Response to Anonymous Referee #2

We thank the reviewer for their comments and suggestions. Accompanying this response letter we provide a revised manuscript, in which we incorporated the reviewers suggestions, as outlined below. In the revised manuscript, suggested changes from the original manuscript are written in blue font. The page and line numbers provided in our answers below refer to the revised manuscript.

General comments

The present paper examines the empirical relationships between carbon fixation and electron transfer measured by fast repetition rate fluorometery (FRRF) and their dependency on diel changes in solar irradiance under low iron availability.
To largest drawback of the FRRF techniques remaining to this day is the conversion of electron transfer to carbon fixation. This has been the focus of multiple recent studies. New algorithms for the direct derivation of reaction centre 2 concentrations from chlorophyll fluorescence measurements (Oxborough et al. 2012) enabled researchers, for the first time since the introduction of the technique, to measure electron transfer in absolute terms. Any conversion of electron transfer to carbon fixation, however, requires that these new algorithms and subsequent conversion factors hold under varying light conditions and nutrient availability. Whether this is actually the case has never been rigorously tested, and thus, the new RCII algorithm should probably be used with a certain degree of caution. A RCII-independent approach may, hence, be an alternative to the present approach that should be included in future work and warrants further in-depth studying. I, therefore, consider the present publication a valuable contribution for the field of fluorescence-based primary productivity measurements.

Specific comments
16805 L7/8 – "more recently" – Kolber & Falkowski 1993 is not exactly recent. Furthermore, Kolber et al. (1998) should probably be mentioned here as well, perhaps in favour of Schreiber's work because the latter deals with multiple rather than single turnover techniques.

We were trying to convey the fact that active chlorophyll a fluorescence based methods (both single and multiple turnover techniques) are recent in relation to "traditional" incubation based methods including 14C-uptake studies. We have changed "more recently" to "Over the past two decades" (page 2, line 39).

16805 L 20-23 – Using a combination of Đd'e,C and 1/nPSII as a conversion factor is rather new to most people working with FRRF. Frankly, I still have somewhat of a hard time getting my head around this conversion factor with regards to its units and absolute values. To ease the reader into this, dould the authors perhaps mention the previous approach of using Đd'e,C alone, emphasize why the new conversion factor is chosen over Đd'e,C and what one would expect as theoretical values, similar to the theoretical minimum of Đd'e,C of 4-5 mol e- (mol C)-1.

We agree with the reviewer and have added the suggested details to the revised manuscript

(page 11, lines 284-287). A discussion about theoretical values of our conversion factor can be found in the original manuscript (page 16820, lines 19-23), and the revised version on (page 18, lines 490-497).

16806 L5-8 References mentioned here did not study the mechanistic underpinning of the uncoupling between ETR and C fixation, but rather the empirical relationships between ETR and Carbon fixation. Either include references that focus on the underlying mechanisms or rephrase the scentence.

The sentence has been rephrased and now reads:

"For example, energy and reducing power (ATP and NADPH) from the photosynthetic light reaction can be used directly for the reduction or assimilation of limiting nutrients rather than for carbon fixation (e.g. Laws, 1991; Myers, 1980), resulting in an increased derived conversion factor K_c/n_{PSII} (e.g. Napoléon et al., 2013)."(page 3, lines 59-62).

16808 L6 – Unfortunate gap in the irradiance data! Could you perhaps just specify when the malfunction occurred and over which times you had to fill the data gap?

The time period for which the PAR data was extrapolated (bases on continuously logged SW radiation data from the NOAA buoy) was 14:00 – 17:00. This has been clarified in the revised manuscript (page 5, line 114).

16810 L 22: Are the four wavelengths of the Soliense FRRF exciting fluorescence one after another or are they used simultaneously?
Please clarify because other (mostly multiple turnover) fluorometers do not allow the user to combine multiple excitation wavelengths at once (instead one has to use them one after another).

The four excitation wavelength in the Soliense FRRF can be triggered separately or in combination, making it a truly versatile instrument. Data presented in the present study was acquired triggering all four wavelengths simultaneously. This has now been clarified in the current manuscript (page 7, line 188).

16811 – Equation 3 is missing the Đd'PSII term. Đd'PSII = 1 mol electrons (mol quanta)-1, and is needed to end up with units of electrons and cancel out the mol quanta. It is often omitted in the literature because it takes a constant value of 1, however, it should be included.

This has been changed in the revised manuscript (page 8, line 212-214).

Also, this whole paragraph on FRR fluorometry makes no mention of a blank measurement. However, the blanks may be very important, especially in waters with low phytoplankton biomass. Please clarify whether blank measurements were carried out and how data were treated for blank correction.

Blank corrections were performed for each sample and values for each wavelength automatically subtracted during the sample run. We added a description of the exact procedure for blank-correction in the revised methods section (page 7, line 181-182).

16812 L 4 – The SI unit for radioactivity is Bequerel (Bq), not Curie. Please convert accordingly.

This has been changed in the revised manuscript (page 9, line 234).

16812 L17 – 10 mL instead of "Ten".

We spelled out this numbers at the beginning of sentences to be consistent with the editorial style of the journal.

16813 L 18-25. The authors may not yet be aware of an improved technique to fit ETR vs. E curves, which was introduced by Silsbe & Kromkamp (2012). One of the assumptions of a regression analysis is that the y-values are independent of the x-values. With irradiance (E) being a factor in the ETR equation (e.g. Eq. 3), this assumption does not hold. Silsbe and Kromkamp addressed this issue in their 2012 paper in L&O methods (Modeling the irradiance dependency of the quantum efficiency of photosynthesis. Limnol. Oceanogr. Methods 10, 645–652). Their approach also reduces the error of the fit at high irradiances (where quantum efficiency values become highly variable due to low variable fluorescence at high light). It may not make much of a difference in the derived P vs. E parameters, but this is certainly something to keep in mind for future work.

We strongly agree with the approach outlined in (Silsbe and Kromkamp, 2012), and will try to implement it in future studies. However, as the reviewer points out, and as is clear from the data presented in Silsbe and Kromkamp (2012), the different approach only marginally changes the derived fit parameters, with the largest effect on the error of the derived fit parameters (P_{max} and alpha). In particular, the derived error for alpha is likely to decrease, while the error of the fit parameter P_{max} is likely to increase since the parameters used to calculate ETR (sigma,in particular) can be measured with much higher accuracy at low relative to high light. During the present study, errors of derived parameters were not large enough to obscure the diurnal patterns we observed. Thus the alternative fitting approach would not have an appreciable influence on our interpretation of our data.

16814 L 10-14 – The authors expect the new RCII algorithm of Oxborough et al. 2012 to not hold under Fe limiting conditions. Could you perhaps elaborate why? As far as I know, the algorithm has never been put to test under low Fe (or any other form of nutrient stress). This needs clarification.

This issue has also been addressed (at length) by reviewer #1. Below we repeat our answer as well as some data analysis, giving evidence that the approach does indeed not work when contrasting iron limited and iron replete conditions. We have changed the wording in the methods section of the revised manuscript, to be less ambiguous about why the Oxborough approach was

not applied in the present study. Additionally, in response to an insightful suggestion by reviewer #3, the revised manuscript now includes estimates of relative diurnal changes in $1/n_{PSII}$ (page 11, lines 292-315; page 14, lines 380-390; page 17, lines 487-488; page 18, lines 494-497).

The inherent assumption to the approach of Oxbrough et al. is that the ratio of the rate constants of photochemistry (kp) and fluorescence (kf) stay within a narrow range. This is not the case under iron limitation, where kp decreases while kf increases (e.g. Vassiliev et al., 1995). Indeed, the original paper by Oxborough et al. cautioned that the approach relied on assumptions which might not hold under nutrient limitation, especially if the limiting nutrient is iron (Oxborough et al., 2012). This potential caveat was recently repeated by Robinson et al., 2014, who say:

"The calculation of [RCII] using the relationship between the minimum fluorescence parameter (Fo) and [RCII] as determined by Oxborough et al. (2012) may be sensitive to nutrient stress (C.M. Moore pers. comm.) which results in the enhanced uncoupling of chlorophyll complexes and PSII reaction centres (…) ."

A recent publication by Silsbe et al. (2015) does include data for cultures grown without added iron, though no specific information is presented on the extent of iron limitation in these cultures. In the assessment of their data, Silsbe et al. note that:

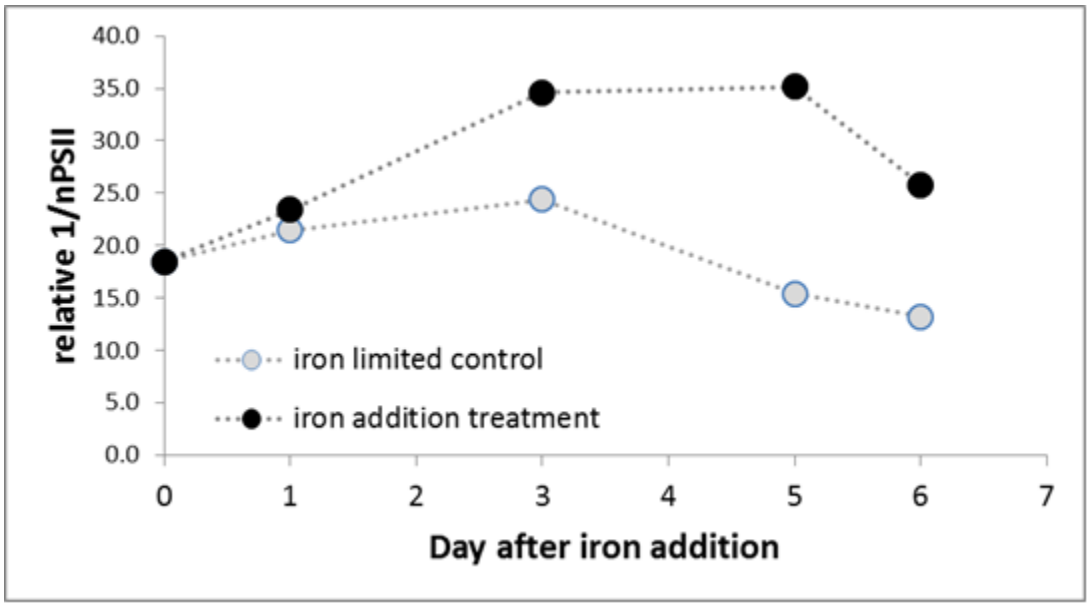
"…,both Tp-Fe and Tw-Fe cultures grown in the absence of iron predicted higher [RCII] than measured. Consequently these cultures yielded lower K_R values than other cultures. This key finding is consistent with the concept that iron limited phytoplankton may accumulate a store of non-energetically coupled chlorophyll-binding complexes that increases the quantum yield of fluorescence (Φ_f) relative to iron replete phytoplankton (Behrenfeld and Milligan 2013; Macey et al. 2014). As K_R is proportional to Φ_P/Φ_f, an increase in Φ_f would diminish KR as observed in this study. Omission of these iron-deplete cultures generally increased the mean K_R value for each instrument and reduced its variance…"

In order to further verify this evidence from the literature, we applied the Oxborough approach to our own data. The data shown below is from an on-board iron addition experiment conducted in the iron-limited NE subarctic Pacific, published in Schuback et al. (2015).

Below, we use values of F_o and σ_{PSII} in the dark-regulated state to derive information on the variability of $1/n_{PSII}$ during our iron-addition experiment (all values are mean of 3 biological replicates). Lacking the instrument specific calibration factor K_R, we were not able to derive absolute values for [RCII], but, since K_R is a constant, changes in of F_o/σ_{PSII} should represent relative changes in [RCII]. Normalized to [chl a] we should be able to derive relative changes in $1/n_{PSII}$. The data below show that $1/n_{PSII}$ increased after iron-addition, which is in contrast to numerous previously published studies showing increased $1/n_{PSII}$ (chla $RCII^{-1}$) in iron limited cells (e.g. Macey et al., 2014; Vassiliev et al., 1995). Similarly, in our laboratory experiment, cultures well acclimated iron-limitation had a significantly lower relative $1/n_{PSII}$ than the iron replete

cultures.

Day	Fe limited CONTROL sig	F0	relative [RCII]	[chla]	relative 1/n_PSII	Fe ADDITION sig	F0	relative [RCII]	[chla]	relative 1/n_PSII
0	963	26	0.03	0.50	18.4	963	26	0.03	0.50	18.4
1	906	25	0.03	0.60	21.5	841	26	0.03	0.72	23.4
3	981	49	0.05	1.22	24.4	743	46	0.06	2.12	34.6
5	910	111	0.12	1.88	15.4	821	182	0.22	7.79	35.1
6	920	121	0.13	1.73	13.2	819	302	0.37	9.49	25.8



16815 L14 – Could the authors please specify how they define the photic zone, i.e. as the 1% or 0.1% light level because different groups of researchers define it differently, and photosynthesis may take place well below the 1% light level (Kirk 1994)

The photic zone was defined as the 0.1% light level, which has been clarified in the revised version of the manuscript (page 12, line 326).

16816 L 6-27 and conversion factors presented in Fig. 1 and 2 – Why do the conversion factors differ so much between the two figures (2000-8000 e- RCII-1/C Chla-1 in Fig. 1, but only 2-10 e-RCII-1 C Chla-1 in Fig. 2)? If one divides the ETRmax by Pmax for carbon fixation (or the corresponding alpha values by one another), one should end up with values of e few thousand e-RCII-1/C Chla-1. Please clarify/fix accordingly.
16817 – Fig. 4 shows conversion factors of 2-12 x104. This is what the axes in Fig. 3 should probably read as well?

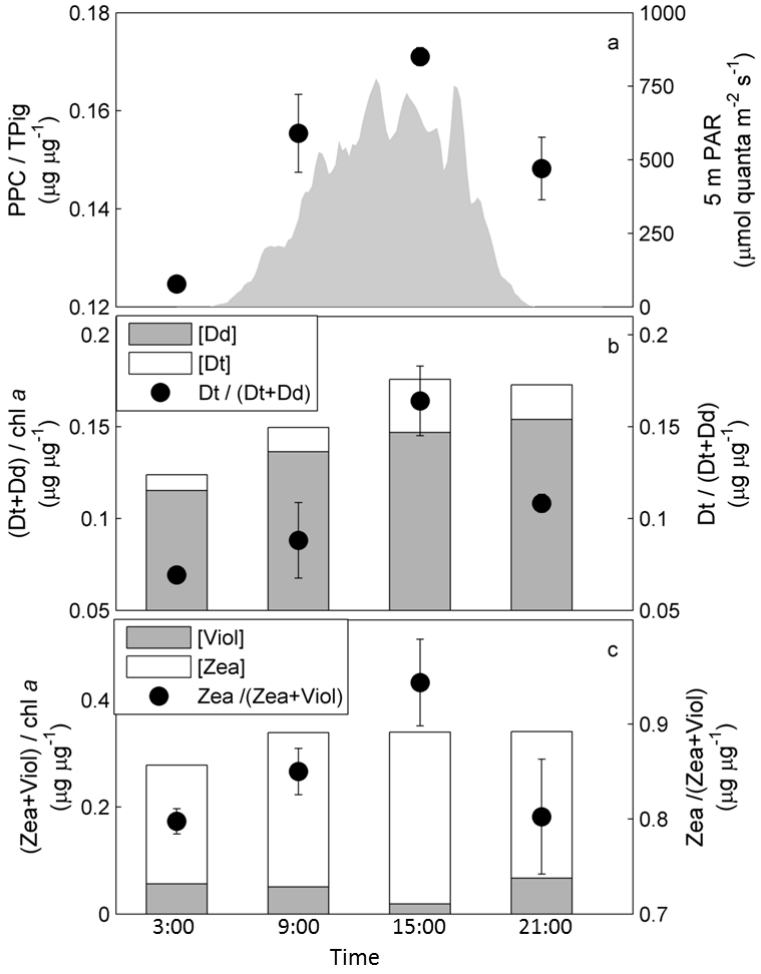
We thank the reviewer for pointing out this mistake and have corrected axes labels on Fig. 2 accordingly.

16817 – The in-text reference to Table 2 is misleading. Table 2 defines PPC, PSC etc. but does not actually present pigment ratios as suggested in the text. I would suggest mentioning this table in the methods and explain how the PPC and PSC were defined there.

We agree and have corrected the revised manuscript according to the reviewers' suggestions (page 6, lines 144-145).

16817 L18-29 – The calculated DES ratios account only for taxa containing a xanthophyll cycle based on diadinoxanthin and diatoxanthin but not for taxa containing a violaxanthin zeaxanthin-based xanthophyll cycle (chlorophytes and prasinophytes). According to the Chemtax results, diadinoxanthin-diatoxanthin containing taxa account for 35 % of the total chlorophyll in the phytoplankton community, chlorophytes and prasinophytes for 28%. Could the authors perhaps also calculate the DES ratios for the "green" group or otherwise explain why they have been left out?

The violaxanthin zeaxanthin- based xanthophyll cycle shows the same trend as the diadinoxanthin-diatoxanthin based xanthophyll cycle. We have incorporated these data into a revised version of Fig 4, as shown below.



Also note that accurate DES calculation requires quick sampling due to rapid epoxidation of the diatoxanthin back to diadinoxanthin (and zeaxanthin to violaxanthin). For such purposes, samples are usually flash-frozen in liquid N2 within 1-2 minutes after their removal from the light source. Given that sampling with Niskin bottles on a rosette and subsequent filtration probably takes on the order of 30 min (?), the ratios presented here may be off. Perhaps the authors could just acknowledge that with one line.

The samples were taken from the underway sampling system of the ship, and HPLC samples were always given priority during the filtration procedure. However, the reviewer's concerns are valid, and we have acknowledged this potential caveat in the revised manuscript (page 14, lines 410-412). Great care was taken to use dark bottles and low light during the filtration procedure. Therefore, it is likely that the observed diurnal trend in DES ratio would have been even larger, if we would have been able to filter samples faster. This would have further confirmed our conclusion that the sampled phytoplankton assemblage experienced, and reacted to, super-saturating light intensities for part of the day.

Also, please note the typo on line 28: de-epoxidation, not de-epoxilation.

Corrected.

16818 L15 – "Fv/Fm (. . .) half of the values expected from nutrient DEplete phytoplankton." Is this correct or should this read half the values expected from nutrient REplete phytoplankton?

We thank the reviewer for pointing out this mistake, which has been corrected in the revised manuscript.

16820 L22, 23 Superscript '-1' is missing in mol e- mol C-1.

Corrected.

168221 L28- 16822 L14 – It is intriguing to conclude that the observed effects on the conversion factor and optical properties are the result of iron limitation. This, to me, seems rather speculative because we do not have a comparison with iron replete conditions, which would need further field or culture work. I understand that this would be beyond the scope of this paper, but perhaps the authors could acknowledge that and insert a "disclaimer" highlighting possible future work to resolve this issue.

The paragraph references our previous study (Schuback et al., 2015), which compares iron deplete and replete field samples as well as laboratory cultures. We rephrased the section to further emphasise this.

16823 L17-29 – Please note that Fv/Fm also shows considerable taxonomic variability / dependency (Suggett et al. 2009 – MEPS Vol. 376:1-19).
Based on the Chemtax results, community composition did not changed throughout the day and, hence, taxonomic dependency of Fv/Fm is probably negligible. Perhaps the authors could acknowledge that with a brief statement.

The section has been rewritten accordingly (page 20 lines 563-565).

16824 L22 and Fig. 7 -Please note that correlation and regression are not the same methods and the two terms should not be used interchangeably (e.g. Field 2006 – Discovering Statistics using SPSS, 2nd edition, Sage Publishing. If the authors aim to establish mathematical relationships between NPQ and the conversion factor (and calculate slopes), then they should use a regression. An appropriate description should also be included in the methods. I am also not convinced that one may not miss some essential information by lumping all the data together into one regression. I looks like the slopes of the regression lines may vary with time of day if the data set was broken up according to the different sampling times. Furthermore, for some sampling time points, the relationship between NPQ and the conversion factor seems to have somewhat of a curvature (e.g. 3:00, 6:00).

The entire section has been rewritten; please see also responses to reviewer #1. We have now more explicitly examined the time-dependence of the correlation and not found any

statistically significant trends.

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