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Roadmaps and Detours: Active Chlorophyll-*a* Assessments of Primary Productivity Across Marine and Freshwater Systems

David J. Hughes,[†] Douglas A. Campbell,[‡] Martina A. Doblin,[†] Jacco C. Kromkamp,[§] Evelyn Lawrenz,[⊥] C. Mark Moore,[#] Kevin Oxborough,[○] Ondřej Prášil,[⊥] Peter J. Ralph,[†] Marco F. Alvarez,[†] and David J. Suggett*[,†]

[†]Climate Change Cluster, University of Technology Sydney, Ultimo, Sydney 2007, New South Wales, Australia

[‡]Department of Biology, Mount Allison University, Sackville, New Brunswick E4L 1E4, Canada

[§]Department of Estuarine and Delta Systems, NIOZ Royal Netherlands Institute for Sea Research and Utrecht University, P.O. Box 140, 4401 NT Yerseke, The Netherlands

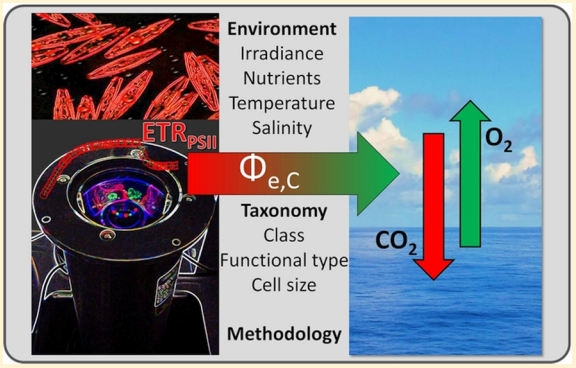
[⊥]Centre Algatech, Institute of Microbiology, Czech Academy of Sciences, Třeboň 379 81, Czech Republic

[#]Ocean and Earth Science, University of Southampton, National Oceanography Centre, Southampton, European Way, Southampton SO14 3ZH, U.K.

[○]CTG Ltd., 55 Central Avenue, West Molesey KT8 2QZ, U.K.

Supporting Information

ABSTRACT: Assessing phytoplankton productivity over space and time remains a core goal for oceanographers and limnologists. Fast Repetition Rate fluorometry (FRRf) provides a potential means to realize this goal with unprecedented resolution and scale yet has not become the "go-to" method despite high expectations. A major obstacle is difficulty converting electron transfer rates to equivalent rates of C-fixation most relevant for studies of biogeochemical C-fluxes. Such difficulty stems from methodological inconsistencies and our limited understanding of how the electron requirement for C-fixation ($\Phi_{e,C}$) is influenced by the environment and by differences in the composition and physiology of phytoplankton assemblages. We outline a "roadmap" for limiting methodological bias and to develop a more mechanistic understanding of the ecophysiology underlying $\Phi_{e,C}$. We 1) re-evaluate core physiological processes governing how microalgae invest photosynthetic electron transport-derived energy and reductant into stored carbon versus alternative sinks. Then, we 2) outline steps to facilitate broader uptake and exploitation of FRRf, which could transform our knowledge of aquatic primary productivity. We argue it is time to 3) revise our historic methodological focus on carbon as *the* currency of choice, to 4) better appreciate that electron transport fundamentally drives ecosystem biogeochemistry, modulates cell-to-cell interactions, and ultimately modifies community biomass and structure.



1. INTRODUCTION

Numerous active chlorophyll-*a* fluorescence techniques and instruments now exist to assay the physiological status and productivity of aquatic phototrophs, notably Pulse Amplitude Modulated fluorometry (PAM, see Supporting Information (SI) Table S1 for an abbreviation list)[1] and recently, PicoF Lifetime Fluorometry.[2] However, Fast Repetition Rate fluorometry (FRRf[3]), its variant, Flash Induction and Relaxation fluorometry (FIRe),[4] and emerging derivatives[5] remain the bio-optical approaches most commonly used to assess photosynthetic activity in natural phytoplankton communities.[6] This is in part because of flexible operational biomass thresholds, which enables sampling from oligotrophic "blue" waters of very low biomass to coastal waters and eutrophic lakes where chlorophyll-*a* (Chl-*a*) concentrations can be several orders of magnitude higher.[7,8]

FRRf was introduced two decades ago, following the earlier Pump and Probe fluorometer,[9] as a bio-optical tool to investigate and potentially quantify aquatic primary productivity (PP). The novel capability to noninvasively probe photosynthetic activities and derive rates *in situ* within seconds freed investigators from long-standing constraints associated with conventional bottle incubations[10,11] and thus offered a means to transform global efforts examining the nature and

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variability of aquatic PP. Indeed, FRRf technology has since been developed for a range of applications, from single microalgal cells[12] to corals and macrophytes,[13] as well as being deployed across all major oceans via research vessels, ships of opportunity, and gliders to assess phytoplankton productivity.[14−17]

Initial adoption of the technology was rapid, with publications based upon FRRf data becoming commonplace post-1998; however, most studies have used FRRf to analyze phytoplankton physiology and community structure (e.g., refs 18 and 19), rather than to derive PP *per se*.[20] All currently available methods for derivation of regional-global scale aquatic PP estimates still contain large uncertainties, due to problems with measurement techniques, chronic undersampling, or both. Ultimately, these deficiencies limit the amount and reliability of discrete photosynthetic rate data sets, which today underpin our best current global estimates of aquatic PP through (semi-) empirically calibrated algorithms applied to satellite-based ocean color observations.[21−23] So, with FRRf instrumentation (and data sets) more accessible than ever, why has FRRf not become the "go-to tool" to fill this data void?

Evolution of FRRf-based research followed a path common to emergent technologies: initial widespread enthusiasm that has, over time, yielded to a pragmatic realization of inherent uncertainties and constraints associated with both concept and application, which together, frequently undermined the interpretation and reconciliation of the data sets generated with other methods. Progress has been hampered by a shifting focus from the power of the technique to understand photosynthetic processes toward the limitations involved in attempting to quantify photosynthesis bio-optically. For FRRf, a recurrent obstacle for estimating PP has been the "currency" in which FRRf quantifies rates of photosynthesis.[24,25]

Carbon (C) is the most common photosynthetic currency used in studies of biogeochemical cycling, yet FRRf does not directly measure C-fixation but rather quantifies an electron transport rate (ETR_{PSII}) per Photosystem II (PSII) (see Section 2).[10,26] Fixed-C is a fundamental currency representing organic matter, energy and reductant exchanges among ecosystem components, and clearly carries broad importance within the Earth system. Generation of electron fluxes could, however, be considered a more fundamental driver of biological metabolism and associated biogeochemical cycles.[27] Our focus upon C as a currency, which stems from foundational studies using ^{14}C to measure photosynthetic production, somewhat obscures the key roles of photosynthetic reductant in driving single-cell nitrogen (N) and sulfur (S) assimilation,[28] reductive biosynthesis of macromolecules such as lipids,[29] and the generation of reactive oxygen species that modulate cell−cell interactions and community structures.[30] Yet, despite such key roles for reductant as currency, the inference of C fluxes from FRRf type measurements nevertheless remains an important goal.

The viability of using FRRf for improved global aquatic PP estimates based upon C hinges on our ability to robustly convert ETR_{PSII} to a rate of C-fixed using a photosynthetic exchange rate, "the electron requirement for C-fixation", termed $\Phi_{e,C}$.[20,25] The value of $\Phi_{e,C}$ may vary considerably due to physiological and taxonomic changes which are driven by external factors (see Section 3) or by errors associated with methodology (e.g., whether the quantity of functional PSII units within a given sample is directly measured or estimated based upon ancillary measurements - see Section 4). While recent efforts have empirically modeled $\Phi_{e,C}$ variation over space and time,[20,31] we continue to lack an integrated understanding of the mechanisms regulating this conversion factor. Thus, much of the observed variability in $\Phi_{e,C}$ across studies or regions remains unexplained.

Despite such persistent uncertainties in how ETR_{PSII} measurements can be scaled to C-fixation rates, oceanographers and limnologists continue to widely incorporate FRRf-based techniques into biogeochemical and ecophysiological studies. The relative ease of semicontinuous data acquisition across immense temporal and spatial scales yields a vast quantity of data that offers unprecedented insight into taxonomic patterns,[19,32,33] physiological processes,[18,34−37] and phytoplankton abundance[16]. However, the diverse array of FRRf protocols deployed ultimately hinders a more systematic exploitation of these ever-expanding data sets to better understand PP across studies, regions, environmental conditions, and taxa. For studies specifically examining $\Phi_{e,C}$, further disparity in methods used to (i) quantify total or particulate C-fixation rates, including incubation lengths, sample volumes, and spectral quality of actinic light, and (ii) report the ancillary data needed to link $\Phi_{e,C}$ to environmental or taxonomic regimes has further confounded our ability to separate methodological biases from true natural variability for published values of $\Phi_{e,C}$.[20]

With this perspective, we begin by describing the principles and techniques of FRR fluorometry and its relation to C-fixation, before examining the range of $\Phi_{e,C}$ measurements reported to date and discussing how these relate to known underlying biological processes. We then evaluate current methodology used to derive $\Phi_{e,C}$ and provide a roadmap by which the aquatic research community can maximize value from FRRf-based platforms, with the ultimate goal of improving broad-scale global evaluation of aquatic PP. We outline "best-practice" protocols, which capitalize upon recent breakthroughs in FRRf technology and theoretical concepts that can be consistently, and immediately, implemented into future observational campaigns. Finally, we discuss how a multidisciplinary research approach, focused upon developing a fully *mechanistic* understanding of electron allocation under various environmental and taxonomic regimes, is essential to develop predictive modeling of $\Phi_{e,C}$ based upon data extracted from FRRf measures, combined with standardized ancillary measures.

2. FAST REPETITION RATE FLUOROMETRY (FRRF) MEASUREMENTS OF PP

Active chlorophyll-*a* fluorometry comprises instrumentation and protocols to actively probe the photochemical status of PSII, the complex that photo-oxidizes water to generate electrons for reductive biosynthesis (for a review of aquatic applications, see ref 38).

Across phytoplankton taxa, photons are initially absorbed by a range of diverse pigment complexes serving PSII, with the resulting exciton passed among the photosynthetic pigments. Following refs 39, 40, the ultimate fates for this exciton can be described by fractional yields (Y), which together sum to 1, comprising (i) re-emission as a longer wavelength photon detectable as fluorescence (YF), (ii) loss as heat through nonphotochemical quenching (NPQ) processes that are both nonregulated (YNO) and regulated (YNPQ), and/or (iii) arrival at a reaction center as the initial photochemical step (YPSII) toward water oxidation and electron transport (Figure

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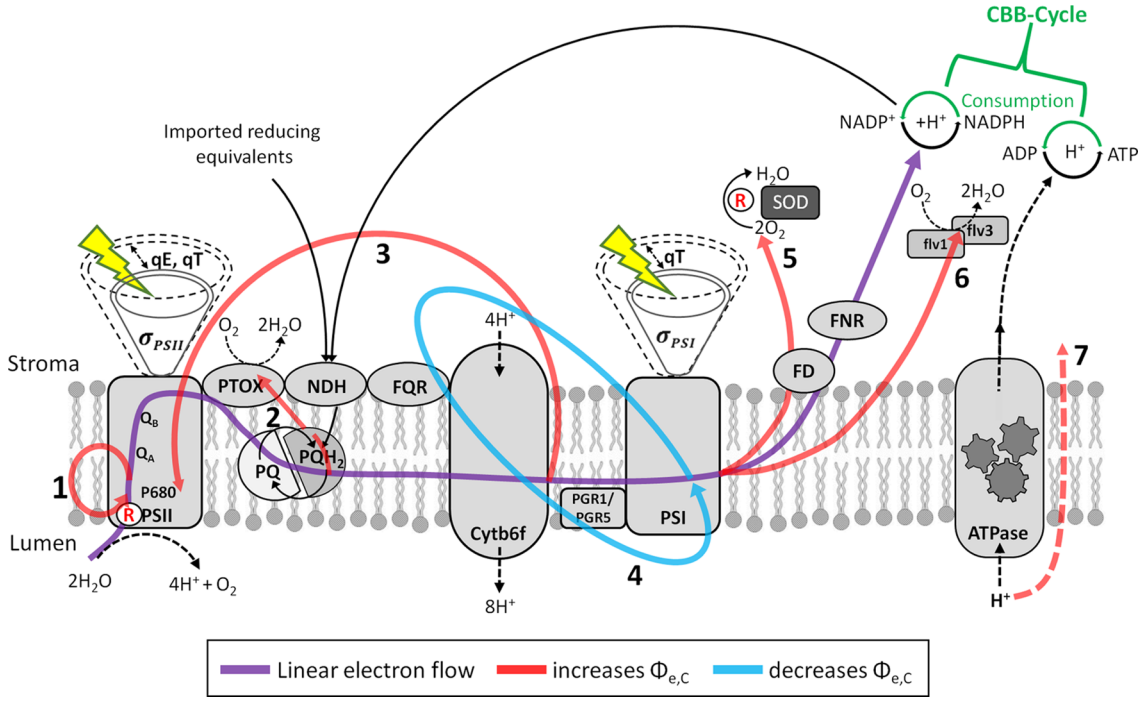

Figure 1. Schematic of photosynthetic linear electron flow (LEF, denoted by purple line) of oxygenic photosynthesis through to ATP/NADPH production and C-fixation via the Calvin-Benson Bassham (CBB) cycle. Also depicted are additional processes which potentially influence $\Phi_{e,C}$, colored according to whether they ultimately increase (red) or decrease (blue) $\Phi_{e,C}$: 1) charge recombination at PSII, 2) plastiquinol terminal oxidase (PTOX), 3) cyclic electron flow around PSII, 4) cyclic electron flow around PSI, 5) Mehler reaction, 6) flavodiiron protein (Flv)-mediated electron flows, and 7) proton slippage reactions. Solid and dashed lines represent electron and proton fluxes, respectively. Energy-dependent nonphotochemical quenching processes (q_E) and state-transitions (q_T) have reversible effects upon the functional absorption cross sections of photosystems I (q_T) and II (q_E, q_T). Generation of reaction oxygen species (ROS) is also indicated via the red R within open circles, together with the superoxide dismutase (SOD) detoxification step following the Mehler reaction.

1). This final step can potentially invoke photooxidation of the P680 chlorophyll of the PSII reaction center thereby generating the $P680^+$Phaeophytin- radical pair. Alternatively, the exciton can return to the antennae pigments (δ) where it is subjected a further time to fates (i)-(iii). Assessing the complementary probabilities of absorbed energy emission via these pathways therefore informs the extent to which excitons are used for photochemistry versus regulated or nonregulated dissipation as heat, generically termed NPQ, or lost through fluorescence emission which accounts for ~7% of absorbed excitation across the oceans.[17] All of these yields are accessible from active Chl-*a* fluorometry measures, although the algebraic extraction of yields from measured fluorescence levels involves assumptions[40] which may be differentially violated depending upon taxa and physiological state. An additional small, but metabolically expensive, exciton fate leads to photoinactivation of PSII through multiple mechanisms.[41,42]

FRRf delivers a series of closely spaced excitation flashlets to cumulatively close all PSII reaction centers typically within 50−200 μs, thereby ensuring that the first acceptor molecule within the photosynthetic electron transport chain (Q_A) is reduced only once for each PSII during a given flashlet series.[3] As PSII reaction centers cumulatively receive a photon they become photochemically "closed" for a period of ~1000 μs until diffusion of the plastoquinone electron carrier (Q_B) removes the photochemically generated electrons. The temporary closure of the photochemical yield stimulates a transient increase in the complementary fluorescence yield, measured as a fluorescence rise (SI Figure S1). Such FRRf protocols are termed "single-turnover"[3,43] which has important implications for the mechanistic interpretation of the resulting fluorescence rise. One of the main advantages of the single turnover protocol is that it does not increase the redox state of the plastoquinone pool, making FRRf measurements less intrusive and simpler to interpret than instrumentation which induces multiple-turnover of PSII (e.g., PAM[6]). FIRe fluorescence is conceptually similar to FRRf, except that a single turnover pulse is provided during which the fluorescence yield is rapidly subsampled to characterize the fluorescence rise. FIRe potentially simplifies FRRf-type technology and can lower power requirements, provided the excitation source is stable.

By fitting the FRRf (or FIRe) rise with a biophysical model describing photochemistry,[3,7,44] we can extract minimal fluorescence, F_0, maximal fluorescence, F_M, the effective absorption cross section for PSII, σ_{PSII}, and a connectivity coefficient, ρ, which describes the probability of an exciton from a closed center being transferred to an open PSII center (SI Table S2, Figure S1). It is important to note that the mechanistic meaning (and correct terminology) for these variables depends upon the state of the sample at the instant of the measurement. For example, if the FRRf rise is imposed upon a sample that is already under a level of actinic illumination, the F_0 extracted from the FRRf fit is actually steady-state fluorescence, F_t. Similarly, the F_m extracted from an illuminated sample will be some version of F_m', the "prime" notation indicating a measurement performed under actinic illumination. From the core FRRf variables a number of derived photosynthetic parameters describing PSII activity can be constructed (SI Table S2, see also ref 45). These parameters

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can, in turn, be used to estimate the rate at which electrons are generated by PSII (ETR_{PSII}), the initial step in both linear photosynthetic electron flow and in multiple forms of pseudocyclic electron fluxes from water back to oxygen to regenerate water.[26,46]

Subsequent to the rapid succession of excitation flashlets that drive PSII closure, FRR fluorometers also allow programming of probe flashes spaced far enough apart to allow progressive reopening of the PSII centers. In this relaxation phase, each probe flash tracks the instantaneous fluorescence yield (intensity) of the PSII pool at that instant and, by complementary inference, tracks the photochemical-reopening of the PSII pool by downstream electron transport processes.[3] These processes can be resolved into a number of kinetic lifetimes, τ (μs), which are the reciprocals of exponential decay rates (μs^{-1}) of the fluorescence signal with time.[47] These lifetimes therefore track the rates and relative amplitudes of processes consuming photochemical electrons. Even so, while routinely measured by chlorophyll-*a* fluorescence induction techniques, τ has been generally underutilized in primary productivity studies despite immense potential to resolve electron turnover processes[48] and productivity.[35]

3. WHAT IS ETR_{PSII} AND ITS RELATIONSHIP TO C-FIXATION?

Photosynthetic electron transfer begins with absorbed photons generating electrons originating from photosynthetic water-splitting, through the PSII reaction centers (RCIIs). Splitting of water at PSII produces O_2, and hence ETR_{PSII} is considered to be proportional to gross O_2 evolution;[49] whereby minimally four successive photochemical electron generating events are required to evolve 1 molecule of O_2.[10] Electrons leaving PSII may flow via PSI photochemistry through to NADPH as photosynthetic linear electron flow (LEF, Figure 1), which supplies the energy (ATP) and reductant (NADPH) needed to fuel the Calvin cycle during C-fixation.[34]

A photosynthetic currency of electrons is rarely considered in PP studies,[25] thus ETR_{PSII} is usually scaled to an equivalent rate of C-fixation via $\Phi_{e,C}$ (mol e^- [mol C]$^{-1}$, i.e. ETR_{PSII}/C-fixation). Thus, algebraically $\Phi_{e,C}{}^{-1}$ accounts for the yield of electron extraction from water during O_2-evolution, theoretically 4 mol e^- [mol O_2]$^{-1}$ when neglecting charge-recombination processes,[42] O_2-consuming processes associated with alternative electron flows,[50] as well as the additional energetic and reductant costs to assimilate CO_2 and other oxidized compounds required for growth for every O_2 generated (the photosynthetic quotient[51]). Assuming that four electrons are extracted from the splitting of two water molecules per molecule of O_2 evolved and using the commonly applied photosynthetic quotient of 1.25 (but see refs 51 and 52), the theoretical minimum reference ratio for $\Phi_{e,C}$ is assumed to be ~5 mol e^- [mol CO_2]$^{-1}$.

While ETR_{PSII} and C-fixation have been repeatedly shown to correlate well for microalgal monocultures in the laboratory[25,53] and phytoplankton assemblages in the field (e.g., refs 54−58), the slopes of the relationships often differ, reflecting combinations of (i) natural variability in $\Phi_{e,C}$ and/or (ii) variability induced by methodological biases between different protocols. Significant divergence from the reference ratio of ~5 mol e^- [mol CO_2]$^{-1}$ appears most common under saturating light[57,59,60] and nutrient limitation.[61,62] Suggett et al.[25] also demonstrated a strong taxonomic influence upon $\Phi_{e,C}$ by comparing ETR_{PSII} and C-fixation rates for six distinct phytoplankton taxa during steady-state growth,[25] reporting $\Phi_{e,C}$ values ranging between 3.63 and 11.5 mol e^- [mol CO_2]$^{-1}$ (see also refs 33, 35, and 58).

Mounting evidence that both environment and taxonomy influence $\Phi_{e,C}$ has turned attention toward resolving, understanding, and hence, ultimately predicting $\Phi_{e,C}$ variation. The undertaking of a comprehensive synthesis of available experimental field data[20] demonstrated that $\Phi_{e,C}$ was highly variable over space and time, ranging from 1.15 to 54.2 (mean 10.9) mol e^- [mol C]$^{-1}$. This analysis showed that $\Phi_{e,C}$ was empirically correlated with key environmental variables known to regulate photosynthesis, including irradiance, temperature, salinity, and nutrients − variables that can also influence phytoplankton composition. Nonetheless, the amplitudes of these effects were highly variable between regions. Environments found to have the lowest $\Phi_{e,C}$ were coastal stations that, presumably, did not often suffer from severe nutrient or light stress. Most variability in $\Phi_{e,C}$ was therefore hypothesized to reflect differences in physiological status and/or taxonomic structure at any given location due to specific, local biotic and abiotic conditions. Importantly, this study focused on largely short-term ^{14}C incubations and somewhat standardized FRRf approaches in an attempt to minimize confounding influences from methodology.[20]

Since then, several further studies have determined $\Phi_{e,C}$ for laboratory cultures[60,63] and natural populations[55−58] reporting values within a similar range. In attempting to reconcile $\Phi_{e,C}$ with environmental conditions, Zhu et al.[57] demonstrated a strong relationship (r^2 = 0.94) between irradiance and $\Phi_{e,C}$ for a specific nutrient-replete location (Ariake Bay, Japan) over a two-year period. Furthermore, Schuback et al.[55,56] recently demonstrated that $\Phi_{e,C}$ was highly covariant with NPQ, suggested to reflect the strong parallel influence of iron-limitation upon both NPQ and electron to C coupling throughout the NE subarctic Pacific (revisited in Section 4). Finally, and in contrast to recent studies demonstrating strong environmental regulation of $\Phi_{e,C}$, Robinson et al.[33] found that phytoplankton community composition instead explained more variance in $\Phi_{e,C}$ within a physically complex coastal system.

To date, these various studies have collectively reinforced the concept that $\Phi_{e,C}$ variability is strongly dependent upon the system in question, with environmental and/or taxonomic control of the underlying physiological processes. However, reconciling the 50-fold variation for published $\Phi_{e,C}$ values across the world's oceans based on empirical approaches alone still seems a step too far − thus a different approach is needed to increase our predictive ability. Instead, we ask if it is possible to better understand variance of $\Phi_{e,C}$ by considering how fluorescence ETR_{PSII} estimators can be biased across taxa or conditions and how microalgae differentially invest electron-derived energy and reductant into cellularly retained C-fixed versus diverse alternative sinks.

3.1. Physiological Regulation of $\Phi_{e,C}$. The parameter $\Phi_{e,C}$ interrelates two photosynthetic currencies (ETR_{PSII} and C-fixed), derived from disparate techniques that evaluate opposing ends of the photosynthetic pathway, operating on significantly different time scales.[64] FRRf probes the initial light-harvesting and photochemical capacity of PSII to estimate ETR_{PSII} with the processes and measurements operating on time scales of ps to ms. ^{14}C- or ^{13}C-methods measure the balance between C-fixation that occurs within the stroma

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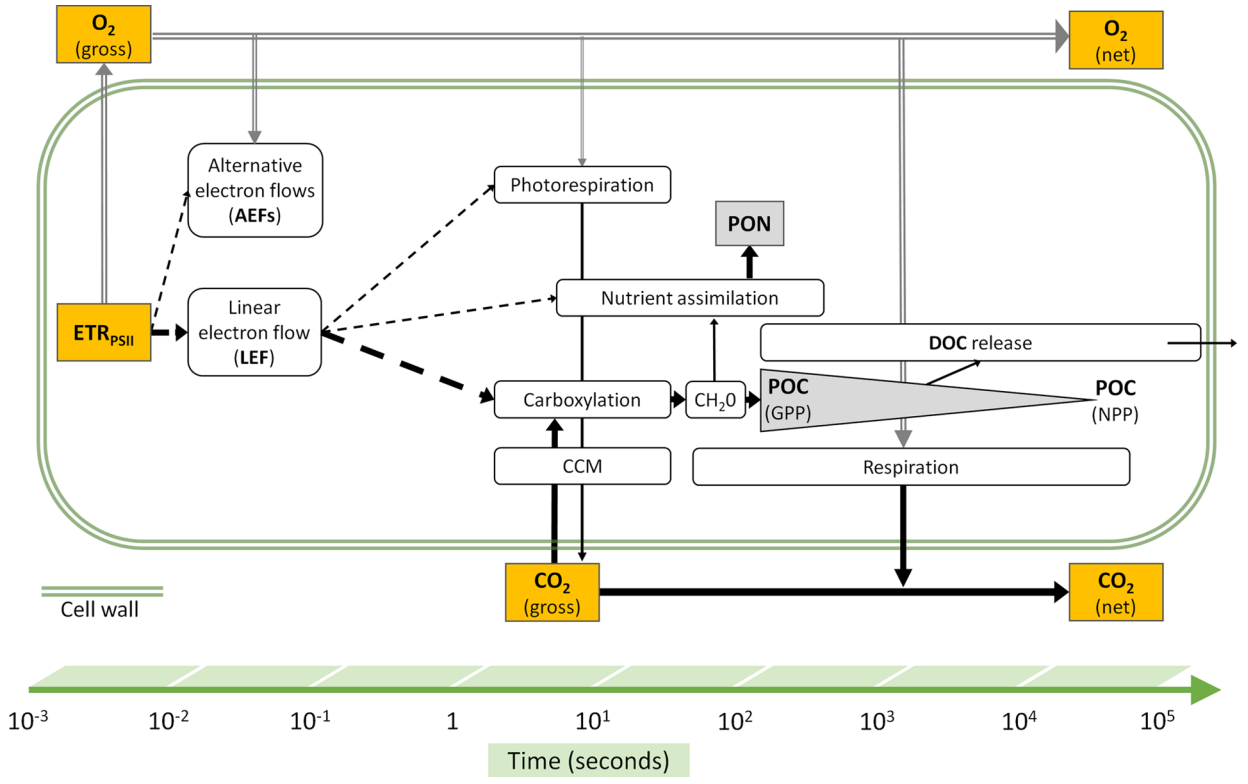

Figure 2. Simplified, conceptual summary highlighting the fluxes of the three major photosynthetic currencies (gold boxes) within a photosynthetic cell: electrons (ETR_{PSII} – dashed lines), oxygen (double gray lines), and carbon (solid black) over a time scale from ms to hours/days, with the direction of arrows indicating production (arrowhead: source) and consumption (arrow tail: sink). Major and minor pathways are indicated by thick and thin lines, respectively. Particulate organic nitrogen (PON) and particulate organic carbon (POC) are indicated by the gray shapes. The direction of the gray triangle (POC) reflects that the fraction of POC measured by ^{14}C-incorporation retained decreases over time due to respiration and/or extracellular release of dissolved organic carbon (DOC), as the photosynthetic rate captured transitions from gross to net primary production (GPP and NPP) with increasing incubation length (e.g., from minutes to hours).

downstream of PSII, during the Calvin Cycle, with the measurement of C-fixation generally extending over scales of min to h;[65] including loss of labeled-C to respiratory processes and extracellular release of dissolved organic C over the incubation period, operating over scales of ms to hours (Figure 2). Critically, because two methods measure processes that are physically, temporally, mechanistically, and kinetically separated (Figure 2), a suite of intervening physiological processes may operate to consume electrons, reductant and/or ATP, thus uncoupling the two measurements.[25,66] Many of these processes dynamically regulate photosynthesis under conditions where instantaneous reductant generation exceeds the instantaneous sink for reductant through C-fixation,[67] including supraoptimal light, suppressed C-fixation capacity due to nutrient stress, or when light-harvesting transiently outpaces the response rate of the regulation of C-fixation under fluctuating light.[68] A variable proportion of electrons may be diverted away from linear electron flow toward alternative electron flows (AEFs[50,69,70]), including the Mehler reaction,[71−73] "Mehler-like" reactions associated with flavoproteins of cyanobacteria and green algae,[74] cyclic electron flow around PSI[71] and PSII,[75−77] and chlororespiration via midstream oxidases such as the plastoquinol terminal oxidase (PTOX,[78−81] Figure 1).

Such AEFs offer photoprotective capacity[50,82] by acting as electron sinks to prevent harmful accumulation of reduced chemical species within the electron transport chain that can lead to damaging reactive oxygen species formation.[42,83,84] They may also activate NPQ processes, increasing thermal-dissipation of excess energy within the antenna bed, to further protect PSII from excess light.[85] AEFs generally increase $\Phi_{e,C}$ (Table S2) and likely explain why $\Phi_{e,C}$ values are often higher when photosynthesis is light-saturated.[57,60] Many AEFs also generate the proton motive force required for ATP synthesis in addition to that generated by linear electron flow (SI Table S3). Certain AEFs therefore allow phytoplankton to fine-tune intracellular ATP:NADPH stoichiometry to balance their respective consumption by various physiological processes[50] and overcome the classic ATP-shortage problem[34] balancing ATP:NAPDH consumption by C-fixation.[86] By decoupling ATP and reductant fluxes within photosynthetic organisms, AEFs provide a potentially important mechanism by which aquatic microbes balance the requirements for these two fundamental products of photosynthetic light reactions.

Cyclic electron flow around PSII is arguably the least-understood AEF but may have significant ramifications for fluorometry-based productivity estimates since it results in the complete disengagement of ETR_{PSII} from the generation of ATP and reductant, akin to a "release valve" to dissipate excess excitation energy under high light.[9,75] Key unknowns include the following: (a) the maximum proportion of electrons that can be diverted to this alternative electron flow, (b) the taxa-dependent variability of this pathway (although it appears especially pronounced in diatoms,[87] and (c) how environmental conditions apart from light may interact to influence this pathway (e.g., N-limitation[88]).

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Ultimately, cellular metabolism requires that the C-fixation pathway must also interact with other electron-consuming processes. Electrons drive reductive assimilation of S and N compounds and reductive biosynthesis of proteins, nucleic acids, and particularly lipids, leading to concurrent increases in $\Phi_{e,C}$ depending upon the proportion of reductant supplied directly by photosynthesis versus that by respiration.[89] Nitrogen is the second-most abundant constituent of phytoplankton dry biomass after C, often requiring the reductive assimilation of inorganic N from an external source,[90] with an associated assimilatory reductant cost that increases roughly in parallel with the oxidation state of the available N source; organic N < NH_4^+ < N_2 < NO_2^- < NO_3^-. The most reduced inorganic form, NH_4^+ is assimilated into biomass at the expense of only ~2 e^- [mol N]$^{-1}$ and 1 ATP, while NO_3^- requires a further investment of 8 mol e^- [mol N]$^{-1}$ for reduction to the level of NH_4^+ prior to assimilation.[91] Utilizing atmospheric N_2 is very costly, and to do so diazotrophs must invest a minimum of 5 e^- [mol N]$^{-1}$, plus at least 16 mol ATP [mol N]$^{-1}$.[92,93] Raising the substantial ATP capital needed to assimilate N_2 inevitably comes at a cost to $\Phi_{e,C}$. In fact, this has been proposed as the mechanism to explain why N_2-fixing cyanobacteria fail to dominate in hypertrophic lakes where self-shading limits light-availability and thus the means to generate sufficient ATP to sustain high diazotrophic growth rates.[94] Similarly, the assimilation of sulfur into organic metabolites is also a metabolically expensive process,[95] requiring that the sulfate anion is activated by ATP sulfurylase before it can be reduced to sulfite at the total cost of 2 e^- [mol S]$^{-1}$ and 1 ATP,[96] prior to further reduction to the level of organic-S at the expense of 6 further e^- [mol S]$^{-1}$ during biosynthesis. Whether cells with intrinsically high N and S quotients exhibit a higher $\Phi_{e,C}$ however remains untested.

Other notable processes that may account for increased $\Phi_{e,C}$ include "enhanced dark" respiration and Rubisco-mediated photorespiration, although the quantitative significance of photorespiration is doubtful for marine phytoplankton due to their C-concentrating mechanisms, which evolved primarily to avoid Rubisco-oxygenation events.[97] Indeed, Claquin et al.[73] demonstrated that light-enhanced respiration in the diatom *Cylindrotheca* was primarily due to Mehler-type reactions and that photorespiration *per se* played only a minor role. More research is, however, required to unlock the relevance of photorespiration in regulating the flow of electrons to C for algae where Rubisco shows a poor CO_2/O_2 discrimination, notably the form II Rubisco of dinoflagellates.[60]

Finally, phytoplankton can release variable, but often large, amounts of dissolved organic C to the environment, thereby altering the ratio of ETR_{PSII} to retained fixed C. This may be missed when just the particulate fraction of C-fixed is measured and thus variably influences measured $\Phi_{e,C}$ depending upon methodology. Dissolved organic C release is a normal function of phytoplankton cells, representing metabolic waste, cellular communication, chemoattractants, chemical defense, and substrate acquisition.[98] Indeed, this release broadly scales with total productivity in marine systems, where it averages 13% of PP[99] but can reach as high as 80%,[100] possibly increasing significantly during periods of environmental stress.[101,102] While it is yet to be conclusively shown which environmental factor(s) primarily influence dissolved organic C release,[98] irradiance has been highlighted as an important factor.[103]

Collectively, these processes operate over wide time scales to influence the ratio between gross O_2 evolution or ETR_{PSII} relative to the concurrently retained fixed C ($\Phi_{e,C}$) (Figure 2). Controlled laboratory or field experiments are therefore needed to unravel the nature and magnitude of the various non-C-fixing pathways but have rarely been performed for phytoplankton (but see[66,104−106]). Interpreting the results of such field experiments, is, however, inherently complicated by the fact that a given sample of seawater may contain many species of phytoplankton with variable cell sizes, metabolic rates, and physiological status.[107]

3.2. Evolutionary (Taxonomic) Divergence. Variability in $\Phi_{e,C}$ has also been considered at a taxonomic level and for both laboratory cultures[25,53,60] and natural phytoplankton communities.[8,33,35] Variance of $\Phi_{e,C}$ relative to taxa present is plausible considering that phytoplankton have evolved over long geological time scales across a broad range of environments.[108] Their elemental composition reflects this, with observations of C:N:P stoichiometry deviating significantly from the canonical "Redfield ratio" of 106:16:1,[109] across latitudinal environmental gradients.[110] A recent meta-analysis by Finkel et al.[29] identified taxon-specific differences in the macromolecular pools of proteins, lipids, and carbohydrates across marine and freshwater phytoplankton which is consistent with the observed variability in C:N. Due to the energetic and reductant cost involved in assimilating N and the differences in reductant requirements to generate retained proteins, lipids, and carbohydrates, differences in the C:N ratio across taxa may ultimately therefore translate into species-specific differences in $\Phi_{e,C}$.

Taxonomically distinct groups of phytoplankton have also adopted very different strategies of photosynthetic energy harvesting and utilization,[111,112] with mechanistic implications for $\Phi_{e,C}$. Diatoms, for example, can express a high capacity for NPQ relative to other taxa[113] through an energy-dependent mechanism (q_E) that diverts excess excitation energy away from PSII before it enters the electron transport chain.[111] Lowering excitation pressure upon PSII alleviates potential demand for AEFs that could otherwise increase $\Phi_{e,C}$. Other taxonomic groups have smaller amplitudes of energy-dependent NPQ (chlorophytes, cryptophytes) or functionally analogous quenching mechanisms which operate upstream of PSII,[114−116] potentially leaving a greater burden for AEFs to dissipate excess excitation energy after PSII, thus increasing $\Phi_{e,C}$. The proportionality between measured changes in NPQ and the downregulation of the effective absorption cross section of PSII (σ_{PSII}) can vary with taxa and conditions,[117] thus environments that select for different taxa may therefore explain spatiotemporal variance in $\Phi_{e,C}$ (e.g., refs 8 and 20).

While the capacities and extents to which specific electron pathways regulate photosynthesis across phytoplankton taxa remain poorly resolved, evidence suggests that specific AEFs are particularly important for certain phytoplankton groups. Flavoproteins have recently been identified as mediating electron flows direct to oxygen from PSII in the cyanobacteria and from PSI to oxygen in all tested photoautotrophs except angiosperms.[74,68] Such electron flows appear particularly important during transitions from dark to light, when electron transport transiently outpaces the regulatory induction of C-fixation. We can therefore hypothesize that flavoprotein-mediated pseudocyclic flows might raise $\Phi_{e,C}$ under fluctuating light regimes in rapidly mixing waters (see ref 63). In another example, PTOX appears important for the picocyanobacteria

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(*Synechococcus* and especially *Prochlorococcus*[81]) and the prasinophyte *Ostreococcus*,[118] allowing these taxa to sustain high ETR_{PSII} and hence presumably high ATP generation rates under conditions where the acceptor-side of PSI is impaired due to iron or N limitation, or excessive light, thereby minimizing potential for photoinhibition. Because cyanobacteria numerically dominate oligotrophic waters, the PTOX pathway can account for a significant proportion of total PSII electron flow in such regions,[50,78] thus measured $\Phi_{e,C}$ values in low-nutrient, oligotrophic regions are expected to sit toward the higher end of the range reported.[20] Even within the same genus, alternate photosynthetic strategies across environmental niches can have markedly different implications for $\Phi_{e,C}$. For example, the prasinophyte *Ostreococcus tauri* preferentially employs a dynamic q_E mechanism to sustain growth rates under very high light (1500 μmol photons m^{-2} s^{-1}),[119] while the low-light adapted *O. tauri* strain RCC809 mainly utilizes PTOX (or analogous pathways) to limit photoinhibition and generate ATP under excessive light,[120] thus adopting a strategy with an intrinsically higher $\Phi_{e,C}$.

Ultimately, variance of $\Phi_{e,C}$ across taxa likely reflects fundamental differences in lifestyle strategies, which have evolved to maintain photosynthetic fitness in terms of requirements for both reductant (NADPH) and energy (ATP) to successfully compete in different environmental niches. These variations in strategies may even extend to C-fixation itself. Notably, evolutionarily older taxa such as cyanobacteria likely need to expend more ATP and reductant to fuel C-concentrating mechanisms to compensate for Rubisco that has (i) lower catalytic efficiency[121] or (ii) lower affinity for CO_2 compared to more recently evolved phytoplankton groups such as diatoms.[122] Indeed, diverse C-concentrating mechanisms may incur a metabolic cost to the cell through ATP consumption, dissipation of chemiosmotic potential or cyclic electron flows,[123] and may thus exert a taxa-specific influence upon $\Phi_{e,C}$. Indeed, in laboratory studies, diatoms, the most recently evolved major phytoplankton taxon, typically exhibit $\Phi_{e,C}$ values within the lower range of reported values.[25,63] Many of the lowest reported values for $\Phi_{e,C}$ in the field (<4 mol e^- mol C^{1-}) however originate from cyanobacterial-dominated communities,[33] perhaps reflecting the distinct macromolecular composition of these prokaryotes compared to eukaryotes[29] or simply that the saturating pulse delivered by single-wavelength fluorometers equipped with a blue LED may not sufficiently drive PSII reaction center closure.[20] With the limited data to date it is hard to infer the extent to which $\Phi_{e,C}$ variability can be attributed to fundamental taxonomic divergence across groups versus physiological plasticity.[19,25] We do know that environmental variability including rapidly fluctuating light, nutrients, and temperature conditions results in the allocation of C, reductant and energy to a range of metabolic pathways to balance cell maintenance and growth with resource availability and acquisition;[66,112] thus, it seems conceivable to expect $\Phi_{e,C}$ to exhibit considerable variability in response to local environmental conditions, irrespective of whether the community assemblage (taxonomy) changes, but this notion clearly warrants future targeted testing.

Lack of $\Phi_{e,C}$ data with which to reconcile true variance across taxa and conditions is of course further compounded by methodological inconsistencies associated with ETR_{PSII} and C-fixation determinations. In particular, values of $\Phi_{e,C}$ less than the "theoretical minimum" of 4 are reported frequently (~30% across FRRf data sets[33]) and are difficult to reconcile on a physiological level with the oxygenic photosynthetic pathway.[25] While resolving the nature and extent of photoheterotrophic metabolism may be a key, but as yet, unexplored factor explaining such low values of $\Phi_{e,C}$, in order to robustly advance programs seeking to better resolve $\Phi_{e,C}$, we next consider potential methodological sources of error or inconsistency associated with over- and underestimation of ETR_{PSIIs} and C-fixation rates.

4. CONSTRUCTION OF *ABSOLUTE* RATES OF ELECTRON TRANSPORT (ETR_{PSII})

Absolute electron transport rates are commonly calculated from a simple mathematical construct: the product of light intensity (E), how much of this light is absorbed by PSII, described by the absorption cross section of PSII light harvesting (σ_{LHII}, units of m^2 $PSII^{-1}$), and the proportion of this absorbed light subsequently used for PSII photochemistry (Φ_{PSII}, unitless). Thus, the PSII specific electron transport rate is given by

$$ETR_{PSII} = E \cdot \sigma_{LHII} \cdot \Phi_{PSII} \quad (1)$$

For FRRf-based measurements, an ETR can be retrieved for each induction curve normalized per unit PSII reaction center (ETR_{PSII}) because the fluorescence-derived parameter F_q'/F_m' (SI Table S2) can provide an estimate of Φ_{PSII},[49] and the absorption cross section of PSII light harvesting can be related to the functional absorption cross section of PSII (σ_{PSII}, units of m^2 $PSII^{-1}$) as measured by the FRRf technique through[3,26]

$$\sigma_{LHII} = \sigma_{PSII}^{(\prime)} \cdot (F_v^{(\prime)}/F_m^{(\prime)})^{-1} \quad (2)$$

Noting that the strict validity of this equivalence under actinic light (indicated by the $^{(\prime)}$ notations) may depend on the degree of connectivity between functional PSII units.[26,124] Consequently, the PSII specific electron transport rate can be estimated from

$$ETR_{PSII} = E \cdot \sigma_{PSII} \cdot (F_v/F_m)^{-1} \cdot F_q'/F_m' \quad (3)$$

Eq 3 expresses ETR_{PSII} per unit PSII reaction center and hence generates fluxes in units of electrons $RCII^{-1}$ s^{-1}. To scale ETR_{PSII} to a more "ecologically meaningful" productivity rate per unit volume of water (see ref 25) we must further account for the number of PSII reaction centers per unit volume of (sea)water, [RCII], units of PSII m^{-3}, through accounting for the total light absorption by all the PSII units within a given volume, often denoted a_{PSII}, where

$$a_{PSII} = \sigma_{LHII} \cdot [RCII] = \sigma_{PSII} \cdot (F_v/F_m)^{-1} \cdot [RCII] \quad (4)$$

thus the volumetric rate of PSII electron transport (which we denote $vETR_{PSII}$, units electrons m^{-3} s^{-1}) can be expressed as

$$vETR_{PSII} = E \cdot a_{LHII} \cdot \phi_{PSII} = E \cdot \sigma_{PSII} \cdot (F_v/F_m)^{-1} \cdot [RCII] \cdot F_q'/F_m' \quad (5)$$

Irradiance and ϕ_{PSII} are both subject to measurement error.[38,125] For example, taxonomically diverse light harvesting complexes show distinct spectral absorbance and fluorescence emission characteristics which impose differential distortions upon the extraction of ϕ_{PSII} from measured fluorescence transients, because the algebraic conversions of fluorescence measures to ϕ_{PSII} assume, incorrectly, that all measured fluorescence arises from PSII (see for example refs 126 and 127). Yet it is the parametrization of a_{LHII} which has likely

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introduced the largest source of error into field measures of $vETR_{PSII}$.

Both σ_{LHII} and a_{LHII} are spectrally dependent properties, hence all methods of estimation are inherently spectrally weighted to the FRRf excitation LED.[26] Therefore, a correction factor is required to account for the spectral quality of the measurement LED versus the spectral quality of the natural light field and the absorbance spectra of the cells.[14] Such corrections require knowledge of lamp and absorbance spectra, thereby limiting the autonomous nature of past $vETR_{PSII}$ determinations[46] (but see ref 8). Newer, multi-wavelength fluorometers, however, enable construction of fluorescence−excitation spectra for any given sample, allowing for correction factors to be generated and applied with relative ease.[125,128] Moreover, several unpublished studies have also opted to incubate radioactive samples within the FRRf cuvette holder itself, using this "dual-incubation" approach to remove the need for spectral corrections between actinic light sources.[20]

Much remaining uncertainty in calculating $vETR_{PSII}$ arises from the need to quantify PSII reaction centers per unit volume of water, termed [RCII]. Direct measurement of [RCII] is conventionally performed using laborious O_2 flash-yield methodology,[129] requiring concentrated biomass (to >1 g m^3 Chl-*a*) and considerable time and effort, rendering this largely impractical for field studies.[26,35] Instead, many studies have thus assumed a constant ratio between the number of Chl-*a* and PSII units (n_{PSII}), relying upon ancillary measurements of Chl-*a* to estimate PSII per unit volume. Values for n_{PSII} of 500 mol Chl-*a* $RCII^{-1}$ for eukaryotes and 300 mol Chl-*a* $RCII^{-1}$ for prokaryotes have commonly been applied by oceanographers,[46] yet evidence indicates this is unlikely to hold true across phytoplankton taxa or growth conditions[130] (but see ref 8). Indeed, use of assumed n_{PSII} values has been identified as the major source of uncertainty in estimates of $vETR_{PSII}$ to date.[131]

The major bottleneck associated with how variance of [RCII] contributes to that of $vETR_{PSII}$ using FRRf may be overcome by the development of a fluorescence-based algorithm by Oxborough et al.[26] Given certain reasonable assumptions, the absolute value of fluorescence emitted from a given volume should be related to the concentration of PSII fluorescing within that volume. The approach proposed by Oxborough et al.[26] thus suggests that following a one-time calibration of the FRRf instrument, independent estimates of [RCII] can be obtained purely from FRRf-derived parameters: specifically, the PSII minimal fluorescence yield (F_o) and functional absorption cross-section (σ_{PSII}). This algorithm has been evaluated for natural communities and laboratory cultures under steady-state growth[26,125] and successfully used to measure absolute $vETR_{PSII}$.[132,133] This approach is attractive since it requires only a fluorometer precalibrated to [RCII] content, which could simply involve cross-instrument calibrations with known differences in excitation power.[26] However, a major constraint to widespread application at present is whether and how this approach will apply under certain specific physiological conditions such as iron-limitation[134,135] or photoinhibition.[84] In particular, the presence of fluorescent pigment complexes, which are not energetically coupled to PSII (notably under iron-limitation[135,136]), could result in a significant overestimation of [RCII]. As such, a rigorous assessment of the algorithm's robustness in the field under various environmental regimes and community compositions is an important step toward understanding the environmental confines under which [RCII] (and thus absolute $vETR_{PSIIs}$) can be confidently estimated via a calibrated FRRf. Such an evaluation has recently been performed in N-limited coastal waters[133] but should be extended to the laboratory, incorporating a wide diversity of strains, conditions, and physiological states to place better confidence intervals on field measurements of [RCII] (e.g., ref 84). Interestingly, recent studies[31,55] have demonstrated for iron-limited conditions that variability in $\Phi_{e,C}$/[RCII], i.e. the empirically derived relationship between $vETR_{PSII}$ and C-fixation, correlated well with an FRRf-based proxy for NPQ (see ref 137). Their approach does, however, maintain [RCII] as a constant, yet recent work suggests that [RCII] may be highly variable in response to relief from N-limitation.[133] Such empirical relationships therefore hold promise for the predictability of [RCII] and $\Phi_{e,C}$ variability within certain environments, but would require further validation to determine how and when they may be applied, and hence could be incorporated routinely into future data-collection campaigns for evaluation purposes. Ultimately, it may prove to be the case that a combination of approaches is required to accurately predict $\Phi_{e,C}$ across global scales, likely depending upon the system in question. Given that both the approaches of Oxborough et al.[26] and Schuback et al.[31] consider variance of [RCII] via inherent FRRf-measured parameters, it may be possible to begin this process by re-evaluating past FRRf data sets.

5. DETERMINATION OF C-FIXATION

Regardless of the photosynthetic currency in question, it is useful to define three components of primary production that can be estimated from measurements in closed systems:[138] Gross Primary Production (GPP), the rate of photosynthesis not adjusted for losses to excretion and respiration, Net Primary Production (NPP), where GPP is adjusted for excretion and photoautotroph respiration, and Net Community Production (NCP), where NPP is adjusted for respiration losses by heterotrophic microorganism and metazoan respiration. FRRf-based $vETR_{PSII}$ measures represent a true, instantaneous GPP (electron generation at PSII through water-splitting) and thus places an upper bound on photosynthetic reductant and energy generation at a given instant. C-Fixation measurements however represent a sliding scale across these definitions depending on the time-scale over which these measurements are made.[65] Importantly, coupling between GPP, NPP, and NCP will not be constant where changes to prevailing environmental conditions and taxa moderate the efficiency with which C is fixed. Consequently, $\Phi_{e,C}$ may be more appropriately defined as $\Phi_{e,C[GPP]}$, $\Phi_{e,C[NPP]}$, etc., and a first step means to provide direct physiological information on the importance of alternative electron flows and respiratory losses; we return to this point later.

Carbon radio- (^{14}C), and more recently stable- (^{13}C), isotope labeled bicarbonate methods are commonly viewed as the gold-standard of phytoplankton productivity measurements since they directly track "C-fixation" and thus the benchmark against which other techniques are commonly evaluated.[138] Nonetheless, isotope uptake has faced considerable methodological scrutiny since its introduction,[139−141] prompting Longhurst et al.[139] to wryly observe, "Rarely, in fact, can a technique have been so persistently criticised, but so consistently used.". A special conference was organized by Williams et al.[142] to celebrate developments 50 years after the

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introduction of the ^{14}C-method; however, a decade later, many of the methodological concerns highlighted by Longhurst et al.[139] persist, particularly in the complex relationship between incubation time and the subsequent cellular residency and metabolic processing of fixed-C (see SI Table S4 for comparative strengths and limitations of various methodological approaches). Theoretically, short-term incubations allow little time for fixed-C to be respired or excreted as dissolved organic C and thus should approximate GPP, whereas longer (e.g., dawn-dusk) incubations inherently allow for a proportion of any fixed-^{14}C to be lost to respiration, such that measurements over a full diel cycle tend to best approximate NPP.[143] In this context, we strictly define NPP as GPP minus dark respiration, light-enhanced mitochondrial respiration and photorespiration,[28] reflecting production rates predominately from the photosynthetic activity of the microbial population. Furthermore, the C-fixed must be strictly defined as that contributing to new particulate production versus that excreted as dissolved organic C (DOC), where in the latter case it may be reincorporated either by heterotrophic bacteria or mixotrophic phytoplankton, as per

$$\mathrm{NPP} = \mathrm{GPP} - \mathrm{R}_{\mathrm{LIGHT+DARK}} \quad (6)$$

$$\mathrm{NPP}_{\mathrm{PARTICULATE}} = \mathrm{NPP} - \mathrm{DOC}_{\mathrm{EXCRETED}} \quad (7)$$

Laboratory experiments regularly show GPP to be on average 3-fold higher than NPP, with a range of 1.2−7-fold,[28] and therefore $\Phi_{e,C}$ would be expected to increase with incubation duration as C-fixation transitions from GPP to NPP, with this transition time influenced by both species, and the dominant growth phase of the population.[144] However, differentiation between short and long incubations (supposedly capturing GPP and NPP respectively) is not always clear-cut, as demonstrated by a series of illustrative papers by Halsey et al.[145−147] Even very short incubations (20 min−1 h) can yield a variable C-fixation rate somewhere between GPP and NPP, influenced by cellular growth rate-dependent differences in the lifetimes of newly fixed C.[146,147] Such studies neatly demonstrate the variable rates and extents with which GPP-derived energy and reductant are used to fuel cellular metabolism. Specifically, when the marine chlorophyte *Duniaella tertiolecta* was maintained under steady-state, N-limited growth (0.12 day^{-1}), newly fixed C predominantly in the form of glyceraldehyde 3-phosphate was rapidly catabolized for cellular processes over a 20 min incubation, yielding a C-fixation rate reflective of NPP. Conversely, at a higher, N-replete growth rate (1.2 day^{-1}) newly fixed C was, instead, invested into longer-lived polysaccharides which have a turnover time of ∼4−6 h, thereby accumulating up to three times more radio-labeled C during the same incubation period (i.e., closer to GPP).

Considerable uncertainty in interpretation may therefore be introduced when $\Phi_{e,C}$ is derived from incubation lengths where it is not possible to determine exactly where a given C-fixation rate sits on the "sliding scale" from GPP to NPP.[65] This holds particularly true for natural phytoplankton communities where growth rates can be highly variable and impractical to quantify. Most studies seeking to derive $\Phi_{e,C}$ values from natural communities have done so using incubation lengths of 1−4 h upon the assumption that the measured C-fixation rates represent GPP[20] and less commonly from longer (12−24 h) incubations aiming to measure NPP rates.[57,58] Given our relatively new insights into the blurred boundaries between GPP and NPP during short incubations, it raises the obvious question: is it even possible to estimate GPP in the field using conventional "short-term" incubations, or is this an inherently flawed approach. To answer this, we compared ^{14}C-fixation rates obtained from 2 and 24 h incubations, using natural samples from a coastal reference station (Port Hacking, NSW, Australia) over an 18-month period. The reasonable equivalency between rates (Slope: 0.902, R^2 = 0.871, Figure 3) provides compelling evidence that 2 h incubations do not reliably determine GPP under the range of specific field conditions sampled but are actually close to NPP.

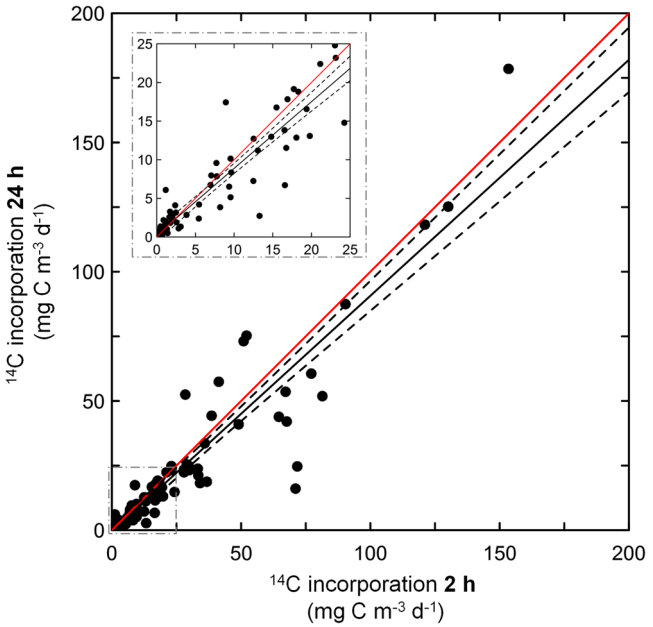

Figure 3. Comparison of C-fixation rates derived from both short (2 h) and long (24 h) ^{14}C-incorporation incubations (250 mL), performed *ex situ* as per Knap et al.[148] Data represents samples collected from natural phytoplankton assemblages (Port Hacking 100 m coastal reference station, NSW, Australia: 34.120° S 151.224° E) over a period of 18 months (April 2014 − September 2015). Linear regression generated r^2 values of 0.86 (slope: 0.88). Breakout panel (inset, dashed gray lines) is used to provide clearer visualization of lower values (0−25 mg C m^{-3} d^{-1}). Black dashed lines show 95% confidence interval, while the solid red line represents a theoretical 1:1 relationship.

Clearly the use of short (in this context defined as 1−4 h), and inconsistent, incubation durations across diverse communities and growth states limits our ability to reconcile the observed variability of $\Phi_{e,C}$ for natural assemblages across broad spatial scales[20] (Figure 3). With inherent uncertainty surrounding the C-fixation rates generated by incubation durations within this time frame, we ask whether future efforts should instead focus on deriving $\Phi_{e,C[NPP]}$ where NPP can perhaps be measured with more confidence than GPP. In the case of Figure 3, 2 h would seem appropriate, but clearly this would be unlikely to commonly apply across systems and taxa. At this stage, additional research is needed to determine the "sweet spot" in terms of incubation length: i.e. long enough to reliably capture NPP but not so long as to introduce sizable artifacts arising from bottle effects. Tentative evidence would suggest that an incubation length of ∼6 h may yield a C-fixation rate equivalent to NPP independent of growth rate,[147]

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but further evaluation of both laboratory cultures and natural communities is needed, together with consideration of potential diel effects. The priority here is to ensure that by advocating lengthier incubation times, we do not introduce variability in $\Phi_{e,C}$ due to bottle-effects, which outweighs that existing from the uncertainty of the balance between GPP and NPP captured by shorter incubation times. Until we can improve our methodological or conceptual ability to retrieve GPP from the field using tracer C-uptake, longer incubations may be the most currently desirable means to begin to better standardize data sets. Alternatively, additional experimentation could be performed to evaluate exactly where a given measured C-fixation rate derived from short-term incubations falls on the spectrum between GPP and NPP as per Halsey et al.[145−147] and Felcmanová et al.[149]

Similarly, for consistency, future laboratory studies investigating $\Phi_{e,C}$ should be mindful of the broader limitations of resolving GPP, such that measurements from phytoplankton cultures can directly inform observations from the field (NPP). This is not to say that GPP should not be the target of laboratory studies. Indeed, developing an understanding of the mechanisms regulating $\Phi_{e,C}$ will require systematically tracking the flow of energy through various photosynthetic substrates, products, and flows (ETR_{PSII}, O_2, and C-fixation), which is only feasible under controlled laboratory settings that track CO_2 uptake (GPP) into assimilation (NPP) and ultimately growth. Regardless, it is important that future studies use terminology, e.g. $\Phi_{e,C[GPP]}$ or $\Phi_{e,C[NPP]}$, to clearly identify the specific aspect of C-metabolism being estimated.

6. A ROADMAP TOWARD WIDESPREAD IMPLEMENTATION OF FRRF FOR PP

A clear long-term goal for FRRf practitioners and data-user communities is to possess sufficiently broad, high-quality data sets that allow us to reconcile natural variability of $\Phi_{e,C}$ over space and time. The short-term priority must be to limit, or compensate for, methodological inconsistencies that have consistently plagued comparison of FRRf and C-fixation data sets to date (outlined in SI Table S5), allowing us to *measure* "real" variability of $\Phi_{e,C}$ across natural assemblages. Unquestionably, applying "easy to fix" sources of error must be central to future study design and execution. For example, lack of spectral corrections[14] sample blanks and/or instrument corrections[7,150,44] can introduce up to 100% error and are thus a first order priority.

Based on Section 5, evidence would suggest that progress might be made in reconciling estimates of $\Phi_{e,C[NPP]}$ through transitioning *toward preferentially longer incubations* that estimate C-fixation rates reflecting NPP, using protocols that minimize bottle artifacts like photoinhibition, while simultaneously ensuring that routine ancillary measurements are collected to inform the key environmental drivers of $\Phi_{e,C}$ and where on a scale from GPP to NPP any given measurement might sit (see SI Table S6). However, to transition to longer incubations that are consistently reflecting some "steady state" NPP[65] will require intensification of studies that integrate time-resolved measurements of C-fixation − an approach that will not be trivial for natural communities where biomass remains low. Photoinhibition might be avoided by mimicking vertical mixing through the mixed layer by using programmable LED light sources, but how well this can be achieved to mimic real-world complex physically dynamic regimes will first require much improved understanding of light-field regulation.

Adoption of clear and standardized terminology when reporting key parameters (for both $vETR_{PSII}$ and C-fixation) will reduce methodological and conceptual ambiguities and facilitate intercomparison across data sets (see ref 43 for recommended fluorometry terminology). Furthermore, calibration of fluorometers to measure [RCII] as per Oxborough et al.[26] should be considered a central step prior to FRRf-based campaigns. On this note, there are numerous approaches to constructing an absolute $vETR_{PSII}$ (equations and units are outlined in SI Table S6), but as yet their relative performance has not been well scrutinized (but see refs 26, 33, and 125). *Reducing uncertainties in fluorescence yields and how they robustly scale to effective quantum yields*, e.g. readsorption of emitted fluorescence and interference from other pigments such as phycobilins, will be needed to reach this goal. Making these steps routine components of future studies, or of retrospective recalculations of existing data sets, would allow us to determine which algorithm is most broadly applicable, or if specific algorithms perform better under certain environmental or taxonomic conditions.

In the medium-term, field-studies can begin a process of algorithm evaluation *assessing the robustness of* more semi-empirical/theoretical[26] and empirical[31] approaches to estimate $\Phi_{e,C}$, across a range of biogeographical provinces. During this validation process, we can continue to evolve region-specific empirical models to predict $\Phi_{e,C}$ (as per ref 20), which *needs critical support from laboratory screening of* $\Phi_{e,C}$ under a diverse selection of taxa/strains and growth conditions to tease apart the respective influence of environment and taxonomy upon $\Phi_{e,C}$. Field-based studies would benefit from measures of phytoplankton functional type data through HPLC pigment, flow cytometry, and microscopy to resolve community composition. In addition to collecting data to support empirical modeling of $\Phi_{e,C}$, *exploitation of functional genomic tools may allow development of mechanistic understanding of electron allocation informed by taxonomic differences in genomic capacities, while, in parallel, use of (meta) transcriptomic and metabolomic tools may improve our understanding of physiologically variable changes in metabolism driven by potential changes in electron allocations and changes in nutrient acquisition strategies such as mixotrophy within a taxon or community* (e.g., refs 151 and 152). Membrane Inlet Mass Spectrometry (MIMS)-based O_2 dynamic measurements that can unambiguously resolve water-splitting,[153] in parallel with transcriptomics and metabolomics to quantify the presence of key markers for alternate electron fluxes, could be used to mechanistically "deconstruct" $\Phi_{e,C}$ for key taxa or oceanographic regions. Few studies have yet attempted to directly quantify the proportion of electrons being diverted into one or more AEFs (e.g., ref 78) relative to C-fixation to explain variability of $\Phi_{e,C}$, and this remains a fundamental knowledge gap. While AEFs have been examined using photosynthetic pathway inhibitors to isolate specific electron pathways,[82,154] *functional genomic platforms would enable these pathways to be examined as an operational network*[155] using flux balance models.

As we begin to generate higher-quality data sets, *the establishment of a centralized data repository (or at least ensuring data is collected according to best available practice and subsequently made publically accessible) dedicated to FRRf-based campaigns* would bring significant added value to data sets and offer considerable research benefits to the wider community. First, by integrating data into a centralized location, disparities in methodology used to derive $\Phi_{e,C}$ can be documented and

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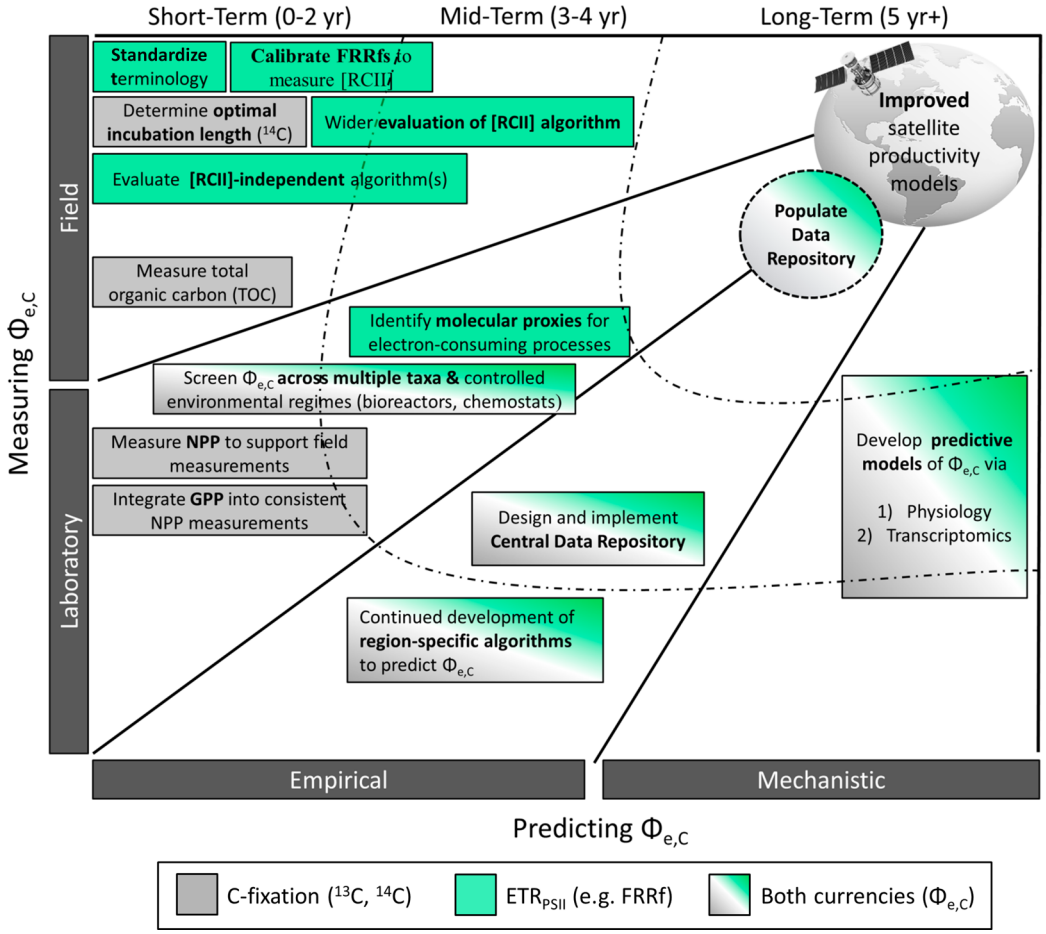

Figure 4. Roadmap for improving Fast Repetition Rate fluorometry (FRRf)-based estimates of primary productivity, focusing upon developing our predictive capability for the critical photosynthetic "exchange rate", $\Phi_{e,C}$. Short- to long-term research objectives are outlined for laboratory and field measurements of both electron transport rate through PSII (ETR_{PSII}) and C-fixation rates, together with recommended steps toward modeling $\Phi_{e,C}$ both empirically and mechanistically. The roadmap culminates in the long term objective of populating a centralized data repository which would provide a dedicated resource from which to "ground-truth" satellite-based models of oceanic primary productivity.

accounted for. These background efforts would enable subsequent data-mining and provide valuable insights into the conditions under which measures of $\Phi_{e,C}$ below the theoretical minimum value are commonly observed.

Second, the accessibility of multiple, quality-controlled data sets from global studies (e.g., refs 156) could be a game-changer for modellers of global PP, providing a central resource for data to feed directly into algorithm development and subsequent ground-truthing. NPP (using ^{14}C) global data sets have been the backbone of ever-improved algorithms to retrieve high resolution marine productivity patterns from satellites.[23] However, models that are built around $vETR_{PSII}$, GPP, and energy and reductant usage would offer a new platform to understand the role of the oceans in global bioenergetic fluxes. Third, and perhaps most critically, such a repository (and its applications) could provide capacity to evolve over time to keep pace with technical developments. For example, archiving of fluorescence induction curves, rather than just key FRRf parameters extracted by any one physiological model, allows the potential for the retrospective application of future FRRf-based algorithm developments or [RCII] calibration to historical data sets.

In conclusion, many of the tools are in place for a more confident evaluation of the accuracy of fluorometric assessment of aquatic productivity and the coupling of this to C-fixation. This can only happen if we can effectively use the growing global pool of FRRf platforms to generate appropriate data sets that allow us to (i) understand and model the critical photosynthetic conversion factor, the "electron requirement for C-fixation", $\Phi_{e,C}$, and (ii) develop a new conceptual understanding of electron flow as the driver of biological metabolism and associated biogeochemical cycles and hence a relevant currency for both marine and freshwater primary productivity studies. The likelihood of such a scenario would be greatly increased by a more coherent and methodologically consistent research approach from the FRRf-community, to systematically address the important gaps in knowledge that still hinder our current understanding of $\Phi_{e,C}$ variability and complexity. In reviewing current gaps in knowledge and advances, we offer a research roadmap to realize this vision (Figure 4). As part of this progression, we as a community may have to overcome long-held views of C as *the* currency of primary productivity, to enable progression toward complementary mechanistic frameworks to capitalize on ever growing data sets of photosynthetic electron transfer, a fundamental driver of aquatic biogeochemical cycles.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b03488.

Figure S1: fluorescence induction transient generated from FRRf showing biophysical model fit; Tables S1−S7: abbreviations used throughout the main text, parameters relevant to FRRf (and synonyms where applicable), information regarding alternative electron flows, comparative strengths and weaknesses of the commonly used methods to measure aquatic primary production (including FRRf), recommended "best-practice" solutions to common methodological issues, recommended ancillary measurements to support future data collection campaign, and common algorithms to calculate electron transport rates from FRRf (+ units) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: David.Suggett@uts.edu.au.

ORCID

David J. Hughes: 0000-0003-3778-7460

David J. Suggett: 0000-0001-5326-2520

Notes

The authors declare no competing financial interest.

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