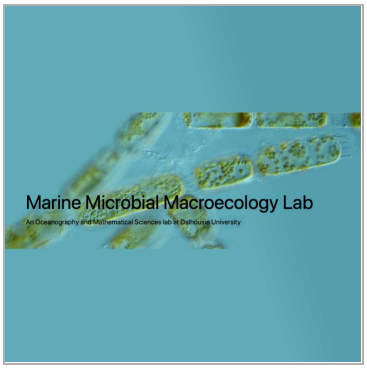

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DAPI-Based Polyphosphate Estimation with Extraction Sufficiency Validation: A Method for Quantifying Polyphosphate from Microalgae Samples

Ying-Yu Hu[1], Zoe V. Finkel[1]

[1]Dalhousie University

Marine Microbial Macroecology Lab
Tech. support email: **ruby.hu@dal.ca**

Ying-Yu Hu
Dalhousie University

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

ABSTRACT

The utilization of DAPI-based fluorometric estimation for polyphosphate (polyP) analysis from microalgae has become increasingly prevalent in field samples since its publication by Martin P. et al. This technique 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.

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.

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

CITATION

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.

LINK

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

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.

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/uL **Fisher Scientific Catalog #AM2239**
- DAPI: 4'6-Diamidino-2-phenylindole dihydrochloride **Fisher Scientific Catalog #D1306**

Sample collection

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

Equipment

Filter forceps	NAME
blunt end, stainless steel	TYPE
Millipore	BRAND
XX6200006P	SKU

2 Rinse sample with filtered artificial seawater (no nutrients)

3 Place sample filters in cryogenic vials

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

5 Flash freeze filters and stored at -20 °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 mM pH 7.0

Note

Budget:
About 250 mL per four samples

7.1 In a 1 L volumetric flask, top 20 mL [M] 1 M pH 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 [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 700 uL to 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.

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 mM pH 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 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 mM pH 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

24 Prepare DAPI working solution [M] 100 uM
Dilute 12.6 µL of [M] 14.3 mM DAPI stock with 1800 µL MilliQ in a foil wrapped microtube and vortex.

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

26 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

27 Shake at room temperature for 00:07:00 7m

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

Equipment

Varioskan LUX Multimode Microplate Reader	NAME
Thermo Fisher	BRAND
VL0L00D0	SKU

29 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.

30 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)

31

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

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

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

Enzyme treated extract

32 Well mix 1~N extract, transfer 12 mL into 15 mL falcon tube, centrifuge 5m

3200 rpm, Room temperature, 00:05:00

33 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.

34 Centrifuge extract "N+1" 3200 rpm, Room temperature, 00:05:00 5m

Note

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

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

36 In Set S, add 18 µL RNase and 18 µL 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 uL RNase and ~400 uL DNase.

37 In Set B, add 15 µL RNase and 15 µL DNase

38 Incubate at 37 °C, shake continuously 10m

Equipment

SHAKING INCUBATOR	NAME
71L	TYPE
Corning® LSE™	BRAND
6753	SKU

Note

Start the timer when temperature reaches 37 °C

39 Thaw proteinase (~700uL)

40 In Set S, add 36 µL Proteinase

41 In Set B, add 30 µL Proteinase

42 Incubate at 37 °C, shake continuously. 30m

Note

Start the timer when temperature reaches 37 °C

Enzyme treated standard amended extract

43 Prepare PolyP working standard [PO3]~ [M] 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 [M] 20 mM pH 7.0

For a final concentration [M] 7.6 uM

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

44 Transfer 840 µL of enzyme treated extract (1~N) into 2 mL tubes (Set A).

Note

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

45 Add 160 µL [M] 7.6 uM polyP working standard to 840 µL of enzyme treated extract, vortex.

46 Prepare DAPI working solution [M] 100 uM

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

Load microtiter plate

7m

47 Load 200 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	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

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

> Note
>
> Forward pipetting

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

50 Shake at room temperature for 00:07:00 7m

51 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

52

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

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

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

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

53

$$Conc(std)_{uM} = \frac{160}{1000} \times C_{PO3\_2nd}$$

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

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

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

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

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