Nutrient deplete/replete algal culture for elemental analysis

Version 2

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Abstract

Purpose: To evaluate the influence of nutrient availability on cellular elemental composition (quotas and stoichiometry) in marine picoeukaryotes.

Summary: Elemental quotas and ratios are assessed under nutrient replete and deplete conditions at the same time to minimize potential variation between experiments. Cells from an exponentially growing culture are concentrated by centrifugation, washed and re-suspended in a small volume of nutrient deplete media. These concentrated cells are used to inoculate triplicate replete and deplete culture flasks at a starting density corresponding to early-exponential growth. Culture growth is monitored daily. When the mean growth rate of the nutrient deplete treatment is half or less of the replete treatment ($GR_{DEP}/GR_{REP} < 0.5$), samples for cell counts and elemental analyses (dissolved nutrients; particulate carbon, nitrogen, and phosphorus) are collected.

Citation: Amy Zimmerman, Susanne Wilken Nutrient deplete/replete algal culture for elemental analysis. **protocols.io** dx.doi.org/10.17504/protocols.io.gukbwuw
Published: 11 Feb 2017

Guidelines

Background

Marine phytoplankton play an important role in coupling multiple nutrient cycles because they live at the interface between the abiotic and biotic components of ecosystems. The quantitative details of how multiple nutrient cycles intersect is determined by cellular elemental composition, including both the quantity and relative ratio of elements that make up a cell. The literature clearly demonstrates that phytoplankton can vary substantially in elemental composition depending on the availability of key nutrients. This variation may reflect physiological responses to ambient conditions (e.g., nutrient availability, temperature, light, etc.) and/or robust inter-specific differences in “average” elemental composition. Likewise, an organism’s ability to modify its elemental composition in response to environmental gradients (“plasticity”) varies among species. Interspecific differences in elemental composition could lead to substantial seasonal or regional differences in community structure as well as biogeochemical variables like algal biomass and export.

Before start

Equipment and Reagents.

Equipment:

BD Accuri flow cytometer
Sorvall RC 26 Plus centrifuge
Large rotor for Sorvall centrifuge
Centrifuge bottles, acid-cleaned and autoclaved
Balance
25 mm glass filtration units (flask, funnel, & support base), acid-cleaned and autoclaved
vacuum pump
Advantec GF-75 25mm glass fiber filters (Sterlitech Corp #GF7525MM), pre-combusted (3 hrs at 450°C)
Forceps
50 mL conical tubes, acid-cleaned
12-well plates or pieces of pre-combusted aluminum foil
Cryovials & cryocanes

Reagents:

Nutrient replete medium
Nutrient deplete medium
25% EM-grade glutaraldehyde
37% formaldehyde

Filter Combustion Procedure.

1. Make a double thick aluminum sheet with several folds.
2. Use ethanol-cleaned forceps to arrange a single layer of 25mm Advantec GF-75 glass fiber filters (0.3 µm nominal pore size) in each fold.
3. Combust in a muffle oven for 3 hours at 450°C (plus time to warm up).
4. Open door and allow to cool overnight before removing packet.
5. Store at room temperature in a clean ziplock bag. Only open in hood.

Acid-cleaning Procedure.

1. After use, rinse equipment with tap water.
2. Soak in 1% Micro soap overnight (*Note*: Keep centrifuge bottles in culture room and filter units and conical tubes in main lab).
3. Rinse 6 times with tap water and 6 times with Milli-Q water, then soak in Milli-Q overnight.
4. Rinse 4 times with Milli-Q.
5. Soak in 1:10 diluted (~3.7%) trace-metal grade HCl overnight.
6. Rinse 6 times with Milli-Q water, then soak in Milli-Q overnight.
7. Rinse 2 times with Milli-Q and air dry covered with absorbent lab mats.
8. Once dry: Wrap filter unit components in foil or sterilization bags and autoclave on gravity cycle; Tightly cap conical tubes and store in plastic bag; Loosely cap centrifuge bottles and autoclave in sterilization bags on gravity cycle.

Protocol

Culture Preparation/Growth

Step 1.

Culture Preparation/Growth Comparative measurements depend on having well-characterized culture growth.

1. Initiate an experimental culture to allow for at least 7 generations of characterized exponential growth (10 generations is the gold standard) prior to centrifugation and resuspension.
2. Monitor growth daily, including transfer/dilution to pre-determined mid-exponential density (i.e., semi-continuous culture) until target biomass is reached. A safe estimate for the target biomass is to assume 10% recovery of cells after centrifugation and washing (*this is strain dependent*).
3. Ensure cell density remains within the exponential range by increasing culture volume (not cell density) when ramping up to target biomass.

Example *Ostreococcus lucimarinus* (CCMP2972A) culture preparation:

Growth conditions: 18°C, 14:10 hour light:dark cycle, light irradiance of 100 µE m^{-2} s^{-1}

Growth media: L1 with natural seawater base

Exponential range: $7x10^5$ – $2x10^7$ cells/mL

Day	1	2	3	4	5	6	7	8	9	10

Initial density (mL^{-1})	$3x10^7$		$1.5x10^7$	$1x10^7$	$9x10^6$	$8x10^6$	$1x10^7$	$9x10^6$	$1x10^7$	$1.4x10^7$
Growth Rate (d^{-1})	NA		0.527	0.628	0.558	0.384	0.720	0.639	0.554	0.261
Dilution/ Transfer	Trans	Skip	Dil	Dil	Trans	Dil	Dil	Dil	NA	
Final density (mL^{-1})	$5x10^6$		$5x10^6$	$5x10^6$	$5x10^6$	$5x10^6$	$5x10^6$	$5x10^6$	$1x10^7$	
Volume (mL)	30 x3		86 x3	172 x3	80 x9	125 x9	257 x9	401 x9	401 x9	***3.6 L (use!)***

7 generations of growth prior to centrifugation

NOTE: Extra-large volumes used for omics experiment

Concentration and Resuspension

Step 2.

Concentration and Resuspension

1. When target culture biomass has been reached, split volume evenly among 2 or 4 acid-cleaned and autoclaved centrifuge bottles.
2. Weigh and balance bottles by removing volume until bottle pairs are within 0.01 g of each other. *Only open bottles in hood to maintain axenicity*!
3. Carry bottles over to Sorvall RC 26 Plus centrifuge.
4. Fit centrifuge with large rotor (accomodates 4 centrifuge bottles). Make sure pins are offset when rotor is placed into the centrifuge.
5. Place paired bottles opposite of each other in the rotor and tighten the lid.
6. Spin at the following settings:

- Rotor = GS-3 (choose option code “03”)
- Speed = 7300 RPM (equivalent to 10,000 rcf with this large rotor)
- Time = 0:32 minutes (includes 2 minute ramp up) **strain specific*
- Temp = +20/+25°C (will try to maintain lower temp, and shut down at max temp)

7. After the spin, carefully transport the bottles back to hood, taking care not to resuspend the cell pellets/smears.
8. Gently pour off the supernatant into a waste container, trying to disturb the cell smear as little as possible.
9. Resuspend/wash cells with a volume of nutrient deplete medium equivalent to the initial culture volume in each bottle.
10. Repeat 2-8.
11. Resuspend cells with a volume of nutrient deplete medium that is 5% of the initial culture volume in each bottle.
12. Run a sample of the cell concentrate in the Accuri to determine density. Store cells at normal growth conditions during Accuri run and calculations.

Experimental Culture Initiation

Step 3.

Experimental Culture Initiation

1. Calculate appropriate volume based on Accuri measurement to inoculate experimental nutrient replete and deplete culture flasks from the cell concentrate at a starting density corresponding to early-exponential growth. *NOTE: It helps to have some amount of media pre-aliquoted to experimental flasks. Adjust as needed to achieve target density and volume*.
2. Once all experimental cultures have been inoculated, mix and sample for FCM and Accuri measurements to monitor growth daily.
3. When the average daily growth rate of the nutrient deplete cultures (GR_{DEP}), is reduced to half or less of the growth rate of the replete cultures ($GR_{DEP}/GR_{REP} < 0.5$), collect samples for FCM (i.e., cell counts) and elemental analyses (i.e., particulate carbon/nitrogen and phosphorus, as well as dissolved nutrients).

Sample Collection

Step 4.

Sample CollectionFlow Cytometry (FCM, for cell counts)

1. Transfer 1 mL of culture to a sterile 1.2 mL cryovial tube.
2. Add 10 µL 25% glutaraldehyde (0.25% final conc) and *gently* vortex to mix.
3. Aliquot 500 µL to a duplicate cryovial.
4. Snap cryovials into cryocanes and incubate at 4°C for 30 minutes in the dark.
5. Flash freeze in liquid nitrogen.
6. Store at -80°C until analysis.

Dissolved and Particulate Elemental Analysis (EA, for cellular elemental composition & dissolved nutrient concentrations)

NOTE: Separate filters are needed from each sample for POC/N and POP analyses! Additional filters must be collected if you want technical replicates for each sample type.

1. Set up an acid-cleaned and autoclaved glass filter unit with pre-combusted 25mm Advantec glass fiber filter. **Use ethanol-cleaned forceps in hood!*
2. Apply 20-40 mL of media or culture to the filter funnel and turn on the vacuum until the filter is dry.
3. Use ethanol-cleaned forceps to fold filter in half and collect in a 12-well plate or piece of combusted aluminum foil.
4. Pour filtrate into an acid-cleaned 50 mL conical tube.
5. Repeat for sample duplicate.
6. Rinse filter tower with MilliQ between samples.
7. Store filters and filtrate at -20°C until further processing.

DAPI (for microscopy to assess axenicity)

1. Transfer 1 mL of culture to sterile 1.2 mL cryovial tube.
2. In the chemical hood, add 100 µL of 37% formaldehyde (3.7% final conc) and mix by inverting. Do not vortex.
3. Snap cryovials into cryocanes and incubate at room temp for 15 minutes (in the dark).
4. Store at 4°C until analysis (can be stored for several days or flash frozen in liquid nitrogen and stored at -80°C).

Sample Processing

Step 5.

Sample Processing Samples for elemental analysis should be sent to Analytical Services at Horn Point Laboratory (University of Maryland Center for Environmental Science, UMCES).

For each sample, request at least the following analyses:

Analysis	Cost per sample	Sample type
Soluble Reactive Phosphate (SRP or PO4)*	$7.90	filtrate
Nitrate plus Nitrite (NO(2+3))*	$7.90	filtrate
Total Particulate Phosphorus (PP)	$19.80	filter
Particulate Carbon and Particulate Nitrogen (CHN)	$17.70	filter

**Dependent on limiting nutrient*

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