variables taken two at a time. Systematic graphical analyses should lead to specific hypotheses and initial selection of alternate statistical models. Graphics can also help identifying observations that appear unique or even outliers. The general structure of relationships can also be identified, such as linear vs. nonlinear relationships as well as the presence of interactions. However, simple X-Y graphics do not correct the observations for the effects of all other variables that can affect Y. The reason is that as the multi dimensions of the data are collapsed into two or possibly three dimension graphics, the unbalance that clearly is an inherent characteristic of meta-analytic data can lead to false visual relationships and not as conclusive evidence. In addition, graphical analyses should also be done in regards to the joint coverage of predictor variables, identifying their possible ranges, plausible ranges, and joint distributions, all being closely related to the inference range.

(v) The meta-analysis design is determined by the structure of the experiments for each of the predictor variables. The experimental design used in each of the studies forming the database must be identified and coded, their relative frequencies calculated and the frequency plots (histograms) can identify areas of focus of prior research. This information can be valuable during the interpretation of the results. One should also consider the intra and inter study variances for the predictor variable. Small intra-study variances reduce the ability of assessing the structural form of the relationship between the predictor and the dependent variable. Large intra-study variances but with only two levels of the predictor variable in all or most of the studies hides completely any potential nonlinear relationships. Another important aspect in the meta-analysis is to determine the significance of the study effect on the predictor variables. We must exert great caution in the interpretation of the results, especially regarding the applicability of these results, because of the relationship between the predictor variable and the dependent variable is dependent on the study, which itself represents the sums of a great many factors such as measurement errors, systematic differences in the methods of measurements of the dependent variable across studies, and, more importantly, latent variables (hidden) not balanced across experiments. In the final, we must graphically investigate the functional form of the relationship between the dependent variable and the predictor variable.

(vi) Because meta-analytic data are extracted from the results of many experiments conducted under many different statistical designs and number of experimental units, the observations (treatment means) have a wide range of standard errors. Intuition and classical statistical theory would indicate that observations should be subjected to some sort of weighing scheme. Under a general linear model where observations have heterogeneous but known variances, maximum likelihood parameters estimates are obtained by weighing each observation by the inverse of its variance. Furthermore, the study effect should be considered random because it represents the sum of the effects of a great many factors, all with relatively small effects on the dependent variable. In general, statistical theory indicates that these effects would be close to Gaussian (normal), thus much better estimated if treated as random effects. In short, the choice of statistical models depends on the sample size (the number of studies in the meta-analysis).

(vii) Lastly, as when fitting conventional statistical models, numerous analyses should follow the fitting of a meta-analytic model. The post-optimization analysis is used to assess the assumptions underlying the model, and to determine whether additional meta-analytic models should be investigated.

Taken into account all the above, meta-analysis can be fruitfully applied to many kinds of phenotypic data in the area of forest ecology, for instance target a larger number of physiological, morphological, chemical, and anatomical plant traits, such as photosynthetic capacity, biomass allocation, nitrogen content and so on. In future analysis, another focal point will be the interaction between different variables among categorical groups. Meta-analysis provides us with a more systematic and formal way to structure information on the response of plants to their environment. This will be advantageous, in understanding both the constraints to plant productivity by limiting factors and the response of plants to global change.

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Chapter 2

Effects of N fertilization on growth and water-use efficiency in hybrid poplar

2.1. Abstract

The effects of nitrogen (N) fertilization on carbon (C) gain in relation to water use were studied in hybrid poplar (*Populus* × *euroamericana*) grown in the greenhouse with three levels of fertilization: high N supply (HN, 100 kg N ha^{-1}), intermediate N supply (MN, 50 kg N ha^{-1}) and control (C, 0 kg N ha^{-1}). There was no significant effect of N fertilization on plant stem height and diameter as well as the properties of leaves, whereas the growth of new leaves was the main effect of N addition and significantly enhanced plant water use, particularly in HN. Leaf N concentration ($N_{\%}$) and N content (N_a) were markedly affected by the diminishing nutrient availability in old sample leaves but not significantly in newly grown leaves. The more severe N deficiency (treatment C) led to decreased growth which was associated with markedly reduced leaf gas exchange characteristics, but CO_2 assimilation rate (A), stomatal conductance (g_s), leaf evapotranspiration rate (E) significantly increased under the N deficient conditions (MN), due to the existence of this response patten in old sample leaves, which was consistent with the increase also observed for plant intrinsic water-use efficiency (ITE, ratio A/g_s) and instantaneous water-use efficiency (TE, ratio A/E); the average values of ITE and TE in all treatments were also positively related to leaf N. Furthermore, there was a pronounced effect on gas exchange parameters between HN and MN in newly grown leaves, except photosynthesis. We speculate that this discrepancy between HN and MN was linked with excess N supply or drought in Mediterranean region altered leaf function for water requirements led to plant-carbon losses through processes like inhibited CO_2 assimilation capacity, and thereby enhanced palnt respiration.

2.2. Introduction

Populus species are potentially the fastest growing trees in North America (McLennan & Mamias 1992) as a bioenergy resource (Luo & Polle 2009) and important material for the pulp and paper industry (Ai & Tschirner 2010). They are associated with a high carbon assimilation rate and consumption of a large amount of water during photosynthesis. Although poplar species are high water-consuming trees, considerable species variability exists in terms of water-use efficiency (WUE), growth performance and hydraulic traits (Monclus et al. 2006; Fichot et al. 2009, 2010; Silim et al. 2009; Yang et al. 2009; Zhang et al. 2010). Therefore, it is of central importance to select appropriate poplar species with relatively high water-use efficiency for growth in semi-arid regions (Cao et al. 2012). *Populus × euroamericana* is well-characterized hybrid of *P. nigra L.* and *P. deltoides*, and was the most drought resistant of five clones compared by Havaux et al. (1988), particularly, is suitable for growing under Mediterranean conditions, where the growth and C sequestration potential of forest ecosystems is severely limited by water availability.

Water constraints could be severely affected by several components of the global change forecasted over the next century: apart from possible changes in precipitation and potential evapotranspiration, an amelioration of the water balance as a result of the steady increase in atmospheric CO_2 concentration is widely expected. What is less commonly considered, however, are the future effects of changes in soil N capital and N availability as a result of atmospheric N deposition. Over the last century, human activities, such as fossil fuel combustion and agricultural fertilizer use, have caused a great increase in anthropogenic N_r emissions, and atmospheric N pollution and deposition over land and oceans (Gruber & Galloway 2008). Soil N availability under a changing climate has the potential to alter plant physiology in terrestrial ecosystems, and might strongly affect the balance between C uptake and water consumption of trees (Vitousek & Howarth 1991; Oren & Sheriff 1995; LeBauer & Treseder 2008). Whilst the impact of N deposition on temperate and boreal forest ecosystems has been the subject of intense research (Magnani et al. 2007; Thomas et al. 2010), the impact of N fertilization and availability in dry climates has been largely neglected. Several studies (Ripullone et al. 2003; Ripullone et al. 2004; Fernandez et al. 2006; Guerrieri et al. 2010; Leonardi et al. 2012), however, suggested that N fertilization could have a positive effect on water-use efficiency (WUE), and therefore on C sequestration in the Mediterranean region (Elhani et al. 2005; Brueck 2008).

Water-use efficiency (WUE) is generally used to evaluate the ability of a plant to use water for biomass production. Furthermore, increased nutrient availability, due to N fertilization, may have potential effects on tree hydraulic properties and ecosystem-level hydrological processes. Water and nutrients often interact to affect tree physiology and growth (Oren & Sheriff 1995). At the leaf level, intrinsic water-use efficiency (ITE) defined as the ratio between net CO_2 assimilation (A) and stomatal conductance (g_s) as estimated from leaf gas exchange is subject to large temporal variability (Flexas et al. 2007; Cao et al. 2012). The impact of N availability on the variation of ITE has been widely investigated, showing quite contrasting results. Livingston et al. (1999), Clearwater & Meinzer (2001), DaMatta et al. (2002), Fernandez et al. (2006) and Forrester et al. (2012) revealed that significant positive correlations of N supply between dry matter production, A/g_s ratio and $\delta^{13}C$ demonstrate which pooled over crosses. However, other studies showed a decrease or absence of any effect. Harvey & van den Driessche (1999), Welander & Ottosson (2000), Ripullone et al. (2003, 2004) and Martin et al. (2010) reported elevated N supply increased both biomass production and water-use efficiency, with positive effects on photosynthetic capacity, but a reduction in stomatal conductance or transpiration was observed in several tree species by gas exchange measurements. Guehl et al. (1995) found increasing water loss control by stomatal conductance, with no influence on assimilation rate. Sheriff & Nambiar (1991), Mitchell & Hinckley (1993), Thomas & Gehlen (1997) and Wang et al. (1998), who found linear relationships between CO_2 assimilation rate and stomatal conductance and, consequently, nearly constant values of c_i/c_a (c_a and c_i are the ambient and intercellular CO_2 partial pressures) and ITE over a broad range of nutrient supplies. On the contrary, Elhani et al. (2005) even showed a negative effect, in contrast to most previous ex situ experiments on young seedlings; the results strengthened the view that N supply does not increase and may reduce WUE in mature stands, at least in the absence of water stress.

In some cases, the reason for these contrasting results remains obscure. Martin et al. (2010) reported high water-use efficiency is achieved at the expense of nitrogen-use efficiency (NUE), as more nitrogen (N) is required for leaf photosynthesis because of leaf chlorophyll concentration and plant growth all increase with the greater N availability (Kutik et al. 1995; Xu et al. 2002; Bondada & Syvertsen 2003; Huang et al. 2008), even though increasing photosynthetic capacity does not compromise C gain, it is unclear how changes in N concentrations affect the coupling between stomatal conductance and CO_2 assimilation (Wong et al. 1979) or the mechanisms underlying the responses of stomatal conductance to altered concentrations of leaf N and intercellular CO_2 (Mitchell & Hinckley 1993; Grassi et al. 2002; Ripullone et al. 2004). In other cases, it is likely that increased productivity and foliar biomass, as a result of increased nutrient availability (Fernandez et al. 2006; Yang et al. 2011; Wang et al. 2012; Yin et al. 2012; Yang et al. 2012) will increase evapotranspiration (E) due to increased transpiration or rainfall interception rates (Linder et al. 1987). Although water and N use are interrelated, especially under water stress conditions (e.g., Mediterranean region), it is often unclear to what extent growth reduction under drought is a consequence of reduced stomatal conductance and CO_2 uptake, or reduced N uptake and accumulation in dry soils (Chaves et al. 2003). Moreover, although experiments mostly indicated consistent N effects on ITE, unproductive C and water loss, there appear to be substantial differences in response of water-use efficiency to N supply among different tree species (DesRochers et al. 2006; Mo et al. 2008; Liu et al. 2011; Cao et al. 2012), environments (Welander

& Ottosson 2000; Lovelock & Feller 2003; Grulke et al. 2005; Tricker et al. 2005; Betson et al. 2007; Guerrieri et al. 2010; Martin et al. 2010; Weih et al. 2011) and treatments (Tjoelker & Luxmoore 1991; Lovelock et al. 2006; Laclau et al. 2008; Watanabe et al. 2008; Forrester et al. 2012) indicating that N effects are not straightforward and plants interactively control water-use efficiency by physiological and morphological processes in a very complex manner.

In the present study, I evaluated growth, gas exchange, and leaf nutrient status of hybrid poplar (*Populus* × *euroamericana*) seedlings in response to N fertilization under greenhouse conditions for 4 months. Current uptake was increased with (NH_4NO_3), which enabled direct quantification of intermediate N (MN) and high N (HN) supply compared with control (C) in growth and water use of hybrid poplars. I tested the hypothesis that (i) fertilization increases photosynthetic rates, stomatal conductance and nutrient uptake in different levels of N fertilizer compared with unfertilized seedlings, and these responses partly explain the greater leaf production and tree growth, (ii) if N influenced intrinsic (ITE) or instantaneous water-use efficiency (TE) through stomatal control of carbon assimilation rates or through changes in tanspirational water loss, (iii) if a relationship exists between SPAD meter readings with N determined by conventional wet-chemistry indices inferring good predictability of leaf N status with SPAD as noted elsewhere.

2.3. Material and methods

2.3.1. Plant material

The experiment was conducted on twenty-one seedlings of hybrid poplar (*Populus* × *euroamericana*) clone AF2, grown in cylindrical pots of 11.3 L capacity containing a mixture of sand and peat (1:1, by volume). The pots were maintained in the greenhouse at the Faculty of Agriculture of the University of Bologna (Italy) with adequate watering conditions (Fig. 1). The temperature and relative humidity was maintained at 25℃ and 50% in the greenhouse during the experiment period, respectively. Clean days prevailed with a photosynthetic photon flux (PPF) of 1000 μmol m^{-2} s^{-1} at the leaf level.

Figure 1 Potted plants of *Populus* × *euroamericana* in the greenhouse.

Populus × *euroamericana* is a widely planted hybrid of *P. nigra* and *P. deltoids*, two species of poplar in the cottonwood (*Aegiros*) section of the genus *Populus*. The two species are widely distributed in the northern hemisphere. It is a medium-sized to large deciduous tree, reaching 20-30 m (rarely 40 m) tall, with a trunk up to 1.5 m diameter (Rushforth 1999). The species is dioecious (male and female flowers on different plants), with flowers in catkins and pollination by wind. The response of water-use efficiency to environmental stress in *P. euroamericana* has also been widely studied (Schmutz et al. 1995; Ibrahim 1998; Harvey & van Den Driessche 1999; Ripullone et al. 2004; Tricker et al. 2005; DesRochers et al. 2006).

2.3.2. Experimental design

On August 22nd 2011， three fertilization treatments were applied: control (C), intermediate (MN) and high (HN) N supply; one of three pots was assigned orderly to each treatment according to the height of hybrid poplars. Nitrogen was supplied as ammonium nitrate (NH_4NO_3) in solution, seven pots were fertilized with 50 kg N ha^{-1} for intermediate (MN) N supply, other seven pots were fertilized with 100 kg N ha^{-1} for high (HN) N supply, whilst the remaining were not fertilized and acted as a control (C). All plants were grown in pots and well watered by adding known amounts of water (below soil saturation) to each pot in order to ensure nutrients remained localized and leaching did not influence trees in other nutrient treatments. We focused on nitrogen (N) because it is the nutrient element most commonly limiting plant growth and because its role in controlling plant growth and metabolism is well understood (Chapin 1980; Epstein & Bloom 2004).

2.3.3. Plants evapotranspiration and soil evaporation measurements

From July to October 2011, daily evapotranspiration (by weight loss every 24 hrs, avoiding the period just after re-watering) of all twenty-one plants were measured by an electronic balance; each plant was covered with an aluminum foil on the top of each pot to minimize evaporation from the soil. From August 8th to 19th 2011, bare-soil evaporation was measured continuously during two weeks on three pots of soil with no plants, each pot was covered an aluminum foil on the top. Transpiration from each pot was calculated as follows:

$$\mathrm{E_T} = \mathrm{I} - (\mathrm{E_S} + \Delta \mathrm{W_S}) \tag{1}$$

where E_T is evapotranspiration, I is water added, E_S is evaporation from the soil and ΔW_S is the change in soil water content over the time interval. Based on ΔW_S values of three pots containing no plants, E_S was found to be negligible. Given the relative plant homogeneity, we assumed that, over the experimental period, plants contributed equally to evapotranspiration from each pot. Therefore, water use of each plant for biomass measurements at the end of the experiment was assumed to be the total evapotranspiration of each plant.

2.3.4. Biomass measurements

The stem height and root collar diameter, and leaf area and retention were measured at the beginning (July 2011), middle (September 2011) and the end (October 2011) of the experiment. All 104 sample leaves, included old sample leaves (n=84, 4 sample leaves per plant in HN, MN and C treatments respectively) were selected at the beginning (July 2011) and new growth leaves (n=20, 10 growth leaves in HN and MN treatments respectively) were selected after harvest (October 2011). Stem root collar diameter and leaf area were measured to the nearest millimeter. Leaf retention was a count of the number of leaves remaining. Data from 4 sample leaves per tree were averaged for each tree, with tree as the independent unit of measure for statistical evaluation. After each measurement, all 104 sample leaves (84 old sample leaves and 20 new growth leaves) were then oven dried at at 70 ℃ for 48 h and dry mass was determined. Specific leaf area (SLA) was calculated as leaf area divided by leaf dry mass, which is one of the most widely accepted key leaf characteristics used for the study of leaf traits.

2.3.5. Leaf biochemical content

Fertilization and nutrient availability have a large effect on the growth and development of plants, largely as a result of changes in chlorophyll and nitrogen content, which together determine the photosynthetic capacity of leaves. But this effect on plant growth is commonly monitored through destructive sampling followed by chemical analysis in the laboratory, which is expensive and time consuming. Instead, there are other methods to measure the nutrient status of plants indirectly, because of the linear relationship often found between optical readings (such as obtained by a SPAD-502 Chlorophyll Meter) and total leaf chlorophyll content. Nitrogen content can be also be indirectly determined by quantifying chlorophyll content (Filella et al. 1995; Moran et al. 2000; Richardson et al. 2002) because of the close correlation between leaf chlorophyll concentration and nitrogen availability (Boochs et al. 1990; Everitt et al. 1985; Yoder & Pettigrewcrosby 1995), making it possible to predict the nutritional status of plants. Moreover, several studies have established the relationship between SPAD-502 readings and total chlorophyll concentration for several plant species (Marquard & Tipton 1987; Schaper & Chacko 1991) reporting simple linear mathematical model fitted in these studies. Some other studies have related SPAD-502 readings with nitrogen content in several plant species (Smeal & Zhang 1994; Peng et al. 1995), making it a promising tool for improving N management. This was confirmed in a previous study by the significantly positive correlation observed in potted *P.* × *euroamericana* seedlings between SPAD-502 reading and both of nitrogen concentration and content.

In the present study, SPAD chlorophyll meter readings were taken in old sample leaves (n=84, 4 sample leaves per plant in HN, MN and C treatments respectively), which were measured each week from July to October 2011 by the portable SPAD-502 chlorophyll meter (SPAD Minolta 502, Minolta LTD., Osaka, Japan), which is a simple and portable diagnostic tool that measures the greenness or the relative chlorophyll content of leaves (Inada 1985) to estimate the effects of fertilization and nutrient availability on plant growth and leaf biochemistry (Chang & Robinson 2003; van den Berg & Perkins 2004). A linear regression mathematical model ($y=0.0502x-0.5489$, $R^2=0.8734$; Fig. 2), between leaf N concentration ($N_{\%}$) and SPAD chlorophyll meter readings for *Populus* × *euroamericana* (derived from previous experimental work in our laboratory by Dr. Ammar Dayyoub in 2011) was used to estimate the leaf N concentration ($N_{\%}$) from the SPAD chlorophyll meter readings. On 21st October 2011, the same model was also used to estimate the leaf N concentration ($N_{\%}$) from the SPAD chlorophyll meter readings in old sample leaves (n=56, 4 sample leaves per plant) and new growth leaves (n=20, 10 growth leaves per treatment) in the different N fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization). Furthermore, the estimated results of the leaf N concentration ($N_{\%}$) were used to calculated leaf N content on a leaf area basis (N_a) from specific leaf area (SLA).

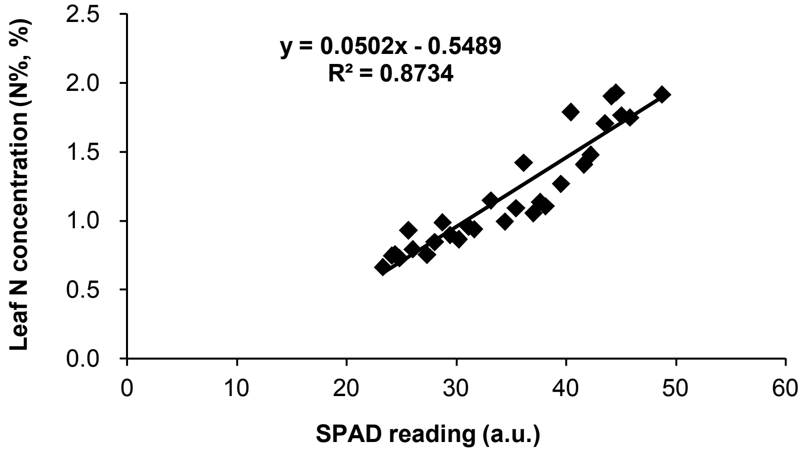


Figure 2 Relationship between leaf N concentration ($N_{\%}$) and SPAD chlorophyll meter readings of *Populus* × *euroamericana* (Data derived from previous experimental work of Dr. Ammar Dayyoub in 2011).

2.3.6. *Gas exchange measurements*

The photosynthetic gas exchange characteristics of a sample of 104 leaves were measured with a portable LiCor Li-6400 gas-exchange analyzer (LI-Cor Inc., Lincoln, NE, USA) in 21 hybrid poplar seedlings (7 pots in HN, MN and C treatments respectively). For old sample leaves (n=84, 4 sample leaves per plant in HN, MN and C treatments respectively), leaf gas exchange characteristics have been measured once a week since August 2011. After the end of the experiment, the same measurements were made on new growth leaves (n=20, 10 growth leaves in HN and MN treatments respectively) on 21st October 2011. Only leaves growing in the upper crown portion of each plant were sampled, fully expanded mature sample leaves exposed to full sunlight (i.e. facing south), were measured with a broadleaf type chamber that enclosed 2×3 cm of leaf surface. All the trees within a particular transect were measured on the same day between 9:30 am and 3:30 pm. Measurements were made on sunny days with little or no cloud cover using the Li-Cor internal halogen lamp source providing a photosynthetic photon flux (PPF) of 1000 μmol m^{-2} s^{-1} at the leaf level, which was close to saturating for photosynthesis under ambient conditions (Ball 1981). The chamber temperature was set at 26±2 °C, this selection of appropriate measurement conditions was based on a study by Ball (1981) that showed maximal assimilation rates of *A. marina* that occurred over a broad temperature range of approximately 20 to 30 °C throughout the year, consistent with the occurrence of daily maximum leaf temperatures. Light-saturated photosynthesis at 380 μmol mol^{-1} (ambient CO_2) was considered as maximum carbon assimilation rate (A_{max}). Moreover, the chamber relative humidity controller maintained a 50% value. The following parameters of leaf gas exchange measurements were recorded under standard conditions to enable comparison across treatments: net carbon assimilation (A), intercellular CO_2 concentration (c_i), stomatal conductance (g_s), transpiration (E), leaf temperature (T) and humidity.

2.3.7. Water-use efficiency

The efficiency of C gain per unit water transpired is termed instantaneous water-use efficiency (TE). A combination of transport equations of maximum carbon assimilation rate at saturating light (A_{max}, $A_{max} = g_{s,c} (c_a - c_i) / p$) and evapotranspiration rate (E, $E = g_{s,w} VPD / p$) gives:

$$\mathrm{TE} = \mathrm{A_{max}/E} = \mathrm{c_a(1 - c_i/c_a)/(1.6\ VPD)} \tag{2}$$

where c_a and c_i are the ambient and intercellular CO_2 partial pressures; $g_{s,c}$ and $g_{s,w}$ are the stomatal conductance for CO_2 and water-vapor transport; VPD is the leaf-to-atmosphere water-vapor partial-pressure difference; p is the atmospheric pressure. Eq. 2 indicates that, for a leaf with constant temperature and a given c_a, an increase of TE can only be achieved by reducing c_i. Leaf temperature and humidity of the atmosphere are highly variable over time confounding comparison of data from different environments or times of a day (Brueck 2008).

A re-arrangement of Eq. 2 gives the so-called intrinsic water-use efficiency (ITE) which is not depending on leaf temperature and humidity effects:

$$\mathrm{ITE} = \mathrm{A/g_{s,c}} = \mathrm{c_a(1 - c_i/c_a)} \tag{3}$$

Eq. 3 indicates that for a given c_a, an increase of ITE can only be achieved by reducing c_i. A decrease in g_s leads to an (almost) linear decline in transpiration, while photosynthesis decreases relatively less. This usually results in a reduction of c_i, and an increase of ITE in response to mild water deficit. Contrarily, an increase in g_s will reduce ITE because of a proportionately greater raise in transpiration than in photosynthetic rate (Chaves et al. 2004).

Assimilation rate can also be expressed in terms of internal conductance (g_m) and the drop of partial pressure of CO_2 between intercellular air space and the site of carboxylation (c_c), $A = g_m (c_i - c_c)$, although it must be considered that, due to photorespiratory CO_2 release, the classical transport equation strictly does not apply and conductances should be expressed by Kirchhoff's second law (Nobel 1999). Intrinsic water-use efficiency (ITE) then is given by:

$$\mathrm{ITE} = A/g_{s,w} = g_m(c_i - c_c)/(0.6\, g_{s,c}) \qquad (4)$$

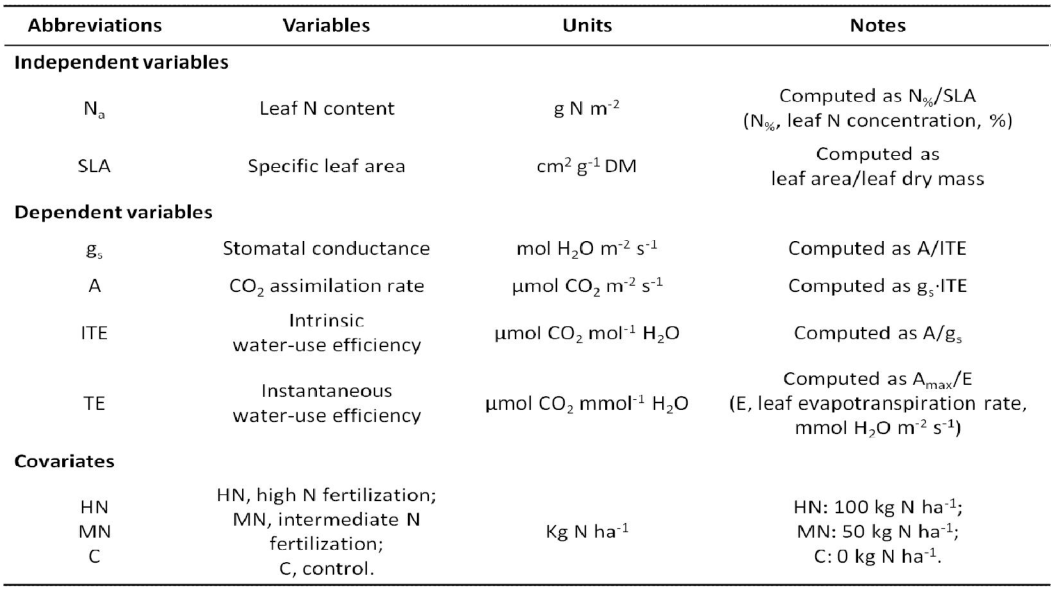
Eq. 4 has the disadvantage that neither c_c nor g_m are easily to measure but allows for a formal differentitation between stomatal and non-stomatal effects on ITE. An increase of ITE can be achieved by a reduction of stomatal conductance, an increase of internal conductance and, for a given intercellular CO_2 partial pressure, by a lower c_c. Variations in g_m and c_c represent non-stomatal effects, which are related to morphological characteristics of the leaf and to photosynthetic capacity (Warren & Adams 2006).

According to the above, for this experiment, water-use efficiency was estimated by TE and ITE that were calculated from gas exchange measurements on individual plants, as the ratio of maximum carbon assimilation rate at saturating light (A_{max}) to leaf evapotranspiration rate (E), and the ratio of CO_2 assimilation rate (A) to stomatal conductance (g_s), respectively (Dang et al. 1991; Zhang & Marshall 1994).

2.3.8. Variable calculations and statistical analysis

All statistics and simulations were performed (Tab. 1) by a split-plot analysis of variance (ANOVA) using the R statistical suite (R Development Core Team, 2012), the same free and open-source software used by Limpens et al. (2011, 2012). Treatment factors were set as N fertilization, and the analysis was blocked by different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization). Residual plots from all ANOVA were inspected to determine the appropriateness of using ANOVA. The data were analysed by a t-test for unequal variances. Results from ANOVA were displayed graphically using adjusted means and standard error bars, or in tabular format using adjusted means and superscripts to identify.

Table 1 Description and interrelation of independent and dependent variables and covariates used in the experiment.



Abbreviations	Variables	Units	Notes
Independent variables			
N_a	Leaf N content	$g\ N\ m^{-2}$	Computed as $N_{\%}$/SLA ($N_{\%}$, leaf N concentration, %)
SLA	Specific leaf area	$cm^2\ g^{-1}$ DM	Computed as leaf area/leaf dry mass
Dependent variables			
g_s	Stomatal conductance	mol $H_2O\ m^{-2}\ s^{-1}$	Computed as A/ITE
A	CO_2 assimilation rate	µmol $CO_2\ m^{-2}\ s^{-1}$	Computed as g_s·ITE
ITE	Intrinsic water-use efficiency	µmol CO_2 mol^{-1} H_2O	Computed as A/g_s
TE	Instantaneous water-use efficiency	µmol CO_2 $mmol^{-1}$ H_2O	Computed as A_{max}/E (E, leaf evapotranspiration rate, mmol $H_2O\ m^{-2}\ s^{-1}$)
Covariates			
HN MN C	HN, high N fertilization; MN, intermediate N fertilization; C, control.	Kg N ha^{-1}	HN: 100 kg N ha^{-1}; MN: 50 kg N ha^{-1}; C: 0 kg N ha^{-1}.

2.4. Results and discussion

2.4.1. Effects of N fertilization on growth

The seedlings in C treatment were stunted with less-developed growth than those in N fertilization treatments (Tab. 2). Stem elongation and root-collar diameter increased with increasing N supply, the difference in stem height was more pronounced in HN (-12%) than in MN (-3%) as compared with C respectively, however, these characteristics were generally not influenced by N fertilization.

Table 2 Stem height and root-collar diameter of *Populus* × *euroamericana* seedlings under the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization) at the end of the experiment.

Treatment	Height (cm)	Diameter (mm)
HN	159.00 ± 11.90^a	14.29 ± 0.93^a
MN	145.29 ± 9.57^b	14.00 ± 0.65^b
C	140.57 ± 8.52^c	12.71 ± 0.83^c
F	1.05	1.25
P	NS	NS

The *F*-value is the analysis of variance test (ANOVA) for the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization). Values are means ± standard error (n=7, 7 trees per treatment) and values with same superscript do not differ significantly (NS) according to the least significant difference test ($P > 0.05$).

Likewise, there were few differences in the properties of leaves between C and N fertilization (Tab. 3). The leaf area, leaf dry mass and specific leaf area (SLA) of new growth leaves were larger in HN, there was also a tendency for all of the properties of old sample leaves to be greater in nutrient enrichment, although these differences were not significant.

Table 3 Old sample and new growth leaf characteristics of *Populus* × *euroamericana* seedlings under the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization) at the end of the growing period.

Treatment	Leaf area (cm^2 per leaf)	Leaf dry mass (g)	SLA (cm^2 g^{-1} DM)
Old sample leaves			
HN	21.27 ± 1.40^a	0.31 ± 0.02^a	68.82 ± 1.06^a
MN	24.22 ± 1.98^b	0.36 ± 0.03^b	66.33 ± 1.21^b
C	22.87 ± 1.93^c	0.34 ± 0.03^c	67.64 ± 1.42^c
F	0.70	1.25	1.05
P	NS	NS	NS
New growth leaves			
HN	27.91 ± 2.53^a	0.31 ± 0.04^a	94.32 ± 4.35^a
MN	22.13 ± 2.01^b	0.26 ± 0.03^b	88.97 ± 4.02^b
F	3.55	1.11	0.91
P	NS	NS	NS

The *F*-value is the analysis of variance test (ANOVA) for the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization). Values are means ± standard error in old sample leaves (n=28, 4 leaves per tree × 7 trees per treatment) and new growth leaves (n=10, 10 leaves per treatment) and values with same superscript do not differ significantly (NS) according to the least significant difference test ($P > 0.05$). SLA: specific leaf area.

Fertilization resulted in a significant increase in number of leaves ($P<0.001$, Fig. 3), the seedlings put forth new leaves in HN and MN obviously, whereas leaves were only lost in C during the whole growing period. Before 27th September, new growth leaves increased rapidly in HN over time and then decreased slightly, nevertheless, the number of newly grown leaves in HN was much higher than in MN at the end of experiment.

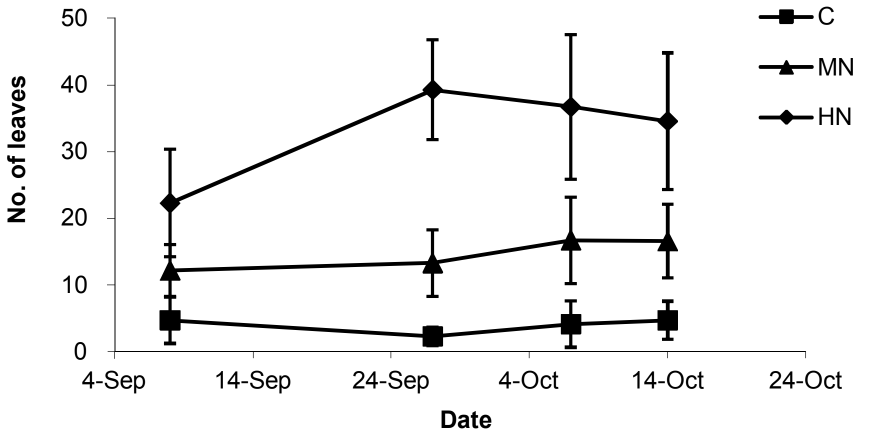


Figure 3 The change in number of leaves of *Populus* × *euroamericana* seedlings under the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization) over the whole growing period. Values are adjusted means from analysis of variance (ANOVA) in each treatment. Error bars represent standard errors (n=7, 7 trees per treatment).

2.4.2. *Effects of N fertilization on leaf biochemistry*

Leaf N concentration ($N_{\%}$, Fig. 4c), as estimated from SPAD readings (Fig. 4a) of old sample leaves and the linear regression mathematical model, was significantly affected by N fertilization as main effects (Tab. 4). The estimated results of the leaf N concentration ($N_{\%}$) could be used to calculate leaf N content (N_a, Fig. 4e) from specific leaf area (SLA). However, since leaf area, leaf dry mass and SLA did not respond to nutrient enrichment as shown in Tab. 3, there was also a significant variation in leaf N content (N_a) as presented in Tab. 4. Overall, the different influcences of N fertilization on leaf chlorophyll content according to SPAD readings were more pronounced in HN (20%) than in MN (16%) compared with C respectively (Fig. 4b). Leaf N concentration ($N_{\%}$) increased to 29% and 23% in response to N supply, for HN and MN treatments respectively (Fig. 4d). Leaf N content (N_a) per unit leaf area differed significantly according to N fertilization and was 24% greater in HN and 20% greater in MN than in C respectively (Fig. 4f).

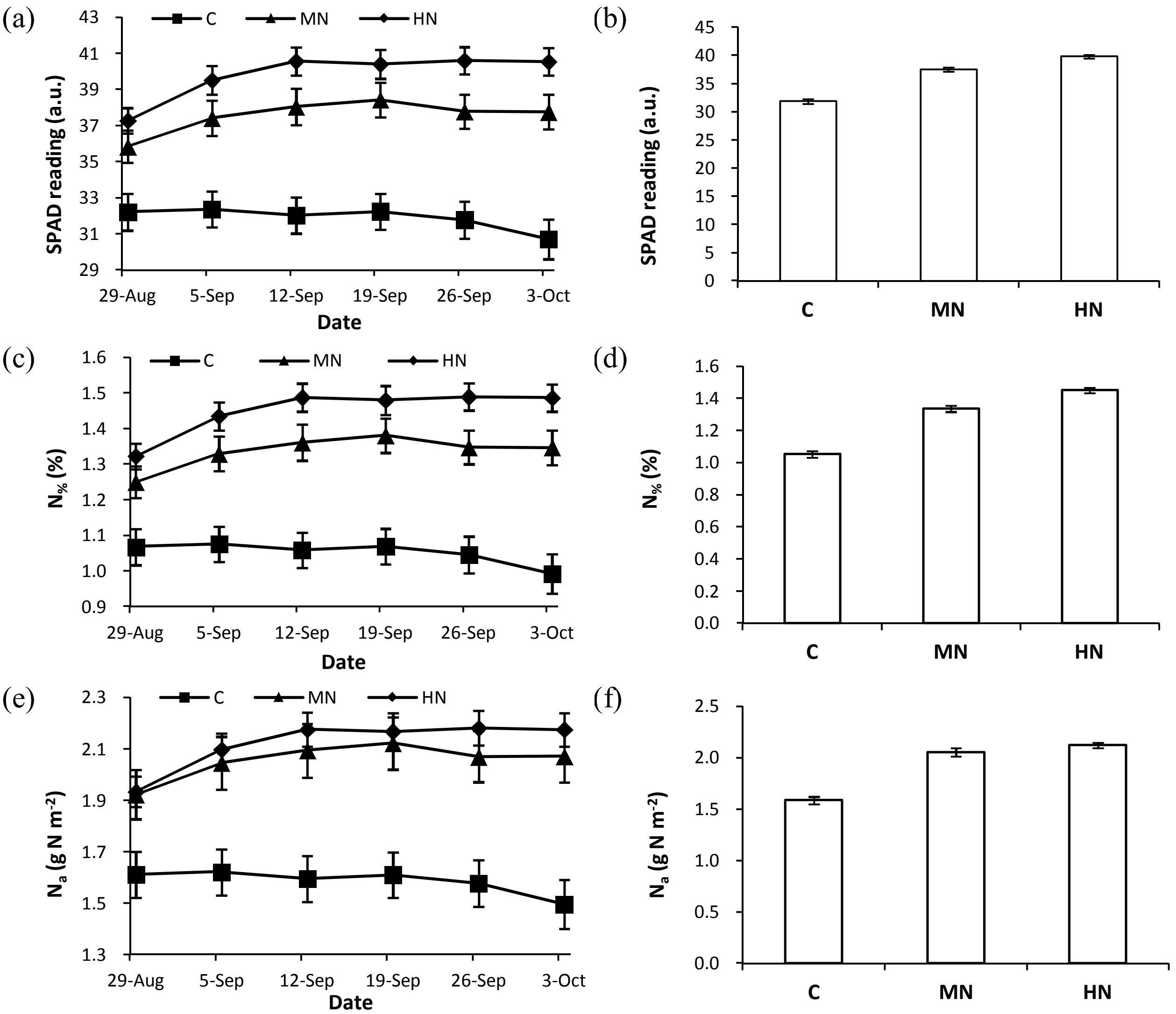


Figure 4 Weekly variation in (a) SPAD reading (a.u.), (c) leaf N concentration ($N_{\%}$, %) and (e) leaf N content (N_a, g N m^{-2}), and the overall influence of fertilization treatment on (b) SPAD reading (a.u.), (d) leaf N concentration ($N_{\%}$, %) and (f) leaf N content (N_a, g N m^{-2}) averaged in old sample leaves of *Populus* ×

euroamericana seedlings under the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization) during the sampling period. Values are adjusted means from analysis of variance (ANOVA) in each treatment. Error bars represent standard errors (n=28, 4 leaves per tree × 7 trees per treatment).

Table 4 The variation of leaf biochemical content in old sample leaves of *Populus* × *euroamericana* seedlings under the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization) during the sampling period.

Variable	Treatment			*F*	*P*
	HN	MN	C		
$N_{\%}$ (%)	1.45 ± 0.04^a	1.34 ± 0.05^b	1.05 ± 0.05^c	121.30	***
N_a (g N m^{-2})	2.12 ± 0.07^a	2.05 ± 0.10^b	1.59 ± 0.09^c	70.74	***

The *F*-value is the analysis of variance test (ANOVA) for the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization). Values are means ± standard error in old sample leaves (n=168, 4 leaves per tree × 7 trees per treatment × 6 measurements) and values with same superscript do not differ significantly (NS) according to the least significant difference test ($P > 0.05$). An asterisk (*) denotes variables differing significantly in the different fertilization treatments using a paired t-test: ***indicates values statistically different at $P < 0.001$, **indicates those different at $P < 0.01$ and *indicates those different at $P < 0.05$. $N_{\%}$: leaf N concentration; N_a: leaf N content.

SPAD readings (data not shown) of individual old sample leaves and new growth leaves were measured under the different N fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization) on 21st October 2011. According to above, leaf N concentration ($N_{\%}$, Fig. 5a) increased with increasing N supply in both old sample leaves and new growth leaves. There was also the same tendency for leaf N content (N_a, Fig. 5b) in new growth leaves but not in old sample leaves. In general, old sample leaves had higher leaf N concentration ($N_{\%}$) and leaf N content (N_a), except leaf N concentration ($N_{\%}$) in HN, which was greater in new growth leaves. However, all of these differences were not significant (Tab. 5) in both old sample leaves and new growth leaves.

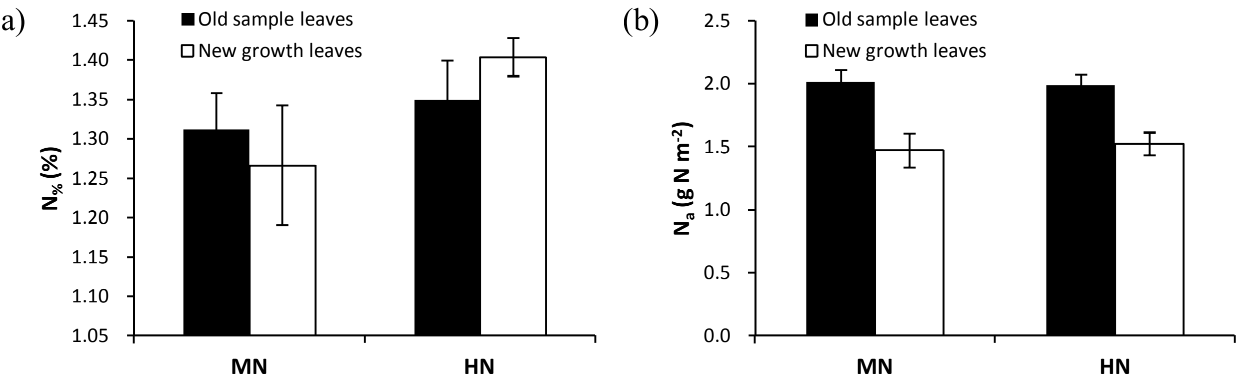


Figure 5 Contrast of (a) leaf N concentration ($N_{\%}$, %) and (b) leaf N content (N_a, g N m^{-2}) between old sample leaves and new growth leaves of *Populus* × *euroamericana* seedlings under the different N fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization) on 21st October 2011. Values are adjusted means from analysis of variance (ANOVA) in each treatment. Error bars represent standard errors in old sample leaves (n=28, 4 leaves per tree × 7 trees per treatment) and new growth leaves (n=10, 10 leaves per treatment).

Table 5 The variation of leaf biochemical content between old sample leaves and new growth leaves of *Populus × euroamericana* seedlings under the different N fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization) on 21st October.

Variable	Old sample leaves				New growth leaves			
	Treatment		*F*	*P*	Treatment		*F*	*P*
	HN	MN			HN	MN		
$N_{\%}$ (%)	1.35 ± 0.05^a	1.31 ± 0.05^b	0.32	NS	1.40 ± 0.02^a	1.27 ± 0.08^b	3.30	NS
N_a (g N m^{-2})	1.99 ± 0.09^a	2.01 ± 0.09^b	0.05	NS	1.52 ± 0.09^a	1.47 ± 0.14^b	0.11	NS

The *F*-value is the analysis of variance test (ANOVA) for the different N fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization). Values are means ± standard error in old sample leaves (n=28, 4 leaves per tree × 7 trees per treatment) and new growth leaves (n=10, 10 leaves per treatment) and values with same superscript do not differ significantly (NS) according to the least significant difference test ($P > 0.05$). $N_{\%}$: leaf N concentration; N_a: leaf N content.

2.4.3. Effects of N fertilization on water use

The evapotranspirational water use of the plants ($P<0.001$, Fig. 6a) increased significantly in response to N fertilization over time. As compared with C as shown in Fig. 6b, plant water consumption was markedly greater in HN than in MN, with a much greater biomass production (i.e. new growth leaves as main effects, $P<0.001$, Fig. 3) under the optimal N condition.

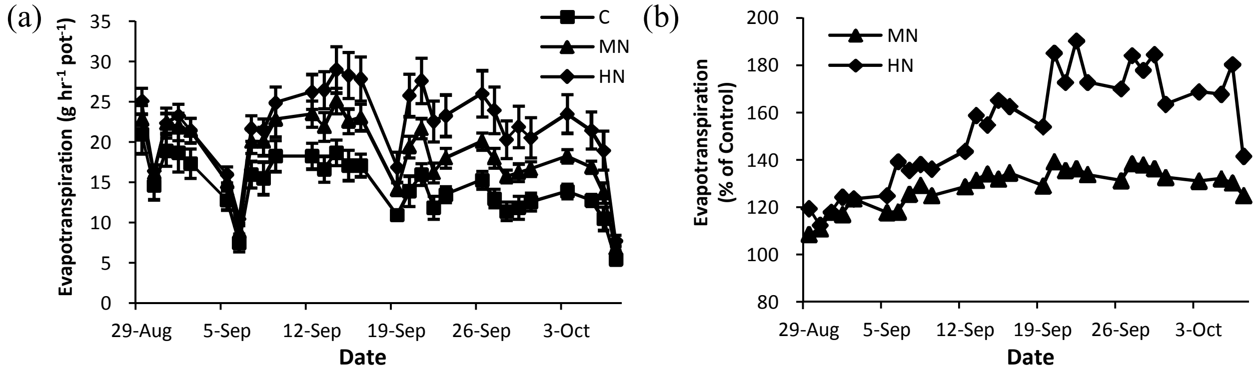


Figure 6 Daily trends of (a) evapotranspiration and (b) the percentage of evapotranspiration between different N fertilization treatments and control of *Populus × euroamericana* seedlings under the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization) during the experiment period. Values are adjusted means from analysis of variance (ANOVA) in each treatment. Error bars represent standard errors (n=7, 7 trees per treatment).

2.4.4. Effects of N fertilization on water-use efficiency

At the leaf level, the weekly variation in gas exchange characteristics were similar under the different fertilization treatments, except for intercellular CO_2 partial pressures (Fig. 7). In general, the most remarkable influences of N addition on gas exchange characteristics were the observed increments in CO_2 assimilation rates (A, Fig. 7a), stomatal conductance (g_s, Fig. 7b) and leaf evapotranspiration rates (E, Fig. 7c) compared with C treatment, whereas intercellular CO_2 partial pressures (c_i, Fig. 7d) was reduced between HN and MN but increased in C. Fertilization with N induced changes in leaf function that also resulted in significantly greater intrinsic water-use efficiency (ITE, the ratio A/g_s, Fig. 7e) and instantaneous water-use efficiency (TE, the ratio A/E, Fig. 7f), as estimated from gas exchange measurements as mentioned above.

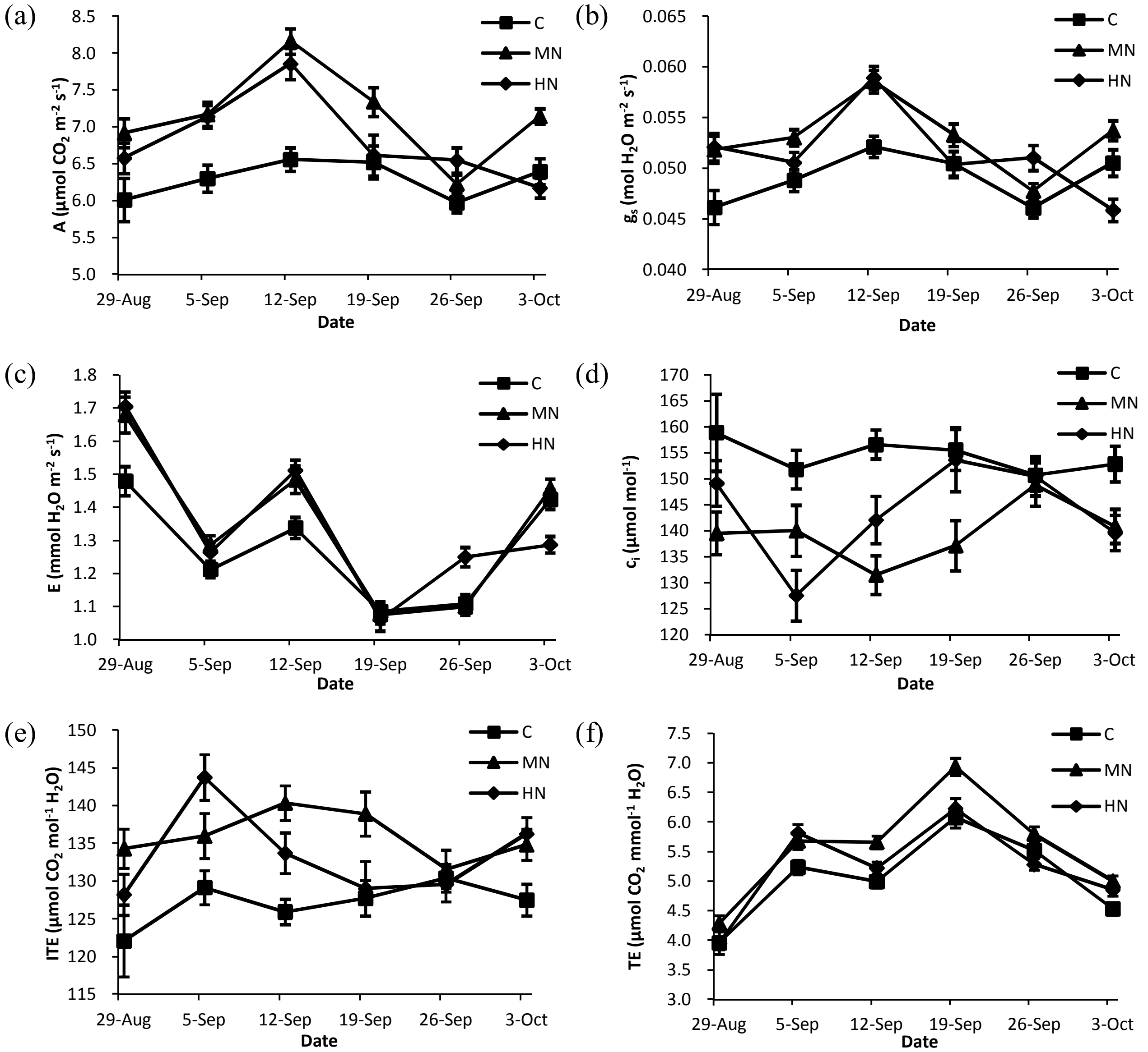


Figure 7 Weekly variation of (a) CO_2 assimilation rate (A, μmol CO_2 m^{-2} s^{-1}), (b) stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), (c) leaf evapotranspiration rate (E, mmol H_2O m^{-2} s^{-1}), (d) intercellular CO_2 partial pressure (c_i, μmol mol^{-1}), (e) intrinsic water-use efficiency (ITE, μmol CO_2 mol^{-1} H_2O) and (f) instantaneous water-use efficiency (TE, μmol CO_2 $mmol^{-1}$ H_2O) in old sample leaves of *Populus* × *euroamericana* seedlings under the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization) during the sampling period. Values are adjusted means from analysis of variance (ANOVA) in each treatment. Error bars represent standard errors in old sample leaves (n=84, 4 leaves per tree × 3 replicates per leaf × 7 trees per treatment).

Gas exchange charateristics varied similarly with N fertilization between HN and MN in CO_2 assimilation rates (A), stomatal conductance (g_s) and leaf evapotranspiration rates (E), but a different pattern was observed in intercellular CO_2 partial pressures (c_i). Generally, CO_2

assimilation rates (A, Fig. 8a), stomatal conductance (g_s, Fig. 8b) and leaf evapotranspiration rates (E, Fig. 8c) were lower in HN than in MN, whereas intercellular CO_2 partial pressures (c_i, Fig. 8d) was higher in HN than in MN but the highest in C. Commonly, intrinsic water-use efficiency (ITE, Fig. 8e) and instantaneous water-use efficiency (TE, Fig. 8f) were less in HN than in MN. However, there was a pronounced effect whereby gas exchange parameters for N fertilization differed significantly in old sample leaves (Tab. 6).

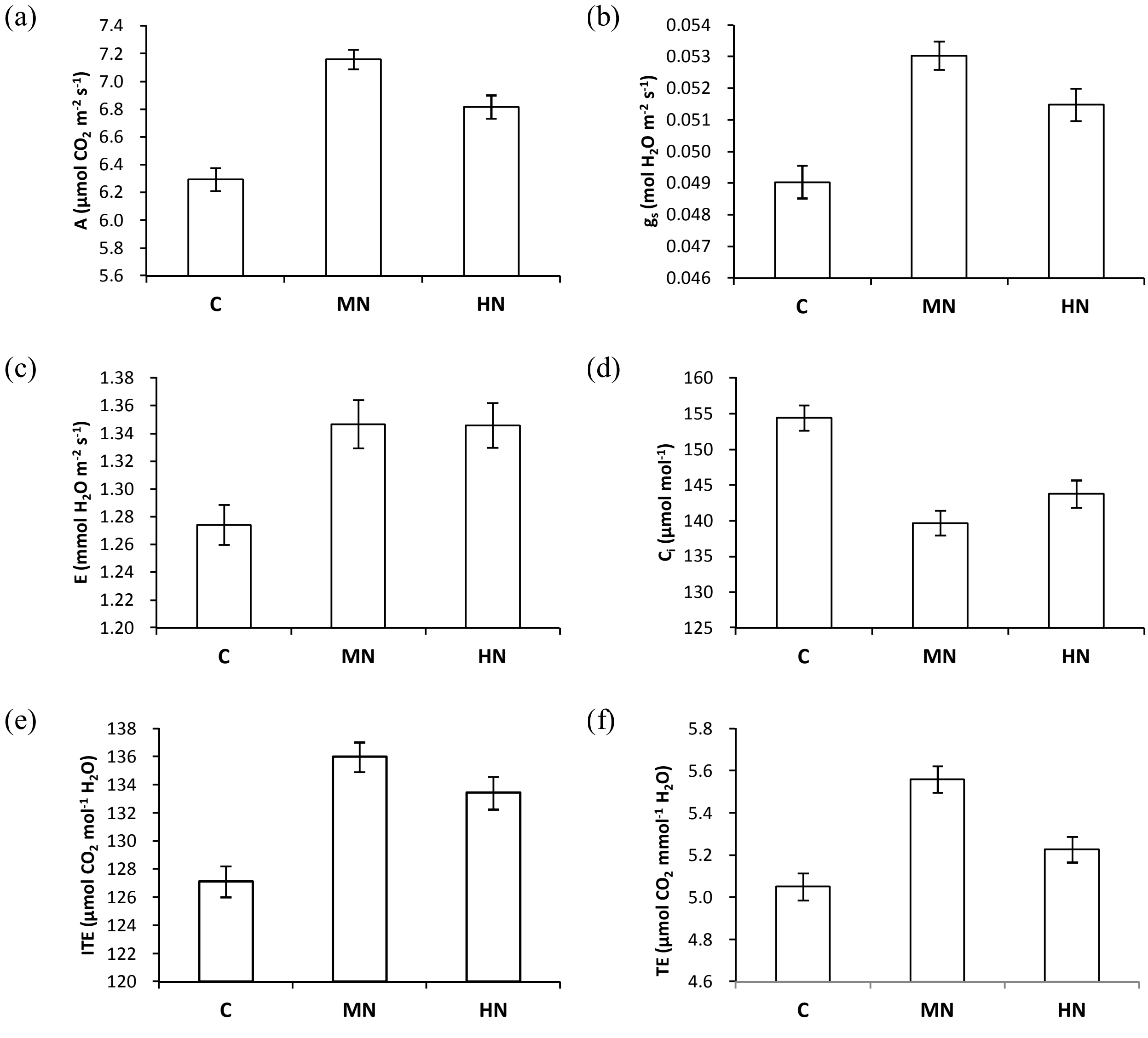


Figure 8 Overall influence of fertilization treatment on (a) CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}), (b) stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), (c) leaf evapotranspiration rate (E, mmol H_2O m^{-2} s^{-1}), (d) intercellular CO_2 partial pressure (c_i, µmol mol^{-1}), (e) intrinsic water-use efficiency (ITE, µmol CO_2 mol^{-1} H_2O) and (f) instantaneous water-use efficiency (TE, µmol CO_2 $mmol^{-1}$ H_2O) averaged in old sample leaves of *Populus* × *euroamericana* seedlings in the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization) during the sampling period. Values are adjusted means from analysis of variance (ANOVA) in each treatment. Error bars represent standard errors in old sample leaves (n=504, 4 leaves per tree × 3 replicates per leaf × 6 measurements per week × 7 trees per treatment).

Table 6 The variation of leaf gas exchange characteristics in old sample leaves of *Populus* × *euroamericana* seedlings in the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization) during the sampling period.

Variable	Treatment			*F*	*P*
	HN	MN	C		
A (μmol CO_2 m^{-2} s^{-1})	6.82 ± 0.08^a	7.16 ± 0.07^b	6.29 ± 0.08^c	30.55	***
g_s (mmol H_2O m^{-2} s^{-1})	51.48 ± 0.52^a	53.03 ± 0.44^b	49.03 ± 0.51^c	16.83	***
E (mmol H_2O m^{-2} s^{-1})	1.346 ± 0.016^a	1.347 ± 0.017^b	1.274 ± 0.014^c	6.73	**
c_i (μmol mol^{-1})	143.75 ± 1.90^a	139.66 ± 1.72^b	154.39 ± 1.76^c	18.01	***
ITE (μmol CO_2 mol^{-1} H_2O)	133.42 ± 1.16^a	135.99 ± 1.05^b	127.13 ± 1.10^c	17.01	***
TE (μmol CO_2 $mmol^{-1}$ H_2O)	5.23 ± 0.06^a	5.56 ± 0.06^b	5.05 ± 0.06^c	17.36	***

The *F*-value is the analysis of variance test (ANOVA) for the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization). Values are means ± standard error in old sample leaves (n=504, 4 leaves per tree × 3 replicates per leaf × 6 measurements per week × 7 trees per treatment) and values with same superscript do not differ significantly (NS) according to the least significant difference test ($P > 0.05$). An asterisk (*) denotes variables differing significantly in the different fertilization treatments using a paired t-test: ***indicates values statistically different at $P < 0.001$, **indicates those different at $P < 0.01$ and *indicates those different at $P < 0.05$. A: CO_2 assimilation rate; g_s: stomatal conductance; E: leaf evapotranspiration rate; c_i: intercellular CO_2 partial pressure; ITE: intrinsic water-use efficiency; TE: instantaneous water-use efficiency.

In old sample leaves, photosynthetic carbon gain for the different fertilization treatments showed a positive linear relationships with both stomatal conductance (g_s, Fig. 9a) and leaf evapotranspiration rates (E, Fig. 9b), reflecting differences in plant intrinsic water-use efficiency (ITE, ratio A/g_s) or instantaneous water-use efficiency (TE, ratio A/E), respectively. The sensitivity of both ITE and TE was greatest in MN, both in terms of changes in A in response to changes in g_s (i.e. the slope of the A vs. g_s relationship) or A to changes in E (i.e. the slope of the A vs. E relationship). The relationship of average A/g_s (i.e. ITE) values of all treatments was expressed by a regression line through the origin ($y=132.09x$, $R^2=0.8485$, $P<0.001$, the solid line shown in Fig. 9a), and also the relationship of average A/E (i.e. TE) values of all treatments was expressed by a regression line through the origin ($y=5.1126x$, $R^2=0.6635$, $P<0.001$, the solid line shown in Fig. 9b). Although there was rarely pronounced differences in both A/g_s and A/E among different treatments, A/g_s was slightly decreased by natural condition (C) and increased by nutrient enrichments (HN and MN) as compared with the average A/g_s values of all treatments, and also the increase in A/g_s in MN was higher than in HN. Likewise, A/E was slightly increased by lowered fertilization (MN) but decreased by increased or suppressed fertilization (HN and C) as compared with the average A/E values of all treatments, and also the decrease in A/E in C was lower than in HN.

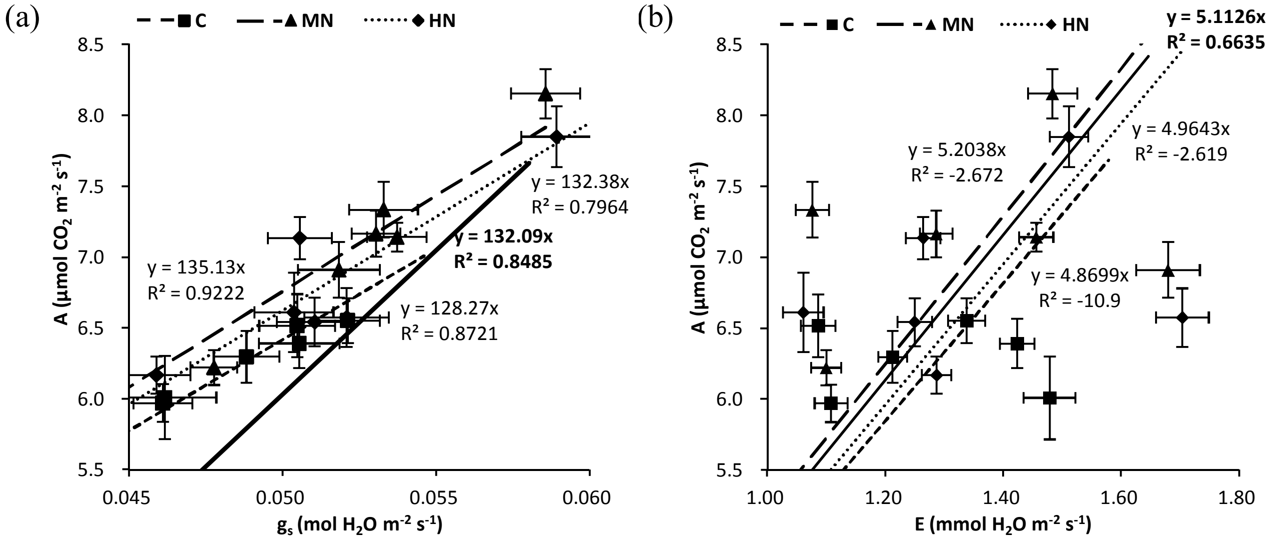
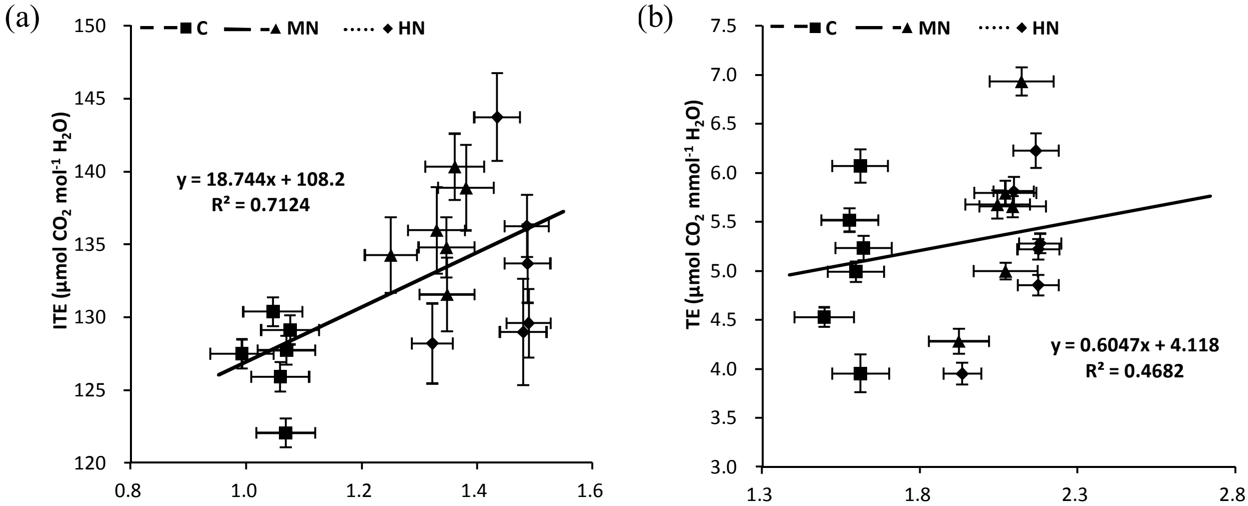


Figure 9 Relationship between CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}) and (a) stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) or (b) leaf evapotranspiration rate (E, mmol H_2O m^{-2} s^{-1}) in old sample leaves of *Populus* × *euroamericana* seedlings under the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization). Regression lines were forced through the origin. The solid lines illustrate average all treatments values of plant intrinsic water-use efficiency (ITE, given in the figure a) or instantaneous water-use efficiency (TE, given in the figure b). The different dashed lines illustrate average each treatment values between A and g_s (given in the figure a) or between A and E (given in the figure b). Values are adjusted means from analysis of variance (ANOVA) in each treatment. Error bars represent standard errors in old sample leaves (n=84, 4 leaves per tree × 3 replicates per leaf × 7 trees per treatment).

The average intrinsic water-use efficiency (ITE, Fig. 10a), CO_2 assimilation rates (A, Fig. 10c) and stomatal conductance (g_s, Fig. 10e) values of all treatments were all positively related to leaf N concentration ($N_{\%}$) in old sample leaves. Also instantaneous water-use efficiency (TE, Fig. 10b), CO_2 assimilation rates (A, Fig. 10d) and leaf evapotranspiration rates (E, Fig. 10f) values of all treatments were all positively related to leaf N content (N_a) in old sample leaves respectively. The increase in ITE in response to all treatments with increasing leaf N concentration ($N_{\%}$) was markedly more pronounced than others.



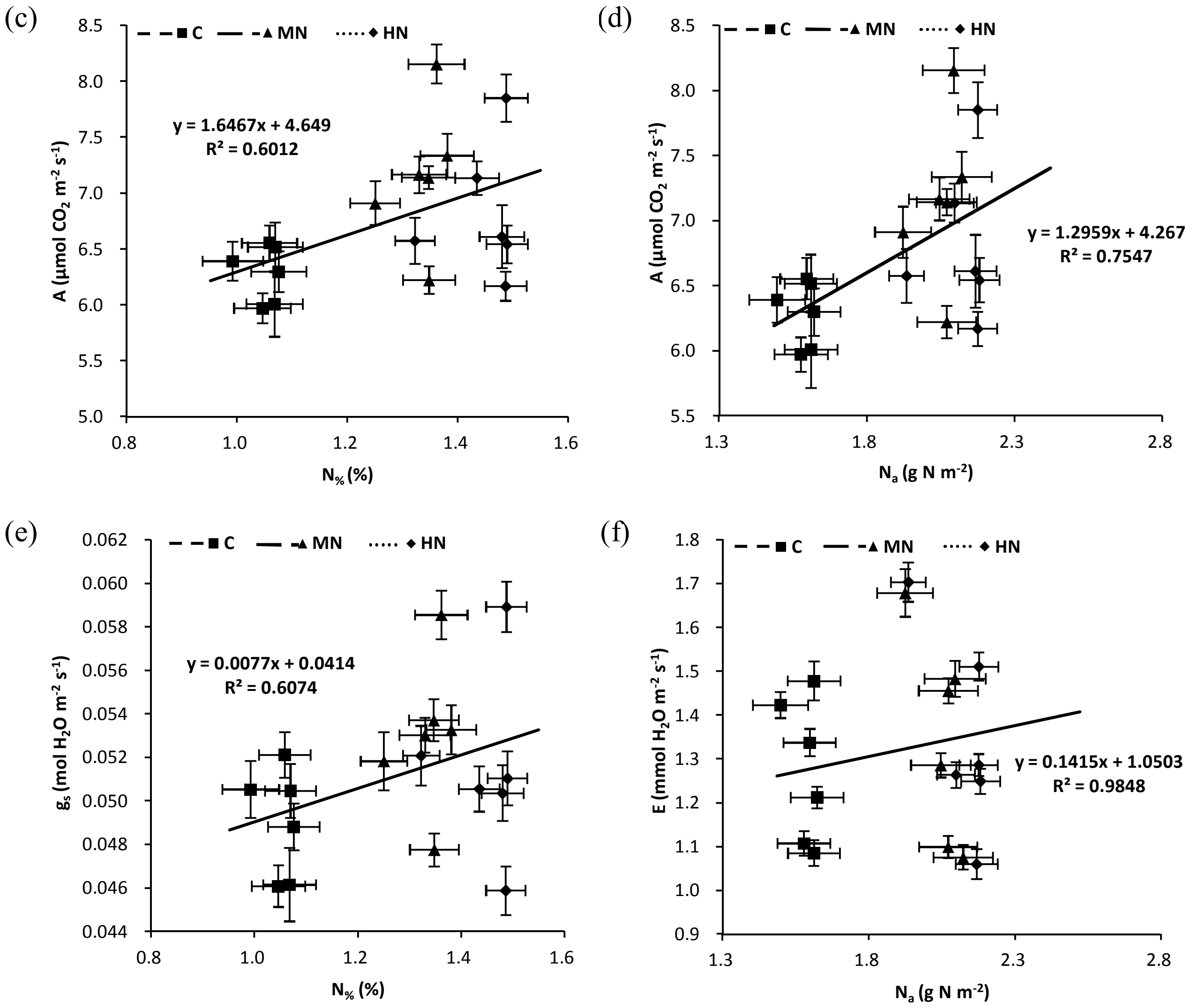


Figure 10 Relationship between (a) intrinsic water-use efficiency (ITE, μmol CO_2 mol^{-1} H_2O) or (c) CO_2 assimilation rates (A, μmol CO_2 m^{-2} s^{-1}) or (e) stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) and leaf N concentration ($N_{\%}$, %); and the relationship between (b) instantaneous water-use efficiency (TE, μmol CO_2 $mmol^{-1}$ H_2O) or (d) CO_2 assimilation rates (A, μmol CO_2 m^{-2} s^{-1}) or (f) leaf evapotranspiration rates (E, mmol H_2O m^{-2} s^{-1}) and leaf N content (N_a, g N m^{-2}) in old sample leaves of *Populus* × *euroamericana* seedlings under the different fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization; C, no fertilization). Regression lines illustrate average all treatments values between ITE and $N_{\%}$ (given in the figure a) or between A and $N_{\%}$ (given in the figure c) or between g_s and $N_{\%}$ (given in the figure e); and between TE and N_a (given in the figure b) or between A and N_a (given in the figure d) or between E and N_a (given in the figure f). Values are adjusted means from analysis of variance (ANOVA) in each treatment. Error bars represent standard errors in old sample leaves (ITE or TE or A or g_s or E: n=84, 4 leaves per tree × 3 replicates per leaf × 7 trees per treatment; $N_{\%}$ or N_a: n=28, 4 leaves per tree × 7 trees per treatment).

Gas exchange charateristics of individual old sample leaves and new growth leaves were measured under the different N fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization) on 21st October 2011. CO_2 assimilation rates (A, Fig. 11a), stomatal conductance (g_s, Fig. 11b) and leaf evapotranspiration rates (E, Fig. 11c) generally increased in new growth leaves but were reduced in old sample leaves with increasing N supply, and were greater in new growth leaves than in old sample leaves. Rather unusually, intercellular CO_2 partial pressure (c_i, Fig. 11d) increased in both old sample leaves and new growth leaves with increasing N fertilization, but was higher in old sample leaves than in new growth leaves. Although intrinsic water-use efficiency (ITE, Fig. 11e) and instantaneous water-use efficiency (TE, Fig. 11f) were reduced in both old sample leaves and new growth leaves with increasing N fertilization, were greater in new growth leaves than in old sample leaves. Changes in gas exchange characteristics were significant to different degrees (Tab. 7), except leaf evapotranspiration rates (E) in old sample leaves and CO_2 assimilation rates (A) in new growth leaves.

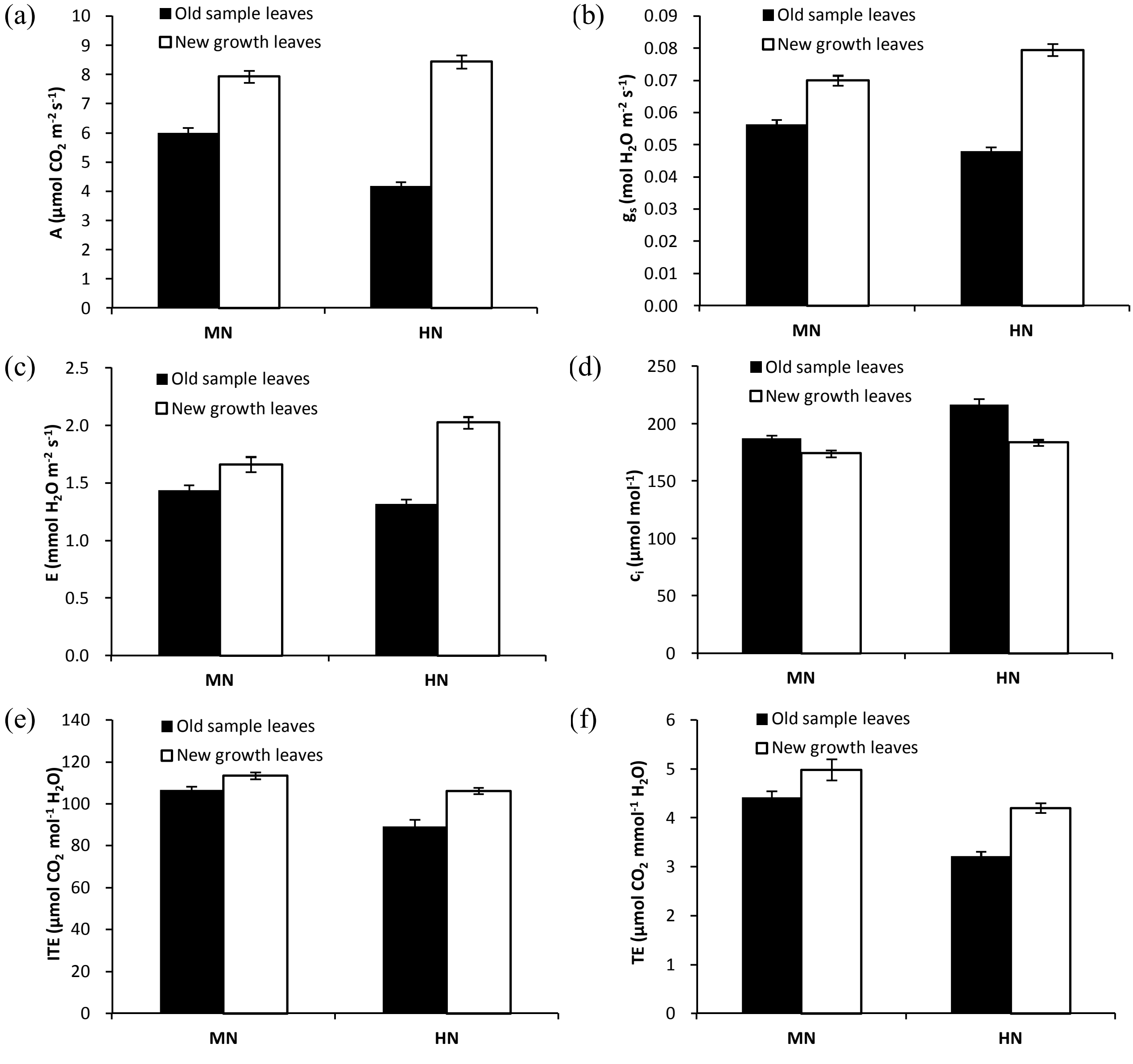


Figure 11 Contrast of leaf (a) CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}), (b) stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), (c) leaf evapotranspiration rate (E, mmol H_2O m^{-2} s^{-1}), (d) intercellular CO_2 partial pressure (c_i, µmol mol^{-1}), (e) intrinsic water-use efficiency (ITE, µmol CO_2 mol^{-1} H_2O), and (f) instantaneous water-use efficiency (TE, µmol CO_2 $mmol^{-1}$ H_2O) between old sample leaves and new growth leaves of *Populus* × *euroamericana* seedlings under the different N fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization) on 21st October 2011. Values are adjusted means from analysis of variance (ANOVA) in each treatment. Error bars represent standard errors in old sample leaves (n=84, 4 leaves per tree × 7 trees per treatment × 3 replicates per leaf) and new growth leaves (n=30, 10 leaves per treatment × 3 replicates per leaf).

Table 7 The variation of leaf gas exchange characteristics between old sample leaves and new growth leaves of *Populus × euroamericana* seedlings under the different N fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization) on 21st October 2011.

Variable	Old sample leaves				New growth leaves			
	Treatment		*F*	*P*	Treatment		*F*	*P*
	HN	MN			HN	MN		
A (μmol CO_2 m^{-2} s^{-1})	4.18 ± 0.14^a	6.00 ± 0.17^b	69.98	***	8.44 ± 0.23^a	7.93 ± 0.21^b	2.80	NS
g_s (mmol H_2O m^{-2} s^{-1})	48.02 ± 1.31^a	56.42 ± 1.39^b	19.69	***	79.53 ± 1.83^a	70.02 ± 1.54^b	16.33	***
E (mmol H_2O m^{-2} s^{-1})	1.32 ± 0.04^a	1.44 ± 0.05^b	3.67	NS	2.03 ± 0.05^a	1.66 ± 0.07^b	19.43	***
c_i (μmol mol^{-1})	216.60 ± 5.17^a	187.35 ± 2.52^b	26.17	***	183.60 ± 2.55^a	174.10 ± 2.97^b	6.09	*
ITE (μmol CO_2 mol^{-1} H_2O)	89.27 ± 3.12^a	106.75 ± 1.54^b	25.55	***	106.14 ± 1.54^a	113.38 ± 1.65^b	10.63	**
TE (μmol CO_2 $mmol^{-1}$ H_2O)	3.22 ± 0.09^a	4.42 ± 0.12^b	64.20	***	4.20 ± 0.10^a	4.99 ± 0.22^b	11.24	**

The *F*-value is the analysis of variance test (ANOVA) for the different N fertilization treatments (HN, high N fertilization; MN, intermediate N fertilization). Values are means ± standard error in old sample leaves (n=84, 4 leaves per tree × 7 trees per treatment × 3 replicates per leaf) and new growth leaves (n=30, 10 leaves per treatment × 3 replicates per leaf) and values with same superscript do not differ significantly (NS) according to the least significant difference test ($P > 0.05$). An asterisk (*) denotes variables differing significantly in the different N fertilization treatments using a paired t-test: ***indicates values statistically different at $P < 0.001$, **indicates those different at $P < 0.01$ and *indicates those different at $P < 0.05$. A: CO_2 assimilation rate; g_s: stomatal conductance; E: leaf evapotranspiration rate; c_i: intercellular CO_2 partial pressure; ITE: intrinsic water-use efficiency; TE: instantaneous water-use efficiency.

2.4.5. Discussion

The remarkable influences of N fertilization on transpirational water use led to enhanced plant water consumption with increasing N supply (Fig. 6), particularly in optimal N conditions (HN); further reducing the N availability (C) also led to a less-pronounced decrease in water consumption. This differential behaviour in transpirational water losses was associated with the existence of (i) significant and substantial production of new growth leaves (Fig. 3) as main effects, in contrast with the fact that there was no significant effect of N addition on stem height and diameter (Tab. 2) and the properties of both old sample leaves and new growth leaves (Tab. 3); (ii) leaf N concentration ($N_{\%}$) and leaf N content (N_a) of old sample leaves increased significantly in HN (Fig. 4 & Tab. 4) and this resulted in reduced CO_2 assimilation capacity whereas enhanced plant respiration, although these differences were not significant in new growth leaves (Fig. 5 & Tab. 5), (iii) although the addition of N modified leaf gas exchange characteristics in both old sample leaves (Fig. 7 & 8, Tab. 6) and new growth leaves (Fig. 11 & Tab. 7) this resulted in the negative variation in instantaneous as well as in intrinsic water-use efficiency in response to decreasing N availability; the responses were transient.

The more severe N deficiency induced by treatment C led to decreased growth which was associated with markedly reduced leaf gas exchange, in terms of CO_2 assimilation rate (A), stomatal conductance (g_s) and leaf evapotranspiration rate (E) in old sample leaves (Fig. 8 & Tab. 6). Considering average treatment values of intercellular CO_2 partial pressure (c_i), for old sample leaves one obtains 143.75, 139.66, 154.39 µmol mol^{-1} in HN, MN and C (Fig. 8d & Tab. 6), respectively. In new growth leaves, these values were 183.60 and 174.10 µmol mol^{-1} in HN and MN (Fig. 11 & Tab. 7), respectively. Thus, decreasing A (Fig. 8a), g_s (Fig. 8b) and E (Fig. 8c) values with increasing N deficiency were associated with increasing c_i (Fig. 8d) in old sample leaves between nutrient enrichments (HN and MN) and natural condition (C), whereas decreasing A (Fig. 11a), g_s (Fig. 11b) and E (Fig. 11c) values with increasing N deficiency were associated with decreasing c_i (Fig. 11d) in new growth leaves between HN and MN. This suggest that CO_2 assimilation capacity was decreased with significantly increasing leaf N deficiency in old sample leaves (Fig. 4 & Tab. 4), as is commonly observed (Field & Mooney 1986, Mitchell & Hinckley 1993, Guehl et al. 1995), however, gas exchange parameters of new growth leaves were not correlated with leaf N which was also not significant variation with N fertilization (Fig. 5 & Tab. 5). The results as we observed indicated that lowered N availability obviously led to a reduced N storage pool in the plants whereas the metabolic pool involved in the processes determining growth might have been less affected (Millard & Proe 1993). In old sample leaves, furthermore, leaf N concentration ($N_{\%}$) and leaf N content (N_a) can be considered as the main driving variable for the differences observed among the different treatments (Fig. 10).

However, increasing CO_2 assimilation rate (A, Fig. 8a), stomatal conductance (g_s, Fig. 8b) and intrinsic water-use efficiency (ITE, Fig. 8e) in simultaneous response to N deficiency in old sample leaves between HN and MN, constitutes an original result that does not conform with the general observation that g_s is reduced in response to N deficiency while ITE (ratio A/g_s) is either (i) decreased as we found between nutrient enrichments (HN and MN) and natural condition (C) here (Fig. 8e) and as also observed in *Ulmus americana* (Reich et al. 1989), or (ii) is maintained constant as observed in *Pseudotsuga menziesii* (Mitchell & Hinckley 1993). Similarly, the differential tendency also for increasing CO_2 assimilation rate (A, Fig. 8a), leaf evapotranspiration rate (E, Fig. 8c) and instantaneous water-use efficiency (TE, Fig. 8f) of old sample leaves in simultaneous response to N deficiency between HN and MN. Theoretically, the imposition of differential N treatments accompanied by any gradual effects on the ratio of exposed mesophyll cell surface to leaf area, and likely also on the surface area of chloroplasts exposed to intercellular airspace, would have important effects on mesophyll conductance to CO_2 diffusion (Evans & Loreto 2000, Ripullone et al. 2004) that would affect instantaneous water-use efficiency (TE). In our study species, however, we previously demonstrated that N supply has no effect on leaf mass per area (Tab. 3) and as also observed between *Pseudotsuga menziesii* and *Populus* × *euroamericana* (Ripullone et al. 2003). Mesophyll conductance, however, was not directly measured in the present experiment. Although the mechanisms underlying the responses of photosynthetic apparatus to N addition remain largely unknown, these responses have been found to be related to plant demand for N. In N-limited conditions N fertilization can meet plant demand for N and improve plant nutrient status, with an increased photosynthetic capacity and stimulation of plant growth (e.g. consistent with previous observations between MN and C treatments in this experiment). However, excess N will result in nutrient imbalance in trees, disturb N metabolism, reduce net photosynthesis, and restrict plant growth (Li et al. 2005, Lu et al. 2006, 2007, Mo et al. 2008). By studying the seedling growth response of two tropical tree species (*Schima superba* and *Cryptocarya concinna*) to N addition, Mo et al. (2008) found that net photosynthetic rate and biomass production were increased by lower N addition (50-100 kg N ha^{-1} yr^{-1}) but both species were negatively affected by higher N addition. In the present experiment, moreover, apart from photosynthesis which was not significantly affected (Fig. 11a), gas exchange characteristics of new growth leaves, such as g_s (Fig. 11b) and transpiration significantly decreased with increasing N deficiency (Fig. 11c and Tab. 7); the N supply effects on c_i (Fig. 11d) was little significant. These parameters were not correlated with leaf N, futhermore, the properties (Tab. 3) and $N_{\%}$ and N_a (Fig. 5 and Tab. 5) of new growth leaves were not significantly modified with N fertilization as mentioned above. However, the mechanisms underlying the response of ITE (Fig. 11e) or TE (Fig. 11f) to N supply were mainly related to the negative effect of N on g_s or E respectively, and the results showed ITE and TE also were significantly increased with increasing N deficiency (Tab. 7). Although the significant extents of ITE and TE were less than g_s and E respectively, this observation for newly grown leaves came as another proof of the negative influences of N excess supply on ITE and TE.

In accord with the differences in stomatal conductance (g_s, Fig. 8b) response patterns in old sample leaves, leaf evapotranspiration rate (E, Fig. 8c) was greater in MN than under optimal N conditions (HN); both parameters were further explored by calculating average intrinsic water-use efficiency (ITE, ratio A/g_s, Fig. 9a) or instantaneous water-use efficiency (TE, ratio A/E, Fig. 9b) for each treatment required to account for measured CO_2 assimilation rate (A, Fig. 8a). The variations for each treatment in old sample leaves respectively provided the average ITE (Fig. 10a), TE (Fig. 10b), A (Fig. 10c & d), g_s (Fig. 10e) and E (Fig. 10f) values of all treatments; all parameters were positively related to leaf N ($N_{\%}$ or N_a), especially ITE vs. $N_{\%}$. As mentioned above, leaf N ($N_{\%}$ and

N_a) could help understand the mechanisms underlying the responses of photosynthetic capacity to significantly altered leaf N status under N fertilizations, although there were complex interactions between resources as determinants of the structure and function of plants remain largely unknown. By combining Fig. 9a and 9b, we obtained the simplified versions of the relationships linking ITE and TE showing convergent patterns and similar relationships with different fertilization treatments; however, we hypothesize that N addition could have an effect on the balance between water loss and carbon gain per unit stomatal (i.e. ITE) or per unit water transpired (i.e. TE). ITE (Fig. 10a) may be the consequence of changes in leaf N content ($N_{\%}$), which can affect the ratio between A (Fig. 10c) and g_s (Fig. 10e), the positive correlations mostly due to nitrogen is essential to photosynthetic metabolism and growth as its role in the synthesis of chlorophyll and proteins such as ribulose-1, 5-biphosphate carboxylase (Rubisco), which is responsible for CO_2 assimilation (Lawlor 2001). Also TE (Fig. 10b) may be the consequence of changes in leaf N content (N_a), which can affect the ratio between A (Fig. 10d) and E (Fig. 10f), the positive correlations mostly due to high leaf-to-air VPD tend to have leaves of low specific leaf area (SLA, area per unit leaf dry mass) and stomatal conductance, but high leaf N content (N_a) for a particular assimilation rate, resulting in correspondingly high TE but low N use (Mooney et al. 1978, MacFarlane et al. 2004, Barker et al. 2006). The relationship between intrinsic water-use efficiency (ITE, Fig.10a) and leaf N concentration ($N_{\%}$) or the relationship between instantaneous water-use efficiency (TE, Fig. 10b) and leaf N content (N_a) were not straightforward, but a positive trend as we found is also generally observed, in ambient air (Guehl et al. 1995, Ripullone et al. 2004) as well as in elevated CO_2 (Tognetti & Johnson 1999). A review on the effects of nutrient supply in crop plants emphasized the importance of N influence on ITE (Brueck 2008).

Although high N fertilization is well know to enhance growth and photosynthetic capacity, the results of the present study show that N limitation contributes strongly to the stomatal conductance, thereby increasing the CO_2 photosynthetic rate and transpiration rate, which can lead to improved CO_2 assimilation capacity of hybrid poplar. The mechanism is unknown, but it is possible that N supply may have increased the leaf chlorophyll content or promoted the growth of leaf structure and function (Rufty et al. 1988), resulting different effects on photosynthetic capacity under the different fertilizations treatments through the differences in soluble protein content as well as in the synthesis and activation of photosynthetic enzymes (i.e. Rubisco; Amy et al. 2006). A, g_s and E were enhanced with increasing N availability in a primary stage; subsequently, there was a negative tendency for A, gs, E, ITE and TE along with N simulated under the optimal N conditions indicated that the significantly facilitative effects induced by limited N conditions on leaf N content were largely dominated by the increase in synthesized leaf chlorophyll content and a greater amount of active Rubisco, which would have been required to account for the gas exchange characteristics of leaves. Under excess N, the activation of Rubisco in assimilation would be reduced at the expense of excessive metabolism consumption and vegetative growth (Manter et al. 2005). Also, it is possible that excessive leaf N concent may have reduced photosynthetic capacity and increased plant respiration through decreasing cell osmotic pressure caused by stomatal limitation.

2.5. Conclusions

In summary, plant biomass production and water requirements were profoundly affected by soil nutrient availability. New growth leaves increased with N fertilization but without resulting in greater light absorption, photosynthetic capacity and foliar N were just positively correlated in old sample leaves. Fertilized plants (HN and MN alike) had significantly greater rates of transpiration than control plants and this led to increased plant water consumption, largely as a result of the production of new leaves. Due to the significant influence of N fertilization on leaf gas exchange characteristics, such as CO_2 assimilation rate (A), stomatal conductance (g_s) and leaf evapotranspiration rate (E), gas exchange measurements revealed that the mechanisms underlying the response of ITE and TE to N supply were mainly related to the positive effect of N on these characteristics in varying degrees. ITE and TE showed convergent patterns and similar relationships between different treatments.

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Chapter 3

Effects of N fertilization on intrinsic water-use efficiency: a meta-analysis

3.1. Abstract

The objective of this study is to provide a synthesis of existing data that comprehensively and quantitatively evaluates how gas exchange characteristics interact with nitrogen (N) fertilization to influence intrinsic wateruse efficiency (ITE). I conducted a meta-analysis to examine the effects of N fertilization on the variability of ITE in woody plants, using data extracted from seventy-three studies previously published over the period 1986-2013. Further, ITE was estimated by tree ring carbon isotope discrimination ($\Delta^{13}C$) as a sensitive long-term monitoring indicator and compared with the literature analytic values of ITE from gas exchange measurements under N fertilization conditions. All statistics and simulations were performed by a split-plot analysis: study types and plant functional types (PFTs) were differentiated between lab and field studies, and conifers and broadleaves studies, respectively, which could be compared with each other. Moreover, the response of functional parameters to leaf nitrogen concentration ($N_{\%}$), used as an intermediate driver directly affected by fertilization. Results showed strongly significant effects of N fertilization (and in a consistent manner between functional types), due to the absence of increasing stomatal conductance and transpiration, in contrast with the sharp rise in photosynthetic rates. ITE obtained by gas exchanges measurement proved completely consistent with what estimated by $\Delta^{13}C$.

3.2. Introduction

One of the most considerable atmospheric changes at the global scale is the anthropogenic perturbation of the nitrogen (N) cycle, due to fossil-fuel combustion and agricultural emissions (Galloway et al. 2008, Gruber & Galloway 2008). Approximately 18×10^6 kg of reactive N is globally deposited into forests each year, with marked regional differences in annual deposition rate (Schlesinger 2009). Together with elevated atmospheric CO_2, N deposition has been recognized as one of the main drivers enhancing the terrestrial carbon 'sink' (Norby 1998, Gruber & Galloway 2008).

However, the carbon (C) and N cycles interact in very complex ways, with mechanisms that can be grouped under two distinct scenarios (Guerrieri et al. 2010). Excess N will result in nutrient imbalance in trees, disturb N metabolism, reduce net photosynthesis (i.e. C uptake), and restrict plant growth (Li et al. 2005, Lu et al. 2006, 2007, Mo et al. 2008a), even increased tree mortality (Magill et al. 2004). However, a beneficial role of N deposition has been hypothesized for N-limited systems (e.g. temperate forests), as N deposition can act as a low-level but sustained input of fertilizer, potentially increasing tree growth and photosynthetic capacity (Högberg 2007, Magnani et al. 2007, Sutton et al. 2008, Thomas et al. 2010). A high carbon assimilation rate is bound to be associated to the consumption of a large amount of water during photosynthesis (Cao et al. 2012). Therefore, we hypothesize that N fertilization could have an effect on the balance between water loss and carbon gain at the leaf level, that is on water-use efficiency (WUE), which is known to have an important role for bridging carbon and water cycling among other functional traits of plant (Jackson et al. 2005).

On an 'instantaneous' time scale, two different definitions of WUE can be made at the photosynthetic organ (e.g. leaf) level (Tambussi et al. 2007). Intrinsic water-use efficiency (ITE) is defined as the ratio between CO_2 assimilation rate (A) and stomatal conductance (g_s); instantaneous water-use efficiency (TE), on the other hand, is defined as the CO_2 assimilated through photosynthesis (A) divided by the water transpired (E). From a methodological viewpoint, both are similarly carried out by gas exchange methods (i.e. infrared gas analyser and porometry), but ITE is not influenced by VPD (the driving force of transpiration rate), and, consequently this parameter is used in comparative studies, where different evaporative demands could be present (Morgan & LeCain 1991, Johnson 1993).

Apart from the method based on direct gas exchange measurements (Flexas et al. 2007), ITE is often estimated from stable carbon isotope composition ($\delta^{13}C$) since there is a positive linear relationship between $\delta^{13}C$ and ITE over the period of biomass accumulation (Farquhar et al. 1982, Farquhar & Richards 1984, Seibt et al. 2008). Most previous N fertilization studies have relied on gas exchange measurements to estimate ITE in young seedlings (Tjoelker & Luxmoore 1991, Schmutz et al. 1995, Roberntz & Stockfors 1996), however, the use of stable carbon isotopes has made the determination of ITE in mature trees easier because the measurement of $\delta^{13}C$ in tree rings provides information on tree physiological responses to environmental changes over the life span of the tree (Francey & Farquhar 1982) and can show the potential of carbon isotopes in cellulose to integrate long-term variations in ITE (Marshall & Monserud 1996, Bert et al. 1997, Duquesnay et al. 1998, Feng 1999). Moreover, tree-ring $^{13}C/^{12}C$ analysis allows a retrospective assessment of fertilization impacts that is impossible with gas exchange measurements or leaf isotopic analysis

(Elhani et al. 2005). We note that the results from mature trees is response to N enrichment are inconclusive and somewhat controversial: increases, decreases or no response in ITE have been observed (Högberg et al. 1993, Lovelock et al. 2006, Betson et al. 2007, Martin et al. 2010, Matsushima et al. 2012, Pascual et al. 2013).

In addition, ITE may be the consequence of changes in leaf N concentration ($N_{\%}$), which can in turn affect the ratio between assimilation and stomatal conductance (Leonardi et al. 2012). The relationship between $N_{\%}$ and ITE is not straightforward, but several studies show a positive trend (Guehl et al. 1995, Tognetti & Johnson 1999b, Ripullone et al. 2004). In general, higher leaf N concentration correlates with greater photosynthetic CO_2 assimilation rates (Salifu et al. 2007, Wang et al. 2012), mostly due to the fact that nitrogen is essential to photosynthetic metabolism and growth because of its role in the synthesis of chlorophyll and proteins such as ribulose-1, 5-biphosphate carboxylase (Rubisco), which is responsible for CO_2 assimilation (Lawlor 2001). Also TE may be the consequence of changes in leaf N content (N_a), which can affect the ratio between assimilation and leaf evapotranspiration rate (E). Plant growing in environments with low water availability and high leaf-to-air VPD generally tend to have leaves of low specific leaf area (SLA, area per unit leaf dry mass) and stomatal conductance, but high leaf N content (N_a) for a particular assimilation rate, resulting in correspondingly high TE but low N use (Mooney et al. 1978, MacFarlane et al. 2004, Barker et al. 2006, Martin et al. 2010).

Numerous individual studies have been conducted to examine how water-use efficiency (WUE) responds to N fertilization or simulated N deposition at the leaf level. Previous studies have demonstrated that intrinsic water-use efficiency which was measured by gas exchange measurement (Wang et al. 2012) or was estimated by carbon isotope composition (Pascual et al. 2013), instantaneous water-use efficiency (Ripullone et al. 2004), maximum assimilation rates (Stephens et al. 2001, Jiao et al. 2011) and stomatal conductance (Graciano et al. 2005) can all be significantly influenced by the external N input. However, experimental results for ITE or TE from various individual studies are highly variable with different (i) tree species (Guehl 1995), (ii) physical treatments, such as thinning or pruning (Forrester et al. 2012), (iii) environments, such as elevated CO_2 (Watanabe et al. 2008, Tricker et al. 2005) or light regimes (Welander & Ottosson 2000) or irrigation (Choi et al. 2005, Fernandez et al. 2006) or elevated O_3 (Tjoelker & Luxmoore 1991) or hypersaline (Martin et al. 2010), (iv) experimental period (Stephens et al. 2001), (v) temperature (Sun et al. 2009), (vi) altitudes (Zhan 2012) have been reported to exhibit an increase (Yang et al. 2011, Yang et al. 2012), a decrease (Hobbie & Colpaert 2004) or an insignificant change (Graciano et al. 2005) in response to external N input. Similarly, the responses to different levels of nitrogen exponential fertilization (i.e. from N-limited or N-saturated) are also highly variable (Wang et al. 2013). At the leaf level, a general understanding of the balance between carbon and water cycling under N fertilization conditions is still unavailable, owing to the lack of a comprehensive understanding of the effects of N fertilization on ITE; this greatly limits our ability to explore the responses of the structure and function of woody plant to N supply.

The highly diverse results from individual experiments are unlikely to reveal a general pattern that can be applied to various woody plants. However, the results across individual studies can be synthesized by meta-analysis to reveal a central tendency of changes in stimulation of growth effects on ITE induced by the additional N fertilization (Hedges & Olkin 1985). To our knowledge, a number of meta-analyses have examined the effects of additional N input in terrestrial ecosystems on C pools (Treseder 2008, Xia & Wan 2008, Liu & Greaver 2010, Limpens et al. 2012), N pools (Lu et al. 2010) and fluxes (Knorr et al. 2005, LeBauer & Treseder 2008, Liu & Greaver 2009, Janssens et al. 2010). Further, Vadeboncoeur (2010) use of meta-analysis indicated multiple limiting nutrients in northeastern deciduous forests according to fertilization experiments. However, little is known about how ITE respond to N fertilization and simulated N deposition at the leaf level, although a review article on the effects of nitrogen supply in crop plants emphasized the importance of N influence on biomass-based water-use efficiency from 90 published data (Brueck 2008). Moreover, a recent study applied generalized additive models and linear mixed-effects models to predict the effects of climatic variables and nitrogen deposition on δ^{13}C and ITE from eighty-nine long-term isotope tree-ring chronologies extracted from the literature, representing 23 conifer and 13 angiosperm species for 53 sites worldwide (Leonardi et al. 2012), suggesting a significant effect of N addition.

In the present study, I conducted a meta-analysis to identify the central tendency of the effects of N addition on ITE by compiling data from seventy-three individual studies in woody plants. Meta-analysis is a powerful statistical tool that allows data compiled from many similar experiments to be used to infer the direction and magnitude of an effect, often with either greater statistical power or broader basis to draw general conclusions than is possible with a single experiment. I also examined whether the effects of fertilization differed significantly between study types such as lab vs. field, and between plant functional types such as conifers vs. broadleaves. Finally, I used relevant subsets of the compiled data to determine whether there were significant synergistic effects when the variables were correlated with foliar N status under N fertilization conditions.

3.3. *Material and methods*

3.3.1. Data mining and selection

Literature searches were first conducted using the ISI Web of Science Database for searching papers published in English and the China Academic Literature Network Publishing Database for searching papers and theses published in Chinese. A total of 73 papers and theses were selected from 3975 peer-reviewed publications that reported water-use efficiency (WUE) at the leaf level in response to N fertilization experiments over the period 1986-2013 (Supporting Information Appendix A). The compiled database included the responses to N fertilization of three independent variables and six dependent variables related to water-use efficiency parameters (Tab. 1). In terms of covariates, study types could be compared by splitting the dataset into two separate groups (Lab and Field); likewise, also plant functional types (PFTs) could be compared by splitting it into two separate functional groups (Conifers and Broadleaves).

Table 1 Description and interrelation of independent and dependent variables and covariates used in this meta-analysis.

Abbreviations	Variables	Units	Notes
Independent variables			
N_a	Leaf N content	$g\ N\ m^{-2}$	Computed as $N_{\%}/SLA$
$N_{\%}$	Leaf N concentration	$g\ N\ g^{-1}\ DM$	Computed as $N_a \cdot SLA$
SLA	Specific leaf area	$m^2\ g^{-1}\ DM$	Computed as 1/LMA (LMA, leaf mass per area, $g\ m^{-2}$)
Dependent variables			
g_s	Stomatal conductance	$mol\ H_2O\ m^{-2}\ s^{-1}$	Computed as A_{max}/ITE
A_{max}	Maximum assimilation rate	$\mu mol\ CO_2\ m^{-2}\ s^{-1}$	Computed as $g_s \cdot ITE$
ITE	Intrinsic water-use efficiency	$\mu mol\ CO_2\ mol^{-1}\ H_2O$	Computed as A_{max}/g_s
Estimated ITE	Estimated intrinsic water-use efficiency	$\mu mol\ CO_2\ mol^{-1}\ H_2O$	Computed as $\triangle^{13}C$
TE	Instantaneous water-use efficiency	$\mu mol\ CO_2\ mmol^{-1}\ H_2O$	Computed as A_{max}/E (E, leaf evapotranspiration rate, $mmol\ H_2O\ m^{-2}\ s^{-1}$)
$\triangle^{13}C$	Carbon-isotope discrimination	‰	Computed as Air carbon isotope ratio (Air $\delta^{13}C$)
Covariates			
—	Study type	—	Lab vs. Field
PFT	Plant functional type	—	Conifers vs. Broadleaves

We believe that this approach could be used to generalize across a wide variety of experiments and conditions. Accordingly, I adopted by default an inclusive approach in considering previously published work. However, I wish to list explicitly the few decisional criteria that were applied consistently to define a final selection of data. (i) I restricted the analysis to studies where only N was used as a fertilizer, but excluded the studies that applied various fertilizer, for instance, Universol Blue® (18N:11P:18K), Osmocote® (14N:8P:14K), Mix fertilizer (N, P, K, Ca, Mg, S, Cu, B & Zn) and Urea/diammonium phosphate mixture, we only focused our attention on N fertilizer, such as: urea ($CO(NH_2)_2$), ammonium nitrate (NH_4NO_3), ammonium sulphate ($(NH_4)_2SO_4$) and sodium nitrate ($NaNO_3$) when the information was explicitly reported in the article. (ii) N fertilizers were directly added to soil rather than anthropogenic NO_x emission or atmospheric pollution (e.g., industrial vs. rural). (iii) To investigate the effects of N fertilization on WUE at the leaf level and at least one of our selected variables was measured. WUE at the stand level was excluded to avoid noise. (iv) Tree-ring $^{13}C/^{12}C$ analysis for estimating ITE allows a retrospective assessment of fertilization impacts that is impossible with gas exchange measurements or leaf isotopic analysis. (v) The N application rate, experimental duration, soil depth and types were clearly recorded and the measurements of treatment and control groups were conducted at the same temporal and spatial scales. (vi) If available data, means, standard deviations or standard errors and sample sizes of the chosen variables in tabular form in the original studies, the values were manually digitized. If provided in graphical form, the values could be indirectly extracted from the related figures using the UN-SCAN-IT 5.0 software (Silk Scientific, Orem, UT, USA) or calculated from the chosen papers if they were not directly reported. Interpolation was not applied in any case. (vii) Measurements for different N application rates were considered as independent observations if more than one level of N fertilization was applied in the same experiment (Curtis & Wang 1998, Liu & Greaver 2009). (viii) The latest sampling was used if more than one measurement at different temporal scales was available for the same experiment (Treseder 2008, Liu & Greaver 2009).

On the basis of N fertilization experiments, the sensitivity to leaf nitrogen concentration ($N_{\%}$) was calculated from the slope of the linear model ($y = a + b(1/D)$) describing the relationship with stomatal conductance (g_s) or maximum assimilation rate (A_{max}) or specific leaf area (SLA) or intrinsic water-use efficiency (ITE) or carbon-isotope discrimination ($\Delta^{13}C$), respectively (Supporting Information Appendix B from Fig. 1 to Fig. 5); likewise, the sensitivity to stomatal conductance (g_s) was calculated from the slope of the linear model ($y = a + b(1/D)$) describing the relationship between intrinsic water-use efficiency (ITE) or carbon-isotope discrimination ($\Delta^{13}C$), respectively (Supporting Information Appendix B from Fig. 1 to Fig. 7), in response to different irrigations (e.g., severe or moderate water stress, precipitation), tree species, environments (e.g., elevated or ambient CO_2, O_3, UV), physiological treatments (e.g., thinning or pruning), temporal and spatial scales (Wullschleger et al. 1998, Samuelson et al. 2007).

3.3.2. Relationship between dependent variables

The potential of carbon isotope chronologies to act as effective physiological archives is a consequence of two independent processes: i) the heavier carbon isotope (^{13}C) depletion in plant material relative to atmospheric CO_2, which is due to isotope fractionations that occur during CO_2 diffusion into the leaf, and during carboxylation by Rubisco; ii) the relationship between carbon isotope discrimination (Δ^{13}C) and the ratio between the concentration of CO_2 in leaf intercellular spaces and in the atmosphere, which is related to changes in assimilation or stomatal conductance (Farquhar et al. 1989). Post photosynthetic fractionations (Cernusak et al. 2009) can partially reduce the degree to which the primary isotope signal imprinted in leaf organic matter is recorded in woody tissues, however, carbon isotope ratio in tree rings still contains useful information on plant gas exchanges, once long-term change in the atmospheric isotope ratio are considered, and the ^{13}C/^{12}C ratio in plant material is expressed as photosynthetic ^{13}C discrimination (Δ^{13}C):

$$\Delta^{13}\mathrm{C} = \frac{\delta^{13}\mathrm{C_a} - \delta^{13}\mathrm{C_p}}{1 + \delta^{13}\mathrm{C_p}/1000} \tag{8}$$

where $\delta^{13}C_a$ and $\delta^{13}C_p$ are the respective isotopic ratios of atmospheric CO_2 and of plant material. Based on a simplified Farquhar equation (Farquhar et al. 1989) carbon discrimination can be expressed as:

$$\Delta^{13}\mathrm{C} = \mathrm{a} + (\mathrm{b} - \mathrm{a})\frac{\mathrm{c_i}}{\mathrm{c_a}} \tag{9}$$

where a is the $^{13}CO_2$ fractionation as a result of diffusion through air (4.4‰), b is the fractionation during carboxylation (27‰) by the CO_2-fixing enzyme Rubisco, c_i is the CO_2 concentration in leaf intercellular spaces, and c_a is the CO_2 concentration in the ambient air.

Consequently, c_i depends on CO_2 flux into the leaf, which is largely regulated by stomatal conductance, and CO_2 flux removed from the leaf for carbon fixation by assimilation. The intrinsic water-use efficiency (ITE) is defined as the ratio between carbon assimilation (A) and stomatal conductance to water vapor ($g_{s,w}$):

$$\mathrm{ITE} = \frac{\mathrm{A}}{\mathrm{g_{s,w}}} \tag{10}$$

where A can be expressed as:

$$\mathrm{A} = \mathrm{g_{s,c}}(\mathrm{c_a} - \mathrm{c_i}) \tag{11}$$

in which $g_{s,c}$ is the stomatal conductance to CO_2.

Substituting Eq. 11 in Eq. 10 and rearranging we obtain:

$$\mathrm{ITE} = \frac{\mathrm{g_{s,c}}}{\mathrm{g_{s,w}}}\mathrm{c_a}\left(1 - \frac{\mathrm{c_i}}{\mathrm{c_a}}\right) = 0.625\ \mathrm{c_a}(1 - \frac{\mathrm{c_i}}{\mathrm{c_a}}) \tag{12}$$

where 0.625 is the ratio between the stomatal conductance to CO_2 ($g_{s,c}$), and the stomatal conductance to water vapor ($g_{s,w}$).

The simplified model of carbon discrimination, which neglects leaf internal conductances, can be problematic for the correct estimate of ITE, as showed by Seibt et al. (2008). However, it is worthy to note that in this study the main focus was not on the actual value of ITE but on the relationship between ITE, atmospheric and climatic factors. Nevertheless, we evaluated the model effects due to uncertainty in ITE.

From Eq. 9:

$$\frac{c_i}{c_a} = \frac{\Delta^{13}C - a}{b - a} \tag{13a}$$

$$c_i = c_a \frac{\Delta^{13}C - a}{b - a} \tag{13b}$$

Substituting Eq. 13a in Eq. 12 we obtain an expression for the intrinsic water-use efficiency as a function of carbon isotope discrimination:

$$\mathrm{ITE} = 0.625\, c_a\left(1 - \frac{\Delta^{13}C - a}{b - a}\right) \tag{14}$$

Therefore, ITE can be estimated from the carbon isotope ratio in tree rings ($\delta^{13}C_p$) taking into account the long-term variation in atmospheric CO_2 concentration (c_a) and its isotopic ratio ($\delta^{13}C_a$) (Leonardi et al. 2012).

The efficiency of C gain per unit water transpired is termed instantaneous water-use efficiency (TE). A combination of transport equations of maximum carbon assimilation rate at saturating light (A_{max}, $A_{max} = g_{s,c}\,(c_a - c_i) / p$) and evapotranspiration rate (E, $E = g_{s,w}\,\mathrm{VPD} / p$) gives:

$$\mathrm{TE} = A_{max}/E = c_a(1 - c_i/c_a)/(1.6\,\mathrm{VPD}) \tag{15}$$

where c_a and c_i are the ambient and intercellular CO_2 partial pressures; $g_{s,c}$ and $g_{s,w}$ are the stomatal conductance for CO_2 and water-vapor transport; VPD is the leaf-to-atmosphere water-vapor partial-pressure difference; p is the atmospheric pressure.

3.3.3. Meta-analysis

The response ratio (RR) was used to reflect the effects of N fertilization on terrestrial ecosystem N pools and fluxes (Hedges et al. 1999). The RR, the ratio of the mean value of the chosen variable in the N addition group ($\overline{X}_T$) to that in the control group ($\overline{X}_C$), is an index of the effect of N fetilization on the corresponding variable:

$$lnRR = ln(\overline{X_t}/\overline{X_c}) = ln(\overline{X_t}) - ln\ (\overline{X_c}) \tag{1}$$

Here, RR is the natural-log proportional change in the means ($\overline{X}$) of a treatment (T) and control group (C). Meta-analysis, when pooling RR from multiple studies, also assigns a weight to each RR that is inversely proportional to its sampling variance:

$$v\ =\ \frac{(SD_C)^2}{N_C\bar{X}_C^2}+\frac{(SD_T)^2}{N_T\bar{X}_T^2} \tag{2}$$

where SD and N are the standard deviation and sample size of $\overline{X}_T$ and $\overline{X}_C$, respectively (Hedges & Olkin 1985).

$$\mathrm{RR}_{++} = \frac{\sum_{i=1}^{m}\sum_{j=1}^{ki} w_{ij}\mathrm{RR}_{ij}}{\sum_{i=1}^{m}\sum_{j=1}^{ki} w_{ij}} \tag{3}$$

The standard error of RR_{++} can be expressed as:

$$\mathrm{S}(\mathrm{RR}_{++}) = \sqrt{\frac{1}{\sum_{i=1}^{m}\sum_{j=1}^{ki} w_{ij}}} \tag{4}$$

where w_{ij} is the weighting factor and also is count backwards of the variance (v):

$$w_{ij} = \frac{1}{v} \tag{5}$$

Mean effect size for each study was calculated, with bias-corrected 95% confidence intervals (CI's) generated by a bootstrapping procedure using meta-analysis software:

$$95\%\mathrm{CI} = \mathrm{RR}_{++} \pm 1.96\ \mathrm{S}(\mathrm{RR}_{++}) \tag{6}$$

means were considered to be significantly different from zero if the 95% confidence interval did not overlap zero, and significantly different from one another if their 95% confidence intervals were non-overlapping (Gurevitch & Hedges 1993, Johnson & Curtis 2001). The percent change in a variable was estimated by (e^{RR++}-1)×100%. In addition, the relationships between lnRR and environmental and/or forcing factors were examined using a single-factor regression approach. The frequency distribution of lnRR was assumed to follow normal distribution and fitted by a Gaussian function (Luo et al. 2006):

$$y = a\exp\left[-\frac{(x-\mu)^2}{2\sigma^2}\right] \tag{7}$$

where x is the mean of lnRR in an individual interval; y is the frequency in an interval; a is the expected number of lnRR values at $x=\mu$; μ and σ^2 are the mean and variance of the normal distribution of lnRR, respectively.

A common effect size metric used to quantify the outcome of experiments for ecological meta-analysis is the response ratio (RR): the log proportional change in the means of a treatment and control group. Estimates of the variance of RR are also important for meta-analysis because they serve as weights when effect sizes are averaged and compared. The varience of an effect size is typically a function of sampling error, however, it can also be influenced by study design. Here, we derive new variances and covariances for RR for several often-encountered experimental designs: when the treatment and control means are correlated; when multiple treatments have a common control; when means are based on repeated measures; and when the study has a correlated factorial design, or is multivariate. These developments are useful for improving the quality of data extracted from studies for meta-analysis and help address some of the common challenges meta-analysts face when quantifying a diversity of experimental designs with the response ratio (Lajeunesse 2011).

Variance among categorical groups was partitioned to determine the effect of each category. Categorical subdivisions could only be compared if they were represented by at least two separate studies. Between-group heterogeneity (Q_b) for each categorical variable was determined for the response variable. If none of the categorical groups displayed significant Q_b, there was no statistical justification for further subdivision of the data. Where significant Q_b was observed, the data were subdivided according to levels of those categorical variables with a significant Q_b (Knorr et al. 2005). In this meta-analysis, the denominator (i.e. the consumed water) of the WUE can be considered in two ways. Water losses may include, despite water transpired by the plant, the direct evaporation from the soil (symbolised as E_s; Oweis et al. 2000). Thus, the estimation of WUE in experiments carried out in pots (where soil evaporation is commonly eliminated) may be sensibly different with respect to plants grown in the field (Tambussi et al. 2007). Therefore, study type could be compared when it was splited into two separate studies (Lab and Field). Also, plant functional type (PFT) bridge the gap between plant physiology and ecosystem processes, providing a powerful tool for studies on global change, vegetation dynamics and vegetation-atmosphere processes (Weng & Zhou 2005). Hence, PFT was related to leaf structure that could be compared when it was splited into two separate structural groups (Conifers and Broadleaves).

3.3.4. Statistical analysis

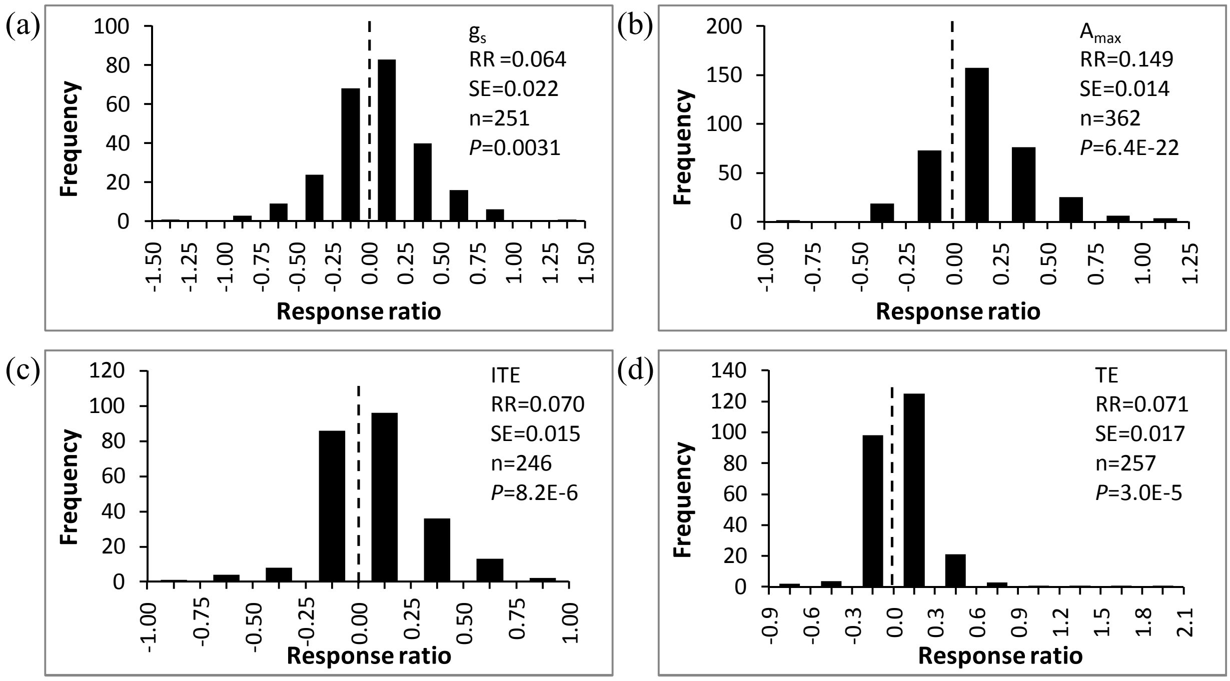
Seventy-three studies reported data sufficient for inclusion in a more rigorous metaanalysis following the methodology outlined by Hedges et al. (1999). All statistics and simulations were performed (Tab. 1) by a split-plot analysis of variance using the R statistical suite (R Development Core Team, 2012), the free and open-source software used by Limpens et al. (2011, 2012). Briefly, RR was ln-transformed and sample size and variance data are used to calculate a weight. The weighted mean logged response ratio and its standard error were then calculated, taking into account within- and between experiment variances. Finally, these results were reported as response ratios by taking the antilog of the means and confidence interval limits. Two-tailed *p*-values for each response ratio were calculated using the standard error of each weighted mean lnRR and

Student’s t-distribution. Results were displayed graphically using adjusted means and standard error bars, or in tabular format using adjusted means and superscripts to identify. Extreme outliers (points exceeding three standard deviations of the mean) were excluded.

3.4. Results and discussion

3.4.1. RR---Overall analysis

N-induced changes in independent and dependent variables exhibited great variability across the studies (Fig. 1), ranging from a minimum lnRR of -1.368 to a maximum of 1.386 in stomatal conductance (g_s), from -0.916 to 1.131 in maximum assimilation rate (A_{max}), from -0.782 to 0.902 in intrinsic water-use efficiency (ITE), from -0.703 to 1.863 in instantaneous water-use efficiency (TE), from -0.189 to 0.027 in carbon-isotope discrimination ($\Delta^{13}C$), from -0.334 to 1.261 in leaf N content (N_a), from -0.320 to 1.586 in leaf N concentration ($N_{\%}$), from -0.467 to 0.619 in specific leaf area (SLA), and from -0.073 to 0.476 in estimated intrinsic water-use efficiency (Estimated ITE). On average, the overall effects of N fertilization on most of variables were positive, with an increase of 6.61% in g_s ($P<0.01$; Fig. 1a), 16.07% in A_{max} ($P<0.001$; Fig. 1b), 7.25% in ITE ($P<0.001$; Fig. 1c), 7.36% in TE ($P<0.001$; Fig. 1d), 16.3% in N_a ($P<0.001$; Fig. 1f), 21.53% in $N_{\%}$ ($P<0.001$; Fig. 1g), 1.11% in SLA ($P>0.05$; Fig. 1h) though no significant, 8.44% in Estimated ITE ($P<0.001$; Fig. 1i), however, the N addition only significantly decreased $\Delta^{13}C$ by 3.34% ($P<0.001$; Fig. 1e). Among all variables, the N-induced increase in Estimated ITE was more than for ITE; together with stomatal conductance (g_s), these were displayed the smallest response to N (Fig. 1j), apart from SLA which did not respond to N fertilization (Tab. 2).



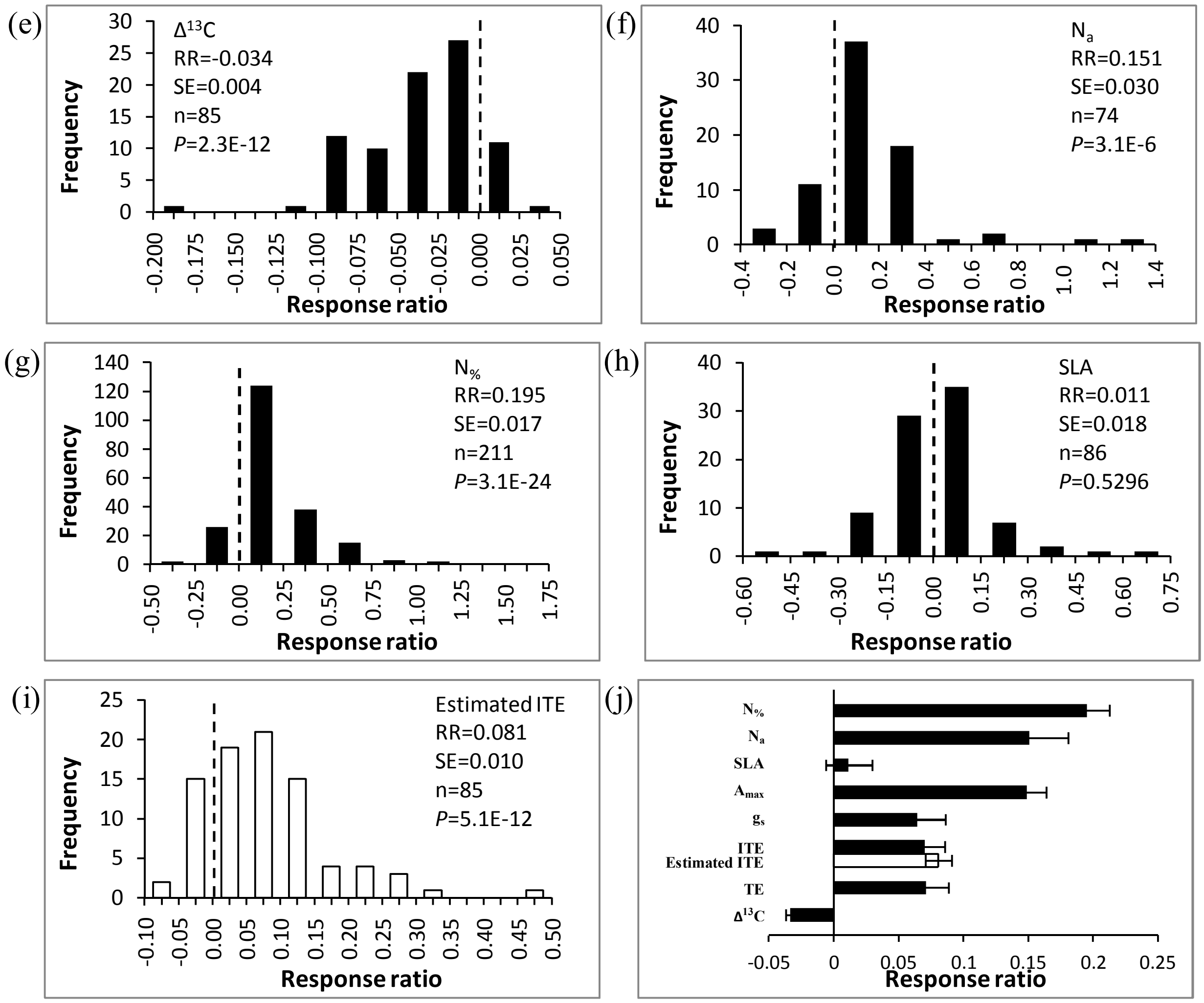
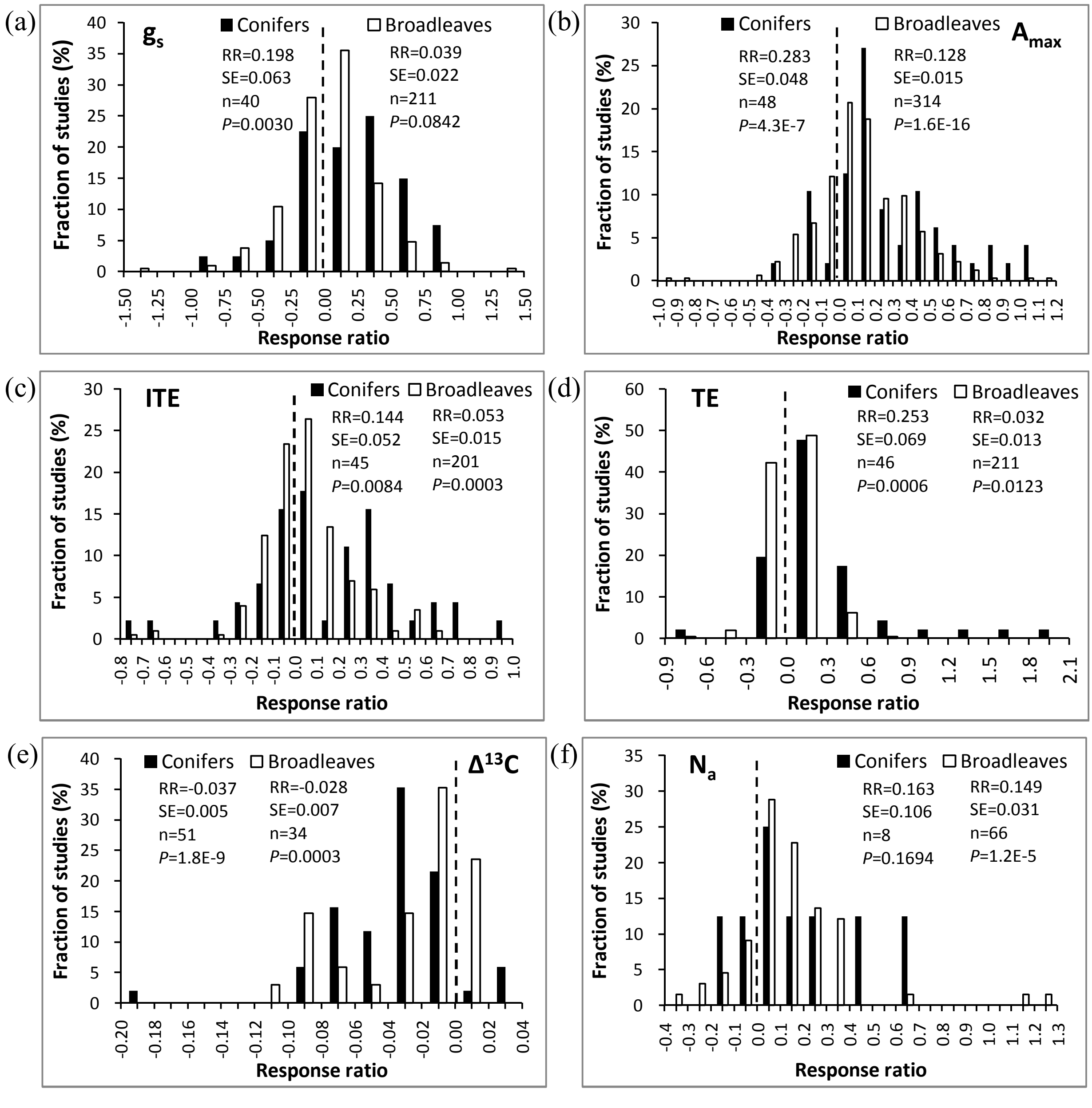


Figure 1 The frequency distributions of the natural logarithm of the response ratio (lnRR) for (a) stomatal conductance (g_s), (b) maximum assimilation rate (A_{max}), (c) intrinsic water-use efficiency (ITE), (d) instantaneous water-use efficiency (TE), (e) carbon-isotope discrimination ($\Delta^{13}C$), (f) leaf N content (N_a), (g) leaf N concentration ($N_{\%}$), (h) specific leaf area (SLA), (i) estimated intrinsic water-use efficiency (Estimated ITE) responses to N fertilization, and (j) the response ratio (lnRR) for the responses to N fertilization of nine variables related to the leaf-level. The vertical dashed line is lnRR=0.

3.4.2. RR---Split analysis by PFT (Conifers and Broadleaves)

N addition significantly decreased carbon-isotope discrimination ($\Delta^{13}C$) by 3.63 and 2.76% (*P*<0.001; Fig. 2e) in both conifers and broadleaves, while maximum assimilation rate (A_{max}), intrinsic water-use efficiency (ITE), instantaneous water-use efficiency (TE), leaf N concentration ($N_{\%}$), and estimated intrinsic water-use efficiency (Estimated ITE) in both conifers and broadleaves significantly increased by 32.71 and 13.65% (*P*<0.001; Fig. 2b), by 15.49% (*P*<0.01; Fig. 2c) and 5.44% (*P*<0.001; Fig. 2c), by 28.78% (*P*<0.001; Fig. 2d) and 3.25% (*P*<0.05; Fig. 2d), by 37.71 and 17.47% (*P*<0.001; Fig. 2g), and by 8.87 and 7.68% (*P*<0.001; Fig. 2i) under N enrichment, respectively. Although N fertilization also increased stomatal conductance (g_s), leaf N content (N_a) and specific leaf area (SLA) in both conifers and broadleaves by 21.89% (*P*<0.01; Fig. 2a) and 3.98%

($P>0.05$; Fig. 2a), by 17.7% ($P>0.05$; Fig. 2f) and 16.07% ($P<0.001$; Fig. 2f), by 6.4 and 0.6% ($P>0.05$; Fig. 2h) respectively, g_s was no significant in broadleaves and N_a was no significant in conifers and SLA was no significant in both conifers and broadleaves (Tab. 2). N-induced changes in independent and dependent variables in conifers were different from those in broadleaves (Fig. 2j). The N fertilization increase $N_{\%}$, N_a, A_{max}, g_s, ITE, Estimated ITE and TE in conifers were larger than those in broadleaves, however, there were no significant differences in N_a increase in conifers and g_s increase in broadleaves ($P>0.05$; Tab. 2). Although N supply increased SLA in both conifers and broadleaves, the changes in SLA did not reveal significant differences between conifers and broadleaves ($P>0.05$; Tab. 2). Moreover, N addition decreased $\Delta^{13}C$ in conifers to a significantly smaller extent than in broadleaves ($P<0.001$; Tab. 2). In addition, ITE was markedly greater than Estimated ITE in conifers ($P<0.01$; Tab. 2), whereas it was markedly lower than Estimated ITE in broadleaves ($P<0.001$; Tab. 2) under N enrichment.



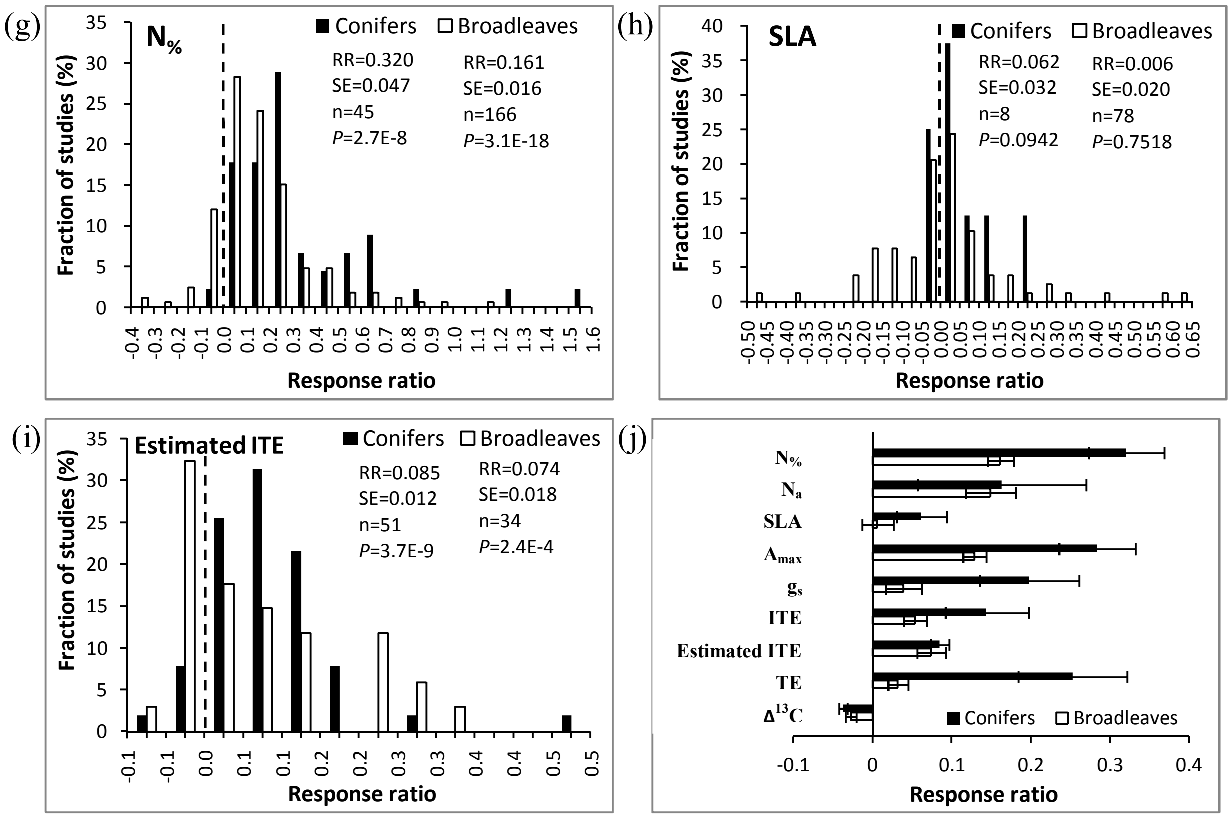
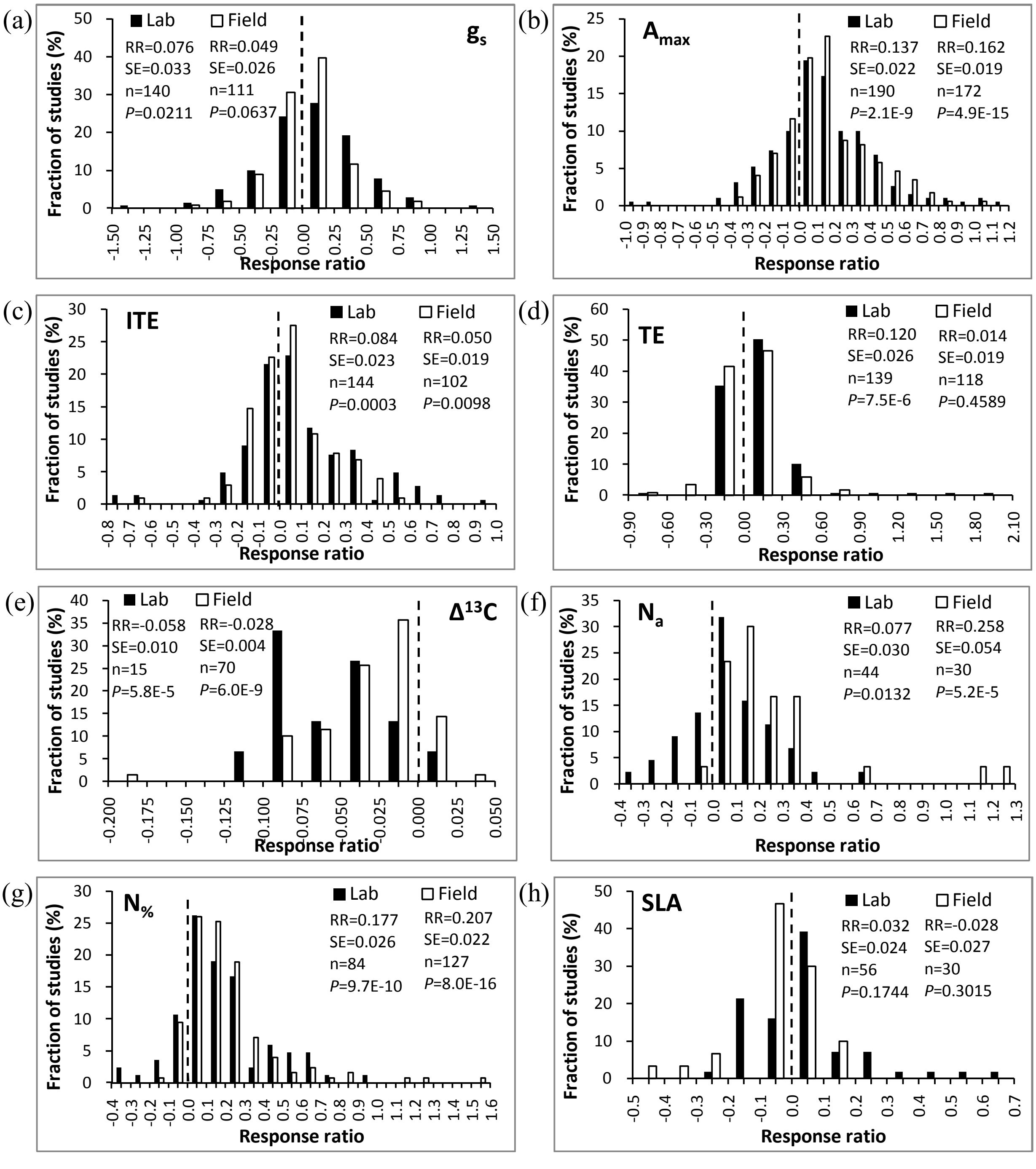


Figure 2 The fraction of studies (%) distributions of the natural logarithm of the response ratio (lnRR) for (a) stomatal conductance (g_s), (b) maximum assimilation rate (A_{max}), (c) intrinsic water-use efficiency (ITE), (d) instantaneous water-use efficiency (TE), (e) carbon-isotope discrimination ($\Delta^{13}C$) , (f) leaf N content (N_a), (g) leaf N concentration ($N_{\%}$), (h) specific leaf area (SLA), (i) estimated intrinsic water-use efficiency (Estimated ITE) responses to N fertilization, and (j) the response ratio (lnRR) for the responses to N fertilization of nine variables related to the leaf-level between different PFT (Conifers and Broadleaves). The vertical dashed line is lnRR=0.

3.4.3. RR---Split analysis by study type (Lab and Field)

N fertilization significantly decreased carbon-isotope discrimination ($\Delta^{13}C$) by 5.63 and 2.76% ($P<0.001$; Fig. 3e) in both lab and field, while maximum assimilation rate (A_{max}), intrinsic water-use efficiency (ITE), leaf N content (N_a), leaf N concentration ($N_{\%}$), and estimated intrinsic water-use efficiency (Estimated ITE) in both lab and field significantly increased by 14.68 and 17.58% ($P<0.001$; Fig. 3b), by 8.76% ($P<0.001$; Fig. 3c) and 5.13% ($P<0.01$; Fig. 3c), by 8% ($P<0.05$; Fig. 3f) and 29.43% ($P<0.001$; Fig. 3f), by 19.36 and 23% ($P<0.001$; Fig. 3g), and by 17.94 and 6.4% ($P<0.001$; Fig. 3i) under N enrichment, respectively. Although N supply also increased stomatal conductance (g_s) and instantaneous water-use efficiency (TE) in both lab and field by 7.9% ($P<0.05$; Fig. 3a) and 5.02% ($P>0.05$; Fig. 3a), and by 12.75% ($P<0.001$; Fig. 3d) and 1.41% ($P>0.05$; Fig. 3d), changes in both g_s and TE were not significant in field studies (Tab. 2). Moreover, the effects of N addition on specific leaf area (SLA) differed, with an increase of 3.25% in lab ($P>0.05$; Fig. 3h) and with a decrease of 2.76% in field ($P>0.05$; Fig. 3h), and changes in SLA was not significant in either lab or field (Tab. 2). N-induced changes in independent and dependent variables in lab were different from those in the field (Fig. 3j). The N fertilization significantly increased $N_{\%}$, N_a and A_{max} in lab, but changes were lower than those in the field; on the contrary, the N fertilization-induced increases in g_s, ITE, Estimated ITE and TE in the lab were larger than those in the field; however, there were no significant differences in both g_s and TE arose in field (*P*>0.05; Tab. 2). Moreover, N addition decreased $\Delta^{13}C$ in lab was significantly smaller than that in field (P<0.001; Tab. 2). Although N supply increased SLA in lab and decreased SLA in field, the changes in SLA did not reveal significant differences between lab and field (P>0.05; Tab. 2). In addition, Estimated ITE was markedly greater than ITE in lab, likewise it was markedly higher than ITE in field under N enrichment.



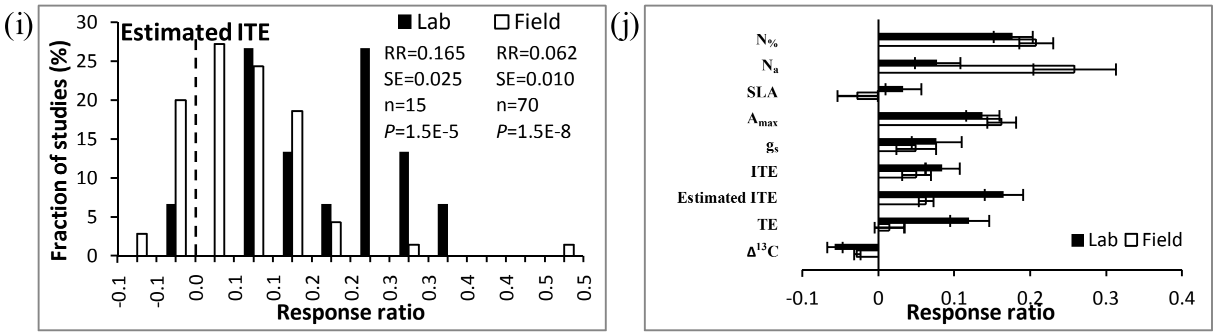


Figure 3 The fraction of studies (%) distributions of the natural logarithm of the response ratio (lnRR) for (a) stomatal conductance (g_s), (b) maximum assimilation rate (A_{max}), (c) intrinsic water-use efficiency (ITE), (d) instantaneous water-use efficiency (TE), (e) carbon-isotope discrimination ($\Delta^{13}C$) , (f) leaf N content (N_a), (g) leaf N concentration ($N_{\%}$), (h) specific leaf area (SLA), (i) estimated intrinsic water-use efficiency (Estimated ITE) responses to N fertilization, and (j) the response ratio (lnRR) for the responses to N fertilization of nine variables related to the leaf-level between different study types (Lab and Field). The vertical dashed line is lnRR=0.

Table 2 Results from statistical analysis for the response ratio (lnRR) of the variables response to N fertilization between different PFTs (Conifers and Broadleaves) and study types (Lab and Field).

Variables	RR-Overall analysis	RR- Split analysis by PFT		RR- Split analysis by study type	
		Conifers	Broadleaves	Lab	Field
$N_{\%}$	***	***	***	***	***
N_a	***	NS	***	*	***
SLA	NS	NS	NS	NS	NS
A_{max}	***	***	***	***	***
g_s	**	**	NS	*	NS
ITE	***	**	***	***	**
Estimate ITE	***	***	***	***	***
TE	***	***	*	***	NS
$\Delta^{13}C$	***	***	***	***	***

An asterisk (*) denotes variables differing significantly using a paired t-test: ***indicates values statistically different at $P < 0.001$, **indicates those different at $P < 0.01$, *indicates those different at $P < 0.05$ and NS indicates the values do not differ significantly according to the least significant difference test ($P > 0.05$). $N_{\%}$: leaf N concentration; N_a: leaf N content; SLA: specific leaf area; A_{max}: maximum assimilation rate; g_s: stomatal conductance; ITE: intrinsic water-use efficiency; Estimate ITE: estimated intrinsic water-use efficiency; TE: instantaneous water-use efficiency; $\Delta^{13}C$: carbon-isotope discrimination.

3.4.4. Slope of correlation with N%---Overall analysis

N-induced changes in the slope of correlation with leaf N concentration ($N_{\%}$) for stomatal conductance (g_s), maximum assimilation rate (A_{max}), specific leaf area (SLA), intrinsic water-use efficiency (ITE), carbon-isotope discrimination ($\Delta^{13}C$) and estimated intrinsic water-use efficiency (Estimated ITE) exhibited great variability across studies, ranging from a minimum slope of -150 to a maximum of 183.75 in g_s vs. $N_{\%}$, from -3682.54 to 3587.74 in A_{max} vs. $N_{\%}$, from -1.86 to 1.97 in SLA vs. $N_{\%}$, from -29762 to 15890 in ITE vs. $N_{\%}$, from -645.55 to 56.93 in $\Delta^{13}C$ vs. $N_{\%}$, and from

-601 to 10792 in Estimated ITE vs. $N_{\%}$. On average, the effects of N fertilization on the overall slope were an increase in g_s vs. $N_{\%}$ (*P*>0.05; Fig. 4a), A_{max} vs. $N_{\%}$ (*P*<0.001; Fig. 4b), SLA vs. $N_{\%}$ (*P*>0.05; Fig. 4c) and Estimated ITE vs. $N_{\%}$ (*P*<0.001; Fig. 4f). In contrast, fertilization resulted in a decrease in ITE vs. $N_{\%}$ (*P*>0.05; Fig. 4d) and $\Delta^{13}C$ vs. $N_{\%}$ (*P*<0.001; Fig. 4e). In addition, Estimated ITE was positively correlated to $N_{\%}$, whereas ITE was negatively related to $N_{\%}$ under N enrichment (Fig. 4g). However, there was no significant correlation in response to N supply between SLA, g_s, ITE and $N_{\%}$ respectively (Tab. 3).

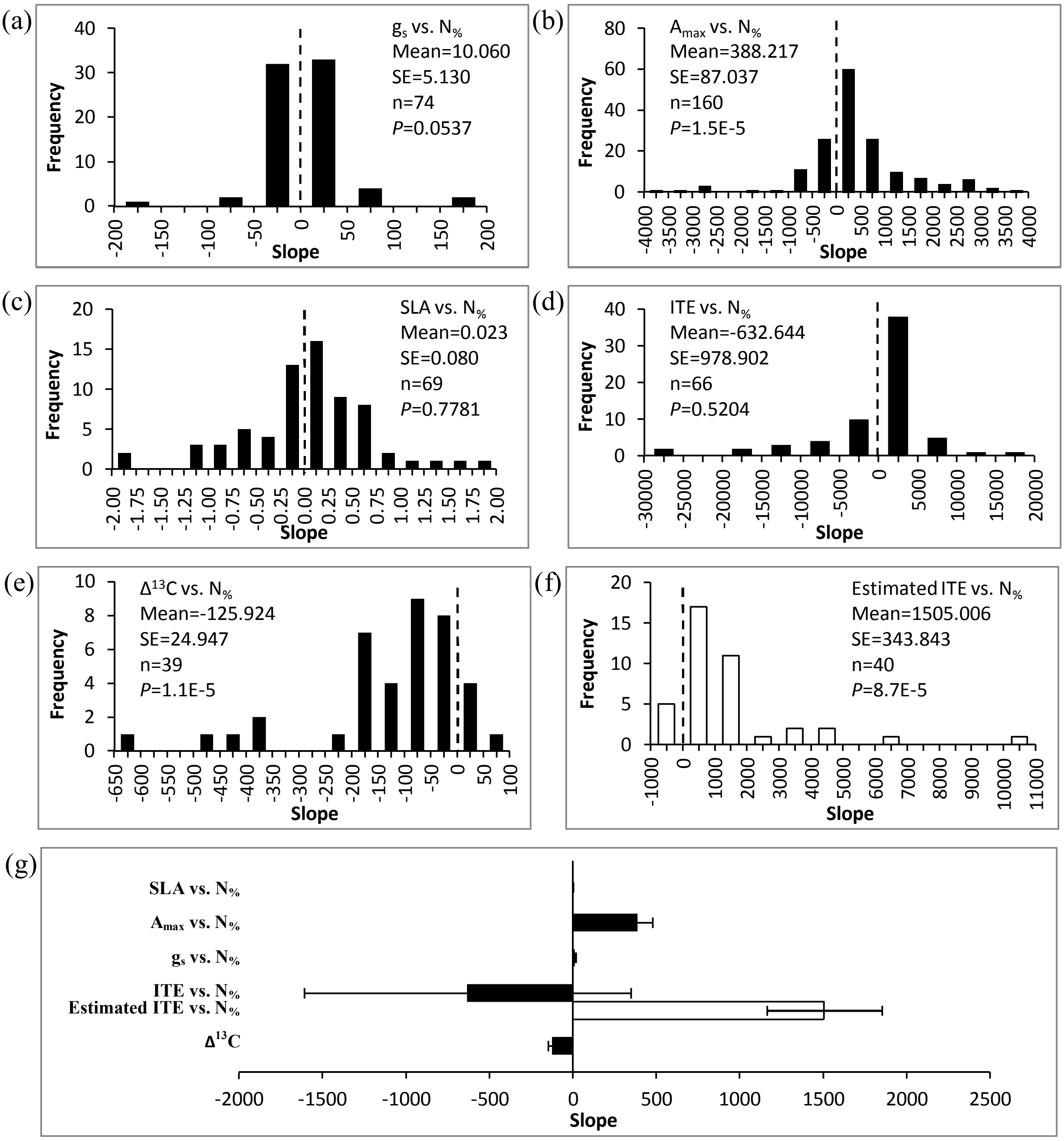
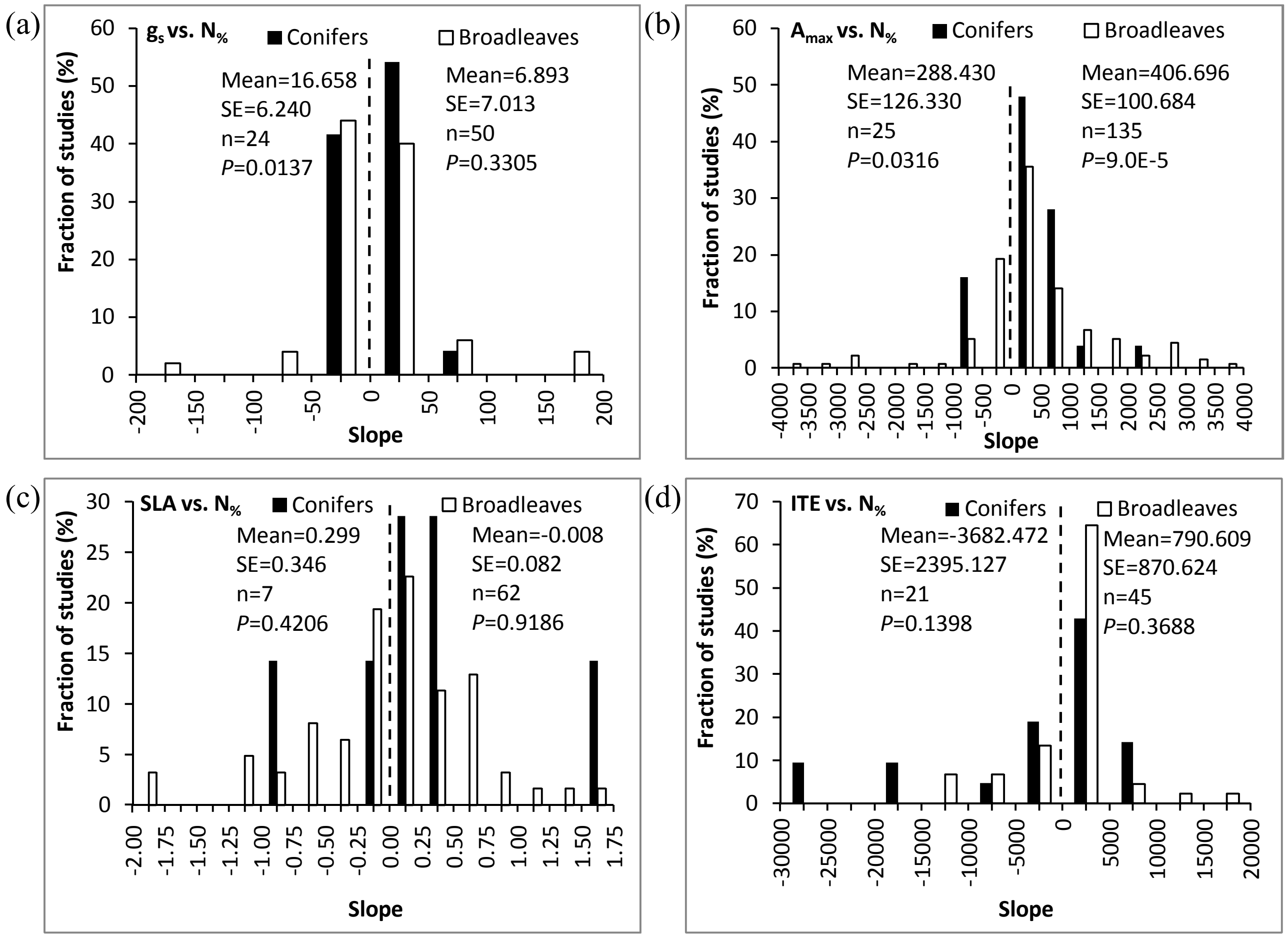


Figure 4 The frequency distributions of the slope of correlation with leaf N concentration ($N_{\%}$) for (a) stomatal conductance (g_s), (b) maximum assimilation rate (A_{max}), (c) specific leaf area (SLA), (d) intrinsic water-use efficiency (ITE), (e) carbon-isotope discrimination ($\Delta^{13}C$), (f) estimated intrinsic water-use efficiency (Estimated ITE) and (g) the slope of the correlation with leaf N concentration ($N_{\%}$) of six variables related to the leaf-level. The vertical dashed line is lnRR=0.

3.4.5. Slope of correlation with N%--- Split analysis by PFT (Conifers and Broadleaves)

On average, the effects of N fertilization on the split-PFT slope differed between conifers and broadleaves with an increase in g_s vs. $N_{\%}$ (*P*<0.05; Fig. 5a), A_{max} vs. $N_{\%}$ (*P*<0.05; Fig. 5b) and Estimated ITE vs. $N_{\%}$ (*P*<0.05; Fig. 5f); in contrast, a significant decrease was observed in $\Delta^{13}C$ vs. $N_{\%}$ (*P*<0.05; Fig. 5e) in both conifers and broadleaves, but g_s vs. $N_{\%}$ (*P*>0.05; Tab. 3) was not significant in broadleaves. In addition, N supply increased SLA vs. $N_{\%}$ (*P*>0.05; Fig. 5c) in conifers and decreased that in broadleaves, whereas, decreased ITE vs. $N_{\%}$ (*P*>0.05; Fig. 5d) in conifers and increased that in broadleaves. However the changes in both SLA and ITE vs. $N_{\%}$ did not reveal significant differences between conifers and broadleaves (Tab. 3). Moreover, Estimated ITE was positively correlated to $N_{\%}$ in both conifers and broadleaves, whereas ITE was negatively related to $N_{\%}$ in conifers and was positively correlated to $N_{\%}$ in broadleaves (Fig. 5g), also Estimated ITE vs. $N_{\%}$ (*P*<0.05; Fig. 5g) was greater than ITE vs. $N_{\%}$ (*P*>0.05; Fig. 5g) in broadleaves under N enrichment, however, there was no significant correlation in response to N supply between ITE and $N_{\%}$ (*P*>0.05; Tab. 3) in both conifers and broadleaves.



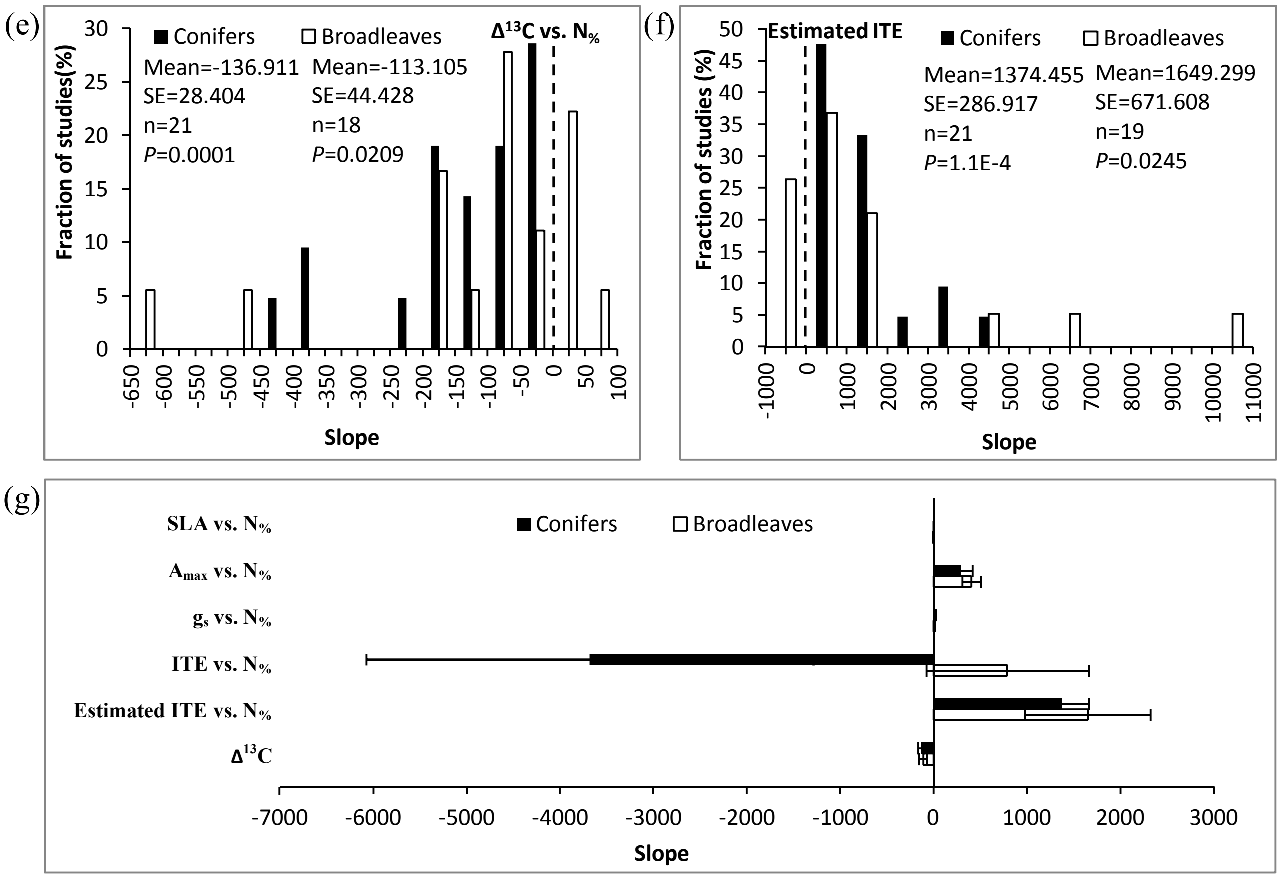


Figure 5 The fraction of studies (%) distributions of the slope of correlation with leaf N concentration ($N_{\%}$) for (a) stomatal conductance (g_s), (b) maximum assimilation rate (A_{max}), (c) specific leaf area (SLA), (d) intrinsic water-use efficiency (ITE), (e) carbon-isotope discrimination ($\Delta^{13}C$), (f) estimated intrinsic water-use efficiency (Estimated ITE) and (g) the slope of the correlation with leaf N concentration ($N_{\%}$) of six variables related to the leaf-level between different PFT (Conifers and Broadleaves). The vertical dashed line is lnRR=0.

3.4.6. Slope of correlation with N%--- Split analysis by study type (Lab and Field)

On average, the effects of N fertilization on the split-PFT slope between lab and field with an increase in g_s vs. $N_{\%}$ ($P>0.05$; Fig. 6a), A_{max} vs. $N_{\%}$ ($P<0.001$; Fig. 6b) and Estimated ITE vs. $N_{\%}$ ($P<0.05$; Fig. 6f), in contrast, with a decrease in ITE vs. $N_{\%}$ ($P>0.05$; Fig. 6d) and $\Delta^{13}C$ vs. $N_{\%}$ ($P<0.05$; Fig. 6e) in both lab and field, however, A_{max} vs. $N_{\%}$ ($P>0.05$; Tab. 3) was no significant in lab as well as both g_s and ITE vs. $N_{\%}$ ($P>0.05$; Tab. 3) was no significant in both lab and field, whereas $\Delta^{13}C$ and Estimated ITE was significantly related to $N_{\%}$ in both lab and field ($P<0.05$; Tab. 3). In addition, N supply increased SLA vs. $N_{\%}$ ($P>0.05$; Fig. 6c) in lab and decreased that in filed, but the changes in SLA vs. $N_{\%}$ did not reveal significant differences between lab and field (Tab. 3). Moreover, Estimated ITE was positively correlated to $N_{\%}$ in both lab and field, whereas ITE was negatively related to $N_{\%}$ in both lab and field (Fig. 6g), also Estimated ITE vs. $N_{\%}$ ($P<0.05$; Fig. 6g) in lab was greater than that in field under N enrichment, however, there was no significant correlation in response to N supply between ITE and $N_{\%}$ ($P>0.05$; Tab. 3) in both lab and field.

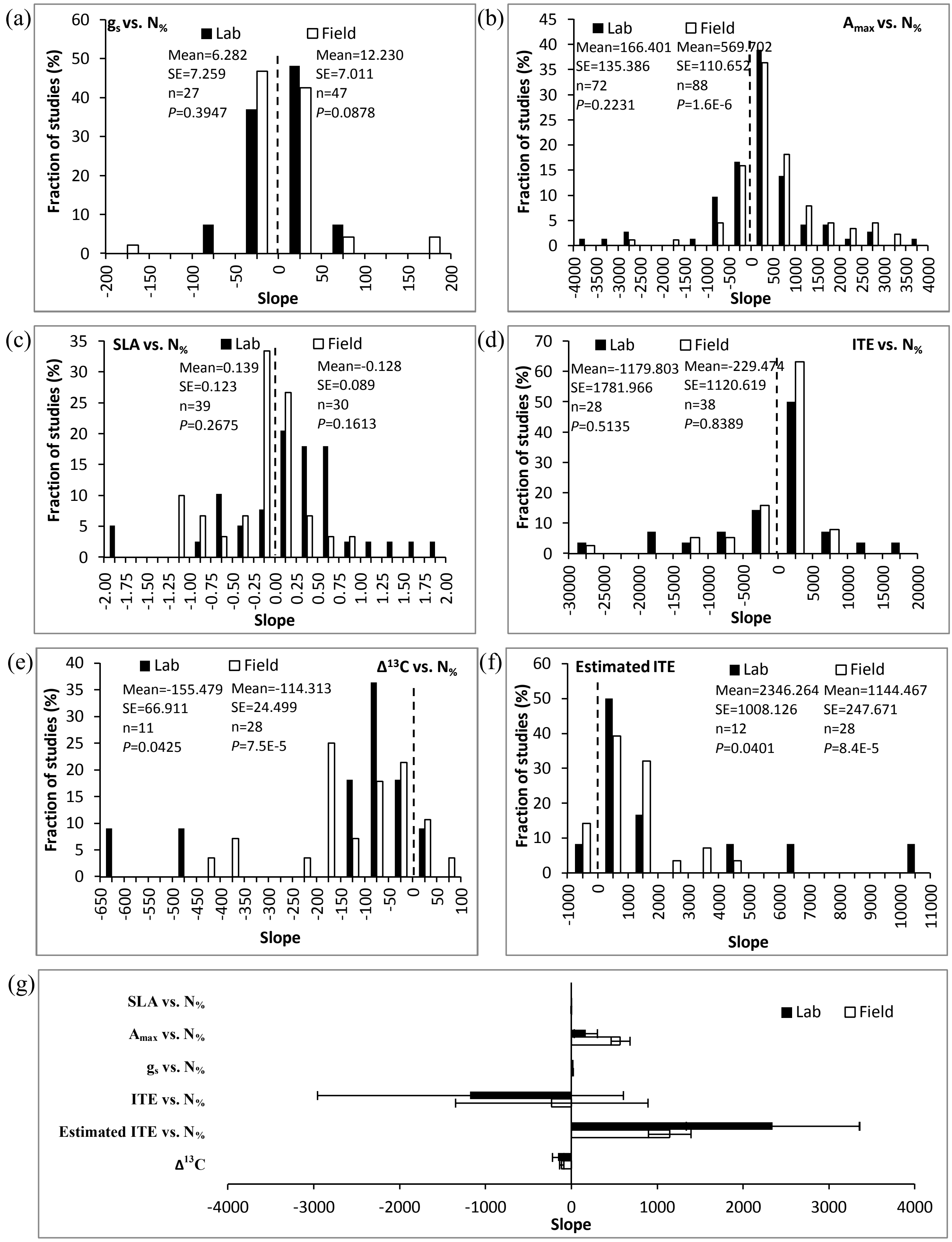


Figure 6 The fraction of studies (%) distributions of the slope of correlation with leaf N concentration ($N_{\%}$) for (a) stomatal conductance (g_s), (b) maximum assimilation rate (A_{max}), (c) specific leaf area (SLA), (d) intrinsic water-use efficiency (ITE), (e) carbon-isotope discrimination ($\Delta^{13}C$), (f) estimated intrinsic water-use efficiency (Estimated ITE) and (g) the slope of the correlation with leaf N concentration ($N_{\%}$) of six variables related to the leaf-level between different study types (Lab and Field). The vertical dashed line is lnRR=0.

Table 3 Results from statistical analysis for the slope of correlation between leaf N concentration ($N_{\%}$) and the variables respectively response to N fertilization between different PFT (Conifers and Broadleaves) and study type (Lab and Field).

Variables	Slope-Overall analysis	Slope- Split analysis by PFT		Slope- Split analysis by study type	
		Conifers	Broadleaves	Lab	Field
SLA vs. $N_{\%}$	NS	NS	NS	NS	NS
A_{max} vs. $N_{\%}$	***	*	***	NS	***
g_s vs. $N_{\%}$	NS	*	NS	NS	NS
ITE vs. $N_{\%}$	NS	NS	NS	NS	NS
Estimated ITE vs. $N_{\%}$	***	***	*	*	***
$\Delta^{13}C$ vs. $N_{\%}$	***	***	*	*	***

An asterisk (*) denotes variables differing significantly using a paired t-test: ***indicates values statistically different at $P < 0.001$, **indicates those different at $P < 0.01$, *indicates those different at $P < 0.05$ and NS indicates the values do not differ significantly according to the least significant difference test ($P > 0.05$). SLA: specific leaf area; A_{max}: maximum assimilation rate; g_s: stomatal conductance; ITE: intrinsic water-use efficiency; Estimate ITE: estimated intrinsic water-use efficiency; $\Delta^{13}C$: carbon-isotope discrimination; $N_{\%}$: leaf N concentration.

3.4.7. Discussion

Overall, we observed a strong photosynthetic responses of plants to N fertilization, which probably triggered also a significant increase in the stomatal diffusive conductance to H_2O (g_s) of plants (Fig. 1a). It is likely that N addition accelerate the transport of photosynthetic CO_2 in the leaves, leading to markedly enhanced maximum assimilation rate (A_{max}) of the plants (Fig. 1b). The photosynthetic capacity responses to N availability have been well documented in hardwood tree species (Wendler & Millard 1996, Wang & Leuning 1998, Tyree et al. 2009), which indicated that the photosynthetic rate of plants might be dependent on soil N availability. In addition, significant increaseing leaf N concent (N_a; Fig. 1f) in response to N addition displayed positive correlation with A_{max} (Fig. 1j) and also with specific leaf area (SLA, area per unit leaf dry mass; Fig. 1h), despite the fact that SLA did not change in response to N fertilization (Tab. 2). We further investigated the changes of leaf N content (N_a) to explain the potential mechanism in leaf photosynthesis. Several studies have highlighted global relationships between leaf-level gas exchange characteristics and N content of the leaves (Field & Mooney 1986, Reich et al. 1998, Niinemets 1999, Reich et al. 1999, Cabrera-Bosquet et al. 2007). In general, higher leaf N content correlates with greater photosynthetic CO2 assimilation rates, mostly due to greater investment in ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco; Evans 1989, Evans & Seemann 1989, Poorter & Evans 1998). However, in halophytes and xerophytes, assimilation rates may not change predictably with leaf N content (N_a). Plants growing in environments with low water availability and high leaf-to-air VPD (water-vapor partial-pressure difference) tend to have leaves of low specific leaf area (SLA) and stomatal conductance (g_s), but high leaf N content for a particular assimilation rate, resulting in correspondingly high water-use efficiency but low nitrogen-use efficiency (Mooney et al. 1978, MacFarlane et al. 2004, Barker et al. 2006). Hence, allocation of N to the photosynthetic apparatus may be constrained by other environmental pressures in halophytes and xerophytes (Martin et al. 2010).

Numerous studies have shown that N fertilization enhances plant productivity by improving ITE (Livingston et al. 1999) by: (1) increasing water loss control by stomatal conductance, with no influence on assimilation rate (Guehl et al. 1995); or (2) increasing assimilation rate as a result of increased N investment in the photosynthetic apparatus (Ranjith et al. 1995) with no counterbalancing effect on stomatal conductance (Harvey & van den Driessche 1999, Welander & Ottosson 2000); or (3) causing a moderate increase in assimilation rate and a slight decrease in stomatal conductance (Livingston et al. 1999). Meanwhile, studies on the N effects on TE in most species showed a decrease when N availability was reduced (Sheriff et al. 1986, Reich et al. 1989, Green & Mitchell 1992). Our results showed that ITE (Fig. 1c) and TE (Fig. 1d) significantly increased with increasing N supply (Tab. 2), and there were also markedly effects of N supply on maximum assimilation rate (A_{max}; Fig. 1b) and stomatal conductance (g_s, Fig. 1a), whereas SLA (Fig. 1h) did not change in response to N fertilization (Tab. 2). However, these contrasting results are not easy to explain, partly because the mechanisms underlying the responses of stomatal conductance to altered concentrations of leaf N and intercellular CO_2 are not fully understood (Mitchell & Hinckley 1993, Guehl et al. 1995, Grassi et al. 2002), and partly because it is unclear how changes in leaf N concentrations ($N_{\%}$) affect the coupling between stomatal conductance and CO_2 assimilation (Wong et al. 1979). As well as the effects of nutrient status on water-use efficiency (i.e. the quotient of biomass production to water use), remain scarce in tree species and practically all refer to leaf instantaneous water-use efficiency (TE). For example, some authors (Stark 1992, Mitchell & Hinckley 1993) have found that TE was not affected by altered leaf N concentration ($N_{\%}$) in *Pseudotsuga menziesii* and *Eucalyptus* trees. Therefore, leaf chlorophyll concentration as a good indicator of photosynthetic capacity is positively correlated with leaf N concentration (chlorophyll complexes being mostly made up of N-containing enzymes and other organic compounds) (Chang & Robison 2003). Also Naumann et al. (2008) observed that low concentrations of chlorophyll limit photosynthetic potential directly and lead to a decrease in biomass production in the plants. In our slope analysis, we found that no significant correlation between SLA (Fig. 4c), g_s (Fig. 4a), ITE (Fig. 4d) and leaf N concentration ($N_{\%}$, Fig. 4g) respectively, only A_{max} (Fig. 4b) was significantly correlated with $N_{\%}$ (Tab. 3). ITE may be the consequence of changes in leaf N concentration ($N_{\%}$), which can in turn affect the ratio between assimilation and stomatal conductance. The relationship between $N_{\%}$ and ITE is not straightforward, but a positive trend is often observed, in ambient air (Guehl et al. 1995, Ripullone et al. 2004) as well as in elevated CO_2 (Tognetti & Johnson 1999). A review on the effects of nutrient supply in crop plants emphasized the importance of N influence on ITE (Brueck 2008).

Tree ring studies, using $^{13}C/^{12}C$ ratio in dated growth rings, provide long-term carbon isotope chronologies to explore the relationship between environmental variability and changes in tree physiology, with special reference to the balance between water loss and carbon gain at the leaf level (Loader et al. 2007, Saurer & Siegwolf 2007). The relationship between TE and ITE and $\delta^{13}C$ has already been extensively studied (Ehleringer et al. 1993, Ennahli & Earl 2005), providing an interesting time-integration for plant WUE over the period of dry matter plant synthesis (Araus 2002). Positive relations between $\delta^{13}C$ in leaves and TE and ITE have been reported in C_3 plants (Farquhar et al. 1989). A limited analysis of data from other Swedish Forest Optimum Nutrition Experiments (Tamm 1985) at Stråsan and Lisselbo (on 5 years of data) showed that on average, N treatments had higher $\delta^{13}C$ values than the control, but that this difference mainly occurred during drier years (Högberg et al. 1993). Leonardi et al. (2012) applied generalized additive models and linear mixed-effects models to predict the effects of climatic variables and nitrogen deposition on $\Delta^{13}C$ and ITE; the results showed a declining $\Delta^{13}C$ trend in the angiosperm and conifer species over the industrial period and a 16.1% increase of ITE between 1850 and 2000, with no evidence that the increased rate was reduced at higher ambient CO_2 values. Our results showed that TE (Fig. 1d), ITE (Fig. 1c) and Estimated ITE (Fig. 1i) significantly increased with increasing N supply (Tab. 2), and there were also markedly effects of N supply on maximum assimilation rate (A_{max}), stomatal conductance (g_s) and carbon-isotope discrimination ($\Delta^{13}C$), though N addition significantly decreased in $\Delta^{13}C$ (Fig. 1e), and the N-induced increased in Estimated ITE more than ITE (Fig. 1j). In our slope analysis, furthermore, we found that no significant correlation between ITE (Fig. 4d) and leaf N concentration ($N_{\%}$, Fig. 4g), whereas both Estimated ITE (Fig. 4f) and $\Delta^{13}C$ (Fig. 4e) was significantly correlated with $N_{\%}$ (Tab. 3), and Estimated ITE and $\Delta^{13}C$ were negatively correlated under N enrichment.

However, Ripullone et al. (2004) appeared to find substantial differences among tree species in the response of ITE to nutritional status. The results of Guehl et al. (1995) showed that there was no uniqueness among forest tree species in the response of TE to nutritional deficiency. Also Leonardi et al. (2012) realised that no single climatic or atmospheric variable had a clearly predominant effect, but $\Delta^{13}C$ and ITE showed complex dependent interactions between different covariates. Therefore, two approach performed in the covariates of our meta-analysis in which split into two groups of plant functional types (PFT) between conifers and broadleaves, and study types between Lab and Field, for assessing interactions in response to N fertilization. One way to investigate inherent differences in allocation among physiologically, ecologically and/or phylogenetically contrasting different tree species is to analyse broad allometric relationships between conifers and broadleaves. This approach, where data typically originate from plants grown under various environmental conditions, focuses on size as the main driver of allocation changes. The analysis proceeds under the assumption that all species of a given group face the same size constraints and that environmental effects are negligible. Another approach is to compare species belonging to different species groups that are grown simultaneously under the same conditions. This minimizes the risk of allocation being confounded with environmentally induced variation (Poorter et al. 2011).

Although we followed these two approach, no clear effect of N fertilization on specific leaf area (SLA) no matter what the results between plant functional types (PFT) and study types (Tab. 2); even in the slope analysis, there was no significant correlation between SLA and leaf N concentration ($N_{\%}$) in conifers and broadleaves group or in lab and field group (Tab. 3). The above implies that an important component of the N-effect mediated through specific leaf area (SLA) cannot be studied until we understand the mechanisms underlying the interaction with SLA better. Plasticity for SLA is known to vary widely, being highest for light, submergence and temperature, and only modest for CO_2, nutrients, drought and salinity (Poorter et al. 2009, 2010). Meanwhile, the changes in SLA can occur through changes in leaf chemistry, changes in concentrations of carbon storage compounds (Waring et al. 1985) or changes in leaf thickness (Konings 1989), Ibrahim (1998) found little evidence of changes in leaf carbon chemistry (unpublished data) and conclude that the changes in SLA were probably associated with the production of thicker leaves.

On the contrary, the response of leaf N concentration ($N_{\%}$) to N application was completely significant in both plant functional types (PFT) and study types (Tab. 2), suggesting that studies on $N_{\%}$ are suitable for detailed physiological investigations on leaf N uptake and CO_2 assimilation capacity, even for indirectly evaluation the effect of adding N on instantaneous water-use efficiency (TE) and intrinsic water-use efficiency (ITE). Within the group of plant functional types (PFT), the response of leaf N concentration ($N_{\%}$; Fig. 5g) to N supply was in strong contrast with Estimated ITE (weakest effect in broadleaves; Fig. 5f) and $\Delta^{13}C$ (weakest effect in broadleaves; Fig. 5e) in conifers as compared with broadleaves. However, such a difference was not found in intrinsic water-use efficiency (ITE, not significant in both conifers and broadleaves; Fig. 5d, Tab. 3), the discrepancies of gas exchange characteristics between conifers and broadleaves when assessing direct and indirect effects of leaf N concentration ($N_{\%}$) on ITE can be found in stomatal conductance (g_s, weakest effect in conifers, not significant in broadleaves; Fig. 5a, Tab. 3) and maximum assimilation rate (A_{max}, weakest effect in conifers; Fig. 5b). Although the relationship between $N_{\%}$ and ITE is not straightforward and also the mechanisms underlying the response of g_s to altered leaf N status remain largely unknown, the results are obtained by Guehl et al. (1995) in pine (conifers) show that these mechanisms do not always bring about a positive coupling between g_s and the CO_2 assimilation capacity as was proposed by Wong et al. (1979). Within the group of study types, we hypothesized that the responses of leaf N concentration ($N_{\%}$; Fig. 6g) in the lab would be modified by the same variables that operate in field experiments. Except some variables, no effect of N fertilization was consistent between approaches: in both lab and field experiments, for instance stomatal conductance (g_s; Fig. 6a, Tab. 3), specific leaf area (SLA; Fig. 6c, Tab. 3), intrinsic water-use efficiency (ITE; Fig. 6d, Tab. 3). Most other variables, such as maximum assimilation rate (A_{max}, not significant in lab; Fig. 6b), estimated intrinsic water-use efficiency (Estimated ITE, weakest effect in lab; Fig. 6f) and carbon-isotope discrimination ($\Delta^{13}C$, weakest effect in lab; Fig. 6e) were of less significance in the lab than in the field (Tab. 3). For example, in a recent meta-analysis on the effects of water and light on plant performance, Holmgren et al. (2011) showed that physiological (direct) effects were very similar between lab and field experiments, whereas seedling survival (indirect effect) was not.

It is worth noting that the reasons behind the lack of sensitivity in the lab remain speculative. Either these variables are mainly controlled for in lab experiments (background N), take effect at time scales beyond the duration of most lab experiments, are mediated through other factors controlled for in lab experiments (temperature, microhabitat), are not fully comparable between the experiment types (temperature, microhabitat), or results reflect a limited amount of data (Limpens et al. 2012). Sensitivity analyses suggested that the absence of a P effect in the lab is driven by a few influential experiments only. The other effects were fairly robust, however, suggesting alternative explanations (Limpens et al. 2012). The most interesting difference with field experiments is the absence of an interaction between N fertilization and temperature in lab experiments. Assuming we can equate the effect of average lab temperature with that of mean July temperature, the absence of a temperature effect in lab experiments suggests that the temperature sensitivity reported for field fertilization experiments (Limpens et al. 2011), is likely an indirect effect mediated through other (environmental) factors and not a direct effect of temperature-induced changes in respiration or photosynthetic activity (Limpens et al. 2011). The above clearly highlights the current lack of experiments targeted at elucidating interactions between N application and other environmental variables (Limpens et al. 2011, 2012) may be one of the prime reasons leading to lab experiments overestimating the effect of adding N compared to field experiments.

The significant variation in carbon isotope discrimination ($\Delta^{13}C$; Fig. 2e & 3e, Tab. 2) translated into a marked effect on estimated intrinsic water-use efficiency (Estimated ITE; Fig. 2i & 3i, Tab. 2) under N fertilization in both plant functional types (PFT) and study types in our meta-analysis. This $\Delta^{13}C$ variation might reflect the capacity of trees to actively adjust leaf gas exchange in conjunction with increasing CO_2 concentration. Indeed, in accordance with significantly increased Estimated ITE in both plant functional types (PFT) and study types, the previous studies based on isotopic records for individual N fertilization experiments that have generally revealed the literature data of intrinsic water-use efficiency (ITE; Fig. 2c & 3c) were also significantly increased in both group of conifers and broadleaves and group of lab and field (Tab. 2), particularly in broadleaves and in lab (Hogberg et al. 1993, Livingston et al. 1999, Clearwater & Meinzer 2001, Choi et al. 2005, Lovelock et al. 2006, Fernandez et al. 2006, Martin et al. 2010, Walia et al. 2010). Therefore, the results indicated that $\Delta^{13}C$ as a robust estimation of predictor factor was possibly free from tree species or study types constraints, and consistently showed a significant and positive association of N supply and ITE. Meanwhile, our observation supported the hypothesis of nitrogen fertilization as a significant factor in the regulation of water loss-carbon gain balance at the leaf level (Leonardi et al. 2012) and long-term soil N accumulation might lead to enhanced plant nutritional status (Aber et al. 2003), which could stimulate photosynthesis and increase ITE.

Furthermore, instantaneous water-use efficiency (TE, weakest effect in broadleaves; Fig. 2d) or intrinsic water-use efficiency (ITE, weaker effect in conifers; Fig. 2c) and $\Delta^{13}C$ (Fig. 2e) were negatively correlated in conifers and in broadleaves (Fig. 2j), respectively. Ripullone et al. (2004) observed the regression between TE and $\Delta^{13}C$ was significant only for Douglas-fir. Thus, the mechanisms underlying the response of WUE to N supply were mainly related to a positive effect of N supply on photosynthetic rates (A_{max}, significant in both PFT and study types; Fig. 2b & 3b). Likewise, TE (not significant in field; Fig. 3d) or ITE (weaker effect in field; Fig. 3c) and $\Delta^{13}C$ (Fig. 3e) were negatively correlated in lab and in field (Fig. 3j), respectively, although the correlation between TE and $\Delta^{13}C$ was not significant in field. This linear negative relationship was in agreement with the results of many studies under both controlled lab and field experiments (Farquhar et al. 1982, 1989, Zhang et al. 1993, Guehl et al. 1994, Aitken et al. 1995, Warren et al. 2001), confirming that $\Delta^{13}C$ is a useful complement to the short-term evaluation of WUE (TE and ITE) by gas exchange measurements.

However, the N fertilization modified the variation in stomatal conductance (g_s, weaker effect in conifers, not significant in broadleaves; Fig. 2a) and leaf N content (N_a, not significant in conifers; Fig. 2f) between conifers and broadleaves, these contrasting results were not easy to explain, partly because the mechanisms underlying the responses of stomatal conductance to altered content of leaf N and intercellular CO_2 are not fully understood (Mitchell & Hinckley 1993, Guehl et al. 1995, Grassi et al. 2002), and partly because it is unclear how changes in N content affect the coupling between stomatal conductance and CO_2 assimilation (Wong et al. 1979). Moreover, there appear to be substantial differences among tree species in the response of WUE to nutritional status (Ripullone et al. 2004). For instance, Guehl et al. (1995) found that increasing g_s in response to N deficiency in pine (conifers), constitutes an original result that does not conform with the general observation that g_s is reduced in response to N deficiency while ITE is either (i) decreased found in oak (broadleaves) and as also observed in *Pinus taeda* (Green & Mitchell 1992) and *Ulmus americana* (Reich et al. 1989) or (ii) is maintained constant as observed in another group of species incuding *Eucalyptus kitsoniana*, *E. globulus* (Stark 1992) and *Pseudotsuga menziesii* (Mitchell & Hinckley 1993). In addition, the similar contrasting results were also observed in stomatal conductance (g_s, weakest effect in lab, not significant in field; Fig. 3a) and leaf N content (N_a, weakest effect in lab; Fig. 3f) between lab and field under N enrichment. A large body of evidence has been generated on elevated CO_2 effects on stomatal conductance and assimilation in short-term experiments, although the applicability of short-term responses to long-term physiological adjustments has been questioned (Körner 2006). Enhanced ITE may imply increased plant transpiration efficiency, and a positive effect on plant carbon balance. However, it has recently been shown that the increased ITE observed during the last half century did not translate to increased tree growth (Peñuelas et al. 2008, Andreu-Hayles et al. 2011, Peñuelas et al. 2011). Nonetheless, enhanced ITE has the potential to aid trees during drought stress conditions associated with climate change (Giuggiola et al. 2010), and to overcome other environmental growth limitations (Leonardi et al. 2012).

3.5. Conclusions

The follwing conclusion can be drawn from this meta-analysis of the literature study on the effect of N fertilization on the ITE of woody seedlings plants through (i) gas exchange measurements, (ii) carbon isotope discrimination ($\Delta^{13}C$) analysis and the application of the Farquhar model on the effects of N fertilization on the ITE at leaf level, which has been shown to respond strongly (and in a consistent manner between plant functional types) to the addition of N due to the absence of increasing stomatal conductance and transpiration as compared with the sharp rise in photosynthetic rates. ITE obtained by gas exchanges measurement have proved completely consistent with what estimated by $\Delta^{13}C$. In the N fertilization experiments, it is worthwhile to note that the effects are overestimated in ambient environment (i.e. lab) as compared with the measures in field.

3.6. References

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Appendix A

A list of 73 papers from which data were extracted for the meta-analysis.

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Cai, X.A., Sun, G.T., Zhao, P., & Liu, X.J. (2008). Nitrogen and low light intensity limit effects on photosynthetic energy use of subtropical forest successional species (In Chinese). *Science in China*, *38(5)*, 464-474.

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DesRochers, A., Van den Driessche, R., & Thomas, B.R. (2006). NPK fertilization at planting of three hybrid poplar clones in the boreal region of Alberta. *Forest Ecology and Management*, *232*, 216-225.

Ding, W.J. (2012). Effects of nitrogen deposition on ecophysiological characteristics and interspecific relationship of *Quercus acutissima* and *Robinia pseudoacacia* (In Chinese). *Published Ph.D. Thesis*.

Fan, Y.Y. (2012). Effects of water and nitrogen availability on carbon, nitrogen metabolism and drought resistance of *Robinia pseudoacacia* (In Chinese). *Published M.Sc. Thesis*.

Fernandez, M., Novillo, C., & Pardos, J.A. (2006). Effects of water and nutrient availability in *Pinus pinaster Ait.* open pollinated families at an early age: growth, gas exchange and water relations. *New Forests*, *31*, 321-342.

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Graciano, C., Guiamet, J.J., & Goya, J.F. (2005). Impact of nitrogen and phosphorus fertilization on drought responses in *Eucalyptus grandis* seedlings. *Forest Ecology and Management*, *212*, 40-49.

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Guo, S.L., Yan, X.F., Bai, B., & Yu, S. (2005). Effects of nitrogen supply on photosynthesis in larch seedlings (In Chinese). *Acta Ecologica Sinica*, *25(6)*, 1291-1298.

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Jassal, R.S., Black, T.A., Cai, T., Ethier, G., Pepin, S., Brummer, C., Nesic, Z., Spittlehouse, D.L., & Trofymow, J.A. (2010). Impact of nitrogen fertilization on carbon and water balances in a chronosequence of three Douglas-fir stands in the Pacific Northwest. *Agricultural and Forest Meteorology*, *150*, 208-218.

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Li, D.J., Mo, J.M., Fang, Y.T., Cai, X.A., Xue, J.H., & Xu, G.L. (2004). Effects of simulated nitrogen deposition on growth and photosynthesis of *Schima superba*, *Castanopsis chinensis* and *Cryptocarya concinna* seedlings (In Chinese). *Acta Ecologica Sinica*, *24(5)*, 876-882.

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Appendix B

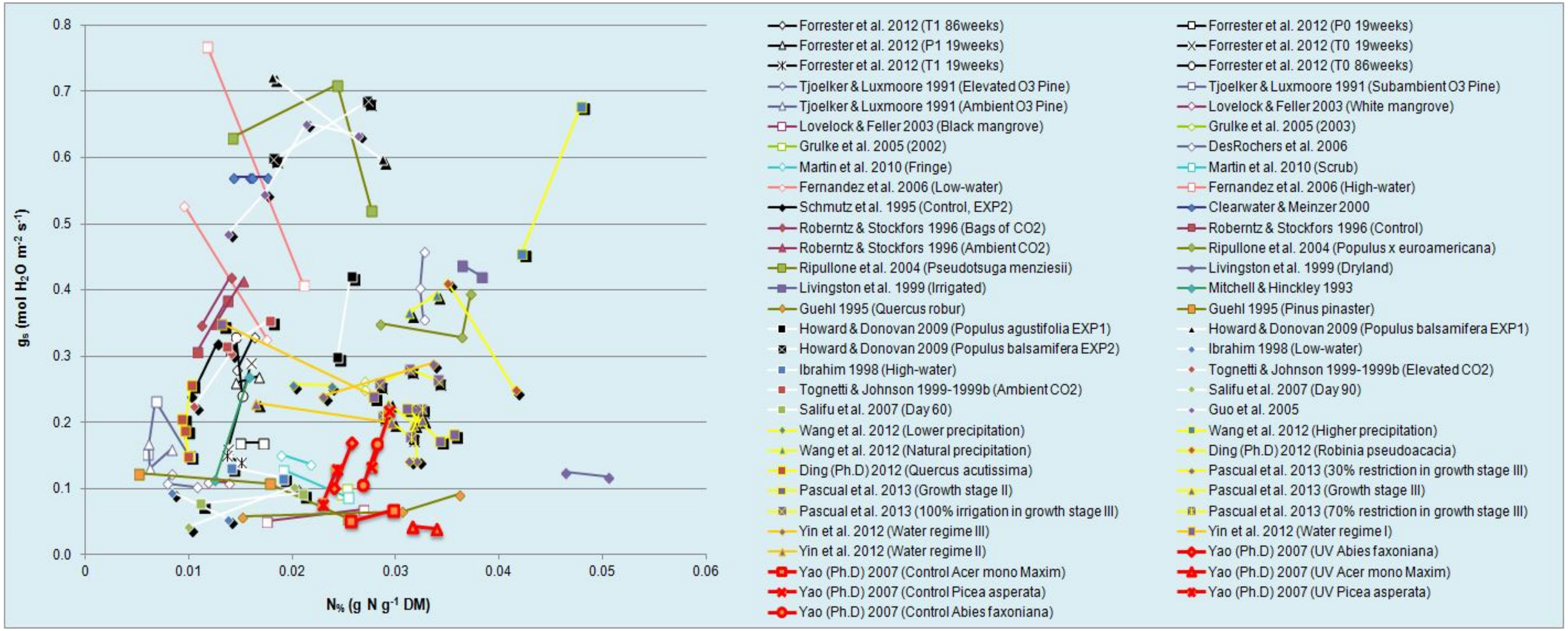


Figure 1 Leaf nitrogen concentration ($N_{\%}$) was calculated from the slope of the linear model ($y = a + b(1/D)$) describing the relationship between stomatal conductance (g_s) in response to different irrigations, tree species, environments, physiological treatments, temporal and spatial scales.

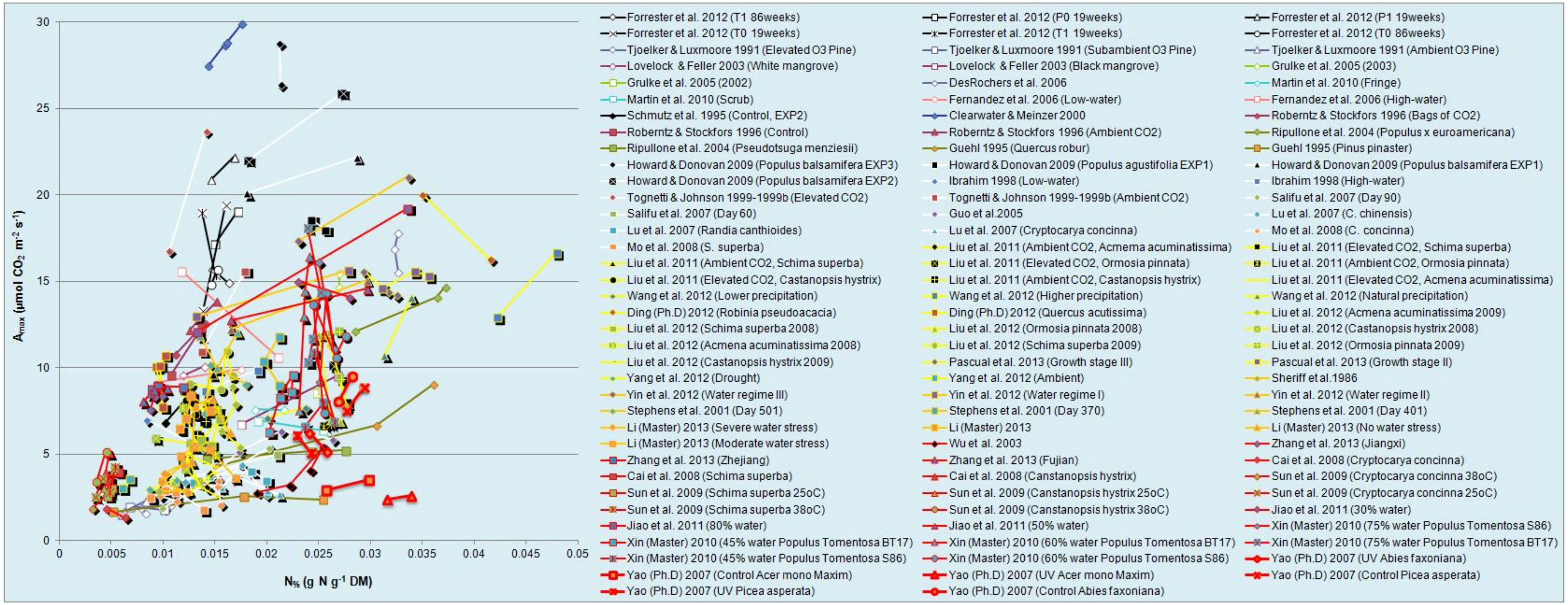


Figure 2 Leaf nitrogen concentration ($N_{\%}$) was calculated from the slope of the linear model (y = a + b(1/D)) describing the relationship between maximum assimilation rate (A_{max}) in response to different irrigations, tree species, environments, physiological treatments, temporal and spatial scales.

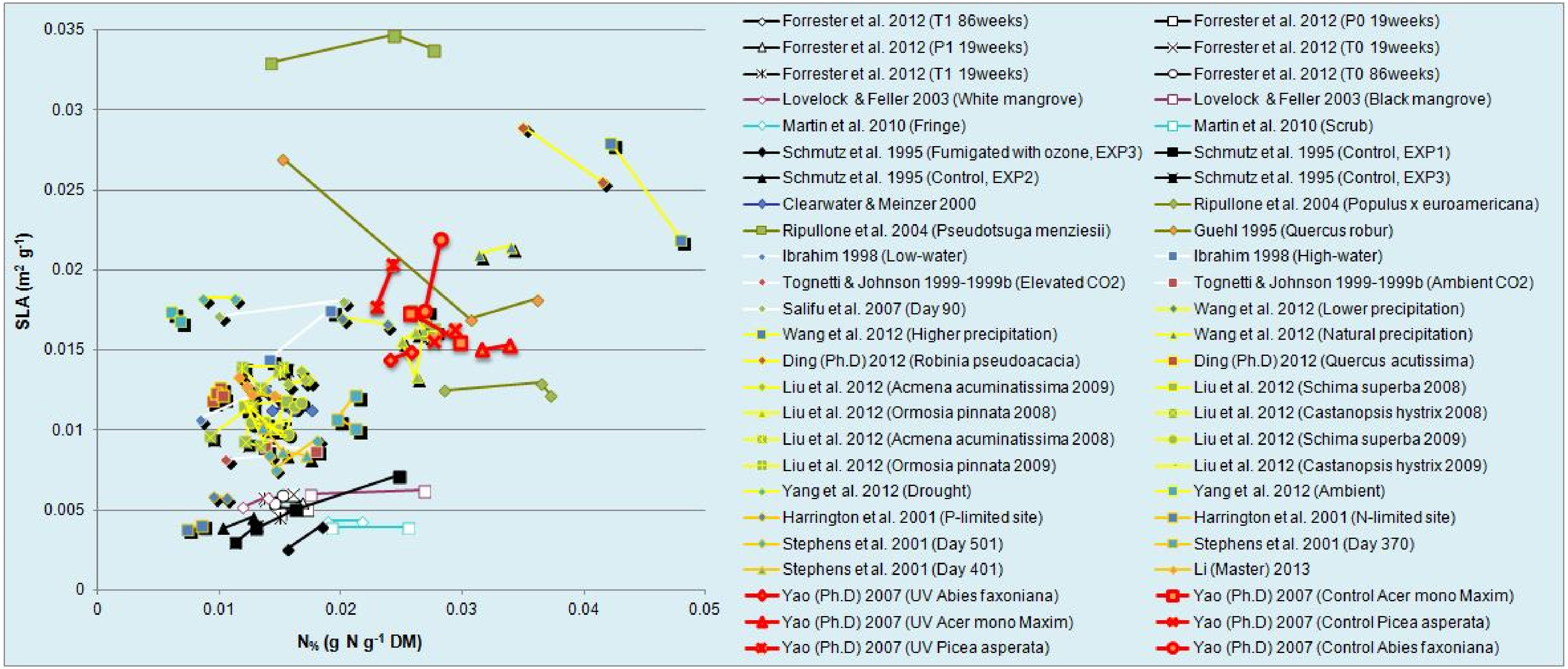


Figure 3 Leaf nitrogen concentration ($N_{\%}$) was calculated from the slope of the linear model ($y = a + b(1/D)$) describing the relationship between specific leaf area (SLA) in response to different irrigations, tree species, environments, physiological treatments, temporal and spatial scales.

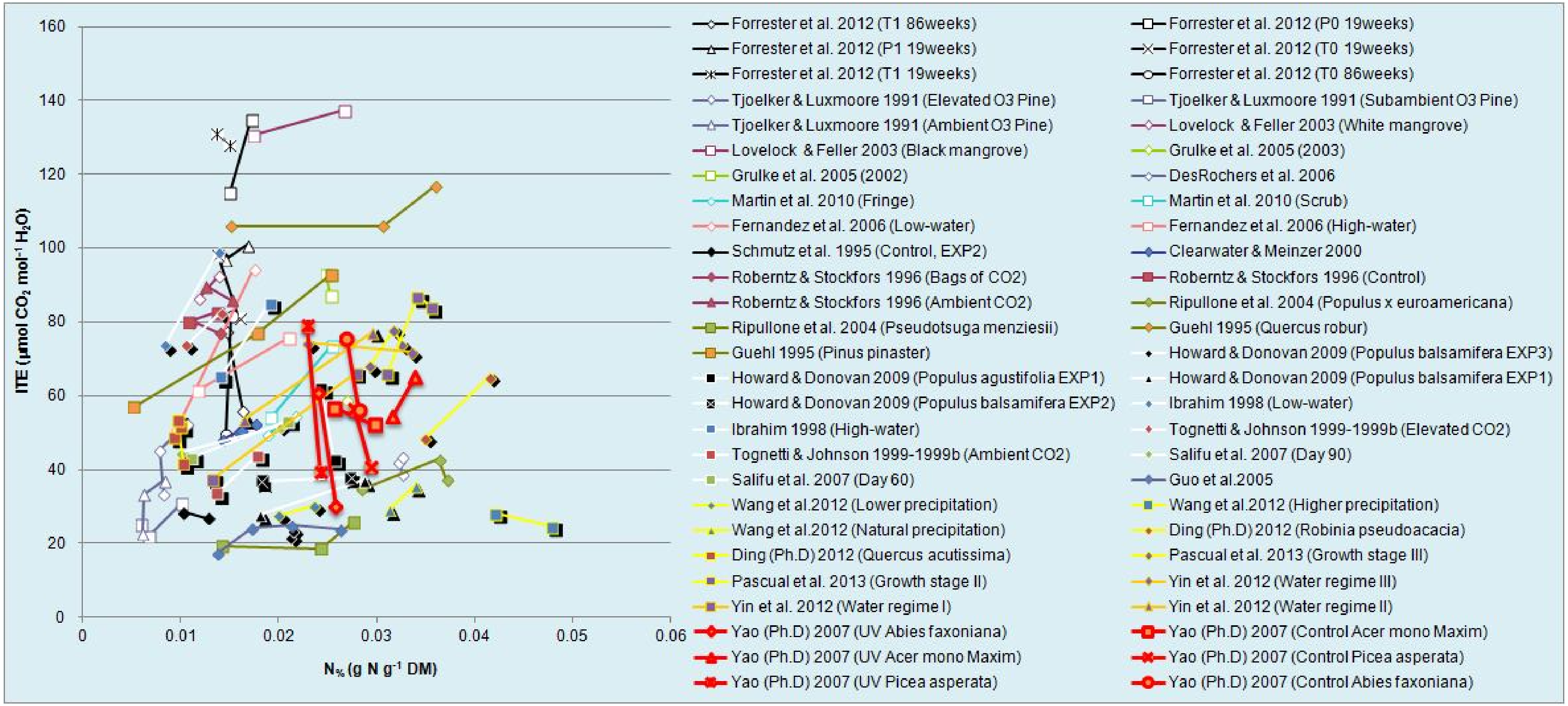


Figure 4 Leaf nitrogen concentration ($N_{\%}$) was calculated from the slope of the linear model ($y = a + b(1/D)$) describing the relationship between intrinsic water-use efficiency (ITE) in response to different irrigations, tree species, environments, physiological treatments, temporal and spatial scales.

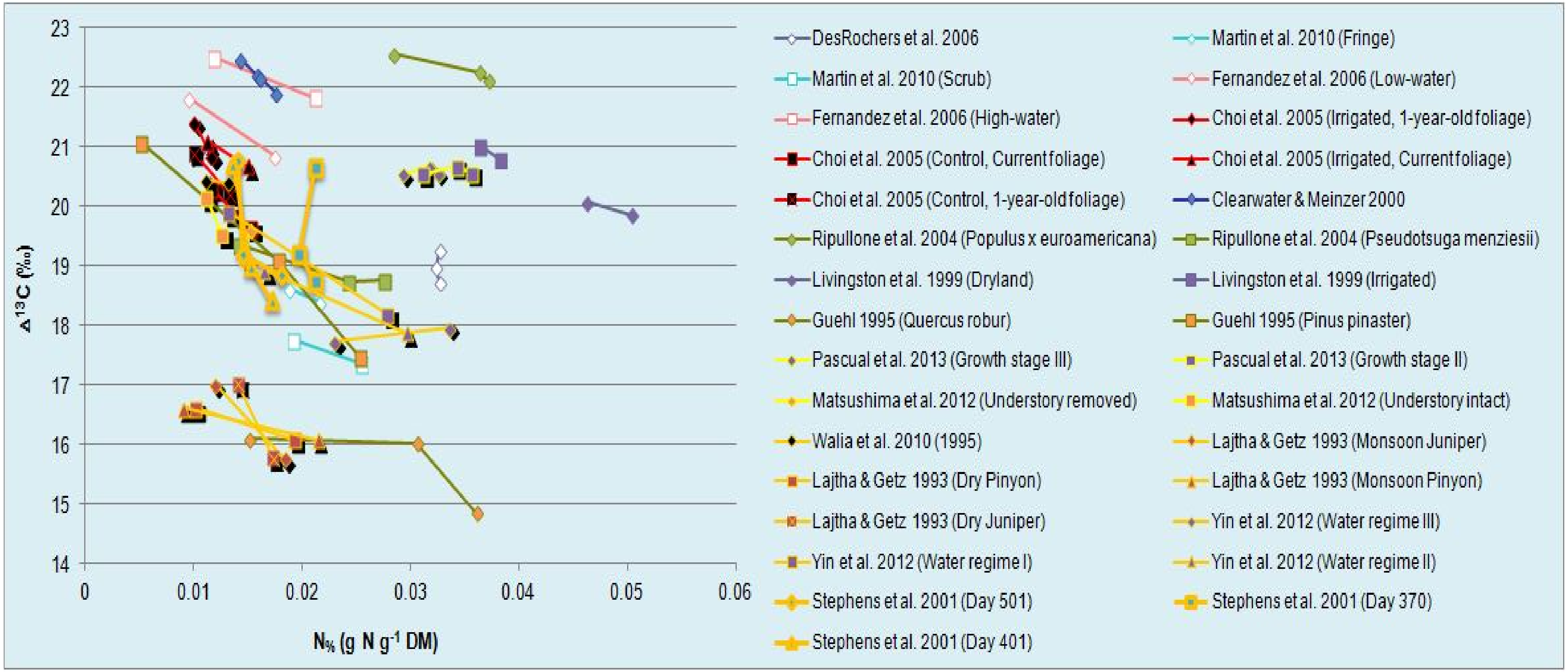


Figure 5 Leaf nitrogen concentration ($N_{\%}$) was calculated from the slope of the linear model (y = a + b(1/D)) describing the relationship between carbon-isotope discrimination ($\Delta^{13}C$) in response to different irrigations, tree species, environments, physiological treatments, temporal and spatial scales.

Figure 6 Stomatal conductance (g_s) was calculated from the slope of the linear model (y = a + b(1/D)) describing the relationship between intrinsic water-use efficiency (ITE) in response to different irrigations, tree species, environments, physiological treatments, temporal and spatial scales.

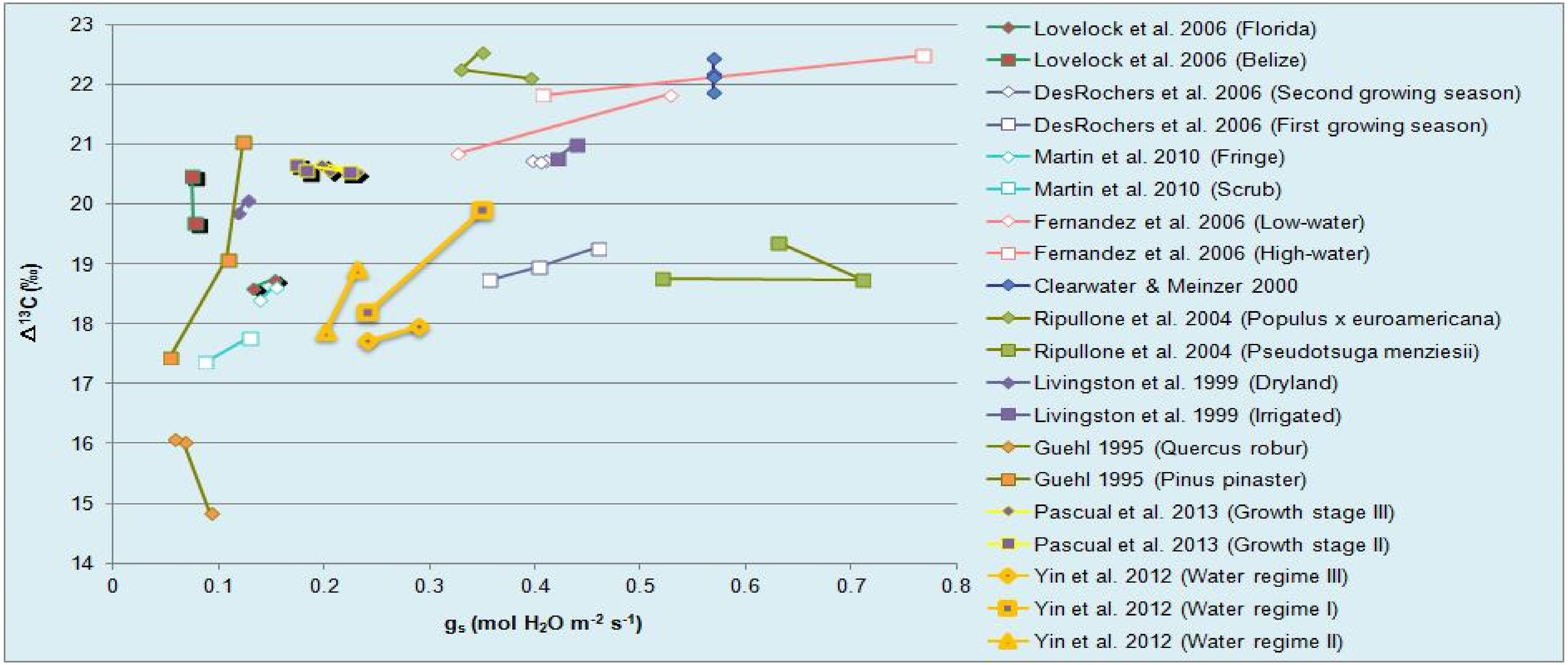


Figure 7 Stomatal conductance (g_s) was calculated from the slope of the linear model (y = a + b(1/D)) describing the relationship between carbon-isotope discrimination ($\Delta^{13}C$) in response to different irrigations, tree species, environments, physiological treatments, temporal and spatial scales.

Chapter 4

Comparison of effects of N fertilization on water use, WUE and growth between poplar experiment and meta-analysis

4.1. Introduction

Whilst the volumes of data generated by scientific instruments such as the previous experiment as already discussed in Chapter 2 for the effects of N fertilization on growth and water-use efficiency in hybrid poplar, a comparable challenge arises with any effort to review and synthesize the primary literature such as the previous meta-analysis as already discussed in Chapter 3 for the effects of N fertilization on intrinsic water-use efficiency. Certainly, the sheer number of published scientific journal articles is astounding. A total of 73 papers and theses were selected from 3975 peer-reviewed publications that reported water-use efficiency (WUE) at the leaf level in response to N fertilization experiments over the period 1986-2013 (Supporting Information Appendix A in Chapter 3). Long gone are the days when tracking a handful of journals would suffice to keep one abreast of important research developments (Curtis & Qeenborough 2012). As such, our meta-analysis as a data-driven approach, taking the results from primary research articles and quantitatively analyzing and synthesizing these data in an attempt to arrive at more robust conclusions.

Meta-analysis approach has become the methodological platform of choice in many areas of empirical science, including ecology. As background, Lajeunesse (2010) put forward general recommendations for a high-quality ecological meta-analysis. Of central importance is transparency in the criteria used to select studies for inclusion in the meta-analysis, with a premium on broad inclusivity. Subsequently, two recent papers from an international group of peatland ecologists (Limpens et al. 2011, Limpens et al. 2012) stood out and performed a meta-analysis over 115 glasshouse experiments and 107 field experiments to investigat whether glasshouse experiments are reliable proxies for field experiments for qualitative and quantitative assessing interactions between N deposition and environment as controls on *Sphagnum* N concentration and production, and also made clear in both their meta-analyses of experimental nitrogen (N) additions to peatlands how the literature was searched and which response variables and covariates were extracted from each study. The same as we made the splited analysis in our meta-analysis as already discussed in Chapter 3. In covariates, study types could be compared when it was splited into two separate studies (Lab and

Field), also plant functional types (PFTs) could be compared when it was splited into two separate functional groups (Conifers and Broadleaves). However, an explicit aim of meta-analysis is not only to provide an overall summary of a wide range of experiments, but also to make the approach quantitative. This may serve as a benchmark for comparison with the evidence of experiments as it can be analysed whether a given species responds more or less strongly compared with the 'average' species. Hence, it should not be neglected, which raised the question: How does the potential N fertilization affect carbon gain in relation to water use, water-use efficiency and growth from the results that have been found between our poplar experiment and meta-analysis? Furthermore, whether the variation of our experiment in concordance with the overall development trend of our meta-analysis for the lab studies and also for the broadleaves trees species? More specifically, how gas exchange characteristics and leaf N concentration varied intrinsic water-use efficiency (ITE) as compared with estimated intrinsic water-use efficiency (Estimated ITE) by carbon isotope discrimination ($\Delta^{13}C$).

In the present study, we conducted a comparison of effects of N fertilization on water use, WUE and growth between poplar experiment and meta-analysis to identify the complex interactions among resources as determinants of the structure and function of woody plants through the central tendency of meta-analysis and the results of individual experiment.

4.2. Material and methods

The comparison included the responses to N fertilization of three independent variables and five dependent variables related to water-use efficiency (WUE) parameters (Tab. 1) which data from the poplar experiment (Chapter 2) and meta-analysis (Chapter 3) as already discussed in our previous work. Owing to the experiment was conducted on the hybrid poplar (*Populus × euroamericana*) in the greenhouse, so for covariates, the results from lab in study types and broadleaves in plant functional types (PFTs) of the split meta-analysis could be selected for comparing with the results from poplar experiment. The results and statistical analysis for the effects of N fertilization on growth and water-use efficiency in poplar experiment please refer to Tab. 3 & 4 & 6 in Chaper 2. Likewise, the results and statistical analysis for the effects of N fertilization on intrinsic water-use efficiency in meta-analysis please refer to Tab. 2 & 3 and Fig. 1-6 in Chapter 3. On the basis of the poplar experiment, the response ratio (lnRR) and the slope of correlation with leaf N concentration ($N_{\%}$) were computed to reflect the effects of N fertilization on leaf gas exchange parameters, the specific data and methods for implementation please refer to the ***Material and methods*** and ***Results and discussion*** sections in Chapter 2 and 3.

Table 1 Description and interrelation of independent and dependent variables and covariates used in this comparison between poplar experiment and meta-analysis.

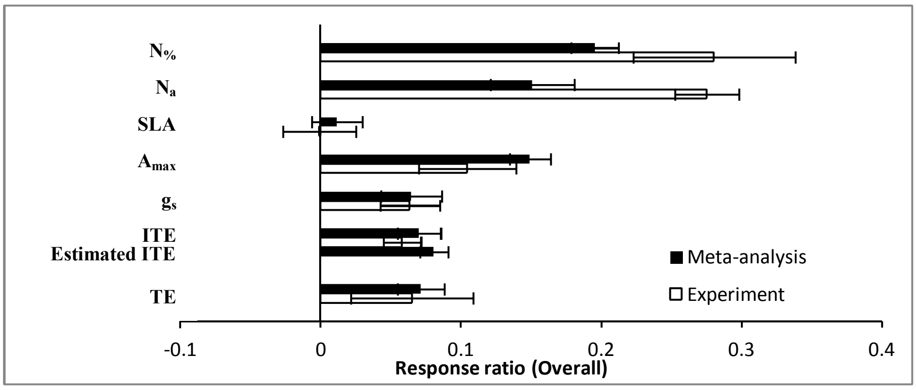
Abbreviations	Variables	Units	Notes
Independent variables			
N_a	Leaf N content	$g\ N\ m^{-2}$	Computed as $N_{\%}/SLA$
$N_{\%}$	Leaf N concentration	$g\ N\ g^{-1}\ DM$	Computed as $N_a \cdot SLA$
SLA	Specific leaf area	$m^2\ g^{-1}\ DM$	Computed as 1/LMA (LMA, leaf mass per area, $g\ m^{-2}$)
Dependent variables			
g_s	Stomatal conductance	$mol\ H_2O\ m^{-2}\ s^{-1}$	Computed as A_{max}/ITE
A_{max}	Maximum assimilation rate	$\mu mol\ CO_2\ m^{-2}\ s^{-1}$	Computed as $g_s \cdot ITE$
ITE	Intrinsic water-use efficiency	$\mu mol\ CO_2\ mol^{-1}\ H_2O$	Computed as A_{max}/g_s
Estimated ITE	Estimated intrinsic water-use efficiency	$\mu mol\ CO_2\ mol^{-1}\ H_2O$	Computed as $\triangle^{13}C$ ($\triangle^{13}C$, carbon-isotope discrimination)
TE	Instantaneous water-use efficiency	$\mu mol\ CO_2\ mmol^{-1}\ H_2O$	Computed as A_{max}/E (E, leaf evapotranspiration rate, $mmol\ H_2O\ m^{-2}\ s^{-1}$)
Covariates			
—	Meta-analysis	—	Lab / Broadleaves
—	Poplar experiment	—	Lab / Broadleaves

Variables for the poplar experiment please refer to Tab. 1 in Chaper 2. Likewise, variables for the meta-analysis please refer to Tab.1 in Chapter 3.

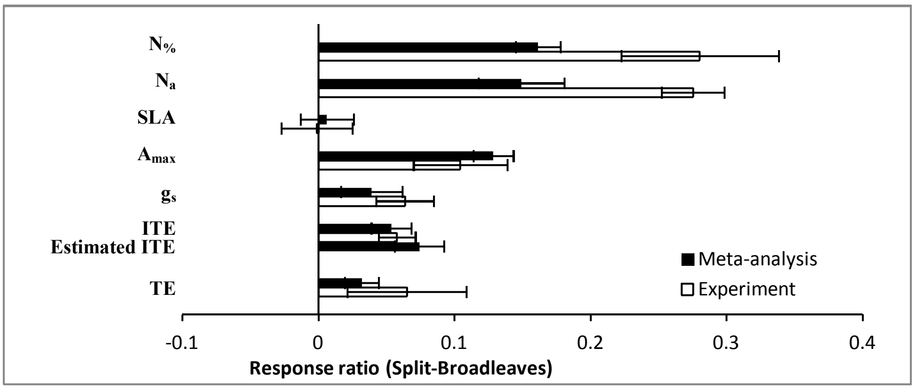
4.3. Results and discussion

N-induced changes in independent and dependent variables exhibited great variability between meta-analysis and poplar experiment (Fig. 1). For independent variables, the significant increments of leaf N concentration ($N_{\%}$; $P<0.001$) and leaf N content (N_a; $P<0.001$) in experiment were larger than those in the overall response ratio ($P<0.001$; Fig. 1a), likewise in the split-broadleaves ($P<0.001$; Fig. 1b) and split-lab ($P<0.05$; Fig. 1c) response ratio. However, N supply increased specific leaf area (SLA) in the overall response ratio in meta-analysis ($P>0.05$; Fig. 1a) and decreased SLA ($P>0.05$; Fig. 1a) in experiment, the changes in SLA did not reveal significant differences in both meta-analysis and experiment ($P>0.05$; Tab. 2), also same as compared with split-broadleaves ($P>0.05$; Fig. 1b) and split-lab ($P>0.05$; Fig. 1c) response ratio. For dependent variables, maximum assimilation rate (A_{max}; $P<0.001$), stomatal conductance (g_s; $P<0.05$) and instantaneous water-use efficiency (TE; $P<0.001$) significantly increased with the cumulative amount of N in the overall ($P<0.01$; Fig. 1a) and split-lab ($P<0.05$; Fig. 1c) of response ratio in meta-analysis were greater than those in experiment ($P<0.001$; Fig. 1a & c). For the split-broadleaves response ratio, A_{max} ($P<0.001$; Fig. 1b) significantly increased under N enrichment in meta-analysis was still higher than that in experiment, whereas g_s ($P>0.05$; Fig. 1b) and TE ($P<0.05$; Fig. 1b) enhanced with N supply in meta-analysis were lower than those in experiment ($P<0.001$; Fig. 1b), and the increases in g_s showed no significant differences in the split-broadleaves response ratio of meta-analysis ($P>0.05$; Tab. 2). Further, the markedly increments of intrinsic water-use efficiency (ITE) in experiment ($P<0.001$; Fig. 1a & c) were smaller than that in the overall ($P<0.001$; Fig. 1a) and split-lab ($P<0.001$; Fig. 1c) of response ratio in meta-analysis, except that in the split-broadleaves response ratio ($P<0.001$; Fig. 1b), while the significantly increments of estimated intrinsic water-use efficiency (Estimated ITE) in meta-analysis ($P<0.001$; Fig. 1a & b & c) always greater than ITE ($P<0.001$; Fig. 1a & b & c) in both meta-analysis and experiment (Tab. 2).

(a)



(b)



(c)

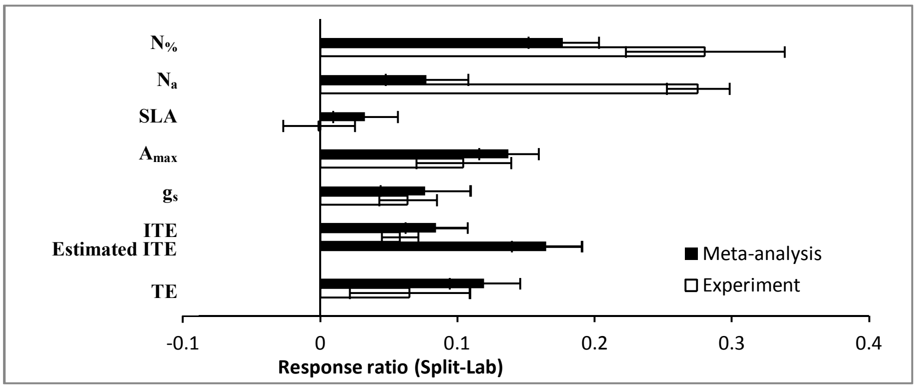


Figure 1 The effects of N fertilization on leaf N concentration ($N_{\%}$), leaf N content (N_a), specific leaf area (SLA), maximum assimilation rate (A_{max}), stomatal conductance (g_s), intrinsic water-use efficiency (ITE), estimated intrinsic water-use efficiency (Estimated ITE) and instantaneous water-use efficiency (TE) for (a) the overall response ratio (lnRR), (b) the split-broadleaves response ratio (lnRR), and (c) the split-lab response ratio (lnRR) between poplar experiment and meta-analysis.

Table 2 Results from statistical analysis for the response ratio (lnRR) of the variables response to N fertilization between poplar experiment and the overall, split-broadleaves and split-lab of meta-analysis.

Abbreviations	Meta-analysis RR-(Overall)	Poplar experiment	Meta-analysis RR-(Split-Broadleaves)	Meta-analysis RR-(Split-Lab)
Independent variables				
N%	***	***	***	***
Na	***	***	***	*
SLA	NS	NS	NS	NS
Dependent variables				
Amax	***	***	***	***
gs	**	***	NS	*
ITE	***	***	***	***
Estimated ITE	***	—	***	***
TE	***	***	*	***

An asterisk (*) denotes variables differing significantly using a paired t-test: ***indicates values statistically different at $P < 0.001$, **indicates those different at $P < 0.01$, *indicates those different at $P < 0.05$ and NS indicates the values do not differ significantly according to the least significant difference test ($P > 0.05$). $N_{\%}$: leaf N concentration; N_a: leaf N content; SLA: specific leaf area; A_{max}: maximum assimilation rate; g_s: stomatal conductance; ITE: intrinsic water-use efficiency; Estimate ITE: estimated intrinsic water-use efficiency; TE: instantaneous water-use efficiency. Statistical analysis for the poplar experiment please refer to Tab. 3 & 4 & 6 in Chaper 2. Likewise, the results and statistical analysis for the meta-analysis please refer to Tab. 2 & 3 and Fig. 1-6 in Chapter 3.

Although N-induced changes in the slope of correlation with leaf N concentration ($N_{\%}$) for specific leaf area (SLA), stomatal conductance (g_s) and intrinsic water-use efficiency (ITE) exhibited great variability among the overall (P>0.05; Fig. 2a), split-broadleaves (P>0.05; Fig. 2b), split-lab (P>0.05; Fig. 2c) response ratio in meta-analysis and poplar experiment (P>0.05; Fig. 2a & b & c), the changes in SLA, g_s and ITE did not reveal significant differences in both meta-analysis and experiment (P>0.05; Tab. 3) under N enrichment. There was no significant correlation in response to N supply between maximum assimilation rate (A_{max}) and $N_{\%}$ in split-lab response ratio of meta-analysis (P>0.05; Fig. 2c) and experiment (P>0.05; Fig. 2a & b & c), however, the effects of N fertilization on the slope with an significant increase in A_{max} vs. $N_{\%}$ in both the overall (P<0.001; Fig. 2a) and split-broadleaves (P<0.001; Fig. 2b) response ratio of meta-analysis (Tab. 3). In addition, Estimated ITE (P<0.05; Fig. 2a & b & c) was always positively correlated to $N_{\%}$ in meta-analysis (Tab. 3).

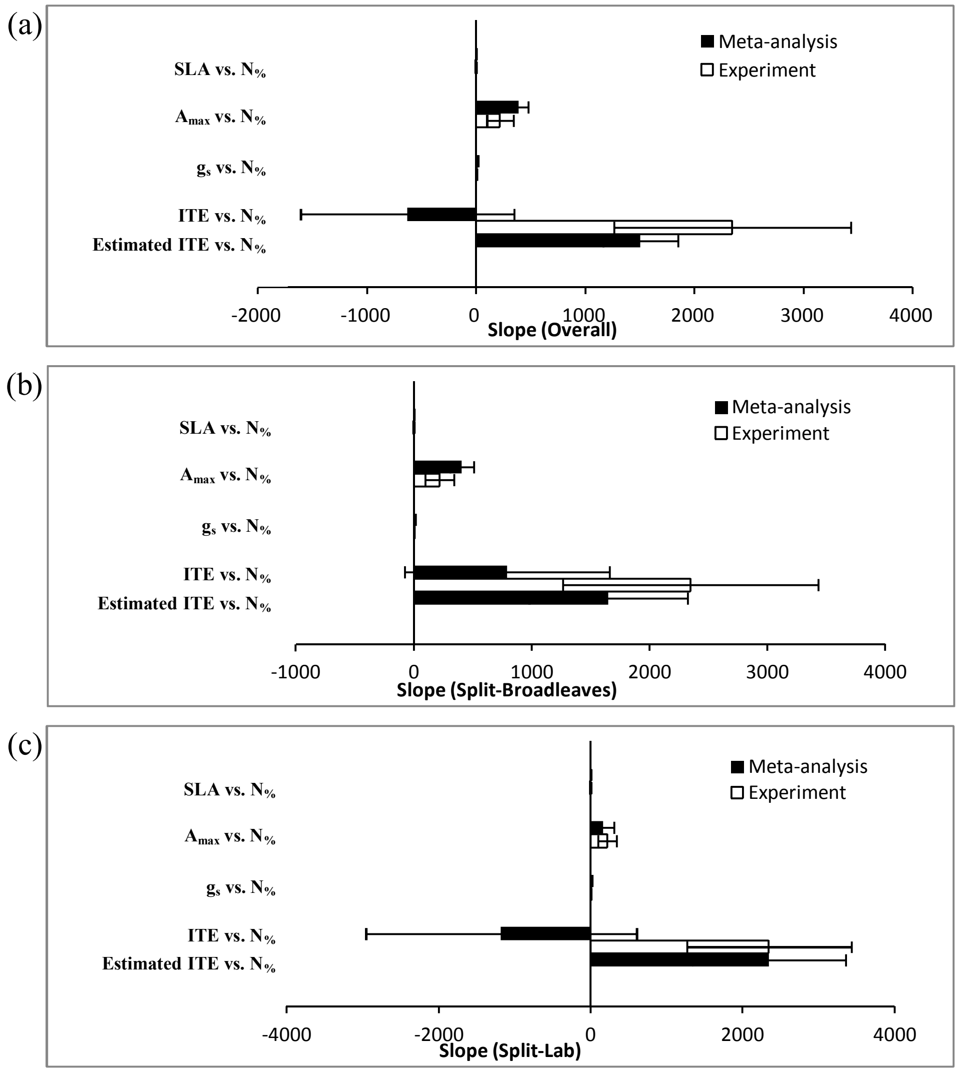


Figure 2 The frequency distributions on specific leaf area (SLA), maximum assimilation rate (A_{max}), stomatal conductance (g_s), intrinsic water-use efficiency (ITE) and estimated intrinsic water-use efficiency (Estimated ITE) (a) the overall slope, (b) the split-broadleaves slope, and (c) the split-lab slope of correlation with leaf N concentration ($N_{\%}$) response to N fertilization between poplar experiment and meta-analysis.

Table 3 Results from statistical analysis for the slope of correlation between leaf N concentration ($N_{\%}$) and the variables respectively response to N fertilization between poplar experiment and the overall, split-broadleaves and split-lab of meta-analysis.

Abbreviations	Meta-analysis Slope-(Overall)	Poplar experiment	Meta-analysis Slope-(Split-Broadleaves)	Meta-analysis Slope-(Split-Lab)
SLA vs. $N_{\%}$	NS	NS	NS	NS
A_{max} vs. $N_{\%}$	***	NS	***	NS
g_s vs. $N_{\%}$	NS	NS	NS	NS
ITE vs. $N_{\%}$	NS	NS	NS	NS
Estimated ITE vs. $N_{\%}$	***	—	*	*

An asterisk (*) denotes variables differing significantly using a paired t-test: ***indicates values statistically different at $P < 0.001$, **indicates those different at $P < 0.01$, *indicates those different at $P < 0.05$ and NS indicates the values do not differ significantly according to the least significant difference test ($P > 0.05$). SLA: specific leaf area; A_{max}: maximum assimilation rate; g_s: stomatal conductance; ITE: intrinsic water-use efficiency; Estimate ITE: estimated intrinsic water-use efficiency; $N_{\%}$: leaf N concentration. Statistical analysis for the poplar experiment please refer to Tab. 3 & 4 & 6 in Chaper 2. Likewise, the results and statistical analysis for the meta-analysis please refer to Tab. 2 & 3 and Fig. 1-6 in Chapter 3.

On the whole, the absence of increasing stomatal conductance and transpiration in response to N fertilization as compared with the sharply rise in photosynthetic rates led to ITE and TE obtained by gas exchanges measurement have proved completely in both meta-analysis and poplar experiment, and also the results in consistent with ITE was estimated by $\Delta^{13}C$ (Fig. 1). The photosynthetic capacity responses to N availability have been well documented in hardwood tree species (Wendler & Millard 1996, Wang & Leuning 1998, Tyree et al. 2009), which indicated that the photosynthetic rate of plants might be dependent on soil N availability. Meanwhile, $\Delta^{13}C$ as a robust estimation of predictor factor indicated a significant and positive association of N fertilization and ITE. This result supported the hypothesis that long-term soil N accumulation might lead to enhanced plant nutritional status (Aber et al. 2003), which can stimulated photosynthesis and increase ITE (Leonardi et al. 2012). However, knowledge is still insufficient since the response is highly dependent on the species (Guehl et al. 1995).

As mentioned above, we observed the response of leaf N concentration ($N_{\%}$) to N application was completely significant in both meta-analysis and poplar experiment (Tab. 2), suggesting that lab studies are suitable for detailed physiological investigations on leaf N uptake and assimilation (Tab. 3), even for indirectly evaluation the effect of adding N on estimated intrinsic water-use efficiency (Estimated ITE; Fig. 2, Tab. 3). However, other variables in response of $N_{\%}$ under N enrichment, such as g_s and ITE were of no significance in meta-analysis and poplar experiment (Tab. 3), even in overall and split-lab of meta-analysis, ITE was negatively correlated with $N_{\%}$ (Fig. 2a & c). Although the relationship between $N_{\%}$ and ITE is not straightforward and also the mechanisms underlying the response of g_s to altered leaf N status remain largely unknown, in a recent meta-analysis on the effects of water and light on plant performance, Holmgren et al. (2011) showed that physiological (direct) effects were very similar between lab and field experiments, whereas seedling survival (indirect effect) was not. Nevertheless, a unique relationship between water-use efficiency and leaf nutrient content cannot always be found (Mitchell & Hinckley 1993, Donovan & Ehleringer 1994); and since it depends on the species (Guehl et al. 1995, Zhang & Cregg 1996), it should be interpreted with some caution. The negative relationships between E or g_s and leaf N concentration ($N_{\%}$) suggests that ITE (A/g_s) increases as nutrient availability increases. Therefore, a further factor to consider at the leaf level, WUE is the ratio of the rates of photosynthetic carbon gain and water loss, with the latter determined by stomatal conductance and the vapour pressure difference (VPD) between leaf and air. For a given photosynthetic capacity, low stomatal conductance reduces water loss and thus increases WUE as we observed; however, low stomatal conductance also constrains carbon gain. Conversely, for a given stomatal conductance, increasing photosynthetic capacity increases the assimilation rate and WUE. Although increasing photosynthetic capacity does not compromise carbon gain, high WUE is achieved at the expense of nitrogen-use efficiency (NUE), as more nitrogen (N) is required for photosynthesis. Hence, the general enhancement of WUE in response to increasing N supply either compromises carbon gain or increases the N cost of carbon gain (Field et al. 1983).

Particularly, no clear effect of N fertilization on specific leaf area (SLA) no matter what the results between meta-analysis and poplar experiment (Tab. 2), even in the slope analysis (Tab. 3), there was no significant correlation between SLA and leaf N concentration ($N_{\%}$). The above implies that an important component of the N-effect mediated through specific leaf area (SLA) cannot be studied until we understand the mechanisms underlying the interaction with SLA better. Owing to plasticity for SLA varied widely, being highest for light, submergence and temperature, and only modest for CO_2, nutrients, drought and salinity (Poorter et al. 2009, 2010). Meanwhile, the changes in SLA can occur through changes in leaf chemistry, changes in concentrations of carbon storage compounds (Waring et al. 1985) or changes in leaf thickness (Konings 1989), Ibrahim (1998) found little evidence of changes in leaf carbon chemistry (unpublished data) and conclude that the changes in SLA were probably associated with the production of thicker leaves.

Moreover, it is worth noting that the reasons behind the lack of sensitivity in the lab remain speculative. Either these variables are mainly controlled for in lab experiments (background N), take effect at time scales beyond the duration of most lab experiments, are mediated through other factors controlled for in lab experiments (temperature, microhabitat), are not fully comparable between the experiment types (temperature, microhabitat), or results reflect a limited amount of data (Limpens et al. 2012). Sensitivity analyses suggested that the absence of a P effect in the lab is driven by a few influential experiments only. The other effects were fairly robust, however, suggesting alternative explanations (Limpens et al. 2012). For instance, the instantaneous character of gas exchange measurements makes the parameters highly influenced by temperature and air water pressure deficit (Teskey et al. 1994, Zhang & Marshall 1994), in spite of the adjustment of stomatal conductance to the gas exchange parameters. It is likely an indirect effect mediated through other (environmental) factors and not a direct effect of temperature-induced changes in respiration or photosynthetic activity (Limpens et al. 2011). The above clearly highlights the current lack of experiments targeted at elucidating interactions between N application and other environmental variables (Limpens et al. 2011, 2012) may be one of the prime reasons led to lab experiments overestimate the effect of adding N compared to field experiments.

4.4. Conclusions

The interesting result has been demonstrated that re-analysis with the literature data on the effects of N fertilization on the ITE at leaf level, which has been shown to respond strongly to the addition of N due to the absence of increasing stomatal conductance and transpiration as compared with the sharply rise in photosynthetic rates. ITE obtained by gas exchanges measurement have proved completely consistent with what estimated by $\Delta^{13}C$. In the N fertilization experiment, it is worthwhile to note that the effects are overestimated in ambient environment (i.e. lab) as compared with the measures in field from our meta-analysis.

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Chapter 5

Effects of N fertilization on C sequestration in forest ecosystems of China: an overview

5.1. Abstract

With increasing scientific and political interest in regional aspects of the global carbon cycle, there is a strong impetus to better understand the carbon balance of forest ecosystems in China. This is not only because China has become one of the three major focus areas on nitrogen deposition in the world, but also because it has experienced regionally distinct climate, community type, geology, land-use history and its duration, which together control the responses of nitrogen deposition on carbon stocks in forest ecosystems of China. This overview we introduced the related researches worldwide about the effects of N fertilization on C sequestration in forest ecosystems (e.g., the long-term NITREX and EXMAN projects in Europe and the experiments in Harvard experimental forest of USA), combined with the status of nitrogen deposition monitoring in China under emissions and atmospheric concentrations of N_r pollutants background, then to assess the impacts of nitrogen deposition on China's forest ecosystems, we found there have been several simulated nitrogen deposition experiments since the 2000s, and most of them were short-term studies or conducted on tree seedlings. Due to lack of long-term and cross-site experimental records in China, we gave up the idea of a meta-analysis on this subject, through data mining and selection; only 2 published Ph.D. theses were selected from 1257 peer-reviewed publications, we summarized them in this overview. However, the good news showed up NEECF: a project of nutient enrichment experiments in China's forests have been started, and is expected to greatly contribute to the scientific base of C sink management of China's forest ecosystems and regulation policy to control the N pollution in the future.

5.2. Introduction

The global carbon cycle has been impacted by human society through three main ways: first, large-scale deforestation to forest land into agricultural land and agricultural farming methods change; second, large-scale using of fossil fuel by human society; third, through the emission of chemical compounds produced by anthropogenic activites. The first two directly led to the loss of the global carbon pool and its transition to carbon source; the third is usually assumed to mainly refer to acid deposition, including deposition of sulfur and nitrogen deposition. The role of both components of acid deposition at the global carbon cycle are relevant to the geographical and the ecological environment.

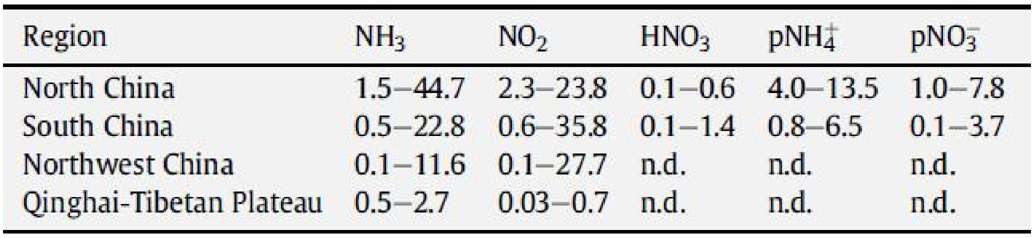
With the development of human society, the negative effects of growing nitrogen deposition on global carbon cycle became more and more serious, so there is a growing research interest in the effects of N deposition on carbon sequestration in forest ecosystems; the large uncertainty on the magnitude of the effects also has aroused an intense debate at global scale. In Europe, Magnani et al. (2007) found that C sequestration was not significantly correlated to temperature and precipitation, and N deposition could be the main driving force on C sequestration with the positive effect value of as high as C:N=200:1 in temperate and boreal forests. Sutton et al. (2008) analyzed the effects of total N deposition on net ecosystem productivity in 22 European forests and found the effect value to be C:N=149:1, but the effect value was reduced to C:N=50~75:1 after excluding the contribution of climatic factors. Hyvönen et al. (2008) analyzed the data from 15 long-term fertilization experiments in northern Europe, found N fertilization for C sequestration in trees were strongly dependent on soil N status, and increased from close to zero at C:N=25 in the humus layer up to 40 kg (C) kg^{-1} (N) at C:N=35 and decreased again to about 20 kg (C) kg^{-1} (N) at C:N=50 when N only was added. Also in the temperate forest ecosystems in north-western and central USA, Thomas et al. (2010) showed that the N deposition with a range of 3 to 11 kg ha^{-1} y^{-1} increased above-ground biomass by 61 kg C per kg N deposited based on forest inventory data. However, other researchers have shown that increased N fertilization through deposition only added marginal or no effects to C sequestration, such as de Vries et al. (2008) found that N deposition had an effect value of C:N=20~40:1 on forest biomass C sequestration in European forests and given an effect value of N deposition on soil C sequestration of C:N=10~30:1, the total effect on whole ecosystem C sequestration would be C:N=30~70:1. In the view of both hemispheres, Ringrose & Neilsen (2005) reported annual applications of N fertilizer, for a period of 13 yr, at 100 kg N ha^{-1} yr^{-1} doubled volume growth of Eucalyptus plantatiuons from 125 to 281 m^3 ha^{-1}, at Age 19 yr in Tasmania (Australia) of the Southern Hemisphere. However, Nadelhoffer et al. (1999) found that soil rather than tree biomass was primary sink of N input that indicated a minor contribution of N deposition to C sequestration in the temperate forests of the Northern Hemisphere. These conflicting results suggest that the effects of N deposition on C sequestration may be regional or site specific.

In China, high nitrogen deposition also occurred in some areas, even some scholars pointed out that China has become one of the world's three major focus areas on nitrogen deposition. In the 1980s, understanding of the environmental effects from N deposition initially arose from the concern of acid deposition (Zhao & Sun 1986). Since the successful controlling of the increase of sulfur deposition from 2000s (Zhao et al. 2009), the growing N deposition due to rapid agriculture and industrial development and urban expansion has aroused great concerns about its ecological impacts on terrestrial and aquatic ecosystems, especially, in eastern and central China (Liu et al. 2011a). During the period 1981-2000, the average C sink capacity of Chinese terrestrial ecosystems was estimated as 0.19-0.26 Pg C year^{-1}, absorbing$\approx$28-37% of C emissions from fossil fuel combustion within the same period (Piao et al. 2009). However, knowledge gaps still exist for the driving strength of N deposition on this C sink. Gradually, some researching projects related to nitrogen deposition have been started, specifically, in connection with the China's forest ecosystems. Some reseachers used the different kinds of ecosystem model to assess the effects of N deposition on C sequestration in Chinese forests, for instance, Wei et al. (2012) assessed by the ecosystem model FORECAST that the contributions of N deposition to C sequestration in all Chinese fir forests in South-East China are 7.4×10^6 Mg C y^{-1} under the current N deposition levels (5 to 10 kg ha^{-1} y^{-1}) and could reach up to 16×10^6 Mg C y^{-1} if N deposition continues increasing and reaches levels of 7.5 to 15 kg N ha^{-1} y^{-1}; Tian et al. (2011) estimated increases in C sequestration under regional variations of N deposition by using the DLEM and TEM models; Zhao et al. (2010) used the GEMS model to estimate increases in C pools along the Yangtze River; Wang et al. (2007) also detected the increase in C sink strength in the latest decades using the InTEC model, an estimation also described by Fang et al. (2001). There also have been several N deposition simulation experiments since the 2000s, however, most of them were short-term studies (Fan et al. 2007a; Mo et al. 2008b; Tu et al. 2011a) or conducted on tree seedlings (Yao et al. 2011; Liu et al. 2012; Ding et al. 2012). So far, we only selected 2 published Ph.D. thesis from 1257 peer-reviewed publications that experimentally analyzed C sequestration in response to N fertilization. Owing to a lack of long-term and cross-site experimental studies, modeling validation and impact assessment of N deposition at a national scale are extremely hindered. It is urgently need to integratively assess the effects of N deposition on ecosystem health and functions in China's forests.

5.3. Emissions and atmospheric concentrations of N_r pollutants in China

Since the early 1980s, NH_3 and NO_x emissions have shown substantial increases. Compared with 1980, NH_3 emissions (13.7 Tg N yr^{-1}) had doubled and NO_x emissions (6.0 Tg N yr^{-1}) had increased by a factor of 4 by the year 2005 (Zhao et al. 2009). The rapid increases in both N_r species emissions are closely related to intensive agricultural and industrial activities. The majority of anthropogenic NH_3 in China is emitted from N fertilizers (e.g., ammonium bicarbonate and urea) and animal/human excreta (Zhao & Wang 1994; Zhang et al. 2010). NO_x emissions are derived mainly from fossil fuel combustion processes including power plants, transportation and industry (Streets & Waldhoff 2000; Streets et al. 2003) with minor contributions from lightning, biomass burning and arable soils (Yan et al. 2003). Concentrations of major gaseous and particulate N pollutants during 1999 and 2009 are summarized in Tab. 1 (Liu et al. 2011a). If this trend continues we can expect the N_r emission induced deposition to make a larger contribution to acid rain than that of sulfur (S) deposition in China in the near future.

Table 1 Reported concentrations of major gaseous and particulate N_r pollutants in China during 1999 and 2009 (μg N m^{-3}).



Region	NH_3	NO_2	HNO_3	pNH_4^+	pNO_3^-
North China	1.5–44.7	2.3–23.8	0.1–0.6	4.0–13.5	1.0–7.8
South China	0.5–22.8	0.6–35.8	0.1–1.4	0.8–6.5	0.1–3.7
Northwest China	0.1–11.6	0.1–27.7	n.d.	n.d.	n.d.
Qinghai-Tibetan Plateau	0.5–2.7	0.03–0.7	n.d.	n.d.	n.d.

Notes: 1) n.d., not determined; 2) $pNH_4^+$and pNO_3^- particulate ammonium and nitrate; 3) Data sources: published journal papers, Ph.D. and M.Sc. thesis and governmental reports.

5.4. *Nitrogen deposition monitoring in China*

Since 2004, China Agricultural University has organized a Nationwide Nitrogen Deposition Monitoring Network (NNDMN) (Liu & Zhang 2009). This network contains about 40 monitoring sites across China, covering cropland, grassland, forest, and urban ecosystems. There are several additional sub-national networks on N deposition monitoring, including the Integrated Monitoring Program on Acidification of Chinese Terrestrial Ecosystems (IMPACT) (Tang et al. 2001) and the World Meteorological Organization Global Atmosphere Watch Precipitation Chemistry Program (WMO/GAW) of China (http://cdc.cma.gov.cn/index.jsp). These networks tend to monitor wet deposition (N input from rainfall and snowfall) but the NNDMN also measures dry deposition of various N_r species (Shen et al. 2009). All these networks have different methodologies and quality control systems. For example, the NNDMN cooperates closely with Centre for Ecology & Hydrology (CEH) Edinburgh, UK and runs the same quality control system as CEH. While the WMO/GAW of China adopts the methods used by WMO protocols. In general, the monitoring networks in China lack long-term continuous data and uniform measuring methods which should be improved in the future.

5.5. *Effects of simulated nitrogen deposition on C sequestration in forest ecosystems of China: two experiments*

China has a large territory with great climatic complexity and spatial variation which sustains a variety of forest ecosystems ranging from boreal forests in the north to tropical rain forests in the south. These forests play an important role in maintaining biodiversity and ecological equilibrium and in providing services for social development. High atmospheric N deposition has been reported in many forest ecosystems (Zhou & Yan 2001; Zhang et al. 2006; Chen & Mulder 2007; Hu et al. 2009, Deng et al. 2009) with N deposition levels commonly exceeding 20 kg N ha^{-1} yr^{-1} in central and east China above which forest health will be seriously threatened (MacDonald et al. 2002; Bobbink et al. 2010). Since 2000s, numerous simulated N deposition experiments have been conducted in different forest types across China (Fig. 1). Most of these studies mainly focus on the effects of elevated N deposition on C, N dynamics in forest soils (Chen et al. 2004; Fang et al. 2006, 2009a,b,c; Fan et al. 2007a,b; Hu et al. 2009; Lu et al. 2009; Ouyang et al. 2008; Xu et al. 2009; Liu et al. 2010; Chen & Duan 2011; Liu et al. 2011b; Chen et al. 2012a,b; Wang et al. 2012), soil acidification (Fan et al. 2007a; Lu et al. 2009; Hu et al. 2010; Jia et al. 2010), plant growth (Li et al. 2005a,b; Lu et al. 2006, 2007; Mo et al. 2008a,b; Duan et al. 2009; Guo et al. 2010; Zhao et al. 2011; Huang et al. 2012a,b), biodiversity (Xu et al. 2005, 2006; Xue et al. 2007; Lu et al. 2008; Lu et al. 2011; Liu et al. 2013), litterfall decomposition (Mo et al. 2006, 2007a, 2008c; Song et al. 2007; Deng et al. 2007; Fang et al. 2007; Fan et al. 2008; Tu et al. 2011b; Zhang & Wang 2012; Guo et al. 2013) and flux of greenhouse gases (Mo et al. 2007b, 2008b; Zhang et al. 2008a,b; Wang 2012).

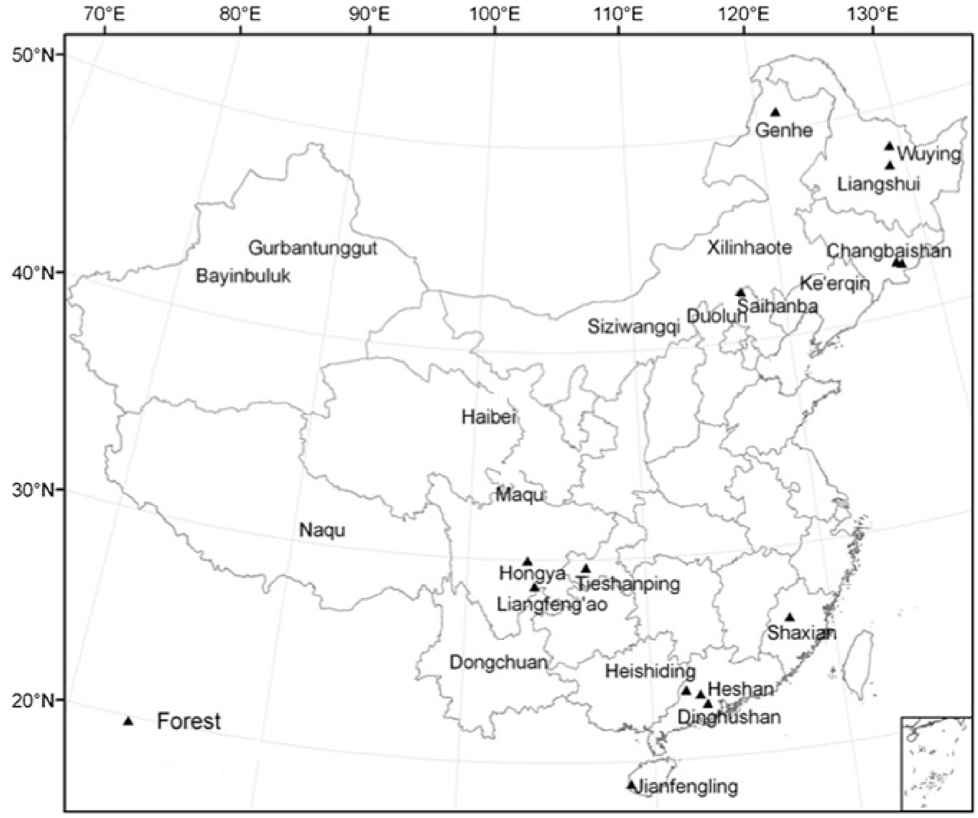


Figure 1 Distribution of N fertilization experiments in forest ecosystems across China based on published journal papers, Ph.D and M.Sc. theses (Mo et al. 2006; Fang et al. 2006; Lin et al. 2007; Fan et al. 2008; Hu et al. 2009; Hu et al. 2010; Song et al. 2009; Xu et al. 2009; Guo et al. 2010; Chen & Duan 2011; Lu et al. 2011; Tu et al. 2011b; Chen et al. 2012; Ding et al. 2012; Huang et al. 2012a,b; Wang et al. 2012; Zhang & Wang 2012; Liu et al. 2013).

However, the long-term response experimental records on C sequestration in Chinese forest ecosystems to N fertilization are far from complete. In this overview, only 2 published Ph.D. theses were selected from 1257 peer-reviewed publications; I summarize them below.

5.5.1. Experiment I

- Ph.D. thesis information

Title: Effeets of simulated nitrogen deposition on carbon cycling processes and characteristics of *Pleioblastus amarus* plantation ecosystem in Rainy Area of West China. (2011c).

Author: Lihua Tu

University: Sichuan Agricultural University

Published paper: Tu, L.H., Hu, T.X., Zhang, J., Li, R.H., Dai, H.Z., Luo, S.H. (2011a). Short-term simulated nitrogen deposition increases carbon sequestration in a *Pleioblastus amarus* plantation. *Plant Soil*, *340*, 383-396.

- Site introduction

The simulated N deposition experiment was conducted in an 8-year old *Pleioblastus amarus* (10 ha) stand in Liujiang, SW, China (29°95′ N, 103°38′ E, altitude 600 m a. s. l.). The region has a mid-subtropical humid mountainous climate (Zhuang & Gao 2002), the annual mean temperature is 14℃-16℃, the annual mean precipitation is 1489.8 mm from 1980 to 2000 and the annual mean relative humidity is 86%. The site was converted from cropland to *P. amarus* plantation since 2000 (National Project of Converting Farmland to Forests). *P. amarus* is one of the bamboo species with the large forestation area in this region. The purple-colored soil is formed from purple sandstone and shale. The aboveground dry biomass was 25.4 kg m^{-2} in November 2007. The surficial organic layer was generally about 1 cm thick before experimental treatments commenced. A survey in the experimental stand was conducted during October 2007 before the N treatments. The forest structure and soil properties were given in Tab. 2. There is very little shrubbery or grass in the understory.

Table 2 Indices of the forest structure and soil properties in a *Pleioblastus amarus* plantation in a rainy region of SW China at the beginning of the study.

Forest structure				Soil properties			
Canopy density	Stem density (1,000 tree ha^{-1})	Height (m)	DHB (cm)	pH	total C (g kg^{-1})	total N (g kg^{-1})	soil depth (cm)
0.9	52.2 (4.5)	5 (0.4)	2.3 (0.2)	4.6 (0.1)	8.9 (0.2)	0.81 (0.01)	~80

Values in the brackets are standard errors.

- Experiment introduction

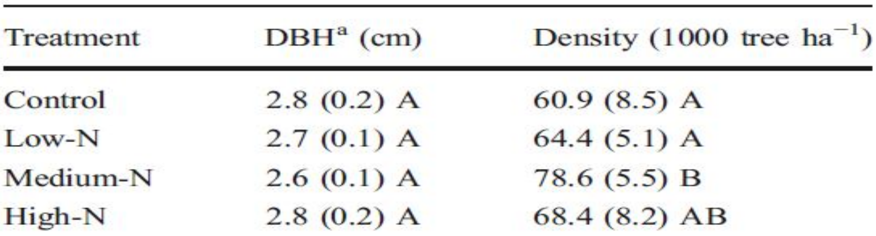
Simulated N deposition experiments were initiated in November 2007. Twelve plots were established and divided into four N addition treatments: control (without N added), low-N (50 kg N ha^{-1} yr^{-1}), medium-N (150 kg N ha^{-1} yr^{-1}), and high-N (300 kg N ha^{-1} yr^{-1}), with three replicates. Each plot measured 3×3 m with >3 m intervals. Plots were randomly selected to receive treatments. Fertilizer additions of NH_4NO_3 occurred monthly in twelve equal applications beginning in November 2007. During each application, the fertilizer was weighed, dissolved in 1 L of water, and applied to each plot using a portable sprayer. The control plot received 1 L water without fertilizer.

Diameters at breast height (DBH, 1.3 m) were recorded on all living stems in each plot in November 2007 and November 2009. The net primary production (NPP, Mg C ha^{-1} yr^{-1}) was calculated as the annual change in living stems plus the amount of litter (including leaf, sheath, and twig) produced that year. Biomass was estimated using a biomass model for the *P. amarus* plantation (Lin et al. 2004). The equation is $M=432.45-479.31\times D+422.83\times D^2$ ($R^2=0.951$, $P<0.01$), where M is the aboveground biomass (g dry weight) and D is DBH (cm). Litterfall was measured outside the treated plots, the results of which only represent the control plots. When the NPP values were calculated, litter production as the same values for all treatments. 50% be used as the C concentration in plant tissue and litter. The annual net ecosystem production (NEP, Mg C ha^{-1} yr^{-1}) as the annual NPP less the annual soil respiration. Since only measured the aboveground biomass turnover, both the NPP and the NEP were underestimated.

- Results

The wet nitrogen deposition in the experimental site was 8.241 g m^{-2}. The mean total litterfall in the *P. amarus* plantation was 691 g m^{-2} yr^{-1} during the experimental period, with the maximum rates from April to June. N treatment greatly increased stand growth, in 2009, the tree densities were significantly higher in the N treated plots than in the control plots (Tab. 3).

Table 3 Influence of N addition on mean (±SE) stand characterisitcs measured in November 2009 in a *Pleioblastus amarus* plantation in a rainy region of SW China.

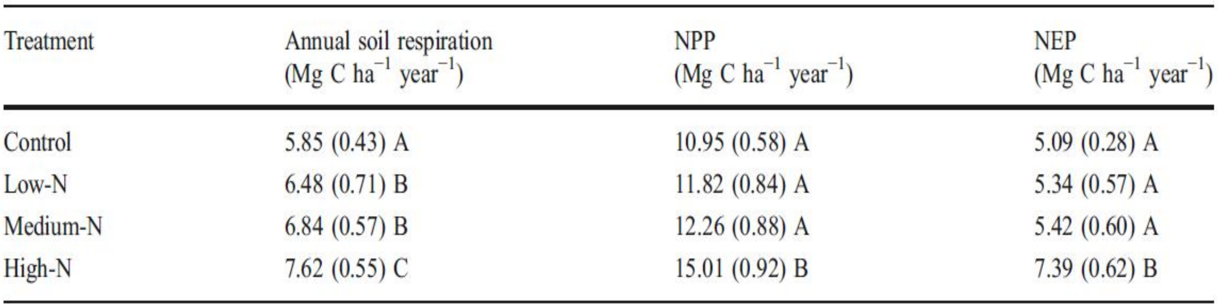


Treatment	DBH^a (cm)	Density (1000 tree ha^{-1})
Control	2.8 (0.2) A	60.9 (8.5) A
Low-N	2.7 (0.1) A	64.4 (5.1) A
Medium-N	2.6 (0.1) A	78.6 (5.5) B
High-N	2.8 (0.2) A	68.4 (8.2) AB

Means of treatments with the same letter are not significantly different (one-Way ANOVA, *N*=3, *P*<0.05). ^{a} DBH diameter at breast height (1.3 m).

The annual cumulative soil respiration was 5.30±0.15 Mg C ha^{-1} in the control plots, exhibited a significant positive linear relationship with NPP. In response to simulated nitrogen deposition, average NPP and NEP increased by 8% to 37% and by 5% to 45%, respectively. The NPP ranged from 10.95 to 15.01 Mg C ha^{-1} yr^{-1} and was higher than the annual soil respiration (5.85 to 7.62 Mg C ha^{-1} yr^{-1}) in all treatments. There was a significant difference between NEP of control and that of high-N, whereas, the difference of NEP among control, low-N and medium-N was not significant (Tab. 4).

Table 4 Mean (±SE) annual soil respiration, net primary production (NPP) and net ecosystem production (NEP) in a *Pleioblastus amarus* plantation in response to N addition in a rainy region of SW China during November 2007 to November 2009.



Treatment	Annual soil respiration (Mg C ha^{-1} year^{-1})	NPP (Mg C ha^{-1} year^{-1})	NEP (Mg C ha^{-1} year^{-1})
Control	5.85 (0.43) A	10.95 (0.58) A	5.09 (0.28) A
Low-N	6.48 (0.71) B	11.82 (0.84) A	5.34 (0.57) A
Medium-N	6.84 (0.57) B	12.26 (0.88) A	5.42 (0.60) A
High-N	7.62 (0.55) C	15.01 (0.92) B	7.39 (0.62) B

Means of treatments with the same letter are not significantly different (one-Way ANOVA, *N*=3, *P*<0.05).

Results suggest that N controlled the primary production in this bamboo plantation ecosystem. Simulated nitrogen deposition increased the C sequestration of the *P. amarus* plantation ecosystem through increasing the plant C pool, though CO_2 emission through soil respiration was also enhance.

5.5.2. Experiment II

- Ph.D. thesis information

Title: Impacts of simulated nitrogen deposition on carbon pool and its chemical mechanism in the Chinese fir plantation. (2009).

Author: Yuzi Huang

University: Fujian Agriculture and Forestry University

- Site introduction

To investigate the response of forest ecosystem to increased nitrogen deposition, the field experiment was conducted in a 12-year-old Chinese fir plantation forest in Sanming City, northwestern Fujian Province, China (26°30'47" N, 117°43'29" E, altitude 200 m a. s. l.). The region has a subtropical monsoon climate, the annual mean temperature is 18.8-19.6℃, the annual mean precipitation is 1606-1650 mm and the soil is the mountain red soil. The site was converted the Chinese fir (*Cunninghamia lanceolata*) plantation since 1992, the total forest area 5.75 ha. The survey in the experimental stand was conducted during December 2003 before the N treatments. There is mainly *Miscanthus floridulus* or *Dicranopteris olichotoma* in the understory.

- Experiment introduction

Simulated N deposition experiments were initiated in January 2004. Fifteen plots were established and divided into four N addition treatments: N0 (without N added), N1 (60 kg N ha^{-1} yr^{-1}), N2 (120 kg N ha^{-1} yr^{-1}), and N3 (240 kg N ha^{-1} yr^{-1}), with three replicates for N1 and N2 treatments and six replicates for N3 treatment (three of the six replicates for the future experiments). Each plot measured 20×20 m, central experiment area measured 15×15 m inside each plot. Plots were randomly selected to receive treatments. Fertilizer additions of urea ($CO(NH_2)_2$) occurred monthly in twelve equal applications beginning in January 2004. During each application, the fertilizer was weighed, dissolved in 20 dm^3 of water, and applied to each plot using a portable sprayer. The control plot received 20 dm^3 water without fertilizer.

- Results

After five years of simulated nitrogen deposition, the stand characteristic value (density, mean DBH, mean height and volume) of Chinese fir plantation in different treatments in Tab. 5 showed no difference after variance analysis, therefore, the following experimental results depended on the different levels of simulated nitrogen deposition could be assumed.

Table 5 Major characteristics of the studied in different treatments.

处理水平 Treatment	林分密度(株数) Density(n・hm^{-2})	平均胸径 D(cm) Mean DBH(cm)	平均树高 H(m) Mean height(m)	蓄积量(m^3・hm^{-2}) Volume(m^3・hm^{-2})
N0	1717	16.1	11.8	525.175
N1	1633	16.0	12.2	510.017
N2	1683	16.3	12.2	545.531
N3	1625	16.0	12.1	503.360

The growth of forest tree layer biomass in plot stands was as follow order: N1> N0> N2> N3. The growth of tree layer biomass were up to 53.690, 60.663, 43.402 and 40.051 t·ha^{-1} respectively in plot stands treated by N0, N1, N2 and N3 (Fig. 2), and compared to the control treatment, respectively, the growth of tree layer biomass increase 12.99%, -19.16% and -23.54% more than that of the plot stands treated by N0. There was significantly different between N1 and the control treatment, and N1 promoted the Chinese fir to increase tree layer biomass. Also N2, N3 and the control treatment reached the level of the difference, but the N2, N3 treatment caused fir tree layer biomass increasing less than that in the control treatment.

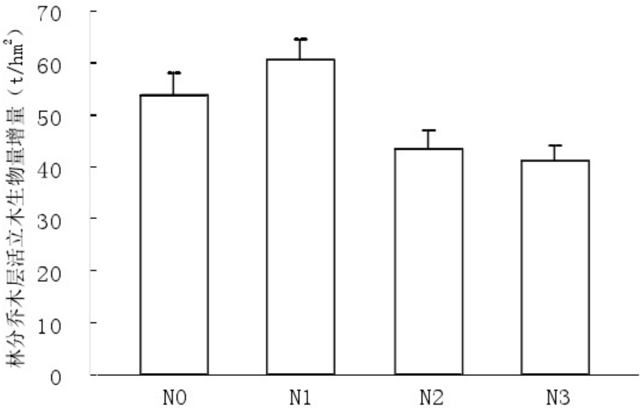


Figure 2 Effects of simulated nitrogen deposition on biomass growth of Chinese fir plantation.

The carbon storage of forest tree layer in treated plot increased in the following order: N1>N0>N2>N3. The carbon storage of tree layer in the plot stands treated with N0, N1, N2 and N3 increased respectively by 24.048, 26.810, 20.215 and 18.746 t·ha^{-1} (Fig. 3), and compared to the control treatment, respectively, the growth of tree layer carbon reserves increase more 11.49%, -15.94% and -22.05% than that of the plot stands treated by N0. There was significant difference between N1, N3 and N0, but no obvious difference between N2 and N3. Compared with the control treatment, N1 promoted the carbon storage growth of tree layer in the plot stands of Chinese fir, and N2，N3 inhibited the carbon storage growth of tree layer in Chinese fir stands.

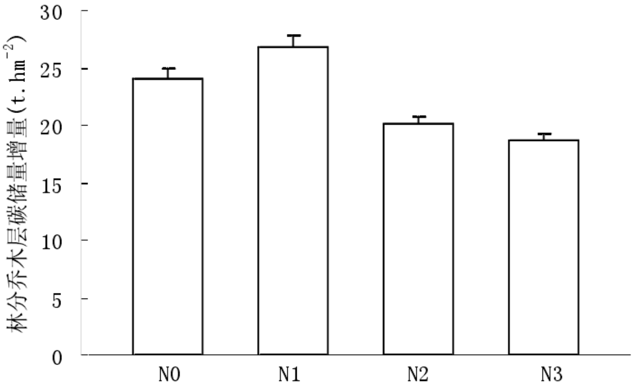


Figure 3 Effects of simulated nitrogen deposition on carbon reserves increment in Chinese fir tree layer.

5.5.3. *Conclusions*

In order to understand the influence of nitrogen (N) deposition on the key processes relevant to the carbon (C) balance, we summarized two simulated nitrogen deposition experiments in this overview involving in C sequestration in forest ecosystems of China. Geographically, these two experiment sites (29°95′N 103°38′E and 26°30'47"N 117°43'29"E) were the same from two of the elevated nitrogen deposition areas in southern China, respectively, according to Fig. 4 (No. 6 and No. 7 areas with black square).

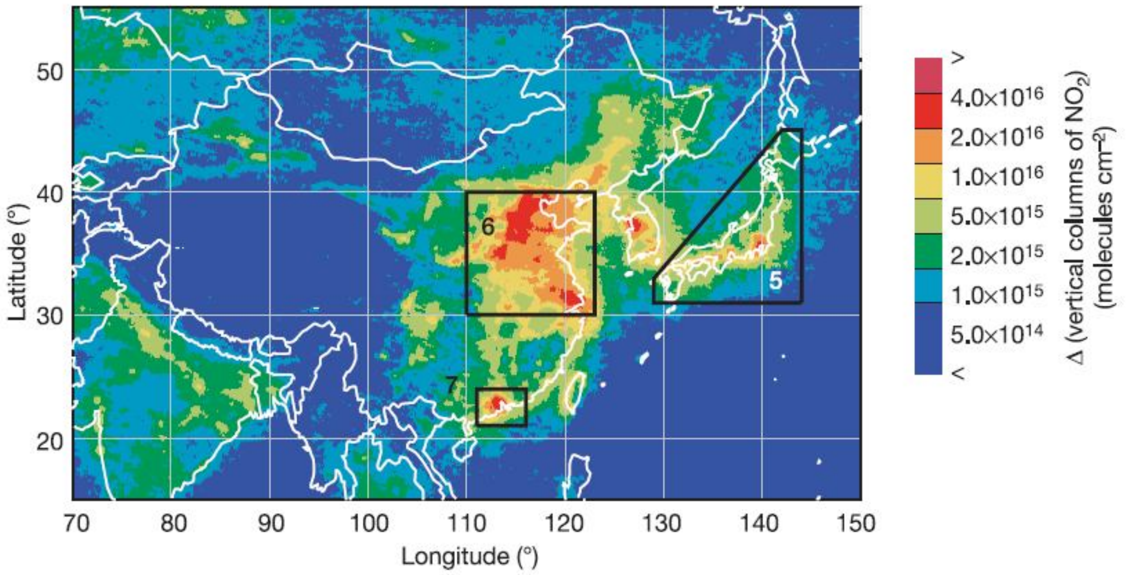


Figure 4 SCIAMACHY tropospheric NO_2 vertical columns averaged between December 2003 and November 2004 for selected industrial regions in China. SCIAMACHY measurements are taken close to 10.00 a.m. **LT**. A nonlinear colour scale has been used because of the large range of NO_2 vertical columns (Richter et al. 2005).

In terms of species, bamboo forest is one of the most important forest types and distributes widely in the world. Currently, China has a bamboo forest area of 5.712×10^6 ha, accounting for 2.94% of the Chinese forest area, which places China first in the world in the area of bamboo forest. In the last eight decades, C stocks in bamboo forests increased, and it contributed about 10% of the C stock in the living biomass of forests in China. Also, Chinese fir, which natural distribution and artificial cultivation is very vast, is one of important timber species in southern China. Chinese Fir grows fast, and its absorption of carbon is also fast, so that it has an obvious effect on reducing atmospheric CO_2 concentration. From this perspective, fir cultivation has a very important role on mitigating the global change.

In *P. amarus* plantation, simulated nitrogen deposition greatly increased stand growth and NPP. Although NPP was not measured, it was greater than annual soil respiration in all treatments. Simulated nitrogen deposition also simulated the C accumulation in the ecosystem by increasing the plant C pool, though CO_2 emission through soil respiration was also enhanced. Currently, this P. amarus plantation ecosystem is a C sink. N controlled the primary production in this bamboo plantation ecosystem. However, due to the soil nutient status controls the plant growth and thus NPP under nitrogen deposition sustainable, there is still uncertainly regarding the soil and plant C pools response to the increased nitrogen deposition in the P. amarus plantation ecosystem in the long term.

In Chinese fir plantation, after five years of simulated nitrogen deposition experiment, the same increased order (N1> N0> N2> N3) showed up from both the biomass and carbon storage of tree layer in N treated plot stands of Chinese fir. The study suggested that nitrogen input promoted the biomass and carbon storage growth of trees at the early stage of experiment, but with continuous nitrogen deposition, it might be evolved from promote into inhibite the biomass and carbon storage growth of trees when nitrogen input reached the saturation in forest ecosystems. It must be emphasized that, at any time, effects of nitrogen deposition on C sequestration in forest ecosystems are cumulative. Such the cumulative results would perhaps lead to misleading people to come to nitrogen deposition has little effect on C sequestration in forest ecosystems, and even not affect.

On the whole, future research is need in order to accurately predict the long-term response of the C balance in forest ecosystems to the sustainable increasing nitrogen deposition, not only can make up China's deficiencies in this area of the research, as well as provides theoretical guidance for the sustainable management of forests, and furthermore makes a base for study the global change.

5.6. NEECF: a project of nutrient enrichment experiments in China’s forests

Anthropogenic nitrogen (N) emissions to atmosphere have increased dramatically in China since 1980s, and this increase has aroused great concerns on its ecological impacts on terrestrial ecosystems. Previous studies have showed that terrestrial ecosystems in China are acting as a large carbon (C) sink, but its potential in the future remains largely uncertain. So far little work on the impacts of the N deposition on C sequestration in China’s terrestrial ecosystems has been assessed at a national scale. Aiming to assess and predict how ecological processes especially the C cycling respond to the increasing N deposition in China’s forests, since 2010 researchers from Peking University and their partners have established a manipulation experimental network on the ecological effects of the N deposition: Nutrient Enrichment Experiments in China’s Forests Project (NEECF). The NEECF comprises 10 experiments at 7 sites located from north to south China, covering major zonal forest vegetation in eastern China from boreal forest in Greater Khingan Mountains to tropical forests in Hainan Island (Du et al. 2013).

5.6.1. Site introduction

The NEECF project currently has 7 sites that cover the typical forests in eastern China from boreal forest in Greater Khingan Mountains to tropical forest in Hainan Island. These 7 sites locate from north to south include Genhe (GH), Wuying (WY), Saihanba (SHB), Donglingshan (DLS), Guniujiang (GNJ), Wuyishan (WYS) and Jianfengling (JFL) (Fig. 5a and Tab. 6).

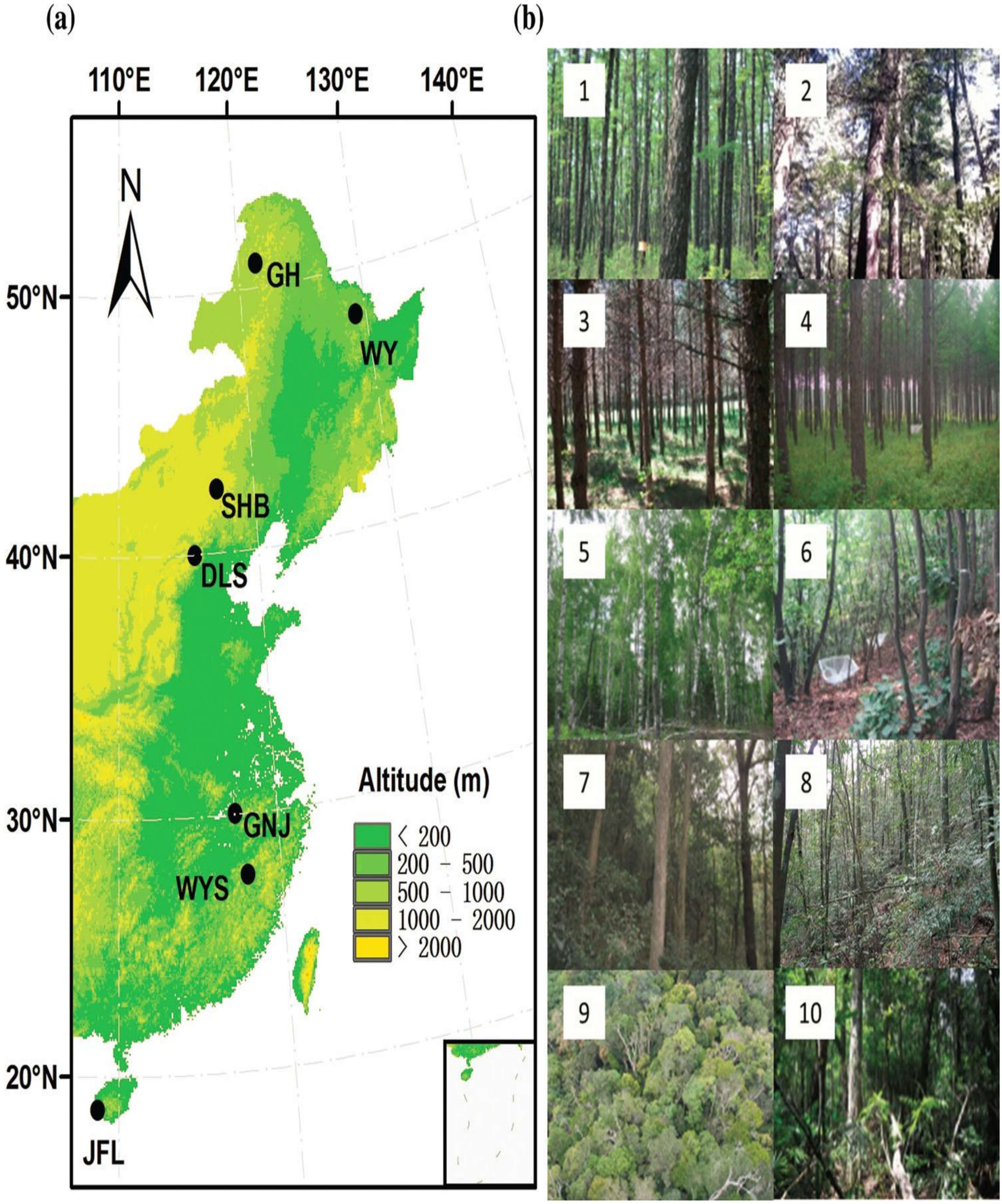


Figure 5 Locations of NEECF sites (a) and photographs of forest structure in each site (b). Seven sites (a) are located from north to south, including Genhe (GH), Wuying (WY), Saihanba (SHB), Donglingshan (DLS), Guniujiang (GNJ), Wuyishan (WYS), and Jianfengling (JFL). Photographs (b) show the view of the ten experimental forests, including: 1 primary larch forest at Genhe, 2 broadleaved Korean pine mixed forest at Wuying, 3 pine plantation at Saihanba, 4 larch plantation at Saihanba, 5 birch forest at Donglingshan, 6 East-Liaoning oak forest at Donglingshan, 7 Sweet Oachestnut forest at Guniujiang, 8 Castanopsis carlesii forest at Wuyishan, 9 primary tropical montane rain forest at Jianfengling and 10 secondary tropical montane rain forest at Jianfengling.

Table 6 General information on NEECF sites.

Site	Location	Altitude (m a.s.l.)	AMT (℃)	AMP (mm)	Growing season	Soil type	PFT
GH	50°56′N, 121°30′E	825	-5.4	481	June-August	Brown coniferous forest soil	Boreal forest
WY	48°07′N, 129°11′E	350	-0.5	654	May-September	Dark brown soil	Temperate broadleaved & conifer mixed forest
SHB	42°25′N, 117°15′E	1400	-1.4	450	May-September	Sandy soil	Plantation
DLS	39°58′N, 115°26′E	1300	5.4	505	May-September	Montane brown soil	Temperate deciduous broadleaved forest
GNJ	30°1′N, 117°21′E	375	9.2	1650	Whole year	Brown earth	Subtropical evergreen broadleaved forest
WYS	27°39′N, 117°57′E	700	18.0	1889	Whole year	Yellow-red soil	Subtropical evergreen broadleaved forest
JFL	18°43′N, 108°53′E	870	24.7	2265	Whole year	Yellow soil	Tropical montane rain forest

Notes: The table shows site location (latitude, longitude and altitude), climate (annual mean temperature (AMT), annual mean precipitation (AMP) and duration of growing season), soil and vegetation type for the seven NEECF sites: Genhe (GH), Wuying (WY), Saihanba (SHB), Donglingshan (DLS), Guniujiang (GNJ), Wuyishan (WYS), and Jianfengling (JFL).

To sum up, the NEECF covers major zonal forests in eastern China with apparent climatic gradients (Fig. 6) and provides an excellent field for experimental research.

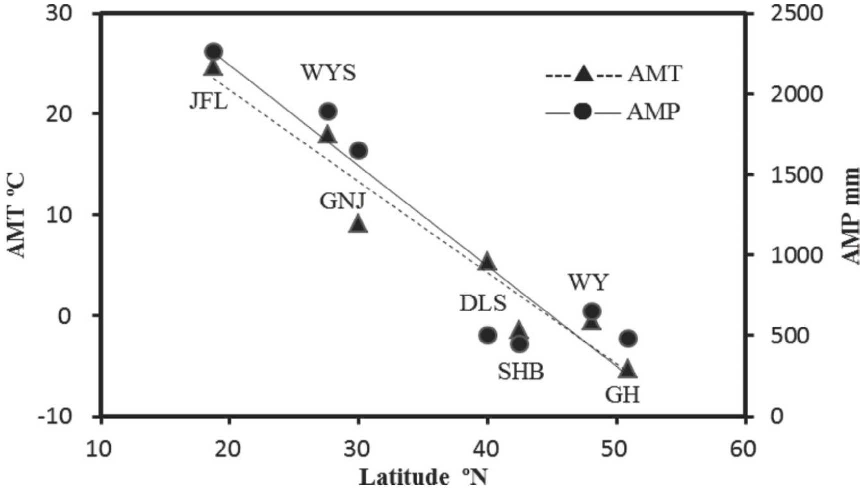


Figure 6 Annual mean temperature (AMT, ℃) and annual mean precipitation (AMP, mm) at NEECF sites. The x-axis, left y-axis and right y-axis indicate latitude, AMT and AMP, respectively.

5.6.2. Forest introduction

Ten representative forests have been chosen for the NEECF Project (Fig.5b). The general information on NEECF forests is shown in Tab. 7. The N deposition ranges from 5.5 to 15.0 kg N ha−1 year−1 in boreal and temperate forests, and from 10.6 to 25.0 kg N ha−1 year−1in subtropical and tropical forests. The relatively low rates of the background N deposition in these forests are an advantage for the nutrient enrichment experiments to track the entire change of the ecosystem processes in response to the simulated N deposition.

Table 7 Experimental forests used in the NEECF project.

Station	Forest type	Forest age (yr)	DBH (cm)	Height (m)	Soil C (g/kg)	Soil N (g/kg)	Soil P (g/kg)	Soil pH	N mineralization (kg N ha^{-1} yr^{-1})	N deposition (kg N ha^{-1} yr^{-1})
GH	Primary larch forest	>300	16.9	15.5	29.73	4.10	1.20	4.70	—	5.5
WY	Broadleaved Korean pine mixed forest	191	23.7	—	—	—	—	—	—	7.0
SHB	Pine plantation	30	12.1	10.1	13.40	1.17	—	6.47	24.80	6.0
	Larch plantation	45	19.8	15.8	29.54	2.23	—	6.29	—	6.0
DLS	East-Liaoning oak forest	70~80	10.9	7.0	—	—	—	—	—	15.0
	Birch forest	70~80	13.6	10.2	—	—	—	—	—	15.0
GNJ	Sweet oachestnut forest	300	13.7	—	10.23	0.81	0.25	4.60	97.14	10.6
WYS	*Castanopsis carlesii* forest	45	11.8	15.0	4.10	0.29	—	—	—	16.0
JFL	Primary tropical montane rain forest	300	5.8	5.4	35.50	1.40	0.12	4.60	105.6	25.0
	Secondary tropical montane rain forest	50	5.2	5.4	35.50	1.40	0.12	4.60	117.4	25.0

Note: The table includes the forest information (forest type, forest age, DBH, Height), soil information (soil C, N and P content, soil pH and soil N mineralization) and ambient N deposition for the forests of NEECF sites. The dashes indicate data not available.

5.6.3. Experiment introduction

Long-term nutrient addition experiments have been started at sites of NEECF since 2010 (Tab. 8).

Table 8 Design of nutrient (N and P) enrichment experiments of each NEECF site.

Station	Forest type	Treatments (kg $m^{-2}yr^{-1}$)	N species	Start time
GH	Primary *Larix gmelinii* forest	N: 0, 20, 50, 100	NH_4NO_3	2010.5
WY	Broadleaved Korean pine mixed forest	N: 0, 20, 50, 100	NH_4NO_3	2010.5
SHB	*Pinus sylvestnis* var. *mongolica* plantation	N: 0, 50, 100, 150	Urea	2009.8
	Larix principis-rupprechtii plantation	N: 0, 20, 50	Urea	2010.5
DLS	*Quercus liaotungensis* forest	N: 0, 50, 100	Urea	2011.7
	Betula platyphylla forest	N: 0, 50, 100	Urea	2011.7
GNJ	*Castanopsis eyrei* forest	N: 0, 50, 100	NH_4NO_3	2011.3
WYS	*Castanopsis carlesii* forest	N: 0, 50, 100	NH_4NO_3	2011.6
JFL	Primary tropical montane rain forest Secondary tropical montane rain forest	N: 0, 25, 50, 100 N+P:50+50 P:50	NH_4NO_3 $Ca(H_2PO4)_2$	2010.9

Notes: The table summarizes the treatments, N species and start time of the nutrient enrichment experiments for each forest type at the seven sites of NEECF.

The research scheme of the NEECF project is shown as Fig. 7. According to the aims of the NEECF project, a series of basic monitoring parameters (Tab. 9) and standard methods of data collection have been implemented.

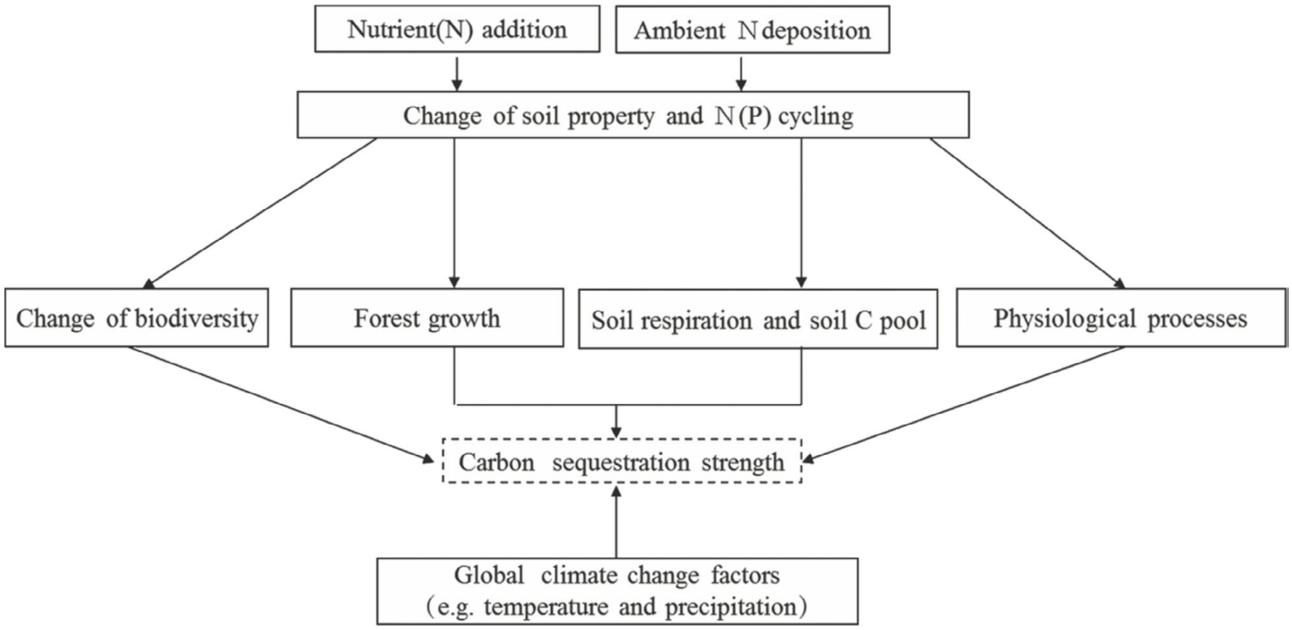


Figure 7 Research scheme of the NEECF project.

Table 9 Mandatory monitoring items of the NEECF project.

No.	Item	Content
1	N deposition	Bulk N deposition
2	Soil N	NH_4^+, NO_3^- and total soil N content
3	Soil P	Soil available P and total P content
4	Soil C	SOC and total C content
5	N mineralization	Rate of N mineralization
6	Soil pH	Soil pH
7	Meteorological factors	Soil and air temperature, and moisture
8	Tree growth	Annual increment of DBH and tree height
9	Grass biomass	Species, coverage, height and biomass
10	Shrub biomass	Species, coverage, BD, height and biomass
11	Root biomass	Root biomass and growth
12	Litter production	Monthly litter production
13	Soil respiration	Rt, Rh and Ra
14	Litter decomposition	Rate of litter decomposition
15	Stoichiometry	Foliar and root C, N and P content

Abbreviations: DBH = diameter at breast height, BD = basal diameter, Rt = total soil respiration, Rh= heterotrophic respiration, Ra= autotrophic respiration.

The NEECF project will be expected to greatly contribute to the scientific base of C sink management of national forest ecosystems and regulation policy to control the N pollution in the future.

5.7. Discussion and conclusions

When nitrogen deposition was first postulated in 2007 as the main mechanism responsible for the large carbon accumulation in Europe's managed forests (Magnani et al. 2007), an intense debate arose over whether such a large nitrogen-induced carbon sink is realistic (De Schrijver et al. 2008). Aber et al. (1998) suggested that net primary production (NPP) may at first increase with increasing N deposition, but then decline with N saturation. Although NPP does not account for respiration and organic matter decomposition, NPP is the main process to acquire atmospheric C and therefore an increase in sequestered C with increasing NPP as a result of enhanced N deposition rates could be expected. After ten years, LeBauer & Treseder (2008) reported that (i) the global N and C cycles interact strongly and that geography can mediate ecosystem response to N within certain biome type; (ii) nitrogen limitation is important in many tropical forests, montane as well as secondary lowland forests; (iii) increasing N deposition, particularly in the most rapidly developing regions, is likely to further stimulate global NPP and slow the accumulation of atmospheric CO_2, which by meta-analysis of 126 nitrogen addition experiments evaluated nitrogen (N) limitation of net primary production (NPP) in terrestrial ecosystems. This study supports the proposition (Aber et al. 1998, Magnani et al. 2007) by demonstrating strong, consistently positive effects of N on NPP. For whether such a large nitrogen-induced carbon sink is realistic, Janssens & Luyssaert (2009) suggested that, to improve the quantification and understanding of variations in forest carbon uptake per unit of nitrogen deposition, we should focus on the spatial differences in the way that photosynthesis, carbon allocation and microbial degradation of soil carbon respond to nitrogen additions.

To sum up, atmospheric deposition of nitrogen can, but does not always, speed up the sequestration of carbon in trees and forest soil. This complexity may arise from climate, community type, geology, the spatial variations and so on by which nitrogen affects carbon storage. It must be emphasized, necessarily, C sequestration or the ecological response of N deposition in forests, which is a long and slow process, depending on the quantity of deposition and the duration. To know exactly how the impacts of N deposition on C sequestration and its ecological mechanism of forests, yet to be positioned for long-term research. As Högberg et al. (2006) believed that even the uniquely long-term simulated N deposition experiment for 30 years in northern Sweden is still young.

5.8. *References*

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Chapter 6

General discussion and conclusions

6.1. Discussion and conclusions

Forests cover approximately one third of the land surface of the earth and account for 80–90% of plant C (Melillo et al. 1990). The impacts on long-term forest growth and C sequestration of N deposition due to human activities over the past 150 years are so large that they have come to perturbing the global C cycle and threatening the Earth system function (Nadelhoffer et al. 1999, Röckstrom et al. 2009). The long-term responses of forests to gradually changing environment are complicated by factors that affect several fluxes at the same time, and by complex interactions among the factors (Gruber & Galloway 2008). For example, the direct response of photosynthesis to an increase in CO_2 will require additional N, and the production response will therefore decline as N limitation progresses (Oren et al. 2001, Reich et al. 2006, Hyvönen et al. 2007). Tree ring techniques offer the opportunity to examine the long-term responses of trees and forests to global climate change and other historical episodes that have already occurred in the past decades (Hietz et al. 2005, Saurer et al. 2004), $\delta^{13}C$ and ITE have been suggested as sensitive long-term monitoring indicators of physiological changes related closely to the gas exchange in the past periods with increased N deposition and altered soil N availability (Francey & Farquhar 1982, Saurer et al. 2004). To understand and predict the effects of N deposition on forest ecosystems, we also have to resort to experimental studies of various kinds. According to several short-term experiments with some factors of large step increases, enhanced N can increase plant photosynthesis and reduce stomatal conductance (Duquesnay et al. 1998, Peñuelas et al. 2008), hence increasing tree growth and C sequestration as well as $\delta^{13}C$ and ITE in forest trees (Oren et al. 2001, Xu et al. 2009). At the same time, it has to be admitted that short-term experiments provide only fragmentary knowledge, the results may not represent longer-term responses of plants to multiple factors in the natural environment (Heimann & Reichstein 2008, Xu et al. 2009). Many N fertilization experiments have been relatively small-scale. What is more, most of the experiments conducted were not set up specifically to study the consequences of atmospheric inputs of N, but primarily to investigate forest productivity. These studies have consequently covered a relatively narrow range of forest types and, furthermore, they have involved patterns of N application which do not tally very closely with atmospheric deposition. Therefore, an improved knowledge of the long-term impact of gradual global climate change and its interaction with N deposition would be urgently required (Gruber & Galloway 2008). However, the only best way is to reduce emissions of nitrogen as rapidly as possible.

6.2. References

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