

Chlorophyll fluorescence and gas exchange measurements in field research: an ecological case study

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Abstract

We tested whether cheap and quick chlorophyll (Chl) fluorescence can be used in ecophysiological field studies as proxies for gas-exchange measurements. We measured net photosynthetic rate at saturating irradiance and ambient atmospheric CO₂ concentrations (P_{Nsat}), maximum carboxylation rate (V_{cmax}), maximum quantum yield of PSII (F_v/F_m), the performance index (PI_{abs}), leaf nitrogen (N_{area}), and carbon isotope discrimination ($\Delta^{13}\text{C}$) within four herbaceous species along two elevational gradients. We analysed the relationship between Chl fluorescence and gas-exchange parameters and their link to indirect assessment of plant performance *via* ecophysiological traits. F_v/F_m showed no relationship to P_{Nsat} and only weak relationships to V_{cmax} . PI_{abs} was positively related to P_{Nsat} and V_{cmax} . PI_{abs} , P_{Nsat} , and V_{cmax} were positively associated with N_{area} and negatively to $\Delta^{13}\text{C}$, whereas F_v/F_m showed no relationship to N_{area} and a positive to $\Delta^{13}\text{C}$. Thus, PI_{abs} might be suitable to characterize the photosynthetic activity when aiming on large numbers of samples.

Additional key words: *Aposeris foetida*, carbon isotope discrimination, *Knautia dipsacifolia*, leaf nitrogen, *Mercurialis perennis*, *Trifolium pratense*.

Introduction

Photosynthesis is the main process in the physiology of all plant species and facilitates life on the Earth as we know it today. Light quanta absorbed by Chl can undergo three different fates: they can be used photochemically driving photosynthesis, be dissipated as heat or they can be reemitted as light, the so called Chl *a* fluorescence (Maxwell and Johnson 2000). Photosynthetic activities of plants are mostly quantified *via* gas-exchange measurements, where the quantity of CO₂ absorbed by leaf tissue per unit area (or mass) and time is measured (Von Caemmerer and Farquhar 1981, Küppers *et al.* 1987). However, Chl *a* fluorescence is increasingly used in

ecology to assess the vitality and performance of plants, since this nondestructive method is fast, cost effective, and requires a minimum of expertise in contrast to the traditional gas-exchange measurements (Schreiber *et al.* 1995, Clark *et al.* 2000, Dias and Brüggemann 2010). The question is, whether Chl *a* fluorescence is linked to gas-exchange measurements under field conditions and whether we can use this faster and easier method to deduce ecophysiological relevant parameters. We explicitly do not focus on enhancing the ecophysiological understanding of the relation between Chl *a* fluorescence and gas-exchange measurements, but on the applicability of

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Abbreviations: Chl – chlorophyll; F_v/F_m – maximum quantum yield of PSII; N_{area} – area based leaf nitrogen content; PI_{abs} – absorption based performance index; P_{Nsat} – net photosynthetic rate at saturating irradiance and ambient atmospheric CO₂ concentrations; V_{cmax} – maximum carboxylation rate; $\Delta^{13}\text{C}$ – carbon isotope discrimination.

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Chl *a* fluorescence parameters in extensive ecological and ecophysiological field studies as proxies for the cost and time intensive gas-exchange measurements.

The Chl *a* fluorescence accounts for 1–2% of the total light absorbed and holds important information on the performance of plants (Maxwell and Johnson 2000, Kalaji *et al.* 2014, 2017a,b). Chl *a* fluorescence of a leaf, which is triggered by a light pulse after dark adaption, rises very fast from a dark-adapted ground fluorescence value (F_0) to a maximum fluorescence value (F_m). It subsequently decreases to a steady-state value due to an increase of electron transport away from PSII and an increased efficiency of energy conversion into heat. This general pattern was described as the Kautsky effect (Kautsky and Franck 1943). Continuous excitation fluorimeters detect this transient light-induced O-J-I-P fluorescence (named after the local maxima of the response curve), where the intensity of Chl *a* fluorescence is displayed against time which is solicited by a saturating light pulse (Strasser *et al.* 2000, Lazár 2006). This transient has been referred to as the barcode for photosynthesis (Tyystjärvi *et al.* 1999) and further parameters can be deduced thereof such as the two parameters, which are often used to answer ecological questions: the maximum quantum yield of PSII (F_v/F_m) and the absorption-based performance index (PI_{abs}). F_v/F_m , where the variable fluorescence F_v is defined as $F_m - F_0$, is an indicator for the potential activity of PSII of a leaf, whereas PI_{abs} is a measure of photosynthetic performance. PI_{abs} comprises information on the density of fully active reaction centres, the probability that an absorbed photon will be trapped by a reaction centre and the efficiency of electron movement by a trapped exciton beyond the primary acceptor quinone Q_A (Kitajima and Butler 1975, Strasser *et al.* 1999, Clark *et al.* 2000, Strasser *et al.* 2000, Živčák *et al.* 2008). PI_{abs} therefore includes the density of fully active radiation centres, the efficiency of electron movement to the electron transport chain and the probability that an absorbed photon will be trapped by the reaction centres. Thus, PI_{abs} encompasses the functionality of PSI and PSII (Strasser and Srivastava 1995, Strasser *et al.* 2000). Chl *a* fluorescence has been used to answer various ecological questions being related to *e.g.* ozone sensitivity (Clark *et al.* 2000), the onset of autumn senescence (Holland *et al.* 2014), water stress (Živčák *et al.* 2008), the determination of frost acclimatisation and effects (Neuner *et al.* 1999, Pflug and Brüggemann 2012) or the effect of temperature on plant vitality and heat stress tolerance under laboratory conditions (Maxwell and Johnson 2000, Neuner and Pramsöhler 2006, Sharma *et al.* 2015).

Maximum light-saturated photosynthetic rates, P_{Nsat} , are photosynthetic rates at saturating light conditions under current atmospheric CO_2 concentration (400 ppm) and optimal humidity (around 60%). Changes in maximum

photosynthetic rates are not reflecting the intrinsic photosynthetic capacity but rather the CO_2 availability at the sites of carboxylation, which depends on the stomatal characteristics and their response to environmental cues (Grassi *et al.* 2005). Thus, most studies rather use the maximum carboxylation rate, V_{cmax} , or the maximum rate of electron transport, J_{max} , as more appropriate parameters to characterize leaf photosynthesis. V_{cmax} is assessed *via* time intensive P_N-C_i curves, where photosynthetic rates, P_N , are measured under varying intercellular CO_2 concentrations, C_i (Farquhar *et al.* 1980, Grassi *et al.* 2005). However, following Wilson *et al.* (2000) and De Kauwe *et al.* (2016), V_{cmax} can also be calculated from the simpler P_{Nsat} measurements *via* the so called ‘one-point method’.

Another way to determine the plant performance, which is gaining more and more attention in ecological field studies, are indirect measurements *via* ecophysiological plant traits (Violle *et al.* 2007, de Bello *et al.* 2010, Pérez-Harguindeguy *et al.* 2013, Bucher *et al.* 2016, Römermann *et al.* 2016). Area-based leaf nitrogen content (N_{area}) is a good proxy for plant photosynthetic capacity, since most of the nitrogen is located in proteins of the Calvin cycle (mainly Rubisco) and thylakoids, which is proportional to Chl content (Evans 1989, Bond *et al.* 1999) and offers an insight to the chemical component of photosynthesis. We expect that besides the well-defined relationship between gas-exchange parameters and N_{area} (Evans 1989, Bond *et al.* 1999), at least PI_{abs} should be positively related to N_{area} since it is a measure of photosynthetic performance. Carbon isotope discrimination ($\Delta^{13}C$) is indicating the gradient between C_i and external CO_2 concentration (C_a) and is thus a proxy for intrinsic water-use efficiency since Rubisco is discriminating against ^{13}C and favours ^{12}C when CO_2 is sufficiently available. $\Delta^{13}C$ is high if C_i/C_a is high, which is the case if either the stomata are open or there is a low demand of CO_2 due to reduced photosynthetic rates (Farquhar *et al.* 1989, Pérez-Harguindeguy *et al.* 2013). Thus, we hypothesise that besides gas-exchange parameters, PI_{abs} should be positively related to N_{area} .

The main aim our case study was to test if Chl *a* fluorescence measurements are suitable in extensive measurements in ecological field studies and how much information on ecophysiological processes can be derived thereof to obtain a better ecological understanding of these different methods applied in the field. More precisely, we asked the following questions:

Are Chl *a* fluorescence parameters related to gas-exchange measurements under field conditions?

Are Chl *a* fluorescence and gas-exchange parameters related to indirect measurements of plant performance *via* ecophysiological traits?

Materials and methods

All measurements were conducted in the Northern Limestone Alps near Garmisch-Partenkirchen along two south facing elevational gradients (“Kramer” and “Kreuzeck”) ranging from 700 to 1,700 and from 800 to 1,700 m a.s.l., respectively. We chose to conduct our research along the elevational gradients since we could find plants adapted to a wide range of abiotic conditions at a relatively small spatial scale. Four herbaceous plant species (all following C₃ metabolism) occurring along a wide elevational range were selected for this analysis, namely *Aposeris foetida* (L.) Less, *Knautia dipsacifolia* Kreutzer, *Mercurialis perennis* L., and *Trifolium pratense* L. Samples were collected every 100-m increase in elevation at altogether 22 sites as far as individuals were abundant. Five mature individuals at full flowering (May for *A. foetida* and *M. perennis* and August for *K. dipsacifolia* and *T. pratense*) were selected per site and species, leading to an overall dataset of more than 100 measurements per species. Chl *a* fluorescence as well as gas-exchange measurements were recorded and ecophysiological traits of the plants were measured following standardized protocols (Pérez-Harguindeguy *et al.* 2013). Fluorescence and gas-exchange measurements were not performed between 12:00 h and 14:00 h in order to avoid midday depressions (Demmig-Adams *et al.* 1989, Lichtenthaler *et al.* 2005, Desotgiu *et al.* 2013).

Chl *a* fluorescence: For each individual, two leaves (one being the same as used for gas-exchange measurements) were selected to measure Chl *a* fluorescence transients on the adaxial side [since Chl *a* fluorescence varies with Chl concentration, which is higher in palisade parenchyma (Lichtenthaler *et al.* 1986)]. We used a plant efficiency analyser, a portable continuous excitation time resolved Chl fluorimeter [*Pocket PEA*, *Hansatech Inst.*, UK; Strasser and Srivastava (1995)]. For an overview on different Chl *a* fluorescence techniques and the parameters which can be derived thereof [see e.g. Maxwell and Johnson (2000), Kalaji *et al.* (2014) or Kalaji *et al.* (2017a)]. Prior to measurements, leaves were dark-adapted for 40 min using black leaf clips. Leaf clips were covered with other leaves to prevent an increase in minimum fluorescence, F_0 , through overheating. For *A. foetida*, *K. dipsacifolia*, and *M. perennis*, veins were avoided for measurements since they adulterate fluorescence measurements (Lichtenthaler *et al.* 2005, Lazár 2006, Giorio 2011). With continuous excitation fluorimeters, the transient light-induced O-J-I-P fluorescence was recorded at 10- μ s intervals and the maximum PSII quantum yield, F_v/F_m , and the Chl *a* fluorescence performance index (PI_{abs}) were calculated thereof (Strasser and Srivastava 1995, Clark *et al.* 2000). The maximum light intensity was 3,500 μ mol(photon) $mm^{-2} s^{-1}$ which was applied for 1 s with a light emitting diode at a 625 nm wavelength. F_0 is defined as the fluorescence after 50 μ s of light emission

(Sušila *et al.* 2004).

The maximum quantum yield of the PSII was calculated as:

$$F_v/F_m = \frac{(F_m - F_0)}{F_m} \quad (1)$$

F_v defines the variable fluorescence, which is the difference between the maximum fluorescence F_m and the minimum fluorescence F_0 of dark-adapted leaves. This fluorescence is mainly emitted by PSII (Kitajima and Butler 1975, Maxwell and Johnson 2000, Sušila *et al.* 2004). Theoretically, the maximum PSII quantum yield would be 1 (1 photon absorbed by PSII leads to a transport of 1 electron). However, most studies report values between 0.70 and 0.85 since energy is dissipated *via* heat and Chl *a* fluorescence (Kalaji *et al.* 2014).

PI_{abs} was calculated following the JIP-test which is based on Chl *a* fluorescence characteristics of the first 2 ms (see Eq. 2; Strasser and Srivastava 1995, Clark *et al.* 2000). It includes the density of fully active radiation centres, the efficiency of electron movement to the electron transport chain, and the probability that an absorbed photon will be trapped by the reaction centres and thus encompasses the functionality of PSI and PSII (Strasser *et al.* 2000). It is calculated *via* the simplified experimental formula:

$$PI_{abs} = \frac{RC}{ABS} \times \frac{F_v}{F_0} \times \frac{1 - V_j}{V_j} \quad (2)$$

Here, RC are the active PSII reaction centres, ABS is the light absorbed by antennae and V_j is the variable fluorescent rise at 2 ms (Strasser and Srivastava 1995, Clark *et al.* 2000). PI_{abs} reflects thus the efficiency of energy conservation of PSII from absorbed photons to reduce the intermediate electron carriers of the plastid electron transport chain.

P_{Nsat} measurements and V_{cmax} estimates: For each individual, net photosynthetic rate at saturating irradiance and at ambient atmospheric CO₂ concentrations (P_{Nsat}) was measured using the *Li-6400XT* (*LI-COR Bioscience*, Lincoln, Nebraska). Irradiance was kept constant at 2,000 μ mol(photon) $m^{-2} s^{-1}$ and CO₂ concentration at 400 ppm. In summer 2013, *K. dipsacifolia* and *T. pratense* were measured at 25°C block temperature. In spring 2014, *M. perennis* and *A. foetida* were measured at 17°C block temperature due to technical limitation, which only allows heating/cooling to a certain extent. Plants were adapted to cuvette conditions until constant photosynthetic rates were reached prior measurements.

We calculated V_{cmax} from P_{Nsat} measurements *via* the ‘one-point method’ since previous studies found that P_{Nsat} is limited by Rubisco carboxylation rather than by the regeneration of ribulose-1,5-bisphosphate (RuBP; Rogers and Humphries 2000, Wilson *et al.* 2000, De Kauwe *et al.*

2016). As we did not measure the leaf mitochondrial respiration in the light (R_{day}) using $P_{\text{N}}-C_i$ curves, we assumed that R_{day} was 1.5% of V_{cmax} even at differing leaf temperatures due to similar temperature dependencies of R_{day} and V_{cmax} (Collatz *et al.* 1991, De Kauwe *et al.* 2016). V_{cmax} was calculated following Collatz *et al.* (1991) as:

$$V_{\text{cmax}} = P_{\text{Nsat}} \times \left(\frac{C_i + K_m}{C_i - \Gamma^*} - 0.015 \right) \quad (3)$$

Here, C_i is the internal CO_2 concentration within the leaf, K_m is the Michaelis-Menten constant of Rubisco, and Γ^* [$\mu\text{mol mol}^{-1}$] is the CO_2 -compensation point in the absence of mitochondrial respiration. K_m was calculated following the parametrization made based on *in vivo* experiments on *Nicotiana tabacum* L. (Bernacchi *et al.* 2001) as:

$$K_m = K_c \left(1 + \frac{O_i}{K_o} \right) \quad (4)$$

Where K_c is the Michaelis-Menten constant for the carboxylation and calculated as:

$$K_c = 404.9 \exp\left(\frac{79,403 \times (T_k - 298.15)}{298.15 \times R \times T_k}\right) \quad (5)$$

Where T_k is the leaf temperature in Kelvin and R is the universal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$) and O_i is the internal concentration of oxygen which is assumed to be $210 \text{ mmol mol}^{-1}$ at sea level, and K_o is the Michaelis-Menten constant for the oxygenase function and calculated as:

$$K_o = 278.4 \exp\left(\frac{36,380 \times (T_k - 298.15)}{298.15 \times R \times T_k}\right) \quad (6)$$

The CO_2 -compensation point in the absence of mitochondrial respiration Γ^* was calculated as:

$$\Gamma^* = 42.75 \exp\left(\frac{37,830 \times (T_k - 298.15)}{298.15 \times R \times T_k}\right) \quad (7)$$

Ecophysiological traits related to plant performance: To measure N_{area} and $\Delta^{13}\text{C}$, the two leaves per individual used for Chl *a* fluorescence analysis and gas-exchange measurements were collected, dried, weighed, and ground using a ball mill after recording the leaf area. Mass-based leaf nitrogen concentration [mg g^{-1}] as well as $\delta^{13}\text{C}$ [‰] were measured simultaneously with an elemental analyser (*NA 1110*, Carlo Erba, Milan, Italy). Leaf nitrogen concentration per unit leaf area was calculated by dividing the mass based concentrations by the specific leaf area (SLA), the ratio of fresh leaf area to dry mass, which was measured prior to grounding. Carbon isotope discrimination ($\Delta^{13}\text{C}$) was measured and calculated from $\delta^{13}\text{C}$ of

the leaves and atmospheric $\delta^{13}\text{C}$ values ($\delta^{13}\text{C}_{\text{air}}$) of the station at Hohenpeißenberg at 35 km distance from our sample location (available at <http://www.esrl.noaa.gov/gmd/dv/data/>) following Farquhar *et al.* (1989). $\Delta^{13}\text{C}$ was used instead of $\delta^{13}\text{C}$, since $\delta^{13}\text{C}_{\text{air}}$ varied between -9.094‰ in February 2013 and -7.946‰ in August 2013. We used $\delta^{13}\text{C}_{\text{air}}$ measured one month before sampling collection assuming that this was the main growth period of the leaves as described in Bucher *et al.* (2016).

Statistical analysis: In a first analysis, we tested how species differed in parameter values (P_{Nsat} , V_{cmax} , F_v/F_m , PI_{abs} , N_{area} , and $\Delta^{13}\text{C}$) using an analysis of variance (ANOVA) followed by pairwise comparisons using *t*-test, with a *p*-value adjustment according to Holm. We then tested for relationships between Chl *a* fluorescence parameters (F_v/F_m , PI_{abs}) and carboxylation rates (P_{Nsat} , V_{cmax}) using linear mixed effect models. In these models, P_{Nsat} and V_{cmax} were the dependent variables. Each of the two Chl *a* fluorescence parameters was included in two separate models together with species as fixed effects allowing two-way interactions; elevation and gradient as well as their interaction were included as random effects to account for spatial autocorrelation.

To analyse each combination of the two Chl *a* fluorescence parameters vs. the two descriptors of carboxylation rates, in total four linear mixed effect models were fitted and simplified *via* backward selection of the least significant variables until the final minimal adequate model obtained a minimal Akaike information criterion (Crawley 2012). Model requirements and assumptions were fulfilled, as variances were homogeneous and residuals normally distributed.

To extract the relative influence of the fixed compared to the random effects, two R^2 values were calculated for each model, the R^2_{marginal} , which is the R^2 value associated with the fixed effects, and the $R^2_{\text{conditional}}$, which includes the fixed and the random effect (Nakagawa and Schielzeth 2013).

The same was done for all bivariate relationships of the parameters related to photosynthesis (P_{Nsat} , V_{cmax} , F_v/F_m , PI_{abs}) and N_{area} as well as $\Delta^{13}\text{C}$, respectively.

All analyses were conducted in *R version 3.2.3* (*R Development Core Team 2016*), for the analysis of the mixed effect models the packages ‘lme4’ (Bates *et al.* 2015), ‘lmerTest’ (Kuznetsova *et al.* 2015) and ‘MuMIn’ (Bartoń 2016) were used, for graphical display we used ‘ggplot2’ (Wickham 2009).

Results

Species-specific differences in parameter values: Gas exchange (Fig. 1A,B), Chl *a* fluorescence (Fig. 1C,D), and ecophysiological trait values (Fig. 1E,F) were highly species-specific. This was especially pronounced in P_{Nsat} ($F_{3,387} = 146.1$, $p < 0.001$) and V_{cmax} values ($F_{3,387} = 249.3$,

$p < 0.001$). All species were significantly different from each other ($M. perennis < A. foetida < K. dipsacifolia < T. pratense$), with the two species measured in the spring ($A. foetida$ and $M. perennis$) displaying lower values than the two species measured in the summer under higher

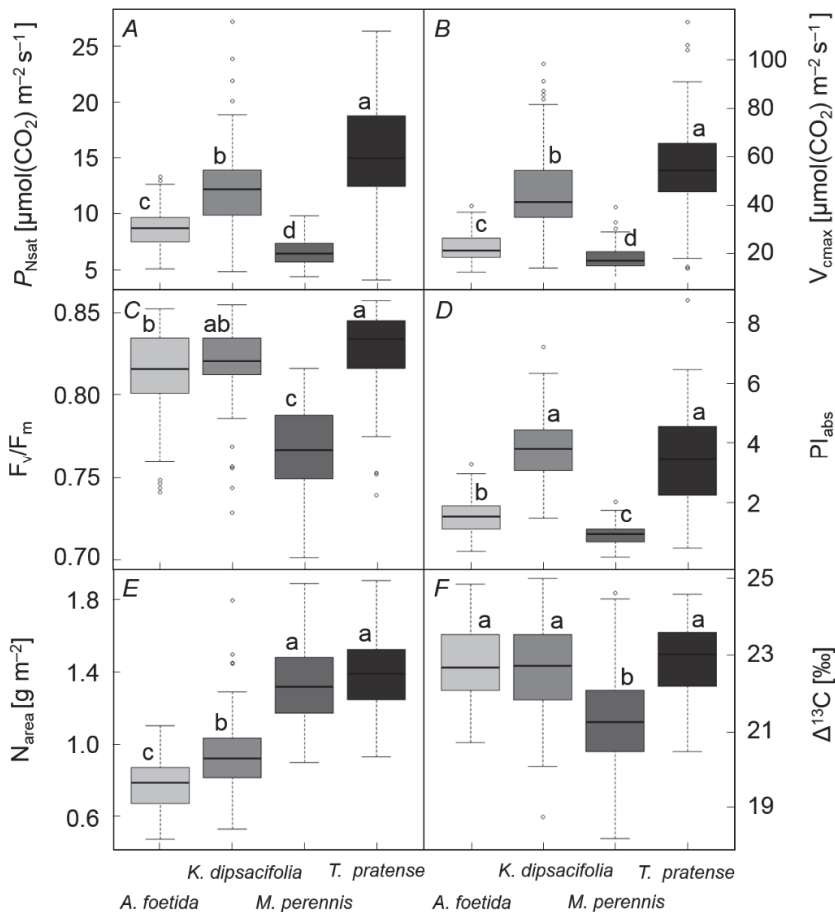


Fig. 1. Boxplots of the parameters displayed per species *A* – net photosynthetic rate at saturating irradiance and ambient atmospheric CO₂ concentrations (P_{Nsat}), *B* – maximum carboxylation rate (V_{cmax}), *C* – maximum quantum yield of PSII (F_v/F_m), *D* – absorption based performance index (PI_{abs}), *E* – area based leaf nitrogen content (N_{area}), and *F* – carbon isotope discrimination ($\Delta^{13}C$). Significant differences between species ($p < 0.001$) are indicated by letters.

temperature conditions (*K. dipsacifolia* and *T. pratense*). F_v/F_m was again species-specific ($F_{3,374} = 107.0$, $p < 0.001$), but these differences were not as pronounced (Fig. 1C), as *A. foetida* did not differ significantly from *K. dipsacifolia*. *M. perennis* displayed the lowest values. In PI_{abs} , there were again species-specific differences ($F_{3,374} = 186.2$, $p < 0.001$) with spring-flowering species displaying lower values than summer-flowering species, yet there was no difference between *K. dipsacifolia* and *T. pratense* (Fig. 1D). Species also differed in their ecophysiological trait values, yet the effect of seasonality was not so clear. Species differed in N_{area} ($F_{3,389} = 180.1$, $p < 0.001$) but there was no difference in N_{area} values between *M. perennis* and *T. pratense* (Fig. 1E). There were also species-specific differences in $\Delta^{13}C$ ($F_{3,390} = 43.0$, $p < 0.001$), yet *A. foetida*, *K. dipsacifolia* and *T. pratense* showed no differences in $\Delta^{13}C$ values, while *M. perennis* displayed lower values than the other species (Fig. 1F).

The relation of P_{Nsat} and V_{cmax} to F_v/F_m : We found no significant association between P_{Nsat} and F_v/F_m ; P_{Nsat} only differed between species ($R^2_{marginal} = 0.51$, $R^2_{conditional} = 0.60$;

Figs. 1A, 2A; Table 1S – *supplement available online*). V_{cmax} was significantly related to F_v/F_m ($R^2_{marginal} = 0.56$, $R^2_{conditional} = 0.68$; Figs. 2B; 1S – *supplement available online*) yet this relationship was highly species-specific. Unexpectedly, the direction of the relationship between F_v/F_m and V_{cmax} differed between species; *M. perennis* showed a positive relation, while *A. foetida*, *K. dipsacifolia*, and *T. pratense* displayed a negative relation (Fig. 2B).

The relation of P_{Nsat} and V_{cmax} to PI_{abs} : As shown in Fig. 2C, P_{Nsat} was positively related to PI_{abs} for all species ($R^2_{marginal} = 0.53$, $R^2_{conditional} = 0.65$; Table 1S). Species differed in their intercept, but not in the slopes.

V_{cmax} was significantly related to PI_{abs} ($R^2_{marginal} = 0.53$, $R^2_{conditional} = 0.69$; Table 1S) and again there was a species-specific difference in the intercept, but not in the slope of the reaction (Fig. 2D).

The relationship of photosynthesis measurements to N_{area} and $\Delta^{13}C$: N_{area} as proxy for Rubisco content was positively related to P_{Nsat} ($R^2_{marginal} = 0.63$, $R^2_{conditional} = 0.69$), V_{cmax} ($R^2_{marginal} = 0.65$, $R^2_{conditional} = 0.73$), and PI_{abs}

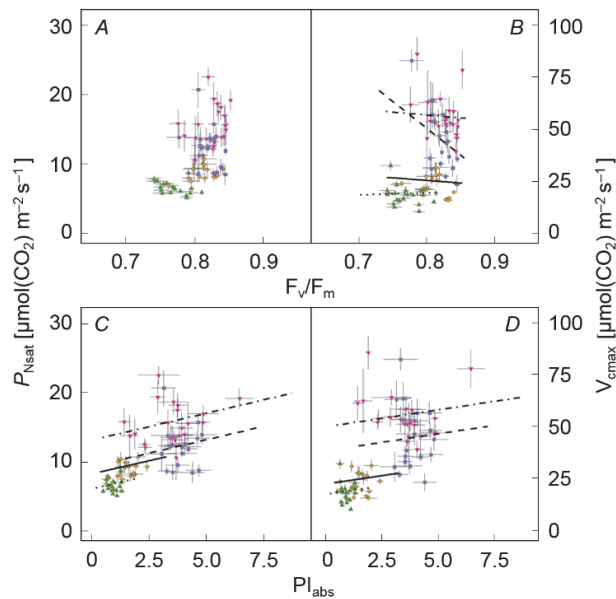


Fig. 2. Relationship between maximum quantum yield of PSII (F_v/F_m) and *A* – net photosynthetic rate at saturating irradiance and ambient atmospheric CO_2 concentrations (P_{Nsat}) and *B* – maximum carboxylation rate (V_{cmax}) as well as the relationship of absorption based performance index (PI_{abs}) and *C* – P_{Nsat} as well as *D* – V_{cmax} . Indicated are the mean values per species, gradient, and elevational band. *Yellow circles and solid lines* display *A. foetida*, *purple squares and dashed lines* *K. dipsacifolia*, *green upwards pointing triangles*, and *dotted lines* represent *M. perennis* and *magenta downwards pointing triangles and dotted-dashed lines* *T. pratense*. Error bars display the standard error of the measurements per elevational band and gradient.

($R^2_{\text{marginal}} = 0.64$, $R^2_{\text{conditional}} = 0.71$), yet it showed no significant relationship to F_v/F_m ($R^2_{\text{marginal}} = 0.46$, $R^2_{\text{conditional}} = 0.55$; Fig. 3A–D, Table 2S – *supplement available online*). For P_{Nsat} , there was a positive influence of N_{area} and a significant interaction between N_{area} and species, so the species differed in the strength of the relationship (Fig. 3A). For V_{cmax} , there was a positive effect of N_{area} and again, species differed in their slopes as well as their intercepts (Figs. 3B; 2S). N_{area} was significantly related to PI_{abs} , there was a significant species effect and a significant interaction between species and N_{area} (Fig. 3C). In general, the two early flowering species, *A. foetida* and *M. perennis*, showed shallower slopes for all relationships described here than the two late flowering species (*K. dipsacifolia* and *T. pratense*).

$\Delta^{13}\text{C}$ was negatively related to P_{Nsat} ($R^2_{\text{marginal}} = 0.59$, $R^2_{\text{conditional}} = 0.64$), V_{cmax} ($R^2_{\text{marginal}} = 0.61$, $R^2_{\text{conditional}} = 0.70$), and PI_{abs} ($R^2_{\text{marginal}} = 0.60$, $R^2_{\text{conditional}} = 0.66$) and there was a positive relationship between $\Delta^{13}\text{C}$ and F_v/F_m ($R^2_{\text{marginal}} = 0.49$, $R^2_{\text{conditional}} = 0.56$; Fig. 3E–H; 2S). The two early flowering species showed a shallower slope for their relationship between $\Delta^{13}\text{C}$ and P_{Nsat} as well as V_{cmax} whereas there was no species-specific slope (*i.e.* interaction with species) for the relationship between $\Delta^{13}\text{C}$ and PI_{abs} . There was a positive relationship between $\Delta^{13}\text{C}$ and

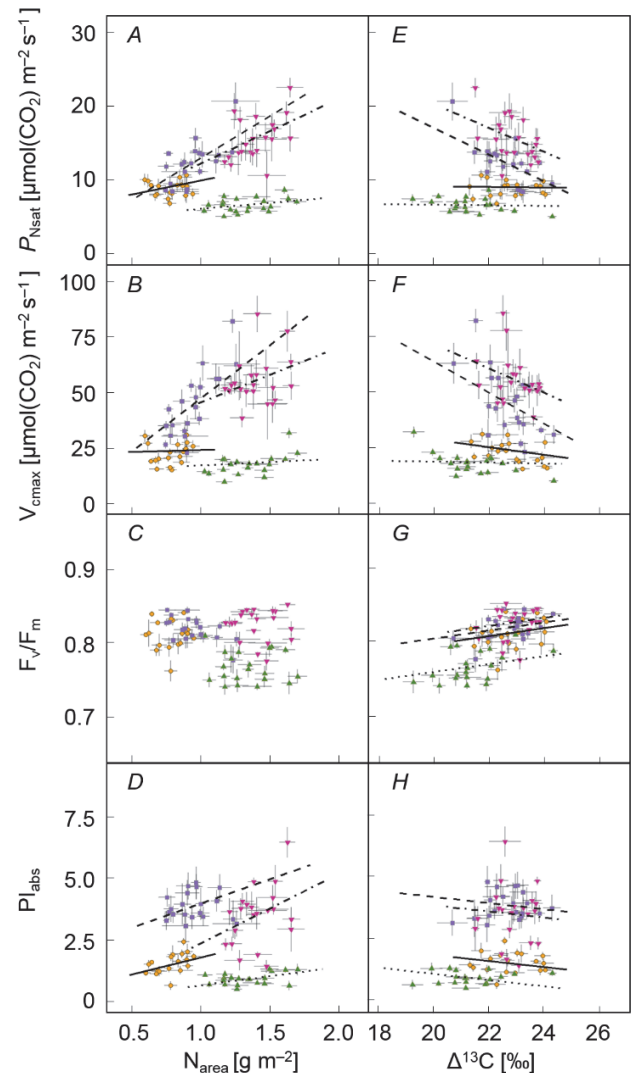


Fig. 3. Relationship between net photosynthetic rate at saturating irradiance and ambient atmospheric CO_2 concentrations (P_{Nsat}), maximum carboxylation rate (V_{cmax}), maximum quantum yield of PSII (F_v/F_m), and absorption based performance index (PI_{abs}) to area based leaf nitrogen content (N_{area} ; left panel, A–D) and carbon isotope discrimination ($\Delta^{13}\text{C}$; right panel, E–H). Indicated are the mean values per species, gradient, and elevational band. *Yellow circles and solid lines* display *A. foetida*, *purple squares and dashed lines* *K. dipsacifolia*, *green upwards pointing triangles and dotted lines* represent *M. perennis*, and *magenta downwards pointing triangles and dotted-dashed lines* *T. pratense*. Error bars display the standard error of the measurements per elevational band and gradient.

F_v/F_m . For P_{Nsat} , there was a negative influence of $\Delta^{13}\text{C}$ and a significant interaction between $\Delta^{13}\text{C}$ and species as well as a significant effect of species (Fig. 3E). For V_{cmax} there was a negative effect of $\Delta^{13}\text{C}$, a significant species effect and a highly significant interaction between $\Delta^{13}\text{C}$ and species (Fig. 3F). $\Delta^{13}\text{C}$ was negatively related to PI_{abs} with a significant species effect (Fig. 3G) and it was significantly positively related to F_v/F_m with a significant species effect (Fig. 3H).

Discussion

In our study based on field measurements, F_v/F_m was a rather poor predictor of assimilation and carboxylation rates, hence we cannot recommend F_v/F_m as a proxy for photosynthetic activity in field studies since F_v/F_m seems to capture different processes. We found a predominantly negative relationship between F_v/F_m and V_{cmax} and no relationship between F_v/F_m and P_{Nsat} contradictory to the laboratory measurements by Sharma *et al.* (2015). Per definition, F_v/F_m should fit well with the quantum yield at low to intermediate light conditions (Ögren and Sjöström 1990) as it measures the quantum yield of PSII. Especially in C_4 plants, with low photorespiration, the link between the quantum yield of PSII and the quantum yield of CO_2 fixation is very tight (Genty *et al.* 1989). But also in C_3 plants, such as the four species of our case study, these parameters should scale since Ögren and Sjöström (1990) reported a relationship between low F_v/F_m and low quantum yield of the photosynthesis in *Salix* species. The discrepancies in between PSII electron transport and CO_2 fixation observed may be caused by changes in relative rates of CO_2 fixations and processes, which are competing for electrons. Amongst these are photorespiration, when Rubisco is acting as oxygenase instead of carboxylase, electrons used in nitrogen metabolism, the Mehler reaction, where electrons originating from PSI reduce O_2 to a superoxide anion, which is disposed *via* H_2O_2 and transformed into water (photosynthetic water-water cycle), and the oxygen photoreduction within the thylakoids, which is important mainly to dissipate excess excitation energy (Maxwell and Johnson 2000, Heber 2002, Kalaji *et al.* 2016, 2017a). Photosynthesis was measured at different temperatures for early and late flowering species and no control of temperature Chl *a* fluorescence was possible due to technical limitations (which explains different levels of P_{Nsat} ; Fig. 1A), yet temperatures were kept constant within species, so relationships to other parameters were not affected. Differences between gas exchange and Chl *a* fluorescence measurements can also be explained with the fact that only the uppermost layers of plant tissue are engaged in Chl *a* fluorescence, so when measuring the adaxial side only, we merely probed the chloroplasts of the palisade parenchyma cells (Vogelmann and Evans 2002, Lichtenthaler *et al.* 2005). Differently, gas exchange engulfs both sides of the leaves. However, due to the anatomy and optical properties of dorsiventral leaves, the adaxial and abaxial side of the leaf differ substantially (Schreiber *et al.* 1977, Vogelmann 1993), thus measuring both leaf sides and calculating a mean proves to be difficult. The species-specific differences could hence arise from the fact that *M. perennis* is functionally hypostomatic, whereas the other species selected in this study are amphistomatic (Bucher *et al.* 2017). Hypostomatic species typically display lower photosynthetic capacities than amphistomatic species (Parkhurst 1994) which can be also seen in our study (Fig. 1A). The

difference in stomatal patterning and consequently leaf anatomy might also affect the quantum yield of PSII and thus the very low level of F_v/F_m in *M. perennis* and the difference in the direction of the relationship between F_v/F_m and V_{cmax} as compared to the other species (Fig. 1C). The relatively low F_v/F_m in *M. perennis* could be also due to the fact, that this species is more affected by low temperatures of the night before the measurements (Fig. 3S – *supplement available online*) which could be due to its erect growth form contrasting the rosette of *A. foetida* (Larcher 1994, Körner 2003).

The performance index PI_{abs} was positively related to P_{Nsat} and V_{cmax} , which indicates its utility as a proxy in large field studies. Even though absolute values in PI_{abs} differed between species, the relation was much less species-specific than the relation of F_v/F_m to P_{Nsat} or V_{cmax} since no species-specific slope in its relationship to P_{Nsat} and V_{cmax} could be observed. Our results confirmed the expected positive relationship between P_{Nsat} and V_{cmax} and PI_{abs} to N_{area} , which is a proxy for Rubisco content (Evans 1989), but not between F_v/F_m and N_{area} . It is interesting to see that these relationships were much weaker in the spring, thus in early flowering species, than in the late flowering species. This is especially the case for *M. perennis*, which is a more shade loving species (Ellenberg's indicator value for light of 2) than the other species [*A. foetida*: 4, *K. dipsacifolia*: 5, and *T. pratense*: 7 (Ellenberg 1974, Ellenberg *et al.* 1991)]. Shade leaves and shade species were previously shown to display lower photosynthetic rates in addition to many other differences in their leaf anatomy (Wild *et al.* 1975, Boardman 1977, Larcher 1994) and partition relatively more nitrogen into the thylakoids, although this is associated with lower photosynthetic capacity per unit nitrogen (Evans 1989). However, our analysis showed that *M. perennis* has similar N_{area} as *T. pratense*, which is a nitrogen fixator (see Fig. 1E). An experimental study using dilute solutions of Chl molecules indicated that fluorescence intensity is related to the quantum yield of photosynthesis and Chl concentration (Lakowicz 2006), whereas Dinç *et al.* (2012) showed that F_m as well as F_0 are not related to Chl content in leaves and only changes in antenna size did have an effect on F_m . The different strength in the relationship could be linked to the species habitat preferences, which in the case of *M. perennis* explains the lower photosynthetic rates (Boardman 1977). This is also reflected in lower PI_{abs} and also lower F_v/F_m and the weaker relationship to N_{area} .

We confirmed our hypothesis and found a negative relationship between $\Delta^{13}C$ and P_{Nsat} , V_{cmax} , and PI_{abs} . Since $\Delta^{13}C$ is lower in case of a strong CO_2 gradient resulting either from closed stomata or high CO_2 demand *via* higher photosynthetic rates (Farquhar *et al.* 1989), the fact that we observed the same pattern in PI_{abs} underlines its strong correlation with the parameters resulting from gas-exchange measurements. In addition to that, hypostomaty,

as in the case of *M. perennis*, leads to a longer diffusion path length between stomata and the sites of carboxylation, which was found by Parkhurst (1994), and it might strengthen this CO₂ gradient and thus lead to the low values in $\Delta^{13}\text{C}$ for this species (Fig. 1F). At the same time, F_v/F_m decreased with lower $\Delta^{13}\text{C}$ which might capture the “stress” component of this parameter. Stress could be induced *via* closed stomata which results in an increased photorespiration activity at the cost of assimilation and temperature stress due to the lack of evaporation which could then lead to a decrease in F_v/F_m (Medrano *et al.* 2002, Kalaji *et al.* 2017b). However, previous research found that F_v/F_m is insensitive towards stomatal changes and other effects occurring under moderate drought stress (Suresh *et al.* 2012, Kalaji *et al.* 2017b). We only found a positive relation of the minimum temperatures during the night before the measurements and F_v/F_m and not to actual leaf temperatures (Fig. 3S), but this field needs further elaboration.

Photosynthesis and electron transport rate is known to vary intraspecifically between seasons, with higher photosynthetic rates in the summer and lower efficiencies in the winter or during senescence and spring (Jenkins and Woolhouse 1981, Troeng and Linder 1982, Larcher 1994, Öquist and Huner 2003, Adams *et al.* 2004, Neuner and Pramsöhler 2006, Holland *et al.* 2014). Thus, a seasonal effect cannot be ruled out also given the fact that gas-

exchange measurements were conducted at different temperatures as outlined above. In our study, all parameters were highly species-specific (Fig. 1) and higher in the summer than in the spring. Nevertheless, the differences of species within the season should be more elaborated.

Our findings give important implications for terrestrial biosphere models (TBMs) since they rely, among others, on correct estimates of V_{cmax} (Zaehle *et al.* 2005) and the relationship between leaf nitrogen content and its scaling with both, photosynthetic rates and V_{cmax} (Kattge *et al.* 2009). Thus, Chl *a* fluorescence might be a fast way to estimate this parameter, when aiming to include aspects of intraspecific variability since we could demonstrate its relationship with traits such as N_{area} and $\Delta^{13}\text{C}$. Further studies need to test whether these correlations also hold on the level of species (interspecific scale). However, the fact that PI_{abs} is positively related to P_{Nsat} and V_{cmax} and also shows the same patterns, when relating it to indirect measurements of plant performance *via* plant traits, is an important finding for future measurements and supports our hypothesis that Chl *a* fluorescence techniques might serve as a proxy for the cost and labour intensive gas-exchange measurements. For field ecologists depending on extensive measurement campaigns, we recommend to use PI_{abs} for a characterisation of photosynthetic rates, but to be careful in relying on F_v/F_m .

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