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TITLE:

Measuring Photophysiology of Attached Stage of *Colacium* sp. by a Cuvette-Type Fast Repetition Rate Fluorometer

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bench-top FRRf, *Colacium* sp., epibiont, epizoic algae, Lake Biwa, photophysiology

SUMMARY:

Fast repetition rate fluorometer (FRRf) is a beneficial method for measuring photosystem II photophysiology and primary productivity. Here we describe a protocol to measure PSII photophysiology of epizoic alga, *Colacium* sp. on substrate zooplankton using cuvette-type FRRf.

ABSTRACT:

Fast repetition rate fluorometer (FRRf) is a beneficial method for measuring photosystem II (PSII) photophysiology and primary productivity. Although FRRf can measure PSII absorption cross-section (σ_{PSII}), maximum photochemical efficiency (F_v/F_m), effective photochemical efficiency (F_q'/F_m'), and non-photochemical quenching (NPQ_{NSV}) for various eukaryotic algae and cyanobacteria, almost all FRRf studies to date have focused on phytoplankton. Here, the protocol describes how to measure PSII photophysiology of an epizoic alga *Colacium* sp. Ehrenberg 1834 (Euglenophyta), in its attached stage (attached to zooplankton), using cuvette-type FRRf. First, we estimated the effects of substrate zooplankton (*Scapholeberis mucronata* O.F. Müller 1776, Cladocera, Daphniidae) on baseline fluorescence and σ_{PSII}, F_v/F_m, F_q'/F_m', and NPQ_{NSV} of planktonic *Colacium* sp. To validate this methodology, we recorded photophysiology measurements of attached *Colacium* sp. on *S. mucronata* and compared these results with its planktonic stage.

Representative results showed how the protocol could determine the effects of calcium (Ca) and manganese (Mn) on *Colacium* sp. photophysiology and identify the various effects of Mn enrichment between attached and planktonic stages. Finally, we discuss the adaptability of this protocol to other periphytic algae.

INTRODUCTION:

Chlorophyll variable fluorescence is a useful tool for measuring algal photosystem II (PSII) photophysiology. Algae respond to various environmental stresses, such as excess light and nutrient deficiency, by altering their PSII photophysiology. Fast repetition rate fluorometer (FRRf) is a common method for measuring PSII photophysiology[1,2] and estimating primary productivity[1,3,4], which enables monitoring phytoplankton PSII photophysiology, as well as primary productivity across wide spatial and temporal scales[5–7]. FRRf can simultaneously measure PSII (σ_{PSII}) absorption cross section, reaction center ([RCII]) concentration, maximum photochemical efficiency (F_v/F_m), effective photochemical efficiency (F_q'/F_m'), and non-photochemical quenching (NPQ_{NSV}) (**Table 1**). In general, F_v/F_m and F_q'/F_m' are defined as PSII activity[8], while NPQ_{NSV} is defined as relative heat-dissipated energy[9].

Importantly, single turnover (ST) flashes of FRRf fully reduce the primary quinone electron acceptor, Q_A, but not the plastoquinone pool. Conversely, multiple turnover (MT) flashes from a pulse amplitude modulation (PAM) fluorometer can reduce both. The ST method has a clear advantage over the MT method when identifying the possible origins of NPQ_{NSV} by simultaneously measuring recovery kinetics of F_v/F_m, F_q'/F_m', NPQ_{NSV}, and σ_{PSII}[10]. To date, several types of FRRf instruments, such as submersible-type, cuvette-type, and flow-through-type, are commercially available. The submersible-type FRRf enables *in situ* measurements in oceans and lakes, while the cuvette-type FRRf is suitable for measuring small sample volumes. The flow-through type is commonly used to continuously measure the photophysiology of phytoplankton in surface waters.

Given the development of PAM fluorometers, including the cuvette-type, for a broad range of subjects[11], PAM fluorometers are still more common than FRRfs in algal photophysiology research[12]. For example, although the sample chamber structure and cuvette capacity between these tools only differs slightly, the cuvette-type PAM has been applied to phytoplanktons[13–15], benthic microalgae[16–18], ice algae[19], and epizoic algae[20], while the cuvette-type FRRf has been applied primarily to phytoplanktons[21–23] and a limited number of ice algal communities[24,25]. Given its effectiveness, cuvette-type FRRf is equally applicable to benthic and epizoic algae. Therefore, expanding its application will provide considerable insight into PSII photophysiology, particularly for lesser-known epizoic algal photophysiology.

Epizoic algae have received little attention, with few studies examining their PSII photophysiology[20,26], most likely due to their minor roles in aquatic food webs[27,28]. However, epibionts, including epizoic algae, can positively influence zooplankton community dynamics, such as increasing reproduction and survival rates[29,30], as well as negatively impact processes, such as increasing sinking rate[29,31] and vulnerability to visual predators[32–36]. Therefore, exploring the environmental and biological factors controlling epibiont dynamics in zooplankton

communities is crucial.

Among epizoic algae, *Colacium* Ehrenberg 1834 (Euglenophyta) is a common, freshwater, algal group[32,37–39] with various life stages, including attached (**Figure 1A–D**), non-motile planktonic (**Figure 1E,F**), and motile planktonic stages[40,41]. During the non-motile planktonic stage, cells live as single-cell planktons, aggregated colonies, or one-layer sheet colonies, covered by mucilage[42]. In the attached stage, *Colacium* sp. uses mucilage excreted from the anterior end of the cell[37,39,41] to attach to substrate organisms (basibionts), particularly microcrustaceans[41,43]. Their life cycle also involves detaching from the molted exoskeleton or dead basibiont and swimming with their flagella to find another substrate organism[39]. Both planktonic and attached stages can increase their population size by mitosis[40]. Although their attached stage is hypothesized to be an evolutionary trait for gathering resources, such as light[44] and trace elements[41,45,46], or as a dispersion strategy[27], little experimental evidence is available about these aspects[37,41,44] and the key attachment mechanisms are largely unknown. For example, Rosowski and Kugrens expected that *Colacium* obtains manganese (Mn) from substrate copepods[41], concentrated in the exoskeleton[47].

Here, we describe how to measure PSII photophysiology of planktonic algae and the related application method for targeting attached algae (attaching to zooplankton) with *Colacium* sp. cells using the cuvette-type FRRf. We use the Act2 system equipped with three light-emitting diodes (LEDs) that provide flash excitation energy centered at 444 nm, 512 nm, and 633 nm[48]. Here, 444 nm (blue) corresponds to the absorption peak of chrophyll *a* (Chl-*a*), while 512 nm (green) and 633 nm (orange) correspond to the absorption peaks of phycoerythrin and phycocyanin, respectively. The fluorescent signal detection peak is 682 nm with 30 nm half bandwidth. Since it is difficult to find the planktonic stage of *Colacium* sp. in natural environments, their attached stage was collected for the experiments. Among the numerous substrate organisms*, Scapholeberis mucronata* O.F. Müller 1776 (Branchiopoda, *Daphniidae*; **Figure 1A,B,G**) is one of the simplest to handle due to their slow swimming speed, large body size (400–650 µm), and unique behavior (hanging upside down on the water surface). Therefore, this protocol uses *Colacium* sp. attached on *S. mucronata* as a case study of the *Colacium*-basibiont system. To avoid fluorescence derived from the gut contents, *S. mucronata* was starved. As a previous study reported that the fluorescence signal from gut contents (ingested algae) displays a five-fold decrease after 40 min[49], we expected that 90 min starvation would be enough to minimize the possibility of gut content fluorescence affecting the FRRf measurement with minimum effects of experimental stress to *Colacium* sp., such as nutrient deficiency. Furthermore, this protocol was applied to clarify the attaching mechanism of *Colacium* sp. and determine how two metals, calcium (Ca) and manganese (Mn) affect the photophysiology of both planktonic and attached stages. Calcium plays key roles in the photosynthetic pathways[50] in multiple ways, and both metals are required to construct the oxygen-evolving complexes of the PSII[51]. As calcium and manganese are highly concentrated in the carapace of crustacean zooplankton[47], we hypothesize that *Colacium* sp. photophysiology might respond more prominently to Ca and Mn enrichment during the planktonic stage if this life stage obtains these elements from *S. mucronata* during the attached stage.

PROTOCOL:

1. Sampling

1.1 Collect lake water from the surface by a bucket. To target *Colacium* sp. attached to *S. mucronata* (**Figure 1A–C**) filter 0.5–10 L of lake water using a 100 µm nylon mesh net[52].

NOTE: *S. mucronata* often densely aggregate in shallow, eutrophic, muddy water, such as among reed (phragmites) areas.

1.2 Store the concentrated samples in 500 mL plastic bottles with 350 mL of lake water. Keep in dark conditions.

1.3 In the laboratory, pour the sample water into a 500 mL beaker and allow it to settle for a few minutes.

1.4 Filter the lake water through a 0.2 µm pore-size filter.

1.5 Pick up *S. mucronata* individuals using a pipette under an optical microscope at 100x magnification. Perform species identification according to Błędzki and Rybak[53].

1.6 Transfer them into a drop of 0.2-µm filtered lake water (FLW) placed on a glass slide.

NOTE: *S. mucronata* may swim to the surface or attach to the beaker wall.

1.7 Check *S. mucronata* under light microscopy.

1.8 Wash *S. mucronata* individuals using FLW (3 drops or more) to prevent contamination from other organisms (**Figure 2**).

1.9 Keep *S. mucronata* at an *in situ* temperature in a growth chamber under 40 µmol photon·m^{-2}·s^{-1}.

2. Effects of *S. mucronata* on baseline fluorescence

2. 1 *S. mucronata* cultivation

2.1.1 Pick up *S. mucronata* individuals using a pipette under an optical microscope at 100x magnification and wash using FLW, as in step 1.8.

2.1.2 Aerate tap water using an electric air pump via an air stone for at least 1 week. Pour 300 mL of aerated tap water into a 350 mL glass jar.

2.1.3 Feed *Chlorella* (1 mg $C \cdot L^{-1}$) and maintain at 20 °C under 40 µmol photon$\cdot m^{-2} \cdot s^{-1}$ in a growth chamber.

2.1.4 After approximately 14 days, pick up 5–30 individuals using a pipette under an optical microscope at 100x magnification and inoculate them into 300 mL of clean, aerated tap water to keep the medium fresh.

2. 2 Setting up FRRf

2.2.1 Launch the Act2Run software.

2.2.2 Click on the **Options** tab and select **Act2 FLC (white LEDs)** in **Mode of Operation** to set the actinic LEDs color.

2.2.3 Click on the value of **Dark** at step **1** of the settings of the fluorescent-light curve in the main window, and type 30 to set the duration of the dark period (**Figure 3A**).

2.2.4 Click on the LED combination **B**, **C**, and **D** to turn off the green and orange LEDs (**Figure 3C**).

2.2.5 Click on the value of **Fets** and **Pitch** under **Sat**, and type 100 and 2, respectively, to set the number and pitch of flashlet in the saturation phase (**Figure 3D**).

2.2.6 Click on the value of **Fets** and **Pitch** under **Rel**, and type 40 and 60, respectively, to set the number and pitch of flashlet in the relaxation phase (**Figure 3E**).

2.2.7 Activate the water jacket pump by clicking on **During FLC** (**Figure 3F**) to control sample temperature during the measurement.

2.2.8 Activate by clicking **Auto-LED** and **Auto-PMT** (**Figure 3F**).

2.2.9 Click on **Synchronize** to connect FRRf-Act2.

2. 3 FRRf measurements

2.3.1 To examine the effects of zooplankton individuals on baseline fluorescence, prepare adult *S. mucronata* (body size 400–650 µm) from the culture in steps 2.1.1–2.1.4 without any attached organisms.

2.3.2 To avoid fluorescence from the gut contents, starve the individuals in FLW at 20°C for at least 90 mins.

2.3.3 Pour 1.5 mL of FLW into a cuvette. Pick up 0, 1, 5, and 10 *S. mucronata* individuals using a pipette under an optical microscope at 100x magnification.

2.3.4 Transfer *S. mucronata* individuals into the cuvette and add FLW to bring the sample up to 2 mL.

2.3.5 Acclimate under low light (1–10 µmol photon·m^{-2}·s^{-1}) at 20 °C for 15 min before FRRf measurement.

2.3.6 Click on **Act2 Run** to start the measurement. Repeat the measurements >3 times per sample.

2.3.7 Read the *Fo* value from the result plot (**Figure 4**).

3. Effects of substrate organism on Chl-*a* fluorescence

3.1 *Colacium* sp. cultivation

3.1.1 Prepare the FLW and AF-6 medium[54] for cultivation (**Table 2**).

3.1.2 Collect *Colacium* sp. attached to *S. mucronata* as in steps 1.1 and 1.2 and, keep at *in situ* temperature in a growth chamber.

3.1.3 Pick up *Colacium* sp. with a molted carapace (**Figure 1D**) using a pipette under an optical microscope at 100x magnification. Wash them with FLW, as in step 1.8.

3.1.4 Aseptically inoculate *Colacium* sp. and AF-6 medium in a 10 mL glass tube on a clean bench.

3.1.5 Maintain the culture at *in situ* temperature under 200 µmol photon·m^{-2}·s^{-1} in a growth chamber. Shake the glass tube gently by hand at least once per day to prevent cell settlement.

NOTE: To keep the attenuation effect of aggregated colonies as low as possible, check the colonies under a microscope prior to FRRf measurement. Cell aggregation may cause dense colonies and affect algal photophysiology[55].

3.2 FRRf measurements

3.2.1 To examine the effects of zooplankton individuals on Chl-*a* fluorescence from *Colacium* sp., prepare adult *S. mucronata* (body size 400–650 µm) without any attached organisms.

3.2.2 To avoid fluorescence from the gut contents, starve the individuals in FLW for at least 90 min.

3.2.3 Set up a cuvette-type fast repetition rate fluorometer (FRRf).

3.2.4 Pour a 1.5 mL subsample of precultured *Colacium* sp. into a cuvette. Transfer 0, 5, 10, and 15 *S. mucronata* individuals into these cuvettes and add 2 µm of filtered medium to bring the sample up to 2 mL.

3.2.5 Acclimate under low light (1–10 µmol photon·m^{-2}·s^{-1}) at 20 °C for 15 min before taking the FRRf measurement.

NOTE: Maintain the samples at incubation temperature during measurements

3.2.6 Click on **Act2 Run** to start the measurement. Repeat the measurements >3 times per sample.

3.2.7 Read the F_O and F_m values from the result plot (**Figure 4**).

NOTE: Check the $R\sigma_{PSII}$ value (**Table 1**), which shows whether the LED power is within the optimal range to estimate the PSII parameters correctly. When the Auto-LED is activated, Act2run system controls the LED power to achieve an optimal $R\sigma_{PSII}$ range (0.042–0.064). The experimental $R\sigma_{PSII}$ cut-off value was defined at 0.03 and 0.08 in a previous study[48].

3.2.8 To correct the baseline fluorescence[22], filter the culture medium using a 0.2-µm pore-size filter and measure the fluorescence. Subtract F_O of the baseline sample from F_O and F_m of *Colacium* sp., or modify the **Blank correction** value in the **Settings** in the **Options** tab.

4. Photophysiology of *Colacium* sp. (attached stage)

4.1 Isolate *S. mucronata* individuals with *Colacium* sp. using a pipette under an optical microscope.

4.2 Wash *S. mucronata* using FLW, as in step 1.8.

4.3 Transfer *S. mucronata* into a 100 mL of FLW. For starvation, keep under dark conditions at *in situ* temperature for 90 min.

4.4 Pour 1.5 mL of FLW into a cuvette.

4.5 Transfer ~10 *S. mucronata* individuals with *Colacium* sp. into a cuvette. For measurements, more than 100 *Colacium* cells per 2 mL are needed. Add FLW to bring the sample up to 2 mL.

4.6 Acclimate under low light (1–10 µmol photon·m^{-2}·s^{-1}) at *in situ* temperature for 15 min. Measure Chl-*a* fluorescence as in steps 3.2.6–3.2.8.

4.7 To enumerate the number of attached cells, fix the sample with glutaraldehyde (2% final volume) after taking the FRRf measurement. Take pictures at several focal depths and positions of *S. mucronata* under a light microscope.

5. Photophysiology of *Colacium* sp. (planktonic stage)

5.1 Cultivate sampled *Colacium* sp. in AF-6 medium at *in situ* temperature as in steps 3.1.1–3.1.5.

5.2 For the stationary phase, take 2 mL of cultured *Colacium* sp. and pour into a cuvette.

5.3 Acclimate under low light (1–10 µmol photon·m^{-2}·s^{-1}) at *in situ* temperature for 15 min. Measure Chl-*a* fluorescence as in steps 3.2.6–3.2.8.

6. Effects of Ca and Mn addition on photophysiology of *Colacium* sp.

6.1 Effects on attached stage

6.1.1 Isolate *S. mucronata* individuals with *Colacium* sp. using a pipette under an optical microscope. Wash using FLW, as in step 1.8.

6.1.2 Transfer six individuals each into 12 glass beakers with 30 mL of FLW. Ensure that each beaker contains >100 *Colacium* sp. cells.

6.1.3 Add 200 µmol·L^{-1} $CaCl_2 \cdot H_2O$ (Ca treatment), 40 µmol·L^{-1} $MnCl_4$ (Mn treatment), or ultrapure water (control) to each beaker. Incubate the samples under 200 µmol photon·m^{-2}·s^{-1} at *in situ* temperature in a growth chamber.

6.1.4 At 3 h and 21 h, transfer all individuals and molted skins into a cuvette with 2 mL of the medium.

6.1.5 To examine the rapid response to increasing light, click on **Up** of the periods of 8 actinic light steps and type 20 (**Figure 3A**) to set the duration of each step in 20 s. To set the stepwise actinic light as 0, 11, 25, 44, 68, 101, 144, and 200 µmol photon·m^{-2}·s^{-1}, click on **High E** and **Step Up** and change the values to 200 and 34, respectively (**Figure 3B**).

6.1.6 After 15 min of dark acclimation, measure Chl-*a* fluorescence of each sample similar to steps 3.2.6–3.2.8.

NOTE: Verify that the $R\sigma_{PSII}$ and $R\sigma_{PSII}'$ values are within the optimal range (0.03–0.08)[48].

6.2 Effects on planktonic stage

6.2.1 Cultivate sampled *Colacium* sp. in AF-6 medium at *in situ* temperature as in steps 3.1.1–3.1.5.

6.2.2 Transfer the cultured *Colacium* sp. into FLW and acclimate at *in situ* temperature less than 200 µmol photon·m^{-2}·s^{-1} for 3 days.

6.2.3 Transfer 1 mL of the acclimated samples into three glass vials with 10 mL of FLW.

6.2.4 Add 200 µmol·L^{-1} $CaCl_2$·H_2O (Ca treatment), 40 µmol·L^{-1} $MnCl_3$ (Mn treatment), or ultrapure water (control) to vials. Incubate the samples under 200 µmol photon·m^{-2}·s^{-1} at *in situ* temperature in a growth chamber.

6.2.5 At 3 h and 21 h, measure Chl-*a* fluorescence of each sample as in steps 3.2.6–3.2.8.

REPRESENTATIVE RESULTS:

There was no significant effect of baseline fluorescence (**Figure 5**) or Chl-*a* fluorescence (**Figure 6**) by *S. mucronata* up to 5 individuals (inds.) mL^{-1}. However, F_v/F_m and NPQ_{NSV} were significantly affected when *S. mucronata* was 7.5 inds·mL^{-1}. Therefore, for measuring the photophysiology of *Colacium* sp. during the attached stage, we chose *S. mucronata* with the higher burden of *Colacium* sp. in order to reach sufficient *Colacium* sp. abundance (>50 cells·mL^{-1}) and a low number of *S. mucronata* (≤5 inds·mL^{-1}) in the cuvette.

Table 3 shows seasonal variation in photophysiology of *Colacium* sp. during the attached stage. Although sampling temperature varied, their photophysiology remained relatively constant. σ_{PSII} varied from 3.42 nm^2 to 3.76 nm^2 (mean 3.60 nm^2), F_v/F_m varied from 0.52 to 0.60 (mean 0.55), and NPQ_{NSV} varied from 0.66 to 0.85 (mean 0.82). To validate these results, we further investigated variations in *Colacium* sp. photophysiology during the planktonic stage for the stationary phase in the AF-6 medium (**Table 4**). Mean F_v/F_m and NPQ_{NSV} for the attached stage were similar to those of the planktonic stage when incubated in AF-6 medium.

To determine the effect of Ca and Mn on *Colacium* sp. photophysiology in both the attached and planktonic stages, we performed Ca and Mn enrichment experiments. Samples were taken from the reed area of Lake Biwa on May 7, 2021. For the attached stage of *Colacium* sp. under dark conditions, there was no significant difference in photophysiological parameters among treatments, except for NPQ_{NSV} between Mn and Ca treatments at 3 h, where Ca < Mn (**Figure 7A,C,E**). Further, σ_{PSII}', F_q'/F_m', and NPQ_{NSV} responses to increasing light during the attached stage showed no clear differences among treatments **(Figure 8A**,**C**,**E** and **Figure 9A**,**C**,**E**). However, NPQ_{NSV} tended to be lower in the Ca treatment than the control at a low light intensity at 21 h (11 and 25 µmol photon·m^{-2}·s^{-1}, **Figure 9E**). For the planktonic stage, σ_{PSII} was significantly lower in the Mn than Ca treatment at 3 h (**Figure 7B**). F_q'/F_m' was significantly higher, but NPQ_{NSV} was lower in the Mn treatment than control at 21 h (**Figure 7D**,**F**). Under increasing light, Mn tended to decrease σ_{PSII} and increase F_q'/F_m' during the planktonic stage, compared to the control at 3 h (**Figure 8D)**. Similarly, Mn significantly reduced NPQ_{NSV} during the planktonic stage compared to the control at 21 h (**Figure 9F**). Similar to the attached stage, calcium slightly improved NPQ_{NSV}

for the planktonic stage under increasing light (**Figure 9F)**. However, Ca decreased F_q'/F_m' and increased NPQ_{NSV} for the planktonic stage compared to Mn treatment under 44–200 µmol photon·m^{-2}·s^{-1} at 3 h (**Figure 8D,F)**.

FIGURE AND TABLE LEGENDS:

Figure 1: *Colacium* sp. and substance organism *Scapholeberis mucronata*. (A) Infected *S. mucronata.* **(B)** Infected *S. mucronata* fixed with glutaraldehyde. **(C)** Attached *Colacium* cells on living *S. mucronata.* **(D)** Attached *Colacium* cells on the molted carapace. **(E, F)** *Colacium* sp. of planktonic (palmella) stage. **(G)** Non-infected *S. mucronata.* Arrows indicate *Colacium* cells. Scale bars: 200 µm (A, B, and G), 10 µm (C, E, and F), and 100 µm (D).

Figure 2: Washing zooplankton by pipetting under filtered lake water (FLW).

Figure 3: Act2Run software user interface. (**A**) Exposure time to each actinic light step; (**B**) Number of steps and photon flux of actinic light; (**C**) Combination of excitation wavelength; (**D**) Saturation and relaxation flashlet sequence; (**E**) Frequencies and intensities of the water jacket and sample mixing pumps; (**F**) Photon flux of excitation flash at 444 (denoted as 450 here), 512 (530), and 633 nm (624), photomultiplier tube (PMT) voltage, and replicates and interval of sequence.

Figure 4: Fluorescence reading of the algal sample on Act2Run software. The red and blue lines indicate raw fluorescence signal by a sequence of flashlet and curve fitting in both saturation and relaxation phases, respectively. See Kolber et al.[2] for more details.

Figure 5: The effect of *S. mucronata* density on baseline fluorescence. The small dots represent replicates (n = 3). The results of the ANOVA test are also shown.

Figure 6: The effects of *S. mucronata* densities on (A) F_O (B) σ_{PSII}, (C) F_v/F_m, and (D) NPQ_{NSV} for *Colacium* sp. during the planktonic stage. The small dots represent replicates (n = 3). *Colacium* sp. was cultured in AF-6 medium. The results of ANOVA and Tukey post-hoc test are also presented.

Figure 7: Responses of (A,B) absorption cross-section, (C,D) PSII photochemistry, and (E,F) non-photochemical quenching of (A,C,E) attached stage and (B,D,F) planktonic stage of *Colacium* sp. at 3 h and 21 h after Ca and Mn addition. The small dots represent replicates (n = 4). The results of ANOVA and Tukey post-hoc test are also presented. * $p < 0.05$.

Figure 8: Rapid-light responses of (A, B) absorption cross-section, (C,D) PSII photochemistry, and (E,F) non-photochemical quenching of *Colacium* sp. in attached and planktonic stages to stepwise light protocol at 3 h after Ca and Mn addition. C, control; Ca, 200 µM Ca; Mn, 40 µM Mn. Significant differences between (a) C and Ca, (b) C and Mn, and (c) Ca and Mn at each PAR flux, with a significance level of $p < 0.05$ shown in each panel. Error bar, Mean SD (n = 4).

Figure 9: Rapid-light responses of (A,B) absorption cross-section, (C,D) PSII photochemistry, and

(E,F) non-photochemical quenching of *Colacium* sp. in attached and planktonic stages to stepwise light protocol at 21 h after Ca and Mn addition. C, control; Ca, 200 µM Ca; Mn, 40 µM Mn. Significant differences between (a) C and Ca, (b) C and Mn, and (c) Ca and Mn at each PAR flux, with a significance level of $p < 0.05$ shown in each panel. Error bar, Mean SD (n = 4).

Table 1: Terms used in this protocol.

Table 2: Recipe for AF-6 medium. Adjust pH to 6.6. Dissolve Fe-citrate and citric acid in warm H_2O separately and add HCl (1 mL·L^{-1}) after mixing both reagents. Contents of trace metals are shown in parenthesis.

Table 3: Photophysiology of *Colacium* sp. attached on *S. mucronata*.

Table 4: Photophysiology of *Colacium* sp. planktonic stage. Each sample was measured during the stationary phase.

DISCUSSION:

This protocol demonstrated for the first time that photophysiology of *Colacium* sp. during the attached stage in a natural environment is comparable to its planktonic stage in AF-6 medium. Additionally, gut contents of starved *S. mucronata* did not affect baseline and Chl-*a* fluorescence when density was ≤5 inds·mL^{-1} (**Figure 5** and **Figure 6**). These results suggest this protocol can measure photophysiology of *Colacium* sp. during the attached stage without correction under low substrate organism abundance. However, results from steps 3.2.1–3.2.8 showed that the highest *S. mucronata* abundance affected F_v/F_m and NPQ_{NSV} significantly, but not F_O and σ_{PSII} (**Figure 4**). Here, it's possible higher organism density exacerbated physical stress on *Colacium* sp. individuals and subsequently decreased photosynthetic activity. For measurements under a high abundance of substrate organisms or other species, the effects of substrate organism density on the baseline and Chl-*a* fluorescence requires further attention.

FRRfs have been used to examine the impact of nutrient manipulation on the linear electron flow and non-photochemical quenching of phytoplankton[22,56,57]. The primary results show that Ca and Mn enrichment differed significantly between *Colacium* sp. life stages (**Figure 7**, **Figure 8**, and **Figure 9**). Specifically, manganese clearly improved the (maximum) photochemical yield of PSII (F_v/F_m and F_q'/F_m') and decreased the heat dissipation (NPQ_{NSV})[50] of planktonic stages under dark (**Figure 7D,F**) and light conditions (**Figure 8D,F** and **Figure 9D,F**). These outcomes can stem from reduced antenna size on PSII, σ_{PSII}, and σ_{PSII}' (**Figure 7B** and **Figure 8B**), which reduces excess light absorption[58,59]. Measuring antenna size in addition to energy flow between PSII complexes would allow more precise measurements of the algal response[10]. This protocol also allows the examination of photosynthesis limitations by other resources. For example, nitrogen and phosphorus limitations have been examined in various phytoplankton communities, but not in epizoic algae, despite predicted effects on *Colacium*[41] and marine epizoic diatoms[60,61]. In addition to nutrients, the light environment can further influence epizoic algae distribution[44].

As shown in **Figure 7**, **Figure 8**, and **Figure 9**, cuvette-type FRRf enables us to simultaneously

examine nutrient and light effects without long incubation times and measurement effort. This stepwise light protocol (step 6.1.5) can also draw rapid-light curves of relative electron transport rates (rETR = F_q'/F_m' × light) vs. light as an analog for production vs. light curves[62]. However, although linear electron flow in PSII can be estimated from photophysiological parameters by the FRRf, it is not necessarily analogous to the carbon fixation rate[63,64]. For estimating carbon-based primary production, electron requirement per CO_2 fixation ($\Phi_{e,C}$), which can vary temporally and spatially[5,48], should be examined when assessing subject communities.

If the plankton net is clogged by debris, prescreen by a larger mesh, such as a 5-mm mesh net, or pick zooplanktons directly from the lake water using a pipette without filtration. It should be noted that some damage might occur to the attached algae even when the filtration is conducted gently using a relatively large (200 µm) mesh size. Although the results show that the standard deviation of the PSII parameters was small (**Table 3**), and the mean values of the parameters were very similar to those of the cultured planktonic stage (**Table 4**), sampling without filtration might be ideal.

Another limitation of this study was deriving σ_{PSII}. Actinic light and Chl-*a* fluorescence attenuation can exert a major influence/distortion on the FRRfs, which relies upon optically thin samples for the accurate σ_{PSII} determination[65]. Although we showed that *S. mucronata* did not affect the σ_{PSII} of planktonic *Colacium* cells, that should be examined related to the σ_{PSII} of the attached *Colacium* cells. Furthermore, a spectral correction factor (SCF) would be needed for the σ_{PSII} *in situ*[66] estimation as the excitation wavelength of the ACT2 system (444 nm) differs from the spectral distribution of the *in situ* light environment. In general, the filter pad technique is used to measure the Chl-*a* specific absorption spectrum to calculate the SCF. This procedure is necessary to estimate algal primary productivity by the FRRfs. As we could not harvest enough attached *Colacium* cells through the study period, the Chl-*a* specific absorption spectrum should be examined in future studies.

Implementing cuvette-type FRRf should depend on substrate size as periphytic algae require a substrate attachment. For example, studies of algae on indestructible substances, such as rocks[67], larger organisms[26,68], or symbiotic algae, including *Symbiodinium* associated with hard corals[10,69,70], might require the submersible-type FRRf[69]. Conversely, if the basibiont is small enough to suspend itself in a cuvette, a cuvette-type FRRf might be sufficient in addition to a cuvette-type PAM, such as benthic algae[16–18]. Indeed, recent studies have explored a cuvette-type FRRf for measuring the photophysiology of ice algae[24,25]. Furthermore, turning on the excitation flash at 512 and 633 nm enables the application to cyanobacteria with different PSII antenna pigments, phycoerythrins and phycocyanins, and thus different absorption peaks with Chl-*a*[71]. As current FRRf models incorporating multi-excitation wavelengths are useful tools for examining cyanobacteria photophysiology and productivity[7,66,72], these ought to be useful methods for assessing benthic cyanobacteria, if the effects of sample thickness on photophysiological parameters would be improved[65]. In future studies, FRRfs should be aimed at a wider range of subject organisms to shed further insight on the complex mechanisms of algal photophysiology across various habitats.

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DISCLOSURES:

The authors have no disclosures to declare.

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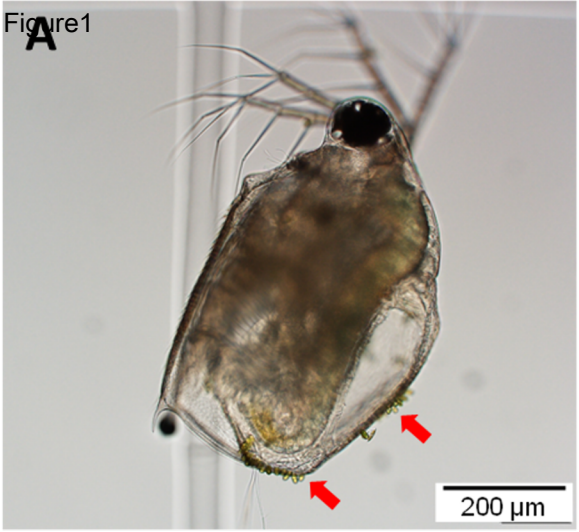
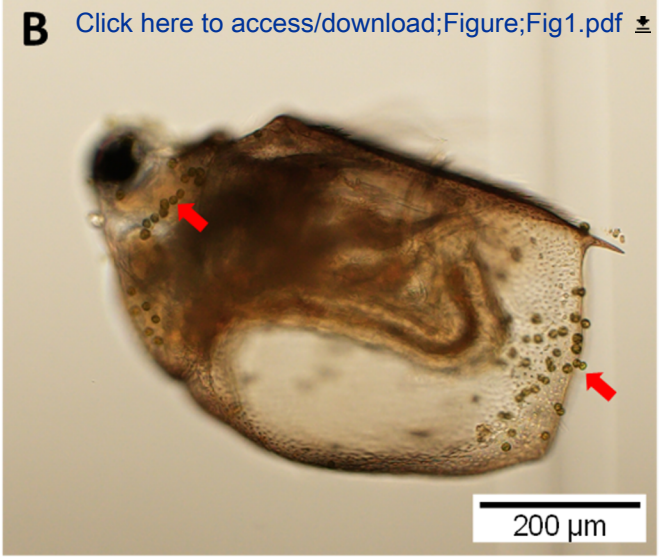
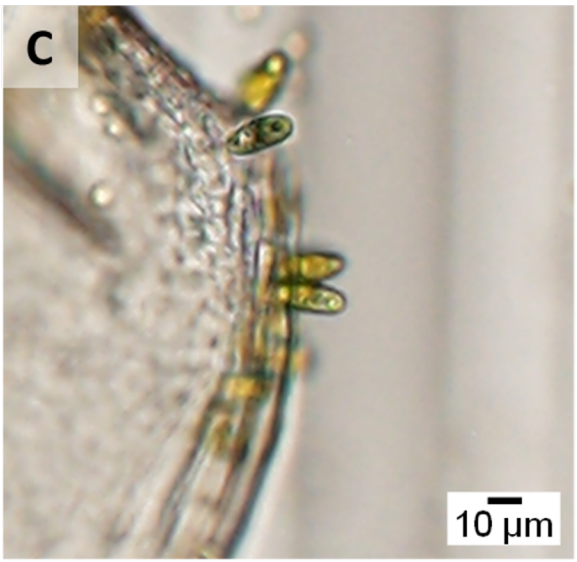
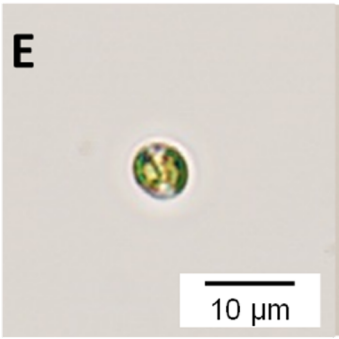
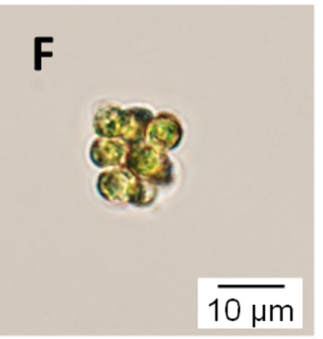
Figure1











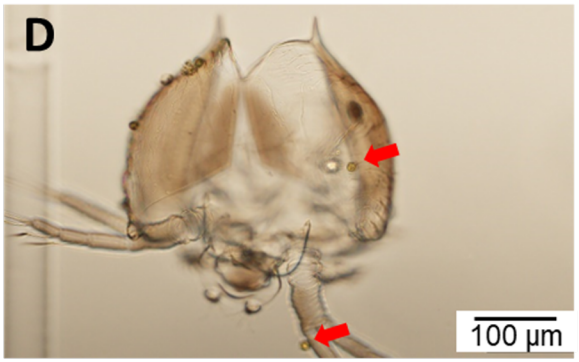


Figure2

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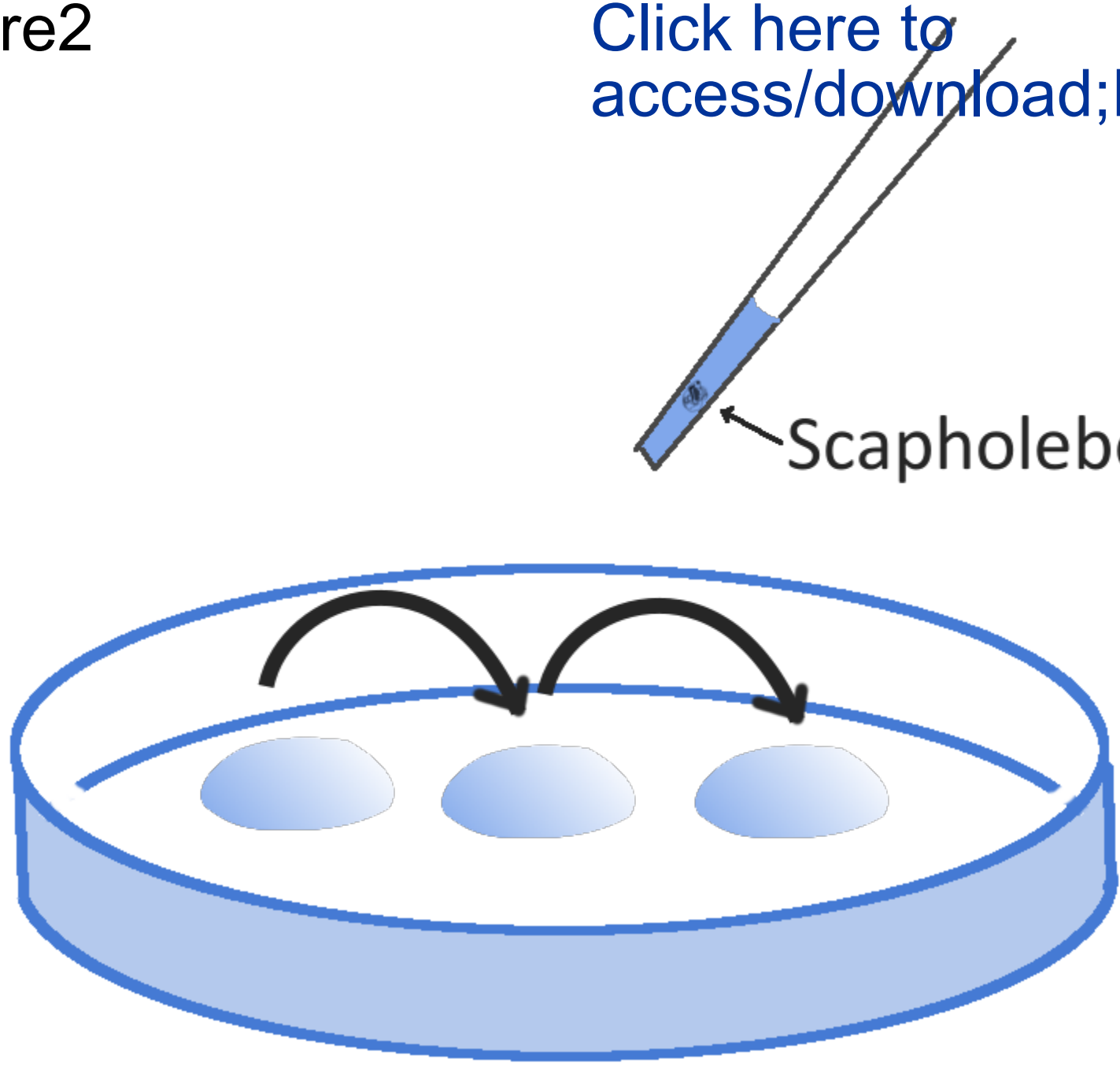

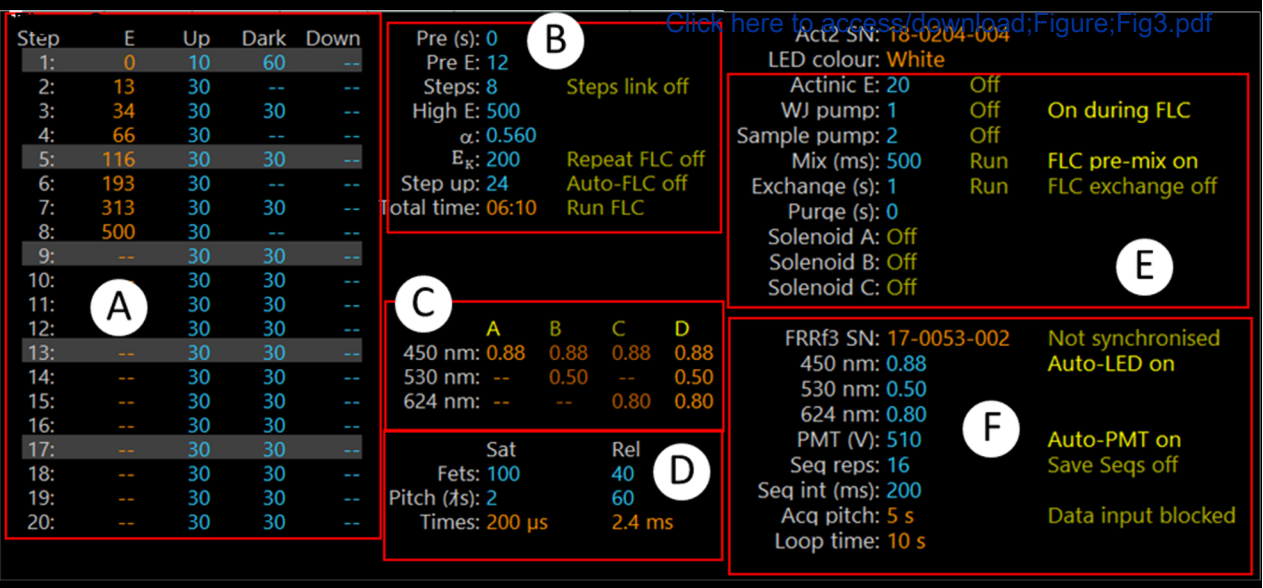

Figure4

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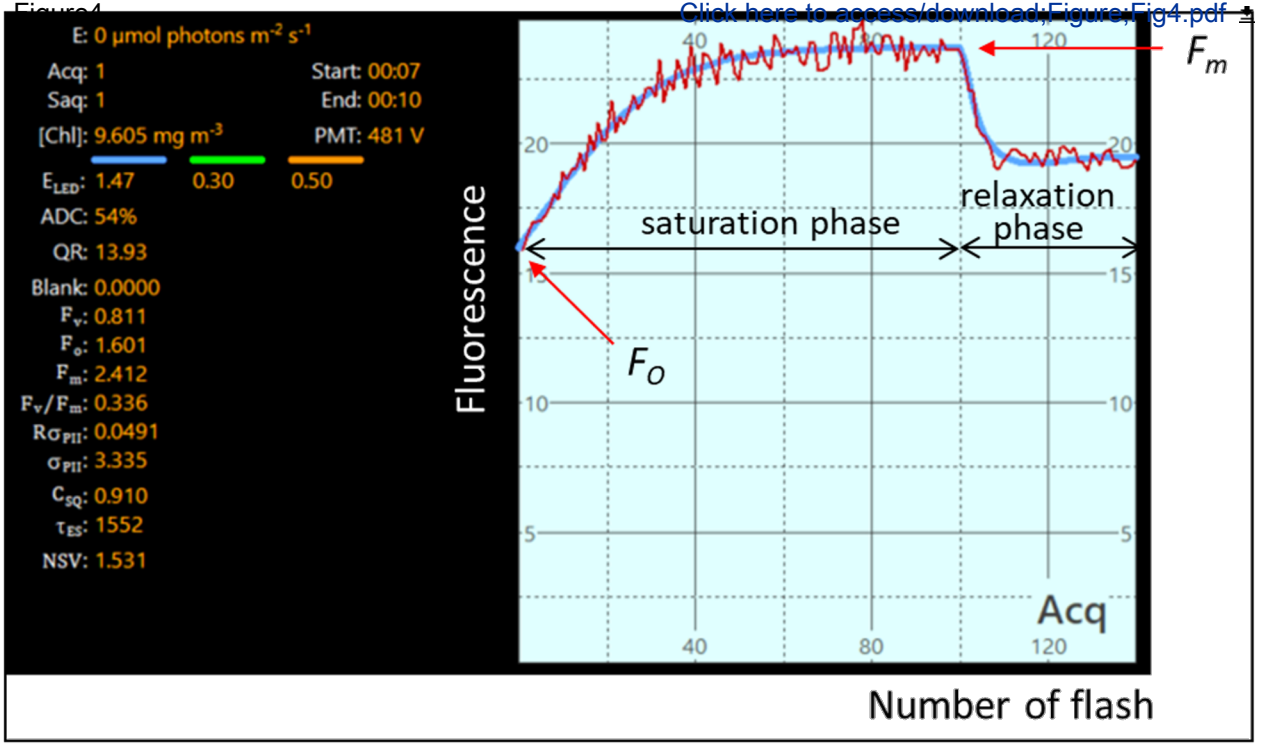

Figure

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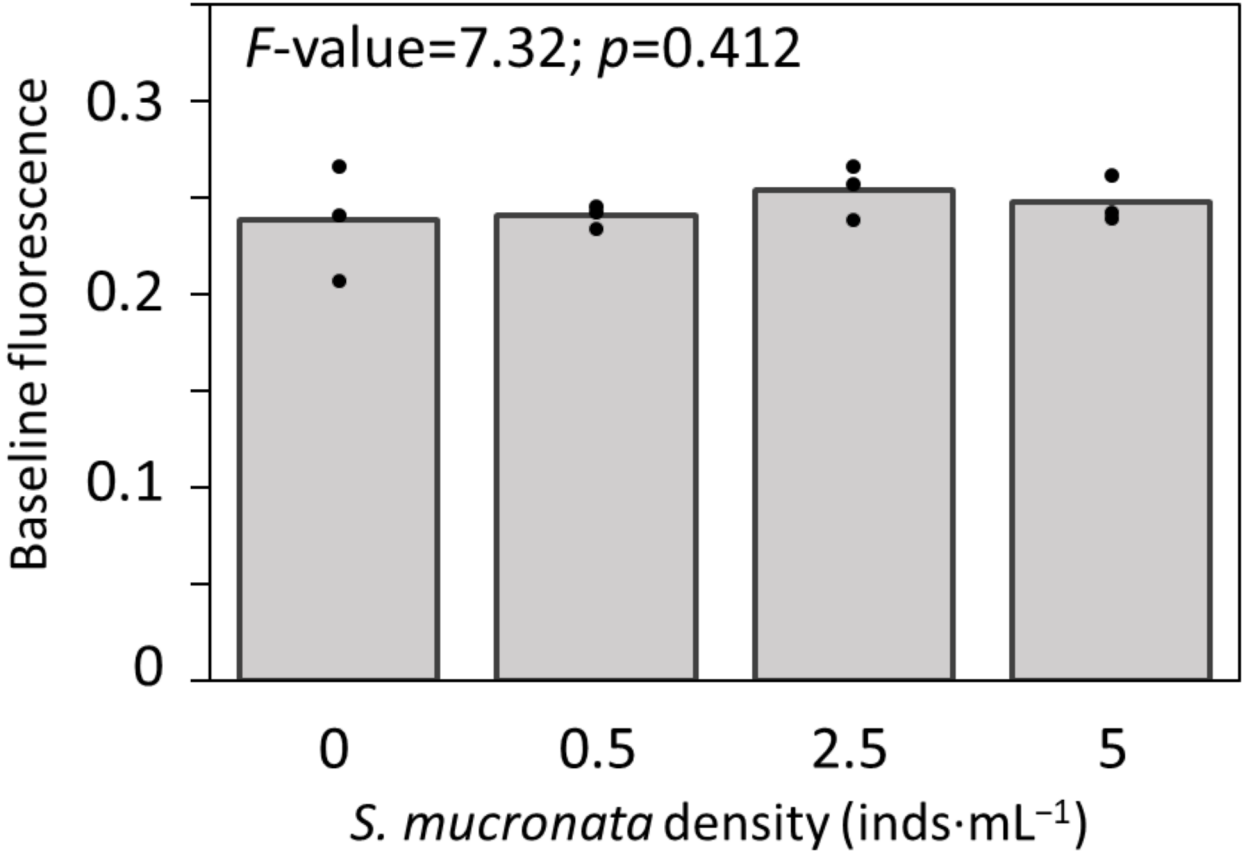

Figure

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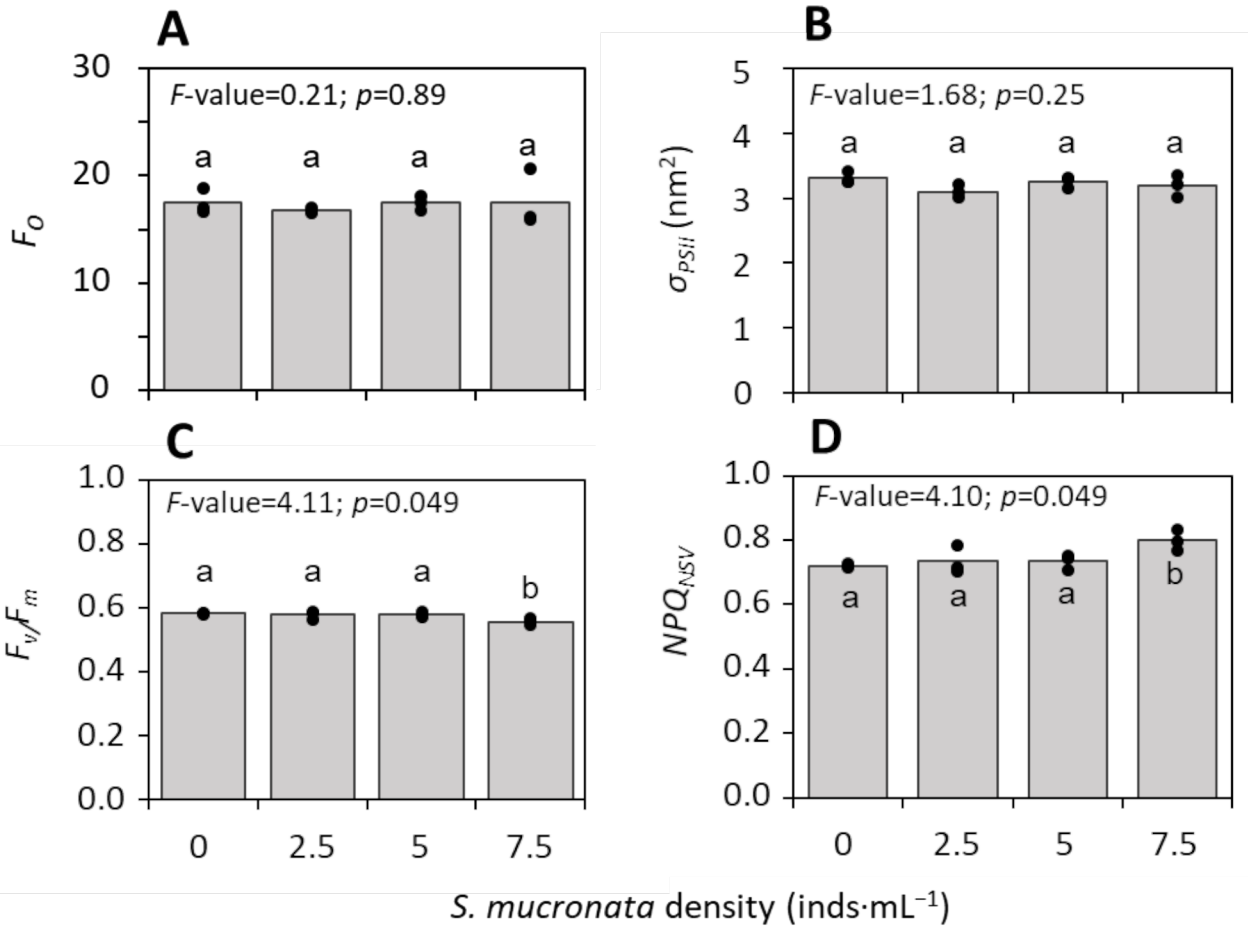

Figure7

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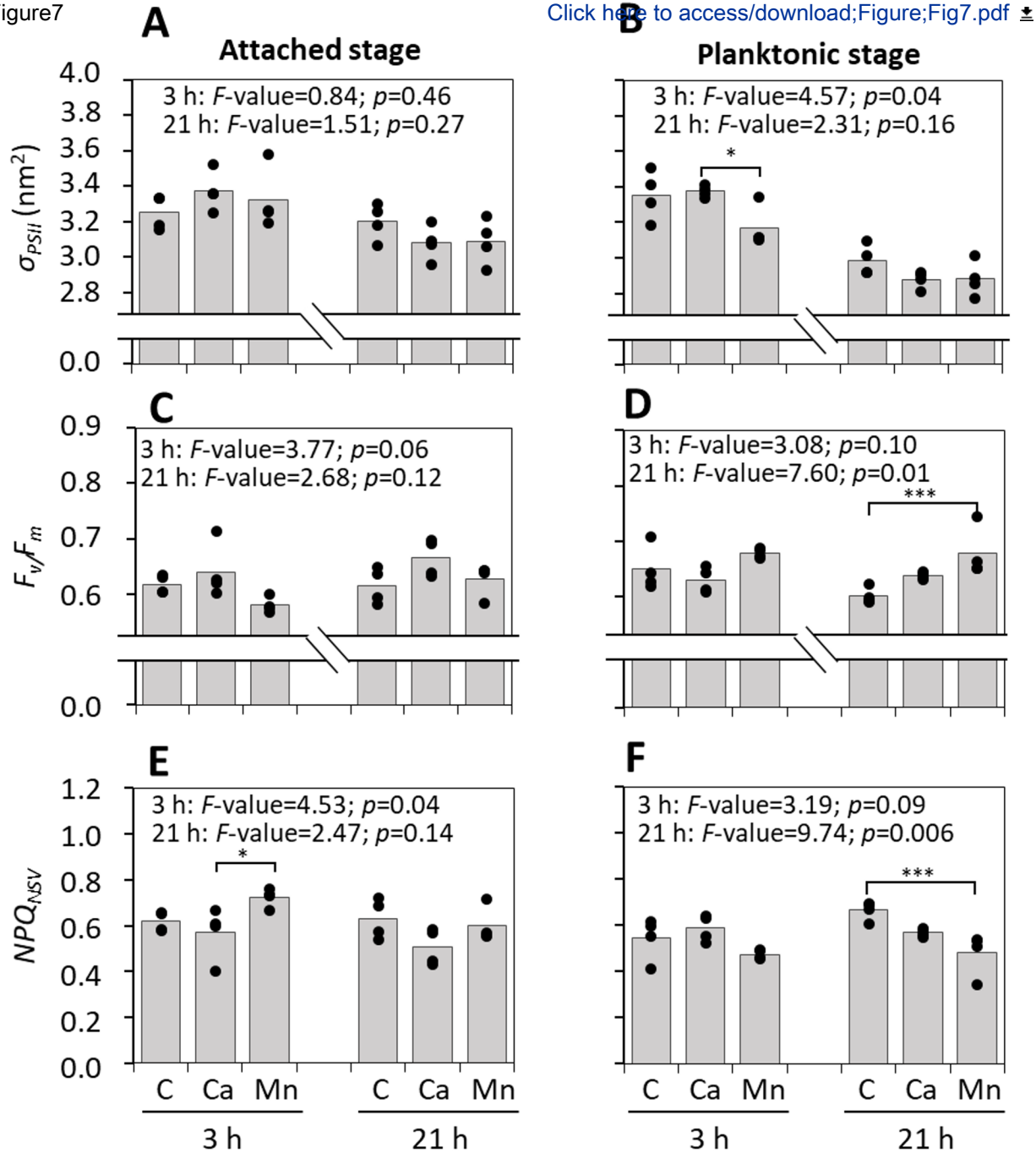
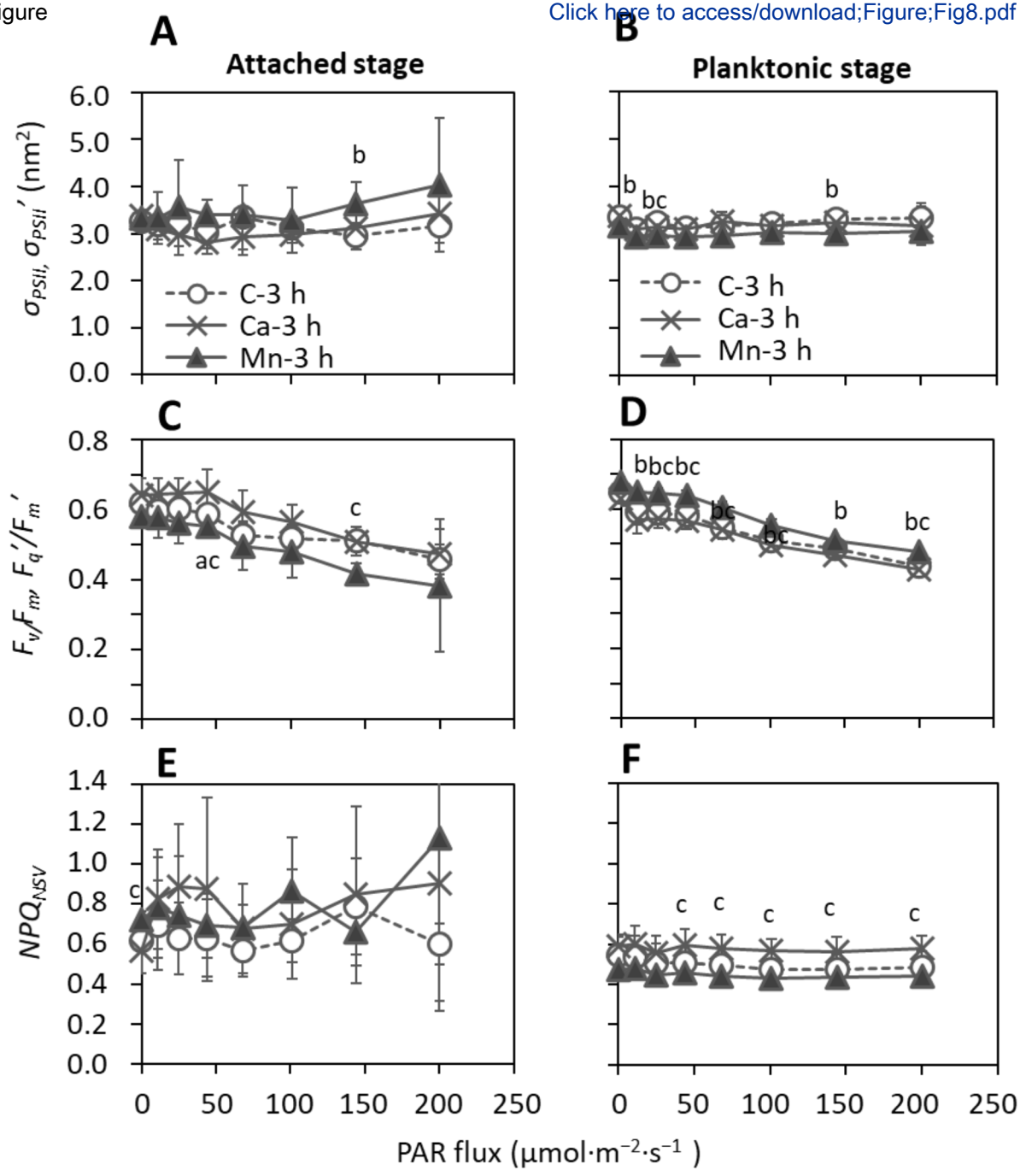


Figure



Figure

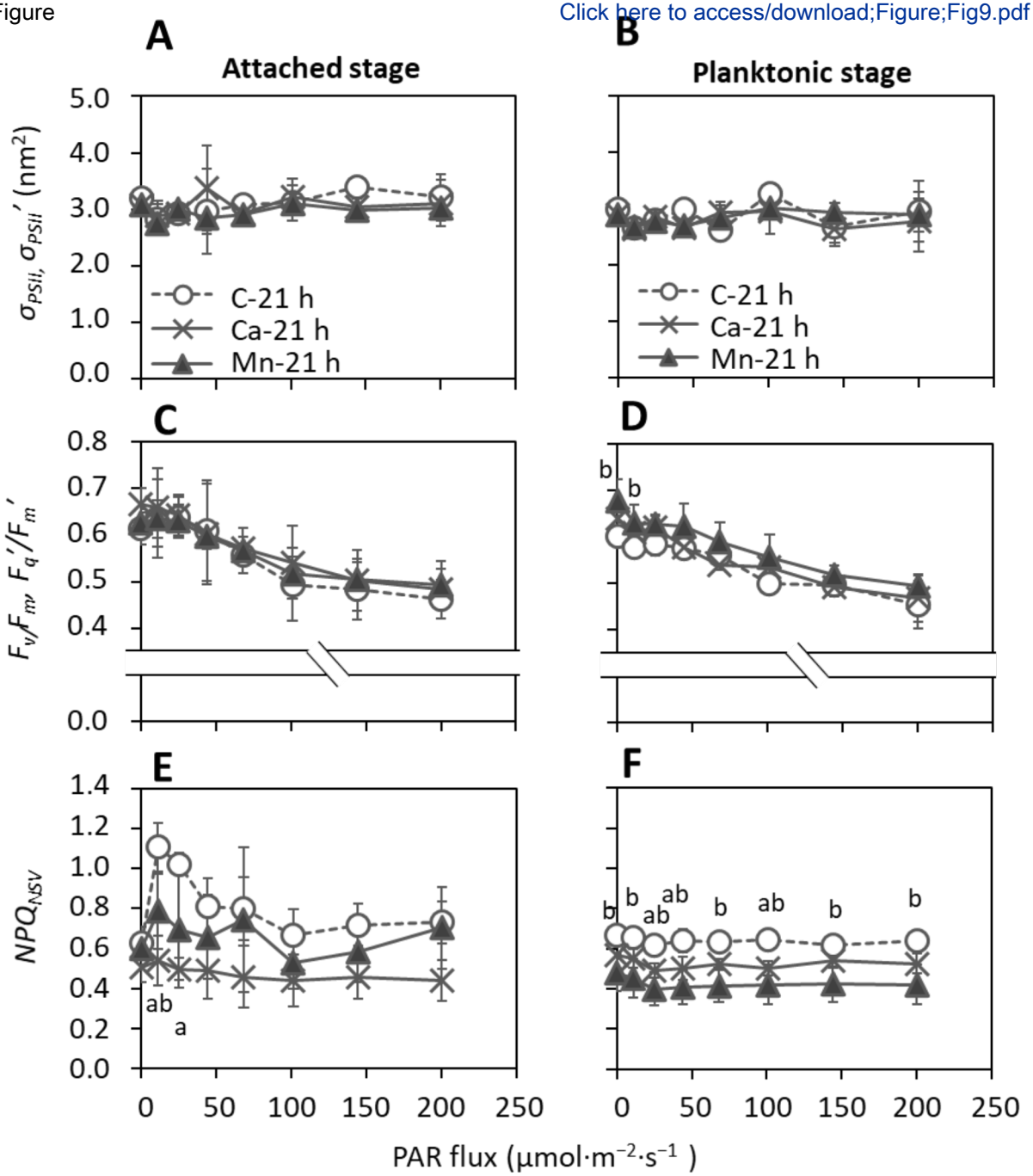


Table 1

Term	Definition	Units
Baseline fluorescence	Fo value without Chl-a fluorescence	
F'	Fluorescence at zeroth flashlet of a single turnover measurement when C>0	
Fo (')	Minimum PSII Fluorescence yield (under background light) at zeroth flashlet	
F_v (')	F_m (') $-$ Fo (')	
F_m (')	Maximum PSII Fluorescence yield (under background light)	
F_v/F_m	Maximum PSII photochemical efficiency under dark	
F_q'/F_m'	Maximum PSII photochemical efficiency under background light, ($F_m' - F$)/(F_m')	
NPQ_{NSV}	Normalized Stern-Volmer quenching, $F_O'/(F_m' - F_O')$	
[RCII]	Concentration of reaction center	
$R\sigma_{PSII}$ (')	Probability of an RCII being closed during the first flashlet of a single turnover saturation phase (under background light)	
σ_{PSII} (')	Functional absorption cross section of PSII for excitation flashlets (under background light)	nm^2

Table 2

Component	Quantity
$NaNO_3$	140 $mg \cdot L^{-1}$
NH_4NO_3	22 $mg \cdot L^{-1}$
$MgSO_4 \cdot 7H_2O$	30 $mg \cdot L^{-1}$
KH_2PO_4	10 $mg \cdot L^{-1}$
K_2HPO_4	5 $mg \cdot L^{-1}$
$CaCl_2 \cdot 2H_2O$	10 $mg \cdot L^{-1}$
$CaCO_3$	10 $mg \cdot L^{-1}$
Fe-citrate*	2 $mg \cdot L^{-1}$
Citric acid*	2 $mg \cdot L^{-1}$
Biotin	0.002 $mg \cdot L^{-1}$
Vit. B_1	0.01 $mg \cdot L^{-1}$
Vit. B_6	0.001 $mg \cdot L^{-1}$
Vit. B_{12}	0.001 $mg \cdot L^{-1}$
Trace metals	1 $mL \cdot L^{-1}$
($FeCl_3 \cdot 6H_2O$)	(1.0 $mg \cdot mL^{-1}$)
($MnCl_3 \cdot 4H_2O$)	(0.4 $mg \cdot mL^{-1}$)
($ZnSO_4 \cdot 7H_2O$)	(0.005 $mg \cdot mL^{-1}$)
($CoCl_2 \cdot 6H_2O$)	(0.002 $mg \cdot mL^{-1}$)
(Na_2MoO_4)	(0.004 $mg \cdot mL^{-1}$)
(Na_2-EDTA)	(7.5 $mg \cdot mL^{-1}$)

Table 3

Sampling date	Sample No.	Water temp. (°C)	*S. mucronata* density (inds.·mL^{-1})	*Colacium* sp. cell density (inds.·mL^{-1})	σ_{PSII} (nm^2)	F_v/F_m	NPQ_{NSV}
April 27/2020	**No. 1**	**14.2**	**4.5**	**77**	**3.42**	**0.60**	**0.66**
	SE				0.22	0.01	0.04
May 21/2020	**No. 2**	**19.4**	**2**	**282.5**	**3.62**	**0.54**	**0.85**
	SE				0.16	0.02	0.06
	No.3	**19.4**	**2**	**250.5**	**3.55**	**0.56**	**0.77**
	SE				0.09	0.01	0.02
	No.4	**19.4**	**5**	**204.5**	**3.76**	**0.52**	**0.94**
	SE				0.12	0.00	0.02
June 18/2020	**No.5**	**22.4**	**2.5**	**474**	**3.62**	**0.54**	**0.85**
	SE				0.16	0.02	0.06
	No.6	**22.4**	**2**	**410**	**3.55**	**0.56**	**0.77**
	SE				0.09	0.01	0.02
	No.7	**22.4**	**2.5**	**441**	**3.76**	**0.52**	**0.94**
	SE				0.12	0.00	0.02
July 20/2020	**No. 8**	**27.5**	**5**	**109**	**3.49**	**0.58**	**0.74**
	SE				0.10	0.00	0.00
				Mean	**3.60**	**0.55**	**0.82**
				S.D.	**0.120349**	**0.03**	**0.10**

Table 4

Sampling date	Sample No.	Medium	Growth temperature (°C)	σ_{PSII} (nm^2)	F_v/F_m	NPQ_{NSV}
May 21/2020	**No. 1**	**AF-6**	**19.4**	**2.72**	**0.65**	**0.53**
	SE			0.03	0.00	0.01
June 18/2020	**No. 2**	**AF-6**	**22.4**	**3.07**	**0.55**	**0.84**
	SE			0.08	0.02	0.07
July 20/2020	**No.3**	**AF-6**	**27.5**	**2.90**	**0.58**	**0.73**
	SE			0.06	0.01	0.02
			Mean	**2.90**	**0.59**	**0.70**
			S.D.	**0.18**	**0.05**	**0.16**

Amit Krishnan, Ph.D.
Review Editor
JoVE
10/9/2021

Dear Dr. Krishnan,
On behalf of my coauthors, I would like to thank you for the opportunity to revise and resubmit our manuscript JoVE63108, entitled “Measuring Photophysiology of Attached Stages of *Colacium* sp. by a Cuvette-Type Fast Repetition Rate Fluorometer.” We found the editor’s comments to be helpful in revising the manuscript and were successful in incorporating the reviewer’s feedback into our revised manuscript.

We have included a response to the editor in which we address each comment the editor made. Corresponding changes can be tracked in “63108-R1TK_track” file.

Thank you again for your consideration of our revised manuscript.

Sincerely,

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Editorial comments:

1. Please note that the manuscript has been formatted to fit the journal standard. Some step numbers have been adjusted. Please check the step numbers referenced in the steps. Comments to be addressed are included in the manuscript. Please review and revise accordingly.

>>>We have checked the manuscript thoroughly and modified along with the comments. Furthermore, we fixed some font issues (italics) and typos ("step" and "steps", and "stage" and "stages").

2. Please use the middle dot to represent multiplication in compound units. Please check if the changes made in the compound units in the manuscript text are correct. If not, please revise. Also, please revise the compound units to include the middle dot in the Figures and Tables. (e.g., "mg L-1" must be "mg·L-1", "µmol m-2 s-1" must be "µmol·m-2·s-1").

>>>We revised the compound units in the manuscript, Figures and Tables. Also, we fixed the values and units in Table 3 ([2 mL]$^{-1}$ to mL^{-1}), which were pointed out by Reviewer 2 but did not modified in the previous version.

3. Please check if reference number 40 is correct.

>>>Reference 40 is correct. This is a chapter written by Rosowski in "Freshwater Algae of North America", published in 2003. This chapter was rewritten by Triemer and Zakryś in the 2nd edition of the book in 2015. The life stages of *Colacium* is only mentioned in the 1st edition.