Mesostats – A multiplexed, low-cost, do-it-yourself continuous culturing system for experimental evolution of mesocosms

Erika M Hansson[1*], Dylan Z Childs[1], Andrew P Beckerman[1]
1 School of Biosciences, The University of Sheffield, Sheffield, United Kingdom
* emhansson1@sheffield.ac.uk

ABSTRACT

Microbial experimental evolution allows studying evolutionary dynamics in action and testing theory predictions in the lab. Experimental evolution in chemostats (i.e. continuous flow through cultures) has recently gained increased interest as it allows tighter control of selective pressures compared to static batch cultures, with a growing number of efforts to develop systems that are easier and cheaper to construct. This protocol describes the design and construction of a multiplexed chemostat array (dubbed "mesostats") designed for cultivation of algae in 16 concurrent populations, specifically intended for studying adaptation to herbicides. We also present control data from several experiments run on the system to show replicability, data illustrating the effects of common issues like leaks and contamination, and outline possible modifications and adaptations of the system for future research.

INTRODUCTION

Microorganisms provide an unparalleled opportunity for the study of evolutionary dynamics due to their combination of short generation time, simple genetics and ability to fit huge population sizes in a small space. The path of evolutionary adaptation can thus be replicated and tightly controlled in real time in the lab, allowing exciting new insights into the mechanisms of adaptive evolution and testing of predictions from theory (Barrick & Lenski, 2013; Good *et al.*, 2017; Kawecki *et al.*, 2012; Lang & Desai, 2014; Van den Bergh *et al.*, 2018).

The most common way of growing microorganisms for experimental evolution is as batch cultures. This involves serial repetition of small cell population subsets being moved to fresh medium and grown to stationary phase before being transferred again to fresh medium to allow new growth and, with time, adaptation. This is a simple, cheap and scalable method, but its drawback is the resulting fluctuating environment as the cells go through “boom and bust”-cycles at every transfer resulting in a complex selective environment (Gresham & Dunham, 2014; Van den Bergh *et al.*, 2018). As the nutrients in the medium gradually run out, the cells will arrest growth and division while waste products build up. Oxygenation, light levels and pH will also fluctuate with population density. All of this affects cellular metabolism and physiology and subsamples taken from such populations will be growth phase specific, making it difficult to define and isolate the selective pressures acting on the populations (Gresham & Dunham, 2014). Furthermore, there is an evolutionary bottleneck at each transfer, where the considerable reduction in population

size associated with transfer to the next batch affects the genetic diversity and mutational space available (Lenski *et al.*, 1991; Van den Bergh *et al.*, 2018; Vogwill *et al.*, 2012; Wahl & Gerrish, 2001) and thus gives an increased role to genetic drift in the evolutionary outcome (Elena & Lenski, 2003).

Chemostats – continuous flow-through, chemically stable cultures where growth medium and treatments are fed into the fixed-volume populations at a constant rate – solve these issues as the specific growth rate of the population at steady state is matched to the dilution rate (Monod, 1950; Novick & Szilard, 1950). The populations are maintained in exponential growth and constant mixing ensures a homogeneous environment, allowing precise control of the relevant selective pressures compared to the complex dynamics present in batch cultures (Gresham & Dunham, 2014). This unique opportunity for experimental manipulation offers a high-throughput chance to pick apart evolution in action and, as a result, chemostats have recently seen a renaissance in experimental evolution and systems biology as new technological advancements make them easier to maintain than ever before (reviewed in Bull, 2010; Gresham & Hong, 2014). Chemostats also allow following population fluctuations and evolutionary dynamics in response to experimental treatments in the long term, where equilibria and population cycles including several species and strains can be described as a function of the flow rate (e.g. Becks *et al.*, 2012; Declerck *et al.*, 2015; Fussmann *et al.*, 2000; Hiltunen *et al.*, 2014; Yoshida *et al.*, 2003). Multiplexed arrays, where the dilution rate is set by a single pump, and medium sources can be shared, further minimise variation between population chambers (Dénervaud *et al.*, 2013; Ekkers *et al.*, 2020; Miller *et al.*, 2013; Skelding *et al.*, 2018; Tonoyan *et al.*, 2020; Toprak *et al.*, 2013; Wong *et al.*, 2018).

Here I describe a multiplexed small-scale DIY chemostat array system (dubbed "mesostats") adapted from the ministat array developed by Miller *et al.* (2013) to suit experimental evolution of herbicide resistance in an algal model species (*Chlamydomonas reinhardtii*). *C. reinhardtii* is an established model species for herbicide resistance evolution (Lagator *et al.*, 2013a,b; Reboud *et al.*, 2007; Vogwill *et al.*, 2012) and molecular analysis of herbicide resistance mutations (Erickson *et al.*, 1984, 1989; Randolph-Anderson *et al.*, 1998), but all studies to date have used batch cultures.

There are three substantive changes from the Miller *et al.* (2013) ministats, two generic and one system specific to algae research. The first change is the introduction of a needle and syringe system to facilitate 1) sampling from the middle of the active culture rather than relying on the overflow which samples only from the top as well as constituting a different environment and 2) addition of cells and compounds throughout the experiment without disconnecting the medium influx or efflux channels. The second change is an increase in the chamber volume to allow larger population sizes and possible future introduction of several trophic levels. Lastly, the flow rate and light sources were adapted to suit the study species *C. reinhardtii*, as well as changes to specific materials to lower the costs.

METHODS

AN OVERVIEW OF THE DESIGN

The mesostat array consists of four main parts: (1) the medium line, (2) the culture chambers, (3) the overflow chambers and the (4) aeration line. Medium is pumped from the medium containers via a peristaltic pump into the culture chambers (mesocosms) where the experimental organisms are grown. Air is pumped through a gas washing bottle into the culture chambers via the aeration needle to ensure mixing and create pressure so liquid flows out through the efflux needle and the culture stays a fixed volume. The efflux line leads to an overflow chamber, and the volume collected in these is regularly measured to ensure equal flow rates between all culture chambers. Samples for analysis can be obtained from the overflow, but this only samples from the top of the culture and the environment in the collection receptacle may differ from the culture chambers. To

allow sampling from lower levels of the culture, sampling needles have been fitted to the culture chambers. The sampling needle can also be used for inoculation or addition of treatments to the chambers. The entire setup can be kept in a controlled temperature room which ensures low levels of evaporation, but the culture temperature can also be maintained by other methods such as a light table or a water bath. When growing photosynthetic organisms such as algae, even light levels for all chambers are best maintained by a light table as well as fitting strip lights around the chambers. An overview of the full design is seen in Figure 1 and Figure 2A, as well as full assembly schematics in item Figure 3–5. The control conditions are summarised in Table 1.

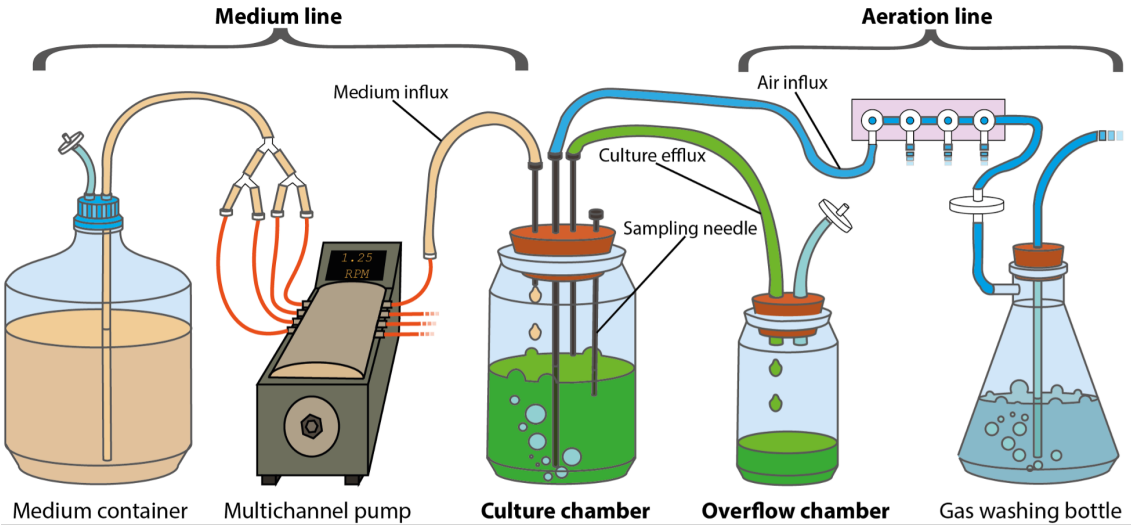

Figure 1: Simplified schematic overview of the mesostat system showing medium containers, the pump, the culture chamber with sampling needle, the overflow bottle, the gas washing bottle along with the medium and air influx lines and the culture efflux line.

THE MEDIUM CONTAINERS

The volume and number of medium containers depends on the experimental design and number of treatments. When the experimental design calls for different treatments applied via the medium, culture chambers sharing a treatment share a medium source. The volume of the medium containers should be chosen to allow sufficient medium to supply all its chambers for at least 5 days, to ensure the time to prepare new sterile medium before changing is needed. The depletion rate of the medium will depend on the number of chambers sharing a container and the flow rate.

The medium container should be sealed with a lid or stopper with a hole that allows air to escape through a filter. If the medium outflow can be through either a tap at the bottom of the container, or tubing through the lid siphoning medium from the bottom.

For the data presented in this thesis, a single 20 L autoclaveable glass container, fitted with a silicone stopper and a filter (0.45 µm) on top and a tap connected to large silicone tubing (inner/wall diameter: 12.5/2.25 mm) at the bottom, is used when all 16 chambers are receiving the same medium.

For data obtained from experiments where different treatment levels were present, 5 L or 2 L autoclaveable glass bottles are used for the treatments, each fitted with medium silicone tubing (inner/wall diameter: 6.5/1.5 mm) siphoning the medium out through holes in the lids alongside tubing ending in a filter to allow air to escape the bottle (Figure 2B). For example, as seen in Figure 2A, 6 different herbicide concentrations are applied through the medium to two chambers each from 2L bottles, and the control treatment is supplied to four chambers from a 5L bottle.

THE PUMP AND THE MEDIUM LINES

The medium is pumped from its container(s) by a multichannel peristaltic pump. Our design employed a Watson-Marlow 205S/CA16 (16 channels) and pumped liquid through small silicone tubing (inner/wall diameter: 3/1 mm), connected to the medium container tubing with a reducing connector. The tubing is split using Y-connectors before the pump so that each culture chamber has its own media line. The same tubing is used throughout the mesostat array (Figure 2A), except for the tubing mounted in the pump itself which is autoclaveable marprene tubing (Watson-Marlow, orange/orange, 0.88 mm bore), connected to the silicone tubing using cut off and blunted hypodermic needles (18 G, 50 mm) inserted into the pump tubing and connected to the silicone tubing using male luer locks (Figure 2C).

THE CULTURE CHAMBERS

Each culture chamber consists of a 500 ml glass jar, sealed with a rubber bung. The rubber bung has four hypodermic needles inserted through it (Figure 2D): a medium influx needle (18 G, 50 mm) connected to the tubing running through the pump, an aeration needle (16G, 203 mm) connected to the aeration system allowing constant mixing of the culture, a sampling needle (16 G, 203 or 101 mm depending on sampling needs), and an efflux needle (16 G, 101 mm) which sets the culture volume.

Tubing is connected to the medium influx, aeration, and efflux needles using male luer locks. A sterile syringe is used with the sampling needle to pull samples out of the chamber or for injections, which is kept sealed with a male luer cap when not in use.

THE AERATION SYSTEM

The culture chambers have a constant influx of air for mixing to prevent the organisms – in our case algae – from sedimenting, and to create pressure for the efflux of liquid so that the culture is kept at a constant volume. Air can be supplied by lab/building infrastructure, e.g. air supply taps, or by an aquarium pump with high enough pressure. The air passes through a filter (0.2 µm) into a gas washing bottle to prevent evaporation. The gas washing bottle consists of a 1L flask with a sidearm, with the incoming air being passed through a glass pipette into distilled water. The air is pushed from the gas washing bottle through 4-port manifold connectors (180° rotation), splitting the air supply to tubing for each culture chamber and passing it through a second filter (0.45 µm). This tubing connects to the aeration needle fitted in the culture chamber bung. The air supply can be controlled using an adjustable clamp fitted to the tubing between the gas washing bottle and the manifold connector.

THE OVERFLOW CHAMBERS AND THE EFFLUX LINE

The efflux line from each culture chamber leads to an overflow chamber consisting of a 175 ml glass bottle sealed with a rubber bung (Figure 2E). The efflux tubing is connected to a hole in the rubber tubing using a female luer lock. A second female luer lock is also fitted on the opposite side of the hole on the underside of the bung, to serve as a funnel for the incoming liquid. A second hole connected with a female luer lock to tubing ending with a filter (0.45 µm) allows air to escape the collection chamber. This chamber should be emptied regularly and can be used to control that the flow rate remains equal between the culture chambers. Samples can be obtained from the overflow chamber, but the environment in the overflow chamber will be different from the culture chambers. With a low flow rate and a high temperature environment, the culture will evaporate quickly when its volume is low. The overflow chamber is also not being diluted with fresh medium,

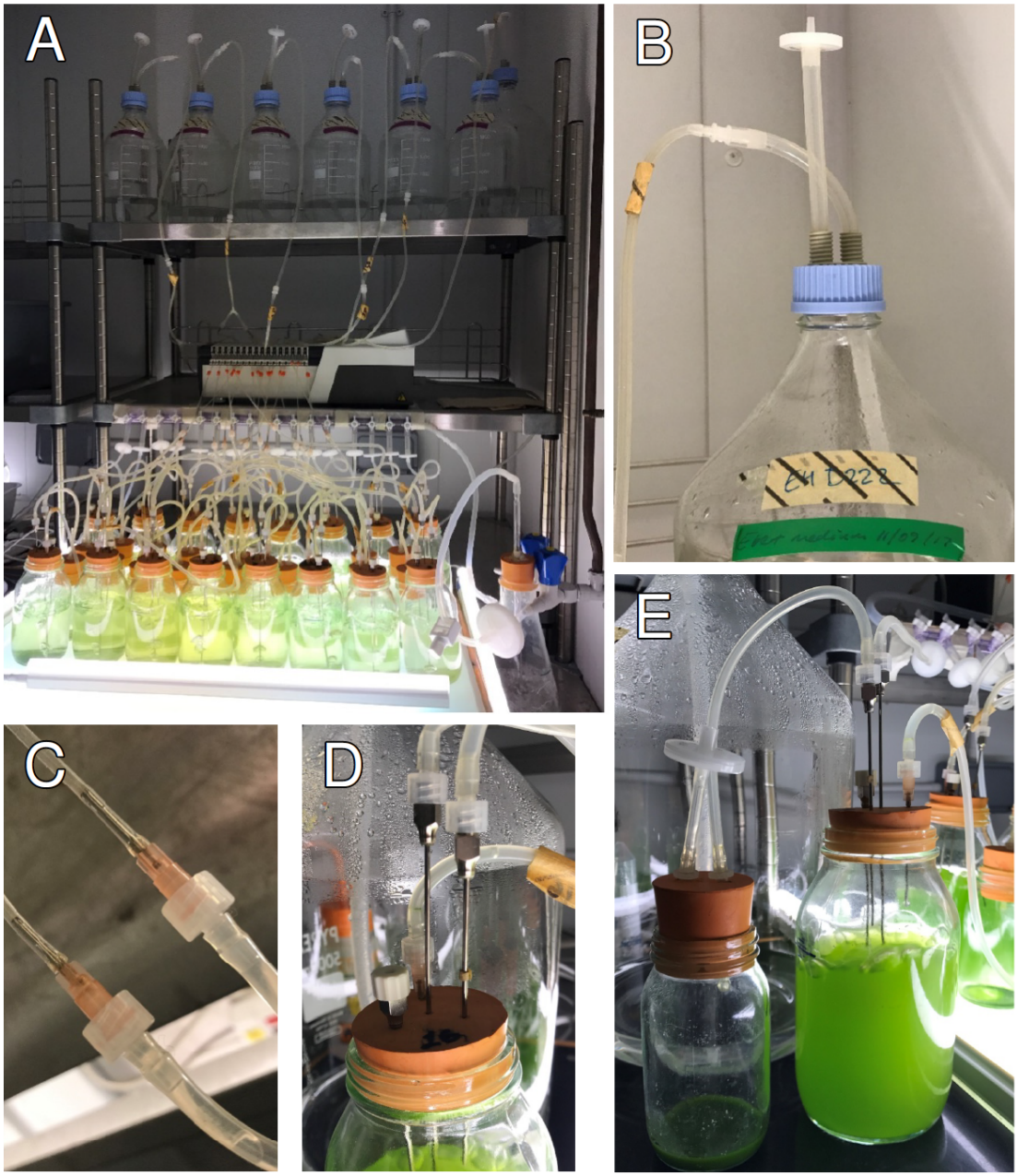

Figure 2: A) The complete setup just after inoculation with algae, running an experiment with six levels of treatments applied through the media lines. B) Close-up of medium siphon through medium container lid. C) Close-up of connection between pump tubing and silicone tubing used throughout array. D) Close-up of culture chambers rubber bung with the four hypodermic needles, capped sampling needle to the left in foreground, steel efflux needle to the right in foreground, steel aeration needle in the middle, and pink plastic medium influx needle in the background. E)The overflow chamber (left) and the culture chamber at steady state (right) with the efflux line running between them.

meaning the cells are no longer kept in exponential growth or connected to the aeration line, often resulting in sedimentation and stratification of the culture.

MATERIALS AND EQUIPMENT

Here we present a complete list of materials required to construct a 16-chamber array. Note that the media containers and associated lids are listed as optional, as exact size and number needed depend on the experimental design. If the company and product code is not listed, the part was not acquired new and the exact same product is no longer sold. All of these are however fairly standard pieces found in many wet labs and similar products can be obtained easily.

1. Small tubing, 20 m (Fisherbrand™ Silicone Tubes; inner/wall diameter: 3.0/1.0 mm, Fisher Scientific, 10111801)
2. Medium tubing, 20 m (Fisherbrand™ Silicone Tubes; inner/wall diameter: 6.5/1.5 mm, Fisher Scientific, 10549201)
3. Large tubing, 10 m (Fisherbrand™ Silicone Tubes; inner/wall diameter: 12.5/2.25 mm, Fisher Scientific, 10726931)
4. Reducing connector, 10 (Reducing Connector PVDF; 1/4" to 1/8", Cole Parmer, EW-30703-50)
5. Male luer lock, 100 (Cole-Parmer ADCF Male Luer to 1/8" L Barb Adapter, Cole Parmer, WZ-30800-24)
6. Female luer lock, 50 (Cole-Parmer ADCF Female Luer to 1/8" L Barb Adapter, Cole Parmer, WZ-30800-08)
7. Y-connector, 40 (Barbed Y Connector; PVDF; 1/8", Cole Parmer, WZ-30633-44)
8. Straight connector, 10 (Barbed fittings; Straight Connector; Kynar; 1/4" ID Cole Parmer, WZ-30703-05)
9. Gas washing bottle, 1 (1L flask with sidearm tubulation)
10. Glass pipette, 1 (10 ml glass pipette)
11. 4-port Manifold, 4 (Polycarbonate individual manifolds with luer locks; 4 ports; 180° rotation Cole Parmer, EW-06464-85)
12. 0.45 µm filters, 100 (PTFE Nonsterile Syringe Filters; 0.45 micron; 25 mm dia, Cole Parmer, WZ-02915-22)
13. 0.2 µm filters, 1 (AcroVent 0.2µm PTFE, Pall Corporation, 4249)
14. Air tubing clamp, 1 (Adjustable tubing clamp)
15. Multiplexed peristaltic pump, 1 (205S/CA16 16 Cartridge pump, Watson-Marlow, 020.3716.00A)
16. Pump tubing, 18 (Autoclaveable marprene manifold pump tubing; orange/orange; 0.88 mm bore, Watson-Marlow, 978.0088.00+)
17. Culture chamber jars, 16 (Clear glass powder jars; 500 ml)
18. Culture chamber rubber bungs, 16 (Fisherbrand™ Solid Rubber Stoppers; 45 mm bottom; 51 mm top Fisher Scientific, 41122502)
19. Aeration needle, 16 (Central Surgical Company™ Stainless Steel Needle; 16 G; 203 mm, Fisher Scientific, 12329259)
20. Media influx needle, 48 (B Braun™ Hypodermic Needles Pink 1.2 mm 18 G 50 mm, Fisher Scientific, 10722784)

21. Efflux needle, 16 (Central Surgical Company™ Stainless Steel Needle; 16 G; 101 mm, Fischer Scientific, 12339259)
22. Sampling needle, 16 (Central Surgical Company™ Stainless Steel Needle; 16 G; 101 mm, Fischer Scientific, 12339259 *or* Central Surgical Company™ Stainless Steel Needle; 16 G; 203 mm, Fisher Scientific, 12329259)
23. Male luer cap, 16 (Male Luer Lock Plug; Nylon, Cole Parmer, WZ-45505-56)
24. Collection chamber jars, 32 (Clear glass powder jars; 175 ml)
25. Collection chamber rubber bungs, 16 (Fisherbrand™ Solid Rubber Stoppers; 37 mm bottom; 42.5 mm top, Fisher Scientific, 41122502)
26. 20 L medium container, 2 *optional*
27. Silicone stopper for 20 L medium container, 2 *optional*
28. 5 L medium container, 2 (Pyrex™ Borosilicate Glass Reagent Bottles with Polypropylene Cap and Pouring Ring; 5000 mL, Fisher Scientific, 12094637) *optional*
29. 2 L medium container, 20 (Pyrex™ Borosilicate Glass Reagent Bottles with Polypropylene Cap and Pouring Ring; 2000 mL, Fisher Scientific, 11922629) *optional*
30. Lids with holes for 5 L and 2 L medium containers, 14 (GL45 Screw cap for Pyrex GL 45 media-lab bottle, Fisher Scientific, 15173927) *optional*
31. Syringes, 100 (BD Discardit™ Eccentric Luer-Slip Two-Piece Syringe, Fisher Scientific, 10152534)

PROTOCOLS

ASSEMBLING THE MESOSTATS FOR THE FIRST TIME

See item Figure 3–5 for visual representation of how the parts connect. A more detailed description with possible variations in design is given below. Place all vessels and machinery in their intended location before cutting the tubing to ensure sufficient lengths and to minimise mistakes.

- Medium container(s):
  1. Prepare the medium container lids.
     (a) If using silicone stoppers:
        i. If the medium container has a tap, the silicone stopper only needs a hole for air to escape. Drill a hole large enough to squeeze medium size tubing through but ensuring a tight seal so that no air can escape around the edges. Attach a short piece of tubing, sticking out about 1 cm on the underside of the stopper and 5 cm on top. Attach a 0.45-micron filter to the tubing.
        ii. If the medium container does not have a tap, a medium siphon is also needed. Drill a second hole in the silicone stopper of the same size and squeeze through medium size tubing long enough to reach the bottom of the container and sticking up about 5 cm on top of the stopper.
     (b) If using screwtop lids with holes, first insert a short piece of medium size tubing through one hole so that it ends just inside the lid and sticks out about 5 cm on top. Attach a 0.45-micron filter to the tubing. For the medium siphon, insert a piece of medium size tubing, long enough to reach the bottom of the container and stick out about 5 cm on top of the lid.

2. Attach the lid or stopper to the medium container.
   (a) If there is a medium siphon, attach the long piece of tubing to a reducing connector.
   (b) If there is no medium siphon and the medium container has a tap, attach a short piece of appropriate size tubing to the tap and attach this to a reducing connector.

- Media lines:

3. Attach a short piece of small size tubing to the other side of the reducing connector and attach it to a Y-connector. Split the medium line into as many channels as needed by connecting short (2—3 cm) pieces of small tubing and Y-connectors. Channels receiving the same treatment should share a medium source.
4. For each channel, attach small size tubing of sufficient length to reach the pump and end with a male luer lock.
5. For each channel, cut off two 18G hypodermic needles to 0.5 cm and insert them into the ends of a piece of pump tubing. Be careful to not cut up the inside of the pump tubing, if pieces detach, they will cause blockages. The ends of the needles may need additional blunting or filing to reduce sharpness. A damaged piece of pump tubing can easily be cut off.
6. Connect the pump tubing to the media lines by screwing the needle luer end to the male luer lock. Screw another male luer lock to the other end of the pump tubing.
7. Cut a piece of small size tubing long enough to reach from the pump to the culture chamber with some slack. Plan carefully where each chamber is going to sit, if multiple treatments are used they should be distributed randomly throughout the array to avoid effects of e.g. differing light level. Attach this piece of tubing to the male luer lock at the end of the pump tubing, and end with another male luer lock.
8. Label the tubing with autoclave tape so you know which chamber it should connect to.

- Culture and overflow chambers:

9. For each chamber, prepare the culture chamber rubber bung by drilling four 1 mm holes, one for each hypodermic needle. This aids pushing the needles through, but the seal around them should still be very tight.
10. Push through one 18 G short needle (medium influx), one 18 G 20 cm needle (air influx) and two 18 G 10 cm needles (culture efflux and sampling needle). A longer sampling needle can be used if desired.
11. Label the rubber bung so you know which chamber it belongs to.
12. Mark the 500 ml glass jar at the desired volume (380 ml) as well as ±19 ml (±5%) to make the magnitude of any volume inconsistencies easier to judge by eye.
13. Attach the media line to the medium influx needle.
14. Cover the sampling needle with a male luer cap.
15. Attach a male luer lock to the culture efflux needle and set the needle at the height required for the desired culture volume, i.e. it should be skimming the surface of the culture.
16. For each culture chamber, prepare an overflow chamber. For each overflow chamber, prepare a rubber bung by drilling two holes large enough to squeeze the lock end of female luer locks into (approximately 2.5 mm in diameter). Connect one hole with a small size of tubing to the male luer lock on the culture efflux needle, and attach another female luer lock on the underside of the bung as a funnel. Attach a short piece of small size tubing to the other hole and end with a 0.2-micron filter.
17. Attach a male luer lock and push the air influx needle down to touch the bottom of the chamber.
18. Mount lights around the chambers and/or place the chambers on a light table, using a light meter to ensure light levels are even.

- Aeration system:

    19. Mount the 4-port manifold(s) above the chambers using clamps or tape so that the aeration tubing will be held up without kinks.
    20. Attach a short piece of small size tubing and a 0.2-micron filter to each active port on the manifold.
    21. Connect a small size tube to the air influx needle long enough to reach the 4-port manifold and attach to the other side of the filter.
    22. Prepare the gas washing bottle rubber bung by drilling a hole large enough to push the long glass pipette through, but tight enough that no air can escape around it. Push the pipette through down to the bottom of the flask. If needed, use a sealant around edges of the hole.
    23. Connect the top of the glass pipette to the air supply (building supply or an aquarium pump) with appropriate size tubing.
    24. Put dH2O in the gas washing bottle. The water level should be so that the water sufficiently covers the air outflow from the pipette, but not so that water enters the sidearm when the air is on. Mark upper and lower water levels on the gas washing bottle.
    25. Connect the sidearm to a 0.2-micron filter with a short piece of medium size tubing and the filter to the manifold with a longer piece of medium size tubing. A clamp can be placed on this part of the tubing to control air flow if needed.

AUTOCLAVING

All parts that will come into contact with the medium need to be sterilised before use:

1. Medium container(s):
    (a) Disconnect the reducing connector on media line between the medium container and the pump tubing.
    (b) Prepare the medium and place in the container as it should be autoclaved with the medium inside.
    (c) Place a filter in the open tubing on the medium container.
    (d) Seal tightly with autoclave bags and autoclave tape around all filters.
    (e) Autoclave at 121 (15psi) for 30 min (longer might be necessary for larger containers).
2. Media line:
    (a) Disconnect at male luer locks to media influx needle.
    (b) Neatly roll up each media line and secure with autoclave tape.
    (c) Place all pieces of tubing in an autoclave bag. Autoclave at 121 (15 psi) for 30 min.
3. Culture chamber and collection chamber bungs:
    (a) Disconnect filters from tubing to 4-port manifold, and disconnect the male luer locks to the efflux line.
    (b) Place the rubber bungs with the needles and tubing in place into autoclaveable trays.
    (c) Place the trays in autoclave bags and seal with autoclave tape, taking care to not let the needles pierce the bag.
    (d) Autoclave at 121 (15 psi) for 30 min.
4. Culture chamber and collection chamber jars:
    (a) These jars are not autoclaveable. Sterilise using 70% IMS and rinse with dH2O.

PREPARING FOR AN EXPERIMENT

1. Reconnect all parts after sterilising. Wear gloves washed with 70% IMS at all times, and wear eye protection when handling the hypodermic needles.
2. When the array is assembled, start the pump to fill up the chambers and turn on the aeration system. At max speed (90 RPM) the flow rate is 2.92 ml/minute, meaning it will take approximately 2 hours and 10 minutes to fill the chambers to 380 ml.
3. When the chambers are full, ensure medium levels are equal and set pump to experiment speed. Adjust the efflux needle as necessary and monitor the overflow bottles to ensure efflux is equal.
4. Turn on the lights and ensure control conditions for light level, internal culture and ambient temperatures are met (see Table 1).

Table 1: Control conditions for *C. reinhardtii* cultures.

Light level	75 µmol m^{-2} s^{-1}, 24 h, all directions
Internal culture temperature	30°C
CT room ambient temperature	25°C
Medium flow rate	0.15/day, 1.25 RPM pump speed
Sampling frequency and volume	1.5 ml day
Medium	Ebert algal medium (Ebert, 2013)

INOCULATING WITH ALGAE

Inoculating with *C. reinhardtii* from static stock culture:

1. Fill up a couple of 15 ml falcon tubes with stock solution and centrifuge at 2000 RPM for approximately 20 minutes. If there is a red layer on top of the green pellet, remove it as this is bacterial contamination. Pour out the supernatant and mix the pellet with fresh, sterile medium. Repeat washing procedure twice.
2. Use a sterile syringe to push an equal amount of freshly washed algal cells into each chamber through the sampling needle. Note: The inoculation volume will vary depending on the amount of stock algae available, the stock density and the desired starting density for the cultures.
3. Allow cultures to reach steady state before applying experimental treatments. See below for methods for how to sample to estimate concentration.

APPLYING TREATMENTS

Treatments can be applied gradually through the medium line by adding the compound directly to the medium or as shock injections through the sampling needle. The two methods can also be combined. For shock injection:

1. Prepare a mixture of medium and compound at as high a concentration as practical to ensure the volume injected into the chamber is as small as possible and reducing the effects of dilution of the culture.

2. Before injection, remove culture at a volume corresponding to the intended injection volume by using a sterile syringe to pull it out through the sampling needle. This so the treatment can be adequately mixed into the culture, and to avoid a sudden rush of culture through the efflux line.

3. Inject the treatment in through the sampling needle using a sterile syringe. All chambers should receive the same injection volume, including controls.

DAILY MAINTENANCE

To ensure equal conditions in all chambers, the mesostat array must be attended to daily according to the daily maintenance protocol:

1. Check water level in gas washing bottle is between max and min markings. Top up with distilled water if running low. Avoid going over the max marking, as this will cause water to bubble into the airflow tubing which can lead to blocked filters.

2. Check culture chamber airflow is satisfactory and equal. If a culture chamber has low airflow, check if the filters are blocked, first the filter on the adjoining collection chamber, then the filter connecting to the manifold, and replace if necessary. Make a note of airflow problems data is being collected if it is possible the problems have been present for more than an hour.

3. Check for leaks around connectors and luer locks. If leaking, first try tightening. If that does not help, replace with a new part (sometimes autoclaving can warp the luer locks), taking care to sterilise the new part with 70% IMS.

4. Check culture levels are even and do not deviate from the 380 ml line. If the medium level is too low, check the media influx tubing and needle for blockages. If medium influx is normal, adjust the efflux needle, and check if the level is back to normal in a couple of hours (time needed to wait dependent on flow rate and total volume deviation). If the level is too high, examine the efflux tubing and needle for blockages, along with the collection chamber filter. When unblocked, the culture chamber level should return to normal volume relatively quickly. Always make a note of culture level changes if data is being collected.

5. Check collection bottle levels are even, and measure volume of a few to ensure the flow rate is correct. Empty the collection bottles and note the time, so that flow rate can be calculated when the bottles are next emptied.

SAMPLING AND MONITORING

1. Temporarily restrict the airflow using the adjustable clamp on the tubing. This prevents liquid from bubbling up through the sampling needle.

2. Wearing gloves washed with 70% IMS, unscrew the cap on the sampling needle and use a sterile syringe to extract liquid.

3. Put the sample in a labelled Eppendorf tube and put the cap back on the sampling needle, ensuring it is screwed on tightly. Clean up any spillage.

4. Repeat for each chamber.

5. Do not forget to turn the airflow back on.

6. The samples may be stored for later processing or counting, either through flash freezing with LN_2 and subsequent storage at -80°C, or by mixing with Lugol's solution and storing in a fridge at 4°C depending on the intended use for the samples.

ASSEMBLY SCHEMATIC

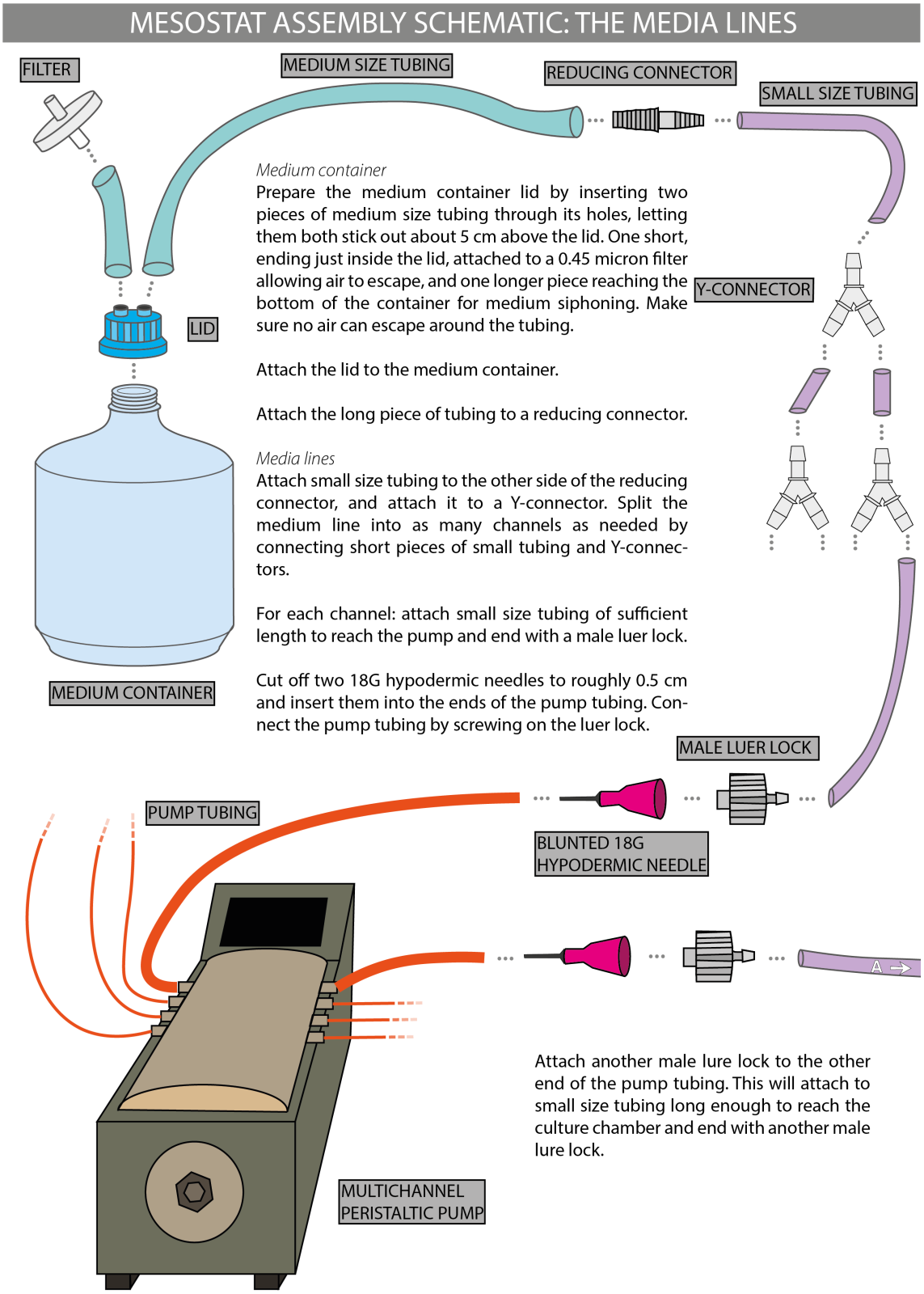

Figure 3: Assembly schematic part 1, the media lines.

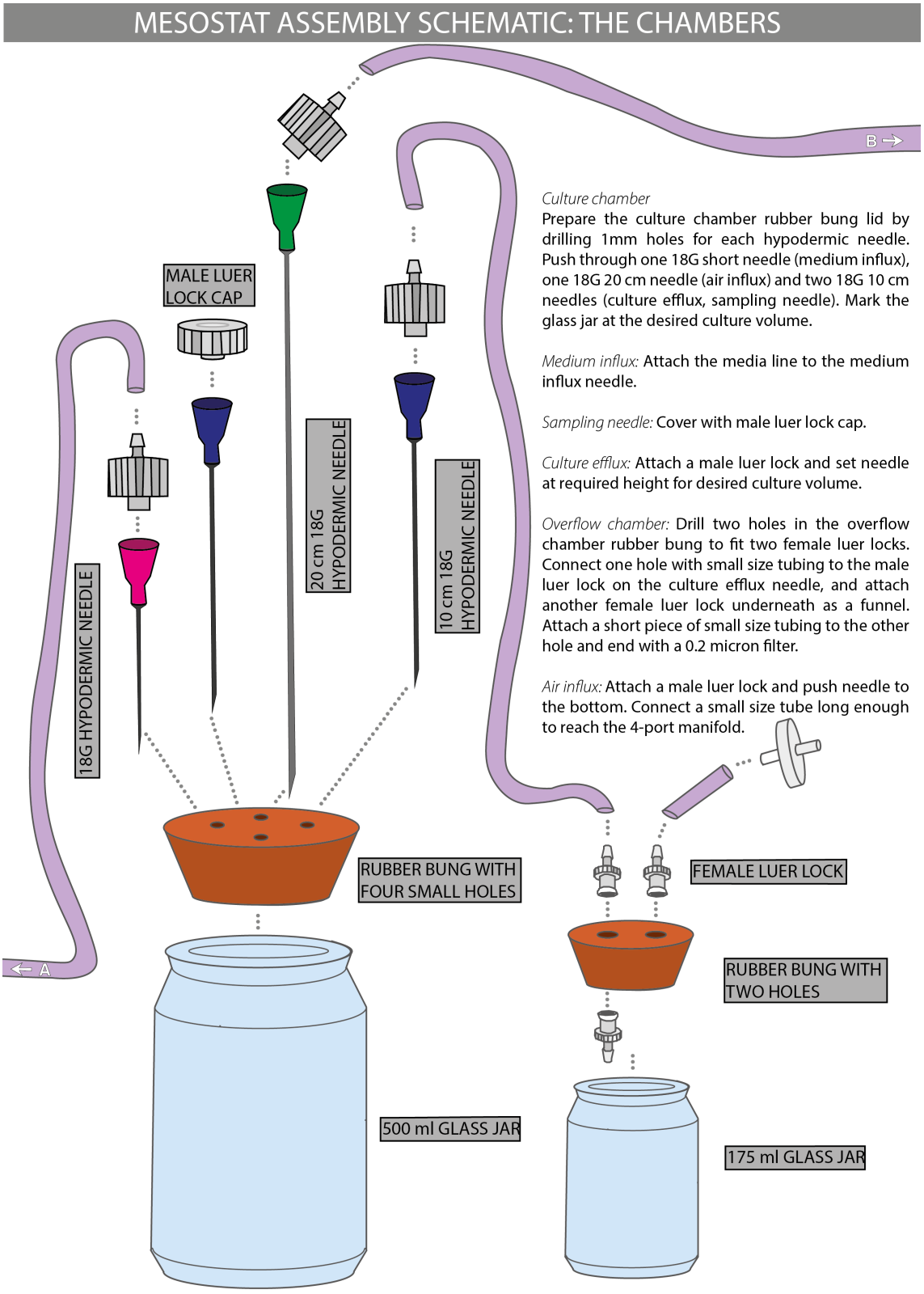

Figure 4: Assembly schematic part 2, the chambers.

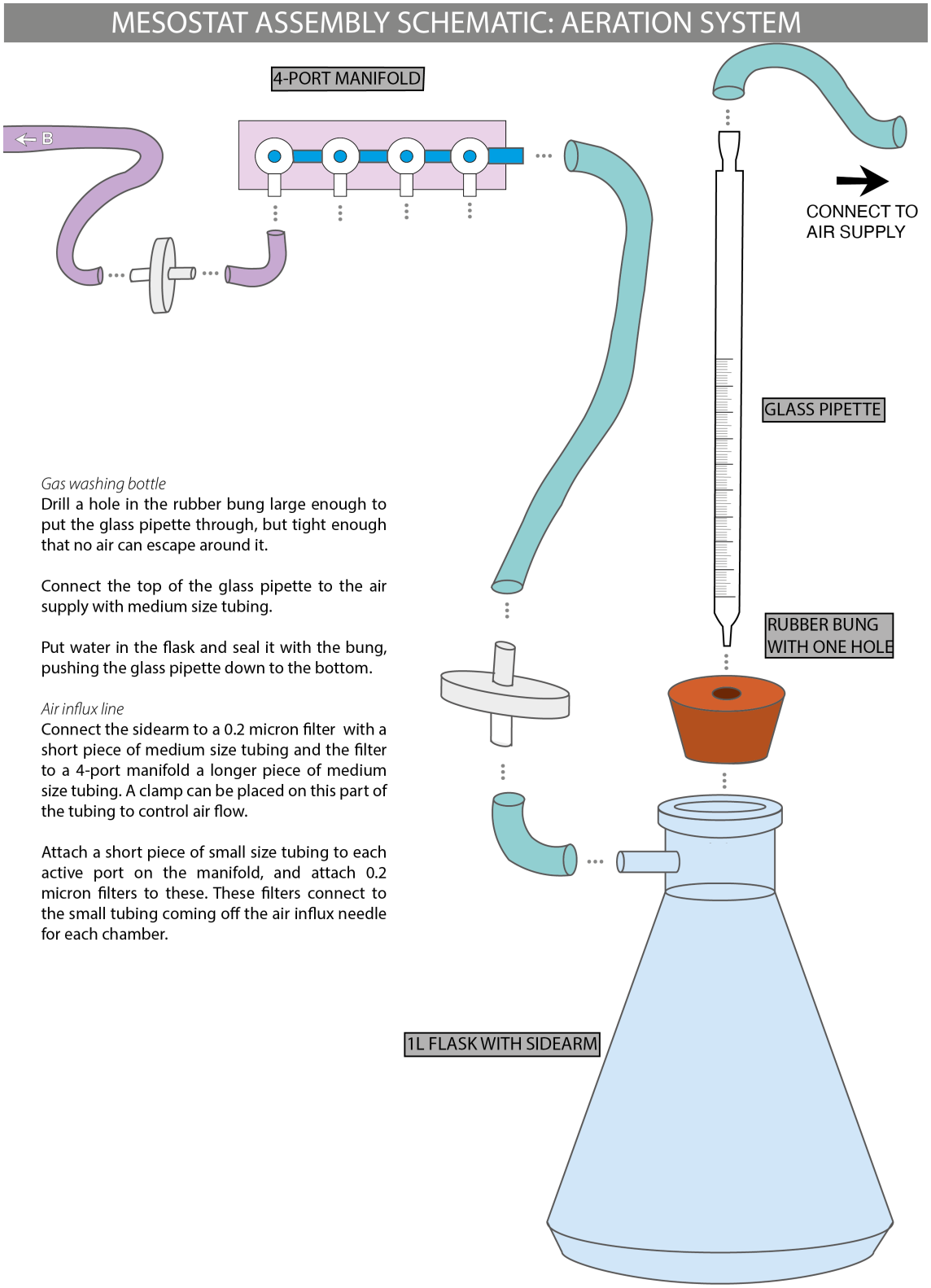

Figure 5: Assembly schematic part 3, the aeration system.

REPRESENTATIVE RESULTS AND OBSERVATIONS

DATA HANDLING

Presented below are control data from four separate experiments to show replicability as well as examples of what the data looks like when common problems like leaks or contamination present. The conditions and relevant differences for these experiments are summarised in Table 2, unless otherwise stated the experimental conditions correspond to those outlined in Table 1. Population density was determined through flow cytometry (Beckman Coulter CytoFLEX), using CytExpert (Beckman Coulter) to gate and count events detected in the PerCP-A channel (Excitation: 488nm, Emission: 690/50 BP). These counts were validated against manual haemocytometer counts. All statistical analyses were carried out in R (version 4.0.5, R Core Team, 2021), using the `lme4` package (Bates *et al.*, 2015) to fit a linear mixed effects model with population density as the response, dilution rate and experiment as fixed effects, and day and chamber as random effects with varying intercepts. The significance of the fixed effects was tested using the Anova() function from the `car` package (Fox & Weisberg, 2019) and confirmed through parametric bootstrapping using the `pbkrtest` package (Halekoh & Højsgaard, 2014).

REPLICABILITY

Across the experiments presented here, there is no difference between the mean population densities after steady state has been reached ($\chi^2 = 2.1$, DF = 3, p = 0.6, Figure 6). Furthermore, there was no difference in steady state population density whether the dilution rate is 0.3 or 0.15/day ($\chi^2 = 0.4$, DF = 1 p = 0.5). The length of the establishment phase before steady state is reached will differ depending on the conditions and inoculate density, however all experiments presented here reached steady state within the first week and it was maintainable for several weeks thereafter.

Table 2: Summary of experimental conditions and properties of data used in Figure 6–8. Data from all four experiments were used to generate Figure 6, whereas data from experiment D were used for Figure 7–8

Experiment	n	Dilution rate	Time window
A	16 (day 1–10) then 4	0.3/day	Days 1–20
B	16 (day 1–7) then 4	0.3/day	Days 1–28
C	7 (day 3–11) then 2	0.15/day	Days 3–35
D	16 (day 5–16) then 6	0.15/day	Days 5–76

COMMON PROBLEMS

LEAKS

One common problem that may occur is leaks as the pump tubing wears out with long term use, which can lead to over or under dilution of the connected chambers. Figure 7 shows the population density in a chamber after a leak resulted in elevated dilution for a couple of hours, the days after which the population density is reduced before returning to steady state. Such events may however affect other traits of the population not visible in the population density data and data collected subsequent to a major leak should thus be excluded or treated with caution.

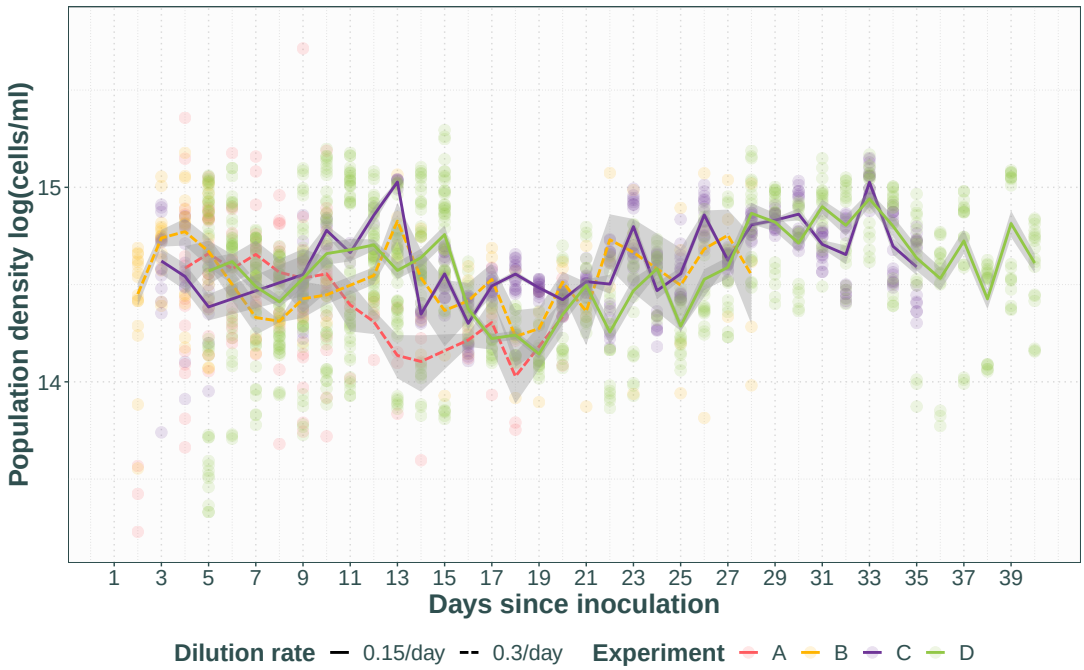

Figure 6: Population density with time in four separate runs of the mesostat system. Experiments A and B had a dilution rate of 0.3/day, whereas experiments C and D had a dilution rate of 0.15/day. Note that all have runs have a brief and rapid decline in population density between day 11 and 16. This corresponds to an injection of additional medium as part of the experiment the data is from.

CONTAMINATION

Bacterial contamination is another common risk in long-running continuous cultures. Figure 8 shows the gradual population density decline in four chambers where bacterial contamination was observed under the microscope. Those chambers, as well as the two plotted where no contamination was found, had been disconnected from the rest of the array six days prior to bacterial contamination being observed, which is likely how contamination was introduced. However, this is the only instance of contamination observed across eight separate experiments each lasting more than a month.

DISCUSSION

Chemostats offer a number of advantages over batch cultures for long-term experimental evolution research. Precise control of selective pressures in a chemically constant environment without evolutionary bottlenecks along with a link between growth rate and dilution rate constitute a useful conceptual framework for modelling evolutionary adaptation and population dynamics. This protocol presents a novel meso-chemostat system for experimental evolution and here its application to the evolution of herbicide resistance in model species *C. reinhardtii*.

This system adds to the small, but growing, number of efforts to produce simple but scalable,

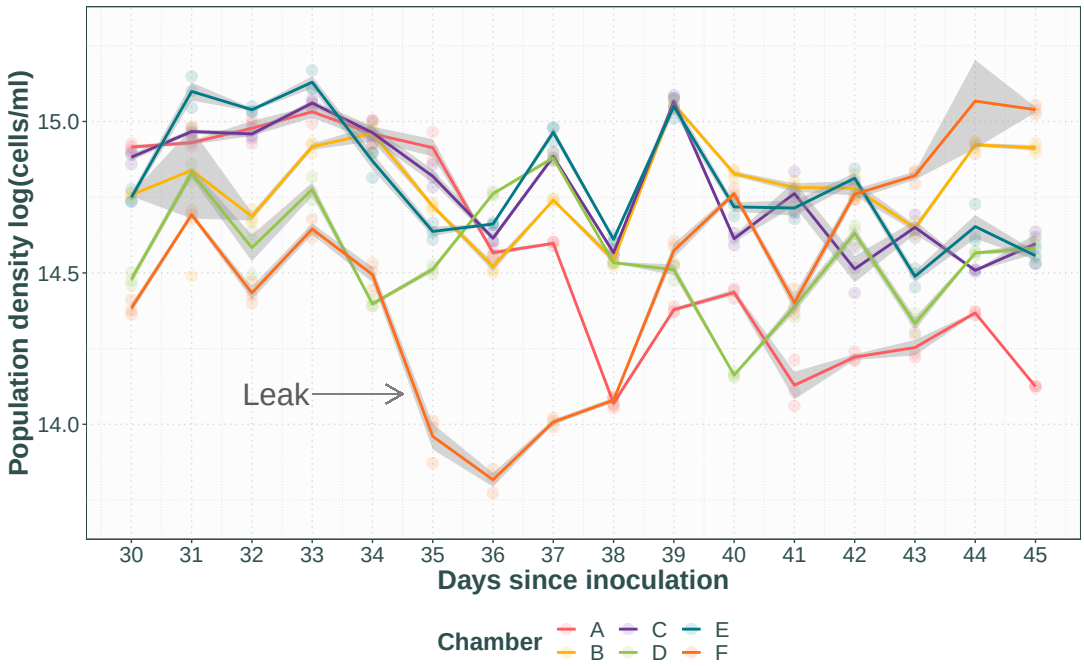

Figure 7: Population density with time after a major leak causing overdilution of chamber F, compared to the unaffected chambers A—E.

multiplexed DIY chemostats from cheaper materials that are possible to build and maintain by a single person. In addition to the Miller *et al.* (2013) ministat system that was designed for yeast, others have applied similar designs to study nutrient limitation in yeast (Skelding *et al.*, 2018) and antibiotic resistance (Tonoyan *et al.*, 2020; Toprak *et al.*, 2013). Others have incorporated multiple sensors or automated controls (Ekkers *et al.*, 2020; Wong *et al.*, 2018), but these constitute a considerably larger initial investment in materials. The setup presented here is the only one so far applying it to algae or herbicide resistance evolution, and uses considerably larger culture chambers than other DIY examples.

LIMITATIONS

Despite the many advantages of chemostat cultures, there are limitations to their application and caveats to how the data may be interpreted. While the system used for this thesis was explicitly designed to be maintainable by one person as well as cheaper than the Miller *et al.* (2013) version it is based on, it is still considerably more expensive than batch cultures. While it is theoretically possible to run very large cultures indefinitely, the cost of the medium or treatment components will limit the lifespan of the experiment as they will be consumed faster than in a batch culture design. One way to conserve medium and treatment components is to lower the dilution rate, which in the experiments presented here had no effect on population density in the chambers. However, this changes the selection pressures experienced by the populations as well as their doubling rate. The logistics and any cost saving measures of the system must therefore be carefully balanced against the resulting biology.

This design introduced sampling needles to allow sampling directly from the culture as an altern-

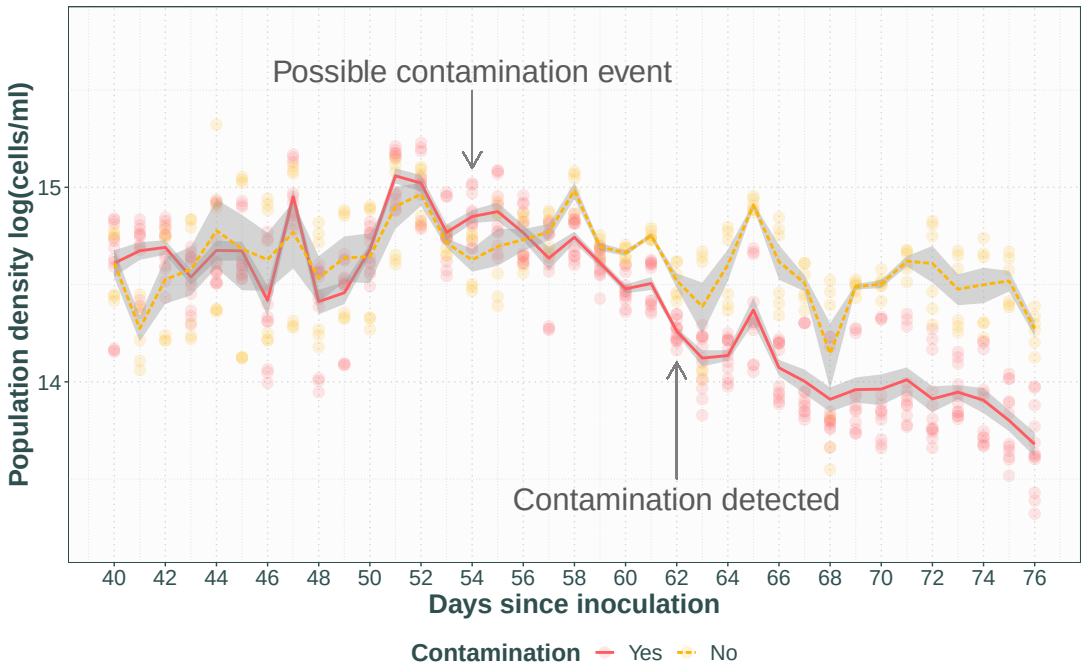

Figure 8: Population density with time after a possible contamination event at day 54, bacterial contamination was found in 4 out of 6 chambers on day 62.

ative to sampling from the overflow chamber, as the environment therein will be different from the culture chamber, or redirecting the overflow, as the low flow rate made sampling a slow process and the high temperature caused high levels of evaporation. However, sampling directly from the culture does perturb the steady state by temporarily reducing the culture volume (Fischer *et al.*, 2014) and thus the frequency and volume of samples have to be carefully considered against the disruption they will cause.

Lastly, in terms of studying the dynamics of adaptive evolution, chemostat systems are highly specific environments. When transplanted out, chemostat populations are often found to grow poorly in their ancestral environment compared to the ancestral strain (Gresham & Hong, 2014; Wenger *et al.*, 2011), as they have had intense selection on a specific part of the growth cycle in an environment of constant dilution that is not reflective of natural populations. However, this is also part of their usefulness and beauty, by keeping the adaptive environment as simple and specific as possible, we can isolate fitness effects and allow fine-tuned investigation of their mechanics and dynamics.

HOW TO IMPROVE OR MODIFY

Several modifications are possible for this system to reduce variation both between chambers and separate experiment runs. A light table that does not transfer heat to the cultures would allow the internal culture temperature to be set solely by the ambient temperature in the controlled temperature room while maintaining low evaporation. Sensors to monitor e.g. pH or CO2 levels, could relatively easily be added to the chambers through additional ports in the chamber lids.

Many parts could also be easily substituted depending on availability or cost constraints. Any

water-tight, sterilisable container can be used for culture chambers if suitable lids can be manufactured, such as falcon tubes (Tonoyan *et al.*, 2020) or commonly available lab glassware (Ekkers *et al.*, 2020). The controlled temperature room can be replaced with waterbaths and portable aquarium pumps can be used instead of building infrastructure.

FUTURE RESEARCH OPPORTUNITIES

This setup is also easily adaptable depending on the research question. The dilution rate and thus the cell growth rate is set by the pump speed, tubing thickness and culture volume, so running chambers with different dilution rates simultaneously would be possible with different pump tubing thicknesses, multiple pumps or multiple culture volumes, depending on the range required. The herbicide treatment could also be applied gradually through the medium or through series of shock injections in a ratchet protocol (Reboud *et al.*, 2007) and investigate to what level the resistance can be pushed and at what speed. Furthermore, the use of multiple light tables with opaque partitions between cultures would allow testing for an interaction with light level, or the chambers could be kept in water baths at different temperatures to determine the effect of temperature.

In addition to testing the effect of abiotic factors such as temperature or light, or manipulating the specific cell cycle stage of the population, a particularly interesting future application would be to use the system to ask focused questions about eco-evolutionary dynamics. In particular, introducing several trophic levels in the culture chambers to study the ecosystem and food web effects of herbicides and evolving herbicide resistance. The predator-prey cycles of rotifer *Brachionus calyciflorus* and *C. reinhardtii* as well as *Chlorella vulgaris* have been successfully studied and modelled using chemostat environments (e.g. Becks *et al.*, 2012; Yoshida *et al.*, 2003) and this setup would allow larger simultaneous replication. Competition could also be introduced to the system through using multiple algal strains and monitoring their frequencies or through expanding the culture ecosystem to include other algal species or bacteria (Raatz *et al.*, 2018).

ACKNOWLEDGEMENTS

EMH was supported by a scholarship from The University of Sheffield. Special thanks to Allison Blake and Lynsey Gregory for technical support in the lab.

AUTHOR CONTRIBUTIONS

EMH, APB and DZC conceived the study. EMH performed the experiments and collected the data. EMH, APB and DZC performed the statistical analyses. EMH wrote the manuscript with contributions from APB and DZC.

RIGHTS RETENTION AND DATA AVAILABILITY

For the purpose of open access, the author has applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising. The data presented in this manuscript, along with relevant code, is available at DOI: 10.5281/zenodo.6472897

DECLARATIONS OF INTEREST

The authors declare no competing interests.

REFERENCES

Barrick, J. E. & Lenski, R. E. (2013). 'Genome Dynamics During Experimental Evolution'. *Nature Reviews Genetics* 14 (12), 827–39.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015). 'Fitting Linear Mixed-Effects Models Using `lme4`'. *Journal of Statistical Software* 67.1, 1–48.

Becks, L., Ellner, S. P., Jones, L. E. & Hairston, N. G. (2012). 'The Functional Genomics of an Eco-Evolutionary Feedback Loop: Linking Gene Expression, Trait Evolution, and Community Dynamics'. *Ecology Letters* 15 (5), 492–501.

Bull, A. T. (2010). 'The Renaissance of Continuous Culture in the Post-Genomics age'. *Journal of Industrial Microbiology and Biotechnology* 37 (10), 993–1021.

Declerck, S. A. J., Malo, A. R., Diehl, S., Waasdorp, D., Lemmen, K. D., Proios, K. & Papakostas, S. (2015). 'Rapid Adaptation of Herbivore Consumers to Nutrient Limitation: Eco-Evolutionary Feedbacks to Population Demography and Resource control'. *Ecology Letters* 18 (6), 553–62.

Dénervaud, N., Becker, J., Delgado-Gonzalo, R., Damay, P., Rajkumar, A. S., Unser, M., Shore, D., Naef, F. & Maerkl, S. J. (2013). 'A Chemostat Array Enables the Spatio-Temporal Analysis of the Yeast Proteome'. *Proceedings of the National Academy of Sciences* 110 (39), 15842–7.

Ebert, D. (2013). *Web-guide to Daphnia Parasites: Culturing Daphnia Food*. Accessed 13-10-2017. URL: `http://evolution.unibas.ch/ebert/lab/algae.htm`.

Ekkers, D. M., Branco dos Santos, F., Mallon, C. A., Bruggeman, F. & Doorn, G. S. van (2020). 'The Omnistat: A flexible Continuous-Culture System for Prolonged Experimental Evolution'. *Methods in Ecology and Evolution* 11.8, 932–42.

Elena, S. F. & Lenski, R. E. (2003). 'Evolution Experiments with Microorganisms: The Dynamics and Genetic Bases of Adaptation'. *Nature reviews. Genetics* 4 (6), 457–69.

Erickson, J. M., Rahire, M., Bennoun, P., Delepelaire, P., Diner, B. & Rochaix, J. D. (1984). 'Herbicide Resistance in *Chlamydomonas reinhardtii* Results from a Mutation in the Chloroplast Gene for the 32-kilodalton Protein of Photosystem II'. *Proceedings of the National Academy of Sciences of the United States of America* 81 (12), 3617–21.

Erickson, J. M., Pfister, K., Rahire, M., Togasaki, R. K. & Mets, L. (1989). 'Molecular and Biophysical Analysis of Herbicide-Resistant Mutants of *Chlamydomonas reinhardtii*: Structure-Function Relationship of the Photosystem II D 1 Polypeptide'. *The Plant Cell* 1, 361–71.

Fischer, R., Andersen, T., Hillebrand, H. & Ptacnik, R. (2014). 'The Exponentially Fed Batch Culture as a Reliable Alternative to Conventional Chemostats'. *Limnology and Oceanography: Methods* 12.7, 432–40.

Fox, J. & Weisberg, S. (2019). 'An R Companion to Applied Regression'. URL: `https://socialsciences.mcmaster.ca/jfox/Books/Companion/`.

Fussmann, G. F., Ellner, S. P., Shertzer, K. & Jr, N. H. (2000). 'Crossing the Hopf Bifurcation in a Live Predator-Prey System'. *Science* 290 (5495), 1358–60.

Good, B. H., McDonald, M. J., Barrick, J. E., Lenski, R. E. & Desai, M. M. (2017). 'The Dynamics of Molecular Evolution over 60,000 Generations'. *Nature* 551 (7678), 45–50.

Gresham, D. & Dunham, M. J. (2014). 'The Enduring Utility of Continuous Culturing in Experimental Evolution'. *Genomics* 104 (6, Part A), 399–405.

Gresham, D. & Hong, J. (2014). 'The Functional Basis of Adaptive Evolution in Chemostats'. *FEMS Microbiology Reviews* 39, 2–16.

Halekoh, U. & Højsgaard, S. (2014). 'A Kenward-Roger Approximation and Parametric Bootstrap Methods for Tests in Linear Mixed Models – The R Package `pbkrtest`'. *Journal of Statistical Software* 59.9, 1–30. URL: `https://www.jstatsoft.org/v59/i09/`.

Hiltunen, T., Hairston, N. G., Hooker, G., Jones, L. E. & Ellner, S. P. (2014). 'A Newly Discovered Role of Evolution in Previously Published Consumer-Resource Dynamics'. *Ecology Letters* 17 (8), 915–23.

Kawecki, T. J., Lenski, R. E., Ebert, D., Hollis, B., Olivieri, I. & Whitlock, M. C. (2012). 'Experimental Evolution'. *Trends in Ecology & Evolution* 27 (10), 547–60.

Lang, G. I. & Desai, M. M. (2014). 'The Spectrum of Adaptive Mutations in Experimental Evolution'. *Genomics* 104 (6), 412–6.

Lenski, R. E., Rose, M. R., Simpson, S. C. & Tadler, S. C. (1991). 'Long-Term Experimental Evolution in *Escheria coli*. I. Adaptation and Divergence During 2,000 Generations'. *The American Naturalist* 138, 1315–41.

Miller, A. W., Befort, C., Kerr, E. O. & Dunham, M. J. (2013). 'Design and Use of Multiplexed Chemostat Arrays'. *Journal of Visualized Experiments* (72), 2–7.

Monod, J. (1950). 'La technique de culture continue: Théorie et applications'. *Annales De l'Institut Pasteur, Paris*, 390–410.

Novick, A. & Szilard, L. (1950). 'Description of the Chemostat'. *Science* 112 (2920), 715–6.

R Core Team (2021). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. Vienna, Austria. URL: `https://www.R-project.org/`.

Raatz, M., Schälicke, S., Sieber, M., Wacker, A. & Gaedke, U. (2018). 'One Man's Trash is Another Man's Treasure – The Effect of Bacteria on Phytoplankton–Zooplankton Interactions in Chemostat Systems'. *Limnology and Oceanography: Methods* 16, 629–39.

Randolph-Anderson, B. L., Sato, R., Johnson, A. M., Harris, E. H., Hauser, C. R., Oeda, K., Ishige, F., Nishio, S., Gillham, N. W. & Boynton, J. E. (1998). 'Isolation and Characterization of a Mutant Protoporphyrinogen Oxidase Gene from *Chlamydomonas reinhardtii* Conferring Resistance to Porphyric Herbicides'. *Plant Molecular Biology* 38 (5), 839–59.

Reboud, X., Majerus, N., Gasquez, J. & Powles, S. B. (2007). '*Chlamydomonas reinhardtii* as a Model System for Pro-Active Herbicide Resistance Evolution Research'. *Biological Journal of the Linnean Society* 91 (2), 257–66.

Skelding, D., Hart, S. F. M., Vidyasagar, T., Pozhitkov, A. E. & Shou, W. (2018). 'Developing a Low-Cost Milliliter-Scale Chemostat Array for Precise Control of Cellular Growth'. *Quantitative Biology* 6.2, 129–41.

Tonoyan, L., Guihéneuf, F., Friel, R. & O'Flaherty, V. (2020). 'Construction and Validation of A Low-Cost, Small-Scale, Multiplex Continuous Culturing System for Microorganisms'. *Bioprotocol* 10.21, e3813.

Toprak, E., Veres, A., Yildiz, S., Pedraza, J., Chait, R., Paulsson, J. & Kishony, R. (2013). 'Building a Morbidostat: An Automated Continuous-Culture Device for Studying Bacterial Drug Resistance Under Dynamically Sustained Drug Inhibition'. *Nature protocols* 8, 555–67.

Van den Bergh, B., Swings, T., Fauvart, M. & Michiels, J. (2018). 'Experimental Design, Population Dynamics, and Diversity in Microbial Experimental Evolution'. *Microbiology and Molecular Biology Reviews* 82 (3), e00008–18.

Vogwill, T., Lagator, M., Colegrave, N. & Neve, P. (2012). 'The Experimental Evolution of Herbicide Resistance in *Chlamydomonas reinhardtii* Results in a Positive Correlation Between Fitness in the Presence and Absence of Herbicides'. *Journal of Evolutionary Biology* 25 (10), 1955–64.

Wahl, L. M. & Gerrish, P. J. (2001). 'The Probability that Beneficial Mutations are Lost in Populations with Periodic Bottlenecks'. *Evolution* 55.12, 2606–10.

Wenger, J. W., Piotrowski, J., Nagarajan, S., Chiotti, K., Sherlock, G. & Rosenzweig, F. (2011). 'Hunger Artists: Yeast Adapted to Carbon Limitation Show Trade-Offs under Carbon Sufficiency'. *PLOS Genetics* 7.8, e1002202.

Wong, B. G., Mancuso, C. P., Kiriakov, S., Bashor, C. J. & Khalil, A. S. (2018). 'Precise, Automated Control of Conditions for High-Throughput Growth of Yeast and Bacteria with eVOLVER'. *Nature biotechnology* 36.7, 614–23.

Yoshida, T., Jones, L. E., Ellner, S. P., Fussmann, G. F. & Hairston, N. G. (2003). 'Rapid Evolution Drives Ecological Dynamics in a Predator-Prey System'. *Nature* 424 (6946), 303–6.