**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 well18.

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 – F0: t-test, df=2.5, t-ratio=-15, p=0.0017\*; Fm: t-test, df=2.1, t-ratio=-16.15, p=0.003\*; Fv/Fm: 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, F0 and/or Fm) 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 104 cfu/ml, so in step 3.7 the initial cells should be serially diluted to 106 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

Fm = 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

Fv/Fm = (Fm-F0)/Fm = potential quantum yield of a dark adapted sample19

*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 Fm 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 Fo and Fm values from which to calculate Fv/Fm.  
  
*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 reviews13,16,17,20,21)