Title:

Dark Adaptation and Ability of Pulse-Amplitude Modulated (PAM) Fluorometry to Identify Nutrient Limitation in the Bloom-Forming Cyanobacterium, *Microcystis aeruginosa* (Kützing)

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Abstract

Harmful algal blooms in inland waters are widely linked to excess phosphorus (P) loading, but increasing evidence shows that their growth and formation can also be influenced by nitrogen (N) and iron (Fe). Deficiency in N, P, and Fe differentially affects cellular photosystems and is manifested as changes in photosynthetic yield (F_v/F_m). While F_v/F_m has been increasingly used as a rapid and convenient *in situ* gauge of nutrient deficiency, there are few rigorous comparisons of instrument sensitivity and ability to resolve specific nutrient stresses. This study evaluated the application of F_v/F_m to cyanobacteria using controlled experiments on a single isolate and tested three hypotheses: i) single F_v/F_m measurements taken with different PAM fluorometers can distinguish among limitation by different nutrients, ii) measurements of F_v/F_m made by the addition of DCMU are comparable to PAM fluorometers, and iii) dark adaptation is not necessary for reliable F_v/F_m measurements. We compared F_v/F_m taken from the bloom-forming *Microcystis aeruginosa* (UTEX LB 3037) grown in nutrient-replete treatment (R) and N-, P-, and Fe-limited treatments (LN, LP, LFe, respectively), using three pulse-amplitude modulated (PAM) fluorometers and the chemical photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and evaluated the effects of dark adaptation prior to PAM measurement. There were significant differences in F_v/F_m estimates among PAM

fluorometers for light- versus dark-adapted cell suspensions over the whole experiment (21 days), which were all significantly higher than the DCMU-based measurements. However, dark adaptation had no effect on F_v/F_m when comparing PAM-based values across a single nutrient treatment. All F_v/F_m methods could distinguish LN and LP from R and LFe treatments but none was able to resolve LFe from R, or LN from LP cultures. These results indicated that for most PAM applications, dark adaptation is not necessary, and furthermore that single measurements F_v/F_m do not provide a robust measurement of nutrient limitation in *Microcystis aeruginosa* UTEX LB 3037, and potentially other, common freshwater cyanobacteria.

Keywords

Cyanobacteria, Pulse-amplitude modulated fluorometry, Nutrient limitation, *Microcystis aeruginosa*, Photosynthetic yield

1. Introduction

Harmful algal blooms in inland waters are widely linked to excess phosphorus (P) loading, but increasing evidence shows their growth can also be limited by nitrogen (N) and iron (Fe) [1]. Nutrient limitation among phototrophs typically manifests as a change in the energy transferred between photosystem I and II (PSI and PSII, respectively) and their associated electron-transport chain [2]. Short-term metabolic activities, including the production of bioactive metabolites such as toxins and volatile organic compounds, and longer-term outcomes such as growth, cellular stoichiometry, and community structure can also be affected [3-6]. Variable chlorophyll-*a* (chl-*a*) fluorescence has been used to better understand changes in PSI and PSII during nutrient enrichment, by measuring the efficiency of light capture and the electron transport rate though the electron transport chain [7-9]. These measurements were first developed for land plants using chemical inhibitors such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and are based on the minimal (F_o) and maximal fluorescence (F_m) of PSI and PSII in dark-adapted plants, and the ratio of ($F_m - F_o$) to F_m (termed F_v/F_m; Table 1). This ratio of chl-*a* fluorescence represents the maximum quantum efficiency of PSII and is correlated to the photosynthetic yield [7-8].

Table 1. Glossary of photosynthetic fluorescence terms used in this study.

Term	Definition
F_o	Minimal chlorophyll fluorescence – dark-adapted for at least 15 minutes
F_m	Maximal chlorophyll fluorescence – dark-adapted for at least 15 minutes
F_v	Difference between F_m and F_o – dark-adapted for at least 15 minutes

F_v/F_m	Maximal quantum yield – dark-adapted for at least 15 minutes
F_o'	Minimal chlorophyll fluorescence – light-adapted
F_m'	Maximal fluorescence – light-adapted
F_v'	Difference between F_m' and F_o' – light-adapted
F_v'/F_m'	Maximal quantum yield – light-adapted: effective quantum yield

Traditional methods for evaluating planktonic nutrient limitation in field populations often involve nutrient addition through bottle experiments and measurements of the resulting growth response. The interpretation of these experiments is complicated by bottle effects, the length of the incubation period, the growth response parameters selected, and the temporal and spatial extrapolation of a limited number of small-scale controlled experiments over the growth period preceding the bloom and across the spatial variations of biotic and abiotic factors in small and large lakes [10]. Total chl-*a* is the most common proxy used to measure algal biomass, but it may largely reflect photoadaptive shifts in cell pigmentation and can be insensitive to changes in the abundance of different taxa within a community. Other endpoints used to detect nutrient limitation include changes in extra- or intracellular enzyme activity such as alkaline phosphatase activity or nitrogenase (i.e., nitrogen fixation). Nutrient limitation induces changes in enzyme activity in multiple members of a community, including bacteria [10], which may benefit other community members [11-12]. Enzyme activity assays are commonly used but assigning the activity to a single species in a complex plankton community can be difficult, but not impossible [13].

93 Active fluorometry has been used in both marine and freshwater environments to
94 measure maximum quantum efficiency (F_v/F_m) as a proxy for photosynthetic efficiency, without
95 the need for chemical inhibitors such as DCMU [10, 14-17]. Active fluorometers use either a
96 single turnover (ST) or multiple turnover (MT) method to measure F_v/F_m. Fast repetition rate
97 (FRR) fluorometers use a ST approach and emit a single short pulse of light that fully reduces
98 the primary electron acceptor in PSII (Q_A). Alternatively, pulse-amplitude modulated (PAM)
99 fluorometers use MT and emit a relatively long pulse (50-1000 ms) of light to fully reduce Q_A,
100 the secondary electron acceptor (Q_B), and plastoquinone (PQ) [18-20]. Both types of
101 fluorometers have been used to assess the physiological state of phytoplankton in laboratory
102 cultures and field experiments [10, 18, 20-23]. To date, however, no studies have used multiple
103 fluorometric methods to measure F_v/F_m and directly compare their output to the traditional
104 DCMU method while also investigating the impacts of nutrient limitation on those data.
105 Traditionally, photosynthetic yield measurements on phytoplankton are made with a
106 period of dark adaptation (15-30 minutes) to allow for the complete oxidation of PSII and the
107 electron transport chain prior to a saturating pulse of light. While this is easy to do in a
108 laboratory setting, there are a number of issues with this practice. *In situ* fluorometers, such as
109 the Turner Designs PhytoFlash, are designed for continuous measurements and do not have a
110 dark adaptation period while sampling. The bbe Moldaenke AlgaeOnlineAnalyzer benchtop
111 fluorometer (often also deployed in the field), has a dark-adaptation period, but this is generally
112 on the order of seconds to minimize particulate settling of the sample within the flow cell (Table
113 S2). Furthermore, the components of mixed phytoplankton samples vary in their response to
114 dark/light cycles, a significant concern which is often overlooked in field applications of these
115 instruments. Importantly, the photosystems in the thylakoids of cyanobacteria are less structured

and more fluid than in eukaryotes. In cyanobacteria, the phycobiliproteins are mobile and can contribute to rapid adaptation of the photosystem to changes in nutrient and light conditions [7, 24]. The above factors should be taken into consideration when assessing the impact of nutrients on mixed phytoplankton assemblages across a spatial-temporal scale, especially when based on a series of spatially- and time-fixed measurements.

The fundamental difference in photosystem structure between cyanobacteria and eukaryotic microalgae is particularly relevant to the traditional application of dark adaptation prior to fluorescence-based measurements. In essence, eukaryotic microalgae have PSI and PSII embedded into a chloroplast. In addition to PSI and PSII, cyanobacteria have mobile phycobilisomes that may be attached to either PSI or PSII and move between the two photosystems depending on light conditions. Cyanobacteria also have extensive non-photochemical quenching mechanisms not found in eukaryotes [25]. The established protocol for measuring F_v/F_m in land plants and eukaryotic algae requires a minimum of 15 minutes of pre-adaptation to dark [8, 26] but there remains considerable debate as to whether dark adaptation is necessary for cyanobacteria or if, more importantly, it can bias the data [27-28]. Potential bias would particularly be an issue for mixed phytoplankton assemblages in the field, especially if the method/instrument used cannot distinguish between different phytoplankton groups or has not been calibrated for the abundant taxa in the sample.

This study addressed these issues by comparing measurements of F_v/F_m collected with different *in situ* field and bench-top fluorometers using cultures of a harmful bloom-forming, toxic cyanobacterium (cyanoHAB), *Microcystis aeruginosa* UTEX LB 3037 (aka LE-3), one of several species of *Microcystis* that co-occur within Lake Erie [29]. Three major hypotheses were tested using cultures grown under a series of nutrient-stress experiments: i) all fluorometers

tested (PhytoPAM (F_v/F_m and F_v'/F_m'), PhytoFlash-Red (F_v'/F_m'), and AlgaeOnlineAnalyser (F_v'/F_m')) can distinguish among the different nutrient deficiencies (N, P, and Fe); ii) measurements of F_v/F_m made following the addition of DCMU [30] are comparable to PAM fluorometers; and iii) dark adaptation is unnecessary prior to F_v/F_m measurements in this ecologically important species.

2. Methods

2.1. Culture Maintenance and Sampling Protocol

Xenic cultures of *Microcystis aeruginosa* UTEX LB 3037 (UTEX 3037) were grown under four different nutrient treatments: control (i.e., nutrient replete, R), low nitrogen (LN), low phosphorus (LP), and low iron (LFe). The growth media and nutrient treatments were based on a modified Z8, prepared without CO_2 bubbling, and adjusted to pH 7.5-7.7 prior to autoclaving [31]. Nutrient-limited treatments contained 1% concentration of the assigned limiting nutrient compared to the control treatment, R (Table S1), and corresponding cationic or anionic salts were not supplemented. A sample of Z8 medium was prepared with no added iron to determine the level of contaminant iron added to and affecting the media during the preparation process. The background concentration was 7 nmol Fe/L as measured by inductively coupled plasma mass spectrometry, ICP-MS (Perkin Elmer Optima 3300DV), and did not significantly affect the final Fe concentration of any treatments. All cultures were maintained at ~26 °C in acid-washed, cotton plugged sterile flasks (500 mL) with ~68 µmol quanta m^{-2} s^{-1} cool white light on a 12 h:12 h light:dark photoperiod. Flasks were gently swirled and moved randomly around the incubator daily to minimize bias introduced by slight variance in light and temperature within the growth chamber. Cells were pre-conditioned in batch cultures under the different nutrient regimes (Table

S1) for three transfers (2-3 weeks each) in the flasks prior to being inoculated into acid-washed, sterile 2.8-L polycarbonate Fernbach flasks, each containing 1.75 L of fresh treatment media (n = 3 per treatment).

Experimental treatments were grown for a period of 28 days and growth was monitored using *in vivo* chl-*a* by removing 6 mL *via* sterile serological pipet on the day of inoculation (t = 1) and every other day at 08:00 until day 21. Nutrients were then added to all treatments on day 22 to restore the media to nutrient-replete levels for Z8 media (R treatments were spiked with N, P, and Fe; LN treatments were spiked with N; LP treatments were spiked with P; and LFe treatments were spiked with Fe) to assess whether the cultures were limited by their respective nutrients.

In vivo chl-*a* was measured for each sample using a Turner Designs TD700 fluorometer with the *in vivo* chlorophyll filter set (excitation: 340-500 nm, emission: > 665 nm, daylight white lamp). This fluorometer was calibrated over a range of 0-200 µg chl-*a*/L using chl-*a* extracted from nutrient-replete UTEX 3037 in exponential growth phase, measured using an in-house modification of US EPA method 445.0 [32]. Samples were filtered onto 47-mm Whatman Nuclepore polycarbonate filters (1-µm pore size) and sonicated in the dark with 7 mL of cold 90% acetone/water (v/v) for 1 hr at 4 ˚C in a water bath. The filters were removed, particles were allowed to settle at -20 ˚C for 2 hr, and soluble chl-*a* was measured in the dark using a second TD700 with the extracted chlorophyll filter set (excitation: 436 nm, emission: 680 nm, mercury vapor lamp) [33]. This fluorometer was calibrated using chl-*a* extracted from spinach (Sigma, USA) in cold 90% acetone/water (v/v) and quantified using a Milton Roy Spectronic 3000 Diode Array Spectrometer and the trichromatic equation (Eq. 1) [34], where A_{630}, A_{647}, A_{664}, and A_{750}

are absorbance values for the sample at those particular wavelengths measured in a 1-cm quartz cuvette.

$$\text{chl-}a\ (\mu g/L) = (11.85(A_{664} - A_{750}) - 1.54(A_{647} - A_{750}) - 0.08(A_{630} - A_{750}))10^6 \quad \text{(Eq. 1)}$$

Final chlorophyll concentrations were corrected for the total volume filtered, the extraction volume, and any dilution factors.

2.2. Photosynthetic Yield: F_v/F_m and F_v'/F_m'

F_v/F_m and F_v'/F_m' were evaluated on days 7, 13, 21, and 28 using four different methods: before and after the addition of DCMU (Sigma, USA; 10 mM in ethanol) using a Turner Designs TD700 (USA; 'TD700'), a PhytoPAM (Heinz Walz PhytoPAM-ED, Germany; 'PhytoPAM'), an AlgaeOnlineAnalyser (bbe Moldaenke, Germany; 'AOA'), and a Turner Designs PhytoFlash-Red (USA; 'PhytoFlash'). The AOA and PhytoPAM use "channels" based on algal pigment classes to measure photosynthetic yield, while the PhytoFlash uses a single wavelength correlating to the excitation maximum of phycocyanin (~642 nm, Table S3). A 200-mL culture volume was collected aseptically from the treatment flasks and transferred into acid-washed polycarbonate bottles for chl-*a* (see above), F_v/F_m, and F_v'/F_m' sub-sampling. Measurements of F_o, F_m, and F_v/F_m were performed in the dark. Five mL subsamples of culture were placed into 13 mm × 100 mm glass screw-top culture tubes and dark-adapted at room temperature for at least 30 min. Samples were diluted with NanoPure™ water when necessary to remain within the range of the instrument. For DCMU measurements, a sample was first measured using a TD700 fluorometer fitted with *in vivo* chl-*a* filters, and recorded. Following this, 5 µL DCMU was

added to the tube (final concentration 10 µM), mixed by inversion 15 times, and the sample was re-read to obtain F_m. The F_v/F_m was calculated using Eq. 2.

$$F_v/F_m = (F_m - F_o)/F_m \qquad \text{(Eq. 2)}$$

PhytoPAM measurements were corrected for background fluorescence (i.e., 'Z-off' measures) for each sample using spent culture media filtered through a 25 mm 0.2-µm reconstituted cellulose syringe filter (Corning, USA). The "blue" algae reference spectrum file for the PhytoPAM was obtained using the manufacturer protocol from a culture of *Microcystis aeruginosa* isolated from Hamilton Harbour, Lake Ontario (*M. aeruginosa* HH011-02; S. Watson, Environment & Climate Change Canada). Although measurements were being taken on a single "blue" taxon, the "brown" and "green" channels were also left on to reflect 'typical' field measurement conditions using the reference spectra collected from commonly occurring representatives of these two groups, *Asterionella formosa* CPCC 692 and *Chlamydomonas reinhardtii* CPCC 243, respectively. Dark-adapted samples (PAM-D) were transferred in the dark to a 3-mL quartz cuvette. Once fluorescence had stabilized, the F_v/F_m was estimated from a saturation pulse of red light (655 nm) at 3600 µmol quanta m^{-2} s^{-1}.

In a separate experimental series, F_v'/F_m' of non-dark-adapted (i.e., light-adapted, PAM-L) samples was measured using the PhytoPAM, following the same procedure as for the dark-adapted samples, but under ambient room lighting and without any dark adaptation period.

Measurements of F_v'/F_m' from the AOA and PhytoFlash were done under ambient light conditions using non-dark-adapted samples, since this represents the conditions typically used with these field instruments. Due to the sample volume required for the PhytoFlash and AOA,

both instruments were blanked with NanoPure™ water instead of culture filtrate. The PhytoFlash was calibrated to zero in a glass beaker covered with black electrical tape. Samples were poured into the beaker and the PhytoFlash was suspended in the sample to record the F_v'/F_m'. Dilutions with NanoPure™ water were made as necessary to adjust to levels within the range of detection for the instrument. The AOA was used with factory calibration settings and blanked with NanoPure™ water prior to measurements. Samples were introduced to the instrument using its peristaltic pump, read in the flow cell, and then returned back to the polycarbonate bottles in which they were collected. Three AOA measurements were collected per bottle (see Table S2 for measurement parameters) to generate an average for each sample. NanoPure™ water was pumped for 2 min through the AOA between each sample, and the measuring cell and tubing were allowed to pump dry between each sample to minimize cross contamination between replicates and the different nutrient treatments. Excitation wavelengths for cyanobacterial pigments and emission wavelengths for each instrument tested can be found in Table S3.

2.3. Statistical Analysis

Analysis of variance (ANOVA) was used to evaluate the results. The response variable was the logarithm of F_v/F_m; its logarithm was used to normalize the residuals. [Day] of measurement (day 7, 13, or 21 of the batch culture growth) was analyzed as a fixed categorical variable, as were [method] (5 categories) and [media] (4 categories). Day 28 data were not included in the statistical analysis because they were taken after the nutrient addition. There were 3 replicate flasks per medium type, so [flask] was considered to be a random effect nested within [media], and [flask] was used as the denominator mean square for the F test of differences among [media] (Table 2).

Flasks were sampled on multiple days; the [flask × day] interaction term provided the denominator mean square for testing [day] and [media × day]. Each day's sample was tested with each method, so the appropriate denominator for the F-tests of [method] and [method × media] was [flask × method]. Finally, [day × method] and [media × day × method] were tested with the residual error (which includes any [flask × day × method] interaction effect) as the denominator of the F-tests. The GLM procedure of SAS (SAS 9.4; SAS Institute, Cary, North Carolina), including its test, contrast, and lsmeans statements, were used to do this analysis. The appropriate error term (Table 2) from the hierarchical (i.e., Type 1) sums of squares table was used to test each effect of interest in the model (*via* test statements) and to calculate (*via* lsmeans statements) and compare (*via* contrast statements) the effects of categories within a given term (e.g., to compare different media types). To compare categories within a term, we used Sheffé's tests, which are well-suited to complex hierarchical designs like the one used in this study [35].

Table 2. Statistical analyses on F_v/F_m measurements were performed using various model terms and interactions as described in this sums of squares table.

Model Term	Type	df	F-test Denominator
media	fixed	3	flask
replicate flask, nested within media	random	8	
day	fixed	2	flask × day
media × day		6	flask × day
flask × day		6	
method	fixed	4	flask × method
media × method		12	flask × method

flask × method		32	
day × method		8	residual
media × day × method		24	residual
residual	random	59	

3. Results

This study used controlled *in vitro* experiments on the monospecific cyanoHAB strain (*Microcystis aeruginosa* UTEX LB 3037) to test three major hypotheses, in order to evaluate the ability of different PAM fluorometers to identify nutrient limitation, assess if DCMU addition results in comparable F_v/F_m output as PAM fluorometers, and test the effects of the traditional dark pre-adaptation period on the data. In general, the results supported the hypothesis that dark adaptation is not necessary in cyanobacteria, but did not support the hypothesis that PAM fluorometers could differentiate among different limiting nutrient conditions, indicating that PAM fluorometry alone cannot be used to reliably identify nutrient limitation. Furthermore, our hypothesis that DCMU-based measurements would be comparable to PAM fluorometry was not supported, but the data show that F_v/F_m obtained using DCMU was the only method able to distinguish between limited and replete cultures.

Patterns of *in vivo* chl-*a* fluorescence were used to follow the overall effects of the different treatments on UTEX 3037, and showed marked decreases in growth in nutrient deficient treatments, but major differences among the different nutrient regimes (Fig. 1). Relative to the nutrient-replete controls (R), growth was significantly lower for both LP and LFe cultures within 1-2 days of inoculation, while LN treatments showed a lagged but similar response after ~1 week. *In vivo* chl-*a* fluorescence of LP and LFe cultures remained consistently low in relation

to both LN and R cultures throughout the growth period. Both LP and LFe cultures recovered after enrichment to levels of *in vivo* chl-*a* comparable to R cultures prior to the enrichment (Fig. 1), while LN cultures did not. This suggested that the decline in the LN cultures after day 13 (Fig. 1) was a result of cultures deteriorating beyond recovery over the growth period of the experiment. Nutrient-amended R cultures showed a decrease in *in vivo* chl-*a* levels, suggesting that another factor (e.g., light) became limiting at the high cell densities (> 1000 µg chl-*a*/L) observed in the later stages of the R treatment (Fig. 1).

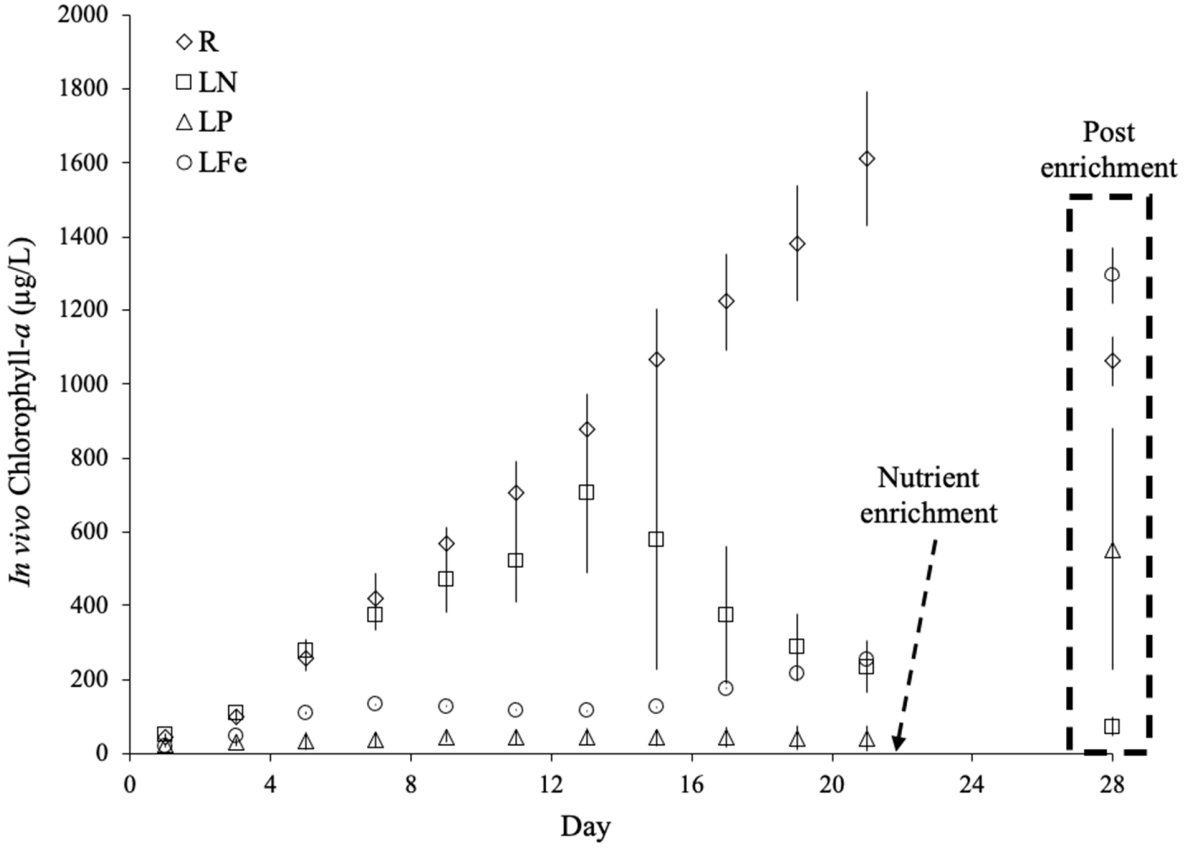


Fig. 1. Average *in vivo* chl-*a* for replete (R), low nitrogen (LN), low phosphorus (LP), and low iron (LFe) cultures of *M. aeruginosa*. Error bars represent one standard deviation of triplicate samples.

However, contrary to our first hypothesis, while *in vivo* growth showed clear differences between R and at least two of the nutrient deficiency treatments (LP and LFe), fluorescence-based F_v/F_m measurements were less clearly defined among treatments (Fig. 2A). ANOVA showed that photosynthetic yield was significantly affected by both the nutrient treatment ($F_{3,8}$=12.18, P=0.0024) and method of measurement (i.e., fluorometer type; $F_{4,32}$=49.78, P<0.001) over the course of the entire experiment (Table S5). There were two significant two-way interactions: [media × method] ($F_{12,32}$=9.05, P<0.001) and [day × method] ($F_{8,59}$=4.54, P=0.002). On average, the F_v/F_m least squares mean ranked as (LN = LP) < (LFe = R) (Fig. 2A), where LN and LP treatments and R and LFe treatments were not statistically different over the course of the entire experiment. These data do not support the first hypothesis that PAM fluorometers can distinguish limitation by different nutrients.

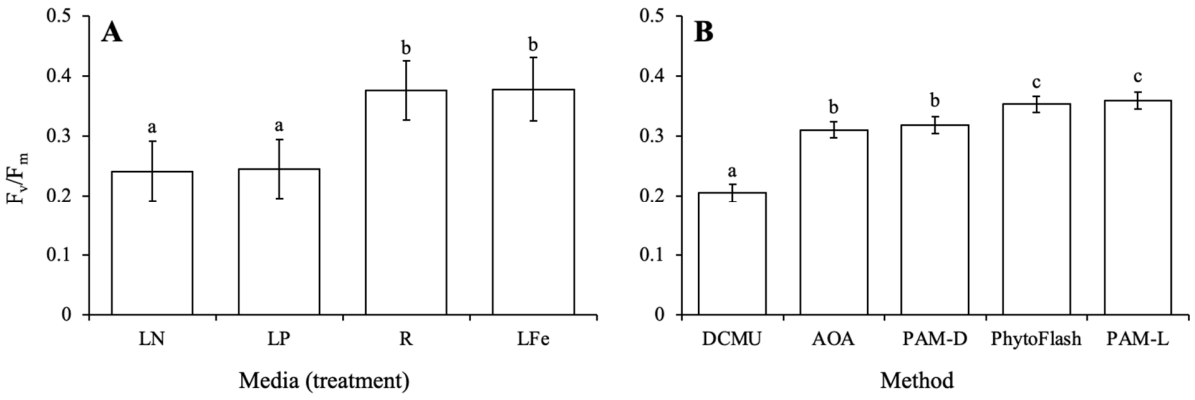
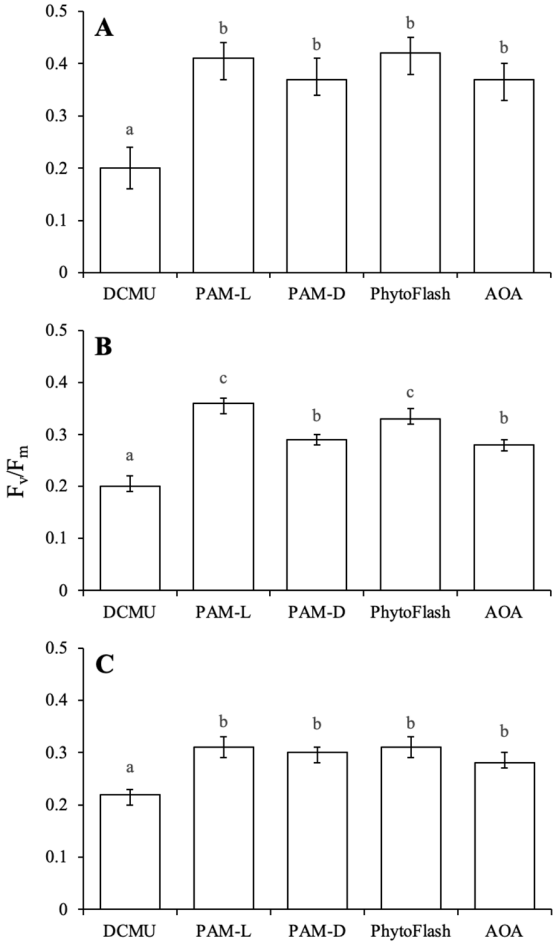


Fig. 2. A: Effects of each treatment on photosynthetic yield of *M. aeruginosa* averaged over 21 days; **B:** Differences in photosynthetic yield measurements using different methods across all treatments. Treatments /methods with the same letter were not significantly different.

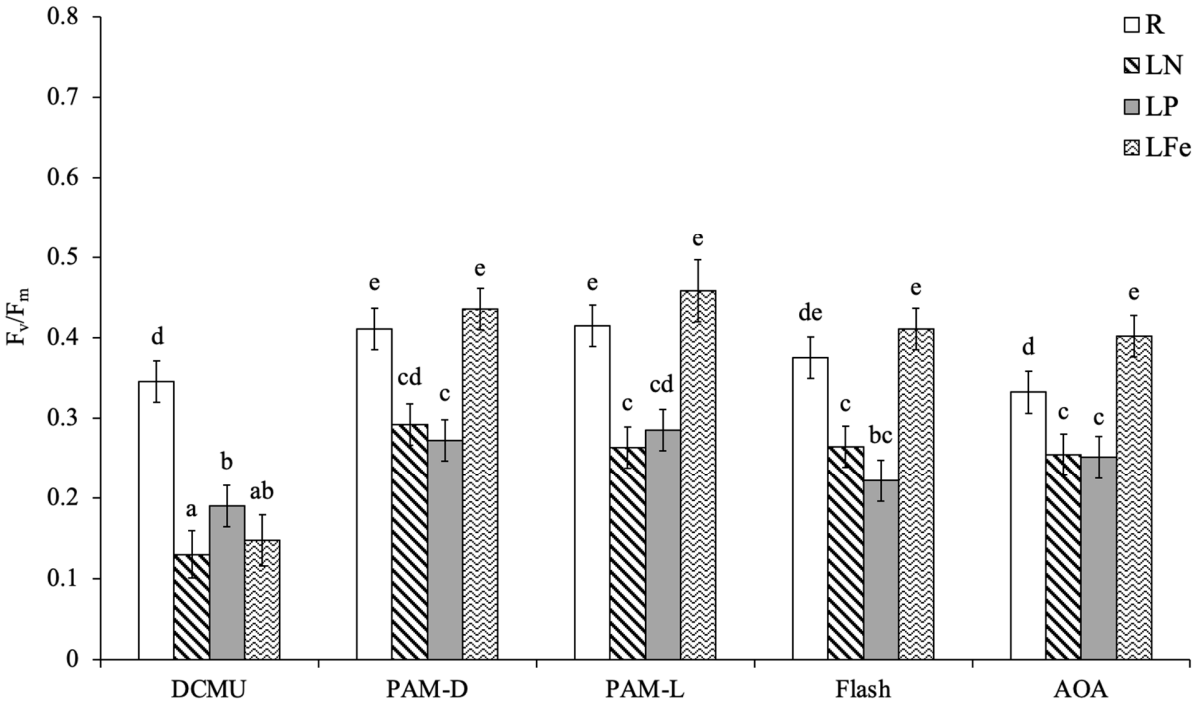
317 Our second hypothesis that DCMU would give comparable F_v/F_m values to the PAM
318 fluorometers was also not supported. DCMU gave the lowest F_v/F_m values in all treatments
319 (0.19-0.23; Fig. 3, Table S4) and could resolve between R and nutrient-limited cultures (Fig. 4).
320 Exploring the [media × method] interaction ($F_{12,32}$=9.05, P<0.001) revealed that F_v/F_m
321 measurements made on LFe treatments using DCMU were significantly lower than
322 measurements made using PAM fluorometers and reinforced the finding that PAM fluorometers
323 could not differentiate between R and LFe treatments and LN and LP treatments (Fig. 4).
324



325

326 **Fig. 3.** Least-squares means of F_v/F_m values taken on days 7 (**A**), 13 (**B**), and 21 (**C**) obtained
327 across all treatments of *M. aeruginosa* using DCMU, light-adapted PhytoPAM (PAM-L), dark-

328 adapted PhytoPAM (PAM-D), PhytoFlash, and AOA methods. Error bars represent the 95%
329 confidence intervals. Methods with the same letter were not significantly different.
330
331 The data support our third hypothesis that dark adaptation is not necessary in
332 cyanobacteria. Light-adapted PhytoPAM (PAM-L) and PhytoFlash measurements were
333 significantly higher than dark-adapted PhytoPAM (PAM-D) and AOA values over the first 21
334 days (Fig. 2), but not significantly different from each other for a given treatment (Fig. 4).
335 However, this significant difference broke down when the data were parsed out by day (Fig. 3).
336 F_v/F_m and F_v'/F_m' were only statistically different on day 13, and not on days 7 and 21. F_v/F_m and
337 F_v'/F_m' measurements across all PAM fluorometers were not statistically significant for a given
338 treatment (Fig. 4), further supporting our premise that dark adaptation does not affect PAM-
339 based F_v/F_m measurements in *M. aeruginosa*.
340



341

342 **Fig. 4.** Least-squares means of F_v/F_m values for replete (R), low nitrogen (LN), low phosphorus
343 (LP), and low iron (LFe) treatments of *M. aeruginosa* obtained across all days using DCMU,
344 light-adapted PhytoPAM (PAM-L), dark-adapted PhytoPAM (PAM-D), PhytoFlash, and AOA
345 methods. Error bars represent the 95% confidence intervals. Treatments with the same letter
346 were not significantly different.
347
348 **4. Discussion**
349 Overall, our results provide important insight into the issues with the application of PAM
350 fluorometry as a diagnostic tool for nutrient stress, and highlights some of the major
351 considerations that need to be taken into account prior to these instruments being deployed.
352 Phytoplankton groups have various accessory pigments that can complicate the measurement of
353 active fluorescence. Three types of light are used in different fluorometers to emit the saturation
354 pulse used to measure F_v/F_m: blue, red, and white. White light was traditionally used before
355 wavelength-specific light-emitting diodes (LEDs) were widely available. Blue-light diodes are
356 generally used for measuring total chl-*a* in phytoplankton populations while red-light diodes are
357 better suited for specifically measuring cyanobacterial populations, as the fluorescence spectrum
358 of their main accessory pigments (phycobiliproteins) absorbs in the red region with the exception
359 of phycoerythrin-rich cyanobacteria [7].
360 Some fluorometers use wavelength-specific LEDs to evaluate the individual F_v/F_m
361 metrics of specific phytoplankton pigment groups in a heterogeneous sample. The PhytoPAM
362 categorizes three broad algal pigment groups on the basis of a four-point reference excitation
363 spectrum calibrated from measurements made on a representative species within each group –
364 (1) green, (2) blue(-green), and (3) brown. The AOA categorizes four algal groups using factory-

365 determined reference spectra and accessory pigment concentrations calculated for each group –
366 chlorophyll-*b* is used for chlorophytes (group 1), phycocyanin (PC) for cyanobacteria (group 2),
367 phycoerythrin (PE) for cryptophytes and PE-rich cyanobacteria (group 3), and the carotenoids
368 peridinin and fucoxanthin for dinoflagellates, chrysophytes, and diatoms (group 4). While using
369 multiple LEDs allows for the estimation of specific F_v/F_m values corresponding to each
370 (assumed) group, each instrument uses slightly different wavelengths to determine the
371 aforementioned groups. To target cyanobacteria, the Walz PhytoPAM and the Turner Designs
372 PhytoFlash Red use 655 nm and 635 nm light, respectively, for the saturation pulse [14, 36],
373 while the AOA uses 650 nm (personal communication, bbe Moldaenke).

374 Active fluorometry has been used to study marine phytoplankton since the early 1990s,
375 but its use for freshwater phytoplankton has only become prominent in the last decade. Several
376 studies have compared fluorometers in a freshwater field setting [14, 37-38], but very few have
377 performed comparisons of freshwater phytoplankton genera under controlled laboratory
378 conditions, where a more rigorous evaluation of instrument performance, light/dark adaptation,
379 and capacity to resolve among different types of nutrient stress can be made [21, 24, 27, 39]. To
380 our knowledge, there have been few studies that have focused solely on cyanobacteria and
381 nutrient limitation [40].

382 Despite the differences in the aforementioned wavelengths, F_v/F_m and F_v'/F_m'
383 measurements made by the three active fluorometers were highly consistent among instruments,
384 unlike observations made in other studies comparing instrument performance [14]. This may, in
385 part, be related to sample density – for our samples, *in vivo* chl-*a* levels (a proxy measure of cell
386 density for our monospecific unicellular cultures held under controlled conditions) were highly
387 variable (20-1100 µg/L; Fig. 1), while other authors have found that readings are less reliable at

388 significantly lower cell abundances [37]. For example, a comparison of F_v/F_m measurements
389 using a DivingPAM, WaterPAM, and PhytoPAM found statistically different readings among
390 the instruments for samples from Lake Erie with total chl-*a* levels less than 10 µg/L, but had
391 comparable readings for the WaterPAM and PhytoPAM with chl-*a* above this level [14].
392 Furthermore, the AOA, in addition to group-specific measurements, provides a weighted average
393 F_v/F_m for all samples but does not provide group-specific F_v/F_m below ~3 µg/L.

394 Discrepancies have been reported between field measurements from different PAM
395 fluorometers for F_v/F_m of diverse phytoplankton assemblages in Lake Erie and Lake Ontario
396 [14]. All of these observations indicate that PAM-based results from phytoplankton communities
397 in natural systems should be analyzed with caution. Clearly, cell density is an important factor,
398 but furthermore, members of these mixed plankton assemblages respond differently to variance
399 in light and nutrient regimes, and in most cases, the sample history (i.e., light, nutrient regimes)
400 is unknown or uncontrolled. Different instruments vary in their ability to resolve among classes
401 of algae, and both community composition and density can both affect PAM measures. Factors
402 that limit phytoplankton growth in natural systems include nutrients, temperature, and light, and
403 these can directly alter the photosystem structure and performance, which are likely to affect
404 PAM-fluorometric measurements. All of these factors can complicate the interpretation of
405 results.

406 Studies have shown that low F_v/F_m values are a common indicator of iron limitation in
407 eukaryotic algae due to the statistically significant and consistent difference between F_v/F_m
408 measurements between replete and Fe-limited cultures. For example, nutrient-replete F_v/F_m
409 values of 0.7 for green algae [41-44] and 0.6-0.7 for diatoms [17, 42, 45-46] have been reported
410 using PAM fluorometry, while iron-limited values of F_v/F_m in eukaryotic algae range from 0.3-

411 0.4 [47-49]. However, in the present study, replete unicellular suspension of *M. aeruginosa*
412 showed lower F_v/F_m values (average of 0.35) than that of eukaryotic algae, regardless of the
413 instrument used (Fig. 4, Table S4). This agrees well with other F_v/F_m data reported for
414 cyanobacteria (~0.4) that were measured under nutrient-replete conditions [28, 50-51]. The lower
415 nutrient-replete F_v/F_m values for cyanobacteria were not statistically different from Fe-limited
416 F_v/F_m measurements in this study (Fig. 2A), making F_v/F_m a poor tool for measuring Fe
417 limitation in *M. aeruginosa*.

418 Several possible factors may contribute to lower F_v/F_m values [21]. Overlapping emission
419 spectra among chl-*a* and cyanobacterial accessory phycobilisome pigments, e.g.,
420 allophycocyanin (APC) and PC, could contribute to an elevated F_o. The phycobilin pigments
421 APC and PC have an emission peak ~650 nm, which corresponds to the measured emission
422 wavelength of many PAM fluorometers [7, 52-53]. Cyanobacteria contain PC and APC as part of
423 their core photosynthetic apparatus and the pigments help to collect photons to funnel into the
424 P680 core [7]. F_o could be higher in cultures with increased phycobilin pigment content due to
425 the APC and PC emission maximum being close to that of chl-*a*.

426 In this study, F_v/F_m measurements made with DCMU were significantly lower for LFe
427 treatments compared to values obtained with PAM fluorometers (Fig. 4), which may reflect
428 changes in the photosystem structure and function of the photosystems. For example, a study of
429 several *M. aeruginosa* isolates reported lower chl-*a* concentrations per cell under iron limitation
430 compared to nutrient-replete conditions [54]. Iron limitation in cyanobacteria also alters the
431 chlorophyll-protein assemblages associated with PSII, which could suppress F_v and should be
432 detectable by PAM fluorometers [7, 55-56]. Any effect of the induced changes in chlorophyll-
433 protein assemblages on F_v/F_m may be difficult to detect, given the generally lower F_v/F_m for

cyanobacteria even under nutrient-replete conditions; nevertheless, a slight decrease was seen in the LFe cultures (Fig. S4), although this change was not statistically significant.

Under iron limitation, ferredoxin may be replaced with the non-iron containing protein flavadoxin in the photosynthetic electron transport system [57-58]. This would necessitate changes in the binding site of the enzyme ferredoxin ubiquinone reductase, in order to recognize flavodoxin [59], which may impact the binding of ubiquinone to PSII. DCMU interacts at this same (or similar) binding site on PSII and any change to the binding site would result in a decrease in F_m due to the inefficient blocking by DCMU. The physical change in enzymes and binding sites within PSII may affect the efficiency of electron transport prior to DCMU addition and raise the F_o values for Fe-limited cultures. This increase in F_o in combination with the decreased F_m would result in a lower F_v/F_m. The increase in F_o would be detected by active fluorometers, but the effect on F_m would not.

Iron limitation has also been shown to induce the production of the *isiA* encoded CP43' (PsbC), a thylakoid-bound chlorophyll protein that can increase F_o [60-61]. When produced in excess, CP43' can help protect PSII from over-excitation and minimize non-photochemical quenching (NPQ) [62]. Further, iron limitation can affect the availability of non-heme iron, used for electron transfer between quinones Q_A and Q_B bound to the D1 (PsbA) and D2 (PsbD) proteins in PSII. The loss of the non-heme iron from the core of PSII blocks the transfer of electrons from Q_A to Q_B and alters the binding pocket for DCMU to Q_B, which prevents DCMU binding efficiently to that site [63]. This would raise F_o and lower F_m, which will result in a lower F_v/F_m, overall.

Dark adaptation has been a foundational step since the discovery of its effects on measurements of F_v/F_m in terrestrial plants in the late 1980s [8, 64]. As with land plants,

457 eukaryotic microalgae require a period of dark adaptation to fully oxidize the quinone pool and
458 optimize electron acceptance and transfer [65]. However, state transitions in PSII and PSI differ
459 for cyanobacteria [18, 23, 66]. The mobility of phycobilisomes on the thylakoid surface
460 facilitates transition from state 2 (light predominantly absorbed by PSI) to state 1 (light
461 predominantly absorbed by PSII) when moving from dark to light conditions, which may reduce
462 NPQ [7, 67-70]. Theoretically, this would allow for measurements of F_v/F_m without a prior dark
463 adaptation period or consideration for NPQ effects. Cyanobacteria also have alternative
464 mechanisms for NPQ (e.g., orange carotenoid protein) that may facilitate relaxation of the
465 excited PSII complex without the emission of typical chlorophyll fluorescence [71]. The orange
466 carotenoid protein (OCP) is a blue-light photoprotective protein that binds to the phycobilisome
467 core (composed of APC) to induce conformational changes, resulting in a reduction of F_m', thus
468 leading to an increase in F_v'/F_m' [67, 69, 72-73].

469 Most studies of PSII fluorescence typically dark-adapt samples prior to measurement, to
470 fully oxidize the electron acceptors within PSII and minimize the influence of NPQ on the
471 measurement of F_o [7]. To estimate F_m, early methods used DCMU to block the energy transfer
472 between the PSII reaction center and its associated electron chain. DCMU binds to Q_B in PSII
473 and prevents the transfer of two electrons from the PSII reaction center to PQ. Thus, excitation
474 energy is not transferred to PSI *via* cytochrome-*b*$_6$*f* and is emitted as maximal fluorescence, F_m
475 [7]. Other studies have argued that because of this state mechanism, photosynthetic yield
476 measurements for cyanobacteria should be carried out without dark adaptation, as this
477 pretreatment affects the capacity for state transitions and thus may produce erroneous
478 measurements (termed F_v'/F_m'; Table 1) [7, 18, 20, 23, 66]. The capacity for state transitions is
479 furthermore affected by nutrient (iron) deficiency [74]. Similarly, the light history and

480 photoadaptation of a cell are important but often overlooked factors that can affect
481 photosynthetic response and confound the interpretation of PAM-fluorometric measurements
482 made using inappropriate illumination (e.g., high or low light environments) [21]. In the present
483 study, ambient light was selected carefully for light-adapted samples (68 µmol quanta m^{-2} s^{-1}) to
484 represent 'typical' subsurface photosynthetically-active radiation (PAR) irradiance under bloom
485 conditions, as seen, for example, in Lake Neatahwanta, New York at 1 m depth [75].

486 While significant differences in F_v/F_m values from PAM fluorometers observed over the
487 course of the 21-day experiment were expected, the similarity in output among PAM instruments
488 across nutrient treatments was not. As conditions in freshwater systems are constantly changing
489 [76], the long-term observations are less relevant to field deployments. These data also
490 strengthen the case that dark adaptation is not necessary for measurements of F_v/F_m for
491 cyanobacteria – at least in cultures grown under low light regimes commonly observed in field
492 conditions (≈70 µmol quanta m^{-2} s^{-1}). Our data supports the hypothesis that dark adaptation does
493 not affect the F_v/F_m of this important cyanoHAB and should be verified in other morphologically
494 different cyanobacterial genera, but these data indicate that dark adaptation *per se* does not
495 introduce significant bias into the analysis of mixed field phytoplankton assemblages.

496 Furthermore, other studies also suggest that dark adaptation may not be necessary for
497 high light regimes as the light intensity masks any effects of nutrient limitation on photosynthetic
498 yield. For example, a FRR fluorometer was used to compare measurements of planktonic F_v'/F_m'
499 with PAR data collected during several Lake Erie cruises, which covered a range of taxonomic
500 assemblages and water quality [16]. These data showed an inverse relationship between PAR and
501 F_v'/F_m', leading the authors to conclude that high-light conditions inhibited photosynthetic
502 efficiency regardless of any nutrient limitation [16]. Similar results have been observed in

503 hypereutrophic systems such as Lake Neatahwanta, New York, where high light conditions
504 observed at midday led to a decrease in their measured photosynthetic yield at the surface and
505 vertical migration of cyanobacteria away from the surface [75].

506 Overall, the results of our study were not consistent with our working hypotheses and
507 demonstrated that single F_v/F_m or F_v'/F_m' measurements made on PAM fluorometers do not
508 provide sufficient information to differentiate between types of nutrient limitation in cultures of
509 *M. aeruginosa* UTEX 3037. Other factors may also impact fluorescence measurements and
510 photosynthetic yield which are particularly relevant to cyanoHABs such as *M. aeruginosa*. Cell
511 and colony morphometry, buoyancy, and distribution within the sample (e.g., evenly distributed,
512 clumped) also affect measurements of fluorescence and hence, estimates of F_v/F_m [16, 77].
513 Moreover, as cyanobacterial colonies grow, cell density and pigment content can decrease, and
514 the colonies may act as discrete optical units rather than as a sum of the optical properties of the
515 individual cells [77]. Thus, differences among these optical units and changes in cell pigment
516 content may affect the F_v/F_m or F_v'/F_m' in these colonial cyanobacteria. To minimize these
517 effects, we used uniform suspensions of unicellular *M. aeruginosa* to avoid the complications of
518 colony morphology, buoyancy, diffractive properties, and non-uniform sample suspensions, and
519 allowed a focus on the response to differences in nutrient regimes at the cellular level.

520

521 **5. Conclusion**

522 We provide important new insight into the interpretation of photosynthetic yield
523 measurements of phytoplankton under different nutrient regimes and light *vs.* dark preadaptation.
524 Using *Microcystis aeruginosa* UTEX LB 3037, a common cyanoHAB, we showed that PAM
525 fluorometers could not distinguish between replete and Fe-limited cultures as well as N- and P-

limited cultures, while DCMU was capable of detecting Fe-limitation. DCMU-based measurements were also significantly lower than PAM-based measurements for all treatments and instruments used. A variety of factors complicate the interpretation of F_v/F_m in cyanobacteria, particularly in mixed field samples where readings are confounded by the variable presence and relative abundance of other algal groups. Our work demonstrates that dark adaptation does not impact F_v/F_m measurements in *M. aeruginosa* on the time scales relevant to freshwater field studies, therefore F_v/F_m of mixed assemblages can be reliably performed under dark adaptation conditions. Based on these results, we do not recommend using single PAM-based F_v/F_m measurements as indicators of nutrient limitation in cyanobacteria due to the inability of PAM fluorometers to discriminate nutrient deficiencies. Whereas nutrient-induced fluorescence transients (NIFTs) and electron transport rate curves (ETRs) may be able to provide more in-depth information and warrant further investigation.

Author Statement

Katherine Perri: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. Schonna Manning: Writing – review & editing. Norma Fowler: Formal analysis, Writing – review & editing. Susan Watson: Methodology, Resources, Writing – review & editing. Gregory Boyer: Conceptualization; Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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