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A small volume bioassay to assess bacterial-phytoplankton co-culture using WATER-Pulse-Amplitude-Modulated (WATER-PAM) fluorometry

--Manuscript Draft--

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Abstract:	Conventional methods for experimental manipulation of microalgae have employed large volumes of culture (20 ml to 5 l), so that the culture can be subsampled throughout the experiment1-7. Subsampling of large volumes can be problematic for several reasons: 1) it causes variation in the total volume and the surface area:volume ratio of the culture during the experiment; 2) pseudo-replication (i.e. replicate samples from the same treatment flask) is often employed rather than true replicates (i.e. sampling from replicate treatments); 3) the duration of the experiment is limited by the total volume; and 4) axenic cultures or the usual bacterial microbiota are difficult to maintain during long-term experiments as contamination commonly occurs during subsampling. The use of microtiter plates enables 1 ml culture volumes to be used for each replicate, with up to 48 separate treatments within a 12.65 x 8.5 x 2.2 cm plate, thereby decreasing the experimental volume and allowing for extensive replication without subsampling any treatment. Additionally, this technique can be modified to fit a variety of experimental formats including: bacterial-algal co-cultures, algal physiology tests, and toxin screening8-10. Individual wells with an alga, bacterium and/or co-cultures can be sampled for numerous laboratory procedures including, but not limited to:

	WATER-Pulse-Amplitude-Modulated (WATER-PAM) fluorometry, microscopy, bacterial colony forming unit (cfu) counts and flow cytometry. The combination of the microtiter plate format and WATER-PAM fluorometry allows multiple rapid measurements of photosynthetic yield and chlorophyll fluorescence with low variability between samples, high reproducibility and avoids the many pitfalls of subsampling a carboy or conical flask over the course of an experiment.
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Question	**Response**
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Dear Editor,

I would like to submit the attached manuscript entitled "A small volume bioassay to assess bacterial-phytoplankton co-culture using WATER-pulse-amplitude-modulated (WATER-PAM) fluorometry" for your consideration. I was approached by JoVE Editor Charlotte Sage at the American Society for Microbiology meeting in San Francisco in 2012 after talking about my novel high-throughput method to test the effects of biological compounds produced by bacteria on microalgae. JoVE's multimedia format is ideal to present this method, as it allows for a much clearer depiction of the steps and a clearer description of the qualitative and quantitative evaluation of how bacteria affect algae physiologically. It is a unique approach, as the small volumes involved and the absence of sub-sampling substantially increase reproducibility and reduce experimental error and biases. I developed the method, while my students Julie and Anna wrote the protocol, and the example data was provided by Leen Labeeuw. All authors were involved in proofreading and editing the protocol. I suggest the following experts to review the protocol:

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TITLE:
A small volume bioassay to assess bacterial-phytoplankton co-culture using WATER-pulse-amplitude-modulated (WATER-PAM) fluorometry.

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Phytoplankton, microalgae, co-culture, bacteria, WATER-Pulse-Amplitude-Modulated (WATER-PAM) fluorometry, photosynthetic yield, chlorophyll, microtiter plate assay, high-throughput bioassay

SHORT ABSTRACT:
The goal of this procedure is to demonstrate the reproducibility and adaptability of using a microtiter plate format for microalgal screening. This rapid screen combines WATER-Pulse-Amplitude-Modulated (WATER-PAM) fluorometry to measure photosynthetic yield as an indicator of Photosystem II (PSII) health with small volume bacterial-algal co-cultures.

LONG ABSTRACT:
Conventional methods for experimental manipulation of microalgae have employed large volumes of culture (20 ml to 5 l), so that the culture can be subsampled throughout the experiment[1–7]. Subsampling of large volumes can be problematic for several reasons: 1) it causes variation in the total volume and the surface area:volume ratio of the culture during the experiment; 2) pseudo-replication (i.e. replicate samples from the same treatment flask[8]) is often employed rather than true replicates (i.e. sampling from replicate treatments); 3) the duration of the experiment is limited by the total volume; and 4) axenic cultures or the usual bacterial microbiota are difficult to maintain during long-term experiments as contamination commonly occurs during subsampling.

The use of microtiter plates enables 1 ml culture volumes to be used for each replicate, with up to 48 separate treatments within a 12.65 x 8.5 x 2.2 cm plate, thereby decreasing the experimental volume and allowing for extensive replication without subsampling any treatment. Additionally, this technique can be modified to fit a variety of experimental formats including: bacterial-algal co-cultures, algal physiology tests, and toxin screening[9–11]. Individual wells with an alga, bacterium and/or co-cultures can be sampled for numerous laboratory procedures including, but not limited to: WATER-Pulse-Amplitude-Modulated (WATER-PAM) fluorometry, microscopy, bacterial colony forming unit (cfu) counts and flow cytometry. The combination of the microtiter plate format and WATER-PAM fluorometry allows for multiple rapid measurements of photochemical yield and other photochemical parameters with low variability between samples, high reproducibility and avoids the many pitfalls of subsampling a carboy or conical flask over the course of an experiment.

INTRODUCTION:
Phytoplankton physiology has traditionally been studied in meso-scale experiments ranging from 20 ml in conical flasks to 5 l in carboys[1–7]. This experimental scale requires subsampling for experimental monitoring, as sacrificing replicate samples for each time point creates an unmanageable experimental setup.

The ability to increase the number of independent experiments while using the same diurnal incubator space by miniaturizing the experimental volume for algal physiology experiments will reduce or eliminate the limitations of subsampling and pseudo-

replication from large volumes. A microtiter plate format has been developed for algal bioassays using a 1 ml culture volume for experimentally manipulating algae in variable conditions. This small experimental volume allows for the number of replicates to be increased, increases experimental reproducibility due to a decreased variability between replicate samples and experiments, and allows true replication while maintaining experimental controls (i.e. axenic algal cultures) for 140 days (Fig. 1)[12].

This microtiter plate format is easily adapted for a variety of experimental questions, such as: does a bacterium have a symbiotic, neutral or pathogenic interaction with its algal host? Is the addition of a compound stimulating or toxic to an alga? These and other questions can be addressed in a rapid high-throughput manner using this new format[9–11].

A 48 well microtiter culture plate allows each 1 ml well to be an independent experimental setup that is sampled at a single time-point. Various parameters can be sampled from this 1 ml volume including, but not limited to: chlorophyll fluorescence and photochemical parameters using WATER-Pulse-Amplitude-Modulated (WATER-PAM) fluorometry (see Materials and Equipment's table)[13]. WATER-PAM fluorometry is a rapid and non-invasive technique that can be used to monitor experiments performed with algae[13]. It allows measurement of photosynthetic efficiency and PSII health from a small culture volume (150-300 μl of culture diluted in medium to a 2-4 ml volume for WATER-PAM)[14,15]. In addition to WATER-PAM fluorometry, this setup can be used to measure a variety of other parameters including, but not limited to: microscopy to visualize the bacteria attached to algal cells and changes in the algal cell morphology; bacterial colony forming unit (cfu) counts; and flow cytometry for algal cell counts and identifying subpopulations.

[Insert Fig. 1 here]

PROTOCOL:

1. Calculations for experimental setup.

1.1) Calculate the volume of algal and/or bacterial cultures needed for controls that will be required for the entire experiment by using Equation 1:

$$V_{control} = (0.5 \text{ ml control culture/well}) \text{ X } (3 \text{ wells/control}) \text{ X } (y \text{ controls/d}) \text{ X } (z \text{ d})$$

Where y equals the number of controls needed per day and z equals the number of days.

1.2) Calculate the volume of algal and/or bacterial cultures that are needed for co-cultures for the experiment by using Equation 2:

$$V_{co\text{-}culture} = (0.5 \text{ ml bacterial and/or algal culture/well}) \text{ X } (3 \text{ wells/co-culture}) \text{ X } (y \text{ co-cultures/d}) \text{ X } (z \text{ d})$$

NOTE: It is possible to replace 'co-culture' experiments with any compound screen; just adjust the final compound, solvent and algal concentrations to fit the experimental design.

1.3) Use Equations 1 and 2 to calculate the final volume of mid-exponential algal culture (commonly 5 d for ~10^4 cells/ml but this should be determined by performing an algal growth curve) required for the experiment (this volume is needed for step: 2.3) using Equation 3:

V_A = volume of mid-exponential algal culture
= $V_{control} + V_{co\text{-}culture}$ + 10 ml

1.4) Use Equations 1 and 2 to calculate the final volume of 10^4 cfu/ml bacterial culture (or desired inoculation concentration) required for the experiment (this volume is needed for step: 4.2) using Equation 4:

V_B = volume of bacterial culture
= $V_{control} + V_{co\text{-}culture}$ + 10 ml

NOTE: Use the additional 10 ml in steps 1.3 and 1.4 to account for pipetting error and any tests that are performed (e.g.WATER-PAM, flow cytometry, microscopy, etc.) on 0 d. Increase this volume if needed to fit the experimental design.

NOTE: For an example calculation of an 8-day experiment see Part 10. See supplemental table for media recipes.

2. Growing algal cells for experimental setup.

2.1) Isolate or obtain an actively growing axenic algal culture.

2.2) Aseptically transfer 10% of the final volume of the algal culture into sterile algal medium (e.g. L1 or similar marine algal medium, see Material and Equipment's table), ensuring a 1:9 dilution of algal culture in fresh medium. Grow the diluted alga in a diurnal incubator using previously determined growth conditions for that strain (e.g. 18˚C with a 16:8 h light: dark cycle is commonly used).

2.3) When the culture has reached mid-exponential phase re-culture the alga in the same sterile algal medium (e.g. L1 or similar marine algal medium), ensure the final concentration is a 1:9 dilution and that the final volume of algae is equal to the V_A calculated (step 1.3).

NOTE: It is important to ensure these cultures are axenic. Test all algal media and algal stock bottles for contamination by plating a 20 μl aliquot onto a general marine bacterial medium (e.g. Marine Broth 2216 supplemented with 1.5% agar or similar).

2.4) Grow algal culture to mid-exponential phase (~10^4 cells/ml).

NOTE: Every algal strain has a unique growth curve depending on culturing conditions. Mid-exponential phase (~10^4 cells/ml) can be determined by performing an algal growth curve based on cell density (i.e. using flow cytometry or microscopy).

3. Preparing bacterial cells for inoculation.

3.1) Pre-determine the bacterial concentration (cfu/ml) and optical density (OD) of the bacterium at stationary phase by doing a growth curve in the chosen bacterial medium and growth conditions (e.g. Marine Broth 2216 at 25 ˚C and 160rpm, or similar). Use this information to grow the cells (step 3.3) and later to dilute the cells correctly in step 3.7.

3.2) Aseptically transfer an isolated and freshly grown bacterial colony from a 1.5% agar plate (e.g. Marine Broth 2216 supplemented with 1.5% agar or similar marine bacterial solid medium) into 5 ml of bacterial liquid medium (e.g. Marine Broth 2216 or similar marine bacterial medium).

3.3) Grow the bacterium to stationary phase on a rolling drum or shaker (~12 to 36 hrs, depending on the bacterium). Plan the experiment so that the bacterium reaches stationary phase (~10^8 -10^9 cfu/ml) at the same time that the algal cells have reached the mid-exponential phase (~10^4 cells/ml).

3.4) Using a pipette, wash down any biofilm attached to the test tube into the medium. Pipette 1 ml of well-mixed bacterial culture into a sterile 1.5 ml microtube. Centrifuge for 1 min at 14000 x g.

3.5) Remove and dispose of the supernatant (bacterial media) without disrupting the pellet (bacterial cells). Add 1 ml of sterile algal media (e.g. L1 or similar) to the microtube (with pellet). Vortex microtube to resuspend pellet in algal media.

3.6) Second wash: repeat step 3.5.

NOTE: It is critical to wash the bacterial cells with algal media in order to thoroughly remove all of the bacterial media, cell detritus, excreted proteins and small molecules from the cells prior to inoculating the algae with them as this could change the nutrient composition of the algal media or introduce bioactive molecules to the screen.

3.7) Serially dilute the washed bacterial cells in algal media, to a final concentration that is 100 fold more concentrated than the desired final bacterial concentration (cfu/ml). Save the microtube containing cells that have been washed and diluted in algal media for step 4.2.

NOTE: Plan the experiment based on having a 1:1 ratio of algae to bacteria on 0 d. To do this the desired initial bacterial concentration for the experiment is 10^4 cfu/ml, so in step 3.7 the initial cells should be serially diluted to 10^6 cfu/ml.

4. Preparing bacteria for experimental setup.

4.1) Prepare 4 sterile autoclaved glass conical flasks and label them: a) 'algal control flask', b) 'diluted bacterial stock flask', c) 'bacterial control flask', and d) 'co-culture flask'.

4.2) Dilute the bacterial suspension from step 3.7 1:99 with sterile algal media to a final volume = V_B (step 1.4). Make the dilution in the flask labeled diluted bacterial stock (step 4.1).

NOTE: In the previous example (step 4.2), this 1:99 dilution gives a final concentration of 10^4 cfu/ml. Swirl flask to mix cells.

4.3) Pipette $V_{control}$ (step 1.1) from the diluted bacterial stock and put it in the bacterial control flask.

4.4) Pipette $V_{control}$ of sterile algal medium into the bacterial control flask (this is a 1:1 dilution). Swirl flask to mix cells and set aside for step 7.3.

4.5) Pipette $V_{co\text{-}culture}$ from the diluted bacterial stock flask to the co-culture flask, set flask aside for step 6.1.

NOTE: When doing multiple co-cultures on the same alga at once (i.e. a co-culture of two different bacterial isolates with one control) it is necessary to re-calculate the $V_{co\text{-}culture}$ for each strain individually and then repeat step 4.5 for each co-culture separately. It is also necessary to increase the V_A calculated in step 1.3 to include both co-cultures shown in Equation 5:

$$V_A = V_{control} + V_{co\text{-}culture\ 1} + V_{co\text{-}culture\ 2} + 10\ ml$$

5. Preparing algae for experimental setup.

5.1) Gently mix the mid-exponential algal culture (from step 2.4) with a wide-mouth pipette tip until cells appear well mixed.

5.2) Pipette $V_{control}$ from the algal stock bottle to the algal control flask. Gently pipette using a 10 ml pipette. Then return the algal stock bottle in the diurnal incubator.

5.3) Pipette $V_{control}$ of sterile algal medium to the algal control flask (this is a 1:1 dilution). Swirl to mix flask and place in the diurnal incubator, until needed for step 7.4.

6. Preparing experimental co-culture.

6.1) Gently pipette $V_{co\text{-}culture}$ from the algal stock flask into the bacterial co-culture flask from step 4.5. Return co-culture flask to the diurnal incubator until needed for step 7.5.

NOTE: The concentration of bacteria in the bacterial control should equal the bacterial concentration in the experimental co-culture. Similarly, the concentration of algae in the algal control should equal the concentration of algae in the experimental co-culture. By having the same initial bacterial and algal concentrations, the population density can be compared throughout the experiment.

7. Setting up microtiter plates.

7.1) Divide a sterile 48-well microtiter plate as per Fig. 2. Label above outer wells containing either sterile diluent or non-photosynthetic samples.

[Place Fig. 2 here]

NOTE: Randomization of the plate wells can be done for samples taken on the same day. For example, quadrant 1 will be sampled at 1 d in the experiment; the wells that should be randomized are B2-4 and C2-4. Leave the perimeter of the plate filled with sterile solution as shown in Fig. 2.

7.2) Pipette 1 ml 1X phosphate buffer solution (PBS) (pH 7.4) or other sterile solution in the appropriate wells as indicated in Fig. 2. Perform this slowly and with care. PBS will change the ionic strength of the algal and/or co-culture medium if it splatters in other wells so handle carefully.

7.3) Pipette 1 ml of bacterial control culture into wells labeled bacterial control (Fig. 2). Swirl the bacterial flask before pipetting each plate to avoid bacterial settling.

7.4) Pipette 1 ml of algal control culture into wells labeled algal control using a wide mouth pipette tip (Fig. 2). Swirl the algal flask as regularly as the bacterial flask. If the alga tends to sink or float, swirl as regularly as is needed to maintain a visually uniform culture.

7.5) Using a wide mouth pipette tip, pipette 1 ml of co-culture in the appropriate wells (Fig. 2). Swirling the co-culture flask as regularly as the algal flask.

7.6) Seal each plate with parafilm and place in a diurnal incubator at the desired temperature and diurnal light cycle (18˚C with a 16:8 hr light: dark cycle is commonly used). Leave the plates in the diurnal incubator until ready to take PAM readings (Part 8). Ensure all plates are oriented in the same direction to allow for consistent light exposure.

7.7) Take a 20 µl aliquot from the PBS, algal media, algal stock and algal control and drop plate onto an appropriate non-selective medium (e.g. Marine Broth 2216 supplemented with 1.5% agar) and incubate plates at 18-25 ˚C for 72 hrs to test for contamination. If growth appears in any of the solutions that should not contain bacteria, then these data should not be used.

7.8) Take PAM fluorometry readings using the remaining sample from the algal control and co-culture flasks for the experimental 0 d PAM fluorometry reading (see step 8.1). Any other 0 d measurements should also be performed with the remaining algal control, bacterial control and co-culture samples.

8. Taking PAM fluorometry readings from stock samples.

8.1) Zero the WATER-PAM with sterile algal media in a clean cuvette before taking readings[8].

8.2) Pipette 300 µl from algal control or co-culture flasks into a clean cuvette containing 2.7 ml of the same algal medium used in the experiment. Mix the sample and diluent gently with a wide mouth pipette tip.

8.3) Wipe-off all fingerprints from outside of cuvette with a tissue (Table 1) before placing the cuvette in the WATER-PAM.

8.4) Place cuvette into WATER-PAM. Cover the sample with the cap and allow it to dark-adapt for 3 min. Dark adaptation times vary depending on the species of algae and must be determined for the specific alga used in the experiment. Avoid lengthy dark adaptation times (>20 min) by taking WATER-PAM readings during the middle of the dark cycle of the algal diurnal incubators[16,17].

8.5) After dark adaption, hit F_0 button. If fluorescence readings are above 3900, dilute sample 1:1 in algal medium. Dark-adapt for an additional 3 min and take a new reading. If the F_0 or F_m readings are still above 3900, continue to dilute the sample 1:1 in algal medium until the F_0 and F_m readings are below 3900.

NOTE: Make sure to account for these dilutions when recording final fluorescence: for instance if the algal sample is diluted 1:9 during the initial transfer from the well to the dilution tube, then the algal fluorescence reading of 500 should be multiplied by the inverse of the dilution factor (in this case 10) and the actual fluorescence of the tube is then 5000.

8.6) After setting F_0, take a saturating pulse (SAT-Pulse) reading every 1 min 30 s by hitting the SAT button to take F_m readings. The time interval between readings may be adjusted depending on algal strain. Discard sample.

8.7) Repeat steps 8.1-8.6 for the remaining samples.

9. Taking PAM fluorometry readings from microtiter plates.

9.1) Label 6 sterile sample tubes of >3 ml volume for wells B2-4 for the algal control and wells C2-4 for the bacterial co-culture (see plate layout in Fig. 2).

9.2) Aliquot 2.7 ml of sterile algal medium into each tube.

9.3) Place tubes in diurnal incubator and allow them to acclimate to the temperature the alga was grown at for 30 min.

9.4) Before removing the microtiter plate from the incubator ensure that light penetration into the dark acclimated wells is limited by covering the plate with aluminum foil (or similar), only remove the foil while actively transferring culture from the wells to the dilution tubes (step 9.5-9.7).

9.5) Aseptically mix the first microtiter plate well (well B2) with a wide mouth pipette tip by slowly pipetting up and down.

9.6) Obtain the dilution tubes from the incubator and aseptically transfer 300 µl from well B2 (Fig. 2) to its corresponding sample tube with a wide mouth pipette tip (this is a 1:9 dilution of the sample in algal medium).

9.7) Repeat steps 9.5-9.6 for the remaining wells (B3-4 and C2-4).

9.8) Cover sample tubes with aluminum foil and return them to the diurnal incubator until ready for WATER-PAM.

9.9) Perform WATER-PAM readings as described in steps 8.1 to 8.7. Seal the microtiter plate with parafilm before returning it to the incubator.

9.10) Repeat readings at planned time intervals for the duration of the experiment. The frequency and length of sampling should be planned at the beginning of the experiment.

10. Sample experiment.

The sample experiment is a 10-day co-culture of a bacterium (*Phaeobacter gallaeciensis* BS107) and a microalga (*Emiliania huxleyi* strain (CCMP3266)). It includes an algal control, bacterial control, and a bacterial-algal experimental co-culture.

10.1) Calculate volumes of bacterial and algal stocks required (step 1.1-1.4).

$V_{control}$ = (0.5 ml algae/well) x (3 wells/day) x (1 control) x (10 d)
= 15 ml bacterial or algal culture for control

$V_{co\text{-}culture}$ = (0.5 ml 10^4 cfu/ml bacteria/well) x (3 wells/d) x (1 experiment) x (10 d)
= 15 ml bacterial or algal culture for co-culture

V_A = volume of mid-exponential algal culture (~10^4 cells/ml)
= $V_{control}$ + $V_{co\text{-}culture}$ + 10 ml
= 15ml + 15 ml + 10 ml = 40 ml

V_B = volume of stationary phase bacterial culture (diluted to 10^4 cfu/ml)
= $V_{control}$ + $V_{co\text{-}culture}$ + 10 ml = 40 ml

10.2) Prepare the algal stock, a V_A of 40 ml, by inoculating 4 ml of alga in 36 ml medium and incubated until mid-exponential growth is achieved with a cell density of ~10^4 cells/ml (step 2.1-2.4).

10.3) Prepare the bacterial stock flask by diluting 400 µl of the 10^6 cfu/ml bacteria obtained in Part 3 to a V_B of 40 ml with algal media (final concentration of bacteria will be 10^4 cfu/ml).

10.4) Transfer half (20 ml) of the bacterial stock to the bacterial control flask and add 20 ml of algal media to make the bacterial control. Transfer half (20 ml) the algal stock to the algal control flask and add 20 ml of algal media to make up the algal control (Part 5). Transfer the remaining 20 ml of bacterial stock to the co-culture flask and mix with the remaining 20 ml of algal stock to establish the co-culture (Part 6).

10.5) Pipette the 1X PBS (pH 7.4), controls and co-culture in two pre-labeled microtiter plates (Fig. 2), 1 ml per well. Use 3 ml of the remaining controls and co-cultures to make 0 d measurements (cfu counts, WATER-PAM (Part 8), microscopic observation of algal cell morphology and algal cell fixation for flow cytometry).

10.6) Take WATER-PAM readings for the algal control and co-culture once a day for 10 d, and always at the same time as the 0 d measurements were taken (Part 8).

11. Other parameters of interest.

11.1) Determine bacterial cfu concentration: perform a serial dilution of the bacterial control and co-culture in sterile 1x PBS (or similar), then drop plate onto Marine Broth 2216 supplemented with 1.5% agar (or similar) and observe the number of cfu that grow to determine the bacterial cfu/ml for each well[18].

11.2) To evaluate the algal cell concentration: fix algal control and co-culture with a 0.15% final concentration of glutaraldehyde. Incubate for 10 min in the dark, then flash freeze in liquid nitrogen, and store at -80 C. Process all samples on a flow cytometer (FACS Calibur or similar) to count algal cells.

11.3) Observe algal cell morphology: algal cultures and bacterial-algal co-culture using microscopy (i.e. light microscopy, epifluorescence or similar).

REPRESENTATIVE RESULTS:

WATER-PAM fluorometry readings.

WATER-Pulse-Amplitude-Modulated (PAM) fluorometry is a quick and efficient method to determine the fluorescence (a proxy for chlorophyll content) and photosynthetic yield (PSII health) of algal cultures. The PAM WinControl software generates a spreadsheet of raw data values for (the following are the basic parameters for dark adapted algal samples):

F_0 = fluorescence of dark-adapted cells
F_m = maximum fluorescence after saturating light-emitting diodes (LED) pulse
F_v/F_m = $(F_m-F_0)/F_m$ = potential quantum yield of a dark adapted sample[19]

PAM fluorometry has many uses and can give a lot of information about the photosystem and health of the algae. In addition, there are other parameters that are important when testing light adapted samples. For further reading these are discussed in detail in these reviews[13,16,20–23]. These data can be transferred to a spreadsheet or graphing software to generate graphs of the initial algal fluorescence (F_0), the maximum algal fluorescence (F_m), and the potential quantum yield (F_v/F_m) (Fig. 1 and 3). The graphs in Fig. 3 depict how the algal fluorescence and F_v/F_m are influenced by the bacterium by comparing the alga grown alone (control) to it being grown with the bacterium in co-culture throughout the 10-day co-culture experiment (Fig. 3C). In this example, a two-sample t-test was used to compare the parameters statistically between the two treatments on each day. Similarly, for experiments in which more than two treatments are present, an ANOVA could be used. The F_m reading (Fig. 3B) is taken directly after the saturating LED pulse, which means the primary PSII electron acceptor QA is fully reduced and cannot accept any more electrons from the PSII reaction centre P680, and as such, all reaction centres are 'closed'[17]. This hinders the photochemical use of light energy, bringing fluorescence emission to a maximum. This then gives the maximum fluorescence (F_m) reading. Fig. 3C illustrates a dramatic decline in PSII health between 5 d and 10 d when co-cultured with the bacterium compared to growing alone (control). The standard error bars are derived from triplicate microtiter wells, which are independent experiments from the same parental bacterial and algal cultures. The consistently small standard error confirms the robustness and reproducibility of the microtiter plate format for algal bioassays as it allows independent experiments to be used as replicates and eliminates the need to subsample the experimental unit over the duration of the experiment.

[Alternant placement for Fig. 1 here]

[Insert Fig. 3 here]

FIGURE LEGENDS:

Figure 1: Representative WATER-PAM fluorometry graphs of a 140 d growth curve of axenic *Emiliania huxleyi* (CCMP3266).

Readings for the initial algal fluorescence (F_0) (A), maximum algal fluorescence (F_m) (B) and potential quantum yield (F_v/F_m) (C) for *E. huxleyi* are shown as black circles. The line of best fit for F_0 and F_m was normal log 3 parameter (SigmaPlot) R^2=0.94, 0.95 respectively. Potential quantum yield (F_v/F_m) is a dimensionless expression of photosynthetic health, which is calculated as $(F_m-F_0)/F_m$. The line of best fit for the F_v/F_m curve was a 3 factor polynomial R^2= 0.6. Error bars represent the standard error between triplicate wells.

Figure 2: Schematic representation of sample placement in a 48-wells microtiter plate.

Wells are to be filled as follows: columns 1 and 6, wells A through F (red) are filled with 1

ml 1X PBS (or other sterile solution). Rows A and F, wells 2-8 (blue) are filled with 1 ml bacterial control; rows B and E wells 2-8 (green) are filled with 1 ml algal control; rows C and D, wells 2-8 (purple) are filled with 1 ml co-culture. The plate is divided into 4 quadrants (A2, A5, D2, and D5). These quadrants are each for different and sequential sampling time points (e.g. 1-4 d). Within each quadrant samples can be randomized using a random number generator. Labeling is on the lid over 1X PBS and/or bacterial control wells to prevent shading of algal cultures.

Figure 3: Representative WATER-PAM fluorometry graphs of a 10 d co-culturing experiment of *Emiliania huxleyi* (CCMP3266) with *Phaeobacter gallaeciensis* BS107.
The initial algal fluorescence (F_0) (A), maximum algal fluorescence (F_m) (B), and potential quantum yield (F_v/F_m) (C) are graphed for control algal (open circles) and alga co-cultured with a bacterium (black circles). Potential quantum yield (F_v/F_m) is a dimensionless expression of photosynthetic health, which is calculated as $(F_m-F_0)/F_m$. Error bars represent the standard error between triplicate wells. An asterisk (*) denotes days on which the parameters for the control and co-culture treatments differed significantly. Differences between treatments for all days were non-significant except for 10 d in all three parameters (10 d – F_0: t-test, df=2.5, t-ratio=-15, p=0.0017*; F_m: t-test, df=2.1, t-ratio=-16.15, p=0.003*; F_v/F_m: t-test, df=-18.68, t-ratio=2.0, p=0.0028*).

Figure 4: WinControl display of several WATER-PAM fluorometry readings of an algal sample being sequentially diluted to achieve the correct dilution.
This figure displays algal fluorescence readings reaching the upper limit of the WATER-PAM machine (red box) and those where a suitable dilution of the sample has been achieved (green box). In addition, this figure depicts some of the other variables that this method calculates, but these are not discussed in detail here (see reviews[13,16,20–22]).

DISCUSSION:

Algal growth in a miniaturized format.
The miniaturization of algal cultures to a 1 ml culture volume in a microtiter plate allows for the replication within an experiment to be increased. It is important to ensure the alga is healthy throughout an experiment; perform a growth curve (Fig. 1), using the microtiter plate format to assess various algal media, to ensure the nutritional requirements of the alga are met. Additionally, it may be important to optimize the diurnal cycle (light and dark periods) and temperature. Proper optimization for a given alga can allow for maintaining healthy algal cultures at peak fluorescence for 26 d and for detecting potential quantum yield after 140 days (Fig. 1).

Minimizing evaporative effects.
It is important to minimize the evaporative effects of liquid based assays as an ‘edge effect’ is commonly observed where there is greater evaporation in wells at the edge of the microtiter plate than in wells located towards the middle of the plate. While evaporation has been observed at the edge of plates, the rate of evaporation does not limit the experimental duration as healthy algal cultures, with peak fluorescence at 26 d has been maintained in this format (Fig. 1). To minimize any potential ‘edge effect’ 1X

PBS (pH 7.4), or other sterile solution, is aliquoted into wells along all four edges (columns A and H, rows 1 and 6, Fig. 2).

Lighting within a diurnal incubator.
Microtiter plates should all have the same orientation in the diurnal incubator. For instance, lengthwise orientation of microtiter plates in an incubator means that the plates are placed along the short axis. If this orientation is used, algal cultures along the long axis B-G (Fig. 2) can experience a slight light and temperature gradient due to the variation in distance from the light source (the light bulb is a source of both light and heat). This has been observed to influence temperature assays on algae at the extreme of their temperature range. Consequently this is unlikely to affect most algae grown at their optimum temperature. To minimize the impact of this light and temperature gradient ensure that larger bottles are not creating shading, place plates at a consistent distance from the lights, use shade cloth to reduce the light level if necessary and check the light intensity across the long axis of the diurnal incubator to ensure that it travels evenly.

Dark adaptation of algal samples for WATER-PAM fluorometry.
Before conducting WATER-PAM readings it is important to dark-adapt the algal samples so that the PSII reaction centers are fully open and the light-induced transthylakoidal pH gradient is fully dissipated, thus giving true F_o and F_m values from which to calculate F_v/F_m. Sampling the alga from the assay for WATER-PAM measurements in the middle of the dark phase of the diurnal cycle (i.e. for a 16:8 hr light: dark cycle, a two hour sampling session would be performed from T(dark)=3-5 hr) makes dark adaptation time shorter (3-5 min) compared with the middle of the light cycle (T(light)=7-9 hr) when dark adaptation is longer (>20 min)[17]. The alga's dark adaption time will also vary depending on the algal species, growth conditions and the light conditions of its natural habitat range.

Sensitivity of WATER-PAM fluorometry readings.
The WATER-PAM was designed to be an ultrasensitive fluorometer capable of detecting F, F_0 and F_m from low chlorophyll samples such as the ocean's surface water[16]. Consequently it is ideally suited to a miniaturized bioassay where samples can be diluted. Due to this sensitivity it is important to understand that the WATER-PAM machine has an upper limit of fluorescence readings (i.e. F, F_0 and/or F_m) if the sample is not sufficiently diluted (Fig. 4). Within the WinControl software the maximum values are first noticeable after pressing F_0 and reading the F_0 (directly after dark adaptation). Consequently the F and F_m are at the maximum value 4056 (Fig. 4, no. 2286). The maximum value of F and F_m depends on the type of algal medium that is used to calibrate the WATER-PAM, but samples above the detection limit can be readily identified because repeated readings with the same maximum value occur until the sample is sufficiently diluted. After a 1:1 dilution in algal media, the less concentrated sample is dark-adapted again and the F_0 measurement was repeated. The F value appears to be measured correctly, but the F_m is still reading the maximum value 4056 (Fig. 4, no. 2287). This sample was again diluted 1:1 in algal media again and dark adapted for an additional 3 min before taking another F_0 reading (Fig. 4, no. 2288) and valid readings were obtained, so the next measurement (F) is taken and the F_v/F_m

calculation is valid. In this example, the sample was diluted twice in a 1:1 ratio with medium, which needs to be factored into the calculation of F_m and F_0. It is important to note that F_v/F_m, as a direct function of F_0 and F_m, is incorrectly calculated if the samples are too concentrated despite being a dimensionless expression of photosynthetic health.

[Insert Fig. 4 here]

Bacterial contamination.
In all algal experiments, algal controls are necessary as a baseline of algal health, so it is imperative that it remains free of bacterial contamination throughout the experiment. Bacterial contamination occurs easily as there is no selection (such as antibiotics) and photosynthesis constantly produces new organic carbon for bacterial growth. The two most important ways to avoid contamination is to ensure sterility of all solutions (e.g. algal media, 1X PBS, pH 7.4) and equipment at T=0 d of the experiment and to maintain aseptic technique while handling the microtiter plates. The experiment should be monitored for contamination at each time point by plating a 20 μl aliquot from all algal control wells. If contamination is observed from any of the algal control wells then those WATER-PAM fluorometry readings should be noted and excluded from data analysis. If a solution used to set up the experiment causes the entire experiment to be contaminated, then discard the experiment and all contaminated reagents and start again. The bacterial controls and bacterial-algal co-cultures can also become contaminated and agar plates used for bacterial cfu counts should be monitored for alternate colony morphologies. Well-to-well cross contamination can occur when removing the microtiter plate lid, but is easily avoided by not tilting or shaking of the plates and employing aseptic technique in a laminar flow hood or near a flame.

Future Applications.
This small volume bioassay provides a rapid screening method for microalgae by combining a microtiter plate format with WATER-PAM fluorometry. Examples of future applications are various, and could include Imaging PAM fluorometry, which provides insight into cell-cell variation of PSII health within a population as it performs PAM fluorometry on individual cells[24]. The bioassay can also be combined with microscopy and flow cytometry as previously discussed. Another combination with the potential to provide further insight is cell staining for flow cytometry and microscopy to elucidate morphological variation within subpopulations of the algal culture.

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DISCLOSURES:
The authors have nothing to disclose.

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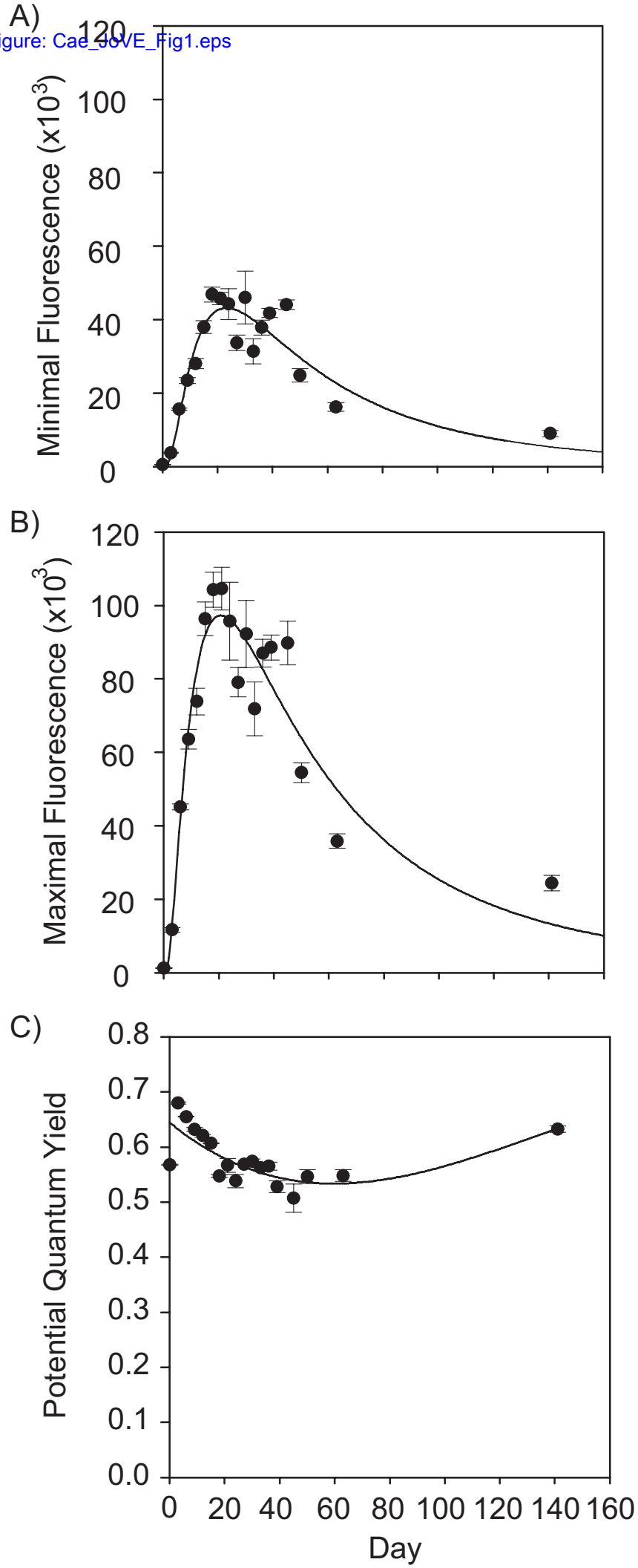

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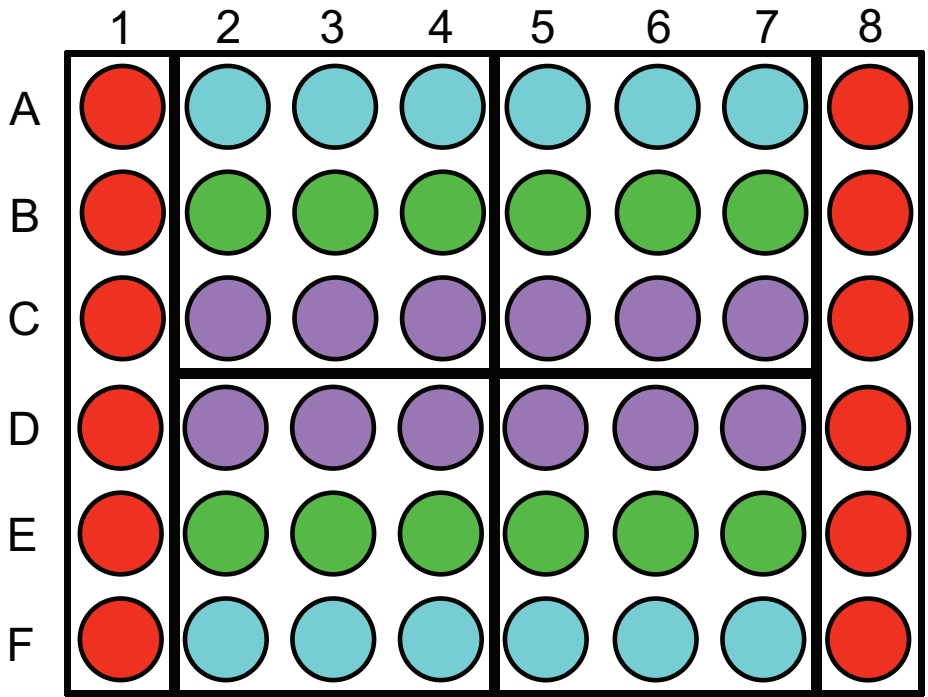

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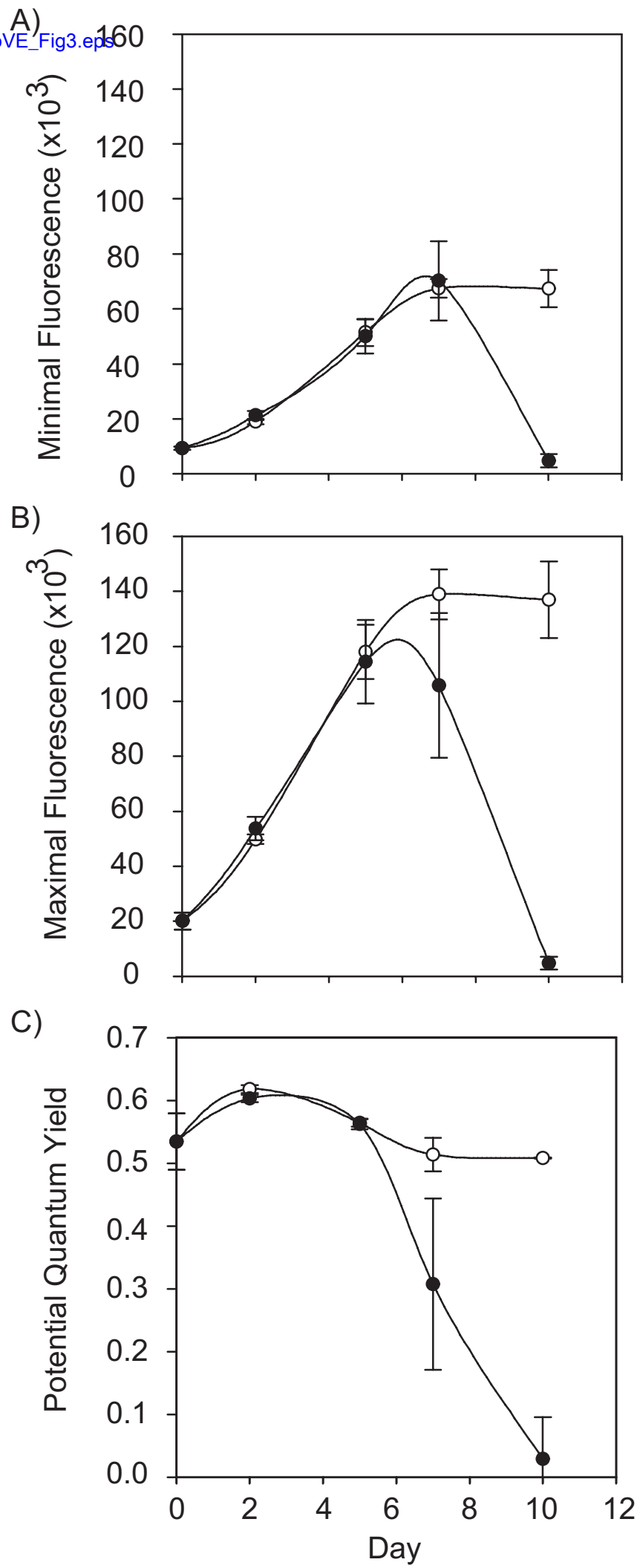

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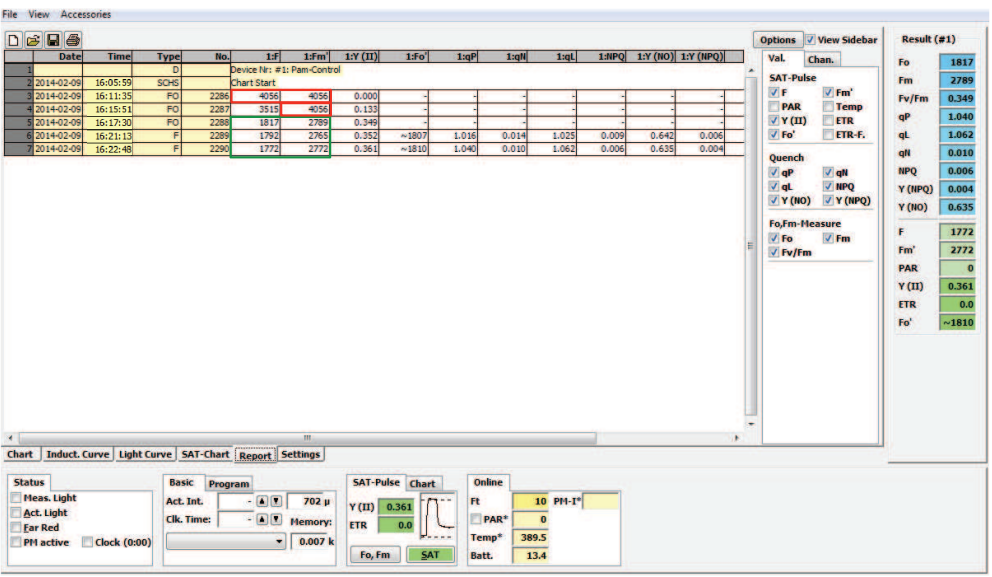

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Nunc EasYFlask 25cm^2, Vent/Close Cap, 7mL working volume, 200	Thermo Fisher Scientific
Multiwell TC Plates – 48 Well	BD Biosciences Discovery Labware
P1000 Gilson The Pipetting Standard—Gilson's Pipetman	Mandel Scientific Company Inc.
P10mL Gilson The Pipetting Standard—Gilson's Pipetman	Mandel Scientific Company Inc.
Wide Orifice Tips nonsterile [100–1250 µL]	VWR International
Ultrafine Tips nonsterile [100–1250 µL]	VWR International
Finntip 10mL [Vol: 1-10mL]	Thermo Fisher Scientific
WATER-Pulse Amplitude Modulation (Water-ED)	Heinz Walz GmbH, Effeltrich, Germany
15 mm diameter quartz glass cuvette (WATER-K)	Caron Corporate
Sodium Chloride (Crystalline/Certified ACS), Fisher Chemical	Thermo Fisher Scientific
BD Difco Marine Broth 2216	BD Biosciences Discovery Labware
BD Bacto Agar	BD Biosciences Discovery Labware
L1 Medium Kit, 50L	NCMA [National Center for Marine Algae and Microbic

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Article Title: A small volume bioassay to assess bacterial-phytoplankton co-culture using WATER-PULSE-amplitude-modulated (WATER-PAM) fluorometry

Signature: R Case

Date: 27 June 2014

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Editorial comments *(in italics)***:**

1.Step 10.1 requires correction to the grammatical tense.
Corrected grammar to: "a 10-day co-culture of a bacterium and a microalga".

2.Step 10.6 Should be expanded into multiple steps giving step-wise instruction.
The text has been expanded as follows:

11. Other parameters of interest (this list is not exhaustive).

11.1) Bacterial cfu concentration: perform a serial dilution of the bacterial control and co-culture in sterile 1x PBS (or similar), then drop plate onto Marine Broth 2216 supplemented with 1.5% agar (or similar) and observe the number of cfu that grow to determine the bacterial cfu/ml for each well[18].

11.2) Algal cell concentration: Fix algal control and co-culture with a 0.15% final concentration of glutaraldehyde. Incubate for 10 min in the dark, then flash freeze in liquid nitrogen, and store at -80 C. Process all samples on a flow cytometer (FACS Calibur or similar) to count algal cells.

11.3) Algal cell morphology: observe algal cultures and bacterial-algal co-culture using microscopy (i.e. light microscopy, epifluorescence or similar).

3.Please discuss Future Applications in the Discussion section.
The text has been added as follows:

Future Applications.
This small volume bioassay provides a rapid screening method for microalgae by combining a microtiter plate format with WATER-PAM fluorometry. Examples of future applications are various, and would include Imaging PAM fluorometry, which provides insight into cell-cell variation of PSII health within a population as it performs PAM fluorometry on individual cells. The bioassay can also be combined with microscopy and flow cytometry as previously discussed. Another combination with the potential to provide further insight is cell staining for flow cytometry and microscopy to elucidate morphological variation within subpopulations of the algal culture.

4. Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.
The text has been proofread.

5. If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or

"Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.
All figures in this manuscript were created for this manuscript and have not been published before.

Reviewers' comments:

Reviewer #1:
Major:
1) Another form of potential pseudo-replication that is perhaps less obvious is the non-random assignment of samples in the microtiter plate and again in the incubator. The authors do acknowledge that the way the plates are aligned in the incubator may cause variation in each wells exposure to the light (line 486). Likewise it is a common (yet rarely cited) problem that evaporation from wells in a microtiter plate is non-uniform (as mentioned on line 475). Thus if the samples are placed as suggested in figure 1 the replicates in contrast to what is stated on line 435 are not independent (i.e. you have pseudo-replication only at a smaller scale). I strongly suggest that the authors add in step that highlights the importance of randomly assigning samples to the various wells (this could easily be done using a random number generator system).
We agree with the reviewer here, and have amended the figure legend and text to suggest randomization of wells within a single time point. Randomization of samples across the plate and between plates is problematic as it would create a large risk of bacterial contamination by repeatedly sampling within a plate. This would also create an edge effect in all the wells surrounding the sampled well, and therefore we only suggest random subsampling within a quadrat that represents a single sampling time point. For larger experiments (for instance when half the plate or the whole plate is used for one sampling day) then we randomization would be across half or the entire plate.

The text has been edited as follows:
Line 286 (protocol)
NOTE: Randomization of the plate wells can be done for samples taken on the same day. For example, quadrant 1 will be sampled at 1 d in the experiment; the wells that should be randomized are B2-4 and C2-4. Leave the perimeter of the plate filled with sterile solution as shown in Fig. 2.

Line 509 (Fig. 2)
Within each quadrant samples can be randomized using a random number generator. Labeling is on the lid over 1X PBS and/or bacterial control wells to prevent shading of algal cultures.

2) Importantly, the authors also make no mention of statistical tests (used in the example or recommended) to determine if the test and control samples are indeed statistically different (above background variation) and with what confidence. Please add this in, providing the reader with a suggestion of the most

appropriate method (i.e. T-Test, ANOVA etc etc). Whist this may be different for the different applications and experimental design used, at the very least this should be done for the example experiment with WATER-PAM.
Statistical tests have been added in a two-way Student T-Test and preformed that on the example experiment (part 10). We have also stated that other statistical testes such as the one-way T-Test or ANOVA could also be preformed.

This text has been added:
Line 472 (results)
In this example, a two-sample t-test was used to compare the parameters statistically between the two treatments on each day. Similarly, for experiments in which more than two treatments are present, an ANOVA could be used.

Line 519 (Fig. 3)
An asterisk (*) denotes days on which the parameters for the control and co-culture treatments differed significantly. Differences between treatments for all days were non-significant except for 10 d in all three parameters (10 d – F_0: t-test, df=2.5, t-ratio=-15, p=0.0017*; F_m: t-test, df=2.1, t-ratio=-16.15, p=0.003*; F_v/F_m: t-test, df=-18.68, t-ratio=2.0, p=0.0028*).

3) Line 90 and again line 480, there is a mention of unpublished data by the authors on the ability of microalgae to be maintained in the microtiter format. Since this appears to be a very important part in determining the feasibility of this experimental procedure, I strongly recommend adding in the data as supporting material.
This data has been added as an additional figure (Fig. 1).

We appreciate your comment here and have added the figure from (Bramucci, A. & Mayers, T., unpublished data) and made Mayers an author on this manuscript. The additional figure is explained in the text.

The other manuscript (Labeeuw, L. and Bramucci, A., unpublished data) is currently under revision and therefore cannot be publish. We have removed the citation.

Minor:
4) In the abstract (line 56) I am surprised by the suggestion that the pseudo-replication in the form that replicates are taken from the same flask is standardly being performed. Do the authors have evidence that this is standard practice in this field and the reasons behind this? If so this would strengthen the benefit of the method proposed.
Yes we added a reference specifically for that, and in the sentence before we state that in larger experiments sub-sampling for replicates is done and those citations are already present as well.

5) The term "maxing-out" is used both on line 459 and 512. Please re-phrase to avoid the use of jargon.
It has been rephrased:
Line 526
This figure displays algal fluorescence readings reaching the upper limit of the WATER-PAM machine (red box) and those where a suitable dilution of the sample has been achieved (green box).

Line 582
Due to this sensitivity it is important to understand that the WATER-PAM machine has an upper limit of fluorescence readings (i.e. F, F_0 and/or F_m) if the sample is not sufficiently diluted (Fig. 4).

6) I recommend that the authors re-phrase the last paragraph in the introduction. Details on WATER-PAM (line 106-109) should be moved up to directly after it is first mentioned (line 103). Then a new sentence can be started to mention the other types of measurements possible. E.g. "In addition to WATER-PAM this set-up can be used for measuring xxxxxx"
It has been rephrased:
Line 113
In addition to WATER-PAM fluorometry, this setup can be used to measure a variety of other parameters including, but not limited to: microscopy to visualize the bacteria attached to algal cells and changes in the algal cell morphology; bacterial colony forming unit (cfu) counts; and flow cytometry for algal cell counts and identifying subpopulations.

7) A recommendation of the "desired" concentration of bacteria would be useful to provide as a starting point (line 204).
This text has been edited to:
NOTE: The authors recommend planning the experiment based on having a 1:1 ratio of algae to bacteria on 0 d. To do this the desired initial bacterial concentration for the experiment is 10^4 cfu/ml, so in step 3.7 the initial cells should be serially diluted to 10^6 cfu/ml.

8) step 7.7 and 7.8 it is not clear where the time 0 readings are taken from. I assume it is from the various flasks used to inoculate the microtiter wells. If so how does one deal with replicates? Line 297- what are the "remaining" algal control etc samples?
The time 0 reading is taken from the various flasks and therefore there is not the replication that subsequent time points have. The reason this is done is because there is no change in reading between sampling the flask and sampling wells immediately after they are aliquoted. So the additional time required to set up a time 0 in wells and then read these wells is not worthwhile as taking the measurements is time consuming and unnecessarily compromises the experimental setup.

9) Step 8.5 describes the use of a particular WATER-PAM model, will these buttons vary depending on the model used? Maybe this should be mentioned here, or described in a way that is universal.
Other models of the PAM have not been tested with this experimental setup and many PAM machines are not sensitive enough to be used with this bioassay. Therefore we think describing PAM universally for this protocol would be misleading. We anticipate the method would work well in other sensitive PAM machines, such as Imaging PAM fluorometry and have added these thoughts into our Future applications section.

Line 624
Examples of future applications are various, and would include Imaging PAM fluorometry, which provides insight into cell-cell variation of PSII health within a population as it performs PAM fluorometry on individual cells.

10) Step 9.4 - 9.10 mentions that plates need to be covered in foil, at what stage is this occurring? I assume it is whilst taking the sample only and then the foil is removed to continue incubation for the remaining time points? Please clarify the "until ready for use" and when the foil should be removed.
Revised as follows:
Line 372
9.4) Before removing the microtiter plate from the incubator ensure that light penetration into the dark acclimated wells is limited by covering the plate with aluminum foil (or similar), only remove the foil when activity transferring culture from the wells to the dilution tubes (step 9.5-9.7).

11) Line 362, Please indicate what algae and what bacteria are being used here.
The organisms names have been added:
Line 397
10.1) The sample experiment is a 10-day co-culture of a bacterium (*Phaeobacter gallaeciensis BS107)* and a microalga (*Emiliania huxleyi* strain (CCMP3266)).

12) Line 432. The statement that the results demonstrate the bacteria is pathogenic do not seem well justified here. What if any bacterium would have a similar effect, just because it is in competition with the algae? I suggest either removing this statement of qualifying it with an indication of what other ways the data could be interpreted and a suggestion of the additional controls that could be in place.
This statement of results has been removed from the text.

Reviewer #3:
Line 77: instead of "…photosynthetic yield and chlorophyll fluorescence…" please write "…photochemical yield and other photochemical parameters…"
The text has been change to:
Line 77

The combination of the microtiter plate format and WATER-PAM fluorometry allows for multiple rapid measurements of photochemical yield and other photochemical parameters with low variability between samples, high reproducibility and avoids the many pitfalls of subsampling a carboy or conical flask over the course of an experiment.

Line 101-1012: instead of "…chlorophyll fluorescence and photosynthetic yield…" please write "…photochemical parameters…"
The text has been change to:
Line 106:
Various parameters can be sampled from this 1 ml volume including, but not limited to: chlorophyll fluorescence and photochemical parameters using WATER-Pulse-Amplitude-Modulated (WATER-PAM) fluorometry (see Materials and Equipment's table)[13].

Line 318: instead of "If either the F or Fm readings are above…" please write "If fluorescence readings are above…"
The text has been change to:
Line 344
If fluorescence readings are above 3900, dilute sample 1:1 in algal medium.

Line 322: which calculations? Please, clarify.
The following note has been added:
Line 349
NOTE: Make sure to account for these dilutions when recording final fluorescence, for instance if the algal sample is diluted 1:9 during the initial transfer from the well to the dilution tube, then the algal fluorescence reading of 500 should be multiplied by the inverse of the dilution factor (in this case 10) and the actual fluorescence of the tube is then 5000.

Line 324: Why did you opted for manual measurements? It would be easy to automate the process using the PAM software Wincontrol.
We do have one of the readings automated.

Line 325: is the actinic light on? At which light irradiance? If the actinic light is on, instead of "…take F readings." Please write "…take F´m readings." (if the actinic light is off, instead of F'm please write Fm).
We are using dark adapted samples so the actinic light is off, we have read through the manuscript and adjusted it as you suggest.

The following note has also been added:
Line 342
NOTE: the actinic light is off while taking readings.

Line 392: The algae in co-culture are in a medium with 50% bacterial medium,

whereas the algal control is 100% algal medium; this seems to be a problem; how do account for this difference in algae growth rate?
The authors respectfully think they have accounted for this in the text. In steps 3.5-3.6 we describe washing the bacteria cells with sterile algal media twice, then in step 3.7 we describe serially diluting the bacterial cells in sterile algal media. This removes the bacterial media and secreted biomolecules from the bacterial cells and replaced the bacterial media with sterile algal media prior to them being mixed with the algae.

However, to avoid confusion we have added this note:
Line 213
NOTE: It is critical to wash the bacterial cells with algal media in order to thoroughly remove all of the bacterial media, cell detritus and excreted proteins and small molecules from the cells prior to inoculating the algae with them as this could change the nutrient composition of the algal media or introduce bioactive molecules to the screen.

Line 394: why did you use PBS? Why not just water?
The text has been change to:
Line 291
7.2) Pipette 1 ml 1X phosphate buffer solution (PBS) (pH 7.4) or other sterile solution in the appropriate wells as indicated in Fig. 2.

Line 416: Are Ft and F synonymous, as well as background and actinic light? You should use only one notation, I suggest "F" and "actinic light"
This has been changed to reflect parameters where the actinic light is off.

Line 417: The WATER-PAM does not use lasers; please substitute "laser" by "light-emitting diodes (LED)".
The text has been change to:
Line 461
F_m = maximum fluorescence after saturating light-emitting diodes (LED) pulse

Line 418: The equation is not correct: the correct equation for the effective quantum yield is:
$Y = (F'm-F)/F´m$
Thank you, we have changed the formulas and added this source to the manuscript.
Line 462
$F_v/F_m = (F_m-F_0)/F_m$ = potential quantum yield of a dark adapted sample[19]

Line 420: Please add "fluorometry" after PAM.
This change has been made at line 420 and elsewhere in the manuscript where needed.

Line 423: Please substitute "photosynthetic yield" by "effective photochemical

yield" (I assume the actinic light is on: this is an important missing information that must be added to the MS: when is the actinic light shut on and what is its intensity).
Line 426-430: Please substitute:

The text has been change to:
Line 475
The F_m reading (Fig. 3B) is taken directly after the saturating LED pulse, which means the primary PSII electron acceptor QA is fully reduced and cannot accept any more electrons from the PSII reaction centre P680, and as such, all reaction centres are 'closed'[17].

Lines 445-447: Why did you opted for this experimental design instead of a randomized distribution of samples?
This has been addressed in our response to reviewer 1.

Lines 498-500: Please substitute:
The text has been change to:
Line 567
Before conducting WATER-PAM readings it is important to dark-adapt the algal samples so that the PSII reaction centers are fully open and the light-induced transthylakoidal pH gradient is fully dissipated, thus giving true F_o and F_m values from which to calculate F_v/F_m.

Line 521: as at this point the actinic light is off, you should refer to Fo instead of F.
This change has been made throughout the text to reflect the actinic light is off.

Line 527: please write F'm instead of Fm.
This change has been made throughout the text

This protocol could take advantage of using IMAGING-PAM instead of WATER-PAM. You should discuss this possibility.
This point has been address in our response to reviewer 1.

Fig. 3: several parameters that were not mentioned in the text are shown; this should be mentioned in the legend.
The following text has been added:
Line 529
In addition, this figure depicts some of the other variables that this method calculates, but these are not discussed in detail here (see reviews[13,16,17,20,21])

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