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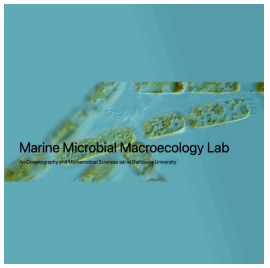
An X-HTDC method for estimating particulate phosphorus from microalgae V.5



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Abstract

Total particulate phosphorus (TPP) is often determined using the High Temperature Dry Combustion (HTDC) method followed by hydrolysis of the ash and then molybdenum colorimetry. Here we show that a higher than traditionally-used combustion temperature, 800 °C vs. 450 - 550 °C, improves phosphorus recovery from several organic phosphorus compounds, marine phytoplankton cultures and particulate samples from the field. In aggregate these improvements to the method double the P recovery from phospholipids to 97%. TPP recovery from laboratory phytoplankton cultures and field samples increased an average of 13%, primarily due to improvements in P recovery from phospholipids, polyphosphates, and nucleic acids. We refer to this new method as the eXtra high temperature dry combustion ash/hydrol method (X-HTDC) and recommend its application for measuring particulate phosphorus from organic compounds in aquatic systems.
The working range of this assay is 1.22 to 500 uM orthophosphate. Minimum sampling biomass is 0.19 ug P/filter. In order to assess the intracellular phosphorus in microalgae, we recommend an oxalate reagent (Tovar-Sanchez 2003) to wash the microalgae collected on the filter to remove surface adsorbed phosphorus.

Citation

P.S. Chen, T.Y. Toribara and Huber Warner. Microdetermination of Phosphorus. Anal. Chem..

https://doi.org/10.1021/ac60119a033 LINK

Citation

AntonioTovar-Sanchez, Sergio A Sañudo-Wilhelmy, Manuel Garcia-Vargas, Richard S Weaver, Linda C Popels, David A Hutchins
. A trace metal clean reagent to remove surface-bound iron from marine phytoplankton. Marine Chemistry.

https://doi.org/10.1016/S0304-4203(03)00054-9 LINK

Protocol materials

- Magnesium sulfate anhydrous **Fisher Scientific** **Catalog #**M65500
- 12 N Hydrochloric acid
- Potassium dihydrogen orthophosphate **ACP Chemicals** **Catalog #**P-4550
- 18M sulfuric acid
- Ammonium molybdate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**09878-100G
- Ascorbic acid **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A5960-100G

Troubleshooting

Safety warnings

Polycarbonate filter can release toxic gas and smoke during combustion. An exhaust system is required for muffle furnace while using the X-HTDC method.

Before start

We have found that crucibles may lose their temperature resistance after acid-washing or long soaks in alkaline detergent. Crucibles tended to shatter in the oven during the initial increase in temperature from room temperature to 500 °C, even when the ramp rate was carefully controlled at 150 °C/h. We recommend not soaking crucibles in acid but instead we suggest the crucibles be filled with 0.2 M HCl and then incubated at 90 °C for 30 minutes as the acid-washing step. It is necessary to inspect the temperature resistance of newly acquired crucibles by combusting them at 500 °C for 6 h (ramp rate: 150 °C/h) after acid-washing. We found that crucibles that pass this inspection do not usually shatter when heated to 800 °C.

Sampling

1 **Sampling microalgae for total particulate phosphorus (i.e. intracellular phosphorus and adsorbed phosphorus)**

1.1 Filter microalgae in liquid media onto polycarbonate filters, using gentle vacuum pressure (130 mmHg).

Equipment

Filter forceps	NAME
blunt end, stainless steel	TYPE
Millipore	BRAND
XX6200006P	SKU

1.2 Rinse samples with filtered seawater

1.3 Place sample filters in 2 mL Cryogenic Vials.

Equipment

Cryogenic Vials with Closures	NAME
Polypropylene, 2 mL	TYPE
Corning®	BRAND
66021-974	SKU

1.4 Filter blank media (without cells) through polycarbonate filter as blank.

1.5 Flash freeze filters and store at -20 °C .

2 Sampling microalgae for intracellular particulate phosphorus

2.1 Filter microalgae in liquid media onto polycarbonate filters , using gentle vacuum pressure (5 inches Hg).

Equipment

Filter forceps	NAME
blunt end, stainless steel	TYPE
Millipore	BRAND
XX6200006P	SKU

2.2 Add 5 mL oxalate reagent onto the filter, and let oxalate reagent sit in the filter funnel for 00:05:00 5m

Protocol

NAME

Preparation of oxalate reagent

CREATED BY

Ying-Yu Hu

Preview

2.2.1 Add 50 mL MilliQ water in a 250 mL beaker.

2.2.2 Weigh 40 g NaOH and slowly pour into the beaker.

2.2.3 Use squeeze bottle to rinse the weighing boat and transfer rinse water into the same beaker.

2.2.4 Use glass rod to gently stir and fully dissolve NaOH.

Note

The solution is very hot and corrosive. It can cause skin burns and eye damage.

2.2.5 Carefully transfer NaOH solution into 100 mL volumetric flask by using glass rod.

2.2.6 Rinse beaker with small amount of MilliQ water three times, transfer rinse water into the flask.

2.2.7 Mix the solution by gently shaking the capped volumetric flask and top to 100 mL with MilliQ water.

2.2.8 Transfer the prepared reagent into a 250 mL PP bottle.

2.2.9 Label the bottle with SDS pictogram.

2.2.10 In a 1000 mL beaker with stir bar, add 600 mL MilliQ water.

2.2.11 Add 18.6 g EDTA, 14.7 g sodium citrate, 0.74 g KCl and 5 g NaCl into the beaker, stir until all ingredients are dissolved. pH 5.7

2.2.12 [M] 10 Molarity (M) NaOH is added dropwise to bring pH in between 6 to 7 by using a transfer pipet

2.2.13 Add 12.6 g oxalic acid to the solution, stir the mixture while heating.

2.2.14 After oxalic acid is completely dissolved, stop heating and let it cool to room temperature. A water bath filled with tap water can be used to speed up cooling. pH 3.3

2.2.15 Add [M] 10 Molarity (M) NaOH dropwise to bring pH to pH 8

2.2.16 Top to 1 L in volumetric flask with MilliQ water.

2.2.17 Filter oxalate reagent by rapid flow to a 1 L PP bottle.

Equipment

Sterile Disposable Filter Units with PES Membrane	NAME
Thermo Scientific™ Nalgene™ Rapid-Flow™	BRAND
5964520	SKU

2.2.18 Label the bottle and keep it at Room temperature.

2.3 Drain and then rinse the sample with filtered seawater once

2.4 Place sample filters in 2 mL Cryogenic Vials.

Equipment

Cryogenic Vials with Closures	NAME
Polypropylene, 2 mL	TYPE
Corning®	BRAND
66021-974	SKU

2.5 Filter blank media (without cells) through polycarbonate filter as blank.

2.6 Flash freeze filters and store at -20 °C.

X-HTDC-ing

3 Mark number at the bottom of each crucible with pencil, log the following information:
(1) The number of crucible
(2) The code of sample in the crucible

Equipment

Porcelain crucibles	NAME
40 mL	TYPE
VWR	BRAND
89037-996	SKU

Equipment

Crucible cover	NAME
VWR	BRAND
71000-146	SKU

4 Transfer sample to crucible with clean filter forceps and lay filter at the bottom.

5 [M] 0.17 M $MgSO_4$ reagent:
Dissolve 1.023 g $MgSO_4$ in 50 mL MilliQ water
Magnesium sulfate anhydrous **Fisher Scientific Catalog #**M65500

6 Add 200 µL [M] 0.17 M $MgSO_4$ directly onto each sample and blank filter.

Note

Sing-use pipet tip to avoid cross-contamination.

7 Cover the crucibles and place in the oven at 90 °C until samples are completely dry.

Equipment

Forced air oven	NAME
VWR	BRAND
89511-410	SKU

Note

Remove samples out of the oven as soon as they are dried. If muffle furnace is not available, keep crucibles in the vacuum desiccator.

8 Combust dried samples at 800 °C for 09:00:00 9h

Equipment

Muffle furnace	NAME
F30428C	TYPE
Thermo	BRAND
10-505-13	SKU

Note

Map the location of crucibles in the oven, in case pencil mark disappears under 800°C.

Note

Ramp rate should be controlled at < 200 °C /hour or follow the instruction provided by manufacture, otherwise the crucibles might shatter.
SP.RAT: 150/PAMPU: hour
Or
SP.RAT: 2.5/PAMPU: minute

9 Allow samples to gradually cool down in the muffle furnace.

10 Pencil mark on crucibles should be still visible, however, it can be easily removed by water. Therefore, when removing samples out of the furnace, label the lid and crucible with sharpie immediately.

Digesting

11 [M] 0.2 M HCl reagent:
In a reagent bottle, dissolve one part of [M] 12 N HCl in 59 parts of MilliQ water
12 N Hydrochloric acid

Note

Volume of HCl_0.2M_mL = (5_mL) X (#Sample + #Blank)

12 Preheat oven to 90 °C

13 Add 5 mL 0.2 M HCl to each crucible.

14 Gently swirl the crucible.

15 Cover the crucibles and place crucibles in the muffin tin pan for easier-handling.

16 Incubate in the oven for 00:30:00 30m

17 Cool samples down to Room temperature

18 Gently swirl the crucible and then transfer 500 uL solution to 2 mL microtube. Duplicate each sample and blank.

Equipment

Maxymum Recovery® Snaplock Microcentrifuge Tube	NAME
2.0 mL, Polypropylene, Clear, Nonsterile,	TYPE
Axygen®	BRAND
MCT-200-L-C	SKU

Preparing standard working solutions 2h

19 Standard working solutions and reagents can be prepared during sample digestion.

20 KH_2PO_4 primary standard stock solution (≈ 1 mM)

Potassium dihydrogen orthophosphate **ACP Chemicals Catalog #**P-4550

20.1 Transfer about 1 g KH_2PO_4 into a beaker, cover the beaker with foil

20.2 Place the beaker into an oven, dry KH_2PO_4 at 110 °C for at least 02:00:00 2h

20.3 Move KH_2PO_4 into a vacuum desiccator, allow KH_2PO_4 to cool to room temperature

20.4 Dissolve around 0.136 g dried KH_2PO_4 in 1 L milliQ water.

- Use 1 L volumetric flask
- Take notes of the actual weight of KH_2PO_4 for final concentration of standard stock solution

20.5 Transfer standard stock solution into a 1 L bottle and store in the fridge.

Note

This stock solution lasts quite a long time, unless there is evidence for growth of algae or other extraneous biotic material.

21 Standard working solution

	KH2PO4	Primary (ul)	Milli Q (ul)
	S1	0	1000
	S2	5	995
	S3	10	990
	S4	20	980
	S5	50	950
	S6	100	900
	S7	150	850
	S8	200	800

22 Transfer 500 uL of each standard working solution to 2 mL microtube.

Preparing working reagents

2h

23

Note

All reagents are freshly prepared before colorimetric measurement.

24 [M] 6 N (3 M) sulfuric acid reagent:
Carefully add 1 part [M] 18 M concentrated sulfuric acid into 5 part MilliQ water
18M sulfuric acid

25 [M] 2.5 % ammonium molybdate reagent:
Weigh 0.25 g ammonium molybdate in a Falcon tube and top to 10 g with MilliQ water.
Cap and shake until totally dissolved.
Ammonium molybdate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**09878-100G

26 [M] 10 % ascorbic acid reagent:
Weigh 1 g ascorbic acid in a Falcon tube and top to 10 g with MilliQ water;
Cap and shake until all dissolved.

Ascorbic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A5960-100G

Note

Wrap the tube with foil if the reagent is not used right after prepared.

27 Calculate the volume of molybdate-ascorbic reagent:
Total volume of reagent_mL = (0.5 mL) X (#standard working solution + #samples + #blanks)

28 Mix the reagents into Falcon tube:

	A	B
	Reagent	Part(s) as in volume

	A	B
	MilliQ	2
	6N sulphuric acid	1
	2.5% ammonium molybdate	1
	10% ascorbic acid	1

Colorimetric measurement

2h

29 Preheat incubator/shaker to 37 °C

Equipment

SHAKING INCUBATOR — NAME

71L — TYPE

Corning® LSE™ — BRAND

6753 — SKU

30 Add 500 µL reagent to each standard, sample and blank, starting from blanks, including blank for standards and blank for samples.

Equipment

Finntip Stepper Tips — NAME

5 mL — TYPE

Thermo Scientific — BRAND

9404200 — SKU

Note

Before dispensing the reagent, wipe or dab the liquid drop on the outside of the tip, avoid wiping the open tip.

31 Vortex each tube.

32 Incubate at 37 °C for 03:00:00 while shaking at 200 rpm 3h

33 Load microplate with 250 uL reactant from each tube, duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	Samples and sample blanks: 40 with duplicate									
B	S2	S2										
C	S3	S3										
D	S4	S4										
E	S5	S5										
F	S6	S6										
G	S7	S7										
H	S8	S8										

Example of loading the microplate

Equipment

96-Well Microplates, Polystyrene, Clear,	NAME
Greiner Bio-One	BRAND
655101	SKU

34 Read plate in microplate reader

	A	B
	Shake duration	00:00:05
	Shaking type	Continuous
	Shaking force	High
	Shaking speed [rpm]	600
	Wavelength [nm]	820
	Use transmittance	No
	Pathlength correction	No
	Measurement Time [ms]	100

Equipment

Varioskan LUX Multimode Microplate Reader NAME

Thermo Fisher BRAND

VL0L00D0 SKU

Calculating

35 Subtract the average absorbance at 820 nm of the blank standard replicates from the absorbance at 820 nm of all other standard working solutions.

36 Subtract the average absorbance at 820 nm of the blank sample (i.e. blank filter) replicates from the absorbance at 820 nm of all other individual samples.

37 Prepare a standard curve by plotting the average blank-corrected 820 nm absorbance for each standard working solution versus its concentration in uM.

38 Use the standard curve to determine the orthophosphate concentration of each unknown sample by using its blank-corrected 820 nm absorbance.

39 (P per sample)_ug = (orthophosphate)_uM X (V_HCl)_mL X (0.001) X (30.97)

Citations

AntonioTovar-Sanchez, Sergio A Sañudo-Wilhelmy, Manuel Garcia-Vargas, Richard S Weaver, Linda C Popels, David A Hutchins. A trace metal clean reagent to remove surface-bound iron from marine phytoplankton
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P.S. Chen, T.Y. Toribara and Huber Warner. Microdetermination of Phosphorus
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