PHD THESIS – TESIS DOCTORAL

PASSIVE DIRECT MEASUREMENT OF SUN-INDUCED CHLOROPHYLL FLUORESCENCE SPECTRUM FROM *IN VIVO* LEAVES

MEDIDA DIRECTA PASIVA DEL ESPECTRO DE FLUORESCENCIA DE LA CLOROFILA INDUCIDA POR RADIACIÓN SOLAR EN HOJAS *IN VIVO*

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Doctorado en Teledetección

Enero 2022

Director:

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PHD THESIS – TESIS DOCTORAL

Programa de Doctorado en Teledetección

Passive Direct Measurement of Sun-Induced Chlorophyll Fluorescence Spectrum from *in vivo* Leaves

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Parque Científico

UNIVERSITAT DE VALÈNCIA – ESTUDI GENERAL

Valencia, enero 2022

Passive direct measurement of Sun-Induced Chlorophyll Fluorescence Spectrum from *in vivo* Leaves

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Tesis Doctoral: PASSIVE DIRECT MEASUREMENT OF SUN-INDUCED CHLOROPHYLL FLUORESCENCE SPECTRUM FROM *IN VIVO* LEAVES

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RESUMEN

La fluorescencia de la clorofila en la vegetación es una señal débil emitida entre los 650 y los 850 nm y que se mezcla con la luz reflejada por la hoja mucho más intensa, por lo que se suele recurrir a métodos activos (mediante la aportación adicional de luz artificial controlada) o a medidas indirectas. Por lo que la medida es solo relativa en el primer caso, o no hay manera de determinar la exactitud de la estimación sin medidas directas en el segundo.

La Tesis presenta un nuevo dispositivo, llamado FluoWat, de medida pasiva que permite obtener de forma directa la emisión de fluorescencia de hojas *in vivo* bajo condiciones naturales en campo con luz solar. Y se enmarca dentro de las actividades de apoyo a la preparación de la misión FLEX de la ESA para la monitorización global de fluorescencia vegetal.

El dispositivo consiste en una pequeña cámara oscura en forma de pinza, de forma que la hoja se pueda alojar en su interior sin dañarla, disponiendo de una abertura con la que apuntar al sol para iluminarla, y en dicha abertura se puede poner y quitar un filtro pasa-baja que bloquea la luz solar del mismo rango espectral que la fluorescencia pero que deja pasar la luz de excitación, de forma que un espectrorradiómetro conectado a la pinza mide el espectro de fluorescencia sin la interferencia de la luz solar.

Además, es posible medir los factores de reflectividad y transmisividad de la hoja, lo que permite determinar la absorbancia, necesaria para poder determinar la radiación fotosintéticamente activa (PAR) que ha sido absorbida (APAR). Un parámetro esencial para interpretar adecuadamente la señal de fluorescencia en relación a la fotosíntesis. De igual manera, los espectros de

reflectividad y transmisividad en el rango visible posibilitan determinar el grado de fotoprotección de la hoja y/o su contenido en clorofila.

Se ha realizado un análisis de sensibilidad de diferentes factores susceptibles de alterar la medida, como por ejemplo la luz residual dejada pasar por el filtro, o efecto de los transitorios en la emisión de fluorescencia, entre otros. Se han desarrollado métodos de procesado para mitigar sus efectos en la medida de la fluorescencia aumentando la precisión de los resultados.

Finalmente, se presentan una serie de experimentos en los que se pone a prueba el sistema y que ilustran como con las medidas proporcionadas por este nuevo dispositivo se puede obtener una mejor comprensión de la dinámica de emisión de la fluorescencia de la vegetación bajo diferentes estados de estrés y de adaptación a condiciones ambientales cambiantes.

SUMMARY

The fluorescence of chlorophyll in vegetation is a weak signal emitted between 650 and 850 nm that is mixed with the much more intense light reflected by the leaf, which is why active methods are commonly used (through the additional contribution of controlled artificial light) or using indirect measurements instead. So, the measurement is provided just in relative units in the first case, or the accuracy of the estimate in the second case is uncertain without proper direct validation.

The Thesis presents a new device, called FluoWat, for passive measurement that allows direct measurement of the fluorescence emission of leaves *in vivo* under natural conditions in the field with sunlight. And it is part of the activities supporting the preparation of ESA's FLEX mission for the global monitoring of vegetation fluorescence.

The device consists of a small dark chamber implemented as a clip, so that the leaf can be housed inside without damaging it, with an opening to illuminate the sample by pointing at the sun, and a sliding filter holder with a low-pass filter that blocks sunlight in the same spectral range as fluorescence is emitted while allowing the excitation light to pass through, then a spectroradiometer connected to the clip measures the fluorescence spectrum without interference from sunlight.

In addition, it is possible to measure the reflectance and transmittance factors of the leaf, which allows determining the absorptance, necessary to determine the photosynthetically active radiation (PAR) that has been absorbed (APAR). An essential parameter to properly interpret the fluorescence signal in relation to photosynthesis. Similarly, the reflectance and transmittance spectra in the

visible range make it possible to determine the degree of photoprotection of the leaf and/or its chlorophyll content.

A sensitivity analysis of different factors likely to affect the measurement has been carried out, such as the residual light that passes through the filter, or the effect of transients on fluorescence emission, among others. Processing methods have been developed to mitigate their effects on the fluorescence measurement, increasing the accuracy of the results.

Finally, a series of experiments are presented in which the system is put to the test and that illustrate how, with the measurements provided by this new device, a better understanding of the dynamics of fluorescence emission while the vegetation adapts to different illumination changes, levels of stress and changing environmental conditions.

Acknowledgments

This has been quite a long journey. A journey that more often than not seemed to have no end. But here it is. And this achievement is not just mine. Many people around me have been part of it in one way or another. But, without any doubt, I wouldn't be where I am now without the guidance, the counseling, and all the knowledge provided by my advisor José Moreno, Pepe. He has always good advice at hand, but also giving the freedom to take my own decisions. Many times, I have left the direct path to explore other possibilities, sometimes leading to new discoveries, others leading to wasting time. Regardless the outcome, Pepe has always been there quietly supporting me, and the group. To me Pepe is safe port I can always come back to. So, Pepe, thank you very much for walking this path with me, and for your patience with a Thesis that was continuously building up, but never seemed to consolidate. Here it is, finally!

Every journey starts with the first steps, and those I took together with Julia, Luis Chova and Joan, when we were naively measuring fluorescence using PVC pipes and CD covers. Feverishly experimenting every new idea on how to improve the measurements. How to link with the PAM. Excited with every new finding. We had so much fun then, and also later on with so many different projects (but those correspond to a different Thesis) being far more than colleagues. Anyway, I am happy and grateful that I could always count on you up to this day.

Those were also the days when I met two stimulating scientists, Ismaël Moya and Secundino del Valle. Much of what this Thesis is, has started from them. My gratitude, and a bittersweet feeling that I have long lost contact with them. Also, thanks, Secundino, for giving us in inheritance the old instruments that are still giving us nice results. And Alasdair MacArthur for promoting the clip among the

field spectrometry community and being a friend. I'd also like to mention Claus Buschmann, whom I briefly met, but who was also an inspiration.

Developing the FluoWat is something more than just implementing some ideas, it is necessary to implement those ideas into a practical design, one that does the work flawlessly, that is easy to operate, and something many people tend to forget, easy to manufacture designing the pieces to facilitate machining and looking for materials that are robust for field work. I am not good at any of that, so, a full load of gratitude to my cousin Ignaciet, that has proven a great deal of engineermanship going through every version of the clip, making into reality the ideas and needs that I came up with. Definitely, the FluoWat would not exist as it is now without him. Thank you, Ignaciet, from my heart.

But what is an instrument worth if it is not put in good use? And that piece falls onto the shoulders of MaPi and Shari, whom have done incredible science with it. Whom have put it to the test, while I've seen them grow from newbie master students to tall standing scientists. It has been, and still is, an honour working with you all this time. Mapi, that left my teachings of "barquitos" to roam around the world, to find her own way. But always with a FluoWat with her (so proud!), and always available for whatever I came with despite of the distance. Someone to count with and to rely on. I am so happy that your way sent you back. And Shari, who was, so to say, in charge of the BIOHYPE project when I met her. Already taking the lead, and already with a head boiling with ideas, both experimental and theoretical, which is a rare quality. The discussions with you are always exciting, and you both know that half of what is inside this manuscript is yours (upps! maybe I shouldn't be writing this down here). And most of all, you two are friend inside and outside of the lab (and I don't mean doing campaigns).

And it is so satisfying to see how you guide Adrian in a similar way I did with you. Adrian, who is the embodiment of cheerfulness. And a great apprentice of everything instrumental.

But one of my greatest frustrations with the FluoWat is not having been able to properly commercialize it, opening it up to a larger community that would exploit all its capabilities for research and applications. And sure, have I tried. To this respect, Javier Calpe has always been there giving me ideas and options, and long discussions about new technologies and their possibilities. He has also been there to hear me complain about how difficult it is to start up in this country. He is a real friend.

Anyway, my work all these years has not been just the clip (otherwise this Thesis would have been finished much earlier, or maybe not…), but also focused on FLEX and on how to measure fluorescence. And here Neus has a very special place for me. Long discussions about absorption bands, aerosols, and how fluorescence stubbornly hides itself in the spectra like a ghost. Excitement! Even though you are so far north, and that we don't talk that often lately, it is always a pleasure when it happens. And to me, when distance and time are of no concern, it means friendship.

And, of course, there is the rest of the group, Jesús, Antonio, Jochem, Caro, Patricia, Xavi, Santi, Eatidal, Helena… and many more compared to when the group started. It is a pleasure and an honour to work with you all every day, with highs and lows, through thin and thick, still it feels more like a family than a team. To all of you, I thank you for being around, and have the patience with me when I get all excited discussing science, and still being there afterwards.

Ah! Antonio, I know you are going through the same ordeal as me, only at a faster pace (but not by much, so don't relax), you got my sympathy and my

support. Moreover, with all the managing of FLEX, which sometimes seems more like juggling (or I should say oftentimes) but no matter what, you still have a nice smile for us; I really admire your managing capability. Even though, I know your scientist flame is still burning deep inside, so, let's see if we could retake at some point in the future the water FluoWat idea!

Pero una Tesis no trata sólo de trabajo. Sin el apoyo y el aliento de mi familia no habría encontrado la fuerza para sentarme y pasar todas esas largas horas y días escribiendo, desenredando la maraña de ideas en mi cabeza para convertirla en una historia. Algo que siempre ha sido muy complicado para mí. Algo para lo que necesito tiempo y paz. Aurora, Javi y David, habéis hecho un gran esfuerzo por equilibrar las necesidades familiares con la tranquilidad que yo necesitaba. Javi, David, sé que para vosotros ha sido difícil reprimir las ganas de jugar conmigo, de hacer legos, de hacer senderismo, de hacer cosquillas y de hacer cualquier cosa divertida. He sido un padre tan aburrido estos últimos meses... y habéis demostrado ser tan maduros. ¡Lo compensaré! Aurora, me has estado apoyando tanto y siendo tan paciente conmigo, cada vez que retrasaba una vez más la conclusión del manuscrito. Dándome el cariño y la sensatez que tanto necesito. ¡Sois mi familia, mi vida, mi amor! me quedo sin palabras!!

Y por supuesto, a mi madre, Inma, llena de cariño, siempre dispuesta a ayudar (a mi y todos los demás), siempre alentándome a seguir o a parar según necesitaba. Eres mi red de seguridad. A mi padre, Alfonso, por tu cariño a tu manera, por sentirte orgulloso. Sin vosotros no sería. Y a mis hermanos José Angel y Jorge, los mejores que se pueden tener. Ya sé que no somos de vernos todas las semanas, ni de hablar a menudo. Pero para mí siempre estáis conmigo. Y gracias también a mi tía Dulce, siempre pendiente de cómo me va.

A mis amigos, Kiko, Jorge, Bea, Laura y Ascen (aplíquese aquí un algoritmo que randomice las permutaciones). Amigos de primer día de facultad. Y Tristán, Arantza y Michael, como quien dice, del "segundo" día. Amigos para toda la vida. Y a todos los demás que dejo sin mencionar pero que están en mi corazón.

Acronyms and Abbreviations

A	Absorptance
APAR	Absorbed Photosynthetically Active Radiation
BRF	Bidirectional Reflectance Factor
CO_2	Carbon dioxide molecule
EEM	Excitation-Emission Matrix
ESA	European Space Agency
F	Fluorescence
$F_{[wvl]}$	Fluorescence at [wvl] nm
F^{ϕ}	Fluorescence Measured Using a Filter
fAPAR	Fraction of Absorbed Photosynthetically Active Radiation
FLD	Fraunhofer Line Depth principle
FLEX	Fluorescence Explorer
FOV	Field of View
FRF	Far-red Fluorescence
F_s	Steady-state Fluorescence
FVC	Fractional Vegetation Cover
FWHM	Full-Width Half-Maximum
H2O	Water molecule
I	Incoming Radiance onto a Surface
I^{ϕ}	Incoming Radiance onto a Surface Measured Using a Filter
IT	Integration Time
L	Radiance Leaving from a Surface
L^{ϕ}	Radiance Leaving from a Surface Measured Using a Filter
NAOC	Normalized Area Over the Curve
ND	Neutral Density (i.e., grey filter)
NIR	Near-Infrared
NPQ	Non-Photochemical Quenching
O_2	Oxygen molecule
O_2-A/B	Absorption Bands A or B from Atmospheric Oxygen Molecule
OD	Optical Density (of a filter)
PAM	Pulsed Amplitude Modulation
PAR	Photosynthetically Active Radiation
PRI	Photochemical Reflectance Index
PSI	Photosystem I
PSII	Photosystem II
QY	Quantum Yield
R	Reflectance
	Apparent Reflectance (including fluorescence)
RAE	Relative Absolute Error
RF	Red Fluorescence
SIF	Sun-Induced chlorophyll Fluorescence
T	Transmittance
$\hat{T}$	Apparent Transmittance (including fluorescence)
UCM	User Consultation Meeting
VIS	Visible
VNIR	Visible and Near-Infrared
WR	White Reference
YF	Yield of fluorescence (apparent)
Φ[wvl]	Low-Pass Filter with Cut-Off wavelength at [wvl] nm
τ	Filter Transmittance

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1 INTRODUCTION

Remote sensing of chlorophyll fluorescence from vegetation is commonplace now in 2022 at canopy, ecosystem, and global levels with diverse degrees of performance. But this was not the case almost two decades ago, when fluorescence studies were dominated by active measuring systems, and only a few highly technologically demanding instruments were capable of measuring the fluorescence of vegetation using just the sunlight. Also, at the time, a portion of the scientific community was sceptical about the possibility of extracting information related to the photosynthetic mechanisms from just steady state fluorescence.

1.1 CHLOROPHYLL FLUORESCENCE

Fluorescence is the re-emission of photons that have been previously absorbed by atoms or molecules. The re-emitted photon is of a lower or similar energy than the absorbed one, i.e., longer or similar wavelengths (although there is a very small provability of emission at shorter wavelengths). This phenomenon was first recognized by Sir G.G. Stokes (1852), although it had been observed before. He was also responsible of naming it as fluorescence.

An absorbed photon can excite an electron of the molecule from its ground state to an excited stated, from which there are different pathways for the de-excitation of the electron (Figure 1-1).

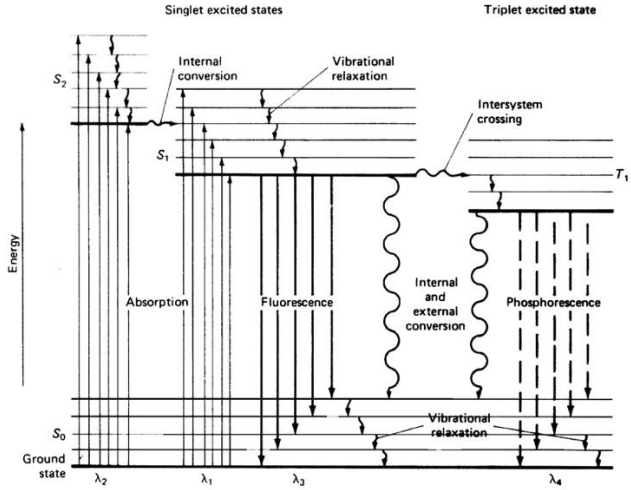

Figure 1-1 Jablonski diagram showing the different pathways of de-excitation of an electron after excitation by photon absorption.

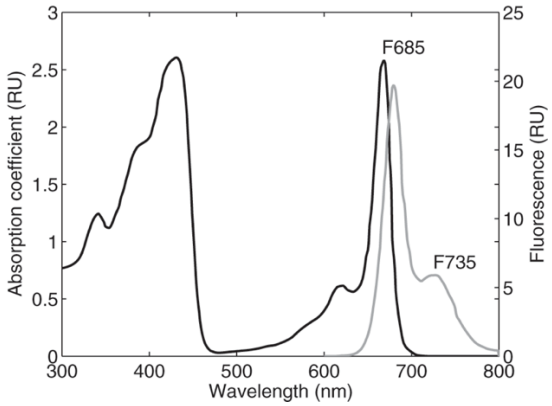

Figure 1-2 Light absorption of chlorophyll-a (black) and the fluorescence emission (grey) at chloroplast level

Chlorophyll fluorescence is produced by absorbed light in the visible spectrum, corresponding to the chlorophyll absorption spectrum from the blue (below 400nm) to the far-red (slightly above 700 nm) (Figure 1-2). The light in this spectral range excites the photosynthetic molecules and it is known as Photosynthetically Active Radiation (PAR) (McCree 1981).

UV light also excites blue-green fluorescence (BGF) in green leaves; however, it has been found that isolated intact chloroplasts and thylakoid membranes exhibit very low levels of this BGF emission (Duysens 1957, Latouche 2000). Instead, most of BGF is produced by phenolic plant substances located in the cell wall and/or vacuoles of leaves (Stober et al. 1994), so it cannot be considered to relate to photosynthesis (Lang et al. 1992) (Buschmann & Lichtenthaler 1998).

Emission of chlorophyll fluorescent light by leaf material represents one way of the de-excitation of photosystems after absorption of a photon, where chlorophyll pigments exhibit red fluorescence emission, with two maxima at 690nm in the red and 740nm in the far-red.

Light absorption can also initiate photosynthesis before photon reemission. Chlorophyll a is one of the main molecules responsible of the absorption of the light energy, that is needed for synthesizing carbohydrates from CO_2 and water. The chlorophyll is located within the photosystems at the thylakoid membranes of the chloroplast. A photosystem consists of a light-harvesting complex and a reaction centre. There are two types of photosystems: photosystem I (PSI) and photosystem II (PSII).

Each photosystem has light-harvesting complexes that contain proteins, 300-400 chlorophylls (a and b), and carotenoids. Most of the pigments in a photosystem act as an energy funnel, passing energy inward to a main reaction centre through a non-radiative mechanism called resonance energy transfer.

The reaction centre of a photosystem contains a unique pair of chlorophyll a molecules, often called special pair that receives the absorbed energy.

Photosystem I and II have different absorption and fluorescence emission properties. PSII absorbs and emits at higher energy than PSI. Thus, PSII and PSI

fluorescence emissions have each particular spectral shapes, and the fluorescence measured at leaf level is actually the combination of both.

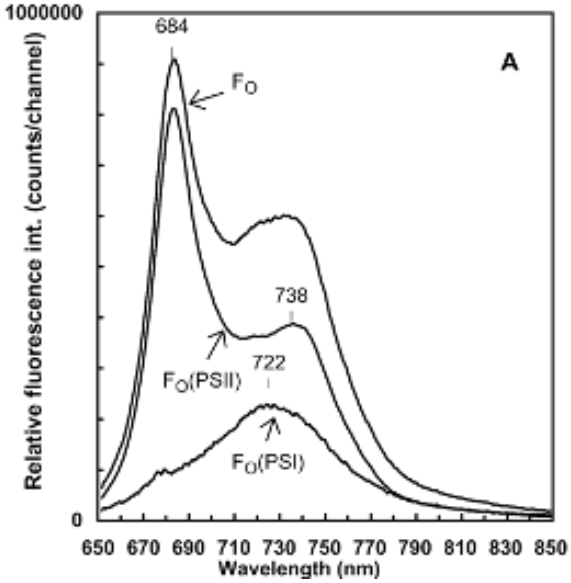

Figure 1-3 Individual fluorescence spectrum from PS-II and PS-I, and the combined emission. From Frank et al. 2002.

The first researchers to relate the variation of fluorescence emission to CO_2 assimilation were Kautsky and Hirsch in 1931 in a short communication (Kautsky et al. 1931). Not too long after this discovery several studies further detailed the relationship between fluorescence and photosynthesis (Franck, French, and Puck 1941).

Some good historical reviews of chlorophyll fluorescence and its basics are those of Moya and Cerovic 2004, and Papageorgiou and Govindjee 2004.

Extensive experimental and theoretical studies demonstrate that chlorophyll fluorescence is a proxy to actual photosynthesis (Rosema et al. 1998 and Baker 2008) and, as such, directly related to light use efficiency and CO_2 uptake (Seaton and Walker 1990). Since fluorescence emission competes with

adaptation/protection mechanisms set-up by the plant (Demming-Adams et al. 2016 and 2012), it also behaves as an indicator of plant vitality and plant stress (Cerovic et al. 1996), (Flexas et al. 2000) susceptible to be monitored from space (Porcar-Castell et al. 2014).

1.2 MEASURING FLUORESCENCE UNDER NATURAL CONDITIONS

So, how can fluorescence be measured under natural conditions? Excitation of fluorescence by an artificial light source has been used for decades at leaf or plant scale to study photosynthetic activity in the laboratory and in the field. A good account of these developments is presented in (Kalaji et al. 2012).

But the fluorescence that a leaf emits is very small compared to the radiance that it reflects in the same spectral range, and trying to separate it from the reflected solar radiance is not an easy feat even at close range, even more so from remote sensing.

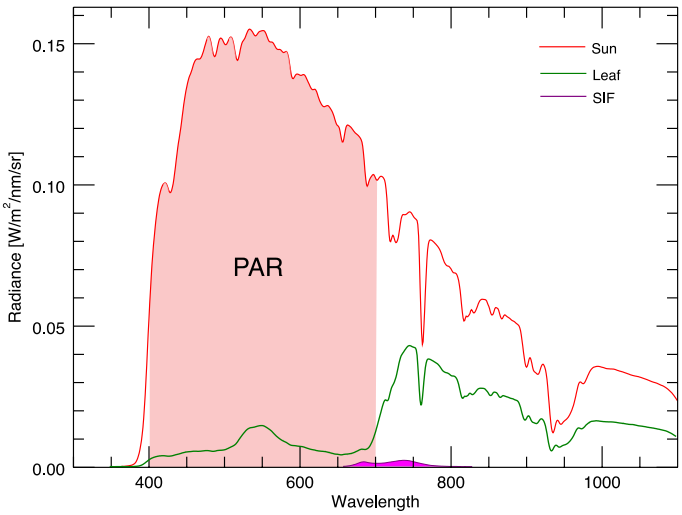

Figure 1-4 Comparison of radiances arriving from the sunlight (red), reflected by the leaf (green) and emitted as fluorescence (purple). Note the overlap between photosynthetically active radiation (PAR) and sun-induced fluorescence (SIF).

Remote sensing of fluorescence first started over water bodies containing phytoplankton (Stoertz, Hemphill, and Markle 1969), since the reflected light by the water and by the luminescent material in the red and near infra-red is rather small in comparison to the emitted fluorescent light making the later easier to detect.

Since then, there have been efforts to detect higher-plant fluorescence under natural conditions from a distance by using two techniques: active (laser induced) and passive (sun induced).

Active methods for fluorescence measurement are based on the fact that the fluorescence emission is, in first order, proportional to the amount of light that reaches the plant. Thus, if a second artificial and controlled light (measurement light) is applied in addition to the natural light received by the sample, the increase in fluorescence emission will be proportional to the total fluorescence emitted. In case that the (pulsed) measurement light is small enough it is assumed that it will not alter the adaptation conditions of the plant.

The first systems were based on laser stimulation or LIF (Laser Induced Fluorescence) and were originally used to detect algae in waters (Friedman and Hickman 1972), and not long after they were used on plants (Brach, Molnar, and Jasmin 1977).

If the measurement light is emitted in the form of modulated pulses (at short on and off intervals of the order of microseconds) and a sensor recording synchronously, it is possible to monitor the evolution of the fluorescence emission. This technique is called PAM (Pulse Amplitude Modulation) and was introduced in the mid-1980s (Schreiber, Schliwa, and Bilger 1986). Furthermore, by applying high intensity light pulses that saturate the photosystems and following a series of protocols, it is possible to determine the partitioning of the absorbed energy between the different pathways: photochemical, regulated heat dissipation and unregulated heat dissipation.

On the other hand, passive measurements are based on the fact that the light spectrum from the Sun has certain wavelengths within the spectral range of chlorophyll fluorescence emission, in which elements of the solar atmosphere

absorb a large number of photons; therefore, in these bands the radiance reaching the Earth is very low compared to the rest of the spectrum. These absorptions are known as Fraunhofer lines, and in particular the H_α absorption at 656 nm was the first to be used due to its width and depth compared to other narrower and weaker ones (Sioris, Courreges-Lacoste, and Stoll 2003; Moya and Cerovic 2004). The technological requirements of this method are very demanding, given the sub-nanometric width of these absorptions, and the very low signal level, to which the disturbances that the Earth's atmosphere introduces must be added. Besides, the Earth's atmosphere has two strong absorptions at 687 nm (O_2-B) and 761 nm (O_2-A) that coincide with red fluorescence (RF) and far-red fluorescence (FRF) emissions, respectively. A sensor measuring precisely in any of these absorption bands will receive a larger proportion of fluorescence than reflected radiance, in contrast with the greater reflected signal outside of the absorption bands.

For the latter case, Plascyk developed a system based on the use of solar Fraunhofer lines to detect SIF, the MK-II Fraunhofer Line Discriminator (Plascyk 1975; Plascyk and Gabriel 1975) that collected the signal at two selectable narrow spectral bands, from which retrieved fluorescence through the Fraunhofer Line Depth principle originally developed by astronomers studying luminescence of the lunar surface; a detailed account of the development of this technique and the Plascyk instrument can be found at (Stoertz, Hemphill, and Markle 1969). It is worth noting that this instrument was originally developed to survey luminescent minerals, pollutant substances and geochemically stressed vegetation (Watson and Hemphill 1976).

An orbital fluorescence sensor was already evaluated for feasibility in (Stacy et al. 1984) but it was considered technologically too challenging.

First reported use of O_2 absorption lines for the measurement of fluorescence was by (Carter et al. 1996) and was based on the O_2-B band. A concept that was followed and improved by (Moya and Cerovic 2004) opening a real possibility of remotely detect chlorophyll fluorescence of the vegetation from space.

However, all these advances in the remote sensing of fluorescence have always lacked a proper validation of the estimations provided by the different retrieval methods and technologies. There has been a need of directly measured fluorescence to be used as ground truth that was not completely fulfilled.

1.3 FLEX: THE FLUORESCENCE EXPLORER MISSION

FLEX (FLuorescence EXplorer) is the first space mission specifically designed for the estimation of vegetation fluorescence on a global scale. The FLEX mission was originally proposed to ESA in 1998 for consideration as candidate in its Earth Explorer program (M.-P. Stoll et al. 1999) and despite it was not selected for phase-A, it was recommended for further investigation due to the high scientific interest of fluorescence as a proxy for photosynthesis. The topics that were found insufficiently mature were, among others, the technological feasibility and the atmospheric perturbations to the measured signal. The mission scientific requirements were reviewed, and instrumental requirements were defined taking into account the weakness of the signal, and the atmospheric perturbations involved (Smorenburg et al. 2002). In a later review of the mission's requirements the O2 absorption bands were presented as an alternative to the Fraunhofer lines (while previously were only considered as a supportive method for the development of the mission due to the strong dependence of the line depth upon the air mass) to overcome the technological challenges to measure the low signal of fluorescence within the H_α line. Besides,

it was remarked the need to stablish reliable knowledge on the fluorescence signal levels (M. P. Stoll et al. 2003)

FLEX was again formally proposed in 2005 as one of seven ESA Earth Explorer 7 (EE7) mission concepts. However, the EE7 winner after the January 2009 community User Consultation Meeting (UCM) was BIOMASS. Subsequently, the FLEX concept was slimmed down and supported as one of two pre-Phase A EE8 mission concepts, along with CarbonSat, beginning in 2011. Finally, FLEX prevailed as the unanimous choice of the ESA review panel and the general science community attending the September 2015 UCM in Krakow, Poland.

In spring 2002 a field campaign, supporting the FLEX mission, took place in Sodankylä (Finland) to observe the fluorescence signal over a conifer forest canopy through the spring recovery with the aim to determine the viability of its remote measurement from space (Davidson et al. 2002). In this campaign dedicated instruments were used for the remote sensing of canopy fluorescence (Moya et al. 2002), in addition an off-the-shelf ASD-FSFR field spectroradiometer was used to characterize the reflectance of the canopy, the understory and some needle mats. It was found that this instrument was sensible to the fluorescence effects on the reflectance (Miller et al. 2002) which led to testing it inside a dark room by exciting the fluorescence of a needle mat with a red laser, realising that the spectrometer was sensible enough to detect the emission spectrum (contrary to the expectations). This opened up the possibility of using such instruments for the measurement of chlorophyll fluorescence and to design a new device that could be attached to the commercial spectroradiometer to provide direct measurement of sun-induced chlorophyll fluorescence at both

sides of the leaf, leaf optical properties, absorptance, PAR and APAR, using physical units instead of the then commonly used relative units.

Those measurements would help to address the (at the time) open questions for the FLEX mission, but also for broad general knowledge:
What is the expected signal level to be measured?
How accurate are the remote sensing retrieval methods?
What is the behaviour of passively measured fluorescence under different environmental conditions and stress levels?
How does passively measured fluorescence relate to the wealth of knowledge already available from active measurements?
How does top-of-canopy fluorescence relate to leaf level fluorescence and ultimately to chloroplast-level fluorescence?
What other sources of information are needed to relate fluorescence to photosynthesis?

2 OBJECTIVES

The work in this Thesis has been conducted within the framework of ESA's Earth Explorer FLEX mission, aiming to respond to some of the needs in the preparation and development of the mission as stated above.

The main objective of this Thesis is to develop a system that enables measuring *in vivo* the fluorescence emission from attached leaves under natural environmental conditions and sunlight in a direct and passive way, as a counterpart to already existing indirect and active methods.

This system aims to satisfy the following objectives:

1) To provide fluorescence measurements in physical units in order to determine the signal levels to be registered by the FLEX mission.
2) To evaluate the accuracy of fluorescence retrieval methods based on O2 absorption bands, applied at leaf level.
3) To provide accurate estimates of the actual energy emitted by the vegetation in form of fluorescence to enable a fair comparison with the fluorescence estimates by remote sensing methods.

And ultimately,

4) To provide an understanding of the emission of fluorescence dynamics of plants under different states of stress and adaptation to changing conditions.

It must be noted that these objectives were set in 2004 when still there were many unknowns regarding the passive measurement of fluorescence, and objectives 1) and 2) were satisfied in an earlier phase of this study, whereas objective 3) and 4) have been addressed over a longer period of time.

3 BACKGROUND

3.1 FLUORESCENCE QUENCHING

Each photon absorbed by chlorophyll excites an electron that has three different main pathways for deexcitation (Figure 3-1). It has a certain probability of being transferred to a reaction centre from where photosynthetic reactions are triggered. It can also lose its excitation energy through internal or external conversion, passing it to the vibrational modes of the molecule (transformation into heat). Ultimately, the electron will be de-excited by the emission of a photon (fluorescence). There is a fourth pathway, shifting from the singlet to the triplet excited state, but it has very small probability and only happens when there is an excess of excitation energy, however, this path is a damaging one.

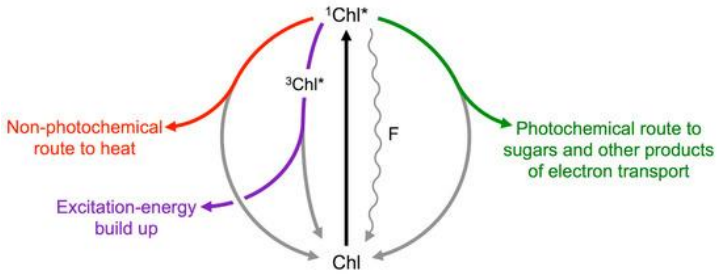

Figure 3-1 De-excitation pathways for an excited electron in chlorophyll embedded in the photosynthetic apparatus. From (Demmig-Adams et al. 2020).

Most of the excited electrons are removed from the chlorophyll to be used in the photosynthetic process, leaving the chlorophyll with an excess of positive charge, which is compensated by the absorption of an electron from the dissociation of water into oxygen and hydrogen (photolysis).

Whether there is a deficit of CO_2 (which uses the electrons yielded by chlorophyll) or H_2O (which provides electrons to chlorophyll), there is an excess

in charge gradient that can end up damaging the photosynthetic apparatus in general and the chlorophyll in particular.

Within the photosystems, chlorophyll is surrounded by carotenoids (a family of pigments grouping carotenes and xanthophylls) that are also capable of absorbing photosynthetic light. These pigments can act as an extended source of electrons to the chlorophylls in the photosystem to be used for photosynthesis, or as protective barrier preventing an excess of photons to reach the chlorophylls. Thus, the main photoprotection mechanism of the plant is based on changes in the molecular structure of the carotenoids, and more specifically, the xanthophylls, and therefore in their absorption spectrum. These pigments, by absorbing visible light (Figure 3-2), shape the appearance of the reflectance and transmittance of the leaf in the green region. Any change in the proportion of pigments within the photosystems will be reflected in the optical properties of the leaves, and therefore, susceptible to be measured, e.g., by detecting changes in the green part of the spectrum with high spectral resolution as it was already suggested by (Gamon et al. 1990; Peñuelas, Filella, and Gamon 1995) where the Photochemical Reflectance Index (PRI) was introduced.

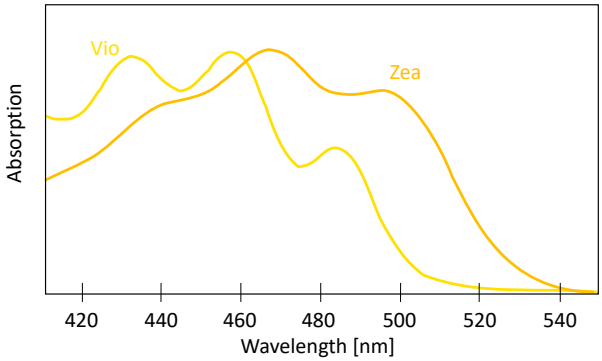

Figure 3-2 Absorption spectrum of Violaxanthin and Zeaxanthin. A change in the leaf content of any of them would translate in a change of the leaf's reflectance and transmittance spectra. Adapted from (Ruban et al. 2001).

The energy stored in the excited electrons that is not used for photosynthesis or dissipated as heat by the carotenoids is released as fluorescence. Therefore, photosynthesis and heat dissipation can be considered quenchers of the fluorescence emission since both remove excited electrons from the de-excitation pathway by photon emission. The first is known as Photochemical Quenching and the second as Non-Photochemical Quenching (NPQ).

Therefore, accounting for the number of photons absorbed, the fluorescence emission, and the level of photoprotection, it would be possible to make an estimate of the photosynthetic efficiency of the plant at a given time. Except for photosynthesis, the other three processes are related to photons, either by emission or dispersion, so they are susceptible to be detected by remote sensing, thus, opening the possibility to estimate photosynthetic activity from afar.

3.2 OPTICAL PROPERTIES OF THE LEAF: REFLECTANCE AND TRANSMITTANCE

In remote sensing, canopy reflectance is a key parameter from which relevant information about its composition and structure is extracted. To make the link to the biophysical properties of the plant, often, the optical properties of the leaves are needed. Information on leaf optical properties can be utilized, for example, as input to radiative transfer models of canopies, or even for the validation of radiative transfer models of the leaves; they can also be used for the training of machine learning models for parameter inversion when the optical properties were measured together with other biophysical parameters independently retrieved (e.g., chlorophyll content by chemical extraction).

According to the Handbook of Optics (Palmer 1995):

“Reflection is the process where a fraction of the radiant flux incident on a surface is returned into the same hemisphere whose base is the surface and which contains the incident radiation. The reflection can be specular (in the mirror direction), diffuse (scattered into the entire hemisphere), retroreflected (or hot-spot, in the direction of the incident beam), or a combination of them.

Transmission is the term used to describe the process by which incident radiant flux leaves a surface or medium from a side other than the incident side, usually the opposite side.”

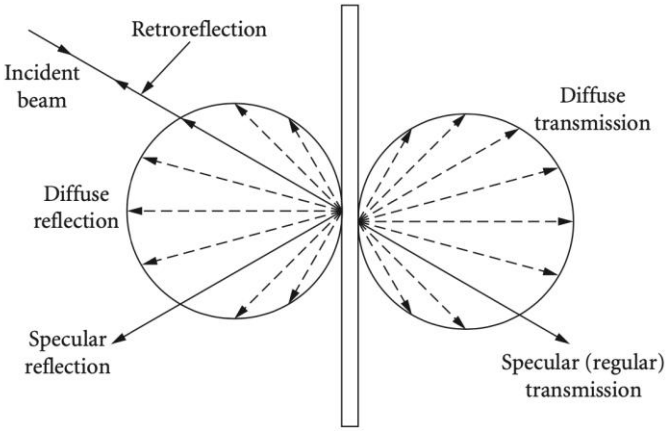

Figure 3-3 Idealized reflection and transmission. Actual behaviour can differ from these but will contain to some extent all of these components. From (Palmer 1995)

Leaf spectral reflectance, and as for any other surface, is defined as the ratio between the reflected spectral flux and incident spectral flux[1]:

$$\rho(\lambda) = \frac{\Phi_r(\lambda)}{\Phi_i(\lambda)} \qquad 3.1$$

The fluxes can be classified on three types: directional, conical, and hemispherical, and reflectance can correspond to any combination of them.

The directional case is an ideal mathematical concept and cannot be measured. Conical is the flux that arrives from a wide light source, or the flux measured by a system with a limited field of view (or solid angle ω_i), e.g., a fibre optic. Hemispherical is when the field of view covers 180° as with cosine corrector optic, or an integrating sphere.

[1] In this work all the terms related to light will be spectrally resolved unless stated otherwise. Therefore, from this point on the wavelength dependency will be implicit in the equations.

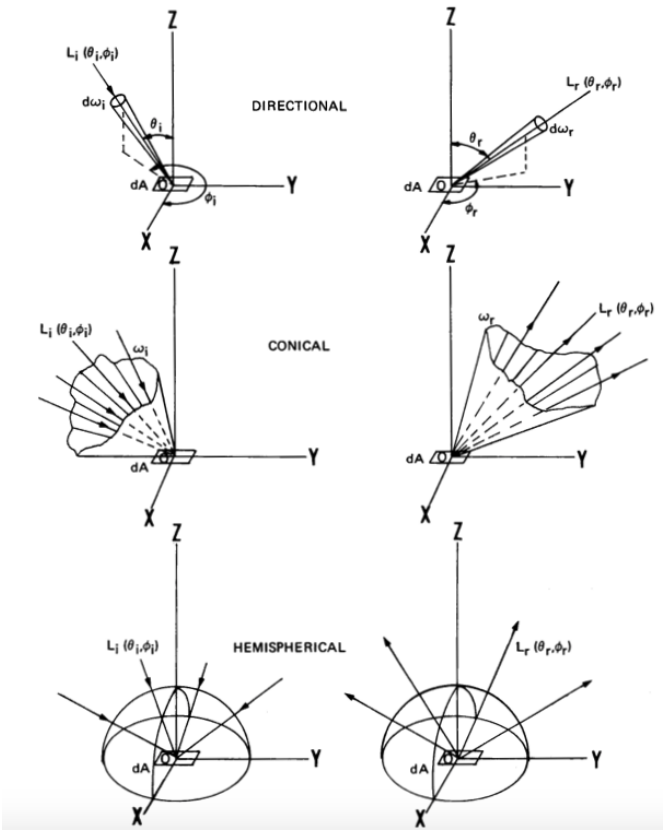

Figure 3-4 Geometry of incident and reflected elementary beams. From (Nicodemus et al. 1977)

The directional case is purely ideal, and only conical and hemispherical are measurable with an instrument. The conical case can correspond to any arbitrary direction, described by the spherical angles (θ, ϕ) and the solid angle of the cone (ω). Therefore, depending on the combination of illumination and observation geometries there will be a corresponding reflectance factor.

$$R(\theta_i, \phi_i, \theta_v, \phi_v) = \frac{L_r(\theta_i,\phi_i,\theta_v,\phi_v)}{L_i(\theta_i,\phi_i,\theta_v,\phi_v)} \qquad 3.2$$

Measurements of leaf reflectance and transmittance spectra are traditionally obtained with integrating spheres. (Hovi et al. 2018). The commercial integrating spheres usually employ a directional-hemispherical measurement geometry, in

which the incidence angle of the incoming collimated light beam is close to surface normal, and reflected or transmitted light are collected over the entire hemisphere. Integrating spheres are relatively large and heavy, and not very easy to transport into remote locations for field measurements. Portable systems such as contact probes (Miller et al. 1992), however, can be applied to measure spectra in situ so that storage of the leaf samples can be avoided), but they cannot measurement transmittance.

Contact probes or leaf clips make use of a reference white panel that, being measured under the same illumination and observation geometry than the sample, provides an estimate of the incident light (assuming that the white has a Lambertian response, and it is non-absorbing and non-transmitting.

Leaf reflectance spectrum can provide plenty of information about its pigments and internal structures, leading to the estimation of photoprotection status. However, it is not sufficient for the interpretation of the fluorescence emission and its relation to photosynthesis. These two directly depend on the number of photons absorbed, thus absorptance must also be determined. And for that it is also necessary to measure the transmittance of the leaf, but this has been a missing parameter in regular field measurements.

3.3 APPARENT REFLECTANCE AND TRANSMITTANCE

Besides, there is a problem that affects the reflectance and transmittance measurements, regardless of the technique used, both in the field or at the laboratory. The definition of reflectance (and transmittance) only accounts for the light that has been reflected by the material, but whenever a material emits fluorescence, the radiance leaving the surface will have two components, the reflected radiance (that has been specularly reflected or scattered) and the fluorescence radiance (that is emitted).

$$L = L_r + L_F \qquad 3.3$$

If one would try and calculate BRF from this radiance the result would be an apparent reflectance instead:

$$\frac{L}{I} = \frac{L_r}{I} + \frac{L_F}{I} = R + \frac{F}{I} \equiv \hat{R} \qquad 3.4$$

Claus Buschmann showed that the contribution of the chlorophyll fluorescence to the reflectance measurements has an impact in the red-edge of the spectrum (Buschmann & Lichtenthaler 1999).

And this apparent reflectance (and transmittance) is what most instruments measure.

Measuring the actual reflectance of vegetation is not an easy feat. Zarco-Tejada did some research in this direction by using monochromatic light above 710 nm, avoiding in this way the excitation of fluorescence emission (Zarco-Tejada et al. 2000). However, this method is not applicable to the spectral range of the red fluorescence, since there is an overlap of absorption and emission.

3.4 DOWNWARD FLUORESCENCE

In the same way that transmittance is the counterpart of reflectance, there is a counterpart to the fluorescence emitted by the illuminated side of the leaf. The fluorescence produced in the interior of the leaf reaches out by the upper side with a larger intensity, since chloroplasts tend to be closer to it; but fluorescence can also exit by the lower side. Upward fluorescence (emitted by the illuminated side) is the one that has been measured and considered in fluorescence research. Downward fluorescence, on the contrary, has been mostly neglected with the exception of (Rinderle and Lichtenthaler 1988, Goulas et al. 2004, Louis 2006). However, when doing the balance of the energy emitted in form of fluorescence, downward fluorescence can account for 30-50% of the total. A contribution that cannot be neglected when using canopy RTM to simulate TOC fluorescence, particularly since most of downward fluorescence is emitted in the NIR, which is highly reflected and transmitted, thus with a high probability of being scattered upwards in a canopy.

3.5 FLUORESCENCE REABSORPTION

Fluorescence is emitted by the chlorophyll molecules that are inside the leaf. Those photons must pass through the leaf tissue and epidermis cells before exiting. Through this passage, the fluorescence light has a high probability of getting scattered and reabsorbed by so the chlorophyll again. Thus, the fluorescence photons that finally make it to the outside of the leaf are less than those produced by the chlorophyll at the chloroplast level.

With higher chlorophyll concentrations the chances of being reabsorbed are higher.

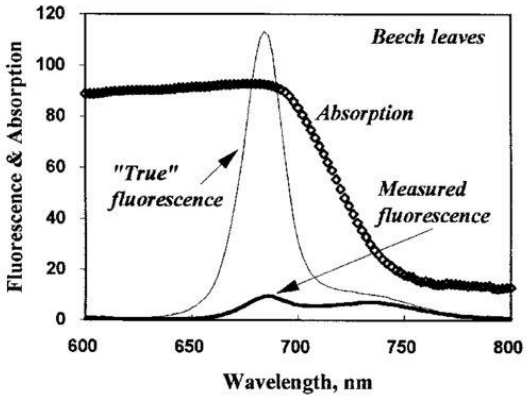

Figure 3-5 The fluorescence emitted at chloroplast level is partially reabsorbed by the chlorophyll before exiting the leaf. From (Buschmann, Gitelson, and Lichtenthaler 1998)

In the early years of research linking fluorescence emission to stress conditions the ratio between the two emission peaks was presented as sensitive estimator of the plant's health. However, even though experimental results supported this link, the generalization of the method was far from being conclusive. Instead, the peak ratio was also highly correlated with chlorophyll content.

Then, (Agati et al. 1993) suggested the reabsorption effect and a method to correct for it in LIF measurements as a way to normalize the fluorescence

emission independently of chlorophyll content. Later on, (Gitelson, Buschmann, and Lichtenthaler 1998) proposed a simpler correction using absorption and reflectance measurements.

3.6 FIELD MEASUREMENT

Measuring optical properties and fluorescence in the field from live samples is difficult most of the time, as it commonly involves the use of integrating spheres, which require in the best-case transportation of bulk instrumentation (tripod, lamps, batteries) and costly set-up, or in case of laboratory measurement, the samples must be cut, and preserved until they reach the laboratory limiting the number of samples; contact probes are more practical but they lack the measurement of absorptance and fluorescence; and fluorometers lack the capability to measure optical properties.

Table 3-1 Comparison of characteristics of different leaf-level instruments for fluorescence and optical properties of leaves

	Integrating Sphere	**Contact Probe**	**PAM**	**FluoWat**
Reflectance	HRF	BRF	–	BRF
Transmittance	HRF	–	–	BRF
True R & T	NO	NO	–	YES
Absorptance	YES	–	–	YES[1]
Measured in Field	NO[2]	YES	YES	YES
Sunlight	NO	NO	YES[3]	YES
F	–	–	Single Band	Spectral
Downward F	–	–	NO	YES
F in Physical Units	–	–	NO	YES
Kautsky effect	–	–	YES	YES[4]
PAR	YES	YES	YES	YES
APAR	YES	–	–	YES
NPQ	R index	R index	Sat. pulse	R index

[1]from BRF, missing specular reflection. [2]Some portable IS can with lower performance. [3]Sun as actinic light, measure with modulated artificial light. [4]performance depends on spectroradiometer sampling rate.

4 DEVELOPMENT OF THE DEVICE AND THE DATA PROCESSING

The main objective in this work is to measure chlorophyll fluorescence under natural conditions, that is, employing only the sun light under which the plant is growing, with as little perturbation as possible; and having a direct measurement of the fluorescence spectrum, so it is possible to accurately quantify the magnitude of the emitted energy under natural conditions. Such a device makes it is possible to study the behaviour of plants under different health status or stress conditions and be able to quantitatively compare the results.

In this chapter the working principles for this instrument are presented, as well as the equations to derive different parameters of interest from the measurements, and a number of considerations regarding the measurements that might affect to the quality and precision of the results.

4.1 THE FLUOWAT LEAF CLIP

4.1.1 WORKING PRINCIPLES

Chlorophyll fluorescence is emitted between 650 nm and 800 nm (with a very weak tail up to 850 nm). Some of the sunlight is reflected by the leaves overlapping the fluorescence emission, which is much weaker. The simplest way to directly observe fluorescence is by removing the overlapping sunlight before it reaches the leaf, but the light that activates photosynthesis must still reach the sample. This can be achieved by means of a low-pass optical filter with high transmission of light with wavelengths shorter than the cut-off wavelength and blocking the light with longer wavelengths.

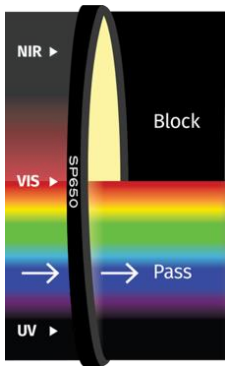

Figure 4-1 Short pass filter with cut-off at 650 nm

Of course, the leaf must not receive any unfiltered light, thus it must be enclosed in a chamber with the opening for the illumination fully covered by the low-pass filter.

4.1.2 FIRST PROTOTYPES

Such a device was built, after a design proposed by Prof. I. Moya during a stage with our group in 2005, enabling the measurement of the actual fluorescence. Since then, and only until recently, the device has been evolving and improving. The basic concept is a low-pass filter that blocks the illumination that overlaps with the fluorescence emission (from 650 nm to 850 nm), while it lets pass the light that excites the photosynthetic molecules.

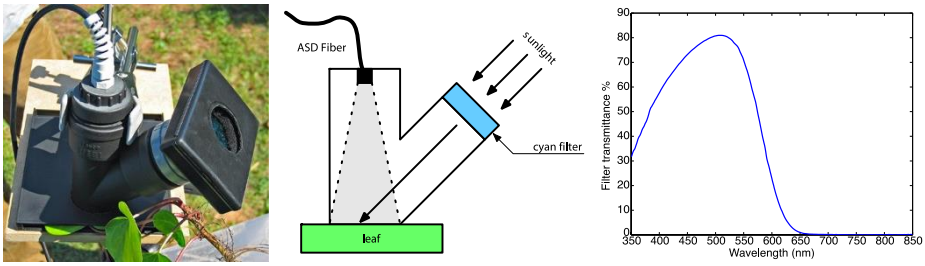

Figure 4-2 First prototype designed together with Prof. I. Moya. The sunlight is used as the excitation source blocking by the cyan low-pass filter the red and NIR light that overlaps with the emitted fluorescence.

This device attaches to a spectroradiometer and allowed to directly measure the actual sun-induced fluorescence emission of plant leaves in their natural environment (see Figure 4-2 for a diagram). The working principle is the following: the sunlight hits the surface of the leaf through a low-pass filter, exciting the plant on the UV and blue-green regions (Figure 4-2 right shows the transmittance of the used filter, originally using a cyan filter). The chlorophyll emits fluorescence in the red and far-red spectral range, which is acquired by a spectroradiometer. As no sunlight reaches the leaf in this spectral range, all the captured radiance is due to the fluorescence emission rather than reflected light. The only light reaching the leaf must pass through the filter, thus, in the field it is necessary to use an enclosing structure that hold the filter and blocks any other ambient light.

A first improvement over the original concept was making the filter to be removable by sliding it in and out of position (Figure 4-3). In this way, it was possible to measure the total radiance coming from the leaves (containing both contributions, reflected and emitted) as it would do any remote sensing system. The device (not a clip yet) was placed on a small optical bench and was hold by a mechanical arm that could slide up and down along the mast that it was affixed. This allowed the device to quickly replace the leaf by a white reference

that provided a measure of the solar radiance reaching the sample. By measuring the solar radiance together with the leaf's radiance it was possible to apply the FLD method to retrieve fluorescence; in comparison with the direct measurement while using the filter.

Besides, a sun finder was added to the device, to ease the proper alignment with the sun, and to get always consistent illumination reaching the target.

Figure 4-3 First evolution introducing novel characteristics, such as removable filter and sun finder, but not yet including transmittance.

Using this device, a first set of experiments were devoted to test if the FLD retrieval method provided reliable estimates of actual fluorescence emission, described and analysed in Sections 5.1.

4.1.3 Leaf Clip Evolution

After performing some experiments using the early prototype, it became evident that it was necessary to think of a redesign of the device, first to overcome some technical problems, and second to add needed functionality. Coping with those requirements (that will be detailed in the following) led to a series of prototypes that concluded in the current FluoWat leaf clip.

The most important requirement for the new design was the capability to measure the absorbed PAR (APAR), enabling the determination of the fluorescence quantum efficiency. Therefore, it is necessary to determine the absorptance of the leaf, and for that transmittance must be measured together with the reflectance. To fulfil this requirement the device transformed into a leaf clip with capability to attach the fibre optic to either end, the top or the bottom (Figure 4-4 left).

Figure 4-4 Pictures of some early prototypes (left, centre) of the FluoWat leaf clip and the final design (right).

Adding a bottom part to the clip solved also a second problem that was detected: the black surface over which the leaf was placed with the original device had a non-negligible reflectance, around 4%, that produced an offset to the reflectance measurement, particularly at the regions with lower signal, i.e., the blue and the red where chlorophyll absorbs most.

The clip also had to become smaller to allow the measurement of a larger variety of leaf types and sizes, and to make it more manageable when measuring inside canopies (Figure 4-4 centre). The fibres needed to be closer to the sample to reduce their footprint to less than 1 cm.

It was also necessary to substitute the original cyan filter by a higher performance one, to reduce the loss of transmitted PAR. Since one of the objectives of the leaf clip is to provide accurate estimates of the actual energy

emitted by the vegetation in form of fluorescence to enable a fair comparison with the fluorescence estimates by remote sensing methods. If the clip measures with notably reduced light intensity, due to the filter, not only would the fluorescence intensity decrease, also the fluorescence yield might change due to the leaf readapting to new illumination conditions, thus modifying the photosynthetic status. Fortunately, filter technology has advanced substantially providing improved blocking capability, reaching transmittances of OD4 or lower (i.e. less than 10^{-4} of the light passes through) with sharp-edge transition.

It was necessary to improve the filter holder to prevent changes in illumination geometry between types of measurements. Former versions had thick filter holders (Figure 4-4 centre) that were merely removed from the place resulting in a different geometry that affected the illumination. The later versions count with a large sliding filter holder (Figure 4-4 right) that has place for one clear opening besides the place for the filter. The clear opening is necessary to maintain the geometry of the light entrance and avoid inconsistent illumination conditions.

4.1.4 FluoWat Final Design

The final design consists of a small portable dark chamber, with cavities on top and bottom to hold the measuring fibre optics, and two opposing opening ports at 45° on the top half (Figure 4-6). One of the openings is used for the natural illumination and can be covered by a low-pass filter. The other can be used to add capabilities to the clip, such as an artificial light.

Figure 4-5 Picture of the FluoWat leaf clip in its final design.

Implementing the capability to measure leaf reflectance and transmittance was necessary first to obtain the absorbed PAR, and second to be able to produce inputs for radiative transfer models of vegetation. In order to do this, the bottom part of the clip had to be symmetrical to the top one, in such a way that the spectroradiometer fibres are looking at the sample from the nadir view, and the illumination reaches at 45°. This angle of incidence of the illumination is the result of a trade of between getting close to nadir and the volume occupied by the slider of the filter holder and the shutter mechanism for the fibre port. This improvement has the added benefit to remove the background plate that was holding the leaf, and that even if painted black had a non-negligible additive effect on the radiance measured from above. Nonetheless, the lower half of the clip can be removed, allowing the upper half, which has a flat profile, to be used on hard thick surfaces (e.g., lichens on soil crust, Section 5.4 below)

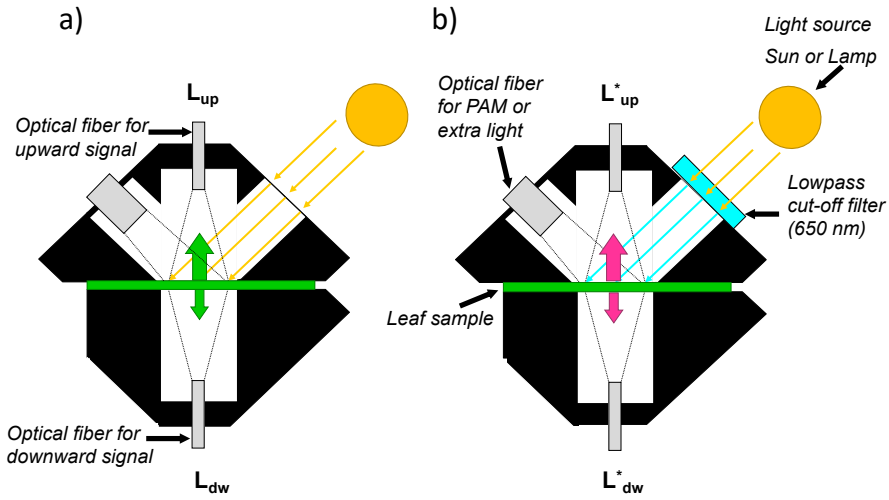

Figure 4-6 Diagram of the FluoWat leaf clip. A) Measuring leaf radiance under full sun light that includes fluorescence mixed with reflected and transmitted radiance. B) Direct measurement of fluorescence by filtering the sun light.

The most relevant modifications and original contributions over the first prototype:

- The filter holder got enlarged to allow accommodating several filters and clear opening for unaltered illumination. The combined use of filters with different cut-off wavelengths allows for a better estimate of the spectral shape and magnitude of the undisturbed fluorescence emission.
- Fast and accurate filter positioning system to ensure repeatability of the measurement, to minimize delays between takes, and to avoid accidental occlusions due to mispositioning of the filter. This system is based on strong magnets strategically placed in the body of the clip and the filter holder.
- High performance filters with a sharp cut-off and OD4 have been used to minimize the loss of PAR reaching the sample (an optical depth of 4 means that the transmittance above the cut-off wavelength is less than 10^{-4}).

- An additional opening in the filter holder permits to place an opaque disk to dark adapt the leaf for Kautsky transient measurements.
- The inclusion of a sun-finder was much needed, to ease pointing to the Sun and to improve measuring accuracy, since the change of incidence angle, even if small, introduces large signal deviations (Section 4.3.4).

Figure 4-7 Dark adapting a leaf with a PAM fibre attached at the back port

- Reduction of sampling area, to accommodate a larger variety of species. Reducing at the same time the overall size of the clip to make it more manageable and practical (e.g., easier to get into dense or closed canopies).
- Added a back port to allow additional capabilities, e.g., artificial light for saturating pulse while illuminating with sun light; or coupling a PAM fibre (Figure 4-7).
- The openings for the fibre optics are compliant with the SMA-905 connector (the most common in field spectrometers) and include a sliding shutter to prevent the entrance of stray light when the fibre is relocated or removed.

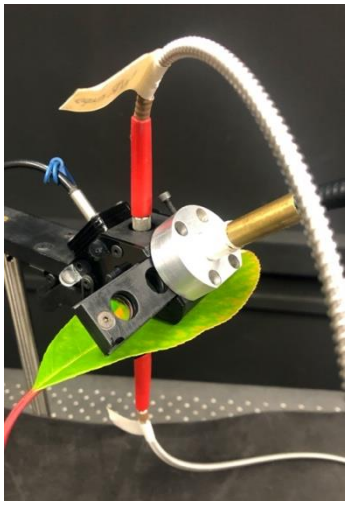

Figure 4-8 The FluoWat leaf clip in one of the optional configurations, with two fibre optics for simultaneous upward and downward measurement, using artificial LED light in the front port and filters, and an additional halogen light in the back port.

The selection of the filter has also been improved. On one hand, the filter must block the light that overlaps with the fluorescence. On the other hand, by blocking the light some red PAR does not reach the leaf reducing the amount of energy compared to the unfiltered natural conditions. So, a compromise must be made between both. There are currently three off-the-shelf filters available, each with a different cut-off wavelength: 650 nm, 675 nm and 700 nm.

- Φ650 allows capturing the full shape of the fluorescence emission at the expense of reduced red PAR (14% less than with full sunlight).
- Φ700 allows full PAR to reach the leaf but only the far-red fluorescence peak is measured.
- Φ675 is a compromise between the two, losing less red light (a reduction of just 6% of total PAR) measuring the full FRF peak while RF peak is only resolved from its maxima, missing the red wing of the peak between 650 nm and 680 nm.

It is possible to use two of them in sequence to get a combined measure.

The developments were done not only on the instrument design, but also on the understanding of the signal, the measurement protocol, and the processing algorithms. The following sections will treat about them.

4.2 MEASURING WITH THE LEAF CLIP

4.2.1 DIRECT MEASUREMENTS

There are six types of measurements in sequence that are necessary to retrieve the fluorescence spectrum.

First of all, the radiance coming from the leaf in both directions: upwards and downwards, with and without setting the filter. Filtered radiances L_{up}^{ϕ} and L_{dw}^{ϕ} provide with a first estimate of the emitted fluorescence (although it needs to be corrected as explained in Section 4.2.3 below). And unfiltered radiance L_{up} and L_{dw} provides the reflectance R and transmittance T factors of the leaf, when normalized by the incoming radiance.

Then, of course, the incoming light I_{tot} needs to be estimated from the radiance reflected by a white reference (WR) I_{up}. Measuring a white panel without using a filter will provide a reference radiance that will be later used to calculate reflectance and transmittance, to obtain the amount of PAR that reaches the leaf sample, and to correct a small contribution of unfiltered light. The measurement of filtered WR (I_{up}^{ϕ}) provides a measure of the actual PAR reaching the leaf when the fluorescence is measured. It also can be used to provide an estimation of the filter transmittance.

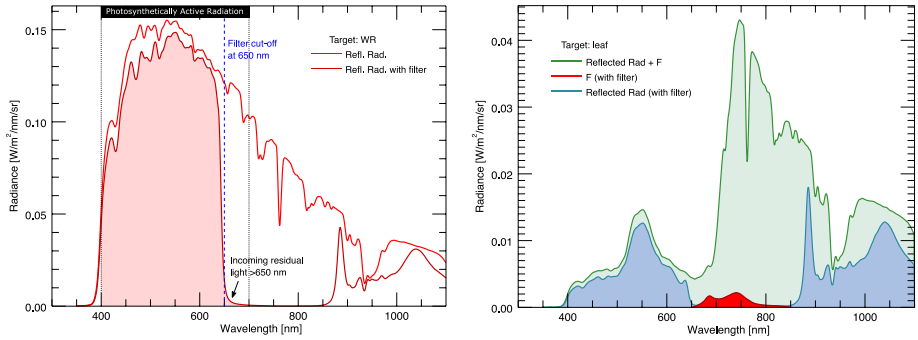

Figure 4-9 Illustration of the basic direct measurements with the clip. Left: radiance from the white reference. Right: and upwelling radiance from the leaf (downwelling radiance is analogous). Spectra collected using the low-pass filter are plotted in different colour. Note the fluorescence between 650 nm and 850 nm, an exceptionally high signal has been selected to facilitate its viewing. Adapted from (Aasen et al. 2019)

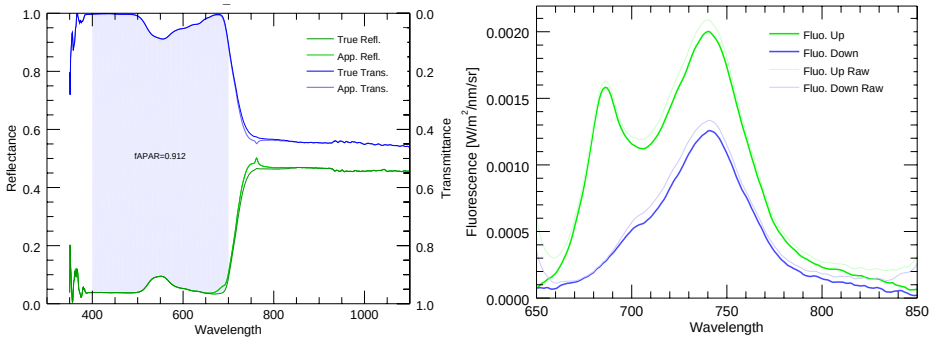

Figure 4-10 Illustration of derived measurements: (left) Apparent and true reflectance in green and transmittance in blue; transmittance is represented as 1-T. (right) Spectra of fluorescence emission, upwelling in green and downwelling in blue with the direct uncorrected measurement in light colours.

Since the WR is not fully reflective, in order to get the total incoming radiance I_{tot} it is necessary to divide I_{up} by the WR reflectance factor. However, provided that the white material used has a negligible absorptance, it is also possible to measure the downward component of the WR I_{dw} and directly get I_{tot} by adding it to I_{up}.

To enable the estimation of uncertainty propagation it is advisable to perform a number n of repetitions during the acquisition of each direct measurement. Then, it is possible to determine for any given parameter x:

the mean value $$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i \quad (4.1)$$

the variance $$\sigma_x{}^2 = \frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2 \quad (4.2)$$

and the covariance $$\sigma_{xy} = \frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y}) \quad (4.3)$$

as a way to characterize at least the random component of the uncertainty, which would include instrumental noise and illumination instability (either by high clouds or unsteady pointing). It is important to note that with the leaf clip clip the different parameters are measured in sequence and not simultaneously, e.g. incoming radiance and reflected radiance to obtain reflectance. Thus, covariance might not always be properly determined.

4.2.2 Directly Retrievable Parameters

When several spectra are collected for each of the measurement types then it is possible to calculate the mean and standard deviation of the set, which will determine the stability and precision of the measurements.

Incoming Radiance:

The radiance reaching the sample is derived from the measurement of the white reference. Since any white reference is not perfectly reflective (even if very close to it for some materials) it is necessary to account for the reflectance factor of the reference R_{wr}.

$$I_{tot} = \frac{I_{up}}{R_{wr}} \quad (4.4)$$

and the corresponding standard deviation would be

$$\sigma_{I_{tot}} = I_{tot} \cdot \sqrt{\left(\frac{\sigma_{R_{wr}}}{R_{wr}}\right)^2 + \left(\frac{\sigma_{I_{up}}}{I_{up}}\right)^2 - 2 \cdot \frac{\sigma_{R_{wr} I_{up}}}{R_{wr} \cdot I_{up}}} \qquad 4.5$$

The reflectance factor of the reference R_{wr} is typically provided by the manufacturer. But in case of it being absent it can be estimated, assuming that the white reference has a negligible absorptance, from the combination of upward and downward WR radiance:

$$I_{tot} = I_{up} + I_{dw} \qquad 4.6$$

with associated error:

$$\sigma_{I_{tot}} = \sqrt{{\sigma_{I_{up}}}^2 + {\sigma_{I_{dw}}}^2 + 2 \cdot \sigma_{I_{up} I_{dw}}} \qquad 4.7$$

and

$$R_{wr} = \frac{I_{up}}{I_{tot}} \qquad 4.8$$

$$\sigma_{R_{wr}} = R_{wr} \cdot \sqrt{\left(\frac{\sigma_{I_{up}}}{I_{up}}\right)^2 + \left(\frac{\sigma_{I_{tot}}}{I_{tot}}\right)^2 - 2 \cdot \frac{\sigma_{I_{up} I_{tot}}}{I_{up} \cdot I_{tot}}} \qquad 4.9$$

allowing to get I_{tot} directly from I_{up} saving time in every measurement set.

This characterization of R_wr will be valid while the white reference remains clean and unscratched, and the reference should be discarded and replaced.

Filtered Incoming Radiance:

$$I_{tot}^{\phi} = \frac{I_{up}^{\phi}}{R_{wr}} \qquad 4.10$$

$$\sigma_{I_{tot}^{\phi}} = I_{tot}^{\phi} \cdot \sqrt{\left(\frac{\sigma_{R_{wr}}}{R_{wr}}\right)^2 + \left(\frac{\sigma_{I_{up}^{\phi}}}{I_{up}^{\phi}}\right)^2 - 2 \cdot \frac{\sigma_{I_{up}^{\phi} R_{wr}}}{I_{up}^{\phi} \cdot R_{wr}}} \qquad 4.11$$

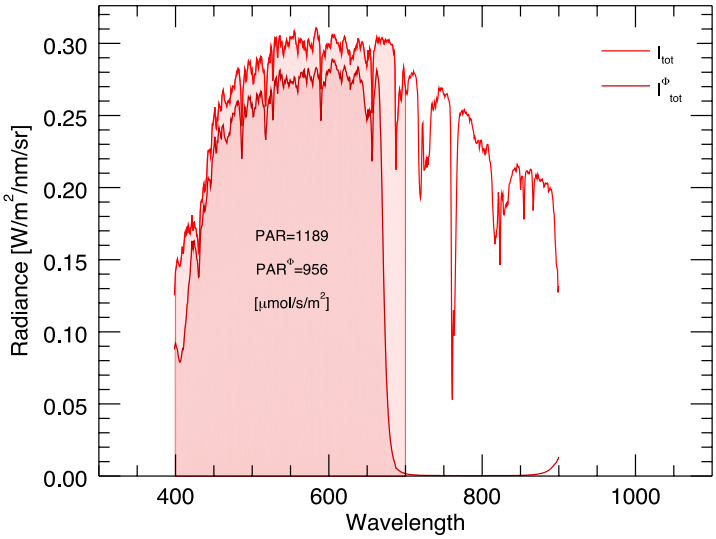

Figure 4-11 Radiance from a white reference measured with a filter (crimson) and without (red), with the corresponding PAR represented by the coloured area below the spectra.

Filter's Transmittance:

$$\tau = \frac{I_{up}^{\Phi}}{I_{up}} \qquad 4.12$$

$$\sigma_{\tau} = \tau \cdot \sqrt{\left(\frac{\sigma_{I_{up}^{\Phi}}}{I_{up}^{\Phi}}\right)^2 + \left(\frac{\sigma_{I_{up}}}{I_{up}}\right)^2 - 2 \cdot \frac{\sigma_{I_{up}^{\Phi} I_{up}}}{I_{up}^{\Phi} \cdot I_{up}}} \qquad 4.13$$

Note that using this estimation of filter transmittance τ is only acceptable for filters with OD4 or higher, or in the case that a proper laboratory filter characterization is not available, since the measurement using the FluoWat introduces an overestimation of the transmittance. See Section 4.4.1 for considerations using this measurement.

Apparent Reflectance Factor:

$$\hat{R} = \frac{L_{up}}{I_{tot}} \qquad 4.14$$

$$\sigma_{\hat{R}} = \hat{R} \cdot \sqrt{\left(\frac{\sigma_{L_{up}}}{L_{up}}\right)^2 + \left(\frac{\sigma_{I_{tot}}}{I_{tot}}\right)^2 - 2 \cdot \frac{\sigma_{L_{up} I_{tot}}}{L_{up} \cdot I_{tot}}} \qquad 4.15$$

Apparent Transmittance Factor:

$$\hat{T} = \frac{L_{dw}}{I_{tot}} \qquad 4.16$$

$$\sigma_{\hat{T}} = \hat{T} \cdot \sqrt{\left(\frac{\sigma_{L_{dw}}}{L_{dw}}\right)^2 + \left(\frac{\sigma_{I_{tot}}}{I_{tot}}\right)^2 - 2 \cdot \frac{\sigma_{L_{dw} I_{tot}}}{L_{dw} \cdot I_{tot}}} \qquad 4.17$$

Apparent reflectance and transmittance factors are calculated from the measured radiance that includes the emitted fluorescence besides the reflected/transmitted radiance. Hence, apparent instead of true factors. These are the parameters commonly measured and used, due to the difficulty to separate the two contributing fluxes.

Photochemical Reflectance Index

The Photochemical Reflectance Index (PRI) is related to variations in the reflectance spectrum in the green caused by changes in the photoprotection mechanisms (Gamon et al. 1990).

From the reflectance measurements:

$$PRI = \frac{R_{570} - R_{531}}{R_{570} + R_{531}} \qquad 4.18$$

$$\sigma_{PRI} = PRI \cdot \sqrt{\frac{{\sigma_{R_{570}}}^2 + {\sigma_{R_{531}}}^2}{(R_{570} - R_{531})^2} + \frac{{\sigma_{R_{570}}}^2 + {\sigma_{R_{531}}}^2}{(R_{570} + R_{531})^2}} \qquad 4.19$$

This index can be also calculated from the transmittance, which might provide even more information about the photoprotective pigments that are located deeper in the leaf.

From the reflectance spectrum other common spectral indices can also be calculated, and more advanced techniques for the determination of leaf photoprotection can also be applied (Van Wittenberghe et al. 2019b).

4.2.3 EMITTED FLUORESCENCE (CORRECTING FOR UNFILTERED LIGHT)

One might be tempted to use the filtered measurement L^{ϕ}, within the filter's blocking spectral range, as the actual measurement of fluorescence F^{ϕ}. It would be so if the filter was an ideal one, completely transparent, with transmittance τ equal 1, for wavelengths below the cut-off wavelength (λ_c), and a zero transmittance in the rejection region above the cut-off wavelength.

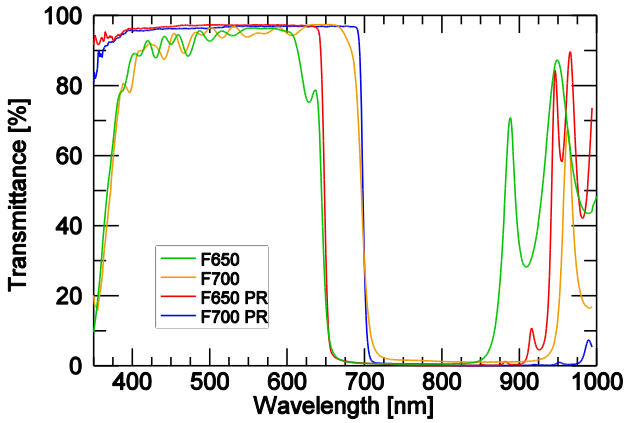

Figure 4-12 Transmittance of several low-pass filters with different cut-off wavelengths and qualities. PR indicate high-performance filter of OD4, otherwise they are OD2.

But most filters present a small slope in the transmittance around λ_c and the transmittance does not reach zero (see Figure 4-12), thus, there is some light passing through the filter, reaching the sample, and being reflected and mixed with the emitted fluorescence F^{ϕ} (ϕ indicates that the PAR exciting the fluorescence was reduced by the filter):

$$L_{up}^{\phi} = F_{up}^{\phi} + I_{tot} \cdot R \cdot \tau \text{ with } \tau \to 0 \qquad 4.20$$

Depending on the quality of the filter and the intensity of the fluorescence, even though filter transmittance is below 1% (OD2), the contamination can go from 5% to 50%. Samples with lower chlorophyll content will have higher reflectance

in the red, and lower fluorescence emission, and they will be most affected by this perturbation.

Taking into account that

$$R \cong \hat{R} = \frac{L_{up}}{I_{tot}} \qquad 4.21$$

an expression can be found for the actual upwelling fluorescence:

$$F_{up}^{\phi} \cong L_{up}^{\phi} - L_{up} \cdot \tau \qquad 4.22$$

$$\sigma_{F_{up}^{\phi}} = \sqrt{\sigma_{L_{up}^{\phi}}{}^2 + \left(L_{up} \cdot \tau\right)^2 \left(\left(\frac{\sigma_{Lup}}{L_{up}}\right)^2 + \left(\frac{\sigma_{\tau}}{\tau}\right)^2\right)} \qquad 4.23$$

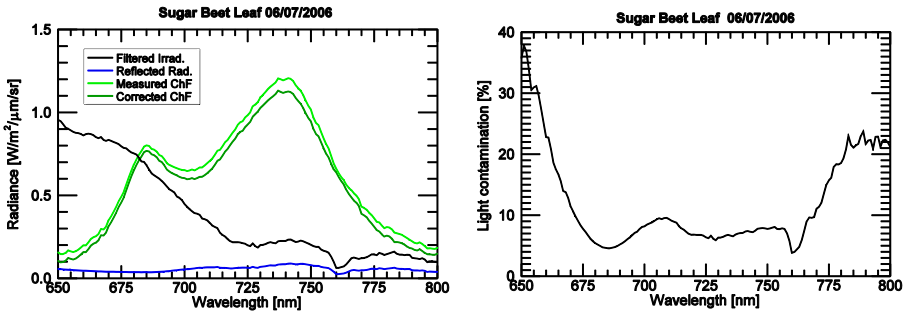

Figure 4-13 Left: Small amount of light unblocked by the filter (black) reaches the leaf and gets reflected (blue) and mixed with the emitted fluorescence (dark green), both contributions are collected by the fibre optic and measured (light green). Right: proportion of unfiltered light with respect to the emitted fluorescence for a particular case; residual light depends on the characteristics of each filter and the reflectance (or transmittance) of the leaf sample. Note that for this example a moderate performance OD2 filter was used.

Similarly, from the downward radiance:

$$L_{dw}^{\phi} = F_{dw}^{\phi} + I_{tot} \cdot T \cdot \tau \text{ with } \tau \to 0 \qquad 4.24$$

$$T \cong \hat{T} = \frac{L_{dw}}{I_{tot}} \qquad 4.25$$

Then the downwelling fluorescence, with the corresponding error:

$$F_{dw}^{\phi} \cong L_{dw}^{\phi} - L_{dw} \cdot \tau \quad 4.26$$

$$\sigma_{F_{dw}^{\phi}} = \sqrt{\sigma_{L_{dw}^{\phi}}{}^2 + (L_{dw} \cdot \tau)^2 \left(\left(\frac{\sigma_{L_{dw}}}{L_{dw}} \right)^2 + \left(\frac{\sigma_{\tau}}{\tau} \right)^2 \right)} \quad 4.27$$

And the total emitted fluorescence, adding both contributions, is

$$F_{tot}^{\phi} = F_{up}^{\phi} + F_{dw}^{\phi} \quad 4.28$$

$$\sigma_{F_{tot}^{\phi}} = \sqrt{\sigma_{F_{up}^{\phi}}{}^2 + \sigma_{F_{dw}^{\phi}}{}^2} \quad 4.29$$

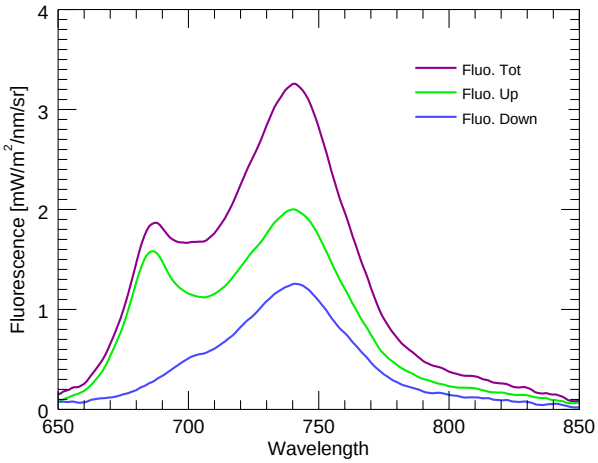

Figure 4-14 Total directional fluorescence, and the upward and downward components.

And, under the assumption of Lambertian emission, the total irradiance emitted as fluorescence can be calculated as

$$E_F = \pi \cdot F_{tot}^{\phi} \quad 4.30$$

4.2.4 Compensating the Filter Reduction of PAR

Chlorophyll absorbs light in the visible spectrum, from the blue (below 400nm) to the far-red (slightly above 700 nm) (Figure 1-2). The light in this spectral range

activates the photosynthesis and it is known as Photosynthetically Active Radiation (PAR) (McCree 1981).

PAR and APAR

The amount of photosynthetically active radiation (PAR) arriving to the sample can be easily obtained integrating the incoming radiance spectrum (I_{tot}) Over the spectral range from 400 to 700 nm.

$$PAR = \int_{400}^{700} I_{tot} \cdot d\lambda \qquad 4.31$$

$$\sigma_{PAR} = \sqrt{\int_{400}^{700} {\sigma_{I_{tot}}}^2 \cdot d\lambda} \qquad 4.32$$

From the total amount of useful radiation arriving, the leaf's chlorophyll only absorbs a part of it.

$$A = 1 - R - T \qquad 4.33$$

$$\sigma_A = \sqrt{{\sigma_R}^2 + {\sigma_T}^2} \qquad 4.34$$

The absorbed PAR is the amount of PAR light that reaches the sample and has not been reflected nor transmitted. Therefore, APAR can be calculated by multiplying the total incoming radiance by the amplitude and integrating.

$$\mathrm{APAR} = \int_{400}^{700} I_{tot} \cdot A \cdot d\lambda \qquad 4.35$$

$$\sigma_{APAR} = \sqrt{\int_{400}^{700} {I_{tot}}^2 A^2 \left(\left(\frac{\sigma_{I_{tot}}}{I_{tot}}\right)^2 + \left(\frac{\sigma_A}{A}\right)^2\right) d\lambda} \qquad 4.36$$

Note that in case that apparent reflectance and transmittance were used instead, then the portion in them due to the fluorescence emission would introduce a very small underestimation in the APAR obtained. This is an acceptable approximation considering other sources of error.

PAR and APAR should be calculated in μmol/m^2/s.

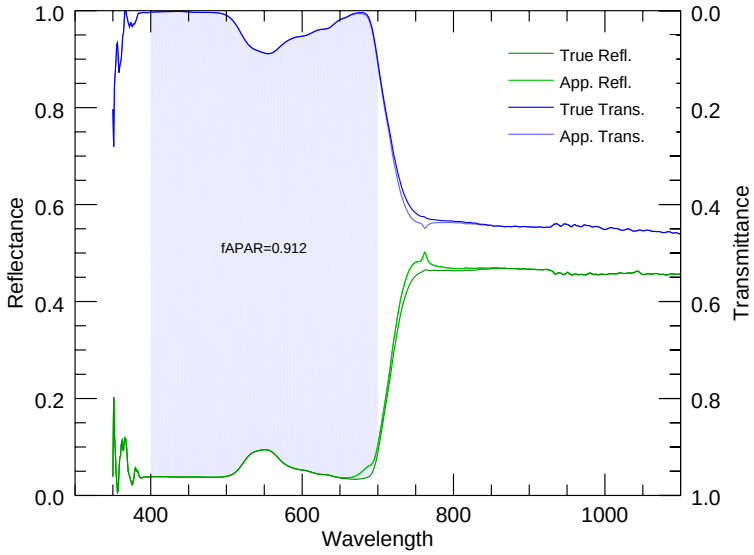

Figure 4-15 Apparent and true reflectance (green) and transmittance (blue). fAPAR is indicated by the area in pale blue. Note the small difference near 680 nm between real and apparent blue areas.

$$\mathrm{APAR} \cong \int_{400}^{700} I_{tot} \cdot \hat{A} \cdot d\lambda \qquad 4.37$$

$$\sigma_{APAR} = \sqrt{\int_{400}^{700} {I_{tot}}^2 \hat{A}^2 \left(\left(\frac{\sigma_{I_{tot}}}{I_{tot}} \right)^2 + \left(\frac{\sigma_{\hat{A}}}{\hat{A}} \right)^2 \right) d\lambda} \qquad 4.38$$

The fraction of APAR (fAPAR) is just the portion of PAR that has been absorbed.

$$fAPAR = \frac{APAR}{PAR} \qquad 4.39$$

APAR can be then used to estimate the fluorescence yield.

In this case, since the fluorescence is measured using the filter, it is important to calculate the corresponding APAR from the filtered irradiance:

$$\mathrm{APAR}^{\phi} \cong \int_{400}^{700} I_{tot}^{\phi} \cdot \hat{A} \cdot d\lambda \qquad 4.40$$

$$\sigma_{APAR^{\phi}} = \sqrt{\int_{400}^{700} {I_{tot}^{\phi}}^2 \hat{A}^2 \left(\left(\frac{\sigma_{I_{tot}^{\phi}}}{I_{tot}^{\phi}} \right)^2 + \left(\frac{\sigma_{\hat{A}}}{\hat{A}} \right)^2 \right) d\lambda} \qquad 4.41$$

Fluorescence Yield (apparent)

Fluorescence quantum yield is the ratio between number of photons emitted as fluorescence by the number of photons absorbed. However, the fluorescence that exits the leaf has gone through a reabsorption process. Thus, the photons measured exiting the leaf is much smaller than what were actually produced (Van Wittenberghe et al. 2021). Thus, the fluorescence yield estimated from leaf measurements can only be considered as an effective or apparent parameter.

Besides, in the same manner that FluoWat measurements of reflectance and transmittance are just an approximation, this fluorescence yield is also approximated, since the measurement is directional (conical) and there is not an exact account of neither the number of photons absorbed, nor the number of photos emitted as fluorescence.

Note that fluorescence yield is typically reported as an integrated value for the total fluorescence. In the case of the FluoWat it is advantageous to maintain the spectral distribution to allow scaling up the fluorescence measured with the filter F^{ϕ} to the one corresponding to the unfiltered light F, as it will be described in the next section.

Then it is possible to calculate the spectrally resolved apparent fluorescence yield:

$$\mathrm{YF}^{\phi}(\lambda) = \frac{F^{\phi}(\lambda)}{APAR^{\phi}} \qquad 4.42$$

$$\sigma_{\mathrm{YF}^{\phi}(\lambda)} = \mathrm{YF}^{\phi}(\lambda) \cdot \sqrt{\left(\frac{\sigma_{\mathrm{F}^{\phi}(\lambda)}}{\mathrm{F}^{\phi}(\lambda)}\right)^2 + \left(\frac{\sigma_{APAR^{\phi}}}{APAR^{\phi}}\right)^2} \qquad 4.43$$

This equation is valid for both upwelling and downwelling fluorescence.

Also, this apparent fluorescence yield is a useful way of normalizing the fluorescence emission spectrum (as long as it is not confused as a truly quantum yield), in the same way that in earlier studies where relative units were used when the fluorescence spectra of different samples were normalized to the maximum peak of RF or FRF to facilitate comparison.

In this case, the normalization is based on physical parameters, enabling fair comparison between measurements performed under unequal conditions. Hence, even thou the retrieved parameter from the leaf clip measurements is an apparent fluorescence yield, it will be named just "fluorescence yield" for brevity.

Fluorescence Scaled to Full PAR

In order to estimate the fluorescence spectrum when the light is not filtered, we make the assumption that fluorescence yield remains constant, at least for the small changes in the illumination caused by the filter. Besides, we also assume that the change in quality (distribution of energy across the colours) of the light does not affect to the shape of the emitted fluorescence.

$$\frac{F}{APAR} \approx \frac{F^{\Phi}}{APAR^{\Phi}} \qquad 4.44$$

Then

$$F = F^{\Phi}\frac{APAR}{APAR^{\Phi}} = YF^{\Phi} \cdot APAR \qquad 4.45$$

$$\sigma_F = F \cdot \sqrt{\left(\frac{\sigma_{YF^{\Phi}}}{YF^{\Phi}}\right)^2 + \left(\frac{\sigma_{APAR}}{APAR}\right)^2} \qquad 4.46$$

It is important to take into consideration that fluorescence yield estimated in this way is merely an approximation, since the measured absorbed PAR does not discriminate between photoactive pigments, and other absorbers within the

structure of the leaf. However, the largest portion of it will be used by the photosystems, so the approximation is an acceptable one.

4.2.5 Alternative Estimation of the Emitted Fluorescence at Full PAR

If we have two measurements, without filter (L) and with filter (L^{ϕ}), then we obtain two equations valid for any given wavelength (wavelength dependence is implicit in the equations):

$$\left.\begin{aligned} L &= R \cdot I_{tot} + F \\ L^{\phi} &= R \cdot I_{tot} \cdot \tau + F^{\phi} \end{aligned}\right\} \qquad 4.47$$

Assuming that the light reduction due to the use of the filter only affects to the magnitude of the fluorescence and not its spectral shape, and using the above equations, then

$$\left.\begin{aligned} R &= \frac{L-F}{I_{tot}} \\ F^{\phi} &= \alpha \cdot F \end{aligned}\right\} \Rightarrow F = \frac{L^{\phi} - \tau \cdot L}{\alpha - \tau} \qquad 4.48$$

But, according to Eq. 4.22 one can find that

$$F \cong \frac{F^{\phi}}{\alpha - \tau} \qquad 4.49$$

$$\sigma_F = F \cdot \sqrt{\left(\frac{\sigma_{F^{\phi}}}{F^{\phi}}\right)^2 + \frac{\sigma_{\alpha}^2 + \sigma_{\tau}^2}{(\alpha-\tau)^2}} \qquad 4.50$$

where

$$\alpha = \frac{APAR^{\phi}}{APAR} \qquad 4.51$$

$$\sigma_{\alpha} = \alpha \cdot \sqrt{\left(\frac{\sigma_{APAR^{\phi}}}{APAR^{\phi}}\right)^2 + \left(\frac{\sigma_{APAR}}{APAR}\right)^2} \qquad 4.52$$

This approach is more correct than the previous one, since here it considers that $L_{up} \cdot \tau$ includes a portion of fluorescence, whereas the formulation in the previous section does not. However, a wrong estimation of τ would have a larger impact in the accuracy of the estimated F than Eq. 4.45.

4.2.6 TRUE REFLECTANCE AND TRANSMITTANCE

Finally, the apparent reflectance and apparent transmittance are obtained respectively from upwelling and downwelling measured radiance, which comprises the light actually reflected (or transmitted) together with the emitted fluorescence. Therefore, in order to get the true reflectance and transmittance of the sample, it is necessary to subtract the fluorescence contribution. In this case, since reflectance and transmittance are measured without the filter, one must use the fluorescence scaled to the corresponding APAR level (F obtained by eq. 4.45 or eq. 4.49).

True Reflectance:

$$R = \frac{L_{up} - F_{up}}{I_{tot}} \qquad 4.53$$

$$\sigma_R = R \cdot \sqrt{\frac{{\sigma_{L_{up}}}^2 + {\sigma_{F_{up}}}^2}{\left(L_{up} + F_{up}\right)^2} + \left(\frac{\sigma_{I_{tot}}}{I_{tot}}\right)^2} \qquad 4.54$$

True Transmittance:

$$T = \frac{L_{dw} - F_{dw}}{I_{tot}} \qquad 4.55$$

$$\sigma_T = T \cdot \sqrt{\frac{{\sigma_{L_{dw}}}^2 + {\sigma_{F_{dw}}}^2}{(L_{dw} + F_{dw})^2} + \left(\frac{\sigma_{I_{tot}}}{I_{tot}}\right)^2} \qquad 4.56$$

It is necessary to set to zero the corrected fluorescence outside the 650 to 850 nm range before calculating true reflectance (or transmittance). And, since the residual light next to the cut-off wavelength (even after correction) affects the shape of the retrieved fluorescence, F should be trimmed from 650 nm to where the filter cut-off is located before calculating true reflectance and transmittance.

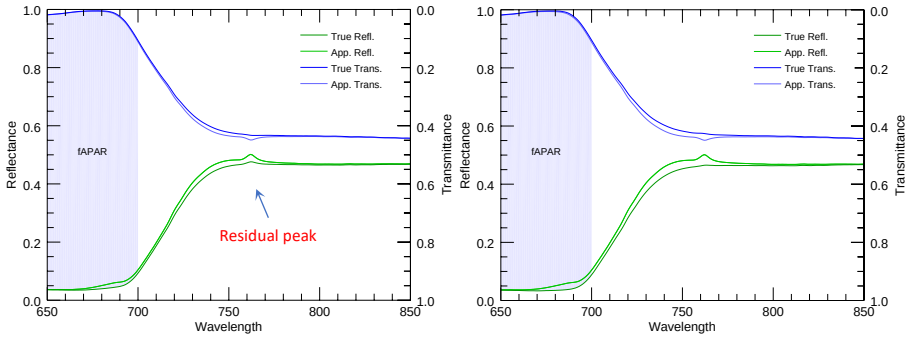

Figure 4-16 spectra of apparent (light colour) and true (dark colour) reflectance (green) and transmittance (blue) of a leaf sample. Left**:** by subtracting the measured fluorescence. Note a residual peak feature at 760 nm. Right**:** true reflectance and transmittance by subtracting the fluorescence after compensating for the filter's APAR reduction. Now the peak feature is gone.

4.2.7 KAUTSKY TRANSIENTS

When photosynthetic samples, kept in darkness (e.g., for 10 min) are illuminated, Chl a fluorescence intensity shows characteristic changes called fluorescence induction, fluorescence transient, or simply the Kautsky effect (Kautsky et al. 1931), named after Hans Kautsky (1891–1966). For higher plants and algae, Chl a fluorescence induction curve measured under continuous light has a fast (within a second) increasing phase, and a slow (within a few minutes) decreasing phase. A detailed account on this effect and its use to retrieve photosynthetic parameters can be found in (Stirbet and Govindjee 2011)

It is possible to measure the Kautsky transient on attached leaves using the FluoWat clip, and depending on the performance of the spectrometer used, capture the fast phase (with ~1KHz sampling rate or better), or just the slow phase (with ~10Hz sampling rate).

To perform this measurement, it is necessary to use an opaque disk on one of the slots of the filter slider to dark adapt the leaf. Then, while pointing to the sun, set the filter to suddenly illuminate the sample and induce the induction

kinetics. In order to properly record this phenomenon, in which the fluorescence emission rises suddenly and decays very fast, the spectrometer should be set to record continuously, with no spectra averaging, and to the minimum integration time possible that allows registering a significant signal with enough sampling rate.

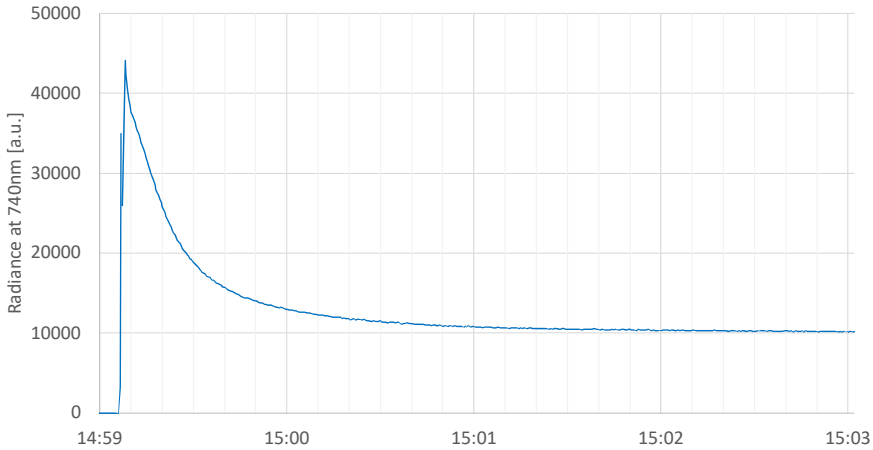

Figure 4-17 Kautsky transient of fluorescence emission at 740 nm after 30 min of dark adaptation, measured using a high sampling rate with an OceanOptics QE-pro.

Dark adapting the leaf should take a minimum of 15min, optimally 30min. For this measurement it is advantageous to set up the filter holder as: black disk, 650nm filter, clear, to easily shift the filter holder to dark adapt, capture fluorescence evolution, and then measure reflectance without breaking the sequence. Setting the instrument to save every spectrum for at least 5min (a full transient can last more than 20min especially if analysing the slow adaptation processes (Van Wittenberghe et al. 2019)) with no spectral averaging. It is very important to actively keep pointing to the sun while the measurement takes place.

This type of measurement implies that the leaf must remain clipped for a long time. This should not be a concern if the leaf is still attached or kept hydrated. But what about the available CO_2 for photosynthesis? The FluoWat has an inside volume larger than 15 ml. Assuming an average photosynthesis rate of 6 μmol/m^2/s (maximum of 40 μmol/m^2/s according to Salisbury and Ross (Salisbury and Ross 1992)), and considering 400 ppm CO2 in air, for a leaf surface of 1 cm^2 it would take 66 minutes to be consumed, or 10 minutes at the maximum rate. Therefore, the use of the leaf clip is acceptable for short measurements, but cannot be used for longer surveys.

Measuring the dynamics of fluorescence transients can also be done without dark adapting the sample, e.g., to analyse the effect of sudden changes of illumination, as with a passing cloud. Such an experiment is described in detail in Section 6.4 below.

4.3 SENSITIVITY ANALYSIS FOR POTENTIAL PERTURBATIONS IN THE MEASUREMENTS

4.3.1 PRELIMINARY CONSIDERATIONS

Despite that the clip provides a direct measurement of the fluorescence emission under natural conditions, the methodology and the design impose some limitations that need to be taken into consideration.

- The illumination port (opening) is set to 45° with respect to the sample. This fixed geometry implies that the sample leaves must be reoriented for the measurement. Besides, the illumination geometry, i.e., the amount of light, remains mostly constant despite the time of the day in which the measurements are taking place. Nonetheless, any change in the plant's fluorescence yield and NPQ resulting from its adaptation to the changes in the environmental conditions will be recorded by the measurements done with the FluoWat clip.
- Provided the geometrical design to accommodate the filter and the fibre tips and to keep the sample enclosed in a dark chamber, the major light source is the direct component of sun light. Therefore, there is a slight decrease of the energy arriving to the sample with respect to the global illumination. This decrease is in the order of 10-15% in PAR for clear days, being larger with high sun elevation, and most of the light dispersion is happening in the blue (Brine & Iqbal 1983). Besides, many leaves do not have full sky view, instead they are surrounded by less reflective objects that occlude the sky view, such as twigs, branches and other leaves. This reduction should not

alter much the photosynthetic efficiency of the sample, thus the fluorescence yield.

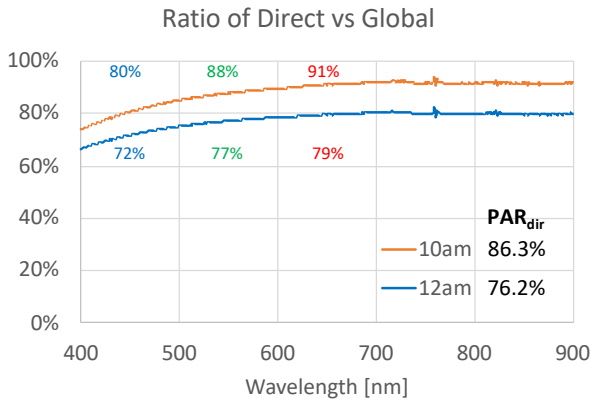

Figure 4-18 Ratio of direct illumination with respect to the global irradiance over a flat surface at 10am and 12am summer. Indicated are the percentages of blue, green, red and total PAR.

Nonetheless, the fact that only direct illumination is used needs to be taken into consideration when estimating the actual fluorescence emission under full illumination based on the FluoWat measurements.

- By placing the filter, the properties of the light reaching the leaf get altered. On one hand, there is the light removal that overlaps with fluorescence and allows the measurement including a portion of PAR. On the other hand, the transmissive portion the filter is not perfectly efficient and there is a reduction, even if small, of the remaining PAR light.

  Both reductions in PAR need to be considered when calculating the corresponding APAR that triggers the measured fluorescence. Last, there is a small amount of light that gets transmitted above the cut-off wavelength and adds to the measured fluorescence. How to remove this unfiltered light from the measurement is address in Section 4.2.5 above.

- When measuring the optical properties of the leaf, i.e., reflectance and transmittance, it must be taken into consideration that, since the illumination is the direct component of the light and a fibre optic is being used to collect the light, the actual properties correspond to direct direct-conical type, instead of the direct-hemispherical that is provided by an integrating sphere (Palmer 1995). Differences based on experimental data are addressed in Section 4.3.6 below.
- When measuring with the FluoWat it is important to take into account the fluorescence transients that are induced when changing the light reaching the leaf. When transients happen, they can last from a few seconds to several minutes. Provided that the FluoWat measurement takes place on sun adapted leaves, that the clipping takes only a few seconds and once in the clip the leaf still receives a similar amount of light, this transient shall not be intense neither too long. Some data supporting this statement is presented in Section 4.3.5.
- Unstable signal due to fibre optic twisting. The transmission of the light through a fibre optic depends on several parameters such as the refractive index (or indices) of the fibre's core, the angle of incidence of the light on the fibre's entrance, and the angle of incidence of the light on the walls of the fibre while traveling through it, among others. When the fibre is bended, the incidence on the fibre's walls changes modifying the propagation of light through the fibre, and it can even produce that the light exits the fibre before reaching the other end. It would be less problematic if two spectrometers are used simultaneously for the upward and downward measurements, avoiding the need to shift the fibre from the top to bottom ports. In any case, it would be impossible to ensure that the fibre will have

the same bending than when the spectrometer was radiometrically calibrated, thus resulting in an unavoidable offset and uncertainty in the measurement (note that this problem is common to any instrument that uses a fibre optic that does not have a fixed disposition, and it is not specific to the FluoWat).

This is one of the possible reasons for overlapping reflectance and transmittance in the NIR (negative absorption) that is found in some FluoWat measurements. Although this overlap can also happen if the leaf's angular response in reflectance and transmittance differs from Lambertian (see Section 4.3.6).

- It is also important setting of proper integration time (IT) in the spectroradiometer to maximize the signal level. However, some compromise must be taken, provided that the six measurements of interest present extremely different dynamic ranges (Figure 4-9). Setting a single IT adjusted for the white reference will be the easiest and fastest option, but it will lead to smaller signals in the detector when measuring filtered reflectance or transmittance. On the contrary, adjusting the IT to every single measurement might get the best signal for each, but it would make the data acquisition slower. Thus, depending on the spectroradiometer being used, its interface for operation and the implemented capabilities to automatically detect the best IT, one solution or the other might be most adequate.
- A frequent dark current measurement and correction must be conducted during any experiment using the leaf clip. A drift in dark current can result in biases in the estimation of all parameters, but particularly in the fluorescence due to its low intensity.

4.3.2 THIN WHITE REFERENCE

In order to measure the radiance reaching the leaf sample, it is necessary to use a reference panel. Ideally Spectralon (or similar material -Zenith polymer- with >99% reflectance) should be used since it is >99% reflective and highly Lambertian, according to the definition of BRF. However, the thickness of the reference panel makes it unpractical for the use with a leaf clip.

Instead, it is possible to use a thin film of the material (by using coatings: Spectraflect or Duraflect, or foils: ODM98), which has also negligible absorptance and Lambertian response, although its reflectance and transmittance will depend on its thickness, but it will be less that the ideal 99%. Thus, it is necessary to transform upward WR measurement to its 100% reflectance equivalent: 1) reflectance calibration, or 2) upward and downward measurement assuming negligible absorptance.

The figure below shows that difference in the measured radiance from a thin white reference (by adding the upward and downward radiances) and a Spectralon refence standard using the FluoWat is below the random error of the measurement at 1σ.

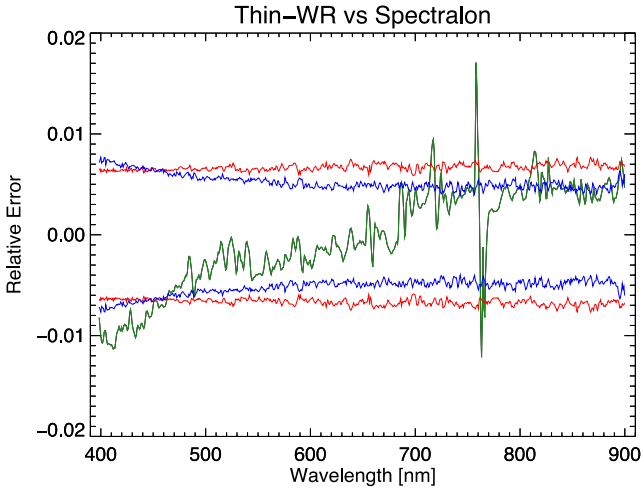

Figure 4-19 Relative error (green) from estimating total irradiance by adding the upward and downward measurements using the thin-WR when compared with the Spectralon. Note that the difference is within the boundary of the measurement stability: In red ±1 standard deviation from the Spectralon measurement (signal stability) and in blue ±1 standard deviation from the thin-WR.

4.3.3 Field of View

The clip was designed such that the sample area viewed by the fibre optic (with a typical numerical aperture of NA=0.22) is smaller than the illuminated area to avoid vignetting effects. These fibres have an approximate field of view of 25° and the fibre's tip is located at 15 mm from the sample, which translates to 6.6 mm of diameter on the surface of the leaf.

To verify this estimate and also obtain a profile on the sensitivity of the fibre the clip was placed on top a TFT-LCD screen (with the bottom part removed) where a colour line was swiping from one end to another, first horizontally (corresponding to the long axis of the clip from handle to tip), then vertically (from side to side of the clip) while the spectrometer was recording continuously. The colour was selected to be a combination of pure blue and pure

red. Blue was on in every scan line and red was on only every two lines. In this way it was possible to obtain a continuous profile from the blue channel, while the red channel also provided a mean to count how many lines had been detected. The line scanning helped to quickly determine the area of sensitivity of the spectrometer. Once identified, a small dot of 3x3 pixels ran across and along the area using the same colour strategy to distinguish the position from the radiometry. The results are shown in Figure 4-20 only for the across-track axis since both are almost identical.

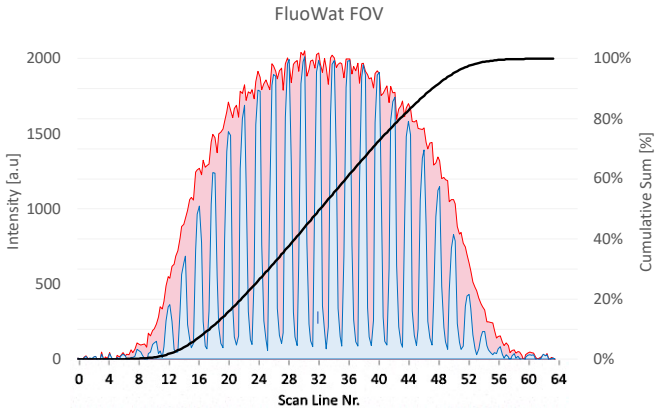

Figure 4-20 Sensitivity for red and blue light across the field of view

The screen used is an IL9342C from an M5Stack CORE2 microcontroller. It is 46.753 mm wide in the horizontal axis with 360 pixels, and 35.064 mm high in the vertical axis with 240 pixels according to specifications, resulting in a pixel size of 0.13 mm/pixel horizontally and 0.146 mm/pixel vertically.

From the graph one can easily determine that the full sensible area cover 50 scan lines, and the FWHM is 35 lines wide. Therefore, the actual diameter of the sampling is ~ 7 mm, with a FWHM diameter of 4.8 mm where more than 90% of the light is collected. Similar numbers are obtained from the bottom fibre, as expected.

4.3.4 SENSITIVITY TO TILTING (MISALIGNMENT WHILE MEASURING)

One thing to be careful about while doing measurements with the FluoWat using the sun as the main light source is to keep a stable pointing throughout the measuring sequence.

To help in keeping an accurate pointing the FluoWat counts with a Sun finder. It helps to point the clip straight to the Sun.

In the previous section we have seen that the FOV of the fibre is smaller than the illuminated area. Therefore, slightly tilting the clip during the measurement will not compromise the target from being fully illuminated. However, the change in the incidence angle of the illumination might cause variations that correspond to the cosine-law and given that the light reaches the sample at 45° the variation is not negligible.

To evaluate the impact of an incorrect pointing, and to check if there is any other effect besides the cosine-law, an experiment was conducted. The FluoWat was fixed to a tripod to ensure a steady measurement and pointed to the Sun with the best alignment. Then, the clip was tilted up and down in increments of 1°, with an offset of 0.5° in the one of the directions to increase the representation of angles. The pointing angle was measured with a mechanical goniometer with 1° markings, so the precision in the angle measurement is limited to 0.5°.

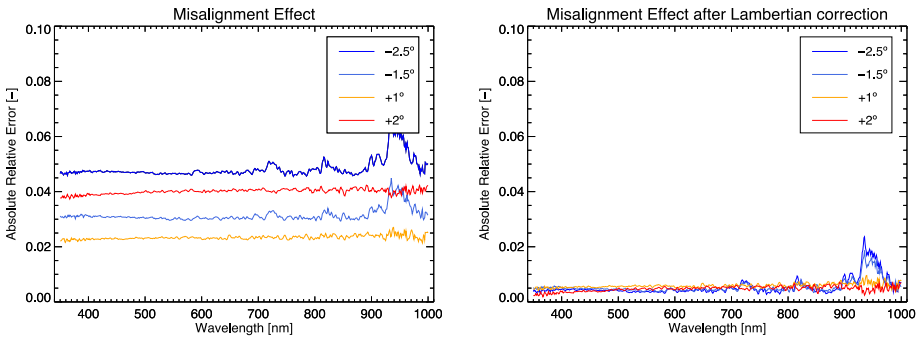

Figure 4-21 Absolute relative error (left) caused by tilting the clip and misaligning the sun and after correcting by the cosine law (right). Note: the peak around 950 nm is due to water vapour.

The results show that pointing errors can lead to substantial radiometric errors of ~2% in radiance for a tilt of 1° and that the deviation is spectrally even. Applying a compensation factor of the respective cosine of the incidence angle the relative radiometric error falls below 0.5% for all four measurements. This indicates that the only effect is due to the cosine-law, and that other effects, like vignetting or unwanted reflections on the clip's internal surfaces, can be discarded.

The inclusion of a sun-finder permits an accurate pointing with misalignments smaller than 0.5°. The use of a tripod is recommended but not always possible in the field, so the sun-finder allows manual operation minimizing the errors.

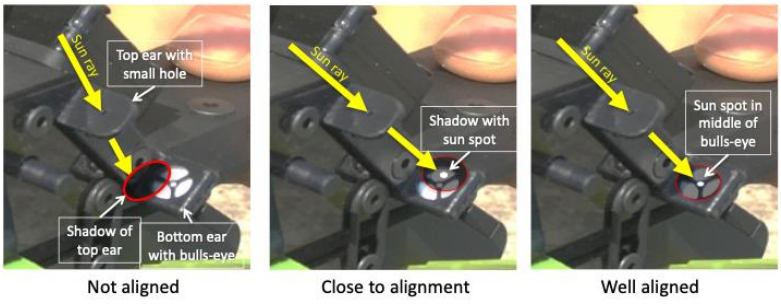

Figure 4-22 Illustration of Sun alignment procedure using the sun-finder

4.3.5 SENSITIVITY TO TRANSIENTS

Whenever the leaf is subjected to a change in the illumination conditions, either naturally or purposely, there is an adaptation of the photoactive mechanisms that ultimately translates in a dynamical response (transient) in the fluorescence emission. Provided that to perform a measuring sequence with the FluoWat it is necessary to place and remove a filter, there will be an induction of transients in each of them. It is, thus, important to quantify the magnitude of those transients and how long do they last, in order to avoid performing the measurement while they are on; otherwise, the recorded fluorescence would not completely correspond to the steady state.

A simple experimental test was performed outdoors on an ivy leaf using an OceanOptics QE-Pro set to fast sampling rate continuously recording selected bands.

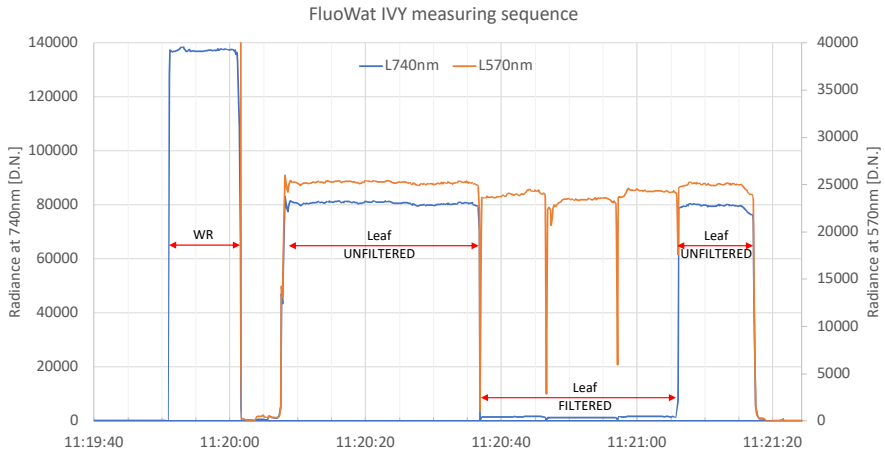

Figure 4-23 Time resolved recording at high frequency of radiance at 570 nm and 740 nm during a full measuring sequence to determine the magnitude and duration of fluorescence transients. The radiance at 570 nm serves to provide a reference to distinguish transients from illumination instability (since there were some thin clouds). Note each radiance is plotted independently in magnitude to facilitate comparison.

Figure 4-23 above shows a whole sequence of the radiance at 740 nm resolved in time at high sampling frequency. Overplot of the radiance at 570 nm for reference of illumination (in)stability.

The sequence shows the measurement of the WR lasting 10 s, then a gap while the WR was replaced by a leaf. The first leaf measurement corresponds to unfiltered light, and the signal is slightly larger than from the WR (i.e., a reflectance >50%). Note the shaky signal at the start while repointing to the Sun, one might be tempted to relate it a fluorescence transient but looking at the radiance at 570 nm (in orange) the same pattern is found, thus, related to illumination change due to realignment. This measurement lasts ~30 s. Then, three filtered measurements of 10 s each (they will be discussed below). Followed by the removal of the filter for a second time. Again, the instability of the signal is related to small variations in the illumination, and there are no detectable signs of a fluorescence transient.

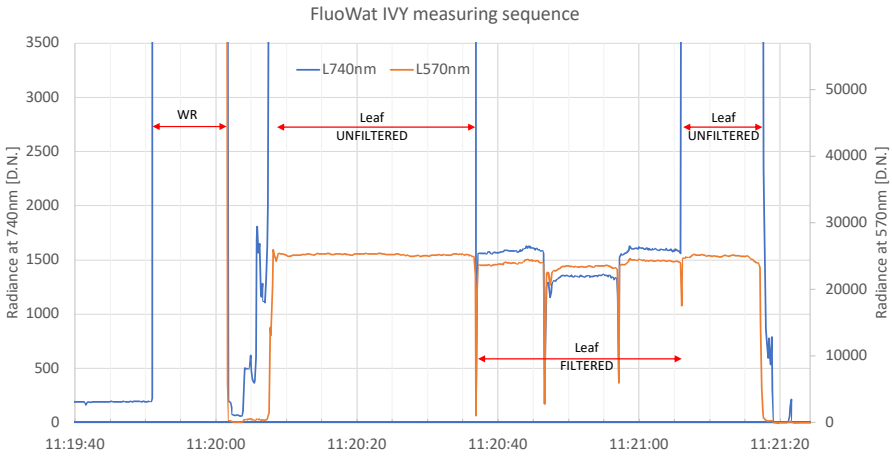

Figure 4-24 Time resolved measuring sequence (as in Figure 4-23) rescaled to show the evolution of the signal while the filters were on.

Figure 4-24 is a rescaled version of the previous one to show the pure fluorescence signal (solar radiance is being blocked). The first filter being placed is the Φ675, followed by the Φ650 and returning to Φ675 before removing the filter. Each measurement lasted 10 s. Any fast transient in fluorescence would not be reflected in other wavelengths. However, here we observe that variations in F_{740} have the same trends than those recorded at L_{570}, indicating that they were due to handheld unstable pointing.

Therefore, even when induction kinetics is a phenomenon to be considered when doing fluorescence measurements with changing light conditions, the change in PAR caused by the filters is sufficiently small to prevent undesirable transients (at least with the current detection threshold). The filters here used are high grade OD4 (or better) with a sharp cut-off and extremely transparent below the cut-off wavelength.

However, this doesn't mean that transients do not happen. A laboratory measurement was conducted under stable light conditions using a high-power multi-LED lamp, that provides 1000 µmoles of white light with a continuous and almost flat spectrum from 400 to 720 nm, composed by 12 individual LEDs, and each one can be adjusted in intensity independently.

An ivy leaf was dark adapted for more than 30 min and then exposed to the light and left undisturbed for its slow adaptation to reach full stability after 2 h, then a sequence of light transitions was performed limiting the light above 675 nm first, and above 650 nm next to observe the fluorescence response to changes similar to the ones performed under natural conditions. Figure 4-25 shows the whole series of the measured radiance at 740 nm of this experiment.

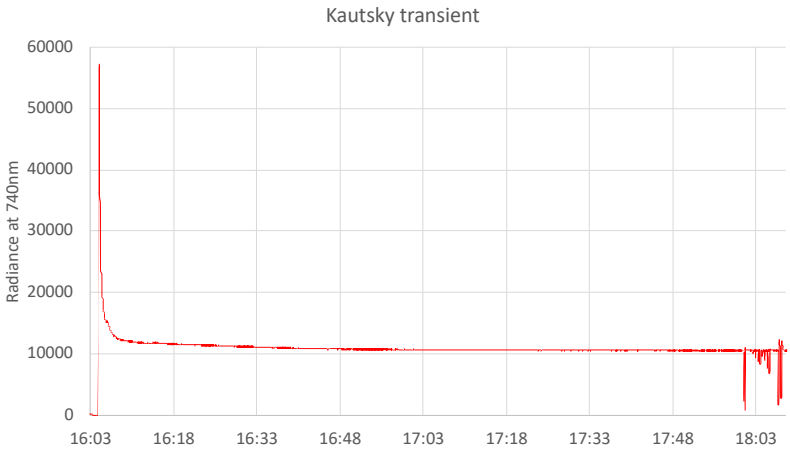

Figure 4-25 Time series of fluorescence at 740 nm from a dark-adapted ivy leaf suddenly exposed to light until reaching steady state after nearly 2h, when it was subjected to light reduction in the far-red.

The expected Kautsky effect appeared when first exposing the dark-adapted leaf to the light. This fast transient reaches a peak of emission almost 5 times larger than the fluorescence at the start of the slow adaptation, and it lasted 4 minutes, as it depicted in Figure 4-26.

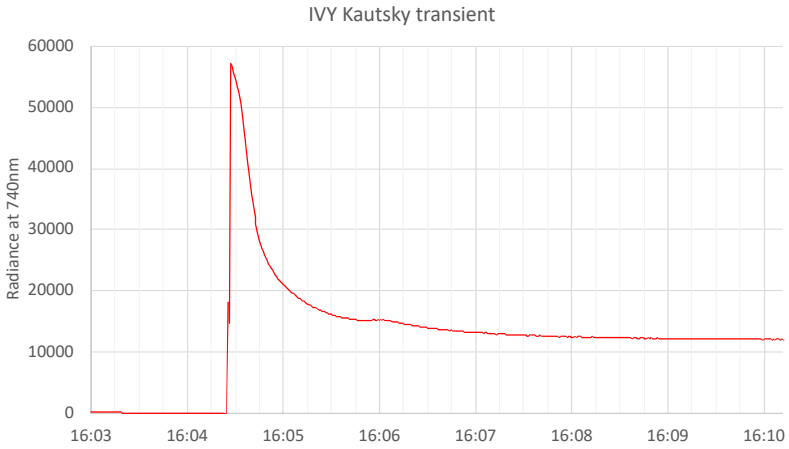

Figure 4-26 Detail of the first minutes of a Kautsky transient of fluorescence emitted at 740 nm on a dark-adapted ivy leaf after sudden exposure to illumination.

Once the photosynthetic apparatus was fully stabilized (after ~2 h) a sequence of light modulation began (Figure 4-27). First a quick (~1 s) dark transition took place causing a small induction kinetic of less than 5% of the steady state fluorescence that lasted ~10 s. Then modulation of the light above 675 nm started by reducing its intensity to 75%, followed by 50%, 25% and finally being completely off. Between each modulation the leaf was exposed back to full PAR. Neither of these light changes caused a transient. Then, the modulation was applied to light above 650 nm following the same pattern. In this case, a small transient was recorded when going back to full PAR after completely removing the light above 650 nm, and even in this case the peak detected was smaller than 2% of F_s lasting ~5 s.

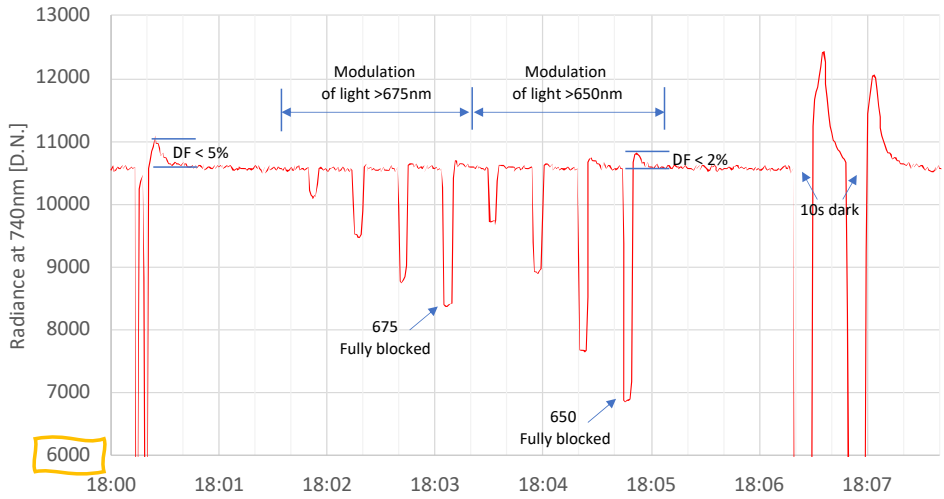

Figure 4-27 Detail of the fluorescence dynamics at 740 nm during a sequence of far-red light decrease at four steps (25%, 50%, 75% and 100% reduction) first on light above 675 nm (18:01:45) followed by light above 650 nm (18:03:15). The sequence ended with two periods of 10s of full darkness. This sequence was designed to evaluate the magnitude and duration of the transients of a light adapted leaf to changes in illumination similar to the ones produced by the FluoWat filters. Note that the y-axis does NOT start at zero to ease viewing the small changes in the fluorescence signal.

The experiment ended by setting the leaf in complete darkness for 10 s twice. In both cases the transient was larger than when the red light was blocked. And also, larger than when a short period (1 s) of darkness was applied at the start of the sequence.

These results indicate that the perturbation of placing the measuring filter is smaller than the natural changes in illumination. But even brief dark conditions (e.g., clipping the leaf and waiting some time until pointing to the sun) translate in measurable and lasting fluorescence transient. Therefore, it is recommended to point to the sun right after clipping the leaf to minimize the fluorescence transient, wait a few seconds and start by the unfiltered measurement (to get reflectance) before doing the filtered measurements (to get fluorescence) to allow for the transient to end.

4.3.6 Leaf Reflectance and Transmittance

The optical properties measured with the FluoWat leaf clip is based on directional illumination and collection. Most models are based on hemispherical measurement of these properties, and thus, it was evaluated how well both approaches matched. It was done on a set of 12 trees of 6 species, both evergreen and deciduous, with 3 leaves per tree, collected during the HYFLEX-US campaign in 2014 (Middleton et al. 2017). The sampled species are white oak (WO), red oak (RO), red maple (RM), hickory (H), poplar (P) and loblolly pine (LP). Leaf reflectance and transmittance were measured, both with an integrating sphere and FluoWat in sequence (so that there was no degradation in between).

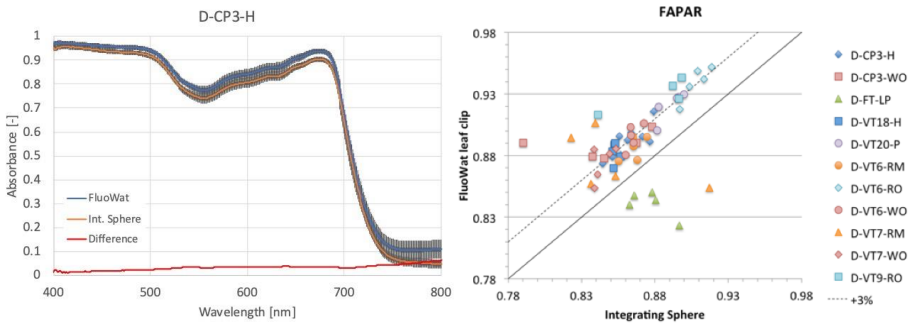

Figure 4-28 Comparison of absorptance (left) and APAR estimation (right) using the FluoWat leaf clip and an integrating sphere.

It is found that the loblolly needle samples (D-FT-LP in Figure 4 28 right) were less matching than broadleaves, but that could be due not only to the differences between direct and diffuse measurements, but also to the effect of the gap between needles (Rajewicz et al. 2019).

There is indeed a difference between the directional-conical measurements with the leaf clip and the directional-hemispherical reflectance and transmittance obtained from an integrating sphere (Hovi et al. 2017). However, from a practical point of view, measuring in the field with the integrating sphere is rather problematic, since it is bulky, requires an artificial light source (this used to be more problematic some years ago) and it is challenging to measure with the leaf still attached to the plant. The advantages provided by the FluoWat in terms of operability in the field might be sufficient to accept the small offset in this measurement.

There is also another bias that appears in some cases in which the absorptance calculated from the reflectance and transmittance measured with the FluoWat results in negative values in the NIR region. This might be caused by some perturbation during the measurement (e.g., illumination instability during the

measuring sequence), but it can also be due to a non-Lambertian angular response of the reflectance and transmittance factors.

Estimating reflectance from directional-conical measurements assumes Lambertian behaviour of the surface (Figure 4-29 left), extrapolating the radiance measured by the sensor (red arrow) to the total reflected flux (circle). If actual reflectance factor does respond to a Lambertian distribution (Figure 4-29 middle and right) the estimated reflected flux (dashed circle) will not correspond to the actual reflected flux (blue ellipse) resulting in an overestimation (Figure 4-29 middle) or underestimation (Figure 4-29 right) of the reflectance factor.

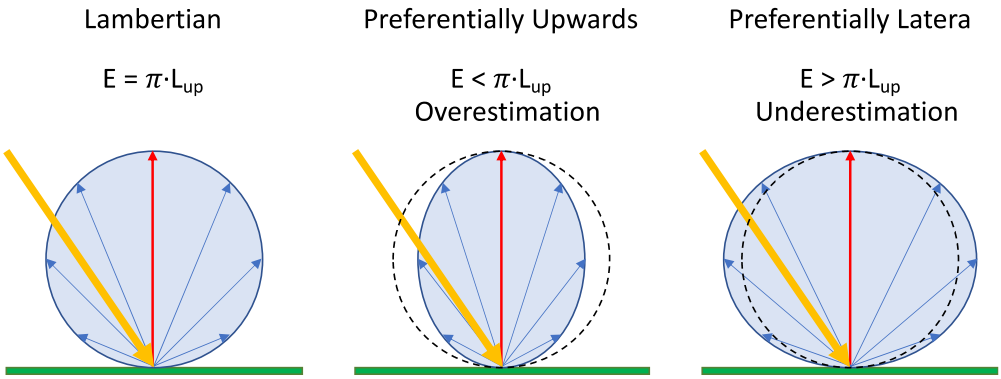

Figure 4-29 The assumption of Lambertian response of the surface (left) allows estimating the reflected flux (blue circle) from the measured radiance (red arrow). In a non-Lambertian surface the estimated reflected flux (dashed circle) will not correspond to the actual reflected flux (blue ellipse) resulting in an overestimation (middle) or underestimation (right) of the reflectance or transmittance factor.

4.3.7 Assessment of the Fluorescence Yield Using Different Filters

To measure fluorescence, it is necessary to use a lowpass cut-off filter. With the increased offer of cut-off wavelengths for the filters it is possible to choose

which one would be the most adequate. Currently there are three available off-the-shelf, each with a different cut-off wavelength: 650 nm, 675 nm and 700 nm. The filter Φ650 allows capturing the full shape of the fluorescence emission at the expense of reduced red PAR (14% less than with full sunlight).

While Φ700 allows almost full PAR to reach the leaf but only the far-red fluorescence peak can be measured.

Φ675 is a compromise between the two, getting a higher amount of red light (losing just 6% of total PAR) and measuring the full FRF peak, while the RF peak is only resolved from the top towards the NIR but losing the starting wing of the RF from 650 nm to ~680 nm. It is possible to use two of them in sequence to get a combined measure.

In order to evaluate the actual performance of each filter, a set of measurements were conducted using all three filters on three different leaves: one ivy leaf, one hibiscus young leaf and one hibiscus old leaf.

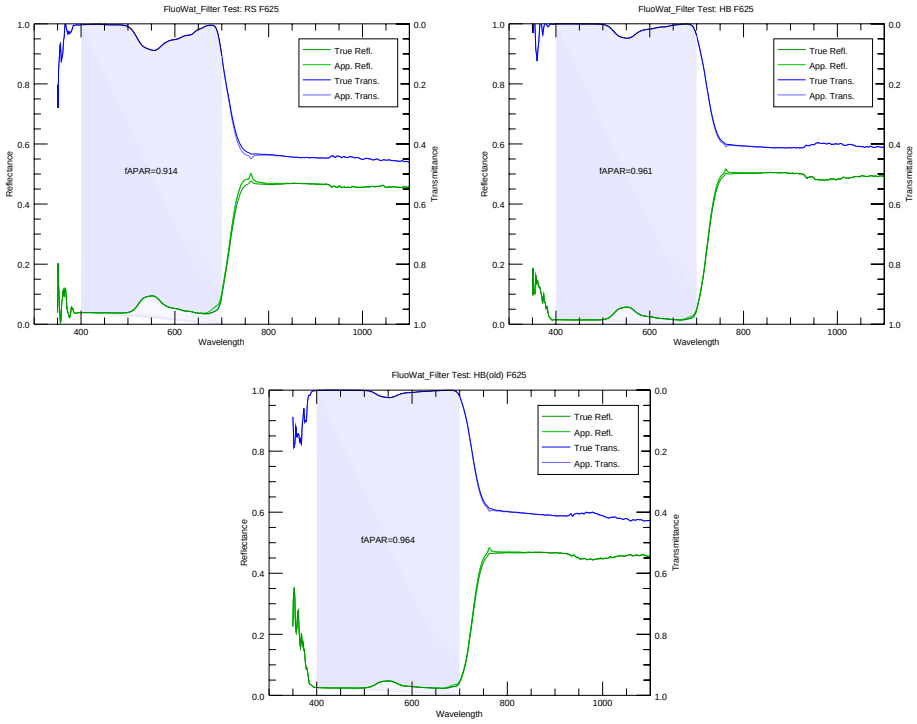

Figure 4-30 Reflectance, transmittance and absorptance from the three leaves.

At the time of this experiments the manufacturing of the filters was still not ideal, so the actual cut-off wavelengths are not exactly located at their nominal values. In this case they had an effective cut-off at 645 nm, 675 nm and 725 nm respectively. Even, the wavelength at which fluorescence can be measured with some accuracy is even further towards the longer wavelengths. Nowadays, available filters have a better build, and their cut-off is closer to specifications.

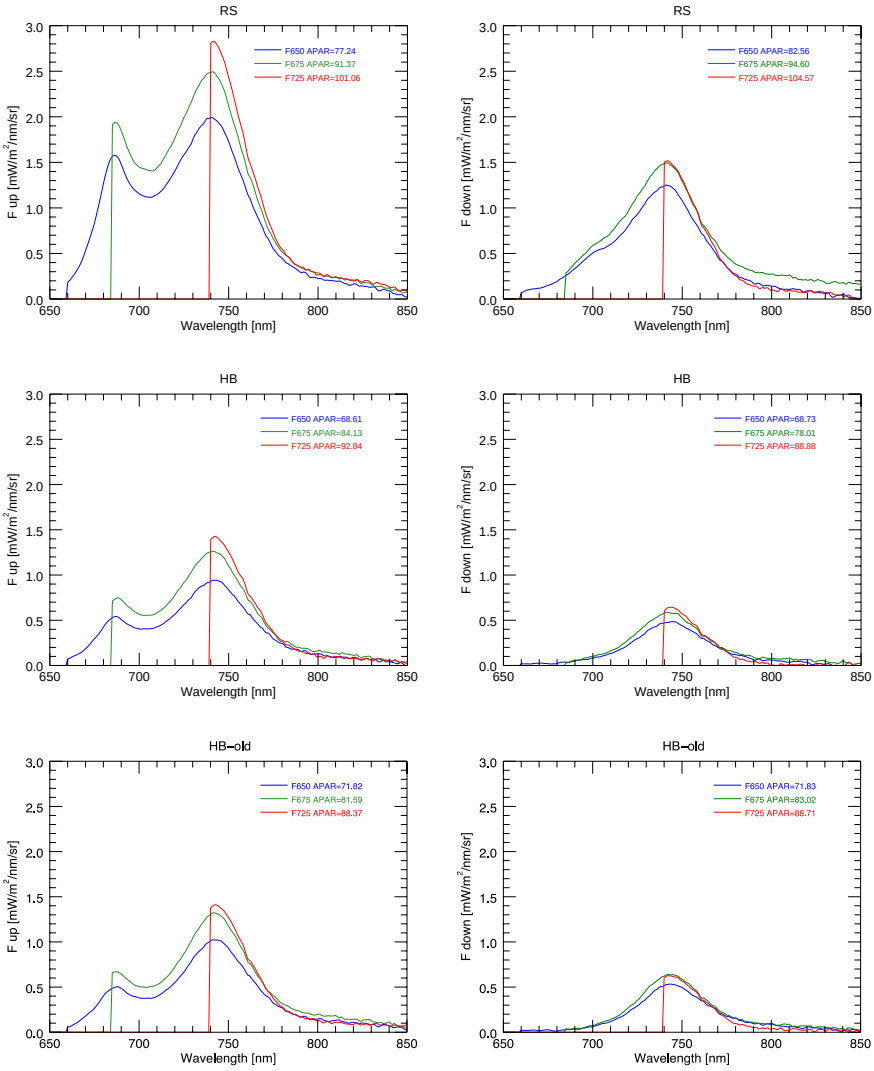

Figure 4-31 Fluorescence as measured with each of the three filters (red for Φ725, green for Φ675, and blue for Φ650) from the adaxial side (left column) and the abaxial side (right column), for three different leaf types (rows)

When looking at the SIF emission, the magnitude increases with each filter (from Φ650 to Φ725) as expected, since more light reaches the sample when the cut-off shift towards the NIR.

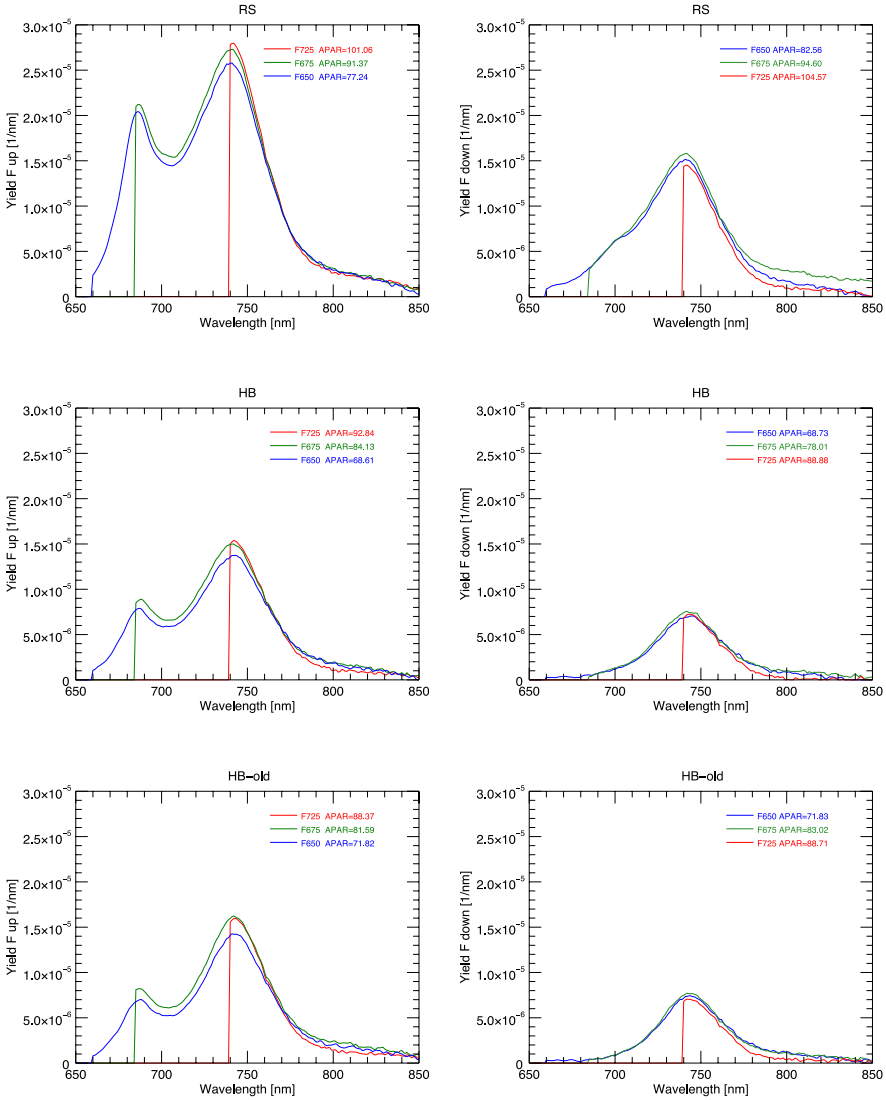

Figure 4-32 Fluorescence yield corresponding to the fluorescence in the previous graph

Calculating the fluorescence yield with the APAR corresponding to each filter, the three spectra come close to each other. For the upward emission, the fluorescence yield obtained with the Φ725 filter gives almost equal values than

with the Φ675. Whereas the yield obtained with the Φ650 filter is systematically slightly smaller than the other two. The downward yield is almost equal for all three filters.

This behaviour of matching yields with Φ675 and Φ725 and a slightly reduced yield with Φ650 is replicated in all the tested leaves.

Moreover, the shapes of the spectral emission are almost identical (within the range where they overlap). The ratio between the three shapes (in Figure 4-33) shows that they are basically the same except for a scaling factor. The constant relationship breaks above 775 nm, where the signal is already sufficiently small to be affected by the inaccuracies of the stray light removal, the dark noise removal and the instrumental noise. This almost flat ratio is indicative that the shape of the fluorescence emission is maintained regardless of the filter used.

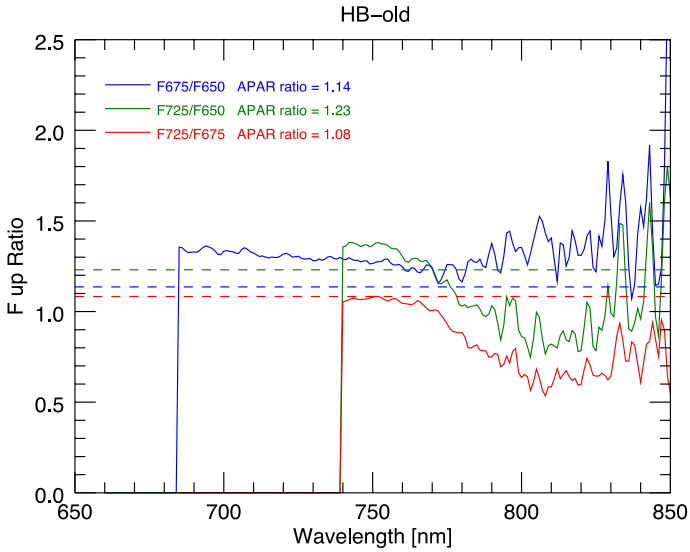

Figure 4-33 Ratio of fluorescence spectra measured with each of the three filters. The flatness of these ratios (up to 775 nm) indicate that the spectral shape has a minimal change regardless of the filter used.

Two conclusions can be brought from these results:

- The apparent fluorescence yield remains constant when the decrease in APAR is small, that it, using filter Φ725 and even Φ675; but it begins to decrease with filter Φ650 that blocks more of the reaching PAR.
- The shape of the fluorescence emission remains unaltered regardless of the filter used, at least down to the RF peak at 685 nm.

Thus, it is most convenient to use the filter with cut-off at 675 nm in order to obtain the actual magnitude of fluorescence yield and fluorescence emission spectrum. Still, it might be suitable to use a filter with cut-off at 650 nm at the expense of underestimating fluorescence within a reasonable amount.

4.4 PERTURBATIONS AND COMPENSATIONS

Besides error propagation from instrumental and random measuring errors, it is also important to take into consideration the variable nature of the elements that participate in the measurement: one being the own dynamic nature of the photosynthetic processes, the other the stability of the illumination under natural conditions. On top of that there are also instrumental perturbations that need being considered.

4.4.1 OVERESTIMATION OF FILTER TRANSMITTANCE AS MEASURED WITH FLUOWAT

Knowing the filter transmittance is necessary to process the measured data, particularly to obtain the clean spectrum of fluorescence emission reed of any residual reflected light.

In principle, it would be possible to get the filter transmittance from the FluoWat measurements, since the WR can be measured with and without the filter under the same illumination conditions and transmittance would be the ratio of both.

However, measuring in this way introduces some distortions that affect the estimated transmittance in the spectral portion that is being blocked (Prabhat and Turan 2017).

This means that the spectrometer is going to report a slightly larger amount of light than what actually passed the filter. Thus, the retrieved filter transmittance would be larger than it should, leading to overcorrection, even negative fluorescence at the IR tail over 800 nm and in extreme cases negative valley between peaks.

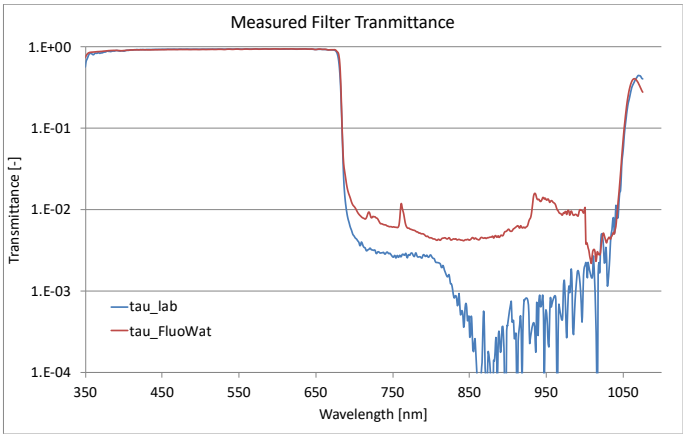

Figure 4-34 Filter transmittance of the same Φ675 filter measured under laboratory conditions (blue) and using the FluoWat (red). Scale is logarithmic to enhance the differences for low transmittance values.

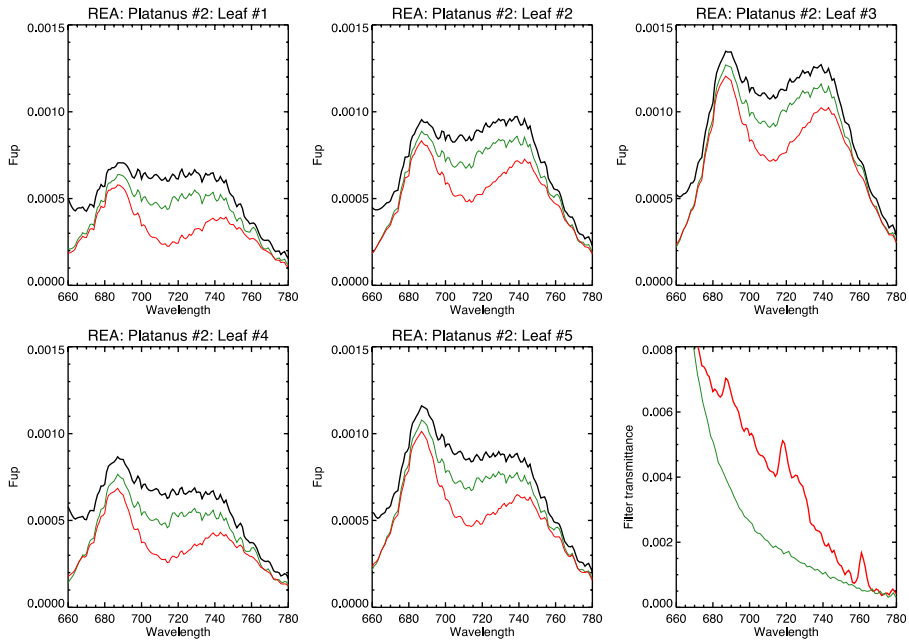

Figure 4-35 Upward fluorescence from five Platanus leaves of the same tree applying filter correction using τ from the field measurements (red) and from laboratory characterization (green); measured upward radiance (black). Bottom-right: filter's transmittance characterized in the laboratory (green) and from the FluoWat measurements (red). Transmittance is below 1% in both cases.

A laboratory characterization of the filter's transmittance is, thus, desirable. And it can be accomplished even with commercial spectrometers and a simple setting (at the expense of accuracy), as described in (Prabhat and Turan 2017). For this work we used exactly the same spectrometers that were used with the FluoWat.

Nonetheless, it might happen that a laboratory characterization of the filter's transmittance is not available, and the filter used is not of very high quality (OD4 or better). Then, since the overestimation depends on the filter transmittance (in the blocking range), it would be more favourable not to apply the filter correction equation (Eq. 4.22) and take the fluorescence directly from the L^{Φ} measurement. Otherwise, the correction will result in an error of a greater magnitude than the stray light itself, as would be the case in Figure 4-35.

It is worth noting, in the above figures, the peaks present in the filter transmittance measured with FluoWat. They correspond to atmospheric absorption features, and one could think of stray light contamination from outside of the dark chamber. However, many tests have been made until ruling out this possibility. The most probable cause being the contribution from the wings of the spectrometer response function (Alonso 2017, Siegman 2019). These features are present in all the outdoors measurements regardless of the spectrometer being used (ASD-FSFR, OO-USB4K and OO-QEpro), although with different magnitude.

It is then recommended to use the filter transmittance as measured in the laboratory for any calculations, when possible.

4.4.2 Compensation of Unstable Illumination Conditions

One limitation of this design is that the illumination reaching the sample is not monitored while the sample is being measured. This implies the assumption that illumination remains stable (and that the pointing is fine and steady) during the rest of the sequence. However, it is not unusual that high cirrus clouds produce a slight change in the sun light intensity that translates to a measured sample radiance that does not fully matches the measured radiance from the WR. This is especially true for long sequences when using several filters and a single spectrometer which fibre needs to be switched from top to bottom position. Having a second WR measurement at the end might help, first, to determine stability (or the lack of it) by comparing it to the former WR measurement; and second, to get a reference that is closer in time to the last measurements from the sample in case that instability is detected.

The main handicap is that there is no easy way to determine how the illumination has evolved while measuring the leaf. A first guess would be to monitor the reflected radiance in the NIR above 800 nm where SIF contribution is minimal. Unfortunately, at these wavelengths the filter's transmittance presents spikes, and it is very variable (see Figure 4-12 in page 42), and since transmission edges vary with incidence angle of the light, any registered changes might be misleading and not necessarily due to unstable environmental illumination but to unstable pointing.

A better option is within the VIS region, particularly towards the blue wavelengths, since the green-red are subjected to changes in absorptance due to photoprotective mechanisms. Besides, the filter's transmittance is very high (close to 100%) and fairly constant from 400 to 650nm (in high performance filters). Regarding the sample's stability in its spectral response, the best option

is using the green bands, even if they are subjected to changes in absorptance due to photoprotective mechanisms since these changes are typically slower than the time required for the full set of measurements. Focusing on the blue band, much more stable, might be suitable for monitoring the reflected radiance, but since the blue light is barely transmitted by the leaf this band is not suitable for the measurements from below, and only the green is susceptible to be used.

To illustrate this, we can look at the set of measurements used in Section 4.3.7 since they actually suffered from illumination changes.

Three samples were measured: one ivy leaf, one new leaf and one old leaf from a Hibiscus plant; and three different filters were used: Φ650, Φ675 and Φ725.

The measuring sequence was: WR→WR+Φ→RLF→RFL+Φ→TRN→TRN+Φ

The filters were place in sequence: Φ725 → Φ675 → Φ650

Measuring each leaf took around 2'30", and the elapsed time to record the three samples was less than 15 minutes. The recorded PAR for each of them was 124 W/m^2, 108 W/m^2 and 100 W/m^2 respectively. Only 2'27" elapsed between finishing the ivy leaf (the first) and starting the hibiscus sequence (the second), and there was already a substantial decrease in light intensity due to cirrus clouds.

If we take as reference the leaf reflected (and transmitted) radiance measured without a filter, assuming that they have the least distortion, then it is possible to apply the available filter/s transmittance (view Section 4.2.2) to get an estimate of what the signal should be when measuring with the filter/s and compare with the actually measured spectrum. In Figure 4-36 below, how the measurements using the three filters (left) are much below the curve of the

unfiltered reflected radiance than what would be expected from the estimated values (right).

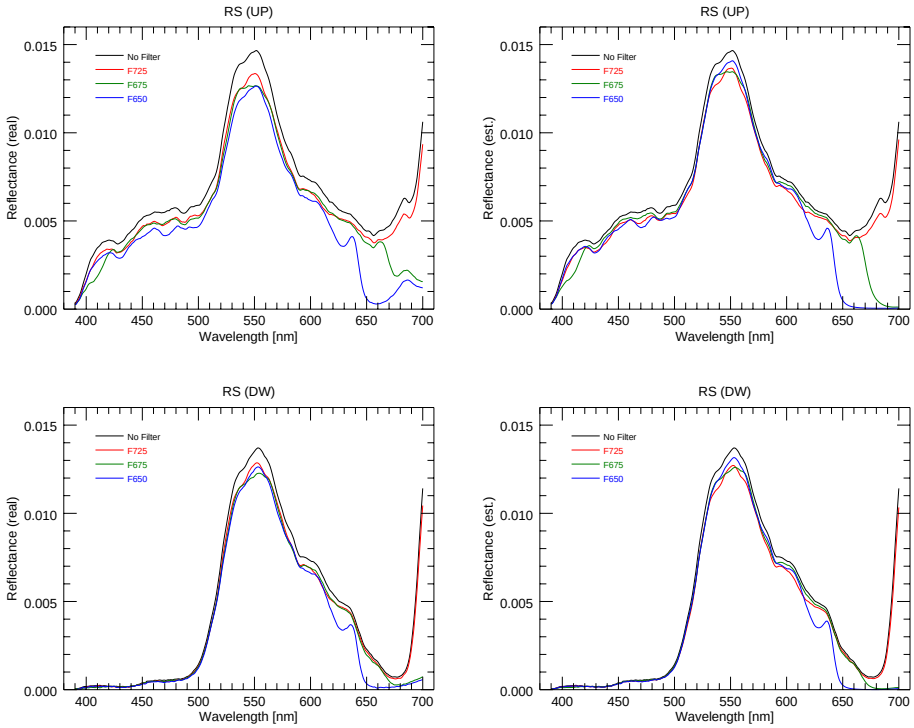

Figure 4-36 Example where was a light decrease during the measuring sequence. Three different filters were used. Left: Spectra as they were collected; reflected radiance (top row) and transmitted radiance (bottom row). Right: estimated radiance spectra as they should have been if there was no change in illumination, obtained by applying the known filter responses to the unfiltered radiance (black line)

A slight change in light intensity would translate in a global increase/decrease of the spectrum without introducing spectral distortions (a different matter would be if the change in illumination is of a greater magnitude, e.g., cloud passing or change in aerosol load, in this case the change in WR measurement from one sample to another show a decrease in intensity more severe in the blue than in the rest of the sun light spectrum).

If we calculate the ratio between each measurement with filter and the unfiltered radiance after applying the corresponding filter transmittance, in the visible, we can check if this assumption holds. In case that the illumination was stable during the measurement sequence the ratio should produce a flat line close to 1. This illustrated in Figure 4-37, where one can see that all the ratios are quite flat below their corresponding cut-off wavelength meaning that there are not substantial spectral distortions (neither from the photoprotection mechanisms). From them, the measurement using the Φ725 filter (red line) is closer to unity, i.e., with lesser change in the light level. This is reasonable since it is the measurement following (or preceding) to the unfiltered one, so unless there is a sharp change the variation should be smaller than for the rest of measurements that are further in time. It is also clear that the largest variation in illumination happened during the measurement from the top (UP), while the measurements from below (DW) were more stable (although not completely).

It is also evident that for the DW measurements, the low signal in the blue (from 400 to 500 nm) is too low to provide a reliable value due to the instrumental noise, thus the need to do the evaluation in the green region.

Note that since the comparison is made between the measurements with and without filters, the ratio only gives useful information well below the cut-off wavelength. In the graphs below, the cut-off is represented by a sudden drop in the ratio values. Besides, provided the low signal in the transmitted blue radiance, the ratio presents a large noise.

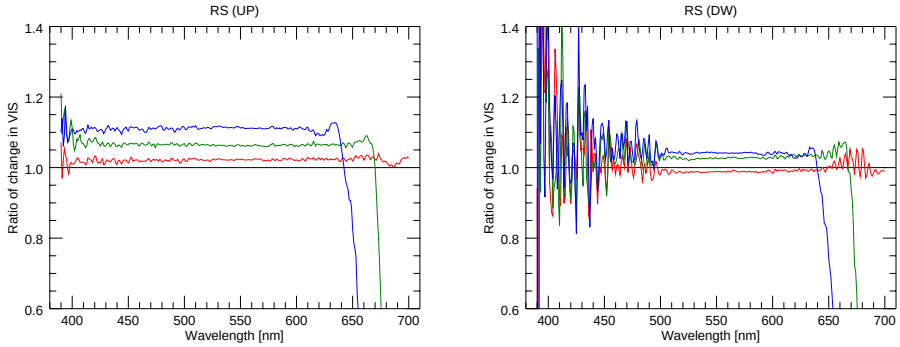

Figure 4-37 Ratio between the filtered spectra and the unfiltered spectrum. Left: results from the reflected radiance. The ratio drops after the cut-off wavelength of each filter. Right results from the transmitted radiance. Note the increased noise levels in the blue where transmitted light is close to zero.

Since the relationship is spectrally flat it is possible to use a simple scaling factor for the whole spectrum. And, in a first impulse, one might be tempted to scale the measurement from the leaf to make it compatible with the white reference radiance. However, even if this option might be suitable for the reflective portion of the measurement (VIS), the fluorescence emission would also be scaled; but fluorescence just does not scale to light variations in the same proportion than the reflected light. Instead, it does so through the fluorescence yield (YF). So, the proper way to proceed is to scale inversely the WR measurement I^*_{tot} and calculate the APAR corresponding to the used filter in order to obtain the YF from that measurement.

$$I^*_{tot}(\lambda) = I_{tot}(\lambda)\frac{L^{\phi}_{VIS}}{L_{VIS}\cdot\tau_{VIS}} \qquad 4.57$$

If we calculate the YF directly from the I_{tot} measurements as they were collected one gets a different level from each filter of the upwelling yield (Figure 4-38 left) and the downwelling yield (Figure 4-38 right). It is worth noting that the up and downward measurements were taken in sequence and not simultaneously, so

illumination changes are different for both. However, the differential efficiency of the different colours exciting the fluorescence emission might account for minor differences in the yield. It can also be seen that the downwelling fluorescence yield (right) is quite similar for the three cases, in concordance with the low estimated ratio of illumination change for the three measurements.

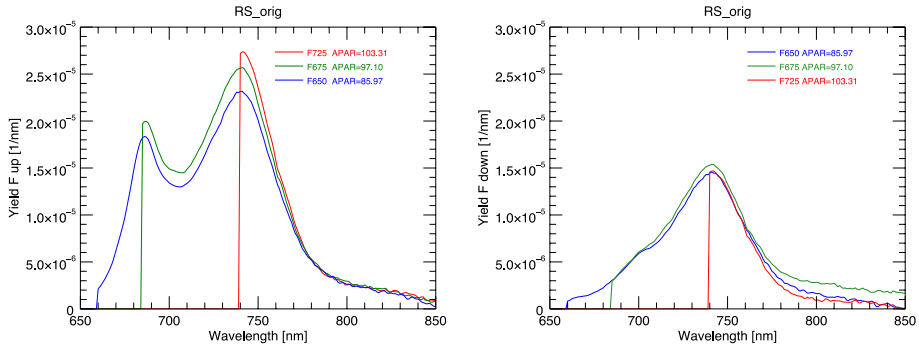

Figure 4-38 Fluorescence yield calculated without compensating the unstable illumination.

Applying the proposed method to the data above, the newly estimated YF shows a better match between the different filters in the case of the upwelling yield, while the downwelling yields remain grouped as before.

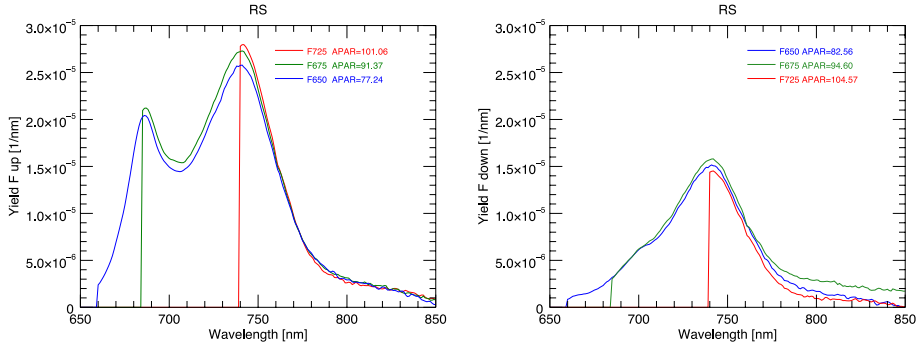

Figure 4-39 Fluorescence yield calculated after compensating the unstable illumination. Note that the three estimates with each filter are now much closer than in Figure 4-38 where unstable illumination was not compensated.

It is important to keep in mind that this correction is a mere approximation, and there is the very strong assumption that the unfiltered reflected/transmittance radiances are unaltered to be used as reference. This, of course, might not be the always the case, and one should pay attention to each particular case while processing the data.

4.4.3 Other Sources of Stray Light

Fluorescence is very small signal in comparison with the reflected/transmitted component of a leaf's radiance. Thus, any inaccuracy in the measurement translates into an error in the estimation of SIF or even preventing the retrieval itself.

Some of the sources of error are:

Erroneous dark current correction (e.g., if not taken frequently, or estimated by software). Dark current is signal reported by the spectroradiometer, but it is not caused by arriving photons, but by the electronics. This DC adds to the signal produced by the light and must be subtracted from the reported measurement in order to get a clear signal. Since fluorescence is small and measured through a blocking filter, an improper DC correction might be sufficient to introduce an offset of the same order of magnitude. Since DC can drift over time with the detector's temperature (among other causes), failing to update the DC estimate will lead to offset results.

Dark measurements can also capture stray light coming from the outside, e.g., by pouring in through the instrument's cooling fan that has not been properly baffled. This might be much more difficult to correct if dark measurement is not taken for every sequence, since it depends on the outside conditions.

Incomplete closure of the ports' shutters. When only one spectrometer (i.e., one fibre) is available, the fibre is placed on one port for the measurement, while the empty port has a shutter that prevents any light from entering the dark chamber. Even when the amount of light is small, it is still sufficient to deform or conceal the fluorescence spectrum, even more so, since this is unfiltered light. The shutters should be periodically checked for wear out that might lead to incomplete closure.

Scratches on the filter's coating. The filters used in the FluoWat are interference filters based on a specially designed coating that produce a sharp cut-on to cut-off transition, better than any absorption filter. This coating is only a few micrometres thick. So, any scratch or damage to its surface will result in a loss of performance, and even the transmittance of a portion of unfiltered light in the blocking region.

In the same way, the edges of the filter might produce unwanted internal reflections of unfiltered light that ends up being transmitted towards the sample. For this reason, the filter holder of the FluoWat is constructed with a thin rim that covers the edge of the filter. But if the filter loses a splinter from the edge e.g., by falling to the ground, the rim might not block the edge anymore.

Leaves with an irregular surface might prevent the clip from full closure. Despite the foam that seals the sides of the clip (and protects the leaf from damage).

Some traits in the data might hint to some of these perturbations in the measurements:

- Missing or negative fluorescence peak at apparent reflectance/transmittance.

- Residual fluorescence peak in true reflectance.
- O2-A absorption feature in the fluorescence measurement (L^{Φ}). Since the filter is completely blocking the sunlight there shouldn't be any absorption in the measurement. Note that after applying the filter correction, a small feature at the 760 nm O_2 absorption might be introduced if τ was measured using the clip. Not a conclusive reason has been found for this unwanted effect, but after discarding stray light from the outside, the most probable reason is sensor cross-talk between elements of the detector or broad ISRF.
- Negative fluorescence or a very deep valley between the RF and FRF peaks is indicative of overcorrection by inaccurate filter transmittance or a strong decrease in illumination between the WR and the filtered leaf measurements (whenever proper dark current correction has been applied).

5 PROOF OF THE MEASURING CONCEPT AND DIFFERENT SET-UPS

In this chapter a number of experiments are presented where the capabilities of the FluoWat leaf clip were tested. They were conducted at different moments during the development of the leaf clip.

First a trial with the very first prototype to test the feasibility of the method.

Then an improved prototype that provided both components of fluorescence emission (upwelling and downwelling) together with a much-needed measurement of absorbed PAR.

One of the limitations of the FluoWat leaf clip is that it needs direct sunlight to measure, which prevents measuring shadowed leaves under diffuse light. An experiment was conducted in which a white LED with adjustable intensity was coupled to the clip to measure fluorescence emission under diffuse light mimicking the illumination.

Also, another experiment tested the capability of measuring fluorescence emission from soil biocrust. Which represents two challenges, first measuring on a solid surface, and second, detecting an even smaller intensity of the fluorescence emission.

A later experiment tested a prototype with capability of measuring absorptance (through reflectance and transmittance) and downwelling fluorescence as well as upwelling. New filters of better performance with sharp cut-off were used. And correction algorithms were developed.

5.1 DIRECT FLUORESCENCE MEASUREMENTS VS. INDIRECT RETRIEVAL

The first experiments took place in 2005 and were mostly aimed at acquiring knowledge about the set-up and methodology, understanding the behaviour of sun-induced fluorescence, and learning how to measure it both directly with the FluoWat and indirectly through the FLD method. This experiment only measured reflectance and upwelling fluorescence, using a cyan filter that greatly reduced the amount of PAR reaching the leaves. Nonetheless, it was sufficient at the time, when this type of measurement was a novelty, to demonstrate the feasibility of the proposed methodology.

Two aims promoted the development of the first prototype:
Determine whether it is possible to detect fluorescence emission with an off-the-shelf spectro-radiometer.
Evaluate the accuracy obtained using the FLD method to estimate fluorescence with an instrument with less-than optimal spectral resolution.

It is important to look at this experiment in the context of the available knowledge and the technological limitations at the time. First, passive measurement of sun-induced chlorophyll fluorescence was incipient, and only a few studies had been published at the time, and only using instrumentation purposely built for the task. So, the knowledge available was not conclusive about the actual magnitude of the fluorescence signal when excited by the sun under natural conditions. And it was not clear that off-the-shelf portable spectroradiometers, such as the ASD-FieldSpec used here, with clearly inferior characteristics (with 3 nm FWHM and 1 nm spectral sampling when other instruments were sub-nanometre), were capable of measuring (or even sensing)

the fluorescence emission. Also, most, if not all, of the attempts to measure SIF had been based on indirect methods, mainly using the FLD technique. But even when there was evidence of the capability to capture the trends of fluorescence dynamics measured with well-established active techniques, there was no verification of the magnitude of the signal.

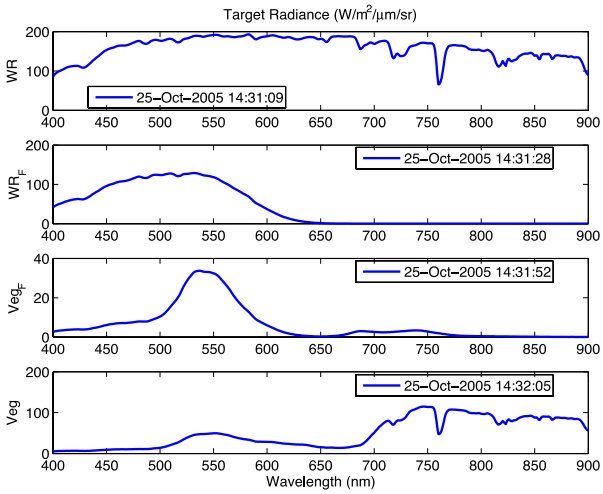

Figure 5-1 Sequence of measurements: radiance from the WR (top), WR with filter (middle up), leaf with filter (middle down), and leaf without filter (bottom).

Four consecutive measurements were taken (Figure 5-1). The first measurement acquires the white reference radiance without filter, and the second acquires the white reference radiance with the cyan filter mounted on the device. The third measurement acquires the spectrum of the leaf when using the filter and the last one acquires the leaf radiance without filtering. The unfiltered measurements (first and fourth) are combined to provide the apparent reflectance of the leaf, and they are also used to estimate the fluorescence with the FLD method. The second measurement (filtered WR) gives the actual PAR intensity associated to the measured fluorescence. Finally, the third

measurement provides the sun-induced chlorophyll fluorescence emission in steady-state, which were used to evaluate the classic FLD method. All these measurements were acquired in less than one minute in order to avoid significant illumination variations between consecutive measurements.

The analysis was performed over a set of 40 leaf samples from three different species: ivy, cabbage, and tobacco.

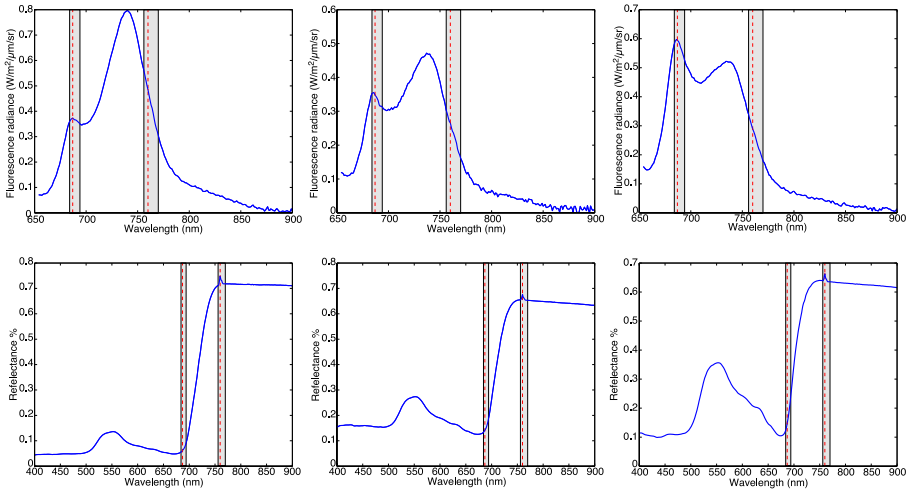

Figure 5-2 Fluorescence (top row) and reflectance (bottom row) as measured from three different leaves: ivy (left), cabbage (middle) and tobacco (right).

Figure 5-2 shows the actual fluorescence emission (upper row) and the retrieved apparent reflectance (lower row) for samples of the three species.

The FLD formulation is based on the assumption (valid in the context under which it was developed) that reflectance and fluorescence remain constant at the wavelengths outside and inside of the absorption band where it is evaluated. Looking at the two O_2 absorption bands (shaded areas), one can see that the fluorescence near 760 nm and reflectance near 687 nm cannot be considered constant as the FLD method assumes.

We have evaluated the accuracy of the fluorescence estimation for the standard FLD method with respect to the directly measured fluorescence emission. The results are reflected in Table 5-1.

It is worth noting that the range of the errors for all methods in the 687 nm band are much greater than in the 760 nm band, despite the similar value (same order of magnitude) of the fluorescence in both bands. This higher overestimation at 687 nm can be attributed to the high curvature of the reflectance around this absorption band, causing the FLD's assumption of constant reflectance to fail dramatically. The overestimation also appears at 760 nm, but to a lesser extent.

Table 5-1 Comparison of retrieved Fluorescence by FLD and direct measurement using a filter.

	Ivy		Cabbage		Tobacco	
Fluorescence mW/m²/nm/sr	O2-B 687 nm	O2-A 760 nm	O2-B 687 nm	O2-A 760 nm	O2-B 687 nm	O2-A 760 nm
FLD	6.93	3.22	10.43	1.99	20.19	1.55
FluoWat	0.37	0.48	0.35	0.27	0.61	0.30
FluoWat PAR compensated	0.64	0.92	0.60	0.52	1.05	0.58
RAE(FLD) PAR compensated	982%	250%	1638%	283%	1823%	167%

This study led to explore different ways of improving the FLD to overcome the limitations of its premises, resulting in the development, among others, of the iFLD method (Alonso et al. 2008); and ultimately in the publication of a comparison of methods and their performances (Meroni et al. 2009)

This experiment was published at the American Institute of Physics (AIP) Conference Proceedings (Gomez-Chova et al. 2006), and while all the published results still hold, it is worth noting that, under the current knowledge and experience, the comparison between measured and estimated fluorescence was

not completely fair. The filter used was causing a drastic reduction in the APAR, hence in the magnitude of the emitted fluorescence. The last rows of Table 5-1 include the corrected fluorescence following the correction method presented in 4.2.4, approximately doubling the originally reported values. Nonetheless, the retrieved fluorescence from the FLD method is still overestimated in both O2 absorption bands.

5.2 TEST OF IMPROVED PERFORMANCE AND ACCURACY

This test aimed at evaluating the performance of the leaf clip with a then-new high-performance OD4 low-pass filter with cut-off wavelength at 650 nm and the formulation for the correction of the filter's residual light above the cut-off wavelength, presented in 4.2.3. For this test four samples were used, two fluorescent (one palm leaf and one hibiscus leaf) and two non-fluorescent (one artificial palm leaf and a piece of green paper).

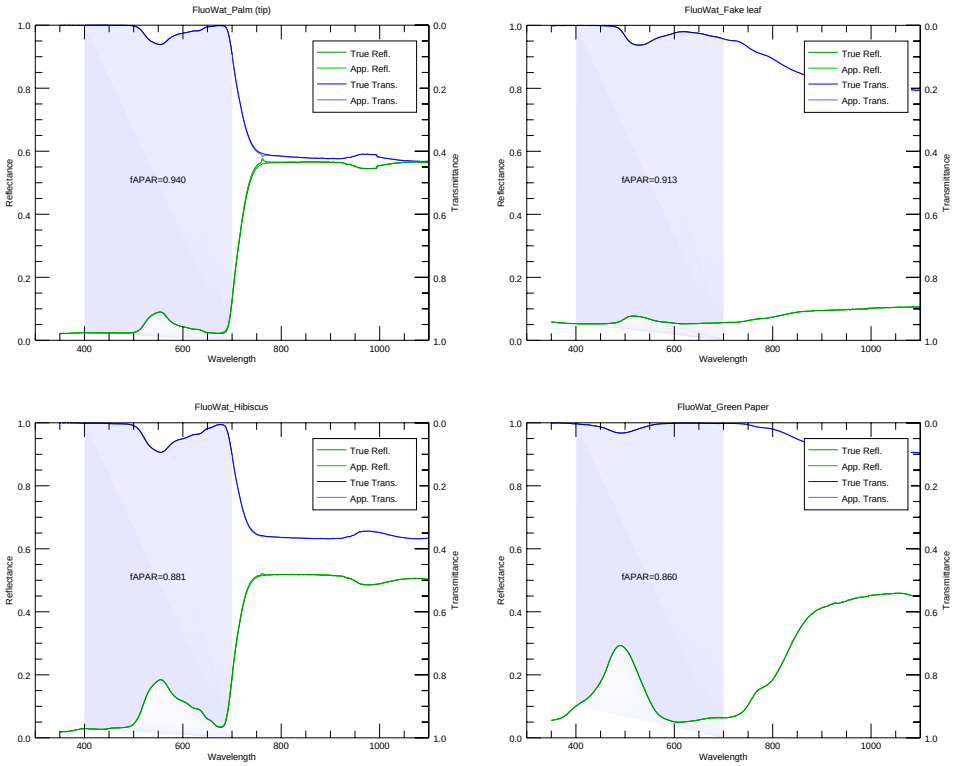

Figure 5-3 Reflectance (green) and transmittance (blue) of the four samples: palm leaf (top-left), artificial palm leaf (top-right), hibiscus leaf (bottom-left) and green paper (bottom-right). Transmittance has been inverted so the area between the curves represents absorbed light. In blue the absorption corresponding to the PAR.

In Figure 5-4 it is shown the raw measurement and the fluorescence after correction. In particular, for the non-fluorescent targets the residual light is completely removed resulting in an estimated fluorescence of zero, without overcorrection. There is however a residual of less than 0.1 mW/m^2/nm/sr between 665 nm (the lower limit of acceptable filter blocking at 0.8% transmittance) and 680 nm (with 0.4% filter transmittance). This residual is also present on the leaf measurements, making the tail of the red fluorescence peak higher than expected.

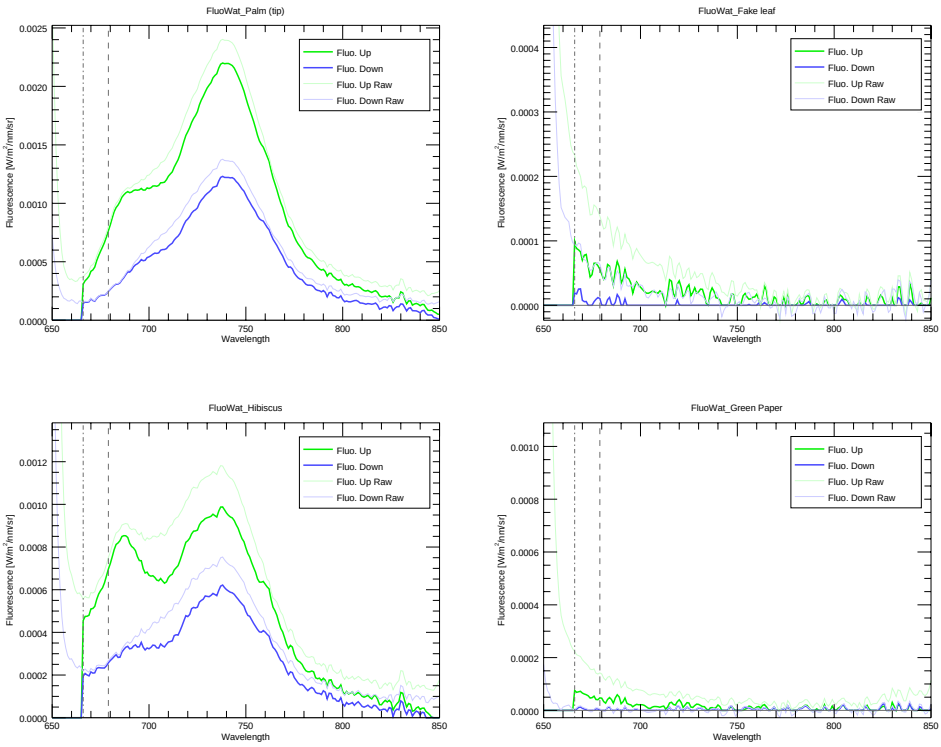

Figure 5-4 Upwelling (green) and downwelling (blue) fluorescence of the four samples: palm leaf (top-left), artificial palm leaf (top-right), hibiscus leaf (bottom-left) and green paper (bottom-right). The uncorrected measurement is plotted in light colour to illustrate the magnitude of the distortion introduced by the filter's residual light. Note that the y-axis are not in the same scale.

It is worth noting how the filter's residual light above the cut-off wavelength is larger in the NIR than in the red in the fluorescent samples due to the red-edge shape of the reflectance/transmittance, while for non-fluorescent targets it is the opposite.

It is also interesting to see how the red peak is more strongly reabsorbed in the palm leaf than in the hibiscus, due to its higher chlorophyll content.

5.3 FLUORESCENCE EMISSION UNDER DIFFUSE ILLUMINATION

Due to design constrains of the FluoWat clip it is not possible measuring under diffuse illumination (as explained in Section 4.3.1 before).

The only way to achieve this task is by diminishing the intensity of the direct sunlight to match the irradiance levels reaching the sample of interest.

Two options were under study: the use of neutral density (ND) filters or the use of polarizing filters.

In the case of ND filters, they are only available at certain values of transmittance. Therefore, they offer little flexibility to reach the desired light intensity, and the option to stack them is complex to operate in the field.

In the case of the polarizing filters, using two sheets and rotating one respect of the other it is possible to select the desired amount of transmittance. But they present an inhomogeneous spectral response, being quite transparent in the NIR. This results in some undesired light overlapping to the fluorescence, that in this case, it is rather difficult to correct.

The adopted solution was to use a white LED light attached to the back port of the clip. The LED being powered with portable batteries and regulated in voltage. By changing the forward voltage, it is possible to regulate to some extent the LED's intensity to match the intensity of the measured diffuse illumination that reach the leaf.

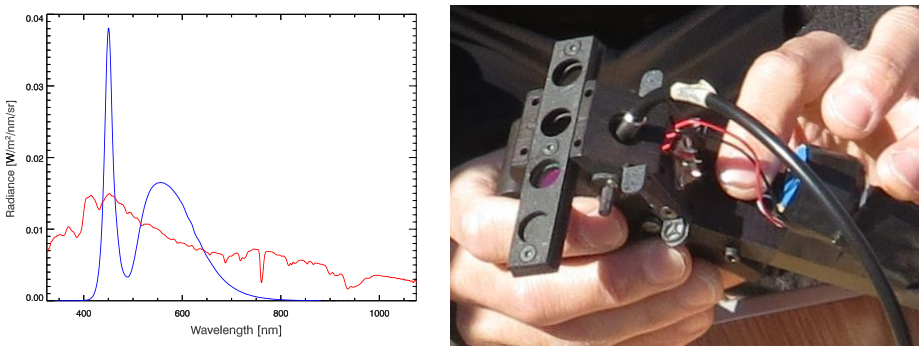

Figure 5-5 Left: Radiance measured on a white reference receiving the same diffuse illumination (red) than the leaf. And the radiance produced by the LED on a white reference inside the FluoWat clip (blue). Regions outside PAR have been greyed out to facilitate comparison. Right: FluoWat with the white LED attached to the back port.

The typical while LED emission is generated by a strong sharp blue peak (produced by the diode itself) and a weaker but much wider emission in the green and red (produced by phosphorescent dyes covering the diode). This shape does not exactly mimic the natural illumination, still, it is possible to adjust its intensity to produce a similar amount of PAR, as seen in Figure 5-5 where both light sources have a PAR of 3.1 W/m2/nm/sr (expressed in photon flux, the LED has 43.9 μmol/s/m^2 and E_{diff} has 42.6 μmol/s/m^2), while PAR from direct sun light was more than 10 times higher. Note that the LEDs used in this proof of concept were the best available at the time. Nowadays there are LEDs with a spectral emission more similar to the sun light.

To test this approach an experiment was conducted under the framework of the OPTIMISE COST Action, during a training course in the experimental site Las Majadas (Spain) in spring 2015. ("ABEL Training School on UAVs and Biogeochemical Cycling 2015 | OPTIMISE Project | COST Action ES1309" 2015)

A *quercus ilex* (holm oak) tree was selected for sampling, leaves were collected from the sunny and the shaded sides. Small branches were cut holding several

leaves, so the sampled leaves were kept attached and away from the cut to minimize the drying effect.

Figure 5-6 Collecting small twigs from the sunny side (left) and shaded side (right) of a *quercus ilex* tree.

The leaves from the sunny side (south) of the tree were measured following the regular protocol pointing to the sun. While the leaves from the shaded (north) side were measured using the LED attached to the back port of the clip, which was previously adjusted to replicate the measured diffuse illumination. The illumination port was shut by setting on the dark disk in the filter holder.

After measuring with the LED, the leaves, still attached to the branch, were placed under direct sun light to get adapted to high-light conditions. Afterwards, a regular measurement using direct sun light was performed. From it, it was possible to obtain the reflectance and transmittance needed to compensate the LED light that was overlapping fluorescence (since there was no cut-off filter on the LED). It also produced a measurement of SIF under high light from a low-light grown leaf and to compare with fluorescence emission under diffuse illumination (low light) of the same leaf.

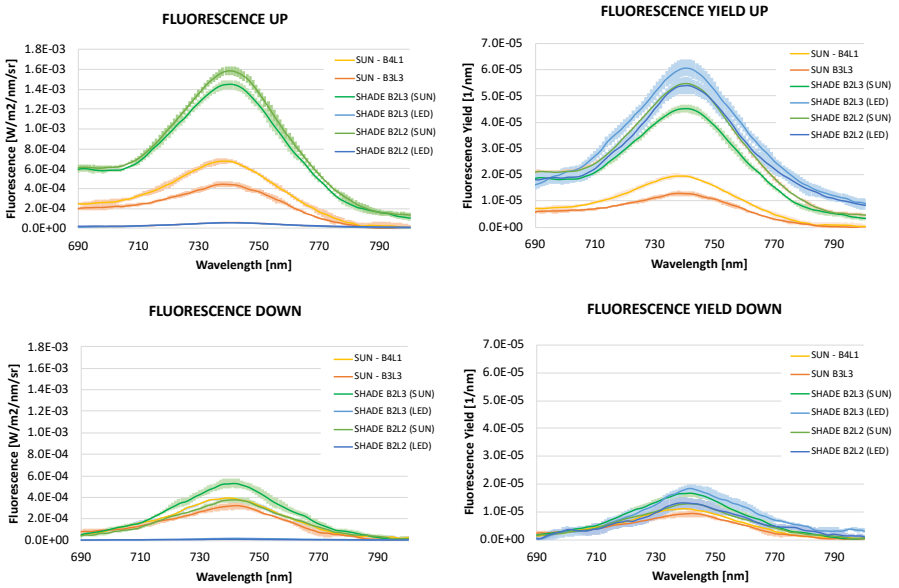

Figure 5-7 Fluorescence (left col.) and yield of fluorescence (right col.) from four leaves from the same *quercus ilex* tree: two grown in the sun-exposed side, and two from the shade side. The two sunny leaves were measured with the FluoWat using the direct sunlight (orange lines), and the two shaded leaves were measured using a white LED adjusted to light levels in the shade (blue lines) and alsoande direct sunlight (green lines).

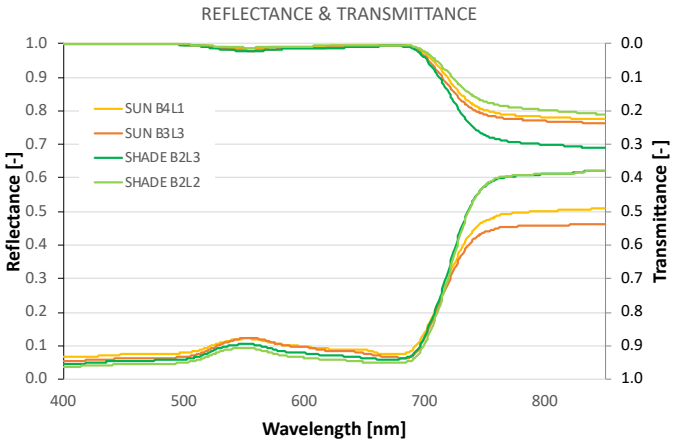

Figure 5-8 Reflectance and transmittance from the two sunny leaves (orange lines) and the two shaded leaves (green lines).

The chlorophyll content of the leaves was measured with a SPAD system. It showed that leaves grown on the shady side had slightly more chlorophyll content than the leaves from the sunny side. This is also reflected in the spectral shape of their reflectance and transmittance.

Table 5-2

	SUNNY B3L3	*SUNNY B4L1*	*SHADE B2L2*	*SHADE B2L3*
SPAD	58.2	59.6	65.8	61.1
ε(SPAD)	1.7	2.1	2.9	0.90

When looking at the SIF emission, one can see that the leaves under diffuse illumination (in the shade) emit one order of magnitude less fluorescence than the leaves under sunlight, whereas the shape is quite similar (same proportion of RF and FRF) [2]. However, the low-light adapted leaves, when exposed to sun light, produce more than twice SIF than the high-light adapted ones.

When looking at the yield (obtained dividing by the estimated APAR) one can see that the shaded leaves produce a similar amount, regardless of being excited by the LED or the full direct sun light. While the sun-adapted leaves show a lower fluorescence yield on the adaxial side. The fluorescence yield from the abaxial side is rather similar for both types of leaves regardless of the illumination type. This experiment served as a trial for an alternative method to measure SIF from leaves under diffuse illumination. It also permitted to obtain some first estimates on the differential behaviour of the fluorescence emission on high-light and low-light adapted leaves under direct and diffuse illumination.

[2] Note that the cue of the RF is not available due to the use of the 675 nm filter, but its maximum peak is retrieved.

5.4 LOW FLUORESCENCE EMITTING SOIL CRUSTS

This piece of work was carried out in collaboration with Cinzia Panigada and Micol Rossini from the University of Milano-Bicocca (UNIMIB) while I was working with Franco Migilietta at the Institute of Biometeorology (IBIMET), Florence.

Desert crust fluorescence emission was measured on five desert crust samples collected in Israel by Dr. Eli Zaady from the Volcani Institute, Agricultural Research Organization, Israel:

- sample 1: Cyanobacterial crusts, 60-100 mm rainfall
- sample 2: Cyanobacterial crust, 200 mm rainfall
- sample 3: Moss crust, 150-200 mm rainfall
- sample 4: Moss + cyanobacterial crust, 250 mm rainfall

The importance of biocrusts resides in the vast areas that they cover in dry landscapes, worldwide (Kuske et al. 2011). Biocrusts represent from 50% to 90% of the soil cover in regions with 400 to 50 mm of annual rainfall (Zaady et al. 2000). Therefore, although they may produce low fluorescence signal (Sarafis, Heintzmann, and Karnieli 2006), they are particularly important as primary producers to the life chains in dry ecosystems.

Figure 5-9 Four samples of soil biocrust

An ASD FieldSpec Pro was coupled to a FluoWat, detaching the lower half of the clip to allow placing it on top of the soil. The measurements were taken in full moisture condition a few hours after wetting the biocrust with the same amount of double distilled water.

Figure 5-10 The FluoWat leaf clip, with the bottom part removed to measure on thick solid samples. The LED in the back port was not used in this experiment.

Five measurements were taken at different positions of the four samples. The graphs show the spectra of the higher and lower emissions of each sample.

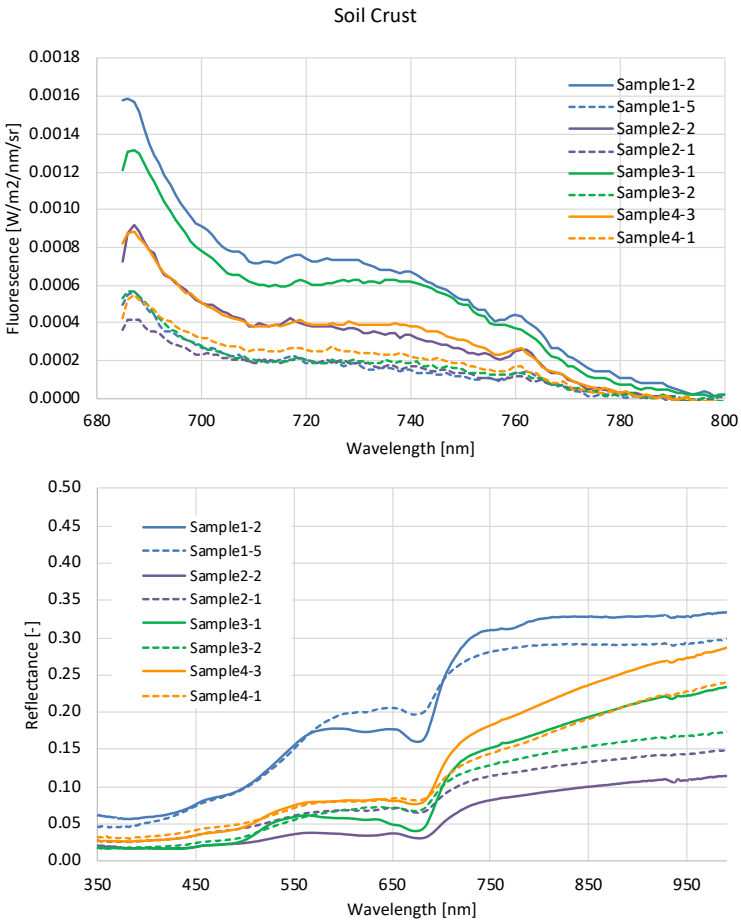

Figure 5-11 Fluorescence emission (top) and reflectance (bottom) from the four samples.

Spectra confirm that moss and cyanobacterial crusts have a higher fluorescence emission in the red than in the far-red region. This may be explained by smaller reabsorption due to low chlorophyll content, but also to the presence of pigment P750 that absorbs radiation around 750 nm (Govindjee and Shevela 2011).

The magnitude of RF is similar or higher to the emission of leaves from higher plants, while the FRF is much weaker and might be in the verge of detectability from remote sensing.

6 APPLIED RESEARCH ON FLUORESCENCE INTERPRETATION

The FluoWat leaf clip, through the different stages of development has contributed to respond some scientific questions. In this chapter some results from broader scientific studies are presented.

6.1 FLUORESCENCE RESPONSE TO STRESS: AN URBAN TREE CASE STUDY

In 2013 an experiment was conducted in Valencia to evaluate the sun-induced fluorescence response to air pollution in urban vegetation. This experiment was carried out within the framework of the BIOHYPE project supported by BELSPO and led by Roeland Samson from University of Antwerp (Belgium). The project had a broader extent of which the FluoWat measurements were just a part. Detailed information and full extension of results can be found in (Van Wittenberghe et al. 2013), (Van Wittenberghe, Alonso, et al. 2014; Van Wittenberghe, Verrelst, et al. 2014), and (Van Wittenberghe et al. 2015).

Four tree species and ten locations of interest were chosen with diverse levels of traffic densities and pollution. Three dicotyledonous deciduous trees, *Celtis australis* L. (European nettle tree), *Morus alba* L. (White mulberry), and *Platanus* × *acerifolia* (Aiton) Willd. (London plane), and one monocotyledonous evergreen tree, *Phoenix canariensis* Chabaud (Canary Island date palm). These four species were selected for being ubiquitous in the city area. At each location, the 4-weekly average NO_2 concentration was monitored during the period May 25th—September 2nd by installation of passive NO_2 diffusive samplers (IVL

Swedish Environmental Research Institute Ltd., Stockholm, Sweden) at 4 m height at a tree trunk and at least 4 m away from other obstacles.

The selected species were classified in either a low or a high local traffic exposure according to the amount of magnetic properties detected in the leaves, caused by deposition of metallic pollutants.

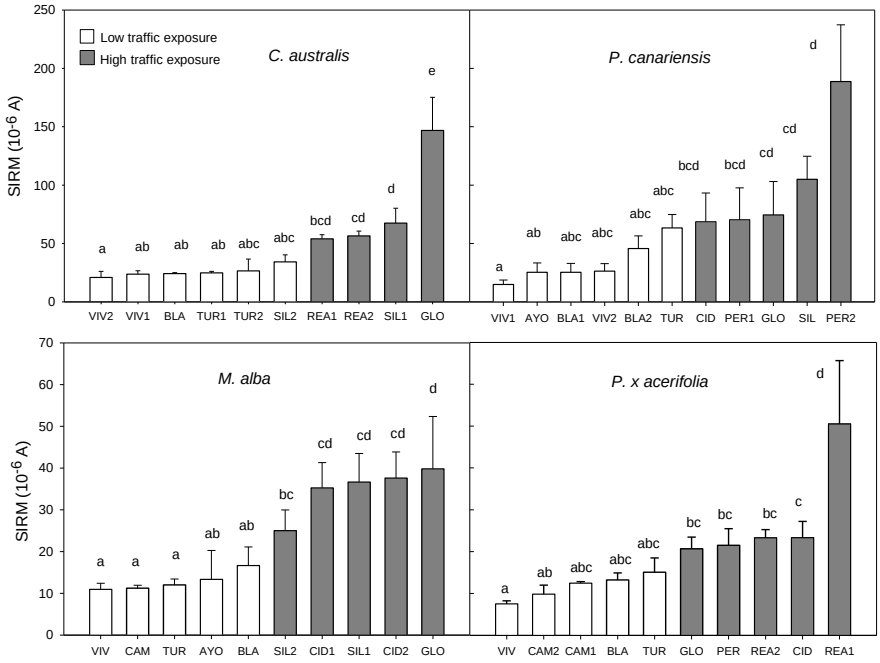

Figure 6-1 Saturation isothermal remanent magnetization (SIRM) with flagged standard deviation for all trees (x-axis: location code) of the four species whereby the different lowercase letters indicate significant ($p<0.05$) differences. The high traffic emissions exposure class consists of all trees with a SIRM value significantly different ($p<0.05$) from the tree with the lowest value. The number of trees in each traffic exposure class was about the same for each species, indicating that the original tree selection was successful in identifying trees for two contrasting traffic exposure conditions (Adapted from Van Wittenberghe et al. 2013).

Steady state fluorescence was measured under natural illumination with clear sky conditions. By positioning the leaf clip opening perpendicular to the sun's

azimuth, the effect of varying incoming radiation by the change in solar elevation angle is substantially reduced facilitating the comparison of samples measured at different times.

When measuring from 12:00 to 20:00 pm, incoming photosynthetic active radiation (PAR) amounts to 234 ± 33 W m^{-2} (or 1068 ± 148 µmol m^{-2} s^{-1}) for the whole measuring period. On a cloudy day LED illumination (MR16 1W 12V 3900–4500 K, Velleman, Gavere, Belgium) was used with a PAR of 209 ± 32 W m^{-2} (or 976 ± 151 µmol m^{-2} s^{-1}).

Fluorescence was further normalized by APAR to get upward and downward fluorescence yield (YF_{up}, YF_{dw}). Additionally, several YF indices based on the upward and downward emission peaks, situated at 687 nm and 741 nm, are evaluated:

$YF_{up}(687)$, $YF_{dw}(687)$, $YF_{up}(741)$, $YF_{dw}(741)$, $YF_{up}(687)/YF_{up}(741)$, $YF_{dw}(687)/YF_{dw}(741)$, $YF_{dw}(687)/YF_{up}(687)$, $YF_{dw}(741)/YF_{up}(741)$.

Since the same filter was used for all the measurements and no comparison was done with other systems, for this study it was sufficient to only use the fluorescence as measured with the filter, so the ϕ symbol is omitted, but it is implicitly understood.

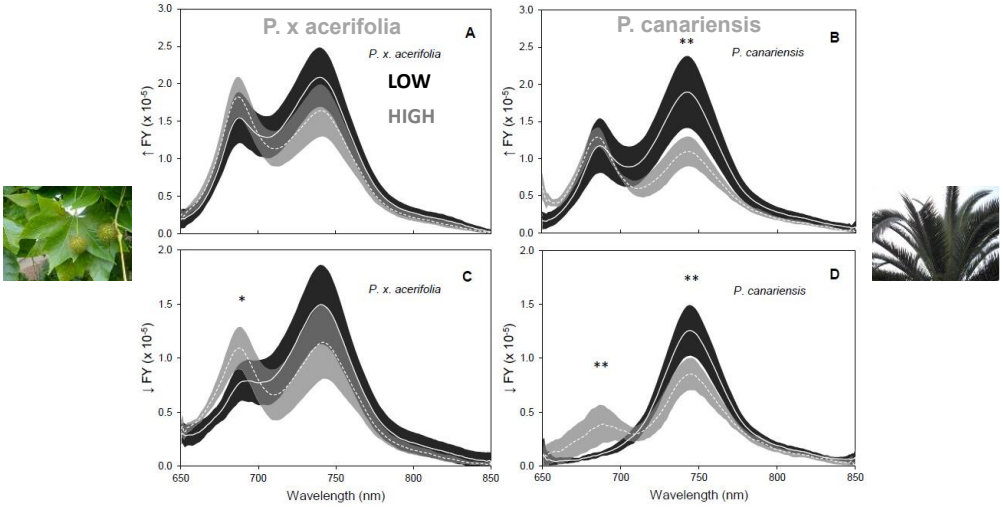

Figure 6-2 Upward and downward fluorescence yield (↑FY, ↓FY) of *P. x acerifolia* (panels A and C) and *P. canariensis* (panels B and D) for Low (—) and High (----) traffic emission exposure; bands indicate standard deviation; significantly different peaks (at 687 or 741 nm) between traffic exposure classes are indicated by * p<0.05 and ** p<0.01 (Adapted from Van Wittenberghe et al. 2013).

Since no significant differences in Chl content were found between the two traffic exposure classes, we suggest that YF indices can indicate early stress before a significant degradation of Chl pigments takes place. This was also stated by (Meroni et al. 2009), saying that steady-state fluorescence is able to detect plant stress before irreversible damage occurs. The significant effect of traffic emissions exposure on the YF indices is, however, only visible for *P. canariensis* and *P. x acerifolia*. Both species have a general lower average Chl content at the higher traffic emissions exposure locations, compared to the lower traffic emissions exposure locations, although this difference was non-significant. Nevertheless, for both species, the upward and downward $YF(687)/YF(741)$ ratio was significantly higher in the high traffic exposure class than in the low traffic exposure class (Table 6-1). Moreover, the red/far-red Fs peak ratio becomes higher than 1 for the high traffic exposure class indicating that the 687

nm peak becomes higher than the 741 nm peak. A higher $YF(687)/YF(741)$ peak ratio under stress conditions has already been demonstrated for cases of N deficiency (Campbell et al. 2007; Campbell et al. 2008; Langsdorf et al. 2000), elevated CO_2 and O_3 levels and elevated ultraviolet radiation. This increase in F_S peak ratio can generally be attributed to a decrease in Chl absorption (Gitelson, Buschmann, and Lichtenthaler 1998; Campbell et al. 2007; Yaryura et al. 2009) and the associated decrease in re-absorption of the red Chl F (Langsdorf et al. 2000).

Table 6-1 Fluorescence yield indices for the four species. The different lowercase letters indicate significant differences (in bold) between the 'Low' and 'High' traffic emission exposure classes (*p < 0.05; **p < 0.01). (Adapted from Van Wittenberghe et al. 2013).

	C. australis		*M. alba*		*P. canariensis*		*P. x acerifolia*	
	Low	High	Low	High	Low	High	Low	High
YF_{up}	1.91 E-3	1.78 E-3	2.00 E-3	2.30 E-3	**1.69 E-3**^{a}	**1.13 E-3**^{b}**	1.77 E-3	1.61 E-3
YF_{dw}	1.02 E-3	9.58 E-4	1.06 E-3	1.16 E-3	**7.97 E-4**^{a}	**6.41 E-4**^{b}**	1.20 E-3	1.08 E-3
$YF_{up}(687)$	1.38 E-5	1.19 E-5	1.56 E-5	1.76 E-5	1.34 E-5	1.28 E-5	1.54 E-5	1.83 E-5
$YF_{dw}(687)$	2.53 E-6	1.66 E-6	2.79 E-6	2.94 E-6	**1.13 E-6**^{b}	**3.82 E-6**^{a}**	**7.58 E-6**^{b}	**1.10 E-5**^{a}*
$YF_{up}(741)$	2.50 E-5	2.37 E-5	2.60 E-5	3.00 E-5	**2.10 E-5**^{a}	**1.09 E-5**^{b}**	2.08 E-5	1.66 E-5
$YF_{dw}(741)$	1.63 E-5	1.56 E-5	1.69 E-5	1.84 E-5	**1.33 E-5**^{a}	**8.32 E-6**^{b}**	1.50 E-5	1.16 E-5
$YF_{up}(687)/YF_{up}(741)$	5.45 E-1	5.04 E-1	5.96 E-1	5.90 E-1	**6.42 E-1**^{b}	**1.24 E+0**^{a}**	**7.64 E-1**^{b}	**1.17E+0**^{a}*
$YF_{dw}(687)/YF_{dw}(741)$	1.55 E-1	1.06 E-1	1.66 E-1	1.68 E-1	**8.57 E-2**^{b}	**4.92 E-1**^{a}**	**5.49 E-1**^{b}	**1.05E+0**^{a}**
$YF_{dw}(687)/YF_{up}(687)$	1.88 E-1	1.42 E-1	1.85 E-1	1.80 E-1	**8.95 E-2**^{b}	**2.93 E-1**^{a}**	**5.01 E-1**^{b}	**6.15 E-1**^{a}*
$YF_{up}(741)/YF_{dw}(741)$	6.54 E-1	6.64 E-1	6.52 E-1	6.38 E-1	6.62 E-1	7.80 E-1	7.11 E-1	6.95 E-1

Both upward and downward total fluorescence yield (YF_{up}, YF_{dw}) were highest for the low traffic intensity class for *P. x acerifolia* and *P. canariensis*, although differences were only statistically significant (p<0.01) for the latter.

Of the four species investigated only *P. x acerifolia* and *P. canariensis* show a change in FY as a response to an increased traffic exposure. This can be rationalized. *P. x acerifolia* is planted in many European cities and is also a common species in the urban area of Valencia. However, it seems to be less adapted to the prevailing climate compared to the other species investigated. In summer defoliation due to drought stress is already reported by the Green Services of the city, though defoliation was not observed for the selected tree individuals in the field campaign. When drought and pollution stress coincide, these stress factors will be at least additive and aggravate the tree crown condition to a higher extent (Klap et al. 2000). So, although *P. x acerifolia* is generally reported as a pollution tolerant tree species, it seems to be susceptible to traffic induced stress in the city of Valencia where the resilience of the species might be weakened due to drought stress. For the lower palm leaves of *P. canariensis*, stress accumulates due to the long lifespan of the leaves (> 3 years) - the other species were deciduous – and thus due to a longer exposure time. Moreover, its leaf architecture seems to be in favour of collecting high amounts of dust, and these both factors might explain the response in FY indices. Species like *C. australis* on the other hand, show a high particle deposition potential, but no significant influence of traffic emission exposure on their functional status was observed.

In this field experiment, it is shown that stress induced by traffic pollution can be detected by steady-state FY indices for leaves at the bottom branches of some urban tree species. Two of the four investigated tree species (*P. canariensis* and *P. x. acerifolia*) show significant differences in FY parameters for the two contrasting traffic emission exposure classes, while Chl content did not

change significantly. Such a change in fluorescence emission indicates a transformation in photosynthesis-related processes or structures. This promotes steady-state fluorescence as a more sensitive indicator of (traffic) pollution stress than the leaf's pigment content as such. It is expected that the mapping of sun-induced Chl fluorescence using hyperspectral sensing data will provide spatially-explicit information of the physiological status of vegetation which can serve in many applications. Monitoring the Fs of urban vegetation as an indicator of traffic pollution impacts will be one of them.

6.2 TOTAL FLUORESCENCE EMISSION: RELATIONSHIP OF RED AND FAR-READ PEAKS

During the preparation of the FLEX mission one point of discussion was the need to include second spectrometer to retrieve the red peak of fluorescence as was requested by the scientific team. It is important to remind that at that moment of the preparation of the mission the knowledge about the behaviour of sun-induced chlorophyll fluorescence in its steady state was not as broad that what was known from active measurements. And there are experiments suggesting that the red and far-red peaks were highly correlated, so that measuring just the far-red peak (less challenging to retrieve than the red one) would be sufficient to obtain all the information related to fluorescence emission, in part following the tradition of a single-band fluorescence from active PAM systems.

This study was prepared for the FLEX mission selection and presented as a Technical Note to ESA in March 2012

Relationship between both Peaks and Total Fluorescence

This work is intended to provide further evidence on the need of estimating the emission on both peaks of the fluorescence spectrum in order to provide an accurate value of total fluorescence emitted radiance, given the recent acquisition of an extensive dataset of field samples.

The data was collected for BELSPO's BIOHYPE project aiming to find a means of estimating urban air pollution from chlorophyll fluorescence emitted from ubiquitous vegetation throughout the city.

The dataset consists of 173 samples collected from 40 urban trees of four distinct species (*Celtis australis* L. (European nettle tree), *Morus alba* L. (White mulberry), *Platanus* × *acerifolia* (Aiton) Willd. (London plane) and *Phoenix*

canariensis Chabaud (Canary Island date palm)) at 10 separate locations throughout Valencia city exposed to different environmental conditions.

The measurements were taken using an ASD FSFR spectroradiometer attached to the FluoWat leaf clip, that allows obtaining the *in vivo* leaf fluorescence spectrum under sunlight illumination, together with reflectance and transmittance, and determination of PAR and APAR.

The fluorescence spectrum is measured from 660 to 840 nm, and the complete dataset is pictured in Figure 6-3 below, where each colour line corresponds to one of the 173 spectra.

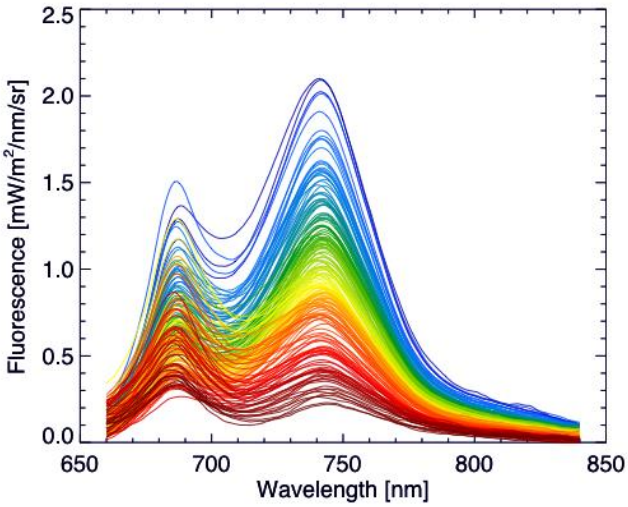

Figure 6-3 Complete collection of fluorescence spectra emitted at leaf level from four different tree species.

The total emitted fluorescence is calculated as the integral of the fluorescence emission spectrum between 660 and 840 nm, using a five-point Newton-Cotes integration formula.

$$F_{tot} = \int_{660}^{840} F(\lambda) \cdot d\lambda \qquad 6.1$$

The analysis has been performed considering two cases: the ideal case, where the data is noise free; and the realistic case, in which we have studied the impact of several levels of noise in the data.

Noise-free Analysis

We have studied the ideal case, in which the fluorescence estimation at any given wavelength is free of noise.

The correlation between punctual fluorescence and total fluorescence has been considered to be linear and has been determined by the least squares method.

In order to estimate goodness of the linear regression we use the squared correlation coefficient R^2, and the relative absolute error (RAE):

$$RAE = \frac{|y' - y|}{y} \cdot 100\% \qquad 6.2$$

where *y* is the true value (F_{tot} in our case) and *y'* is the estimated value.

The linear fit between the fluorescence measured within O2-A at 761 nm (F_{761}) and the total fluorescence (F_{tot}) results in a R^2 of 0.920, with more than 40% of the samples resulting in a relative absolute error (RAE) higher than 10% of the actual total fluorescence, as seen in the histogram in Figure 6-4 top left.

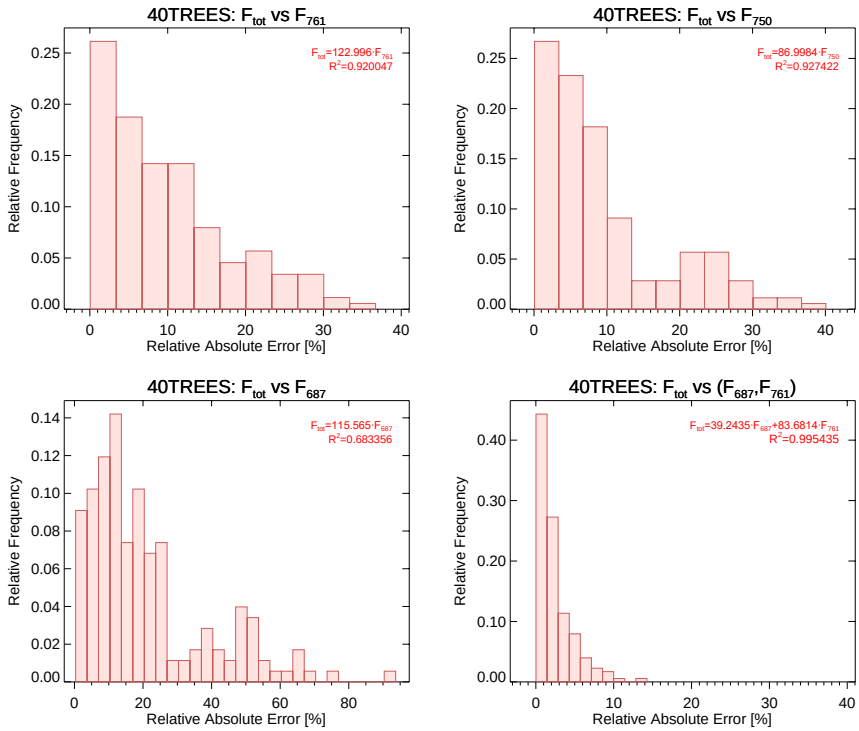

Figure 6-4 Histogram of the error committed at estimating total fluorescence radiance from each of the four cases studied: linear regression with Fs measured at 761 nm (O2-A) (top-left); linear regression with Fs measured at 750 nm (top-right); linear regression with Fs measured at 687 nm (O2-B) (bottom-left); and bi- linear regression with F_{687} and F_{761} (bottom-right).

A linear fit between the fluorescence measured at 750 nm (F_{750}) -closer to the fluorescence peak- and the total fluorescence (F_{tot}) results in a R^2 of 0.927, still with more than 32% of the samples resulting in an absolute relative error (RAE) higher than 10% of the actual total fluorescence, as seen in Figure 6-4 top right. A similar regression between the fluorescence measured within O2-B at 687 nm (F_{687}) and the total fluorescence (F_{tot}) results in a R^2 of 0.683, with more than 2/3 of the samples resulting in an absolute relative error (RAE) higher than 10% of the actual total fluorescence, as seen above in Figure 6-4 bottom left.

However, despite the poor correlation between F_{687} and F_{tot}, including it together with F_{761} in a bilinear fit with F_{tot} improves the results to R^2 of 0.995

and almost 98% of the samples fall below the RAE threshold of 10% and more than 80% of the samples are below a RAE of 4.5%, as seen in Figure 6-4 bottom right.

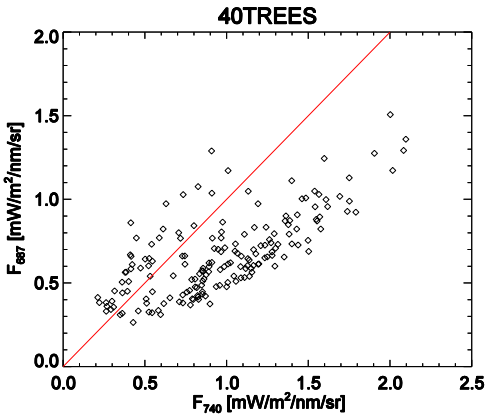

Figure 6-5 Scatter plot between measurements at F_{687} and F_{740} showing that both fluorescence peaks are partially correlated, but this relationship is more complex than simply linear.

Therefore, the use of a single value of fluorescence might provide an estimate of the total fluorescence emission, being better using the second peak than the first, but not with sufficient accuracy in either case, even in the ideal situation of noise-free signal.

On the contrary, the use of measurements at both peaks, even with a simple bilinear regression, it is possible to achieve the accuracy required (less than 10% error).

Considering Noise in Data

We have also analysed the effect of noise in the fluorescence measured at given wavelengths on the estimation of total fluorescence. We have applied gaussian multiplicative noise with a mean of one to the measured fluorescence spectra.

We applied 15 levels of noise, from 5% to 19%, performing 1000 runs at each level randomizing the standard deviation of the gaussian noise.

At each noise level, the mean of the relative absolute error (RAE) from all the samples of the database is calculated, together with the corresponding standard deviation.

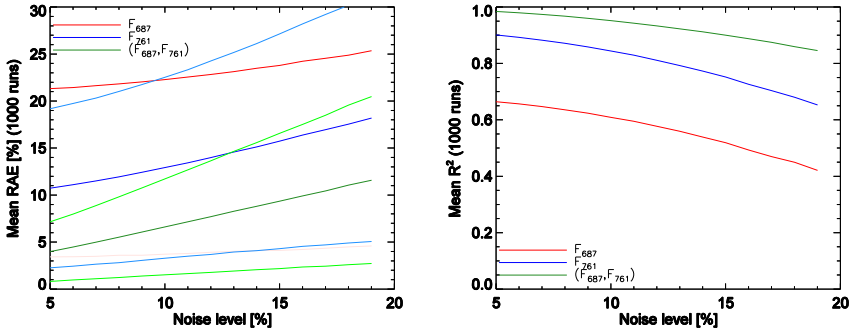

Figure 6-6 Error in total fluorescence estimation in terms of noise in the measured fluorescence, after 1000 simulations of random Gaussian noise, from linear regression with F_{687} (red), F_{761} (blue), and bilinear with F_{687} and F_{761} –using equal noise levels in both– (green). Left: Mean RAE with corresponding plus/minus standard deviation (light colour lines). Right: Mean squared correlation coefficient R^2.

Figure 6-6 shows the results obtained, where the bilinear case represented used identical noise level at both F_{687} and F_{761}. Below a 10% of noise in the signal, the bilinear regression provides a mean RAE lower than 6.5% of F_{tot} with R^2 larger than 0.95; while using a single measurement of fluorescence in 761nm at a noise of 5% presents a mean RAE already higher than 10% with R^2 below 0.9, and the results from F_{687} is far worse in all senses. In all cases the error in estimating F_{tot} increases with increasing noise.

Complete results of the bilinear regression with different combinations of noise levels at F_{687} (x-axis) and F_{761} (y-axis) are shown in Figure 6-7; where it can be seen that the accuracy is more sensitive to the noise in the second peak of fluorescence (F_{761}) than in the first one (F_{687}). Also, the mean RAE of the

estimated does not reach the 10% level until the noise in F_{761} is already larger than 16% (Figure 6-7 top left); however, the standard deviation of the mean RAE is almost in the same order than the mean RAE itself (Figure 6-7 top right), indicating a decreasing precision in the estimation of F_{tot} with increasing noise. Therefore, if the intention is to assure that most of the studied cases fulfil with the requirement of being within 10% of the error, then it is necessary to consider the mean RAE plus its standard deviation (here denoted Total RAE) as a better indicator (Figure 6-7 bottom left) of the acceptable tolerance in measuring fluorescence at 761 and 687 nm. In this case, the precision required at 761 nm is below 9%, while at 687 nm should be lower than 10% (although at this band this limit is not very strict, since the retrieved F_{tot} is less sensible to this input). Further analysis is required in order to determine the proportion of samples that would still comply with the requirements while increasing the noise in the input fluorescence measurements.

Using a dataset of experimental measurements of samples under various levels of stress from diverse tree species we have verified that:

In order to retrieve the total fluorescence radiance emitted by vegetation from single band measurements of the fluorescence emission spectrum it is not sufficient to use just one band, neither from the first peak nor the second peak; but at least a combination of both. This is due to the loose correlation between the amplitudes of the peaks.

A simple bilinear relationship of measurements from each of the fluorescence peaks is sufficient to achieve a high accuracy in the estimation of the total fluorescence emitted radiance. However, it must be noted, that a more

sophisticated approach (e.g., non-linear regression) might be more robust to noise in the input measurements.

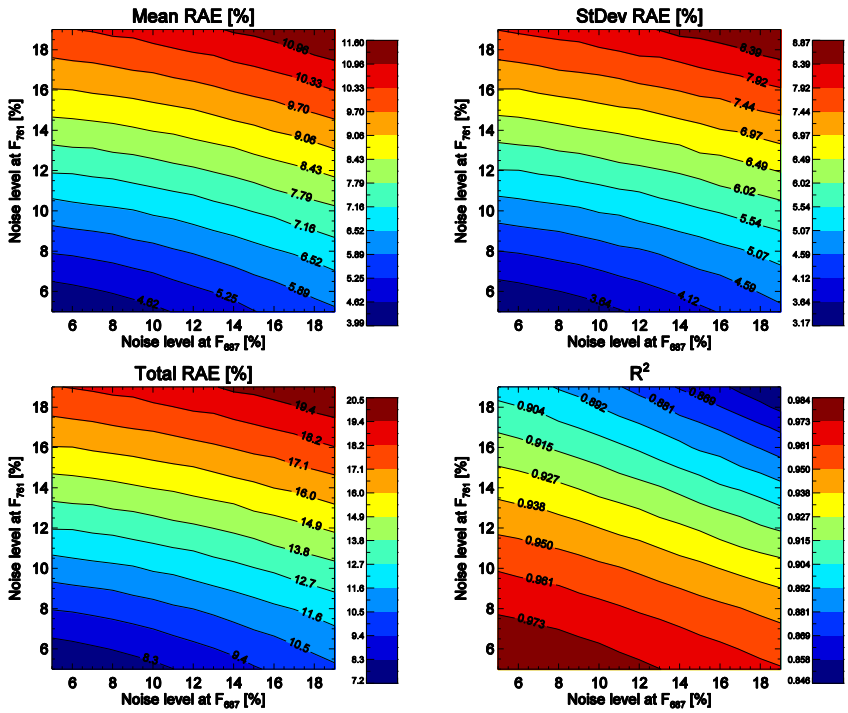

Figure 6-7 Error in total fluorescence estimation in terms of noise in the measured fluorescence, after 1000 simulations of random Gaussian noise, from bilinear regression with F_{687} and F_{761} using separate noise levels in both measurements. Top left: mean RAE; top right: standard deviation of the RAE; bottom left: Total RAE as the mean plus one standard deviation of RAE; and bottom left: Mean squared correlation coefficient R^2.

The mean relative absolute error in the estimation of F_{tot} using a bilinear regression is below 10% for almost all of the noise range in either spectral band. However, the standard deviation of the mean RAE is in the same order of magnitude than the mean RAE. Therefore, a conservative approach has been taken, considering the actual error of the estimation to be the mean RAE plus its standard deviation. This has been done to assure that most of the samples

comply with the requirement of an error smaller than 10% in the retrieval of F_{tot}.

The noise allowed on the input fluorescence measurements, based on the bilinear correlation, is at most 9% at 761 nm and 10% at 687 nm, in order to achieve an error smaller than 10% in the estimation of total fluorescence emitted radiance. However, these requirements might be relaxed if a better correlation function is found.

6.3 FLUORESCENCE RESPONSE OF CHLOROPHYLL MUTANT SPECIES

Within the preparatory activities supporting the FLEX mission the SoyFLEX field campaign took place in 2015 (Schüttermeyer, 2018; "SoyFLEX 2015 - Earth Online" n.d.). The campaign aimed at measuring the fluorescence emission of two soybean varieties and identifying their distinctive characteristics and how to extract the information from FLEX-like measurements.

In this experiment the FluoWat was used to characterize the sun-induced chlorophyll fluorescence emission of leaves from two soybean varieties provided by the University of Minnesota: MinnGold, a chlorophyll deficient mutant which contains about one-fourth the chlorophyll of the natural variant WildType but it is suspected to have the same photosynthetic efficiency (Figure 6-8)

Figure 6-8: Soybean mutant MinnGold (light green) and Wild Type (dark green).

The plants were grown in a greenhouse, and they were subsequently planted outside to an agricultural field at Campus Klein-Altendorf (Germany).

Figure 6-9 Field plots of MinnGold (back) and WildType (front). Plot size was 5m x7m plots, with 20cm space in between the plants.

Within each field three plots were marked for continuous monitoring and gas-exchange measurements (W1, W2, W3 and M1, M2, M3). Besides, two smaller plots, one of each variety, were kept apart (EM2 and EW2).

Figure 6-10 Gas exchange collar with MinnGold (left) and WildTyp (right).

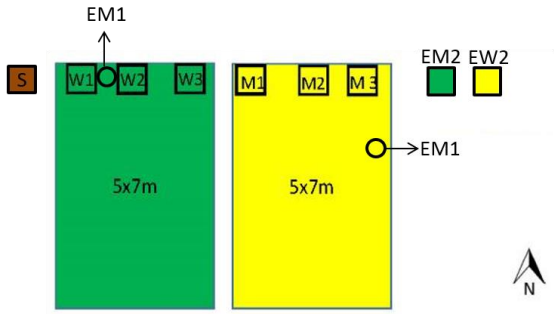

Figure 6-11 Naming of the measurement plots WildType in green and MinnGold in yellow.

Leaf-level reflectance and fluorescence emission was measured with an ASD FieldSpec and the FluoWat leaf clip. Three to five leaves of each soybean plot (Figure 6-11) were measured for three days. Table **6-2** given in overview of the acquired Fluowat measurements.

Table 6-2 Date, time, number of leaves and location of the FluoWat measurements.

Date	Time (local)	No. leaves	Endmember	Plot 1	Plot 2	Plot 3
06/30	16:30-18:15	5	EM2/EW2	W1/M1	W2	
07/01	12:50-13:50	3	EM2/EW2	W1/M1		
07/02	14:30-16:00	3		W1/M1	W2/M2	W3/M3

For each variety, a similar spectral signature could be found across the different plots (Figure 6-12). The WildType showed higher chlorophyll content

([3]NAOC=0.39 ~35.87 μg/cm^2) than MinnGold (NAOC=0.29 ~25.68 μg/cm^2). This is presented in a sharp green peak for the WildType, while the MinnGold variety showed a wider green peak, with a higher reflectance and transmittance in the yellow and red wavelengths, providing the yellow-green tint of the MinnGold leaves. Therefore, the albedo in the visible spectral range is higher for the MinnGold (27 %) than for the WildType (16 %). The near-infrared part of the spectrum is similar between both varieties, which indicated similar composition of e.g., dry matter and water content (Figure 6-13).

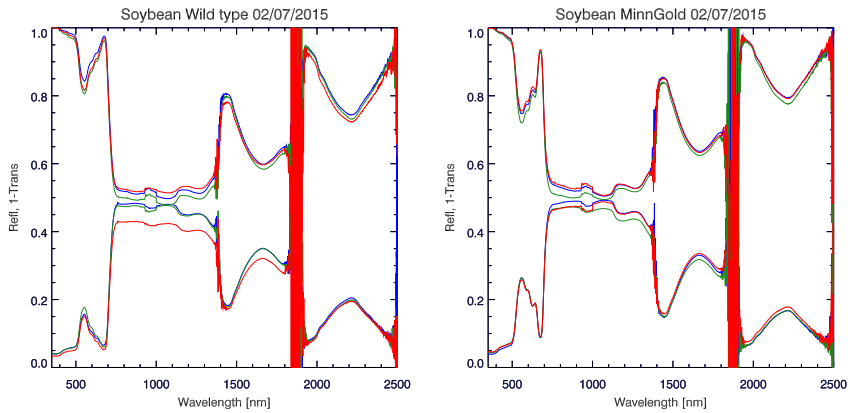

Figure 6-12 Reflectance and transmittance spectra Wild Type (left) and MinnGold (right). Each line represents the mean of all leaves measured on each variety and plot, blue for plot 1, green of plot 2 and red plot 3.

[3] NAOC stands for Normalized Area Over the Curve (Delegido et al. 2010). It is a chlorophyll content index that is more sensible than others like MTCI, since it calculates the integral (not just taking individual bands). If you were to use MTCI or NDVI to calculate chlorophyll content over these amounts, since they are based on RED and NIR, and in this case RED is similar in both. The main difference between both varieties appears in the green and yellow portion of the spectrum.

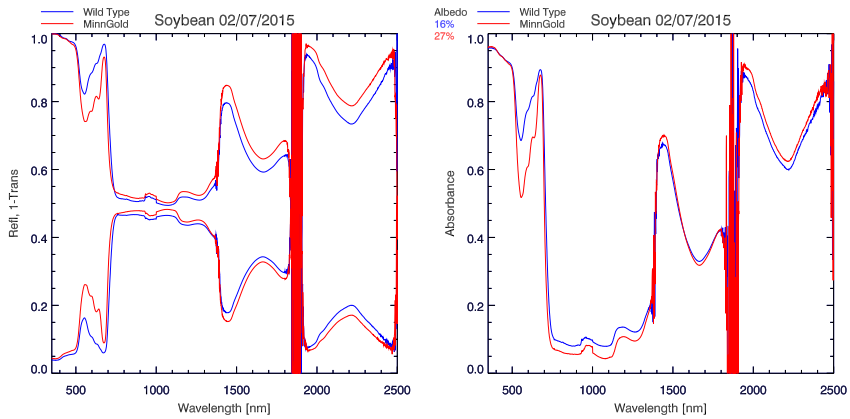

Figure 6-13 Averaged spectral reflectance and transmittance (left) and absorptance (right) of two soybean varieties Wild type (blue line) and MinnGold (red line)

The fluorescence emission of both soybean varieties showed a different shape (Figure 6-14, left). WildType shows larger emission in the far-red peak, reaching 1.95 mW/m^2/nm/sr; while MinnGold only emits 1.13 mW/m^2/nm/sr in the same far-red peak, whereas both varieties have a similar emission level in the red-fluorescence peak of around 1.4 mW/m^2/nm/sr.

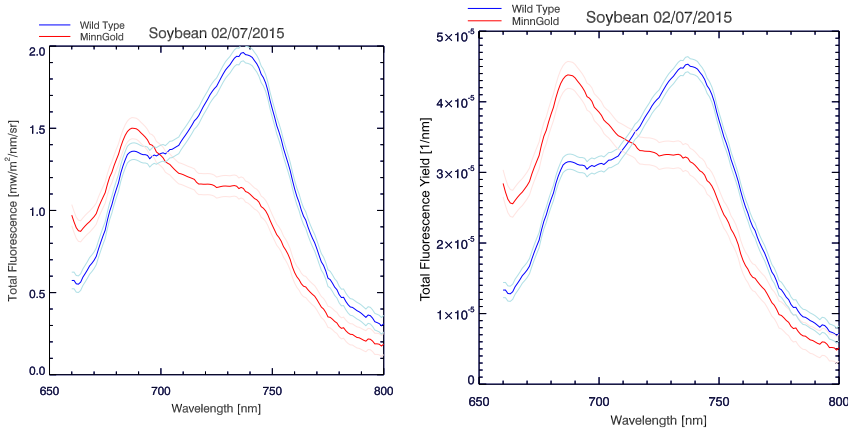

Figure 6-14 Averaged spectral emission of total sun-induced fluorescence (left) and corresponding fluorescence yield (fluorescence normalized by the absorbed photosynthetic active radiation) of the two soybean varieties Wild Type (blue line) and MinnGold (red line).

The fluorescence yield, the emitted fluorescence normalized by absorbed photosynthetic active radiation, is resented in Figure 6-14, right. The far-red peak of the fluorescence yield is higher in the WildType than in the MinnGold. In contrast to the total fluorescence emission, the red peak of the fluorescence yield is not the same for both varieties, but higher in the MinnGold mutant than in the WildType. From the fluorescence yield it can be concluded that both soybean varieties have a similar fluorescence efficiency. However, it is differently distributed within the emission spectrum, as the smaller chlorophyll content of MinnGold results in less reabsorption of red-fluorescence.

The upwelling and downwelling fluorescence emission of both soybean varieties is shown in Figure 6-15. The upwelling fluorescence emission shows a similar shape as the total fluorescence emission for both varieties. The WildType shows a higher far-red fluorescence peak that the MinnGold, but a reversed behaviour for the red fluorescence peak. The downwelling fluorescence emission of MinnGold has the same shape as the upwelling emission with higher fluorescence values in the red peak. The downwelling emission of the WildType shows higher fluorescence values in the far-red peak and for some spectra the emission in the red is so low, that no red peak signal is visible, due to the reabsorption of the red fluorescence in the WildType.

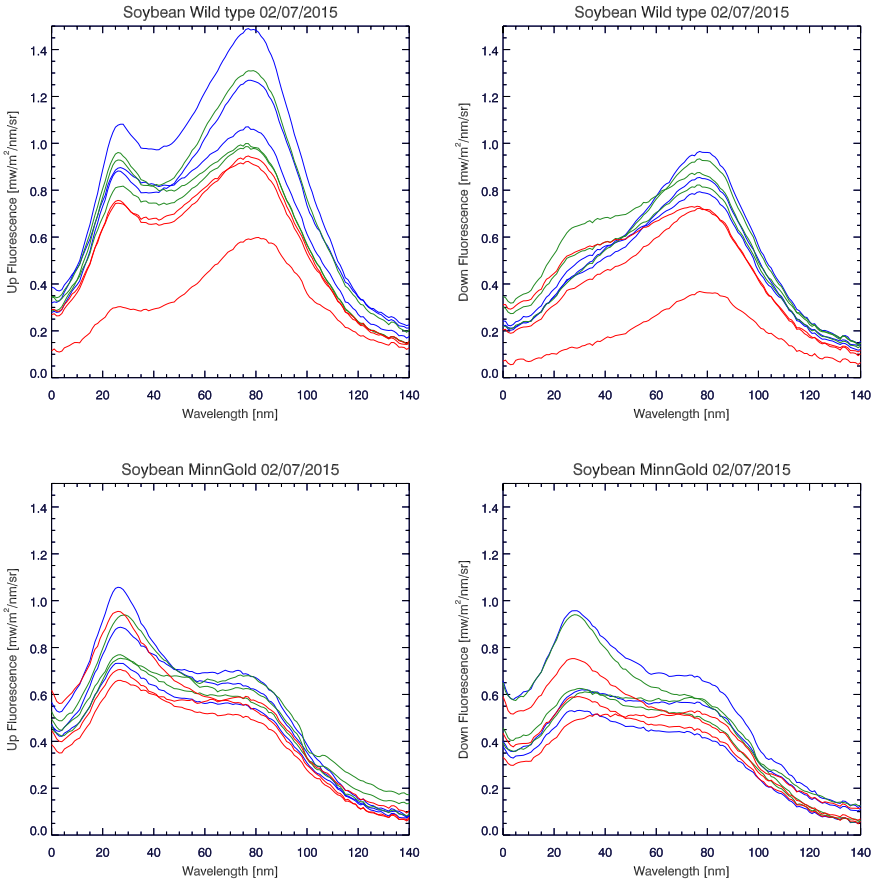

Figure 6-15 Contribution of the upwelling (left) and downwelling (right) fluorescence of WildType (upper panel) and MinnGold (lower panel) leaves illuminated by sunlight from the top. Blue line indicated leaves measured in plot 1, green represents plot 2, and red plot 3.

Further research inspired by these first results was conducted by (Sakowska et al. 2020) where a more comprehensive data collection allowed to link the fluorescence emission to photosynthetic activity, showing that both varieties present similar net photosynthesis and gross primary production. For this later experiment the FluoWat measurements were used to invert the FLUSPECT leaf model to calculate the quantum efficiency of the fluorescence, including the effect of reabsorption (Cendrero-Mateo et al. 2022).

6.4 ADAPTATION TO NATURAL CHANGES IN ILLUMINATION UNDER HEAT STRESS

During the SoyFLEX-2015 campaign a side experiment was set to investigate the influence of shadowing effect on photosynthesis and fluorescence. An experiment similar to the one conducted by (Gamon et al. 1990), except that, instead of completely darkening the canopy, the aim was to emulate the passage of a cloud and study how the vegetation adapts to the illumination change.

To perform the experiment a 6 x 30 m net was put 0.50 m above a sugar beet canopy that reduced the incoming radiation by 50% to emulate the illumination under cloudy conditions, and its passage when removing the net. The vegetation underneath was adapted to the reduced light condition for at least one hour.

Plants react at sudden illumination changes, first by releasing the excess of absorbed photos through increased fluorescence emission, rapidly followed by an activation of photoprotective pigments until the reorganization of reaction centres to be able to cope with the increase of available energy.

This experiment was performed in a sugar beet field in Campus Klein-Altendorf in Germany during a heat wave that lasted three days.

Figure 6-16 Set-up of the virtual could experiment in sugar beet on 1 July 2015.

During the virtual cloud experiment the FluoWat leaf clip was used to measure SIF and absorptance, capturing its dynamics after a sharp change in illumination, such as the one found after a passing cloud. The measurements were repeated three times, one in the afternoon of July 1st; and two on July 2nd, in the morning and the afternoon respectively, so the plants were at various stages of stress due to the heat wave. Table **6-3** gives on overview of the FluoWat data acquisition.

Table 6-3 FluoWat acquisition information of the virtual cloud experiment in sugar beet.

Series	Date	Time frame	Duration
Day 1 PM	1 July	19:29-20:01	20 min
Day 2 AM	2 July	10:53-11:37	12 min
Day 2 PM	2 July	18:04-18:27	8 min

The sugar beet leaves were different between the measurements but were selected from the same location to let the measurements be comparable. On each occasion the measurement lasted between 10 and 20 minutes, with spectra being continuously collected with a rate of at least one per second. Only the afternoon of the second day was shorter due to the intrusion of high thin clouds that altered the illumination conditions.

The clip was placed on a tripod to ensure the stability throughout the measurement and the tracking of the sun was done by hand being continuously corrected. Besides, in pre-processing, the residual changes due to tracking were removed by using a reference band around 870 nm, where leaf reflectance is highly stable and there is no influence of fluorescence allowing to sense slight changes in light levels that can only be attributable to the pointing of the clip.

Provided that for the monitoring of SIF the short-pass filter must remain in place for the duration of the sequence, leaf reflectance and transmittance in the full spectrum was measured either at the beginning or the end in order to determine absorptance and APAR (Figure 6-17). In addition, the reflectance spectrum in the

visible region was continuously recorded to follow the changes of photoprotective pigments over time.

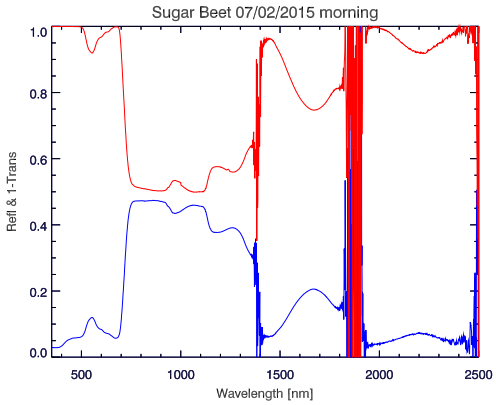

Figure 6-17 Reflectance (in blue) and transmittance (in red, reversed) of one sample. Absorptance is the area between both lines.

The measurements were able to capture the fast and slow relaxation phases of the Kautsky transient once the shading net was removed. However, the spectrometer was not fast enough to capture the fast induction kinetics, but just the maximum fluorescence emission (Figure 6-18).

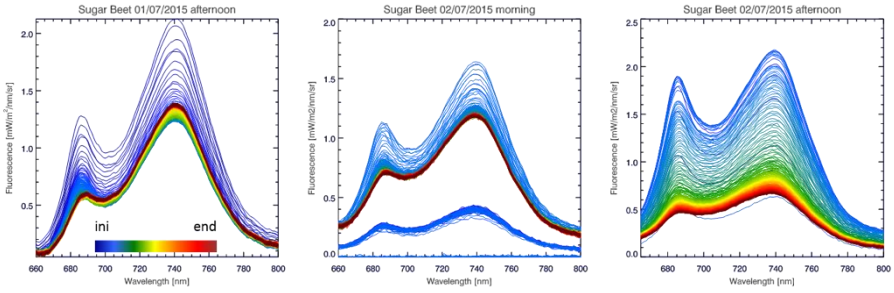

Figure 6-18 Dynamics of fluorescence emission spectra, from the start of the sequence (blue) to the end (red). Note that the second series (morning of 2nd day) also shows the emission spectrum before removing the net (spectra in vivid blue) with a mean far-red fluorescence of 0.4 mW/m^2/nm/sr.

All three series of measurements show high fluorescence emission values after the removal of the net and a decrease until the end of the measurements. The third series, which was recorded in the afternoon of 2 July shows a slightly different behaviour than the first two measurement series. It seems that the decrease (relaxation) occurs much more slowly. This becomes more evident when plotting each peak of fluorescence emission as a function of time in Figure 6-19.

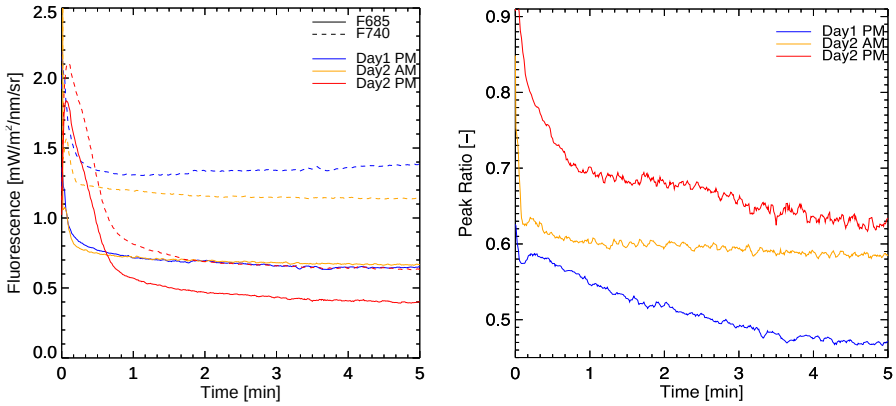

Figure 6-19 Left: Fluorescence emitted as a function of time, at the red (F685, solid line) and far-red (F740, dashed line) peaks. Right: F687/F740 Peak ratio as a function of time.

From the fluorescence emission graph (Figure 6-19) one can see that the fast relaxation transient takes 10 to 15 seconds for the first two series (Day 1 PM and Day2 AM) and about 1 to 2 minutes to reach steady state; while the third series (Day 2 PM) takes about 50 second for the fast relaxation, and almost 4 minutes to arrive to steady state, thus presenting a much smaller dynamic while achieving a higher maximum fluorescence.

Fluorescence Peak Ratio

Another way to look at this transient phase is to plot the emission of red fluorescence (F_{685}) versus the far-red fluorescence emission (F_{740}) (Figure

6-20). For all three series a change in the slope appears during the first 10-15 seconds. For all three measurement-series a change in the slope appears during the first 10-15 seconds. This reflects the fact that the maximum emission for the red peak is reached almost immediately, whereas the far-red peak maximum happens with a delay of few seconds. This is followed by a change in slope at 50 seconds since the exposure to higher light level (reflecting a different change in the relaxation rate of each peak). At this point, the first series keeps a decreasing trend of peak ratio (caused by the increasing far-red fluorescence emission); the second series (next morning) reaches a stable situation in which the proportion of both peaks is maintained; and in the afternoon the peak ratio is more or less stable but begins decreasing after 2.5 minutes due to a slightly faster red peak decrease.

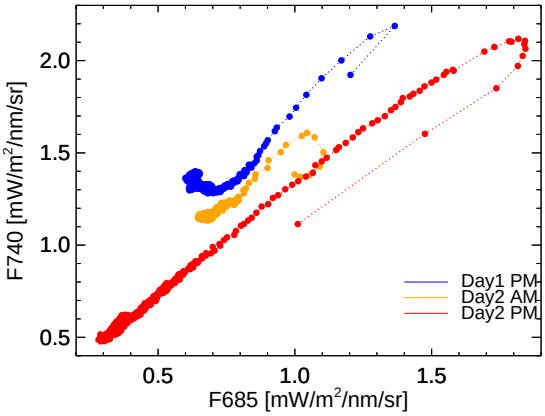

Figure 6-20 Far-red fluorescence emission as a function of red fluorescence. Each dot representing a measurement with a sampling rate of 0.5 s.

In addition, it can be observed that the far-red fluorescence becomes less relevant from one series to the other, resulting in a higher ratio. It is important to note that the steady state of the red peak presents a similar level in the afternoon of day 1 than the morning of day 2, with a slight decrease in the last

afternoon. On the other hand, far red fluorescence shows already a decrease between the afternoon of day 1 than the morning of day 2, that becomes larger towards the afternoon of day 2. This development over the course of the two measurement days might be related to sustained hot temperatures of the two measurement days. However, as not the same leaf was taken for the FluoWat measurement such a conclusion cannot be drawn.

Non-Photochemical Quenching Mechanism

There is, however, another mechanism that is activated when a plant needs to quench excessive absorbed photosynthetic active radiation, namely Non-Photochemical Quenching (NPQ). The energy stored in the excited state of the antennas is transferred to photoprotective pigments, instead of transferring it to the reaction centres, before it can produce damage. These pigments have a strong absorption in the blue and green regions of the visible spectrum, thus, their evolution can be tracked using this portion of the spectrum, by calculating the Photochemical Reflectance Index (PRI). In principle, this index is calculated from reflectance, but in this experiment, it was not possible to monitor solar irradiance simultaneously to the leaf transient. Therefore, the index has been calculated from the radiance data.

The results in Figure 6-21 show how the PRI sharply drops in less than 1 minute after the leaf is exposed to full sunlight, indicating the activation of the NPQ photoprotective process. During the first series (in the afternoon of the first day) the PRI level return to its original state. The second series (next morning) PRI starts at a lower value and it drops less, which indicating that the leaf was already in a photoprotective state before the exposure, and the recovery is only slight so, it remains in photoprotective state. During the third measurement in the afternoon, the results indicate that the sugar beet leaf may have entered a

fully protective state. Its initial PRI value is smaller than in the morning and suffers a very deep drop, although at a slower pace. The protection mechanisms seem not to react as quickly as in the previous series and after 2 min the situation is maintained constantly readapting the levels, which may again be interpreted as a physiological response to heat stress.

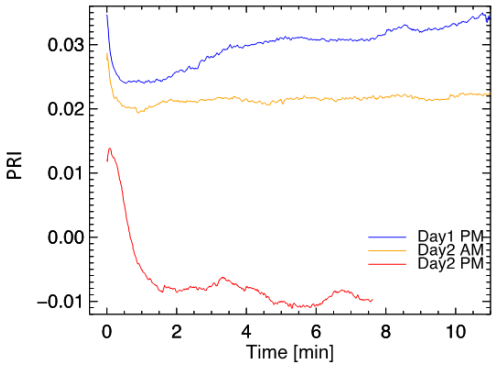

Figure 6-21 Photochemical Reflectance Index (PRI) evolution during the adaptation phase.

The results of the FluoWat make it evident that both quenching mechanisms, fluorescence and NPQ, work at different speeds and time scales. This is best illustrated in Figure 6-22, where PRI is plotted against the far-red fluorescence (F_{740}).

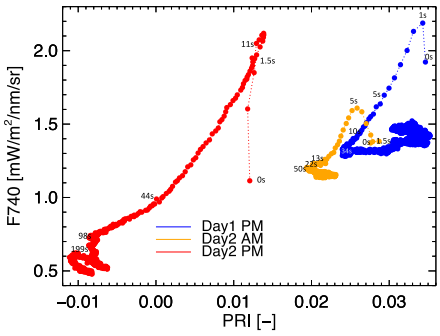

Figure 6-22 Relationship between far-red fluorescence (F_{740}) and photochemical reflectance index (PRI) during the adaptation phase.

Fluorescence is quenched with the faster reaction reaching its maximum value before PRI begins to change. Subsequently, after fluorescence reaches its maximum, it decreases together with PRI until it reaches the steady state, while PRI continues to evolve. The times required for each of the quenching processes is however different. Fluorescence needs between 30s (Day 1 PM) and 100s (Day 2 PM) to return to a relaxed state; while PRI takes up to 12 minutes, and it might not even return to its original state.

These results show how fluorescence has the fastest response after sudden increase of PAR, followed by a slower activation of photoprotection mechanisms reflected by the decrease of PRI while fluorescence emission decreases. This type of behaviour of fluorescence and NPQ to stress conditions has already been described using active measurements, but the experiments carried out in this thesis have shown that a similar type of monitorization of the stress is possible using passive measurements of fluorescence and reflectance of the vegetation.

6.5 RESULTS FROM COLLABORATIVE STUDIES USING FLUOWAT

Besides the works presented in the previous chapters, I have collaborated with other researchers using the FluoWat, of which some relevant results are listed here.

Of particular importance, due to its impact, is the work performed by van der Tol, in which the FluoWat was used to recalibrate the widely used leaf radiative transfer model FLUSPEC. Moreover, he remarked the importance of the downwelling (forward, according to his naming convention) fluorescence emission to the overall radiative transfer at canopy level. A type of measurement that is novel from the FluoWat leaf clip and was first presented during the 4th Intl. Workshop on Remote Sensing of Vegetation Fluorescence that was held the 15-17 of November 2010 in Valencia. (Alonso and Moreno 2010)

"The scattering and re-absorption of red and near-infrared chlorophyll fluorescence in the models Fluspect and SCOPE"

In this work by Van der Tol, the FluoWat was used to recalibrate the FLUSPEC leaf fluorescence model, leading to a better understanding the effects of leaf optical properties on fluorescence, in particular it's anisotropic emission (the ratio of backward to forward emitted fluorescence). Leaf optical properties explain a large part of the observed variation of leaf SIF. Their effect can be estimated by making use of concurrent measurements of reflectance and leaf transmittance. By means of model simulation, further we studied the effects of the anisotropy of leaf fluorescence on top-of-canopy SIF and found that the leaf structure parameter N and leaf chlorophyll content Cab mostly determine the anisotropy, while the escape probability from the canopy is mostly determined by brown pigments and dry matter, and leaf orientations. Although we were not

able to validate these findings at the canopy level, we believe this knowledge may nevertheless serve further studies on remote sensing observations of SIF. (van der Tol et al. 2019)

"Gaussian processes retrieval of leaf parameters from a multi-species reflectance, absorptance and fluorescence dataset"

Biochemical and structural leaf properties such as chlorophyll content (Chl), nitrogen content (N), leaf water content (LWC), and specific leaf area (SLA) have the benefit to be estimated through nondestructive spectral measurements. In this research, leaf characteristics were estimated from a field-based multi-species dataset collected with the FluoWat leaf clip, covering a wide range in leaf structures and Chl concentrations. Parameter retrieval was conducted with the machine learning regression algorithm Gaussian Processes (GP), which is able to perform adaptive, nonlinear data fitting for complex datasets. Moreover, insight in relevant bands is provided during the development of a regression model. Consequently, the physical meaning of the model can be explored. Interestingly, spectral features related to biochemicals with a structural or carbon storage function (e.g., 1090, 1550, 1670, 1730 nm) were found important not only for estimation of SLA, but also for LWC, Chl or N estimation. It is shown that leaf parameter retrieval by GP regression is successful, and able to cope with large structural differences between leaves. (Van Wittenberghe, Verrelst, et al. 2014)

"Plant chlorophyll fluorescence: active and passive measurements at canopy and leaf scales with different nitrogen treatments"

In this study led by Cendrero-Mateo, we investigated the potential for interchanging ChlF measurements using active techniques with passive

measurements at different temporal and spatial scales. The ultimate objective was to determine the limits within which active and passive techniques are comparable. The results presented in this study showed that active and passive measurements were highly correlated over the growing season across nitrogen treatments at both canopy and leaf-average scale. (Cendrero-Mateo et al. 2016)

"Leaf-level spectral fluorescence measurements: Comparing methodologies for broadleaves and needles"

In this work Rajewicz did a first comparison of protocols for measuring leaf-level ChlF spectra: a custom-made system designed to measure ChlF spectra at ambient and 77 K temperatures (optical chamber, OC), the widely used FluoWat leaf clip (FW), and an integrating sphere setup (IS). We tested the three methods under low-light conditions, across two broadleaf species and one needle-like species. The comparison of needle arrangements indicated that needle mats produced more reproducible results and higher signals than single or sparse needles. (Rajewicz et al. 2019)

"Exploring the scattering and reabsorption of chlorophyll fluorescence: implications for remote sensing of photosynthesis"

SIF signal measured at the leaf level or at higher scales is affected by several processes, including wavelength dependent scattering and reabsorption, which may need to be considered when linking SIF data and photosynthetic CO2 assimilation. To address this question, we conducted a multi-scale and multi-technique study that considered measurements of photosynthetic (GPP), optical (SIF, reflectance, and transmittance), physiological (NPQ) and biophysical (APAR) parameters of two soybean varieties: the MinnGold mutant, characterized by

significantly reduced chlorophyll content (Chl), and the wild type, non-Chl deficient Eiko. We then used the SCOPE model to investigate the reabsorption and scattering of SIF. The study revealed that despite the major difference in Chl content (the ratio of Chl between MinnGold and Eiko was nearly 1:5), similar leaf and canopy photosynthesis rates were maintained in both varieties. This phenomenon was not captured by traditional spectral vegetation indices related to canopy greenness, nor by SIF measured in-situ. However, the modelling simulations based on FluoWat leaf measurements (Cendrero-Mateo et al. 2022) revealed that when correcting for leaf and canopy scattering and reabsorption processes both varieties presented similar SIF yield (SIF/APAR). (Sakowska et al. 2020)

7 CONCLUSIONS

This Thesis presents the development of a new measuring device, called FluoWat, for directly measuring the fluorescence emitted in-vivo by leaves under sunlight, including a protocol and data processing that minimises perturbations, increasing the precision of the measured fluorescence.

It consists of a leaf clip connected to a spectroradiometer and the measurement is based on placing a filter that blocks the light in the spectral range that overlaps the fluorescence emission, allowing to pass the light in the rest of the spectral ranges to still produce the excitation for the fluorescence emission.

A set of measuring configurations have been established, from which it is possible to obtain, besides fluorescence itself, a number of biophysical parameters that are necessary for the interpretation of the fluorescence variability, in particular the amount of absorbed light to compute the fluorescence quantum efficiency.

The FluoWat leaf clip measures the fluorescence emitted by both sides of the leaf, the illuminated and the opposite sides, to make it possible the computation of the total balance of energy emitted in form of fluorescence. The opposite-side fluorescence can account for 30-50% of the total, mostly emitted in the near-infrared, which is highly reflected and transmitted, thus with a high probability of being scattered upwards in a canopy, so that it cannot be neglected for canopy-level studies.

With such device, and the data processing developed during this Thesis, it has been possible to address the different objectives originally stated to support the development of ESA's FLEX mission:

1) To provide the measurements in physical units in order to determine the signal levels to be registered by the FLEX mission.
2) Evaluate the accuracy of fluorescence retrieval methods based on O2 absorption bands, applied at leaf level.

These two objectives were already accomplished in the initial stages of the instrument development, as presented in Sections 5.1 and 1.1. Even though the analysis at the time were lacking the current knowledge on perturbations and physiological response of the fluorescence, the results were already relevant to support the FLEX mission in terms of expected signal level and to justify the need to develop newer retrieval algorithms that would be more accurate and less sensitive to signal perturbations.

3) Provide accurate estimates of the actual energy emitted by the vegetation in form of fluorescence to enable a fair comparison with the fluorescence estimates by remote sensing methods.

And ultimately,

4) Provide an understanding of the emission of fluorescence dynamics of plants under different states of stress and adaptation to changing conditions.

These last two objectives took a much longer time to achieve since it required to refine the leaf clip design and data processing to achieve the best accuracy possible, presented in most of Chapter 4. They also required to measure diffdiverse typesvegetation under various environmental and health conditions, reported in Chapters 5 and 6.

Moreover, there has been several additional scientific and technical knowledge gathered during the elaboration of the Thesis. The main conclusions derived from these studies can be summarized as follows:

- The simple direct measurement is not sufficient to get an accurate estimate of the fluorescence emission. A number of perturbations affect the accuracy and the quality of the measured signal. The most important perturbation comes from the filter itself. The magnitude of the filter's transmittance (τ) in the fluorescence range is in the order of 10^{-3} (~0.1%), and the light that is not fully blocked will be reflected by the leaf and reach the sensor mixed with the fluorescence. Since fluorescence is small and reflectance is high in the NIR, this contribution to the signal is not negligible. Depending on the quality of the filter and the intensity of the fluorescence, the contamination can go from 5% to 50%. In this thesis these perturbations have been analysed, and correction methods have been developed when possible. This correction makes use of τ, so, a precise characterization is necessary. It has been found that measuring τ with the FluoWat might provide erroneous overestimation of the transmittance that double the actual transmittance. It is worth noting that, however, this overestimation translates in an overcorrection of the fluorescence. For filters of lesser quality, e.g., OD2, the error introduced by the overcorrection can be larger than the error of using the uncorrected radiance.

- Misalignment to sunlight while measuring can lead to a substantial radiometric error of ~2% for a pointing tilt of 1°. These variations

correspond to the cosine law. The inclusion of a sun-finder permits an accurate pointing with misalignments smaller than 0.5°, reducing the radiometric errors. The use of a tripod is recommended but not always possible when measuring in the field, so the sun-finder allows manual operation minimizing the errors.

- The dynamical nature of the fluorescence signal and the plant's adaptation to changing environmental conditions cause the fluorescence signal to vary in time. The effect of clipping the leaf and follow a measuring sequence has been analysed by monitoring the signal at high frequency sampling (~100Hz). Under controlled conditions in the laboratory, it was found that shifting from lower to higher light intensities produces a larger transient than the other way around. Using a filter with cut-off at 675 nm or above produces a transient of less than 1% in the far-red fluorescence, and for a filter with cut-off at 650 nm the transient is around 2%, lasting less than 10s until returning to the unperturbed level. These transient variations were smaller than the variations due to thin cirrus clouds while measuring outdoors in field conditions. The largest perturbation is produced by placing the leaf in the dark, even for the shortest period of time. Its transitory is larger than 5% and lasts longer than 10s. Therefore, the protocol to measure was adapted to reduce the impact of fluorescence transients.

- A method has been developed to identify illumination instability when a measuring sequence has been affected by such changes, and to compensate the effect when calculating the apparent fluorescence yield

(yield at leaf level). However, this method is limited by the assumption that reflectance and transmittance measurements are little or not affected by the change in illumination, which might not always hold for some cases under stress conditions.

- The leaf optical properties measured with the FluoWat have been compared with those using an integrating sphere. Some differences are found and can be explained by the fact that the FluoWat only measures the radiance in the nadir direction, and from this measurement the irradiance (the integrated radiance over the hemisphere) is approximated by the assumption of Lambertian response by the leaf, leaving out the contribution of the specular reflection and other angular effects. The absorptance measured by the FluoWat was found a positive bias of slightly less than 0.04 for 6 species of evergreen and deciduous samples.

- Measurement of leaves from the same tree under global and diffuse illumination (each one adapted to their light conditions) present a difference of one order of magnitude in emission, but similar "fluorescence yield". This has strong implications for the interpretation of fluorescence measurements at the canopy level.

- Besides fluorescence, NPQ was tracked through the PRI from leaf reflectance. Results show how fluorescence has the fastest response after sudden increase of PAR, followed by a slower activation of photoprotection mechanisms reflected by the decrease of PRI while

fluorescence emission decreases. This type of behaviour of fluorescence and NPQ to stress conditions has already been described using active measurements, but the experiments carried out in this thesis have shown that a similar type of monitorization of the stress is possible using passive measurements of fluorescence and reflectance of the vegetation.

- The measurements carried with the FluoWat leaf clip have provided new insights in the characteristics of fluorescence emission by chlorophyll from different vegetation types and under various environmental conditions, contributing to a better understanding of the variability of the fluorescence signal.

Therefore, it can be concluded that the fluorescence analysis and protocols presented in this thesis, together with the FluoWat device, have contributed to the development of the FLEX mission in the determination of signal levels, development of retrieval methods, assessment of accuracy and understanding of the fluorescence signal. But they have also contributed to many vegetation health studies and applications. The next section highlights the impact of the results of this Thesis to the scientific community.

7.1 RELEVANCE OF THE RESULTS

The FluoWat has been mentioned in more than 70 scientific contributions, in some of them with my participation, others without. The areas of research of those studies are varied, and have been summarized in form of diagram in Figure 7-1

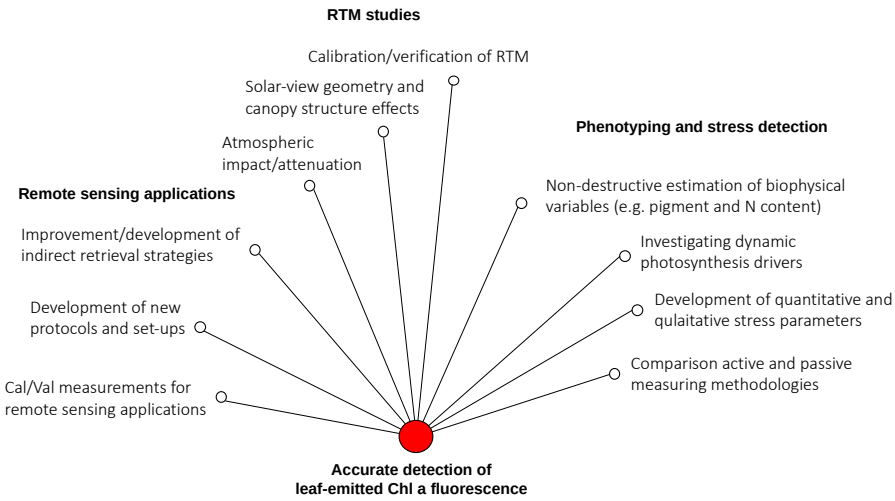

Figure 7-1 Areas of research of the 71 publications that included the FluoWat.

The FluoWat leaf clip has been mentioned in five review papers on various aspects of chlorophyll fluorescence, which I have co-authored with substantial contribution (except for Mohammed et al. 2019):

- "Remote sensing of solar-induced chlorophyll fluorescence: Review of methods and applications" (Meroni et al. 2009)
- "Sun-induced chlorophyll fluorescence II: review of passive measurement setups, protocols, and their application at the leaf to canopy level" (Aasen et al. 2019)

- “Remote sensing of solar-induced chlorophyll fluorescence (SIF) in vegetation: 50 years of progress” (Mohammed et al. 2019)
- “Early Diagnosis of Vegetation Health from High-Resolution Hyperspectral and Thermal Imagery: Lessons Learned from Empirical Relationships and Radiative Transfer Modelling” (Hernández-Clemente et al. 2019)
- “Towards the quantitative and physically-based interpretation of solar-induced vegetation fluorescence retrieved from global imaging" (Van Wittenberghe et al. 2021)

USE IN THESIS

There have been four Doctoral Thesis based on the use of the FluoWat clip (even though I have only participated in the first two):

- Cendrero-Mateo M.P., (2013), “Chlorophyll fluorescence response to water and nitrogen deficit”, University of Arizona, USA
- Van Wittenberghe S., (2014), “Hyperspectral solar-induced chlorophyll fluorescence of urban tree leaves: Analyses and applications”, Universiteit Antwerpen, Belgium. [c:irua:117410]
- Zhang C., (2017), “Optical cues reveal the photosynthetic spring recovery in Scots pine needles”, CREAF-CSIC, Universitat Autònoma de Barcelona, Spain
- Olascoaga B., (2018), “Leaf optical properties and dynamics of photosynthetic activity”, University of Helsinki, Finland. DOI: 10.14214/df.247

And two Master Thesis (participating only in the second one):

- Busch C, (2019), “Untersuchungen von nicht-destruktiven Verfahren zur Messung von Kohlenstoffakkumulation in Pflanzen: Sonneninduzierte Chlorophyllfluoreszenz und Eddy-Kovarianz”, Universität zu Köln, Germany
- Wincott C., (2020), “Characterising and analysing the solar induced fluorescence spectrum under different illumination conditions”, Imperial College London, UK

PATENT

The originality of the FluoWat leaf clip has been recognized by a granted patent in both design and methodology.

PATENT nr: ES-2400411

TITLE: Device and Methodology for the measurement of reflectance, transmittance, and fluorescence. [Dispositivo y método de medición de reflectividad, transmisividad y fluorescencia.]

INVENTORS: Luis Alonso-Chordá; José F. Moreno-Méndez; Ignasi Chordá-Carrasco

APPLICATION nr: P201031670 **COUNTRY:** ESPAÑA **APPLICATION DATE:** 2010

PATENTEE: Universitat de València

RESUMEN EN CASTELLANO

INTRODUCCIÓN

La teledetección de la fluorescencia emitida por la clorofila de la vegetación tanto a nivel de dosel, de ecosistema, como global es ya algo común ahora en 2022, pero con diversos grados de eficacia y precisión. Pero este escenario era distinto hace casi dos décadas, cuando dieron comienzo los estudios para esta Tésis. En aquel momento los estudios de fluorescencia estaban dominados por sistemas de medición activos, y solo unos pocos instrumentos de alta tecnología tenían la suficiente precisión de medir la fluorescencia de la vegetación usando únicamente la luz del sol. Además, en aquel momento, una parte importante de la comunidad era escéptica sobre la posibilidad de extraer información relacionada con los mecanismos fotosintéticos tan solo a partir de la fluorescencia en estado estacionario, la que se obtiene por métodos pasivos de medida.

Fluorescencia de la Clorofila

La fluorescencia es la reemisión de fotones que han sido previamente absorbidos por átomos o moléculas. El fotón reemitido es de una energía menor (o similar) que el absorbido (es decir, con longitudes de onda más largas o similares). Este fenómeno fue reconocido como tal por primera vez por Sir G.G. Stokes (1852), aunque ya se había observado antes. También fue el responsable de darle a este fenómeno el nombre de fluorescencia.

Un fotón absorbido por una molécula puede excitar un de sus electrones desde su estado fundamental a un estado excitado, a partir del cual existen diferentes vías para la desexcitación del electrón (Figura 1).

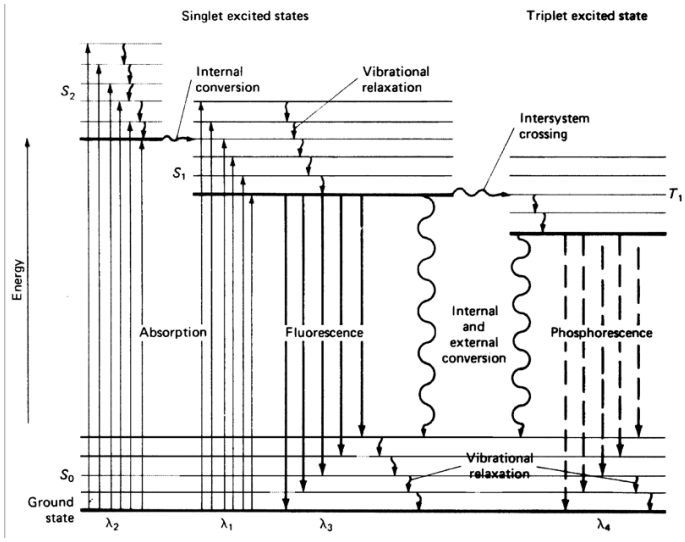

Figura 1 Diagrama de Jablonski que muestra las diferentes vías de desexcitación de un electrón después de la excitación por absorción de fotones.

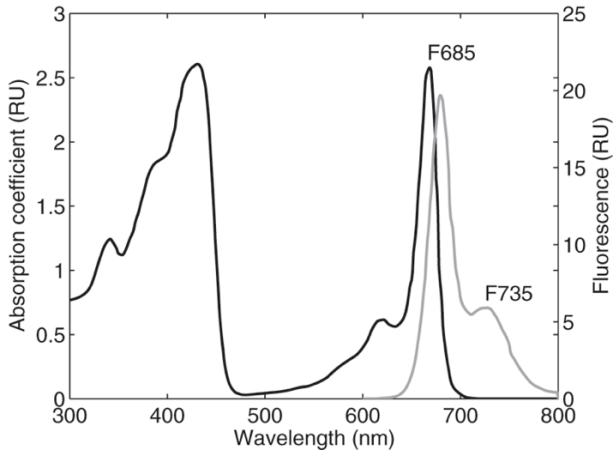

Figura 2 Absorción de luz de la clorofila-a (negro) y emisión de fluorescencia (gris) a nivel de cloroplasto

La fluorescencia de la clorofila se produce por la luz absorbida en el espectro visible, correspondiente al espectro de absorción de la clorofila desde el azul

(por debajo de 400 nm) hasta el rojo lejano (ligeramente por encima de 700 nm) (Figura 2, línea negra). La luz en este rango espectral excita la fotosíntesis y se conoce como Radiación Fotosintéticamente Activa (PAR) (McCree 1981).

La luz ultravioleta también excita la fluorescencia verde azulada en las hojas verdes; sin embargo, se ha encontrado que los cloroplastos intactos aislados y las membranas tilacoides no muestran esta emisión. En cambio, es producido por sustancias vegetales fenólicas ubicadas en la pared celular y/o vacuolas de las hojas (Stober et al. 1994), por lo que no puede considerarse relacionada con la fotosíntesis (Lang et al. 1992; Buschmann & Lichtenthaler 1998).

La emisión de luz fluorescente por parte del material foliar representa una forma de desexcitación de los fotosistemas después de la absorción de un fotón, donde los pigmentos de clorofila exhiben una emisión de fluorescencia roja (Figura 2, linea gris), con dos máximos a 690 nm en el rojo y 740 nm en el infrarrojo cercano (NIR).

La absorción de luz también puede iniciar el proceso de fotosíntesis antes de la reemisión de fotones. La clorofila *a* es una de las principales moléculas responsables de la absorción de la energía luminosa, necesaria para sintetizar carbohidratos a partir de CO_2 y agua. Los primeros investigadores en relacionar la variación de la emisión de fluorescencia con la asimilación de CO_2 fueron Kautsky y Hirsch en 1931 en una breve comunicación (Kautsky et al. 1931). No mucho después de este descubrimiento, varios estudios detallaron aún más la relación entre la fluorescencia y la fotosíntesis (Franck, French y Puck 1941).

Algunas buenas revisiones históricas de la fluorescencia de clorofila y sus fundamentos son las de (Moya y Cerovic 2004) y Papageorgiou y Govindjee (2004).

Amplios estudios experimentales y teóricos demuestran que la fluorescencia de la clorofila es un indicador de la fotosíntesis real (Rosema et al. 1998) y, como tal, está directamente relacionado con la eficiencia del uso de la luz, la absorción de CO_2 (Seaton y Walker 1990) y también se comporta como un indicador de la vitalidad y el estrés de la planta ya que la emisión de fluorescencia compite con los mecanismos de adaptación/protección desarrollados por la planta (Cerovic et al. 1996; Flexas et al. 2000).

Medición de la Fluorescencia en Condiciones Naturales

Pero la fluorescencia que emite una hoja es muy pequeña en comparación con la radiancia que refleja en el mismo rango espectral, y tratar de separarla de la radiancia solar reflejada no es tarea fácil ni siquiera a corta distancia, y más aún por teledetección.

Entonces, ¿cómo se puede medir la fluorescencia en condiciones naturales? La excitación de la fluorescencia por una fuente de luz artificial se ha utilizado durante décadas a escala de hoja o planta para estudiar la actividad fotosintética en el laboratorio y en el campo. Una buena descripción de estos desarrollos se presenta en (Kalaji et al. 2012).

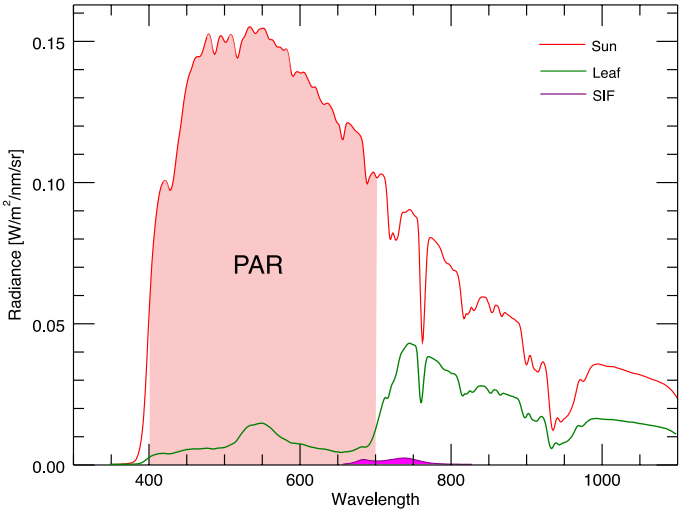

Figura 3 Comparación de los resplandores que llegan de la luz solar (rojo), reflejados por la hoja (verde) y emitidos como fluorescencia (púrpura). Tenga en cuenta la superposición entre la radiación fotosintéticamente activa (PAR) y la fluorescencia inducida por el Sol (SIF).

La detección remota de fluorescencia comenzó sobre aguas (Stoertz, Hemphill y Markle 1969), ya que la luz reflejada por el agua y por la materia luminiscente en el rojo y el infrarrojo cercano es bastante pequeña en comparación con la luz fluorescente emitida que hace que sea más fácil de detectar.

Desde entonces, se han realizado esfuerzos para detectar la fluorescencia de plantas superiores en condiciones naturales a distancia mediante dos técnicas: activa (inducida por láser) y pasiva (inducida por el sol).

Los métodos activos para medir la fluorescencia se basan en que la emisión de fluorescencia es, en primer orden, proporcional a la cantidad de luz que llega a la planta. Así, si a la luz natural que recibe la muestra se le aplica una segunda luz artificial y controlada (luz de medición), el aumento de la emisión de fluorescencia será proporcional a la fluorescencia total emitida. En caso de que la luz de medida sea lo suficientemente pequeña se supone que no alterará las condiciones de adaptación de la planta.

Los primeros sistemas se basaron en estimulación láser o LIF (Laser Induced Fluorescence) y se usaron originalmente para detectar algas en el agua (Friedman y Hickman 1972), y no mucho después se usaron en plantas (Brach, Molnar y Jasmin 1977).

Si la luz de medida se emite en forma de pulsos modulados (a intervalos cortos de encendido y apagado del orden de microsegundos) y un sensor registrando sincrónicamente, es posible monitorizar la evolución de la emisión de fluorescencia. Esta técnica se denomina PAM (Pulse Amplitude Modulation) y se introdujo a mediados de la década de 1980 (Schreiber, Schliwa y Bilger 1986). Además, aplicando pulsos de luz de alta intensidad que saturan los fotosistemas y siguiendo una serie de protocolos, es posible determinar el reparto de la energía absorbida entre las diferentes vías: fotoquímica, disipación de calor regulada y disipación de calor no regulada.

Por otro lado, las medidas pasivas se basan en que el espectro de luz del Sol tiene ciertas longitudes de onda (algunas dentro del rango espectral de emisión de fluorescencia de la clorofila), en el que los elementos de la atmósfera solar absorben una gran cantidad de fotones; por lo tanto, en estas bandas la radiación que llega a la Tierra es muy baja en comparación con el resto del espectro. Estas absorciones se conocen como líneas de Fraunhofer, y en particular la absorción H_α en 656 nm fue la primera en utilizarse debido a su anchura y profundidad en comparación con otras más estrechas y débiles (Sioris, Courreges-Lacoste y Stoll 2003; Moya y Cerovic 2004). Los requisitos tecnológicos de este método son muy exigentes, dado el ancho subnanométrico de estas absorciones, y el bajísimo nivel de señal, a lo que hay que sumar las perturbaciones que introduce la atmósfera terrestre. Además, la atmósfera terrestre tiene dos fuertes absorciones en 687 nm (O_2-B) y 761 nm (O_2-A) que

coinciden con las emisiones de fluorescencia roja (RF) y fluorescencia roja lejana (FRF) respectivamente. Un sensor que mida con precisión en cualquiera de estas bandas de absorción recibirá una mayor proporción de fluorescencia que de radiación reflejada, en contraste con la mayor señal reflejada fuera de la absorción.

Plascyk desarrolló un sistema basado en el uso de líneas solares de Fraunhoffer para detectar SIF, el MK-II Fraunhofer Line Discriminator (Plascyk 1975; Plascyk y Gabriel 1975) que recogía la señal en dos bandas espectrales estrechas seleccionables, de las cuales recuperó la fluorescencia a través del principio de Profundidad de la Línea de Fraunhofer desarrollado originalmente por astrónomos que estudiaban la luminiscencia de la superficie lunar; se puede encontrar una descripción detallada del desarrollo de esta técnica y del instrumento de Plascyk en (Stoertz, Hemphill y Markle 1969). Vale la pena señalar que este instrumento se desarrolló originalmente para estudiar minerales luminiscentes, sustancias contaminantes y vegetación estresada geoquímicamente (Watson y Hemphill 1976).

Se evaluó la viabilidad de un sensor de fluorescencia orbital en (Stacy et al. 1984), pero se consideró demasiado exigente tecnológicamente.

El primer uso informado de las líneas de absorción de O_2 para la medición de la fluorescencia fue realizado por (Carter et al. 1996) y se basó en la banda O_2-B. Un concepto que fue seguido y mejorado por Ismael Moya (Moya y Cerovic 2004) abriendo una posibilidad real de detectar remotamente la fluorescencia de la clorofila de la vegetación desde el espacio.

Sin embargo, todos estos avances en la teledetección de fluorescencia siempre han carecido de una adecuada validación de las estimaciones proporcionadas por los diferentes métodos y tecnologías de estimación. Ha habido una

necesidad de fluorescencia medida directamente para ser utilizada como verdad básica que no se cumplió por completo.

La Misión FLEX (EXplorador de FLuorescencia)

FLEX (Fluorescence EXPlorer) es la primera misión espacial diseñada específicamente para la estimación de la fluorescencia de la vegetación a escala global. La misión FLEX se propuso originalmente a la ESA en 1998 para su consideración como candidata en su programa Earth Explorer (M.-P. Stoll et al. 1999) y, a pesar de que no fue seleccionada para la Fase A, se recomendó la financiación para profundizar en su investigación dado el alto interés científico de la fluorescencia como indicador de la fotosíntesis. Los temas que se encontraron insuficientemente maduros fueron, entre otros, la viabilidad tecnológica y las perturbaciones atmosféricas a la señal medida. Se revisaron los requisitos científicos de la misión, se definieron los requisitos instrumentales y se estudiaron las perturbaciones atmosféricas (Smorenburg et al. 2002). En una revisión posterior de los requisitos de la misión, las bandas de absorción del O_2 se presentaron como una alternativa a las líneas de Fraunhofer (mientras que anteriormente éstas solo se consideraban como un método de apoyo para el desarrollo de la misión debido a la fuerte dependencia de la profundidad de la absorción con la masa de aire) para superar los desafíos tecnológicos de medir la tenue señal de fluorescencia dentro de la línea H_α. Además, se resaltó la necesidad de establecer un conocimiento confiable sobre los niveles de la señal de fluorescencia (M. P. Stoll et al. 2003)

FLEX se propuso formalmente de nuevo en 2005 como uno de los siete conceptos de misión del Earth Explorer 7 (EE7) de la ESA. Sin embargo, el ganador del EE7 después de la Reunión de Consulta de Usuarios (UCM) de enero

de 2009 fue BIOMASS. Posteriormente, el concepto FLEX se redujo en complejidad y fue aceptado como uno de los dos conceptos de misión EE8 pre-Fase A, junto con CarbonSat, a partir de 2011. Finalmente, FLEX prevaleció con el apoyo unánime del panel de revisión de la ESA y la comunidad científica en general que asistió al UCM de Septiembre de 2015 en Cracovia, Polonia.

Siguiendo la recomendación de ahondar en el estudio de la fluorescencia, en la primavera de 2002 se llevó a cabo una campaña de campo en Sodankylä (Finlandia), en apoyo de la misión FLEX, para observar la señal de fluorescencia sobre la cubierta de un bosque de coníferas durante la recuperación de primavera y para determinar la viabilidad de la medición remota desde el espacio (Davidson et al. 2002). En esta campaña se utilizaron instrumentos dedicados para la teledetección de la fluorescencia de la cubierta vegetal (I. Moya et al. 2002) y se utilizó un espectrorradiómetro de campo ASD-FSFR comercial para caracterizar la reflectancia de la cubierta, del sotobosque y de las agujas de los pinos. Encontramos que este instrumento era capaz de detectar los efectos de fluorescencia en la reflectancia (J. Miller et al. 2002), lo que llevó a probarlo en una habitación oscura excitando la fluorescencia de una estera de agujas con un láser rojo, encontrando que era lo suficientemente sensible como para detectar el espectro de emisión (contrariamente a las expectativas). Esto abrió la posibilidad de utilizar tales instrumentos para la medición de la fluorescencia de clorofila y diseñar un nuevo dispositivo que podría acoplarse al espectrorradiómetro que fuera capaz de proporcionar información muy necesaria, como la medición directa de la fluorescencia de clorofila inducida por el Sol en ambos lados de la hoja, las propiedades ópticas, la absorción, PAR y

APAR, utilizando unidades físicas en lugar de las unidades relativas que eran de uso común en aquel entonces.

Esas mediciones ayudarían a abordar las preguntas abiertas (en ese momento) para la misión FLEX, pero también para el conocimiento general:

¿Cuál es el nivel de señal que se espera medir?

¿Cómo de precisos son los métodos de estimación de fluorescencia por teledetección?

¿Cuál es el comportamiento de la fluorescencia medida pasivamente bajo diferentes condiciones ambientales y niveles de estrés?

¿Cómo se relaciona la fluorescencia medida pasivamente con todo el conocimiento ya disponible a través de las medidas activas?

¿Cómo se relaciona la fluorescencia de la parte superior del dosel con la fluorescencia a nivel de hoja y, en última instancia, con la fluorescencia a nivel de cloroplasto?

¿Qué otras fuentes de información se necesitan para relacionar la fluorescencia con la fotosíntesis?

MOTIVACIÓN Y OBJETIVOS

El trabajo de esta Tesis se ha realizado en el marco de la misión Earth Explorer FLEX de la ESA, con el objetivo de dar respuesta a algunas de las necesidades para la preparación y el desarrollo de la misión, tal y como se ha señalado anteriormente.

El principal objetivo de esta Tesis es desarrollar un sistema que permita medir *in vivo* la emisión de fluorescencia de hojas aun adheridas a la planta en condiciones ambientales naturales y luz solar de forma directa y pasiva, como contrapartida a los métodos indirectos y activos ya existentes.

Este sistema pretende dar respuesta a las siguientes cuestiones:

1) Proporcionar las medidas en unidades físicas para determinar los niveles de señal a registrar por la misión FLEX.
2) Evaluar la precisión de los métodos de restimación de fluorescencia basados bandas de absorción de O_2, aplicados a nivel de hoja.
3) Proporcionar estimaciones precisas de la energía real emitida por la vegetación en forma de fluorescencia para permitir una comparación justa con las estimaciones de fluorescencia por métodos de teledetección.

Y en última instancia,

4) Proporcionar una comprensión de la dinámica de emisión de la fluorescencia de la vegetación bajo diferentes estados de estrés y de adaptación a condiciones cambiantes.

Cabe señalar que estos objetivos se establecieron en 2004, cuando aún había muchas incógnitas sobre la medición pasiva de la fluorescencia, y los objetivos 1) y 2) se cumplieron al principio del estudio, mientras que los objetivos 3) y 4) se han abordado durante un período de tiempo más largo.

METODOLOGÍA

El dispositivo consiste en una cámara oscura de reducido tamaño, que puede abrirse como una pinza para alojar una hoja vegetal (o cualquier otro material fino, como pueden ser discos de filtros con cianobacterias o algas). La pinza tiene una apertura a 45° de la horizontal por la que puede pasar la luz para iluminar la muestra y dispone de un portafiltros deslizante con el que modular o bloquear la luz entrante según se necesite. Tiene además dos conectores, uno en la parte superior y otra en la parte inferior, donde acoplar sendas fibras ópticas, apuntando a la muestra, con las que realizar las medidas.

Con la pinza se puede medir propiedades de las hojas tales como la emisión de fluorescencia, la reflectividad y transmisividad, o la radiación fotosintéticamente activa (PAR) absorbida, así como otros índices derivados de estas, p. e. el PRI, o el ratio entre picos de fluorescencia.

Por último, dispone de otra apertura a 45° de la horizontal y opuesta a la de iluminación pudiendo conectar otros dispositivos que añadan funcionalidad al FluoWat. Por ejemplo, la fibra óptica de un sistema PAM para medidas activas simultaneas a las pasivas, o una lámpara LED para aplicar pulsos saturantes, u otra fibra para medir la reflexión especular.

El sistema FluoWat se ha adaptado para su uso tanto en el campo con luz solar directa y con las plantas en su estado natural, como para su uso en laboratorio con luz artificial no modulada y con las plantas en un entorno controlado.

Se ha establecido un protocolo de medida de los parámetros básicos de medida: radiancia incidente, radiancia reflejada y transmitida, adquiridos con y sin filtro. A partir de ellos se establecen las relaciones para poder obtener los parámetros de interés: fluorescencia (hacia arriba y hacia abajo), reflectividad,

transmisividad (aparentes y reales), absorbancia, PAR, APAR y rendimiento de fluorescencia.

Se ha realizado un análisis de sensibilidad a diferentes potenciales fuentes de distorsión en las medidas. Entre ellas:

- el efecto de usar una referencia blanca delgada, de respuesta lambertiana pero de reflectividad inferior al 99%, necesaria para poder ser insertada en la pinza.
- La sensibilidad a la precisión y estabilidad del apuntamiento al Sol.
- El efecto de los transitorios de fluorescencia inducidos por cambios de iluminación al colocar y retirar el filtro.
- La reducción del PAR por el uso del filtro

Se ha desarrollado una metodología en el procesado de los datos para asegurar la mayor precisión en las medidas, eliminando ciertas perturbaciones intrínsecas al método de medida:

- Eliminación de la luz residual que el filtro deja pasar en la zona de bloqueo, y que se solapa a la fluorescencia.
- La compensación en la reducción de la magnitud de la fluorescencia debida a la disminución del PAR causada por el uso del filtro.
- Detección y compensación de las variaciones en la iluminación durante la secuencia de medidas. Tanto las debidas a nubes altas como a un apuntamiento inestable.

RESULTADOS

Durante esta tesis se han realizado una serie de experimentos de distinta índole, unos que han permitido corroborar que el concepto de medida propuesto es válido, otros que han proporcionado el conocimiento sobre las características de la señal y sobre diferentes perturbaciones cuya corrección es necesaria para obtener una medida de gran precisión.

Además, se han llevado a cabo experimentos que han permitido caracterizar la respuesta de la vegetación bajo distintas condiciones naturales, como son:

- Emisión de fluorescencia por hojas en sombra bajo iluminación difusa. (Sección 5.3). Se realizaron medidas de emisión de fluorescencia en hojas de la parte exterior de la cubierta de una encina en la zona sombreada. Dado que el FluoWat no puede medir usando luz difusa, se usó en su lugar una lámpara LED de intensidad regulable ajustando la cantidad de PAR emitida a la que estaban recibiendo las hojas en ese momento. Seguidamente, se midieron esas hojas con luz solar directa dejando previamente que se adaptaran a ese nivel de luz. Así mismo, se midió la emisión de fluorescencia de hojas del lado soleado para poder comparar las medidas.

  Se encontró que: 1) las hojas en sombra emiten un orden de magnitud menos fluorescencia que las hojas en Sol por lo que su contribución a la emisión de cubierta es muy baja; aunque 2) el rendimiento de fluorescencia es más del doble en las hojas en sombra que en las soleadas. 3) En las hojas en sombra el rendimiento de fluorescencia de la cara superior es tres veces mayor que el de la cara inferior; mientras que en las hojas en Sol esta diferencia se reduce a apenas 1.5 veces. Y 4) como cabe esperar, al exponer las hojas adaptadas a la sombra a condiciones de luz

directa la emisión de fluorescencia es muy superior al de las hojas aclimatadas a luz alta, pero su rendimiento de fluorescencia no cambia (al menos en corto plazo).

- Se probó la pinza para medir la emisión de líquenes y cianobacterias sobre corteza de suelos (soil crust) de zonas áridas con poca precipitación (Sección 5.4), demostrando que su nivel de emisión es entre 10 y 20 veces más pequeña que la de las plantas superiores, aunque susceptible de ser medida; y que la fluorescencia sufre un nivel menor de reabsorción por lo que el pico de emisión dominante es el rojo.
- Se estudió el efecto de la polución atmosférica sobre la vitalidad de la arboleda urbana y su reflejo en las características de la fluorescencia (Sección 6.1). Se midió la emisión de fluorescencia de cuatro especies de árboles presentes en diversas áreas de la ciudad de Valencia con diferentes grados de contaminación atmosférica (mayormente debida al tráfico rodado), encontrándose una diferenciación significativa en la forma del espectro de emisión de fluorescencia en reen relación con elo de polución, así como diferencias entre la emisión hacia arriba y hacia abajo.
- Este mismo conjunto de datos fue utilizado para caracterizar la relación entre los picos de emisión en 685 nm y en 735 nm y la emisión total de fluorescencia (Sección 6.2), mostrando que existe una correlación parcial pero no completa, por lo cual es necesario medir ambos para poder describir de forma completa la emisión de fluorescencia, siendo insuficiente medir uno solo de ellos. Lo cual es relevante al planear misiones de teledetección de fluorescencia.

- Se determinaron las diferencias en la emisión de fluorescencia de dos variedades de la soja (la salvaje y un mutante con déficit de clorofila) creciendo bajo idénticas condiciones ambientales (Sección 6.3).
- Se realizó un experimento sobre la adaptación de las hojas a cambios súbitos de iluminación (Sección 6.4). Durante una ola de calor, se simuló la transición de la sombra de una nube sobre un cultivo de remolacha. Se dispuso sobre el cultivo una red de sombreo que reducía la cantidad de luz que llegaba a las plantas al 50%, retirando posteriormente la red, simulando el efecto de paso de una nube. Poco antes de retirar la red se comenzó a medir de forma continua sobre una hoja con el FluoWat durante al menos 10 minutos después del cambio de exposición. Estas medidas se realizaron en tres ocasiones, en la tarde del primer día de ola de calor, y a la mañana y tarde del segundo día de ola de calor. Se observó como la fluorescencia tiene una respuesta casi inmediata, como forma de eliminación del exceso de luz, y poco después se detectó a través de los cambios en la radiancia visible la activación de los mecanismos de fotoprotección. También fue posible determinar la progresiva disminución en la capacidad de recuperación de los mecanismos de fotoprotección y su contrapartida en la emisión de fluorescencia.

CONCLUSIONES

Esta Tesis presenta el desarrollo de un nuevo dispositivo de medición, denominado FluoWat, para medir directamente la fluorescencia emitida *in vivo* por las hojas bajo la luz solar, incluyendo un protocolo y procesado de datos que minimiza las perturbaciones, aumentando la precisión de la fluorescencia medida.

Consiste en una pinza para hojas conectada a un espectrorradiómetro y la medida se basa en colocar un filtro que bloquea la luz en el rango espectral que se solapa con la emisión de fluorescencia, dejando pasar la luz en el resto de los rangos espectrales para seguir produciendo la excitación de la emisión de fluorescencia.

Se han establecido un conjunto de configuraciones de medida a partir de las cuales es posible obtener, además de la propia fluorescencia, una serie de parámetros biofísicos que son necesarios para la interpretación de la variabilidad de la fluorescencia, en particular la cantidad de luz absorbida para calcular la eficiencia cuántica de la fluorescencia.

El FluoWat mide la fluorescencia emitida por ambos lados de la hoja, el lado iluminado y el lado opuesto, para hacer posible el cálculo del balance total de energía emitida en forma de fluorescencia. La fluorescencia emitida desde el lado opuesto puede representar del 30 al 50 % del total, en su mayoría emitida en el infrarrojo cercano, que es altamente reflejado y transmitido, por lo tanto, con una alta probabilidad de ser dispersado hacia arriba en un dosel vegetal, por lo que no puede ser despreciado en estudios a nivel del dosel.

Con dicho dispositivo, y el procesamiento de datos desarrollado durante esta Tesis, ha sido posible abordar los diferentes objetivos planteados originalmente para apoyar el desarrollo de la misión FLEX de la ESA:

1) Proporcionar las medidas en unidades físicas para determinar los niveles de señal a registrar por la misión FLEX.
2) Evaluar la precisión de los métodos de recuperación de fluorescencia basados en bandas de absorción de O2, aplicados a nivel de hoja.

Estos dos objetivos se lograron en las primeras etapas del desarrollo del instrumento, tal y como se presenta en las Secciones 5.1 y 1.1. Aunque los análisis en ese momento carecían del conocimiento actual sobre las perturbaciones y la respuesta fisiológica de la fluorescencia, los resultados ya eran relevantes para respaldar la misión FLEX en términos del nivel de señal esperado y para justificar la necesidad de desarrollar nuevos algoritmos de recuperación que serían más precisos y menos sensibles a las perturbaciones de la señal.

3) Proporcionar estimaciones precisas de la energía real emitida por la vegetación en forma de fluorescencia para permitir una comparación justa con las estimaciones de fluorescencia por métodos de detección remota.

Y en última estancia,

4) Proporcionar una comprensión de la emisión de la dinámica de fluorescencia de las plantas bajo diferentes estados de estrés y adaptación a condiciones cambiantes.

Estos dos últimos objetivos tardaron mucho más en lograrse, ya que requerían refinar el diseño de la pinza y el procesado de los datos para lograr la mayor precisión posible, presentado en la mayor parte del Capítulo 4. También

requerían medir diferentes tipos de vegetación bajo diversas condiciones ambientales y de salud, reportadas en los Capítulos 5 y 6.

Además, ha habido una serie de conocimientos científicos y técnicos adicionales recopilados durante la elaboración de la Tesis. Las principales conclusiones derivadas de estos estudios se pueden resumir de la siguiente manera:

- La simple medición directa no es suficiente para obtener una estimación precisa de la emisión de fluorescencia. Varias perturbaciones afectan la precisión y la calidad de la señal medida. La perturbación más importante proviene del propio filtro. La magnitud de la transmitancia del filtro (τ) en el rango de fluorescencia es del orden de 10^{-3} (~0,1 %), y la luz que no queda completamente bloqueada será reflejada por la hoja y llegará al sensor mezclada con la fluorescencia. Dado que la fluorescencia es pequeña y la reflectancia es alta en el NIR, esta contribución a la señal no es despreciable. Dependiendo de la calidad del filtro y de la intensidad de la fluorescencia, la contaminación puede ir del 5% al 50%. En esta Tesis se han analizado estas perturbaciones y se han desarrollado métodos de corrección cuando ha sido posible. Esta corrección hace uso de τ, por lo que es necesaria una caracterización precisa. Se ha encontrado que la medición de τ con el FluoWat podría proporcionar una sobreestimación errónea de la transmitancia que puede llegar a duplica la transmitancia real. Vale la pena señalar que esta sobreestimación se traduce en una sobrecorrección de la fluorescencia. Para filtros de menor calidad, por ejemplo, OD2 ($\tau \sim 1\%$),

el error introducido por la sobrecorrección puede ser mayor que el error de usar la directamente la medida de radiancia sin corregir.

- La desalineación con la luz solar durante la medición puede generar un error radiométrico sustancial de ~2% para una inclinación en el apuntamiento de 1°. Estas variaciones corresponden a la ley del coseno. La inclusión de un apuntador solar permite un apuntamiento preciso con desalineaciones menores a 0,5°, reduciendo los errores radiométricos. Se recomienda el uso de un trípode, pero no siempre es posible cuando se mide en campo, por lo que el apuntador solar permite la operación manual minimizando los errores.

- La naturaleza dinámica de la señal de fluorescencia y la adaptación de la planta a condiciones ambientales cambiantes hacen que la señal de fluorescencia varíe con el tiempo. El efecto de pinzar la hoja y realizar una secuencia de medición se ha analizado monitorizando la señal en un muestreo de alta frecuencia (~100 Hz). Bajo condiciones controladas en laboratorio, se encontró que cambiar de intensidades de luz más bajas a más altas produce un transitorio más grande que al revés. El uso de un filtro con corte a 675 nm o superior produce un transitorio menor del 1 % en la fluorescencia de rojo lejano, mientras que, para un filtro con corte a 650 nm, el transitorio es de alrededor del 2 %, con una duración de menos de 10 s hasta que recupera el nivel original. Estas variaciones transitorias fueron más pequeñas que las variaciones debidas a cirros delgados mientras se medían al aire libre en condiciones de campo. Sin embargo, la mayor perturbación se produce al colocar la hoja en la

oscuridad, incluso durante el menor tiempo posible. Su transitorio es superior al 5% de la fluorescencia estacionaria y dura más de 10 s. Por lo tanto, el protocolo de medida se adaptó para reducir el impacto de los transitorios de fluorescencia.

- Se ha desarrollado un método para identificar cuando una secuencia de medición se ha visto afectada por inestabilidad en la iluminación, y para compensar su efecto al calcular el “rendimiento de fluorescencia". Sin embargo, este método está limitado por la suposición de que las mediciones de reflectancia y transmitancia no se ven afectadas o se ven poco afectadas por el cambio en la iluminación, lo que no siempre se cumple en algunos casos bajo condiciones de estrés.

- Las propiedades ópticas de la hoja medidas con el FluoWat se han comparado con aquellas en que se utiliza una esfera integradora. Se encuentran algunas diferencias que pueden explicarse por el hecho de que el FluoWat solo mide la radiancia en la dirección del nadir y, a partir de esta medición, la irradiancia (la radiancia integrada sobre el hemisferio) se aproxima mediante la suposición de respuesta Lambertiana de la hoja, sin contabilizar la contribución de la reflexión especular y otros efectos angulares anisotrópicos. La absorbancia medida por el FluoWat mostró un sesgo positivo de poco menos de 0,04 para muestras de hojas de 6 especies perennes y caducifolias.

- La medición de hojas de un mismo árbol unas bajo iluminación solar directa y otras bajo luz difusa (cada una adaptada a sus condiciones de

luz) presenta una diferencia de un orden de magnitud en la emisión, pero un "rendimiento de fluorescencia" similar. Esto tiene fuertes implicaciones para la interpretación de las mediciones de fluorescencia a nivel del dosel.

- Además de la fluorescencia, el NPQ se monitorizó a través del PRI obtenido a partir de la reflectancia de la hoja. Los resultados muestran cómo la fluorescencia muestra la respuesta más rápida después de un aumento repentino de PAR, seguida de una activación más lenta de los mecanismos de fotoprotección reflejada por la disminución del PRI (valores más bajos indican mayor fotoprotección) mientras la emisión de fluorescencia disminuye. Este tipo de comportamiento de la fluorescencia y el NPQ ante condiciones de estrés ya ha sido descrito utilizando medidas activas, pero los experimentos realizados en esta tesis han demostrado que es posible un tipo similar de monitorización del estrés utilizando medidas pasivas de fluorescencia y reflectancia de la vegetación.

- Las medidas realizadas con la pinza FluoWat han brindado nuevos conocimientos sobre las características de la emisión de fluorescencia por la clorofila de diferentes tipos de vegetación y en diversas condiciones ambientales, contribuyendo a una mejor comprensión de la variabilidad de la señal de fluorescencia, y también han contribuido a desarrollar varias aplicaciones relacionadas.

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A) ANNEX

A-1 FLUOWAT MEASURING PROTOCOL

During the development of the FluoWat leaf clip the measuring protocol has evolved following the knowledge being acquired.

The latest protocol is defined to minimize the errors during the measuring process, while maximizing the efficiency in terms of required time and precision. In the following Filter #1 is the one with the longer cut-off wavelength, and Filter #2 the one with the shorter. Filter #1 should be placed in the filter holder next to the empty slot, followed by Filter #2 (if any). WR stands for white reference.

1) The clip should be aligned to the sun, for this the sun finder shall be used (this is explained below). The clip can be fixed to a tripod or held by hand. In either case, the alignment to the sun must be constantly checked every to ensure consistent measurements.
2) First, one must place the WR to measure it with no filter (empty slot in the filter holder) from the top.
3) Only once per session, the WR should be also measured from below. This will allow estimating total irradiance. Since the optical properties of the WR remain constant, it is only necessary to measure once the transmitted component. If the WR reflectance has been characterized, then this measurement is not necessary
4) Second, one must place the leaf, checking that the clear opening is set and the sun alignment is proper.
5) One must wait for 20-30s to avoid Kautsky effect
6) Then the measurement for reflectance is taken

7) Set filter #1, checking alignment, wait for 15s and measure (since it receives slightly less light the time for adaptation is shorter)
8) Set filter #2 (if any), check alignment, wait 15s and measure
9) In case that only one spectrometer/fibre is available, then add steps 9 to 12:
10) Move the fibre to the bottom port and check alignment
11) Filter should be still in place. Wait for 15s and measure with filter #2 (if any)
12) Set filter #1, check alignment, wait 15s and measure
13) Set the empty slot, check alignment, wait 15s and measure for transmittance
14) The measurement sequence ends by an additional WR measurement:
15) Release the leaf, place the white reference, and measure it. This measurement can be used as the beginning of the next leaf (if it is measured immediately after).

In order to be able to make an estimate of the uncertainty and/or stability of the results it was established that a minimum of 5 consecutive spectra should be recorded for each measurement. Although a larger number (10 or 15) would be desirable it is important to balance error reduction with the time required. In this case, it is important to disable spectra averaging in the spectrometer.

Note that white reference (WR) measurements should be taken twice, before reflectance and after transmittance measurements, in order to check illumination stability. In case of unstable illumination (while using a single spectrometer), the first WR measurement should be used to process the reflectance data and the last WR measurement for the transmittance, and the mitigation procedure described in Section 4.4.2 should be applied.

In case the filter has been previously characterized and it remains in mint condition it is possible to skip the filtered WR measurements, thus reducing the time required for a whole acquisition.

A-2 EFFECTS DUE TO FILTER'S OPTICAL CHARACTERISTICS (NUMERICAL APPROACH)

The use of a filter that blocks the incoming light in the NIR allows the direct measurement of the fluorescence emission; but it also reduces the amount of PAR that reaches the plant, (affecting the fluorescence emission) in two ways. First, because PAR and fluorescence partially overlap between 650 and 700 nm, the region of maximum absorption by the chlorophyll. So, setting the cut-off wavelenght at 650 nm would allow measuring both peaks simultaneously, but at the expense of significantly reducing the amount of PAR. But even when the filter's cutoff wavelenght is 700 nm its transmittance in the passband region is close but smaller than 1, thus slightly attenuating PAR in whole.

In order to estimate the impact of using the filter on the level of fluorescence emitted we have used an Excitation-Emission Matrix (EEM) that relates the luminescence spectral emission intensity of fluorescent compounds as a function of the excitation wavelength.

The EEM used in this work has been measured and gently provided by Elisabeth M. Middleton from the Biospheric Sciences Branch, NASA Goddard Space Flight Center and Lawrence A. Corp from USDA-ARS Hydrology and Remote Sensing Laboratory (Corp et al. 2006).

The EEM is an average of two crops and three deciduous trees just for a rough generalization of vegetation response. The excitation source used to measure the EEM is a xenon lamp which has a similar distribution as solar energy but a wavelength dependent correction to simulate solar illumination was made (assuming a linear response to changes in illumination intensity).

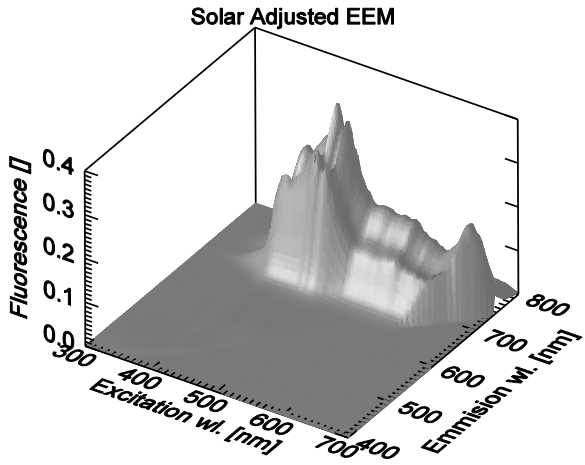

Figure A-1 Solar Adjusted EEM

The filters used with FluoWat have changed through the various development stages. During the early development of the clip the availability of cut-off filters was extremely limited (actually, the first filter was cyan with a very long transition from fully transmitting to fully blocking). Soon high-performance filters appeared in the off-the-self market, with increased optical density (OD) first at OD3 and later OD4 (an OD of 4 indicates that the transmittance is 10^{-4} at the blocking region), improving the quality of the measurements. Also, adding different options for selecting the cut-off wavelength.

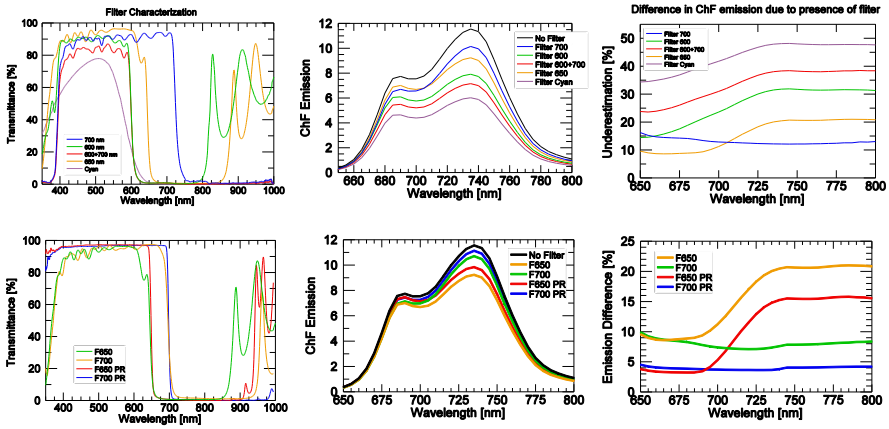

Figure A-2 Left: Transmittance of the filters used in the analysis. Centre: fluorescence emission corresponding to each filter, calculated from the EEM. Right: Relative difference of the fluorescence measured with a filter with respect to unfiltered excitation light.

Nonetheless, these results need to be contrasted with experimental evidence, since they have been obtained from a EEM that has missing elements in the region corresponding to the red emission from red and far-red excitation. Most probably this would result in an underestimation of the red fluorescence peak. Besides, there are some concerns about the representativeness of EEM as they are measured using monochromatic light, since it is known that the fluorescence emission differs when the excitation is monochromatic and when it is broad-band polychromatic or white; and there is also a difference in the illumination level that could change the state of the photosynthetic apparatus. Therefore, the estimation of the filter effect presented in Section 4.2.3 is preferred.

A-3 FILTER REDUCTION OF PAR. COMPENSATION WITH A LENS

Low pass filters should have a high transmittance (close to 1) in the bands below the cut-off wavelength. But for some filters of low performance this is not the case, and there is a significant reduction of the PAR reaching the sample, in contrast to the PAR without the filter (see Figure 4-11 for such a case).

Despite the filter's high transmittance factor below the cut-off wavelength (>90%) there is a slight loss of energy arriving to the sample, but enough as to change the activity of the chlorophyll. To compensate for reduction on the irradiance a lens can be placed after the filter, in order to collect the light rays in a smaller area, thus increasing the intensity.

This solution was originally implemented by I. Moya (see Section 1.1) but was discarded when the sliding filter holder was first introduced, since the lens had to be removed together with the filter, so both irradiances would have the same intensity. But then, there would be a mismatch of illumination conditions between the measurements involved in the correction of the residual light.

Besides, for each filter used it would be necessary to use a specific lens that would not be interchangeable, what makes this solution less practical.

Nonetheless, here are presented the equations needed to determine the focal length of the lens.

We need a system such that from the intensity of the filtered irradiance at the entrance area it is obtained the unfiltered intensity at the smaller sampling area:

$$\tau \cdot I \cdot \pi R^2 = I \cdot \pi r^2 \qquad \text{A.1}$$

therefore

$$r = R \cdot \sqrt{\tau} \qquad \text{A.2}$$

Since the radius *R* of the entrance pupil of the clip is 5.3 mm, and the radius *r* of the measuring area is 5 mm, the least filter transmittance that can be compensated by using a simple lens is $\tau = 0.89$

Once we have the inner radius, it is possible to make use of the relationship of similar triangles

$$\frac{R}{f} = \frac{r}{f-d} \qquad \text{A.3}$$

in order to derive the required focal length of the lens:

$$f = d \cdot \frac{R}{R-r} \qquad \text{A.4}$$

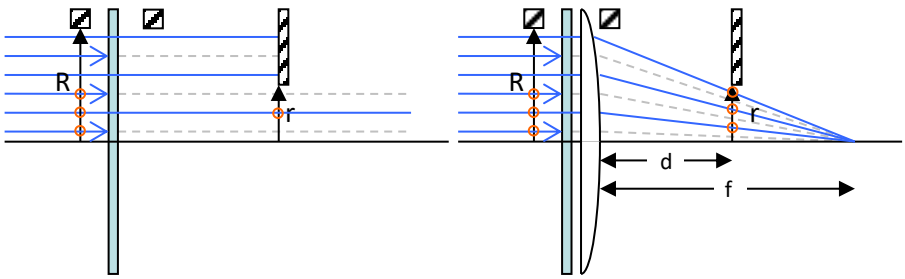

Figure A-3 Diagram of how the lens compensates the loss of light

From equations A.2 and A.4 we obtain:

$$f = d \cdot \frac{1}{1-\sqrt{\tau}} \qquad \text{A.5}$$

For the final design of the clip, the distance *d* between the bottom of the filter holder and the centre of the measuring area is 23.9 mm. The typical transmittance of the filter is 90%, resulting in a lens with a focal length f=466 mm.

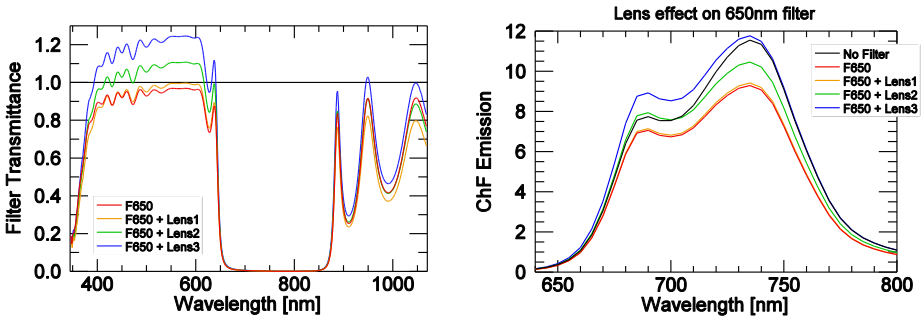

Figure A-4 Left: Effective transmittance when coupling the filter and lenses of different power. Right: resulting fluorescence for each combination, as calculated from the EEM.

When using a lens, it is important to place it below the filter, and not above it, since the filter performance depends on the angle of incidence of the rays and how parallel they are. The lens changes the angle of the rays with respect to the optical axis. Thus, if placed before the filter, the rays will pass through the filter with a variety of incidence angles, producing a distortion in the filter transmittance especially at the cut-off wavelength.

However, this solution is not fully satisfactory since the lens would depend on the properties of the filter used. That is, each particular filter must have its own lens with the adequate focal length. This implies that custom made lenses are necessary, with the extra cost that they represent. Alternatively, it could be possible using affordable off-the-shelve lenses with standard focal lengths at the expense of inaccurate compensation of the light intensity.

Another handicap of this approach comes from the need, for some calculations, of using together filtered and unfiltered measurement; but using the lens changes the illumination geometry and conditions of the two, making them incompatible.

Besides, the reduction of the illuminated area imposes a requirement of very accurate pointing, since a small tilting would shift the illuminated are out of the

measuring area, which in field conditions is not always easy to achieve (see Section 4.3.4). Thus, the use of lenses with the current design of the leaf clip is not recommended except when using filters with lower transmittances in the VIS region, such as the old ones.

High performance filters have become available in recent years at a reasonable price which provide transmittances higher than 95% for the PAR bands, and transmittances of 10^{-4} (OD-4) in the rejection region for the fluorescence bands, with a very sharp transition from fully transmitting to fully blocking around the cut-off wavelength of just a few tens of nanometres. It is possible to use these filters without any lens, and the PAR reduction is small enough to use the constant-yield approximation used in Section 4.2.4 before.