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Measuring Photophysiology of Attached Stages of *Colacium* sp. by a Cuvette-Type Fast Repetition Rate Fluorometer V.2

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FRRf in Lake Biwa

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

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

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SUMMARY:
Fast repetition rate fluorometer (FRRf) is a beneficial method for measuring photosystem II photophysiology and primary productivity. Here we describe how 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[1,4] and estimating primary productivity[1–3], which enables monitoring phytoplankton PSII photophysiology, as well as primary productivity, across wide spatial and temporal scales[5–7]. FRRf can simultaneously measure absorption cross section of PSII (σ_{PSII}), concentration of reaction center ([RCII]), maximum photochemical efficiency (F_v/F_m), effective

photochemical efficiency (F_q'/F_m'), and non-photochemical quenching (NPQ_{NSV}) (**Table 1**). Generally, 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, two types of FRRf instruments are commercially available, the submersible-type and cuvette-type. The submersible-type FRRf enables *in situ* measurements in oceans and lakes, while the cuvette-type FRRf is suitable for measuring small sample volume.

Given the development of PAM fluorometers, including the cuvette-type, for a broad range of subjects[11], PAM fluorometers are still more common than the FRRf 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 phytoplankton[13–15], benthic microalgae[16–18], ice algae[19], and epizoic algae[20], while the cuvette-type FRRf has been applied primarily to phytoplankton[21–23] and a limited number of ice algae species[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 algae 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 epibionts 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 an attached stage (**Figure 1A–D**), non-motile planktonic stage (**Figure 1E,F**), and motile planktonic stage[40,41]. In the non-motile planktonic stage, cells live as single-cell plankton, an aggregated colony, or as a one-layer sheet colony, which are covered by mucilage[42]. In the attached stage, *Colacium* sp.use mucilage excreted from 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 evolutionally trait for gathering resources, such as light[44] and trace elements[41,45,46], or as a dispersion strategy[27], there is little experimental evidence[37,41,44] and the key attachment mechanisms are largely unknown. For example, Rosowski and Kugrens expected that *Colacium* obtains Mn from substrate copepods[41], which

concentrated in the exoskeleton[47].

Here, we describe how to measure PSII photophysiology of planktonic algae and its application method for targeting attached algae (attaching to zooplankton) with *Colacium* sp. cells using the cuvette-type FRRf. Since finding the planktonic stage of *Colacium* sp. in natural environments is difficult, we collected their attached stage for our experiments. Among the many substrate organisms, *Scapholeberis mucronata* O.F. Müller 1776 (Branchiopoda, *Daphniidae*; **Figure 1A,B,G**) is one of the simplest organisms 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. Further, we applied this protocol to clarify the attaching mechanism of *Colacium* sp. and determine the effects of two metals, calcium (Ca) and manganese (Mn) on the photophysiology of both planktonic and attached stages. Calcium plays multiple, key roles in photosynthetic pathways[48] and both metals are required to construct oxygen-evolving complexes of PSII[49]. Because calcium and manganese are highly concentrated in the carapace of crustacean zooplankton[47], we hypothesize that *Colacium* sp. photophysiology may 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. Collect lake waterfrom surface by bucket. To target *Colacium* sp.attached to *S. mucronata* (**Figure 1A–C**), filter 0.5 to 10 L of lake water using a 100 µm nylon mesh net. **NOTE**: *S. mucronata* often densely aggregate in shallow, eutrophic, muddy water, such as among reed (phragmites) areas.
2. Store concentrated samples in 500 mL plastic bottles with some lake water. Keep in dark conditions.
3. In the laboratory, pour the sample water into a 500 mL beaker and allow to settle for a few minutes.
4. Filter lake water through a 0.2 µm pore-size filter.
5. Pick up *S. mucronata* individuals by pipette. Transfer them into a drop of 0.2 µm filtered lake water (hereafter, FLW) on a slide glass. **NOTE**: *S. mucronata* may swim to the surface or attach to the beaker wall.
6. Check *S. mucronata* under light microscopy.
7. Wash *S. mucronata* individuals using FLW (3 drops or more) to prevent contamination from other organisms (**Figure 2**).
8. Keep *S. mucronata* atan *in situ* temperature in a growth chamber.

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

2. 1. *S. mucronata* cultivation

1. Pick up *S. mucronata* individuals by pipette under an optical microscope and wash using FLW, as in step 1.7.
2. Pour aerated tap water into a glass jar.
3. Feed *Chlorella* (1 mg C L^{-1}) and maintain at 20° under dim light in a growth chamber.
4. After approximately 14 days, inoculate a sub-culture with clean aerated tap water to keep the medium fresh.

2. 2. FRRf measurements

1. To examine the effects of zooplankton individuals on baseline fluorescence, prepare adult *S. mucronata* (body size 400–650 µm) without any attached organisms. To avoid fluorescence from

the gut contents, individuals must be starved in FLW at 20° for at least 90 mins.

2. Pour 1.5 mL of FLW into a cuvette. Transfer 0, 1, 5, and 10 *S. mucronata* individuals into the cuvette and add FLW to bring the sample up to 2 mL.
3. Acclimate under low light (1–10 µmol photon m^{-2} s^{-1}) at20° for 15 mins before FRRf measurement.
4. Set the operation software to apply a ST method by blue excitation wavelength[50,51]. Measurements should be repeated >3 times per sample. **NOTE**: Check the $R\sigma_{PSII}$ value is within the optimal range (0.03–0.08)[52].

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

3. 1. *Colacium* sp. cultivation

1. Prepare FLW and AF-6 medium[53] for cultivation (**Table 2**).
2. Keep sampled *Colacium* sp. on *S. mucronata* at *in situ* temperature in a growth chamber.
3. Pick up *Colacium* sp. with molted carapace (**Figure 1D**) by pipette under an optical microscope. Wash them with FLW, as in step 1.7.
4. Aseptically inoculate *Colacium* sp. and AF-6 medium ina 10 mL glass tube on a clean bench.
5. Maintain 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.

3. 2. FRRf measurements

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. To avoid fluorescence from the gut contents, individuals must be starved in FLW for at least 90 mins.
2. Set up a cuvette-type fast repetition rate fluorometer (FRRf).
3. 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.
4. Acclimate under low light (1–10 µmol photon m^{-2} s^{-1}) at20° for 15 mins before taking the FRRf measurement.
5. Set the operation software to apply a ST method by blue excitation wavelength[50,51]. Measurement should be repeated >3 times per sample. **NOTE**: Maintain samples at incubation temperature during measurements
6. To correct baseline fluorescence[22], filtrate culture medium using a 0.2 µm pore-size filter and measure fluorescence. Subtract F_O of the baseline sample from F_O and F_m of *Colacium* sp., or calculate using the operation software.

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

1. Isolate *S. mucronata* individuals with *Colacium* sp. by pipette under an optical microscope.
2. Wash *S. mucronata* using FLW, as in step 1.7.
3. Transfer *S. mucronata* into a 100 mL of FLW. For starvation, keep under dark conditions at *in situ* temperature for 90 mins.
4. Pour 1.5 mL of FLW into a cuvette.
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.
6. Acclimate under low light (1–10 µmol photon m^{-2} s^{-1}) at *in situ* temperature for 15 mins. Measure Chl-*a* fluorescence as in step 3.2.5–3.2.6.

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 light microscope.

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

1. Cultivate sampled *Colacium* sp. in AF-6 medium at *in situ* temperature as in steps 3.1.1–3.1.5.
2. For the stationary phase, take 2 mL of cultured *Colacium* sp. and pour into a cuvette.
3. Acclimate under low light (1–10 µmol photon m^{-2} s^{-1}) at *in situ* temperature for 15 mins. Measure Chl-*a* fluorescence as in step 3.2.5–3.2.6.

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

6.1. Effects on attached stage

1. Isolate *S. mucronata* individuals with *Colacium* sp. by pipette under an optical microscope. Wash using FLW, as in step 1.7.
2. Transfer six individuals into 12 glass beakers with 30 mL of FLW. Each beaker must contain >100 *Colacium* sp. cells.
3. Add 200 µmol L^{-1} $CaCl_2 \cdot H_2O$ (Ca treatment), 40 µmol L^{-1} $MnCl_4$ (Mn treatment), or MiliQ water (control) to each beaker. Incubate samples under 200 µmol photon m^{-2} s^{-1} at *in situ* temperature in a growth chamber.
4. At 3 and 21 h, transfer all individuals and molted skins into a cuvette.
5. After 15 min of dark acclimation, measure Chl-*a* fluorescence of each sample as in steps 3.2.5–3.2.6. To examine the rapid response to increasing light, expose samples to 20 s periods of 8 actinic lights increasing stepwise in intensity from 0 to 200 µmol photon m^{-2} s^{-1} after 30 s measurement in dark conditions. **NOTE**: Verify $R\sigma_{PSII}$ and $R\sigma_{PSII}'$ values are within the optimal range (0.03–0.08)[52]

6. 2. Effects on planktonic stage

1. Cultivate sampled *Colacium* sp. in AF-6 medium at *in situ* temperature as in steps 3.1.1–3.1.5.
2. Transfer cultured *Colacium* sp. into FLW and acclimate at *in situ* temperature less than 200 µmol photon m^{-2} s^{-1} for three days.
3. Transfer 1 mL of acclimated samples into three glass vials with 10 mL of FLW.
4. Add 200 µmol L^{-1} $CaCl_2 \cdot H_2O$ (Ca treatment), 40 µmol L^{-1} $MnCl_3$ (Mn treatment), or MiliQ water (control) to vials. Incubate samples under 200 µmol photon m^{-2} s^{-1} at *in situ* temperature in a growth chamber.
5. At 3 h and 21 h, measure Chl-*a* fluorescence of each sample as in step 6.1.5.

REPRESENTATIVE RESULTS:

There was no significant effect of background fluorescence (**Figure 3**) or Chl-*a* fluorescence (**Figure 4**) by *S. mucronata* up to 10 inds. $[2\ mL]^{-1}$. However, F_v/F_m and NPQ_{NSV} were significantly affected when *S. mucronata* was 15 inds. $[2\ mL]^{-1}$. 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 (>100 cells $[2\ mL]^{-1}$) and a low number of *S. mucronata* (≤10 inds. $[2\ mL]^{-1}$) 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 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 5A,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 6A,C,E** and **Figure 7A,C,E**). However, NPQ_{NSV} tended to be lower in the Ca treatment than the control at low light intensity at 21 h (11 and 25µmol photon m^{-2} s^{-1}, **Figure 7E**). For the planktonic stage, σ_{PSII} was significantly lower in the Mn than Ca treatment at 3 h (**Figure 5B**). F_q'/F_m' was significantly higher, but NPQ_{NSV} was lower in the Mn treatment than control at 21 h (**Figure 5D,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 6D)**. Similarly, Mn significantly reduced NPQ_{NSV} during the planktonic stage compared to the control at 21 h (**Figure 7F**). Similar to the attached stage, calcium slightly improved NPQ_{NSV} for the planktonic stage under increasing light (**Figure 7F)**. 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 6D,F)**.

FIGURE AND TABLE LEGENDS:

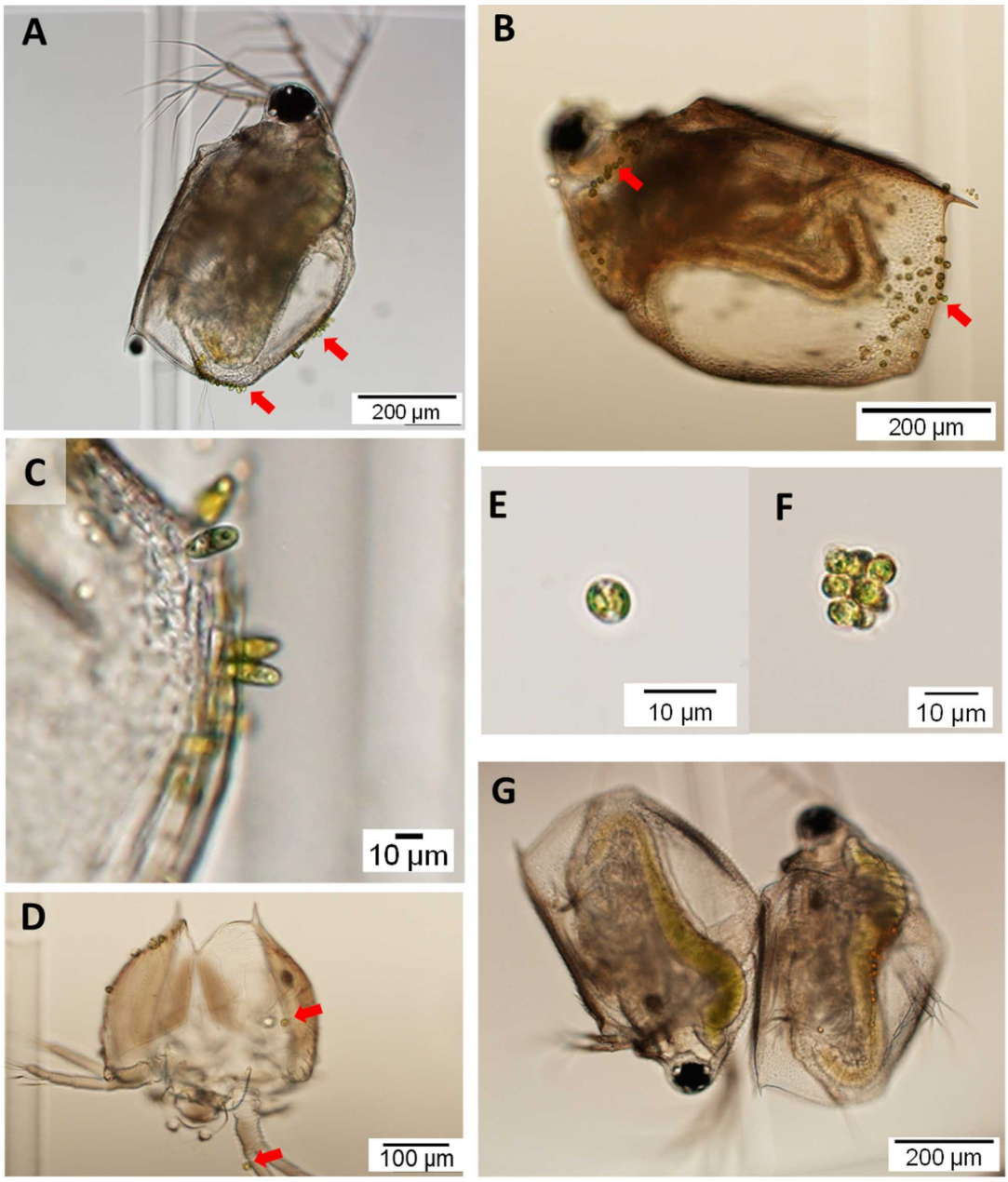

Figure 1: *Colacium* sp. and substance organism *Scapholeberis mucronata*.
(A) Infected *S. mucronata*.**(B)** Infected *S. mucronata* fixed with glutaraldehyde. **(C)** Attached *C*olacium cells on living *S. mucronata*.**(D)** Attached *C*olacium cells on molted carapace. **(E, F)** *Colacium* sp.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

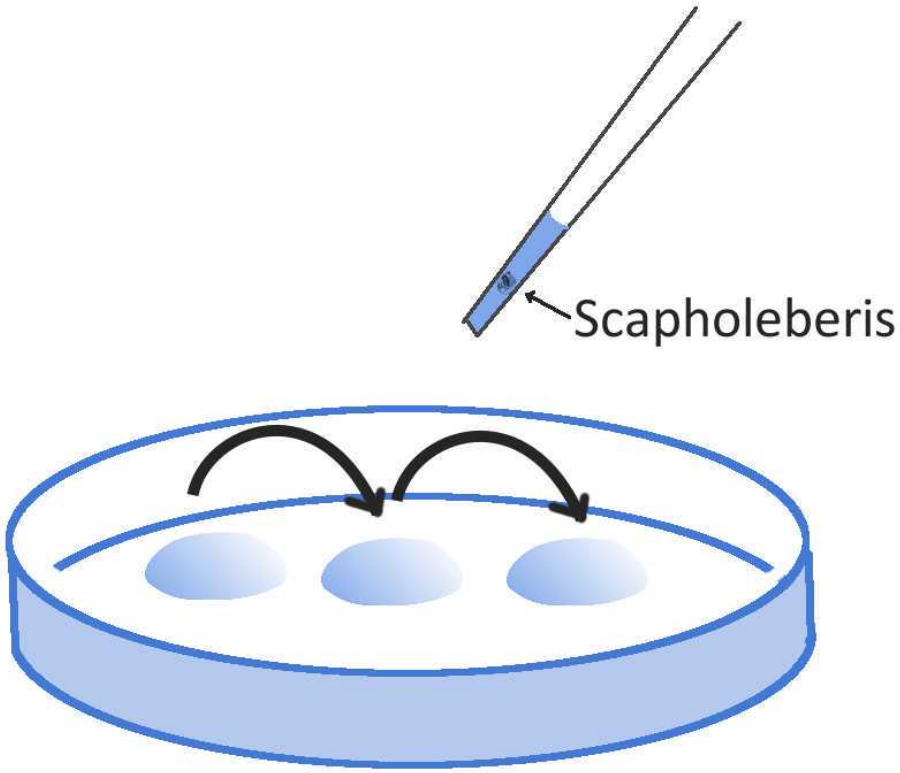

Figure 2: Washing zooplankton by pipetting under filtered lake water (FLW).

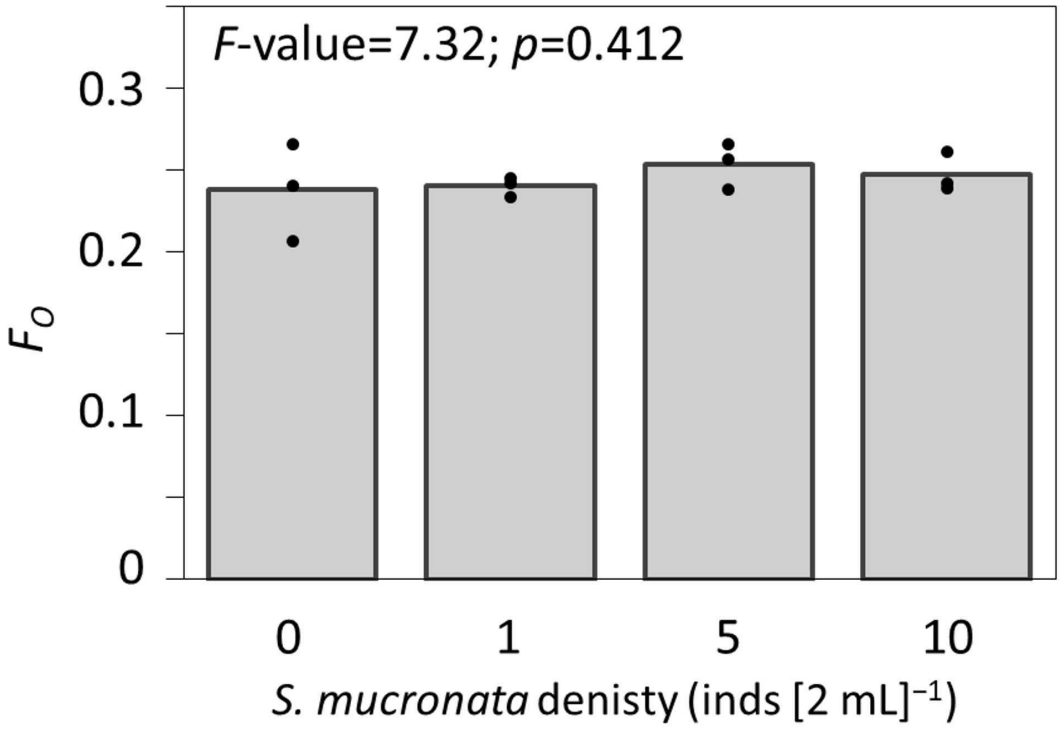

Figure 3: The effect of *S. mucronata* density on background fluorescence.

Small dots represent replicates. The results of ANOVA test are also shown.

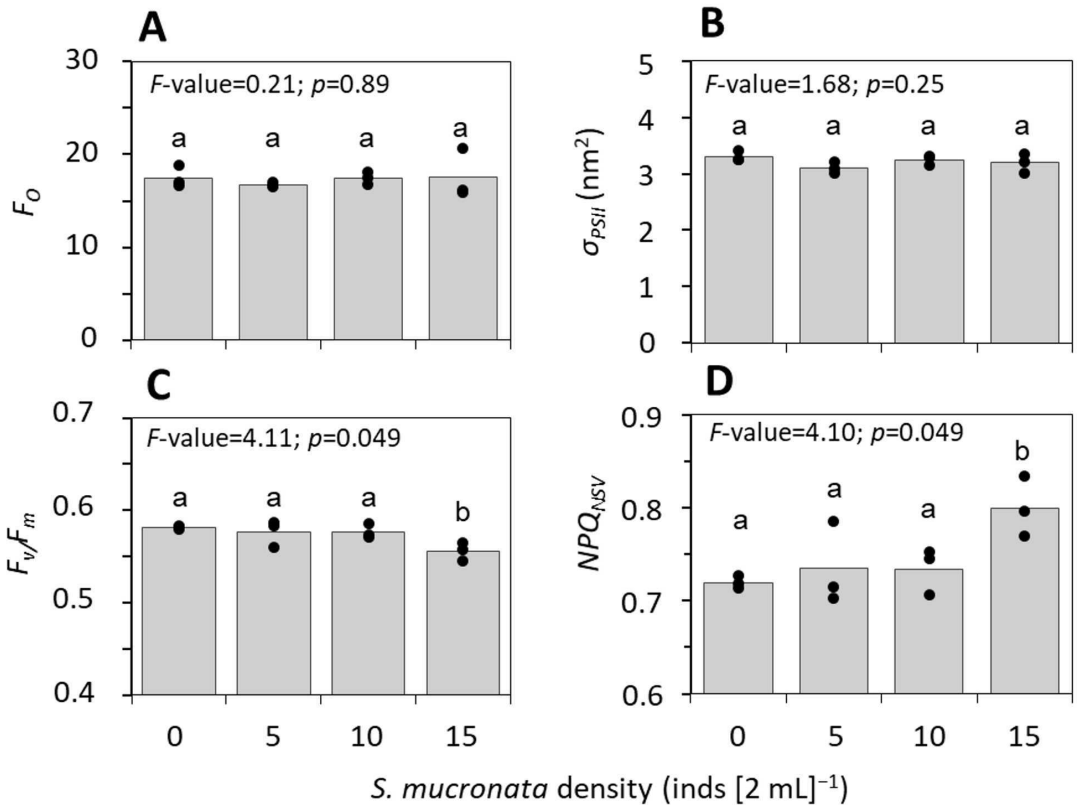

Figure 4: The effects of *S. mucronata* densities on (A), (B) σ_{PSII}, (C) F_v/F_m, and (D) NPQ_{NSV} for *Colacium* sp. during the planktonic stage.

Small dots represent replicates. *Colacium* sp. was cultured in AF-6 medium. The results of ANOVA and Tukey post-hoc test are also presented.

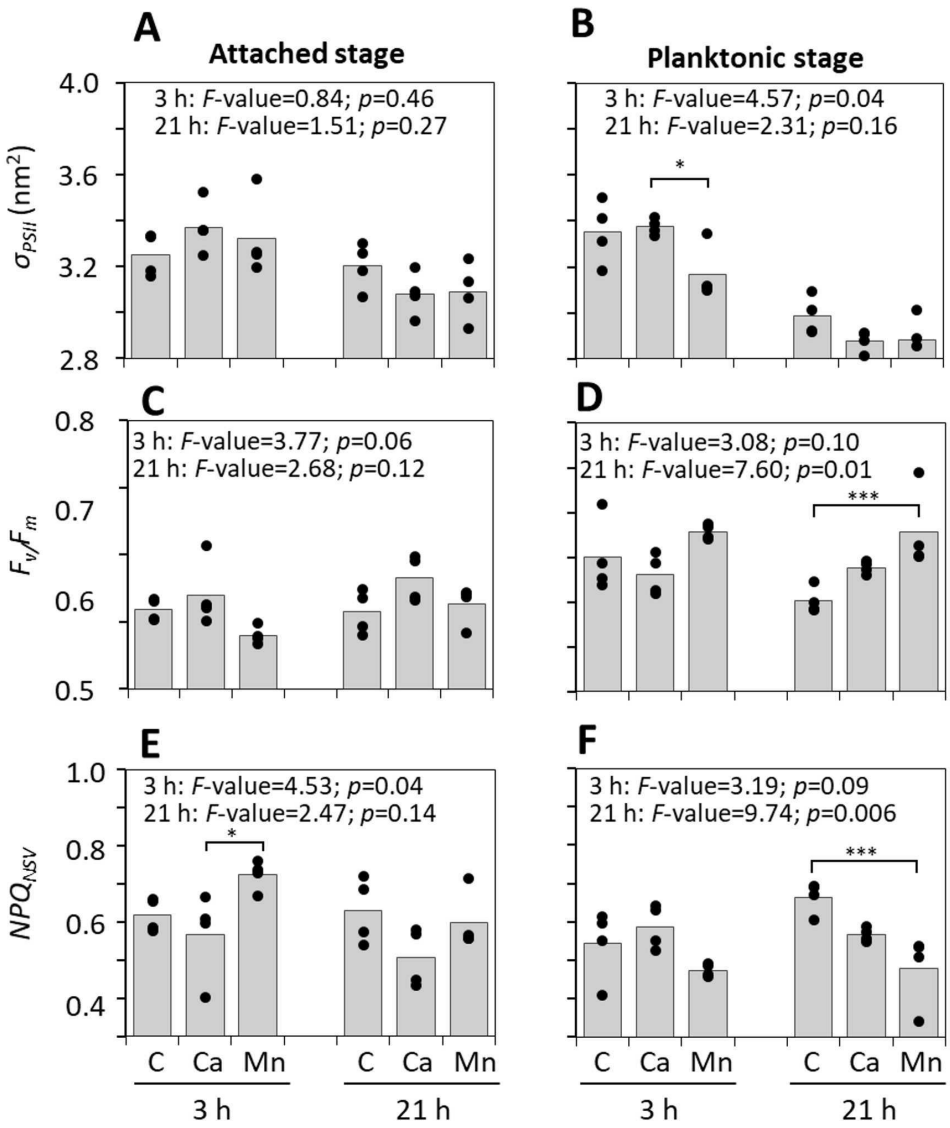

Figure 5: Responses of (A, B) absorption cross section, (C, D) PSII photochemistry, and (E, F) non-photochemical quenching of (A, C, E) attached stage and (B, D, F) planktonic stage of *Colacium* sp. at 3 h and 21 h after Ca and Mn addition.

Small dots represent replicates. The results of ANOVA and Tukey post-hoc test are also presented. *, $p < 0.05$.

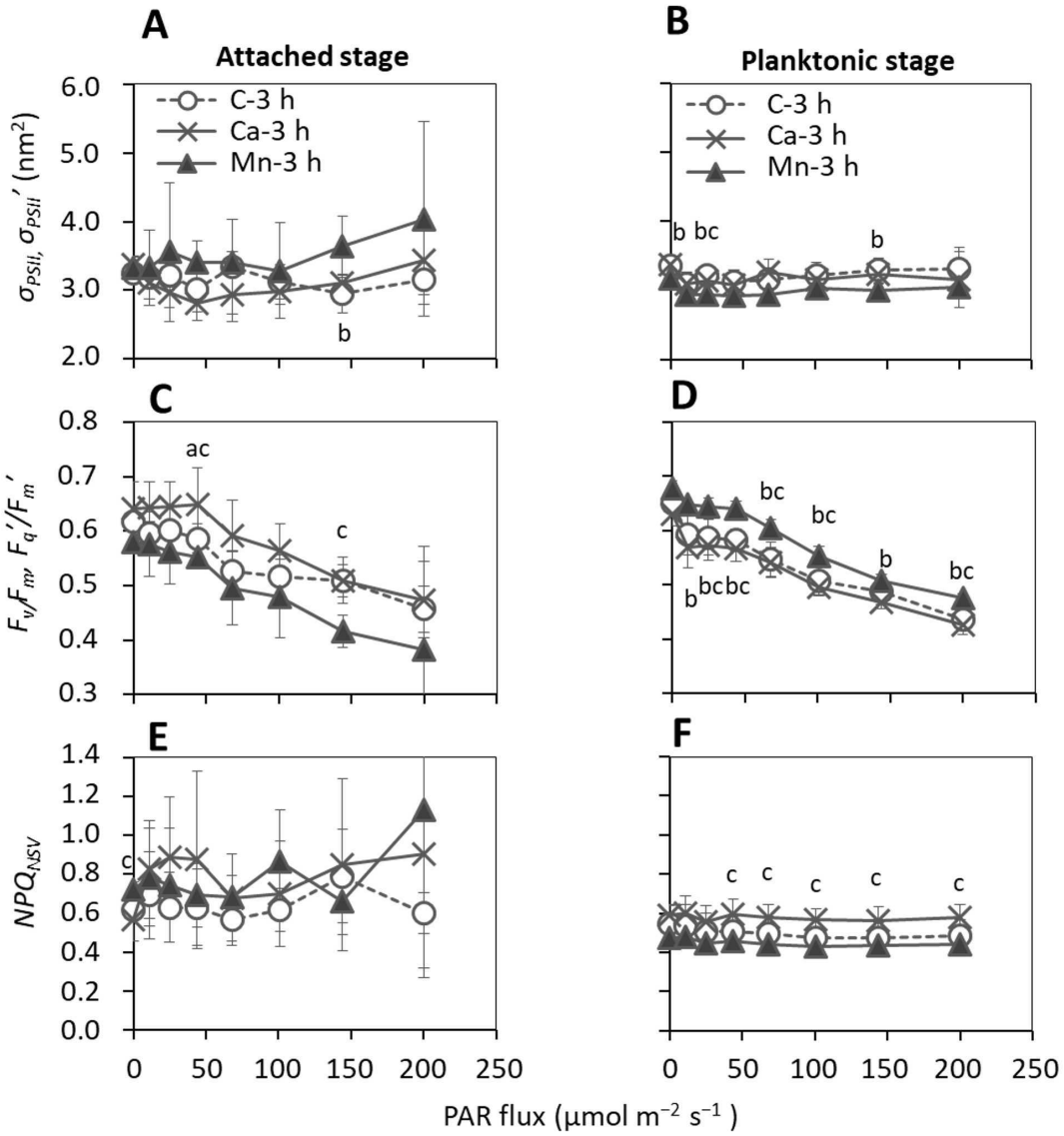

Figure 6: Rapid-light responses of (A, B) absorption cross section, (C, D) PSII photochemistry, and (E, F) non-photochemical quenching of *Colacium* sp. in attached and planktonic stages to stepwise light protocol at 3 h after Ca and Mn addition.

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.

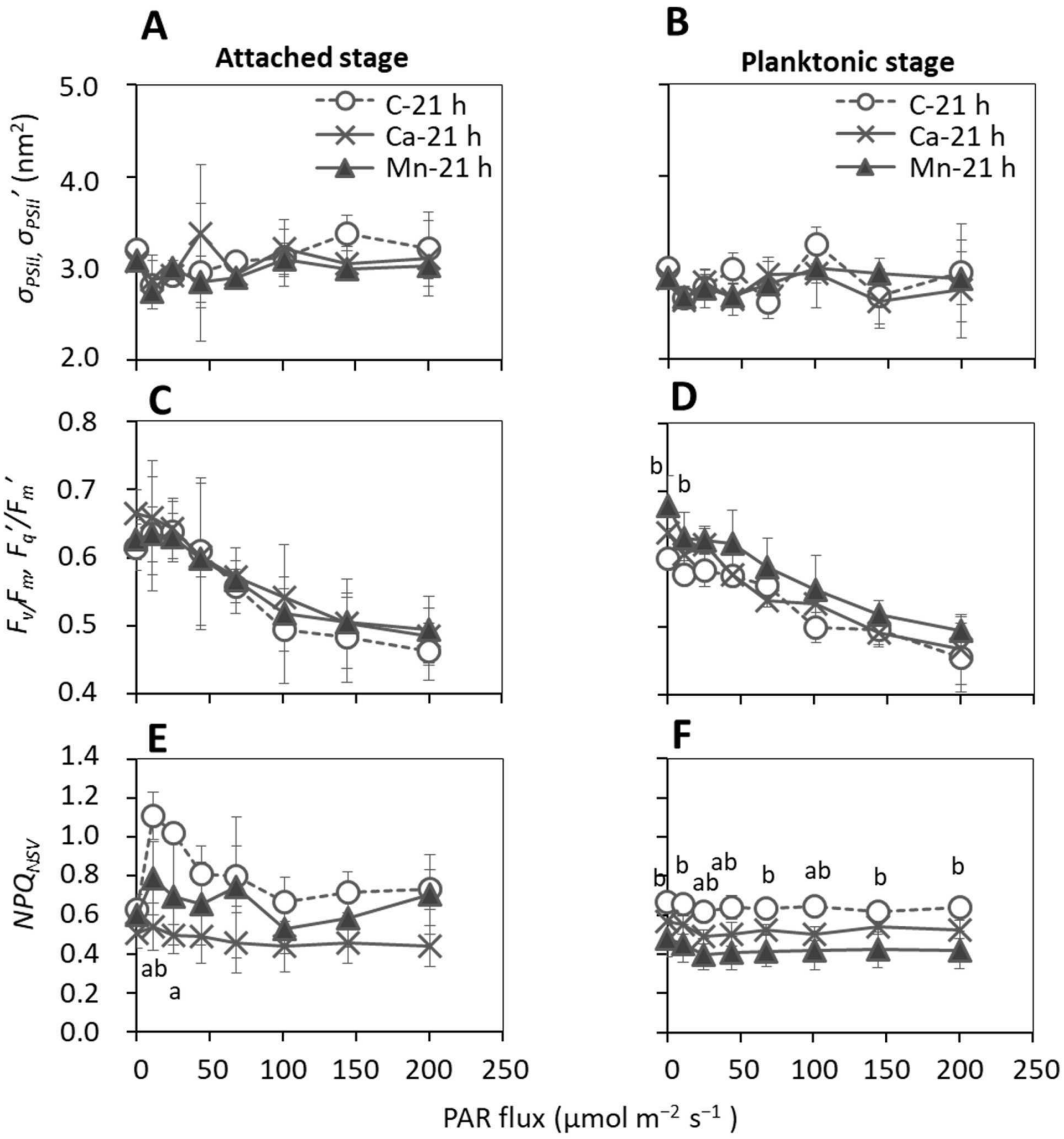

Figure 7: Rapid-light responses of (A, B) absorption cross section, (C, D) PSII photochemistry, and (E, F) non-photochemical quenching of *Colacium* sp. in attached and planktonic stages to stepwise light protocol at 21 h after Ca and Mn addition.
C, control; Ca, 200 µM Ca; Mn, 40 µM Mn. Significant 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.

A	B	C
Term	Definition	Units
F'	Fluorescence at zeroth flashlet of a single turnover measurement when C>0	
Fo (')	Minimum PSII Fluorescence yield (under background light)	
Fv (')	Fm(') − Fo(')	
Fm (')	Maximum PSII Fluorescence yield (under background light)	
Fv/Fm	Maximum PSII photochemical efficiency under dark	
Fq'/Fm'	Maximum PSII photochemical efficiency under background light, (Fm' − F)/(Fm')	
NPQ_{NSV}	Normalized Stern-Volmer quenching	
[RCII]	Concentration of reaction center	
RσPSII (')	Probability of an RCII being closed during the first flashlet of a single turnover saturation phase (under background light)	
σPSII (')	Functional absorption cross section of PSII for excitation flashlets (under background light)	nm^2

Table 1: Terms used in this protocol.

A	B
Component	**Quantity**
$NaNO_3$	140 mg L^{-1}
NH_4NO_3	22 mg L^{-1}
$MgSO_4 \cdot 7H_2O$	30 mg L^{-1}
KH_2PO_4	10 mg L^{-1}
K_2HPO_4	5 mg L^{-1}
$CaCl_2 \cdot 2H_2O$	10 mg L^{-1}
$CaCo_3$	10 mg L^{-1}
Fe-citrate*	2 mg L^{-1}
Citric acid*	2 mg L^{-1}
Biotin	0.002 mg L^{-1}
Vit. B_1	0.01 mg L^{-1}
Vit. B_6	0.001 mg L^{-1}
Vit. B_{12}	0.001 mg L^{-1}
Trace metals	1 mL L^{-1}
($FeCl_3 \cdot 6H_2O$)	(1 mg L^{-1})
($MnCl_3 \cdot 4H_2O$)	(0.4 mg L^{-1})
($ZnSO_4 \cdot 7H_2O$)	(0.005 mg L^{-1})
($CoCl_2 \cdot 6H_2O$)	(0.002 mg L^{-1})
(Na_2MoO_4)	(0.004 mg L^{-1})
(Na_2-EDTA)	(7.5 mg L^{-1})

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 1 mL HCl L^{-1} after mixing both reagents.Contents of trace metals are shown in parenthesis.

A	B	C	D	E	F	G	H
Sampling date	Sample No.	Water temperature (°C)	*S. mucronata* density (inds. [2 mL]$^{-1}$)	*Colacium* sp. cell density (cells [2 mL] $^{-1}$)	*σPSII* (nm^2)	*Fv/Fm*	*NPQ$_{NSV}$*
April 27/2020	**No. 1**	**14.2**	**9**	**154**	**3.42**	**0.60**	**0.66**
	SE				0.22	0.01	0.04
May 21/2020	**No. 2**	**19.4**	**4**	**565**	**3.62**	**0.54**	**0.85**
	SE				0.16	0.02	0.06
	No.3	**19.4**	**4**	**501**	**3.55**	**0.56**	**0.77**
	SE				0.09	0.01	0.02
	No.4	**19.4**	**10**	**409**	**3.76**	**0.52**	**0.94**
	SE				0.12	0.00	0.02
June 18/2020	**No.5**	**22.4**	**5**	**948**	**3.62**	**0.54**	**0.85**
	SE				0.16	0.02	0.06
	No.6	**22.4**	**4**	**820**	**3.55**	**0.56**	**0.77**
	SE				0.09	0.01	0.02
	No.7	**22.4**	**5**	**882**	**3.76**	**0.52**	**0.94**
	SE				0.12	0.00	0.02
July 20/2020	**No. 8**	**27.5**	**10**	**218**	**3.49**	**0.58**	**0.74**
	SE				0.10	0.00	0.00
				Mean	**3.60**	**0.55**	**0.82**

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

A	B	C	D	E	F	G
Sampling date	Sample No.	Medium	Growth temperature (°C)	*σPSII* (nm^2)	*Fv/Fm*	*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**

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 natural environment is comparable to its planktonic stage in AF-6 medium. Additionally, the effects of a substrate organism of fluorescence was negligible when density was ≤10 inds. $[2\ mL]^{-1}$. 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 to 3.2.6 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 background and Chl-*a* fluorescence requires further attention.

FRRf have been used to examine the impacts of nutrient manipulation on linear electron flow and non-photochemical quenching of phytoplankton[22,54,55]. Our primary results show Ca and Mn enrichment differed significantly between *Colacium* sp. life stages (**Figure 5–7**). Specifically, manganese clearly improved linear electron flow ($F_v/F_m$and F_q'/F_m')and decreased heat dissipation (NPQ_{NSV})[48] of planktonic stages under dark (**Figure 5D,F**) and light conditions (**Figure 6D,F** and **Figure 7D,F**). These outcomes can stem from reduced antenna size on PSII, σ_{PSII},and σ_{PSII}'(**Figure 5B and Figure 6B**), which reduces excess light absorption[56,57]. Measuring antenna size in addition to energy flow between PSII complexes will allow more precise measurements of algal response[10]. Our protocol can also examine photosynthesis limitation by other resources. For example, nitrogen and phosphorus limitation have been examined in various phytoplankton communities, but not in epizoic algae, despite predicted effects on *Colacium*[41] and marine epizoic diatoms[58,59]. In addition to nutrients, the light environment can further influence epizoic algae distribution[44].

As shown in **Figure 5–7**, 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[60]. However, although linear electron flow in PSII can be estimated from photophysiological parameters by FRRf, it is not necessarily analogous to carbon fixation rate[61,62]. For estimating production rates based on carbon levels, electron requirement per CO_2 fixation ($\Phi_{e,C}$), which can vary both temporally and spatially[5,52], is recommended when assessing subject communities. Another limitation of our study was deriving $\sigma_{PSII}$to represent background light. Because the wavelength of excitation light for FRRf varies, a spectral correction factor should be calculated from a Chl-*a* specific absorption spectrum of algae, as well as spectral distribution of background light[63].

Implementing cuvette-type FRRf should depend on substrate size because periphytic algae require a substrate attachment. For example, studies of algae on indestructible substances, such as rocks[64], larger organisms[26,65], or symbiotic algae, including *Symbiodinium* associated with hard corals[10,66,67], may require the submersible-type FRRf[66]. Conversely, if the basibiont is small enough to suspend itself in a cuvette, a cuvette-type FRRf may 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 photophysiology of ice algae[24,25]. Because current models of cuvette-type FRRf incorporating multi-excitation wavelengths are useful tools for examining cyanobacteria photophysiology and productivity[7,63,68], these ought to be useful methods for assessing benthic cyanobacteria. In future studies, FRRf 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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The authors have nothing to disclosure.

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