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TITLE:

Operation of Laboratory Photobioreactors with Online Growth Measurements and Customizable Light Regimes

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Photobioreactor, oxygen production, microalgae, cyanobacteria, biotechnology, photosynthesis

SUMMARY:

This publication describes the design of laboratory photobioreactors (PBRs) with customizable light regimes. The growth of cyanobacteria or microalgae, using bicarbonate as their carbon source, is monitored continuously by measuring volumetric oxygen production. These PBRs facilitate rapid, replicated laboratory growth comparisons with little user intervention during experiments.

ABSTRACT:

The laboratory study of microalgae can be experimentally challenging. In addition to the cultivation requirements of non-photosynthetic microorganisms, phototrophs also require illumination. Routinely, researchers seek to provide custom light supplies, i.e., vary the light intensity and time over which it is delivered. Such flexibility is difficult with standard benchtop lights. Usually, cultivation studies also require growth comparisons between experimental treatments. Frequently, growth is assessed over an extended duration, e.g., multiple times a day over a week-long trial. Manual measurements can be time-consuming and lack data resolution. Therefore, photobioreactors (PBRs) with automatic growth monitoring and customizable light supply are useful for replicated experiments with multiple treatments. The current work presents the design, construction, and operation of laboratory PBRs. The materials are easily sourced and relatively inexpensive. The design could be duplicated with moderate skill. Each structure has a footprint of ~40 cm^2 and hosts three 1 L glass bottles for triplicate replication. Bottles rest upon platforms containing magnetic stirrers and are arranged vertically within a 1 m high and 15 cm diameter polyvinyl chloride (PVC) pipe. The pipe interior is lined with light-emitting diodes (LEDs). These LEDs produce continuous light intensities from 0–2400 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR). Users design a custom lighting program. The light intensity can be adjusted each second or held constant for longer durations. Oxygen produced

from photosynthesis exits each bottle *via* a one-way volumetric gas sensor. Software is used to record gas sensor data. The amount of oxygen produced can be correlated to biomass growth. If biomass samples are required, a syringe can be used to extract culture. The method is suited for microalgae grown with bicarbonate as the carbon source. These PBRs are valuable to a laboratory that requires replicated experiments, light regime flexibility, and continuous high-resolution growth data.

INTRODUCTION:

Microalgae and cyanobacteria, collectively called microalgae for simplicity, are championed for their potential in sustainable biotechnology. They are attractive candidates due to their rapid growth, ability to be cultivated on non-arable land, and for their use of sunlight to drive the conversion of carbon dioxide to biomass[1–3]. Microalgal biomass can be converted into products such as bioenergy in the form of oil or gas, food dyes and nutritional supplements, and materials such as biopolymers[1,4–7]. In addition, they can be used to treat wastewater or remediate waterbodies by consuming excess nutrients[8,9]. Given this, microalgal research is widespread and established. The field grows as society reconsiders the carbon intensity and environmental sustainability of current manufacturing and energy generation approaches.

Three fundamental requirements of laboratory-based microalgal studies are a culture vessel, light source, and method to quantify growth. The term photobioreactor (PBR) describes a set-up in which culture vessels are illuminated[10]. Commonly, studies of microalgae aim to compare growth between two or more treatments, e.g., different growth media, light regimes, or species[11–13]. For statistical relevance, each condition, e.g., treatment and control, should be replicated. If control and treatment are run simultaneously, this means many PBRs must be monitored and sampled for the duration of an experiment. The challenge with operating multiple PBRs is two-fold. Firstly, supplying a uniform light intensity to each PBR is essential for reproducibility but can be difficult. The amount of light incident on the vessel surface is influenced by its distance from the light source, shading from adjacent vessels, and background light fluctuations[14]. Secondly, a method to accurately quantify growth must be selected.

Growth is commonly measured by cell count, optical density (OD), chlorophyll A content, dry weight (DW) density, and ash-free dry weight (AFDW) density[15]. Cell counts, chlorophyll A content, and gravimetric methods are manual processes that produce discrete data points. OD can be measured continuously and non-invasively with a spectrophotometer, provided it is well-calibrated against another method such as AFDW density[15]. However, OD measurements and Chlorophyll A content can be unreliable as results vary under different culture conditions, e.g., between species and throughout the growth cycle[15,16]. For Chlorophyll A, the extraction method can also impact pigment yield[17]. Chlorophyll A content is particularly useful in tracking the growth of microalgae within microbial communities which also contain non-photosynthetic organisms[17,18]. When choosing a method to determine growth, it is essential to consider the morphology of the suspension. When organisms clump and are not well mixed, OD and cell counts aren't possible[15]. A single method is not suited to all experimental applications—researchers must decide which methods are practical and relevant to their experimental aims.

AFDW is a reliable method that enables growth comparisons among various culture conditions, notably, between species and culture media[15,19–20]. To calculate AFDW, a sample of microalgal culture is first concentrated, either by filtration or centrifugation, and dried. At this stage, the DW can be determined. Usually, the DW sample contains at least 8%–10% ash—inorganic material such as salts and particulate matter[15]. DW tracks growth trends but can be skewed if the contribution of inorganics varies. To determine AFDW density, dry biomass is combusted at high temperature; this vaporizes the organic or useful portion while leaving ash (inorganics) behind[19]. To calculate the AFDW, the weight of the ash fraction is subtracted from that of the DW fraction. Typically, in microalgal suspensions, AFDW ranges from 0.1–3 g/L[12,21,22]. Small volumes of dilute suspensions yield little dry biomass, <10 mg. After combustion, ash may only weigh 1 mg. Therefore, depending on the culture density, this method requires volumes between 5–100 mL and analytical scales accurate to 0.1 mg[12,15,19,22]. Laboratory PBRs are typically small, a couple of liters at most, hence every liquid sample depletes culture volume. Further, the AFDW method is manual and takes 2–3 days. For replicated and repetitive experiments, an automated and continuous process is preferable.

For microalgae that use bicarbonate as the carbon source, two additional growth metrics can be measured continuously. Photosynthesis consumes bicarbonate and produces oxygen. Bicarbonate consumption drives up medium pH[23]. An immersed pH probe can measure this change. Photosynthetic oxygen production increases the medium's dissolved oxygen (DO) concentration until the medium is saturated. Beyond saturation, oxygen exists as bubbles. Oxygen production is measured by many different techniques: probes gauge DO concentration, manometric devices assess headspace pressure, gas chromatography measures headspace composition, and volumetric sensors record gas outflow[24–27]. When oxygen is used as a growth proxy, culture vessels must be fully sealed or allow only gas outflow. For pH and oxygen measurements, carbon must be supplied in the form of bicarbonate, not by CO_2 sparging. CO_2 sparging decreases the medium pH[23] and, as a gas, can disturb oxygen measurements. One advantage of pH and oxygen over optical density is that the method is not compromised if microalgae form clumps. Although indirect, both pH and oxygen are effective in comparing growth between treatments.

PBRs in use today range in complexity. Laboratories may use simple benchtop flasks, custom prototypes, or commercially available products. For research groups seeking to upgrade from flasks, the cost of commercial PBRs or technical skill and part fabrication required to build many prototypes may be a barrier. This manuscript aims to describe the step-by-step design, construction, and operation of laboratory PBRs that bridge this gap. These PBRs have a customizable light regime and monitor growth continuously by recording volumetric oxygen production. This design houses three culture vessels for triplicate replication and can be built with moderate skill and easily accessible materials. This PBR is a valuable addition to a laboratory looking to expand its capacity for microalgal research without investing in very technical or expensive products. When choosing to acquire or build a PBR, researchers must consider the suitability of a design to their culture conditions, financial position, and research questions.

PROTOCOL:

1. Construction of the PBR stand

1.1. With a handheld hacksaw, cut five 380 mm lengths and two 200 mm lengths of angled slotted steel. Fasten together with bolts and large corner braces to make a stand's base (**Figure 1A**). Glue on safety end caps.

1.2. Connect two uncut (1220 mm) vertical lengths of angled slotted steel to the base. Secure with bolts and metal corner gussets (**Figure 1B**). Glue on safety end caps.

1.3. Cut four 65 mm flat lengths of slotted steel. Bolt these at 90° angles onto the vertical supports—attach two to each support, one 130 mm up from the base (**Figure 1C**) and one 60 mm down from the top.

1.4. Fasten vertical supports across their top with a horizontal 140 mm length of flat slotted steel (crossbeam) bolted to the back of the frame (**Figure 2A**).

2. Construction of the light chamber

2.1. Cut a white 153 mm diameter polyvinyl chloride (PVC) pipe to a length of 1070 mm. Cut the pipe in half lengthwise with a bandsaw. Sand all edges.

2.2. Evenly space and center four aluminum heat sink channels vertically along with the interior of the pipe. Do not attach channels within 20 mm from the pipe's cut edge. With small bolts, secure the channels in place at their top and bottom (**Figure 2B**).

2.3. Bolt one-half of the pipe to the stand using the horizontal supports fashioned in step 1.3.

2.4. Lay the reactor down and reunite the pipe halves by taping them together. Center the piano hinge along one cutline. Trace the hinge holes and drill the pipe accordingly. Use a rivet gun and medium-length rivets to fasten the hinge to the pipe.

2.5. Use a small bungee cord (see **Table of Materials**) to hold the pipe shut (**Figure 1C**).

2.6. Consult an electrician to wire in the LED lights and install the following four components: LED driver, digital multiplex (DMX) decoder, DMX lighting controller, and switch box (see **Table of Materials**). Fix all the components to the rear of the PBR according to **Figure 2A**.

3. Construction of the bottle platforms

3.1. Cut platform shapes (**Figure 3A**) out of hard plastic, e.g., high density polyethylene (HDPE) (see **Table of Materials**), using computer numerical control (CNC) milling. Make three of each shape.

NOTE: Cutting spare top and bottom layers is recommended.

3.2. Tape the bottom and top layer together. Mark and drill five small holes, 6 mm in diameter, through both shapes (**Figure 3A**). Using a larger drill bit, carefully expand the surface of these holes so that bolt heads can be recessed (**Figure 3B**).

3.3. For each of the three platforms, bolt two small corner braces to the back half of the pipe.

NOTE: The distance between the top of the braces should be 350 mm.

3.4. Center each bottom layer on top of its braces. Mark the location of the drill holes from beneath the braces. Drill two 6 mm diameter holes. Using a larger drill bit, carefully expand the surface of the holes so that bolts can be recessed.

3.5. Bolt bottom layers to their braces (**Figure 3B,C**).

3.6. Cut fifteen pieces of 12 mm long 6.35 mm outside diameter (OD) rigid tubing. Sandwich five pieces of the rigid tube between each top and bottom layer. Fasten layers and tubing together with long narrow bolts according to **Figure 3B–D**.

3.7. Drill a large hole in the PVC pipe behind each platform. Insert each micro magnetic stirrer into its platform. Thread each stirrer's electrical cable through these newly cut holes (**Figure 3C–E**). Connect each stirrer to its respective control unit as well as a power outlet.

3.8. Place a 1 L bottle on each platform. Add eye bolts in the rear pipe at bottle neck level. Wrap a small bungee cord around each bottle neck to add stability (**Figure 4A**).

NOTE: Throughout the protocol, color coding platforms, bottles, magnetic stirrers, and all associated cables and sensors will be helpful.

4. Construction of the liquid sampling ports (optional)

4.1. Cut three 60 mm lengths of 6.35 mm OD rigid tubing. Using a 5 mm drill bit, drill holes through each rubber stopper. Push rigid tube lengths through the stopper.

4.2. Cut three 60 mm lengths of 3.18 mm OD rigid tubing. Connect these with a straight reducer to the protruding tube on the underside of each stopper.

4.3. Insert a one-way stopcock valve (e.g., port 1 = female Luer, port 2 = male slip Luer) in the protruding tube on each stopper surface (**Figure 4A**).

4.4. Cut three 30 mm lengths of 3.18 mm OD flexible tubing. Insert Luer fittings (e.g., male Luer to hose barb and female Luer to hose barb) on either end.

4.5. Connect the pieces made in step 4.4 to the stopcock valves on the surface of each rubber stopper (**Figure 4A**).

NOTE: Many combinations of stopcocks and Luer fittings can produce the same result. The design should allow liquid to be drawn out or inserted *via* a syringe.

5. Hooking up volumetric gas sensors

5.1. Prepare gas sensors according to the manufacturer's instructions.

NOTE: This mainly involves filling gas sensors with packing liquid (**Figure 4B**).

5.2. To make the gas lines, cut three 1000 mm lengths of 3.18 mm OD flexible tubing.

5.3. Drill three 4 mm diameter holes in the rear PVC pipe. Position holes next to the hinge at bottle neck height. Thread gas lines through these holes (**Figure 4A**).

5.4. To the end of the gas line inside the PVC pipe, add a Luer fitting (e.g., hose barb to male Luer) and connect a one-way stopcock valve (e.g., port 1 = female Luer, port 2 = male Luer).

NOTE: The valve is only required when liquid sampling ports are also installed.

5.5. Join the other end of the gas line to the inlet port of the gas sensor using a straight reducer. Secure this connection with a zip tie.

5.6. Connect all gas sensors to the digital input module (DIM) with jack plug cables and the DIM to a nearby computer.

5.7. Install the data acquisition software (see **Table of Materials**) on a Windows operating system and plug in the licensing key dongle. Add sensor calibration files to the software's calibration directory.

6. Programming of the light regime

6.1. Using the rear on/off switch, turn on the PBR and connect the DMX lighting controller to a computer *via* a micro-USB cable.

6.2. Download Store Upgrade Tools (SUT) and the LED control software (see **Table of Materials**). Register the DMX lighting controller online.

6.3. Open the LED control software and select **Click here to work with the USB-DMX interface: SUSHI-RB-RJ**.

6.4. Under the **Set-up** tab in the **ScanLibrary** box, select the **Generic** folder and **Single Channel**.

Change **ScanLibrary** settings to DMX universe 1, the number of fixtures to 4, and the index number to 1. In the upper-right corner, change the drop-down box to **List View**. Lastly, click on **Patch** (**Supplementary Figure 1**).

NOTE: In the DMX universe, one box tests each LED strip's control by sliding the dimmer buttons or entering a numerical value into the text box.

6.5. Make a standard curve that relates the digital light setting in the lighting control software to the light intensity experienced in the center of the PVC tube (**Figure 5**). Measure internal light intensity with a small spherical probe (see **Table of Materials**) suspended in the center of the PVC pipe.

6.6. Advance to the **Editor** tab. To build a custom light program, create a new **Scene** and begin adding **Steps**. Refer to **Supplementary Table 1** for an example 16:8 h diurnal program. Set the scene to loop.

NOTE: Steps break down scenes into blocks of time, each of which can be set to different light intensity. Steps range from 1 s to 43 min. Here, 30 min steps are most convenient. Multiple scenes can be loaded onto one DMX lighting controller device.

6.7. Create an additional helper scene that is immediately recognizable, e.g., two of the four LEDs on.

NOTE: Scenes can be cycled through manually using the button on the side of the DMX lighting controller. If the desired light program begins during the night, it will be impossible to distinguish whether the light program has already started. The helper scene serves as an indicator that the DMX lighting controller is functioning correctly.

6.8. Save the scenes and advance to the **Stand Alone** tab. Write the memory of the DMX lighting controller and disconnect the device from the computer.

6.9. Connect the DMX lighting controller to its power source using a micro-USB.

6.10. Before starting an experiment, test the light program by logging the internal light intensity for a 24 h duration. If the liquid temperature is of interest, log this simultaneously with a submerged temperature probe (**Figure 6**).

7. Starting an experiment

7.1. Sterilize media, bottles, stir bars, rubber stoppers, sampling ports, threaded aperture screw caps, and tubing.

NOTE: All components used in this design are autoclavable except the valves and Luer fittings—there are autoclavable alternatives from other manufacturers.

7.2. Open the data acquisition software and fill in the configuration page (**Supplementary Figure 2**). Assign calibration files to their respective sensors.

7.3. Under **Directory File Name** select the corresponding DIM port number folder. Click on **Save** and repeat for all ports.

7.4. Click on **OK** to move on to the logging page.

7.5. Fill bottles to the desired volume with cultivation medium (**Supplementary Tables 2,3**).

NOTE: Each of these bottles will hold a maximum of ~1.1 L with a small headspace (~80 mL, including the gas line).

7.6. Centrifuge the stock culture in three balanced 50 mL tubes for 15 min at 4500 x *g* to yield three pellets. Add one pellet to each bottle.

NOTE: The culture density of day 0 is also known as the initial biomass concentration (IBC). For measuring the IBC in $g_{AFDW}.L^{-1}$, an additional 50 mL tube may be centrifuged in step 7.6. The resulting pellet can then be dried and combusted[15,19]. Step 7.6 will likely require modification based on the individual aims of users and their experiments.

7.7. Drop a magnetic stirrer, 25 x 8 mm, into each bottle.

7.8. Seal each bottle opening with a rubber stopper and threaded aperture screw cap (**Figure 4A**). If optional sampling ports are installed, close the valves.

7.9. Locate the end of each gas line inside the PVC pipe (built in step 5.4) and attach a needle to the valve's male Luer port.

7.10. Connect each bottle to its gas sensor by piercing each rubber stopper with the corresponding needle.

7.11. Launch gas sensors by checking the tick boxes on the left side of the screen and clicking on **Start** (**Supplementary Figure 3**).

NOTE: While logging, do not exit the data acquisition window. Set power and sleep settings of the computer to **never** and postpone computer updates for the duration of the experiment.

7.12. Switch on the PBR and ensure that the DMX lighting controller is plugged into a power supply. The first programmed scene will automatically commence. Refer to step 6.8 to confirm that the DMX lighting controller is functioning correctly.

8. Bottle sampling (optional)

8.1. Prepare an additional 500 mL of the fresh medium before commencing the experiment (**Supplementary Table 2**).

NOTE: If a 24 h light program with a 16:8 h diurnal cycle were started at 9 am, then sampling times before dusk and dawn would fall at 8 am and 4 pm (**Supplementary Table 1**). Here, dawn and dusk refer to 30 min steps that transition lights from ON to OFF and vice versa.

8.2. Close the valve on the gas line.

8.3. Connect a syringe (10 mL) to the sampling port valve (**Figure 4A**).

8.4. Open the sampling port valve and withdraw 8 mL of culture.

NOTE: Between 5–10 mL is recommended. Removing liquid generates a vacuum in the headspace, making volumes >10 mL difficult to extract.

8.5. Close the sampling port valve and disconnect the syringe.

8.6. Connect a syringe containing 8 mL of fresh medium (from step 8.1) to the sampling port valve.

8.7. Open the sampling port valve and inject fresh medium.

NOTE: Replacing the volume of sampled culture with fresh medium serves to maintain an equal headspace volume and pressure and flush the sampling port line.

8.8. Close the sampling port valve before disconnecting the syringe.

8.9. Repeat steps 8.2–8.8 at each sampling time.

9. Ending an experiment

9.1. Check all active port tick boxes in the data acquisition window and click on **Stop**.

9.2. To export data, select **File** and **Offline Data**. Select all relevant log files. Export the data to spreadsheet software and save.

9.3. For each bottle, convert the total volume of oxygen measured in mL to moles using the ideal gas law. Predict the weight of biomass grown (gAFDW) if 1.05 mole of O_2 is generated for each mole of biomass produced. Take the molar weight of biomass as 24.6 g mol^{-1}.

9.4. Manually curate the flow rate data. Use units of mL/h and a 3-point moving average.

REPRESENTATIVE RESULTS:

Here the oxygen flow rate is a measure of the culture's photosynthetic rate. Higher rates of photosynthesis, and hence carbon fixation, translate to higher growth rates. This means the user can compare oxygen flow rates between different treatments and operational days as a proxy for growth. Briefly, the gas sensor works by trapping and releasing gas bubbles in a dual-chamber measurement cell (**Figure 4B**). Gas bubbles from the inlet at the base of the sensor travel up through the packing liquid. Bubbles accumulate in one chamber of the measurement cell to a volume of ~3.2 mL. Once this threshold is reached the measurement cell tips. This releases the gas and resets the system. Each tip is recorded by the data acquisition software.

In the example data, the growth rate of three treatments with varying daytime light intensities and initial biomass concentrations (IBCs) were compared. These treatments were chosen arbitrarily for demonstrative purposes. They were (A) 300 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ and 0.03 $g_{AFDW}\ L^{-1}$, (B) 600 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ and 0.13 $g_{AFDW}\ L^{-1}$, and (C) 600 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ and 0.40 $g_{AFDW}\ L^{-1}$. These irradiances were measured with a spherical probe in the center of the PVC pipe before bottles were placed on the platforms. Culture depth and density affect light attenuation. Hence the actual light intensity experienced by microalgae can vary from those reported. Each treatment was performed in triplicate—within one PBR containing three bottles.

Here, a successful experiment was characterized by closely replicated diurnal patterns of gas production (**Figure 7A–C**). During illuminated hours (day), gas production steadily increased, and over non-illuminated hours (night), gas production stopped (**Figure 7A–C**). Two gases are produced by microalgae, oxygen from photosynthesis and carbon dioxide from respiration[28]. Photosynthesis is restricted to illuminated hours, whereas respiration occurs continuously but is most active at night[28]. Photosynthesis builds, whereas respiration catabolizes biomass[28]. Initially, the composition of headspace gas is identical to that of the atmosphere. With each flip of the measurement cell, O_2 displaces atmospheric gas. Therefore, gas sensor readings were attributed to the production of O_2 even if the outgoing gas was not pure O_2. The minimum gas inlet pressure for the gas sensor is extremely low, 8–9 mbar, rendering bottle headspace pressure only slightly over atmospheric (1.01 bar at sea level). Hence, gas sensor readings commence shortly after O_2 bubbles leave the medium.

CO_2 released from respiration does not contribute to gas sensor readings for two reasons. Firstly, in the alkaline medium, CO_2 reacts to bicarbonate, decreasing the pH (**Figure 8**). Secondly, if CO_2 does escape, the gas sensor packing liquid, Silox, dissolves CO_2 bubbles before they can reach the measurement cell, outgassing CO_2 at the liquid surface[29]. This is supported by the lack of overnight gas sensor readings. Those that did occur were recorded shortly after lights were turned off, indicating that readings represented residual daytime oxygen release (**Figure 7**).

In the experimental set-up (using local temperature and pressure data), an 80 mL headspace at ambient pressure required 340 mL of exsolved O_2 to establish an O_2 partial pressure of 99%. Here, the total volume of oxygen produced over 4 days ranged from 316 (SEM ± 11) mL in treatment A to 902 (SEM ± 51) mL in treatment C (**Table 1**). Therefore, by the end of the experiment, the headspace of all bottles would have contained primarily O_2. The increased concentration of

headspace O_2, and thus decreased concentration of N_2, would have impacted these gases' partial pressure and saturation. With a 99% O_2 headspace, a 5x increase in DO was calculated. For the 1.1 L cultures, this translated to an additional 23 mL of DO. Conversely, it was estimated that the shift to a 1% N_2 headspace would have caused 15 mL of N_2 to exsolve. This means that under a near pure oxygen headspace, more O_2 dissolved than N_2 was displaced. Thus, because more O_2 remains in the medium, this effect would have led to slight underestimations in the amount of photosynthetic oxygen produced.

The principal challenge of this method arose when cultures became dense. With more biomass, and hence more respiration, the demand for O_2 increased. Nighttime O_2 consumption generated a headspace under-pressure. This caused gas sensor packing liquid to travel up through the gas line. When O_2 production resumed, packing liquid had to be driven back into the gas sensors. This caused a delay in the first gas sensor reading. However, on the fourth night, the magnitude of this under-pressure caused packing liquid to reach and drip into two of the three replicates of treatment B, generating a surface oil slick. Due to the reduced packing liquid level, the gas sensors short-circuited, releasing unmeasured O_2 directly to the atmosphere. This caused data collection to flatline (**Figure 7B**).

Under-pressure can also be caused by a temperature-induced contraction of headspace volume. However, the effect here was minimal. Heat sink channels and airflow adequately dissipated excess heat. Of the two light regimes trialed, the maximum temperature change reduced the headspace volume by 1% or less, equivalent to an 800 µL packing liquid displacement in an 80 mL headspace. The maximum diurnal temperature swing was 1.4 °C for the 300 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ regimes (**Figure 6**) and 3.2 °C for the 600 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ regimes. The average daytime temperature rise for the 300 and 600 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ regimes was 0.7 and 1.8 °C, respectively. Culture temperatures returned to baseline overnight (**Figure 6**).

High-resolution growth rate data can reveal trends that otherwise may go unnoticed. Consider treatments B and C. Despite their differing IBCs, both generated the same amount of total biomass (g_{AFDW}), which caused an identical shift in medium pH (**Table 1**). Given only starting and final data points, an individual may rightly assume no difference in the average growth rate between the two treatments (**Table 1**). However, online oxygen flow rate data revealed that each treatment had varying daily growth rates. These variations were also reflected in twice-daily pH measurements (**Figure 8**). On day one, treatment B's growth rate was lower than that of treatment C. By day three, this reversed with treatment B's growth rate surpassing that of treatment C (**Figure 7B,C**). Oxygen flow rate data indicated that the highest growth rate occurred on day three in treatment B (**Figure 7B**).

The total volume of oxygen generated by each bottle in the three treatments was used to estimate their respective change in total biomass (g_{AFDW}). This was achieved using a generic equation for photosynthetic biomass synthesis: $CO_2 + 0.2\ NH_3 + 0.6\ H_2O = CH_{1.8}\ O_{0.5}\ N_{0.2} + 1.05\ O_2$. The increase in headspace O_2 partial pressure and subsequent rise in DO saturation was expected to cause a slight underestimation of biomass growth. This was true for five out of seven examples (**Table 2**). On average, estimated biomass growth was within 10% of measured biomass

485 growth. Some estimates differed by only 1–3 mg from measured growth. Two examples
486 overestimated growth, i.e., more oxygen was produced than biomass growth could account for.
487 Any O_2 consumed by respiration overnight should be reflected in the lag in O_2 production the
488 following day. Here, experiments were terminated at night's end. In this way, overnight biomass
489 catabolism during the final 8 h of each experiment goes unmeasured. This may cause
490 overestimations of biomass growth, especially in dense cultures. As such, it is recommended that
491 experiments are terminated at the end of illuminated hours.
492
493 **FIGURE AND TABLE LEGENDS:**
494
495 **Figure 1: Reactor stand base.** (**A**) Dimensions of base components in mm. (**B**) Orientation of
496 metal corner gussets that secure the two vertical supports. (**C**) One of four short steel lengths
497 connects the back half of the PVC pipe to the reactor stand.
498
499 **Figure 2: Electrical components.** (**A**) Rearview of the PBR showing top cross beam and
500 configuration of electrical components. (**B**) Front view of PBR after light installation.
501
502 **Figure 3: Bottle platform details.** (**A**) Dimensions of top and bottom layer in mm. (**B**) Recessing
503 bolt heads into both layers. (**C**) Braces connect the bottom layer directly to the rear half of the
504 PVC pipe. (**D**) Five short pieces of rigid tubing fitted over narrow bolts hold the top and bottom
505 layers apart. (**E**) When the bottle platform is complete, the surface should be flush.
506
507 **Figure 4: Gas line and optional sampling port.** (**A**) Gas lines connect each bottle headspace to
508 exterior gas sensors. If the sampling port is required, gas lines should include a one-way valve
509 immediately downstream of the needle. (**B**) Volumetric gas sensor. The liquid packing level
510 should touch the tracing screw.
511
512 **Figure 5: Standard curve relating LED control software settings to internal light intensity.** White
513 circles and gray triangles each represent an individual PBR. For each light setting, all four fixtures
514 were set to an identical value.
515
516 **Figure 6: Culture temperature change for the 300 $\mu mol_{photons}$ m^{-2} s^{-1} light regimes.** During the 24
517 h, 16:8-h diurnal program, LEDs increased the daytime culture temperature. The blue arrow
518 indicates the difference between the minimum and maximum temperature. A light program
519 error caused the temperature dip before dusk; this was corrected before the experiment
520 commenced.
521
522 **Figure 7: Oxygen production for three unique experimental conditions.** Each reactor received a
523 different combination of light intensity and initial biomass concentration (IBC); (**A**) 300 $\mu mol_{photons}$
524 m^{-2} s^{-1} and IBC 0.03 g_{AFDW} L^{-1}, (**B**) 600 $\mu mol_{photons}$ m^{-2} s^{-1} and IBC 0.13 g_{AFDW} L^{-1}, (**C**) 600 $\mu mol_{photons}$
525 m^{-2} s^{-1} and IBC 0.40 g_{AFDW} L^{-1}. Top graphs display cumulative oxygen production (mL) and the gas
526 flow rate (mL/h). Solid black lines, dashed blue lines, and dotted red lines are replicates. The
527 runtime for each experiment was 104 h, which included four complete 16:8 h day-night cycles.
528 Dark orange shading represents nighttime hours and light orange daytime hours. Note that in

treatment B, oxygen production flatlines on day 4 for two of the three replicates.

Figure 8: pH response. Each reactor received a different combination of light intensity and IBC; (**green diamonds**) 300 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ and IBC 0.03 $g_{AFDW}\ L^{-1}$, (**red triangles**) 600 $\mu mol_{photons}\ m^{2}\ s^{-1}$ and IBC 0.13 $g_{AFDW}\ L^{-1}$, (**purple circles**) 600 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ and IBC 0.40 $g_{AFDW}\ L^{-1}$. Dark orange shading represents nighttime hours and light orange daytime hours. Error bars represent the standard error of the mean.

Table 1: Growth metric shift from hour 0 to 104. Brackets represent the standard error of the mean.

Table 2: Growth estimates based on total measured oxygen. Only one replicate from the 300 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ and IBC = 0.13 $g_{AFDW}\ L^{-1}$ treatment ran to completion.

Supplementary Figure 1: Screenshot from LED control software. Each of the four light fixtures can be controlled independently by sliding the dimmer buttons or entering a numerical value into the text box.

Supplementary Figure 2: Screenshot of the data acquisition software configuration window.

Supplementary Figure 3: Screenshot of the data acquisition software logging window. Bright green rectangles indicate online gas sensors. Data is displayed in real-time.

Supplementary Table 1: Example 24 h light regime. For a 16:8 h diurnal program, there are 48 sets of 30 min each. Asterisks indicate suggested sampling times.

Supplementary Table 2: High alkalinity high pH medium composition.

Supplementary Table 3: Trace element solution. Add to a final concentration of 1 mL/L to the base medium.

DISCUSSION:

Within this protocol, focus on the following steps increases the likelihood of generating reproducible, high-quality data. When constructing the reactor stand (step 1), the base must be sturdy with well-alligned vertical supports. Slotted steel has sharp edges, so the addition of safety caps is essential. Bottle platform surfaces need to be completely flat, the magnetic stirrer and bolt heads should both sit below the top layer surface (steps 3.2–3.6). According to the manufacturer's instruction, the gas sensor packing liquid should be filled to the "tracing screw for liquid level" for accurate oxygen measurements. This liquid level should be checked regularly as evaporation of the packing liquid can short-circuit the measurement cell. All three gas lines made in step 5.2 should be the same length; this enures that replicates have identical headspace volumes. Before commencing an experiment, it is advisable to test the programmed light regime by logging light intensity over a 24 h period (step 6.11). If increases in liquid temperature are of concern, this test should also include a sealed bottle with an internal temperature probe (step

6.11). When logging, do not exit the data acquisition software window; this will terminate logging. If taking culture samples, be careful not to release headspace gas by opening valves in the wrong sequence (steps 8.2–8.8). When reviewing experimental data be advised that the data acquisition software automatically generates a moving average of the flow rate. This inflates the value of the one or two flow rate readings generated overnight. Curate gas sensor logs manually to rectify this.

The most common setback with this method is the potential to short circuit the gas sensor if the liquid packing level declines. There are two ways this can occur. Firstly, evaporation can slowly reduce the liquid level. However, this is unlikely over a short-term (<7 day) experiment[29]. Secondly, high respiration rates can pull oxygen into the solution and generate a headspace under-pressure. When light energy is unavailable, microalgae use aerobic respiration to supply the energy required for cellular maintenance and repair[28]. Hence, in dense cultures during non-illuminated hours, oxygen consumption, and the resulting under-pressure, can be substantial. This sucks packing liquid from the gas sensors into the gas line. The distance the packing liquid travels is proportional to the amount of nighttime respiration. If the packing liquid enters the bottles, this generates an oil slick on the liquid surface.

If high nighttime respiration rates are expected, modifications to the protocol can be made. The simplest way to avoid under-pressure is to leave the bottle headspaces open overnight. This also has the advantage of easing DO levels by decreasing the partial headspace pressure of O_2. High DO concentrations are believed to be detrimental to growth as O_2 can impede the activity of Rubisco and may trigger oxidative stress[30,31]. It is not uncommon for culture suspensions to reach 4x oversaturation even when in contact with the atmosphere[25,32]. To open the headspace, disconnect the gas line from the needle spanning the rubber stopper. Nighttime hours can serve as a window to top up gas sensor packing liquid or manipulate continuous experiments with little impact on data collection. For example, one may alter the culture density, refresh nutrients, add an amendment, or introduce a pathogen. Bottles must be re-sealed, and the gas sensor line reconnected before lights turn back on. The oxygen measurements collected from experiments with closed versus open nighttime headspaces will differ.

When bottles remain sealed, nighttime oxygen consumption reduces the number of moles of O_2 in the headspace. This causes packing liquid to creep up the gas sensor line to maintain headspace pressure. When lights turn on, oxygen production resumes. Packing liquid must be pushed back into the gas sensor before flow rate readings commence. This lag is therefore proportional to the degree of nighttime respiration. In this way, when the headspace remains closed, O_2 readings represent net O_2 production (photosynthetic production – respiratory consumption). Conversely, when the headspace is open at night, atmospheric gas replaces what headspace O_2 is consumed, and no packing liquid enters the gas line. The result is that respiratory O_2 consumption is not accounted for in O_2 production data. This may reduce the accuracy of AFDW biomass growth estimates. However, it should not impact the utility of using daytime O_2 production as a metric to compare growth between treatments.

All laboratory PBRs are afflicted by the same limitation; artificial lights cannot replicate the solar

spectrum. Microalgae use wavelengths of light between 400–700 nm for photosynthesis. This region is referred to as photosynthetically active radiation (PAR)[33]. Sunlight and artificial light vary in their relative contribution of wavelengths within this range. This, alongside favorable temperatures and a constant light supply, means that laboratory growth data often cannot be reliably extrapolated to outdoor conditions. These PBRs can, however, address one of the limitations of laboratory PBR light supply. Sunlight intensity is highly variable throughout the day, with cloud cover generating transient fluctuations in incident PAR. The lighting control software and DMX lighting controller can provide light intensities from 0 to 2400 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ and beyond. Light regimes can be broken down into individual increments as short as 1 s. Tunable light intensity allows the user to mimic outdoor light patterns more closely than standard PBR set-ups. Here, simulated 30 min dawn and dusk intervals fade day and night cycles together (**Supplementary Table 1**).

Although AFDW density has become the standard measure of growth, this method can require substantial culture volumes, a 2–3-day processing period, and generates one data point at a time. Further, if conditions become unfavorable and cells die, AFDW density does not discriminate between actively photosynthesizing cells and those that are decomposing. Quantifying the rate of photosynthetic oxygen production serves as an alternative growth proxy. This PBR design can record oxygen production continuously with little user intervention while conserving culture volume. Data resolution could be improved by selecting a gas sensor with a lower measurement cell volume, e.g., 1 mL. Further, if cultures are well-mixed, users may decide to install a spectrophotometer for continuous optical density readings. If temperature control of the medium is desired, a recirculating chiller could be added. These PBRs are a valuable addition to a laboratory looking to expand its microalgal research capacity without heavy financial investment. They are particularly suitable for those working with high alkalinity, high pH species like *Spirulina*. These PBRs offer light regime flexibility and are valid for rapid, replicated, laboratory growth comparisons.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

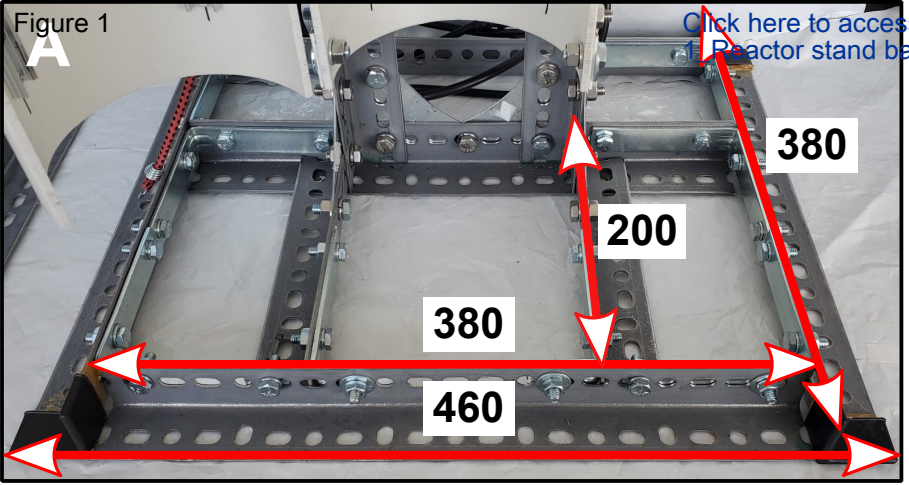


Figure 2

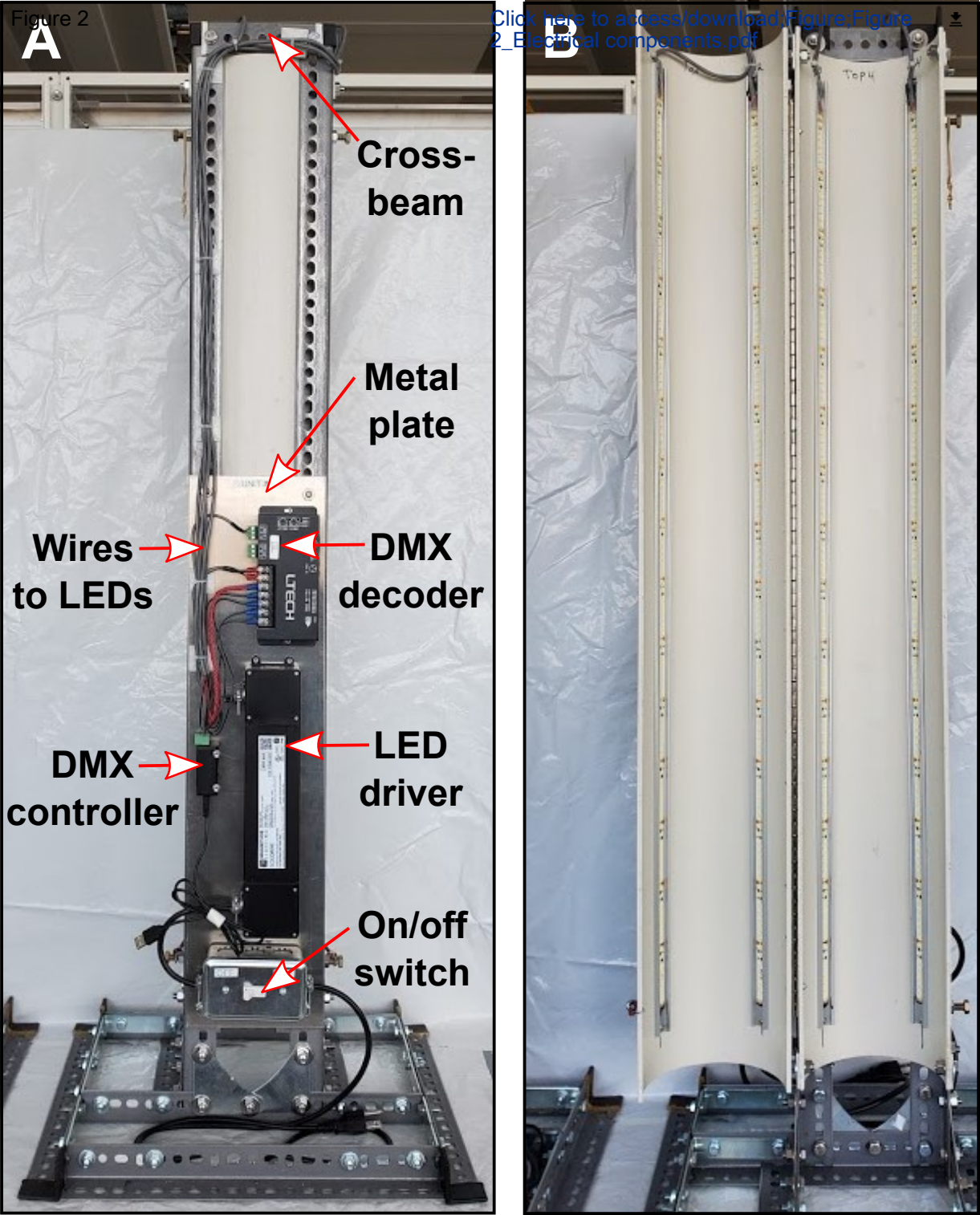
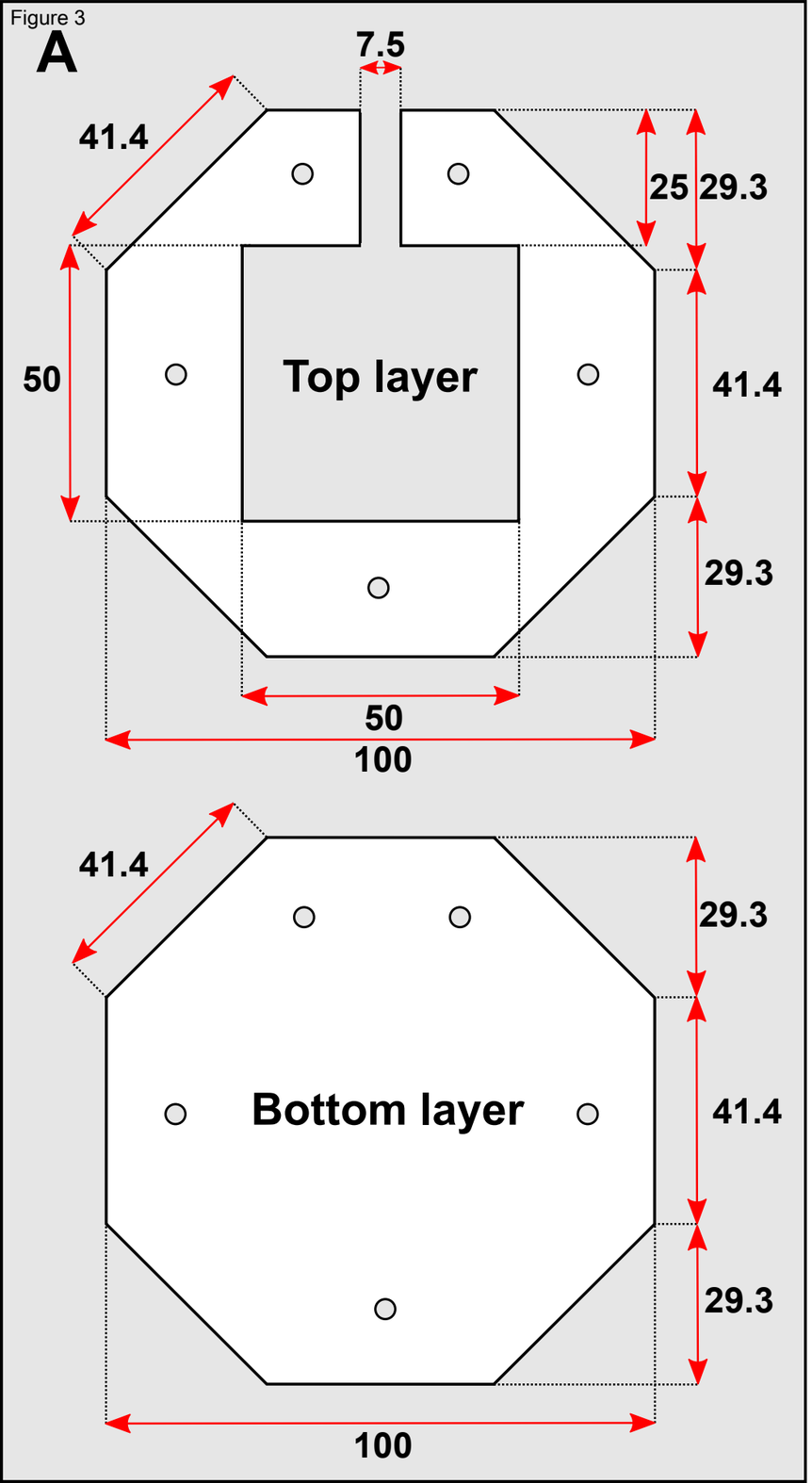
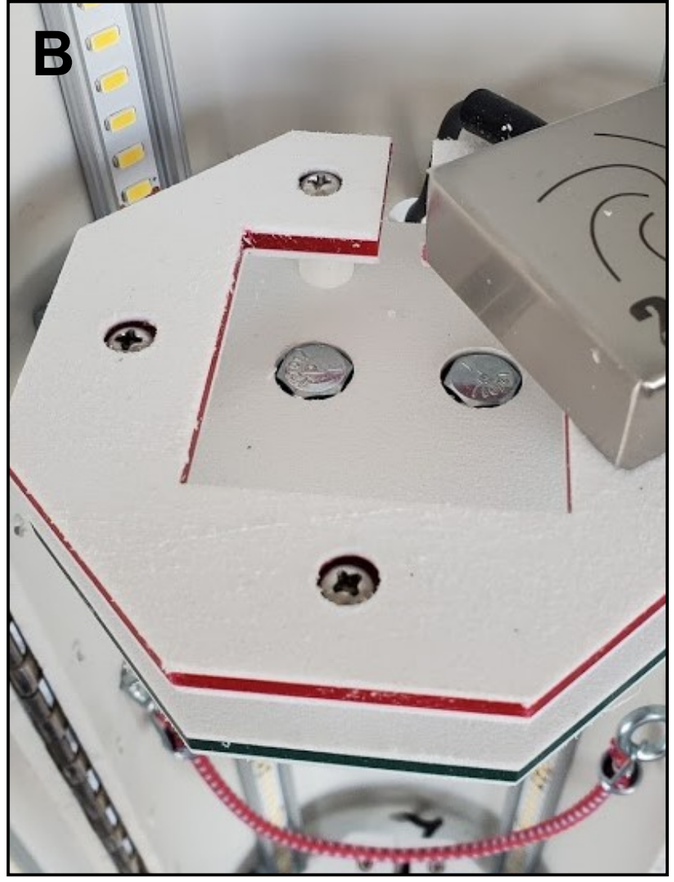
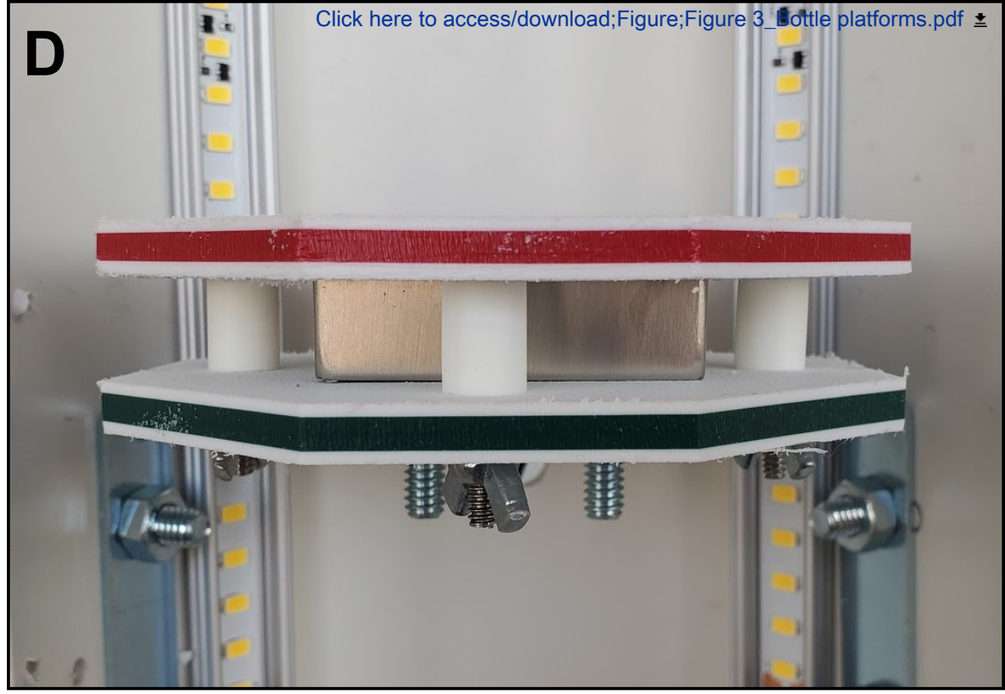
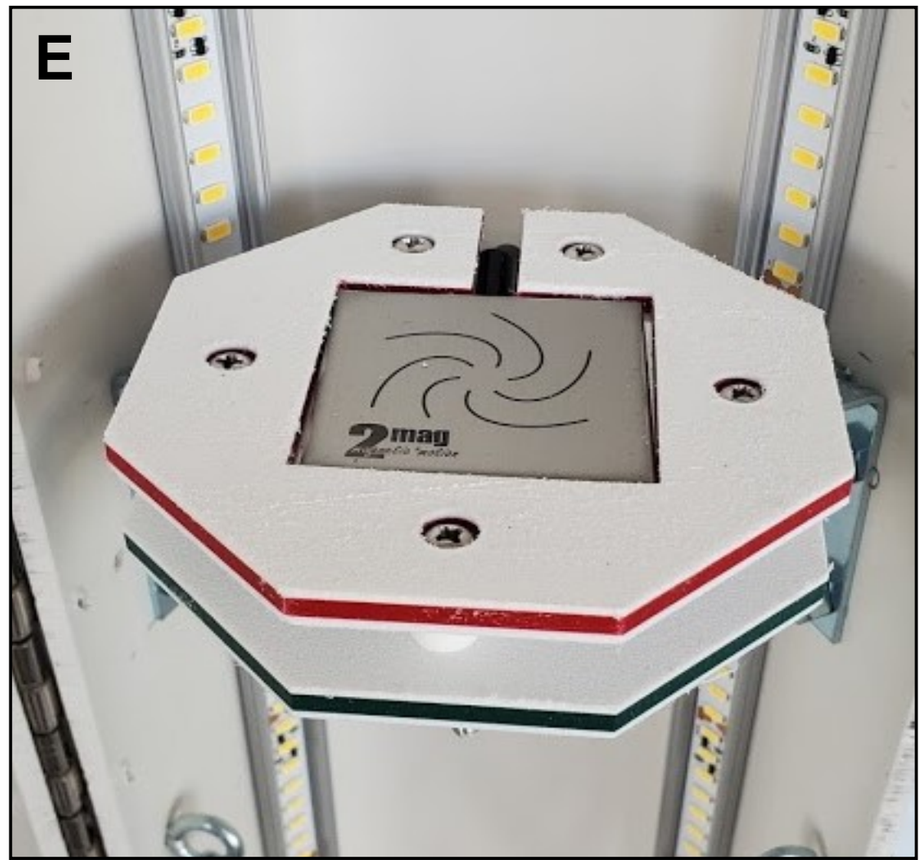


Figure 3









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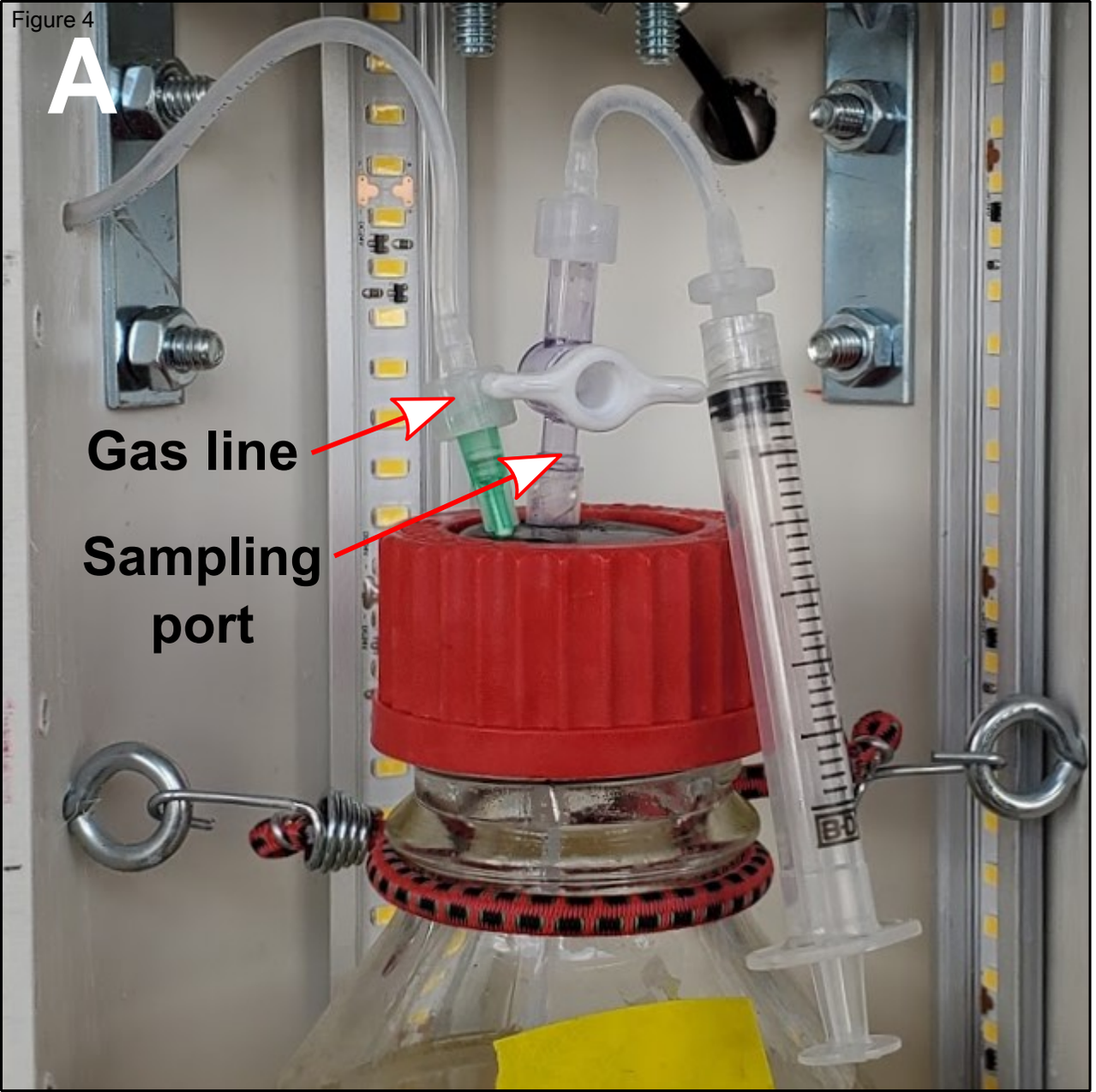


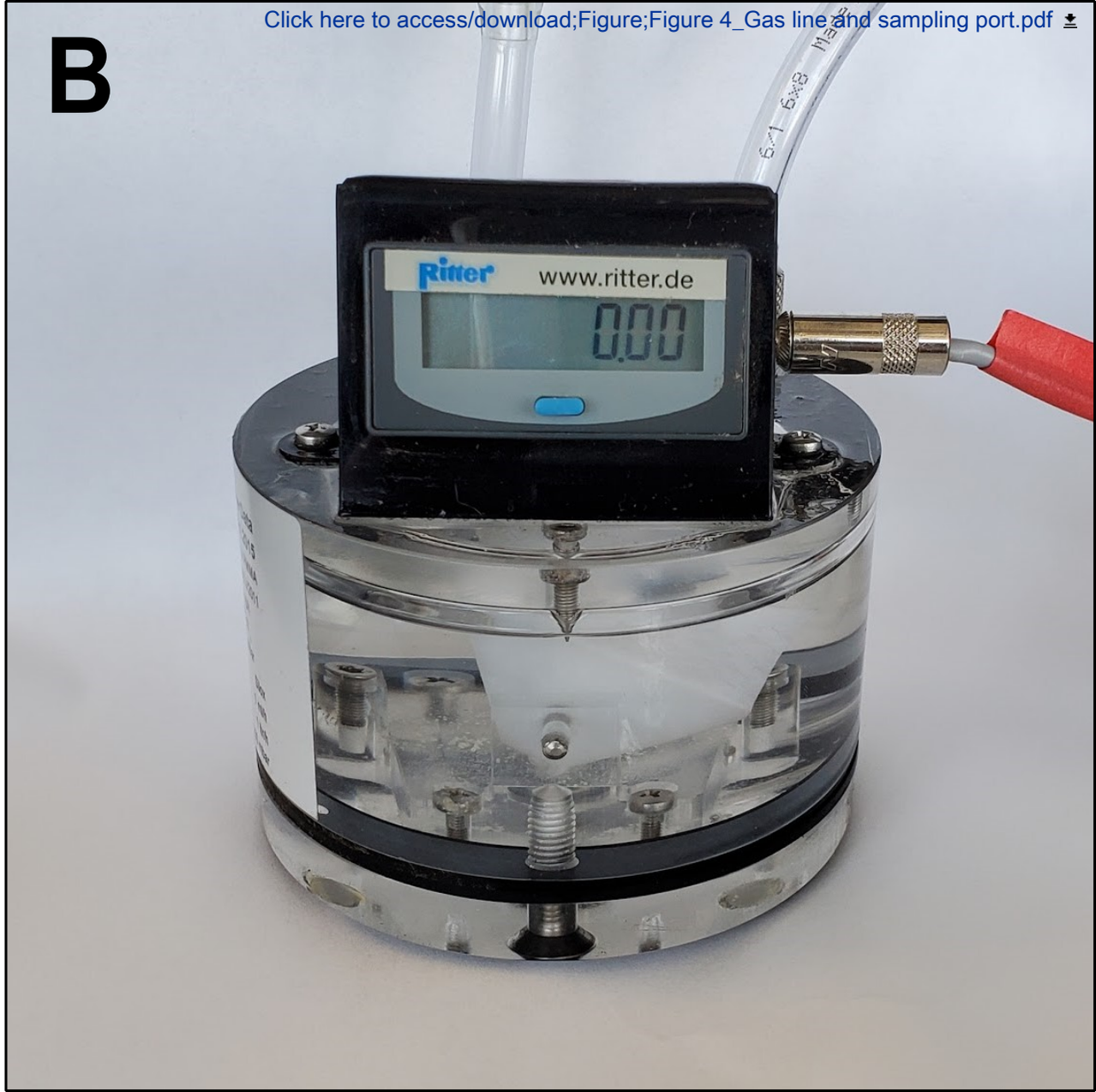


Figure 3

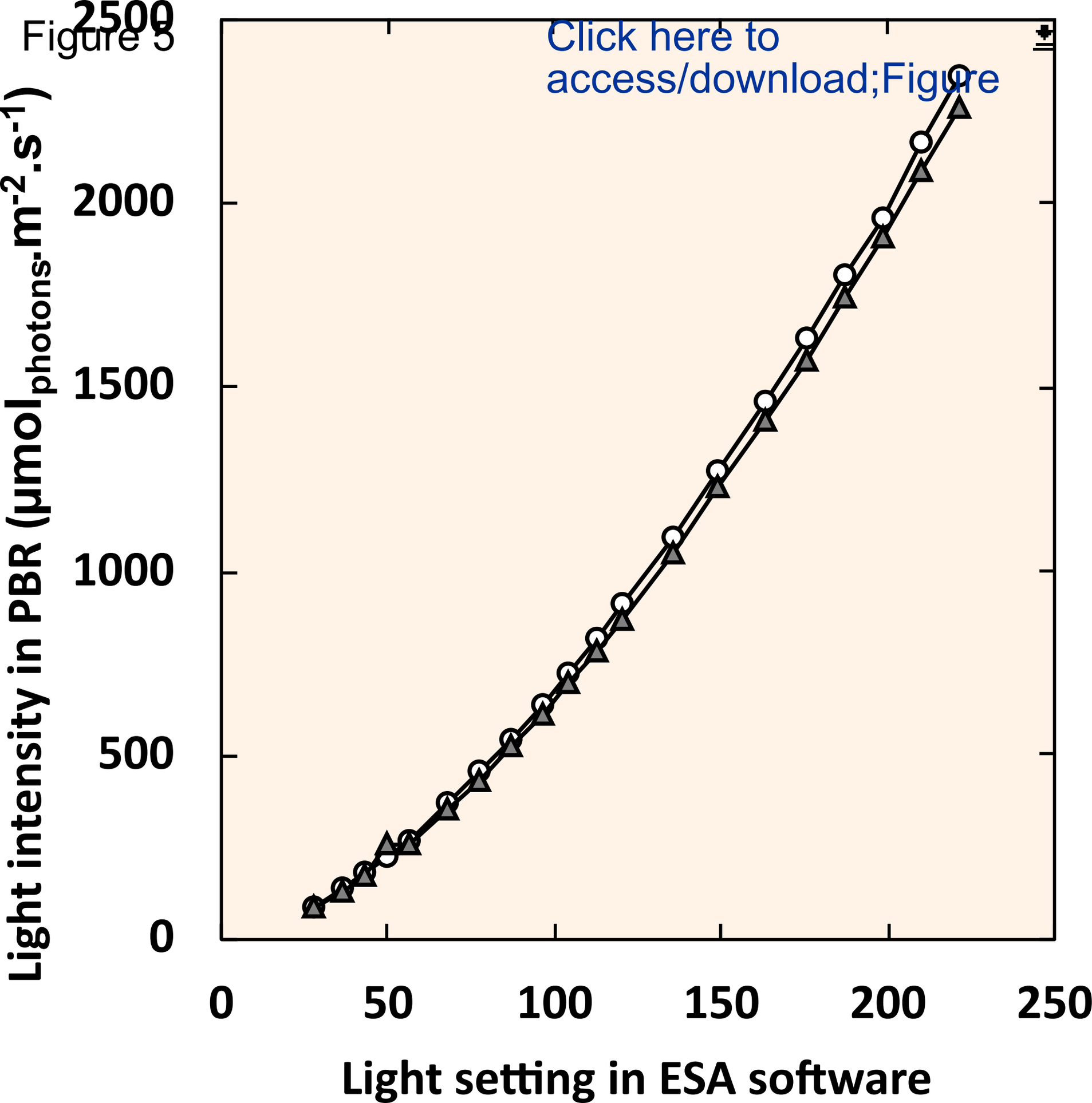


Figure 6

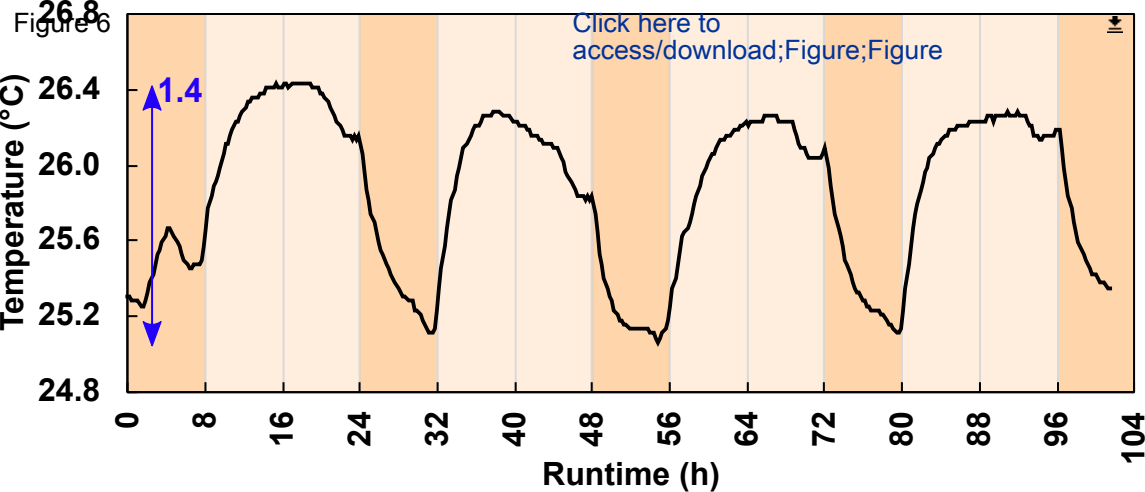

Click here to
access/download;Figure;Figure
1.4
Temperature (°C)
26.8
26.4
26.0
25.6
25.2
24.8
0
8
16
24
32
40
48
56
64
72
80
88
96
104
Runtime (h)

Figure 7

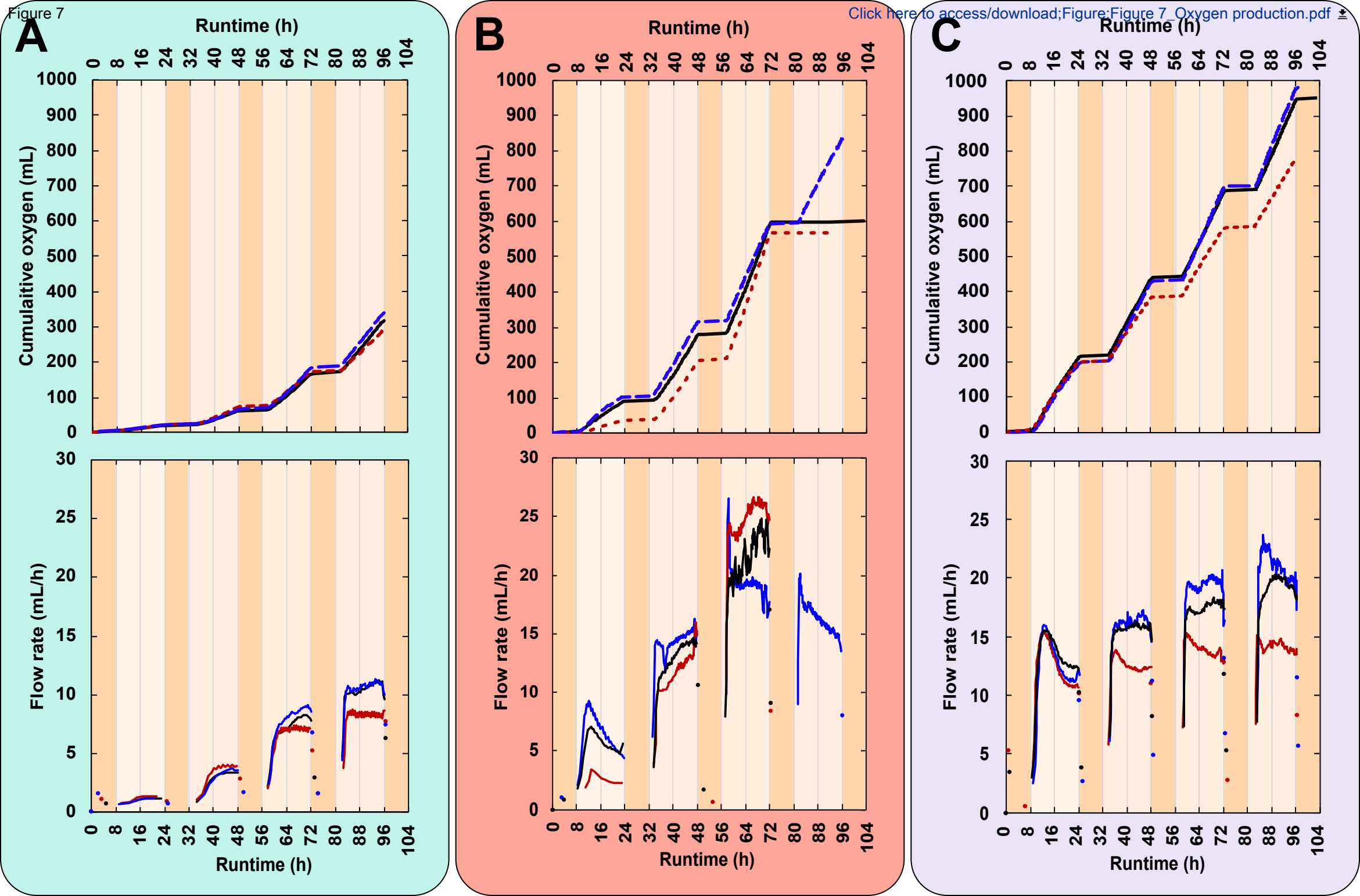


Figure 8

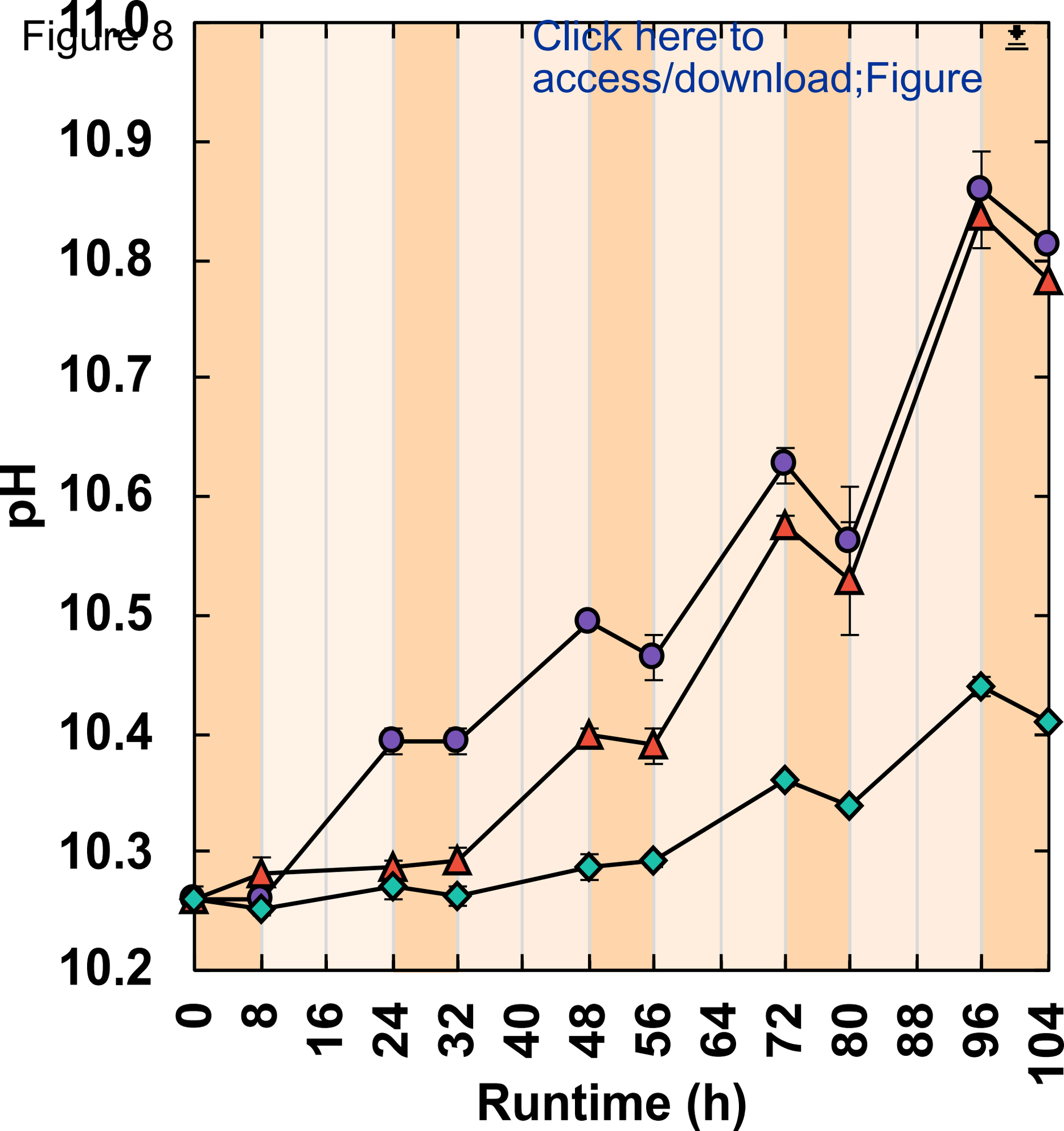


Table 1

Treatment	Light Intensity ($\mu mol_{photons} m^{-2} s^{-1}$)	IBC ($g_{AFDW} L^{-1}$)
A	300	0.031
B	600	0.130
C	600	0.400

* Only one of the three replicates was successful

Δ total biomass (g_{AFDW})	Δ pH	Total oxygen produced (mL)
0.289 (± 0.01)	0.15 (± 0.01)	316.2 (±11.4)
0.674 (± 0.02)	0.52 (± 0.27)	834.6*
0.675 (± 0.02)	0.55 (± 0.03)	902.2 (±50.5)

Table 2

Treatment	Light Intensity ($\mu mol_{photons}$ m^{-2} s^{-1})	IBC (g_{AFDW} L^{-1})	Biomass growth measured (g_{AFDW})	Biomass growth predicted (g_{AFDW})
A	300	0.031	0.289	0.288
A	300	0.031	0.311	0.270
A	300	0.031	0.268	0.247
B	600	0.13	0.708	0.705
C	600	0.4	0.718	0.796
C	600	0.4	0.640	0.830
C	600	0.4	0.668	0.659

Underestimate (%)
0.5
13.1
7.9
0.4
-10.9
-29.7
1.3

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use and use American English.

The document was proofread to correct spelling and grammar. All abbreviations were defined upon first use, e.g., outer diameter (OD) in step 3.5. and digital multiplex (DMX) in step 2.7. The text has been converted to American English.

2. Please reduce your summary's word count to <50.

The summary is now 47 words. Summary - "This publication describes the design of laboratory photobioreactors (PBRs) with customizable light regimes. The growth of cyanobacteria or microalgae, using bicarbonate as their carbon source, is monitored continuously by measuring volumetric oxygen production. These PBRs facilitate rapid, replicated, laboratory growth comparisons with little user intervention during experiments."

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Rigamo; DMX; ESA etc.

Thank-you for clarification. I was unaware that software should also be included in the Table of Materials. This has been amended. DMX stands for Digital MultipleX. It is not a commercial product but rather a type of lighting control, thus this remains throughout the text. The text has been updated so that commercial names are mentioned only once when software is first introduced as suggested. Generic terms like 'data acquisition software' and 'LED control software' are used throughout the remainder of the text.

Line 244 - "5.7. Install the data acquisition software, Rigamo, on a Windows operating system and plug in licensing key dongle. Add sensor calibration files to the software's calibration directory."

Line 252 – "6.2. Download Store Upgrade Tools (SUT) and the LED control software, Easy Stand Alone (ESA). Register the DMX lighting controller online."

4. Obviously, if you have optimized your protocol with certain instruments/reagents/software, you will need to mention them, but include just the bare minimum information (e.g., name of product) in the manuscript and come up with a generic term to refer to the product (include this

in the comments column in the Table of Materials) after the first mention so that you don't keep repeating commercial terms throughout the paper. You can mention products or software in the discussion if you wish to compare them to what you have used.

Refer to the response to comment #3. The Table of Materials has been updated to include the generic term in the comments section. Further, all URL links for software have also been shifted to the Table of Materials.

5. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Text has been revised and personal pronouns have been removed.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Steps within the protocol that were not in the imperative sense have been modified, e.g., step 2.4. was modified to remove "The fit should be snug". Notes have been revised to be more concise. Three notes have been removed altogether, with the information they conveyed instead shifted to the adjacent steps. In particular, the protocol now only recommends colour coding of materials once after step 3.7.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Additional details were added to the protocol to better describe how each step is performed. For example, in step 1.1. and 1.2. it is now specified that fastening/securing is achieved with bolts. In step 2.5, it is now detailed that "medium length rivets" are used to attach the piano hinge. In step 7.8. the text has been updated from "Insert rubber stoppers into each bottle opening and seal with threaded aperture screw caps" to "Seal each bottle opening with a rubber stopper and threaded aperture screw cap." Further, references for determining the ash-free dry weight of microalgal cultures was added to the note after step 7.6.

8. Please sort the Materials Table alphabetically by the name of the material.

The Table of Materials is now sorted alphabetically.

Reviewers' comments:

Reviewer #1:

The manuscript Design, construction, and operation of laboratory photobioreactors with online growth measurements and customisable light regimes provides a detailed protocol for building a DIY PBR and a thorough discussion of specifications.

Clarity: The introduction needs a bit of rephrasing, but the protocol for building the PBR and discussion is clear.

Usefulness: the usefulness of the described DIY PBR is obvious despite some details mentioned in comments 12 and 13.

My comments and suggestions:

1. Please improve the English. The introduction and abstract are a bit difficult to follow.

We agree with reviewer #1s comment and thank them for this advice. The abstract and introduction have been extensively reworked with an emphasis on reader comprehension.

2. Avoid using slash e.g. in and/or, cyanobacteria/microalgae, species/strains...

We have heeded reviewer #1's suggestion and defined microalgae as including both cyanobacteria and microalgae. See line 52 – "Microalgae and cyanobacteria, herein collectively referred to as microalgae for simplicity, are championed for their potential in sustainable biotechnology." This eliminated the need for slashes in this context. Further, all and/or and species/strains have now been removed.

3. It might be better to define microalgae as algae including cyanobacteria in the introduction. That would make it easier to refer to all photosynthesizing microbes (phototrophs, microalgae, microbes, cyanobacteria) at once as well as avoid using a bit unusual terms like microbial photosynthesis.

See the response to comment #2.

4. Line 22 and 34: "...using bicarbonate." - using bicarbonate for what? Probably as a carbon source? Please rephrase.

These sentences have been updated to specify that bicarbonate is used as a carbon source. Line 21 now reads “The growth of cyanobacteria or microalgae, using bicarbonate as their carbon source, is monitored continuously by measuring volumetric oxygen production.” Line 46 now reads “The method is suited for microalgae grown with bicarbonate as the carbon source.” Line 104 now reads “For microalgae that use bicarbonate as the carbon source, there are two additional growth metrics that can be measured continuously.”

5. Line 41: "...and can be adjusted on fine time scales (1 sec)." - not clear what you mean, probably photoperiod? It would be better if this part of the sentence was put after the following sentence dealing with light regimes.

The abstract has been updated for clarity. The segment “…and can be adjusted on fine time scales (1 sec).” has been updated to “Light intensity can be adjusted each second or held constant for longer durations.” This refers to the duration of ‘steps’ within the LED control software. Each step can be set to last between 1 second and 43 minutes and from 0-2400 $\mu mol_{photons}.m^{-2}.s^{-1}$. This is further detailed in protocol step 6.7 and the subsequent note.

6. Line 53: "...and for using sunlight to drive carbon dioxide conversion." - conversion to what? Please specify.

The introduction has been altered to clarify that CO_2 can be converted into products. Line 53-55 - “They are attractive candidates due to their rapid growth, ability to be cultivated on non-arable land, and for their use of sunlight to drive carbon dioxide conversion to biomass. Microalgal biomass can be converted into products like…”

7. Lines 74-82 talk about biomass assessment by measuring DW and the need for large sample volumes. BUT, biomass can be (and very often is) nicely estimated by measuring OD against calibration to DW or pigment content. This can be done within the cultivation vessel if sensors are present (no need to extract samples) or measured by an external spectrophotometer needing usually just 250-1000 uL of sample volume. This makes your paragraph on large volumes a bit obsolete.

This is valuable feedback which has prompted the inclusion of additional information about common growth metrics. It has been noted in line 72 that “a suitable method to accurately quantify growth must be selected.” The text goes on to discuss cell counts, chlorophyll A content, dry weight (DW), ash-free DW (AFDW), and optical density (OD) between lines 74 and 86. OD is indeed a powerful non-invasive continuous method for measuring culture growth. We have previously explored it as an option in our laboratory, however due to the clumping nature of the organisms we work with it was unsuitable. This disadvantage is highlighted in line 83-84. In lines 104-117 we highlight the advantage of pH and O_2 measurements as alternative growth metrics due to their insensitivity to changes in microalgal morphology. We emphasize in lines

84-86 that researchers should choose methods that are both practical and relevant to their experimental aims.

8. Line 84: "When the target organisms use bicarbonate as their source of CO2, no external supply of CO2 is needed." - Please rephrase e.g. external CO2 supply can be replaced by addition of bicarbonate into the cultivation media...

This paragraph containing this sentence has been reordered and rephrased for clarity. Instead, line 112-114 now reads “For pH and oxygen measurements, carbon must be supplied in the form of bicarbonate, not by CO_2 sparging.”

9. It would be nice to mention the list of needed material before you start describing how to build the PBR.

The materials list is provided as a supplementary file. This format was requested by JoVE.

10. Figure 1B - pls change the background of letter B so that the letter is visible.

Figure 1B has been updated.

11. It would be nice to have a picture of the fully equipped PBR, open, including bottles filled with algae culture, preferentially with lights on. But this is just a non-essential detail.

The accompanying video should display the PBR in its entirety during operation.

12. Just a note: The PBR design is good. Nevertheless, I would miss temperature controller and the possibility to control light for each vessel independently.

Temperature control would indeed be a nice addition to the design. We have added a comment in the discussion at line 633-634 – “If temperature control of medium is desired, a recirculating chiller could be added.” The design *could* be altered to facilitate individual lighting control. However, we have refrained from suggesting this as the PBR is intended to facilitate equal lighting intensities to all three bottles for the sake of triplicate replication.

13. It would be good to include information on the possibility to autoclave (or sterilize otherwise) the materials that will get into contact with algae culture. Some tubes or plastic parts might not be autoclavable (rubber stoppers, tubing, sampling port valve...).

Thank-you for pointing out this oversight, we have amended section 7 of the protocol, Starting an experiment’. Now the first step, 7.1, reads “If required, sterilize media, bottles, stir bars, rubber stoppers, sampling ports, threaded aperture screw caps, and tubing.” This is followed by

a subsequent note, “NOTE: All components used in this design are autoclavable except the valves and luer fittings—there are autoclavable alternatives from other manufacturers.”

14. The authors might want to include some comparison of their PBR design with some other existing commercial or DIY PBRs and highlight the advantages of their product preferably in the introduction and also in the conclusion.

Thank-you for this suggestion. We are aware that there are copious numbers of commercial PBRs and DIY prototypes available. What we believe is lacking, is a PBR that bridges the gap between simple benchtop flasks and very expensive, highly technical PBRs. We hope that this design is accessible to individuals or groups without a fabrication or engineering background. The JoVE format aids this by providing a step-by-step construction guide–something that is missing in published DIY PBR protocols. Given the sheer magnitude and variety of PBRs (each suited to a different experimental application) we believe a comparison of PBRs is best suited to a more extensive review article. We have added a paragraph at the end of the introduction which explains the gap this PBR hopes to fill. Further, we recommend that readers consider the suitability of a PBR design to their own culture conditions, financial position, and research questions.

Lines 119-130- “PBRs in use today range in complexity. Laboratories may use simple benchtop flasks, custom prototypes, or commercially available products. For research groups seeking to upgrade from flasks, the cost of commercial PBRs or technical skill and part fabrication required to build many prototypes, may be a barrier. The goal of this manuscript is to describe the step-by-step design, construction, and operation of laboratory PBRs that bridge this gap. These PBRs have a customizable light regime and monitor growth continuously by recording volumetric oxygen production. This design houses three culture vessels for triplicate replication, can be built with moderate skill, and is made with easily accessible materials. This PBR is a valuable addition to a laboratory looking to expand their capacity for microalgal research without investing in very technical or expensive products. When choosing to acquire or build a PBR, researchers must consider the suitability of a design to their own culture conditions, financial position, and research questions.”

In the conclusion, we have focused primarily on the Protocol, as direct by the Instructions for Authors document. Further, the discussion is limited to 6 paragraphs.

Reviewer #2:

Comments for JoVE62910

Design, construction, and operation of laboratory photobioreactor with online growth measurements and customisable light regimes

Keywords: photobioreactor, oxygen production, microalgae, cyanobacteria, biotechnology, photosynthesis.

The JoVE MS showed a lot of interesting information. However, the work just lacks some specific quantitative data ranges. Some comments are as below:

1. All the pages need consecutively numbering.

All pages have been numbered.

2. Line 51 - 53, "Microbial phototrophs……carbon dioxide conversion" What is sustainable biotechnology? How fast is the development?

We welcome the reviewer's suggestion to elaborate on the definition of sustainable biotechnology and comment on the speed of development. However, we feel the introduction adequately conveys how technology which uses microalgal biomass is sustainable. Especially in lines 53-54 – "They are attractive candidates due to their rapid growth, ability to be cultivated on non-arable land, and for their use of sunlight to drive the conversion of carbon dioxide to biomass." We believe that commenting on the speed of development is beyond the scope of this paper and is perhaps instead best suited to a review article.

3. Line 54 - 57, " Microbial phototrophs…….water remediation", more references are needed to clarify their application in making these products. Moreover, how specifically is the application in water and wastewater treatment?

Additional references have been added to the product examples. We have clarified the application briefly in water remediation and wastewater treatment in lines 57-58 – "In addition, they can be used to treat wastewater or remediate waterbodies by consuming excess nutrients".

4. Line 62 - 63, " In cyanobaterial/microalgal……, and method to quantify growth", needs adding references to clarify how the growth of cyanobacteria/microalgae in laboratory studies.

We have added a paragraph in the introduction which outlines the most common ways that microalgal growth is measured. Lines 74-86 – "Growth is commonly measured by cell count, optical density (OD), chlorophyll A content, dry weight (DW) density, and ash-free dry weight (AFDW) density. Cell counts, chlorophyll A content, and gravimetric methods are manual processes which produce discrete data points. OD can be measured continuously and non-invasively with a spectrophotometer, providing it is well calibrated against another method like AFDW density. However, OD measurements and Chlorophyll A content can be unreliable as

results vary under different culture conditions, e.g., between species and throughout the growth cycle. For Chlorophyll A, the extraction method used can also impact pigment yield. Chlorophyll A is typically used to assess the microalgal composition of microbial communities which also include non-photosynthetic organisms. When choosing a method to assess growth, the morphology of the suspension is also important. When organisms clump and are not-well mixed, OD and cell counts aren't possible. Any one method is not suited to all experimental applications—researchers must decide which methods are both practical and relevant to their experimental aims."

The references for this information are available within the manuscript.

5. The sentence "AFDW quantifies the organic or "useful" portion of the biomass by excluding contaminating inorganic salts" (Line 77-78) What is AFDW? How is this quantification?

Ash-free dry weight (AFDW) is defined in the introduction. Paragraph 4, lines 88-102 of the introduction has been expanded to include a thorough overview of the AFDW calculation.

"AFDW enables growth comparisons among a range of culture conditions, notably, between species and culture media. To calculate AFDW, first a sample of microalgal culture is concentrated, either by filtration or centrifugation, and dried. At this stage the DW can be determined. Usually, the DW sample contains at least 8-10% ash—inorganic material like salts and particulate matter. DW tracks growth trends but can be skewed if the contribution of inorganics varies. To determine AFDW density, dry biomass is combusted at high temperature, this vaporizes the organic or "useful" portion while leaving ash (inorganics) behind. To calculate the AFDW the weight of the ash fraction is subtracted from that of the DW fraction. Typically, microalgal suspensions range from 0.1-3 g_{AFDW} per liter. Small volumes of dilute suspensions yield little dry biomass, < 10 mg. After combustion ash may only weigh 1 mg. Therefore, depending on the culture density, this method requires volumes of 5-100 mL, and analytical scales accurate to 0.1 mg. Laboratory PBRs are typically small, a couple of liters at most, hence every liquid sample depletes culture volume. Further, the AFDW method is manual and takes 2-3 days. For replicated and repetitive experiments, an automated and continuous method is preferable."

The references for this information are available within the manuscript.

6. Line 374 - 377, Please explain the basis for choosing this daylight intensity and initial biomass concentration (IBC) for comparison?

For the basis of providing representative results these IBCs and light intensities were chosen arbitrarily for demonstrative purposes only. This information has been added at line 403.

7. The statement, "In our experimental setup (using local temperature and pressure data) we

calculated that an 80 mL headspace required 340 mL of exsolved O2 to establish an O2 partial pressure of 99% of the ambient pressure", additional basis for calculating the amount of O2 needed to establish the partial pressure in this study?

The reason this calculation is important, is that over the course of an experiment, the gas composition of the bottle headspaces will change. Initially the headspace gas composition will be identical to that of the atmosphere (~ 80% N_2 and ~20% O_2). "With each flip of the measurement cell, O_2 displaces atmospheric gas" – Lines 416-417. This means that with time, the headspace partial pressure of O_2 will increase, also increasing the DO of the medium. Here, the 340 mL of O_2 required to reach 99% O_2 partial pressure, is compared to the actual values produced in the study. This demonstrates to the reader, that the headspace gas composition can shift to almost pure oxygen. We have added an additional sentence to line 433 to clarify this – "Therefore, by the end of the experiment, the headspace of all bottles would have contained mostly O_2." The disadvantage of high DO to microalgal cultures and a potential solution is discussed in lines 578-597.

Reviewer #3:

Manuscript Summary:
The paper "Design, construction, and operation of laboratory photobioreactors with online growth measurements and customisable light regimes" nicely presents the build-up of laboratory photo-bioreactor that can be useful to work triplicates in parallel and that may monitor with high resolution the growth of cyanobacterial/microbial biomass. The construction is well detailed, some interesting results and discussion are also provided. The paper is well organized, interesting to be read and the discussion valuable for the following research and it will be very valuable for the filming.

Major Concerns:
My main doubt is that the scientific literature is not well considered, for example there are many examples regarding manometric (or other methods, like microcalorimetric, etc.) that have been previously applied (some systems are also commercial ones) and are not included in this manuscript. Even though the proposed system is more flexible, etc. this comparison is lacking. Most of the presented literature is updated but only demonstrates the need of the system, not the comparison with other systems.

Thank-you for this suggestion. In the introduction we have added a paragraph which considers common methods of growth measurement.

Lines 74-86 – "Growth is commonly measured by cell count, optical density (OD), chlorophyll A content, dry weight (DW) density, and ash-free dry weight (AFDW) density. Cell counts, chlorophyll A content, and gravimetric methods are manual processes which produce discrete

data points. OD can be measured continuously and non-invasively with a spectrophotometer, providing it is well calibrated against another method like AFDW density. However, OD measurements and Chlorophyll A content can be unreliable as results vary under different culture conditions, e.g., between species and throughout the growth cycle. For Chlorophyll A, the extraction method used can also impact pigment yield. Chlorophyll A is typically used to assess the microalgal composition of microbial communities which also include non-photosynthetic organisms. When choosing a method to assess growth, the morphology of the suspension is also important. When organisms clump and are not-well mixed, OD and cell counts aren't possible. Any one method is not suited to all experimental applications—researchers must decide which methods are both practical and relevant to their experimental aims."

It is our opinion that microcalorimetric methods are not commonplace in this field and that their inclusion would be beyond the scope of this manuscript. Methods to measure photosynthetic oxygen production, including manometry, are included in lines 110-111 - "Oxygen production is measured by many different techniques: probes gauge DO concentration, manometric devices assess headspace pressure, gas chromatography measures headspace composition, and volumetric sensors record gas outflow."

We are aware that there are copious numbers of commercial PBRs and DIY prototypes available. What we believe is lacking, is a PBR that bridges the gap between simple benchtop flasks and very expensive, highly technical PBRs. We hope that this design is accessible to individuals or groups without a fabrication or engineering background. The JoVE format aids this by providing a step-by-step construction guide–something that is missing in published DIY PBR protocols. Given the sheer magnitude and variety of PBRs (each suited to a different experimental application) we believe a comparison of PBRs is best suited to a more extensive review article. We have added a paragraph at the end of the introduction which explains the gap this PBR hopes to fill. Further, we recommend that readers consider the suitability of a PBR design to their own culture conditions, financial position, and research questions.

Lines 119-130 - "PBRs in use today range in complexity. Laboratories may use simple benchtop flasks, custom prototypes, or commercially available products. For research groups seeking to upgrade from flasks, the cost of commercial PBRs or technical skill and part fabrication required to build many prototypes, may be a barrier. The goal of this manuscript is to describe the step-by-step design, construction, and operation of laboratory PBRs that bridge this gap. These PBRs have a customizable light regime and monitor growth continuously by recording volumetric oxygen production. This design houses three culture vessels for triplicate replication, can be built with moderate skill, and is made with easily accessible materials. This PBR is a valuable addition to a laboratory looking to expand their capacity for microalgal research without investing in very technical or expensive products. When choosing to acquire or build a PBR,

researchers must consider the suitability of a design to their own culture conditions, financial position, and research questions."

Minor Concerns:
Some minor comments, that the authors may consider for improving the paper quality, are given below.

1) Line 78: "contaminating inorganic salts", I suggest deleting "contaminating.

The word "contaminating" has been removed.

2) Line 79: "in our experience", information from the literature should be provided. The same for similar sentences.

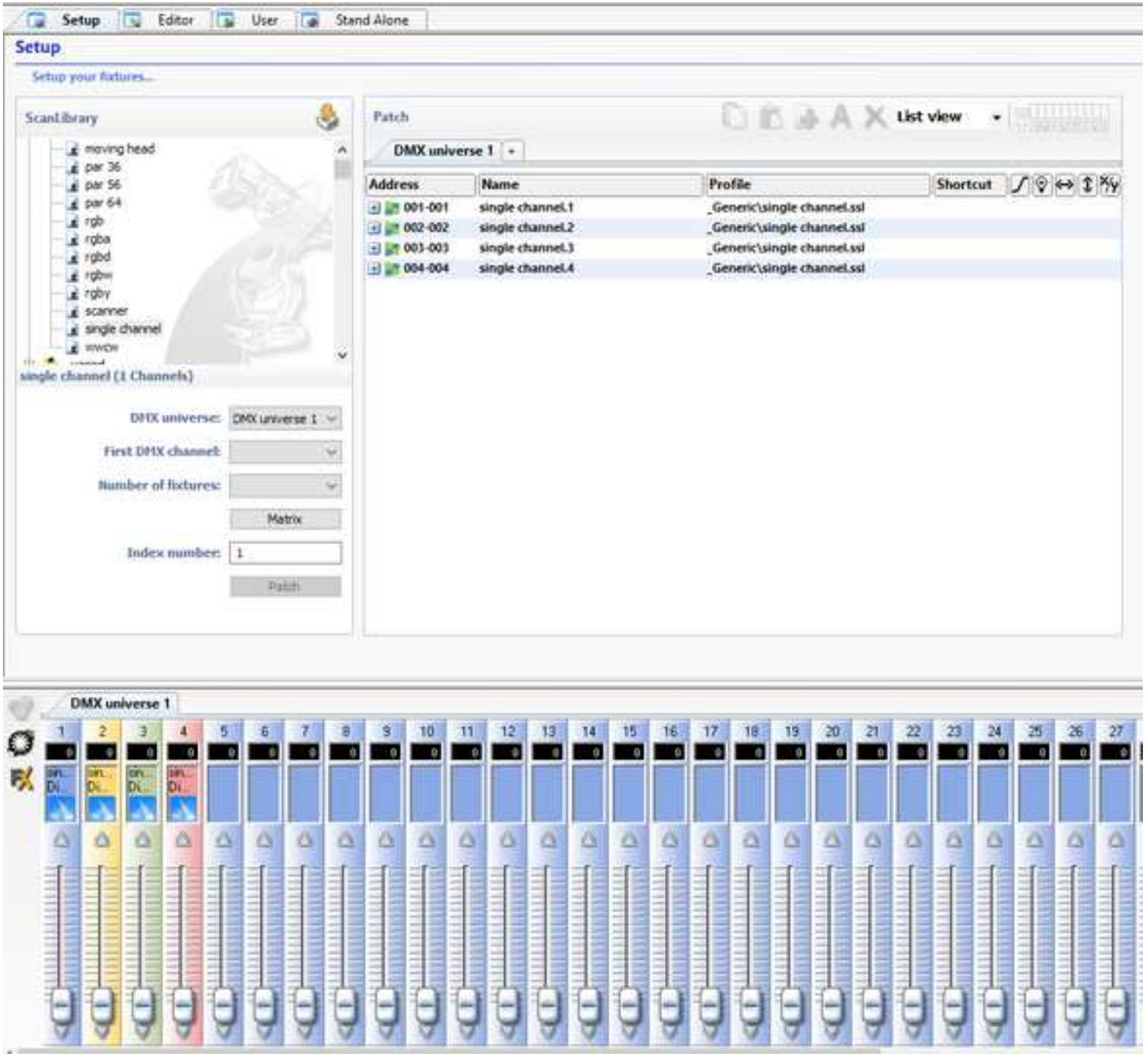
We have rectified this by providing additional referenced information, particularly with regards to the volume of culture required for accurate AFDW determination. The number of cited articles for this manuscript has been increased from 14 to 27.

3) Some parts in chapters 4, 5, 7 and 8 are highlighted in yellow, why?

The journal asks that the portions of the protocol that are intended to be filmed be highlighted this way.

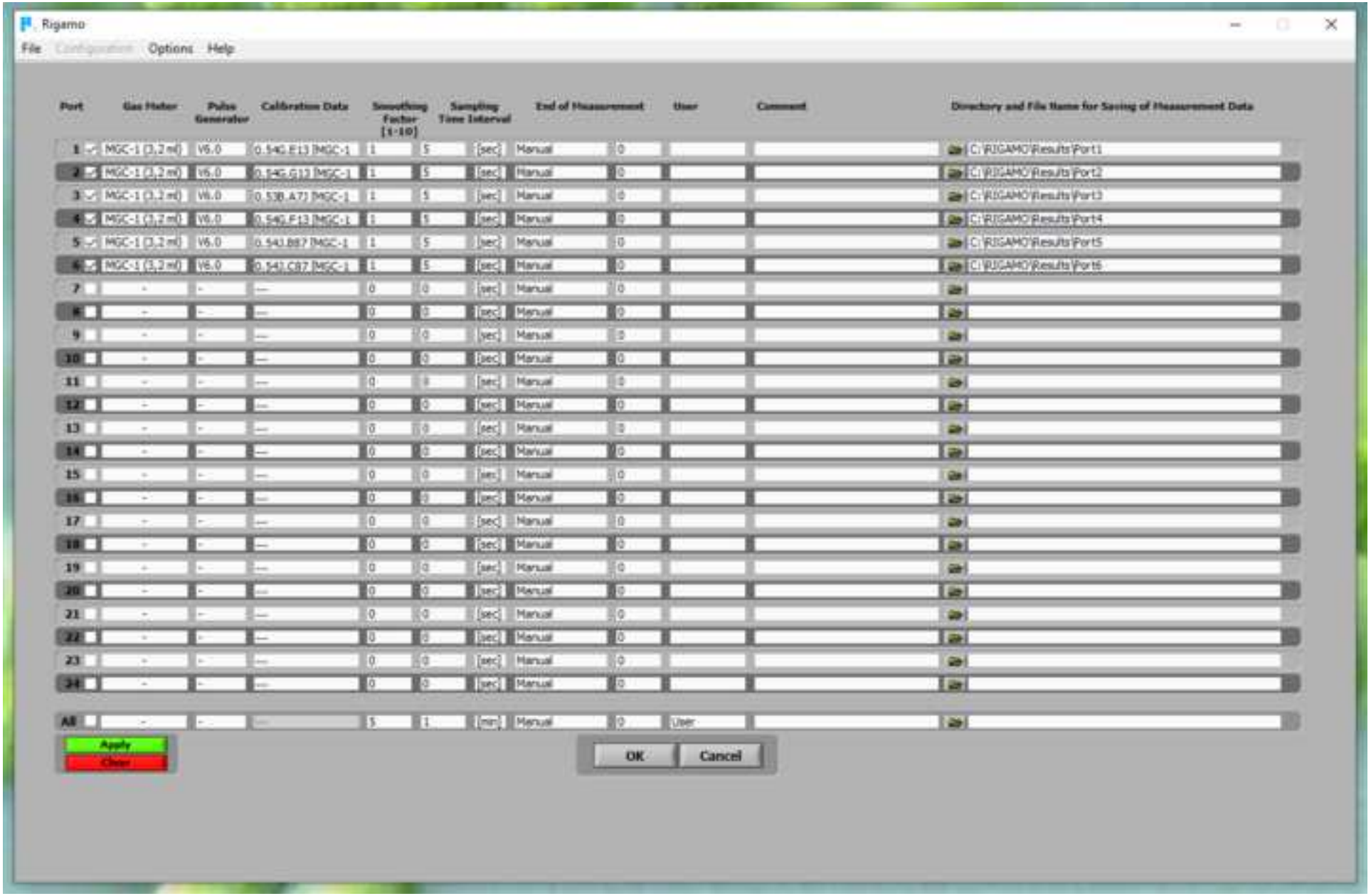
Supplementary Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary Figure 1_Software screenshot_Lighting control.png



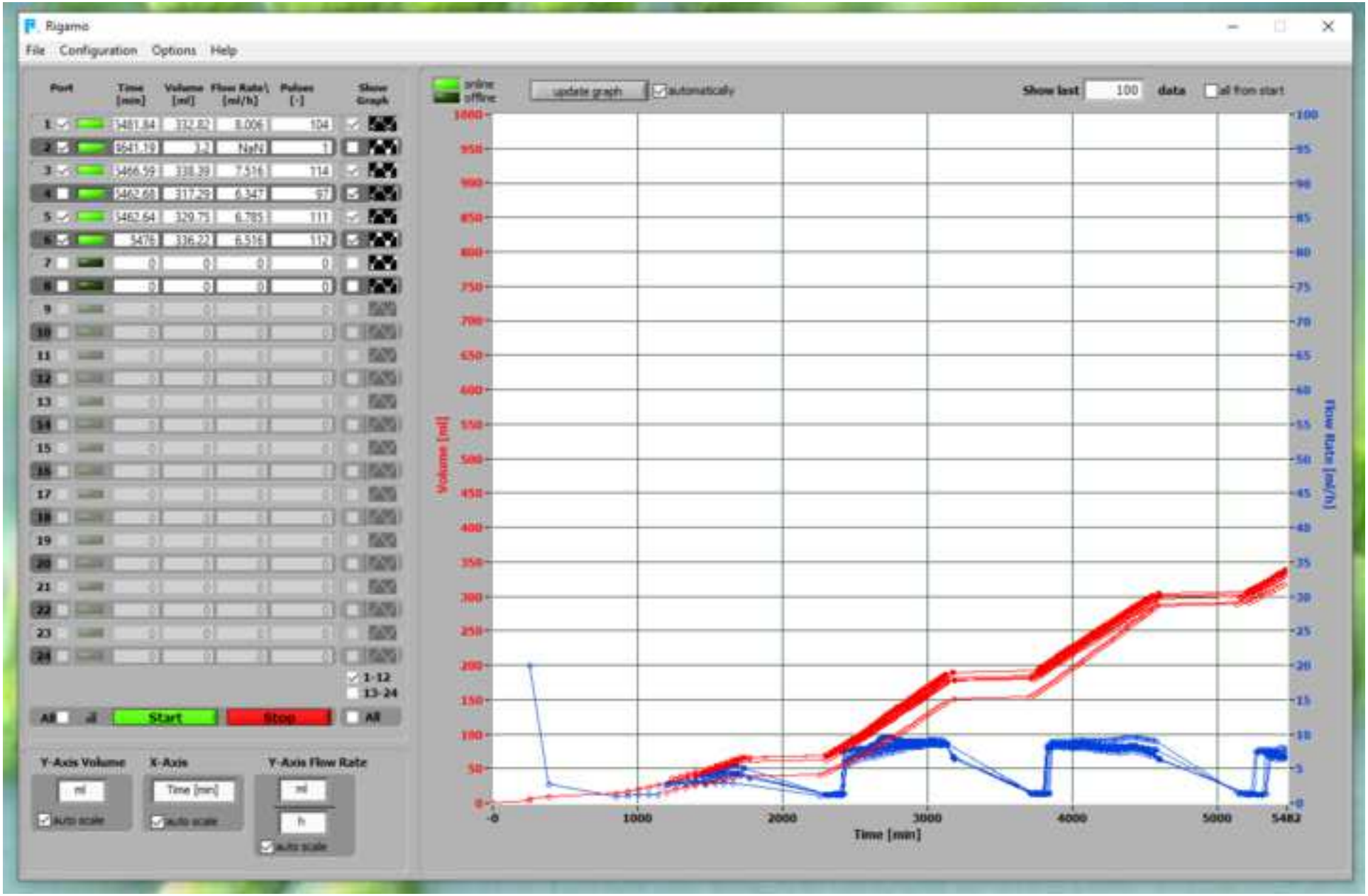
Supplementary Figure 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary Figure 2_Software screenshot_Data aq._Config..PNG



Supplementary Figure 3

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary Figure 3_Software screenshot_Data aq._Logging.PNG



Supplementary Table 1

Set #	Fade	Hold	Dimmer	Time	Light status	Runtime (hr)
1	0 s	30 m	0	9:00 AM	OFF	0.5
2	0 s	30 m	0	9:30 AM	OFF	1
3	0 s	30 m	0	10:00 AM	OFF	1.5
4	0 s	30 m	0	10:30 AM	OFF	2
5	0 s	30 m	0	11:00 AM	OFF	2.5
6	0 s	30 m	0	11:30 AM	OFF	3
7	0 s	30 m	0	12:00 PM	OFF	3.5
8	0 s	30 m	0	12:30 PM	OFF	4
9	0 s	30 m	0	1:00 PM	OFF	4.5
10	0 s	30 m	0	1:30 PM	OFF	5
11	0 s	30 m	0	2:00 PM	OFF	5.5
12	0 s	30 m	0	2:30 PM	OFF	6
13	0 s	30 m	0	3:00 PM	OFF	6.5
14	0 s	30 m	0	3:30 PM	OFF	7
15	0 s	30 m	0	4:00 PM	OFF	7.5
16	30 m	0 m	100	4:30 PM	FADE IN/DAWN	8
17	0 s	30 m	100	5:00 PM	ON	8.5
18	0 s	30 m	100	5:30 PM	ON	9
19	0 s	30 m	100	6:00 PM	ON	9.5
20	0 s	30 m	100	6:30 PM	ON	10
21	0 s	30 m	100	7:00 PM	ON	10.5
22	0 s	30 m	100	7:30 PM	ON	11
23	0 s	30 m	100	8:00 PM	ON	11.5
24	0 s	30 m	100	8:30 PM	ON	12
25	0 s	30 m	100	9:00 PM	ON	12.5
26	0 s	30 m	100	9:30 PM	ON	13
27	0 s	30 m	100	10:00 PM	ON	13.5
28	0 s	30 m	100	10:30 PM	ON	14
29	0 s	30 m	100	11:00 PM	ON	14.5
30	0 s	30 m	100	11:30 PM	ON	15
31	0 s	30 m	100	12:00 AM	ON	15.5
32	0 s	30 m	100	12:30 AM	ON	16
33	0 s	30 m	100	1:00 AM	ON	16.5
34	0 s	30 m	100	1:30 AM	ON	17
35	0 s	30 m	100	2:00 AM	ON	17.5
36	0 s	30 m	100	2:30 AM	ON	18
37	0 s	30 m	100	3:00 AM	ON	18.5
38	0 s	30 m	100	3:30 AM	ON	19
39	0 s	30 m	100	4:00 AM	ON	19.5
40	0 s	30 m	100	4:30 AM	ON	20
41	0 s	30 m	100	5:00 AM	ON	20.5
42	0 s	30 m	100	5:30 AM	ON	21
43	0 s	30 m	100	6:00 AM	ON	21.5
44	0 s	30 m	100	6:30 AM	ON	22
45	0 s	30 m	100	7:00 AM	ON	22.5

46	0 s	30 m	100	7:30 AM	ON	23
47	0 s	30 m	100	8:00 AM	ON	23.5
48	30 m	0 m	0	8:30 AM	FADE OUT / DUSK	24

Supplementary Table 2

Chemical	g/L
Sodium Nitrate	0.34
Magnesium Sulfate Heptahydrate	0.246
Calcium Chloride Dihydrate	0.019
Sodium Chloride	0.025
Potassium Phosphate Dibasic	0.25
Potassium Chloride	0.45
Ferric (III) Ammonium Citrate	0.01
Sodium Bicarbonate	6.54
Sodium Carbonate	22.36

Supplementary Table 3

Chemical	g/L
Titriplex III (EDTA)	10.00
Iron (II) Sulfate Heptahydrate	4.00
Zinc Sulfate Heptahydrate	0.20
Manganese (II) Chloride Tetrahydrate	0.06
Boric Acid	0.60
Cobalt (II) Chloride Hexahydrate	0.40
Copper (II) Chloride Dihydrate	0.02
Nickel (II) Chloride Hexahydrate	0.04
Sodium Molybdate Dihydrate	0.06