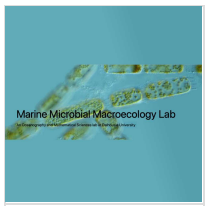

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Apr 12, 2022

An X-HTDC method for estimating particulate phosphorus from microalgae V.4

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protocol .

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Here we describe a protocol to estimate particulate phosphorus associated with microalgae using an extra-high temperature dry combustion method (X-HTDC). An 800°C-combustion can completely decompose intracellular macromolecules, improving estimates of particulate phosphorus relative to methods that use lower combustion temperatures.
We recommend microalgae be collected on polycarbonate filters and then combusted at 800 °C before digestion by 0.2 M HCl for 30 minutes at 90°C. The resulting orthophosphate is detected by mixing the digested sample with a mixture of molybdate and ascorbic acid to produce molybdenum blue (Chen 1956).
The working range of this assay is 0.074 to 200 uM orthophosphate. Minimum sampling volume (mL)=130/Chl-a_ug/L.
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.

P.S. Chen, T.Y. Toribara and Huber Warner. Microdetermination of Phosphorus. Anal. Chem..
https://doi.org/10.1021/ac60119a033

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

Yingyu Hu, Zoe V. Finkel 2022. An X-HTDC method for estimating particulate phosphorus from microalgae. **protocols.io**
https://protocols.io/view/an-x-htdc-method-for-estimating-particulate-phosph-b7g3rjyn
Yingyu Hu

Crucible cleaning

particulate phosphorus, intracellular phosphorus, phosphomolybdenum-ascorbic reduction, orthophosphate, oxalate reagent, adsorbed phosphorus, X-HTDC, High temperature dry combustion

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

(1) Wash
- For new crucibles: Gently brush new crucibles with phosphate free detergent and then rinsed with reverse osmosis water. Do not soak crucibles in detergent due to the low resistance to alkaline solution.
- For crucibles after hydrolysis: Dump hydrolysate to soda solution for neutralization, rinse crucible with reverse osmosis water.

(2) Fill crucibles with 0.2 M HCl (about 80% full), put lids on, keep in the oven at **90 °C** for **00:30:00** .

(3) Collect acid in a bottle to reuse, rinse crucibles with reverse osmosis water and milliQ water for the last rinse.

(4) Try to drain water as much as possible, then cover the crucibles, dry in the oven completely.

(5) Combust dry crucibles at **500 °C** for **06:00:00** at ramp-rate **150 °C** /h. Some crucibles may shatter, but the remainders can intact after being combusted with samples at **800 °C** . Crucibles are numbered with pencil at the bottom of crucible cups.

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 (5 inches Hg).

Filter forceps
blunt end, stainless steel
Millipore **XX6200006P**

1.2 Rinse samples with filtered seawater

1.3 Place sample filters in 2 mL Cryogenic Vials.

Cryogenic Vials with Closures
Polypropylene, 2 mL
Corning® **66021-974**

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).

Filter forceps
blunt end, stainless steel
Millipore **XX6200006P**

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

Preparation of oxalate reagent
by Yingyu Hu,
Dalhousie University

PREVIEW RUN

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.

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.

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

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**

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

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

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**

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

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

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

Sterile Disposable Filter Units with PES Membrane	
Thermo Scientific™ Nalgene™ Rapid-Flow™	**5964 520**

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.

Cryogenic Vials with Closures Polypropylene, 2 mL	
Corning®	**66021-974**

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

Porcelain crucibles
40 mL
VWR 89037-996

Crucible cover
VWR 71000-146

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.

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.

Forced air oven
VWR 89511-410

Remove samples out of the oven as soon as they are dried. If muffle furnace is not

available, remove crucibles to vacuum desiccator.

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

Muffle furnace
F30428C
Thermo **10-505-13**

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

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 **Contributed by users**

Volume of HCl 0.2M, mL = (5 mL) X (#Sample + #Blank)

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 oven for **00:30:00** 30m

16 Cool samples down to **Room temperature**

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

Maxymum Recovery® Snaplock Microcentrifuge Tube
2.0 mL, Polypropylene, Clear, Nonsterile,
Axygen® **MCT-200-L-C**

Preparing standard working solutions 2h

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

19 KH_2PO_4 primary standard stock solution (≈ 1 mM)

Potassium dihydrogen orthophosphate **ACP**

Chemicals Catalog #P-4550

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

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

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

19.4 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

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

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

20 Standard working solution

KH2PO4	Primary (ul)	MilliQ (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

21 Transfer 500 ul of each standard working solution to 2 mL microtube.

Preparing working reagents 2h

22

All reagents are freshly prepared before colorimetric measurement.

23 [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 **Contributed by users**

24 [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 **Sigma Aldrich Catalog #09878-100G**

25 [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 **Sigma Aldrich Catalog #A5960-100G**

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

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

27 Mix the reagents into Falcon tube:

A	B
Reagent	Part(s) as in volume
MilliQ	2
6N sulphuric acid	1
2.5% ammonium molybdate	1
10% ascorbic acid	1

Colorimetric measurement 2h

28 Preheat incubator/shaker to **37 °C**

SHAKING INCUBATOR
71L
Corning® LSE™ **6753**

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

Finntip Stepper Tips
5 mL
Thermo Scientific **9404200**

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

30 Vortex each tube.

31 Incubate at **37 °C** for **03:00:00** while shaking at 200 rpm

3h

32 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

96-Well Microplates, Polystyrene, Clear,

Greiner Bio-One **655101**

33 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

Varioskan LUX Multimode Microplate Reader

Thermo Fisher **VL0L00D0**

Calculating

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

35 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.

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

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

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