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Growing *Prochlorococcus* in nitrogen-limited chemostats at low cell abundances

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The Aquatic Microbial E...



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We use this protocol and it's working

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Abstract

This protocol is designed to help researchers culture *Prochlorococcus* in nutrient-limited chemostats at relatively low cell abundances. Because dilute *Prochlorococcus* can be extremely sensitive, this protocol outlines means to reduce potential stressors during continuous culturing. These methods are based off of extensive work conducted in the Zinser lab at the University of Tennessee, Knoxville where *Prochlorococcus* was cultured in nitrogen-limited chemostats at relatively low cell abundances through much trial and error. While our experiments were focused on nitrogen, we believe these methods can be applied to culture *Prochlorococcus* under a variety of nutrient limitations.

Attachments



[Image1.pdf](#)

194KB



[Image2.pdf](#)

314KB

Materials

Notable materials are listed in the steps of the protocol.

Preparing *Prochlorococcus* Cells

- 1 *Prochlorococcus* cells should be well acclimated to experimental conditions prior to starting the chemostat. This includes factors such as light level, light wavelengths, light cycle, and temperature. For example, when *Prochlorococcus* grown in white light was inoculated into chemostats under similar intensities of blue light, cells were washed out due to low initial growth rates. Even vessel headspace and material should be considered. Cells growing in small glass tubes may be stressed when transferred to larger volume glass bottles, leading to washout once the chemostat pump is activated. Acclimation typically requires one to two transfers of late log phase cells which can take weeks.
- 2 Nutrient concentrations in starter cultures should also be considered. We have had success running chemostats using both culture methods listed below:
 - 2.1 One option is to first acclimate cells in batch culture using the same nutrient limited medium that will be used in the chemostat. Cells will not reach typical abundances seen in replete media ($\sim 10^8$ cells mL⁻¹), so they will need to be grown in larger volumes for chemostat inoculation. Additionally, because they are at low abundances, they may be more vulnerable to oxidative stress. The benefit of growing starter cultures in the nutrient limited medium beforehand is that these cells may reach steady state faster since nutrient concentrations will not increase due to carryover when the chemostat is inoculated. In our experiments, cells pre-acclimated to the nutrient limited medium reach steady state in approximately two weeks.
 - 2.2 The alternative option is to grow cells in a nutrient replete medium. These cells will reach higher concentrations, will be more resistant to stressors, and chemostats will require a lower inoculation volume. However, inoculating from a nutrient replete medium will result in a temporary increase in nutrient concentration for the limiting nutrient at the start of culturing. This results in increased time before cells reach steady state, as they typically grow to higher abundances, then slowly decline to steady state abundances set by the original concentration of the limiting nutrient. In our experiments, cells inoculated from a nutrient replete medium reach steady state in approximately four weeks.

Preparing Chemostat Materials: Glassware and Stir Bars

- 3 Following the recommendations of Moore et al. for culturing *Prochlorococcus* [1], all glassware should be acid washed prior to use then rinsed with 18Ω MilliQ (MQ) water. Bottles for reservoirs should be large enough to hold media for several days of culturing. The goal is to minimize the number of reservoir exchanges required over the course of the experiment. The experimental vessels can be any desired size but should include ample headspace. For example, 300 mL cultures were grown in 500 mL glass bottles.

- 4 Stir bars should also be acid washed prior to use then rinsed with MQ water. Stir bars with signs of rust or metal leaching from the plastic casing should not be used. To prevent this, do not leave stir bars in the acid bath for more than one day. Stir bars can be sterilized by autoclaving them with tubing in a closed bin or by placing them into chemostat vessels when the medium is initially autoclaved.

Preparing Chemostat Materials: Lids

- 5 Chemostat bottle lids have ports for the insertion of chemostat tubing. Media reservoir lids and experimental vessel lids will likely have different numbers of ports. Reservoir lids will require one port per experimental vessel connected and one port for pressure release. Experimental vessel lids will require four ports – one for inflow, one for outflow, one for a sampling port, and one for the aquarium air pump line that maintains vessel pressure. Unused ports should be tightly plugged so that the chemostat maintains pressure and therefore a steady dilution rate.
- 6 Lids do not need to be acid washed.
- 7 Lids should be connected to tubing prior to autoclaving to make final assembly as simple as possible to decrease odds of contamination.
- 8 No lid is required for the waste collection bottle. Outflow tubing can sit in an open flask or bottle as contamination is not a concern. If a lid is used for the waste bottle, ensure this lid is loose so that pressure is released at this point. Tight lids on the waste bottle can result in stop or reversal of chemostat flow.

Preparing Chemostat Materials: Aquarium Air Pumps

- 9 Chemostats will not flow properly unless they are pressurized. To do so, 5-15 gallon aquarium air pumps (Aqua Culture) are utilized to flow filtered air into the headspace of the experimental vessels. Each experimental vessel requires its own aquarium air pump.

Preparing Chemostat Materials: General Tubing Guidelines

- 10 Silicone tubing utilized for continuous culturing is commonly cured with hydrogen peroxide. Because *Prochlorococcus* is notoriously vulnerable to hydrogen peroxide, even at nanomolar concentrations [2], platinum cured silicone tubing (VWR; Cat. No. MFLX96410-14) should be used instead. This is critical for any tubing that will interact with reservoir media or growing cells, such as the inflow and sampling tubes.
- 11 The silicone tubing will be attached to hard, PTFE tubing (Cole-Parmer; Cat. No. EW-21942-76) which will go through the lid ports and make an air-tight seal to maintain

pressure within the chemostat. The PTFE tubing can and should be autoclaved. The lengths of the various PTFE tubes are critical to the function which they will serve:

- 11.1 In the reservoir: Tubes for moving media from the reservoir to the experimental vessels should reach down to the bottom of the reservoir to maximize media available for uptake. The tube for pressure release should be very short and sit several inches above the medium.
- 11.2 In the experimental vessels: There will be four total tubes - inflow, outflow, sampling, and the aquarium air pump tube. The inflow tube should be 2-3 inches above the medium, so that medium flow can be visualized. The waste out tube should sit immediately above the medium. Wherever the end of this tube sits will dictate the volume of the experimental vessel. Pressure will cause this line to drain until it can no longer reach the medium. The sampling tube should be long enough to reach into the middle of the vessel. The aquarium air pump tube, similar to the pressure release tube in the reservoir, can be very short and should not be in the medium.
- 12 Chemostat tubes should be thoroughly rinsed with MQ water to remove any salt residue from previous use. Washing with detergent should be avoided unless there is significant build-up within the tubing. Using detergent can result in increased nutrients entering media upon pump activation, especially if using ammonia-based cleaners. Rinse tubes extensively if detergent is required. Tubes should not be, and do not need to be, acid washed. This may degrade the tubing from within and cause leaching into the chemostat medium.
- 13 See Image 1 for a visual on how long each PTFE tube should be in relation to the medium level.

Preparing Chemostat Materials: Inflow Tubing

- 14 The inflow tubing is most critical as it interacts directly with fresh media and cells. Generation of leachates or hydrogen peroxide must be avoided to successfully culture *Prochlorococcus* in chemostats at low abundances.
- 15 If peristaltic pump tubing is able to be autoclaved, ensure all water is removed from tube interiors prior to autoclaving. Autoclaving can result in the generation of hydrogen peroxide if water is present. A vacuum line can be used to remove water from chemostat tubes.
- 16 An alternative sterilization method is to use ethanol. This is the preferred method as the peristaltic pump tubing used in these experiments was unable to be autoclaved. Do NOT autoclave peristaltic pump tubing listed as non-autoclavable – this will alter the tube shape and may result in inaccurate flow rates, plastic leachates entering the medium, and production of hydrogen peroxide at concentrations between ~100-200 nM. To sterilize tubes with ethanol, detach the silicone and pump tubing portions of the tubes



from the PTFE portions, leaving the PTFE tubing attached to the lids. Push several milliliters of 70% ethanol through the silicone and pump tubing then fill with 70% ethanol and allow to sit overnight in a laminar flow hood. Ends of tubes can be placed in a container of ethanol overnight to ensure openings are sterilized. Next, rinse tubing with sterile MQ water several times using a sterile syringe. If time permits, fill tubes with MQ and allow to sit overnight to ensure all ethanol has been removed. Once rinsed with MQ water, these tubes can be connected to the rest of the autoclaved tubing prior to final assembly.

- 17 Exposure to light can result in abiotic generation of hydrogen peroxide in media. This occurs primarily as media travels through the tubing, even if it is platinum cured. To resolve this, inflow tubing should be covered in aluminum foil after final assembly. Substances that stick to tubing such as tape should be avoided as they may leave residues that may impact tube chemistry, especially after autoclaving.
- 18 To minimize media interactions with tubing, silicone tubes should be made as short as possible without increasing tension. Tubing under tension can easily pop off the lids, resulting in possible contamination.

Preparing Chemostat Materials: Outflow, Sampling, and Aquarium Air Pump Tubing

- 19 Tubing for other aspects of the chemostat, including the outflow lines, sampling ports, and aquarium air pump lines do not require such special care. These should be autoclaved to prevent contamination:
- 19.1 All tubes (except inflow tubing if non-autoclavable) and lids should be assembled together then placed in an autoclavable bin with a lid. Place lines in carefully and untangled so that assembly can be done quickly without introducing contaminants. This bin should also include any Luer-lock port caps, such as those that screw into the sampling ports.
- 20 Once all tubing and caps are sterilized, they can be assembled onto the reservoirs and experimental vessels containing the finished medium. This should be done in a laminar flow hood to ensure sterility.
- 21 Finally, place 0.2 μm Luer-lock filters on the ends of aquarium air pump lines and reservoir pressure release ports and tightly attach autoclaved Luer-lock caps onto the sampling ports of experimental vessels.
- 22 See Image 2 for a visual of a fully assembled chemostat.

Media

- 23 Nitrogen limited chemostat studies for *Prochlorococcus* were conducted using the artificial seawater medium AMP-MN [3]. To reduce possible sources of additional

ammonia, this medium was made using BioUltra salts from Sigma-Aldrich (NaCl: Cat. No. 71376; MgSO₄•7 H₂O: Cat. No. 63138; MgCL₂•6 H₂O: Cat. No. 63068; KCl: Cat. No. 60128; CaCl•2 H₂O: Cat. No. 21097).

Inoculation and Activation of the Peristaltic Pump

- 24 *Prochlorococcus* cells should be inoculated from log phase. Cells inoculated from stationary phase will experience longer lag phases and may die outright if not well acclimated to chemostat conditions.
- 25 Cells should be inoculated at concentrations well below steady state abundances to prevent starvation. However, extremely low inoculation concentrations can result in cell death due to stress. In AMP MN chemostats, *Prochlorococcus* typically reaches steady state abundances of $3\text{--}5 \times 10^6$ cells mL⁻¹. We have had success with starting inoculations of $1\text{--}5 \times 10^5$ cells mL⁻¹. These starting concentrations allow for multiple days of growth before reaching steady state abundances.
- 26 *Prochlorococcus* growth should be monitored prior to starting the chemostat pump. It is not uncommon for cells to experience one to two days of lag phase, especially if transferred to a new medium or different volume. Activating the pump during lag phase will likely result in washout. Once at least two consecutive days of increased cell abundances are observed, the pump can be turned on with reasonable confidence that cells will not wash out. The pump must be activated before cells reach steady state abundances to prevent starvation.
- 27 We have had success with dilution rates of ~ 0.24 day⁻¹ (+/- 0.02), though faster or slower rates are likely feasible. A Watson Marlow 205U peristaltic pump fitted with either PVC or Marprene pump tubing with bore sizes of 0.76 mm were used to achieve desired dilution rates.

Sampling the Chemostat

- 28 Because the experimental vessel is under pressure from the aquarium air pump, bottle lids should be slightly loosened prior to sampling to prevent rapid outflow of cells from the sampling port.
- 29 Sampling ports should be fitted with a female Luer-lock end and male Luer-lock cap. To sample, use a male Luer-lock syringe of desired volume. Sampling should be done as quickly as possible to prevent contamination. Caps can be dipped in a tray of ethanol before being reattached to sterilize the port after sampling.
- 30 Make sure lids are closed tightly after sampling, or the chemostat will not drain properly.



Checking the Flow Rate

- 31 Different peristaltic pump models and tubing makes will have different rpm to flow rate ratios. These values can often be found on the pump manufacturer's website. Because these values are often estimates, it is important to check the actual flow rate of the chemostats throughout the experiment.
- 32 To do so, collect chemostat outflow for 24 hours and measure the volume. To calculate the true flow rate, divide this volume by the total volume of your experimental vessel. Repeat these measurements occasionally throughout the duration of the experiment as flow rate can slightly vary day to day.
- 33 If flow rate dramatically changes over the course of the experiment, it may be because the pump tubes have become deformed. To alleviate this, move the peristaltic pump tubing so that pressure points are in new locations, or replace the pump tube under sterile conditions.

Checking for Contamination

- 34 Because experimental vessels are being opened regularly, it is important to check for contamination of the chemostat throughout the experiment. To do so, 1 mL samples from chemostats can be inoculated into 5 mL liquid YTSS medium [4] and incubated in the dark for several weeks. These cultures should be routinely checked for increases in turbidity, signifying contamination.

Upon Experiment Completion

- 35 Once the experiment is complete, tubing should be disconnected and rinsed with MQ water as soon as possible to prevent salt build up. Outflow tubing typically has high levels of salt build-up. It is often easiest to just replace this tube to prevent future clogs.
- 36 A final contamination check should be conducted as outlined above.



Protocol references

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