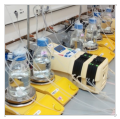

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Building up chemostats for experimental eco-evolutionary studies

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ABSTRACT

Chemostats and other flow through culture systems are a powerful tool for the study of microbial and plankton communities in experimental ecology and evolutionary studies. Commercially available chemostat systems allow the control of a large number of parameters (e.g., ph, pressure, CO_2 concentration) but are often expensive and offer a high level of control that is often not needed for many experimental studies. Non-commercial chemostats are more cost efficient, easily set up and flexible in volumes used. Different from semi-continuous culture systems, the flow through conditions of chemostats allow a constant inflow of resources from a reservoir (medium bottle) and outflow of unused nutrients, waste products and organisms, which are all collected in a waste bottle. Nutrient levels in the reservoir and the flow rate of the chemostat system (often referred to as dilution rate and presented as the fraction of the volume of the chemostat that is replaced per day) determines population growth rates and dynamics (for the theory behind chemostats see Smith & Waltman, 1995 and Weitz, 2015).

Chemostats have been used in a number of studies and with different organisms and combinations of organisms. For example, Boer et al. (2010) studied how growth-limiting intracellular metabolites control yeast growth under diverse nutrient availability (*Saccharomyces cerevisiae* growing under five different nutrient supply of nitrogen: carbon: phosphorus). Becks et al. (2005) used chemostat systems to show how changes in the flow rate of a chemostat system influences the population dynamics of a three species microbial system. Frickel et al. (2016) used chemostats to investigate eco- evolutionary dynamics in a coevolving host-virus system (algae *Chlorella variabilis* and Chlorovirus strain PBCV-1) for 90 days.

We present here an instruction for a cost efficient and flexible chemostat systems. These chemostats are composed of four main parts: a syringe unit, a glass bottle (i.e. the chemostat), a medium bottle and a waste bottle, all connected by tubing. A peristaltic pump and a low overpressure in the system allow the flow of medium from the reservoir to the chemostat bottle and of unused nutrients, waste products and organisms to the waste bottle. Chemostats are put on stirring plates to create a homogenosus environment within.

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Boer, V.M., Crutchfield, C.A., Bradley, P.H., Botstein, D., Rabinowitz, J.D., 2010. Growth-limiting Intracellular Metabolites in Yeast Growing under Diverse Nutrient Limitations 21, 198–211. https://doi.org/10.1091/mbc.E09

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MATERIALS TEXT

1.Syringe (50 mL)
2.Luer Lock syringe adapter CT62.1, Ø 1.6 mm (Carl Roth International).
3.Tube connector CT45.1, Ø 1,6 - 4,8 mm (Carl Roth International).
4.PVC tubes (10 mm length, Ø 6 mm).
5.Ruber plugs with two holes (Ø 6 mm)
6.Glass tubes (12 cm, Ø 6 mm of the holes)
7.Acrodisc CR 25 mm syringe filters (0.2 µm, PTFC membrane)
8.Tube holder/clamp
9.PharMed BPT tubing for pumps (Ø 5 mm inner size, Ø 1.6 wall size, 50 cm length each piece, but it will depends on the pump model)
10. Silicone tubing (Ø 0.5 mm, variable length)
11.Tube connector CT33.1, Ø 1,6 mm (Carl Roth International).
12. Clear glass laboratory bottle (500 mL, experimental vessel used as the chemostat, volume is flexible depending on your experimental set up).
13. Cannula Luer-lock (1.00 x 200 mm)
14. Pipette tip 1-10 µL
15. Glass tube for chemostats outflow (8 cm length)
16. Lid for screw cap with 3 holes (two holes of Ø 0,4 mm and one of Ø 0.5 mm)
17. Screw cap with 3 holes (two holes of Ø 0,4 mm and one of Ø 0.5 mm)
18. Stirring bar (5 cm)
19. Clear glass laboratory bottle (5 L) as medium bottle
20. Clear glass laboratory bottle (5 L) as waste bottle
21. Tripod
22. Lab clamps to hold syringes on the tripod
23. Stiring plates
24. Air pressure (i.e. aquarium air pumps or other systems)
25. Pumps

Others
26. Lab torch burner
27. Autoclavable silicone
28. Cable ties
29. Ethanol
30. Gloves

SAFETY WARNINGS

Use silicone in well-ventilated area and wear lab coat and gloves. Leave material to dry in well-ventilated area.

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BUILDING CHEMOSTATS

1

This section for building chemostats is divided into preparing the four main parts of a chemostat (see below). Before you start assembling the different parts you should have an idea of the space you want to use and where you want to put the peristaltic pumps as some of the tubing needs to be cut according to the distances between medium bottle – pump – syringe – chemostat – waste bottle. All parts will be assembled by the connectors and some parts sealed with silicone. You can assemble all parts of the chemostats, autoclave and then put them in the space where the experiment will take place.

Parts of a chemostat:

1/4 Syringe
2/4 Chemostat bottle
3/4 Medium bottle
4/4 Waste bottle

1/4 PREPARING SYRINGE

Materials:

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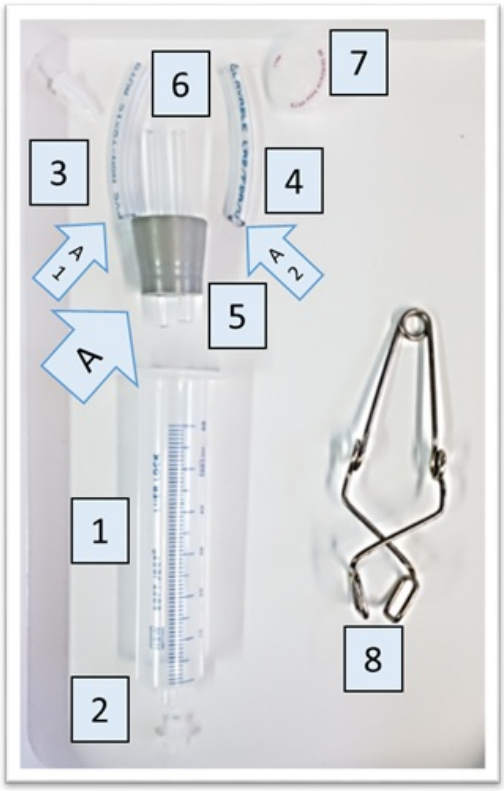

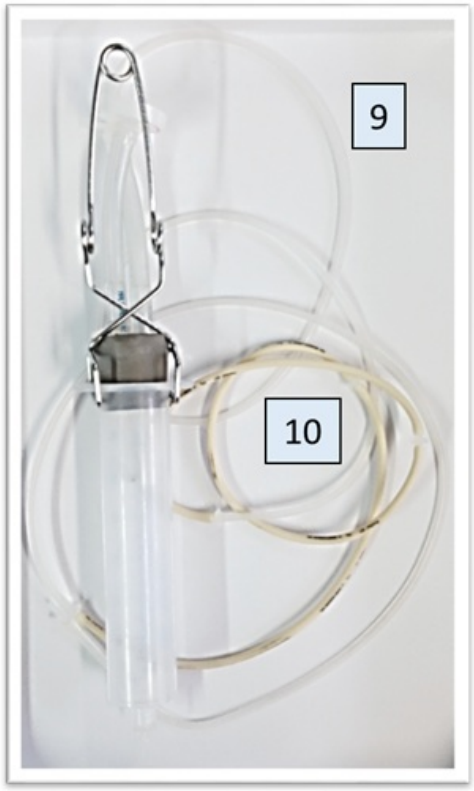

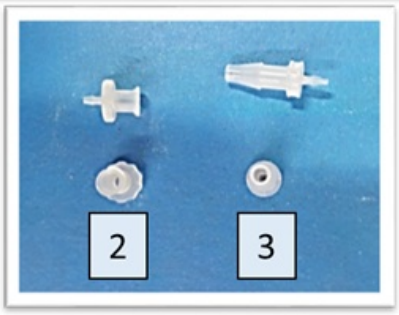

A. Syringe plug
A1. Connection with the medium
A2. Connection with the air pressure

1.Syringe (50 mL)
2.Luer Lock syringe adapter CT62.1, Ø 1.6 mm.
3.Tube connector CT45.1, Ø 1,6 - 4,8 mm.
4.PVC tubes (10 mm length, Ø 6 mm).
5.Ruber plugs with two holes (Ø 6 mm)
6.Glass tubes (12 cm, Ø 6 mm of the holes)
7.Acrodisc CR 25 mm syringe filters (0.2 µm, PTFC membrane)
8.Tube holder/clamp
9. Silicone tube (Ø 0.5 mm, variable length)
10. PharMed BPT tubing for pumps (Ø 5 mm inner size, Ø 1.6 wall size, 24 cm length each piece, but it will depends on the pump model)
11.Tube connector CT33.1, Ø 1,6 mm.

You need to build up a syringe plug (A) containing two critical entries: one for the medium (A1) and the other for air inflow (A2) to create overpressure in the system.

Be aware of the available space, location of air connections and hight of benches because it will be needed to estimate tubes

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length.

Steps:

* Push the glass tubes (6) through the rubber plug (5) until the glass tubes ends stick out of the plug (2 cm).

* Push the PVC tubes (10 cm length, Ø 6 mm) (4) onto the glass tubes (6) on the part that will be outside of the syringe. Push them down until they reach the rubber plug. Autoclaving the PVC tubes (10 cm length, Ø 6 mm) (4) before using makes them more flexible to be pushed onto the glass tubes (6).

* Attach the acrodisc syringe filter (0.2 µm, PTFC membrane) (7) to the end of one of the PVC tubes (10 cm length, Ø 6 mm) (4).

* Attach a tube connector CT45.1, Ø 1,6 - 4,8 mm (3) to the end of the other PVC tube (10 cm length, Ø 6 mm) (4).

* Close the syringe with the now prepared rubber plug (5) with the glass tubes inside (6) and the now prepared PVC tubes (4). Hereinafter, it will be named as the syringe plug (A).

* Attach luer lock syringe adapter CT62.1, Ø 1.6 mm (2) to the lower end of syringe. Connect it with a silicone tube (Ø 0.5 mm, variable length) long enought to reach your chemostats inflow (see later: B, B1).

* Use a tube holder/clamp (8) to hold the rubber plug (5) on the syringe.

* Cut one piece of silicone tube (Ø 0.5 mm, variable length) (9) in the length of the distance between syringe and the pump.

* Connect this silicone tube with the tube connector CT45.1, Ø 1,6 - 4,8 mm (3) on the PCV tube (A1) from the rubber plug.

* Connect the end of the silicone tube (Ø 5 mm, variable length) (9) with a tube connector CT33.1 (Ø 1,6 mm) (11).

* Connect it with the PharMed BPT tube for pumps (Ø 5 mm, 24 cm length each piece) (10) that will go inside the peristaltic pump. We suggest to add three pieces of these PharMed BPT tube for pumps (10) because they can be damaged during the experiment, in such a case, it can be easily removed.

* Put a tube connector CT33.1 (Ø 1,6 mm) (11) on the other end of the three pieces of PharMed BPT tube for pumps (Ø 5 mm, 24 cm length each piece, but it will depends on the pump model) (10) and connect a silicone tube (Ø 5 mm, variable length) (9) that is long enough to reach from your pump to the bottle with media (see later: C, C1).

2/4 CHEMOSTATS

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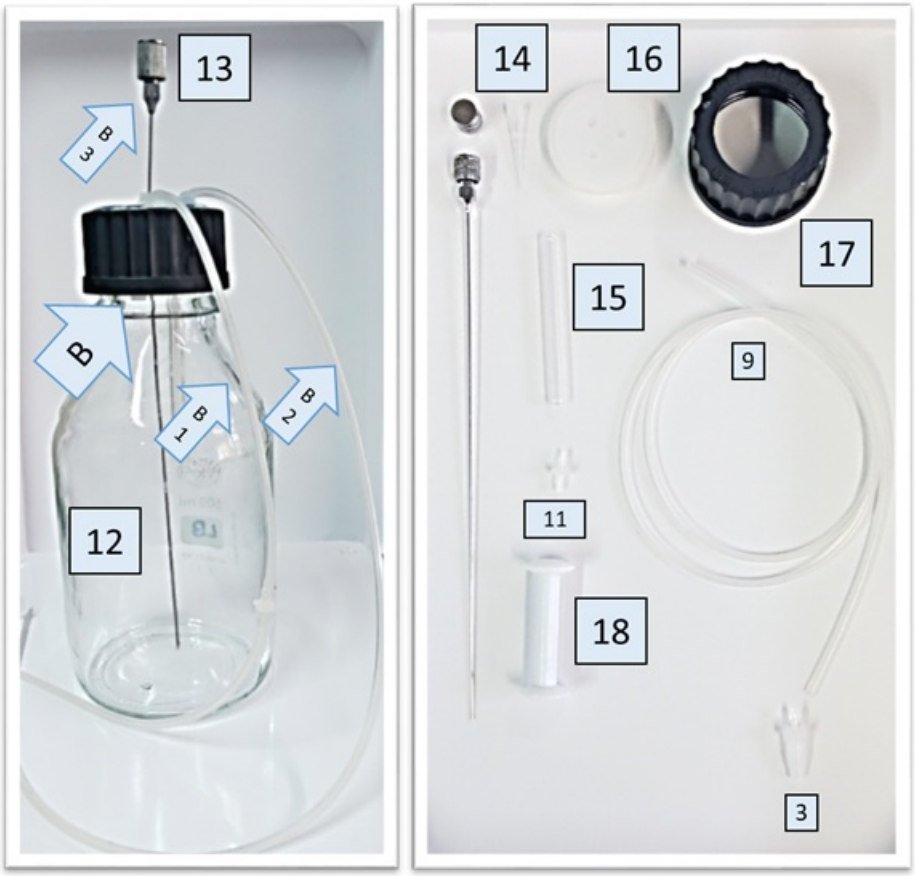

12. Clear glaboratory bottle
(500 mL, experimental vessel)
13. Cannula neoLab Luer lock (1.00 x 200 mm)
14. Pipette tip 1-10 µL
15. Glass tube for chemostats outflow
chemostats (8 cm lenght)
16. Lid for screw cap with 3 holes (two
holes of Ø 0,4 mm and one of Ø 0.5 mm)
17. Screw cap with hole (Ø 4 cm)
18. Stirring bar

You need to build up the experimental vessel used as the chemostats. The most important part is the chemostat cap (B) with the inflow (B1), the outflow (B2) and sampling needle (B3) connections corresponding to the inflow of resourses from the medium bottle, the outflow of unused nutrients and a cannula neoLab Luer lock for sampling respectively.

Steps:

* Add stirring bar (17) to the chemostat bottle (12). We noted in some experiments that stirring bars degrade quickly which can interfere with the experiments. Degradation can depend on the production batch and/or the culture conditions.

* The length of the glass tube for chemostats outflow (8 cm length) (15) attached to the screw cap with hole (Ø 4 cm) (17) that closes the chemostat determines the volume of the chemostat. The volume can be choosen and changed according to the volume needed in the experiment.

* Assemble the lid for screw cap with 3 holes (two holes of Ø 0,4 mm and one of Ø 0.5 mm) (17) and lid for screw cap with hole (Ø 4 cm) (16). Hereinafter, it will be named the chemostats cap (B).

* Cut two pieces of silicone tube ~ 25 and 20 cm (9), one is for the INFLOW (B1) and the other one for the OUTFLOW (B2). Fiddle

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both tubes (B1, B2) through the smaller holes (Ø 0,4 mm) in the lid for screw cap with 3 holes (16):

INFLOW (B1): Connect the shorter silicone tube (~ 20 cm) (9) to a tube connector CT45.1, Ø 1,6 - 4,8 mm (3). The adapter has to face the inside of screw cap (17). The other side of the silicone tube is connected with the silicone tube coming from the syringe (mentioned in step 1) with a tube connector CT33.1 (Ø 1,6 mm) (11).

OUTFLOW (B2): Put the longer BPT tube (~ 25 cm) (9) through the glass tube for chemostats outflow (15) and connect a tube connector CT33.1 (Ø 1,6 mm) (11) to prevent that the glass tube falls off. Connect the other end of the ~ 25 cm silicone tube (9) with a tube connector CT45.1, Ø 1,6 - 4,8 mm (3), this tube will be connected with a tube from the waste bottle (see later: D, D1).

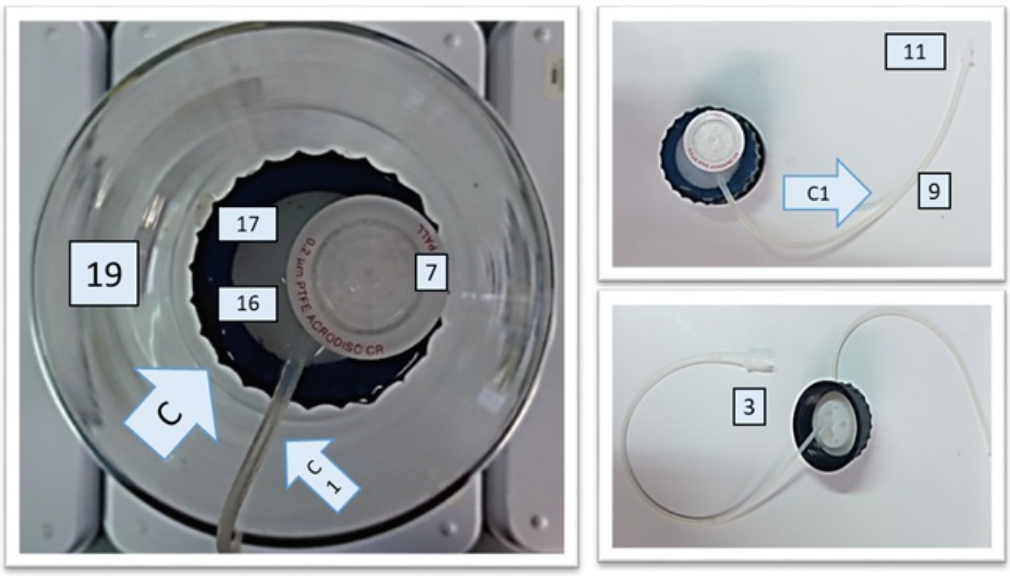
* Close the chemostats bottle (12) with the chemostats cap (B).

SAMPLING NEEDLE (B3): Take the pipette tip (14) and cut the lower tip off. Add it in the biggest hole of the lid for screw cap (Ø 0.5mm) (16). Put Cannula neoLab needle (13) through the pipette tip. The pipette tip is supposed to hold the needle in place without it falling into the bottle and at the same time allow for the needle to be moved up and down for taking samples in the running chemostat.

* Add a tube connector CT33.1 (Ø 1,6 mm) (11) on the two ends of tubes coming from chemostats cap (B, so from B1 and B2).

* Seal the screw cap and the lid with silicone (16, 17) which forms the chemostats cap (B). Wait for 24 hours for it to dry. Silicone is used to ensure that all holes are sealed as the system should be airtight to maintain the system under overpressure conditions.

3/4 MEDIUM BOTTLE



19. Clear glass laboratory bottle (5 L, size variable depending on dilution rate, volumen and lenght of the experiment) as medium bottle

You need to build up a medium cap (C). In the lid for screw cap that we use, there are three holes (two holes of Ø 0,4 mm and one of Ø 0.5 mm) (16). Actually, only two are needed but we have lid for screw cap with three holes so that they can be used for the chemostats (B), the medium (C) or the waste (D) bottles.

Steps:

* Cut a piece of silicone tube (Ø 0.5 mm, variable length) (9) that is long enough (~ 25 cm) to reach the bottom of the medium

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bottle plus additional 10 cm.

* Put this silicone tube through one of the 0.4 mm holes in the lid.

* Connect a tube connector CT45.1, Ø 1,6 - 4,8 mm (3) to the end of the silicone tube (Ø 0.5 mm, variable length) that will go inside the bottle (9). Put a tube connector CT33.1 (Ø 1,6 mm) (11) on the other end of the BPT tube outside of the bottle (9). This tube (C1) will be connected with the tube (A1) going into the pump to make the medium flow into the chemostats. It closes the circuit continuing the flow from the pump to the syringe by the already assembled tube connector CT45.1, Ø 1,6 - 4,8 mm (3) corresponding to tube A1 (A, see step 1/5 and its figures). And, it brings the medium into the chemostats by the inflow tube B1.

* Cut another piece, ~4 cm, of silicone tube (9) to block the other small (Ø 0,4 mm) hole of the lid (17). Make a knot in the tube on both sides of the lid. Or use lids with only 2 holes.

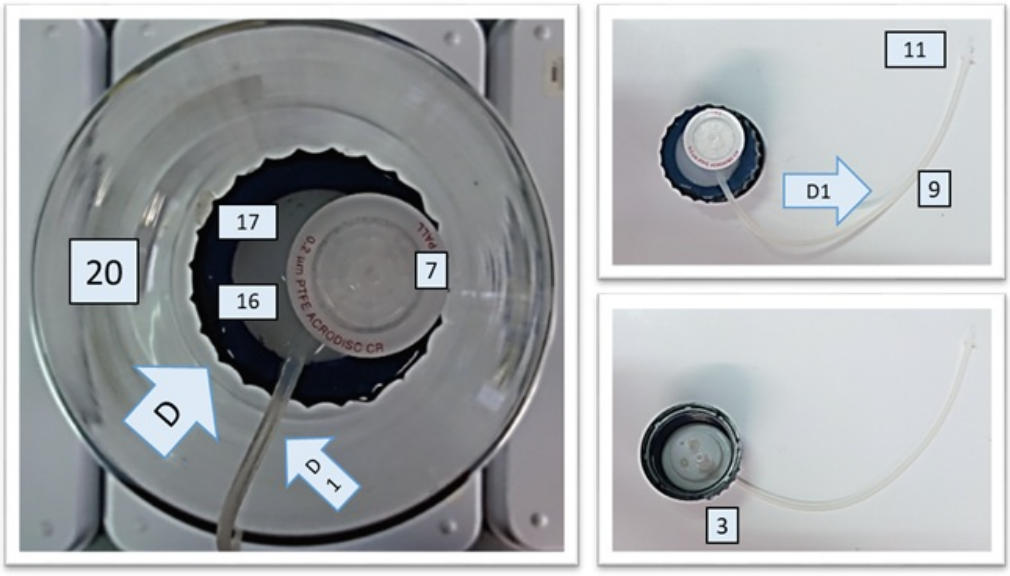
* Assemble the lid for screw cap with 3 holes (17) into the screw cap with hole (16) and close the clear glass laboratory bottle (5 L) that will be used as the medium bottle (19).

* On the top of the lid (17), fit one acrodisc syringe filter (7) by its slip outlet side on the big hole (Ø 0.5 mm) of the lid for screw cap (16).

* Seal it all with silicone, paying attention to not cover the air filter with silicone.

You can choose the size of your medium bottle according to the size, flow rate and length of your experiment. I would recomment that the medium bottle lasts for your whole experiment if possible.

4/4 WASTE BOTTLE



20. Clear glass laboratory bottle (5 L, size variable depending on dilution rate, volumen and lenght of the experiment) as waste bottle

You need to build up a medium cap (D). In the lid for screw cap that we use, there are three holes (two holes of Ø 0,4 mm and one of Ø 0.5 mm) (16). Actually, only two are needed but we have lid for screw cap with three holes so that they can be used for the chemostats (B), the medium (C) or the waste (D) bottles.

Steps:

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* Cut a piece of silicone tube (Ø 0.5 mm) (9) that is long enough (~ 14 cm) to be connected later with the outflow tube coming from the chemostat (16).

* Put this silicone tube through one of the 0.4 mm holes in the lid.

* Connect tube connector CT45.1, Ø 1,6 - 4,8 mm (3) to the silicone tube (Ø 0.5 mm, variable length) (9) that will go inside the bottle. On the other side of the tube, connect the tube connector CT33.1 (Ø 1,6 mm) (11) to the end of the silicone tube (9). This tube (D1) coming from the waste bottle will be connected with the silicone tube ~ 25 cm coming from the glass-tube connection of the for chemostats outflow (B2).

* Cut another piece, ~4 cm, of silicone tube (9) to block the other small (Ø 0,4 mm) hole of the lid (17). Make a knot in the tube on both sides of the lid. Or use lids with only 2 holes.

* Assemble the lid for screw cap with 3 holes (17) into the screw cap with hole (16) and close the clear glass laboratory bottle (5 L) that will be used as the waste bottle (19).

* On the top of the lid (17), fit one acrodisc syringe filter (7) by its slip outlet side on the big hole (Ø 0.5 mm) of the lid for screw cap (16).

* Seal it all with silicone, paying attention to not cover the air filter with silicone.

You can choose the size of your waste bottle according to the size, flow rate and length of your experiment. I would recomment that the waste bottle last for your whole experiment if possible.

ASSEMBLY AND AUTOCLAVE CHEMOSTATS

2

* Connect all parts of the chemostat so that the whole circuit is close from the medium to the waste (A, B, C and D).

* When all part are connected autoclave it. After autoclaving, locate the chemostat at its designated place. Make sure all connections are tight, especially the air filters on the syringes.

* Chemostats (12) are on top of stirring plates (23). It intends to create and homogenosus environment within the chemostats, set up the speed of the stirring plate for a gentle homogenization.

* In order to make the chemostats flow working, we must create overpressure conditions. Therefore, set the syringe (1) on a tripod (21) behind the chemostats (12) and connect it to an air pump (24) through the PVC tube (A2) with the acrodisc filter (4) on the syringe plug.

* Adjust the air pressure to create overpressure but not too strong to prevent he plugs of the syringes pop off.

* Check all connections and ensure all parts are tied, reinforce the connections where needed with cable ties.

* When all tubes are connected, fill the chemostats with the medium by starting the pump (25).

* Let the system run for 24 hours to ensure that the in- and outflow are working properly. We recommend that the volume is completely replaced before starting the experiments.

* For a working flow-through system such as the chemostat the dilution rate with which the medium will be exchanged in the chemostat (in- and outflow) is crucial. The dilution rate is depending on the volume inside the chemostat and the volume pumped in and out per day (volume pumped per day depends on tube width used for the peristaltic pump and the pump speed). With a dilution rate of, for example, d=0.1 you exchange 10% of the medium in 24h. Check the pump manual to set up the intended dilution rate. In addition, consider the growth rate of your organisms to prevent extinction, if the dilution rate is higher than the growth rate the organisms will be washed out.

A example for a specific dilution rate:

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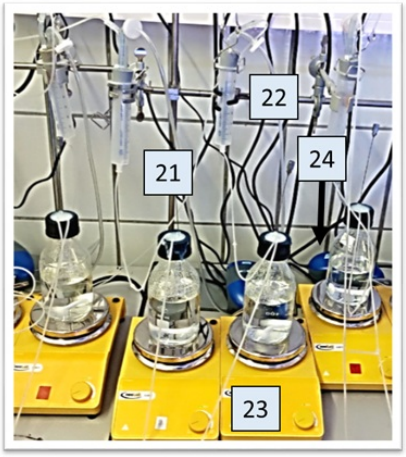
Total chemostat volumen: 400 ml
Dilution rate: 0.5
Pump tube diameter: 0.8 mm
RMP: this depends on the pump
Medium renewal per day: 200 ml/day

OVERVIEW OF EXPERIMENTAL CHEMOSTATS



21. Tripod
22. Lab clamps to hold syringes on the tripod
23. Stiring plates
24. Air pressure (i.e. aquarium air pumps or other systems)
25. Pumps

SAMPLING CHEMOSTATS

3 * Turn off the air inflow (or the needles will bubble and spill medium when they are opened to take a sample).

* Before taking the samples, clean the needles with ethanol 70% and flame the upper part outside the chemostat with a lab torch burner.

* After needles are sterile, you can push them into the chemostat medium (always same depth).

* Open the needles cap and take the sample with syringes.

* After sample is collected, take the needle out of the medium, clean again with ethanol 70% and flame them with the lab torch burner.

* Do not forget to restart the air inflow!

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