Application of Single Turnover Active Chlorophyll Fluorescence for Phytoplankton Productivity Measurements

Version 2.0, June, 26, 2023

Centric and pennate diatoms from the Ross Sea showing red chlorophyll fluorescence
Photo by Philippe Tortell
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Preface

This document represents the collective efforts of SCOR Working Group 156, ‘Active Chlorophyll Fluorescence for Autonomous Measurements of Global Marine Primary Productivity’. The group was established in 2019, bringing together researchers and instrument manufacturers from 10 countries and 6 continents to develop standards of best practice in the application of single turnover active chlorophyll fluorescence (ST-ChlF) for phytoplankton photo-physiology and productivity measurements. We focused our efforts on single turnover ChlF methods, which are most prevalent in phytoplankton research, while recognizing that other approaches, including Pulse Amplitude Modulation (PAM) fluorescence, are also commonly employed with macro-algae, corals and terrestrial plants. Some of the material in this document will apply to those related measurement techniques.

During the period between 2019-2022, our group worked to build consensus around best practice for the collection, analysis, interpretation and archiving of ST-ChlF data from a variety of aquatic environments. This work was aimed at facilitating wide-spread use of ST-ChlF methods by the international research community, focussing on two key objectives outlined in the Working Group’s terms of reference:

1. To develop, implement and document internationally-agreed best practice for the acquisition and analysis of ST-ChlF data to retrieve photosynthetic parameters and primary productivity estimates.

2. To develop, implement and document standardised ST-ChlF data output formats and archiving approaches.

As a first step towards these objectives, we published a review article (Schuback et al. 2021), outlining applications, opportunities and current limitations of ST-ChlF measurements. This high-level review was aimed at readers wishing to apply ST-ChlF methods and interpret the resulting data in the most robust manner possible. Journal length restrictions precluded in-depth treatment of many important topics, including instrument calibration, data fitting and spectral correction. In this User Guide, we expand on the material presented by Schuback et al. (2021), providing hands-on guidance for both experts and new users alike. With this document, we aim to provide both a strong theoretical background for ST-ChlF methods, and a practical handbook to inform the application of these methods across a wide range of aquatic environments. We assume that readers of have a basic understanding of aquatic photosynthesis, as outlined, for example, by Falkowski and Raven (2013).

This document represents the first complete version of the SCOR ST-ChlF user guide. It is being released to the international research community for a period of open consultation. We encourage comments and suggestions, which can be addressed to the working group co-chairs, Philippe Tortell and David Suggett. We expect the contents of this User Guide to evolve significantly over time, through our own efforts and with input from other experts and end-users. Revised versions of the User Guide will be posted on the Ocean Best Practices site (https://www.oceanbestpractices.org/), and interested readers are encouraged to check periodically for updates. In follow up work, we aim to further develop existing open-source software (e.g. Ryan-Keough and Robinson, 2021) to facilitate ST-ChlF data analysis from a range of different instruments and formats.
Acknowledgements

We thank the SCOR Executive Directors Ed Urban who helped bring our group together, and Patricia Miloslavic, who supported our work over the past four years. Pauline Simpson and the IOC Ocean Best Practices team provided valuable assistance in making this document publicly available through the OBP repository. We also wish to acknowledge the international ST-ChlF research community, whose work laid the foundation for the ideas and concepts in this field, and whose future contributions will refine subsequent iterations of this User Guide.
Dedication

We dedicate this work to the memory of Jacco Kromkamp, who passed away on October 5, 2020. Jacco was a pioneer in the application of ST-ChlF measurements to assess phytoplankton productivity, and a founding member of SCOR WG 156. His ideas and insights contributed greatly to our field, and his warmth and friendship helped bring our community together. Much of the knowledge presented in this document was shaped by Jacco’s research, and his passion for scientific monitoring of aquatic ecosystem health. He will be greatly missed.
How to use this document

This document is intended to serve the needs of a wide range of users, from novices (first year graduate students, for example) to expert researchers with decades of experience using ST-ChlF methods. We anticipate that some readers will require only high-level information on key operational parameters needed for successful ST-ChlF measurements, while others will be interested in further exploring the underlying ideas and concepts. For this reason, the document includes appendices that provide greater detail on some topics presented in the main document. In subsequent versions of this User Guide, we aim to include linked Jupyter notebooks, with hands-on tutorials and data processing tools. These software tools will help facilitate the inter-comparison of measurements collected by different users and instruments, supporting the development of globally-coherent ST-ChlF data compilations.
## Document History

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<tr>
<td>2.0</td>
<td>June 26, 2023</td>
<td>First full-length version for broad community input</td>
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1. INTRODUCTION

1.1 Photosynthetic Production

Photosynthetic primary productivity is the light-driven process of extracting reducing power from water to drive CO₂ reduction to carbohydrates (i.e., CO₂ ‘fixation’). The process consists of several steps, including light harvesting, primary charge separation in the photosynthetic reaction centers, stripping of electrons and O₂ from water, and the transfer of electrons through a series of redox carriers to produce NADPH and ATP. In addition to CO₂ fixation, the reducing power generated through photosynthesis is used to drive other redox reactions, including NO₃⁻ reduction, SO₄²⁻ assimilation, and the dissipative photo-reduction of O₂ to water. In nitrogen-fixing (diazotrophic) organisms, a portion of the acquired reducing power and ATP is also utilized in the process of nitrogen fixation.

Global primary productivity is the ultimate source of O₂ for the atmosphere and oceans, and sets the planetary redox state over geological time-scales (Falkowski and Godfrey, 2008). Marine primary productivity also provides a critical source of organic matter for aquatic food webs, and plays an important role in carbon (C) sequestration to the deep ocean through the so-called biological carbon pump (Ducklow et al. 2001). Global climate change has created a pressing need to better understand the environmental controls on aquatic primary productivity, its variability over space and time, and its potential response to various natural and anthropogenic perturbations (Behrenfeld et al. 2006, Moore et al. 2018). Addressing these questions requires consistent and coherent primary productivity measurements across a range of time and space scales.

1.2 Measuring Aquatic Primary Productivity

Measurements of aquatic primary productivity (PP) date back more than a century, with early approaches (Gaarder and Gran, 1927) based on measured changes in O₂ concentrations in dark and light bottles to assess net and gross productivity (NPP and GPP, respectively). GPP quantifies the total amount of C fixed (or photosynthetic O₂ evolved) by primary producers, while NPP quantifies the amount of ‘excess’ C fixed (or evolved O₂), after accounting for autotrophic respiration. A major advance occurred in the years following WWII, with the development of the now classic Steeman-Nielsen incubation method (Steeman-Nielsen, 1952), where the incorporation of ¹⁴CO₂ into particulate organic matter by phytoplankton is quantified in seawater samples. Depending on the length of the incubation period, ¹⁴C measurements capture a signal somewhere between GPP and NPP, with shorter measurements more closely approximating GPP (Halsey and Jones, 2015). In the decades following the advent of the ¹⁴C method, other incubation approaches were developed, including ¹⁵NO₃⁻ and ¹⁵NH₄⁺ uptake experiments to estimate ‘new production’ and ‘regenerated’ production, respectively (Dugdale and Goering, 1967), and H₂¹⁸O incubations to quantify the photosynthetic water splitting reaction as a metric of GPP (Bender et al. 1987). In these latter experiments, the photosynthetically-derived ¹⁸O₂ is diluted in a large background of unlabelled O₂, and is thus not significantly affected by respiration.

Our understanding of oceanic PP variability has largely been based on results from discrete incubation experiments, with ¹⁴C measurements also used to benchmark satellite-based global productivity algorithms (Behrenfeld and Falkowski 1997, Bouman et al., 2018). Such discrete PP
measurements are significantly constrained in time and space, as they require dedicated personnel, the use of radioactive tracers (for $^{14}$C incubations) and / or complex instrumentation (mass spectrometers for $^{15}$N and $^{18}$O measurements). These factors have limited the spatial and temporal coverage and sampling resolution of PP measurements (Halsey and Jones 2015). Moreover, the need for sample incubation creates potential containment artefacts, with adverse effects on some components of the plankton community (Quay et al. 2010).

To move beyond the limitations of discrete bottle incubation methods, several approaches have been developed to estimate PP from mixed layer chemical tracers, including nutrients, dissolved inorganic carbon and oxygen. Over the past decade, for example, there has been increasing use of O$_2$/Ar as a mixed layer productivity tracer (Craig and Hayward, 1987). In this approach, net community production (NCP; defined as GPP minus community-wide respiration) is assessed from the biologically-induced oxygen saturation anomaly, after accounting for air-sea O$_2$ fluxes (Kaiser et al. 2005). Similarly, measurements of the triple oxygen isotope composition of water have provided information on gross oxygen production in the mixed layer (e.g. Jaurnek and Quay 2013). These tracer-based methods provide bulk productivity estimates on time-scales of mixed layer ventilation (~ 1 – 3 several weeks), but they do not resolve shorter-term (e.g. sub-daily) PP variability resulting from a range of environmental and physiological drivers. Tracer-based methods also rely on a steady-state assumption, which may not apply in dynamic ocean systems.

1.3 Active Chlorophyll Fluorescence as a real-time primary productivity measurement tool

During the early to mid-1990s, a new technique was introduced for measurements of aquatic photosynthesis based on the analysis of single turnover chlorophyll fluorescence (ST-ChlF) transients. This approach exploits the inverse relationship between chlorophyll fluorescence and PP (see Schuback et al. 2021). Seminal papers described methods to quantify photosynthetic electron transport rates at photosystem II (ETR$_{PSII}$), by resolving the rapid changes in ChlF following exposure to a strong excitation light source (Kolber and Falkowski 1993, Kolber et al. 1998). As a component of the photosynthetic process, ETR$_{PSII}$ – here defined as the rate of charge separation in photosystem II – are inherently coupled to the rate of light-dependent water splitting, O$_2$ evolution and the production of ATP and NADPH. Quantification of ETR$_{PSII}$ can thus provide an estimate of gross primary productivity. Indeed, early studies demonstrated that ETR$_{PSII}$ derived from ST-ChlF correlated well with parallel measurements of short-term $^{14}$C-uptake (Kolber and Falkowski 1993) and gross O$_2$ evolution (Suggett et al. 2003). Other studies applied ST-ChlF measurements to understand the physiological status of phytoplankton in situ, including cellular responses to iron limitation (Kolber et al. 1994).

In the decade following the pioneering application of aquatic ST-ChlF measurements, the field expanded significantly, with improved instrumentation and increased understanding of underlying photo-physiological processes. The first commercially available Fast Repetition Rate fluorometers (FRRf) and related Fluorescence Induction and Relaxation (FIRe) instruments for ST-ChlF were released in the early 2000s (Chelsea Technologies Group Ltd., Satlantic Inc., Soliense), and quickly found increasing use in laboratory experiments and on oceanographic vessels. Significant efforts were aimed at further comparing ETR$_{PSII}$ with $^{14}$C-uptake (e.g. Corno et al. 2006, Suggett et al. 2006, Moore et al. 2006), and growing data sets repeatedly demonstrated strong covariance between parallel ETR$_{PSII}$ and $^{14}$C-uptake measurements in marine and freshwater systems (e.g.
Kolber and Falkowski, 1993). However, results from this work also demonstrated that the relationship between ST-ChlF and other productivity metrics varied widely depending upon the prevailing phytoplankton taxa and/or environmental conditions (see Suggett et al. 2009, Lawrenz et al. 2013). Within a decade, ST-ChlF instruments became standard on many large-scale oceanographic programs (e.g. Atlantic Meridional Transect; Suggett et al. 2006, Hawaii Ocean Time-Series; Corno et al. 2006) and biogeochemical studies of ocean productivity (e.g. Behrenfeld et al. 2006).

Over the past decade, ST-ChlF measurements have become increasingly common, and several approaches have been developed to estimate \( ETR_{\text{PSII}} \) from the resulting data (Oxborough et al. 2012, Boatman et al. 2019, Gorbunov and Falkowski 2021). At the same time, instrument sensitivity has increased significantly, permitting robust measurements in the most oligotrophic waters, while multi-wavelength instruments have been used to resolve the spectral signatures of different phytoplankton taxa (e.g. Gorbunov et al., 2020). These technological developments have further stimulated interest in the use of ST-ChlF for oceanographic and freshwater research (e.g. Schuback et al. 2017, Zhu et al. 2017, Hughes et al. 2020), with a growing range of commercial and custom-built instruments (e.g. Hoadley and Warner 2017, Fujiki et al. 2020) deployed on a range of sampling platforms, including ships, gliders, moorings and floats. These theoretical and operational advances, coupled with the advent of satellite-based passive chlorophyll fluorescence measurements, present an exciting opportunity for global-scale ST-ChlF measurements. Such global measurements would revolutionize our understanding of marine primary productivity, and its future response to rapid climate change.

1.4 Opportunities and Challenges

The field of ST-ChlF measurements now sits at a critical crossroads. As the application of these methods continues to grow, conceptual, operational and computational approaches to collect and interpret ChlF data are rapidly diverging (e.g. Hughes et al. 2018). An increasing number of sensors, protocols and data processing algorithms are now being used to obtain primary productivity estimates from ST-ChlF measurements, yet there has been little direct inter-comparison of methods and approaches, and no standards of best practice have been adopted by the international research community. As a result, the advantages and limitations of different methods are presently unclear, as is the influence of operational context and key environmental and taxonomic variables on the choice of optimal ST-ChlF protocols. Rapidly growing data sets may soon become increasingly difficult (if not impossible) to reconcile, thus limiting our ability to build global ST-ChlF compilations and examine large-scale patterns in photo-physiological properties, \( ETR_{\text{PSII}} \) and their response to environmental forcing.

SCOR working group 156 was created to address this fundamental challenge, bringing together experts from across the globe to produce recommendations for best-practices in the acquisition, interpretation and archiving of ST-ChlF data. This User Guide represents a key deliverable of our work, providing a framework for the development and expansion of ST-ChlF measurements for aquatic primary productivity research. In this document, we highlight critical considerations for the use of ST-ChlF methods, presenting the current state of knowledge, and gaps in understanding that must be addressed going forward. Based on this analysis, we provide recommendations to support robust application and interpretation of ST-ChlF methods under a range of conditions. We
also provide practical instructions to facilitate the processing and analysis of ST-ChlF data. Finally, we discuss the requirements for data quality control, analysis and archiving, with the objective of establishing a framework for the global synthesis of ST-ChlF measurements. Such a synthesis of observations will be of significant value in understanding broad-scale patterns in aquatic productivity, and in supporting robust interpretation of satellite-based passive fluorescence measurements.

1.5 Primary Productivity as an Essential Climate Variable

The goals of SCOR WG 156 reflect back to one of the first SCOR Working Groups, more than half a century ago. In the late 1950s, SCOR Working Group 3, ‘Biological Production of the Sea’, was established to ‘appraise and recommend methods for world-wide comparisons of organic productivity and standing crops’. Today, this remains a critical challenge facing marine scientists, and one that has taken on increasing urgency considering global climate change and other anthropogenic pressures impacting aquatic ecosystems. Despite notable progress over the past decade, significant discrepancies persist among Earth System Model projections of future NPP changes (Kearny et al. 2021). This highlights a gap in our understanding and ability to accurately represent a major planetary carbon flux (Canadell et al., 2021). The United Nations Decade of Ocean Science for Sustainable Development (2021-2030), provides a unique opportunity to stimulate international collaboration and cooperation in understanding global-scale patterns in critical ocean properties such as primary productivity. Our Working Group efforts are meant to align with these initiatives, improving the monitoring and management of aquatic ecosystems in support of the UN’s Sustainable Development Goals.

In an effort to improve global environmental monitoring programs, the World Meteorological Organization established the Global Climate Observing System, defining a series of Essential Climate Variables (ECVs) that are needed to understand the state of Earth’s climate system. To qualify as an ECV, a variable must be: 1) critical for characterizing the climate system and its changes; 2) cost effective and reliant on coordinated observing systems using proven technology and historical datasets; and 3) technically feasible for global-scale application using established and scientifically-proven methods.

At present, ECVs relating to primary production in the oceans include nutrients, surface and subsurface temperature, plankton biomass and ocean colour, the latter of which includes satellite-based measurements of water leaving radiance and chlorophyll a (Chl a) concentration. Measurements of phytoplankton biomass are critical to understanding ocean health and climate, but biomass alone does not provide a full understanding of photosynthetic production without information on physiological rates. For this reason, there is a critical need to develop standardized methods to assess photosynthetic rates in aquatic environments at ecologically-relevant temporal and spatial scales. We believe that ST-ChlF techniques have the potential to achieve this goal, and that ETR$_{PSII}$, in particular, is a possible candidate for an ECV. The relevance of ETR$_{PSII}$ to the functioning of aquatic ecosystems is beyond doubt, and cost effectiveness is not a significant issue, as the required instrumentation is not particularly expensive. The primary challenge relates to the feasibility of developing scientifically-proven approaches to measure and interpret ETR$_{PSII}$ and related photo-physiological variables. In this respect, much work remains to be done, as we are far from consensus on the relevant methodology, application and interpretation of this approach. We
hope that the material presented in this User Guide will provide an important step in this direction, helping to stimulate robust, inter-comparable application of ST-ChlF methods on a global scale.
2. CONCEPTS AND FOUNDATIONS

2.1 Terminology and definitions

The use of multiple terms and notation to describe various photo-physiological processes can be one of the most challenging aspects for new users of ST-ChlF methods. Here we provide detailed definitions of key concepts and variables that are used throughout this User Guide. Where possible, we provide explanations for the existence of multiple terms, which are often used interchangeably in the literature. Previous work has discussed the parallel nomenclature systems describing variables associated with chlorophyll fluorescence measurements (van Kooten and Snel 1990; Maxwell and Johnson 2000; Kromkamp and Forster 2003; Cosgrove and Borowitzka 2010; Schuback et al. 2021). In tables 2.2.1 – 2.2.8, we provide a list of variables and parameters commonly used in the ST-ChlF literature, alongside a ‘thesaurus’ of synonyms. In this section we provide a more detailed discussion of particular terms, parameters and their definitions. Our group was not able to reach a consensus on the use of any one specific nomenclature, recognizing that multiple terms continue to be used with some justification. Given the likely persistence of multiple nomenclatures, we strongly recommend that users always provide clear definitions of chosen parameters in their publications.

Chlorophyll a fluorescence (ChlF)

Fluorescence occurs when atoms or molecules absorb energy from incident light, promoting electrons to higher energy states, and subsequently emit light at longer wavelengths upon the return of electrons to energetic ground states. The molecule chlorophyll a, a light harvesting pigment in all photosynthetic organisms, absorbs light in the range of 450–700 nm (photosynthetically available radiation, PAR) with distinct absorption peaks at 450-475 nm and 650-675 nm, and emits fluorescence with a peak around 680 nm. There is no known biological function of ChlF, but the phenomenon has been used for over a century in the study of photosynthetic organisms.

In vivo ChlF methods

In vivo ChlF methods include all ChlF measurements in living cells exposed to natural or artificial excitation light sources. In aquatic sciences, ‘conventional’ in vivo Chl a fluorometers are frequently used to monitor ChlF as a proxy for Chl a concentration, and thus for phytoplankton biomass. In these measurements, ChlF is induced by a constant artificial light source, and physiologically-driven changes in the detected ChlF signal per Chl a (‘ChlF quenching’) needs to be corrected for (see section 5.2.1). In contrast, ST-ChlF methods use rapid pulses or ‘flashlets’ of very high intensity excitation light to specifically induce changes in ChlF yields, which are interpreted in terms of underlying photo-physiological processes (see sections 2.2 and 2.3).

‘Active’, ‘Variable’ and/or ‘Induced’ ChlF
Methods including ST-ChlF are interchangeably referred to as ‘active’, ‘variable’, or ‘induced’ ChlF approaches. Arguably, fluorescence is always a passive process; the term ‘active’ refers to the use of artificial light sources to intentionally (actively) ‘induce’ the ‘variable’ ChlF signal. In order to avoid confusion, we recommend that the term ‘active Chl \( a \) fluorometry’ be reserved for instruments that induce and measure changes in ChlF yields, and not for fluorometers with a constant excitation source, which are used to derive Chl \( a \)-based biomass estimates.

‘Active’ versus ‘passive’ ChlF

In vivo ChlF can also be detected without the use of artificial light sources. The ChlF signal induced by the absorption of natural sunlight is generally referred to as ‘sun induced’ or ‘passive’ ChlF, and can be interpreted in parallel with active ChlF approaches.

Fluorescence yield

The absolute value of fluorescence signals measured by the ST-ChlF technique are dependent on the optical and electronic design of any particular instrument, the units of the measured signal, and the excitation power used to induce fluorescence. To account for this variability, the ST-ChlF transients can be recorded as a fluorescence yield, rather than an absolute value, by normalizing the measured fluorescence signal to the applied excitation power. This approach is used in most ST-ChlF instruments and allows the comparison of data collected with different excitation power during the saturation and the relaxation phase. To further correct for instrument-specific variability, the fluorescence yield can be reported in the Standard Referenced Units (SRU) through the use of an appropriate fluorescence standard (section 3.1.4).

Quantum yield of fluorescence

The quantum yield of the ChlF (\( \Phi_F \), table 2.1.3) is the ratio of the fluorescence signal emitted to the light absorbed by Light Harvesting Complex II (LHII), the pigment-protein complex functionally associated with photosystem II (PSII). Routine assessment of the true quantum yield of ChlF in vivo is very challenging, requiring specialized instrument to examine pico-second fluorescence lifetimes (Lin et al., 2016). Fortunately, absolute quantum yields are not required for application of the ST-ChlF method, which focuses on relative changes in ChlF in response to varying light.

Other approaches utilizing in vivo ChlF sometimes use alternate fluorescence quantum yield definitions, which can lead to confusion. For example, measurements of in vivo ChlF as a phytoplankton biomass proxy define the quantum yield of ChlF as the measured fluorescence yield per unit Chl concentration (measured spectrophotometrically or by other methods). Studies concerned with the interpretation of sun-induced ChlF sometimes define the quantum yield of fluorescence as the ChlF detected normalized to incident light or light absorbed by all pigments (the apparent quantum yield of fluorescence). While acknowledging the use of other terminology in the literature, the interpretation of ST-ChlF methods requires a strict definition of ‘quantum yields’ as a process normalized per quantum absorbed by LHII.
**Quantum yield of photochemistry**

In measurements of ST-ChlF, the quantum yield of photochemistry in PSII ($\Phi_{\text{PSII}}$) is defined as the fractional allocation (ranging from 0 to 1) of absorbed light that is directed to photochemical charge separation, in competition with fluorescence emission and heat dissipation. This definition implies that the commonly derived parameter $F_v/F_m$ can only be interpreted as the quantum yield of photochemistry if the minimum and maximum fluorescence yield ($F_o$ and $F_m$, respectively) are fully attributable to LHII associated with functionally-active PSII reaction centers. This, in turn, implies a need for blank and baseline fluorescence corrections (section 3.3).

**Non-photochemical quenching (NPQ)**

Non-photochemical quenching (NPQ) refers to the decrease (quenching) of ChlF due to allocation of absorbed energy to processes other than photochemistry, including heat dissipation. When the photochemical pathway is saturated under conditions of excess absorbed light energy, the upregulation of heat dissipation becomes necessary to protect the photosynthetic apparatus from damage. Reallocation of light harvesting complexes from PSII to PSI (i.e. state transition) can also alleviate excess excitation pressure, leading to a decrease in ChlF. As discussed in section 3.4, the term NPQ is used to denote a suite of physiological processes, as well as a specific derived quantity obtained from ST-ChlF data.

**The dark-regulated state: Dark ‘adapted’, dark ‘acclimated’, dark ‘regulated’**

In order to derive physiological parameters of interest from ST-ChlF measurements, it is often necessary to conduct measurements of a sample when NPQ is active (the light-regulated state), and also when NPQ is fully relaxed/absent (the dark-regulated state) (see section 3.4). Within the literature, the term ‘regulated’ is used interchangeably with ‘acclimated’ or ‘adapted’. However, these latter terms are less appropriate in a strict biological sense, as ‘acclimation’ implies de novo synthesis and accumulation of molecules (e.g. Chl a synthesis under low light acclimation), while ‘adaptation’ involves genetic changes, which can only occur over multiple generations. Neither of these processes is involved in the short period (minutes) of low light exposure used to relax NPQ and achieve dark-regulated states for ST-ChlF measurements. Nonetheless, the process of dark or low-light exposure of phytoplankton to relax NPQ (see section 3.4) is commonly referred to as ‘dark-adaptation’ or ‘dark-acclimation’. A better term, however, is ‘NPQ-relaxation’.

**Light absorption: absorption cross sections and absorption coefficients**

Accurate quantification of light absorption is fundamental to derive photosynthetic rates from ST-ChlF measurements (section 2.3). Unfortunately, different ETR$_{\text{PSII}}$ algorithms have used a range of abbreviations for different light absorption properties, resulting in some confusion in the literature. We list parallel nomenclature in table 2.1.7 and provide below some explanation for their existence.
By definition, an absorption cross section represents the probability that a molecule will absorb radiation of a particular wavelength, with units of area per molecule. In ST-ChlF methods, an important parameter is the absorption cross section serving photochemistry in PSII, which can be obtained from the initial rise of the ST-ChlF transient (section 2.2). By convention, absorption cross sections are given the symbol $\sigma$, and the absorption cross section of photochemistry in PSII, which is also known as the ‘functional’ or ‘effective’ absorption cross section, has frequently been denoted as $\sigma_{\text{PSII}}$ in the literature. In this document, we use the term $\sigma_{\text{PSII}}$, but note that the term $\sigma_{\text{PSII}}$ has recently been introduced into the literature to achieve consistency with subscripts used for absorption coefficients, and to emphasise that only the absorption cross section of photochemistry (rather than the absorption-cross section of PSII itself) is described. Units of $\sigma_{\text{PSII}}$ have been reported as area, area quanta$^{-1}$, or area PSII$^{-1}$. Here we adopt units of area PSII$^{-1}$.

The absorption cross section of all pigments functionally associated with PSII, regardless of whether or not the absorbed energy is used for photochemistry, has traditionally been described as the ‘optical’ absorption cross section. This term can be estimated from ST-ChlF measurements as the ratio $\sigma_{\text{PSII}}/\Phi_{\text{PSII}}$ (see tables 2.1.1-2.1.3 and section 2.3). In older literature, the ‘optical’ absorption cross section was often abbreviated as $a_{\text{PSII}}$. More recent papers used the symbol $\sigma$ in the form of $\sigma_{\text{PSII}}^{\text{opt}}$ (e.g. Gorbunov et al. 2020) or $\sigma_{\text{LHII}}$ (e.g. Boatman et al. 2019), where the subscript ‘LHII’ identifies the absorption cross-section as related to antenna pigments functionally associated with PSII reaction center.

In contrast to the absorption cross sections, absorption coefficients quantify the amount of light energy of a certain wavelength absorbed per unit distance traveled through an absorbing medium. Absorption coefficients are typically expressed in units of inverse length, such as m$^{-1}$. In the fields of ocean optics and remote sensing, absorption coefficients attributable to different constituents of seawater are traditionally abbreviated as ‘a’, with a subscript denoting the primary source of absorption (e.g. $a_{\text{phy}}$ for phytoplankton-specific absorption see table 2.1.7.). Absorption coefficients can be calculated as the product of the absorption cross section of a molecule and its concentration within a medium. Thus, the absorption coefficient of photochemistry in PSII, with units of m$^{-1}$, can be derived as the product of $\sigma_{\text{PSII}}$ (m$^2$ PSII$^{-1}$) and the volumetric concentration of PSII (PSII m$^{-3}$). Following the rational described above, the absorption coefficient of photochemistry in PSII should thus be $a_{\text{PSII}}$ (m$^{-1}$) or, to avoid confusion with old terminology for absorption cross sections, $a_{\text{PSII}}$ (m$^{-1}$).

**Photosynthetic rates: ‘Photosynthetic electron transport rate’, ‘PSII photochemical flux’**

ST-ChlF methods can be used to estimate photosynthetic activity by quantifying rates of charge separation in PSII (electron PSII$^{-1}$ s$^{-1}$). In the literature, this term is frequently referred to as the electron transport rate (ETR$_{\text{PSII}}$), and it represents the rate of electron transport from the donor side of PSII (water) to the acceptor side of PSII (QA).

Another term used in the literature to quantify photosynthetic rates is the PSII photochemical flux, J (photons m$^{-2}$ s$^{-1}$), which can be expressed per unit PSII (J$_{\text{PSII}}$, photon PSII$^{-1}$ s$^{-1}$) or expressed volumetrically (J$_{\text{VPSII}}$, photon PSII$^{-1}$ s$^{-1}$). Since every photon absorbed and used for
photochemistry will lead to one stable charge separation, $J_{\text{PSII}}$ can be directly converted to electron m$^{-3}$ s$^{-1}$.

**Perturbations in fluorescence state: Fluorescence transients and chlorophyll fluorescence induction (curve)**

ST-ChlF methods induce and interpret fluorescence ‘transients’, in which the recorded fluorescence yield, ChlF, is rapidly altered over time in response to excitation light that transiently shifts the balance between charge separation and QA reoxidation. The excitation power- and time-dependent changes in ChlF are related to underlying photo-physical responses, which are, in turn, conditioned by the growth environment experienced by the organisms investigated. In ST-ChlF approaches, strong excitation power is applied for a brief ($\sim$ 100-200 $\mu$s) period (saturation phase), which can be followed by a 100 – 200 ms period of decreasing excitation power (relaxation phase). The resulting ST-ChlF response is measured and interpreted as described in section 2.2.

**Excitation and actinic light**

To examine photosynthetic rates, ST-ChlF data are collected in the presence of a background ‘actinic’ light source, delivered within the PAR spectrum. Whereas excitation power is varied to induce short-term changes in ChlF, the actinic light source is applied constantly (over time scales of multiple seconds to minutes) to drive photochemistry. The high intensity ‘excitation light’ is provided at one, or several, narrow-waveband wavelengths (section 3.5), and used to rapidly induce fluorescence transients (section 2.2). In contrast, the background ‘actinic’ light source provides constant light at environmentally relevant intensities, and can be of a broader spectral distribution, mimicking the underwater light quality (section 3.5).
Table 2.1.1: Primary ST-ChlF parameters obtained from ST-ChlF transients and associated curve fits, as described in section 2.2. Note that more than one time constant for QA re-oxidation (τ) can be derived from the relaxation phase of ST-ChlF transients. Note also that the absolute values of $F_o$, $F_m$, $F'$, $F_{m'}$, $F_o'$ are wavelength-dependent (i.e. $F(\lambda_{ex}, \lambda_{em})$).

<table>
<thead>
<tr>
<th>Primary ChlF parameter</th>
<th>Parameter</th>
<th>Synonym(s)</th>
<th>Meaning</th>
<th>Derivation</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_o$</td>
<td>$F_0$</td>
<td>$F_{min}$</td>
<td>Minimum ChlF</td>
<td>Retrieved by processing ST-ChlF transients acquired under conditions of fully oxidized QA in the dark-regulated state.</td>
<td>Unitless, but can be expressed in standard-normalized units (section 3.1.4)</td>
</tr>
<tr>
<td>$F_m$</td>
<td>$F_{max}$</td>
<td>Maximum ChlF</td>
<td>Retrieved by processing ST-ChlF transient acquired under conditions of fully reduced QA in the dark-regulated state.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F'$</td>
<td>$F$</td>
<td>$F_t$</td>
<td>Steady-state ChlF</td>
<td>Similar to derivation of $F_o$, based on ST-ChlF transient acquired in the light-regulated state. (note that as the model fit parameter is $F_o$, many instruments report the biological parameter $F'$ as $F_o$)</td>
<td></td>
</tr>
<tr>
<td>$F_{m'}$</td>
<td></td>
<td>Maximum ChlF in the light-regulated state.</td>
<td>Similar to derivation of $F_m$, based on ST-ChlF transient acquired in the light-regulated state. (Note that as the fit parameter is $F_m$, many instruments report the biological parameter $F_{m'}$ as $F_m$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F_o'$</td>
<td></td>
<td>Minimum ST-ChlF in the light-regulated state.</td>
<td>Similar to derivation of $F_o$ based on ST-ChlF transient acquired in the light-regulated state after a brief (1-2 s) period of darkness to promote the oxidation of all QA. Alternatively estimated as: $F_o/F_o/F_m + F_o/F_m'$.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\sigma_{PSII}(\lambda)$</td>
<td>$\sigma_{PII}(\lambda)$</td>
<td>Absorption cross section of photochemistry in PSII or ‘functional’</td>
<td>Derived by fitting the ST-ChlF transient to a theoretical</td>
<td>$A^2$ PSII$^{-1}$ or $m^2$ PSII$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>( \sigma_{PSII}(\lambda) )</td>
<td>( \sigma_{PSII'}(\lambda) )</td>
<td>( \sigma_{PSII} ) in the light-regulated state.</td>
<td>Measured ( \sigma_{PSII} ) is wavelength-specific; e.g. ( \sigma_{PSII(445)} ). To express ( \sigma_{PSII} ) with absolute units, the energy (quanta m(^{-2})) of the excitation flash(lets) must be known. ( \sigma_{PSII} ) at a given waveband can be extrapolated to other wavebands if the ChlF excitation spectrum is known.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( p )</td>
<td>( \rho )</td>
<td>Probability of excitation transfer from a closed PSII reaction center to another PSII reaction center, open or closed(^1) (excitonic connectivity factor).</td>
<td>Derived by fitting the ST-ChlF transient to a model of the fluorescence vs photosynthesis relationship. Controls the sigmoidicity of the ST-ChlF rise during the saturation phase. Unitless, ranging from 0 to 1, but rarely exceeding the value 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( p' )</td>
<td>( \rho' )</td>
<td>( p ) in the light-regulated state.</td>
<td>Derived by fitting the ST-ChlF transient acquired in the light-regulated state. Unitless, with values usually lower compared to that observed in dark-regulated state</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau_n )</td>
<td>( \tau_{QA,n} )</td>
<td>Time constants QA(^{-}) reoxidation kinetics in the dark-regulated state.</td>
<td>Derived by fitting a multi-component exponential decay curve to the relaxation phase of ST ChlF transients. The numerical subscript indicates different components of the exponential decay model, corresponding to different relaxation processes occurring on different time-scales. ( \mu s, ms )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau_n' )</td>
<td>( \tau_{QA,n'} )</td>
<td>( \tau_n ) in the light-regulated state.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

\(^1\) Following original formulation by Joliot and Joliot, 1964.
Table 2.1.2: Variable ChlF values derived directly from primary ChlF parameters in table 2.1.1. See section 2.2 for a description of ST-ChlF transients and derived ChlF parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Synonym(s)</th>
<th>Physiological Meaning</th>
<th>Derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_v$</td>
<td></td>
<td>Variable ST-ChlF in the dark-regulated state.</td>
<td>$F_m - F_o$</td>
</tr>
<tr>
<td>$F_{q'}$</td>
<td>$\Delta F'$</td>
<td>Variable ST-ChlF in the light-regulated state.</td>
<td>$F_{m'} - F'$</td>
</tr>
<tr>
<td>$F_{v'}$</td>
<td></td>
<td>Maximum variable ST-ChlF in the light-regulated state.</td>
<td>$F_{m'} - F_{o'}$</td>
</tr>
</tbody>
</table>
Table 2.1.3: Quantum yields derived from primary ChlF in Table 2.1.1. Note that $\Phi_{NPQ} + \Phi_{NO} + \Phi_{PSII} = 1$. See section 2.2 for a description of ST-ChlF transients and derived ChlF parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Synonym(s)</th>
<th>Physiological Meaning</th>
<th>Derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Phi_{PSII}$</td>
<td>$F_v/F_m, \Phi_{PH}$</td>
<td>The maximum quantum yield of PSII photochemistry. The maximum fraction of light energy absorbed by PSII, which can be used for photochemistry under given environmental conditions. Note that a strict interpretation of this term as the maximum quantum yield of PSII photochemistry explicitly assumes all measured ChlF comes from pigments functionally associated with photochemically-active PSII (see Section 5.2.1).</td>
<td>$(F_m-F_o) / F_m$</td>
</tr>
<tr>
<td>$\Phi_{PSII}'$</td>
<td>$\Phi_{PH}'$</td>
<td>The realized quantum yield of photochemistry. The fraction of photons absorbed by PSII used for photochemistry under given background irradiance. Note that a strict interpretation of this term requires the same assumptions needed for $\Phi_{PSII}$ (see Section 5.2.1).</td>
<td>$(F_m'-F') / F_m' = F_q' / F_m'$</td>
</tr>
<tr>
<td>$\Phi_{NPQ}$</td>
<td>Y(NPQ)</td>
<td>Quantum yield of regulated energy dissipation processes (NPQ). (see section 3.4).</td>
<td>$F'/F_m' - F'/F_m$</td>
</tr>
<tr>
<td>$\Phi_{NO}$</td>
<td>Y(NO)</td>
<td>Quantum yield of non-regulated energy pathways through ChlF and heat dissipation.</td>
<td>$F'/F_m$</td>
</tr>
<tr>
<td>$\Phi_F$</td>
<td></td>
<td>Quantum yield of ChlF. Note that $\Phi_F$ is part of $\Phi_{NO}$ and that $\Phi_{NO} + \Phi_{NPQ} + \Phi_{PSII} = 1$</td>
<td></td>
</tr>
<tr>
<td>$\Phi_{PSII, reg}$</td>
<td></td>
<td>The quantum yield of photochemistry of open RCII in the light-regulated state. Quantifies the extent to which PSII photochemistry is limited by competition with thermal dissipation processes.</td>
<td>$F_v'/F_m' = (F_m'-F_o') / F_m'$</td>
</tr>
</tbody>
</table>
Table 2.1.4: Photochemical quenching coefficients calculated from the primary ChlF parameters in table 2.1.1. See section 2.2 for a description of ST-ChlF transients and derived primary ChlF parameters, and section 2.3 for secondary ChlF parameters. The coefficient of photochemical quenching quantifies the drop in $F'$ below $F_m'$ attributable to photochemistry, and under certain assumptions may therefore be interpreted as an estimate of the fraction of open RCII. By definition, this coefficient is 1 in the dark-regulated state, and decreases with increasing background light. Although the parameter $q_P$ has been applied most commonly in the literature and can be calculated most consistently, none of the quenching coefficients presented below provides a perfect quantification of the fraction of open RCII.

### Secondary ChlF parameter – quenching coefficients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Synonym(s)</th>
<th>Meaning</th>
<th>Derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$q_P$</td>
<td>(1-C)</td>
<td>photochemical quenching coefficient assuming no connectivity between PSII units (‘puddle’ model).</td>
<td>$F_q'/F_v'$ = $(F_m'-F')/(F_m'-F_o')$</td>
</tr>
<tr>
<td>$q_L$</td>
<td>(1-C)</td>
<td>photochemical quenching coefficient assuming full connectivity between PSII units (‘lake’ model).</td>
<td>$F_q'/F_v' \cdot F_o'/F' = \left[\frac{(F_m'-F')}{(F_m'-F_o')}\right] \cdot F_o'/F'$</td>
</tr>
<tr>
<td>$q_J$</td>
<td></td>
<td>photochemical quenching coefficient assuming an intermediate level of connectivity between PSII units.</td>
<td>$(F'-F_o')/(F_m'-F_o) + \rho \cdot (F' - F_o)$</td>
</tr>
</tbody>
</table>
Table 2.1.5: PSII photochemical flux or electron transport rates. See section 2.3 for a description of different approaches to estimate electron transport rates from primary ChlF parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Synonym(s)</th>
<th>Physiological Meaning</th>
<th>Derivation</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$ETR_{PSII}$</td>
<td>$J_{PSII}$, $J_{PII}$, $ETR_{RCII}$, $PSII_{ETR}$</td>
<td>PSII-normalized rates of charge separation</td>
<td></td>
<td>electrons PSII$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>$rETR_{PSII}$</td>
<td>$r_P$</td>
<td>Relative photosynthesis, or relative $ETR_{PSII}$. Photochemical flux through a population of PSII complexes with a combined absorption cross section for PSII photochemistry ($\sigma_{PSII}$) of 1 m$^2$.</td>
<td>see Section 2.3</td>
<td>$\mu$mol photons m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>$ETR_{VOL}$</td>
<td>$J_{VPSII}$, $J_{VPHI}$, $LET$, $PET$, $P_e$</td>
<td>Volumetric rates of photosynthesis at the level of PSII; a product of $ETR_{PSII}$ and the concentration of PSII units</td>
<td></td>
<td>electrons m$^{-3}$ s$^{-1}$</td>
</tr>
</tbody>
</table>
Table 2.1.6: Estimates of non-photochemical quenching (NPQ). See section 3.4 for a discussion on NPQ.

<table>
<thead>
<tr>
<th>Secondary ChlF parameter – NPQ</th>
<th>Parameter</th>
<th>Synonym(s)</th>
<th>Physiological Meaning</th>
<th>Derivation</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>NPQ</em>&lt;sub&gt;SV&lt;/sub&gt;</td>
<td><em>NPQ</em></td>
<td></td>
<td>Regulated (<em>i.e.</em> light-induced) energy dissipation quantified using the Stern – Volmer ratio of quenched ChlF to unquenched fluorescence. Algebraically, ( \text{NPQ}<em>{\text{NSV}} = \Phi</em>{\text{NPQ}} / \Phi_{\text{NO}}. )</td>
<td>( (F_m - F_m') / F_m' )</td>
<td>Dimensionless, unbound ratio</td>
</tr>
<tr>
<td><em>NPQ</em>&lt;sub&gt;NSV&lt;/sub&gt;</td>
<td><em>NSV</em></td>
<td></td>
<td>A measure of non-photochemical energy dissipation including regulated (<em>i.e.</em> light-induced) processes and increases in energy dissipation in the dark-regulated state (<em>e.g.</em> photoinhibition). Referred to as normalized Stern – Volmer quenching.</td>
<td>( F_0' / F_v' )</td>
<td>Dimensionless, unbound ratio</td>
</tr>
</tbody>
</table>
Table 2.1.7: Parameters used to describe light absorption properties of phytoplankton. Note that all parameters listed below are wavelength-dependent (as indicated by \((\lambda)\)). Additional detailed explanations are provided in section 2.1. Note that we consistently use ‘\(\sigma\)’ for absorption cross-sections and ‘\(a\)’ for absorption coefficients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Synonym(s)</th>
<th>Physiological Meaning</th>
<th>Derivation</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\sigma (\lambda))</td>
<td></td>
<td>Absorption cross-section</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\sigma_{\text{PSII}} (\lambda))</td>
<td>(\sigma_{\text{PII}} (\lambda))</td>
<td>Absorption cross-section for PSII photochemistry in the dark-regulated state. Also referred to as the ‘functional’ or ‘effective’ absorption cross-section of PSII.</td>
<td>Derived from initial ST-ChlF rise during the saturation phase of a ST-ChlF transient.</td>
<td>(\text{Å}^2\ \text{PSII}^{-1}) or (\text{m}^2\ \text{PSII}^{-1})</td>
</tr>
<tr>
<td>(\sigma_{\text{LHII}} (\lambda))</td>
<td>(a_{\text{PSII}} (\lambda))</td>
<td>Absorption cross-section for all antenna pigments functionally associated with PSII, also known as the ‘optical’ absorption cross section.</td>
<td>(\sigma_{\text{PSII}} / \Phi_{\text{PSII}} = \sigma_{\text{PSII}} / (F_v/F_m))</td>
<td>(\text{Å}^2\ \text{PSII}^{-1}) or (\text{m}^2\ \text{PSII}^{-1})</td>
</tr>
<tr>
<td>(a (\lambda))</td>
<td></td>
<td>Absorption coefficient</td>
<td></td>
<td>(\text{m}^{-1})</td>
</tr>
<tr>
<td>(a_w (\lambda))</td>
<td></td>
<td>Absorption coefficient of pure water</td>
<td></td>
<td>(\text{m}^{-1})</td>
</tr>
<tr>
<td>(a_{\text{CDOM}} (\lambda))</td>
<td></td>
<td>Absorption coefficient of chromophoric dissolved organic matter</td>
<td>Filtered water in a spectrometer</td>
<td>(\text{m}^{-1})</td>
</tr>
<tr>
<td>(a_P (\lambda))</td>
<td></td>
<td>Absorption coefficient of particles</td>
<td></td>
<td>(\text{m}^{-1})</td>
</tr>
<tr>
<td>(a_{\text{NAP}} (\lambda))</td>
<td></td>
<td>Absorption coefficient of non-algal particles</td>
<td>Filter-pad technique</td>
<td>(\text{m}^{-1})</td>
</tr>
<tr>
<td>(a_{\text{phy}} (\lambda))</td>
<td>(a_{\Phi} (\lambda))</td>
<td>Absorption coefficient of phytoplankton</td>
<td></td>
<td>(\text{m}^{-1})</td>
</tr>
<tr>
<td>(a_{\text{ppc}} (\lambda))</td>
<td></td>
<td>Absorption coefficient of photoprotective carotenoids</td>
<td>Spectral reconstruction from pigment concentration</td>
<td>(\text{m}^{-1})</td>
</tr>
<tr>
<td>(a_{\text{psp}} (\lambda))</td>
<td></td>
<td>Absorption coefficient of photosynthetic pigments</td>
<td></td>
<td>(\text{m}^{-1})</td>
</tr>
<tr>
<td>(a_{\text{LHII}} (\lambda))</td>
<td></td>
<td>Absorption coefficient of antenna pigments functionally associated with PSII. (K_a) represents an instrument-specific coefficient. See section 2.3.3 for details.</td>
<td>(\sigma_{\text{PSII}} \cdot F_m/F_v \cdot [\text{PSII}]) or (K_a \cdot (F_m \cdot F_o)/F_v)</td>
<td>(\text{m}^{-1})</td>
</tr>
</tbody>
</table>
Table 2.1.8: Fit parameters derived from ‘photosynthesis vs incident light’ (PE) curves. Traditionally, such curves have been measured using carbon uptake or oxygen evolution as proxies for the rate of photosynthesis under varying light levels. ST-ChIF approaches allow for high-resolution acquisition of PE curves, where the rate of photosynthesis is reported as ETR$_{psii}$. In the column of units, ‘P’ stands for ‘productivity’, e.g. ETR$_{vol}$, or volumetric CO$_2$ fixation / O$_2$ evolution per time. Note that both $\alpha$ and $E_k$ are spectrally-dependent (section 3.5). Different models to fit PE curves are discussed in section 2.5.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Meaning &amp; Derivation</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha(\lambda)$</td>
<td>The initial slope of the PE curve. The photosynthetic light-use efficiency before light saturation, i.e. the increase of photosynthetic rate per increase in incident light under conditions where photosynthesis is light-limited.</td>
<td>P (µmol photon m$^{-2}$ s$^{-1}$)$^{-1}$</td>
</tr>
<tr>
<td>$P_{max}$</td>
<td>The maximum rate of photosynthesis achieved under light saturation.</td>
<td>P</td>
</tr>
<tr>
<td>$E_k(\lambda)$</td>
<td>The light saturation parameter. The incident light intensity at which photosynthesis begins to be light saturated. Calculated as $\alpha/P_{max}$</td>
<td>µmol photon m$^{-2}$ s$^{-1}$</td>
</tr>
</tbody>
</table>
2.2 Deriving primary parameters from ST-ChlF transients

As a first step in determining photosynthetic rates from ST-ChlF measurements, non-linear biophysical models (e.g. Kolber et al. 1998) are fit to fluorescence transients to retrieve a number of primary ChlF parameters (Table 2.1.1). Here, we briefly consider the conceptual basis of these model fits, with specific details of practical implementation for different instruments outlined in appendices A, B and C. Section 3.2 discusses operational considerations for the generation of robust ST-ChlF transients.

Irrespective of the particular instrument used, several common principles apply to the generation and analysis of ST-ChlF data. ST-ChlF induction comprises a ‘saturation phase’, where the fluorescence yield increases from a minimum (~ $F_o$) to a maximum (~ $F_m$), as the primary electron acceptor, $Q_A$, becomes progressively reduced. Upon reduction, $Q_A$ cannot accept further electrons, and becomes functionally ‘closed’ to incoming excitation until electrons are moved through the downstream electron transport chain. The subsequent ‘relaxation phase’ reflects the return of fluorescence to an initial level, as $Q_A$ is progressively re-oxidised and PSII reaction centres ‘re-open’ (Fig. 2.2.1). In practice, the true value of $F_o$ and $F_m$ can be closely approximated, but not fully realized, due to a number of background photo-physiological processes (Schuback et al. 2021).

![Hypothetical example of a ST ChlF transient showing the primary photosynthetic parameters derived from the analysis of a sample measured under dark-regulated conditions. See the text and table 2.1.1 for an explanation of the terms. Note that true $F_o$ and $F_m$ values are not directly measured, but rather derived from model fits.](image)

The excitation protocols most commonly used to induce ChlF transients during the saturation phase are based on a sequence of short, high-frequency, sub-saturating flashlets (e.g. FRRf technique, Appendix A) or a single, high power flash (e.g. FIRe, LabSTAF, PSI; Appendix B, C). The shape of the ChlF transient during the saturation phase is governed by the photon flux density (excitation power), the absorption cross section of photochemistry in PSII ($\sigma_{PSII}$), the extent of connectivity between PSII units ($p$) and the concurrent rates of $Q_A$ reoxidation. Accounting for connectivity requires some assumption regarding pigment organization. Some models apply
relatively simple ‘lake’ models with extended connectivity between PSII reaction centers (Appendix A), while others assume dimerization of PSII centers (Appendix B). Both these models can account for varying levels of connectivity.

During the relaxation phase of a ST-ChlF transient, excitation power is decreased, or removed, shifting the balance from charge separation towards QA reoxidation. This enables the kinetics of QA reoxidation by downstream electron transfer (QA-Qb-PQ, etc.) to be monitored as a change in ChlF yields. Some measurement approaches require an excitation sequence to capture the kinetics of fluorescence decay, and these applied flashlets lead to partial QA reduction, which must be accounted for in the biophysical model (see appendix A, B and C). Different protocols used to measure the relaxation phase are described in section 3.2.2. Regardless of the exact measurement protocol, the relaxation phase serves to asses QA reoxidation as multi-exponential decay in ChlF parameterized with time constants $\tau_1, \tau_2, \tau_3$ etc. These time constants are determined by the fastest to slowest electron transport steps downstream of QA. Users may need to define the number of relaxation time constants used in the model fit, requiring inherent assumptions about the underlying processes captured by the respective parameters.

In summary, all ST-ChlF approaches are designed to transiently modify the redox state of QA to extract information about the dynamics of photosynthetic electron flow in and out of PSII. Various mathematical formulations are then used to estimate the primary parameters determining rates of QA reduction and oxidation. In order to robustly interpret the resulting data, it is important to report the protocols used to generate the ST-ChlF transient (see section 3.2), and the specific model fitting routines and their associated assumptions. This reporting requirement will become more important as models become increasingly sophisticated in their efforts to capture additional biophysical processes (e.g. processes regulating the excited state of PSII-associated Chl a, such as carotenoid and p680+ quenching).

A common (open source) software platform would greatly facilitate inter-comparison among research groups, enabling different models to be fit to a range of independent data sets. This would eliminate sources of variability associated with different numerical fitting approaches, allowing the community to compare results across instruments and users. A common data analysis platform would also support sensitivity studies examining the effects of different biophysical models on the resulting parameter derivations. The Python-based package, Phytotools, provides a recent example of a platform-independent software (Ryan-Koegh and Robinson, 2021), building on the previous package PSIWORX (https://sourceforge.net/projects/psiworx/). SCOR WG 156 is working towards the development of updated and expanded versions of such open software tools.

### 2.3 Secondary ChlF parameter and estimates of electron transport rates (ETRPSII).

The primary parameters derived from ST-ChlF transients (section 2.2) are used to calculate secondary ChlF parameters of physiological interest (see tables in section 2.1). These secondary properties can then be integrated into equations quantifying photosynthesis as the rate of initial charge separation in PSII reaction centers (referred to as PSII photochemical flux, $J_{PSII}$, or electron transport rate, ETRPSII). Various approaches have been developed for these calculations, including the ‘sigma algorithm’ (Kolber and Falkowski 1992; Suggett et al. 2010), the ‘absorption algorithm’ (Oxborough et al. 2012; Boatman et al. 2019), and the ‘kinetic’ approach (Gorbunov and
Falkowski 2020). All of these approaches rely on accurate retrieval and interpretation of primary ChlF parameters from ST-ChlF transients, and they all have advantages and limitations. In this section, we briefly introduce the secondary ChlF parameters, which are listed in section 2.1. We then briefly explain the three most common approaches of calculating ETR$_{PSII}$, and discuss their relative advantages and limitations, particularly in the context of field measurements in aquatic systems. The relationship between ETR$_{PSII}$ and other PP proxies (e.g., O$_2$ evolution and CO$_2$ uptake) is addressed in section 5.1.

2.3.1 Secondary ChlF parameters

Tables 2.1.2 – 2.1.5 list the secondary ChlF parameters that are commonly derived from ST-ChlF measurements. Importantly, the interpretation of these parameters as photo-physiological properties in ETR$_{PSII}$ calculations does not necessarily hold under all conditions. For example, the interpretation of F$_{v}$/F$_{m}$ as the maximum quantum yield of PSII photochemistry assumes that measured ChlF is derived exclusively from Chl a functionally associated with a RCII. However, certain conditions (most notably iron limitation) can lead to the formation of energetically-decoupled pigment (Chl a or phycobilin) molecules, which contribute to background ChlF but not photochemistry, thus decreasing measured F$_{v}$/F$_{m}$ (see also section 3.3 and 5.2.2). Moreover, the use of q$_{P}$ = F$_{q}'$/F$_{v}'$ as a measure of the fraction of open RCII assumes no connectivity between photosynthetic units, which may not always hold in practice. Finally, estimates of the absorption-cross section of photochemistry in PSII ($\sigma_{PSII}$) may be problematic in mixed phytoplankton assemblages, since ‘bulk’ $\sigma_{PSII}$ measurements do not necessarily reflect the weighted average of individual $\sigma_{PSII}$ values for each species present (Suggett et al. 2004; Laney 2010). With these caveats in mind, we briefly introduce three approaches to estimating ETR$_{PSII}$, providing references to more detailed descriptions for readers wishing to delve more deeply. After introducing the three approaches, we discuss their respective advantages and limitations, with a focus on field-based applications of ST-ChlF for primary productivity estimates.

2.3.2 The sigma algorithm

Over the past three decades, most studies quantifying phytoplankton ETR$_{PSII}$ from ST-ChlF measurements have utilized versions of the so-called ‘sigma algorithm’. This approach and its derivation have been discussed in detail by a number of authors (e.g. Gorbunov et al. 2001, Suggett et al. 2010). In the simplest terms, the sigma algorithm estimates ETR$_{PSII}$ per PSII reaction center (ETR$_{PSII}$, photons PSII$^{-1}$ s$^{-1}$ or electrons PSII$^{-1}$ s$^{-1}$) from the product of photon flux ($E$, $\mu$mol photons m$^{-2}$ s$^{-1}$), the fraction of light absorbed by a PSII unit ($\sigma_{LHII}$, m$^{2}$ PSII$^{-1}$) and the quantum yield of photochemistry in PSII ($\Phi_{PSII}'$).

$$ETR_{PSII} = E \cdot \sigma_{LHII} \cdot \Phi_{PSII}' = E \cdot \sigma_{PSII}/(F_{v}/F_{m}) \cdot F_{q}'/F_{m}'$$  \hspace{1cm} (Equation 2.3.1)

The term $\sigma_{LHII}$ reflects the absorption cross-section of PSII light harvesting complexes, and is also often referred to as the optical absorption cross-section of PSII and abbreviated as $\sigma_{PSII}^{opt}$ or $\alpha_{PSII}$ (see section 2.1). The term $\Phi_{PSII}'$ reflects the portion of absorbed photons that lead to a charge separation in PSII.
Alternatively, the sigma algorithm can be written as

$$ETR_{PSII} = E \cdot \sigma_{PSII}' \cdot q_P = E \cdot \sigma_{PSII}' \cdot F_q'/F_v'$$  \hspace{1cm} (Equation 2.3.2)

In equation 2.3.2, $\sigma_{PSII}'$ represents the absorption cross section of photochemistry at each open PSII, and $q_P$ provides an estimate of the fraction of RCII in the open state.

The two equations are equivalent under the assumption that light-induced changes in the absorption cross section for PSII photochemistry occur to the same extent as light-induced changes in the quantum yield of photochemistry at open RCII (Equation 2.3.3).

$$\frac{\sigma_{PSII}'}{\sigma_{PSII}} = \frac{F_v'/F_m'}{F_v/F_m}$$  \hspace{1cm} (Equation 2.3.3)

Note that sample heterogeneity in terms of $\sigma_{PSII}$ can invalidate this assumption (Section 2.4).

From equation 2.3.3, it follows that the realized quantum yield of photochemistry ($\Phi_{PSII}' = F_q'/F_m'$) is the product of the quantum yield of photochemistry at open RCII ($F_v'/F_m'$) multiplied by the fraction of RCII in the open state ($q_P = F_q/F_v'$; Equation 2.3.4).

$$F_q'/F_m' = F_v'/F_m' \cdot F_q'/F_v'$$  \hspace{1cm} (Equation 2.3.4)

### 2.3.3 The absorption algorithm

The absorption algorithm can provide estimates of $ETR_{PSII}$ per volume rather than per PSII unit, avoiding the need to estimate volumetric PSII concentrations. This algorithm avoids the need for $\sigma_{PSII}$ measurements, which can be ambiguous in mixed phytoplankton communities, as noted above and explained further in section 2.4. To quantify light absorption, the algorithm relies on the parameter $a_{LHII}$ ($m^{-1}$), which represents the absorption coefficient of PSII light harvesting in a volume of water. This parameter is the product of the concentration of PSII in a volume of water and the absorption cross section of light harvesting pigments functionally associated with RCII:

$$a_{LHII} = \sigma_{LHII} \cdot [PSII] = \frac{\sigma_{PH}}{F_v/F_m} \cdot [PSII]$$  \hspace{1cm} (Equation 2.3.5)

Substituting $a_{LHII}$ for $\sigma_{LHII}$ in equation 2.3.1 thus provides $ETR_{VOL}$ with units of photons $m^{-3}$ s$^{-1}$ (or electrons $m^{-3}$ s$^{-1}$).

$$ETR_{VOL} = E \cdot a_{LHII} \cdot \Phi_{PH}'$$  \hspace{1cm} (Equation 2.3.6)

An approach to directly estimate [PSII] from ST-ChlF measurements was introduced by Oxborough et al. (2012) and refined by Boatman et al. (2019).

$$[PSII] = K_a \cdot \frac{F_o}{\sigma_{PH}}$$  \hspace{1cm} (Equation 2.3.7)

The parameter $K_a$ is an instrument-specific constant, with units of $m^{-1}$ (Oxborough et al. 2012; Boatman et al. 2019; Oxborough 2022).

Incorporation of equation 2.3.7 into 2.3.5 thus yields
\[ a_{LHI} = \frac{\sigma_{\text{PSII}}}{F_0/F_m} \cdot K_a \cdot \frac{F_0}{\sigma_{\text{PSII}}} = K_a \cdot \frac{F_0 - F_m}{F_0} \]  
(Equation 2.3.7)

such that ETR_{PSII} can be estimated independently of \( \sigma_{\text{PSII}} \) as

\[ ETR = E \cdot a_{LHI} \cdot \Phi_{P\text{H}'} = E \cdot K_a \cdot \frac{F_0 - F_m}{F_0} \cdot \frac{F_q'}{F_m'} \]  
(Equation 2.3.8)

### 2.3.4 The kinetic approach

Recent work has provided an approach to quantify ETR_{PSII} based on estimates of the time of Q\text{A re-oxidation (}\tau_1), derived from the relaxation phase of a ST-ChlF transient (sections 2.2, 2.3, and 3.2.2; Gorbunov and Falkowski 2020). In practice, \( \tau_1 \) is derived from fitting a multiple-component exponential decay function to the relaxation phase of a ST-ChlF transient. As explained in detail by Gorbunov and Falkowski (2020), the derived value of \( 1/\tau_1 \) under saturating background light represents the maximum rate of photosynthetic unit turnover (1/\( \tau_1 \), s\(^{-1}\)), which sets an upper limit on ETR_{PSII}. Use of the kinetic approach requires quantification of the light-dependence of the PSII quantum yield of photochemistry (\( F_q'/F_m' \)):

\[ ETR_{PSII} = \frac{1}{\tau} \cdot \left( E \cdot \frac{F_q'}{F_m'}(E) / (E_{\text{max}} \cdot \frac{F_q'}{F_m'}(E_{\text{max}})) \right) \]  
(Equation 2.3.9)

Here, \( E \cdot F_q'/F_m' (= r\text{ETR}_{PSII}) \) at a given sub-saturating irradiance is normalized to its maximum value (\( E_{\text{max}} F_q'/F_m'(E_{\text{max}}) \)) recorded at saturating irradiance (\( E_{\text{max}} \)). The value of \( E_{\text{max}} \) is typically assumed to be achieved at a light level 3-fold higher than \( E_k \). Multiplication of \( r\text{ETR}_{PSII} \) by the maximum photosynthetic turnover rate (1/\( \tau \)) measured under saturating light provides values of ETR_{PSII}. Operationally, the approach does not require estimation of \( \sigma_{\text{PSII}} \), but it does rely on measurement of a rapid light response curve (RLC), which can be conducted over several minutes.

### 2.4 Recommendations on the use of different approaches to estimate ETR_{PSII}

For new users of ST-ChlF instruments, the different approaches to estimate ETR_{PSII} and their associated literature can appear rather confusing. As a SCOR working group, we do not recommend a particular approach or instrument type. Rather, we recognize that each approach has different strengths and caveats, which must be weighed against project-specific research objectives and operational constraints. Over time, inter-comparison of ST-ChlF data across instruments and experimental protocols will yield insight into differences between the various ETR_{PSII} algorithms (Sezginer et al., 2021), and their coherence with other primary productivity metrics. Such comparative studies will also provide insights into the physiological and taxonomic controls on different ST-ChlF parameters. Below, we outline the most important factors that should be considered when selecting one or more approaches to calculate ETR_{PSII} from ST-ChlF measurements.

**Baseline fluorescence**

As discussed in section 3.3, ‘baseline’ fluorescence emanating from pigments that are not functionally associated with PSII can affect the primary ChlF parameters \( F_0, F_o', F', F_m, \) and \( F_m' \), and the subsequent interpretation of derived secondary parameters such as \( q_P \) and \( \Phi_{PSII} \) (section 2.1). Baseline fluorescence can be high under conditions of iron limitation (section 5.2.2), and can
vary as a function of environmental conditions, diel cycles and taxonomic composition (Schuback et al. 2021). Approaches to correct for baseline fluorescence exist (Boatman et al. 2019), but these require further validation for mixed phytoplankton assemblages under a range of environmental conditions. All the ETRPSII algorithms outlined above are affected by baseline fluorescence, with the Sigma algorithm (equation 2.3.2) being the least sensitive, since baseline fluorescence does not impact $\sigma_{\text{PSII}}$, and has a minimal effect on $F_q'/F_v'$.

**NPQ relaxation**

The Sigma and Absorption ETRPSII algorithms require ST-ChlF measurements in the dark-regulated state, *i.e.* after relaxation of any NPQ processes (see section 3.4). Achieving a fully dark-regulated state in mixed phytoplankton assemblages is challenging, as the optimal time and treatment for relaxation will vary for different phytoplankton species and environmental conditions. Among the different ETRPSII derivations, the Kinetic algorithm is the least influenced by the effects of NPQ relaxation (equation 2.3.9, and 2.3.2, when $F_o'$ is measured rather than derived).

**Signal to noise (low biomass)**

While the sensitivity of new generation ST-ChlF instruments allows for data acquisition in even the most oligotrophic regions, accurate retrieval of the primary ST-ChlF parameters from ST-ChlF transients can require significant data averaging (section 2.6). In particular, accurate retrieval of the primary light-regulated ChlF parameters $\sigma_{\text{PSII}}'$ and $\tau_{\text{QA}}'$ can be challenging, as the amplitude of ST-ChlF transients are decreased by quenching processes and PSII closure in the presence of background irradiance. The ETRPSII algorithms most affected by these issues are the Sigma (equation 2.3.2) and Kinetic (2.3.9) approaches, as these rely most heavily on accurate derivation of $\sigma_{\text{PSII}}'$ and $\tau_{\text{QA}}'$.

**Heterogeneity**

The Sigma and Kinetic approaches are both sensitive to heterogeneity within the sample. Heterogeneity of PSII complexes will always be present in mixed natural phytoplankton assemblages, but also occurs within single species cultures. Sensitivity of derived ChlF parameters to heterogeneity is particularly evident under nutrient illumination. The signal used to derive $\sigma_{\text{PSII}}'$, is generated by PSII complexes that remain in the open state at the point of measurement. As complexes with higher $\sigma_{\text{PSII}}$ are more likely to be closed (all other things being equal), they are less likely to contribute to the measured value of $\sigma_{\text{PSII}}'$. Connectivity among PSII complexes can also generate heterogeneity. As a proportion of PSII complexes closes, the $\sigma_{\text{PSII}}$ of the remaining open complexes (which contribute to the measured value of $\sigma_{\text{PSII}}$) is potentially increased.

**Pigment packaging**

An important assumption of the absorption algorithm is that the ratio of light energy used for PSII photochemistry *vs.* that emitted as fluorescence stays within a narrow range (Oxborough et al. 2012). However, this ratio can be significantly affected by the so-called ‘pigment package’ effect, which results from the self-shading of Chla molecules tightly packed inside phytoplankton cells (Bricaud et al. 1995). As the red absorption peak of Chla partly overlaps with the emission peak of fluorescence, re-absorption of fluorescence in large or highly pigmented cells can lead to a reduction of the detected fluorescence. Boatman et al. (2019) first introduced an approach to correct for pigment packaging based on detection of fluorescence emission at two wavebands. This
dual waveband correction uses the ratio of fluorescence detected at 730 nm (low re-absorption) and 682 nm (high re-absorption) as a proxy for pigment packaging, thus improving ETR$_{PSII}$ estimates from the Absorption algorithm.

2.5 Light-dependence of ETR$_{PSII}$: Protocols, curve fits and derived fit parameters

The Photosynthesis-Irradiance (PE) response curve is fundamental to parameterizing light-dependent photosynthetic performance (e.g. Sakshaug et al. 1997, Bouman et al. 2018), and empirically predicting environmental controls on PP (e.g. Behrenfeld & Falkowski 1997). Traditionally, the light-response function of aquatic primary producers has been characterized in terms of carbon fixation or O$_2$ evolution. However, ST-ChlF measurements also hold significant potential in this respect, by retrieving rapid and non-invasive information on the light-dependence of ChlF parameters, including ETR$_{PSII}$. To fully realize this potential, some attention is required to the protocols used to generate ETR$_{PSII}$ vs E curves, and also to the statistical methods used to extract meaningful light-dependent parameters from the resulting data. Both of these topics are addressed below.

2.5.1 Statistical considerations of fitting PE curves from ST-ChlF data

Early efforts to model the light-dependence of ChlF parameters focused on comparing light-limited and light-saturated electron transport rates ($\alpha$, ETR$_{PSII}^{MAX}$) with parallel light-dependent O$_2$ evolution and carbon fixation parameters. However, as noted by Silsbe & Kromkamp (2012), the PE approach is statistically problematic for ETR$_{PSII}$ since the response variable (ETR$_{PSII}$) is explicitly dependent on the predictor variable (E). Furthermore, the signal-to-noise ratio for ChlF parameters generally decreases with increasing light levels (the opposite of what happens for O$_2$ evolution and carbon fixation), such that models must be able to resolve the ETR$_{PSII}$ response curve under high light in the presence of potentially significant data noise (Boatman et al. 2019). To overcome these challenges, ChlF light-response parameterisation is more robustly determined through non-linear models describing the light-dependence of derived ChlF parameters, such as $F'_q/F'_m'$. A list of these models is given in Table 2.5.1 below, which is reproduced from Silsbe & Kromkamp (2012).
Table 2.5.1. Equations that can be used to describe the irradiance (E) response of ETR_{PSII} (left column) and Fq'/Fm' (right column). The term $\alpha$ describes the initial light-dependent slope of a particular photosynthetic parameter under light-limiting conditions, while $E_k$ represents the ‘light-saturation’ parameter, when photosynthetic rates transition from light-limitation to light-saturation. The maximum rate of photosynthesis, $P_M$ is equivalent to the maximum electron transport rate (ETR_{PSII}^{MAX}), where P (photosynthesis) is in the currency of electrons. $E_k$ can be derived as $P_M/\alpha$. The term $P_S$ represents the maximum rate of photosynthesis that would be observed in the absence of photo inhibition ($\beta$), while $E_{OPT}$ is the optimum light intensity. References are 1. Webb et al. 1974; 2. Jassby & Platt 1976; 3. Platt et al. 1980; 4. Eilers & Peters (1988).

<table>
<thead>
<tr>
<th>Ref</th>
<th>Functions to derive ETR_{PSII}</th>
<th>Functions to derive Fq'/Fm'</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$\alpha \cdot E_k \cdot [1-\exp(-E \cdot E_k)]$</td>
<td>$\alpha \cdot E_k \cdot [1-\exp(-E \cdot E_k)] \cdot E^{-1}$</td>
</tr>
<tr>
<td>2</td>
<td>$\alpha \cdot E_k \cdot \tanh(E \cdot E_k^{-1})$</td>
<td>$\alpha \cdot E_k \cdot \tanh(E \cdot E_k^{-1}) \cdot E^{-1}$</td>
</tr>
<tr>
<td>3</td>
<td>$P_S \cdot [1-\exp(-\alpha \cdot E \cdot P_S^{-1}) \cdot \exp(-\beta \cdot E \cdot P_S^{-1})]$ Where $P_M = P_S(\alpha/\alpha + \beta) \cdot (\beta/\alpha + \beta)^{\beta/\alpha}$</td>
<td>$P_S \cdot [1-\exp(-\alpha \cdot E \cdot P_S^{-1}) \cdot \exp(-\beta \cdot E \cdot P_S^{-1})] \cdot E^{-1}$</td>
</tr>
<tr>
<td>4</td>
<td>$E \cdot (a \cdot E^2 + b \cdot E + C)^{-1}$ Where $a = (\alpha \cdot E_{OPT}^2)^{-1}$, $b = P_M^{-1} - 2 \cdot (\alpha \cdot E_{OPT})^{-1}$, and $c = \alpha^{-1}$</td>
<td>$(a \cdot E^2 + b \cdot E + c)^{-1}$</td>
</tr>
</tbody>
</table>

To address the issue of increasing ChlF data variability under high light, Boatman et al. (2019) suggested an alternative curve fitting approach. As illustrated in Table 2.5.2, these authors proposed a modified version of the Webb model (equation 1 in Table 2.5.1) for light-dependent Fq'/Fm' curves, using a two-step weighting function to ensure that both low and high light data are well resolved.
Table 2.5.2: Modified PE equation fits, using a two-step weighting function to resolve both low and high light portions of the data. Equations taken from Boatman et al. (2019).

<table>
<thead>
<tr>
<th>Step</th>
<th>Equation</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$F_{q'}/F_{m'} = \alpha \cdot E_K \cdot [1-\exp(-E \cdot E_K)] \cdot E^{-1}$</td>
<td>The initial points (low actinic E values) are weighted by multiplying each square of the difference by $(F_{q'}/F_{m'})$. This generates a good fit up to $E_K$, but overshoots beyond this point. As such, derived $P_{MAX}$ values are generally too high. This weighting process minimizes the sum of squares of the difference between observed and fit values.</td>
</tr>
<tr>
<td>2</td>
<td>$F_{q'}/F_{m'} = (\alpha \cdot E_K \cdot [1-\exp(-E \cdot E_K^{-1})]) - \beta \cdot E_{K\beta} \cdot [1-\exp((E-E_K)/E_{K\beta})] \cdot E^{-1}$</td>
<td>To improve the value of $P_{MAX}$, a second exponential is only applied to data points at or above the $E_K$ value generated by step 1. The sum of squares of the difference between observed and fit values is not weighted.</td>
</tr>
</tbody>
</table>

Moving beyond statistical curve fitting approaches, mechanistic models have also been used to describe the light-dependent dynamics of photosynthetic electron transport (e.g. Ye et al. 2013, Kolber & Falkowski 1993, Gorbunov & Falkowski 2020). Early models were based on principles of biochemical reactions, describing the absorption and processing of excitation energy (Ye et al. 2013) in a manner equivalent to the maximum rates of RuBP carboxylase/oxygenase activity governing CO$_2$ uptake. These approaches are inevitably more computationally demanding than statistical curve fits, and they still rely on robustly retrieving a range of ST-ChlF parameters for model input. Yet, the models have the capacity to provide more mechanistic insight into the factors governing light-dependent photosynthetic processes.

### 2.5.2 Experimental considerations for the collection of PE ST-ChlF data

In carbon and oxygen-based PE curves, it is typical for parallel samples to be incubated simultaneously under a range of light intensities – for example in a photosynthetron. In contrast, ST-ChlF data are normally obtained using a single instrument, such that light-dependent measurements must be made sequentially on individual samples. This leads to the important question of which ST-ChlF protocols should be employed to capture inherent light-response features. Most commonly, measurements are made using ‘sequential light-response curves’, which employ systematic increases in E to drive changes in ChlF parameters. These sequential light-curves leave the user with some important operational choices, including: 1) the number of light steps, 2) the duration of each step, and 3) the order of light exposure (low to high vs. high to low).
There is general agreement that sequential light-response curves should be run from low to high light, as the reverse order could create challenges associated with prolonged quenching ‘carry over’ from the initial high light exposure treatment. Light steps that are too short in duration do not allow samples to reach ‘steady state’ ChlF parameters, as dissipation pathways re-equilibrate to each new light intensity \( (e.g. \text{Suggett et al. } 2003, 2015) \). In cases where cells are not able to reach appropriate short-term light-regulated states, they may be prone to photo-inhibition. In contrast, where light steps are too long, acclimation processes will become increasingly important, including changes in pigment transfer efficiencies. Either scenario can significantly alter the shape of the PE response function (and hence associated parameterisation of \( \alpha, P_{\text{MAX}}, \text{etc.} \)). How these processes manifest may also be dependent upon the interval between light steps, \( i.e. \) larger light step intervals may induce longer re-equilibrations times.

To date, many light-response protocols have employed a constant, but arbitrary, step duration \( (e.g. 100s, \text{Aardema et al. } 2019) \), whereas others have employed a time step interval that is demonstrated to be long enough to reach steady state based on examination of retrieved ST-ChlF parameters \( (e.g. \text{Suggett et al. } 2003) \). The ‘steady-state’ approach will typically increase the time needed for each light curve (thus reducing measurement frequency), but this can be optimised by analysing data to identify the time-scales needed to achieve steady-state at different light intensities \( (\text{Suggett et al. } 2003) \). Moving forward, a more elegant approach would be to adjust the PE time-steps in real time, based on an assessment of variability in sequentially-measured ChlF parameters at any given light step. Such an approach requires instrument software that is able to analyse data and adjust measurement protocols in a real-time feedback loop (see Appendix D for an example of such an approach).

Another important consideration in PE analysis is the low light acclimation of samples \( (\text{see section 3.4}) \) prior to measurements. Low-light (recommended over fully dark) exposure is meant to ensure that all quenching pathways are ‘relaxed’ \( (\text{see section 3.4.3}) \). Alternatively, users may prefer to run light-curves with no pre-acclimation time, in order to capture ‘instantaneous’ light curve characteristics that reflect the short-term light history of samples. Finally, users may wish to incorporate brief (on the order of seconds) dark intervals between each light step as a means of estimating minimal fluorescence in the light-acclimated state \( (F_o'\text{ – section 2.1}) \). This measurement is needed to estimate non-photochemical quenching \( (\text{NPQ}_s, \text{section 3.4}) \) and examine more complex quenching characteristics that influence the light-response parameterisation \( (\text{Aardema et al. } 2019) \).

No single PE protocol will be optimal under all situations, and the choice of a particular approach should be informed by the goals of the study. Irrespective of the methods chosen, it is critical that users specify their particular PE protocols as meta-data \( (\text{section 4.2}) \). Importantly, few studies have inter-compared PE approaches to quantify how different light-response protocols impact the resulting parameterisations. Until more data are available to better inform ST-ChlF light-response protocols, we recommend that users, at a minimum, report the light-response protocol, including the number of steps, duration of each step, dark intervals and any acclimation times, and the light-response model fitting routine, including the numerical fitting procedures and constraints used for parameter retrieval.
2.6 Precision, accuracy, and error quantification in ST-ChlF data analysis

As discussed in previous sections, primary and secondary photo-physiological properties are derived by numerically fitting ST-ChlF transients to theoretical models of fluorescence-photosynthesis relationships. Having introduced a range of these models and key elements of the model fitting procedures, here we identify sources of errors and uncertainties in ST-ChlF analysis, and discuss several metrics that can be used to quantify the statistical quality of retrieved data. These topics are addressed in the context of instrument sensitivity and operating conditions that exacerbate errors, such as low phytoplankton biomass and high background irradiance. We introduce techniques for quantifying the noise content of ST-ChlF transients, and discuss the relationship between this noise and the statistical data quality of the retrieved parameters. We also discuss some best practices aimed at optimizing data quality, and describe how error analysis techniques can be used to assess the robustness of numerical fitting procedures. Finally, we emphasise the importance of reporting and archiving uncertainty estimates alongside primary ST-ChlF data.

As error quantification in ST-ChlF analysis is not trivial, and implemented differently in various instruments, we provide only a brief overview of methods here. Readers are referred to Appendix D for more instrument-specific details. While recognizing that some of the material presented below and in Appendix D may be of greater interest to advanced users, we believe that all users of the ST-ChlF technique should be aware of basic data quality considerations.

2.6.1. Signal-to-Noise Ratio in the raw FRRf Transients and error propagation

Data acquired from ST-ChlF transients are subject to instrument-specific background noise and photon-flux variability. Although the magnitude of instrument-specific errors is determined by hardware design and performance, their contribution to fitting errors can be minimised by the selection of appropriate operational parameters. In contrast, noise associated with the intrinsic photon-flux variability is controlled only by the intensity of the measured fluorescence signal, which is mostly determined by the sample biomass and by the efficiency of fluorescence light collection. Nevertheless, this type of measurement variability can also be controlled (within limits) by selection of the excitation power, as discussed below.

Error quantification in ST-ChlF data analysis requires a simple metric of raw data quality that captures all error sources described above. One such metric is the signal-to-noise ratio in the raw fluorescence transients, SNR\textsubscript{raw}, defined as a ratio of the effective signal amplitude \( \text{e.g. } F_v = F_m - F_o \) relative to the noise content in the fluorescence transient. The noise content can be derived by assessing point-to-point variations in the relatively stable plateau phase of the ST-ChlF transient – see Appendix D). The SNR\textsubscript{raw} quantifies the fundamental quality of raw data, which directly controls the quality of all ST-ChlF derived parameters. This property can be assessed in real-time prior to data fitting, enabling operating parameters to be adjusted to maximize its value. The measurement of SNR\textsubscript{raw} using an accepted fluorescence standard can also quantify instrument sensitivity.
Assessing the relationship between SNR$_{\text{raw}}$ and the statistical quality of derived parameters using analytical rules of error propagation is not trivial. As an alternative, empirical relationship between SNR$_{\text{raw}}$ and data quality (e.g. standard deviation, standard error, or SNR$_{\text{data}}$) can be established experimentally (Fig. 2.6.1.) using automated data acquisition and data processing (Appendix D.2). In this approach, data are collected from a single sample at varying levels of SNR$_{\text{raw}}$, controlled by sample dilution, changes in detector gain, and/or by varying number of transients averaged prior to data fitting. Repeated measurements (~100 acquisition at each SNR$_{\text{raw}}$ setting) yield estimates of both SNR$_{\text{data}}$ and SNR$_{\text{raw}}$ allowing their co-variability to be assessed. This relationship is likely to be instrument-specific, and it may vary between samples depending on the physiological properties of the organisms being studied. Nonetheless, this empirical approach can be used as a well-constrained predictor of the statistical quality of the retrieved parameters. As explained in appendix D, this analysis can be extended to include other error metrics (e.g. standard deviation, etc.).

![Figure 2.6.1](image)

**Figure 2.6.1.** Empirical relationship between statistical variability in raw fluorescence transients (SNR$_{\text{raw}}$) and retrieved ST-ChlF parameters (SNR$_{\text{data}}$) for several derived properties. Open circles represent SNR$_{\text{data}}$ assessed from 80 acquisitions at each SNR$_{\text{raw}}$ level controlled by changes in instrument gain (first three data points) and number of running averages (the last three data points), with lines depicting residual-weighted second order regressions of these data, forced to zero at plot origins. The underlying formalism, procedures, and experimental data are presented in Appendix D.

The relationship between SNR$_{\text{raw}}$ and SNR$_{\text{data}}$ (Fig. 2.6.1) can be used to: (1) guide the selection of experimental protocols toward SNR$_{\text{raw}}$ levels that satisfy an acceptable quality of ST-ChlF data; and (2) discriminate between the instrument-related vs. biological or environmental drivers of data variability. This analysis also provides important insight into the relative sensitivity of different
ST-ChlF parameters to underlying noise in the raw transients. For example, the statistical quality of the time constants of QA re-oxidation appears to be lower (i.e. lower SNR_{data}) than derived values of \( \sigma_{PSII} \) and \( F_v/F_m \) at comparable levels of SNR_{raw}. Indeed, the robustness of \( F_v/F_m \) and \( \sigma_{PSII} \) derivations under low biomass conditions was the main motivation for the original implementation (FRRf) of the ST-ChlF method.

2.6.2. Approaches to increasing SNR_{raw} and data quality

Given the well-defined relationship between SNR_{raw} and the statistical quality of retrieved ST-ChlF parameters, maximizing SNR_{raw} represents the best strategy for optimizing ChlF data quality. As mentioned earlier, the ultimate limits of noise performance are determined by the optical and electrical configuration of a particular instrument. Nevertheless, there are several factors under user control that determine actual performance within these limits. Here, we identify these factors and offer some advice for maximizing data quality. Importantly, the discussion below applies to any ST-ChlF instrument or technique.

The most direct factor controlling SNR_{raw} is the excitation power used to produce fluorescence transients. Increasing excitation power will proportionally increase the observed fluorescence signal, the corresponding SNR_{raw} and the subsequent quality of the retrieved data. However, an excessive excitation power will also lead to overly rapid saturation of the ST-ChlF signal, decreasing the ability to resolve the initial portion of the fluorescence transient (see section 3.2.1). The resulting errors in \( F_0 \) and \( \sigma_{PSII} \) estimates may propagate to other derived photosynthetic properties. Among these, estimates of the kinetics of QA re-oxidation are particularly sensitive to excessive excitation power. Nevertheless, the use of high excitation power may be justified under conditions of extremely low phytoplankton biomass, where the benefits of high quality \( F_v/F_m \) and \( \sigma_{PSII} \) outweigh potentially erroneous estimates of QA re-oxidation kinetics (\( \tau_{QA} \)). These trade-offs should be examined under well-defined laboratory conditions before field campaigns.

Instrument gain is another important factor to consider. Generally, an increase in the instrument gain does not change the relative noise content in fluorescence transients, as both the useful signal and the instrument noise are amplified. However, the resulting increase in the electrical signal decreases the relative contribution of errors related to analog-to-digital (A/D) conversion. Unfortunately, the efficacy of gain adjustments diminishes at very low fluorescence levels, when the noise associated with photon flux variability becomes dominant. These two effects are instrument-specific, and users are encouraged to experiment with gain adjustments at progressively decreasing biomass concentrations. To assist in these efforts, some instruments implement automatic gain control to operate at an optimal setting.

A final approach to increasing SNR_{raw} is based on averaging sequential ChlF transients prior to curve fitting. As an example of this approach, Fig.2.6.2 shows a sequence of ST-ChlF measurements at different levels of SNR_{raw} controlled by increasing the number of the averaged ST-ChlF transients. The averaging procedure reduces noise content of the final fluorescence transients roughly in proportion to square root of running averages, improving the quality of the of ST-ChlF data by a similar factor.
Figure 2.6.2. Fluorescence transients recorded with the centric diatom, *T. pseudonana* (0.34 μg L⁻¹ Chl a). (A) measurements based on single acquisition yielding an average SNR<sub>raw</sub> of 8.17; (B) measurements based on 10 running averages yielding an average SNR<sub>raw</sub> of 23.7); (C) measurements based on 100 running averages yielding an average SNR<sub>raw</sub> of 64.3. Both the noise and fit residuals, amplified by a factor of 5, are displayed along the horizontal line in the plot’s centers. The instantaneous SNR<sub>raw</sub> values are calculated and displayed in real-time prior to fitting (red ovals). The corresponding statistical quality of the retrieved data (SNR<sub>data</sub> and σ<sub>fractional</sub>), assessed automatically after completion of the acquisition sequence, is displayed in the inserts. The red color code indicates an unacceptable quality of the retrieved parameters (SNR<sub>data</sub> < 2), yellow indicates marginal quality (2 <= SNR<sub>data</sub> < 4), and green indicates acceptable quality (SNR<sub>data</sub> >= 4).

The use of running averages, although very effective in controlling data quality, may increase the sample acquisition time to unacceptable levels, thus limiting the frequency of data collection. Increasing sampling frequency to over once per second may alleviate this problem, but the cumulative effects of prolonged excitation may make the measured photo-physiological properties deviate from their native status. To minimize these effects, the running averages protocols should be performed with the least number of acquisitions that satisfy the required levels of SNR<sub>raw</sub>. Adaptively adjusting the number of running averages satisfies this requirement, while producing measurements with consistent statistical quality across a wide range of biomass conditions (Appendix D). This approach becomes particularly effective when performing rapid light curve (RLC) measurements, as data collected under low light have a higher SNR<sub>raw</sub> (high F<sub>v</sub>) than those collected under high light. Where available, users are encouraged to experiment with different modes of adaptive acquisition under well-defined laboratory conditions.

2.6.3 Quantifying adequacy errors in model fits and optimizing model parameter sets

Beyond quantifying errors in individual ST-ChlF parameters, it is also possible to examine the overall quality and the appropriateness of biophysical models used to fit the fluorescence transients. This is defined by the numerical quality of the fit (a measure of how closely the model fits reproduce the experimentally derived ST-ChlF transients), and by the resolving power of these fits (the ability to retrieve progressively complete sets of photo-physiological properties at varying levels of SNR<sub>raw</sub>).
The most common measures of the model fit quality are the mean-square error:

\[
\text{mse} = \frac{1}{n} \sum_{i=1}^{n} (\hat{f}_i - f_i)^2,
\]

and the variance-normalized Chi-square, \(\chi^2_v\):

\[
\chi^2_v = \frac{1}{(n-m+1)} \sum_{i=1}^{n} \frac{(f_i - \hat{f}_i)^2}{\sigma_i^2},
\]

where \(\hat{f}_i\) is the \(i^{th}\) sample in the fluorescence transient, \(f_i\) is the corresponding fit value, \(n\) is the length of the transient, \(m\) is the number of free parameters in the fitting model (model degree of freedom), and \(\sigma_i^2\) is the noise variance at the \(i^{th}\) sample of the transient.

Numerical fitting procedures can be driven by minimizing either the \(\text{mse}\) or \(\chi^2_v\). Both can be minimized to arbitrarily low levels by increasing the number of free parameters in the fitting model. This, however, comes with the risk of over-fitting the experimental data. The \(\chi^2_v\) measure, by converging to a threshold value of 1 at the limits of model resolving power, guards against this danger. This statistical metric is controlled by both the number of free parameters \textit{and} by the noise level in the fluorescence transients. A \(\chi^2_v\) level less than 1 indicates that the variance in fit residuals falls below the noise variance, signalling progressive fitting to the noise. When present, such over-fitting leads to large variations in the values of so-called ‘evaporating parameters’\(^2\), often observed in model-derived time constants of QA’ re-oxidation at low levels of SNR\(_{\text{raw}}\) (Fig. 2.6.2). On the other hand, \(\chi^2_v\) levels much higher than 1 indicate the inability of a model to adequately account for some features present in the fluorescence transients. Such a situation is usually encountered under conditions of high SNR\(_{\text{raw}}\) where the information content of the high-quality data exceeds the resolving power of the applied model. Users should strive to use models that produce \(\chi^2_v\) levels close to, but no smaller than 1, with the \textit{fewest number} of free parameters.

Use of the \(\chi^2_v\) metric requires quantifying the noise signal along the entire length of the fluorescence transient (Appendix D). This cost is partially accounted for when quantifying the SNR\(_{\text{raw}}\). If that is not possible, \(\text{mse}\) may be used as the measure of the fit quality, but without the benefits of quantifying the model adequacy in terms of under, or over-fitting.

\textbf{2.6.4. Reporting errors and uncertainties for data QC and archiving}

In reporting ST-ChlF data, it is important to include SNR\(_{\text{raw}}\) as well as the statistical quality of derived parameters. This will enable end-users of archived data to gauge the confidence with which particular interpretations can be made (\textit{e.g.}, judging the apparent significance of differences in

\(^2\) Parameters that cannot converge to their expected values at repetitive measurement and and/or data averaging. The retrieved value of such parameters is driven by the noise when the noise level in fitted data exceeds the model capability to consistently retrieve the parameter value.
2.6.5 Quantifying sensitivity of the ST-ChlF technique

The error analysis introduced here can be applied to quantify instrument sensitivity. By definition, sensitivity quantifies the ability of a particular instrument to extract a useful signal in the presence of background noise, irrespective of the noise and signal source. The SNR\text{raw}, when measured with a well-defined fluorescence standard, will objectively quantify this property. Fluorescent compounds with a stable, temperature-independent fluorescence yield and a short (~ single nano-second) fluorescence lifetime such as Rose Bengal, Nile Blue, Fluorescein, or Rhodamine B can serve as such a ‘gold standard’. These standards can also be used to verify and correct the instrument response function (see section 3.1.3), and to calibrate instrument gain (section 3.1.4). As the instrument sensitivity is determined to a large extent by the optical configuration of the instrument (particularly the size and the geometry of the sample measurement chamber), a water-soluble liquid-phase standard would be most desirable. SCOR WG 156 is working towards the production and distribution of such a SCOR-adopted fluorescence standard.
3. OPERA TION

3.1 Instrument Characterization, Calibration, and Standardization

As the number of different ST-ChlF instruments and protocols increases, it is critical to ensure that common standards and benchmarks are available to facilitate comparison of data across different research groups. In this respect, robust and traceable calibration of the intensity and spectral properties of excitation light sources and detectors is critical (e.g., Laney 2003, 2010). Unfortunately, such calibrations are not trivial, and are often seen as the responsibility of manufacturers rather than end-users. Going forward, it is desirable to increase instrument users’ awareness of the importance of calibration, and provide practical methods for periodic checks of instrument calibrations. User oversight of calibration procedures will enhance data robustness, and facilitate inter-comparability across instruments that may be equipped with light sources of varying spectral properties and intensities. Calibration files with information on the intensity and spectral distribution of light sources should be easily accessible to the end-user and reported as metadata, with changes documented over time. In this section, we outline calibration requirements, and the general approaches available to users, including the use of commercial fluorescent dyes and calibration standards. We also outline protocols to assess the intensity and spectral properties of light sources and detectors in ST-ChlF instruments. This latter information will be particularly useful for advanced users who wish to develop custom-built instruments with robust and traceable calibrations.

3.1.2. Control and Calibration of Excitation-Emission Properties

Induction of ST-ChlF transients requires high excitation power. To achieve this, high-power commercial LED sources are usually driven with current levels far above their nominal levels, albeit at low duty cycle. Although there is little evidence (thus far) that these operating conditions are detrimental to the long-term stability of the LED’s, the ability to periodically test and recalibrate excitation power by the user is paramount to ensure the reliability of ST-ChlF data. The photosynthetic property most affected by the exact calibration of excitation power is the functional absorption cross section of photochemistry in PSII, $\sigma_{PSII}$. This parameter is a critical measure of photosynthetic performance, and holds the potential to discriminate between different phytoplankton taxa based on spectral composition of their photosynthetic pigments (Gorbunov et al., 2020). The increasing availability of multi-spectral ST-ChlF instruments further highlights the need for proper calibration of excitation power sources for accurate determination of spectrally-resolved $\sigma_{PSII}$ values. Well-calibrated excitation power is also critical to accurately assess the kinetics of QA$^-$ reoxidation.

The excitation power required for substantial reduction of QA within the short length of the saturation flash (~ 200 μs) is on the order of 20,000 to 60,000 μmol photons m$^{-2}$ s$^{-1}$. This is well above the range of most commercial light meters. In the FRRf technique, the character of the excitation signal, usually a sequence of 1 μs long pulses, requires a measurement response time of about 100 ns, which is not attainable with commercial light meters. Fortunately, there are techniques for calibrating such short, high-intensity light flashes that overcome this limitation. One
such technique relies on making a continuous train of excitation pulses at low duty cycle and integrating (or averaging) the emitted excitation energy over time. For instance, producing 10,000 equally spaced flashlets per second and scaling the reading of the light meter (in photons m$^{-2}$ s$^{-1}$) down by 10,000 provides a direct measure of energy produced by a single flashlet. Assuming flashlet length of 1 μs, the peak power can be calculated by scaling up the meter reading by a factor of 100. More detailed procedures for these calibrations are typically provided in the instrument manuals.

For multi-wavelength emission instruments that spectrally resolve the fluorescence signal, all the emission channels need to be inter-calibrated against each other, based on the product of the spectral transmission of the emission filters and the spectral response of the detector. This inter-calibration needs to satisfy an assumption that a spectrally flat excitation signal should produce a spectrally flat instrument response across all emission channels (see section 3.1.3 below). As spectrally flat light sources do not exist (with the rare exception of laser-excited plasma devices), a second-best option is to ‘flatten’ the spectrally resolved emission response using a calibrated tungsten lamp operating at well-defined emission temperature. Because of the complex nature and high cost of the required equipment, such calibration can only be performed at the manufacturer's site.

The above-described procedures can also be used to calibrate the reference channel (the detector that monitors the excitation light), thus providing a real-time measure of the excitation power. If the long-term stability of this channel can be guaranteed by the manufacturer, the requirements for the periodic recalibration of the instrument excitation power can be greatly relaxed.

3.1.3. Instrumental Response Function (IRF), Gain and Bandwidth

The fidelity of recorded fluorescence yields in any ChlF transient is a critical factor defining the quality and inter-comparability of retrieved ChlF data. Unless the intensity of the excitation signal remains constant along the length of the excitation protocol (a requirement that is surprisingly difficult to satisfy), the fluorescence yield, calculated as the ratio of fluorescence signal to the excitation signal intensity, needs to be recorded. The stability of the recorded fluorescence yield can be quantified in terms of ‘flatness’ of the instrument response function, IRF, acquired with short-lifetime and constant fluorescence yield fluorophore such as Rhodamine B, Nile Blue or Rose Bengal, or with ethanol/acetone Chl $a$ extracts. The choice of a particular standard should be driven by convenience and other operational factors (e.g., stability of the standard solutions). The solid-state, Rhodamine-B based quantum counters, although inherently stable, are rather expensive and not amenable to different geometries of the instrument optical configurations. In contrast, liquid standards provide adequate performance, and can be applied in any size or shape cuvette. However, such liquid standards need to be well characterized in terms of their concentration and stability. Regular users may wish to retain their own fluorescence standards, including an extracted Chl $a$ reference. Exchange of standards between laboratories would be useful for cross-validation of data across different user groups. Once a standard has been chosen, users should implement a procedure for assessing and reporting the IRF quality (e.g., percentage of maximum deviation from the IRF mean). We recommend that such-defined IRF quality information be archived with accompanying data files. Adherence to these practices should make ST-ChlF data more inter-comparable across different methods and instruments (see Appendix E for more details).
3.1.3. Control and Calibration of the Instrument Gain

Perhaps somewhat surprisingly, calibration of ST-ChlF instrument gain (in units of fluorescence signal per unit of biomass) is not straightforward. Fortunately, all the photosynthetically relevant properties are independent of the absolute gain calibration (see section 2.1). Still, there is value in quantifying the instrument gain relative to a selected fluorophore standard, and relative to the calibrated excitation power. The instrument gain is defined by the spectral properties of the emission filter/detector channel, and by the gain of detector electronics. In instruments with multiple excitation and emission channels, this gain should be defined for each of the excitation and emission wavelength. In addition, emission channels should be calibrated against each other to properly record changes in the spectral emission of samples (see section 3.1.2).

The following should be considered best-practices for the calibration of instrument gain. Users should calibrate the instrument gain in all excitation channels against an appropriate fluorescence standard (see section 2.6.5). In multi-wavelength instruments, all the emission channels should be calibrated against each other, based on the product of the spectral transmission of the emission filter and the spectral response of the detector. Finally, it is recommended that raw fluorescence data be stored in standard-relative units to satisfy requirements that the recorded signal is independent of the excitation power, emission optics, and the actual electronic gain of the instrument. There is also the potential to adopt standard-relative fluorescence yield units, with values normalized to a known concentration of standard (see Appendix E for details).

3.2 ST-ChlF Induction Protocols

The quality of derived ST-ChlF parameters (section 2.1 and 2.2) depends on the use of excitation protocols that achieve a close-to single-turnover (ST) closure of PSII reaction during the saturation phase, while resolving the kinetics of QA re-oxidation in the relaxation phase. Several approaches towards these objectives are discussed below.

3.2.1. The saturation phase of the ST-ChlF transient

The ‘single-turnover’ (ST) mode of the saturation flash requires sufficient excitation power to achieve near complete reduction of QA over a time-scale of less than 200 μs, which is short enough to minimize the concomitant QA re-oxidation and the associated reduction of downstream electron carriers. Longer saturation phases will allow subsequent reduction of re-oxidized QA, progressively changing the character of the observed ChlF transients to a multiple turnover (MT) flash. Such transitions from ST to MT protocols change the shape of the ChlF transient, introducing significant variability in derived primary and secondary parameters. The most affected parameters are Fm and σPSII due to progressive reduction of PQ pool and subsequent changes in Qb-site occupancy. On the other hand, significant physiological insights can be derived from MT-ChlF protocols, especially when the ST and MT fluorescence transients are combined in a single ST-MT-excitation protocol (Kolber et al. (1998), Prášil et al. (2018), Brown et al. (2019).
In devising protocols for the ST saturation phase, it is important to note that continuous re-oxidation of some fraction of QA molecules prevents complete reduction of QA unless electron transport inhibitors (e.g., DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea) are added. Nevertheless, QA reduction levels of 80% - 95% are sufficient for robust estimates of the ChlF signal attributable to full reduction of QA, based on the knowledge of $\sigma_{PSII}$ and the kinetics of QA$^{-}$ re-oxidation (Kolber et al. 1998). When quantified, the level of QA reduction attained by the end of saturation phase should be logged in the archived data.

The excitation light responsible for QA reduction during the saturation phase of a ST-ChlF transient can be applied as a sequence of flashlets (fast repetition rate fluorometers, FRRf) or as a single pulse (e.g., FiRe, PSI and CTG-STAF instruments). The photon flux provided during the saturation phase can be adjusted by either changing the length, number and/or the intensity of the flashlets or pulse. As mentioned above, ST excitation protocols require that the saturation phase of the ChlF transient be shorter than 200 $\mu$s. As a general rule of thumb, the total photon dose provided during such a 200 $\mu$s saturation phase should be 3-10 photons per RCII. This value is derived from the intensity of the excitation light source ($E_{ex}$ ($\lambda$), $\mu$mol photon m$^{-2}$ s$^{-1}$), the length of the excitation pulse or flashlets ($\mu$s), and the absorption cross section for PSII photochemistry ($\sigma_{PSII}$ ($\lambda$), nm$^2$). (Kolber et al. 1998)

As shown in figure 3.2.1a, the ideal saturation phase of a ST-ChlF transient shows a clear plateau, with the maximum observed ChlF reached approximately two thirds through the saturation phase. Note that this maximum level does not necessarily equal Fm, due to simultaneous QA$^{-}$ reoxidation. Rather, this maximum level, an apparent Fm, reflects the point of dynamic balance between charge separation and QA re-oxidation. If too little excitation energy is provided, the saturation phase will not reach a plateau, resulting in erroneous primary ST-ChlF parameter estimates (Figure 3.2.1b). Similarly, too much excitation energy provided during the saturation phase of a ST-ChlF transient can affect the derived primary ChlF parameters (Figure 3.2.1d).

The provision of sufficient excitation energy is easily achieved when ChlF is excited in the 410 to 500 nm spectral range, which is strongly absorbed by most eukaryotic phytoplankton species, using commercially available LEDs with high photon flux. However, when excitation power is delivered at wavelengths poorly absorbed by PSII in the phytoplankton taxa being measured, or at wavelengths served by less effective LEDs, the photon flux achievable during the short ST saturation phase may be insufficient for near complete QA reduction (Courtecuisse et al. 2023). Users of instruments with multiple excitation wavelengths should be aware of this potential caveat and should confirm sufficient QA reduction (ST-ChlF transient saturation) in their measurements, through visual inspection of derived ChlF transients.
Figure 3.2.1: The saturation phase of the ST-ChlF transient. (A) During the saturation phase of a ST-ChlF transient, strong excitation power applied over a timescale of < 200 μs induces an increase in ChlF from $F_o$ to $F_m$. (B) Hypothetical example where the excitation power is insufficient to saturate the saturation phase of the ST-ChlF transient. (C) Hypothetical example of optimal excitation power provided during the saturation phase of the ST-ChlF transient. (D) Hypothetical example where too much excitation power is provided during the saturation phase of the ST-ChlF transient, leading to the induction of quenching.

On a practical level, most current ST-ChlF instruments are capable of adjusting the intensity of excitation power to optimize the shape of the saturation phase of the ST-ChlF transient. Users should confirm that appropriate levels of QA reduction have been achieved, particularly during when excitation light is applied at wavelengths that are less well absorbed by PSII; 500 – 630 nm in most eukaryotic phytoplankton. Appendix F provides an approach to quantify the level of QA reduction during ST-ChlF transients. Once an optimal excitation protocol is achieved, details of this protocol (i.e. lengths of saturation phase, power and waveband of excitation light source, etc.) should always be reported and archived alongside ST-ChlF data.

3.2.2. The relaxation phase of the ST-ChlF transient

The ‘relaxation phase’ of a ST-ChlF transient follows the decrease of ChlF back to its initial level at increasing intervals of darkness between the excitation flashlets (FRRf method) or flashes (double flash method). The time dependence of ChlF relaxation largely reflects QA re-oxidation kinetics through downstream photosynthetic electron transport. The progressive decrease in ChlF can be kinetically resolved into a number of time-constants, $\tau_i$ (μs), which are the reciprocals of exponential decay rates (μs$^{-1}$) of the ChlF signal.

Several approaches exist to observe the ChlF decrease during the relaxation phase. In one approach, a series of excitation flashlets is applied at progressively increasing time intervals to record the kinetics of QA re-oxidation (Figure 3.2.2, Kolber et al. 1998). To quantify these kinetics, the entire fluorescence transient (both the saturation and the relaxation phase) is fit
with a theoretical model based on a dynamic equilibrium between the rate of charge separation and QA⁻ reoxidation (Appendix A). An alternative approach is the use of paired ST-ChlF transient saturation phases, with gradually increasing time intervals between them (Figure 3.2.3). Here, the apparent F₀ level measured in the second ST-ChlF transient in a pair resolves the decrease in ChlF from the apparent Fₘ level towards F₀ over the time-interval between the paired measurements. A series of such paired ST-ChlF saturation phases over an increasing time interval can thus be used to reconstruct the kinetics of QA⁻ reoxidation. A ‘multi-flash’ protocol of this kind (comprising five ST saturation phases) was implemented in a single-cell FRRf (Gorbunov et al. 1999). A similar ‘dual-pulse’ protocol has been incorporated within CTG-STAF instruments (Oxborough 2022, Appendix B). In all cases, the time-dependent decrease in ChlF after saturation is fit to a multi-component exponential decay curve to resolve the time constant(s) (τ) of QA⁻ re-oxidation (Gorbunov and Falkowski 2021; Oxborough 2022).

Proper analysis of QA⁻ re-oxidation kinetics during the relaxation phase requires measurements of the ChlF decrease over periods 4-5 times longer than the slowest reported time constants of the QA⁻ re-oxidation. Furthermore, the number and spacing of data points must be optimized to resolve the multiple decay components. For example, it was recently suggested that the use of a three-component kinetic analysis is critical for the most accurate description of QA⁻ re-oxidation kinetics (Gorbunov and Falkowski 2021). However, the fitting of three (or more) components requires a high signal-to-noise ratio, which is not always achievable in oligotrophic regions, or instruments with lower sensitivity.

Figure 3.2.2: ST-ChlF decay after the saturation phase, resolved through the application of a series of excitation flashlets at progressively-increasing time intervals.
Figure 3.2.3: ST-ChlF decay after the saturation phase, resolved through a series of paired ST saturation protocols with gradually increasing spacing. Here, the $F_o$ level measured during the second of the paired saturation phases represents the ChlF decay from $F_m$ over the time interval between the paired saturation phases.

As with details of the ChlF induction protocols, derived values of $\tau$ should always be reported and archived alongside details of the relaxation protocol and fitting procedure used. Raw data of ST-ChlF transients, including both saturation and relaxation phases, should be archived and made available as a supplement to any published data sets and analyses.
3.3 Blanks and baseline fluorescence

The characterization and quantification of blanks and background signals is of great importance in optical oceanography, particularly in oceanic regions with low phytoplankton biomass, where blanks can be of a similar magnitude to the measured properties of interest. This is certainly true for phytoplankton ST-ChlF measurements, where the proper quantification and correction of blanks and baseline fluorescence is critical for robust data interpretation. In this section, we discuss analytical blanks resulting from hardware-specific properties, and from fluorescent dissolved materials. We also describe different sources of baseline non-variable fluorescence emanating from phytoplankton cells, and discuss the importance of correcting for this signal.

As shown in Figure 3.3.1, both blanks and baseline fluorescence contribute to a non-inducible (i.e. invariant) fluorescence signal measured with ST-ChlF protocols. It is important to distinguish between these two sources of fluorescence. The analytical blank is related to instrument specific properties (Bint in Fig. 3.3.1) and non-living dissolved material in the sample matrix (F\textsubscript{diss}). This blank signal should be quantified regularly and subtracted from all measurements. By comparison, baseline fluorescence contains potential information about phytoplankton physiological state, and may also be influenced by taxonomic composition. Baseline fluorescence is much more difficult to quantify, and can exert a significant, though poorly known, effect on retrieved ST-ChlF parameters. The various components making up blanks and baseline fluorescence are described further below, and illustrated in Fig. 3.3.1.
Figure 3.3.1: Components of the non-inducible fluorescence signal affecting ST-ChlF measurements. The top part of the figure (green shading) shows a ST-ChlF induction curve, where the measured ChlF rises from a minimum, $F_0$, to maximum, $F_m$, level. Such inducible ChlF originates from photosynthetically-active PSII complexes in the sample. The analytical blank in ST-ChlF measurements (pink shading at bottom), generally consists of an instrument-specific blank, $B_{inst}$, and optically-active fluorophores in the dissolved phase, $F_{diss}$. The baseline fluorescence (blue shading) is composed of fluorescence from non-living phytoplankton ($F_{deadstuff}$), and various sources of non-inducible fluorescence from living phytoplankton. These include non-inducible fluorescence from pigments other than Chl $a$ ($F_{phycobi}$), photosynthetically-inactive PSII ($F_{damaged}$), energetically decoupled light-harvesting complexes or chromophores ($F_{edLHC}$) and fluorescence from PSI ($F_{PSI}$), which is not necessarily negligible as often assumed. The subscript $c$ for the values $F_{m,c}$ and $F_{0,c}$ indicate that these values are corrected for both blank and baseline fluorescence.

3.3.1 Analytical Blank

The ‘analytical blank’ consists of $B_{inst}$ and $F_{diss}$. The value of $B_{inst}$ is defined by the properties of an instrument’s optical design (e.g., wavelength overlap between the excitation and emission bands), and by the quality of the optics employed (e.g., the amount of stray light scattered from the optics). As such, $B_{inst}$ varies among instruments both within and between different manufacturers. Fortunately, $B_{inst}$ can be relatively easily quantified through measurement of high quality Milli-Q water, with the resulting value subtracted from all raw data. It is recommended that $B_{inst}$ be monitored regularly (at least daily), as a drift in this signal can indicate biofouling in the sampling cuvette. Ideally, $B_{inst}$ values should be low relative to measured signals of interest – i.e. $< 5\%$ of $F_m$ in oligotrophic waters and $< 1\%$ of $F_m$ in mesotrophic and eutrophic waters. This condition is typically met in most newer instruments. In systems with high $B_{inst}$ values, the use of a constant correction factor for all data can be problematic, as the blank may increase when highly-scattering cells (e.g. haptophytes or some diatoms) are present in the sample, resulting in a significant source of variability among measurements. In this case, it is not possible to distinguish $B_{inst}$ and dissolved fluorescence ($F_{diss}$), and both components of the analytical blank should be measured and subtracted together, as described for $F_{diss}$ below.

Non-variable fluorescence in the background sample matrix, $F_{diss}$, is caused by colored dissolved organic matter (CDOM) and various biological degradation products (pheophytin, etc.). The magnitude of $F_{diss}$ is likely to vary significantly in both time and space, along both lateral and vertical dimensions. In oligotrophic regions with low phytoplankton biomass, $F_{diss}$ can contribute significantly to the ST-ChlF signal, and correction is therefore more crucial than in eutrophic regions or lab cultures, where the dissolved contribution is generally negligible. Even within a single location, the relative contribution of $F_{diss}$ to the measured signal can vary significantly, as relative concentrations of CDOM and other degradation products typically increase strongly with depth (below the deep Chl $a$ maximum). As a result, it is not sufficient to use a single $F_{diss}$ measurement for the correction of entire depth profiles of ST-ChlF measurements.

In principle, the measurement of $F_{diss}$ is relatively simple. Yet the procedure requires some time and attention to detail, and is not practical for high frequency underway operation. The most common approach is to measure fluorescence signals in samples that have been gently filtered through a fresh 0.2 $\mu$m filter. A number of research groups use polycarbonate (or similar) membrane filters for these blanks, as they have a well-defined porosity. Other groups, however, have used glass fiber filters (e.g. GF/F, nominal pore size of $\sim 0.7$ um). On balance, the membrane filters are likely preferable as they are less likely to allow particles to pass through. On the other
hand, these membranes can develop higher back-pressure, and care must be taken to avoid rupturing cells, which would artificially increase the CDOM pool and associated $F_{diss}$ signal. In addition, rapid filtration can create microbubbles, which could affect the measurement through strong elastic scattering of excitation light. In practice, gentle filtration of ~10 ml of water using a plastic or glass syringe through an in-line filter holder is effective at minimizing air bubbles. The absence of any living phytoplankton in the filtrate can be confirmed by the lack of ChlF induction (i.e. $F_0 = F_m$). By subtracting $B_{inst}$ values from all measurements prior to quantifying $F_{diss}$, values of $F_{diss}$ (as % of $F_m$) can be compared between sampling environments and instrument types.

3.3.3 Example from underway operations and depth profiles

Based on available measurements, it appears that the $F_{diss}/F_m$ ratio in ocean surface waters is typically between 0.02 – 0.04, implying that $F_{diss}$ represents less than 5% of the maximum analytical signal of interest. However, higher relative blank values can be observed in some regions, including hydrographic fronts and upwelling eddies. As an example, Fig. 3.3.2, shows ST-ChlF collected along a 600 km transect across the California continental shelf. The average errors in $F_v/F_m$ associated with $F_{diss}$ were less than 2% for much of the sampling transect, but increased to 4.5% at one of the offshore hydrographic fronts. Assuming that $F_{diss}$ can be measured once per day (a regular maintenance period for cleaning the sample chamber of the underway instrument), the blank-related errors in $F_v/F_m$ should be less than 2%.
Figure 3.3.2: Upper panel: data from an ST-ChlF instrument deployed in underway mode along a 600 km transect across the California continental shelf where surface [Chl a] varied from 8 μg/L at the coast down to 0.08 μg L⁻¹ offshore. Middle panel: [Chl a], Fₘ, and fluorescence blanks (Fₘₐₓ = 0.2 μm filtrate from which Bₘₐₓ has been subtracted) measured at surface on discrete surface samples. Lower panel: estimated error in the measured Fᵥ/Fₘ (Fₘₐₓ-corrected minus Fₘₐₓ-uncorrected Fᵥ/Fₘ relative to Fᵥ/Fₘ uncorrected).

The Fₘₐₓ signal can lead a to much larger interference in depth profile measurements. As shown in Fig. 3.3.3, Fₘₐₓ values are typically homogenous throughout the mixed layer. Below the deep Chl a max (DCM), however, the Fₘₐₓ contribution to the raw Fₘ signal usually increases rapidly. Whereas Chla concentrations decrease to undetectable levels at depths below ~200 – 300 m, Fₘₐₓ remains detectable at depths down to 500 - 700 m, reflecting the presence of refractory fluorescence dissolved organic matter. As shown in Fig. 3.3.3, the Fₘₐₓ/Fₘ ratio increases rapidly below the DCM. In the absence of blank correction, the apparent decrease in Fᵥ/Fₘ estimates could be easily misinterpreted, as a sign of nutrient limitation for example.
Figure 3.3.3: Left panel: [Chl $a$] and $F_m$ (raw, and blank-corrected) measured in discrete water samples from a vertical profile at an open ocean station 600 km west off the California coast. Middle panel: blanks ($F_{diss}$) and their factional contribution to the $F_m$ signal. Right panel: $F_v/F_m$ (raw and blank-corrected), and the $F_v/F_m$ error in the absence of blank correction.

In summary, both $B_{inst}$ and $F_{diss}$ should be monitored regularly during deployments of ST-ChlF instruments. For instruments with a low $B_{inst}$, a constant value can be subtracted from all $F_0$ and $F_m$ values, enabling specific determination of $F_{diss}$ (as a percentage of $F_m$). Systematic examination of $F_{diss}$ values is important to characterize conditions where non-corrected ST-ChlF measurements (e.g. from autonomous platforms) may be subject to significant error. As an ancillary benefit, $F_{diss}$ measurements can also provide useful information on CDOM distributions in aquatic environments.

3.3.4 Baseline fluorescence

The ‘baseline fluorescence’ signal measured in ST-ChlF methods is distinct from the analytical blank ($B_{inst}$ and $F_{diss}$), but nonetheless contributes significantly to non-inducible fluorescence, and must be considered in the interpretation of data. Variations in baseline fluorescence can be rather complex, reflecting differences in the taxonomic composition and physiological state of phytoplankton assemblages. The prevalence of baseline fluorescence in phytoplankton, and the uncertainty around the sources and correct interpretation of this signal, presents challenges for the application of ST-ChlF instruments in aquatic environments.
Variations in baseline fluorescence and their effect on \( F_v/F_m \) are frequently observed in the context of nutrient stress. For example, low measured \( F_v/F_m \), which is a well-established diagnostic for iron limitation, can be partially explained by the presence of energetically-decoupled light harvesting complexes (edLHC), which absorb light and emit ChlF, but do not transfer energy toward photochemistry (e.g., Behrenfeld and Milligan, 2013; Macey et al., 2014). These complexes contribute to the baseline fluorescence signal, increasing measured values of \( F_o \) and \( F_m \) by equal amounts, leaving \( F_v \) unchanged, and thus lowering \( F_v/F_m \). Other sources of baseline fluorescence include the presence of phycobilin-containing phytoplankton (e.g. cyanobacteria; Campbell et al. 1998), as well as the fluorescence contribution from Photosystem I (\( F_{PSI} \); Franck et al. 2002), which is not always negligible, as is often assumed.

Going forward, it will be important to develop approaches to distinguish different sources of baseline fluorescence, and to interpret these signatures in terms of phytoplankton taxonomy and physiology. In particular, robust approaches for the correction of baseline fluorescence need to be developed in order to accurately interpret ST-ChlF data in terms of phytoplankton productivity. Correction for baseline fluorescence is necessary for the strict interpretation of \( F_v/F_m \) as the photosynthetic quantum efficiency of PSII. This, in turn, is necessary for accurate calculation of ETR\(_{PSII} \) using the algorithms outlined in section 2.3. Moreover, a number of NPQ derivations (section 3.4) also require that data be corrected for baseline fluorescence. Approaches to correct for baseline fluorescence have been suggested (Boatman 2019), but require further validation under a range of environmental conditions. One known, but laborious, method to determine the origin of baseline fluorescence is the use of low temperature (77K) emission spectroscopy that distinguishes the emission of Photosystem II from that of phycobilisomes, uncoupled antenna or Photosystem I (e.g., Hill et al., 2012).

### 3.4 Non-photochemical quenching (NPQ) and NPQ relaxation

The term non-photochemical quenching (NPQ) is used to represent a diverse range of taxonomically-variable and environmentally-dependent physiological processes that influence heat dissipation of absorbed energy from PSII. NPQ affects the quantum yields of ChlF and photochemistry, and must be characterized and accounted for when relating changes in the quantum yield of ChlF to changes in the quantum yield of photochemistry. In the discussion below, we briefly review the definition of NPQ, and describe the effects of NPQ on various primary and secondary ST-ChlF parameters. We conclude by recommending practical operational procedures to achieve NPQ relaxation and obtain ST-ChlF measurements from phytoplankton in the dark-regulated state.

#### 3.4.1 Definition and parameterization of NPQ

Non-photochemical quenching (NPQ) is a decrease in the quantum yield of ChlF (\( \Phi_F \)) that is not attributable to photochemical quenching of excitation by open PSIIIs. Confusingly, the acronym ‘NPQ’ is also widely used to denote one particular parameterization of the NPQ processes, the so-called Stern-Volmer formulation (Bilger and Björkman 1990; Holzwarth et al. 2013, see also table 2.1.6):
\[ NPQ_{SV} = \frac{(F_m - F_m')}{F_m'} \]  

(Equation 3.4.1)

This unfortunate homonym leads to conceptual confusion between the general phenomenon of NPQ, and one particular parameterization of this process within an organism or assemblage. To avoid this confusion, we recommend denoting the Stern-Volmer NPQ formulation as NPQ_{SV}, though we recognize that this is widely termed ‘NPQ’ in the literature. A key limitation of NPQ_{SV} is that it is expressed relative to some ideal maximum ChlF, \( F_m \), which is not affected by any quenching. The challenge lies in achieving accurate measures of true \( F_m \) values, since relaxation of different NPQ components can occur over a range of timescales, from fractions of a second to many hours. Moreover, the time-scale of NPQ relaxation can be sensitive to environmental conditions and phytoplankton taxonomy, and specific light treatments may be required to allow full relaxation in some taxa (e.g. Campbell and Öquist 1996; Chukhutsina et al. 2014) (Figures 3.4.1 and 3.4.2). Further confusion arises from the fact that NPQ measured in different phytoplankton groups can result from fundamentally different underlying processes. Researchers familiar with the dominant mechanisms and patterns of NPQ in one taxa or physiological state may thus incorrectly assume that NPQ in another taxa or physiological state arises from the same mechanisms. As a result, accurately quantifying and interpreting NPQ in mixed phytoplankton assemblages is not trivial. Nevertheless, recent work has demonstrated that observed patterns in NPQ can provide useful information to constrain mechanistically distinct, but correlated, parameters used to estimate phytoplankton primary productivity from ST-ChlF measurements (section 5.2.3).

3.4.2 Effects of NPQ on primary ChlF parameters

Induction and relaxation of NPQ mechanisms can significantly and differentially influence measurements of \( F_o' \), \( F_m' \), and \( \sigma_{PSII}' \). These primary ST-ChlF parameters are, in turn, used to derive secondary parameters needed to estimate ETR_{PSII} (section 2.3) and thus photosynthetic rates. In this section, we outline general approaches to detect and control the effects of NPQ induction and relaxation on ST-ChlF parameters used for photosynthetic rate estimates. Within this discussion, we stress the importance of recognising confounding effects of photo-inhibition and species composition when measuring or correcting for NPQ under field conditions. Approaches to analyse the underlying mechanisms of NPQ, per se, are covered extensively elsewhere (e.g. Gorbunov et al. 2011; Holzwarth et al. 2013; Ruban 2016; Xu et al. 2018).
Figure 3.4.1: Typical ST-ChlF transient including saturation and relaxation phase. See section 2.1 for nomenclature and definition of derived parameters, and sections 2.2 and 3.2 for details on fitting procedures. In the dark-regulated state, all NPQ mechanisms are relaxed and the QA pool is oxidised (‘open’) at the beginning of the ChlF transient. The application of strong excitation light during the saturation phase results in an increase of ChlF from $F_0$ to $F_m$. In the light-regulated state, the minimum ChlF at the beginning of the saturation phase is increased from $F_0$ to $F'_0$, due to partial reduction of QA and an associated decrease in photochemical quenching. NPQ induced during the light regulated state generally results in a decrease of $F_0$ to $F'_0$ and $F_m$ to $F'_m$. The absorption cross-section of photochemistry in PSII, $\sigma_{PSII}$, generally decreases in the light-regulated state, and this decrease can be partially attributed to NPQ. The decrease in amplitude of the ST-ChlF transient in the light-regulated state can interfere with estimation of all ChlF parameters, in particular $\sigma_{PSII}$, $\tau'$, and $\rho$.

The induction of NPQ can suppress $F'_0$, with relaxation times ranging from seconds, for state transitions in cyanobacteria (Campbell et al. 1998), to hours for high-light or dark-induced NPQ in diatoms (Lavaud 2007). In contrast, photo-inhibition of PSII, which overlaps kinetically and phenomenologically with NPQ induction, can cause a significant rise in $F'_0$ (Ware et al. 2015), reflecting the accumulation of inactive PSII repair cycle intermediates, which contribute to $F'_0$ but not to $F'_0$ (Li et al. 2016). This photo-inhibitory effect on $F'_0$ relaxes over time scales of minutes to hours, particularly under low light. Importantly, the rate of recovery from photo-inhibition can vary widely with temperature across taxa, with longer recovery times typically observed under lower temperatures (Ni et al. 2017). Confounding these interpretations, strong increases in $F'_0$ (and in $F'_0$, $F'_m$ and $F'_n$) can also be caused by iron limitation, where the increase in ChlF is attributable to energetically uncoupled antenna complexes (section 5.2.2, Behrenfeld and Milligan 2013; Macey et al. 2014; Sherman et al. 2020). Thus, significant care must be taken to not falsely attribute taxonomic and environmental influences on primary ChlF parameters as indicators of NPQ.

Similar to $F'_0$, $F'_m$ is generally suppressed by NPQ induction, with relaxation times varying from seconds to hours. Suppression of $F'_m$ below $F_m$ can be extreme in some taxa, including diatoms (Lavaud 2007). In cyanobacteria, however, $F'_m$ can decrease significantly in darkness, while reaching maximum levels under the acclimated growth light level (Campbell and Oquist 1996). In principle, $F'_m$ is not affected by photo-inhibition per se, although photo-inhibitory conditions generally coincide with parallel induction of NPQ.

NPQ-related effects on $\sigma_{PSII}$ are somewhat more complicated. Certain NPQ mechanisms suppress $\sigma_{PSII}'$, but the extent of suppression is not necessarily proportional to the measured induction of NPQ, as quantified by NPQ$_{SV}$, for example (Vassiliev et al., 1994; Koblizek et al., 2001; Gorbunov
et al. 2011; Holzwarth et al. 2013; Xu et al. 2018). Furthermore, induction of NPQ suppresses the amplitude of $F_v' (= F_m' - F_o')$, which leads to greater uncertainty in the estimation of $\sigma_{PSII}'$ from the rise of $F_o'$ towards $F_m'$. As with $F_o'$ and $F_m'$, the relaxation time-scale of NPQ effects on $\sigma_{PSII}'$ ranges from seconds to hours. Photo-inhibition can drive an increase in $\sigma_{PSII}'$ as the antenna bed serves a decreasing number of active excitonically connected PSII.

Unlike the parameters discussed above, $\tau'$ is not, in principle, expected to be directly influenced by NPQ. However, factors that induce or relax NPQ can cause kinetically overlapping changes in electron transport that influence $\tau'$. Furthermore, as with $\sigma_{PSII}'$, induction of NPQ can interfere with the estimation of $\tau'$, which is derived from fitting an exponential decay function to describe $F_m'$ relaxation towards $F_o'$.

### 3.4.3 Recommendations for NPQ relaxation protocols

During measurements of ST-ChlF, it is standard practice to expose samples to a period of NPQ-relaxation (commonly referred to as dark-adaptation or dark-acclimation) prior to measurements. In practice, it is often impossible to define one ideal NPQ-relaxation time for taxonomically diverse phytoplankton assemblages under field conditions. This limitation results from the large variety of taxon-specific NPQ mechanisms and associated relaxation times, the confounding effects of other environmental parameters (e.g., temperature) on NPQ dynamics, and the overlapping effects of NPQ relaxation and recovery from photo-inhibition. Notwithstanding this complexity, the SCOR WG156 consensus is that NPQ relaxation should be conducted under low light (~ 10 – 20 µmol quanta m$^{-2}$ s$^{-1}$; Figure 3.4.2), rather than complete darkness. This recommendation differs from the suggested protocols used in some higher plant systems and early protocols for marine phytoplankton. The need to avoid fully dark NPQ relaxation is based on the fact that low light induces electron transport and a $\Delta$pH gradient across the thylakoid membranes, which helps accelerate recovery from the energy-dependent NPQ in some taxa, particularly diatoms (Lepetit et al. 2012; Lavaud and Goss 2014). In diatoms and cyanobacteria, complete darkness can actually lead to induction of NPQ (Jakob et al. 1999, 2001). Furthermore, low light is also critical to promote recovery from photo-inhibition through the de novo synthesis of D1 protein of the PSII reaction centres (Lavaud et al. 2016; Theis and Schroda 2016).
Figure 3.4.2: The effect of low light vs. dark exposure on the recovery kinetics of ChlF and photosynthetic characteristics in phytoplankton previously exposed to high light. (A) The maximum ChlF, Fm and (B) the maximum quantum yield of photochemistry in PSII, Fv/Fm. Open dots show the recovery kinetics observed under dim light (10 μmol quanta m⁻² s⁻¹), solid dots signify recovery in darkness. The sample was collected from the surface at noon (the Northern Pacific) and was subject to photo-inhibition and induction of non-photochemical quenching (NPQ) by strong sunlight, which resulted in low initial Fm and Fv/Fm. Exposure to low-light stimulated recovery from photo-inhibition and accelerated relaxation of NPQ, whereas darkness largely prevented recovery from photo-inhibition. Note that Fm values are reported in arbitrary units (a.u.).

When designing NPQ-relaxation protocols for field campaigns, it is critical to consider the goals of the measurement, and the physiological information needed to answer the research questions of interest. For example, examining the effects of iron limitation on Fv/Fm requires full relaxation of all NPQ mechanisms and achieving complete recovery from photo-inhibition. Measurement of primary ChlF parameters under dark-regulated states (as needed for some ETRPSII algorithms – see section 2.3) also requires full NPQ relaxation, though perhaps not complete recovery from long-term photo-inhibition. On the other hand, studies measuring the instantaneous electron transport capacity of phytoplankton under ambient light conditions seek to measure cells under their in situ eco-physiological state, including relevant NPQ levels without relaxation.

The lack of an ideal or ‘standard’ NPQ relaxation time might seem discouraging to new users of ST-ChlF instruments. On the other hand, there is valuable information that can be derived from the variability of NPQ dynamics. For example, it has recently been shown that iron-limitation can be tracked over regional scales based on stronger NPQ observed in phytoplankton present in these regions (Ryan-Keogh and Thomalla 2020; Schallenberg et al., 2020).

In conclusion, it is important to explicitly consider NPQ relaxation time-scales in ST-ChlF measurements. Whenever possible, users should conduct experimental trials to determine these time-scales in their systems, with NPQ relaxation conducted under low light rather than complete darkness. In such experiments, samples are repeatedly analysed under low light conditions to examine the dynamics of primary ChlF parameters over the time-course of NPQ relaxation. Information derived from such experiments can be used to decide when an appropriate level of NPQ relaxation has been achieved, and this information can be built into analysis protocols. Details of NPQ-relaxation protocols should be reported in the metadata, as these will inform downstream interpretation.
3.5 Spectral correction and multi-excitation-wavelengths measurements

Photosynthetic light harvesting depends on a wide range of pigments, each of which has unique spectral absorption characteristics. As such, all light-dependent photosynthetic processes exhibit spectral dependence, which must be accounted for during the interpretation of ST-ChlF measurements and their comparison with other productivity metrics (e.g. \(^{14}\)C incubations) (Suggett et al. 2001; Moore et al. 2006). In this section, we discuss the importance of spectral correction for ST-ChlF data and describe recommended approaches. We first provide a conceptual background on the light absorption characteristics of phytoplankton relative to the spectral variability of *in situ* light fields and instrument light sources. We then discuss the necessary ancillary information needed to implement spectral correction, and the use of multi-excitation-wavelength ST-ChlF instruments to automate these corrections. The application of multi-excitation-wavelength ST-ChlF measurements to examine the contribution of different phytoplankton taxa to total biomass and productivity is discussed in section 5.2.4.

### 3.5.1. Spectral dependence of photosynthetic properties

Photosynthetic organisms absorb and photosynthetically utilize light in the visible part of the spectrum (400 – 700 nm; referred to as photosynthetically available radiation, PAR). In studies of photosynthesis, light intensity is typically integrated over this spectral region and reported in units of \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\). Integration to a single number simplifies calculations, and is justified since all light absorbed energy within the PAR spectrum can equally drive photochemistry. However, significant variability exists in: 1) the light absorption capabilities of phytoplankton within the PAR spectrum (section 3.5.2); 2) the spectral distribution of light in aquatic systems (section 3.5.3); and 3) the spectral properties of light sources used in different ST-ChlF instruments (section 3.5.4). In order to collect environmentally-relevant and inter-comparable ST-ChlF data, it is critical to consider these spectral differences, and apply corrections when necessary.

The tables provided in section 2.1 indicate which ST-ChlF parameters are spectrally dependent. These spectrally-dependent parameters include all measures of incident light (E(\(\lambda\))), light absorption (e.g., a(\(\lambda\)), \(\sigma(\lambda)\)), rates derived for sub-saturating light intensities (ETR\(_{\text{PSII}}(\lambda)\)) and the derived photosynthetic parameters \(\alpha(\lambda)\) and E\(_{A}(\lambda)\). Absolute values of the primary ST-ChlF parameters F\(_{o}\), F\(_{m}\), F\('\), F\(_{m}'\), F\(_{o}'\) are spectrally-dependent in terms of both excitation and emission (i.e. F(\(\lambda_{\text{ex}},\lambda_{\text{em}}\))). However, these parameters are most commonly reported in relative units, so that wavelength-dependent changes in their absolute values do not affect the interpretation of results. In contrast, all rates measured at light saturation (ETR\(_{\text{PSIImax}},\ P_{\text{max}}\)) and the time-constants of QA re-oxidation (\(\tau_{1}\)) are not spectrally-dependent.

### 3.5.2 Spectral variability in phytoplankton light absorption

Light absorption spectra of phytoplankton can vary significantly as a function of environmental conditions and taxonomic composition. This spectral dependence can vary significantly depending on which absorption coefficient is measured or derived (e.g., particulate absorption a\(_{p}(\lambda)\),...
phytoplankton pigment-specific absorption $a_{\text{phy}}(\lambda)$, absorption specific to photosynthetic pigment $a_{\text{psp}}(\lambda)$, etc.; Table 2.1.5; Figure 3.5.1).

For the correction of ST-ChlF data, knowledge of the spectral distribution of light used to drive photosynthetic reactions is ideal. This can be approximated from ChlF excitation spectra (typically measured with a spectro-fluorometer, e.g. Silsbe et al. (2015)), but these measurements are error prone, not suitable for routine application, and have rarely been applied in practice. More commonly, spectral correction has been performed using phytoplankton light absorption $a_{\text{phy}}(\lambda)$, derived from one of several measurement or modelling approaches. The total light absorption coefficient of natural waters ($a(\lambda)$, m$^{-1}$) is an additive inherent optical property of all light absorbing components within seawater. Values of $a_{\text{phy}}(\lambda)$ is thus estimated after correcting for the contribution from other light absorbing constituents (optically active components), including water molecules ($a_{w}(\lambda)$), colored dissolved organic matter ($a_{\text{cdom}}(\lambda)$) and non-algal particles ($a_{\text{nap}}(\lambda)$) (table 2.1.5).

$$a_{\text{phy}}(\lambda) = a(\lambda) - a_{w}(\lambda) - a_{\text{cdom}}(\lambda) - a_{\text{nap}}(\lambda)$$

Equation 3.5.1

A number of automated sensors (e.g. AC-9 or AC-S by Wetlabs/Seabird, integrating cavity absorption meters (ICAM), point-source integrating-cavity absorption meters (PSICAM) can be used to acquire high spatial or temporal resolution measurements of $a(\lambda)$ (unfiltered water) and $a_{w}(\lambda) + a_{\text{cdom}}(\lambda)$ (filtered water). Particulate absorption $a_{p}(\lambda)$ ($= a_{\text{phy}}(\lambda) + a_{\text{nap}}(\lambda)$) can be derived from these measurements, but no generally-applicable method currently exists for a robust deconvolution of derived $a_{p}(\lambda)$ into $a_{\text{phy}}(\lambda)$ and $a_{\text{nap}}(\lambda)$.

Using empirical modelling approaches, $a_{\text{phy}}(\lambda)$ can be estimated using simple statistical functions of Chl $a$ concentrations (Bricaud et al. 1995). More complex bio-optical models and numerical simulations can directly compute $a_{\text{phy}}(\lambda)$ from in situ or remote sensing radiometric data (e.g., Dierssen et al. 2021). While these approaches can provide good estimates of $a_{\text{phy}}(\lambda)$ at high resolution, more direct measurements, described below, are recommended for spectral correction of ST-ChlF data.

Direct measurements of $a_{\text{phy}}(\lambda)$ can be performed on discrete samples using the so-called filter-pad approach (Mitchell et al., 2000), where a thin layer of phytoplankton is collected on a filter and scanned on a spectrophotometer before and after solvent extraction of pigments. The difference in the absorption spectrum before and after de-pigmentation is assumed to represent bulk light absorption by phytoplankton pigments, $a_{\text{phy}}(\lambda)$. This method provides useful hyperspectral measurements, but is rather laborious and time consuming, and not readily amenable to automation. It also requires a number of correction terms (e.g. path-length amplification due to scattering in the filter, Werdell et al. 2018), which can introduce significant error. A recent compilation of all direct methods for phytoplankton absorption measurements is found in Neely and Manino (2018).

Another approach to derive $a_{\text{phy}}(\lambda)$ is based on the reconstruction of absorption spectra from pigment concentrations measured by High Performance Liquid Chromatography (HPLC), following the original work by Bidigare et al. (1990) and Hoepffner and Sathyendranath (1991). This approach allows for the reconstruction of absorption spectra attributable to photosynthetic
pignments only $a_{\text{psp}}(\lambda)$, excluding light absorption by photoprotective carotenoids ($a_{\text{ppc}}(\lambda)$) (Figure 3.5.1). However, the method requires correction for changes in light absorption properties of pigments in solution relative to pigments arranged in the light harvesting antenna of phytoplankton, which can be subject to so-called ‘pigment packaging’ and other effects (Le et al. 2009; Letelier et al. 2017; Robinson et al. 2021). Furthermore, as with the filter-pad approach, pigment-based absorption spectra require sampling and analysis of discrete samples, and are thus not suited to high-resolution, automated data collection needed for ST-ChlF data correction.

As discussed further in section 3.5.6, it is also possible to use multi-excitation-wavelength ST-ChlF instruments to obtain spectrally-resolved light absorption properties of PSII.

**Figure 3.5.1:** The spectral absorption coefficient ($a^*$, m$^2$ mg Chl $^{-1}$) of phytoplankton as determined using the quantitative filter-pad technique (QFT $a^*$phy, red line) and reconstructed from HPLC pigments (HPLC $a^*$phy, black line). Using the HPLC pigment reconstruction approach, it is further possible to determine absorption spectra specific to photosynthetic pigment ($a^*$psp, dotted black line) and specific to photoprotective carotenoids ($a^*$ppc, dashed line). The sample was collected in the coastal NE subarctic Pacific (44.3°N, 124.4°W) in August, 2017 at 16:00 local time (Schuback and Tortell 2019).

### 3.5.3 Variability in the subsurface light spectrum

In addition to quantifying the spectral absorption characteristics of phytoplankton, it is also necessary to understand the spectral quality of *in situ* light. This information is required if
photosynthetic rates acquired with a bench-top ST-ChlF instrument are used to determine in situ rates at the sampling location.

In the global oceans, water colour can vary from deep blue to green, brown, and even red, depending on the quantity and nature of light-absorbing and scattering substances. Ideally, the spectral distribution of in situ light is measured directly using multi- or hyper-spectral radiometers. In the absence of direct measurements, in situ light quality can be modelled with a reasonably high degree of accuracy, particularly in offshore (Case 1) waters, where the light field can be described as a function of phytoplankton abundance. More complex modelling approaches can also be used in coastal systems, where a greater range of dissolved and particulate material will influence both inherent and apparent optical properties.

In offshore regions, phytoplankton cells are the main drivers for both ocean colour (i.e. spectral reflectance) and vertical spectral light attenuation (Morel 1988; Kirk 2010). These apparent optical properties (AOPs) depend on the sum of inherent optical properties (IOPs, e.g. light absorption, and scattering) of water molecules and coloured constituents (Gordon 1988), and on the geometrical distribution of the radiance field (Preisendorfer 1961). As a result, ocean light fields in Case 1 waters can be reasonably well modelled using the surface mixed layer Chl a concentration as the main input variable (e.g. Morel 1988). Near the coast, the IOPs of sediments and CDOM exert a much stronger influence on the spectral quality of the light field (IOCCG, 2006). However, the strong relationships between IOPs and AOPs has allowed the development of semi-analytical models (Gordon 1988), as well as numerical methods that solve the Radiative Transfer Equation (e.g. Mobley 1994; Mobley et al. 2002; Chowdhary et al. 2019). These computational approaches can be used to estimate spectral irradiance as a function of depth through the water column. As these bio-optical models explicitly consider the phytoplankton contribution to water column AOPs (alongside water molecules, non-algal particles, and CDOM), they can be used to derive estimates of $a_{\text{phyt}}(\lambda)$ (section 3.5.2).

![Figure 3.5.2](image)

**Figure 3.5.2:** Relative downwelling irradiance spectrum at the sea surface, and at 3 and 10 m depth for a coastal and an open ocean location. The coastal station is modelled after data from the Gulf of Finland, Baltic Sea. The open ocean condition is modelled after data from the tropical Pacific Ocean collected during the 2007 SORTIE cruise and obtained from SeaBASS.
3.5.4 Spectral quality of light sources used in ST-ChlF instruments

Two categories of light sources are used in ST-ChlF instruments. In all instruments, strong ‘excitation’ (i.e. measurement) light is used to induce ST-ChlF transients (Figure 3.5.3a). In addition, many instruments apply background or actinic light to obtain PE curves (Section 2.5, Figure 3.5.3b). In most instruments, the spectral quality of these two light sources is not identical. Spectral correction is thus necessary to account for differences in the spectral distribution of excitation and actinic light used to derive primary or secondary ChlF parameters in the light-regulated state (e.g. $\sigma_{PSII}'(E(\lambda))$, $\Phi_{PSII}'(E(\lambda))$, $ETR_{PSII}(E(\lambda))$. Spectral correction is also necessary to account for differences between the actinic light in ST-ChlF instruments and that of in situ light fields. The required calculations are outlined in section 3.5.5.

In modern ST-ChlF instruments, excitation light is typically provided by strong LEDs that emit in the blue part of the spectrum (Figure 3.5.3). High-power blue LEDs are readily available, and blue light is absorbed efficiently by most phytoplankton, making it possible to supply sufficient energy to fully and rapidly saturate the ST-ChlF transient (see section 3.2.1). Most contemporary ST-ChlF instruments also provide excitation light at additional wavelengths, enabling users to assess the contribution of different phytoplankton groups to observed ST-ChlF signals (see section 5.2.4). However, as noted in section 3.2.1, the use of these less well absorbed excitation light sources can make it challenging to induce full saturation of the ST-ChlF transient (Gorbunov et al. 2020).

![Figure 3.5.3](image)

**Figure 3.5.3**: (A) Excitation light source of a typical ST-ChlF instrument with a blue LED (red line) and a typical phytoplankton absorption spectrum (grey patch). (B) Actinic light of a typical ST-ChlF instrument, provided by a blue-enhanced white LED (red line), and a typical phytoplankton absorption spectrum (grey patch). All spectra are normalized to unity at the peak.

3.5.5 Application of spectral correction to ST-ChlF measurements

A first step of spectral correction is necessary when the excitation light used for the induction of ST-ChlF transients does not spectrally match the background actinic light used for PE curves.
The spectral correction factor (SCF) required for this correction can be calculated following equation 3.5.1:

$$SCF = \frac{\int_{400}^{700} a_{\lambda}(\lambda) E_{BG}(\lambda) d\lambda \cdot \int_{400}^{700} E_{EX}(\lambda) d\lambda}{\int_{400}^{700} a_{\lambda}(\lambda) E_{EX}(\lambda) d\lambda \cdot \int_{400}^{700} E_{BG}(\lambda) d\lambda} \quad \text{(Equation 3.5.1)}$$

Here, $a_{\lambda}(\lambda)$ is the absorption spectrum (e.g. $a_{\text{phy}}(\lambda)$, $a_{\text{sp}}(\lambda)$, $a_{\text{LHII}}(\lambda)$), $E_{BG}(\lambda)$ is the background actinic light during PE curves, and $E_{EX}(\lambda)$ is the excitation light. This SCF needs to be either applied to all estimates of ETR$_{PSII}$ before the fitting of PE curves, or applied to the derived fit parameters $\alpha$ and $E_k$ after curve fitting (see section 2.5).

A second step of spectral correction is required to estimate ETR$_{PSII}$ values or PE fit parameters relevant to in situ light quality ($E_{IS}(\lambda)$).

$$SCF = \frac{\int_{400}^{700} a(\lambda) E_{BG}(\lambda) d\lambda \cdot \int_{400}^{700} E_{IS}(\lambda) d\lambda}{\int_{400}^{700} a(\lambda) E_{IS}(\lambda) d\lambda \cdot \int_{400}^{700} E_{BG}(\lambda) d\lambda} \quad \text{(Equation 3.5.2)}$$

Here, $a(\lambda)$ is the absorption spectrum (e.g. $a_{\text{phy}}(\lambda)$, $a_{\text{sp}}(\lambda)$, $a_{\text{LHII}}(\lambda)$), $E_{BG}(\lambda)$ is the background light during ST-ChlF PE curves, and $E_{IS}(\lambda)$ is the measured or modelled in situ light at the depth and time of sampling. As above, the SCF needs to be applied to values of E before fitting PE curves, or to the derived fit parameters $\alpha$ and $E_k$ after curve fitting. This spectral correction should also be applied when ST-ChlF measurements of ETR$_{PSII}$ are compared against independent productivity estimates (e.g. $^{14}$C-uptake data) measured with a different irradiance source (e.g. ‘photosynthetron’ incubation chambers). In this scenario, the $^{14}$C-uptake measurements should also be corrected to match in situ light fields, with $E_{BG}(\lambda)$ representing the spectral quality of light in the incubation chamber. Alternatively, a correction can be made to directly align ST-ChlF measurements to other lab-based measurements by replacing relevant spectra in place of $E_{BG}$ and $E_{IS}$ in Equation 3.5.2.

### 3.5.6 Multi-excitation-wavelengths ST-ChlF for automated spectral correction

As outlined in section 3.5.2, estimates of light absorption spectra $a_{\lambda}(\lambda)$ required for spectral correction of ST-ChlF data often rely on discrete measurements, and are therefore not easily obtained at high sampling resolution (e.g. Moore et al., 2006; Silsbe et al., 2015). In recent years, new instruments have provided opportunities to measure ST-ChlF parameters specific to a range of wavelengths across the PAR spectrum (Gorbunov et al. 2020, Courtecuisse et al., 2023). Interpolation between measurements at multiple wavelengths allows the construction of light absorption profiles specific to PSII photochemistry, which are akin to ChlF excitation spectra. Collection of such data can be automated, providing high resolution measurements. Going forward, it is likely that routine application of multi-excitation wavelength ST-ChlF instruments will greatly simplify the spectral correction of data. Moreover, high resolution measurements of the spectral variability in phytoplankton light absorption ($a_{\text{phy}}$) will be valuable for further development of many ocean colour remote sensing approaches.
4. DATA REPORTING AND ARCHIVING

4.1 Overview

The full potential of ST-ChlF measurements for regional and global monitoring can only be realized through the compilation of inter-comparable data across the international research community. This requires the specification and adoption of standard reporting procedures for ST-ChlF data and associated metadata, for both new observations and, where possible, for archived datasets. Taking inspiration from the remote sensing research community, this section describes an approach for reporting ST-ChlF data in a series of well-defined processing levels. In this approach, measurements across all processing levels remain accessible in archived formats to facilitate down-stream re-processing as algorithms evolve. This ensures traceability of processed data products, and allows derived variables to be updated from raw measurements based on new conceptual developments. Within this framework, we also recommend essential metadata to accompany ST-ChlF measurements at each data processing level. Explicit versioning of data and metadata, alongside calibration and processing procedures, ensures backwards compatibility in an evolving research field.

4.2 Data archiving

In this section, we provide recommendations to guide ST-ChlF data archiving efforts, building on established protocols and conventions where possible, and suggesting improvements over existing efforts, where needed. We also suggest appropriate terminology for data processing and metadata, as well as intermediate file formats and naming conventions that would help to ensure consistency and clarity in data repositories.

4.2.1. Interoperability

Interoperability is achieved by making data self-describing, versioned, and readily accessible, with the purpose and terms of use described alongside the data. Metadata (information describing various attributes of the primary measurements) are key to ensuring the correct use of the data regardless of when, where, or by whom they are accessed. Metadata can also be important to facilitate interpretation of the primary observations, as in the case where key environmental variables (e.g. PAR) are reported.

The state of the art in interoperable geospatial data sharing is developed and maintained through the Open Geospatial Consortium (OGC; https://www.ogc.org/), which details a series of self-describing and versioned data formats to support robust data exchange. The OGC standards provide solutions to structure reported data and submit observations directly from instruments to data repositories. These standards include the Sensor Observation Service (SOS) or the more recently developed Sensor Things application programming interface.

Instruments collecting ST-ChlF data are often deployed at remote locations with potential constraints on power consumption and network connectivity. This makes it impractical to produce OGC-compliant data packages in real-time. In such cases, it is likely that data will be collected
offline, annotated with the required metadata, converted to calibrated records and quality checked before they are archived. These data treatment steps, which can be automated, require careful documentation of the processing history before they are offered through interoperable data stores.

4.2.2. Processing levels

The levels of data processing can be defined as follows, with specific reference to ST-ChlF observations:

Level 0: Raw instrument data (*i.e.* detector voltage outputs or numbers from an analog-to-digital converter), which generally have no value to users without access to instrument characterisation records and calibration files. At this stage of processing, calibration functions describing the intensity and spectral properties of excitation light sources and detectors (Section 3.1) have not been applied. Manufacturers of the currently available ST-ChlF instruments do not include such uncalibrated data in basic data outputs, with users interacting only with Level 1 or 2 data. There are, nevertheless, essential metadata associated with this processing level, such as the instrument serial number, measurement configuration, date and time of the observations and measurement artefacts such as detector saturation. These metadata are important to produce data quality flags and Level 1 data using the appropriate calibration records.

Level 1: Calibrated observation data, where each data point corresponds to a single measurement in the L0 data, following the application of calibration coefficients and characterization procedures specific to the instrument. In ST-ChlF measurements, L1 data include time-series of measured fluorescence yields (*i.e.* ChlF transients) expressed as a function of excitation/emission wavelength. Information concerning the instrument and its calibration constants should be included in L1 metadata. If measurement uncertainty is known (*e.g.* signal to noise ratio of the fluorescence transient – see section 2.6.1), this should also be added to the data presented at this level. Blanks and samples are both individually presented at this level, and should be linked using a common identifying attribute. Since blank-corrections are intrinsic to the calibration procedure of ST-ChlF data, the inclusion of blank-corrected data at L1 is acceptable, as long as these are distinguished from uncorrected data using an appropriate naming convention. Calibration records are a specific type of L1 data, as they link the uncalibrated instrument output to physical units. We recommend the storage of L1 data in dedicated repositories to facilitate future re-processing using evolving physiological models of photosynthetic parameters.

Level 2: Primary photo-physiological parameters derived from fitting blank-corrected ST-ChlF transients to a physiological model (section 2.2). If multiple transients are combined to improve the signal-to-noise ratio prior to model fitting, the resultant averaged parameters should also be considered L2 data. Typical L2 data include fit parameters (*e.g.* $F_0$, $F_m$, $F_v$, $F_v/F_m$, $\sigma_{PSII}$, $\rho$), actinic irradiance if present, and a statistical evaluation of model fit (*e.g.* RMSE or $\chi^2$, see section 2.6.3). Providing fit parameters implies a reduction of the dimensionality of the data from L1. We recommend that end users consider the open-source software PhytoPhotoTools ([https://gitlab.com/tjryankeogh/phytophotoutils](https://gitlab.com/tjryankeogh/phytophotoutils)) for data analysis. This Python-based tool can read L1 data from a variety of instruments and apply the biophysical model of Kolber et al. (1998), with functionality to control induction curve averaging and blank corrections. Moving forward, our group is working to expand this software to incorporate other physiological models. We
recommend the storage of L2 data in dedicated repositories from which spatial and temporal data subsets can be extracted to support regional and global studies of primary productivity.

Level 3: At this level, representations of multiple samples are combined to provide a new data dimension. For example, water-column average productivity, areal productivity or annual production. Metadata at this level should include reference to the selection of relevant input data, and to the procedures used to derive values from these inputs. Multiple observations (repeat measurements, blanks, actinic light intensity) and supporting attributes (correction factors, measurement timing, etc.) can be included in the reporting at Levels 0-2 to support modelling to L3.

Re-processing from Level 0 to higher levels of interpretation will always be possible, as long as instrument characterization factors are known. In contrast, it is not possible to reverse the data reduction processes that occur from L3 to L2 or from L2 to L1. As a result, data processing levels 1-3 should include all metadata that were used in their derivation (e.g. blanks, correction factors, protocols and reference to the input data) so that any user can reproduce the successive data processing steps.

4.2.3. Metadata

At each data processing level, metadata are important for describing the underlying measured phenomena and the data treatment steps. Metadata can also be used to inform downstream applications and interpretation of the data. State-of-the-art environmental data repositories work towards FAIR data principles which render their records Findable, Accessible, Interoperable and Reusable. Metadata records must be complete to support these four FAIR criteria, and should be contained within the basic unit of data storage, either within the same file or as part of a file bundle, making the data inherently self-describing. The use of inter-related file or folder names should not replace the functional association of data and metadata within individual or linked files.

The first level of metadata is used to define the observed phenomena (measurands), supporting the Reusability of the data. They must also be identifiable for a specific purpose of reuse, ideally without the need for human interpretation of their suitability. It is, therefore, important to use accepted naming conventions and standard units of measurement for each variable (see Tables in section 2.1 for SCOR WG 156 recommendations). In the case of Essential Climate Variables, the Climate and Forecast (CF) metadata conventions are used as the accepted standard for variable naming and units. Because ST-ChlF data do not contribute to any currently accepted Essential Climate Variables, there are few CF-compliant names already adopted for ST-ChlF data.

To optimise data Findability, several standards are used to categorize data along set keywords, making it possible to find relevant data sets from environmental data catalogues. Commonly used identifiers are those listed in the General Environmental Multilingual Thesaurus (GEMET) and the Global Change Master Directory (GCMD), including descriptors of the type of environment sampled, the type and purpose of the data.
Metadata describing observed variables can be of little value without information on how data have been or should be interpreted, or how they are licensed for further use. These ‘functional metadata’ include geospatial descriptors such as location, time stamp and sampling depth. Other less obvious descriptors support automated downstream processing, quality control and integration with other data. Examples of these descriptors include references to calibration records, the source of time or location information, measurement protocols, instrument platform details and data ownership and licensing. Such metadata are relatively easy to add at the point of data capture, but increasingly difficult to retrace by users who are further removed from the original collection effort. Adding such metadata at the source can significantly facilitate the coupling of data across sensor platforms. This enables data users to identify quality control issues affecting a larger sampling effort, and supports the compilation of larger collective data sets for climate modelling or satellite validation, which may take place years after the original observations were collected. Providing such metadata also encourages wider sharing and collaboration between research groups. As an example of current standards of practice, the table in Appendix E provides the proposed metadata reporting requirements for the H2020-MONOCLE project, which includes water quality data originating from instruments ranging from automated spectroradiometers to citizen-science kits.

4.2.4. File-based exchange

An important factor for efficient data exchange and communication among diverse research groups is the ability to write raw data and derived results to stand-alone file formats, which can be readily exchanged among end-users, ingested in data analysis software packages and used for long-term archiving. Text-based and machine-based data formats, as well as hybrid formats, may all be considered as acceptable standards. Each is briefly discussed below.

Text-based data formats have the advantage of not requiring knowledge of specialist software. On the other hand, a limitation of human-readable file formats is the lower efficiency of data storage, as numerical characters use more space than binary representation. This may, in turn, lead to loss of precision due to truncation of data values to limit file size.

Recommended practises for human-readable data files include:

- Comma Separated Value (csv) format
- UTF8 or UTF16 encoding (specified in the first line of the file)

File naming (rather than folder structure) to provide uniqueness based on essential metadata, for example:

- [sensor_id]_[sample_id]_[variable_name].csv
- All metadata included in the file header, before any data records

An example of a compliant self-describing file header is:

```
# -*- coding: utf-8 -*-
[metadata]
sensor_id = ABCD123
sample_id = 9689fd4a-ceae-11eb-b8bc-0242ac130003
```
variables = \( F_o, F_m, F_v \)
processing_level = 2

data_header = date_time,\( F_o,F_m,F_v \)

...[data]
2021-06-16T14:11:21.101Z,0.122,0.333,0.211

Human-readable file formats provide a clear structure, which will be generally understandable to non-specialist users. It is possible to standardize the use of such formats, but also easy to break agreed specifications. The value of human-readable file formats thus lies primarily in the ease with which users can rapidly inspect data for errors, rather than the efficiency of long-term storage or data exchange.

In contrast to text-based data formats, hybrid formats contain data written in native (binary) encoding. Access to these data requires specialized software, along with human-readable headers describing the file contents. Self-describing file types are a particularly useful sub-set of hybrid data formats, consisting of blocks of binary data following a single header. Such self-describing file formats allow data to be stored efficiently in multiple dimensions, and benefit from well-established standards for their encoding and structure, with readily available software to explore the file contents and read data. Among self-describing data formats, the Hierarchical Data Format (HDF) is commonly used to store large datasets, such as model output or satellite data. NetCDF (Network Common Data Form) is also widely adopted, and has become the standard format for CF-compliant data storage. This format contains a header and a block of data, providing a high degree of machine-to-machine data interoperability, and eliminating data loss from truncation. A wide range of netCDF file reading tools are available across computing platforms, including a number of open source options (e.g. Python, R etc.), and resources to get started reading and writing NetCDF file formats (e.g. [https://www.unidata.ucar.edu/software/netcdf/docs/index.html#what_is_netcdf](https://www.unidata.ucar.edu/software/netcdf/docs/index.html#what_is_netcdf)).

The header of a netCDF file will typically include three sections of information. The first section contains information about data dimensions, which describe the organisation of data along specified axes. In the case of ST-ChlF data, this would include flash number, excitation colour, actinic light intensity, emission band and replicate number for a typical FRRf record. If multiple records are written to a single file, dimensions such as sample time, latitude, longitude or depth would be added to separate them. Secondly, netCDF files contain a list of observed variables, their attributes (such as units of measurement), and a (sub)set of the dimensions to describe how the data in each variable are structured. Finally, files contain global metadata, such as keywords, licensing, identifiers, which apply to all variables contained in the file.

One of the main advantages of NetCDF is that it can be structured to contain any number of dimensions, with practically unlimited size. Because this file format is self-describing, the potential complexity of a data set does not prevent automated processes from extracting specific data of interest. Moreover, as the header specifies all data dimensions, a spatial or temporal ‘slice’ of data can be extracted rapidly without the need to read all data. As a result, NetCDF files are commonly used in data services, and we recommend their use as a standard method to record ST-ChlF data at all processing levels. However, it is important note that some software (e.g. most GIS
packages) will struggle to visualize netCDF files that index data in non-monotonous or discontinuous steps, whereas in situ data tend to be collected at irregular time or spatial intervals. In this case, a data reduction step is needed to aggregate the observation data into regular grids, likely at L3 processing level.

4.2.5. Service-based data exchange

The exchange of data between computers largely takes place over the internet, and offers fine-grained data retrieval mechanisms. Standards include the Web Map Service, a protocol developed by the OGC in 1999 for serving georeferenced map images over the Internet, which can be much faster than providing all underlying data. The Web Feature Service (WFS) is an Interface Standard that allows requests for discrete geographical features (measurements), and is generally suitable to represent data from environmental sensors. The WFS allows a user to constrain a data request in space and time, while also specifying a desired range for any of the requested data fields. For example, a WFS server could be queried for all ST-ChlF data obtained in 2008 from the Pacific Ocean where Fv/Fm was in the 0.5-0.6 range. Currently, there is no established data service for ST-ChlF data.

4.2.6. Existing data Repositories

In addition to selecting appropriate file formats and database structures, it is also important to identify potential repositories for a future collection of global ST-ChlF data. One candidate is the NASA SeaBASS repository, a site that already contains some ST-ChlF data. In addition (or alternatively), ST-ChlF data could be archived in the NSF-sponsored BCO-DMO (Biological and Chemical Oceanography Data Management Office). This repository also currently hosts some ST-ChlF data. While neither service presently offers the level of data interoperability needed to meet the recommendations given above, these repositories provide a starting point for discussions of best-practices in ST-ChlF data archiving.

The SeaWiFS Bio-Optical Archive and Storage System is part of the NASA Ocean Biology Distributed Active Archive Center (https://earthdata.nasa.gov/obdaac). This archive currently serves as the permanent public repository for all ocean data collected under the auspices of the NASA Ocean Biology and Biogeochemistry Program. The SeaBASS database archives data exclusively in the SeaBASS data file format, a self-describing ASCII format with a metadata header section followed by a delimited data matrix. The file format specification is described in Chapter 3 of the NASA Technical Memorandum, and is compliant with the ASCII File Format Guidelines for Earth Science Data and approved for use in NASA’s Earth science data systems. Importantly, all data submitted into SeaBASS must strictly adhere to predefined variable names conventions. The five current ST-ChlF-related parameters stored in SeaBASS are shown in Table 4.2.1, and an example ASCII dataset of ST-ChlF data is provided in Appendix E.
### Table 4.2.1: FRRf Parameters currently in SeaBASS

<table>
<thead>
<tr>
<th>Parameter Name</th>
<th>Units</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{1}\text{F-initial}$</td>
<td>unitless</td>
<td>Minimum fluorescence in darkness. Fast Repetition Rate Fluorescence measurement, commonly abbreviated as F0.</td>
</tr>
<tr>
<td>Fm</td>
<td>unitless</td>
<td>Maximum fluorescence in darkness. Fast Repetition Rate Fluorescence measurement</td>
</tr>
<tr>
<td>Fv_Fm</td>
<td>unitless</td>
<td>Maximum photochemical efficiency. Fast Repetition Rate Fluorescence measurement, commonly abbreviated as Fv/Fm</td>
</tr>
<tr>
<td>sigma_PSII</td>
<td>angstrom $^2$</td>
<td>Effective absorption cross-section of photosystem II in darkness</td>
</tr>
<tr>
<td>TauAv</td>
<td>ns</td>
<td>Fluorescence kinetics decay time (i.e., fluorescence lifetime of the excited photosynthetic unit)</td>
</tr>
</tbody>
</table>

1Note that SeaBass reserves FO to denote extraterrestrial solar radiation, and this has sometimes been used incorrectly for FRRf data (see Appendix 4).

### 4.3 Conclusions and recommendations

Storage and dissemination of new and recovered ST-ChlF data should adhere to common standards. When datasets are described using a common vocabulary, and the purpose and ownership of datasets is adequately described, they can serve a wide range of purposes for the global research community. The provision of standard metadata will support data quality control and the ability to reprocess primary observations using evolving conceptual models and numerical fitting procedures. There is no current repository that efficiently handles the complexity of ST-ChlF data, and this is something that should be explored as a next step. Existing data stores could fulfil this role, or new ones could be created. The use of data cataloguing services and harmonized data vocabularies within OGC-compliant data stores is essential to ensure the widest possible Findability, Accessibility, Interoperability and Reusability of environmental data. With appropriate attention to robust data archiving practices, large scale compilations of inter-comparable ST-ChlF data could significantly enhance our understanding of global-scale variability in phytoplankton productivity and photo-physiology.
5. INTERPRETATION AND INTEGRATION

5.1 Comparing $E_{TRPSII}$ to other photosynthetic currencies

A primary motivation for ST-ChlF measurements is the acquisition of high resolution, real-time phytoplankton primary productivity estimates (e.g. Hughes et al. 2018). At best, ST-ChlF data can be used to provide a robust measure of photosynthetic electron transport rates ($E_{TRPSII}$, section 2.3). These rates should scale stoichiometrically with gross O$_2$ evolution from PSII, with 4 electrons extracted from every molecule of O$_2$ released from the water splitting reaction. In practice, a number of interacting taxonomic and environmental factors complicate the relationship between $E_{TRPSII}$, O$_2$ evolution and other primary productivity metrics, including carbon fixation (Lawrenz et al. 2013). This section discusses how ST-ChlF-based $E_{TRPSII}$ estimates relate to other, more commonly reported measures of phytoplankton photosynthesis, with a particular focus on O$_2$ evolution and C fixation. We discuss the underlying physiological processes that can decouple the photosynthetic ‘currencies’ of electrons, O$_2$ and C, briefly review information on $E_{TRPSII}$ : O$_2$ : C stoichiometry, and provide recommended procedures for direct comparison of $E_{TRPSII}$ estimates against other productivity measures. We discuss the use of $E_{TRPSII}$ as an empirical proxy for $^{14}$C / O$_2$ cycling, while also considering the physiological insights that can be gained by examining differences and decoupling between these productivity metrics.

5.1.1 Photosynthetic currencies

Electrons are the fundamental currency of biochemical reactions. Consequently, photosynthetic electron transport rates ($E_{TRPSII}$) provide inherently meaningful estimates of primary productivity in their own right. ST-ChlF measurements can be used to estimate the rate of charge separation in PSII, commonly referred to as $E_{TRPSII}$, but also known as a photochemical flux, $J_V^{PSII}$ (section 2.3). This rate quantifies the generation of low-potential reductant in aquatic ecosystems (e.g. Falkowski et al. 2008). Not surprisingly, a major goal in aquatic biology in recent decades has been to predict O$_2$ production or C fixation from $E_{TRPSII}$, thus enabling high resolution productivity estimates in these ecologically-important currencies. However, there is no single conversion factor to reliably interrelate $E_{TRPSII}$ with rates of O$_2$ production or C fixation across taxa or different environmental conditions. While individual sampling campaigns have often reported good correlations between $E_{TRPSII}$ and simultaneous measures of O$_2$ evolution or C fixation (Kolber and Falkowski 1993; Lawrenz et al. 2013 and references therein), the nature of the relationship is influenced by the physiological states (e.g. Halsey et al. 2011; Brading et al. 2013) and/or community taxonomic composition (e.g. Suggett et al. 2009; Hughes et al. 2021; Fisher et al. 2020) of phytoplankton assemblages, reflecting acclamatory or adaptive strategies that determine the flow of electrons through various metabolic processes.

The theoretical value for the ratio of $E_{TRPSII}$ to gross O$_2$ evolution is 4 e\textsuperscript{−} for each O$_2$ produced in PSII. In the case of $E_{TRPSII}$:C, also known as the electron requirement for C fixation ($\Phi_{e,C}$, Lawrenz et al. 2013; or $K_c$, Hancke et al. 2015), values of 4.8 to 5.6 or higher are expected, assuming a photosynthetic quotient (O$_2$:C) of $>$1, where photosynthetically generated reductant and ATP are used in metabolic processes other than C fixation. $E_{TRPSII}$:C values of this magnitude
have been observed in phytoplankton cultures (Halsey and Jones 2015) and field-based campaigns (Juranek and Quay 2013), though most studies have reported higher and more variable values than these theoretical numbers. A meta-analysis of field-based measurements of the ETP_{PSII}:C relationship yielded a global mean of 10.9 ± 6.9, with values ranging from <5 to >50 (Lawrenz et al. 2013). Low values of ETP_{PSII}:C indicate tight coupling of PSII electron transport and C fixation, leading to high efficiency in the conversion of photosynthetic energy to biomass. Such low values are usually observed in phytoplankton cells operating at their physiological optimum. In contrast, higher ETP_{PSII}:C values result from the diversion of photochemically generated reductant away from C assimilation, and are typically observed under conditions of transient stress (e.g. nutrient limitation or high light stress). Thus, higher ETP_{PSII}:C values might be expected in dynamic ocean environments where continuous optimum resource availability is rare (Lawrenz et al. 2013).

Given the number of interacting factors that can potentially drive ETP_{PSII}:C variability, this parameter cannot be easily reconciled with prevailing nutrient conditions or taxonomy. Predicting ETP_{PSII}:C from other oceanographic properties therefore remains problematic, and is likely best avoided until more data become available. However, recent work suggests that it may be possible, in some cases, to estimate ETP_{PSII}:C from other metrics of phytoplankton physiology, including NPQ SV (Schuback et al. 2016). More field campaigns collecting parallel ETP_{PSII} and C fixation (O2 evolution) measurements are needed to overcome this current challenge, while continued laboratory experiments will deepen mechanistic understanding of variability in ETP_{PSII}:C (and ETP_{PSII}:O2) stoichiometry. As discussed below, such future work should seek to employ (and document) common methodological approaches to avoid introducing unnecessary sources of variability into the resulting data.

5.1.2 Towards a more mechanistic understanding of electron-oxygen-carbon coupling

As noted above, the electron transport rate at PSII (ETP_{PSII}) can be assumed to be directly related to gross O2 production. However, a wide range of metabolic processes, occurring on various timescales, may consume photosynthetically-generated electrons and O2, or oxidize photosynthetically fixed C, thus changing net ETP_{PSII}:O2 and ETP_{PSII}:C stoichiometry. These metabolisms can be grouped into two general processes: (1) alternative electron flows, including water-water cycles (Asada 1999; Allahverdiyeva et al. 2013) affecting ETP_{PSII} and O2 budgets; and (2) C assimilation and respiration affecting C and O2 budgets. Brief descriptions of how these metabolic processes influence the photosynthetic energy budget are given below, and represented schematically in Fig. 5.1.1. Other processes, including nitrogen and sulfur reduction, carbon excretion and photorespiration should also be considered to fully complete the photosynthetic energy budget, but these are not discussed further here. The key point is that the ETP_{PSII}:C and ETP_{PSII}:O2 stoichiometry depend on the degree to which cells rely on different metabolic pathways, either through evolutionarily-conserved, taxon-specific metabolisms, or under the transient influence of one or more environmental variables.

1. Short water-water cycles refer to a collection of biochemical pathways that function only in the light, do not involve a C intermediate, and operate to maintain proper cellular balance of ATP and NADPH. Electrons produced at PSII (derived from water) are energized and passed through a series of electron carriers, creating a proton motive force to drive ATP generation. The photochemical Z-scheme describes how these electron carriers function not only to generate
energy, but also to bridge the two photosynthetic reaction centers in PSII and PSI. However, some electrons can be diverted away from the intersystem electron transport chain, or be consumed before or after PSI to reduce O₂ to H₂O or H₂O₂ to H₂O₂ (Figure 5.1.1). These ‘short water-water cycles’ are sometimes associated with sudden high light exposure and are thought to function as ‘overflow valves’ to prevent photodamage to PSII (Niyogi 2000). However, water-water cycles are also measurable at low light intensities, and are thus recognized as an important additional source of ATP generated through the flow of protons from the lumen to the stromal side of the thylakoid membrane (e.g. Fisher and Halsey 2016). There is some evidence that regulatory control over these pathways varies across phytoplankton taxa (Fisher et al. 2020). Furthermore, an increased reliance on ATP production via water-water cycles may be an important strategy to conserve fixed C by decreasing rates of C respiration for ATP production (Fisher and Halsey 2016). Importantly, ‘cyclic’ electron flows mediated by PSI alone can also achieve similar outcomes without directly affecting the ETRPSII:C ratio (Halsey et al. 2014; Hughes et al. 2018).

2. Carbon fixation and respiration: In cells growing under optimal physiological conditions, a significant proportion of photosynthetic electron flow is allocated to C fixation. Culture studies suggest that as much as 50-75% of electrons produced at PSII are used to reduce NADP⁺ to NADPH, which is subsequently used to reduce CO₂ into organic C. However, direct measurements of gross C fixation rates are challenging, as fixed C can be rapidly (within seconds) catabolized for energy generation or during biosynthesis of amino acid, nucleotide, lipid, and other biochemical intermediates (e.g. Halsey et al. 2011; Hughes et al. 2018, Figure 5.1.1). Thus, experimentally determined ETRPSII:C ratios will vary depending on the timescale over which C fixation is integrated (e.g. Halsey et al. 2013; Hughes et al. 2018, Figure 5.1.1), with ETRPSII:C increasing over time as C fixation rate measurements increasingly reflect as signature of respiration (i.e. approaching net, rather than gross photosynthesis).

Fixed C that is respired via glycolysis and the TCA cycle yields CO₂ and NADH. The NADH is transferred to the respiratory electron transport chain, terminating with O₂ reduction to H₂O. As such, respiration affects the ratio of ETRPSII and net C fixation and O₂ production. As a general rule of thumb, about half of the total pool of daily fixed C is respired back to CO₂. Historically, C respiration rates were considered to be independent of time of the day, but it is now apparent that some species use alternate forms of ATP production during day and night, or during periods of intense resource limitation (Halsey and Jones, 2015).

5.1.3 Precise definition of derived stoichiometries

A major challenge in reconciling ETRPSII estimates with other primary productivity measures is the need to precisely define all relevant quantities, and understand what exactly is being measured by the specific methods employed. In the latter case, the integration time of measurements is often a key factor. As noted above, ETRPSII:C can have very different values and variability depending on the timescales of C assimilation experiments (Table 5.1.1). These methodological differences will drive variability because the turnover time of cellular C varies in response to a number of factors, including time of day, cell physiological status, etc. This variability has been observed in culture experiments where C fixation demonstrates a transition from gross towards net C uptake as the length of measurements increase (Halsey et al. 2011, 2013; Felcmanová et al. 2017). This
time-scale dependence raises challenges in the field, where longer-term incubations are required to capture meaningful change using $^{14}$C or $^{13}$C labeled samples.

**Table 5.1.1** definition of measured quantities related to photosynthetic electron, carbon and oxygen cycling

<table>
<thead>
<tr>
<th>Stoichiometry</th>
<th>Definition</th>
<th>Value dependent on</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{ETR}<em>{\text{PSII}} : C</em>{(\text{gross})}$</td>
<td>electrons produced at PSII / electrons invested into the Calvin cycle</td>
<td>magnitude of water-water cycles, Cyclic Electron Flow-PSII</td>
</tr>
<tr>
<td>$\text{ETR}<em>{\text{PSII}} : C</em>{(\text{net})}$</td>
<td>electrons produced at PSII / electrons in biomass after C catabolism</td>
<td>magnitude of water-water cycles, carbon respiration, and biosynthetic investments</td>
</tr>
<tr>
<td>$\text{ETR}<em>{\text{PSII}} : O_2</em>{(\text{net})}$</td>
<td>electrons produced at PSII / electrons remaining after respiration</td>
<td>magnitude of water-water cycles and carbon respiration</td>
</tr>
</tbody>
</table>

**Figure 5.1.1:** Conceptual summary of the coupling of electrons (dotted lines), O$_2$ (thick gray lines), and C (solid black lines) within a photosynthetic cell over timescales from milli-seconds to days. The direction of arrows indicates production and consumption. Major and minor pathways are indicated by the size of the arrows. Particulate organic nitrogen (PON) and particulate organic carbon (POC) are indicated as grey shapes. In the case of POC, the grey triangle reflects the decrease in POC retained within a cell over time (gross vs net C assimilation). Figure adapted from Hughes et al. (2018)
5.1.4 Key recommendations

Conceptual frameworks need to be developed to interpret variation in ETR$_{PSII}$:C and ETR$_{PSII}$:O$_2$ resulting from environmental and/or taxonomic controls on cellular metabolism. Towards this goal, larger datasets of parallel ETR$_{PSII}$ and C fixation and O$_2$ evolution measurements are required to better understand their co-variability. Such datasets, if collected using consistent and well-documented methodologies, will further support the development of algorithms describing the electron requirement for carbon fixation.

Reporting of ETR$_{PSII}$ but also O$_2$ and C fixation must be standardized, including information on the time scales of measurements, and ancillary data required to interpret environmental, physiological or taxonomic influences. Measurements of ETR$_{PSII}$ and C fixation (or O$_2$ evolution) should be made simultaneously, whenever possible. In the ideal case, measurements should be conducted on the same sample at the same time, for example by quantifying C fixation (or O$_2$ evolution) within an ST-ChlF instrument optical chamber (e.g. Hughes et al. 2021; Hupp et al. 2021). Such dual measurements avoid the need for spectral correction factors and ancillary data, which can propagate errors. As outlined in section 4, it is desirable that parallel measures of primary productivity are archived, alongside primary ChlF parameters, to a centralized data repository.

5.2 Beyond productivity: Other ST-ChlF applications in aquatic environments

The application of ST-ChlF measurements in aquatic research has often been centered around direct estimates of phytoplankton primary productivity, as quantified by ETR$_{PSII}$ (sections 2.3 and 5.1). Beyond this important goal, ST-ChlF measurements can also yield additional insight into phytoplankton physiological ecology. Below, we outline how high-resolution ST-ChlF measurements can advance our understanding of phytoplankton physiological ecology, and taxonomic composition.

5.2.1 ChlF as a proxy for chlorophyll a concentration

Conventional in vivo Chl a fluorometers specifically induce and measure ChlF from living phytoplankton with a constant excitation source. Instruments of this kind have a long history in aquatic research (Lorenzen 1966), and are routinely deployed for real-time monitoring of phytoplankton biomass in surface waters along a cruise track (drawing samples from a continuous seawater supply), or alongside other sensors during CTD-rosette depth profiles. These instruments can also be deployed autonomously, and are a core part of BGC-Argo floats (e.g. Claustre et al. 2020). As a result, large global datasets of in vivo ChlF measurements are widely available.

The widespread use of in vivo Chl a fluorescence as a proxy for Chl a concentration (which is, itself, a proxy for phytoplankton biomass) assumes that the ratio between the ChlF signal and Chl a concentration is constant. However, it is well known that this ratio varies widely over space and time, in response to numerous taxonomic and environmental influences, thus necessitating correction approaches (e.g. Proctor and Roesler 2010; Xing et al. 2017; Roesler et al. 2013;
Thomalla et al. 2018). In contrast to conventional in vivo fluorometers, ST-ChlF instruments are specifically designed to induce changes in the ratio of ChlF and Chl $a$ concentration, and exploit the information inherent in variable ChlF quantum yields (see section 2.1 for definitions of quantum yield). By providing information on ChlF quantum yield variability, ST-ChlF measurements can provide information required to improve Chl $a$ biomass estimates from conventional in vivo fluorometers.

There are three main factors affecting the ratio of the ChlF signal to Chl $a$ concentration: 1) Non-photochemical quenching (NPQ, section 3.4); 2) the distribution of Chl $a$ molecules within a phytoplankton cell (‘pigment packaging’, e.g. Bricaud et al. 1995, Huan 2022); and 3) fluorescence from sources other than Chl $a$ (e.g. other pigments or CDOM). We discuss these factors below, and provide suggestions on how ST-ChlF measurements can be used to address them.

Excess light availability in surface waters is well known to induce NPQ and thus reduce the ChlF detected per Chl $a$ concentration (e.g. Marra and Langdon 1993; Morrison 2003; Serôdio and Lavaud 2011; Roesler and Barnard 2013 - see also section 3.4). Previous work has been directed towards correcting NPQ effects on in vivo ChlF-based phytoplankton biomass estimates (e.g. (Xing et al. 2012; Thomalla et al. 2018), yet no consensus on a best approach currently exists. One issue for many current NPQ correction approaches is the complex nature of factors regulating NPQ variability, including light history, nutrient status, and taxonomic composition of phytoplankton communities. ST-ChlF approaches are well suited to provide high resolution data on NPQ dynamics in phytoplankton in the field and under controlled single-species laboratory conditions. Through the collection of large, geographically-distributed datasets and laboratory culture experiments, ST-ChlF measurements could be used to examine the relationship between NPQ, nutrients, temperature, light and phytoplankton taxonomy across different ocean regions. These measurements would provide empirical data and new mechanistic understanding to improve NPQ-correction of phytoplankton biomass estimates from conventional Chl $a$ fluorometers.

The pigment ‘package effect’ results from a reduction in Chl $a$ absorption efficiency due to self-shading. This effect is particularly significant in larger cells, leading to a decrease in $a^*(\lambda)$, and increased re-absorption of ChlF (Bricaud et al. 1995, Huan et al. 2022). Pigment packaging can thus generate systematic error in Chl $a$ concentrations estimated with conventional in vivo fluorometers. Boatman et al. (2019) recently developed an approach to estimate the extent of pigment packaging based on changes in ChlF re-absorption. The approach, further explained in Oxborough (2022), quantifies changes in the ratio of ChlF at 680 nm (strong re-absorption) and ChlF at 730 nm (weak re-absorption). If validated with in situ data from a range of oceanographic conditions, the approach could provide useful data to improve the parameterization of pigment packaging and associated changes in $a^*_\text{ph}(\lambda)$. Improved estimates of pigment packaging and its effects on $a^*_\text{ph}(\lambda)$ would also be desirable for deriving the quantum yield of fluorescence from remotely sensed sun induced fluorescence (Gupana et al. 2021), and for the improvement of remote sensing algorithms quantifying Chl $a$ concentration in the surface ocean (Naik et al. 2013; Lee et al. 2020).

Finally, the ChlF signal detected with conventional in vivo fluorometers can be affected by fluorescence from sources such as CDOM or other pigments (Proctor and Roesler 2010; Xing et al. 2017). In this case, multi-spectral excitation and emission bands available in many current ST-
ChlF instruments can provide information regarding the presence of pigments and other compounds that may interfere with the ChlF signal detected by conventional in vivo fluorometers.

In summary, ST-ChlF instruments are well suited to examine the factors decoupling ChlF from Chl concentrations, thus improving phytoplankton biomass estimates from in vivo Chl \( a \) fluorometers. Importantly, ST-ChlF data can be acquired autonomously and at high resolution, thus providing large datasets suitable for the development of empirical modeling approaches.

In addition to contributing to the correction approaches described above, the primary ST-ChlF parameters \( F_o \) and \( F_m \) (see section 2.1) are, themselves, both suitable as proxies for Chl \( a \) biomass. By definition (see section 2.1), both \( F_m \) and \( F_o \) are measured in the dark-regulated state in which NPQ processes are relaxed. These two parameters are typically reported in relative units, but they can be readily calibrated against Chl \( a \) concentration from low resolution discrete measurements. Even without calibration, relative changes in \( F_o \) or \( F_m \) can be reliably used to track relative changes in phytoplankton biomass.

5.2.2 ChlF as an indicator for iron-limitation

The availability of iron limits phytoplankton primary productivity in up to one third of oceanic waters, most notably in the Southern Ocean and Subarctic Pacific (Boyd et al. 2007; Moore et al. 2013). Iron plays a fundamental role in the photosynthetic electron transport chain, and therefore directly affects the conversion of light energy to organic carbon (e.g. Yruela 2013). A number of studies have used the ST-ChlF-derived parameter \( F_v/F_m \) as a sensitive indicator for iron limitation in phytoplankton assemblages. Here we discuss current understanding of iron limitation effects on phytoplankton photo-physiology, and the influence of iron limitation on ST-ChlF signatures, including \( F_v/F_m \). We also describe how iron-dependent changes in ChlF may affect the interpretation of ST-ChlF parameters in terms of photosynthetic rates and productivity.

Iron limitation significantly affects phytoplankton ChlF properties (Behrenfeld and Milligan 2013, Schallenberg et al., 2022), leading to an increase in ChlF per Chl \( a \) concentration, and an increase in ChlF per absorbed light. This iron-dependent increase in ChlF is easily and rapidly detectable as a decrease in measured \( F_v/F_m \) using ST-ChlF approaches (e.g Suggett et al. 2009), and also as an increase in the apparent quantum yield of ChlF from remote sensing (Behrenfeld et al. 2009) and BGC Argo data (Schallenberg et al., 2022). The mechanistic basis for the diagnostic increase on ChlF under iron stress was initially attributed to the presence of damaged reaction centers (Greene et al. 1994; Kolber et al. 1994; Falkowski and Kolber 1995), but it is now widely accepted to be caused by the presence of so-called energetically-disconnected light harvesting complexes (edLHCII, Schrader et al. 2011; Behrenfeld and Milligan 2013; Macey et al. 2014; Park et al. 2017; Sherman et al. 2020).

Iron-dependent changes in ChlF have important implications for the interpretation of \( F_v/F_m \) as the maximum quantum yield of PSII photochemistry (\( \Phi_{PSII} \), table 2.1.3), and the interpretation of \( F'_q/F'_m' \) as the realized quantum yield of photochemistry in PSII (\( \Phi_{PSII}' \), table 2.1.3). Both of these parameters are commonly used to estimate ETR\(_{PSII} \) (section 2.3). The interpretation of \( F_v/F_m \) and \( F'_q/F'_m' \) as quantum yields of PSII photochemistry explicitly assumes that all measured ChlF is emitted from pigments functionally associated with photochemically-active PSII. However, under
iron-limiting conditions, a significant fraction of ChlF is emitted from edLHCII (or a mixture of edLHCII and damaged PSII reaction centers). This increase in non-variable ChlF is more appropriately recognized as an increase in baseline fluorescence (section 3.3.4), which must be properly accounted for in the derivation of ETR$_{PSII}$ estimates (e.g. Boatman et al. 2019). All approaches for ETR$_{PSII}$ estimation outlined in section 2.3 are, to some extent, affected by baseline fluorescence, and no robust correction approaches have thus far been established and tested for a wide range of species and environmental conditions.

In summary, low F$_{v}$/F$_{m}$ and its rapid increase after iron addition represent a reliable and sensitive proxy for iron limitation in phytoplankton. The value of such a well-established proxy is immense, and the concept can be extrapolated to ocean basin and even global scales (Behrenfeld et al. 2009, Lin et al., 2016). At the same time, however, iron-dependent changes in ST-ChlF measurements can increase baseline ChlF, which must be accounted for in order to derive robust ETR$_{PSII}$ estimates.

5.2.3 Using NPQ to constrain ETR$_{PSII}$:C stoichiometry

To convert ST-ChlF-derived ETR$_{PSII}$ into ecologically-relevant carbon uptake estimates, the stoichiometry of the two rates must be known (section 5.1). Given that ETR$_{PSII}$:C stoichiometry is highly variable across environments and taxonomic groups (section 5.1.1), a proxy for the conversion factor is needed to estimate rates of carbon uptake from ETR$_{PSII}$. Ideally, such a proxy can be acquired at high resolution, and without additional measurements.

As suggested by Schuback et al. (2015, 2016), the extent of NPQ$_{SV}$ as measured by ST-ChlF measurements can provide such a proxy. Empirical evidence has demonstrated a strong correlation between NPQ$_{SV}$ and ETR$_{PSII}$:C, with both NPQ$_{SV}$ and ETR$_{PSII}$:C increasing under conditions of excess energy and/or limited downstream photosynthetic capacity (e.g. iron limitation). Under such conditions, excess energy can be dissipated upstream of the photosynthetic electron transport chain (increased NPQ), or downstream by shunting electron flow (and thus reducing power) to alternative sinks (e.g. Niyogi 2000), increasing the ETR$_{PSII}$:C ratio. In this way, NPQ and ETR$_{PSII}$:C provide parallel, phenomenologically-linked pathways to dissipate excess absorbed light energy, making NPQ a potential proxy for photosynthetic electron : carbon stoichiometry. Importantly, the relationship between NPQ$_{SV}$ and ETR$_{PSII}$:C appears to vary across oceanic environments, suggesting that regional and time-specific validation of this relationship may be needed to fully exploit NPQ$_{SV}$ measurements as a proxy for ETR$_{PSII}$:C. This will require the simultaneous measurement of ST-ChlF and C fixation across a range of oceanic regions over different portions of the seasonal cycle.

5.2.4 Spectral deconvolution of communities

In addition to measuring bulk phytoplankton photo-physiological properties, multi-spectral ST-ChlF data can provide information on the taxonomic composition of mixed phytoplankton assemblages (e.g. Gorbunov et al., 2020, Courtecuisse et al., 2023), based on group-specific differences in light absorption properties. Although the variable ChlF at ~680 nm stems primarily from Chl a associated with PSII, other pigments present in the PSII light harvesting antenna can
pass absorbed energy on to Chl \(a\), thus contributing to the measured ChlF signals. Current ST-ChlF instruments are not yet able to fully resolve entire ChlF emission spectra, as is possible with fluorescence spectrometers (e.g. Seppälä and Balode, 1998; Seppälä and Olli, 2008). However, some ST-ChlF instruments can measure ChlF excitation at 5-8 wavebands, which is sufficient to resolve characteristic spectral profiles. Importantly, such profiles do not merely track ChlF emission, but provide a suite of wavelength-specific primary and secondary ST-ChlF parameters. Additionally, different bandpass filters can be used to detect fluorescence emission at multiple wavelengths, further increasing the taxonomic resolution of ST-ChlF measurements. For example, fluorescence emission at 650-670nm arising from phycobiliproteins and phycobilisomes found in cyanobacteria and cryptomonads can be used to monitor the occurrence and distribution of these groups (Seppälä et al., 2007). Looking forward, exciting possibilities exist for the interpretation of multi-wavelength ST-ChlF data in the context of phytoplankton community composition. However, some words of caution are also necessary.

An important caveat in all pigment-related taxonomic identification is the fact that specific pigments are rarely unique to individual taxa. Moreover, the relative concentrations of pigments within even a single group will change in response to light and nutrient availability. These caveats limit the taxonomic specificity of pigment-related analysis, and introduce sources of variability that can be difficult to constrain. Another limitation is the effect of ‘pigment packaging’, where variations in intracellular pigment concentration affect light absorption and ChlF (Bricaud et al. 1995, Huan 2022). Variability in spectral shape due to pigment packaging is likely greater among species of the same phytoplankton group under different environmental conditions, than among different spectral groups. In the specific case of ST-ChlF measurements, systematic error can be introduced into spectrally-resolved ST-ChlF parameters if less well-absorbed wavelengths do not provide sufficient energy for full saturation of a ChlF transient during the ST saturation phase (section 3.2.1).

Notwithstanding these caveats, ST-ChlF instruments have great potential for fine-scale monitoring of phytoplankton taxonomic succession or early detection of harmful algal blooms. In the future, it will be particularly important for the research community to identify the most useful excitation and emission wavelengths for the detection and discrimination of specific phytoplankton groups. In the interest of compiling globally-coherent and inter-comparable datasets, an effort should be made to match the wavelengths used in different instrument types. The development of multi-spectral ST-ChlF instruments coincides with the increasing use of spectrally-resolved approaches in remote sensing and biogeochemical modelling. Further development and application of ST-ChlF instruments should consider the wavebands measured by satellites, or target key taxa as identified by biogeochemical modelling.

5.2.5. Phenotyping: Resolving functional diversity within and between species

A more recent growth area of interest for ST-ChlF measurements is the use of retrieved photophysiological properties to study functional diversity (i.e. phenotypes) across natural samples or species isolates, with the goal of predicting environmental changes in phytoplankton community form and functioning. Phenotyping can be achieved by examining various aspects of productivity
(e.g. ETR$_{PSII}$, carbon uptake, growth rate, e.g. Suggett et al. 2015), but it is often desirable to examine underlying photo-physiological properties driving productivity differences across phenotypes (Fisher et al. 2020; Suggett et al. 2022). For example, diatoms can be separated into different phenotypes, which are inherently adapted to be more competitive under either variable or stable light environments (Fisher et al. 2020). Recent phenotyping using ChlF (including ST-based platforms) has been used in agriculture and forestry to develop high-throughput screening for stress-resistant plants (e.g. Keller et al. 2019). A similar ‘prospecting’ approach has recently been proposed to identify stress-tolerant corals (Suggett et al. 2022), and also for specific microalgae with phenotypes desirable for a particular industry (Behrendt et al. 2020). Regardless of the particular application or research question, ST-ChlF phenotyping rests on accurate and consistent parameterization of multiple photo-biological traits, which can then be applied to complex computational platforms, including machine learning. Identifying meaningful traits that separate functional variants of interest can then be integrated into longer-term deployments within the framework of ‘routine’ ST-ChlF data collection and ETR$_{PSII}$ estimates.
6. OPPORTUNITIES AND CHALLENGES FOR FIELD-BASED MEASUREMENTS

6.1 Introduction

Over the past two decades, oceanic ST-ChlF data have been collected from various sampling platforms, including research vessels (Behrenfeld and Kolber, 1999), coastal observatories (Tortell et al. 2014), and, more recently, autonomous floats, gliders and moorings (Fujiki et al. 2008, Carvahlho et al. 2020). Each of these data collection platforms has unique strengths, limitations and operational constraints, which must be carefully weighed in the context of a particular research objective. In this section, we discuss the main operational issues affecting field deployments of ST-ChlF measurements, and recommend sampling and analysis approaches relevant to different applications. Key factors include uncertainty regarding the light history of samples prior to measurements, the need to monitor blanks and biofouling of optical surfaces, and potential cell damage during sample collection. As with other aspects of ST-ChlF measurements, optimal approaches will depend on the research questions of interest, as well as the environmental conditions and taxonomic composition of phytoplankton assemblages under investigation.

The discussion below is organized by sampling method. We begin with the most common approach used presently, where ST-ChlF measurements are made on oceanographic vessels with bench-top instruments for discrete or continuous analysis. We then consider the challenges of unattended deployment of ST-ChlF instruments on vessels of opportunity (e.g. ferries and cargo ships), which are becoming increasingly common as a means of expanding ocean data coverage (Rosa et al. 2021). Finally, we discuss in situ (i.e. underwater) deployment of ST-ChlF instruments, including depth profile measurements on CTD casts, and truly autonomous in situ measurements (e.g. on moorings and gliders). We conclude by considering how ST-ChlF measurements can most effectively leverage other oceanographic data. In this respect, we describe critical ancillary measurements needed for the robust interpretation of ST-ChlF data, and opportunities to combine these data with other oceanographic measurements of phytoplankton biomass, taxonomic composition and productivity. As discussed below, such multi-instrument measurements can provide a rich context to understand ocean productivity and biogeochemical cycles.

6.2 ST-ChlF measurement on research vessels

6.2.1 Sample Collection and Handling

Discrete samples—Research vessels provide many advantages for the collection of ST-ChlF data, with significant infrastructure to support scientific observations, including reliable electrical power and the possibility of operator oversight. On the other hand, ship-based sampling requires water collection and ex situ analysis, which can introduce measurement artefacts. Most oceanographic vessels are equipped with one or more types of sampling bottles that are appropriate for the collection of discrete samples for ST-ChlF measurements. As these bottles are opaque, seawater is exposed to a period of darkness prior to analysis, which can be relatively long in cases where
water is collected at multiple depths. At the same time, deck-board processing of samples can expose phytoplankton to surface high irradiance levels and increased temperature. To minimize such light and temperature shock, we recommend that sub-samples be collected into opaque bottles, and maintained close to *in situ* ocean temperature (*e.g.* in a cooler, or a flow-through incubator). Protocols may include a 15 min exposure to low light before measurements if dark-regulated primary ST-ChlF parameters are required (see section 3.4.3 and below).

Several other considerations are important during the collection of discrete samples. First, where measurements are compared across multiple sampling stations, it is advisable to collect water at consistent times of the day (*e.g.* near sunrise) to eliminate variability introduced by strong diel cycles (*e.g.* Schuback and Tortell, 2019). For example, samples collected and analysed near mid-day would be more prone to photo-inhibition than those collected at dawn. Irrespective of sampling time, it is recommended that samples be exposed to a period of low light in order to relax NPQ processes (see section 3.4 and below). In many cases, it will also be necessary to minimize trace metal contamination of samples (Fitzwater et al. 1982), with sampling bottles typically acid-cleaned before use (Cutter et al. 2017). This is particularly true in cases where samples are incubated for an extended period to monitor responses to nutrient additions or light manipulations. Notably, trace metal-dependent effects can exert a strong influence on ST-ChlF signatures prior to any detectable growth rate or biomass response (Ryan-Keogh et al., 2013).

*Underway sampling*—Continuous ‘underway’ seawater sampling allows real-time data collection, providing measurements at the spatial and temporal resolution needed to resolve fine-scale oceanographic variability. Such continuous sampling has been routinely used to measure seawater hydrography (temperature and salinity) and bulk phytoplankton biomass (from in vivo Chl *a* fluorescence), and is also well suited to exploit the rapid and non-invasive capabilities of ST-ChlF measurements. As underway observations do not require vessels to stop at sampling stations, these measurements are also less affected by weather and logistical constraints.

Research vessels typically have dedicated scientific seawater supplies, but the nature and flow dynamics of these systems varies widely. Both the type of pump used to deliver the seawater to the ship’s laboratories and the residence time of seawater in the supply lines are particularly relevant for ST-ChlF measurements. These factors affect the potential for cell damage during sampling, and determine the time of dark exposure prior to analysis.

Significant attention has been given to the nature of seawater supply lines on research vessels. In the case of dissolved gas measurements, and for O₂ in particular, the accumulation of biofilms on the walls of the seawater plumbing can affect measurements by introducing an additional signal of bacterial respiration (*i.e.* O₂ consumption). It is unclear how the presence of such biofilms might affect ST-ChlF measurements, though it is possible that they could produce higher background DOC concentrations, which could influence fluorescence blanks (section 3.3). Similarly, any non-biological particulates, such as small rust particles, derived from the seawater supply line could increase optical back-scatter and blanks. For these reasons, it is important to use the cleanest supply lines possible. Juranek *et al.* 2010 presented an approach for periodically cleaning the seawater sampling lines on research vessels, typically during a port call.
The pumps used to deliver seawater to ship-board laboratories can also directly affect ST-ChlF and other photo-physiological measurements. Certain pumps, including impeller and gear pumps, introduce significant shear during water collection, potentially leading to cell damage and rupture, and a decrease in apparent phytoplankton biomass and/or physiological activity. Particular phytoplankton taxa, such as diatoms with cell walls and opal shells, may be less sensitive to damage from sampling pumps, whereas more fragile cells such as nanoflagellates may be disproportionately affected, creating a taxonomic bias in the resulting measurements. A number of sampling pumps are likely to be less damaging to phytoplankton, including diaphragm pumps, peristaltic pumps, screw pumps and progressive cavity pumps. Discussions with the ship’s engineers are important to understand the nature of the pumps used in the seawater supply system. At the same time, users should examine potential sampling artefacts by comparing ST-ChlF measurements in samples obtained simultaneously from the seawater line and discrete bottles at a matching depth (typically 5 – 7 m for most underway systems). Good agreement between these measurements provides evidence that the continuous sampling system is not introducing significant cell damage. For more information on the selection of appropriate seawater pumps for phytoplankton measurements, readers are referred to Boss et al. (2019).

In practice, water for ST-ChlF analysis is not directly drawn from a ship’s underway system. Rather, a secondary water supply system is typically used to provide a lower and more constant flow into the measurement cuvette. For example, the ship’s primary seawater supply can be used to pump water through a container that serves as a gravity feed source for ST-ChlF measurements. In other cases, a secondary sampling loop (often using a peristaltic pump) can be connected, via T-junction, to a primary seawater supply. This allows more precise control and lower flow rates of water to the sampling cuvette, and also helps minimize the introduction of bubbles, which substantially increase optical backscatter. To ensure adequate flushing of samples through the measurement chamber, water should be introduced near the bottom of a ST-ChlF sampling cuvette (avoiding interference with any optical detectors), with a return flow coming from an exhaust port near the top. For continuous underway analysis, users must choose an appropriate flow rate to achieve a desired sample residence time in the measurement cuvette. Automated pumps and/or valves can be used to achieve a combination of continuous flow-through measurements and discrete analysis (e.g. in the case where periodic PE curves will be run on discrete samples; Sezginer et al. 2021, Oxborough 2022).

6.2.2 Light Exposure History

As discussed in section 3.4, the light history exposure of phytoplankton has a direct effect on ST-ChlF measurements, with notable differences between cells measured following NPQ relaxation as compared to light-regulated states. As most ST-ChlF instruments are equipped with background actinic LEDs, light-regulated measurements can be obtained with relative ease, since photochemical and non-photochemical processes appear to reach steady-state levels quickly. By comparison, the relaxation of NPQ processes needed for dark-regulated measurements requires significantly longer, with time-scales ranging from 10 minutes to up to an hour or more (Kolber and Falkowski 1993; Gorbunov et al. 2001, section 3.4). Exposure of samples to dim light (<10 μmol quanta m⁻² s⁻¹) can accelerate the relaxation of non-photochemical quenching and recovery from photo-inhibition (Fig. 3.4.3), but dark-regulated states may, nonetheless, be difficult to achieve during short measurement windows.
For measurement of discrete samples, users typically implement a low-light NPQ-relaxation period prior to measuring cells in dark-regulated states. The challenge, however, is that different natural samples may require varying low-light acclimation times, depending on the taxonomic composition of the phytoplankton assemblages, and environmental factors such as temperature (NPQ relaxation is slower in cold water; Guangyan et al. (2017). When sampling in relatively homogeneous environments, it may be feasible to find a representative low-light NPQ relaxation period that is suitable for the majority of samples. The time-scale of this NPQ relaxation period can be assessed by holding samples in low light and making repeated measurements of Fv/Fm until a plateau is reached (see section 3.4). Where significant heterogeneity exists among sampling locations, it may be necessary to repeat such experiments with higher frequency to characterize the varying time-scales of NPQ relaxation.

The challenge of achieving dark-regulated states is particularly significant during continuous underway data collection, when cells experience relatively short periods of darkness in transit through the seawater supply lines. Under these conditions, photochemical quenching may be fully relaxed prior to measurements, but NPQ effects are still expected. As a result, phytoplankton will display physiological characteristics intermediate between light and dark-regulated states, making the resulting data difficult to interpret. The simplest approach to this problem is for samples to be isolated in the measurement cuvette (or an external chamber) for a period of low-light acclimation prior to data acquisition. As with discrete sample analysis, pilot experiments can be run to determine optimal time of NPQ relaxation. Such an approach makes data interpretation more robust, but significantly decreases the sampling resolution (4 data points per hour in the case of a 15-minute NPQ relaxation time, and less if PE curves are run). This challenge may be partially addressed by using a continuous-flow light acclimation loop, where samples are passed through a long length of darkened tubing, with an appropriate residence time under low light prior to introduction to the cuvette. Under this scenario, users will need to account for time offsets between ST-ChlF measurements and other underway ancillary data (see section 6.5).

6.2.3 Blanks

As discussed in section 3.3, correct interpretation of ST-ChlF data requires accurate determination of blanks, which derive from several sources. For discrete sample analysis, instrument blanks (Binst) can be routinely monitored using measurements of ultra-pure Milli-Q water, with the resultant fluorescence signals subtracted from all measured samples. Similarly, background fluorescence attributable to non-living dissolved compounds (Fdis) can be quantified by analysing filtered samples, thus accounting for any constituents in the seawater matrix. These filtered measurements are particularly important in oligotrophic regions and in samples taken from below the deep Chl a max, where blanks can contribute significantly to the total measured fluorescence signal (for details, see section 3.3). For bench-top analysis of depth profile samples, a number of blanks should be run to characterize the depth-dependent variability in Fdis.

The measurement of blanks is more complicated during continuous underway analysis. At a minimum, it is recommended that users measure at least one blank per day (more, if regions of obvious heterogeneity in phytoplankton biomass are encountered) and subtract this value from subsequent measurements. More sophisticated approaches are possible, using automated fluidic
systems (e.g. syringe pumps and values) to periodically introduce filtered water into the flow-through measurement cuvette.

### 6.2.4 Biofouling

As with all ocean optical measurements, biofouling is of significant concern for ST-ChlF data. The development of biofilms on optical windows affects the quality and quantity of light produced from excitation sources and received by detectors. Fortunately, the presence of biofouling can be routinely monitored through measurements of $B_{\text{inst}}$, which will tend to increase as optical windows become contaminated. Typically, the progress of biofouling is faster within the euphotic zone and in more productive ocean regions (Manov, 2004). For continuous underway operations, optical windows should be cleaned regularly after blanks are run, by gently wiping the cuvette walls with a damp smooth-fibered tissue or pad. In general, the cleaning process is simple and rapid, and should be incorporated into routine maintenance protocols. We recommend that underway data acquisition be stopped once per day, and blank measurements with Milli-Q water run prior to and after cleaning the cuvette. In cases where a blank signal appears to be drifting upward during the course of measurements, blank values can be interpolated to the measurement frequency of data.

### 6.3 Vessels of opportunity

With significant limits on the availability of dedicated research vessels and/or scientific berths, it is becoming increasingly common to deploy automated sensors on various ships of opportunity, or to send unattended instruments to the field. The operational principles described above for bench-top ST-ChlF measurements on research vessels apply equally to such opportunistic deployments. However, a key difference with research vessels is the lack (or significantly reduced capacity) of dedicated personnel to oversee instrument operation. Under these conditions, it is important to define a minimum level of acceptable data quality, and establish procedures to ensure that this is met. One possibility is the use of remote access to the ST-ChlF instruments and/or data through iridium-based connections to vessels at sea. A minimal set of remotely-accessible data can be periodically acquired to assess the status of underway measurements. More ambitiously, it may be possible in some cases for remote users to connect to ST-ChlF instruments through a ship’s server. Even in cases where remote oversight of instruments and/or data is possible, this does not preclude the need for on-site personnel to provide basic support, including periodic cleaning of optical windows and putting instruments into stand-by mode during port calls. Ultimately, the strategies used for unattended underway data collection must be acceptable for both the scientific users and ship operators, subject to a range of logistical constraints.

### 6.4 In situ (submerged) instruments

In situ deployment of ST-ChlF instruments provides several advantages over shipboard deployment and other *ex situ* methods. For example, submerged instruments can capture phytoplankton under ambient temperature and light conditions (but see caveats below), and often do not require water sampling systems that may damage cells. On the other hand, *in situ* deployments have unique logistical challenges, with users less able to oversee and validate data quality and measure blanks. Below, we focus our discussion on underwater light fields, blanks and
biofouling, with specific reference to instruments that measure ChlF in ambient seawater, rather than enclosed submerged sampling chambers.

### 6.4.1 Shadows and red-light effects

For in situ ST-ChlF data obtained from a water column without sample enclosure, measurements are sensitive to perturbations of the underwater light field. For this reason, it is important to mount ST-ChlF instruments towards the top of any platform systems (e.g. a mooring or CTD frame) to avoid shadows from other sensors and sampling equipment. Whenever possible, ST-ChlF instruments should be deployed from the sunward side of the ship. By placing a PAR sensor beside the ST-ChlF instrument, perturbations of the underwater light field can be examined from the vertical profile of ambient irradiance (Moore et al. 2005).

A further consideration for in situ ST-ChlF measurements in near-surface waters is the so-called red-light effect (RLE), which results when scattered ambient red light reaches the instrument detector, increasing the apparent fluorescence signal (Raateoja et al. 2004). The magnitude of the RLE depends on the ambient light level and the optical properties of the water. Due to the rapid attenuation of red light with depth, the RLE is most pronounced in upper layers, with maximum impact between ~ 2–5 m upper layer in coastal waters (Moore et al. 2003; Raateoja et al. 2004) and 5–10 m in the open ocean (Suggett et al. 2001; Sosik and Olson 2002). To correct for the RLE, the background fluorescence can be measured prior to the application of excitation flashes and subtracted from the subsequent ST-ChlF transient. If such corrections are not possible (i.e. not supported by a particular instrument) RLE-influenced data may need to be excluded in the near-surface layers. Under this scenario, surface values can be estimated using light response curves from vertical profile data (i.e. interpolated from measurements at deeper waters using a depth-dependent derived PE curve (e.g., Laws et al. 2002; Smyth et al. 2004). Even with appropriate correction, the RLE will lower the effective dynamic range of ST-ChlF measurements and reduce the signal to noise ratio of the acquired data (SNRraw). As noted in section 2.6, several approaches can be used to adjust the SNRraw to acceptable levels, including changes in detector gain and increasing the number of running averages from sequential ST-ChlF transients. Ultimately, the challenges of producing acceptable ST-ChlF measurements under increasing levels of ambient red light will be similar to those under low biomass concentrations.

### 6.4.2 In situ blanks and biofouling

One of the main challenges of unattended long-term ST-ChlF measurements is sedimentation and biofouling of the instrument sample chambers and optical surfaces. Different manufacturers have devised various strategies to address this problem, including mechanical wipers and copper gaskets. However, the effectiveness of these approaches can only be assessed through periodic measurement of instrument blanks (Binst), which is non-trivial for submerged instruments. As discussed above for underway analysis, various automated approaches could be developed to run in situ blanks using micro-fluidic systems, though the technical challenges (e.g. need to minimize power consumption) would be significantly greater. In cases where instruments are installed on mobile autonomous platforms (e.g. profiling moorings, floats or gliders), sensors can be moved below the euphotic zone outside of measurement periods. This deep water ‘parking’ is commonly
used for Argo floats to minimize sensor biofouling. However, this requires sophisticated deployment infrastructure, and significantly limits the temporal resolution of surface measurements. In many cases, prolonged ST-ChlF measurements in the euphotic zone will be of interest to capture the high-frequency dynamics of phytoplankton blooms and photo-physiological processes over diel cycles. In these instances, it will be important to develop strategies to ensure data quality control with respect to blanks and biofouling.

6.5 Integration with other oceanographic measurements.

The true potential of field-based ST-ChlF measurements can only be realized when these data are combined with other oceanographic observations. Some ancillary data, such as PAR, surface temperature, salinity and nutrient concentrations, provide information on dominant hydrographic features and the environmental history of phytoplankton, and are thus needed for robust interpretation of ST-ChlF data. Other important ancillary data include information on the taxonomic composition of phytoplankton assemblages and their spectral absorption properties, which will influence ST-ChlF signatures. At the same time, opportunities exist to leverage ST-ChlF alongside other measurements of phytoplankton biomass and productivity. Most notably, new approaches have been developed to estimate net primary productivity (NPP) and net community production (NCP) from underway measurements of ocean optics and dissolved gas tracers. These measurements, when combined with ST-ChlF-based estimates of photosynthetic electron transport rates, provide the opportunity to examine the balance between photosynthesis and respiration in ocean surface waters. We briefly discuss these measurements below.

6.5.1 Ancillary environmental data

Sea surface temperature and the availability of light and nutrients can all influence ST-ChlF measurements through their impact on cellular metabolism and the balance of light harvesting and downstream electron sinks, including carbon fixation. Simultaneous measurement of these environmental variables thus provides an important context for the interpretation of ST-ChlF signatures.

Light levels and their spectral quality exert a particularly strong effect on ST-ChlF by regulating photo-physiological processes over different time-scales. Over diel cycles, \( ETR_{PSII} \) shows light-dependent regulation, which can be decoupled from carbon fixation (Schuback and Tortell, 2019). As a result, measurement variability can be introduced by sampling at different times of the day. On shorter time-scales, ST-ChlF measurements can reveal near instantaneous photo-physiological responses to transient changes in upper ocean light fields (e.g., cloud cover), whereas longer-term seasonal variability will reflect the photo-acclimation state of phytoplankton. For these reasons, it is important to characterize the light exposure history of phytoplankton prior to ST-ChlF measurements. This requires measurements of surface irradiance (PAR), and information on mixed layer depth and water column extinction coefficients (derived from depth profiles of PAR, Secchi disk deployments or estimated empirically from Chl \( a \) concentrations), which will determine the
mean irradiance fields experienced by phytoplankton. As discussed in section 3.5, it is also important to characterize the spectral quality of mixed layer light fields, in order to spectrally-correct ST-ChlF data to in situ values. A number of instruments can be used to obtain spectrally-resolved light absorption measurements, including hyperspectral radiometers for depth profiles. In the absence of direct measurements, it is also possible to estimate spectral properties of a water column based on bio-optical model calculations (see, section 3.5.3).

Measurements of temperature and salinity (in surface underway mode or in CTD depth profiles) are now a standard part of all oceanographic expeditions. The required instrumentation is relatively inexpensive, and robust enough for deployment on ships of opportunity. Seawater temperature and salinity data provide important information on key mixing processes and water mass distributions. For example, the presence of cold, saline surface waters can be used as a diagnostic signature of coastal upwelling in mid-latitude regions, which can significantly affect phytoplankton productivity and ST-ChlF signatures. Conversely, low salinity can be indicative of river inputs or sea ice/glacial melt events, which can influence phytoplankton photo-physiology through nutrient inputs, changes in the light field and enhanced mixed layer stratification. Over the past decade, direct measurements of seawater nitrate have become increasingly common, using automated UV absorption measurements in flow-through mode (e.g. Satlantic SUNA). These data can reveal nutrient limitation in surface waters, which can exert a significant effect on photo-physiological properties. Iron is another important limiting micronutrient in some oceanic regions, with strong effects on ST-ChlF measurements (section 5.2.2). At present, there are no readily-available commercial systems for underway dissolved iron measurements, and at-sea iron measurements are subject to significant contamination artefacts. However, the persistence of high nutrient low chlorophyll (HNLC) conditions in large extensions of the oceans can be taken as a good proxy for iron limitation of phytoplankton assemblages. Notably, Fv/Fm data, themselves, have been used to infer iron limitation in surface waters (Kolber et al. 1994).

### 6.5.2 Phytoplankton biomass and productivity measurements

As noted in section 5.2.1, Chl a fluorescence is widely used as a metric of phytoplankton biomass. At the same time, measurements of particulate absorption (rather than fluorescence) centered at 675 nm (using a Wetlabs AC-s, for example), have been shown to correlate strongly with extracted Chl a concentrations derived from HPLC analysis (the current gold standard). As discussed below, these measurements can be coupled with automated optical measurements of particulate back-scatter (Graff et al. 2015) to estimate phytoplankton carbon and C:Chl a ratios, which can be related to phytoplankton growth rates and net primary productivity (NPP), as well as photo-physiological adjustments in pigment packaging (Baird et al. 2013).

The true power of ST-ChlF measurements lies in their ability to yield high spatial and temporal resolution measurements of photo-physiological processes. Notwithstanding a number of assumptions and potential caveats, ETR\textsubscript{PSII} estimates can be correlated to gross primary productivity (GPP), and several studies have shown good agreement between ST-ChlF-based GPP estimates and those derived from other ‘bench-mark’ methods, including H\textsubscript{2}\textsuperscript{18}O and (short-term) \textsuperscript{14}C incubations (Suggett et al. 2003, Corno et al. 2006). Such measurements, when combined with other high-resolution productivity metrics, can yield deeper insight into ocean metabolism.
The use of optically-derived C:Chl \(a\) ratios to infer NPP in surface ocean waters is based on well-established relationships between phytoplankton growth rates, temperature, light and nutrients. These variables are all known to influence C: Chl \(a\) ratios in a systematic manner that can be described using physiological models (Westberry et al. 2008). Irradiance levels are the primary driver of C: Chl \(a\) variability, both in natural phytoplankton assemblages and in laboratory cultures. When light-dependent variability is accounted for using an appropriate model, the residual C: Chl \(a\) variability can then be ascribed to nutrient-control of phytoplankton growth rates, which are scaled to a maximum, temperature-controlled value. This approach, underlies the Carbon-based Production Model (CbPM, Behrenfeld et al. 2005). The model, along with other satellite approaches, can provide highly resolved NPP estimates along a ship track or from satellite measurements (Burt et al. 2018). Application of the CbPM alongside ST-ChlF measurements can thus be used to estimate GPP/NPP ratios, providing information on autotrophic respiration in surface waters, an important, though poorly quantified, term in the ocean carbon cycle.

Biological productivity can be also obtained from dissolved gas measurements in surface ocean waters. Most notably, underway ship-board systems have been used to measure biological oxygen saturation with high spatial resolution. In this approach, changes in oxygen concentrations are normalized to Ar (\(\Delta\text{O}_2/\text{Ar}\)) to correct for any physical processes affecting \(\text{O}_2\) saturation state, including bubble injection and temperature and salinity-dependent solubility changes (Craig and Hayward, 1987). In the steady state, the sea-air flux of excess biologically-produced \(\text{O}_2\) is equivalent to net community production (NCP), which represents the balance of GPP and community respiration (Kaiser et al. 2005). This method yields NCP estimates integrated over the residence time of mixed layer \(\text{O}_2\), typically 1 – 3 weeks. Recent improvements to oxygen-based NCP measurements have enabled corrections for vertical mixing biases in the surface \(\Delta\text{O}_2/\text{Ar}\) mass balance (Cassar et al. 2014, Izett et al. 2018), and the development more economical and robust sensors to use \(\Delta\text{O}_2/\text{N}_2\) as an alternative to \(\Delta\text{O}_2/\text{Ar}\) (Izett and Tortell 2020). These methods have begun to significantly expand data coverage and understanding of NCP distributions in the oceans. Going forward, there are significant opportunities to combine underway NCP measurements with optically-derived NPP (from C: Chl \(a\) ratios) and ST-ChlF-based GPP estimates to yield new insights into the metabolic balance of ocean ecosystems. In comparing these different measurements, it is important to consider the different space and time averaging of the various methods employed. In particular, near instantaneous variability observed with ST-ChlF measurements along a ship-track may not be reflected in \(\Delta\text{O}_2/\text{Ar}\) -based NCP estimates, which integrate biological signatures over longer space and time-scales.

6.5.3 Leveraging satellite observations

Ship-based measurement of oceanographic properties provides a useful context to interpret ST-ChlF data, but these measurements provide only a small snap-shot in space and time. By comparison, satellite-based remote sensing yields broader-scale synoptic information on oceanographic conditions during and prior to ship-based sampling. For example, satellite data can be used to characterize the timing of phytoplankton bloom initiation (from Chl \(a\) observations), and the extent of prior sea ice cover or upwelling for a particular sampling region. Satellite observations of sea surface height anomalies have also been used to identify the presence of meso-scale eddies, which can transport significant quantities of nutrients into the surface layer and create...
significant spatial heterogeneity in surface water properties (Benitez-Nelson et al. 2007). These eddies, and other small-scale hydrographic fronts, will influence phytoplankton physiological ecology and affect the interpretation of ST-ChlF data (Moore et al. 2003, Bibby et al. 2008). As discussed by Schuback et al. 2021, ST-ChlF measurements can be used to help interpret Sun Induced Fluorescence (i.e. passive fluorescence) measured by satellite-based radiometers. Beyond this important application, ST-ChlF data can be examined in the context of other remote sensing data products, including Chl \(a\), particulate organic carbon, SST, wind speed, ice cover, etc. Other derived quantities, including various primary productivity algorithms such as the vertically-resolved general production model (VGPM, Behrenfeld and Falkowski 1997), Carbon-based production model (CBPM; Westberry et al., 2008), and other regionally-tuned models (e.g. Arrigo et al. 1998) can also provide valuable comparative data to contextualize ST-ChlF observations. Although the absolute values of these productivity metrics are subject to significant uncertainty, the broad-scale spatial and temporal patterns likely provide an important context for ship-based physiological data. Going forward, global scale deployment of ST-ChlF measurements will help to constrain the distribution of phytoplankton physiological activity, significantly benefiting the future development of remote sensing productivity algorithms.

6.5.4 Lagrangian vs. Eulerian approaches

As a final consideration, we turn now to the challenge of separating spatial and temporal components of ST-ChlF (and other oceanographic) variability. Ship-based observations in a typical Eulerian framework (i.e. based on a moving sampling platform) face a particular challenge in this respect, as they sample simultaneously through both time and space. In physically-dynamic coastal waters, research vessels may transit across hydrographic frontal regions separating distinct productivity regimes, while at the same time capturing the temporal development (or decline) of a regional phytoplankton bloom. Strong diel cycles in photo-physiology may also be superimposed on other sources of variability, such as tidally-driven nutrient inputs. Under such conditions, it can be challenging to identify the primary drivers of observed gradients in phytoplankton physiological ecology.

One approach to address the ocean ‘space-time conundrum’ involves the use of fixed sampling locations, typically at shore-based coastal observing facilities or tethered moorings. Such fixed location measurements have been deployed across many ocean regions, and have, in some cases, been sites of long-term (several decades) data collection (Benway et al. 2019). The resulting observations provide detailed and highly resolved temporal measurements within a given oceanographic region, yet they are also subject to lateral advection, tidal cycles and other physical disturbances, which can introduce additional variability into measured ocean properties. A more robust (though more logistically complex) approach involves the use of Lagrangian drifters to track water masses moving with prevailing currents. This can be done in ship-based studies by following a GPS-equipped drogue buoy. Application of such Lagrangian methods largely eliminates the signature of spatial heterogeneity, allowing better resolution of temporal variability within a single water mass (Abbott et al. 1990, Zaiss et al. 2021). This is particularly useful in the case of diel studies, where light-dependent processes are examined over successive day-night cycles. As an example, results from such studies have demonstrated significant decoupling of ETR\(_{PSII}\) and C fixation over diurnal cycles (Schuback and Tortell, 2019). With the increasing
availability multi-instrumented ocean drifters and gliders, such Lagrangian approaches for ST-ChlF measurements may become more common. To fully exploit this future opportunity, it will be necessary to devise instrumentation and deployment protocols that facilitate robust data collection, subject to the considerations outlined at the beginning of this chapter. At the same time, ship-based surveys across distinct water masses will remain important in documenting the factors driving spatial variability in phytoplankton photo-physiology and productivity. Achieving truly global coverage of ST-ChlF data will also require capacity building for researchers across the world, including those in developing nations whose access to the required equipment and infrastructure remains limited at present.
Appendix A

ST-ChlF transient fitting and interpretation in a Soliense FRRf instrument

Fluorescence yield transients recorded by all ST-ChlF instruments are determined, to first order, by changes in the level of reduction of the first stable electron acceptor, QA, and by the probability of energy transfer between PSII reaction centers (RCII), $p^3$. Assuming the simplest, “lake” model of RCII distribution, this relationship can be expressed as described by Kolber et al. (1998):

$$F(t) = F_o + (F_m - F_o) \frac{C(t) \cdot (1 - p)}{1 - C(t) \cdot p}$$

(A.1)

Where $F(t)$ is the time-dependent fluorescence yield (i.e. transient), $F_o$ is the fluorescence yield in the native state (i.e., prior to applying an FRRf excitation signal), $F_m$ is the fluorescence yield under conditions of fully reduced QA, and $C(t)$ is the extent of QA reduction within the fraction of reaction centers that are oxidized in the native state. It is only this subset of oxidized reaction centers that contribute to the variable components of the ST-ChlF transient.

Changes in $C(t)$ are controlled by the dynamic balance between rates of charge separation, $R_{cs}(t)$ and QA reoxidation, $R_{reox}(t)$:

$$\frac{∂C(t)}{∂t} = \frac{∂C_{cs}(t)}{∂t} - \frac{∂C_{reox}(t)}{∂t} = R_{cs}(t) - R_{reox}(t),$$

(A.2)

where the first term increases levels of QA reduction, and the second term decreases QA reduction. The first term can be quantified as:

$$\frac{∂C_{cs}(t)}{∂t} = \frac{∂C_{cs}}{∂E} \frac{∂E(t)}{∂t}$$

(A.3)

Where $E(t)$ is the incident excitation energy. In the presence of excitation transfer between PSII reaction centers

---

3 Based on the original formulation by Joliot and Joliot, 1964.
4 Note that $C(t)$ reflects the QA reduction level in both the statistical sense (as a fraction of QA in the reduced state within the large population of reaction centers) and at a stochastic level (the fraction of time the reaction center spends in the reduced state). The QA of a single reaction center is either fully oxidized, of fully reduced.
\[
\frac{\partial C_{\text{Res}}}{\partial E} = \sigma_{\text{PSII}}[(1 - C) + Cp(1 - C) + (Cp)^2(1 - C) + \cdots ] \\
= \sigma_{\text{PSII}}[(1 - C) \sum_{\text{step}=0}^{\text{step}=n}(Cp)^{\text{step}} \approx \sigma_{\text{PSII}} (1 - C) \frac{1}{1-Cp}], \\
\text{(A.5)}
\]

where \( C \) is the average level of QA reduction in the population of RCII that contributes to the variable fluorescence, \( n \) is the size of the pigment domain (lake) accessible for the excitation transfer. Step 0 in Eqn. A5 quantifies excitation trapping by the first reaction center (RCII) associated with the open PSII unit that absorbed the excitation energy. If this RCII is closed, step 1 quantifies the process of excitation de-trapping from this RCII, followed by excitation transfer and trapping to another open PSII unit. If the second encountered RCII is closed, step 2 quantifies the process of excitation de-trapping and transfer from this RCII to yet another RCII unit, and so on. At each of these steps, the excitation can be (1) thermally de-activated, (2) re-radiated as fluorescence to influence the shape of the ChlF transient, or (3) trapped by an open RCII. Usually, these fates are fully resolved within 4-5 steps at the highest levels of experimentally retrieved \( p \) values (\( p < 0.5 \)).

Based on these derivations, rates of charge separation are expressed as:

\[
R_{cs}(t) = I_{E}(\lambda, t) \cdot \sigma_{\text{PSII}}(\lambda) \frac{1-C_{QA}(t)}{1-C_{QA}(t) \cdot p}, \quad \text{(A.6)}
\]

where \( I_{E}(\lambda, t) \) is the excitation power (or quanta flux) [quanta m\(^{-2}\) s\(^{-1}\)].

The reoxidation rates are determined by the actual level of QA reduction, and by the rate constant(s) of electron transport from QA\(^{-}\) into the PSII \( \rightarrow \) PSI electron transport chain, \( K_{\text{reox}} \).

\[
R_{\text{reox}}(t) = C_{QA}(t) \cdot K_{\text{reox}}. \quad \text{(A.7)}
\]

The \( K_{\text{reox}} \) is a multi-parameter function that describes the relationship between the measured fluorescence transient and the properties of photosynthetic electron transport. The simplest form of this relationship amenable to efficient numerical procedures assumes a multi-exponential form of the QA\(^{-}\) reoxidation:

\[
R_{\text{reox}}(t) = C_{QA}(t) \sum_{i=1}^{n} \alpha_i \exp \left( -\frac{t}{\tau_i} \right), \quad \text{(A.8)}
\]

\[ \sum_{i=1}^{n} \alpha_i = 1, \]
where \(1/\tau_i\) are rate constants and \(\alpha_i\) are their fractional contribution to the kinetics of \(Q_A^-\) reoxidation. With these assumptions, \(C(t)\) can be expressed as (Kolber et al., 1998):

\[
C_{QA}(t) = \left[ I_E(\lambda, t) \cdot \sigma_{PSII}(\lambda) \frac{1 - c_{QA}(t)}{1 - c_{QA}(t) \cdot p} \right] \ast \sum_{i=1}^{n} \alpha_i \exp \left( -\frac{t}{\tau_i} \right),
\]

(A.9)

where the star symbol denotes a convolution operation between the two terms in the equation above. This convolution integral, in a time-discrete form, is substituted into Eqn. A.1 and fitted against the experimentally retrieved transients to retrieve the desired photosynthetic parameters. The discretization is performed in 100 ns steps (identical to the Analog-to Digital discretization of the recorded fluorescence transients in FRRf technique) during the 1 \(\mu\)s long period of flashlets ON time, extending to a time period between flashlets during flashlets OFF time.

It assumed here that all the parameters (i.e. \(\sigma_{PSII}, p, \tau_i,\) and \(\alpha_i\)) remain constant over the length of the excitation protocol (usually 100 - 200 \(\mu\)s). This assumption may not always hold; for example, these parameters may change in response to progressive PQ pool reduction during FRRf excitation as observed during Multiple Turnover (MT) flash. They may also change in response to abrupt changes in the ambient irradiance during the excitation protocol.

A.1. Kinetics of \(Q_A^-\) Oxidation

Fitting Eqn. 1 against experimentally acquired ChlF transient is usually performed using least squares regression, or maximum likelihood minimization techniques. A typical ST-ChlF excitation protocol involves a saturation phase, applied with high excitation power to shift the balance in Eqn. A.2 toward fast reduction of \(Q_A\) within \(~100\ \mu s\) (see section 3.2.1 in the main User Guide), followed by a relaxation phase with gradually decreasing excitation power (increasing time interval between flashlets) to shift this balance toward \(Q_A^-\) reoxidation. Any excitation protocol capable of shifting the balance between rates of charge separation and \(Q_A^-\) reoxidation can produce fluorescence transients to retrieve photosynthetic properties related to light harvesting, charge separation and electron transport, as long as this balance is quantified along the entire length of the excitation protocol.
Erroneous estimates of $F_v/F_m$, $\sigma_{PSII}$ and the kinetics of QA$^-$ reoxidation result when partial reoxidation of QA$^-$ is not accounted for in the saturation phase, and partial reduction of QA is not accounted for in the relaxation phase. The first type of error can be minimized by operating the saturation phase with excitation power high enough to saturate fluorescence transient at time scales much shorter than the fastest component of QA$^-$ reoxidation. This, however, creates problems in resolving the initial portion of the saturations phase (User Guide section 3.2.1). The errors caused by neglecting partial reduction of QA in the relaxation phase can be minimised by operating with very low excitation power during this phase, but at the cost of a low signal-to-noise ratio.

The performance of the FRRf technique is examined based on experimental ChlF data with high signal to noise ratio (SNRraw level of 720; see Appendix D), acquired with the centric diatom, *T. pseudonana*, grown under low light conditions. The sample was dark-acclimated for two minutes before the measurements, and the 100th transient in a series of 1,600 records was selected for analysis (Fig. A1). The key question is the selection of the number of time constants of QA$^-$ re-oxidation (Eqn. A.8) required for adequate fitting of the recorded fluorescence transients. The $\chi^2$ measure of fit quality, and the uniformity of residual distribution (the differences between the fit and the experimental data) provide information on the statistical robustness of the model fit (see Appendix D). Fig. A.1 demonstrates progressive improvement in the fit quality at increasing number of exponential components in QA$^-$ re-oxidation kinetics. The $\chi^2$ value approaching one indicates that the four-exponential fully describes experimental data within the limits of the signal-to-noise ratio, and the uniform distribution of residuals shows that all features in the recorded fluorescence transient have been accounted for.
Figure A.1. An example of ST-ChlF data processing with one (A), two (B), three (D) and four-exponential (D) models of QA- reoxidation kinetics, based on a 100-th transient in a series of 1,600 consecutive records (Fig. A.2). The residuals and the noise traces are amplified by a factor of five to better illustrate fit deviations from the experimental data. Increasing the number of exponentials, from one to four, offers progressively better fits, approaching limits of resolution with the four-exponential model. Both the $\chi_0^2$ approaching 1, and the statistics of residual distribution (green oval in (D) approaching that of noise distribution in the ChlF transient (red oval in (D) indicate fully resolved power on the 4-exponential fit. Retrieved data are shown in Table A.1.
Table A.1. Results of the FRRf fit of fluorescence transient shown in Fig. A.1 analysed with one, two, three, and four-exponential models of QA reoxidation. The single-exponential model appears to be inadequate in retrieval of all photosynthetic properties, while progressively better retrieval is observed as the numbers of exponentials in QA reoxidation increases. The slowest rate constant of about 100 ms revealed by the four-exponential model, although contributing to only 2% of relaxation kinetics, still affects the retrieval of most properties because of its ‘longevity’.

<table>
<thead>
<tr>
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<th>Two-exponential</th>
<th>Three-exponential</th>
<th>Four-exponential</th>
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<tr>
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Fitting the ChlF transient with as many as 15 free parameters (each amplitude of Q\textsubscript{A}\textsuperscript{+} reoxidation kinetics is a dependent variable) raises the possibility of over-fitting. This possibility is demonstrated in User Guide, section 2.6.2, Fig 2.6.2, where so-called evaporating parameters\textsuperscript{5} are flagged with a red background to highlight insufficient SNR\textsubscript{data} in their retrieved values. In addition, a corresponding $\chi^2_p < 1$ indicates partial fitting to the noise. Elimination of over-fitting requires improving the quality of the transient by increasing the SNR\textsubscript{raw} (e.g., by increasing the number of running averages), and/or eliminating the evaporating parameters (Section D4 of this Appendix). Progressive fitting to the noise will also be apparent when the standard deviation of the fit residuals falls below the standard deviation of the noise in the processed fluorescence transient. None of these indicators are present in Fig. A.1.D. As stated in the section 2.6.3 of User Guide, users should strive to use models that produce $\chi^2_p$ levels close to, but no smaller than 1, with the fewest number of free parameters’. This appears to be followed in the four-exponential fit in Fig. A1.D.

Verification and interpretation of retrieved data in terms of the underlying biophysics is more complicated. Sometimes previous knowledge can support data/model compliance with the biophysical reality based on ‘expected’, or ‘reasonable’ pattern in acquired data in response to stimuli (e.g., light, temperature, etc.,). A simple, 20-minute experiment using T. pseudonana grown under low light conditions, subjected to step-wise changes in actinic light (475 nm) from 0-100 $\mu$E m\textsuperscript{-2} s\textsuperscript{-1} illustrates this point (Fig. A2 below). All data were acquired with relatively high SNR\textsubscript{raw} above 400, fitted with 15 degrees of freedom, with average $\chi^2_p$ values of ~2 (except during transient changes in the actinic light). All transients displayed ‘well-behaving’ saturation levels in range 0.65 to 0.75 (User Guide, section 3.2.1) and high SNR\textsubscript{data} ranging from ~10 (the longest time constant of Q\textsubscript{A}\textsuperscript{+} reoxidation) to 400 (the primary F\textsubscript{v}/F\textsubscript{m} and $\sigma$\textsubscript{PSII}). These metrics all suggest good data quality matched with high model quality. Interestingly, all of retrieved parameters displayed strong responses to relatively small changes in the actinic light (see the initial 0 to 10 $\mu$E transition), with the most dramatic responses in the properties related to the kinetics of electron transport. The ‘reasonable’ responses recorded in this dataset are the decrease in the F\textsubscript{v}/F\textsubscript{m}, and the increase in F\textsubscript{o} to in response to increasing actinic light levels. The decrease in $\sigma$\textsubscript{PSII} may indicate a state transition in response to the blue actinic light, but that would be inconsistent with the observed initial increase in the F\textsubscript{m} fluorescence yield. Alternatively, the initial anti-correlation between $\sigma$\textsubscript{PSII} and F\textsubscript{m} may indicate rebalancing of excitation energy trapping by RCII and radiative energy loss in the pigment bed. Across the entire experiment, strong anti-correlation between F\textsubscript{m} and the probability of energy transfer, p, and well-correlated changes in $\mu$ and $\sigma$\textsubscript{PSII} support the notion of transient increases in radiative deactivation following step-wise changes in the actinic light. This reduces the chance of a successful transfer finding an open reaction center.

\textsuperscript{5} The term used in the numerical fitting terminology for parameters that cannot converge to their expected values at repetitive measurement and and/or data averaging. The retrieved value of such parameters is driven by the noise in situations when the noise level in fitted data exceeds the model capability to consistently retrieve the value of the parameter.
The dynamics of QA⁻ reoxidation also demonstrate interesting patterns. Each light step induces a transient increase in $\tau_2$, a time constant that most likely reflects QA/QB→PQ pool electron transport. This transient increase may result from the initially slow rates of PQ pool reoxidation in the dark, as indicated by the long $\tau_3$ and $\tau_4$ during the first 200 seconds of the dark period. After exposure to 10 $\mu$E light, PQ oxidation is quickly re-activated, and then up-regulated within 30 seconds following each light step. The steady-state level of $\tau_3$ and $\tau_4$ gradually increases with increasing light intensity, indicating a gradual decrease in rates of PQ pool reoxidation, possibly due to the progressive reduction of electron carriers downstream of the PQ pool. As a result, the levels of PQ pool reduction increase, gradually slowing rates of QA⁻ reoxidation, as manifested by the increase in $\tau_2$ and, to a lesser extent, $\tau_1$. Exposure to a final period of darkness transiently re-oxidizes the PQ pool, as indicated by a short-term decrease in $\tau_3$ due to reoxidation of electron carriers downstream of PQ pool. This allows transiently faster QA→PQ pool electron transport, which is manifested by a transient decrease in both $\tau_1$ and $\tau_2$. Within the next 30 seconds, the entire electron transport chain becomes fully oxidized, restoring the initial conditions.
Figure A.2. Performance of the four-exponential model of $Q_{A}$' reoxidation along 1,600 flashes sequence at 1 second time intervals in response to small, step-wise changes in background irradiance in $0 - 100 \mu E$ range. The experiment was performed with low-light grown $T. pseudonana$ exposed to darkness for two minutes prior to the experiment. (A) Data properties of the transient: the saturation coefficient of the FRRf transients (User Guide, section 3.2.1), $SNR_{raw}$ in these transients (Appendix D.1), the $\chi^2_f$ measure of the fit quality (User Guide, Section 2.6.3), and the maximal level of QA reduction attained in the saturation phase. (B) the primary ST-ChlF properties (fluorescence yields $F_o$ and $F_m$ in relative units (User Guide section 3.1.4, referenced against solid-state Rhodamine B quantum counter), $F_v/F_m$, and $\sigma_{PSII}$. (C and D) time constants of $Q_{A}$' reoxidation with their corresponding amplitudes. (E) Probability of energy transfer between PSII reaction centers assuming lake model of pigment organization, the amplitude and time constant of carotenoids quenching ($\alpha_{CarQ}$ and $\tau_{CarQ}$), and the amplitude of $P_{680}^+$ quenching (Appendix A.2), with the time...
constant 600 µs. The statistical quality of retrieved data, calculated over the period marked by the yellow rectangle, is shown in the statics panels on the right. All properties were retrieved with SNR_{data} higher than 8, and with $\chi^2_p$ greater than 2, indicating absence of over-fitting despite the significant degrees of freedom (15 free parameters) in the applied model.

There may exist other explanations of the observed dynamics shown in Fig. A.2. What matters, however, is that high-quality data (if available), coupled with adequate models operating at the highest resolving power, allow a range of coherent and testable hypotheses to be verified or refuted by subsequent scientific inquiry. Decreasing the degrees of freedom in such well coupled data/model comes with some risks, as described below using tri-exponential model of QA-reoxidation (Fig. A.3).

![Figure A.3](https://example.com/image.png)

**Figure A.3.** Effects of processing experimental data with decreasing degrees of freedom in the fitting model. (A) fitting with three-time constants of QA-reoxidation. The $\tau_2$ and $\tau_3$ in the three-exponential kinetic are retrieved with relatively high noise (SNR$_{\tau_2} = 11.8$, compared with SNR$_{\tau_2} = 57$ in the four-exponential model), possibly reflecting difficulty of the fitting algorithm to map the reduced set of four free parameters ($\alpha_2$, $\tau_2$, $\alpha_3$, $\tau_3$) into variable space previously occupied by six free parameters ($\alpha_2$, $\tau_2$, $\alpha_3$, $\tau_3$, $\alpha_4$, $\tau_4$.) This difficulty is further demonstrated by highly variable $\chi^2_p$ values along the dataset and the rather inconsistent fitting during the terminal dark period. (B) fitting with two-exponential model of QA-reoxidation. Both $\tau_1$ and $\tau_2$ are retrieved with very high fidelity (SNR$_{\tau_1}, \tau_2 \sim 100$), with $\tau_1$ displaying weak, but consistent increase to increasing light levels, with and much clearer trend shown in $\tau_2$. 

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The three-exponential model applied to the same dataset, although still statistically valid, produces much noisier results with $\chi^2$ spiking from 5 to 20 between adjacent records, possibly reflecting model difficulty in mapping $\tau_2$ and $\tau_3$ parameters in the variable space previously occupied by $\tau_2$, $\tau_3$ and $\tau_4$ in the four-exponential model. The $\tau_1$ retrieved in the three-exponential model remains similar to that retrieved by 4-exponential one, affording the same biophysical interpretation (i.e., QA/QB$\rightarrow$PQ pool electron transport), but the biophysical interpretation of $\tau_2$ and $\tau_3$ is less clear.

A much different picture is presented by the two-exponential model (Fig. A3.B). Both $\tau_1$ and $\tau_2$ are retrieved with extremely high precision. The $\tau_1$ can be reasonably interpreted as the composite rate of electron transport along QA$\rightarrow$QB$\rightarrow$PQ pool electron transport chain, with a value that increases at increasing irradiance levels. Likewise, $\tau_2$ can be reasonably interpreted as representing the composite rates of electron downstream of the PQ pool. The relatively high $\chi^2$ levels, at about 20, provide evidence of under-fitting, possibly indicating that the derived time constants may deviate from their corrected values.

The high precision of $\tau_1$ and $\tau_2$ retrievals in the 2-exponential model suggests a good performance when processing low-SNR$_{raw}$ ChlF transients. This feature should prove beneficial under conditions of low biomass, and in the analysis of Rapid Light Curves, where raw data quality deteriorates significantly at high actinic light levels (Fig. A.4). Indeed, the two-exponential model performs exceptionally well in retrieving the primary photosynthetic properties (Fig. A.4.A) despite of the 10-fold drop in SNR$_{raw}$ at the highest irradiance level. This model is also quite capable of observing the dynamics of the electron transport in the entire RLC experiment (Fig. A.4.B). Assuming that $\tau_1$ mostly quantifies kinetics of QA$\rightarrow$PQ pool electron transport, and $\tau_2$ mostly quantifies electron transport downstream of the PQ pool, the observed interplay between these two parameters (an increase in $\tau_1$ and decrease in $\tau_2$) with increasing irradiance reflects progressive reduction of the PQ pool, which is partially relieved by acceleration of electron transport from the PQ pool to PSII at increasing actinic light levels. These trends quickly reverse at decreasing irradiance levels, and most likely reflect homeostatic mechanisms optimising the entire PSII$\rightarrow$PSI electron transport chain in response to varying irradiance levels. Beyond these steady-state responses, there appears to be a range of other responses to abrupt changes in the actinic light operating on various time scales. Interestingly, the amplitudes of these responses are most prominent at the lower range 0 - 200 $\mu$E range of light. This dynamic presents an obvious problem in interpreting the RLC data, but also an easily deployable tool for studying the mechanisms of photosynthetic electron transport. Arguably, the 4-exponential kinetics (Fig A3. C and D) offers much sharper tool, but this tool breaks down at irradiances above 300 $\mu$E due to insufficient sensitivity of the instrument and increased data noise.
Users should understand the opportunities and limitations offered by models with different degrees of freedom (i.e., different numbers of free parameters). Experimenting with different models, under well-constrained laboratory conditions with varying biomass concentration, should serve as the best guide towards routine field applications. Based on the analysis of the FRRf fitting technique discussed above, the most reasonable, multi-exponential models are:

- four-exponential models when using high quality data (SNR\text{raw} above 500)
- two-exponential models for data with low- to-moderate quality (SNR\text{raw} of 10 – 500)
- single-exponential models at extremely low chlorophyll biomass, using quality control flags to record the quality of retrieved data (section D5).

### A.2. Accounting for Carotenoid and P_{680}^{+} Quenching

Most observed FRRf fluorescence transients show evidence of two types of rapid quenching phenomena (Fig. A.5). The first phenomenon, presumably caused by the donor side p680\(^{+}\) accumulation, manifests itself as a declining slope of the fluorescence transient during the saturation phase. This quenching, denoted here as P680Q, appears to relax with a time constant of 400 to 800 µs, consistent with the turnover time of the donor side of PSII. The second type of quenching manifests itself as a transient increase in the fluorescence yield at the beginning of the
relaxation phase. This phenomenon, attributable to carotenoid quenching and denoted here as CarQ, generally relaxes with a time constant of 8 to 30 $\mu$s.

Figure A.5. Effects of P680Q quenching and CarQ quenching recorded in fluorescence transient acquired with *T. pseudonana*. (A) P680Q quenching is manifested as a gradual decrease in the fluorescence yield after a transient saturation. The CarQ quenching is best manifested as a short, transient increase in the fluorescence yield at the start of the relaxation phase. (B) The failure to account for P680Q results in a downward trend of residuals along the length of the saturation phase (blue rectangle). The failure to account for CarQ shows as a transient increase in the residuals at the beginning of the relaxation phase, and residuals inflection at the beginning of the saturation phase (red ovals). Both failures deteriorate fit quality, as demonstrated by the increase of $\chi^2$ from 2.43 to 39. The effects of these failures on the retrieved parameters are summarized in Table A.2.

Failure to account for these phenomena in the FRRf data analysis worsens the quality of the fit, as demonstrated by the increase of $\chi^2$ from 2.4 to 39, and deteriorates the quality of recovered $F_v/F_m$, $\sigma_{P575}$, and $p$ (Table A.2). The most detrimental effect of not accounting for CarQ is the inability to resolve the $\tau_1$ and $\tau_2$ time constants of QA reoxidation (Table A1, Fig. A6), impairing the interpretation of the QA→PQ pool electron transport rates. Moreover, as the time constant of CarQ relaxation is ~ 16 $\mu$s, the effects of this quenching gradually accumulate over the first 16 $\mu$s of the transient, resulting in a characteristic inflection during the initial portion of the transient. This effect is similar to that produced by energy transfer between PSII reaction centers, although the maximum inflection point of the latter is usually observed at the mid-point of the saturation curve.

Table A.2. Results of the FRRf fit of fluorescence transient with and without accounting for of P680Q and CarQ quenching, based on record 100 of the dataset in Fig. A.6.
To account for P680Q and CarQ quenching in the fitting procedure, Eqn. A.1 is modified as:

\[ F(t) = F_{nat}(1 - CarQ) + (F_m(1 - P680Q) - F_{nat}(1 - CarQ)) \frac{C_{QA}(t) \cdot (1 - p)}{1 - C_{QA}(t) \cdot p}, \]

(A.6)

where CarQ and P680Q are integrated along the length of the fluorescence transient:

\[ \frac{\partial CarQ(t)}{\partial t} = E(\lambda, t) \cdot \sigma_{PSII}(\lambda) \cdot \alpha_{CarQ} - CarQ(t) \cdot \exp\left(-\frac{t}{\tau_{CarQ}}\right) \]

(A.7)

\[ \frac{\partial P680Q(t)}{\partial t} = E(\lambda, t) \cdot \sigma_{PSII}(\lambda) \cdot \alpha_{P680Q} - P680Q(t) \cdot \exp\left(-\frac{t}{\tau_{P680Q}}\right). \]

(A.8)
The terms $\alpha_{\text{CARQ}}$ and $\alpha_{\text{P680Q}}$ describe the efficiency of production of the corresponding quenching species, and $\tau_{\text{CARQ}}$ and $\tau_{\text{P680Q}}$ are time constants of their relaxation. The combined effects of failing to account for these quenching phenomena (increase of $\chi^2$ from 2.4 to 39) far exceed the effects of reducing the number of time constants of QA re-oxidation from 4 to 2 (increase of $\chi^2$ from 2.43 to 21.2).

The relatively short (8 - 30 $\mu$s) time constant of CarQ relaxation makes the transient ChlF increase at the beginning of the relaxation phase (Fig. A.5.A) ‘disappear’ when data recording begins more than 40-150 $\mu$s after the end of the saturation phase. With such long delays, the relaxation of the fluorescence yield caused by forward electron transport masks the CarQ effects. However, whether or not they are masked, the effects of this quenching remain present in the ChlF transient, as it is being produced by each flashlet along the excitation protocol. Similar problem exists with detection and quantification of P680Q quenching. The appearance of this quenching becomes less clear in fluorescence transients acquired with the saturation coefficient of less than 0.5 (User Guide, Fig. 3.2.2 C). Under such conditions of relatively low QA reduction, the relatively slow rise in fluorescence yield masks the relatively weak downward trend due to P680Q evolution. Regardless, this quenching still affects the shape of the ST-ChlF, influencing the accuracy of the retrieved parameters. In particular, the $\tau_1$ and $\tau_2$ time constants of the 4-exponential model cannot be resolved (Table A3, Fig A.6). This issue underscores the importance of achieving the maximum possible saturation of QA during fluorescence transients, as described in the main user Guide (section 3.2.1).

In the discussion above, it is assumed that carotenoid quenching operates at a level of excitation delivery to PSII reaction centers, while P680Q modulates the $F_m$ signal at a level of excitation trapping by the PSII reaction centers. There is, at present, little mechanistic underpinning for these assumptions, other than the improved model fit that results from their implementation (Eqn. A.6). The FRRf fitting procedure allows the user to optionally reassign any combination of these two quenching terms to $F_o$ and/or $F_m$, allowing more detailed studies of the underlying phenomena.
Appendix B

ST-ChlF transient fitting in a Chelsea LabSTAF instrument

Chelsea LabSTAF instruments use a custom software, RunSTAF, to control all elements of data acquisition and analysis.

RunSTAF software is continuously being updated and improved, with new functions added based on demand of instrument users and improved understanding of primary ChlF parameter retrieval and interpretation. A comprehensive handbook covering STAF instruments and RunSTAF software is openly available on the Ocean Best Practice Repository (Oxborough 2022)

https://repository.oceanbestpractices.org/handle/11329/1531.4

The handbook is regularly updated, and reader are referred to the latest available version of the handbook for details of data fitting within the RunSTAF software. Below we provide only a brief overview of the current RunSTAF ST-ChlF transient fitting routines and options.

The ST-ChlF excitation protocol

The default RunSTAF excitation pulse, used to induce a ST-ChlF transient, is 100 µs long. Data are acquired at 1 MHz, beginning 20 µs before the ST pulse is triggered.

If the fitting of ST-ChlF relaxation is desired, such excitation pulses can be applied to the sample in a ‘dual-pulse’ approach, outlined in section 3.2.2 of the main User Guide and described in detail in the RunSTAF handbook.

The ST-ChlF transient saturation phase fit

Two fitting routines for the saturation phase of the ST-ChlF transient are available within the RunSTAF software. The ‘Rho - fit’, based on equations first presented in Kolber et al. (1998), is equivalent to the fitting procedures applied in most other commercially available ST-ChlF instruments, including Chelsea Technologies FastOcean FRRF instruments. The ‘Dimer - fit’ was recently developed based on the concept that photochemically-active PSII complexes form structural dimers within thylakoid membranes (e.g. Nield and Barber 2006; Watanabe et al. 2009; Umena et al. 2011). The underlying model assumes that:

- All functional PSII reaction centres are incorporated within dimeric complexes and the two reaction centres within each dimer effectively share a single light harvesting system.
- PSII dimer complexes are completely isolated from each other, preventing the transfer of photons from one dimer to another.
Results of both fitting routines can be visualized and exported using RunSTAF software.

The ST-ChlF transient relaxation phase (τ) fit

As mentioned above, STAF instruments utilize the ‘dual-pulse’ method to record the gradual decrease of ChlF after the saturation phase of a ST-ChlF transient. Number and lengths of gaps can be adjusted by the user, with guidance on optimal dual-pulse protocols for given conditions provided in the handbook.

Two phases of ChlF relaxation are fit by RunSTAF (i.e. two time-constants of ChlF relaxation, τ, are derived). It is assumed that the fast relaxation phase tracks the reopening of RCIIIs with plastoquinone or semi-plastoquinone bound at the QB site at the end of the ST pulse, while the slow relaxation tracks the reopening of RCIIIs with an empty QB site at the end of the first ST pulse.

Three different relaxation phase fits are generated by RunSTAF: Fv, Rho and Dimer. The Fv fit tracks the recovery of variable ChlF during the gap between the first and second ST-ChlF excitation pulses in a series of dual-pulse pairs (see figure 3.2.2 in the main User Guide). The Rho and Dimer relaxation phase fits track the reopening of RCIIIs during the first and second ST-ChlF excitation pulses in a series of dual-pulse pairs using the Rho and Dimer models. Results of all three fitting routines can be visualized and exported using RunSTAF software.
Appendix C

ST-ChlF transient fitting in mini-FIRe instruments and upgraded Satlantic FIRe instruments

Induction of fluorescence during a saturating single-turnover (ST) flash is driven by the accumulation of closed reaction centers C(t) over time, and the resulting increase in fluorescence yield F(t) from its minimum (F_o) to maximum (F_m) values. The rate of increase in C(t) is driven by the rate of photochemical charge separation in PSII reaction centers, and determined by $\sigma_{PSII}$, the photon flux density (E) and the extent of energy transfer between reaction centers (the so-called “connectivity factor”, $\rho$):

$$ \frac{dC(t)}{dt} = \sigma_{PSII} E \frac{(1-C(t))}{(1-\rho C(t))} \quad \text{(Equation C.1)} $$
$$ F(t) = F_o + F_v C(t) \frac{(1-\rho)}{(1-\rho C(t))} \quad \text{(Equation C.2)} $$

$F_v (= F_m - F_o)$ is the variable fluorescence yield. These equations assume that the rate of photochemistry in PSII induced by the saturating single turnover flash is much faster than the rate of $Q_a^-$ reoxidation, and the latter can be ignored. In the case when the rate of fluorescence induction is comparable to the rate of $Q_a^-$ reoxidation, the $Q_a^-$ reoxidation kinetics can be taken into account, at the expense of more complex mathematical formalism, as described below.

The relaxation kinetics of fluorescence yield after a saturating ST flash is driven by the kinetics of $Q_a^-$ reoxidation and the resulting conversion of RCs back to the open (i.e. oxidized) state. However, the measured kinetics are also affected by the rate of photochemistry produced by measuring flashes and by actinic (background) light. These two processes slow down re-opening of reaction centers, and thus distort the measured fluorescence relaxation kinetics. This must be taken into account while retrieving the actual kinetic rates of $Q_a^-$ reoxidation. Below we explain how and why these two processes must be incorporated into mathematical formalism for the analysis of ST-ChlF transients.

The kinetics of $Q_a^-$ reoxidation are recorded by applying a sequence of relatively weak relaxation flashlets at progressively increasing intervals (Gorbunov and Falkowski 2021). Under such conditions, the observed ST-ChlF kinetics of C(t) relaxation between the flashlets are driven by two competing processes: $Q_a$ re-oxidation and the actinic effect of ambient light. The former leads to conversion of reaction centers to the open state, while the latter closes a fraction of centers. A change in C(t) between the flashlets is described as follows:

$$ C(t_i + \Delta T) = C(t_i) \{ \alpha_1 \exp(-\Delta T/\tau_1) + \alpha_2 \exp(-\Delta T/\tau_2) + \alpha_3 \exp(-\Delta T/\tau_3) \} + $$
$$ + \sigma_{PSII} E \Delta T \frac{(1-C(t_i))}{(1-C(t_i)) \rho} \quad \text{(Equation C.3)} $$
Here, $\Delta T$ is the interval between relaxation flashes, $\tau_1$, $\tau_2$, $\tau_3$ are the time constants fast, medium, and slow components of Q$_A$ re-oxidation, $\alpha_1$, $\alpha_2$, $\alpha_3$ are fractional contributions of these components ($\alpha_1+\alpha_2+\alpha_3 = 1$), and $E$ is the intensity of ambient photosynthetically available radiation. This equation assumes multi-component kinetics of Q$_A$ re-oxidation. Repeated data analysis has demonstrated that at least three components are usually needed to describe these kinetics with sufficient accuracy (Gorbunov and Falkowski 2021). The interval between relaxation flashes is assumed to be short ($\Delta T \ll (\sigma_{PSII} E)^{-1}$). The first term in this equation describes opening the reaction centers due to Q$_A$ re-oxidation, while the second term reflects the partial closure of reaction centers due to photochemistry. The incorporation of irradiance, $E$, in the above equation is important for accurate retrievals of the rates of Q$_A$ re-oxidation measured under ambient light (Fig. C.1).

If the intensity of measuring flashes during the relaxation phase were negligible, the measured relaxation kinetics in darkness would precisely follow the kinetics of Q$_A^-$ reoxidation. However, the induced fluorescence signals would be extremely weak and noisy. To achieve a suitable signal-to-noise, the intensity of the measuring flashlet is set at ca. 0.02 $\mu$mole quanta m$^{-2}$ per flash with 1 $\mu$s duration, which corresponds to ca. 0.05 hits per reaction center per flash. This number is not negligible; each flashlet produces a small, but significant, increase in the fraction of closed reaction centers. This increase in $C(t)$ by the measuring relaxation flashlet is numerically described as follows:

$$\Delta C = \sigma_{PSII} E \Delta t_{relax} (1-C(t)) / (1-\rho C(t))$$  \hspace{1cm} \text{ (Equation C.4)}$$

where $\Delta t_{relax}$ is the duration of the relaxation flashlet (1 $\mu$s for FIRe instruments), $E$ is the peak intensity of measuring excitation light, and $C(t)$ is the fraction of closed centers at the beginning of the relaxation flashlet. The measured FIRe relaxation kinetics are processed by numerically fitting these profiles to the above equations to retrieve corrected time constants ($\tau_1$, $\tau_2$, $\tau_3$) of Q$_A$ re-oxidation kinetics (Fig. C.1). Note that this mathematical procedure for the analysis of relaxation kinetics recorded under ambient light differs from that in FRR instruments (Fig. C.1). This fitting procedure has been implemented in mini-FIRe instruments and in the upgraded Satlantic FIRe instruments.
Figure C.1. The effect of ambient photosynthetically available radiation (PAR) on the time of QA reoxidation (solid dots), in relation to PAR-driven alterations in the redox state of the PQ pool (PQox, open dots). These time constants were retrieved from the kinetics of fluorescence relaxation in the diatom, *T. pseudonana*, following a saturating ST flash by using two different mathematical models, the FRR model (Kolber et al, 1998) (panel A) and the new mini-FIRe (Gorbunov and Falkowski 2001; panel B) (see section 2.2.c). As PAR increases and the PQ pool becomes reduced, the actual rate of electron flow away from PSII is expected to slow down, and the rate of fluorescence relaxation kinetics expected to decrease (panel B). The FRR retrieved relaxation times remain virtually unchanged with an increase in PAR (panel A), while the mini-FIRe analysis reveals an increased lifetime for QA re-oxidation as PAR increases and the PQ pool becomes more reduced (panel B). At saturating irradiance, QA re-oxidation lifetime plateaus, closely approaching the photosynthetic turnover time (τ) (panel B). (Data from Gorbunov and Falkowski 2021).
Appendix D

Error quantification in ST-ChlF data analysis with the Soliense LIFT instrument and software

Based on the general principles outlined in section 2.6 of the main User Guide, this appendix explains the numerical techniques used in Soliense LIFT instruments to quantify noise in the ST-ChlF transients, and the subsequent errors and uncertainties in the retrieved photo-physiological properties. All the techniques described here are built into the instrument software, enabling error calculation in real-time to support adaptive sampling and data acquisition. The main approaches are described at a level of detail that should allow their implementation for any ST-ChlF instrument, using stand-alone software packages such as R, Python, or MATLAB. The discussion below focuses on: 1) calculating signal-to-noise ratios in raw ST-ChlF transients (SNR$_{raw}$); 2) quantifying the relationship between SNR$_{raw}$ and the statistical quality of derived photo-physiological properties; 3) best practices for data acquisition to optimize data quality; and 4) the application of error analysis to inform the selection of biophysical models used to retrieve phytoplankton photosynthetic properties. All the techniques described here apply to the analysis of both single and multiple-turnover fluorescence transients.


In electrical engineering, Signal-to-Noise Ratio (SNR) is expressed as the ratio of the signal power to noise power, in logarithmic units of decibels. In optical measurements, where the range of measured SNR doesn’t exceed a few orders of magnitude, SNR is usually calculated as:

\[
SNR = \frac{rmssignal}{rmsnoise} = \frac{\frac{1}{n} \sum_{i=1}^{n} \hat{s}_i^2}{\frac{1}{n} \sum_{i=1}^{n} e_i^2}, \tag{D.1}
\]

where \(rmssignal\) is the root mean square of the measured signal, \(rmsnoise\) is the root mean square of the noise, \(\hat{s}_i\) is the noise-free signal, and \(e_i\) is the noise. In case of stationary signals, with no time-dependent variability (i.e., \(\hat{s}_i = s\)), SNR can be expressed in terms of signal statistical properties:

\[
SNR \approx \frac{\frac{1}{n} \sum_{i=1}^{n} \hat{s}_i^2}{\frac{1}{(n-1)} \sum_{i=1}^{n} (s_i-s)^2} = \frac{\bar{s}_n}{\sigma_s^2}. \tag{D.2}
\]
where $\bar{\mu}_s$ and $\bar{\sigma}_s$ are the respective mean and standard deviation of measurements. In fluorescence transient techniques, SNR$_{raw}$ calculations apply to the variable component of the recorded fluorescence yield, $f_{vi}$:

$$f_{vi} = f_i - f_o = (f_{vi} + e_i) - f_o$$  \hspace{1cm} (D.3)

where $f_{vi}$ is the noise-free, variable component of the FRRf transient at the $i^{th}$ sample, $e_i$ is the corresponding noise, and $f_o$ is the fluorescence yield in the native state at the beginning of the transient. To accommodate the non-stationary character of $f_{vi}$, we assume that a relatively-stable portion of the fluorescence saturation transient can be approximated by a 3rd order polynomial (Fig. D.1), allowing SNR$_{raw}$ to be expressed as:

$$SNR_{raw} = \frac{\sqrt{\frac{1}{n} \sum_{i=1}^{n} f_{vi, regr}^2}}{\sqrt{\frac{1}{n-4} \sum_{i=1}^{n} (f_{vi} - f_{vi, regr})^2}}$$  \hspace{1cm} (D.4)

where $f_{vi, regr}$ is the variable part of the fluorescence transient regressed over the last $n$ last samples of the saturation transient, (Fig. D.1, Insert A1). The $f_o$ term in Eqn. D.3 is estimated from the third-order regression of the first 20 data-points of the ChlF saturation phase, extrapolated to a zero excitation power. SNR$_{raw}$ is calculated in real-time following each data acquisition prior to data fitting (Fig. D.1, S/N in a red rectangle). When performing running averages, the user can observe a progressive increase in the SNR$_{raw}$, usually in proportion to the square root of the number of averaged transients.

Replacing $f_{vi}$ with $f_i$ in Eqn. D.4 provides estimates of instrument performance (SNR*) at the $F_m$ signal level (Fig. D.1, S/N* in green rectangle). When recorded using an accepted fluorescence standard, this property quantifies instrument sensitivity.
D.2. Error propagation from SNR\(_{\text{raw}}\) to calculated photosynthetic properties

The statistical quality of ST-ChlF parameters (e.g. \(F_v/F_m\)), in terms of standard deviation \((\sigma_{\text{data}})\) or Signal-to-Noise (SNR\(_{\text{data}}\)), is directly related to SNR\(_{\text{raw}}\). When quantified, this relationship can be used to (1) guide the selection of experimental protocols to achieve acceptable SNR\(_{\text{raw}}\) levels, and (2) to discriminate between the instrument-related noise and the biological or environmental drivers of data variability. Although analytical rules of error propagation allow such quantification based on the covariance matrix of partial derivatives, this approach rarely yields reproducible results because of the nonlinear, complex relationship between fluorescence transients and the retrieved fit parameters. The highly variable contribution of instrument-related noise (of mostly normal distribution) and photon flux-related noise (of Poisson distribution) further affects the reliability of this approach. As an alternative, an empirical relationship between SNR\(_{\text{raw}}\) and SNR\(_{\text{data}}\), derived from sufficiently long data records acquired at varying levels of SNR\(_{\text{raw}}\) (Fig. D.2), offers a more reliable, empirical approach to quantifying the statistical quality of ST-ChlF parameters.

![Figure D.2](image)

**Figure D.2.** Fluorescence data acquired at 6 different levels of the SNR\(_{\text{raw}}\) with 100 data records at each level. Changes in instrument gain controlled the first three SNR\(_{\text{raw}}\) levels, while increased data averaging (4, 8, and 32 running averages, respectively) controlled the last three levels. Derived values of SNR\(_{\text{raw}}\) (Eqn. D.4) are shown as a light-blue line. The SNR\(_{\text{data}}\) and STD\(_{\text{data}}\) values for the retrieved variables (e.g. \(F_o\), \(F_m\), \(F_v/F_m\) and \(\sigma\)) at the highest SNR\(_{\text{raw}}\) level are shown in green background insert. Because of short-term (10-20 seconds) variation in the recorded data following step changes in experimental conditions (vertical dashed lines), the first 20 data points in each 100-long step were excluded from the analysis (grey patches). The remaining 80 data points displayed slow systematic trends (see Fig. D.3), resulting from physiological responses to a series of excitation flashes.

Because of the generally non-stationary character of biological data, SNR\(_{\text{data}}\) is calculated as:

\[ \text{SNR}_{\text{data}} = \frac{\text{Signal}}{\text{Noise}} \]

\[ \sigma_{\text{data}} = \frac{\text{Standard Deviation of Signal}}{\text{Signal}} \]

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where $d_i$ is the set of the retrieved ST-ChlF parameters (F$_{v}$/F$_{m}$, $\sigma_{PSII}$, etc.) at given level of SNR$_{raw}$, and $d_{i, regr}$ represents $k^{th}$ order regressed data (a proxy for the trendline in these properties, Fig. D3). The regression order needs to be sufficiently high to capture the observed non-linearity of the underlying signal trend. With most ST-ChlF data sets, these trends can be satisfactorily approximated by second order polynomials, as shown in Fig. D.3.

In the absence of trends in the acquired data, a zero-order regression applied to calculate SNR$_{data}$ yields:

$$SNR_{data} = \frac{\frac{1}{n} \sum d_i d_{i, regr}}{\sqrt{\frac{1}{n} \sum (d_i - \mu_d)^2}} = \frac{\bar{\mu}_d}{\sigma_d},$$  \hspace{1cm} (D.6)$$

where $\bar{\mu}_d$ and $\sigma_d$ are the mean and standard deviation of FRRf parameters derived from sequential data fits. Subsequently,

$$\frac{1}{SNR_{data}} = \frac{\sigma_d}{\bar{\mu}_d} = \sigma_{fractional},$$  \hspace{1cm} (D.7)$$

where $\sigma_{fractional}$ quantifies the relative noise in the acquired data, also known as the coefficient of variation, and expressed as a fraction of the measured parameter. The $rms_{noise}$, as defined by the denominator of Eqn. D.5, provides a general expression for sample standard deviation in both stationary and non-stationary signals.

Following the acquisition of a representative dataset (Fig. D.2), the SNR$_{data}$ (Eqn. D.6) are regressed against SNR$_{raw}$ (Eqn. D.4) to produce an empirical relationship, as shown in Fig. D.4.

The procedure for acquiring ST-ChlF data at varying levels of SNR$_{raw}$ and the subsequent regression
analysis of SNRdata vs SNRraw is performed automatically from a user-editable script. The resulting empirical relationship can then quantify the statistical quality of the retrieved data based on the underlying SNRraw values. The results of the data error analysis described above, although specific to a particular organism, offer some general observations:

1. The statistical quality of \( F_v/F_m \) and \( \sigma_{\text{PSII}} \) remains relatively robust even at high data noise levels (low SNRraw).
2. Statistical quality of the time constants of QA\(^{-}\) reoxidation (\( \tau_1 \) and \( \tau_2 \)) is lower than \( F_v/F_m \) and \( \sigma_{\text{PSII}} \), hampering efforts to reliably estimate rates of electron transport under low biomass concentrations.

The procedure for assessing error propagation presented above typically takes several hours to complete, using automated user-programmable scripts. The analysis serves to establish the empirical rules of error propagation, which can then be applied to downstream analysis. The results from this analysis also identify SNRraw as a key factor controlling data quality, providing a strategy for optimizing experimental protocols during adaptive data acquisition techniques, as discussed below.

**D.3. Noise distribution along fluorescence transients: fit quality and fit statistics**

As stated in Section 2.6 of the main User Guide, noise properties in the fluorescence signal change with signal amplitude. This holds true for data acquired at different biomass concentration, but also affects signal-dependent noise distribution in individual transients. For example, under conditions of \( F_v/F_m \sim 0.5 \), both the signal amplitude and the electronic noise change by a factor of two between \( f_o \) and \( f_m \) levels. The ‘shot noise’ introduced by variability in the photon flux further exacerbates this effect. To compensate for differences in the noise content along the fluorescence transient, calculation of \( \chi^2 \) (User Guide section 2.6.3), as well as calculation of partial derivatives in the fitting procedure, requires assessing noise variance at each point of the fluorescence transient. To quantify this variance, noise calculation is performed at both the saturation plateau of the fluorescence transient (\( f_{\text{high noise}} \)), and also over 50 points of the relaxation portion of the fluorescence transient (\( f_{\text{low noise}} \), Fig D.5). The noise standard deviation calculated at these two regions (\( \sigma^* \) and \( \sigma \) in Fig. D.5) is then interpolated along the entire fluorescence transient:

\[
\sigma_i = \sigma + (f_i - f(\sigma)) \left( \frac{\sigma^* - \sigma}{f(\sigma^*) - f(\sigma)} \right)
\]  

(D.8)
where \( f(\sigma^*) \) and \( f(\sigma) \) are the means of the fluorescence yield at the respective points of the ChlF transients (Fig. D.5). This approach requires noise independence along the length of the fluorescence transient. Compliance with this requirement is monitored using Durbin-Watson tests of noise autocorrelation at three sections of the fluorescence transient, and is flagged as a warning (with a yellow background) in presence of negative autocorrelation, or as an error (with a pink background) in presence of positive autocorrelation\(^6,7\). Although negative autocorrelation in the reconstructed noise doesn’t invalidate the approach described here, positive autocorrelation may signal significant problems with the acquired ST-ChlF transients, such as inadequate bandwidth, systematic errors, or a bad Instrument Response Function. If pervasive, these conditions affect the accuracy of the retrieved photosynthetic properties.

Using \( \chi^2 \) as a measure of fit quality and weighting partial derivatives with the reciprocals of noise variance improves the precision and accuracy of fitting procedure, while also providing an indicator of under-, and over-fitting.

The acquired noise information can also be used to quantify the statistical quality of the ST-ChlF retrieved parameters in real time, based on a single transient acquisition. This approach uses a series of synthetic transients, all with the shape and noise characteristics identical to the experimentally acquired transient. Fitting these synthetic transients and processing such retrieved synthetic data reproduces data statistics similar to that calculated in a series of repeated measurement using a real sample (Fig D.4), while eliminating the need for a rather lengthy experiment protocol as in Fig. D.2). There are several caveats, however. Data acquired with real

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\(^7\) Durbin-Watson Significance Tables.
samples will display some drift in photo-physiological parameters due to repetitive flashes (Fig. D.3). The synthetic, computer-generated transients, will be free of such systematic drift (which may actually help discriminate between the biological, and the instrument-related variability in the acquired data), and will use a single parent noise distribution to produce the family of synthetic transients. Finally, as the fractional contribution of the normal and the Poissonian noise in the acquired transient is difficult to assess, the Gaussian random number generator with normal noise only approximates the actual noise character in real data. Despite these caveats, estimates of data quality derived from computer-generated synthetic transients based on a single acquisition compare relatively well with those based on the 80-record long datasets (Fig. D.4).

The numerical procedure for producing the family of synthetic FRRf transients and performing statistical analysis on these transients is implemented in a FitStats procedure in the instrument control software (see below). The number of the synthetic transients and fit iterations are controlled from the user interface.

D.4. FitStats Procedure: quantifying data quality and model adequacy

Noise distribution along FRRf transients (Eqn. D.8), is calculated by default following acquisition of each fluorescence transient. It is used by the ‘FitStats’ procedure to produce a number of synthetic fluorescence transients (FitStats runs in Fig. D.5) and to estimate their statistical quality (SNRdata, σdata). These measures of data quality, and the corresponding quality flags (good, marginal, or bad) are automatically logged alongside the ST-ChlF parameters in the archived data files.

Noise distribution in ST-ChlF transients is also calculated by default when retrieving archived data, allowing users to assess, or re-assess the quality of previous measurements and experiments. Such post-processing can also be used to re-evaluate the adequacy of previously applied fitting models. An example of such exercise is shown in Fig. D.6, where the statistical quality of an archived fluorescence transient (Fig. D.6A) is re-assessed using different fitting models. As shown in this example, processing a relatively low quality (SNRraw = 15.7) transient using a three-exponential kinetics of QA− reoxidation results in a significant over-fitting, as manifested by the prevalence of the red and yellow quality flags (Fig. D.6.B). Fitting with two time constant of QA− reoxidation appears to perform relatively well (Fig D.6.C), but the CarQ terms, αCarQ and τCarQ, are still retrieved with low quality (yellow quality flags). Single-exponential fit leads to under-fitting, manifested by the increase in χ², deterioration of residual distribution (Fig. D.6.D), and further reduction in the quality of αCarQ and τCarQ. Allowing τ back in to the model and removing αCarQ and τCarQ improves the χ² somewhat, but produces unreasonable values of τ1 and τ2. As stated in section A2 of this Appendix, such practice should be avoided.
One difficulty in assessing $\alpha_{\text{CarQ}}$ and $\tau_{\text{CarQ}}$ at low (< 30) SNR$_{\text{raw}}$ levels is the relatively low precision in their retrieval. While these two parameters are independent of each other, their composite effect on the shape of fluorescence transient is roughly proportional to their product. Therefore, at low SNR$_{\text{raw}}$ levels, the FRRf fitting can reliably retrieve the product of $\alpha_{\text{CarQ}}$ and $\tau_{\text{CarQ}}$, but usually fails to recover these parameters separately with sufficient precision. Based on experience, most marine phytoplankton show $\tau_{\text{CarQ}}$ values range from 8 $\mu$s to 30 $\mu$s. Fixing the value of this parameter at 20 $\mu$s (Fig. D.6.F) and allowing the free $\alpha_{\text{CarQ}}$ compensate for the possibly erroneous guess usually accounts for the effects of CarQ on the overall fit accuracy, while improving statistical fit quality (Fig D.6. C, F). Users are encouraged to experiment with this approach using their archived data.

Each statistic in Fig D.6, panels B-F) was calculated using a sequence of 32 synthetic transients. Although generating these transients is computationally inexpensive, processing all of them takes several seconds. Performing the FitStats operation after each acquired ChlF transient will therefore reduce data throughput in proportion to the number of synthetic transients used, while improving the precision of assessed statistics in proportion to the square root of their number. The criteria for selecting the number of synthetic transients in the FitStats are similar to selecting the number of repetitive measurement when experimentally assessing the statistics of acquired data.
D.5. Adaptive modes of data acquisition

In adaptive modes of data acquisition, the length of experimental protocols (the number of sequential running averages or consecutive data acquisitions) is controlled by the user-specified criteria of data quality. These modes can optimize the frequency of data collection under conditions of low but variable phytoplankton biomass in the open ocean, while ensuring uniform data quality. All adaptive modes presented in this section operate in real-time, in a fully automated mode.

D.5.1 AUTO-SNR adaptive mode

In the AUTO-SNR mode, the running averages acquisition is terminated upon reaching the preset level of SNR_{raw}, preserving uniform statistical data quality under low, but variable biomass concentration (e.g., depth profiles in the open ocean) and under the variable irradiance regime used for rapid light curve (RLC) experiments. This mode can be initiated directly from the instrument control menu, or from an automated script. Following completion of the AUTO-SNR acquisition, the FitStats procedure, when engaged, calculates the statistics of the acquired data. The FitStats-based, real-time assessment of statistical data quality allows model properties (the number of free parameters) to be adjusted to retrieve the sought-after photo-physiological properties within the safe limits of model resolving power (absence of over-fitting). The uniform quality of retrieved data is satisfied at the minimal number of adaptively-adjusted running averages. However, this mode of data acquisition does not allow users to specify the required statistical quality of the retrieved data.
In the AUTO-StErr mode, sequence of data acquisitions is terminated upon satisfying the pre-set level of standard errors in the user-selected Quality Control (QC) parameters. Data are fit after each flash, and the retrieved parameters are sequentially added to a list of data records. Following each acquisition, the incrementally growing list of records is detrended (Eqn. D.5), and their statistical quality is re-analysed in terms of means, SNR_{data}, and standard deviations of all fitted parameters. Such calculated SNR_{data} and standard deviation are normalised with the square root of the number of records in the list to update their standard errors, StErr, and the Signal-to-Noise ratios of the means, SNR_{data}, as follows:

**D.5.2 AUTO-StErr adaptive mode**

In the AUTO-StErr mode, sequence of data acquisitions is terminated upon satisfying the pre-set level of standard errors in the user-selected Quality Control (QC) parameters. Data are fit after each flash, and the retrieved parameters are sequentially added to a list of data records. Following each acquisition, the incrementally growing list of records is detrended (Eqn. D.5), and their statistical quality is re-analysed in terms of means, SNR_{data}, and standard deviations of all fitted parameters. Such calculated SNR_{data} and standard deviation are normalised with the square root of the number of records in the list to update their standard errors, StErr, and the Signal-to-Noise ratios of the means, SNR_{data}, as follows:
\[ SNR_{\text{data}} = SNR_{\text{data}} \sqrt{n}, \]  
\[ StErr = \sigma_{\text{data}} = \frac{\sigma_{\text{data}}}{\sqrt{n}}, \]  
\[ StErr\% = 100 \frac{StErr}{Val_{\text{data}}} \]

where \( Val_{\text{data}} \) is the parameter value based on detrended data records, and \( StErr\% \) is the relative precision of the retrieved parameters, assessed in real-time after each flash, in the progressively-growing list of acquired records. In the example shown in Fig. D.10, a target statistical quality of three QC-parameters (\( F_v/F_m \), \( \sigma_{\text{PSII}} \) and \( \tau_1 \)) was specified. These parameters and their required precision are user selected from the instrument control interface, or pre-programmed with user-editable scripts. Flash-to-flash evolution of all fitted parameters with their statistical quality and their visual flags are displayed in real-time until all specified QC-conditions (identified by their cyan background) are satisfied. At that time, the marking of the last holding QC-parameter parameter changes (see the red border in Fig D.8, right panel) to mark the QC parameters with the most stringent quality criteria.
D5.3 AUTO-Progr adaptive mode

The AUTO-Progr adaptive mode is the combination of the AUTO-SNR and AUTO-StErr modes, where each step of AUTO-StErr is preceded by a single step of AUTO-SNR acquisition. The AUTO-Progr mode is useful under conditions of extremely low biomass, with single flash SNR_{raw} < 2. Under these conditions, some of the retrieved parameter may not converge to the required QC levels specified in AUTO-StErr mode. Boosting the SNR_{raw} level to 5-10 before engaging the AUTO-StErr mode described in the previous section usually resolves this problem.

Figure D.8. Operation of the AUTO-StErr acquisition mode. Three sets of 50 records, at progressively increasing levels of statistical quality in three QC-parameters (F_{v}/F_{m}, \sigma_{PSII} and \tau_1, highlighted by cyan background in the StErr column, right panel) were acquired using a sample with SNR_{raw} of ~6.8. The results of three acquisition with progressively stringent statistical requirements (records #50, #100, and #150) are shown. When more than one QC-parameter is specified, the acquisition is terminated after all pertaining conditions are satisfied. The last holding QC-parameter is marked by the cyan background across the mean, SNR, and the StErr columns red rectangle). The Auto-StErr mode, the QC-parameters, and their quality requirements are selected from the user interface, or can be pre-programmed using user-editable scripts.
D.5.4. Summary

Theoretically, all the adaptive modes of data acquisitions described in section D.5 are equivalent in terms of data quality relative to experimental effort (number of repetitive flashes). Selection of a particular mode will depend on the type of measurement, on the experimental condition, and on the desired speed and efficiency of numerical procedures for data processing. For example, the Auto-SNR procedure requires a single analysis step after completion. This mode allows pre-setting the required SNR\textsubscript{raw} levels in the averaged fluorescence transients, but doesn’t allow direct control of SNR\textsubscript{data}. The AUTO-StErr mode removes this limitation, but requires data processing and statistical processing after each flash at a higher numerical effort compared to AUTO-SNR mode. Practice shows that reasonably fast laptop computers can complete the required computation within the idle period between flashes (~1 second). Finally, under conditions of extremely low phytoplankton biomass, where many flashes are required to pull the signal from the noise, the

Figure D.9. Operation of the AUTO-Prog acquisition mode. Three sets of 50 records, at progressively increasing levels of QC in three properties (F\textsubscript{v}/F\textsubscript{m}, σ\textsubscript{PSII} and t\textsubscript{1}, highlighted by cyan background in the StErr column, right panel) were acquired under conditions of single-flash SNR\textsubscript{raw} of ~1.26. Data recovery under such low SNR\textsubscript{raw} is aided by an initial AUTO-SNR step to boost the quality of the fluorescence transient to an SNR\textsubscript{raw} level of 5 before engaging the AUTO-StErr mode. This mode can be initiated from the user interface, or pre-programmed with user-editable scripts.
AUTO-Progr mode may offer the best performance at reasonable computational effort. Users are encouraged to verify the performance of different modes of adaptive data acquisition in well-controlled laboratory conditions, to select the most appropriate method for a particular application.

D.6. Data logging and error reporting

Information regarding the quality of the raw fluorescence transients, including Durbin-Watson flags, the ST-ChLF saturation coefficient, SNR_{raw}, as well as the $\chi^2_0$ metrics of the fit quality and QA reduction level are logged automatically by default in a transient-specific section of archived data. The statistical quality of the retrieved data (standard deviation/errors, and data quality flags), when acquired using any of the adaptive acquisition methods described in this section, are also logged alongside the retrieved parameters in the data-specific part of the archived records.

Figure D.10. Deployment of the AUTO-StErr mode in RLC experiments. (A) Presence of slow trends in the acquired RLC signals. (B) Data reporting based on the last end-point of the second-order regression line in the 300-350 data record (pink background).
Appendix E

Instrument response function (IRF), bandwidth and gain

The fidelity of recorded fluorescence yields, and the ability to inter-compare data across instruments, demands that the shape of acquired fluorescence transients is controlled solely by photo-physiological processes, as opposed to instrument-specific effects. In practice, this requirement is rather difficult to satisfy, as a number of instrument artifacts can introduce systematic errors in the retrieved data. Such artifacts include, for example, mismatch of time response between excitation and emission channels, detector and amplification nonlinearity, inadequate bandwidth and power supply noise. Instrument makers continuously strive to reduce these artifacts, but users must be aware of them, and able to quantify and report their extent, and make appropriate corrections.

E.1. Instrument response function

In order to accurately capture time-varying ChlF yields associated with physiological processes, it is important to characterize the background ‘flatness’ of the instrument response function, IRF, in the absence of any physiology. The IRF can be characterized with a fluorescence standard (section 2.6.5) using experimental protocols identical to those used to acquire ChlF transients with samples. Alternatively, ethanol/acetone extracts of Chl a can also be used for this purpose, as these will have no physiologically-induced changes in ChlF. Deviation of the IRF from a flat line indicates the presence of one or more instrument artifacts that can influence data quality. While the IRF can be quantified and reported (in units of % deviation from the IRF mean), the effects of such deviation on the retrieved fluorescence properties are more difficult to quantify. Qualitatively, a variable IRF during the saturation phase of the excitation protocol will be more detrimental for assessing \( \sigma_{\text{PSII}}, \) \( p, F_v/F_m, \) while IRF variability during the relaxation phase will be more detrimental for assessing the kinetics of QA reoxidation. These effects can be corrected, to a large extent, using the procedures described in the following section. It is recommended that IRF quality information be archived with accompanying data files, together with the information on relevant correction procedures. Adherence to these practices should make ST-ChlF data more inter-comparable across different methods and instruments.

E.2. Instrument Bandwidth

Instrument bandwidth determines the ability to capture and record the dynamics of fluorescence yield changes with adequate time resolution. The fastest component of these dynamics is the change in the fluorescence yield due to light absorption, charge separation, and the reduction of QA, which all occur on time-scales of about 1 ns. Assuming that ~ 2 – 4% of the QA is reduced per \( \mu \text{s} \) of excitation light, an artifact-free recording of fluorescence transients requires a temporal response function with a rise-time of about 100 ns, and a corresponding bandwidth of ~3 MHz. The Nyquist criterion to adequately sample such signals requires digitization sampling rates of at least 6 MHz. Fluorescence transients acquired with lower bandwidth will display a range of artifacts in both the saturation and the relaxation portion of the recorded fluorescence transients. ST-ChlF transients acquired with a series of short excitation flashlets in both the saturation and
relaxation phase (FRR\textsubscript{f} mode of operation) will display progressive dampening of the fluorescence yield signal at increasing levels of Q\textsubscript{A} reduction, affecting retrieval of all fluorescence properties. ST-Chl\textsubscript{f} transients acquired with a single pulse in the saturation phase will display initial signal dampening, gradually relaxing by the end of the saturation phase, with the resulting signal inflection mimicking the effects of energy transfer between PSII reaction centers. Although the F\textsubscript{m} can be retrieved correctly, the F\textsubscript{o} is likely to be under-estimated. ST-Chl\textsubscript{f} transients acquired with a combination of a single pulse in the saturation phase, and a sequence of short flashlets in the relaxation phase, will suffer from both effects. The most detrimental artifact will be an abrupt decrease of the recorded fluorescence transient during transition from the saturation phase to the relaxation phase.

Inadequate bandwidth has significant consequences for ST-Chl\textsubscript{F} data analysis. Fortunately, satisfying the bandwidth requirement is relatively easy using widely available, moderate-speed electronics.

E.3. Control and Calibration of the Instrument Gain

Although all photosynthetically-relevant properties derived from ST-Chl\textsubscript{F} transients are calculated using fluorescence yield, there is some value in archiving the absolute intensity of the recorded fluorescence signals. Comparing these absolute intensities with independently measured pigment concentration may allow development of more robust methods for estimating chlorophyll biomass from ST-Chl\textsubscript{F} signal. The major obstacle in achieving this objective stems from instrument-specific variability in the measured intensity of the fluorescence signal. This signal is controlled by the excitation power (hopefully resolved by proper calibration), instrument gain, optical design of sample chamber, the spectral properties of the emission filter/detector channel, and the detector quantum yield. The inherent instrument-to-instrument variability in the last three of these factors makes it extremely difficult to directly compare absolute fluorescence signals acquired by different instruments. However, this challenge can be addressed by recording the ‘Standard Referred Fluorescence’ yield (SRF) acquired with a fluorescence standard:

$$SRF_{\text{Eng\ Units}} = \frac{\text{Signal}_\text{RefEng\ Units}(\lambda_{EM})}{Ex(\lambda_{EX})}[REF],$$  

(E.1)

where $SRF_{\text{Eng\ Units}}$ is the fluorescence yield recorded with the fluorescence standard in engineering units (Volts or counts), $\text{Signal}_\text{RefEng\ Units}(\lambda_{EM})$ is the absolute fluorescence signal acquired with this standard, $Ex(\lambda_{EX})$ is the excitation power used to acquire this signal, and $[REF]$ is the concentration of the standard. The excitation power and the excitation/emission channels ($\lambda_{EX}$ and $\lambda_{EM}$) should be identical to those used for acquisition of the ST-Chl\textsubscript{F} transients. Referencing the Chl\textsubscript{F} transient against the SRF transients...
allows the acquired ChlF transients to be expressed in Standard Referenced Units (SRU), which are directly interchangeable among all instruments that are cross-referenced to the same standard. Moreover, expressing the ChlF transients in SRF units (Eqns. E.1 and E.2) can be used to correct for most IRF-corrupting instrument artifacts (with the exception of non-linearity and the insufficient bandwidth).
Appendix F
Quantifying Q_A reduction during ST-ChlF transients

As noted in the main User Guide (section 3.2.1), the ‘single-turnover’ (ST) mode of the saturation flash requires close-to-full reduction of Q_A over a time-scale short enough to minimize the concomitant Q_A re-oxidation and the associated reduction of downstream electron carriers. Most current ST-ChlF instruments are capable of adjusting the excitation power to achieve this objective, but this requires a quantitative metric of the extent of Q_A reduction. The saturation level of Q_A reduction is rather ill-suited as such a metric, as it can only be assessed after data fitting. Moreover, the accuracy of such fit-based estimates is sensitive to the shape of acquired transients, especially at the low-end of the excitation power (Fig. F.1.A).

Instead, a ‘saturation coefficient’ ($sat_{coeff}$) can provide an objective measure of the saturation level in ChlF transients:

$$sat_{coeff} = \frac{area_{Hi}}{area_{Lo}},$$  \hspace{1cm} (F.1)

where $area_{Hi}$ is the area of the saturation shape above the diagonal between the lowest and the highest point of the saturation profile, and the $area_{LO}$ is the area below this diagonal (Fig. F.1.C). The $sat_{coeff}$ can be assessed in real-time, prior to the fitting procedure, and is model-independent. As judged from the statistical quality of the retrieved parameters (Fig. F.1.A), fluorescence transients with $sat_{coeff}$ in the range of 0.6 - 0.8 allow reliable retrieval of most of the photosynthetic parameters. The $sat_{coeff}$ below 0.5 (Fig. F.1. A) is likely to result in incorrectly-retrieved F_v/F_m and $\sigma_{PSII}$, while increasing $sat_{coeff}$ above 0.9 is likely to cause unpredictable trends on derived time constants of Q_A re-oxidation, albeit with increased precision of F_v/F_m and $\sigma_{PSII}$ estimates. Under conditions of very low biomass, it may be advisable to increase the excitation power above the 0.8 $sat_{coeff}$ to acquire more accurate estimates of F_v/F_m and $\sigma_{PSII}$, while sacrificing the accuracy of the retrieved Q_A reoxidation rates. In most cases, however, automatic adjustment of excitation power toward the target value of $sat_{coeff}$ in 0.6-0.8 range will produce most consistent results. The saturation coefficient, together with the saturation Q_A reduction level, when available, should be logged in the archived data.
Appendix G

Functional metadata recommendations

The table below provides an example of meta-data that can accompany a series of ST-ChlF measurements.

<table>
<thead>
<tr>
<th>Category &amp; time</th>
<th>Element</th>
<th>Description</th>
<th>Data type, conventions, units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>latitude</td>
<td>Geographic location</td>
<td>Floating point decimal, degrees, north positive</td>
</tr>
<tr>
<td></td>
<td>longitude</td>
<td>Geographic location</td>
<td>Floating point decimal, degrees, east positive</td>
</tr>
<tr>
<td></td>
<td>elevation</td>
<td>Height above reference ellipsoid</td>
<td>Integer in meters</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Alternative: height above ground)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>reference</td>
<td>coordinate system</td>
<td>Default WGS84</td>
</tr>
<tr>
<td>time</td>
<td></td>
<td>Date and time in Coordinated Universal Time (UTC)</td>
<td>Character string formatted according to ISO8601</td>
</tr>
<tr>
<td>location_source</td>
<td></td>
<td>Source of the Geodetic information</td>
<td>e.g. GNSS</td>
</tr>
<tr>
<td>time_source</td>
<td></td>
<td>Source of the Time information</td>
<td>e.g. GNSS, internet time pool</td>
</tr>
</tbody>
</table>

Data Processing

<table>
<thead>
<tr>
<th>Element</th>
<th>Description</th>
<th>proc_level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Processing level, indicated by an integer or string including sublevels such as 1A, 1B, 1C. Level 0 is uncalibrated sensor output and not distributed; Level 1 is calibrated data prior to any corrections or interpretation; Level 2 is interpreted</td>
<td>Sensor-specific</td>
</tr>
</tbody>
</table>
data; Level 3 is aggregated or re-gridded data.

<table>
<thead>
<tr>
<th>Field</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>proc_procedure</td>
<td>Reference to protocols and algorithms describing data processing steps</td>
</tr>
<tr>
<td>proc_version</td>
<td>Version of the data processing software</td>
</tr>
<tr>
<td>proc_revision</td>
<td>Incremental version of the processed data</td>
</tr>
<tr>
<td>cal_procedure</td>
<td>For calibrated data: documentation describing the calibration procedure. Can be the same as Processing procedure reference</td>
</tr>
<tr>
<td>cal_reference</td>
<td>Identifier of calibration information</td>
</tr>
<tr>
<td>cal_time</td>
<td>Date/time stamp of applicable or applied sensor calibration</td>
</tr>
<tr>
<td>cal_version</td>
<td>Version of the calibration processing software</td>
</tr>
<tr>
<td>Identifiers</td>
<td></td>
</tr>
<tr>
<td>sensor_id</td>
<td>Unique identifiers used to prevent duplication in the records of data users. Sensor serial number (manufacturer decides format)</td>
</tr>
<tr>
<td>platform_id</td>
<td>Sensor_id and sample_id are required as a minimum. Other identifiers are used to Platform serial number or global unique identifier (UUID) used with all connected sensors. May be left empty if not applicable.</td>
</tr>
<tr>
<td>Field</td>
<td>Description</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>deployment_id</td>
<td>identify data as belonging to the same or associated sources.</td>
</tr>
<tr>
<td></td>
<td>UUID can be automatically generated and assure global uniqueness of the identifier.</td>
</tr>
<tr>
<td>sample_id</td>
<td></td>
</tr>
<tr>
<td>observer_id</td>
<td></td>
</tr>
<tr>
<td>Licensing</td>
<td></td>
</tr>
<tr>
<td>owner_contact</td>
<td>A permanent email address where the owner of the data can be contacted</td>
</tr>
<tr>
<td>operator_contact</td>
<td>An email address where the current operator can be contacted</td>
</tr>
<tr>
<td>license</td>
<td>A licence string or coding that is either self-explanatory, or detailed in the License_reference field</td>
</tr>
<tr>
<td>license_reference</td>
<td>A reference describing the details of the data license</td>
</tr>
<tr>
<td>embargo_date</td>
<td>A date following which the data may be used according to the specified license. Used, for example, to hide the data record in near-real time visualization until quality control is completed.</td>
</tr>
</tbody>
</table>
A sample ST-ChlF data set currently stored in SeaBASS

The dataset references the file SeaBASS_ReadMe_for_Behrenfeld_NAAMES_3.docx, which is partially reproduced below.

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/begin_header
/identifier_product_doi=10.5067/SeaBASS/NAAMES/DATA001
!/received=20180914 fixed incorrectly scaled PAR values
/received=20181126
/investigators=Michael_Behrenfeld
/affiliations=Oregon_State_University_Department_of_Botany_and_Plant Pathology
/contact=mjb@science.oregonstate.edu
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/cruise=NAAMES_3
/data_file_name=Behrenfeld_FRR_PAR_NAAMES3
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/end_date=20170922
/start_time=00:05:29[GMT]
/end_time=23:59:17[GMT]
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/south_latitude=40.23[DEG]
/east_longitude=-38.784[DEG]
/west_longitude=-70.0795[DEG]
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/water_depth=NA
/wave_height=NA
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/cloud_percent=NA
/missing=-999
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/data_status=final
/delimiter=comma
/fields=date,time,lat,lon,SST,F0,Fm,Fv_Fm,Sigma_PSII,PAR
```
SeaBASS ReadMe for Behrenfeld_FRR_PAR_NAAMES_3
NAAMES (North Atlantic Aerosols and Marine Ecosystem Study)

This dataset contains Fast Repetition Rate (FRR) ST-ChlF data, incident Photosynthetically active radiation (PAR) data, and sea surface temperature data for the third expedition of the NASA NAAMES Earth Venture Mission (August 31 to September 22, 2017) in the North Atlantic. Michael Behrenfeld is the lead PI for the NAAMES Mission and served as chief scientist for this field campaign on the RV Atlantis cruise AT38.

FRR and SST data were collected continuously during the cruise using sample water drawn from the ship’s flow through seawater system. Prior to the cruise, the ship’s science seawater system was flushed with bleach to clean the lines. For NAAMES, a Teflon lined diaphragm pump was installed in the ship’s seawater system to provide clean and relatively undisturbed sample to the in-line instruments. PAR data reported here were collected with a Licor cosine collector positioned on the top rail of the port side aerosol van on the upper forward weather deck of the Atlantis, providing measurements relatively free of any ship shading. PAR data are reported in units of uM quanta/cm^2/s.

The FRR was characterized by the manufacturer, Zbignew Kolber. The Licor sensor was calibrated by Licor shortly before the cruise. Data submitted to SeaBASS from the FRR include initial
fluorescence (F0), Maximum Fluorescence (Fm), Variable Fluorescence (Fv/Fm), and the functional cross section of photosystem II (Sigma-PSII). These properties were derived from the single turnover flash sequence from the FRR. 16 individual flash sequence results were averaged into each reported value. Seawater sample analyzed by the FRR was exposed to darkness from the time the water was drawn into the ship to the time of measurement (estimated as a few minutes).

In addition to FRR and PAR data, co-located samples for phytoplankton carbon and particulate organic carbon and nitrogen (POC/PON) are or will be available in SeaBASS, along with HPLC pigments. Additional in-water and above water optical parameters and aerosols were measured during this campaign and should also be available in SeaBASS or by contacting the primary lead for the parameters of interest. A NAAMES participant list with area of expertise can found through the NAAMES website http://naames.larc.nasa.gov/participants.html

References


Seppälä, J., P Ylöstalo, S Kaitala, S Hällfors, M Raateoja, P Maunula. 2007. Ship-of-opportunity based phycocyanin fluorescence monitoring of the filamentous cyanobacteria bloom dynamics in the Baltic Sea. Estuarine, Coastal and Shelf Science 73 (3-4), 489-500


