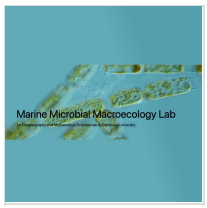

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Apr 04, 2022

Inorganic polyphosphate from microalgae: A DAPI-based estimation in microtiter plate V.6

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protocol .

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The DAPI-based fluorometric estimation of polyphosphate from microalgae has been widely used in field samples since the method was published by Martin P. et al., where fluorescence of DAPI-stained samples is analyzed in quartz cuvettes by spectrofluorometer. In order to minimize the photobleaching of DAPI and reduce the consumption of reagent, time and labor, we have now scaled this method to 96-well black microtiter plate. Regarding to the matrix effects in microplate, the calculation has been modified accordingly.
Our method permits processing nine samples by using only 250 uL of extracted sample, 500 uL of RNase, 500 uL of DNase, 1000 uL of proteinase and <2000 uL of DAPI (100 uM). A lid with black film can protect all DAPI-stained samples from photobleaching.

Martin, Patrick & Van Mooy, Benjamin. 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

Yingyu Hu, Zoe V Finkel 2022. Inorganic polyphosphate from microalgae: A DAPI-based estimation in microtiter plate . **protocols.io**
https://protocols.io/view/inorganic-polyphosphate-from-microalgae-a-dapi-bas-b64brgsn
Yingyu Hu

DAPI, polyphosphate, microtiter plate, microplate, microalgae, fluorescence

protocol ,

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1. Total particulate phosphorus (TPP) measurement is recommended prior to the extraction of polyphosphate. The level of TPP helps to estimate the volume of extraction solution.
2. Different species or different sample locations (for field samples) require different numbers of extraction. A preliminary extraction efficiency test helps to obtain optimized number of extraction for extracting the most amount of polyphosphate with the least number of extraction.
3. Extracted polyphosphate must be measured on the same day. Polyphosphate loss has been observed if the extraction is processed days after.
4. The polyphosphate standard aliquot can only be thawed and used once. Do not refrozen and thawed multiple times.

Chemicals

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

Sodium phosphate glass type 45 **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/uL **Fisher Scientific Catalog #AM2239**

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

Budget of enzyme for every nine samples

- RNase A (AM2288): half package
- DNase (AM2239): one package
- Proteinase-K: two tubes of aliquot (about 600 uL/tube)

Sample collection

1 Filter microalgae in liquid media onto precombusted GFF filters, using gentle vacuum pressure (5 inches Hg).

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

2 Rinse sample with filtered seawater

3 Place sample filters in cryogenic vials

4 Filter blank media (without cells) through precombusted GFF filter as blank.

5 Flash freeze filters and stored at **-20 °C**

6 Freeze dry before measurement.

FreeZone® 2.5 L Benchtop Freeze Dryers
Labconco® **700202000**

Preparation of reagents

7 Tris buffer **20 mM** **7.0**

Budget:
About 400 mL per nine samples

7.1 In a 1 L volumetric flask, top **20 mL** **1 M** **7.0** Tris buffer to 1 L with MilliQ

7.2 Filter through Rapid-flow and store at **Room temperature**

If Tris buffer is to be used right away, this step is not necessary.

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

8 PolyP primary standard stock

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

Microbalance
Cubis series
Sartorius **MSE6.6S-000-DM**

8.2 Transfer the pellet into a 100 mL graduated cylinder.

8.3 Dilute to 100 mL with Tris [M]**20 mM** 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 [M]**20 mg/ml**

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

10.2 Aliquot 600 uL to microtubes (around 45 microtubes) and keep frozen at

-20 °C

11 DAPI primary stock [M]14.3 mM

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

Preliminary extraction efficiency test

12 Prepare boiling bath.

VWR® Advanced Hot Plates
VWR 97042-658

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

Tube rack
Simport MultiRack™ CA48648-606

13 Transfer sample into glass centrifuge tube.

Disposable Glass Screw-Cap Centrifuge Tubes
10 mL
Corning® 99502-10

14 If the sample has less than 3 ug total particulate phosphate, use **2 mL** Tris Buffer **20 mM** pH **7.0** for each extraction.
Otherwise, use **4 mL** Tris Buffer **20 mM** pH **7.0** for each extraction.

15 Add **2 mL** or **4 mL** Tris buffer **20 mM** pH **7.0** , vortex and then sonicate. 15s

Specific Pipette Tips 5mL
Thermo Scientific™ Finntip™ 21-377-304

16 Keep in boiling bath. 5m

17 Sonicate 15s

18 Vortex and then transfer extract to a 20 mL scintillation vial, label the vial with number of extraction.

Disposable Soda-Lime Glass Pasteur Pipets
5 3/4"
Fisherbrand **13-678-6A**

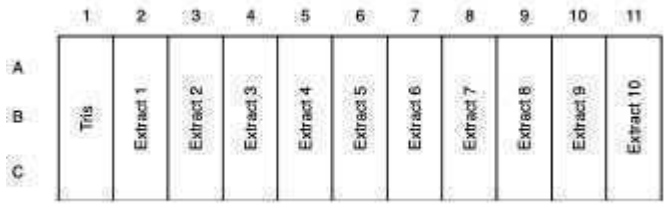
VWR® Vials, Borosilicate Glass, with Phenolic Screw Cap
22.18 mL
VWR **66012-044**
24-400 cap: VWR 89076-764

19 **Repeat until total extract number reaches 10.**

20 Transfer 2 mL of extract to a 2 mL microtube.
13300 rpm, Room temperature, 00:05:00

21 Load black microtitre plate with **250 µL** extract (triplicate).
Tris buffer **20 mM** **pH 7.0** is used as blank.

96-Well Black Microplates
Polystyrene
Greiner Bio-One **655076**



22 Prepare DAPI working solution **100 uM**
Dilute **2.1 µL** of **14.3 mM** DAPI stock with **300 µL** MilliQ in a foil wrapped microtube and vortex.
Volume of total 100 uM DAPI = 30 X (10 X 3 X N + 3), where N is the number of culture samples tested.

23 In the dimmed room with only red light bulb on, by using either stepper or pipette, add **30 µL** **100 uM** DAPI to each sample in the plate.

Finntip™ Stepper Pipette Tips
500 uL
Thermo Scientific™ **9404170**

Finnpipette Stepper Pipette
Thermo Scientific™ **4540000**

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

Black Vinyl Films for Fluorescence and Photoprotection
VWR **89087-692**

25 Shake at room temperature 7m

26 Read fluorescence: excitation at 410 nm and emission at 550 nm

Varioskan LUX Multimode Microplate Reader
Thermo Fisher **VL0L00D0**

27 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.

Extraction of polyphosphate from samples

28 Prepare boiling bath. 45m

29 Prepare **37 °C** incubator.

30 Transfer samples into glass centrifuge tubes.

31 Add same amount of Tris buffer **20 mM** **7.0** as preliminary test, vortex and then sonicate 15s

32 Place vials in boiling bath 5m

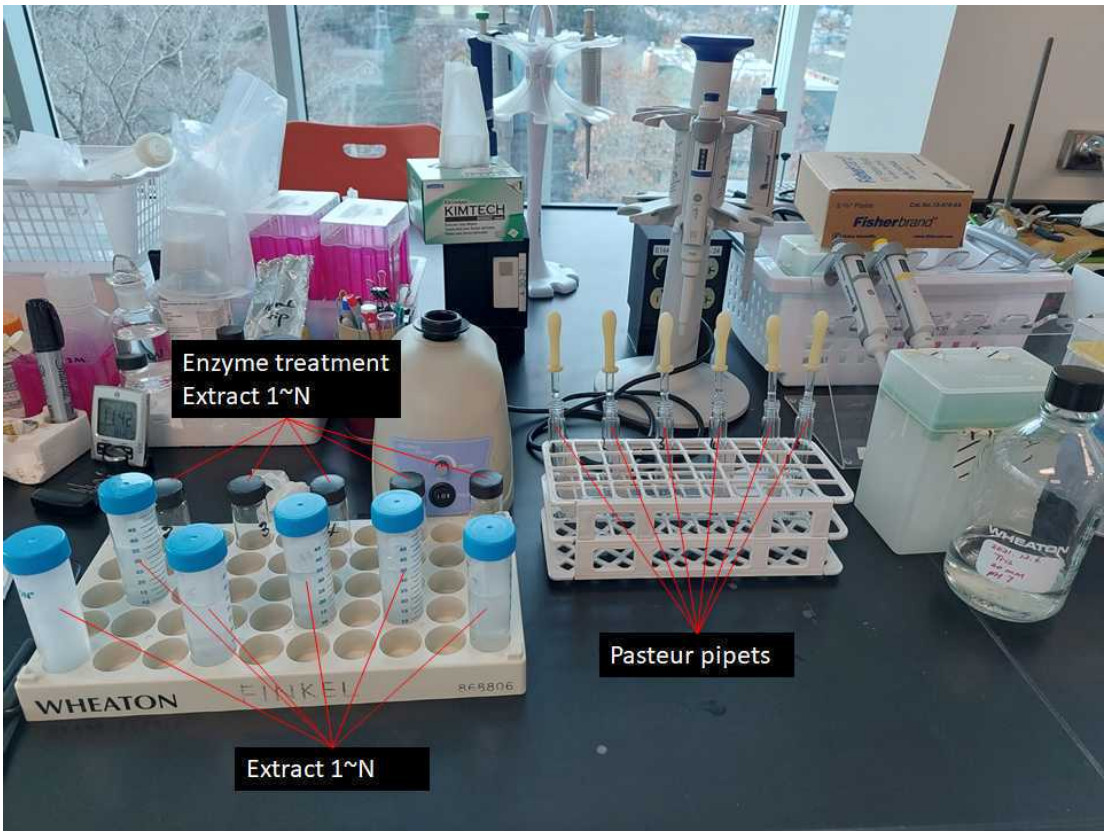
33 Sonicate 15s

34 Vortex and then remove extract to a 50 mL Falcon tube, and then **until total extract reaches N+1** .
Keep using the same pasteur pipet

Falcon® Centrifuge Tubes
Polypropylene, Sterile, 50 mL
Corning® **352070**

35 Combine extract 1~N into the same Falcon tube, keep extract N+1 in the centrifuge tube.

36



Main setup

Enzyme treated extract

37 Centrifuge the mixture of 1~N extract **3200 rpm, Room temperature, 00:05:00**

General-purpose benchtop centrifuge
IEC CENTRA CL2
Thermo **00427 0F**

38 Transfer **4 mL** supernatant to a scintillation vial, add **40 µL** RNase and **40 µL**

DNase

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.

39 Incubate at **37 °C** , shake continuously 10m

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

40 Add **80 µL** Proteinase

41 Incubate at **37 °C** , shake continuously. 30m

Enzyme treated N+1 extract

42 Centrifuge extract "N+1" (in the centrifuge tube)
3200 rpm, Room temperature, 00:05:00

43 Transfer **1.5 mL** supernatant into a 2 mL tube, add **15 µL** RNase and **15 µL** DNase

44 Incubate at **37 °C** , shake continuously 10m

Thaw proteinase during the 10-minute incubation.

45 Add **30 µL** Proteinase

46 Incubate at **37 °C** , shake continuously 30m

Enzyme treated standard amended extract

47

Prepare DAPI working solution **100 uM**

Dilute **2.1 µL** of **14.3 mM** DAPI stock with **300 µL** MilliQ in a foil wrapped microtube and vortex.
Total volume = 30 X 63 (ul) for one microplate
12.6 ul **14.3 mM** DAPI stock with **1800 µL** MilliQ

48 Prepare PolyP working standard **7.6 uM**

Based on the actual concentration of PolyP (45) primary or secondary standard stock, dilute a certain volume of stock with Tris buffer **20 mM** **7.0**

For a final concentration **7.6 uM**

Total volume = 320 X N (ul)
N = sample number

FW(45Na2O.55P2O5)=10600
Mol of PO3 per mol of PolyP (45) = 110

49 Load each enzyme treated sample **250 µL** (no need to have replicates) to microplate.

50 In a dimmed room with only red bulb on, add **30 µL** DAPI working solution **100 uM** to each sample in the microplate

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

52 Shake at room temperature. 7m

53 Read fluorescence: excitation at 410 nm and emission at 550 nm

54 Transfer **1680 µL** of enzyme treated extract into a scintillation vial.

Reverse pipetting

If fluorescence of the enzyme treated samples is higher than 5, dilute samples into 50% or more with Tris buffer until the estimated fluorescence is lower than 5 (this dilution factor must be added into the final calculation).

55 Add **320 µL** **[M]7.6 uM** polyP working standard to **1680 µL** of enzyme treated extract, vortex.

Load microtiter plate

56 Load **250 µL** 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	B1	B2	B3	S1	S2	S3	A1	A2	A3	B1	B2	B3
B	B1	B2	B3	S1	S2	S3	A1	A2	A3	B4	B5	B6
C	B4	B5	B6	S4	S5	S6	A4	A5	A6	B7	B8	B9
D	B4	B5	B6	S4	S5	S6	A4	A5	A6	S1	S2	S3
E	B7	B8	B9	S7	S8	S9	A7	A8	A9	S4	S5	S6
F	B7	B8	B9	S7	S8	S9	A7	A8	A9	S7	S8	S9
G												
H	A1	A2	A3	A4	A5	A6	A7	A8	A9			

Reverse pipetting

If samples have been diluted in **go to step #54** , load diluted samples in this step

57 In a dimmed room with only red bulb on, add **30 µL** DAPI working solution **100 uM** to each sample in the microplate **except for those labelled with UN**.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B1	B2	B3	S1	S2	S3	A1	A2	A3	B1 (UN)	B2 (UN)	B3 (UN)
B	B1	B2	B3	S1	S2	S3	A1	A2	A3	B4 (UN)	B5 (UN)	B6 (UN)
C	B4	B5	B6	S4	S5	S6	A4	A5	A6	B7 (UN)	B8 (UN)	B9 (UN)
D	B4	B5	B6	S4	S5	S6	A4	A5	A6	S1 (UN)	S2 (UN)	S3 (UN)
E	B7	B8	B9	S7	S8	S9	A7	A8	A9	S4 (UN)	S5 (UN)	S6 (UN)
F	B7	B8	B9	S7	S8	S9	A7	A8	A9	S7 (UN)	S8 (UN)	S9 (UN)
G												
H	A1 (UN)	A2 (UN)	A3 (UN)	A4 (UN)	A5 (UN)	A6 (UN)	A7 (UN)	A8 (UN)	A9 (UN)			

B: N+1
S: 1~N
A: Amended sample

Use stepper to dispense DAPI is faster but show bigger deviation.

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

59 Shake at room temperature. 7m

60 Read fluorescence: excitation at 410 nm and emission at 550 nm

Calculation

61 Definition of symbol

Symbol	Definition
M	Concentration of standard in one microplate well (nmol) $M = 7.6 \times (\frac{160}{1000}) \times 0.25 \times 0.001 \times 1000$
DF_{amend}	Dilution factor in amended extract $V_{extract}/(V_{extract} + V_{std})$
N	extraction times
V_{Tris}	Volume of Tris per extraction (mL)
V_s	Volume of sample loaded in microplate, $V_s = 0.25\ mL$
MW	Molecular weight of polyP, i.e. 101.9617
DF_{enzyme}	Dilution factor in enzyme treatment $DF_{enzyme} = (4 + 0.04 + 0.04 + 0.08)/4$

62

Signal	Color of wells
$SignalA(DAPI)$	
$SignalA(DAPI + std)$	
$SignalB(DAPI)$	
$SignalA(unstained)$	
$SignalA(+std,\ unstained)$	
$SignalB(unstained)$	

63

$$Signal(polyP) = [SignalA(DAPI) - SignalB(DAPI)] - [SignalA(unstained) - SignalB(unstained)]$$

64

$$Signal(polyP + std) = [SignalA(DAPI + std) - SignalB(DAPI)] - [SignalA(+std, unstained) - SignalB(unstained)]$$

65

$$polyP/well\_nmol = M \cdot DF_{amend} \cdot \frac{Signal(polyP)}{Signal(polyP+std) - DF_{amend} \cdot Signal(polyP)}$$

66

$$polyP/\text{filter\_}\mu g = 101.96 \cdot (0.001) \cdot V_{Tris} \cdot N \cdot DF_{enzyme} \cdot (polyP\ /\text{well})/V_s$$

If samples have been diluted in **go to step #54** , add dilution factor into the calculation.