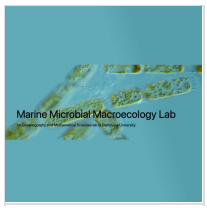

5

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

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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.

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Yingyu Hu

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

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60447

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

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).

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

1.2 Rinse samples with filtered seawater

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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

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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.

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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.

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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

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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

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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

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**

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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.

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

Preparing standard working solutions 2h

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

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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.

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

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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

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

Preparing working reagents 2h

23

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 **Contributed by users**

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 **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.

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Ascorbic acid **Sigma**

Aldrich Catalog #A5960-100G

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
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**

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

30 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**

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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

96-Well Microplates, Polystyrene, Clear,

Greiner Bio-One **655101**

34 Read plate in microplate reader

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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

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)

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