Multiple drivers of seasonal change in PRI: Implications for photosynthesis

1. Leaf level

Anatoly A. Gitelson^{ε*}, John A. Gamon^{μβ}, Alexei Solovchenko^ϕ

Abstract

The goal of this study was to explore the relationships between the photochemical reflectance index (PRI) at the leaf level and pigment pools, focusing on the constitutive role of pigments in influencing PRI over seasonal or ontogenetic time frames. The purpose was to re-evaluate the role of PRI as an indicator of seasonally shifting pigment (chlorophyll, carotenoid and anthocyanin) contents, and hence photosynthetic activity, across a range of tree and crop species. We studied natural vegetation - three tree species (maple, chestnut and beech) and two managed irrigated and rainfed crop species (maize and soybean), contrasting in photosynthetic

pathway and leaf structure, and having wide variation of pigment content and composition. In anthocyanin-free leaves, PRI related to both total chlorophyll (Chl) and carotenoid (Car) contents, however, much closer relationships were found between PRI and Car to Chl ratio (Car/Chl). The sensitivity of PRI to Car/Chl varied widely in tree species with the degree of secondary carotenoids. In crop leaves where the Car vs. Chl relationship was very close, the slopes of PRI vs. Car/Chl relationships for maize and soybean were almost identical. PRI vs. Car/Chl relationships for leaves of different tree species formed a significant, uniform relationship with PRI. Two crops also formed a significant, uniform PRI vs. Car/Chl relationship with a slope half the value found for trees. In anthocyanin-containing leaves, PRI did not clearly relate to any pigment content because reflectance values at both PRI wavebands are affected by anthocyanin content. The findings of a strong link between leaf level PRI and Car/Chl over seasonal and ontogenetic time spans supports recent findings calling for a more careful evaluation of the relationship between PRI and either LUE or photosynthetic activity. In particular, studies that contrast short-term (e.g. diurnal) vs. long-term (e.g. seasonal) pigment, PRI, and photosynthetic responses in contrasting vegetation types are needed.

Key words: chlorophyll, carotenoids, anthocyanin, xanthophyll cycle.

1. Introduction

The photochemical reflectance index (PRI) was originally derived as an optical indicator of xanthophyll cycle activity and photosynthetic light-use efficiency (LUE) over diurnal time scales (Gamon et al., 1992, 1993, Peñuelas et al., 1995). More recently, a large body of research has demonstrated that PRI is also strongly influenced by pigment pool sizes, particularly in studies

spanning seasons (Stylinski et al., 2002, Filella et al., 2009), multiple species (Sims and Gamon, 2002, Garrity et al., 2011, Gamon and Berry, 2012) or canopy positions (Gamon et al., 2001, Gamon and Berry, 2012). These two facets of PRI that reflect the rapid xanthophyll cycle activity and the slower, seasonal or ontogenetic pigment pool size adjustments have been termed the “facultative” and “constitutive” responses, respectively (Gamon and Berry, 2012, Wong and Gamon, 2015A, 2015B). Both are of interest because they represent physiological adjustments of photosynthesis to prevailing environmental conditions, one over short (minutes or diurnal) time spans, and another over long (ontogenetic or seasonal) time spans. Both have been shown to correlate with light-use efficiency, but the mechanistic explanations of these correlations are likely to be different (Sims and Gamon, 2002, Solovchenko, 2010, Gamon, 2015). This dual nature of PRI along with different adjustments of the photosynthetic pigments occurring over disparate time scales has led to considerable confusion in the PRI literature. Most remote sensing studies of PRI have not directly tested mechanism at the level of pigment responses, assuming implicitly that the xanthophyll cycle operation is the primary driver of the changing PRI signal.

The issue of the underlying mechanism(s) driving PRI responses has a larger significance. While the majority of studies report positive PRI-LUE correlations for individual circumstances (Garbulsky et al., 2011), some studies suggest that the PRI-LUE relationship is not constant across all locations (Nichol et al., 2002, Goerner et al., 2011) and ontogenetic stages (Solovchenko et al., 2010), and it is likely that the different underlying mechanisms discussed above could be partly responsible. Understanding the mechanistic relationships between PRI, LUE, and photosynthetic activity across a range of time scales is clearly important for developing accurate, predictive photosynthesis models from remote sensing approaches involving pigment-based reflectance indices.

Much of the recent carotenoid (Car) research in higher plants has focused on the role of the Car involved in operation of xanthophyll cycle pigments (specifically, violaxanthin, antheraxanthin and zeaxanthin) in the regulation of light energy conversion and photoprotection under stress. This “downregulation” of photosynthesis is balanced against the need for light harvesting and photochemical utilization, served predominantly by lutein and, to a certain extent, β -carotene. Another photoprotective function is served by Car and other pigments that are not directly involved in the xanthophyll cycle or core photosynthetic light reactions but operate mainly via light screening (for reviews, see Solovchenko, 2010, 2013). All of these pigments can influence the vegetation reflectance spectra (and hence PRI). It is conceivable that the PRI response would reflect the diversity of functional roles of Car in plants, particularly in the context of different plant types having different pigment responses.

Recently, Garbulsky et al., (2011) and Gamon, (2015) have proposed that the PRI-LUE relationship should vary not only with time scale, but also with vegetation type. According to this view, particularly in annual or deciduous species where green canopy display drives the seasonal photosynthetic response, PRI should be driven primarily by pigment transformations associated with ontogeny and senescence as modulated by environmental conditions, and not the xanthophyll cycle *per se*. On the other hand, over short time scales in evergreens, where green canopy display changes little, and the chlorophyll (Chl) content is relatively stable, PRI is likely to more closely follow changes in photosynthetic activity mediated in part by rapid operation of xanthophyll cycle. However, across seasons, the PRI changes are more likely to reflect Car/Chl ratio shifts (Wong and Gamon, 2015A and 2015B). This predicted complementary behavior of functionally different vegetation types recently called the “complementarity hypothesis” (Gamon, 2015) offers a framework for evaluating PRI responses across different vegetation

types, but has not yet been fully tested. Since it suggests that the role of pigment pool sizes in driving PRI should vary with vegetation type, condition, and time scale in ecologically significant ways, it provides an ecological and evolutionary framework for testing our understanding of PRI responses across multiple ecosystems and biomes.

One way to examine these different perspectives is through the concept of pigment stoichiometry. In many cases, the ratios of Car/Chl pigment pools seem to be largely invariant suggesting accessory light harvesting and its regulation as a predominant functional role for the Car closely associated with the photosynthetic apparatus (Feret et al., 2011). On the other hand, stresses that limit photosynthesis often increase Car/Chl ratios (see Solovchenko, 2010 and references therein). Thus temporal changes in the stoichiometry of plant pigments can give an insight into their functional roles, and stoichiometric variation can be detectable with PRI (Wong and Gamon, 2015A and B). In the context of this study, “stress” refers to any condition that leads to an excess of radiation beyond what can be used for photosynthesis. Conditions leading to such stress can include temperature extremes, drought, or nutrient stress. A well-documented short-term response of leaves to stress is the conversion of violaxanthin to zeaxanthin affecting PRI. In the long term, leaves respond to stress by changing the pigment levels and ratios that we refer to as constitutive changes.

A further reason for examining the PRI-pigment relationship is that the LUE model (a primary modeling framework for relating PRI to photosynthetic activity) can be parameterized or defined in multiple ways, and this has implications for the interpretation of PRI. For example, some authors have defined the LUE based on incident radiation, and some on absorbed radiation, while others define it solely on the radiation absorbed by green (photosynthetic) canopy material (see references in Gitelson and Gamon, 2015). These different definitions can have significant

consequences for the conclusions that can be drawn regarding the function of PRI, a topic that has added to the confusion in the PRI literature. Changes in canopy structure and/or pigmentation occurring over a growing season, can lead to the conclusion that LUE, defined or measured via PRI, is changing seasonally, when actually these changes in *apparent LUE* are being driven by gradual changes in canopy structure and pigmentation associated with plant development, stress acclimation or senescence, among other factors. Functionally, these changes in PRI due to pigment pools and canopy structure are distinct from activity of the xanthophyll cycle that is associated with PSII LUE or non-photochemical quenching (NPQ), and they may or may not coincide with this activity. Without further information to constrain the problem, PRI by itself cannot resolve these underlying mechanisms.

The goal of this study was to quantify the relationships between leaf level PRI and pigment pools, focusing on the constitutive role of pigments in influencing PRI over seasonal or ontogenetic time frames. A growing body of evidence suggests a primary role for pigment pools in driving the seasonal PRI responses. However, most studies have been done in evergreens and very few (if any) papers have explored the link between pigment (chlorophyll, carotenoid and anthocyanin) contents and their composition in deciduous species and managed crops (see Garbulsly et al., 2011 for review). The purpose was to re-evaluate the role of PRI as an indicator of pigment (chlorophyll, carotenoid and anthocyanin) pools, and hence photosynthetic activity, across a range of tree and crop species.

2. Methods

Plant material. Juvenile, mature and senescent leaves of Norway maple (*Acer platanoides* L.) and horse chestnut (*Aesculus hippocastanum* L.) were collected in a park at Moscow State

University campus in the spring, summer and fall of 1992-2000 (Table 1). Second-flush beech (*Fagus sylvatica* L.) leaves grown on the University of Karlsruhe campus were taken in August and September 1996. Leaves were visually selected according to their difference in color. Leaves healthy and homogeneous in color without anthocyanin pigmentation or visible symptoms of damage were used in the experiments (Table 1, details are in Gitelson et al., 2003).

Anthocyanin-containing leaves of Norway maple were collected in the park at Moscow State University campus (1992-2000) in spring and fall. In maple, the red pigmentation determined by the presence of anthocyanins was especially expressed during cold seasons. The anthocyanin pigmentation was normally observed in sunlit leaves whereas shaded leaves lacked the anthocyanin pigmentation being green to yellow in color (details are in Gitelson et al., 2001). Growing conditions of tree species were typical for these species in the regions - Moscow, Russia (maple and chestnut), and Karlsruhe Germany (beech).

Maize (*Zea mays* L.) and soybean (*Glycine max* L.) leaves (Table 2) were collected in irrigated and rainfed sites at Mead Nebraska (USA) in 2003 through 2005 with varying weather conditions. Leaves were collected across the growing season in maize and in the middle of the reproductive stage in soybean when wide variability of pigment content occurred. To study temporal change in leaf pigment content, leaves were collected across the growing season at the rainfed maize site in 2003 with a period of drought lasted from the 190th through 220th day of year, DOY (Peng et al., 2011). For more detail see Gitelson et al., 2005; 2006).

Pigment analysis. The leaf pigment content was determined from the same leaf samples that were used for reflectance measurement. Leaf disks were cut from the leaves and extracted with 100% acetone or methanol by grinding the disks in a mortar and a pestle. The pigment homogenates were centrifuged for 3-5 min at 3000 g in glass tubes to obtain the fully transparent

supernatant which was immediately assayed spectrophotometrically. Specific absorption coefficients of Chl *a*, Chl *b* and total carotenoids reported by Lichtenthaler (1987) were used. The accepted average molecular weight of carotenoids was 540 g mol⁻¹.

Anthocyanin content was determined after extract acidification with concentrated HCl. Absorbance at 530 nm was corrected for pheophytin contribution: pheophytins *a* and *b* were obtained from corresponding chlorophylls (Fluka Chemie AG) and their absorption coefficients at 530 nm in acid methanol were found to be 8.17 and 6.35 mM⁻¹ cm⁻¹, respectively. Anthocyanin absorption coefficient of 30 mM⁻¹ cm⁻¹ at 530 nm (Strak Wray 1989) was used (details in Gitelson et al., 2001). Pigment content was expressed on leaf area basis in mg m⁻² (Tables 1 and 2).

Reflectance measurements. Adaxial reflectance (ρ) spectra of the leaves were taken in a spectral range between 400 and 800 nm with a spectral resolution of 2 nm with a Hitachi 150-20 spectrophotometer equipped with a 150 mm integrating sphere for reflectance measurements (maple and chestnut leaves), a Shimadzu 2101 PC spectrophotometer equipped for reflectance measurements with an integrating sphere (beech leaves). Maize and soybean leaves' reflectance spectra were measured using an Ocean Optics radiometer (US92000, Ocean Optics, Dunedin FL, USA) with an Ocean Optics LS-1 light source with a Mini Leaf Clip (UNI501, PP Systems, Amesbury MA, USA) with a 2.3-mm diameter bifurcated fiber-optic attached to both radiometer and light source. In each leaf at least five reflectance spectra were measured and median value of reflectance was used for analyses. A 99% reflectance standard (Spectralon, LabSphere, North Sutton NH, USA) was scanned before and after each leaf measurement. The reflectance was calculated as the ratio of upwelling leaf radiance to the upwelling radiance of the standard.

186 Table 1. Pigment content (in mg m^{-2}) and Car/Chl ratio in tree leaves studied; n is the number of
187 leaves in each data set.

	min	max	mean
Chestnut $n = 27$			
Chl	117.90	562.50	346.39
Car	30.85	83.20	55.30
Car/Chl	0.09	0.46	0.20
Beech $n = 37$			
Chl	144.45	675.00	431.62
Car	43.51	137.18	91.55
Car/Chl	0.15	0.44	0.24
Maple (<i>AnC-free</i>) $n = 61$			
Chl	3.00	570.00	189.16
Car	14.60	82.60	41.18
Car/Chl	0.09	0.50	0.16
Maple (<i>AnC-containing</i>) $n = 23$			
Chl	103.2	399.8	268.7
Car	29.81	101.9	55.67
Anth	10.73	298.34	130.1
Car/Chl	0.11	0.69	0.21

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189 Table 2. Pigment content (in mg m^{-2}) and ratio in the crop leaves studied; n is the number of
190 leaves in data set.

	min	max	mean
Maize $n = 20$			
Chl	2.80	775.21	229.21
Car	45.53	143.16	64.61
Car/Chl	0.18	18.84	0.24
Soybean $n = 20$			
Chl	105.33	859.05	424.01
Car	43.81	174.46	88.30
Car/Chl	0.19	0.45	0.21

3. Results and Discussion

3.1 Chlorophyll vs. carotenoid content relationship

Three main pigment groups, Chl, Car, and AnC, affect reflectance at the two wavelengths used for PRI. To quantify the effect of the pigments on PRI, we firstly analyzed PRI-Chl and PRI-Car relationships in AnC-free leaves. It is often difficult to discern the specific behavior of these relationships against the background of an inherently close correlation between Chl and Car content, because Chl and primary (photosynthetic) Car are bound in the pigment-protein complexes of the photosynthetic apparatus according to a conserved stoichiometry that is often species-specific. At the same time, absolute amounts of these pigments and their ratios can be adjusted within genetically programmed limits in response to environmental stimuli. By contrast, pigments that do not participate in assimilation of light energy (exemplified by secondary Car) are not so strictly controlled genetically and may vary over a much wider range e.g. during senescence and/or under stress (Green and Durnford, 1996; Young, 1993).

For the species studied, the Chl-Car relationships were very close ($p < 0.001$), with R^2 above 0.7 for leaves of the three tree species and above 0.94 for both crops (Fig. 1 for beech and maize). A similar close relationship ($R^2 = 0.86$) was found in seventeen independent datasets including a wide range of leaf spectral, chemical, and structural properties collected all over the world (Feret et al., 2011). This largely conserved stoichiometry has led to the perspective of an invariant Chl/Car ratio in many modeling studies (Jacquemoud and Baret, 1992).

Figure 1. Relationships between carotenoid and chlorophyll contents in beech and maize leaves.

PRI uses reflectance at 531 nm in the so-called green edge and at 570 nm in the green range of the spectrum. The green edge is a transition region where *in situ* absorption by Chl *a* and *b* and different Car drops sharply from a high value at 480 nm to an almost negligible amount above 531 nm (Gitelson et al., 2002; Feret et al., 2016). Reflectance in the green edge range is governed by Chl *a*, Chl *b*, Car and AnC absorption. In some studies, the reflectance at 570 nm was invariant with respect to Car content due to very small (if any) Car absorption (Lichtenthaler, 1987; Gitelson et al., 2002; Feret et al., 2008; 2011, 2016). Thus, it was expected that PRI in AnC-free leaves would be affected by both Car and Chl pigments, and we found significant PRI-Chl and PRI-Car relationships in all tree and crop leaves studied (Fig. 2 for beech and maize). PRI-Chl relationships were closer than PRI-Car (R^2 was 0.73 for Chl vs. 0.47 for Car in beech, and 0.62 vs. 0.53 in maize, respectively; $p < 0.001$). Multiple regression analysis of PRI vs. Car and Chl relationships showed that both pigments together were responsible for more than 74% of PRI variation in maize and 76% in beech.

Figure 2. Relationships between PRI and pigment contents in beech (A) and maize (B) leaves.

To illustrate how the deviation of points from Chl-PRI and Car-PRI relationships (Fig. 2) is affected by the close Chl-Car relationships (Fig. 1), we plotted residuals of the PRI-Chl relationship versus residuals of the PRI-Car relationship (Fig. 3). The relationships between residuals were very close for both tree and crop leaves and, as for the Chl-Car relationship (Fig. 1), R^2 was higher for crop than for tree leaves. Relationships for tree and crop leaves were very close to each other with R^2 above 0.79 for tree leaves and above 0.95 for crops.

Figure 3. Relationships between residuals of Chl vs. PRI and Car vs. PRI relationships for beech and maize leaves.

The reasoning for such close relationships may be the tightly related Chl and Car contents that often vary synchronously during ontogeny and senescence. Since both pigments affect reflectance at 530 nm, PRI-pigment relationships are quite scattered (Fig. 2), but the deviations from best-fit functions (i.e., the residuals) of Chl-PRI and Car-PRI relationships are inherently linked. In other words, if a certain Chl-PRI data point departs from a linear trend, the corresponding Car-PRI data point has a high probability of diverging from its corresponding linear trend by a certain value which is ‘hardcoded’ by the close Car-Chl relationship. The absolute deviation of these departures may be different, but their ratios show a similar strength as the Chl-Car relationship. Thus, the close relationship shown in Fig. 3 is an inevitable consequence of close Car-Chl relationship for these particular species and growing conditions

(Fig. 1). However, a close Car-Chl relationships might not necessarily hold for the wide range of leaf traits, species, or conditions existing in nature or under all cultivation conditions. As could be seen in Fig 1, the tree leaves studied did not show as tight a stoichiometry as crop leaves, suggesting that ontogenetic and environmental factors may be at play (Wright et al., 2004), suggesting that pigment ratios were responding in a different way to the tight stoichiometric ratios seen in crops, as further discussed below.

3.2 Departure from a strong stoichiometric relationship

Reasons for deviations from the main Car/Chl trend could be divided, based on time scale, into life-long and short-term responses, and many of these reflect a leaf's response to the amount of excess energy (Björkman and Demmig-Adams 1994, Gamon 2015). Over longer periods, acclimation to elevated solar irradiances have been known to increase the Car/Chl ratio due to decline in Chl content against the background of Car retention (Young, 1993). Shifts in the Car/Chl ratio over long time scales in response to environmental conditions (Wong and Gamon, 2015A), or during senescence-induced Car transformation (Merzlyak et al., 1999; Gamon et al., 2001), lead to gradual spectral changes that influence the PRI signal. Furthermore, differences in the amount of excess radiation can influence the composition of distinct Car having slightly different spectral properties (Britton, 1995) and thus affect the PRI-Car relationship. Thus, the change in the proportion between light-harvesting (e.g. lutein) and photoprotective Car (e.g. violaxanthin cycle xanthophylls) might also displace both the Car/Chl ratio and PRI.

Over long time spans, additional disturbing factors include the accumulation of so called “secondary carotenoids” and/or acclimatory rearrangements of the pigment apparatus. In contrast to “primary” or photosynthetic Car, which are tightly linked with photosynthetic apparatus

(PSA), being involved in light harvesting and photoprotection, secondary Car are structurally and functionally uncoupled from PSA (Young, 1993, Solovchenko, 2010). As a result, secondary Car do not obey the strict stoichiometry linking the primary Car and Chl contents, and this appears to be the case for the deciduous tree leaves. In contrast, very close Car-Chl relationships evident in crops (Fig. 1) indicate leaves with a negligible content of secondary Car. This situation is clearly illustrated by the temporal behavior of Chl content, Car/Chl ratio and PRI as a function of day of year, DOY (Fig. 4) in rainfed maize site during 2003 with period of drought around DOY 180-200 (Peng et al., 2013). The PRI closely followed the increase in Chl early in the growing season as well as the decrease of Chl in the reproductive stage beyond DOY 210. However, in a period of decrease in Chl content due to water deficiency (DOY 180-205) PRI did not follow Chl content. Due to the very close relationship between Chl and Car (R^2 above 0.97, $p < 0.0001$), the Car/Chl ratio in this period of stress increased very slightly. It underlines the limitation of using seasonal PRI changes as an indicator of plant stress in cases where there is a strict stoichiometry of Chl and Car.

Figure 4. Temporal change of PRI and Chl content (A) and PRI and Car/Chl ratio (B) in leaves collected at maize rainfed site in year 2003 with drought event between day of year (DOY) 190-220.

In contrast to crops, where the Car-Chl ratio is conserved, we observed a much weaker Car-Chl relationship in tree leaves (Fig. 1), most likely due to an increased accumulation of

secondary Car. The chloroplasts of beech leaf cells (Steinmüller and Tevini, 1985, Tevini and Steinmüller, 1985) as well as number of other plant species (Merzlyak et al., 2005, Han et al., 2003, Hormaetxe et al., 2005) often contain lipid inclusions serving as a depot for secondary Car.

Figure 5. Relationships between PRI and Car to Chl ratio in crop (A) and tree (B) leaves. Also shown are summary relationships comparing all crops and trees investigated (C) where PRI explained 73% of Car/Chl variation in crops and 50% in trees ($p < 0.05$).

Due to the close relationship between the residuals (Fig. 3), it is not surprising that PRI was found to be closely related to the Car/Chl ratio (Fig. 5). As a result of the strong stoichiometric relationship between Chl and Car for crops (Fig. 1), the range of PRI variation was narrow and the slope of the PRI vs. Car/Chl relationship was very small (Fig. 5A). By contrast, the PRI vs. Car/Chl relationship for tree leaves illustrates how the departure from this strong stoichiometric relationship affects the sensitivity of PRI to Car/Chl ratio (Fig. 5B). The PRI sensitivity to Car/Chl in tree leaves was higher than in crops, which emerges as a steeper slope of the PRI vs. Car/Chl relationship.

In two crops (soybean and maize) having different photosynthetic pathways (C_3 vs. C_4) and related leaf structure, the slopes of PRI vs. Car/Chl relationships were quite close to each other. In contrast, the slopes of PRI vs. Car/Chl relationships for three unrelated tree species were different depending on the closeness of Car vs. Chl relationships and the dynamic range of each pigment content (Fig. 5B). Despite these differences, a significant uniform the PRI vs. Car/Chl

relationship was formed for three tree species with PRI explaining more than 73% ($p < 0.05$) of Car/Chl variation (Fig. 5C). The same was the case for crop leaves also forming a uniform (but distinct from the trees leaves with two-fold smaller slope) significant PRI vs. Car/Chl relationship where PRI explained about 50% ($p < 0.05$) of Car/Chl variation (Fig. 5C).

Rahimzadeh-Bajgiran et al. (2012) evaluated PRI for assessing plant photosynthetic performance throughout the plant life cycle in eggplant. PRI was significantly correlated with plant pigments estimated via vegetation indices (VI) retrieved from leaf reflectance. They found that photosynthetic capacity and steady-state PRI could be better explained by Chl content than by xanthophylls, arguing that PRI reflects pigment content over long time spans. To test how the VIs used in their study relate to leaf pigment content, we calculated the VIs using measured reflectance and compared them to analytically determined pigment contents. The relationships were very close with $p < 0.0001$ (Fig. S1). Thus, our findings on the dominant role of pigment pools over long time spans closely correspond to Rahimzadeh-Bajgiran et al., (2012) results taken at different species.

Thus, in AnC-free tree and crop leaves PRI was related to both Chl and Car contents, but much more closely to the Car/Chl ratio. The sensitivity of PRI to Car/Chl depends strongly on the fidelity (i.e. stoichiometry) of the Chl/Car relationship. These processes might not all be immediately or equally linked with LUE and this may confound the interpretation of PRI in remote sensing studies that have often assumed a single mechanism of PRI response linked to the xanthophyll cycle. These findings illustrate the importance of assessing leaf-level pigment levels when evaluating PRI or other similar vegetation indices. Variation in PRI-LUE responses in remote sensing studies spanning dates or species could in fact reflect different functional roles of the pigments influencing PRI, leading to misinterpretation of the underlying function. Profound

changes in Car/Chl ratio and Car composition take place during ontogenesis during the maturation and senescence of the leaf photosynthetic apparatus (Merzlyak et al., 1998, Lichtenthaler and Babani, 2004). This trend of Car/Chl ratio is genetically programmed and further modulated by acclimation to long-term stresses (Solovchenko et al., 2006, Lichtenthaler and Babani, 2004). Similarly, Car/Chl ratios, and hence PRI values, vary with canopy position and irradiance (e.g., Hilker et al., 2008; Gamon and Berry, 2012). These processes reflect ontogenetic and acclimatory responses to prevailing conditions, and obviously exert a dominant contribution to the changes in PRI observed over seasonal timescales. What is less clear (and requires further study) is how closely PRI reflects underlying photosynthetic activity or LUE across this range of pigment responses, and to what degree the varying PRI-LUE relationships often reported in the literature (Nichol et al., 2002, Goerner et al., 2011) reflect these different mechanisms.

Behavior of PRI in *AnC-containing leaves* was very different from that in AnC-free leaves. Anthocyanins absorb *in situ* in a wide range around 550 nm (Gitelson et al., 2001, Peters and Noble, 2014; Feret et al., 2016) affecting reflectance at both wavelengths used in PRI. In AnC-containing leaves, reflectances at 530 and 570 nm are governed mainly by AnC absorption (i.e., AnC content). When AnC and Chl contents did not relate closely (as in our data), *there was no significant relationship between PRI and any pigment (Chl, Car and AnC) content as well as with Car/Chl* (Fig. S2). The disturbance of the close Car/Chl relationship upon appearance of AnC can be explained by a strong photoprotective effect of anthocyanins (Hatier and Gould, 2009, Gould, 2004). When AnC accumulated in appreciable amounts, it takes over the photoprotective function of Car resulting in a weakening of its tight relationships with PRI. The presence of anthocyanins led to a strong interference of AnC absorption with the PRI-Car/Chl

relationship, and indicates an additional leaf photoprotective strategy from the xanthophyll cycle that is available to some species (e.g. *Quercus* spp. Gamon and Surfus 1999). Additional studies are needed to further elucidate alternative leaf strategies for photoprotection over different time scales and their overall effects on leaf optical signals.

Conclusions

Analysis of *seasonal constitutive changes* in foliar pigment content and composition of three tree species and two crop species lead to the following conclusions.

- In anthocyanin-free leaves, PRI related to both total Chl and Car content. Each pigment (Chl and Car) content explained from 43 to 73% of PRI variation. Both pigments together were responsible for more than 74% of PRI variation.
- In the tree and crop leaves, PRI was closely related to the Car/Chl ratio over long time spans, most likely affecting physiological and ontogenetic factors.
- PRI vs. Car/Chl relationships for leaves of three different tree species formed a significant uniform relationship with PRI explaining more than 73% of Car/Chl variation.
- The crop leaves displayed a strict stoichiometric relationship between Chl and Car, and also formed a significant PRI vs. Car/Chl relationship (distinct from that observed in the trees) where PRI explained about 50% of Car/Chl variation.
- The sensitivity of PRI to Car/Chl in the tree leaves was at least 2-fold higher than in the crops. It was boosted by less close Chl vs. Car relationships in the tree leaves stemming likely from the increased contribution of secondary carotenoids.
- In anthocyanin-containing leaves, PRI did not clearly relate to any pigment content due to the effect of AnC absorption on reflectance at both PRI wavelengths. This indicates both a confounding effect of anthocyanins on reflectance and an alternative leaf photoprotective strategy.

These findings are consistent with other recent research in other vegetation types indicating that constitutive pigment effects, not the xanthophyll cycle, are the primary cause of PRI variation over long time spans, and illustrate the importance of assessing leaf pigments when evaluating vegetation indices. Finally, the strong link between leaf-level PRI and Car/Chl, as

well as PRI and separate Chl and Car contents suggest the need for a more careful evaluation of the relationship between PRI and LUE and photosynthetic activity. In particular, studies that clarify PRI, pigments, and photosynthetic responses over short-term (e.g. diurnal) vs. long-term (e.g. seasonal) time periods for contrasting vegetation types are needed.

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References

- Britton, G. (1995). UV/visible spectroscopy. In G. Britton, S. Liaaen-Jensen, & H. Pfander (Eds.), *Carotenoids* (pp. 13–62). Basel: Birkhauser Verlag.
- Bilger, W., Björkman, O., & Thayer, S. S. (1989) Light-induced spectral absorbance changes in relation to photosynthesis and the epoxidation state of xanthophyll cycle components in cotton leaves. *Plant Physiology*, 91, 542–551.
- Björkman, O., and Demmig-Adams, B. (1994) Regulation of photosynthetic light energy capture, conversion, and dissipation in leaves of higher plants, in: *Ecophysiology of Photosynthesis*, edited by: Schulze, E.-D., Caldwell, M. M., and Pospisilova, J., Springer, Berlin Heidelberg New York, pp. 27-47.

418 Ciganda, V., Gitelson, A.A. and Schepers, J. (2009). Non-destructive determination of maize
 419 leaf and canopy chlorophyll content. *Journal of Plant Physiology*, 166,157-167.

420 Féret, J.-B., Gitelson, A.A., Noble, S.D. and Jacquemoud, S. (2016). PROSPECT-D: modeling
 421 leaf optical properties through a complete lifecycle. *Submitted to Remote Sensing of*
 422 *Environment*.

423 Féret, J.-B., François, C., Gitelson, A.A., Barry, K.M., Panigada, C., Richardson, A.D., and
 424 Jacquemoud, S., (2011). Optimizing spectral indices and chemometric analysis of leaf
 425 chemical properties using radiative transfer modeling, *Remote Sensing of Environment*,
 426 115, 2742-2750.

427 Feret, J.-B., François, C., Asner, G. P., Gitelson A.A., Martin, R.E., Bidel, L.P.R., Ustin, S.L., le
 428 Maire, G., and S. Jacquemoud. (2008). PROSPECT-4 and 5: Advances in the Leaf
 429 Optical Properties Model Separating Photosynthetic Pigments, *Remote Sensing of*
 430 *Environment*, 112, 3030-3043, doi:10.1016/j.rse.2008.02.012

431 Filella, I., Porcar-Castell, A., Munne-Bosch, S., Back, J., Garbulsky, M. F., and Peñuelas, J.
 432 (2009). PRI assessment of long-term changes in carotenoids/chlorophyll ratio and short-
 433 term changes in de-epoxidation state of the xanthophyll cycle, *International Journal of*
 434 *Remote Sensing*, 30, 4443-4455, doi: 10.1080/01431160802575661,.

435 Hilker, T., Coops, N.C., Hall, F.G., Black, T.A., Wulder, M.A., Nesic, Z., & Krishnan, P.
 436 (2008). Separating physiologically and directionally induced changes in PRI using BRDF
 437 models. *Remote Sensing of Environment*, 112, 2777-2788.

438 Gamon, J.A. (2015). Optical sampling of the flux tower footprint. *Biogeosciences*, 12, 4509-
 439 4523. doi:10.5194/bg-12-4509-2015

- Gamon, J.A., Berry, J.A. (2012). Facultative and constitutive pigment effects on the Photochemical Reflectance Index (PRI) in sun and shade conifer needles. *Israel Journal of Plant Sciences*, 60, 85-95. DOI: 10.1560/IJPS.60.1-2.85
- Gamon, J.A., Filella, I., Peñuelas, J. (1993). The dynamic 531-nanometer Δ reflectance signal: a survey of twenty angiosperm species. Yamamoto HY , Smith CM (Eds). *Photosynthetic Responses to the Environment*. American Society of Plant Physiologists, Rockville. pp. 172-177.
- Gamon, J.A., Peñuelas, J., Field, C.B. (1992). A Narrow-Waveband Spectral Index that Tracks Diurnal Changes in Photosynthetic Efficiency. *Remote Sensing of Environment*, 41, 35-44.
- Gamon, J.A., Field, C.B., Fredeen, A.L., Thayer, S. (2001). Assessing photosynthetic downregulation in sunflower stands with an optically-based model. *Photosynthesis Research*, 67,113-125.
- Gamon, J.A., Serrano, L., and Surfus, J. (1997). The photochemical reflectance index: an optical indicator of photosynthetic radiation use efficiency across species, functional types, and nutrient levels. *Oecologia* 112(4), 492-501.
- Gamon, J.A., Surfus, J.S. (1999). Assessing leaf pigment content and activity with a reflectometer. *New Phytologist*, 143, 105-117.
- Garbulsky, M. F., Peñuelas, J., Gamon, J., Inoue, Y., and Filella, I. (2011). The photochemical reflectance index (PRI) and the remote sensing of leaf, canopy and ecosystem radiation use efficiencies: A review and meta-analysis, *Remote Sensing of Environment*, 115, 281-297, doi:10.1016/j.rse.2010.08.023, 2011.

Garrity, S. R., Eitel, J. U. H., and Vierling, L. A. (2011). Disentangling the relationships between plant pigments and the photochemical reflectance index reveals a new approach for remote estimation of carotenoid content, *Remote Sensing of Environment*, 115, 628-635, doi:10.1016/j.rse.2010.10.007.

Gitelson A.A., Gamon, J.A. (2015). The need for a common basis for defining light-use efficiency: implications for productivity estimation. *Remote Sensing of Environment* 156, 196-201.

Gitelson, A.A., and Merzlyak, M. N. (1997). Remote Estimation of Chlorophyll Content in higher Plant Leaves. *International Journal of Remote Sensing*, 18(12), 291-298.

Gitelson, A.A., Merzlyak, M.N., and Chivkunova, O.B. (2001). Optical properties and non-destructive estimation of anthocyanin content in plant leaves, *Photochemistry and Photobiology*, 74(1), 38-45.

Gitelson, A.A., Zur, Y., Chivkunova, O.B. and Merzlyak, M.N. (2002). Assessing Carotenoid Content in Plant Leaves with Reflectance Spectroscopy, *Photochemistry and Photobiology*, 75(3), 272-281.

Gitelson, A.A., Gritz, U. and Merzlyak M.N. (2003). Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves. *Journal of Plant Physiology*, 160(3), 271-282.

Gitelson, A.A., Viña, A., Rundquist, D.C., Ciganda, V., Arkebauer, T.J. (2005). Remote Estimation of Canopy Chlorophyll Content in Crops, *Geophysical Research Letters*, 32, L08403, doi:10.1029/2005GL022688

- Gitelson, A. A., Keydan, G. P. and Merzlyak, M. N. (2006). Three-band model for noninvasive estimation of chlorophyll, carotenoids, and anthocyanin contents in higher plant leaves, *Geophysical Research Letters*, 33, L11402, doi:10.1029/2006GL026457.
- Gitelson A.A., Gamon, J.A. and Solovchenko. (2016). Multiple drivers of seasonal change in PRI: Implications for photosynthesis. 2. Canopy level. Submitted to *Remote Sensing of Environment*.
- Goerner A, Reichstein M, Tomelleri E, Hanan N, Rambal S, Papale D, Dragoni D, Schimullius C. (2011). Remote sensing of ecosystem light use efficiency with MODIS-based PRI. *Biogeosciences*, 8, 189-202.
- Gould, K. (2004). Nature's Swiss army knife: the diverse protective roles of anthocyanins in leaves. *Journal of Biomedicine and Biotechnology*, 5, 314–320.
- Green, B. R., and D. G. Durnford. (1996). The chlorophyll-carotenoid proteins of oxygenic photosynthesis. *Annual review of plant biology* 47.1, 685-714.
- Han, Q., Shinohara, K., Kakubari, Y., & Mukai, Y. (2003). Photoprotective role of rhodoxanthin during cold acclimation in *Cryptomeria japonica*. *Plant, Cell and Environment*, 26, 715–723.
- Hatier, J.-H. B., & Gould K. S. (2009). Anthocyanin function in vegetative organs. In *Anthocyanins* (pp. 1–19). Heidelberg, New York: Springer Science+Business Media.
- Hormaetxe, K., Becerril, J., Fleck, I., Pinto, M., & Garcia-Plazaola, J. (2005) Functional role of red (retro)-carotenoids as passive light filters in the leaves of *Buxus sempervirens* L.: increased protection of photosynthetic tissues? *Journal of Experimental Botany*, 56, 2629–2636.

505 Horton, P. (2014). Developments in research on non-photochemical fluorescence quenching:
 506 emergence of key ideas, theories and experimental approaches. In B. Demmig-Adams, G.
 507 Garab, W. Adams III, and Govindjee (Eds.), *Non-photochemical quenching and energy*
 508 *dissipation in plants, algae and cyanobacteria* (pp. 73–95). Dordrecht: Springer
 509 Netherlands.

510 Jacquemoud, S., and Baret, F. (1990). PROSPECT: A model of leaf optical properties spectra.
 511 *Remote Sensing of Environment*, 34, 75–91.

512 Jahns, P., Holzwarth, A. R. (2012). The role of the xanthophyll cycle and of lutein in
 513 photoprotection of photosystem II. *Biochimica et Biophysica Acta—Bioenergetics*, 1817,
 514 182–193.

515 Nichol CJ, Lloyd J, Shibistova O, Arneth A, Röser C, Knohl A, Matsubara S, Grace J. (2002).
 516 Remote sensing of photosynthetic light-use efficiency of a Siberian boreal forest. *Tellus*
 517 54B, 677-687.

518 Lichtenthaler, H., & Babani, F. (2004). Light adaptation and senescence of the photosynthetic
 519 apparatus. Changes in pigment composition, chlorophyll fluorescence parameters and
 520 photosynthetic activity. In G. Papageorgiou, & G. Govindjee (Eds.), *Chlorophyll a*
 521 *fluorescence. A signature of photosynthesis* (pp. 713–736). Heidelberg, New York:
 522 Springer.

523 Maxwell, K., & Johnson, G. (2000). Chlorophyll fluorescence—a practical guide. *Journal of*
 524 *Experimental Botany*, 51, 659–668.

525 Merzlyak, M. N., Gitelson, A. A., Chivkunova, O. B., and Rakitin, V. Y. (1999). Non-
 526 destructive optical detection of pigment changes during leaf senescence and fruit
 527 ripening. *Physiologia Plantarum*, 106, 135–141.

528 Merzlyak, M. N., Solovchenko A. E., Smagin A. I., & Gitelson A. A. (2005). Apple flavonols
 529 during fruit adaptation to solar radiation: spectral features and technique for non-
 530 destructive assessment. *Journal of Plant Physiology*, 162, 151–160.

531 Merzlyak, M., Gitelson, A., Pogosyan, S., Lekhimena, L., & Chivkunova, O. (1998). Light-
 532 induced pigment degradation in leaves and ripening fruits studied in situ with reflectance
 533 spectroscopy. *Physiologia Plantarum*, 104, 661–667.

534 Merzlyak, M., Solovchenko, A., & Pogosyan, S. (2005). Optical properties of rhodoxanthin
 535 accumulated in *Aloe arborescens* Mill. leaves under high-light stress with special
 536 reference to its photoprotective function. *Photochemical and Photobiological Sciences*, 4,
 537 333–340.

538 Rahimzadeh-Bajgiran, P., Munehiro, M., and Omasa, K. (2012). Relationships between the
 539 photochemical reflectance index (PRI) and chlorophyll fluorescence parameters and plant
 540 pigment indices at different leaf growth stages. *Photosynthesis Research*, 113, 261–271.

541 Peñuelas, J., Filella, I., Gamon, J.A. (1995). Assessment of photosynthetic radiation-use
 542 efficiency with spectral reflectance, *New Phytologist* 131, 291-296.

543 Peters, R.D., and Noble, S.D. (2014). Spectrographic measurement of plant pigments from 300
 544 to 800nm. *Remote Sensing of Environment*, 148, 119-123. doi:10.1016/j.rse.2014.03.020

545 Sims DA, Gamon JA. (2002). Relationships between leaf pigment content and spectral
 546 reflectance across a wide range of species, leaf structures and developmental stages.
 547 *Remote Sensing of Environment* 81, 337-354.

548 Solovchenko, A. E. (2013). Physiology and adaptive significance of secondary carotenogenesis
 549 in green microalgae. *Russian Journal of Plant Physiology*, 60, 1–13.

550 Solovchenko, A. E., Merzlyak, M. N., & Pogosyan, S. I. (2010). Light-induced decrease of
 551 reflectance provides an insight in the photoprotective mechanisms of ripening apple fruit.
 552 *Plant Science*, 178, 281–288.

553 Solovchenko, A., Avertcheva, O., & Merzlyak, M. (2006). Elevated sunlight promotes ripening-
 554 associated pigment changes in apple fruit. *Postharvest Biology and Technology*, 40, 183–
 555 189.

556 Solovchenko, A. (2010). *Photoprotection in plants: optical screening-based mechanisms*.
 557 Heidelberg, New York: Springer.

558 Steinmüller, D., & Tevini, M. (1985). Composition and function of plastoglobuli. *Planta*, 163,
 559 201–207.

560 Strac, D., Wray, V. Anthocyanins. Harborne, J.B., Dey, P.M. editors. Methods in Plant
 561 Biochemistry, Vol. 1. New York: Academic Press; 1989. p. 326–352.

562 Stylinski, C.D., Gamon, J.A., and Oechel, W.C. (2002). Seasonal patterns of reflectance indices,
 563 carotenoid pigments and photosynthesis of evergreen chaparral species. *Oecologia*, 131,
 564 366-374.

565 Tevini, M., & Steinmüller, D. (1985). Composition and function of plastoglobuli. *Planta*, 163,
 566 91–96.

567 Wong, C.Y.S., Gamon, J.A. (2015A). Three causes of variation in the Photochemical
 568 Reflectance Index (PRI) in evergreen conifers. *New Phytologist*, 206, 187–195, doi:
 569 10.1111/nph.13159

570 Wong, C.Y.S., Gamon, J.A. (2015B). The Photochemical Reflectance Index (PRI) provides an
 571 optical indicator of spring photosynthetic activation in conifers. *New Phytologist*, 206,
 572 196–208, doi: 10.1111/nph.13251

Young, A. J. (1993). Factors that affect the carotenoid composition of higher plants and algae.
In: A. J. Young, & G. Britton (Eds.) *Carotenoids in photosynthesis* (pp. 160–205).

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Figure 1. Relationships between carotenoid and chlorophyll contents in beech and maize leaves.

Figure 2. Relationships between PRI and pigment contents in beech (A) and maize (B) leaves.

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Figure 4. Temporal change of PRI and Chl content (A) and PRI and Car/Chl ratio (B) in leaves
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Figure 5. Relationships between PRI and Car to Chl ratio in crop (A) and tree (B) leaves. Also
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explained 73% of Car/Chl variation in crops and 50% in trees ($p < 0.05$).