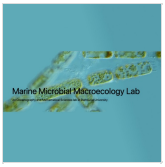

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X-HTDC method for better estimation of particulate phosphorus in microalgae

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1 *Works for me* This protocol is published without a DOI.

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ABSTRACT

Here we describe a protocol using extra-high temperature dry combustion method (X-HTDC) to estimate particulate phosphorus in microalgae. The 800°C-combustion can completely decompose intracellular macromolecules. Thus the estimation of intracellular particulate phosphorus can be increased 10% compared with traditional 500 °C-combustion; while the estimation of total particulate phosphorus can be increased about 5%. Microalgae is collected on PC filter and then combusted at 800 °C. Phosphate is then digested by 0.2 M HCl for 30 minutes incubated at 90°C. The resulted orthophosphate reacts with the mixture of molybdate and ascorbic acid to produce molybdenum blue (Chen 1956).
The working range of this assay is 0 ~ 200 uM orthophosphate. The recommended maximum phosphorus per sample is about 30 ug.
In order to assess the intracellular phosphorus quotas in microalgae, an oxalate reagent (Tovar-Sanchez 2003) is used to wash the sample 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

PROTOCOL CITATION

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https://protocols.io/view/x-htdc-method-for-better-estimation-of-particulate-bhjkj4kw

KEYWORDS

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

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SAFETY WARNINGS

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

ABSTRACT

Here we describe a protocol using extra-high temperature dry combustion method (X-HTDC) to estimate particulate phosphorus in microalgae. The 800°C-combustion can completely decompose intracellular macromolecules. Thus the estimation of intracellular particulate phosphorus can be increased 10% compared with traditional 500 °C-combustion; while the estimation of total particulate phosphorus can be increased about 5%. Microalgae is collected on PC filter and then combusted at 800 °C. Phosphate is then digested by 0.2 M HCl for 30 minutes incubated at 90°C. The resulted orthophosphate reacts with the mixture of molybdate and ascorbic acid to produce molybdenum blue (Chen 1956).
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https://doi.org/10.1016/S0304-4203(03)00054-9

Collect sample

1 Total particulate phosphorus (i.e. intracellular phosphorus and adsorbed phosphorus)

1.1 Filter sample onto PC filters, using gentle vacuum pressure (5 inches Hg).

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

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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 through PC filter as blank.

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

2 Intracellular particulate phosphorus

2.1 Filter sample onto PC 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.

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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 burn 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 to 6~7 by using a transfer pipet

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

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2.2.14 After oxalic acid is completely dissolved, stop heating and let it cool to room temperature (tap water bath can 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.

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

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.

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

2.5 Filter blank media through PC filter as blank.

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

X-HTDC

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.

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** $MgSO_4$ to each sample and blank filter.

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

Forced air oven
VWR 89511-410

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.

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Ramp rate should be controlled at < **200 °C** /hour, otherwise the crucibles will shatter.

SP.RAT: 150
PAMPU: hour

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

10 Pencil mark 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.

Digest

11 **[M]0.2 M** HCl reagent:

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)

12 Preheat oven to **90 °C**

13 Add 2.5 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.

Prepare standard working solutions 2h

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

KH_2PO_4 primary standard stock solution (≈ 1 mM)

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19 ⊠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.

Prepare working reagents 2h

22 [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**

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23 [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

24 [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.

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

26 Mix the reagents into Falcon tube:

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

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

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

28 Add **500 µl** reagent to each standard, sample and blank

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 tip open.

29 Vortex each tube.

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

3h

31 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										

96-Well Microplates, Polystyrene, Clear,
Greiner Bio-One **655101**

32 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**

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Calculate

33 Subtract the average absorbance at 820 nm of the blank standard replicates from the absorbance at 820 nm of all other individual standard working solution.

34 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 sample.

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

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

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

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