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🌐 Sample Preparation for Elemental Analysis of *Auxenochlorella protothecoides* (UTEX 250) Cells and Spent Media by Inductively Coupled Plasma Mass Spectrometry (ICP-MS/MS) and Total Organic Carbon (TOC). V.1

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We use this protocol and it's working

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Abstract

This protocol describes a method for quantifying the elemental composition of *Auxenochlorella protothecoides* (UTEX 250) cells and the spent HP medium (Camacho, 2024). Elements measured include C (as total organic C), Na, Mg, P, S, K, Ca, Mn, Fe, Cu, Zn, Se, and Mo. Samples are collected, washed, digested, and analyzed for total organic carbon (Shimadzu) and other elements by ICP-MS/MS (Agilent 8900). Elemental measurements can be normalized to volume of cultures, cell number, sulfur, or total organic carbon.

Materials

Materials

1. Metal free 15 mL tubes - Globe Scientific Inc. Centrifuge, high performance, red screw cap, assembled, polypropylene, printed graduations, sterile, 25 tubes / bag, 20 bags/ case. Catalog No. 6295, with a maximum rating of 17,000 $\times g$
2. 1 L HDPE bottles
3. Trace metal grade 1 mM Na₂EDTA washing solution
4. Ultra-pure ICP-MS grade Milli-Q H₂O
5. ICP-MS grade Optima 70% HNO₃
6. Diluted 2.1% HNO₃
7. Ultra-pure 12 M HCl
8. Kimtech Kimwipes™

Equipment

1. RAININ P1000, P200, P20 pipettes and tips
2. 10 mL and 25 mL serological pipets
3. Fume Hood
4. Microscope and Hemocytometer
5. Beckman Coulter Avanti JXN-26 Centrifuge
6. Fixed angle centrifuge rotor JA-14.50 with 15 mL adapters
7. Eppendorf 5810 R centrifuge
8. Eppendorf A-4-81 swinging bucket rotor with 15 mL adapters
9. Liquid waste container
10. Solid trash container
11. 65 °C - 80°C shaker water bath
12. Fisherbrand CPX2800 ultrasonic bath 2.8 L with heater
13. Agilent 8900 ICP-MS/MS
14. Shimadzu Total Organic Carbon Analyzer (TOC-L)

Safety warnings

-  Nitric acid (HNO₃) and hydrochloric acid (HCl) are corrosive and toxic acids. Use a lab coat, closed toe shoes, long pants, gloves, and eye protection when handling HNO₃ and HCl. Work with concentrated HCl and HNO₃ should be done in the fume hood. Gloves should also be used when handling ultra-pure reagents. Never mix HCl and HNO₃. Never mix bleach with ammonia, acids, alcohol, hydrogen peroxide, or other household cleaners.

Before start

1. Acid wash all stock bottles with fresh (unused) [IM] 6 Mass Percent . Do not use glass containers as glass may leach metals. Use high density polyethylene (HDPE) bottles instead.

1.1. Dilute fresh (unused) [IM] 12 Mass Percent HCl to [IM] 6 Mass Percent . Remember to add acid to water and never water to acid.

1.2. Add  100 mL of [IM] 6 Mass Percent HCl to  1 L stock bottles. Swirl [IM] 6 Mass Percent HCl in the bottle to clean every internal surface. Transfer the used [IM] 6 Mass Percent HCl to the next bottle and repeat.

1.3. Rinse with ICP-MS grade ultra-pure H₂O at least seven times. See next section for a detailed description of the Milli-Q H₂O.

1.4. Neutralize the used [IM] 6 Molarity (M) HCl.

1.4.1. Add  1 L of H₂O to a  4 L beaker placed in a larger, chemically compatible secondary container, such as an autoclavable polypropylene  12 L tray (Cat. no. S37253, Fisher Scientific).

1.4.2. Slowly and carefully add  1 L of [IM] 6 Molarity (M) HCl to water. Always add acid to water. The solution may heat up, so take your time.

1.4.3. Neutralize [IM] 3 Molarity (M) HCl by adding NaHCO₃ (Arm & Hammer pure baking soda) slowly, scoop by scoop until no foam is formed.

1.4.4. Use a pH indicator strip to verify that the acid is safely neutralized ().

1.4.5. Neutralized acid may be discarded down the drain.

2. The ICP-MS/MS is extremely sensitive and contaminants may confound your measurements. Use trace metal grade reagents and ICP-MS grade ultra-pure Milli-Q H₂O. The production of ICP-MS grade ultra-pure Milli-Q H₂O relies on a Q-POD® Element containing a Quantum® ICP filter (REF QTUM00ICP).

2.1. ICP-MS grade ultrapure Milli-Q H₂O will be referred to as Milli-Q H₂O throughout the rest of this protocol.

2.2. Before using the Milli-Q H₂O, verify that the resistivity is 18.2 MΩ • cm and the total organic carbon (TOC) reading is <10 ppb.

2.3. Keep  1 L of Milli-Q H₂O in an acid washed  1 L high density polyethylene (HDPE) bottle.

2.4. Keep the bottle closed to avoid dust from entering.

2.5. Replace your Milli-Q H₂O before each experiment.

3. Be mindful of metal contamination from tubes, plastic bottles, pipets, and dust.

3.1. Ensure all work areas are thoroughly wiped to be free of dust. If a clean room is not available, work can be carried out in a clean fume hood and a room where the paint is not deteriorated.

3.2. Keep tubes and reagents closed to avoid metal contamination from dust. Zn is abundant in dust.

3.3. Most  15 mL and  50 mL conical tubes contain residual metal contamination. We recommend acid washing tubes with  12 Mass Percent HCl (step 5 in this section). Tube manufacturers may change their sources of materials over time, so make sure to check that the tubes you are using are metal free using the ICP-MS. Record the lot numbers for all tubes.

3.3.1. Metal free  15 mL tubes - Globe Scientific Inc. Catalog No. 6295 were specially chosen because they consistently contained less metals and can withstand a force of 17,000 ×g, which is needed to collect *Auxenochlorella* cells. These tubes however contain a large amount of Ca and therefore need to be washed if you are interested in the Ca content of cells and supernatants.

3.4. Disposable  10 mL and  25 mL serological pipets Cat. No. 13-676-10J and 13-676-10K, respectively, will introduce a detectable amount of Zn contamination to your samples. We advise rinsing each newly opened pipet by pipetting Milli-Q H₂O to the maximum volume and disposing the water before pipetting any samples or reagents.

4. Pre-label a set of  15 mL tubes for cell pellets and another set for spent media (optional).

4.1. Label tubes with the date, your initials, sample name, and experiment ID.

4.2. If measuring - Fe, - Cu, or - Zn media, prepare five  15 mL tubes for each supernatant sample.

4.3. Include six or more method blanks for cell pellets and three method blanks for supernatant sample processing. Treat the method blanks as if they contained cells or supernatants, using the same reagents for digestion and dilution. Use a different set of three method blanks for each digestion method.



5. Acid-wash all  15 mL tubes with  5 mL of fresh  12 Mass Percent in the fume hood. Cap and invert the tube gently. Re-use the  5 mL of  12 Mass Percent HCl for the next 15 tubes.

5.1. Neutralize used  12 Mass Percent HCl by diluting to  3 Mass Percent HCl in the fume hood. Refer to steps 1.4 to 1.4.5 but adjust volumes for  12 Mass Percent instead of  6 Mass Percent HCl.

5.2. Rinse tubes with ICP-MS grade Milli-Q H₂O at least seven times. Rinse the exterior of the capped tube to prevent exposure to residual HCl. It is very important to ensure there is no residual HCl. HCl will discolor some plastics and corrode metals.

5.3. Dry the exterior of the tube with a Kimwipe™ and centrifuge the empty tubes at  3000 x g, 22°C, 00:02:00 using the 5810 R centrifuge fitted with a swinging bucket rotor (rotor A-4-81) and  15 mL tube adapters. Rinse the rotors and buckets with Milli-Q H₂O when you are finished.

5.4. Use a P200 pipette to carefully remove remaining water in the tube. Avoid touching the inner wall of the tube with the pipette.

6. Prepare the centrifuges, rotors, and adapters.

6.1. Set the JXN-26 centrifuge to  17000 x g, 22°C, 00:02:00, rotor A-4-81 with  15 mL tube adapters.

6.2. Set the 5810 R centrifuge to  3220 x g, 22°C, 00:02:00, rotor JA-14.50 with  15 mL tube adapters.

7. Determine the density of the culture by counting cells with a hemocytometer (Camacho & Merchant, 2024). Calculate and record the volume of culture required to collect 1×10^8 - 3×10^8 total cells.

Procedure

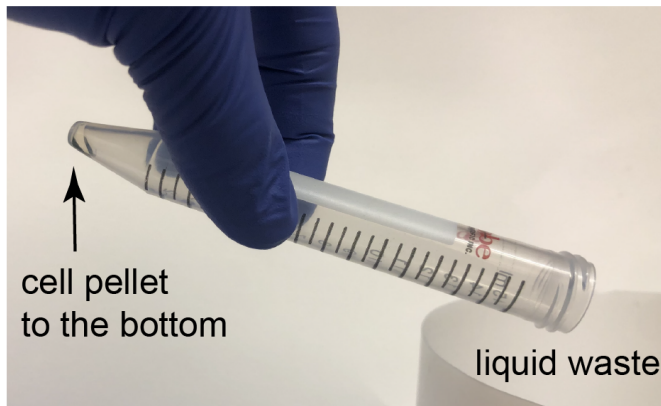
- 1 Collect 1×10^8 - 3×10^8 cells at 17000 x g, 22°C, 00:02:00 in red screw cap 15 mL tubes using the JXN-26 centrifuge. Do not disturb the pellet. The pellet will partially adhere to the side of the tube at a 45-degree angle.

If cells are sticking to the side of the tube and not collecting to the bottom of the tube, centrifuge for an additional 00:05:00 - 00:10:00 .

- 2 Carefully transfer 0.5 mL of supernatant to each prelabeled 15 mL tube without disturbing the pellet. If measuring Fe, Cu, or Zn deficient media, then aliquot 0.5 mL into each of the five tubes.

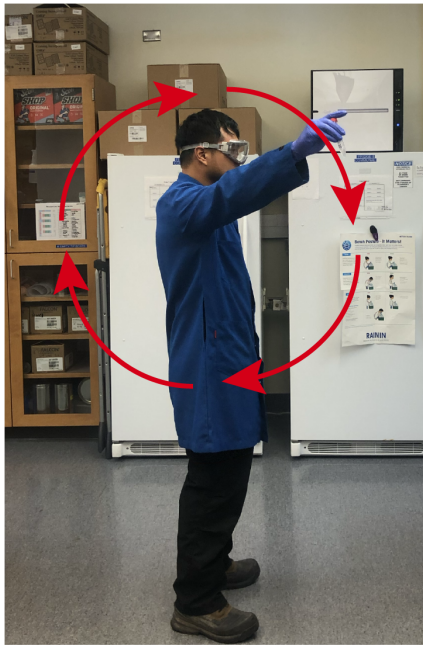
- 3 Slowly and carefully decant the remaining supernatant into a liquid waste container in one motion. Keep the side with the pellet to the bottom and make sure that no cells are decanted. You may keep about 100 µL of liquid to avoid decanting cells.

Do not mix liquid waste with ammonia, acids, alcohol, hydrogen peroxide, or other household cleaners. You will add bleach to the liquid waste later.



Cell pellet is oriented to the bottom while decanting. Some liquid is left to avoid decanting cells.

- 4 Pipette 1 mL of 1 millimolar (mM) Na₂EDTA into the tube containing the pellet. Resuspend the pellet by closing the cap and swinging the tube in a circular motion until the pellet is fully resuspended.



Swinging motion to re-suspend cell pellets in washing solutions.

- 5 Centrifuge the mixture  17000 x g, 22°C, 00:02:00 and discard the supernatant by carefully decanting, again with one motion and the pellet to the bottom.
- 6 Resuspend the cell pellet in  1 mL of Milli-Q H₂O by closing the cap and swinging the tube in a circular motion.
- 7 Centrifuge again at  17000 x g, 22°C, 00:02:00 .
- 8 Remove the supernatant by slowly decanting in one motion. Do not disturb the pellet.
- 9 Transfer the tubes to the swinging bucket rotor on the Eppendorf 5810 R centrifuge and pellet the cells at  3220 x g, 22°C, 00:02:00
- 10 Carefully remove the supernatant with a P200 pipette. Do not take up any cells. If you accidentally take up cells into the tip, dispense the liquid back into the tube and centrifuge again.

Add bleach to the liquid waste to make a 10% bleach and 90% waste solution. Wait 01:00:00 and dispose down the drain. Do not mix bleach with ammonia, acids, alcohol, hydrogen peroxide, or other household cleaners.

11 (Optional) Freeze the pellet and supernatant samples at -20 °C until 1 day before the ICP-MS/MS analysis.

12 Digest cells with 143 μL of 70% Optima® grade or similar ICP-MS grade HNO_3 and incubate at 65 °C while shaking at 175 RPM (setting 5 on New Brunswick Gyrotory Water Bath Shaker, Model G76) for 16:00:00. Fill the water bath before leaving to ensure that the water does not fully evaporate.

12.1 Cell pellets that appear larger than 100 μL will require an additional 143 μL of HNO_3 . For instance, fatty mixotrophic or heterotrophic cells grown with 2% glucose are larger in diameter so although the number of cells is the same, the biomass of these cells will be greater than that of photoautotrophic cells. Lipid rich cells are also harder to digest.

12.2

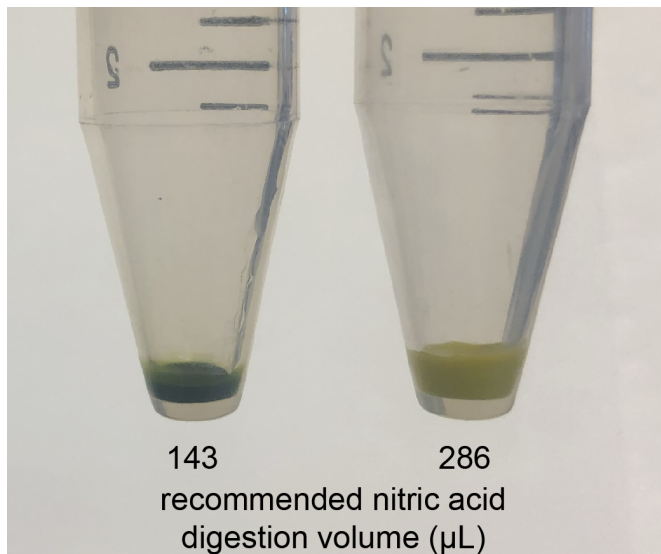


Image of 15 mL tubes containing cell pellets. Photoautotrophic (left) and mixotrophic (right) cell pellets and corresponding recommended digestion volume.

The mixture should be clear with absolutely no solids present. If cell pellets are not fully digested, you may take either one (or a combination) of the following actions until the

mixture is clear with no precipitates.

1. Incubate for an additional  02:00:00 at  80 °C while shaking at 175 RPM.
2. Incubate samples in a  65 °C ultra-sonic water bath for  01:00:00 at maximum power. Use floats or a strainer tray to make sure that nothing touches the bottom of the ultra-sonic bath tank. The cavitation effect will be diminished and the transducers may break if an object is placed on the bottom of the tank or if the water level is too low. Ensure that the water level is maintained precisely at the operating level.
3. Add fresh  50 µL of 30% hydrogen peroxide to the pellet and incubate at  85 °C for  02:00:00 with shaking at 175 RPM.
4. Use a dedicated microwave (800 W) in a fume hood to denature samples. Heat samples in increments of  00:00:15 and slightly twist open tubes to avoid fume pressurization. Let the tubes cool before placing back in the microwave. Do not exceed  00:01:30 total.

Tubes may leach material and metals as they encounter HNO₃, H₂O₂, are heated, sonicated, and/or microwaved. Use a different set of method blanks for each digestion method or combination of digestion methods. Only analyze blanks that have undergone the same digestion method(s) as the samples.

- 13 Fill to  5 mL with Milli-Q H₂O if  143 µL 70% HNO₃ was used. Fill to  10 mL if  286 µL of HNO₃ was used. Final HNO₃ concentration should be 2%. Record the volume of 70% HNO₃ and Milli-Q H₂O added.

If filled to  5 mL , the dilution factor is 5.

If filled to  10 mL , the dilution factor is 10.

	A	B	C
	Nitric acid (µL)	Fill to (mL)	Dilution factor
	143	5	5
	286	10	10

Volumes required for the digestion of cell pellets and the resulting dilution factors.

- 14 Cell pellet samples are now ready for the ICP-MS/MS analysis.

Samples in capped tubes may be stored at room temperature in a cabinet equipped with an exhaust system to remove HNO_3 fumes.

- 15 To prepare spent media from cultures grown in replete conditions, add  9.5 mL of 2.1% HNO_3 to each tube containing  0.5 mL of sample, for a final HNO_3 concentration of 2%. The dilution factor is 20.

- 16 To prepare - Fe, - Cu, or - Zn supernatant samples for measurement, perform a standard addition of Fe, Cu, and/or Zn to each  0.5 mL sample.

- 16.1 Prepare 4 acid washed  15 mL tubes. Label the tubes standard addition stock (SAS) 1x, SAS 0.1x, SAS 0.01x, and SAS 0.001x.

- 16.2 Aliquot  7 mL of Milli-Q H_2O into the tube labeled SAS1x and  9 mL into each of the remaining tubes.

- 16.3 Pipette  1 mL of [M] 2 millimolar (mM) Cu, [M] 20 millimolar (mM) Fe, and [M] 10 millimolar (mM) Zn stocks from the HP medium into the 1x tube.

- 16.4 Execute a serial dilution by transferring  1 mL of 1x solution into the tube labeled 0.1x which contains  9 mL of Milli-Q H_2O . Invert the tube to mix and transfer  1 mL of the 0.1x solution to  9 mL of Milli-Q H_2O in the tube labeled 0.01x. Repeat for 0.001x using the 0.01x solution.

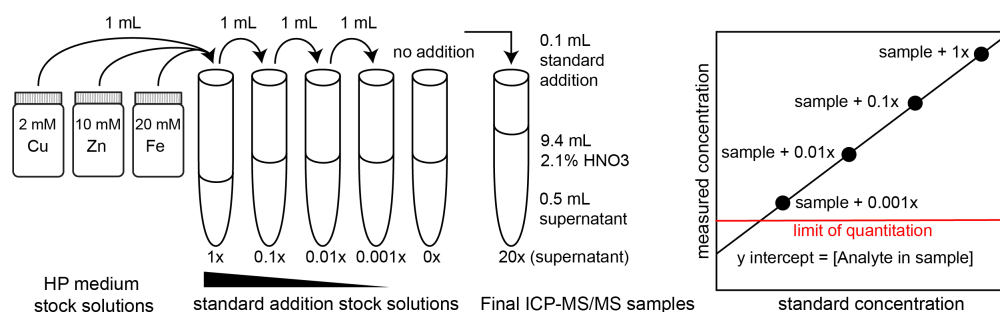
Label the five supernatant samples 0, 1x, 0.1x, 0.01x, and 0.001x

Do not add any standard into the tube labeled 0. Aliquot  100 μL of the standard addition stocks into their corresponding supernatant samples. Repeat for all supernatant samples.

Add  9.5 mL of 2.1% HNO₃ to all supernatant samples without standard additions.

Add  9.4 mL of 2.1% HNO₃ to all supernatant samples with standard additions.

The dilution factor for all supernatant samples is 20x.



- 17 To obtain total organic carbon measurements from ICP-MS/MS cell samples, transfer  500 μ L of digested 2% HNO₃ sample to a TOC vial containing  14.4 mL of Milli-Q H₂O and  135 μ L of [M] 3 Molarity (M) HCl. Measure carbon only and include a set of method blanks from step 12.2. Samples are now ready for TOC measurement. The dilution factor for cell pellets is 30 $\times$ ICP-MS/MS dilution factor.

- 18 For measurement of ICP-MS/MS supernatants from step 15 – 16, transfer  500 μ L of 2% HNO₃ sample to a TOC vial containing  14.4 mL of Milli-Q H₂O and  135 μ L of [M] 3 Molarity (M) HCl. The total dilution factor is 600.

If you are measuring ICP-MS/MS supernatants which originally contained 2% glucose, then transfer  180 μ L of ICP-MS/MS samples to a vial containing  14.7 mL of Milli-Q H₂O and  135 μ L of [M] 3 Molarity (M) HCl. The total dilution factor is 1666.67



Protocol references

Dimitrios Camacho, Charles Perrino, Sabeeha Merchant 2024. HEPES-Phosphate Medium, Suitable for Studies of Trace Element Nutrition in Photoautotrophic and Heterotrophic Auxenochlorella protothecoides.. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.kxygxyzdzl8j/v1>

Dimitrios Camacho, Sabeeha Merchant 2024. Determination of Auxenochlorella protothecoides Cell Density With a Hemocytometer. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.rm7vzjk12lx1/v1>