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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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Disclaimer

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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 ×*g*
2. 1 L HDPE bottles
3. Trace metal grade 1 mM Na_2EDTA washing solution
4. Ultra-pure ICP-MS grade Milli-Q H_2O
5. ICP-MS grade Optima 70% HNO_3
6. Diluted 2.1% HNO_3
7. Ultra-pure 12 M HCl
8. Kimtech Kimwipes^{TM}

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 (HNO_3) and hydrochloric acid (HCl) are corrosive and toxic acids. Use a lab coat, closed toe shoes, long pants, gloves, and eye protection when handling HNO_3 and HCl. Work with concentrated HCl and HNO_3 should be done in the fume hood. Gloves should also be used when handling ultra-pure reagents. Never mix HCl and HNO_3. Never mix bleach with ammonia, acids, alcohol, hydrogen peroxide, or other household cleaners.

Before start

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

1.1. Dilute fresh (unused) [M] 12 Molarity (M) HCl to [M] 6 Molarity (M) . Remember to add acid to water and never water to acid.

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

1.3. Rinse with ICP-MS grade ultra-pure H_2O at least seven times. See next section for a detailed description of the Milli-Q H_2O.

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

1.4.1. Add 1 L of H_2O 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 [M] 6 Molarity (M) HCl to water. Always add acid to water. The solution may heat up, so take your time.

1.4.3. Neutralize [M] 3 Molarity (M) HCl by adding $NaHCO_3$ (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 (pH 7).

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 H_2O. The production of ICP-MS grade ultra-pure Milli-Q H_2O relies on a Q-POD® Element containing a Quantum® ICP filter (REF QTUM00ICP).

2.1. ICP-MS grade ultrapure Milli-Q H_2O will be referred to as Milli-Q H_2O throughout the rest of this protocol.

2.2. Before using the Milli-Q H_2O, 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 H_2O 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 H_2O 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 [M] 12 Molarity (M) 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 H_2O 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 [M] 12 Molarity (M) in the fume hood. Cap and invert the tube gently. Re-use the 5 mL of [M] 12 Molarity (M) HCl for the next 15 tubes.

5.1. Neutralize used [M] 12 Molarity (M) HCl by diluting to [M] 3 Molarity (M) HCl in the fume hood. Refer to steps 1.4 to 1.4.5 but adjust volumes for [M] 12 Molarity (M) instead of [M] 6 Molarity (M) HCl.

5.2. Rinse tubes with ICP-MS grade Milli-Q H_2O 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 H_2O 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

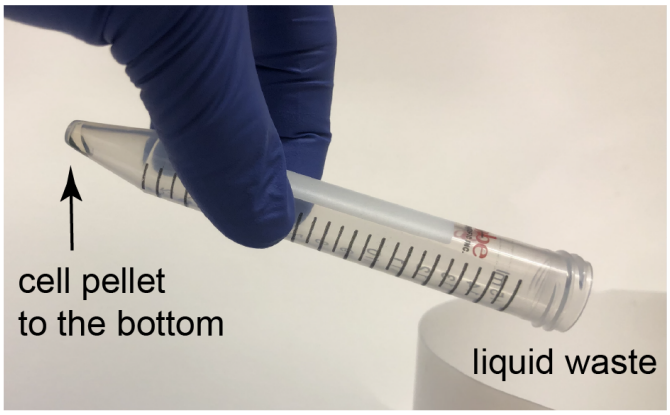
1 Collect 1 × 10^8 - 3 × 10^8 cells at 17000 x g, 22°C, 00:02:00 in red screw cap 15 mL tubes using the JXN-26 centrifuge. Do not disturb the pellet. The pellet will partially adhere to the side of the tube at a 45-degree angle.

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.

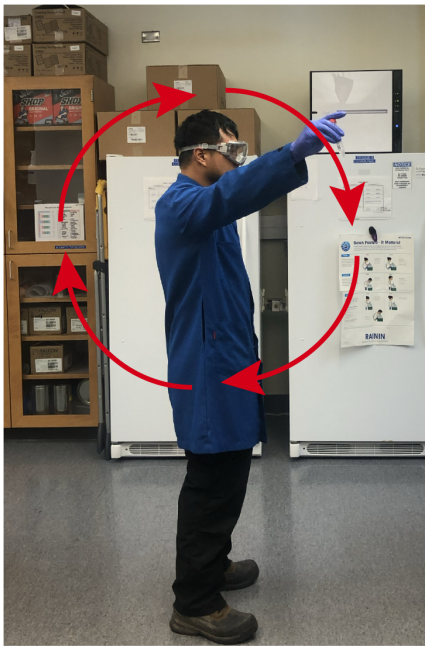
3 Slowly and carefully decant the remaining supernatant into a liquid waste container in one motion. Keep the side with the pellet to the bottom and make sure that no cells are decanted. You may keep about 100 µL of liquid to avoid decanting cells.

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 [M] 1 millimolar (mM) Na_2EDTA 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 H_2O 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

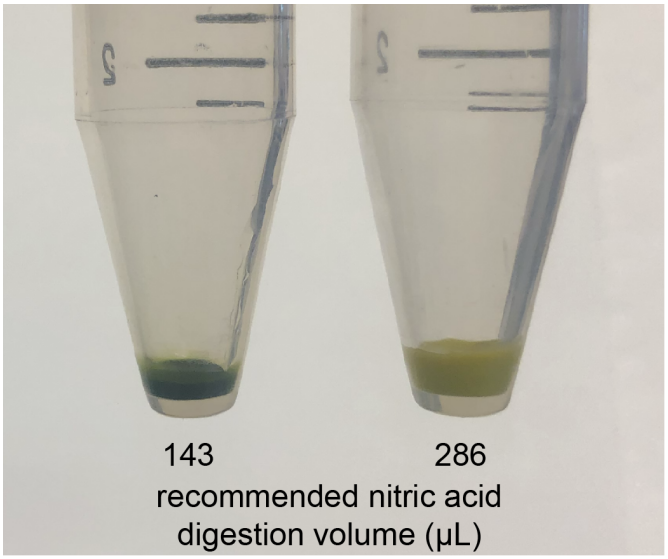


Image of 15 mL tubes containing cell pellets. Photoautotrophic (left) and mixotrophic (right) cell pellets and corresponding recommended digestion volume.

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 HNO_3, H_2O_2, 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 H_2O if 143 µL 70% HNO_3 was used. Fill to 10 mL if 286 µL of HNO_3 was used. Final HNO_3 concentration should be 2%. Record the volume of 70% HNO_3 and Milli-Q H_2O 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.

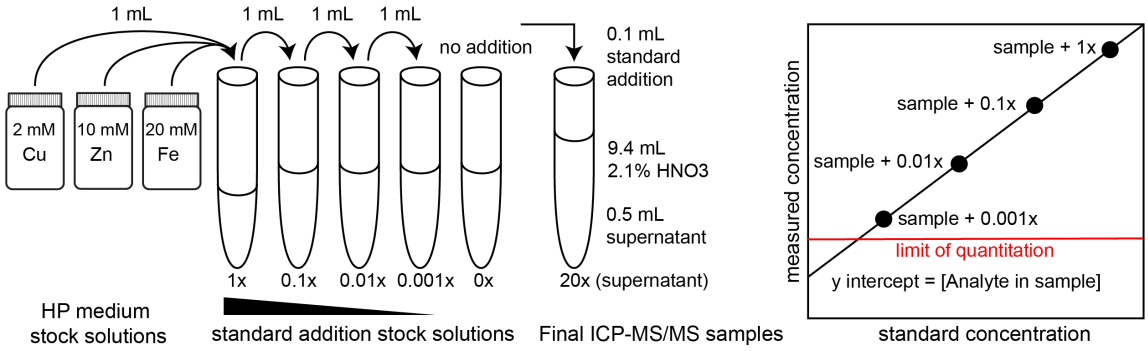
Label the five supernatant samples 0, 1x, 0.1x, 0.01x, and 0.001x

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% HNO_3 to all supernatant samples without standard additions.

Add 9.4 mL of 2.1% HNO_3 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 µL of digested 2% HNO_3 sample to a TOC vial containing 14.4 mL of Milli-Q H_2O and 135 µL 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 × ICP-MS/MS dilution factor.

18 For measurement of ICP-MS/MS supernatants from step 15 – 16, transfer 500 µL of 2% HNO_3 sample to a TOC vial containing 14.4 mL of Milli-Q H_2O and 135 µL 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 µL of ICP-MS/MS samples to a vial containing 14.7 mL of Milli-Q H_2O and 135 µL 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

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