Supporting Information for

Quantification of polyphosphate in environmental planktonic samples using a novel fluorescence dye JC-D7

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This 14-page document includes additional description of experiment details and methods (S1-S3), 5 supporting figures (Figs. S1- S5), 8 supporting tables (Tables S1-S8), and supporting references:

S1. Verification of polyP concentrations in standard solutions

S2. Preparation of exopolyphosphatase (PPX)

S3. Quantification of PO_4^{3-} after PPX enzymatic degradation of polyP

Figure S1 Fluorescence intensity (Fluo, in arbitrary unit) vs. polyP concentration for polyP of various chain lengths (n =14, 45, 60 and 130) under various concentrations of JC-D7 (20-40 μmol L^{-1}). PolyP is stained using JC-D7 solutions dissolved in 3% of DMSO and 12.5 mmol L^{-1} of HEPES buffer. Fluorescence measurements are measured under 405 nm excitation and 535 nm emission.

Figure S2 Comparing polyP quantified using PPX, DAPI, and JC-D7. PolyP was extracted by using the phenol-chloroform method from cultures of **A)** a wild-type strain of algae *Chlamydomonas reinhardtii* (polyP accumulating), treated using DNase and RNase for 30 minutes after extraction, **B)** similar to (A) but 2-hour DNase and RNase treatment, **C)** a *vtc-1* mutant strain of algae *Chlamydomonas reinhardtii* (not accumulating polyP), 30-min DNase and RNase treatment, **D**) similar to (C) but 2-hour DNase and RNase treatment, **E**) bacteria *V. perlucida*, 2-hour DNase and RNase treatment, **F**) similar to (E) but 4-hour DNase and RNase treatment.

Figure S3 The responses of JC-D7-polyP fluorescence intensity (Fluo) to the concentrations of polyP under the influence of the lysis solution composition. Results are for tests under the conditions in a Taguchi orthogonal array (see Table S5).

Figure S4 Fluorescence spectra of **A**) polyP stained by JC-D7 and DAPI, **B**) Chondroitin sulfate stained by DAPI and JC-D7, and **C**) the ratios of polyP and chondroitin fluorescence signals. The fluorescence spectra were obtained under 405 nm excitation**.**

Figure S5 Fluorescence spectra of chondroitin sulfate stained by DAPI and JC-D7 in tris buffer of various level of EDTA concentrations**.**

Table S1 Fluorescence intensity of various concentrations of polyP stained by various concentrations of JC-D7 (405 nm excitation and 535 nm emission)..

Table S2 Slope of the linear calibration curve for polyP-JC-D7 fluorescence vs. polyP concentrations.

Table S3 Fluorescence intensity of various concentrations of polyP of various chain lengths stained by JC-D7 (405 nm excitation and 535 nm emission).

Table S4 Concentration levels used for testing the effects of lysis solution composition on the linear response of JC-D7-polyP fluorescence intensity to polyP concentration.

Table S5 The Taguchi orthogonal array of experimental conditions for testing the effects of lysis solution composition on the linear response of JC-D7-polyP fluorescence intensity to polyP concentration, and the test results in terms of detection limit, linearity (R^2 of the linear curve), and sensitivity (slope of the linear curve).

Table S6 Detection limits of the JC-D7-polyP fluorescence quantification under the various conditions tested using the 4-parameter-3-level Taguchi orthogonal array (see Tables S4-5).

Table S7 Linearity (R^2 of the linear calibration curve) of the JC-D7-polyP fluorescence quantification under the various conditions tested using the 4-parameter-3-level Taguchi orthogonal array (see Tables S4-5).

Table S8 Sensitivity (slope of the linear calibration curve) of the JC-D7-polyP fluorescence quantification under the various conditions tested using the 4-parameter-3-level Taguchi orthogonal array (see Tables S4-5).

Table S9 Conditions used to test the effects of chondroitin sulfate on polyP quantification using DAPI and JC-D7.

S1. Verification of polyP concentrations in standard solutions

The concentrations of polyP in the standard solutions were verified by hydrolyzing the polyP into PO_4^{3-} and subsequently measuring the PO_4^{3-} concentration: 200 μL of polyP standard solutions was mixed with 400 μL of H_2SO_4 solution (0.25 N) and 0.005 g of potassium persulfate, and the mixed solutions were autoclaved at 120 °C and 15 psi for 2 hours to digest polyP into PO_4^{3-} [1]. The PO_4^{3-} in the samples was then measured using either the molybdenum blue assay [2] or the malachite green assay [3]. For the molybdenum blue assay, samples were mixed with ammonium molybdate (final concentration 8 mmol L^{-1}), potassium antimony tartrate (final concentration 0.2 mmol L^{-1}), L-ascorbic acid (final concentration 30 mmol L^{-1}), and sulfate acid (final concentration 2.5 N) and reacted for more than 30 minutes before measuring the absorbance at 805 nm. For the malachite green method, the samples were mixed with ammonium heptamolybdate (final concentration 16 mmol L^{-1}), malachite green (final concentration 0.175 mmol L^{-1}) and sulfuric acid (final concentration 1.2 N), reacted for 10 minutes and measured for the absorbance at 620 nm. Absorbance measurements were performed using transparent 96-well plates and a microplate reader (BMG ClarioStar Plus Microplate Reader). PO_4^{3-} in the polyP standards without persulfate digestion was also measured to check for contamination of PO_4^{3-} in the standards, which was found to be less than 0.4%. Therefore, the concentration of PO_4^{3-} measured in the polyP standards after digestion, which breaks down polyP into PO_4^{3-}, accurately reflects the original polyP concentration (in P units).

S2. Preparation of exopolyphosphatase (PPX)

S2.1. Transformation of *E. coli* and induction of PPX expression – To prepare the yeast *Saccharomyces cerevisiae* PPX, we first transformed *E. coli* BL21(DE3) competent cells (ThermoFisher, # EC0114) using the plasmid with PPX gene (pKM263-ScPPX, Addgene plasmid #38327, Florian Freimoser; tagged with 3XHis on the N terminal; http://n2t.net/addgene:38327) by applying heat shock: 100 μL of the Bl21(DE3) competent cells (stored at −80 °C) was defrosted on ice, gently mixed with 10 ng of plasmids, incubated on ice for 20 minutes, placed into a water bath at 42 °C for 30 seconds, and placed back on ice for 2 minutes. The transformed *E. coli* competent cells were cultured in 500 μL of LB medium (10% (w/v) Tryptone, 5% (w/v) Yeast Extract, and 10% (w/v) NaCl) at 37 °C for 30 minutes before being spread on an LB agar plate containing 0.1 mg mL^{-1} of ampicillin and incubated overnight. A single colony of the *E. coli* BL21(DE3) competent cells containing the plasmid pKM263-ScPPX was then picked and inoculated in 200 mL of LB medium containing 0.1 mg mL^{-1} of

ampicillin. This culture was grown at 37ºC until log phase (OD_{600} = 0.8). To induce the expression of PPX in the culture, Isopropyl β- d-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, #70527-3) was added to the culture to a final concentration of 1 mmol L^{-1}, which was then incubated at 25 °C for 3 hours [4].

S2.2 Collection and purification of PPX— To collect the PPX produced, *E. coli* cells were first pelleted using centrifugation at 3000 g for 10 minutes to remove the supernatant containing the medium. The cells were then frozen at -20 °C and later resuspended in NEBExpress *E. coli* Lysis Reagent (New England Biolabs, # P8116S; lysis buffer volume (mL) = OD600 x culture volume x 0.05). The resuspended cells were incubated on an orbital shaker at room temperature for 30 minutes. The lysate was then centrifugated under 16000g for 15 minutes to collect the supernatant that contained the PPX, which was then purified using HisPur™ Ni-NTA Spin Columns (ThermoFisher, # 88226). The supernatant was mixed with the same volume of Equilibrium Buffer (20 mmol L^{-1} sodium phosphate, 300 mmol L^{-1} sodium chloride, and 10 mmol L^{-1} imidazole; pH = 7.0), added into the spin columns, incubated for 1 hour, and centrifuged at 700 g for 1 minute to remove the liquids. The column was then washed by adding 6 mL of Wash Buffer (20 mmol L^{-1} sodium phosphate, 300 mmol L^{-1} sodium chloride, and 25 mmol L^{-1} imidazole; pH = 7.0) and centrifuge at 700 g for 1 minute to remove the Wash Buffer. Finally, the PPX (with 3XHis tag) that bound to the columns was eluted off using 3 mL of Elution Buffer (20 mmol L^{-1} sodium phosphate, 300 mmol L^{-1} sodium chloride, 250 mmol L^{-1} imidazole; pH = 7.0) and collected by centrifugation at 700 g for 1 minute. The concentration of the protein PPX in the eluted solution was determined to be ~ 1.2 μg μL^{-1} by measuring the absorbance at 280 nm using a Nanodrop Spectrophotometer [5].

To ensure accurate determination of polyP concentrations using the enzyme PPX, which relies on measuring the release of PO_4^{3-} from the enzymatic degradation of polyP, it is necessary to remove any PO_4^{3-} contamination from the eluted solution. We used dialysis to resolve this. In brief, approximately 3 mL of the eluted solution containing PPX was sealed into a dialysis bag (pre-cleaned by boiling for 30 minutes and thoroughly washing with deionized H_2O). The bag was then immersed in a TBS buffer (20 mmol L^{-1} Tris, 150 mmol L^{-1} NaCl, pH = 7.6) and dialyzed for 4 hours at 4°C. The dialysis was repeated by transferring the bag containing the PPX solution into fresh TBS buffer solution for another 4 hours at 4°C. After dialysis, the solution containing PPX was transferred, mixed with an equal volume of glycerol, and stored at -20 °C for future usage (final PPX concentration ~ 600 ng μL^{-1} for the PPX stock).

S3. Quantification of PO_4^{3-} after PPX enzymatic degradation of polyP

The samples and polyP standards after PPX digestion were determined for PO_4^{3-} produced using the molybdate-based methods[2,3]. We first tried the molybdenum blue assay for its wider linear range. In brief, PPX digested samples were mixed with ammonium molybdate (final concentration 8 mmol L^{-1}), potassium antimony tartrate (final concentration 0.2 mmol L^{-1}), L-ascorbic acid (final concentration 30 mmol L^{-1}), and sulfate acid (final concentration 2.5 N) and reacted for more than 30 minutes before measuring the absorbance at 805 nm. For the samples with a low polyP concentration (<0.5 μM) that were difficult to measure using the molybdenum blue method, the malachite green method was used. The samples were mixed with ammonium heptamolybdate (final concentration 16 mmol L^{-1}), malachite green (final concentration 0.175 mmol L^{-1}) and sulfuric acid (final concentration 1.2 N), reacted for 10 minutes, and measured for the absorbance at 620 nm. Absorbance measurements were performed using transparent 96-well plates and a microplate reader (BMG ClarioStar Plus Microplate Reader).

To account for any PO_4^{3-} originally present in the polyP samples or introduced during the treatment, each sample was treated with denatured PPX (heated at 85 °C for 5 min) as a control following the same procedure. The PO_4^{3-} concentration measured in the control was subtracted, if higher than the detection limit (>0.05 μmol L^{-1}), from that measured in the samples treated with active PPX to calculate the polyP concentration in the sample.

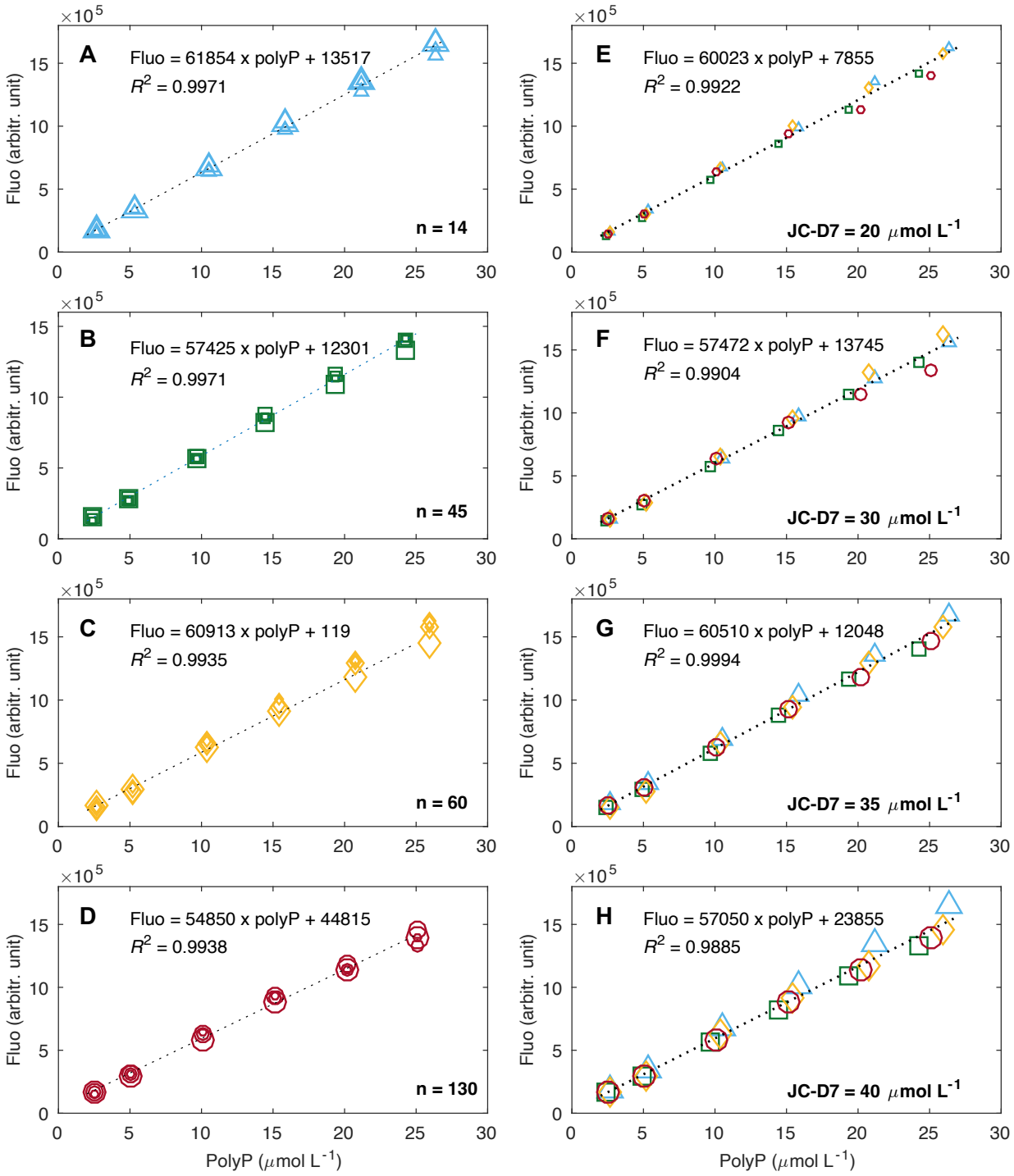

Figure S1 Fluorescence intensity (Fluo, in arbitrary unit) vs. polyP concentration for polyP of various chain lengths (n =14, 45, 60 and 130) under various concentrations of JC-D7 (20-40 μmol L^{-1}). PolyP is stained using JC-D7 solutions dissolved in 3% of DMSO and 12.5 mmol L^{-1} of HEPES buffer. Fluorescence measurements are measured under 405 nm excitation and 535 nm emission.

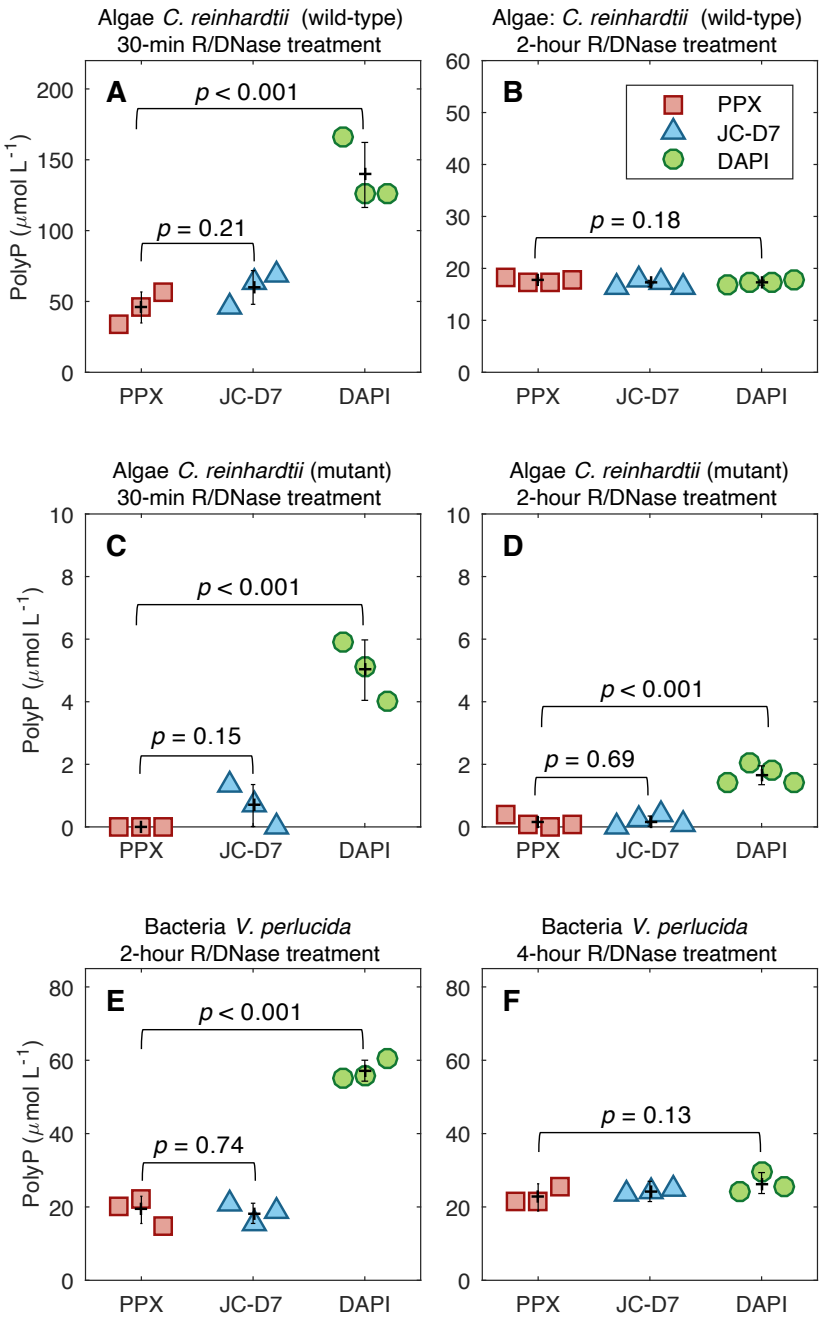

Figure S2 Comparing polyP quantified using PPX, DAPI, and JC-D7. PolyP was extracted by using the phenol-chloroform method from cultures of **A)** a wild-type strain of algae *Chlamydomonas reinhardtii* (polyP accumulating), treated using DNase and RNase for 30 minutes after extraction, **B)** similar to (A) but 2-hour DNase and RNase treatment, **C)** a *vtc-1* mutant strain of algae *Chlamydomonas reinhardtii* (not accumulating polyP), 30-min DNase and RNase treatment, **D**) similar to (C) but 2-hour DNase and RNase treatment, **E**) bacteria *V. perlucida*, 2-hour DNase and RNase treatment, **F**) similar to (E) but 4-hour DNase and RNase treatment. Cross markers indicate average values and error bars represent the standard deviations from the means. *p* values for ANOVA tests are shown in the figure.

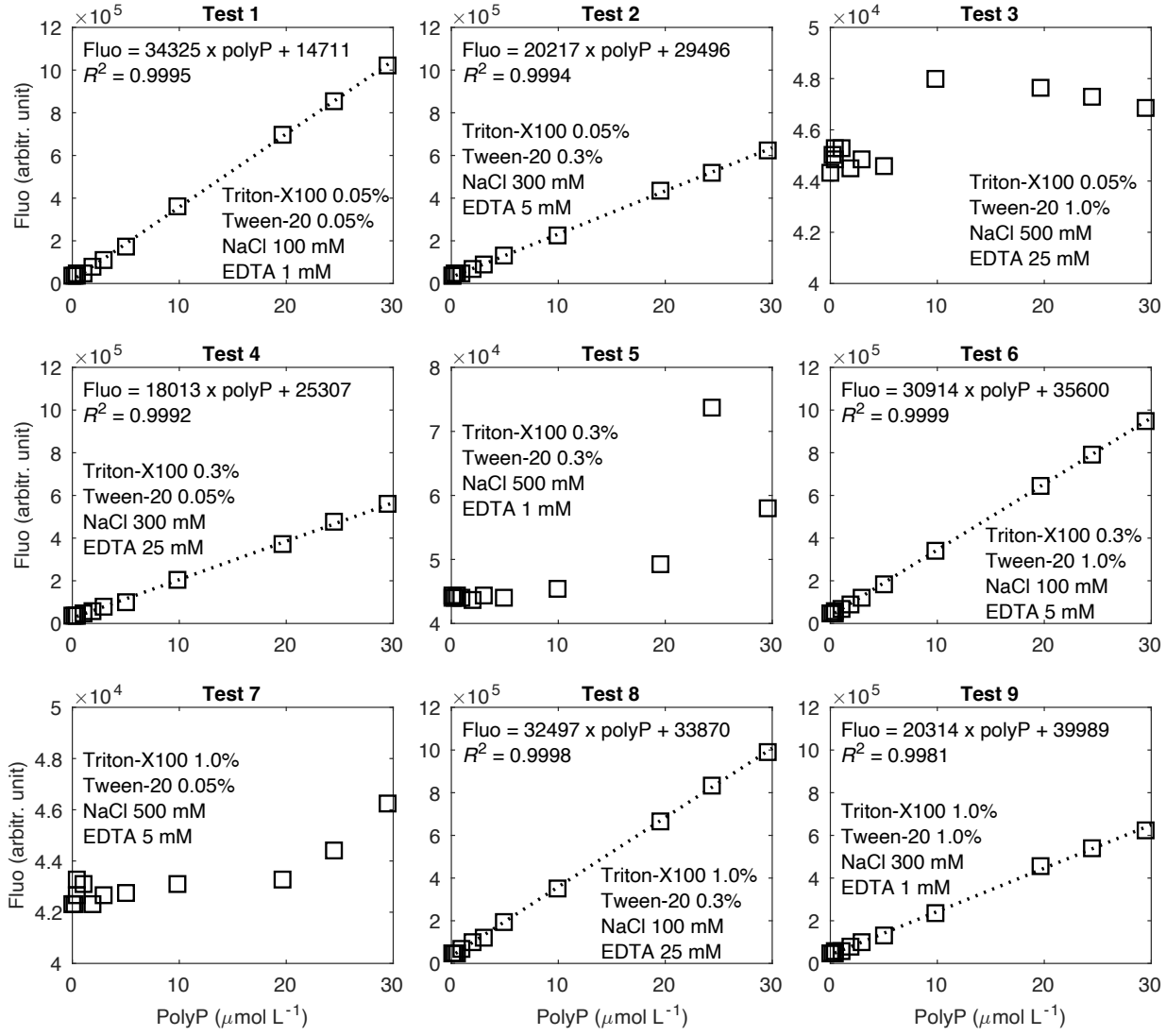

Figure S3 The responses of JC-D7-polyP fluorescence intensity (Fluo) to the concentrations of polyP under the influence of the lysis solution composition. Results are for tests under the conditions in a Taguchi orthogonal array (see Table S5).

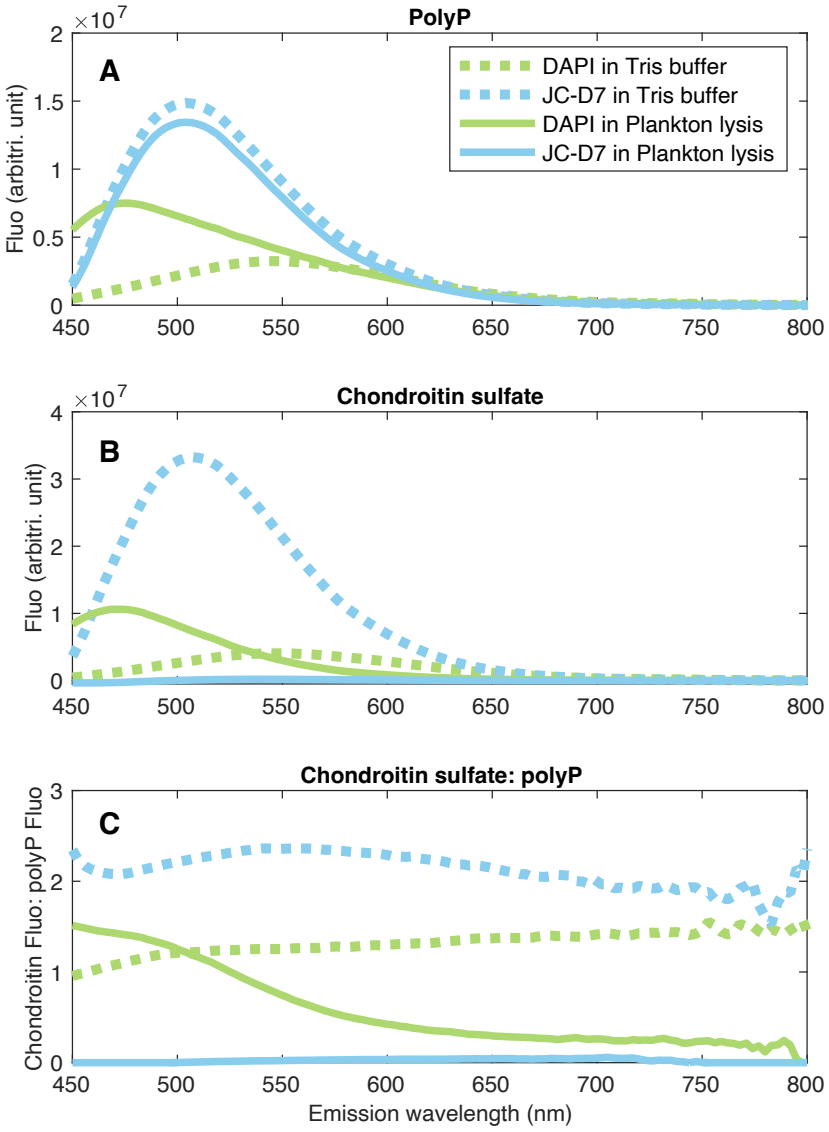

Figure S4 Fluorescence spectra of A) polyP stained by JC-D7 and DAPI, B) Chondroitin sulfate stained by DAPI and JC-D7, and C) the ratios of polyP and chondroitin fluorescence signals. The fluorescence spectra were obtained under 405 nm excitation.

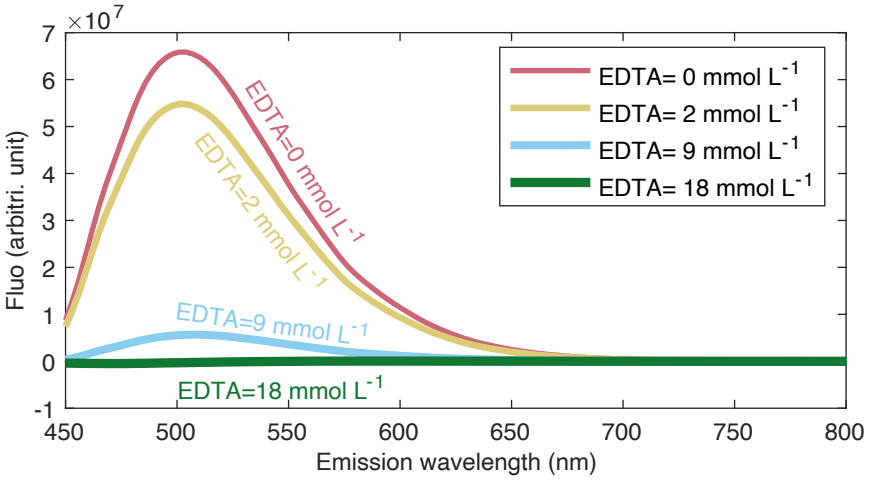

Figure S5 Fluorescence spectra of chondroitin sulfate (final concentration 10 μg mL^{-1}) stained by DAPI and JC-D7 in Tris buffer (final concentration 20 mmol L^{-1}; pH=7) of various levels of EDTA**.** Fluorescence intensities are measured under 405 nm excitation and emission spectra across 450–800 nm was measured. All experiments were conducted in triplicates and average values were plotted here.

Table S1 Fluorescence intensity of various concentrations of polyP (2−30 μmol L^{-1}) stained by various concentrations of JC-D7 (20−40 μmol L^{-1}). Under each condition, fluorescence intensities were determined using calibration curves obtained for polyP of four different chain lengths (n=14, 45, 60, and 130) (Fig. S1E-H). Variability of results caused by the different concentrations of JC-D7 is reported as the standard deviation from the mean (STD). Fluorescence intensities are measured under 405 nm excitation and 535 nm emission.

PolyP (μmol L^{-1})	PolyP-JC-D7 fluorescence intensity (arbitr. unit)						
	JC-D7 concentration (μmol L^{-1})				Average	STD	%STD
	20	30	35	40			
2	127901	131089	133068	137955	132503	4212	3.2%
5	307970	307105	314598	309105	309695	3370	1.1%
10	608085	600465	617148	594355	605013	9848	1.6%
15	908200	893825	919698	879605	900332	17406	1.9%
20	1208315	1187185	1222248	1164855	1195651	25085	2.1%
25	1508430	1480545	1524798	1450105	1490970	32801	2.2%
30	1808545	1773905	1827348	1735355	1786288	40533	2.3%
Average							**2.1%±0.6%**

Table S2 Slope of the linear calibration curve for polyP-JC-D7 fluorescence vs. polyP concentrations.

PolyP chain length	Slope of the linear curve for fluorescence vs polyP concentration						
	JC-D7 concentration (μmol L^{-1})				Average	STD	%STD
	20	30	35	40			
n=14	62792	59077	63149	62561	61895	1894	3.1%
n=45	59127	58251	58347	53972	57424	2335	4.1%
n=60	62045	63705	62069	55832	60913	3475	5.7%
n=130	55042	56228	57377	54318	55741	1345	2.4%
Average	59752	59315	60236	56671			**3.8±1.4%**
STD	3515	3162	2804	4009			
%STD	5.9%	5.3%	4.7%	7.1%	**5.7±1.0%**		

Table S3 Fluorescence intensity of various concentrations of polyP (2−30 μmol L^{-1}) of various chain length (n=14, 45, 60, and 130) stained by JC-D7. Under each condition, fluorescence intensities were determined using calibration curves obtained for polyP stained using various JC-D7 concentrations (20−40 μmol L^{-1}; Fig. S1A-D). Variability of results caused by the different chain length of polyP is reported as the standard deviation from the mean (STD). Fluorescence intensities are measured under 405 nm excitation and 535 nm emission.

PolyP (μmol L^{-1})	PolyP-JC-D7 fluorescence intensity (arbitr. unit)						
	PolyP chain length				Average	STD	%STD
	n=14	n=45	n=60	n=130			
2	137225	127151	121945	154515	135209	14349	10.6%
5	322787	299426	304684	319065	311491	11208	3.6%
10	632057	586551	609249	593315	605293	20221	3.3%
15	941327	873676	913814	867565	899096	34835	3.9%
20	1250597	1160801	1218379	1141815	1192898	50392	4.2%
25	1559867	1447926	1522944	1416065	1486701	66231	4.5%
30	1869137	1735051	1827509	1690315	1780503	82189	4.6%
Average							**5.0%±2.5%**

Table S4 Concentration levels used for testing the effects of lysis solution composition on the linear response of JC-D7-polyP fluorescence intensity to polyP concentration (see the testing conditions in Table S5).

Level	Triton-X100 (%)	Tween-20 (%)	NaCl (mmol L^{-1})	EDTA (mmol L^{-1})
1	0.05	0.05	100	1
2	0.3	0.3	300	5
3	1	1	500	25

Table S5 The Taguchi orthogonal array of experimental conditions (4 parameters x 3 levels) for testing the effects of lysis solution composition on the linear response of JC-D7-polyP fluorescence to polyP concentration, and the test results in terms of detection limit, linearity (R^2 of the linear curve), and sensitivity (slope of the linear curve) (see Fig. S3 for the calibration curves). The detection limits of tests # 3, 5, and 7 are set to be the same number (5 μmol L^{-1}), for these tests all yield bad results (see Fig. S3); assigning them with the same value can avoid the misinterpretation that one is better than the others.

Test	Triton-X100 (%)	Tween-20 (%)	NaCl (mmol L^{-1})	EDTA (mmol L^{-1})	Detection limit (μmol L^{-1})	Linearity R^2	Sensitivity (arbit. unit)
1	0.05	0.05	100	1	0.4	0.9995	34325
2	0.05	0.3	300	5	0.4	0.9994	20217
3	0.05	1	500	25	5	0.5661	97.18
4	0.3	0.05	300	25	0.4	0.9992	18013
5	0.3	0.3	500	1	5	0.6321	264
6	0.3	1	100	5	0.4	0.9999	30950
7	1	0.05	500	5	5	0.7155	90
8	1	0.3	100	25	0.4	0.9998	32606
9	1	1	300	1	0.4	0.9981	20314

Table S6 Detection limits (in μmol L^{-1}) of the JC-D7-polyP fluorescence quantification under the various conditions tested using the 4-parameter-3-level Taguchi orthogonal array (see Tables S4-5). Results (detection limit of polyP measurement) are averaged for tests of each parameter at each level; for example, Triton-X100 level 1 results are average polyP detection limit of Test #1, 2, and 3 (see Table S5, all experiments in which Triton-X100 was set to be level 1, 0.05%). The difference between maximum and minimum values (Δ) for each parameter across three levels are reported to show how results respond to the variability of the parameters.

Level	Triton-X100	Tween-20	NaCl	EDTA
1	1.9	1.9	0.4	1.9
2	1.9	1.9	0.4	1.9
3	1.9	1.9	5.0	1.9
Δ	0.0	0.0	**4.6 (238%)**	0.0

Table S7 Linearity (R^2 of the linear calibration curve) of the JC-D7-polyP fluorescence quantification under the various conditions tested using the 4-parameter-3-level Taguchi orthogonal array (see Tables S4-5). Results are averaged for each parameter at each level; for example, Triton-X100 level 1 results are average of Test #1, 2, and 3 (Table S5). The difference between maximum and minimum values (Δ) for each parameter across three levels are reported to show how results respond to the variability of the parameters.

Level	Triton-X100	Tween-20	NaCl	EDTA
1	0.8550	0.9047	0.9997	0.8766
2	0.8771	0.8765	0.9989	0.9049
3	0.9045	0.8547	0.6379	0.8550
Δ	0.0495 (5.6%)	0.0500 (5.7%)	**0.3618 (41%)**	0.0499 (5.7%)

Table S8 Sensitivity (slope of the linear calibration curve) of the JC-D7-polyP fluorescence quantification under the various conditions tested using the 4-parameter-3-level Taguchi orthogonal array (see Tables S4-5). Results are averaged for each parameter at each level; for example, Triton-X100 level 1 results are average of Test #1, 2, and 3 (Table S5). The difference between maximum and minimum values (Δ) for each parameter across three levels are reported to show how results respond to the variability of the parameters.

Level	Triton-X100	Tween-20	NaCl	EDTA
1	18213	17476	32627	18301
2	16409	17696	19515	17086
3	17670	17120	150	16905
Δ	1804 (10%)	575 (3.3%)	**32477 (186%)**	1396 (8.0%)

Table S9 Conditions used to test the effects of chondroitin sulfate on polyP quantification using DAPI and JC-D7. Tris = Tris-HCl buffer of final concentration 20 mmol L^{-1} (pH=7); polyP = polyP (n=45) of final concentration 20 μmol L^{-1}; chondroitin sulfate = chondroitin sulfate of final concentration 10 μg mL^{-1}. Fluorescence intensities are measured under 405 nm excitation and emission spectra across 450–800 nm was measured. All experiments were conducted in triplicates.

Test	Buffer	Compound	Dye
A	Tris	PolyP	DAPI
B	Tris	PolyP	JC-D7
C	Tris	Chondroitin sulfate	DAPI
D	Tris	Chondroitin sulfate	JC-D7
E	Plankton lysis	PolyP	DAPI
F	Plankton lysis	PolyP	JC-D7
G	Plankton lysis	Chondroitin sulfate	DAPI
H	Plankton lysis	Chondroitin sulfate	JC-D7

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