Dissertationes Forestales 330

Methodological and mechanistic context for the interpretation of leaf-level spectral chlorophyll-*a* fluorescence

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Academic dissertation

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ABSTRACT

Boreal forests assimilate a substantial fraction of global atmospheric CO_2 and thus play a key role in the global carbon cycle. However, due to the prevalence of evergreen species, monitoring photosynthetic dynamics of boreal forests is challenging when using conventional greenness- or vegetation-indices. Fortunately, an increasing body of evidence suggests that chlorophyll-*a* fluorescence (ChlF) – a weak red-to-far-red radiation emitted by the chlorophyll *a* molecules nanoseconds after light absorption – can enhance our capacity to assess photosynthetic dynamics in evergreen-dominated ecosystems. However, before extracting complete information embedded in the ChlF, comprehensive understanding and quantitative characterization of the mechanisms that connect the measured ChlF to photosynthesis across various scales are essential.

In this thesis, I discuss several challenges that we currently need to face to leverage the full potential of ChlF. I present a roadmap through these challenges, towards a more comprehensive interpretation of ChlF. The main focus is laid on the challenges concerning ChlF measured at a leaf-level in methodological and mechanistic contexts. In other words, this thesis contributes to the interpretation of ChlF by contextualizing the influence that methodological and mechanistic factors have on leaf-level spectral ChlF.

An impact of methodological factors, measuring geometry and sample arrangements, on spectral ChlF was analysed. Results indicate that ChlF shape is less dependent on measuring geometry as compared to ChlF magnitude and that if needle-mats are used, measuring geometry does not lower the comparability between studies using different setups. Mechanical factors were investigated in terms of their effect on spatial and temporal variation in spectral ChlF. The diversity of species and light environments within an ecosystem was shown to generate a temporarily-invariant, baseline variation in leaf spectral ChlF, as well as contrasting seasonal photosynthetic acclimation patterns. Consequently, I suggest the need for considering both the methodological and mechanistic contexts in the interpretation of ChlF.

Keywords: Boreal forest, Conifer, Foliar Morphology, Foliar Pigments, Non-photochemical Quenching, Photosynthesis

TIIVISTELMÄ

Pohjoinen havumetsävyöhyke on avainasemassa ilmakehän hiilidioksidin sidonnassa ja täten globaalissa hiilenkierrossa. Havupuiden yhteyttämisdynamiikan seuranta on haastavaa perinteisillä kasvien vihreyttä mittaavilla kasvillisuusindekseillä. Klorofyllifluoresenssin (ChlF), eli klorofylli–*a* molekyylin lähettämän punaisella aallonpituudella havaittavan heikon säteilyn, potentiaalista havupuuvaltaisten ekosysteemien yhteyttämisdynamiikan seuraamisessa on jatkuvasti enemmän näyttöä. Ennen kuin on mahdollista saada irti kaikki informaatio ChlF – signaalista, on tärkeää ymmärtää ne mekanismit joilla mitattu ChlF kytkeytyy yhteyttämiseen eri mittakaavoilla.

Käsittelen tässä väitöskirjassa niitä monia haasteita, jotka estävät meitä hyödyntämästä ChlF-signaalin koko potentiaalia ja rakennan teoreettisen suunnitelman kohti kokonaisvaltaisempaa tulkintaa ChlF-signaalista. Suurin painoarvo asetetaan niille haasteille, jotka käsittelevät ChlF-signaalia metodologisessa ja mekanistisessa kontekstissa. Tämä väitöskirja edesauttaa ChlF-aineiston tulkitsemista käsittelemällä niitä vaikutuksia, joita metodologisilla ja mekanistisilla tekijöillä on ChlF-signaaliin lehtitason mittauksissa.

Väitöskirjassa analysoidaan mittausgeometrian ja mittausjärjestelyiden metodologisten tekijöiden vaikutusta ChlF-signaaliin eri mittausasetelmissa havunneulasilla ja lehtipuiden lehdillä. Tulokset osoittavat, että käytettäessä havuneulasmattoja, ChlF-signaalin muoto on vähemmän riippuvainen mittausgeometriasta kuin signaalin vahvuudesta, ja neulasmattoja mitattaessa, mittausgeometria ei vähennä eri näytejärjestelyjä käyttävien tutkimusten vertailtavuutta. Väitöskirjassa tutkitaan miten eri mekaaniset tekijät vaikuttavat ChlF-signaalin muutoksiin ja huomioitiin fysikaaliset tekijät, kuten lehden morfologia, ja fysiologiset tekijät, kuten pitkäkestoinen ei-fotokemiallinen sammutus. Huomattiin, että ekosysteemin sisäinen vaihtelu lajeissa ja valo-olosuhteissa tuotti sekä muuttumattoman variaation ChlF-signaalin perustasoon, että poikkeamia yhteyttämisen kausittaisen sopeutumisen malleihin.

Tämän väitöskirjan tulosten tukemana perustelen tarvetta huomioida sekä metodologiset, että mekanistiset kontekstit ChlF-signaalin tulkinnassa.

Avainsanat: Avupuu, Ei-fotokemiallinen sammutus, Fotosynteesi, Havumetsävyöhyke, Lehtien morfologia, Lehtipigmentit

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Helsinki, December 2022
Paulina

LIST OF ORIGINAL ARTICLES

This thesis consists of an introductory part and three original articles. Two articles have been published in peer-reviewed journals and one article is a manuscript submitted to a peer-reviewed journal. Two articles are research articles and one is a perspective/discussion article. The articles are referred to in the text by their Roman numerals.

I. Rajewicz P.A., Atherton J.M., Alonso L., Porcar-Castell A. (2019). Leaf-level spectral fluorescence measurements: Comparing methodologies for broadleaves and needles. *Remote Sensing*, 11(5), pp. 532, doi:10.3390/rs11050532

II. Rajewicz P.A., Zhang C., Atherton J.M., Van Wittenberghe S., Riikonen A., Magney T., Fernandez-Marín B., Garcia Plazaola J.I., Porcar-Castell A. The photosynthetic response of spectral chlorophyll fluorescence differs across species and light environments in a boreal forest ecosystem. Manuscript submitted to *Agricultural and Forest Meteorology*, doi: 10.2139/ssrn.4170451 [Preprint]

III. Porcar-Castell A., Malenovský Z., Magney T., Van Wittenberghe S., Fernández-Marín B., Maignan F., Zhang Y., Maseyk K., Atherton J., Albert L.P., Robson T.M., Zhao F., Garcia-Plazaola J.I., Ensminger I., Rajewicz P.A., Grebe S., Tikkanen M., Kellner J.R., Ihalainen J.A., Rascher U., Logan B. (2021). Chlorophyll-a fluorescence: illuminating the path connecting plant molecular biology to Earth-system science. *Nature Plans*, 7, pp. 998–1009, doi: 10.1038/s41477-021-00980-4

AUTHOR'S CONTRIBUTION

Paulina Rajewicz was the author of this thesis summary. In **Study I**, Rajewicz participated in the design of the study, collected the data of spectral chlorophyll-*a* fluorescence, processed, analysed the data, and was the leading writer of the article. In **Study II**, Rajewicz participated in the design of the study, collected the data of spectral chlorophyll-*a* fluorescence and light environment, processed and analysed the data, and was the leading writer of the article. In **Study II**, several co-authors contributed to field materials collection and pre-analysis of the data. In both studies, data analysis, data interpretation, and writing were supported by supervisors. Rajewicz was responsible for organizing the *Fluorescence Across Space and Time* Workshop, which was the ground for creating the underlying ideas and concepts for **Study III**. She took part in discussing, commenting, and preparing this article for submission. All co-authors participated in the discussion and commented on the three articles included in this thesis. The three articles have not been used previously and, to the author's current knowledge, will not be used in another dissertation(s).

TABLE OF CONTENT

ABBREVIATIONS

1N: one needle arrangement3N: three needles arrangement
A_{1200}: Assimilation (net exchange of CO_2) at PAR of 1200 μmol $m^{-2}s^{-1}$
A_{max}: Maximum assimilation factor
$APAR_g$: Absorbed photosynthetically active radiation
Cab: Chlorophyll *a+b*
[Cab]: Chlorophyll *a+b* concentration
Car: Carotenoids
ChlF: Chlorophyll-*a* fluorescence
DHP: Digital Hemispherical Photography
F_0: Minimal chlorophyll a fluorescence measured in a dark-adapted leaf after a saturating light pulse
F_M: Maximal chlorophyll a fluorescence measured in a dark-adapted leaf after a saturating light pulse
F_{MR}: Reference level of F_M, maximum F_M recorded for a given sample in a given time period
F_V/F_M: Maximum quantum yield of Q_A reduction, i.e., maximum quantum yield of PSII photochemistry
F_V: Variable chlorophyll a fluorescence, calculated as a difference between F_M and F_0
f$APAR_g$: Fraction of the light is absorbed photosynthetically active radiation
$f_{esc}(\lambda)$: wavelength-dependent escape probability of chlorophyll a fluorescence, at leaf-level, $f_{esc}(\lambda)$ indicates the probability that ChlF emitted at the photosystem level escapes the leaf and reaches a sensor
FOV: field of view
FR: Far-red chlorophyll a fluorescence maximum, located around 740nm
FW: FluoWat
FWHM: Full width at half maximum
G_F: Gap fraction
GLI: Global light index, parameter of local light environment
GPP: Gross primary productivity
IS: Integrating Sphere
IT: Integration time
LMA: leaf mass per area
LUE: Light use efficiency
LUE_{max}: Maximum level of light use efficiency
NM: needles mat arrangement
NPP: Net primary production
NPQ: Non-photochemical quenching of the chlorophyll a fluorescence signal
NPQ_S: sustained form of the non-photochemical quenching
OC: Optical Chamber
P*app*: apparent photosynthesis, i.e., true photosynthesis minus photorespiration
P680: Chlorophyll of PSII with absorption peak at 680 nm
P700: Chlorophyll of PSI with absorption peak at 700 nm
PAM: Pulse-Amplitude-Modulated fluorescence
PAR: Photosynthetically active radiation
PCA: Principal components decomposition
PC: Principal component

PPFD: photosynthetic photon flux density
PQ: Photochemical quenching of the chlorophyll a fluorescence signal
PQ_S: sustained form of the photochemical quenching
PSI: Photosystem I
PSI: Photosystem I
Q_A: Primary quinone acceptors (quinone A)
R/FR: Ratio of *red* and *far-red* chlorophyll a fluorescence maxima
R: Red chlorophyll a fluorescence maximum, located around 685 nm
SIF: Solar-induced fluorescence
SLA: Specific leaf area
SMEAR-II: Station for Measuring Ecosystem-Atmosphere Relations, Hyytiälä, Finland
SNR: Signal to noise ratio
SVD: Singular value decomposition
SV: Singular value, component of singular value decomposition
VAZ: xanthophyll cycle pigments
Zea: Zeaxanthin
$\Phi F(\lambda)$: wavelength-dependent quantum yield of chlorophyll-*a* fluorescence
$\Phi F_{PSI(\lambda)}$: quantum yield of chlorophyll-*a* fluorescence associated with photosystem I
$\Phi F_{PSII(\lambda)}$: quantum yield of chlorophyll-*a* fluorescence associated with photosystem II
$\Phi P(\lambda)$: quantum yield of photochemistry

1. INTRODUCTION

1.1. Boreal forest

Boreal forest, named after the Greek god of the North Wind, *Boreas*, constitutes about one-third of all the global forest area (FAO, 2010; Brandt et al. 2013). Ranging from 50º to 70º North (Johnson and Miyanishi, 2012), the biome is characterized by strong seasonality of temperature and irradiance. In certain regions, the temperature can vary from as low as -70ºC in winter to as high as 30ºC in summer, and the irradiance can alter from polar night to polar days (Baldocchi et al. 2000). Such strong seasonal dynamics influence yearly patterns of photosynthetic activity in the boreal vegetation.

Photosynthesis is a metabolic process carried out by photoautotrophic organisms, e.g., plants, in which carbon dioxide (CO_2) in the atmosphere is turned into glucose with the use of visible radiation as an energy source. Photosynthesis, influencing atmospheric CO_2 concentration, is a key element in the carbon cycle. Globally, about 50% of the total net primary production (NPP), i.e., the net amount of carbon fixed through photosynthesis, can be assigned to terrestrial biomes (Falkowski et al. 2000; Falkowski and Raven 2007). However, terrestrial biomes differ in terms of photosynthetic activity (intensity and seasonal dynamics (Xiao et al. 2004)) and, thus, do not contribute equally to the global NPP. Encompassing about 30% of the global forest area (Brandt et al. 2013), boreal forests assimilate a substantial fraction of global atmospheric CO_2 (Beer et al. 2010; Thurner et al. 2014, 2016) and play a key role in the global carbon cycle (Anav et al. 2015).

Boreal forest vegetation is dominated by conifers, with evergreen genera of pine (*Pinus* L.), spruce (*Picea* L.), and fir (*Abies* L.) being the most abundant (Esseen et al. 1997). Other conifers, as well as deciduous genera, e.g. birch (*Betula* L.), aspen (*Populus* L.), and willow (*Salix* L.) can be also found (Soja et al. 2007). The dominance of conifers is well-grounded in the evolutionary adjustments of these cold-resistant species, which developed small, waxy, light-collimating, and water-retaining needle-like leaves (Thomas, 2014). The photosynthetic activity of deciduous, mostly broadleaves species is limited to the growing season (spring-autumn), after which they drop their leaves. In contrast, evergreen species keep their leaves/needles for many years, remaining green throughout the seasons (thus called evergreens (Bäck et al. 1994; Dengel et al. 2013)). Therefore, the foliage of evergreen species must undergo a series of biochemical and physiological adjustments over the course of a year in order to cope with a substantial variation in temperature, irradiance, and their relative (im)balance.

Monitoring the dynamics of photosynthetic activity can be performed at a scale of leaves/needles, shoots, and whole plants with gas exchange-based methods (Long et al. 1996), e.g., using chambers (Long and Bernacchi, 2003) or eddy covariance towers (Baldocci, 2003; Mammarella et al. 2007). While this relatively small scale monitoring is applicable across various biomes, boreal forests introduce challenges at larger scales, where remote sensing techniques using optical data are needed. Unlike in deciduous biomes, where covariation between greenness and photosynthesis is strong (Yang et al. 2015), conventional greenness- or vegetation-indices are not optimal in tracking the seasonality of photosynthesis in regions with relatively little seasonal variation in greenness (Magney et al. 2019a). Fortunately, an increasing body of evidence suggests that seasonal photosynthetic dynamics

of these regions can be accurately assessed with chlorophyll-*a* fluorescence (Walther et al. 2016; Magney et al. 2019a).

Chlorophyll-*a* fluorescence (hereafter denoted as ChlF) has long been used by biophysicists, molecular biologists, and ecophysiologists, as a method to investigate the structure and function of the photosynthetic apparatus (Govindjee, 1995; Tikkanen et al. 2017). Active methods, i.e. methods that use artificial light sources, have been successfully applied to measure ChlF at small scales (Kolber et al., 2005; Amoros-Lopez et al., 2008). Recently, means to measure ChlF expanded from the subcellular or leaf-level (Oxborough and Baker, 1997) to passive remote sensing techniques retrieving Solar-induced ChlF (SIF) from towers, unmanned aerial vehicles, aircrafts, and satellites (Davidson et al. 2003; Moya et al. 2004; Zarco-Tejada et al. 2009; Guanter et al. 2012). In short, remotely sensed SIF is the integrated ChlF signal emitted by various leaves within a considered area, complicated by canopy structure as well as dependent on the effects of methodological and mechanistic factors (Figure 1). Crucially, although ChlF is emitted across a wavelength range of 650 – 850 nm, SIF is retrieved only within narrow, distinct Fraunhofer or atmospheric absorption bands (see Figure 3 for more details and visualization of ChlF and SIF) (Meroni et al. 2009).

ChlF can provide acquirable data on the photosynthetic dynamics of plants across various spatio-temporal scales, especially in the case of evergreen-dominated biomes. However, the relationship between ChlF and photosynthesis (or at a larger scale, between SIF and gross primary productivity, GPP) is not trivial, as it depends on various factors. Crucially, the effect of these factors can change depending on the measuring methodology (i.e., on methodological context) and operate differently in spatial and temporal scales (i.e., depending on spatial and temporal context). Consequently, the measured ChlF will change as well. Comprehensive understanding and quantitative characterization of the processes or mechanisms that connect the measured ChlF to photosynthesis across various scales are, therefore, essential before we will be able to extract the complete information embedded in the ChlF signal. Further work and investigation towards scaling and standardizing methods for ChlF interpretation are required. Nevertheless, the complexity of the ChlF-photosynthesis relationship introduces a series of challenges in interpreting ChlF at the range of different scales. A *roadmap* is needed for future studies to navigate across these challenges and to join efforts towards the same overarching goal of a more precise and complete interpretation of ChlF in terms of photosynthesis across scales. The need for this roadmap is urgent: with recent improvements in remote sensing techniques (Aasen et al. 2019; Mohammed et al. 2019), the spatial and temporal resolution of SIF retrievals is rapidly expanding, but we are not yet ready to convert this increasing volume of data into meaningful information and engage SIF into new applications.

This thesis discusses several of the current challenges that stop us from leveraging the full potential of ChlF. The main emphasis is laid on the challenges found at the leaf-level. Specifically, this thesis aims at identifying, contextualizing, and characterizing the influence that *methodological* and *mechanistic* factors have on leaf-level spectral ChlF. At leaf-level, methodological factors include measuring geometry or sample arrangement. Characterizing the effects that these factors have on ChlF constitutes a *methodological challenge*. Mechanistic factors include physiological and physical factors (described in sections *1.3* and *1.4*, respectively). Characterizing the effects that these factors have on the variation in ChlF in a spatial and temporal context, constitutes a *mechanistic challenge*. Solving these two challenges, occurring at the leaf-level, is an important step on the way towards the more precise and complete interpretation of ChlF across scales.

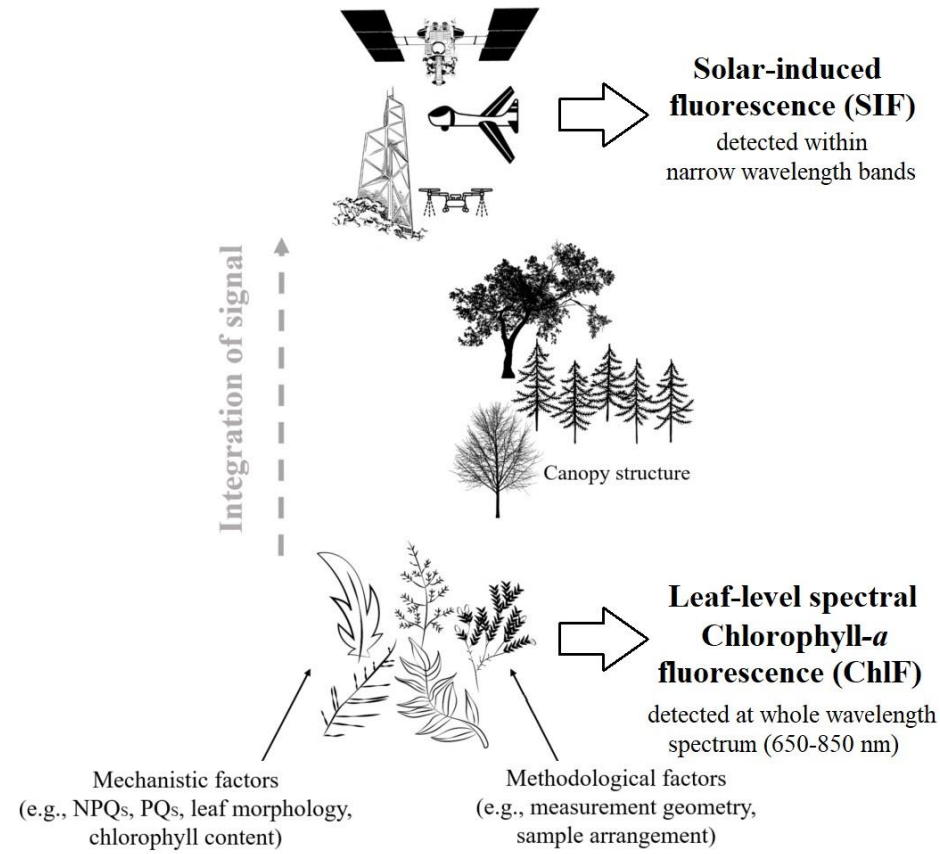

Figure 1. Leaves in the perspective of remotely sensed SIF. An ecosystem is a dynamic mixture of leaves of different species, developed under different light environments. What is observed with remote sensing techniques is an integrated ChlF signal emitted by various leaves, further complicated by canopy structure and dependent on measuring approaches. Consequently, ChlF and its relationship with the photosynthesis process will depend on a series of factors and their effect will vary depending on the methodological and mechanistic context of ChlF measurements and interpretation.

1.2. Chlorophyll-*a* fluorescence: theoretical background and leaf-level measuring approaches

When photosynthetically active radiation (PAR) reaches the surface of a leaf, part of it is reflected, part is transmitted, and part is absorbed (Figure 2). The fraction of the light that is absorbed ($fAPAR_g$, where g stands for the greenness of the absorbing pigment) depends not only on the chlorophyll concentration itself and its distribution within the leaf (Asner et al. 1998) but also on the leaf surface structures, like presence of epicuticular waxes or trichomes (Olascoaga et al. 2014). However, not all absorbed light ($APAR_g$, $APAR_g = PAR \times fAPAR_g$) is used in photosynthesis. $APAR_g$ can actually undergo three fates, which constitute a theoretical base for using ChlF in assessing photosynthetic dynamics, as described in the "Butler model" (Butler, 1978).

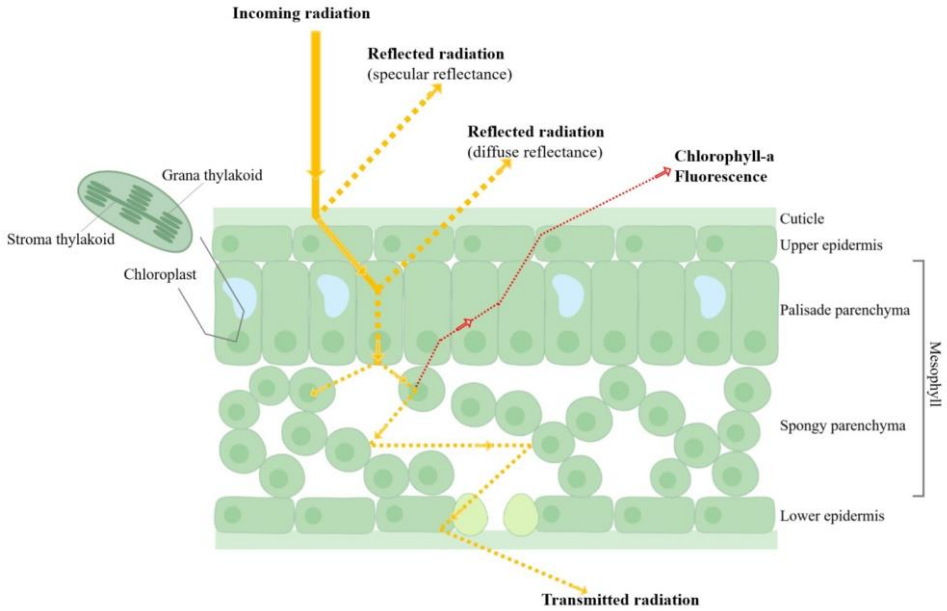

Figure 2. Radiation energy partitioning in a leaf: reflectance, transmittance, and absorption. Once absorbed, light can be emitted as ChlF (red), dissipated as heat, or used in photosynthesis. The main elements of a broadleaf cross-section and a chloroplast are presented.

In the model, excitation energy can be bound chemically (photosynthesis), thermally (heat loss, or non-radiative thermal energy dissipation), and optically (ChlF). The chemical and thermal processes limit, or quench, the number of photons that can be bound as ChlF, and thus are called photochemical (PQ) and non-photochemical (NPQ) quenching, respectively. The Butler model states that ChlF can be used to monitor changes in photochemistry, provided that the rate constant of NPQ does not change (Butler, 1978). However, the rate constant of heat loss from the photosystem II (PSII) antenna does change (Kramer et al. 2004), and it can, in fact, change substantially both on a temporal (Ensminger et al. 2004; Porcar-Castell et al. 2008a) and spatial scale (Porcar-Castell et al. 2008b). Therefore, ChlF can track the PSII photochemistry, but only if quenching processes are determined (Baker, 2008). At the leaf-level, the rate constants of the quenching mechanisms can be determined using Pulse-Amplitude-Modulated (PAM) fluorescence.

1.2.1. Pulse-Amplitude-Modulated (PAM) fluorescence

PAM fluorescence, combined with a saturating pulse technique (Schreiber 1986, 2004), allows investigating the energy partitioning into photochemical and non-photochemical quenching. PAM fluorescence technique is based on light-saturating pulses (Baker 2008). When a leaf is kept in the dark, its primary quinone acceptors (quinone A, Q_A) are maximally oxidized and the PSII reaction centers are “open”, i.e., ready to a perform photochemical reduction of Q_A. When this dark-adapted leaf is exposed to a weak, non-actinic measuring beam, the minimum level of ChlF (F_0) is recorded. When the leaf receives a short, actinic, high-intensity light pulse, leading to maximal reduction of Q_A, i.e. blockage of photochemistry, a maximum level of ChlF (F_M) can be recorded. If a leaf is “stressed”, it

cannot perform photochemistry at the maximum level. Therefore, when the light pulse is applied at a dark-adapted stressed leaf, processes that thermally dissipate the excitation energy are activated, quenching the ChlF signal and resulting in a smaller increase from F_0 to F_M.

The two F values can be used to estimate the maximum quantum yield of Q_A reduction, i.e., the maximum quantum yield of PSII photochemistry (widely used F_V/F_M parameter, where variable F, $F_V = F_M - F_0$, Kitajima and Butler (1975)). In the literature, F_V/F_M values for non-stressed leaves are remarkably consistent at around 0.83 (Björkman and Demmig, 1987; Malenovsky et al. 2009; Atherton et al. 2017; Solanki et al. 2019), while F_V/F_M values of stressed leaves are comparatively smaller (Adams and Demmig-Adams, 2004; Adams and Demmig-Adams, 2006). Moreover, the two F values can be also used to estimate the rate constants of PQ and NPQ (Porcar-Castell et al. 2011).

As will be described in more detail in section *1.3.1*, when leaves in a boreal forest are exposed to low temperatures combined with relatively high irradiance, winter downregulation of photosynthesis is engaged (Míguez et al. 2015; Pierrat et al. 2022). This downregulation, represented by, e.g., damage or loss of PSII reaction centers (Savitch et al. 2002; Ensminger et al. 2004), leads to a decrease in the photochemical capacity and thus to an increase in F_0. In response to the imbalance between light absorption and its decreased utilization, the upregulation of photo-protective mechanisms connected with NPQ is promoted (Demmig-Adams and Adams III, 2006). These mechanisms will be reflected in the decrease in F_M. In this thesis, only these long-term dynamics in the *sustained* form of the two quenching mechanisms (PQ_S and NPQ_S, where S stands for sustained) will be considered (see section *3.4.1* for PQ_S and NPQ_S calculation principles), being relevant to the remote sensing of photosynthetic seasonality.

When PAM fluorescence is used in long-term studies, the maximum F_M recorded for a sample in a certain time span is referred to as reference maximum F, F_{MR} (Porcar-Castell, 2011). This reference level, when NPQ_S is assumed to be zero (fully non-stressed leaf), can be used to estimate the dynamics of PQ_S and NPQ_S over a given time period. This is especially useful in studies on boreal forests, where the seasonality of photosynthetic activity is strong because evergreen vegetation downregulates the photosynthesis in response to exposure to low temperatures combined with relatively high irradiance (Míguez et al. 2015; Pierrat et al. 2022). From the perspective of PAM fluorescence, this downregulation will be reflected as a decrease in F_V/F_M along with a decrease in PQ_S and an increase in NPQ_S (Ensminger et al. 2004; Porcar-Castell et al. 2008a). Consequently, PAM fluorescence has been widely used and, on the seasonal scale, evidence for its strong correlation with photosynthesis in evergreens has been reported (Ottander and Öquist, 1991; Ensminger et al. 2004; Zarter et al. 2006; Soukupová et al. 2008; Kolari et al. 2014; Springer et al. 2017). However, there is a mechanistic gap between PAM fluorescence and SIF (Figure 3) which makes it difficult to translate the results of numerous PAM-based studies into SIF data.

PAM fluorescence comprises ChlF signal across a range of ChlF emission (usually a band of 50-60 nm (Schreiber, 2004)). In turn, SIF is retrieved at discrete, narrow wavelength bands, which corresponds to Fraunhofer or atmospheric absorption bands (Meroni et al. 2009; Rascher et al. 2015) and thus SIF is, unlike PAM, dependent on the wavelength at which it is being retrieved.

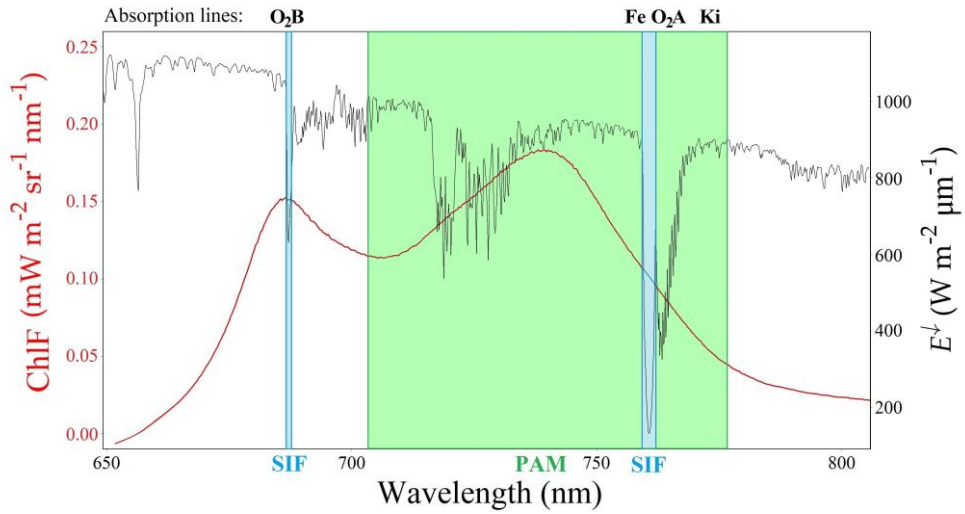

Figure 3. Mechanistic differences between PAM fluorescence, SIF and spectral ChlF shown together with Sun down-welling irradiance, E↓ (black line). The full ChlF spectrum (red line) covers a wavelength range from 650 up to 850 nm and is characterized by two maxima around 685 nm (*R*) and 740 nm (*FR*) (Franck et al. 2002). When ChlF is measured as SIF with remote sensing techniques, it is convolved with the radiance reflected (reflectance) by the vegetation. The level of reflectance is large as compared to SIF itself. Consequently, to retrieve SIF from spectroradiometric measurements, absorption features in the solar or Earth atmosphere have to be exploited. Principally, two solar Fraunhofer lines, Fe (758.8 nm) and Ki (770.1 nm) (Joiner et al. 2011), and two Earth O_2 absorption features, O_2B (687 nm) and O_2A (760 nm) bands, are used because of their spectral proximity to the ChlF emission maxima (Rascher et al. 2015) (blue windows). In contrast, when ChlF is measured as PAM fluorescence, it is comprised across a broader wavelength range (green window). The presented ChlF spectrum (red line) was measured at leaf-level under laboratory conditions. The presented irradiance (black line) was measured under the Sun on a clear day of a crop-field experiment (Xu et al. 2021).

Therefore, despite the fact that both PAM and SIF essentially measure the same (i.e., level of ChlF) and that SIF is, in principle, proportional to the product of steady-state PAM fluorescence and $APAR_g$ (Magney et al. 2017), absolute comparison between the two metrics is difficult. Due to the differences between active and passive techniques to measure ChlF, validation of SIF using PAM fluorescence is very challenging. This mechanistic gap that restricts the use of PAM in investigating the spectral dependency of the ChlF-photosynthesis relationship can be overcome with spectral ChlF, i.e., data retrieved across the whole wavelength range of the ChlF emission.

1.2.2. Spectral chlorophyll-a fluorescence

The spectral ChlF has two maxima: red (*R*) at 685–690 nm and a far-red (*FR*) at 740–750 nm (Lichtenthaler and Rinderle, 1988; Papageorgiou, 2007). Consequently, spectral ChlF can be characterized with the levels of the two maxima (i.e., ChlF magnitude) and their ratio (*R/FR*), i.e., ChlF shape. Variation in these ChlF properties might be affected by a series of factors, like i. leaves responsiveness to environmental conditions and/or stress, e.g., to temperature (Agati et al. 1996) or to water stress (Valentini et al. 1994); ii. the architecture of the photosynthetic apparatus and the relative contributions of PSI and PSII to the ChlF emission

(Pfündel, 1998; Franck et al. 2002; Strasser et al. 2004; Porcar-Castell et al. 2014); and iii. light absorption and scattering within a leaf, which are associated with chlorophyll concentration (Buschmann, 2007; Van Wittenberghe et al. 2014) and leaf architecture (Johnson et al. 2005). Consequently, these factors will affect the ChlF – photosynthesis relationship. The relationship can be described by the equation below, which is more commonly used for SIF-GPP relationship, but can be applied at the leaf-level as well (Guanter et al. 2014; van der Tol et al. 2014; Damm et al. 2015; Frankenberg and Berry, 2018):

$$P_{app} = \frac{LUE}{\Phi F(\lambda)} \times \frac{1}{f_{esc}(\lambda)} \times \mathrm{ChlF}(\lambda) \quad (1)$$

where P*app* is apparent photosynthesis, following the nomenclature proposed by Wohlfahrt and Gu (2015), hereafter denoted simply as photosynthesis; LUE stands for light use efficiency, ΦF(λ) for a wavelength-dependent quantum yield of ChlF, with spectral properties relating to the relative contribution of photosystem II ($\Phi F_{PSII(\lambda)}$) and photosystem I ($\Phi F_{PSI(\lambda)}$), and $f_{esc}(\lambda)$ for a wavelength-dependent escape probability of ChlF photons within a leaf or a plant canopy (Buschmann, 2007; Porcar-Castell et al. 2014; Romero et al. 2018). It is worth noting that $APAR_{g,}$ although fundamental to both photosynthesis and ChlF, is not included in *Equation 1*. The reason is that $APAR_g$ appears in equations describing both photosynthesis ($P_{app} = APAR_g \times LUE$) and the ChlF ($\mathrm{ChlF}(\lambda) = APAR_g \times \Phi F(\lambda) \times f_{esc}(\lambda)$), thus it cancels out in *Equation 1*. Please note that APAR (total light absorption of a leaf) does not equal $APAR_g$, as other non-green pigments can also absorb light.

Various factors can either couple or decouple ChlF from photosynthesis, having a similar or different effect on ChlF and photosynthesis, respectively. These factors can be divided in, at least, two ways. First, considering the nature of the effect they carry on different elements of *Equation 1*, factors can be divided into physiological and physical groups. Physiological factors affect LUE and ΦF(λ) via the regulation of light and carbon reactions in photosynthesis. Along with chlorophyll concentration, which is the fundamental driver of $APAR_g$, physiological factors are represented by PQ, NPQ, and the relative contributions of photosystems to ChlF emission, as discussed in section *1.3.* Physical factors affect $f_{esc}(\lambda)$, via reabsorption and scattering processes within a leaf. In this group, leaf architecture and foliar chlorophyll concentration are the most crucial factors, as described in section *1.4.* It is important to note that chlorophyll concentration can act both as a physiological and physical factor.

The second classification is based on the scale (context) of their variation. Some factors vary on a temporal scale – either in a short (minutes to days) or long (across seasons, years, and millennia) timeframe. Their dynamics can be observed in plants circadian rhythms (García-Plazaolaet et al. 2017), seasonal patterns (Sveshnikov et al. 2006), responsiveness to environmental conditions and the recurring presence of stressors (Jaleel et al. 2009; Fernandez-Marin et al. 2020), or through evolutional adjustments to climatic changes (Nakamura et al. 2011; Anderson et al. 2012). These factors will influence ChlF emission on various temporal scales. On the other hand, there are factors that vary on a spatial scale – among leaves of different species (Li et al. 2018) and light environments, e.g. between different canopy positions (Niinemets et al. 2002), and these factors can also influence spectral ChlF. Factors that express little temporal variation and differ on spatial scale consistently over time, can be called “baseline factors”, causing the temporally-invariant, baseline variation in ChlF (Atherton et al. 2017).

As presented in the following sections, the roles of individual factors in controlling spectral ChlF have been widely studied over the years. However, the mechanisms that connect or disconnect the measured ChlF to photosynthesis are still not quantitatively characterized or even fully understood. In fact, in nature, the effects of various factors are firmly tangled, contributing together and simultaneously to the overall spatio-temporal variation in ChlF. Therefore, it is important to undertake a comprehensive approach to investigate the variation in spectral ChlF and characterize factors that control it simultaneously in the spatial and temporal contexts (mechanistic challenge). Providing enough data to investigate controls of the ChlF-photosynthesis relationship across various spatio-temporal scales, a series of long-term, comprehensive, and multi-traits studies should be implemented, to create a coherent dataset of ChlF emitted by leaves of different species, developed under different environmental conditions, and exposed to different stressors. However, so far, very few studies have embraced such a comprehensive approach to provide a mechanistic interpretation of the ChlF spatio-temporal variation.

1.3. ChlF variation due to physiological factors

1.3.1. ChlF variation due to photochemical and non-photochemical quenching mechanisms

In boreal forests, leaves show substantial temporal variation in $\Phi F(\lambda)$ (Porcar-Castell et al. 2008a) in response to a strong seasonality of photosynthesis (Tanja et al. 2003; Kolari et al. 2014). In winter and early spring, the boreal vegetation is exposed to low temperatures combined with relatively high irradiance and, thus, it engages a downregulation in photosynthesis (Míguez et al. 2015; Pierrat et al. 2022). This downregulation is expressed as organizational changes in the photosystems (Ottander et al. 1995; Ensminger et al. 2004; Zarter et al. 2006), as well as a reduction in the size antennas and in the number of chlorophylls (Vogg et al. 1998). Moreover, the decrease in the photosynthetic enzyme activity decrease (Lundmark et al. 1988) and the damage or loss of PSII reaction centers (Savitch et al. 2002; Ensminger et al. 2004) lead to a downregulation in PSII functionality. Simultaneously, the energy imbalance between light absorption and its decreased utilization via PQ_S promotes the upregulation of photo-protective mechanisms. Both PsbS protein concentration (Öquist and Hüner 2003) and the de-epoxidation state of the xanthophyll cycle pigments (VAZ) increase (Adams et al. 2004; Ensminger et al. 2004; Demmig-Adams and Adams III, 2006). These mechanisms built up a sustained form of thermal dissipation, which is the only form of thermal dissipation that will be discussed in this thesis. Sustained thermal dissipation occurs on a long-term, seasonal scale and is independent of the trans-thylakoid ΔpH (Öquist and Hüner 2003; Demmig-Adams and Adams 2006; Verhoeven 2014), therefore not relaxing on a short timescale, during low light intensities or complete darkness. Later on, during the spring recovery of photosynthesis, PQ_S gradually increases and NPQ_S decreases in response to raising temperature and irradiance (Ensminger et al. 2008; Porcar-Castell et al. 2008a).

Because $APAR_g$ is partitioned between quenching mechanisms and fluorescence (Baker, 2008), a decrease in either PQ_S or in NPQ_S will result in an increase in $\Phi F(\lambda)$. However, PQ_S and NPQ_S have opposite seasonal trends and, thus, opposite effects on the temporal variation in $\Phi F(\lambda)$ (Porcar-Castell et al. 2011). Multiple studies have shown that $\Phi F(\lambda)$ is lower in winter than in summer (Ensminger et al. 2004; Linkosalo et al. 2014; Porcar-Castell et al. 2014), suggesting that it is more sensitive to the effect of increasing NPQ_S, rather than to the

effect of decreasing PQ_S. In the case of evergreens, the dynamics of PQ_S and NPQ_S were also shown to vary between leaves of different light environments (Porcar-Castell et al. 2008b). For example, higher NPQ_S was reported for upper canopy needles of Scots pine, due to higher exposure to excessive light during winter and early spring (Porcar-Castell et al. 2008b). Therefore, PQ_S and NPQ_S are expected to affect the variation in $\Phi F(\lambda)$ not only on the temporal, but also on the spatial scale. However, how will these variations be reflected in *spectral* ChlF?

In response to higher NPQ_S, both ChlF magnitude and *R/FR* are expected to be lower in winter, as compared to summer (Ensminger et al. 2004; Porcar-Castell et al. 2014) and in sun-exposed leaves, as compared to more shaded leaves (Porcar-Castell et al. 2008b). The effect of NPQ_S suppressing ChlF emission especially in the red-ChlF region will be explained in the next section (*1.3.2*) as it is associated with the relative contribution of two photosystems to ChlF emission. What remains uncertain is how the physiological factors interact in their effects on spectral ChlF. Namely, does the role that physiological factors have on spectral ChlF remain constant in space and time?

1.3.2. ChlF variation due to the relative contribution of PSI and PSII to the ChlF emission

Photosystems are pigment-protein complexes, located in the thylakoid membranes of chloroplasts, whose role is to absorb and transfer light energy. In plants, two types of these functional photosynthetic units can be found – photosystem I (PSI) and photosystem II (PSII). Pigments associated with the two photosystems are capable of absorbing photons of PAR, but their specific absorption spectra differ. The most common pigments of the photosystems, the chlorophylls, have the absorption maxima in blue (~450 nm) and red (~650 nm) regions and the absorption minimum in the green light region (~550 nm). In higher plants, two types of chlorophylls are found – chlorophyll *a* and chlorophyll *b*. For chlorophyll *a*, the absorption maxima lay around 410, 440, and 660 nm while for chlorophyll *b* around 420, 460, and 640 nm (Papageorgiou, 2007; Antal et al. 2013).

Due to the efficient transfer of excitation energy from chlorophyll *b* to chlorophyll *a* within light-harvesting antennae (Bittner et al. 1994), essentially all ChlF is emitted by chlorophyll *a*. Chlorophyll *a* can be found in both PSI and PSII and both photosystems can emit ChlF (Farooq et al. 2018), but the signal is largely dominated by the emission from PSII (Krause and Weis, 1991). The reason behind this is that P700, i.e. the chlorophyll associated with the PSI reaction center, is relatively stable in the oxidized state and acts as a trap for excitation energy (which is dissipated as heat), therefore, represents a fluorescence quencher. In other words, as the reaction center of PSI lacks the back-transfer mechanisms of excitation into the antenna system (Franck et al. 2002; Hasegawa et al. 2010), $\Phi F_{PSI(\lambda)}$ remains low even upon closure of the PSI retraction centers (Mimuro et al. 1988). In contrast, P680, i.e. chlorophyll associated with the PSII reaction center, is more rapid to return from oxidized to the ground stage by electron donation (Krause and Weis 1991) and thus does not quench ChlF, leading to a relatively higher $\Phi F_{PSII(\lambda)}$.

To summarize, PSII quantum yield is more responsive to quenching mechanisms (Genty et al. 1990; Pfundel, 1998; Franck et al. 2002), while PSI remains largely unaffected, as $\Phi F_{PSI(\lambda)}$ appears to be independent of the state of its reaction center (Butler 1978; Briantais et al. 1986)). Consequently, the relative contribution of the two photosystems to the total ChlF emission will differ (Peterson et al. 2014) with spatio-temporal variation in PQ or NPQ. Moreover, the relative contribution of the two photosystems to ChlF emission will also

depend on an absorption cross-section between the photosystems. Despite often being assumed to be 1:1 (Maxwell and Johnson 2000), the relative number of the two photosystems reaction centers is not equal, nor fixed (Chow et al. 1988; Fan et al. 2007). Moreover, it remains unknown whether the absorption cross-section between the photosystems varies among species, canopy positions, or with time.

Because PSII presents higher emission across the whole wavelength range, which is especially evident in the red range (Govindjee, 1995), and PSI has only one emission maximum (around 740 nm (Papageorgiou, 2007)), variation in relative PSI:PSII contribution to the ChlF emission will be reflected in the ChlF magnitude and especially in the ChlF shape. Nevertheless, separating the ChlF signal to the two photosystems is difficult because of a large degree of spectral overlap between the two emission peaks (Pfündel, 1998; Franck et al. 2002; Strasser et al. 2004). This problem can be overcome by measuring spectral ChlF at 77 Kelvin, when the red-ChlF corresponds to PSII and the far-red-ChlF corresponds to PSI, so that the ChlF emission of the two photosystems can be separated (Björkman and Demmig, 1987; Govindjee, 1995). However, an efficient and reliable method to measure leaf-level 77 Kelvin-ChlF is demanding to achieve, which constitutes a methodological challenge.

1.3.3. ChlF variation due to foliar chlorophyll concentration acting as a physiological factor

On a temporal scale, many studies reported a decrease in chlorophyll *a+b* concentration [Cab] towards winter. For example, lower [Cab] in mass (measured in grams) was found in winter as compared to summer in Scots pine (Linder, 1972) or, at a larger scale, in pine-dominated forests (Hernández-Clemente et al. 2017). At the same time, a seasonal variation was also reported for chlorophyll based on the area. For *Pinus sylvestris L.*, Ottander, Campbell, Öquist (1995) and Oquist (2003) showed around 40% decrease in [Cab]/area in winter as compared to summer, and even stronger decrease trends were found in other studies (approx. 50% and 70% decrease in Sofronova et al. (2016) and Sveshnikov et al. (2006), respectively). Interestingly, [Cab] can also vary on a short timescale of hours, as demonstrated for bean and cotton plants examined under controlled environmental conditions by García-Plazaola et al. (2017).

On a spatial scale, [Cab] varies among species, as shown by Li et al. (2018) for 823 plant species of natural (cold-temperate, temperate, subtropical, and tropical) forests, as well as within the canopy vertical light gradient (Niinemets et al. 2002). However, the effect of the light environment (canopy position) on the chlorophyll concentration depends on whether it is expressed on the area or dry mass. As this aspect is firmly related to the distribution of chlorophyll molecules within a leaf and thus is strongly dependent on the leaf architecture, it will be discussed in more detail in the section on chlorophyll concentration effect on $f_{esc}(\lambda)$ (section *1.4.1*).

An increase in [Cab] leads to an increase in $APAR_g$ and thus to an increase in ChlF emission across all wavelengths (i.e. ChlF magnitude), although only to a certain threshold [Cab] level. For ethanol chlorophyll suspensions of different concentrations, Gitelson et al. (1998) showed that at low [Cab] levels, where ChlF reabsorption is small, $APAR_g$ indeed increases linearly with the rise in [Cab] and with the increase in both *R* and *FR*. However, in high [Cab], the reabsorption of red photons is relatively larger than the increase in the total ChlF taking place in response to augmented $APAR_g$ (Gitelson et al. 1998). Consequently, in relative terms, *R* will be decreasing while *FR* – being unaffected by reabsorption – will be increasing in response to higher [Cab]. The threshold level of [Cab] at which the

compensation between increasing $APAR_g$ and ChlF reabsorption occurs, will depend on the distribution and packing of the chlorophyll molecules across the leaf tissues, i.e., the leaf anatomy (Liu et al. 2019). In summary, both ChlF magnitude and shape are strongly dependent on [Cab], but this relation is not trivial and has a dual nature. On one hand, [Cab] can be considered a physiological factor, due to its effect on $APAR_g$. On the other hand, it can be considered a physical factor, given its effect on reabsorption within a leaf (as discussed in the next section, *1.4.1*)

1.4. ChlF variation due to physical factors

1.4.1. ChlF variation due to foliar chlorophyll concentration as a physical factor

At the leaf scale, the $f_{esc}(\lambda)$ indicates the probability that ChlF emitted at the photosystem level escapes the leaf and reaches a sensor (Buschmann, 2007; Yang and van der Tol, 2018). There are two important factors affecting the leaf-level $f_{esc}(\lambda)$: foliar chlorophyll $a+b$ concentration [Cab] and leaf architecture (*1.4.2*), which, importantly, both can vary in space and time. However, in nature, it is difficult to discuss the effect that [Cab] has on ChlF emission separately from the leaf architectural features, as chlorophyll has been shown to be distributed unevenly throughout the leaf.

With paradermal sections analysis on *Spinacia oleracea* leaves, Cui et al. (1991) showed that [Cab] increased in the palisade cells located deeper within the leaf as compared to the leaf surface (Cui et al. 1991), while it remained almost constant throughout the spongy mesophyll (Vogelmann and Evans, 2002, see Figure 2 for visualization of palisade and spongy mesophyll layers). At the same time, as chlorophyll molecules located on the leaf surface layer absorb photons, primarily of red and blue wavelength, gradually less light is left to be absorbed by chlorophyll molecules located at the deeper layers (Van Wittenberghe et al. 2013). The chlorophyll molecules distribution and absorption performance will be reflected in ChlF magnitude and shape. For example, in their later study, Van Wittenberghe et al. also reported differences between upwards- and downwards-measured ChlF of four broadleaves species, demonstrating that both [Cab] and its distribution affect the within-leaf light scattering properties (Van Wittenberghe et al. 2015). Fortunately, ChlF has been also measured from isolated chlorophyll solutions (Lichtenthaler and Rinderle, 1988), where the effect of leaf architecture disappears and [Cab] itself can be discussed as control of spectral ChlF.

As mentioned in the previous section, [Cab] can vary in time (e.g., Linder, 1972; Ottander et al. 1995; Öquist 2003; Hernández-Clemente et al. 2017) and in space, among species (Li et al. 2018) and canopy positions (Niinemets et al. 2002). However, the effect of the light environment depends on the way the chlorophyll concentration is expressed, based on mass or area. For four broadleaf species, Niinemets et al. (1998) showed that [Cab]/mass (mmol/g DW) was high in very low irradiance, and then it remained at a relatively stable level in middle and high light. Lukeš et al. (2013) showed that [Cab]/mass (μg/g) was higher in shaded as compared to sun-exposed leaves in *Pinus sylvestris L.*, *Picea abies* (L.) Karst. and *Betula pendula* Roth., with the biggest difference found in broadleaves species. In the same study, Lukeš also reported higher specific leaf area (SLA, m^2/kg) for shaded as compared to sun-exposed leaves in all three species. Indeed, leaf mass per area (LMA, kg/m^2, i.e. an inverse of SLA) was shown to decrease with canopy depth (Niinemets, 2010). However, the

levels of variation and interaction between [Cab]/mass and LMA can result in contrasting across-canopy patterns of [Cab]/area. With increasing relative photosynthetic photon flux density (PPFD, %), either an increase or a decrease in [Cab]/area were reported by Hansen et al. (2002) in *Quercus robur L.* or *Pinus sylvestris L.*, respectively. Sveshnikov et al. (2006) showed a negative correlation between light intensity and [Cab]/area, comparing exposed and shaded needles of *Pinus sylvestris L.* seedlings. On the other hand, almost no variation on a relative diffuse irradiance scale was reported by Hallik et al. (2008) in three broadleaves species (*Betula pendula* Roth., *Populus tremula* L., *Tilia cordata* Mill.). Finally, in the meta-analysis of plant responses to light intensities, Poorter et al. (2019) confirmed that one clear pattern of how [Cab] responds to irradiance cannot be drawn if [Cab] is presented based on the area. This leads to a question: what effect will the distribution of chlorophyll molecules within a leaf have on the variation in absorption, i.e. ChlF magnitude and re-absorption, i.e. ChlF shape? To establish that, we first need to know how the leaf architecture itself varies across space and time.

1.4.2. ChlF variation due to leaf architecture

Just like walls and interior design form a house, leaf morphology (external shape/cross-section) and leaf anatomy (internal arrangements) can be together termed as leaf architecture. The leaf architecture determines the distribution of chlorophyll molecules within a leaf (Borsuk and Brodersen, 2019) and constitutes an important factor affecting leaf-level $f_{esc}(\lambda)$. In different biomes, leaf architecture can vary in time (Marchi et al. 2008), but its variation is especially pronounced in space, among leaves of different species and canopy positions (light environments) (Niinemets et al. 1995; England and Attwill, 2006; Afas et al. 2007).

Sun-exposed leaves are usually smaller and ticker as compared to shaded ones (Poorter et al. 2019) and present well-developed layers of palisade parenchyma. The palisade cells scatter less light as compared to spongy cells and work as “light pipes”, letting the light penetrate deeper into a leaf (Cui et al. 1991; Vogelmann and Martin, 1993). Therefore, a more equal distribution of light penetration through the leaf provided by well-developed palisade layers appears as significant anatomical adjustments allowing the light to all the layers within a thicker leaf. In contrast, shaded leaves have little to no palisade layer and, instead, develop more spongy parenchyma (Vogelmann, 1993). Spongy cells work very well in scattering light within a leaf (Fukshansky et al. 1993; Vogelmann, 1993), securing an optimal use of limited light resources in more shaded parts of a canopy.

Conifers typically have a strong morphological response to light exposure and develop ticker, rounder, or more rhomboid needles in upper canopies, as compared to thinner and flatter needles in lower ones (Niinemets et al. 2002). The more cylindrical needle might constitute a solution for avoiding high sunlight, and therefore protect against photoinhibition, by reducing the level of incident light with the cosine effect on surface reflectance (Johnson et al. 2005). Moreover, a more circular cross-section might enhance internal light propagation, allowing photons to penetrate deeper into the needle (Smith et al. 1997). Finally, a more cylindrical needle eliminates the necessity for mesophyll cell differentiation that is found in broadleaves species or even in species with flatter needles (e.g., *Abies fraseri* or genus of *Tsuga*) (Johnson et al. 2005). Clearly, leaf architecture differs not only among species, especially comparing broadleaves and conifers (Johnson et al. 2005), but also across canopy light gradients (Niinemets et al. 2015).

Considering its role in light absorption and scattering, both on the surface and inside a leaf, leaf architecture can be expected to affect the spectral ChlF. However, despite strong

theoretical background for such expectation, there is little information about the specific and direct effect of leaf morphology and anatomy on ChlF spectra (Van Wittenberghe et al. 2014). There are many studies that considered other leaf optical properties, namely reflectance and transmittance, and their variation among species (Baldini et al. 1997; Knapp and Carter, 1998; Hovi et al. 2018), canopy positions (Lukeš et al. 2013), age classes (Homolová, 2007), or as a function of species-specific epicuticular structures, like waxes (Olascoaga et al. 2014). Studies that focus on chlorophyll-*a* fluorescence are mostly based on PAM fluorescence, from where the full ChlF spectrum cannot be resolved (Vogelmann and Han, 2000; Johnson et al. 2005). Consequently, the relation between leaf architectural features and spectral ChlF, as well as its variation in space and time, remains underexplored, especially in the case of conifers.

Although conifers constitute a significant fraction of terrestrial ecosystems (FAO, 2010) and ChlF is especially useful in tracking photosynthesis of boreal forests, where conventional greenness indices are not optimal, (Springer et al. 2017), the methodology for measuring spectral ChlF of needles remains unstandardized (methodological challenge). Indeed, measuring the ChlF of thick and small needles, as compared to broadleaves, is technically arduous, as placing a needle in a secured position towards the light source and a sensor is difficult. Moreover, measuring only one needle potentially leads to a very small measured signal and, thus, to a reduction of the signal-to-noise ratio (SNR) (Daughtry et al. 1989). On the other hand, measuring a set of multiple needles would increase the probability of mutual shading by adjacent needles, multiple scattering, and/or reabsorption (Yáñez-Rausell et al. 2014). When a single needle is measured, the ChlF emitted in lateral directions has no chance to reach a sensor. In contrast, when a two-dimensional needle configuration is measured, the ChlF emitted in lateral directions can be scattered by adjacent needles and, in result, it can reach a sensor and contribute to the measured signal. Moreover, the ChlF photons, especially red-ChlF, can be also reabsorbed by these adjacent needles.

To conclude, how spatio-temporal variation in physical factors, which affect the leaf-level $f_{esc}(\lambda)$, will be reflected in the dynamics of spectral ChlF remains unexplored. For example, considering that the distribution of chlorophyll molecules within a leaf is dependent on leaf architecture and that the leaf architecture varies in space, can we assume that the effect that chlorophyll concentration has on ChlF spectra remains similar among the leaves of different species and light conditions? This question is especially challenging to answer in the case of needles, as measuring needle-level spectral ChlF remains difficult to perform due to their complicated morphology.

All questions raised in sections *1.3* and *1.4*, regarding the role that physiological, physical factors, and, especially, their mutual interactions, have on the variation in ChlF magnitude and shape in the spatial and temporal context, clearly point to the urgent need for a mechanistic interpretation of ChlF variation in terms of photosynthesis. This challenge calls for long-term, comprehensive, and multi-traits studies to allow the establishment of a coherent dataset of ChlF emitted by leaves of different species, canopy positions, and exposed to different stressors.

2. AIM OF THE STUDY

The main goal of this thesis is to contribute to the interpretation of ChlF data by identifying, characterizing, and contextualizing the influence of methodological and mechanistic factors on the leaf-level spectral ChlF. To reach this goal, this thesis covers several specific aims listed below.

1. To identify and discuss challenges that make the ChlF-photosynthesis (or SIF- GPP) link difficult to disentangle across space, time, and a range of scales at which ChlF can be measured, as well as to draft a theoretical roadmap to navigate through these challenges (**Study III)**;

2. To investigate whether and how measurement geometry affects the spectral ChlF across samples of leaves with different morphologies and in different needles arrangements; and to examine how these needles arrangement affect the spectral ChlF in setups of different viewing and illumination geometry (**Study I**, *methodological challenge*);

3. To establish which factors affect spatial variation in spectral ChlF among leaves in a boreal forest and whether these effects vary over the period of spring recovery of photosynthesis; and to establish which factors affect temporal variation in spectral ChlF and whether these effects differ between species and canopy positions (**Study II**, *mechanistic challenge*).

3. MATERIALS AND METHOD

3.1. Fluorescence Across Space and Time workshop

This thesis includes two research articles (**Study I** and **II**) and one perspective/discussion article (**III**). Therefore, the materials and methods chapter of this thesis primarily regards **Studies I** and **II**. However, background information on how **Study III** was created is also provided.

Study III identifies and characterizes current challenges in ChlF/SIF studies and suggests a theoretical roadmap to guide the future efforts of the scientific community. Consequently, **Study III** could not be created without well-orchestrated cooperation between scientists representing different fields of interest, from plant physiologists to modellers, and different scales of measuring ChlF/SIF, from a single photosystem to a global level. Arranging the ground for discussion about challenges and the future direction of ChlF/SIF research was the main goal of the *Fluorescence Across Space and Time* workshop. The workshop was organized in February 2019 at the Station for Measuring Ecosystem-Atmosphere Relations (SMEAR-II), Hyytälä forestry field station, Finland. Around 50 scientists from around the world participated in the workshop and shared knowledge from their scale- and discipline-specific perspectives. The 3-days-long workshop consisted of a series of presentations and brainstorming sessions. The sessions focused on identifying present knowledge gaps and research questions that could be solved with currently available data, as well as with data arising from future collaborative efforts. Three brainstorming groups were created to focus on: the mechanistic interpretation of leaf-level ChlF, current methodological issues, and

leaf/canopy radiative transfer models. At the end of the workshop, conclusions, doubts, and new ideas were shared among all the participants during a plenary discussion and the concept of **Study III** was drafted. The discussion was further carried out online. Creating a common ground for discussion was essential to gain a full-ranged perspective on the current and future challenges which lie ahead of the scientific community.

3.2. Study sites and plant material

The measurements described in the materials and methods chapter of this thesis were performed either under laboratory conditions (e.g., chlorophyll-*a* fluorescence) or under field conditions (e.g., light environment). An intensive, 2 weeks-long laboratory experiment of **Study I** was performed at Viikki Campus (Helsinki, Finland) and based on leaf material collected in the vicinity of the Campus. **Study II** was carried out at the SMEAR-II Hyytälä forestry field station (61º 31' North 24º17' East, southern Finland) from mid-February to mid-July and measurements were collected every two to three weeks, with 10 measuring points in total.

Both studies focused on boreal species, including deciduous (silver birch, *Betula pendula* Roth., **Study I**) and evergreen species (lingonberry, *Vaccinium vitis-idaea L.* in **Study I** and **II**; Scots pine, *Pinus Silvestris* L. in **Study I** and **II**; and Norway spruce, *Picea abies* L. Karst., in **Study II**). In both studies, measurements of evergreen species were conducted on 1-year-old leaves developed during the previous growing season. In **Study I**, leaves were sampled from exposed, south-facing branches of trees or from shrubs growing on sun-exposed areas. In **Study II**, lingonberries were sampled like in **Study I,** while the leaves of trees were sampled from two positions, upper and lower canopy, representing an across-canopy vertical gradient of light environments. A combination of three species and two sampling locations within the canopy light vertical gradient was used in **Study II** to investigate spatial variation among leaves of the boreal forest. Moreover, corresponding data collected across 10 measuring points were used in **Study II** to explore temporal variation during the spring recovery of photosynthesis.

3.3. Leaf-level spectral chlorophyll-*a* fluorescence

3.3.1. Setups for measuring spectral ChlF

In **Study I**, three setups for measuring the full spectrum of chlorophyll-*a* fluorescence (spectral ChlF) were tested and compared:

i. Optical chamber (OC). In OC, ChlF was measured at the nadir. OC consisted of a 3-D printed PLA plastic cuvette and a set of detachable plates, on which foliar samples were arranged with the use of black, non-fluorescence vinyl tape (the tape was also used as the samples background). The cuvette had two little tabs, between which a fiber holder (RPH-1, Ocean Optics Inc., Largo, FL, USA) was placed so that the field of view remained the same between measurements of ChlF and reflectance, between different samples and, in **Study II,** also between the 10 measuring points. In the fiber holder, the bifurcated reflectance probe (600 µm; R600-7-VIS-125F, Ocean Optics) was mounted always at the same depth, to further secure the stability of the field of view. The second end of the bifurcated reflectance probe

was mounted into a filter carrier (FHS-UV Ocean Optics®), where a short-pass 650 nm filter (ThorLabs®, OD=4) was set to allow measurement of ChlF from 650 to 850 nm. The filter carrier was connected to a halogen light source (HL-2000, Ocean Optics®) through a fiber bundle cable (BF20HSMA, ThorLabs®). Finally, the third end of the bifurcated probe was connected to a spectrometer (USB2000+, Ocean Optics, FWHM at 600–800 nm: 1.5–1.8 nm), with a spectral range of 339–1020 nm. It is important to highlight that although the main aim of developing OC was to allow measuring ChlF in both ambient and 77 Kelvin temperatures, only ambient temperature ChlF results will be discussed in this thesis.

ii. FluoWat (FW). A customized and portable FW clip can be used to measure reflectance, transmittance (out of scope of this thesis), and ChlF, both from the adaxial (as in **Study I**) and abaxial surface of a leaf (Alonso et al. 2007; Van Wittenberghe et al. 2013). In FW, the leaf was illuminated at a 45º angle and ChlF is measured at the nadir view (Alonso, 2022). FW shared most of the elements of the setup with OC, with the exception of a short-pass filter (Edmund Optics®, Barrington, NJ, US, OD=4), and fiber optics (here, 600 µm, ASD Inc., Boulder, CO, USA).

iii. Integrating sphere (IS). In the three-inch integrating sphere (ASD RTS-3ZC, ASD Inc., Boulder, CO, USA), ChlF was estimated as total hemispherical ChlF. Unlike the two previous methods, IS had a dedicated light source (10 W, 6 V, Model 64225, Osram, Munich, Germany), a dedicated collimator lens, and a filter carrier. The same fiber optics as for FW was connected to the sphere north pole port, transmitting radiation to the spectrometer. After placing a leaf in front of one of the sphere ports and illuminating it at the nadir, ChlF was measured with the use of a 650 nm cut-off filter, which was found in the filter carrier in front of the light source.

To increase comparability, the same light sources of halogen type (Ocean Optics®HL-2000 in OC and FW or ASD®RTS-3ZC in IS), the same cut-off wavelength 650 nm filters of the corresponding optical density (OD) = 4 (Thorlabs® in OC and FW or Edmund Optics® in IS), the same spectrometer (Ocean Optics®USB2000+), and the same software (Ocean Optics®) were used in all three methods. For each method, the light sources were adjusted to yield similarly low (38–50 µmol PAR) light intensities at the leaf surface, in order to minimize potential differences in ChlF due to light-induced photochemical and non-photochemical quenching. Out of the methods tested in **Study I**, the OC was used in **Study II**.

3.3.2. Needles arrangement in measuring spectral ChlF

While leaves of *Betula pendula* Roth. (**Study I**) and *Vaccinium vitis-idaea L.* (**Study I** and **II**) were big enough to cover the measuring area in OC, FW, and sample-port in IS (**Study I**), different arrangements were possible for conifers. In **Study I**, three needle arrangements were measured and compared: a single needle (1N); a set of three needles with an approximately one-needle-wide gap in between (3N); and a continuous needle mat with as minimal gaps as technically possible (NM, Figure 4). Measurements were conducted with a background consisting of a photon trap (FW and IS) or a non-fluorescing black tape in OC (the same tape was used in **Study I** and **II**).

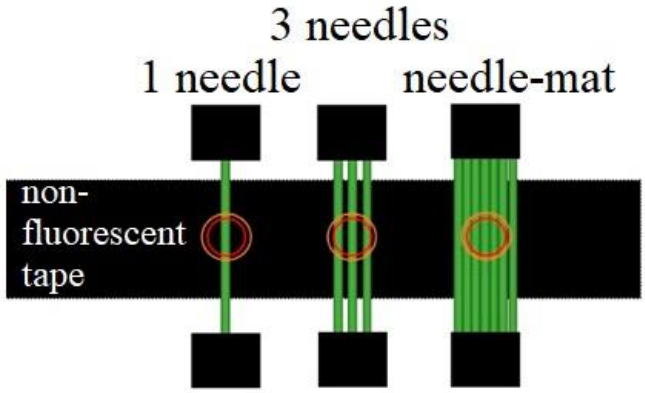

Figure 4. Three different needle arrangements tested for spectral ChlF measurements: 1N – single needle, 3N – set of 3 needles with gaps, NM – needle mat. The black tape, used as a background in OC, is represented as a black rectangle. Analogous arrangements were used in FW, but with light trap as background. Circles indicate an approximate field of view in OC (red) and FW (orange). Figure adapted from **Study I**.

3.3.3. Protocol for measuring spectral ChlF

The protocol (i.e., order of steps) for measuring spectral ChlF with the Optical Chamber was similar in both **Studies I** and **II**. Leaf samples were dark-adapted at room temperature (**Study I**) or 10ºC (**Study II**) for at least 45 min prior to measurement of spectral ChlF. The measurement was done at room temperature. For each sample, dark current and a white reference (with a Spectralon® panel, Diffuse Reflectance Standards, Labsphere®, North Sutton, NH, USA) were recorded, and then ChlF was recorded during the dark-to-light induction for 300 seconds (integration time, IT, set to 300 ms). For other setups in **Study I**, the integration times and time of dark-to-light induction were adjusted. Steady-state ChlF spectra, i.e. average of the last 10 seconds of each ChlF measurement, were used for further analysis after two corrections. First, a correction for stray light (as discussed in **Study I** and further in this thesis, see section *3.7.1*) and then a correction for light input variation were applied, to address potential changes in incident PAR, due to assembly/disassembly of our measuring setup between measuring days (only needed in **Study II**). The resulting spectra were further smoothed with a Savitzky–Golay filter (sgolayfilt function from the R package "signal", with order 3 and averaging interval 21). Values from 684.77 to 686.12 nm and from 739.71 to 741.03 nm were averaged to represent the red (*R*) and far-red (*FR*) ChlF maxima, respectively. For Singular Value Decomposition analysis (section *3.7.2*), ChlF spectra from 660 to 850 nm were used.

3.4. Leaf physiological factors

Along with spectral ChlF, a series of foliar and environmental factors were investigated in **Study II**, as described in sections *3.4* to *3.6*.

3.4.1. PAM florescence-based photosynthetic parameters

PAM fluorescence measurements were conducted using a PAM-2500 portable fluorometer (Heinz Walz GmbH, Effeltrich, Germany). In each sampling shoot, leaves (n=5) were dark-adapted using leaf clips for 90 min prior to the measurements. The fast kinetics of the fluorescence rise in response to a saturating pulse (600 ms) were recorded at 20 µsec resolution. The fast kinetics results were used to determine the minimal (F_0) and maximal (F_M) fluorescence levels, as an average of 50 fluorescence recordings over 1 ms both prior to the pulse and around the point of maximal fluorescence, respectively. Finally, the maximum quantum yield of PSII photochemistry (the maximum quantum yield of Q_A reduction) was calculated as:

$$\frac{F_V}{F_M} = \frac{F_M - F_0}{F_M} \quad (2)$$ Kitajima and Butler (1975)

The reference level of F_{MR}, i.e. maximum F_M recorded within the whole study period (i.e., with NPQ_S assumed to be zero) was selected separately for each sampling location (i.e. species × canopy position) and used to estimate rate constants of the sustained forms of quenching mechanisms:

$$PQ_s = \frac{F_{MR}}{F_0} - \frac{F_{MR}}{F_M} \quad (3)$$ Porcar-Castell (2011)

$$NPQ_s = \frac{F_{MR}}{F_M} - 1 \quad (4)$$ Porcar-Castell (2011)

As the use of *Equations 3* and *4* implies a constant level of leaf PAR absorption within each sampling location during the study period, this condition was confirmed (ASD HH spectrometer (FieldSpec HH VIS-NIR; ASD Inc., Boulder, USA; AdaptaSphere, LabSphere Inc., NewHampshire, UK), after Olascoaga et al. (2016)).

3.4.2. Gas exchange-based photosynthetic parameters

Sampling shoots were brought indoors 5–15 min prior to the gas exchange measurements. CO_2 exchange was measured with an infra-red gas analyser (IRGA, Walz GFS-3000, Heinz Walz GmbH, Effeltrich, Germany). For the measurements, the flow rate through the cuvette was set at 600 µmol s^{-1}, inflow CO_2 at 400 ppm, RH at 50%, and cuvette temperature at 20°C. For spruce, the entire shoot was enclosed in the conifer cuvette chamber; for pine, 8 individual needles were used; and for lingonberry, 1-2 leaves were placed in the leaf cuvette. Light response of photosynthesis was measured with stepwise changes in light levels (0, 400, 100, 50, 25, 800, and 1200 µmol m^{-2} s^{-1} PAR). The stabilization period between each level was 150-450 s and data were recorded for 25 s (5 s intervals, 6 measurements) at the end of each light level. Following the light response, photosynthetic assimilation was estimated at 1500 ppm CO_2 and 1500 µmol $m^{-2}s^{-1}$ PAR after ca. 600 s stabilization. In **Study II**, the following parameters were used: assimilation (net exchange of CO_2, µmol $m^{-2}s^{-1}$) at PAR of 1200µmol $m^{-2}s^{-1}$ (A_{1200}) and assimilation at high PAR (1500 µmol $m^{-2}s^{-1}$) and high CO_2 level (1500 ppm), i.e., maximum assimilation factor (A_{max}). Lastly, the slope of the initial part of the light response curve was used as a proxy of maximum light use efficiency (LUE_{max}), after Kolari et al. (2014).

3.5. Foliar pigments and morphological factors

3.5.1. Foliar pigments

In **Study II**, chlorophyll *a+b* concentration, [Cab], total carotenoids to chlorophyll *a+b* ratio, and zeaxanthin to chlorophyll *a+b* were measured. It is worth noting that the zeaxanthin to chlorophyll *a+b* ratio parameter was used in **Study II** instead of the more commonly used DEPS (de-epoxidation of xanthophyll cycle pigments, calculated as (zeaxanthin + 0.5 antheraxanthin) / (zeaxanthin + antheraxanthin + violaxanthin)) due to issue with antheraxanthin analysis.

Leaf material was frozen with liquid nitrogen immediately after being collected and kept at -80°C until analysis. Freeze-dried material was ground (Tearor 985370, BioSpec, Bartlesville, OK, USA) with 0.5 mL of acetone (95%), buffered with 0.5 gL^{-1} $CaCO_3$ and centrifuged for 10 min at 12000 g and 4°C. The resulting pellet was re-extracted with 0.5 mL pure acetone, centrifuged again, and both supernatants were pooled and adjusted to 1000 µL. The resulting extract was then filtered (0.2-µm PTFE filters, Teknokroma, Barcelona, Spain) and injected into a Waters HPLC system (Milford, Massachusetts, USA). Finally, the pigments were separated via high-performance liquid chromatography (HPLC) with a reversed-phase C18 column (Waters Spherisorb ODS1, 4.6 × 250 mm) and peaks were analysed (photodiode array detector, Waters model 996) at 445 nm, by comparison of spectral properties with pure standards (DHI, Hørsholm, Denmark). A more detailed description of the analysis can be found in Fernández-Marín et al. (2018).

3.5.2. Specific leaf area

Specific leaf area (SLA) was the only architectural, and more precisely morphological, factor included in **Study II.** For each species × canopy position × measuring point, fresh leaf samples were scanned, oven-dried (60 ºC), and their dry mass was measured. SLA ($cm^2\ g^{-1}$ DW) was calculated as a ratio of projected leaf area (cm^2, estimated with ImageJ software (Schneider et al. 2012)) and dry mass (g).

3.6. Light environment estimates

One of the main objectives of **Study II** was to investigate spatial variation in spectral ChlF together with various foliar factors. This spatial variation was defined as variation among leaves of different species and different canopy positions (for tree species), i.e., contrasting light environments across the canopy vertical gradient. Therefore, the light environments for each sampling location (3 species × 3 biological replicates per each × 2 canopy positions for trees) had to be precisely estimated, which was done using Digital Hemispherical Photography (DHP, Figure 5). DHPs were taken with a Canon EOS 70D camera and Sigma 4.5 mm fish-eye lens, mounted on a gimbal (HemiView, Delta-T Ltd., Cambridge, UK), which allowed a secured and horizontal position of the camera. Upper canopies were sampled from scaffolding towers. Due to the location of two spruce trees out of reach from any available tower, two sampling locations were approached using a drone-based spherical photography technique (Ribas-Costa et al. 2022).

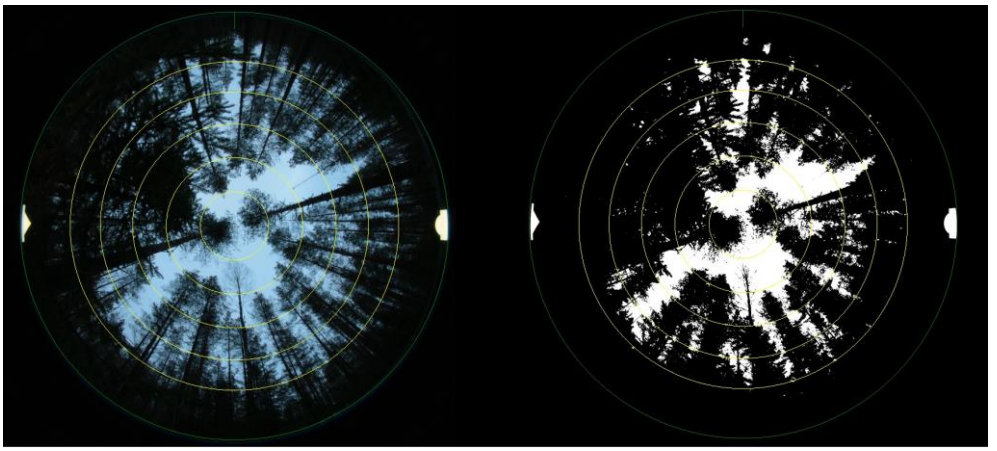

Figure 5. Example Digital Hemispherical Photography used in HemiSfer software for estimating the light environment experienced by one of the lingonberries sampled in **Study II**. The photography was taken in April (2017), before the leaves burst. The visible white marks are used to orient the photography in the correct direction towards North, which is required by the software. (a) Raw photography implemented into HemiSfer software with centered position, set North, and set radius of the informative part of the photography; (b) Result of black-and-white threshold analysis, where pixels are assigned to non-sky and sky. Detailed description of the analytical method can be found in Schleppi et al. (2007).

All resulting photos were analysed with Hemisfer software (WLS, Birmensdorf, Switzerland) following the instructions in Schleppi et al. (2007). The software simulated direct and diffuse radiation, as a function of time-of-day and day-of-year, for each sampling location. The direct and diffuse radiation was summed (PAR_{hour}), averaged over day-time (7 a.m. to 8 p.m., PAR_{day}), and finally averaged over a period of 10 days preceding each of the 10 measuring points, to represent the seasonal variation in the light environment (PAR_S, where S stands for seasonal).

Next to PAR_S, Hemisfer delivers a parameter of a global light index (GLI), i.e., weighted annual average representing the fraction of diffuse and direct light transmitted through the canopy to a specific sampling location. GLI can be considered a "local light environment", reflecting the irradiance conditions of a certain sampling location.

It is important to note that, although both PAR_S and GLI characterize light environments, they differ with respect to their expressed variation. PAR_S varies both in space (between sampling locations, i.e. individuals, species and canopy positions) and time (across the analysed period, with a gradual increase of PAR from winter to summer). Therefore, in **Study II**, PAR_S was primarily used to investigate the sources of temporal variation in spectral ChlF and other foliar factors. In turn, GLI expresses no temporal variation and was therefore used for assessing the spatial variation only.

3.7. Processing of spectral ChlF and statistical analyses

3.7.1. Baseline correction

In **Study I**, I investigated and tested a correction for a potentially significant and wavelength-dependent element of the spectra that can affect both the magnitude and the shape of the measured ChlF, i.e., "baseline". The presence of baseline level arises from three technical/measuring issues:

i. Saturation issue. Because the ChlF signal emitted by a leaf can be as low as three orders of magnitude smaller than the incoming radiation, its detection at a level higher than the background noise might require increasing the level of the incoming radiation or increasing the integration time of the measurement. However, the first of these options is not always applicable, as it might promote photochemical and non-photochemical quenching. Alternatively, if the reflectance dynamics are not of interest, the integration time (IT) can be increased, leading to enhanced SNR across the ChlF spectral range. However, it can push the digital counts of the measured spectra over the threshold of saturation for the spectral region below the cut-off filter (here, 650 nm). Simultaneously, signal saturation can also affect other regions of the spectra by generating a component of "saturation-induced current" in the detector, which may require correction.

ii. Filter transmittance issue. Under certain measuring conditions, some photons of incoming radiation can be let through a filter and potentially interfere with the measured ChlF, as ChlF is relatively low compared to the incoming radiation. That can happen, for example, if halogen light sources (high output in the red and near-infrared regions) and cut-off filters with a low OD are used. This issue is especially relevant in the red-ChlF range (Alonso, 2022), near the filter cut-off region, where filter transmittance can still be significant. In both **Study I** and **II**, filters of OD=4 (transmission of 0.0001) were used, and thus a correction for the filter transmittance issue was assumed to be required only around the cut-off zone (650–660 nm).

iii. Spectrometer spectral resolution issue. Spectral features assessed with spectrometers follow a normal probability distribution centered on the line emission peak. Therefore, spectral features will be visible as counts for a variable range of wavelengths (dependent on the spectrometer resolution, intensity of incoming radiation, and integration time) rather than a sharp line. A similar phenomenon can appear for a cut-off filter, that rapidly reduces the intensity of radiation reaching the detector within a narrow cut-off region (e.g., from 74% transmittance at 644 nm to less than 1% at 654 nm, as shown in **Study I**). Therefore, the spectral resolution of the spectrometer is expected to interact with the measured ChlF spectra, especially in the red edge. In **Study I**, this limitation was suggested to interfere with the measured ChlF spectra, especially when long ITs are saturating the spectral region prior to the cut-off filter.

In **Study I**, I presented and implemented a preliminary and simple baseline correction to correct for the issues i, ii, and iii described above:

$$Baseline(\lambda) = REF(\lambda) \times \overline{\rho_{AB}} \quad (5)$$

where REF(λ) is a level of the signal measured with Spectralon® white reference panel, using a cut-off filter and with the same IT as used for ChlF measurements; and $\overline{\rho_{AB}}$ is the leaf reflectance of a given sample averaged for a region between A and B wavelengths, prior to the cut-off filter (550 to 650 nm).

The same baseline correction was applied to all results of spectral ChlF in **Study II**. Please note that applying the baseline correction in the form of *Equation 5* requires separate measurements of reflectance for every considered sample. It is also important to note that filters of high optical density were used in **Study I** (as well as **Study II**). In contrast, an additional correction for filter transmittance would be required if filters of higher transmittance were used.

It is important to highlight that a baseline correction, which regards the baseline that is being discussed in this section and corrected with *Equation 5*, is not by any means, except the name, connected with baseline variation (Atherton et al. 2017), which regards the temporally-invariant element of the overall variation in ChlF, not arising from measuring methodology.

3.7.2. Singular Value Decomposition, spatial and temporal correlations between spectral ChlF components and analysed factors

Singular Value Decomposition (SVD) analysis is a statistical method of factoring data into underlying, uncorrelated components (Steward, 1993). In **Study II**, SVD was used to decompose leaf-level spectral ChlF results and investigate their variation across space and time. Accordingly, two SVD analyses were executed, one on the spatial and one on the temporal scale. In both analyses, spectral ChlF was decomposed into three components, SV1, 2, and 3 (details in section *4.3.2*). These were used along with or instead of the *R*, *FR*, and *R/FR* to investigate the covariation between ChlF and the measured physiological, physical, and environmental factors.

i. Spatial variation, i.e., variation among leaves of different species and canopy positions; SVD was performed on observations clustered within each of the 10 measuring points; Spearman correlations were tested to explore the effect of different factors on the spatial variation in spectral ChlF, as well as the evolution of these effect over time.

ii. Temporal variation, i.e., variation across the 10 measuring points over the period of spring recovery of photosynthesis, SVD was performed on observations clustered within each one of 5 species × canopy position combinations; Spearman correlations were tested to explore the effects of different factors on the temporal variation in spectral ChlF, as well as differences of these effects among species and canopy positions.

All the analyses presented in this thesis and in the included articles were conducted with R (Rstudio version 1.3.959; 2020).

4. RESULTS AND DISCUSSION

4.1. Leaf-level spectral ChlF in the context of current challenges of ChlF/SIF research

Chlorophyll-*a* fluorescence, either as PAM, spectral ChlF (full wavelength range), or SIF can be retrieved across scales: from isolated chlorophyll molecules (Gitelson et al. 1998), leaves (Van Wittenberghe et al. 2014), canopies (Liu et al. 2019), up to global scale (Frankenberg et al. 2011; Guanter et al. 2012). However, the relationship between ChlF and photosynthesis (or SIF and GPP) depends on various factors, which can operate differently on a spatial and temporal scale (mechanistic context) and whose effects can also change with measuring methodology (methodological context). While we know that the linearity of the ChlF-

photosynthesis (SIF-GPP) relationship changes, the mechanisms behind this change are not yet fully understood (Meroni et al. 2009; Porcar-Castell et al. 2014; Mohammed et al. 2019). Consequently, in order to reach the full informative potential of ChlF/SIF signal, we need to understand and quantitatively characterize processes or mechanisms that connect measured ChlF/SIF with photosynthesis/GPP across various scales. In **Study III**, several challenges towards a comprehensive interpretation of ChlF are identified and discussed and a roadmap for future studies to navigate through these challenges is proposed.

The first challenge is related to $APAR_g$. As mentioned alongside *Equation 1*, $APAR_g$ conceptually connects ChlF and photosynthesis. While imprecise estimations of $APAR_g$, performed with currently available methods (Olascoaga et al. 2016; Zhang et al. 2020), do not necessarily disrupt the ChlF-photosynthesis relationship, they can lead to inaccurate quantifications of the energy flux entering the photosynthetic process and, thus, to misinterpretation of ChlF. The second challenge relates to energy partitioning between PSI and PSII, which can vary in space and time, resulting in changes in the relative contribution of the two photosystems to the total ChlF emission (Pfündel 1998; Peterson et al. 2014). However, the extent of this variation and, most importantly, its effect on the ChlF-photosynthesis link remain largely underexplored. That is primarily because ChlF emissions from PSI and PSII overlap spectrally and, therefore, are very difficult to measure at ambient temperature. The ChlF emission can be assigned specifically to the two photosystems when measured at 77 Kelvin, i.e., using liquid nitrogen. Although 77 Kelvin ChlF and partitioning of PSI:PSII ChlF are not included in this thesis, it is here worth mentioning that the Optical Chamber (setup tested in **Study I** and used in **Study II**) constitutes a convenient method to measure leaf-level 77 Kelvin ChlF of both broadleaves and needles.

The third challenge identified in **Study III** regards estimating the quantum yield of photochemistry (ΦP) from remotely sensed SIF. At the leaf-level, ΦP can be estimated with PAM fluorometry, but this technique cannot be applied at a larger scale and, thus, approaching ΦP values from SIF is difficult. Interpretation of SIF in terms of ΦP requires the use of process-based models that can quantitatively characterize the energy partitioning in PSII, but the development and implementation of such models remain challenging (Van der Tol et al. 2014; Gu et al. 2019; Raczka et al. 2019). In fact, the development and fine-tuning of process-based models require a great amount of data concerning energy partitioning between excitation energy fates. Fortunately, such data can be gathered at the leaf-level. However, the stream of new data, coming from long-term, comprehensive, leaf-level field studies is currently not sufficient to meet the rapidly expanding needs of modellers. The lack of such studies and possible guidelines to perform them will be further discussed along with mechanistic challenge and **Study II** conclusions. On the other hand, ChlF data that are available or currently delivered, are usually difficult to compare between different studies due to methodological challenge, as will be discussed along with **Study I** (section *4.2*).

The next challenge relates to ChlF scattering and reabsorption. At the leaf-level, this problem has been already mentioned in section *1.4* and will be further discussed along with the results of **Study II**. However, this challenge is also relevant for SIF measurements at larger scales, i.e., scales where remote sensing is used (Migliavacca et al. 2017; Kallel, 2020; Zhang et al. 2020). Remote sensing techniques measure ChlF signal that is integrated across millions of leaves within an ecosystem. All these leaves can differ in terms of physical and physiological factors and the signal is further complicated by the canopy structure (Figure 1). While SIF sensors are usually positioned above the canopy and have only a limited field of view (FOV) (Mu et al. 2017), SIF can escape from the canopy anisotropically, in multiple

directions (Zhao et al. 2016). Consequently, on its way from the chlorophyll molecule to the sensor, SIF can be both absorbed and scattered. The fraction of the emitted SIF that escapes the canopy and can be detected (canopy-level $f_{esc}(\lambda)$), depends on the geometry of SIF measurement, but most importantly on the physical factors, like canopy structure, density, or proportion of non-fluorescent (soil, bark) to fluorescent elements (leaves) in the forest (Romero et al. 2018). For instance, more SIF photons will be reabsorbed on the way from a leaf to a sensor in a dense, as compared to a sparse forest. However, the scattering and reabsorption properties of individual leaves might also play a role here. For example, high-[Cab] leaves will reabsorb more SIF photons as compared to paler leaves. In that sense, leaf-level $f_{esc}(\lambda)$ might also be relevant from the canopy-level $f_{esc}(\lambda)$ perspective. Importantly, many of the factors that affect canopy-level, as well as leaf-level, $f_{esc}(\lambda)$, vary in space and time. Because $f_{esc}(\lambda)$ affects SIF, but not GPP (Zeng et al. 2019), it becomes evident that we need to determine what fraction of the SIF photons is "lost" on the way to the sensor, before we can quantitatively interpret remotely sensed SIF in terms of GPP. This issue is the target of radiative transfer models (RTMs). RTMs simulate how photons travel through leaves and canopies and can be used as a quantitative framework to account for the impact of various physical factors on remotely sensed SIF (Kallel, 2020; Zhang et al. 2020). Despite recent advancements in leaf- and canopy-level RTMs, the models still need improvements in the parameterization of foliar factors, like pigment concentration or leaf architecture. In that sense, leaf-level studies can constitute a crucial element in RTM development, by providing information on how ChlF or SIF emission is affected by various physical foliar factors via leaf-level $f_{esc}(\lambda)$.

The sensitivity, strength, and linearity of SIF-GPP relation is not universal, as it depends on a series of factors (Magney 2020). However, the effect of different factors, acting as couplers and decouplers of ChlF-photosynthesis (SIF-GPP) relationship, can change across scales: at coarser spatio-temporal scales, the linearity of SIF-GPP relationship increases, as both parameters accommodate the physical and physiological processes within a canopy and neglect many of the nonlinearities observed at smaller scales (Magney 2020). At leaf-level, the ChlF-photosynthesis is not linear (Magney et al. 2017; Gu et al. 2019; Magney et al. 2019b), as it is strongly dependent on the spatio-temporal variation in a series of different factors. The effect of these factors can be, therefore, evaluated at leaf-level. Consequently, it is here important to highlight the importance of leaf-level spectral ChlF studies and contextualize them on the roadmap of current and future efforts for integrating and disentangling ChlF-photosynthesis relationship across space, time, and in response to different environmental stressors.

Unlike remotely sensed SIF, which can be retrieved only within discrete, narrow wavelength bands (Meroni et al. 2009; Rascher et al. 2015), leaf-level ChlF can be measured at the whole spectrum (Zarco-Tejada et al. 2009). Being the smallest scale at which ChlF can be measured *in vivo*, leaf-level also allows investigation of the role of various physiological and physical factors in controlling the variation in spectral ChlF. Importantly, by measuring separate leaves, we can examine ChlF emitted by various elements of an ecosystem, free from canopy-scale disturbances. At the leaf-level, disentangling the effect that various physiological and physical factors have on spectral ChlF is possible not only at a given point in time (summer, winter, event of stress like a sudden cold spell, period of drought) but also at a given point in space (single leaf of a certain species, developed under given light environment within the canopy). In sum, leaf-level sets a foundation for interpreting remotely sensed SIF (Malenovsky et al. 2009; Porcar-Castell et al. 2014), as it constitutes a convenient scale not only to investigate variations in ChlF, but also their controls, and finally the

connection between ChlF and photosynthesis. However, leaf-level spectral ChlF studies are not free from challenges. Two of these challenges have been evaluated in **Study I** (methodological challenge) and **Study II** (mechanistic challenge) and will be discussed in the following sections of this thesis.

4.2. *Methodological challenge*: characterizing the methodological factors and their effect on leaf-level spectral ChlF

4.2.1. Effect of measurement geometry

There are several methods to measure leaf-level spectral ChlF, but none of them is considered a "golden standard". As the measuring method is always selected or adjusted to a specific study purpose, it is extremely difficult to develop one method that would meet all the possible objectives of various experiments. However, the lack of standardization of methods to measure leaf-level, and especially needle-level ChlF, has led to issues with replicability and reproducibility of different experiments and, finally, to incomparability of ChlF results across studies. Consequently, overcoming the methodological challenge would be a breakthrough in assessing how variation in spectral ChlF, observed at the spatial and temporal scale, can be attributed to physical and physiological factors, and, consequently, in understanding the quantitative link between ChlF and photosynthesis. For example, methodological standardization would allow to create a dataset of comparable ChlF results, measured from leaves of different species, developed under different light conditions, as well as adapted to different environmental conditions and the presence of different stressors.

The methodological challenge was addressed in **Study I**, with a particular focus laid on conifer needles. Specifically, the study investigated how two methodological factors: geometry of measurements (viewing and illumination angles) and needle arrangements affect spectral ChlF. Three setups, Optical Chamber, FluoWat, and Integrating Sphere, were compared in measuring thin leaves of birch (*Betula pendula* Roth.), thick leaves of lingonberry (*Vaccinium vitis-idaea*), and pine needles (*Pinus sylvestris* L.) in three needles arrangements (section *4.2.2*).

Spectral ChlF differed among tested setups (Figure 5 in **Study I**), with Integrating Sphere always showing the lowest ChlF magnitude and the lowest ratio between two ChlF maxima (*R/FR*). These results were interpreted in terms of the signal attenuation and the enhanced reabsorption of red-ChlF inside the sphere, respectively. In general, because of the very low signal (as low as 25-time smaller compared to other tested setups using the same level of incoming PAR), Integrating Sphere was found not optimal to measure ChlF.

In the two remaining setups, Optical Chamber and FluoWat, differences in ChlF magnitude were recorded (Figure 5a in **Study I**): the highest ChlF magnitude was found in the Optical Chamber for all samples, except needle-mat (NM). Simultaneously, ChlF shape remained remarkably similar in both setups (Figure 5b therein). These results have important implications in terms of the standardization of leaf-level ChlF measurements, as they suggest that ChlF recorded with different measuring methods can be comparable, even if the setups represent contrasting viewing and illumination angles. Because ChlF shape was shown to be independent of measurement geometry, it can constitute a reliable parameter when results from studies using different measuring setups are compared. Consequently, **Study I** suggests the importance of recording not only ChlF magnitude but the full-spectrum ChlF, as ChlF

shape can deliver additional information that is not always accessible when considering ChlF magnitude alone. However, will different setups remain similarly comparable when samples of complicated architecture are measured?

4.2.2. Effect of needle arrangements

In the case of conifer needles, optimal sample arrangements have been studied for reflectance and transmittance (Daughtry et al. 1989; Middleton et al. 1997; Olascoaga et al. 2016), mostly using integrating spheres and more than one needle. Therefore, previous studies had to consider the effect of gaps between the needles (gap fraction, G_F). In terms of transmittance, small G_F led to unrealistic negative values in the PAR region (Middleton et al. 1996; Olascoaga et al. 2016) and the effect of G_F was suggested to be stronger in transmittance, as compared to reflectance (Mesarch et al. 1999). However, the effect of G_F on spectral ChlF has not been previously characterized.

In the case of ChlF, not only the multiple scattering of photons, important for reflectance and transmittance but also their reabsorption by adjacent needles has to be considered. Due to the chlorophyll absorption spectrum with the maximum at 681 nm (Buschmann, 2007), reabsorption primarily affects red-ChlF and, thus, needle arrangement and G_F could be expected to affect both ChlF magnitude and shape. **Study I** presents the first comparison of the effect of needle arrangements on spectral ChlF and discusses their applicability in different measuring setups. Three needle arrangements were tested: one needle (1N), three needles with approx. needle-size gaps in between (3N), and a needle mat (NM).
In both Optical Chamber and FluoWat, measuring ChlF at nadir and at 45º, respectively, the ChlF magnitude increased with the number of needles (Figure 6). This was expected as an effect of a larger proportion of the field of view being occupied by tissues absorbing and then emitting photons as ChlF. The highest ChlF magnitude was recorded in Optical Chamber for 1N and 3N, but not for NM. This finding suggests that in the NM arrangement, G_F causes a smaller effect when viewed at 45º, as less PAR is "lost" through the gaps as compared to the nadir. Simultaneously, a decreasing, although not significant, trend was found in *R/FR* when more needles were measured. The trend was found in both measuring setups and can be primarily explained by enhanced reabsorption (affecting *R* more than *FR* and, thus, leading to the *R/FR* decrease).

When a single needle is measured, ChlF emitted in lateral directions has no chance to reach a sensor. In contrast, when a needle-mat is measured, ChlF emitted in a lateral direction can be scattered by adjacent needles and, as result, can reach a sensor and contribute to the measured signal. However, ChlF photons, especially red-ChlF, can be also reabsorbed by these adjacent needles. Therefore, the contribution of the laterally scattered ChlF, enriched in the far-red photons, will reduce the *R/FR* of the overall ChlF. This is why in **Study I**, *R/FR* values of NM were smaller, as compared to 1N and 3N. Consequently, the potential effect of artificially low *R/FR* has to be considered when the NM arrangement is used in a study.

Using the standard deviation of *R/FR* across replicates and needle arrangements, replicability and reproducibility of measuring setups were evaluated. In both OC and FW, NM showed the highest replicability and reproducibility, as compared to 1N and 3N. Therefore, even considering the potential effect of needles self-shading and scattering or reabsorption of ChlF, NM was suggested to be a reliable and convenient approach to assess variation in spectral ChlF of needle-leaved species, irrespectively of the measuring geometry.

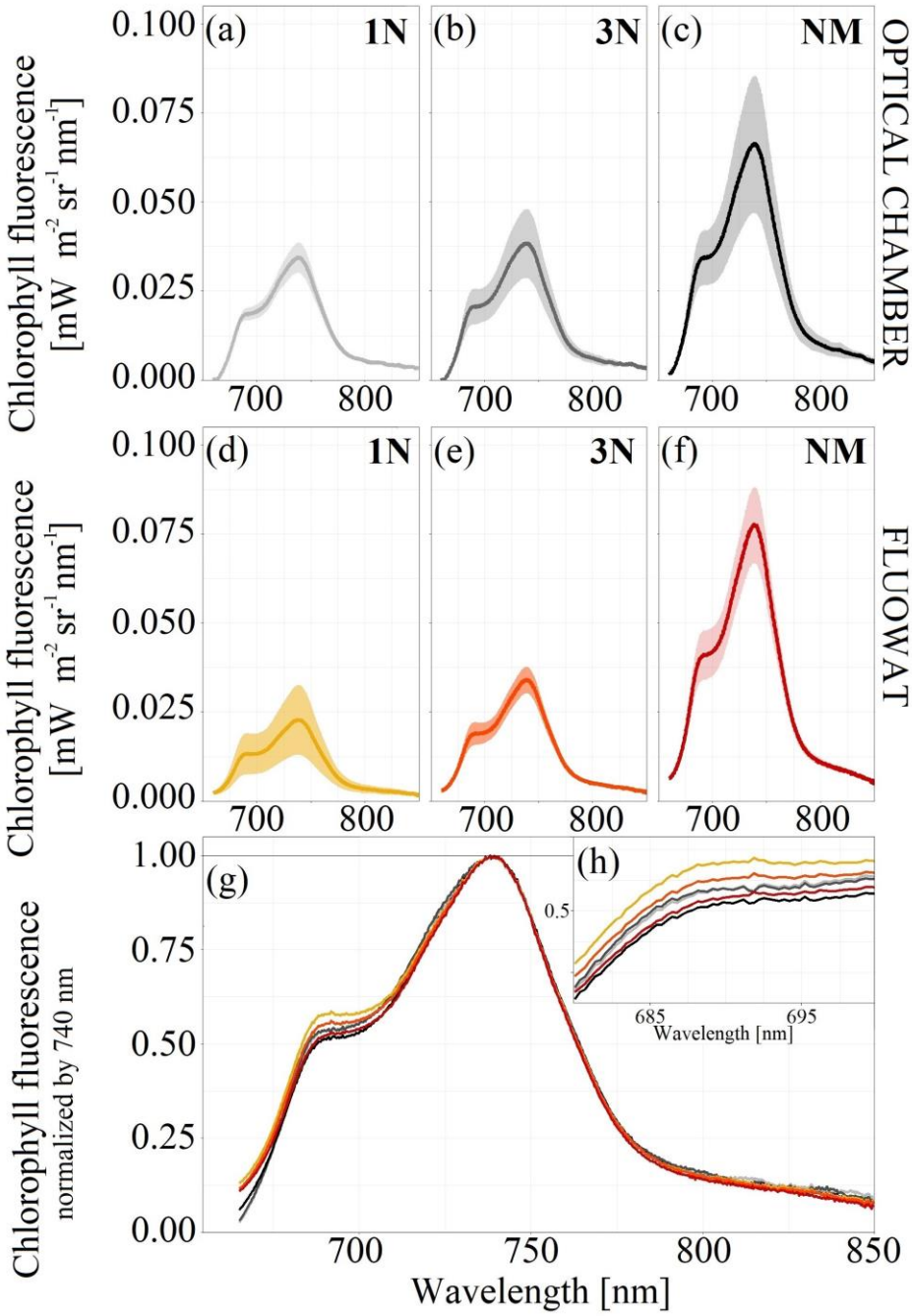

Figure 6. Chlorophyll-*a* fluorescence spectra of pine needles measured in the Optical chamber (a: 1N, light grey; b: 3N, dark grey; c: NM, black) and FluoWat (d: 1N, yellow; e: 3N, orange; f: NM, red). Spectral ChlF represented by the colors corresponding to a-f, were then normalized to F740 (g), also shown in zoom at the 675–700 nm range (h). Please note that Integrating Sphere was not considered in the tests of needles arrangement due to a very low ChlF signal delivered. Figure adapted from **Study I**.

Keeping in mind the potential underestimation of *R/FR* in NM arrangement when quantitatively comparing spectral ChlF of needles and broadleaves, NM was used to measure spectral ChlF throughout the experiment of **Study II**.

4.3. *Methodological challenge*: post-processing and decomposition of spectral ChlF

4.3.1. Baseline correction

Errors can appear while measuring spectral ChlF. For example, remounting the setup in long-terms studies (like **Study II**), might introduce inconsistencies in the field of view (e.g., due to misallocation of the fiber in a fiber holder) or in incoming radiation (e.g., due to different depths of screwing the bifurcated reflectance probe connecting the light source to the filter carrier). Moreover, significant dissimilarities between studies can be introduced by specific technical setup features. For example, spectral ChlF might vary with the used light source, as the depth to which photons can penetrate within a leaf and, thus, the reabsorption or scattering phenomena, depend on the emission spectrum of a light source (Buschmann and Lichtenthaler, 1998; Vogelmann and Evans, 2002; Van Wittenberghe et al. 2014, 2015). Likewise, spectral ChlF can be affected by the OD of the used filter. Lastly, depending on the aim of the study, an IT for ChlF measurement has to be chosen. In a perfect scenario, IT allows measurement of ChlF with a high signal-to-noise ratio, simultaneously keeping the signal in the wavelength range prior to the cut-off filter and below the saturation level. However, as ChlF is orders of magnitude smaller than reflectance, such a scenario is difficult to obtain, especially when not using a spectrometer with a high dynamic range.

Although the list of potential errors that can occur while measuring ChlF in different studies is much longer, the few examples listed above clearly point to the need for methodological standardization. Many of the issues can be avoided by e.g., using the best available filters of high OD, but some issues are very difficult to overcome, thus decreasing comparability between studies. Measurement geometry that promotes specular reflectance of a sample, long IT (used to increase SN, leading to signal saturation), spectrometers that present a limited dynamic range, or not optimal filters, can all lead to contamination of spectral ChlF with a wavelength-dependent baseline component. The level of the baseline component and, thus, the strength and consequences of its presence, might vary depending on measuring setup, light source, or certain foliar factors. Consequently, baseline correction can be critical in comparing datasets from different studies, regarding various species, leaf architectures, and measuring setups, and is an important element of the methodological challenge.

In **Study I**, a simple baseline correction was implemented to correct for potentially significant and wavelength-dependent element of the spectra that can affect both the magnitude and the shape of the measured ChlF, as a combined effect of: i. saturation ("saturation-induced current"), ii. filter transmittance, and iii. spectrometer spectral resolution issues. The presence of the baseline component was proven by non-zero values of the signal measured from Spectralon® panel across the whole measuring range. As the considered issues have the largest impact on the spectral ChlF near the filter, baseline correction is especially useful in recovering the spectra near the filter cut-off. Indeed, in **Study I**, the baseline correction had the biggest effect on the red wavelength range. Consequently, the baseline effect was shown to interfere not only with ChlF magnitude but also its shape: as *R* is closer to the filter cut-off region than *FR*, the baseline effect on the

R/FR was expected (Van Wittenberghe et al. 2014). Considering that *R/FR* has been widely used in assessing [Cab] (Hak et al. 1990; Lichtenthaler et al. 1990), in tracking physiological stress responses (Agati et al. 1995; Agati et al. 1996; Agati et al. 2000; Buschmann, 2007), or in investigating the relative PSI:PSII contribution to total ChlF (Genty et al. 1990; Palombi et al. 2011), the results of **Study I** suggest that a baseline correction should be addressed in future studies.

The level of baseline component varied among leaves and between measuring setups, primarily due to differences in leaf reflectance ($\overline{\rho_{AB}}$ in *Equation 5*, see section *3.7.1*). The reflectance of broadleaves was higher in OC as compared to FW, which was expected due to higher specular reflectance (see Figure 2) while measuring at nadir. Interestingly, larger reflectance of needle in NM was found in FW, not OC, suggesting that the effect of small G_F could enhance reflectance while measured at 45º as compared to nadir. Although only two measuring setups (Integrating Sphere was here excluded from the consideration due to very low ChlF signal), and only three species were tested, results of **Study I** imply that the effect of baseline component should be considered in future ChlF studies. Precise baseline correction will help in the efforts towards solving the methodological challenge, as it will make the ChlF results from different studies more comparable.

4.3.2. SVD components and their correlation with ChlF-affecting factors

In **Study II**, SVD (Steward (1993)) was used as a convenient statistical method to decompose ChlF spectrum (Magney et al. 2019b). In the literature, Principal Component Analysis (PCA) was also used (e.g., Zhang et al. 2019). The two methods are very similar and only differ in subtracting the mean: PCA subtracts the mean and SVD does not. While the analytical approach is adjustable to specific study aims, some method to decompose the spectral ChlF results should be used because, as shown below, it can be an important step in making the results more comparable.

Below, the results of **Study II** (SVD) are discussed in comparison with the results of two studies from 2019: Magney et al. (SVD) and Zhang et al. (PCA). It is here important to note the context of variation being decomposed with SVD (and likewise, PCA), which always has to be adapted to the nature of ChlF variation in a certain study. Consequently, in **Study II**, the spatial (Figure A6 in **Study II**) and temporal (Figure 3 therein) context of variation were investigated separately.

Magney et al. (2019b) used SVD to decompose the spectral ChlF variation observed among a range of 27 species (Figure 1 a,c,e therein). Although Magney et al. considered a much higher number of species and did not consider light environments, the SVD of this study is compared with Figure A6 in **Study II** (specifically, the dark-red line representing summer). In turn, in a study on upper canopy pine needles, sampled in the Hyytiälä forest, Zhang et al. (2019) used PCA to investigate the variation in spectral ChlF observed over the spring recovery of photosynthesis (Figure 5 a-c therein). Consequently, results of Zhang et al. can be compared with temporal variation in **Study II** and Figure 3 therein (specifically, the solid blue line representing upper pine). In general, **Study II** showed that results of SVD performed in temporal and spatial contexts are similar, but some differences were found which, in fact, aligned very well with the results reported by Zhang et al. and Magney et al. on the two different scales of variation, as described in more details below.

In all three studies, the first spectral component (PC1/SV1) had two maxima, around the usual location of *R* and *FR* (Figure A6a and Figure 3a in **Study II**). Therefore, PC1/SV1

reflects a variation in the "basic/background" ChlF magnitude, which is dependent on the total PAR absorption (correlation of 51% shown Magney et al. 2019b), or on the NPQ_S (correlation of 83% shown by Zhang et al. 2019). Apparently, different factors can affect PC1/SV1, or, in general, variation in ChlF magnitude, depending on the context of the analysed variations. This was also shown in **Study II,** where the set of factors correlating with variation in SV1 was different on the spatial (Figure 4 in **Study II**) and temporal (Figure 5 therein) scale (see section *4.4* for details). In the second component, the difference between the three analysed studies became more visible. Comparing PC/SV2 on the spatial scale (**Study II** and Magney et al. 2019b), two corresponding features at around 685 and 740 nm were clear but swapped in direction. In PC/SV2 on the temporal scale (**Study II** and Zhang et al. 2019), a corresponding minimum was found at a typical location of *R* in both studies. Affecting ChlF shape, the second component (PC2/SV2) reflects the variation in the mechanisms of within-leaf reabsorption. However, the foliar factors affecting this within-leaf reabsorption were different when the spatial and temporal scales were considered (section *4.4*).

Finally, the SV3 reported by Magney on the spatial scale was very similar to SV3 in **Study II** (Figure A6c in **Study II**), with all three spectral features appearing in the corresponding locations. On the temporal scale, PC3/SV3 were similar, but the whole shape of the component shifted towards longer wavelengths in **Study II** (Figure 3c therein), as compared to Zhang et al. 2019. While no explanation for PC3 on the temporal scale was provided by Zhang et al. Magney et al. demonstrated how the spatial scale SV3 can be reproduced by adjusting the relative contribution of PSI and PSII to the overall ChlF signal. Similarly, in **Study II**, SV3 was also suggested to reflect the variation in the relative contribution of photosystems, both on the spatial and temporal scale.

Therefore, there are at least two components of variation in ChlF shape: SV2, associated with reabsorption, and SV3, associated with PSI:PSII relative contribution to total ChlF. The fact that the two components were found in all three considered studies, shows the advantage of decomposing spectral ChlF (with SVD, PCA, or any other applicable method) over using the simple *R/FR* parameter. In fact, SV2 and SV3 in **Study II** were found to correlate with more factors, as compared to *R/FR* (see Figure 5 in **Study II**), as will be discussed further in section (*4.4*). In short, from the point of view of the methodological challenge, it appears advisable to apply decomposition analysis to any collected spectral ChlF data, in order to make the results not only more comparable between various studies but also more precisely interpretable in terms of ChlF-photosynthesis relationship in the spatial and temporal context.

4.4. *Mechanistic challenge:* characterizing the mechanistic factors and their effect on leaf-level spectral ChlF in the spatial and temporal context

In boreal forests, temporal and spatial variation in spectral ChlF is expected due to strong seasonality of physiological factors (e.g. NPQ_S, Ensminger et al. 2004) and large differences in physical factors among species and canopy positions (e.g. chlorophyll concentration, Niinemets, 2002). However, at a given point in time, physiological factors might differ among species and canopy positions, whereas at a given point in space, physical factors can differ across time. For example, differences in the rate of spring recovery of photosynthesis among boreal species can be found in the literature: Merry et al. (2017) showed that spruce reached summer values of F_V/F_M earlier in a year as compared to pine. Similarly, in the

shading experiment from Porcar-Castell et al. (2008b), summer F_V/F_M values were reached earlier in shaded pine seedlings, as compared to sun-exposed ones.

This is why an interpretation of the variation in ChlF in terms of photosynthesis has to be mechanistic, i.e., it has to consider factors that affect variation in ChlF simultaneously in space and time. In other words, interpreting the variation in ChlF separately on the spatial or temporal scale will not be complete and might be incorrect if the simultaneous effect that different factors have on ChlF is neglected. Disentangling the effects that these factors have on the spatiotemporal variation in spectral ChlF is, however, difficult, and requires comprehensive and long-term studies that regard diverse foliar representatives in an ecosystem.

So far, studies focusing on spectral ChlF, where both ChlF magnitude and shape were defined, considered exclusively either the temporal (Zhang et al. 2019) or spatial (Van Wittenberghe et al. 2014; Atherton et al. 2017; Magney et al. 2019b) context. In contrast, **Study II** constitutes an example of a comprehensive approach to analysing variation in spectral ChlF on both the spatial and temporal scale and can be a hint for future studies to provide data for the mechanistic interpretation of the variation in the leaf-level spectral ChlF.

In **Study II**, ChlF magnitude (presented in Figure 7 as *R* and *FR*) and shape (presented as *R/FR*) varied both in space (among species and canopy positions) and in time (across spring recovery of photosynthesis, from mid-February to mid-July). Lower canopies of trees consistently showed higher *R, FR,* and *R/FR* across the study period, with the exception of pine in summer. Species and canopy positions clearly differed both in the levels of ChlF parameters (*R, FR, R/FR*) and in their seasonal variation. Importantly, the spatial differences among leaves did not remain the same over time. All these results lead to a question: what lies behind the spatial and temporal patterns found in spectral ChlF? In **Study II**, at least some mechanisms influencing these patterns were revealed.

4.4.1. Effect of physiological factors and their spatio-temporal variation

In boreal forests, physiological factors are expected to affect both temporal and spatial variation in $\Phi F(\lambda)$ (Porcar-Castell et al. 2012). However, depending on the relative importance of PQ_S and NPQ_S, winter downregulation of photosynthesis can have a variable effect on $\Phi F(\lambda)$. Seasonal trends of PQ_S and NPQ_S have opposite directions, decreasing and increasing towards winter, respectively (visualization can be found in Figure 3 of Porcar-Castell et al. 2008a). Because quenching mechanisms primarily affect PSII, leading to a relative decrease in red-ChlF, as compared to far-red-ChlF (Farooq et al. 2018, see section *1.3.2* for details), these variable effects will be reflected as changes in ChlF magnitude but also in ChlF shape. If winter downregulation was dominated by a decrease in PQs, winter ChlF magnitude would increase. In contrast, if the downregulation of photosynthesis was dominated by increasing NPQ_S, ChlF magnitude and *R/FR* would decrease in winter, and then increase towards summer as NPQ_S relaxes.

On the spatial scale, such a dominant role of NPQ_S would be reflected in a decrease in ChlF magnitude and *R/FR* on upper, compared to lower canopies, as more exposed leaves are known to develop higher NPQ_S (Porcar-Castell et al. 2008b). In nature, a combination of both quenching mechanisms is most likely, which results in complex relationships between spectral ChlF and photosynthesis.

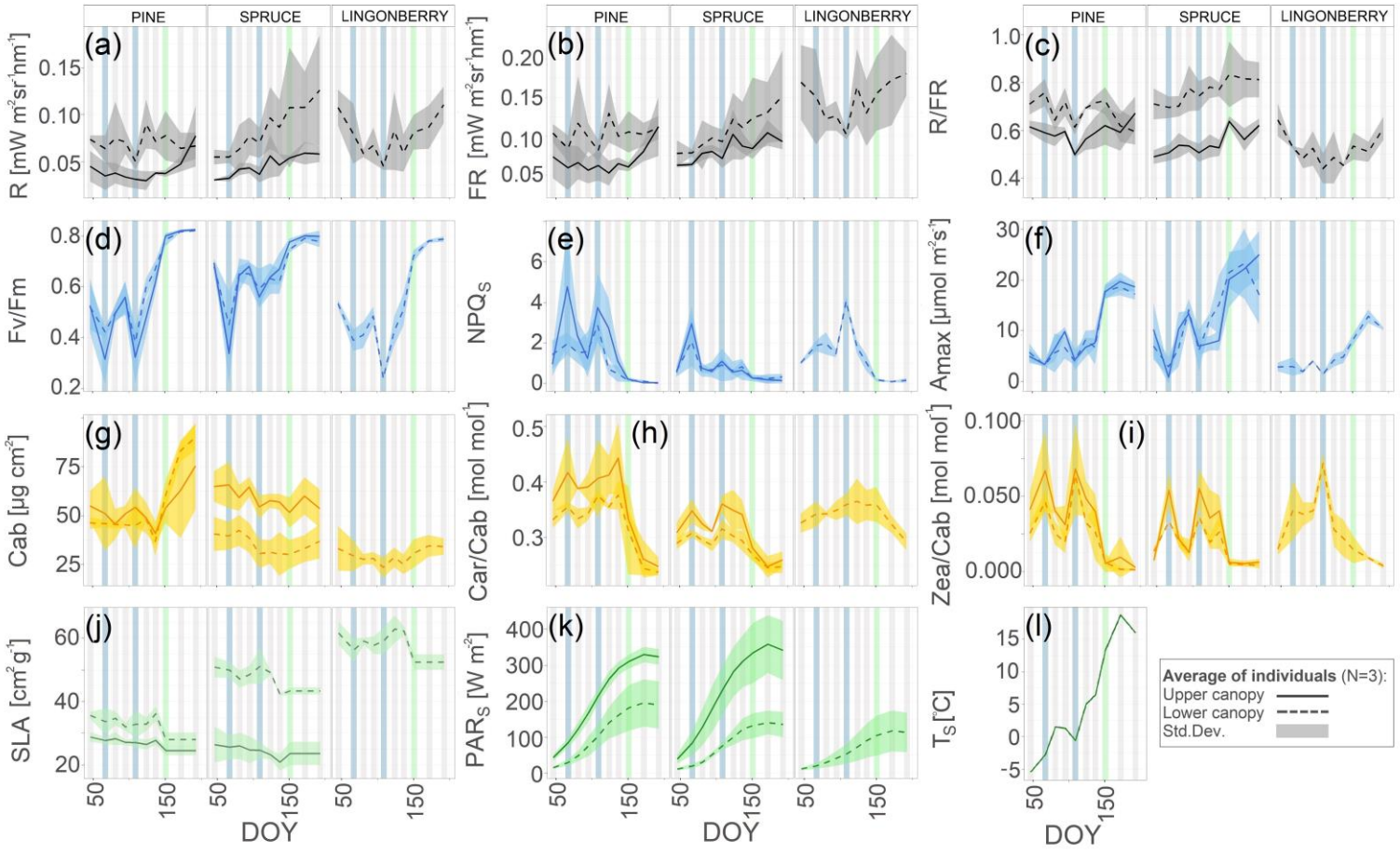
PINE
SPRUCE
LINGONBERRY
(a)
R [mW m-2sr-1nm-1]
(b)
FR [mW m-2sr-1nm-1]
(c)
R/FR
(d)
Fv/Fm
(e)
NPQS
(f)
Amax [µmol m-2s-1]
(g)
Cab [µg cm-2]
(h)
Car/Cab [mol mol-1]
(i)
Zea/Cab [mol mol-1]
(j)
SLA [cm2g-1]
(k)
PARS [W m-2]
(l)
TS [°C]
DOY
Average of individuals (N=3):
Upper canopy
Lower canopy
Std.Dev.

Figure 7 (facing page). Comprehensive approach on analysing variation in spectral ChlF on the spatio-temporal scale with foliar and environmental factors. The spatial variation scale is represented by species (separate boxes) and canopy positions (line types, solid for upper and dashed for lower canopy) together with foliar physiological and physical factors, as well as environmental factors. Temporal variation is represented by 10 measuring points marked as shading (blue for two cold spells, i.e., points 2 and 5, green for the first point after spring recovery of photosynthesis, i.e., point 8). Next to spectral ChlF parameters R, FR, and R/FR (a-c), foliar and environmental factors are presented – (d): Fv/Fm, maximum quantum yield photochemistry; (e): NPQ_S, sustained non-photochemical quenching; (f): Amax, a proxy of maximum assimilation rate at 1500 µmol $m^{-2}s^{-1}$ PAR and 1500 ppm CO_2; (g): Cab, total chlorophyll *a+b*; (h): Car/Cab, total carotenoids to chlorophyll *a+b* ratio; (i): Zea/Cab, zeaxanthin to chlorophyll *a+b* ratio; (j): Specific leaf area (SLA); (k): PAR_S, seasonal PAR obtained from the digital hemispherical images as a 10-day average of daily mean PAR preceding each measuring point; and (l): T_S, seasonal temperature obtained as described for PAR_S.

Results of **Study II** support previous findings of lower ΦF(λ) in winter than in summer (Ensminger et al. 2004; Porcar-Castell et al. 2008a; Linkosalo et al. 2014), implying that temporal variation in spectral ChlF is indeed mostly dependent on the seasonal dynamics in NPQ_S (decrease towards summer) rather than in PQ_S (increase towards summer). This suggestion was further supported by the negative correlation between photo-protection-related pigments (Zea/Cab, Car/Cab) and ChlF magnitude (SV1) or shape (i.e., positive correlation with SV2/3, Figure 5 in **Study II**). However, no significant correlation between ChlF magnitude and NPQ_S or PQ_S was found for the lower canopy needles (Figure 5cd therein), suggesting that the effects that NPQ_S and PQ_S have on ChlF magnitude in more shaded parts of the canopy might actually compensate and cancel each other out. Indeed, on lower canopies, the seasonal range of the variation in NPQ_S (Figure 7e) and PQ_S (Figure A3a in **Study II**) remains largely complementary both in terms of levels and patterns. This finding can appear crucial from the perspective of rapidly increasing spatial and temporal resolution of SIF data, especially considering recent advances in SIF imaging systems (Rascher et al. 2015; Siegmann et al. 2019). Namely, when a SIF imaging system is positioned to capture the whole canopy profile, the retrieved signal will be a mixture of SIF affected by different factors. Therefore, interpretation of the new stream of SIF data will require us to provide enough understanding of the mechanisms behind the spatio-temporal variation in ChlF emitted by various leaves within an ecosystem.

While ChlF magnitude (SV1) was not able to capture variation in photosynthetic factors in lower canopies (especially lower pine, Figure 5 in **Study II**), ChlF shape (especially SV2) showed a significant correlation with at least some factors (spruce) or even all of them (pine). Here, it is important to discuss the previously mentioned (*4.3.2*) difference between variation in *R/FR* and variation in ChlF shape-related SV components (SV2 and SV3). In the temporal variation analysis, only the lower spruce showed a correlation between [Cab] and *R/FR*. In contrast, [Cab] correlated with SV2 (variation in ChlF shape associated with reabsorption) in the upper and lower pine and upper spruce, as well as with SV3 (variation in ChlF shape associated with PSI:PSII relative contribution to total ChlF) in lingonberry. These results imply that there are indeed at least two components that affect variation in ChlF shape and that the mechanisms underlying this variation cannot be captured while using *R/FR* alone. What is more, the correlations between [Cab] and SV2 were positive (pine) or negative (spruce), depending on the species, suggesting that on the seasonal scale, not only [Cab]

adjustments but also other processes can affect variation in reabsorption. For example, reorganization at the level of thylakoid membrane in response to NPQ was suggested by Zhang et al. (2019), who found that the increase in NPQ_S, in response to cold spell experienced by overwintering upper canopy Scots pine needles, was reflected by a strong increase in ChlF reabsorption and decrease in ChlF magnitude without changes in [Cab].

At the same time, the changes in NPQ_S can affect the relative contribution of PSI and PSII emission to the total ChlF signal, because PSII is expected to be more responsive to quenching mechanisms, as compared to PSI (Farooq et al. 2018). Therefore, with the increase in NPQ_S, *R/FR* will decrease, irrespectively of the stable level of [Cab]. In **Study II**, the variation in ChlF shape associated with PSI:PSII relative contribution to total ChlF was represented by SV3 (Figures 7c,f and 8c,f,, also Magney et al. 2019b). Although there were only some correlations found between physiological factors and SV3, seasonal re-distribution of excitation energy between PSII and PSI in overwintering evergreens was recently reported (Bag et al. 2020; Grebe et al. 2020), supporting the explanation of mechanisms behind SV3.

Differences in seasonal trends found between separate species and canopy positions were supported by an analysis of spatial variation at separate points in time (Figure 4 in **Study II**). In general, for the majority of measuring points, a limited number of correlations was found between physiological factors and SV component representing spatial variation in ChlF magnitude (SV1). In other words, for the majority of the analysed period, the effect that photosynthetic activity had on ChlF magnitude was similar among leaves in the boreal forest (i.e., no spatial variation within a single measuring point). The measuring points when SV1 correlates with the highest number of physiological factors were in mid-February (low temperatures in measuring point 1) and in late May (first point after spring recovery of photosynthesis, measuring point 8). Results from these measuring points suggest that leaves of different species and canopy positions can have different recovery rates (i.e., spatial variation across time), which is in agreement with previous studies (Matsubara et al. 2002; Linkosalo et al. 2014; Merry et al. 2017) and with conclusions from temporal-scale analysis (Figure 5 in **Study II**).

In summary, the effect that physiological factors have on the temporal variation in spectral ChlF can vary among species and canopy positions, so depend primarily on leaf architecture and foliar pigment concentration. The local light environment is also important as it affects these two foliar factors. At the same time, the effect that physiological factors have on spatial variation in spectral ChlF can vary in time, in response to seasonal changes in environmental conditions, temperature, and incoming irradiance. In conclusion, results from our two spectral ChlF variation analyses on i. temporal scale, considered separately for diverse foliar representatives of an ecosystem, and on ii. spatial scale, considered separately for different measuring points across spring recovery of photosynthesis, are complementary and pointing together to the need for a mechanistic interpretation of the variations in spectral ChlF in terms of photosynthesis.

4.4.2. Effect of physical factors and their spatio-temporal variation

Next to physiological factors, which affect $\Phi F(\lambda)$ and LUE, physical factors affect $f_{esc}(\lambda)$. At the leaf-level, the $f_{esc}(\lambda)$, i.e., a probability that ChlF emitted at the photosystem level escapes the leaf and reaches a sensor, depends on reabsorption and scattering properties within a single leaf and, thus, on foliar [Cab] and leaf architecture (*1.4*).

[Cab] is generally expected to decrease towards winter (Linder, 1972; Öquist, 2003; Sveshnikov et al. 2006; Sofronova et al. 2016; Hernández-Clemente et al. 2017). In **Study**

II, temporal variation in [Cab] was not pronounced, except for pine. [Cab] also varied between canopy positions, but only in spruce. It is important to note that despite variations in [Cab], more on the temporal scale in pine and more on the spatial scale in spruce, $APAR_g$ did not show strong temporal or spatial variation (Figure A1 in **Study II**). Therefore, the effect that [Cab] had on variation in spectral ChlF was expected to be primarily physical, i.e., affecting $f_{esc}(\lambda)$, rather than physiological, i.e., affecting $APAR_g$. Finally, it has to be remembered that in the spatio-temporal patterns described here (and in **Study II**) [Cab] is represented based on its area. The variation in [Cab]/area can fluctuate in time because the seasonal changes in [Cab]/mass and in leaf architecture can differ. Therefore, it is difficult to discuss the general effect that [Cab] has on the spatio-temporal variation in spectral ChlF without considering foliar architectural features.

In **Study II**, SLA was estimated as a representative of foliar architectural or, more precisely, morphological features. As expected, SLA was temporarily stable and expressed large differences on the spatial scale. Higher SLA levels (i.e., thinner leaves) were found for more shaded conditions, which was consistent with summer values of SLA reported by Atherton et al. (2017) for the same forest. The differences in SLA between the upper and lower canopy were much more pronounced in spruce. The lower needles of spruce were much thinner and flatter (i.e., higher SLA), as compared to more exposed, thick, and rhomboid ones, and the anatomical differences across the canopy were pronounced. These phenomena could explain why spruce, but not pine, showed big differences in [Cab]/area between upper and lower canopies.

Considering their relatively small temporal and substantial spatial variation, physical factors were expected to affect variation in spectral ChlF primarily on the spatial scale, specifically to affect the temporarily-invariant, baseline variation. Moreover, considering that physical factors strongly influence reabsorption and scattering within the leaf, these factors were expected to correlate with variation in ChlF shape rather than in ChlF magnitude.

Temporal analysis showed large differences in the correlations between ChlF variation components (SVs) and all the analysed factors on the spatial scale, i.e. among species and canopy positions. However, in order to identify controls of baseline variation in spectral ChlF more precisely, it was essential to investigate the correlation between spatial variation in ChlF magnitude and shape in each of the 10 measuring points, as well as the evolution of these correlations across the whole study period (Figure 4 in **Study II**).

SLA and GLI (local light environment) showed remarkable consistency in correlation with the variation in ChlF magnitude (SV1) across the whole study period. These results confirm the important role of leaf morphology and local light environment in the baseline variation in ChlF magnitude, even if only conifers are considered (Figure A7 in **Study II**). In terms of variation in ChlF shape (SV2 and SV3), especially the component related to ChlF reabsorption (SV2), more correlations are found with physical factors when lingonberry was excluded from the analysis (Figure A7 in **Study II**). GLI and SLA strongly correlated with SV2 in all measuring points except in summer. Apparently, baseline variation in spectral ChlF can be dependent on the morphological or anatomical differences that appear not only between ground vegetation and trees or between broadleaves and conifer, but also between needles of different species or between needles of the same species but developed under different light conditions.

All these findings lead a question of what underlies the strong and temporarily consistent correlation between leaf morphology, or perhaps architecture, and spectral ChlF. Surprisingly, this aspect was not a central scope of previous studies and the effect of leaf

architecture on spectral ChlF remains underexplored. Although **Study II** did not include any estimates of leaf anatomy, as only the SLA was measured, some theoretical background can be framed to understand how leaf architecture, including morphology and anatomy, influences the spectral ChlF.

When light reaches a thinner leaf with a flatter surface, developed under low-light conditions, it is absorbed on a relatively wider area, as compared to sun-exposed leaves which are generally thicker and smaller (Knapp and Carter, 1998; Niinemets et al. 2014; Poorter et al. 2019). Therefore, most of the light can be absorbed by the abundant spongy parenchyma cells (Fukshansky et al. 1993; Vogelmann, 1993) and emitted as ChlF without reaching deeper layers of a leaf. That, in turn, will lead to higher ChlF magnitude, but also higher *R/FR*, due to the limited probability of reabsorption. In contrast, when light reaches a thick leaf or a thick and round/rhomboid needle developed on the sun-exposed branches, it will penetrate deeper into a leaf, thanks to well-developed palisade layers (Cui et al. 1991; Vogelmann and Martin, 1993). Therefore, relatively fewer photons will be left to be emitted as ChlF, leading to a smaller ChlF magnitude. Because of the deeper light penetration, ChlF has a longer way back to the detector and, thus, is more prone to be reabsorbed. In sum, increased leaf thickness leads to lower ChlF emission, as well as enhanced reabsorption and lower *R/FR* in sun-exposed, as compared to shaded leaves. These mechanisms, although not supported by direct measurements of leaf anatomy, could explain the spatial variation in spectral ChlF found in **Study II**.

The chlorophyll concentration ([Cab]/area, Figure 7g) was found to significantly affect the spatial variation in ChlF magnitude only in 4 measuring points. For ChlF shape, no correlations were found (Figure 4 in **Study II**). Even fewer correlations with SV components were found for [Cab] when expressed on a mass basis (data not shown). When lingonberry was excluded from the analysis and only conifers were considered (Figure A7 in **Study II**), [Cab]/area correlated with the variation in ChlF shape associated with reabsorption (SV2) in the majority of measuring points, especially in spring. Therefore, when more architecturally similar samples are considered, the role of chlorophyll concentration in affecting ChlF shape increases.

To summarize, the results of **Study II** imply that when interpreting spatial variation in spectral ChlF, not only chlorophyll concentration alone, but leaf morphological or, if possible, architectural features should be considered. However, this aspect has been somehow neglected in the literature, which points to the mechanistic challenge in designing leaf-level spectral ChlF studies and interpreting spectral ChlF in terms of photosynthesis. If the variation in spectral ChlF is not considered simultaneously in its spatial and temporal context, parametrization of factors that drive this variation within a leaf and within a canopy will be difficult, or, potentially, incorrect. This will lead to inaccuracies in RTMs, as well as process-based models. Such inaccuracies could decelerate future efforts in following the roadmap drawn in **Study III**.

5. CONCLUSIONS

The main goal of this thesis was to contribute to the interpretation of ChlF data by identifying, characterizing, and contextualizing the influence of methodological and mechanistic factors on leaf-level spectral ChlF. In this thesis, I aimed at highlighting the importance of leaf-level studies of spectral ChlF in the context of the current and future work of the ChlF research community and beyond. I also suggested that spectral ChlF should always be interpreted considering the methodological and mechanistic context.

In **Study III**, challenges that make the link between ChlF and photosynthesis difficult to disentangle were identified, and the theoretical roadmap to navigate through these challenges was drawn. In this thesis, I contextualized the leaf-level spectral ChlF on this roadmap. I suggested that the leaf-level sets a foundation for interpreting remotely-sensed SIF (Malenovsky et al. 2009; Porcar-Castell et al. 2014), as it allows to investigate not only the variations in ChlF, but also controls of these variations, and the connection between ChlF and photosynthesis. A precise understanding of how different factors affect ChlF emission and, thus, the mechanistic interpretation of spatio-temporal variation in spectral ChlF, will help in a better parametrization of RTMs (accounting for, e.g., heterogeneous and dynamic distribution of Cab within the leaf, chloroplast, and thylakoids) and process-based models (accounting for, e.g., differences in light and temperature history to resolve the dynamics in physiological processes). Development and fine-tuning of models, together with the implementation of SIF imaging systems, will lead us closer to extracting the full potential of SIF measured across scales. However, in order to collect a reliable amount of complementary and comparable data, providing ground for mechanistic interpretation of ChlF, methodological challenges have to be solved.

In **Study I**, the methodological context was discussed and the effect that methodological factors have on spectral ChlF was investigated. I tested the effect of measuring geometry and showed that ChlF shape is less dependent on measuring geometry than ChlF magnitude. Therefore, the importance of recording not only ChlF magnitude, but the full-spectrum ChlF was suggested. This methodological challenge is especially relevant for needles, which represent complicated foliar architecture and, thus, are very difficult to measure. Needle arrangement as a methodological factor was also investigated in **Study I**. I showed that needle-mats delivered the most reproducible and replicable results of spectral ChlF. Consequently, I suggest that needle mats should be used to secure the best comparability of spectral ChlF results between studies, where different leaves are measured with diverse viewing and illumination angle setups.

The complete informative potential carried by spectral ChlF will not be extracted without mechanistic interpretation of its spatio-temporal variation. In **Study II**, this mechanistic context was discussed and the effect that various mechanistic factors have on spectral ChlF was investigated. **Study II** showed that spatial variation in spectral ChlF was dependent on different factors across the period of spring recovery of photosynthesis. Leaf morphology (represented by SLA) and local light environment (GLI) were remarkable exceptions, showing a strong correlation with the variation in ChlF magnitude across the whole study period. The presence of this baseline, consistent, time-independent element in the variation in ChlF magnitude clearly shows that foliar factors (SLA, but perhaps also other factors that are dependent on GLI) should be considered when interpreting variations in ChlF. Simultaneously, temporal variation in spectral ChlF was dependent on different factors across

leaves within the forest. For exposed needles and lingonberry, ChlF magnitude followed the seasonal patterns of NPQ_S, while a compensation effect between NPQ_S and PQ_S was found for shaded needles. Consequently, with the results of **Study II**, I propose, that the effect that a certain factor has on this relationship should not be considered in a single context alone, as factors operate simultaneously across space and time and their effect is never free from the influence of other factors. Disentangling this complex relationship, dependent on multiple factors that operate simultaneously on the spatial and temporal scale, will never be possible without long-term, comprehensive studies delivering comparable results of spectral ChlF from different species, light environments, or biomes. Although considering only boreal forest, **Study II** can be treated as an example to encourage similar studies in different biomes.

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