Procedures for culturing *Prochlorococcus*, *Synechococcus*, SAR11, and marine heterotrophic bacteria

Described here are commonly used protocols and media recipes used to maintain *Prochlorococcus*, *Synechococcus*, SAR11, and marine heterotrophic bacteria. *Prochlorococcus* and *Synechococcus* are routinely grown in natural seawater-based (undefined) and artificial seawater-based (defined) media[1-3]. Recipes for growing Sar11 described in Carini et al. (2013)[4] and marine heterotrophic bacteria[5-7] are also included. Descriptions of the intricacies of plating both *Prochlorococcus* and *Synechococcus*, the measures taken to remove metals from cultureware, suggestions for media and bottle modification for growing *Prochlorococcus* in large volumes (1-20L), descriptions of methods to cryopreserve *Prochlorococcus* for long-term storage and shipment are provided. Also discussed are methods for preserving samples for flow cytometry, how cultures are measured, how growth rates are calculated, and lists of what incubators and tools used to maintain and monitor cultures. Finally, tips for successfully growing *Prochlorococcus* and *Synechococcus* are described.

CONTENTS:

[1] Moore et al. (2002) Limnol. Oceanogr. 47:989-996.
[2] Moore et al. (2007) *Limnol. Oceanography: Methods* 5: 353-362
[3] Waterbury et al. (1986) Photosynthetic Picoplankton. Can. Bull. Fish. Aquatic Sci. 214: 71-120
[4] Carini et al. (2013) *ISME* 7(3): 592-602
[5] Saito et al. (2002) Limnol. Oceanogr. 47:1629-1636
[6] Morris et al. (2008) Appl. Environ. Microbiol. 74: 4530-4534
[7] Berube et al. (2015) *ISME* 9: 1195-1207

Cultureware for growing *Prochlorococcus* and *Synechococcus* (Moore et al, 2007)

Prochlorococcus and some strains of *Synechococcus* are highly sensitive to trace metal concentrations, so special care should be given in choosing and preparing media containers and culture vessels. We have had success with the disposable and reusable cultureware, though reusable cultureware requires cleaning with acid and autoclaving (see protocol below). Avoiding flasks with reusable foam or cheese cloth stoppers is generally a good idea as they could become contamination risks. Single-use stoppers, foam, or filters that can be autoclaved are okay to use.

A. TYPES OF CONTAINERS

Media containers

Acid washed Nalgene polycarbonate (1L #2015-1000) or Teflon bottles (1L #1630-0032) work well. Take caution when using glass bottles for natural seawater media if you plan to autoclave the media as this could cause silicate precipitation (visible crystal shards), but may be dependent on the type of glass or seawater source.

Culture vessels

We use a range of vessel types and sizes, but prefer to use acid washed Nalgene polycarbonate bottles (large volume cultures), borosilicate test tubes, tissue culture-grade polycarbonate and polystyrene plasticware, or tissue culture-grade 96 well plates.

Commonly used cultureware (single-use plastics should be sterile, no acid wash required):

VWR borosilicate test tubes 25mm (#47729-586) and 13mm (#47729-572)

Kim-Kap polypropylene test tube caps 25mm (#73660-25) and 13mm (#73660-13)

Falcon round bottom polystyrene tubes 14ml (#352051) and 5mL (#352054)

96 well Falcon clear plates (#353072) and Corning black walled plates (#3603)

Greiner Bio-One CELLSTAR® Filter Cap Cell Culture Flasks with filter screw cap (#690 175)

B. LIQUID TRANSFER AND MEASUREMENT DEVICES

For small volumes, use sterile filter pipette tips and serological pipettes. For larger volumes, use scales that we can place in the culture hood to weight out large volumes of media and cultures.

C. PROTOCOL FOR ACID WASHING CULTUREWARE

NOTE: under no circumstances should a brush be used to clean the glassware, as this is a significant source of metal and bacterial contamination. Be sure that there are no rusty objects anywhere near your sink or bench. This includes test tube racks. Use Nalgene ResMer resin unwire racks (#5970-0425) for cleaning and autoclaving as these will not rust after multiple autoclave runs. Use epoxy coated test tube racks (#60916-870) for growing as these allow for more light penetration.

- Soak cultureware overnight in tap water w/ ~2% Micro-90 (Intl. Products Corp.) detergent or Pyroneg liquid detergent (~0.1% v/v) (ThermoFisher Scientific, # DIV12291)
- Rinse 6X with deionized or reverse-osmosis water
- Soak overnight in 1N HCl (trace metal free; we use Mallinckrodt #2062); this 1N HCl can be reused multiple times as the acid will 'hold' a lot of metal.
- Rinse 6X with Milli-Q water (18MΩ water)
- Fill with ultrapure (Milli-Q) water if autoclaving

Incubators and Lighting

We grow our cultures in either single or double door incubators such as a Percival I-36LLVL or we will grow them in large Harris Environmental growth chamber rooms (called "warm rooms") which are custom ordered and require elaborate plumbing, electrical, and ventilation systems. Infors HT Multitron incubator shakers or other brand incubators wired with fluorescent or LED lights also work well.

Light bulbs are specific to each incubator and you should check the specs on your incubator for ordering information. We commonly use daylight or cool white fluorescent bulbs (for example, F48T12/D/VHO or F30T12/CW/RS). Window mesh/screen is best for shading as it allows for more airflow in the incubators. Rolls of these can be purchased at your local hardware store. Adding lighting film (Lee Filters or Roscolux) to light bulbs directly is best for shading large areas. To do this, roll film just slightly bigger than the bulb into a tube, secure with clear tape, and slide over the lighting. Putting lighting film directly on shelving or around racks reduces air flow and circulation which will create patches of warm areas throughout the incubator. Lighting films can also filter out certain wavelengths and are ideal if you wish to grow your cells in colored light (like blue light). Green light has been shown to have the least effect on *Prochlorococcus* gene expression (Steglich et al. 2006) and is therefore used when we need to sample in the dark during a night cycle, for light deprivation experiments, or with assays that require darkness. We simply apply the green lighting film on our overhead lights or additional spot lights to create a "dark" room environment.

Light levels can be checked using a light meter that measures PAR (Photosynthetically Active Radiation) and has an output in µmol quanta m^{-2} s^{-1} which translates into µmol photons m^{-2} s^{-1}. There are many options for different light meters, but the best measurements come from a meter that has a Teflon coated spherical detector which will measure light levels from almost all angles. We prefer using a LI-COR LI-250A handheld reader coupled with a Walz US-SQS/L probe. We attach the probe to the end of a telescoping wand which we extend for measurements and retract for storage. This allows us to get access to the back of the incubators without blocking light with our body and allows us to shut the door as much as possible to reduce ambient light that will affect the measurements. We also have a Biospherical QSL-2100 attached to a laptop, but we find this awkward and cumbersome to carry and use so we prefer the Walz probe for our measurements. A "light logger" which logs light, temperature, and humidity over time (every minute) is helpful. Our instrument was custom designed and the instructions for creating your own device are available upon request with the Chisholm Lab. This monitor is ideal for measuring light flux in incubators with dimmable controllers, like the ones available from Percival. Programs can be developed to have the light gradually increase after sunrise, peak at mid-day for several hours, and then decrease until sunset. While these incubators are more expensive, the lighting is more representative of the ocean and therefore worth considering when purchasing your incubator.

Average growth rates (day^{-1}) of various *Prochlorococcus* strains and associated clades at different light intensities. All replicate cell cultures were grown at 24°C on a 14:10 light:dark cycle. All data from Moore and Chisholm, 1999, denoted with ¥, except data for MED4 and SS120, denoted with †, from Moore et al. 1995, and data for NATL2A, denoted with ‡, from Zinser et al. 2007. *Prochlorococcus* clades are defined by the evolutionary relationships determined from the 16S-23S rDNA spacer, also called the internal transcribed spacer (ITS) sequence.

Light Intensity (μmol photons m^{-2} s^{-1})	MED4† HL(I)	MIT9201¥ HL(II)	MIT9202¥ HL(II)	MIT9215¥ HL(II)	MIT9302¥ HL(II)	MIT9312¥ HL(II)	NATL2A‡ LL(I)	SS120† LL(II)	MIT9211¥ LL(III)	MIT9303¥ LL(IV)	MIT9313¥ LL(IV)
1	–	–	–	–	–	–	–	0.00 ± 0.00	–	–	–
2	–	–	–	–	–	–	–	–	0.00 ± 0.00	–	–
3	–	–	–	–	–	–	–	0.22 ± 0.04	–	–	–
4	–	–	0.08 ± 0.02	0.08 ± 0.01	–	–	–	–	0.16 ± 0.03	0.07 ± 0.00	0.01 ± 0.00
5	–	0.00 ± 0.00	–	–	–	–	0.19 ± 0.03	–	–	–	–
7	–	–	–	–	0.00 ± 0.00	0.00 ± 0.00	–	–	–	–	–
8	0.00 ± 0.00	–	–	–	–	–	0.25 ± 0.01	–	–	–	–
10	–	0.12 ± 0.01	0.15 ± 0.04	0.15 ± 0.03	0.23 ± 0.01	–	–	–	0.27 ± 0.07	–	0.20 ± 0.01
11	–	–	–	–	–	–	0.27 ± 0.02	–	–	0.20 ± 0.01	–
14	–	–	–	–	–	0.23 ± 0.15	–	–	–	–	–
15	–	–	–	–	–	–	0.50 ± 0.08	0.40 ± 0.05	–	–	–
18	0.25 ± 0.01	–	–	–	–	–	–	–	–	–	–
20	–	–	–	–	0.46 ± 0.05	–	0.52 ± 0.08	–	–	–	–
21	–	–	–	–	–	–	–	–	–	0.45 ± 0.05	–
23	–	–	–	–	–	–	–	–	–	–	0.45 ± 0.00
25	–	–	–	–	–	–	0.54 ± 0.03	–	–	–	–
26	–	0.19 ± 0.00	–	0.23 ± 0.00	–	–	–	–	–	–	–
27	–	–	–	–	–	–	–	–	0.42 ± 0.01	–	–
29	–	–	0.30 ± 0.08	–	–	–	–	–	–	–	–
33	0.47 ± 0.03	–	–	–	–	–	–	–	–	–	–
35	–	–	–	–	–	–	–	0.59 ± 0.07	–	–	–
36	–	–	–	–	–	–	–	–	–	0.53 ± 0.01	–
38	–	–	–	–	–	–	–	–	0.41 ± 0.05	–	–
39	–	–	–	0.36 ± 0.01	–	–	–	–	–	–	–
40	–	–	–	–	–	–	0.58 ± 0.01	–	–	–	–
43	–	–	0.41 ± 0.02	–	–	–	–	–	–	–	–
48	–	0.26 ± 0.01	–	–	–	–	–	–	–	–	–
53	–	–	–	–	–	–	–	–	–	0.41 ± 0.02	–
55	–	–	–	–	–	–	–	–	–	–	0.55 ± 0.01
56	–	–	–	0.45 ± 0.03	–	–	–	0.52 ± 0.05	–	–	–
60	–	–	–	–	–	–	0.54 ± 0.05	–	0.59 ± 0.06	–	–
62	0.56 ± 0.04	–	–	–	–	–	–	–	–	–	–
63	–	–	–	–	–	0.73 ± 0.09	–	–	–	–	–
64	–	0.41 ± 0.02	0.48 ± 0.03	–	–	–	–	–	–	–	–
66	–	–	–	–	0.66 ± 0.08	–	–	–	–	–	–
73	–	–	–	–	–	–	–	–	–	0.28 ± 0.02	–
77	–	–	–	–	–	–	–	0.49 ± 0.04	–	–	–
80	–	–	–	–	–	–	0.52 ± 0.01	–	–	–	–
100	–	–	–	–	–	–	0.46 ± 0.05	–	–	–	–
110	–	–	–	–	–	–	–	0.44 ± 0.04	–	–	–
120	–	–	–	–	–	–	0.38 ± 0.01	–	–	–	–
121	–	0.57 ± 0.03	–	0.64 ± 0.04	–	–	–	–	–	–	–
122	–	–	–	–	–	–	–	–	0.64 ± 0.02	–	–
128	–	–	0.74 ± 0.05	–	–	–	–	–	–	–	–
129	0.60 ± 0.07	–	–	–	–	–	–	–	–	–	–
144	–	–	–	–	–	–	–	0.00 ± 0.00	–	–	–
148	–	–	–	–	–	–	–	–	–	–	0.42 ± 0.00
149	–	–	–	–	–	–	–	–	–	0.00 ± 0.00	–
155	–	–	0.71 ± 0.01	–	–	–	–	–	–	–	–
158	–	–	–	0.77 ± 0.04	0.77 ± 0.01	–	–	–	–	–	–
159	–	0.80 ± 0.03	–	–	–	–	–	–	–	–	–
164	–	–	–	–	–	0.78 ± 0.00	–	–	–	–	–
173	0.63 ± 0.01	–	–	–	–	–	–	–	–	–	–
206	–	0.69 ± 0.03	–	–	–	–	–	–	–	–	–
211	–	–	0.85 ± 0.06	–	–	–	–	–	0.46 ± 0.01	–	–
222	–	–	–	0.77 ± 0.06	–	–	–	–	–	–	–
235	–	–	–	–	–	–	–	–	–	–	0.00 ± 0.00
236	0.61 ± 0.02	–	–	–	–	–	–	–	–	–	–
254	–	0.78 ± 0.05	–	–	–	–	–	–	–	–	–
272	–	–	0.80 ± 0.02	–	–	–	–	–	–	–	–
291	–	0.78 ± 0.05	–	–	–	–	–	–	–	–	–
314	–	–	–	0.65 ± 0.00	–	–	–	–	–	–	–
366	–	–	0.65 ± 0.01	–	–	–	–	–	–	–	–
402	–	0.63 ± 0.05	–	–	–	–	–	–	–	–	–
407	–	–	–	0.54 ± 0.04	–	–	–	–	–	–	–
418	–	–	0.68 ± 0.04	–	–	–	–	–	–	–	–
457	0.52 ± 0.03	–	–	–	–	–	–	–	–	–	–
465	–	–	–	–	0.46 ± 0.03	–	–	–	–	–	–
492	–	–	–	–	–	0.48 ± 0.08	–	–	–	–	–

= Highest Observed Growth Rate

– No data available

Average growth rates (day^{-1}) of *Prochlorococcus* strains and associated clades at different temperatures.
All replicate cell cultures were grown in a 14:10 light:dark cycle at 66 ±1 mmol Q m-2 s-1. Data for MED4ax, MIT 91515, MIT9215, and MIT9312, denoted with †, from Johnson et al. 2006; data for MIT9321, ASS9601, NATL2A, and MIT9313, denoted with ‡, from Zinser et al. 2007. *Prochlorococcus* clades are defined by the evolutionary relationships determined from the 16S-23S rDNA spacer, also called the internal transcribed spacer (ITS) sequence.

Temperature (°C)	MED4ax† HL(I)	MIT9515† HL(I)	MIT9215† HL(II)	MIT9312† HL(II)	MIT9321‡ HL(II)	AS9601‡ HL(II)	NATL2A‡ LL(I)	MIT9313‡ LL(IV)
11	0.09 ± 0.02	0.10 ± 0.03	–	–	–	–	–	–
13	0.20 ± 0.01	0.19 ± 0.01	–	–	–	–	0.00 ± 0.00	–
14	0.24 ± 0.04	0.21 ± 0.03	–	–	–	–	0.18 ± 0.01	–
15	0.23 ± 0.03	0.24 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	–	–	–	–
17	0.30 ± 0.02	–	0.16 ± 0.03	0.28 ± 0.06	–	–	0.27 ± 0.01	–
18	0.30 ± 0.03	0.27 ± 0.03	0.17 ± 0.03	0.29 ± 0.06	–	0.00 ± 0.00	–	–
19	0.32 ± 0.07	0.33 ± 0.05	0.22 ± 0.02	0.32 ± 0.02	–	0.25 ± 0.02	0.31 ± 0.03	–
21	–	–	–	0.36 ± 0.12	–	0.30 ± 0.03	–	–
22	0.37 ± 0.02	–	0.36 ± 0.03	0.49 ± 0.01	–	–	0.43 ± 0.01	0.37 ± 0.05
23	0.40 ± 0.02	–	0.40 ± 0.02	0.54 ± 0.02	–	–	0.47 ± 0.02	0.39 ± 0.05
24	0.39 ± 0.02	0.46 ± 0.03	0.44 ± 0.02	0.58 ± 0.03	–	–	0.48 ± 0.01	0.43 ± 0.02
25	0.40 ± 0.01	0.47 ± 0.01	0.50 ± 0.01	0.62 ± 0.03	–	–	0.47 ± 0.03	0.44 ± 0.02
26	0.38 ± 0.01	0.43 ± 0.03	0.43 ± 0.01	0.58 ± 0.03	–	–	0.42 ± 0.02	0.45 ± 0.04
27	0.34 ± 0.02	–	0.47 ± 0.03	0.61 ± 0.01	–	–	0.36 ± 0.01	0.47 ± 0.04
28	0.00 ± 0.00	0.28 ± 0.04	0.43 ± 0.01	0.47 ± 0.08	0.47 ± 0.47	–	0.00 ± 0.00	0.38 ± 0.04
29	–	0.00 ± 0.00	0.43 ± 0.01	0.33 ± 0.04	0.44 ± 0.44	0.43 ± 0.02	–	0.00 ± 0.00
30	–	–	0.45 ± 0.01	0.00 ± 0.00	0.37 ± 0.37	0.42 ± 0.04	–	–
31	–	–	0.43 ± 0.01	–	–	0.39 ± 0.01	–	–
33	–	–	0.00 ± 0.00	–	0.00 ± 0.00	0.00 ± 0.00	–	–

= Highest Observed Growth Rate

– No data available

NATURAL SEAWATER-BASED PRO99 MEDIUM (Moore et al., 2002)

NOTE: Maximal growth has been observed with open ocean seawater (i.e. Sargasso seawater, Eastern Australian Current seawater, etc.), though most *Prochlorococcus* strains can grow in coastal seawater (Woods Hole, Gulf of Maine, etc.) and on Red Sea salts (cat # R99344; https://www.redseafish.com/red-sea-salts/) as well. All chemicals were purchased from Sigma (except Red Sea Salts) at the highest purity available.

Nutrient additions to 1L filtered, autoclaved seawater

Nutrient	Grade	Primary Stock (M)	Dilution Factor	Final Conc. (µM)
$NaH_2PO_4 \cdot H_2O$	SigmaUltra	0.025	1:500	50
NH_4Cl	SigmaUltra	0.50	1:625	800
$Na_2EDTA \cdot 2\ H_2O$	99%	0.012	$1:10^4$	1.17
$FeCl_3 \cdot 6\ H_2O$	Analytic	0.012	$1:10^4$	1.18
$ZnSO_4 \cdot 7\ H_2O$	>99.5%	0.080	$1:10^7$	0.008
$CoCl_2 \cdot 6\ H_2O$	Analytic	0.050	$1:10^7$	0.005
$MnCl_2 \cdot 4\ H_2O$	Analytic	0.900	$1:10^7$	0.090
$Na_2MoO_4 \cdot 2\ H_2O$	ACS	0.030	$1:10^7$	0.003
Na_2SeO_3	~98%	0.100	$1:10^7$	0.010
$NiCl_2 \cdot 6\ H_2O$	Analytic	0.100	$1:10^7$	0.010

PREPARATION:

1. Prepare a glass filter funnel and flask by cleaning with acid and Milli-Q water, as described in the cultureware section (clean the system after every 10 filtrations). Filter raw seawater through 47mm Whatman GF/F stacked on top of a 47mm 0.2µm polycarbonate filter (make sure there are no bubbles/creases) OR through a Pall 0.2µm Supor filter.

   Autoclave seawater in a Teflon or polycarbonate bottle (60 minutes for 2L) and allow to cool to room temperature before adding nutrients. Glass bottles may or may not cause precipitation after autoclaving.

2. Prepare primary stocks of NH_4Cl, $NaH_2PO_4 \cdot H_2O$, and trace metals as follows:

   A. 0.5M NH_4Cl
   - Weigh out 2.67g NH_4Cl using dust-free weigh paper
   - Transfer into 100mL volumetric flask filled with about 60mL Milli-Q water
   - Dissolve NH_4Cl by swirling flask several times
   - Adjust volume to 100mL mark with Milli-Q water
   - Filter with a sterile 0.2µm nylon filtration unit
   - Store sterile stock at 4°C

B. 0.025M $NaH_2PO_4 \cdot H_2O$

- Weigh out 0.345g $NaH_2PO_4 \cdot H_2O$ using dust-free weigh paper
- Transfer into 100mL volumetric flask filled with about 60mL Milli-Q water
- Dissolve NaH_2PO_4 by swirling flask several times
- Adjust volume to 100mL mark with Milli-Q water
- Filter with a sterile 0.2µm nylon filtration unit
- Store sterile stock at 4°C

C. Trace Metal Stocks

i. **Primary trace metal stocks**

- Using dust-free weigh paper, weigh out:

2.30g	$ZnSO_4 \cdot 7H_2O$
1.19g	$CoCl_2 \cdot 6H_2O$
17.81g	$MnCl_2 \cdot 4H_2O$
0.726g	$Na_2MoO_4 \cdot 2H_2O$
1.73g	Na_2SeO_3
2.38g	$NiCl_2 \cdot 6H_2O$

- Transfer each trace metal into separate 100mL volumetric flasks containing about 60 mL Milli-Q water
- Dissolve contents by swirling flask several times
- Adjust volume to 100mL mark with Milli-Q water
- Store each stock in a cleaned Teflon, polycarbonate, or polystyrene bottle at 4°C

ii. **Trace metal working stock**

- Weigh out 0.435g $Na_2EDTA \cdot 2\ H_2O$ using dust-free weigh paper
- Transfer into 100mL volumetric flask filled with 60mL Milli-Q water
- Dissolve Na_2EDTA by swirling flask several times
- Weigh out 0.32g $FeCl_3 \cdot 6\ H_2O$ using dust-free weigh paper
- Dissolve $FeCl_3$ into same volumetric flask by swirling several times
- Individually add and dissolve 100µl of the Primary trace metal stocks
- Adjust volume to 100mL mark with Milli-Q water
- Filter with a sterile 0.2µm nylon filtration unit
- Store sterile stock at 4°C

3. To make up the media, add following volumes of sterile nutrients and trace metal stock to one liter of the autoclaved or 0.2µm filtered seawater. It is important to *dissolve each nutrient sequentially*.

Filtered Seawater		1000.0mL
0.5M	NH_4Cl	1.6mL
0.025M	$NaH_2PO_4 \cdot H_2O$	2.0mL
Trace metal working stock		100µL

4. Store at room temperature for up to two months.

Notes: Acid-clean nylon or plastic syringes (no rubber ended plungers) and 0.2µm Supor syringe filters can be used in place of the filtration units. Same concentration of sodium nitrate (Sigma #71755) can be substituted for NH_4Cl.

ARTIFICIAL SEAWATER BASED AMP1 MEDIUM (Moore et al., 2007)

Sea Salt and nutrient additions for 1L

Nutrient	Grade	Primary Stock (M)	Dilution Factor	Final Conc.
NaCl	SigmaUltra	n/a	n/a	481 mM
$CaCl_2 \cdot 2\ H_2O$	SigmaUltra	n/a	n/a	10 mM
KCl	SigmaUltra	n/a	n/a	9 mM
$Mg\ SO_4 \cdot 7\ H_2O$	SigmaUltra	n/a	n/a	28 mM
$MgCl_2 \cdot 6\ H_2O$	SigmaUltra	n/a	n/a	27 mM
NaH_2PO_4	SigmaUltra	0.025	1:500	50 µM
$(NH_4)_2SO_4$	SigmaUltra	0.4	1:1000	400 µM
$NaHCO_3$	SigmaUltra	0.6	1:100	6 mM
TAPS	SigmaUltra	0.5	1:133	3.75 mM
$Na_2EDTA \cdot 2H_2O$	99%	0.012	$1:10^5$	0.1170 µM
$FeCl_3 \cdot 6\ H_2O$	Analytic	0.012	$1:10^5$	0.1180 µM
$ZnSO_4 \cdot 7\ H_2O$	>99.5%	0.080	$1:10^8$	0.0008 µM
$CoCl_2 \cdot 6\ H_2O$	Analytic	0.050	$1:10^8$	0.0005 µM
$MnCl_2 \cdot 4\ H_2O$	Analytic	0.900	$1:10^8$	0.0090 µM
$Na_2MoO_4 \cdot 2\ H_2O$	ACS	0.030	$1:10^8$	0.0003 µM
Na_2SeO_3	~98%	0.100	$1:10^8$	0.0010 µM
$NiCl_2 \cdot 6\ H_2O$	Analytic	0.100	$1:10^8$	0.0010 µM

NOTE: All chemicals should be the highest purity available. We purchase our chemicals from Sigma. To avoid metal contamination, dry nutrients and metals should be removed with plastic or teflon coated spatulas (not metal!). If concerned about removing residual carbon from sterile filtration units, pre-wash the filter 2-3 times with 100mL Milli-Q. When filtering use 1 inch of Hg.

1. Prepare nutrient stocks as following using Sigma Ultra grade chemicals:

A. 25mM NaH_2PO_4 (pH 7.5)

- Weigh out 0.300g NaH_2PO_4 on dust-free weigh paper
- Transfer into 100mL volumetric flask filled with about 60mL Milli-Q water
- Dissolve NaH_2PO_4 by swirling flask several times
- Adjust to pH 7.5 with NaOH (Mallinckrodt #7708)
- Adjust volume to 100mL mark with Milli-Q water
- Filter with a sterile 0.2µm nylon filtration unit
- Store sterile stock at 4°C

B. 0.4M $(NH_4)_2SO_4$

- Weigh out 5.284g $(NH_4)_2SO_4$ on dust-free weigh paper
- Transfer into 100mL volumetric flask filled with about 60mL Milli-Q water
- Dissolve $(NH_4)_2SO_4$ by swirling flask several times
- Adjust volume to 100mL mark with Milli-Q water
- Filter with a sterile 0.2µm nylon filtration unit
- Store sterile stock at 4°C

C. 0.5M TAPS (pH 8)

- Weigh out 60.82g TAPS on dust-free weigh paper
- Transfer into 1L glass beaker with stir bar and 300mL Milli-Q water
- Dissolve TAPS by stirring
- Fill remaining volume to 500mL mark with Milli-Q water
- Check pH by pipetting out small amount onto pH paper for rough estimate
- Turn stirrer on and **SLOWLY** add NaOH, check pH by pipetting out small amount onto pH paper for rough estimate periodically
- When pH is around 8 use a calibrated pH meter probe that has been Milli-Q water rinsed and soaked several times (add NaOH as needed to obtain pH 8)
- Transfer to volumetric flask or graduated cylinder and adjust volume to 500mL
- Filter with a sterile .2µm nylon filtration unit
- Store sterile stock at 4°C

NOTE: TAPS buffer has been shown to produce less H_2O_2 (Morris et al, 2011), but if working with high density cell concentrations and you are not concerned about H_2O_2 effects using HEPES (pH 7.5) will also work. Adjust the pH of the buffer as needed for experimental requirements.

D. Working trace metal stock

Use the working trace metal stock from the Natural Seawater-based PRO99 medium recipe, **but ten times LESS of the stock will be added to AMP1 compared to PRO99.**

2. Prepare "Turks Island Salt Mix" (Merck Index 9954) by adding the following:

Dissolve each ingredient sequentially in 2/3 final volume of Milli-Q water

Milli-Q water	1.0L
NaCl	28.10g
$MgSO_4 \cdot 7 H_2O$	6.90g
$MgCl_2 \cdot 6 H_2O$	5.49g
KCl	0.67g
$CaCl_2 \cdot 2 H_2O$	1.47g

- Fill to appropriate volume with Milli-Q water
- *Optional:* To reduce metals from the salts, run Turks Salt Mix through a Chelex 100 (200-400 mesh, cat no. 1421253, Bio-Rad) resin column inside a 20mL Econo-Rac Chromatography Column (cat. No 7321010, Bio-Rad). Pretreat the resin with Aquil (see protocol from Neil M. Price et al. (1989) Preparation and Chemistry of the Artificial Algal Culture Medium Aquil, Biological Oceanography, 6:5-6, 443-461)
- Autoclave or 0.2µm filter sterilize salt mix and allow to cool

3. To sterile Turks Island Salt Mix add the following nutrients stocks:

	1L
$NaH_2PO_4 \cdot H_2O$	2mL
$(NH_4)_2SO_4$	1mL
TAPS	7.5mL
Working trace metal stock	10µl
$NaHCO_3$	see below

4. Store this AMP1 basal medium at room temperature for up to two months.

5. Prepare 0.6M $NaHCO_3$ stock fresh on day of medium inoculation (**do not store**)
   - Weigh out 2.52g $NaHCO_3$ on dust-free weigh paper
   - Transfer into 50mL volumetric flask filled with 30mL Milli-Q water
   - Dissolve $NaHCO_3$ by swirling flask several times
   - Adjust volume to 50mL mark with Milli-Q water
   - Filter with a sterile 0.2µm nylon filtration unit

6. $NaHCO_3$ addition to AMP1 basal medium:
   10 mL of 0.6M NaHCO3 per 1L basal mediumOR directly to culture tubes (calculate based on final tube volume). **Do not add bicarb to 1L bottle if you will not use all of the media immediately.** The concentration of bicarb can be adjusted as needed for experimental requirements. For example, in chemostats, the bicarbonate can be adjusted to ~2mM which is similar to the concentration in natural seawater. Note: references for chemostats are located at the end of this manual.

Note: Same concentration of sodium nitrate $NaNO_3$ (Sigma #71755) can be substituted for $(NH4)2SO4$.

SN Medium

Waterbury et al. (1986) proposed three media for culturing marine *Synechococcus* and this is the most popular version. If a solid medium (agar) is prepared, see Waterbury et al. (1986) for a method to wash the agar before use to remove contaminants.

Prepare stocks using Milli-Q water and add quantities listed in the table below. Filter sterilize all solutions listed in tables below using a 0.2µm nylon filtration unit or a 0.2µm Supor syringe filter. To prepare media, autoclave 750 mL of 0.2µm filtered natural seawater in a Teflon-lined bottle and separately autoclave 236 mL of double distilled dH_2O. Aseptically combine the two solutions. Aseptically, add 10 mL of sterile sodium nitrate solution and 1 mL of the other five sterile stock solutions (see table below).

References:

Waterbury, J.B., Watson, S.W., Valois, F.W. and Franks, D.G. 1986. Biological and ecological characterization of the marine unicellular cyanobacterium *Synechococcus*. *In* Platt, T. and Li, W.K.I. (eds.) *Photosynthetic Picoplankton*. *Can. Bull. Fish. Aquatic Sci.* 214: 71-120

Waterbury, J. B. and J. M. Willey. Isolation and growth of marine planktonic cyanobacteria. Methods in Enzymology 167: 100-105 (1988)

Component	Stock Solution	Add to 1L media	Molar Concentration in Final Medium
$NaNO_3$	76.5 g L^{-1} dH_2O	10 mL	9.0 x 10^{-3}M
K_2HPO_4 (anhydrous)	15.68 g L^{-1} dH_2O	1 mL	9.9 x 10^{-5}M
$Na_2EDTA \cdot 2H_2O$	5.58 g L^{-1} dH_2O	1 mL	1.5 x 10^{-5}M
Na_2CO_3	10.70 g L^{-1} dH_2O	1 mL	1.0 x 10^{-4}M
cyanocobalamin (vit. B12)	1.0 mg L^{-1} dH_2O	1 mL	7.38 x 10^{-10}M
trace metal solution	(see recipe below)	1 mL	-

Trace metal solution

Compound	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
$C_6H_8O_7 \cdot H_2O$	-	6.250 g	3.25 x 10^{-5}M
$C_6H_8FeNO_7$		6.000 g	~2 x 10^{-5}M
$MnCl_2 \cdot 4 H_2O$	-	1.400 g	7.08
$Na2MoO_4 \cdot 2 H_2O$	-	0.390 g	1.61
$ZnSO_4 \cdot 7 H_2O$	-	0.222 g	7.72 x 10^{-7}M
$Co(NO_3)_2 \cdot 6 H_2O$	-	0.025 g	8.59 x 10^{-8}M

Note: While *Synechococcus* is generally grown on SN media, some strains can be maintained on Pro99 amended natural, AMP1, ASW media (Wilson et al. (1996) J. Phycol 32, 506-516), and PCR-S11 Red Sea medium (RCC website, http://roscoff-culture-collection.org/medium-id/pcr-s11-red-sea)

Additional notes on *Synechococcu*s media

SNAX Medium

SNAX is an enrichment medium for Synechococcus that is a ten fold dilution of medium SN augmented with 100 μM NH_4Cl added from a filter sterilized stock after the medium has been autoclaved.

Sterilization

A. Autoclaving

Autoclaving is routinely used to sterilize Synechococcus media. Problems may arise if chemical additives are used to prevent corrosion in high pressure boilers. These additives may carry over and contaminate liquid media.

B. Microwaving preceded by filtration (Modern 'Tyndalization')

In theory microwaving by itself will not kill heat resistant spores. However it can be successfully used to sterilize liquid media if it is preceded by successive low pressure filtration through 1μm and then 0.2 μm Millipore Durapore filters. Following filtration the medium or seawater is microwaved in Teflon bottles that are brought to a rolling boil, swirled and brought to a boil a second time.

C. Filter Sterilization (A Caution)

Filter sterilization using commercially available cartridges of various capacities works well. Filter sterilization through irradiated membrane filters should be avoided because multiple hits in one location often result in pore sizes larger than those specified by the manufacturer.

Prochlorococcus and *Synechococcus* MEDIA COMPARISON

Amounts for 1L media	*Prochlorococcus and Synechococcus*			*Synechococcus*	
Salt base (g/L)	**PRO99[1]**	**PCR-S11[2]**	**AMP1[3]**	**SN[4]**	**ASW[5]**
NaCl	-	-	28.1	-	25
$MgCl_2 \cdot 7\ H_2O$	-	-	5.49	-	2
$MgSO_4 \cdot 6\ H_2O$	-	-	6.9	-	3.5
KCl	-	-	0.67	-	0.5
$CaCl_2$	-	-	1.47	-	0.5
Red Sea Salts	-	33.3	-	-	-
seawater	n/a	-	-	n/a	-
Macronutrients	-	-	-	-	-
$NaNO_3$ (mM)	-	-	-	9	8.8
NH_4Cl (mM)	0.80	-	-	-	-
$(NH4)_2SO_4$ (mM)	-	0.40	0.40	-	-
K_2HPO_4 (mM)	-	-	-	0.099	0.172
NaH_2PO_4 (mM)	0.05	0.05	0.05	-	-
Buffers	-	-	-	-	-
TRIS-base, pH 8 (mM)	-	-	-	-	9.08
Na_2CO_3, pH 8 (mM)	-	-	6	10	-
TAPS, pH 7.5 (mM)	-	1	3.75	-	-
Vitamins	-	-	-	-	-
Vit B12 (µg/L)	-	1	-	-	-
Trace Metals (µM)	-	-	-	-	-
EDTA	1.17	2	0.117	15	1.3
$FeCl_3 \cdot 6\ H_2O$	1.18	2	0.117	-	11.1
$C_6H_8FeNO_7$	-	-	-	~20	-
H_3BO_3	-	0.3008	-	-	46.3
$MnCl_2 \cdot 4\ H_2O$	0.09	-	0.009	7.08	9.15
$MnSO_4 \cdot H_2O$	-	0.0598	-	-	-
$ZnSO_4 \cdot 7\ H_2O$	0.008	0.006	0.0008	0.77	0.77
$Na_2MoO_4 \cdot 2\ H_2O$	0.003	-	0.0003	1.61	1.61
$(NH_4)_2Mo_7O_{24}$	-	0.0004	-	-	-
$CuSO_4 \cdot 5\ H_2O$	-	0.003	-	-	0.03
$Co(NO_3)_2 \cdot 6\ H_2O$	-	0.003	-	0.0859	0.17
$CoCl_2 \cdot 6\ H_2O$	0.005	-	0.0005	-	-
Na_2SeO_3	0.01	-	0.001	-	-
SeO_2	-	0.003	-	-	-
$NiCl_2 \cdot 6\ H_2O$	0.01	0.003	0.001	-	-
$Na_2WO_4 \cdot 2\ H_2O$	-	0.0006	-	-	-
KBr	-	0.006	-	-	-
KI	-	0.003	-	-	-
$Cd(NO_3)_2 \cdot 6\ H_2O$	-	0.003	-	-	-
$Cr(NO_3)_3 \cdot 9\ H_2O$	-	0.0006	-	-	-
$VOSO_4 \cdot 5\ H_2O$	-	0.0006	-	-	-
$KAl(SO_4)_2 \cdot 12\ H_2O$	-	0.0059	-	-	-
$C_6H_8O_7 \cdot H_2O$	-	-	-	32.5	-

References:

[1]Moore et al. (2002) Limnol. Oceanogr. 47:989-996

[2]Rippka et al. (1992) J. Syst. Evol. Microbiol. 50:1833–47 (note: Roscoff adjusted original recipe. As of Jan 2020, this is the current protocol Roscoff uses)

[3]Moore et al. (2007) Limnol. Oceanography: Methods 5: 353-362
For a comparison of all *Prochlorococcus* media variants see Table 1

[4]Waterbury et al. (1986) Photosynthetic Picoplankton. Can. Bull. Fish. Aquatic Sci. 214: 71-120

[5]Wilson et al. (1996) J. Phycol 32, 506-516

Recipe for AMS1 media for growing SAR11 (Carini et al., 2013)

Prepare the following nutrients (3), vitamin (1), organic stocks (3), and salts:

A. 0.5M $(NH_4)_2SO_4$

-Weigh out 6.61g $(NH_4)_2SO_4$ using dust-free weight paper
-Transfer to acid washed and rinsed 100mL volumetric flask filled with approximately 60mL Milli-Q water
-Swirl to dissolve and then bring up to 100mL with Milli-Q water
-Sterile filter by 0.2µm through sterile filtration unit and store at 4˚C

B. 0.025M NaH_2PO_4

-Weigh out 0.3g NaH_2PO_4 using dust-free weight paper
-Transfer to acid washed and rinsed 100mL volumetric flask filled with approximately 60mL Milli-Q water
-Swirl to dissolve and then bring up to 100mL with Milli-Q water
-Adjust pH to 7.5 using NaOH (1M solution made with MQ - 40g/L, or 2g in 50ml). This usually takes approximately 1.5mL NaOH to get 100mL NaH_2PO_4 to pH 7.5
-Sterile filter by 0.2µm through sterile filtration unit and store at 4˚C

C. Trace Metal Working Stock

NOTE: this is identical to 1/10th the Pro99 trace metal working stock **<u>without</u>** EDTA added

-Weigh out 32mg $FeCl_3 \cdot 6H_2O$ using dust free weight paper
-Transfer to acid washed and rinsed 100mL volumetric flask filled with approximately 60mL Milli-Q water
-Swirl to dissolve
-Individually add each of the other 6 trace metals from the liquid stocks in the fridge. Add the correct volume of primary liquid stocks based off the concentration written on each bottle to end up with 1/10th the working concentration for Pro99 (i.e. instead of adding 100µl of each primary metal stock as the Pro99 recipe calls for, add 10ul).
-Bring up to 100mL mark with Milli-Q water
-Sterile filter by 0.2µm through sterile filtration unit and store at 4˚C

D. AMS1 Va Vitamin solution

Note: This identical to Va vitamin solution if used at 2X, but with 10X thiamine

The 2,000X Va Vitamin solution in the fridge that we use for ProMM is fine for this if diluted 1,000-fold (want final 2X concentration), except that we need 10 times the vitamin B1 than what is contained in that solution.
If you already have a 2,000X Va vitamin solution, take 100mL into clean beaker, add and dissolve 180mg of thiamine-HCl, and then filter through 0.1µm filteration unit or syringe filter. Dilute the 2000x stock to 2x by taking 1mL of the stock and adding 999mL Milli-Q and then re-sterilizing through a 0.2µm filtration unit.

E. 0.025M Glycine

-Weigh out 187.7mg glycine using dust-free weight paper
-Transfer to acid washed and rinsed 100mL volumetric flask filled with approximately 60mL Milli-Q water
-Swirl to dissolve and then bring up to 100mL with Milli-Q water
-Sterile filter by 0.2µm through sterile filtration unit and store at 4˚C

F. 0.025M Sodium Pyruvate

-Weigh out 275.1mg sodium pyruvate using dust-free weight paper
-Transfer to acid washed and rinsed 100mL volumetric flask filled with approximately 60mL Milli-Q water

-Swirl to dissolve and then bring up to 100mL with Milli-Q water
-Sterile filter by 0.2µm through sterile filtration unit and store at 4˚C

G. 0.01M L-Methionine

-Weigh out 149.2mg L-methionine using dust-free weight paper
-Transfer to acid washed and rinsed 100mL volumetric flask filled with approximately 60mL Milli-Q water
- Swirl to dissolve and then bring up to 100mL with Milli-Q water
-Sterile filter by 0.2µm through sterile filtration unit and store at 4˚C

Base Salts

-Weigh empty acid-washed PC bottle with normal cap (but have acid washed ported cap/tubing for sparging prepped as well).
-Weigh out the following amounts of each salt on dust-free weight paper and transfer & dissolve each sequentially into Milli-Q water in the following order:

Salt	**grams per 1000mL**
NaCl	28.10
$MgSO_4 \cdot 7H_2O$	0.69
$MgCl_2 \cdot 6H_2O$	5.49
$CaCl_2 \cdot 2H_2O$	1.47
KCl	0.67
$NaHCO_3$	0.51

-Sterilize by autoclaving in a 1 or 2L polycarbonate bottle with a normal cap on liquid cycle (note, that it will likely turn cloudy, but should clear up by just 10 minutes of CO_2 sparging).
-Assemble bubble tops and tubing (see *Prochlorococcus* bubbling protocol for order information). From the air in port, attach 3 inline air filters starting at size 0.45µm (HEPA), followed by 0.2µm, and then 0.1µm Polydisc TF filters. The largest size filter will connect to either the CO_2 tank or the aquarium pump and the smallest size will connect with the bubble cap. On the vent port, add a 0.45µm (HEPA). Place entire apparatus inside an autoclave bag, roll up, and tape closed. Autoclave for 30min on a dry cycle.
-Switch out the normal bottle cap with a bubble top in the hood and then sparge autoclaved water with filtered CO_2 for at least 5hr, followed by filtered air (aquarium pump) for at least 10hr (overnight is fine).
-While sparging, re-autoclave the normal caps again in autoclave bags on a dry cycle.
-In the hood, switch back to normal caps and weigh full bottles and subtract from empty weight to calculate volume using density of seawater (1.025g/ml)

Nutrient, Vitamin, and organic stock additions:
-In the hood, add the following to 1L of sterile sparged base salt mix in the following order:

1mL	$(NH_4)_2SO_4$
2.67mL	NaH_2PO_4
100µl	Trace Metal Mix
1mL	2000X ASM1 Va Vitamin Solution
2mL	0.025M Glycine
2mL	0.025M Sodium Pyruvate
1mL	0.01M L-methionine

Reference: Final concentrations for all ingredients are listed in Table 1 of Carini et al. *ISME* 2013, except for glycine (50uM), L-methionine (10uM), and pyruvate (50uM).

ProMS media for growing SAR11 and co-cultures of *Prochlorococcus* and SAR11 (Becker et al., 2019)

Note: ProMS medium is a natural seawater based variant of Pro99 with additional organics added for SAR11. This medium has been used for viable co-culture of *Prochlorococcus* and SAR11.

Prepare the AMS1 vitamin solution, 0.025M glycine, 0.025M sodium pyruvate, and 0.01M L-methionine stock solutions as described above.

Prepare Sargasso-seawater based Pro99 medium as per lab protocols and add organics as follows (assuming 1,000ml):

Vol to add	Stock Conc	Nutrient stock	Final concentrations
40µl	0.025M	Sodium Pyruvate	1µM
40µl	0.025M	Glycine	1µM
20µl	0.01M	L-methionine	0.2µM
20µl	2000X	AMS1 vitamin solution	1/50th Vit conc listed in Table 1, Carini et al. 2013

Growing *Prochlorococcus* and *Synechococcus* in large volume (1-20L)

A. METHOD 1: BUFFERING AND BICARBONATE:

- Acid wash Nalgene polycarbonate container. Add appropriate volume of media
  - Supplement 1L (or more) medium with 3.75mM TAPS and 6mM sodium bicarbonate. Bicarbonate can be increased to 12mM for 20L volume, if desired.
  - Incubate with cap loosened

Note: 3.75mM TAPS and 0.6 M sodium bicarbonate stocks should be filter- sterilized with a 0.2 µm filter. The TAPS can be stored at 4°C for several months, but the bicarbonate should be made fresh the day of inoculation. We haven't (as of 3/24/15) done extensive testing on whether more TAPS is needed for 10L or more, but we have been successful in growing 20L volumes with only 3.75mM. Stir bars are NOT needed as there is not much settling during exponential growth. If you have concerns about circulating 20L cultures, you can add a stir bar, but have it spinning on the absolute lowest setting. Stirring too fast could shear the cells and they generally prefer not to be stirred. Stirring in 20L square Nalgene polycarbonate carboys could shear plastic particles from the carboy. This should be avoided.

B. METHOD 2: PUMPING STERILE AIR OR CO_2 INTO SYSTEM:

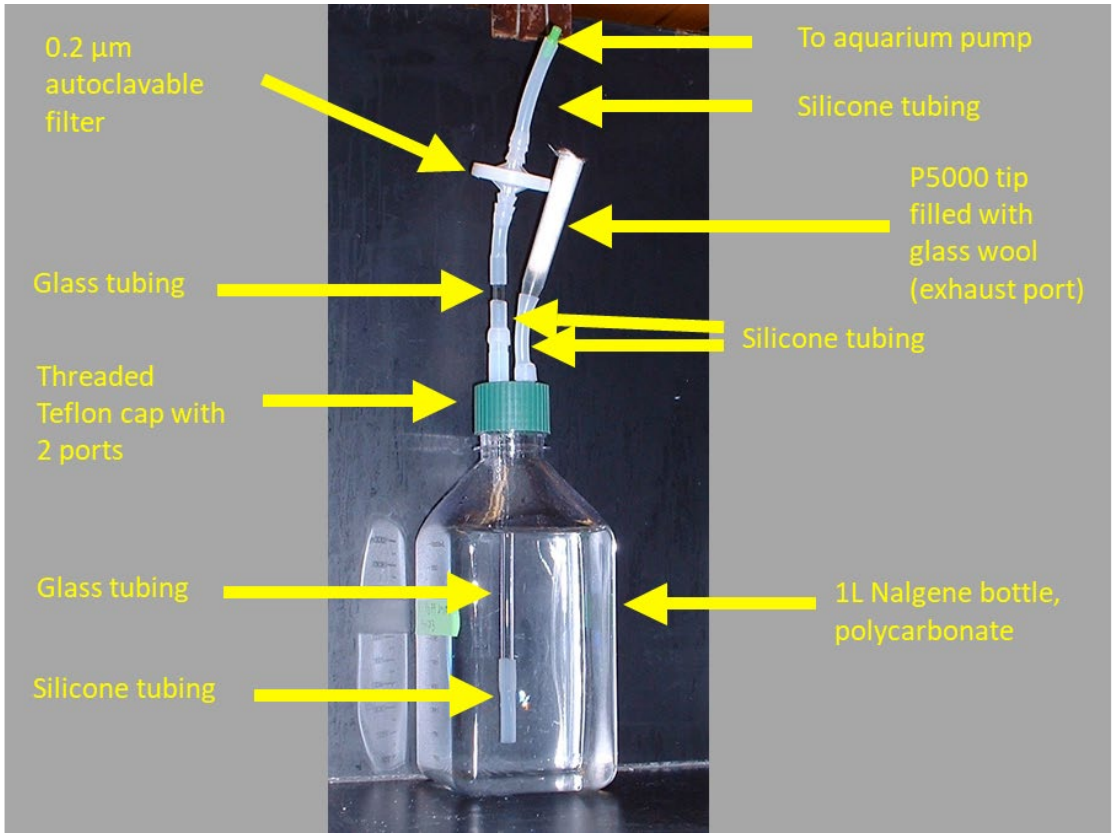
- Acid wash a 1L or 10L Nalgene polycarbonate container. Add appropriate volume of media.
- Fix top with filtered air system (see figure, next page)
- Incubate with cap tightened, low rate of airflow (1-4 bubbles/second). A higher airflow rate will cause higher evaporation and the culture may crash as a result. Bubbling will also help circulate a large volume culture.

Note: Air source can be an aquarium pump, or compressed air, or CO_2 tank.

System components (for 1L flask):

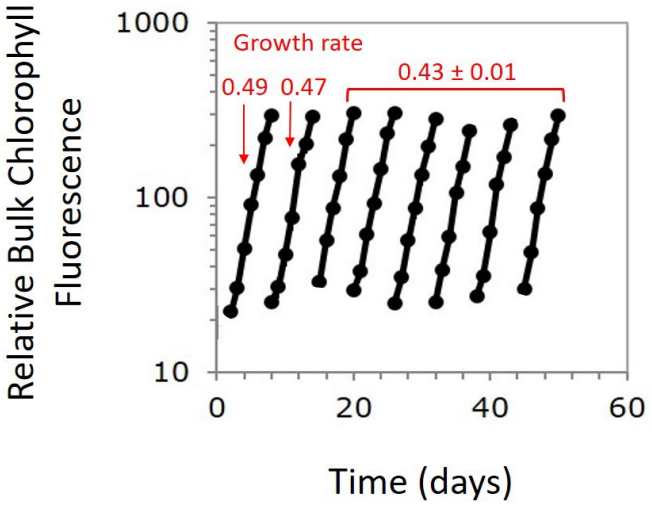
Part	Vendor	Part number
Nalgene 50 silicone tubing (0.187 ID, 0.312 OD)	VWR	8060-0040
6mm Pyrex glass tubing	Carolina Biol. Suppl.	WW-71-1146
Autoclavable 0.2µm filter Acro 37 TF	Pall Gelman	4464
Threaded Teflon cap for 1L flasks (1/4 inch tube diameter)	Savillex	0738-4-2

Bubbling system for *Prochlorococcus*



The entire system as shown is autoclavable. To increase longevity of tubing, we autoclave the tubing, including the cap and import and exhaust filters, separate from the 1L flask. We use an autoclave bag to sterilize the cap/tubing setup and aseptically exchange the cap on the media bottle with the tubing setup. Glass tubing with a bubble stone at the bottom can be used instead, but we find that stones are difficult to clean. They often stain the color of the culture regardless of cleaning with acid or bleach. It is also more difficult to maintain consistency of bubble rates between replicate bottles. We regulate the air for multiple 1L bottles using plastic gang valves (Penn Plax Lok Tite plastic gang valve). These can accommodate up to 5 air lines, but generally we find that using no more than 3 lines for one gang valve is best for consistency of bubble rates. As of Jan 2020, these can be purchased at most aquarium supply stores and Amazon.

Measuring and calculating culture growth



Bulk chlorophyll fluorescence and growth rates of sequential transfers as cells acclimate to growth conditions. After the third transfer, the growth rate remains the same and the cells are in balanced growth.

Balanced Growth

Balanced growth can be defined as cultures that have the same growth rate each time they are transferred. The concept of balanced growth is important for physiological studies to ensure that the results are due to specific experimental conditions. Experiments done without ensuring balanced growth will give results - for instance, transcriptomics - but the results may not be accurate for the test conditions and instead reflect changing physiological state. Of course, if you are studying what happens to cells as they are responding to a change in growth condition, then balanced growth is not required.

In order for cells to be in balanced growth, they should be transferred during exponential phase into the same growth conditions (e.g. light, temperature, media) at least once, but likely for 3-4 transfers. Cells are in balanced state when:

1. the flow cytometric signature shows a tight population of cells with little to no cells with lower chlorophyll fluorescence
2. the geometric mean of the chlorophyll fluorescence per cell is the same throughout exponential growth phase, and from transfer to transfer in exponential growth phase
3. the exponential growth rate is the same from transfer to transfer for at least 2 transfers with 5 doublings each

Note that without a flow cytometer, the same exponential growth rate (criteria #3) determined with bulk fluorescence or OD measurements for 3 transfers is usually sufficient. When balanced growth conditions are satisfied, the experiment can begin, and you can be confident that your results are truly due to the experimental condition(s) being tested.

Definition of growth phases, calculating growth rates, and transferring guidelines

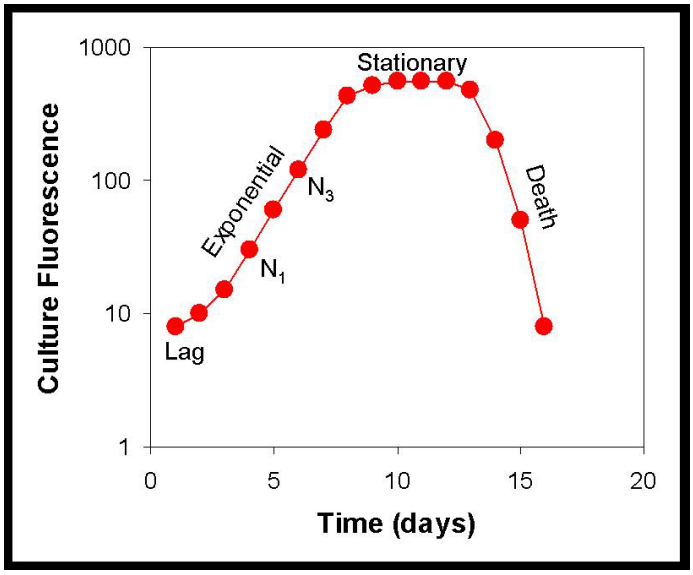


Fig 1. General pattern of fluorescence over time in cultures. Points during the Exponential growth phase (for example, N_1 and N_3) should be used to calculate growth rate.

Lag Phase

The condition of the starting inoculum has a strong bearing on the duration of the lag phase. An inoculum taken from a healthy exponentially growing culture in balanced growth is unlikely to have a lag phase when transferred to fresh medium under similar growth conditions of light, temperature, etc. Assuming the transfer will survive, the length of the lag phase will be proportional to the length of time the inoculum has been in stationary and death phases. A lag phase may also occur if the inoculum is transferred from one set of growth conditions to another.

Exponential phase, calculating growth rates, and transfer guidelines

During the exponential (or logarithmic) growth phase, a bacterial culture mimics a first-order chemical reaction, i.e. the rate of increase of cells is proportional to the number of bacteria present at that time. The constant of proportionality, **μ**, is an index of the growth rate and is called the growth rate constant. The best way to calculate growth rates is by using a flow cytometer (see flow cytometry section for more details), but these machines are expensive and often inaccessible. Bulk chlorophyll fluorescence measurements can also be used to monitor cultures and estimate growth rates because these measurements represent a **PROXY** for growth. Bulk chlorophyll fluorescence represents the summed chlorophyll fluorescence of each cell in a liquid sample and, thus, increases as biomass increases assuming the culture in is balanced growth (see below). However, it is important to measure cultures with a flow cytometer occasionally because correlations between fluorescence measurements may become inaccurate as cultures move into stationary phase. Additionally, fluorescence cannot be used as a substitute for cell counts where an estimate of final cell yield is needed. Several instruments can be used for measuring chlorophyll fluorescence, including the following:

- Turner 10-AU and TD700 fluorometer with chlorophyll a optical filters
- BioTek Synergy2 or Pherastar FS plate readers with 488nm excitation and 575 and 690nm emission filters for chlorophyll a and phycoerythrin (additional pigment for *Synechococcus* detection)

A similar principle applies if using spectrophotometric optical density (OD) to measure growth rates - OD will increase as the biomass increases. However, using OD has two disadvantages. One, the limit of detection for *Prochlorococcus* is around 1 x 10^7 cells/mL, 1-2 orders of magnitude higher than possible with fluorometry. And two, the presence of heterotrophs in non-axenic cultures can also be detected. Using chlorophyll fluorescence gets around both of these issues as fluorescence detection is more sensitive (can detect down to ~10^5 cells mL^{-1} on fluorometers) and does not detect non-chlorophyll containing biomass. Although these issues make using OD less desirable, it is still possible if fluorometers are not available.

Growth rates are only **one** important way of expressing the relative ecological success of a species or strain in adapting to its natural environment or the experimental environment imposed upon it. Using flow cytometry will determine cell density (collected over time will also generate a growth rate), size, chlorophyll per cell, and many other responses. Flow cytometry samples should always be taken throughout your experiment. We usually excite with a blue (488nm) laser and have filters for chlorophyll a (690nm), phycoerythrin (588nm), and green fluorescence (530nm) when staining with Sybr Green. It is important to take flow cytometry samples as correlations between fluorescence measurements may become inaccurate as cultures move into stationary phase. Fluorescence cannot be used as a substitute for cell counts where an estimate of final cell yield is needed. The duration of exponential phase in cultures depends upon the size of the inoculum (use transfer equation listed below), the growth rate, and the capacity of the medium and culturing conditions to support growth. Once the growth phase has been plotted (time on x-axis and fluorescence on logarithmic y-axis) careful determination of the exponential (straight line) phase of growth is needed. Two points, N1 and N3, are taken during this linear phase (see figure above) and substituted into the equation:

Growth rate:

$$\mu = \frac{\mathrm{Ln}\left[\frac{N_3}{N_1}\right]}{(t_3 - t_1)}$$

Where N_1 and N_3 = chlorophyll fluorescence or OD at time (t_1) and (t_3) respectively.

Recommended Growth Rate Calculation

It is best to calculate growth rates from a linear regression of at least 5 data points within the exponential growth phase for the results to have strong statistical significance. This can be done in a variety of statistical and spreadsheet software programs. In Microsoft Excel, the growth rate can be calculated using a statistical Add-on package that provides error calculations for each parameter. Alternatively, it can be obtained by plotting cell density or fluorescence (logarithmic y-axis) vs time (x-axis), fitting an exponential trendline to all the points in exponential growth, and

selecting “display equation on chart”. The slope (which equals the growth rate, μ) can be found in the exponent of the exponential equation:

$$N_t = N_0 e^{\mu t} \quad (y = be^{mx})$$

where m = slope (= growth rate, μ) and b = y-intercept.

Divisions per day and the generation or doubling time can also be calculated once the specific growth rate, μ, is known.

Divisions per day:

$$D^{-1} = \frac{\mu}{Ln2}$$

Doubling time (aka Generation time):

$$t_d = \frac{Ln2}{\mu}$$

It is important that cells growing at the same growth rate for multiple transfers (aka balanced growth) are used to start experiments. This means the cells are fully adapted to the environmental conditions. Changing from lower volumes to higher volumes will likely affect the cells (changes in surface area to volume, carbon limitations of larger cultures, etc.) so it is important to adapt the cells to changes before starting your experiment.

When transferring cultures, small inoculums equal to 10-20% of the volume of the new culture will normally generate new healthy cultures, but it is ideal to calculate your transfers using a set cell density or target fluorescence to maintain consistency from transfer to transfer (see equation below). A media blank is recommended and required for the following equation:

Transfer Equation with relative fluorescence unit (RFU):

$$V_t = \frac{V_f * (\text{target RFU} - \text{blank})}{(\text{current RFU} - \text{blank})}$$

Transfer Equation with cell density

$$V_t = \frac{V_f * \text{target density}}{\text{current density}}$$

V_t = Volume of transfer
V_f = Final volume of culture
Target = Target RFU or cell density
Current = Current RFU or cell density
Blank = media blank relative fluorescence unit (RFU)

Stationary phase

Cultures enter stationary phase when net growth is zero. The nature of the changes depends upon the growth limiting factor (i.e. Phosphate, Nitrogen, Carbon, etc). Cells can fluctuate in fluorescence for a while during this phase before ultimately dying. Cultures with heterotrophic bacteria are able to maintain in stationary phase for much longer (months) than axenic cultures. If the cultures are far enough into stationary phase, then the transfer efficiency is quite low. When maintaining stock cultures, it is best to transfer **before** the cultures reach stationary phase or within a few days of entering stationary phase.

Death phase

When cell metabolism can no longer be maintained in stationary phase they will enter the "death phase". Response with axenic cultures during this phase is generally very rapid and the term "culture crash" is often used. Cultures can lose their pigmentation and appear washed out or cloudy. Some *Prochlorococcus* co-cultures with heterotrophic bacteria can survive stationary and death phase for extended periods of time and often have an undulating increase and decrease of cell growth over time (typically always lower fluorescence/cell density than in exponential growth phase). Most of these co-cultures can be revived after extended periods of darkness by transferring a large inoculum to fresh media.

Isolation Strategies for *Prochlorococcus* and *Synechococcus* from the wild

Protocol

- Collect seawater in Go-Flo bottles using "acid-clean" tubing and amber Teflon bottles to protect seawater from high sunlight exposure. Isolating from CTD bottles that are not acid-clean is possible, but chances for success are higher with acid-clean bottles
- Isolation procedures should be initiated immediately following water collection to avoid loss of viability or overgrowth of other microorganisms
- You can try different media or other growth conditions (i.e. different light, temperatures, nutrient, CO_2 or O_2 sources, etc) to increase the chance of isolating a specific ecophysiological subtype of *Prochlorococcus* or *Synechococcus*
- Collect an additional 250-1000 mL of seawater from the location of isolation for making media and transferring isolates as they are being established
- Two different types of isolation strategies are described below (flow cytometric sorting is also an option, but not described here):

Option 1

- Gravity filter 250 mL of seawater through 0.8 or 1µm filters for *Prochlorococcus* OR 1µm filters for *Synechococcus* using polycarbonate, Supor, or Nucleopore filters and acid-cleaned glass or plastic filtration funnels, frits, and sidearm flasks. Double stacking the same sized filter can also be effective
- Add nutrients aseptically and mix gently. See below for Pro2 recipe for *Prochlorococcus* or add nutrients based on targeted strains/clades (i.e. add nitrate to isolate nitrate users). For *Synechococcus* use SN, SNAX, ASW, or F/40 + 10µM ammonium chloride
- Using sterile serological pipettes, transfer enriched seawater to one or more of the following sterile containers: acid-washed glass or polystyrene culture tubes, polystyrene flasks, or multi-well plates

Option 2

- Immediately run seawater on flow cytometer to estimate targeted cell populations
- Calculate media requirements and serially dilute (dilution to extinction) into desired enrichment media (see rendering cultures axenic section for more details). Keep the higher dilutions in case the lower dilutions do not grow up
- Place into sterile tubes or multi-well plates in a light and temperature environment that closely matches the conditions of the native environment of the isolates. If a mixed population occurs (such as *Prochlorococcus* AND *Synechococcus*), we often shift to higher or lower light to allow for one or the other population to out compete the other or try other growth conditions that you think might work for your targeted isolates
- Monitor growth via fluorescence and flow cytometry. Avoid spectrophotometry as it is too insensitive and doesn't separate signal of mixed bacterial populations
- Most of the cultures should be free of larger phytoplankton, but they will not be free of heterotrophic bacteria
- Remove heterotrophic bacteria by plating (see plating protocol section), serial dilution (see rendering cultures axenic section), and/or flow cytometric sorting (Moore et al, 2005)
- It can take weeks-to-months to see growth and likely many transfers before isolates are unialgal

NOTE: This is ONE media recipe option for isolating *Prochlorococcus*. You can and should try many different variations of nutrients

PRO2 recipe

Nutrient	Concentration
NH_4Cl	50 µM
$(NH_2)_2CO$ (urea)	100 µM
NaH_2PO_4	10 µM
Trace metal mix	
EDTA	1.17 µM
$ZnCl_2$	8 nM
$CoCl_2$	5 nM
$MnCl_2$	90 nM
Na_2MoO_4	3 nM
Na_2SeO_3	10 nM
$NiCl_2$	10 nM
$FeCl_3$	1.17 µM

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Agar Purification –*Synechococcus* plating (Waterbury et al, 1986)

I. Equipment
   1. (4) 10x15 Pyrex baking dishes
   2. 8 inch diameter Buchner funnel, 2L vacuum filter flask and Whatman #1 filter paper
   3. (4) 2L glass beakers

II. Protocol
   1. Start with 500g of Difco Bacto agar
   2. Split into four equal portions and wash each portion 4 to 5 times with 1.5L of Milli-Q water, decanting between washes
   3. Filter each portion onto #1 Whatman filter paper and then scrape the filtered portion into a 2L glass beaker
   4. Add 1.5L of 95% ETOH to each portion and let stand for two hours
   5. Filter as above, resuspend in a second 1.5L of 95% ETOH and leave over night at room temperature
   6. Filter and resuspend each portion in 1.5L of acetone for 1 hour
   7. Repeat step #6
   8. Filter and spread each portion in a glass baking dish and dry at 60˚C over night.
   9. Wash each portion with 1L of Milli-Q water, filter, and dry overnight at 60˚C in baking dishes. This last step prevents clumping.

III. Recovery from the original 500g should be approximately 375g. Streakable plates will require between 0.5 and 0.7 % purified agar.

Synechococcus plating

- Prepare SN media (see recipe previously described) and autoclave
- Add 0.3% (wt/vol) purified agar to SN media
- equilibrate to 37˚C in water bath
- In sterile hood, add cells at desired concentration
- Pour ~40mL into 100mm petri dish
- Allow plates to cool in sterile hood
- Transfer plates to 10 μmol photons m^{-2} s^{-1} for 24hrs and then move to a higher light intensity (20-30 μmol photons m^{-2} s^{-1})

Note: If plates are to be spread, the agar concentration would be 0.6% (wt/vol). The agar purification method is what is currently used (as of Jan 2020) and has been slightly altered from the original publication.

Alternative method for plating *Synechococcus* (similar to below method by Laurenceau et al. 2019)

This method was developed from personal communication with David Kehoe for plating *Synechococcus* that have been transformed with *E. coli* to generate mutants. As written, the method describes making 0.4% agar plates in 75% seawater media with 100% nutrient levels. It can be used with different media, such as ASW and PCRS11-RS. Na_2SO_3 (1.86 mM final conc), which serves as an antioxidant, is added to the media just prior to plating. For plating cells to obtain mutants, ~10^9-10^{10} cells mL^{-1} are used; thus, the plates are pigmented when poured. For obtaining individual colonies, lower concentrations can be used. Growth of colonies from lower concentrations, such as 10^6 cells mL^{-1}, can take several weeks.

The following procedure makes 40 mL for 1 pour plate in 100 mm petri dish (or 4 pour plates in small, 60 mm petri dishes, 10 mL each). It can be scaled up or down depending on the amount and kind of plates being used.

1. Make media and agarose

- Prepare 1.33X ASW by adding the nutrients for 1 L of media in 750 mL of seawater.
- Dispense media: 30 mL into 50 mL polypropylene centrifuge tubes
- add Na_2SO_3 and any antibiotics to the media in the 50 mL polypropylene centrifuge tube such that the final amount is for 40 mL of media
- Place tubes with media in 31.5 °C water bath (a slightly lower temperature, such as down to 28 °C, can be used if plating will occur immediately; otherwise, the agarose can start to solidify at this temperature)
- Make 1.6% Ultrapure LMP agarose (0.16 g in 10 mL QH_2O):
    - add agarose to sterile QH_2O in acid-clean, autoclaved glass flask covered with filter top used for autoclaving (also, a 50 mL polypropylene centrifuge tube with loose cap can also be used for small volumes)
    - microwave to boiling 3 times, ~20 sec each
    - can make ~10% extra so as not to run out as some sticks to the sides of the container and the outside of the pipet
- Place flask in 31.5 °C water bath, securing it so as not to tip into water bath

2. Make pour plates

- add ~1 mL of concentrated cells to media, cap and tip gently to mix
- add 12.5 mL of molten 1.6% agarose to 37.5 mL of cells & media, cap and tip gently to mix
- immediately pipet or pour ~10 mL into 5 petri dishes
- allow to solidify on the lab bench at low light ~2 - 5 µmol Q m^{-2} s^{-1}. Solidification time varies depending on ambient temperature and humidity, but usually occurs within 30 min.

3. Incubating plates

- put plates in a clear, sealed plastic container to keep plates from drying out
- plates can be kept right-side up (lid on top) if the agar appears soft and might fall if plates are upside-down (lid on bottom)
- maintain at normal growth temperature
- keep at low light (~5 µmol Q m^{-2} s^{-1}) for 2-3 days then shift to ~10 µmol Q m^{-2} s^{-1}
- depending on starting concentration of (viable) cells, growth may be apparent immediately or can take up to 8 weeks

Protocol for Pro99-agarose pour plates (Tolonen et al. 2006)

Note: This is a protocol for embedding cells in agar plates and makes 6 plates with a final agar concentration of 0.4% after cells are added to seawater agar solution. Moore et al. 2007 assessed multiple LMP agarose concentrations and determined that 0.28% LMP was the optimal concentration to achieving relatively fast and long-lasting lawns of Prochlorococcus. Either concentration is sufficient in achieving lawns of Prochlorococcus and the recipe below can be easily adjusted.

- filter 200mL sea water through 0.2µm supor filter

- add 1g UltraPure low-melting-point agarose (product 15517-014; Invitrogen Corp.) (this results in a 0.5% concentration)

- autoclave

- allow solution to equilibrate to 28˚C in water bath (but not solidify)

- add Pro99 nutrients according the Pro99 recipe

- add 50mL of exponentially growing cultures at ~10^7 cells mL^{-1}

- in a sterile hood, pour ~40mL into 100mm petri dish and repeat with remaining volume

- allow plates to solidify in sterile hood

- transfer plates to desired light intensity

Note: See the Morris et al. 2008 publication on using "helper" heterotrophic bacteria to facilitate growth of Prochlorococcus colonies on agar plates. Saito et al. 2001 also describes methods for streaking agar plates that contained spent media, but our success using this method has been low.

Both Tolonen et al. 2006 and Moore et al. 2007 call for autoclaving the media. Research in T. Tanaka et al. 2014 showed that autoclaving agar creates reactive oxygen species, which we know *Prochlorococcus* is sensitive to since it does not produce catalase. We outline a newer pour-plating method below (Laurenceau et al., 2019), that eliminates autoclaving and reduces oxidative stress.

Development of an improved pour-plating procedure for axenic Prochlorococcus *cultures* (Laurenceau et al., 2019)

"Our strategy was to develop a plating medium that would replace the need for helper cells to reduce the reactive oxygen stress experienced by *Prochlorococcus.* We approached this in two ways. First, based on findings that autoclaving agar produces reactive oxygen species in plating media (Tanaka et al. 2014), we sterilized a 3% low melting point agar solution in Milli-Q water by three rounds of boiling in a microwave, avoiding boiling-over and loss of media, before adding to Pro99 media. Second, we included pyruvate in the media. Pyruvate can serve to quench reactive oxygen species, and has been shown to enhance the survival of liquid axenic *Prochlorococcus* cultures at low densities (Sher et al. 2011). With these modifications, we were able to successfully obtain pour plates containing axenic *Prochlorococcus* colonies. Such colonies typically took 2-3 months to become visible, and could be picked using a sterile pipet tip and transferred into small (~5 mL) volumes of liquid Pro99 media, where they grew as expected and were verified as axenic. Although microwave-based boiling was not a perfectly reliable sterilization method, the plates were able to yield axenic cultures as any contamination we encountered, when it occurred, propagated very little in nutrient-poor medium. Thus, the combination of microwave sterilization and addition of pyruvate to the plating medium allowed us to reproducibly obtain all axenic colonies of *Prochlorococcus*." (Laurenceau et al. 2019)

- filter (0.2µm supor) seawater (volume calculated based on desired number of plates)

-autoclave and allow to cool

- in sterile hood add sterile Pro99 nutrients, and supplement with sterile final concentrations of 0.05% (wt/vol) Pyruvate and 3.75 mM TAPS (pH 8) (working stocks of these are described in ProMM and AMP1 recipes above)

- sterilize a 3% LMP agar solution in Milli-Q water independently by three rounds of boiling in a microwave (~20 sec each, to avoid boiling-over and loss of media)

- in sterile hood, add the melted LMP agar 1:10 in the Pro99 media base supplemented with pyruvate and TAPS

- allow solution to equilibrate to 28˚C in water bath (but not solidify)

- add 400ul of concentrated cells (pellet 50mL of exponentially growing cells to concentrate) to 40mL Pro99 agar solution

- in a sterile hood, pour ~40mL into 100mm plates and allow to cool

- place in desired light level and allow to grow

ProAC, MPTB, and ProMM recipes for axenicity screening and marine heterotrophic growth media

NOTE: Due to differences in growth properties among heterotrophs, axenicity must be tested in all 3 media AND purity should be screened using flow cytometry (Sybr stain) as well!

FINAL concentrations for 1L media

Components	ProAC	MPTB	ProMM
AC Difco Broth	Add 1.7g	Add 17 g	none
Natural or artificial seawater	750 ml seawater +250 ml Milli-Q H_2O (=75% seawater)	1L Milli-Q H_2O	1 L seawater
Pro99 nutrients (N,P,trace metals)	$NaH_2PO_4 \cdot H_2O$ 50 µM NH_4Cl 800 µM 1X trace metals	none	$NaH_2PO_4 \cdot H_2O$ 50µM NH_4Cl 800 µM 1X trace metals
Organic Carbon	none	none	0.05% each lactate, pyruvate, glycerol, acetate
Va vitamin mix	1X Va vitamins	none	1X Va vitamins
Salt	none	20 g NaCl	none
Calcium Chloride	none	1.5 g $CaCl_2*2H_2O$	none
Magnesium Sulfate	none	8 g $MgSO_4*7H_2O$	none
	pH: n/a	pH: 6.24	pH: 7.87

Instructions for making 1L ProAC media (Morris et.al, 2008)

- Prepare a glass filter funnel, frit/base, and sidearm flask by cleaning with 1N HCL overnight followed by rinses with Milli-Q water.
- Filter 750mL raw seawater through 47mm Whatman GF/F stacked on top of a 47mm 0.2µm polycarbonate filter (make sure there are no bubbles/creases) OR through a 0.2µm Supor filter.
- Add 250mL Milli-Q water to seawater to bring volume up to 1L (producing 75% seawater stock)
- Add 1.7g AC Difco Broth (note: this is 10 fold less than in MPTB)
- Pour into an acid washed Teflon or Polycarbonate bottle and autoclave (50 minutes for 2L).
- Allow to cool overnight before adding nutrients described below
- Prepare primary stocks of NH_4Cl, $NaH_2PO_4 \cdot H_2O$, and trace metals as follows:

(note: these are the SAME nutrient stocks as are made for natural based Pro99 media)

A. **0.5M NH_4Cl**

- Weigh out 2.67g NH_4Cl using dust-free weigh paper
- Transfer into 100mL volumetric flask filled with about 60mL Milli-Q water
- Dissolve NH_4Cl by swirling flask several times
- Adjust volume to 100mL mark with Milli-Q water
- Filter with a sterile 0.2µm nylon filtration unit
- Store sterile stock at 4°C

B. **0.025M $NaH_2PO_4 \cdot H_2O$**

- Weigh out 0.345g $NaH_2PO_4 \cdot H_2O$ using dust-free weigh paper
- Transfer into 100mL volumetric flask filled with about 60mL Milli-Q water
- Dissolve NaH_2PO_4 by swirling flask several times
- Adjust volume to 100mL mark with Milli-Q water
- Filter with a sterile 0.2µm nylon filtration unit
- Store sterile stock at 4°C

C. **Trace Metal Stocks**

i. **Primary trace metal stocks**

Using dust-free weigh paper, weigh out:

2.30g	$ZnSO_4 \cdot 7H_2O$
1.19g	$CoCl_2 \cdot 6H_2O$
17.81g	$MnCl_2 \cdot 4H_2O$
0.726g	$Na_2MoO_4 \cdot 2H_2O$
1.73g	Na_2SeO_3
2.38g	$NiCl_2 \cdot 6H_2O$

- Transfer each trace metal into separate 100mL volumetric flasks containing ~60 mL Milli-Q H_2O
- Dissolve contents by swirling flask several times
- Adjust volume to 100mL mark with Milli-Q water
- Store each stock in a cleaned Teflon bottle at 4°C

ii. **Trace metal working stock**

- Weigh out 0.435g $Na_2EDTA \cdot 2\ H_2O$ using dust-free weigh paper
- Transfer into 100mL volumetric flask filled with 60mL Milli-Q water
- Dissolve Na_2EDTA by swirling flask several times
- Weigh out 0.32g $FeCl_3 \cdot 6\ H_2O$ using dust-free weigh paper
- Dissolve $FeCl_3$ into same volumetric flask by swirling several times
- Individually add and dissolve 100µl of the Primary trace metal stocks.
  Note: Always double check that primary stocks are the correct concentration (see final concentration table for values) before assuming to add 100µl. If concentrations are different, calculate correct volume of addition. Make sure to remove the metal ejector from the pipettor to avoid contamination.
- Adjust volume to 100mL mark with Milli-Q water
- Filter with a sterile 0.2µm nylon filtration unit
- Store sterile stock at 4°C

3. Prepare 2000x Va Vitamin Stock by adding the following to 100mL volumetric flask filled with 60mL Milli-Q water

A. 100mg of Inositol
B. 20mg Thiamine·HCL
C. 0.1mL of a 1g/L Vitamin B_{12} (cyanocobalamin) stock
D. 1mL of a 0.1g/L Biotin stock
E. 0.1mL of a 2g/L Folic Acid stock
F. 0.5mL of a 2g/L p-aminobenzoic acid stock

G. 10mL of a 1g/L Niacin stock
H. 10mL of a 2g/L Ca d-pantothenate stock
I. 10mL of a 1g/L Pyridoxine stock

- Adjust volume to 100mL mark with Milli-Q water. Filter sterilize (0.2µm filter unit or syringe filter) and store at 4°C in polycarbonate bottle.

Reference for Va Vitamin mix: (Davis and Guillard, 1958; derived from V8 vitamins, Provasoli *et al.* 1957)

4. Add nutrients and Va Vitamin mix to 1L of cooled autoclaved 75% seawater

0.5M NH_4Cl	1.6mL
0.025M $NaH_2PO_4 \cdot H_2O$	2.0mL
Trace metal working stock	100µL
Va Vitamin mix	500µL

Instructions for making Marine Purity Test Broth (MPTB)

(Saito et.al 2002)

To make 400 ml (adjusted from the 1L recipe listed in main table above):

	ingredients	amount	dissolve ingredient in:
Bottle 1 – 250mL size	$MgSO_4 \cdot 7H_2O$	3.2 g	100 ml QH_2O
Bottle 2 – 250mL size	$CaCl_2 \cdot 2\ H_2O$	0.6 g	100 ml QH_2O
Bottle 3 – 500mL size	NaCl	8 g	100 ml QH_2O
	AC Difco Broth	6.8 g	slowly add to NaCl solution while stirring; when dissolved, bring volume to 200ml

- Clean three glass bottles (2 - 250mL for Mg and Ca stocks and 1 - 500mL bottle for NaCl/broth stock) and Milli-Q water to make solutions
- Make and autoclave $MgSO_4$, $CaCl_2$ and NaCl/broth solutions separately – this prevents precipitation
- Let cool
- Mix all solutions together in a sterile hood– solution should be a transparent brownish color. Store at room temperature

Instructions for making 1L ProMM media

(Berube et al., 2015)

1. Prepare a glass filter funnel, frit/base, and sidearm flask by cleaning with 1N HCL overnight followed by rinses with Milli-Q water. Filter 1L raw seawater through 47mm Whatman GF/F stacked on top of a 47mm 0.2µm polycarbonate filter (make sure there are no bubbles/creases) OR through a 0.2µm Supor filter. Pour into an acid washed Teflon or Polycarbonate bottle and autoclave (50 minutes for 2L). Allow to cool overnight before adding nutrients described below.

2. Prepare primary stocks of NH_4Cl, $NaH_2PO_4 \cdot H_2O$, and trace metals as follows:
(note: these are the SAME nutrient stocks as used in natural based Pro99 media and in the ProAC listed above)

A. **0.5M NH_4Cl**
- Weigh out 2.67g NH_4Cl using dust-free weigh paper
- Transfer into 100mL volumetric flask filled with about 60mL Milli-Q water
- Dissolve NH_4Cl by swirling flask several times
- Adjust volume to 100mL mark with Milli-Q water
- Filter with a sterile 0.2µm nylon filtration unit
- Store sterile stock at 4°C

B. **0.025M $NaH_2PO_4 \cdot H_2O$**
- Weigh out 0.345g $NaH_2PO_4 \cdot H_2O$ using dust-free weigh paper
- Transfer into 100mL volumetric flask filled with about 60mL Milli-Q water
- Dissolve NaH_2PO_4 by swirling flask several times
- Adjust volume to 100mL mark with Milli-Q water
- Filter with a sterile 0.2µm nylon filtration unit
- Store sterile stock at 4°C

C. **Trace Metal Stocks**

i. **Primary trace metal stocks**

Using dust-free weigh paper, weigh out:

2.30g	$ZnSO_4 \cdot 7H_2O$
1.19g	$CoCl_2 \cdot 6H_2O$
17.81g	$MnCl_2 \cdot 4H_2O$
0.726g	$Na_2MoO_4 \cdot 2H_2O$
1.73g	Na_2SeO_3
2.38g	$NiCl_2 \cdot 6H_2O$

- Transfer each trace metal into separate 100mL volumetric flasks with about 60 mL Milli-Q water
- Dissolve contents by swirling flask several times
- Adjust volume to 100mL mark with Milli-Q water
- Store each stock in a cleaned Teflon bottle at 4°C

ii. **Trace metal working stock**

- Weigh out 0.435g $Na_2EDTA{\cdot}2\ H_2O$ using dust-free weigh paper
- Transfer into 100mL volumetric flask filled with 60mL Milli-Q water
- Dissolve Na_2EDTA by swirling flask several times
- Weigh out 0.32g $FeCl_3{\cdot}6\ H_2O$ using dust-free weigh paper
- Dissolve $FeCl_3$ into same volumetric flask by swirling several times
- Individually add and dissolve 100µl of the Primary trace metal stocks
  Note: Always double check that primary stocks are the correct concentration (see final concentration table for values) before assuming to add 100µl. If concentrations are different, calculate correct volume of addition. Make sure to remove the metal ejector from the pipettor to avoid contamination.
- Adjust volume to 100mL mark with Milli-Q water
- Filter with a sterile 0.2µm nylon filtration unit
- Store sterile stock at 4°C

3. Prepare FOUR separate 50mL 5% organic carbon stocks by adding the following to <u>separate</u> 100mL beakers filled with 35mL of Milli-Q water

   A. 2.5g Sodium Acetate
   B. 2.5g Sodium Lactate
   C. 2.5g Sodium Pyruvate
   D. 2.5mL Glycerol

   Top off volume to 50mL and filter sterilize with 0.2µm 25mm Supor syringe filter into a sterile 50mL "Falcon" centrifuge tube. Store sterile stocks at 4°C.

4. Prepare 2000x Va Vitamin Stock by adding the following to 100mL volumetric flask filled with 60mL Milli-Q water

   A. 100mg of Inositol
   B. 20mg Thiamine·HCL
   C. 0.1mL of a 1g/L Vitamin B_{12} (cyanocobalamin) stock
   D. 1mL of a 0.1g/L Biotin stock
   E. 0.1mL of a 2g/L Folic Acid stock
   F. 0.5mL of a 2g/L p-aminobenzoic acid stock
   G. 10mL of a 1g/L Niacin stock
   H. 10mL of a 2g/L Ca d-pantothenate stock
   I. 10mL of a 1g/L Pyridoxine stock

Adjust volume to 100mL mark with Milli-Q water. Filter sterilize (0.2µm filter unit or syringe filter) and store at 4°C in polycarbonate bottle.

Reference Va Vitamin mix: (Davis and Guillard, 1958; derived from V8 vitamins, Provasoli *et al.* 1957)

5. Add the following ingredients to 1L of cooled autoclaved 100% artificial or natural seawater. Store at room temperature.

0.5M	NH_4Cl	1.6mL
0.025M	$NaH_2PO_4 \cdot H_2O$	2.0mL
Trace metal working stock		100µL
5% glycerol		10mL
5% acetate		10mL
5% lactate		10mL
5% pyruvate		10mL
Va Vitamin mix		500µL

Instructions for using the purity media broths and agar plating techniques

- Add ~0.3ml of culture to tubes of 3ml of EACH purity media in sterile plastic snap cap tubes, leaving the cap in the breathable position.
- Let sit at room temperature (on lab bench) to allow time for any heterotrophic bacteria to grow.
- Contaminated samples will become cloudy with bacteria as early as the next morning, though sometimes it can take several days or weeks. Check for contamination for 1-3 months before confirming purity and disposing of samples.

Agar Plating

- If you would like to grow bacteria on plates, you can add 14g of Difco Bacto Agar to 1L of any of the media recipes above. Place in a 50-55°C water bath after autoclaving to prevent solidification before you are ready to plate. Pour plates in a sterile hood and store unused plates at 4°C.
- To streak bacteria use the T method:

Using sterile inoculation loop (disposable or flame sterilized glass), obtain specimen by dipping into a liquid culture or by picking a colony from a plate. Drag the inoculation loop across the surface of the agar back and forth in a zigzag motion until approximately 30% of the plate is covered. Dispose of the plastic loop (or sterilize glass loop with flame), unwrap a new sterile plastic loop, and turn the plate 90 degrees. Starting in the previously streaked section, drag the new loop through the previous section two to three times continuing the zigzag pattern until 30% of the plate is covered. Dispose of the plastic loop (or sterilize glass loop with flame), unwrap a new sterile plastic loop, and turn the plate 90 degrees. Be cautious to not touch the first streaked sector and repeat (using instructions listed above) until the remaining 30% of the plate has a zigzag pattern. Each time the loop gathers fewer and fewer bacteria until it gathers just single bacterial cells that can grow into a colony. The plate should show the heaviest growth in the first section. The second section will have less growth and a few isolated colonies, while the final section will have the least amount of growth and many isolated colonies.

Rendering *Prochlorococcus* and *Synechococcus* axenic (aka dilution to extinction) (Berube et al. 2015)

General principle – dilute non-axenic culture in 96-well plates to 1-5 *Prochlorococcus* or *Synechococcus* cells/well, using ProMM medium, which contains pyruvate. Pyruvate allows *Prochlorococcus* to grow from one cell, whereas it will not do so in normal Pro99 medium. Further, the other carbohydrates in ProMM allow any heterotrophs to grow rapidly, making the wells cloudy and thereby providing the ability to eliminate contaminated wells.

- Run non-axenic culture on flow cytometer to count targeted population. Sybr stain to count heterotrophic cells to determine if they are more or less abundant. This will affect your dilution to extinction calculations. Generally, heterotrophs are 1:5 to 1:10 of the *Prochlorococcus* population.
- Serially dilute the non-axenic culture into ProMM medium in a 96 well plate (final 200-250µl volume/well), targeting a theoretical final concentration of 1 OR 5 cells/well and no heterotrophs. In practice, aim for 1 OR 5 cells/well in as many wells as possible, to enhance the chances that some will grow up.
- Seal lids of 96 well plates with breathable tape or parafilm on the sides of the plate. This will reduce evaporation.
- Grow plates in relatively low light (~15uE) for as long as three months. Monitor by eye every week to see if wells turn green.
- Wells that grow cloudy, usually relatively quickly, indicate heterotroph growth. Do not use these wells even if they eventually turn green. Mark an "x" over the cloudy wells. Occasionally some of these can turn green, but if they were originally cloudy, they are NOT axenic.
- Transfer (~150-200µl) green wells AS SOON AS THEY ARE VISIBLY GREEN, first into ~2-3mL ProMM in a plastic falcon (snap-cap) culture tube. Ensuring the cap is in the breathable position. Transferring the wells as they are still growing enhances the chance of the culture growing up after dilution.
- After the transfer, add 200uL ProMM back into the original well (well will usually turn green again and can serve as backup if the first attempt to transfer into Pro99 does not work).
- Gradually transfer cultures that grow (turn green) in test tubes into increasing volumes of Pro99. During the initial transfers make sure not to dilute more than 1:10.
- Test newly purified green cultures for axenicity repeatedly, using ProMM, MPTB and flow cytometry.

Note: Most *Synechococcus* strains can also be diluted into SN media without the organic carbon sources, but determining heterotrophic growth will require sybr staining with flow cytometry. Rendering *Synechococcus* axenic can also be done on agar plates (see plating protocol) and both *Prochlorococcus* and *Synechococcus* can be rendered axenic by flow sorting (Moore et al., 2005), but it is advisable to either add pyruvate to the designated media or use ProMM for this as well.

Filter Plating Method for Rendering Picocyanobacteria Cultures Free of Heterotrophic Bacterial Contaminants and Clonal

Sean M. Kearney, Allison Coe, Kurt Castro, and Sallie W. Chisholm (2022) *Frontiers* 13. doi 10.3389/fmicb.2022.821803

In advance of filter plating:

1. Prepare a culture of your helper heterotroph so that it is in exponential growth phase for inoculation into pour plates. *Henriciella, Alteromonas*, and other copiotrophs are likely okay to use for this

2. Prepare xenic culture of picocyanobacteria so it is in exponential growth phase

3. 0.2µm filter and then autoclave natural seawater

4. 1M Acid wash and autoclave 250 or 500mL glass Erlenmeyer flask

On the day of filter plating:

1. Prepare 1M Na_2SO_3 stock in Milli-Q water and filter-sterilize through a 0.2µm syringe filter into a sterile container (10mL of the stock will be sufficient for several attempts)

2. Count picocyanobacteria cells in the culture via flow cytometry using SYBR staining to confirm heterotroph counts are less than 50% (ideally less than 10%) of the total number of cells in the culture; otherwise the protocol is unlikely to work. For SYBR staining, prepare freezer stocks of a 30X concentrate (3 uL 10,000X SYBR green in 1 mL sterile TE buffer made in the natural seawater base). Cultures of Syn/Pro are diluted to ~500 cells/uL in sterile natural seawater base (190 uL final volume, so for instance, if you dilute a culture expected to be at 5e4 cells/uL, this would be 2 uL into 188 uL natural seawater), then add 10 uL 30X SYBR to the 190 uL diluted culture. After SYBR is added, incubate in the dark for 30 min to 1 hr, before counting on flow cytometer. Do the same SYBR protocol for the helper heterotroph to determine concentration.

3. Prepare agar plates as follows:
   (a) add 0.3% (w/v) noble agar (UV treat in the biosafety cabinet for 5-10 mins) to ~100 mL or 20% of the final volume of filtered and autoclaved natural seawater in a sterile glass container (volume will depend on how many final plates are needed)
   (b) heat the agar in microwave until it comes to a boil (careful not to overboil) and dissolves in the natural seawater
   (c) Add remainder natural seawater volume (to reach final volume) and cool to 45°C. Once cooled, add Pro99 nutrient amendments, plus add final volume 1 mM Na_2SO_3 from freshly made stock (i.e. add 100µl to 100mL)
   (d) cool to 35°C, then add 1e5 cells/mL (so for 100 mL, this would be 1e7 cells total) of the helper heterotroph to the medium, and immediately pour 15-20 mL into each agar per plate
   (e) cool for at least 10 min

4. Using the cell counts for the picocyanobacterial culture, confirm the heterotrophs are sufficiently low to proceed (again, ideally less than 10% of cells in the culture), then prepare 10-fold serial dilutions in 1 mL natural seawater of the culture, aiming for 1e6, 1e5, 1e4, 1e3, and 5e2 cells per mL.

5. Using ethanol sterilized tweezers, transfer 47 mm 0.2 um Supor filter to the surface of the solidified agar and immediately proceed to step 6.

6. Using the 5x 10^2 cells/mL dilution, make twelve 10 µL spots on the surface of the filter on a plate labeled with the appropriate dilution (final concentration 5cells/spot). Let spots dry (lid resting tilted on the side) in laminar/biosafety hood for 30min. Cover the plate, and seal with cut titer tops (or parafilm if titer tops not available). Repeat to create 5-10 plates at this dilution. If desired, use the 1e3 dilution and repeat process (final concentration 10 cells/spot). Drying will take significantly longer if filter is placed on agar well before spotting.

7. Using the 1e6 dilution, make twelve 10 µL spots on the surface of a filter on a plate labeled positive control (two of these plates are sufficient). You should expect growth on this filter if the cells are able to tolerant conditions.

8. Incubate the plates at 10-20 µmol photons m^{-2} s^{-1}. Colonies should appear within 2-3 weeks. The plates should be kept upright in a sealed container (Tupperware or a Ziploc) with something to maintain the humidity (i.e. small beaker of water or a wet paper towel). It is super important not to put the plates directly over a heat source (i.e. light bulb) because this will generate a temperature gradient and condensation on the lids and everything will die. Two ways to avoid this are: (1) place the plates directly on the ground and (2) place the plates on the top of a tube rack so that the convective medium is air (rather than a solid surface separated from the light bulb, which will facilitate heat transfer to the plates and condensation).

9. Once well-separated individual colonies are visible on the filters, use a 10 uL pipettor to transfer individual colonies into 200µL Pro99 medium in a 96 well plate. After picking all the colonies into Pro99, remove 20µL of the culture and place into a new 96 well plate with 180µl of purity broth. Keeping the same well format for both plates will help with organization when identifying potential axenic wells in the next step. For broths, options are MB 2216 medium, MPTB, or ProAC, but the latter two can take several days before visible growth, and frequently show inconsistent results from each other; plus the vast majority of our heterotrophs appear to be copiotrophs, and seem to grow well in MB 2216 (Sigma #76448-500G, follow instructions on container to make using filtered seawater as base and sterilizing by microwave).

10. Monitor both the culture and the purity plate for growth. For cultures, you can monitor by fluorescence or by eye. For purity plates, mark wells (“X” on lid) that appear cloudy as these are contaminated with heterotrophs. Identify remaining uncontaminated wells in purity broth and confirm axenicity using flow cytometry and SYBR staining as described in step 2. Transfer all hopeful axenic wells to larger volumes of Pro99, as sometimes low-level contaminants grow out in the next step – confirm axenicity at least one more time after cells have reached exponential phase in a larger volume of Pro99.

CYROPRESERVATION METHODS (Moore et al., 2007)

A. REUSABLE *Prochlorococcus* and *Synechococcus* FROZEN STOCK (CONCENTRATED CELLS):

Note: DMSO should be filter sterilized before using. Make sure to use DMSO-safe 0.2µm syringe filters. DMSO will eat through Supor syringe filters.

1. Preparation of concentrated cells

- Grow 50mL (or more) cultures in light levels between 10-15 µmol Q m^{-2} s^{-1} and 20-22°C temperature
- At late exponential growth phase, transfer volume of cells (50mL) into sterile centrifuge tubes/bottles.
- Spin at 10,000g for 15 minutes at 20-22°C.
- Carefully decant liquid into waste container in sterile hood
- Resuspend pellet in 1ml fresh medium
- Withdraw concentrated cells (1mL) and transfer into 1.2mL cryogenic vial (Nalgene #5000-0012).
- Add 75 µl DMSO (7.5% final concentration) to cryogenic vial and invert several times.
- Immediately place into liquid nitrogen to flash freeze and store at -80˚C (or colder)

2. Recovery of cells in fresh medium

- Reduce ambient light in work area as much as possible.
- Remove cryogenic vials one at a time from liquid nitrogen or freezer and transport vials under cover to sterile hood as quickly as possible.
- With a sterile toothpick or pipet tip, immediately scrape up a small amount of the frozen cells (¼" or smaller) and place into 25 mL sterile medium. Alternatively, slowly thaw the whole vial and add to sterile medium (see method below for unconcentrated cells). **Thawing the whole concentrated vial yields the best recovery rates.**
- Once scrape is completed, flash freeze with liquid nitrogen to flash freeze and store at –80°C
- Incubate cultures at 20-22°C under low light (10-15 µmol photons m^{-2} s^{-1}).
- Note: **Recovery under low light is absolutely critical**.
- Monitor growth with daily fluorescence measurements. Growth can be detected within one week after thawing, but may take as long as 3-4 weeks.

B. Cryopreservation of Heterotrophs

- Make 50% glycerol stock (50:50 solution with Milli-Q water)
- Filter sterilize with 0.22µm filtration unit or syringe filter into sterile container
- Store at 4°C while not in use
- Concentrate your cells using the same above method for *Prochlorococcus*, adding 800µl of your concentrated culture to a 1.7mL cryogenic vial
- In sterile hood, add 200µl of your sterile 50% glycerol stock to make a **final 10% glycerol** preserved stock
- Flash freeze in liquid nitrogen and store in –80°C freezer. Follow same recovery instructions as listed above

C. SINGLE USE *Prochlorococcus* FROZEN STOCK (UNCONCENTRATED CELLS):

1. **Preparation of unconcentrated cells**

- Grow cultures in light levels between 10-15 µmol Q m^{-2} s^{-1} and 20-22°C temperature
- At late exponential growth phase, transfer 1 ml cells into a 1.2mL cryogenic vial (Nalgene #5000-0012).
- Add 75 µl DMSO (7.5% final concentration) to cryogenic vial and invert several times.
- Flash freeze with liquid nitrogen and store in –80°C freezer.

2. **Recovery of cells in fresh medium**

- Reduce ambient light in work area as much as possible.
- Remove cryogenic vials from liquid nitrogen or freezer and transfer to a cold water bath with lid to partially thaw the cells slowly. (this can also be done at 37˚C water bath, but you could accidentally heat shock them if you don't catch them as soon as they partially thaw)
- Once half of liquid has thawed and small ice chunk remains, transport to sterile hood
- Transfer whole vial into culture tube containing 25 mL sterile medium.
- Incubate cultures at 20-22°C under low light (10-15 µmol photons m^{-2} s^{-1}).
- Note: **Recovery under low light is absolutely critical**.
- Monitor growth with daily fluorescence measurements. Growth can be detected within one week after thawing, but may take as long as 3-4 weeks.

Note: We have found that cells frozen in continuous light can be recovered into 14:10 LD photoperiod (and vice versa) as long as they are recovered in low light. We have also found that both PRO99 and AMP1 media are suitable for preparing cells for freezing and for recovery after thawing. Cells can also be recovered into different media from which it was originally cultured in (i.e. grown in AMP1 can be recovered in PRO99 or via versa). We also found that adding pyruvate and organic carbon sources (at ProMM concentrations) to AXENIC strains only can help speed up recovery. We have tried using the DMSO cryopreservation technique on unfiltered raw seawater samples and have been successful recovering/isolating *Synechococcus*, but not *Prochlorococcus* cells.

Sorbitol cryopreservation protocol

NOTE: This is unpublished and tested with a few AXENIC *Prochlorococcus* cultures. Some cultures recovered quicker, some recovered the same, and some recovered slower than in DMSO. This is an alternative to using DMSO. We have not tested this on all strains or with non-axenic cultures. Glycerol is also a common preservative and used with *Synechococcus* strains.

Preparation of **sterile** solutions

- Make Pro99 media following the Pro99 protocol
- Make 1M Sorbitol buffer by dissolving sorbitol into Pro99 media followed by filtering (0.2µM) the solution
- Make Pro99 media enriched with 0.2µM filtered solutions of 5mM Sodium Pyruvate and 5mM Glucose

Cryopreservation

- Grow **AXENIC** cultures in 50 ml Pro99 (2x25 ml test tubes) to late exponential phase (note: addition of 6mM sodium bicarbonate added at onset of culture preparation showed a positive effect on the recovery)
- Concentrate cells by centrifugation (7000g, 15 min, 20°C)
- Decant liquid to remove Pro99 from pellet
- OPTIONAL: Wash pellet in 2mL 1M Sorbitol buffer in Pro99 and follow concentration steps again
- Resuspend pellet in a final volume of 100µl 1M Sorbitol buffer in Pro99
- Transfer to a 1.2 ml cryo vial
- Flash freeze in liquid nitrogen
- Store at – 80°C

Recovery

- Immediately add 1 ml Pro99 enriched with 5mM Sodium pyruvate and 5mM Glucose to frozen vial containing 100µl sorbitol preserved cells
- Thaw sample to room temperature (this happens quickly)
- Transfer to small glass tube with 4mL Pro99 enriched with 5mM Sodium pyruvate and 5mM Glucose
- Place culture in light level slightly less (10-20%) than mother culture
- After approximately 24 hours, start a subculture in Pro99 (suggested 1:10 dilution) without pyruvate or glucose amendments
- Place culture in same light level as original 4mL recovery attempt
- Use purity test broths and flow cytometry to confirm axenicity once culture has recovered

Notes: Changing light regimes has not be tested. For example, if cells were grown on diel, we do not know if they can recover in continuous light under same number of photons/day. We have not tested this method with AMP1 artificial media. We have not tested this method with our entire *Prochlorococcus* and *Synechococcus* axenic culture collection. We have not tested this method for longevity freezer storage. Do NOT use this method with non-axenic cells.

Flow Cytometry

Flow cytometry is a laser-based instrument that allows for rapid, automatic counting of single cells in liquid. Cells are hydrodynamically focused to flow single-file past a laser beam. The laser light can be scattered off the cells and absorbed by the cells. If the cells contain fluorophores (e.g. molecules, such as pigments or DNA stains), then the cells can fluoresce light at a longer wavelength than the laser light exciting the fluorophore. Most flow cytometers have multiple lasers and detectors allowing for the detection of multiple parameters for each cell. These parameters allow for more detailed understanding of the physiological status of the culture than microscopy, bulk fluorescence, or OD measurements. Thus, not only does flow cytometry enable quantification of cells in natural populations or in a culture for calculation of growth rates, but flow cytometric parameters can provide chlorophyll and phycoerythrin fluorescence per cell, scatter properties which provide information about cell size and shape, and DNA per cell (when a DNA stain is used). Additionally, some flow cytometers are capable of sorting cells within a population based on user defined parameters. A good practical guide and reference for using flow cytometry for cyanobacteria analysis is Ch. 17 (Marie et al.) of Algal Culturing Techniques book by R. Anderson, which is available free online.

Prochlorococcus and *Synechococcus* can be analyzed flow cytometrically using the same instrument configuration. There are a variety of flow cytometers that work well for measuring cyanobacteria and marine heterotrophic bacteria, ranging from modestly priced, benchtop cytometers (e.g. Millipore Guava EasyCyte HT and Beckman Coulter Cytoflex S) to high-end, high-priced, research cytometers (e.g. Becton Dickinson Influx Cell Sorter and Bio-rad ZE5). Flow cytometers typically have a blue laser (488 nm) that excites chlorophyll a (and divinyl chlorophyll a of *Prochlorococcus*), phycoerythin, and some DNA stains (e.g. SYBR Green) and three detectors with filters for red fluorescence (690nm) to measure chlorophyll a, orange fluorescence (588nm) to measure phycoerythrin, and green fluorescence (530nm) when staining with SYBR Green to measure DNA. Some flow cytometers have additional lasers (i.e red laser (640nm), violet (405nm), etc.) that be helpful with fluorescent probes, assays, etc. One of the advantages of using flow cytometry for monitoring *Prochlorococcus* and *Synechococcus* cultures is the condition of the culture can easily be determined by how tight or spread out the flow cytometric signature is of the culture. It is important to keep in mind that the small size and low chlorophyll fluorescence of *Prochlorococcus* cells requires high gain levels on the chlorophyll and scatter detectors in order to get the *Prochlorococcus* chlorophyll fluorescence and scatter signal above the noise. This generally requires the flow cytometer be maintained at a level of cleaning that is more stringent than required for larger cells such as yeast or mammalian cells that might be run on an instrument in a facility. Also note that on some flow cytometers, the signal to noise level of forward scatter for *Prochlorococcus* is too low to accurately determine forward scatter, and thus, cell size of *Prochlorococcus* cells.

Preserving cells and thawing for Flow Cytometry

Cryopreservation methods for flow cytometry of phytoplankton have been examined since the 1980 when the basic method was worked out by Vaulot et al. (1989). Two major aldehyde-based preservatives, glutaraldehyde and paraformaldehyde, are routinely used for cryopreserving cyanobacteria cultures and natural populations for later flow cytometric analysis (Marie et al. 2014, 1999). The final concentration of glutaraldehyde has varied somewhat in publications over the years, but the general consensus is to use a low final concentration of glutaraldehyde, such as 0.125% indicated in the following procedure, to keep the pH closer to that of seawater for better preservation of *Prochlorococcus* and fragile picoeukaryotes (Marie et al. 1999).

Step 1 depends on how dense the culture is, how dense you want your sample, what the concentration restrictions are for your flow cytometer, etc. If the sample is for a culture in early exponential growth phase, you might want to consider NOT diluting it. You can always dilute your samples after you thaw them and before you run them on the flow cytometer. It is important to have separate pipettors for "live" work (cell culturing) and "dead" work (preservatives) and if possible use filtered pipette tips. All tips should go into a jar for chemical solid waste and all vial holders should be labeled "dead" and used only for "dead" work.

1. Dilute (if necessary) your sample with the same media used for culturing and add 1mL to a 1.2mL cryovial (Nalgene, cat# 5000-0012).
2. Add 5µl 25% glutaraldehyde (electron microscopy grade, Sigma, cat# G5882) to the inside of the cap. Close and invert to mix.
3. Store in the dark (wrapped in foil) and at room temp for 10min
4. Flash freeze in liquid nitrogen and store in -80°C freezer. Flash freezing is important to prevent damage to the cell walls
5. Thaw on ice or a cold water bath and in the **dark** until ready to use. If you do not use all of the sample, flash freeze the remaining sample before placing back in the -80°C freezer

If you are going to **SYBR green stain** your cells, it is recommended to take the 10,000x SYBR green stock and make multiple 100µl aliquots at a concentration of 1,000x by diluting into TE buffer for single-use in a sterile hood or environment. Refreeze and store the 1,000x stocks at -20°C. Multiple freeze/thaws for sybr should be avoided and using TE buffer to store your sybr dilutions is very important for stability (**do not dilute into water**). The final sybr concentration in your sample should be **1x.** Staining should be done in the dark and the time optimized for your cells and flow cytometer, but generally anytime between 10-60min works for *Prochlorococcus*, *Synechococcus*, and marine heterotrophs. In our experience, SAR11 needs at least 60min staining time.

Tips for culturing *Prochlorococcus*
(most tips are applicable to growing *Synechococcus* too)

- When isolating *Prochlorococcus*, consider what clade you are striving to obtain. Know the depth at which it could be in higher densities, the nutrients it would prefer, the light levels it optimally grows at in the lab, and what the environmental (light, temp, nutrients) conditions it was collected from. Using this knowledge, try as many different conditions that would be optimal for this clade. The more conditions you have, the better your chances are at isolating the cells. You must also be diligent about monitoring and transferring them. Using flow cytometry will allow you to monitor the changes in the populations over time. Adjusting the light or temperature with a mixed population could a population to outcompete the other. Once you believe you have obtained a unialgal culture, try rendering them axenic by filter plating or dilution to extinction methods listed previously.

- While it is ideal to immediately work with seawater when attempting isolations, it is possible to isolate cells from water that is kept in the dark for several days or from cryopreserved seawater.

- Cells should be in balanced growth (same growth rate for multiple generations) prior to the start of any experiment. This could take upwards of 3 or more transfers to achieve. Planning well in advance is highly suggested.

- Always have a media blank tube or bottle when analyzing with fluorescence. Subtract your blank from your fluorescence values before plotting.

- Do not rush cultures when switching their growth conditions. It is ideal to take "baby steps" when transitioning to different light levels, media, etc. For example, if you need to move your culture from a low light to a high light, try gradually increasing the light. If you need to transition from natural sea water to artificial sea water, try ratios of the two media (50:50, 25:75, etc.). **Short cuts do not work for growing *Prochlorococcus***.

- Check light and temperature conditions with reference published data for their optimal growth conditions. *Prochlorococcus* are extremely sensitive to change. Adding tubes or bottles next to your cultures might shade them and remember that light bulbs dim over time. Make sure to check your light levels AND temperature regularly and always before and after an experiment.

- When setting up for an experiment, make sure you measure light levels for every test tube and have the least amount of difference in light levels between biological replicates (ideally < 1 µmol photons m^{-2} s^{-1}). If there are large differences in growth between your biological replicates, check the light and temperature. This will likely be the cause. The lower the light level you are growing at, the lower the difference should be between replicates.

- The pH of natural seawater after autoclaving is around 8.2. This will increase as the cells grow and reach over pH 9 in late exponential growth phase. If you have an assay that is sensitive to the pH, you should add TAPS to buffer the media. If you are growing in unbuffered media and are having difficulty growing cells, the pH could be affecting your cultures. Measuring the pH is easy and should be considered as you troubleshoot cell growth problems.

- Pro cultures do NOT need to be stirred or shaken. In fact, they prefer not to be. If you are growing in large volumes (like 20L) and are worried about circulating the culture, you can stir the culture, but it has to be at the lowest possible setting. If you are using a polycarbonate bottle with a seam on the bottom, stirring can shave off small pieces of plastic into the culture so we suggest avoiding this technique. Instead you can manually gently shake the culture up 1-2x/day or more often if needed.

- Make sure the cells have proper air circulation. For example, covering racks of tubes or tubes themselves in foil (for light deprivation experiments) or neutral density light film for shading will increase the temperature AND prevent proper air exchange in the cultures. Find a separate incubator for dark work, use window screening, or cover the light bulbs with neutral density film to reduce the light.

- Test tubes with screw tops can create differences in air circulation. If you are using smaller screw topped tubes (13-16mm diameter) and find that your biological replicates are not identical, but you are certain your growth conditions are, then you are likely having issues with differences in the cap threading that could be restricting air exchange for certain tubes. We typically do not have this problem in larger screw cap polycarbonate bottles.

- Do not try to grow *Prochlorococcus* cells in the exterior wells of a 96 well plate. Smaller volumes (200-250µl per well) are more sensitive to evaporation issues. Filling the exterior wells with media is suggested and using a black walled plate will prevent light scatter bias in your fluorescence measurements. Use the suggested plates (see cultureware section) and either parafilm or cut strips of breathable tape (Titer Tops, Diversified Bio) that will secure the lid, but provide air exchange and reduce evaporation. For long time growth in plates (1+month), seal using the breathable tape for the least amount of evaporation.

- If working with axenic cultures, check purity with ALL three broths and flow cytometry (sybr green stained) at the beginning and end of an experiment It is also ideal to sequence the cells (i.e. Sanger sequencing with *Prochlorococcus* ITS primers) to confirm they are the strains you assume you are working with every year and prior to every large scale experiment.

- When shipping cultures, include a small book light to help them survive the darkness. Using a book light with a switch that is more difficult to turn on and off will prevent it from

accidentally switching off during transit. If the cells are axenic, you can also add sterile glucose and pyruvate (see Coe et al, 2016) to increase their odds of surviving the transit in dark. Do NOT add glucose and/or pyruvate if the cells are xenic. The heterotrophs will take over the culture and your *Prochlorococcu*s cells will likely not survive.

- When removing samples from your experimental test tubes or bottles over a growth curve, keep in mind you should never remove more than 50% of your starting volume throughout the experiment or you could risk changing the environment too much.

- Early exponential cells do not pellet well. If you need to concentrate early exponential cells, try filtering with 0.2µm Supor or Polycarbonate filters or using concentrators like Amicon filters. If you use polycarbonate filters, you can easily "shake" the cells off of filters submerged in liquid using a vortexer or bead beater (no beads). What you use will depend on how intact you need the cells (bead beater could shear the cells). If it does not interfere with the samples downstream, we have had success adding Pluronic F-68 Polyol in 10% solution (100x) (cat. No. 2750049, MP Biomedicals) to help achieve better cell pellets. Do not add Pluronic if you are going to isolate compounds your cells might be producing as this might interfere with your analysis.

- If your cells are growing in a light:dark cycle, read their fluorescence at the same time every day. These cells are synchronized (those that can divide will ~2hrs after sunset) and their chlorophyll fluorescence will change over the day. If you have both continuous light and light:dark incubators, consider your question and whether sampling at a specific time of day will matter. Cells in continuous light are not synchronized (dividing when they are ready) and sampling can be done at any time of the day and is more of a "bulk" response for the cells. Cells grown in a light:dark incubator will be in different cell states an hour after sunrise vs. an hour before sunset. Consider what you are sampling for, when you are sampling, and how this will affect your analysis.

Additional references

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For PCR-S11 Red Sea medium:
a modification of PCRS11 media originated by Rippka et al. 2000; recipe available at RCC website, http://roscoff-culture-collection.org/medium-id/pcr-s11-red-sea

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