

The increase of atmospheric CO₂ affects growth potential and intrinsic water-use efficiency of Norway spruce forests: insights from a multi-stable isotope analysis in tree rings of two Alpine chronosequences

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Abstract

Key message Relevant CO₂ increase affects iWUE and growth potential of Alpine Norway spruce forests due to triggering of photosynthetic capacity. Minor effect on iWUE of tree size/age ontogenetic factors.

Abstract An increase in European forest productivity has been widely reported, but evidences on its causal relationship with climate change are still scarce, though they are crucial to understand the mitigation potential of forests and their future dynamics. In the present study, we first assessed the changes in forest productivity of two even-aged Norway spruce forests. Consequently, we investigated the role of several environmental drivers, such as atmospheric CO₂ levels, temperature, and precipitation regimes

on the intrinsic water-use efficiency (iWUE) temporal patterns of the above-mentioned forests. We applied a chronosequence approach, combining it with a multi-stable isotope analysis, including $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$, to infer tree responses to climate change over time in terms of iWUE changes. By this innovative methodology, we were able to separate environmental and age/size-related factors on iWUE changes. Results showed an increase in forest productivity in both sites, paralleled by a significant increase of iWUE, mainly triggered by a CO₂-driven increase in photosynthetic capacity, rather than by a reduction of stomatal conductance. The paramount role of the increase in photosynthetic capacity was confirmed by a strong correlation between atmospheric CO₂ concentration and iWUE temporal patterns. The effect of size/age of trees on iWUE temporal changes resulted to be less defining than that of climate change.

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Introduction

In the last decades, several authors (Spiecker et al. 1996; Karlsson 2000; Jandl et al. 2007; Lopatin 2007; Bontemps et al. 2009; Pretzsch et al. 2014a, b) have reported an increase in productivity of European forests in response to climate change. Increased temperature (Allen et al. 2010), higher atmospheric CO₂ concentration, and nitrogen depositions as well as their combined fertilising effect (Hyvönen et al. 2007; Kahle et al. 2008) were considered the most important environmental drivers of the observed forest growth increase. Especially, Alpine forests seem to

be particularly sensitive to climate change (Gottfried et al. 2012) and to gain knowledge on how they respond to the above-mentioned environmental drivers is of paramount importance to predict their climate change mitigation potential. As highlighted by Peñuelas et al. (2011), plants could react to climate change and more specifically to rising levels of atmospheric CO₂ and temperature by increasing the water-use efficiency (WUE), through an increased photosynthetic rate or by a reduced water transpiration related to a higher vapour pressure deficit (VPD). Both these physiological processes result in carbon (C) and oxygen (O) isotopic fractionation. In this regard, the response of tree growth to increased CO₂ is far from being a straightforward one and seems to be strongly dependant on site conditions, as it can interact with other drivers, such as warming-induced drought and physiological acclimation to high carbon dioxide levels, potentially reducing the ability of forest to act in the long-term as carbon sinks (Linares and Camarero 2011). In particular, tree growth and intrinsic water-use efficiency (iWUE) have been observed not to increase as expected or even decline (Waterhouse et al. 2004; Andreu-Hayles et al. 2011; Peñuelas et al. 2011), for instance, due to a drought-induced critical reduction of stomatal conductance. Moreover, the increase in tree mortality observed in some studies could further reduce the carbon sequestration by European forests (Allen et al. 2010, 2015).

Stable isotope signatures in wooden tissues have been successfully used, both in natural and controlled conditions, to detect and understand the physiological causes of iWUE changes (Grams et al. 2007; Barnard et al. 2012; Lévesque et al. 2014; Guerrieri et al. 2015). In this respect, Scheidegger et al. (2000) suggested a conceptual physiological model that links changes in C and O isotopic ratios to clarify the role of photosynthetic capacity (*A*) and stomatal conductance (*g_s*) in determining iWUE changes, based on the response of the plant to different VPD scenarios. More recently, other authors (Saurer et al. 2004; Gagen et al. 2010; Battipaglia et al. 2013) used this conceptual model to better understand the effect of the environmental drivers and climate change on iWUE changes. Tree size and age can also affect iWUE, probably because of a drop in stomatal conductance with increasing tree height (Marshall and Monserud 1996; Ryan and Yoder 1997). In this respect, the effect of tree age and size on iWUE can be a confounding factor when the estimation of iWUE is based on the retrospective analysis of long-term trends in δ¹³C and δ¹⁸O in tree rings. In the present study, to disentangle the effects of the environmental factors on iWUE from those related to the tree age and size, a multi-sites chronosequence approach coupled with a multi-stable isotope retrospective analysis (δ¹³C and δ¹⁸O) of tree rings of dominant plants was adopted. Correlation

between δ¹³C-inferred iWUE and past CO₂, temperature and precipitation trends, as well as with tree age and tree size was investigated in two even-aged Norway spruce forests growing on non-water-limited sites. Primarily, the use of dominant height (Hd) as a reliable proxy of forest productivity potential (Skovsgaard and Vanclay 2008) was chosen to investigate the temporal trends of forest growth potential by the stem analysis method. The aim was to gain insight on the causal relationships between environmental drivers and the above-mentioned increase in forest productivity and iWUE, while taking into account also the effects of tree size/age.

Materials and methods

Study sites

The present study was carried out in two Norway spruce chronosequences located at Nova Levante (South Tyrol, Italy; 46°27'N, 11°35'E) and Traunstein (Bavaria, Germany; 47°56'N, 12°40'E). The former site is located at an altitude of 1750 m a.s.l. Mean annual temperature is 3.3 °C and mean annual precipitation is 1233 mm. The latter site is at a lower elevation of about 590 m a.s.l. The mean annual temperature is 7.3 °C and mean annual precipitation is equal to 1200 mm. Given the abundant precipitation, these sites were chosen also to study tree growth devoid of the effects of water shortage and drought stress. Each chronosequence was composed of three different aged stands, corresponding to pole stage, adult stage and mature stage of an even-aged high-forest, as derived from the management plans of the respective forests. In Nova Levante, stands were, respectively 55, 125, and 180 years old, whereas in the Traunstein site were 30, 70, and 130 years old. In accordance to the management type, the structure of these stands was single-layered and even-aged, with Norway spruce being the sole species or dominant one with a percentage always higher than 60 %. The key feature of a chronosequence is that the selected stands should have similar site conditions and have been managed in a similar way (Walker et al. 2010). This prerequisite was respected in the present study, as in both sites the stands of the chronosequence are concentrated in a very small area with homogeneous slope, orientation, soil properties, and managed as a forest unit by clear-cutting of small patches (<1000 m²).

Sampling and stem analysis

To avoid unnecessary falling of trees for the stem analysis, the sampling procedure was split into two steps. In 2012, ten dominant trees per stand were chosen and two cores per

tree were sampled orthogonally at breast height. These cores were sanded with increasingly fine-grained sandpaper, measured for tree-ring width to the nearest 0.01 mm and cross-dated by means of a Rinntech LINTAB 6 measuring device coupled with TSAP-Win Scientific software (Rinntech, Heidelberg, Germany). Each series was standardised by a negative exponential curve through ARSTAN software (Cook 1985) to remove the age-related growth trend. A growth suppression analysis was performed, by applying a 10-year-long moving median and a 100 % growth reduction threshold to each standardised series (Rubino and McCarthy 2004). This procedure allowed us to select six of the initial ten trees per stand, i.e. those that were not showing any suppression period in radial growth. Given that these trees were already preselected on a dominant height basis, with this further selection only trees that were always dominant throughout their entire lifespan were identified and then felled. This double-step sampling design allows to obtain data that are better representing the forest productivity potential. In addition, the use of dominant height in place of radial growth strongly reduces the effects related to factors such as competition and forest management, avoiding the biases recently highlighted by Nehrass-Ahles et al. (2014). Following the tree felling, a 5 cm thick cross-section was taken every 4 m along the stem starting from the cutting height, sanded, and the number of annual rings counted. Stem analysis was then performed and the age-height curve of each tree reconstructed (Rossi et al. 2009), by assigning at each sampling height the corresponding cambial age as derived from tree-ring measures. Among the growth functions that can be used to model height development the following 3-parameter Richards Eq. (1) (Richards 1959) was chosen to transform raw age-height data into modelled growth curves:

$$H = a \times (1 - \exp(-b \times t))^c, \quad (1)$$

where H is the predicted height at a given age t and a , b , and c are parameters corresponding to the upper asymptote, slope, and inflexion point, respectively. Non-linear regression was performed to estimate the three parameters of Richards equation and the Marquardt algorithm was chosen for curve fitting. Initial values were set based on both experimental data and the literature (Deleuze and Houllier 1995).

Stable isotope analysis

An increment core was taken with a 5-mm increment borer (Haglöf, Sweden) from each sampled dominant tree (i.e. 6 cores per age class). Increment cores were mounted on a channeled cardboard and air-dried. A small portion of the surface of each core was removed with a scalpel to allow the

identification of annual rings and 5-year-long sections were cut, finely ground with a mill (MM400, Retsch, Germany) and the resulting powder sealed into Teflon filter bags (F57 filter bags, Ankom Technologies, United States), to extract cellulose for C and O isotope analyses according to Loader et al. (1997). Bags were treated first with a 5 % NaOH solution to remove tannins, resins, fats and hemicellulose and then with a 7 % NaClO₂ solution in acetic acid for 30 h to remove lignin. Samples were successively dried for 12 h at 60 °C. About 1 mg of cellulose was weighted and enclosed in tin capsules for carbon isotope analysis, whereas 0.4–0.5 mg were placed in silver capsules and pyrolysed for oxygen isotope analysis. The isotopic analyses were done using a TC/EA elemental analyser (Flash 2000, Thermo Scientific) connected to an isotope ratio mass spectrometer (Delta V Advantage, Thermo Scientific) via a continuous flow interface (ConFlo IV, Thermo Scientific). Isotope ratios were expressed as permil δ notation ($\delta = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$) relative to VPDB and VSMOW international standards for ¹³C and ¹⁸O, respectively. Moreover, $\delta^{13}\text{C}$ values were corrected for the “Suess effect” (Francey et al. 1999), i.e. the decrease of atmospheric $\delta^{13}\text{C}$ due to emissions of ¹³C depleted carbon dioxide since the onset of industrialization. Data for correction were retrieved from McCarroll and Loader (2004) up to 2003 and NOAA mean annual CO₂ data from 2004 to 2011 (<http://www.esrl.noaa.gov/gmd/ccgg/trends>).

Carbon and oxygen stable isotope data interpretation

As isotopic discrimination against ¹³C occurs during photosynthesis; plant tissues are generally depleted in ¹³C in comparison to the atmospheric CO₂. $\delta^{13}\text{C}$ in tree rings could reflect changes in the c_i/c_a ratio, which is the ratio between CO₂ concentration in the leaf intercellular spaces (c_i) and in the atmosphere (c_a), that are, in turn, linked to changes in both photosynthetic capacity (A) and stomatal conductance (g_s). These relations are described in the simplified Farquhar equation (Farquhar 1989) according to which the plant $\delta^{13}\text{C}$ is described as:

$$\delta^{13}\text{C}_p = \delta^{13}\text{C}_a - a - (b - a)(c_i/c_a) \quad (2)$$

where $\delta^{13}\text{C}_a$ is the isotopic signature of atmospheric CO₂, a is the fractionation for ¹³CO₂ during diffusion through air (4.4 ‰), b is the fractionation occurring during carboxylation (27 ‰) by the Rubisco enzyme. From this equation it is possible to derive c_i as follows:

$$c_i = c_a((\delta^{13}\text{C}_a - \delta^{13}\text{C}_p - a)/(b - a)). \quad (3)$$

Therefore, given the values of CO₂ atmospheric concentration and those of atmospheric and plant $\delta^{13}\text{C}$, it is

possible to estimate the iWUE (A/g_s) by the following equation:

$$\begin{aligned} \text{iWUE} &= A/g_{\text{H}_2\text{O}} = (c_a - c_i)/1.6 \\ &= (c_a - (c_a(\delta^{13}\text{C}_a - \delta^{13}\text{C}_p - a)/(b - a)))/1.6. \end{aligned} \quad (4)$$

Hence, taking into account the long-term variation of atmospheric CO_2 concentration and its isotopic signature, $\delta^{13}\text{C}$ in tree rings allows to estimate the iWUE (Saurer et al. 2004).

$\delta^{18}\text{O}$ in plant tissues are related to transpiration rate and to the ratio between the atmospheric and intercellular vapour pressure (e_a/e_i), as described below according to Craig and Gordon (1965), later modified by Dongmann et al. (1974):

$$\begin{aligned} \delta^{18}\text{O}_e &= \delta^{18}\text{O}_s + \varepsilon^* + \varepsilon_k \\ &+ (\delta^{18}\text{O}_v - \delta^{18}\text{O}_s - \varepsilon_k)(e_a/e_i), \end{aligned} \quad (5)$$

where $\delta^{18}\text{O}_e$ is the isotopic signature at the evaporative site, which is directly related to the ratio e_a/e_i ($\sim\text{RH}$, relative humidity), whereas $\delta^{18}\text{O}_s$ and $\delta^{18}\text{O}_v$ are isotopic signatures of soil water and atmospheric water vapour, respectively. ε_k is the kinetic fractionation during leaf water evaporation and ε^* is the equilibrium fractionation, which occurs during the transition from the liquid to the gaseous phase, depending on leaf temperature (Bottinga and Craig 1969). However, also the Péclet effect should be taken into account (Farquhar and Lloyd 1993), which reduces the evaporative site enrichment due to input of not enriched water during transpiration. This effect increases the role of $\delta^{18}\text{O}_s$ in determining $\delta^{18}\text{O}_{\text{cel}}$, as described by Barbour and Farquhar (2000) and Roden et al. (2000):

$$\delta^{18}\text{O}_{\text{cel}} = \delta^{18}\text{O}_s(P_{\text{ex}} \times P_x) + \delta^{18}\text{O}_1(1 - P_{\text{ex}} \times P_x) + \varepsilon_{\text{wc}}, \quad (6)$$

where ε_{wc} is the equilibrium fractionation between water and carbonyl groups (Sternberg and Deniro 1983), whereas P_{ex} and P_x are, respectively, the proportion of exchangeable oxygen in cellulose and the proportion of xylem water in the meristematic tissue where cellulose is synthesised. This equation points out the inverse relationship between $\delta^{18}\text{O}_{\text{cel}}$ and stomatal conductance, as explained by Scheidegger et al. (2000) and Grams et al. (2007), particularly when $\delta^{18}\text{O}_s$ and $\delta^{18}\text{O}_v$ do not vary, e.g. when trees grow in the same environment and with the same relative humidity. This condition is fulfilled by the chronosequence approach adopted within this study, as in each site the three age classes were growing in the same location and relying on the same surface water for uptake, also considering that Norway spruce is a shallow-rooted species. Moreover, $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ can be related with each other and their knowledge suggests which physiological trait contributed the

most to the variations in iWUE, as proposed by Scheidegger et al. (2000) and Grams et al. (2007). In fact, according to the conceptual model, it is possible to infer changes in A from variations in the $\delta^{13}\text{C}$, with a positive relationship between the two variables, due to decreasing intercellular CO_2 concentration (c_i). On the other hand, changes of g_s could be traced back through $\delta^{18}\text{O}$ variations, with a negative relationship between the two variables, as the evaporative enrichment in ^{18}O that takes place when plants grow in drier environments could be related to a decrease of g_s , as quantified by the Péclet effect. However, some concerns about this approach have been recently risen, mainly regarding the $\delta^{18}\text{O}$ interpretation due to the different isotopic signatures of different water sources (i.e. rain water and groundwater) and to the dampening of the isotopic signal from the leaves to tree rings (Roden and Siegwolf 2012; Gessler et al. 2014). Given the conditions previously mentioned about the chronosequence approach, in particular with regard to the water source, in our study it is reasonable to interpret the variability of the two stable isotope ratios in tree rings to understand which factors brought to the change in iWUE.

Climate data, correlation with iWUE temporal trends and relationship with dominant height trends

The differences in the iWUE patterns among the different aged stands over time were analysed by a Repeated Measures ANOVA considering them as a between-subject factor (IBM SPSS, United States). The analysis was performed only when all three stands were established, hence from 1955 in Nova Levante and from 1990 in Traunstein. Given the length of the obtained stable isotope series and the lack of long-term historical climate data, monthly and yearly temperature and precipitation data were retrieved from the CRU TS 3.1 gridded data set for the period 1901–2009 (Mitchell and Jones 2005). The influence of climate change on trees' physiological responses was inferred first by performing a forward stepwise regression, with iWUE as dependent variable and CO_2 (retrieved from McCarroll and Loader 2004 and NOAA/ESRL database), yearly and growing season (from April to September) temperature and precipitation as independent variables, all averaged in 5-year periods to be comparable with stable isotope-derived data. The relation among these drivers and iWUE was investigated in both sites also through a Principal Component Analysis (PCA), to overcome the limitations connected to the autocorrelation of some of drivers, namely CO_2 and temperature. All the variables were transformed to make them comparable. The transformation was carried out by subtracting the mean value of the whole sample (i.e. the single age class) from each single value, and then by dividing it by the

sample standard deviation. Furthermore, as both the stepwise regression analysis and the PCA highlighted the prevailing role of CO₂ on iWUE change, we decided to implement the following linear model to distinguish the effect of climate change (i.e. atmospheric CO₂ concentration) on iWUE from the ontogenetic ones (i.e. tree age and size).

$$\text{iWUE} = (a \times \text{Age}) \times (b \times \text{Size}) + c \times \text{CO}_2, \quad (7)$$

a , b and c are the parameters of each independent variable, set with initial values of 0.3, 0.1 and 0.5, respectively. In this model, an additive effect of CO₂ as environmental driver and a multiplicative effect of tree size (i.e. dominant height) and age on iWUE was postulated. Age, size and CO₂ concentration were expressed in years, metres and ppm, respectively.

Results

Dominant height growth trends

Modelled dominant height (Hd) growth trends of the two studied chronosequences (Fig. 1) show that pole stage stands (P) have grown faster in height than adult stands (A) and mature (M) ones in both sites. Comparing the two sites, it is possible to observe that in Traunstein all the three stands had a stronger Hd increment, with an earlier inflexion point and steeper slope (Fig. 1b) in comparison with the Nova Levante site (Fig. 1a).

$\delta^{13}\text{C}$ temporal trends

In the Nova Levante site, the $\delta^{13}\text{C}$ values increased over the time for all the three stands of the chronosequences (Fig. 2a). For instance, P stand $\delta^{13}\text{C}$ values ranged from -22.18 ‰ in 1955 to -21.54 ‰ in 2010, from -23.70 ‰ in 1890 to -21.14 ‰ in 2010 in A stand and from -22.16 ‰ in 1860 to -20.88 ‰ in 2010 in M stand. However, since 1985 a decrease of the $\delta^{13}\text{C}$ values was observed in all the three stands, and it was stronger in the youngest stand in comparison with the others. The $\delta^{13}\text{C}$ values of M and A stands were always higher than those of P stand. In Traunstein site, $\delta^{13}\text{C}$ values increased from -23.71 ‰ in 1875 to -20.57 ‰ in 2010 in M stand and from -23.61 ‰ in 1935 to -21.85 ‰ in 2010 in A stand. In the youngest P stand the pattern was not clear. This is probably due to the short time span (1990–2010) that overlaps with the above-mentioned (since 1985) period in which a decrease in the $\delta^{13}\text{C}$ values was observed also in the other chronosequence. In addition, as it occurred in the

former site, the $\delta^{13}\text{C}$ values were higher in M stand than A and P ones (Fig. 2b).

$\delta^{18}\text{O}$ temporal trends

The values of $\delta^{18}\text{O}$ in Nova Levante increased in P stand from 25.85 to 27.65 ‰ (1955–2010) and in A stand from 25.75 to 27.19 ‰ (1890–2010), with a noticeable raise taking place from the 1980 decade. In the M stand, we did not observe such increase, as the $\delta^{18}\text{O}$ values were more stable over time (from 26.10 to 26.43 ‰) (Fig. 3a). In Traunstein only M -stand $\delta^{18}\text{O}$ values increased from 26.08 to 27.61 ‰ (1875–2010), whereas both P - and A -stand $\delta^{18}\text{O}$ values remained constant in time (Fig. 3b).

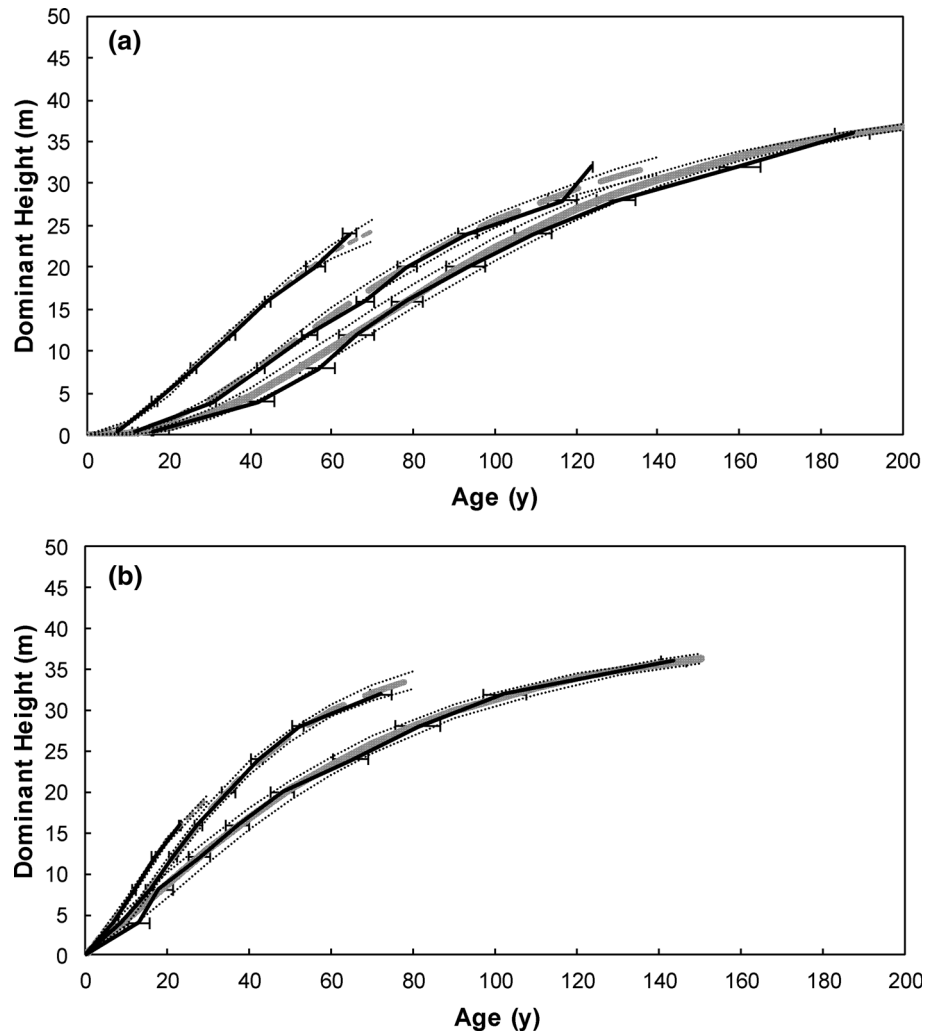
iWUE temporal trends

The $\delta^{13}\text{C}$ -inferred iWUE showed increasing trends in both sites (Fig. 4). In Nova Levante site, it rose from 89.29 to 98.71 $\mu\text{mol mol}^{-1}$ in the period 1860–1950 in the M stand, and from 79.12 to 101.18 $\mu\text{mol mol}^{-1}$ in the period 1890–1950 in the A stand. Since 1950, the period in which the whole P stand temporal trend is included, a steeper slope in $\delta^{13}\text{C}$ -inferred iWUE was observed in all the stands. In the period 1955–2010, P -stand iWUE values increased from 97.93 to 129.18 $\mu\text{mol mol}^{-1}$, the A -stand values from 98.94 to 133.51 $\mu\text{mol mol}^{-1}$ and the M -stand values from 103.91 to 136.32 $\mu\text{mol mol}^{-1}$. The $\delta^{13}\text{C}$ -inferred iWUE values were constantly higher in the older stands. Regarding the Traunstein site, the increase of $\delta^{13}\text{C}$ -inferred iWUE is more constant in time than in the former site, but also in this site a stronger increase was observed starting from 1950. The $\delta^{13}\text{C}$ -inferred iWUE values rose from 77.78 to 139.35 $\mu\text{mol mol}^{-1}$ for the M stand (1875–2010), from 83.68 to 126.73 $\mu\text{mol mol}^{-1}$ for the A stand (1935–2010) and from 117.73 to 123.82 in P stand (1990–2010).

Climate sensitivity

Results from the forward stepwise regression between $\delta^{13}\text{C}$ -inferred iWUE and 5-year mean annual temperature (Ty), growing season temperature (Tg), annual precipitation (Py), growing season precipitation (Pg) and CO₂ atmospheric concentration values are shown in Table 1. In both sites changes in CO₂ explained almost the whole variability of iWUE in all three age classes. The remaining variables accounted for less than 1 % of the variability in iWUE (Table 1). In both sites the principal components 1 and 2 combined explained more than 85 % of the total variability (Figs. 5, 6). Both in Nova Levante and Traunstein, the variables split into two groups represented by

Fig. 1 Modelled growth curves after Richards equation of the three age classes (Pole stage = grey short dashed line, Adult stage = grey long dashed line, Mature stage = grey bold line) and corresponding raw data from stem analysis (black line; age standard errors are shown) of **a** Nova Levante and **b** Traunstein. 95 % confidence intervals of modelled data showed in dotted lines



iWUE, CO₂ concentration and yearly and growing season temperature, associated with component 1, and yearly and growing season precipitation, related to component 2. The distribution of the samples considering the 5-year periods shows that starting from 1985 they are grouping together regardless of the age class. Particularly, 1985–2000 are grouping closer to iWUE and CO₂ in all age classes of Nova Levante (Fig. 6a), whereas in Traunstein only A and M classes group in those years (Fig. 6). 2005 and 2010 appear to be related more to growing season temperature in all age classes of both sites (Figs. 5, 6). All the previous 5-year periods, from 1905 to 1980, are either grouping closer to precipitation, though without a clear time-related pattern, or are scattered far from the considered variables. The outcome of the Repeated Measures ANOVA showed that the values of iWUE were always differing among the three stands of each chronosequence during the considered time span, as the effect of the between-subject factor was statistically significant both in Nova Levante and Traunstein ($P < 0.05$). The iWUE linear model was able to

explain 94.16 % of the variability in Nova Levante and 77.02 % in Traunstein (Table 2). Estimates for the three parameters of both sites are reported in Table 2.

Discussion

A number of studies tried to infer the ecophysiological response of trees to climate change using stable isotope data, and an increase in iWUE was reported in both broadleaf (Duquesnay et al. 1998; Guerrieri et al. 2010; Battipaglia et al. 2013) and coniferous forest (Saurer et al. 2004; Gagen et al. 2010; Leonardi et al. 2012; Frank et al. 2015). Nevertheless, some studies (Peñuelas et al. 2011; Gómez-Guerrero et al. 2013) reported a discrepancy between the above-mentioned increase in iWUE and forest growth, pointing out the fact that rising atmospheric CO₂ levels do not always imply enhanced photosynthetic rate and tree growth (Nock et al. 2010). However, in the present study, rising iWUE values were observed in different aged

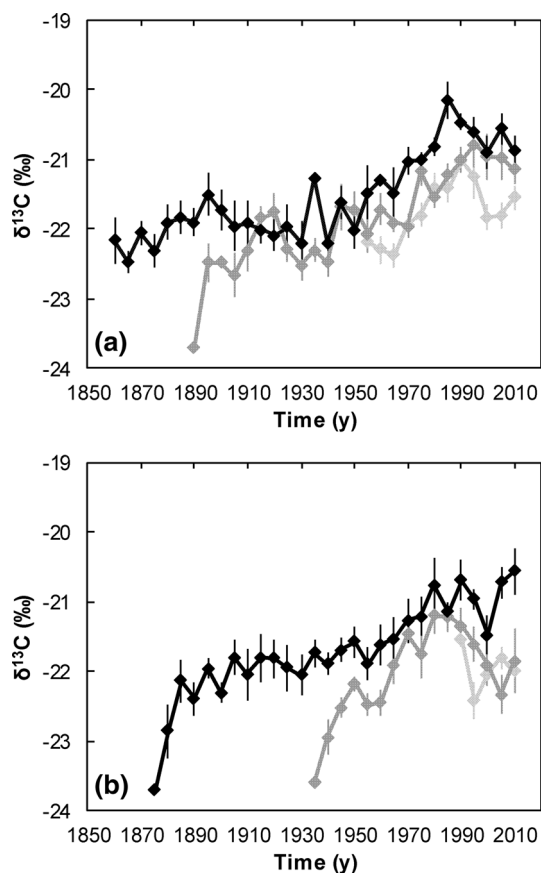


Fig. 2 $\delta^{13}\text{C}$ temporal trends of the three stands in **a** Nova Levante and **b** Traunstein (P stand = light grey line; A stand = dark grey line; M stand = black line). Standard errors are shown in bars

stands of two chronosequences, which were paralleled by substantial changes in tree growth, suggesting a connexion between *i*WUE increase and tree growth in these environmental conditions (Fig. 4). This is a contrasting result compared to other recent studies that investigated the relation between the increase in *i*WUE and tree growth. This can be explained at least partially by the favourable site conditions, with high water availability and a gentle slope. Most of these studies, moreover, assessed tree growth through radial increment trends, whereas here we focus on dominant height trends as indicators of stand productivity, which is based on different assumptions, as explained in the materials and method section. Among the environmental drivers of the *i*WUE change, the atmospheric CO_2 concentration increase proved to be the most relevant one in our study. Temperature trends appear to have been also relevant at least in the last decades, as highlighted by the PCA performed on the two sites. In evaluating the relationship between *i*WUE and CO_2 , it must be stated that c_a is part of the equation that has been used to calculate *i*WUE. Nevertheless, c_i affects the final *i*WUE value, depending on the physiological response of the plant

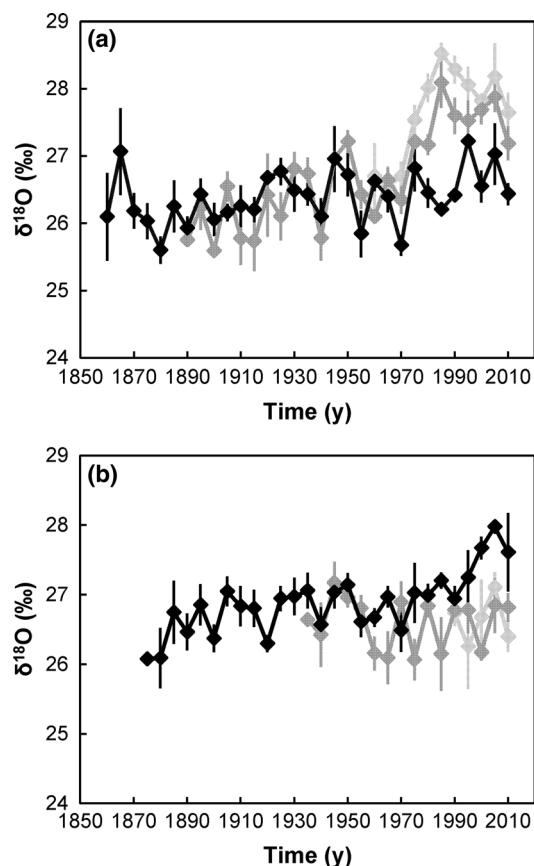


Fig. 3 $\delta^{18}\text{O}$ temporal trends of the three stands in **a** Nova Levante and **b** Traunstein (P stand = light grey line; A stand = dark grey line; M stand = black line). Standard errors are shown in bars

to rising CO_2 (Voelker et al. 2015). Increasing trends in $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values were observed, although for the latter the increase was generally less pronounced. If the increase in $\delta^{13}\text{C}$ can be linked to the increase in atmospheric CO_2 concentration through an enhanced photosynthetic capacity (A), the interpretation of $\delta^{18}\text{O}$ changes may be less straightforward. The reduction in stomatal conductance that brings about the increase in $\delta^{18}\text{O}$ is not necessarily related to changes in VPD and to evaporative enrichment. Our results suggest that the changes in g_s and thus in $\delta^{18}\text{O}$ are a result of increasing CO_2 concentration, rather than changes in the VPD. In fact, when stomatal conductance decreases, either due to increased VPD or CO_2 , the lamina leaf water gets proportionally enriched in ^{18}O due to a lower input of non-evaporatively enriched xylem water, which is then imprinted on the organic matter (Barbour et al. 2002; Farquhar et al. 2006; Gessler et al. 2009). It must be taken into account that post-photosynthetic processes can further dampen the original leaf stable isotope signature. For instance, alteration of the leaf $\delta^{13}\text{C}$ are supposed to take place as sugars can mix with different pools during transportation through the phloem and

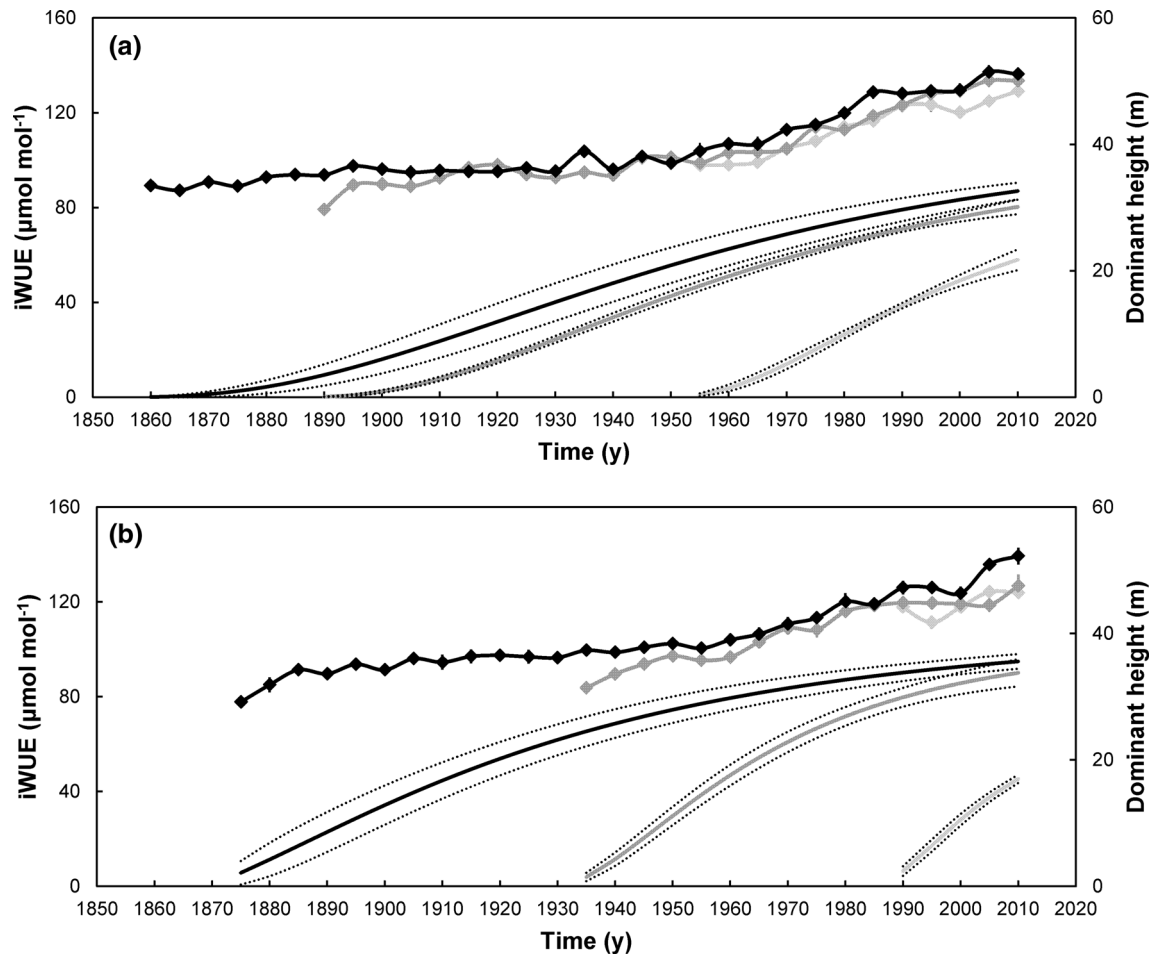


Fig. 4 $\delta^{13}\text{C}$ inferred iWUE and dominant height temporal trends of the three stands of **a** Nova Levante and **b** Traunstein (P stand = light grey line; A stand = dark grey line; M stand = black line). Standard

errors of iWUE are shown in bars; 95 % confidence intervals of the modelled dominant height curves are shown as dotted lines

Table 1 Forward stepwise regression estimates, R^2 values and P values ($P < 0.05$), $\delta^{13}\text{C}$ inferred iWUE being the dependent variable and 5-year mean atmospheric CO_2 concentrations (CO_2),

yearly (Ty) and growing season (Tg) temperature and yearly (Py) and growing season (Pg) precipitation the independent variables

Stand	NOVA LEVANTE				iWUE	TRAUNSTEIN			
	Parameter	Estimate	$P (<0.05)$	$R^2 \%$		Parameter	Estimate	$P (<0.05)$	$R^2 \%$
P	CO_2	0.533082	0.0000	99.88	P	CO_2	0.318679	0.0000	99.94
	Tg	-6.90367	0.0187	+0.08		Tg	-4.20607	0.0385	+0.09
A	CO_2	0.431846	0.0000	99.74	A	CO_2	0.464328	0.0000	99.69
	Ty	14.1049	0.0020	+0.11		Tg	-3.67214	0.0006	+0.11
M	Tg	-10.2377	0.0000	+0.03	M	Pg	-0.02748	0.0034	+0.03
	CO_2	0.5971	0.0000	99.72		Tg	-8.41359	0.0000	+0.16

remobilization at the beginning of the growing season, whereas leaf $\delta^{18}\text{O}$ signal could be dampened by non-enriched xylem water during cellulose synthesis, as well as remobilization (Gessler et al. 2014). Nevertheless, our data

clearly suggest that, in an environment with significantly increasing CO_2 concentration, the observed iWUE increase was mainly triggered by an improved photosynthetic capacity and to a lesser extent by changes in stomatal

Fig. 5 PCA biplots for Nova Levante, showing the distribution of the 5-year periods divided by age class and the relationship with intrinsic water-use efficiency (iWUE), yearly and growing season temperature (Ty and Tg), yearly and growing season precipitation (Py and Pg) and carbon dioxide concentration (CO₂). Age classes are: pole stage (*dots*), adult stage (*squares*) and mature stage (*triangles*). Percentages of variability explained by PC 1 and 2 are written in *brackets*

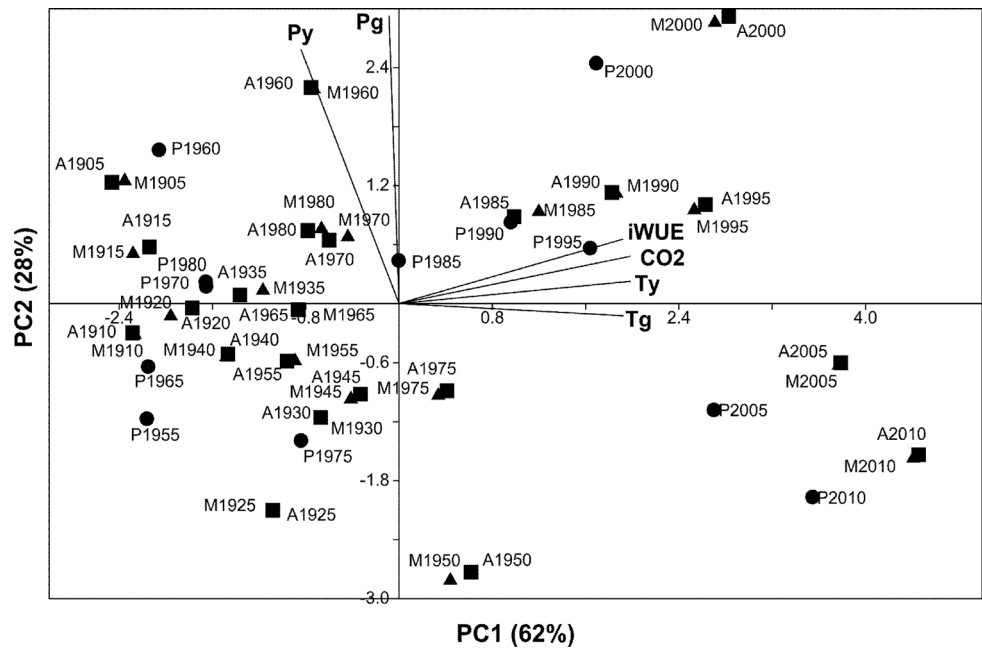


Fig. 6 PCA biplots for Traunstein, showing the distribution of the 5-year periods divided by age class and the relationship with intrinsic water-use efficiency (iWUE), yearly and growing season temperature (Ty and Tg), yearly and growing season precipitation (Py and Pg) and carbon dioxide concentration (CO₂). Age classes are: pole stage (*dots*), adult stage (*squares*) and mature stage (*triangles*). Percentages of variability explained by PC 1 and 2 are written in *brackets*

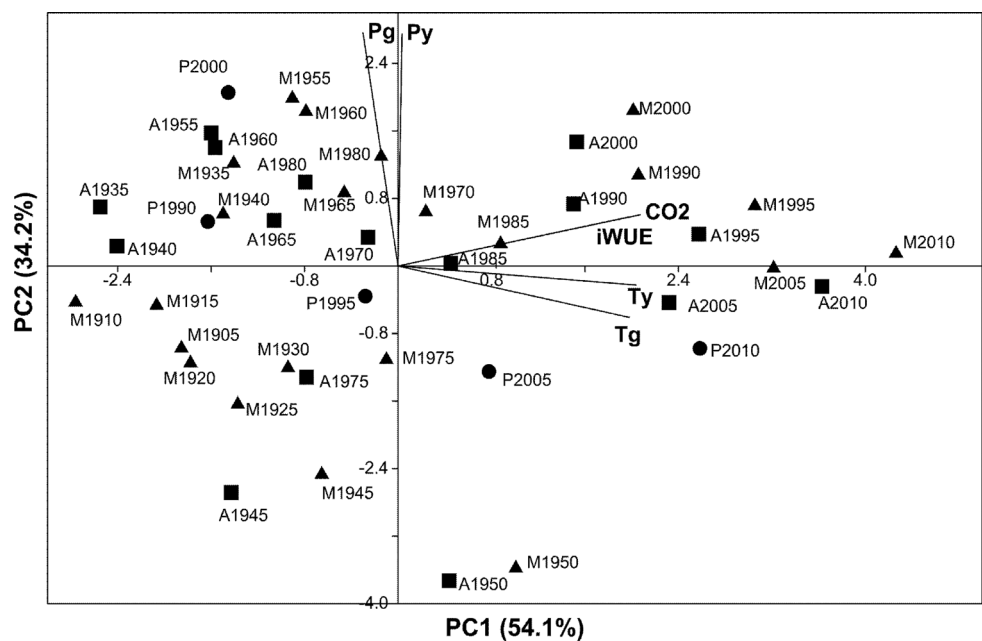


Table 2 Estimates of the parameters for the iWUE linear model for both Nova Levante and Traunstein

	NOVA LEVANTE	TRAUNSTEIN
<i>a</i>	+0.103 (±0.006)	+0.087 (±0.016)
<i>b</i>	+0.034 (±0.002)	+0.030 (±0.005)
<i>c</i>	+0.312 (±0.021)	+0.321 (±0.011)
Model <i>R</i> ²	94.16 %	77.02 %

Independent variables are Age (parameter *a*), Size (parameter *b*) and CO₂ (parameter *c*). Standard errors in brackets

conductance. Hence, photosynthesis could have increased in time, affecting the iWUE more than CO₂-related changes in *g_s*. The selection of the two chronosequences in non-water-limited environments could have significantly affected these findings, as in xeric environments no significant change in forest productivity has been usually found because of iWUE increase (Lévesque et al. 2014). A slight modification in the stable isotope trends of both sites in the last 20 years was also observed (Figs. 2, 3), but our analyses do not show any significant shift in iWUE in the

same period, nor changes in the forest productivity. For instance, the recent changes in the $\delta^{18}\text{O}$ could be explained by a decrease in stomatal conductance due to the onset of a warming-induced water stress, or by a higher effect of hydraulic limitations for what concerns taller trees. However, further investigation might be needed to better understand if trees are adjusting their ecophysiological responses to the ongoing climate change.

Comparing the iWUE with dominant height temporal trends, older stands, and hence taller dominant trees, always display higher absolute values of iWUE (Fig. 4), a difference that is statistically significant according to the results of the Repeated Measures ANOVA. Therefore, in addition to the effect of the environment, also the effect of tree size and/or tree age on iWUE must be considered in retrospective studies. Size-related hydraulic limitations reducing stomatal conductance have been proposed as causes of the increase in iWUE in taller trees (Ryan and Yoder 1997; Bond 2000; Day et al. 2002), and grafting experiments showed that tree growth decline was related to size rather than to age (Martínez-Vilalta et al. 2007; Mencuccini et al. 2005). However, not only changes in the xylem structure are thought to influence WUE and growth as function of age and/or size, but also leaf nitrogen content (Gower et al. 1996; Niinemets 1997) and morphological changes in the foliar structure of older trees were proposed (Niinemets 2002). Hence, although there is a good understanding of the processes involved, it is very difficult to disentangle the senescence from size-related effects on iWUE, and to clarify which of them are more defining. Nevertheless, in the model implemented in this study, in which age and size were considered to act together through a multiplicative relationship, they had a much lower influence than CO_2 concentration on iWUE change. In fact, the c parameter value, associated with CO_2 concentration, was much higher than the parameters (a and b) associated with tree age and size in both sites, hinting to an effect of CO_2 stronger than any ontogenetic factor on the shift in iWUE. For instance, if according to the model we take into consideration a 25 m tall and 60 years old tree in Nova Levante site, and the corresponding iWUE increase of 29.88 ($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$), the relative contribution to iWUE of age and size is 5.25, whereas the contribution of the sole CO_2 is 24.63. If the effect of age is removed by plotting iWUE against dominant height (Fig. 7), the paramount role of climate change is once again confirmed, as the younger stands, which established after the 1950 and grew under higher CO_2 concentrations, all show higher iWUE compared to the older ones at the same dominant height. The same patterns can be observed when plotting iWUE trends against cambial age (Fig. 8). To this end, the prevalence of the environmental drivers over the effect of age/size

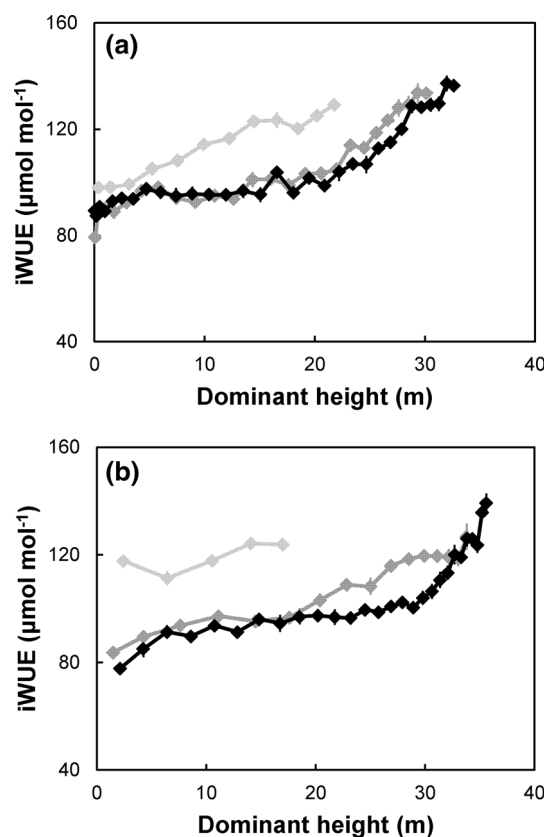
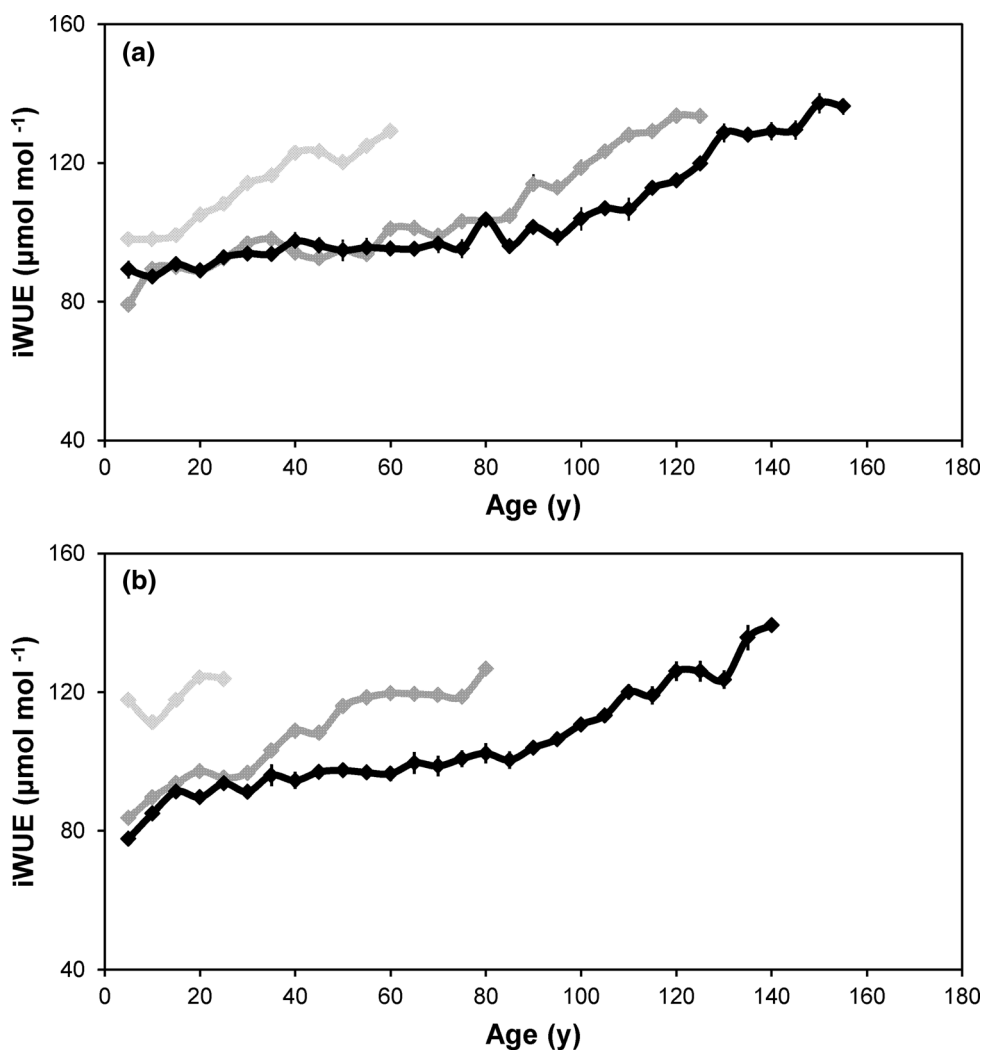


Fig. 7 iWUE values against dominant height of the three stands in **a** Nova Levante and **b** Traunstein (P stand = light grey line; A stand = dark grey line; M stand = black line). Standard errors are shown in bars

was emphasised also by the PCA, where in the last three decades data were grouping closer to the main variables based on calendar years and not on the age class.

Full comprehension of the long-term effects of the CO_2 increase on water-use efficiency and tree growth has still to be achieved, and the nature of these effects is not a straightforward one (Nowak et al. 2004). Besides the observation of decreased iWUE and growth under increasing CO_2 , Körner (2015) highlighted the importance of considering the factors affecting plant tissue formation activity in defining growth levels, such as temperature and water availability, rather than the sole effect of CO_2 . Nevertheless, in the present study, the CO_2 , along with increasing temperatures, seemed to be the most important environmental driver behind the shift in iWUE, as previously suggested by other authors in different studies (Battipaglia et al. 2013; Keenan et al. 2013). In addition, the role of high precipitation levels and water availability could also have been of paramount importance, to avoid drought-induced responses to increased temperatures. However, other concurring environmental drivers that were not included in this

Fig. 8 iWUE values against cambial age of the three stands in **a** Nova Levante and **b** Traunstein (P stand = light grey line; A stand = dark grey line; M stand = black line). Standard errors are shown in bars



study, namely nitrogen depositions (Strömgren and Linder 2002), could act together with the rising atmospheric CO₂ and temperature, playing a significant role in determining the increase in forest productivity in the two studied sites.

Conclusions

In this study, we adopted an innovative methodology composed by stem analysis combined with stable isotope analyses, within the framework of a chronosequence. This approach enabled us to gain insight on the ecophysiological responses of Norway spruce Alpine forests to climate change and their relation with the recent increase in forest productivity, while taking into account also size/age-related ontogenetic effects by including different aged classes. In both sites, higher iWUE was found to have been driven by increased atmospheric CO₂ concentration mainly due to an improvement of the

photosynthetic capacity of trees rather than to changes in stomatal conductance. A minor effect of tree size/age on iWUE was discernible too.

Author contribution statement FG set up the research, carried out fieldwork, samples preparation and analysis, data analysis and interpretation and wrote the manuscript. PC contributed to stable isotope analysis, data interpretation and manuscript revision. HP provided the Traunstein site and contributed to the growth trends assessment analysis. GT set up the research, carried out data analysis and interpretation, wrote and revised the manuscript.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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