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Extraction and Measurement of Polyphosphate and Inorganic Phosphorus from Microalgae Samples

 Forked from [DAPI-Based Polyphosphate Estimation with Extraction Sufficiency Validation: A Method for Quantifying Polyphosphate from Microalgae Samples](#)

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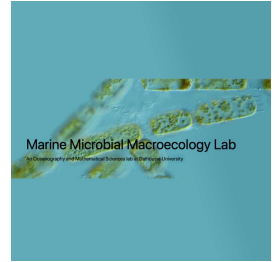
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We use this protocol and it's working

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Abstract

Polyphosphate and inorganic phosphate are extracted by hot Tris buffer. After centrifugation, 500 uL of extract is used for inorganic phosphate measurement, 200 uL of extract is used for polyP extraction sufficiency test.

Testing the number of treatment rounds necessary to extract all polyP is crucial. However, even when collecting samples from the same field location or cultivation condition, there can be high variability in treatment rounds among replicates, leading to significant background fluorescence and rendering the polyP from the sample undetectable. This challenge is especially prominent when measuring polyP from field samples. Limited sample availability and insufficient polyP extraction, combined with high background fluorescence, make the laborious measurement unpredictable and hinder accurate polyP measurement. This obstacle is a significant hurdle in polyP measurement. In our assay, we overcome the challenge by validating the sufficiency of extraction for each sample and then measuring the polyP values.

The utilization of DAPI-based fluorometric estimation for polyphosphate (polyP) analysis from microalgae involves evaluating the fluorescence of DAPI-stained samples in quartz cuvettes using a spectrofluorometer. To reduce the consumption of reagent, time, and labor while minimizing DAPI photobleaching, we have adapted this method to a 96-well black microtiter plate with a black film-covered lid. Additionally, the calculation method has been modified to account for matrix effects in microplates.

To conduct the polyP assay, roughly 400 uL RNase, 400 uL DNase, and 700 uL proteinase are required for four samples.

Inorganic P is measured by molybdate/ascorbic acid/sulphuric acid reagent following Chen et al. (1956).

Citation

P. S. Chen, T. Y. Toribara, and Huber. Warner (2023). Microdetermination of Phosphorus. Anal. Chem..

<https://doi.org/10.1021/ac60119a033>

LINK



Citation

Martin, Patrick & Van Mooy, Benjamin (2026)

. Fluorometric Quantification of Polyphosphate in Environmental Plankton Samples: Extraction Protocols, Matrix Effects, and Nucleic Acid Interference.

Applied and Environmental Microbiology.

<http://doi.org/10.1128/AEM.02592-12>

LINK

Guidelines

1. Extracted polyphosphate must be measured on the same day. Polyphosphate loss has been observed if the extraction is processed days after.
2. The polyphosphate standard aliquot can only be thawed and used once. Do not refrozen and thawed multiple times.
3. Inorganic phosphate extract can be stored in the fridge and measured the next day.

Materials

Chemicals

☒ Tris Buffer 1M pH 7.0 **Fisher Scientific Catalog #BP1756-500**

☒ Sodium phosphate glass type 45 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S4379-500MG**

☒ Proteinase-K **Fisher Scientific Catalog #BP1700-500**

☒ RNase A: 500 U/mL; RNase T1: 20000 U/mL **Fisher Scientific Catalog #AM2288**

☒ TURBO DNase 2 U/μL **Fisher Scientific Catalog #AM2239**

☒ DAPI: 4'6-Diamidino-2-phenylindole dihydrochloride **Fisher Scientific Catalog #D1306**



Protocol materials

✕ RNase A: 500 U/mL; RNase T1: 20000 U/mL **Fisher Scientific Catalog #AM2288**

✕ TURBO DNase 2 U/μL **Fisher Scientific Catalog #AM2239**

✕ DAPI: 4'6-Diamidino-2-phenylindole dihydrochloride **Fisher Scientific Catalog #D1306**

✕ Tris Buffer 1M pH 7.0 **Fisher Scientific Catalog #BP1756-500**

✕ Sodium phosphate glass type 45 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S4379-500MG**

✕ Proteinase-K **Fisher Scientific Catalog #BP1700-500**

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

✕ Ammonium molybdate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #09878-100G**

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

Sample collection

- 1 Filter microalgae in liquid media onto GF/F or PC filters, using gentle vacuum pressure (130 mmHg).

Equipment

Filter forceps

NAME

blunt end, stainless steel

TYPE

Millipore

BRAND

XX6200006P

SKU

- 2 Rinse sample with filtered saline (no nutrients)
- 3 Place sample filters in cryogenic vials
- 4 Filter same volume of the blank media (without cells) through GF/F or PC filter as blank.
- 5 Flash freeze filters and stored at  -80 °C
- 6 Freeze dry before measurement.



Equipment

FreeZone® 2.5 L Benchtop Freeze Dryers

NAME

Labconco®

BRAND

700202000

SKU

Preparation of reagents

7 Tris buffer [M] 20 Mass Percent  7.0

Note

Budget:
About 250 mL per four samples

7.1 In a 1 L volumetric flask, top  20 mL [M] 1 Mass Percent  7.0 Tris buffer to 1 L with MilliQ

7.2 Store at  Room temperature

8 PolyP primary standard stock

8.1 Weigh one glass pellet of polyP (45) and write down the weight.



Equipment

Microbalance

NAME

Cubis series

TYPE

Sartorius

BRAND

MSE6.6S-000-DM

SKU

8.2 Transfer the pellet into a 100 mL graduated cylinder.

8.3 Dilute to 100 mL with Tris IMJ 20 Mass Percent pH 7.0

8.4 Aliquot primary stock into 10~50 uL per microtube with Stepper and store at 🌡️ -20 °C

9 PolyP secondary standard stock

If the pellet is far more than 10 mg, dilute primary to secondary to bring down the concentration before preparing working standard

10 Proteinase K IMJ 20 Mass Percent

10.1 Add 🧪 25 mL MilliQ directly into the original package of Proteinase K, vortex to mix

10.2 Aliquot 700 uL to microtubes and keep frozen at 🌡️ -20 °C

11 DAPI primary stock IMJ 14.3 Mass Percent

Add 🧪 2 mL MilliQ directly into the original package and keep frozen at 🌡️ -20 °C

Preliminary extraction efficiency test

12 Prepare boiling bath.

Equipment

VWR® Advanced Hot Plates	NAME
VWR	BRAND
97042-658	SKU

Equipment

Hollow Polypropylene (PP) Ball Bath Covers, 20 mm	NAME
Cole-Parmer	BRAND
UZ-06821-04	SKU

Equipment

Tube rack	NAME
Simport MultiRack™	BRAND
CA48648-606	SKU

13 Prepare  37 °C incubator/shaker.



14 Transfer sample into glass centrifuge tube

Equipment

Disposable Glass Screw-Cap Centrifuge Tubes^{NAME}

10 mL

TYPE

Corning®

BRAND

99502-10

SKU

15 Label centrifuge tube for different samples, place one Pasteur pipet into the tube for transferring extract from the same sample

16 Label 15 mL Falcon tube from 1 to 15 for each one sample.

17 Add  4 mL Tris buffer [M] 20 Mass Percent  7.0 , vortex and then sonicate.

15s

Equipment

Specific Pipette Tips 5mL

NAME

Thermo Scientific™ Finntip™

BRAND

21-377-304

SKU

18 Keep in boiling bath.

5m

**Note**

Make sure the tube rack is in the middle of the boiling bath and covered with PP balls. Tris solution in the tube should be boiling during the 5 minutes' incubation.

19 Sonicate

15s

20 Vortex and then transfer extract to 15 mL Falcon tube, according to the extract number.

Note

Do not push filter to the bottom. Use Pasteur pipet, gently lift the filter upwards, and then transfer as much extract as possible. Gently press the extract out of the filter.

Equipment

Disposable Soda-Lime Glass Pasteur Pipets

NAME

5 3/4"

TYPE

Fisherbrand

BRAND

13-678-6A

SKU

21 Repeat Step 17 to Step 20 until complete 15 times' extraction in total.

22 Centrifuge the extract

5m

3200 rpm, Room temperature, 00:05:00

23 Transfer 500 μ L supernatant to a 2 mL microtube for Inorganic P measurement.

Label each tube as

1-1, 1-2, ..., 1-N

2-1, 2-2, ..., 2-N

3-1, 3-2, ..., 3-N



4-1, 4-2, ..., 4-N

- 24 Use forward pipetting, load black microtitre plate with  200 μL supernatant from the extract (one well for one extract, no need to load replicates).

Tris buffer [M] 20 Mass Percent  7.0 is used as blank.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1-1	1-9	2-1	2-9	3-1	3-9	4-1	4-9				
B	1-2	1-10	2-2	2-10	3-2	3-10	4-2	4-10				
C	1-3	1-11	2-3	2-11	3-3	3-11	4-3	4-11				
D	1-4	1-12	2-4	2-12	3-4	3-12	4-4	4-12				
E	1-5	1-13	2-5	2-13	3-5	3-13	4-5	4-13				
F	1-6	1-14	2-6	2-14	3-6	3-14	4-6	4-14				
G	1-7	1-15	2-7	2-15	3-7	3-15	4-7	4-15				
H	1-8	Tris	2-8		3-8		4-8					

Equipment

96-Well Black Microplates

NAME

Polystyrene

TYPE

Greiner Bio-One

BRAND

655076

SKU

- 25 Prepare DAPI working solution [M] 100 Mass Percent

Dilute  12.6 μL of [M] 14.3 Mass Percent DAPI stock with  1800 μL MilliQ in a foil wrapped microtube and vortex.

- 26 In the dimmed room with only red light bulb on add  24 μL [M] 100 Mass Percent DAPI to each sample in the plate.



- 27 Adhere black film on the top of a microplate lid and cover the plate with this lid.

Equipment

Black Vinyl Films for Fluorescence and Photoprotection NAME

VWR

BRAND

89087-692

SKU

- 28 Shake at room temperature for  00:07:00

7m

- 29 Read fluorescence: excitation at 410 nm and emission at 550 nm

Equipment

Varioskan LUX Multimode Microplate Reader NAME

Thermo Fisher

BRAND

VL0L00D0

SKU

- 30 Plot fluorescence intensity versus number of extraction.
The number of extract (N) is the stationary point where the fluorescence of stained extract stops decreasing or the derivative of the fluorescence after that point is close to zero.



If $RFU(15) - RFU(Tris) > 1$, proceed to extract five additional times. And then measure the stained extract following the previous steps.

- 31 Combine Extraction 1 to Extraction N into a falcon tube.

Note

Try to transfer all solution including debris from each tube.
If the total volume is over 50 mL, use a beaker instead.

	Sample code	N	V(Tris) per extract (mL)

- 32

$$C_{extract} = \frac{\sum_{i=1}^N C_i V_i}{V_T} = \frac{\sum_{i=1}^N C_i \times V_{Tris}}{N \times V_{Tris}} = \frac{\sum_{i=1}^N C_i}{N}$$

$$C'_{extract} = \frac{\sum_{i=1}^N C_i (V_i - V)}{V_T - N \times V} = \frac{\sum_{i=1}^N C_i \times (V_{Tris} - V)}{N \times V_{Tris} - N \times V} = \sum_{i=1}^N C_i \times \frac{V_{Tris} - V}{N \times V_{Tris} - N \times V} = \frac{\sum_{i=1}^N C_i}{N}$$

$\Rightarrow C_{extract} = C'_{extract}$ Assuming the volume of extract from each vial is precisely removed for preliminary test.

Enzyme treated extract

- 33 Well mix 1~N extract, transfer 12 mL into 15 mL falcon tube, centrifuge

3200 rpm, Room temperature, 00:05:00

5m



34 Transfer  1.8 mL supernatant to a 2 mL tube (Set S).

Note

Sample is triplicated into S1a, S1b and S1c; S2a, S2b, S2c...etc.

35 Centrifuge extract "N+1"  3200 rpm, Room temperature, 00:05:00

5m

Note

Blank is duplicated into B1a and B1b; B2a and B2b... etc.

36 Transfer  1.5 mL supernatant into a 2 mL tube (Set B).

37 In Set S, add  18 μ L RNase and  18 μ L DNase

Note

RNase tends to leave residue in the tip. However one package has only 1 mL RNase, it will be a waste to use reverse pipetting. After dispensing RNase into the vial, use the same tip to draw the solution and gently dispense it back into the solution for about three time, so that there is no residue remaining in the tip. Replace a new tip for the next vial.

Note

Require ~400 μ L RNase and ~400 μ L DNase.

38 In Set B, add  15 μ L RNase and  15 μ L DNase

39 Incubate at  37 $^{\circ}$ C , shake continuously

10m



Equipment

SHAKING INCUBATOR

NAME

71L

TYPE

Corning® LSE™

BRAND

6753

SKU

Note

Start the timer when temperature reaches  37 °C

40 Thaw proteinase (~700uL)

41 In Set S, add  36 µL Proteinase

42 In Set B, add  30 µL Proteinase

43 Incubate at  37 °C , shake continuously.

30m

Note

Start the timer when temperature reaches  37 °C

Enzyme treated standard amended extract

44 Prepare PolyP working standard [PO3]~  7.6 Mass Percent



Based on the actual concentration of PolyP (45) primary or secondary standard stock, dilute a certain volume of stock with Tris buffer [M] 20 Mass Percent  7.0

For a final concentration [M] 7.6 Mass Percent

Note

Total volume = 160 X N (ul), where N = sample number

Note

FW(45Na2O.55P2O5)=10600

Mol of PO3 per mol of PolyP (45) = 110

- 45 Transfer  840 μ L of enzyme treated extract (1~N) into 2 mL tubes (Set A).

Note

Forward pipetting, aspire and dispense for three times to mix.

- 46 Add  160 μ L [M] 7.6 Mass Percent polyP working standard to  840 μ L of enzyme treated extract, vortex.

- 47 Prepare DAPI working solution [M] 100 Mass Percent

Dilute  12.6 μ L of [M] 14.3 Mass Percent DAPI stock with  1800 μ L MilliQ in a foil wrapped microtube and vortex.

Load microtiter plate

7m

- 48 Load  200 μ L blanks (B: N+1), samples (S: 1~N) and amended samples (A: Amended 1~N) to the microplate. Organize samples as shown in the following scheme:

	1	2	3	4	5	6	7	8	9	10	11	12
A	B1a	B1a	B2a	B2a	B3a	B3a	B4a	B4a	B1a (UN)	B2a (UN)	B3a (UN)	B4a (UN)
B	B1b	B1b	B2b	B2b	B3b	B3b	B4b	B4b	B1b (UN)	B2b (UN)	B3b (UN)	B4b (UN)
C	S1a	S1a	S2a	S2a	S3a	S3a	S4a	S4a	S1a (UN)	S2a (UN)	S3a (UN)	S4a (UN)
D	S1b	S1b	S2b	S2b	S3b	S3b	S4b	S4b	S1b (UN)	S2b (UN)	S3b (UN)	S4b (UN)
E	S1c	S1c	S2c	S2c	S3c	S3c	S4c	S4c	S1c (UN)	S2c (UN)	S3c (UN)	S4c (UN)
F	A1a	A1a	A2a	A2a	A3a	A3a	A4a	A4a	A1a (UN)	A2a (UN)	A3a (UN)	A4a (UN)
G	A1b	A1b	A2b	A2b	A3b	A3b	A4b	A4b	A1b (UN)	A2b (UN)	A3b (UN)	A4b (UN)
H	A1c	A1c	A2c	A2c	A3c	A3c	A4c	A4c	A1c (UN)	A2c (UN)	A3c (UN)	A4c (UN)

Note

Reverse pipetting

- 49 In a dimmed room with only red bulb on, add  24 µL DAPI working solution [M] 100 Mass Percent to each sample in the microplate **except for those labelled with (UN)**.

Note

Forward pipetting

- 50 Adhere black film on the top of a microplate lid and cover the plate with this lid.

- 51 Shake at room temperature for  00:07:00

7m

- 52 Shake duration: 1 min
Shaking type: continuous



Shaking speed and force: 600 rpm/High

Fluorescence: excitation at 410 nm and emission at 550 nm

Measurement time: 300 ms

Excitation bandwidth: 5 nm

Calculation

53

In the 1~N extraction	In the N+1 extraction	In the amended 1~N extraction
$PolyP_{1\sim N}(uM) = \frac{\sum_{i=1}^N C_i \times V}{V \times N} = \frac{1}{N} \sum_{i=1}^N C_i$ $Blank_{1\sim N}(uM) = \frac{\sum_{i=1}^N C_{bi} \times V}{V \times N} = \overline{C_{bi}}$	$Blank_{N+1}(uM) = C_{b(N+1)}$	$PolyP_{amend}(uM) = \frac{840}{1000} \times \frac{1}{N} \sum_{i=1}^N C_i$ $Blank_{amend}(uM) = \frac{840}{1000} \times \overline{C_{bi}}$
Tube set S: 1.8 mL extract 18 uL RNase 18 uL Dnase 36 uL Proteinase	Tube set B: 1.5 mL extract 15 uL RNase 15 uL Dnase 30 uL Proteinase	Tube set A: 840 uL from Tube set S 160 uL ~ 2 nmol standard
SignalA(DAPI) SignalA(unstained)	SignalB(DAPI) SignalB(unstained)	SignalA(DAPI+std) SignalA(+std, unstained)

$$Signal(extract + enzyme) = [SignalA(DAPI) - SignalB(DAPI)] - [SignalA(unstained) - SignalB(unstained)]$$

$$Signal(extract + enzyme + std) = \left[SignalA(DAPI + std) - \frac{840}{1000} \times SignalB(DAPI) \right] - \left[SignalA(+std, unstained) - \frac{840}{1000} \times SignalB(unstained) \right]$$

$$Signal(std) = Signal(extract + enzyme + std) - \frac{840}{1000} \times Signal(extract + enzyme)$$

54

$$Conc(std)_{uM} = \frac{160}{1000} \times C_{PO3_2nd}$$

$$\frac{Signal(std)}{Conc(std)} = \frac{Signal(extract + enzyme)}{Conc(extract + enzyme)}$$

$$\Rightarrow Conc(extract + enzyme)_{uM} = \frac{Signal(extract + enzyme)}{Signal(std)} \times Conc(std)$$

$$Conc(extract)_{uM} = Conc(extract + enzyme) \times \frac{1800 + 18 + 18 + 36}{1800}$$

$$polyP_{umol/filter} = Conc(extract) \times 0.001 \times V_{Tris/extraction_mL} \times N_{extraction}$$

$$NaPO3_{ug/filter} = polyP_{umol/filter} \times 101.96$$



Inorganic P measurement

5h

55 KH_2PO_4 primary standard stock solution (≈ 1 mM)

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

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

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

2h

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

55.4 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

55.5 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.

56 Standard working solution

	KH_2PO_4	Primary (uL)	MilliQ (uL)
	S1	0	1000
	S2	5	995
	S3	10	990
	S4	20	980
	S5	50	950
	S6	100	900



	KH ₂ PO ₄	Primary (uL)	MilliQ (uL)
	S7	150	850
	S8	200	800

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

58 Remove extract from the fridge and warm up to room temperature.

59 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**

60 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**

61 6 N (3 M) sulfuric acid reagent:
Carefully add 1 part 18 M concentrated sulfuric acid into 5 part MilliQ water

Note

Save the pipet tip for final reagent preparation.

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

63 Mix the reagents into Falcon tube:

	Reagent	Parts in volume
	MilliQ	2



Reagent	Parts in volume
6N sulphuric acid	1
2.5% ammonium molybdate	1
10% ascorbic acid	1

64 Preheat incubator/shaker to 37 °C

65 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.

66 Vortex each tube

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

3h

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

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

Example of loading the microplate

69 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

70 Calculation

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

70.2 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.

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



- 70.4 Use the standard curve to determine the orthophosphate concentration of each unknown sample by using its blank-corrected 820 nm absorbance.
- 70.5 22.5% polyP can be measured by molybdate assay, possibly due to the partial hydrolysis in acid condition

$$Pi_{\mu mol} = \sum_{j=1}^n (p_{j_{\mu M}} \times V_j) - 0.225 \times polyP_{\mu mol}$$

Where, $Pi_{\mu mol}$ is the concentration of inorganic P, $P_{j_{\mu M}}$ is inorganic P from each extract, V_j is the volume of Tris, n is the total extraction times, $polyP_{\mu mol}$ is the polyP concentration in the sample.

Citations

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

Martin, Patrick & Van Mooy, Benjamin. Fluorometric Quantification of Polyphosphate in Environmental Plankton Samples: Extraction Protocols, Matrix Effects, and Nucleic Acid Interference
<http://doi.org/10.1128/AEM.02592-12>